Optimization of Media Components for the Over Production and Enhanced Fibrinolytic Activity of Recombinant Msak – RGD – Hirulog from *E.Coli* GJ1158

Seetha Ram Kotra[1*], N. Prudvi[1#], JB Peravali[2], Anmol Kumar[1#], K. R. S. Sambasiva Rao[1] and K. K. Pulicherla[3**]

[1]*Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh, India*
[2]*Department of Biotechnology, Bapatla Engineering College, Bapatla, Guntur, Andhra Pradesh, India*
[3]*Department of Biotechnology, RVR & JC College of Engineering, Chowdavaram, Guntur, Andhra Pradesh, India*

[*] *Corresponding author: kotraseetharam@gmail.com*
+919491475860
[**] *Co – first author*
[#] *Authors have equal contribution*

Abstract

*Fibrinosis, platelet aggregation and thrombosis were the leading hurdles in the patients alleged from myocardial infarction and stroke. Various nutritional factors were influencing the production of multifunctional recombinant staphylokinase variant (**M**sak – **R**GD – **H**irulog) from salt inducible E.coli* GJ1158*, which was constructed in our lab. One-factor-at-a-time method was used to investigate the constituents like carbon and nitrogen sources for the best medium composition. Initial optimization was carried out by using L_{27} - orthogonal array method. The significance of each factor with respect to MRH production was identified by Taguchi (Dextrose Mono Hydrate (DMH), Na_2HPO_4, KH_2PO_4, TMM, NH_4Cl, Yeast extract and 1M $MgSO_4$) and the specific fibrinolytic activity was increased 1.34 folds (20,058 to 22,763 U/mL). The outcome of taguchi results indicates the dextrose mono hydrate, yeast extract, Na_2HPO_4 and KH_2PO_4 are influencing in the production of MRH. Final optimization was carried out using response surface methodology (Central Composite Rotatable Design) and the activity of MRH was significantly enchanced to 1.17 folds (22,763 to 26,758 U/mL). For the first time, cost effective media constituents for enhanced production (960 mg/L) of multi-functional MRH and its activity (26,758 U/mL) was studied.*

Keywords: *MRH, E.coli* GJ1158*, fibrinolytic activity, One factor–at–a–time method, Orthogonal array, Central composite design*

1. Introduction

Stroke and other cerebrovascular diseases are major threating to human health because of thrombosis [1, 2]. Staphylokinase was produced by certain species of *Staphylococcal* strains, is to become a promising blood clot dissolving agent in future [3]. Sak is not an enzyme but forms 1:1 stoichiometric complex with plasminogen that, after conversion to plasmin, activates other plasminogen molecules to plasmin [4]. Due to its specific mechanism of

action, now-a-days sak and its variants has gained a lot of significance in thrombotic complications with less side effects like bleeding and reocclusion.

Still now no investigations have been carried out on statistical methods to optimize the media components for the enhanced production of newly constructed Msak – RGD – Hirulog. Different nutritional requirements may be manipulated by conventional or statistical methods [5]. From a huge number of factors, a statistical design enables easy selection of important parameters. Initially, one-factor-at-a-time (classical) method was used to investigate the effect of media constituents, such as carbon and nitrogen source. Intial optimization was carried out by using orthogonal matrix and final optimization was carried out by Design Expert Version 6.0.10 version. Among response surface methodology (RSM), Central composite rotatable design (CCRD) is the most widely used in fermentation media optimization [6, 7, 8] because of a desirable property, *i.e.*, rotability [9].

The present study aims on economical production and enhanced activity of recombinant staphylokinase variant (MRH) fusion protein within affordable cost by using the classical method one-factor-at-a-time, Orthogonal matrix method and response surface methodology (RSM).

2. Materials and methods

2.1. Medium components

All nutrient components like Dextrose mono hydrate, sugar, lactose, fructose, xylose, maltose, beef extract, yeast extract, soya peptone, meat peptone, corn steep liquor, mycological peptone and other chemicals like $MgSO_4$, KH_2PO_4, Na_2HPO_4, NH_4Cl, H_3BO_4, $Al_2(SO_4)_3.7H_2O$, $CuSO_4.H_2O$, $Na_2MoO_4.2H_2O$, $MnCl_3.4H_2O$, $NiCl_2.6H_2O$, $ZnSO_4.7H_2O$, $NaNO_3$, $FeSO_4$, NH_4NO_3 and $(NH_4)_2HPO_4$were 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 500 ml conical flasks with a working volume of 100 ml. The fermentation studies were carried out in modified GYE medium containing Na_2HPO_4 – 6g/L, KH_2PO_4 - 3 g/L, NH_4Cl – 1 g/L, Yeast extract – 5 g/L, Dextrose mono hydrate – 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 (100 µg/µl). The initial pH of the medium was not adjusted to any value before autoclaving at 121 ^{0}C for 15 to 20 min (resulting in an initial pH value in the range of pH 7.0 ± 0.2). The autoclaved medium was inoculated aseptically with 3% of overnight fresh culture at 37 ^{0}C.

2.3. Optimization of components 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 modified GYE medium, DMH was substituted with five different types of carbon sources viz., galactose, fructose, maltose, lactose and sucrose. The carbon sources were used at 5 g/L.

2.3.2. Effect of Nitrogen Source - In the modified GYE medium, yeast extract was substituted with meat peptone, soya peptone, beef extract, mycological peptone and corn steep liquor. The nitrogen sources were used at 5 g/L.

