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Enhanced Production Of Streptokinase By Chemical Mutagenesis Of *Streptococcus agalactiae* EBL-20

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HIGHLIGHTS

- Streptokinase isolated from *Streptococcus agalactiae* is an active thrombolytic agent
- Ethyl methanesulfonate is an effective mutagenic agent for *Streptococcus* spp.
- Response surface methodology resulted in 1.92-fold increased streptokinase activity

Abstract: Streptokinase (SK) is an enzyme that is used for the treatment of cardiovascular diseases. The current study focused on the enhanced production of SK by inducing mutation in *Streptococcus agalactiae* EBL-20 and optimization of medium components and culture conditions for the maximum growth of mutant derived strain. *S. agalactiae* EBL-32 was selected as a potent mutant after exposure of *S. agalactiae* EBL-20 to EMS for 180 minutes. SK activity obtained from mutant derived strain was found to be 1.6 fold higher as compared to the activity achieved by wild strain. Nutritional requirements of the mutated strain were optimized by single factor analysis method suggesting glucose as the optimum carbon source; yeast extract and peptone as a suitable nitrogen sources and corn steep liquor (CSL) as an appropriate substrate for the maximum SK production. The culture conditions determined by response surface methodology (RSM) suggested that a temperature value of 37.5°C and pH 7 of the fermentation medium with 2.50 mL inoculum size for 36 hours of incubation was optimum for maximum yield of SK. Hence the optimization studies resulted

into 1.92 fold increase in the yield of SK suggesting the new isolate suitable for commercial scale production of SK.

Keywords: Ethyl methanesulfonate, fermentation, response surface methodology, Streptokinase

INTRODUCTION

Cardiovascular diseases (CVDs) and their outcomes are a major cause of mortality worldwide. Complications associated with cardiovascular disorders are due to imbalance in the maintenance of homeostasis between the development of clot and its breakdown [1, 2]. Among these diseases, thrombosis is most common within the elderly population which is due to obstruction of blood circulation caused by blood clots [3]. A variety of thrombolytic agents with varied pharmacodynamic and pharmacokinetic properties are available for treating diseases like stroke, acute myocardial infarction (AMI), deep vein thrombosis (DVT) and pulmonary embolism. Streptokinase (SK) is one of those agents that are frequently used to treat such type of complications [4] mainly in world's poor health care systems due to its low cost [5].

SK (EC 3.4.99.22) is the first FDA approved thrombolytic drug emerging as a highly potential thrombolytic agent for treating different cardiovascular disorders [6]. It is an extracellular protein of 47 kDa which is composed of 414 amino acids. The isoelectric pH of the protein is 4.7 showing its best activity at pH range of 7.0 to 7.5 [7, 8]. Activation of plasminogen by fibrinolytic drugs is used to treat blood clots. Administration of SK in cases of early stage thrombosis can avert heart muscle impairments [9]. SK acts in a fibrin independent manner and binds with both the circulatory and thrombus-bound plasminogen in a stoichiometric 1:1 ratio to convert plasminogen into plasmin. Plasmin is the active fibrinolytic constituent of the blood circulatory system which neutralizes the mesh of fibrin in blood clots leading to dissolution of thrombus [9]. SK possesses high affinity for plasminogen and has a long half-life as compared to other plasminogen activators [11].

It has been demonstrated that β hemolytic *Streptococcus* of Lancefield group A, C and G produces the enzyme streptokinase [12-14]. Moreover, Group B *Streptococcus* (*S. agalactiae*) has also recently been reported to produce SK [15]. Different strategies have been introduced to enhance the production of streptokinase. Strain improvement studies have been performed for inducing modifications in *Streptococcus* spp. resulting into high production of SK by submerged and liquid state fermentation. Ultraviolet (UV) and gamma radiations have been successfully used for physical mutagenesis; whereas, N-methyl-N'-nitro-N-nitroso guanidine (NTG), ethyl methanesulfonate (EMS), ethidium bromide and sodium azide were used as a chemical mutagens to induce mutations in *Streptococcus* spp. All these mutagenic agents are highly efficient to induce mutations in *Streptococcus* which has the ability to produce SK with improved activity [16-18]. In addition, it has been observed that production cost of the enzyme by microorganism can be reduced by optimizing the growth conditions and medium components. Regarding the production of SK, different factors played a significant role to increase or decrease the yield of the end product.

Fermentation conditions including pH, temperature of the fermentation medium, inoculum size, fermentation time, aeration rate, and agitation speed significantly affects the production of SK [19, 20]. Moreover, carbon source, nitrogen source and selection of substrate and other necessary salts also exert a noteworthy effect on enzyme production [21-23]. Response Surface Methodology is an influential process that is composed of statistical and mathematical skills for planning experiments and analyzing the effect of different factors (independent variables) [24].

