Miranda House
University of Delhi

INSPIRE INTERNSHIP PROGRAMME 2024

Innovation in Science Pursuits for Inspired Research
An Initiative of DST, Govt of India

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DNA and Enzymes:
Tiny but Mighty Players

Offered by:
Zoology Department

Inspire Internship Programme

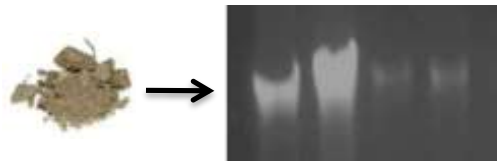
Innovation in Science Pursuit for Inspired Research
An initiative of DST, Government of India

Workshop

DNA and Enzymes: Tiny but Mighty Players

The deoxyribonucleic acid or DNA is the hereditary material in humans and most other organisms. The complete DNA of each organism is unique. Although DNA is the blue print of life, it controls the cell activity by encoding various enzymes. The transfer of information is accurate and determines the functioning of cell. The aim of the workshop is to provide basic understanding of the master molecule and how enzymes work.

Isolation of DNA by home kit & its detection: **Come and see how you can isolate DNA from any soil!**



To study the biological oxidation in liver homogenate: **Learn and see enzyme kinetics in action.**

Liver Catalase- The Housekeeper of Body: **Test the activity of the enzyme catalase from fresh liver.**

Prof. Rekha Kumari, Dr. Monika Sharma , Dr. Simran Jit, Dr. Deepak Yadav, Dr. Joni Yadav

DNA and Enzymes: Tiny but Mighty Players

ISOLATION OF DNA BY HOME KIT & ITS DETECTION

REKHA KUMARI, SIMRAN JIT & DEEPAK YADAV

Aim:

This practical exercise aims to provide hands on experience on DNA isolation using common reagents and visualize it using electrophoresis.

Objective:

At the end of this exercise the student should be able to understand the basic process of isolation of DNA from various sources eg., blood, tissue, bacteria. The student should also realise that different types of DNA require different methods of isolation and that the method used is dependent upon the final application.

Background:

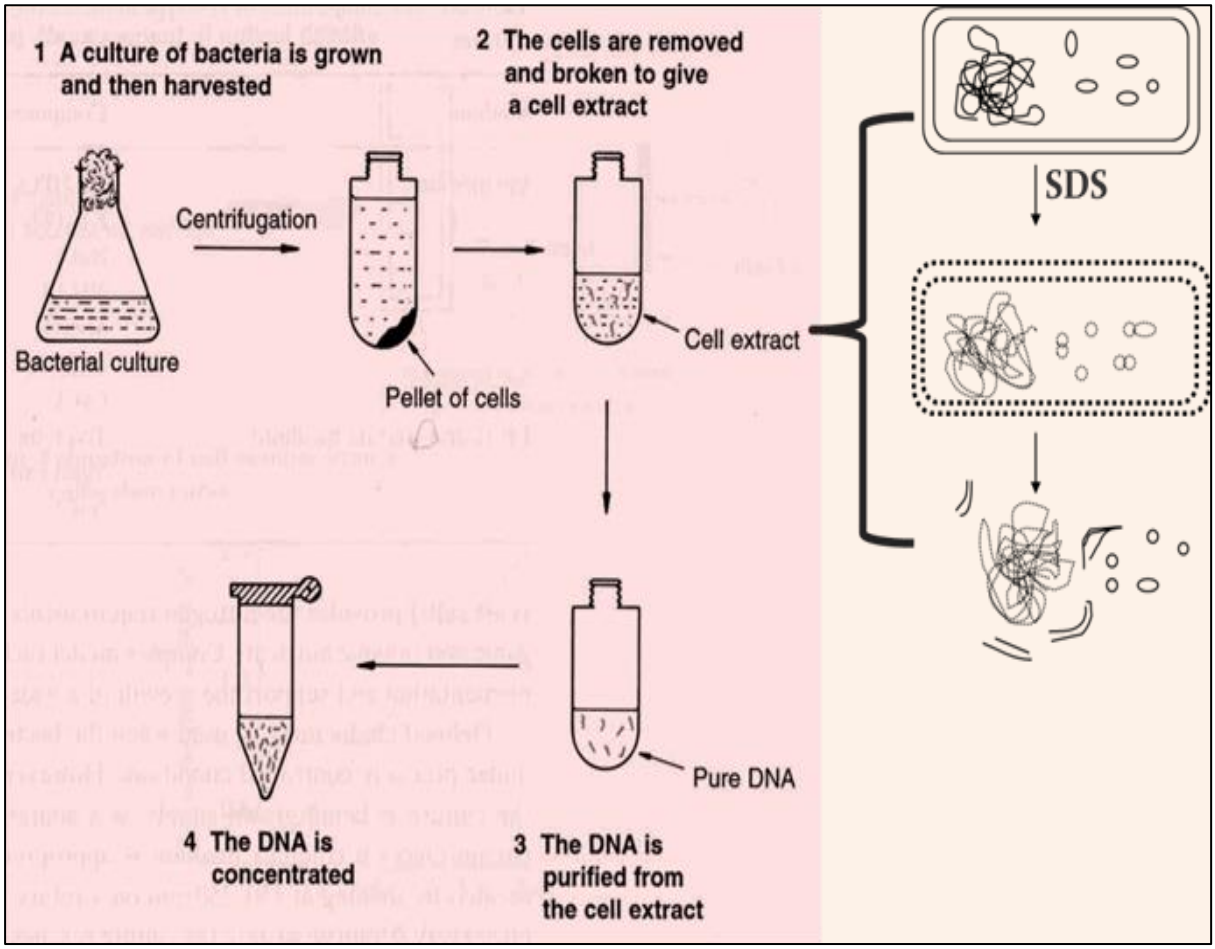
DNA is present in most of the biological samples. Before it can be sequenced, it must beisolated and purified. Details of DNA extraction protocol vary from lab to lab, and different procedures are standardized according to the source of DNA.However all protocols have the same basic steps of:

1. Cell lysis (to free the DNA): The first step is to break the cell membrane and pull out the cell extracts. For this a detergent is used to destabilize the bacterial cell wall (makes holes in the cell membrane) and allows the release of the cellular components. Addition of alkali NaOH denatures (separates DNA strands) irreversibly the cell chromosome.
2. Nuclease inactivation (to prevent DNA breakdown): various enzymes are present in the cell that can degrade the DNA. To prevent this, EDTA, Proteinase K, SDS are added which inactivate nucleases.
3. DNA purification (to remove non-DNA molecules): the DNA sample after the two steps will also contain lipids, polysaccharides, proteins which need to be removed. This is usually done by phenol-chloroform extraction and then precipitating by ethanol. A glass rod can be used to spool long strands of DNA out of the solution.

The procedure described here is adapted to gram negative bacteria. For this, a fine suspension of cells is prepared in buffer. In this, experiment we are preparing a crude lysate wherein proteins and other contaminants are precipitated from the cell lysate using high concentrations of salt. The precipitates are removed by centrifugation, and the DNA is recovered by alcohol precipitation. Removal of proteins and other contaminants using this method may be inefficient, and RNase treatment, dialysis, and/or repeated alcohol

precipitation are often necessary before the DNA can be used in downstream applications. DNA yield and purity are highly variable using this method.

DNA Isolation: an overview



Equipment/Chemicals/Solutions:

- DNA source material (such as bacterial pellet)
- Extraction mix (120 ml DW + 4 tsp Detergent +pinch of Table salt)
- Ethanol
- Glass rod
- EtBr
- TBE buffer
- Agarose
- Bromophenol blue dye
- Falcon tubes
- Micropipette&Micropipette tips
- Resuspension buffer (such as GTE mix or 0.9% saline)

Pre Lab Questions:

1. What is DNA?
2. What is the structure of cell membrane?

Procedure:

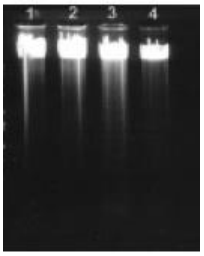
DNA isolation

1. Suspend the source material in 100 ul of suspension buffer.
2. Add200ul of Extraction mix.
3. Gently invert
4. To this add chilled absolute alcohol drop by drop almost double the amount of aqueous solution.
5. Dip a tooth pick and spool out DNA.
6. Air dry the spooled DNA and dissolve it in DW.
7. Take the supernatant in a fresh microfuge tube and subject it to gel electrophoresis. Follow the same procedure from DNA extraction from spleen.

