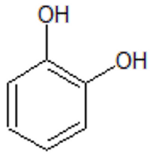
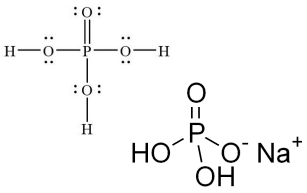
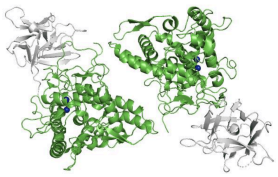
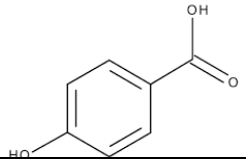
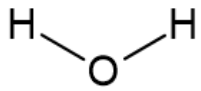
Enzyme Kinetics October 08, 2024
Alfonso, Roxas, Sanguyo, Uy

Objectives:
This experiment aims to:

I. determine the Michaelis-Menten constant (K_m) and the maximum rate of reaction done with tyrosinase (V_{max}) via spectrophotometry
II. determine the type of inhibition done (competitive, non-competitive, or mixed) by p-hydroxybenzoic acid and a selected household inhibitor on tyrosinase

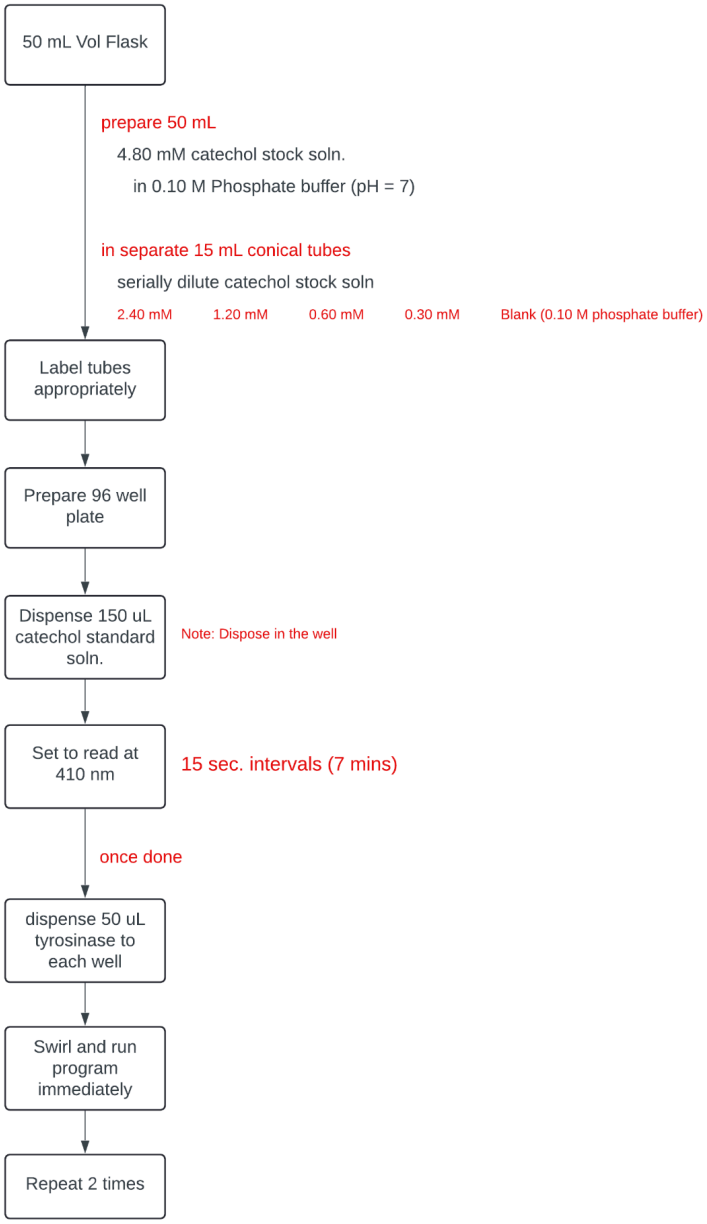
Reagents:

