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Towards the use of Alcohol dehydrogenases as biocatalysts for stereoselective isotope labeling of aromatic alcohols.

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Abstract

The enzyme ADH-A and one of its mutants ADH-A C1B1 from *Rhodococcus ruber,* have in previous studies been proved to act as proper biocatalysts, fully capable of performing redox reactions. Two redox reactions were studied during this project, were those enzymes act as catalysts. For that matter, ADH-A wild type and ADH-A C1B1 genes were expressed in *E. coli* and the encoded enzymes were purified and used for kinetic studies with a final goal on studying the kinetic isotope effect that is generated between them and the molecules that contain deuterium. HPLC analysis on these products showed that the reactions were not thermodynamically favored and conclusions on the best reaction conditions for both enzymes as well as for further improvements are discussed.

Contents

Abbreviations

ACN	Acetonitrile
ADH	Alcohol dehydrogenase
E. coli	Escherichia coli
HPLC	High Performance Liquid Chromatography
IMAC	Immobilized Metal Ion Affinity Chromatography
LB	Lysogeny broth
MeOH	Methanol
NAD	Nicotinamide Adenine Dinucleotide
NMR	Nuclear Magnetic Resonance
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
Wt	Wild Type
2TY	2x Tryptone Yeast extract, microbial growth medium

1. Introduction

1.1 Purpose

The aim of this project was to study the enzyme alcohol dehydrogenase A, ADH-A,which catalyses the reduction of ketones in their corresponding alcohols .[1] Two reactions were studied in parallel for ADH-A wild type and ADH-A C1B1, a F43H, Y54L double mutant (further discussed in 1.3): one where acetophenone acted as a substrate together with isopropanol and one where 2-hydroxy-1-phenylethanone did (Figure 1).

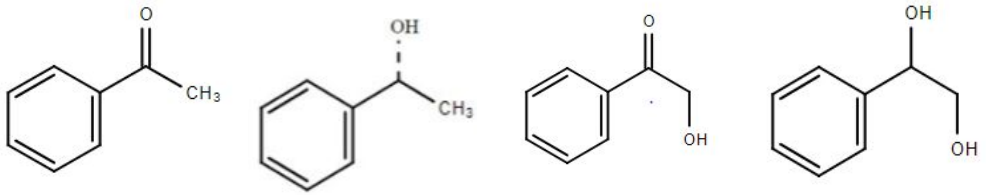

Figure 1: Structures of acetophenone, 1-phenylethanol, 2-hydroxy-1-phenylethanone and 1-phenyl-1,2-ethanediol from the left side to the right side.

In both cases isopropanol was present, minimising the cost of the chemicals needed for the study and contributing to relatively greener conditions for the reactions to occur (further discussed in 1.2).

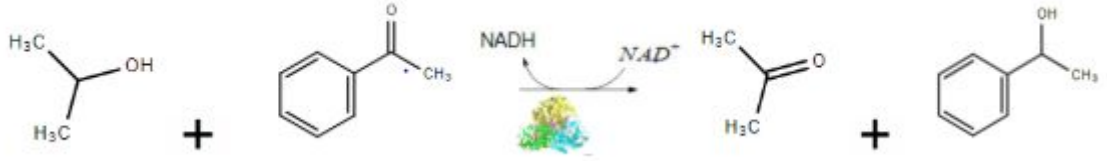

Figure 2: Reaction 1. Reduction of acetophenone to 1-phenylethanol following reduction of the cofactor NAD^+ by isopropanol.

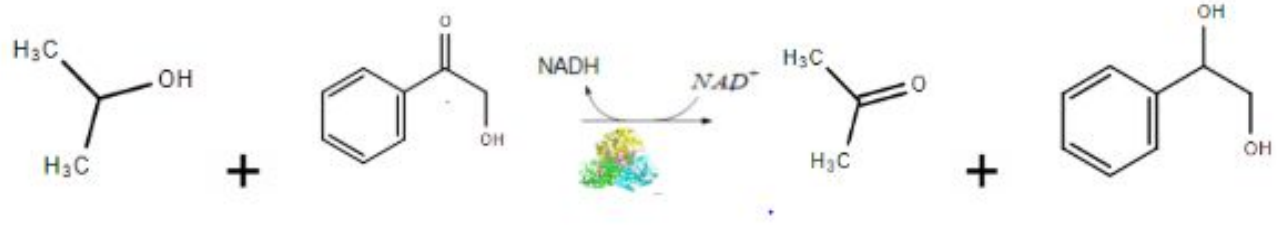

Figure 3: Reaction 2. Reduction of 2-hydroxyacetophenone to 1-phenyl-1,2-ethanediol following reduction of the cofactor NAD^+ by isopropanol.

In both cases, a redox reaction is carried out, where an alcohol is oxidised to its corresponding ketone and a ketone is reduced to its corresponding alcohol. The main focus of this study has been on the

reactions presented in Figures 2 and 3, towards the formation of 1- or 1,2-substituted (vicinal) alcohols.

Looking closely on reaction 1 and splitting it into two half-reactions (Figures 4 and 5), catalysed by the same enzyme, it is worth to take two aspects into consideration. Those aspects are vital for the enzyme and therefore for the upcoming study and are described below.

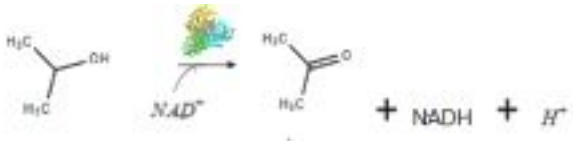

Figure 4: Reaction A. Oxidation of isopropanol to acetone,catalysed by ADH-A wt or ADH-A C1B1 upon an electron transfer by the cofactor NAD^+ to its reduced form, NADH

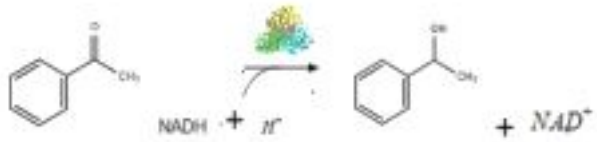

Figure 5: Reaction B. Reduction of acetophenone to 1-phenylethanol by ADH-A wt or ADH-A C1B1 upon electron transfer by the cofactor NADH, to its oxidized form, NAD^+.

According to previous studies where the enzyme's catalytic parameters k_{cat} and K_M were determined, the reduction of acetophenone is a much easier reaction for the enzyme than the oxidation of isopropanol is. The specificity constant $\frac{k_{cat}}{K_M}$ for the reduction of acetophenone is almost 10 fold higher than that of the oxidation of isopropanol ($30 * 10^3\ M^{-1}\ s^{-1}$ and $4.2\ 10^3\ M^{-1}\ s^{-1}$ respectively).[1] NADH is produced only during the first half-reaction, making the second half-reaction fully dependent on it and the overall reaction (1) particularly interesting.

The goal is to study the kinetic isotope effect, (Figure 6) and therefore the determination of the right concentration of the starting material needed that will give the highest yield of 1-phenylethanol,will be in focus during this study. In this way, the lowest concentration of isopropanol with deuterium will give the highest concentration of 1-phenylethanol with deuterium (Figure 7).

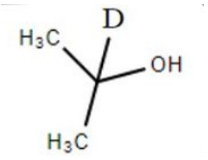

Figure 6: Isopropanol, where α-carbonyl hydrogen is exchanged with deuterium

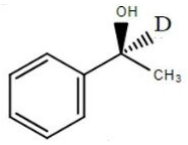

Figure 7: 1-Phenylethanol, where α-carbonyl hydrogen is exchanged deuterium.

After this study, information about the mechanism of the overall reaction (1 or 2) will be obtained and hopefully the rate limiting step of those reactions will be possible to be studied closely.

1.2 Biocatalysis, Green Chemistry and the role of ADH-A

Redox reactions, described in 1.1, are carried out by enzymes as biological catalysts, like ADH-A, under environmentally friendly and energy-saving conditions. Enzymes, after expression and purification are readily available to catalyse such reactions. The use of biological catalysts is one of the principles of green chemistry according to which, a molecule that is both selective about which substrate it can transform to the desired product and also capable of reducing the reaction steps of that particular reaction, is to be preferred over a stoichiometric reagent. In this way, side products of a reaction can be avoided, the activation energy of the reaction is lowered and thereby the reaction rate is enhanced .[2] The environment of the reaction becomes a non-toxic one, the temperature is much lower and water can be used as a solvent.

