Surname..... Date .....

Name..... Degree program name.....

Exercise B33

Enzymatic Catalysis – Study of Catalase Kinetics Using the Oxygen Sensor

Temperature in the laboratory t = ... °C, T = ... K

Volume of the reaction vessel v = ... ml

Table I: Calculation of the initial reaction rates involving catalase

Measurement	$\Delta C = C_2 - C_1$	$\Delta t = t_2 - t_1$	$V_0 = \frac{\Delta C}{\Delta t}$
with 0.25% H_2O_2			
with 0.5% H_2O_2			
with 0.75% H_2O_2			
with 1% H_2O_2			

Table II: Conversion of percentage concentration C_p of H_2O_2 to molar concentration $C_{m[S]}$

Percentage concentration C_p H_2O_2 [%]		**Molar concentration $C_{m[S]}$ H_2O_2 [mol/dm^3]**
0.25	$\boldsymbol{C_{m[S]} = \frac{C_P * d}{M * 100\%}}$	
0.5		
0.75		
1		

*where **M** – the molar mass of H_2O_2 = 34.0147 g/mol, **d** – density of H_2O_2 = 1.45 g/cm^3

Table III: Parameters of catalytic reaction

$\boldsymbol{\frac{K_m}{V_{max}} = a}$	$\boldsymbol{\frac{1}{V_{max}} = b}$	**Maximum reaction rate V_{max} in enzymatic reactions**	**Michaelis-Menten constant K_M**

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OBJECTIVE

The main idea of the exercise is to study the enzymatic kinetics of catalase by analyzing the rate of hydrogen peroxide (H_2O_2) decomposition. For this purpose, an oxygen sensor designed for the quantitative measurements of molecular oxygen can be used to determine the Michaelis-Menten constant (K_m) and the maximum catalytic reaction rate (V_{max}).

THEORY

Catalase is one of the most important and efficient enzymes found in living organisms. It plays a key role in protecting cells from oxidative stress, and particularly from the toxic effects of reactive oxygen species (ROS – reactive oxygen species). As a result of various biochemical reactions, especially those related to cellular respiration (e.g., oxidative phosphorylation, oxidation in peroxisomes), potentially harmful free radicals and hydrogen peroxide are generated. H_2O_2 can undergo further conversion into more reactive hydroxyl radicals (●OH) for example, in Fenton reaction. Catalase quickly and efficiently protects against this threat by catalyzing the decomposition of hydrogen peroxide into water and oxygen.

Catalase is a tetrameric heme protein, meaning it consists of four identical subunits, each containing a heme group within its structure, surrounded by a protein (apoenzyme). The heme active site, with a centrally located iron atom, is crucial for the enzyme's catalytic activity. The heme group, as a non-protein prosthetic group, is also found in hemoglobin, where it is responsible for binding and activating hydrogen peroxide.

