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Access to Optically Active Aryl Halohydrins Using a Substrate-Tolerant Carbonyl Reductase Discovered from *Kluyveromyces thermotolerans*

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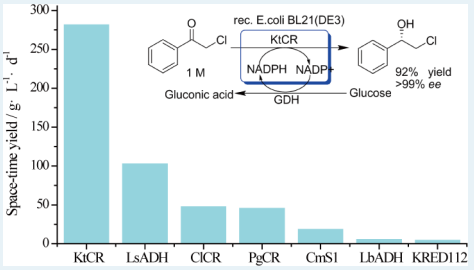
Supporting Information

ABSTRACT: By genome data mining, a carbonyl reductase tool box was designed and developed for chiral alcohol synthesis. On the basis of systematic comparison of the specific activity and substrate tolerance toward α-chloroacetophenone among reductases in this tool box, KtCR, a highly substrate-/product-tolerant carbonyl reductase from *Kluyveromyces thermotolerans*, was identified. The reduction of a series of substituted aryl ketones was investigated using this newly mined biocatalyst. Almost all of the ketones tested were asymmetrically reduced into corresponding chiral alcohols in 99% ee. Substrates with substituents adjacent to the carbonyl group or those with substituents on the para position of the phenyl ring were easier to reduce. For α-choloacetophenone as a representative substrate, as much as 154 g/L (1.0 M) of the substrate was asymmetrically reduced within merely 12 h by lyophilized cells of *Escherichia coli*/pET28-KtCR, resulting in an isolated yield of 92%, an enantiopurity of >99% ee, and a total turnover number of 5000, which was five times higher than the highest record reported so far. These results indicate the great potential of KtCR in practical synthesis of valuable aryl halohydrins as versatile chiral synthons.



KEYWORDS: *carbonyl reductase, asymmetric hydrogen transfer, aryl halohydrin, Kluyveromyces thermotolerans, genome data mining, substrate/product tolerance*

INTRODUCTION

Enantiomerically pure aryl halohydrin, an important class of chiral compounds, could be easily converted to chiral 1-phenyl-1,2-ethanediol[1−3] or 2-amino-1-arylethanol.[4] Optically active 1-phenyl-1,2-ethanediol can serve as a key intermediate for the synthesis of a variety of pharmaceutically important compounds, such as fluoxetine and β-lactam antibiotics to treat psychiatric disorders and metabolic problems.[5,6] They also work as precursors for the production of chiral biphosphines and chiral initiators for stereoselective polymerization.[7,8] Chiral phenylethanolamines have been widely used as precursors in the synthesis of β-adrenergic receptor-blocking agents, popularly known as β-blockers.

With the difference in physiological properties of optically active compounds, constant interest has been stimulated in exploring new ways to obtain single-isomer compounds. Various technologies for harvesting the single enantiomers of halohydrins have been summarized in a few recent reviews.[9−13] These optically active alcohols are commonly produced via the asymmetric transfer hydrogenation (ATH) using isopropyl alcohol, ethanol, or formic acid as the hydrogen source, at the price of adding precious metals, such as iridium, rhodium, and ruthenium as catalysts.[14−17] Although some innovative studies have been reported on the development of less expensive, more abundant, and less toxic chiral metal catalysts, such as iron nanoparticles,[18] the enantiomeric excesses of products are not yet very satisfactory. A biocatalyst-mediated reaction, among all the explored strategies, is practical and environmentally friendly because of its high enantioselectivity, mild reaction conditions, and environmental compatibility.[19−22] The most prospective enzymatic method is asymmetric transfer hydrogenation to carbonyl groups catalyzed by reductases.[23−26] In this process, not only could a high yield and an excellent enantiomeric excess be achieved but also metal waste and extreme reaction conditions for chemical catalysis could be avoided.

