

**DERACEMIZATION OF SECONDARY ALCOHOLS
USING A BIOCATALYTIC APPROACH**

BY

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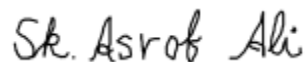
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This Thesis is dedicated to Almighty Allah and to the memory of my late wife
(QUEEN KARIMOT ADEYEYE BINT AHLIM)

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LIST OF ABBREVIATIONS

ADH	:	Alcohol dehydrogenase
ADPR	:	Adenosine diphosphoribose
CALB	:	<i>Candida antarctica</i> Lipase B
DKR	:	Dynamic kinetic resolution
ee	:	Enantiomeric excess
FDH	:	Formate dehydrogenase
GC-MS	:	Gas chromatography mass spectrometry
HLADH	:	Horse liver alcohol dehydrogenase
KR	:	Kinetic resolution
LkADH	:	<i>Lactobacillus kefir</i> alcohol dehydrogenase
NAD ⁺	:	Nicotinamide adenine dinucleotide
NADP ⁺	:	Nicotinamide adenine dinucleotide phosphate
NADPH	:	Nicotinamide adenine dinucleotide phosphate reduced
NMR	:	Nuclear magnetic resonance
<i>rac</i>	:	racemic
TeSADH	:	<i>Thermoanaerobacter ethanolicus</i> secondary alcohol dehydrogenase
TbSADH	:	<i>Thermoanaerobium brockii</i> secondary alcohol dehydrogenase
TMOS	:	Tetramethyl orthosilicate
Tris-HCl	:	tris(Hydroxymethyl)aminomethane hydrochloride
W110G	:	Tryptophan at position 110 was mutated to glycine
W110V	:	Tryptophan at position 110 was mutated to valine
YADH	:	Yeast alcohol dehydrogenase

ABSTRACT

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A one-pot, two-step deracemization approach using W110G and W110V mutants of *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase (*TeSADH*) was developed. This approach was implemented in deracemization of phenyl-ring containing racemic alcohols. (*S*)-Configured alcohols were obtained in up to >99% enantiomeric excess. The marginally stereoselective W110G *TeSADH* enables a non-stereoselective oxidation of alcohol racemates to their corresponding prochiral ketones, followed by heat treatment to denature W110G *TeSADH*, which in turn followed by addition of W110V *TeSADH* to catalyze enantioselective reduction of the resulted prochiral ketones to their corresponding (*S*)-configured alcohols. The heat treatment step was eliminated by using sol-gel encapsulated W110G *TeSADH* to oxidized racemic alcohols. This bi-enzymatic approach was implemented in the stereoinversion of (*R*)-configured alcohols, and (*S*)-configured alcohols were obtained from their (*R*)-alcohols in up to >99% enantiomeric excess. We further developed this approach to a one-pot concurrent deracemization strategy via stereoinversion using two mutants of *TeSADH* that exhibit opposite stereo-preferences towards secondary alcohol substrates and achieved up to >99% enantiomeric excess and quantitative yields of (*S*)-alcohols. The stereochemical identities of the products of the deracemization and stereoinversion reactions were determined by using a Gas Chromatogram equipped with a chiral column, and their chemical identities were

confirmed by mass spectrometry and ^1H as well as ^{13}C nuclear magnetic resonance spectroscopy.

ملخص الرسالة

لقد طورنا طريقة لتحضير كحولات نشطة ضوئياً من كحولاتها الراسيمية وذلك باستخدام W110G و W110V من طفرات *Thermoanaerobacter ethanolicus* ديهيدروجينيز الكحول (TeSADH). وقد تم استخدام هذه الطريقة الثنائية الانزيم في انتاج كحولات نشطة ضوئياً من كحولاتها الراسيمية بفاعلية وانتقائية عالية. تعتمد هذه الطريقة على أكسدة الكحولاتها الراسيمية بطريقة غير انتقائية باستخدام W110G TeSADH إلى كيتونات. يتبع هذه الخطوة معالجة حرارية لإفساد نشاط W110G TeSADH، والتي تتبعها أيضاً إضافة W110V TeSADH لتحفيز الاختزال الانتقائي للكيتونات الناتجة من الخطوة الأولى إلى الكحولات النشطة ضوئياً وانتقائية عالية. وللتخلص من خطوة المعالجة الحرارية تم استخدام W110G TeSADH المغلف بطريقة sol-gel. هذا وتم تنفيذ هذا النهج الثنائي الأنزيمي لتحويل الكحولات (R) إلى (S) بانتقائية عالية. قمنا أيضاً بتطوير هذه الطريقة لاستراتيجية تتضمن توظيف الاختزال والأكسدة بطريقة متزامنة وبخطوة واحدة بدل اثنتين. تم التشخيص الكيميائي للكحولات الناتجة باستخدام الرنين المغناطيسي النووي وتم تشخيص مدى الانتقائية الضوئية للمركبات الناتجة باستخدام جهاز كروماتوجراف الغاز.

CHAPTER 1

INTRODUCTION

1.1 Background

Optical active alcohols are very important building blocks used in the preparation of high-quality fine chemicals including pharmaceuticals, food and agrochemicals. It is well established that optically active drugs are more effective in action than their corresponding racemic mixtures [1-2]. For instance, the (*S*)-configured Ibuprofen drug metabolized faster in blood compared to the racemic Ibuprofen drug [1]. The use of racemic mixture of 3,4-dihydroxyphenylalanine (DOPA) for the Parkinson's disease treatment initiate adverse effects like vomiting, revulsion, granulocytopenia, loss of appetite and uncontrolled movements [2]. These effects can be prevented when patients use L-DOPA enantiomer. Moreover, (*S*)-4-(4'-hydroxyphenyl)-2-butanol, also known as (*S*)-rhododendrol, has been employed in cosmetics as a bleaching agent [3], likewise in prevention of hepatotoxicity [4]. Biological asymmetric synthesis of prochiral ketones to optically active alcohols has been explored widely. For instance, biocatalytic reduction of 6-keto buspirone was employed to synthesize both (*S*)-6-hydroxybuspirone and (*R*)-6-hydroxybuspirone enantiomers [5]. Optically active 4-phenyl-2-butanol has been reported in the literature as the building block for the synthesis of protease inhibitors such as VX-478 and SC-52151 (Figure 1), which are potential therapeutic agents for the treatment of acquired immunodeficiency syndrome (AIDS) [6]. The commonly used industrial method for the

synthesis of enantiomerically pure alcohols is kinetic resolution (KR) [7], but a limited yield of not more than 50% with high enantioselectivity is a challenging drawback of this strategy (Scheme 1). One of the proposed alternatives to the KR approach is known as deracemization of racemic alcohols, which involves obtaining up to >99% of enantiopure alcohols from their racemates. Several deracemization strategies have been proposed in the last two decades. This includes dynamic kinetic resolution, cyclic deracemization, enantioconvergence, and deracemization via stereoinversion [8-11].

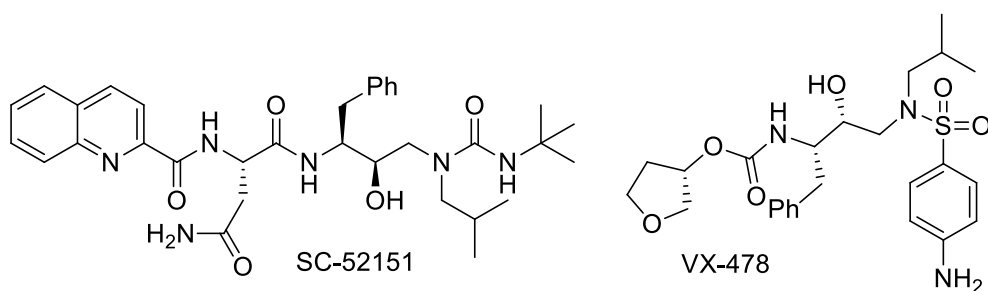
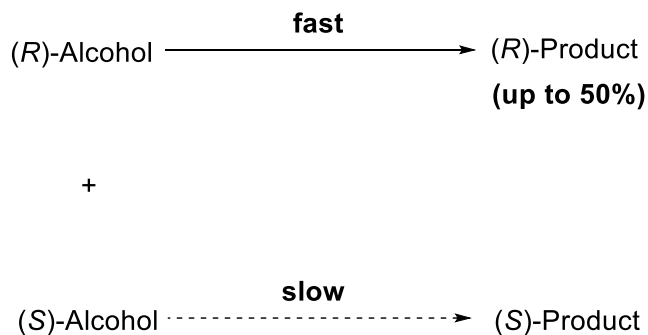


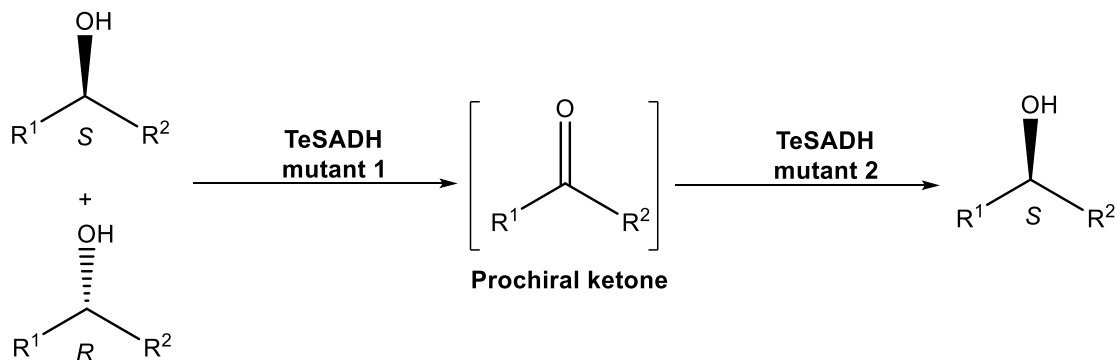
Figure 1. Structures of Human immunodeficiency virus (HIV) protease inhibitors



Scheme 1. Kinetic resolution of racemic alcohols.

Although, several enzymatic deracemization strategies of alcohols have been reported, however, these required the use of more than one enzyme [12-13], which restricts their use.

Herein, a deracemization strategy of alcohols that involves a non-stereospecific oxidation of alcohols combined with a stereoselective reduction of the resulted ketones in one pot and two steps without the need to isolate the prochiral ketone intermediate is investigated. The two steps are conducted using two mutants of *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase (TeSADH), as shown in Scheme 2.



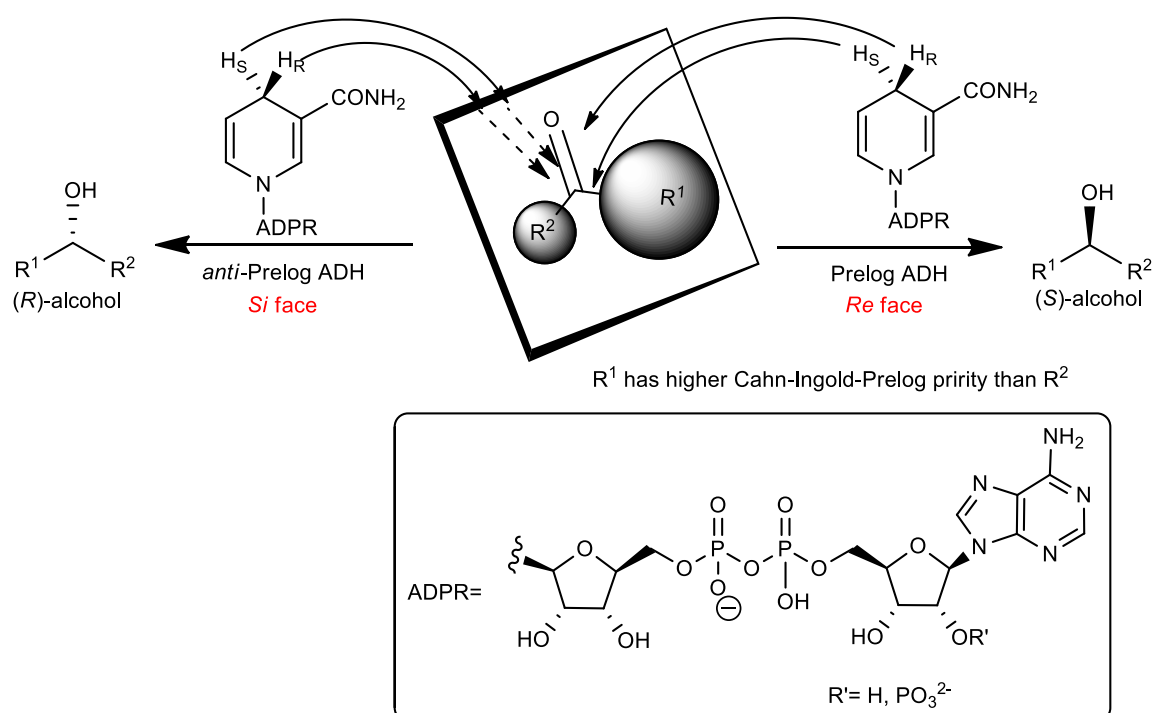
Scheme 2. One pot deracemization of alcohols using two mutants of TeSADH. R^1 is more sterically hindered and has higher Cahn-Ingold-Prelog priority than R^2 .

1.2 Alcohol Dehydrogenases (ADHs) in asymmetric redox reactions

Alcohol dehydrogenases (ADHs, EC 1.1.1.X, X = 1 or 2) catalyze the reversible reduction of carbonyl compounds to corresponding alcohols. ADHs require a co-enzyme, which is usually nicotinamide adenine dinucleotide (NADH) or its phosphate (NADPH) as a hydride source for reduction and their oxidized forms, $NADP^+$ or $NADP^+$, to mediate oxidation of alcohols. The oxidized and reduced forms of co-enzyme can be regenerated by using acetone and 2-propanol cosubstrates, respectively.

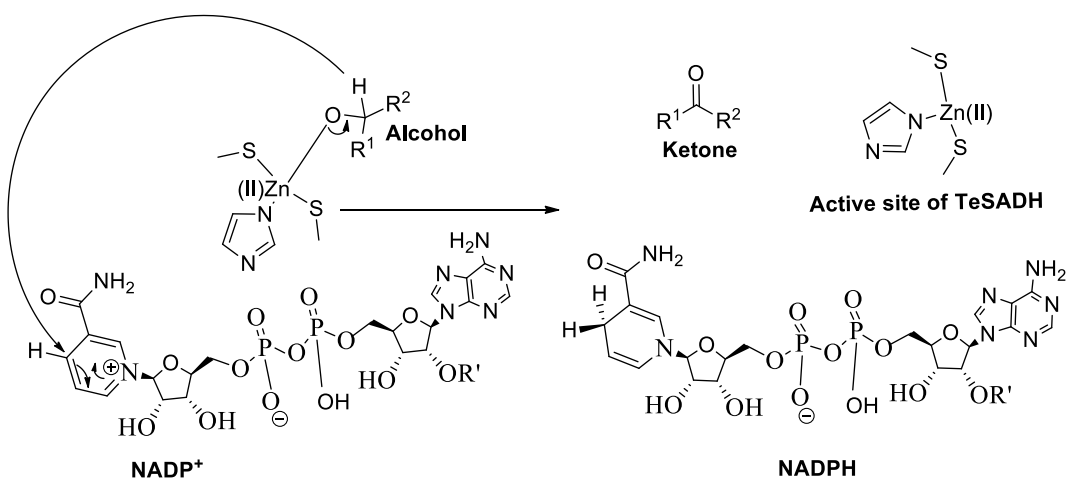
Two hydrogen atoms are added to a prochiral ketone to facilitate the asymmetric reduction and lead to the formation of *S*- or *R*-configured alcohols. The pro-(*R*) or pro-(*S*) hydride of the NAD(P)H is delivered from the *Re* or *Si* faces of the prochiral ketone to facilitate the

synthesis of *S*- or *R*-configured alcohols, respectively, as long as the large group has higher Cahn-Ingold-Prelog priority than the small group of the ketone [14]. This stereochemical outcome of the reaction is regulated by Prelog's rule [14], where the NAD(P)H pro-*R* hydride is delivered through the *Re* face of the prochiral ketone generating alcohols with *S* configuration virtually in all cases. For instance, Horse liver ADH (HLADH), *Thermoanaerobium brockii* ADH (TbADH), *Thermoanaerobacter ethanolicus* secondary ADH (TeSADH), *Rhodococcus ruber* ADH (ADH-A) and Yeast ADH (YADH) are known as Prelog ADHs. Meanwhile, *Lactobacillus kefir* ADH (LkADH) is recognized as an *anti*-Prelog ADHs [14]. The stereochemistry of hydride addition in ADH-catalyzed reduction of ketones is summarized in Scheme 3.



Scheme 3. Hydride addition stereochemistry in asymmetric reduction of ketones catalysed by ADH. ADPR = adenosine diphosphoribose

The mechanism of TeSADH-catalyzed oxidation of alcohols involves the coordination between the Zn ions present at the catalytic site of TeSADH together with the hydroxyl oxygen of alcohol through an electrostatic interaction. This increases the electrophilicity of methine carbon and thus the latter becomes more prone to nucleophilic attack from the hydride source and mediate the formation of prochiral ketone intermediate. The coordination between the coenzyme and the active site is achieved as a result of intermolecular hydrogen bond connecting the hydroxyl groups present in both amino acids and the ribose. This mechanism is shown in Scheme 4



Scheme 4. Proposed mechanism of TeSADH-catalyzed secondary alcohol oxidation.

Biocatalytic asymmetric redox reactions mediated by ADHs is an important class of reactions. The application of ADHs for deracemization of racemic alcohols to obtain enantiopure alcohols provides a greener alternative to the metal-catalyzed deracemization. NADH or its corresponding phosphate (NADPH) serve as a hydride source. Some

examples of ADHs reported for asymmetric redox reactions are TbADH, YADH, HLADH [15], as well as TeSADH [16-17].

1.3 *Thermoanaerobacter ethanolicus* secondary ADH (TeSADH)

Asymmetric reduction of ketones has been successfully achieved using a thermostable TeSADH and recently secondary alcohols has been deracemized using the same alcohol dehydrogenase [18]. This enzyme gained compelling interests due to its high thermal stability besides its high tolerance to organic media [19]. Substrate selectivity rely upon the character of the aryl/ alkyl groups connected to alpha carbon of the secondary alcohol substrates or their corresponding ketones. Series of TeSADH mutants were developed to broaden substrate specificity and thus accommodate sterically hindered groups such as phenyl rings and various alkyl chains, which are not substrates for wild-type TeSADH [20]. TeSADH have high thermostability up to 85 °C, and tolerates organic solvents with high concentrations [19, 21]. It is NADP-dependent and obeys Prelog's rule [14]. This enzyme is identical to TbADH, another example of thermostable ADHs [19]. In 1986, Keinan and coworkers designed a model that proved that TbADH possesses two distinct hydrophobic pockets, which differ in size and affection for the alkyl groups affix directly to the carbonyl carbon (Figure 2). They showed that the small pocket has a greater affinity for alkyl groups when compared to the large pocket after screening various aliphatic ketones [15]. The crystal structure of TbADH was investigated and confirmed a tetramer in which each repeating unit comprises of 352 amino acids with the presence of Zinc ion in the active site [22]. It has also been reported previously that the small pocket of TeSADH contain Ile-86 and Cys-295. Whereas, the large pocket shows the presence of Trp-110 at the active site

[17a]. The active site can be expanded by replacing some amino acids present at these two pockets with less bulky amino acids, thus giving room for the accommodation of many substrates that cannot be accepted before modification, which in turn improve the substrate scope of the modified enzyme [17a].

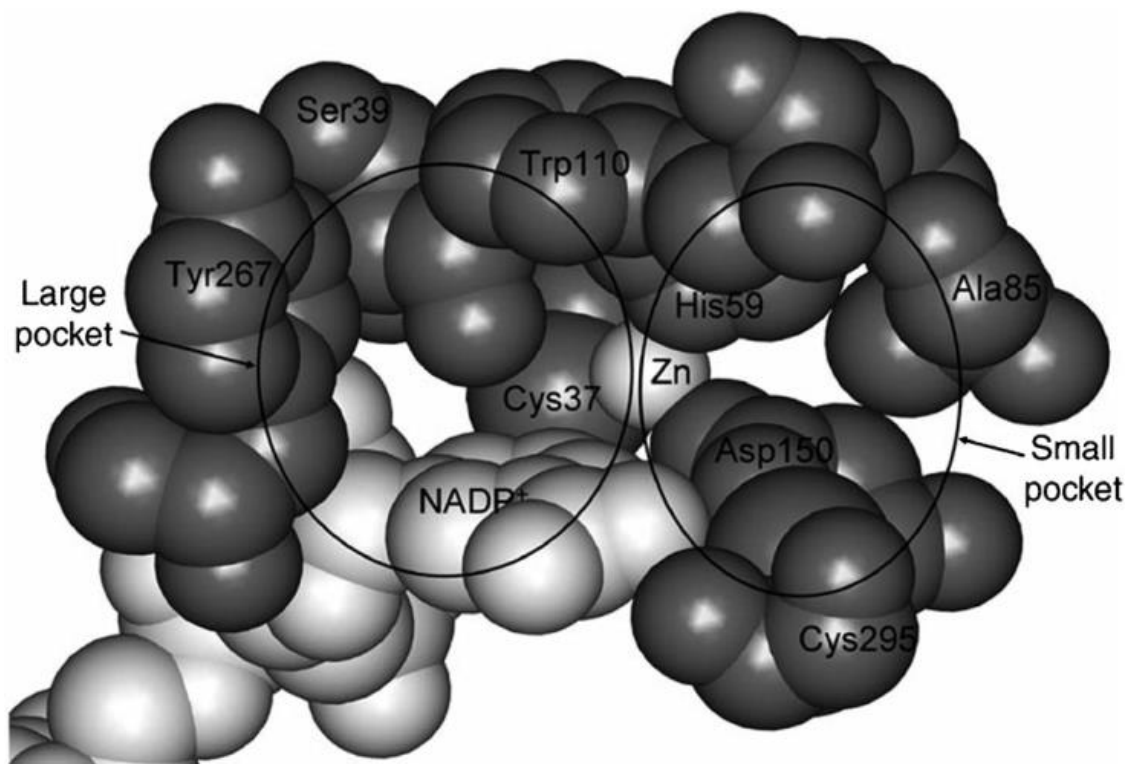
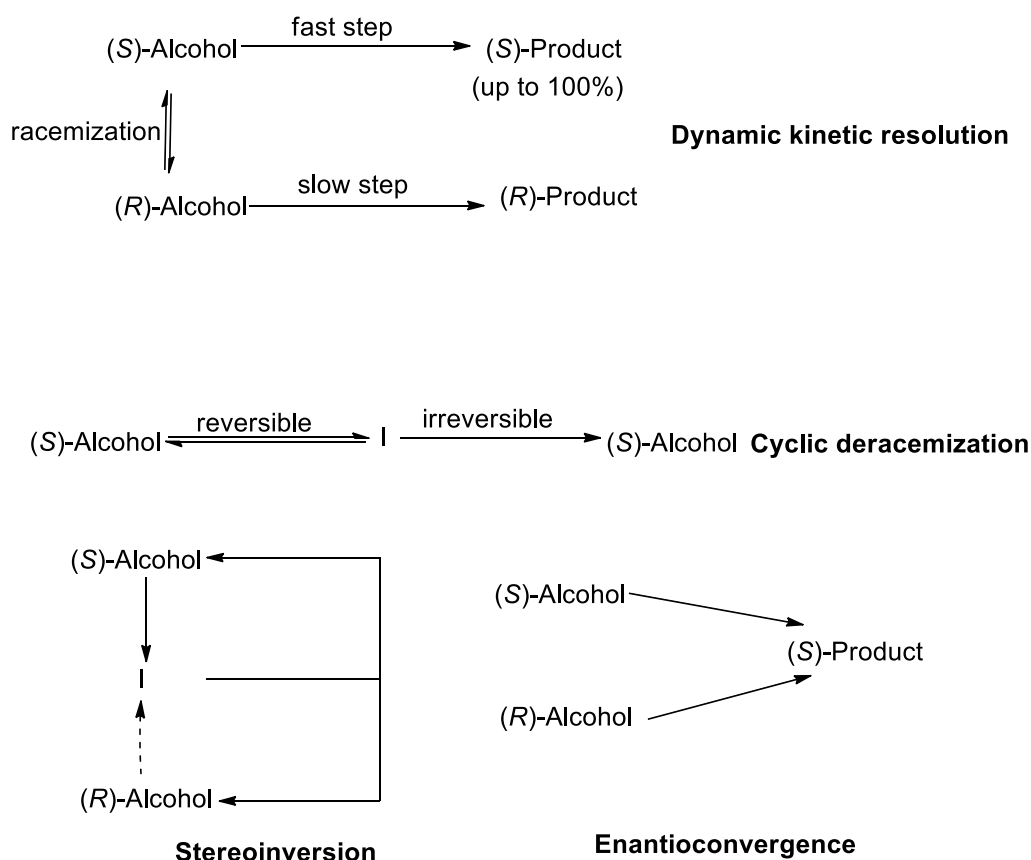


Figure 2. Catalytic site of TbADH showing NADP⁺ and Zn. Used with permission from [*Protein Eng. Des.* **2007**, 20, 47-55]

1.4 Deracemization of alcohols

Deracemization involves the production of a single enantiomer from its racemate. It is the optimum alternative to the well-known KR, which suffers from the drawback of being limited to a maximum yield of 50% with high enantiomeric excess. Among the deracemization strategies discussed in the literature are cyclic deracemization, dynamic

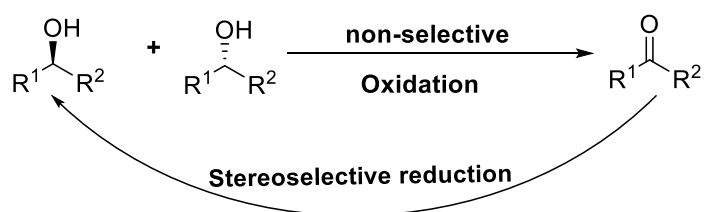
kinetic resolution (DKR), stereoinversion and enantioconvergence (Scheme 5) [8-9]. Chemical methods of alcohol deracemization using one [23-24] or two [25] catalysts have been reported. However, biocatalytic deracemization is preferable due to the following reasons: firstly, enzymes possess high stereo-, regio- and chemoselectivities. Secondly, due to their ability to overcome challenges like profitability and sustainability in fine chemical industries [26-27], and thirdly, the use of enzymes does not create any negative effects on the environment, because they are biological catalysts, which perform well under moderate conditions, and help to eliminate any side products that could result from isomerization or epimerization.



