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Original Research Article

Optimization of culture media for extracellular expression of streptokinase in Escherichia coli using response surface methodology in combination with Plackett-Burman Design

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Abstract

Purpose: *To investigate the enhancement of streptokinase extracellular expression in Escherichia coli by adjusting culture media.*
Methods: *Screening of 10 chemical factors (EDTA, peptone, glycine, triton X-100, glycerol, K_2HPO_4, KH_2PO_4, Ca^{2+} (calcium chloride), yeast and NaCl) in order to increase the secretion of extracellular protein was carried out by response surface methodology (RSM). The method was also employed to optimize the concentrations of critical factors that had been determined in the screening step.*
Results: *The results indicate that glycine, triton X-100 and Ca^{2+} were the most effective chemical factors in terms of increase in extracellular expression of streptokinase with optimum levels of 0.878, 0.479 and 0.222 %, respectively. Expression of streptokinase under optimum concentrations of critical permeabilizing factors led to a 7-fold increase in the quantity of secreted recombinant protein (5824 U/mL) compared to the initial level (802 U/mL).*
Conclusion: *The results show that medium optimization using RSM is effective in improving extracellular streptokinase expression. The optimization medium is considered fundamental and useful for efficient production of streptokinase on a large scale.*

Keywords: *Streptokinase, Response surface methodology, Membrane permeabilization, Extracellular secretion*

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INTRODUCTION

Streptokinase (SK) is a single chain (47 kDa) protein that has received much attention as a commonly used thrombolytic agent for treating cardiovascular disease such as heart attack or stroke [1]. SK is not originally a protease; however on complexation with plasminogen it gives rise to plasmin that can dissolve the fibrin network of a blood clot and solubilize degradation products [2,3]. Heterologous production of SK in *Escherichia coli* (*E. coli*), as the most widely used prokaryotic system is useful method for industrial applications [4].

Although heterologous expression in *E. coli* could bring about numerous advantages, accumulation of the expressed protein in the cytosol as a

biologically inactive protein aggregate (inclusion body) is a significant problem that could be overcame by extracellular expression (periplasmic space or culture medium) of recombinant protein [5,6]. However, recombinant proteins most often fail to translocate across the inner and outer membranes of *E. coli* cells [7,8]. Medium optimization is one of the most commonly applied approaches to facilitate translocation through membranes. Some medium supplements such as lysozyme, magnesium, calcium, EDTA, glycine, and triton X-100 could increase membrane permeability and subsequent protein secretion to the culture medium [9-11]. The existence certain values of triton X-100 and glycine in the culture medium had a strong effect on extracellular production and could greatly increase both inner and outer cell membrane permeability [12]. Divalent cations, such as Ca^{+2} and Mg^{+2} in medium rich with glycine can effectively protect cells from lysing [13]. EDTA and lysozyme could also be led to enhancement of *E. coli* outer membrane permeability [7]. Use of statistical models such as Response Surface Methodology (RSM) could help to determine effective factors, optimum amounts and possible interactions that are related to permeability of a bacterial membrane. An RSM designs the least number of experiments to achieve these goals.

In a previous study, the synthetic SK gene (GenBank Acc. No. KT156726.1) of *Streptococcus pyogenes* was successfully cloned into pET21b expression vector, while PelB signal peptide was added to the 5' end of the gene to deliver the expressed protein into the periplasm. The conditions required for optimum periplasmic expression were determined using RSM. Experiments were done to determine optimum settings for volume, temperature, agitation speed, cell density, IPTG concentration and duration of induction. The aim of the present study was to screen the main culture supplements involved in membrane permeability of *E. coli* and to optimize their concentrations using RSM.

