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*Original Article*

# Production, Screening and Purification of a Fibrinolytic Enzyme (Actinokinase) from *Streptomyces* species

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## Abstract

**Aim:** Cardiovascular diseases associated with thrombosis are one of the main causes of death all around the world. Urokinase, streptokinase, and tissue plasminogen activator are the major thrombolytic agents used to treat thrombosis. The fact that these agents have several side effects and expensive, has driven researchers to search for safer and more economically viable compounds for the treatment of cardiovascular diseases. Thus, the aim of this study is to evaluate the potential of Streptomyces isolated from local soil to produce fibrinolytic enzyme.

**Materials and methods:** Three hundred total of soil samples were collected from different areas in Sudan, the isolates were identified using molecular markers. The enzyme was extracted from fermentation broth. The extract was concentrated by precipitating with (30%- 90%) ammonium sulfate salt, the precipitate fractions were obtained by dialysis membrane and Sephadex G-75 gel

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permeation chromatography. The purified enzyme was characterized in term of pH, temperature and time of storage. The time of complete lysis of blood clot *in-vitro* was also calculated.

**Results:** The study revealed that 68% of the isolates were positive for thermophilic *Streptomyces sp*. (41%) of the positive isolates could product actinokinase. Identification of the isolate confirmed it is a thermophilic *Streptomyces megasporus*. The pH had dropped toward acidic (5.7) at the fermentation broth as an indicator of successful actinokinase production. Purification of actinokinase could produce 5.7mg/mL of total protein, with a specific activity of 101U/mg$^{-1}$, and yield of (70%). The enzyme was stable at a broad range of pH ranging from 6 to 10, and had high thermostable activity at 37ºC for 6 months. SDS-PAGE showed that the molecular mass of actinokinase was approximately 35 KDa. The enzyme kinetic revealed that the $V_{max}$ and $K_m$, were found to be 8.02 µmol /ml/min and 0.56 µmol /ml/min, respectively. *In vitro* fibrin degradation showed that complete clot lysis was attained within 20 minutes.

**Conclusion** The study findings indicated that the isolates Streptomyces from local soil in Sudan were capable of producing fibrinolytic enzyme actinokinase that specifically act on fibrin and could lyse blood clot within 20 minutes.

**Keywords:** Cardiovascular diseases, Fibrinolytic enzyme, Actinokinase, , *Streptomyces spp*. and thrombosis

## INTRODUCTION

Cardiovascular disease (CVD) and circulatory diseases are now recognized as the leading causes of death in the world. In 2013 there were more than 54 million deaths (95% uncertainty interval [UI], 53.6-56.3 million) globally and 32% of these deaths, or 17 million (95% UI, 16.5-18.1 million), were attributable to CVD. The majority of these CVD deaths were attributable to ischemic heart disease IHD [1]. Over the years, thrombolytic therapies via injecting or orally administrating thrombolytic agents to lyse thrombi in blood vessels have been extensively investigated [2] which is a secretory serine protease, that is generated from inactive precursor plasminogen via limited cleavage by plasminogen activator (PA). Worldwide, over (29%) of the total mortalities are due to thrombosis. By the year 2020, The prevalence of cardiovascular diseases is progressively rising and remains at the top of 15 leading diseases as shown by the global mortality projection for 2002 to 2030 [3]. The increase in heart-related diseases has increased the significance of fibrinolytic enzymes from microbes [4]. Fibrinolytic enzymes, such as urokinase [5], streptokinase [6], staphylokinase [7] and tissue plasminogen activators have been widely used in the treatment of thrombosis. These enzymes are very expensive [8] and sometimes have immunologic side effects [9], which has led to a search for novel fibrinolytic enzymes as therapeutic agents in thrombosis, so the fibrinolytic enzymes are of interest and the search for new enzymes continues. Thermophilic actinomycetes are producers of thermostable enzymes. These enzymes exhibit several advantages due to high processing temperature which leads to increased reaction rates, solubility and contamination chances are less. Fibrinolytic enzymes lysis fibrin, the major protein component of blood clots. An ideal thrombolytic drug should have the following

characteristics: reasonable cost, has no antigenicity, no effect on blood pressure, pro coagulant effect, lower occlusion rate and low incidence of intracranial and system bleeding. It should be fibrin selective, effective in dissolving older thrombi and resistant to plasminogen-activator inhibitor. Moreover, an ideal drug should be suitable to administer as an intravenous bolus and should execute reperfusion in 100% of patients and rapid and complete coronary flow [10], but these agents have some disadvantages such as hemorrhagic effect, immunogenicity, and high cost, due to which their uses are limited. There is still scope to search new agents which overcome these drawbacks [11]. Therefore, there is a continuing need to screen more organisms to obtain strains capable of producing a new, potential source and high yield of actinokinase with minor side effects. This study deals with production of a purified new fibrinolytic enzyme from a thermophilic *Streptomyces megaspore*, isolated from soil, along with its efficiency towards dissolving blood clotting.

**Materials and Method**

**Sampling**

Three hundred soil samples (300) were collected randomly from different areas in Sudan. Samples were collected at 5-10 cm depth after removing approximately 3 cm of earth surface and were enriched with $CaCo_3$ [12]. After removing the surface, the soil gathered in sterilized plastic bag then transferred into sterilized bottles to the laboratory according to the standard soil collection method [13]. Each sample weighed 200 g and its collecting location had a distance of 500 meter separated from others and stored at - 4$^{o}$C refrigerator before used.

**Isolation of microorganism**

Ten gm of each soil samples were mixed with 90ml sterile water, and were shaking at 180 rpm for 30 min. After standing for 30 min. the supernatant was separated and serially

diluted with sterile water to $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ $10^{-6}$ times. Then 200 µl soil suspension of the dilutions with, $10^{-4}$, $10^{-5}$ $10^{-6}$ were drawn on by pipette, and spread on the screening medium plates. Each dilution was poured in sterilized petri-dishes which prefilled with selective media (Glucose yeast extract peptone agar (GYP) containing (g/L.): 5gm of glucose, 5gm peptone, 3gm yeast extract, 20gm Agar and completed to 1000 ml of distilled water, the pH was adjusted to 8.0 with 2M NaOH, after gently rotating the plates they were incubated at 55°C for 7 days. Colonies showed morphological characteristic of *Streptomyces* that appeared in the incubated plates, were re-cultured in nutrient agar. [14]

**Primary screening of the enzyme**

**The enzyme was screened** on the screening medium plates, which were composed of: peptone 5gm, yeast extract 3gm, bacteriological agar 18gm, and skim-milk 250ml. The pH of the medium was adjusted to 7.4 [15]. The plates were incubated at 55°C for 48 hrs., where a clear zone created by the hydrolysis of skim-milk was observed indicating the presence of fibrinolytic-producing strains. Each colony having a clear zone on the plate was streaking on the plate of re-screening medium containing fibrin 5gm, ammonium sulfate 2gm, $CaCl_2$ 1.0gm, $K_2HPO_4$ 0.1gm, $KH_2PO_4$ 0.1gm, $MgSO_4 7H_2O$ 0.2gm, bacteriological agar 20gm per liter, with the medium pH of 8.0 [16]. A small amount of each colony was transferred by inoculating loop into a test tube including 10ml liquid growth medium (Without bacteriological agar), and cultured with shaking at 140 rpm at 55°C, and the fermented broth which filtered out the bacteria cell by membrane of 0.22µm was used to determine the fibrinolytic activity. To assure that the locally isolated thermophilic Streptomyces are producing actinokinase, drop in pH was used as quantitative method.