A lot of work has been done on the preparation of enantiopure aryl halohydrin using biocatalysts, mostly focusing on the *R* configuration by the culture of bacteria,[27,28] yeasts,[29] or plants[30] and by heterogeneously expressed ketoreductases.[31−35] The mutant of an NADH-dependent zinc-containing medium-chain alcohol dehydrogenase (PAR) was reported to catalyze 71 g/L α-chloroacetophenone into (*R*)-aryl halohydrin in pure form in 24 h, with a space-time yield of 50.4 $g{\cdot}L^{-1}{\cdot}d^{-1}$ and a total turnover number (TTN) of 461.[35] The crude enzyme of Adzuki bean was shown to reduce 15.4 g/L α-chloroacetophenone with a full conversion and >99% ee, resulting a space−time yield of 61.6 $g{\cdot}L^{-1}{\cdot}d^{-1}$.[30] Because optically active aryl halohydrins or styrene oxides, either the *S*

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or *R* configuration, are important synthetic building blocks, several enzymes with the *S* configuration preference were also reported.[32,34,36] An *S* configuration preferring alcohol dehydrogenase LsADH purified from styrene-assimilating strain *Leifsonia* sp. strain S749 was able to reduce α-chloroacetophenone at 144 g/L, resulting in a space−time yield of 104 $g{\cdot}L^{-1}{\cdot}d^{-1}$ and a TTN of 935.[35]

As an alternative to chemical catalysts, an ideal biocatalyst for the reduction of prochiral ketones should satisfy the following criteria: (i) excellent reactivity and enantioselectivity, (ii) broad substrate scope, and (iii) tolerance against a high concentration of substrate/product in a reaction mixture. Even though an ideal biocatalyst has been successfully developed, additional research on process development is still needed, such as the selection and optimization of an efficient cofactor regeneration system.[37−41]

Several biocatalysts with aryl ketone reductase activities have been identified in our laboratory by traditional screening from soil samples or plant tissues.[27,30,42] Fostered by the progress in bioinformatics and protein engineering, promising tools for biocatalysts discovery have emerged, and the discovery period of an ideal biocatalyst with desired traits was sharply shortened.[43] Herein, we report the development of a carbonyl reductase tool box with activities toward aryl ketones by a strategy of so-called genome mining. Among the reductases in this tool box, a carbonyl reductase KtCR from *Kluyveromyces thermotolerans* with a wide substrate scope and high substrate tolerance was identified. The potential of KtCR in organic synthesis was subsequently evaluated by the asymmetric synthesis of (*S*)-aryl halohydrin in a glucose dehydrogenase-coupled NADPH regeneration system.

RESULTS AND DISCUSSION

A genome mining approach was adopted to search for robust reductases that might be able to asymmetrically reduce prochiral aryl ketones to corresponding chiral alcohols with high substrate loads. In total, 30 oxidoreductases owning 45−85% identities of amino acid sequence with functionally known carbonyl reductases were selected from the NCBI database and overexpressed in *Escherichia coli* BL21 (DE3) to form a carbonyl reductase tool box. After testing their activities toward α-chloroacetophenone as a representative substrate (Supporting Information Table S1), three putative carbonyl reductases (KtCR from *K. thermotolerans* CGMCC 2.1492, PgCR from *Pichia guilliermondii* CGMCC 2.1801, and ClCR from *Clavispora lusitaniae* CGMCC 2.1597) showed relatively higher activities and excellent enantioselectivity.

Further comparison was performed among the three reductases under elevated substrate concentrations. At a low concentration (10 mM, 1.54 g/L), α-chloroacetophenone could be reduced with >99% conversion and >99% ee by KtCR, PgCR, and ClCR; however, as the substrate loading was increased up to 200 mM (30.8 g/L), 99% conversion could be achieved only with KtCR in 12 h, whereas 77% and 81% conversions were observed for PgCR and ClCR using the same amount of biocatalyst, as shown in Figure 1. More importantly, the ee of the product was kept above 99% with KtCR, but it dropped to 60% and 80% with PgCR and ClCR, indicating a higher substrate tolerance of KtCR. Therefore, the carbonyl reductase KtCR was chosen for further studies.

A protein BLAST search was done again, indicating that KtCR has moderate similarities to known carbonyl reductase; that is, 57% identity with CPADH from *Candida parapsilosis*

