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Comparative Studies on the Enhanced Production of Heparinase by Physical and Chemical Mutagen

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Abstract: ***This experiment was deigned check the comparative studies on the enhanced production of heparinase by physical and chemical mutagen. Heparin is a combined sulfated polysaccharide that has certain characteristics and dispersions. It was initially discovered in the liver and was thus called heparin. In this experiment the growth of heparin was seen under the adverse physical conditions whereas under the chemical conditions heparin couldn't survive. Physical condition of heparin was kept under UV radiations for different time duration i.e. 2min, 4min and 6min. likewise under chemical condition heparin was added with EtBr i.e. 1µl, 2µl and 3µl. After that in purification the sample collected from my ultraviolet light plate were processed in down streaming. In downstream processing the sample in dialysis bag which was submerged in Tris HCl. By this process the salt content in the bag was excreted out which is called salt precipitation. Finally, Observed the optical density of the sample at 620 nanometre and I found the OD 0.23 on spectrophotometer.***

Keywords: Heparinase, UV radiation, EtBr mutation, Down streaming, Optical density

1. Introduction

Heparin, commonly known as heparin non-fractional (UFH) is a medicine and glycosaminoglycan which occurs naturally. Has been used as an anticoagulant as a medicine. It is also prescribed for the treatment of heart attacks and hypotension in particular. It is administered in a vein or beneath the skin via injection (**Hirsh, 2011**). Other applications include within the test tubes and equipment for renal dialysis. Common adverse effects include bleeding, injection site discomfort and low platelets in the blood. Serious adverse effects include thrombocytopenia caused by heparin. In individuals with weak kidney function, more attention is required. **(Warkentin, 2015)**

Heparin is a combined sulfated polysaccharide that has certain characteristics and dispersions. It was initially discovered in the liver and was thus called heparin. Heparin levels in the bronchi, ileum and skin of the majority of the species examined are rather high. While heparin is extensively utilized in medicine, it does not yet have its true biological function. Polysaccharide molecules have excellent immunological control, anti-tumour, anti-coagulation and anti-inflammatory characteristics and are used for the pharmaceutical sector. (**Warkentin, 2015**) NMH is a tiny percentage of heparin generated via physical, chemical, or enzymatic heparin hydrolysis. Low-molecular heparin has reduced anti-factor IIa activity and decreased risks of bleeding compared to normal-molecular heparin. Current techniques for preparing low molecular heparin include physical, chemical, biological and synthetic. Based on the benefits of moderate circumstances, high Selectivity and minimal pollution, the bioenzymatic technique has become a developing method. **(Hirsh, 2011**).

In suspected instances of pro-thrombotic immune thrombocytopenia (VIPIT) caused by vaccine subsequent to SARS-CoV-2, heparin is against recommended as the risk of bleeding, in favor of other medicines, in an anti-PF4/heparin compound autoimmune, may be further increased by heparin (such as argatroban or danaparoid). **(Levine, 2012)**

Heparin is administered parenterally because of its strong negative charge and big size, it is not absorbed in the stomach. It may be delivered intravenously or intravenously (under the skin); the risk for hematomas prevents intramuscular (into the muscle) injections. Heparin has to be administered often or as a continuous infusion because of its short biologic half-life of approximately an hour. **(Gray, 2018)** The half-life of non-fractional heparin is approximately one to two hrs after infusion, while the life of LMWH is about four to five hours. The use of LMWH enabled once-a-day dosing and therefore no continuous infusion of the medicine. When long-term anticoagulation is needed, heparin is typically only used to begin anticoagulation treatment, until oral anticoagulants, such as warfarin, take action. **(Levine, 2012)**

Heparin seems very safe for usage during pregnancy and nursing. Heparin is generated in all animals by basophils and mast cells. Heparin is a polymer with different chain sizes in nature. Heparin as a pharmaceutical non-fractionated (UFH) is heparin which has not been divided into the fraction of low molecular weight molecules. (**Gray, 2018)** In contrast, low molecular heparin (LMWH) has been fractionated to enhance the predictability of its pharmacodynamics. UFH or LMWH may often be utilized; either or other is preferred in certain cases.

