Biology Higher Level: Internal Assessment

Research Topic:

Effect of ethanol (a non-competitive inhibitor) on the enzymatic activity of tyrosinase from *Agaricus bisporus* mushroom

Research Question:

What is the effect of varying ethanol concentrations (10%, 35%, 55%, 75%) on the concentration of oxygen evolved from the reaction between hydrogen peroxide and tyrosinase enzyme extracted from Indian edible mushroom *Agaricus bisporus* measured by an oxygen sensor over a period of 100 seconds?

Examination Session: May 2021

1: Introduction

I've loved eating mushrooms for a long time, but never once did I wonder what exactly in it is so important that it has been of scientific research interest. Reading about the wide range of real-life applications of tyrosinase to humans and the environment piqued my curiosity. More specifically, the fact that the properties of mushroom tyrosinase help predict those of human tyrosinase, thereby increasing its scope for research, urged me to delve deeper into this topic.

What fascinated me most about tyrosinase activity was when I read an article[1] explaining the key role of tyrosinase in melanin synthesis – especially how tyrosinase might play a significant role in neuromelanin formation in the human brain and cause neurodegeneration associated with Parkinson's and Huntington's disease. Its usage in various other fields of study like bioremediation[2] and biosynthesis of drugs also fascinated me. Additionally, tyrosinase inhibitors have recently gained scientific interest too as they are essential in the prevention of hyperpigmentation[3]. Experiments regarding the inhibitory effects of ethanol on tyrosinase activity have not been conducted even though it has been predicted to be an effective tyrosinase inhibitor. This stoked my curiosity, and I embarked on this experiment to determine if this prediction is, in fact, accurate.

2: Investigation

2.1: Background Information

Tyrosinase in daily applications

Tyrosinase, also known as polyphenol oxidase, is a copper-containing enzyme, which is found in microorganisms, animals, and plants. It is useful in different industries such as food, biomedical, pharmaceutical and cosmetics. The melanin pigment formed under the activity of tyrosinase is a good protection for mammals against UV radiations. It can be further applied for detoxification of water and soil (bioremediation), in biosynthesis of L-DOPA (a drug highly recommended for Parkinson's disease patients), and also as food additives.

Tyrosinase in Mushrooms

Among different sources of tyrosinase, mushroom tyrosinase from *Agaricus bisporus* has become increasingly popular as it is a cheap source of tyrosinase with a high homology to human tyrosinase. Because of its structural, functional and biochemical properties, mushroom tyrosinase has been studied extensively as a model system for catalytic activity, inhibitory effects and melanogenic studies.

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[1] Authors, A., & Pillaiyar, T. (n.d.). Skin whitening agents: Medicinal chemistry perspective of tyrosinase inhibitors. Retrieved January 14, 2021, from https://www.tandfonline.com/doi/full/10.1080/14756366.2016.1256882

[2] A process involving the use of microbes to remove contaminants from soil and water

[3] A condition in which skin patches become darker than the normal surrounding skin

When left for too long, mushrooms turn brown due to the accumulation of brown pigments known as melanins. Melanins are formed through the enzymatic oxidation of phenols (such as L-tyrosine) followed by a series of spontaneous oxidations and polymerizations. In mushrooms, melanins help the organism protect themselves from external biotic and abiotic factors.

Reaction Mechanism

Tyrosinase is an oxidoreductase[4] that has two activities in its catalytic reaction: (1) the reduction of monophenols (e.g. tyrosine), which is referred to as cresolase activity, and (2) the oxidation of diphenols (e.g. catechol), which is referred to as catecholase activity. Diphenols are better substrates of mushroom tyrosinases than monophenols. Monophenolase and diphenolase activity is demonstrated in *Figure 1*[5].

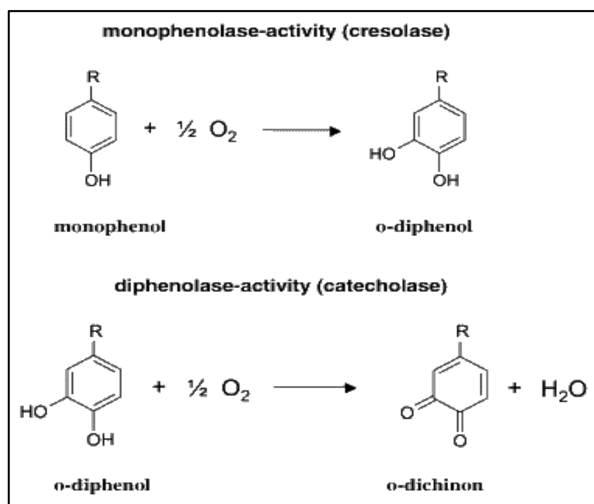


Figure 1: Cresolase and Catecholase activities of tyrosinase

Mushroom tyrosinase is found to exhibit activity similar to the catalase enzyme with hydrogen peroxide as the substrate. Hydrogen peroxide is able to act as both an oxidising and a reducing substrate, which makes it a highly suitable substrate for tyrosinase to carry out both its reduction and oxidation activities on.

Figure 2 displays the chemical reaction of the decomposition of hydrogen peroxide into water and oxygen.

