The aim:

To determine the effect of the environmental conditions (temp. and pH) on the velocity of enzymatic catalysis using amylase as model

Enzyme

Enzyme - a protein acts as biological catalyst for specific chemical reactions.

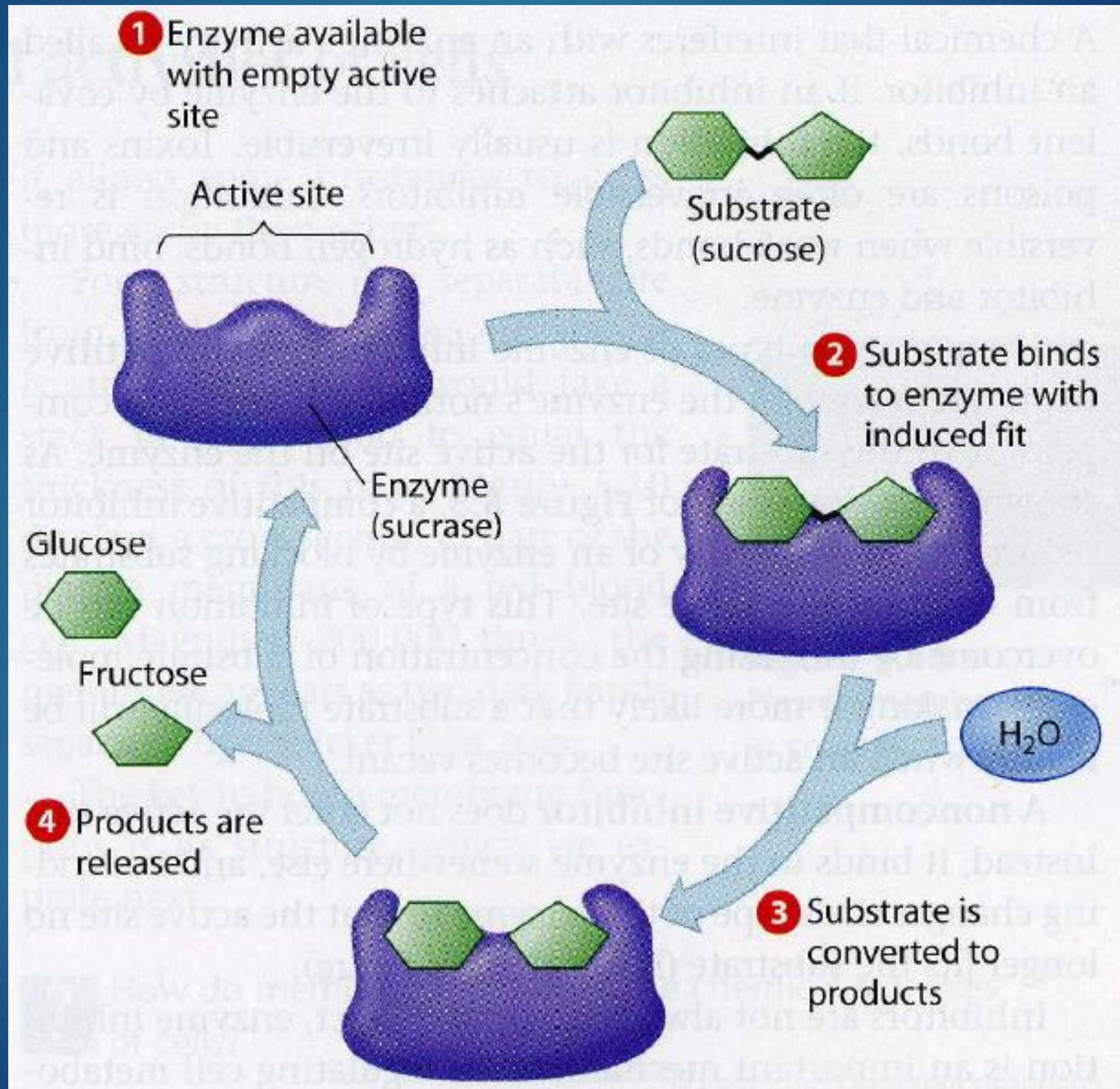
The mechanism of enzyme action:

Enzyme, like any protein molecule, has 3D structure. The region that contains catalytic residues (around 3–4 amino acids with polar side chains) is known as the **active site.**

Active site is a cleft or crevice to which the specific set of reactants (called substrates) bind, and then, they are converted into specific products.