Heparin, which contains the closely related molecule heparan sulfate, is a component of the glycosaminoglycan family of carbohydrates. It comprises a variably sulphated repeated disaccharide unit. **(Warkentin, 2015)**

Heparin is being used to diagnose or manage various diseases of the blood vessel, heart and lung. Heparin can also be used to avoid blood clotting, bypass operations, renal dialysis and blood transfusions when having open-heart operations. (**Levine, 2012**) It is used in small dosages to

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prevent blood clots from forming in certain individuals, particularly those who need specific kind of treatment or who need to be in bed for a lengthy period of time. Heparin also may be used for the diagnosis and treatment of a severe blood disease termed intravascular dissemination. **(Hirsh, 2011**).

Two kinds of heparins exist: low molecular heparins and high molecular heparins.

The aPTT must be monitored regularly using high molecular weight heparins. Low molecular weight heparins provide a greater response to anticoagulant and do not require regular blood testing. Heparin is a polymeric with different chain sizes in nature. **(Gray, 2018)** Heparin as a pharmaceutical nonfractionated (UFH) is heparin which has not been divided into the fraction of low molecular weight molecules. In contrast, LMWH underwent a fractionation in order to make its pharmacodynamics more predictable. Often UFH or LMWH may be utilized; either one is better in certain circumstances. **(Levine, 2012)**

Heparin is extremely acidic and consists of equivalent portions of sulfated D-glucuronic acid and D-glucosamine with sulfamin bridge. The weight varies between 3000 and 30 000 daltons. It is derived from the mast cells liver, lung, and other vertebrate cells. It is a recognized and widely utilized anti-thrombotic anticoagulant. Heparin inhibits processes leading to blood clotting and development of fibrin coagulations together in vivo and in vitro. Small doses of heparin in conjunction with heparin cofactor antithrombin III may prevent thrombosis via Factor Xa and thrombin inactivation. Having established active thrombosis, higher quantities of heparin may block further coagulation via thrombin-inactivation and fibrin conversion. (**Warkentin, 2015**) It also inhibits the development of a steady fibrin coagulation by blocking fibrin stabilization factor activity. Heparin extends many tests of coagulation. The most clinically significant result is activated partial prothrombin time (aPTT) for all coagulation tests. **(Levine, 2012)**

Uses of Heparin

- Heparin works as an anticoagulant which inhibits the development of clots and the spread of active blood clots. While heparin alone does not disassemble previously formed clots (unlike the activator of tissue plasminogen), it enables natural clot lysis processes to function properly to disassemble clots. **(Gray, 2018)** Heparin is usually used for the following disorders in the anticoagulation: all acute coronary syndrome, for example,

a) NSTEMI and all atrial fibrillations.
b) Deep-vine thrombosis and lung embolism
c) Cardiopulmonary bypass for cardiovascular surgery
d) ECMO support circuit for extracorporeal life
e) Hemofiltration
f) Centrally or peripherally indwelling venous catheters

- Heparin and its low molecular derivative are effective in avoiding thrombosis in the deep veins and pulmonary emboli in at-risk individuals, but no evidence has shown that one is more successful than the other in preventing death. **(Hirsh, 2011**).
- Can be used to prevent and treat lung/leg blood clots. It can also be used to treat some abnormalities of blood coagulation. It may also be used to avoid blood clots following surgery, dialysis, blood transfusions, blood collection or those unable to function for a long period. Heparin helps to maintain smooth blood circulation by improving the function of a particular natural ingredient (anti-clotting protein) in your body. It is referred to as an anticoagulant. **(Warkentin, 2015)**

In addition, heparinase is extensively employed in low-molecular heparin production. LMWH is more consistent and secure than non-fractionated heparin and is extensively used for preventing and treating venous thromboembolism and treating myocardial infarction. HepI is also the main technique to analyze the structure of heparin and eliminate heparin in blood. In lab heparin analysis and in low-molecular-weight commercial heparin manufacturing, however, the original synthesis of P. heparinusHepI is expensive, hampering much of the use of HepI. While HepI in Escherichia coli was heterologously produced, the yield was extremely low owing to the existence of integrative bodies. HepI's expensive price, poor output and low activity significantly restrict its industrial use.

