EFFECTS OF TEMPERATURE AND PRESSURE ON ENANTIOSPECIFICITY OF NOVEL SECONDARY ALCOHOL DEHYDROGENASE MUTANTS FROM *THERMOANAEROBACTER ETHANOLICUS*

by

JAY MAYANK PATEL

(Under the Direction of ROBERT S. PHILLIPS)

ABSTRACT

Alcohol dehydrogenases (ADHs) are enzymes that catalyze the reversible reduction of carbonyl compounds to their corresponding alcohols. In chapter 2, we study the effect of hydrostatic pressure on stereospecificity of secondary ADH (SADH) from *Thermoanaerobacter ethanolicus* catalyzed oxidation of alcohols. Under high pressure conditions of 137.5 MPa and at 298K, the enantiomeric ratio (E) can be enhanced to 13.5 compared to 3.9 at room temperature and pressure for (S)-2-hexanol over (R)-2-hexanol. In chapter 3, site saturation mutagenesis approach was adopted in creating a comprehensive SADH mutant library at W110; and we used phenylacetone as a model substrate to study the effectiveness of our library. We are pleased to note that five of our mutants gave reductions at >99.9% e.e. and two of the mutants showed an E of over 100 for (S)-1-phenyl-2-propanol.

INDEX WORDS: [Biocatalysis, Alcohol dehydrogenase, *Thermoanaerobacter ethanolicus*, Asymmetric synthesis]

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May 2013

DEDICATION

To my parents for their love, patience and support over the years

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
BY	Baker's Yeast
Conv.	Conversion
DMF	Dimethyl formamide
E. coli	Escherichia Coli
e.e.	Enantiomeric Excess
E	Enantiomeric Ratio
GC	Gas chromatography
gm	gram
HLADH	Horse Liver Alcohol Dehydrogenase
KR	Kinetic Resolution
Me	Methyl
mg	miligram
mM	milimole
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide Hydride
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydride
nm	nanometer
ph	Phenyl
RT	Room Temperature
SADH	Secondary Alcohol Dehydrogenase

Tris-HCl	Tris(hydroxymethyl)aminomethane Hydrochloride
μmol	micromol
μL	microlitre

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Biocatalysis

The use of microorganisms to make fermented food products has been known for thousands of years. The earliest examples are using yeast to make beer, wine and bread. Those can be described as the earliest precursors to the modern field of biocatalysis, which is essentially using whole cell microorganisms and isolated enzymes to carry out synthesis of organic molecules (Woodley, 2008). There are many advantages to this approach including: 1) Enzymatic reactions have very a high degree of chemo-, regio-, and stereospecificity, and this is usually the biggest reason why this reaction methodology is used; 2) These reactions are carried out at mild reaction conditions such as room temperature and pressure, and hence can be used for synthesis of sensitive molecules; 3) The natural solvents for most of this enzymatic systems is water; this can be considered an advantage because of the recent push for "green" strategies, and this water based systems would be obviously greener then reactions in organic solvents. The advantages described above are also the reasons why biocatalysis has had limited acceptance among organic and process chemists, and frequently, organic chemists viewed it as method of last resort. Some of the common disadvantages are: 1) Most enzymes do not tolerate organic solvents well, and many of the organic compounds desired have low to no solubility in water, hence their synthesis via this method becomes challenging; 2) Most wild type enzymes accept a very narrow range of compounds as substrates and even smallest functional group changes makes them inactive; 3) Most enzymes operate under a very narrow range of optimum conditions such as pH, temperature and atmospheric

pressure (Musa and Phillips, 2011). This becomes a disadvantage for organic synthesis where more flexibility is often needed for physical reaction conditions. In the last twenty years, advancements in molecular biology techniques such as mutagenesis and recombinant DNA, high throughput screening etc, have eliminated most of this disadvantages, and through the work in this thesis we have tried to design a very robust biocatalytic system comparable to traditional process chemistry systems. In the subsequent pages, we will just introduce different biocatalytic systems, and specifically a brief history of alcohol dehydrogenase catalyzed reactions.

1.2 Whole cell biocatalysis

Whole cell biocatalysis is an older method, and it essentially uses live cells to carry out desired bio-transformations. Due to obvious similarities to the food fermentation, it was very popular earlier because it incorporates a lot from the techniques developed for food fermentation. Its advantage is that it doesn't require designing expensive and cumbersome co-factor recycling systems, which is especially an advantage for NADP cofactor dependent systems in which there is a dearth of effective recycling options (Kroutil et al., 2004). Whole cell biocatalysts recycle cofactors using the usual cell metabolic pathways. Disadvantages of this methods are that usually: 1) Cells cannot tolerate high substrate density in the reaction media, leading to high reaction volume which becomes very difficult for scale up; 2) Usually there is some form of product inhibition observed, which slows down the reaction considerably as the conversion increases; 3) Optimum growth conditions for cells in whole cell biocatalysts may not be

optimum reaction conditions, which leads to additional problems and may lead to either low conversions or cell death depending on how sensitive the reaction conditions are. 4) Usually whole cell biocatalysts have a mixture of enzymes with differing enantiospecificity, which, in cases when we want an enantiopure product, this method becomes unable to give us products in high e.e.; 5) Typically this method has longer reaction times which can be on order of days; 6) Due to low substrate density and consequently low product density, the separation of product from the reaction media becomes very cumbersome and hence expensive. As is apparent, there are far too many disadvantages for such a biocatalyst system, and hence, this method was used for very few reaction systems, and there was added impetus to develop more robust isolated enzyme methods with efficient cofactor recycling systems. In subsequent sections we will talk about reactions catalyzed by baker's yeast, which in many ways is the most widely accepted biocatalytic system.

1.3 Isolated enzyme biocatalysis

These methods use a purified enzyme or cell extract containing the enzyme responsible for the concerned biocatalytic transformation, and there is a recycling system along with it which recycles the expensive co-factors (Faber, 2004). Broadly there two cofactor recycling approaches, the coupled enzyme approach in which a set of two enzymes are used, usually this is done in NAD recycling systems and there are ample approaches to having independent recycling systems with separate enzyme responsible for recycling. The second method is coupled substrate approach, in which a cheap substrate in high

excess is used which is set up in reverse direction to the chief reaction desired, and that makes the system very convenient to use; this is frequently the case with NADP-dependent systems.

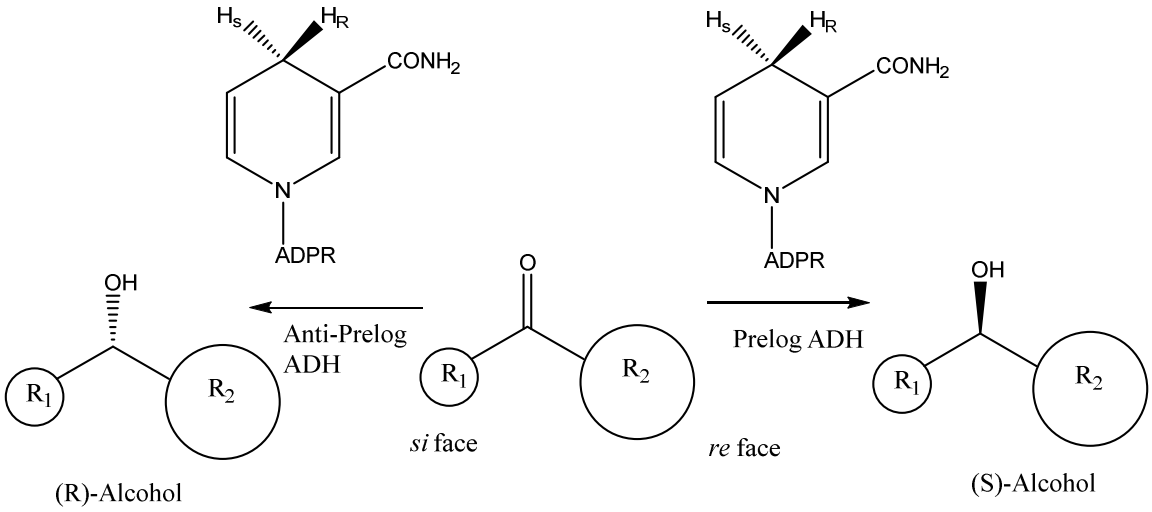
The advantages of using isolated enzymes include the flexibility in designing tailor-made systems which with the right combination of enzyme and reaction conditions can give 1) high substrate density, 2) faster reaction times usually in order of hours, 3) easier product separation.

1.4 Alcohol dehydrogenase

Alcohol dehydrogenases (ADH) are a class of NAD(P) dependent enzymes which catalyze the reversible oxidation of alcohols to corresponding ketones or aldehydes. These enzymes are extremely important in biocatalysis because frequently these reactions are highly regio- and stereo-specific, and that makes it highly attractive to synthesize alcohols with high enantiopurity (Keinan et al., 1986, 1990). Prelog came up with an empirical rule to predict the stereospecificity for the alcohol formed based on four ways with which a hydride can be delivered from NAD(P)H to the substrate (Prelog, 1964), as shown in **Scheme 1**; According to Prelog's law, the *pro*-(R) or *pro*-(S)-hydride will attack from the *re* face of a prochiral ketone, to produce the (S)-alcohol. Most ADHs like HLADH, Baker's yeast, etc follow this rule; however, there are some ADHs like that from *Lactobacillus kefir* in which the attack occurs from the *si* face of a ketone to produce the (R)-alcohol, and then the ADH is said to follow the anti-Prelog rule.

One of the ways to classify ADHs is based on whether their substrates are primary alcohol or secondary alcohols; and they are then called primary ADH or secondary ADH (SADH) respectively. The earliest ADH based biocatalytic systems were baker's yeast and horse liver alcohol dehydrogenase (HLADH). Both of these were primary ADHs and typical drawbacks included low substrate density and consequent difficulty in product separation, low activity for acyclic ketones, low thermal stability, etc.

Scheme 1: Illustration of Prelog's law (Prelog, 1964)



1.4.1 Baker's yeast

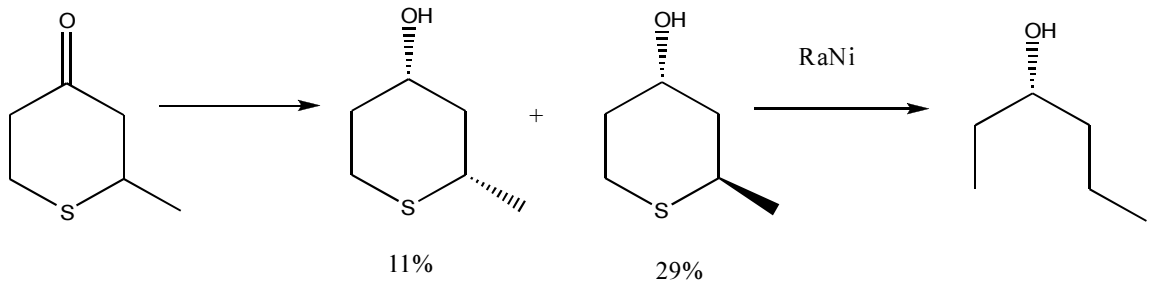
Baker's yeast (BY) has been used for food applications like beer and bread for thousands of years. The yeast alcohol dehydrogenase (YADH) is the enzyme responsible for most of the desired transformations, and its natural function in cells is to reduce acetaldehyde to ethanol. Due to such a long history with applications using BY, it is probably the most readily available microorganism, and this was a huge advantages to chemists who didn't require any expertise to perform complicated fermentations, and hence this became one

of the first biocatalytic systems to be studied extensively. It was observed that BY systems can reduce a wide range of aldehydes and ketones to give chiral primary and secondary alcohols (Matsuda et al., 2009). These reactions are usually carried out at room temperature with a pH of about 8-9, and it has low tolerance for temperature variations. The reaction rates decrease with increase in chain length; however, it is found that unsaturated straight chain ketones are very good substrates and they show high rates with good e.e. even with longer chain lengths.

