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Purification and characterization of carbonyl reductase from *Candida krusei* SW 2026 involved in enantioselective reduction of ethyl 2-oxo-4-phenylbutyrate

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ABSTRACT

Optically active ethyl (*R*)-2-hydroxy-4-phenylbutyrate [(*R*)-HPBE] is widely used as a key chiral building block in the synthesis of a class of angiotensin-converting enzyme (ACE) inhibitors. A highly enantioselective carbonyl reductase responsible for the reduction of ethyl 2-oxo-4-phenylbutyrate (OPBE) was identified and characterized from *Candida krusei* SW 2026. The enzyme was purified to homogeneity through three chromatography columns. The relative molecular mass of the enzyme was estimated to be around 45,500 by gel filtration and 46,000 by SDS-polyacrylamide gel electrophoresis. The enzyme yielded (*R*)-enantiomer product and utilized NADPH as the cofactor. The purified enzyme exhibited maximum activity at pH 6.0 and 30 °C, and retained over 80% of its activity over an acidic pH range of 4.5–7.0. The maximum reaction rate (V_{max}) and apparent Michaelis–Menten constant (K_m) for OPBE and NADPH were 18.7 μmol/(min mg) protein and 0.319 mmol, 14.9 μmol/(min mg) protein and 0.306 mmol, respectively.

1. Introduction

Angiotensin-converting enzyme (ACE) inhibitors such as enalapril and lisinopril are useful for the treatment of hypertension because ACE (peptidyl-dipeptidase A, EC 3.4.15.1) catalyzes both the production of vasoconstrictor angiotensin and inactivation of vasodilator bradykinin [1,2].

Ethyl (*R*)-2-hydroxy-4-phenylbutanoate [(*R*)-HPBE] is an important intermediate for the synthesis of ACE inhibitors. Iwasaki et al. reported an efficient and practical method for the preparation of enalapril and lisinopril from (*R*)-HPBE [3]. Various approaches for the preparation of (*R*)-HPBE have been reported, such as chemical resolution of racemic HPBE [4,5], chemical synthesis of (*R*)-HPBE [6,7], microbial or enzymatic resolution of racemic HPBE [8–10]. However, the applications of chemical/microbial/enzymatic resolution methods are limited by theoretical maximum yield of only 50%, and chemical synthesis methods always require stringent reaction conditions.

Recently, enzymatic or microbial reduction has been used for the preparation of (*R*)-HPBE [11–14], and also an attractive methodology for the synthesis of a variety of optically active alcohols. Carbonyl reductases belong to the class of oxidoreductase enzymes which are generally cytosolic enzymes, of low molecular weight, capable of catalyzing the NAD(P)H-dependent reduction of various natural and unnatural carbonyl compounds to corresponding alcohols [15,16]. More than 200 organisms have been reported for the production of enantiopure alcohols by asymmetric reduction of carbonyl group [17]. These chiral alcohols are key intermediates of high value-added chiral synthons for the production of pharmaceuticals, agrochemicals, inhibitors, and pheromones [18–22]. There are a few reports on the carbonyl reductases capable of producing (*R*)-HPBE, the applicability is however limited by their low enantioselectivity or stability. Kaluzna et al. purified two putative dehydrogenases Ypr1p and Gre2p from *Saccharomyces cerevisiae*, which catalyzed the reduction of ethyl 2-oxo-4-phenylbutyrate (OPBE) to (*R*)-HPBE and (*S*)-HPBE with 97% and 90% *e.e.*, respectively [12]. Bai and Yang constructed the polyethyleneimine–enzyme–cell aggregates to improve the stability of the carbonyl reductase [11]. Previously, a highly potent carbonyl reductase producing yeast strain *Candida krusei* SW 2026 was isolated in our laboratory, which catalyzes the enantioselective reduction of OPBE to optical pure (*R*)-HPBE and has good stability over a wide pH range. In this work, we describe the purification and biochemical characterization of the carbonyl reductase from *C. krusei* SW 2026.

2. Materials and methods

2.1. Chemicals

OPBE (purity 99%) and (*R*)-HPBE (purity 99% and over 97% *e.e.*) were purchased from Sigma Chemical Company (St. Louis, USA).

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Other ketones and aldehydes were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). NADPH disodium salt, dithiothreitol (DTT), β-mercaptoethanol, Bradford reagent, and different inhibitors were purchased from Jingchun Chemical Company and Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Racemic HPBE was prepared by using $NaBH_4$ as the reducer. All other reagents and chemicals were of analytical grade and obtained from local companies. Matrices for protein purification were purchased from GE Healthcare Life Science (Piscataway, NJ, USA).

2.2. Microorganism and growth conditions

The organism used in this study was isolated in our laboratory and identified as *C. krusei* SW 2026 [23]. The organism was grown aerobically at 28 °C for 12 h in fermentation broth (pH 6.0) comprising of (w/v) 4.5% glucose, 3% peptone, 1.5% beef extract, and 0.05% Mn^{2+}. The culture (10% inoculum, v/v) was then transferred to the same medium for further growth at 28 °C (200 rpm). Growth of microorganism was estimated by measuring optical density at 660 nm using a UV–vis spectrophotometer (Spectrum 752, Shanghai Spectrum Instruments Co., Ltd., China) with fresh medium as blank. After specified time period (48 h), the cells were harvested by centrifugation (10,800 × *g*, 10 min, 4 °C), and then were washed with saline (0.85%) and suspended in phosphate buffer (20 mM, pH 7.0) containing 1 mM DTT (buffer A).