Production of heparinase

1) **By fungus:** heparinases are enzymes that are produced by bacteria that break down heparins, which are sulfated glycosaminoglycans, in order to produce low-molecular-weight heparins (LMWHs). Aspergillus oryzae was isolated from a soil specimen and found to be a heparinase-producing fungus. During its middle log phase of growth, the fungus produced heparinase both extracellularly and intracellularly, indicating that it was actively producing the enzyme. (**Hirsh, 2011**). The synthesis of heparinase and the growth kinetics of the enzyme in complex protein digest medium were studied using heparin as the inducer. Heparinase activity has been found to be reliant on the quantity of heparin consumed during the course of development. **(Warkentin, 2015)**
2) **By anaerobic bacteria:** The synthesis of heparinase by a broad variety of anaerobic bacteria isolated from clinical specimens was studied. None of the 29 strains of Bacteroides fragilis generated heparinase. Of 62 additional Bacteroides examined, only 2 of four variants of B ovatus, three different strains of B thetaiotaomicrons, and two of 4 strains of B uniformis were heparinase manufacturers. Neither of the 48 strains of fusobacteria or 7 strains of Veillonella generated heparinase. (**Gray, 2018)** The anaerobic cocci were likewise negative for heparinase synthesis as were 46 Clostridium spp examined. It was determined that heparinase synthesis by anaerobic bacteria was expected to have a role in the localized thrombophlebitis that occasionally happens in anaerobic infections.

2. Literature Survey

(Damus, 2013) stated that Heparin, commonly known as heparin non-fractional (UFH) is a medicine and glycosaminoglycan which occurs naturally. Has been used as an anticoagulant as a medicine. It is also prescribed for

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the treatment of heart attacks and hypotension in particular. It is administered in a vein or beneath the skin via injection Other applications include within the test tubes and equipment for renal dialysis.

(**Hirsh, 2011**) stated that Heparin is a combined sulfated polysaccharide that has certain characteristics and dispersions. It was initially discovered in the liver and was thus called heparin. Heparin levels in the bronchi, ileum and skin of the majority of the species examined are rather high. While heparin is extensively utilized in medicine, it does not yet have its true biological function. Polysaccharide molecules have excellent immunological control, anti-tumour, anti-coagulation and anti-inflammatory characteristics and are used for the pharmaceutical sector.

3. Methodology

Sample Collection

The soil sample was collected from slaughterhouse and then weighed it. Then discarded rest of sample and took 1 gram of soil sample which had blood stains on it.

After that the sample was packed in polystyrene bag in order to avoid contamination. Then the sample was taken to lab and kept it at room temperature.

Bacterial Isolation by Serial Dilution Method

The sample was weighed and again took 1 gram and serially diluted it up to 10^{-9}.

Sequential dilution is stepwise dilution that is performed to convert a thick solution into more usable concentration. In other words, sequential dilution is step wise dilution of solution. Sequential dilution is associated with the reduction of concentration of cells in cultures. Diluting a sample means that it is feasible to acquire incubated culture plates with an effective countable number of settlements and then compute the quantity of microorganisms present in the sample.
After performing serial dilution, Spreading technique was performed.

Figure 1: Serial Dilution

Spread Plate Technique

Spread-plate strategy is the technique for detachment and identification of different-different microbes in mixed cultures. After identification, next step is circulating it evenly on entire plate in uniformly circular motion.

Spread-plate technique comprises of sterilized spreader, the surface can be of glass or metal which is used to pick limited quantity of microbes suspended in our solution. Plates must be sterilized well before to avoid any possible contamination, it must be dried at room temperature so the agar can retain the bacteria more readily.

For performing spread-plate technique, Firstly dispensed 0.1 ml of sample in petri plate which contains nutrient agar media.

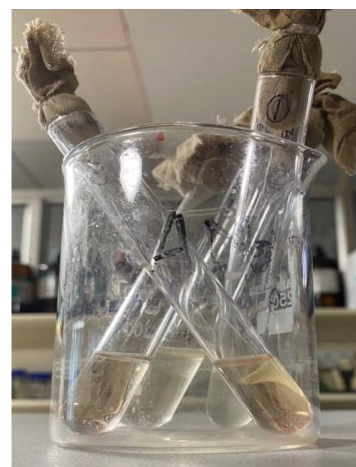

Figure 2: Heparin solution used in media

After dispensing, sterilized spreader over the flame, slightly cool it and then did clockwise rotation on the entire plate and from the last spot, Then did anticlockwise rotation on plate. Spreaded the sample evenly on entire plate by using our sterile spreader.

