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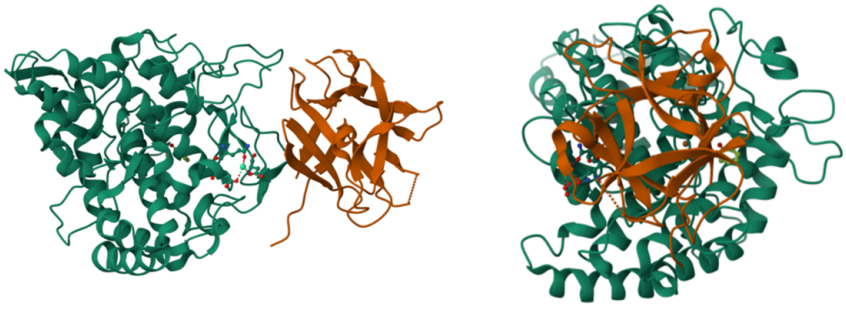
**Abstract**

The kinetics of the oxidation of catechol by mushroom tyrosinase-PPO3 (MT-PPO3) and the effects of p-hydroxybenzoic acid (p-HBA) and a commercial vinegar sample on MT-PPO3 kinetics was studied through visible spectrophotometry at 410 nm. Results showed that the reaction had $v_{max}$ = 3.99 (± 0.73) × $10^{-5}$ mM/s and $K_m$ = 3.69 (± 0.69) mM, both of which are consistent with ranges observed in literature. Both p-HBA and vinegar were found to significantly increase reaction rate but affect the enzyme's activity similar to mixed inhibitors.

**Introduction**

Mushroom Tyrosinase-PPO3 (MT-PPO3) is a heterodimeric polyphenol oxidase that utilizes copper. MT-PPO3 can be found in mushrooms, although tyrosinase can be found in other organisms and plays a role in the creation of melanin. It catalyzes the ortho-hydroxylation of phenols to catechols and the dehydrogenation of the produced catechols to ortho-quinones via two copper ions within the active site. (Duckworth & Coleman, 1970) Due to this function, it can catalyze the oxidation of tyrosine to dopaquinone in a 2-step reaction.



(i) (ii)

*Figure 1.* Heterodimeric Structure of MT-PPO3 in (i) Front View and (ii) Side View with Two Subunits, Polyphenol Oxidase 3 (PO3) and Lectin-like Fold Protein (LFP), Highlighted in Teal and Orange, Respectively (*PDB 2y9x,* n.d.)

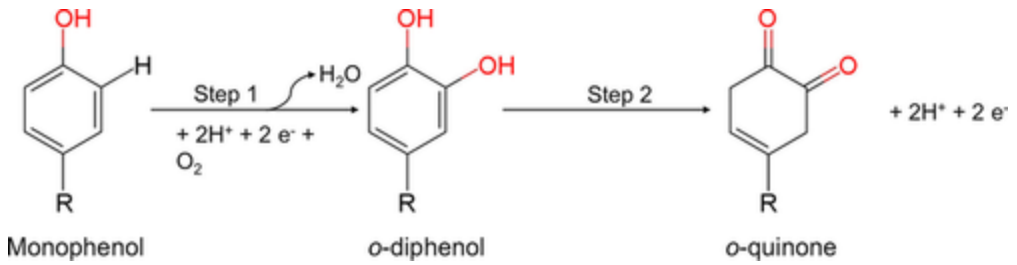



*Figure 2.* Tyrosinase-Catalyzed Oxidation of Catechol to Benzoquinone (Lai *et al*., 2017)

Enzymes catalyze a reaction by lowering the activation energy required for the specified reaction to proceed but they also change its kinetics. The Michaelis-Menten Equation is used to determine the kinetics of an enzyme-catalyzed reaction (equation 1).

$$V_0 = \frac{V_{max}[S]}{K_M + [S]} \qquad (1)$$

This equation is composed of different terms, where $V_0$ is the initial velocity of the reaction, $V_{max}$ is the maximum reaction velocity, $K_M$ is the Michaelis-Menten constant and [S], the concentration of the substrate. The kinetics of tyrosinase vary with pH, oxygen concentration and the presence of inhibitors.

