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IN VITRO STUDIES ON ISOLATION, SCREENING AND CHARACYERIZATION OF STREPTOKINASE FROM BACILLUS SPECIES

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ABSTRACT

The clot in the blood vessels is the result of coagulation of fibrin molecules and leads to many consequences like blockage of blood vessels, myocardial infarction, cerebral infarction. The drug which is available in the present time for anti-thrombotic diseases has several side effects and shows restricted mechanism. Therefore, the enzyme 'Streptokinase' is used to thin the blood in order to prevent from the formation of a thrombus. Streptokinase degrades the fibrin molecules into tyrosine and was isolated from Bacillus species.

Chapter-1

INTRODUCTION

INTRODUCTION

Enzymes are the biological catalyst which catalyzes the biochemical reactions. Enzymes are produced by almost all living cells to bring about specific biochemical reactions and is responsible for the occurring of all metabolic processes within the cell.

All enzymes are protein but all proteins are not enzyme. These are the macromolecules which catalyzes the chemical reactions. Enzymes are highly specific in their action and binds only with its substrate on active site. A substrate is a chemical species which will bind with a reagent to generate a product.

E (Enzyme) + S (Substrate) ===| ES Complex (Enzyme Substrate)

ES (Enzyme Substrate) Complex ===| EP (Enzyme Product)

EP (Enzyme Product) ===| E (Enzyme) + (Product)

Many different enzymes are required to bring about the sequences of all the metabolic reactions in the living cell. All enzymes are almost protein in nature and may or may not possess a non- protein prosthetic group. Enzyme increases the rate of reaction by lowering its activation energy. Some enzymes can make their conversion of substrate to product occur many millions of time faster. Enzyme specificity usually comes from their 3D structure and therefore used in practical application and at industrial level. They are used from many centuries and was practiced long before the nature or function of enzymes was understood. Enzymes from barley are released during the mashing stage of beer production. They degrade starch and proteins to produce simple sugar, amino-acids and peptides that are used by yeast for fermentation. The use of barley malt for starch conversion in brewing and of dung in leather making, are examples of ancient use of enzymes. In the same way, Streptokinase is an extra-cellular protein which is an enzymatic, produced by the strain of β- hemolytic streptococci. By activating plasminogen, the streptokinase facilitates the lysis of blood clots. The streptokinase made up of 414 amino acid and have molecular weight 47kda without the bridge of di-sulphur with different functional properties. The streptokinase made up of free structure domain like alfa, beta and gamma. The plasminogen binds with each domain, even though none can activate plasminogen. In the plasminogen activation, the streptokinase plays an important role.

The streptokinase producing streptococci which were found in 1874 by Billorth in exudate of infected wounds. After that similar microorganisms which were shown in the blood of scarlet fever patients. After based on type of hemolytic reaction, the streptococcus species classified into alfa, beta and gamma variants. The plasminogen activators that is streptokinase include into the fibrinolytic therapy. Streptokinase is a non

- fibrin which is a specific drugs, which convert into plasmin both circulating the plasminogen and lysis of fibrin and also an important systemic fibrinogenolysis, and fibrinogenemia and the circulating products increased from the fibrin degradation. Because of the plasminogen fibrin binary complex, the fibrin lysis on the clot surface given by the fibrin-specific activators without affect the circulating. Streptokinase is a potent activator of the fibrinolytic enzyme system in man. In the treatment of occlusive vascular disease, its principle mechanism of action in believed to involve the activation of both circulating plasminogen and intrinsic thrombus plasminogen through the formation of an activator complex with the plasmin molecule itself. At that time the streptokinase may also induce a coagulation defect due to the induction of a hyperplasminemic state.

The streptokinase is a complex mechanism for the activation of the human plasminogen. This activator consists of 1/1 complex of streptokinase and human plasminogen. It has been studied about the streptokinase that induced the activation of plasminogen in the specific species respect to plasminogen. About all species, the plasminogen of only human, monkey, cat, dog, and rabbit are activated by the streptokinase.

The equimolar complex can form by streptokinase and human plasminogen, catalyzes the conversion of plasminogen to plasmin of different mammalians species. The blood cols dissolution causes by the plasmin. To relieve the thromboembolic blockage in blood vessels, the streptokinase used in the thrombolytic agent such as acute myocardial infarction.

As a carbon source under the soya bean, the bacillus licheniformis used for the production of streptokinase. Streptokinase naturally produced and secreted by various strain of hemolytic streptococci.

The streptokinase is not an enzyme but rather a potent activator for plasminogen.

For the activation of plasminogen the coiled region of the streptokinase gamma-domain is supposed as the necessary and similarly, the β-domain is involved in the formation of the streptokinase plasminogen complex which is responsible for the activating the plasminogen.

The fibrinolytic activity of the streptokinase originates in its ability to activate plasma plasminogen. The mechanism of plasminogen activation by streptokinase is of particular interest for two reasons. First, the increased therapeutic use of streptokinase provokes a better understanding of its mode of action. Second, unlike most of plasminogen by the formation of a strong stoichiometric complex with plasminogen.

The efficacy of streptokinase in the treatment of thrombolic disorder is determine by several parameters including the ability of streptokinase to form an activator complex with plasminogen, the plasma concentration of antibody to streptokinase before streptokinase infusion, the ability of streptokinase to react with antibody.

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with antibody.In human, streptokinase used as a thrombolytic agent especially for the treatment of acute myocardial infarction as it is a costeffective for a both animal and human disorder. This agent is used to treat heart attack, stroke, deep vein thrombosis, pulmonary embolism.

The clinical importance of Streptokinase was first noted by Tillet and Garner (Tillet and Garner,1933) who discovered that this bacterial protein caused the lysis of human blood clots. The hydrolysis of fibrin is also known as fibrinolysis (Rashmi and Lini,2013). Fibrinolytic enzymes are one of the largest groups of proteolytic enzymes involved in numerous regulatory process related to fibrinolytic action (Jayalakshmi et al.,2012). These enzymes are currently used in managing the heart diseases, effectively prevent in cardiovascular disease because it is a blood clot dissolving agent. However, this enzyme is often expensive thermo labile and produce unwanted and undesirable side effect (Rashad et al., 2012).

