Determining the influence of temperature on the rate of dehydrogenase enzyme activity in yeast using a microplate reader

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1. AIM AND RESEARCH QUESTION

This experiment investigates the rate at which the dehydrogenase enzyme catalyses the oxidation of nicotinamide adenine dinucleotide (NADH) found in yeast when methylene-blue is added. The change in absorbance of the solution using a microplate reader will be used to observe the reaction.

Research question: *How does increasing the temperature (25°C, 30°C, 35°C, 40°C, 45°C, 50°C) of a methylene-blue and yeast solution affect the rate at which the dehydrogenase enzyme catalyses the oxidation of NADH molecules found in yeast, determined by the reduction of methylene-blue and observed through its color change and change in absorbance?*

2. INTRODUCTION

When learning about enzyme-catalysed reactions, I discovered an important redox reaction involving NADH molecules catalysed by enzyme dehydrogenase. The NADH plays a crucial role in driving the production of adenosine triphosphate (ATP) in cellular respiration, as it donates electrons to the electron transport chain (NADH, n.d.). I became interested in how climate change would influence the rate of cellular respiration in poikilotherms, animals whose body temperature varies depending on the outside conditions (Poikilotherm, 2024), since enzymatic activity depends on the temperature. Consequently, I explored a dehydrogenase-driven reaction between NADH and methylene-blue, which accepts the electrons and changes its color when reduced.

3. BACKGROUND INFORMATION

3.1 THEORETICAL BACKGROUND

Enzymes are substances that act as catalysts in organisms (Enzyme, n.d.). Reactions involving enzymes are characterised by lower activation energy as they provide an additional reaction pathway (Figure 1).

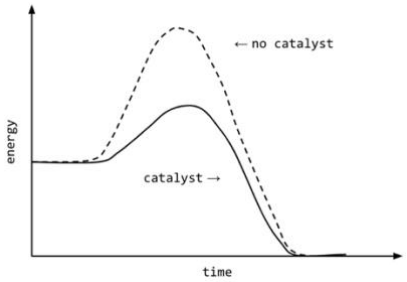

Figure 1: Activation energy with and without a catalyst. (source: Activation energy, n.d.)

Enzymes consist of a specific structure of proteins with active sites on the surface that bind to substrates, the reactants involved in the reaction (Figure 2). The resulting structure—the

enzyme-substrate complex—enables the substrate to undergo a reaction, converting it into the product, which is subsequently released from the active site (Active site, 2025).

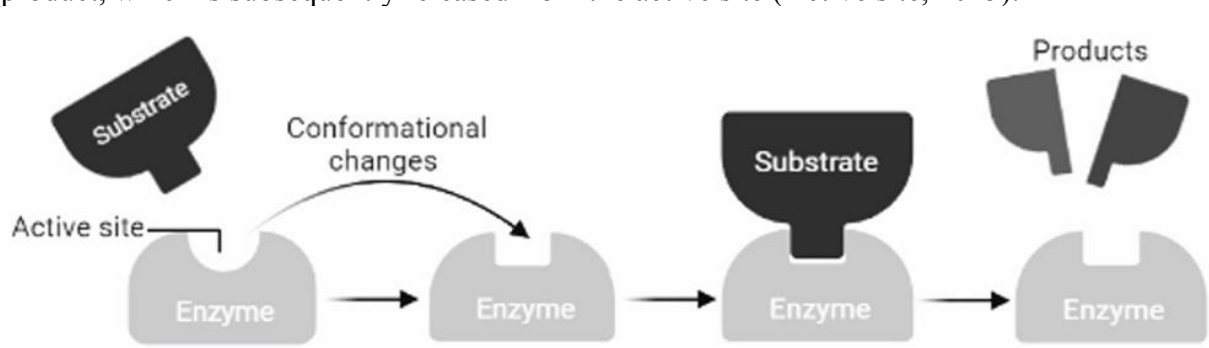

Figure 2: Step-by-step enzyme-catalysed reaction (source: Active site, 2025)

The dehydrogenase enzyme oxidation of NADH is a redox reaction, catalysed by the dehydrogenase enzyme, in which NADH (nicotinamide adenine dinucleotide) molecules are oxidised and lose two electrons and a proton. The reaction involves two primary reagents in an acidic environment: NADH ($C_{21}H_{29}\ N_7O_{14}P_2$) and an ionic redox indicator methylene-blue ($C_{16}H_{18}ClN_3S$), the cation of which accepts the electrons and a proton from NADH. H^+ (acidic medium), dehydrogenase and NADH are found in yeast cells; methylene-blue is added separately. In the reduction process, the color of methylene-blue changes from blue to colorless, which helps determine the reaction rate.
The overall reaction between the ionic methylene-blue and NADH molecules is presented in (1):

$$C_{21}H_{29}\ N_7O_{14}P_{2(aq)} + C_{16}H_{18}\ N_3\ S^+_{(aq)} + Cl^-_{(aq)} \overset{\text{dehydrogenase, } H^+}{\rightleftharpoons} C_{21}H_{28}N_7O_{14}P_2{}^+ + C_{16}H_{19}\ N_3\ S_{(aq)} + Cl^-_{(aq)} \quad (1)$$

Firstly, the compound methylene-blue dissociates into ions when water dissolves it, with Cl^- as spectator ion (Eq. 2).

$$C_{16}H_{18}ClN_3S_{(s)} \longrightarrow C_{16}H_{18}\ N_3S^+_{(aq)} + Cl^-_{(aq)} \quad (2)$$

In the solution, the NADH molecules are oxidised by dehydrogenase enzyme to NAD^+. Two electrons and a proton are lost, as presented in Eq. (3).

$$NADH \rightleftharpoons NAD^+ + 2e^- + H^+ \quad (3)$$

One of the two donated electrons forms a bond between nitrogen in the cation of methylene-blue and the donated hydrogen proton. The other neutralises the positive charge on the sulfur atom in the central ring of methylene-blue, as presented in Eq.(4).

$$C_{16}H_{18}\ N_3\ S^+ + 2e^- + H^+ \rightleftharpoons C_{16}H_{19}\ N_3\ S \quad (4)$$

The structural formula of methylene-blue shows that two double bonds are broken to obtain its reduced form after the addition of hydrogen and electrons (*Figure 3*). The molecule exhibits resonance in the cation form and has delocalised electrons across the structure, creating blue color. In the reduced form, the structure with more defined single and double bonds is formed, and the color changes.