Desintegration of the structure of the enzyme changes the distance between the aa residues, which build the active site. It leads to a change in the ability to bind to the substrate and thereby affect the velocity of catalytic reaction.

Scheme of enzymatic catalysis



Enzyme Classification

An official commission of the International Union of Biochemistry and Molecular Biology (IUBMB) has classified enzymes in the following six categories, based on **the type of catalyzed reaction**:

1. Oxidoreductases	catalyzing dehydrogenation or other oxidation and reduction reactions; one substrate is oxidixed, the another reduced
2. Transferases	transfer of certain groups from one substrate to the other
3. Hydrolases	catalyzing transfer of groups to the hydroxide ion of H_2O; hydrolytic cleavage of different bonds
4. Lyases	promoting addition to double bonds or the reverse
5. Isomerase	catalyzing rearrangement reactions
6. Ligases / Synthetases	catalyze condensation with simultaneous cleavage of ATP and related reactions

Common names for enzymes begin with some description of its action and end with the –*ase* suffix, eg.:

lactate dehydrogenase (EC 1.1.1.27.)

The commision (IUBMB) has also developed a numerical system for classifying enzymes (to avoid errors occurring during translation into other languages).

The names begin with EC (for Enzyme Commission) and end with four numbers, separated by decimal points, that describe the enzyme. The code is unique for each enzyme.

Enzyme Nomenclature

EC **1**.1.1.27.
1st number – **class**
tells us which of the six major enzyme classes the enzyme belongs to. In this case, the 1, designates the enzyme as a oxidoreductase

EC 1.**1**.1.27.
2nd number - **subclass**
indicates what group the enzyme acts on (CH-OH; dehydrogenation)

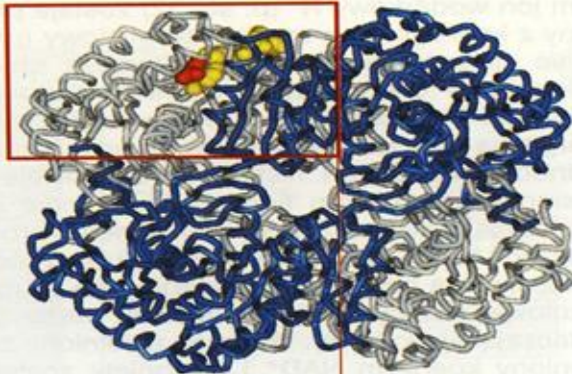
EC 1.1.1.27.

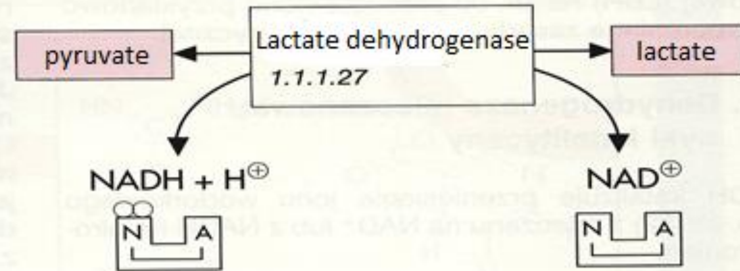
EC 1.1.**1**.27.
3rd number - **sub-subclass**
indicates what is the acceptor of hydrogen atoms (NAD)

