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Estimation of the kinetic parameters (K_m & V_{max}) and the optimization of yeast alcohol dehydrogenase (ADH) assay

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Abstract

Alcohol dehydrogenases (ADHs) are zinc-containing enzymes that catalyze the oxidation of alcohols to aldehydes or ketones. The enzymes also play a critical role in the metabolism of a number of drugs and metabolites containing alcohol functional groups. Kinetic parameters of yeast alcohol dehydrogenase (ADH) was estimated using spectrophotometer, optimum temperature and pH for ethanol, as well as ADH specificity to other substrates was determined. Results from the assay showed value for the initial velocity of reaction of the enzyme estimated as 0.297. Change in concentration of NADH which correspond to ADH activity for ethanol was calculated as 48nanokatals (4.8×10^{-5} $mol^{-1}l$), the results also showed average values of the K_m and V_{max} of ADH for ethanol as estimated from Michaelis-Menten and Line weaver-Burk double reciprocal plots, to be 21.5mM and 0.426 respectively. Optimum temperature and pH of ADH for ethanol were estimated to be 25 °C and 8 respectively. Although, reaction specificity of ADH to other substrates showed gradation between propan-2-ol and ethanol with ethanol showing highest activity and methanol least. 2-propene-1-ol also presented high enzyme activity relative to ethanol. In most of enzyme assays, enzyme activity is determined by measuring the rate of conversion of substrate or rate of production of products within a given period of time. In this experiment, the rate of oxidation of NADH was monitored since NADH has a known maximum light absorbance at 340nm.

Keywords: Alcohol dehydrogenase (ADH); Optimum temperature; Optimum pH; Enzyme activity; Maximum velocity (Vmax); Michaelis constant (Km)

1. Introduction

Alcohol dehydrogenases (ADHs) are zinc-containing enzymes that catalyze the oxidation of alcohols to aldehydes or ketones, which is the first step in the ethanol metabolism by the liver, where nicotinamide adenine dinucleotide and its reduced form (NAD^+/NADH) are the coenzymes involved (Bhuiya *et al*., 2017). Although less known, ADH also play a critical role in the metabolism of a number of drugs and metabolites containing alcohol functional groups such as abacavir (HIV/AIDS), hydroxyzine (antihistamine), and ethambutol (anti-tuberculosis) (Di *et al*., 2021). In recent developments, ADHs have been found to play important roles in catalyzing asymmetric reactions, and have been applied widely in fine chemical synthesis, particularly in the production of chiral alcohols and hydroxyl compounds that are key elements in the synthesis of active pharmaceutical ingredients (API) employed in the pharmaceutical industries (An *et al.*, 2019). ADHs found in higher eukaryotic organisms are generally dimeric in nature, whereas in the case of prokaryotes and lower eukaryotes (yeasts), the enzymes exist as tetramer (Bhuiya *et al*., 2017). Yeast (ADH) is a tetramer composed of four identical subunits each of which is made up of single polypeptide chain of 36kDa with 347 amino acid residues, each subunit has one binding site for coenzyme and a tightly bound zinc ion (Begüm *et al*, 2013). ADH is responsible for the reversible oxidative breakdown of alcohols to their corresponding aldehydes, coupled with the reduction of coenzyme nicotinamide adenine dinucleotide NAD^+ to NADH (Crabb, *et al*, 2004; Boeckx *et al*., 2017). Investigation on the kinetic parameters of ADH has been done extensively using different buffer, for instance in 0.5M

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pyrophosphate buffer at pH 8.7 and 25 °C, pure ADH presented specific activity of 380-430µmol/min while commercial ADH has 200-250µmol/min (Schopp & Aurich, 1975). Using tris buffer, ADH presented highest stability at pH 8.5 and 35 °C (Juliana *et al*, 2007), and in pyrophosphate buffer the optimum pH and temperature are found to be 8.8 and 25 °C respectively (Madhsudhan *et al*, 2007).