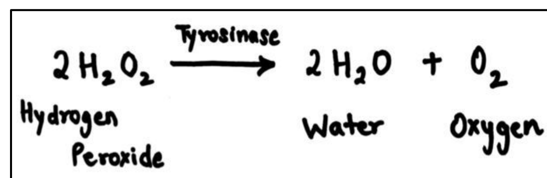


Figure 2: Decomposition of Hydrogen Peroxide

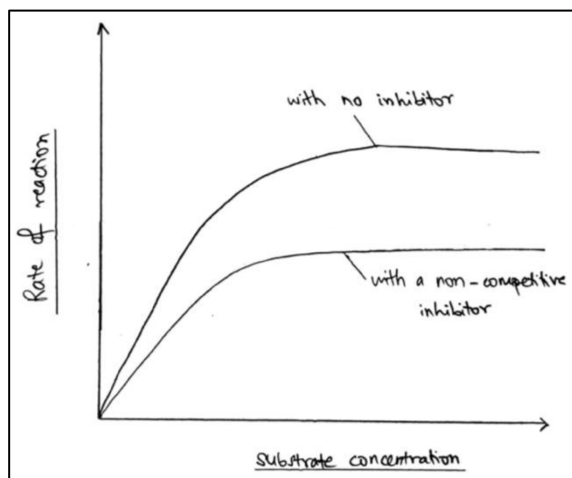
Tyrosinase Inhibition

Enzyme inhibitors are molecules that disrupt the normal reaction pathway between an enzyme and a substrate by binding to the enzyme, thereby reducing the rate of enzyme activity.

[4] Enzyme that catalyses the transfer of electrons from one molecule (electron donor) to another (electron acceptor)

[5] Melanin Pigment. (n.d.). Retrieved January 2, 2021, from http://2009.igem.org/Team:Cambridge/Project/Melanin

Alcohols like p-hydroxybenzyl alcohol (4HBA) inhibit the monophenolase activity of mushroom tyrosinase. When 4HBA binds with the enzyme, the conformation of the enzyme is altered and enzymatic activity decreases. This happens via non-competitive inhibition as conformational changes do occur. This type of inhibition is called non-competitive inhibition because the substrate and inhibitor are not competing for the same site. The inhibitor binds to the enzyme at the allosteric site[6], leading to changes in shape of the active site of the enzyme. As a result, the substrate cannot bind to the enzyme anymore. Therefore, even at high substrate concentrations, the enzyme activity is low. *Graph 1* shows the reaction pathway of non-competitive enzyme inhibition in comparison to a reaction pathway of no inhibition.



Graph 1: Non-competitive Enzyme Inhibition

Ethanol is a type of alcohol, that has proven to be one of the best inhibitors of catalase. Since catalase exhibits similar enzymatic activities as tyrosinase, ethanol is considered to be an inhibitor of tyrosinase as well. Therefore, ethanol has been used in this IA as the inhibitor between the reaction of tyrosinase and hydrogen peroxide, to test the effect of increasing concentrations of ethanol on the rate of tyrosinase inhibition.

2.2: Hypotheses

Null Hypothesis (H_0): As ethanol concentration increases, there is no effect on the concentration of oxygen evolved from hydrogen peroxide.

Alternate Hypothesis (H_1): As ethanol concentration increases, the concentration of oxygen evolved from hydrogen peroxide decreases.

2.3: Variables

Independent variable: Concentration of ethanol (0%, 10%, 35%, 55%, 75%)

Dependent variable: Concentration of oxygen evolved from the reaction between hydrogen peroxide as the substrate, tyrosinase as the enzyme and ethanol as the enzyme inhibitor.

[6] A site on the surface of the enzyme that is different from the active site

Controlled Variables:

Controlled Variable	Possible Effect on Investigation	Method of Control
Type of Mushroom	Different types of mushrooms would have different amounts and concentrations of tyrosinase in them	All tyrosinase extracts were made from the same species (*Agaricus bisporus*) and brand of mushrooms ('Champ fungi – white button mushroom')
Concentration and Volume of hydrogen peroxide	- Higher concentration of hydrogen peroxide would be too corrosive and dangerous to work with. - Varying concentrations and volumes would also interfere with results and give an unfair conclusion	All tests were conducted using 1 mL of 10% hydrogen peroxide solution
Volume and temperature of tyrosinase extract	Needs to remain constant for fair comparison of results	All tests were conducted using 15mL of tyrosinase extract from the same mushroom at room temperature
Volume and temperature of ethanol	Volume and temperature of ethanol need to be constant for the comparison of *only* the variable being investigated i.e. concentration	All tests were carried out using 1 mL of ethanol at room temperature
Time	The amount of time the reaction is allowed to proceed must remain constant so as to obtain conclusive results and make fair comparisons	All reactions were carried out for 100 seconds
pH	Since pH of the solution affects enzyme activity, it must be kept constant so only the inhibitory effect can be observed	2 mL of pH 7 buffer solution (optimum pH for tyrosinase)

