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Characterization of two carbonyl reductases from *Ogataea polymorpha* NBRC 0799

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Abstract The enzyme responsible for the enantioselective production of (*S*)-1,1,1-trifluoro-2-propanol ((*S*)-TFP) from 1,1,1-trifluoroacetone (TFA) has been identified in *Ogataea polymorpha* NBRC 0799. We purified two carbonyl reductases, *Op*CRD-A and *Op*CRD-B from this strain, and revealed their characteristics. Both enzymes were specific to NADH, but the following characteristics were different: The molecular mass of subunit *Op*CRD-A was 40 kDa and that of *Op*CRD-B was 43 kDa. Amino acid sequences of both enzymes were only 21% identical. *Op*CRD-B contained 4 mol of zinc per mole of enzyme, but *Op*CRD-A did not. The optimal pH, temperature, pH stability, thermostability, and inhibitor specificity were also remarkably different. With regard to substrate specificity, both enzymes exhibited high reductase activity toward a wide variety of ketones, aldehydes and fluoroketones, and dehydrogenase activity toward 2-propanol and 2-butanol. The reductase activity was much higher than the dehydrogenase activity at acidic pH. *Op*CRD-A enantioselectively produced (*S*)-TFP from TFA, but *Op*CRD-B preferentially produced (*R*)-TFP. Thus, we concluded that *Op*CRD-A plays the main role in the production of (*S*)-TFP by a reaction of *O. polymorpha* NBRC 0799 cells and that *Op*CRD-A has great potential for efficient production of (*S*)-TFP, as it is an *S*-specific enzyme and does not catalyze the dehydrogenation of (*S*)-TFP.

Keywords 1,1,1-Trifluoroacetone · (*S*)-1,1,1-Trifluoro-2-propanol · Carbonyl reductase · Alcohol dehydrogenase · *Ogataea polymorpha*

Introduction

Chiral secondary alcohols and their derivatives are valuable building blocks for synthesis of pharmaceuticals, agrochemicals, or fine chemicals. Stereospecific alcohol dehydrogenases (ADHs) and carbonyl reductases (CRDs), which catalyze asymmetric reduction of prochiral ketones, are widely used for synthesis of chiral alcohols (Nakamura et al. 2003; Ni and Xu 2012; Yamada-Onodera et al. 2004). Among the chiral secondary alcohols, (*S*)-1,1,1-trifluoro-2-propanol ((*S*)-TFP) is one of the most useful optically active alcohols, and chemical methods for the production of (*S*)-TFP have been developed using a ruthenium phosphine complex (Puentener and Waldmeier 2008). Biochemical methods for enantioselective production of (*S*)-TFP from 1,1,1-trifluoroacetone (TFA) were also reported, utilizing baker's yeast (Bucciarelli et al. 1983; Doswald et al. 2007), and ADH (Rosen et al. 2006) or a crude enzyme solution of ADH from *Rhodococcus erythropolis*, *Arthrobacter paraffineus*, or *Phichia farinose* (Hayashi et al. 2007). Our group also screened microorganisms capable of enantioselectively producing (*S*)-TFP from 1,1,1-trifluoroacetone and discovered that *Ogataea polymorpha* (formerly *Hansenula polymorpha*), *Candida mycoderma*, and *Pichia naganishi* are producers of (*S*)-TFP (Asano et al. 2011). Among these strains, *O. polymorpha* NBRC 0799 exhibited the highest conversion yield of (*S*)-TFP. However, the

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enzyme catalyzing this reaction has not yet been identified. During experiments to purify the enzyme, we found that this strain produces two enzymes that catalyze the reduction of TFA. Therefore, we have identified both of the enzymes from *O. polymorpha* NBRC 0799 that are responsible for the enantioselective production of (*S*)-TFP (Fig. 1a). The present paper describes the purification and characterization of two CRDs produced by *O. polymorpha* NBRC 0799.

Materials and methods

Materials

TFA was purchased from Merck Millipore (MA, USA). (*S*)- and (*R*)-TFP were a gift from Central Glass Co., Ltd. (Tokyo, Japan). 1,1-Difluoroacetone and acetone were purchased from INDOFINE Chemical Company Inc. (NJ, USA) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. Fluoroacetone, 1,3-difluoroacetone, and 4,4,4-trifluoro-2-butanone were from Sigma-Aldrich Inc. (MO, USA). β-NADH, β-NADPH, and β-NAD^+ were from Oriental Yeast Co., Ltd. (Osaka, Japan). Trypsin (sequence grade modified trypsin) and ammonium bicarbonate were from Promega (Madison, WI, USA) and Nacalai Tesque Inc. (Kyoto, Japan), respectively. All other chemicals used were of analytical grade and commercially available.

Cultivation of *O. polymorpha* NBRC 0799

O. polymorpha NBRC 0799 was first cultivated in a test tube containing 5 mL of YM medium (1.0% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, 0.3% KH_2PO_4, 0.2% K_2HPO_4, pH 6.5), at 30 °C for 24 h, with shaking at 300 strokes per minute. The culture (2 mL) was then inoculated into a 500-mL shaker flask containing 200 mL of YM medium, and the second culture was incubated at 30 °C for 24 h. Ten milliliters of the second culture was transferred into a 2-L shaker flask containing 1 L of YM medium. After the third cultivation was performed at 30 °C for 24 h, the cells were harvested by centrifugation, washed with 0.85% saline, and stored at − 20 °C until use.

Assay of enzyme activity

CRD activity was assayed at 30 °C and pH 6.0 or 6.5 using TFA and NADH, as follows: the standard reaction mixture contained 50 mM TFA, 0.1 mM NADH, and 170 mM potassium phosphate buffer (KPB) at pH 6.5 (*Op*CRD-A) or pH 6.0 (*Op*CRD-B) and appropriate amounts of enzyme in a final volume of 1.0 mL. The reaction was initiated by the addition of TFA and followed spectrophotometrically at 30 °C by measuring the absorbance at 340 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the reduction of 1 μmol of TFA per minute, which coincides with the oxidation velocity of NADH. The molar absorption efficiency of NADH, $6.22 \times 10^3\ M^{-1}\ cm^{-1}$, was used for the calculation of the enzyme activity.