Scheme 5. Deracemization approaches. I: prochiral ketones that corresponds to the alcohol used.

1.4.1 Cyclic deracemization of alcohols

Generally, cyclic deracemization involves the combination of two redox reactions in one-pot as shown in Scheme 6. In most cases reported in the literature, cyclic deracemization involves an enantioselective enzymatic reaction step in combination with a non-stereoselective step in one-pot. A careful look at the cyclic deracemization approach indicates that the desired alcohol is being oxidized by the non-stereospecific oxidation reaction, and this is accompanied by a stereoselective reduction (Scheme 6). Cyclic deracemization can also be achieved by combining a non-stereoselective reduction with a stereospecific oxidation reaction. This approach has the intrinsic disadvantage of unavoidable futile cycles, which reduces the efficiency of this approach.

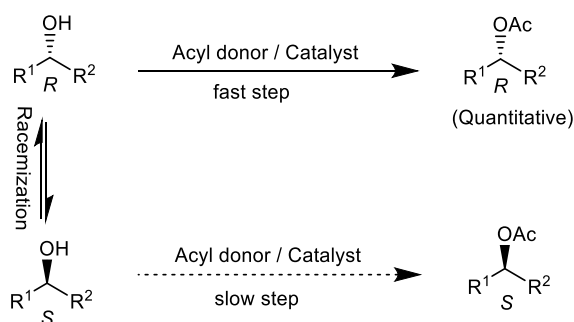


Scheme 6. Cyclic deracemization of alcohols.

1.4.2 Dynamic kinetic resolution (DKR) of alcohols

Dynamic kinetic resolution (DKR) involves a simultaneous KR and racemization of the unreactive alcohol (Scheme 7). A successful DKR requires an efficient KR, an efficient racemization catalyst, and the challenging requirement of compatibility of the two systems. This makes it challenging to develop new DKR methods; especially the commonly used KR methods are enzyme-based, and this requires mild racemization conditions to

accomplish the compatibility requirement. Conventionally, deracemization of alcohols by DKR is achieved by hydrogenation-based racemization catalysts using mainly organometallic catalysts [8], and more recently using enzyme-based racemization [28-29]. Alcohol racemization by these catalysts involves redox reactions that incorporate the corresponding prochiral ketones. DKR can also be achieved by catalysts that permit a dehydration mechanism, such as VOSO_4 and AlMe_3 [8]. These catalysts make it possible to deracemize tertiary alcohols by DKR, which are practically impossible to deracemize by catalysis involving hydrogen transfer. They are also cheaper than the Pd-, Ru- or Ir-based catalysts. ADHs were also shown effective in racemization of enantiopure alcohols in absence of co-factor regeneration [28-29].



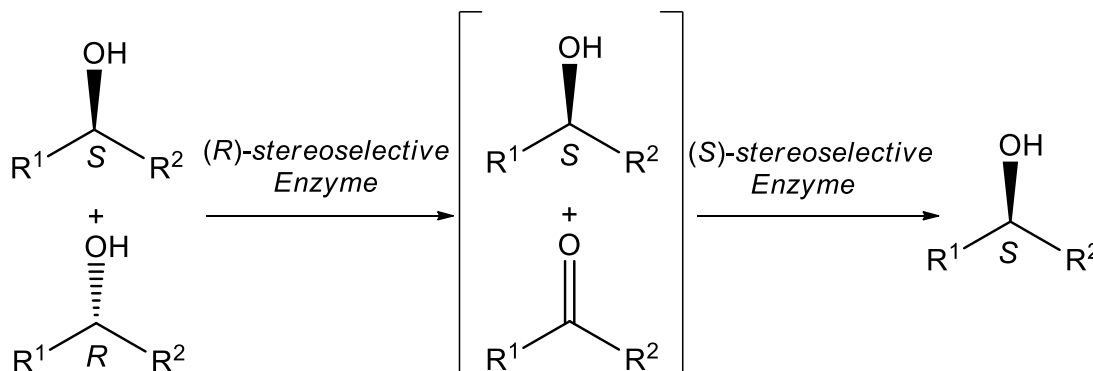
Scheme 7. Dynamic kinetic resolution (DKR) of alcohols.

1.4.3 Deracemization of alcohols by stereoinversion approach

Deracemization of alcohols via stereoinversion includes two redox reactions that exhibit opposite stereo-preferences (Scheme 8). The deracemization in this sense has to be accomplished using a stereospecific oxidation accompanied by a stereoselective reduction reaction, which results in leaving one enantiomer untouched while conducting a

stereoinversion of the other. This approach is very attractive; however, identifying two catalysts that can operate on the same substrate with opposite stereo-preferences in one pot is a challenge.

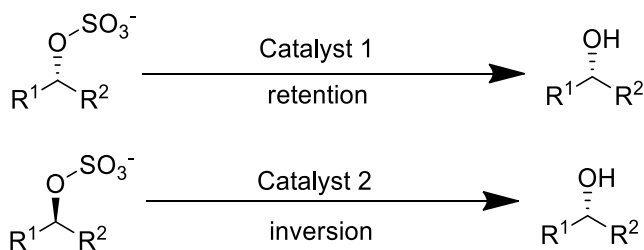
Deracemization of alcohol racemates via stereoinversion in one-pot was reported by Kroutil and coworkers [30]. This involves the use of two ADHs, in which one must be NAD-dependent and the other is NADP-dependent to make sure that the two systems are orthogonal; two more enzymes were also needed for the cofactor regeneration. Chemo-enzymatic deracemization of racemic alcohol via stereoinversion was also reported [31-33]. This involves a non-stereoselective oxidation with TEMPO/I₂ and selective reduction to enantiopure alcohols by an ADH.



Scheme 8. Deracemization of racemic alcohols by stereoinversion approach.

1.4.4 Enantioconvergent deracemization of Alcohols

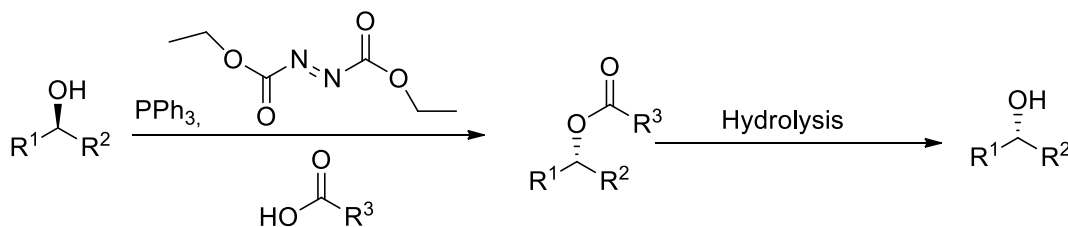
This process involves two operations that have opposite stereo-preferences, the undesired enantiomer of a starting material undergoes a transformation via stereoinversion to the desired alcohol enantiomer, and the desired enantiomer undergoes a transformation with retention of configuration, as shown in Scheme 9 [34].



Scheme 9. Deracemization of sulfonate ester racemates via enantioconvergent approach

1.5 Mitsunobu reaction

Mitsunobu reaction involves a chemical transformation that leads to the inversion of stereochemistry of alcohols (Scheme 10) [35]. This methodology has been well established in the literature for the stereoinversion of many organic compounds, most especially alcohols [36]. Major limitations of conventional Mitsunobu reactions includes generation of unwanted by-products and difficulty in separation of the reaction products [37-38]. These shortcomings call for alternative stereoinversion reactions.

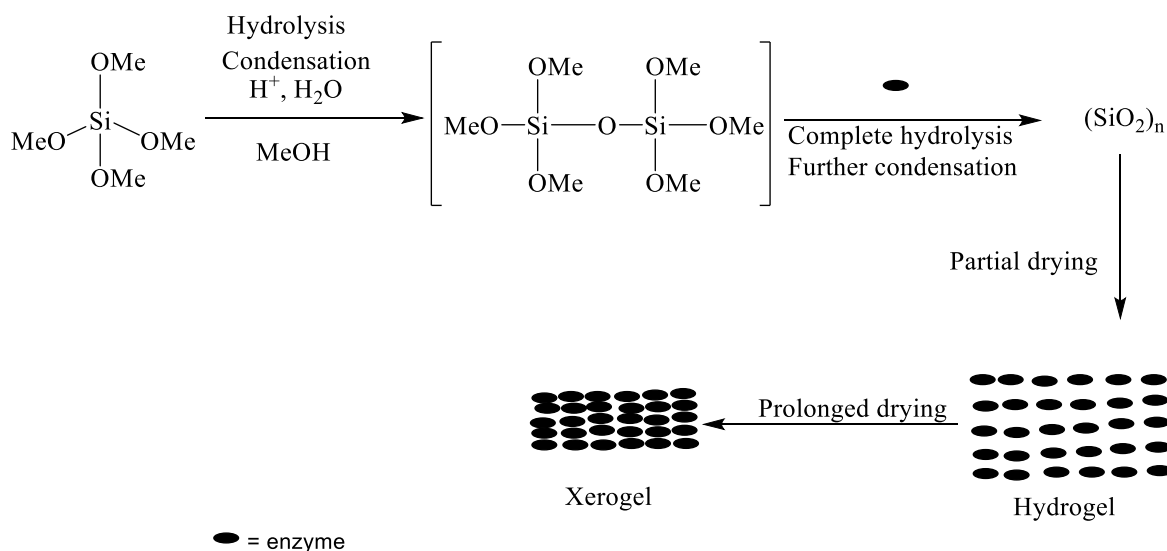


Scheme 10. Mitsunobu stereoinversion reaction of alcohols.

1.6 Enzymes Encapsulation

This involves the entrapment of enzyme molecules in a chemically inert support. Some of the advantages offered by the enzymes immobilization are improved stability, recyclability and easy handling of the immobilized enzymes [39]. For example, *Candida antarctica*

lipase B (CaLB), also known as Novozyme-435, was immobilized on the surface of an acrylic resin and that makes it widely applicable as a catalyst for the esterification reactions in the presence of organic solvents [40]. ADHs have been immobilized successfully using the sol-gel encapsulation approach and using silica glasses [39]. Sol-gel encapsulation method is very simple and cost effective, which makes it widely acceptable at both laboratory and industrial scales. Sol-gel synthesis follows a reaction mechanism of hydrolysis in acidic or basic medium to form a monolayer silane called sol, and further subjected to condensation to form $(\text{SiO}_2)_n$ framework. This can be used as hydrogel, or dried to form xerogel [41]. Due to the porosity of the sol-gel, substrates can move to and from the encapsulated enzymes. Moreover, enough water molecules need to be retained in order to keep the enzymatic activity of the immobilized enzymes intact [42]. Tetramethyl orthosilicate (TMOS) is the most widely employed silane for the sol-gel encapsulation of enzymes (Scheme 11).



Scheme 11. Sol-gel encapsulation of enzymes.

1.7 Objectives

The major objectives of this work are to:

- (1) optimize the W110G TeSADH-catalyzed oxidation reaction conditions for 1-phenyl-2-propanol and 4-phenyl-2-butanol substrates,
- (2) optimize the deracemization reaction using bi-enzymatic approach,
- (3) determine the stereochemical identities of the reaction products by GC furnished with a chiral column, and
- (4) confirm the chemical identity of the reaction products by GC-MS and NMR (^1H and ^{13}C) spectroscopy.

1.8 Significance of the study

The use of TeSADH to produce enantiopure alcohols from their racemates through deracemization provides a greener reaction system than those conducted using metal catalysts; nevertheless, they also have drawbacks. For instance, the use of a single mutant of TeSADH in deracemization of secondary alcohols sometimes gave low enantiomeric excesses (*ee*). In order to solve the problem of low *ee* encountered in single enzymatic deracemization of secondary alcohols, we herein use the marginally stereoselective W110G mutant of TeSADH to oxidized alcohol racemates and the enantioselective W110V mutant of TeSADH to reduce the prochiral ketones to enantiopure alcohols (Scheme 2). Upon optimizing the redox reactions using racemic mixtures of 1-phenyl-2-propanol and 4-phenyl-2-butanol, this study was extended to other phenyl-ring-containing secondary alcohol substrates (Figure 3).]

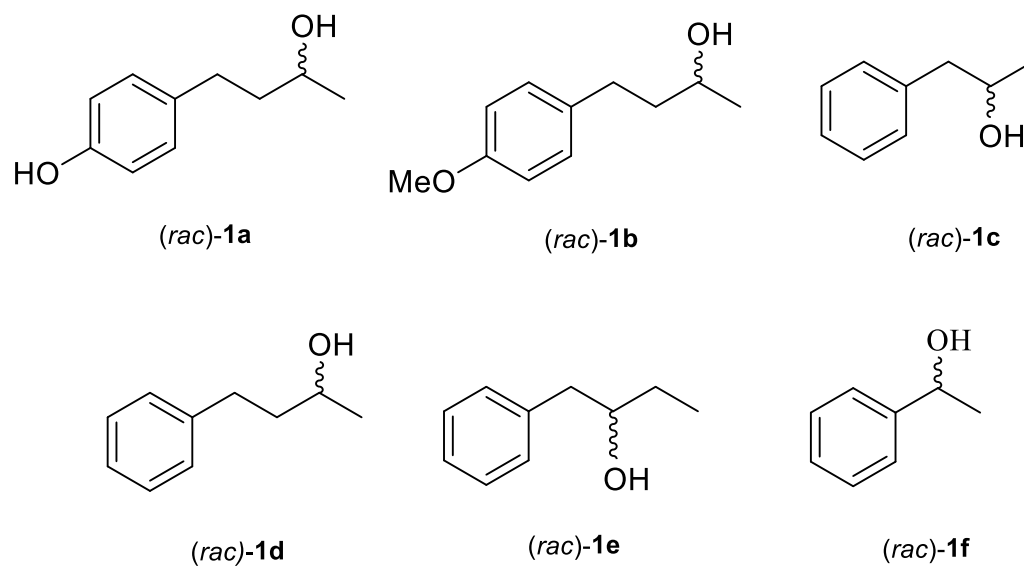


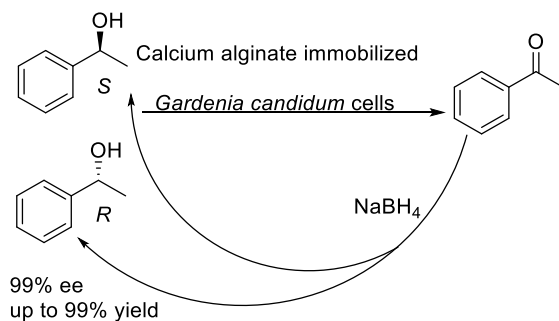
Figure 3. Phenyl-ring containing secondary alcohols used in this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Biocatalytic deracemization of secondary alcohols

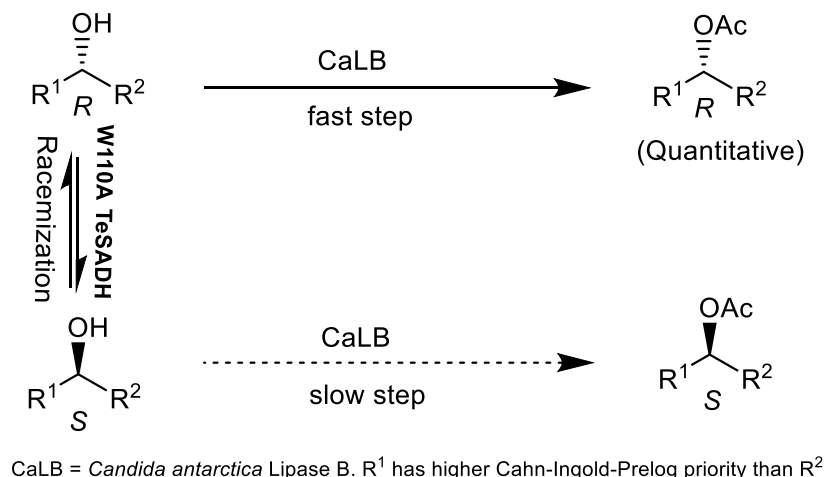
Among the biocatalyzed deracemization strategies discussed in the literature are cyclic deracemization, DKR, stereoinversion and enantioconvergence. For instance, chemo-enzymatic cyclic deracemization approach was used to synthesize optically active *R*-configured alcohols by combining *Gardenia jasminoides* cells supported with calcium alginate with sodium borohydride [43]. Likewise, cells of *Geotrichum candidum* and NaBH_4 were used to deracemize racemic alcohols with high enantiomeric excess (Scheme 12) [44].



Scheme 12. Chemo-enzymatic deracemization of alcohols.

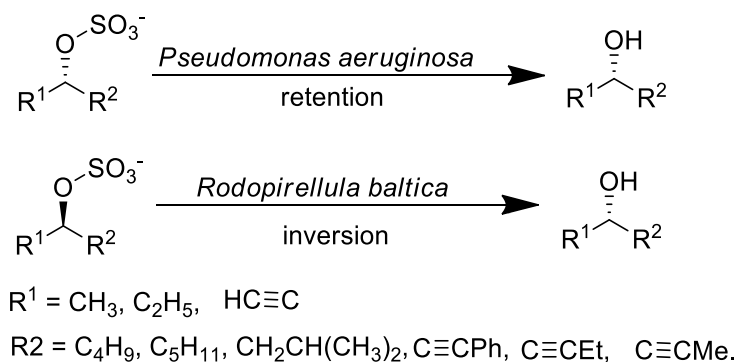
Kroutil and coworkers reported the use of *Rhodococcus ruber* ADH and *Lactobacillus kefir* ADH for the deracemization of series of optically active alcohols via a sequential KR and racemization [45], this was in an effort to mimic a DKR. It is worth noting that racemization

reaction coupled with KR using hydrolase as a catalyst does not proceed in aqueous media, because it favors the hydrolysis of the ester product. Therefore, Musa et al. employed xerogel-encapsulated W110A mutant of TeSADH [46-47] to racemize series of alcohols in non-aqueous reaction medium [28], and thus accomplished a bi-enzymatic DKR. This DKR approach utilized lipase catalyst for KR reaction step (Scheme 13).



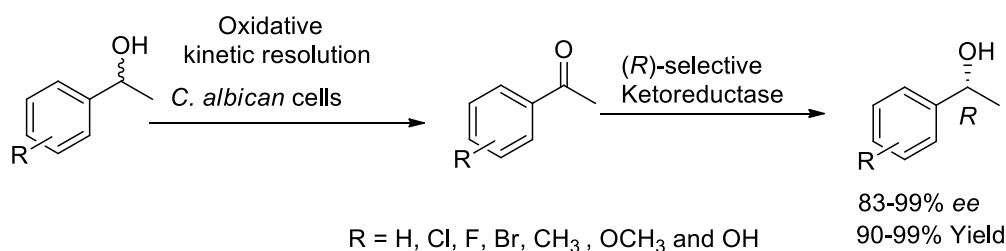
Scheme 13. Dynamic kinetic resolution (DKR) of alcohols.

Mastumoto and coworkers reported a chemo-enzymatic deracemization via enantioconvergent concept [48-49]. They subjected racemic acetate esters to lipase-catalyzed KR followed by a chemical Mitsunobu reaction to obtain *S*-configured 2-acetoxysulfates. Optically active alcohols were also generated via enantioconvergent approach from the sulfate ester racemic mixtures using enantioselective alkyl sulfatases as catalyst (Scheme 14) [50-52]. Epoxide hydrolases were also used to deracemize styrene oxide via Markovnikov (inversion) or *anti*-Markovnikov (retention) mode to produce optically active 1,2-diol [53]. Isolated enzymes have also been employed for deracemization via enantioconvergent [54-55].



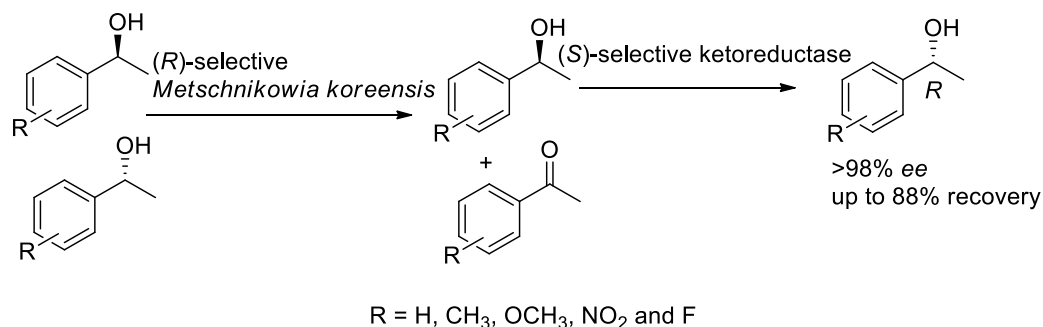
Scheme 14. Enantioconvergence deracemization of sulfonate esters using alkyl sulfatases catalysts.

In 2019, Nasário et al. employed a ketoreductase and the whole cells of *Candida albicans* CCT 0776 immobilized on calcium alginate beads to deracemize secondary alcohols via stereoinversion approach to obtain *R*-configured alcohols in the range of 83-99% and 90-99% *ee* and percentage recovery, respectively (Scheme 15) [56].



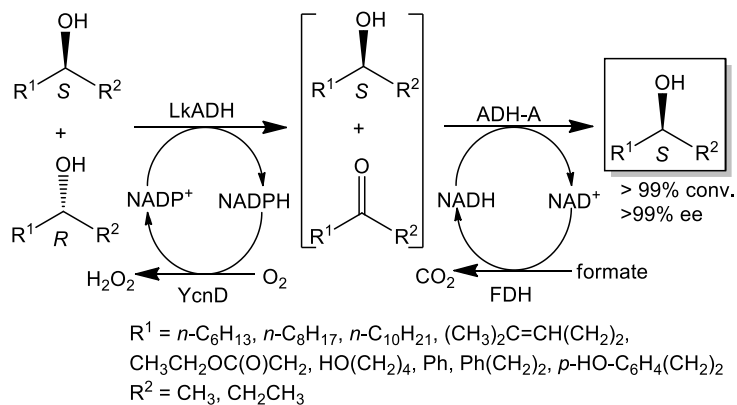
Scheme 15. Sequential deracemization of alcohol using *C. albicans* cells.

The commercially available *Metschnikowia korensis* biocatalyst was used to deracemize secondary alcohols via stereoinversion approach and achieved up to >98% *ee* of *S*-configured secondary alcohols with maximum of 88% percentage recovery (Scheme 16) [57].