1.4.2 Horse Liver Dehydrogenase (HLADH)

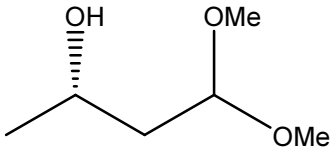
HLADH has been one of the most extensively studied ADHs for its ability to catalyze reactions with very high stereo- and regio-specificity (Davies and Jones, 1979). HLADH has good stability, and is stable at room temperature without substantial reduction in activity for over a week. It is also pretty stable over a wide range of pH 5-10, although usually the optimum pH for HLADH based systems is pH 7-9. Enantioselective oxidation of primary alcohols catalyzed by HLADH provides a tangible route to enantiopure aldehydes (Wong et al., 1985). HLADH shows low enantiospecificity for oxidation of bridged systems containing secondary alcohol groups. HLADH catalyzes reduction of wide range wide cyclic ketones (**figure 1**), and also tolerates heterocycles with oxygen and sulfur; However, cyclic heterocycles with nitrogen are not good substrates due to apparent coordination with Zn in active site (Jones and Takemura, 1984). Acyclic ketones are usually poor substrates for HLADH; However, Davies and

Jones (1979) performed reduction of thiopyranones followed by desulfurization **(Scheme 2)** to give enantiopure acyclic alcohols.

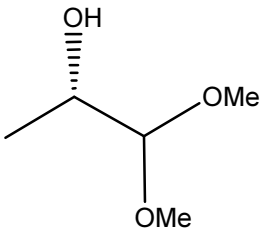


Scheme 2

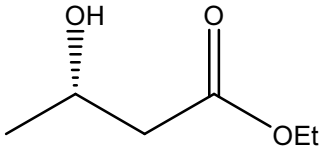
Figure 1: Representative sample of product alcohols formed by using HLADH as biocatalyst with their corresponding e.e.



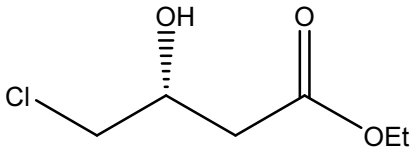
89% e.e., Wong et al., 1985



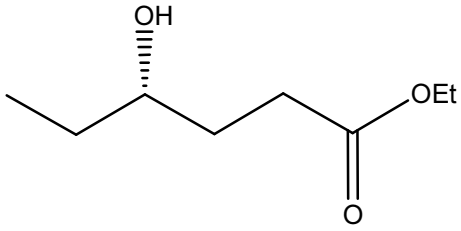
>99.9% e.e., Wong et al., 1985



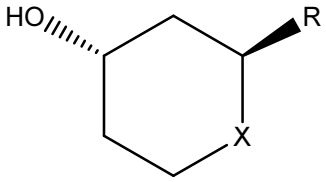
84% e.e., Wong et al., 1985



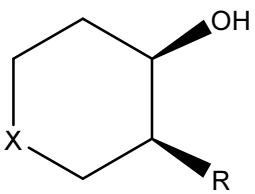
98% e.e. Wong et al., 1985



98% e.e. Wong et al., 1985



X = -CH_2-, S, O
R = $(CH_3)_3C$-, $(CH_3)_2CH$-
>99.9% e.e.
Davies et al., 1979



X = -CH_2-, S
R = -CH_3, -CH_2CH_3
45-85% e.e.
Jones et al., 1984

1.4.3 Thermoanaerobacter ethanolicus ADH

Bryant et al. (1988) isolated and characterized two alcohol dehydrogenases from a thermophilic bacterium, *Thermoanaerobacter ethanolicus*, which was isolated from hot springs at Yellowstone National Park. It was observed that while one of them preferred primary alcohol, the other ADH was more active towards ketones and secondary alcohols then it was towards ethanol, and henceforth, we refer to this enzyme as secondary alcohol dehydrogenase (SADH), which is an NADP dependent Zn^{2+} tetrameric oxidoreductase. One of the most obvious advantages of using a SADH from a thermophilic bacteria is that the enzyme has very good tolerance for high temperature, and this SADH has been found to be highly stable at temperatures above 70 °C; this is substantial improvement to traditional enzyme systems whose stability rapidly declines past 42 °C, and allows more control on this important physical parameter.

Pham et al. (1989, 1990) found a strong temperature dependence on enantiospecificity of *T. ethanolicus* SADH, and it was observed that for 2-butanol, there was a reversal of stereospecificity for (S)-2-butanol below 26 °C to (R)-2-butanol above that temperature. (S)-2-Pentanol was found to be the preferred substrate at temperatures up to 60 °C. This was one of the earliest reports which conclusively established the importance of temperature in stereospecificity of SADH, and showed great potential for its practical applications. Values of k_{cat}/K_m were also obtained for cyclic substrates like cyclobutanol, cyclopentanol and cyclohexanol, and it was found that the values were at least an order of magnitude less than that for acyclic secondary alcohols like 2-propanol. Zheng et al. (1992, 1994) subsequently studied the reduction of ketones to get chiral alcohols as this was a more interesting application of SADH, and it was observed that aliphatic saturated

ketones like 3-hexanone, 2-heptanone, 2-octanone, and 4-methyl cyclohexanone were very good substrates and gave (S)-alcohols in >95% e.e. It was found that aromatic ketones like acetophenone and diketones like 2,4- pentanedione gave no reaction with SADH. These results were encouraging, considering that earlier known enzyme systems like HLADH could not reduce some of these substrates with high enantiopurity and at high temperatures.

Secundo and Phillips (1996) studied the effect of pH on enantiospecificity of SADH on 2-butanol, and they observed that E for 2-butanol increases from 2.5 at pH 9 to 4.2 at pH 5.5. This represented yet another correlation of physical parameter change to stereospecificity, and it potentially imparted one more tool in hands of synthetic chemists to influence stereospecificity.

However, there were just a narrow range of substrates which showed good stereospecificity, and there were still some other drawbacks to use this *T. ethanolicus* based SADH, chiefly among them were that is *T. ethanolicus* is an obligate anaerobe requiring N_2 or Ar atmosphere, they grow very slowly and to low cell density of about 25-33 gm cells in 20 L carboy fermenter. Due to the low weight of cells obtained, they scaled up the procedure and used a 400L fermenter. The protein purification procedure was pretty labor intensive, because it involved separating the primary and secondary ADH by using a Red Agarose column and elute the protein out by using 0.5mM of NADP in the Tris buffer. The next step involved purification of SADH by using an Octyl Sepharose column, and the pooled fractions from this which were active with 2-propanol were then subsequently further purified using Hydroxyapatite chromatography, then dialyzed and ultrafiltered. All the proteins in previous chromatography steps were eluted

using NADP which makes the elution buffer very expensive due to the high cost of NADP.

1.4.3.1 Cloned *T. ethanolicus* SADH

The next big development came when Burdette et al. (1994, 1996) were able to clone, sequence, and express the SADH gene in *Escherichia coli*. This substantially simplified the steps involved, because now the cells can be grown in 1L batches in Erlenmeyer flasks under aerobic conditions using simple media like LB media and usually the cell density was about 10 g/L. SADH is stable at 70 °C but the majority of other *E. coli* cell proteins are not, and this fact was used for purification. After cell lysis, the extract was incubated for 30 mins at 70 °C to precipitate all the other proteins; it was then centrifuged to get a partially purified cell extract. After this step, a Red Agarose column can be used to get the NADP dependent SADH; However, unlike what was done previously, here instead of eluting with NADP, we can use sodium perchlorate, which can elute out everything bound to the column, and this makes this purification substantially easier and cheaper than what was done before.

Tripp and Phillips (1998) then used this cloned WT SADH and then studied the mutation of S39T in stereospecificity of 2-butanol and 2-pentanol. **Figure 2** shows the crystal structure of *Thermoanerobium brockii* ADH, which is completely identical to *T. ethanolicus* SADH which we use for our studies. The S39T mutation was chosen because it doesn't disrupt the hydrogen bonding in ADH which forms a necessary part in the enzyme activity, and hence the amino acid residue needs to have a side chain hydroxyl

group; and it has been well known in other enzymes that threonine residues can perform identical role as serine in facilitating enzyme function. It was observed that this mutation makes the enzyme active site pocket smaller, and this results in an increase in enzyme activity compared to wild type SADH on 2-propanol. Another interesting effect of this mutation is that it increases the preference for (R)-2-butanol and (R)-2-pentanol, and this represented a potential for accessing both enantiomers of same alcohols with a *T. ethanolicus* based system. However, the stereospecificity of S39T SADH was not nearly as good for a synthetic utility, and better mutants were needed before this system can have any practical utility.

In summary, Phillips and co-workers had studied the effects of temperature and pH on stereoselectivity, and next obvious milestone would be to study the effect of hydrostatic pressure, which we have done in chapter 2 of this thesis. We selected S39T SADH because of the higher activity of the enzyme compared to WT, and because we had already studied the temperature dependence of this enzyme hence this study would serve as a final piece in the puzzle of how physical parameters influence stereoselectivity.

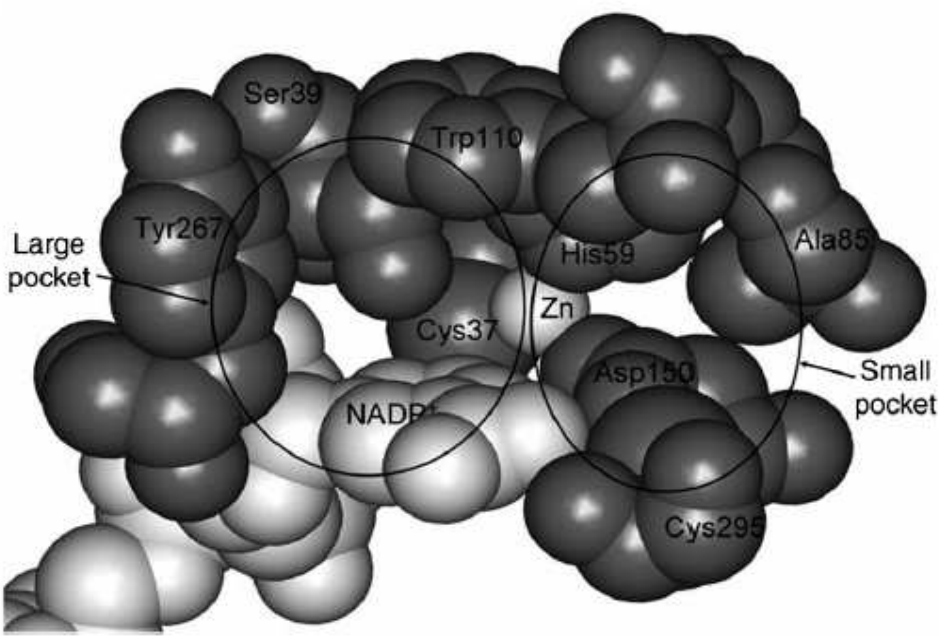


Figure 2: Active site of *Thermoanerobium brockii* ADH with bound $NADP^+$ and Zn^{2+}. Reprinted from [Ziegelmann, K.J.; Musa, M.M.; Phillips, R.S.; Zeikus, J.G.; Vieille, C. *Protein Eng., Des. Sel.* **2007**, 20, 47–55.] by permission from Oxford University Press.

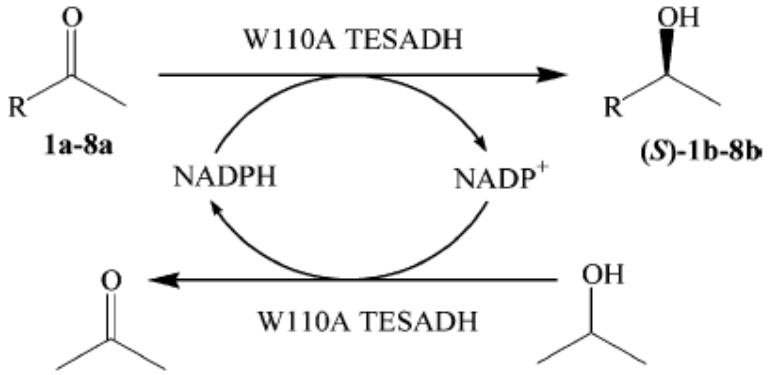
1.4.3.2 W110A SADH

In subsequent years, some more mutations were studied in this lab such as C295A, I86A, (Heiss et al., 2000, 2001) and W110A SADH (Musa et al., 2007, 2008) and those immensely expanded the substrates of SADH into aliphatic ketones, substituted aromatic ketones, alkynyl ketones, etc. Out of all those mutant SADH, W110A SADH will be discussed in detail here as that's the site of mutation which is of special interest for the purpose of this thesis. Musa et al. (2007, 2008) studied the W110A in detail, and as shown in **Table 1**, it can reduce a variety of ketones into (S)-alcohols in high enantiopurity. It was also observed that W110A was stable in organic solvents like acetonitrile, and some of the hydrophobic alcohols were synthesized using xerogel encapsulated W110A (Musa et al., 2007), and this was probably the mutant SADH with the widest variety of substrates and with very high stereoselectivity. A very unexpected result was obtained when reduction of 7 (a), phenylacetone was attempted, which gave the corresponding (S)-alcohol in very low e.e. of 37%. This was contrary not only to what was observed with other similar sized substrates, but also suggested that phenylacetone somehow fits in the large pocket of enzyme active site in an alternative mode. As a result of this intriguing result, we decided that we will select this enzyme site, and create a library of enzyme mutants which can not only accept a wider range of substrates with higher stereoselectivity and higher predictability. We decided to screen those enzymes against (R)- and (S)-1-phenyl-2-propanol in the oxidizing direction and phenylacetone in the reduction direction as model substrates because those were the ones which performed poorly with one of the best SADH mutants studied in this lab i.e. W110A.

