Statistical Optimization of Media Components for Enhanced Production of the Recombinant Staphylokinase Variant from Salt Inducible *E.Coli* GJ1158

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Abstract

Different types of nutritional factors are influencing the production of recombinant staphylokinase variant from Msak - Hirulog p^{RSET-A} GJ1158, which was constructed in our lab. The optimal conditions for the production of recombinant staphylokinase variant by using submerged fermentation at 37^0C for 6 hours after induction with 300 mM NaCl at flask level with a working volume of 100 ml. Initially, all nutritional factors were selected by one factor- at-a-time method. The significance of each factor with respect to recombinant staphylokinase production was identified by Taguchi (7 factors viz.,glucose, K_2HPO_4, TMM, KH_2PO_4, NH_4Cl, yeast extract and $MgSO_4$)and the enzymatic activity was increased remarkably by 1.32 folds compared to the test tube level (7580 to 10,081 U/mL). Based on taguchi results glucose, K_2HPO_4, TMM and KH_2PO_4 are the most influencing parameters. The outcome of taguchi design showed that the further optimization using response surface methodology (Central Composite Design) with 30 experiments increased the yield of 1.98 folds (10,081to 19,928 U/ml). For the first time we optimized the components concentration for enhanced production of sak variants and having maximum activity.

Keywords: *recombinant staphylokinase, one factor–at–a–time method, Taguchi, central composite design, enzyme activity*

1. Introduction

Staphylokinase (Sak), an extracellular protein produced by *Staphylococcus aureus* strains, is a promising blood clot dissolving agent [1]. Sak is a profibrinolytic agent that forms a 1:1 stoichiometric complex with plasminogen that, after conversion to plasmin, activates other plasminogen molecules to plasmin [2]. Now-a-days sak has gain lot of importance in thrombotic complications due to its mechanism of action and less side effects.

Still now no investigations have been performed on use of statistical methods for optimization of media components for the enhanced production of recombinant staphylokinase.Optimization by one-factor-at-a-time method (classical method) involves changing individual variable while fixing the others at a certain arbitrary levels [3]. From large number of factors, a statistical design enables easy selection of important parameters. Initially, one-factor-at-a-time method was used to investigate the effect of media constituents, such as carbon and nitrogen source. Later, the concentration of the medium components were optimized by using orthogonal matrix method and by Design Expert Version 6.0.10 version.

The present study aims to investigate the enhanced production of recombinant staphylokinase variant fusion protein within low cost by using the classical method one-factor-at-a-time, Plackett–Burman design, Orthogonal matrix method and response surface methodology (RSM).

2. Materials and Methods

2.1 Medium Components

All nutrient components like glucose, fructose, sugar, lactose, maltose, xylose, yeast extract, beef extract, soya peptone, corn steep liquor, meat peptone, mycological peptone and other chemicals like $MgSO_4$, K_2HPO_4, KH_2PO_4, NH_4Cl, $Al_2(SO_4)_3.7H_2O$, $CuSO_4.H_2O$, H_3BO_4, $MnCl_3.4H_2O$, $NiCl_2.6H_2O$, $Na_2MoO_4.2H_2O$, $ZnSO_4.7H_2O$, $FeSO_4$, $NaNO_3$ $(NH_4)_2HPO_4$ and NH_4NO_3were procured from Hi- Media Limited, Mumbai, India. Ampicillin was procured from Ranbaxy, India.

2.2 Media Preparation and Culture Conditions

All growth experiments were carried out in 1000 ml conical flasks with a working volume of 250 ml. The fermentation studies were carried out on production medium containing K_2HPO_4 – 6g/L, KH_2PO_4 - 3 g/L, NH_4Cl – 1 g/L, Yeast extract – 5 g/L, Glucose – 5g/L, 1M $MgSO_4$ – 2 mL, TMM – 1 mL ($Al_2(S0_4)_3.7H_2O$ – 10mg/L, $CuSO_4.H_2O$ – 2 mg/L, H_3BO_4 – 1 mg/L, $MnCl_3.4H_2O$ – 20 mg/L, $NiCl_2.6H_2O$ – 1 mg/L, $Na_2MoO_4.2H_2O$ – 50 mg/L, $ZnSO_4.7H_2O$ – 50 mg/L, $FeSO_4$ – 50 mg/L). The medium was further supplemented with appropriate amounts of ampicillin (100µg/µl). The initial pH of the medium was not adjusted to any value before autoclaving at 121 °C for 15 to 20 min (resulting in an initial pH value in the range of pH 6.9 to 7.2). The autoclaved medium was inoculated aseptically with4% of overnight fresh culture. Allow the flask on rotary shaker and maintain the temperature at 37^0C.