**Enzyme activity and growth profile**

Extraction of the crude enzyme from the fermentation broth was carried out. *Streptomyces* was grown on selective media containing GYP medium at pH of 8.0, and the medium was sterilized for 15 mins and cooled to room temperature. The fermentation conditions were carried out at 55 ºC for 24hrs. Under a controlled condition, the agitation was kept at 140 rpm in an orbital shaker incubator. From the five fermentation flasks, a sample was taken at zero time and then samples were removed periodically every 6 hours for 24 hrs. to measure the enzyme activity at 405 nm and at 600 nm for bacterial growth using U.V spectrophotometer. The cells were harvested by centrifugation at 4°C and agitated at 10,000 rpm for 10mins. The free cell supernatant was used as a crude enzyme. [14]

The activity of actinokinase (Umole/min) = (Increase in Ab of fibrin at 405 nm)

(Molar extinction of fibrin at 405 nm) [17]

Actinokinase activity U is defined as the amount of actinokinase that catalysis the formation of 1.0 µmol of substrate fibrin /min.

**Molecular characterization of the microorganism**

**DNA extraction**

DNA was extracted from *Streptomyces sp.* using boiling method where nutrient broth was prepared according to HIMEDIA procedure and incubated overnight at (37° C), loop full of cultured bacteria was suspended in each falcon tube containing the broth and again incubated overnight, the mixture was centrifuged at 1000 rpm for 10 min, the supernatant layer was discarded then (200µl) water for injection was added to the precipitate and boil at 95ºC for 20 mins in the water bath, after 20 mins the tube was directly thermally shocked and placed in the ice to cool down for (10 min.), again centrifuged but this time the ice was put on

the supernatant layer which contain the extracted DNA, the were placed in Eppendorf tube to be ready for the PCR [18].

**Polymerase Chain Reaction**

The PCR was performed by processing the extracted DNA from *Streptomyces* with 16s r-RNA gene' specific set of primers

Forward primer: (5-´GGTGGCGAAGGCGGA-3´).

Reverse primer:(5´-GAACTGAGACCGGCTTTTTGA-3´). [19]; [20]; [21].

The reaction was performed in (20µl) volume using Maxime PCR series "Next Generation of Premix", the volume included: 1µl of forward primer, 1µl of reverse primer, 2 µl of extracted DNA and 16 µl distilled water. The vials were vortexed and amplified in thermo-cycling conditions using PCR machine PeQlab where program was initiated by denaturation for 5min at 95ºC, PCR comprised 30 cycles as follow 45 seconds at 94ºC, 45 secs at 65 ºC and 1 min at 72 ºC, and finished with an extension step of 10 min. at 72 ºC. The gel was prepared for analyzing amplified product for gel electrophoresis

**Purification of crude actinokinase**

**Preparation of seed culture**

The 50ml GYP medium pH 8.0 containing 0.02% CaCl2, inoculated with 25 µl spore suspension (1.7×108 spores) and grown at 55°C for 18 hrs. The 5% seed culture was used as an inoculum for 1-L.

**Ammonium sulphate precipitation**

The extracted enzyme was precipited with 30% saturated ammonium sulphate. Then centrifuged at 6000rpm for 15min. Further the supernatant layer was precipitated by 60% ammonium sulphate, then centrifuged at 6000rpm and the last volume of the solution was precipitated with 90% ammonium sulphate and centrifuged at 6000 rpm (Encore bio sulfate calculation) the desired protein

remained at the bottom of the glass flask as sulfate pellets. The method of concentration by ammonium sulphate precipitation and water removal involves osmotic force.

**Filtration by dialysis membrane**

Dialysis membrane size 5 inf Dia 24/32'' - 19.0 mm: 30 M (Approx). MW CO ranged from 12-14000 Daltons was used. The membrane was tightly closed and placed in 400ml 0.1M potassium phosphate buffer(PB) pH of 8.6 at 4°C . Then filled with 4.0 ml ammonium sulfate extract and passed through the 50% sucrose solution all overnight. The buffer was changed every 2 hours and the dialysate was tested using sliver nitrate solution to ensure that it was free of ammonium sulfate and kept for further experiments. Proteins molecular weight higher than 14 KDa will be trapped on the surface and that with molecular weight less than 14 KDa will pass through the tube.

**Anion exchange chromatography**

The filtrate was loaded on the UNO sphere strong Anion-Q column (41 h×1.6 cm diameter) of the bed height 20 cm with 13g of SephadexG-75. According to the manufacturer specifications the exclusion limit for the Sephadex C-75 and Sephadex G 75 is ~200,000 KDa. The column tap at the bottom was closed with cotton wool and inert sand to prevent the loss of Sephadex, then, sephadex was wetted by mobile phase. The column was filled with mobile phase, allowed to run through cotton wool, and sand to remove air bubbles. Sephadex was allowed to settle gently so they would pack tightly. The column was equilibrated with two column volume of the 10 mM Tris buffer of pH of 7.8 then the cell- free broth was passed through the column with flow rate
of 1.5 ml/min. The column was then washed with 10 mM Tris buffer of pH 7.8 and then eluted with 10 mM Tris buffer containing

0.5 to 1.0 M NaCl and of pH 8.0. The first fraction was discarded while fractions 2, 3 and 4 were collected and enzyme activity was measured. Estimation of the protein concentration was assayed by two different methods: absorption at 280 nm and Lowry Assay using BSA as the standard [22].

**Molecular weight determination of protein by Sodium - dodecyl Sulphate - polyacrylamide gel (SDS - PAGE.**

The purity of the purified enzyme of the fractions recovered from the column and its molecular mass were determined by SDS – PAGE using laemmLi system. Laemmli (1970). After preparation of the gel tank, components of separating gel (pH 8.8) were mixed in a 50 ml flask, TEMED (Tetra methyl ethylene di amine) and APS (Ammonium per sulfate) were added at the end, they initiate polymerization. After pouring TEMED, the gel solution was immediately transferred to the gel cassette after mixing until it reached one cm away from the bottom of the comb. To ensure the smoothness of gel surface, a drop of isopropanol was added at the top of the gel. It took almost 30 minutes for polymerization of the gel when the separating gel was set, it was poured off the overlying isopropanol and washed with water. Once more, TEMED and APS were added in the end. After mixing, the solution was transferred to the separating gel till it had reached the cut-way edge of the plate. A comb was placed in this solution and left to set. This took about 20 minutes. The comb was carefully removed from the stacking gel. Wells formed were washed with distilled water to rinse out unpolymerized gel. Rubber was removed from the gel plates and the cassette was assembled in the electrophoretic tank. The top reservoir and the bottom tank were filled with Tris-Glycine-SDS buffer system (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3) developed by [23] Then it was checked for

any leaks, and check the apparatus to remove any bubbles caught under the gel. The fraction 5 µl were loaded on SDS-PAGE with 3 pl of protein ladder (Gang Nam-stain TM) and run at a constant current of 3.0 mA and 140mH volts. Electrophoresis was performed in a mini-Protein Dual slab cell. The samples were run till the dye front reached the bottom of the gel. The proteins were visualized by staining with (0.25% Coomassie Brilliant Blue R250 in 50% methanol: 10% acetic acid: 40% distilled water). The gel was kept in the staining solution overnight and subsequently de-stained in (5% methanol: 10% acetic acid: 85% distilled water) until the bands were almost clear. Finally, the gel was pictured using a regular smart phone camera, and Image software was then used to analyze the images of the gels. SDS molecular mass marker consists of different color each had a particular molecular weight starting from 5.0 kDa to 245.0 kDa.

**Characterization of the purified actinokinase Effect of temperature stability on actinokinase activity**

Purified actinokinase temperature stability was determined by assessing the enzyme activity at different temperatures for 15 min ranging from 35, 45, 55, 65, and 75° C. at pH 8.0. Thermo stability was determined by measuring the residual activities after incubating the enzyme for 30 min at different temperatures and compared with the activity of the enzyme solution kept at 4$^{o}$C as a control.