Table 1: Asymmetric synthesis of (S)-alcohols catalyzed by W110A SADH (Musa et al, 2006)

substrate	R	product	Conv (%)	e.e. (%)
1a	$PhCH_2CH_2$	(*S*)-**1b**	99	>99
2a	$Ph(C{=}O)CH_2$	(*S*)-**2b**	98	>99
3a	(*E*)-Ph–HC=CH	(*S*)-**3b**	64	>99
4a	*p*-$MeOC_6H_4(CH_2)2$	(*S*)-**4b**	87	91
5a	$PhOCH_2$	(*S*)-**5b**	>99	>99
6a	*p*-$ClC_6H_4CH_2CHCl$	(2*S*,3*R*)-**6b**	83	>99
7a	$PhCH_2$	(*S*)-**7b**	95	37
8a	*p*-$MeOC_6H_4CH_2$	(*S*)-**8b**	97	>99

Figure 3: Reduction of ketones to give secondary alcohols catalyzed by *T. ethanolicus* SADH (TeSADH) along with the cofactor recycling system.



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CHAPTER 2

EFFECT OF HYDROSTATIC PRESSURE ON ENANTIOSPECIFICITY OF OXIDATION OF (R)- AND (S)-ALCOHOLS CATALYZED BY SECONDARY ALCOHOL DEHYDROGENASE FROM *THERMOANAEROBACTER ETHANOLICUS*[1]

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Abstract: Alcohol dehydrogenases (ADHs) are enzymes that catalyze the reversible reduction of carbonyl compounds to their corresponding alcohols. We have been studying a thermostable, NADP dependent, secondary ADH (SADH) from *Thermoanaerobacter ethanolicus* for some time now, and it has been shown that our library of SADH has high tolerance of nonaqueous media as well as the ability to accept a variety of secondary alcohols and their corresponding ketones as substrates with high activities. It has been of great interest to study the effects of physical variables such as pressure and temperature on stereospecificity of biocatalytic reactions. We studied the effect of hydrostatic pressure (up to 150MPa) and temperature (293 - 326 K) on the stereospecificity of S39T mutant SADH in oxidation of secondary alcohols. We are pleased to show that under high pressure conditions of 137.5 MPa and at 298K, the enantiomeric ratio can be enhanced to 13.5 compared to 3.9 at room temperature and pressure for (S)-2-hexanol over (R)-2-hexanol. This direct correlation of stereospecificity with hydrostatic pressure is just a preliminary step in enhancing the utility of our SADH library in synthesis of enantiopure alcohols.

2.1 Introduction

The modern field of biocatalysis essentially includes using whole cell microorganisms and isolated enzymes to carry out synthesis of organic molecules (Woodley, 2008). There are many advantages to this approach including: 1) Enzymatic reactions have very a high degree of chemo-, regio-, and stereospecificity, and this is usually the biggest reason why this reaction methodology is used; 2) These reactions are carried out under mild reaction conditions such as room temperature and pressure, and hence can be used for synthesis of sensitive molecules; 3) The natural solvents for most of these enzymatic systems is water; this can be considered an advantage because of the recent push for "green" strategies, and this water based systems would be obviously greener then reactions in organic solvents. The advantages described above are also the reasons why biocatalysis has had limited acceptance among organic and process chemists, and frequently, organic chemists viewed it as method of last resort (Faber, 2004). Some of the common disadvantages are: 1) Most enzymes do not tolerate organic solvents well, and many of the organic compounds desired have low to no solubility in water, hence their synthesis via this method becomes challenging; 2) Most wild type enzymes accept a very narrow range of compounds as substrates and even smallest functional group changes makes them inactive; 3) Most enzymes operate under a very narrow range of optimum conditions such as pH, temperature and atmospheric pressure (Musa and Phillips, 2011). This becomes a disadvantage for organic synthesis where more flexibility is often needed for physical reaction conditions.

In the last twenty years, advancements in molecular biology techniques such as mutagenesis and recombinant DNA, high throughput screening etc, have eliminated most of this disadvantages, and through the work in this paper we attempt to showcase how biocatalytic systems are becoming comparable to traditional process chemistry systems.

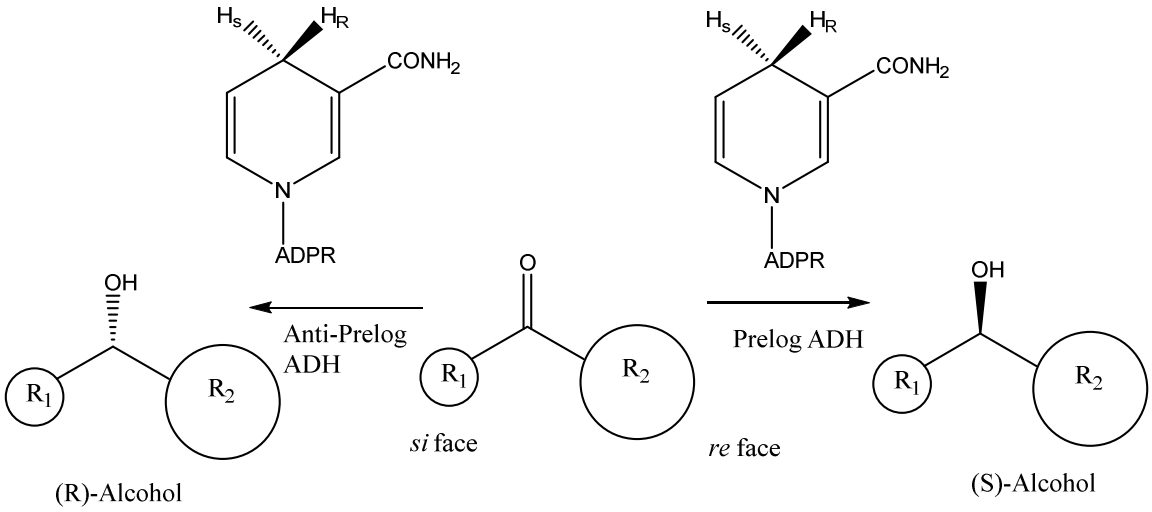
2.1.1 Alcohol dehydrogenase

Alcohol dehydrogenases (ADH) are a class of NAD(P) dependent enzymes which catalyze the reversible oxidation of alcohols to corresponding ketones or aldehydes. These enzymes are extremely important in biocatalysis because frequently these reactions are highly regio- and stereospecific, and that makes it highly attractive to synthesize alcohols with high enantiopurity (Keinan et al., 1986, 1990). Prelog came up with an empirical rule to predict the stereospecificity for the alcohol formed based on four ways with which a hydride can be delivered from NAD(P)H to the substrate, as shown in **Scheme 1**; According to Prelog's law, the *pro*-(R) or *pro*-(S)-hydride will attack from the *re* face of a prochiral ketone, to produce the (S)-alcohol. Most ADHs like HLADH, Baker's yeast, etc follow this rule, however, there are some ADHs like that from *Lactobacillus kefir* in which the attack occurs from the *si* face of a ketone to produce the (R)-alcohol, and then the ADH is said to follow the anti-Prelog rule.

One of the ways to classify ADHs is based on whether their substrates are primary alcohol or secondary alcohols; and they are then called primary ADH or secondary ADH (SADH) respectively. Some of the earliest examples of ADH based systems are baker's yeast (Matsuda et al., 2009), and horse liver ADH (Davies and Jones, 1979). Both of

these are primary ADHs and typical drawbacks include low substrate density and consequent difficulty in product separation, low activity for acyclic ketones, low thermal stability, etc.

Scheme 1: Illustration of Prelog's law (Prelog, 1964)



2.1.2 *Thermoanaerobacter ethanolicus* ADH

Bryant et al. (1988) isolated and characterized two alcohol dehydrogenases from the a thermophilic bacterium, *Thermoanaerobacter ethanolicus*, which was isolated from hot springs at Yellowstone National Park. It was observed that while one of them preferred primary alcohol, the other ADH was more active towards ketones and secondary alcohols then it was towards ethanol, and henceforth, we refer to this enzyme as secondary alcohol dehydrogenase (SADH), which is an NADP-dependent Zn^{2+} tetrameric oxidoreductase. One of the most obvious advantages of using a SADH from a thermophillic bacteria is that the enzyme has very good tolerance for high temperature, and this SADH has been found to be highly stable at temperatures above 70 °C; this is a substantial improvement to traditional enzyme systems whose stability rapidly declines past 42 °C, and allows more control on this important physical parameter.

Pham et al. (1989, 1990) found a strong temperature dependence on enantioselectivity of *T. ethanolicus* SADH, and it was observed that for 2-butanol, there was a reversal of stereospecificity for (S)-2-butanol below 26 °C to (R)-2-butanol above that temperature. (S)-2-Pentanol was found to be the preferred substrate at temperatures up to 60 °C. This was one of the earliest reports which conclusively established the importance of temperature in stereospecificity of SADH, and showed great potential for its practical applications. Values of k_{cat}/K_m were also obtained for cyclic substrates like cyclobutanol, cyclopentanol and cyclohexanol, and it was found that the values were at least an order of magnitude less than that for acyclic secondary alcohols like 2-propanol. Zheng et. al (1992, 1994) subsequently studied the reduction of ketones to get chiral alcohols as this

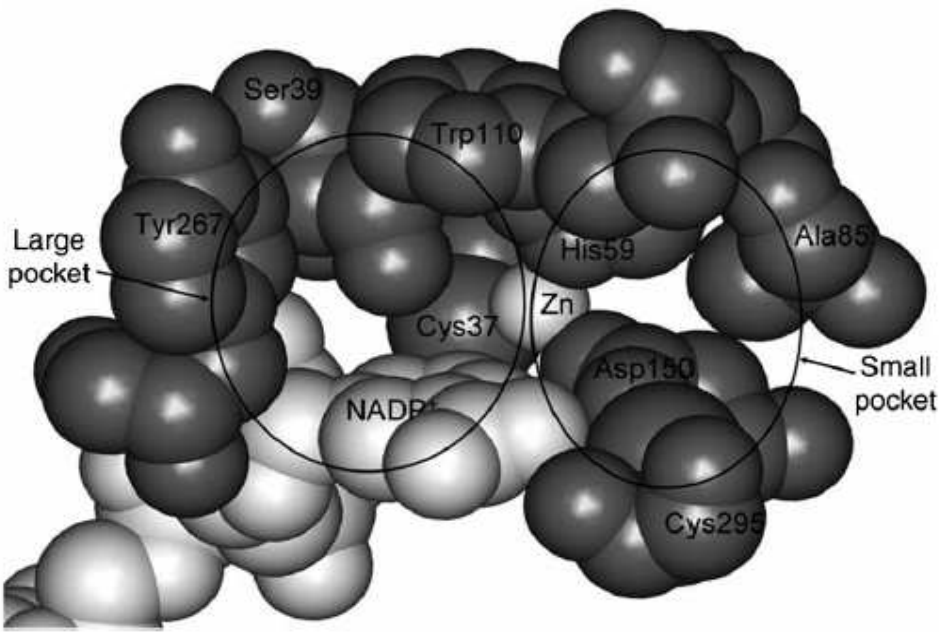
was a more interesting application of SADH, and it was observed that aliphatic saturated ketones like 3 hexanone, 2 heptanone, 2-octanone, and 4-methyl cyclohexanone were very good substrates and gave (S)-alcohols in >95% e.e. It was found that aromatic ketones like acetophenone and diketones like 2,4- pentanedione gave no reaction with SADH. These results were encouraging, considering that earlier known enzyme systems like HLADH could not reduce some of these substrates with high enantiopurity and at high temperatures.

Secundo and Phillips (1996) studied the effect of pH on enantiospecificity of SADH on 2-butanol, and they observed that E for 2-butanol increases from 2.5 at pH 9 to 4.2 at pH 5.5. This represented yet another correlation of physical parameter change to stereospecificity, and it potentially imparted one more tool in the hands of synthetic chemists to influence stereospecificity.