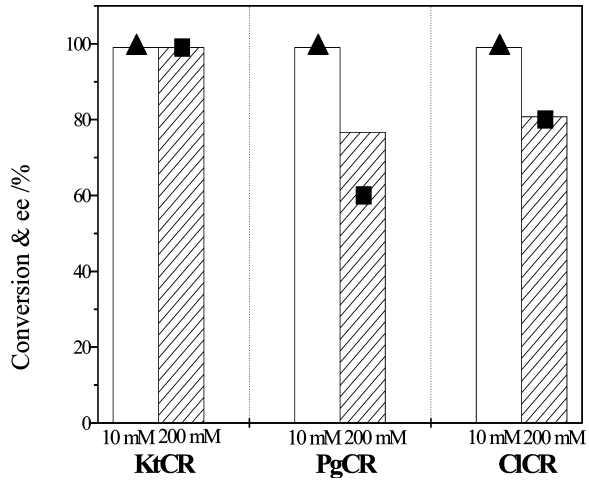
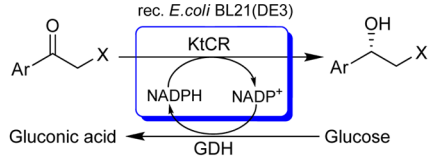


Figure 1. Conversion and enantioselectivity of KtCR-, PgCR-, and ClCR-catalyzed asymmetric reductions of α-chloroacetophenone at 10 and 200 mM. White column bar: the conversion of substrate at 10 mM; Stripped column bar: the conversion at 200 mM; triangles: ee values at 10 mM; squares: ee value at 200 mM.

CCTCC M203011[44] and PsCR from *Pichia stipitis* CBS 6054,[45] 55% identity with SCR1 from *C. parapsilosis* CCTCC M203011,[46] and 53% identity with CMS1 from *Candida magnoliae* AKU4643.[47] They all shared the characteristic sequence motifs of SDR,[48] including the glycine-rich motif Gly-X-X-X-Gly-X-Gly (X denotes any amino acids) for cofactor binding and the catalytic triad of Ser-Tyr-Lys, as shown in Supporting Information Figure S1. Therefore, KtCR was identified from the database by genome mining as a new member of the short-chain carbonyl reductase family.

To map the substrate spectrum of this newly mined carbonyl reductase KtCR, various aryl ketones of diverse structure were employed, including those with substituents adjacent to the carbonyl group (i: $\mathbf{S_1}$−$\mathbf{S_{10}}$) or with substituents on the phenyl ring (ii: $\mathbf{S_{11}}$−$\mathbf{S_{26}}$) and heteroaryl ketones (iii: $\mathbf{S_{27}}$−$\mathbf{S_{31}}$), as shown in Chart 1. Activity assay of the control, using a cell-free

Chart 1



i

	X	Ar
S_1	H	Ph
S_2	CH_3	Ph
S_3	CH_2CH_3	Ph
S_4	Cl	Ph
S_5	CH_2Cl	Ph
S_6	CH_2CH_2Cl	Ph
S_7	F_3	Ph
S_8	OH	Ph
S_9	Br	Ph
S_{10}	CN	Ph

ii

	X	Ar
S_{11}	H	2'-Cl-Ph
S_{12}	H	3'-Cl-Ph
S_{13}	H	4'-Cl-Ph
S_{14}	Cl	4'-Cl-Ph
S_{15}	Cl	2',4'-2Cl-Ph
S_{16}	Cl	3',4'-2Cl-Ph
S_{17}	H	2'-Me-Ph
S_{18}	H	3'-Me-Ph
S_{19}	H	4'-Me-Ph
S_{20}	H	2'-MeO-Ph
S_{21}	H	3'-MeO-Ph
S_{22}	H	4'-MeO-Ph
S_{23}	H	2'-CF_3-Ph
S_{24}	H	3'-CF_3-Ph
S_{25}	H	4'-CF_3-Ph
S_{26}	H	3',5'-$2CF_3$-Ph

iii

	X	Ar
S_{27}	H	pyridin-2-yl
S_{28}	H	pyridin-3-yl
S_{29}	H	pyridin-4-yl
S_{30}	H	thiophen-2-yl
S_{31}	H	thiophen-2-yl

extract obtained by expression of the pET28a vector without the KtCR gene in *E. coli* BL21 (DE3), did not show any activity toward all the substrates tested.