2.4. Optimization using L_{27} - Orthogonal array

L_{27} – Orthogonal array was designed, developed and analyzed by using MINITAB 14.00 software. All the experiments were carried out in duplicates. The L_{27} – orthogonal array design was shown in Table 1.

Table 1. L_{27} – Orthogonal design

S. No.	A	B	C	D	E	F	G	FA 1 (U/mL)	FA 2 (U/mL)
1	1 (4.0)	1 (5.0)	1 (4.0)	1 (2.0)	1 (0.5)	1 (1.5)	1 (0.5)	16782	16924
2	1 (4.0)	1 (5.0)	1 (4.0)	1 (2.0)	2 (1.0)	2 (2.0)	2 (1.0)	17825	17926
3	1 (4.0)	1 (5.0)	1 (4.0)	1 (2.0)	3 (1.5)	3 (2.5)	3 (1.5)	20815	20194
4	1 (4.0)	2 (6.0)	2 (5.0)	2 (3.0)	1 (0.5)	1 (1.5)	1 (0.5)	16984	17106
5	1 (4.0)	2 (6.0)	2 (5.0)	2 (3.0)	2 (1.0)	2 (2.0)	2 (1.0)	18642	18428
6	1 (4.0)	2 (6.0)	2 (5.0)	2 (3.0)	3 (1.5)	3 (2.5)	3 (1.5)	19248	19006
7	1 (4.0)	3 (7.0)	3 (6.0)	3 (4.0)	1 (0.5)	1 (1.5)	1 (0.5)	20079	20272
8	1 (4.0)	3 (7.0)	3 (6.0)	3 (4.0)	2 (1.0)	2 (2.0)	2 (1.0)	21562	21652
9	1 (4.0)	3 (7.0)	3 (6.0)	3 (4.0)	3 (1.5)	3 (2.5)	3 (1.5)	21458	21378
10	2 (5.0)	1 (5.0)	2 (5.0)	3 (4.0)	1 (0.5)	2 (2.0)	3 (1.5)	19658	19794
11	2 (5.0)	1 (5.0)	2 (5.0)	3 (4.0)	2 (1.0)	3 (2.5)	1 (0.5)	18653	18486
12	2 (5.0)	1 (5.0)	2 (5.0)	3 (4.0)	3 (1.5)	1 (1.5)	2 (1.0)	19756	19569
13	2	2	3	1	1	2	3	19986	20102

	(5.0)	(6.0)	(6.0)	(2.0)	(0.5)	(2.0)	(1.5)		
14	2 (5.0)	2 (6.0)	3 (6.0)	1 (2.0)	2 (1.0)	3 (2.5)	1 (0.5)	21572	21496
15	2 (5.0)	2 (6.0)	3 (6.0)	1 (2.0)	3 (1.5)	1 (1.5)	2 (1.0)	22189	22226
16	2 (5.0)	3 (7.0)	1 (4.0)	2 (3.0)	1 (0.5)	2 (2.0)	3 (1.5)	20785	20684
17	2 (5.0)	3 (7.0)	1 (4.0)	2 (3.0)	2 (1.0)	3 (2.5)	1 (0.5)	21578	21684
18	2 (5.0)	3 (7.0)	1 (4.0)	2 (3.0)	3 (1.5)	1 (1.5)	2 (1.0)	22342	22275
19	3 (6.0)	1 (5.0)	3 (6.0)	2 (3.0)	1 (0.5)	3 (2.5)	2 (1.0)	21846	21769
20	3 (6.0)	1 (5.0)	3 (6.0)	2 (3.0)	2 (1.0)	1 (1.5)	3 (1.5)	22514	22486
21	**3 (6.0)**	**1 (5.0)**	**3 (6.0)**	**2 (3.0)**	**3 (1.5)**	**2 (2.0)**	**1 (0.5)**	**22763**	**22679**
22	3 (6.0)	2 (6.0)	1 (4.0)	3 (4.0)	1 (0.5)	3 (2.5)	2 (1.0)	21753	21846
23	3 (6.0)	2 (6.0)	1 (4.0)	3 (4.0)	2 (1.0)	1 (1.5)	3 (1.5)	22132	22234
24	3 (6.0)	2 (6.0)	1 (4.0)	3 (4.0)	3 (1.5)	2 (2.0)	1 (0.5)	21624	21539
25	3 (6.0)	3 (7.0)	2 (5.0)	1 (2.0)	1 (0.5)	3 (2.5)	2 (1.0)	22078	22143
26	3 (6.0)	3 (7.0)	2 (5.0)	1 (2.0)	2 (1.0)	1 (1.5)	3 (1.5)	21524	21687
27	3 (6.0)	3 (7.0)	2 (5.0)	1 (2.0)	3 (1.5)	2 (2.0)	1 (0.5)	22156	22254

A – Dextrose mono hydrate, B – Na_2HPO_4, C – Yeast extract, D – KH_2PO_4, E – TMM, F – $MgSO_4$, G – NH_4Cl, FA – Fibrinolytic Activity

2.5. Fibrinolytic assay

Fibrinolytic activity was determined by using fibrin plate method [10, 11]. 4 mL of 1 % (w/v) fibrinogen solution in 40 mM sodium phosphate buffer (pH 7.4) was mixed with the equal volume of 1 % (w/v) agarose solution containing 20 µl of a thrombin solution (200 NIH U/mL) and 10 µL of plasminogen (10 U/mL). The mixture was poured in petri dish and allowed to solidify at room temperature to form a fibrin clot layer. 20 µL of equimolar concentration of MRH was loaded on the wells in fibrin plate and incubated for 6 – 7 hrs at 37 ^{0}C. Enzyme activity of recombinant proteins in terms of Units (U/mL) was estimated by measuring the mean diameter of zone of clearance around the well, using clinically approved streptokinase as standard.