However, there were just a narrow range of substrates which showed good stereospecificity, and there were still some other drawbacks to use this *T. ethanolicus* based SADH, chiefly among them were that is *T. ethanolicus* is an obligate anaerobe requiring N_2 or Ar atmosphere, they grow very slowly and to low cell density of about 25-33 gm cells in 20 L carboy fermenter. Due to the low weight of cells obtained, they scaled up the procedure and used a 400L fermenter. The protein purification procedure was pretty labor intensive, because it involved separating the primary and secondary ADH by using a Red Agarose column and elute the protein out by using 0.5mM of NADP in the Tris buffer. The next step involved purification of SADH by using an Octyl Sepharose column, and the pooled fractions from this which were active with 2-propanol were then subsequently further purified using Hydroxyapatite chromatography, then

dialyzed and ultrafiltered. All the proteins in previous chromatography steps were eluted using NADP which makes the elution buffer very expensive due to the high cost of NADP.

Figure 1: Active site of *Thermoanerobium brockii* ADH with bound $NADP^+$ and Zn. Reprinted from [Ziegelmann, K.J.; Musa, M.M.; Phillips, R.S.; Zeikus, J.G.; Vieille, C. *Protein Eng., Des. Sel.* **2007**, 20, 47–55.] by permission from Oxford University Press.



2.1.3 Cloned *T. ethanolicus* SADH

The next big development came when Burdette et al. (1994, 1996) were able to clone, sequence, and express the SADH gene in *Escherichia coli*. This substantially simplified the steps involved, because now the cells can be grown in 1L batches in Erlenmeyer flasks under aerobic conditions using simple media like LB media and usually the cell density was about 10 g/L. SADH is stable at 70 °C but the majority of other *E. coli* cell proteins is not, and this fact was used for purification. After cell lysis, the extract was incubated for 30 mins at 70 °C to precipitate all the other proteins; it was then centrifuged to get a partially purified cell extract. After this step, a Red Agarose column can be used to get the NADP-dependent SADH; However, unlike what was done previously, here instead of eluting with NADP, we can use sodium perchlorate, which can elute out everything bound to the column, and this makes this purification substantially easier and cheaper than what was done before.

Tripp and Phillips (1998) then used this cloned WT SADH and then studied the mutation of S39T in stereospecificity of 2-butanol and 2-pentanol. **Figure 1** shows the crystal structure of *Thermoanerobium brockii* ADH, which is completely identical to *T. ethanolicus SADH* which we use for our studies. The S39T mutation was chosen because it doesn't disrupt the hydrogen bonding in ADH which forms a necessary part in the enzyme activity, and hence the amino acid residue needs to have a side chain hydroxyl group; and it has been well known in other enzymes that threonine residues can perform identical role as serine in facilitating enzyme function. It was observed that this mutation makes the enzyme active site pocket smaller, and this results in an increase in enzyme

activity compared to wild type SADH on 2-propanol. Another interesting effect of this mutation is that it increases the preference for (R)-2-butanol and (R)-2-pentanol, and this represented a potential for accessing both enantiomers of same alcohols with a *T. ethanolicus* based system. However, the stereospecificity of S39T SADH was not nearly as good as needed for synthetic utility, and better mutants were needed before this system can have any practical utility. Hence, other mutant SADH such as C295A, I86A, (Heiss et al., 2000, 2001) and W110A (Musa et al., 2007, 2008) were subsequently studied.

In summary, Phillips and co-workers had studied the effects of temperature and pH on stereoselectivity and next obvious milestone would be to study the effect of hydrostatic pressure on stereoselectivity, which we have done in this paper. We selected S39T SADH because of the higher activity of the enzyme compared to WT, and because we had already studied the temperature dependence of this enzyme hence this study would serve as a final piece in the puzzle of how physical parameters influence stereoselectivity.

Morita and Haight (1962) showed that malic dehydrogenase from *Bacillus stearothermophilus* was inactive at 101 ˚C from 0.1 to 70 MPa. However, there was activity observed at 70MPa with optimal activity at 130MPa at 101 ˚C. This showed that pressure can influence enzyme activity, and not many reports were published until Dallat and Legoy (1996) showed that thermostable *Thermoanaerobium brockii* ADH was activated by pressure up to 100MPa. Cho and Northrop (1999) also studied the kinetics of yeast ADH under high pressure. However, to best of our knowledge, no one has studied the effect of high pressure to stereospecificity, and this work represents one of the first investigations into that area.

2.2 Materials and Methods

2.2.1 Growth of Cells

E. coli DH5α containing the recombinant *adhB* gene coding for wild-type or mutant S39T SADH was grown aerobically for 24 hours in rich complex medium containing 20 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 25 μg/L kanamycin at 37 °C. Cells were collected by centrifugation at 10000 g for 15 min (Tripp and Phillips, 1998).

2.2.2 Purification of Secondary Alcohol Dehydrogenase (SADH)

The wet cells were resuspended (0.5 g cells per mL of buffer) in 50 mM Tris HCl at pH 8.0 containing 5 mM DTT (buffer A) according to the procedure of Burdette et al. (1996). The cells were then lysed by sonication at 0 °C in three 3-min intervals. Cell debris was removed by centrifugation at 15000 g for 45 min. The resulting supernatant was incubated at 70 °C for 15 min and centrifuged at 25000 g for 30 min to remove the thermally denatured impurities. The crude supernatant solution of SADH was applied to a 10 mL Red Agarose column that had been preequilibrated and washed with buffer A. Impurities were eluted with buffer A containing 0.07 M $NaClO_4$, followed by elution of SADH fractions with buffer A containing 0.2 M $NaClO_4$. The purified SADH solutions can be stored at -77 °C for several months without loss of activity, and were used in the subsequent kinetics studies.

2.2.3 Secondary Alcohol Dehydrogenase Assay

SADH was assayed spectrophotometrically at 50 °C by following the production of NADPH (extinction coefficient 6220 $M^{-1}cm^{-1}$ at 340 nm) in a solution containing 200 mM 2-propanol as the substrate and 1.25 mM NADP in 100 mM Tris.HCl buffer (pH 8.9).7 One unit of activity is the amount of SADH that reduces or oxidizes 1 *μmol* of NADP or NADPH per minute, respectively.

2.2.4 Enzyme kinetics under hydrostatic pressure

The effects of hydrostatic pressure on the rates and absorption spectra were measured using a Cary 14 UV/Vis spectrophotometer modified by OLIS, Inc. to contain a high pressure cell from ISS (Champaign, Illinois, USA), equipped with a manual pressure pump from High Pressure Equipment Co., using spectroscopic grade ethanol as the pressurizing fluid. The cell temperature was controlled with an external circulating water bath. The enzyme solutions were contained in 1 mL quartz bottles with a 9 mm path length, capped with Teflon tubing. The quartz bottle contained 1mM of $NADP^+$, 15 mM of (R) or (S)-2-butanol, 2-pentanol, and 2-hexanol, 50mM of Tris.HCl (pH 8.9 at 323K) in the final volume of 1 ml. The initial reaction rates were measured by following the production of NADPH spectrophotometrically at 340 nm. Enantiomeric ratio was determined as the ratio between initial rates of (R) and (S) alcohols.

2.2.5 Stability with organic and ionic solvents

S39T SADH activity was measured at 50 °C under different organic solvents such as acetonitrile, dimethyl formamide, and 1-butyl-3-methylimidazolium tetrafluoroborate, [bmim][BF_4] and activity was calculated according to the definition stated above.

2.3 Results and Discussion

Tripp and Phillips (1998) had shown that S39T SADH prefers the (R)-isomer as substrate for 2-butanol and 2-pentanol, and we got results consistent with what was observed before. Apart from that, we studied 2-hexanol which was not studied as a substrate before, and we found that in case of 2-hexanol, the preference switched from (R)-alcohol to (S)-alcohol. We postulate the reason for this might be that the alkane chain had finally gotten so big that it could not fit in the smaller pocket of the enzyme site anymore and it was forced to go into the larger pocket, thus becoming a Prelog type substrate. We did not study any higher alcohols, such as 2-heptanol, because those were not very soluble in water, and S39T SADH was found to rapidly lose activity in organic solvents past 5% concentration.

S39T SADH was found to have high stability for hydrostatic pressure up to 150 MPa, and that itself is a pretty interesting result because this is the first time anyone even studied the stability of this enzyme under high pressure. The enantiomeric ratio for 2-butanol and 2-pentanol is calculated as R/S whereas for 2-hexanol it was calculated as S/R due to preference switch.

As shown in **Table 1**, the enantiomeric ratio (from specific activity) for 2-butanol at both 298K and 318K slowly decreases with increase in pressure, with a preference for (S)-alcohol as pressure increases. The ratio is more strongly influenced at lower temperatures then that at higher temperature.

In **Table 2**, we show the relationship between hydrostatic pressure and initial rates and the enantiomeric ratio for 2-pentanol. At 298K, the rates for (R) and (S)-2-pentanol are almost the same at 0.1 MPa, and with the increase in pressure, the rates for both S and R alcohols decrease, although at 150 MPa, the S isomer has a faster rate than R, and this is pretty interesting considering that we shifted the preference from R alcohol to S alcohol by varying pressure alone. At a higher temperature of 318K, there is more appreciable preference of about 1.7 for R isomer at 0.1 MPa, however with the increase in pressure, this slowly decreases to about 1 at 150 MPa.

In **Table 3**, we show the relationship between hydrostatic pressure and initial rates and the enantiomeric ratio for 2-hexanol; and this substrate showed the most change in enantiomeric ratio with increase in temperature. We did it at four different temperatures so as to understand this substrate more completely. The general trend was that the reaction rates increase with increasing temperature. At 0.1 MPa, the enantiomeric ratio at 293K was 5.3, and this decreases with increase in temperature and at 325K it goes down to 2.2. The reaction rates typically increased with higher pressure just that it increased faster for (S) isomer then (R), and that's why we observed an overall increase for enantiomeric ratio with increase in pressure. The best improvement of ratio was observed at 298K at 137.5 MPa when it went to 13.5 from 3.9 and this represents almost 3 fold improvement by the effect of pressure alone, and this has never been shown before.

Table 1: Pressure dependence of stereospecificity of 2-butanol with S39T SADH

Enantiomer	Temperature (K)	Pressure (Mpa)	Initial Rate (A/min)	R/S
R	298	0.1	0.00442 ± 0.0004	2.36 ± 0.26
S	298	0.1	0.00186 ± 0.00009	
R	298	50	0.004 ± 0.00004	2.44 ± 0.05
S	298	50	0.001638 ± 0.00003	
R	298	100	0.002958 ± 0.00009	1.98 ± 0.13
S	298	100	0.00149 ± 0.00007	
R	298	150	0.0019 ± 0.000005	1.48 ± 0.09
S	298	150	0.00128 ± 0.00008	

Table 1 (Cont.): Pressure dependence of stereospecificity of 2-butanol with S39T SADH

Enantiomer	Temperature (K)	Pressure (MPa)	Initial Rate (A/min)	R/S
R	318	0.1	0.01813 ± 0.00004	2.23 ± 0.02
S	318	0.1	0.00811 ± 0.0001	
R	318	50	0.01126 ± 0.00003	2.19 ± 0.03
S	318	50	0.00513 ± 0.00007	
R	318	100	0.01197 ± 000006	2.18 ± 0.02
S	318	100	0.00549 ± 0.00004	
R	318	150	0.0109 ± 0.00004	1.98 ± 0.01
S	318	150	0.00548 ± 0.00002	

Table 2: Pressure dependence of stereospecificity of 2-pentanol with S39T SADH

Enantiomer	Temperature (K)	Pressure (MPa)	Initial Rate (A/min)	R/S
R	298	0.1	0.00589 ± 2.9x10-6	1.031 ± 0.011
S	298	0.1	0.0057 ± 1.9x10-5	
R	298	50	0.002336 ± 8.9x10-6	1.052 ± 0.0089
S	298	50	0.0015314 ± 3.8x10-6	
R	298	100	0.0026043 ± 6.9x10-6	1.345 ± 0.015
S	298	100	0.001936 ± 1.0x10-5	
R	298	150	0.002059 ± 1.9x10-5	0.925 ± 0.0099
S	298	150	0.002225 ± 1.0x10-5	

Table 2 (Cont.): Pressure dependence of stereospecificity of 2-pentanol with S39T SADH