EXPERIMENTAL

Microorganism and culture conditions

Recombinant *E. coli BL21* cells harboring SK gene in pET21b (+) expression vector (Novagen, Madison, USA) were used for the protein over-expression [14]. The gene was cloned between *Nde*I and *Bam*HI restriction sites with PelB as the leader sequence. A single colony of *E. coli* BL21 (DE3) cells with recombinant plasmid was inoculated into 5 mL Luria - Bertani (LB) medium containing 100 µg/mL ampicillin and grown at 37 °C overnight and used for inoculation. The expression was done in 5 mL of LB culture medium supplemented with 100 µg/mL ampicillin, at 37 °C, 200 rpm, 0.28 mM of IPTG addition at cell density of OD600 = 3.4 and 10 h of expression induction. Total (using the cells and the culture medium as the specimen) and extracellular (using the culture medium as the specimen) activity of the SK was assayed by chromogenic substrate method [15]. Streptokinase converts plasminogen to plasmin in solution in the absence of fibrin. S-2251 chromogenic substrate (H-D-valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride; (Sigma, USA)) was used in the chromogenic assay method. The substrate solution included a mixture of 1 mL of Tris–HCl (0.5 M, pH = 7.4), 1 mL of S-2251 (3 mM) and 5 µL of Tween 20 (10 %). This solution was kept at 37 °C, but immediately before use, 45 µL of human-plasminogen solution (1 mg/mL) was added to the solution. Dose-response curve for streptokinase was drawn using streptokinase dilution preparation in 10 mM Tris–HCl (pH = 7.4) by adding 0.1 mM NaCl and 1 mg/mL albumin. Different dilutions containing 0.5, 1.0, 2.0 and 4.0 IU/mL were assayed at 37 °C in a microtiter plate. In the test samples, the reaction was performed by adding 60 µL SK solution onto 40 µL of substrate solution. The final concentration of substrate S-2251 was 0.59 mM and the final strength of buffer was 103.7 mM. After 20 min, the reaction was stopped and the end point absorbance was measured at 405 nm.

Data analysis

Minitab 16 software (Minitab Inc., USA) and Design Expert 7.0.0 were employed to design the experiment. Plackett–Burman design was employed to screen 10 different variables during 20 experiments. The results were used to accentuate the factors with the highest affectivity. ANOVA was used to assess the statistical parameters. The variables were analyzed by RSM at five different levels. The experimental design matrix consisted of a central composite design with six replications of the central points (all factor levels at 0). The analysis of experimental data was carried out statistically by regression method. The resulting equation was used to calculate the predicted amount of SK activity. The predicted and actual quantities of SK were compared using MiniTab software to analyze the reliability of the results. Using Design Expert 7.0.0 software, two-dimensional contour plots of the obtained results were produced. These plots were used to display the effect of different variables on the permeability of the

membrane and hence, SK activity. Finally, the results of the experiments and analyses were fed into MiniTab software to predict the optimum concentrations of the critical factors and the resulting maximum SK activity.

Experimental design

To have a realistic modelling approach for higher secretory expression, the effecting factors should be known. In this regard, literature review revealed 10 factors (EDTA, peptone, glycine, triton X-100, glycerol, K_2HPO_4, KH_2PO_4, $CaCl_2$, yeast extract and NaCl) to be effective enhancers of the permeability of bacterial membrane and hence increase SK release into the culture medium. Each experiment was followed by an enzyme activity assay. SK activity was assayed using the chromogenic substrate method which is an end point method.

Optimization of screened factors

To enhance membrane permeability towards expressed recombinant SK, a design with five levels of study was used. In this regard, a RSM-based on the central composite design (CCD) was used to achieve optimized levels of glycine, triton X-100 and Ca^{2+}. Three replicated experiments were performed for each section. Recombinant *E. coli* host cells (transformed with SK gene containing pET21b vector) without induction were used as negative control. The SK activity of the secreted recombinant protein was measured in different conditions by using the chromogenic substrate method (section 2.2). The results of enzyme activity assays was recorded in Table 5 in the column, 'Actual'. Finally, the activity of the recombinant SK was assayed in the culture medium at the optimum levels of glycine, triton X-100 and Ca^{2+}.

RESULTS

Protein expression

The recombinant bacterium was successfully constructed and cultured according to results of the SK over expression optimization experiment. Enzyme activity analysis of the total and extracellular (802 U/ml) SK activity revealed that the extracellular SK comprised only 10 % of the total produced SK.