Reagent Name and Structure	***Physical and Chemical Properties***	***Safety Precautions***
4.80 mM catechol stock solution 	Brown powder MP: 100ºC Density: 1.34 g/cm^3 at 15ºC MW = 110.11 g/mol Absorption maximum at 280 nm; does not absorb significantly at 410 nm	Causes serious skin and eye irritation if in contact Wear protective gears (i.e. protective gloves, lab gown) Rinse immediately with water if in contact Disposal: G704
0.10 M pH 7 Phosphate (H_3PO_4 and NaH_2PO_4) 	- odorless liquid - density: 1.91 g/cm^3	Rinse contaminated skin and eyes with running water Disposal: B204 (if excess is <pH7)
Tyrosinase 	Solid state MP: 89-94ºC catalyzes the oxidation of catechol to 1,2-benzoquinone, of which absorbs 410 nm strongly	Rinse eye and skin with running water if contaminated Avoid inhaling the compound Disposal: M503
0.30 mM p-hydroxybenzoic acid 	Beige powder solid Odorless MP: 214ºC MW = 138.12 g/mol	Hazardous combustion compounds Rinse cautiously with water if ever the skin and eyes are contaminated Disposal: B208

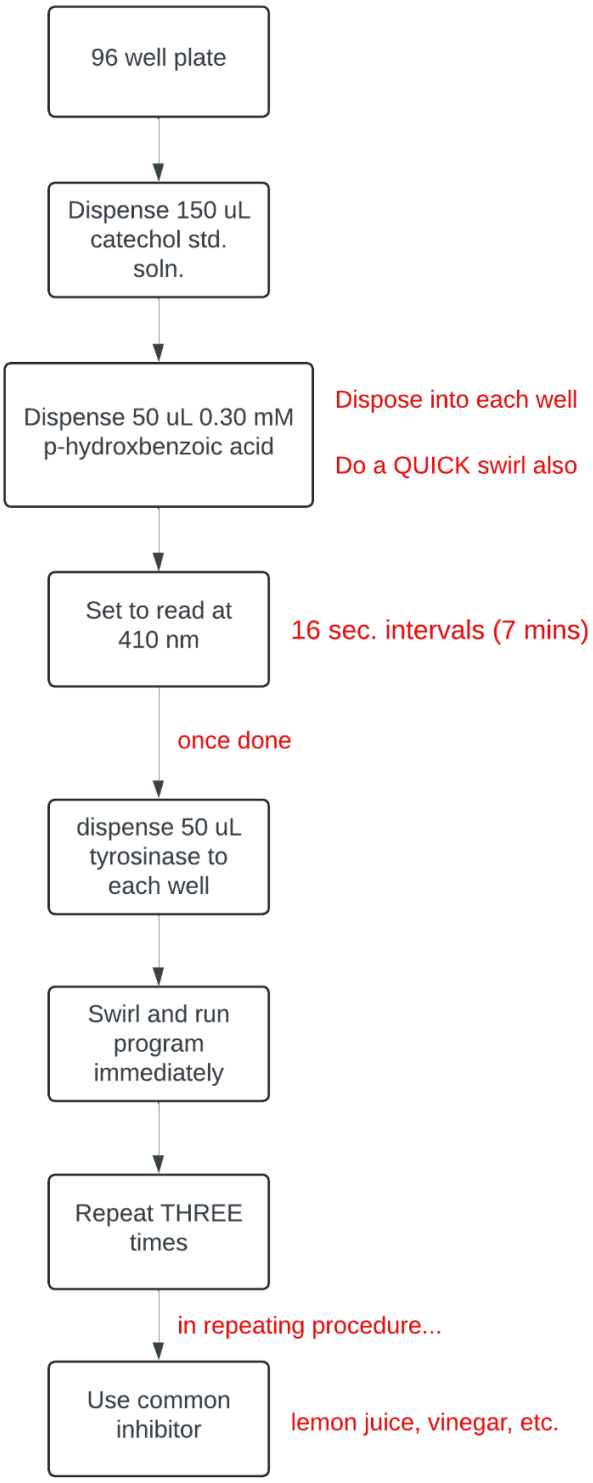
Deionized water 	- colorless, odorless liquid - BP: 100ºC - MW: 18.02 g/mol	Non-irritant, generally safe to handle Disposal: sink

