American Journal of Microbiological Research, 2015, Vol. 3, No. 5, 165-170
Available online at http://pubs.sciepub.com/ajmr/3/5/3

DOI:10.12691/ajmr-3-5-3

Optimization of Culture Conditions to Enhance Nattokinase Production Using RSM

Kawther Isam Eldeen[1], Hanan Moawia Ibrahim[1,*], Elrashied Elimam Elkhidir[2], Hassan Beshir Elamin[3]

[1]Central Lab. Ministry of Higher Education and Scientific Research, Sudan
[2]Faculty of Agriculture, University of Khartoum, Ministry of Higher Education and Scientific Research, Sudan
[3]Commission of Biotechnology and Genetic Engineering, National Centre for Research, Sudan
*Corresponding author: hara111@yahoo.com

Received August 27, 2015; Revised September 13, 2015; Accepted September 28, 2015

Abstract This study was conducted with the aim to isolate, characterize and optimize of *Bacillus subtilis* that produce nattokinase with highest activity under optimal culture conditions. The study was carried out on 25 samples collected from different regions in Khartoum. Primary screening and characterization of the microorganism showed that seven out of 25 samples (28%) were presumptively considered *Bacillus subtilis* according to microscopic and biochemical analysis. The promising isolates produced an extracellular crude nattokinase indicating by heamolytic and fibrinolytic activity. Selective medium was prepared for the extraction and production of nattokinase from these new isolates. Optimization of enzymatic production using statistical experimental design was carried out using central composite design (CCD) in response to surface methodology (RSM) to achieve the maximum nattokinase yield such as temperature and pH in shake flask culture from *Bacillus* spp. The optimal conditions for maximum enzyme production were found to be of 45°C and pH of 5.0. This study reveals that under optimized conditions, nattokinase yield was significantly increased which was higher than earlier reports and promises the use of RSM as an efficient tool for nattokinase production

Keywords: *optimization, nattokinase, RSM, fibrinolytic activity*

Cite This Article: Kawther Isam, Hanan Moawia Ibrahim, Elrashied Elimam Elkhidir, and Hassan Beshir Elamin, "Optimization of Culture Conditions to Enhance Nattokinase Production Using RSM." *American Journal of Microbiological Research*, vol. 3, no. 5 (2015): 165-170. doi: 10.12691/ajmr-3-5-3.

1. Introduction

Nattokinase is an enzyme that finds a wide range of applications in Pharmaceutical industry, health care and Medicine, Its digests fibrin both directly and indirectly. Indirectly, it activates pro- urokinase 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 [1]. Nattokinase was discovered in Natto, a fermented cheese-like food that has been used in Japan for over 1000 year [2].

Nattokinase is a potent fibrinolytic enzyme produced by a fermentation process by adding *Bacillus* natto, a benefical bacteria, to boiled soybeans, it is only the natto preparation that contains the specific nattokinase [3]. Nattokinase a particularly potent treatment, it enhances the body's natural ability to fight blood clots in several different ways; because it so closely resembles plasmin, it dissolves fibrin directly. In addition, it also enhances the body's production of both plasmin and other clot-dissolving agents, including urokinase (endogenous) [4]. Nattokinase produces a prolonged action (unlike antithrombin drugs that wear off shortly after IV treatment is discontinued) in two ways: it prevents coagulation of blood and it dissolves existing thrombus. Both the efficacy and the prolonged action of NK can be determined by measuring levels of euglobulinfibrinolytic activity (EFA) and fibrin degradation products (FDP), which both become elevated as fibrin is being dissolved [3].