Enantiomer	Temperature (K)	Pressure (MPa)	Initial Rate (A/min)	R/S
R	318	0.1	0.009155 ± 1.3x10-5	1.721 ± 0.01
S	318	0.1	0.005319 ± 2.9x10-5	
R	318	50	0.009004 ± 2.6x10-5	1.42 ± 0.005
S	318	50	0.006338 ± 1.6x10-5	
R	318	100	0.011843 ± 1.0x10-5	1.36 ± 0.003
S	318	100	0.008658 ± 1.4x10-5	
R	318	150	0.013493 ± 8.3x10-6	1.000 ± 0.0013
S	318	150	0.0134833 ± 1.4x10-5	

Table 3: Pressure dependence of stereospecificity of 2-hexanol with S39T SADH

Enantiomer	Temperature (K)	Pressure (MPa)	Initial Rate (A/min)	R/S
R	293	0.1	0.0003 ± 0.00001	0.187 ± 0.008
S	293	0.1	0.0016 ± 0.00004	
R	293	50	0.00016 ± 0.00005	0.069 ± 0.02
S	293	50	0.0023 ± 0.00002	
R	293	100	0.00037 ± 0.00009	0.078 ± 0.02
S	293	100	0.0047 ± 0.00004	
R	293	137.5	0.00051 ± 0.00009	0.10 ± 0.02
S	293	137.5	0.0053 ± 0.00001	

Table 3 (Cont.): Pressure dependence of stereospecificity of 2-hexanol with S39T SADH

Enantiomer	Temperature (K)	Pressure (MPa)	Initial Rate (A/min)	R/S
R	298	0.1	0.00114 ± 0.000002	0.256 ± 0.002
S	298	0.1	0.00445 ± 0.00004	
R	298	50	0.0013 ± 0.00003	0.138 ± 0.003
S	298	50	0.0094 ± 0.00006	
R	298	100	0.0025 ± 0.00002	0.137 ± 0.001
S	298	100	0.01825 ± 0.00009	
R	298	137.5	0.00128 ± 0.00004	0.073 ± 0.002
S	298	137.5	0.0174 ± 0.00003	

Table 3 (Cont.): Pressure dependence of stereospecificity of 2-hexanol with S39T SADH

Enantiomer	Temperature (K)	Pressure (MPa)	Initial Rate (A/min)	R/S
R	318	0.1	0.0059 ± 0.00004	0.430 ± 0.004
S	318	0.1	0.0137 ± 0.0001	
R	318	50	0.0035 ± 0.00003	0.21 ± 0.02
S	318	50	0.0167 ± 0.00005	
R	318	100	0.0063 ± 0.00009	0.165 ± 0.002
S	318	100	0.0381 ± 0.00001	
R	318	137.5	0.0042 ± 0.0004	0.085 ± 0.008
S	318	137.5	0.0492 ± 0.00003	

Table 3 (cont.): Pressure dependence of stereospecificity of 2-hexanol with S39T SADH.

Enantiomer	Temperature (K)	Pressure (MPa)	Initial Rate (A/min)	R/S
R	325	0.1	0.0066 ± 0.00006	0.446 ± 0.005
S	325	0.1	0.0148 ± 0.00009	
R	325	50	0.0067 ± 0.0004	0.20 ± 0.01
S	325	50	0.0323 ± 0.0005	
R	325	100	0.0122 ± 0.0007	0.23 ± 0.01
S	325	100	0.0521 ± 0.0004	
R	325	137.5	0.0118 ± 0.00005	0.133 ± 0.0005
S	325	137.5	0.0881 ± 0.00003	

2.4 Conclusions

T. ethanolicus SADH has been known to be a highly regio- and stereo-specific ADH which is thermostable at high temperatures and its mutants can accept a wide range of substrates. In this paper we have shown that SADH is not only highly stable at very high pressure of 150 MPa, but it actually makes the reaction more stereospecific, and the enantiomeric ratio at 137.5 MPa can be enhanced to 13.5 compared to 3.9 at room temperature and pressure for (S)-2-hexanol over (R)-2-hexanol. We believe that this is the first paper which has shown a strong correlation between stereospecificity and hydrostatic pressure, and this potentially makes this system even more attractive not only for small scale lab scale reductions but also for doing it on industrial setting.

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CHAPTER 3

DESIGNING NOVEL SECONDARY ALCOHOL DEHYDROGENASE FOR ENANTIOSPECIFIC OXIDATION OF 1-PHENYL-2-PROPANOL USING SITE SATURATION MUTAGENESIS[1]

[1]Patel J.M., Rodriguez L, Sutton D.A., Popik V.V., Phillips R.S., Manuscript to be submitted to Biochemistry

Abstract: Alcohol dehydrogenases (ADHs) are enzymes that catalyze the reversible reduction of carbonyl compounds to their corresponding alcohols. We have been studying a thermostable, NADP-dependent, secondary ADH (SADH) from *Thermoanaerobacter ethanolicus* for some time now, and it has been shown that our library of SADH has high tolerance of nonaqueous media as well as the ability to accept a variety of secondary alcohols and their corresponding ketones as substrates with high activity and stereoselectivity. We went out to further improve our library of SADH and adopted the site saturation mutagenesis approach in creating a comprehensive mutant library at W110; and we used phenylacetone as a model substrate to study the effectiveness of our library. We are pleased to note that five of our mutants gave reductions at >99.9% e.e. and two of the mutants have an enantiomeric ratio (E) of over 100. We consider this an immense improvement over our previous best mutant, W110A, which could carry out the same reduction at 37% e.e.; and we are sure that this new library will have very high synthetic utility in future.

3.1 Introduction

The modern field of biocatalysis essentially includes using whole cell microorganisms and isolated enzymes to carry out synthesis of organic molecules (Woodley, 2008). There are many advantages to this approach including: 1) Enzymatic reactions have very a high degree of chemo-, regio-, and stereospecificity, and this is usually the biggest reason why this reaction methodology is used; 2) These reactions are carried out under mild reaction conditions such as room temperature and pressure, and hence can be used for synthesis of sensitive molecules; 3) The natural solvents for most of this enzymatic systems is water; this can be considered an advantage because of the recent push for "green" strategies, and this water based systems would be obviously greener then reactions in organic solvents. The advantages described above are also the reasons why biocatalysis has had limited acceptance among organic and process chemists, and frequently, organic chemists viewed it as method of last resort (Faber, 2004). Some of the common disadvantages are: 1) Most enzymes do not tolerate organic solvents well, and many of the organic compounds desired have low to no solubility in water, hence their synthesis via this method becomes challenging; 2) Most wild type enzymes accept a very narrow range of compounds as substrates and even the smallest functional group changes makes them inactive; 3) Most enzymes operate under a very narrow range of optimum conditions such as pH, temperature and atmospheric pressure (Musa and Phillips, 2011). This becomes a disadvantage for organic synthesis where more flexibility is often needed for physical reaction conditions.

In the last twenty years, advancements in molecular biology techniques such as

mutagenesis and recombinant DNA, high throughput screening etc, have eliminated most of these disadvantages, and through the work in this paper we attempt to showcase how biocatalytic systems are becoming comparable to traditional process chemistry systems.

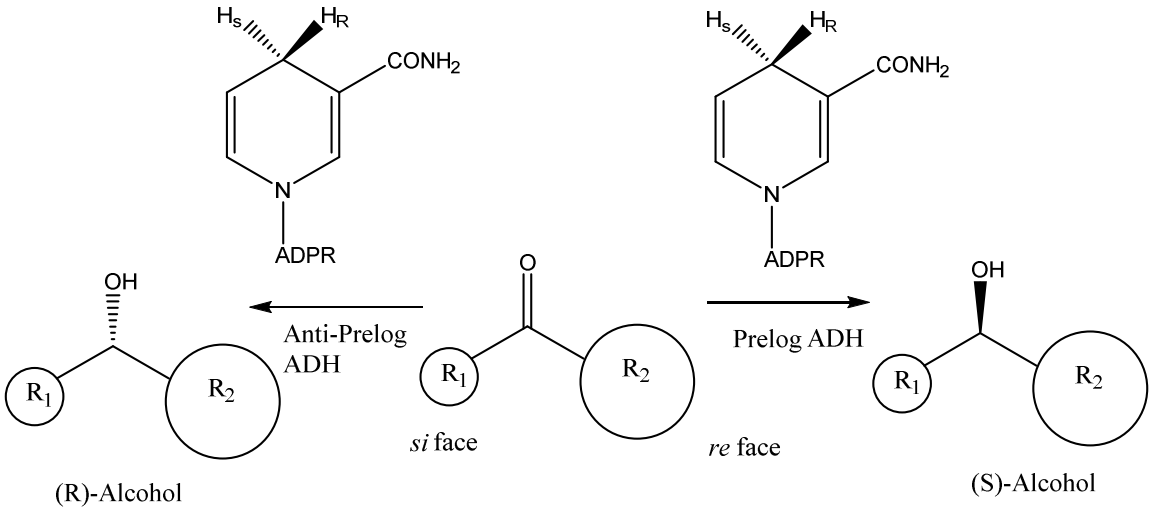
3.1.1 Alcohol dehydrogenase

Alcohol dehydrogenases (ADH) are a class of NAD(P) dependent enzymes which catalyze the reversible oxidation of alcohols to corresponding ketones or aldehydes. These enzymes are extremely important in biocatalysis because frequently these reactions are highly regio- and stereo-specific, and that makes it highly attractive to synthesize alcohols with high enantiopurity (Keinan et al., 1986, 1990). Prelog came up with an empirical rule to predict the stereospecificity for the alcohol formed based on four ways with which a hydride can be delivered from NAD(P)H to the substrate, as shown in **Scheme 1**; According to Prelog's law, the *pro*-(R) or *pro*-(S)-hydride will attack from the *re* face of a prochiral ketone, to produce the (S)-alcohol. Most ADHs like HLADH, Baker's yeast, etc. follow this rule; however, there are some ADHs like that from *Lactobacillus kefir* in which the attack occurs from the *si* face of a ketone to produce the (R)-alcohol, and then the ADH is said to follow the anti-Prelog rule.

One of the ways to classify ADHs is based on whether their substrates are primary alcohol or secondary alcohols; and they are then called primary ADH or secondary ADH (SADH) respectively. Some of the earliest examples of ADH based systems are baker's yeast (Matsuda et al., 2009), and horse liver ADH (Davies and Jones, 1979). Both of these are primary ADHs and typical drawbacks include low substrate density and

consequent difficulty in product separation, low activity for acyclic ketones, low thermal stability, etc.

Scheme 1: Illustration of Prelog's law (Prelog, 1964)



3.1.2 Thermoanaerobacter ethanolicus ADH

Bryant et al. (1988) isolated and characterized two alcohol dehydrogenases from the a thermophilic bacterium, *Thermoanaerobacter ethanolicus*, which was isolated from hot springs at Yellowstone National Park. It was observed that while one of them preferred primary alcohol, the other ADH was more active towards ketones and secondary alcohols then it was towards ethanol, and henceforth, we refer to this enzyme as secondary alcohol dehydrogenase (SADH), which is an NADP dependent Zn^{2+} tetrameric oxidoreductase. One of the most obvious advantages of using an SADH from a thermophilic bacteria is that the enzyme has very good tolerance for high temperature, and this SADH has been found to be highly stable at temperatures above 70 °C; this is substantial improvement to traditional enzyme systems whose stability rapidly declines past 42 °C, and allows more

control on this important physical parameter.

Pham et al. (1989, 1990) found a strong temperature dependence on enantiospecificity of *T. ethanolicus* SADH, and it was observed that for 2-butanol, there was a reversal of stereospecificity for (S)-2-butanol below 26 °C to (R)-2-butanol above that temperature. (S)-2-Pentanol was found to be the preferred substrate at temperatures up to 60 °C. This was one of the earliest reports which conclusively established the importance of temperature in stereospecificity of SADH, and showed great potential for its practical applications. Values of k_{cat}/K_m were also obtained for cyclic substrates like cyclobutanol, cyclopentanol and cyclohexanol, and it was found that the values were at least an order of magnitude less than that for acyclic secondary alcohols like 2-propanol. Zheng et al. (1992, 1994) subsequently studied the reduction of ketones to get chiral alcohols as this was a more interesting application of SADH, and it was observed that aliphatic saturated ketones like 3 hexanone, 2 heptanone, 2-octanone, and 4-methyl cyclohexanone were very good substrates and gave (S)-alcohols in >95% e.e. It was found that aromatic ketones like acetophenone and diketones like 2,4- pentanedione gave no reaction with SADH. These results were encouraging, considering that earlier known enzyme systems like HLADH could not reduce some of these substrates with high enantiopurity and at high temperatures.