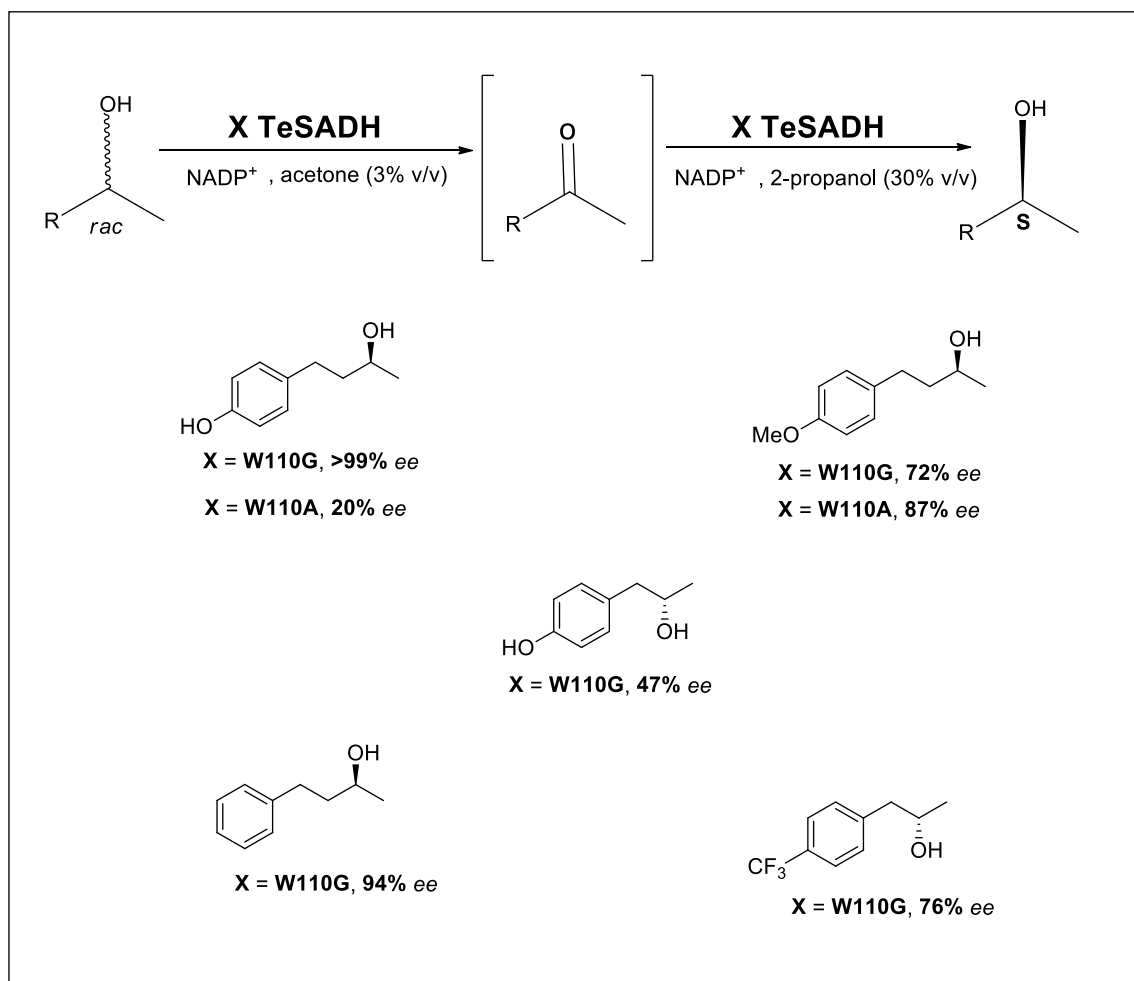
Scheme 16. Sequential deracemization of alcohol using *Metschnikowia koreensis* cells.

Voss et al. reported a deracemization strategy for alcohols via stereoinversion. They used four enzymes, two ADHs with opposite stereo-preferences were employed (LkADH and ADH-A) and YcnD and formate dehydrogenase (FDH) for cofactor regeneration. *S*-Configured alcohols were obtained in up to >99% ee (Scheme 17). *R*-Configured alcohols were obtained by using another set of enzymes. However, the use of two different ADHs makes this approach suffer from the problem of compatibility of enzymes, besides the challenge of having the two enzymes used to catalyze the redox reaction to accommodate the same substrate with opposite stereopreferences. Moreover, the use of four enzymes is not economical [12, 30].



Scheme 17. One-pot deracemization of secondary alcohols via stereoinversion. LkADH: *Lactobacillus kefir* ADH, ADH-A: ADH from *Rhodococcus ruber* DSM 44541.

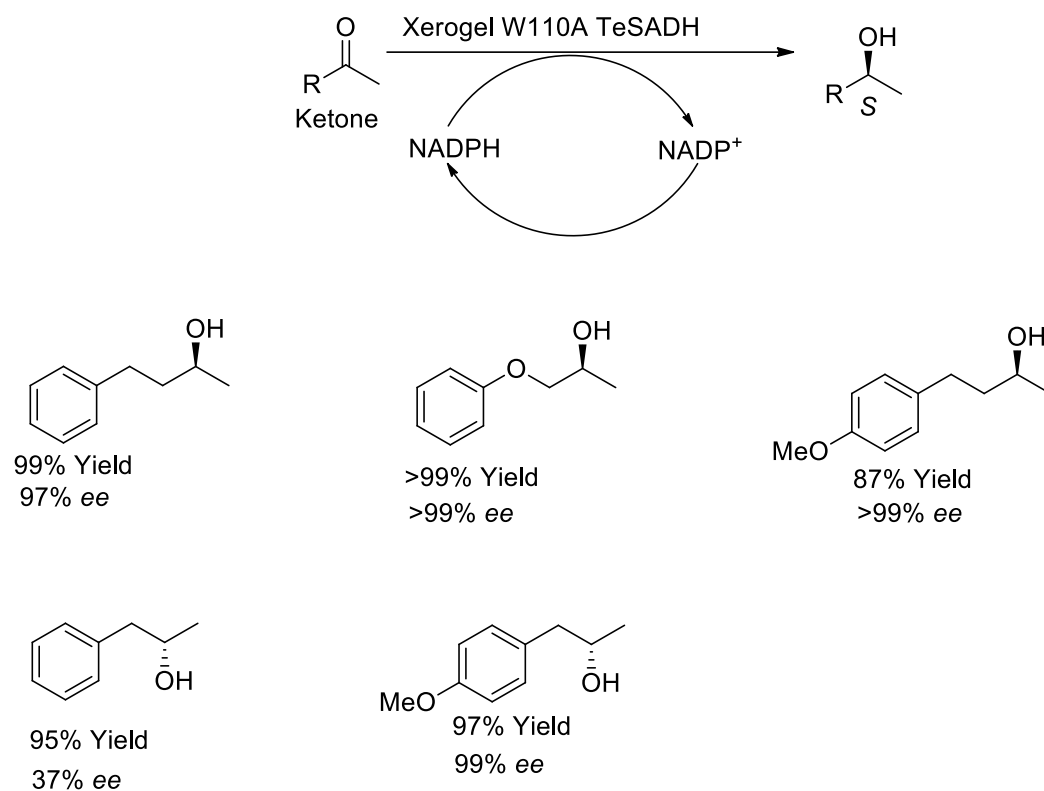
Recently, Musa and coworkers developed a one-pot, two-step single enzymatic deracemization for phenyl-ring-containing secondary alcohols [18]. They employed either W110G or W110A mutants of TeSADH and achieved up to >99.5% recovery and >99% *ee*, the results are presented in Scheme 18. This approach relies on non-stereospecific oxidation of alcohols, followed by an enantioselective reduction of the resulted prochiral ketone in the first step; both steps are catalyzed by the same mutant of TeSADH. The enantioselectivity of the redox reactions in this approach was controlled by tuning the amounts of acetone and 2-propanol cosubstrates used in the oxidation and reduction steps, respectively. They also used this approach to obtain *S*-configured alcohols from their corresponding *R*-configured alcohols (i.e., Mitsunobu-like stereoinversion reaction) [58]. Their results were comparable with the multiple enzymes deracemization approaches previously reported by Voss et al. in 2008 [12].



Scheme 18. TeSADH catalyzed single enzymatic deracemization of secondary alcohols.

2.2 Xerogel-encapsulated W110A TeSADH in asymmetric reduction of ketones

In 2007, Musa et al. investigated the asymmetric reduction of ketones using xerogel-encapsulated W110A TeSADH in organic media such as methyl *tert*-butyl ether (MTBE) and *n*-hexane [17a] as shown in Scheme 19.



Scheme 19. Asymmetric reduction of hydrophobic ketones with Xerogel-encapsulated W110A TeSADH

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and materials

Compounds 4-(4'-hydroxyphenyl)-2-butanone (**2a**), 4-(4'-methoxyphenyl)-2-butanone (**2b**), 4-phenyl-2-butanone (**2d**), 1-phenyl-2-butanone (**2e**), acetophenone (**2f**), (*R*)-1-phenyl-propanol [(*R*)-**1c**], (*S*)-1-phenyl-propanol [(*S*)-**1c**], (*R*)-4-phenyl-2-butanol [(*R*)-**1d**], (*S*)-4-phenyl-2-butanol [(*S*)-**1d**], (*rac*)-1-phenyl-propanol [(*rac*)-**1c**], (*rac*)-1-phenylethanol [(*rac*)-**1f**], NaBH₄, FDH, CaLB, sodium formate, NAD⁺ and NADP⁺ were used as acquired from commercial sources; (*rac*)-4-(4'-hydroxyphenyl)-2-butanol [(*rac*)-**1a**], (*rac*)-4-(4'-methoxyphenyl)-2-butanol, [(*rac*)-**1b**], (*rac*)-4-phenyl-2-butanol [(*rac*)-**1d**] and (*rac*)-1-phenyl-2-butanone [(*rac*)-**1e**] were obtained by NaBH₄ reduction of their corresponding commercially accessible ketones, as previously described [59]. Whereas, (*R*)-4-(4'-hydroxyphenyl)-2-butanol [(*R*)-**1a**] was synthesized by CaLB-catalyzed KR of racemic alcohols. All solvents were used as received from commercial sources.

3.2 Reduction of ketones with Sodium borohydride

Ketones were reduced using a slightly modified version of a method reported in the literature [59]. In summary, ketone (2 mmol) was dissolved in 5.0 mL of methanol. NaBH₄ (2 mmol) was added to the solution while stirring at room temperature. The reaction mixture was stirred for 15 minutes before methanol was evaporated. Diethyl ether was added to the residue, and then the reaction mixture was washed with 5 mL of each of

saturated NH₄Cl, distilled water and brine in this order. The organic solvent was evaporated under vacuum to obtain the racemic alcohols.

3.3 Optimization of W110G-catalyzed oxidation of alcohols

Alcohol racemate [(*rac*)-**1c** or (*rac*)-**d**] (0.03 mmol) was introduced into a mixture of W110G TeSADH (0.2 mg) and NADP⁺ (2.0 mg) in Tris-HCl buffer solution (970 μ L, 50 mM, pH 8.0) and acetone (30 μ L) in a 2.0-mL Eppendorf tube. The mixture was stirred at 50 °C at 180 rpm for about 24 h until both enantiomers were oxidized; reactions were monitored by Gas chromatography (GC). The oxidized products were extracted using diethyl ether (2 $\times$ 1.0 mL). The resulted combined organic layer was subjected to drying with Na₂SO₄ and then concentrated. For *ee* determination, the reaction products were derivatized by adding two drops of acetic anhydride and three drops of pyridine and incubated for 6 h at room temperature prior to analysis of their esters by using a GC equipped with a chiral column [60].

3.4 Bi-enzymatic deracemization of secondary alcohols

Alcohol racemates [(*rac*)-**1a-e**] (0.03 mmol) were introduced into a mixture of W110G TeSADH (0.2 mg) and NADP⁺ (2.0 mg) in Tris-HCl buffer solution (970 μ L, 50 mM, pH 8.0) and acetone (30 μ L) in a 2.0-mL Eppendorf tube. The mixture was stirred at 50 °C at 180 rpm for about 24 h until both enantiomers were oxidized; reactions were monitored by GC. The solution obtained after the oxidation reaction was heated for 45 min at 80 °C to denature any remaining W110G TeSADH. W110V TeSADH (0.2 mg) and 2-propanol (50 μ L) were added to the same reaction vessel, and further subjected to shaking for about 24 h at 180 rpm at 50 °C. The reaction mixture was then extracted with diethyl ether (2 $\times$ 1.0

mL), and the resulted organic layer was subjected to drying with Na₂SO₄ and further concentrated to get rid of the solvent. The percent recovery and the *ee* of the resultant alcohol were subsequently evaluated using a GC equipped with a chiral column.

3.5 Preparation of xerogel-encapsulated W110G TeSADH

Sol-gel encapsulated W110G TeSADH was prepared according to a previously reported method with little modification [17b, 20]. The Sol was prepared by mixing 2.10 g of tetramethyl orthosilicate (TMOS), 0.47 g of distilled water and three drops of 0.05 M HCl. The mixture was mixed thoroughly until a homogeneous phase was achieved. The gels were then processed by adding the previously prepared sol (1.5 mL) to a solution containing W110G TeSADH and NADP⁺ in a 15 mL Eppendorf tube. The W110G TeSADH and NADP⁺ were prepared by adding Tris-HCl buffer solution (50 mM, pH 8) to attain a final concentration of 0.43 and 3.0 mg.mL⁻¹ for the enzyme and coenzyme, respectively. The sol-gel tube was subsequently sealed and allowed to stand undisturbed for 48 h to allow complete formation of a sol-gel encapsulated W110G TeSADH. The sol-gel was then air-dried at room temperature to form xerogel.

3.6 Deracemization of alcohols using xerogel-encapsulated W110G TeSADH in the oxidation step

Alcohol racemates [(*rac*)-**1a** or (*rac*)-**1b**] (0.03 mmol) were introduced into a mixture of xerogel-encapsulated W110G TeSADH and NADP⁺ (2.0 mg) in Tris-HCl buffer solution (970 μ L, 50 mM, pH 8.0) and acetone (30 μ L) in a 2.0-mL Eppendorf tube. The mixture was stirred at 50 °C at 180 rpm for about 24 h until both enantiomers were oxidized; reactions were monitored by GC. The encapsulated W110G was removed from the reaction

mixture before W110V TeSADH (0.2 mg) and 2-propanol (50 μ L) were added to the same reaction vessel, and further subjected to shaking for about 24 h at 180 rpm at 50 $^{\circ}$ C. The resulted organic layers were subjected to drying with Na_2SO_4 and further concentrated to dryness. The percent recovery and the *ee* of *S*-configured alcohols were subsequently evaluated using a GC equipped with a chiral column.

3.7 Stereoconversion of *R*-configured secondary alcohols

Alcohols [(*R*)-**1a**, (*R*)-**1c** or (*R*)-**1d**] (0.03 mmol) were added into a mixture containing 0.4 mg and 2.0 mg of W110G TeSADH and NADP^+ , respectively, in Tris-HCl buffer solution (970 μ L, 50 mM, pH 8.0) and acetone (3%, v/v) in a 2.0-mL Eppendorf tube. The mixture was stirred for about 36 h at 50 $^{\circ}$ C at 180 rpm. The solution obtained after the oxidation reaction was heated for 45 min at 80 $^{\circ}$ C to denature any remaining W110G TeSADH. A fresh W110V TeSADH (0.2 mg) and 2-propanol (5%, v/v) were added to the same reaction vessel, and further subjected to shaking for about 24 h at 180 rpm at 50 $^{\circ}$ C. The reaction mixture was extracted with diethyl ether (2 $\times$ 1.0 mL), and the resulted organic layers were subjected to drying with Na_2SO_4 and further concentrated to dryness. The percent recovery and the *ee* of *S*-configured alcohols were subsequently evaluated using chiral GC.

3.8 Concurrent deracemization of alcohols via cyclic deracemization approach

Alcohol racemates (0.01 mmol) were introduced into a mixture of W110G TeSADH (0.2 mg), W110V/G198D TeSADH (0.2 mg), sodium formate (40.8 mg), NAD^+ (2.0 mg), NADP^+ (2.0 mg) and formate dehydrogenase FDH (1 mg) in Tris-HCl buffer solution (980 μ L, 50 mM, pH 8.0) and acetone (20 μ L) in a 2.0-mL Eppendorf tube. The mixture was

stirred at 25 °C at 180 rpm for about 24 h. The reaction mixture was extracted with diethyl ether (2×1.0 mL), and then the combined organic layer was subjected to drying with Na_2SO_4 and further concentrated. The remaining residue was converted to the corresponding acetate ester as mentioned above. The percent recovery and *ee* of resulted alcohols were subsequently evaluated using a GC equipped with a chiral column.

3.9 Concurrent deracemization of alcohols via stereoinversion approach

Alcohol racemates (0.01 mmol) were introduced into a mixture of I86A TeSADH (0.2 mg), W110V/G198D TeSADH (0.2 mg), sodium formate (40.8 mg), NAD^+ (2.0 mg), NADP^+ (2.0 mg), ZnCl_2 (to a final concentration of 0.0028 μM), and formate dehydrogenase FDH (1.0 mg) were added to Tris-HCl buffer solution (980 μL , 50 mM, pH 8.0) and acetone (20 μL) in a 2.0-mL Eppendorf tube. The mixture was stirred at 25 °C at 180 rpm for about 24 h. The mixture was then extracted with diethyl ether (2×1.0 mL). The combined organic layer was dried with Na_2SO_4 and then concentrated. The percent recovery and *ee* of the resulted alcohols were subsequently evaluated using a GC equipped with a chiral column.

3.10 Determination of the stereochemical identities of the products

Capillary gas chromatography (GC) measurements of the products of deracemization were executed on an Agilent 7890A gas chromatograph furnished with FID and an Agilent HP-Chiral-20B column 30 m long, with 0.32 mm inner diameter and 0.25 μm film thickness employing helium as the carrier gas. The following set up was adopted for the oven: initial temperature of 70 °C, hold for 10 min and finally increased to 180 °C and subsequently

hold for 20 min at a rate of 5 °C/min. Likewise, the injector and detector temperature were maintained at 220 and 230 °C, respectively. The flow rates of air was set at 300 mL/min, 30 mL/min for H₂ and 15 mL/min for the Helium gas. The split mode of 10:1 ratio was used. The absolute configurations of the alcohols synthesized by deracemization of alcohols were demonstrated by co-injection of their acetate ester derivatives on a chiral column equipped GC with either the commercially purchased enantiopure alcohols (*S*- or *R*-configured) or those synthesized by asymmetric reduction of the prochiral ketones catalyzed by W110A mutant of TeSADH, which are confirmed in the literature to produce (*S*)-configured alcohols [61] or those prepared by CaLB-catalyzed KR of racemic alcohol to give *R*-configured alcohols [62].

3.11 Determination of the chemical identity of the products

Gas chromatography-mass spectrometry analyses was used to elucidate the structures of the products of deracemization. They were accomplished using Agilent 7890A GC instrument coupled with an Agilent 5975C inert mass-selective detector with a triple-axis detector. The following set up was adopted for the oven: initial temperature of 60 °C, hold for 10 min and finally increased to 240 °C and subsequently hold for 20 min at a rate of 5 °C/min. Likewise, the injector and detector temperatures were maintained at 300 and 310 °C, respectively. The flow rates of air, H₂, and Helium were set at 300 mL/min, 30 mL/min, and 15 mL/min, respectively. The split mode of 10:1 ratio was used. NMR spectra were recorded on a JOEL JNM-LA500 FT NMR operated at 500 MHz and 125 MHz for ¹H and ¹³C, respectively, at ambient temperature, employing deuterated chloroform (CDCl₃) peak as an internal standard.

CHAPTER 4

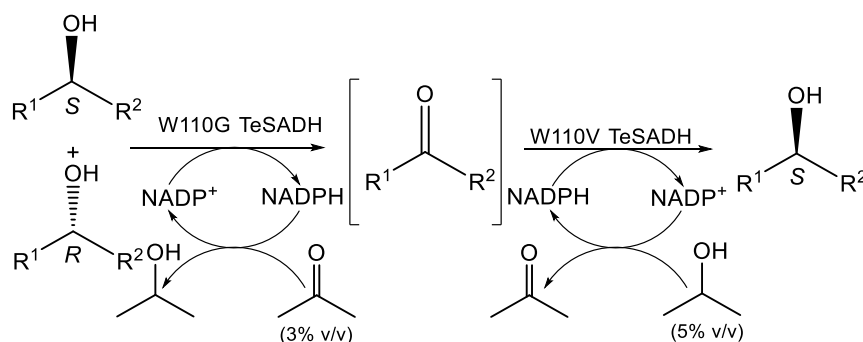
RESULTS AND DISCUSSION

4.1 Optimization of W110G TeSADH-catalyzed oxidation of alcohols

The intended deracemization approach relies on non-stereospecific oxidation of alcohol followed by a stereoselective reduction of the resultant prochiral ketone. Thus, depletion of both enantiomers of an alcohol is crucial for the success of this method. W110G TeSADH, which is known for its marginal enantioselectivity with phenyl-ring-containing secondary alcohols [62], was used to conduct the oxidation reaction of 1-phenyl-2-propanol [(*rac*)-**1c**] to ensure the depletion of both enantiomers because it was deracemized with low efficiency by the previously reported single enzymatic approach using W110G or W110A TeSADH [18]. Quantitative conversion of (*rac*)-**1c** to the corresponding ketone was achieved using 3% of acetone (v/v) [18], which was used as a co-substrate and as a co-solvent at the same time. The high conversion of racemic alcohol can be explained by the relatively low stereoselectivity of W110G TeSADH when the oxidation reaction was carried out using 3% (v/v) acetone. The complete depletion of both enantiomers of (*rac*)-**1c** is not strange because a previous study showed that the *E*-values [$(k_{\text{cat}}/K_{\text{m}})_{\text{S}}/(k_{\text{cat}}/K_{\text{m}})_{\text{R}}$] of W110G and W110V TeSADHs are 9 and 134, respectively [62], which reflects the marginal stereoselectivity of W110G TeSADH.

4.2 Bi-enzymatic deracemization of secondary alcohols

Upon full conversion of (*rac*)-**1c** to its ketone using W110G TeSADH, a heat treatment to deactivate this mutant was applied before proceeding with the enantioselective reduction step; this step was necessary to prevent action of W110G TeSADH in the reduction step [18], which will result in a reduced enantioselectivity in the reduction step because of the marginal enantioselectivity of this mutant. W110V TeSADH was used for enantioselective reduction of the intermediate ketone; this mutant was reported to have high enantioselectivity in the reduction of the selected phenyl-ring-containing ketones [62]. The reduction step was affected by using 2-propanol (5%, v/v), which is used as a co-substrate and a co-solvent. Deracemization of (*rac*)-**1c** under these conditions resulted in formation of (*S*)-**1c** in 95% *ee*, with a significant improvement when compared with the single enzymatic deracemization approach previously reported using a single enzymatic deracemization [18]. This bi-enzymatic deracemization approach was then applied to deracemize (*rac*)-**1a**, (*rac*)-**1b**, (*rac*)-**1d**, and (*rac*)-**1e**; these results are presented in Table 1. These substrates were deracemized and their (*S*)-configured alcohols were obtained with high *ee*. The presently reported bi-enzymatic deracemization approach gave better results in percentage recovery and *ee* than the previously reported single enzymatic approach developed in our laboratory [18]. It is worth mentioning that the percent of ketone obtained at the end of deracemization process did not exceed 0.5%. This method allows deracemization of alcohols to be conducted in two steps and without the need to isolate the ketone intermediate. The superior efficiency of this method justifies the use of two mutants of the same enzyme instead of using one.

Table 1. One-pot two-step bi-enzymatic deracemization of secondary alcohols.

Substrate	R ¹	R ²	Ketone [%]	ee [%] ^[b]
(rac)- 1a	<i>p</i> -HO-C ₆ H ₄ CH ₂ CH ₂	CH ₃	<0.5	>99
(rac)- 1b	<i>p</i> -MeO-C ₆ H ₄ CH ₂ CH ₂	CH ₃	<0.5	98
(rac)- 1c	C ₆ H ₅ CH ₂	CH ₃	<0.5	95
(rac)- 1d	C ₆ H ₅ CH ₂ CH ₂	CH ₃	<0.5	>99
(rac)- 1e	C ₆ H ₅ CH ₂	CH ₃ CH ₂	<0.5	>99

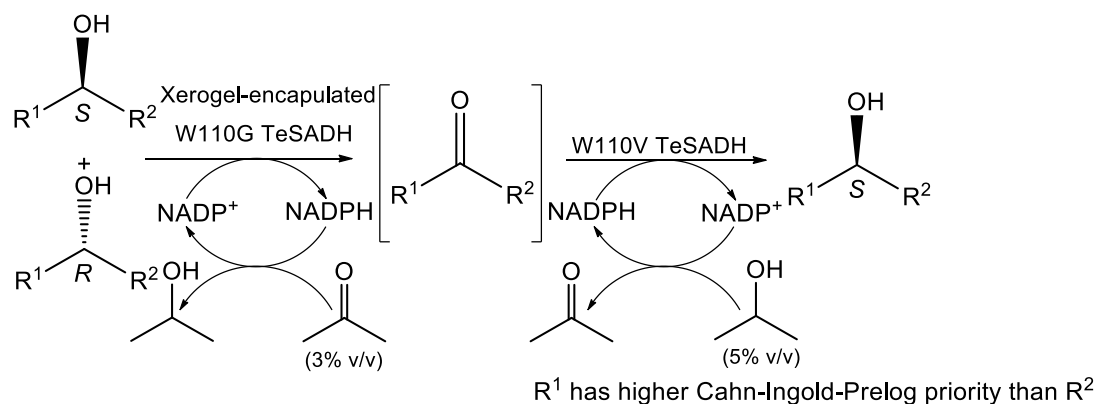
[a] For the oxidation reactions, 0.03 mmol of alcohol racemates (rac)-**1a–e**, 0.2 mg of W110 G mutant of TeSADH, 1.0 mg of $NADP^+$ and 3% (v/v) acetone were used; Meanwhile, 0.2 mg of W110V TeSADH and 5% v/v of 2-propanol were employed to facilitate reduction reactions. [b] The percentage *ee* was investigated using a chiral column equipped GC.