Fibrinolytic enzymes such as Nattokinase, Streptokinase and Urokinase, used as thrombolytic agent but too costly and also used through intravenous instillation, needs large scale production by some alternative methods and high purity. So, isolation, production, purification, assay and characterization of fibrinolytic enzymes from bacterial sources are very effective and useful [5]. The selection of optimal conditions is usually based on a combination of the different variables, which involved the optimization of one factor at a time technique. This approach is tedious, time consuming and expensive, more over it does not guarantee the determination of optimum conditions [6]. For a commercial use of nattokinase, it is, therefore, necessary to develop an efficient fermentation process that results in maximum production of the enzyme at high yield, high titer and high productivity. The statistical experimental design constitutes an efficient tool and well adopted for treating problems with large number of variables, and allows simultaneous, systemic and efficient analysis of all

variables [7]. The conventional methods for multifactor experimental design are time-consuming and incapable of detecting the true optimum, due especially to the interactions among the factors, this can be eliminated using response surface methodology (RSM). RSM is a statistical technique for the modeling and optimization of multiple variables, which determines the optimum process conditions through combining experimental designs with interpolation by first- or second-order polynomial equations in a sequential testing procedure [8]. The goals of this study were to recommend an established statistical methodology as an important tool for optimum points and to optimize some culture conditions in term of temperature and pH to enhance Nattokinase production using this statistical experimental design.

2. Materials and Method

25 soil samples were collected into sterilized plastic bags from different locations in Khartoum state, Sudan. Samples were taken from 15-20 cm depth after removing approximately 3 cm of earth surface. During February - April 2015.

2.1. Isolation and Identification of Microorganism

Isolation of microorganism was performed by the soil dilution plate technique [9]. In this technique: 1g of each soil sample was taken in 9 ml of sterilized distill water in pre- sterilized test tube. Serial aqueous dilutions were prepared by transferring 1 ml of the soil suspension into 9 ml of sterilized distill water in sterilized test tubes. Different aqueous dilutions (10^{-7}) of the soil suspension were applied separately into sterilized petri-dishes containing sterilized nutrient agar (which has been prepared by taken 28 g of agar base and pour it into 1 L of water, sterilized by using autoclave, poured it into petri-dishes until cooling and incubated for 24 hrs at 37^0C and checked it for contamination), and incubated for 24 hr at 37 ^{o}C *Bacillus spp*. were determined according to the morphology of the colony. The Identification and characterization of the isolate were performed [10].

2.2. Enzyme Extraction

B. subtilis was grown on basal medium containing (g/lit.) Soya peptone10, K_2HPO_4 2, $MgSO_4$ 1, Maltose 20, Yeast extract 10, Glucose 2 in 1000 ml distilled water. The pH was adjusted to 7.2 with 2 M acetic acid and 2 M NaOH Medium was sterilized by autoclaving at 121°C for 35 min and cooled to room temperature. One ml of uniformly prepared suspension of *B. subtilis* used as inoculums; incubated at 37°C and 150 rpm in an orbital shaker. After 2 days of fermentation, cells were removed by centrifugation [6,11].

2.3. Enzyme Assay

Fibrinolytic activity was determined by conducted mixing of (0.2) mL of human plasma and (0.8) mL of normal saline with (0.25) mL of (0.25%) of calcium chloride, (0.5) mL of liquid bacterial growth in the test tube and (0.5) mL of normal saline in the control tube then mixed and incubated for one hours at 37°C. The positive result is heamolytic the clot formed. The number of units was determined according to standard curve by using bovine serum albumin. The A595 nm for the supernatant was measured and converted to the amount of protein equivalent. One unit of Fibrinolytic Activity (FU) was defined as the amount of enzyme releasing 0.2 ml of soluble plasma equivalent per one hour [12].

2.4. Time Course of Enzyme Production

The colonies that showed large zone on blood agar media were used for inoculum preparation. A volume of 200 ml of brain heart infusion broth taken in a 250-ml Erlenmeyer flask was inoculated with a loop full of cells from a 24-hr. old slant and kept at 37 ºC in a rotary shaker. After 18 hour of incubation, 10 ml of this broth culture was used as the inoculums.

A 10% inoculum was added to 100 ml of production medium into 250 ml Erlenmeyer flask. The cultures were incubated at 37^{o}C and 140 rpm on a rotary shaker for 72h. Samples were removed periodically every 12h and cell growth as well as nattokinase activity was determined [13].