The objective of this experiment is to determine (i) the kinetics of crude MT-PPO3 or tyrosinase with a catechol substrate without inhibitors, (ii) the effect of p-hydroxybenzoic acid (p-HBA) and a commercial vinegar sample on the kinetics of MT-PPO3, and (iii) the $v_{max}$ and $K_m$ values for the oxidation of catechol by MT-PPO3.

## Methods

A 4.80 mM catechol solution was prepared and serially diluted to prepare five different solutions. Table 1 collates the different concentrations within each well of the 96 well plate. Along with that, the absorbance was measured in a UV-Visible spectrophotometer at 410 nm. A multichannel pipettor was employed to deliver 50 µL of MT-PPO3-containing tyrosinase extract.

*Table 1.* Information Related to Sample Preparation

| Category | # Trials per Conc. | # Conc. Values | Initial mM Catechol | µL Catechol Added | µL Tyrosinase Extract Added | µL p-HBA or Vinegar Added | Diluted mM Catechol |
|---|---|---|---|---|---|---|---|
| Tyrosinase-only Samples | 3 | 6 | 4.8, 2.4, 1.2, 0.6, 0.3 | 150 | 50 | - | 3.6, 1.8, 0.9, 0.45, 0.225 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Tyrosinase-p-HBA Samples | 3 | 6 | 4.8, 2.4, 1.2, 0.6, 0.3 | 150 | 50 | 50 (p-HBA) | 2.88, 1.44, 0.72, 0.36, 0.18 |
| Tyrosinase-Vinegar Samples | 3 | 6 | 4.8, 2.4, 1.2, 0.6, 0.3 | 150 | 50 | 50 (Vinegar) | 2.88, 1.44, 0.72, 0.36, 0.18 |

*Table 2.* Summary of Mathematical Models Used for Determination of Reaction Rate and Michaelis-Menten Parameters

| Model # | X-Value | Y-Value | Equation | Description and Legend |
|---|---|---|---|---|
| 1 | Elapsed time since addition of tyrosinase (s) | Absorbance of Sample (A.U.) | $y = A - Be^{-x/C}$ | Exponential fitting of absorbance at 410 nm vs time data of each sample (*A, B, C* = expo. parameters) |
| 2 | Elapsed time since addition of tyrosinase (s) | Absorbance of Sample (A.U.) | $\frac{dy}{dx} = \left(\frac{B}{C}\right) e^{-x/C}$ | Equation used for estimating the absorbance rate (*dy/dx*) at *x* = 300 s and 410 nm wavelength from model #1 (*B, C* = expo. parameters in model #1) |
| 3 | Reaction rate at 300 s (mM/s) | Absorbance rate (A.U./s) | $y = \frac{xy_{max}}{A}$ | Equation for calculating reaction rate *y* from absorbance rate *x* (*A* = expo. parameter in model #1) ($y_{max}$ = max. conc. of product) |
| 4 | Initial concentration of catechol (mM) | Reaction rate at 300 s (mM/s) | $y = A' - B'e^{-x/C'}$ $v_0 = A' - B'e^{-c/C'}$ | Exponential fitting of reaction rate at 300 s vs initial concentration of catechol (*A', B', C'* = expo. parameters) ($v_o$ = initial reaction velocity) (*c* = concentration of catechol) |
| 5 | Reciprocal of the initial concentration of catechol ($mM^{-1}$) | Reciprocal of the reaction rate at 300 s (s/mM) | $y = \left(\frac{1}{v_{max}}\right) + \left(\frac{K_M}{v_{max}}\right)(x)$ $\frac{1}{v_0} = \left(\frac{1}{v_{max}}\right) + \left(\frac{K_M}{v_{max}}\right)\left(\frac{1}{c}\right)$ | Equation for the Lineweaver-Burk plot under the Michaelis-Menten model for tyrosinase kinetics ($v_{max}$ = maximum reaction velocity) ($K_M$ = Michaelis-Menten constant) ($v_o$ = initial reaction velocity) (*c* = concentration of catechol) |

**Results**

This portion of the paper describes the results taken from the experiment on enzyme kinetics. Further, the data was calculated using a program from Python 3.7 created by Sanguyo (2024). The full calculations can be found in the appendix where the spreadsheet link is located.

