

Enhanced production of streptokinase from *Streptococcus agalactiae* EBL-31 by response surface methodology

Arooj Arshad^{1*}, Muhammad Anjum Zia¹, Muhammad Asgher¹,
Faiz Ahmad Joyia² and Muhammad Arif³

¹Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan

²Center of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad, Pakistan

³Department of Mathematics and Statistics, University of Agriculture, Faisalabad, Pakistan

Abstract: Streptokinase (SK) is a fibrinolytic protein used for the treatment of cardiovascular disorders. In the present study, enhanced production of SK was achieved by determining the optimum fermentation conditions for the maximum growth of *Streptococcus agalactiae* EBL-31 using response surface methodology (RSM). Four process variables (pH, temperature, incubation time and inoculum size) with five levels were evaluated in 30 experimental runs. Central composite rotatable design (CCRD) was employed to predict the effect of independent variables on SK activity. The statistical evaluation by ANOVA showed that the model was fit as the effect of single factors, quadratic effects and most of the interactions among variables. The value of R^2 (0.9988) indicated the satisfactory interaction between the experimental and predicted responses. Furthermore, the model F value (902.67) and coefficient of variation (1.92) clearly showed that the model is significant ($p \Rightarrow >0.0001$). The functional activity of SK was determined by spectrophotometric analysis and maximum SK production was obtained at pH-7.0, temperature- 37.5°C, an incubation time of 36 hours and 2.5 mL inoculum size. Hence it was concluded that the optimization of culture conditions through RSM increases the production of SK by 2.01-fold. Production of SK by fermentation is an economical choice to be used for the treatment of cardiovascular diseases.

Keywords: Cardiovascular disorders, Fermentation, Response surface methodology, Streptokinase.

INTRODUCTION

Thrombosis is the development of blood clot which results into occlusion of blood vessels and eventually circulatory disorders including ischemic stroke, deep vein thrombosis, pulmonary embolism and acute myocardial infarction (AMI) (Collen, 1998). These cardiovascular disorders and their outcomes are increasingly becoming the reason of mortality worldwide. Treatment of these disorders requires clinical interventions including the administration of fibrinolytic agents (Banerjee *et al.*, 2004; Furie and furie, 2008). A number of clot-dissolving agents are available such as streptokinase (SK), urokinase, prourokinase, alteplase (t-PA), reteplase (r-PA), reptilase and brinase. All these agents mediate the conversion of plasminogen to its active form plasmin, which eventually causes the lysis of blood clot by dissolving the network of fibrin in the clot (Rae *et al.*, 2017). Among these agents, SK was the first drug to be used for the therapy of AMI. The basic foundation for SK as a successful thrombolytic agent was laid in 1933 and the drug is now included in the list of essential medicines in World Health Organization (Ghosh, 2012). In comparison to other activators of plasminogen, SK has long half-life and also proved to have high affinity with circulatory plasminogen (Mucklow, 1995).

SK (E.C. 3.4.99.22) is an extra cellular monomeric protein having a molecular weight of 47kDa. The protein creates an active 1:1 stoichiometric complex with plasminogen and triggers the opening of an active site in plasminogen to convert it into active plasmin (Bajaj and Castellino, 1977). The plasminogen present in SK-plasminogen complex displays the proteolytic activity leading to the change of plasminogen molecule to plasmin by proteolysis (Zhang, 1999). It has been hypothesized that SK does not have intrinsic proteolytic activity; instead, it activates plasminogen with which it forms a rigid complex (Castellino, 1981). SK is naturally secreted by a number of streptococcal strains. It has been demonstrated that β -hemolytic streptococci of Lancefield group A, B, C and G produce streptokinase (Kumar *et al.*, 2012; Bhardwaj and Angayarkanni, 2005). Moreover, Group B of *Streptococcus* (*S. agalactiae*) has also recently been reported to produce SK (Babu and Devi, 2015). The growth of microorganisms is always influenced by process optimization which can improve the production considerably and significantly reduce the production cost. In enzyme extraction processes, even small improvements in culture conditions can have a significant effect on commercially successful production (Reddy *et al.*, 2008). The biological processes can be optimized by designing experiments using a statistical approach; one example is the response surface methodology (RSM) which helps in analyzing the simultaneous effect of different factors to

*Corresponding author: e-mail: elegant_aroj@hotmail.com

determine the optimum conditions (Bhardawaj *et al.*, 2017).