2.3 Optimization by using one Factor-at-a-time

Mainly the following factors like carbon and nitrogen were studied for the optimization of medium components.

2.3.1. Effect of Carbon Source

In the production medium, glucose was substituted with six different types of carbon sources viz. fructose, galactose, lactose, maltose, xylose and sucrose. The carbon sources were used at 5 g/L.

2.3.2. Effect of Nitrogen Source

In the production medium, yeast extract was substituted with soya peptone, meat peptone, mycological peptone, beef extract, and corn steep liquor were used at 5 g/L.

2.3.3 Effect of pH

In order to study the effect of pH on staphylokinase production, fermentation runs were carried out at different pH values ranging between 5to 9.

2.4 Optimization of Media Components by using L_8 - Orthogonal Array

L_8 - Orthogonal array was designed, developed and analyzed by using MINITAB 13.00 software. All the experiments were carried out in duplicates. The L_8 – orthogonal array design was shown in Table 1.

Table 1. L_8 – Orthogonal Design

S. No	A	B	C	D	E	F	G	OD 1	OD 2	AA 1 (U/ml)	AA 2 (U/ml)
1	1 (0.75)	1 (2)	1 (0.75)	1 (4)	1 (5)	1 (1.5)	1 (4)	0.77	0.78	10077	10079
2	1 (0.75)	1 (2)	1 (0.75)	2 (6)	2 (7)	2 (2.5)	2 (6)	0.76	0.77	10075	10078
3	1 (0.75)	2 (4)	2 (1.25)	1 (4)	1 (5)	2 (2.5)	2 (6)	0.73	0.75	10070	10075
4	1 (0.75)	2 (4)	2 (1.25)	2 (6)	2 (7)	1 (1.5)	1 (4)	0.79	0.8	10077	10081
5	2 (1.25)	1 (2)	2 (1.25)	1 (4)	2 (7)	1 (1.5)	2 (6)	0.79	0.76	10079	10076
6	2 (1.25)	1 (2)	2 (1.25)	2 (6)	1 (5)	2 (2.5)	1 (4)	0.78	0.79	10078	10081
7	**2 (1.25)**	**2 (4)**	**1 (0.75)**	**1 (4)**	**2 (7)**	**2 (2.5)**	**1 (4)**	**0.79**	**0.81**	**10079**	**10083**
8	2 (1.25)	2 (4)	1 (0.75)	2 (6)	1 (5)	1 (1.5)	2 (6)	0.74	0.76	10074	10078

A-TMM, B-KH_2PO_4, C-NH_4Cl, D-Yeast extract, E- K_2HPO_4, F-$MgSO_4$, G-Glucose
AA – Acitivity Analysis

2.5 Estimation of r-SAK Assay

r-SAK and r-SAK variant (Sak–Hirulog (SH)) activity was determined by using plasminogen coupled chromogenic substrate assay according to the method as previously described [4]. The Sak activity was assayed by mixing 4µl plasminogen (0.025 U/µl) in 20µl of 20 mM Tris-HCl buffer pH 7 and 20 µl of appropriate dilution of rSak (30 ng) in 50 mM potassium phosphate buffer (pH 7) and incubated at 37^0C for 30 min. The plasminogen sak complexes were mixed with 80 µl of 1 mM chromogenic substrate AAS in 50 mM potassium phosphate buffer (pH 7) and incubated at 37^oC for 15 min. The reaction was terminated by adding 20 µl of 0.5 M acetic acid and the absorbance was measured at 405 nm. One unit of Sak was defined as one unit of standard streptokinase from Sigma-Aldrich Co., liberating a standard clot of fibrinogen, plasminogen, and thrombin at pH 7.5 and 37^oC for 10 min. The units of Sak were calculated by using standard curve of pure standard streptokinase from Sigma-Aldrich.

2.6 Optimization of Components of the Selected Medium by RSM

To examine the combined effect of four independent variables A: Glucose; B: KH_2PO_4; C: TMM; D: K_2HPO_4 on maximum production of recombinant staphylokinase variant (SH), media was optimized by one factor at-a-time and Taguchi method was used. Each variable in the design was studied at two different levels, with four variables taken at a central coded value of zero. The experiments were designed using the software, Design Expert Version 6.0.10 version (Stat Ease).Accordingly, a factorial experimental design, with an axial point (α=2) and six replicates at the center point, with a total number of 30 experiments, was

employed. The CCRD matrix in terms of coded and actual values of independent variables is given in Table 2.