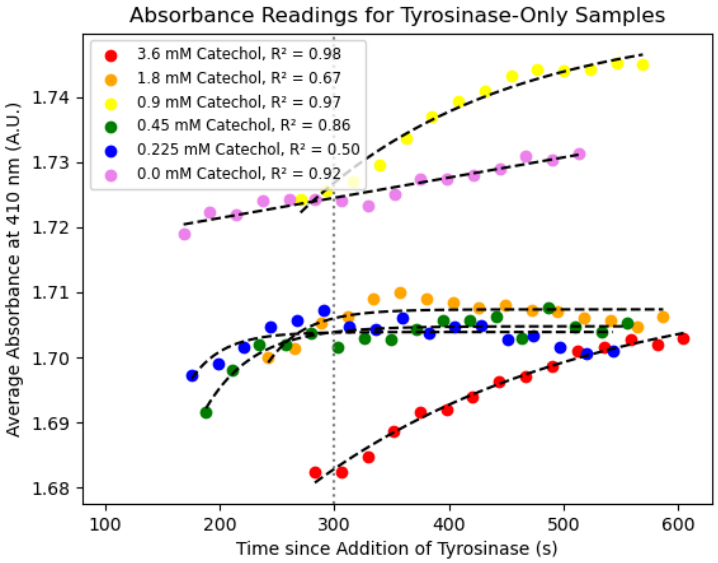



*Figure 3.* Trial-Averaged Absorbance-Time Plot for Tyrosinase-only Samples at 410 nm Wavelength and Differing Catechol Concentrations (Note: Black dashed lines refers to model #1.)

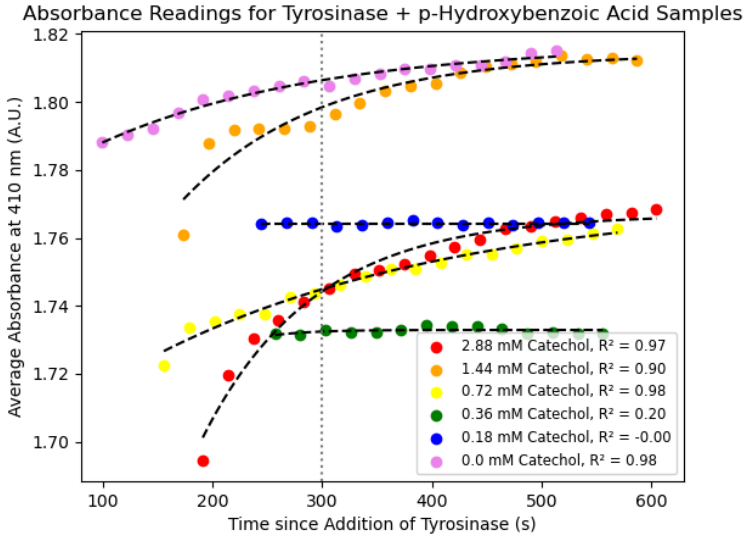



*Figure 4.* Trial-Averaged Absorbance-Time Plot for Tyrosinase-p-HBA Samples at 410 nm Wavelength and Differing Catechol Concentrations (Note: Black dashed lines refers to model #1.)