Procedures:

Part A. DETERMINATION OF Km and Vmax



Part B. ENZYME INHIBITION STUDIES



Calculations:

I. Calculations for the Preparation of Catechol Stock Solutions

To prepare 2.40, 1.20, 0.60, and 0.30 mM catechol solutions from 4.80 mM stock catechol, given that each solution is limited to 15 mL:

- For 2.40 mM solution (A): 7.5 mL stock + 7.5 mL DI H_2O
- For 1.20 mM solution (B): 7.5 mL soln. A + 7.5 mL DI H_2O
- For 0.60 mM solution (C): 7.5 mL soln. B + 7.5 mL DI H_2O
- For 0.30 mM solution (D): 7.5 mL soln. C + 7.5 mL DI H_2O

These values are obtained from the dilution equation:

$$M_1V_1 = M_2V_2$$

Example for 2.40 mM solution (A):

$V_1 = M_2V_2/M_1$ = (2.40 mM)(15 mL) / (4.80 mM) = 7.5 mL Stock

II. Calculation of Molar Concentration for Each Batch of Solutions

Assuming solution volumes are additive, the volumes of each standard solution in the 96-well plate are:

- For tyrosinase-only batch: V = 150 µL + 50 µL = 200 µL
- For tyrosinase-and-inhibitor batch: V = 150 µL + 50 µL + 50 µL = 250 µL

Because 150 µL of the catechol solution is added per well, and that the solution is diluted to 200 or 250 µL, the concentration of catechol is decreased, also in accordance to the dilution equation:

$$M_1V_1 = M_2V_2$$

Example for 2.40 mM solution (A) in Tyrosinase-and-Inhibitor Batch:

$M_2 = M_1V_1/V_2$ = (2.40 mM)(150 µL) / (250 µL) = 1.44 mM Catechol

III. Notes and Formulas for Data Analysis

- The Beer-Lambert law can be used to quantify the molar concentration c of catechol before the reaction and 1,2-benzoquinone after the reaction. Since the absorbance A at 410 nm is measured, and 1,2-benzoquinone, but not catechol, absorbs light at 410 nm (large molar absorptivity ε), the reacted tyrosinase-only solutions obtained after 7 minutes can be set as the external standard solutions from which an external calibration curve may be obtained. It is assumed here that all the catechol is transformed to 1,2-benzoquinone and that the original concentration of catechol is equal to the final concentration of 1,2-benzoquinone. Note that b is path length through each well and A_0 is systematic error.

$$A = A_0 + \varepsilon bc$$

- Using the above equation, the molar concentrations of 1,2-benzoquinone for each well at each time value may be calculated. Once the concentration is plotted as y against time t for a particular solution (tyrosinase with or without inhibitor), the points may be fitted with an exponential model (in the form $y = A - Ae^{-kt}$) and the reaction velocity v may be calculated as the magnitude of the slope, $|dy/dt|$, as time x approaches zero.

$$v = \left| \lim_{t \to 0} \left[\frac{dy}{dt} \right] \right|$$

- The initial molar concentration of substrate, $[S]_0$ is equal to the initial concentration of catechol in each well, which is calculated from the concentrations of standards used based on the volume of the solution (see calculations II and table 3).