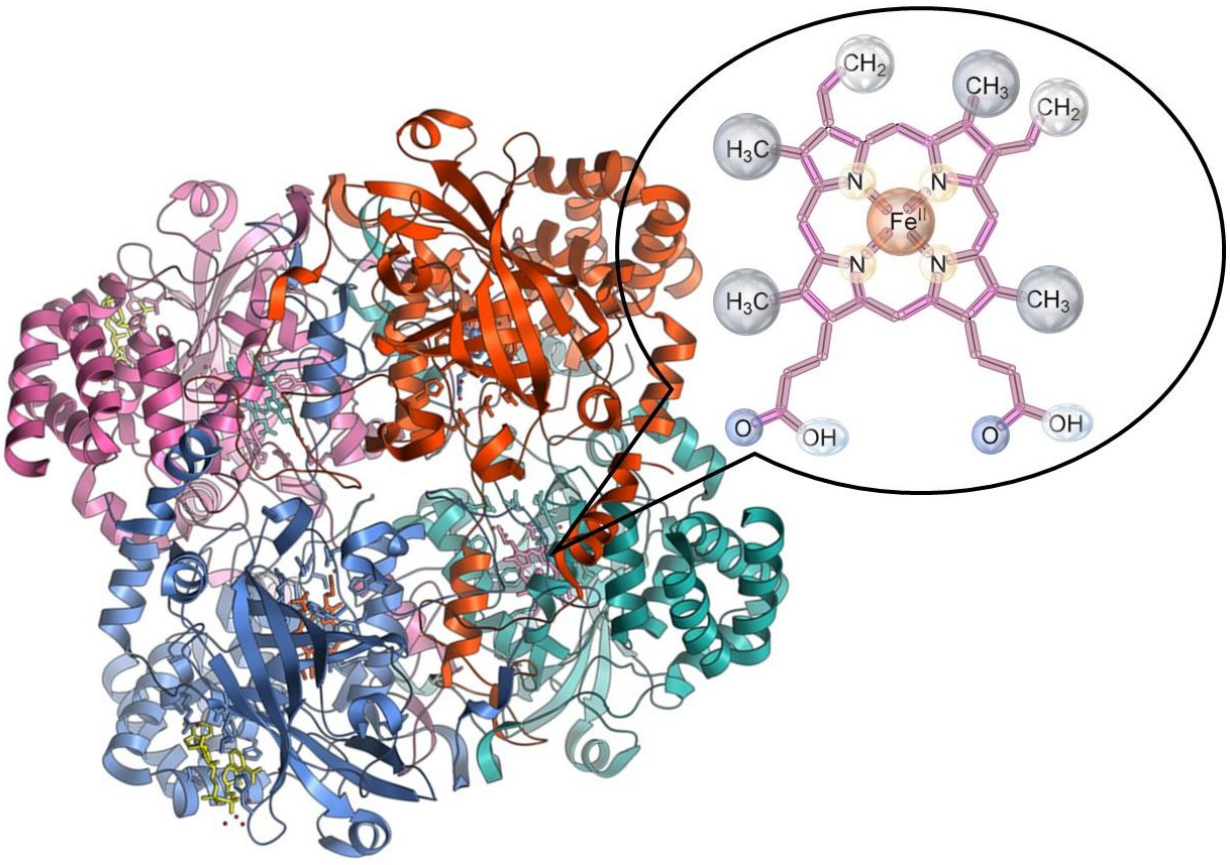


Fig. 1 Quaternary structure of catalase and iron-porphyrin, the so-called heme prosthetic group, located in the active site of the enzyme.

The catalytic mechanism of catalase can be divided into two main stages:

I. In the first stage of the process, as shown in Fig. 2, a hydrogen peroxide molecule (the substrate) binds to the enzyme's active site, resulting in the heme group transitioning into an intermediate state known as oxyheme. In this phase, one molecules of H_2O_2 is decomposed, producing a molecule of water and atomic oxygen, which remains bound to the iron atom in the heme group.

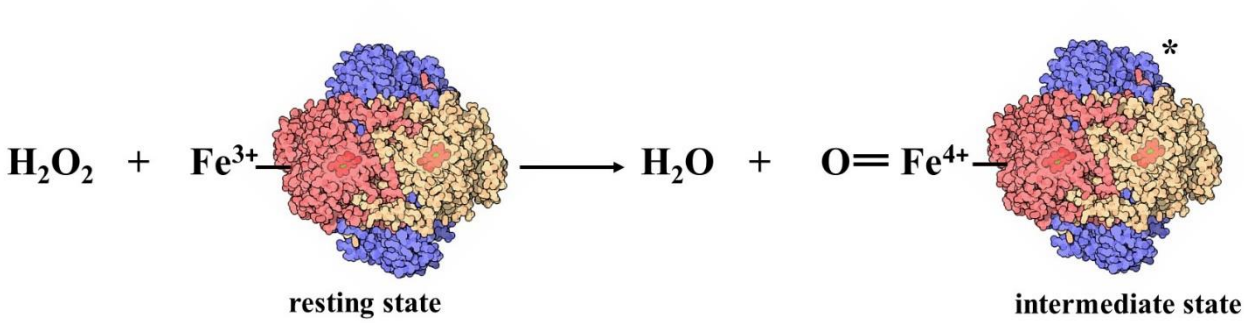


Fig. 2 Schematic representation of the first stage of the catalytic mechanism of hydrogen peroxide decomposition by catalase. In the resting state of catalase, the iron atom in the heme group is in the +3 oxidation state. In the intermediate state, oxygen binds to the heme group, and the oxidation state of the iron atom increases to +4.

II. In the **second** stage of the process, as shown in Fig. 3, another hydrogen peroxide molecule reacts with the oxyheme complex. This leads to its reduction, resulting in the formation of water and molecular oxygen (O_2), and catalase returns to its resting state.