The specific activities on different aromatic ketones are presented in Figure 2; the conversion and enantiomeric excess of product are shown in Figure 3. Almost all the tested aryl ketones and heteroaryl ketones could be asymmetrically

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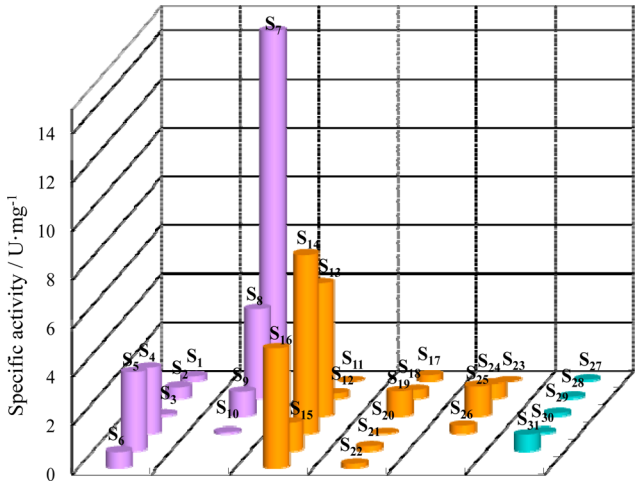


Figure 2. Specific activity of KtCR toward various aryl ketones. The unit of specific activity was μmol·min^{-1}·mg^{-1} purified protein. The activity was determined by standard assay protocol. (i) $\mathbf{S_1}$–$\mathbf{S_{10}}$, (ii) $\mathbf{S_{11}}$–$\mathbf{S_{26}}$, (iii) $\mathbf{S_{27}}$–$\mathbf{S_{31}}$.

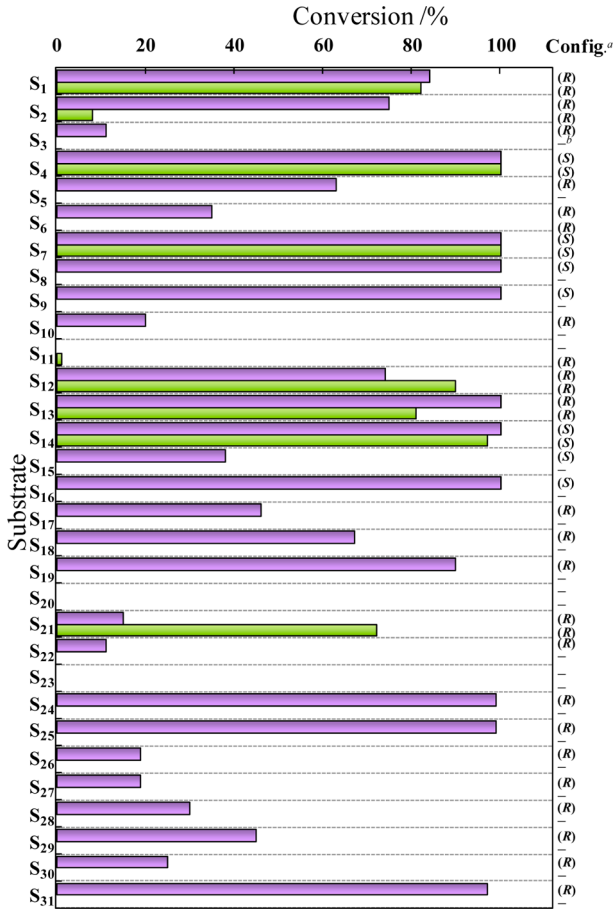


Figure 3. Conversion and enantiomeric excess of KtCR-catalyzed asymmetric H-transfer reduction of aryl ketone derivatives, compared with those of LsADH. The data of LsADH were cited from the literature.[35,37] Reactions for KtCR were carried out as follows: 10 mM substrate, 10 U purified KtCR, 10 U GDH, 15 mM glucose, and 0.5 mM $NADP^+$ were mixed, incubated and shaken at 900 rpm and 30 °C for 12 h. Purple bar, KtCR; green bar, LsADH. The ee value of all the detected products was more than 99%. [a]The absolute configuration was determined as shown in experimental section. [b]No product was detected or the related data of LsADH were not availiable.

reduced by KtCR, following the anti-Prelog's rule with an *R* preference (note that products of $\mathbf{S_4}$, $\mathbf{S_7}$, $\mathbf{S_8}$, $\mathbf{S_9}$, $\mathbf{S_{14}}$, $\mathbf{S_{15}}$, and $\mathbf{S_{16}}$ were of the *S* configuration because of the Cahn–Ingold–Prelog priority). Moreover, the catalytic activity of KtCR was dependent on the substrate structure and the electronic effects of the relevant substituents.

