Journal of Applied Sciences

ISSN 1812-5654

science alert

ANSInet
an open access publisher
http://ansinet.com

OPEN ACCESS Journal of Applied Sciences

ISSN 1812-5654
DOI: 10.3923/jas.2020.35.43

Research Article
Optimization of Physical Conditions of Actinokinase Synthesis from Local Isolate Using Response Surface Methodology (RSM)

[1]Mawada Elzein Alawad Alkhider, [2]Elrashied Elimam Elkhidir and [3]Hanan Moawia Ibrahim

[1]Ahfad University for Women, Omdurman, Sudan
[2]Faculty of Agriculture, University of Khartoum, Khartoum, Sudan
[3]Central Laboratory, Ministry of Higher Education and Scientific Research, Khartoum, Sudan

Abstract

Background and Objective: Actinokinase is a new fibrinolytic enzymes that dissolve fibrin clots. These fibrinolytic agents have potential use to treat cardiovascular diseases, such as heart attack and stroke. This study aimed to optimize physical conditions of actinokinase extracted from local isolate in batch fermentation. **Materials and Methods:** Isolation and identification of the isolates were carried out using conventional technique. Enzyme was extracted and hemolytic activity of the crude enzyme and time for complete clot lysis was tested by blood agar media and test tubes containing clotted blood. Physical conditions as pH and temperature were optimized using statistical experimental design, response surface methodology (RSM). **Results:** About 32% of the total samples were found to be a promising enzyme producer according to hemolytic activity and large inhibition zone. Characterization of the isolates were considered as *Streptomyces* spp. according to microscopic and biochemical analysis. Maximum *Streptomyces* growth and actinokinase activity reached at 18 h of incubation. The time for complete clot lysis revealed that 20 min is the time for complete lysis of blood clot. Statistical analysis is significant with model F-value of 93.36 and R^2 value of 98.53%. Optimum growth conditions of actinokinase production was at high pH of 10.6 and temperature of 36.89°C that achieve the maximum actinokinase activity to be of 47.37%. **Conclusion:** The optimum pH of 10.6 and optimum temperature of 36.89°C enhance three-fold actinokinase production.

Key words: Fibrinolytic enzymes, response surface methodology, *Thermophilic streptomyces*, batch fermentation, optimization, actinokinase synthesis

Citation: Mawada Elzein Alawad Alkhider, Elrashied Elimam Elkhidir and Hanan Moawia Ibrahim, 2020. Optimization of physical conditions of actinokinase synthesis from local isolate using response surface methodology (RSM). J. Applied Sci., 20: 35-43.

Corresponding Author: Hanan Moawia Ibrahim, Central Laboratory, Ministry of Higher Education and Scientific Research, Khartoum, Sudan
Tel: +249-9-11171941

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

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]. Thrombosis is a critical event and a common pathology underlying ischemic heart disease, myocardial infarction, ischemic stroke and venous thromboembolism disorders account for considerable morbidity and mortality. Moreover, venous thrombosis is the second leading cause of death in patients with cancer[2]. The patho-physiological process of thrombosis was found to be due to the accumulation of fibrin when injury on blood vessels occurs. Thrombin convert fibrinogen to fibrin which is lysed by plasmin, which is a secretory serine protease, that is generated from inactive precursor plasminogen via limited cleavage by plasminogen activator (PA). There are two widely used as biological medicines for the treatment of cardiovascular disease which are Urokinase (u-PA) and tissue-type plasminogen activator (t-PA), but these agents have some undesirable side effects and have drawbacks such as bleeding complications, secondary immune response gastrointestinal bleeding, toxicity and allergic reactions. Microbial fibrinolytic agent such as streptokinase produced by *Streptococcus hemolyticus* and staphylokinase produced by *Staphylococcus aureus* have attracted medical attention during decades that were proved to be effective for thrombolytic therapy[3]. 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 complete coronary and rapid flow[4]. Although microbial fibrinolytic enzymes have been extensively studied, only few reports are available concerning statistical medium optimization[5-7]. Actinokinase E.C. # 3.4.21.23. is a new fibrinolytic enzyme that was found to be reported from thermophilic *Streptomyces megasporus*[8]. Actinokinase is a promising anti thrombotic drug with high fibrin selectivity that finds a wide range of applications in pharmaceutical industry, health care and medicine, Its digests fibrin both directly and indirectly. Indirectly, it activates prourokinase and tissue plasminogen activator (t-PA), supporting the fibrinolytic activity of plasmin. These combined actions promote healthy platelet function, contribute to the regular healthy function of the heart and cardiovascular system by maintaining proper blood flow, thinning the blood and preventing blood clots[9]. The ideal drug for prophylaxis and treatment of thrombotic disease remains an agent that will inhibit thrombosis but not hemostasis[2]. It was found to be plasminogen independent which acts directly on fibrin and subsequently lysed the fibrin clot. The enzyme spectrum is closely similar to commercial available urokinase enzyme spectrum. Although there is high prevalence of cardiovascular disease and thrombosis there is no ideal antithrombotic drug till now. Strain of *Streptomyces* found to require specific conditions for maximum growth as well as bioactive metabolite production[10], due to that the need for optimization of culture conditions of *Streptomyces* for production of actinokinase will be of high benefits for its large scale production. Traditional approach to optimization of biological systems based on one factor at a time does not include the interactive effects among the variables studied[11]. This approach is tedious, time consuming and expensive, more over it does not guarantee the determination of optimum conditions[12]. Response surface methodology (RSM), which is a compilation of statistical and mathematical techniques widely used to determine the effects of several parameters and to optimize various biotechnological processes[13]. There is a need to search potent and safe fibrinolytic agent which can be used to treat cardiovascular diseases[14] with reasonable cost, no antigenicity, no effect on blood pressure, proagulant effect, minimum occlusion rate and minimum incidence of intracranial and system bleeding are the characteristics of the ideal thrombolytic drug. Although microbial actinokinase have been extensively studied, only few reports are available concerning statistical experimental design for optimization of physical conditions. Therefore, main objective of this study was to optimize the physical conditions of actinokinase synthesis from local isolate using response surface methodology.