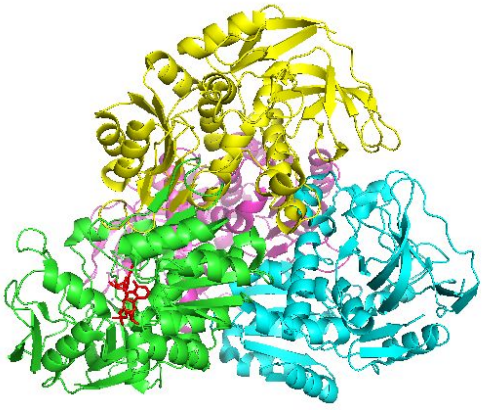

Figure 8: Structural representation of ADH-A wild type (PDB 3jv7)[1] generated in Pymol. Each subunit is marked with a different colour. The enzyme's cofactor NADH is marked with sticks in red colour.

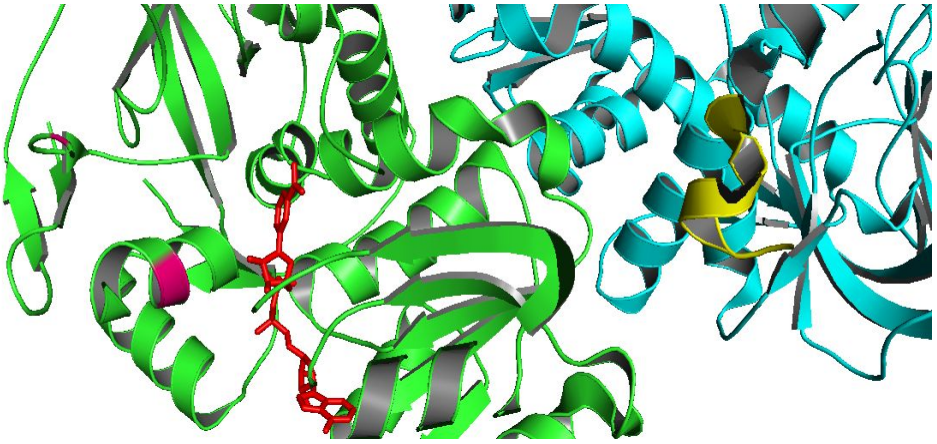

Figure 9 : Close up of the active site of ADH-A C1B1 with both mutations F43H and Y54L (pdb: 6ffz)[1] (marked with pink) generated in Pymol. The enzyme's cofactor NADH is marked with sticks in red colour.

Alcohol dehydrogenase A from the bacterium *Rhodococus ruber* DSM 44541 is an enzyme capable of conducting redox reactions.This enzyme is dependent on the cofactor Nicotinamide Adenine Dinucleotide (NAD^+), which during the reaction is reduced to the corresponding NADH upon an electron transfer from the substrate containing a hydroxyl group. This electron transfer occurs due to

the presence of a zinc atom in the active site of the enzyme, which polarises the carbonyl oxygen of the ketone .[3]

In previous studies the enzymes tolerance towards organic solvents has been highlighted ,[1] as well as the fact that aryl-substituted compounds can act as enzymes substrates ,[4] making the acetophenone and 2-hydroxy,1-phenylethanone (Figure 1) two perfectly suitable substrates. It has been shown that the enzyme is both regioselective, favoring secondary over primary alcohols, and also stereoselective .[1]

In this study, wild type ADH-A as well as the mutant ADH-A C1B1 were studied. Site-directed mutagenesis is a technique that has been performed during earlier laboratory work, according to which, specific amino acids in the active site of the enzyme were replaced to another, giving the enzyme a modified local structure and most importantly, different activity .[4] In the case of ADH-A C1B1, the mutations in the active site were F43 → H43 and Y54 → L54 (Figure 9). Having studied the stereoselectivity of these two enzymes, the wild type and the mutant C1B1, they have been proven to favor (S)-1-phenylethanol (Figure 1) and (R)-1-phenylethanethiol, the corresponding 2-hydroxy derivatives, as substrates .[1]

1.3 Protein Purification

In order to purify a recombinant protein several techniques can be used, the choice of which depends on the different features of the protein of interest. In the limits of this study, affinity chromatography will be used, a purification technique that exploits the distinct distribution of proteins between a stationary and a mobile phase. In the stationary phase several non-porous beads with a ligand will interact with the protein flowing through them in the mobile phase. The interaction will occur because of the special affinity that the protein has to this ligand .[5] IMAC, a specific type of affinity chromatography, will be used in this case, where the stationary phase will consist of beads with nickel(II) nitrilotriacetic acid (NTA) complexes attached to them. These will bind the Histidine tag of the protein ADH-A (attached on the C-terminus of the protein), letting non-Histidine-tagged proteins pass through .[6] Eventually the desired protein ADH-A will be eluted from the column, when the interaction between the column and the bound protein is outcompeted with an other, stronger interaction. Therefore an Elution Buffer that contains Imidazole will be used at the final steps of the purification process (Appendix 1, Table 4).

1.4 Kinetics

Kinetic measurements can be performed in order to determine an enzyme's activity during a reaction as well as in order to study the time course of a reaction. Two components of reaction 1, the starting material for reaction 1, acetophenone and the reduced form of its cofactor, NADH, absorb light in different wavelengths (244 nm and 340 nm, respectively)[6 7] and therefore the absorbance of those two components can be measured with a spectrophotometer. In this way the reaction taking place can be followed and the rates calculated .[8]

1.5 HPLC

HPLC, High Performance Liquid Chromatography, is a technique used for separating and quantifying the different components of a sample. In this study, HPLC will be used in order to quantify the amount of the starting materials acetophenone and 2-hydroxyacetophenone (Figure 1) that were transformed into the products 1-phenylethanol and 1-phenyl-1,2-ethanediol (Figure 1).

Since ketones and alcohols differ in polarity, a reversed phase HPLC was performed using a hydrophobic stationary phase and a C18 column .[9] Because of this difference in polarity between the components that were to be separated, a gradient program with a final mixture of 30% (v/v) to 80% (v/v) methanol and 50 mM sodium phosphate, pH 3.0, was used .[9] In this way, well-separated peaks within a reasonably long elution time will be obtained .[10] Because of the difference in polarity, the least hydrophobic-most polar alcohol is expected to elute from the column first and the most hydrophobic-least polar ketone last.

Reaction 1 was analysed in a small scale in order to find the most effective reaction conditions for the reaction to occur. The same study was performed at a bigger scale, using higher amounts of the starting material for both reactions 1 and 2.

2. Experimental

Recipes for the buffers, the gels and growing media that were used during the experimental part of this project, are presented in Appendix 1, page 20.

2.1 *Enzyme expression and purification*

Plasmids encoding for ADH-A wild type and ADH-A C1B1 were transformed in electrocompetent *E.coli* BL21-AI pREP4 cells with electroporation.
positive samples: 600 µL of *E.coli* BL21-AI cells were mixed with 1 mL 2TY Media and 600 µL of plasmid ADH-A wt or plasmid ADH-A C1B1.
negative sample: 600 µL of *E.coli* BL21-AI cells were mixed with 1 mL 2TY Media and 600 µL H_2O. The applied voltage was 1.25 kV.

These solutions were afterwards incubated for 1 h and then, after a serial dilution of 10-, 100- and 1000- fold, applied on LB plates. The LB Media contained 250 µL ampicillin of a stock sample of 100 ug/mL and 250 µL kanamycin of a stock sample of 30 ug/mL. After 17 h of incubation in 37 °C, colonies were successfully grown on the plates (1600 on 10x C1B1, 420 on 100x C1B1, 66 on 1000x C1B1, uncountable on 10x and 100x wt and 720 on 1000x wt).

