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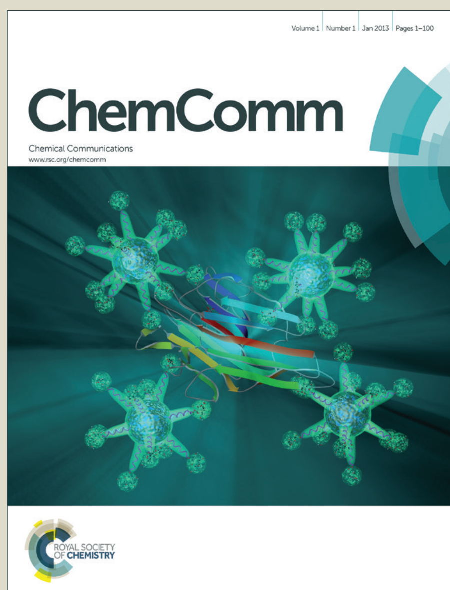

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Identification of key residues in *Debaryomyces hansenii* carbonyl reductase for highly productive preparation of (*S*)-aryl halohydrins

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Key residues of *Debaryomyces hansenii* carbonyl reductase in dertermination the reducing activity towards aryl haloketones were identified through combinantorial mutation of conserved residues. This study provides a green and efficient biocatalyst for the synthesis of (*S*)-aryl halohydrins.

Optically active aryl halohydrins are well known as important building blocks for the synthesis of pharmaceutical agents, agrochemicals and natural products.[1] Based on the three key functional groups (*i.e.*, hydroxyl, halogen and phenyl groups), aryl halohydrins can be readily converted into a broad range of useful building blocks, such as epoxides, hydroxyl acids, hydroxynitriles and azides.[2,3]

The asymmetric reduction of prochiral ketones in the presence of a biological or chemical catalyst is one of the most straightforward and efficient approaches for direct synthesis of chiral halohydrins, with theoretical yields of up to 100%.[4] Halohydrins are generally prepared by transfer hydrogenation of the corresponding α-haloketone in the presence of a transition metal catalyst such as iridium, ruthenium or rhodium.[5,6] However, the enantioselectivities of these processes are usually moderate and the halogen groups can be easily reduced under the catalytic conditions to give the corresponding des-halo products. Bioreduction of aryl haloketones employing stereospecific carbonyl reductases appears to be a much more effective strategy for the preparation of chiral halohydrins based on its inherent advantages, including its stereoselectivity, ambient reaction condition and environmental friendliness.[7,8] Among these reported enzymes, the short-chain dehydrogenase/reductase from *Leifsonia* sp. S749 has been reported to display the highest activity ever recorded towards α-aryl haloketone (2.97 U mg_{prot}^{-1}).[9] We've recently discovered one carbonyl reductase, namely *Dh*CR from *Debaryomyces hansenii*, exhibited a specific activity of 2.1 U mg_{prot}^{-1} towards α-aryl haloketone. As much as 100 g L^{-1} of α-aryl haloketone could be asymmetrically reduced to (*S*)-aryl chlorohydrin using 30 g L^{-1} of recombinant *Dh*CR dry cells with a productivity (substrate-to-catalyst ratio, S/C) of about 3.3 $g_{substrate}$ $g^{-1}_{biocatalyst}$. The low catalytic efficiency of this system would therefore require large quantities of catalyst to achieve full conversion at high substrate loading. To be amenable to the large-scale manufacture of chiral building blocks, an ideal biocatalyst should possess certain properties, including a wide substrates scope, high enantioselectivity, robust tolerance towards organic compounds and high activity (most importantly).[9] Research directed towards engineering the activity of *Dh*CR to allow for the efficient reduction of α-aryl haloketone is therefore urgently required. Since most of the naturally evolved reductases displayed low activity to α-aryl haloketone, we supposed that conserved residues in substrate binding pocket were key residues in determining catalytic efficiency.