2.3. Activity assay

Carbonyl reductase activity for OPBE was assayed spectrophotometrically. The standard assay mixture, comprising 100 mM potassium phosphate buffer, 1 mM OPBE, 1 mM NADPH, and the enzyme solution in a total volume of 250 μl, was monitored for decrease in absorbance at 340 nm under 30 °C. One unit of the enzyme activity was defined as the amount of enzyme that catalyzing the oxidation of 1 μmol of NADPH per minute. Specific activity was expressed as units per milligram of protein. Protein content of different samples was estimated by Bradford protein–dye binding method [24] using bovine serum albumin as standard and the absorbance was monitored at 595 nm in a UV–vis spectrophotometer.

2.4. Purification procedure

Protein purification was performed using a fast performance liquid chromatography (FPLC) system (AKTApurifier, GE Healthcare Life Science, Piscataway, NJ, USA). All purification steps were carried out at 0–4 °C in buffer A.

Step 1. Preparation of cell-free extract. Washed cells (53 g) from a 1000 ml culture broth was suspended in 160 ml buffer A. Cells were disrupted by ultrasonication (285 W, pulse 2 s, pause 4 s) for 15 min. Cell debris was removed by centrifugation at 12,000 × *g* for 15 min at 4 °C which led to clear supernatant, designated as cell-free extract (CFE).

Step 2. Ammonium sulfate fractionation. Initially, ammonium sulfate was added to CFE to a final concentration of 40% and the mixture was stirred on ice for 10 h. The precipitate was removed by centrifugation at 17,300 × *g* for 20 min at 4 °C. Clear supernatant was subjected to increased concentration of ammonium sulfate fractionation to 90%. The solution was further stirred for 10 h on ice. The resulted precipitate was collected by centrifugation at 17,300 × *g* for 20 min and was then resuspended in buffer A and dialyzed against the same buffer at 4 °C overnight.

Step 3. Anion exchange chromatography. The dialyzed fraction was filtered through 0.45 μm cellulose membrane, and was then loaded on HiPrep DEAE FF column (1.6 cm × 10 cm, GE Healthcare Life Science, Piscataway, NJ, USA). The column was first balanced with phosphate buffer (20 mM, pH 8.0) containing 1 mM DTT until no protein was eluted. The enzyme was then eluted with a linear gradient of NaCl (from 0 to 0.7 M) in the same buffer over 7 column volume. Fractions containing carbonyl reductase activity were pooled and concentrated with Amicon Ultra-15 (10 kDa, Millipore, Bedford, MA, USA). The concentrated protein solution was dialyzed against buffer A at 4 °C overnight.

Step 4. Affinity chromatography. The dialyzed solution was then loaded on HiTrap Blue HP column (5 ml, GE Healthcare Life Science, Piscataway, NJ, USA). The column was first balanced with buffer A until the basic line was plane. The enzyme was then eluted with a linear gradient of NaCl (from 0 to 1.5 M) in buffer A over 10 column volume. Fractions containing carbonyl reductase activities were treated the same as described in step 3.

Step 5. Gel filtration. Protein solution after step 4 was loaded on Superdex^{TM} 75 column (1.0 cm × 30 cm, GE Healthcare Life Science, Piscataway, NJ, USA) which had been pre-equilibrated with buffer A. The active fraction was used as purified enzyme for characterization. The apparent molecular mass of the purified carbonyl reductase of *C. krusei* SW 2026 was then determined by the same column using phosphate buffer (50 mM, pH 7.0) containing 0.15 M NaCl as the mobile phase at 0.4 ml/min. The column was pre-calibrated with standard molecular mass markers: BSA (67.0 kDa), Ovalbumin (43.0 kDa), Ribonuclease A (13.7 kDa), Aprotinin (6.512 kDa), Vitamin B12 (1.355 kDa).

2.5. Effect of pH and temperature

For the determination of optimal pH, activities were assayed in 100 mM buffers of different pH (3.5–9.0): acetate for a pH range of 3.5–6.0, phosphate for pH 6.0–8.0, and Tris–HCl for pH 8.0–9.0. The optimal temperature was determined by incubation over the range from 20 to 70 °C. The pH stability was determined after incubating the purified enzyme in same buffers with various pH as above for 30 h at 4 °C. For thermal stability, aliquots of the purified enzyme were incubated at temperatures over the range of 10–70 °C in 100 mM phosphate buffer (pH 6.0) for 1 h followed by cooling on ice before activity assay. All the activity was measured under the standard conditions as described in Section 2.3.

2.6. Substrate specificity

Biotransformation of different ketones and aldehydes was tested with purified enzyme to assess the substrate specificity of carbonyl reductase of *C. krusei* SW 2026. The enzyme activity was determined under above standard condition.

2.7. Effect of various compounds

Influence of various compounds on the catalytic activity of purified carbonyl reductase of *C. krusei* SW 2026 was determined by pre-incubating the enzyme with different compounds in 100 mM phosphate buffer for 25 min. The enzyme activity was then determined under the standard assay condition. Relative activity was expressed as a percentage of the activity in the absence of test compounds.

2.8. Time course of enzymatic reduction of OPBE

The bioreduction of OPBE with the isolated enzyme was performed in a 1.5 ml reaction mixture containing 10 mM OPBE, 10 mM NADPH, and approximate 0.2 U of purified enzyme in phosphate buffer (100 mM, pH 6.0). The reaction was carried out at 30 °C

Table 1
Summary of purification steps of carbonyl reductase of *C. krusei* SW 2026.

