Journal of Molecular Catalysis B: Enzymatic 105 (2014) 66–73

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Genomic mining-based identification of novel stereospecific aldo-keto reductases toolbox from *Candida parapsilosis* for highly enantioselective reduction of carbonyl compounds

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ARTICLE INFO

Article history:
Received 6 January 2014
Received in revised form 18 March 2014
Accepted 5 April 2014
Available online 16 April 2014

Keywords:
Aldo-keto reductase
Genomic mining
Toolbox
Enantioselectivity
Asymmetric reduction

ABSTRACT

Biocatalytic reduction of prochiral ketones offers significant potential in synthesis of optically active alcohols. However, so far the application of aldo-keto reductases (AKRs) in asymmetric reduction has been hampered due to limited availability of AKRs with high enantioselectivity and catalytic efficiency. Based on the genome sequence of *Candida parapsilosis*, a versatile bioresource for asymmetric reduction, eight open reading frames encoding putative AKRs were discovered and expressed, and the resulted enzymes (CPARs), comprising an AKR toolbox, were evaluated toward various carbonyl substrates. The CPARs were active to the selected substrates, especially 2-hydroxyacetophenone and ethyl 4-chloro-3-oxobutyrate. Additionally, most of them were obviously enantioselective to the substrates and gave alcohol products with optical purity up to 99%e.e. Of the enzymes, CPAR4 was outstanding with excellent enantioselectivity and broad substrate spectrum. All these positive features demonstrate that genomic mining is powerful in searching for novel and efficient biocatalysts of desired reactions for pharmaceuticals and fine chemicals synthesis.

1. Introduction

Optically active alcohols are useful chiral intermediates for the synthesis of pharmaceuticals, agricultural chemicals, and specialty materials [1,2]. Compared with the conventional chemical process, biocatalytic asymmetric reduction is one of the efficient ways due to its high chemo-, enantio-, and regioselectivities [3–5]. For the production of chiral alcohols from the corresponding prochiral aldehydes and ketones, the aldo-keto reductases (AKRs) including aldehyde reductase (EC 1.1.1.21) and carbonyl reductase (EC 1.1.1.184) have the inherent advantages over other enzyme systems in terms of their effectiveness, pertinence, and diversity in catalyzing reduction [6,7].

The NAD(P)H-dependent AKRs with broad physiological roles have been found in vertebrates, invertebrates, plants, protozoa, fungi, eubacteria, and archaebacteria [8]. Several microbial enzymes have already been cloned and used for the asymmetric synthesis of chiral alcohols, such as ARI from *Sporobolomyces salmonicolor* [9], Conjugated polyketone reductase (CPR-C1 and CPR-C2) from *Candida parapsilosis* [10], and KER from *Penicillium citrinum* [11]. However, so far the resources of functional enzymes are yet not sufficient and the application of AKRs in the reduction of aldehydes or ketones has been hampered due to the limited availability [12]. Therefore, discovery and identification of AKRs with the application potential should be critical for bio-mediated asymmetric synthesis.

With the rapid development of genomics, proteomics, and bioinformatics, the candidates of ideal biocatalysts could be discovered and characterized significantly [13,14]. One typical example is that 18 key reductases from bakers' yeast were overexpressed and tested for their abilities of reducing α- or β-ketoesters after analysis of the yeast genome [15]. Within this area, the genus *Candida* spp. has been taken as an important source of oxidoreductases for biocatalytic redox reactions including enantioselective keto-reductions [16]. Of them, *C. parapsilosis* has been described as a highly efficient biocatalyst for kinds of asymmetric reductions [17–19], involving stereospecific alcohol dehydrogenases and

Abbreviations: AKR, aldo-keto reductase; ORF, open reading frame; IPTG, isopropyl-β-D-thiogalactopyranoside; TCEP, Tris (2-carboxyethyl) phosphine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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http://dx.doi.org/10.1016/j.molcatb.2014.04.003
1381-1177/

carbonyl reductases [20,21]. Besides the functional conjugated polyketone reductase [22], however, the available AKRs are yet limited for practical application and it would be necessary to discover and identify new enzymes systematically from the promising functional microorganism of *C. parapsilosis* [23].

The disclosure of microbial genome sequence allows scientists to search for novel enzymes with potential applications [24,25]. The genome sequence of *C. parapsilosis* (http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/) provides us a research avenue to dig biocatalytic resource of new enzymes from the microorganism by genomic mining [26]. In this report, by analyzing the genome sequence of *C. parapsilosis*, a biocatalytic toolbox was discovered, comprising eight open reading frames (ORFs) encoding putative AKRs. After expression of these ORFs, the encoded proteins were purified and functionalized as stereospecific AKRs catalyzing enantioselective reduction toward various carbonyl compounds including aryl ketones, aliphatic ketones, and ketoesters. This study would represent a systematic investigation on *C. parapsilosis* enzymes catalyzing stereoselective carbonyl reductions, which would serve as a useful guideline for future development of new AKRs and also the enzymatic processes for synthesis of optically pure alcohols.

2. Materials and methods

2.1. Materials

C. parapsilosis CCTCC M203011 was obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China). *Escherichia coli* strain XL-10 Gold was used for gene cloning. *E. coli* BL21 (DE3) was used for gene expression. The plasmid pET21c was obtained from Novagen (USA) served as expression vector. All enzymes used for DNA manipulations were obtained from TaKaRa Biotechnology Co., Ltd (Dalian, China). The cofactors including NAD(P)H and NAD(P)$^+$, the substrates including aryl ketones (acetophenone, 2-hydroxyacetophenone, *o*-chloroacetophenone *m*-chloroacetophenone, and *p*-chloroacetophenone), aliphatic ketones (2-octanone and 2-hexanone), and ketoesters (ethyl 4-trifluoro-3-oxobutyrate, methyl 3-oxobutyrate, ethyl 3-phenyl-3-oxopropionate, and ethyl 4-chloro-3-oxobutyrate), and the standard samples of chiral alcohol products corresponding to the above carbonyl substrates were purchased from the Sigma–Aldrich Chemical Co. (USA). All other used chemicals were of analytical grade and commercially available.