Streptokinase known as the plasminogen activator which is activated both by fibrin dependent and fibrin independent mechanism. The multiple domains help to streptokinase to interact with plasminogen. There are two identified plasminogen binding sites of the streptokinase. The C- terminal domain of streptokinase which help in substrate recognition and activation of the plasminogen. The streptokinase binds firstly to the extended conformation of plasminogen through the lysine binding site which help to trigger the conformational activation of plasminogen (Anirban Banerjee et. al., 2003; Yusuf Chishti et. al., 2003).

Streptokinase used for the treatment of the acute myocardial infarction and ischemic stroke. It is crucial to develop a production process with high yield. The pathogenicity is the main reason for the low production yields from natural host which is investigated by recombinant DNA technology for the production of this important proteins. In the several grams positive and gram-negative bacteria, the streptokinase expressed in these bacteria. Heavy glycosylated protein is the disadvantages of producing recombinant protein which secreted into the culture medium by pichia pastoris and low cell density obtained by lactococcus lactic due to growth inhibition coused by acidification of the medium lactic acid (Shima Mahmoudi et. al., 2012; Hamid Abtahi et. al., 2012).

The nature of streptokinase activation of plasminogen has been the subject of intense study over the last ten years become of the unique effect of streptokinase on activities arising from plasminogen like plasmin and activator activities. The evidence has been presented by the kline and fishman that is the streptokinase react directly with plasmin to form a complex with increased affinity for certain synthetic substrates (e.g., lysine methyl ester) and decreased affinity for certain protein substrates (e.g. casein). The plasmin streptokinase complex was termed as activator because it was an efficient activator of bovine plasminogen alone was not and it was an effective activator of human plasminogen whereas plasmin alone was not (Fletcher B. Taylor et. al., 1968; Jean. Botts et. al., 1968).

By the Ethiopian, the strain isolated which have the various streptococcal disease expression, there are reported that the streptokinase genotype which distributed, have no association to disease patterns. By considering the strain possibility which is secreted the protein by differing in their ability, level of streptokinase activity in culture supernatant of these strain were determine by a plasminogen activation assay that are synthetic tripeptide, H-O-valyl-leucyl- lysine-p-nitroaniline, as a substrate. The streptococcal

disease expression does not correlate directly with streptokinase genotype and in vitro streptokinase production (Wezenet Tewodras et. al., 1995; Mari Norgren et. al., 1995).

The novel bacterial enzyme that streptokinase which binds to the plasminogen to activate it. The streptokinase produced by the many different species of streptococcus. There are the different method like fibrin clot method, casein hydrolysis and chromozym test to determine the streptokinase activity. By using batch culture obtaining high production of the streptokinase. In the continuous culture, the production of streptokinase increase 2-3 times. The different species of the streptococcus which is selected for the optimum production of streptokinase by using the different concentration of substrates such as CSL, molasses, sugarcane bagases, rich polishing. The activity of streptokinase enhanced by using the RSM at the temperature 37^{o}C and pH-7 which is the physical parameters. To enhance the enzyme production which depends on nitrogen source, C- source, incubation time, growth factor such as glysine, thiamine and traces element like FeSO4 and MgSO4whichis uses n limited amount (Hadia Naseem et. al., 2021; Abdul Jabbar et. al., 2021).

Streptokinase is the best microbial plasminogen activator, it is not only one. Staphylokinase sourced from staphylococcus species is a potential alternative plasminogen activator. The structure and mechanism of the action of staphylokinase becoming better understood. Recombinant streptokinase has been produced in bacteria such as E. coli and shown to induce fibrin specific clot lysis in human plasma milieu in vitro. Another activator in clinical use is the acylated plasminogen streptokinase activator complex, in human plasminogen with an acylated active site has been complexed with bacterial streptokinase. The acylated streptokinase activator complex has an extended therepeutical effective half-life in circulation relative to streptokinase (Anirban Banerjee et. al., 2003; Yusuf Chisti et. al., 2003).

The bacterial activator means streptokinase which contains three-domain (α, β, γ) and staphylokinase which contain one-domain (α) both have the ability to activate the plasminogen of the mammalian protease. These activator act as the plasmin cofactor and the initiation of the proteolytic activity of host plasminogen by the resulting complex which facilitates the bacterial colonization of host organism. The plasminogen activation have the kinetic mechanism which mediated by a novel streptokinase which have two-domains (α, β) which is isolated from the streptococcus uberis with specificity towards bovine plasminogen. The plasminogen and streptococcus uberis intraction occurs in two step – firstly the protein association which occurs rapidly and second is to activate the complex streptococcus uberis – plasminogen activator the transition occurs slowly. The following parameters Km ≤ 1.5 μM and kcat = 0.55 s^{-1} which help to the streptococcus uberis – plasminogen activator to convert the plasminogen into plasmin (Laust B. Johnsen et.al., 20000; Sergaey N. Fedosov et. al., 20000).

The pharmaceutical drug that is streptokinase which have the high thrombolytic activity. The technology developed which allow for the synthesize membrane with relative elongation of 25- 165% and the tensile strength of 20-55MPa. The biodegradable membrane, in n isotonic solution the polymer degraded at the rate of the 0.5-1.0% per day. As the separate film samples, when the polymer were implanted the only trces of the PGLA were detected in the tissues two month after surgery. The implantation of strent coated with

streptokinase-infused polymers resulted in the formation of a mature and thick connective tissue capsule (A.S. Baikin and K.V. Segienko).