Table 2. The CCRD Matrix of Independent Variables in Coded Form with their Corresponding Response from Experiments

Std	Run	Block	A:Glucose g/L	B:K_2HPO_4 g/L	C:TMM ml	D:KH_2PO_4 g/L	Experimental[a] U/mL	Predicted value U/mL
1	13	Block 1	2	5	1	5	19810	19784.79
2	7	Block 1	6	5	1	5	19861	19864.25
3	3	Block 1	2	9	1	5	19675	19679.58
4	19	Block 1	6	9	1	5	19616	19608.29
5	2	Block 1	2	5	1.5	5	19371	19368.91
6	27	Block 1	6	5	1.5	5	19810	19832.62
7	9	Block 1	2	9	1.5	5	19190	19183.95
8	30	Block 1	6	9	1.5	5	19510	19496.91
9	1	Block 1	2	5	1	9	19320	19365.25
10	28	Block 1	6	5	1	9	19160	19126.45
11	10	Block 1	2	9	1	9	19596	19533.79
12	29	Block 1	6	9	1	9	19110	19144.25
13	24	Block 1	2	5	1.5	9	19770	19738.12
14	5	Block 1	6	5	1.5	9	19856	19883.58
15	22	Block 1	2	9	1.5	9	19798	19826.91
16	25	Block 1	6	9	1.5	9	19836	19821.62
17	26	Block 1	0	7	1.25	7	19820	19840.62
18	**20**	**Block 1**	**8**	**7**	**1.25**	**7**	**19928**	**19914.79**
19	23	Block 1	4	3	1.25	7	19796	19789.29
20	17	Block 1	4	11	1.25	7	19608	19622.12

21	15	Block 1	4	7	0.75	7	19380	19396.95
22	14	Block 1	4	7	1.75	7	19668	19658.45
23	18	Block 1	4	7	1.25	3	19243	19251.12
24	21	Block 1	4	7	1.25	11	19157	19156.29
25	8	Block 1	4	7	1.25	7	19350	19374.66
26	4	Block 1	4	7	1.25	7	19365	19374.66
27	16	Block 1	4	7	1.25	7	19380	19374.66
28	11	Block 1	4	7	1.25	7	19389	19374.66
29	12	Block 1	4	7	1.25	7	19379	19374.66
30	6	Block 1	4	7	1.25	7	19385	19374.66

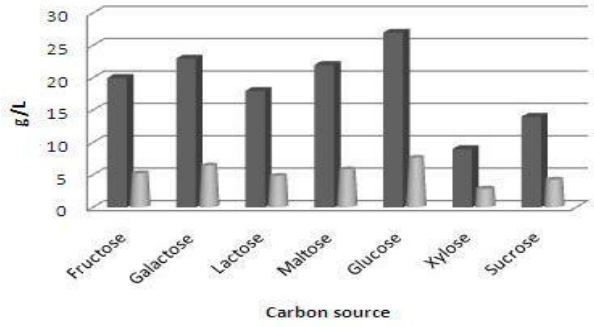
[a] values are mean±SD of two determinations.

3. Results & Discussion

3.1 One Factor-at-a-time Method

During the microbial fermentations the carbon source not only acts as a major constituent for synthesis of cellular material, but also used in synthesis of polysaccharide and as energy source [5, 6].

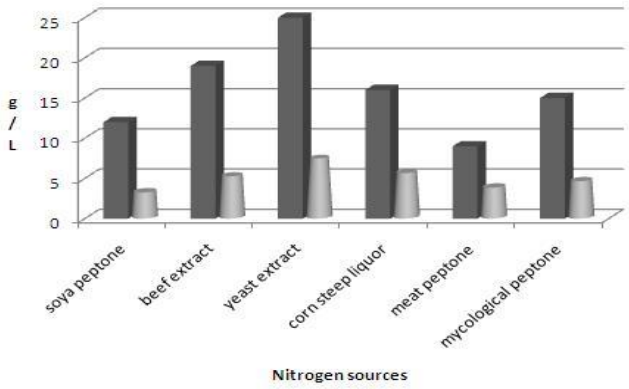
Figure 1 shows the effect of different carbon sources on dry cell weight (DCW). The medium was supplemented with carbohydrates as carbon sources. Different carbohydrates such as fructose, Galactose, lactose, maltose, glucose, xylose and sucrose were used as carbon sources but only glucose, Galactose and maltose were found to be promising. Glucose supported maximum enzyme production of 7.6 g/l and gave maximum biomass.