Purification steps	Protein (mg)[a]	Activity (U)[a]	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	490	20.3	0.0410	100	1.00
$(NH_4)_2SO_4$ fractionation	256	15.2	0.0590	74.8	1.40
HiPrep DEAE FF	109	13.2	0.120	64.8	2.90
HiTrap Blue HP	0.330	2.71	8.28	13.3	200
Superdex^{TM} 75	0.0950	1.19	12.6	5.90	304

[a] The assay conditions are given in Section 2.3.

and 200 rpm for 6 h, and samples were taken at regular intervals to monitor the progress of the reaction. The supernatant was separated by centrifugation (12,000 × *g*, 15 min) and extraction using ethyl acetate. The solvent was dried using anhydrous magnesium sulfate, and the supernatant obtained was subjected to chiral GC analysis to determine the product yield and *e.e.* value.

2.9. Analytical methods

2.9.1. Gel electrophoresis (SDS-PAGE)

Gel electrophoresis was performed on 12% SDS-polyacrylamide gel with Tris–glycine buffer system. Protein bands were visualized by staining the gel with Coomassie blue stain. The molecular mass of the carbonyl reductase subunit was estimated from relative migration of the standard proteins (β-Galactosidase 116.0 kDa, BSA 66.2 kDa, Ovalbumin 45.0 kDa, Lactate dehydrogenase 35.0 kDa, REase Bsp981 25.0 kDa, β-Lactoglobulin 18.4 kDa, Lysozyme 14.4 kDa) on the gel.

2.9.2. Chiral GC

The enantiomeric excess of (*R*)-HPBE was determined using Varian CP 3900 gas chromatograph (USA) equipped with Chirasil-Dex CB column (CP 7502, 25 m × 0.25 mm × 0.25 μm, VARIAN, USA). Temperature program was as follows: 140 °C for 2 min, increasing to 240 °C at 10 °C/min, holding 240 °C for 2 min. Retention times for OPBE, (*R*)-HPBE, and (*S*)-HPBE were 6.804, 7.431, and 7.546 min, respectively. The *e.e.* value was defined as the ratio of $[([R]-[S])/([R]+[S])] \times 100$.

3. Results and discussion

3.1. Purification of carbonyl reductase of C. krusei SW 2026

The purification of carbonyl reductase of *C. krusei* SW 2026 was achieved by four complementary separation techniques including ammonium sulfate precipitation, ion exchange chromatography on HiPrep DEAE FF column, affinity chromatography on HiTrap Blue HP column, and finally gel filtration on Superdex^{TM} 75 column (Table 1). The cell-free extract was precipitated with ammonium sulfate fractionation where the active protein was precipitated in the range of 40–90%. This precipitate was resuspended and dialyzed against buffer A overnight, and was loaded on HiPrep DEAE FF column. Among four peaks observed, the third peak eluted at NaCl concentration of 0.19–0.37 M was found to be active. This active fraction was concentrated with Amicon Ultra-15 and dialyzed against buffer A overnight before loaded on HiTrap Blue HP column. The third peak eluted at NaCl concentration of 0.65–0.82 M, also the highest one among four main peaks, was detected to be active. The active fraction was concentrated and dialyzed as above. Finally, the sample was loaded on Superdex^{TM} 75 column where the purified protein was eluted at 10.4 ml. The total purification factor was calculated to be 304 corresponding to a yield of about 5.90%. The purified enzyme exhibited a single band on SDS-PAGE and a specific activity of 12.6 U/mg (Table 1). The specific activities of carbonyl reductases from different microorganisms are compared and listed in Table 2. The results clearly indicated that the specific activity of carbonyl reductases originated from different microorganisms lies in a broad range over 0.890–590 U/mg and preferably utilizing NADPH as cofactor. The disparity in activity is likely due to their different affinity and catalytic efficiency towards ketone substrates.

3.2. Enzymatic characterization of carbonyl reductase of C. krusei SW 2026

3.2.1. Molecular mass and subunit size

The purified carbonyl reductase of *C. krusei* SW 2026 showed a single peak in Superdex^{TM} 75 gel filtration chromatography, and the molecular mass was estimated to be 45.5 kDa in comparison with the elution volume of standard proteins (supplementary Fig. 1). The molecular mass of the subunit was estimated approximately to be 46 kDa by SDS-PAGE. The results of gel filtration chromatography together with SDS-PAGE (Fig. 1) suggested that enzyme is a monomer enzyme.

Dehydrogenases or reductases generally have low molecular weight and homomeric subunit. This result is coincident with SAKER-II and SAKER-III from *Streptomyces avermitilis* [26], and carbonyl reductase from *Candida parapsilosis* ATCC 28474 [32] which were all reported to be monomer. Some other carbonyl reductases have homodimeric or homotetrameric subunits, such as alcohol dehydrogenase from *Rhodococcus ruber* DSM 44541 [33] and carbonyl reductase from *Candida viswanathii* MTCC 5158 [34]. One unusual example of heterodimeric carbonyl reductase has been reported in *Geotrichum capitatum* JCM 3908 with two subunits of 39 and 41 kDa [35].