**Effect of pH stability on actinokinase activity**

pH stability of the purified actinokinase was determined at different pHs, ranging from 5.0 – 9.0 using citrate phosphate buffer pH 9.0, sodium phosphate pH 6.0-8.0, and Tris Cl pH 5.0. Stability to pH was determined by measuring the residual activity after exposure to different pHs. Enzymatic activity for each

one was measured and the highest remaining activity is expressed as 100% of the activity.

**Effect of different storage times on actinokinase activity**

The stability of the purified actinokinase storage time was determined by measuring the residual activities after storage for 6 months at temperatures 35$^{o}$ C. The enzyme solution in 10 mM phosphate buffer pH 8.0, and compared with the activity of the enzyme solution (as 100%) kept at 4$^{o}$C as control.

**Determination of kinetic parameters of purified actinokinase**

**Monod Equation fit to observed data**

To determine $V_{max}$ and $K_{m,}$ different substrate concentration was used (0.0 - 0.92 µ mol). The data was plotted in arithmetic way, where X - axis represent substrate concentration and Y– axis represents the initial calculated enzyme velocity using Monod equation as seen in Figure (1).

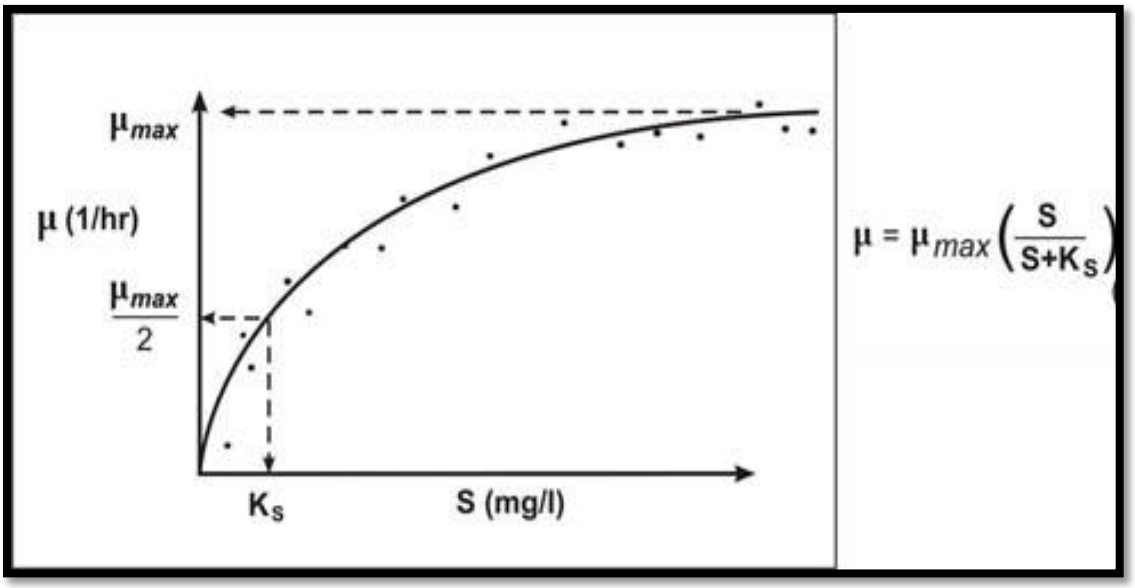



**Figure (1): Monod Equation fit to observed data [24].**

The illustrated $V_{max}$ and $K_m$ ($K_m$ is the substrate concentration at half maximal velocity $V_{max}$ / 2) are used to calculate the $V_0$ using the following Monod equation:

$$v_0 = \frac{V_{max}[S]}{K_m + [S]}$$

$V_0$: The initial reaction rate.

$V_{max}$: Maximum reaction rate, i.e. reaction rate when the enzyme is saturated with the substrate.

$K_m$: Michaelis constant, which is only determined by the nature of the enzyme and has no connection with the concentration of the enzyme. It could be used to identify different enzymes.

[$S$]: Substrate concentration.

**The line weaver – Burk reciprocal plot**

The line weaver – Burk reciprocal plot for (1/S) and (1/μ) is widely used primary diagnostic plot as seen in the Figure (2) below.

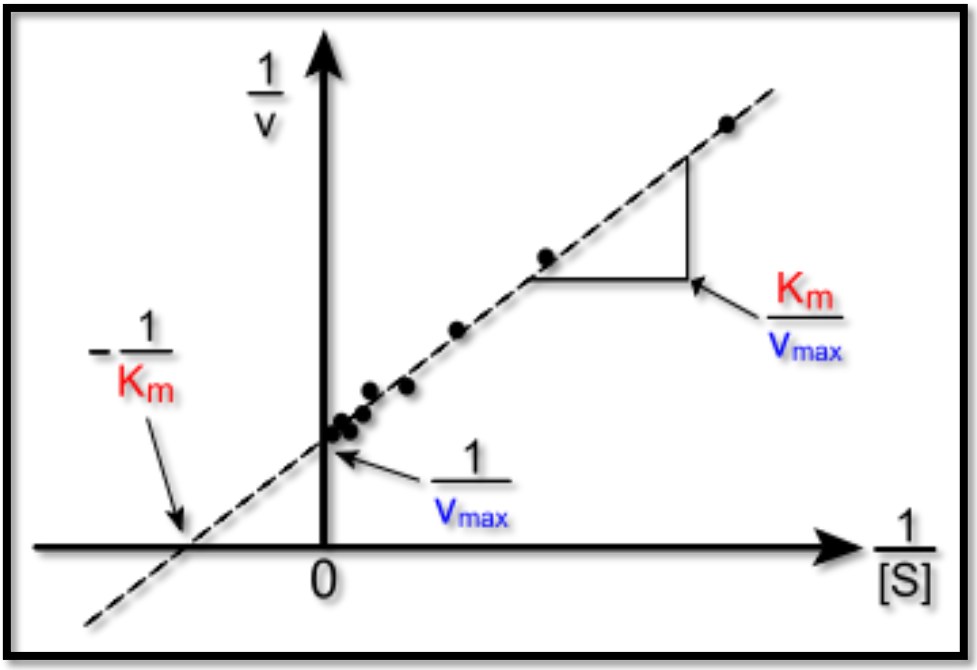



**Figure (2) Example of a Lineweaver-Burk plot [25].**

***In- vitro* Fibrin Degradation**

10 mg of clotted blood spread on slide and test tubes then a 20 µl of the purified enzyme was added, then the time was calculated until clot completely lyses, and the same steps on glass tube.

## Results

### Sample collection and isolation of *Streptomyces?? Or The growth rate of Streptomyces*

Two hundred and seventy isolates (270) out of 300 (90%) of sample were positive thermophilic *Streptomyces spp.* by the growth of the microorganism in GYP as a selective medium and incubated at 55°C. The growth of the isolate in GYP media indicating the percent of thermophilic *Streptomyces spp.* as shown in Figure (3).