ADH activity was assayed at 30 °C and at pH 6.5 and 9.0 (*Op*CRD-A) or pH 6.0 and 10.0 (*Op*CRD-B), using 2-propanol and NAD^+, as follows: the standard reaction mixture contained 50 mM 2-propanol, 1.0 mM NAD^+, and 170 mM KPB at pH 6.0 or 6.5, or 170 mM glycine-NaOH buffer at pH 9.0 or 10.0, and appropriate amounts of enzyme in a final volume of 1.0 mL. The reaction was initiated by the addition of 2-propanol and followed spectrophotometrically at 30 °C by measuring the absorbance at 340 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the dehydrogenation of 1 μmol of 2-propanol per minute, which coincides with the formation velocity of NADH.

Purification of enzyme

All procedures were performed at 4–10 °C, and the buffer used was KPB, pH 7.0, unless otherwise stated.

Preparation of cell-free extract: Cells from 5 L of culture broth (34.8 g wet weight) were resuspended in 174 mL of 10 mM buffer and disrupted with glass beads (φ0.5 mm) at 2700 rpm for 6 min (time-on 1 min, time-off 1 min, 6 cycles) using a Multi-beads Shocker (Yasui Kikai, Osaka, Japan). The

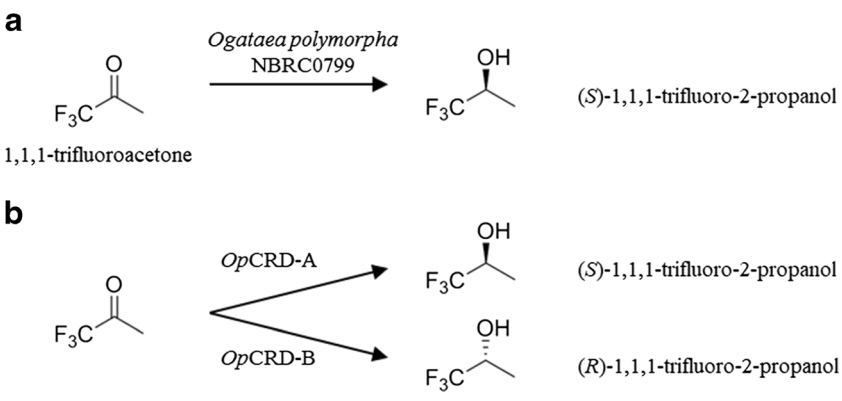

Fig. 1 The enantioselective production of (*S*)- or (*R*)-TFP from TFA. **a** Whole-cell reaction of *O. polymorpha* NBRC0799. **b** Enzyme reaction with *Op*CRD-A or *Op*CRD-B

supernatant (230 mL) obtained by centrifugation at 20,000×*g* for 10 min was used as a crude enzyme solution.

Ammonium sulfate fractionation: Solid ammonium sulfate (40.5 g) was added to the crude enzyme solution (final 30% saturation), and the resulting precipitates were removed by centrifugation at 20,000×*g* for 30 min.

Phenyl-Toyopearl column chromatography: The supernatant from the 30% ammonium sulfate precipitation was applied to a Phenyl-Toyopearl column (14 × 2.8 cm diameter), equilibrated with 10 mM buffer containing 1.33 M ammonium sulfate. After the column was washed with 10 mM buffer containing 1.33 M ammonium sulfate, the adsorbed enzyme was eluted by a linear gradient of 10 mM buffer containing 1.33 M ammonium sulfate and 10 mM buffer (400 ml each), followed by 10 mM buffer (200 ml). Since the enzyme activity was separated in this step, *Op*CRD-A which eluted at a higher conductivity and *Op*CRD-B which eluted at lower conductivity were separately collected and each enzyme solution was dialyzed against 10 mM buffer.

Q-Sepharose column chromatography: The dialyzed *Op*CRD-A solution was applied to a Q-Sepharose column (7 × 2.8 cm diameter) equilibrated with 10 mM buffer. Since *Op*CRD-A did not adsorb to this resin, unadsorbed active fractions were collected. On the other hand, *Op*CRD-B adsorbed to this resin and the enzyme was eluted by a linear gradient of 10 mM buffer and 10 mM buffer containing 0.15 M NaCl (150 mL each). The active fractions were combined and dialyzed against 10 mM buffer.

Hydroxyapatite column chromatography: Solutions of *Op*CRD-A and *Op*CRD-B were applied to a hydroxyapatite column (6 × 1.6 cm diameter) equilibrated with 10 mM buffer. After the column was washed with 10 mM buffer, the adsorbed enzyme was eluted by a linear gradient of 10 mM buffer and 0.3 M buffer (100 mL each). The active fractions were collected and concentrated by ultrafiltration (Amicon Ultra-15, Merck Millipore) with 10 mM buffer.

Gel filtration on a Superdex 200 column: The concentrated *Op*CRD-A solution was applied to a Superdex 200 10/300GL column (GE Healthcare UK Ltd., Buckinghamshire, UK) equilibrated with 10 mM buffer containing 0.3 M NaCl.

Identification of reaction products

A 56 mM TFA sample was incubated with 10 mM NADH, 50 mM d-glucose, 1.0 U of glucose dehydrogenase, and an appropriate amount of *Op*CRD at 30 °C for 24 h in 0.2 M KPB, pH 7.0 (total volume, 1 mL). The reaction products were identified by gas chromatography using a capillary column (Rt-βDEXsa, Restek Corporation, PA, USA), in which helium was used as the carrier gas and the pressure was 100 kPa. The temperature of the column was kept at 80 °C for 25 min, then increased to 200 °C at 20 °C/min, followed by 200 °C for 4 min. The temperature of the vaporizing chamber and the flame ionization detector was kept at 230 °C. Under these conditions, the retention time for TFA, (*R*)-TFP, and (*S*)-TFP was 2.5, 7.7, and 8.4 min, respectively (Fig. S1a). The optical purity of the reaction products was calculated using the peak area obtained under the above analyzing conditions.

Amino acid sequence of the enzyme proteins

The N-terminal amino acid sequence of each enzyme was determined using a gas-phase protein sequencer equipped with an online reverse-phase chromatography system, for identification of PTH-amino acids.

The internal amino acid sequence was analyzed as follows: After separating the purified enzyme using 12% SDS-PAGE, the protein was excised and incubated with trypsin (sequence grade modified trypsin). The trypsin-digested peptides were separated by a nanoUPLC equipped with a trap column (nanoACQUITY UPLC Symmetry C18 Trap Column, Waters, Milford, MA, USA) and a reverse-phase capillary column (ACQUITY UPLC Peptide CSH C18 nanoACQUITY Column 10 K psi, Waters), using acetonitrile water containing 0.1% formic acid. The molecular mass of each peptide was determined using an electrospray ionization/quadrupole time-of-flight mass spectrometer (nanoACQUITY UPLC-SYNAPT G2-Si, Waters). The sequence of each peptide was analyzed using the Biolynx software suite (Waters).