Boxes indicating the total biomass and partial pyramid indicating the enzyme production

Figure 1. Effect of Different Carbon Sources on Biomass and Enzyme Production

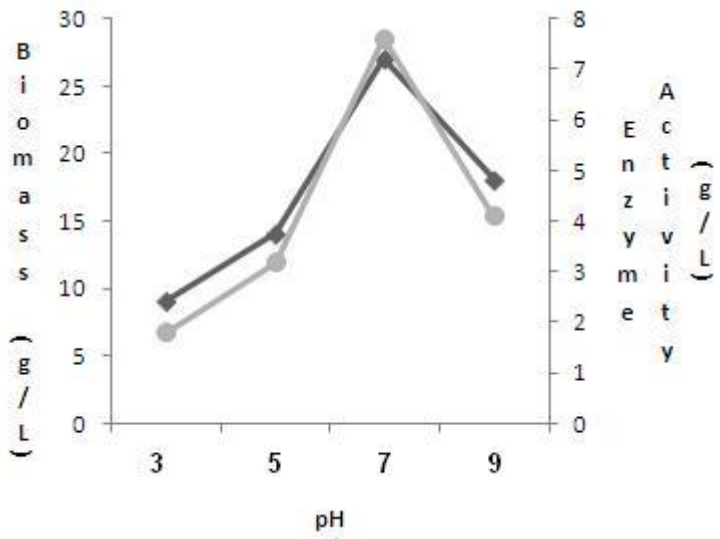
Figure 2 shows the effect of different nitrogen sources on recombinant staphylokinase variant production. Among the six nitrogen sources yeast extract gave the maximum biomass and enzyme production of 25 g/L and 7.4 g/L respectively. Beef extract and corn steep liquor gave maximum biomass production after yeast extract.



Boxes indicating the total biomass and partial pyramid indicating the enzyme production

Figure 2. Effect of Different Nitrogen sources on biomass and enzyme production

Figure 3 shows the effect of pH for the production of sak variant. 7 is the ideal pH for the production of maximum biomass and enzyme production. At low pH and



Squares (■) indicating the total biomass and rounded symbol (●) indicating the enzyme production

Figure 3. Effect of Biomass and Enzyme Production at Different pH

3.2 Optimization using L_8-orthogonal Array

Once the best carbon and nitrogen sources were selected, the medium was subjected to further optimization using L_8-orthogonal array. The parameters optimized involved concentrations of glucose, KH_2PO_4, KH_2PO_4, TMM, yeast extract, $MgSO_4$ and NH_4Cl. Tables 1 & 2 represents the response table for means (larger is better) and for signal to noise ratio obtained with L_8-orthogonal array. The last two rows in the tables document the delta values and ranks for the system. Rank and delta values help to assess which factors have the greatest

effect on the response characteristic of interest. Delta measures the size of the effect by taking the difference between the highest and lowest characteristic average for a factor. A higher delta value indicates greater effect of that component. Rank orders the factors from the greatest effect (based on the delta values) to the least effect on the response characteristic.

The order in which the individual components selected in the present study effect the fermentation process can be ranked as glucose > KH_2PO_4 > TMM > K_2HPO_4 suggesting that glucose has a major effect and K_2HPO_4 had least effect on staphylokinase production. Figures 4 and 5 represent the main effect plots for the system. Main effects plots show how each factor affects the response characteristic. A main effect is present when different levels of a factor affect the characteristic differently. MINITAB creates the main effects plot by plotting the characteristic average for each factor level. These averages are the same as those displayed in the response Tables 3 & 4. A line connects the points for each factor. When the line is horizontal (parallel to the x-axis), then there is no main effect present. Each level of the factor affects the characteristic in the same way and the characteristic average is the same across all factor levels. When the line is not horizontal (parallel to the x-axis), then there is a main effect present. Different levels of the factor affect the characteristic differently. The greater the difference in the vertical position of the plotted points (the greater the deviation from the parallel x - axis), the greater is the magnitude of the main effect was shown in the Figures 4 & 5.

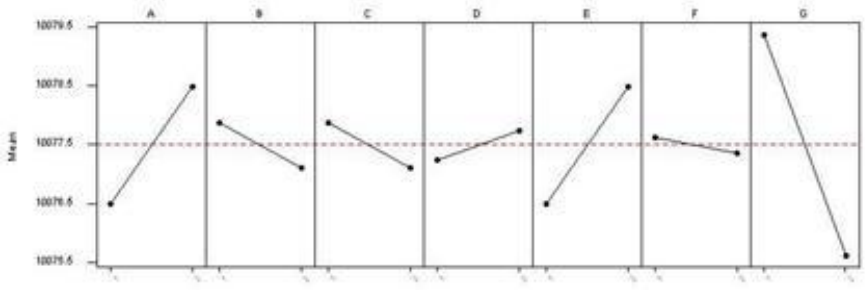
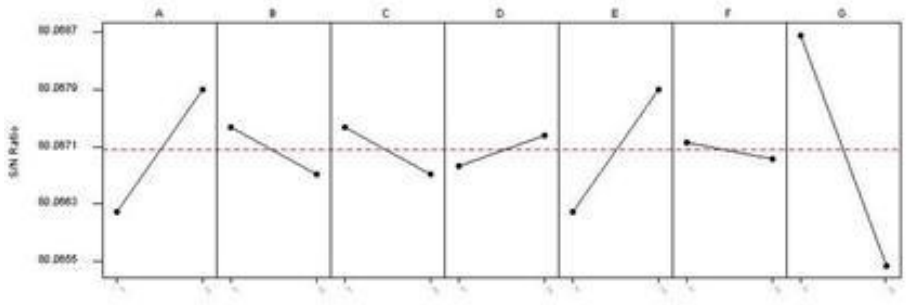