2.2. Search for potential genes and sequence analysis

Discovery of potential AKRs was carried out by NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BLAST against the complete *C. parapsilosis* genome (http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/) [26]. Multiple sequence alignment was performed using Clustal X software [27]. The phylogenetic trees were deduced from the alignment using the neighbor-joining method of MEGA [28].

2.3. Cloning and expression of genes encoding AKRs

The genes encoding the eight AKRs were amplified from *C. parapsilosis* genome using appropriate primer pairs with the restriction sites of *Nde*I and *Xho*I (Table 1). The purified fragments were digested with these restricted enzymes and ligated into pET21c expression vector. Then the verified recombinant plasmids were transformed into the *E. coli* BL21 (DE3) competent cells. The transformants were grown in Luria–Bertani (LB) medium (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl, 5 g L^{-1}) containing 100 μg mL^{-1} ampicillin at 37 °C.

Table 1
Oligonucleotide primers for amplification of the genes encoding CPARs from *C. parapsilosis* genome, involving restriction sites of *Nde*I and *Xho*I underlined.

Gene	Primer sequence
cpar1	F: 5′-CCCGCCCGCATATGACTCCACAACCAATTGAG-3′
	R: 5′-GCCCGCTCGAGCTGAAACAATGAGCCTTCACTTTG-3′
cpar2	F: 5′-CCCGCCCGCATATGTCTACTACAATCAAGAAAGCC-3′
	R: 5′-GCCCGCTCGAGATCAAATTCTTTACTCAAAGTGTCTC-3′
cpar3	F: 5′-CCCGCCCGCATATGACCCATCAGCGCAGTCTTC-3′
	R: 5′-GCCCGCTCGAGCTCATCCTTATACAATGTTGGATC-3′
cpar4	F: 5′-CCCGCCCGCATATGTCAGCTCAATTGAAAGTAAAC-3′
	R: 5′-GCCCGCTCGAGGTCATTGAAGTTGTTGAAGCCTG-3′
cpar5	F: 5′-CCCGCCCGCATATGTCATATAGACTAATTCAATTAAAC-3′
	R: 5′-GCCCGCTCGAGTGGGGCATTGGTACATTCCC-3′
cpar6	F: 5′-CCCGCCCGCATATGAGCTCTCCTTTACCCTCAC-3′
	R: 5′-GCCCGCTCGAGCAGGTGACCGCTTGGCCAC-3′
cpar7	F: 5′-CCCGCCCGCATATGACTCAAAGTAACTTACTACC-3′
	R: 5′-GCCCGCTCGAGCAAATCTTTAAATTGCTCATGGAAG-3′
cpar8	F: 5′-CCCGCCCGCATATGTCAATTGAGCTCAAGTACAAT-3′
	R: 5′-GCCCGCTCGAGAGAGAGGGACTGTTTGGGTTTA-3′

The recombinant cells were cultivated in 4 mL LB liquid medium containing 100 μg mL^{-1} ampicillin at 37 °C and 200 rpm for 12 h. Then the culture was inoculated into a 250-ml Erlenmeyer flask containing 50 ml fresh LB medium supplemented with 100 μg mL^{-1} ampicillin. When the culture turbidity (OD_{600nm}) increased to the level between 0.6 and 0.8, the expressions of target recombinant proteins were initiated with the optimization of the following conditions, where isopropyl-β-D-thiogalactopyranoside (IPTG) (0.1, 0.5, and 1.0 mM) or lactose (2%, 4%, and 6%) was added as inducer and the culture was incubated under different temperatures (17, 20, 25, 30, and 35 °C) at 200 rpm for additional 12 h. The yield of target protein was evaluated by calculating the amount of purified protein obtained from the corresponding culture broth.

2.4. Purification of recombinant enzymes

The cells were suspended in binding buffer (20 mM Tris-HCl, pH6.5, 0.3 M NaCl, 40 mM imidazole, 1× protease inhibitors, 1 mM Tris (2-carboxyethyl) phosphine (TCEP)) and disrupted on ice with an ultrasonic oscillator (VCX750, Sonic). The supernatant of the cell lysate was collected by centrifugation at 26,000 × *g* for 40 min at 4 °C and purified by an AKTAxpress system using HisTrap HP affinity column (GE Healthcare, USA). Elution was carried out with 300 mM imidazole in the same buffer at a flow rate of 2.0 mL min^{-1}. Then the purified fractions were exchanged into low salt buffer (10 mM Tris–HCl, pH 6.5, 0.1 M NaCl, 0.02% NaN_3, 5 mM D,L-dithiothreitol) using disposable PD-10 desalting columns (GE Healthcare, USA) [29]. The final recombinant enzymes were purified to homogeneity as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% polyacrylamide gels [29]. The amount of purified protein obtained from the corresponding culture broth was used to evaluate the yield of target protein. These final preparations of purified enzymes were used in all of the experiments in this study.

2.5. Enzyme activity assay

The enzyme activity of AKR was measured by a continuous spectrophotometric assay using the standard assay mixture containing 0.1 M potassium phosphate buffer (pH 6.5), 0.5 mM NAD(P)H, 5 mM substrate, and the appropriate enzyme in a total volume of 100 μL. The involved substrates of carbonyl compound included aryl ketones (acetophenone, 2-hydroxyacetophenone, *o*-chloroacetophenone *m*-chloroacetophenone, and *p*-chloroacetophenone), aliphatic ketones (2-octanone and 2-hexanone), and ketoesters (ethyl 4-trifluoro-3-oxobutyrate, methyl 3-oxobutyrate, ethyl

3-phenyl-3-oxopropionate, and ethyl 4-chloro-3-oxobutyrate). One unit of enzyme activity was defined as the amount of enzyme required to catalyze the oxidation of 1 μmol NAD(P)H per minute under the given assay conditions. The decrease in the amount of the coenzyme was measured spectrophotometrically at 340 nm (extinction coefficient $[e] = 6.22\,mM^{-1}\,cm^{-1}$). Protein concentration was determined using Bradford reagents (Bio-Rad) with bovine serum albumin as a standard. All the values of enzymatic activities were averaged from three replicates and significant differences ($p < 0.05$) were measured.