Gene cloning of the enzyme

As mentioned in the "Results" section, the N-terminal and internal amino acid sequences of *Op*CRD-A and *Op*CRD-B were similar to those of ADH 2, alcohol dehydrogenase, and a putative threonine dehydrogenase/Zn-dependent dehydrogenase-like protein from *O. parapolymorpha* DL-1, respectively. Therefore, the DNA for *Op*CRD-A was cloned by PCR with the following two primers that were synthesized based on the N-terminal and C-terminal DNA sequences of ADH 2 of *O. parapolymorpha* DL-1. The forward and reverse primers were 5′-gaaggagatatacatATGACTTCCATTCCAAAGAC TCAAA-3′ and 5′-ttagcagccggatccCTATTTGGAAGTGT CAAGAACG-3′, respectively. The nucleotides denoted by lower case letters indicate the 15-bp overlap sequence of pET11a (Merck KgaA, Darmstadt, Germany), which is necessary for use of the In-Fusion HD Cloning kit (Takara Bio Inc., Shiga, Japan). The following two primers, which were prepared using a sequence 200–300 bases away from the N-terminus and C-terminus of the DNA sequence from ADH.2 were also used. The forward and reverse primers were 5′-gaaggagatatacatTGTGAAATACCCCGCCACGGCCATC-3′ and 5′-ttagcagccggatccACTGGGACAGGTGCCGGAGTCG-

3′, respectively. PCR was performed for 35 cycles, consisting of 10 s of denaturation at 98 °C, 10 s of annealing at 55 °C, and 15 s of extension at 72 °C, using PrimeSTAR Max DNA Polymerase (Takara Bio Inc.). Genomic DNA of *O. polymorpha* NBRC0799 was extracted by using a Wizard Genomic DNA Purification Kit (Promega Co., Wisconsin, USA), according to the manufacturer's protocol. The PCR product was inserted into a linearized pET11a vector using the In-Fusion HD Cloning Kit. The linearized vector was amplified by inverse PCR using the following two primers; 5′-ATGTATATCTCCTTCTTAAAGTTAAAC-3′, and 5′-GGATCCGGCTGCTAAC-3′. The sequence was analyzed using an Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, CA, USA).

The DNA for *Op*CRD-B was also cloned under the same conditions as that of *Op*CRD-A, using the N-terminal and C-terminal DNA sequences of a putative threonine dehydrogenase/Zn-dependent dehydrogenase-like protein from *O. parapolymorpha* DL-1. The forward primer of 5′-gaaggagatatacatATGATGAAAGCATTGTGTTACTTA-3'and reverse primer of 5′-ttagcagccggatccCTATTCCGAGGTCA GAACGACC-3′ were used to amplify the *Op*CRD-B gene. The primers 5′-gaaggagatatacatGTGCCTCGCATGTT CCACGTGAG-3′ and 5′-ttagcagccggatccATCCGCAA GCACGCGTGCTGCAC-3′ were used as the amplifiers of a region outside of the *Op*CRD-B gene.

The recombinant plasmids pET11a-*Op*CRD-A and pET11a-*Op*CRD-B, named pOpCRD-A and pOpCRD-B, respectively, were used to transform *Escherichia coli (E. coli)* BL21 (DE3) cells (Agilent Technologies Inc., Santa Clara, CA, USA).

Expression of the genes for the enzymes

E. coli BL21 (DE3) cells harboring either pOpCRD-A or pOpCRD-B plasmids were inoculated into 5 mL of Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 L of water) containing 100 μg/mL of ampicillin, and then incubated at 37 °C for 18 h with reciprocal shaking at 300 strokes per min. The culture (50 μL) was then inoculated into 5 mL of the same medium and incubation continued at 37 °C, with shaking at 300 rpm. When the optical density of the medium at 600 nm reached 0.5–1.0, isopropyl β-d-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the incubation was further continued at 30 or 20 °C for 24 h. Cells from 4 mL of the culture were harvested by centrifugation (10,000×*g*, 5 min, 4 °C) and washed with 20 mM KPB, pH 7.0. Wet cells (ca. 0.1 g) were suspended in the same buffer, and then disrupted with glass beads (φ0.5 mm) at 2700 rpm for 6 min using a Multi-beads Shocker. The supernatant was obtained after centrifugation (20,000×*g*, 15 min, 4 °C) and used to assay the CRD activity and the reaction products from TFA.

Other analytical methods

The molecular mass of native enzymes was calculated by gel filtration on a Superdex 200 10/300GL column using the following marker proteins: glutamate dehydrogenase (290 kDa), lactate dehydrogenase (140 kDa), enolase (67 kDa), myokinase (32 kDa), and cytochrome c (12.4 kDa). The molecular mass of denatured enzymes was calculated using SDS-PAGE, with the markers myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa). SDS-PAGE was carried out according to the method of Laemmli (Laemmli 1970).

Protein concentration was measured by the method of Bradford (Bradford 1976) using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and bovine serum albumin as a standard protein.

The carbohydrate content of the enzyme was assayed with phenol/sulfuric acid, according to the method of Hodge and Hofreiter (Hodge 1962). The reaction mixture, containing 0.5 mL of the purified enzyme or mannose, 0.5 mL of 5% phenol, and 2.5 mL of concentrated sulfuric acid, was allowed to stand at room temperature for 20 min. The absorbance value was then measured at 490 nm.

The metal species and their content in the enzymes were analyzed by ICP-MS 7700× (Agilent Technologies, Inc., CA, USA) using the ICP multi-element standard solution I (Merck Millipore, MA, USA).

The isoelectric point (pI) was determined using an isoelectric focusing apparatus (Nippon Eido, Tokyo, Japan) in 1% Pharmalyte (GE Healthcare UK Ltd., Buckinghamshire, UK), pH 3.0–10.0, with a sucrose gradient at 400 V for 2 days at 4 °C. The pI was also determined using a Novex pH 3–10 IEF Gel (Thermo Fisher Scientific, MA, USA), according to the manufacturer's instructions.