The increasing potential of SK administration as a thrombolytic agent triggers the development of quantitative enhancement strategies. Similarly, medium composition and growth parameters are necessary to be optimized for enhanced production of highly active SK. In the present study, indigenously isolated *S. agalactiae* EBL-20 has been used for enhanced production of highly active SK by EMS induced mutations and optimizing the nutritional requirements as well as culture conditions for the growth of microorganism. Previously cited reports are available demonstrating the enhanced production of SK by inducing mutations in wild Streptococcal strains by physical or chemical means. However, there is no data available presenting the use of *S. agalactiae* for overproduction of SK by inducing mutations and optimization of culture conditions and nutritional requirements. With regards to enhanced SK production; response surface methodology proved to be an efficient attempt.

MATERIAL AND METHODS

Microorganism and Culture Conditions

S. agalactiae EBL-20 was previously isolated and identified by API-20-Strep kit (BioMerieux, France) in Enzyme Biotechnology Lab., University of Agriculture, Faisalabad, Pakistan. The culture was maintained on blood agar medium and nutrient agar medium to be used in the present study.

Chemical Mutagenesis of *S. Agalactiae* Ebl-20 by Ethyl Methanesulfonate (Ems)

S. agalactiae EBL-20 was exposed to EMS for 0-200 minutes with an interval of 20 minutes to induce mutation. Spore suspension of wild *S. agalactiae* EBL-20 was prepared in tryptone broth which was then washed and suspended in 100 mM potassium phosphate buffer (pH 7.5). 1mL of spore suspension was added aseptically into sterile test tube and introduced with 12 μ L of EMS [25]. The aliquots were obtained after every 20 minutes, washed and poured on nutrient agar plates after serial dilution. The plates were incubated at 37°C for 24 hours. The optimum dose of EMS exposure was selected by formulating 3-log kill curve (Figure 1) and the potent mutant was screened out from the selected colonies by following the procedures described below.

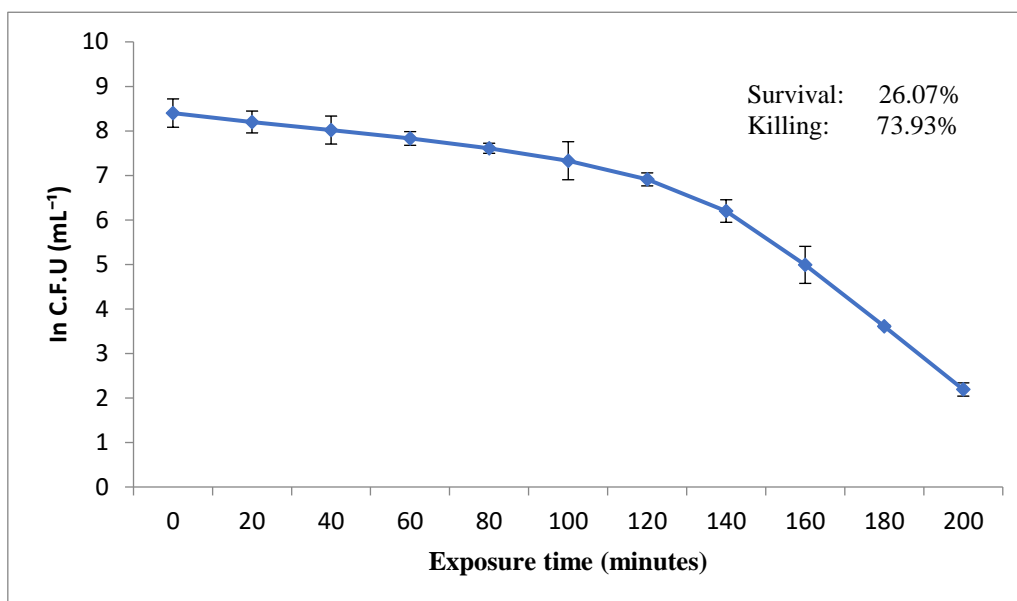


Figure 1 - Formulation of kill curve after mutagenesis of *S. agalactiae* EBL-20 by EMS

Screening of Mutants

The potent mutated bacterial colonies were screened out by plating bacterial culture on nutrient agar plates supplemented with triton X-100. The colonies grown on triton X-100 were further analyzed on skimmed milk agar plates. For this purpose, isolated colonies were allowed to grow on plates containing agarose mixture composed of 0.8% agarose, 200uL human plasma, 10% skimmed milk, 150 mM NaCl and 50 mM Tris-HCl (pH 8.0) [15]. The plates were incubated at 37°C for 12–16 h. The activity of SK was determined by measuring the clear zones around the colonies occurred due to the proteolytic activity of SK [26, 27]. Final selection was accomplished by examining the clot lysis ability of the colonies exhibiting maximum zone of clearance.

The thrombolytic activity of crude enzyme extracted from wild type and mutant derived strain was examined by *in vitro* blood clot lysis assay [15, 18, 28]. The clot lysis (%) ability of the selected colonies was determined as:

Clot weight = weight of tube containing clot – weight of empty tube.

Clot lysis (%) = (weight of the lysed clot / weight of the clot before lysis) × 100.

One unit of streptokinase is the concentration of enzyme which converts 1 micro-mole of substrate into product in one minute.