2.6. Optimization of components of the selected medium by RSM

To examine the combined effect of four independent variables A: DMH; B: Yeast extract; C: Na_2HPO_4; D: TMM on maximum production of MRH, media constituents were optimized by orthogonal array was used and to study their interactions using CCRD to achieve enhanced activity. 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 ($\alpha = 2$) 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

Std	Run	Block	A: DMH (g/L)	B: YE (g/L)	C: Na_2HPO_4 (g/L)	D: TMM (mL)	Experimental [a] U/mL	Predicted value U/mL
1	15	Block 1	3	3	3	0.75	24064	24051.29167
2	30	Block 1	9	3	3	0.75	25296	25273
3	12	Block 1	3	9	3	0.75	24758	24752.33333
4	7	Block 1	9	9	3	0.75	26318	26280.29167
5	4	Block 1	3	3	7	0.75	24849	24849.33333
6	27	Block 1	9	3	7	0.75	25289	25261.29167
7	14	Block 1	3	9	7	0.75	25487	25452.125
8	2	Block 1	9	9	7	0.75	26150	26170.33333
9	23	Block 1	3	3	3	2.25	24358	24330.66667

10	24	Block 1	9	3	3	2.25	25378	25358.625
11	18	Block 1	3	9	3	2.25	25443	25416.45833
12	**1**	**Block 1**	**9**	**9**	**3**	**2.25**	**26758**	**26750.66667**
13	26	Block 1	3	3	7	2.25	24447	24430.45833
14	22	Block 1	9	3	7	2.25	24650	24648.66667
15	25	Block 1	3	9	7	2.25	25402	25418
16	5	Block 1	9	9	7	2.25	25984	25942.45833
17	8	Block 1	0	6	5	1.5	23489	23512.04167
18	6	Block 1	12	6	5	1.5	25220	25258.20833
19	17	Block 1	6	0	5	1.5	22953	22986.20833
20	19	Block 1	6	12	5	1.5	24953	24981.04167
21	9	Block 1	6	6	1	1.5	26667	26716.20833
22	10	Block 1	6	6	9	1.5	26694	26706.04167
23	20	Block 1	6	6	5	0	25961	25990.875
24	29	Block 1	6	6	5	3	26011	26042.375
25	28	Block 1	6	6	5	1.5	25613	25598.16667
26	3	Block 1	6	6	5	1.5	25584	25598.16667
27	13	Block 1	6	6	5	1.5	25601	25598.16667
28	21	Block 1	6	6	5	1.5	25594	25598.16667
29	16	Block 1	6	6	5	1.5	25589	25598.16667
30	11	Block 1	6	6	5	1.5	25608	25598.16667

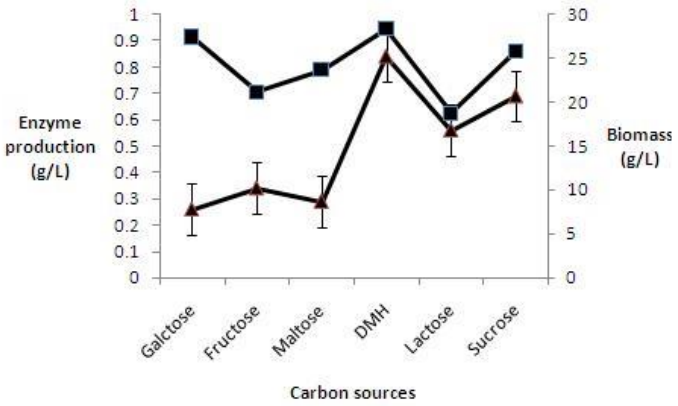
[a] values are mean ± SD of three determinations.

3. Results & Discussion

3.1. One factor-at-a-time method

The carbon source acts as a major constituent for synthesis of cellular material, also used in synthesis of polysaccharide and as energy source [12, 13]. The modified medium was supplemented with different carbohydrates as carbon sources including dextrose mono hydrate. Different carbohydrates like, Galactose, fructose, maltose, DMH, lactose and sucrose

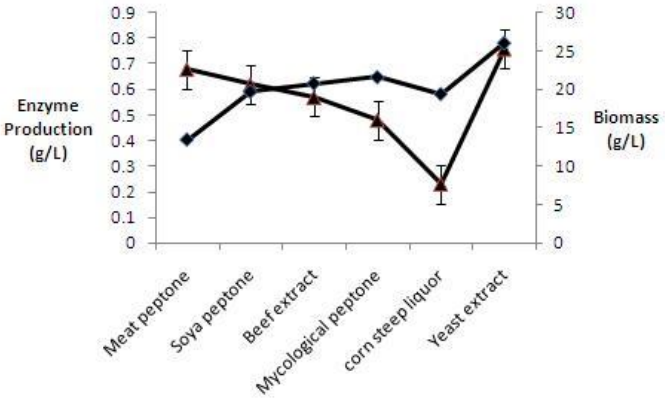
were used as carbon sources. But DMH was only found to be promising carbon sources and supported maximum biomass of 28.5 and 0.84 g/L of MRH.