Accession numbers

The amino acid sequences of *Op*CRD-A and *Op*CRD-B from *O. parapolymorpha* NBRC 0799 are deposited at DDBJ as LC275242 and LC275243, respectively.

Results

Enzyme production

O. polymorpha NBRC 0799 was incubated in a 2-L shaker flask containing 1 L of YM medium at 30 °C for 48 h, and CRD activity was assayed every 6 h. The strain grew well in the YM medium, and enzyme production reached a maximum at 24 h of cultivation, which did not decrease until 48 h

(Fig. 2). We therefore incubated the strain with YM medium at 30 °C for 24 h to obtain sufficient enzyme for purification.

Purification and molecular mass

Before purification of the enzymes catalyzing the reduction of TFA, we discovered that *O. polymorpha* NBRC 0799 produced two CDRs, *Op*CRD-A and *Op*CRD-B, by isoelectric focusing analysis (Fig. S2a). Both enzymes were efficiently separated by Phenyl-Toyopearl column chromatography and finally purified to a homogeneous state with a specific activity of 1.36 U/mg of protein and 2.57 U/mg of protein, respectively (Table S1, Fig. S3). The purified enzyme solution of *Op*CRD-A exhibited no absorption maxima in the visible region, but *Op*CRD-B exhibited absorption maxima at 275 nm and around 650 nm (data not shown).

The molecular mass of the native form of *Op*CRD-A and *Op*CRD-B was estimated to be 115 and 69 kDa on a Superdex 200 10/300GL column and that of the denatured form was 40 and 43 kDa by SDS-PAGE, respectively (Fig. S3). As mentioned in the section on amino acid sequences, molecular masses of 36.6 kDa (*Op*CRD-A) and 39.3 kDa (*Op*CRD-B) were also estimated from the deduced amino acid sequences. These results indicated that *Op*CRD-A and *Op*CRD-B might be composed of three and two identical subunits, respectively.

Substrate specificity and kinetic values

Substrate specificity in the reduction reaction of both enzymes was assayed under standard assay conditions at each optimal pH (*Op*CRD-A, pH 6.5; *Op*CRD-B, pH 6.0, as shown below) using 50 mM ketones or aldehydes. Both enzymes exhibited reductase activity toward all substrates tested, and the specific activity of *Op*CRD-B was much higher than that of *Op*CRD-A for most of the substrates. In addition, both enzymes exhibited remarkably different relative activity toward ketones and aldehydes.

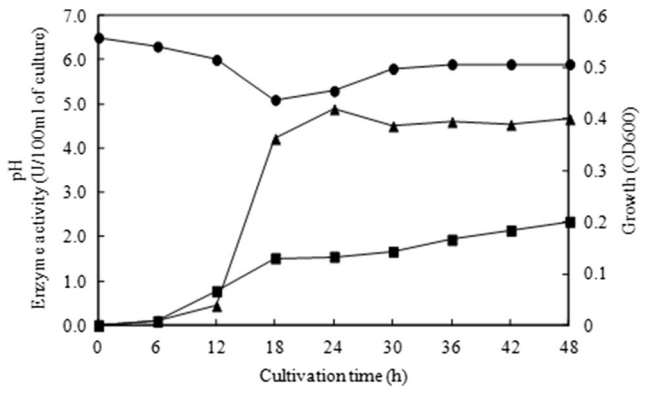

Fig. 2 Production of CRD from *O. polymorpha* NBRC 0799. The CRD activity toward TFA was assayed every 6 h using cell-free extracts. Closed triangles, closed quadrangles, and closed circles indicate CRD activity toward TFA, cell growth, and the pH of the culture broth, respectively

For example, enzyme activity of *Op*CRD-A toward fluoroacetone, formaldehyde, and acetoaldehyde was more than 10 times higher than that toward TFA, and that toward 4,4,4-trifluoro-2-butanone was 30% of TFA. On the other hand, *Op*CRD-B exhibited more than 20 times higher activity toward acetone, 2-butanone, fluoroacetone, 4,4,4-trifluoro-2-butanone, acetoaldehyde, propionaldehyde, and butyraldehyde than that toward TFA (Table 1a).

The substrate specificity in the dehydrogenation reaction was assayed under standard assay conditions at the optimal pH of both enzymes (*Op*CRD-A, pH 6.5 and 9.0; *Op*CRD-B, pH 6.0 and 10.0), using 50 mM alcohols and 1.0 mM

Table 1 Substrate specificity of CRDs from *O. polymorpha* NBRC 0799

Substrate	*Op*CRD-A		*Op*CRD-B	
	Specific activity (U/mg)		Specific activity (U/mg)	
(a) Reduction				
Acetone	4.1		51.4	
2-Butanone	2.0		67.8	
Fluoroacetone	21.2		109	
1,1-Difluoroacetone	4.5		29.8	
1,3 -Difluoroacetone	0.9		26.7	
TFA	1.6		2.57	
4,4,4-Trifluoro-2-butanone	0.5		117	
Formaldehyde	67.7		8.2	
Acetoaldehyde	33.0		170	
Propionaldehyde	10.1		72.5	
Butyraldehyde	6.0		99.7	
(b) Dehydrogenation				
	pH 6.5	pH 9.0	pH 6.0	pH 10.0
2-Propanol	1.15	8.85	10.8	29.5
2-Butanol	0.05	0.44	12.0	30.0
Methanol	0	0.15	0	0.1
Ethanol	0.01	0.37	0.4	2.6
1-Propanol	0.01	0.42	0.7	5.0
1-Butanol	0	0.40	0.2	1.2
(*S*)-TFP	0	0	0	0
(*R*)-TFP	0	0	0	0
2-Methyl-2-propanol	0	0	0.1	0.2
2-Methyl-2-butanol	0	0	0.6	3.7
1,2-Propanediol	0	0.01	1.3	4.7
1,3-Propanediol	0	0.05	0	0
Glycerol	0	0	0	0

(a) Reductase activity was assayed under standard assay conditions using 50 mM ketones or aldehydes and 0.1 mM NADH at pH 6.5 (CRD-A) or pH 6.0 (CRD-B). (b) Dehydrogenase activity was assayed under standard assay conditions using 50 mM alcohols and 1 mM NAD^+ at pH 6.5 and 9.0 (CRD-A) or 6.0 and 10.0 (CRD-B). The values are shown as the average of three-time assay

NAD^+. Both the enzymes exhibited high activity at pH 9.0 or 10.0, and secondary alcohols, such as 2-propanol and 2-butanol, were good substrates, while the reaction rates of both enzymes were different from each other. *Op*CRD-A exhibited higher activity toward 2-propanol than 2-butanol, whereas *Op*CRD-B exhibited similar activity toward 2-propanol and 2-butanol. Short-chain aliphatic primary alcohols were also dehydrogenated by both enzymes, but their reaction rates were much slower than for 2-propanol. (*S*)-TFP and (*R*)-TFP were not substrates for either enzyme (Table 1b). When reaction velocity for the reduction of ketones and aldehydes was compared with that for dehydrogenation of corresponding alcohols, the former reaction was much faster than the latter reaction at pH 6.0 and 6.5 (Table S2).