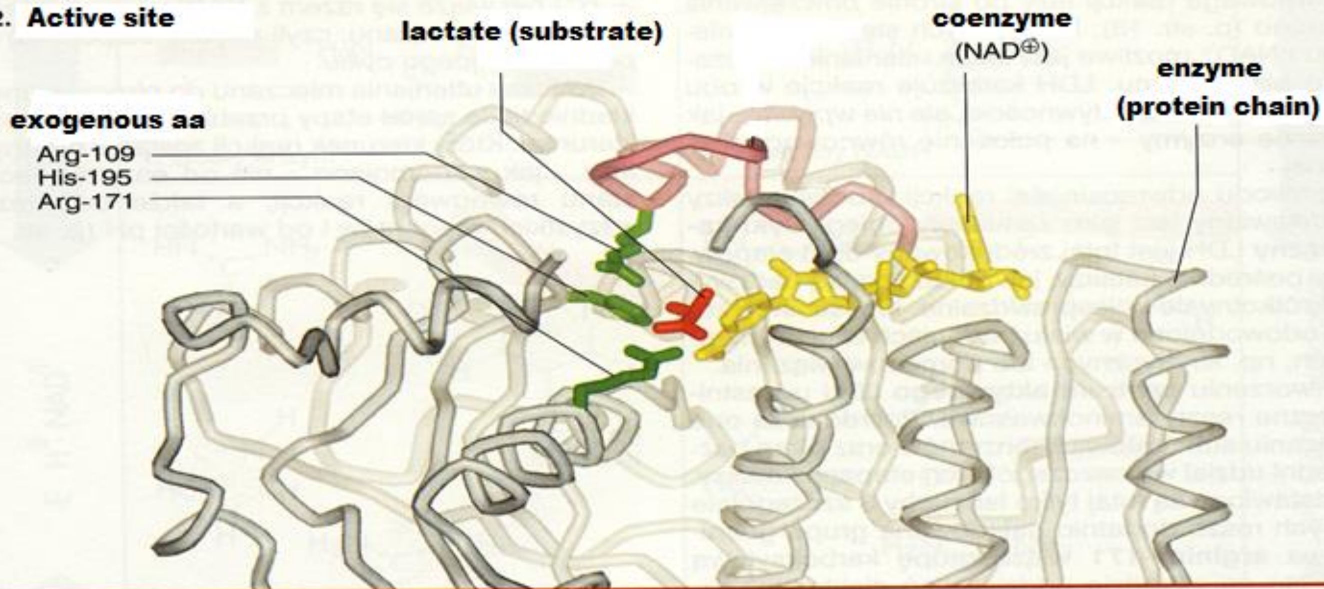
EC 1.1.1.**27**.
4th number -
number within the sub-subclass

Structure and function of lactate dehydrogenase (LDH)

A. Chemical structure of LDH







The effects of external factors on the velocity of enzymatic catalysis

Several factors have the influence on:

- the distribution of charges in a protein molecule
- the ability to create hydrophobic interactions, hydrogen bonds and van der Waals forces

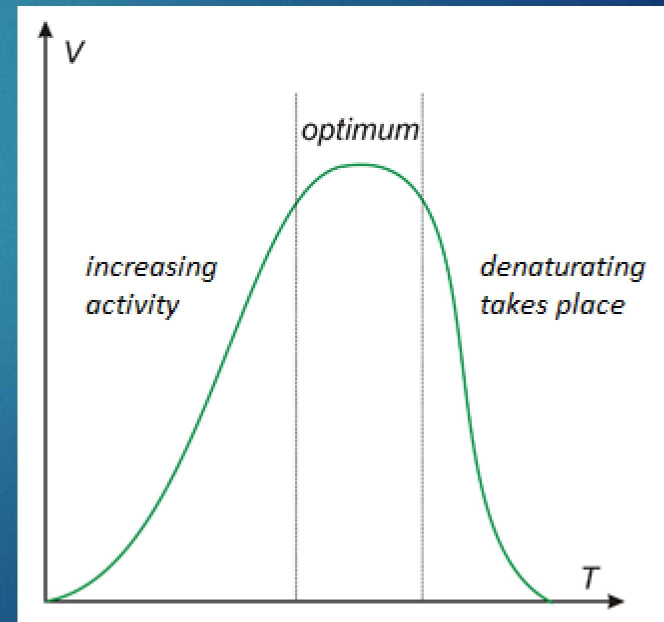
These factors are as follows:

- the presence of **mono-** and **bivalent ions**
- **pH** of the solution
- **temperature**

The effect of the TEMPERATURE

- Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases as the temperature is raised.
- A ten degree Celsius (10°C) rise in temperature increases the activity of most enzymes for 2-3 times
- However, most animal enzymes rapidly become denatured at temperatures above 40°C

- The tolerance of the various enzymes against the temperature is different
- For enzymes of animal origin body temperature **37°C** is optimal



The effect of pH

The velocity of an enzymatic reaction depends on the H^+ ion concentration.

