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Thermoanaerobacter ethanolicus secondary alcohol dehydrogenase mutants with improved racemization activity

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ABSTRACT

Controlled racemization of enantiopure alcohols is a key step in dynamic kinetic resolution. We recently reported the racemization of enantiopure phenyl-ring-containing alcohols using W110A *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase (W110A TeSADH), which relies on selectivity mistakes. Trp-110 lines the large pocket of the active site of TeSADH, which allows W110A TeSADH mutant to accommodate phenyl-ring-containing substrates in Prelog mode, albeit with selectivity mistakes. Here, we report the racemization of enantiopure phenyl-ring-containing alcohols using several Trp-110 mutants of TeSADH in the presence of the oxidized and reduced forms of nicotinamide–adenine dinucleotide. We observed a noticeable enhancement in racemization efficiency when W110G TeSADH was used compared with W110Q, W110M, W110L, W110I, and W110V. This observation was anticipated because the W110G mutation is expected to open the large pocket of the active site to a greater extent compared to other mutants of TeSADH at W110. Both enantiomers of 1-phenyl-2-propanol and 4-phenyl-2-butanol were fully racemized by W110G TeSADH. We also constructed a triple mutant of TeSADH, W110A/I86A/C295A, by site-directed mutagenesis with the aim of opening the two pockets of the active site of TeSADH. The W110A/I86A/C295A mutant was employed to racemize enantiopure phenyl-ring-containing alcohols. The current study demonstrates that W110G and W110A/I86A/C295A TeSADH are more efficient catalysts for the racemization of enantiopure secondary alcohols than the previously reported mutant W110A TeSADH [6].

1. Introduction

Deracemization of enantiomers is an ideal alternative to the well-known industrial kinetic resolution (KR) approach, which is limited to 50% chemical yield with high enantiomeric purity and therefore requires separation of starting materials from products. By contrast, dynamic kinetic resolution (DKR) is a deracemization method that involves KR and in situ racemization of the slowly reacting enantiomer, thus enabling complete conversion of a racemic starting material to a single enantiopure product. Developing mild controlled racemization approaches is crucial to enabling the development of new DKR methods. Metal catalysts have been used for controlled racemization in DKR [1,2]. Most established racemization methods involve harsh conditions that use acids or bases and are therefore unsuitable for use in DKR. Modern advances in biotechnology allow enzymes to be altered to improve their effectiveness in organic synthesis [3]. Because nature has a limited requirement for racemization, there is no known enzyme with physiological racemase activity for secondary alcohols that is not accompanied by another functionality [4]. Alcohol dehydrogenases (ADHs) have been employed to racemize enantiopure alcohols [5–8]. Enzyme-catalyzed racemization represents a greener alternative to metal-catalyzed racemization. For effective racemization, an ADH with low stereoselectivity is required. However, only a few non-selective ADHs are known because the primary goal was to search for highly selective enzymes [5].

Secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (TeSADH; EC 1.1.1.2), a nicotinamide–adenine dinucleotide phosphate ($NADP^+$)-dependent ADH, has high thermal

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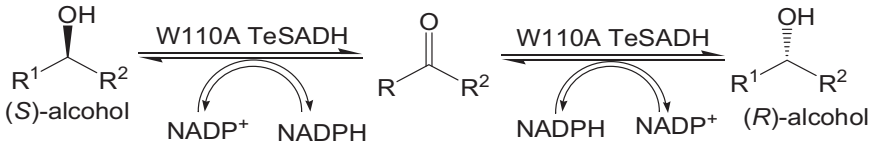


Scheme 1. W110A TeSADH-catalyzed racemization of enantiopure phenyl-ring-containing secondary alcohols.

stability and tolerance to elevated concentrations of organic solvents, making it a favorable biocatalyst for organic synthesis [9–12]. Several mutants of TeSADH have been designed with expanded substrate specificity compared to the wild type enzyme [13,14]. Temperature [15,16], pressure [17], mutation [18,19] and reaction medium [20] influence the stereospecificity of TeSADH. We recently reported a racemization approach for enantiopure phenyl-ring-containing alcohols using W110A TeSADH in the presence of reduced and oxidized forms of cofactor (i.e., NADPH and $NADP^+$), shown in Scheme 1 [6]. TeSADH follows Prelog's rule; the stereoselectivity toward *S*-configured alcohols is higher if the bulkier alkyl group of the alcohol has higher Cahn–Ingold–Prelog priority. Although ADHs are known for their high enantioselectivities, selectivity mistakes can occur, leading to lower enantioselectivities. The accumulation of selectivity mistakes leads to the formation of racemic mixtures under appropriate conditions and time. Racemization is a thermodynamically downhill process ($\Delta G \approx -RT \ln 2$) because of the entropy increase associated with the formation of two enantiomers from one [21].