After that, It was the plates were incubated at 37 degrees Celsius for twenty four hours.

This whole procedure is performed under Laminar air flow to avoid any possible contamination.

Figure 3: Spreading plate

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Pure Culture Preparation by Streaking-Plate Technique
Streaking plate technique is used for the separation of unmixed strain from a single species of bacteria. In streaking plate method, a sterilized loop is used to get uncontaminated microorganism's culture.

After 24 hours of incubation of spreading plate, observed mix bacterial strains. Then marked colonies from the plate.

After marking colonies on plate, Streaking was performed for getting pure bacterial strains. Basically, streaking technique is used to get pure strain from the mixed bacterial strains.

Streaking plate process is achieved by using a sterile inoculation loop which was sterilized it over the flame in zigzagging manner.

Then marked the plates and kept it in incubation chamber for twenty-four hours.

The streaking cycle will weaken the sample that was initially set in the underlying area of the Agar. The cycle is designated as picking colonies when it's done from from an agar plate with colonies isolated and it is shifted to a new agar plate using a sterile loop.

This whole procedure was performed under Laminar air flow to avoid any possible contamination.

Screening-Firstly and Secondary Screening for Heparin Producing Bacteria:
In primary screening, plates were firstly observed after 24 hours of incubation for any possible contamination or bacterial growth.

Figure 4: Screening Plate

In order to perform secondary screening, I prepared (45ml) media which contained media such as:

Table 1: Media Preparation

Reagents	Concentration
KH_2PO_4	6G/L
NaH_2PO_4	3G/L
NaCl	5G/L
NH_4Cl	2G/L
DEXTROSE	8G/L
$MgSO_4$	0.2G/L
AGAR-AGAR	20G/L
HEPARIN	10G/L

First mixed this and then putted in autoclave along with fresh petri-dishes for sterilization to be completed. Then took bacteria from pure cultures plate and then streaked on the plates to get secondary screening results.

Preparation of SMEAR

After performing screening, took some colonies from the screening plate.

Heat fixed a slide over the flame.

Wire-loop was picked up and sterilized it over the flame. Then with the help of wire-loop, took colonies from the screening plate and kept it over the slide and rotated it in uniform circular motion so that it spreads evenly.

Heat fixed slide and kept for drying.

After preparing smear, the slide was placed over the staining rack and performed Gram's staining.

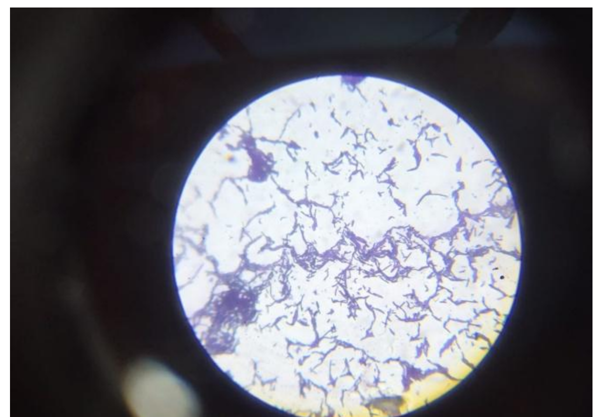

Figure 5: Bacillus Species

Gram Staining
- Gathered all the necessary reagents required to perform staining.
- Covered the slide with methyl violet and kept for 2 minutes.
- Methyl violet is mainly used for color presentation. After 2 minutes, washed the slides under tap water and kept for drying.
- Covered the slide with Gram's Iodine and kept for 2 minutes and then washed it under tap water.
- Covered the slide with acetone and washed it immediately under tap water. Acetone is used for color fixation.
- Covered the slide with Safranin and kept it for 2 minutes. Safranin is used for counter staining.

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- Washed the slide and kept it for air dry.
- Observed the slide under microscope by using seedar wood oil.

Mutation– By Physical and Chemical Method

After performing screening, I then performed mutation using Ultraviolet radiation and ethidium bromide (ETBR).