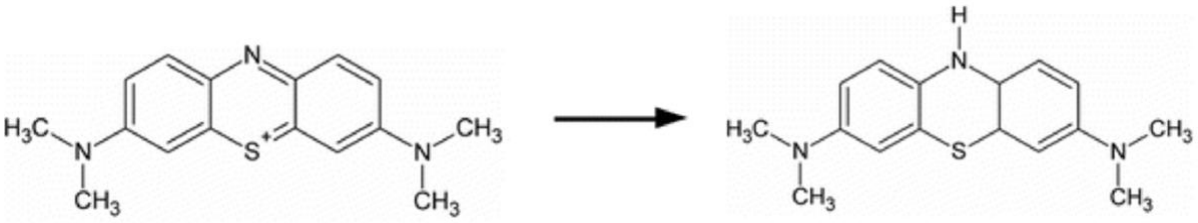

Figure 3: Structural formula of methylene blue in its charged and neutral form (Source: Molecular neurobiology, n.d.)

The reaction rate shows the speed of reactant/product concentration change over time, depending on stoichiometric coefficients (Eq. (5)).

$$aA + bB \rightarrow cC + dD$$

$$\text{Rate} = \frac{-1}{a}\frac{d[A]}{dt} = \frac{-1}{b}\frac{d[B]}{dt} = \frac{1}{c}\frac{d[C]}{dt} = \frac{1}{d}\frac{d[D]}{dt} \quad (5)$$

When all coefficients equal one (as in this experiment), this equation symplifies to:

$$\text{Rate} = -\frac{d[A]}{dt}. \quad (6)$$

When enzymes are not saturated, raising the temperature enlarges reaction rates (more collisions through increased particle kinetic energy ($E_k = \frac{mv^2}{2}$)). However, temperatures around 40°C lead to the enzymes' denaturation, terminating their activity and reducing the reaction rate (Denatured enzyme, n.d.).

Absorbance: The solution color change can be quantified by the change in absorbance -the quantity of light absorbed by a solution (Absorbance technology, n.d.)- as the ratio between the incident light I_0 and transmitted light I and is a unitless quantity. According to the Beer-Lambert Law, the absorbance depends on molar absorptivity, the sample's path and the solutes' concentration (Absorbance technology, n.d.).

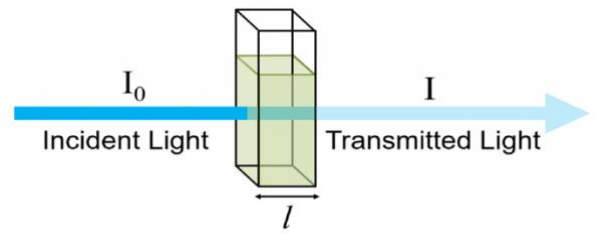

Figure 4. Absorbance is defined as the ratio between the transmitted and original beam intensity. (source: Beer-Lambert, n.d.)

A microplate reader is a laboratory instrument used to determine the absorbance of specific wavelength of incident light. Unlike spectrophotometer with a similar function, it consists of multiple wells, allowing simultaneous measurements of samples.

The variables:

Independent variable: temperature *(25°C, 30°C, 35°C, 40°C, 45°C, 50°C)* of the methylene-blue and yeast solution

Dependent variable: the absorbance of the sample-well (mg/10ml min), concentration of methylene-blue (mg/10ml)

Controlled variables:

Table 1: Controlled variables and explanations

Controlled variable	**How is it controlled?**	**Why is it controlled?**
Yeast-solution concentration in the mixture	- using same stock solution	- avoid variations in the number of enzymes
	- using precise pipettes	- avoid affecting the reaction rate
		- avoid affecting solution absorbance
Methylene-blue-solution concentration in the mixture	- using same stock solution	- avoide enzyme independent absorbance variation
	- using precise pipettes	
The path length of the sample	- measurements in the same microtitre-plate	- assure consistency with Beer-Lambert's Law,
	- wells of the same path length	- avoid influence on absorbance
The wavelength of transmitted light	- maximum determined before experiment	- avoid methylene-blue absorbing less light
	- wavelength kept constant by microplatereader	- provide consistent results

3.2 HYPOTHESIS

The dehydrogenase will not be saturated with the NADH molecules in this reaction. Consequently, the rate of NADH oxidation (i.e. the observed reaction) is anticipated to increase with rising temperature. The rate is expected to decline following enzyme denaturation, so the optimal activity is predicted right before, at 35°C. The rate of the observed reaction will be monitored by the rate of change in absorbance of methylene blue (related to concentration) due to its reduction. The anticipated changes in NADH concentration will mirror the trend seen in methylene-blue concentration, as NADH is oxidised at the same rate as methylene-blue is reduced.

3.3 MATERIALS AND EQUIPMENT

Table 2: Materials

Materials	
● Fresh yeast, brand Fala 5.0g	● Methylene-blue 0.01g
● Distilled water	
● Pipette filler, wash bottle, spatula	

Table 3: Equipment

Equipment	
• Volumetric pipette 1x 50 mL ± 0.05 mL	• 96- well transparent microtitre-plate 2x
• Volumetric pipette 1x 10 mL ± 0.02 mL	• Incubator ± 0.5°C
• Plastic test tube with a screw 2x 100 mL ± 5 mL	• Root analysis framework
	• Multimeter (0.1mV)
• Pipette tips, 11x 150 µL ± 2.25 µL	• A connected thermistor
• Pipette tips 4x 300µL ± 2.7 µL	• Scale (range 250 g, ± 0.0001 g), brand Mettler Toledo
• Pipette tips 8X 30 µL ± 1.2 µL	• Test tube stand
• Pipette tips 1x 270 µL ± 2.6 µL	• Multichannel pipette with eight channels
• Weighting papers 2x (size 4 cm × 4 cm)	• Biotek 800-TS Absorbance microplate-reader (96 samples/4 seconds) ± 0.001

3.4 METHODOLOGY

The peak absorbance wavelength (600nm) at which methylene-blue absorbs the most strongly is determined using UV-Vis Spectrophotometer by scanning distilled water to account for its absorbance, zeroing the spectrophotometer, and scanning methylene-blue solution. It is used in further experimentation using a microplate reader.