Table 1: Controlled Variables in the Experiment

3: Procedure

3.1: Apparatus and Materials

Chemical/Apparatus	Quantity	Uncertainty
White *Agaricus bisporus* mushrooms	-	-
Knife	1	-
Data logger	1	± 4%
Hydrogen peroxide solution (concentration 10%)	10mL for each trial	-
Chilled ethanol of concentrations – 10%, 35%, 55%, 75%	1mL of each concentration	-
100 mL beakers	2	± 1 mL
Blender	1	-
Oxygen Sensor	1	± 4%
pH 7 buffer solution	2 mL	-
Conical Flask	1	-
50 mL measuring Cylinder	1	± 0.05 mL
Electronic balance	1	± 0.01g
DI water		-
Stirrer	1	-
3 mL droppers	1	-
Water bath	1	± 0.15 °C
Para film	-	-

Table 2: Apparatus and Materials used in the Experiment

3.2: Methodology

1. Collection of mushroom
    - Obtain 50g of frozen white mushrooms (*Agaricus bisporus)*
2. Preparation of the mushroom tyrosinase paste:
    - Cut them into thin slices
    - Cut the slices further into smaller pieces
    - Add them into a blender containing DI water
    - Leave the blender on for 45 seconds (amount of time it takes to form the paste)
    - Transfer it into a beaker and cover it with para film
3. Dilution of hydrogen peroxide (from 20% to 10%)
    - Measure out 50 mL of hydrogen peroxide of 20% concentration in a measuring cylinder
    - Add 50 mL of DI water to the measuring cylinder containing the hydrogen peroxide of 20% concentration
    - The result will be 100 mL of hydrogen peroxide of 10% concentration
4. Control experiment – Reaction between hydrogen peroxide and tyrosinase, without the use of ethanol
    - Warm the water bath to 35 degrees Celcius
    - Transfer 10 mL of the tyrosinase paste into conical flask **1**
    - Add 2 mL of pH 7 buffer solution into the same conical flask

- Connect data logger apparatus with the oxygen sensor and calibrate the devices
- Do not add the inhibitor (ethanol) for the first 5 trials
- Add 10 mL of freshly prepared 10% hydrogen peroxide solution into the conical flask placed in the water bath and immediately attach the oxygen sensor
- Start data collection for 100 seconds

5. Begin experimentation with varying ethanol concentrations
    - Transfer 10ml of the tyrosinase extract into conical flask **2**
    - Prepare 5 mL of 10% concentration of ethanol from 96% by dilution with DI water[7]
    - Add this to conical flask **2** containing tyrosinase, and swirl it in the water bath for 5 minutes
    - Add 10 mL of 10% hydrogen peroxide solution into conical flask **2** while it is in the water bath
    - Connect the conical flask to the oxygen sensor, and start data collection for 100 seconds
    - Conduct this again with increasing concentrations of ethanol (35%, 55% and 75%)

3.3: Risk Assessment

1. **Safety Issues:**
    - Cutting of mushrooms using a knife was slightly risky, therefore gloves were worn
    - Usage of goggles and gloves while handling hydrogen peroxide; storing it away from sources of ignition, heat and moisture, in a tightly closed container
2. **Ethical Issue:** It was ensured that there was minimal wastage of mushrooms, and only the required amount was utilized
3. **Environmental Issue:** The spoilt mushrooms were deposited in the bio-compost of our school

4: Raw Data

4.1: Qualitative Data

Ethanol Concentration	Observation
Control (0%)	Increase in height of froth column was maximum
10%	Increase in height of froth column was large
35%	Increase in height of froth column was moderate
55%	Increase in height of froth column was minimal
75%	Very less froth produced

Table 3: Qualitative Data (Observations)

[7] Calculation: (5*0.1 the whole divided by 0.96 = 0.521mL of 96%. Add 4.479mL of water to make the total volume 5mL)

Figure 3: Froth at 10% Ethanol Concentration

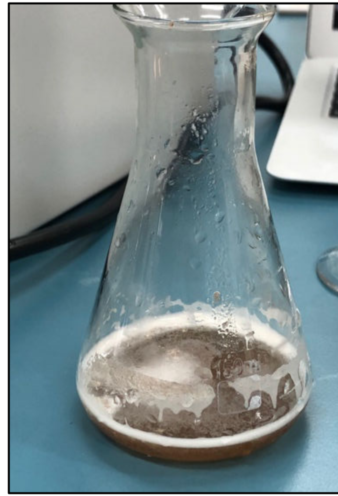
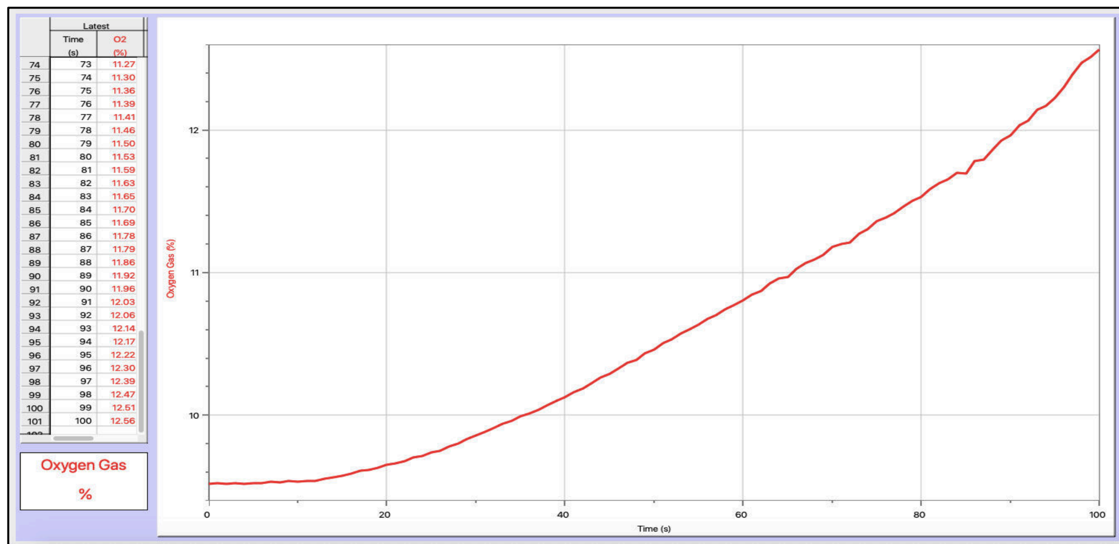