2.5. Experimental Design

To optimize the culture conditions for nattokinase production. The surface response for enzyme production as a function of selected key variable was determined. A two-level full fractional factorial design with two variables consisting of two blocks and with 14 runs (8 combinations with 6 replications of the center points) were employed as in Table 1. The Minitab (release 13.2) was used to describe the response surface method.

Table 1. Variables in experimental level of nattokinase production

RunOrder	Blocks	Temp	pH
1	1	45.0000	5.00000
2	1	45.0000	9.00000
3	1	37.0000	7.00000
4	1	29.0000	5.00000
5	1	37.0000	7.00000
6	1	37.0000	7.00000
7	1	29.0000	9.00000
8	2	25.6863	7.00000
9	2	37.0000	4.17157
10	2	48.3137	7.00000
11	2	37.0000	9.82843
12	2	37.0000	7.00000
13	2	37.0000	7.00000
14	2	37.0000	7.00000

The optimization of the temperature and pH for nattokinase activity, the surface response for enzyme production as a function of selected key variable has to be predetermined. Therefore, according to the literature review, the temperature range tested in the final optimization step was (29- 45°C) and pH (5- 9). The minimum and maximum ranges of variables were investigated Table 1 and the full experimental plan with respect to their values in actual and coded form was listed in (Table 2). A 2^2 factorial design and 6 replications at the centre point, with number of 14 experiments were employed.

Table 2. Process variables and their levels in the two variables-two levels response surface design

Independent variables	symbol		levels	
	coded	decoded	coded	uncoded
Temperature	X_1	°C	1	45
			0	37
			-1	29
pH	X_2	-	1	9
			0	7
			-1	5

The empirical formula to find the optimal nattokinase yield is given by:

$$Y = \alpha + \beta_1 X_1 + \beta_1 X_1 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

where A, B, C, D are the coded forms of pH and temperature, respectively. AB, AC, AD, BC, BD, and CD are the interaction terms, and A^2, B^2, C^2, and D^2 are the squared terms of the independent variables. Three dimensional surface plots were drawn to show the effects of independent variables on response and a quadratic polynomial equation was proposed to describe the mathematical relationship between the variables and the response. The significance of the model was evaluated by determination of R^2 and adjusted R^2 coefficient. An experiment was also conducted to confirm the predicted optimum response using the selected optimum values of the two variables.

3. Results and Discussion

3.1. Isolation and Identification of Microorganisms

Seven out of 25 samples (28%) were shown to be as *Bacillus subtilis* These organisms showed gram positive and endospore forming bacteria,. also showed positive result for casein hydrolysis, starch hydrolysis, blood hemolysis and even motility test was motile, but negative results for oxidase and indole production test. Nattokinase can now be produced in batch culture, rather than relying on extraction from Nattō [14]. Nattokinase is traditionally produced by fermentation of various microorganisms, among which the genus *Bacillus Subtilis* natto is the preeminent nattokinase producer [15].

3.2. Presence of Nattokinase Produced by *Bacillus substilis*

3.2.1. Fibrinolytic Activity:

The fibrinolytic activity of nattokinase was measured by casein and fibrin plate. It is an excellent qualitative performance. The measurement of the dimension of the clear zone around each organism indicates nattokinase activity. Table 3 shows the clear zone of *Bacillus substilis*. Figure 1 and Figure 2 showed the primary screening of the sample using blood hemolysis and Casein hydrolysis. However, [16] reported that intravenous administration did not show any clear thrombolytic effect but oral administration enhanced fibrinolytic activity. Nattokinase degrades fibrin clots both directly and indirectly by affecting plasmingen activator inhibitor. Researchers suggest that nattokinase may promote normal blood pressure, reduce whole blood viscosity and increase circulation being an effective supplement to support cardiovascular health [17]. In another study [3] reported that when nattokinase 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.