We recently created six mutants of TeSADH at the Trp-110 site to enhance the enantioselectivity of the reduction of phenyl-ring-containing ketones [22]. In this study, we evaluated the effect of these small alterations in the active site of TeSADH on the efficiency of the racemization reactions of enantiopure phenyl-ring-containing secondary alcohols. We also report a triple mutant of TeSADH and demonstrate its effectiveness in the racemization of enantiopure alcohols.

2. Experimental

2.1. General

Capillary gas chromatographic measurements were performed on a gas chromatograph (GC) equipped with a flame ionization detector and an HP chiral-20B column (30 m, 0.32 mm [i.d.], 0.25 μm film thickness) using Helium as the carrier gas. Nuclear Magnetic Resonance spectra were recorded on a 500 MHz spectrometer at 500 MHz (^{1}H) and at 125 MHz (^{13}C) at room temperature using the solvent peak as an internal standard. Details of GC and GC–MS analysis have been provided in supplementary information. Commercial grade solvents were used without further purification. *Candida antarctica* lipase B (CALB, Novozyme 435), $NADP^+$, NADPH, isopropenyl acetate, (*R*)-1-phenyl-2-propanol [(*R*)-**1a**] (>99% *ee*), (*S*)-1-phenyl-2-propanol [(*S*)-**1a**] (>99% *ee*), (*rac*)-1-phenyl-2-propanol [(*rac*)-**1a**], (*rac*)-4-phenyl-2-butanol [(*rac*)-**2a**], (*R*)-4-phenyl-2-butanol [(*R*)-**2a**] (>99% *ee*), (*S*)-4-phenyl-2-butanol [(*S*)-**2a**] (>99% *ee*), 4-(4′-methoxyphenyl)-2-butanone, and 1-phenyl-2-butanone were used as purchased from commercial sources. (*R*)-**2a** (>99% *ee*), (*S*)-**2a** (72.7% *ee*), and (*R*)-1-phenyl-2-butanol [(*R*)-**4a**] (98% *ee*) were prepared by lipase-catalyzed kinetic resolution of their racemates as reported [22]. (*S*)-4-(4′-methoxyphenyl)-2-butanol [(*S*)-**3a**] (91% *ee*) was prepared by the W110A TeSADH-catalyzed reduction of the corresponding ketone as reported [23].

2.2. Methods

2.2.1. Preparation, gene expression and purification of mutants

All point mutations were introduced by PCR-amplified oligonucleotide-directed mutagenesis using a modified QuikChange site-directed mutagenesis protocol (Agilent) and confirmed by DNA sequencing. The wild-type *T. ethanolicus adhB* gene for TeSADH in the pADHB1M1-kan plasmid (a pBluescriptII KS(+)-kanamycin derivative, Burdette et al.) was used as the template [11,24]. W110G, W110V, W110I, W110Q, W110L, W110M TeSADH (W: tryptophan, G: glycine, V: valine, I: isoleucine, Q: glutamine, L: leucine, M: methionine) were prepared, expressed and purified as reported [22]. To prepare the double mutant, I86A/C295A TeSADH, a C295A mutation (C: cysteine) was introduced in the single mutant I86A [14] using the forward mutagenic primer 5′-ATAAAAGGCGGGCTA**GCC**CCCGGTGGACGTCT and its reverse complement as the reverse mutagenic primer (the mutated nucleotides are bold and underlined). To prepare the triple mutant, W110A/I86A/C295A TeSADH, an additional mutation (W110A) was introduced in the double mutant using the forward mutagenic primer 5′-TGGAATGCTGGCAGGC**GCG**AAATTTTCGAATGTA and its reverse complement as the reverse mutagenic primer. W110A/I86A/C295A TeSADH was then expressed and purified as reported [22], with the exception that the heat treatment was performed at 65 °C instead of 70 °C. Briefly, the TeSADH gene was expressed in *E. coli* DH5α under the control of the *lacZ* promoter on the pBluescriptII KS(+) plasmid. Enzyme concentrations were determined by the Bradford protein assay [25].