MATERIALS AND METHODS

Study area: This is an experimental designed study. The practical work was carried out at Central Laboratory, Sudan, during August-December, 2017.

Samples collection: Fifty samples of soil were collected from different area in Khartoum State, samples were taken from 5-10 cm depth after removing 3 cm of surface soil. The samples were collected into sterilized plastic bags and delivered to Central Lab.

Isolation and screening of the microorganism: Isolation of microorganism was performed using serial dilution plate technique[15]. One gram of sample dissolved in 9 mL of sterile distilled water in pre sterile falcon tube. Dilution process was continued till 10^{-9}. Different aqueous dilutions of soil suspension were applied separately into pre-sterilized petri dishes with sterilized glucose yeast extract peptone agar media (GYPA) of pH 8. The plates were rotated gently and incubated at 45°C for 5 days, colonies that had *Streptomyces* morphological characteristics were sub cultured for further study and the purified isolates were maintained as spore suspension[16].

Preliminary screening of the enzyme

Casein hydrolysis: Casein hydrolysis media was prepared from two solutions: A and B. Solution A prepared by dissolving 10 g of skimmed milk powder in 100 mL distilled water and solution B prepared by dissolving 2 g of agar in 100 mL distilled water. Then the two solutions were autoclaved separately, allowed to cool to 50°C, mixed together and poured into sterile plates. The plates were inoculated with the isolates and incubated at 45°C for 3 days, appearance of clear zone around each colony indicate positive casein test[17].

Blood hemolysis: Ten milliliter of fresh blood were added to 100 mL of sterilized nutrient agar in 250 mL conical flask and mixed gently. Then the media was poured in sterilized petri dishes and incubated for one night at 37°C. The un-contaminated plates were inoculated and incubated for 24 h at 47°C. Hemolysis was indicated by the presence of clear zone around colonies[18]. Isolates that exhibited large inhibition zone were identified and characterized[19].

Extraction of fibrinolytic enzyme: Five promising isolates of positive blood hemolysis test were used for the production of actinokinase. Hundred microliter of spore suspension was inoculated in 100 mL of sterile GYP medium, containing (% w/v) glucose 1%, yeast extract 0.5%, peptone 0.5% and CaCl 0.02%. The pH was adjusted to 8.0 with 2 M NaOH. Sterilization of the media was done by autoclaving at 121°C for 35 min in 250 mL conical flask and incubated for 24 h at 55°C in orbital shaker incubator. After 24 h cells were harvested and removed by cold centrifugation at 10,000 rpm for 10 min. The supernatant was used as is an extra-cellular crude actinokinase enzyme[20].

Enzyme assay: Fibrinolytic activity was carried out as follow[4]:

Drop in pH: Shake flask fermentation was carried out using orbital shaker incubator, pH of the samples was measured at 0, 6, 12, 18 and 24 h of incubation, a drop in pH indicate enzyme production. There is no report on the development of acidic pH during the production of other available fibrinolytic enzymes.

Hemolytic activity: Hemolytic activity or appearance of a clear zone was carried out using blood agar medium that prepared by dissolving 12 g of blood agar base in 250 mL distilled water, the media was autoclaved at 121°C for 15 min, 12 mL of fresh blood were added after cooling. Then the media poured into sterile petri dishes and incubated for 24 h at 37°C. One centimeter diameter well was performed in the center of the blood agar plate and inoculated with crude enzyme and incubated for 24 h at 37°C.