By using dynamic light scattering like small angle X-ray scattering and circular dichroism spectroscopy, structure of the streptokinase has been studied in the solution. The radius of stock is 3.58nm and the radius of gyration is 4.03nm of the protein monomers. The 14nm is the maximum intra particle distance of the molecule organized more than half of the amino acid. The organization of the regular folded secondry-structure, by using X-ray scattering curve, the result from dynamic light scattering and finding that at least 50% of the amino acid residues which are consisting with the following model. The four compact, separately folded, domains linked by mobile segment of the protein chain are consisting by the streptokinase. The conformation of a flexible string-of-beads in solution exhibited by the molecule (Klaus Gast et.al, 1992; Heinz Welfle et.al., 1992).

The streptokinase activates the plasminogen to plasmin which allowing the GAS to disseminate into deeper tissue or lyse fibrin clots in which they may be entrapped. Streptococal infection in the human body usually behave in characteristic manner. They tend to spread diffusely through the tissue s which seem unable to wall them off as they typically do with streptococcoi. In addition, pus when it is formed is often thin and watery. Over the number of the years which are responsible for this characteristic behavior have been discovered (Selwyn Taylor et.al., 1950).

Chapter- 2

REVIEW OF LITRATURE

REVIEW OF LITRATURE

J. Am coll cardial et al. (2009) studied that the effect of intracoronary streptokinase that the primary percutaneous coronary intervention is given before giving the low dose of intracoronary streptokinase. The primary percutaneous coronary invention which significantly improves the microvascular perfusion, decrease the long terms infarct size means a small localized are of dead tissues resulting from failure of blood supply and improves the left ventcular volume and function. The change in long term clinical outcomes on the determination of positive effect promises parallel.

Noogh et al. (2014) reported that in 46.6% of patients who take the streptokinase, significantly increases the enzymes of liver after 48 hours, although increasing enzymes which did not result in the jaundice, the enzymes were close to reaching the upper 2-3 times normal. He found that the change in transaminases, slowly revolved reduce to the normal volve by the 7thday after patients. Additionally, no change in total or direct bilirubin was noted due to streptokinase utilization.

Kim et al. (2006) investigated that by the help of Asp41 – His48 in streptokinase plasminogen binary complex play important role in binding to a substrate plasminogen. The plasminogen substrate recognized and activated by involving the C-terminal domain of streptokinase. The streptokinase participating in virgin enzyme induction and stabilizing the activator complex for the activation of human plasminogen.

Adinarayana et al. (2006) concluded that for the streptokinase activation of bovine plasminogen, the gamma domain is not required.

Spann et al. (2009) reported their experience with high dose intravenous therapy by the use of intravenous streptokinase in myocardial infarction in 13 patients under the age of 70 years. Their review indicated that, the thrombolysis with streptokinase, either by intracoronary or intravenous administration, appear promising in the treatment of acute myocardial infarction.

Reddy & Markus et al. (1974) find out that the methyl esters of lysine, acetyllysine and tosylarginine which hydrolyzing by the help of streptokinase, at almost one-half the rates characteristics for the fully developed streptokinase plasmin complex.

Nitzsche & Rasenheinrich et al. (2008) reported that human contact system is activated by the action of streptokinase. The role of secreted and surface-bound streptokinase in this process was investigated by comparing on M49 S.pyogenes ska mutant, which is enable to trigger plasmin activity in human.

Mansouri & Tashroie et al. (2014) studied that aim to describe the quantitative method for detecting new drug safety signals noted that this drug was leading agent in the number of report. They present the data of

activities of the adverse drug reaction monitoring center from 1998 to 2008. In this study streptokinase was ranked as the third most frequent couse of adverse drug reactions reported to this center.

Javidee & Kargar et al. (2014) reported in 14 studies and was commonly observed adverse event following cardiovascular adverse drug reactions. Minor bleeding (epistaxis, hematuria, local blood oozing) and mild to moderate hematoma were most frequent reported adverse drug reactions in two studies that used streptokinase for mechanical valve thrombosis in adult and femoral artery thrombosis in infants and young children.

Noha Basher et al. (2015) had studied the screening of isolates for their potency to produce streptokinase was an important criterion of this research. The study was carried out on 60 throat swabs collected from patients with acute tonsillitis. Primary screening and characterization of the specimens from the infected throat can be an excellent source for the isolation of haemolytic organisms. Three bacterial isolates (5%) demonstrated - haemolysis. Based on the results of radial caseinolysis assay and blood clot dissolving assay, isolate SK-2 demonstrated the highest streptokinase activity. When subjected to morphological and biochemical characterization based on Bergey s criteria, it was identified as Streptococcus pyogenes. The thrombolytic potential of this particular isolate indicated that it could extract a promising streptokinase with potent activity also it may be utilized for large scale production of streptokinase.

Daksha P. Mehta et al. (1991) investigated that the association of the heparin-induced thrombocytopenia and thrombosis by significantly incidence of morbidity and mortality. The recognition of this tangle and instant withdrawal of heparin therapy are imperative. The thrombocytopenia with major vascular inadequacy of the extremities and heparin-induced thrombosis are the description of a case report.

Laura Q. Rogers et al. (2004) studied in 70% of patients at the lower extremity reveals complete lysis thrombi by the evaluating streptokinase in the treatment of deep venous thrombosis. Before treatment the degree of lysis which is affected by the duration of symptoms, degree of occulation, thrombus location and development of a plasma proteolytic state. When Streptokinase patient compared with heparin-treated patient, the streptokinase patient have not been found to have a greater incidence of major hemorrhagic complications. If the patients are properly selected and carefully managed than the streptokinase is most beneficial in the treatment of deep venous thrombosis.

Gabor Markus et al. (1960) concluded that the fibrinolytic effect decrease very slowly than the increasing amount of human anti-plasmin, caseinolytic effect decrease much more rapidly when the streptokinase activated or spontaneously activated human plasmin which is mixed with the anti-plasmin and caseinolytic effect by increasing its amount. The caseinolytic effect completely disappear at the concentration of the anti-plasmin which is considered as fibrinolytic activity persist. Author suggest that in this condition other patient may be less effective but the fibrin can compete for plasmin with anti-plasmin. In the presence of inhibitor, the fibrin compete for plasmin with anti-plasmin which explain the specificity of the plasmin for fibrin in vivo. For the protection of plasma protein, the anti-plasmin play an impotent physiological role when allowing the lysis of fibrin clots by plasmin take place.