2.6. Kinetic parameters analysis

Kinetic parameters of the purified enzyme were assayed by measuring initial velocity at various concentrations of substrates and cofactors [30]. To determine the apparent K_m value, the concentration of carbonyl compounds was varied from 0.5 to 4 mM with a fixed concentration of NADPH at 0.05 mM, 0.1 mM, and 0.25 mM, respectively. Apparent kinetic parameters were further calculated from double reciprocal Lineweaver–Burk plots. All the data were averaged from three replicates for each substrate and cofactor concentration and significant differences ($p < 0.05$) were measured.

2.7. Asymmetric reduction of carbonyl compounds

Asymmetric reductions of various carbonyl compounds by the purified enzymes were carried out at 30 °C for 8 h with mild shaking in a reaction mixture containing 0.1 M potassium phosphate buffer (pH 6.5), 1 g L^{-1} substrate, 10 mM NADPH, and the purified enzyme of appropriate amount in a total volume of 2 mL. In order to determine the absolute configuration of chiral alcohols, the reaction products were extracted with ethyl acetate or hexane and the organic layer was used for analysis. The optical purity of the reaction products were determined by chiral HPLC (HP 1100, Agilent, USA) equipped with Chiralcel OB-H column (4.6 mm × 250 mm; Daicel Chemical Ind. Ltd., Japan) or chiral GC (7890A, Agilent, USA) equipped with FID detector and Chrompack Chirasil-Dex CB chiral capillary column (25 m × 0.25 mm; Varian, USA) [21].

2.8. Nucleotide sequence accession number

The nucleotide sequences for the stereospecific AKR genes have been deposited in the GenBank database with accession numbers JX512911, JX512912, JX512913, JX512915, JX512916, JX512917, JX512918, and JX512919, respectively.

3. Results and discussion

3.1. Identification of putative AKR-encoding genes

With the amino acid sequence of conjugated polyketone reductase CPR-C1 as the template [22], genomic mining based on sequence similarity was carried out against the genome sequence of *C. parapsilosis*. Then eight homologous ORFs, named here as *cpar*, were revealed, which comprise around 1000 base pairs in length and uninterruptedly encode the putative proteins of CPARs with different theoretical molecular mass, respectively (Table 2), where the entire gene sequences can be translated into the corresponding amino acid sequences without non-coding regions and thus no intron was found in the encoding sequences of the eight homologous genes. Of them, CPAR2, CPAR3, CPAR4, CPAR5, and CPAR7 have a higher sequence identity ranging from 25% to 42% to the conjugated polyketone reductase C1, while somewhat lower identity around 15% was found between C1 and other sequences including CPAR1, CPAR6, and CPAR8. The multiple sequence alignment of these sequences indicated that the newly discovered ORFs all code for putative AKRs (Fig. 1). From the amino acid sequence and secondary structure prediction, the putative enzymes exhibit a classic $(\alpha/\beta)_8$ structure containing the NADP-binding motif and the catalytic tetrad DxxxxY, K, and H. Two AKR family signature sequences (PROSITE accession numbers PS00798 and PS00062) and an AKR family putative active site signature sequence (PROSITE accession number PS00063) could be observed in all the sequences of putative enzymes (Fig. 1). For AKRs, the IPKS motif, comprised of the first four amino acid residues in the AKR family putative active site signature sequence, has been reported to play an important role in cofactor binding [22]. While in CPARs, some residues were observed to be varied in the IPKS motif and also the AKR family putative active site signature sequence described as the pattern of [ILVM]-[PAIV]-[KR]-[ST]-{EPQG}-{RFI}-x(2)-R -{SVAF}-x-[GSTAEQK]-[NSL]-x-{LVRI}-[LIVMFA], indicating that the CPARs would be different in catalytic properties except for the common nature of reducing aldehyde and ketone [31]. Therefore, the putative CPARs encoded by the newly discovered ORFs in *C. parapsilosis* genome sequence would be members of the AKR family, although perform distinct characteristics in catalyzing keto reductions due to the diversity of functional fragment in the primary structure. Additionally, the enantioselective oxidoreductases belonging to different superfamilies in classification were identified from the same functional microorganism of *C. parapsilosis*, of which the previously reported carbonyl reductases, SCRs, share sequence motif characteristic of the short-chain dehydrogenase/reductase (SDR) superfamily with the highly conserved regions including the cofactor binding motif Gly-x-x-x-Gly-x-Gly and the catalytic triad of Ser-Tyr-Lys [21]. Thus the AKRs discovered in this study would exhibit distinct characteristics from the reported SCRs in sequence homology, substrate specificity, and enantioselectivity.

To understand the evolution relationship and classification of CPARs, a phylogenetic tree was constructed to describe the homologous relevance of CPARs and other reported AKRs. As shown in Fig. 2, the putative proteins of CPAR2, CPAR3, CPAR4, CPAR5, and CPAR7 would be the members of the AKR3 subfamily, where CPAR3 and CPAR5 would be predicted to have a closer relationship. On the other hand, CPAR1, CPAR6, and CPAR8 were supposed to phylogenically belong to the subfamilies of AKR8, AKR7, and AKR10, respectively. From the map of the phylogenetic tree, additionally, it could be observed that the subfamilies from AKR1 to AKR5 cluster as one major branch with close evolution relationship, while the rest of the subfamilies comprise another phylogenetic branch. Since the evolutionary position may contribute to the difference of enzymatic characteristics, the CPARs would be diverse in physiological property and catalytic function.

3.2. Expression and purification of recombinant CPARs

The genes encoding CPARs were expressed in recombinant *E. coli* as His-tagged fusion proteins. Because the change of the external expression conditions has the potential to significantly increase expression yield of genetically stable recombinant system, various factors involved in the expression optimization of the target proteins were investigated, including cultivation temperature from 17 to 37 °C and inducer of 0.1 mM to 1.0 mM IPTG or 2–6% lactose. Under the optimized expression conditions involving varied temperature and inducer concentration, the recombinant CPARs could be obtained in soluble form with different expression levels and specific activities (Table 3). Of them, CPAR2, CPAR3, CPAR4, and CPAR7 were expressed at somewhat higher yields of target protein, while CPAR1, CPAR5, CPAR6, and CPAR8 gave relatively lower levels of target protein yield in heterologous expression (Table 3). Then the recombinant enzymes with His-tag at C-terminal were purified by nickel affinity chromatography, showing distinct bands