Percentage of clot lysis: Venous blood drawn from healthy volunteers was transferred in different pre -weighed sterile micro-centrifuge tube (l.0 mL/tube) and incubated at 37°C for 45 min. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed) and each tube having clot was again weighed to determine the clot weight:

$$\text{Clot weight (g)} = \text{Weight of clot containing tube-Weight of empty tube}$$

Each micro centrifuge tube containing clot was properly labeled and 200 µL of crude actinokinase was added. Water was also added to one of the tubes containing clot and this serves as a negative thrombolytic control. All the tubes were then incubated at 37°C and observed for clot lysis. After incubation, fluid obtained was removed and tubes were weighed again to observe the difference in weight after clot disruption[21]:

$$\text{Clot lyses (\%)} = \text{Clot weight before-}\left(\frac{\text{Clot weight after}}{\text{Clot weight before}}\right)\times 100$$

Time course of bacterial growth: The colonies that showed large zone on blood agar media were used for inoculum preparation. Ten milliliter inoculum was added to 90 mL of production medium into 250 mL Erlenmeyer flask. The cultures were incubated at 37°C and 140 rpm on a rotary shaker incubator for 72 h. Samples were removed periodically every 6 h and cell growth estimation using UV spectrophotometer at 600 nm, as well as actinokinase activity was determined[22].

***In vitro* clot lysis:** Ten mg of clotted blood were taken in a test tube, 20 µL of the extracted crude actinokinase was added, therefore the time for complete clot lysis was recorded.

Table 1: Experimental design of the two level (2^2) full factorial design

Standard order	Run order	Type	Block	pH	Temperature
8	1	-1	1	8.5	58.1
7	2	-1	1	8.5	36.9
5	3	-1	1	6.4	47.5
12	4	0	1	8.5	47.5
6	5	1	1	10.6	47.5
11	6	0	1	8.5	47.5
4	7	1	1	10.0	55.0
10	8	0	1	8.5	47.5
9	9	0	1	8.5	47.5
3	10	1	1	7.0	55.0
2	11	1	1	10.0	40.0
13	12	0	1	8.5	47.5
1	13	1	1	7.0	40.0

Optimization of physical conditions of actinokinase synthesis: Response surface methodology[23,11] was used for the optimization of the variables, the variables were temperature and pH. From the literature the level of pH chosen is 7.0 which is low level and 10.0 is high level and the temperature 40°C is low level and 55°C is high level. The variables (coded) of each constituent are given in Table 1.

The surface response for enzyme production as a function of selected key variables was determined. A two-level full fractional factorial design with two variables, namely, pH and temperature ($-\alpha$, -1, 0, +1, $+\alpha$) consisting of one block and with 13 runs (8 combinations with 5 replication of the center points) were used. The Minitab package version 16.0 was used to describe the response. The experiments were conducted in duplicates and the mean value (%) of fibrinolytic activity was taken as the response (Y).

RESULTS

Samples collection and bacterial isolation: Forty five out of 50 (90%) of soil samples were exposed growth of different types of microorganism as shown in Fig. 1, while 32% were positive casein hydrolysis with large inhibition zone as shown in Fig. 2 and also positive hemolytic zone in blood agar as in Fig. 3, which indicates promising isolates that could be able to produce actinokinase. Identification of the isolates revealed that the isolates were *Thermophilic streptomyces* and could be able to produce actinokinase.

Fibrinolytic activity: The fibrinolytic activity of actinokinase was measured by casein hydrolysis method and drop in pH. It is an excellent qualitative performance. The measurement of the dimension of the clear zone around each organism indicates actinokinase activity. Figure 4 shows the activity of actinokinase and pH of the media against time (h).

Fig. 1: Soil culture of different types of colonies

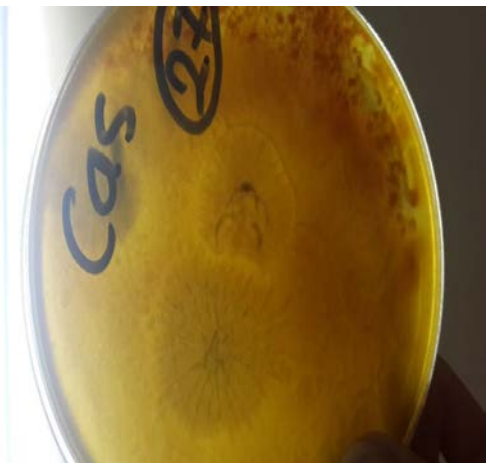

Fig. 2: Primary screening by casein hydrolysis test

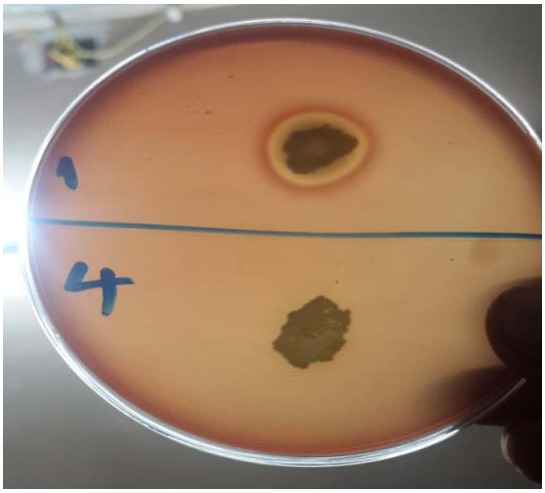

Fig. 3: Hemolysis of blood agar media (inhibition zone)