To determine the precise relationship between absorbance and concentration, eight samples are prepared by dilution of methylene-blue solution with known concentration and their absorbances measured. A calibration curve connecting the two quantities is drawn to identify further results.

A constant temperature throughout the reaction is ensured by inserting the microplate reader into an incubator (Figure 5), where the temperature can be adjusted. Before the reaction, all solutions are also preheated.

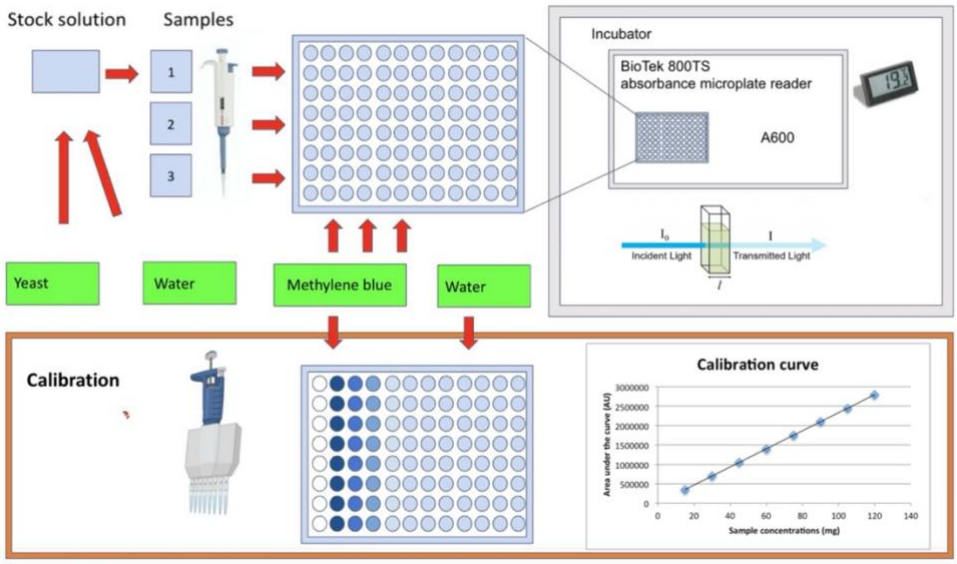

Figure 5: The procedure

3.5 SAFETY NOTES

Methylene-blue is classified as an irritant (Irritant agent, 2024). Its immediate, prolonged, or repeated contact with living tissue induces a local inflammatory reaction (GHS, n.d.). It can harm aquatic life, thus presenting a long-term hazard (MB, n.d.) for a living species and should not be released to living-spieces environment. Bakery yeast is not a hazardous substance (Yeast, 2014) However, since both may cause skin-irritation (MB overview, n.d.), eye-redness (yeast) or severe eye damage (methylene-blue), appropriate lab coats, goggles, and gloves should be worn.

No ethical concerns existed since no living species were involved in the experiment. All materials were used efficiently to avoid unnecessary waste.

3.6 PROCEDURE

Yeast and methylene-blue stock solutions

1. Weigh 5.00g of active yeast, transfer it to the 100ml plastic test tube;
2. Use a 50ml volumetric pipette to measure 50.0ml of distilled water, add it to the yeast;
3. Close the test tube, mix;
4. Weight 0.01g of methylene-blue, transfer it to the second 100ml test tube;
5. Pipette 10.0ml of distilled water with methylene-blue using 10ml volumetric pipette.

Measurements:

1. Before measurements, insert solutions into the incubator at the set temperature (25°C-50°C, 5°C interval). Monitor their temperature with a thermistor until reaching the desired temperature;
2. Pipette 3x 300 µl of yeast solution using a 300 µl multichannel pipette. Transfer each to one microtitre-plate well;
3. Using 30µl pipette tips, pipette 3x 30µl of methylene-blue into the same wells;
4. Insert the microtitre-plate into the microplate reader. Start the measurement;
5. Record measurements each minute until colorless;
6. Repeat for all desired temperatures.

Calibration curve measurements

1. Pipette 8x 30µl of methylene-blue stock solution into microtitre-plate-wells using 30µl multichannel pipette;
2. Add 270µl of distilled water to each;
3. Take 150µl of diluted solution from each and transfer it to the neighbouring wells;
4. Add 150µl of distilled water;
5. Repeat 3. and 4. five times, using the solution from the previous well;
6. Insert the microtitre-plate into microplate-reader. Record the measurements of absorbance;
7. Plot a graph relating the absorbance and concentration.

4. RESULTS

4.1 QUALITATIVE OBSERVATIONS

Methylene blue stock solution was dark blue, while yeast solution was almost colorless. The color of the mixture was initially blue, but gradually disappeared over the time. The final solution resembled the initiall yeast solution (Figure 6)

Solutions exposed to temperatures of 35°C and 40°C discoloured the fastest, whereas the solution at 50°C took the longest to discolour.