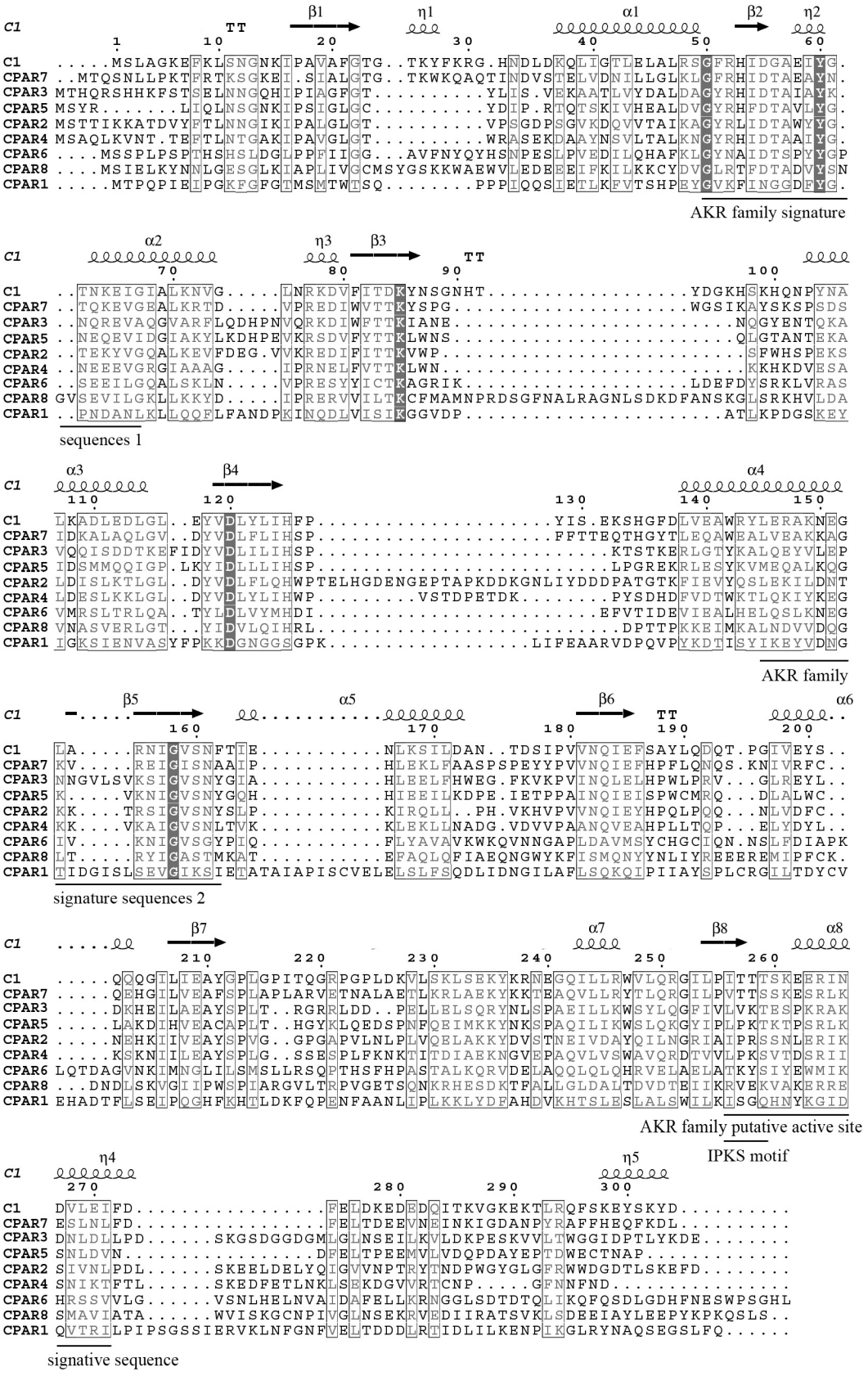

Fig. 1. Amino acid sequence alignment of C1 and putative CPARs from *C. parapsilosis*. Gaps in the aligned sequences are indicated by dashes. The AKR family signature sequence 1 (PROSITE accession number PS00798) and sequence 2 (PROSITE accession number PS00062) and the IPKS motif are underlined.

Table 2
Properties of potential genes and their coding products discovered from *C. parapsilosis* genome by BLAST search.

Gene	GenBank accession number	Length of open reading frame (bp)	Residue number of encoded protein	Theoretical molecular weight of encoded protein (Da)
cpar1	JX512911	1059	352	38,927.4
cpar2	JX512912	987	328	36,904.8
cpar3	JX512913	933	310	35,170.9
cpar4	JX512915	888	295	32,761.1
cpar5	JX512916	852	283	32,382.0
cpar6	JX512917	996	331	37,167.2
cpar7	JX512918	924	307	34,650.4
cpar8	JX512919	1047	348	39,439.4

Table 3
Optimized conditions for expression of CPARs in recombinant *E. coli*.

Construct	Expression conditions			Target protein yield (mg L^{-1})[a]	Specific activity (μmol min^{-1} mg^{-1})[b]
	IPTG concentration (mM)	Lactose concentration (%)	Induction temperature (°C)		
CPAR1	1	0	30	2 ± 0.23	2.51 ± 0.15
CPAR2	1	0	17	40 ± 1.32	3.23 ± 0.21
CPAR3	1	0	17	52 ± 0.74	4.72 ± 0.24
CPAR4	1	0	17	42 ± 1.18	3.57 ± 0.09
CPAR5	0	2	17	9 ± 0.73	14.02 ± 2.10
CPAR6	1	0	17	4 ± 0.34	1.50 ± 0.78
CPAR7	1	0	17	48 ± 0.88	0.82 ± 0.05
CPAR8	0	2	17	5 ± 0.37	0.67 ± 0.12

[a] The target protein yield (mg L^{-1}) was evaluated by calculating the amount of purified protein from the corresponding culture broth.
[b] Activity assay was carried out at the pH 6.5 for each enzyme with ethyl 4-chloro-3-oxobutyrate as the substrate.

on SDS-PAGE, with the size in agreement with the theoretically calculated molecular weight (Fig. 3).