Over day (o/d) culture: One colony from a LB plate with ADH-A wt and one form a LB plate with ADH-A C1B1 were transferred to 2 mL culture tubes and mixed with 2 mL 2TY media, 2 µL ampicillin of a stock sample of 100 mg/mL and 2 µL kanamycin of a stock sample of 30 mg/mL and were incubated at 30 °C, 200 rpm for 18 h.

Overnight (o/n) culture: 35mL from the (o/d) cultures were transferred to 35 mL pre-autoclaved 2TY media in E-flasks for ADH-A wt and ADH-A C1B1 respectively. 35 µL ampicillin of a stock sample of 100 mg/mL and 35 µL kanamycin of a stock sample of 30 mg/mL were also added in both of them and the E-flasks were incubated at 30 °C, 200 rpm for 18 h.
750 mL cultures: 7.5 mL from the (o/n) cultures were transferred to 750 mL pre-autoclaved 2TY media in E-flasks for ADH-A wt and ADH-A C1B1 respectively. 375 µL ampicillin of a stock sample of 100 mg/mL and 375 µL kanamycin of a stock sample of 30 mg/mL were also added in both of them and the E-flasks were incubated at 30 °C, 200 rpm for 5 h. 7.5 mL L-arabinose and 750 µL 1M IPTG were added in each E-flask. The E-flask were afterwards incubated at 30 °C, 200 rpm for 18 h.

The cultures were then transferred to centrifuge bottles and centrifuged at 5000 g for 15 min with a JA-14 rotor. Since the volume of the cultures for both ADH-A wt and ADH-A C1B1 was 750 mL, the centrifugation step was repeated three times where the pellet was kept after each centrifugation and the supernatant was discarded. 50 mL of lysis buffer was then added to each of the pellets until they were completely resuspended. Sonication was performed afterwards on the pellets in order to resuspend the cells and then they were centrifuged at 15 000 rpm, 4 °C with a JA-25.50 rotor for 60 min. The lysates, the supernatant of this last centrifugation, were to be used for the rest of the protein purification procedure.

A $NiCl_2$ buffer was prepared. This buffer was equilibrated in an empty gravity flow column with binding buffer and H_2O and eventually added as a gel form into the lysates. They were then incubated at 4 °C on a vipping table for 60 min. 25 mL H_2O was then added to the lysate-gel solution, that were also centrifuged until the the pellets were totally resuspended. The lysate-gel solution was then transferred to a PD-10 column, that had been equilibrated with washing buffer in advance (Appendix 1, Table 6), and the solution was left to flow through it. The flow through was collected in a 50 mL falcon tube and the column was washed three times with washing buffer (the flow throughs were collected similarly). Elution buffer was then added to the column and the flow throughs were collected this time as well in 15 mL falcon tubes. The two last named flow throughs were then mixed together in concentration tubes, concentrated to 2.5 mL and lastly, centrifuged for 15 min. They were then desalted, by being first added in the column and then eluted with elution buffer (Appendix 1, Table 4) into the *Final* 15 mL falcon tube.

The final volume of both ADH-A wt and ADH-A C1B1 was approximately 5 mL.

Eventually, samples of each fraction of the purification procedure were analysed with SDS-PAGE electrophoresis. 2 µL of each solution were mixed with 8 µL H_2O and 10 µL SDS buffer.10 µL of each of the resulting samples (Lysate, Pellet, Flow through, Wash 1, Wash 2, Wash 3, Elution 1, Elution 2 and Final) were then loaded on the stacking gel and a voltage of 200 V was applied for 55 min for the SDS-PAGE electrophoresis analysis. The gels were prepared in advance and destaining buffer was used afterwards in order to destain the proteins from them.

2.3 Kinetic Measurements

Since the catalytic parameters of the enzyme were already determined in previous studies ,[1] the focus here was on ensuring that reaction 1 takes place and also on following the reaction over time.

A reaction sample with 1.0 mM acetophenone, 1.2 mM isopropanol, 0.2 mM NAD^+ and 0.8 μM of ADH-A wt in sodium phosphate Buffer 0.1 M, pH 8, 10 μL $ZnSO_4$ were added in a 1.0 mL cuvette. The absorbance was measured at three different wavelengths, at 244 nm for the starting material acetophenone and at 340 nm for the reduced form of the cofactor, NADH. Sodium phosphate Buffer 0.1 M, pH 8, 10 μL $ZnSO_4$ was kept in 30 °C and the cofactor NAD^+ together with the enzyme ADH-A wt were kept on ice before their addition to the reaction solution. The measurements were performed every 5 min during 3 h and 10 min. The same study was performed using 0.8 μM ADH-A C1B1 as the reaction catalyst this time and absorbance measurements were done at the same wavelengths every 5 min during 2 h and 5 min. In this way the time course on the reaction could be studied and conclusions about the different parameters of the reaction could be made.

2.4 HPLC

The study on reactions 1 and 2 was to be expanded by the use of one more method, the instrumentation of which was available in the lab and therefore a HPLC analysis was performed. Reaction samples with either enzymes ADH-A wt and ADH-A C1B1 were analysed by analytical reverse phase chromatography, using a C18 column (Ascentis). The flow-rate was 0.5 ml/min and 3 or 5 μL in small scale and 10 μL in big scale of centrifuged and filtered samples were injected. The compounds were detected at 220 nm with a diode array detector SPD-M20A from Shimadzu.

Small scale: 10 mM isopropanol and 1.0 mM acetophenone mixed with 0.2 mM NAD^+ and 3.0 μM ADH-A wt and ADH-A C1B1, respectively, in glass vials of 2.0 mL total volume. Same set up of reaction samples was performed with 20 mM isopropanol this time.Measurements were repeated with changing the concentration of isopropanol, [1.0, 2.0, 3.0, 4.0, 5.0 and 20.0 mM] without modifying the concentration of acetophenone, the enzyme and the cofactor. Before the analysis, the samples were transferred to Eppendorf tubes and centrifuged for 10 min at 13,300 rpm 30 °C and then filtered in filtration devices in order to precipitate the enzyme. Afterwards an amount of 200 μL of the supernatant was filtered and added to HPLC vials. Those measurements were performed after 3 and 7 h of reaction time during which the reaction vials were left with a magnetic stirrer and wrapped in foil.

Large scale: 300 mM isopropanol and 200 mM acetophenone mixed with 0.2 mM NAD^+ and 4.0 μM ADH-A wt or ADH-A C1B1, respectively, in glass vials of 10.0 mL total volume. Similarly, 200 mM 2-hydroxyacetophenone were mixed with 300 mM isopropanol and the same amount of the cofactor and the enzyme, reaction 2. An addition amount of isopropanol (780 μL) was added in all reaction samples, after 3 h, making the final concentration of isopropanol to 13.6 M. The samples were transferred to Eppendorf tubes and centrifuged for 10 mins at 13,300 rpm 30 °C, then filtered in filtration devices in order to precipitate the enzyme and finally 20 μL of them were added to HPLC vials, diluted in Methanol (10 dilution factor).

Reaction 1 was studied also separately, when 10mM acetophenone was added over time into a reaction solution containing 300 mM isopropanol, 4.0 μM of the respective enzyme and 0.2 mM NAD^+ (10 additions in total, until 100 mM of final acetophenone concentration). The reaction vials were incubated in 30 °C and samples of 200 μL were collected every 20 min before each addition of

10 mM acetophenone. Those samples were centrifuged at 13,300 rpm, filtered and diluted afterwards in methanol (20 dilution fold).

Table 1: Preparation of samples in the study 10 mM acetophenone added every 20 min., both for ADH-A wt and for ADH-A C1B1.