Critical factors

All factors considered to have an effective role in enhancing membrane permeability are listed in Table 1. Minimum-level (-1) and maximum-level (+1) determinations of each factor are listed in Table 1 as a percentage of the culture medium.

Table 1: Ten nutrient screening using a Plackett-Burman design

Variable		Percentage (%)	
Nutrient Code	Nutrient	Low (-1)	High (+1)
A	EDTA	2×10^{-4}	10×10^{-4}
B	Peptone	0.5	1.5
C	Glycine	0.6	2.25
D	Triton X-100	0.25	0.75
E	Glycerol	0.2	0.8
F	K_2HPO_4	1	2
G	KH_2PO_4	0.1	0.4
H	$CaCl_2$	0.11	0.33
J	Yeast Extract	0.5	2.5
K	NaCl	0.5	1.5

Table 2 lists the 20 experiments designed to screen for the most effective variables and results for enzyme activity of each experiment under the last column labelled 'results'. These results were further examined with MiniTab software. The minimum and maximum amounts of each factor were derived from results of previous studies. Factor with P value evaluation of $0.001 \leq P \leq 0.05$ (according to the software defaults) was assigned as very effective factor, while factor with P-value of $0.05 \leq P \leq 0.1$ was assigned as effective one.

The results of statistical analyses (Table 3) introduced Glycine (C) (P-value = 0.026), triton X-100 (D) ($p = 0.035$) and Ca^{2+} (H) ($p = 0.095$) as the most effective factors in membrane permeability to release SK into the culture medium. Other factors showed the *p*-values > 0.1 and hence were not effective on SK secretion.

Optimized culture

Following selection of the critical factors for SK secretory expression (glycine, triton X-100 and Ca^{2+}), it was necessary to determine optimum concentration of these selected factors. In this regard, the experimental range of each of the three variables was presented in five levels (-α, -1, 0, +1, +α) (Table 4). The variables could be investigated in three levels (-1, 0, +1) but analysis of the factors in five levels can demonstrate the correctness of each factor range. Thereafter, 20 experiments, including six replications of the central points, were designed to optimize the screened factors (Table 5). In this step, it was tried to find the most effective concentration of three different factors (glycine, triton X100 and $CaCl_2$) on extracellular SK expression.

Table 2: Screening of effective factors for secreted streptokinase activity by using Plackett-Burman experimental design matrix

Run order	A	B	C	D	E	F	G	H	J	K	Result
	EDTA	Peptone	Glycine	Triton X-100	Glycerol	K_2HPO_4	KH_2PO_4	Ca^{2+}	Yeast	NaCl	
1	1	-1	1	1	-1	-1	-1	-1	1	-1	1095
2	1	1	-1	1	1	-1	-1	-1	-1	1	1020
3	-1	1	1	-1	1	1	-1	-1	-1	-1	825
4	-1	-1	1	1	-1	1	1	-1	-1	-1	1260
5	1	-1	-1	1	1	-1	1	1	-1	-1	1065
6	1	1	-1	-1	1	1	-1	1	1	-1	600
7	1	1	1	-1	-1	1	1	-1	1	1	1320
8	1	1	1	1	-1	-1	1	1	-1	1	405
9	-1	1	1	1	1	-1	-1	1	1	-1	960
10	1	-1	1	1	1	1	-1	-1	1	1	1215
11	-1	1	-1	1	1	1	1	-1	-1	1	720
12	1	-1	1	-1	1	1	1	1	-1	-1	1065
13	-1	1	-1	1	-1	1	1	1	1	-1	855
14	-1	-1	1	-1	1	-1	1	1	1	1	660
15	-1	-1	-1	1	-1	1	-1	1	1	1	480
16	-1	-1	-1	-1	1	-1	1	-1	1	1	570
17	1	-1	-1	-1	-1	1	-1	1	-1	1	315
18	1	1	-1	-1	-1	-1	1	-1	1	-1	555
19	-1	1	1	-1	-1	-1	-1	1	-1	1	360
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	165