Table 3. Illustrates the dimension of clear zone around casein hydrolysis and blood hemolysis

Microorganism	Dimension of the clear zone (mm) using casein hydrolysis	Dimension of the clear zone (mm) using Blood hemolysis
Sample 2 *(Bacillus substilis)*	21	20

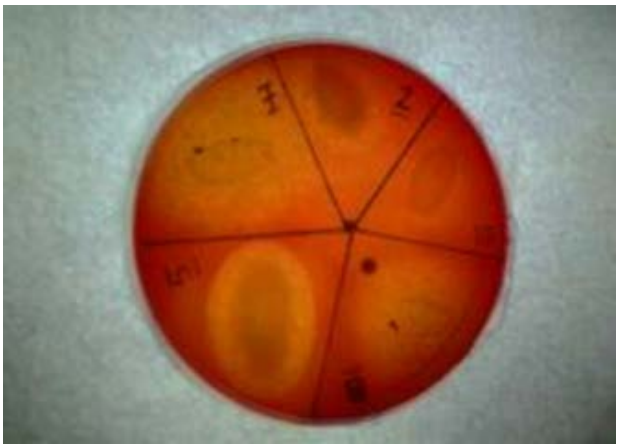

Figure 1. Primary screening of sample (2) using blood hemolysis

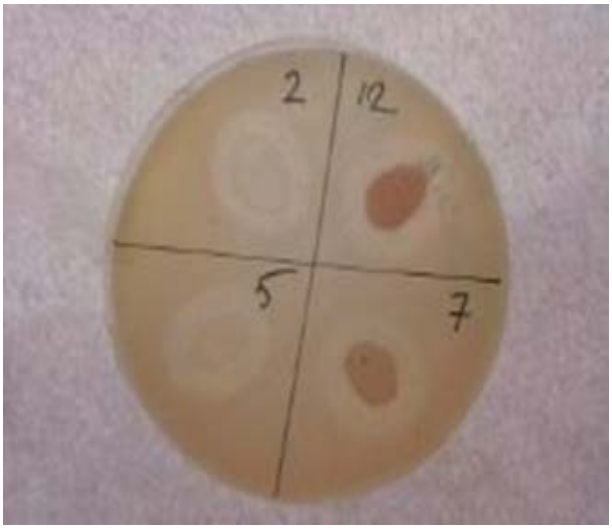

Figure 2. Primary screening of sample (2) using casein hydrolysis

The enzyme activity and the bacterial growth kinetics are depicted on Figure 3 Fibrinolytic Enzyme production from the local isolated *Bacillus substilis* in different time periods (0-72) hr, Figure 3 Show that the growth profile during the 72-hr cultivation Maximum growth of the bacterium was obtained with 24hr of cultivation. The activity of the enzyme reached a maximum within 24 hr after inoculation, beyond 24hr of growth, no increase in enzyme activity was recorded. The two profiles were similar and show that the fermentation kinetics of nattokinase production by *Bacillus substilis* might be

classified as growth associated enzyme, production was found to be concomitant with growth.