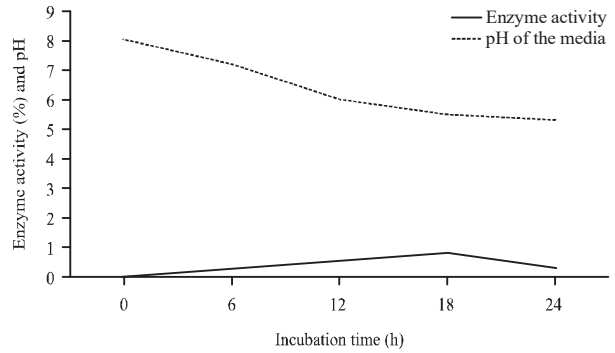

Fig. 4: Activity of actinokinase and pH against incubation time (h)

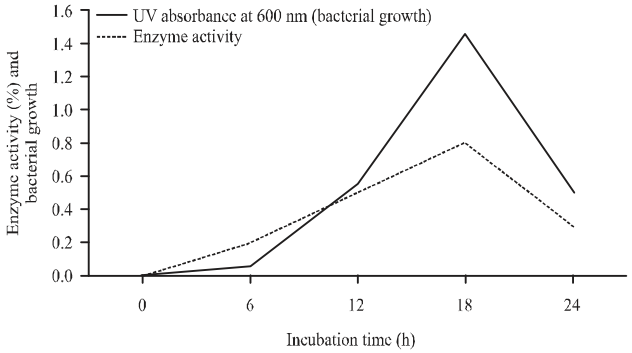

Fig. 5: Actinokinase activity profile and *Streptomyces* growth at different incubation time

Bacterial biomass and enzyme activity: Fibrinolytic activity and maximum growth of the bacterium from the local isolated *Streptomyces* in different time periods 0-24 h (Fig. 5). The activity of the enzyme reached a maximum within 18 h of inoculation, beyond 18 h of growth, no increase in enzyme activity was recorded. The two profiles were similar and show that the fermentation kinetics of actinokinase production by *Streptomyces* might be classified as growth associated. Enzyme production was found to be concomitant with growth.

Time of clot lysis: Blood clot was slowly began to dissolve with the time until complete lysis occur within 20 min (Fig. 6).

Response surface methodology analysis: RSM allow the calculation of maximum production based on a set of experiments in which all the factors were varied within chosen ranges. Enzyme activity achieved for each variables concentration and the experimental combination for the two factors two level 2^3 response surface analysis are shown in Table 2.

The analysis was done using un-coded units. The matrix developed by the two-level full factorial design and the results were shown in Table 2. The fibrinolytic enzyme production varied from 0.14-20.6%. The analysis of variance (ANOVA) was used to analyze the main effects as shown in Table 3. The

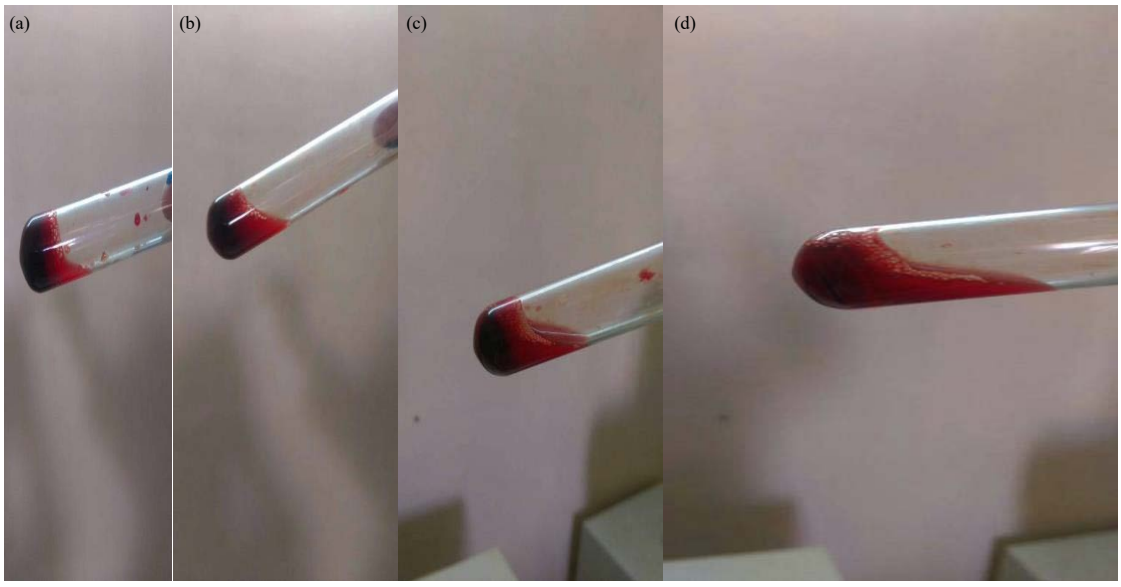

Fig. 6(a-d): Time profile of thrombolytic activity of the crude actinokinase at, (a) 5 min, (b) 10 min, (c) 15 min and (d) 20 min