Saxena et al. (2017) reported that the vector constructed by joining the E. coli plasmid by replication in streptococcus spp. and E. coli. The chimeric plasmid which have been designated resulting inn pSA3 (chloramphenicol, erythromycin and tetracycline resistance) which have seven unique restriction site. In the presence of erythromycin, the pSA3 was transformed and stable in the streptococcus sanguis and streptococcus mutans. The library of the S. mutans GS5 genome which is constructed by using the pSA3 in E. coli, and the heterologous host have confirmed with S. mutans antiserum by the expression of surface antigen. Previously, it is determined that the species streptokinase was sub-cloned into the pSA3 and in the presence of pressure and expression of streptokinase activity in E. coli, the recombinant plasmid was stable. For increasing production of heterologous recombinant proteins, the culture strategy often used in E.coli.

CHAPTER – 3
MATERIAL AND METHODOLOGY

1. COLLECTION OF SOIL SAMPLES

The soil samples has been collected from seven different sources (Slaughter houses) to isolate those bacteria which are able to produce streptokinase enzyme. The soil samples are collected by using a spatula and transported to the microbiology laboratory for weighing and further analysis and the remain soil are kept stored.

Sample	Location
Sample-1	Mushipuliya, Lucknow
Sample- 2	Tedhipuliya, Lucknow
Sample-3	Bijnour, Lucknow

2. ISOLATION OF BACTERIA FROM COLLECTED SAMPLE BY SERIAL DILUTION METHOD

Materials Required:

- Soil sample
- Test tubes
- Test tubes stand
- Weighing balance
- Measuring cylinder
- Distilled water
- Micropipette
- Micro-tips

- Nutrient Agar Media(NAM)
- Laminar Air Flow
- Spirit Lamp
- Autoclave.

Principle:

It is based on the principle that we serially dilute our soil sample in order to isolate pure colonies over the NAM plates. The soil we collected cannot be directly spread over NAM plates as a number of bacterial colonies grows together which will create difficulty for us to select the different colonies.

Serial Dilution:

Serial dilution technique is one of the most commonly used methods for the isolation and the growth of fungi, bacteria and other microbes. It is the stepwise dilution of a substance in solution. Serial dilution helps us to decrease the number of colony progressively in the soil suspension which results in the isolated colonies of the microbes. As we use the NAM medium for their growth and the viable microbes will grow and from the isolated colonies over the NAM plates.

In microbiology, serial dilutions are used to decrease a bacterial concentration to required concentration for a specific test method, or to a concentration which is easier to count when plated an agar plate.A serial dilution method is the stepwise dilution of the substance. Serial dilution is a sequential dilutions which used to decrease a dense culture of cells to a more usable concentration. In serial dilution, a small amount of mixed solution which is transferred into new test tube, and in which adding the water or other solvent.

Procedure:

- First we took eleven test tubes, washed and autoclaved at 121^0C and 15 psi.
- After that, the autoclaved test tubes took away in the laminar and leave it for some time to cool down.
- Then we added 9ml distilled water in each test tubes.
- All test tubes marks as 10^{-1},10^{-2},10^{-3},10^{-4},10^{-5},10^{-6},10^{-7},10^{-8},10^{-9},10^{-10}
- After that, we added 1gm soil sample in the 10^{-1} test tube and mixed it gently.
- From the 10^{-1} test tube we transferred 1ml sample into 10^{-2} test tube and mixed it.
- From the10^{-2} test tube we transferred 1 ml sample into the 10^{-3} test tube and so on.
- After this we have inoculated 50µm in each petri plates and named as 10^{-1}, 10^{-2}, 10^{-3} so on.

Colony Morphology Study:-

Large number of bacterial colonies grow on the agar plates to distinguish between the different colonies, colony morphology of the bacterial colonies were studies. Colony morphology represents the important information regarding the identification of the organism. Important criteria include:

S. No.	Characteristics	Culture AZSN2021 01	Culture AZSN2021 02	Culture AZSN2021 03	Culture AZSN2021 04
1.	Shape	Filamentous	Irregular	Circular	Rhizoidal
2.	Margin	Entire	Curled	Discrete	Curled
3.	Elevation	Flat	Convex	Raised	Pulvonate
4.	Pigmentation	Pale yellow	Cream	Off white	Orange
5.	Surface	Rough	Smooth	Smooth	Rough
6.	Texture	Hard	Gummy	Soft	Hard
7.	Opacity	Transparent	Transparent	Opaque	Opaque

3. PURIFICATION OF THE OBTAINED MIXED CULTURE BY STREAKING (CONTINUOUS QUADRANT)

Materials Required:

- Source sample of bacteria
- NAM media plate
- Spirit lamp
- Spreader
- Micropipette
- Micro-tips
- Test-tubes
- Inoculation loop
- Mixed culture
- Laminar Air Flow
- Spirit Lamp

Principle:

A pure culture is usually isolated from the mixed culture by picking up the inoculation loop, isolated marked colonies from the mixed colonies and then spread into the NAM plates. The principle behind the streaking is that the individual colonies are allowed to grow into the new media giving them the chance to grow into individual colony. Hence, pure colonies are obtained after 24 hrs of incubation in incubator.

Quadrant streaking is a type of streaking which is done in order to obtain well isolated colonies. This allowed the sequential isolated of original microbial material over the entire surface of the plate. As the original sample diluted by streaking it over successive quadrant so that the number of organism decreases finally till the third or fourth quadrant.