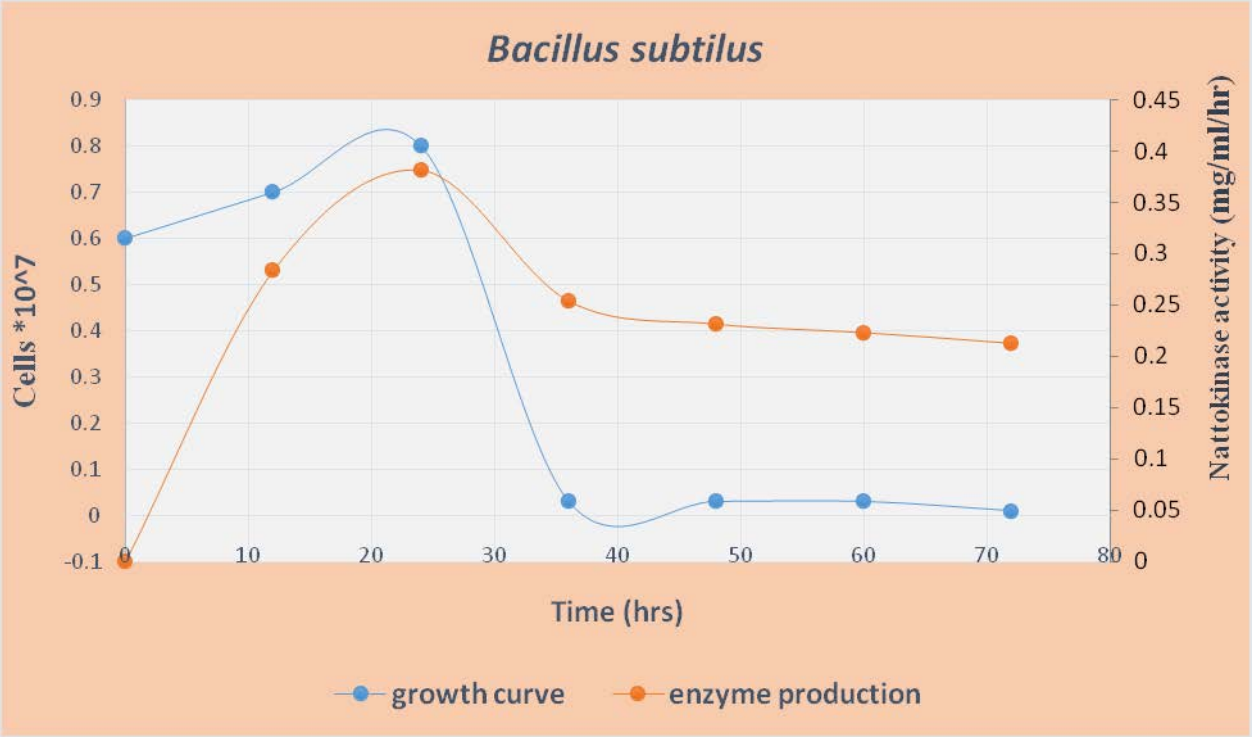

Figure 3. Nattokinase activity profile during the growth of *B. substilis*

3.3. CCD Design

The overall effect of experimental operation factors; temperature and pH on nattokinase production is shown in Table 4. It was found that those factors all exerted certain effect on response and percent variability explained adj (R^2) was 76.55 %. The adj R^2 value also indicates that only 23.45 % of the total variation is not explained by the model. This indicated that the variation in those selected factors could explain the variation in nattokinase activity up to 76.55 %.

The regression coefficient, standard error, t- values, p-values, for theb full quadratic model of nattokinase production are presented in Table 4, made the fitted second-order polynomial more acceptable.

Table 4. Estimated regression coefficients for concentration mg/ml

Term	Coef	SE Coef	T	P
Constant	-0.433626	0.840962	-0.516	0.622
Block	-0.038407	0.018229	-2.107	0.073
temp	0.064566	0.032771	1.970	0.089
pH	-0.093691	0.118670	-0.790	0.456
temp*temp	-0.000457	0.000392	-1.165	0.282
pH*pH	0.022692	0.006275	3.616	0.009
temp*pH	-0.006299	0.002131	-2.955	0.021

S = 0.0682077 PRESS = 0.304386, R^2 = 87.38% R^2(pred) = 0.00% R^2(adj) = 76.55%.

Table 5. Analysis of variance for the regression model of nattokinase production: Analysis of variance for concentration mg/ml

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Blocks	1	0.020751	0.020651	0.020651	4.44	0.073
Regression	5	0.204745	0.204745	0.040949	8.80	0.006
Linear	2	0.093533	0.029778	0.014889	3.20	0.103
temp	1	0.090908	0.018059	0.018059	3.88	0.089
pH	1	0.002626	0.002900	0.002900	0.62	0.456
Square	2	0.070583	0.070583	0.035292	7.59	0.018
temp*temp	1	0.009742	0.006310	0.006310	1.36	0.282
pH*pH	1	0.060841	0.060841	0.060841	13.08	0.009
Interaction	1	0.040628	0.040628	0.040628	8.73	0.021
temp*pH	1	0.040628	0.040628	0.040628	8.73	0.02
Residual Error	7	0.032566	0.032566	0.004652		
Lack-of-Fit	3	0.032566	0.032566	0.010855	*	*
Pure Error	4	0.000000	0.000000	0.000000		
Total	13	0.257961				