Preparation of gel

1. Add 0.8 g agarose powder in a flask or glass bottle containing 100ml of 0.5X TBE buffer. Heat the mixture in a boiling water-bath or microwave oven until the agarose dissolves and the solution becomes clear.
2. Take clean gel casting mold, place it horizontally on the table and level it using the equilibrium bubble. Position the comb 0.5-1.0 mm above the plate so that a complete well is formed when the agarose is added.
3. Pour the warm agarose solution into the mold. There should not be any air bubble between or under the teeth of the comb.
4. After the gel is completely solidifies (30-45min. at room temperature) carefully remove the comb.
5. Add electrophoresis buffer (0.5x TBE) to cover the gel.
6. Mix the samples of DNA (15µl) with the tracking dye bromophenol blue dye (1.5µl of 10X stock). Slowly load the mixture into slots of the submerged gel using disposable microtips.
7. Attach the electrical leads so that the DNA will migrate toward the anode (red lead). Apply a voltage of 60-80V.
8. Run the gel until the bromophenol blue dye has migrated the appropriate distance (3/4th) through the gel.
9. Turn off the electric current and remove the gel from the gel tank.
10. Stain the gel with EtBr stain (for 100ml stain add 5µl of the stock) for 20-30 min.
11. Analyse the gel under UV light.

Expected Results:



Agarose gel analysis of the genomic DNA purification procedure.

Discussion:

- When you add the soap, the mixture should get very thick. The soap is destroying cell membranes, allowing the cell contents to spill out (cell lysis). We often think that the cytoplasm of cells is quite watery. In fact, the cellular milieu is quite thick composed of hundreds of thousands of proteins, nucleic acid molecules and other cell components.
- DNA is only soluble at a pH near physiological levels.
- Since the supernatant is thick from the cellular contents, carefully pouring the alcohol on top of thesupernatant leaves two distinct layers. DNA is soluble in water, but not in alcohol; thus, the DNA present at the water-alcohol interface precipitates out of solution, allowing it to be seen.

Analysis Questions:

1. What observation do you make from this exercise?
2. Can you use the same method for other sources?
3. How can you purify DNA further?

TO STUDY THE BIOLOGICAL OXIDATION IN LIVER HOMOGENATE

MONIKA SHARMA & JONI YADAV

Aim:

This practical exercise aims to study the biological oxidation by succinate dehydrogenase present in mitochondria of liver.

Objective:

To familiarize students with:

- Enzyme functioning: Formation of new products by the action of enzymes. Active sites are the catalytic regions of enzymes.
- Role of inhibitor on the activity of enzymes.

Introduction:

The mitochondrion is often referred to as the powerhouse of the cell. It contains all of the machinery needed to provide the cell and its components with energy to carry out cellular processes. The matrix of the mitochondrion is where the TCA cycle occurs, in which pyruvate (oxidized from glucose in gycolysis) is converted into acetyl-CoA, then fed into the pathway to be oxidized to CO2 and its energy conserved. Succinate dehydrogenase(SDH) is the only enzyme of the TCA cycle that is also part of the electron transport system, thus, it is located in the inner membrane. Succinate dehydrogenase and its conenzyme flavin adenine dinucleotide (FAD), represented as the complex E-FAD, oxidize the metabolite succinate to fumarate. Succinate dehydrogenase removes electrons from succinate, which reduces FAD, thus reducing the enzyme complex to E-FADH2. The reduced coenzyme then transfers the electrons to coenzyme Q to be taken through the rest of the electron transport chain.

For the experiment, students will prepare chicken liver homogenate, and assay it to find out the activity of SDH. An artificial electron acceptor: methylene blue will be used for detection of SDH activity.