Spreading:

- The spread plate method is a technique to plate sample containing a number of bacteria so that the bacteria are easy to count and isolate. A successful spread plate will have a countable number of isolated bacterial colonies evenly distributed on the plate.
- The sample taken from diluted mixture, which is placed on the surface of the agar plate and spread evenly over the surface by using spreader.
- In this technique, the culture is not mixed with agar medium. Instead it is mixed normal saline and serially diluted.
- A successful spread plate will have countable number of isolated bacterial colonies evenly distributed on the plate.

Procedure:

- Firstly, we took 11 petri plates and incubated at 121^0C and 15 psi for 60 minutes.
- Then, we prepared 110ml nutrient agar media and incubated.
- After incubated, the media poured in petri plates and leaved it for solidify.
- After inoculation we have used on L- shape spreader to spread thoroughly on each petri plates.
- Then we used paraffin to cover each petri plates in order to prevent from contamination.
- Then incubate it at 37^oC

Streaking:

- The streak plate technique is the most popular method for isolating specific bacteria from a sample containing a mixture of microorganisms. The technique essentially dilutes the number of organisms and reduce their density.
- Streak plate technique is used to grow bacteria on a growth media surface so that individual bacterial colonies are isolated.

- Streak plate technique is used for the isolation into a pure culture of the bacteria from a mixed population.
- The inoculum is streaked over the agar surface in such a way that it "thins out" the bacteria.
- Some individual bacterial cells are separated and well space from each other.

Materials Required:

- Spread plate culture
- NAM media plate
- Inoculation loop
- Paraffilm

Procedure:

- Firstly, we have flamed a loop and allow it to cool down in sterile area.
- Then, we have taken a developed bacterial culture and open it near the flame to pick a colony and undergoes development.
- We have streaked the bacterial colony on agar plate in the zig-zag motion with the help of loop.
- Then, we have labelled each petri plate to indicate the colony on agar plate number date of culture and name.
- Then the petri plate wrapped with parafilm.
- At the last, we have incubated the petri plate at 37^0 C in invert position to reduce chances of droplets of condensation falling on the media.

4. PRIMARY AND SECONDARY SCREENING FOR STREPTOKINASE PRODUCING BACTERIA

Materials Required:

Petri-plates, Beaker, Autoclave, Skimmed Milk Agar Media (30g/l), Inoculation loop, Spirit lamp etc.

Principle:

Screening is the procedure to check whether the selected microbes have the potential to produce the desired secondary metabolites or not. Streptokinase producing bacteria is detected in the screening media in which carbon source is limited and the additional substrate has been provided. The isolated pure strain was screened for the production of streptokinase. This is visualized by the formation of clear zone after the utilisation of the skimmed milk agar as a substrate.

Primary Screening:- It is to check the growth of bacteria in skimmed milk agar by visual identification.

Procedure:

- We have prepared Skim Milk Agar media and autoclave it at 121^0C and 15psi.

- Then we have poured the media in petri plate and leave for solidify.
- After solidify the media, we have streaked the bacterial culture in the center of the media in zig-zag motion.
- After that, we have leave it at room temperature for 24 hours in order to determine growth of bacteria.

Secondary Screening:-It is used to differentiate endospores forming bacteria and streptokinase producing bacteria by the formation of clear zone.

Procedure:

- We have prepared Skim Milk Agar media and autoclave it at 121^0C and 15psi.
- Then we have poured the media in petri plate and leave for solidify.
- After solidify the media, we have streaked the bacterial culture in the center of the media in zig-zag motion.
- After that we have leave it at room temperature for 48 hours in order to determine growth of bacteria
- After 48 hours, we have seen clear zone in the center of the plate which indicate the zone of inhibition.
- The zone of inhibition indicate that the bacillus bacteria consumed the protein for its growth.

5. IDENTIFICATION OF SELECTED BACTERIA

The identification of bacteria capable of producing streptokinase is carried out by Gram Staining Method.

Materials Required:

- Pure culture plate
- Crystal violet
- Iodine solution
- 95% Ethanol
- Safranin
- Slide
- Spirit lamp
- Inoculation loop
- Distilled water
- Microscope

Principle:

Gram staining is a process used for the identification and classification of bacteria into two categories. Gram positive and Gram negative. Simultaneously, provide infective regarding the cellular morphology and arrangements of cells. The functions are subjected in the different chemicals.

- Crystal violet(Primary stain).
- Iodine solution(Mordant).
- Ethyl alcohol(Decolorizing agent).' ➢ Safranine(Counter strain).

Gram positive cells have thick peptidoglycan cell wall that is able to retain crystal violet iodine complex, while gram negative cell have only thin layer of peptidoglycan and is not able to retain crystal violet, when treated with ethyl alcohol, beside the gram positive cells have cross like peptidoglycan layer hence preventing the cells from decolorizing by ethyl alcohol. And safranine gives purple colour.

Gram staining also called gram's method, is a method of staining used to distinguish and classify bacterial species into two large group: gram-positive and gram-negative bacteria. Gram's staining technique was used to differentiate bacteria on the basis of their cell wall composition. The bacteria culture which appeared purple color are known as gram positive while the bacteria which appeared pink are gram negative. Gram-positive cells have a thick peptidoglycan cell wall that is able to retain the crystal violate-iodine complex that occurs during staining.

Procedure:

- We have taken a sterile slide to make smear of isolated bacteria and fixed smear by heat After that, we have stained the slide by flooding it with crystal violet for 30 seconds.
- Then poured off access dye and washed it gently in tap water and drained the slide.
- We have added Iodine and leave it on the smear until the 25 minutes.
- Then washed with tap water and drained.
- After that, we have washed with 95% alcohol for 30 seconds.
- Then washed with tap water at the end of 30sec to stop decolorization and drained.
- We have added counter stain with 0.25% safranin for 30 seconds.
- Then washed, drained, blot, and examine under compound microscope at 40Xand 100X objectives.