Figure 4: Froth at 35% Ethanol concentration

4.2: Quantitative Data



	Latest	
	Time (s)	O2 (%)
74	73	11.27
75	74	11.30
76	75	11.36
77	76	11.39
78	77	11.41
79	78	11.46
80	79	11.50
81	80	11.53
82	81	11.59
83	82	11.63
84	83	11.65
85	84	11.70
86	85	11.69
87	86	11.78
88	87	11.79
89	88	11.86
90	89	11.92
91	90	11.96
92	91	12.03
93	92	12.06
94	93	12.14
95	94	12.17
96	95	12.22
97	96	12.30
98	97	12.39
99	98	12.47
100	99	12.51
101	100	12.56

Image 1: Graph obtained from data logger (Control: Trial 3); Refer to appendix for all graphs

4.3: Raw Data Table

Table 4: Initial and final oxygen concentrations over 100 seconds for each of the 5 ethanol concentrations

		Oxygen Concentration/(%)	
Concentration	**Trial**	**Initial**	**Final**
0 (Control)	1	9.60	13.60
	2	9.53	14.65
	3	9.52	12.56
	4	9.54	12.72
	5	9.54	12.39
10.00	1	8.89	10.57
	2	8.93	10.51
	3	9.29	13.33
	4	9.23	14.56
	5	9.25	13.71
35.00	1	8.60	9.93
	2	8.41	9.12
	3	8.86	9.47
	4	8.86	9.41
	5	8.84	9.42
55.00	1	8.30	9.10
	2	8.96	9.79
	3	8.56	8.99
	4	8.20	8.68
	5	8.47	8.87
75.00	1	8.64	8.85
	2	8.66	8.85
	3	8.62	9.09
	4	8.30	8.79
	5	8.28	8.75

5: Processed Data

5.1: Calculations

The change in concentration of oxygen evolved was calculated as follows (using Trial 1 from control condition from *Table 1* as an example):

$$\text{Change in Oxygen Concentration} = \text{Final Concentration} - \text{Initial Concentration} = 13.60 - 9.60 = 4.0$$

Ethanol concentration/ %	Increase in Concentration of Oxygen/%						
	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Average**	**Standard Deviation**
0 (Control)	4.00	5.12	3.04	3.18	2.85	3.64	0.94
10.00	1.68	1.58	4.04	5.33	4.46	3.42	1.70
35.00	1.33	0.71	0.61	0.55	0.58	0.76	0.33
55.00	0.80	0.83	0.43	0.48	0.40	0.59	0.27
75.00	0.21	0.19	0.47	0.49	0.47	0.37	0.15

Table 5: Change in Oxygen Concentration over 100s

The standard deviation (σ) for each condition was calculated as follows (using Control condition from Table 5 as an example):

Number of samples, N = 5 Sum, $\sum x = 4.00 + 5.12 + 3.04 + 3.18 + 2.85 = 18.19$

Mean, $\bar{x} = \frac{18.19}{5} = 3.64$

Standard Deviation, $\sigma = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \bar{x})} = \sqrt{\frac{(4.00-3.64)^2+\cdots+(2.85-3.64)^2}{5-1}} = 0.94$

5.2: Statistical Test

Analysis of Variance (ANOVA) is the statistical test that will be used to test the differences among group means in a sample. Through the results of the ANOVA test, we can deduce which hypothesis is true – $\mathbf{H_0}$ or $\mathbf{H_1}$.

Descriptive statistics of your k=5 independent treatments:

Treatment →	A	B	C	D	E	Pooled Total
observations N	5	5	5	5	5	25
sum $\sum x_i$	18.1900	17.0900	3.7800	2.9400	1.8300	43.8300
mean $\bar{x}$	3.6380	3.4180	0.7560	0.5880	0.3660	1.7532
sum of squares $\sum x_i^2$	69.6909	69.9409	3.2840	1.9042	0.7621	145.5821
sample variance s^2	0.8789	2.8818	0.1066	0.0439	0.0231	2.8641
sample std. dev. s	0.9375	1.6976	0.3265	0.2095	0.1519	1.6924
std. dev. of mean $SE_{\bar{x}}$	0.4193	0.7592	0.1460	0.0937	0.0679	0.3385

Table 6: Summary of data used for ANOVA

[8]**p-value** = 3.45e-06

One-way ANOVA of your k=5 independent treatments:

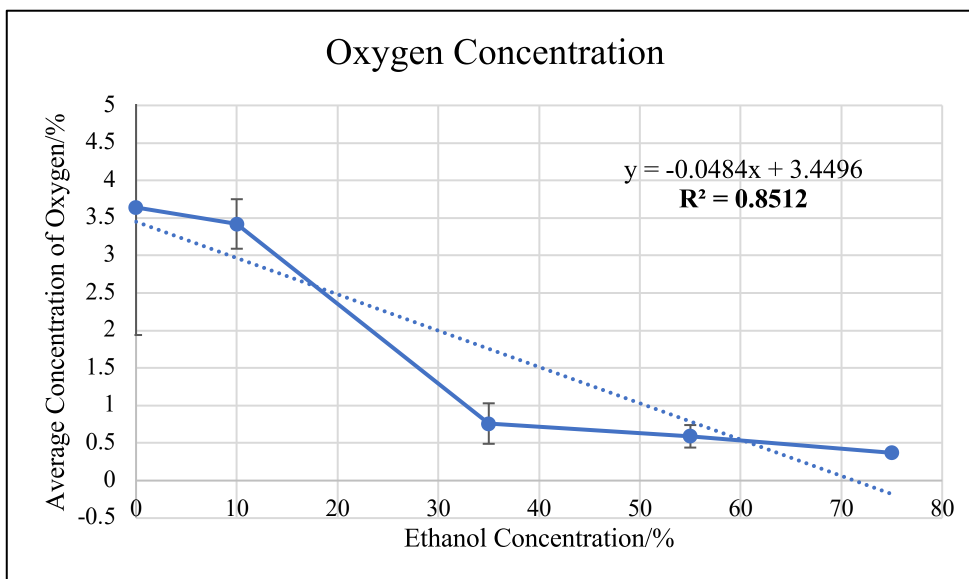
source	sum of squares SS	degrees of freedom ν	mean square MS	F statistic	p-value
treatment	53.0023	4	13.2506	16.8399	3.4454e-06
error	15.7371	20	0.7869		
total	68.7393	24			

Table 7: ANOVA test calculations

Ethanol concentration/%	**Average Concentration of Oxygen/%**
0	3.64
10.00	3.42
35.00	0.76
55.00	0.59
75.00	0.37

Table 8: Oxygen Concentrations at the 5 ethanol concentrations

[8]Vasavada, N. (n.d.). One-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey HSD (Honestly Significant Difference) Test Calculator for comparing multiple treatments. Retrieved January 3, 2021, from https://astatsa.com/OneWay_Anova_with_TukeyHSD/



Graph 2: Oxygen Concentration at different ethanol concentrations

6: Analysis and Conclusion

This investigation tested the inhibitory effect of ethanol on tyrosinase from *Agaricus Bisporus* using hydrogen peroxide as the substrate. As seen in *Table 8* and *Graph 2,* as ethanol concentration increases from 0% to 75%, the average oxygen concentration reduces from 3.64% to 0.37%. The most rapid reduction in oxygen concentration took place when the ethanol concentration rose from 10% to 35%. All ethanol concentrations beyond 35%, resulted in the liberation of less than 1% oxygen, indicating that at and above 35% ethanol concentration, the inhibition of tyrosinase was extremely high. The highest ethanol concentration (75%) produced an 89.8% rise from the control condition (0% ethanol). Additionally, *Graph 2* has a high R^2 value of 0.85. R^2 is a statistical measure of how close the data values are to the regression line. Therefore, it can be deduced that there is a strong negative correlation between the concentration of ethanol used and the concentration of oxygen produced.

The one-way ANOVA is a single test to determine the significance of the difference between the mean values of each of the 5 conditions. This helps in testing the null and alternate hypotheses. The p-value corresponding to the F-statistic of my ANOVA test is lower than 0.01 which strongly suggests that the there is a statistically significant effect in varying the concentrations of ethanol on the oxygen concentration of the reaction between tyrosinase and hydrogen peroxide.

This supports my alternate hypothesis that as ethanol concentration increases, the concentration of oxygen evolved from the hydrogen peroxide reaction decreases. This occurs because ethanol is a non-competitive inhibitor of the tyrosinase enzyme. This means that it changes the shape of the active site of tyrosinase by attaching itself to an allosteric site. As a result, hydrogen peroxide is unable to bind to its active site on tyrosinase and therefore, unable to give the product – oxygen. As ethanol concentration increases, there is a greater inhibitory effect on the reaction between tyrosinase and hydrogen peroxide, thereby reducing the concentration of oxygen produced.

Since mushroom tyrosinase exhibits similar enzymatic activity to catalase, the result of this investigation was compared to that of a study[9] conducted using ethanol (in wine) as an inhibitor of catalase. Catalase was found to be completely inhibited via non-competitive inhibition by ethanol in wine, thereby inhibiting the reaction between catalase and hydrogen peroxide (substrate).

In conclusion, from the results of my experiment, I can reject my null hypothesis and accept my alternate hypothesis.