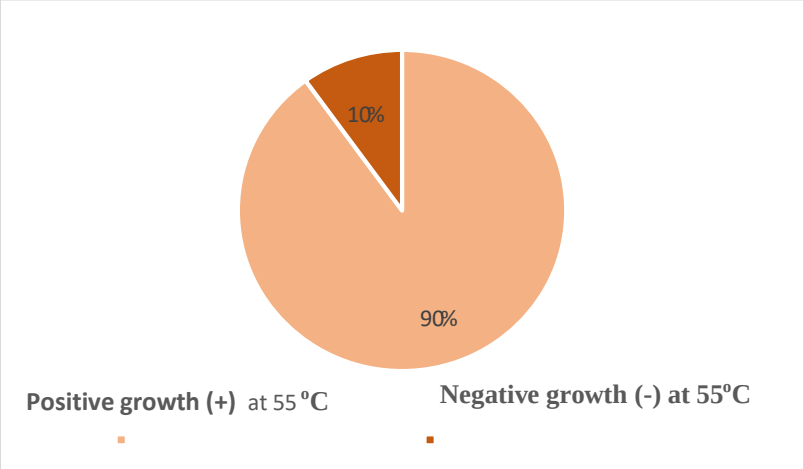



**Figure (3): Percentage of *Streptomyces* grown in GYP medium at 55°C**

90% = (+ve) thermophilic *Streptomyces sp.*

10% = (-ve) no growth of thermophilic *Streptomyces sp.*

On the other hand 186 out of 270 isolates (68%) of the samples showed gram positive filamentous bacteria while (32%) showed no growth as shown in (Figure 4). The specific morphology of the isolate with dark yellow is indicating that the isolate is mainly *Streptomyces spp.*

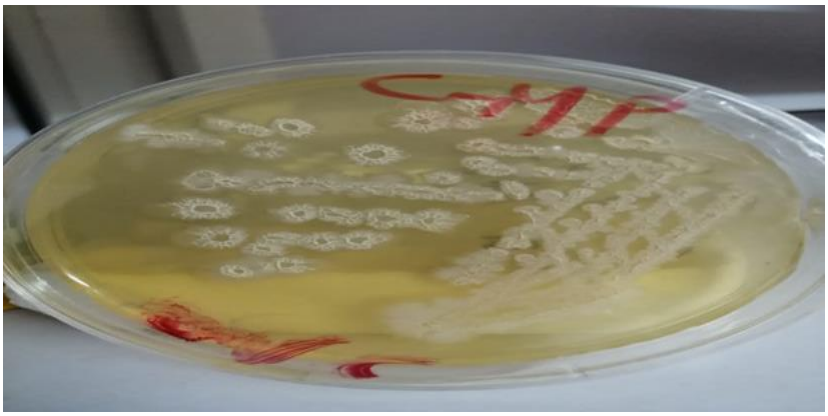
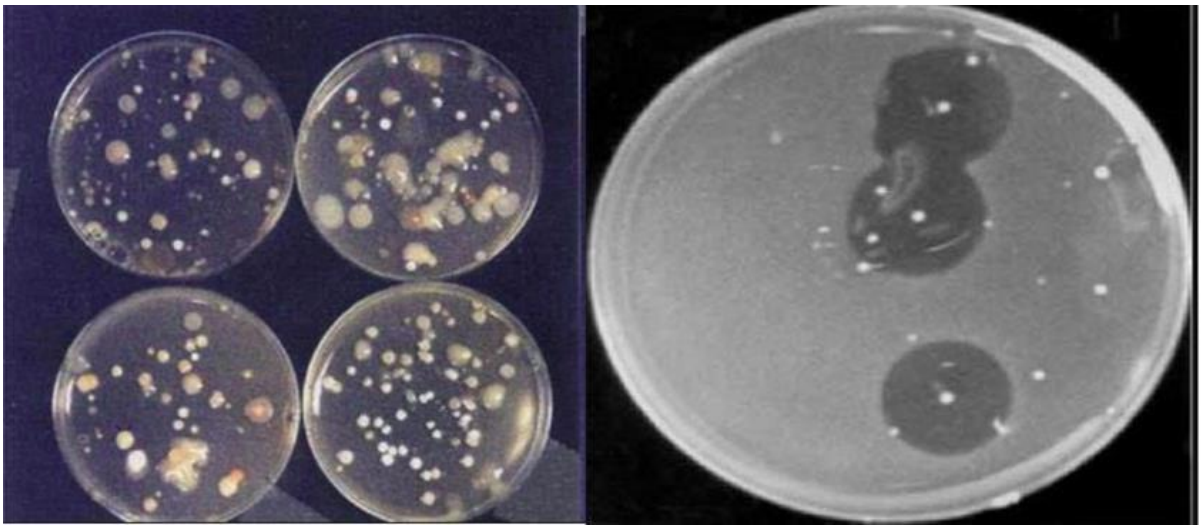



**Figure (4): Pure colony of Streptomyces *spp*. in GYP medium**

**Primary screening of the enzyme**

The measurement of the dimension of the clear zone around each organism indicates fibrolytic activity. It is an excellent qualitative performance method. Figures (5 and 6) below show the clear inhibition zone of the samples indicating that the heamolytic effect of the crude enzyme range from 4-25 mm. Seventy-eight (78) out of (186) isolates (41%) of the positive *Streptomyces* were actinokinase producer using fibrin heamolysis zone by plate fibrin method.



**Figure(5) Isolation and screening of bacteria producing fibrinolytic enzymes by fibrin plate method**

Left: Screening of soil samples at GYP media;

Right: Screening of soil samples using Fibrin Plate Method

Explain figure 6

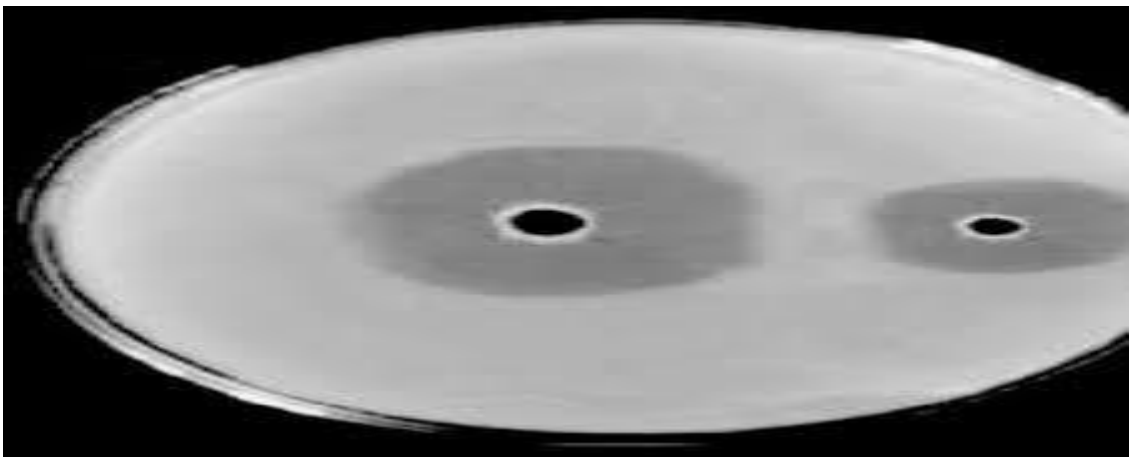


**Figure (6) Screening *Streptomyces sp* producing fibrinolytic enzymes by the clear zone**

**Enzyme activity and growth profile**

Figure (7) below shows the growth profile of *Streptomyces.* The bacterial growth increased gradually from zero time as log phase till 18 hrs. Maximum growth (1.45) was obtained within 18 hr. of incubation at pH 8.0. While the activity of the enzyme reached a maximum (0.8 U/ml) within 18 hr. using fibrinolytic activity, and plateau off for the 6 hr., beyond 18 hr. of growth no increase in enzyme activity was recorded. The two profiles were paralleled .The Initial media pH at zero time is of (8.0) after incubation. It is shown that the pH of fermentation process dropped towards acidic side 5.5 after 18 hr. of incubation as in Figure (8).The enzyme was an actinokinase as evidenced by drop in pH. The actinokinase enzyme resists to broad pH range**.**