UV RADIATION-Bright (UV) light is the piece of electromagnetic range somewhere in the range of 200-400 nm, with more limited frequencies than violet of the apparent range (consequently the name, bright). This reach is additionally separated into short wave (200-280nm, UV-C), Center wave (280-315nm, UV-B) And long wave (315-400nm, UV-A) light. The more limited frequency UV-C light conveys with it significantly more energy than its long wave UV-A partner and is considerably more harming to DNA.

UV-B and UV-C beams are two sorts of high-energy radiation that are equipped for ionizing (eliminating electrons) from atoms in an interaction considered a photochemical response that prompts new subatomic items. UV harm happens through two particular sorts of changes.
1) Dimerizing changes
2) Oxidative changes

For performing mutation, I used total of 8 plates (Four plates for physical and another four for chemical mutation). In physical mutation I took four plates and labelled them.
- Kept the first plate in room temperature.
- Second plate was kept for 2 minutes in UV light for mutation.
- Third plate was kept for 4 minutes in UV light.
- Plate 4 was then kept for 6 minutes in UV light.

After performing mutation, the results were observed.

Second, third plate showed no results and heparin could not survive by the action of UV light. Fourth plate which was kept for 6 minutes showed possible growth.

ETHIDIUM BROMIDE-CHEMICAL MUTATION

Ethidium bromide is the most ordinarily utilized color for DNA and RNA identification in gels. Ethidium bromide is a DNA intercalator, embedding itself between the bases sets in the two-fold helix.

Ethidium bromide has UV absorbance maxima at 300 and 360nm, and an emission max at 590nm. The identification furthest reaches of DNA bound to ethidium bromide is 0.5 to 5.0ng/band.

For performing chemical mutation,
- Took 4 petri dishes and labelled them.
- After that, Took 4 test tubes and created a mixture of heparin and ethidium bromide.
- In the first tube, took 10ul of heparin and 1ul ETBR, in the second tube, took 2ul ETBR and 10ul heparin, in third tube, took 3ul ETBR and 10ul heparin and in the last one, took 4ul ETBR and 10ul heparin.
- After creating a mixture of ETBR and Heparin, the test tubes were placed in orbital shaker for 24 hours.
- Two wells were created in each media plate with the help of sterilized scalpel.
- After that, Poured the mixture from test tube-1 with the help of pipette into wells of petri plate-1 and kept it in incubator.
- After that Poured the mixture from test tube-2 into the wells of petri plate-2 that it was kept in for incubation.
- Then poured the mixture from test tube-3 to petri plate-3 and kept it in incubator
- At last, Poured the mixture from test tube-4 to petri plate-4 and kept in incubator for 24 hours.
- After 24 hours, results were observed.

Figure 6: Ethidium Bromide mutation

Fermentation

Usually, fermentation is performed for production of enzymes. The type of fermentation I performed is called Submerged fermentation. For performing fermentation, I prepared media containing.

Table 2: Media Preparation

Reagents	Concentration
KH_2PO_4	6G/L
NaH_2PO_4	3G/L
NaCL	5G/L
NH_4Cl	2G/L
DEXTROSE	8G/L
$MgSO_4$	0.2G/L
AGAR-AGAR	20G/L
HEPARIN	10G/L

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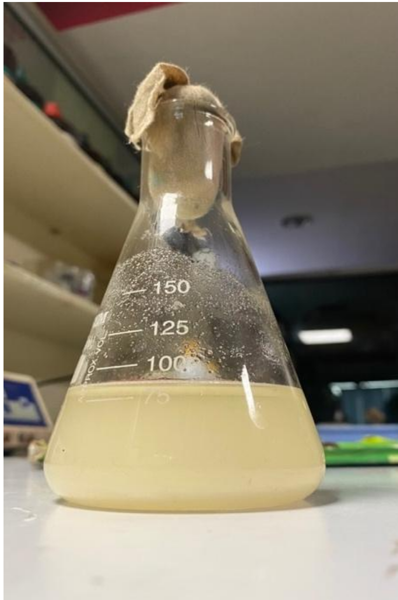


Figure 7: Media

Submerged fermentation is a technique for assembling biomolecules in which chemicals and other responsive mixture are lowered in a fluid like alcohol, oil or a supplement stock.