Table 2: Experimental design and results of actinokinase activity using two methods

Standard order	Run order	Type blocks	Blocks	pH	Temperature	Actinokinase activity using drop in pH	Actinokinase activity using percentage of clot lysis
8	1	-1	1	8.5	58.1	8.23	14.43
7	2	-1	1	8.5	36.9	24.70	3.92
5	3	-1	1	6.4	47.5	7.84	0.85
12	4	0	1	8.5	47.5	10.70	7.51
6	5	-1	1	10.6	47.5	22.59	20.60
11	6	0	1	8.5	47.5	11.29	8.20
4	7	1	1	10.0	55.0	18.50	10.88
10	8	0	1	8.5	47.5	9.41	7.60
9	9	0	1	8.5	47.5	9.41	7.53
3	10	1	1	7.0	55.0	11.40	4.11
2	11	1	1	10.0	40.0	32.00	14.32
13	12	0	1	8.5	47.5	10.47	8.51
1	13	1	1	7.0	40.0	8.57	0.14

Table 3: Regression results of a polynomial model for actinokinase

Terms	Coefficient	SE coefficient	T-value	p-value
Constant	60.13	32.32	1.86	0.105
pH	6.21	4.22	1.47	0.185
Temperature	-3.37	0.88	-3.82	0.007
pH×pH	1.11	0.20	5.57	0.001
Temperature×Temperature	0.07	0.01	8.69	0.000
pH×Temperature	-0.44	0.05	-8.45	0.000

S: 1.18, Press: 54.60, R-Sq: 98.53%, R-Sq(pred.): 91.81%, R-Sq(adj.): 97.4%, SE: Standard error

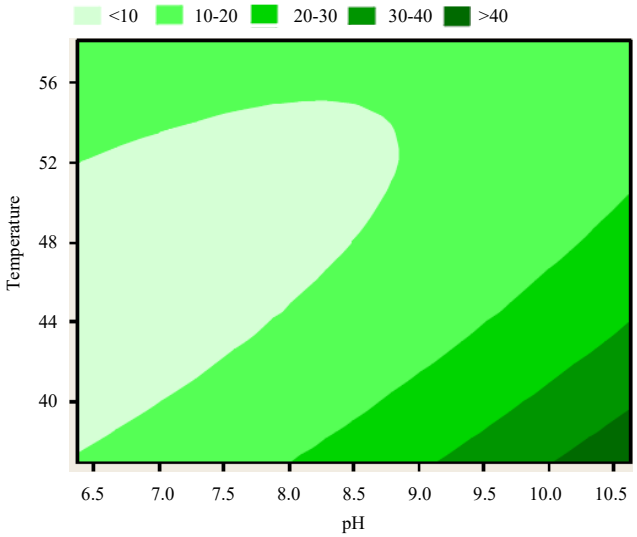

Fig. 7: Contour plot of enzyme activity (%) of drop in pH vs. temperature

estimated model F-value of 93.63 with error <0.05 indicates that the model terms are significant. The predicted R-squared of 91.81% is in reasonable agreement with the adjusted R-squared of 97.4%. The R^2 value (98.53%) also indicates that only (1.47%) of the total variation was not explained by the estimated model. This indicates that the variation in those selected factors could explain the variation in actinokinase activity up to 98.5%. Adequate precision (PRESS) measures the signal-to-noise ratio. A ratio greater than 4 is desirable. In this estimated model, the ratio of 54.6 indicates an adequate signal. For each term in the model, there is a coefficient. In this estimated model, enzyme production was directly and highly significantly affected by temperature ($p<0.01$). The temperature coefficient was negative (-3.37), which states that the lower levels of temperature would benefit fibrinolytic enzyme production. The interaction effects revealed a p-value of 0.000 for the pH by temperature interaction is less than 0.05. Therefore, there is a significant interaction effect. That is, the effect of pH on fibrinolytic enzyme production depends on the temperature. Squared effects which representative by squared terms in the model are used to evaluate whether or not there is curvature in the response surface. The p-value of 0.001 for the squared effects is less than 0.05. Therefore, there is significant evidence of a quadratic effect. The individual p-values for pH×pH and Temperature×Temperature are 0.000 and 0.001, respectively, indicating that the relationships between pH and fibrinolytic enzyme production and temperature and fibrinolytic enzyme production follows a curved line. The estimated model of this study has a good performance, which explained by the variation due to model inadequacy (Lack-of-Fit). The p-value of 0.132 is not less than 0.05. Therefore, there is no evidence that the estimated model of this study does not adequately explain the variation in the responses. Neglecting the insignificant variable, the estimated model equation for optimal actinokinase yield is as follows:

$$\text{Yield} = 60.13+6.21\ \text{pH}-3.37\ \text{temperature}+1.11\ \text{pH}^2+0.07\ \text{temperature}^2 -0.44\ \text{pH}\times\text{Temperature}$$