4.3 Deracemization using xerogel-encapsulated W110G TeSADH

In order to overcome the heat treatment step and make sure that no enzyme remaining from the oxidation step, we used xerogel-encapsulated W110G TeSADH to perform the oxidation step [17, 63]. Encapsulation of enzymes in porous sol-gel offers unique properties such as large surface area and porosity [64]. Affecting the oxidation of (rac)-**1a** and (rac)-**1b** by xerogel-encapsulated W110G TeSADH enabled the complete oxidation to their corresponding ketones. The reduction of the intermediate ketones was then followed by using free W110V TeSADH after removal of the xerogel-encapsulated W110G TeSADH from the reaction mixture, thus avoiding the heat treatment after the oxidation step. It was observed that deracemization of these two substrates using the xerogel-

encapsulated W110G TeSADH gave the same *ee* values obtained by the deracemization using free enzyme; these results are presented in Table 2. This sol-gel encapsulated enzymatic deracemization approach could be beneficial in deracemization of many racemic alcohols that are heat labile without decomposition.

Table 2. Bi-enzymatic deracemization of (*rac*)-**1a** and (*rac*)-**1b** with sol-gel encapsulated W110G TeSADH.



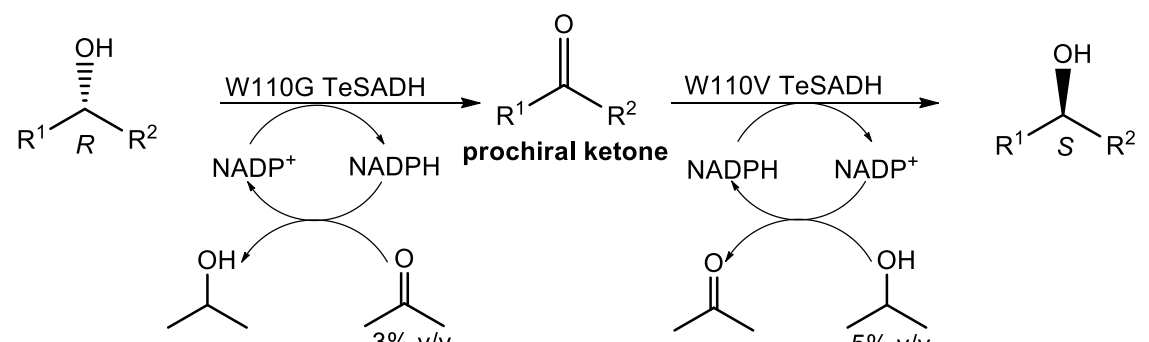
Substrate	R^1	R^2	Ketone [%]	<i>ee</i> [%] ^[b]
(<i>rac</i>)- 1a	<i>p</i> -HO-C ₆ H ₄ CH ₂ CH ₂	CH ₃	<0.5	>99
(<i>rac</i>)- 1b	<i>p</i> -MeO-C ₆ H ₄ CH ₂ CH ₂	CH ₃	<0.5	98

[a] For the oxidation reactions, 0.03 mmol of alcohol racemates (*rac*)-**1a–e**, xerogel- encapsulated W110 G mutant of TeSADH, 1.0 mg of NADP⁺ and 3% (v/v) acetone were used; Meanwhile, 0.2 mg of W110V TeSADH and 5% v/v of 2-propanol were employed to facilitate reduction reactions. [b] The percentage *ee* was investigated using a chiral column equipped GC.

4.4 Stereoconversion of *R*-configured secondary Alcohols

The successful deracemization of alcohols using the two mutants of TeSADH with tuned enantioselectivities encouraged us to conduct stereoconversion of (*R*)-alcohols (i.e., Mitsunobu-like reaction). This strategy relies on the ability of W110G TeSADH to accommodate the (*R*)-alcohols, evident by the complete depletion of both enantiomers in deracemization of alcohols. This bi-enzymatic stereoconversion was conducted for (*R*)-**1a**, (*R*)-**1c** and (*R*)-**1d** and up to >99% *ee* of (*S*)-configured alcohols was achieved as shown in Table 3. Whereas, (*S*)-**1c** and (*S*)-**1d** were obtained previously in 33% *ee* and 80% *ee*, respectively, using the single enzymatic stereoconversion approach [58]. Although the current approach uses two mutants of TeSADH instead of one, however, there is significant enhancement in *ee* when the current approach is used. The use of two mutants of the same enzymes, at least, ensures their compatibility, unlike when two different enzymes are used.

Table 3. Bi-enzymatic stereoconversion of *R*-configured secondary alcohols.



Substrate	R ¹	R ²	Ketone [%]	<i>ee</i> [%] ^[b]
(<i>rac</i>)- 1a	<i>p</i> -HO-C ₆ H ₄ CH ₂ CH ₂	CH ₃	<0.5	>99
(<i>rac</i>)- 1c	C ₆ H ₅ CH ₂	CH ₃	<0.5	88
(<i>rac</i>)- 1d	C ₆ H ₅ CH ₂ CH ₂	CH ₃	<0.5	99

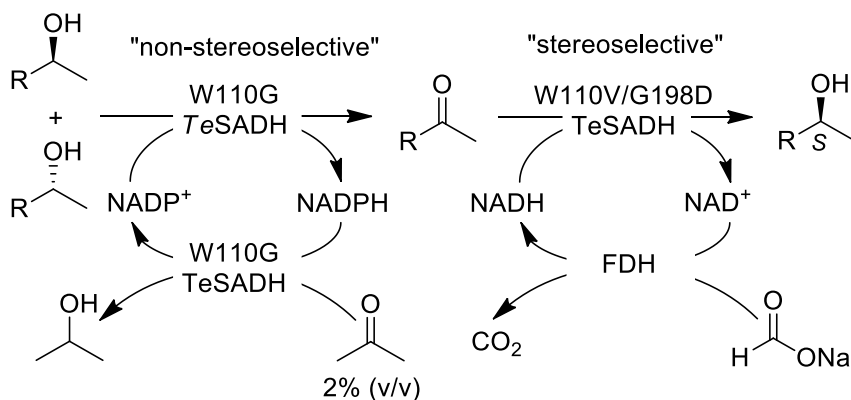
[a] For the oxidation reactions, 0.03 mmol of (*R*)-**1a**, (*R*)-**1c** and (*R*)-**1d**, 0.4 mg of W110 G mutant of TeSADH, 1.0 mg of NADP⁺ and 3% v/v acetone were used; Meanwhile, 0.2 mg of W110V TeSADH and 5% v/v of 2-propanol were employed to facilitate reduction reactions. [b] The percentage *ee* was investigated using a chiral column equipped GC.

4.5 Concurrent deracemization of alcohols via cyclic deracemization approach

The successful deracemization of alcohols using the one-pot two-step approach described above encouraged us to conduct a concurrent one-pot deracemization of alcohols using the two mutants of TeSADH in one-step. In order to design a concurrent cyclic deracemization strategy, the following requirements should be satisfied: first, a non-stereoselective mutant of TeSADH, second, a stereoselective mutant of TeSADH, third, the oxidation and reduction reactions have to be orthogonal in terms of cofactor regeneration. W110G TeSADH, which is an NADP-dependent enzyme, was selected as a marginally stereoselective catalyst to conduct the oxidation step, and W110V/G198D TeSADH, which is an NAD-dependent catalyst was selected to accomplish the stereoselective reduction. The concurrent implantation of these redox reactions is expected to work in one pot (i.e., cyclic deracemization) assuming orthogonal coenzyme regeneration systems. This strategy relies on the ability of W110G TeSADH to oxidize both enantiomers of alcohols without high discrimination using large excess of acetone as a cosubstrate to shift the equilibrium in the oxidation step towards the formation of ketone. To simultaneously affect the stereoselective reduction of the resulted ketone, even in the presence of acetone needed for the oxidation reaction, an orthogonal system for coenzyme regeneration was used for the reduction reaction (i.e., NAD regeneration system). A single mutation of TeSADH at G198, which is not part of the active site, by D was shown recently to switch the NADP-dependent TeSADH to an NAD-dependent enzyme [65]. Thus, W110V/G198D TeSADH was constructed to be used as an NAD-dependent reduction catalyst for the second step. Since this extra mutation is not part of the active site, we expect that the substrate scopes

and enantioselectivities of W110V TeSADH and those of W110V/G198D TeSADH are identical.

Deracemization reactions for (*rac*)-**1a-c** were conducted using W110G TeSADH and W110V/G198D TeSADH in the presence of NADP⁺ and NAD⁺ cofactors as well as acetone and sodium formate as sacrificial substrates; moreover, formate dehydrogenase (FDH) was used for NAD⁺ regeneration. Multiple conditions were tried in the deracemization of the aforementioned substrates and up to 40% *ee* of (*S*)-alcohol was obtained as shown in Table 4. The low *ee*'s obtained with this cyclic deracemization approach could be explained by the unavoidable futile cycles (i.e., oxidation of the desired enantiomer), which inactivates enzymes with unnecessary redox cycles; this is evident by the enhanced *ee* that was observed when the amount of enzyme used was doubled. Although W110G TeSADH is marginally stereoselective, when compared with other known TeSADH, however its *E*-value [$(k_{\text{cat}}/K_{\text{m}})_{\text{S}}/(k_{\text{cat}}/K_{\text{m}})_{\text{R}}$] of 9 in the oxidation reaction of **1c** (previously described) [62] indicates that it still prefers the *S* enantiomer. Designing a completely non-stereoselective TeSADH mutant will, for sure, improve the performance of this oxidative cyclic deracemization approach.

Table 4. Concurrent cyclic deracemization of alcohols.^[a]

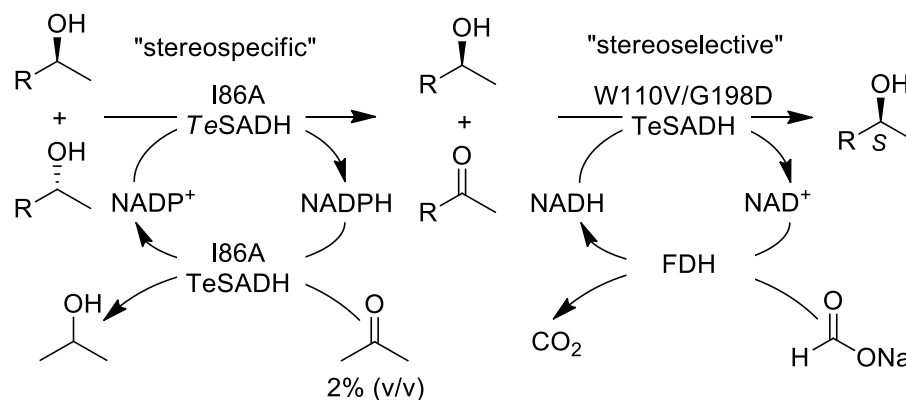
Substrate	R ¹	R ²	Ketone [%]	ee [%] ^[b]
(<i>rac</i>)- 1a	<i>p</i> -HO-C ₆ H ₄ CH ₂ CH ₂	CH ₃	4.2	6 (<i>R</i>)
(<i>rac</i>)- 1a	<i>p</i> -HO-C ₆ H ₄ CH ₂ CH ₂	CH ₃	11.7	4 (<i>R</i>) ^[c]
(<i>rac</i>)- 1b	<i>p</i> -MeO-C ₆ H ₄ CH ₂ CH ₂	CH ₃	0.7	12 (<i>S</i>)
(<i>rac</i>)- 1b	<i>p</i> -MeO-C ₆ H ₄ CH ₂ CH ₂	CH ₃	20	40 (<i>S</i>) ^[d]
(<i>rac</i>)- 1c	C ₆ H ₅ CH ₂	CH ₃	6.2	32 (<i>S</i>)
(<i>rac</i>)- 1c	C ₆ H ₅ CH ₂	CH ₃	7.7	28 (<i>S</i>) ^[e]
(<i>rac</i>)- 1c	C ₆ H ₅ CH ₂	CH ₃	6.1	28 (<i>S</i>) ^[f]
(<i>rac</i>)- 1c	C ₆ H ₅ CH ₂	CH ₃	1.2	16 (<i>S</i>) ^[g]
(<i>rac</i>)- 1c	C ₆ H ₅ CH ₂	CH ₃	1.0	6 (<i>R</i>) ^[c]
(<i>rac</i>)- 1c	C ₆ H ₅ CH ₂	CH ₃	1.3	24 (<i>S</i>) ^[h]

[a] Unless stated otherwise, reactions were performed by using racemic alcohol [(*rac*)-**1a–c** (0.01 mmol)], W110G TeSADH (0.2 mg), W110V/G198D TeSADH (0.2 mg), NAD⁺ (2.0 mg), NADP⁺ (2.0 mg), sodium formate (40.8 mg), FDH (1.0 mg) and acetone (2%, v/v) in Tris-HCl buffer (50 mM, pH 8.0). [b] The percentage of ee was determined by using GC with a chiral stationary phase. [c] Tris-HCl buffer (50 mM, pH 6.5) was used. [d] W110V/G198D TeSADH (0.4 mg) was used. [e] Cyclohexanone (2%, v/v) was added. [f] Acetonitrile (2%, v/v) was added. [g] Sodium formate (81.6 mg) was used. [h] W110A/I86A TeSADH was used instead of W110G TeSADH.

4.6 Concurrent deracemization of alcohols via a stereoinversion approach

Alcohol deracemization via stereoinversion, which involves two stereoselective redox reactions that exhibit opposite stereopreferences, could be looked at as one extreme of cyclic deracemization. It is considered among the efficient deracemization approaches;

however, the design of two catalytic systems that act on the same substrate with opposite stereopreferences is challenging. The low *ee* encountered in the above-described concurrent one-pot cyclic deracemization could be due to futile cycles as a result of the use of the marginally stereoselective W110G TeSADH mutant in the oxidation step. This unnecessary oxidation of *S*-configured alcohols, the desired one in this case, generated from reduction of the prochiral ketones (i.e., futile cycles) using W110V/G198D TeSADH leads to enzyme deactivation; note that the *S*-configured alcohols are still the preferred enantiomers for W110G TeSADH. After an extensive search for two enzymes that exhibit opposite stereopreferences on the same substrate, we found that I86A TeSADH, which was described as an *anti*-Prelog TeSADH [66-67], can be used in the oxidation step. Therefore, we used NADP-dependent, (*R*)-stereoselective I86A TeSADH to oxidize the (*R*)-alcohols to their corresponding ketones leaving the *S*-alcohols untouched. The reduction of the produced prochiral ketones was then concurrently facilitated by using NAD-dependent W110V/G198D TeSADH. Deracemization of (*rac*)-**1a**, (*rac*)-**1b**, (*rac*)-**1c** and (*rac*)-**1f** was conducted using this approach, Table 5. The best results were obtained in the deracemization of (*rac*)-1-phenylethanol, which is a good substrate for I86A TeSADH. Future efforts should be directed towards constructing enzyme mutants with *anti*-Prelog stereopreference that exhibit wide substrate scope to further improve the deracemization efficiency of this approach. The current study shows a proof of concept for concurrent deracemizations via cyclic and stereoinversion approaches using two mutants of TeSADH.

Table 5. Concurrent deracemization of alcohols via stereoinversion approach.^[a]

Substrate	R ¹	R ²	Ketone [%]	ee [%] ^[b]
(rac)- 1a	<i>p</i> -HO-C ₆ H ₄ CH ₂ CH ₂	CH ₃	3.0	14 (<i>R</i>)
(rac)- 1b	<i>p</i> -MeO-C ₆ H ₄ CH ₂ CH ₂	CH ₃	21	22 (<i>R</i>)
(rac)- 1c	C ₆ H ₅ CH ₂	CH ₃	10	22 (<i>S</i>)
(rac)- 1c	C ₆ H ₅ CH ₂	CH ₃	1.7	76 (<i>S</i>) ^[c]
(rac)- 1f	C ₆ H ₅	CH ₃	15	85 (<i>S</i>)
(rac)- 1f	C ₆ H ₅	CH ₃	<0.5	>99 (<i>S</i>) ^[c]

[a] Unless stated otherwise, reactions were performed by using racemic alcohol [(rac)-**1a**, (rac)-**1b**, (rac)-**1c** or (rac)-**1f**] (0.01 mmol), I86A TeSADH (0.2 mg), W110V/G198D TeSADH (0.2 mg), NAD⁺ (2 mg), NADP⁺ (2 mg), sodium formate (40.8 mg), FDH (1 mg), ZnCl₂ (0.0028 μM) and acetone (2%, v/v). [b] The percentage of ee was determined by using GC with a chiral stationary phase. [c] W110V/G198D (0.4 mg) was used.

4.7 Stereochemical identities of the reaction products

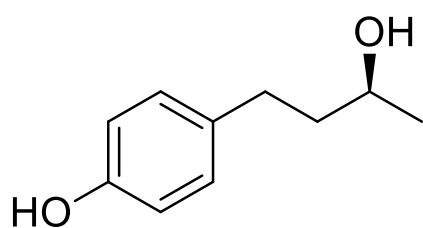
The stereochemical identities of the products of deracemization of racemic alcohols were confirmed by co-injection of their acetate ester derivatives using a chiral column equipped GC with standard samples that are either commercially available or prepared by CaLB-catalyzed KR or W110A TeSADH-catalyzed reduction of their ketones. Analysis of racemic alcohols synthesized by sodium borohydride reduction or commercially available ones was crucial to make sure that the two enantiomers are separated by the chiral column.

All GC chromatograms for the deracemization and stereoinversion reactions as well as those for racemic alcohols are shown in the Appendix A (Figures S1-S28)

4.8 Chemical identities of the reaction products

Gas chromatography-mass spectrometry as well as NMR (^1H and ^{13}C) analyses were conducted for deracemization and stereoinversion products to ascertain their chemical identities. GC-MS and NMR data are shown in Appendix B (Figure S29-S33) and Appendix C (Figure S34-S43), respectively.

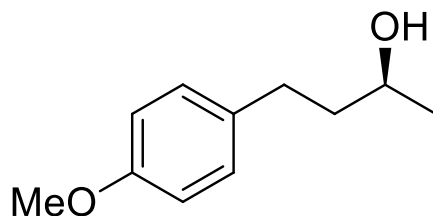
(S)-4-(4'-Hydroxyphenyl)-2-butanol [(S)-1a]: ^1H NMR (CD_3OD): $\delta = 1.18$ (d, $J = 6.1$



Hz, 3 H), 1.60-1.75 (m, 2 H), 2.50-2.59 (m, 1 H), 2.60-2.71 (m, 1 H), 3.32 (s, 1 H), 3.68-3.76 (m, 1 H), 6.70 (d, $J = 8.3$ Hz, 2 H), 7.01 (d, $J = 8.3$ Hz, 2 H) ppm. ^{13}C NMR (CD_3OD): $\delta = 23.5, 32.3, 42.4, 67.9, 116.1,$

130.2, 134.4, 156.3 ppm. Both the ^1H and ^{13}C NMR data were consistent with the previously reported spectroscopic data [3].

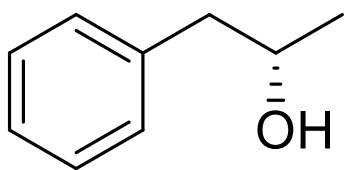
(S)-4-(4'-Methoxyphenyl)-2-butanol [(S)-1b]: ^1H NMR (CD_3OD): $\delta = 1.21$ (d, $J = 6.1$



Hz, 3 H), 1.46 (br s, 1 H), 1.69-1.78 (m, 2 H), 2.57-2.70 (m, 2 H), 3.76 (s, 3 H), 3.77-3.81 (m, 1 H), 6.81 (d, $J = 8.3$ Hz, 2 H), 7.10 (d, $J = 8.3$ Hz, 2 H) ppm. ^{13}C

NMR (CD_3OD): $\delta = 23.6, 31.2, 41.0, 55.2, 67.5, 113.8, 129.2, 134.1, 157.7$ ppm. Both the ^1H and ^{13}C NMR data were consistent with the previously reported spectroscopic data [61].

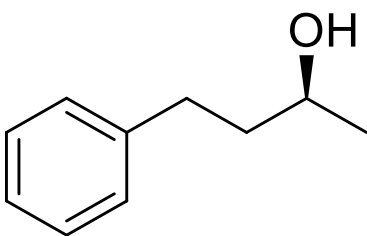
(S)-1-Phenyl-2-propanol [(S)-1c]: ^1H NMR (CD_3OD): δ = 1.27 (d, J = 6.1 Hz, 3 H), 1.61



(br s, 1 H), 2.71 (dd, J = 13.6, 8.1 Hz, 1 H), 2.81 (dd, J = 13.4, 4.9 Hz, 1 H), 4.01-4.08 (m, 1 H), 7.22-7.35 (m, 5 H) ppm. ^{13}C NMR (CD_3OD): δ = 23.0, 46.0, 69.1, 126.8, 128.9,

129.7, 138.8 ppm. Both the ^1H and ^{13}C NMR data were consistent with the previously reported spectroscopic data [68].

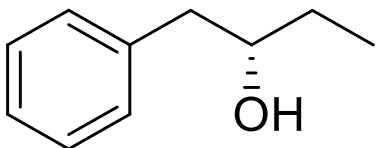
(S)-4-Phenyl-2-butanol [(S)-1d]: ^1H NMR (CD_3OD): δ = 1.23 (d, J = 6.1 Hz, 3 H), 1.59



(s, 1 H), 1.74-1.85 (m, 2 H), 2.67-2.81 (m, 2 H), 3.79-3.86 (m, 1 H), 7.17-7.31 (m, 5 H) ppm. ^{13}C NMR (CD_3OD): δ = 23.8, 32.3, 41.1, 67.8, 126.1, 128.6, 128.7, 142.4 ppm.

Both the ^1H and ^{13}C NMR data were consistent with the previously reported spectroscopic data [68].

(S)-1-Phenyl-2-butanol [(S)-1e]: ^1H NMR (CD_3OD): δ = 1.01 (t, J = 7.3 Hz, 3 H), 1.49-



1.60 (m, 3 H), 2.60 (dd, J = 13.5, 8.4 Hz, 1 H), 2.85 (dd, J = 13.5, 4.3 Hz, 1 H), 3.71-3.78 (m, 1 H), 7.21-7.63 (m, 5 H) ppm. ^{13}C NMR (CD_3OD): δ = 10.0, 29.6, 43.6, 74.0,

126.4, 128.5, 129.4, 138.6 ppm. Both the ^1H and ^{13}C NMR data were consistent with the previously reported spectroscopic data [69].

CHAPTER 5

CONCLUSION AND RECOMMENDATION

A marginally stereoselective W110G TeSADH was employed to mediate the oxidation of alcohol racemates and a highly stereoselective W110V TeSADH for the reduction of the corresponding ketones to enantiopure alcohols, and thus enable a sequential deracemization of secondary alcohols in one-pot and two-steps. This strategy was adopted to deracemize phenyl-ring-containing secondary alcohol substrates in high efficiencies (up to >99% *ee*). It gave better results in term of percentage recovery and *ee* than those previously reported using a single enzymatic approach [18]. It was observed that deracemization using the xerogel-encapsulated W110G TeSADH gave similar results in term of *ee* when compared to the results obtained by the free enzymatic deracemization approach, which in turn help to eliminate a heat treatment step that was necessary otherwise. This bi-enzymatic approach was also employed for the stereoinversion reaction (Mitsunobu-like reaction) of *R*-configured alcohols to give up to >99% *ee* of *S*-configured alcohols. This approach was improved on step further to a one-pot, one-step (concurrent) deracemization strategy via stereoinversion using two mutants of TeSADH , this approach involved two TeSADH mutants that exhibit opposite stereo-preferences towards secondary alcohol substrates. Up to >99.5% enantiomeric excess and quantitative yields of (*S*)-alcohols was achieved using this approach. This method is an enticing deracemization approach at industrial level for the synthesis of alcohols with very high enantiomeric excess from their racemates because it uses an environmentally benign robust catalyst. Bi-enzymatic concurrent

deracemization of more phenyl-ring containing secondary alcohols should be investigated. More mutants of TeSADH with tuned enantioselectivities should be designed, which can be achieved through extensive molecular dynamic simulation and protein engineering.