Table 3: Statistical analysis from the results of Plackett-Burman design for screening of medium components

Factor	Medium component	Effect	T-value	*P*-value
A	EDTA	180.00	1.69	0.124
B	Peptone	-27.00	-0.25	0.805
C	Glycine	282.00	2.65	**0.026**
D	Triton X-100	264.00	2.49	**0.035**
E	Glycerol	189.00	1.78	0.109
F	K2HPO4	180.00	1.69	0.124
G	KH2PO4	144.00	1.36	0.208
H	Ca^{2+}	-198.00	-1.86	**0.095**
J	Yeast Extract	111.00	1.04	0.323
K	NaCl	-138.00	-1.30	0.226

0.001 ≤ P value ≤ 0.1 is more effective factors, hence C = Glycine and D=Triton X-100 and H=Ca^{2+} are more important factors

Table 4: Experimental range of variables and coded values of three variables used in Central Composite Design

Variable level	Component	Level				
		-α	**-1**	**0**	**+1**	**+α**
A	Glycine (%)	0.039	0.6	1.425	2.25	2.811
B	Triton X-100 (%)	0.08	0.25	0.50	0.75	0.92
C	$CaCl_2$ (%)	0.0352	0.11	0.22	0.33	0.4048

This table provides results for SK activity under the ‘Actual’ column, while predicted amounts are shown under the ‘Predicted’ column. Predicted levels of SK activity, as functions of glycine (A), triton X-100 (B) and Ca^{2+} (C) were fitted with a second-order equation which provided the levels of SK activity (Eq 1).

Y (Streptokinase activity U/mL) = + 5516.48– (375.2A) – (73.6017B) + (22.7601C) – ($248.755A^2$) – ($423.234B^2$) – ($498.011C^2$) – (1.86435×10^{-14} AB) – (6.82826×10^{-14} AC) + (2.54278×10^{-14} BC) …….. (1)

Table 5: CCD with measured an--d predicted responses with transcription level of SK as a response

Run no.	Factor A	Factor B	Factor C	Variations of streptokinase activity (U/ml)	
	Glycine percentage	Triton X-100 percentage	$CaCl_2$ percentage	Actual	Predicted
1	-1	-1	-1	3447	3182
2	1	-1	-1	2757	2681
3	-1	1	-1	3180	3084
4	1	1	-1	2491	2583
5	-1	-1	1	3400	3212
6	1	-1	1	2710	2712
7	-1	1	1	3133	3114
8	1	1	1	2444	2614
9	-1.68	0	0	3337	3629
10	1.68	0	0	2945	2788
11	0	-1.68	0	2695	2962
12	0	1.68	0	2930	2797
13	0	0	-1.68	2554	2713
14	0	0	1.68	2789	2764
15	0	0	0	3651	3678
16	0	0	0	3739	3678
17	0	0	0	3672	3678
18	0	0	0	3616	3678
19	0	0	0	3678	3678
20	0	0	0	3733	3678

Analysis of variance (ANOVA) for the response surface demonstrate that the regression is statistically significant at 99 % ($p < 0.05$) confidence level. If the values of "Prob > F" was less than 0.0500 it means that model terms are significant. But values greater than 0.1000 are not significant. In this case A, A^2, B^2 and C^2 are significant model terms (Table 6). So B, C and all interactions are not significant. It means that there are no effective interactions between these three different factors.

Model F-value of 10.01 indicates that the model is significant. The chance that a "Model F-Value" this large could occur due to noise is just 0.06%. Comparison between actual and predicted response values show agreement ($R^2 = 0.9$) (Figure 1).