2.2.2. General procedure for mutant TeSADH-catalyzed racemization

Enantiopure alcohol (0.015 mmol), $NADP^+$ (0.5 mg), NADPH (1.0 mg), mutant TeSADH (0.2 mg in Tris–HCl buffer solution, 50 mM, pH 8.0), Tris–HCl buffer solution (950 μL, 50 mM, pH 8.0), and acetonitrile (50 μL) were mixed in a 1.5-mL plastic tube. The reaction mixture was shaken at 50 °C and 200 rpm for 48 h, followed by extraction with ethyl acetate (2 × 500 μL). The combined organic layer was dried with sodium sulfate and concentrated to dryness. The remaining residue was treated with pyridine (two drops) and acetic anhydride (one drop) for 1 h to convert the alcohols to their corresponding acetates. The acetate products were diluted with $CHCl_3$ prior to analysis on a GC equipped with a chiral column to determine *ee*.

2.2.3. Determination of the absolute configuration of alcohols

The racemization products were converted to the corresponding acetate derivatives, then injected in a GC equipped with a chiral stationary phase. The resulting retention time was then compared with the *R*- or *S*-acetate derivatives of standard samples of alcohols. The acetate derivatives of the racemization products of enantiopure **1a** and **2a** were co-injected with the acetate derivatives of standard samples of enantiopure alcohols. The derivatized racemization products of (*S*)-**3a** were co-injected with a sample of (*S*)-**3a** prepared by W110A TeSADH-catalyzed reduction of the corresponding ketone, which is known to produce (*S*)-**3a** [23]. The acetate derivatives of racemization products of (*R*)-**4a** were co-injected with a

sample prepared by lipase-catalyzed kinetic resolution of (*rac*)-**4a**. Details of chiral GC analysis, GC–MS analysis have been provided in supplementary information.

3. Results and discussion

3.1. Racemization of (R)-1-phenyl-2-propanol and (S)-1-phenyl-2-propanol using different mutants of TeSADH at the W110 site

We examined racemization of enantiopure phenyl-ring-containing alcohols using W110X TeSADH, where X = V, L, I, Q, M, or G. We utilized these mutants to racemize (*R*)-1-phenyl-2-propanol [(*R*)-**1a**] and (*S*)-1-phenyl-2-propanol [(*S*)-**1a**], as shown in Table 1, to study the effect of different mutations at W110 on the efficiency of racemization. Because the enzyme and its W110 mutants obey Prelog's rule, the active site of TeSADH can be modeled as two pockets, large and small [13,14,18,26]. These pockets play an essential role in aligning substrates to achieve stereospecific reactions. W110 lies within the large pocket of TeSADH, and thus substrates such as (*S*)-**1a** are expected to fit with the benzyl moiety in the large pocket and the methyl group in the small pocket [13]. We selected the enantiopure forms of alcohol **1a** as models in this study because we previously determined that the enantioselective reduction of phenylacetone using W110A TeSADH produces (*S*)-**1a** in only 37% *ee* [23]. This poor enantioselectivity could be explained by the ability of phenylacetone to fit within the large pocket in alternating modes, thus increasing "selectivity mistakes".

The racemization reactions of (*S*)-**1a** and (*R*)-**1a** were performed in Tris–HCl buffer solution (50 mM, pH 8.0) containing acetonitrile (5%, v/v) to enhance the solubility of the alcohol substrates. W110V TeSADH decreased the *ee* of (*S*)-**1a** and (*R*)-**1a** from >99% to 77.5% and 82.6%, respectively. In a previous report [22], kinetic assays revealed that this mutant has a k_{cat}/K_m value of 45,300 M^{-1} s^{-1} for (*S*)-**1a** and an *E* value of 134.5 [$E = (k_{cat}/K_m)_S/(k_{cat}/K_m)_R$], an indication that even a highly stereospecific mutant for (*S*)-**1a**, such as W110V TeSADH, can lead to racemization if sufficient time is allowed. Under the same conditions and using W110L TeSADH, a negligible decrease in *ee* was observed for (*S*)-**1a** and no change for (*R*)-**1a** after 48 h. Thus, the *E* value is likely not the only factor influencing racemization, and the stability of the mutant may also be important. Racemization using W110I, W110Q, and W110M TeSADHs yielded results similar to those obtained with W110V TeSADH. Surprisingly, even though W110M has a significantly lower *E* value than W110V, W110I and W110Q, they all yielded similar racemization results (Table 1). Among the six W110 mutants tested, W110G TeSADH achieved the best racemization results with a racemic mixture obtained from the racemization of (*S*)-**1a** and (*R*)-**1a** after 48 h. This performance is explained by the bigger size of the larger pocket of the active site in this mutant when compared to others, which enables it to recognize both enantiomers of **1a** (leading to more "selectivity mistakes"). The improved efficiency of W110G TeSADH in racemization is consistent with the kinetic parameters shown in Table 1 because it exhibits the lowest *E* value among the six W110 mutants evaluated in the current study. W110G TeSADH-catalyzed racemization reactions of (*S*)-**1a** and (*R*)-**1a** are also more effective than those reported using W110A TeSADH [6], as shown in Table 1.