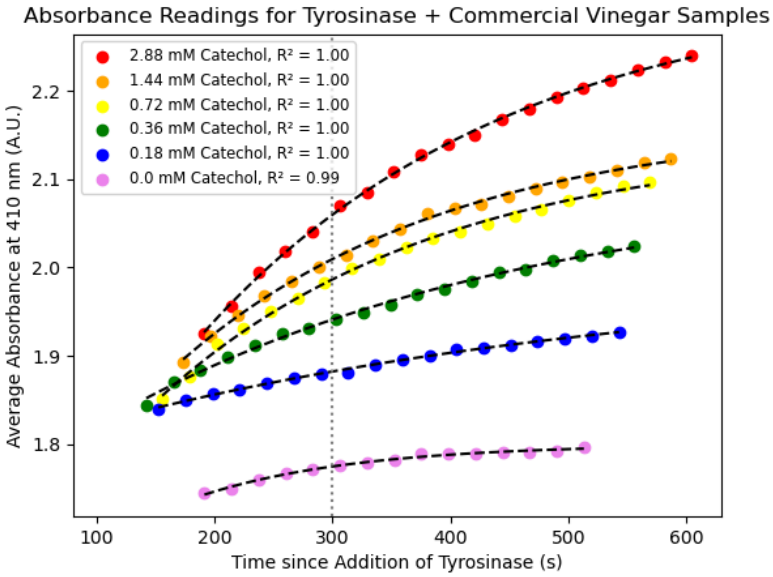



*Figure 5.* Trial-Averaged Absorbance-Time Plot for Tyrosinase-Vinegar Samples at 410 nm Wavelength and Differing Catechol Concentrations (Note: Black dashed lines refers to model #1.)

*Table 3.* Reaction Rates at 300 s for Samples at Differing Catechol Concentrations Estimated Using Models #1, 2, and 3

| Category | Variable | Data Point 1 | Data Point 2 | Data Point 3 | Data Point 4 | Data Point 5 |
|---|---|---|---|---|---|---|
| Tyrosinase-only Samples | Reaction rate (mM/s) [c] | 0.000253 | $4.44 \times 10^{-5}$ | $7.34 \times 10^{-5}$ [a] | $5.52 \times 10^{-6}$ | $4.12 \times 10^{-7}$ [a] |
| | mM Catechol | 3.6 | 1.8 | 0.9 [a] | 0.45 | 0.225 [a] |
| Tyrosinase-p-HBA Samples | Reaction rate (mM/s) [c] | 0.000360 | $9.93 \times 10^{-5}$ | $3.91 \times 10^{-5}$ | $2.65 \times 10^{-6}$ [a] | 0.00 [b] |
| | mM Catechol | 2.88 | 1.44 | 0.72 | 0.36 [a] | 0.18 [a] |
| Tyrosinase-Vinegar Samples | Reaction rate (mM/s) [c] | 0.00123 | 0.000448 | 0.000222 | $7.67 \times 10^{-5}$ | $2.12 \times 10^{-5}$ |
| | mM Catechol | 2.88 | 1.44 | 0.72 | 0.36 | 0.18 |

[a] Data points were excluded from the Lineweaver-Burk plot due to not following a linear trend.
[b] Data point was excluded from analysis since reaction rates cannot be zero for the Lineweaver-Burk Plot.
[C] Reaction rates were computed by computing the absorbance rate via model #2 and converting to reaction rate via model #3. This process involves relating reaction rate to the enzyme activity of tyrosinase (Chikezie, 2015; Flurkey & Inlow, 2016).

*Table 4.* Parameters for Model #4 in Fitting Reaction Rate with Catechol Concentration

| Category | Parameter A' | Parameter B' | Parameter C' | $R^2$ Value |
|---|---|---|---|---|
| Tyrosinase-only Samples | $-1.77 \times 10^{-5}$ | $-2.48 \times 10^{-5}$ | -1.51 | 0.942 |
| Tyrosinase-p-HBA Samples | $-7.78 \times 10^{-5}$ | $-7.14 \times 10^{-5}$ | -1.59 | 0.998 |
| Tyrosinase-Vinegar Samples | -0.00075 | -0.00074 | -2.93 | 0.999 |

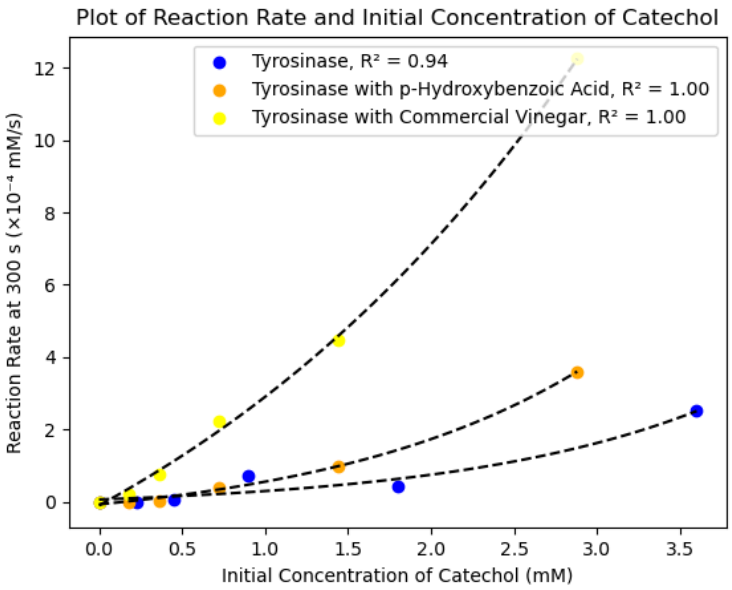



*Figure 6.* Plot of Reaction Rate at $t$ = 300 s with Respect to Initial Concentration of Catechol (Note: Black dashed lines refers to model #4.)