Total volume (µL)	Acetophenone concentration (mM)	Added Volume of Acetophenone (µL)
5000	10	50
4896	20	96
4837	30	141
4822	40	185
4853	50	231
4932	60	279
5063	70	331
5252	80	389
5507	90	455
5962	100	530

3. Results and Discussion

3.1 Protein Expression and purification

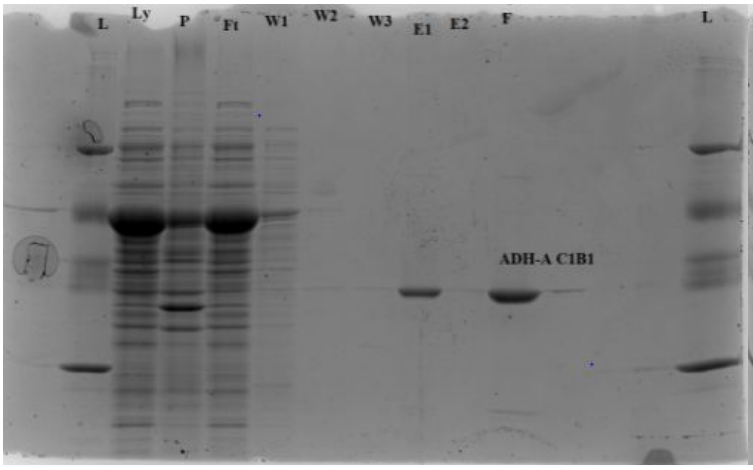

Figure 10: SDS-PAGE electrophoresis on the different fractions of ADH-A C1B1 purification. The different fractions stated with the abbreviations on the top of each column are the following: (L) Ladder, (Ly) Lysate, (P) Pellet, (W1, W2, W3) Wash 1, 2, 3, (E1, E2) Elution 1, 2 and (F) Final.

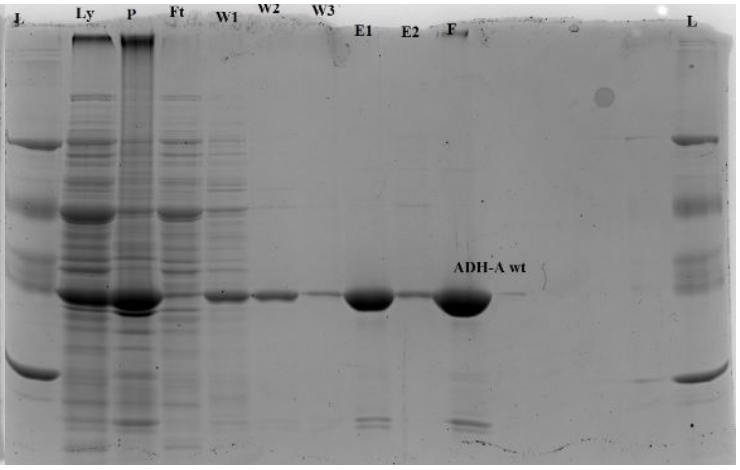

Figure 11: SDS-PAGE electrophoresis on the different fractions of ADH-A wt purification. The different fractions stated with the abbreviations on the top of each column are the following: (L) Ladder, (Ly) Lysate, (P) Pellet, (W1, W2, W3) Wash 1, 2, 3, (E1, E2) Elution 1, 2 and (F) Final.

After protein purification, SDS-PAGE analysis was done on the different fractions, in order to ensure that the desired proteins were purified and present in the final fractions.

The purification was successful for both ADH-A wt and ADH-A C1B1 (Figures 10 and 11) and the Absorbance of light of them at 280 nm was measured on Shimadzu spectrophotometer to A_{280} = 0.626 for ADH-A wt and A_{280} = 0.071 for ADH-A C1B1.

$A = c * \varepsilon * l$ Beer's Law *(Equation 1)*

Using Equation 1, the calculated concentrations are $c_{ADH-A\,wt} = 199$ μM and $c_{ADH-A\,C1B1} = 24$ μM , where the molar extinction coefficient ε, calculated from amino acid compositions is $\varepsilon_{ADH-A\,wt} = 31517\ M^{-1}\ cm^{-1}$ and $\varepsilon_{ADH-A\,C1B1} = 30097\ M^{-1}\ cm^{-1}$,the light pathway distance, l, was 1 cm for the cuvettes used and the dilution factor in the cuvettes for the measurements was 10. The exact volume of each final protein fraction was unfortunately not noticed and therefore the amount of the protein variants that were obtained can not be calculated. The concentration of the proteins was not particularly high, indicating in that protein must have been lost in the pellets formed after the centrifugation steps during the purification process. Though the amount collected was sufficient for performing the next steps of this study.

3.2 Kinetics

Kinetic measurements on reaction 1 with both ADH-A wt and ADH-A C1B1 showed that acetophenone was consumed and the concentration of reduced form of the cofactor, NADH was increased over time.

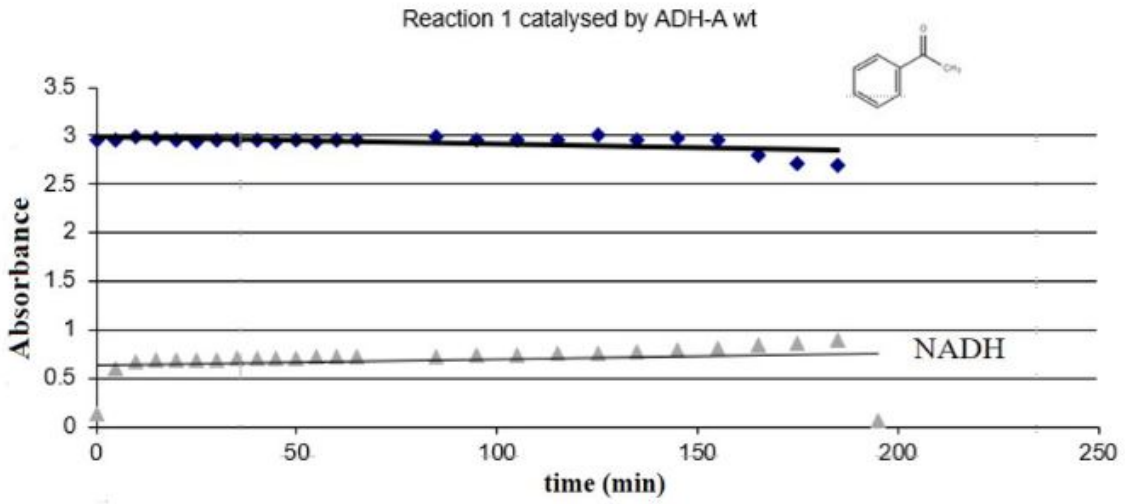

Figure 12: Kinetic study of reaction 1. The measured absorbance values where plotted against time, for acetophenone (blue color) and the reduced form of the enzyme's cofactor, NADH (grey color).

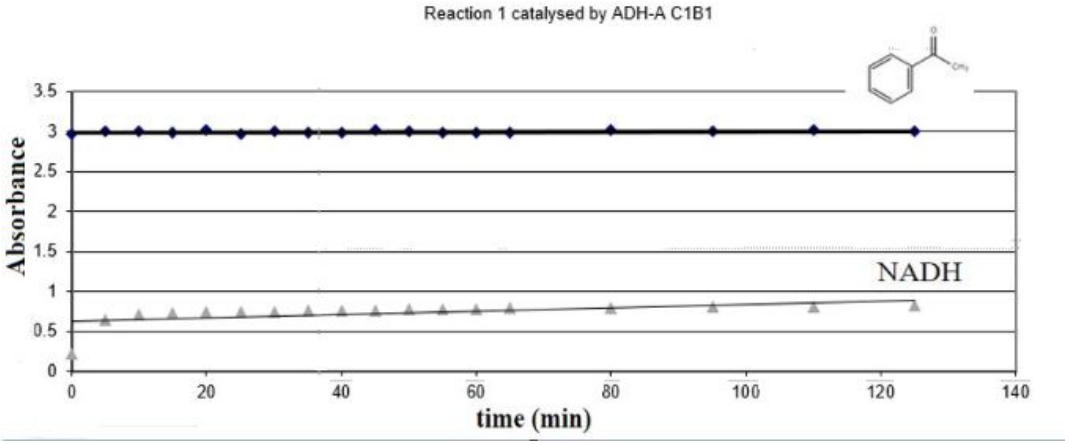

Figure 13: Kinetic study of reaction 1. The measured absorbance values where plotted against time, for acetophenone (blue color), the reduced form of the enzyme's cofactor, NADH (grey color).