3.2. Racemization of enantiopure phenyl-ring-containing alcohols using W110G TeSADH and W110V TeSADH

Encouraged by the results obtained with W110G TeSADH in the racemization of (*R*)-**1a** and (*S*)-**1a**, we next used this mutant to racemize other phenyl-ring-containing enantiopure alcohols, as shown in Table 2. Under the same conditions used for the racemization of enantiopure **1a** in Table 1, (*S*)-4-phenyl-2-butanol [(*S*)-**2a**] and (*R*)-4-phenyl-2-butanol [(*R*)-**2a**] were fully racemized using W110G TeSADH. In a previous study, W110A TeSADH-catalyzed racemization of (*S*)-**2a** and (*R*)-**2a** resulted in reductions of *ee* from 72.7% and 99% to 51.8% and 82.4%, respectively [6]. The *ee* of (*S*)-4-(4′-methoxyphenyl)-2-butanol [(*S*)-**3a**] was reduced from 91% to 1.4% using W110G TeSADH compared to 44% when W110A TeSADH was used. The W110G TeSADH-catalyzed racemization of (*R*)-1-phenyl-2-butanol [(*R*)-**4a**] reduced the *ee* from 98% to 74%.

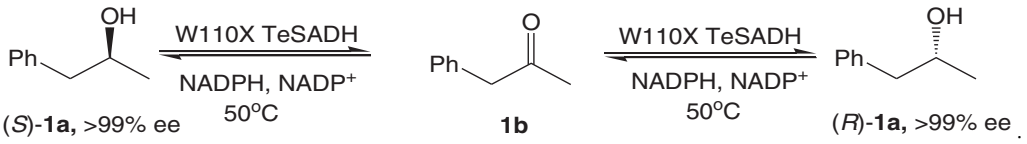
To validate the efficiency of W110G TeSADH for racemization, we conducted W110V TeSADH-catalyzed racemization for the same enantiopure alcohol substrates under the same reaction conditions used for W110G TeSADH. W110V TeSADH reduced the *ee* of (*S*)-**2a** from 72.7% to 64.3% and that of (*R*)-**2a** from >99% to 92%, as shown in Table 2. The *ee* of (*S*)-**3a** was reduced from 91% to 73.3% in the W110V TeSADH-catalyzed racemization, whereas the *ee* of (*R*)-**4a** was reduced from 98% to 94.8%. The results shown in Table 2 demonstrate that W110G TeSADH is a remarkable catalyst for the racemization of phenyl-ring-containing enantiopure alcohols. These results also indicate that a highly stereospecific mutant for (*S*)-configured alcohols, such as W110V TeSADH, can still racemize enantiopure alcohols, albeit less efficiently than W110G TeSADH.

3.3. Racemization of enantiopure phenyl-ring-containing alcohols using W110A/I86A/C295A TeSADH

To further lower the stereospecificity of W110A TeSADH toward phenyl-ring-containing alcohols and thus improve its performance in racemization reactions, we constructed a triple mutant, W110A/I86A/C295A TeSADH. This mutant was designed to expand both pockets of TeSADH and consequently was predicted to increase the occurrence of selectivity mistakes. As expected, using this mutant, the *ee* of (*R*)-**1a** and (*S*)-**1a** were reduced from >99% to approximately 3% (Table 3) under the same conditions described for the other mutants in Tables 1 and 2. The W110A/I86A/C295A TeSADH-catalyzed racemization resulted in a decrease of *ee* from 72.7% to 21.7% for (*S*)-**2a** and >99% to 8.2% for (*R*)-**2a**. The improved racemization of (*R*)-**2a** in comparison with (*S*)-**2a** was previously observed when W110A TeSADH was used with (*S*)-**2a** and (*R*)-**2a** but not (*R*)-**1a** and (*S*)-**1a** [6]. This is explained by the Prelog stereopreference for this enzyme and the significantly higher *E* value of **2a** compared to **1a** [6]. The enzymatic oxidation of (*R*)-**2a** to the corresponding ketone (a difficult process) followed by its enantioselective reduction to (*S*)-**2a** is expected to result in accumulation of the latter (i.e., more effective racemization). The W110A/I86A/C295A TeSADH-catalyzed racemization of (*S*)-**3a** lowered its *ee* from 91% to 25.3%. The lower performance of W110A/I86A/C295A TeSADH in the racemization of enantiopure **1a**, **2a**, and **3a** compared to W110G TeSADH could be explained by differences in the stability of the two mutants. The triple mutant is expected to be less stable because the active site may be unable to bind Zn, which is essential for enzymatic activity. However, the triple mutant TeSADH reduced the *ee* of (*R*)-**4a** from 98% to 47.7% (Table 3, entry 6), superior to the racemization result obtained using W110G TeSADH (entry 4 of Table 2). W110A/I86A/C295A TeSADH is a more efficient catalyst for the racemization of (*S*)-**1a**, (*R*)-**1a**, (*S*)-**2a**, (*R*)-**2a**, and (*S*)-**3a** than the previously reported mutant W110A TeSADH [6].