Figure 4. Main Effects Plot for Means



Figures 5. Main Effects Plot for S/N Ratios

Response tables can also be used to predict the optimal levels of each component used in the study. To obtain the optimized levels or composition of each factor, the predictive analysis based on statistical calculations is shown in Tables 3 & 4.

Table 3. Response Table for S/N Ratio

Level	A	B	C	D	E	F	G
1	80.0662	80.0674	80.0674	80.0668	80.0662	80.0672	80.0687
2	80.0679	80.0667	80.0667	80.0673	80.0679	80.0669	80.0654
Delta	0.0017	0.0006	0.0006	0.0004	0.0017	0.0002	0.0032
Rank	3	4	5	6	2	7	1

A-TMM, B-KH_2PO_4, C-NH_4Cl, D-Yeast extract, E- K_2HPO_4, F-$MgSO_4$, G-Glucose

Table 4. Response Table for Means

Level	A	B	C	D	E	F	G
1	10076.5	10077.9	10077.9	10077.3	10076.5	10077.6	10079.4
2	10078.5	10077.1	10077.1	10077.8	10078.5	10077.4	10075.6
Delta	2.0	0.8	0.8	0.5	2.0	0.3	3.8
Rank	2.5	4.5	4.5	6.0	2.5	7.0	1.0

A-TMM, B-KH_2PO_4, C-NH_4Cl, D-Yeast extract, E- K_2HPO_4, F-$MgSO_4$, G-Glucose

3.3 Optimization of Concentrations of the Selected Medium Components by RSM

The combined effect of four independent variables A: Glucose; B: K_2HPO_4; C: TMM; D: KH_2PO_4 for production of recombinant staphylokinase variant was examined by using RSM. The CCRD gave quadratic model for the given set of experimental results. The following equation represents the mathematical model relating for the production of recombinant staphylokinase variant with the independent process variables, A to D and the second order polynomial coefficient for each term of the equation determined through multiple regression analysis using the Design Expert 6.0.10. The experimental and predicted values of yields of r-sak variant were given in Table 2. The results were analyzed by using ANOVA, i.e., analysis of variance suitable for the experimental design used. The ANOVA of the quadratic model indicated that the model is significant. The model F-value of 132.78 implies the model to be significant and is calculated as ratio of mean square regression and mean square residual. Model P-value (Prob> F) was very low (<0.0001), again signifying the model to be significant.

The smaller the magnitude of the P, the more significant is the corresponding coefficient. values of P less than 0.05 indicate the model terms to be significant. The coefficient estimates and the corresponding P values suggests that, among the test variables used in the study, A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD and CD (where A = Glucose B = KH_2PO_4, C = TMM and D = K_2HPO_4) are significant model terms

Final equation in terms of coded factors for Staphylokinase activity (U/mL) = 19374.66667 + 18.54166667 X Glucose - 41.79166667 X K_2HPO_4 + 65.375 X TMM -23.70833333 X KH_2PO_4 + 125.7604167 X $(Glucose)^2$ + 82.76041667 X $(K_2HPO_4)^2$ + 38.26041667 X $(TMM)^2$-42.73958333 X $(KH_2PO_4)^2$-37.6875 X Glucose X K_2HPO_4+96.0625 X Glucose X TMM -79.5625 X Glucose X KH_2PO_4-19.9375X K_2HPO_4X TMM + 68.4375 X K_2HPO_4X KH_2PO_4 + 197.1875 X TMM X KH_2PO_4.