(i) It is interesting to notice that the activities toward α-substituted aryl ketones ($\mathbf{S_2}$–$\mathbf{S_{10}}$, 0.08–14 U/mg), with either electron-withdrawing or electron-donating groups, were higher than the nonsubstituted acetophenone ($\mathbf{S_1}$, 0.22 U/mg), except for butyrophenone ($\mathbf{S_3}$, 0.09 U/mg) and benzoyacetonitrile ($\mathbf{S_{10}}$, 0.08 U/mg). The reduction activity of KtCR was also affected by the length of the side chain, with a tendency of C3 > C2 > C4 ($\mathbf{S_1}$, $\mathbf{S_2}$, $\mathbf{S_3}$ or $\mathbf{S_4}$, $\mathbf{S_5}$, $\mathbf{S_6}$). Electron-withdrawing chloro group substitution in the α-position of the carbonyl group makes it easier for the substrate to accept hydrogen ($\mathbf{S_1}$, $\mathbf{S_4}$; $\mathbf{S_2}$, $\mathbf{S_5}$; $\mathbf{S_3}$, $\mathbf{S_6}$), even though the β- or δ-halogenated structure is not so stable.

(ii) Different substituents on the aryl ring of substrates endue the aryl ketones with different electronic effects. Among *o*-, *m*-, and *p*-substituted acetophenones, the *o*-substituted derivatives were always the poorest substrates for KtCR. The substitution at the *o*-position might have steric effects on the hydrogen attack from the electron donator, NADPH, on the carbonyl group. Consequently, KtCR shows the lowest activity toward the substrates with the *o*-substituent on aryl ring ($\mathbf{S_{11}}$, $\mathbf{S_{20}}$, $\mathbf{S_{17}}$, and $\mathbf{S_{23}}$, 0.01–0.27 U/mg), which was in agreement with the report of SCR1.[46] The activity was higher for aryl ketones with a *p*-substitutent than that with an *o*- and *m*-substituent, which is similar to YMR226c from *Saccharomyces cerevisiae*.[49] In view of the electronic effect, substrates with electron-withdrawing groups are generally better for KtCR than those with electron-donating substituents, with relatively higher activities.

With regard to multisubstituted aryl ketones, higher specific activity was detected for the substrate with 1-(4′-chlorophenyl)-2-chloroethanone ($\mathbf{S_{14}}$, 7.4 U/mg), and relatively lower activities were observed for substrates with another substitutent at the ortho or meta position ($\mathbf{S_{15}}$ and $\mathbf{S_{16}}$, 1.2 and 4.9 U/mg). A substituent at the para position of the aryl ring usually facilitates the hydrogenation process, whereas another substitutent at the ortho or meta position may have a negative effect due to the steric hindrance.

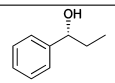
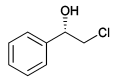
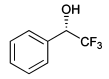
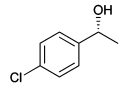
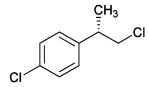
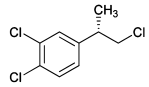
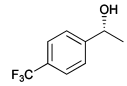
(iii) Heteroaryl ketones ($\mathbf{S_{27}}$–$\mathbf{S_{31}}$) could also be asymmetrically reduced with lower activity (0.08–0.7 U/mg) but excellent enantioselectivity. The farther the acetyl group was away from the heteroatom, the more accessible the substrate was for reductases to reduce.

It is worth noting that almost all of the tested aryl ketones, especially aryl α-haloketones, could be reduced to essentially optically pure alcohols with excellent enantioselectivity. Some of the products have been used as valuable intermediates for the synthesis of biologically active compounds. For example, (*S*)-$\mathbf{P_2}$ is used for the β-blocker Sotalol;[50] (*R*)-$\mathbf{P_{10}}$ is for antidepressant fluoxetine, tomoxetine, and nisoxetine;[51] (*S*)-$\mathbf{P_{26}}$ is for Aprepitant;[52] (*R*)-$\mathbf{P_{25}}$ is for the fungicide Econazole,[53] and a derivative of $\mathbf{P_{28}}$ is needed for the synthesis of PNU-142721, a drug candidate for treatment of AIDS.[54]

To test the potential of KtCR in organic synthesis, eight carbonyl compounds that showed relatively higher specific activity were selected for asymmetric reduction at a higher substrate load of 0.2 M. With the assistance of glucose dehydrogenase from *Bacillus subtilis*[55] for cofactor regeneration, all the substrates tested were easily reduced to the

corresponding chiral alcohols with >99% conversion and >99% ee at 30 °C within 12 h (Table 1). The products were isolated from the reaction mixture and further validated through ^{1}H NMR (see the Supporting Information).