The apparent K_m values of *Op*CRD-A for TFA, acetone, and 2-propanol were estimated to be 35.5, 256, and 338 mM, respectively. Those of *Op*CRD-B for these substrates were estimated to be 67.8, 0.13, and 0.56 mM, respectively (Table 2). Thus, *Op*CRD-A exhibited high affinity to TFA compared with acetone, whereas *Op*CRD-B exhibited high affinity to acetone compared with TFA. The results indicate that *O. polymorpha* NBRC 0799 can properly utilize *Op*CRD-A and *Op*CRD-B in the cells, when the different concentration of acetone or 2-propanol is contained in a medium; *Op*CRD-B reacts with a low concentration of acetone or 2-propanol, and NAD^+ or NADH is efficiently formed, respectively. On the other hand, *Op*CRD-A reacts with a high concentration of acetone or 2-propanol.

Table 2 The K_m values of CRDs from *O. polymorpha* NBRC 0799

Substrate cofactor	K_m (mM)	
	*Op*CRD-A	*Op*CRD-B
TFA	35.5	67.8
Acetone	256	0.13
2-Propanol	338	0.56
NADH	0.012	0.011
NAD^+	0.176	0.22

The K_m values for TFA and acetone were obtained by assay of the enzyme activity using 2.5–100 mM TFA or 25–400 mM acetone and 0.1 mM NADH at pH 6.5 (*Op*CRD-A), and 5.0–200 mM TFA or 0.015–2.5 mM acetone and 0.1 mM NADH at pH 6.0 (*Op*CRD-B). Those for 2-propanol were obtained by assay of the enzyme activity using 25–600 mM 2-propanol and 1.0 mM NAD^+ at pH 9.0 (*Op*CRD-A) and using 0.05–12.5 mM 2-propanol and 1.0 mM NAD^+ at pH 10.0 (*Op*CRD-B). The K_m values for NADH was obtained by assay the enzyme activity using 300 mM acetone and 0.0075–0.25 mM NADH at pH 6.5 (*Op*CRD-A) and using 0.5 mM acetone and 0.001–0.05 mM NADH at pH 6.0 (*Op*CRD-B). Those for NAD^+ were obtained by assay the enzyme activity using 200 mM 2-propanol and 0.01–1.0 mM NAD^+ at pH 9.0 (*Op*CRD-A) and using 5.0 mM 2-propanol and 0.025–2.0 mM NAD^+ at pH 10.0 (*Op*CRD-B). Those values are shown as the average of three-time assay

Stereospecificity

TFA was incubated with *Op*CRD-A or *Op*CRD-B, and the reaction products were analyzed by GC. *Op*CRD-A enantioselectively converted TFA into (*S*)-TFP with 99% ee. On the other hand, *Op*CRD-B converted TFA into (*R*)-TFP with 40% ee (Fig. S1). In addition, (*R*)- and (*S*)-TFP were not dehydrogenated by either enzyme as mentioned above. These results indicated that *Op*CRD-A can enantioselectively produce (*S*)-alcohols with high yields. Therefore, we concluded that *Op*CRD-A might contribute to the production of (*S*)-TFP in *O. polymorpha* NBRC 0799 cells (Fig. 1).

Cofactor specificity

Cofactor specificity of both enzymes was analyzed under standard assay conditions using 50 mM TFA and 0.1 mM NADH or NADPH. *Op*CRD-A exhibited an enzyme activity of 1.02 U/mL by incubation with NADH, but no activity was detected with NADPH. In the case of *Op*CRD-B, enzyme activities of 4.62 and 0.06 U/mL were obtained with NADH or NADPH, respectively (data not shown). Thus, it was revealed that both the enzymes were NADH-dependent.

The K_m values for NADH and NAD^+ were analyzed using acetone and 2-propanol, respectively, because both enzymes did not react with (*R*/*S*)-TFP. Both enzymes showed much lower apparent K_m values for NADH than for NAD^+ (Table 2), indicating that they have high affinity for NADH, and the reduction of ketones or aldehydes proceeds well compared with the dehydrogenation of alcohols under a low concentration of cofactor.

Effects of pH and temperature on enzyme activity and stability

The optimal pH of TFA reduction was assayed under standard assay conditions, except that the reaction pH was varied between 5.0 and 11.0. *Op*CRD-A exhibited reductase activity over a wide pH range, with highest activity at pH 6.0–6.5. On the other hand, *Op*CRD-B exhibited reductase activity at acidic but not alkaline pH, and the highest activity was obtained at pH 6.0. (Fig. 3a).

Since neither enzymes exhibited dehydrogenase activity toward (*S*)-TFP and (*R*)-TFP, the optimal pH of the dehydrogenation reaction was analyzed using 2-propanol and NAD^+. Both enzymes exhibited dehydrogenase activity over a wide pH range. *Op*CRD-A exhibited the highest activity at pH 9.0, and 85% activity at pH 10.5. On the other hand, *Op*CRD-B exhibited the highest activity at pH 10.0, which was drastically decreased at pH 11.0 (Fig. 3b).