Secundo and Phillips (1996) studied the effect of pH on enantiospecificity of SADH on 2-butanol, and they observed that E for 2-butanol increases from 2.5 at pH 9 to 4.2 at pH 5.5. This represented yet another correlation of physical parameter change to stereospecificity, and it potentially imparted one more tool in hands of synthetic chemists to influence stereospecificity.

However, there were just a narrow range of substrates which showed good stereospecificity, and there were still some other drawbacks to use this *T. ethanolicus* based SADH, chiefly among them were that is *T. ethanolicus* is an obligate anaerobe requiring N_2 or Ar atmosphere, they grow very slowly and to low cell density of about 25-33 gm cells in 20 L carboy fermenter. Due to the low weight of cells obtained, they scaled up the procedure and used a 400L fermenter. The protein purification procedure was pretty labor intensive, because it involved separating the primary and secondary ADH by using a Red Agarose column and elute the protein out by using 0.5mM of NADP in the tris buffer. The next step involved purification of SADH by using an Octyl Sepharose column, and the pooled fractions from this which were active with 2-propanol were then subsequently further purified using Hydroxyapatite chromatography, then dialyzed and ultrafiltered. All the proteins in previous chromatography steps were eluted using NADP which makes the elution buffer very expensive due to the high cost of NADP.

3.1.3 Cloned *T. ethanolicus* SADH

The next big development came when Burdette et al. (1994, 1996) were able to clone, sequence, and express the SADH gene in *Escherichia coli*. This substantially simplified the steps involved, because now the cells can be grown in 1L batches in Erlenmeyer flasks under aerobic conditions using simple media like LB media and usually the cell density was about 10 g/L. SADH is stable at 70 °C but majority of other *E. Coli* cell proteins is not, and this fact was used for purification. After cell lysis, the extract was

incubated for 30 min at 70 °C to precipitate all the other proteins; it was then centrifuged to get a partially purified cell extract. After this step, a Red Agarose column can be used to get the NADP dependent SADH; However, unlike what was done previously, here instead of eluting with NADP, we can use sodium perchlorate, which can elute out everything bound to the column, and this makes this purification substantially easier and cheaper than what was done before.

Tripp and Phillips (1998) then used this cloned WT SADH and then studied the mutation of S39T in stereospecificity of 2-butanol and 2-pentanol. **Figure 3** shows the crystal structure of *Thermoanerobium brockii* ADH, which is completely identical to *T. ethanolicus SADH* which we use for our studies. The S39T mutation was chosen because it doesn't disrupt the hydrogen bonding in ADH which forms a necessary part in the enzyme activity, and hence the amino acid residue needs to have a side chain hydroxyl group; and it has been well known in other enzymes that threonine residues can perform identical role as serine in facilitating enzyme function. It was observed that this mutation makes the enzyme active site pocket smaller, and this results in an increase in enzyme activity compared to wild type SADH on 2-propanol. Another interesting effect of this mutation is that it increases the preference for (R)-2-butanol and (R)-2-pentanol, and this represented a potential for accessing both enantiomers of same alcohols with a *T. ethanolicus* based system. However, the stereospecificity of S39T SADH was not nearly as good for a synthetic utility, and better mutants were needed before this system can have any practical utility.

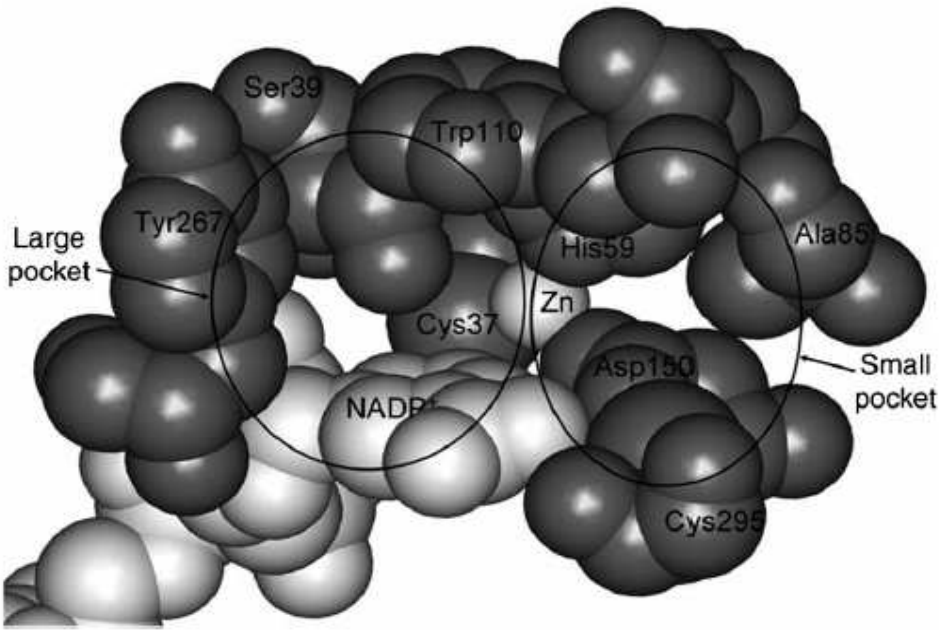


Figure 1: Active site of *Thermoanerobium brockii* ADH with bound $NADP^+$ and Zn^-. Reprinted from [Ziegelmann, K.J.; Musa, M.M.; Phillips, R.S.; Zeikus, J.G.; Vieille, C. *Protein Eng., Des. Sel.* **2007**, 20, 47–55.] by permission from Oxford University Press.

3.1.4 W110A SADH

In subsequent years, some more mutations were studied in this lab such as C295A, I86A, (Heiss et al., 2000, 2001) and W110A SADH (Musa et al., 2007, 2008) and those immensely expanded the substrates of SADH into aliphatic ketones, substituted aromatic ketones, alkynyl ketones, etc. Out of these mutant SADHs, W110A SADH will be discussed in detail here as that's the site of mutation which is of special interest for the purpose of this thesis. Musa et al. (2007, 2008) studied the W110A mutant in detail, and as shown in **Table 1**, it can reduce a variety of ketones into (S)-alcohols in high enantiopurity. It was also observed that W110A was stable in organic solvents like acetonitrile, and some of the hydrophobic alcohols were synthesized using xerogel encapsulated W110A, Musa (2007), and this was probably the mutant SADH with the widest variety of substrates and with very high stereoselectivity .

A very unexpected result was obtained when reduction of 7 (a), phenylacetone was attempted, which gave the corresponding (S)-alcohol in very low e.e. of 37%. This was contrary not only to what was observed with other similar sized substrates, but also suggested that phenylacetone somehow fits in the large pocket of enzyme active site in an alternative mode. As a result of this intriguing result, we decided that we will select this enzyme site, and create a library of enzyme mutants which can not only accept a wider range of substrates with higher stereoselectivity and higher predictability. We decided to screen those enzymes against (R)- and (S)-1-phenyl-2-propanol in the oxidizing direction and phenylacetone in the reduction direction as model substrates because those were the ones which performed poorly with one of the best SADH mutants studied in this lab i.e.

W110A. We chose to create a mutant library at W110 using an approach of degenerate primers, which is frequently called site saturation mutagenesis, and this gives us access to all the mutants at that site by doing a PCR just once instead of doing it for each mutant as done in site directed mutagenesis.

Table 1: Asymmetric synthesis of (S)-alcohols catalyzed by W110A SADH

substrate	R	product	Conv (%)	e.e. (%)
1a	$PhCH_2CH_2$	(*S*)-**1b**	99	>99
2a	$Ph(C{=}O)CH_2$	(*S*)-**2b**	98	>99
3a	(*E*)-Ph–HC=CH	(*S*)-**3b**	64	>99
4a	*p*-$MeOC_6H_4(CH_2)_2$	(*S*)-**4b**	87	91
5a	$PhOCH_2$	(*S*)-**5b**	>99	>99
6a	*p*-$ClC_6H_4CH_2CHCl$	(2*S*,3*R*)-**6b**	83	>99
7a	$PhCH_2$	(*S*)-**7b**	95	37
8a	*p*-$MeOC_6H_4CH_2$	(*S*)-**8b**	97	>99

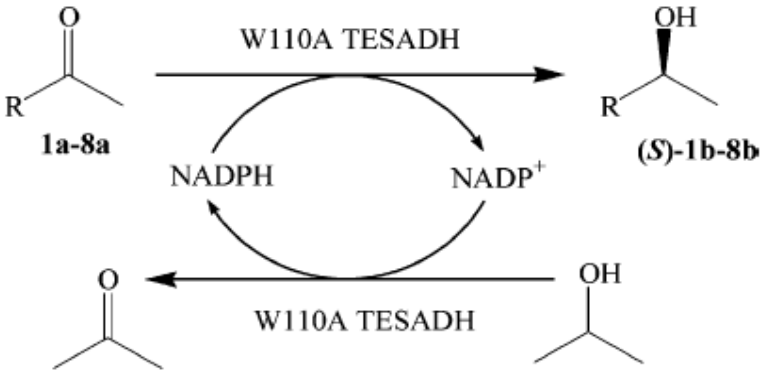


Figure 2: Reduction of ketones to give secondary alcohols catalyzed by *T. ethanolicus* SADH (TeSADH) along with the cofactor recycling system.

3.2 Materials and Methods

3.2.1 Site saturation mutagenesis

The codon NNK (N=GATC and K=GT) was used at W110 site to get all the possible mutations using site saturation mutagenesis based on Quikchange (Stratagene) kit. This degenerate primer design approach gives 32 codons, and theoretically gives access to all 20 amino acids. Primer design was done using broad guidelines from Liu and Naismith (2008) and Zheng et al. (2004). We used partially overlapping primer design in this case, and their guidelines stated that there should be at least 8-18 overlapping bases, targeted mutation in both forward and reverse primers, and at-least one G or C should be at each of the terminus. Using this, we designed the primers as shown below:

GGCAGGCNNKAAATTTTCGAATGTAAAAGATGGTGTTTTTG fprimer (melting temp 62.5 degC to 65.5 degC)

GAAAATTTMNNGCCTGCCAGCATTCCACCGGAGTGCTGGTG rprimer (melting temp 69.5 degC to 72.5 degC)

The PCR reaction was done in a 50 µL containing 1 µL of template (2-10ng), 1 µM of primer pair, 200 µM of dNTP and 3 units of Pfu DNA polymerase. The PCR cycle was initiated by at 95 ^{O}C for 5 min to denature the template DNA, followed by 16 amplification cycles, each of 95 ^{O}C for 1 min, 52 ^{O}C for 1 min, and 68 ^{O}C for 24 min, followed by incubation at 68 ^{O}C for 1 hr.

3.2.2 Digesting the products

The PCR reaction was then digested by 1 μL of Dpn I restriction enzyme (10U/μL) for one hour at 37 ^{O}C to digest the entire parental methylated supercoiled DNA. After going onto the further steps, we realized that we were having very high background of wild type mutants, and that was attributed to incomplete DNA digestion. In order to minimize the WT background, we ended up digesting the PCR mixture 4 times using the above procedure, and then we were able to bring down the WT background to more acceptable 40-50% from 95%.

3.2.3 Cell transformation

Supercompetent DH5α *E. coli* cells were prepared from a method modified from Inoue et al. (1990) where we used SOC media instead of SOB media. About 50 μL of these cells were thawed on ice and 1 μL of Dpn-I treated DNA was transferred to this tube, and it was incubated on ice for 30 min. This was heat pulsed for 45 seconds at 42 ^{O}C, and was then placed on ice for 2 min. SOC media (250 μL) was added to this and this was incubated at 37 ^{O}C for about 1 hr with shaking at 180 rpm. A 50 μL aliquot was plated on an agar plate. It was observed that if higher volume was used then the density of colonies on the plate was too high, and it was almost impossible to pick them out for later steps. This plate was grown overnight at 37 ^{O}C, and then it was stored for up to 2 months at 4 ^{O}C for later use.

3.2.4 Screening of colonies

The agar plate prepared in the earlier step was taken, and a colony was picked using a 10 µL micropipette tip, and that was transferred into a 10 ml tube containing LB media with ampicillin (100mg/L) in it. This was grown overnight and about one ml of this grown culture was taken and a glycerol stock (50:50 v/v) was prepared. This was stored in a -78 ^{0}C freezer, hence in the case of a positive hit, we will have both the plasmid as well as a culture of the mutant. Plasmid prep was subsequently performed on the remaining amount of culture using a Qiagen plasmid prep kit. We also used Invitrogen kit for about one third of our samples, but often their kit didn't yield enough plasmids for us to proceed further, and hence we switched back to Qiagen. The Plasmids prepared were then run using DNA gel which was made by taking 1% agar (0.5gms) in 50 ml 1% TAE solution, it was heated until agar was melted, and 5 µL ethidium bromide was added to this, and it was left to set. The samples were prepared by taking 10 µL plasmid and 2 µL dye, and for making DNA ladder sample we added 1 µL DNA ladder concentrate, 4 µL water, 1 µL dye. This samples were pipetted into gel wells and the gel was run for about 1.5 hours at 70 V. The gel was visualized under a UV lamp, and the bands from each plasmid prep were noted. The ones which we were able to see were then sent for sequencing. We screened about 160 colonies using the method outlined above.