The behavior of the system was explained by the following second-order polynomial equation predicted by the model for maximum nattokinase and by applying the regression analysis on the experimental data the equation was found to represent the nattokinase production

Nattokinase production

$$= -\mathbf{0}.433626 + 0.064566\text{temp} - 0.093691\ \text{pH} \quad (2)$$
$$-0.000457\text{temp}^2 + 0.022692\text{pH}^2 - 0.006299\text{temp} * \text{pH}$$

Where Y is the measured response in nattokinase yield. A, B, C, and D are the coded independent inputs. β_0 is the intercept term and β_1, β_2, β_3, and β_4 are the coefficients showing the linear effects. β_5, β_6, β_7, β_8, β_9, and β_{10} are the cross-product coefficients showing the interaction effects. β_{11}, β_{12}, β_{13}, and β_{14} are the quadratic coefficients showing the squared effects [19].

To obtain the maximum optimum activity the factor levels was set as values given by MINI TAB Multiple Response Optimizer under global solution of desirability equal to one. That is the temperature would be 45°C and pH at 5. As shown in Table 6.

Table 6. The derived optimum levels of the physical environment within context

Number	Temp	pH	Response	Desirability=1	
1	45.0000	5.00	2.23647	0.779	Selected
2	45.0000	9.00	1.7926	0.564	

The three-dimensional graphs and contour graphs are the common graphical representation of the regression equation which shows the optimal values of each dependent variables [16]. Figure 4 and Figure 5 represent the surface and response contour plots for the optimization of temperature and pH on nattokinase production.