Reactions involved:

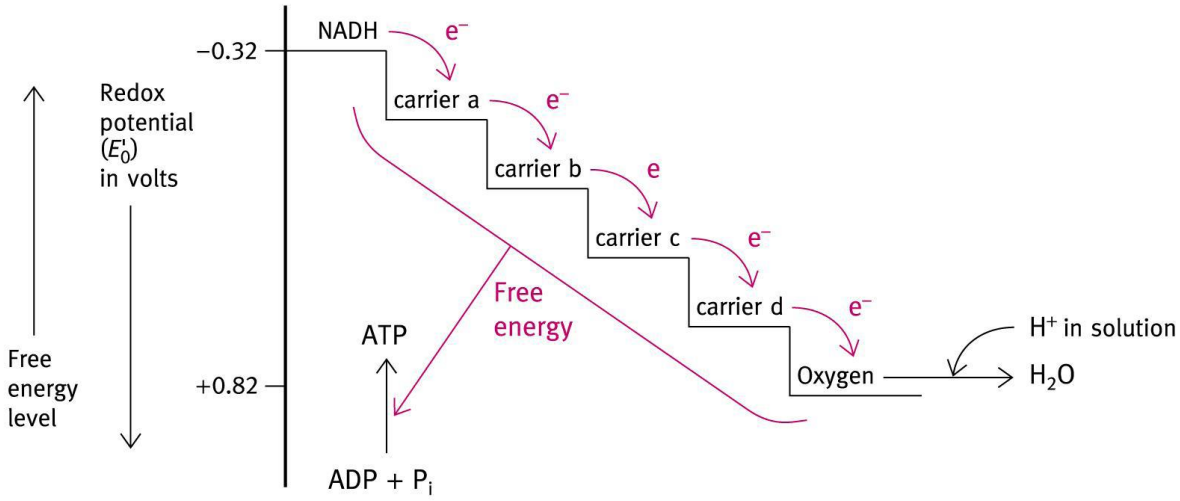
succinate + succinate-dehydrogenase-FAD ---> fumarate + succinate-dehydrogenase-FADH2

succinate-dehydrogenase-FADH2 + Methylene blue ---> succinate-dehydrogenase-FAD + methylene blue-H_2

BACKGROUND

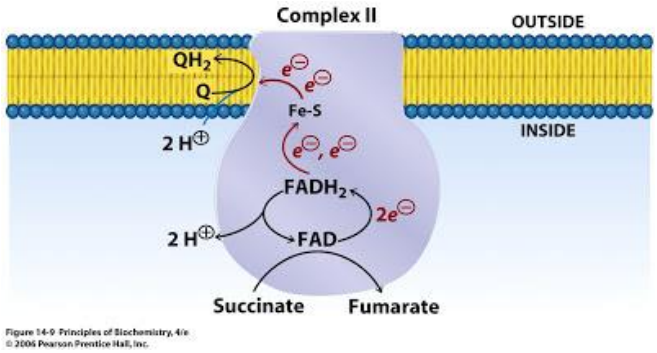
Succinate Dehydrogenase, a flavoprotein and the only enzyme of Krebs cycle bound to the inner mitochondrial membrane, catalyses the reversible oxidation of succinate to fumarate. It is also the component of protein complexnamed succcinateubiquinone reductase, the elemrnt of electron transport chain. Using this complex, electrons and protonsfrom $FADH_2$ linked with succinate dehysrogenase are transferred onto respiratory chain. An artificial electron acceptor used to detect the activity of SDH is methylene blue(MB). Standard redox potential for MB is +0.01V. this redox potential is higher than redox potential of $FAD/FADH_2$, which is -0.06V. Therefore, protons from $FADH_2$can be transferred to MB.

PRINCIPAL OF ELECTRON TRANSPORT CHAIN



Oxygen is the ultimate oxidiser in the aerobic cell because it is electrophilic with a high affinity for electrons. It accepts electrons, protons join up from the solution,and water is formed.Oxygen does not have to be added to oxidise a molecule.The removal of electrons alone, or electronsandprotonsfromamoleculeconstitutesoxidation.