Squares (■) indicating the biomass production and triangle (▲) indicating the enzyme production

Figure 1. Effect of different carbon sources on biomass and enzyme production

Figure 2 shows the effect of various nitrogen sources on recombinant MRH production. Among the different nitrogen sources yeast extract gave the maximum biomass and enzyme production of 26.1 g/L and 0.76 g/L respectively.



Squares (■) indicating the biomass production and triangle (▲) indicating the enzyme production

Figure 2. Effect of different nitrogen sources on biomass and enzyme production

3.2. Optimization using L_{27}-orthogonal array

After the selection of best carbon and nitrogen sources, the medium was subjected to initial optimization using L_{27} – orthogonal array. The parameters optimized involved the different concentrations of DMH, Na_2HPO_4, TMM, KH_2PO_4, Yeast extract, $MgSO_4$ and NH_4Cl. Table 1 & 2 represents the response table for means (Larger is better) and for S/N ratio was obtained with L_{27} – orthogonal array. The last two rows in both tables indicating the delta values and ranks for the designed system. These ranks and delta values help to assess

the factor which has the greatest effect on the desired response. The size of the effect was measured by delta by taking the difference between the highest and lowest characteristic average for each and every factor. A high delta value specifies the greater effect of that component. Rank orders the factors from the maximum effect 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 DMH > Yeast extract > Na_2HPO_4 > TMM suggesting that DMH has a major effect and TMM has the least effect on MRH production. Figures 3 and 4 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 Figures 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 3 & 4.

