# IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF ACTINOKINASE' PRODUCING STREPTOMYCES IN KHARTOUM STATE IN SUDAN

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**Abstract** - Streptomyces species are abundant micro-organisms in soil, play an important role in mineralization process in nature and well known for their ability to produce biologically active secondary metabolites, particularly antibiotics such as Streptomycin and Erythromycin. Interestingly, only a small number of Streptomyces species are known to be plant or animal pathogens, Streptomyces characterized by producing fibrinolytic and caseinolytic enzyme known as actinokinase which is an extracellular plasminogen independent prokaryotic protein that directly degrade fibrin filament and dissolve clot. A total of (50) soil samples were collected from different areas in Khartoum State, laboratory tests were performed to identify the isolate using morphological and biochemical tests and Polymerase Chain Reaction (PCR) technique. The findings of this study indicated that the collected soil samples is (28%) Streptomyces that capable of producing actinokinase which act as fibrinolytic enzyme.

**Key words** - Actinokinase, Cardiovascular diseases, Molecular characterization, Streptomyces.

## I. INTRODUCTION

Cardiovascular and cerebrovascular diseases are listed in the top (15) leading causes of death both at present and in future decade. The mortality rate caused by vascular disorders such as myocardial infarction, stroke, deep-vein thrombosis, and pulmonary embolism is rapidly mounting, and thrombosis is the proximate cause of these vascular disorders. Thrombosis can occur when the blood vessels are blocked by a blood clot, where fibrin is the main protein component of the blood clot [1], and it is normally formed from fibrinogen by the action of thrombin after trauma or injury. Accumulation of fibrin in blood vessels usually increases thrombosis, leading to myocardial infarction and other cardiovascular diseases (CVDs) [2]. Worldwide, over (29%) of the total mortalities are due to thrombosis. By the year 2020, cardiovascular diseases (CVDs) may cause an estimated (25) million deaths per year, thus antithrombotic therapy is of great interest. Over the years, thrombolytic therapies via injecting or orally administrating thrombolytic agents to lyses thrombi in blood vessels have been extensively investigated [3]. An ideal thrombolytic drugs should consider reasonable cost, has no antigenicity, no effect on blood pressure, pro-coagulant effect, lower occlusion rate and low incidences of intracranial and system bleeding, should be fibrin selective, effective in dissolving older thrombi and resistant to plasminogen-activator inhibitor also able to be administered as intravenous bolus [4]. The major thrombolytic agents are classified into two types based on their mechanisms. One is the plasminogen activators, such as urokinase, tissue-type plasminogen activator and streptokinase, which activate plasminogen to plasmin, and then eventually hydrolyze fibrin. The other is plasmin-like proteins that directly degrade fibrin [5], but plasminogen activators have some undesirable side effects such as gastrointestinal bleeding, toxicity and allergic reactions in addition to the high cost that why several studies have focused on researching of safer and less expensive thrombolytic agents from diverse sources such as fermented food, earthworms, mushrooms and microbial sources [6], microorganisms have attracted researchers' attention for their fast growth rate and strong enzyme productivity, such as bacteria, actinomycetes, fungi and algae are reported to produce fibrinolytic enzyme with few reports on the use of Streptomyces sp [3]. Streptomyces species (spp) are abundant micro-organisms in soil are well known for their ability to produce biologically active secondary metabolites, particularly antibiotics. Interestingly, only a small number of Streptomyces spp are known to be plant or animal pathogens [7]. Nattokinase is a fibrinolytic enzyme produced by Bacillus subtilis (B.subtilis). Streptomyces were also studied for the secretion of various serine proteases ,part of these serine proteases have strong fibrinolytic activity which could be promising candidates screen for fibrinolytic enzymes [1].

## II. MATERIALS AND METHODS

### A.Sampling:

Total of 50 soil samples were collected from different area and districts in Khartoum State, Sudan. Soil

samples were taken from 5-10 cm depth after removing approximately 3 cm from the earth surface; these samples were collected into sterilized plastic bags and then transferred to labeled screw bottles, each soil sample divided into two, one fortified with CaCO3 to increase the growth of Streptomyces spp, (alkaliphilic).

**B. Isolation and identification of microorganism:**

Isolation of microorganism was performed by dilution plate technique [8]. In this technique, 1gm of each soil sample was taken in 9 ml of sterilized distilled water in a pre-sterilized test tube. Dilution process was continuing until 10-4 obtain. Different aqueous dilutions (10-3, 10-5, and 10-7) of the soil suspension were applied separately into sterilized Petri- dishes with glucose yeast extract peptone (GYP) agar medium (pH 8.0), After gently rotating the plates they incubated at 45˚C for 7 days. Colonies, with Streptomyces morphological characteristic that appeared in the incubated plates, were repeatedly sub-cultured for further studies.