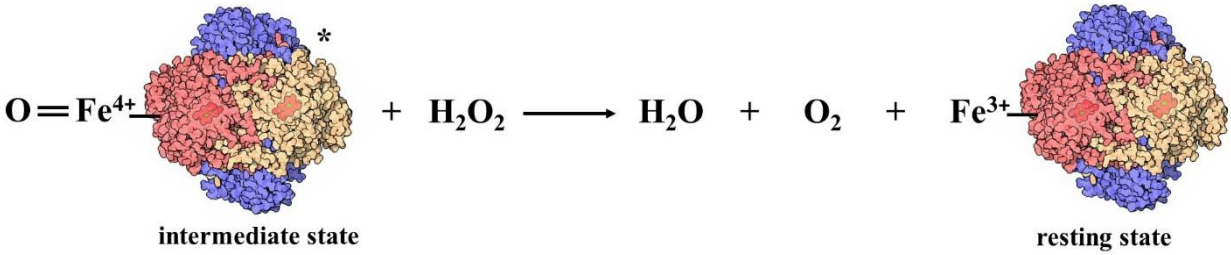


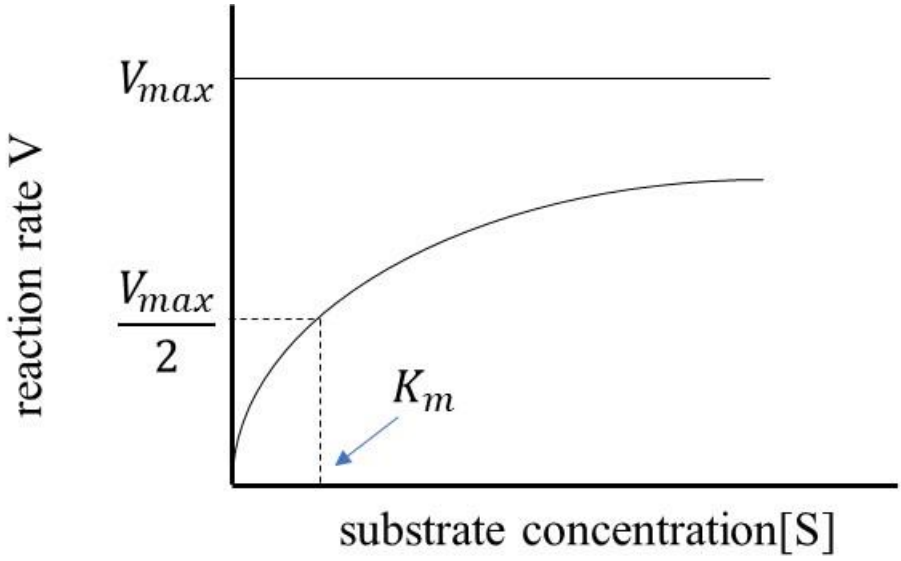
Fig. 3 Schematic representation of the second stage of the catalytic mechanism of hydrogen peroxide decomposition by catalase.

The entire process occurs extremely rapidly – catalase can convert millions of hydrogen peroxide (H_2O_2) molecules per second, making it one of the most efficient enzymes known in biology. Additionally, the catalytic reaction is a redox process, as both hydrogen peroxide and the heme group of the enzyme undergo oxidation and reduction. This precisely why catalase belongs to the group of enzymes known as oxidoreductases.

Enzymatic reactions, such as the one catalyzed by catalase, can be represented using Michaelis-Menten kinetics. It serves as the fundamental mathematical model used to describe the rate of enzymatic reactions as a function of substrate concentration. The relationship of the kinetics and the Michaelis-Menten equation is illustrated in Fig. 4, where:

- V_0 represents the initial reaction rate, which is the speed of the reaction at the beginning of the process when the substrate concentration is still high,
- V_{max} is the maximum reaction rate, which is reached when all enzyme molecules are engaged in the process, and further increases in substrate concentration do not lead to a higher reaction rate,
- [S] represents the substrate concentration,

- K_m is the Michaelis-Menten constant, which reflects the enzyme's affinity for the substrate. Specifically, it represents the substrate concentration at which the reaction rate reaches half of V_{max}.