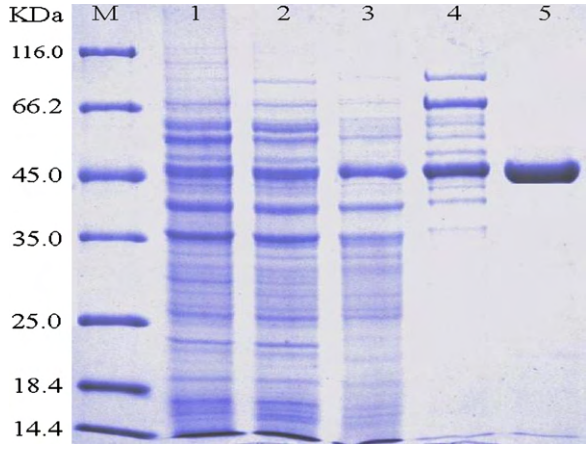

Fig. 1. SDS-polyacrylamide gel (12%) electrophoresis of purified carbonyl reductase of *C. krusei* SW 2026. Lane M: β-Galactosidase 116 kDa; BSA 66.2 kDa; Ovalbumin 45 kDa; Lactate dehydrogenase 35 kDa; REase Bsp981 25 kDa; β-Lactoglobulin 18.4 kDa; Lysozyme 14.4 kDa. Lane 1: cell-free extract. Lane 2: ammonium sulfate fraction. Lane 3: HiPrep DEAE FF fraction. Lane 4: HiTrap Blue HP fraction. Lane 5: fraction of Superdex^{TM} 75. Gel was stained with 0.1% Coomassie Blue R-250.

Table 2
Specific activity of carbonyl reductases from different microorganisms.

Microorganism	Substrate	Coenzyme	Specific activity (U/mg)	Reference
Candida krusei SW 2026	OPBE[a]	NADPH	12.6	This study
Escherichia coli K12	OPBA[b]	NADPH	18.0	[25]
Williopsis saturnus var. *mrakii* AJ-5620	HPPO[c]	NADPH	0.890	[16]
Streptomyces avermitilis – SAKER-I	α,β-Keto esters	NADPH	13.0	[26]
Streptomyces avermitilis – SAKER-II	α-Keto esters	NADPH	5.10	[26]
Streptomyces avermitilis – SAKER-III	α-Keto esters	NADPH	32.1	[26]
Sporobolomyces salmonicolor AKU 4429	COBE[d]	NADPH	10.4	[27]
Candida magnoliae KFCC 11023	Erythrose	NAD(P)H	19.6	[28]
Kluyveromyces lactis NRIC 1329	COBE[d]	NADPH	50.8	[29]
Cylindrocarpon sclerotigenum IFO 31855	COBE[d]	NADPH	125	[30]
Geotrichum candidum	1-Acetonaphthone	NADH	590	[31]

[a] OPBE means ethyl 2-oxo-4-phenylbutyrate.
[b] OPBA means 2-oxo-4-phenylbutanoic acid.
[c] HPPO means 3-hydroxy-1-phenylpropane-1-one.
[d] COBE means ethyl 4-chloroacetoacetate.

3.2.2. Effect of pH and temperature

The effect of pH on the enzyme activity was studied in different buffer systems (100 mM) in the pH range of 3.5–9.0 (Fig. 2). The maximum enzyme activity was observed in pH 6.0 phosphate buffer. The enzyme showed over 80% activity in pH ranging from 5.5 to 6.5, indicating an acidic pH optimum. In previous reports, carbonyl reductase (optimal pH of 4.5) from *Candida parapsilosis* CCTCC 203011 [36] and phenylacetaldehyde reductase (optimal pH of 6.0–6.5) from *Corynebacterium* sp. strain ST-10 [37] also showed acidic or nearly the same pH optimum, while some had alkaline pH optima such as carbonyl reductase from *Candida parapsilosis* DSM 70125 (pH 8.5) [38].

The carbonyl reductase of *C. krusei* SW 2026 showed maximum activity at 30 °C. The activity dropped rapidly when temperature was increased from 35 to 60 °C, and nearly no activity was detected at above 70 °C (Fig. 3). The optimum temperature of the purified enzyme is lower than some others, such as *Williopsis saturnus* var. *mrakii* AJ-5620 [16], *Geotrichum candidum* [31] and *C. viswanathii* MTCC 5158 [34] which fall in the range of 50–65 °C.

Biocatalysts are inherently labile and therefore their operational stability is important for any bioprocess. The pH stability was determined by incubating the purified carbonyl reductase of *C. krusei* SW 2026 in 100 mM buffer of various pH ranging from 3.5 to 9.0 at 4 °C for 30 h (Fig. 4). The enzyme showed highest stability at pH 5.5. Over 80% of activity was retained between pH 4.5 and 7.0. The stability profile of the carbonyl reductase of *C. krusei* is broader compared with other reported carbonyl reductases such as *G. candidum* [31] and *C. viswanathii* MTCC 5158 [34], which showed good stability at pH 4.4–6.4 and pH 6–7.5, respectively. Different stability region was also observed in some reductases, for example, the carbonyl reductase of *C. parapsilosis* DSM 70125 [38] showed comparatively higher stability in alkaline region while that of *Rhodococcus erythropolis* DSM 743 [39] is stable in acidic region.

The thermostability of the purified carbonyl reductase of *C. krusei* was assayed at various temperatures from 10 to 70 °C. The enzyme was extremely fragile at temperatures higher than 40 °C as demonstrated in Fig. 5. Like majority of the carbonyl reductases reported in literatures [32,40], the enzyme was quite labile under higher temperature (50–70 °C), retaining less than 40% of its activity after 1 h.