With regards to increasing demand of SK as a therapeutic agent, there is an urge to select a high-potential microbial source for SK production. In addition, culture conditions are equally important for the growth of the microorganism and eventually the yield of the end product. The present study was conducted with *S. agalactiae* which is a novel source of SK and has the ability to secrete highly active protein molecules into the extracellular medium with a well-established fermentation technology. For this purpose, cultivation conditions were optimized by RSM which is a preferred method for designing experiments to optimize the natural processes. RSM is preferred over the conventional method of optimization when studying the effect of interaction among the individual parameters. As a result, a cost-effective thrombolytic agent was obtained to be used for the treatment of cardiovascular complications.

MATERIALS AND METHODS

Microorganism and culture conditions

Streptococcus agalactiae EBL-31 was obtained from Enzyme Biotechnology Lab. (EBL) University of Agriculture, Faisalabad (Pakistan). The strain was previously isolated from bovine milk and identified by using API 20 STREP system in EBL. The bacterial culture was maintained on blood agar medium and nutrient agar plates.

Production of streptokinase

The inoculum of *S. agalactiae* EBL-31 was prepared in Todd Hewitt Broth at 37°C for 24 h. The growth of microorganism was observed by liquid state fermentation in Erlenmeyer flask containing 50 mL of pre-optimized production media (g/100mL: Glucose-0.5, Yeast Extract-0.5, K₂HPO₄-0.25, KH₂PO₄-0.25, NaHCO₃-0.1, MgSO₄·7H₂O-0.04, CH₃COONa·3H₂O-0.1, MnCl₂·4H₂O-0.002, and FeSO₄·7H₂O-0.002, pH 7.5) (Baewald *et al.*, 1975). A total of 1mL inoculum was introduced into the production media and allowed to grow by incubating at 37°C for 24 hours. After incubation, the culture was centrifuged at 10,000g for 30 minutes to obtain the supernatant which contained the crude enzyme (Hamid *et al.*, 2012).

Experimental design for the optimized production of streptokinase

The fermentation/cultivation conditions for the production of microorganism were optimized by RSM. A quadratic polynomial design was organized for the process optimization of SK production using *S. agalactiae* EBL-31. The Design-Expert software (version 7.0.0) developed by State-Ease Inc., Minnesota, USA was used to formulate the response surface design for the process optimization of SK. Four factors (pH, temperature,

incubation time and inoculum size) with five levels were analyzed in 30 experimental runs in triplicate to minimize the errors. All the four factors were designated the codes as A, B, C and D, respectively.

STATISTICAL ANALYSIS

Model validation

The calculation of predicted response was completed with the help of second order polynomial equation (Eq. 1), which involves all the interacting terms of the experiment.

$$SK\ yield = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 + \epsilon$$

where,

X₁, X₂ and, X₃ and X₄ are independent variables; β₁, β₂, β₃ and β₄ are coefficients of the polynomial for linear terms; β₁₁, β₂₂, β₃₃ and β₄₄ are coefficient of polynomials for quadratic terms; and β₁₂, β₁₃, β₁₄, β₂₃, β₂₄ and β₃₄ are coefficients of polynomials for the combined effect of variables.

The statistical significance of the observed experimental data was evaluated by analysis of variance (ANOVA). Student's *t*-test was employed to determine the significance of regression coefficient. The interactive effect of the four process variables was analyzed by three-dimensional surface plots to eventually determine the optimum levels of these parameters for enhanced SK production (Umer *et al.*, 2009; Vellanki *et al.*, 2013). The fitness of the regression model obtained was given by the coefficient of determination, i.e., R² (Bhardawaj *et al.*, 2017). The upper and lower limits of independent variables for the production of SK are presented in table 1.

Activity of streptokinase

Casein digestion method was used to analyze the activity of streptokinase by calculating the amount of tyrosine released from hydrolyzed casein after the activation of plasminogen (Mounter and Shipley, 1957). The reaction mixture (2 mL) was prepared by adding 10 mg casein to 50mM Tris-HCl of pH 8.0. After the addition of 0.1 mL supernatant, the temperature was maintained at 37°C and reaction was performed for 20 minutes. After incubating the reaction mixture, 0.4 mL of 3.3 M HCl and 2.6 ml 5% w/v trichloroacetic acid (TCA) was added to terminate the reaction. The reaction mixtures were filtered by Whatman paper after keeping on ice for 30 mins. The absorbance of the filtrate obtained was taken at 280 nm. A calibration curve for SK was used to determine the enzyme activity (El-Mongy and Taha, 2012).

