LAB MANUAL

ENZYME ENGINEERING LAB

CODE: RBT-453

IMS ENGINEERING COLLEGE

A NAAC Accredited & ISO 9001:2008 Certified Institution

(Approved by AICTE and affiliated to APJ Abdul Kalam Technical University, Lucknow)

NH-24, Delhi-Hapur by Pass Road, Adhyatmik Nagar, Ghaziabad-201 009 (U.P.)

PRACTICAL - 1 **Date:**

AIM: **Isolation of Enzyme from plant sources**

PRINCIPLE:

The first step in purifying **intracellular** (inside the cell) proteins is preparation of a **crude extract**. The extract will contain a complex mixture of all the proteins from the cell cytoplasm, and some additional macromolecules, cofactors and nutrients. Crude extract may be used for some applications in biotechnology, however, if purity is an issue, subsequent purification steps must be followed. Crude protein extracts are prepared by removal of cellular debris generated by cell lysis, which is achieved using chemicals and enzymes, sonication or a French Press. The debris are removed by centrifugation and the supernatant is recovered. Crude preparations of extracellular proteins may be obtained by simply removing the cells by centrifugation.

REQUIREMENTS:

Material and Reagents:

1. **Experimental material**: Barley seeds which have been kept for germination for 2-3 days at 25^{o}C.
2. Phosphate buffer (0.05 M , pH.7.0) containing 0.5 M NaCl.

Equipments: Balance, water bath, spectrophotometer.

Glassware: test tubes, beakers, measuring cylinders, glass pipettes etc.

PROCEDURE:

1. Extraction of the enzyme should be done in cold at 0-4^{0}C.
2. Macerate 500 mg of washed germinating seeds in a chilled pestle and mortar with 5ml of 0.05 M phosphate buffer (pH.7.0) containing 0.5 M NaCl.
3. Centrifuge the homogenate at 10,000 X g for 15 min in a refrigerated centrifuge at 4^{0}C.
4. Decant the supernatant and use it as the enzyme preparation.
5. Store at 4^{0}C.

RESULTS:

CONCLUSION / INTERPRETATION

[Signature of Student] **[Signature of Instructor]**

PRACTICAL - 2 **Date:**

AIM: **Partial purification of isolated enzymes**

PRINCIPLE:

A common second step to purifying a protein from a crude extract was by **precipitation** in a solution with high osmotic strength (i.e. salt solutions). Nucleic acids in the crude extract can be removed by precipitating aggregates formed with streptomycin sulfate or protamine sulfate. Protein precipitation is usually done using ammonium sulfate as the salt. Different proteins will precipitate in different concentrations of **ammonium sulfate**. In general, proteins of higher molecular weight precipitate in lower concentrations of ammonium sulfate. Salt precipitation does not usually lead to a highly purified protein, but can assist in eliminating some unwanted proteins in a mixture and concentrating the sample. Salts in the solution are then removed by dialysis through porous cellulose tubing, filtration, or gel exclusion chromatography.

REQUIREMENTS:

Material and Reagents:

Experimental material: Enzyme extract kept at $4^{o}C$.

Sodium alginate

Equipments: Balance, water bath.

Glassware: test tubes, beakers, measuring cylinders, glass pipettes etc.

PROCEDURE:

1. Clarify the protein solution (in most cases the lysates) by centrifugation.
2. Transfer the supernatant into a nice cold beaker with a magnetic bead.
3. Note the exact amount of the supernatant.
4. Keep the beaker chilled by placing it in an ice tray. Transfer the beaker with the ice tray onto a magnetic stirrer.
5. Weigh the amount of ammonium sulfate to be added. The amount depends on the volume of the solution and the percentage saturation of the salt needed. Refer to the precipitation chart. In case of protein purification, a step precipitation is carried out. Slowly add the ammonium sulphate with stirring. One needs to be careful as the addition of the salt should be very slow. Add a small amount at a time and then allow it to dissolve before further addition.
6. Keep it on the stirrer for 1hr precipitation to occur in ice.
7. Centrifuge at 10,000g for 15min at 4˚C.
8. The pellet contains the precipitated protein which could be dissolved in a suitable buffer for further analysis and purification.
9. For a second round of precipitation of a different protein, the supernatant is again used and the above same steps are followed.