3.2.3. Substrate specificity

Carbonyl reductases belong to oxidation and reduction enzyme family, which consists of aldo–keto reductase (AKR), short-chain dehydrogenases/reductase (SDR), and medium-chain dehydrogenases/reductases (MDR) superfamily [41]. Carbonyl reductases belonging to different family usually exhibit different substrate specificity. The activity of purified carbonyl reductase of *C. krusei* SW 2026 in catalyzing the reduction of different carbonyl compounds was investigated (Table 3). The enzyme showed little or no activity towards acetophenone and its various derivatives. It

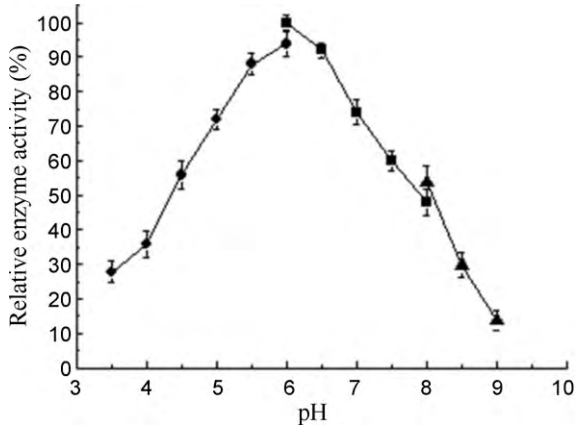

Fig. 2. Effect of pH on the activity of carbonyl reductase of *C. krusei* SW 2026. Enzyme assay was performed using standard assay procedure: 1 mM OPBE, 1 mM NADPH, and appropriate quantity of enzyme in different buffers (100 mM) from 3.5 to 9.0 in total 250 μl at 30 °C. Buffers used: (♦) 100 mM sodium acetate; (■) 100 mM potassium phosphate; (▲) 100 mM Tris–HCl. The maximum activity in tested buffers was taken as 100% and used as control, relative activity of other pHs was expressed as a percentage of control.

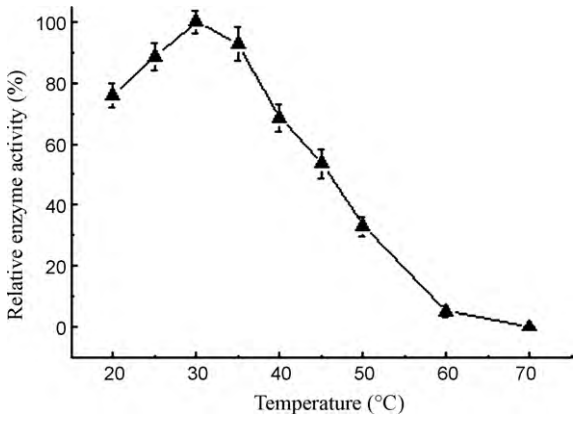

Fig. 3. Effect of temperature on the activity of carbonyl reductase of *C. krusei* SW 2026. Enzyme assay was performed using standard assay procedure: 1 mM OPBE, 1 mM NADPH, and appropriate quantity of enzyme at various temperatures from 20 to 70 °C in phosphate buffer (100 mM, pH 6.0) in total 250 μl. The maximum activity in tested temperatures was taken as 100% and used as control, relative activity of other temperatures was expressed as percentage of control.

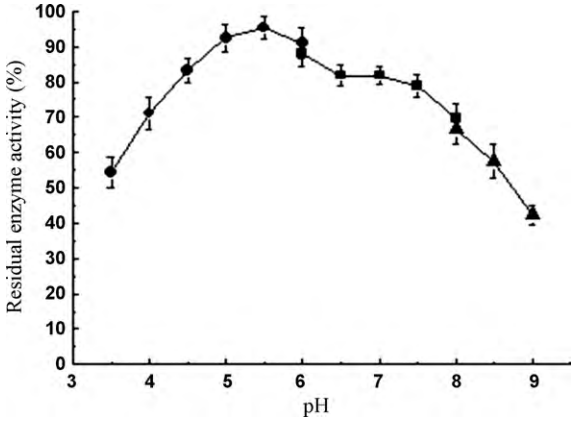

Fig. 4. Effect of pH on the stability of carbonyl reductase of *C. krusei* SW 2026. The purified protein was incubated against various buffers of different pH from 3.5 to 9.0 for 30 h at 4 °C. Buffers used: (♦) 100 mM sodium acetate; (■) 100 mM potassium phosphate; (▲) 100 mM Tris–HCl. Reaction condition: 1 mM OPBE, 1 mM NADPH, and appropriate quantity of enzyme in total 250 μl at 30 °C. The activity in pH 6.0 at 4 °C without pre-incubation was taken as 100% and used as control, and the residual activity was expressed as a percentage of control.

is noticed that, acetophenone and its derivatives with electron-donating substitute group amino- on benzene ring, specifically acetophenone, 3-aminoacetophenone, and 4-aminoacetophenone, could not be reduced by the enzyme at all, while some activity was observed in derivatives substituted with electron-withdrawing groups, for example, nitro- and halogen-. Higher activity was observed for the esters substrates such as phenylbutyric acid esters, phenylpyruvic acid esters, benzoyl formic acid esters, and acetoacetic acid esters, which have similar structure to the original substrate OPBE. The results suggested that the carbonyl reductase of *C. krusei* exhibited higher reduction activity towards carbonyl compounds with ketone ester structure.