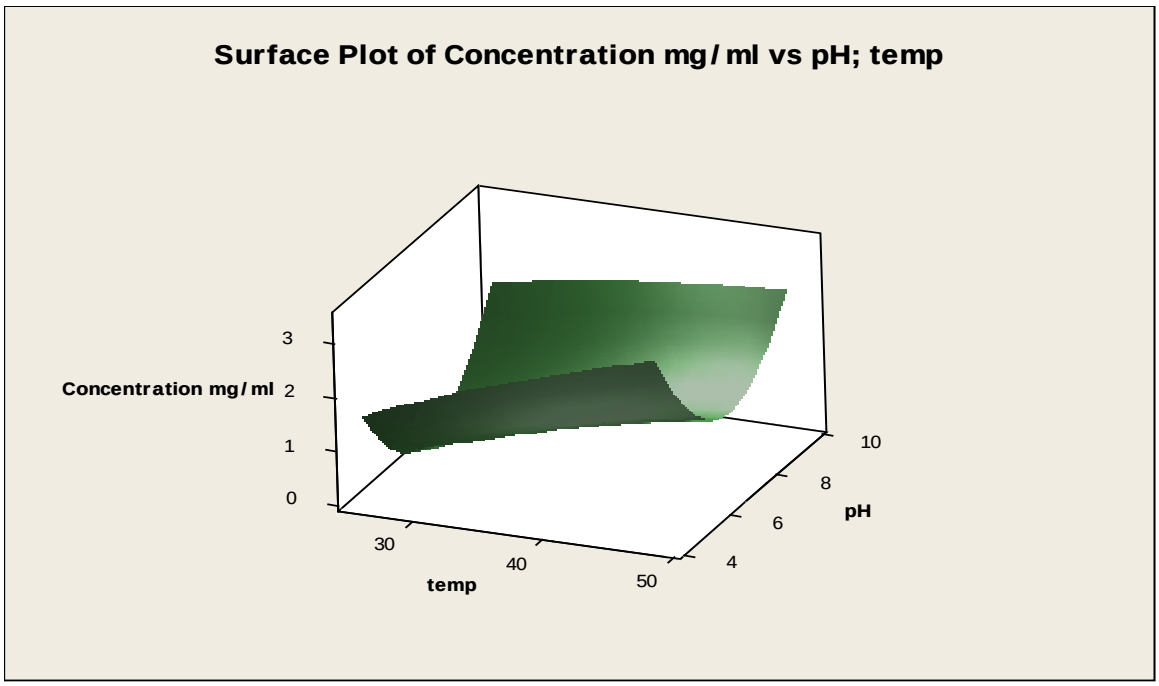

Figure 4. Fitted surface temperature-pH-enzyme activity. Three dimensional surface plot

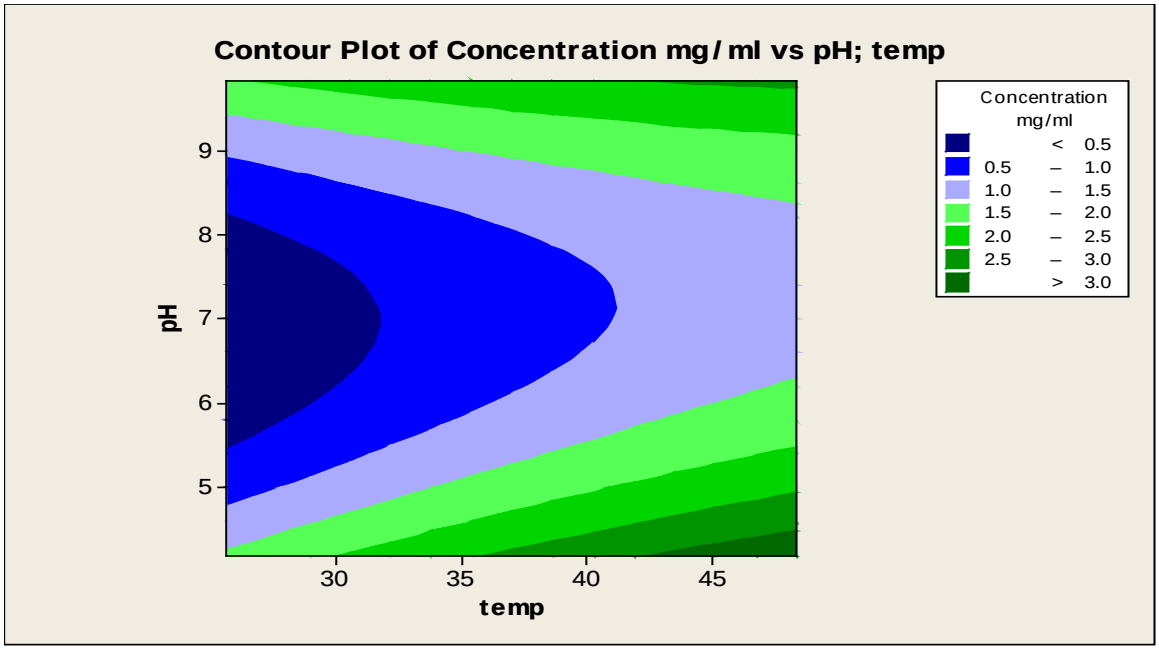

Figure 5. Contour plot of nattokinase activity

4. Conclusion

Twenty five soil samples brought from different locations in Sudan. Seven out of 25 samples (28%) were considered as *Bacillus subtilis* according to Microscopic and Biochemical analysis. The promising isolates also could produce an extracellular crude nattokinase indicating by heamolytic and fibrinolytic activity.

The optimization of the physical conditions as temperature and pH enhanced the production of nattokinase by *Bacillus* sp. The optimal conditions for *Bacillus substilis* were found to be 45°C and pH at 5 that gave a maximum yield using RSM.

Acknowledgment

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

References

[1] Borah, D.; Yadav, R.N.S.; Sangra, A.; Shahin, L. and Chaubey, A.K. (2012). Production, purification and characterization of nattokinase from *Bacillus*. Asian Journal of Pharmaceutical and Clinical Research. 5(3): (124-125).