7: Evaluation

7.1: Strengths of Experiment

1. The tyrosinase extract was freshly prepared before every trial so as to ensure it does not get degraded
2. The conical flask containing ethanol and tyrosinase was swirled in the water-bath for 5 minutes before adding hydrogen peroxide so as to ensure that the enzyme gets accustomed to the new chemical environment before the reaction starts
3. The standard deviation error bars in *Graph 2* did not overlap each other, indicating a small random error
4. An oxygen sensor was used instead of a gas pressure sensor to ensure only the oxygen concentration is measured, and not any other gases present in the surroundings.

7.2: Weaknesses of Experiment

Limitation	Improvement
Only 5 trials were carried out at each trial condition	Conduct a greater number of trials (8-10) to increase precision of results
Time lag between placing the oxygen sensor in the conical flask and reaction commencing, leading to loss of some oxygen gas	Get someone else to pour in the last reagent added (hydrogen peroxide) and immediately once this is done, make sure you place the oxygen sensor
Inhibitory effect of only a few ethanol concentrations was investigated	Investigate the concentration of oxygen evolved for a greater number of ethanol concentrations in the range of 10% and 35%

Table 9: Limitations and Improvements of Experiment

7.3: Extension to Investigation

Using kojic acid, a *competitive* inhibitor of tyrosinase, could be an extension to this investigation. Conducting this experiment could help compare the inhibitory effects of two different *types* of inhibitors, thereby revealing more about the various reaction mechanisms and properties of tyrosinase.

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[9] Temple, D., & Ough, C. (1975, January 01). Inhibition of Catalase Activity in Wines. Retrieved January 15, 2021, from https://www.ajevonline.org/content/26/2/92

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Appendices

Appendix 1: Raw Data (Quantitative)

Control: No inhibitor

Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)
0	9.60	0	9.53	0	9.52	0	9.54	0	9.54
20	9.72	20	9.64	20	9.65	20	9.65	20	9.62
40	10.20	40	10.14	40	10.13	40	10.22	40	10.05
60	10.87	60	10.98	60	10.81	60	10.96	60	10.71
80	11.84	80	12.39	80	11.53	80	11.79	80	11.57
100	13.60	100	14.65	100	12.56	100	12.72	100	12.39

10% inhibitor (ethanol)

Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)
0	8.89	0	8.93	0	9.29	0	9.23	0	9.25
20	8.98	20	8.93	20	9.40	20	9.38	20	9.57
40	9.27	40	9.24	40	9.85	40	9.99	40	10.29
60	9.67	60	9.66	60	10.60	60	11.09	60	11.19
80	10.12	80	10.12	80	11.95	80	12.8	80	12.47
100	10.57	100	10.51	100	13.33	100	14.56	100	13.71

35% inhibitor (ethanol)

Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)
0	8.60	0	8.41	0	8.86	0	8.86	0	8.84
20	9.20	20	8.51	20	8.90	20	8.88	20	8.86
40	9.45	40	8.66	40	8.93	40	8.99	40	9.00
60	9.62	60	8.83	60	9.16	60	9.14	60	9.17
80	9.75	80	8.98	80	9.31	80	9.29	80	9.31
100	9.93	100	9.12	100	9.47	100	9.41	100	9.42

55% inhibitor (ethanol)

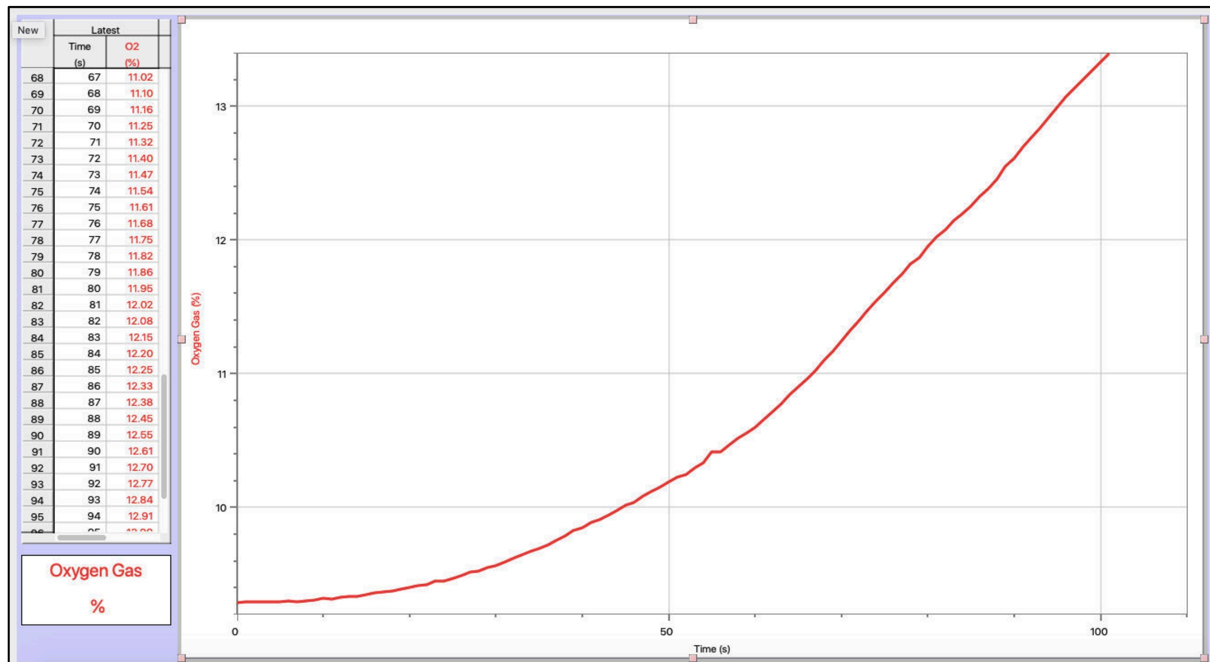
Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)
0	8.30	0	8.24	0	8.08	0	7.87	0	7.87
20	8.26	20	8.22	20	8.02	20	7.78	20	7.87
40	8.26	40	8.32	40	8.08	40	7.86	40	8.01
60	8.31	60	8.51	60	8.24	60	7.96	60	8.18
80	8.33	80	8.72	80	8.40	80	8.09	80	8.33
100	8.38	100	8.96	100	8.56	100	8.20	100	8.47