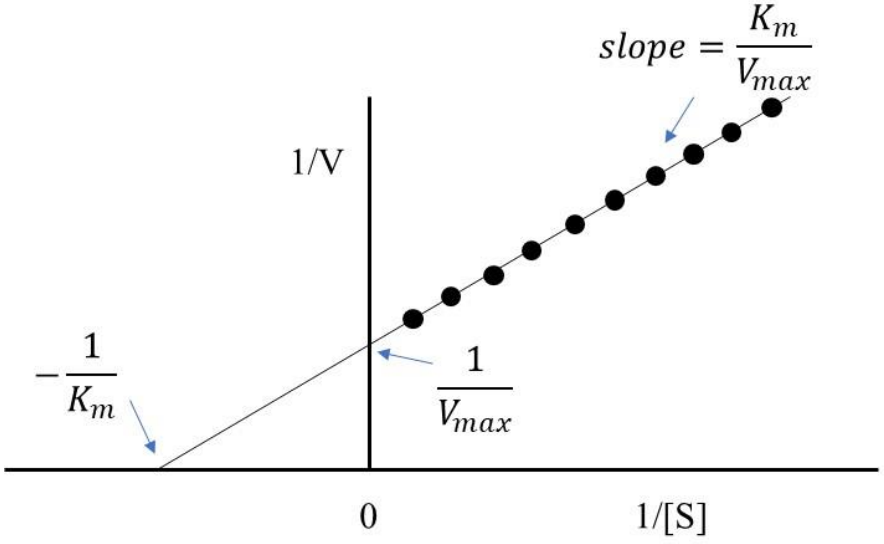


$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$

Fig. 4 Kinetic dependence of the reaction rate V on substrate concentration [S] and the Michaelis-Menten equation.

The Michaelis-Menten constant K_m reflects how strongly and specifically the enzyme binds to the substrate. High affinity (high K_m) indicates that enzyme molecules readily react with the substrate, even at low substrate concentration, whereas low affinity (low K_m) suggests that the enzyme requires higher substrate concentrations to bind effectively.

The Michaelis-Menten constant K_m and the maximum catalytic reaction rate V_{max} can be determined by fitting the relationship between the initial reaction rate V_0 and substrate concentration [S] to the Michaelis-Menten equation. However, a more practical approach is to use the Lineweaver-Burk plot, which is a linear transformation of the Michaelis-Menten equation (Fig.5):



$$\frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

Fig. 5 Kinetic relationship and the Lineweaver-Burk equation.

Fun fact

Biosimilar drugs are intended to mimic the function of endogenous proteins or enzymes, demonstrating therapeutic efficacy equivalent to that of a reference drug. The kinetic experiments conducted in this exercise can be used to compare their activity with that of natural counterparts.

An example is *Enbrel*, a biologic immunosuppressive drug containing the active ingredient *etanercept*, used in the treatment of severe, active, and progressive rheumatoid arthritis. Its more cost-effective biosimilar available on the market is *Benepali*, which demonstrates high similarity in structure, efficacy, safety, and quality.

PERFORMING THE EXERCISE

Exercise B33: Enzymatic Catalysis – Study of Catalase Kinetics Using the Oxygen Sensor

EQUIPMENT	
• PC with „SPARKvue" software	• Magnetic stirrer with stir bar
• O_2 sensor - PASCO wireless O_2	• Glass bottle, graduated cylinder, plastic spoon
• Plastic bottle with tap	• Catalase (powder)
• Syringe with tubing	• Aqueous hydrogen peroxide solutions

Record all data and calculations in the table and report.

Computer setup – do not save changes to files (DON'T SAVE)

1. Turn on the table power (see the table's dashboard – located near your left leg when sitting directly in front of the computer), turn the red knob in the direction of the arrows (it should pop up), turn the key as you would in a car, then release. Finally, turn on the computer.

2. The *Windows* operating system will start automatically. Log in by selecting the user icon labeled „**B33"**. Turn on the PASCO wireless **O_2** sensor. The sensor will turn off automatically when not in use. If the sensor battery is low, connect it directly to the computer using a cable. On the center of the desktop, you will find a shortcut labeled "**B33**" – this opens the „SPARKvue" program. Expand the window to full screen.

3. Click the Bluetooth icon (upper right corner of the window). Connect the **O_2** sensor (236-170 O2) by selecting it from the devices window. Once added correctly, click "Done". Proper sensor connection is shown in Fig. 6. **Do not connect any other sensors!**