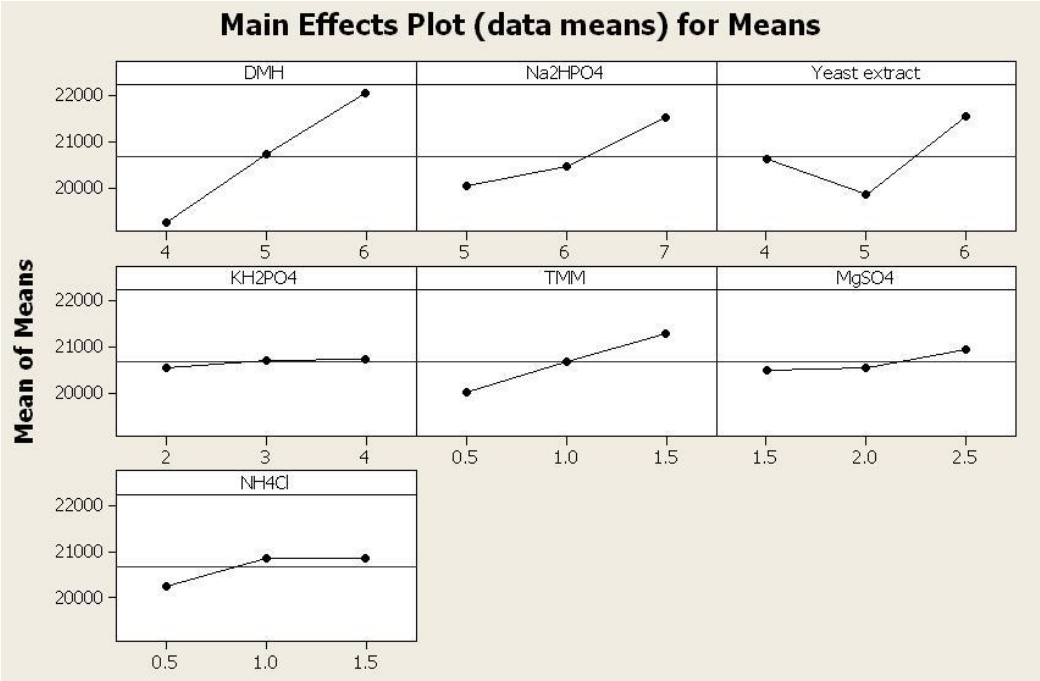


Figure 3. Main effects plot (data means) for means

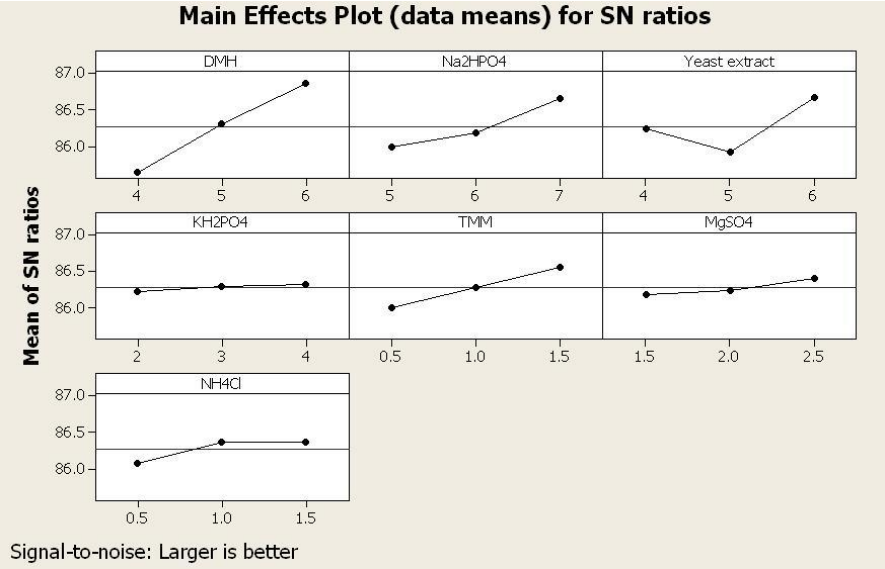


Figure 4. Main effects plot (data means) for S/N Ratios

The use of the response tables was to predict the optimal levels of each constituent. To gain 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	85.65	85.99	86.24	86.22	86	86.19	86.08
2	86.31	86.18	85.92	86.29	86.28	86.23	86.37
3	86.87	86.66	86.66	86.32	86.55	86.41	86.38
Delta	1.22	0.67	0.74	0.11	0.56	0.22	0.3
Rank	1	3	2	7	4	6	5

A-DMH, B-Na_2HPO_4, C-Yeast extract, D-KH_2PO_4, E- TMM, F-$MgSO_4$, G-NH_4Cl

Table 4. Response Table for Means

Level	A	B	C	D	E	F	G
1	19238	20024	20608	20549	20033	20505	20257
2	20713	20451	19843	20712	20671	20559	20879
3	22057	21533	21557	20747	21304	20945	20871
Delta	2819	1508	1714	198	1271	440	622
Rank	1	3	2	7	4	6	5

A-DMH, B-Na_2HPO_4, C-Yeast extract, D-KH_2PO_4, E- TMM, F-$MgSO_4$, G-NH_4Cl

3.3 Optimization of concentrations of the selected medium components by RSM

The combined effect of four independent variables A: DMH; B: Yeast extract; C: Na_2HPO_4; D: TMM for production of recombinant MRH 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 MRH 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 fibrinolytic activity of MRH was 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 1375.11 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 indicates the model terms are 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 = DMH, B = Yeast extract, C = Na_2HPO_4 and D = TMM) are significant model terms.

Final equation in terms of coded factors for fibrinolytic activity (U/mL) of MRH = 25598.17 + 436.54 X DMH + 498.71 X Yeast extract – 2.54 X Na_2HPO_4 + 12.87 X TMM – 303.26 X $(DMH)^2$ – 403.64 X $(Yeast\ extract)^2$ + 278.24 X $(Na_2HPO_4)^2$ + 104.61 X $(TMM)^2$ + 76.56 X DMH X Yeast extract – 202.44 X DMH X Na_2HPO_4 – 48.44 X DMH X TMM – 24.56 X Yeast extract X Na_2HPO_4 + 96.19 X Yeast extract X TMM – 174.56 X Na_2HPO_4 X TMM.

Final equation in terms of actual factors for fibrinolytic activity (U/mL) of MRH = 21527.58073 + 699.80903 X DMH + 609.71875 X Yeast extract – 295.30729 X Na_2HPO_4 – 86.23611 X TMM – 33.69560 X $(DMH)^2$ – 44.84838 X $(Yeast\ extract)^2$ + 69.55990 X $(Na_2HPO_4)^2$ + 185.98148 X $(TMM)^2$ + 8.50694 X DMH X Yeast extract – 33.73958 X DMH X Na_2HPO_4 – 21.52778 X DMH X TMM – 4.09375 X Yeast extract X Na_2HPO_4 + 42.75000 X Yeast extract X TMM – 116.37500 X Na_2HPO_4 X TMM.

The fit of the model was also expressed by the coefficient of regression (R^2), which was found to be 0.9992, indicating that 99.92 % of the confidence level of the model to predict the response. The ‘‘Pred R-Squared” of 0.9956 is in reasonable agreement with the ‘‘Adj R-Squared” of 0.9985. ‘‘Adeq Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, the ratio of 155.287 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. Figure 5 to 10 illustrated the response surface plot for fibrinolytic activity of MRH. Figure 11 illustrated the parity plot for the distribution of predicted and experimental values of enhanced fibrinolytic activity of fusion protein.

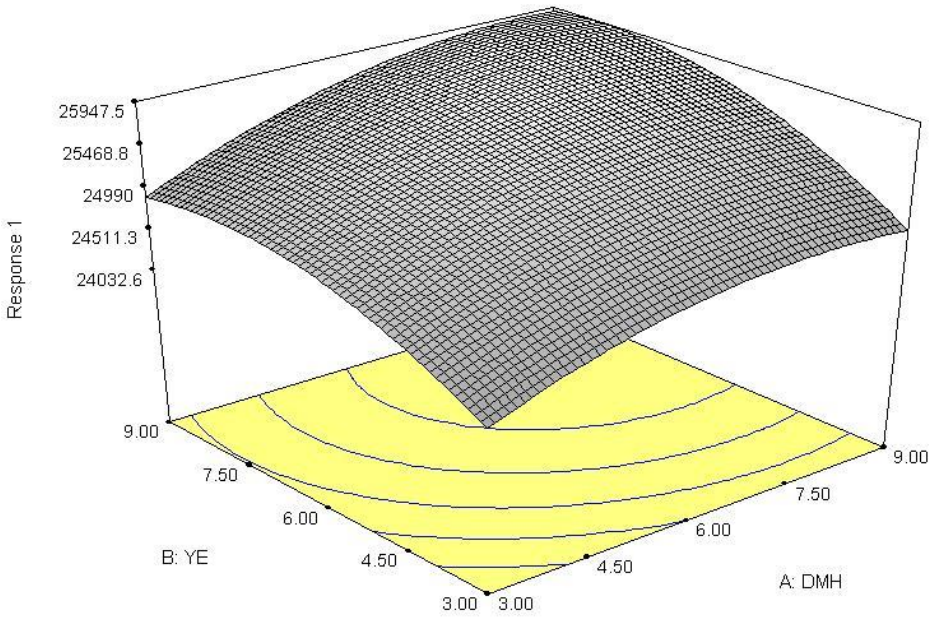


Figure 5. Illustrated the response surface plot for fibrinolytic activity of MRH (Msak – RGD – Hirulog); Effect of DMH and Yeast extract