RESEARCH EXTENSION:

6. INOCULATION OF NUTRIENT BROTH

Material Required:

- Test tubes
- Nutrient broth
- Culture plate
- Incubator
- Distilled water
- Conical flask
- Pured culture
- Autoclave
- Cotton plug
- Pipette
- Shaker
- Autoclave
- Cotton plug
- Aluminum foil

Procedure:

- 5ml NB was prepared by adding 0 .065gm of NB on 5ml of d/w. It was then autoclaved at 121^0C at 15 psi for 15 minutes.
- The bacterial colonies were picked up from pure culture (Streaking plates) showing positive result & inoculated into the respective test-tubes.
- Then the test-tube were kept incubated at 37^0C for 24 hrs in the Shaker incubator.

7. FERMENTATION OF MEDIA

Materials Required:

Conical flask, Cotton plug, Aluminium foil, Casein(0.2gm), NaCl(0.16gm), KH_2PO_4(0.08gm), Dextrose(0.2gm), NH_4Cl(0.6gm).

Procedure:

- Prepare 40ml of production media by adding Casein, NaCl, KH_2PO_4, Dextrose, NH_4Cl.
- This media is prepared in 40 ml of d/w.
- Autoclave the Media.
- Cool it.
- Then inoculate it with 50µl of NB.
- Incubate the media in the shaking incubator for 24 hrs.

8. DOWNSTREAM PROCESSING

Principle:

It is the process refers to the recovery and purification of biosynthetic products, particularly pharmaceuticals, from natural sources such as animal or plant tissue or fermentation broth. It includes following:

1. Extraction of enzyme:

- Extraction is the first step to isolate the enzymes. The technique which employed either to separate enzyme from solid substrate culture or the enzyme release from the inner of microbial cells.
- Enzymes are relatively large molecule which separated based on the size or mass of the molecules which favor for purification of enzyme.
- The enzyme extracted by centrifuge by recovering the cell debris, cell organelles which lead to the partial purification of enzyme.
- The enzyme will move at a definite speed through the solution and occupy the characteristics position and the centrifuge tubes.

Materials Required:

- Optimized media
- Eppendorf
- Micro-centrifuge tube
- Centrifuge
- Measuring cylinder
- Pipette

Procedure:

- First, we have prepared 5ml nutrient broth in test tube and autoclave it at 121^{o}C and 15psi.
- In the laminar, we have taken a sterile loop and inoculated pure culture into the nutrient broth.
- After that, we have incubated the nutrient broth at 37^{o}C for 24 hours.

- After fermentation, we have taken the fermentation media out of the shaker.
- Transfer 1.5ml of media in each 20 micro-centrifuge tube.
- Then, we have centrifuged it at 5000 rpm for 5 min.
- Now we have collected the supernatant.
- Then measured the volume.

Enzymatic Activity Test:

Materials Required:

Na2CO3

NaOH

CuSO45H2O

- 1% KNaC4H4O6.4H2O
- Distilled water
- Test tubes
- Beaker

Lowry Method:

- The principle of Lowry method is based on two reaction leading to color complex formation.
- The first reaction is the reduction of copper ions under alkaline conditions, which forms a complex with peptide bonds.
- The second is the reduction of Folin-Ciocalteu reagent by copper-peptide bond complex, which subsequently causes a color change of the solution into blue with an absorption in the range of 650- 750 nm detectable with a spectrophotometer.
- Lowry greatly increased the sensitivity of the determination by pretreatment with the copper reagent in a basic medium.
- The method used to prepare the following reagents-

Reagent A: 2% Na2CO3 + 0.1%N NaOH for 10ml and was prepared by adding Na2CO3 (0.2gm) and NaOH (0.04gm) in 10ml of distilled water.

Reagent B: 0.5% of CuSO4 + 1% KNaC4H4O6.4H2O for 5ml and was prepared by adding CuSO4 (0.025gm) and KNaC4H4O6.4H2O (0.05gm) in 5ml of distilled water.

Reagent C: Reagent A + Reagent B, 10.2ml of reagent C was prepared by adding 0.2ml of reagent B in 10ml of reagent A.

Reagent D: Folin ciocalteu reagent + Distilled water, 1ml of Folin's reagent was prepared by adding 0.5ml of FC in 0.5ml of distilled water.

Procedure:

We have taken 1ml of enzyme in an Eppendorf and centrifuged at 5000 rpm for 5min.

We have taken 2 test tubes.

The first test tube is kept as blank.

- 0.5ml crude enzyme extract have taken in the second test tube.
- Then added casein (0.5ml) as a substrate to both test tubes.
- Now, we have incubated both test tubes at 37^0C for 20min.
- After that we have added 5ml reagent C.
- Then incubated the test tubes for 10min.
- Now, we have added 0.5ml reagent D Then, incubated test tubes in dark for 30min.
- After that, we have taken OD at 680nm.

2. Salt Precipitation:

- The crude was extracted in present in the solution forming the homogenous mixture with the help of the hydrogen bond so making it difficult for us to extract our protein for mixture.
- In the salt precipitation we use the other compound that have the greater ability to out.
- At low salt concentration the present of the salt stabilizer change the various group solubility of our protein. This is commonly known as salting in.
- However, as the salt concentration is increased a point of maximum solubility is usually reached. Further increase in the salt concentration imply that there is less water available to solubilize protein.
- Finally protein start to precipitate when there are not sufficient water molecules available to interact with the protein molecule. This phenomena of protein precipitation in the presence of the excess of salt is known as salting out.

- Enzyme are protein, enzyme purification can be carried out by following the same set of the procedure as those of the protein, except that some attention must be paid to the consideration the loss of permanent loss of enzyme activity due to the denaturation under adverse condition.

Materials Required:

- Beaker

Magnetic stirrer

Centrifugal tube

Centrifuge

- Micro-pipette

Procedure:

- A beaker containing the crude extract (24ml) was kept on the magnetic stirrer.
- 18gram of ammonium sulphate was added pinch by pinch after every 5-7 min.
- When the total salt gets dissolved then mixture was kept overnight at 4^0C.