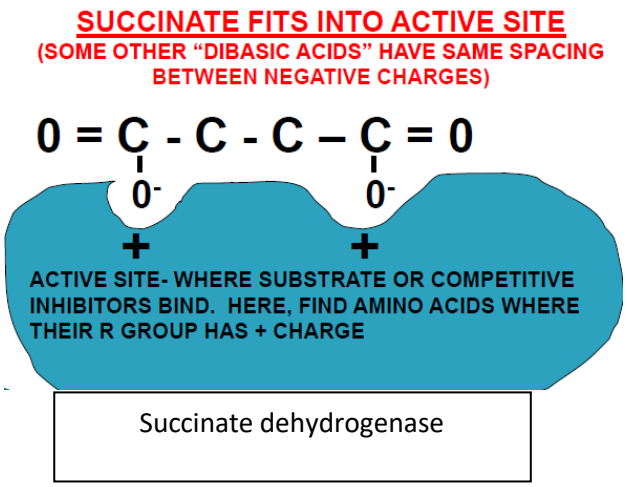
SuccinateDehydrogenase(SDH): This enzyme is coupled with the coenzyme FAD is bound to the inner membrane of the mitochondria.



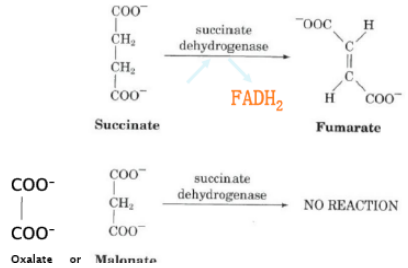
- **SDH has a size of 100,000 daltons.**
- **Also contains 8 iron atoms, Fe (iron) atoms help in the transfer of electrons from succinateto FAD.**
- **Has two subunits (so it has quarternarystructure).**
- **Has higher activity than any other TCA cycle enzyme**

COMPITITIVE INHIBITION

Inhibitor resembles Substrate. This is not how the body/cell regulates enzymes (typically) -some medicines work this way. Competitive inhibitor used in this experiment is sodium oxalate. The inhibitor can bind to the active site (preventing the normal substrate from binding) but the inhibitor cannot form the product.So, both the inhibitor and Substrate competefor the active site of the enzyme. If the substrate is in excess, the inhibitor will not inhibit.



·In the reaction succinate → fumarate, oxalate (or malonate) has a structure similar to succinate and competes with it for the active site on succinate dehydrogenase.



Experimental Setup:

In this experiment we put methylene blue solution to liver homogenate. The enzymatic activity of SDH will decolourize the blue colour of MB gradually. This discolouration will be due to reduction of oxidized form of dye (MB). Also, a competitive inhibitor sodium oxalate will be added to the reaction mixture for studying its effect on SDH. In the presence of sodium oxalate MB will not get reduced thus remains blue.

Requirements:

- Phosphate buffer (pH 7.4) or 0.9% NaCl(NS, normal saline)
- 0.2 M Succinatesolution
- 0.5 M Oxalate solution
- 0.02% Methyleneblue (MB)
- Test tubes
- Mortar& pestle
- Mitochondria preparation:SDH is bound to the inner membrane of the mitochondria. Rat liver is an ideal source for functional intact mitochondria but we will be using fresh chicken liver.

Pre Lab Questions:

1. What is oxidation?
2. Why liver is called metabolic hub?
3. What is power house of a cell?
4. What is the location of succinate dehydrogenase in mitochondria?

Procedure:

1. Take 1gm of liver tissue in mortar and pestle and make a paste by grinding in PBS. Raise the volume of the homogenate 10 times by PBS.
2. Take 3 test tubes and add 1 ml of tissue homogenate to each tube.

3. Also add 0.5 ml of methylene blue (0.02%) to each tube.
4. Added 1 ml of 0.2M sodium succinate to test tube 1, 1ml buffer to test tube 2 and 1 ml 0.5M sodium oxalate to test tube 3.
5. Keep the tubes in water bath at 37^0 Cfor about 15-20 minutes, taking care to note down the tissue taken for change in colour.