3.2.5 Growth of Cells

Cell cultures from each of the positive hits were taken and grown in a 10 ml LB media

overnight at 37 °C with shaking at 180 rpm. This was subsequently used as a preculture inoculum, and was transferred to 400 ml LB media, where the cells were grown overnight at 37 °C with shaking it at 180 rpm. Cells were collected by centrifugation at 4000 rpm for 15 min.

3.2.6 Purification of Secondary Alcohol Dehydrogenase

The wet cells were resuspended in 10 ml of 50 mM Tris HCl at pH 8.0 (buffer A). The cells were then lysed by sonication at 0 °C in three 3-min intervals. Cell debris was removed by centrifugation at 4000 rpm for 1 hr 50 min. The resulting supernatant was incubated at 70 °C for 15 min and centrifuged at 4000 rpm for 1 hr 50 min to remove the thermally denatured impurities. The crude supernatant solution of SADH was applied to a 15 mL Red Agarose column that had been regenerated, pre-equilibrated and washed with buffer A. Impurities were eluted with buffer A containing 0.01 M $NaClO_4$, followed by elution of SADH fractions with buffer A containing 0.2 M $NaClO_4$. The eluted SADH solution was then concentrated by ultrafiltration. This was stored at -77 °C for several months without loss of activity, and was used in the subsequent kinetics studies. Protein concentration was determined by Bradford (1976) method.

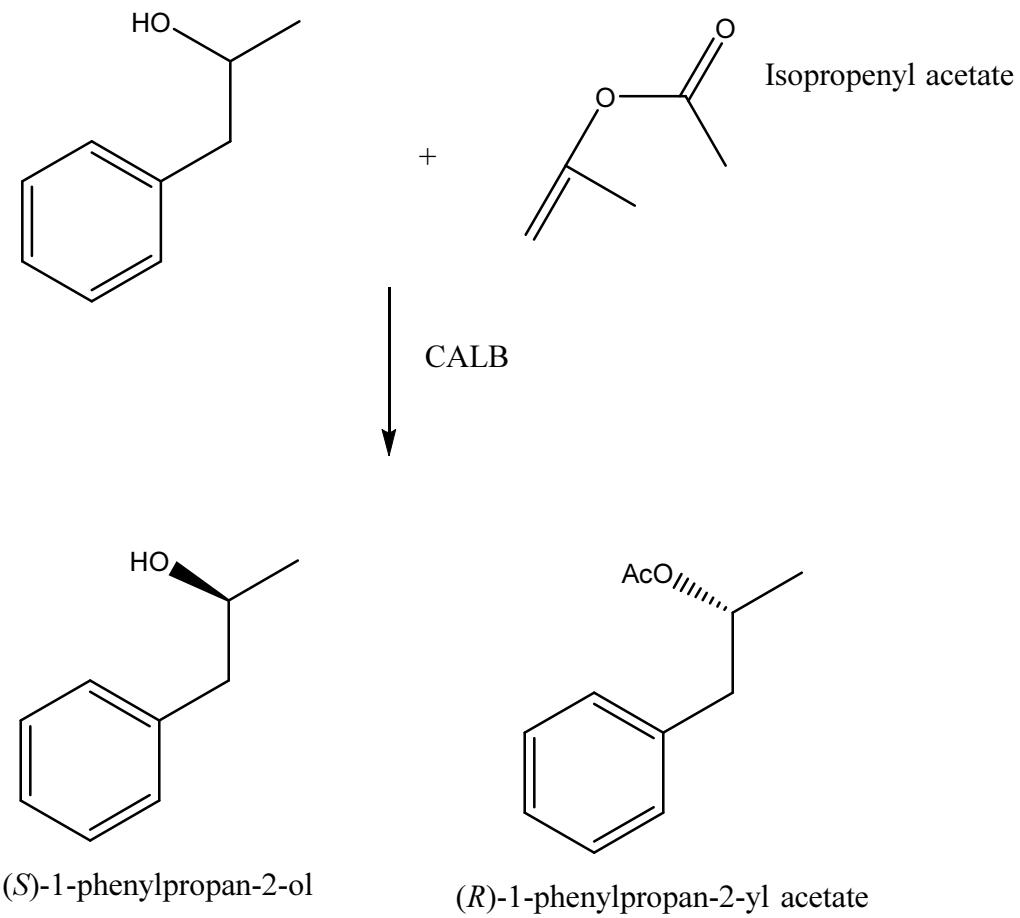
3.2.7 Regeneration of Red Agarose column

The Red Agarose column was regenerated was washing it at a flow rate of 1 ml/min with two column volumes each of 4M Guanidine.HCl solution, distilled deionized water, 4M

NaCl solution, distilled deionized water, and lastly, buffer A.

3.2.8 Kinetic resolution of 1-phenyl-2-propanol

Enantiomerically pure (R) and (S)-1-phenyl-2-propanol was obtained via *Candida antarctica* lipase B (CALB) catalyzed kinetic resolution of racemic alcohol with isopropenyl acetate (1.5eq). The reaction was carried out at 1 gm scale in about 12 ml toluene and about 80 mg CALB (**Scheme 2**). The reaction was monitored on a chiral GC, and after 8 days, the reaction was quenched by filtering out the immobilized CALB, and S-alcohol and R-ester were separated using column chromatography. The (R)-ester was then subsequently hydrolyzed by 4 eq KOH in 1:1 water/methanol to get the R-alcohol. These were subsequently used as substrates for all the kinetic experiments.



(*S*)-1-phenylpropan-2-ol (*R*)-1-phenylpropan-2-yl acetate

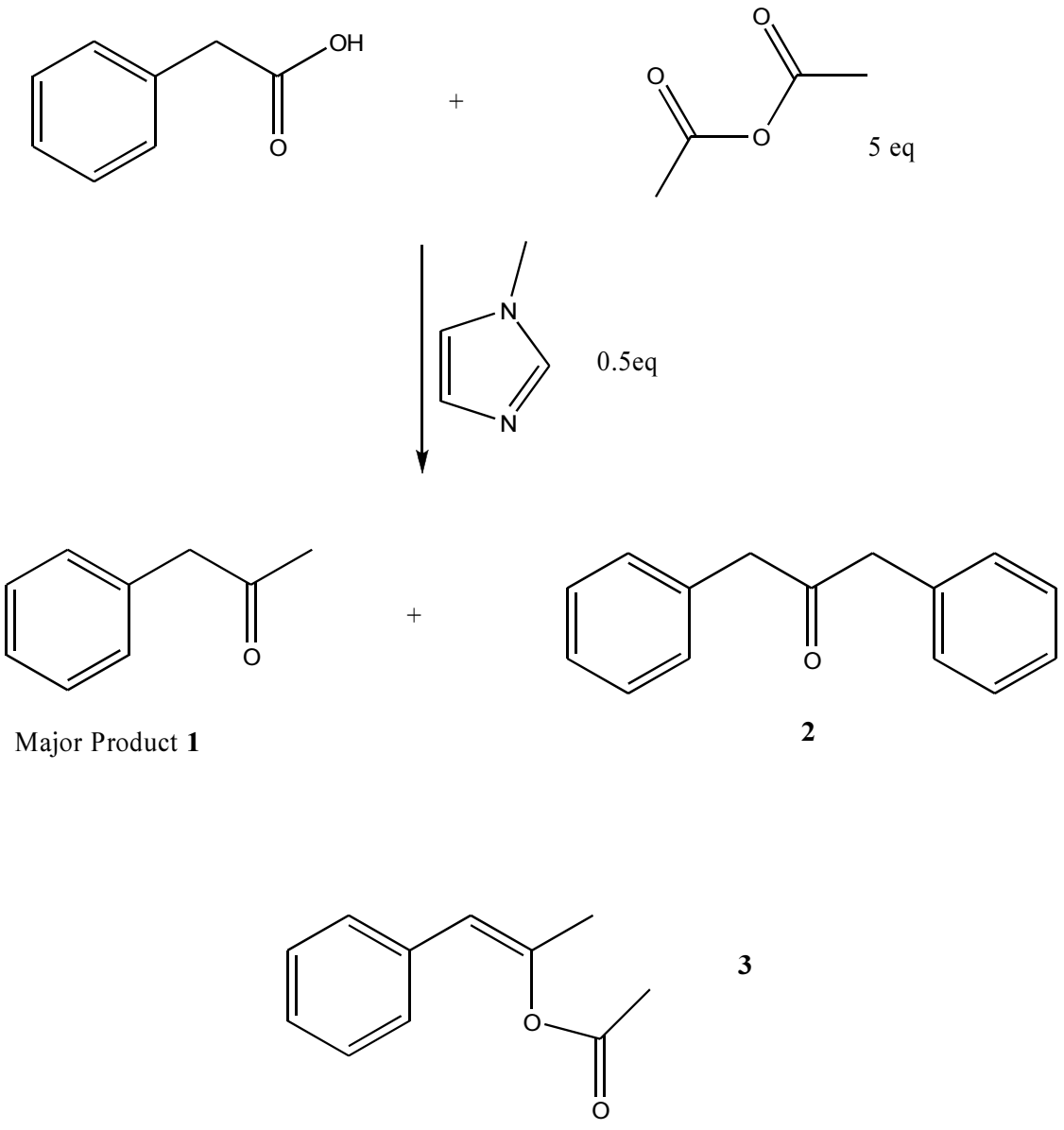
Scheme 2

3.2.9 Synthesis of phenylacetone

Phenylacetone was synthesized using the Dakin-West synthesis procedure as modified by Tran and Bicker (2006). The reaction (**Scheme 3**) done on 5 gm scale was run overnight and was quenched by adding 10 ml water to hydrolyze acetic anhydride, and it was extracted with ethyl acetate (3 x 50 ml), and the extracts were then washed with sodium bicarbonate (2x50ml) followed by water (2x50 ml) and it was dried over sodium sulfate, filtered, and evaporated. We got about 50% conversion to **1**, with other products formed being **2** and **3**. The purification of **1** proved challenging because there were numerous

other products being formed which all were running very close on TLC, and **1** and **3** ran together on TLC in spite of trying out all the possible solvent mixtures. We decided to do a short path vacuum distillation with a small packed column because the boiling points on them were substantially different.

Scheme 3



3.2.10 k_{cat}/K_m determination for oxidation of (R) - and (S)-1-phenyl-2 propanol

Reaction rates were determined by taking cuvettes containing 0.4 mM NADP, 50 mM Tris buffer pH 8.9, 0.33 mM – 4 mM (R)- and (S)-1-phenyl-2 propanol at 50 ºC in a total volume of 0.6 ml. The cuvettes were incubated for at least 15 min before SADH was added to initiate reaction. The reaction rates were measured by following the production of NADPH spectrophotometrically at 340 nm. Values of k_{cat} and k_{cat}/K_m were calculated for each enantiomer by doing at least three repetitions.

3.2.11 Reduction of phenylacetone by mutant SADH

Reaction mixtures were prepared in 50 mM Tris buffer pH 8.0, 5 mg NADP, 0.5ml 2-propanol, 0.35mg SADH, 5 µL phenylacetone in final reaction volume of 10 ml. The total reaction time was 6 hours and the product was extracted with methylene chloride, dried over sodium sulfate and then evaporated in vacuo to get the (S)-alcohol. This was then acetylated with acetic anhydride and pyridine (1:3) for the reaction time of one hour. The resulting product was injected in a chiral GC and e.e. was subsequently calculated. The GC parameters were:

Initial column temperature	90 ºC
Initial column hold time	25 min
Final column temperature	170 ºC
Column rate	3 ºC/min
Column hold time	50 min
Injector temperature	200 ºC
Detector temperature	250 ºC
total time	101.66 min

3.3 Results and discussion

We screened about 160 colonies for mutations from the site saturation mutagenesis and from them we isolated 9 new mutants. All of them were active with 1-phenyl-2-propanol except W110R, W110S, and W110F. We took those six active mutants and purified SADH from them, and subsequently did the enzyme kinetics studying the oxidation of (R) and (S) 1-phenyl-2-propanol. We obtained a total protein concentration of about 1.7 mg/ml – 2.2 mg/ml by Bradford (1976) method. In order to correlate the enantiomeric ratio (E) values to e.e., we also did reduction of phenylacetone and then analyzed that with chiral GC to get the e.e. The results are shown in **Table 2**. We obtained complete conversion in our reduction reaction, and the e.e. from that corresponded very well to the E values. All of the mutants, except W110G gave E value >10 and >99.9% e.e. and the best mutant SADH was found to be W110V which gave an E of 134.5 and its k_{cat}/K_m for (S) alcohol was about 45300 showing that it's binding similar to natural substrates. We did docking studies using Autodock Vina, and we found that phenylacetone docks closer to site 110 and Zn^{2+} in the mutants, however for wild type SADH it stays further away from site W110 because probably the large size of tryptophan doesn't allow it to go in. This is consistent to the results we got here because it is already known that phenylacetone is not a substrate for wild type SADH (Musa et. al., 2007), whereas it is a substrate for the other mutant SADHs.