*Table 5.* Parameters for Model #5 and Michaelis-Menten Parameters for Lineweaver Burk Plot

| Category | Slope | Intercept | $R^2$ Value | Apparent $v_{max}$ *(mM/s)* | Apparent $K_m$ *(mM)* |
|---|---|---|---|---|---|
| Tyrosinase-only Samples | 9.26 (± 0.34) $\times 10^4$ | -2.51 (± 0.46) $\times 10^4$ | 0.9986 | 3.99 (± 0.73) $\times 10^{-5}$ | 3.69 (± 0.69) |
| Tyrosinase-p-HBA Samples | 2.19 (± 0.03) $\times 10^4$ | -4.97 (± 0.03) $\times 10^3$ | 0.9998 | 2.01 (± 0.12) $\times 10^{-4}$ | 4.41 (± 0.27) |
| Tyrosinase-Vinegar Samples | 8.96 (± 0.11) $\times 10^3$ | -5.73 (± 0.32) $\times 10^3$ | 0.9552 | 1.75 (± 0.98) $10^{-4}$ | 1.56 (± 0.90) |

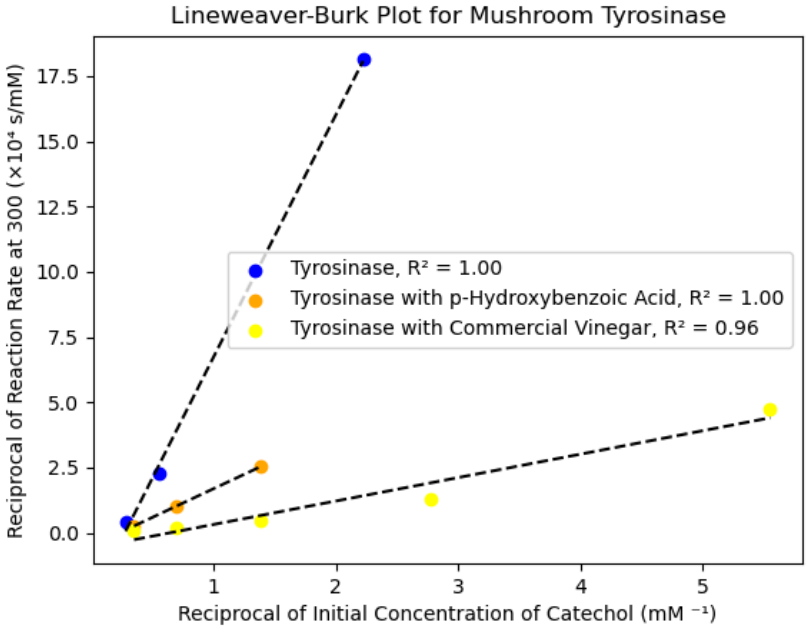



*Figure 7.* Lineweaver-Burk Plot for Tyrosinase-catalyzed Oxidation of Catechol with Outliers at Low Catechol Concentrations Excluded (Note: Black dashed lines refers to model #5.)