A homology model of *Dh*CR was constructed based on the structures of the gluconate 5-dehydrogenase from *Thermotoga maritime* MSB8 (PDB no. 1VL8), carbonyl reductase from *Candida parapsilosis* (3CTM) and NADP-dependent mannitol dehydrogenase from *Cladosporium herbarum* (3GDF).[10] The substrate binding pocket consisted of 15 residues, including P122, W123, E125, S174, N179, V180, S214, P215, G217, Y218, I223, S224, D225, F226 and V227, with all of the residues lying in the flexible loop regions. The consensus was analysed using 20 published or putative reductases with less than 40% sequence identity. Residues E125, D225, F226 and V227 were found to be variable, whereas the other residues showed consensus, especially the S214, P215, G217 and Y218 residues, which were highly conserved (**Fig. 1**). To obtain variants with remarkably improved activity, the 10 relative consensus residues were regarded as potential hot spots for the combinatorial active site semi-saturation test and then divided into five groups, including group A (P122 & W123), group B (S174, N179), group C (S214 & P215), group D (G217 & Y218) and group E (I223 & S224), based on their locations in the loops. Five mutant libraries were constructed with more than 600 variants in each library. All of the libraries were tested by high throughput screening and mutant

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with greater activity than that of the wild type *Dh*CR were selected and shown in **Fig. 1(A-F)**.

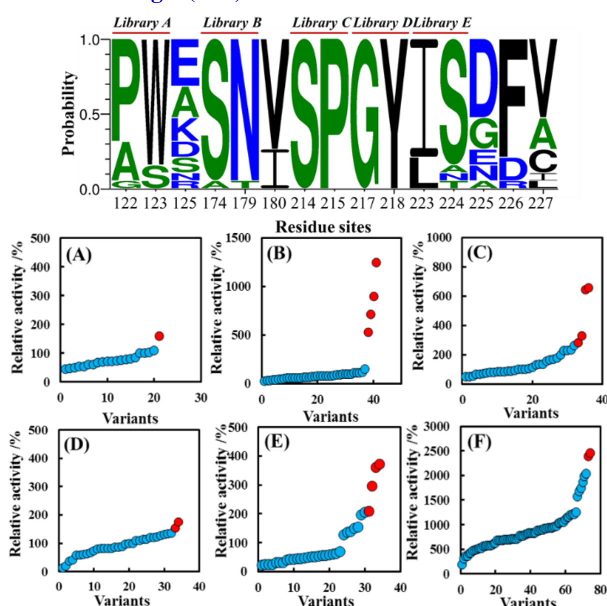

Fig. 1 Consensus analysis of residues around substrate tunnel and HTS result of CASTing and shuffling libraries.

As shown in **Fig. 1(A)** for library A, only 21 mutants were identified with the ability to reduce of α-chloroacetophenone. The best mutant (P122T/W123C), designated as $Dh\mathrm{CR_{V1}}$, displayed the highest catalytic efficiency (**Table 1**), with specific activity of 17.7 U mg^{-1} purified $Dh\mathrm{CR_{V1}}$. Residues P122 and W123 were part of the large substrate binding pocket. The mutation provided a much larger space and a polar hydroxyl group for the binding of the α-chloroacetophenone.