Production of Streptokinase From Mutant Derived Strain

The colonies of the selected mutant named as *S. agalactiae* EBL-32 grown on nutrient agar plate were picked and inoculated into Todd Hewitt Broth at 37°C for 24 h. Liquid state fermentation was performed in triplicate in Erlenmeyer flask containing 50 mL of pre-optimized production media (g/100mL: Glucose-0.5g, Yeast Extract-0.5g, K₂HPO₄-0.25g, KH₂PO₄-0.25g, NaHCO₃-0.1g, MgSO₄·7H₂O-0.04g, CH₃COONa·3H₂O-0.1g, MnCl₂·4H₂O-0.002g, and FeSO₄·7H₂O-0.002g, pH 7.5) [29]. A total of 1mL inoculum was introduced into the production media and allowed to grow by liquid state fermentation by incubating at 37°C for 24 hours. After incubation the culture was centrifuged at 10,000 g for 30 minutes to obtain

the supernatant which was supposed to be the crude enzyme [30]. The nutritional requirements of the microorganism were optimized by analyzing single factor at a time, whereas the fermentation/cultivation conditions were optimized by RSM.

Selection and Optimization of Nutritional Requirements for SK Production

Effect of different carbon sources (glucose, fructose, sucrose, lactose and starch) on the production of streptokinase by mutant derived *S. agalactiae* EBL-32 was studied. The concentration of the carbon sources was maintained at 0.2%, 0.4%, 0.6%, 0.8% and 1.0% while keeping the concentration of all other components constant and activity of SK was monitored [17, 22].

Streptokinase production was analyzed by using different organic nitrogen sources (tryptone, peptone, beef extract, casein and yeast extract) with varied concentrations ranging from 0.2-1.0% and keeping the concentration of all other components at constant level [19].

Similarly, a variety of substrates including corn steep liquor, sugarcane bagasse, rice polishing and molasses at different concentrations ranging from 0.1-1.0% were analyzed for the better production of streptokinase from mutant derived *S. agalactiae* EBL-32 [21, 31].

Optimization of Culture Conditions for SK Production

Four variables including pH, temperature, and incubation/fermentation time and inoculum size were analyzed for their interactive influence on the yield of SK by using a statistical approach. A central composite design (CCD) of response surface methodology (RSM) was employed for five levels of four different variables, and a total of 30 ($= 2^k + 2k + 6$) experimental runs were conducted (Table 1), where k is the number of independent factors/variables. The design consisted of sixteen factorial and eight axial experimental runs with six replicates at the centre point to calculate the pure error. All the experiments were performed in triplicate. For each equation term "X", the second order polynomial coefficient was estimated through multiple regression and the analysis of variance (ANOVA) [32, 33]. Design-Expert 7.0 was used to generate response surfaces and contour plots.

Table 1 - Central Composite Design (CCD) for the optimization of four process variables for the enhanced production of streptokinase

Runs	pH	Temperature (°C)	Incubation time (h)	Inoculum size (mL)
1	5	25.00	72	4.00
2	7	37.50	48	3.25
3	7	37.50	48	2.50
4	5	50.00	72	1.00
5	6	37.50	48	2.50
6	7	37.50	48	2.50
7	5	25.00	72	1.00
8	8	37.50	48	2.50
9	5	50.00	72	4.00
10	5	50.00	24	4.00
11	5	25.00	24	1.00
12	7	43.75	48	2.50
13	9	50.00	24	4.00
14	7	31.25	48	2.50
15	9	25.00	24	4.00
16	7	37.50	48	2.50
17	7	37.50	48	2.50
18	9	25.00	24	1.00
19	9	50.00	72	4.00
20	7	37.50	60	2.50
21	9	25.00	72	1.00
22	9	25.00	72	4.00
23	7	37.50	36	2.50
24	5	25.00	24	4.00
25	9	50.00	72	1.00
26	7	37.50	48	1.75
27	7	37.50	48	2.50
28	5	50.00	24	1.00
29	9	50.00	24	1.00
30	7	37.50	48	2.50

Determination of Streptokinase Activity

Activity of streptokinase was determined by using casein digestion method in which liberated tyrosine was determined from digested casein after plasminogen activation [33]. Reaction mixture (2 mL) was prepared by adding 10 mg casein, 50 mM Tris-HCl (pH 8.0) to 0.1 ml of supernatant. The reaction process was carried out at 37°C for 20 minutes and the reaction mixtures were kept on ice for 30 minutes after terminating the reaction by adding 2.6 mL of 5% trichloroacetic acid (TCA) and 0.4 mL of 3.3 M HCl. The mixture was then

filtered using Whatman paper and absorbance was measured at 280 nm. A standard SK curve was plotted to determine the activity of streptokinase [34].

Statistical Analysis

Two factorial complete randomized design (CRD) was used for the optimization of nutritional requirements of the bacterial strain. Data obtained for the optimization of four process variables by RSM was used to determine the regression coefficients of the second-order multiple regression models.

$Y_{yield} = b_0 + \sum_{i=1}^k b_i X_i + \sum_{i=1}^k b_{ii} X_i^2 + \sum_{i>j}^k \sum_j^k b_{ij} X_i X_j + e$ where, Y_{yield} is the predicted response variable (SK activity); k is the number of variables being considered in the experiment; b_0 , b_i , b_{ii} , and b_{ij} are regression coefficients and e is the random error.