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APPENDIX A

GC CHROMATOGRAMS OF THE PRODUCTS OF DERACEMIZATION AND STEREOINVERSION REACTIONS

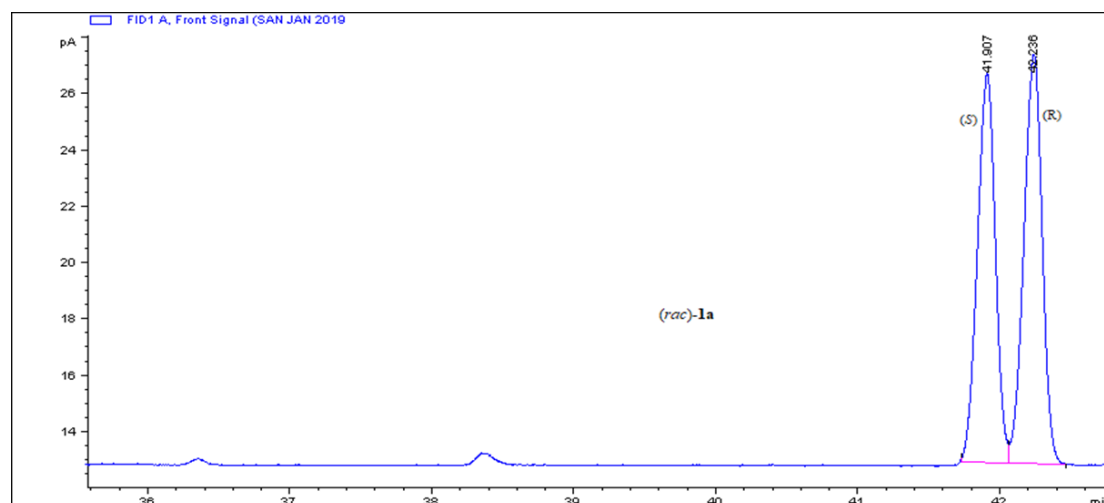


Figure S1. Gas chromatogram of the acetate derivative of (*rac*)-**1a**.

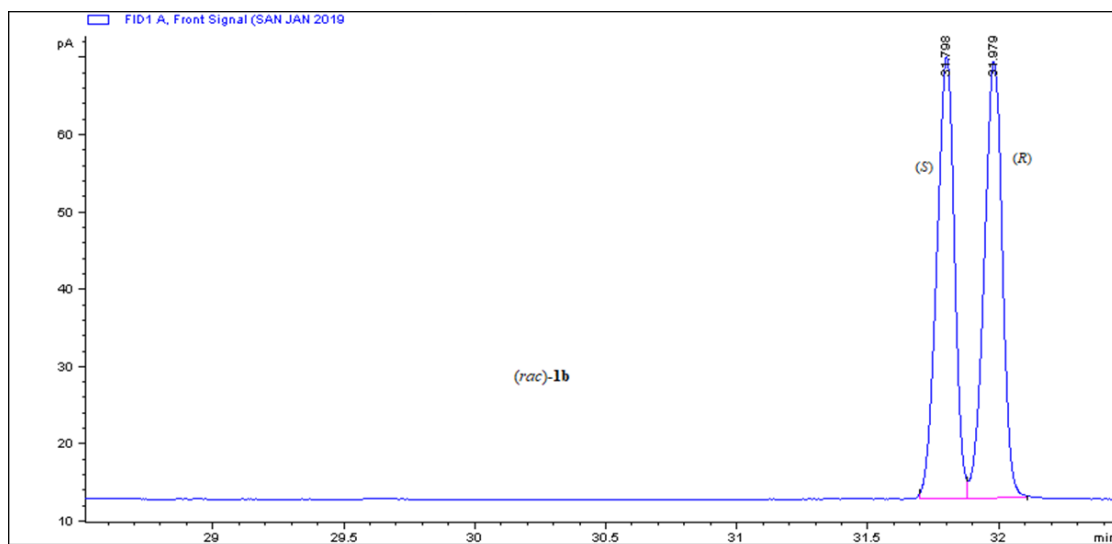


Figure S2. Gas chromatogram of the acetate derivative of (*rac*)-**1b**.

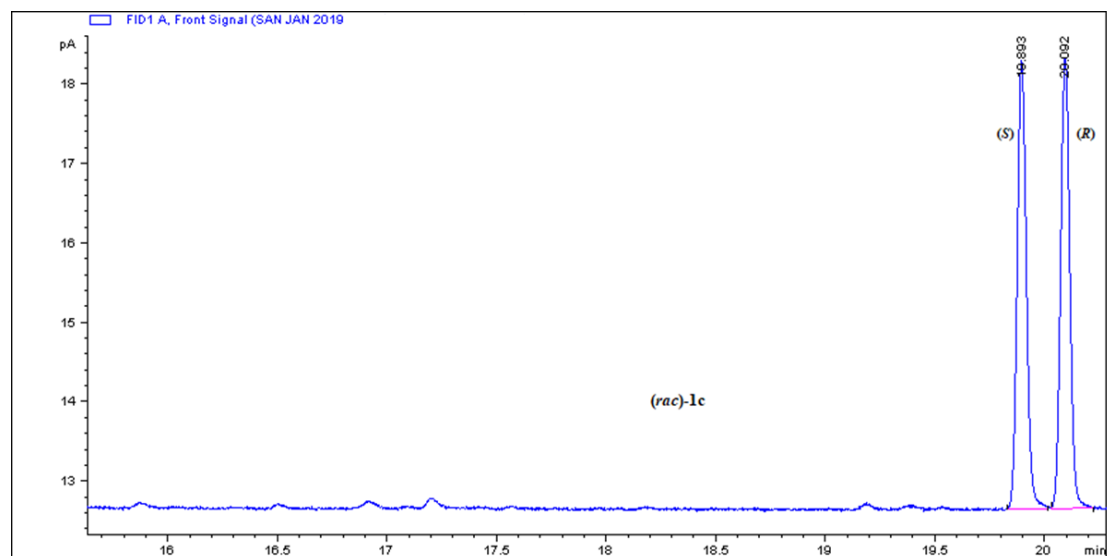


Figure S3. Gas chromatogram of the acetate derivative of (*rac*)-**1c**.

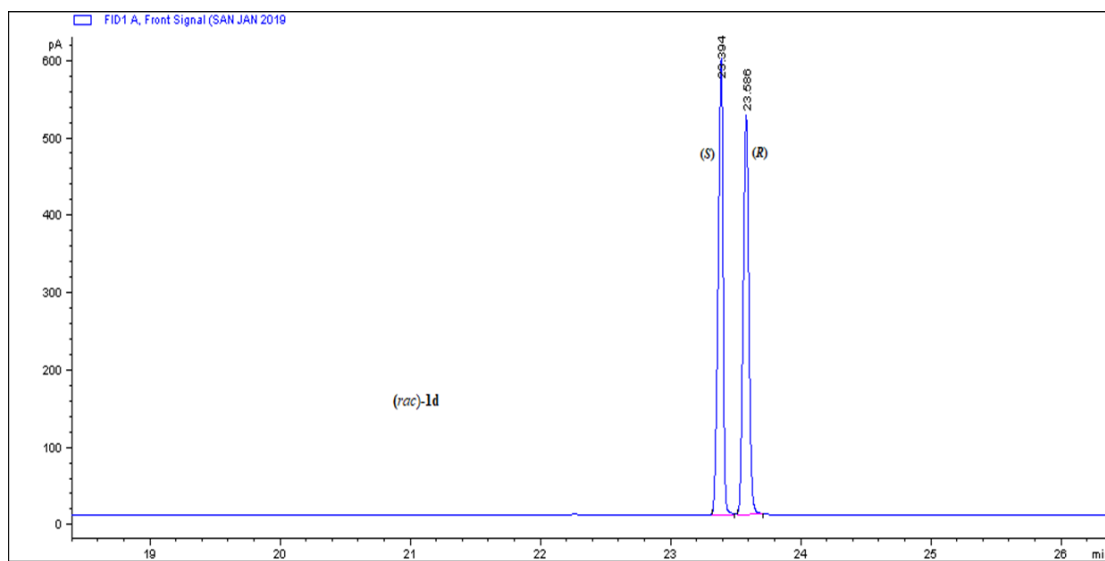


Figure S4. Gas chromatogram of the acetate derivative of (*rac*)-**1d**.

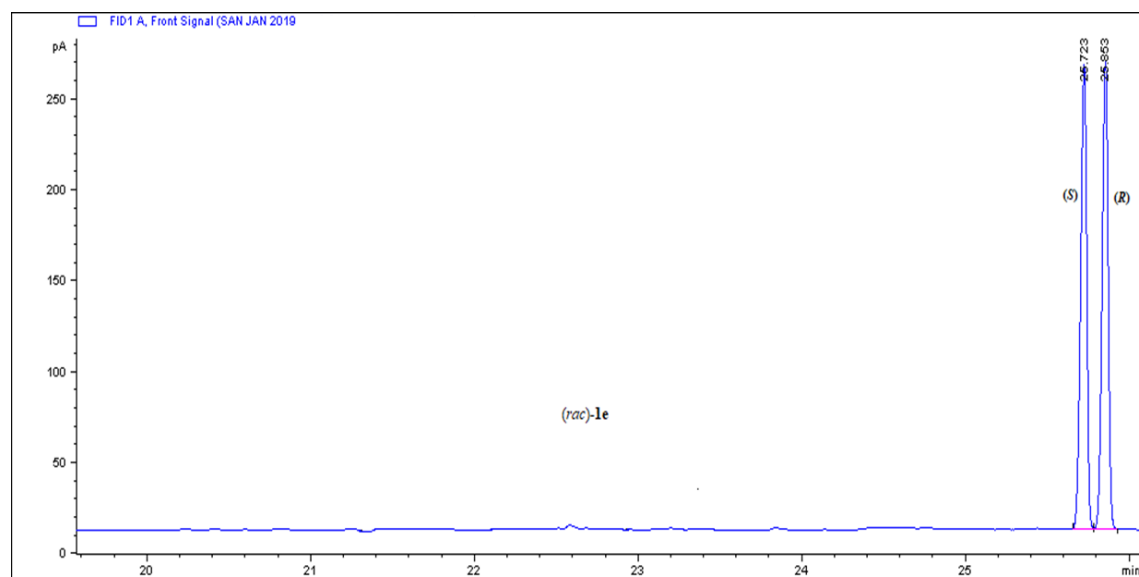


Figure S5. Gas chromatogram of the acetate derivative of (*rac*)-**1e**.

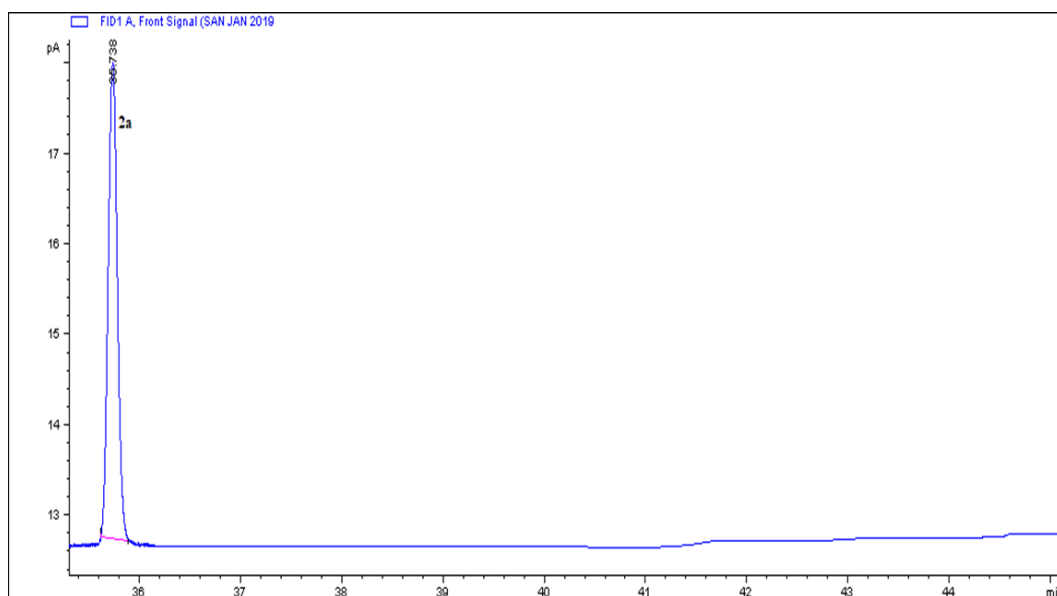


Figure S6. Gas chromatogram of the acetate derivative of the oxidation product of (*rac*)-**1a** using W110G mutants TeSADH.

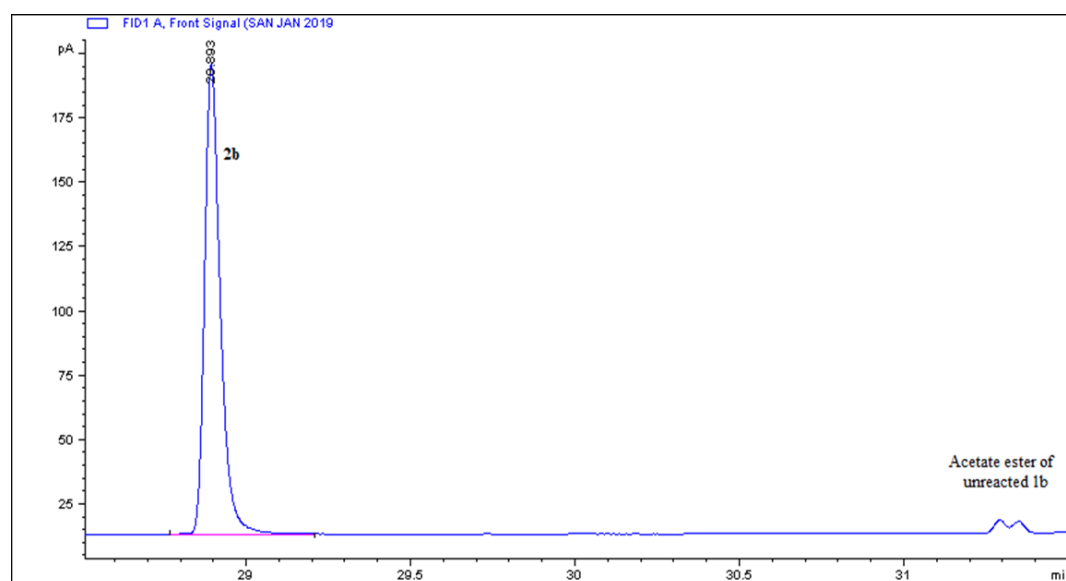


Figure S7. Gas chromatogram of the acetate derivative of the oxidation product of (*rac*)-**1b** using W110G mutants TeSADH.

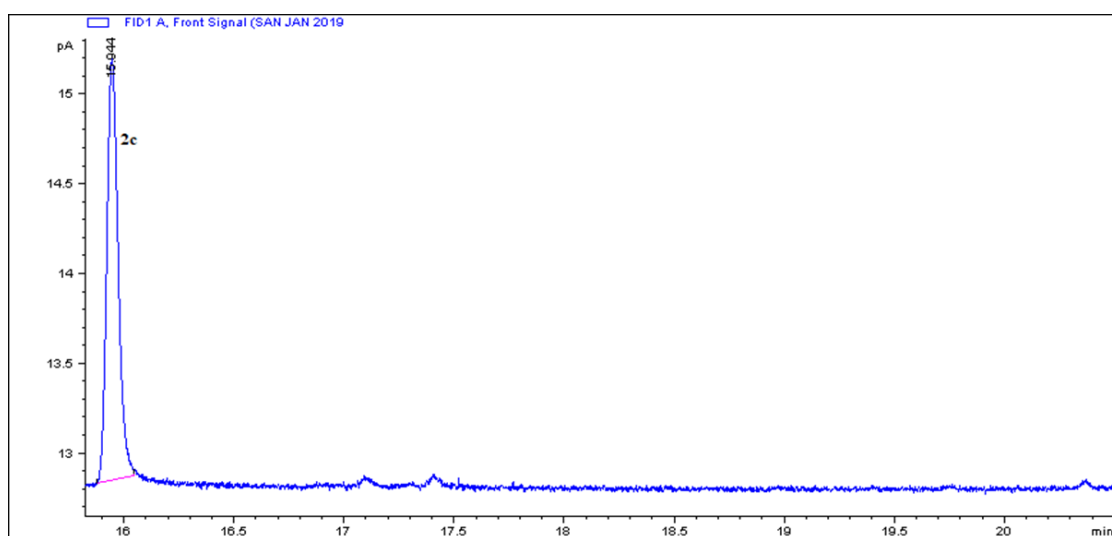


Figure S8. Gas chromatogram of the acetate derivative of the oxidation product of (*rac*)-**1c** using W110G mutants TeSADH.

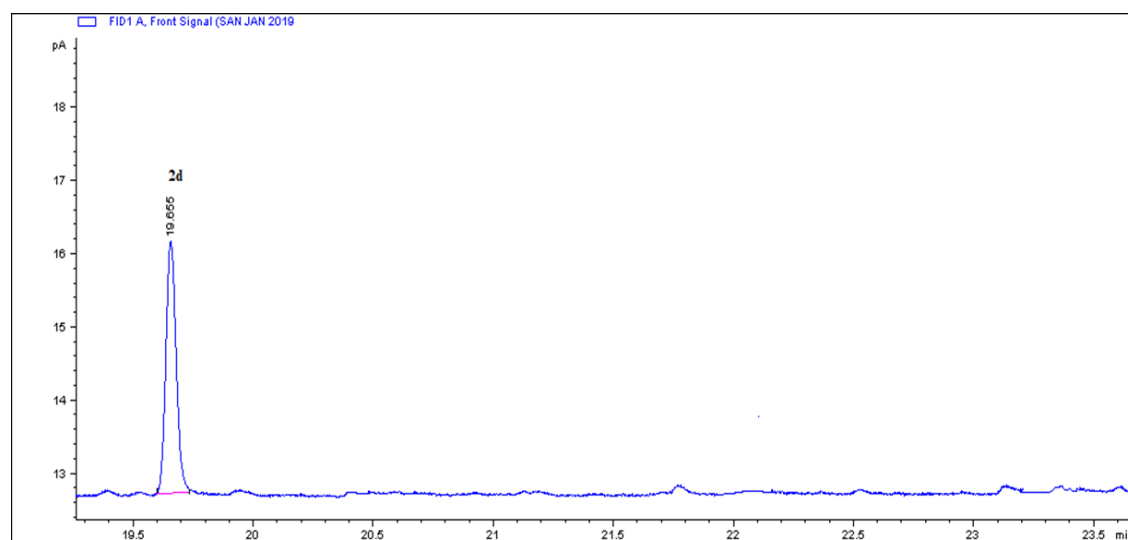


Figure S9. Gas chromatogram of the acetate derivative of the oxidation product of (*rac*)-**1d** using W110G mutants TeSADH.

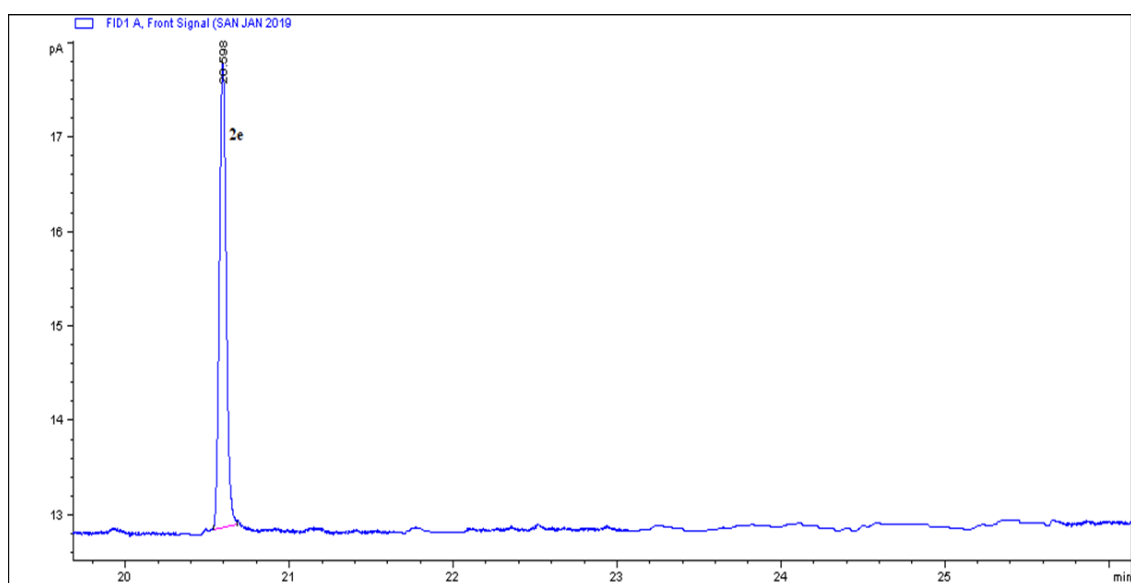


Figure S10. Gas chromatogram of the acetate derivative of the oxidation product of (*rac*)-**1e** using W110G mutants TeSADH.

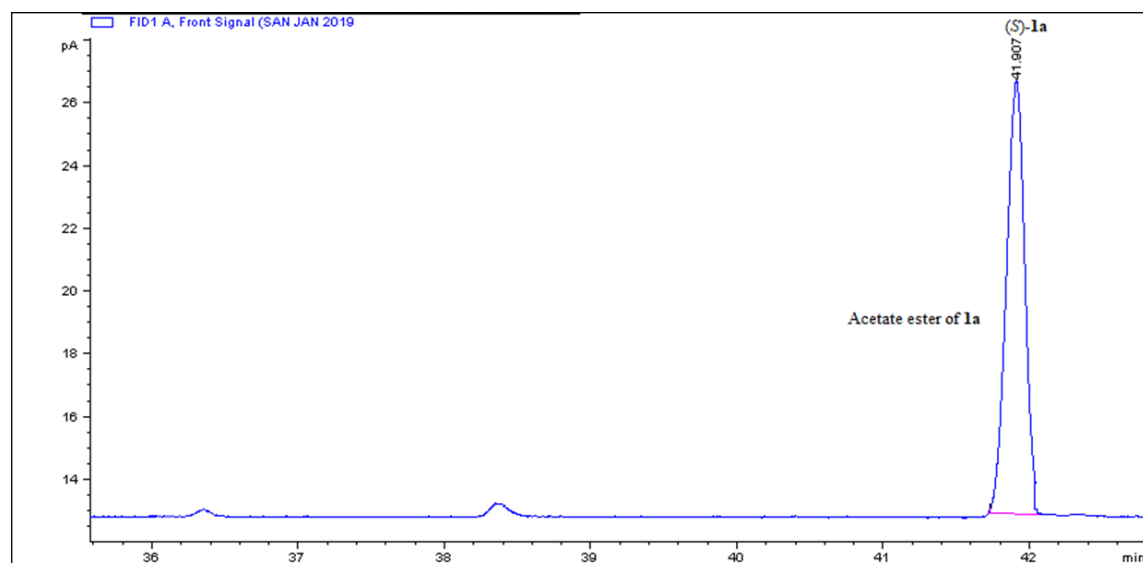


Figure S11. Gas chromatogram of the acetate derivative of (*rac*)-**1a** of the deracemization product using W110G and W110V Mutants of TeSADH (Table 1, entry 1).

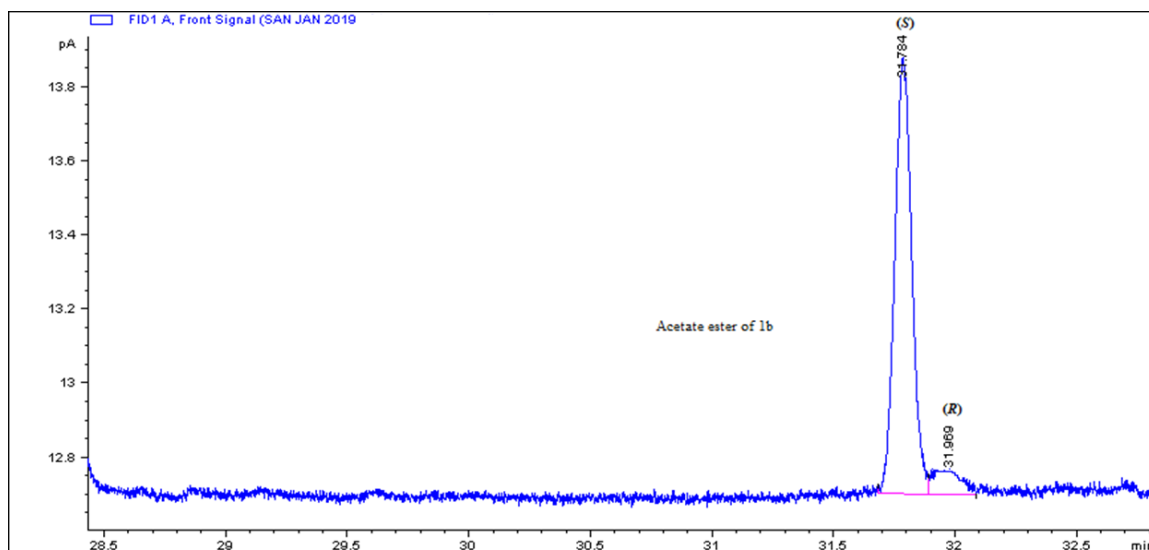


Figure S12. Gas chromatogram of the acetate derivative of (*rac*)-**1b** of the deracemization product using W110G and W110V Mutants of TeSADH (Table 1, entry 2).