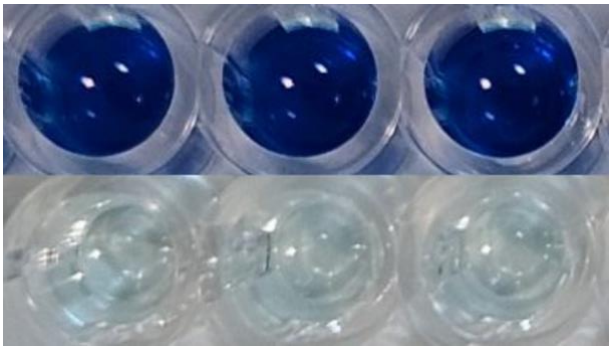

Figure 6: Initial (above) and final (below) color of the samples

4.1 CALIBRATION DATA

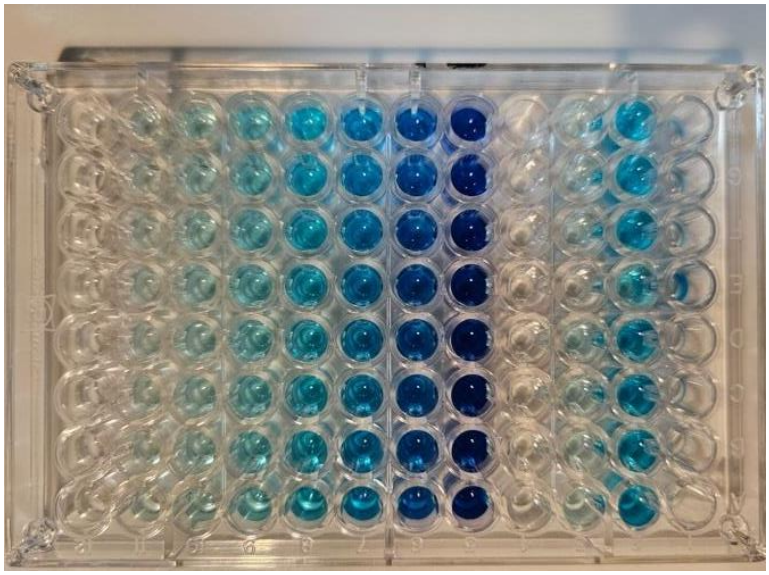

Figure 7: Calibration solutions of methylene-blue in a microtitre plate

Before the experiment, a calibration curve was created by measuring the absorbance of eight known methylene-blue concentrations. The concentrations, prepared by diluting a stock solution of methylene-blue, were determined using equation, e.g. for the first dilution $c_2 = \frac{c_{stock} \cdot V_{stock}}{V_2} = \frac{\frac{10.0000mg}{10.00ml} \cdot 30.0\mu l}{300.0\mu l} = 1.00mg/10ml$ (the unit mg/10ml is unconventional, but here used because such small values are considered).

The uncertainty propagation formula was used to determine concentration uncertainties. An example of the calculation of the stock and first dilution (C1) is presented below, and all measurement uncertainties are seen in Table 4.

STOCK SOLUTION:

- mass uncertainty (0.01 g ±0.0001 g) → relative uncertainty $\frac{0.0001}{0.0100} \cdot 100 = 1\%$
- volume uncertainty (10 mL pipette, ±0.02 mL) → relative uncertainty $\frac{0.02}{10.00} \cdot 100 = 0.2\%$

total uncertainty: $U_s = \sqrt{(1\%)^2 + (0.2\%)^2} = \sqrt{1.04}\,\% = 1.02\% \approx 1\%$

C1 SOLUTION:

- U_{30} pipette uncertainty (30 µL pipette, ±1.2 µL) → relative uncertainty $\frac{1.2}{30.0} \cdot 100 = 4\%$
- U_{300} pipette uncertainty (300 µL pipette, ±2.7 µL) → relative uncertainty $\frac{2.7}{300.0} \cdot 100 = 0.9\%$

total uncertainty: $U_{C1} = \sqrt{{U_s}^2 + {U_{30}}^2 + {U_{300}}^2} = \sqrt{1^2 + 4^2 + 0.9^2}\ \% = 4.24\% \approx 4\%$

Table 4: Concentrations of diluted methylene-blue stock solution

Solution	STOCK	C1	C2	C3	C4	C5	C6	C7
Concentration (mg/10ml)	10.0 (1±1%)	1.00 (1±4%)	0.500 (1±5%)	0.250 (1±5%)	0.125 (1±5%)	0.0625 (1±6%)	0.0313 (1±6%)	0.0156 (1±7%)

The microtitre-plate with the prepared solutions is shown in Figure 7. Each concentration was tested eight times and rounded to three significant figures (same as least precise equipment). The measured absorbance values can be seen in Table 5, with each trial labelled A0, A1, A2, A3, A4, A5, A6, or A7. The absorbance was measured using a microplate reader, with range of absorbance measurements (0.040 - 3.687)±0.001 (uncertainty of microplate reader).

Table 5: Absorbance for known concentrations

Concentration (mg/10ml)	Absorbance (precision±0.001)							
	A0	A1	A2	A3	A4	A5	A6	A7
1(1±4%)	3.687	3.609	3.607	3.651	3.625	3.631	3.573	3.466
$5.00\ 10^{-1}$ (1±5%)	2.059	1.846	1.683	1.766	1.923	1.854	1.830	1.870
$2.50\ 10^{-1}$ (1±5%)	1.050	0.938	0.853	0.918	0.954	0.975	0.938	1.002
$1.25\ 10^{-1}$ (1±5%)	0.545	0.498	0.505	0.476	0.515	0.494	0.514	0.482
$6.25\ 10^{-2}$ (1±6%)	0.283	0.274	0.272	0.259	0.280	0.275	0.282	0.279
$3.13\ 10^{-2}$ (1±6%)	0.157	0.152	0.153	0.149	0.160	0.152	0.155	0.152
$1.56\ 10^{-2}$ (1±7%)	0.095	0.099	0.096	0.093	0.101	0.096	0.095	0.095
0 (1±0%)	0.041	0.040	0.041	0.041	0.043	0.041	0.041	0.040

The absorbance was notably lower at lower concentrations of methylene-blue. The minimum was obtained for distilled water ($c = 0\text{mg}/10\text{ml}$), as water absorbs some light at 600 nm. The least light successfully passed through the sample with the highest concentration of 1mg/10ml.

4.2 REACTION DATA

The experiment comprised three trials—1, 2, and 3—each involving five samples of the same mixture solution exposed to different temperatures. The absorbance values of all samples were measured every minute to assess the reaction rate. Measurements were halted once the samples lost their blue colour. The results are displayed in Table 6.