Final equation in terms of actual factors for Staphylokinase activity (U/mL) = 25269.875 - 277.21875 X Glucose - 342.7916667 X K_2HPO_4 - 4518.916667 X TMM - 395.4375 X KH_2PO_4 + 31.44010417 X (Glucose)^2 + 20.69010417 X (K_2HPO_4)^2 + 612.1666667 X (TMM)^2 - 10.68489583 X (KH_2PO_4)^2-9.421875 X Glucose X K_2HPO_4 + 192.125 X Glucose X TMM-19.890625 X Glucose X KH_2PO_4 - 39.875 X K_2HPO_4 X TMM + 17.109375 X K_2HPO_4 X KH_2PO_4 + 394.375 X TMM X KH_2PO_4.

The fit of the model was also expressed by the coefficient of regression (R^2), which was found to be 0.9920, indicating that 99.2% of the confidence level of the model to predict the response. The ‘‘Pred R-Squared” of 0.9564 is in reasonable agreement with the ‘‘Adj R-Squared” of 0.9845. ‘‘Adeq Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, the ratio of 35.449 indicates an adequate signal.

Accordingly, three-dimensional graphs were generated for the pair-wise combination of the four factors, while keeping the other two at their center point levels. From the central point of the contour plot or from the bump of the 3D plot the optimal composition of medium components was identified. Figures 6 to 11 illustrated the response surface plot for staphylokinase activity of sak variant. Figure 12 illustrated the parity plot for the distribution of predicted and experimental values of enhanced staphylokinase variant activity.

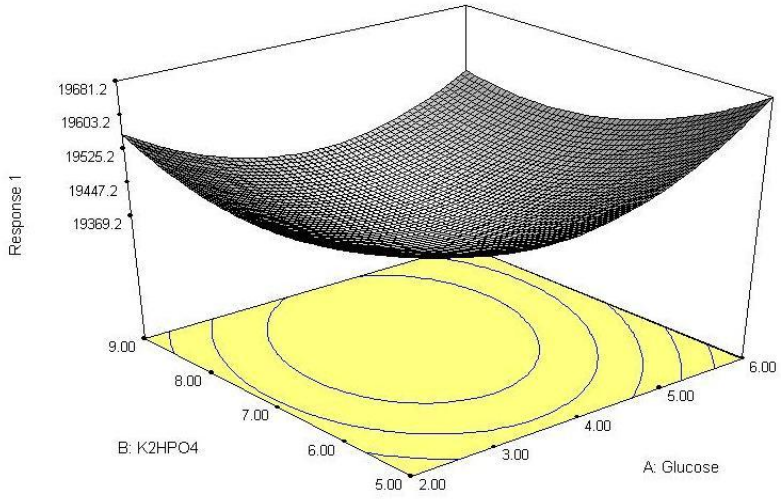


Figure 6. Illustrated the Response Surface Plot for Staphylokinase Activity of sak variant (Staphylokinase - Hirulog); Effect of Glucose and K_2HPO_4

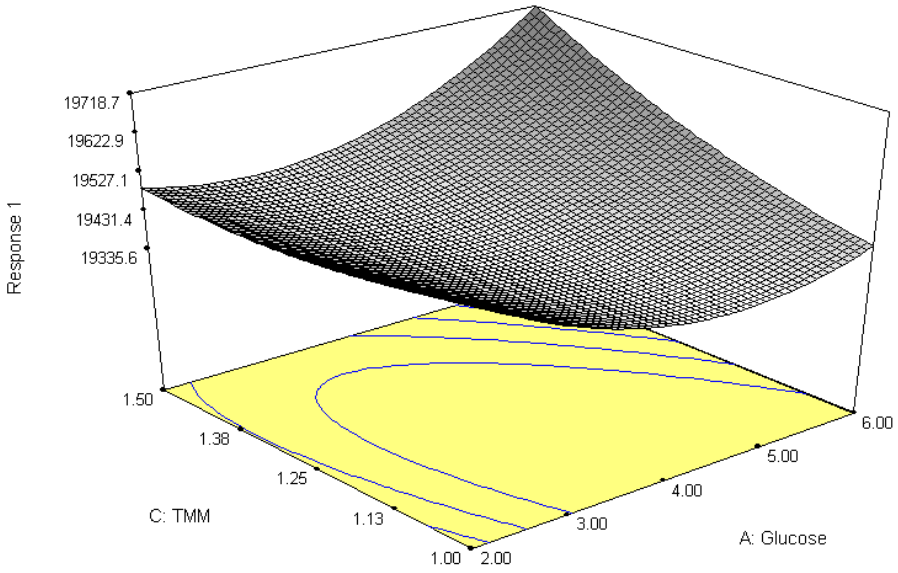


Figure 7. Illustrated the Response Surface Plot for Staphylokinase Activity of sak variant (Staphylokinase - Hirulog); Effect of Glucose and TMM