*Table 6.* T-Test Results for Identifying Significant Differences in Slope and Intercept of Lineweaver-Burk Plots at 95% Confidence Level

| **Condition 1** | **Condition 2** | **t Value for Slopes** | **p Value for Slopes** | **Significantly different slopes?** | **t Value for Intercepts** | **p Value for Intercepts** | **Significantly different intercepts?** | **Inhibition Type** |
|---|---|---|---|---|---|---|---|---|
| Tyrosinase | Tyrosinase with p-HBA | 35.41 | $3.80 \times 10^{-6}$ | Yes | 7.581 | 0.00162 | Yes | Mixed |
| Tyrosinase with p-HBA | Tyrosinase with Vinegar | 24.25 | $3.23 \times 10^{-7}$ | Yes | 0.5243 | 0.619 | No | N/A |
| Tyrosinase with Vinegar | Tyrosinase | 40.81 | $1.45 \times 10^{-8}$ | Yes | 6.425 | 0.000672 | Yes | Mixed |

## Discussion

Figures 3, 4, and 5 show plots of model #1 and the time-averaged absorbances $A_{av}$ of the tyrosinase-catechol samples under different conditions. Upon applying the method of Chikezie (2015), the reaction rates $v_{300}$ at 300 s since addition of tyrosinase for all catechol concentrations $c_{catechol}$ are summarized in Table 3. Table 4 and figure 6 show the relationship between $v_{300}$ and $c_{catechol}$. As indicated by the high $R^2$ values for model #1 in figures 3, 4, and 5, $A_{av}$ followed a trend of exponential approach toward maximum absorbance at $A_{av} = A$. Thus at 300 seconds, the reaction behaves as a first-order reaction but approaches completion. However, table 4 and figure 6 suggest that $v_{300}$ follows an exponential trend with $c_{catechol}$, rather than a hyperbolic trend as suggested by the Michaelis-Menten model. One explanation is that dihydric phenols such as catechol gradually activate tyrosinase over a lag period, resulting in a sigmoidal relationship for reaction progress in both time and concentration (Falguera *et al.,* 2010). According to Oyama *et al.,* (2023), this sigmoidal relationship reflects the strength of the interactions of the two phenolic -OH groups with $H_2O$, $Cu^{2+}$, and polar uncharged residues on the enzyme's active site. It may be inferred that $v_{300}$ for tyrosinase might follow a sigmoidal relationship with $c_{catechol}$, of which may appear to be an exponential trend at low concentrations as observed.

The Lineweaver-Burk plot for the tyrosinase-catalyzed oxidation of catechol, the maximum reaction velocity $v_{max}$, and Michaelis-Menten constant $K_m$ under each condition are summarized in figure 7 and table 5, respectively. The type of inhibition and the results of the two-samples *t*-test between the slopes and intercepts in the Lineweaver-Burk plot are shown in table 6. From figures 6 and 7, the addition of p-HBA and vinegar sample to the tyrosinase-catechol mixture raised $v_{300}$ and significantly lowered the slope of the reaction's Lineweaver-Burk plot ($t > 35$, $p << 0.05$ at 95% level). Additionally, the reaction was found to have $v_{max} = 3.99\ (\pm\ 0.73) \times 10^{-5}$ mM/s and $K_m = 3.69\ (\pm\ 0.69)$ mM, both of which change when p-HBA or vinegar is added. In comparison with Pretzler & Rompel (2024), the $K_m$ value for MT-PPO3 is within the range of 1.7 to 4.7 mM for small dihydric phenols. The magnitude of $v_{max}$ is consistent with Shahpar *et al.* (2024), which reported $v_{max}$ ranging from 3.3 to $8.2 \times 10^{-5}$ mM/s for MT diphenolase. Nevertheless, the rate-accelerating behaviors of p-HBA and vinegar are inconsistent with the inhibiting behaviors of stilbenes, Kojic acid, and catechol derivatives (Flurkey & Inlow, 2017; Oyama *et al.,* 2023; Xie *et al.,* 2007). One reason is that p-HBA lowers pH and can be oxidized by MT. According to Maria-Solano *et al.* (2016), lowering the pH causes a Glu residue on its active site to be protonated. This promotes the entry and subsequent oxidation of catechol and p-HBA into light-absorbing quinones due to enhanced H-bonding. Vinegar may also contain phenols that contribute to the detected quinones and lowering the pH to alter the enzyme's catalytic activity, resulting in an effect similar to mixed inhibition.

For this experiment, there are notable sources of error that might affect the reliability of the results. These include the improper delivery of liquids during sample preparation especially at low concentrations, the lack of analyte selectivity in quantification of product at 410 nm wavelength, and non-homogeneity of the samples (Flurkey & Inlow, 2017).