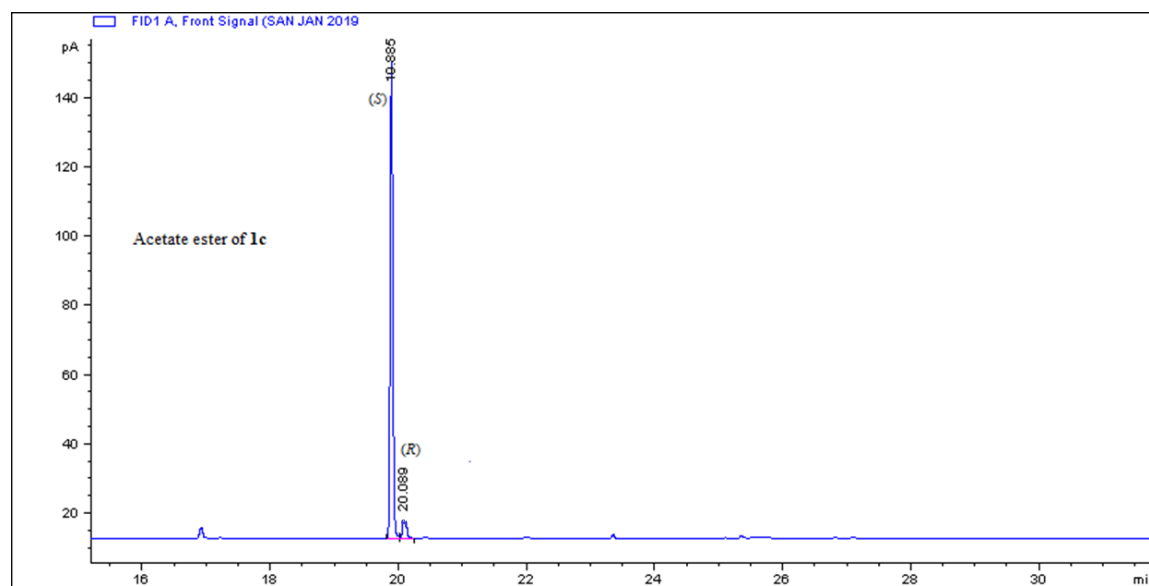


Figure S13. Gas chromatogram of the acetate derivative of (*rac*)-**1c** of the deracemization product using W110G and W110V Mutants of TeSADH (Table 1, entry 3).

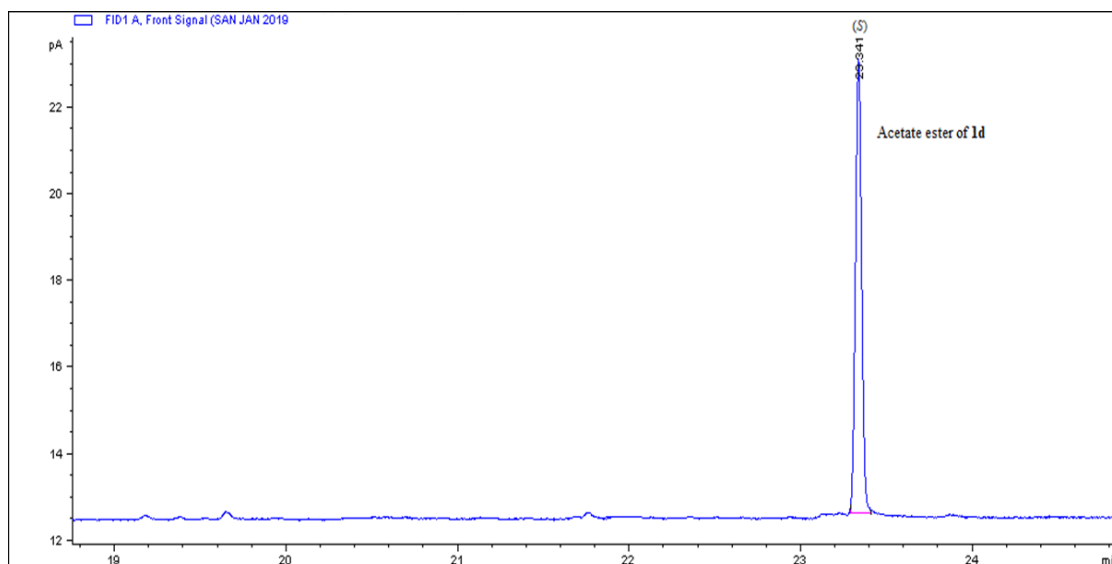


Figure S14. Gas chromatogram of the acetate derivative of (*rac*)-**1d** of the deracemization product using W110G and W110V Mutants of TeSADH (Table 1, entry 4).

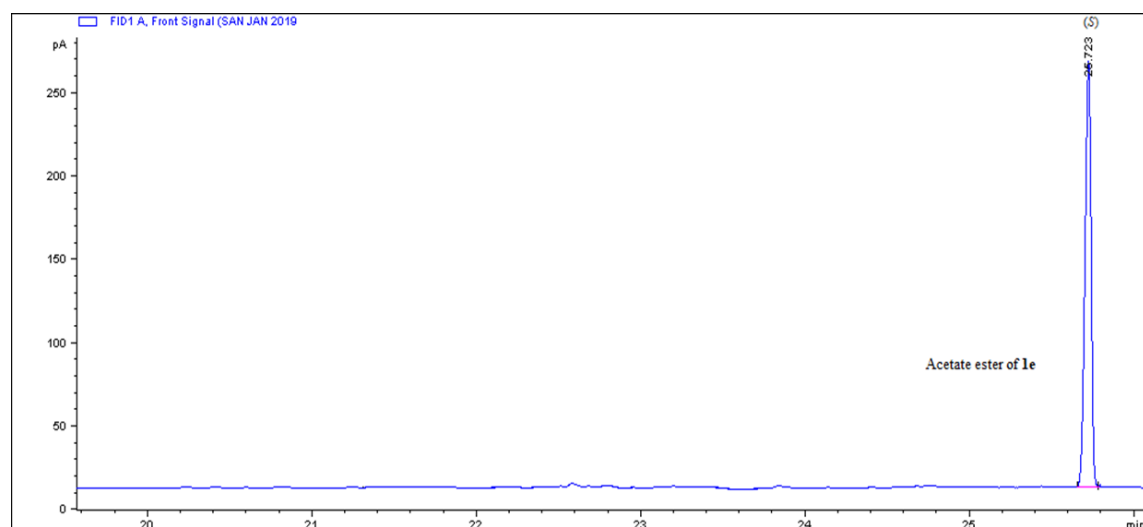


Figure S15. Gas chromatogram of the acetate derivative of (*rac*)-**1e** of the deracemization product using W110G and W110V Mutants of TeSADH (Table 1, entry 5).

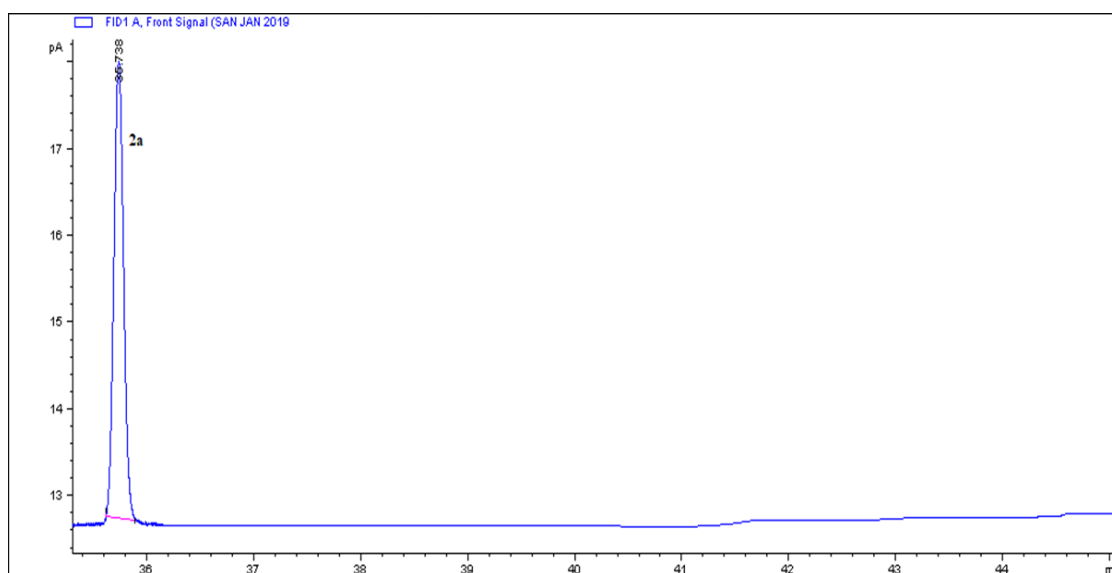


Figure S16. Gas chromatogram of the acetate derivative of the oxidation product of (*rac*)-**1a** using xerogel-encapsulated W110G mutant of TeSADH.

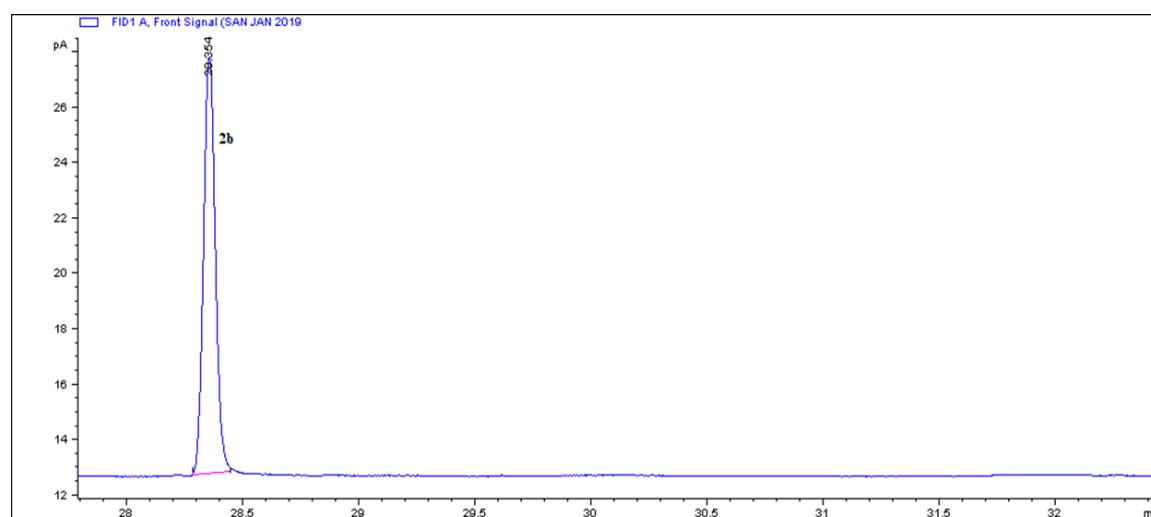


Figure S17. Gas chromatogram of the acetate derivative of the oxidation product of (*rac*)-**1b** using xerogel-encapsulated W110G mutant of TeSADH.

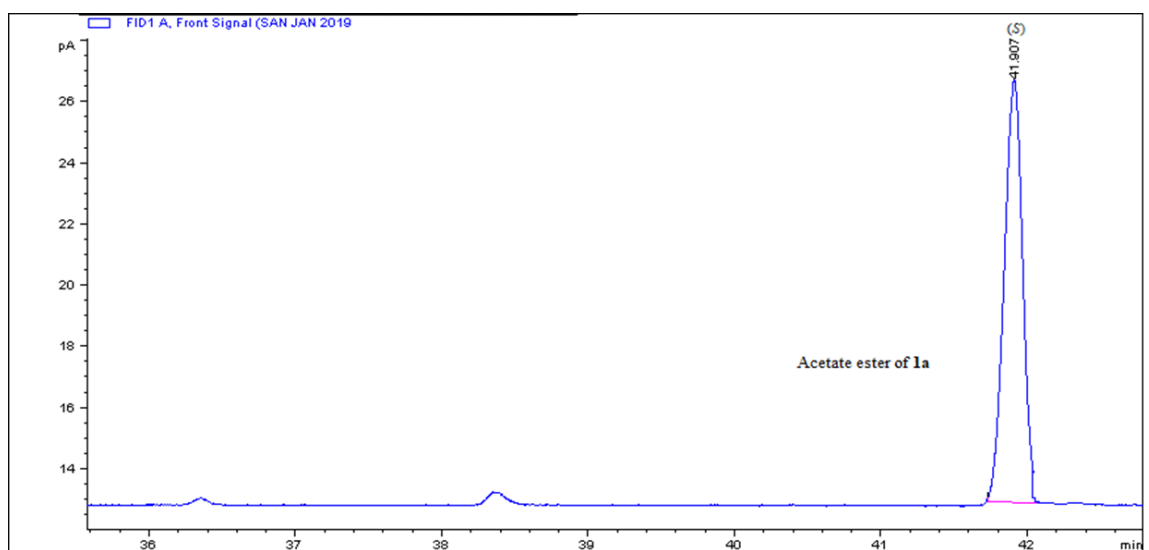


Figure S18. Gas chromatogram of the acetate derivative of (*rac*)-**1a** of the deracemization product using xerogel-encapsulated W110G and free W110V Mutants of TeSADH (Table 2, entry 1).

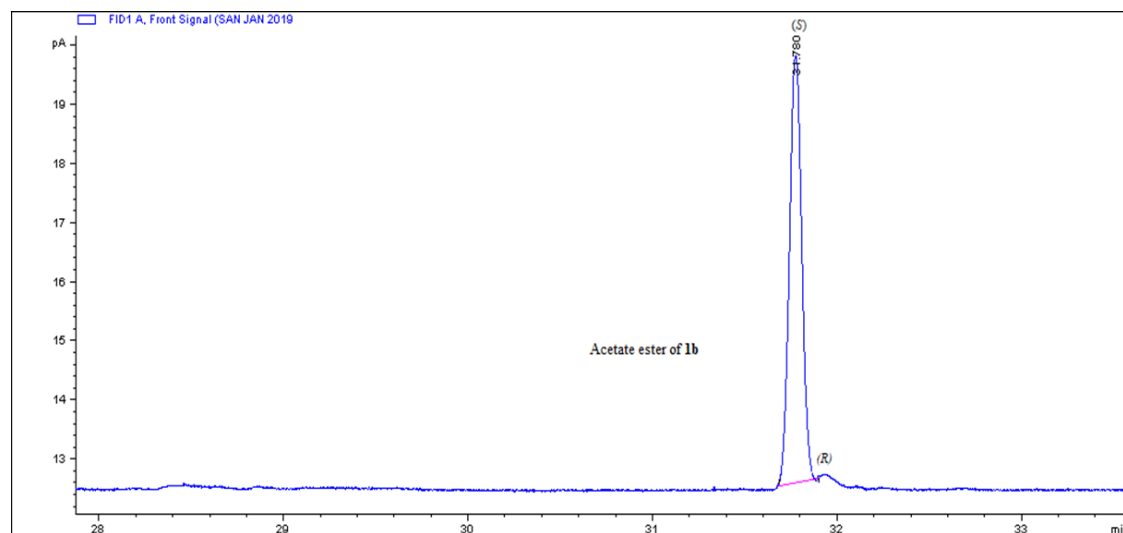


Figure S19. Gas chromatogram of the acetate derivative of (*rac*)-**1b** of the deracemization product using xerogel-encapsulated W110G and free W110V Mutants of TeSADH (Table 2, entry 2).

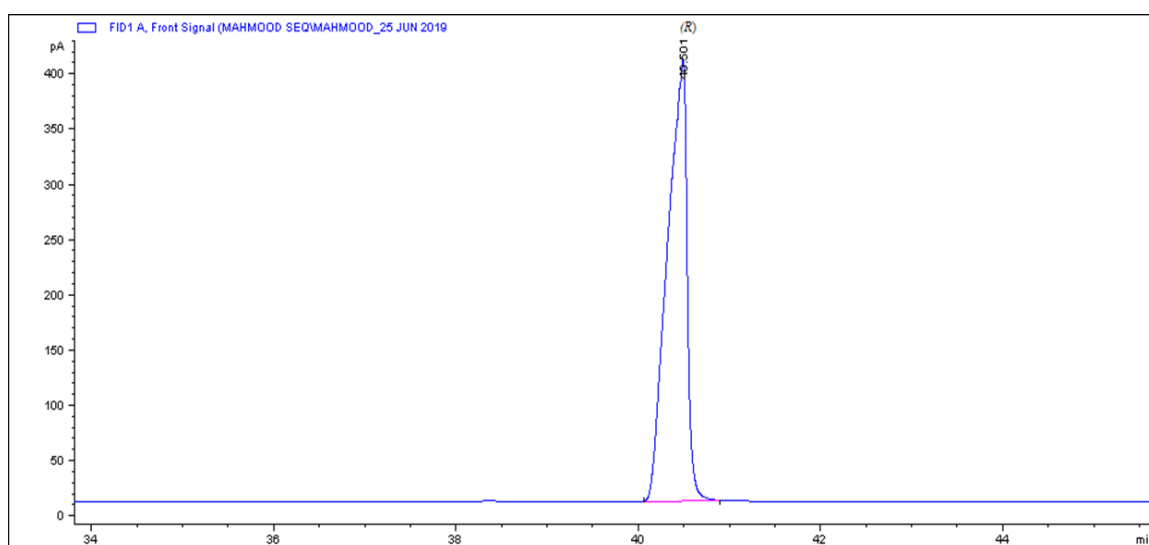


Figure S20. Gas chromatogram of reference (*R*)-**1a**.

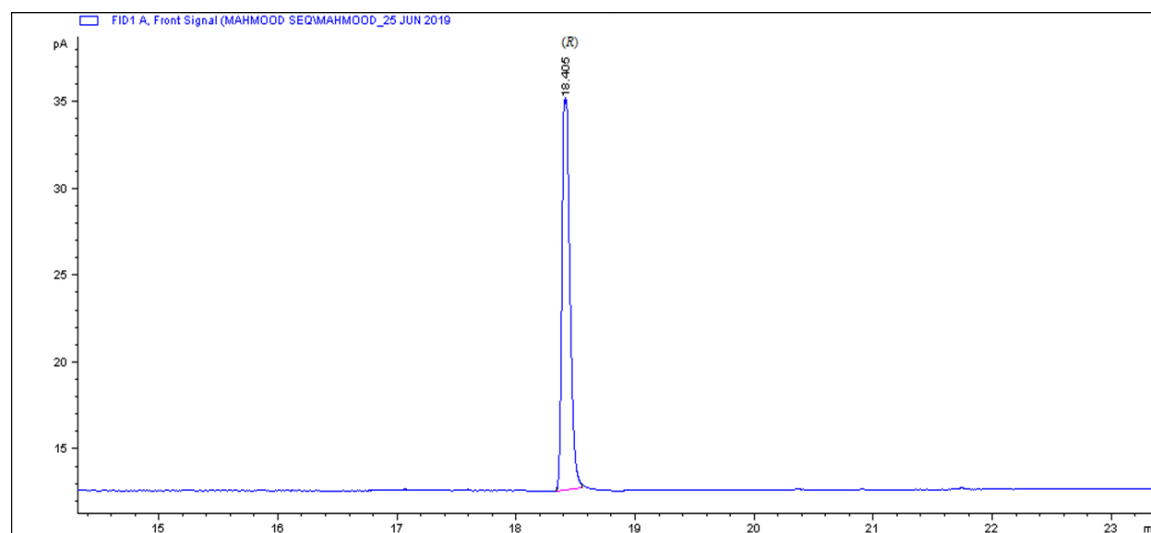


Figure S21. Gas chromatogram of reference (*R*)-**1c**.

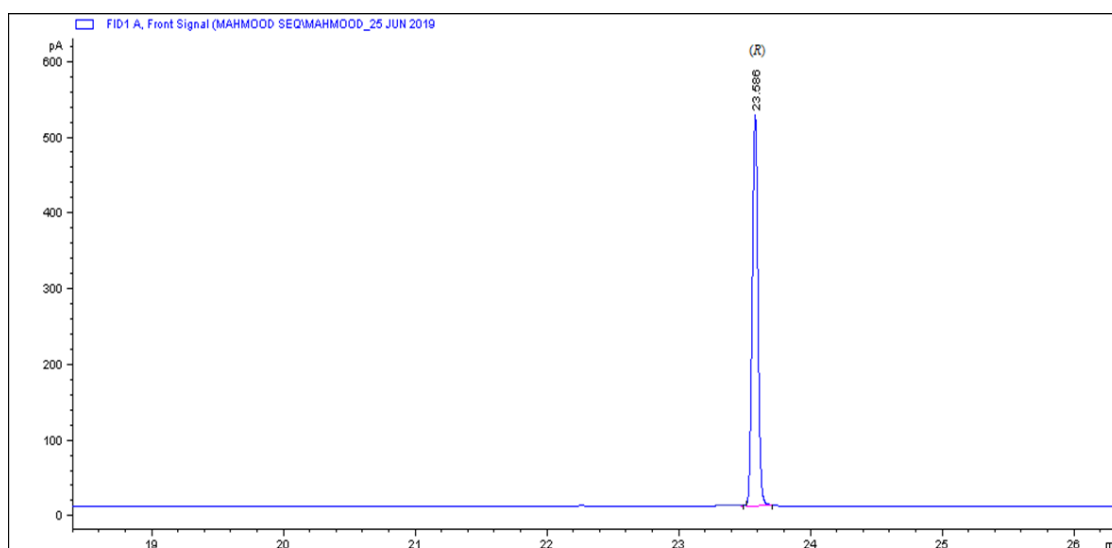


Figure S22. Gas chromatogram of reference (*R*)-**1d**.

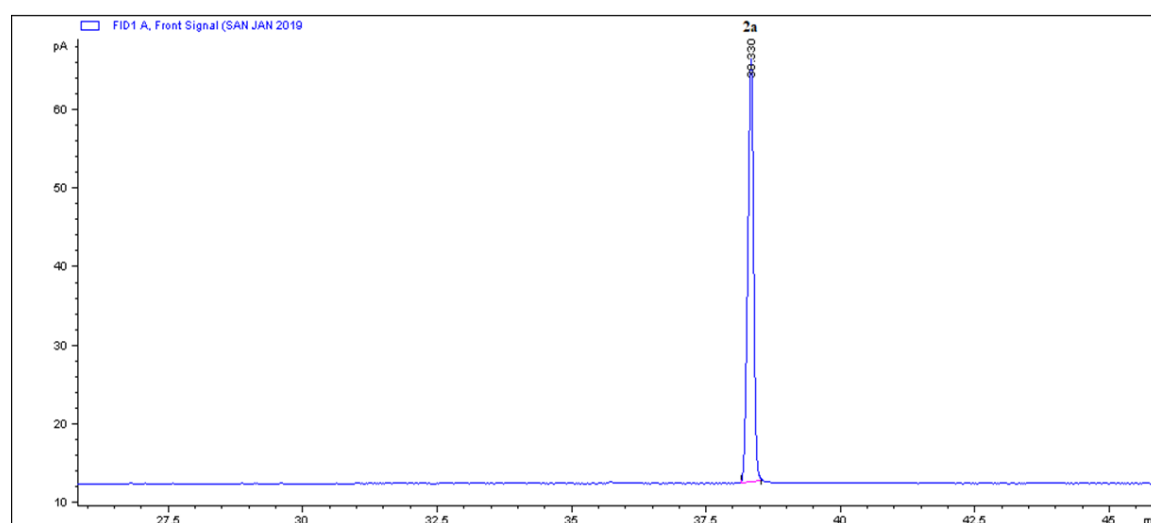


Figure S23. Gas chromatogram of the oxidation product of (*R*)-**1a** using W110G mutant of TeSADH.