The pH stability of both enzymes was analyzed by incubating them at 30 °C for 60 min in a pH ranging from 5.0 to 11.5, without substrate. *Op*CRD-A was stable in the pH range

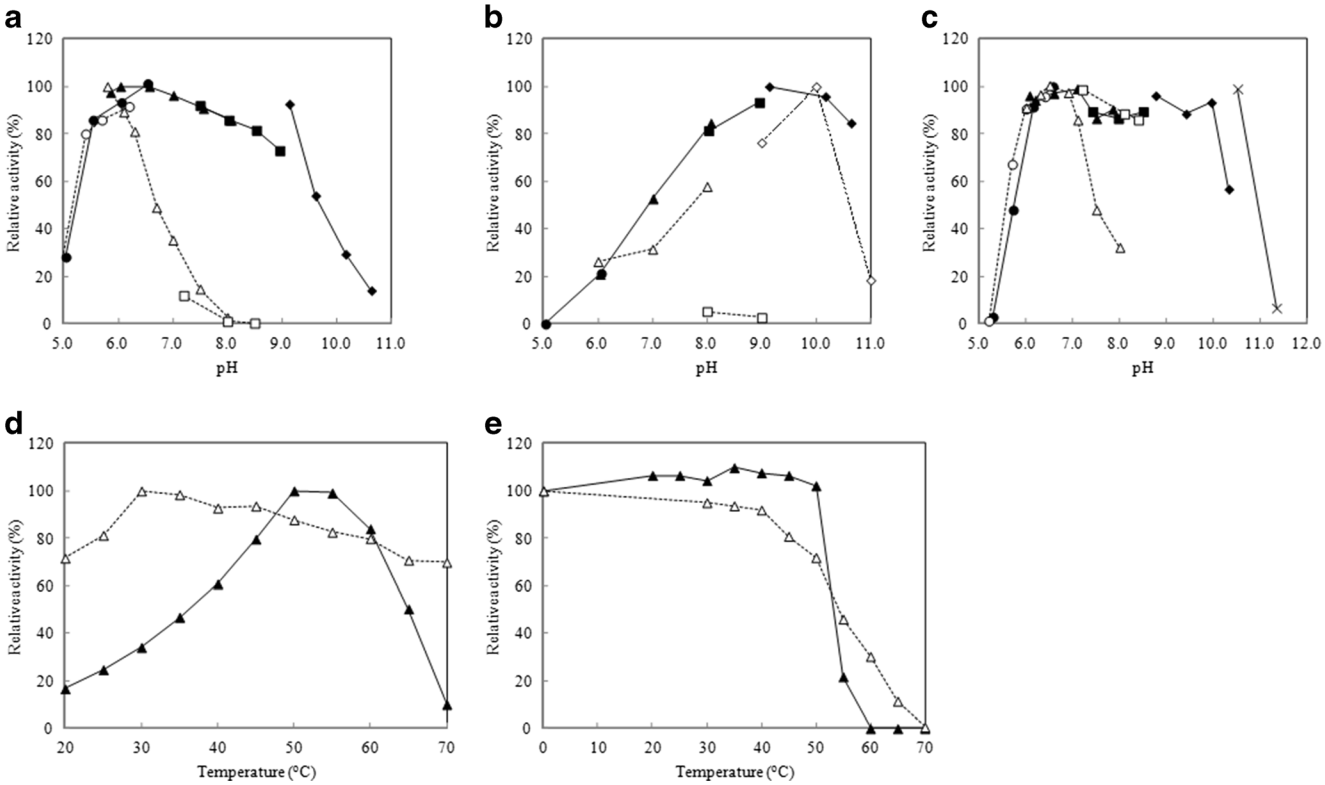

Fig. 3 Effects of pH and temperature on enzyme activity and stability. **a** Optimal pH of the reductase reaction: enzyme activity was assayed under standard assay conditions using 170 mM citric acid-Na_2HPO_4 (pH 5.0–6.5), KPB (pH 5.8–8.0), Tris-HCl (pH 7.2–8.5), glycine-NaOH (pH 9.0–10.5), and NaH_2PO_4-NaOH (pH 11.0–11.9). **b** Optimal pH of the dehydrogenase reaction: the enzyme activity was assayed under standard assay conditions using 2-propanol and the same buffers as (**a**). **c** pH stability: the enzyme was incubated at 30 °C for 60 min with 40 mM buffers as described above (**a**), and the remaining activities were assayed under the standard assay conditions for CRD activity measurement. **d** Optimum temperature: CRD activity was assayed under standard assay conditions, except that the reaction temperature was varied between 20 and 70 °C. **e** Thermostability: enzyme activity was assayed after incubation at 30–70 °C for 60 min at pH 6.5. The remaining activity was calculated by the ratio to the enzyme activity without heating. In **a**–**d**, solid lines are *Op*CRD-A and dotted lines are *Op*CRD-B

from 6.5 to 10.5. *Op*CRD-B was stable in the pH range from 6.0 to 8.4 (Fig. 3c).

The effect of temperature on TFA reduction was assayed under standard assay conditions at the optimal pH of both enzymes, except that the reaction temperature was varied between 20 and 70 °C. *Op*CRD-A exhibited the highest activity at 50–55 °C, and enzyme activity at 20 and 70 °C was less than 20 and 10% of the highest activity, respectively. On the other hand, *Op*CRD-B exhibited the highest activity at 30 °C and more than 70% activity was obtained at 20–70 °C (Fig. 3d).

Thermostability was analyzed after the enzymes were incubated at 20–70 °C for 60 min at pH 6.5, without substrate. The enzyme activity of *Op*CRD-A remained intact even after incubation at 50 °C, but dramatically decreased at 55 °C. For *Op*CRD-B, more than 70% of the activity remained after incubation at 50 °C and 30% at 60 °C (Fig. 3e).

Effects of compounds on enzyme activity

Effects of compounds on the CRD activity were analyzed by adding 1 mM carbonyl reagents, chelating reagents, sulfhydryl reagents, or metals. The enzyme activity of *Op*CRD-A was strongly inhibited by Cu^{2+}, but not by other metals, carbonyl reagents, chelating reagents, sulfhydryl reagents, or chelating reagents. On the other hand, the enzyme activity of *Op*CRD-B was strongly inhibited by chelating reagents, such as *o*-phenanthroline, α,α-dipyridyl and 8-hydroxyquinoline, and Cu^{2+} and Fe^{3+}. Both enzymes were slightly activated by Mg^{2+} (Table 3).

Metal content and isoelectric point

*Op*CRD-B contained 2 mol of zinc per mole of subunit, but *Op*CRD-A did not (data not shown). This result is in accordance with the inhibitor specificity, whereby the enzyme activity of *Op*CRD-B was inhibited by chelating reagents, but that of *Op*CRD-A was not.

*Op*CRD-A and *Op*CRD-B showed a pI of 6.7 and 5.6, respectively, using a Novex pH 3–10 IEF Gel (Fig. S2b). These results are similar to the values obtained by an isoelectric focusing apparatus using Pharmalyte, pH 3.0–10.0, as shown in Fig. S2a.