The isolates are maintained as spore suspension (Hopwood et al., 1985) and used for the further study. The identification of purified isolates was carried out according to Cowan and Steel [9].

**C. Blood hemolysis:**

Hundred ml of sterilized nutrient agar was prepared in 250 ml-conical flasks. 10 ml of filter-sterilized blood were added to each flask and contents were mixed gently to avoid any bubble formation. The medium was then poured in sterilized petri-dishes and incubated over night at room temperature. Clean uncontaminated plates were inoculated each with one of test organism isolates, and incubated for 24 hr at 37 ˚C. Hemolysis was indicated by the presence of clear zones around the colonies [10].

**D. Molecular characterization:**

**1. 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 Ċ), loop full of cultured bacteria was suspended in each falcon tube containing the broth and again incubated overnight, the mixture was centrifuged at (1000) rotation per minute (rpm) for (10 mins), the supernatant layer was discarded then (200µl) water for injection was added to the precipitate and boiled at (95 Ċ) for (20 mins) in the water bath, after the (20 mins) the tube was directly thermally shocked and placed in the ice to cool down for about (10 mins), again centrifuged but this time the eyes was put on the supernatant layer which contain the extracted DNA, the placed in Eppendorf tube to be ready for the PCR [11].

**2. 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' )* |

[12], [13], [14].

The reaction was performed in (20µl) volume using Maxime PCR series "Next Generation of Premix", the volume included: (1µl) forward primer, (1µl) 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 (5 mins) at (95 Ċ), PCR comprised (30 cycles) as following, (45 seconds) (secs) at (94 Ċ), (45 secs) at (65 Ċ) and (1 mins) at (72 Ċ), and finished with an extension step of (10 mins) at (72 Ċ). The gel was prepared for analyzing amplified product for gel electrophoresis.

**3. Gel preparation:**

(0.75 g) of agarose powder (iNtRON Biotechnology) was weighed, 1× Tribase Boricaid EDTA so that the concentration of agarose gel to be (1%), the mixture was heated by microwave until clear solution is obtained, then (0.15%) ethidium bromide was added for staining, poured onto a gel tray where the combs were introduced to create the wells that will be used for loading the PCR product and then the gel was allowed to solidify.

**4. loading and electrophoresis:**

The gel was placed in electrophoresis apparatus containing 1×TAE buffer, (5 µl) of amplified DNA and (3 µl) of DNA marker from Solis Biodyne was inserted into combs, the gel was emerged in TBE buffer at (120 volt) for (40 mins) using analytic Jena standard Power P25T device to enable running of DNA, he gel was visualized under ultraviolet (UV) trans-illumination using UV Solo TS imaging system software. The expected size of 16s r-RNA gene of the Streptomyces was (600) base-pair according to ([12], [13].

**E. Enzyme extraction:**

Extraction of enzyme was carried out using fermentation broth using GYP medium pH of 8.0. The fermentation was carried out at 45°C for 18 hr. under controlled conditions. The agitation was maintained at 140 rpm. Streptomyces was grown on selective media containing (g/lit.) 5gm of glucose, 5gm peptone, 3gm yeast extract and 1000 ml distilled water. The pH was adjusted to 8 with 2 M NaOH. Medium was sterilized by autoclaving at 121°C for 15

min. and cooled to room temperature. One ml of uniformly prepared suspension of Streptomyces used as inoculums; incubated at 45°C and 140 rpm in an orbital shaker incubator. After 18 hr of fermentation, cells were harvested by centrifugation at 4°C and 10,000 rpm for 10 min [15].

**F. Enzyme Activity and growth profile:**

To assure that the thermophilic Streptomyces bacteria are producing the actinokinase enzyme and to determine its activity four indicators were used (Quantitative method):

**1. Drop in pH:**

Five fermentation batches for the production of actinokinase enzyme were successfully carried out using shaker. Five ml samples were taken to measured pH after 18 hr. of incubation.

**2. Growth profile and enzyme activity:**

From the five fermentation flasks, a sample was taken at zero time and then samples were removed periodically every 6 hr. for 24 hr. to measure the enzyme activity at 405 nm and at 600 nm for bacterial growth using U.V spectrophotometer.

**G. Hemolytic activity (Clear zone):**

Using blood agar media which prepared by weighing 5g of blood agar base and completed to 100 ml by distilled water and introduced to autoclave at 121 °C for 15 min and then 5 ml of human blood was added and leave until cool and incubated for 24 hr. to ensure that the media is sterilized, then a loopful taken from ISSN 2320-5407 International Journal of Advanced Research (2016), Volume 4, Issue 1, 869- 878 871 Streptomyces subculture plate and cultured into blood agar plate and incubated at 45 °C for 24 hr. which later show a clear zone indicted of the hemolytic effect of the enzyme.