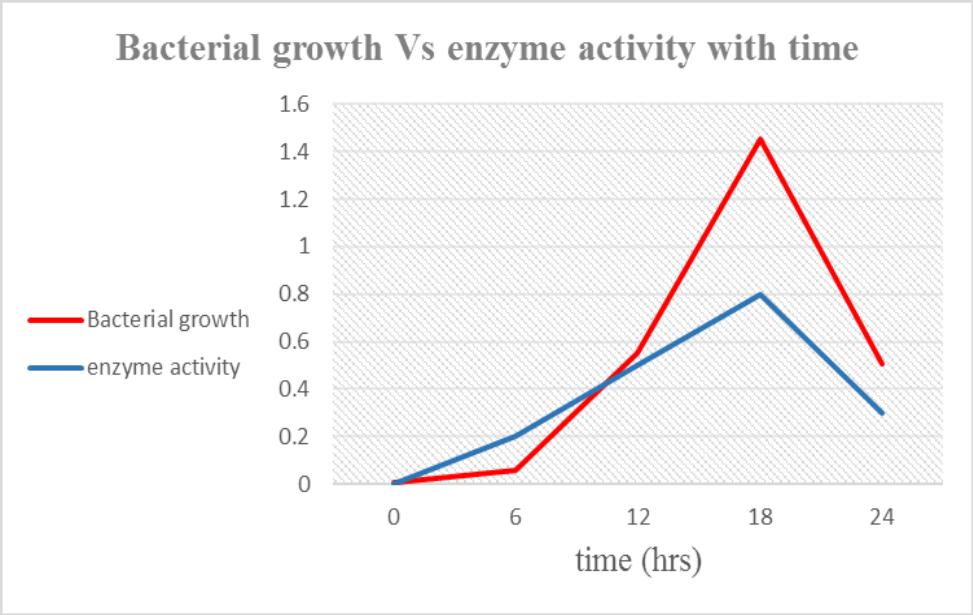



**Figure (7) Crude actinokinase activity and growth profile curve during incubation time**

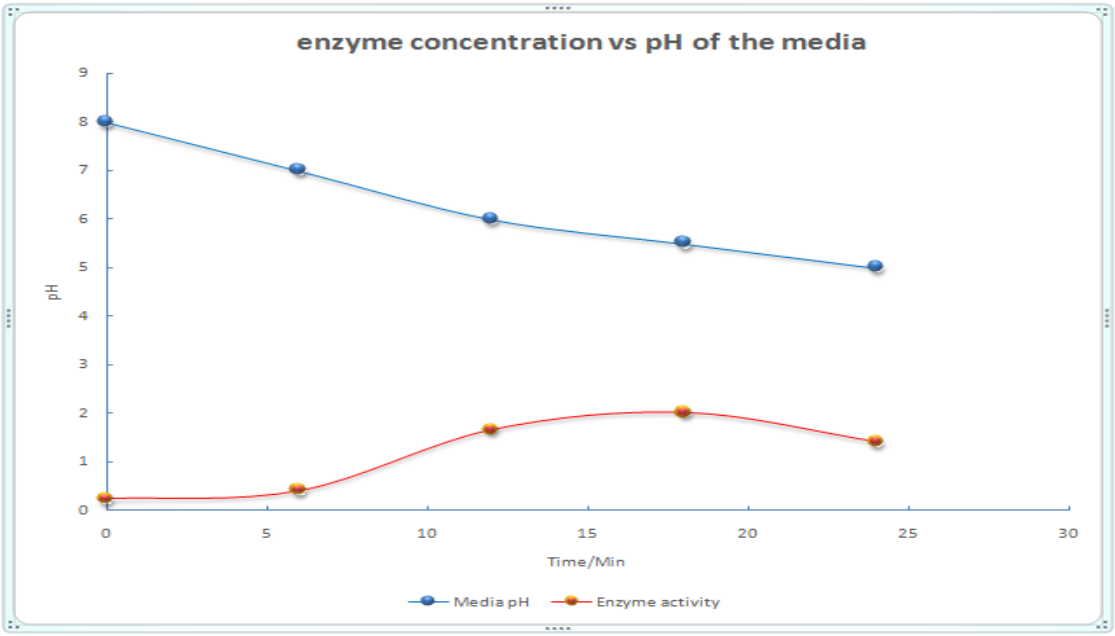



**Figure (8) The activity of crude actinokinase against pH of the media during incubation time**

**Molecular characterization of the microorganism**

Identification by 16S Ribosomal RNA (r-RNA) gene. DNA was being extracted by physical thermal shock to lysis cell membrane and expose DNA, it has been centrifuged in-order to separate clumped cellular debris (Lipid, protein and RNA) and then introduced into PCR machine in-order to amplify single copy of DNA segment to generate thousand to million copies of DNA sequence, by amplifying 25 samples (36%) of total samples, where the size of DNA fragments that have been amplified was 600 base pair which determined by gel electrophoresis using DNA ladder (Molecular weight marker) that contains DNA fragment of defined length for sizing the bands of PCR products, visualized under UV light and photographed using gel documentation system as shown below Figure(9).

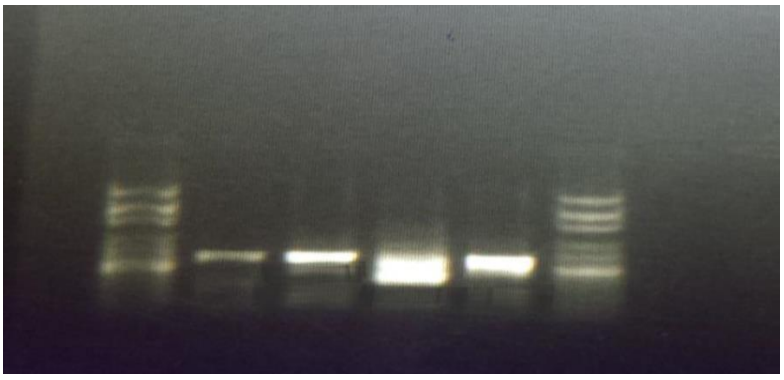


**Figure (9): PCR amplified products run in an agarose gel using ladder as a marker and visualized under UV light.**

**Lane (1 and 6)**: DNA marker, **Lane (2, 3, 4, 5)**: (+ve) Sample

**Purification of actinokinase**

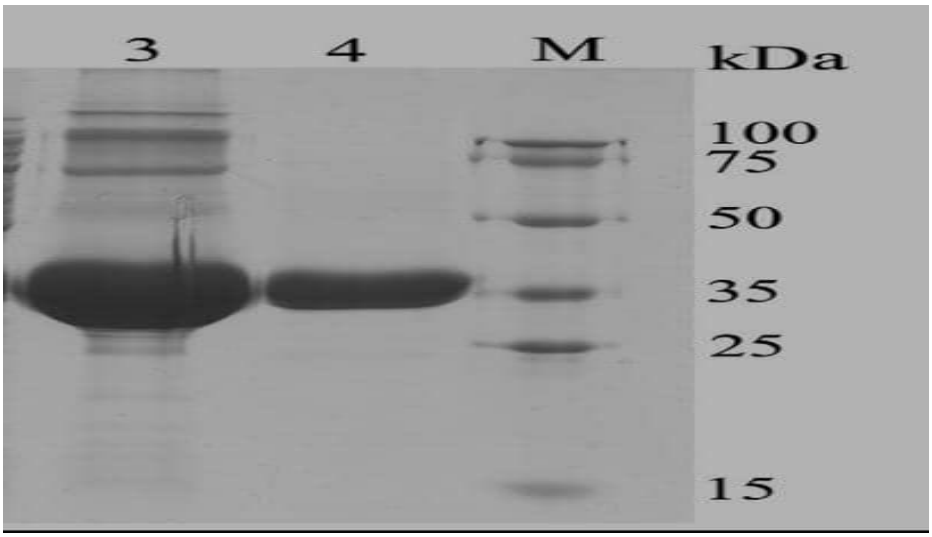
Purification of the actinokinase enzyme was achieved by ammonium sulphate precipitation followed by dialysis technique and chromatography. The enzyme activity of each purification level was calculated as the substrate absorbance increased at the protein –substrate mixture. The total protein of the crude actinokinase was 420.0 mg and the total activity was 824.0 U/ml, while the total protein fractions in anion exchange column was5.7mg/ml and the total activity was 578.0 U/ml with enzymes specific activity of 101.4 U/mg$^{-1}$ and a yield of 70.1% and 53%-fold (Table1).