3.3. Substrate specificity of recombinant CPARs

Predicted as members of AKRs based on the primary structure analysis, the capability of CPARs for catalyzing reduction of carbonyl compounds was investigated toward different kinds of substrates including aryl ketones

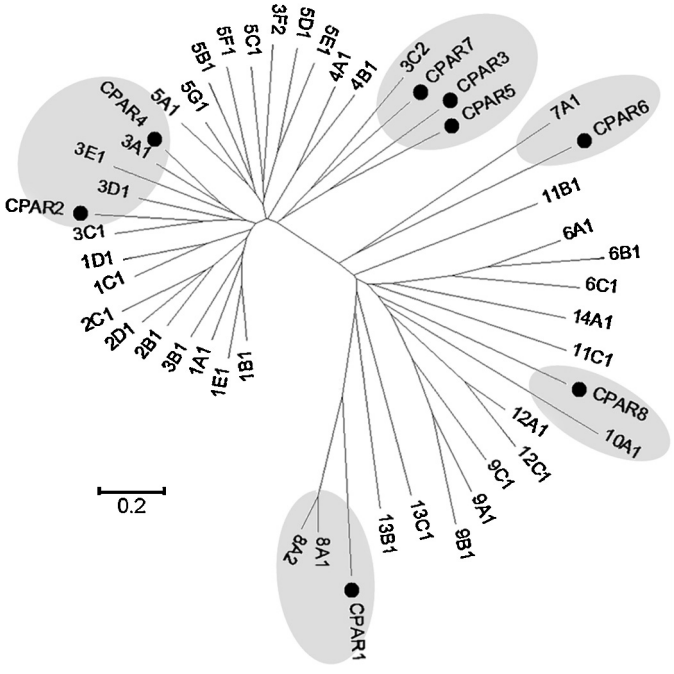

Fig. 2. Unrooted phylogenetic tree of CPARs and relevant AKRs. The amino acid sequences of relevant AKRs were referred to AKR homepage (http://www.med.upenn.edu/akr/).

(acetophenone, 2-hydroxyacetophenone, *o*-chloroacetophenone *m*-chloroacetophenone, and *p*-chloroacetophenone), aliphatic ketones (2-octanone and 2-hexanone), and ketoesters (ethyl 4-trifluoro-3-oxobutyrate, methyl 3-oxobutyrate, ethyl 3-phenyl-3-oxopropionate, and ethyl 4-chloro-3-oxobutyrate), from which the corresponding alcohol products are valuable building blocks and intermediates for the synthesis of important pharmaceuticals and functional materials, for example, semisynthetic β-lactam antibiotics and cholesterol-lowering drug atorvastatin [32–34]. The CPARs all performed catalytic activity with NADPH as the coenzyme, but no obvious activities toward NADH (data not shown), indicating that these newly discovered enzymes all play the role of NADPH-dependent oxidoreductases.

Then the substrate specificity of the interested enzymes involved in the discovered AKR toolbox was assessed by measuring the reductive activity with NADPH. For the carbonyl substrates with various chemical structures, it could be observed that the enzymes generally exhibited obviously higher activities to 2-hydroxyacetophenone of aryl ketones and ketoesters, especially ethyl 4-chloro-3-oxobutyrate (Table 4). Compared with other carbonyl substrates including acetophenone derivatives and aliphatic ketones, from the viewpoint of chemical structure of the substrates, ethyl 4-chloro-3-oxobutyrate and 2-hydroxyacetophenone both possess an electron-withdrawing substituent neighboring the carbonyl group, such as chlorine or hydroxyl, indicating that substituent at α-carbon has a great effect on the enzyme activity by forming hydrogen bond between active site residues of the enzyme and such electron-withdrawing groups at the α-position of substrates, which would be favorable for the reaction involving electron transfer.

In addition, the specific activity and the catalytic efficiency of the enzymes were evaluated by calculating the steady-state kinetics via double reciprocal plots, using ethyl 4-chloro-3-oxobutyrate, 2-hydroxyacetophenone, acetophenone, and 2-octanone as the exemplary substrates (Fig. 4). Except for the observed trends of the CPARs toward various carbonyl compounds, some enzymes performed distinct specificity to certain kind of the tested substrates. Of them, CPAR2 and CPAR4 exhibited obvious activities toward

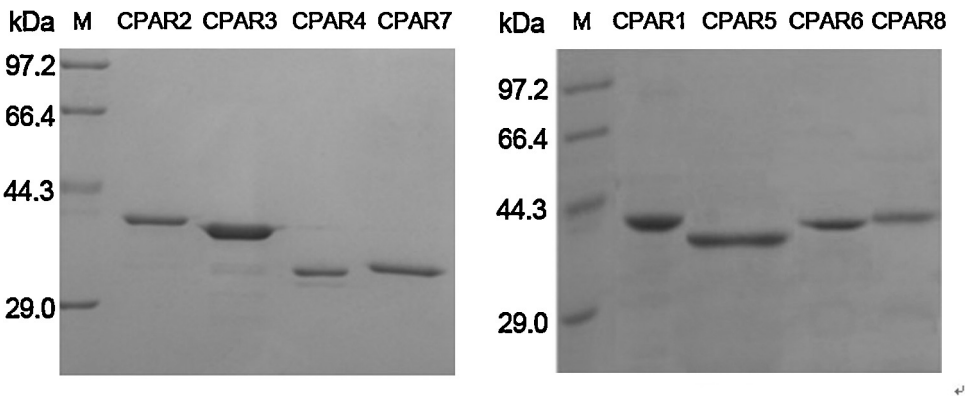

Fig. 3. SDS-PAGE analysis of purified CPARs. Lanes 1 and 6, molecular mass standard; Lane 2, purified CPAR2; Lane 3, purified CPAR3; Lane 4, purified CPAR4; Lane 5, purified CPAR7; Lane 7, purified CPAR1; Lane 8, purified CPAR5; Lane 9, purified CPAR6; Lane 10, purified CPAR8.

acetophenone, and CPAR2, CPAR3, CPAR4, and CPAR5 were observably active to 2-octanone. On the contrary, CPAR6, CPAR7, and CPAR8 were almost inefficient in catalyzing reduction of both acetophenone and 2-octanone. Associating the catalytic efficiency regarding the value of k_{cat}/K_m, except for CPAR2 to 2-hydroxyacetophenone, CPAR4 generally performed relatively higher efficiency of catalyzing reduction of carbonyl compounds with various chemical strictures, indicating that the enzyme possesses a somewhat broad spectrum of carbonyl substrates and has the potential for the synthesis of pharmaceuticals and fine chemicals from the application point of view.