Distinct improvements in the activity were found in library B, with some variants providing a 5-fold increase in activity relative to the *Dh*CR. For short chain dehydrogenases/reductases, the catalytic triad is made up of the S172, Y187 and K191. The S174 and N179 residues were located in close proximity to the catalytic amino acids, where they formed a small pocket for the binding of the substrate. Mutations in these sites could therefore have a significant effect on the catalytic activity of the enzyme. The improved variants were rechecked and named as $Dh\mathrm{CR_{V2}}$ (S174G) and $Dh\mathrm{CR_{V3}}$ (N179S). The specific activity of purified $Dh\mathrm{CR_{V3}}$ with single mutation was determined to be 84.1 U $\mathrm{mg_{prot}}^{-1}$, which was about 40-fold greater than that of *Dh*CR. This variant also displayed a high affinity for α–chloroacetophenone, with a K_M value of 0.41 mM, which was much lower than that of *Dh*CR. The k_{cat} and k_{cat}/K_M values for $Dh\mathrm{CR_{V3}}$ were 119 s^{-1} and 295 s^{-1} mM^{-1}, respectively. Based on the modelled structure of this enzyme, residue N179 was situated at the bottom of a small substrate binding pocket in close proximity to the catalytic residues S172 and Y187. It has been reported that the SCRI from *C. parapsilosis* reacted efficiently with acetophenone derivatives and exhibited a specific activity of 4.23 U $\mathrm{mg_{prot}}^{-1}$ towards α-hydroxyacetophenone, which was structurally similar to that of α-chloroacetophenone, making it one of the most robust reductases currently known for the asymmetric reduction of α-hydroxyacetophenone.[11] The consensus analysis results are shown in **Fig. 1(B)** for the N179 residue in *Dh*CR and the corresponding T179 residue in SCRI, which represent the only differences between the 20 amino acid residues in the tunnels and active sites of these reductases. The substrate binding energy of the N179S mutant was calculated using molecular docking, which showed a decrease from −4.8 to −5.9 kcal/mol compared with *Dh*CR. This increased binding ability of the N179S mutant for the asymmetric reduction of α-chloroacetophenone inspired us to introduce similar mutation into other carbonyl reductases. *Kt*CR was previously reported by our group with stable performance in the reduction of α-chloroacetophenone and the amino acid sequence identity compared with *Dh*CR was 60%. However, the specific activity of *Kt*CR was only 2.73 U $\mathrm{mg_{prot}}^{-1}$. The subsequent site directed mutation of the N181 residue of *Kt*CR to Ser to form $Kt\mathrm{CR_{V1}}$ (N181S) led to an increase in the specific activity and k_{cat}/K_M values towards α-chloroacetophenone to 65.4 U $\mathrm{mg_{prot}}^{-1}$ and 247 s^{-1} mM^{-1}, respectively (**Table 1**).

Table 1 Kinetic parameters of wide-type *Dh*CR and its variants.

Enzyme	Activity [U mg^{-1}]	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} mM^{-1}]
*Dh*CR	2.13±0.05	2.25±0.03	3.95±0.08	1[illegible]
$Dh\mathrm{CR_{V1}}$(P122T/W123C)	17.2±0.1	0.79±0.01	22.2±1	27.5
$Dh\mathrm{CR_{V2}}$(S174G)	14.2±0.1	1.31±0.01	21.8±0.1	14.2
$Dh\mathrm{CR_{V3}}$(N179S)	84.1±0.4	0.40±0.01	119±1	2[illegible]
$Dh\mathrm{CR_{V4}}$(I214F/S215G)	9.67±0.11	6.21±0.05	14.8±0.1	2.39
$Dh\mathrm{CR_{V5}}$(I214F/S215C)	5.88±0.22	6.80±0.05	10.8±0.1	1.59
$Dh\mathrm{CR_{V6}}$(I214F/S215V)	12.9±0.1	10.2±0.1	30.0±0.2	2.95
$Dh\mathrm{CR_{V7}}$(I223F)	4.02±0.03	2.31±0.02	6.20±0.02	2.69
$Dh\mathrm{CR_{V8}}$(I223F/G242R)	8.42±0.06	2.36±0.01	13.0±0.1	5.5[illegible]
$Dh\mathrm{CR_{V9}}$ (N179S/I214F/S215G)	104±1	1.45±0.01	204±1	140
*Kt*CR	2.73±0.04	2.08±0.02	7.31±0.12	3.52
$Kt\mathrm{CR_{V1}}$(N181S)	65.4±0.2	0.49±0.01	105±1	247

Some of the variants in library C showed a 4- to 6-fold increase in activity compared with *Dh*CR. The specific activities of $Dh\mathrm{CR_{V4}}$ (I214F/S215G), $Dh\mathrm{CR_{V5}}$ (I214F/S215C) and $Dh\mathrm{CR_{V6}}$ (I214F/S215V) were 5.88–12.9 U mg^{-1}. However, these three variants displayed much higher K_M values (6.21–10.2 mM) than *Dh*CR (2.25 mM), which highlighted the importance of the S215 residue in binding of substrate possibly by the interaction between hydroxyl side chain of S215 and chloro group of α-chloroacetophenone.