RESULTS AND DISCUSSION

Although a number of plasminogen activators are available for thrombolytic therapy, streptokinase has widely been used in developing countries mainly due to its high effectiveness and low cost. It is used as a thrombolytic agent for AMI in about half of the countries worldwide where patients do not have rapid access to catheterization [35]. Due to its wide application in cardiovascular diseases and their outcomes, there is an urge for reduction in its production cost. Native SK can easily be produced inexpensively by optimizing the production process of fermentation [15, 36]. The comparison of our findings with previous studies shows that strain improvement can be achieved by exposing the wild *Streptococcus* spp. to certain mutagenic agents and one advantage of using customary procedures of inducing mutations by using different mutagens is the cost effectiveness.

Mutagenesis

The study was conducted to enhance the yield of SK from *S. agalactiae* by exposing the wild strain to EMS. The kill curve was formulated using colony forming units and 180 minutes exposure time was selected as the optimum dose where percentage survival and killing of bacterial strain was 26.07% and 73.93%, respectively (Figure 1). The colonies showing restriction against Triton X-100 were analyzed for SK production. High SK producing colonies of *S. agalactiae* were screened out by Casein-plasminogen overlay and percentage clot lysis methods, respectively. The zone of clearance of selected colonies was determined on skimmed milk agar plates and the colonies showing maximum zone of clearance (named as EMS-1 to EMS-11) were considered as the best mutants and further subjected to clot lysis assay as shown in Table 2. It was analyzed that the activity of streptokinase shown by EMS-5 was highest with 8.2mm zone size (Figure 2) and 54.37% ability of clot lysis. The selected strain was named as *S. agalactiae* EBL-32 which was further exploited for enhanced production of SK by optimizing the conditions. Wild *S. agalactiae* EBL-20 exhibited a zone size of 4mm (Figure 2) with 32.65% clot lysis. Strain improvement study resulted into 1.6 fold increased production of SK from mutant derived *S. agalactiae* EBL-32 as compared to the wild strain. This result revealed that chemical mutagenesis of wild bacterial strain increases the yield of end product. One of the previous findings demonstrated that SK yield from *S. equisimilis* mutated by UV radiations was 120% more than that of the wild strain and the SK yield from *S. equisimilis* exposed to NTG was 146%

more as compared to the wild strain [16]. In another study, 3 fold increased production of SK was reported from *S. equisimilis* by exposing the wild strain to ultraviolet radiations [17].

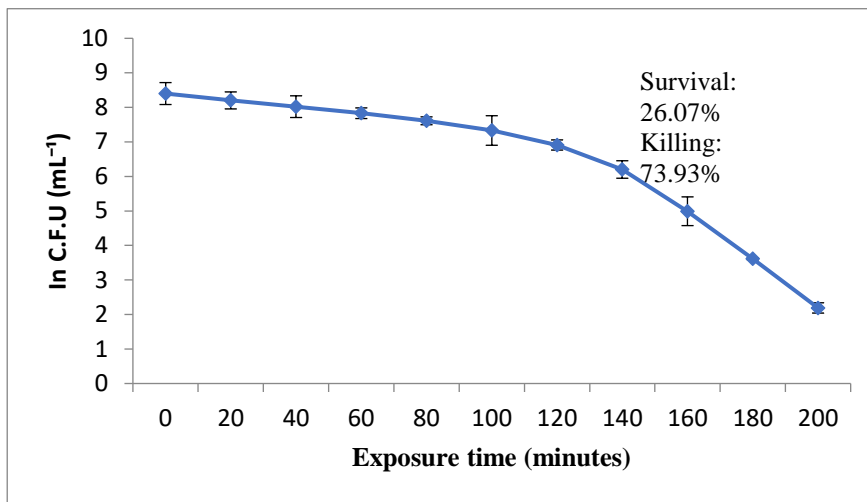


Figure 2 - Formulation of kill curve after mutagenesis of *S. agalactiae* EBL-20 by EMS