Those results were an indication of reaction 1 taking place, though in the case of ADH-A C1B1 the consumption of acetophenone was not as effective as it is in the case of ADH-A wt. This can possibly be because acetophenone is not does not act as a good substrate for the ADH-A C1B1 mutant as it does for the ADH-A wt. The formation of one of the products of the reaction, the cofactor NADH, was though clear in both cases and therefore the study was continued further in analysis of the product components with HPLC.

3.3 HPLC

HPLC analysis on reaction 1 in small scale indicated that the desired product, 1-phenylethanol, was produced and the starting material acetophenone was being consumed. (Appendix 3, Table 20). The best yield of the desired product, 1-phenylethanol was proven to be obtained with 10 mM isopropanol for both enzymes ADH-A wt and ADH-A C1B1 (Figure 14).

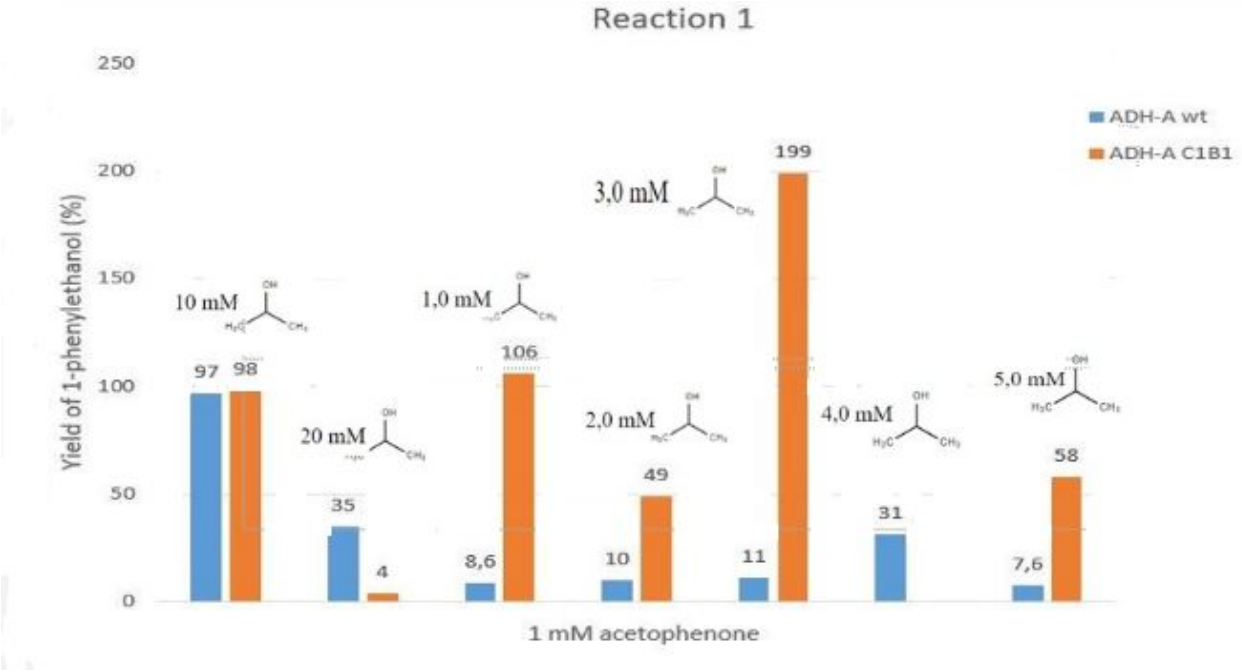

Figure 14: The yields obtained of the desired product 1-phenylethanol when reaction 1 was performed in a a small scale, with 1.0 mM acetophenone, 0.2 mM of the cofactor NAD^+, 3.0 μM of ADH-A wt and ADH-A C1B1 respectively and different concentrations of isopropanol.

The high yield obtained in the presence of 3.0 mM isopropanol is a problem worth to be discussed. The yield of reaction 1 obtained when catalysed with C1B1 is higher than 100%, something that theoretically can not be achieved and probably indicates to an instrumentation problem. The pressure in the C18 column was during these analysis really high, around 19 kPa something that most of the times led to that the instrument was shut down. The ineffective separation of the components inside the column is believed to have led to a bigger area of 1-phenylethanol in the HPLC chromatogram, which when used in the calculations gave such a high value. Therefore, these measurements with isopropanol concentration between 1.0-5.0 mM should be repeated several times, in order to present a statistically representative study. Though, when 10 mM isopropanol was used as starting material the reaction proceeded as expected and the yield of it was high enough to continue studying reaction 1 in large scale.

When the concentrations of the starting material isopropanol and acetophenone used were higher, the reaction did not proceed as expected. Acetophenone was not fully consumed and the amounts of 1-phenylethanol formed were really low (Appendix 3, Table 21). Given the fact that isopropanol was used at a higher concentration, acetone was produced at higher amounts as well, reaction 1. The ketone could then be considered to be competing the enzyme, preventing the production of the desired product, 1-phenylethanol. Another factor contributing into that reaction 1 was not favored, is

the fact that 1-phenylethanol is a better substrate for the enzyme than isopropanol is .[1] Since in all measurements with HPLC, 1-phenylethanol were clearly produced, it can be assumed that it also can act as a substrate for the enzymes and turn the reaction to the opposite direction, getting oxidized and leading into higher amount of acetophenone.

In order to look closely into those aspects, reaction 1 was studied when increasing the concentration of acetophenone over time from 10 mM to 100 mM while keeping the rest of reaction parameters unmodified. The results shown in figure 15, (and Appendix 3, table 22) indicate that the best yield of the 1-phenylethanol was produced when a concentration of 20-30 mM acetophenone and 10-50 mM acetophenone was used for ADH-A wt and ADH-A C1B1 respectively.

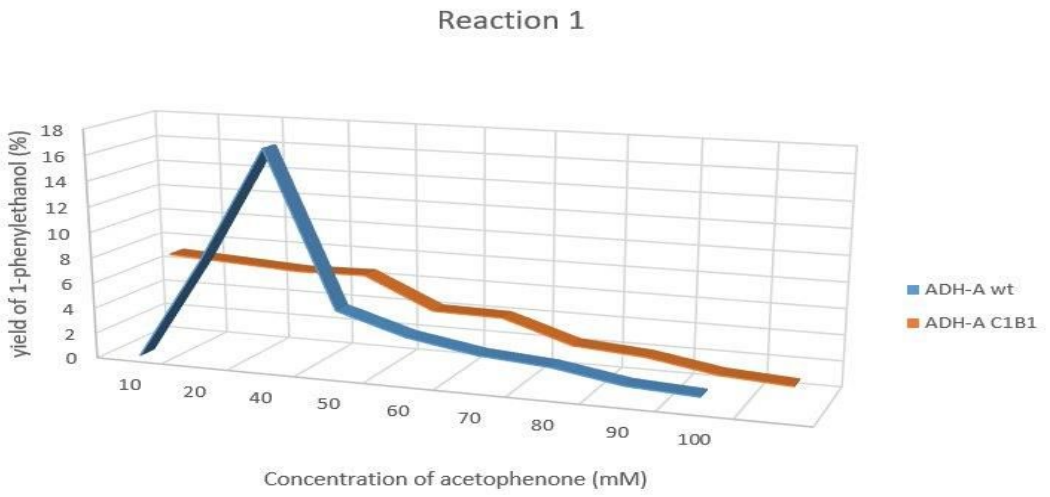

Figure 15: The yields obtained of the wished product 1-phenylethanol when reaction 1 was performed in a large scale, with 10 mM to 100 mM acetophenone, 300 mM isopropanol, 0.2 mM of the cofactor $NAD^+$and 4.0 µM of ADH-A wt and ADH-A C1B1 respectively.

For these results, the amount of chemical substance divided by the area of each component's peak in the chromatogram for the reference samples was used to calculate the amount of each component in the product samples that were analysed with HPLC. Each component's area was multiplied to the amount of chemical substance of this substance that was injected in the column and then divided by that of the corresponding reference sample (Appendix 3, Table 23). The measurements were performed in all cases only once because of difficulties with the dilution factor in the samples and the high pressure in the column. Therefore no standard deviation between these values is calculated.

A parameter that is worth to take consideration to for reaction 1 is that of time. Probably 20 min. was not a sufficient time period for the additional amount of acetophenone to be consumed and the enzyme might have been in the need of a longer time period to convert the added ketone to its corresponding alcohol efficiently.