3. Dialysis of salt precipitated protein:

- Protein can be separated from small molecules such as salt by dialysis through a semipermeable membrane such as cellulose membrane with pores.
- Protein mixture is placed inside the dialysis bag which is submerged in buffer solution or pure water.
- Molecules having dimensions significantly greater than the pore diameter are retained inside the dialysis bag
- Smaller molecule and ions capable of passing through the pores of the membrane diffuse down their concentration gradient and emerge in the solution outside the bag.
- Dialysis is a technique which operates on the diffusion and osmosis.
- Osmosis is a process in which the solute molecule move from higher concentration to lower concentration by the help of semi-permeable membrane.

To equalize the solvent concentration of both side.

Materials Required:

- Dialysis bag
- Enzyme beaker
- Tris buffer
- Centrifuge tube
- 0.1% SDS

Procedure:

Pre-treatment of dialysis bag:

First the bag have boiled in the 100ml distilled water for 2min.

Then boiled in o.1% SDS for 2min.

Again boiled in 100ml distilled water for 2min.

Procedure:

- The salt precipitated protein in first centrifuged at 10000 rpm for 10min. and discard the supernatant.
- Pellet dissolve in the 100mM Tris.
- The protein is dissolved in the 100mM Tris is packed in dialysis bag, were suspended in the beaker containing 100mM Tris.
- After that, 1 & half hour, the Tris buffer was again changed and the diffusion, kept overnight at 4^{o}C.
- Then in next morning, the buffer was again changed and the protein collected in the beaker.

9. CHARACTERIZATION OF STREPTOKINASE:

1. Effect of Temperature:Material used:

- 5 test tubes
- Incubator
- Freezer
- Water bath
- Calorimeter

Procedure:

- We have taken 1 test tubes as a blank.
- Added 0.1ml extracted enzyme in each other 4 test tubes.
- Added 0.5ml Casein protein in each 5 test tubes respectively.
- Then incubated 1 test tube at room temp., 2 at 37^{o}C, 50^{o}C, 22^{o}C respectively.
- After that added 5ml Reagent C.

Then incubated it for 10 min.

- Then added Reagent D and incubated for 30 min.
- And then obtained O.D at 680nm.

2. Effect of pH Materials Required:

- 5 Test tubes
- Incubator
- Calorimeter
- pH meter
- Strips

Procedure:

- We have taken 1 test tube as blank.
- Added 0.1ml extracted enzyme in each other 4 test tubes.
- Added 0.5ml Casein protein in each 5 test tubes respectively.
- After that set pH of test tube 2, 3, 4, 5 at 5, 7, 9, 11 respectively.
- Then added 5ml Reagent C and incubated for 10min.
- After that added Reagent D and then incubated for 30min.
- After that we have obtained O.D at 680nm.

CHAPTER – 5

RESULTS AND DISCUSSION

1. COLLECTION OF SOIL SAMPLE:

- The soil sample have used for isolation of bacteria which have collected from different locations which are as follows:

Sample	Location
Sample-1	Mushipuliya, Lucknow
Sample- 2	Tedhipuliya, Lucknow
Sample-3	Bijnour, Lucknow

Fig -1: soil sample

2. ISOLATION OF BACTERIA FROM COLLECTED SAMPLE:

Serial Dilution:

- The sample has serially diluted prior to use for the inoculation purpose.
- Serial dilution is a simple yet efficient technique to determine the number of cells or organisms in a concentrated sample.
- Serial dilution has helped to obtain diversified colonies on the nutrient agar media plate.

Fig-2: Serial dilution of soil

3. PURIFICATION OF OBTAINED MIXED CULTURE:

SPEADING:

- The isolation of bacteria has done through serially diluted sample. The sample has inoculated on the nutrient agar media plate by spread plate method and the plate has incubated for 24 hours at 37^0C.
- After incubation the plate show the colonies of bacteria on them.

Fig-3: Spreading plate showing colonies of bacteria

STREAKING:

- In the streak plate method, we have picked a single colony from the spread plate and draw zig-zag pattern on the fresh nutrient agar media plate and incubated for 24 hours at 37^0C.
- To produce isolated colonies of bacteria on an agar plate. This is useful, when we need to separate bacteria inn a mixed culture (to purify particular strain from contaminants) or when we need to study the colony morphology of bacteria.
- Streak plate technique is used to grow bacteria on a growth media surface so that individual bacterial colonies are isolated.
- Streak plate technique is used for the isolation into a pure culture of the bacteria from a mixed population.

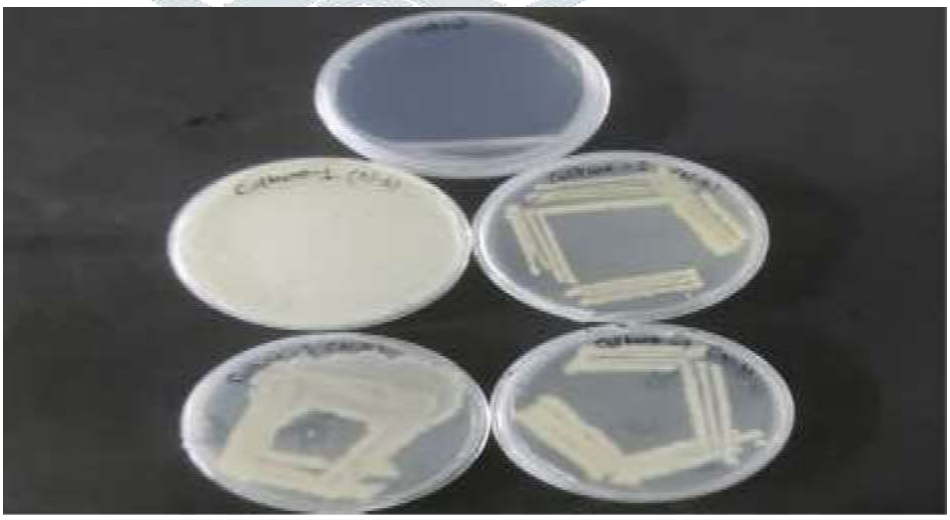

Fig-4: Streaking plate showing pure culture of bacteria

4. SCREENING OF BACTERIA:

- There are two types of screening-
- **Primary screening –** In primary screening, we have observed the growth of bacteria in 24 hours on SM agar media.