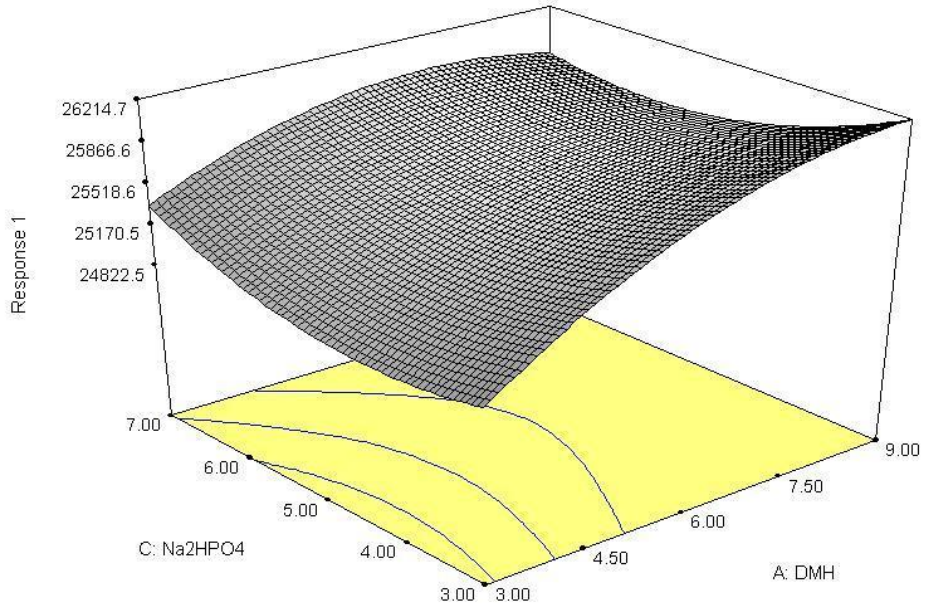


Figure 6. Illustrated the response surface plot for fibrinolytic activity of MRH (Msak – RGD – Hirulog); Effect of DMH and Na_2HPO_4

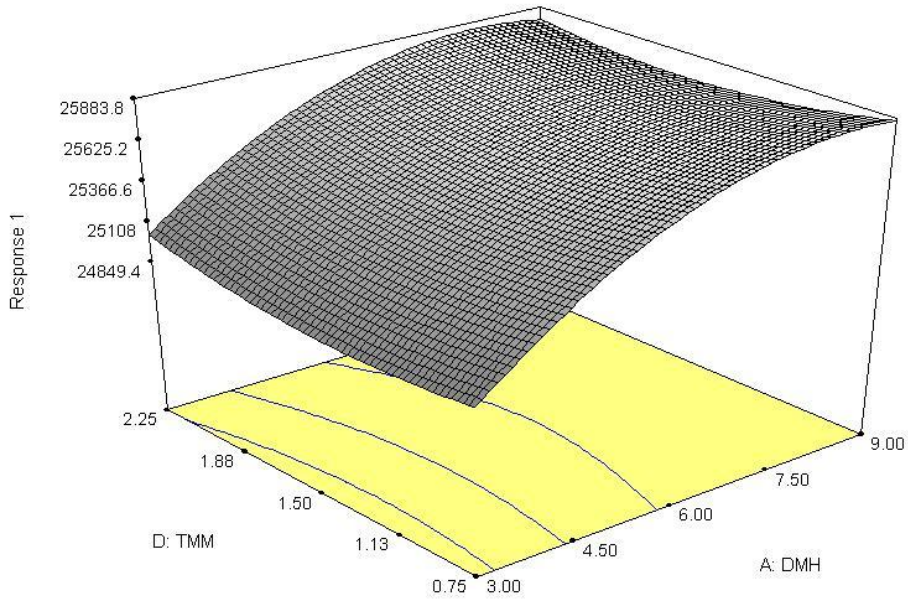


Figure 7. Illustrated the response surface plot for fibrinolytic activity of MRH (Msak – RGD – Hirulog); Effect of DMH and TMM