3.4. Enantioselective reduction of carbonyl compounds

The enantioselectivity of CPARs catalyzing asymmetric reduction was evaluated toward the investigated substrates including aliphatic ketones, aryl ketones, and ketoesters (Table 4). On the one hand, although almost all of the CPARs performed stereoselectivity to the carbonyl compounds, they did not follow the same pattern of asymmetric reduction, according to the stereo-configuration outcomes of the products generated from the reduction of carbonyl substrates. Of them, CPAR2 and CPAR6 were supposed to catalyze asymmetric reduction in anti-Prelog type, while the

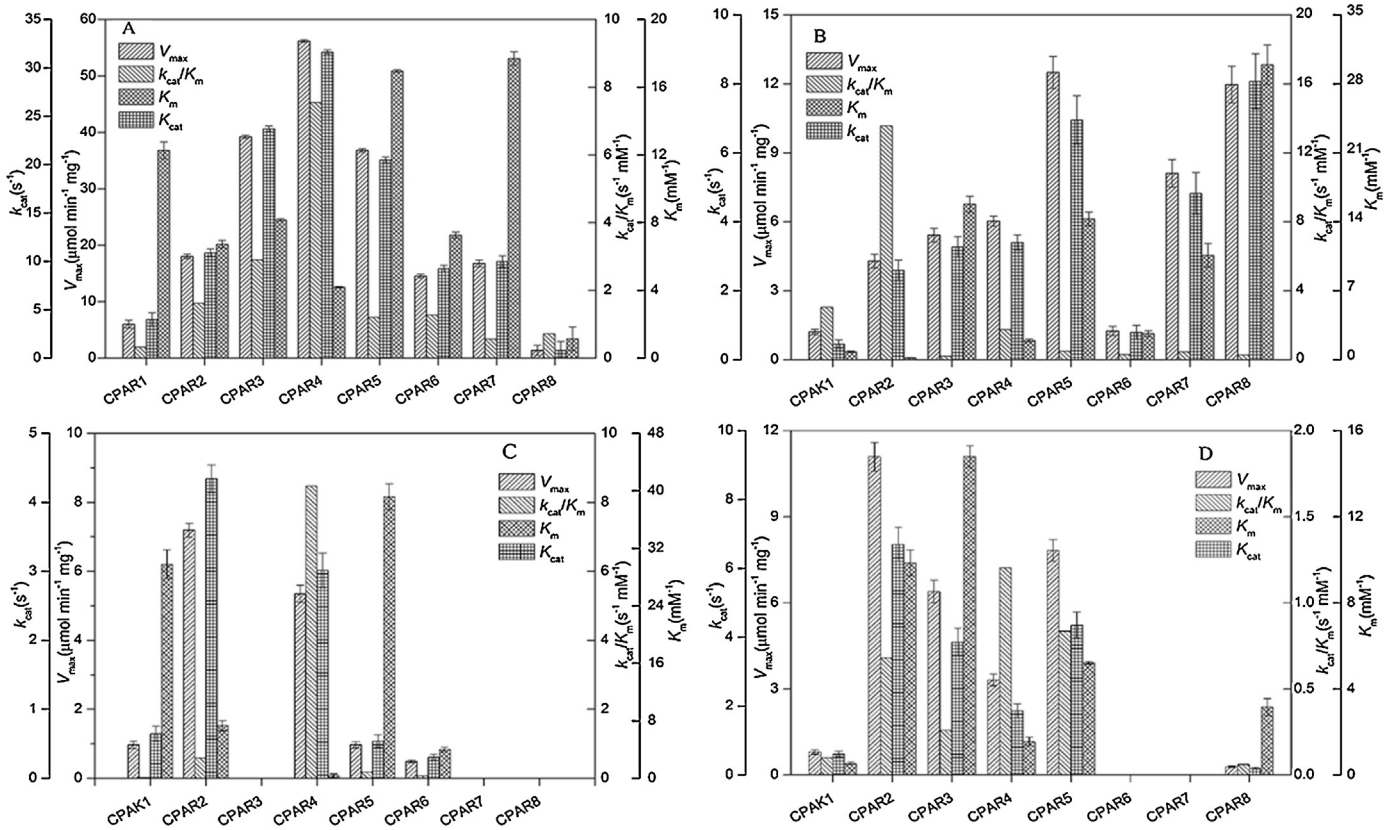

Fig. 4. Specific activities and apparent kinetic parameters of CPARs toward various carbonyl substrates including (A) ethyl 4-chloro-3-oxobutyrate, (B) 2-hydroxyacetophenone, (C) acetophenone, and (D) 2-octanone. All reactions involved in the calculation of activities and kinetic parameters were carried out as described in the text.

Table 4
Enantioselective reduction of carbonyl compounds catalyzed by purified CPARs, respectively.