Table 6: Raw data Absorbance measurements for three parallel samples for six temperatures

Temperature (°C) ± 0.1°C		25	30	35	40	45	50
	t (min) ± 0.08 min						
Trial 1	0	3.591	3.615	3.610	3.630	3.580	3.625
	1	3.252	3.206	2.700	2.850	3.200	3.351
	2	2.89	2.509	1.710	2.130	2.710	3.121
	3	2.409	2.051	0.670	1.380	2.250	2.809
	4	2.056	1.305		0.690	1.810	2.503
	5	1.689	1.031			1.440	2.251
	6	1.251				0.910	2.009
	7						1.704
	8						1.121
	9						0.849
	10						0.615
Trial 2	0	3.598	3.601	3.610	3.600	3.610	3.601
	1	3.201	3.185	2.750	2.910	3.190	3.401
	2	2.851	2.458	1.680	2.110	2.690	3.105
	3	2.451	1.955	0.690	1.400	2.210	2.841
	4	2.109	1.359		0.700	1.790	2.459
	5	1.651	0.951			1.480	2.306
	6	1.206				0.890	1.953
	7						1.653
	8						1.089
	9						0.851
	10						0.554
Trial3	0	3.615	3.593	3.630	3.600	3.610	3.591
	1	3.189	3.159	2.740	2.940	3.230	3.421
	2	2.869	2.551	1.650	2.160	2.650	3.159
	3	2.389	1.987	0.710	1.410	2.310	2.794
	4	2.124	1.380		0.710	1.830	2.482
	5	1.693	1.039			1.400	2.205
	6	1.275				0.930	2.071
	7						1.679
	8						1.183
	9						0.895
	10						0.515

5. DATA ANALYSIS

5.1 CALIBRATION CURVE ANALYSIS

The calibration curve is drawn using data from Table 5.

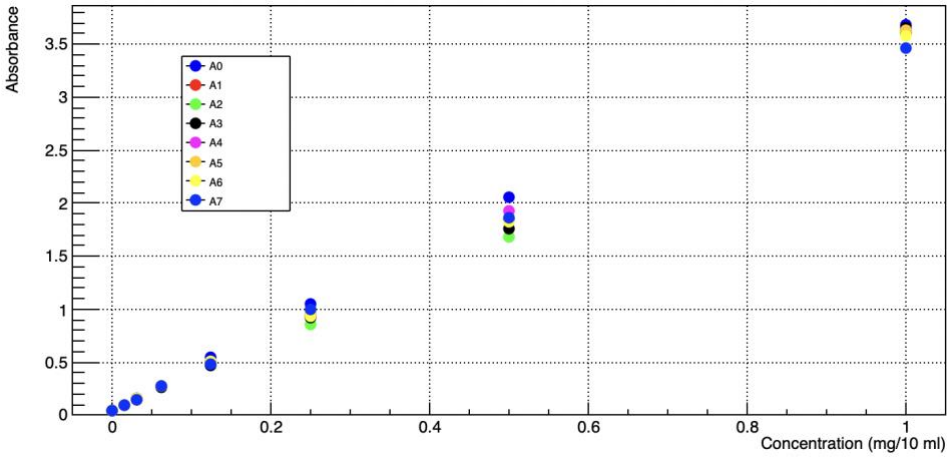

Figure 7: Absorbances corresponding to concentration

The values for absorbance are more dispersed with the increasing concentration. The average value and standard deviation for each concentration are calculated to quantify this dispersion. The calculation follows the equations for average value and standard deviation using $A_m = \frac{1}{N}\sum_{i=1}^{N} A_i \ \ and \ \sigma = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(A_i - A_m)^2}$, where A_m is mean, σ standard deviation, n the number of samples, and A_i the measurement from Table 5. An example of a calculation for c=0.5mg/10ml is shown below.

$$A_m = \frac{2.059 + 1.846 + 1.683 + 1.766 + 1.923 + 1.854 + 1.830 + 1.870}{8} = 1.850$$

$$\sigma = \sqrt{\frac{(2.059 - 1.850)^2 + (1.846 - 1.850)^2 + \cdots + (1.870 - 1.850)^2}{7}}$$

$$\sigma = 0.110$$

The values obtained are shown in Table 7 below:

Table 7: Concentrations, mean absorbance and standard deviation

C (mg/10ml)	A_m	σ
1.00	3.612	0.066
0.500	1.850	0.110
0.250	0.952	0.059
0.125	0.506	0.022
0.0625	0.284	0.008
0.0313	0.154	0.003
0.0156	0.108	0.003
0.00	0.041	0.001

Figure 8 shows mean absorbance values to determine the relationship between absorbance and concentration, a vertical error representing the standard deviation of absorbance and a horizontal value using % uncertainty for concentration.

A linear function is fitted on to the measurement points (A=kC+n), where k is the slope of the line (3.63) and n is the y-axis offset (absorbance at C=0) (0.04).

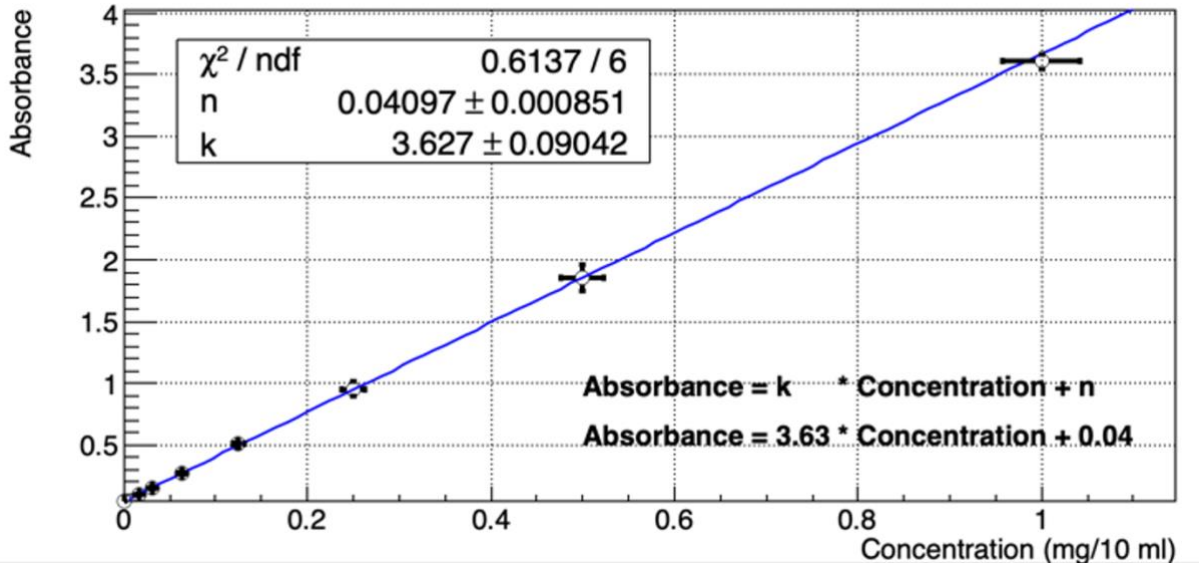

Figure 8: Calibration of the Absorbance and Concentration. The linear fit through the point for six concentrations