75% inhibitor (ethanol)

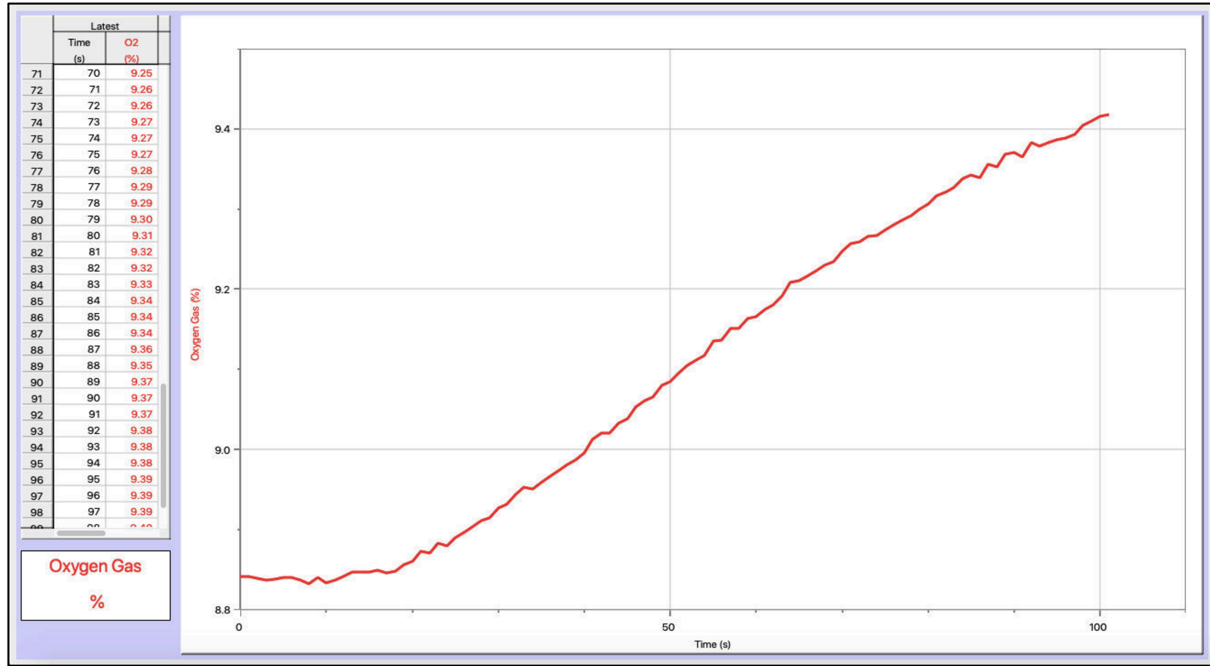
Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)	Time (s)	Oxygen (%)
0	8.64	0	8.66	0	8.62	0	8.30	0	8.28
20	8.69	20	8.71	20	8.73	20	8.42	20	8.41
40	8.74	40	8.75	40	8.84	40	8.55	40	8.50
60	8.78	60	8.80	60	8.94	60	8.63	60	8.58
80	8.82	80	8.83	80	9.02	80	8.71	80	8.66
100	8.85	100	8.85	100	9.09	100	8.79	100	8.75