Substrate	Specific activity (μmol min^{-1} mg^{-1})/product configuration and optical purity (%e.e.)							
	CPAR1	CPAR2	CPAR3	CPAR4	CPAR5	CPAR6	CPAR7	CPAR8
Acetophenone	0.15 ± 0.01/68.34 ± 0.31 (*S*)	0.87 ± 0.14/61.52 ± 0.21 (*R*)	–	0.67 ± 0.04/>99.90 (*S*)	0.13 ± 0.01/75.20 ± 0.22 (*S*)	0.02 ± 0.01/>99.90 (*R*)	–	-
2-Hydroxyaceto-phenone	0.63 ± 0.13/>99.90 (*R*)	0.74 ± 0.03/>99.90 (*S*)	1.48 ± 0.05/18.81 ± 0.17 (*R*)	2.16 ± 0.15/>99.90 (*R*)	5.32 ± 0.71/61.30 ± 0.13 (*R*)	0.83 ± 0.13/–	1.59 ± 0.07/20.39 ± 0.18 (*R*)	5.34 ± 0.11/>99.90 (*R*)
o-Chloroaceto-phenone	0.35 ± 0.08/78.22 ± 0.59 (*S*)	0.92 ± 0.11/68.17 ± 0.38 (*R*)	0.04 ± 0.01/39.55 ± 0.42 (*S*)	1.35 ± 0.09/>99.90 (*S*)	0.19 ± 0.02/68.69 ± 0.31 (*S*)	0.05 ± 0.01/>99.90 (*R*)	0.05 ± 0.01/31.45 ± 0.59 (*S*)	-
m-Chloroaceto-phenone	0.12 ± 0.06/>99.90 (*S*)	1.54 ± 0.12/56.34 ± 0.19 (*R*)	–	1.88 ± 0.21/>99.90 (*S*)	0.27 ± 0.01/62.56 ± 0.52 (*S*)	0.04 ± 0.01/>99.90 (*R*)	0.06 ± 0.01/55.95 ± 0.24 (*S*)	-
p-Chloroaceto-phenone	0.39 ± 0.09/>99.90 (*S*)	1.57 ± 0.02/83.23 ± 0.24 (*R*)	–	1.21 ± 0.07/>99.90 (*S*)	0.24 ± 0.01/58.91 ± 0.36 (*S*)	0.03 ± 0.01/>99.90 (*R*)	0.06 ± 0.01/40.37 ± 0.39 (*S*)	-
2-Hexanone	0.07 ± 0.01/55.56 ± 0.41 (*S*)	0.65 ± 0.07/>99.90 (*R*)	0.14 ± 0.03/88.71 ± 0.18 (*S*)	1.77 ± 0.08/>99.90 (*S*)	4.14 ± 0.09/86.13 ± 0.16 (*S*)	–	–	-
2-Octanone	0.05 ± 0.01/86.43 ± 0.25 (*S*)	0.56 ± 0.09/>99.90 (*R*)	0.13 ± 0.01/68.53 ± 0.24 (*S*)	1.26 ± 0.07/>99.90 (*S*)	3.25 ± 0.12/85.14 ± 0.20 (*S*)	–	–	0.02 ± 0.01/>99.90 (*S*)
Ethyl 4-trifluoro-3-oxobutyrate	0.75 ± 0.08/65.43 ± 0.45 (*R*)	1.13 ± 0.10/>99.90 (*S*)	1.31 ± 0.18/8.65 ± 0.43 (*R*)	1.52 ± 0.05/>99.90 (*R*)	3.52 ± 0.52/92.43 ± 0.74 (*R*)	0.47 ± 0.03/14.81 ± 0.81 (*S*)	1.14 ± 0.23/16.70 ± 0.13 (*R*)	0.19 ± 0.06/>99.90 (*R*)
Methyl 3-oxobutyrate	0.68 ± 0.11/78.51 ± 0.68 (*S*)	0.99 ± 0.08/>99.90 (*R*)	1.42 ± 0.15/10.21 ± 0.56 (*S*)	1.48 ± 0.06/>99.90 (*S*)	3.96 ± 0.61/78.56 ± 0.48 (*S*)	0.21 ± 0.04/17.56 ± 1.05 (*R*)	0.88 ± 0.16/38.82 ± 0.41 (*S*)	0.21 ± 0.05/>99.90 (*S*)
Ethyl 3-phenyl-3-oxopropionate	–	–	0.38 ± 0.12/9.34 ± 0.81 (*R*)	0.97 ± 0.11/>99.90 (*R*)	–	–	–	-
Ethyl 4-chloro-3-oxobutyrate	2.51 ± 0.15/–	3.23 ± 0.21/>99.90 (*S*)	4.72 ± 0.24/8.50 ± 0.12 (*R*)	3.57 ± 0.09/>99.90 (*R*)	14.02 ± 2.10/82.30 ± 0.18 (*R*)	1.50 ± 0.78/11.84 ± 0.27 (*S*)	0.82 ± 0.05/8.78 ± 0.14 (*R*)	0.67 ± 0.12/>99.90 (*R*)

others were suggested to follow Prelog's rule [35,36]. On the other hand, not only in stereoselectivity, the enzymes but also exhibited diversity in stereopreference to prepare the corresponding chiral alcohol products with different optical purities, presented as enantiomeric excess (e.e.) value. Except for CPAR3 and CPAR7 with somewhat lower stereopreference to the tested substrates, most of the newly discovered CPARs were able to catalyze highly enantioseletive reduction of certain kinds of carbonyl compounds, even resulting in the production of optically active alcohols with the e.e. value over 99%, such as CPAR6 to acetophenone derivatives, CPAR2 to aliphatic ketones, and CPAR2 and CPAR8 to ketoesters.

It is worth noting that, of the enzymes involved in the newly discovered AKRs toolbox, CPAR4 was found to be outstanding in catalyzing asymmetric reduction due to its excellent enantioselectivity to all of the examined substrates, even producing the chiral alcohols of absolute stereo-configuration (>99% e.e.). Correlating with the catalytic efficiency, therefore, CPAR4 was supposed to be a promising catalyst with high stereopreference and broad substrate specificity for highly efficient and enantioseletive reduction of carbonyl compounds, and hence has the application potential for the synthesis of pharmaceutical intermediates and fine chemicals.

4. Conclusion

In this work, a biocatalytic toolbox of novel stereospecific AKRs was identified through genomic mining. Eight putative AKRs encoded by the ORFs from *C. parapsilosis* genome were expressed, purified, and characterized. The resulted CPARs exhibited catalytic activities to the investigated carbonyl compounds, especially 2-hydroxyacetophenone and ethyl 4-chloro-3-oxobutyrate, and most of them were stereoselective to the substrates and even performed the capability of catalyzing highly enantioselective reduction of ketoesters or acetophenone derivatives to the corresponding alcohols with optical purity over 99%e.e. Of them, CPAR4 was impressive with remarkable enantioselectivity and broad substrate specificity, indicating its practical application potential. Additionally, the approach of genomic mining would be promising to discover novel and efficient enzymes for pharmaceutical and fine chemical industries.

Acknowledgements

Financial supports from the National Key Basic Research and Development Program of China (973 Program) (2011CB710800), the National Hi-Tech Research and Development Program of China (863 Program) (2011AA02A210 and 2011AA02A209), the National Natural Science Foundation of China (NSFC) (21376107 and 21336009), the Program of Introducing Talents of Discipline to Universities (111 Project) (111-2-06), the High-end Foreign Experts Recruitment Program (GDW20133200113), and the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions are greatly appreciated.