To calculate concentration from absorbance in further analysis ($C = \frac{A-n}{k}$), the error of the calibration curve (the absolute uncertainty of k and n) is considered, determined using a computer. The absolute uncertainties (δk) are then converted to relative uncertainties (k=3%, n= 2%), the calculation for slope uncertainty is shown below.

$$\delta k = 0.09$$

$$\%k = \frac{\delta k}{k} \cdot 100 = \frac{0.09}{3.63} \cdot 100 = 2{,}579\% \approx 3\%$$

5.2 REACTION DATA ANALYSIS

From Table 6, average absorbances for specific time and temperature are calculated. For example:

$$\mathrm{t} = 0\ \mathrm{min}, \mathrm{T} = 25°\mathrm{C} \rightarrow A_{mean} = \frac{3.591 + 3.598 + 3.615}{3} = 3.601$$

Here, absolute uncertainty is determined instead of standard deviation due to small number of measurements. An example is continued, all values are presented in Table 8.

$$\delta A_{0;25} = \frac{A_{max} - A_{min}}{2} = \frac{3.615 - 3.591}{2} = 0.012$$

Table 8: Mean values of absorbance of the yeast-methylene-blue solution with absolute uncertainties

t (min) ± 0.08min	Temperature (°C) ± 0.5°C					
	Absorbance					
	25	30	35	40	45	50
0	3.601±0.012	3.603±0.011	3.617±0.010	3.610±0.015	3.600±0.015	3.606±0.017
1	3.214±0.031	3.183±0.024	2.730±0.025	2.900±0.045	3.207±0.020	3.391±0.035
2	2.870±0.020	2.506±0.047	1.680±0.030	2.133±0.025	2.683±0.030	3.128±0.027
3	2.416±0.031	1.998±0.048	0.690±0.020	1.397±0.015	2.257±0.050	2.815±0.024
4	2.096±0.034	1.348±0.038		0.700±0.010	1.810±0.020	2.481±0.022
5	1.678±0.021	1.007±0.044			1.440±0.040	2.254±0.051
6	1.244±0.035				0.910±0.020	2.011±0.059
7						1.679±0.026
8						1.131±0.047
9						0.865±0.023
10						0.561±0.050

Concentration can be calculated from absorbance using the line equation $C = \frac{A-n}{k}$ and averages from Table 8 and shown in *Table* 9. Thus the uncertainties are calculated using the rules for absolute, relative uncertanties and the propagation formula, as follows:

$$For\ t = 0, T = 25\ ℃$$

$$\delta(A+n) = \delta A_{0;25} + \delta n = 0.012 + 0.000851 = 0.0129 \approx 0.013$$

$$\%unc.(A+n) = \frac{\delta(A+n)}{A+n}100 = \frac{0.013}{3.601 + 0.04097}100 = 0.353\% = 0.4\%$$

$$\%unc.C = \sqrt{(\%k)^2 + (\%A+n)^2} = \sqrt{(3\%)^2 + (0.4\%)^2} = 3.03\% \approx 3\%$$

Table 9: Changing concentrations of methylene-blue at different temperatures

t (min) ± 0.08min	Temperature (°C) ± 0.1°C					
	25	30	35	40	45	50
	Concentration (mg/10ml)					
0	0.992(1±3%)	0.999(1±3%)	0.997(1±3%)	1.002(1±3%)	0.989(1±3%)	1.002(1±3%)
1	0.897(1±3%)	0.884(1±3%)	0.743(1±3%)	0.785(1±4%)	0.882(1±3%)	0.925(1±3%)
2	0.796(1±3%)	0.689(1±4%)	0.465(1±4%)	0.582(1±3%)	0.744(1±3%)	0.860(1±3%)
3	0.661(1±3%)	0.561(1±4%)	0.174(1±4%)	0.372(1±3%)	0.617(1±4%)	0.773(1±3%)
4	0.562(1±4%)	0.352(1±4%)		0.180(1±4%)	0.493(1±3%)	0.687(1±3%)
5	0.459(1±3%)	0.275(1±5%)			0.390(1±4%)	0.617(1±4%)
6	0.336(1±4%)				0.240(1±4%)	0.549(1±4%)
7						0.463(1±4%)
8						0.300(1±5%)
9						0.224(1±4%)
10						0.158(1±9%)

Concentrations of methylene-blue decline as the reaction proceeds. The fastest decrease occurs at T = 35°C, and the reaction rate declines as the temperature deviates from 35°C. The data is plotted against time for a better representation (Figure 9).

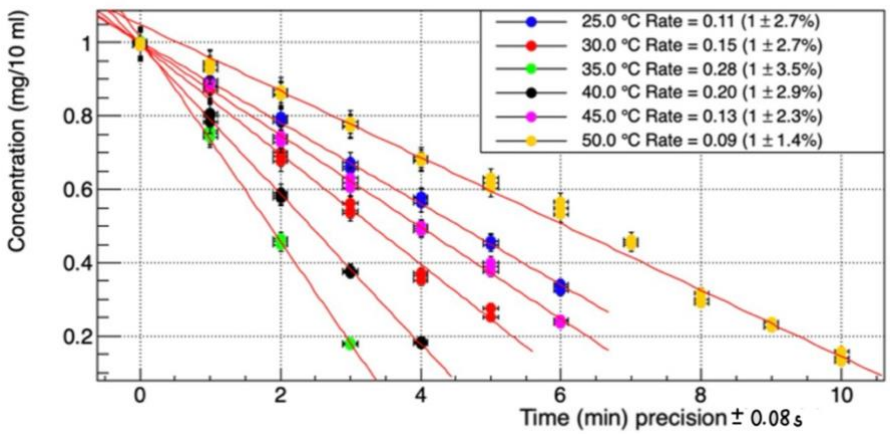

Figure 9: Average concentrations of methylene-blue as a function of time for different temperatures.