3.2.4. Kinetic parameters

The kinetic parameters of the carbonyl reductase of *C. krusei* SW 2026 were estimated over a range of OPBE (0.05–2.25 mM) and NADPH (0.05–2.0 mM) concentration at 30 °C in phosphate buffer (100 mM, pH 6.0), keeping both NADPH and OPBE concentration in excess at 3.0 mM. The maximal reaction rate (V_{max}) and apparent Michaelis–Menten constant (K_m) of the enzyme for OPBE and NADPH calculated from Lineweaver–Burk plot were 18.7 μmol/min per milligram of protein and 0.319 mM, and 14.9 μmol/min per milligram of protein and 0.306 mM, respectively. Compared with other carbonyl reductases (Table 4), the purified carbonyl reductase of *C. krusei* exhibited lower K_m towards substrate OPBE than those from *W. mrakii* AJ-5620 [16], *Candida magnoliae* [28] and *G. candidum* [31], while higher K_m and reaction rate than those from *C. viswanathii* MTCC 5158 [34] and *G. capitatum* JCM 3908 [35]. The comparison results in Table 4 indicated that the carbonyl reductase of *C. krusei* has low K_m towards OPBE and moderate reaction rate compared with some previous reported reductases [16,28]. The K_{cat} and K_{cat}/K_m for OPBE and NADPH were calculated to be 14.2 S^{-1}, 44.5 mM^{-1} S^{-1} and 11.3 S^{-1} and 36.9 mM^{-1} S^{-1}, respectively.

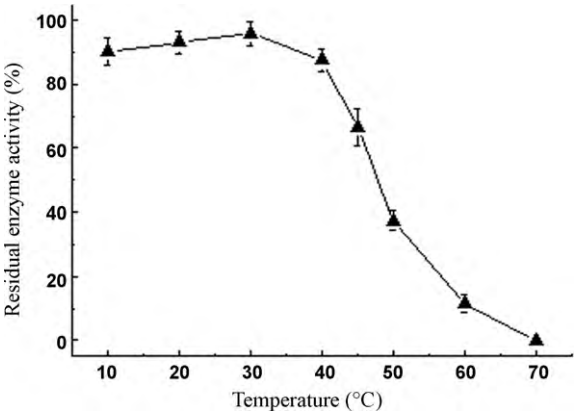

Fig. 5. Thermostability of carbonyl reductase of *C. krusei* SW 2026. The purified enzyme was incubated at different temperatures from 10 to 70 °C in phosphate buffer (100 mM, pH 6.0) for 1 h. Reaction condition: 1 mM OPBE, 1 mM NADPH, and appropriate quantity of enzyme in total 250 μl at 30 °C. The activity in pH 6.0 at 4 °C without pre-incubation was taken as 100% and used as control, and residual activity was expressed as a percentage of control.

Table 3
Reduction of various carbonyl compounds by carbonyl reductase of *C. krusei* SW 2026.

Substrate[a]	Relative activity (%)[b]
Ethyl 2-oxo-4-phenylbutyrate	100
Methyl 2-oxo-4-phenylbutyrate	88.5 ± 2.31
2-Oxo-4-phenylbutanoic acid	77.1 ± 3.02
Ethyl 2-oxo-3-phenylpyruvate	97.7 ± 1.99
Methyl 2-oxo-3-phenylpyruvate	86.3 ± 2.62
2-Oxo-3-phenylpyruvic acid	58.8 ± 3.14
Ethyl benzoylformate	90.8 ± 1.68
Methyl benzoylformate	79.4 ± 2.59
Benzoylformic acid	45.0 ± 2.36
Ethyl 4-chloroacetoacetate	76.9 ± 2.01
Ethyl 4,4,4-trifluoroacetoacetate	40.6 ± 1.64
Acetophenone	ND
3-Aminoacetophenone	ND
4-Nitroacetophenone	18.5 ± 1.15
4-Chloroacetophenone	18.5 ± 1.69
4-Aminoacetophenone	ND
α-Chloroacetophenone	21.8 ± 2.35
α-Bromoacetophenone	23.5 ± 2.67
4-Dimethylaminobenzaldehyde	6.41 ± 1.06
4-Methylpropiophenone	8.61 ± 2.27

ND means not detected. Enzyme activity was measured as described in Section 2.3.
[a] The substrate concentration was 10 mM.
[b] To calculate the relative activity, the activity for ethyl 2-oxo-4-phenylbutyrate was taken as 100%. All activities were measured in at least three separate experiments and expressed as means ± standard deviations.

3.2.5. Effect of various compounds

The effect of various compounds on the purified carbonyl reductase of *C. krusei* SW 2026 was also studied. The effect of metal ion was assessed by incubating the enzyme with salts of different metal ions at a final concentration of 1 mM. The enzyme showed high sensitivity towards both thiol binding and heavy metal ions such as Cu^{2+}, Ag^{+}, and Ba^{2+} (Table 5). In the presence of these metal ions, the enzyme exhibited little or no activity at all. Carbonyl reductases are generally known to be thiol enzymes and therefore activity was found to be severely affected by thiol specific metal ions such as Cu^{2+}, Ag^{+}. These results are in good accordance with carbonyl reductase from *C. viswanathii* MTCC 5158 [34]. Other metal ions did not have apparent adverse effect on enzyme activity, except that Fe^{2+} and Fe^{3+} caused a marked decrease in activity. This could possibly be attributed to the alteration of the active site, either substrate or cofactor bonding site. Interestingly, a few metal ions, such as Mn^{2+}, Co^{2+} and Ni^{2+}, exhibited positive effect on the activity of carbonyl reductase of *C. krusei*. According to the statistic analysis, the activation effect of Mn^{2+} was statistically significant. This observation is consistent with our previous report [23], in which Mn^{2+} was identified as a key component in the fermentation of *C. krusei* SW 2026 for higher carbonyl reductase activity. It is speculated that Mn^{2+} might be an important metal ion for the catalytic center of carbonyl reductase of *C. krusei*. Previous studies indicate that stereospecific oxidoreductases have dissimilar sensitivity towards various metal ions. The carbonyl reductases from *C. parapsilosis* and

Table 4
Kinetic constants of carbonyl reductases from different microorganisms.