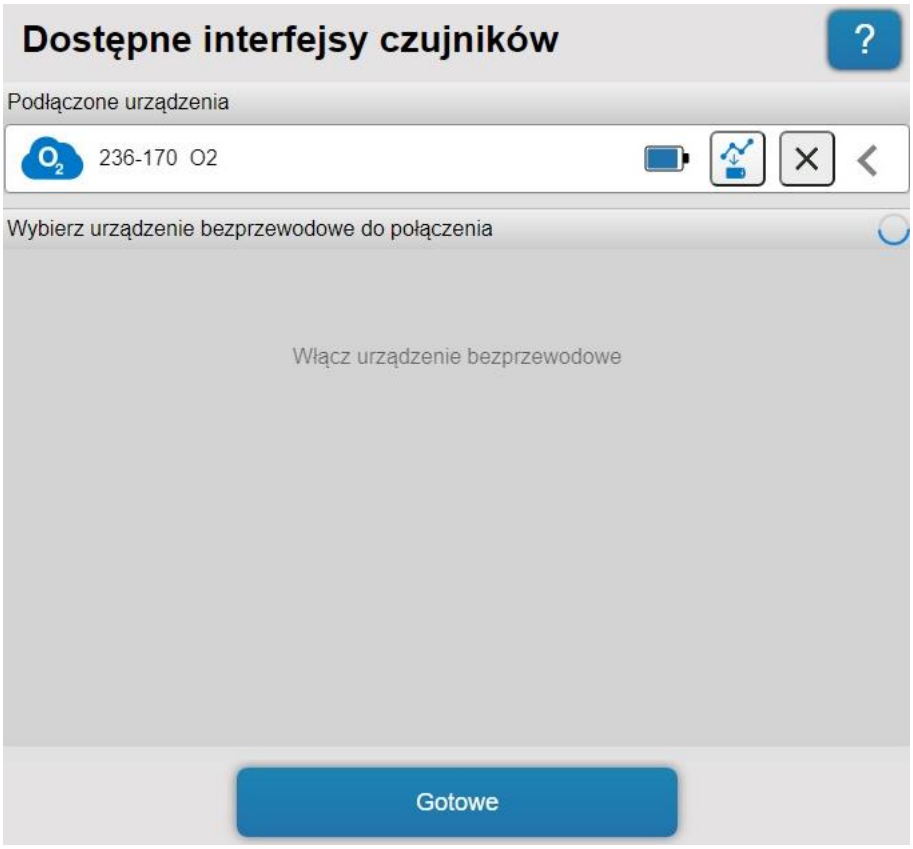


Fig. 6 Wireless Connection Window for the Pasco Oxygen Sensor

Experiment window **B33**

The main window „**Pomiar stężenia tlenu/Oxygen concentration measurement**" (Fig. 7) contains control buttons and a graph of the O_2 concentration (%) versus time (s). the axis ranges will adjust automatically during measurements.

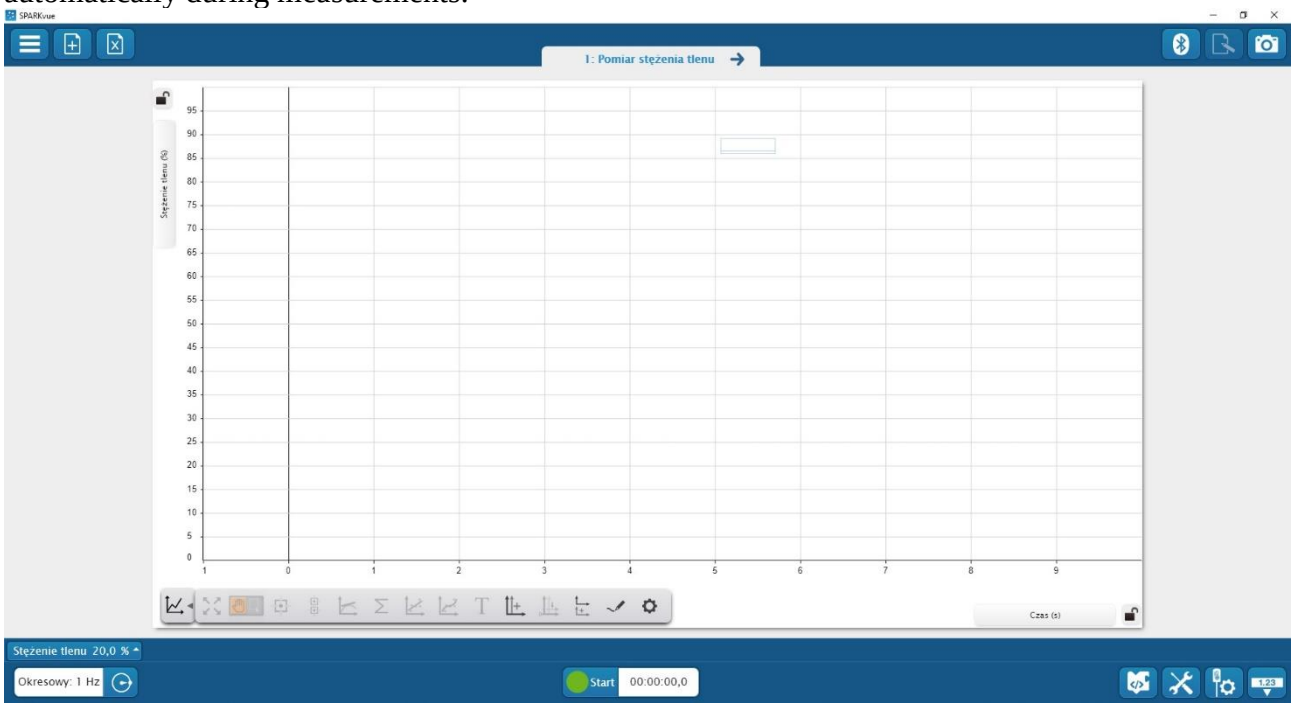


Fig. 7 Main measurement window „Pomiar stężenia tlenu/Oxygen concentration measurement".