The best-fit line for each temperature is drawn to determine the relationship between time and concentration. The highest and lowest reaction rates, respectively, are determined at T=35°C (the steepest line), and T=50°C and T=25°C (the two least steep lines). Gradients of the best-fit lines are examined (Table 10) and plotted as a function of temperature in Figure 10, showing how the reaction rate gradually increases until it peaks and starts declining. Additionally, a data analysis program identifies the absolute (Table 10) and relative (Figure 9) uncertainties of the best fitting slope.

Table 10: Reaction rate as a function of temperature

Temperature (° C) (±0.5)	Reaction rate (mg/10ml min)	Absolute uncertainty (mg/10ml min)	% uncertainty
25	0.1093	0.0017	2.7%
30	0.1521	0.0060	2.7%
35	0.2753	0.0064	3.5%
40	0.2048	0.0020	2.9%
45	0.1249	0.0021	2.3%
50	0.0868	0.0025	1.4%

The reaction rates range from $0.0868\, mg/10ml$ min at 50° C to $0.2753\, mg/10ml$ min at $35° C$. The absolute uncertainties are minor and attributed to a random error in measurement. The most significant uncertainty is identified at 35 °C rate, most likely due to lower number of measurements (4) at that temperature (due to the fastest progressing reaction). The slowest reaction (at 50°C) is the most precise, including 10 measurements.

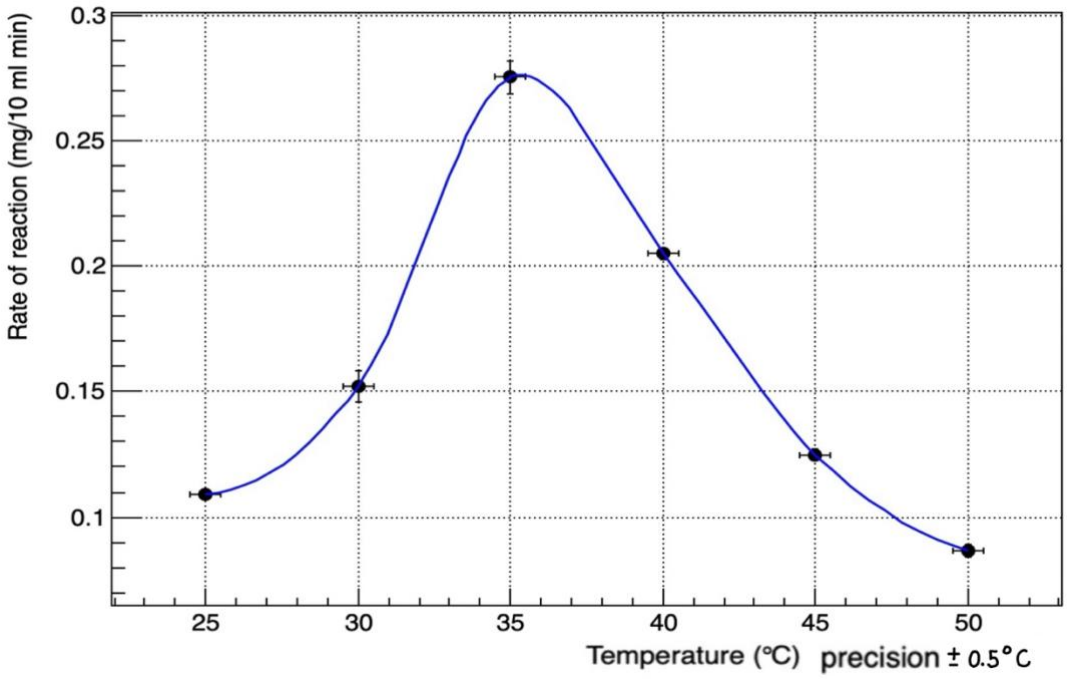

Figure 10: Reaction rate as a function of temperature

These results coincide with literature values (Jgam, 1981), which imply that the optimal activity for dehydrogenase enzyme is highest between 35°C and 40°C and starts denaturing after this temperatures are reached.

6. CONCLUSION

This exploration aimed to determine the rate of NADH oxidation in the presence of enzyme dehydrogenase at different temperatures and to identify its optimum temperature. This was done by exposing a mixture of yeast (source of NADH and dehydrogenase) and methylene-blue solutions to different temperatures. In the process, the methylene-blue, a redox indicator, was reduced by accepting electrons and a proton from NADH, and the sample color changed from blue to colorless. The rate of reaction was monitored every minute by measuring absorbance.

Firstly, a linear relationship between concentration and absorbance was determined by measuring known concentrations of diluted solution of methylene-blue. The line equation was identified as $A=3.63C+0.04$. The absolute uncertainty of the calibration curve was determined as uncertainty on the gradient (± 0.09) and y-intercept (± 0.0004) consistent with the uncertainties propagated through dilution (ranging between 4% and 7%).

Mean values of the absorbance results were translated to the concentration of the samples. Result uncertainties were determined by combining the calibration curve and absorbance uncertainties, which again showed small random errors (Table 9). A best-fit line for changing concentration over time for specific temperatures was shown (Table 9), and the reaction rates were identified as the gradient of lines, indicating the fastest rate at 35°C, followed by 40°C, and the slowest at 50°C. The results align with the hypothesis and indicate that the reaction rate increases due to increased kinetics and decreases due to dehydrogenase enzyme denaturation. This happens after the optimal temperature is reached, between 35°C and 40°C, unprecise due to measurements being 5°C apart. The uncertainties of the gradients were obtained using a

computer fitting algorithm, again small random error was observed. The uncertainty showed an increase in the higher reaction rate due to less obtained values.
Lastly, the results were compared to the literature values (Jgam, 1981) and showed a similar trend for dehydrogenase activity in yeast, further confirming the hypothesis.

