Lab Report on Enzyme Activity and Kinetics

Harsh Agrawal[1,1*]

[1*]Molecular Bioengineering, Imperial College London.

Corresponding author(s). E-mail(s): ha1822@ic.ac.uk;

Abstract

The purpose of this set of experiments was to determine and study the activity of enzyme action under various conditions. These included pH action under different pH, temperatures, and in the presence of a competitive inhibitor.

1 Experiment 1

1.1 Generation of Standardization curve for p-nitrophenol

Different serial dilutions were prepared for p-nitrophenol and the absorbances were recorded at 410 nm to obtain the following standardization curve. The

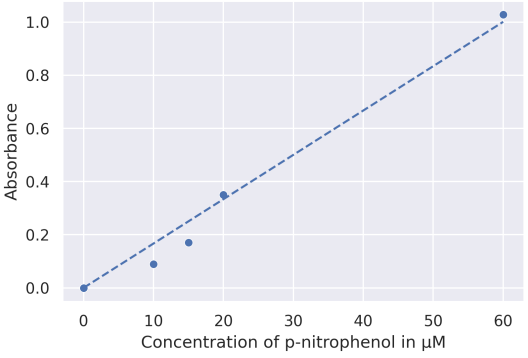

Fig. 1 Standardization Curve of p-nitrophenol.

line of best fit was plotted by adjusting the regression line to pass through the origin. The equation of the line plotted is:

$$y = 0.0171x + 0.00222$$

1.2 Determination of optimum pH of phosphatase

Citrate buffers with five different pH values (3 - 7) were added to the different vials containing p-nitrophenyl phosphate. While keeping controls for every pH, 1 mL of phosphatase solutions was added to each vial. After **exactly 40 minutes**, the reactions were stopped by adding NaOH. The absorbance was then read at 410nm and the product concentration was inferred from the calibration curve generated above.

Table 1 Velocity of Enzyme Activity vs pH

Vial Number	pH	Vial Type	A_{410} (OD)	Product Conc.[1] (μM)	Velocity (μM/min)
1.	3.0	Test	0.226	22.60	0.56
2.	3.0	Control	0.000	-	-
3.	4.0	Test	0.425	42.50	1.06
4.	4.0	Control	0.000	-	-
5.	5.0	Test	0.723	72.30	1.80
6.	5.0	Control	0.000	-	-
7.	6.0	Test	0.702	70.20	1.75
8.	6.0	Control	0.000	-	-
9.	7.0	Test	0.378	37.80	0.94
10.	7.0	Control	0.000	-	-

[1]The concentration of the product was doubled since the assay was diluted two-fold by the addition of 3mL HCl.

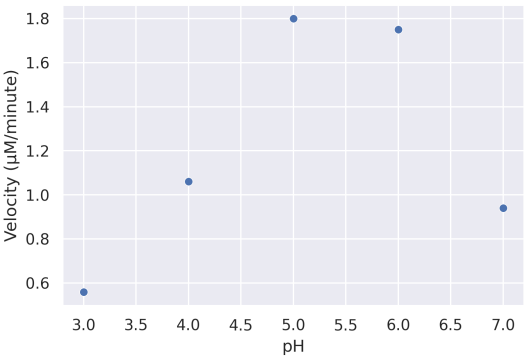

Fig. 2 Scatterplot of the velocity of reaction (μM of product liberated per minute) vs. pH.

We can clearly observe that the velocity first increases within the increase in pH and then falls off after pH 5.0. To determine the two principal ionizations, the two inflection points need to be determined from the curve above. A Gaussian distribution curve was fit on top of the curve to obtain the gaussian function of the above graph:

$$y = 1.86201e^{-\frac{(x-5.395395)^2}{2(1.42176)^2}}$$

The first-order differential of the function was taken and set to zero to obtain the critical points. Followingly, a second-order derivative was taken to infer the point of change of sign to obtain the inflection points. The inflections points obtained were:

$$P1 = (3.97, 1.12);\ P2 = (6.81, 1.12)$$

Hence, these points represent the two principal ionizations. The pH at these ionizations (x-value) are the $\mathrm{pK}_a values$.

$$pK_a(1) = 3.97;\ pK_a(2) = 6.81$$

1.3 Phosphate as a competitive inhibitor of phosphatase

When phosphate is added, it competes for the binding site of acid phosphatase, thus preventing it to bind to p-NPP. This results in an inhibition of product formulation overall. Phosphate was used as a competitive inhibitor for a total of five different dilutions of p-nitrophenyl phosphate - 0.0001M, 0.0004M, 0.002M, 0.004M, and 0.006M.