**H. Time of clot lysis:**

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

**I.** Identification and Characterization of the Microorganism:

When you submit your final version, after your paper has been accepted, prepare it in two-column format, including figures and tables.

## III. RESULTS AND DISCUSSION

Among a total of (50) soil samples collected from different area in Khartoum State (Omdurman, Khartoum, Soba, Toti and Bahri), (90%) showed positive growth and (10%) showed negative growth at (45 °C) while (60%) of the samples showed gram positive filamentous bacteria while (40%) showed negative result and 46 % showed non-motile bacteria indicating the characteristic of Streptomyces sp. where the percentage was calculated by dividing the number of positive result of each area over total number of samples (50) then multiply it by 100.

$$\left(\frac{y}{50} * 100\right)$$

**Table (1): Percentage of positives culture growth at $45^0$ C, gram stain and motility test of the growing bacteria.**

| Samples | Culture growth at 45 °C (%) | Gram stain test (%) | Motility test (%) |
|---|---|---|---|
| Omdurman | 20 | 16 | 11 |
| Khartoum | 18 | 12 | 9 |
| Soba | 20 | 15 | 11 |
| Toti | 18 | 10 | 8 |
| Bahri | 14 | 7 | 7 |
| Total (%) | 90 | 60 | 46 |

Table below show the percentage of the positivity of the biochemical hydrolysis tests, blood hemolysis and PCR for the bacterial isolates from the five different areas.

**Table (2): Percentage of positive test on bacterial isolates.**

| Samples | Casein test (%) | Starch test (%) | Hemolytic zone (%) | PCR (%) |
|---|---|---|---|---|
| Omdurman | 12 | 12 | 10 | 10 |
| Khartoum | 6 | 6 | 4 | 4 |
| Soba | 10 | 10 | 8 | 8 |
| Toti | 8 | 8 | 6 | 4 |
| Bahri | 6 | 6 | 4 | 2 |
| Total (%) | 42 | 42 | 32 | 28 |



**Fig (1) Colonies of Streptomyces**



**Fig (2) Colonies of Streptomyces**

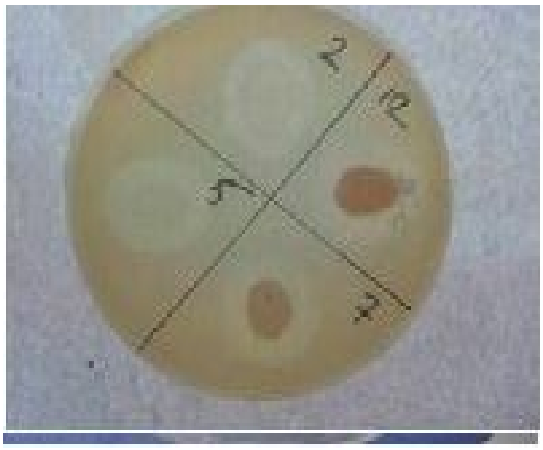
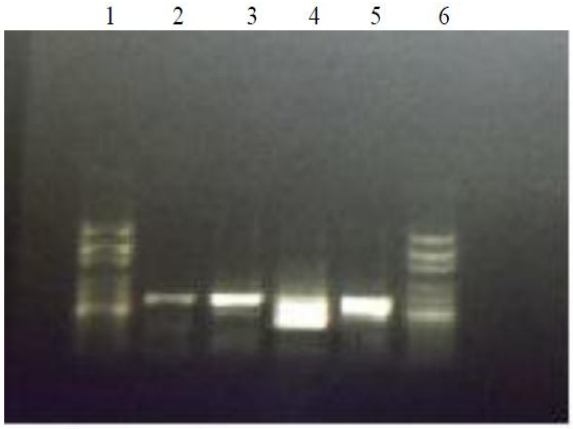


**Fig (3) Clear zone around Streptomyces colonies**

### A. Identification by 16S Ribosomal RNA (r-RNA) gene:

After DNA 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 14 samples (28%) 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:




Lane (1): DNA marker
Lane (2): Sample 1
Lane (3): Sample 2
Lane (4): Sample 3
Lane (5): Sample 4
Lane (6): DNA marker

**Figure (4): PCR amplified products run in an agarose gel using ladder as a marker and visualized under UV light.**

### B. Activity of Actinokinase on Blood agar media:

Enzyme extracted at different time interval (6,12,18 and 24 hrs.) in blood agar media were resulted in variant inhibition zone diameter (0.2, 0.5,0.8 and 0.3 cm) respectively which showed the larger diameter after 18 hours as the optimum time needed for actinokinase activity as shown below:



**Figure (5): Inhibition zone of actinokinase enzyme extracted after 6, 12, 18 and 24 hours**

### C. Actinokinase activity on Clot lysis:

Showed time course of thrombolytic activity of the enzyme actinokinase produced by Streptomyces at different time ranged from (0-20 mins), where clot lyses started after (5 mins) and continues until (20 mins) although actinokinase is crude and not purified yet.