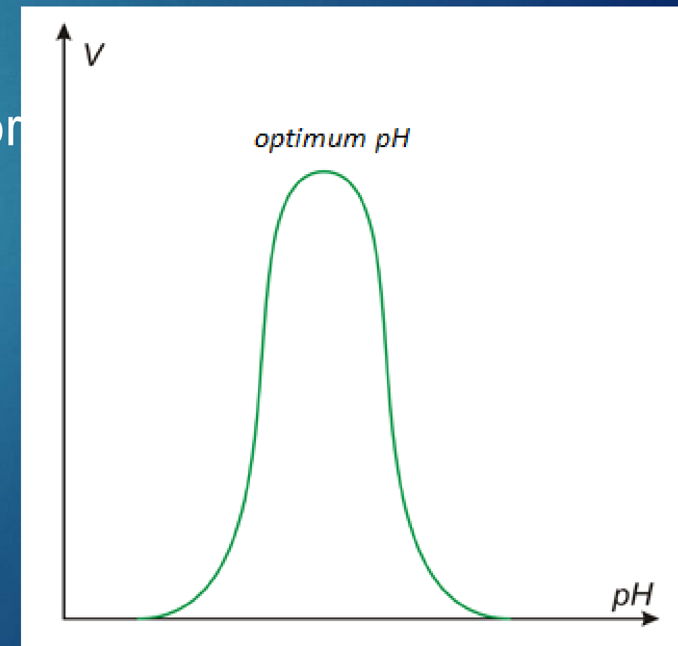
The most favorable pH value - the point where the enzyme is most active - is known as the **optimum pH** (unique for each enzyme).

Changes in the pH value (↑ and ↓) alter:

•the chain conformation of the enzyme protein, or

•the electric charge of either the substrate or the enzyme and by this the binding of substrate.

These effects result in the decrease of the velocity of the reaction.



The effect of ions

Activators increase the velocity of enzymatic reactions. The mechanism of this activation is yet unclear. It is supposed that the activators may loosen the bonds of the substrate.

1. Mg^{2+} (phosphatases, phosphokinases, synthetases)
2. Zn^{2+} (carbonic anhydrase, lactate dehydrogenase, alcohol dehydrogenase, proteases)
3. Mn^{2+} (peptidases)
4. Ca^{2+} (lipase)
5. Cu^{2+} (oxydases)
6. Seldom: Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Na^{+}, K^{+}

- Anions have little effect on enzyme activity, except **Cl^{-}, acting as an amylase activator**
- Heavy metal cations (Pb^{2+} , Hg^{2+}) have **inhibitory effects.**

Salivary α-amylase (EC 3.2.1.1)

Amylase belongs to the 3rd class of enzymes (Hydrolases).

Reaction:

- it cleaves **(1→4)-α-D-glucosidic linkages** in polysaccharides (starch and glycogen) and products of their gradual hydrolysis,

Products:

- oligosaccharides containing 6-7 glucose units (dextrins),
- trisaccharides
- maltose

Methods for determining the activity of various enzymes

Measuring of the activity of an enzyme can be performed by measuring:

1) the decrease in the concentration of the substrate

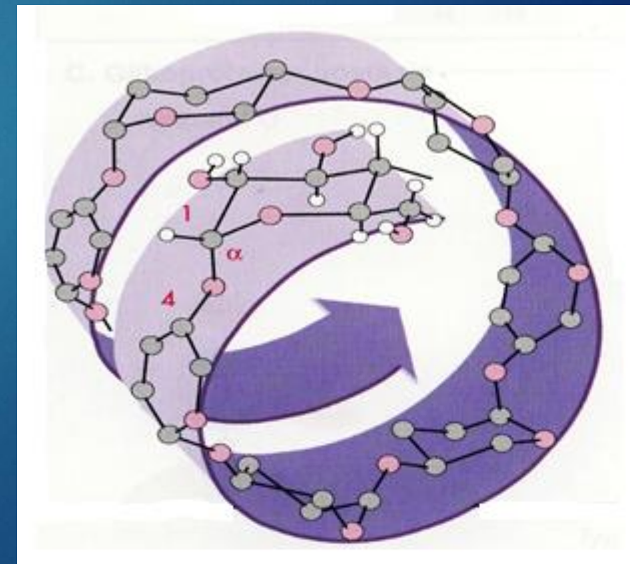
2) the increase in the concentration of the new product

Reaction of starch digestion – method 1
Iodine test

- The non-hydrolysed starch gives a positive result in the test with iodine solution (blue color). Blue color indicates the presence of undigested starch.