Table 1. Preparation of Chiral Aryl Alcohols with KtCR

entry	ketone	alcohol	time [h]	conv [%]	yield [%]	ee [%]
1	S_2	 P_2	12	>99	92	>99
2	S_4	 P_4	6	100	90	>99
3	S_7	 P_7	3	100	92	>99
4	S_{13}	 P_{13}	8	>99	87	>99
5	S_{14}	 P_{14}	3	>99	93	>99
6	S_{16}	 P_{16}	12	>99	87	>99
7	S_{25}	 P_{25}	12	>99	86	>99
8	S_{31}	 P_{31}	12	>99	89	>99

[a]Conditions: substrate (2.0 mmol), D-glucose (3.0 mmol), GDH (400 U), DMSO (5%), KtCR (400 U) and $NADP^+$ (5.0 μmol), 10 mL phosphate buffer (pH 6.5, 0.5 mmol). Reactions were carried out at 30 °C until completion. [b]Conversion and ee were determined by GC analysis as shown in the Supporting Information.

Furthermore, the bioreaction of α-chloroacetophenone (S_4) was optimized at the gram scale, aiming to meet the industrial demands of ≥100 g/L substrate loading and ≤0.5 mM $NADP^+$ addition.[56] Without external cofactor addition to the reaction system (Table 2, entry 3), the conversion of S_4 was only 84%, and it could not be improved by extending the reaction time. This may be ascribed to the enzyme inactivation caused by a high concentration of the substrate and product.[28] On the other hand, the reaction temperature may also account for the enzyme inactivation, which is easily regulated.[57] As expected, the reaction conversion was improved up to >99% when the reaction temperature was lowered from 30 to 25 °C. This inspired us to raise the substrate load to 100 g/L (0.65 M), resulting in 94% conversion (Table 2, entry 5). Therefore, the reaction temperature was further decreased down to 20 °C (Table 2, entry 6), affording >99% conversion after 12 h. When the substrate load was further increased up to 154 g/L (1.0 M) (entry 7), 92% conversion was observed at 20 °C. Although further decrease of temperature might give a complete conversion, too low a temperature was considered disadvantageous for industrial production, so we tried to regulate the biocatalyst and cofactor loads. When the concentration of KtCR and $NADP^+$ were slightly increased to 1.2 kU/mL and 0.2 mM, as shown in entries 8 and 9 of Table 2, the reaction was completed within 12 h, giving an isolated yield of 92%,

Table 2. Asymmetric Reduction of α-Chloroacetophenone (S_4) with KtCR[a]

entry	ketone (M)	$NADP^+$ (mM)	KtCR (kU/L)	temp (°C)	time (h)	conv (%)[b]	yield (%)	ee (%)[b]
1	0.20	0.5	40	30	6	100	90	>99
2	0.50	0.5	100	30	6	100	87	>99
3	0.50	0.0	100	30	24	84	74	>99
4	0.50	0.0	100	25	6	100	93	>99
5	0.65	0.0	100	25	8	94	80	>99
6	0.65	0.0	100	20	12	99	94	>99
7	1.0	0.0	100	20	24	92	69	>99
8	1.0	0.0	120	20	16	94	69	>99
9	1.0	0.2	120	20	12	99	92	>99

[a]Conditions: Substrate S_4 (2−10 mmol), D-glucose (1.5 equiv vs substrate), GDH (1.0 equiv vs KtCR), DMSO (5%), KtCR (0.33−1.0 g, 1.2 kU/g DCW) and $NADP^+$ (0−5 μmol), 10 mL phosphate buffer (pH 6.5, 0.5 mmol). Reactions were carried out at varied temperatures until no further conversion. [b]Conversion and ee were determined by GC analysis.