The effect of each factor on SK activity was depicted by two-dimensional contour plot (Figure 2, 3 and 4). Optimum levels of the critical factors (glycine= 0.878 %, triton X-100= 0.479 % and $CaCl_2$ = 0.222 %), along with the predicted maximum SK activity (5661.3747 U/ml) are presented in Figure -5. The optimum levels are acceptable because all of them are in selected ranges. Finally, activity assay of SK (5824 U/ml) cultured in optimum levels of glycine, triton X - 100 and Ca^{2+} confirmed the maximum activity predictions and it shows highest level of extracellular SK activity, in comparison to other studies (Table 7).

Table 6: Analysis of variance (ANOVA) for response surface quadratic model for the streptokinase production

Source	Sum of squares	DF	F-value	Prob > F
Model	8.012E+006	9	10.01	0.0006
A-Glycine %	1.923E+006	1	21.63	**0.0009**
B-Triton X-100 %	73858.37	1	0.83	0.3835
C-CaCl2 %	7112.91	1	0.080	0.7831
AB	0.000	1	0.000	1.0000
AC	0.000	1	0.000	1.0000
BC	0.500	1	5.624E-006	0.9982
A^2	8.906E+005	1	10.02	**0.0101**
B^2	2.580E+006	1	29.01	**0.0003**
C^2	3.575E+006	1	40.20	**< 0.0001**
Residual	8.891E+005	10		
Lack of Fit	8.635E+005	5	33.70	0.0007
Pure Error	25622.83	5		
Cor Total	8.901E+006	19		

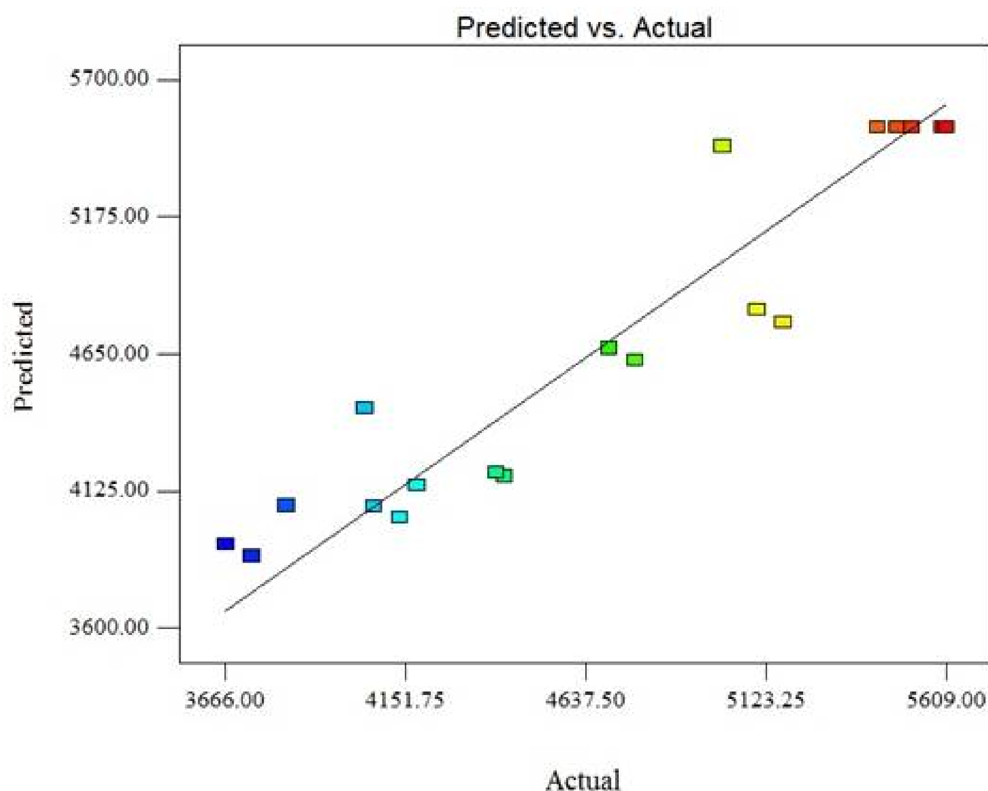
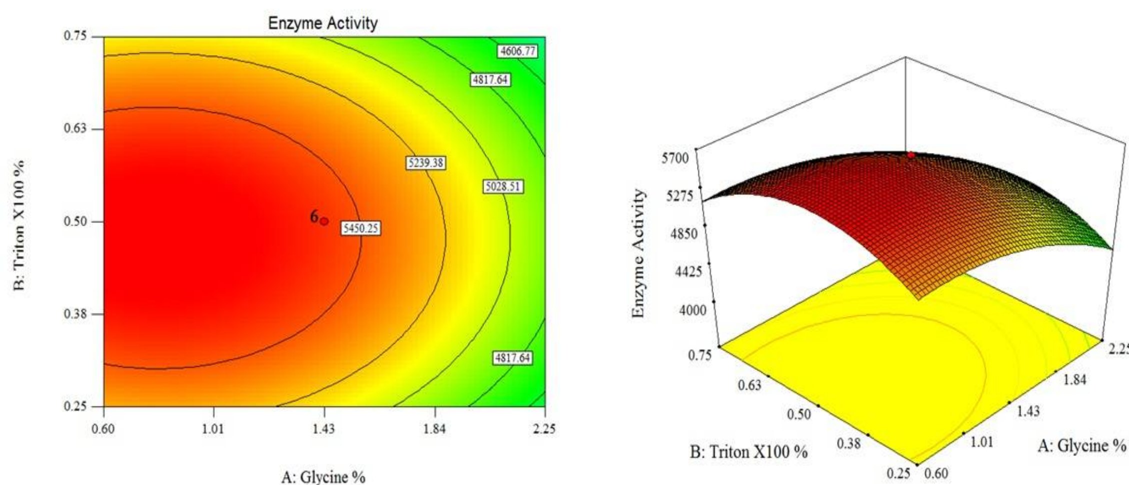