Almost all of the variants in library D were inactivated towards α-chloroacetophenone or showed, at best, similar activity to *Dh*CR. As shown in **Fig. 1(D)**, the G217 and Y218 residues were highly conserved among the short-chain dehydrogenases/reductases, with a mutation in either of these two residues leading to a loss of activity.

In library E, $Dh\mathrm{CR_{V7}}$ (I223F) and $Dh\mathrm{CR_{V8}}$ (I223F/G242R) performed more effectively than *Dh*CR. The G242R mutation was caused by the misreading of the polymerase. The specific activity of $Dh\mathrm{CR_{V7}}$ was 4.02 U mg^{-1}, which was 2-fold greater than that of *Dh*CR, while the K_M of $Dh\mathrm{CR_{V7}}$ (2.3 mM) was the same as *Dh*CR.

A gene shuffling library (library F) was developed based on the templates of the best variants in each library. In most cases, the shuffling variants showed higher activity than *Dh*CR. Rescreening revealed that the relative activity of the best variant, $Dh\mathrm{CR_{V9}}$ (N179S/I214F/S215G), was 24-fold higher than that of *Dh*CR. The specific activity and k_{cat} of purified $Dh\mathrm{CR_{V9}}$ were 104 U $\mathrm{mg_{prot}}^{-1}$ and 204 s^{-1}. The I214F and S215G mutations contributed to improvements in the catalytic activity of the enzyme compared with $Dh\mathrm{CR_{V3}}$ (N179S), while also led to an increase in the K_M from 0.4

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to 1.45 mM. Hence, k_{cat}/K_M of *Dh*CR$_{V9}$ to α-chloroacetophenone was decreased to 140 s^{-1} mM^{-1}.

The enzymatic properties of *Dh*CR$_{V1}$, *Dh*CR$_{V3}$ and *Dh*CR$_{V9}$ were fully characterized. The optimum pH values for all three enzymes were around 6.5. There was very little difference between *Dh*CR and the three variants in optimum temperature and thermostability (details in ESI). All of the mutations in these enzymes were positioned around their internal tunnel structures, which indicated that they could be having a significant influence on the substrate specificities. The substrate spectra of *Dh*CR and its variants were compared using 20 different ketone substrates, including aryl ketones, heteroaryl ketones, aliphatic ketones and keto-esters. From *Dh*CR to *Dh*CR$_{V1}$, *Dh*CR$_{V3}$ and *Dh*CR$_{V9}$, the areas covered by the substrate profiles in the radar scheme expended increasingly, which indicated the enhanced reducing ability of the enzymes towards the prochiral ketones tested in the current study. In a similar manner to *Dh*CR, the substrate specificity of *Dh*CR$_{V1}$ displayed higher activity towards keto-esters than aryl ketones. However, *Dh*CR$_{V3}$ and *Dh*CR$_{V9}$ showed a preference for aryl ketones over keto-esters, as shown in **Fig. S7**. *Dh*CR$_{V9}$ was found to be more active towards aryl ketone substrates bearing a substituent at the *p*- or *m*-position on their phenyl ring compared with aryl ketones bearing an *o*-substituent or no substituent at all. The most suitable substrates were found to be different among the four reductases. The maximum specific activity towards α-chloroacetophenone was exhibited by *Dh*CR$_{V1}$ and *Dh*CR$_{V3}$, whereas *Dh*CR$_{V9}$ and *Dh*CR showed a preference for 4-chloroacetophenone and ethyl 4-chloro-3-oxobutanate, respectively.