[2] Haritha, M.and Meena, V. (2011). Nattokinase: A review of fibrinolytic enzyme. Chemical, environmental and pharmaceutical research 2(1) 61-66.

[3] Sumi, H.; Hamada, H.; Nakanishi, K. and Hiratani, H. (1990). Enhancement of the fibrinolytic activity in plasma by oral administration of Nattokinase.Acta.Haematol. 84, 139-143.

[4] Martin Milner, N.D. and KouheiMakise, M.D. (June 2002).Natto and Its Active Ingredient Nattokinase, Alternative and complementary therapies.

[5] Dubey R, J. Kumar, D. Agrawala, T. Char and P. Pusp.(2011) Isolation, production, purification, assay and characterization of fibrinolytic enzymes (Nattokinase, Streptokinase and Urokinase) from bacterial sources. African Journal of Biotechnology. 10(8), pp. 1408-1420.

[6] Ibrahim, H. M. & Elrashied Elimam Elkhidir (2011) " Response Surface Method as an Efficient Tool for Medium Optimization" Trents in Applied Sciences Research 6: 121-129.

[7] Ibrahim, H. m. Wan Yusoff, W.M.;, Hamid, A.A, A.A.;Illias and Omar, O. (2003) "Optimization of the Process Conditions for Cyclodextrin Glucanotransferase Production using Response Surface Methodology" Asian Journal of Microbiology, Biotechnology and Environmental Sciences. 5(3): 297-300.

[8] Myers, R. H., D. C. Montgomery, and C. M. Anderson-Cook, Response Surface Methodology: Process and Product Optimization Using Designed Experiments, Wiley, New York, NY, USA, 3rd edition, 2009.

[9] You, K. M. and Prak,Y.K.(2004). A new method for the selective isolation of Actinomycetesfrom soil. Biotechnol. Techn.10:541-546

[10] Barrow, G.I. and Feltham, R.K.A. (1993). Cowan and Steel's Manual for the Identification of Medical Bacteria, (3rd ed). Cambridge University Press, Cambridge, p.352.

[11] Atlas, R.M. (1997). Handbook of Microbiological Media; 2nd ed.,CRC press,Boca Raton, New York, London, Tokyo, 1706 pp.

[12] Tillett, W.S. and Garner, R.L. (1933). The fibrinolytic activity of heamolytic *Streptococci*. J. Exp. Med., 58: 485.

[13] Ibrahim, H. M.; Wan Yusoff, W.M.; Hamid, A.A.;Illias, R.M.; Hassan, O. and Omar, O. (2004).Optimization of Medium for the Production of β-Cyclodextrin glucanotransferase using Central Composite Design (CCD).*Process Biochemistry* 40(2): (753-758).

[14] Kwon, E. Y.; Kim, K. M.; Kim, M. K.; Lee, I. Y.; Kim, B. S. (2011). "Production of nattokinase by high cell density fed-batch culture of Bacillus subtilis".Bioprocess and Biosystems Engineering 34 (7): 789-793.

[15] Kim, S.H. and Choi, N.S. (2000). Purification and characterization of subtilisin DJ-4 secreted by *Bacillus sp.* strain DJ-4 screened from Doen-Jang, Biosci. Biotechnol.Biochem. 64 1722-1725.

[16] Sumi, H. Hamada, H. Tsushima, H. Mihara, H. Muraki, H. 1987. A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet. Experientia 43(10):1110-1.

[17] Japanese Nattkinase Association (JNKA) 2004. http://www.j-nattokinase.org.

[18] Chen X, Wei D, Liu D (2008) Response surface optimization of biocatalytic biodiesel production with acid oil. Biochem Eng J 40:423-429.

[19] Nadyaini WN, Omar W, Aishah N, Amin S (2011) Optimization of heterogeneous biodiesel production from waste cooking palm oil via response surface methodology. Biomass Bioenergy 35:1329-1338.