**Table (1): Summary of actinokinase purification**

| Fractions | Total volume (ml) | protein concentration (mg) | Total Protein (mg/ml) | Enzyme activity (U/ml) | Specific activity (Umg$^{-1}$) | Yield % | Purification Fold% |
|---|---|---|---|---|---|---|---|
| **Ammonium sulphate precipitation** | **700** | **0.63** | **420** | **824** | **1.9** | **100** | **1** |
| **Filtration by dialysis membrane** | **100** | **0.57** | **39.5** | **748** | **18.9** | **90.7** | **9.9** |
| **Ion exchange chromatography** | **17.5** | **0.44** | **5.7** | **578** | **101.4** | **70.1** | **53** |

**Molecular weight determination of protein by Sodium - dodecyl Sulphate -polyacrylamide gel (SDS – PAGE)**.

The activated fractions were collected and concentrated, the concentrated enzyme was subjected to SDS-PAGE to test the purity. The molecular weight of actinokinase was estimated to be 35 kDa. As shown in the Figure (10), a single band can be observed at the gel, indicating the purity of actinokinase




**Figure(10) The Bands of Actinokinase Enzymes Using 10% Sodium Dodecyle Sulphate Polyacramide Gel (SDS -PAGE)**

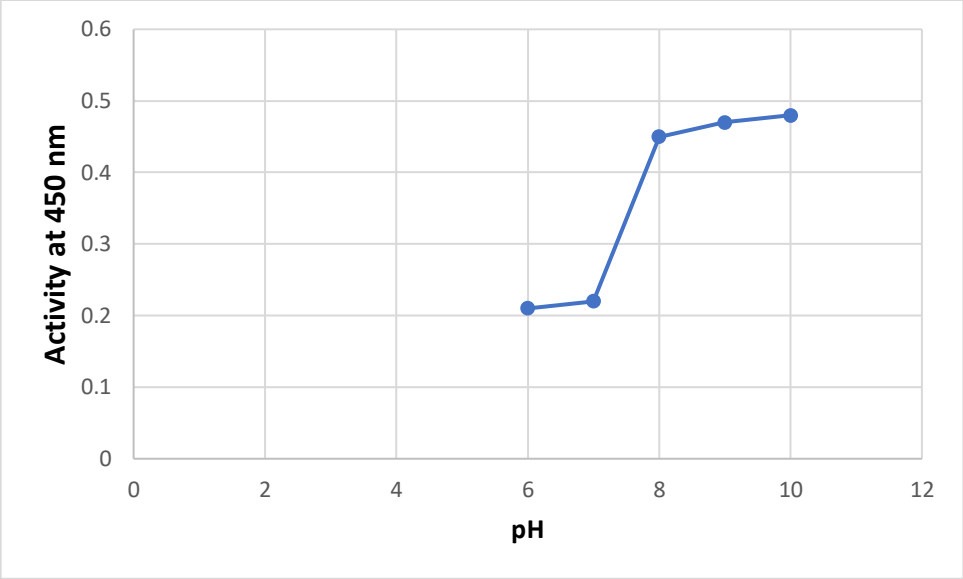
3: Crude enzyme

4: Signal band of actinokinase

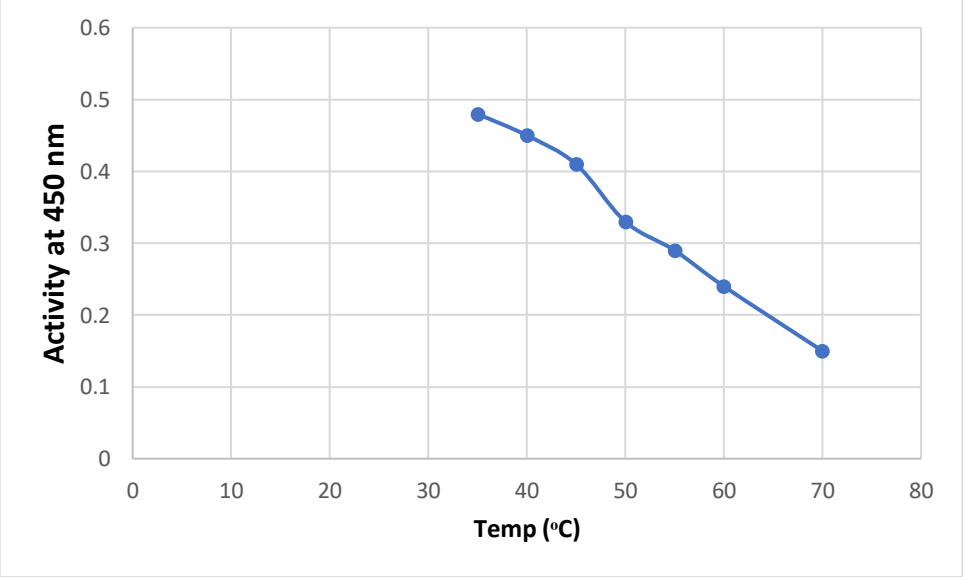
M: Protein marker

**Characterization of the purified actinokinase**

As indicated in Figure(11,12,and 13) pH had a great influence on activity and stability. pH increased gradually from 8.0–10.0. The residual values revealed that the enzyme retained 51% activity even at pH 6.0. The enzyme was stable for 6 months at 40 $^{o}$C., 25% of the activity was reduced after storage at 55$^{o}$ C. More than 60% activity was lost within 3 months and gradually decreased linearly with the storage time. At 70$^{o}$C most of the activity was lost, within one month, only 22% activity was retained.