Preparation Table:

Reagents	**Test tube 1**	**Test tube 2**	**Test tube 3**
Tissue homogenate	1ml	1 ml	1 ml
Methylene blue (0.2%)	0.5 ml	0.5 ml	0.5ml
Sodium Succinate	1ml	-	1 ml
Buffer	-	1ml	-
Sodium Oxalate	-	-	1ml
Observation (Colour of Methylene blue)			

*Observation and Result***:**

T**est Tube**	Reaction Mixture	Colour Change
1	Liver homogenate + sodium succinate + methylene blue	
2	Liver homogenate + buffer + methylene blue	
3	Liver homogenate + sodium succinate + sodium oxalate + methylene blue	

Clinical Significance

Succinate dehydrogenase is quite stable and found at high concentration only in the mitochondrion of eukaryotes, succinate dehydrogenase is useful as a marker enzyme for mitochondria.

The usefulness of the most important pharmaceutical agents, antimetabolites, is based on the concept of competitive enzyme inhibition. The antimetabolitesare structural analoguesof normal biochemical compounds.As competitive inhibitors they compete with the naturally substratefor the active site of enzyme and blockthe formation of undesirable metabolic products in the body.

Post Lab Questions?

1. What are the characteristic features of SDH?
2. What is the role of sodium oxalate in the experiment?
3. Which electron acceptor is used in the experiment?
4. What is the clinical significance of SDH ?

LIVER CATALASE- THE HOUSEKEEPER OF BODY

MONIKA SHARMA & JONI YADAV

Aim:

To test the activity of the enzyme catalase from fresh liver.

Objective:

- Extract the enzyme catalase from fresh (or frozen) liver
- Use of Liver to break down hydrogen peroxide
- Test the activity of catalase under different conditions

In this experiment, you will extract the enzyme catalase from fresh (or frozen) liver, use it to break down hydrogen peroxide, and test the activity of catalase under different conditions.

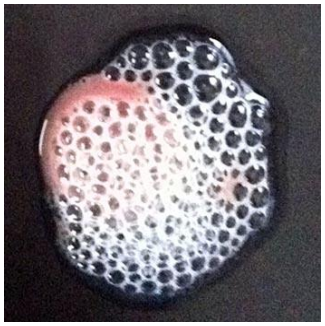
Introduction:

Chemical reactions can occur more quickly by using enzymes. Enzymes are made out of protein and they speed up the rate of a chemical reaction by acting as a catalyst. A catalyst provides the necessary environment for the chemical reaction to occur, which speeds up the reaction. Certain catalysts work for certain kinds of reactions. In other words, each enzyme has a particular type of reaction that it can activate.

Enzymes are very large and dynamic and need to be in certain conditions to work, and the ideal conditions are usually reflective of where the enzyme must normally function in the body. In other words, different tissues are home to different enzymes, and an enzyme should be able to function in the conditions that surround it in the tissue it's supposed to function in. Some enzymes can be damaged under certain conditions, such as heat. A damaged enzyme will no longer work to catalyze a chemical reaction. Catalase is one enzyme from liver that breaks down harmful hydrogen peroxide into oxygen gas and water. Catalase speeds up a reaction which breaks down hydrogen peroxide, a toxic chemical, into 2 harmless substances--water and oxygen.

The reaction is: $2\ H_2O_2$ ----> $2\ H_2O + O_2$

This reaction is important to cells because hydrogen peroxide (H_2O_2) is produced as a byproduct of many normal cellular reactions.



When a cut liver is exposed to hydrogen peroxide, the catalase enzyme in the liver reacts with the hydrogen peroxide to form oxygen gas, which creates the visible bubbles in this photograph, and water.

Background:

Metabolism is the sum total of chemical reactions in the body that are necessary to the maintenance of life. Enzymes are biological catalysts that can speed up, and control, chemical reactions that would otherwise virtually never occur at normal body temperature, 37°C. Thousands of chemical reactions are occurring in the human body every moment of life, and each of these reactions is controlled by a particular enzyme.