Figure 1: Predicted response versus actual value



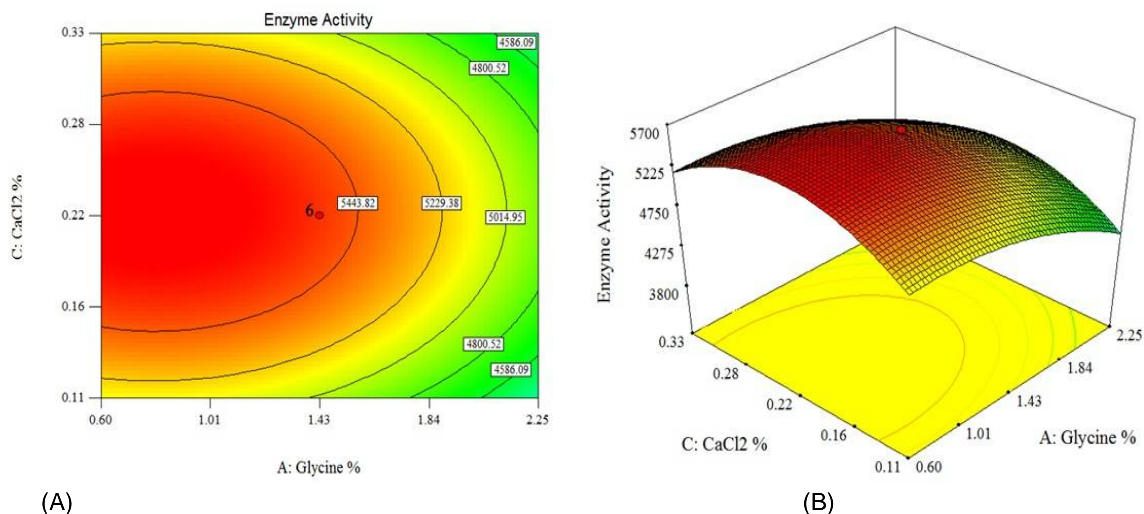
(A) (B)

Figure 2: Contour plot (A) and 3D response surface (B) for Streptokinase production by *E. coli* that show the effect of glycine and triton X-100 concentration on secreted SK activity.