The shorter intermediary products of the starch degradation show blue-brownish (amylodextrin), red (erythrodextrin) or no colour (achrodextrin) with I_2 solution.

The time at which color is no longer visible, indicating the disappearance of iodine-starch complex, is defined as **<u>achromatic point</u>**.



Reaction of starch digestion – method 2
Tests for reducing sugars

In general, the following reactions are used for the detection of the reducing aldehyde groups:

- Benedict's test
- Fehling's test
- Trommer's test
- Tollens' test

These tests allow to show the presence of monosaccharides or maltose - the end products of starch hydrolysis.

Principles of enzyme activity determination in tested samples

1. Prepare a solution in which the reaction will take place (incubation mixture) and which most commonly consists of:

- a buffer of optimum pH
- an appropriate substrate

2. Insert the incubation mixture into a water bath to ensure a constant, desired temperature - usually 37°C

3. Add an enzyme solution. The reaction starts when the enzyme is joined to the substrate, so the reaction time is counted from that moment: time „0" (not from the insertion of the incubation mixture into the water bath!)

**Sometimes the enzyme can be added to the buffer solution first. Then the addition of substrate starts the reaction.*

Task 1. Procedure for determining the achromatic point

The aim: to measure the enzyme (amylase from saliva) activity

I stage: We should establish optimal concentration of saliva (source of the enzyme) to obtain the achromatic point within 3-15 minutes

Achromatic point - the time at which the products from the hydrolysis of starch are released and the color of iodine-starch complex is no longer visible

Preparation of enzyme solution:

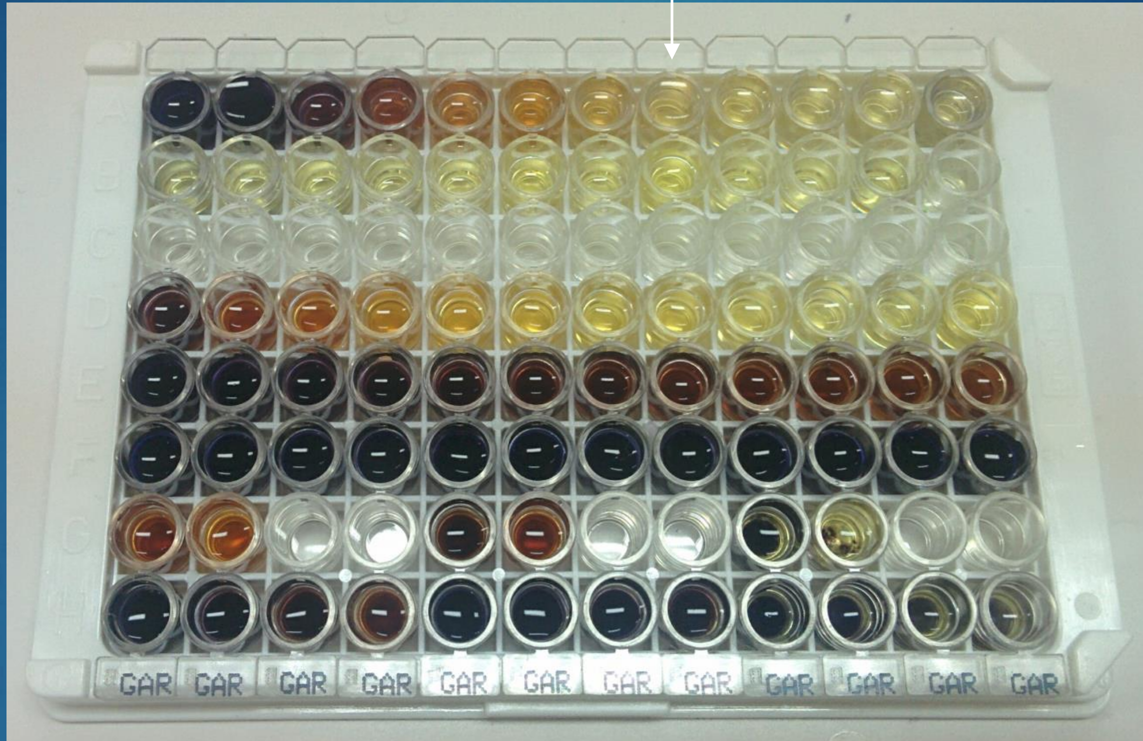
1. Expectorate the saliva into a clean beaker (or test tube). Collect about 5 ml of saliva.
2. Add 1 ml of saliva to the graduated cylinder, dilute it with distilled water (1:100 or a 0.1% enzyme solution) and mix.