RESULTS

In the present study, indigenously isolated *S. agalactiae* EBL-31 has been exploited for the production of SK by liquid state fermentation to meet the increasing demand of

Table 1: Experimental ranges and levels of independent process variables

Factors	-1	Ranges and levels 0	+1
A, pH	5	7	9
B, Temperature	25	37.50	59
C, Incubation time	24	48	72
D, Inoculum size	1	2.50	4

Table 2: Analysis of variance (ANOVA) for the process optimization of streptokinase from mutant derived strain *S. agalactiae* EBL-31

Sources	Sum of Squares	df	Mean Square	F Value	p-value Prob> F
Model	37956.03	14	2711.15	902.67	< 0.0001
A-pH	1096.38	1	1096.38	365.04	< 0.0001
B-Temperature	17.52	1	17.52	5.83	0.0290
C-Incubation time	3550.51	1	3550.51	1182.14	< 0.0001
D-Inoculum size	2736.74	1	2736.74	911.20	< 0.0001
AB	189.06	1	189.06	62.95	< 0.0001
AC	1387.56	1	1387.56	461.99	< 0.0001
AD	76.56	1	76.56	25.49	0.0001
BC	0.063	1	0.063	0.021	0.8872
BD	351.56	1	351.56	117.05	< 0.0001
CD	52.56	1	52.56	17.50	0.0008
A ²	953.81	1	953.81	317.57	< 0.0001
B ²	289.47	1	289.47	96.38	< 0.0001
C ²	493.47	1	493.47	164.30	< 0.0001
D ²	0.014	1	0.014	4.789E-003	0.9457
Residual	45.05	15	3.00		
Lack of Fit	34.31	10	3.43	1.60	0.3157
Pure Error	10.74	5	2.15		
Cor Total	38001.08	29			
C.V=1.91%R2=0.9988 R2 _{Adj} =0.9977 Predicted R2=0.9934					

fibrinolytic agents. The individual and interactive effects of four independent variables (pH, temperature, incubation time and inoculum size) was investigated by the central composite design of RSM. The second order polynomial coefficient for each term of equation 1 was calculated by means of multiple regression analysis using Design Expert 7.0.0.

$$\text{SK activity (U mL}^{-1}\text{)} = 124.96 + 8.15A - 1.03B - 14.67 C - 12.88D - 3.44 AB + 9.31AC - 2.19 AD + 0.063 BC - 4.69 BD + 1.81 CD - 75.66 A^2 - 41.66 B^2 + 54.34 C^2 + 0.34 D^2 \dots\dots\dots(1)$$

ANOVA table indicates that the model is significant as the F-value for the model is 902.67. The value of "Prob > F" (p) less than 0.0500 indicated that the model terms are significant. The significance of all the determinants was analyzed by p -values shown in table 2. The signal to noise ratio is depicted by adequate precision and the value of 103.50 suggested an adequate signal. It is evident that the precision of experimental data is relatively reasonable. The plot between predicted and actual values and normal probability plot have been shown in fig.1. Adequacy of the model for optimized production of SK was predicted

by normal probability plot. A straight line graph proved that assumptions of normality were satisfactory (Myers and Montgomery, 2002).

The 3-D response surface plots are depicted in fig. 2 (a-f) showing the trend of response at variable levels of factors. Regarding the linear terms, all the four factors involved in the experiment; pH, incubation time, inoculum size ($p < 0.0001$) and reaction temperature ($p = 0.0290$) made the significant contributions to the fitted model. All the quadratic terms except the inoculum size were also found to be significant. Moreover, all the first order interactive effects of the independent variables except the temperature and incubation time were statistically significant.

Based on the experimental results, the maximum production SK with 147.08 U mL⁻¹ from mutant derived *S. agalactiae* EBL-31 was obtained at pH 7; temperature 37.5°C; incubation time 36 hours and inoculum size 2.50mL. In conclusion, an overall 2.01-fold increase in the production of SK was observed after optimization of culture conditions. Our results demonstrated that

optimization of the cultivation conditions has significant impact on the production of SK.