DISCUSSION

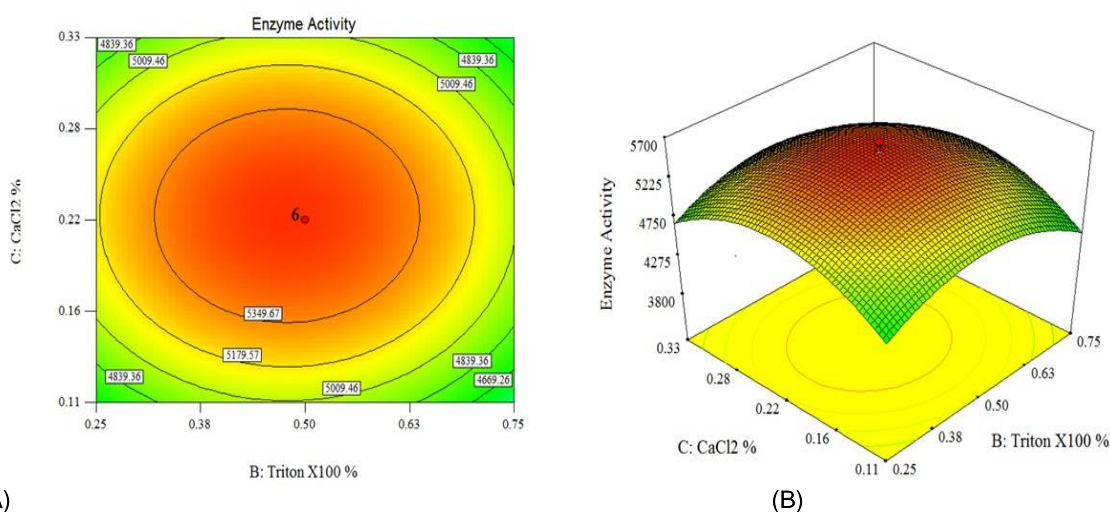
Production yield of a recombinant protein can be affected by several factors. Developing novel methods for enhancement of secreted active recombinant SK production yield have important financial significance. Accordingly, the analysis in this study were done to determine the most effective chemical factors and their optimum amounts to permeabilize the *E. coli* cell membrane for secretion of a recombinant SK protein. The process was successfully optimized for periplamic expression.

Extracellular release of recombinant proteins from the periplasm seems to be a relatively ideal and rational solution for extracellular protein production. The reportedly low rate of SK secretion (10 %) in this expression system accentuates the necessity of increasing SK translocation from the cytoplasm to the culture medium. The inner membrane can be crossed using various signal sequences such as PelB,



(A) (B)

Figure 3: Contour plot (A) and 3D response surface (B) for Streptokinase production by *E. coli* that show the effect of glycine and $CaCl_2$ concentration on secreted SK activity.



(A) (B)

Figure 4: Contour plot (A) and 3D response surface (B) for Streptokinase production by *E. coli* that show the effect of triton X-100 and $CaCl_2$ concentration on secreted SK activity.