Fig-5: In primary screening showing result after 24 hours

- **Secondary screening –** In secondary screening, we have determined the zone of inhibition in 48 hours on SM agar media.

Fig-6: In secondary screening showing result after 48 hour

5. IDENTIFICATION OF BACTERIA:

Gram's staining:

- Gram staining is a technique which is used to differentiate the group of bacteria based on their different cell wall.
- Gram positive bacteria which give the violate stain because of gram positive bacteria

have thick layer of peptidoglycan which retain the crystal violate in the cell wall.

- Alternatively, gram negative bacteria which give the red stain due to thin peptidoglycan layer which does not retain the crystal violate throughout the decolorizing proces.

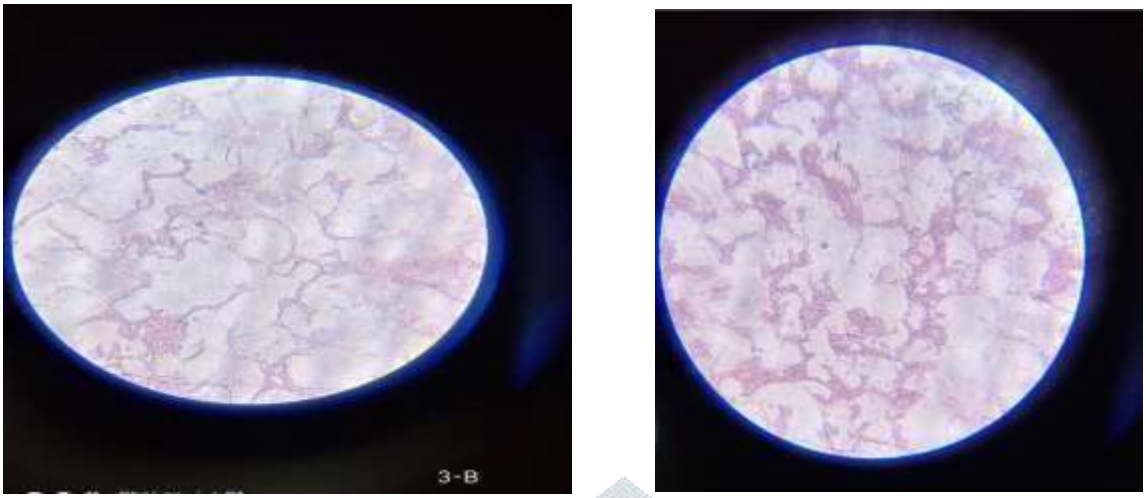

Fig-7: Gram staining of isolated bacteria showing Bacillus spp.

CONCLUSION

In this project work, the enzyme Streptokinase had been isolated from Bacillus species which exhibited fibrinolytic activity. It was concluded from the performed procedures at the laboratory scale that the maximum activity was obtained after 48 hrs. The fermentation media had kept for 48 hrs in the shaking incubator after inoculation in order to grow the bacteria in an efficient manner. The enzyme obtained after downstream processing was only 40-60% pure. The enzyme had exhibited good thrombus degrading property and therefore, may be considered for further scale up and commercial production. The enzyme had high degree of specificity and therefore use as an alternative to treat thrombolytic diseases.

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FUTURE PROSPECTS

Research has focused on structurally modifying streptokinase to extend the half-life, reduce or eliminate immunogenicity and improve plasminogen activation. Any structural change need to be informed by a structural-function analysis of streptokinase domains and this has been the subject of extensive investigation. Structurally modified streptokinase have been produced in several ways including genetic mutation, recombinant DNA technology and chemical or enzymatic modification of native streptokinase. This knowledge has been used to engineer variant of streptokinase.

At last we can say that the future prospect of this study is to scale up the production trials in fermenters. It can further be used for the Identification purpose and the enzyme can be more purified by Column Chromatography.

INSTRUMENTATION

(1). Shaker Incubator:

A shaking incubator work by evenly distributing nutrients throughout your sample. Also, incorporate oxygen throughout the culture sample. Shaking incubator also provide consistence temperature conditions. Finally, they make use of an orbital agitation at different speed. An orbital shaker has a circular shaking motion with a slow speed (25-500rpm).

It can be modified by placing it an incubator to create an incubator shaker due to its low temperature and

(2). Centrifuge:

Centrifuge works on the principle of "Centrifugal Force "where the centrifugal acceleration causes denser substances to separate out (the side –bottom of the tube), by the same taken lighter objects will tend to move to the top (supernatant

(3). Weighing Balance:

An analytical balance (often called a "lab balance") is a class of balance designed to measure small mass in the sub-milligram range. The measuring pan of an analytical balance (0.1 mg or better) is inside a transparent enclosure with doors so that dust does not collect and so any air currents in the room do not affect the balance's operation. This enclosed.

(4). Pipette:

Micropipette is common equipment used in Laboratories. It works on the principle of suction pressure or vacuum.

(5). Autoclave:

An autoclave is a pressure chamber used to sterilize equipment and supplies by subjecting them to high pressure saturated steam at 1210 C (2940F) for around 15-20 minutes depending on the size of the load and the contents.

(6). Laminar Air Flow:

A laminar flow cabinet or laminar flow closet or tissue culture hood or tissue culture hood is a carefully enclosed closet designed to prevent contamination of semiconductor wafers, biological samples or any particle sensitive.

(7). pH Meter:

The digital pH meter (metza mode) was used to determine the pH of the sample. It has an electrode which is to be dipped in distilled water always and thereby in sample solution, pH is displayed on the screen.

(8). Compound Microscope:

The basic principle on which a simple (compound microscope) works is focusing the light rays which come from a definite source onto a specimen which results in the magnified image of the specimen. It comprises of two lenses, objective and eye piece.

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