7. EVALUATION

7.1 STRENGHTS

The choice of equipment and methodology ensured reliable results. The system's boundaries were well defined, provided by a consistent and controled environment conditions, controlled variables e.g. concentrations, pressure, consistent use of the same type of wells.
The concentrations and volumes were measured with caution and **precise equipment** to limit the random errors. Moreover, a high-precision microplate reader was used (±0.001), enabling acurate absorbance measurements.
Furthermore, to assure the most precise results, the environmental conditions were controlled. An incubator provided a **constant temperature**, fluctuating by 0.5°C, enabling the reaction to proceed at a desired temperature. Also, thermistor was used to verify the temperature of each solution before mixing.
Lastly, **sufficient trials** for calibration curve were conducted to provide accurate results about the best fit line. After calculation of uncertainties, no serious deviations from expected trends were observed as the uncertainties of all measurements were less than 10%.

7.2 WEAKNESSES AND LIMITATIONS

Some weaknesses were observed in the execution of the experiment. Random errors could have resulted from inconsistencies and equipment uncertainties in the pipetting and mass measuring. Also, even though precise volumes of yeast solution were measured, **yeast metabolic variations** may have affected the results, as each yeast cell processes reactants differently. This led to random errors. Secondly, as the solutions were preheated, some enzymes at higher temperatures might have **denaturated** even before the measurements started. This led to a possible systematic error in reaction rate.
Moreover, while the incubator provided a constant temperature, it was **opened every time** the measurements were made, introducing random error leading to slight variations in temperature. Also, the microplate reader was **not zeroed** at the start of the measurements introducing an offset in the callibration curve, which had to be taken into account when creating the linear fit. Lastly, the **standard deviation** of absorbance values for methylene blue was done using only eight samples (instead of the usuall 12-15) which might have provided inacurate results.

7.3 IMPROVEMENTS AND APPLICATIONS

However, the experiment and its results could be improved taking into account the following comments. The data was acquired in intervals of 5°C, which limited the investigation into the optimum temperature. In case of repetition of the experiment, a **better range of temperatures** would be used (between 35°C and 40°C) at smaller intervals to ensure a more precise result about the optimum temperature. Also, the absorbance could be measured **more frequently** to expand the range of values and deduce more accurate results. Improvements in equipment could be made; a **spectrophotometer** measuring one sample at a time could offer even more precision in absorbance than microplate-reader.

Lastly, the three trials of measurements allowed for the determination of absorbance mean values. However, to improve accuracy of the results, **more than three subsequent trials** at each temperature could be executed and the impact of random errors reduced.

To conclude, the experiment provides insight into enzyme kinetics, particularly for dehydrogenase in yeast. The findings are highly applicable and could be extended to studies of metabolic processes, possibly investigating dehydrogenase activity under varying conditions.

8. BIBLIOGRAPHY

Activation energy (n.d.). Ness Labs. Retrieved March 30, 2024, from https://nesslabs.com/activation-energy

Active site. Active site in enzymes (n.d.). Unacademy. Retrieved March 23, 2024, from https://unacademy.com/content/neet-ug/study-material/biology/active-site/

Absorbance technology (n.d.). Molecular Devices. Retrieved December 5, 2024, from https://www.moleculardevices.com/technology/absorbance

Beer/Lambert. The Beer-Lambert law (n.d.). Edinst. Retrieved December 30, 2024, from https://www.edinst.com/resource/the-beer-lambert-law/

Denatured enzyme. Denatured enzyme, definition, causes and effect (n.d.). Studi.com. Retrieved March 22, 2024, from https://study.com/learn/lesson/denatured-enzyme-overview-causes.html#:~:text=What%20does%20it%20mean%20for,the%20chemical%20rate%20of%20reaction

Enzyme. (n.d.). Encyclopædia Britannica. Retrieved March 22, 2024, from https://www.britannica.com/science/enzyme

GHS. Methylene blue GHS classification**.** (n.d.). PubChem. Retrieved December 10, 2024, from https://pubchem.ncbi.nlm.nih.gov/compound/6099#section=GHS-Classification

Irritant agent. (n.d.). ScienceDirect. Retrieved December 10, 2024, from https://www.sciencedirect.com/topics/pharmacology-toxicology-and-pharmaceutical-science/irritant-agent#:~:text=An%20irritant%20is%20any%20noncorrosive,produces%20a%20local%20inflammatory%20reaction

MB. Methylene blue**.** (n.d.). PubChem. Retrieved December 11, 2024, from https://pubchem.ncbi.nlm.nih.gov/compound/6099#section=Hazards-Identification

MB overviw. Methylene blue overview. (n.d.). Edvotek. Retrieved December 11, 2024, from https://www.edvotek.com/site/pdf/Methylene%20Blue.pdf?srsltid=AfmBOopSniqHP9brJ3nCMr4VRQnwGFTzvXx47n9bcwbGDrcsx2mcIRPe.

Molecular neurobiology. (n.d.) Research Gate. Retrived December 27,2024, from https://www.researchgate.net/figure/Chemical-structure-of-methylene-blue-and-leucomethylene-blue_fig1_319275161

Jgam (1981) NADH Dehydrogenase. Temperature dependence and distribution of nadh dehydrogenase in a psychrophilic bacterium vibiro sp. Stran abe/1 Retrieved Februar 15, 2025, from https://www.jstage.jst.go.jp/article/jgam1955/27/4/27_4_327/_pdf/-char/en.

NADH. Understanding the role of NADH in cellular respiration. (n.d.). Drip Hydration. Retrieved on December 10, 2024, from https://driphydration.com/blog/nadh-cellular-respiration/

Poikilotherm. (2024). Wikipedia. Retrieved Januar 20, 2025, from https://en.wikipedia.org/wiki/Poikilotherm

Yeast. *Safety data sheet* (2014). *(SDS)*. Retrieved December 15, 2024, from https://www.fishersci.com/content/dam/fishersci/en_US/documents/programs/education/regulatory-documents/sds/chemicals/chemicals-y/S25632A.pdf