Global solution: According to the two-level full factorial design, the optimum physical conditions were as follows: pH of 10.5 and temperature of 37°C. Predicted responses enzyme activity (%) = 47.37, desirability = 1.000. Composite desirability = 1.000 (Fig. 7 and 8).

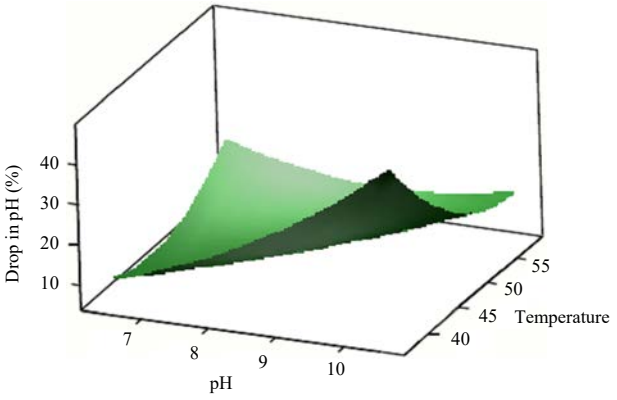

Fig. 8: Surface plot for the optimization of temperature and pH on actinokinase

DISCUSSION

The present study revealed that 90% were positive thermophilic bacterial isolate, gram positive filamentous bacteria, non-motile and confirmed by biochemical and morphological characteristics to be *Thermophilic streptomyces*. About 32% was found to be fibrinolytic bacteria that produce actinokinase which confirmed by the clear zone around Streptomyces colonies when cultured in a blood agar media, which was found to be a high percentage when compared with other study where they found only 14% of total isolates were found to be actinokinase producer[4] and than previous study[24] who stated that, 50% from total isolates was found to be *Thermophilic streptomyces*, these differences may be due to difference in the source of the sample and soil type, The results indicate that the soil-sourced Khartoum possesses high level of actinokinase productivity and belongs to *Thermophilic streptomyces*. The batch fermentation of production of actinokinase were successfully carried out using fermentation broth, the pH of the fermentation broth was dropped toward the acidic pH (5.5) after 18 h of incubation, change in pH is an indicator of actinokinase production there is no report for the development of acidic pH during the production of other fibrinolytic enzymes and these results were in accordance with reported study of actinokinase[4,25]. Maximum growth of the *Streptomyce*s was obtained after 18 h of cultivation also actinokinase activity reached a maximum within 18 h after inoculation beyond 18 h of cultivation no increase in bacterial growth or actinokinase activity was recorded. The two profiles were found to be similar indicating that the fermentation kinetics of actinokinase production by *Streptomyce*s is growth associated, that agreed with previous study[4] but disagreed with study that nattokinase enzyme produced by *Bacillus natto* strain within 22-120 h of incubation time at 37°C[26]. A novel fibrinolytic enzyme from *Rhizopu schinesis* requires 5 days of incubation at 30°C under different culture conditions[5]. Previous reported study found that the maxima of fibrinolytic and caseinolytic activities by *Streptomyces rimosus* were reached at 84 hr. incubation[27], while another study showed that production of actinokinase began after 72 h of cultivation (1.3 U mL^{-1}) and reached to maximum level (3.9 U mL^{-1}) after 120 h of incubation[28]. In this study the *in vitro* fibrinolytic activity of actinokinase produced by *Streptomyces* studied at different incubation time ranged from 0-20 min. It showed clot lysis start after 5 min and continue until 20 min interestingly the actinokinase is still a crude enzyme not purified yet, this agree with study that found that actinokinase could dissolve the blood clot within 20 min[29], while urokinase and streptokinase took 40 and 80 min and also in accordance with other study[4]. The requirement of ideal biotechnological process is isolation of industrial microorganism of high yield production, so to satisfy this requirement the knowledge of various fermentation parameters are necessary, for this purpose we optimized physical conditions in term of pH and temperature. The extracellular pH has a strong influence on the pathways of metabolism and product generation by micro-organism, the temperature also of importance that affects the conversion efficiency of substrate to cell mass which affect the product particularly when the product is growth associated, in addition, the temperature also affect oxygen concentration and mass transfer rate in fermentation broth and, therefore influenced microbial metabolism[30]. The most significant factors such as pH and temperature were selected for this optimization study to increase the yield of actinokinase extracted from local *Streptomyces*. The limitations of a single factor optimization process can be eliminated by optimizing all contributing process parameters collectively using statistical experimental design which constitutes an efficient tool and is well adopted for treating problems with large number of variables of all component[23,31]. In the present article the optimum pH for actinokinase production from local *Streptomyces* was found to be 10.6 revealed that enzyme produced under alkaliphilic conditions, the pH of the substrate greatly affected enzyme production, which agree with previous study[32]. The optimum temperature in this study was found to be of 36.89°C that gave maximum crude action kinase yield of 47.3%. This result result was not in accordance of the results that found that the optimum temperature condition for *S. megasporus* SD5 for enzyme production is