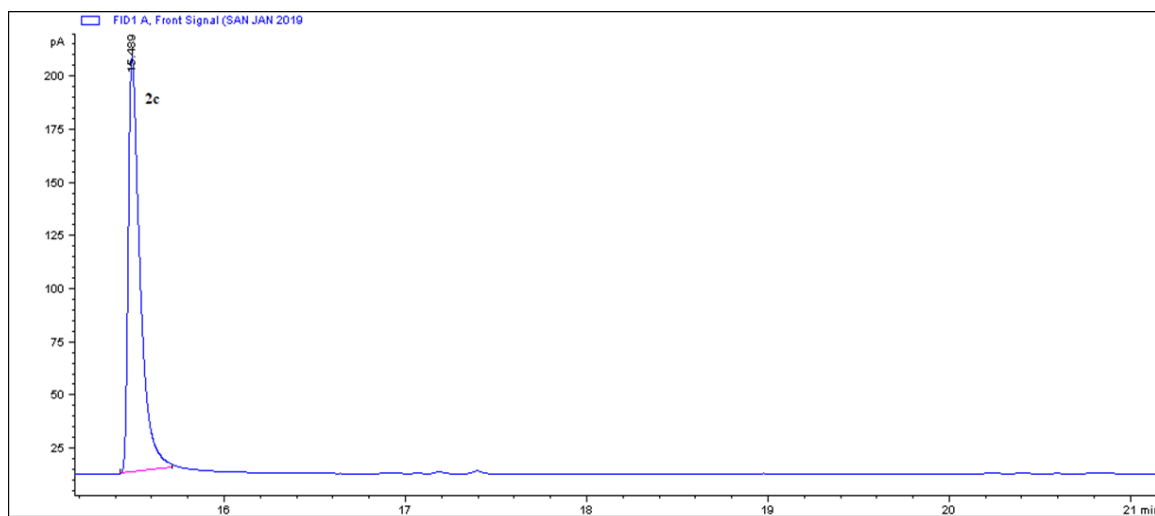


Figure S24. Gas chromatogram of the oxidation product of (*R*)-**1c** using W110G mutant of TeSADH.

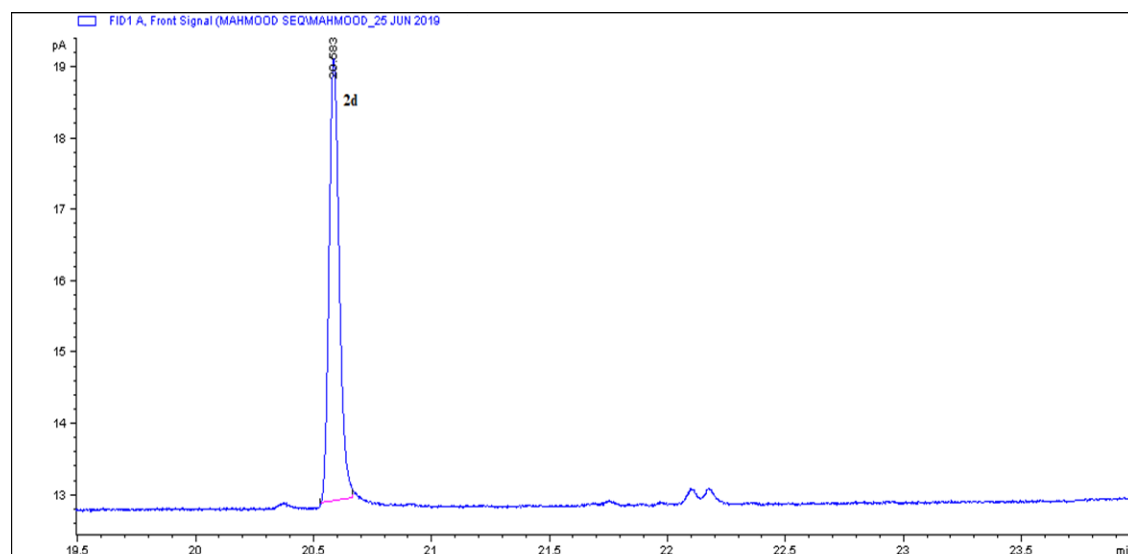


Figure S25. Gas chromatogram of the oxidation product of (*R*)-**1d** using W110G mutant of TeSADH.

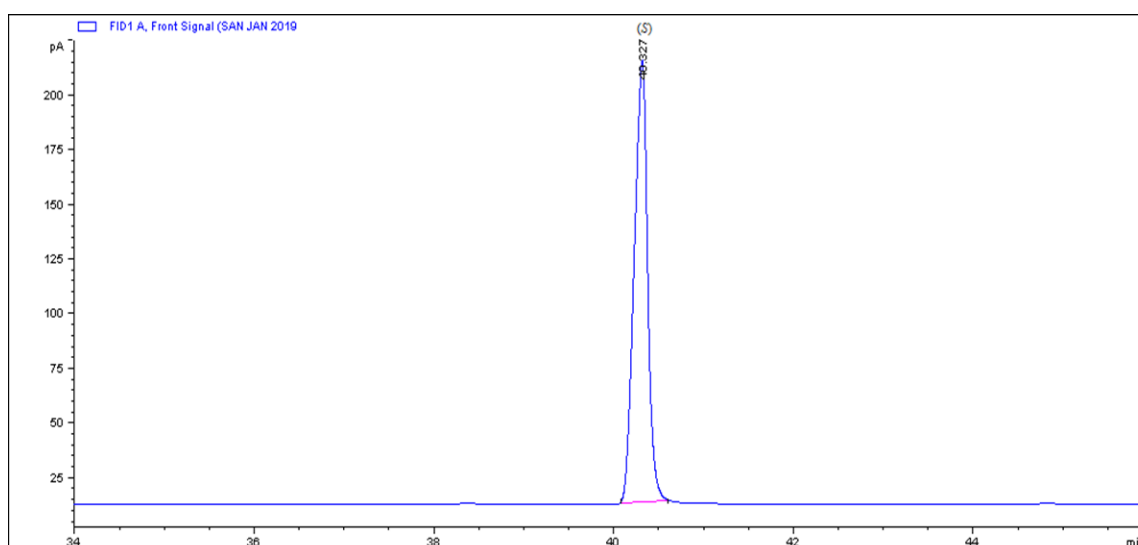


Figure S26. Gas chromatogram of the stereoinversion product of (*R*)-**1a** using W110G and free W110V Mutants of TeSADH (Table 3, entry 1).

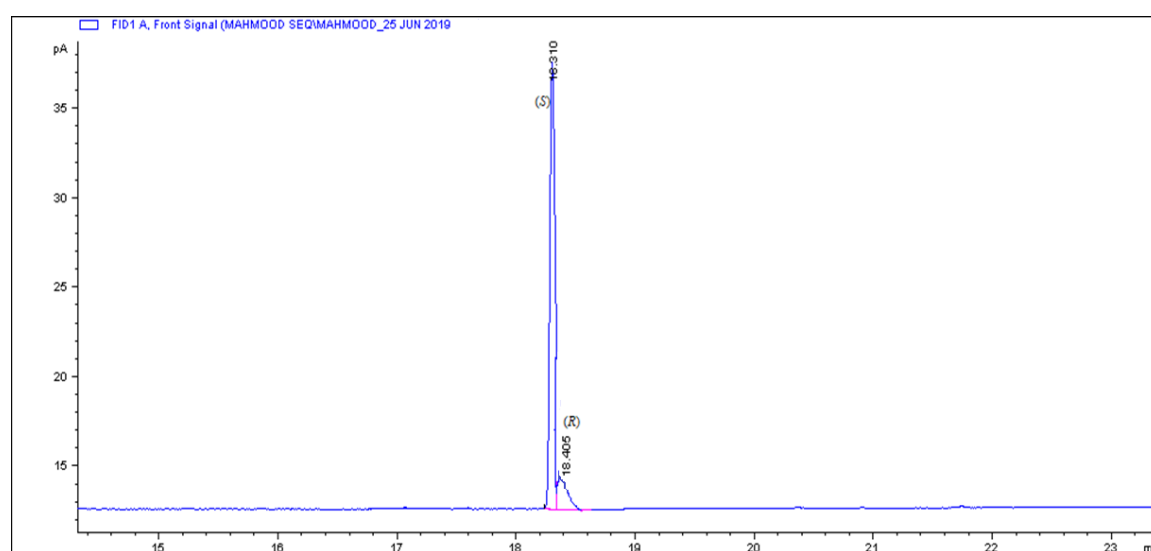


Figure S27. Gas chromatogram of the stereoinversion product of (*R*)-**1c** using W110G and free W110V Mutants of TeSADH (Table 3, entry 2).

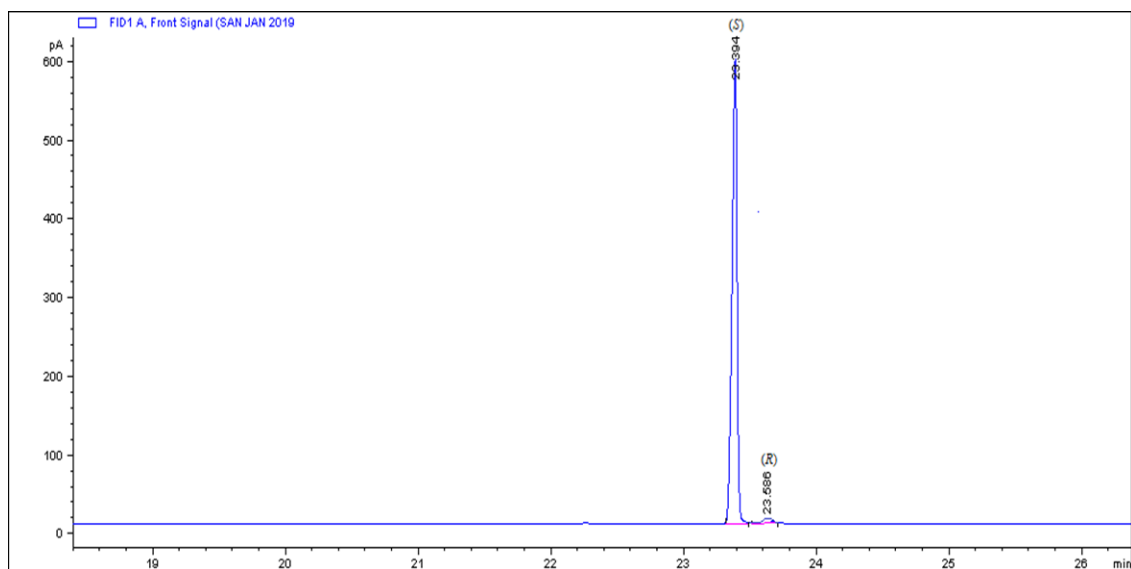


Figure S28. Gas chromatogram of the stereoinversion product of (*R*)-**1d** using W110G and free W110V Mutants of TeSADH (Table 3, entry 3).

APPENDIX B

GC-MS SPECTRA OF THE PRODUCTS OF DERACEMIZATION REACTIONS

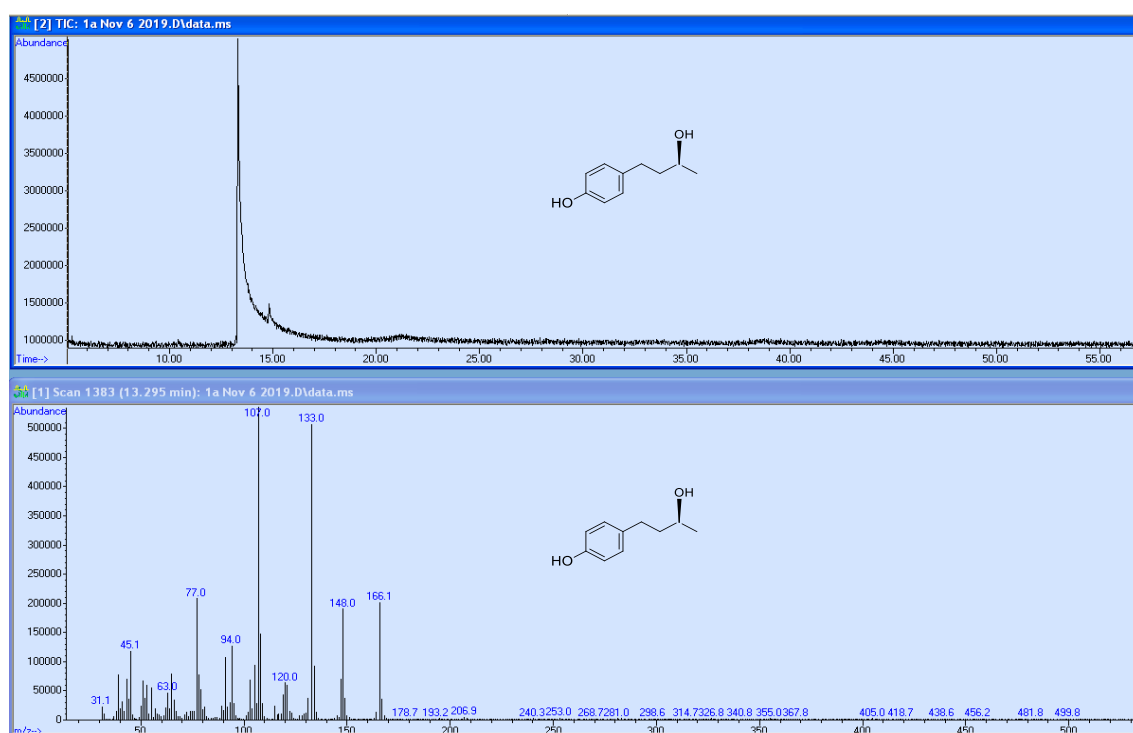


Figure S29. GC-MS spectra of *(rac)*-1a deracemization product using W110G and W110V Mutants of TeSADH.

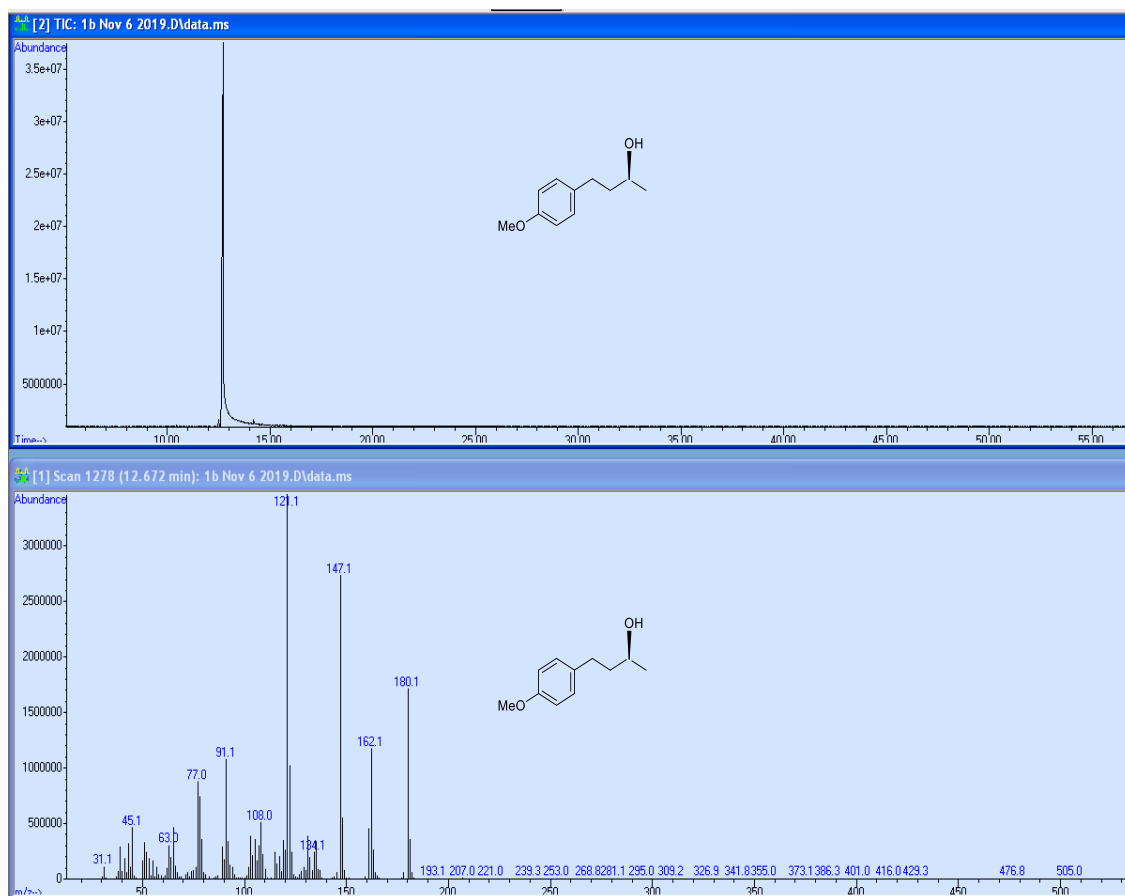


Figure S30. GC-MS spectra of (*rac*)-**1b** deracemization product using W110G and W110V Mutants of TeSADH.

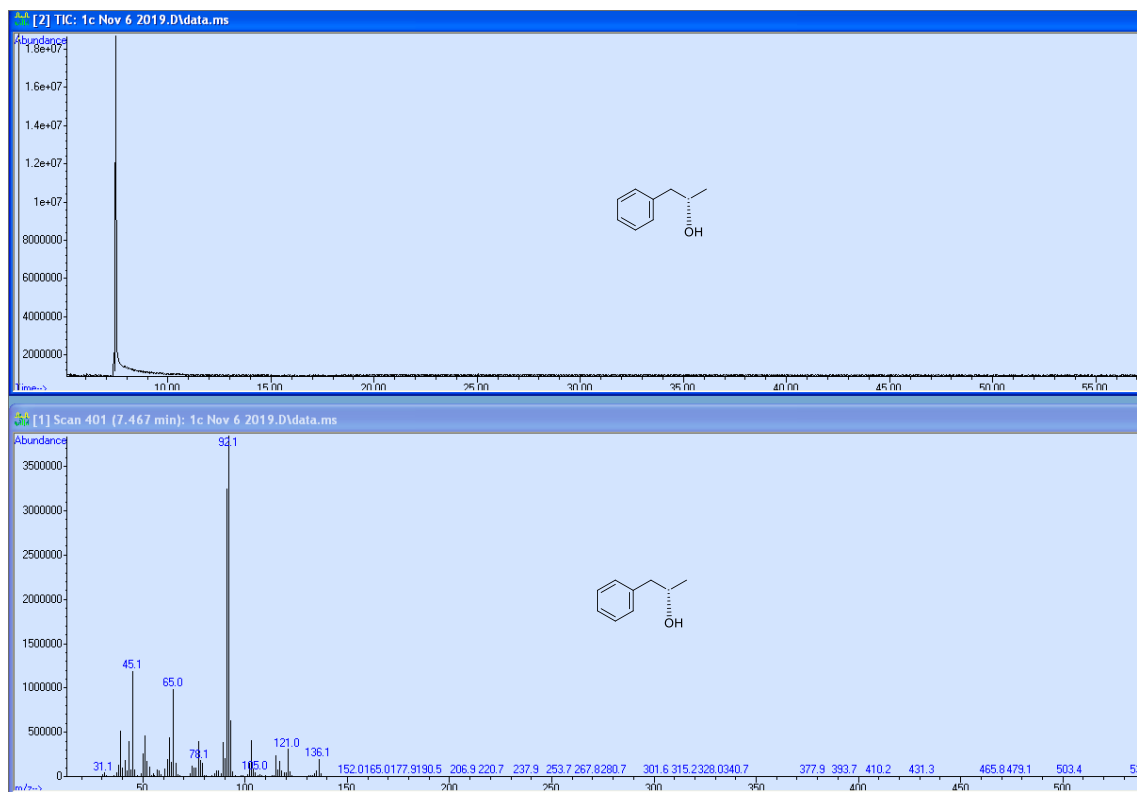


Figure S31. GC-MS spectra of (*rac*)-1c deracemization product using W110G and W110V Mutants of TeSADH.

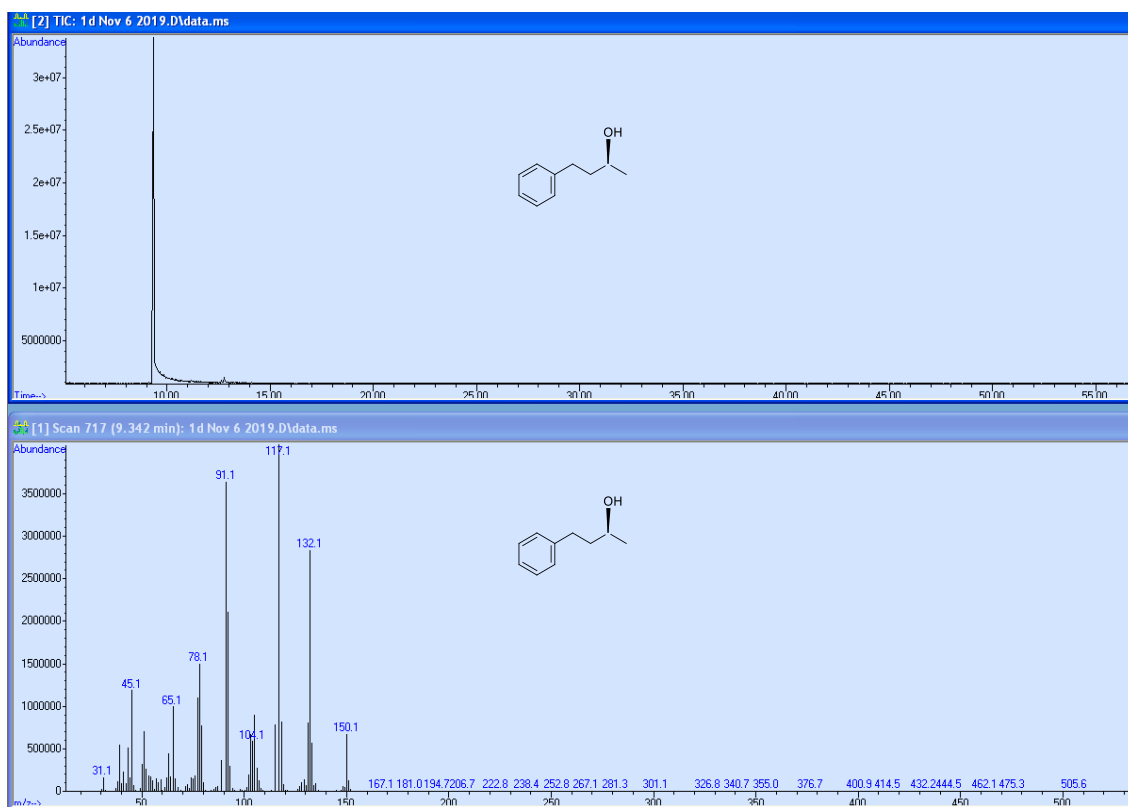


Figure S32. GC-MS spectra of (*rac*)-1d deracemization product using W110G and W110V Mutants of TeSADH.

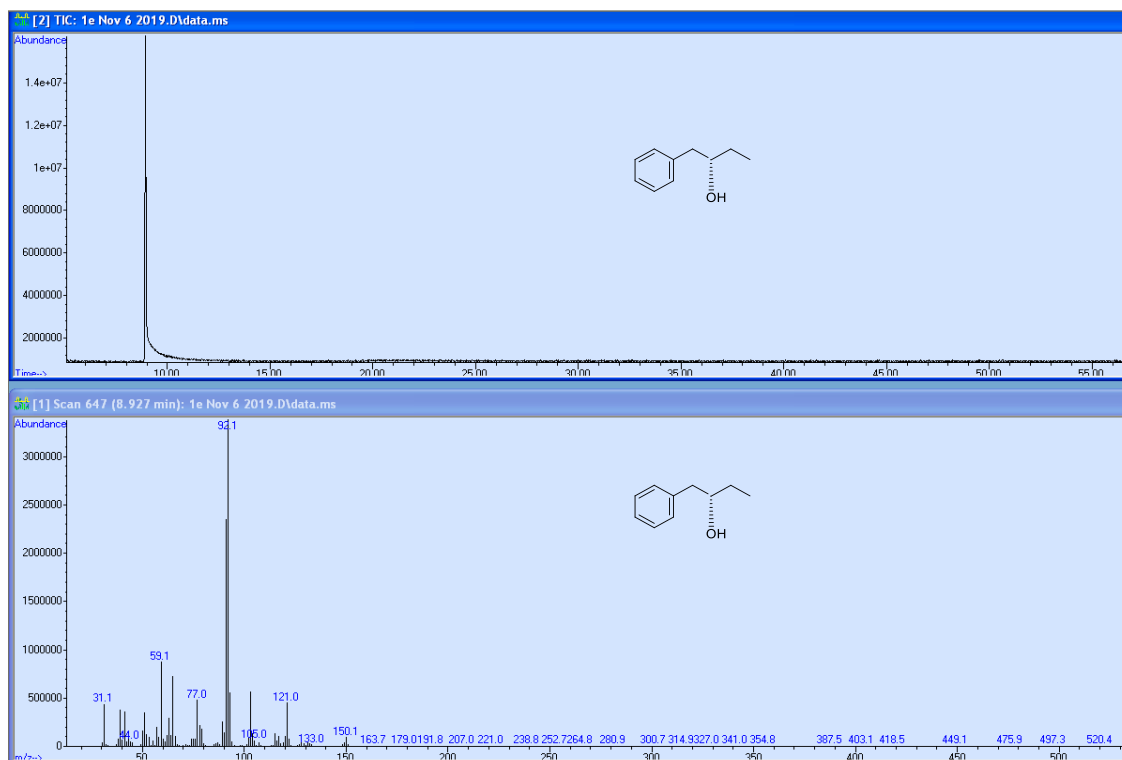


Figure S33. GC-MS spectra of *(rac)*-1e deracemization product using W110G and W110V Mutants of TeSADH.