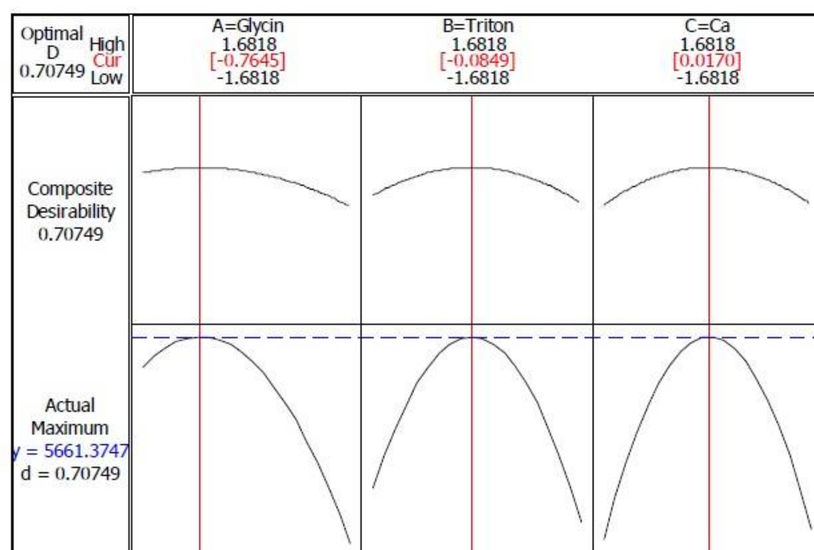


Figure 5: Optimization plot for highest secreted SK activity

which could translocate the protein to the periplasm through a sec-dependent secretion pathway [7]. The outer membrane barrier could be dealt with by chemical permeabilization.

For determination of the most effective components of the culture medium, RSM method was used to design the experiments. Optimization of medium supplements could be achieved by various approaches. This method has been widely utilized in biotechnological processes for bioprocess optimization exploring several interplaying factors [16]. Placket Burman software was selected to design these experiments because it minimises the number of experiments, is realistic and could analyze different factors and their interactions simultaneously.

It has been demonstrated that glycine induces morphological changes and cell lysis [17,18]. Hammes *et al* reports that glycine could be incorporated into the peptidoglycan precursors and peptidoglycan [19]. Research suggests that since glycine-containing precursors are poor substrates in transpeptidation reactions, a high percentage of muropeptides remains uncross-linked [19]. Therefore, it increases membrane permeability for recombinant protein. Fu *et al* reports that the chemical triton X-100 could release recombinant protein to the extracellular medium by permeabilization of the cells [20]. Triton X-100 is reported to inhibit synthesis of membrane phospholipids, which would promote extracellular translocation of recombinant proteins by affecting integrity of the cell membrane [12].

Several lines of evidence indicate that triton X-100 mainly effects the inner membrane [21]. Aside from their individual ability to permeate the *E. coli* membrane, Bin Lee *et al* reveals a potential mechanism in the interaction effect of glycine and triton X-100 [12]. Research has shown that combination of glycine and triton X-100 could result in drastically increased permeability of both inner and outer membranes [12]. More interestingly, Li *et al* reveals that glycine and Ca^{2+} can markedly enhance secretion of recombinant proteins to extracellular medium [13]. However, Li *et al* demonstrates that Ca^{2+} did not alter directly total translocation rates of the recombinant protein through the membrane barriers. Ca^{2+} helped to compensate for the adverse effects of glycine treatment through reduced cell number and viability by promoting healthy cell growth by maintaining sufficient membrane permeability [13].

In light of these observations, it can be concluded that SK secretion was mostly effected by glycine, triton X-100 and Ca^{2+} due their specific mechanism of interactions. It seems that glycine and triton X-100 were responsible for inner and outer membrane permeability, while Ca^{2+} apparently provided support for healthy cell growth and cell viability, which was harmed by the adverse effects of glycine and triton X-100. The collective effect of the interplay between these factors is successful permeability of the *E. coli* membrane for SK secretion that was confirmed by enzyme activity assays. Moreover, permeability of the inner membrane may facilitate translocation of expressed SK into the preplasmic space and lead to more soluble SK production and increased enzyme activity.

There are several reports on different approaches to extracellular expression of SK. However, the significant increment of the extracellular SK activity, in comparison to other studies [22-26], confirms robustness of the method employed in these experiments (Table 7). Although translocation of recombinant SK across the bacterial membrane was significantly increased (> 7-fold) following optimization, the problem of non-specific protein secretion and adverse effects of selected chemical factors on produced enzyme activity needs further investigation.

CONCLUSION

Optimization of media conditions for secretory expression of streptokinase in *E. coli* has been successfully conducted by RSM. Glycine, triton X-100 and Ca^{2+} are the most effective chemical factors in terms of increase in membrane permeability, with a 7-fold increase in the amount of secreted recombinant protein. To the best of our knowledge, this is the first study to determine the essential chemicals of a culture medium for SK release and optimization.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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