- Values of $1/v$ and $1/[S]_0$ are plotted against each other as y and x axes, respectively. This is called a Lineweaver-Burk plot. Then, the maximum velocity v_{max} and Michaelis-Menten constant K_M may be calculated according to the Lineweaver-Burk equation. The first equation is used for the tyrosinase-only samples, whereas the second equation is used for samples with tyrosinase and inhibitor (Atkins & de Paula, 2006)

$$\frac{1}{v} = \frac{1}{v_{\max}} + \left(\frac{K_{\mathrm{M}}}{v_{\max}} \right) \frac{1}{[\mathrm{S}]_0}$$

$$\frac{1}{v} = \frac{\alpha'}{v_{\max}} + \left(\frac{\alpha K_{\mathrm{M}}}{v_{\max}} \right) \frac{1}{[\mathrm{S}]_0}$$

- Then, the *1/v* vs *1/*$[S]_0$ plots of tyrosinase-only (TO) and tyrosinase-and-inhibitor (TI) samples may be compared. The type of inhibition may be identified as follows (Atkins & de Paula, 2006):
    - If the slope of plot TI is significantly greater than the slope of plot TO, the inhibitor is a **competitive inhibitor** for tyrosinase.
    - If the intercept of plot TI is significantly greater than the intercept of plot TO, the inhibitor is a **non-competitive inhibitor** for tyrosinase.
    - Finally, if both the slope and intercept of plot TI are significantly greater than the slope and intercept of plot TO, the inhibitor is a **mixed inhibitor** for tyrosinase.

Gantt Chart:

Each portion must be done after the previous step is finished; Each portion has its own allocated time

Procedure	Time Slot														
Preparation of Glassware															
Obtain Reagents															
Prep Catechol Stock Solution and Blank															
Serial Dilution of Stock Solution															

Insert Solutions + Inhibitor to Well Plates															
Travel Time															
Addition of Tyrosinase															
Spectrophotometry															

Data and Observations:

- Setup of the 96-Well Plate (indicates where each solution is located in)

Table 1. *Measurements Taken During Preparation of Catechol Solutions*

Preparation of Catechol Stock Solution	
Parameter	Measurement
Solution pH	

Table 2. *Setup of The 96-Well Plate*

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Table 3. *Legend of Catechol-containing External Standard Solutions to be Prepared*

Aspect	*Label*	*Solution A*	*Solution B*	*Solution C*	*Solution D*

Catechol	150 µL mM Catechol Added	2.40	1.20	0.60	0.30
	Initial mM Catechol for Tyrosinase-Only Batch	1.80	0.90	0.45	0.225
	Initial mM Catechol for Tyrosinase-and-Inhibitor Batch	1.44	0.72	0.36	0.18
Total Volume of Solution	Total Volume (µL) for Tyrosinase-only Batch	200	200	200	200
	Total Volume (µL) for Tyrosinase-and-Inhibitor Batch	250	250	250	250

Table 4. *Absorbance of Catechol-1,2-Benzoquinone Reaction Mixtures at 410 nm for Tyrosinase-Only Batch*

	Absorbance at 410 nm (Tyrosinase Only)			
Elapsed Time (s)	*Solution A*	*Solution B*	*Solution C*	*Solution D*
0				
15				
30				
45				
60				
⋮	⋮	⋮	⋮	⋮
420				

Table 4. *Absorbance of Catechol-1,2-Benzoquinone Reaction Mixtures at 410 nm in the Presence of Tyrosinase and 0.30 M p-Hydroxybenzoic Acid*

	Absorbance at 410 nm (Tyrosinase and p-HBA)			
Elapsed Time (s)	*Solution A**	*Solution B**	*Solution C**	*Solution D**
0				
16				
32				

48				
64				
⋮	⋮	⋮	⋮	⋮
432				

Table 5. *Absorbance of Catechol-1,2-Benzoquinone Reaction Mixtures at 410 nm in the Presence of Tyrosinase and a Household Inhibitor*

	Absorbance at 410 nm (Tyrosinase and _____)			
Elapsed Time (s)	*Solution A'*	*Solution B'*	*Solution C'*	*Solution D'*
0				
16				
32				
48				
64				
⋮	⋮	⋮	⋮	⋮
432				

Observations:

References:

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