APPENDIX C

**NMR SPECTRA OF THE PRODUCTS OF DERACEMIZATION
REACTIONS**

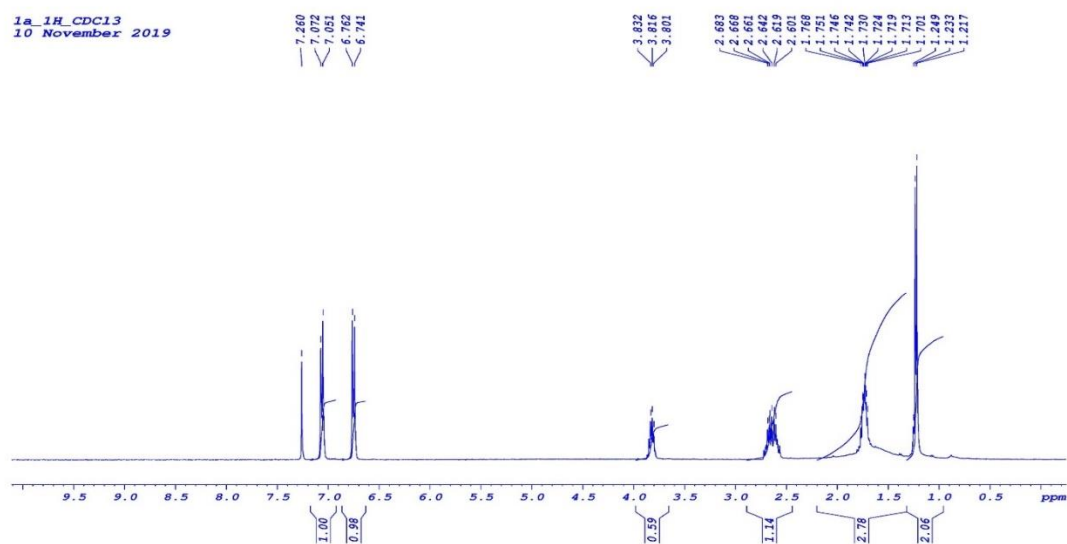


Figure S34. ^1H NMR spectrum of (*rac*)-**1a** deracemization product using W110G and W110V Mutants of TeSADH.

1a_C13_CDCl3
10 November 2019

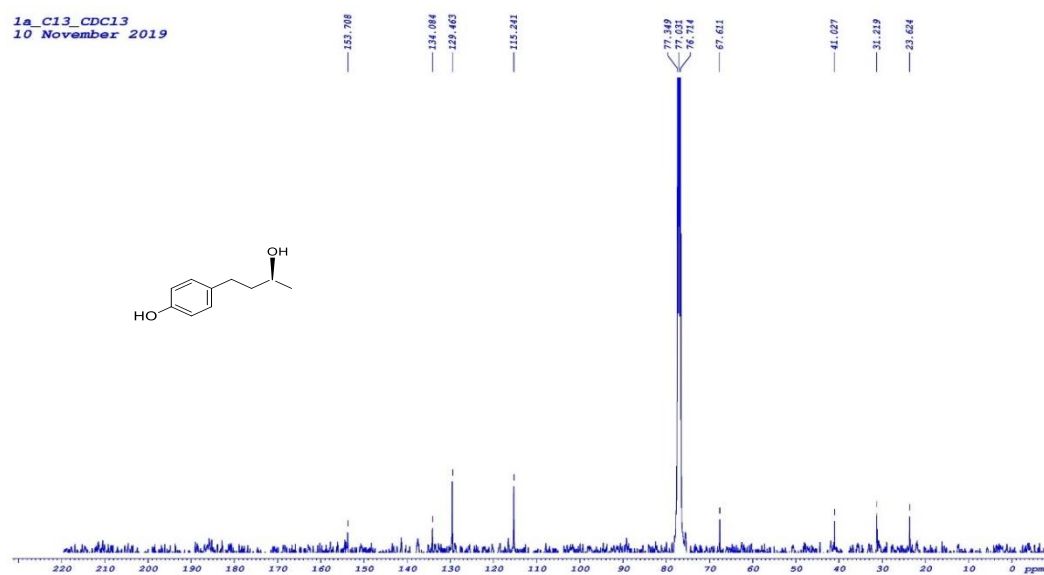


Figure S35. ^{13}C NMR spectrum of (*rac*)-**1a** deracemization product using W110G and W110V Mutants of TeSADH.

1b 1H_CDCl3
10 November 2019

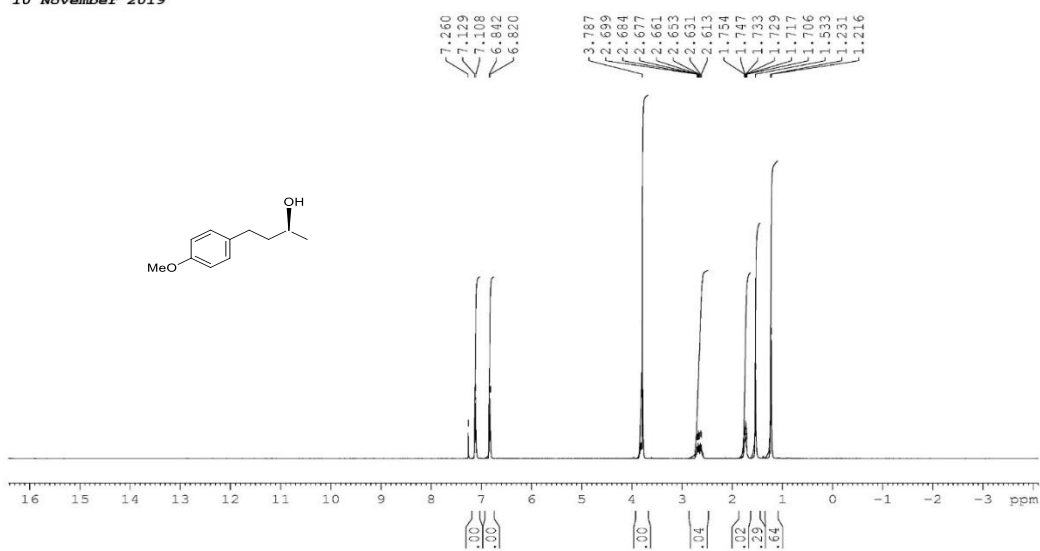


Figure S36. ^1H NMR spectrum of (*rac*)-**1b** deracemization product using W110G and W110V Mutants of TeSADH.

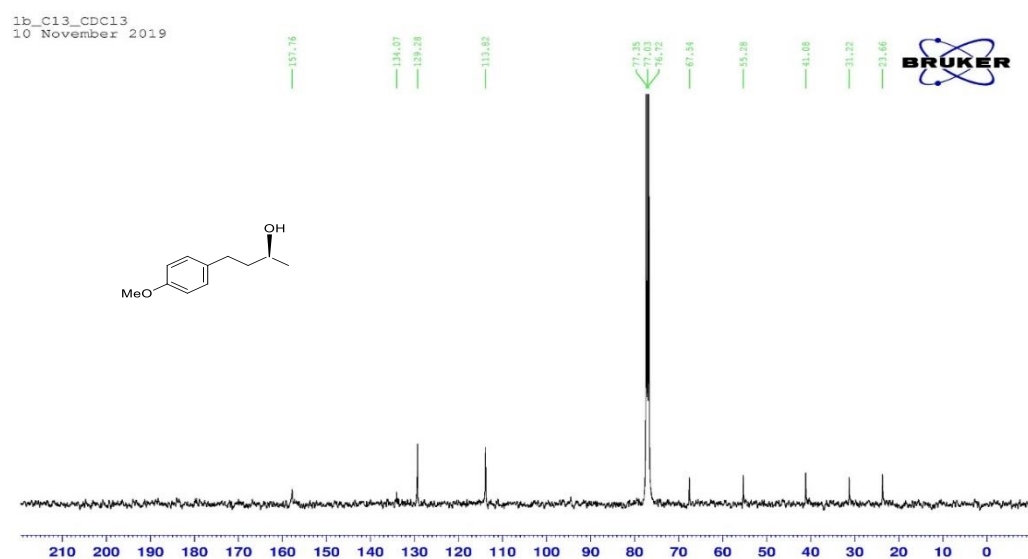


Figure S37. ^{13}C NMR spectrum of (*rac*)-**1b** deracemization product using W110G and W110V Mutants of TeSADH.

1C_1H_CDCl3
10 November 2019

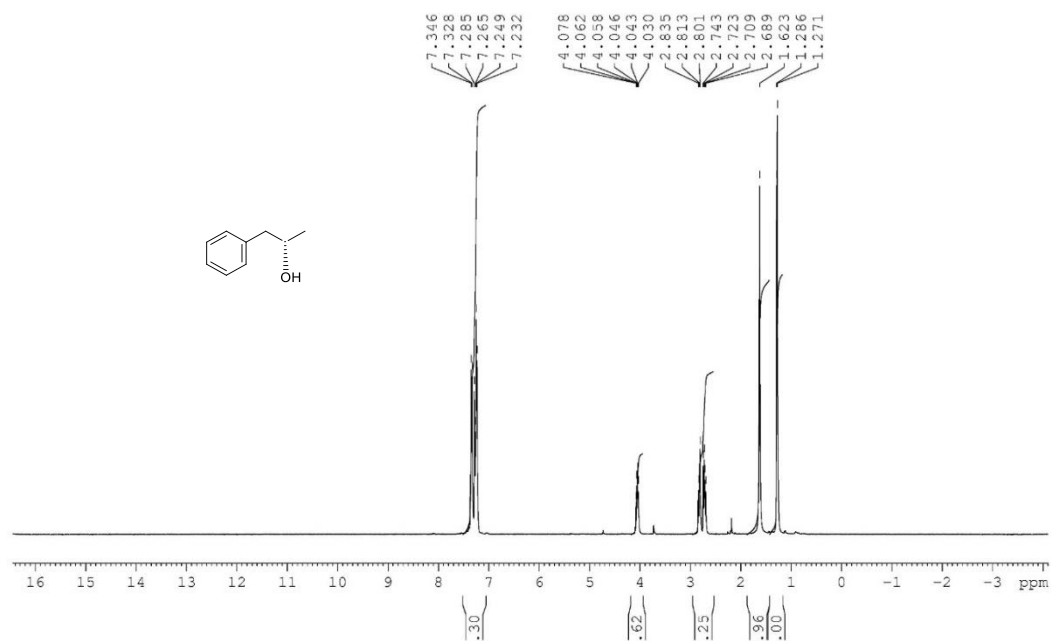


Figure S38. ¹H NMR spectrum of (*rac*)-**1c** deracemization product using W110G and W110V Mutants of TeSADH.

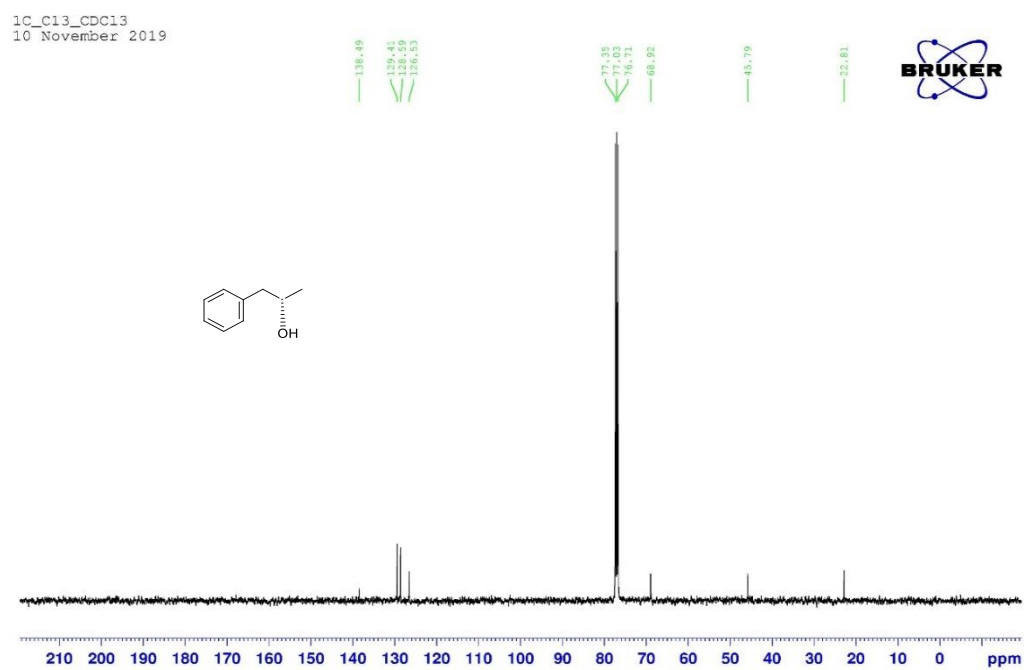


Figure S39. ^{13}C NMR spectrum of (*rac*)-**1c** deracemization product using W110G and W110V Mutants of TeSADH.

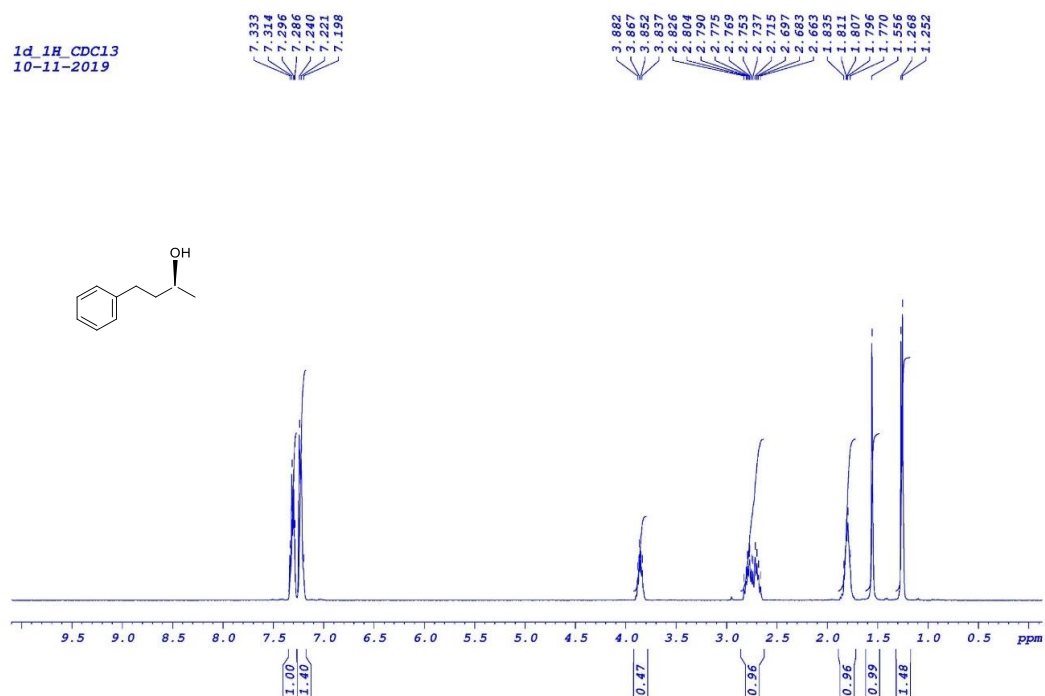


Figure S40. ^1H NMR spectrum of (*rac*)-**1d** deracemization product using W110G and W110V Mutants of TeSADH.

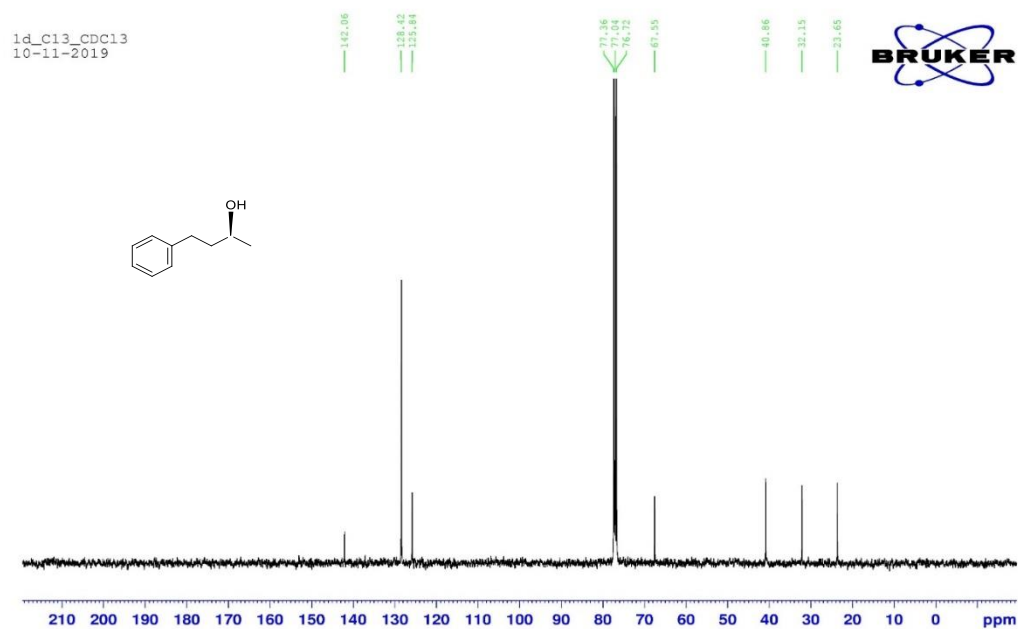


Figure S41. ^{13}C NMR spectrum of (*rac*)-**1d** deracemization product using W110G and W110V Mutants of TeSADH.

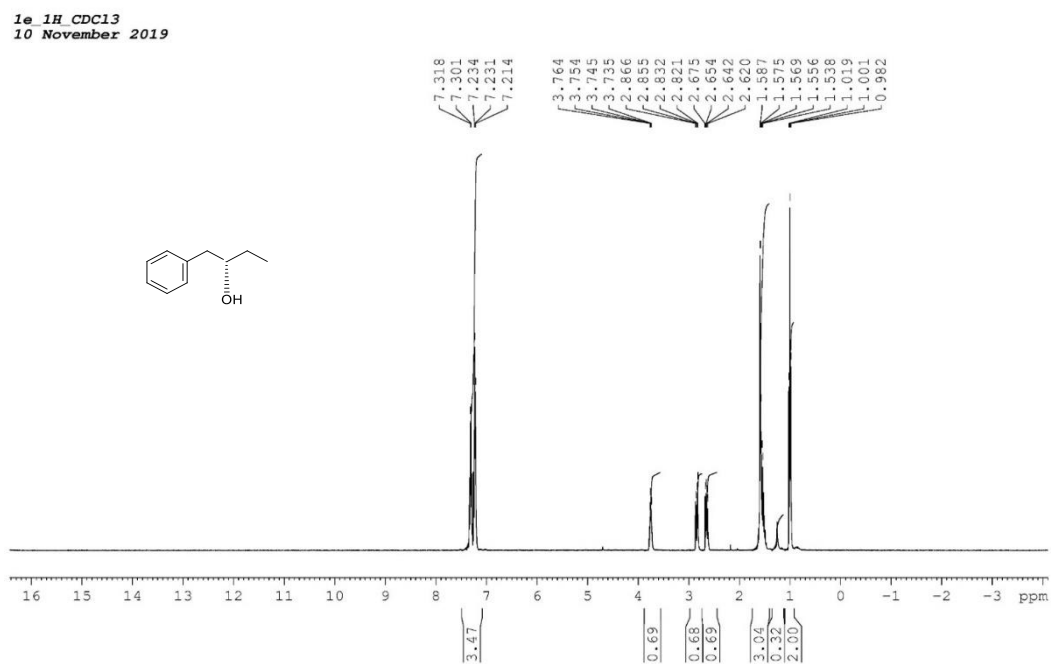


Figure S42. ^1H NMR spectrum of (*rac*)-**1e** deracemization product using W110G and W110V Mutants of TeSADH.

1e_C13_CDCl3
10 November 2019

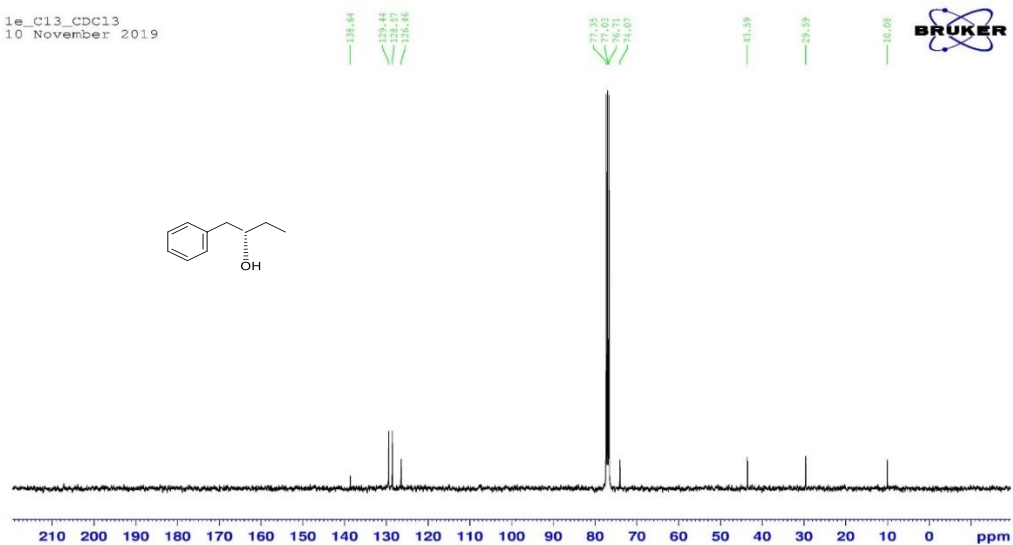


Figure S43. ^{13}C NMR spectrum of *(rac)*-1e deracemization product using W110G and W110V Mutants of TeSADH.

CURRICULUM VITAE

PERSONAL INFORMATION

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Graduated with First class honours

Secondary School Certificate, June, 2008 Iwo School of Science, Iwo, Osun State, Nigeria

Primary School leaving Testimonial, 2002

RESEARCH EXPERIENCE

July 2018 to April 2020: **Deracemization of Secondary Alcohols using a Biocatalytic Approach**

Department of Chemistry, King Fahd University of Petroleum and Minerals (KFUPM), Dhahran, Saudi Arabia

January 2018- April 2018: **Deracemization of Alcohols Catalyzed by Two Mutants of Secondary Alcohol Dehydrogenase from *Thermoanaerobacter ethanolicus***

Department of Chemistry, King Fahd University of Petroleum and Minerals (KFUPM), Dhahran, Saudi Arabia

August 2014- June 2015: **Effect of drying on the Chemical constituents of citrus sinensis peels essential oils**

Department of Chemistry, University of Ilorin, Ilorin, Nigeria

Instrumentation skills: Have hands-on experience in

Nuclear magnetic resonance spectroscopy

Gas chromatography

Mass spectrometry

IR spectroscopy

Ultraviolet-visible spectroscopy

Familiar with most laboratory bench techniques

RESEARCH INTERESTS

Organic synthesis

Biocatalysis

Natural product chemistry

Medicinal chemistry

Polymer synthesis

Nanomaterial synthesis and applications

RESEARCH PUBLICATIONS AND PRESENTATIONS

Manuscripts under preparation

“Deracemization and Stereoinversion of Alcohols Using a Bi-enzymatic Approach of Two Forms of Secondary Alcohol Dehydrogenase Mutants from *Thermoanaerobacter pseudoethanolicus*” **Sodiq A. Nafiu**, Masateru Takahashi, Etsuko Takahashi, Samir M. Hamdan, and Musa M. Musa

“Bi-enzymatic Concurrent Deracemization of Alcohols by Tuning the Enantioselectivity of *Thermoanaerobacter pseudoethanolicus* Secondary Alcohol Dehydrogenase” **Sodiq**

A. Nafiu, Masateru Takahashi, Etsuko Takahashi, Samir M. Hamdan, and Musa M. Musa

AWARD

Ministry of Higher Education Award, Saudi Arabia, 2018 till date

Department of Chemistry, University of Ilorin best graduating student Award, 2015

Best Student in Chemistry Award, Iwo School of Science, Iwo, 2007-2008

REFEREES

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