>99% ee, a space−time yield of 285 $g{\cdot}L^{-1}{\cdot}d^{-1}$, and a TTN of 5000, which is pretty high as compared with the literature.[35]

Because of the severe toxicity of aromatic ketones to microbial cells, the substrate loads in the bioreductions reported were usually very low, typically less than 10 g/L, such as LBADH, CMCR, KRED112 (Table 3).[31,33,34] In addition, the enantiopurity of products might have decreased to some extent as the substrate loading was increased if the reductase, such as PgCR and ClCR, could not tolerate a high concentration of aromatic ketones (Table 3). In contrast, LsADH could catalyze the asymmetric reduction of α-chloroacetophenone at a substrate load of 144 g/L with a high enantiomeric excess but a lower yield of 72%. In this work, KtCR could catalyze the asymmetric reduction of α-chloroacetophenone at a maximal load of 154 g/L without any loss of enantiomeric excess, making it very competitive and promising for practical applications in asymmetric bioreduction of various carbonyl compounds.

Both LsADH and KtCR displayed excellent performance in the asymmetric reduction of α-chloroacetophenone. Their catalytic difference in the preparation of chiral aryl alcohols arouses our interest. To the best of our knowledge, the substrate scope of LsADH toward aryl ketones had been systematically investigated by Itoh et al.[35,58] A comparison of the substrate preference between KtCR and LsADH was carried out, as shown in Figure 3. Both reductases could catalyze the asymmetric bioreduction of aryl ketones with very high enantioselectivity following the anti-Prelog rule; however, there still exist some differences between them in the substrate preference. For instance, LsADH prefers acetophenone (S_1) to propiophenone (S_2), and m-substituted acetophenone to p-substituted ones (S_{13}, S_{14}), whereas completely reversed results were observed for KtCR. Relatively higher conversion rates were obtained in the reductions of propiophenone (S_2) and 4′-chloroacetophenone (S_{14}) with KtCR. In addition, with regard to the cofactor recycling, a substrate-coupled mode was employed for LsADH, and an enzyme-coupled system was used for KtCR. However, the coexpression of cofactor regeneration enzymes (e.g., glucose dehydrogenase) and carbonyl reductase together could also facilitate the enzyme-coupled bioreduction with in situ cofactor regeneration.[59] Hence, a novel carbonyl reductase, KtCR, was successfully

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Table 3. Comparison on Asymmetric Reduction of α-Chloroacetophenone

entry	enzyme	substrate load (g/L)	time (h)	conv (%)	ee (%)/ config	STY[a] ($g \cdot L^{-1} \cdot d^{-1}$)	TTN	ref
1	LBADH	7.7	24	90	99 (*S*)	6.9	50	31
2	CMS1	10	overnight	100	99 (*S*)	20	270	36
3	KRED 112	4	overnight	72	99 (*S*)	5.8	216	34
4	LsADH	144	24	72[b]	>99 (*S*)	104	935	35
5	KtCR	154	12	99 (92)[b]	>99 (*S*)	283	5000	this work
6	PgCR	30.8	12	77	60 (*S*)	47	154	this work
7	ClCR	30.8	12	81	80 (*S*)	49	162	this work

[a]STY is an abbreviation of space−time yield ($g \cdot L^{-1} \cdot d^{-1}$). [b]This value refers to isolation yield.

discovered and heterogeneously overexpressed in *E. coli* BL21(DE3), with a great potential for preparation of chiral aryl alcohols.

CONCLUSION

In summary, a tool box of carbonyl reductases was developed by the genome data mining strategy. Three carbonyl reductases were discovered with relatively higher activity on α-chloroacetophenone as a model substrate. Further tests at 0.2 M substrate loading revealed that KtCR, a new NADPH-dependent carbonyl reductase mined from *K. thermotolerans* CGMCC 2.1492, showed the highest substrate tolerance. The substrate spectrum of this recombinant reductase was systematically evaluated using aryl ketones with diverse structures. KtCR exhibits varied activities (0.01−14 U/mg protein) toward nearly all the tested aryl ketones and heteroaryl ketones, obeying the anti-Prelog's rule. Among them, eight derivatives of acetophenone were reduced with excellent enantioselectivity. After a simple optimization of the reaction temperature, as much as 1.0 M (154 g/L) of α-chloroacetophenone could be asymmetrically reduced with 92% isolated yield and >99% ee, indicating the great potential of KtCR for preparative synthesis of (*S*)-aryl halohydrins.

ASSOCIATED CONTENT

Supporting Information

Experimental details, genome data mining results, catalytic performance of KtCR, analytical methods, and ^{1}H NMR data. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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