55°C as the microorganism was isolated from a hot spring[32] and optimum pH of 8.0 with maximum pure enzyme yield of 12.5%. Previous study showed that high enzyme activity was recorded at pH range 7.0-9.0 and temperature range 37-70°C[33].

CONCLUSION

Sixteen sample (32%) of the total samples were considered as *Thermophilic streptomyces* according to microscopic and biochemical characteristics, that could produce a promising an extracellular crude actinokinase. Twenty minutes was the time required for complete clot lysis of blood using a crude actinokinase. Maximum activity and maximum bacterial growth reached after 18 h incubation, indicated that the enzyme was found to be growth associated. Response surface methodology reveals that the optimum pH of 10.6 and optimum temperature of 36.89°C achieve the maximum actinokinase activity of 47.37%.

SIGNIFICANCE STATEMENT

This study reveals that Statistical Experimental Design is an efficient tool for physical conditions optimization to increase enzyme production. This study will help the researchers to uncover the critical area of statistical optimization that many researchers were not able to explore.

ACKNOWLEDGMENT

Authors acknowledge Bacteriology Department/Central Lab. Ministry of Higher Education and Scientific Research/ Sudan for their help and support during the lab. work.

REFERENCES

1. Roth, G.A., M.D. Huffman, A.E. Moran, V. Feigin, G.V. Mensah, M. Naghavi and C.J. Murray, 2015. Global and regional patterns in cardiovascular mortality from 1990 to 2013. Circulation, 132: 1667-1678.
2. Furie, B. and B.C. Furie, 2008. Mechanisms of thrombus formation. N. Engl. J. Med., 359: 938-949.
3. Silva, G.M.M., R.P. Bezerra, J.A. Teixeira, T.S. Porto, J.L. Lima-Filho and A.L.F. Porto, 2015. Fibrinolytic protease production by new *Streptomyces* sp: DPUA 1576 from Amazon lichens. Electron. J. Biotechnol., 18: 16-19.
4. Ibrahim, H.M. and M.A.E. Ahmed, 2017. A potential new isolate for actinokinase production. EC Microbiol., 10: 179-185.
5. Liu, J., J. Xing, T. Chang, Z. Ma and H. Liu, 2005. Optimization of nutritional conditions for nattokinase production by *Bacillus natto* NLSSE using statistical experimental methods. Process Biochem., 40: 2757-2762.
6. Deepak, V., S. Ilangovan, M.V. Sampathkumar, M.J. Victoria, S.P.B.S. Pasha, S.B.R.K. Pandian and S. Gurunathan, 2010. Medium optimization and immobilization of purified fibrinolytic URAK from *Bacillus cereus* NK1 on PHB nanoparticles. Enzyme Microbial Technol., 47: 297-304.
7. Mahajan, P.M., S. Nayak and S.S. Lele, 2012. Fibrinolytic enzyme from newly isolated marine bacterium *Bacillus subtilis* ICTF-1: Media optimization, purification and characterization. J. Biosci. Bioeng., 113: 307-314.
8. Dey, S. and R.R. Chitte, 2001. A new thrombolytic enzyme for acute myocardial infarction. Med. Chem. Res., 10: 507-515.
9. Borah, D., R. Yadav, A. Sangra, L. Shahin and A.K. Chaubey, 2012. Production, purification and characterization of nattokinase from *Bacillus subtilis*, isolated from tea garden soil samples of Dibrugarh, Assam. Asian J. Pharm. Clin. Res., 5: 124-125.
10. Bundale, S., D. Begde, N. Nashikkar, T. Kadam and A. Upadhyay, 2015. Optimization of culture conditions for production of bioactive metabolites by *Streptomyces* spp. isolated from soil. Adv. Microbiol., 5: 441-451.
11. Bezerra, M.A., R.E. Santelli, E.P. Oliveira, L.S. Villar and L.A. Escaleira, 2008. Response Surface Methodology (RSM) as a tool for optimization in analytical chemistry. Talanta, 76: 965-977.
12. Ibrahim, H.M. and E.E. Elkhidir, 2011. Response surface method as an efficient tool for medium optimisation. Trends Applied Sci. Res., 6: 121-129.
13. Khuri, A.I., 2017. A general overview of response surface methodology. Biometr. Biostatist. Int. J., Vol. 5, No. 3. 10.15406/bbij.2017.05.00133.
14. Chitte, R.R., 2013. Microbial Source of Fibrinolytic Protease and Its Biotechnological Potential. In: Proteases in Health and Disease, Chakraborti, S. and N. Dhalla (Eds.)., Advances in Biochemistry in Health and Disease, Vol. 7. Springer, New York, pp: 79-88.
15. You, K.M. and Y.K. Prak, 2004. 2004. A new method for the selective isolation of *Actinomycetes* from soil. Biotechnol. Techn., 10: 541-546.
16. Hopwood, D.A., M.J. Bibb, K.F. Chater, T. Kieser and C.J. Bruton *et al*., 1985. Genetic Manipulation of *Streptomyces*: A Laboratory Manual. John Innes Foundation, Norwich, UK., ISBN: 9780708403365, Pages: 356.
17. Williams, S.T. and T. Cross, 1971. Actinomycetes. In: Methods in Microbiology, Booth, C. (Ed.). Vol. 4, Academic Press, London, UK., ISBN-13: 9780080860305, pp: 295-335.
18. Collins, C.H., P.M. Lyne and J.M. Grange, 1995. Collins and Lynes Microbiological Methods. 7th Edn., Butterworth-Heinman, Oxford, Pages: 493.