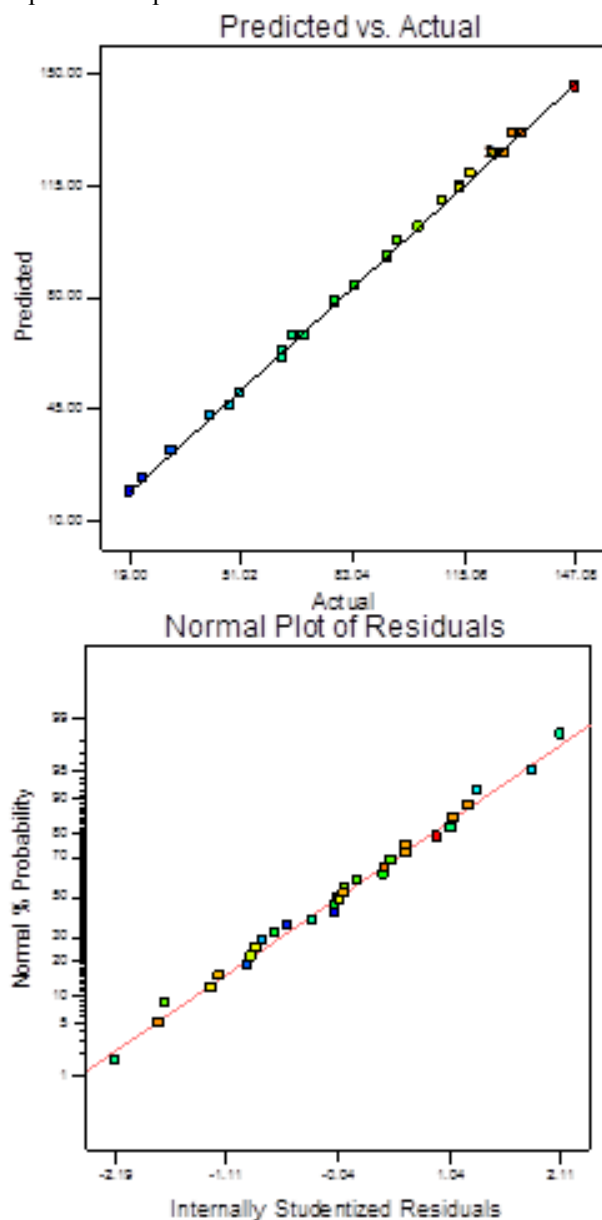


Fig. 1: Plot of predicted versus actual value and normal probability plot for parental *S. agalactiae* EBL-31

DISCUSSION

A non-significant difference was observed between the predicted and actual response values (activity $U\ mL^{-1}$). The ANOVA revealed the comparison between the variation due to the treatment (change in the combination of variable levels) and the variation due to the random errors. Smaller p -value indicates the significance of the independent variables (Khuri *et al.*, 1987). The predicted R^2 (0.9934) is in accordance with adjusted R^2 (0.9977). The divergence between these two values indicates the presence of some non-significant terms in the model. The

higher the values of R^2 and R^2_{adj} , the closer will be the correlation between the experimental and predicted values.

Table 2 indicates that coefficient of variation (C.V) is low, suggesting the reliability precision of the performed experiments (Box *et al.*, 1978). It is evident that the precision of experimental data is relatively reasonable. The plot demonstrating the correspondence between actual and predicted values estimates the accuracy of central composite design for optimization of SK production. A significant and fit model gives approximate values of the actual experiment. The present investigation clearly indicates that the selected variables exert a significant impact on SK production. Vellanki *et al.* (2013) optimized the culture conditions and nutritional requirements for the production of streptokinase from *Saccharomyces cerevisiae*. Student's t -test and p -values were observed to determine each coefficient. It was demonstrated that larger t -value and smaller p -value were more significant to determine the coefficients. With regards to the growth of *S. agalactiae* EBL-31, a very strong interaction was observed between pH and temperature which can be seen by the curvature of the plots (fig. 2a). An increase in SK activity was observed as the pH and temperature of the culture medium was increased, and maximum yield of the protein was achieved at pH 7 and temperature $37.5^\circ C$. The experimental runs revealed that by a further increase in both the variables, there was a gradual decline in SK activity. This might be due to the fact that the production of acidic products at the end of the metabolism leads to the decrease in pH of the production medium. In addition, degradation of SK usually takes place at high temperature as the temperature range of $27-45^\circ C$ is most suitable to keep the enzyme in active state (Dubey *et al.*, 2011). A moderate interaction between pH and incubation time was observed while keeping the other two parameters at constant levels (fig. 2b). Fig. 2c illustrates a noteworthy combined effect of pH and inoculum size, which indicates that SK activity increased by increasing the inoculum size upto 2.50mL at pH 7 but a decreased activity of SK, was observed by a further increase in the volume of the inoculum as well as pH of the culture medium. It was observed that SK shows the best activity at neutral pH (Kumar *et al.*, 2012). A significant increase in response was observed by increasing the incubation time and temperature gradually, suggesting a strong interaction between both the variables. However, lowered temperature resulted in a sudden decline in SK activity (fig. 2d). A strong individual effect of temperature and incubation time was found on SK yield, but the interactive influence of the two variables was non-significant (fig. 2e). Fig. 5f illustrates that incubation time and inoculum size exerted a positive combined effect on SK productivity while keeping the remaining two variables constant. However, there is a negative effect of inoculum