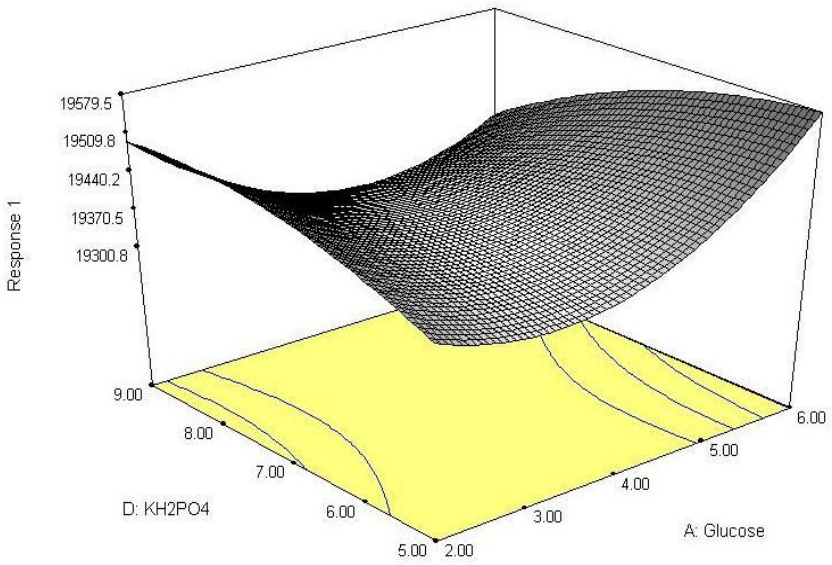


Figure 8. Illustrated the Response Surface Plot for Staphylokinase Activity of sak variant (Staphylokinase - Hirulog); Effect of Glucose and KH_2PO_4

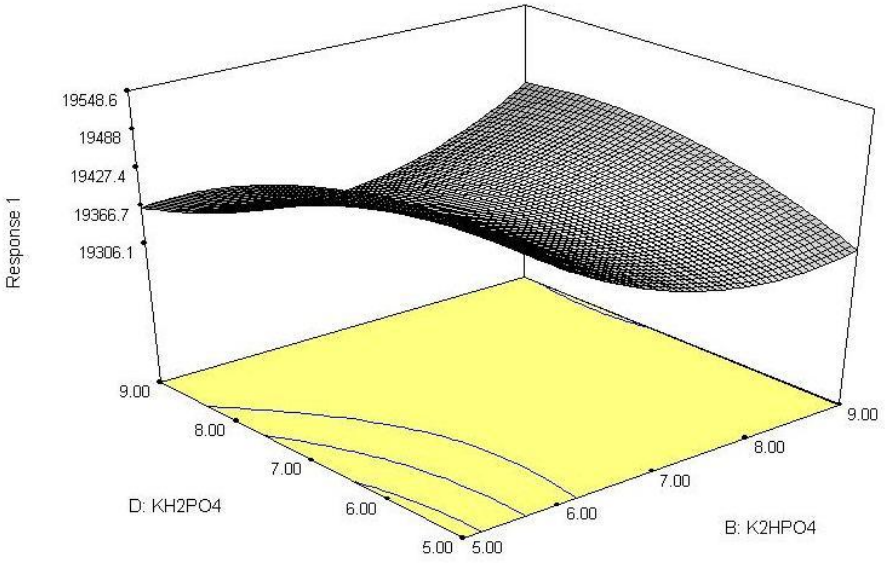


Figure 9. Illustrated the Response Surface Plot for Staphylokinase Activity of sak variant (Staphylokinase - Hirulog); Effect of KH_2PO_4 and K_2HPO_4

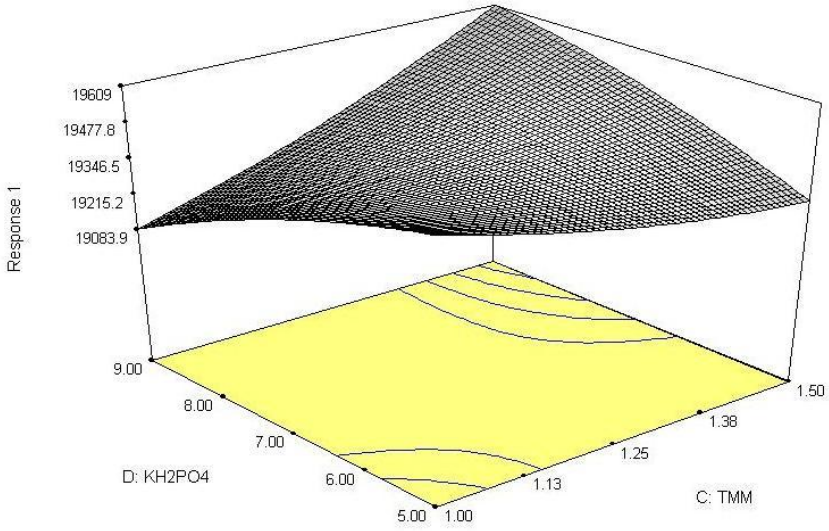


Figure 10. Illustrated the Response Surface Plot for Staphylokinase Activity of sak variant (Staphylokinase - Hirulog); Effect of KH_2PO_4 and TMM