Appendix 2: Data Logger Graphs

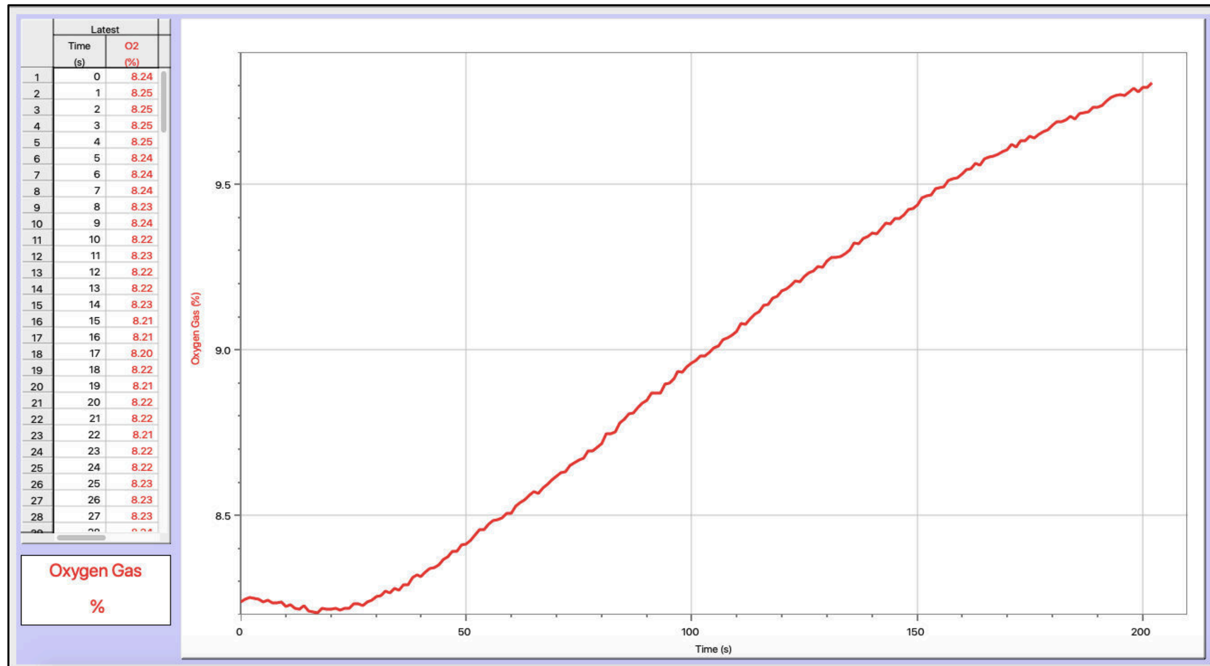
10% Ethanol Concentration



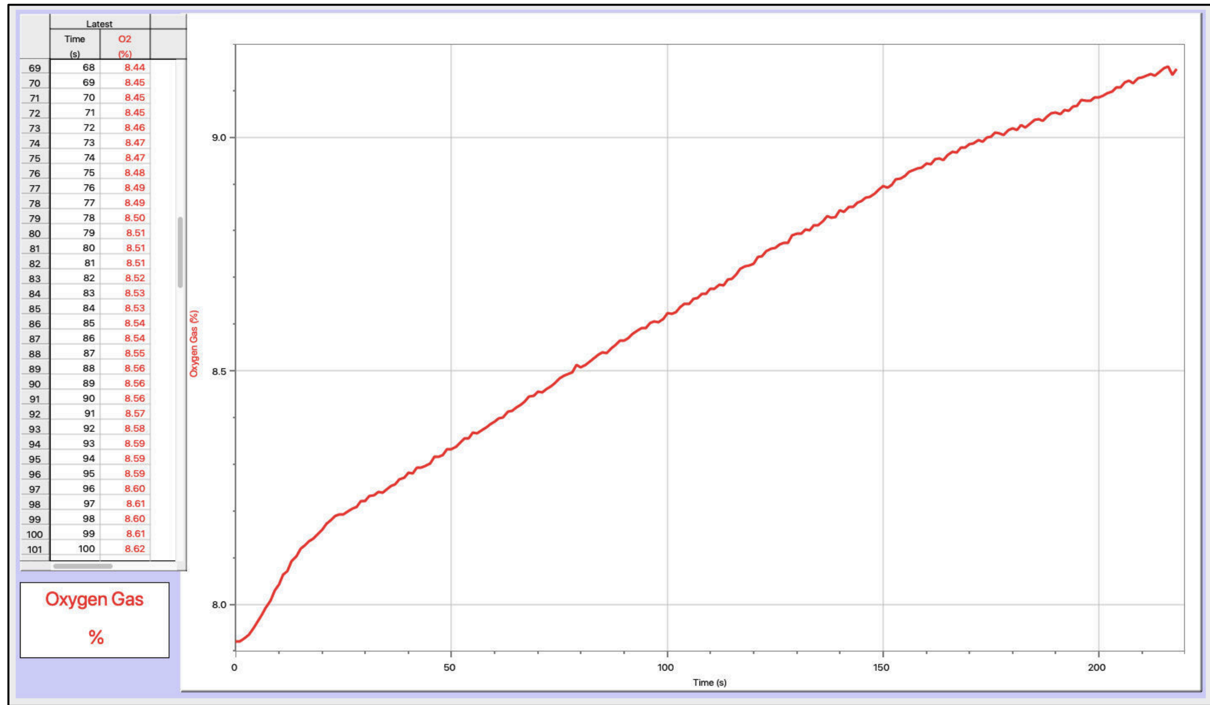
35% Ethanol Concentration



55% Ethanol Concentration



75% Ethanol Concentration



	Latest Time (s)	O2 (%)
69	68	8.44
70	69	8.45
71	70	8.45
72	71	8.45
73	72	8.46
74	73	8.47
75	74	8.47
76	75	8.48
77	76	8.49
78	77	8.49
79	78	8.50
80	79	8.51
81	80	8.51
82	81	8.51
83	82	8.52
84	83	8.53
85	84	8.53
86	85	8.54
87	86	8.54
88	87	8.55
89	88	8.56
90	89	8.56
91	90	8.56
92	91	8.57
93	92	8.58
94	93	8.59
95	94	8.59
96	95	8.59
97	96	8.60
98	97	8.61
99	98	8.60
100	99	8.61
101	100	8.62