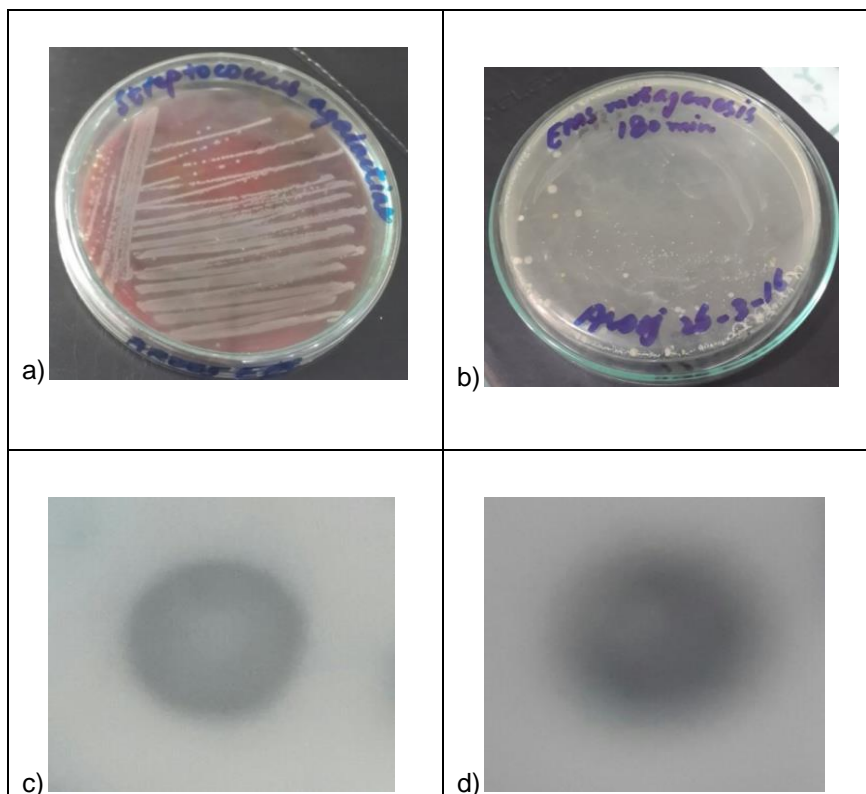


Figure 2 (a): Culture of *S. agalactiae* EBL-20 maintained on blood agar media, (b) Selected plate after exposure to EMS, (c and d) maximum zone of casein hydrolysis observed by wild *S. agalactiae* EBL-20 (4 mm) and mutant derived *S. agalactiae* EBL-32 (8.2 mm), respectively.

Table 2 - Measurements of zone size and clot lysis (%) ability of selected colonies after exposure to EMS

<i>S. agalactiae</i> strains	Zone size (mm)	Clot lysis (%)
Wild/control	4.0	32.65
EMS-1	5.8	36.83
EMS-2	6.5	39.74
EMS-3	5.9	34.11
EMS-4	6	36.33
EMS-5	8.2	54.37
EMS-6	7.9	47.63
EMS-7	6.5	35.43
EMS-8	7.0	40.54
EMS-9	7.0	39.26
EMS-10	7.3	41.28
EMS-11	7.1	40.22

Selection and optimization of carbon and nitrogen source and substrate

A noteworthy increase in enzyme production can be obtained by optimizing the nutritional requirements of the microorganism. The present study describes the optimum concentrations of substrate, carbon and nitrogen sources for enhanced SK production mutant derived strain of *S. agalactiae*. Among the different carbon sources, the mutant derived *S. agalactiae* EBL-32 showed the best SK activity of 93.54 UmL^{-1} with 0.6% glucose. The production of SK was not promoted by using lactose and starch in the medium; however the production was satisfactory by using 0.4% fructose (Figure 3a). Regarding the nitrogen source, the improved production of SK was observed by using peptone (0.6%) in the fermentation medium showing 95.43 UmL^{-1} SK activity (Figure 3b). These results demonstrate that carbon and nitrogen sources played a significant role in obtaining high yield of SK⁵. Previous findings suggested glucose as the best carbon source for SK production from *Streptococcus* spp. It has been reported that 0.25% glucose and 1.5% casein are the optimum concentrations of carbon and nitrogen sources, respectively for better growth of *S. pyogenes* and *S. equisimilis* [22]. In comparison, our findings revealed that peptone is the best organic nitrogen source for growth of mutant derived *S. agalactiae* EBL-32. However, the growth was found to be stable in the presence of yeast extract and typtone but it was decreased in the presence of beef extract. In addition, maximum SK

activity (96.21 U mL^{-1}) was observed by adding 0.2% CSL in the production medium. However, the production of SK was found to be lowered in the presence of rice polishing and sugarcane bagasse (Figure 3c). CSL exerts a positive effect on the production of SK [20, 21]. Analysis of variance revealed the significant impact of all the nutritional sources with $p < 0.01$ (as given in supplementary Table S1-S3).