Study of the structures and catalysis of ADH from different sources has grouped the enzyme into three (3) basic types. This include medium-chain zinc dependent type which consist of 350 amino acid residues per subunit (horse liver & *S. cerevisiae* ADH); short-chain zinc independent ADH found in *Lactobacillus brevis* which consist of 250 amino acid per subunit and the long-chain Iron (Fe) activated ADH which consist of 385 amino acid residues per subunit, common in *Saccharomyces cerevisiae* (Juliana *et al*, 2007). Study reveals that the molecular weight of ADH from yeast doubles that of the mammalian by two folds, and about a hundred times more active. ADH specificity is restricted to primary alcohols with linear aliphatic chains with ethanol being the best substrate, also alcohols increasingly become less effective as substrate to ADH as their chain length increases and on moving from primary to secondary alcohols (Sidney *et al*, 2011, Dickinson & Monger, 1973). Also, ADH from Baker's yeast in heptane solution have been found to oxidize unsaturated alcohols, 3-methyl-2-buten-1-ol to the corresponding unsaturated aldehyde, 3-methyl-2-butenal (Yang & Russell, 1993).

Due to its wide distribution in animal tissues, plants, microorganisms, broad substrate and stereoselectivity ADH has attracted major scientific attention (Juliana, *et al*, 2007), and has found an important application in research, biochemical industries and forensic science for estimating concentration of primary alcohols, NAD^+, ethylene glycol, numerous aldehydes, enzymatic breakdown of organic solvents, and in biosensors (Madhusudhan et al, 2007). Optimization of enzyme assays is essential in studying and validating the behavior of that enzyme, it ensures high enzyme yield and performance, enzyme sensitivity and specificity. In this paper, the kinetic parameters (K_m) and (V_{max}) of Yeast Alcohol Dehydrogenase (ADH) for ethanol was estimated. Also, yeast ADH activity was optimized for different conditions (temperature and pH), and specificity to different substrates was determined.

2. Materials and methods

2.1. Optimization of Enzyme Concentration required to determine the Kinetics of Yeast ADH

A pyrophosphate buffer (pH 9.0) containing 0.01M sodium pyrophosphate decahydrate, 0.34 mM EDTA disodium salt dehydrate was prepared. Spectrophotometer cuvettes were set up as shown in table I.

Table 1 Spectrophotometer Cuvettes Setup

Reaction Mixture	Volume
Pyrophosphate buffer (0.001M, PH 9)	1Ml
Fresh deionized water	1Ml
NAD+ (0.0030M)	200µl
Cysteine	200µl
ADH solution (17 units per mL)	100µl
Total volume	2.5Ml

The contents of the cuvettes were mixed by stretching a small piece of paraffin over the top of the cuvette and inverted several times. A Thermo Genesys 10S spectrophotometer was calibrated with deionized water and the wavelength set at 340nm. The cuvettes were placed in the spectrophotometer to begin the kinetics. After 20 seconds, the reaction was initiated by adding 500µL of 2.0M ethanol, the reaction mixture was mixed as earlier described, and the cuvette immediately replaced in the spectrophotometer (this brought the total volume of solution in the cuvette to 3mL). After 3minutes, the absorbance change per minute (ΔA/min) was found to be 0.601. The enzyme was diluted (1:1) and protocol repeated. This time, the absorbance change per minute (ΔA/min) was 0.297 and this, according to Vallee and Hoch (1955), can be inferred to be the initial velocity (rate) of the enzyme reaction.

The experimental procedure was validated by running ethanol blank and enzyme blank assays. Ethanol blank assay was performed by adding 500µL of water instead of ethanol into the cuvette containing the mixture as described in Table 1

and no absorbance was detected after 3minutes. Also, enzyme blank control assay was performed by replacing 100µL of ADH (Table 1) with 100µL of water and no absorbance was detected after 3minutes.

2.2. The Estimation of K_m and V_{max} of ADH for Ethanol

A series of dilution was made on the 2M ethanol to produce solutions with a range concentration and their corresponding concentrations in 3mL (working concentration) was worked out as shown in table 2.

Table 2 A Range of Ethanol Concentration and Working Concentration

Ethanol Concentration(M)	Working Concentration (mM)
0.05	8
0.12	20
0.15	25
0.20	33
0.50	83
1.00	166
1.50	250
2.00	330

Assays were carried out on 500µL of each working concentration using the protocol previously described in section 2.1.

2.3. The Effect of Temperature on ADH

Four (4) cuvettes containing the reaction mixture were incubated in water baths pre-set to 25 °C, 35 °C, 45 °C and 55 °C for 10minutes and the change in absorbance was recorded for the different temperatures accordingly.

2.4. The Effect of pH on ADH

Seven (7) different solutions of pyrophosphate buffer of varying pH range between 6 and 12 were prepared. 1mL of each of the solutions was added to the assay mix and the absorbance values were recorded.