Calibration of PASCO wireless O_2 sensor (236-170 O2).

1. Click on the „Stężenie tlenu/Oxygen concentration” bar (bottom left corner of the „SPARKvue” window).
2. Select „Skalibruj pomiar/Calibrate measurement”, then click „Kontynuuj/Continue”.
3. In the second calibration point, enter the value 20.9 in the „Standardowa wartość/Standard value” field, then click „Odczytaj z czujnika/Read from sensor” and confirm with „Ok”. The value of 20,9% represents the oxygen concentration in the atmosphere.

Preparation of the measuring system

1. Ask the instructor for vials containing aqueous hydrogen peroxide solutions with different concentrations (0.25%, 0.5%, 0.75%, 1%).
2. Prepare an aqueous catalase solution. Weigh 5 g of catalase powder on an analytical balance – use a plastic spoon and a piece of paper for accuracy. Transfer the measured catalase into a glass bottle, then add 200 ml of cold water. Secure the bottle cap and mix the contents the enzyme is fully dissolved.
3. Carefully pour 15 ml of the hydrogen peroxide solution with the lowest concentration (0.25%) into the plastic bottle with a tap, then dry the upper part of the bottle with a paper towel.
4. Place the magnetic stir bar into the bottle containing the hydrogen peroxide solution.
5. Secure the top of the bottle containing hydrogen peroxide solution with the PASCO oxygen sensor.

Fig. 8 Properly prepared measurement setup.

Procedure and data recording

Spontaneous decomposition of hydrogen peroxide

1. During the experiment, the bottle sealed with the sensor should remain stable and upright on the laboratory table.
2. Wait 5 minutes to allow measurement conditions to stabilize.
3. Press the green „START” button (a red square will appear, and a timer will begin when data collection is active). The measurement will last for 7 minutes and will end automatically.
4. If the experiment does not end automatically after 7 minutes, stop it manually by pressing the red „STOP” square.
5. Retain the prepared measurement setup for the next experiment and follow the instructions below.

Decomposition of hydrogen peroxidase in the presence of catalase

1. Place the prepared measurement setup securely and upright on the magnetic stirrer.
2. Connect the power cable of the magnetic stirrer to an outlet and set the stirrer to maximum speed. Adjust the bottle position to ensure uniform and even mixing.
3. Draw 15 ml of the aqueous catalase solution into a plastic syringe (to make it easier, pour the enzyme solution into a beaker). Then attach tubing to the syringe tip and connect it to the bottle's tap.
4. Press the green „START" button. The measurement will last for 7 minutes and will end automatically. **NOTE! At the 2-minute mark, quickly but smoothly inject 15 ml of the catalase solution into the bottle over approximately 4 seconds.**
5. If the experiment does not end automatically after 7 minutes, stop it manually by pressing the red „STOP" square.
6. After completing the measurement, disconnect the syringe, remove the oxygen sensor from the bottle, and pour out its contents. Then, rinse the bottle thoroughly twice with cold tap water and dry the upper part with a paper towel.
7. Wait until the PASCO oxygen sensor displays an oxygen detection level of 20.9%.
8. Repeat the catalase experiment using 0.5%, 0.75% i 1% hydrogen peroxide solutions. Follow again the instructions in the section titled „**Decomposition of hydrogen peroxidase in the presence of catalase"**

Fig. 9 Measurement setup required for conducting experiments in the presence of catalase.

Data analysis

1. Based on the recorded oxygen concentration versus time data during the H_2O_2 decomposition in the presence of catalase, determine the initial reaction rate V_0 for each tested hydrogen peroxide solutions. For this purpose, select the linear segment of the curve representing the sudden increase of oxygen concentration (starting around the 2-minute mark of the measurement).
2. Use the „Scale to fit" button to adjust the obtained data to fit the window.
3. From the selected linear section of the curve, select two points (Fig. 10) labeled as (**t_1, C_1**) and (**t_2, C_2**), and record their values: where:
   - **t_1** and **t_2** represent the time values corresponding to these points,
   - **C_1** and **C_2** are the oxygen percentage concentration values at these points.
4. Calculate the slope of the linear section of the curve, which corresponds to the initial reaction rate V_0, using the following equation:

$$slope = \frac{\Delta C}{\Delta t} = \frac{C_2 - C_1}{t_2 - t_1}$$

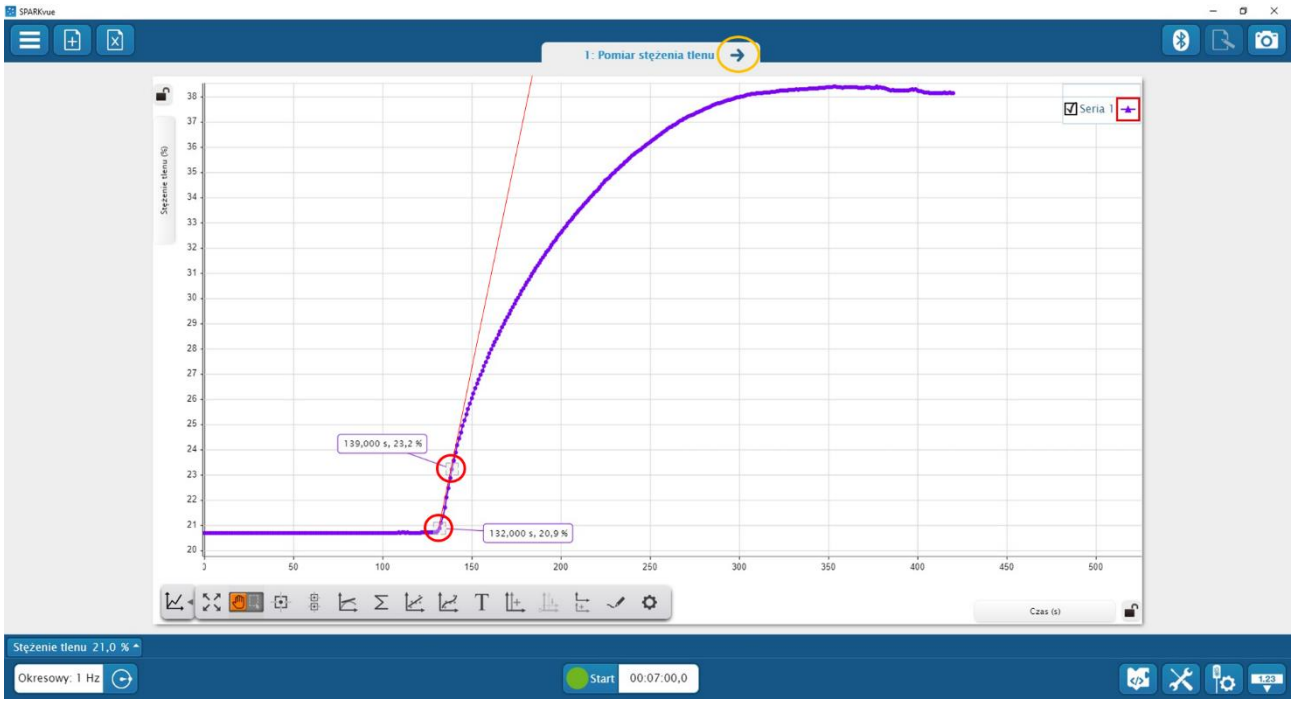


Fig. 10 Selection of measurements points required to determine the initial reaction rate.

5. Perform the calculations of the initial reaction rate $\mathbf{V_0}$ for all recorded curves (for all hydrogen peroxide concentrations) and enter the results into the measurement table I „*Calculation of the initial reaction rates involving catalase*".
6. Using the calculated initial reaction rates $\mathbf{V_0}$, create a Lineweaver-Burk plot and fit a straight line to the resulting data, as shown in Figure 5.
7. For this purpose, navigate to the next tab in the application by clicking the arrow highlighted with a yellow circle (Fig. 10).
8. Using the equation provided below, convert the percentage concentrations (0.25%, 0.5%, 0.75%, 1%) of hydrogen peroxide into molar concentrations **[S]**, where the density $\mathbf{d}H_2O_2$ is 1.45 g/cm^3, and the molar mass **M** is 34.0147 g/mol:

$$\boldsymbol{C_{m[S]}}\left[\frac{mol}{dm^3}\right] = \frac{\boldsymbol{C_p}[\%] * \boldsymbol{d}\left[\frac{g}{cm^3}\right]}{\boldsymbol{M}\left[\frac{g}{mol}\right] * \mathbf{100}}$$

9. In the window shown in Figure 11, enter the reciprocals of the calculated initial reaction rates ($\mathbf{1/V_0}$) and the corresponding reciprocals of hydrogen peroxide concentrations ($\mathbf{1/C_{m[S]}}$) into the appropriate columns of the table. Then, fit a straight-line equation by clicking the icon highlighted with a red circle (Fig. 11) in the application. The determined slope coefficient „*a*" of the fitted line „$y = ax + b$" is equal to the ratio $\mathbf{K_M/V_{max}}$.