W110G TeSADH and W110A/I86A/C295A TeSADH are more efficient catalysts for racemization of enantiopure phenyl-ring-containing alcohols than the previously reported W110A TeSADH [6]. They are also more efficient racemization catalysts for these substrates than ADH-"A", *Lactobacillus kefir* ADH, and KRED-118 in racemization of (*R*)-2-octanol using the single enzymatic approach reported by Gruber et al. [5].

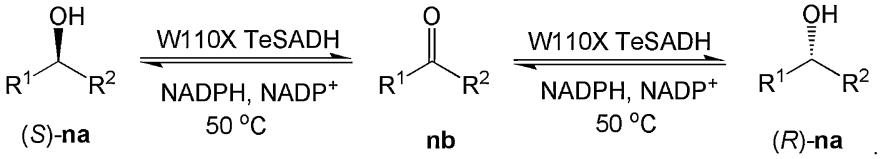
Table 1
W110X TeSADH-catalyzed racemization of enantiopure 1-phenyl-2-propanol (X = V, L, I, Q, M, or G).[a]



Entry	Mutant TeSADH	Substrate	*ee* (%)[b]	*E* value[c]
1	W110V	(*S*)-**1a**	77.5 (*S*)	134.5 ± 27.7
2		(*R*)-**1a**	82.6 (*R*)	
3	W110L	(*S*)-**1a**	98.4 (*S*)	104.4 ± 42.1
4		(*R*)-**1a**	>99 (*R*)	
5	W110I	(*S*)-**1a**	87.4 (*S*)	80.3 ± 16.2
6		(*R*)-**1a**	92.6 (*R*)	
7	W110Q	(*S*)-**1a**	83.6 (*S*)	80.0 ± 17.5
8		(*R*)-**1a**	82.3 (*R*)	
9	W110M	(*S*)-**1a**	61.4 (*S*)	16.3 ± 3.5
10		(*R*)-**1a**	72.8 (*R*)	
11	W110A	(*S*)-**1a**	6.3 (*S*) [d]	17.4 ± 4.7
12		(*R*)-**1a**	10.3 (*R*) [d]	
13	W110G	(*S*)-**1a**	0.2 (*R*)	9.0 ± 2.6
14		(*R*)-**1a**	1.5 (*R*)	

[a] *Conditions*: Reactions were performed at 50 °C for 48 h using enantiopure **1a** (2.0 mg, 0.015 mmol), $NADP^+$ (0.5 mg), NADPH (1.0 mg), W110X TeSADH (0.2 mg), Tris–HCl buffer solution (950 μL, 50 mM, pH 8.0), and acetonitrile (50 μL).
[b] *ee* values were determined by chiral stationary phase GC of the corresponding acetate derivatives.
[c] *E* values were obtained from Ref. [22].
[d] Results for W110A TeSADH-catalyzed racemization were obtained from Ref. [6].