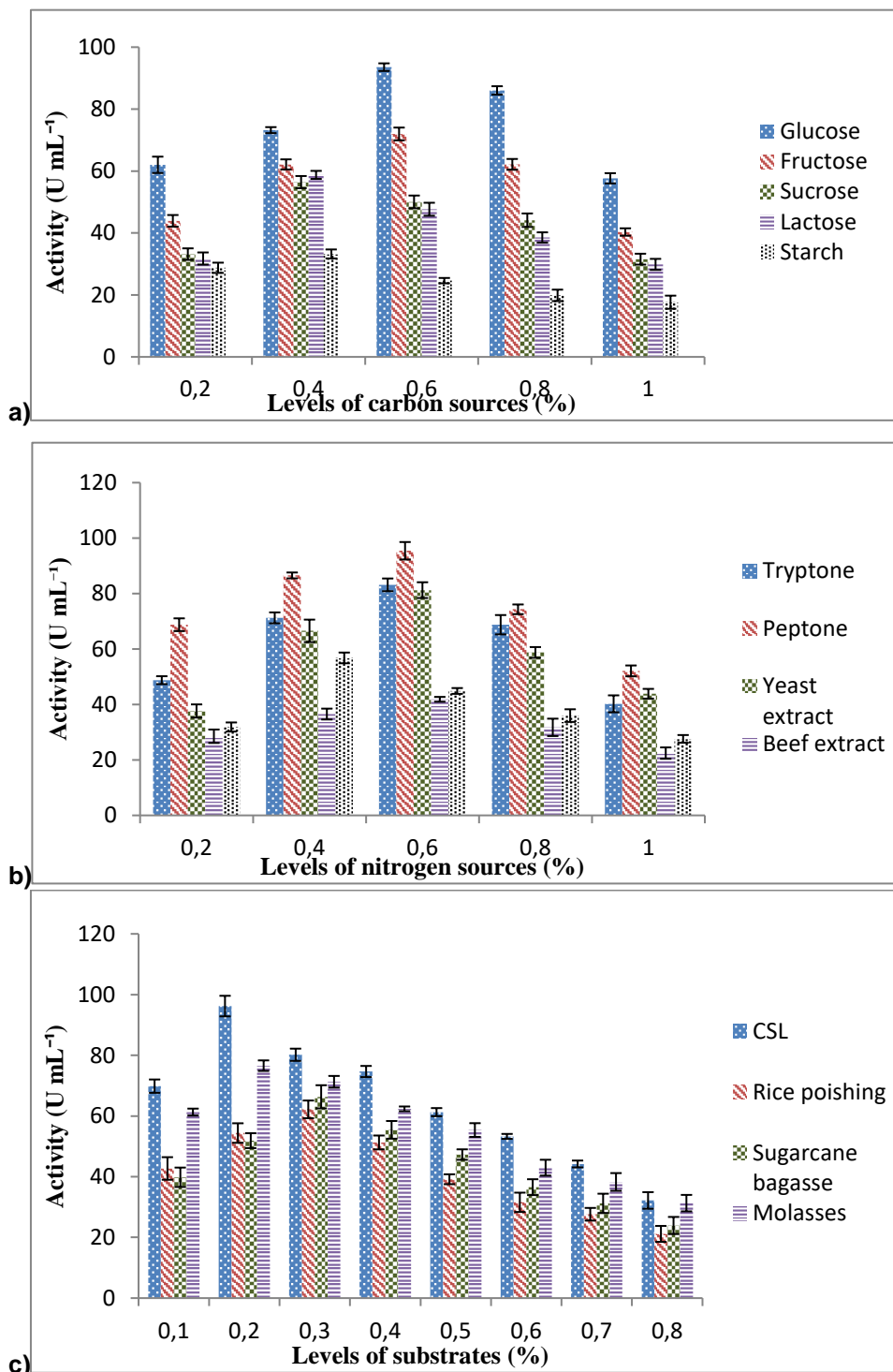


Figure 3 - Selection and optimization of (a) carbon source, (b) nitrogen source and (c) substrate for the production of SK from mutant derived *S. agalactiae* EBL-32. Optimization of SK production by experimental designs and RSM

Analysis of variance (ANOVA) of the data obtained for second order response surface model is presented in Table 3. The high F value, $F_{\text{model}}=860.66$ indicates that complete quadratic regression model is significant; whereas, the non-significant lack of fit shows that second-order model is a satisfactory approximation to the exact response surface. Adjusted coefficient of determination (R_{adj}^2) evaluated the peculiarity of fit of the polynomial model equation. For mutant derived *S. agalactiae* EBL-32, linear terms; incubation time and pH, whereas, quadratic terms; pH and incubation time significantly contributed to the fitted model. The significance of $R_{adj}^2 = 0.9976$ indicates that 99% variation in response has been explained by the terms involved in the quadratic model [37].

The statistical signification of the fitted model of parameters being studied for response (SK yield) was evaluated by the *F* ratio and its equivalent *p* values; mentioned in Table 3. The *p* value indicates the relative significance of the terms involved with the particular parameter and analyzes the significance of coefficients. The smaller *p* value for a parameter indicates that there is more influence of the observed parameter [38]. It was observed that the first order interactive influence of all the analyzed variables is significant ($p < 0.0001$) with the non-significant interactive effect of temperature and incubation time. For valid application of Analysis of Variance, residuals of the model must be normally distributed. Figure 4 shows the normal probability plot for residuals; normality of residuals is substantiated by the points in close proximity to the straight line.