Table 2: k_{cat}/K_m values for oxidation of enantiomers of 1-phenyl-2-propanol with mutant SADH and e.e. for reduction of phenyl acetone.

Mutant	Enantiomer	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	E = $(k_{cat}/K_m)_S$ / $(k_{cat}/K_m)_R$	e.e. (%)
W110I	S	18.8 ± 1.9	15200 ± 2300	80.3 ± 16.2	>99.9
	R	0.46 ± 0.05	188 ± 25		
W110Q	S	2.3 ± .2	551.4 ± 31.2	80.0 ± 17.5	>99.9
	R	.025 ± .006	6.9 ± 1.5		
W110M	S	4.5 ± 0.4	1990 ± 230	16.3 ± 3.5	>99.9
	R	0.045 ± .003	121.0 ± 22.1		
W110V	S	38.6 ± 3.06	45300 ± 4500	134.5 ± 27.7	>99.9
	R	1.2 ± .3	336.5 ± 61.0		
W110G	S	17.8 ± 3.1	5800 ± 940	9.02 ± 2.6	79
	R	1.4 ± .3	639.0 ± 149.0		
W110L	S	0.65 ± .03	2510.0 ± 560	104.4 ± 42.1	>99.9
	R	.0080 ± .00081	24.0 ± 9.0		

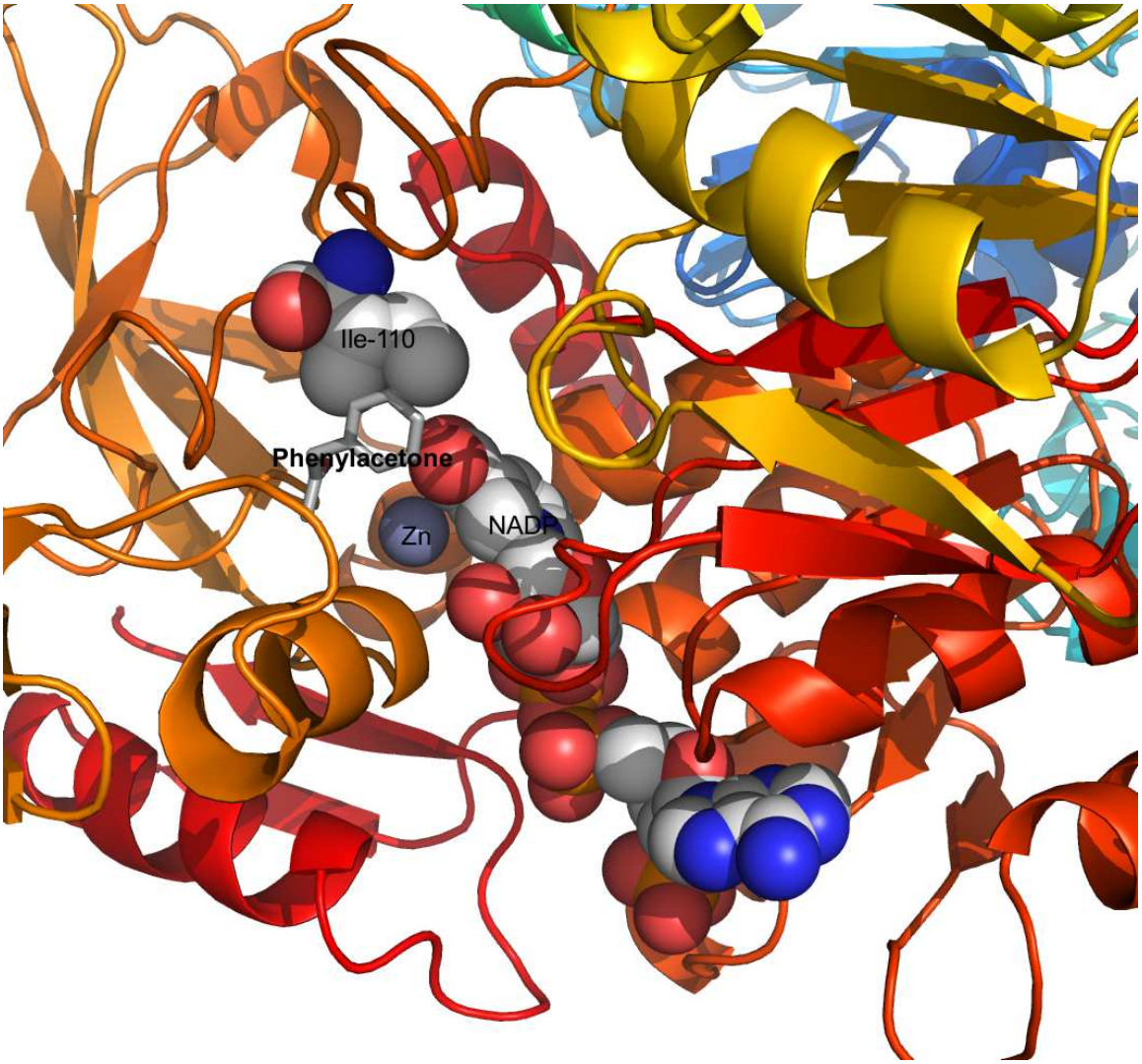


Figure 2: Docking Phenylacetone to W110I SADH using Autodock Vina on holoenzyme form of *Thermoanaerobacter brockii* ADH (PDB: 1YKF)

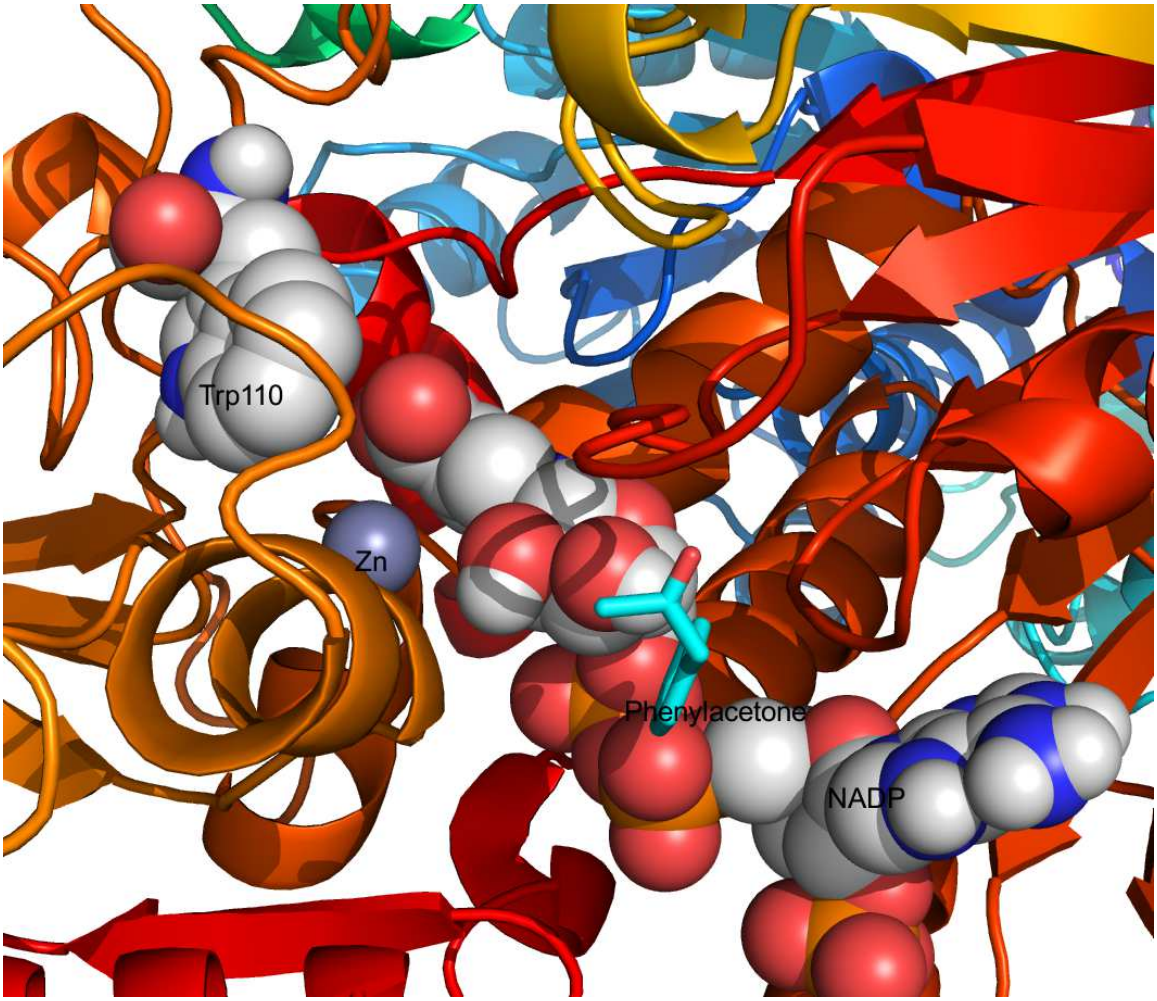


Figure 3: Docking Phenylacetone to Wild Type SADH using Autodock Vina on holoenzyme form *Thermoanaerobacter brockii* ADH (PDB: 1YKF)

3.4 Conclusions

SADH from *T. ethanolicus* has been shown as a very good biocatalyst with high stereoselectivity with broad substrate acceptability and high thermostability. In this paper, we use site saturation mutagenesis to create a library of mutant SADH at site W110 and we were able to successfully get six very interesting mutants, five out of them gave 99.9% e.e., all of which represent immense improvement over previously reported mutant SADH, W110A; which reduced the same substrate with 37% e.e. One of our new mutant SADH, W110V shows extremely high E and k_{cat}/K_m values of 134.5 and 45300 respectively for (S)-1-phenyl-2-propanol. I think this mutant SADH library will prove to be extremely useful in future, and it will allow to further optimization of the SADH by doing more mutations at other amino acid residues, making it even more attractive for synthetic utility.

3.5 References

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CHAPTER 4: CONCLUSIONS

The focus of this thesis was to study the effects of physical conditions like temperature and pressure on enantiospecificity of mutant SADH, and subsequently create new SADH mutants so as to further improve the stereospecificity.

In chapter 2, we showed that SADH is highly stable under pressures over 150 MPa, and no loss of activity was observed under experimental conditions. We observed slow increase in the comparative preference for (S) isomer with 2-butanol and 2-pentanol with increase in pressure. We are pleased to show that under high pressure conditions, the enantiomeric ratio at 137.5 MPa can be enhanced by 13.5 fold compared to 3.9 fold at room temperature and pressure for (S)-2-hexanol over (R)-2-hexanol. This direct correlation of stereospecificity with hydrostatic pressure is just a preliminary step in enhancing the utility of our library of SADH in synthesis of enantiopure alcohols. We believe that this is the first paper which has shown a strong correlation between stereoselectivity and pressure, and this potentially makes this system even more attractive not only for small scale lab scale reductions but also for doing it on industrial setting.

In chapter 3, we use site saturation mutagenesis to create a library of mutant SADH at site W110 and we were able to successfully get six very interesting mutants, five out of them gave 99.9% e.e., all of which represent immense improvement over previously reported mutant SADH, W110A; which reduced the same substrate with 37% e.e. One of our mutant SADH, W110V shows extremely high E and k_{cat}/K_m values of 134.5 and 45300 respectively for (S)-1-phenyl-2-propanol. I think this mutant SADH library will prove to be extremely useful in future, and it will allow to further optimization of the

SADH by doing more mutations at other amino acid residues, making it even more attractive for synthetic utility.