**Figure(11) Effect of pH on the activity and stability of Actinokinase**




**Figure(12) Effect of temperature on activity and stability of actinokinase**

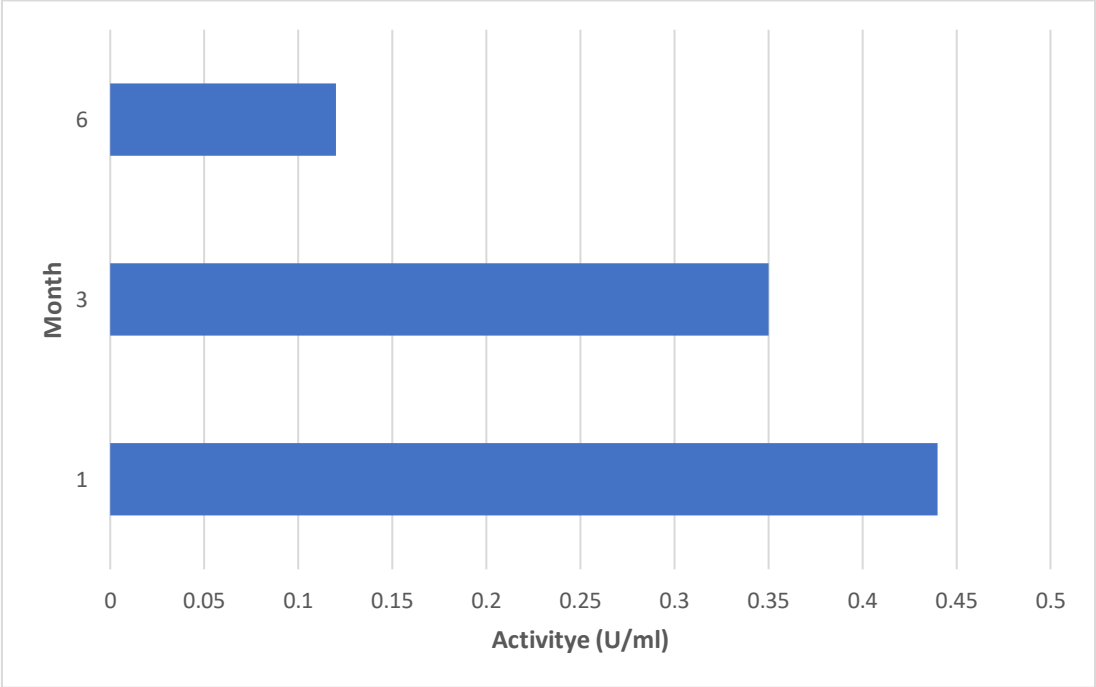



**Figure (14) Effect of storge time on activity and stability of actinokinase**

**Actinokinase kinetics study**

Regarding the enzyme kinetic activity, indicated that the $V_{max}$ and $K_m$, were found to be 8.02 µ mol /ml and 0.56 (µ mol /ml/min) respectively. Generally, determination of $V_{max}$ and $K_m$ was explained by the fact as small error in the determination of $V_{max}$ are magnified where reciprocal are taken . For practical purposes, $K_m$ is the concentration of substrate which permits the enzyme to achieve half $V_{max}$. An enzyme with a low $K_m$ has a high affinity for its substrate, and requires a greater concentration of substrate to achieve $V_{max}$. In the study, it was observed that actinokinase fits the Michaelis– Menten[26] , kinetic model, Line weaver–Burk [5] plots at different fixed concentrations, were linear and plotted of straight lines that intersect X-axis at the same point, $K_m$ was calculated to be (0.56), the slope was 14.33, and V

$_{max}$ 8.02 m moles L/ sec. Table (2) and Figure (14).

| S (substrate concentration (µ mol/ml) | U Enzyme unit (µ mol/ml/min) | 1/S | 1/µ |
|---|---|---|---|
| **0** | **0** | **0.0** | **0.0** |
| **5.0** | **0.23** | **0.2** | **4.5** |
| **10.0** | **0.29** | **0.1** | **3.5** |
| **11.1** | **0.32** | **0.09** | **3.1** |
| **20.00** | **0.37** | **0.05** | **2.7** |
| **33.33** | **0.41** | **0.03** | **2.4** |
| **50.00** | **0.55** | **0.02** | **1.8** |

**Table (2): Data used to Plot the Line Weaver – Burk Reciprocal Plot Graph**

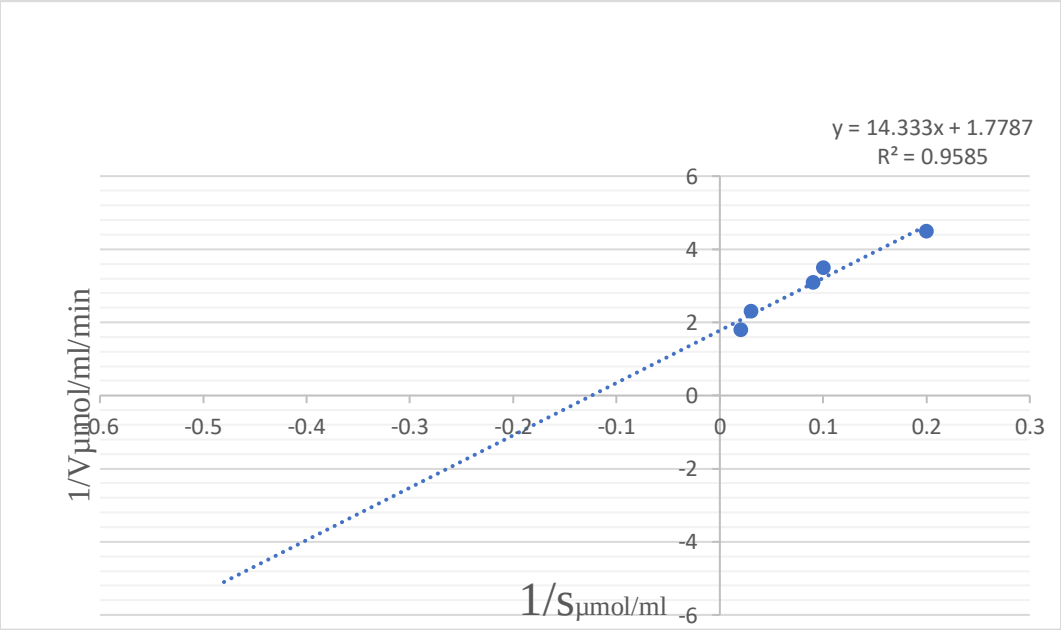



**Figure: (15)The Lineweaver–Burk plot for actinokinase**

M =14.33

B =1 .77

$V_{max}$=0.56

$K_m$ =8.02

### *In- vitro* Fibrin Degradation

Figure (16) below showed that the clot of blood start to lysis slowly at 5 min and began to dissolve with the time until complete lysis occurs within 20 min.

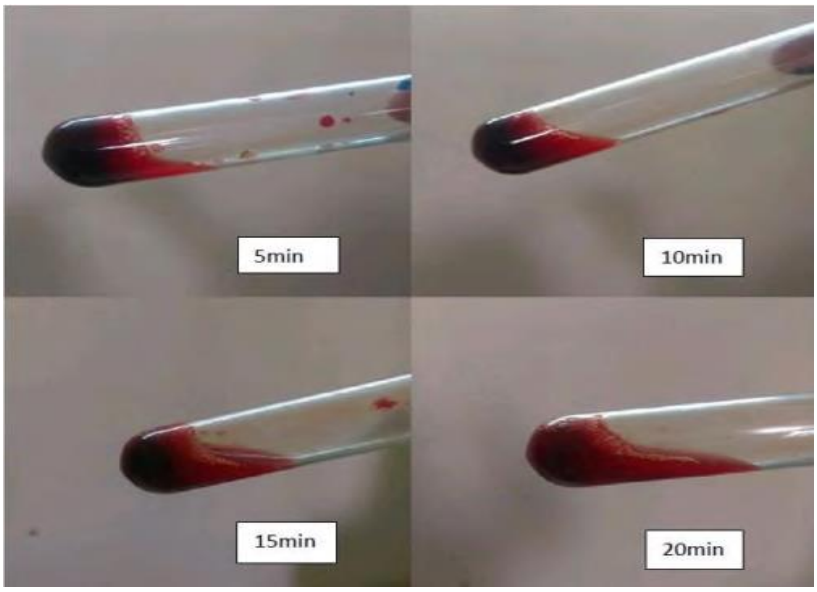



**Figure (16):** Time profile of thrombolytic activity of actinokinase at: 5 min, 10 min, 15 min and 20 min.

Increasing of clot lysis with time, indicated that the action of actinokinase enzyme is different than that of streptokinase and urokinase in that it's a plasminogen independent where it directly cleaves the fibrin network and completely lysis the blood clot within only (20 mins), While Urokinase and Streptokinase need longer time, 40 mins

and 80 mins respectively [27],[28] for clot lysis as shown in Table (3).

| Time (min.) | 5 | 10 | 15 | 20 | 30 | 40 | 60 | 80 | Reference |
|---|---|---|---|---|---|---|---|---|---|
| **Actinokinase % clot lysis** | **22** | **65** | **89** | **100** | | | | | Findings of this study |
| **Urokinase %clot lysis** | **25** | **50** | **60** | **80-90** | **95** | **100** | | | [5], [27], [40] |
| **Streptokinase % clot lysis** | **5** | **5** | **10** | **15** | **40** | **40** | **90** | **100** | [6], [28],[41] |