Enzymes are extremely efficient. Some of the chemical reactions that take place in the body produce toxic by-products, which must be quickly degraded or converted. For example, certain reactions in the liver produce hydrogen peroxide, which is extremely poisonous. Under the influence of an enzyme called catalase, the hydrogen peroxide is broken down into water and oxygen. Catalase acts quickly; one molecule of it can deal with six million molecules of hydrogen peroxide in one minute. This same reaction can be catalyzed by iron. However, to achieve the same speed there would need to be about six tons of iron.

Enzymes have properties that you should know:

1. They are always proteins.

2. They are specific in their action. Each enzyme controls one particular reaction, or type of reaction. Thus sucrase degrades sucrose and only sucrose (table sugar).

3. They are not altered by the reaction. This means that an enzyme can be used repeatedly. It also means that enzymes appear neither in the reactants nor in the products of a chemical equation.

4. They are destroyed by heat. This is because enzymes are proteins, and all proteins are destroyed by heat. Destruction of protein by heat (or under any extreme conditions of pH or salt concentration) is called denaturation.

5. They are sensitive to pH. The term pH refers to the degree of acidity and alkalinity of a solution. Most intracellular enzymes work best in neutral conditions, i.e. conditions that are neither acidic nor alkaline.

Pre Lab Questions:

1. Why are enzymes important for making chemical reactions happen?
2. Keeping in mind the normal temperature of the human body, how hot do you think an enzyme needs to be for it to become damaged and not function?
3. What does catalase do?

4. Under which conditions does it work best?
5. Why do we need catalase in our liver?
6. Why are bubbles made when catalase reacts with hydrogen peroxide? What are the bubbles made out of?
7. Aside from break down harmful substances, what else does the liver do?

Requirements:

- Chicken liver, fresh or frozen
- Scalpel
- Pasteur Pipette
- Petri dishes
- 3%Hydrogen peroxide (Freshly made)
- Measuring teaspoon

Procedure:

1. Obtain 2 test tubes and pour 2 ml of 3% hydrogen peroxide solution into one of them
   Caution: hydrogen peroxide is corrosive and can irritate the skin.
   Pour 2ml of water into the other test tube.
2. Drop a small piece of liver into each test tube. Liver contains considerable catalase. Watch the reaction and record your observations in a data table.
3. Repeat the experiment using a piece of liver which has been boiled for three minutes. Record your observations.
4. Repeat the experiment using muscle and potato to find out if catalase occurs elsewhere

Expected Results & Observations:

In your lab notebook, you can record this in a data table like Table 1 below. The cut liver you just tested is your "untreated" sample and it will be your positive control. Use a scale of 0-5, with 5 representing the "most bubbling" and 0 representing "no bubbling." Write down any other observations you make in your lab notebook.

Treatments	**Bubbles**
Untreated liver+3% hydrogen peroxide solution	
Untreated Liver +Water	
Heated Liver + hydrogen peroxide solution	

Knowing that the amount of bubbles made reflects how active the catalase enzyme is, which condition, or conditions, does it look like the enzyme works best under? Which condition, or conditions, makes it work the worst? Why do you think this is?

Discussion:

Metabolism is the sum total of chemical reactions in the body that are necessary to the maintenance of life. Enzymes are biological catalysts that can speed up, and control, chemical reactions that would otherwise virtually never occur at normal body temperature, 37°C. Thousands of chemical reactions are occurring in the human body every moment of life, and each of these reactions is controlled by a particular enzyme.

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5. They are sensitive to pH. The term pH refers to the degree of acidity and alkalinity of a solution. Most intracellular enzymes work best in neutral conditions, i.e. conditions that are neither acidic nor alkaline.

Analysis Questions:

1. The primary reaction catalyzed by catalase is the decomposition of hydrogen peroxide to form water and oxygen, which occurs spontaneously, but not at a very rapid rate. Write a balanced equation for this reaction. (Remember that catalase is not a reactant or a product and can be written over the arrow separating the reactant from the products.)

2. Explain why, in your first trial, you used two test tubes, one with hydrogen peroxide and one with water.

3. What effect did boiling the liver have on the reaction? Why?

4. Explain the results you obtained using a piece of intestinal muscle.

5. What effect would acetic acid have on the reaction? Why?