2.5. ADH Specificity

Ethanol in the assay mix was replaced by 2M solutions of 6 different alcohols, one at a time and the absorbance for each of the alcohols was recorded.

3. Results

3.1. Determination of the Catalytic Activity of ADH

The value for the initial velocity (rate) of reaction of the enzyme which was found to be 0.297 was used to calculate the catalytic activity of ADH (nanokatal/gram). This rate of reaction was converted to change in concentration of NADH in nanomoles per second (nanokatals) using Beer-Lambert Law and was found to be 48nanokatals.

3.2. Estimation K_m and V_{max} of ADH

Table 3 shows the absorbance values for different working concentrations of ethanol. The values generated were used to draw Michaelis-Menten and Lineweaver-Burk double reciprocal plots on a graph sheet and with a computer software (Enzfiller).

Table 3 Working Concentrations and Their Corresponding Absorbance Values

Working Concentration (mM)	Absorbance
8	0.130
20	0.216
25	0.220
33	0.250
83	0.308
166	0.344
250	0.400
330	0.425

The graphs were used to estimate the values for K_m and V_{max} of ADH for ethanol. Table 4 shows a comparison of these values for the two plots drawn by hand and with a computer. The average K_m for both plots was 21.5mM while the average V_{max} was 0.426.

Table 4 K_m and V_{max} Values Estimated from a Graph Sheet and a Computer

	Michaelis-Menten (Hand Drawn)	Michaelis-Menten (Computer Generated)	Lineweaver-Burk (Hand Drawn)
$K_{m\ (Mm)}$	22.00	21.70	22.20
$V_{max}\ (min^{-1})$	0.42	0.42	0.41

3.3. The Effect of Temperature on ADH

Table 5 shows the effect of temperature on ADH while keeping other conditions constant. The optimum temperature was found to be 25 ˚C while the least favorable temperature was 55 ˚C.

Table 5 Effect of Temperature on ADH

Temperature (˚C)	Absorbance
25	0.250
35	0.171
45	0.008
55	0.002

3.4. The Effect of pH on ADH

Figure 1 shows the effect of pH on ADH while keeping other conditions constant. The optimum pH was found to be 8 while at pH 12, the enzyme showed no activity.

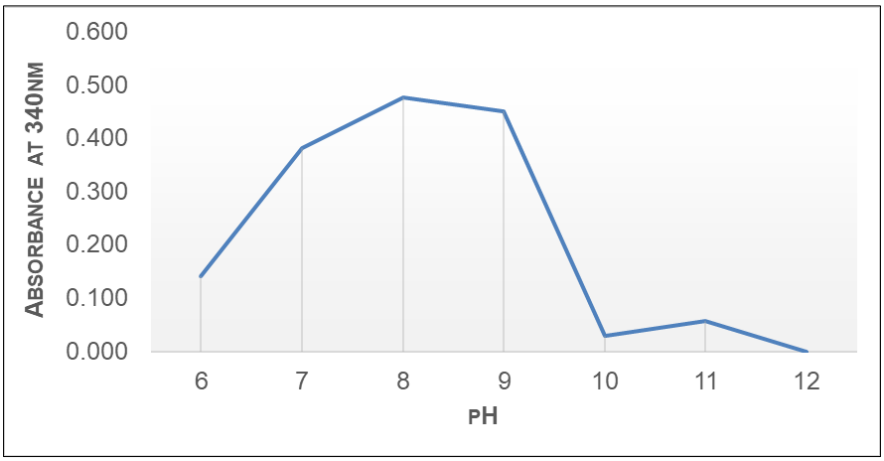

Figure 1 The Effect of pH on ADH

3.5. The ADH Specificity to other substrates

Figure 2 shows the result of the reaction of ADH with 6 other alcohols (shown in blue) compared with ethanol (brown). The enzyme showed high activity for 2-propanol-1-ol, moderate activity for propanol but very little or no activity for glycerol and methanol.