Based on its high catalytic efficiency and wide substrate scope, *Dh*CR$_{V9}$ was selected for further investigation to evaluate its ability in asymmetric reduction of α-chloroacetophenone. *Dh*CR$_{V9}$ and glucose dehydrogenase from *Bacillus megaterium* were coexpressed in *E. coli* BL21(DE3) by tandem ligation in one plasmid to achieve internal cofactor regeneration. A biphasic toluene/buffer system was used to improve the dispersion of α-chloroacetophenone and reduce its cytotoxicity. As shown in **Fig. 2**, 100 g L^{-1} of α-chloroacetophenone was converted to (*S*)-α-chloroacetophenol using *Dh*CR and *Dh*CR$_{V9}$ without the addition of external $NADP^+$. The use of this newly identified *Dh*CR$_{V9}$ provided several advantages for the preparation of chiral (*S*)-aryl chlorohydrin. First, only 7.0 g L^{-1} of the *Dh*CR$_{V9}$ catalyst was required to achieve this transformation, compared with 30 g L^{-1} of *Dh*CR. However, when the amount of *Dh*CR$_{V9}$ catalyst added was further decreased to 5.0 g L^{-1}, the conversion was reduced to only 80%. The internal amount of $NADP^+$ in the *E. coli* cells was estimated to be about 1.86 μmol/g_{dcw}.[12a] Based on this estimation, there would be 13.0 μmol·L^{-1} of $NADP^+$ in 7.0 g L^{-1} of *Dh*CR$_{V9}$ and 55.8 μmol·L^{-1} in 30 g L^{-1} of *Dh*CR. The low conversion may therefore have been caused by the low concentration of $NADP^+$, which would work as the driving currency in this system, since it was not supplemented with external $NADP^+$. Although the addition of $NADP^+$ could promote the reaction, it would also lead to a significant increase in the production costs. Consequently, considering the high price of $NADP^+$, 100 g L^{-1} of α-chloroacetophenone versus 7.0 g L^{-1} of *Dh*CR$_{V9}$ was regarded as the best S/C ratio (14.3 $g_{substrate}$ $g^{-1}_{biocatalyst}$) (**Table 2**). Second, only 6 h was needed for *Dh*CR$_{V9}$ to achieve >99% conversion, which was much less than the 24 h required for *Dh*CR to reach the same level of conversion. Third, the optical purity of the (*S*)-α-chloroacetophenol (>99% *ee*) produced by *Dh*CR$_{V9}$ was the same as that produced by *Dh*CR, which indicated that the mutation had no discernible impact on the stereospecificity of the enzyme (details in ESI).

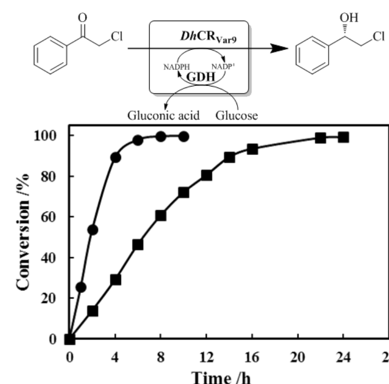

Fig. 2 Asymmetric reduction of 100 g L^{-1} α-chloroacetophenone employing *Dh*CR (30 g L^{-1}, ■) and *Dh*CR$_{V9}$ (7 g L^{-1}, ●).

To further improve the utilization efficiency of *Dh*CR$_{V9}$ towards the asymmetric synthesis of (*S*)-aryl chlorohydrins, we investigated the immobilization of recombinant *E. coli* BL21/pET28-*bmgdh-dhcrv9* for repeated use. Given that this system is a two-enzyme coupled and cofactor-dependent, immobilization cells was considered to be a feasible strategy. [13] Following optimization, the PEI and 2% glutaraldehyde were added to the fermentation broth and the mixture was further shaken for the cross-linking (**Fig. S9**). After about 0.5 h, the color of the *E. coli* cells turned from yellow to red and the cross-linked cells were collected by centrifugation at 4000 × *g* (**Fig. 3**). The activity recovery was about 48.6% of the free cells. The optimal temperature for cross-linked *E. coli* BL21(DE3)/pET28-*bmgdh-dhcrv9* (*clDh*CR$_{V9}$) was 55 ℃. Furthermore, *clDh*CR$_{V9}$ was found to be stable at 30 ℃ and 900 rpm for 96 h without any significant loss in its activity. Under the same condition, the optimal temperature of free whole cells of *E. coli* BL21(DE3)/pET28-*bmgdh-dhcrv9* (*freeDh*CR$_{V9}$) was 30 ℃, and the cells could only be active at this temperature for 48 h (**Fig. S10**).