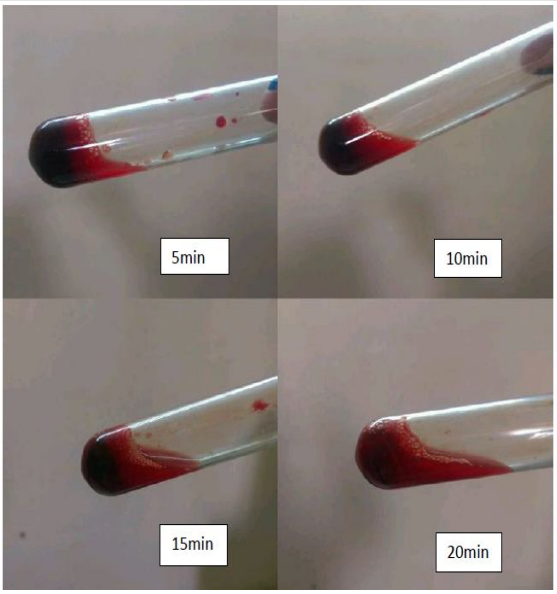



**Figure (6): Thrombolytic activity of the actinokinase enzyme determined by measuring time needed for complete clot lysis**

### D.Drop in pH:

By measuring pH of the five fermentation flasks which incubated at different time interval, the flask at zero time is of pH (8.0) after incubation the pH is (7.2, 6, 5.5 and 5.3) after (6, 12, 18 and 24 hrs.) respectively, the drop in pH toward acidic side is an indicator of actinokinase production.

### E. Enzyme activity and growth profile curve:

Chart below showed that the bacterial growth measured by absorbance at 600 nanometer (nm) and enzyme activity by measuring diameter (cm), reached to the maximum after (18 hrs.) as (1.45Au and 0.8cm) respectively while they decreased to (0.5Au and 0.3cm) after 24 hrs.

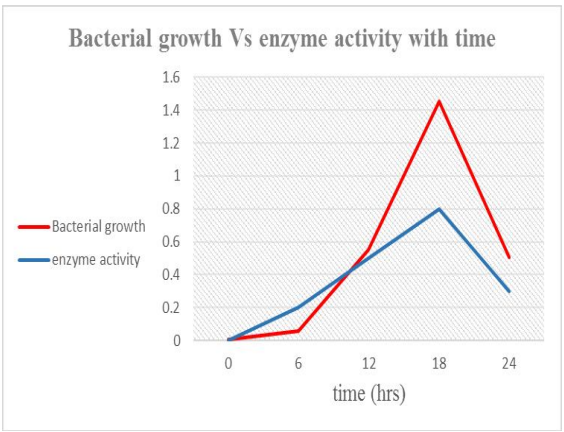



**Chart (1): Change in bacterial growth and actinokinase activity along with incubation time.**

### Enzyme activity and pH curve:

Chart below provided that actinokinase reach the maximum activity after (18 hrs.) by (0.8 cm) for diameter of enzyme activity with the dropping of pH toward acidic side (5.5-5).

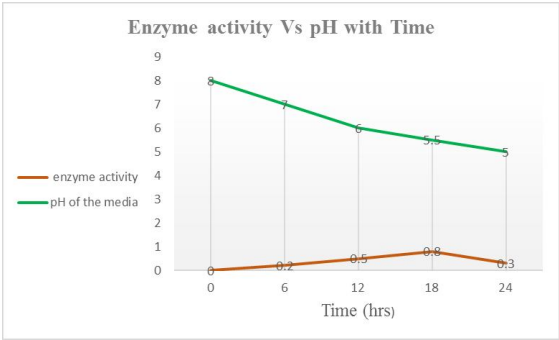



**Chart (2): Actinokinase activity and changes in pH along with incubation time**

## CONCLUSION

Among morphological test, 90% positive culture samples and (60%) gram positive stain resulted in (46%) non-motile filamentous bacteria and (42%) positive starch and casein test which forming clear zone where the cultured soil' samples was (28%) verified to be Streptomyces (by using PCR and gel electrophoresis resulting in the same bands size at 600 nm) that capable of producing actinokinase which act as fibrinolytic enzyme by hydrolyzing the blood forming a transparent zone by (32%) of total samples.

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