RESULTS:

CONCLUSION / INTERPRETATION

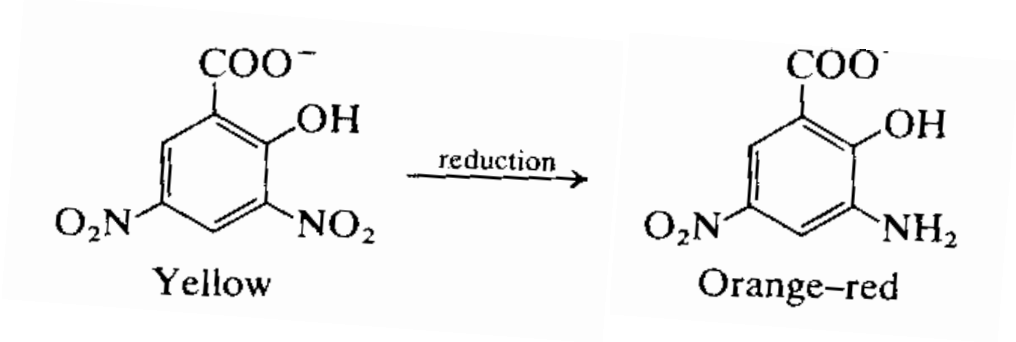
[Signature of Student] **[Signature of Instructor]**

PRACTICAL - 3 **Date:**

AIM: Preparation of standard curve for maltose for the estimation of enzyme activity

PRINCIPLE:

Under alkaline condition reducing sugar is converted to furfural, which reduces one of the nitro group (-NO_2) of Dinitro Salicylic Acid (DNSA) to amino group (-NH_2) to produce orange-brown colored 3-amino-5-nitrosalicylic acid. The orange-brown colored compound can be measured spectrophotometrically at 540 nm. Since the chemistry of the reaction is complicated standard curves do not always go through the origin and different sugars give different color. The method is therefore not suitable for the determination of complex mixture of reducing sugars.



3, 5 Dinitrosalicylic acid **3-amino-5-nitrosalicylic acid**

REQUIREMENTS

Reagents:

1. **Standard Sugar solution** (maltose 1mg/ml): Dissolve 100mg of maltose and make up the final volume 100 ml with D/W.
2. **DNSA reagent:** Dissolve 10g DNSA powder in 250 ml of 2N sodium hydroxide. Allow it to cool. Add 300 g sodium potassium tartarate, and make final volume 1000ml with D/W. Stored in brown bottle.

Equipments: Balance, Water bath, Spectrophotometer,
Glassware: Test tubes, conical flasks, glass pipette etc……

Range of the method: 100-1000 µg/ml
Working concentration: 1mg/ml.

PROCEDURE:

1. Take clean and dried test tubes. Mark all the tubes as per the protocol table.
2. Take all the reagents along with maltose (std) with 100, 200, 300 up to 1000 µg and test solution sequentially as per the protocol table.
3. Make up the final volume 1ml with D/W along with the blank tube.
4. Add 1ml DNSA reagent in all tubes. Mix all the reagents properly and incubate the mixture in BWB as mentioned in the protocol table.
5. After cooling under running tap water, add 8.0ml D/W in all tubes.
6. Measure the color complex spectrophotometrically at 540 nm.
7. Draw the standard curve of Concentration of maltose vs Optical Density (O.D.)

8. Put the O.D. of the unknown solution and calculate out the concentration of the maltose present in given sample from standard graph and formula (given below) and express your result in mg%.

FORMULA:

$$\frac{\textbf{OD (test)}}{\textbf{OD (std)}} \times \frac{\textbf{Conc (std)}}{\textbf{Aliquot (test)}} \times \textbf{100}$$

PROTOCOL TABLE

Volume of maltose sol. (ml)	Conc. of maltose sol. (mg/ml)	Volume of water added (ml)	Total volume (ml)	Absorbance (540nm)
0.2		0.8	1.0	
0.4		0.6	1.0	
0.6		0.4	1.0	
0.8		0.2	1.0	
1.0		-	1.0	
-		1.0 (blank)	1.0	