19. Barrow, G.I. and R.K.A. Feltham, 1993. Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd Edn., Cambridge University Press, Cambridge, UK.
20. Chitte, R.R. and S. Dey, 2003. Streptomyces megasporus SD5, process for the isolation thereof, novel fibrinolytic enzyme prepared therefrom, process for the production of said enzyme and method of treatment of thrombolytic disorders using said enzyme. USA Patent No. US6638503B2.
21. Prasad, S., R.S. Kashyap, J.Y. Deopujari, H.J. Purohit, G.M. Taori and H.F. Daginawala, 2006. Development of an *in vitro* model to study clot lysis activity of thrombolytic drugs. Thrombosis J., Vol. 4. 10.1186/1477-9560-4-14.
22. Ibrahim, H.M., W.M.W. Yusoff, A.A. Hamid, R.M. Illias, O. Hassan and O. Omar, 2005. Optimization of medium for the production of β-cyclodextrin glucanotransferase using Central Composite Design (CCD). Process Biochem., 40: 753-758.
23. Box, G.E.P., W.G. Hunter and J.S. Hunter, 1978. Statistics for Experimenters: An Introduction to Design, Data Analysis and Model Building. John Wiley and Sons Inc., New York, USA., ISBN-13: 9780471093152, Pages: 653.
24. Rama, T. and R. Vibhuti, 2011. Isolation and biochemical characterization of thermophilic Streptomyces from soil of Raipur district of Chhattisgarh, India. Res. J. Pharm. Biol. Chem. Sci., 2: 795-808.
25. Chitte, R.R., S.V. Deshmukh and P.P. Kanekar, 2011. Production, purification and biochemical characterization of a fibrinolytic enzyme from thermophilic *Streptomyces* sp. MCMB-379. Applied Biochem. Biotechnol., 165: 1406-1413.
26. Wang, C., M. Du, D. Zheng, F. Kong, G. Zu and Y. Feng, 2009. Purification and characterization of nattokinase from *Bacillus subtilis* natto B-12. J. Agric. Food Chem., 57: 9722-9729.
27. Gesheva, V., 2009. Production of fibrinolytic enzyme by *Streptomyces rimosus* at conditions of nitrogen limitation. J. Microbiol. Biochem. Technol., 1: 57-58.
28. El-Sabbagh, S.M., N.H. El-Batanony and T.A. Salem, 2013. L-Asparaginase produced by *Streptomyces* strain isolated from Egyptian soil: Purification, characterization and evaluation of its anti-tumor. Afr. J. Microbiol. Res., 7: 5677-5686.
29. Peng, Y., X. Yang and Y. Zhang, 2005. Microbial fibrinolytic enzymes: An overview of source, production, properties and thrombolytic activity *in vivo*. Applied Microbiol. Biotechnol., 69: 126-132.
30. Ju, S., Z. Cao, C. Wong, Y. Liu and M.F. Foda *et al*., 2019. Isolation and optimal fermentation condition of the *Bacillus subtilis* Subsp. natto Strain WTC016 for Nattokinase Production. Fermentation, Vol. 5, No. 4. 10.3390/fermentation5040092.
31. Davis, L., 1993. Efficiency in Research Development and Production: The Statistical Design and Analysis of Chemical Experiment. Royal Society of Chemistry, Cambridge.
32. Chitte, R.R. and S. Dey, 2002. Production of a fibrinolytic enzyme by thermophilic *Streptomyces* species. World J. Microbiol. Biotechnol., 18: 289-294.
33. Chitte, R.R. and S. Dey, 2000. Potent fibrinolytic enzyme from a thermophilic *Streptomyces megasporus* strain SD5. Lett. Applied Microbiol., 31: 405-410.