Table 3 - Analysis of variance for the quadratic model for mutant derived *S. agalactiae* EBL-32

Source of variation	Sum of Squares	df	Mean Square	F Value	p-value
Model	36426.85	14	2601.92	860.66	< 0.0001
Residual	45.35	15	3.02		
Lack of Fit	37.73	10	3.77	2.48	0.1644
Pure Error	7.62	5	1.52		
Cor Total	36472.19	29			

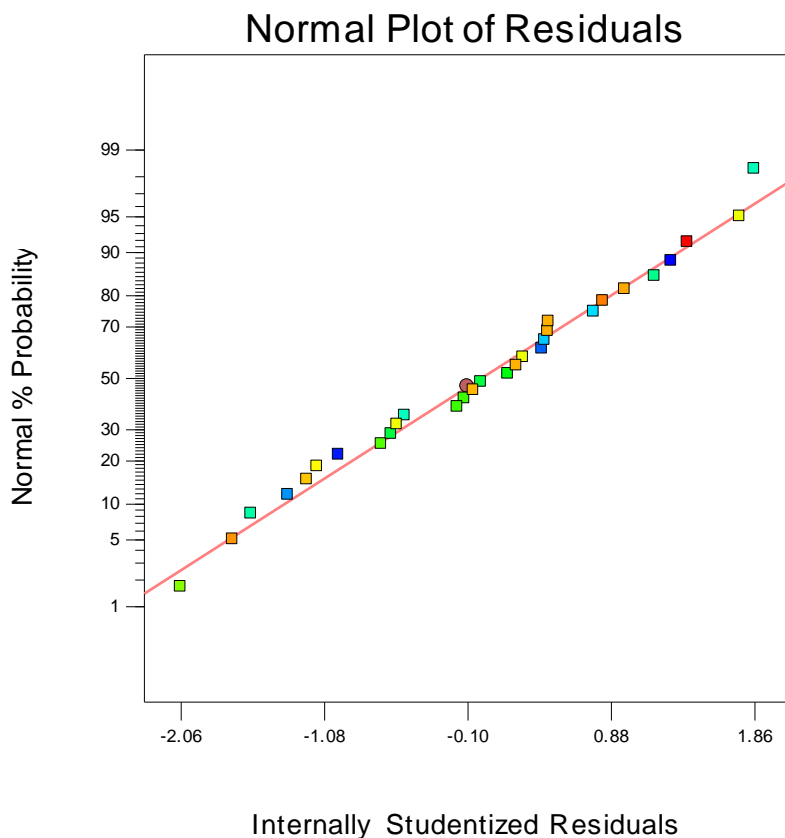


Figure 4 - Normal probability plot of residuals

Response surface analysis

Using the design of experimental data, the second degree polynomial model for SK yield from mutant derived *S. agalactiae* EBL-32 was obtained (in terms of actual factors) as: $Y = -862.59 + 242.43X_1 + 22.41X_2 - 10.69X_3 + 8.38X_4 - 0.12X_1X_2 + 0.17X_1X_3 - 0.80X_1X_4 - 5.58X_2X_3 - 0.23X_2X_4 + 0.05X_3X_4 - 17.16564X_1^2 - 0.28008X_2^2 + 0.09X_3^2 - 1.05891X_4^2$ where Y is the response variable for *S. agalactiae* EBL-32; X₁, X₂, X₃, and X₄ are the actual factors, namely, pH, temperature, incubation time and inoculum size, respectively.

3-D surface plots were generated by considering an infinite number of combinations of two test variables simultaneously, while keeping other two variables constant. The predictor variables are displayed on the x- and y-scales, and the response variable is represented by a smooth surface. Figure 5 represents the surface plots for two variables keeping other variables at specified levels. Curvature in the surface plots indicates the interaction between variables holding other variables constant at some pre-specified level.

A very strong interaction can be seen between temperature and pH for the growth of *S. agalactiae* EBL-32 which is indicated by the curvature of the plots (Figure 5a). It was observed that SK activity increases as the pH and temperature of the culture medium increases and maximum SK yield was obtained from both the strains at pH 7 and temperature 37.5°C while by a further increase in both the parameters, there was a gradual decline in SK activity. This may be due to the decline in pH of the fermentation medium over

time by the production of acidic products at the end of the metabolism. Degradation of SK usually takes place at high temperature as this fibrinolytic enzyme is active between the temperature range of 27-45^oC [39]. In addition, it was observed that there is a moderate interaction between pH and inoculum size, while keeping the other two variables constant (Figure 5b). Figure 5c indicates a significant interaction between pH and inoculum size and shows that SK activity is increased by increasing the inoculum size upto 2.50 mL at pH 7 but a decreased activity of SK was observed by further increase in the volume of inoculum as well as pH of the culture medium. An increased SK yield was obtained by increasing the incubation time and temperature gradually, revealing a noteworthy interaction between the two variables. In addition a sudden decreased SK activity was observed at lower temperatures (Figure 5d). No significant interaction was observed between temperature and incubation time, but the two variables have a strong individual affect on the yield of SK (Figure 5e). Figure 5f interprets that incubation time and inoculum size acts as a function of SK productivity, while keeping the remaining two variables constant. However, inoculum size exerts a negative effect on SK productivity [20].

Based on the statistical results, the maximum production of streptokinase with 168.46 U mL⁻¹ from mutant derived *S. agalactiae* EBL-32 was obtained at pH 7; temperature 37.5 ^oC; incubation time 36 hours and inoculum size 2.50mL as presented in the figures.