References

[1] A. Schmid, J.S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, Nature 409 (2001) 258–268.
[2] H.E. Schoemaker, D. Mink, M.G. Wubbolts, Science 299 (2003) 1694–1697.
[3] R. Wohlgemuth, Curr. Opin. Microbiol. 13 (2010) 283–292.
[4] F. Hollmann, I.W.C.E. Arends, D. Holtmann, Green Chem. 13 (2011) 2285–2313.
[5] R. Wohlgemuth, J. Chem. Technol. Biotechnol. 82 (2007) 1055–1062.
[6] E.M. Ellis, FEMS Microbiol. Lett. 216 (2002) 123–131.
[7] G.W. Huisman, J. Liang, A. Krebber, Curr. Opin. Chem. Biol. 14 (2010) 122–129.
[8] K. Yamamoto, D.K. Wilson, Arch. Biochem. Biophys. 538 (2013) 156–163.
[9] K. Kita, K. Matsuzaki, T. Hashimoto, H. Yanase, N. Kato, M.C.M. Chung, M. Kataoka, S. Shimizu, Appl. Environ. Microbiol. 62 (1996) 2303–2310.
[10] A.R.G.D. Hidalgo, M.A. Akond, K. Kita, M. Kataoka, S. Shimizu, Biosci. Biotechnol. Biochem. 65 (2001) 2785–2788.
[11] H. Asako, M. Shimizu, N. Itoh, Appl. Microbiol. Biotechnol. 80 (2008) 805–812.

[12] J.B. van Beilen, E.G. Funhoff, Curr. Opin. Biotechnol. 16 (2005) 308–314.
[13] M. Ferrer, F. Martinez-Abarca, P.N. Golyshin, Curr. Opin. Biotechnol. 16 (2005) 588–593.
[14] I.A. Kaluzna, T. Matsuda, A.K. Sewell, J.D. Stewart, J. Am. Chem. Soc. 126 (2004) 12827–12832.
[15] I.A. Kaluzna, B.D. Feske, W. Wittayanan, I. Ghiviriga, J.D. Stewart, J. Org. Chem. 70 (2005) 342–345.
[16] D. Gamenara, P.D. de María, Biotechnol. Adv. 27 (2009) 278–285.
[17] Y. Nie, Y. Xu, X.Q. Mu, Org. Process Res. Dev. 8 (2004) 246–251.
[18] Y. Nie, Y. Xu, T.F. Lv, R. Xiao, J. Chem. Technol. Biotechnol. 84 (2009) 468–472.
[19] Q. Hu, Y. Xu, Y. Nie, J. Chem. Technol. Biotechnol. 85 (2010) 252–257.
[20] Y. Nie, Y. Xu, X.Q. Mu, H.Y. Wang, M. Yang, R. Xiao, Appl. Environ. Microbiol. 73 (2007) 3759–3764.
[21] Y. Nie, R. Xiao, Y. Xu, G.T. Montelione, Org. Biomol. Chem. 9 (2011) 4070–4078.
[22] M. Kataoka, A.R.G.D. Hidalgo, M.A. Akond, E. Sakuradani, K. Kita, S. Shimizu, Appl. Microbiol. Biotechnol. 64 (2004) 359–366.
[23] T. Matsuda, R. Yamanaka, K. Nakamura, Tetrahedron: Asymmetry 20 (2009) 513–557.
[24] G.S. Nguyen, M.L. Thompson, G. Grogan, U.T. Bornscheuer, R. Kourist, J. Mol. Catal. B: Enzym. 70 (2011) 88–94.
[25] A. Riebel, G. de Gonzalo, M.W. Fraaije, J. Mol. Catal. B: Enzym. 88 (2013) 20–25.
[26] G. Butler, M.D. Rasmussen, M.F. Lin, M.A.S. Santos, S. Sakthikumar, C.A. Munro, E. Rheinbay, M. Grabherr, A. Forche, J.L. Reedy, I. Agrafioti, M.B. Arnaud, S. Bates, A.J.P. Brown, S. Brunke, M.C. Costanzo, D.A. Fitzpatrick, P.W.J. de Groot, D. Harris, L.L. Hoyer, B. Hube, F.M. Klis, C. Kodira, N. Lennard, M.E. Logue, R. Martin, A.M. Neiman, E. Nikolaou, M.A. Quail, J. Quinn, M.C. Santos, F.F. Schmitzberger, G. Sherlock, P. Shah, K.A.T. Silverstein, M.S. Skrzypek, D. Soll, R. Staggs, I. Stansfield, M.P.H. Stumpf, P.E. Sudbery, T. Srikantha, Q. Zeng, J. Berman, M. Berriman, J. Heitman, N.A.R. Gow, M.C. Lorenz, B.W. Birren, M. Kellis, C.A. Cuomo, Nature 459 (2009) 657–662.
[27] M.A. Larkin, G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson, D.G. Higgins, Bioinformatics 23 (2007) 2947–2948.
[28] B.G. Hall, Mol. Biol. Evol. 30 (2013) 1229–1235.
[29] R. Xiao, S. Anderson, J. Aramini, R. Belote, W.A. Buchwald, C. Ciccosanti, K. Conover, J.K. Everett, K. Hamilton, Y.J. Huang, H. Janjua, M. Jiang, G.J. Kornhaber, D.Y. Lee, J.Y. Locke, L.C. Ma, M. Maglaqui, L. Mao, S. Mitra, D. Patel, P. Rossi, S. Sahdev, S. Sharma, R. Shastry, G.V.T. Swapna, S.N. Tong, D. Wang, H. Wang, L. Zhao, G.T. Montelione, T.B. Acton, J. Struct. Biol. 172 (2010) 21–33.
[30] X.Q. Mu, Y. Xu, M. Yang, Z.H. Sun, J. Mol. Catal. B: Enzym. 43 (2006) 23–28.
[31] W. Dyrka, J.-C. Nebel, BMC Bioinformatics 10 (2009) 323.
[32] H. GroÈger, Adv. Synth. Catal. 343 (2001) 547–558.
[33] J.M. Patel, J. Mol. Catal. B: Enzym. 61 (2009) 123–128.
[34] A.M. Thayer, Chem. Eng. News 84 (2006) 26–27.
[35] A. Manzocchi, A. Fiecchi, E. Santaniello, J. Org. Chem. 53 (1988) 4405–4407.
[36] V. Prelog, Pure Appl. Chem. 9 (1964) 119–130.