In the case of reaction 2, the starting material 2-hydroxyacetophenone was not fully consumed and and the amount of (R)-1-phenylethane-1,2-diol formed was not enough to state that the reaction is favored in this case either. An explanation to that might be the fact that the aryl-substituted vicinal diol (R)-1-phenylethane-1,2-diol can inhibit the enzyme (especially the mutant ADH-A C1B1).[1]

Moreover the elution of another component from the column during HPLC analysis, probably 1-phenylethenol (Figure 16) is another factor contributing to the low final amount of (R)-1-phenylethane-1,2-diol formed.

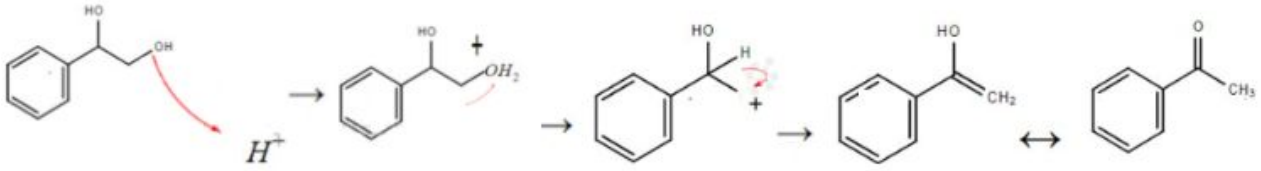

Figure 16: Possible reaction mechanism for the formation of 1-phenylethenol when (R)-1-phenylethane-1,2-diol is dehydrated by the solvent Methanol. In the second step of the reaction, water is also generated.

Returning to the analysis of reaction 1, the need of a thermodynamic study of the overall reaction, by studying the two half-reactions separately (Figures 4 and 5) might be a new topic of interest that rises at this point. The difference in Gibbs free energy between them two, equation 2, describes their relation and has a positive value for reaction 1, catalysed by enzyme ADH-A wt. In other words, the enzyme's efficiency is much higher when acetophenone acts as its substrate than when isopropanol does.

$$\Delta\Delta G = -RT\ln \frac{(k_{cat}/K_M)^{A}}{(k_{cat}/K_M)^{B}} \qquad \textit{(Equation 2)}$$

Since the kinetic parameters k_{cat} and K_M for ADH-A wt are already determined, the same study is to be done in the future for the other mutants of ADH-A C1B1, something that would explain the results obtained on this study.

4. Conclusion

As long as the concentrations of the starting material for reaction 1, were low, the reaction was successfully performed for the ADH-A wt and not for the ADH-A C1B1, giving the best results when 1.0 mM acetophenone and 10 mM isopropanol were used. When increasing the concentration of these, while keeping the overall reaction time and the amount of the enzymes cofactor stable, the reaction was not performed successfully any longer. The same issue occurred when studying reaction 2 at a larger scale. The high concentration of the produced molecules acting in an inhibiting manner on the enzymes is one of the the most possible explanations. In a closer study of reaction 1, it was also found that the best yield of the 1-phenylethanol was produced when a concentration of 20-30 mM acetophenone and 10-50 mM acetophenone was used for ADH-A wt and ADH-A C1B1 respectively.

Acknowledgements

I would like to thank Professor Mikael Widersten for giving me the opportunity to do this project, and for being apart from the supervisor of my experiment also a great teacher, answering all my questions, discussing about my project and handling the difficulties I had in the greatest possible way. I would also like to thank Jie Yang for his assistance and help with the HPLC instrumentation and lastly my friend Patricia Kindström whose presence in the lab made my time there enjoyable and pleasant.

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Appendix

In all cases deionized water was used.

Appendix 1: Protein Purification

Table 2: Preparation of 2TY Media used for ADH-A expression

2TY Media			
Amount 2TY (mL)	**Tryptone (g)**	**Yeast extract (g)**	**NaCl (g)**
750	12	7.5	3.75
300	4.8	3	1.5
35	0.56	0.35	0.175
Diluted to the corresponding volumes with H_2O and autoclaved.			

Table 3: Preparation of LB Media used for ADH-A expression

LB Media				
Amount (mL)	**Tryptone (g)**	**Yeast extract (g)**	**NaCl (g)**	**Agar (g)**
250	2.5	1.25	2.5	3.75
Diluted to 250 mL with H_2O and autoclaved.				

Table 4: Preparation of elution buffer used for ADH-A purification

ADH-A Elution buffer, 500 mL			
Chemical	**Start concentration (M)**	**V/m**	**End concentration (mM)**
Imidazole		10.20 g	300
NaCl		14.61 g	500
NaPi Buffer 0.1 M pH 8 10 µL $ZnSO_4$	1 M	10 mL	20
$H_2 0$		400 mL	
pH corrected to 7.5 and diluted to 500 mL with H_2O			

Table 5: Preparation of desalting buffer used for ADH-A purification

ADH-A Desalting buffer, 1L			
Chemical	**Start concentration (M)**	**V/m**	**End concentration (mM)**
NaPi Buffer 0.1 M pH 8 10 µL $ZnSO_4$	1 M	100 mL	100 mM
H_2O		800 mL	
$ZnSO_4$	100 mM	100 mL	10 µM
pH corrected to 7.4 and diluted to 1 L with H_2O			

Table 6: Preparation of washing buffer used for ADH-A purification

ADH-A Washing buffer, 1L			
Chemical	**Start concentration (M)**	**V/m**	**End concentration (mM)**
Imidazole		6.81 g	100
NaCl		29.22 g	500
NaPi Buffer 0.1 M pH 8 10 µL$ZnSO_4$	1 M	20 mL	20
H_2O		800 mL	
pH corrected to 7.5 and diluted to 1 L with H_2O			

Table 7: Preparation of binding buffer used for ADH-A purification

ADH-A Binding buffer, 1L			
Chemical	**Start concentration (M)**	**V/m**	**End concentration (mM)**
Imidazole		1.36 g	20
NaCl		29.22 g	500
NaPi Buffer 0.1 M pH 8 10 µL $ZnSO_4$	1 M	20 mL	20
H_2O		800 mL	
pH corrected to 7.5 and diluted to 1 L with H_2O			

Table 8: Preparation $NiCl_2$ buffer used for ADH-A purification.

$NiCl_2$ buffer, 50 mL, 0.1 M	
Chemical	**V/m**
$NiCl_2$	0.648 g
diluted to 50 mL with H_2O	

Table 9: Preparation of Lysis buffer used for ADH-A purification.

Lysis buffer 50 mL
50 mL Binding buffer
1 complete tablet EDTA free
100 µL of 10 mg/mL DNase I

Table 10: Preparation of Separation and Stacking Gel for SDS-PAGE Gel Electrophoresis. The amounts used were for making two separation and two stacking gels, for ADH-A wt and ADH-A C1B1.

	Separation Gel	Stacking Gel
H_2O	3.28 mL	6.0 mL
1.5 M Tris pH 8.8	2.5 mL	-
0.5 M Tris 6.8	-	2.5 mL
10 % SDS	100 µL	100 µL
10 % APS	100 µL	100 µL
30 % Acrylamide	4 mL	1.33 mL
TEMED	10 µL	10 µL

Appendix 2: Kinetics

Table 11: Preparation of the stock solutions for the two substrates and the cofactor of reaction 1.

Stock Solutions			
Chemical	**Amount**	**Dilution Factor**	**Concentration**
Isopropanol	18 µL	928 µL H_2O	240 mM
Acetophenone	23 µL	977 µL ACN	200 mM
NAD^+	2.65 mg	997.6 NaPi Buffer 0.1 M pH 8 10 µL $ZnSO_4$	4.0 mM

Enzyme	-	-	ADH-A wt: 326.2 µM ADH-A C1B1 : 215.3 µM

Table 12 : Preparation of reaction vials studying reaction 1. The study was conducted first with ADH-A wt and later with ADH-A C1B1. The enzymes used were those available in the lab, with concentrations of 362.2 µM for ADH-A wt and 215.3 for ADH-A C1B1.

