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Research Article

A Potential New Isolate for Actinokinase Production

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Received: May 02, 2017; **Published:** August 09, 2017

Abstract

The study aimed to produce a potent fibrinolytic enzyme actinokinase from local isolate in batch fermentation, culture of identified thermophilic *Streptomyces* spp, grown on glucose yeast extract peptone medium pH of 8.0. The haemolytic activity of the crude enzyme and time spend for complete lysis was tested using blood agar media, and test tubes containing clotted blood. The *in-vivo* clot lyses of the crude enzyme was found to be faster (20 min) compared to the other commercial fibrinolytic enzyme (90 min). The enzyme was stable at a broad range of pH ranging from 5 to 9. This particular isolate could produce a promising actinokinase with thrombolytic potent activity.

Keywords: *Fibrinolytic Activity; Actinokinase; Thermophilic Streptomyces and Thrombosis*

Introduction

Actinokinase is a new fibrinolytic enzyme reported from thermophilic *Streptomyces megasporus*. 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 [1]. The key reason of cardiovascular diseases is the formation of blood clots The clot formation and lysis are kept in balance by homeostasis inside human body, but in unbalanced conditions the clots are not hydrolyzed that leads to thrombosis Administration of thrombolytic drugs to dissolve the clot is the only alternative of surgery to remove or by bypass the blockage [2]. The development of anti-thrombotic drug is either activation of coagulation factors or platelets aggregation [3,4]. Now researchers developed several types of thrombolytic drugs that categorized into three generations for the therapeutic purposes, these are urokinase-type plasminogen activator, tissue plasminogen activator (t-PA) and bacterial plasminogen activator streptokinase are commonly used. However, an ideal drug is not yet developed.

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 [5]. Different enzymes such as streptokinase, urokinase, staphylokinase, and nattokinase are applied to treat thrombosis, but these agents have some disadvantages such as hemorrhagic effect, immunogenicity, and high cost, due to which their uses are limited. There are still research focuses to search of a new drug which overcome the drawbacks [6]. There are several thrombolytic agents discovered till today but still a need to investigate new fibrin-specific fibrinolytic enzymes to overcome these drawbacks, side effects. The downstream processing and the cost of the enzyme production are the major concern for successful application of fibrinolytic enzymes in the industry. Nattokinase, lubrokinase, and third generation of fibrinogen activator were act directly on the fibrin etc., of the presently available drugs. In this study, we attempted to explore a potent thrombolytic agent from the microbial source. Isolation, extraction and identification and thrombolytic activity of a fibrinolytic enzyme actinokinase produced by a locally *Streptomyces* strain.

The present study is carried out for bench scale production of the fibrinolytic enzyme with validation of batches, its production and activity.

Materials and Methods

This study was done at the Bacteriology Department/Central Laboratory, during March - August 2015.

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* sp, (alkaliphilic).

Screening, isolation and identification of the locally microorganism were performed using dilution plate technique [7]. One gm of each soil samples were taken in nine 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 contain glucose yeast extract peptone agar medium (GYP) at pH of 8.0, after gently rotating the plates they incubated at 45°C for 7 days. Colonies showed morphological characteristic of *Streptomyces* that appeared in the incubated plates, were sub-cultured for further investigations. The isolates are maintained as spore suspension [8] and used for the further study.

The identification of purified isolates were carried out according to Cowan and Steel [9].

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. The presence of clear zones around the colonies indicated the hemolysis [10].

Enzyme extraction

Extraction of the crude enzyme was carried out from the fermentation broth using GYP medium at pH of 8.0. The fermentation conditions were carried out at 45°C for 18 hr under controlled conditions. The agitation (RPM) was kept at 140 rpm. *Streptomyces* was grown on selective media containing (g/lit.) 5 gm of glucose, 5 gm peptone, 3 gm 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 [6].

Enzyme activity and growth profile

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

Drop in pH: The 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.

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.

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 introduced to incubator for 24 hr to ensure that the media is sterilized, then a loopful taken from *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.

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.

Results and Discussion

Samples that streak on selective media showed: 43 out of 50 samples (86%) were grown at 45°C, while 7 sample (14%) showed no growth at 45°C. 31 out of 50 sample (62%) isolated and grow in the soil that fortified with $CaCO_3$, while 19 out of 50 samples (38%) showed no growth of the microorganism. Gram stain test of all the cultured bacteria revealed that 7 out of 50 samples (14%) were spore forming, negative motility, positive starch hydrolysis, positives casein hydrolysis and positive filamentous bacteria indicating the characteristics of *Streptomyces* species (Figure 1, 2). Confirm by the clear zone around *Streptomyces* colonies (Figure 3). Compared to study done by Rama and Vibhuti [11] stated that 50% from total isolates was found to be thermophilic *Streptomyces* according to morphological and biochemical characteristics which grown at the same conditions.