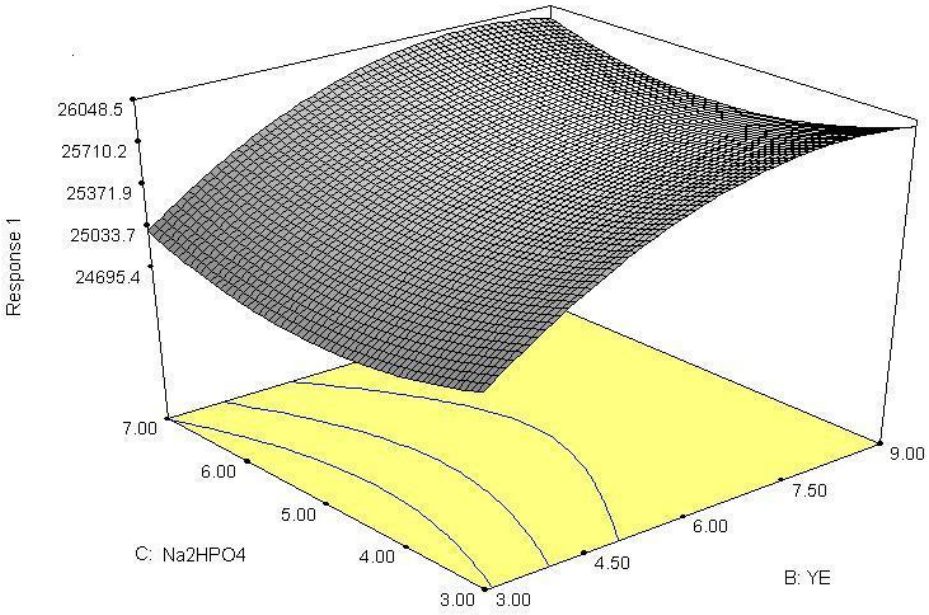


Figure 8. Illustrated the response surface plot for fibrinolytic activity of MRH (Msak – RGD – Hirulog); Effect of Yeast extract and Na_2HPO_4

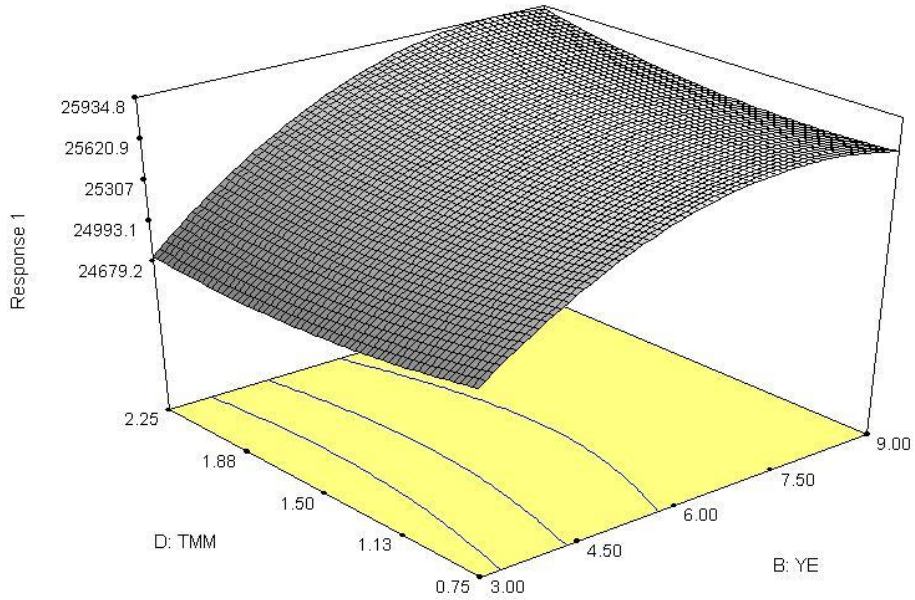


Figure 9. Illustrated the response surface plot for fibrinolytic activity of MRH (Msak – RGD – Hirulog); Effect of Yeast extract and TMM

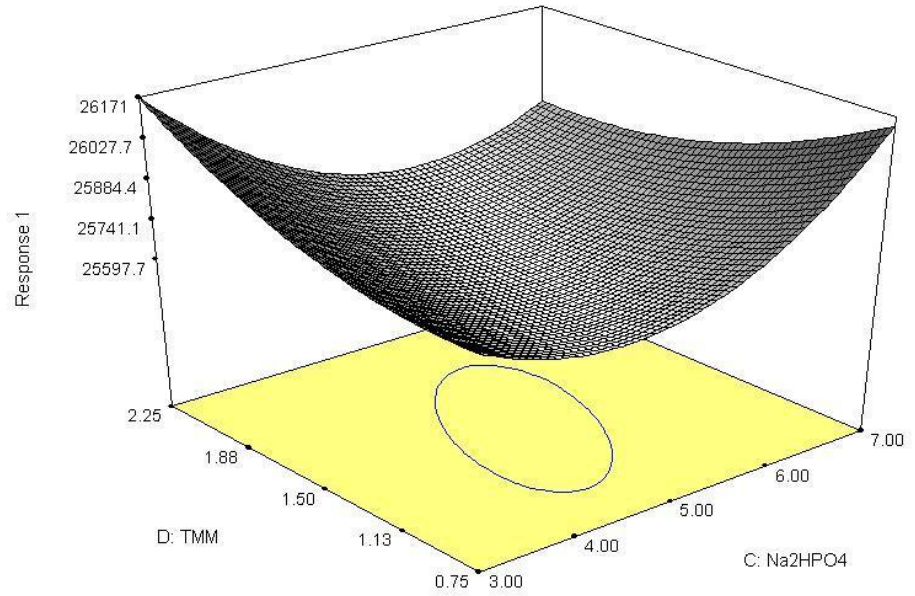


Figure 10. Illustrated the response surface plot for fibrinolytic activity of MRH (Msak – RGD – Hirulog); Effect of Na_2HPO_4 and TMM