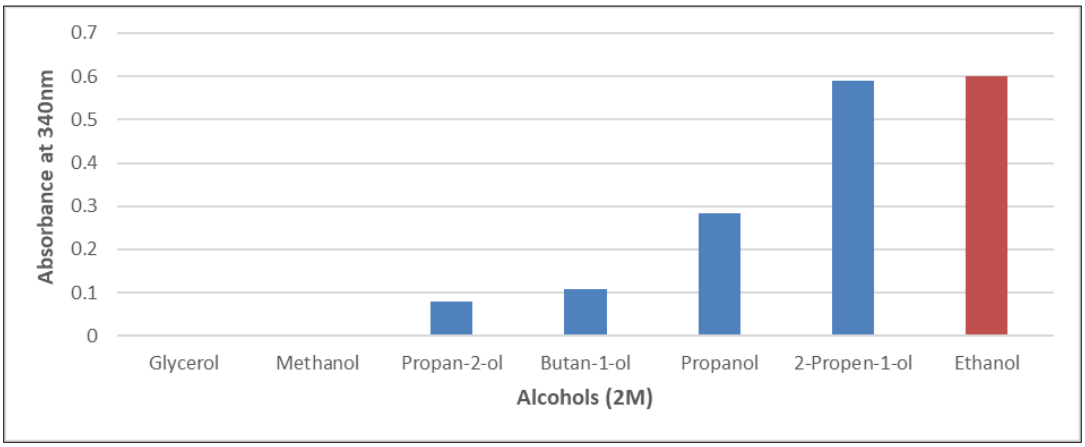

Figure 2 The ADH Specificity to different substrates

4. Discussion

In most of enzyme assays, enzyme activity is determined by measuring the rate of conversion of substrate or rate of production of products within a given period of time. However, in this experiment, the rate of oxidation of NADH was monitored since NADH has a known maximum light absorbance at 340nm (Boeckx *et al*., 2017). Alcohol dehydrogenase (ADH) from yeast was optimized successfully. The value of the initial rate of enzyme reaction was found to be 0.299, and using the Beers Law the rate of change in concentration of NADH was found to be 4.7 X 10^{-5} mol^{-1}/l/min, rate of ADH reaction was calculated to be 23.73 nanokatals. This is equivalent to 1.4 unit or 1.4µmol and is in line with the specification of ADH provided standard. The values of V_{max} and K_m obtained from Michaelis-Menten were 3.2mMs^{-1} and 0.4mM, and from Lineweaver Burk double reciprocal curve were 0.33mMs^{-1} and 0.4mM for ethanol. This result is in agreement with the value reported by Crabb *et al*., (2004). The optimum temperature obtained for yeast ADH in this assay was 25 °C where the enzyme activity was at the maximum. This is in line with the report on similar assay (Schopp & Aurich, 1975), (Juliana *et al*; and Madhsudhan *et al*, 2007). Temperature above the optimum causes denaturation of enzyme which results in loss of conformational structure and subsequent loss of activity (Stryer, Berg & Tymoczko, 2012; Nelson & Cox, 2008).

The pH at which ADH presented maximum level of activity was 8.5 which is defined as the optimum pH for yeast ADH. This agrees with the report from similar experiment (Madhsudhan *et al*, 2007) and (Schopp & Aurich, 1975). Extreme

pH causes loss of structure as a result of breaking of hydrogen and disulfide bonds which stabilizes the secondary protein structure, and also affects the ionic behavior of enzymes by altering the charge of amino acids present in the active sites of the enzymes (Nelson & Cox, 2008).

Result showed that ethanol and 2-propen-1-ol presented the highest specificity to yeast ADH, while activity of ADH declined among propan-1-ol, butan-1-ol, and propan-2-ol through glycerol. This result concur with the report that ADH specificity is restricted to primary alcohols having linear carbon chains, with ethanol as the best substrate, specificity decrease with increase in chain length (Sidney *et al*, 2011, Dickinson & Monger, 1973). The ability of yeast ADH to oxidize unsaturated alcohol in this case 2-propen-1-ol was reported by Yang & Russell (1993).

The variation in substrate specificity may be the result of configuration of the active site of ADH which exhibits geometric specificity allowing binding to different substrates that are geometrically isomeric. It may also be related to the ease with which different alcohol structures fit into active site of the enzyme.

5. Conclusion

ADH has attracted major scientific attention, and has found an important application in research and biochemical industries, forensic science for estimating concentration of primary alcohols, NAD^+, ethylene glycol, numerous aldehydes and enzymatic breakdown of organic solvents, and in biosensors. Optimization of enzyme assays is essential in studying and validating the behavior of enzymes, it also ensures high enzyme yield and performance, enzyme sensitivity and specificity. Results from this work has shown that temperature and pH affects the kinetic behavior of the enzyme ADH, results also revealed that ADH presents broad spectrum of activity but with high specificity to ethanol and 2-propen-1-ol.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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