**Table (3) Comparasion between *in vitro* clot lysis of actinokinase with known fibrolytic enzymes**

**Discussion**

The study revealed that 90% of the total isolates (300) were positive thermophilic bacteria isolate, 68% of them were gram positive filamentous *Streptomyces* as described early. 41% was found to be that actinokinase producer according to the clear zone around *Streptomyces* colonies when cultured in fibrin plate agar media, this production rate is higher than what was reported by [29] that only 14% of the total isolates was found to be actinokinase producer, and less than that of [1] who stated that, 50% from total isolates was found to be thermophilic *Streptomyces* according to morphological and biochemical characteristics which grown at the same conditions, that may be due to different habitat and type of soil. Recently, [30] reported fibrinolytic potential of exopolysaccharide synthesized by *Pseudomonas spp*. from the deep-sea sediments. In this study the local isolate Streptomyces could produce a fibrinolytic enzyme under alkalophilic conditions at pH of 10.0 which is in accordance with the results of [29] and also agree with the result of [11] in that optimum pH for actinokinase production was found to be 10.6. Previous studies showed that high enzyme activity was recorded at pH range of 7.0 to 9.0 [14]. In this study, actinokinase was produced in the log phase after 18 hr. of incubation by *Stroptomyces megaspores* which is shorter than a study which found that 48 hrs. is optimum of mesophilic Streptomyces [31] and is comparable to that of streptokinase and staphylokinase by *Streptococcus heamolyticus* and *Staphylococcus aureus*, respectively [32]. One study revealed that the biomass increased rapidly during 72 hrs. and declined after 84 hrs. [33], when compared

with fibrolytic enzyme (nattokinase) produced by *Bacillus natto* strain within 22–120 hrs. at 37 °C [34] and the novel fibrinolytic enzyme from *Rhizopus chinesis* 12 requires 5 days of incubation at 30 °C under different culture conditions [35]. Organism isolated in this study appeared to be the best producer of fibrinolytic enzyme compared to those reported previously. The study agrees with [32] that the production time is 18 hrs. incubation, these might be that our actinokinase is classified as growth associated enzyme. The pH of the fermentation broth was dropped toward the acidic pH (5.5) after 18 hrs. of incubation as described in (Figure 8). The change in pH is an indicator of enzyme production where there are no reports on the development of acidic pH during the production of other available fibrinolytic enzymes this agree with [36]. Purification is critical step objective behind deciding the strategy for purifications to obtain the greatest possible purity. Extra purification of enzyme may result in loss of enzyme activity as many proteins are excluded by additional separation steps. The activity of the crude actinokinase from of *Streptomyces* spp. decreases at each purification step till reaching 578 U/ml indicating that the purification step was corrected and enabled to increase specific activity to 101 $U/mg^{-1}$ with purification fold reaching to 53 and enzyme recovery arrived at 70.1%, this decline in activity is imputed to freezing, thawing, and freeze-drying processes in comparison to others [37]. In parallel study [14] the strain could produce 450mg/L total protein with specific activity of 80 U/mg and 64% yield incubation at 55 $^{o}$C for 18 hrs. in GYP medium. *Streptomyces-CS 684*) isolated from Korean soil for 48 hrs. in glucose /oat at 28 $^{o}$ C, the enzyme purified by gel permeation using Sepharose CL- 6 B Colum yield 79% with specific activity 4 U/mg, both studies showed the protein content of cell-free broth was about 420 mg/L

This result agrees with [11]. The molecular weight was estimated to be 35 kDa by SDS-PAGE, The molecular mass of actinokinase was similar to fibrinolytic enzymes from *Streptomyces sp*. CS684 (35 kDa) [38], and higher than a marine bacterium *Bacillus subtilis A26* (28 kDa) [15], *Bacillus subtilis LD-8547* (30 kDa) [39] and lower than *Perenniporia fraxinea mycelia* (42 kDa) [5] With respect to the effect of pH and temperature, the maximum production of the enzyme by *S. megasporus* was at a pH range of 6–10 with an optimum pH 8.0 The residual activity of the enzyme after exposure to different pH values revealed that the enzyme retained 51% activity even at pH 5.0. The residual values revealed that the enzyme retained 51% activity even at pH 5.0. Thermal stability was tested by measuring the residual activities at different temperatures ranged from 35–70 $^{o}$C for 6 months. While at 70 $^{o}$C, most of the activity was lost within one month, and only 25% activity was retained, and the enzyme was quite stable up to 60$^{o}$C. Both in terms of thermostability and resistance to pH, the enzyme was better than other enzymes produced by [40]and [41] Thrombolytic therapy is still the best way to achieve recanalization in thrombosis diseases nowadays. Despite some thrombolytic agents' widespread use, all of them have drawbacks [42]. Therefore, it is indispensable to screen new thrombolytic agents from diverse sources. Increasing of clot lysis with time, indicated that the action of actinokinase enzyme is different than that of streptokinase and urokinase in that it's a plasminogen independent where it directly cleaves the fibrin network and completely lysis the blood clot within only 20 mins, our results agree with [43]. and[28] also take 20 min to completely lyses the clot.

The studies indicate that the enzyme actinokinase could be an effective thrombolytic agent against cardiovascular

diseases. This result indicated that the $V_{max}$ and $K_m$, were found to be 8.02 µ mol /ml and 0.56 (µ mol /ml/min respectively. Generally, determination of $V_{max}$ and $K_m$ was explained by the fact as small error in the determination of $V_{max}$ are magnified were reciprocal are taken (47), the apparent $K_m$ and $V_{max}$ for the fibrinolysis were calculated to be 3.2 mg/ml and 249 U/ml, respectively. The $K_m$ value, which means an apparent binding affinity for a substrate, was either higher than that of the alkaline protease (1.3 mg/ml) reported by [44]or lower than that of the alkaline protease (8.2 mg/ml) reported by [45]The present results showed that the purified enzyme had a relatively high affinity for fibrin hydrolysis. This result agrees with [46]. The purified enzyme had a low $K_m$ value for fibrin hydrolysis, fibrin degradation product which converted from colorless to yellow color solution mixture, the change in color allows measurement of the progress of the enzymatic reaction in the UV-VIS light range with a good sensitivity, Meanwhile the result indicated that the enzyme exhibited an efficient anticoagulant effect *in- vitro*. Therefore, actinokinase directly digested fibrin and not by plasminogen activators such as streptokinase, urokinase and tissue plasminogen activator. Proteolytic activity of actinokinase was assayed with fibrin as specific substrate. The actinokinase cleaves the peptide bond of the substrate fibrin. This result agrees with [47] This study indicates that our actinokinase enzyme will be a promising thrombolytic agent against cardiovascular diseases. Studies suggest that nattokinase may reduce whole blood viscosity, promote normal blood pressure, and increase circulation being an effective supplement to support cardiovascular health [48], However reported that intravenous administration did not show any clear thrombolytic effect but oral administration enhanced fibrinolytic activity. Another study [49] reported that when fibrinolytic enzymes

were given to human subject by oral administration, fibrinolytic activity and the amount of Tissue plasminogen activator (t-PA) and fibrin degradation product in plasma increased about two folds.

**Conclusions**

Seventy eight percent (78%) of the total isolates (300) were considered as thermophilic Streptomyces according to molecular identification. The promising isolates also could produce an extracellular crude actinokinase according to primary screening of the enzyme. Maximum activity and maximum bacterial growth reached after 18 hrs. incubation, indicating that the enzyme was growth associated. Purification of the crude actinokinase revealed that the specific activity of purified actinokinase is 101.4 U/mg and yield of 70.1%. The molecular weight of the purified actinokinase was estimated to be 35kD. The effect of pH, temperature and storage time on the stability of the purified actinokinase were 10.0, 37 $^{o}$C. and 6 months respectively. The enzyme kinetic activity, indicated that the $V_{max}$ and $K_m$, were found to be 8.02 µ mol /ml and 0.56 (µ mol /ml/min) respectively. 20 minute is the time for complete lysis of blood clotting using an actinokinase isolated from local thermophilic *Streptomyces*. The experiments confirmed that *Streptomyces* could be a good source of actinokinase for medical applications in Sudan

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