Table 3 Effects of chemicals and metals on the CRD activity

Chemical	Relative activity (%)	
	*Op*CRD-A	*Op*CRD-B
None	100	100
Phenylhydrazine	92	93
Hydrazine	92	77
Hydroxylamine	84	94
Semicarbazide	90	42
EDTA	88	86
o-Phenanthroline	74	16
α,α'-Dipyridyl	77	37
8-Hydroxyquinoline	70	15
Sodium azide	85	76
Potassium cyanide	90	95
N-Ethylmaleimide	88	75
Iodoacetic acid	77	80
$MgCl_2$	111	115
$MnCl_2$	108	74
$CoCl_2$	101	64
$NiCl_2$	103	67
$ZnCl_2$	96	89
$CuCl_2$	0	0
$FeCl_3$	96	29

Enzyme activity was assayed under standard assay conditions using 0.27 U of *Op*CRD-A and 0.62 U of *Op*CRD-B by the addition of 1 mM carbonyl reagents, chelating reagents, sulfhydryl reagents, or metals. Relative activity was calculated as percentage to the enzyme activity without chemicals

Carbohydrate analysis

The carbohydrates were not detected in *Op*CRD-A, but 20 mol of mannose were estimated in 1 mol of *Op*CRD-B by the phenol-sulfuric acid method (data not shown), although carbohydrate-binding motifs were present in the deduced amino acid sequence of both CRDs as described below.

Amino acid sequence

The N-terminal amino acid sequences of *Op*CRD-A and *Op*CRD-B were found to be PSIPKTQKAIVFET and MKALCYLGQNKGIRW, respectively, in which Cys was identified using the nanoACQUITY UPLC-SYNAPT system. The internal amino acid sequences of six trypsin-digested peptides from *Op*CRD-A and five peptides from *Op*CRD-B were determined by mass spectrometry (Table S3). Homologous sequence analysis by BLAST, using above sequences, showed that *Op*CRD-A is homologous to ADH 2 (Genbank accession No. ESW99796.1) and that *Op*CRD-B is homologous to a putative threonine dehydrogenase/Zn-dependent dehydrogenase-like protein (Genbank accession No. ESW96437.1) from *O. parapolymorpha* DL-1. Cloning of *Op*CRD-A and *Op*CRD-B genes was performed as described in the "Materials and methods" section, and the whole deduced amino acid sequence of both enzymes was determined as shown in Fig. 4 (sequence data of *Op*CRD-A and *Op*CRD-B were submitted to the DDBJ databases under the accession numbers LC275242 and LC275243, respectively).

The deduced amino acid sequence of *Op*CRD-A was only 22% identical to that of *Op*CRD-B (DNA sequence similarity was 46%). The deduced amino acid sequences of *Op*CRD-A and *Op*CRD-B were approximately 99% identical to those of ADH 2 and a putative threonine dehydrogenase/Zn-dependent dehydrogenase-like protein from *O. parapolymorpha* DL-1, respectively (DNA sequence similarity was 96 and 95%, respectively). Recently, completely identical DNA sequences of *Op*CRD-A and *Op*CRD-B were reported in the genome sequence of *O. parapolymorpha* NCYC 495 leu1.1 (Riley et al.

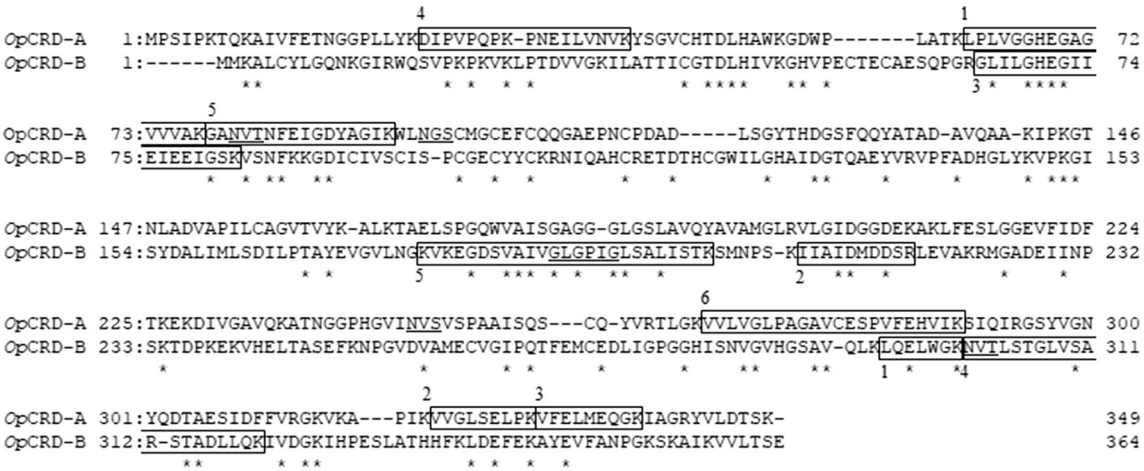

Fig. 4 Deduced amino acid sequence of *Op*CRD-A and *Op*CRD-B. Identical amino acid residues are indicated by asterisks. The sequences of peptides obtained by trypsin digestion are framed in boxes. Motifs for carbohydrate binding at asparagine residues (N-X-T/S) are underlined. The NAD^+-binding motif (**G**-X-**G**-X-X-**G**) is double lined

2016). The alcohol dehydrogenase of that strain (Genbank accession No. OBA15472.1) was identical to *Op*CRD-A, and a hypothetical protein, OGAPODRAFT_22591 (Genbank accession No. OBA18267), was identical to *Op*CRD-B (Fig. S4).

In the sequence of *Op*CRD-A, the typical NAD^+-binding motif (**G**-X-**G**-X-X-**G**) was not recognized, but the motif was present in the *Op*CRD-B sequence at position 188–193 (G-L-G-P-I-G). Motifs for carbohydrate binding at an asparagine residue (N-X-T or S) were confirmed at positions 80–82 (N-V-T), 96–98 (N-G-S), and 246–249 (N-V-S) in *Op*CRD-A and at position 301–303 (N-V-T) in *Op*CRD-B, indicating that carbohydrates can bind at these positions in both CRDs.