Reaction Vials		
Chemicals	**Amount (µL)**	**Concentration**
Isopropanol	5	1.2 mM
Acetophenone	5	1.0 mM
NAD^+	50	0.2 mM
Enzyme	ADH-A wt: 50 ADH-A C1B1: 50	ADH-A wt: 0.8 µM ADH-A C1B1: 0.8 µM
NaPi Buffer 0.1 M pH 8 10 µL $ZnSO_4$	890	-

Appendix 3: HPLC

Table 13: Preparation of the reference sample for the HPLC measurements in a small scale.

Reference samples			
Chemicals	**Amount (µL)**	**Dilution**	**Final Concentration (mM)**
Acetophenone	0.25	999.75 µL MeOH	2.0
1-Phenylethanol	2.5	997.5 µL MeOH	20

Table 14 : Preparation of the stock solutions for the two substrates and the cofactor of reaction 1 in a small scale.

Stock Solutions			
Chemical	**Amount**	**Dilution Factor**	**Concentration**
Isopropanol	18 µL	928 µL H_2O	240 mM
Acetophenone	23 µL	977 µL ACN	200 mM
NAD^+	2.65 mg	997.6 µL NaPi Buffer 0.1 M pH 8 10 µL $ZnSO_4$	4.0 mM

Table 15: Preparation of reaction vials for the different concentrations of isopropanol for reaction 1 in a small scale. The enzymes used were ADH-A wt purified earlier during the study and ADH-A C1B1 that already available in the lab, with concentrations of 198.6 µM for ADH-A wt and 215.3 for ADH-A C1B1.

Reaction vials														
	Amount (µL)							Concentration						
Chemical	**10 mM isopropanol**	**20 mM**	**1,0 mM**	**2,0 mM**	**3,0 mM**	**4,0 mM**	**5,0 mM**	**10 mM**	**20 mM**	**1.0 mM**	**2.0 mM**	**3.0 mM**	**4.0 mM**	**5.0 mM**
Isopropanol	83	166	8,3	16	25	33	42	10 mM	20 mM	1.0 mM	2.0 mM	3.0 mM	4.0 mM	5.0 mM
Acetophenone	10	10	10	10	10	10	10	1.0 mM	1.0 mM	1.0 mM	1.0 mM	1.0 mM	1.0 mM	1.0 mM
NAD^+	100	100	100	100	100	100	100	0.2 mM	0.2 mM	0.2 mM	0.2 mM	0.2 mM	0.2 mM	0.2 mM
ADH-A wt	30	30	30	30	30	30	30	3.0 µM	3.0 µM	3.0 µM	3.0 µM	3.0 µM	3.0 µM	3.0 µM
ADH-A C1B1	250	250	250	250	250	250	250	3.0 µM	3.0 µM	3.0 µM	3.0 µM	3.0 µM	3.0 µM	3.0 µM
NaPi Buffer 0.1 M pH 8 10 µL $ZnSO_4$	1777 with wt 1557 with C1B1	1694 with wt 1474 with C1B1	1851,7 with wt 1731,7 with C1B1	1844 with wt 1724 with C1B1	1835 with wt 1715 with C1B1	1827 with wt 1707 with C1B1	1818 with wt 1698 with C1B1	-	-	-	-	-	-	-

Table 16: Preparation of the reference sample for the HPLC measurements for reaction 1 and 2 in a large scale.

Reference samples			
Chemicals	**Amount (µL)**	**Dilution**	**Final Concentration (mM)**
Acetophenone	0.25	999.75 µL MeOH	2.0
1-Phenylethanol	2.5	997.5 µL MeOH	20
2-Hydroxy-1-phenylethanone	0,0027	999.99 µL MeOH	20
1-Phenyl-1,2-ethanediol R enantiomer	0.00276	999.99 µL MeOH	20
1-Phenyl-1,2-ethanediol S enantiomer	0.00276	999.99 µL MeOH	20

Table 17: Preparation of the stock solutions for the two substrates and the cofactor of reaction 1 in a large scale.

Stock Solutions

Chemical	Amount	Dilution Factor	Concentration
Isopropanol	306 µL	3694 µL ACN	1.0 M
Acetophenone	700 µL	5300 µL ACN	1.0 M
2-hydroxyacetophenone	0.545 g	3500 µL ACN	1.0 M
NAD^+	2.65 mg	997.6 µL NaPi Buffer pH 3 $ZnSO_4$	4.0 mM

Table 18: Preparation of reaction vials for HPLC analysis of reaction 1 in large scale The enzymes used were ADH-A wt purified earlier during the study and ADH-A C1B1 that already available in the lab, with concentrations of 198.6 µM for ADH-A wt and 215.3 for ADH-A C1B1.

Reaction Vials		
Chemicals	**Amount**	**Concentration**
Isopropanol	3.0 mL	300 mM
Acetophenone	2.0 mL	200 mM
NAD^+	500 µL	0.2 mM
ADH-A wt	201,4 µL	4.0 µM
ADH-A C1B1	185,8 µL	4.0 µM
NaPi Buffer 0.1 M pH 8 10 µL $ZnSO_4$	4298,6 µL with wt 4314,2 with C1B	-

Table 19: Preparation of reaction vials for HPLC analysis of reaction 2 in large scale. The enzymes used were ADH-A wt purified earlier during the study and ADH-A C1B1 that already available in the lab, with concentrations of 198.6 µM for ADH-A wt and 215.3 for ADH-A C1B1.

Reaction Vials		
Chemicals	**Amount**	**Concentration**
Isopropanol	3.0 mL	300 mM
2-hydroxyacetophenone	2.0 mL	200 mM
NAD^+	500 µL	0.2 mM
ADH-A wt	201.4 µL	4.0 µM
ADH-A C1B1	185.8 µL	4.0 µM
NaPi Buffer 0.1 M pH 8 10 µL $ZnSO_4$	4298.6 µL with wt 4314.2 with C1B	-

Table 20: HPLC measurements of reaction 1 in large scale. 10 mM acetophenone was added every 20 min. to a total concentration of 100 mM. The same additions were performed with both ADH-A wt and ADH-A C1B1. The enzymes used were ADH-A wt purified earlier during the study and ADH-A C1B1 that already available in the lab, with concentrations of 198.6 μM for ADH-A wt and 215.3 for ADH-A C1B1. A concentration of 4.0 μM of each enzyme was added in the reaction vials.

Reaction Samples					
Reaction vial	**Isopropanol (mL)**	NAD^+ **(μL)**	**Amount of Acetophenone (μL)**	**Concentration of Acetophenone (mM)**	**NaPi Buffer 0.1 M pH 8 10 μL $ZnSO_4$ (μL)**
1	1.5	250	50	10	3014.12 for C1B1 3090 for wt
2	1.5	250	96	20	3014.12 for C1B1 3090 for wt
3	1.5	250	141	30	3014.12 for C1B1 3090 for wt
4	1.5	250	185	40	3014.12 for C1B1 3090 for wt
5	1.5	250	231	50	3014.12 for C1B1 3090 for wt
6	1.5	250	279	60	3014.12 for C1B1 3090 for wt
7	1.5	250	331	70	3014.12 for C1B1 3090 for wt
8	1.5	250	389	80	3014.12 for C1B1 3090 for wt
9	1.5	250	455	90	3014.12 for C1B1 3090 for wt
10	1.5	250	530	100	3014.12 for C1B1 3090 for wt

Table 21: Results from HPLC measurements of reaction 1 in small scale. *In all reactions samples, 1.0 * 10^{-8} mol acetophenone was added and therefore 1.0 * 10^{-8} mol phenylethanol is used for the calculations, as the maximum possible molarity. All reaction samples are diluted 10 times in MeOH.