Submerged fermentation (SmF) / Liquid fermentation (LF) smF uses free-streaming fluid substrates, like molasses and stocks. The process is utilized for an assortment of purposes, generally in mechanical assembling.

Principle of Submerged Fermentation

Submerged fermentation includes the development of the microorganisms as a suspension in a fluid medium wherein different supplements are either broken down or suspended as particulate solids in numerous commercial media.

Submerged fermentation is a cycle including the improvement of microbes in fluid stock. The fluid stock contains supplements, and it brings about the creation of mechanical proteins, Anti-toxins, or different items. The cycle includes taking a particular microorganism, for example, growth and setting it in a little shut jar containing the rich supplement stock. A high volume of oxygen is additionally needed for the cycle. The creation of proteins then, at that point happens when the microbes interact with supplements on the stock bringing about them for being separated.

After preparing media, I performed rest of experiment in Laminar air flow to avoid any possible contamination. I took my screening plate on which I had performed UV-mutation.

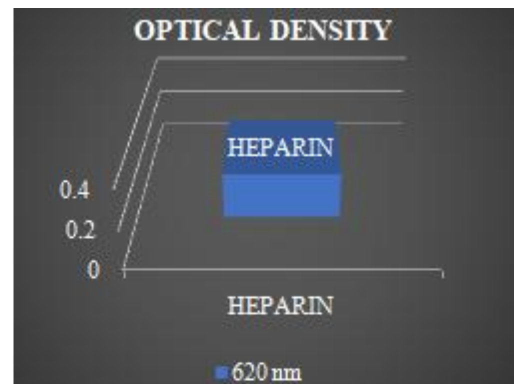
- Then sterilized wire loop over the flame.
- I took some colonies from the plate and submerged it in the media which I prepared.
- Shaked it for few minutes
- Re-sterilized wire loop and closed laminar air flow.
- After that, I took media in orbital shaker and kept it for overnight.

Down Streaming

- Transfer media in 6 microcentrifuge tubes.
- Centrifuge it at 10000 RPM for 10 minutes.
- After centrifugation, transfer supernatant in measuring cylinder.
- After transferring supernatant, measure volume.
- Take 20% ammonium sulphate of total volume and place it on magnetic stirrer on hot plate
- Place ice pack around the beaker to maintain temperature.
- Now add pinch by pinch ammonium sulphate and keep stirring.
- Keep stirring for around 10 minutes.
- Now keep it at 4°c for overnight.
- On the next day, transfer 1.5 ml sample in microcentrifuge tube.
- Centrifuge it at 10, 000 RPM for 10 minutes.
- After centrifugation, discard supernatant.
- Dissolve pellet in Tris-buffer to maintain optimal PH.
- Now transfer in dialysis bag.
- Dip dialysis bag in Tris-buffer and keep it at 4°c for overnight.

4. Result & Discussion

Bacterial plate of heparin which was kept for 6min under UV rays in which growth of heparin was seen whereas there was no growth seen in EtBr culture.



Graph 1: Result showing OD vs Heparin

In purification positive sample that we got from a UV plate were sent to downstream processing. After that, visualized optical density on spectrophotometer at 620nm and found the optical density to be 0.23.

5. Conclusion

As the topic says comparative studies on the enhanced production of heparinase by physical (UV rays) and chemical (EtBr) mutagen, The result seen in physical mutation i. e UV rays, the growth was actively increased as compared to the chemical mutagen i. e using Ethidium Bromide.

The culture plate which was placed in UV rays at 2 minutes, 4 minutes and 6 minutes. The observations was that 2 min and 4 min plates couldn't survive. But the plate which was kept for 6 min survived as the heparin in the plates has

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higher growth capacity. The heparin present in the 6 min plates got mutated to survive under such physical conditions.

After that in purification the sample was collected from my ultraviolet light plate and were processed in down streaming. In downstream processing the sample were placed in dialysis bag which was submerged in Tris HCl. By this process the salt content in the bag was excreted out which is called salt precipitation.

Finally, observed the optical density of the sample at 620 nanometre and I found the OD 0.23 on spectrophotometer.

Figure 8: Result

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