Microorganism	V_{max} (μmol/min mg)	K_m (mM)	Substrate	Reference
C. krusei SW 2026	18.7	0.319	OPBE	This study
W. mrakii AJ-5620	2.80	18.3	HPPO	[16]
C. magnoliae KFCC 11023	20.1	7.90	Erythrose	[28]
G. candidum	5.73	0.740	1-Acetonaphthpone	[31]
C. viswanathii MTCC 5158	59.2	0.153	Acetophenone	[34]
G. capitatum JCM 3908	–	0.130	N-benzyl-3-pyrrolidinone	[35]

Table 5
Effect of metal ions on the activity of carbonyl reductase of *C. krusei* SW 2026.

Metal ions	Relative enzyme activity (%)[a]
Control	100
$CaCl_2$	88.8 ± 2.56[b]
$CuSO_4$	32.5 ± 1.53[b]
$FeSO_4$	49.9 ± 2.09[b]
$MgSO_4$	92.8 ± 3.13[b]
$ZnSO_4$	85.7 ± 4.02[b]
$CoCl_2$	102 ± 2.76[c]
$NiCl_2$	103 ± 1.77[c]
$BaCl_2$	ND
$AgNO_3$	18.2 ± 2.14[b]
$MnCl_2$	110 ± 1.65[d]
$FeCl_3$	29.5 ± 2.53[b]

ND means not detected.
[a] Enzyme activity was spectrophotometrically measured in the presence of additional test substance (1 mM) under the standard conditions. The activity in absence of test compound was taken as 100%. All activities were measured in at least three separate experiments and expressed as means ± standard deviations.
[b] $P < 0.01$ metal ion vs. control.
[c] $P > 0.05$ metal ion vs. control.
[d] $0.01 < P < 0.05$ metal ion vs. control.

R. erythropolis require Zn^{2+} for its catalytic activity site [32,39], Mg^{2+} is an essential metal ion for the activity of alcohol dehydrogenase from *Lactobacillus kefir* [42], The α-keto ester reductase enzyme from *Streptomyces thermocyaneoviolaceus* IFO 14271 however contain no essential metal ions at all [43].

The inhibitory effect of different reagents was also assessed by incubating the enzyme with tested compounds at a final concentration of 1 mM (Table 6). Strong metal chelating agents such as hydroxyquinoline and o-phenanthroline have significant inhibitory effect on enzyme activity while only appreciable effect was noticed for a relatively weaker metal chelating agent EDTA, suggesting that a tightly bound metal ion might exist in carbonyl reductase of *C. krusei*. Similar inhibitory effect of metal chelating reagents was observed for carbonyl reductases from *C. parapsilosis* DSM 70125 [38], *R. erythropolis* DSM 743 [39] and *C. viswanathii* MTCC 5158 [34]. The thiol specific reagents, 2-nitrobenzoate and N-ethylmaleimide, showed drastic effect on the enzyme activity, suggesting the importance of thiol group in the enzyme. The deleterious effect of histidine reagent, such as diethylpyrocarbonate, capable of destroying the imidazole of histidine, indicates that histidine may play an important role in the catalytic activity of carbonyl reductase of *C. krusei* SW 2026. When incubated with carbonyl reagent 2,4-dinitrophenyl hydrazine, the enzyme almost showed no catalytic activity because of its strong inhibition to carbonyl function site of enzyme. The results are in good agreement with the carbonyl reductase from *C. viswanathii* MTCC 5158 [34].

The effect of various reducing agents on carbonyl reductase activity was also examined (Table 6). The purified carbonyl reductase of *C. krusei* was incubated with different reducing agents at a final concentration of 1 mM. Among different chemicals tested, only β-mercaptoethanol and DTT showed positive effect on the enzyme activity. Other reducing agents listed in Table 6 had deleterious effect on enzyme activity. The results of β-mercaptoethanol and DTT were in good agreement with previous reports in which they were used as protection agents during the purification step [30,33,34]. Most carbonyl reductases showed enhanced activity in the presence of β-mercaptoethanol or DTT, while less pronounced effect with other reducing agents, suggesting a similarity in the properties of their active sites.

Table 6
Effect of various inhibitors and reducing agents on the activity of carbonyl reductase of *C. krusei* SW 2026.

Inhibitors and reducing agents		Relative enzyme activity (%)[a]
Control		100
Chelators	EDTA	92.8 ± 2.37[b]
	o-Phenanthroline	58.2 ± 2.33[c]
	Hydroxyquinoline	45.0 ± 1.75[c]
Sulfydryl agents	2-Nitrobenzoate	8.61 ± 6.11[c]
	N-Ethylmaleimide	5.31 ± 4.95[c]
Histidine specific agents	Diethylpyrocarbonate	32.8 ± 2.23[c]
Carbonyl agents	2,4-Dinitrophenyl hydrazine	0.910 ± 4.04[c]
	Hydroxylamine	29.5 ± 2.78[c]
Others	Oxalate	42.8 ± 2.32[c]
	Citrate	48.3 ± 1.81[c]
Reducing agents	β-Mercaptoethanol	105 ± 2.23[b]
	Dithiothreitol	109 ± 1.88[b]
	Ascorbic acid	14.1 ± 2.47[c]
	Glutathione	32.8 ± 2.09[c]
	L-Cysteine	18.5 ± 2.41[c]

[a] Enzyme activity was spectrophotometrically measured in the presence of additional test substance (1 mM) under the standard conditions. The activity in absence of any test compound was taken as 100%. All activities were measured in at least three separate experiments and expressed as means ± standard deviations.
[b] $0.01 < P < 0.05$ inhibitor/reducing agent vs. control.
[c] $P < 0.01$ inhibitor/reducing agent vs. control.