Overexpression of *Op*CRD-A and *Op*CRD-B genes

Each gene of *Op*CRD-A and *Op*CRD-B was cloned into the pET11a vector and transformed into *E. coli* BL21 (DE3) cells. Both transformants grew well in LB medium at 20 and 30 °C and produced the CRD in a soluble fraction. However, the transformants showed different optimal cultivation temperatures for enzyme production. The transformant harboring the p*Op*CRD-A gene produced 0.22 U/mL of broth (specific activity of the cell-free extract was 0.40 U/mg of protein) at 30 °C, and 0.12 U/mL of broth (specific activity of the cell-free extract was 0.18 U/mg of protein) at 20 °C. On the other hand, the transformant harboring the p*Op*CRD-B gene showed high enzyme productivity at 20 °C, compared with 30 °C. The transformant produced 0.26 U/mL of broth (specific activity of the cell-free extract was 0.48 U/mg of protein) at 20 °C and 0.02 U/mL of broth (specific activity of the cell-free extract was 0.05 U/mg of protein) at 30 °C. Thus, it was revealed that recombinant *Op*CRD-A was efficiently produced by incubation at 30 °C, and *Op*CRD-B, at 20 °C.

Production of 1,1,1-trifluoro-2-propanol

The conversion of TFA to TFP was performed using a crude recombinant enzyme solution, in which recombinant *Op*CRD-A and *Op*CRD-B were prepared from the cells cultivated at 30 and 20 °C, respectively. When 50 mU of recombinant *Op*CRD-A was incubated with 56 mM TFA under the same conditions as described in the section of "Identification of reaction products" in "Materials and methods," 39.1 mM (*S*)-TFP was produced with 99% ee. In the case of recombinant *Op*CRD-B, 16 mM (*R*)-TFP was produced with 40% ee. These results agree with the results of the purified *Op*CRD-A and *Op*CRD-B enzymes (shown in Fig. S1).

Discussion

(*S*)-TFP is one of the most useful optically active alcohols, and biochemical methods for production of (*S*)-TFP have been developed by enantioselective reduction of TFA using baker's yeast (Bucciarelli et al. 1983; Doswald et al. 2007) or a crude enzyme solution of ADH from *R. erythropolis*, *A.paraffineus*, or *P. farinose* (Hayashi et al. 2007). These methods were useful for enantioselective production of (*S*)-TFP, but not sufficient for production of enantiomerically pure (*S*)-TFP (> 99% ee). Therefore, they were utilized under the limited condition to obtain the pure (*S*)-TFP (Bucciarelli et al. 1983). When the optical purity of (*S*)-TFP in the reaction mixture is low, some chemical methods such as a simulated moving bed chromatography are also performed to obtain the pure (*S*)-TFP. Thus, the enzyme with higher enantioselectivity is important to obtain enantiomerically pure (*S*)-TFP. Recently, Asano et al. reported that *O. polymorpha* NBRC 0799 is useful for the production of enantiomerically pure (*S*)-TFP from TFA, with high yields (Asano et al. 2011). However, the enzyme responsible for this reaction had not yet been identified. Therefore, we have now purified the enzymes catalyzing this reaction in this strain and elucidated their characteristics.

The strain produced two enzymes catalyzing the reduction of TFA, *Op*CRD-A with pI 6.7 and *Op*CRD-B with pI 5.6, by isoelectric focusing analysis. Since the pI values were rather high, separation of the enzymes was difficult by ion exchange chromatography, but they were efficiently separated by a Phenyl-Toyopearl column chromatography.

Both *Op*CRD-A and *Op*CRD-B exhibited reductase activity toward not only TFA but also other fluoroacetone derivatives, acetone, 2-butanone, and short-chain primary aldehydes (Table 1a). In addition, high CRD activity was obtained by incubation with NADH, but no activity with NADPH. Both enzymes also exhibited dehydrogenase activity toward certain alcohols, although these were much lower than toward the corresponding ketones and aldehydes at pH 6.0 or 6.5 (Table 1, Table S2). Thus, *Op*CRD-A and *Op*CRD-B are classified into the same group of NADH-dependent CRDs.

While both CRDs are classified into the same enzyme group and exhibit broad substrate specificity, their enzymatic properties are significantly different from each other. *Op*CRD-A exhibited high CRD activity and stability over a wide pH range compared with *Op*CRD-B (Figs. 3a, c). The optimal temperature, thermostability, and inhibitor specificity were also remarkably different from each other (Figs. 3d, e; Table 3). *Op*CRD-A did not contain zinc, but *Op*CRD-B contained 4 mol of zinc per mole of enzyme. Furthermore, the amino acid sequence of *Op*CRD-A was only 21% identical to that of *Op*CRD-B. The amino acid sequences of *Op*CRD-A and *Op*CRD-B were approximately 99% identical to those of ADH 2 and a putative threonine dehydrogenase/Zn-dependent dehydrogenase-like protein from *O. parapolymorpha* DL-1, respectively. We confirmed that *Op*CRD-B did not exhibit threonine dehydrogenase activity. Thus, we demonstrated that *O. polymorpha* NBRC 0799 produces two CRDs. Recently, completely identical DNA sequences of *Op*CRD-A and

*Op*CRD-B were reported by genome sequencing of *O. parapolymorpha* NCYC 495 leu1.1 (Riley et al. 2016). These results indicate that strains belonging to *O. polymorpha* can produce different types of ADHs or CRDs.

The two CRDs from *O. polymorpha* NBRC 0799 catalyze the reduction of ketones and dehydrogenation of alcohols, and the former reaction is much faster than the latter reaction at acidic pH. However, stereoselectivity of both enzymes was different. *Op*CRD-A can produce (*S*)-TFP from TFA with high enantioselectivity, whereas *Op*CRD-B exhibits low enantioselectivity in the reduction of TFA. Therefore, we concluded that *Op*CRD-A greatly contributes to the production of (*S*)-TFP from TFA in *O. polymorpha* NBRC 0799 cells. Rosen et al. isolated two ADHs, ADH RS1 and ADH RS2, from *Rhodococcus* sp. and applied them to produce chiral alcohols. The ADH RS1 exhibited reductase activity toward TFA and enantioselectively produced (*S*)-TFP. However, the activity toward TFA was much lower than that toward acetone, and production of (*S*)-TFP was 93% ee (Rosen et al. 2006). On the other hand, our *Op*CRD-A exhibited similar activity toward TFA and acetone and efficiently produced (*S*)-TFP with 99% ee by *E. coli*. Thus, *Op*CRD-A from *O. polymorpha* NBRC 0799 is useful for the efficient production of enantiomerically pure (*S*)-TFP from TFA.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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