RESULT:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

PRACTICAL - 4 **Date:**

AIM: Quantitative estimation of enzyme activity (amylase activity in the given sample by calculating units (U) of enzyme)

PRINCIPLE:

Amylases hydrolyse starch and glycogen into sugars. There are two types of amylases: α-amylase and β-amylase. α-amylase is an endohydrolase which hydrolyses α-1,4-linkage within the chain and breaks these polymers into small fragments. In contrast β-amylase is an exohydrolase and it starts acting from non-reducing end removing two glucose molecules at a time. The hydrolytic action of β-amylase results in increasing reducing power of the starch solution due to the release of a large number of molecules of the reducing sugar (maltose).The reducing sugars can be determined quantitatively by their reaction with DNSA reagent which undergoes reduction to a red-brown coloured product with absorption maxima at 540nm.

REQUIREMENTS:

Material and Reagents:

3. **Experimental material**: use barley seeds which have been kept for germination for 2-3 days at 25^{o}C.
4. **DNSA Reagent**
5. **Sodium Potassium Tartarate (40%):** Take 40g of sodium potassium tartarate and dissolve it in small amount of water and make the final volume to 100 ml with D/W.
6. **Substrate (1% Starch, w/v):** Weigh 1g of starch and add 100ml of boiling water to it. Continue boiling for 2-3 min. Let the solution cool down and then filter it. Use the filtrate as the substrate solution.
7. **Sodium phosphate buffer (0.05M, pH 7.0)** containing 0.5M NaCl.
8. **Standard maltose solution (**1 mg/ml**):** Dissolve 100 mg of maltose in 100ml of D/W.

Equipments: Balance, water bath, spectrophotometer.
Glassware: test tubes, beakers, measuring cylinders, glass pipettes etc.

PROCEDURE:

1. Extraction of the enzyme should be done in cold at 0-4^{0}C. Macerate 500 mg of washed germinating seeds in a chilled pestle and mortar with 5ml of 0.05 M phosphate buffer (pH.7.0) containing 0.5 M NaCl.
2. Centrifuge the homogenate at 10,000 X g for 15 min in a refrigerated centrifuge at 4^{0}C. Decant the supernatant and use it as the enzyme preparation.
3. Pipette 1 ml of starch solution and 1 ml of an appropriately diluted enzyme preparation into two tubes (control and experimental).
4. In one of the tubes, which serve as a zero min control, terminate the reaction immediately by adding 2ml of dinitrosalicylic acid (DNSA) reagent.
5. Incubate the other test tube at 37^{0}C for 15 min and then stop the reaction by adding 2 ml dinitrosalicylic acid reagent.
6. Keep both the tubes in a boiling water bath for 5 min and then add 1 ml of sodium potassium tartarate to each of the tubes.
7. Cool the tubes under running tap water. Make the volume to 10 ml with water.
8. Set the colorimeter to zero absorbance at 540nm with the zero min. control and then record the absorbance of the second tube.

Protocol Table:

Test tube	Enzyme (ml)	Substrate (ml)	Buffer (ml)
Enzyme Blank	-	1.0	1.0
Substrate Blank	1.0	-	1.0
Test	1.0	1.0	-

RESULTS:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

PRACTICAL -5 **Date:**

AIM: Study of time course of enzyme catalyzed reaction.

PRINCIPLE:

The longer an enzyme is incubated with its substrate, the greater the amount of product that will be formed. However, the rate of formation of product is not a simple linear function of the time of incubation. All proteins suffer denaturation, and hence loss of catalytic activity, with time. Some enzymes, especially in partially purified preparations, may be noticeably unstable, losing a significant amount of activity over the period of incubation. If the activity of the enzyme is such that much of the substrate is used up during the incubation, then, even if the concentration of substrate added was great enough to ensure saturation of the enzyme at the beginning of the experiment, it will become inadequate as the incubation proceeds, and the formation of product will decrease. Enzyme catalysed reactions are reversible. Initially, there is little or no product present, and therefore the reaction proceeds only in the forward direction. However, as the reaction continues, so there is a significant accumulation of product, and there is a significant rate of back reaction. As a result, the rate of formation of product slows down as the incubation proceeds, and if the incubation time is too long, then the measured activity of the enzyme is falsely low. Selecting an appropriate incubation time depends on a compromise between these various factors. As a general rule, the incubation should be long enough to permit a moderate amount of product to be formed, and long enough that the error in timing is insignificant, but not so long that there is detectable levelling off of the curve. You need to be sure that when you determine the rate of reaction (in mol of product formed / minute) the enzyme has been active at a more or less constant rate throughout your incubation.