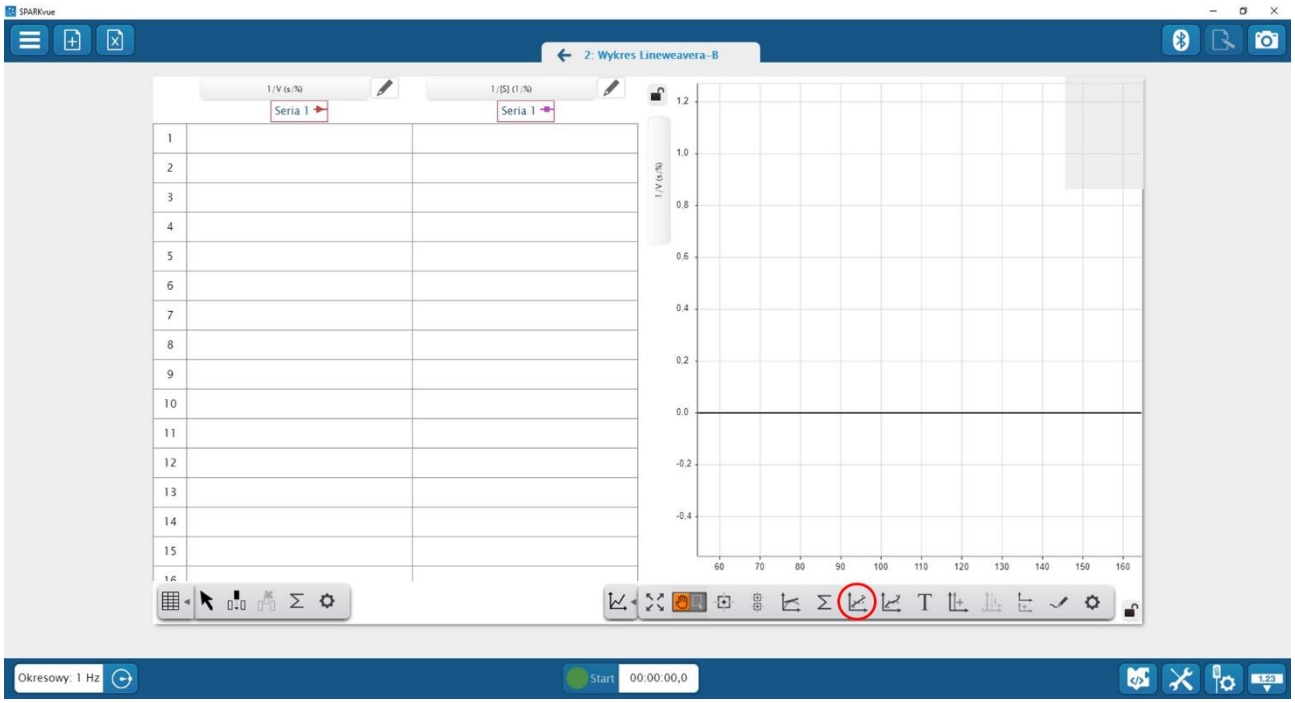


Fig. 11 Table and Lineweaver-Burk plot window.

10. Determine the intersection point of the fitted line with the Y-axis (the value of this point is $1/V_{max}$ and corresponds to the intercept term „b" in the equation „y = ax + b"). Use this value to calculate the maximum catalytic reaction rate V_{max}.
11. Identify the intersection point of the fitted line with the X-axis (the value of this point is $-1/K_m$ and calculate the Michaelis-Menten constant K_m as a positive value.
12. Create the Lineweaver-Burk plots and the graphs showing the relationships between the initial reaction rate V_0 and the molar substrate concentration $C_{m[S]}$ (Michaelis-Menten hyperbola) at home, using Excel or graph paper. On the graphs, mark the characteristic points, including the maximum catalytic reaction rate V_{max}, the Michaelis-Menten constant K_m, as well as $1/K_M$, $1/V_{max}$ and K_M/V_{max} in accordance with Figures 4 and 5.

Questions for Discussion:

1. How does the initial reaction V_0 change with the concentration of hydrogen peroxide, and what is the experimental significance of the obtained maximum catalytic reaction rate V_{max}?
2. In what units is the Michaelis-Menten constant K_m expressed, and what does it represent in the context of this experiment?
3. What parameters and factors might contribute to inaccuracies in the experimental measurements?