HPLC measurements of reaction 1 in small scale					
Chemical	**Concentration (mM)**	**Injected Volume (** μL)	**Amount of the chemical substance (mole)**	**Area (m A^2)**	**Percentage (%)**
Standard acetophenone	2.0	5.0	1.0 * 10^{-8}	6458970	-
Standard	20	5.0	1.0 * 10^{-7}	10502722	-

Phenylethanol					
wt, with 10mM isopropanol	*	10	$1.0 * 10^{-8}$	ketone: 359041 alcohol: 102121	ketone:5.5 alcohol: 97
C1B1, with 10mM isopropanol	*	10	$1.0 * 10^{-8}$	ketone: 358413 alcohol: 1028375	ketone: 5.5 alcohol: 98
wt, 20mM isopropanol	*	10	$1.0 * 10^{-8}$	ketone: 67064 alcohol: 365998	ketone: 1 alcohol: 35
C1B1, 20mM isopropanol	*	10	$1.0 * 10^{-8}$	ketone: 97712 alcohol: 510192	ketone: 1.5 alcohol: 4
Standard acetophenone	2.0	5.0	$1.0 * 10^{-8}$	33824841	-
Standard Phenylethanol	20	5.0	$1.0 * 10^{-7}$	35694948	-
wt, 1.0mM isopropanol	*	10	$1.0 * 10^{-8}$	ketone: 503964 alcohol: 307553	ketone: 1.5 alcohol: 8.6
C1B1, 1.0mM isopropanol	*	10	$1.0 * 10^{-8}$	ketone: 11912606 alcohol: 3793658	ketone: 35 alcohol: 106
wt, 2.0mM isopropanol	*	10	$1.0 * 10^{-8}$	ketone: 370058 alcohol: 368880	ketone: 1 alcohol: 10
C1B1, 2.0mM isopropanol	*	10	$1.0 * 10^{-8}$	ketone 3314547 alcohol: 1740463	ketone: 9.8 alcohol: 49
wt, 3.0mM isopropanol	*	10	$1.0 * 10^{-8}$	ketone: 403604 alcohol: 388653	ketone: 1.2 alcohol: 11
C1B1, 3.0mM isopropanol	*	10	$1.0 * 10^{-8}$	ketone: 7508670 alcohol: 7136929	ketone: 22 alcohol: 199
wt, 4.0mM isopropanol	*	10	$1.0 * 10^{-8}$	ketone: 465430 alcohol: 1093257	ketone: 1.4 alcohol: 31
C1B1, 4.0mM isopropanol	*	10	$1.0 * 10^{-8}$		
wt, 5.0mM isopropanol	*	10	$1.0 * 10^{-8}$	ketone: 107470 alcohol: 272482	ketone: 0.32 alcohol: 7.6
C1B1, 5.0 mM isopropanol	*	10	$1.0 * 10^{-8}$	ketone: 777066 alcohol: 2069992	ketone: 2.3 alcohol: 58

Table 22: Results from HPLC measurements of reaction 1 and 2 in large scale. *In the first two reactions samples, 2000 * 10^{-9} mol acetophenone was added and therefore 2000 * 10^{-9} mol phenylethanol is used for the calculations, as the maximum possible molarity. For the last two samples, reaction 2 was studied and because of the fact that 200 mM 2-hydroxyacetophenone was added, 200mM of both the R-and the S-enantiomer of 1-Phenyl-1,2-ethanediol is used for the calculations, as the maximum possible molarity. Reaction samples were diluted 20 times in MeOH for reaction 1 and 10 times in MeOH for reaction 2.

HPLC measurements of reactions 1 and 2 in large scale					
Chemical	**Concentration (mM)**	**Injected Volume (μL)**	**Amount of the chemical substance (mole)**	**Area (m A^2)**	**Percentage (%)**
Standard acetophenone for reaction 1	2.0	5.0	1.0 * 10^{-8}	31735735	-
Standard 1-Phenylethanol for reaction 1	20	5.0	1.0 * 10^{-7}	23557437	-
Standard 2-hydroxyacetophenone for reaction 2	20	5.0	1.0 * 10^{-7}	31233116	-
Standard 1-Phenyl-1,2-ethanediol for reaction 2	20	5.0	1.0 * 10^{-7}	24899553 for R-enantiomer 25306921 for S enantiomer mean value: 25103237	-
wt, for reaction 1	*	10	2000 * 10^{-9}	ketone: 58095059 alcohol: 3662926	ketone: 0.9 alcohol: 0.8
C1B1, for reaction 1	*	10	2000 * 10^{-9}	ketone: 51573638 alcohol:6758940	ketone: 0.8 alcohol: 1.4
wt, for reaction 2	*	10	2000 * 10^{-9}	ketone:9987951 alcohol:33940796	ketone: 1.5 alcohol: 6.7
C1B1, for reaction 2	*	10	2000 * 10^{-9}	ketone: 6354019 alcohol:56613717	ketone: 0.98 alcohol: 11

Table 23: Results from HPLC measurements of reaction 1 in large scale in the study with the adding amount of 10 mM acetophenone over time. 300 mM isopropanol, 0.2 mM NAD^+ and 4.0 μM of each enzyme were added in reaction vials of total 5.0 mL volume. The sample for reaction vial wt 3, was not successfully filtered and therefore not included in this study. Reaction samples were diluted 20 times in MeOH.

HPLC measurements of reactions 1 in large scale. Study with the adding 10 mM acetophenone over time.					
Reaction vial	**Concentration (mM)**	**Injected Volume (μL)**	**Amount of the chemical substance (mole)**	**Area (m A^2)**	**Percentage (%)**
Standard acetophenone for	2.0	5.0	1.0 * 10^{-8}	31735735	-

reaction 1					
Standard 1-Phenylethanol for reaction 1	20	5.0	$1.0 * 10^{-7}$	23557437	-
wt 1	10	10	$1.0 * 10^{-7}$	ketone : 20135029 alcohol :1410517	ketone : 0.67 alcohol : $5.98 * 10^{-7}$
wt 2	20	10	$200 * 10^{-9}$	ketone :7890688 alcohol : 3819333	ketone : 1.2 alcohol : 8
wt 4	40	10	$400 * 10^{-9}$	ketone : 34230264 alcohol :16281268	ketone :0.27 alcohol :17
wt 5	50	10	$500 * 10^{-9}$	ketone : 40419382 alcohol : 5940012	ketone : 25 alcohol :5
wt 6	60	10	$600 * 10^{-9}$	ketone : 43015625 alcohol :4841881	ketone : 2.3 alcohol :3.4
wt 7	70	10	$700 * 10^{-9}$	ketone :57117804 alcohol :4061524	ketone : 2.6 alcohol :2.5
wt 8	80	10	$800 * 10^{-9}$	ketone : 59522198 alcohol : 3906536	ketone : 2.3 alcohol :2.1
wt 9	90	10	$900 * 10^{-9}$	ketone :48310542 alcohol : 2482787	ketone : 0.17 alcohol :1.2
wt 10	100	10	$1.0 * 10^{-6}$	ketone : 50674320 alcohol : 2088929	ketone : alcohol : 0.9
C1B1 1	10	10	$1.0 * 10^{-7}$	ketone : 1979987 alcohol :1669977	ketone : 0.62 alcohol :7.1
C1B1 2	20	10	$200 * 10^{-9}$	ketone : 6299625 alcohol :3206024	ketone : 10 alcohol :6.8
C1B1 3	30	10	$300 * 10^{-9}$	ketone :14862905 alcohol : 4625523	ketone : 1.6 alcohol : 6.5
C1B1 4	40	10	$400 * 10^{-9}$	ketone : 29920306 alcohol : 6185540	ketone : 2.4 alcohol :6.6
C1B1 5	50	10	$500 * 10^{-9}$	ketone : 35724703 alcohol : 4914452	ketone : 2.3 alcohol : 4.2
C1B1 6	60	10	$600 * 10^{-9}$	ketone : 44928627 alcohol :5746649	ketone : 2.4 alcohol :4.1
C1B1 7	70	10	$700 * 10^{-9}$	ketone : 56306966 alcohol :3893559	ketone : 2.5 alcohol :2.4
C1B1 8	80	10	$800 * 10^{-9}$	ketone : 48367786 alcohol :3610930	ketone : 2.0 alcohol : 2.0
C1B1 9	90	10	$900 * 10^{-9}$	ketone : 47557983 alcohol : 2387235	ketone : 1.7 alcohol : 1.1
C1B1 10	100	10	$1.0 * 10^{-6}$	ketone : 48780761 alcohol :1804449	ketone : 1.5 alcohol : 0.76