After exactly 26 minutes, the reactions were stopped with NaOH and the absorbances were read. The following table summarizes the absorbance for every vial along with the concentration and velocity inferred with the help of the calibration curve created earlier.

Fig. 3 Michaelis Menten plot of Substrate concentration (M) vs Velocity of Rection (μM/min).

Table 2 Velocity of Enzyme Activity vs pH

Vial Number	p-NPP conc. (M)	Phosphate Added	A_{410} (OD)	Product Conc.[1] (μM)	Velocity (μM/min)
1.	0.0001	Yes	0.014	1.38	0.05
2.	0.0001	No	0.139	16.00	0.62
3.	0.0001	Yes	0.000	-	-
4.	0.0004	Yes	0.058	6.52	0.25
5.	0.0004	No	0.256	29.68	1.14
6.	0.0004	Yes	0.000	-	-
7.	0.002	Yes	0.176	20.33	0.78
8.	0.002	No	0.351	40.79	1.57
9.	0.002	Yes	0.000	-	-
10.	0.004	Yes	0.249	28.86	1.11
11.	0.004	No	0.377	43.83	1.69
12.	0.004	Yes	0.000	-	-
13.	0.006	Yes	0.180	20.79	1.22
14.	0.006	No	0.401	46.64	1.79
15.	0.006	Yes	0.000	-	-

[1]The concentration of the product was doubled since the assay was diluted two-fold by the addition of 3mL HCl.

[2]Due to contamination, the reaction in vial 13 was redone. While all reactions were stopped after 26 minutes, this reaction was stopped after 17 minutes due to time constraints.

The next step was to calculate V_{max} and K_m from the Micheales Menten plot. A rectangular hyperbola was fit and the equations for the two different curves (inhibition and non-inhibition) were obtained.

With competitive inhibitor $\Rightarrow$

$$y = \frac{1.725x}{0.00237 + x}$$

$$V_{\max} = 1.725\,\mu\text{M/min}, K_m = 0.00237\,\text{M}$$

Without competitive inhibitor $\Rightarrow$

$$y = \frac{1.789x}{0.000211 + x}$$

$$V_{\max} = 1.789\,\mu\text{M/min}, K_m = 0.000211\,\text{M}$$

To ensure that the above-inferred values are correct, a Lineweaver-Burk plot was plotted using the values obtained.

In the Lineweaver-Burk plot, there is a noticeable difference in the y-intercept values of both curves (0.2224 vs. 0.574) suggesting that the line of best fit for the Lineweaver-Burk plot has a lesser fit than the Michaelis Menton plot and thus is less accurate in this context. Moreover, in the Michaelis Menton plot, the y-intercepts were almost similar (1.725 vs 1.789) which should

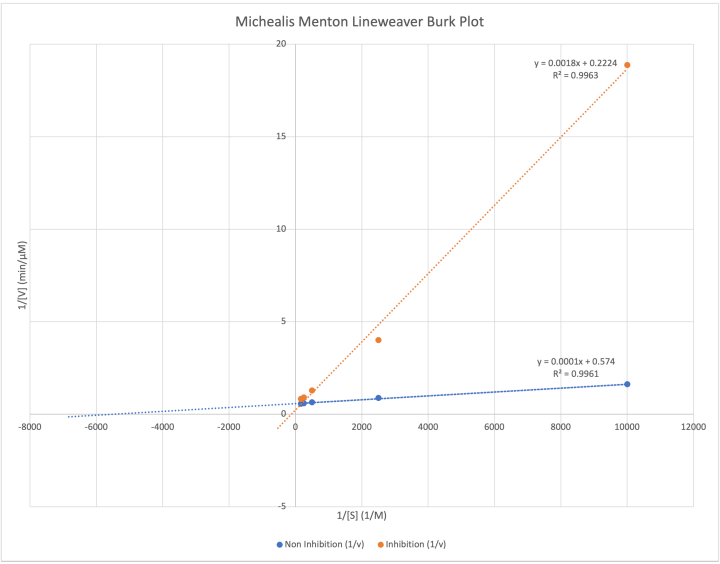

Fig. 4 Lineweaver Burk plot of Substrate concentration (M) vs Velocity of Rection (μM/min).

ideally be the case as $1/\mathrm{V_{max}}$ of both the curves (inhibition vs. non-inhibition) should be equal.

Since we already have the two $\mathrm{K_m}$ values, we only need to calculate the two $\mathrm{K_i}$ values which we can obtain using the following equation:

$$\alpha = 1 + \frac{[I]}{K_i}$$

Where,

$$\alpha = \frac{K'_m}{K_m}$$

Where the K'_m is the value of the Michaelis constant of the inhibitor reaction and K_m is the value at the non-inhibitor reaction. By plugging,

$$K'_m = 0.00237, K_m = 0.000211 \Rightarrow \alpha = 11.23$$

We know that 1 mL of 0.033 M of KH_2PO_4 was added to 10 mL of citrate buffer to make 11 mL of solution. The resulting concentration of Potassium Phosphate ([I]) = 0.003 M.