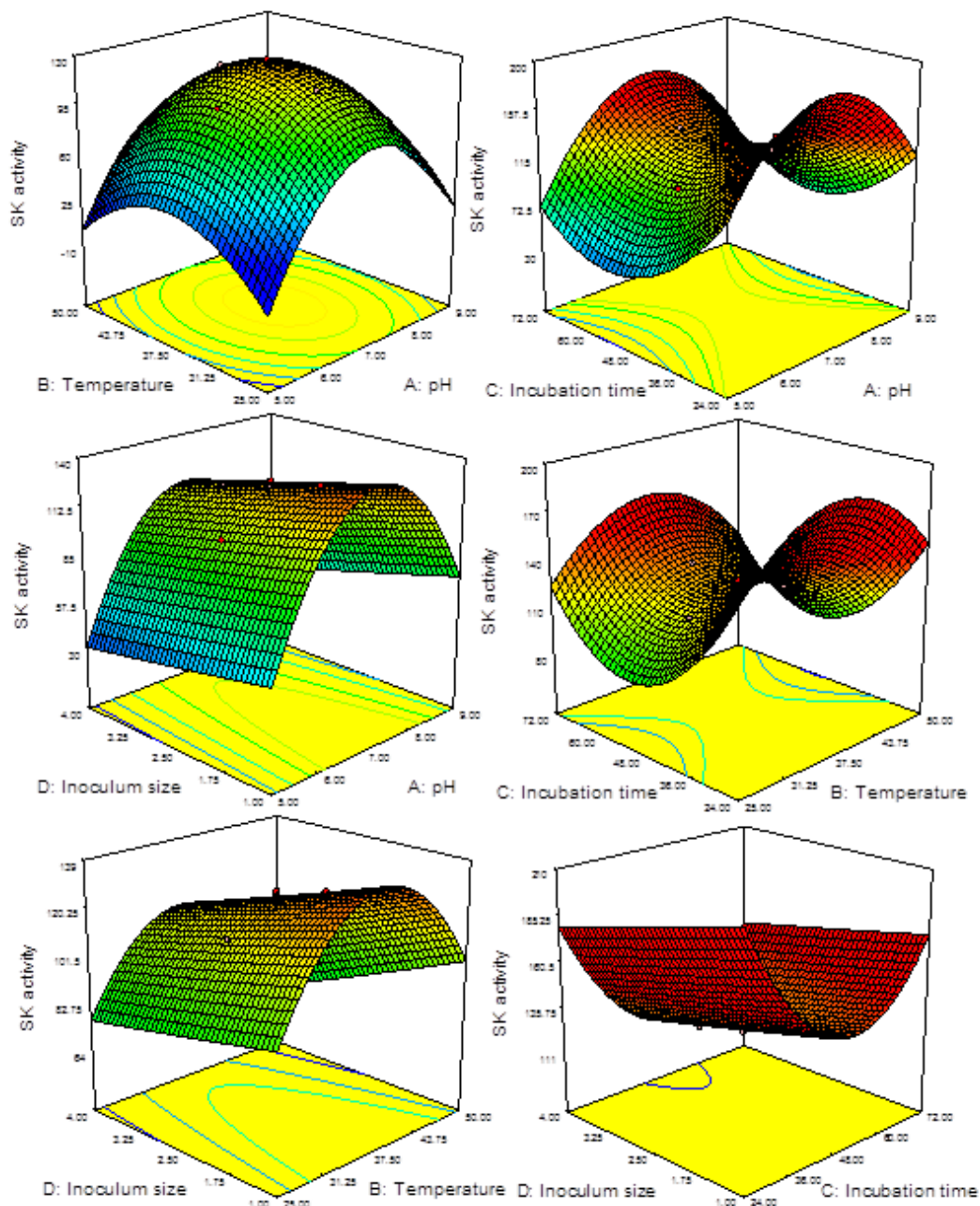


Fig. 2: 3-D surface plots for the production of streptokinase from *S. agalactiae* EBL-31

size on SK yield (Patel *et al.*, 2011). Hence it is evident that the production of SK is remarkably influenced by the combined effect of individual variables.

CONCLUSION

The study indicated that optimization studies could be the better option to obtain cost effective SK for the treatment of cardiac patients. *S. agalactiae*, a novel source of SK was found to be as suitable as other species of β -hemolytic *Streptococcus*. The present study was planned to meet the increasing demands of SK in near future. Thrombolytic therapy by using native SK is useful but its

administration usually results into allergic reactions. Therefore, the production of genetically modified SK to have extended half-life in blood circulation and reduced immunogenicity should be an important consideration in the future.

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