REQUIREMENTS:

Material and Reagents:

1. **Experimental material**: use barley seeds which have been kept for germination for 2-3 days at 25^{o}C.
2. **DNSA Reagent**
3. **Sodium Potassium Tartarate (40%):** Take 40g of sodium potassium tartarate and dissolve it in small amount of water and make the final volume to 100 ml with D/W.
4. **Substrate (1% Starch, w/v):** Weigh 1g of starch and add 100ml of boiling water to it. Continue boiling for 2-3 min. Let the solution cool down and then filter it. Use the filtrate as the substrate solution.
5. **Sodium phosphate buffer (0.05M, pH 7.0)** containing 0.5M NaCl.
6. **Standard maltose solution (**1 mg/ml**):** Dissolve 100 mg of maltose in 100ml of D/W.

Equipments: Balance, water bath, spectrophotometer.
Glassware: test tubes, beakers, measuring cylinders, glass pipettes etc.

PROCEDURE:

1. Extraction of the enzyme should be done in cold at 0-4^{0}C. Macerate 500 mg of washed germinating seeds in a chilled pestle and mortar with 5ml of 0.05 M phosphate buffer (pH.7.0) containing 0.5 M NaCl.
2. Centrifuge the homogenate at 10,000 X g for 15 min in a refrigerated centrifuge at 4^{0}C. Decant the supernatant and use it as the enzyme preparation.

3. Pipette 1 ml of starch solution and 1 ml of an appropriately diluted enzyme preparation into two tubes (control and experimental).
4. In one of the tubes, which serve as a zero min control, terminate the reaction immediately by adding 2ml of dinitrosalicylic acid (DNSA) reagent.
5. Incubate the other test tube at 37^0C for 5-15 min and then stop the reaction by adding 2 ml dinitrosalicylic acid reagent.
6. Keep the tubes in a boiling water bath for 5 min and then add 1 ml of sodium potassium tartarate to each of the tubes.
7. Cool the tubes under running tap water. Make the volume to 10 ml with water.
8. Set the colorimeter to zero absorbance at 540nm with the zero min. control and then record the absorbance of the second tube.

RESULTS:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

PRACTICAL -6 **Date:**

AIM: Effect of enzyme concentration on rate of enzyme catalyzed reaction.

PRINCIPLE:

In order to study the effect of increasing the enzyme concentration upon the reaction rate, the substrate must be present in an excess amount; i.e., the reaction must be independent of the substrate concentration. Any change in the amount of product formed over a specified period of time will be dependent upon the level of enzyme present. Graphically this can be represented as:

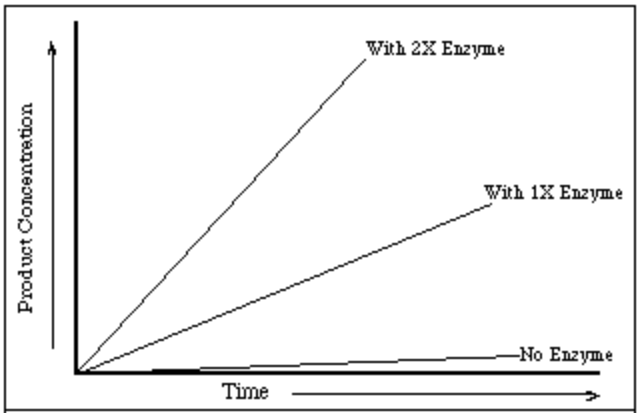


FIGURE 4
"Zero order" reaction rate is independent of substrate concentration.

REQUIREMENTS:

Material and Reagents:

1. **Experimental material**: use barley seeds which have been kept for germination for 2-3 days at 25^{o}C.
2. **DNSA Reagent**
3. **Sodium Potassium Tartarate (40%):** Take 40g of sodium potassium tartarate and dissolve it in small amount of water and make the final volume to 100 ml with D/W.
4. **Substrate (1% Starch, w/v):** Weigh 1g of starch and add 100ml of boiling water to it. Continue boiling for 2-3 min. Let the solution cool down and then filter it. Use the filtrate as the substrate solution.
5. **Sodium phosphate buffer (0.05M, pH 7.0)** containing 0.5M NaCl.
6. **Standard maltose solution (**1 mg/ml**):** Dissolve 100 mg of maltose in 100ml of D/W.