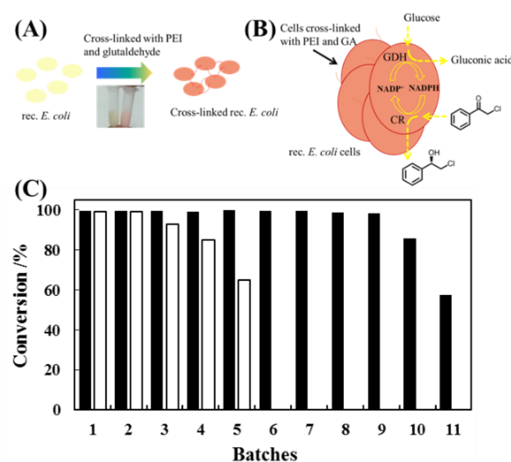

Fig. 3 Scheme for the cross-linking of recombinant *E. coli* for preparation of (*S*)-aryl chlorohydrins and comparison of operational stability between *freeDh*CR$_{V9}$ (□) and *clDh*CR$_{V9}$ (■) in repeated batch reactions.

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Table 2 Comparison of biocatalyst efficiency in asymmetric reduction of α-chloroacetophenone.

Entry	Enzyme	Activity [U mg^{-1}] [a]	Sub. concn. [g L^{-1}]	Time [h]	Conv. [%]	*ee* [%]/[*R*/*S*]	S/C [g g^{-1}] [b]	STY [g L^{-1} d^{-1}] [c]	TTN of cofactor [d]	Ref.
1	LBADH	–	7.7	24	90	99/*S*	–	6.9	–	[14a]
2	CMCR	0.818	10	12	100	99/*S*	–	20	270	[14b]
3	KRED112	1.424	4	12	72	99/*S*	–	5.8	216	[14c]
4	*Ls*ADH	2.97	144	24	72	>99/*S*	–	104	935	[9]
5	*Kt*CR	2.73	154	12	>99	>99/*S*	1.93	308	5000	[12b]
6	*Dh*CR	2.13	100	24	>99	>99/*S*	3.33	100	11637 [f]	This work
7	*Dh*CR$_{V9}$	104	100	6	>99	>99/*S*	14.3	400	49873 [f]	This work
8	*clDh*CR$_{V9}$	104	450	36	>99	>99/*S*	22.5 [e]	300	73550 [f]	This work

Note: [a] specific activity of purified enzyme; [b] g g^{-1}: g$_{substrate}$ g$^{-1}_{biocatalyst}$; [c] STY: space-time yield; [e] Total turnover number per cofactor (NADPH) of reductase; [e] biocatalyst amount was calculated using dried cells; [f] NADP$^+$ content was *ca.* 1.86 μmol·g^{-1} DCW.

We investigated the repeated batch operation of the *freeDh*CR$_{V9}$ and *clDh*CR$_{V9}$ in a 100-mL biphasic toluene/buffer system with mechanical agitation. Each batch was carried out with 5.0 g of α-chloroacetophenone in 50 mL toluene, 10.0 g of *clDh*CR$_{V9}$ and 1.5 equiv. of glucose in 50 mL of KPB (pH 6.5, 100 mM) at 30 ℃ and 120 rpm for 4 h. The reaction was terminated by separating the organic and aqueous phases. Reactions with 99% conversion were treated with an additional 5.0 g of α-chloroacetophenone in 50 mL of toluene and 1.0 equiv. of glucose. Only three repeated batches with >90% conversion could be achieved with *freeDh*CR$_{V9}$, while the use of *clDh*CR$_{V9}$ allowed for nine consecutive runs with >99% conversion and >99% *ee* for (*S*)-aryl chlorohydrin (**Fig. 3**). The space-time yield and TTN of the cofactor were 300 g L^{-1} d^{-1} and 78550, respectively. The use of *clDh*CR$_{V9}$ in batch mode allowed for a further improvement in the productivity to 22.5 g$_{substrate}$ g$^{-1}_{biocatalyst}$ compared with 14.3 g$_{substrate}$ g$^{-1}_{biocatalyst}$ of *freeDh*CR$_{V9}$.

In summary, key residues in determination the reducing activity of short chain dehydrogenases/reductases towards α-chloroacetophenone were identified and applied in similar protein. These results confirmed the potential feasibility of engineering *Dh*CR for the highly productive synthesis of enantiopure (*S*)-aryl chlorohydrins.

Notes and references

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