Since we know [I], and α, we can solve for the equation to obtain K_i:

$$K_i = \frac{[I]}{\alpha - 1}$$

$$K_i = \frac{0.003}{11.23 - 1} = 0.000293\,\mathrm{M}$$

1.4 Effect of Temperature on Enzymatic Activity

The Arrhenius equation is used to relate the first-order rate constant for a reaction (k) to the activation energy (Ea) for a chemical reaction.

$$k = Ae^{-Ea/RT}$$

After taking the natural log of both sides, we have:

$$ln(k) = ln(A) - (Ea/R).(1/T)$$

Where, y = $ln(k)$; x = $(1/T)$; slope (m) = $-(Ea/R)$; and y-intercept (b) = $ln(A)$. To obtain V_{max}, the following equation can be used: $V_{max} = k_{cat}[E]_T$. We have,

⇒ Molecular weight of enzyme: 150,000 g/mol.

⇒ Concentration of Enzyme (mg/L): 24.5 mg/L.

⇒ Concentration of Enzyme (mol/L): $24.5 \times 10^{-3}/150,000\,\text{mol/L} = 1.63 \times 10^{-7}\,\text{M} = 0.163\,\mu\text{M}$

We can then plot $\ln(K_{cat})$ vs 1/T to obtain the slope and intercept of the Arrhenius equation. To ensure we don't get very small values in the graph, the value of 1/T was multiplied by 1000 to obtain the following graph.

To obtain slope and intercept, algebraically, two points were taken and the equation was solved for two variables. The following equation of line was obtained:

$$y = -7235x + 26.355$$

Where,

$$\text{slope} = -7235;\ \text{intercept} = 26.355$$

We know that slope = $-\frac{E_a}{R}$ and Intercept = $\ln(A)$; Plugging, the values of $R = 8.314\,\text{J}\,\text{K}^{-1}\,\text{mol}^{-1}$ and slope = −7.235,

$$E_a = -(-7235 \times 8.314) = 6.01 \times 10^4\,\text{J/mol}$$

Similarly,

$$\ln(A) = 26.355$$

Table 3 Enzyme Activity at different Temperatures

Temp. (K)	Absorbance with Enzyme (OD)	Absorbance without Enzyme (OD)	Absorbance adjusted[1] (OD)	Velocity (μM/s)	K_{cat} (s^{-1})
288	0.110	0.010	0.100	0.57	3.49
293	0.170	0.012	0.158	0.91	5.58
298	0.190	0.006	0.184	1.06	6.50
303	0.388	0.016	0.372	2.16	13.2
308	0.576	0.014	0.562	3.27	20.0
313	0.690	0.028	0.662	3.86	23.6

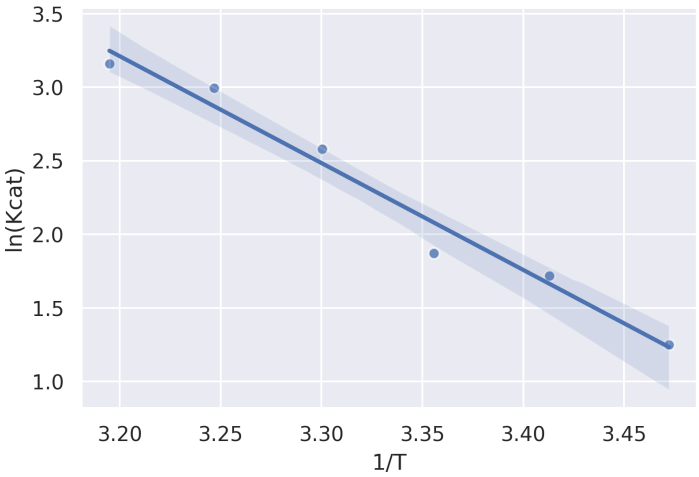

Fig. 5 The plot of $\ln(k_{\text{cat}})$ (s^{-1}) vs. $1/T$ (K^{-1})

$$A = 2.71 \times 10^{11}\,\text{s}^{-1}$$

1.5 Sources of Error

1. One of the most prominent sources of error is the inaccuracy of measurement of time of incubation to calculate the velocity of the reaction.
2. Followingly, due to fewer observations, the calibration curve obtained might not be perfect.
3. Similarly, due to fewer data points, generating a line or curve of best fit could be difficult.