**Answers to Questions**

The slope for each concentration and each enzymatic reaction can be shown in the sample calculation portion. The slope and the intercept of each reaction can also be seen in the excel file (refer to appendix 1 and sample calculation 1, 2 and 3) (AtQ#1). The Michaelis-Menten parameters (refer to Table 5) and Lineweaver-Burk plot (refer to Figure 7) were also generated and the plot was placed in the results section of the paper (AtQ#2, AtQ#3). Through the two plots, the $K_m$ and $V_{max}$ were calculated. The Lineweaver-Burk plot gave the slope and intercept of the reactions which were essential in determining $K_m$ and $V_{max}$. Both values are summarized in table 5 and calculated in sample 1, 2 and 3 for tyrosinase, tyrosinase with p-hydroxybenzoic acid, and tyrosinase with commercial vinegar respectively. The type of inhibition was identified based on significant differences in the slopes and intercepts of the Lineweaver-Burk plots, as shown in Table 6 (AtQ#4).

**Sample Calculations**

Most of the calculations regarding the kinetics of mushroom tyrosinase $PPO_3$ were performed in a program developed by Sanguyo (2024) using the programming language Python 3.7. The data was summarized in CSV files and processed into spreadsheets via MS Excel.

*Link to Program Used for Computation (Sanguyo, 2024):*
https://github.com/NotAMadTheorist/Data-Analysis-for-Kinetics-of-Mushroom-Tyrosinase

The slope, intercept, $V_{max}$, $K_m$ along with their corresponding uncertainty values can be viewed in the excel and the link shown above. Additionally, the computation process is outlined to provide a better process on how the values were computed.

*Outline of Computation Process*

1. For each category and concentration:
    a. Get the average absorbance of the three trials at each time they were recorded.
    b. Add the computed delay between the actual addition of tyrosinase and time of absorbance measurement to each time value.
    c. Exclude the first *n* trials that do not fit into model #1, including decreasing absorbance values over time.
    d. Using $x$ = time value and $y$ = average absorbance, fit points $(x, y)$ into model #1 via "scipy.optimize", and compute parameters $A$, $B$, and $C$.
2. For each category:
    a. Plot model #1 and its respective data points to yield Figures 1, 2, and 3.
    b. Using model #2, estimate the absorbance rate at each concentration at 300 s.
    c. Convert absorbance rate to reaction rate at 300 s using model #3.
    d. With $x$ = catechol concentration and $y$ = reaction rate at 300 s, fit points $(x, y)$ into model #4 via "scipy.optimize", and compute parameters $A'$, $B'$, and $C'$.
    e. Plot model #4 and its respective data points to yield Figure 4.

f. Exclude trials which do not fit into model #5, including data points that have zero reaction rates and/or concentrations.
g. Compute the reciprocals of catechol concentration and of reaction rate at 300 s.
h. With $x$ = 1 / catechol concentration and $y$ = 1 / reaction rate at 300 s, fit points ($x$, $y$) into model #5 via "scipy.optimize", and compute slope, intercept, apparent $v_{max}$, apparent $K_m$, and their respective uncertainties.
i. Plot model #5 and its respective data points to yield Figure 5.

3. For each pair of conditions:
   a. Perform a two-samples t-test between the slopes and intercepts from Model #5, and compute the $t$ and $p$ values.
   b. Test if $p > 0.05$. If true, there is a significant difference in the slopes or intercepts. Otherwise, there is no significant difference.

***Sample Calculation of Initial Reaction Rate***

Parameters: A = 1.712059508, B = 0.10037595, C = 242.9362381, x = 3.6 mM

$$\frac{dy}{dx} = (\frac{B}{C})e^{-x/C} \; at \; x \; = \; 300 \; s$$

$$\frac{dy}{dx} = (\frac{0.10037595}{242.9362381})e^{-300/242.9362381}$$

$$\frac{dy}{dx} \; = \; 1.20 \; x \; 10^{-4}$$

$$y \; = \; \frac{xy_{max}}{A} \; ; \; where \; x \; is \; concentration \; of \; catechol$$

$$y \; = \; \frac{3.6 \; x \; 1.20 \; x \; 10^{-4}}{1.712059508}$$

$$y \; = \; 2.52 \; x \; 10^{-4} \; mM/s$$

***Sample Calculation of Michaelis-Menten Parameters $K_m$ and $v_{max}$***

In calculating the Michaelis-Menten parameters, the slope and the intercept in the form of y = bx + a, where b is the slope and a is the y-intercept. The calculations listed below are divided into three different samples: (1) Tyrosinase only, (2) Tyrosinase with p-Hydroxybenzoic acid, and (3) tyrosinase with commercial vinegar.