Equipments: Balance, water bath, spectrophotometer.
Glassware: test tubes, beakers, measuring cylinders, glass pipettes etc.

PROCEDURE:

1. Extraction of the enzyme should be done in cold at 0-4^{0}C. Macerate 500 mg of washed germinating seeds in a chilled pestle and mortar with 5ml of 0.05 M phosphate buffer (pH.7.0) containing 0.5 M NaCl.

2. Centrifuge the homogenate at 10,000 X g for 15 min in a refrigerated centrifuge at 4^0C. Decant the supernatant and use it as the enzyme preparation.
3. Pipette 0.1-1 ml of starch solution and 1 ml of an appropriately diluted enzyme preparation into two tubes (control and experimental). Make up the volume to 2 ml by buffer.
4. In one of the tubes, which serve as a zero min control, terminate the reaction immediately by adding 2 ml of dinitrosalicylic acid (DNSA) reagent.
5. Incubate the test tube at 37^0C for 15 min and then stop the reaction by adding 2 ml dinitrosalicylic acid reagent.
6. Keep the tubes in a boiling water bath for 5 min and then add 1 ml of sodium potassium tartarate to each of the tubes.
7. Cool the tubes under running tap water. Make the volume to 10 ml with water.
8. Set the colorimeter to zero absorbance at 540nm with the zero min. control and then record the absorbance of the second tube.

RESULTS:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

PRACTICAL -7 **Date:**

AIM: Investigate the effect of temperature on amylase activity and find out the temperature optima for amylase.

PRINCIPLE: All the enzymes have narrow temperature range for their efficient functioning. To determine enzyme activity in the reaction mixture, assay should be carried out at optimum temperature, where enzyme and substrate binding are more feasible compare to rest of the temperature.

REQUIREMENTS: Same as in the Practical 6

PROCEDURE:

1. Carried out the similar steps 1-4 as mentioned in the Practical 6
2. Incubate the different reaction mixture tubes at different temperature such as, 20^0C, 25^0C, 30^0C, 37^0C and 45^0C for 15 min.
3. Stop the reaction by adding 2 ml DNSA reagent.
4. After that the process is remain the same as mentioned in the Practical 6.
5. Plot graph with Enzyme activity vs temperature and determine temperature optima.

RESULT:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

PRACTICAL - 8 **Date:**

AIM: Determination of optimum pH of a particular enzyme.

PRINCIPLE:
Since enzymes are proteins, they are very sensitive to changes in pH. Each enzyme has its own optimum range for pH where it will be most active. This is the result of the effect of pH on a combination of factors: (1) the binding of the enzyme to substrate, (2) the catalytic activity of the enzyme, (3) the ionization of the substrate, and (4) the variation of protein structure.

REQUIREMENTS: Same as in the Practical 6

PROCEDURE:

1. Carried out the similar steps 1-4 as mentioned in the Practical 6
2. Incubate the different reaction mixture tubes at different pH such as, 5, 6, 7, 8, 9 etc. for 15 min.
3. Stop the reaction by adding 2 ml DNSA reagent.
4. After that the process is remain the same as mentioned in the Practical 6.
5. Plot graph with Enzyme activity vs pH and determine temperature optima.

RESULT:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**

PRACTICAL - 9 **Date:**

AIM: Immobilization of enzymes

An **immobilized enzyme** is an enzyme that is attached to an inert, insoluble material such as calcium alginate (produced by reacting a mixture of sodium alginate solution and enzyme solution with calcium chloride). This can provide increased resistance to changes in conditions such as pH or temperature. It also allows enzymes to be held in place throughout the reaction, following which they are easily separated from the products and may be used again - a far more efficient process and so is widely used in industry for enzyme catalysed reactions. An alternative to enzyme immobilization is whole cell immobilization

Immobilization of an Enzyme

There are three different ways by which one can immobilize an enzyme, which are the following, listed in order of effectiveness:

- **Adsorption on glass, alginate beads or matrix**: Enzyme is attached to the outside of an inert material. In general, this method is the slowest among those listed here. As adsorption is not a chemical reaction, the active site of the immobilized enzyme may be blocked by the matrix or bead, greatly reducing the activity of the enzyme.
- **Entrapment**: The enzyme is trapped in insoluble beads or microspheres, such as calcium alginate beads. However, this insoluble substances hinders the arrival of the substrate, and the exit of products.
- **Cross-linkage**: The enzyme is covalently bonded to a matrix through a chemical reaction. This method is by far the most effective method among those listed here. As the chemical reaction ensures that the binding site does not cover the enzyme's active site, the activity of the enzyme is only affected by immobility. However, the inflexibility of the covalent bonds precludes the self-healing properties exhibited by chemoadsorbed self-assembled monolayers. Use of a spacer molecule like poly(ethylene glycol) helps reduce the steric hindrance by the substrate in this case. Enzymes may also be immobilized to a surface using non-covalent or covalent Protein tags.

PRACTICAL - 10 **Date:**

AIM: Entrapment method of enzyme immobilization

PRINCIPLE:

Alginic acid can be either water soluble or insoluble depending on the type of the associated salt. The salts of sodium, other alkali metals, and ammonia are soluble, whereas the salts of polyvalent cations, e.g., calcium, are water insoluble, with the exception of magnesium. The alginate polymer itself is anionic (i.e., negatively charged) overall. Polyvalent cations bind to the polymer whenever there are two neighboring guluronic acid residues. Thus, polyvalent cations are responsible for the cross-linking of both different polymer molecules and different parts of the same polymer chain. The process of gelation, simply the exchange of calcium ions for sodium ions, is carried out under relatively mild conditions. Because the method is based on the availability of guluronic acid residues, which will not vary once given a batch of the alginate, the molecular permeability does not depend on the immobilization conditions. Rather, the pore size is controlled by the choice of the starting material.

$$2\ Na(Alginate) + Ca^{++} \text{-------} > Ca(Alginate)_2 + 2\ Na^{+}$$

The ionically linked gel structure is thermostable over the range of 0-100ºC; therefore heating will not liquefy the gel. However, the gel can be easily redissolved by immersing the alginate gel in a solution containing a high concentration of sodium, potassium, or magnesium. Maintaining sodium:calcium <= 25:1 will help avoid gel destabilization. In fact, it is recommended by alginate vendors to include 3mM calcium ions in the substrate medium. On the other hand, citrate or phosphate pH buffers cannot be effectively used without destabilizing the alginate gel.

REAGENTS AND INSTRUMENTS

Equipment: Beakers, Graduated cylinder, Balance, Pipets, Syringe

Reagents: Alginic acid, sodium salt, $CaCl_2$, Enzyme

PROCEDURE

1. Dissolve 30g of sodium alginate in 1 liter to make a 3% solution.
2. Mix approximately 0.015 g of enzyme with 10 ml of 3% (wt.) sodium alginate solution. The concentration of sodium alginate can be varied between 6-12 % depending on the desired hardness.
1. The beads are formed by dripping the polymer solution from a height of approximately 20 cm into an excess (100 ml) of stirred 0.2M $CaCl_2$ solution with a syringe and a needle at room temperature. The bead size can be controlled by pump pressure and the needle gauge. A typical hypodermic needle produces beads of 0.5-2 mm in diameter. Other shapes can be obtained by using a mold whose wall is permeable to calcium ions. Leave the beads in the calcium solution to cure for 0.5-3 hours.
2. Sodium alginate solution is best prepared by adding the powder to agitated water, rather than vice versa, to avoid the formation of clumps. Prolonged stirring may be necessary to achieve the complete dissolution of sodium alginate. After sodium alginate is completely dissolved, leave the solution undisturbed for

30 minutes to eliminate the air bubbles that can later be entrapped and cause the beads to float.

3. Although not necessary, the beads may be hardened by mixing some amines in the sodium alginate solution and cross-linking with glutaraldehyde.

RESULT:

CONCLUSION / INTERPRETATION:

[Signature of Student] **[Signature of Instructor]**