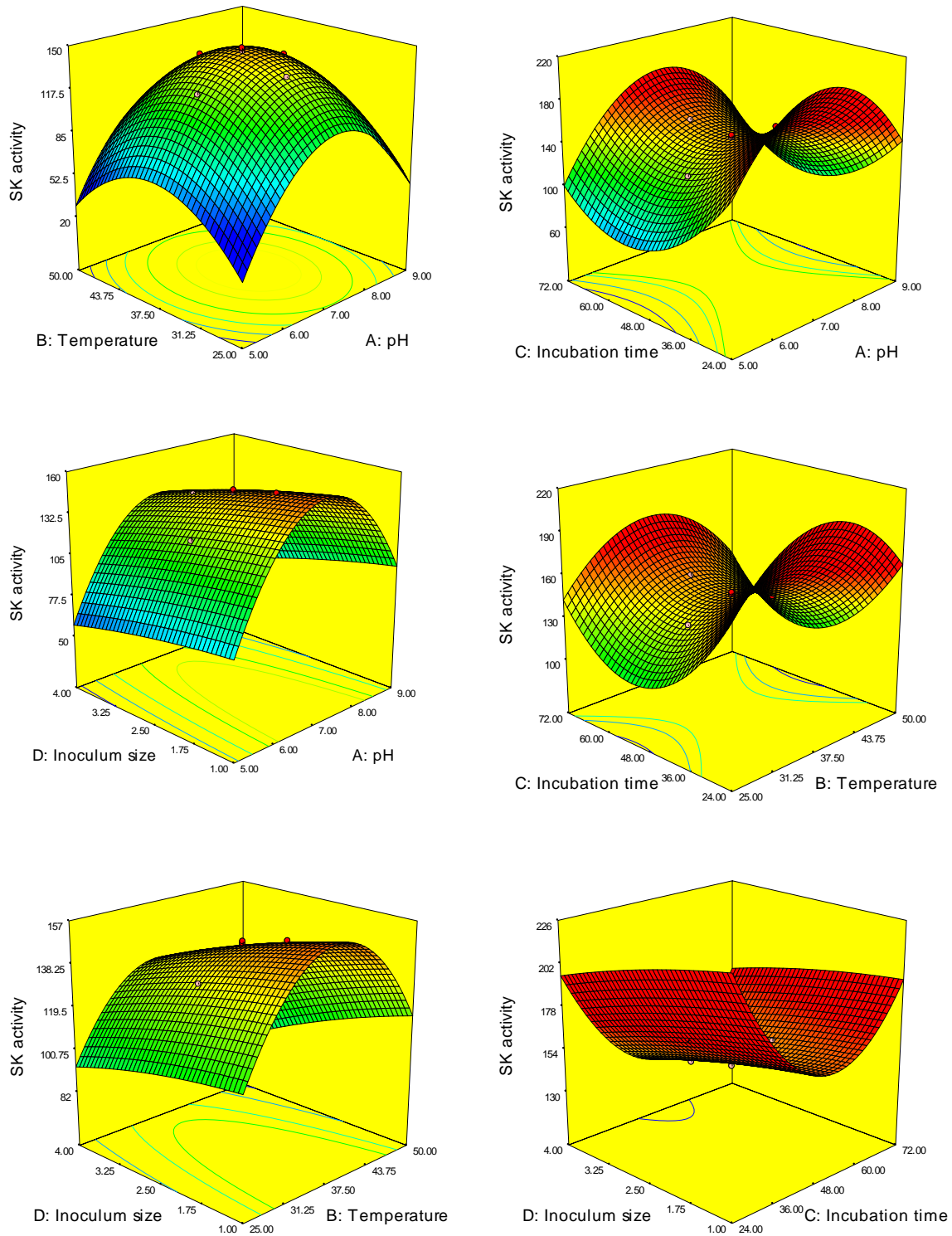


Figure 5 - 3-D surface plots for the production of SK from mutant derived *S. agalactiae* EBL-32.

After the optimization studies, enhanced production of streptokinase was observed from mutant derived *S. agalactiae* EBL-32 with 168.46 U mL^{-1} activity suggesting that mutating the wild bacterial strain by EMS is an effective technique to obtain over-production of SK. The optimization study by RSM concludes that production of SK from mutant derived *S. agalactiae* EBL-32 was increased by 1.92 fold after optimization of culture conditions and

nutritional requirements. Based on the literature cited, 110% increase in SK yield was observed after optimizing the culture conditions and nutritional requirements [19].

CONCLUSION

The study revealed that induction of random mutations in the genome of microorganism is an efficient technique to improve the production of streptokinase. The most remarkable achievement of this study was the highly potent mutant *S. agalactiae* EBL-32 and enhanced production of streptokinase by fermentation process. These results suggest that EMS is an effective mutagen for strain improvement of *S. agalactiae* EBL-20. Moreover, optimization studies could be the better option to make it economically feasible for the treatment of poor heart patients in developing countries. Hence, the study concludes that *S. agalactiae* can be exploited for large scale production of streptokinase. Although thrombolytic therapy by using native streptokinase is useful; however its administration provokes allergic reactions. In future, more focus should be made for the production of genetically modified streptokinase to extend its half life in blood circulation and reducing immunogenicity.

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