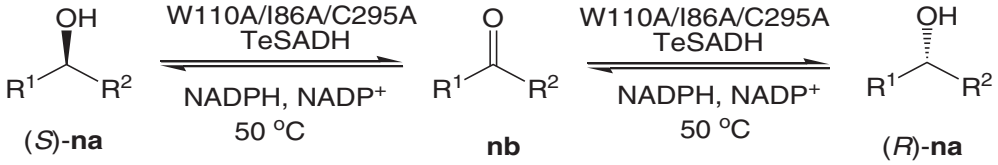
Table 2
Racemization of enantiopure phenyl-ring-containing alcohols using W110G TeSADH and W110V TeSADH.[a]



Entry	R^1	R^2	Substrate	*ee* (%)[b] Before	*ee* (%)[b] After	
					X = G	X = V
1	$PhCH_2CH_2$	CH_3	(*S*)-**2a**	72.7	0.1 (*R*)	64.3 (*S*)
2	$PhCH_2CH_2$	CH_3	(*R*)-**2a**	>99	0.2 (*R*)	92 (*R*)
3	*p*-MeO-$C_6H_4(CH_2)_2$	CH_3	(*S*)-**3a**	91	1.4 (*S*)	73.3 (*S*)
4	$PhCH_2$	CH_3CH_2	(*R*)-**4a**	98	74 (*R*)	94.8 (*R*)

[a] *Conditions*: Reactions were performed at 50 °C for 48 h using enantiopure **na** (0.015 mmol), $NADP^+$ (0.5 mg), NADPH (1.0 mg), W110X TeSADH (0.2 mg), Tris–HCl buffer solution (950 μL, 50 mM, pH 8.0), and acetonitrile (50 μL).
[b] *ee* values were determined by chiral stationary phase GC of the corresponding acetate derivatives.

Table 3
W110A/I86A/C295 TeSADH-catalyzed racemization of enantiopure phenyl-ring-containing alcohols.[a]



Entry	R^1	R^2	Substrate	*ee* (%)[b] Before	*ee* (%)[b] After
1	$PhCH_2$	CH_3	(*S*)-**1a**	>99	3.7 (*S*)
2	$PhCH_2$	CH_3	(*R*)-**1a**	>99	3.4 (*R*)
3	$PhCH_2CH_2$	CH_3	(*S*)-**2a**	72.7	21.7 (*S*)
4	$PhCH_2CH_2$	CH_3	(*R*)-**2a**	>99	8.2 (*R*)
5	*p*-$MeOC_6H_4(CH_2)_2$	CH_3	(*S*)-**3a**	91	25.3 (*S*)
6	$PhCH_2$	CH_3CH_2	(*R*)-**4a**	98	47.7 (*R*)

[a] *Conditions*: Reactions were performed at 50 °C for 48 h using enantiopure **na** (0.015 mmol), $NADP^+$ (0.5 mg), NADPH (1.0 mg), W110A/I86A/C295A TeSADH (0.2 mg), Tris–HCl buffer solution (950 μL, 50 mM, pH 8.0), and acetonitrile (50 μL).
[b] *ee* values were determined by chiral stationary phase GC of the corresponding acetate derivatives.

In all racemization reactions described in this report, the percentage of ketone formed due to equilibrium with alcohols did not exceed 3%. Generating more mutants of TeSADH will be crucial to alter its stereospecificity and improve its racemization performance. TeSADH, with its remarkable thermal stability and organic solvent tolerance [7–10,18], can thus be used in situ with a KR method to achieve successful DKR.

4. Conclusion

In this study, the racemization of phenyl-ring-containing enantiopure alcohols was studied using seven mutants of TeSADH. Among the tested mutants, six were at the W110 site (W110V, W110L, W110I, W110Q, W110M, and W110G). W110G TeSADH exhibited a remarkable improvement in the racemization of phenyl-ring-containing enantiopure alcohols compared to the other five W110 mutants tested in this study, and racemic mixtures were obtained from enantiopure (*S*)-1-phenyl-2-propanol, (*R*)-1-phenyl-2-propanol, (*S*)-4-phenyl-2-butanol, (*R*)-4-phenyl-2-butanol, and (*S*)-4-(4′-methoxyphenyl)-2-butanol. W110G TeSADH is also more efficient than the previously reported mutant W110A TeSADH in the racemization of enantiopure phenyl-ring-containing alcohols. The current results indicate that the W110 site plays a very important role in controlling the stereospecificity of TeSADH-catalyzed redox reactions of phenyl-ring-containing alcohols and their corresponding ketones. We also evaluated the racemization ability of W110A/I86A/C295A TeSADH using the same enantiopure alcohols and obtained results similar to those obtained with W110G TeSADH. Two of the mutants reported in the current study, W110G TeSADH and W110A/I86A/C295A TeSADH, are more efficient catalysts in racemization of enantiopure alcohols than the previously reported W110A TeSADH. This enhancement of the efficiency of the environmentally benign TeSADH-catalyzed racemization may enable its use in situ with a KR method to achieve DKR.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.02.012.

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