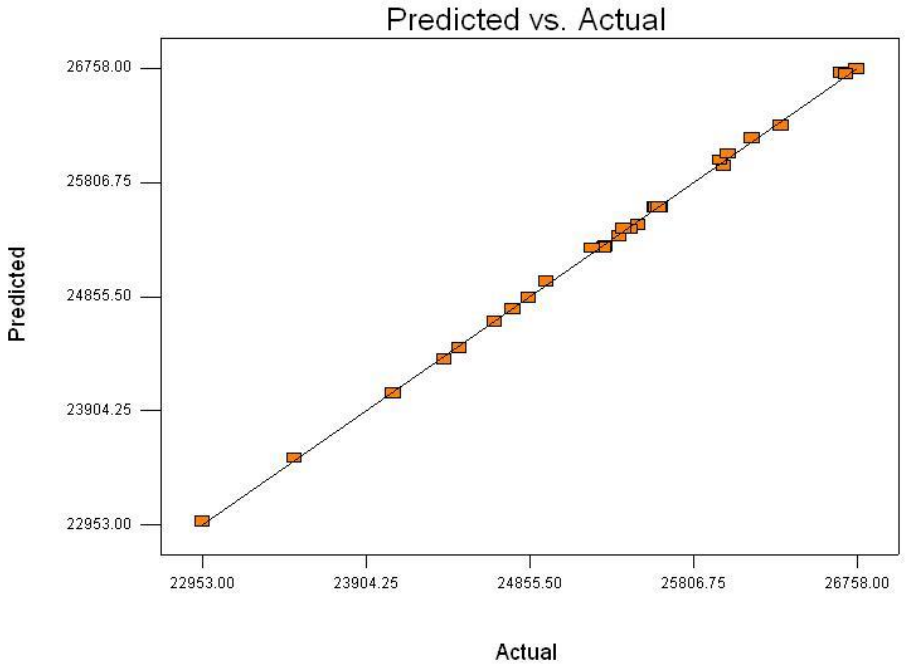


Figure 11. Illustrated the parity plot for the distribution of predicted and experimental values of fibrinolytic activity of MRH

4. Conclusion

Third generation molecule, staphylokinase (sak) and its variant like, Sak – hirulog and Msak – RGD – Hirulog having less side effects like reocclusion. The sak and its variants expression was carried out in different expression host systems like *E.coli* [14, 15, 16], *Bacillus subtilis* [17], *Streptomyces lividans* [18].

In the present study, 960 mg/L of recombinant staphylokinase variant was produced from salt inducible *E.coli* GJ1158 is much higher than in other studies i.e., 20 mg/L [19], 200 mg/L fermentation broth [20], 300 mg/L [21] and 70 to 500 mg/L [22]. On the other hand, low production levels were recorded when compared to 1 g/L [23] and 2.8 g/L of fermentation broth [24].

In this study, cost effective media composition was determined for the production of MRH using *E.coli* by one-factor-at-a-time method, Taguchi design and CCRD. The classical one-factor-at-a-time method indicated DMH and yeast extract are high yielding media components. The Taguchi design was found to be useful for identifying the most influential components like DMH, yeast extract, Na_2HPO_4 and TMM in modified GYE medium. The taguchi design showed DMH was the most significant parameter and it plays a major role in the production of MRH. Yeast extract and Na_2HPO_4 were other significant factors followed by DMH. The concentrations of significant media components and their interactions were studied by final optimization, CCRD. The fibrinolytic activity of MRH was same like sak and sak-hirulog of our previous study. The final fibrinolytic activity of MRH was increased 1.17 folds over Taguchi results in this study. The fibrinolytic activity of 26,758 U/mL was

recorded as one of the highest units of sak variant till today, produced from salt induction system.

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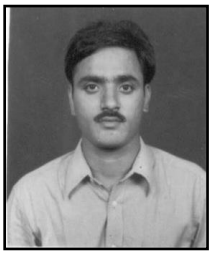
Authors

Seetha Ram Kotra

Currently pursuing Ph.D as a full time scholar in Department of Biotechnology, Acharya Nagarjuna University, Guntur.

N. Prudvi

Curently pursuing M.Tech in Department of Biotechnology, Acharya Nagarjuna University, Guntur.



J. B. Peravali

Assistant Professor in Deparatment of Biotechnology, Bapatla Engineering College, Bapatla. He has 10 years of teaching (UG and PG level) and 4 years of research experience in the areas of Biochemistry, Immunology, Molecular Biology and Genetic Engineering. He is a good advisor.

Anmol Kumar

Currently pursuing Ph.D as a full time scholar in Department of Biotechnology, Acharya Nagarjuna University, Guntur.

K. R. S. Sambasiva Rao

Professor & Head, Department of Biotechnology, Acharya Nagarjuna University, Guntur. His 20+ years of teaching and research experience includes different streams of Biotechnology, Zoology and Pharmaceutical Sciences. Number of central and state government projects are added to his credentials and he is General Secretary for Association of Biotechnology and Pharmacy. He is a good administrator.

K. K. Pulicherla

Professor and Head, Department of Biotechnology, R.V.R. & J.C. College of Engineering, Guntur. His 8 years of teaching and research areas includes Genetic Engineering, Molecular Biology, Fermentation Technology, Genomics, Bioinformatics and Cancer Biology. He has four patents to his credits. He contributed a number of book chapters for UG and PG level students. He is a good mentor as well as good administrator.