Task 1. Procedure for determining the achromatic point

Procedure:

1. Add 3 drops of iodine solution (I_2 in KI; 0,001 mol/dm^3) and 1 drop of HCl to 15 wells of the plate.
2. Add 5 cm^3 of starch solution, 2 cm^3 of NaCl and 2 cm^3 of phosphate buffer solution (pH=6.6) to a test tube and mix.
3. Incubate it for 5 minutes in water bath (37°C).
4. Add 1 cm^3 of prepared enzyme solution (diluted saliva). This is the zero time.
5. After 1 minute transfer 0.2 cm^3 of incubation mixture to the first well containing the iodine solution.
6. From this point on continue this procedure at **one minute intervals** until the achromatic point is reached. This well will have the same color as iodine without starch (yellow).

achromatic point



In case of too short time (less than 3 minutes) or too long (more than 15 minutes), repeat the test with **another dilution of saliva**.

Task 2. Evaluation of the effect of pH on the amylase activity

Procedure:

1. Add 3 drops of iodine solution (I2 in KI; 0,001 mol/dm^3) and 1 drop of HCl to the wells of the plate creating 3 rows.
2. Prepare 3 test tubes with 5 cm^3 of starch solution and 2 cm^3 of NaCl.
3. Then, add 2 cm^3 of phosphate buffer of pH=6.6 into the 1st tube, 2 cm^3 of phosphate buffer of pH=8.0 into the 2nd tube and 2 cm^3 of phosphate buffer of pH=5.0 into the 3rd one.
4. Incubate them for 5 minutes in water bath (37°C) and add 1 cm^3 of prepared enzyme solution (prepared in the first task) to each tube.
5. Every one minute transfer 2 drops of incubation mixture to the following wells until yellow color is reached.

Task 3. Evaluation of the effect of chloride ions on the amylase activity

Procedure:

1. **1.** Add 3 drops of iodine solution (I_2 in KI; 0,001 mol/dm^3) and 1 drop of HCl to the wells of the plate creating 2 rows.
2. Prepare 2 tubes with 5 cm^3 of starch solution and 2 cm^3 of phosphate buffer of optimal pH (the result of Task 2).
3. Add 2 cm^3 of 1% NaCl to the 1st tube and 2 cm^3 of distilled water to the 2nd.
4. Incubate them for 5 minutes and add 1 cm^3 of prepared enzyme solution to each tube.
5. Every one minute transfer 2 drops of incubation mixture to the following wells.
6. Note color changes and achromatic points for each row.

Task 4. Evaluation of the effect of the temperature on the amylase activity

Procedure:

1. Add 3 drops of iodine solution (I_2 in KI; 0,001 mol/dm^3) and 1 drop of HCl to the wells of the plate creating 3 rows.
2. Prepare 3 test tubes with 5 cm^3 of starch solution, 2 cm^3 of 1% NaCl and 2 cm^3 of phosphate buffer of optimal pH (the result of Task 2).
3. Incubate the tubes as folllows: 1st tube in water bath (37°C), 2nd tube at RT, and 3rd tube on ice
4. After 5 minute incubation add 1 cm^3 of prepared enzyme solution to each tube.
5. Every one minute transfer 2 drops of incubation mixture to the following wells. Note achromatic points for each row.