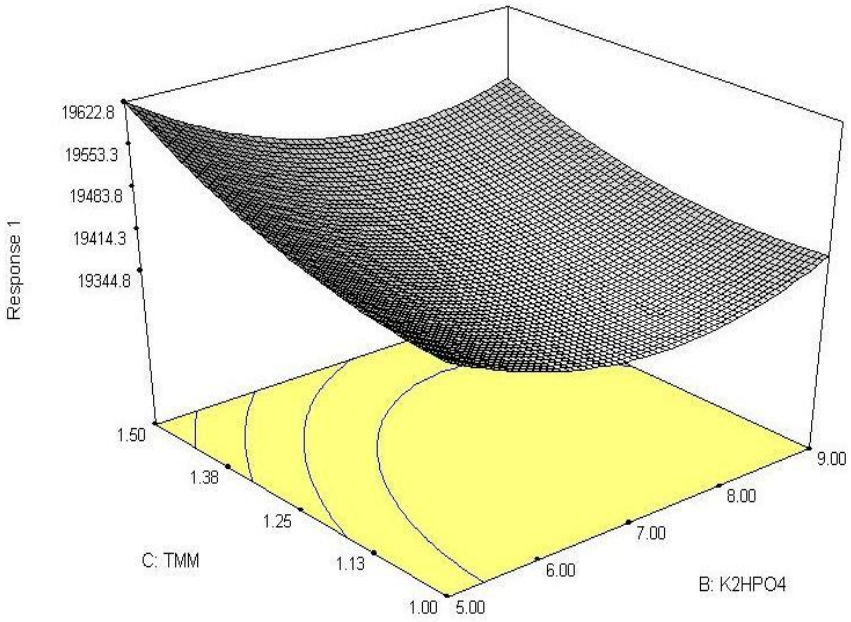


Figure 11. Illustrated the Response Surface Plot for Staphylokinase Activity of sak variant (Staphylokinase - Hirulog); Effect of K_2HPO_4 and TMM

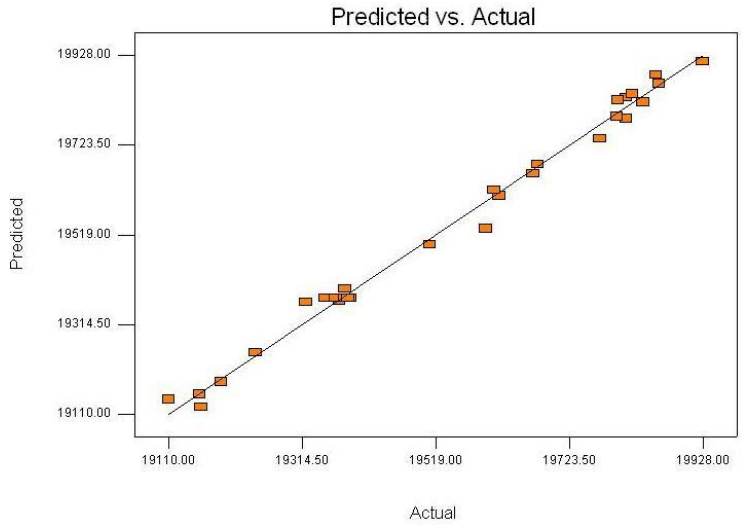


Figure 12. Illustrated the Parity Plot for the Distribution of Predicted and Experimental Values of Staphylokinase Activity of sak variant (Staphylokinase - Hirulog)

4. Conclusion

Thrombotic complications requires a third generation molecule, staphylokinase (sak) and its variant (SAK-Hirulog) which is having less side effects like reocclusion. But in production point of view, the best medium composition was optimized instead of nutrient broth to get the

maximum enzyme activity. The production medium was optimized in the present study with different statistical approaches like PB, Taguchi and response surface methodology was useful for production at large scale. The biomass and enzyme activity of sak variant was same like sak. The final yield (SH) was increased remarkably by 2.62 folds over the test tube results. So, in future lot applications have to be done for the production of ideal therapeutics against to different disorders with in low cost where the industrial needs are satisfied.

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