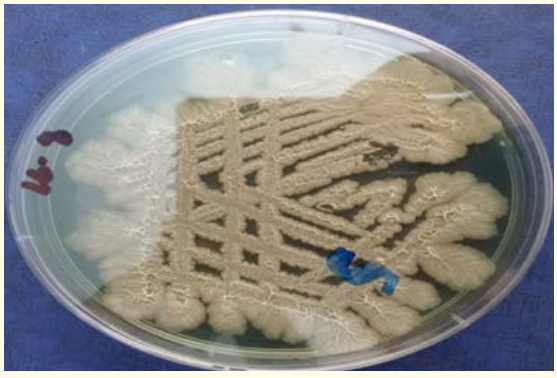

Figure 1: *Colonies of Streptomyces.*

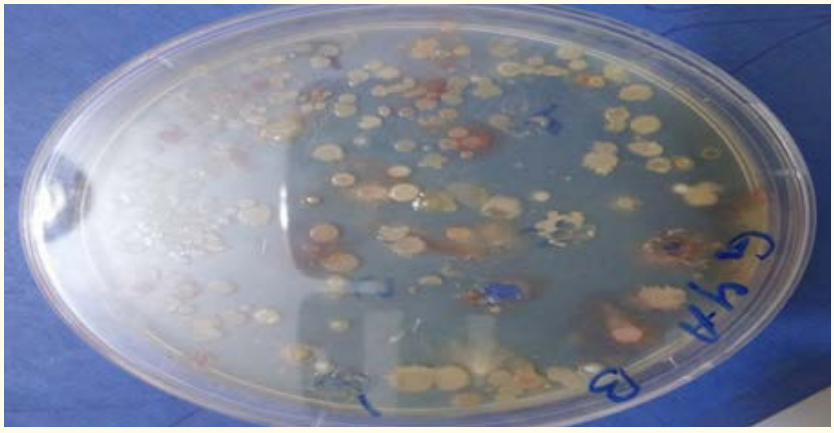

Figure 2: *Colonies of Streptomyces.*

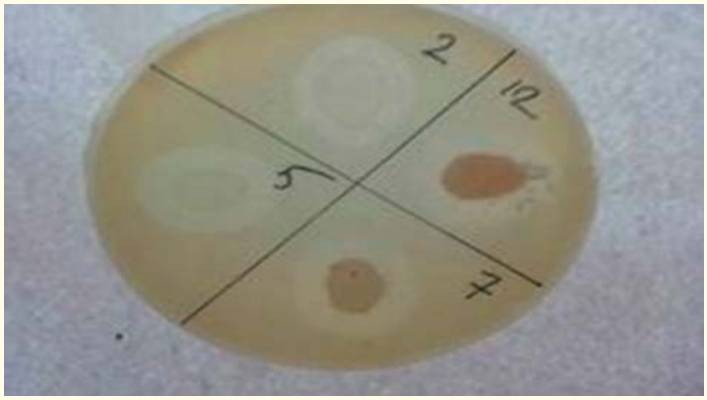

Figure 3

Enzyme Activity

The activity of actinokinase was determined by four indicators:

Drop in pH: Five ml samples were taken to measure the pH after 18 hr of incubation, It is shown that the pH of fermentation process dropped towards acidic side (5 - 5.5) after 18 hr of incubation figure 4. The change in pH is the indicator for enzyme production. There is no report on the development of acidic pH during the production of other available fibrinolytic enzymes. The actinokinase enzyme resists to broad pH range.

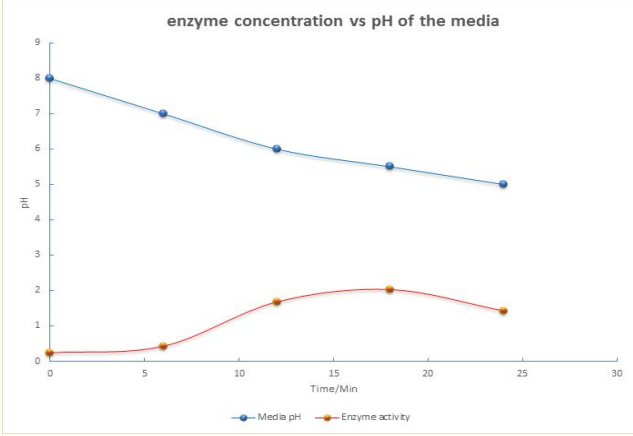

Figure 4: *pH profile against enzyme activity.*

Growth profile and enzyme activity: The activity of crude actinokinase and the *Streptomyces* growth kinetics are depicted on figure 5. Fibrinolytic Enzyme production from the local isolated *Streptomyces* throughout different time periods (0 - 24) hr, Figure 3 shows that the growth profile during a 24-hr cultivation. Maximum growth of the *Streptomyces* was obtained within 18 hr of cultivation also actinokinase activity reached a maximum within 18 hr after inoculation, beyond 18 hr of growth, no increase in actinokinase activity was recorded. The two profiles were similar indicating that the fermentation kinetics of actinokinase production by *Streptomyces* might be classified as growth associated. Enzyme production was found to be concomitant with growth. The novel fibrinolytic enzyme from Rhizopus chinesis 12 requires 5 days of incubation at 30°C [12]. *Bacillus natto* strain produced the fibrinolytic enzyme within 22 - 120h of incubation time at

37°C under different culture conditions [13]. Gesheva [14] produce a fibrinolytic enzyme using culture of *Streptomyces rimosus* and under nitrogen limitation in the production medium. The biomass increased rapidly during 72h and declined after 84h.