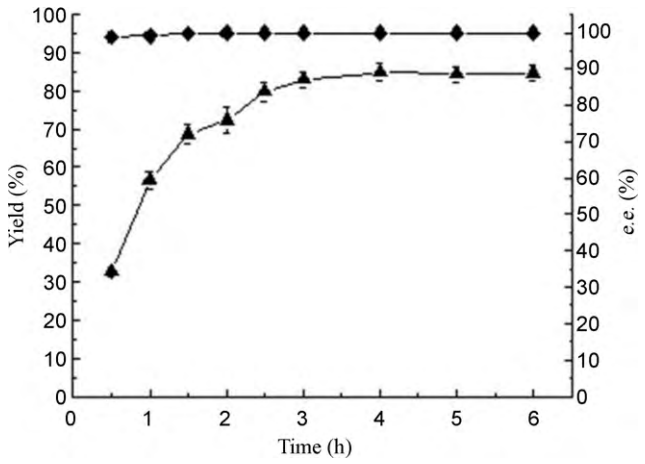

Fig. 6. The time courses of the enzymatic reduction of OPBE in aqueous medium. (▲) Yield. (♦) *e.e.* value. Reaction conditions: 10 mM OPBE, 10 mM NADPH, and approximate 0.2 U of purified enzyme in phosphate buffer (100 mM, pH 6.0) at 30 °C (200 rpm) for 6 h.

The enzymatic transformation of hydrophobic compounds in aqueous solution is generally limited by its low solubility. The purified carbonyl reductase of *C. krusei* could catalyze the reduction of hydrophobic OPBE in aqueous medium with good efficiency. The tolerance of carbonyl reductase against different organic solvents, such as dimethyl sulfoxide, dimethyl formamide, isopropanol, tetrahydrofuran, ethanol, and pyridine, was also investigated (data not shown). It was observed that, in the presence of organic solvents at 10% (v/v) level, the enzyme activity decreased to varying extend. Among the solvents tested, the highest tolerance was observed with ethanol, exhibiting residual enzyme activity of approximately 77.9%. Similar operational instability in organic solvent was reported for carbonyl reductases from *C. parapsilosis* DSM 70125 [38], *R. erythropolis* DSM 743 [39], *C. viswanathii* MTCC 5158 [34], *R. ruber* DSM 44541 [33] and *Rhodotorula* sp. AS 2.2241 [44]. According to our result, heterocyclic organic solvents, pyridine for example, significantly affected the enzyme activity, in which only 15.0% residual activity was observed. It is hypothesized that pyridine might have competitive inhibition effect on the enzyme activity due to the structural similarity between heterocycle of pyridine and benzene ring of OPBE.

Different detergents like Tween 80, Triton X-100 and SDS were also tested for their effect on carbonyl reductase activity. All these detergents had deleterious effect on the enzyme activity, especially SDS (data not shown).

Taken together, our results indicate that certain functional structures, such as intact thiol group, disulfide linkage, imidazole group of histidine, and substrate/cofactor binding site, have important roles in the catalytic activity of carbonyl reductase of *C. krusei*. This study also demonstrates that the activity of the enzyme was strongly inhibited in the presence of some heavy metal ions, strong metal ion chelating agent, heterocyclic organic solvents, and protein denaturant.

3.2.6. Time course of enzymatic reduction of OPBE to (R)-HPBE

Optical pure (*R*)-HPBE was used as an important intermediate for the synthesis of several angiotensin-converting enzyme (ACE) inhibitors such as enalapril, lisinopril, etc. The time course of enzymatic reduction of OPBE by purified carbonyl reductase of *C. krusei* was carried out to confirm the catalytic activity of this enzyme (Fig. 6). As expected, the purified enzyme produced (*R*)-HPBE in an enantiomeric excess of more than 99.9% as determined by chiral GC (supplementary Fig. 2). After 4 h of reaction, approximately 84.0% yield and nearly 100% *e.e.* were achieved. Compared with our previous study [23], the enzymatic reaction showed higher product optical purity than that of the whole cell bioreduction (99.7% *e.e.* at 2.5 g/l of OPBE; 87.5% *e.e.* at 20 g/l of OPBE).

Further investigations on the exploitation of this enzyme along with construction of recombinant strain for desired preparative scale reactions are in progress in our laboratory.

4. Conclusions

A highly active carbonyl reductase from a yeast strain *C. krusei* SW 2026 has been purified for up to 304-fold with a yield of 5.9%. The carbonyl reductase of *C. krusei* SW 2026 catalyzed the reduction of OPBE to (*R*)-HPBE in high enantioselectivity of more than 99.9% *e.e.* This is so far, to our knowledge, the only carbonyl reductase with almost absolute enantioselective in the reduction of OPBE. The enzyme exhibited optimal pH and temperature at 6.0 and 30 °C, and was relatively stable over an acidic pH range of 4.5–7.0 and temperature range of 10–40 °C, respectively. The process can further be improved by cloning and coexpression of carbonyl reductase of *C. krusei* and cofactor regeneration system in a heterologous host, which could potentially be used in the biocatalytic reaction to produce the important intermediate of ACE inhibitors, (*R*)-HPBE.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.05.008.

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