**Sample 1.** Tyrosinase

Slope: 92617.2
Intercept: -25082.1

$V_{max}$ calculation

$$\frac{1}{V_{max}} = intercept$$

$$\frac{1}{V_{max}} = |-25\,082.1|$$

$$\frac{1}{25\,082.1} = V_{max}$$

$$V_{max} = 3.98\,x\,10^{-5}\,mM/s$$

$K_m$ calculation

$$\frac{K_m}{V_{max}} = slope$$

$$\frac{K_m}{3.98\,x\,10^{-5}} = 92\,617.2$$

$$K_m = 92\,617.2\,x\,3.98\,x\,10^{-5}$$

$$K_m = 3.6926\,mM$$

**Sample 2.** Tyrosinase with p-Hydroxybenzoic Acid
Slope: 21932.47
Intercept: -4969.6

$V_{max}$ calculation

$$\frac{1}{V_{max}} = intercept$$

$$\frac{1}{V_{max}} = |-4969.6|$$

$$\frac{1}{4969.6} = V_{max}$$

$$V_{max} = 2.01\,x\,10^{-4}\,mM/s$$

$K_m$ calculation

$$\frac{K_m}{V_{max}} = slope$$

$$\frac{K_m}{2.01\,x\,10^{-4}} = 21932.47$$

$$K_m = 21932.47 \; x \; 2.01 \; x \; 10^{-4}$$

$$K_m = 4.4133 \; mM$$

**Sample 3.** Tyrosinase with Commercial Vinegar

Slope: 8963.437

Intercept: -5728.36

$V_{max}$ calculation

$$\frac{1}{V_{max}} = intercept$$

$$\frac{1}{V_{max}} = |-5728.36|$$

$$\frac{1}{5728.36} = V_{max}$$

$$V_{max} = 1.75 \; x \; 10^{-4} \; mM/s$$

$K_m$ calculation

$$\frac{K_m}{V_{max}} = slope$$

$$\frac{K_m}{1.75 \; x \; 10^{-4}} = 8963.437$$

$$K_m = 8963.437 \; x \; 1.75 \; x \; 10^{-4}$$

$$K_m = 1.5647 \; mM$$

## Conclusion

The kinetics of the oxidation of catechol into 1,2-benzoquinone by mushroom tyrosinase-PPO3 (MT-PPO3) and the effects of p-hydroxybenzoic acid (p-HBA) and a commercial vinegar sample was studied at initial catechol concentrations from 0.36 mM to 3.6 mM while employing quantification of product via visible spectrophotometry at 410 nm. Results showed that the reaction rate at 300 seconds after addition of crude MT-PPO3 had an exponential trend with catechol concentration ($R^2 \geq 0.94$). The reaction had a maximum velocity of $v_{max}$ = 3.99 (± 0.73) × $10^{-5}$ mM/s and a Michaelis-Menten constant of $K_m$ = 3.69 (± 0.69) mM, both of which are consistent with typical ranges observed in literature. Finally, p-HBA and vinegar were found to have significantly increased the reaction rate with effects similar to that of a mixed inhibitor. These results were related to several features of the active site in the MT-PPO3 enzyme, including the binding of phenolic -OH to $H_2O$, bound $Cu^{2+}$, and polar residues, the

protonation of a Glu residue at lowered pH, and their effects on enzyme activity. As recommendation, future studies may explore on determining the kinetics of MT-PPO3 on other phenolic substrates, employing the use of more accurate sample preparation and quantification methods in determining enzyme kinetics, and relating further the effects of environmental factors such as pH on the enzyme activity of MT-PPO3.

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## Appendix I

Link to the Full Calculations of the Data:

[CHEM 151.02 LAB] Alfonso, Roxas, Sanguyo, Uy - Kinetics Data Sheet for Expt 3