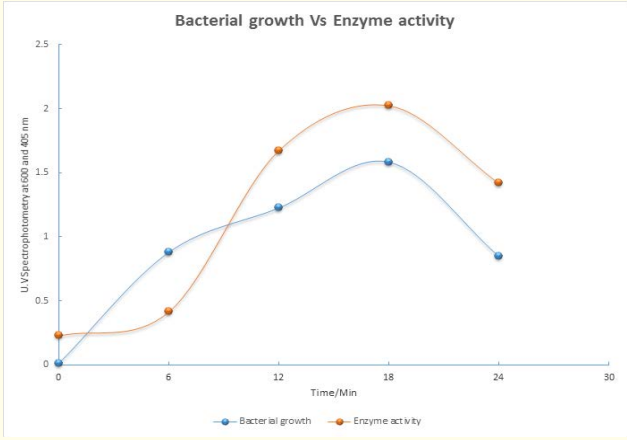

Figure 5: *Actinokinase activity profile during the growth of Streptomyces.*

Hemolytic activity (Clear zone): It is an excellent qualitative performance method. The measurement of the dimension of the clear zone around each organism indicates actinokinase activity. Figure 6 below shows the clear inhibition zone of the samples indicted of the hemolytic effect of the crude enzyme. Studies suggest that nattokinase may reduce whole blood viscosity, promote normal blood pressure, and increase circulation being an effective supplement to support cardiovascular health [15]. However, Sumi., *et al*. [16] reported that intravenous administration did not show any clear thrombolytic effect but oral administration enhanced fibrinolytic activity. In another study Sumi., *et al*. [16] reported that when fibrinolytic enzymes was given to human subject by oral administration, fibrinolytic activity and the amount of tPA and fibrin degradation product in plasma increased about two folds.

Figure 6: *Primary screening of the samples using blood agar media (blood hemolysis).*

Time of clotting: By applying 1g of clotted blood to the tube or slide and addition of 200 µl of crude actinokinase, clot will slowly begin to dissolve with the time until complete lyses occur within 20 min figure 7 below. The fibrinolytic activity of the crude enzymes reported during different time per min. Our results agree with Peng., *et al*. [17] who found that actinokinase could dissolve the blood clot within 20 min while urokinase and streptokinase took 40 and 80 min, respectively. Streptokinase is obtained from the pathogenic strain like *Streptococcus* so the enzyme is antigenic in nature, which leads to immunological reaction in the patient. Of the enzyme, actinokinase produced by *Streptomyces* at different time ranged from 0 - 20 min, it showed clot lyses start at 5 min and follow until 20 min although actinokinase is a crude enzyme not purified yet. Our results agree with study of [18] also take 20 min to completely lyses the clot [19].

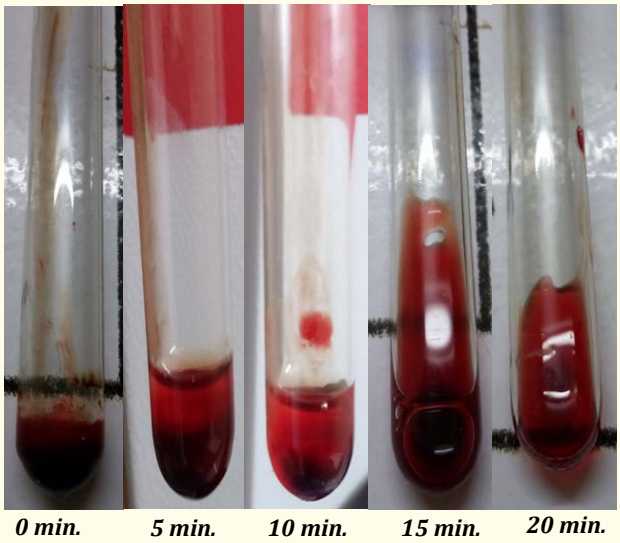

Figure 7: *Time profile of thrombolytic activity of the crude actinokinase.*

Conclusion

Seven samples (14%) were considered as Thermophilic *Streptomyces* according to microscopic and biochemical characteristics, the promising isolates also could produce an extracellular crude actinokinase indicating by haemolytic and fibrinolytic activity. 20 minute is the time for complete lysis of blood clotting using a crude actinokinase isolated from local thermophilic *Streptomyces*.

Acknowledgment

Authors acknowledge Bacteriology Unit. Central Lab. Ministry of Higher Education and Scientific Research for their help and support during lab. Work, thanks also extended to University of Medical Science and Technology for their help.

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Volume 10 Issue 5 August 2017