Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 3 (6): 405-413, 2008
ISSN 1816-4935

Non-Conventional Method for Evaluation and Optimization of Medium Components for Rapamycin Production by *Streptomyces hygroscopicus*

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Abstract: An evaluation and optimization study of medium components for the production of the immunosuppressant compound rapamycin by *Streptomyces hygroscopicus* was addressed. Plackett-Burman experimental design was applied for screening of the most significant variables affecting production, where $FeSO_4$, mannose, fructose and lysine-HCl were the most positive significant factors. In order to find out the combination among the most significant variables that brings maximum yield, Response Surface Methodology (RSM) was applied and the rapamycin yield increased to reach a theoretical value of 93 mg rapamycin per liter in the following medium (g L^{-1}): Fructose, 90; mannose, 18.9; lysine-HCl, 16.8 and $FeSO_4$, 0.1. Experimental verification of the polynomial model revealed a rapamycin yield of 95 mg L^{-1}, which is an evidence of more than 98% accuracy of the model under the investigated conditions.

Key words: *Streptomyces hygroscopicus*, rapamycin optimization, statistical experimental design

INTRODUCTION

Many antibiotics are used commercially or are potentially useful in medicine or agriculture for activities other than their antibiotic action. These include anti-tumor agents, immunosuppressive agents, hypocholesterolemic agents, enzyme inhibitors, antimigraine agents, herbicides, antiparasitic agents, ruminant growth promoters and bioinsecticides (Liu and Reynolds, 1999).

Rapamycin (sirolimus), a macrocyclic polyketide, was first reported as an antifungal agent by Vezina *et al*. (1975) and Singh *et al*. (1979). It is produced from *Streptomyces hygroscopicus* isolated from the Easter Island soil sample. Although rapamycin was originally isolated as an antifungal agent, its immunosuppressive effect has recently attracted more attention (Balter, 1996). Rapamycin is of interest for the clinical treatment of autoimmune disease and in the prevention of organ rejection and skin allografts. Recently, researchers have found that rapamycin plays the role of a molecular marriage broker, linking two proteins that normally ignore each other into a complex, called a heterodimer. Rapamycin binds to FKBP in a fashion similar to tacrolimus (cyclosporin A). However, sirolimus and tacrolimus affect different and distinctive sites in the signal transduction pathway. Sirolimus and tacrolimus compete for the occupation of the same cellular receptor; thus, they are pharmacological antagonists *in vitro*. However, *in vivo* they interact to produce immunosuppression that is additive or synergistic. Rapamycin has been shown to prolong the survival of histoincompatible organ allografts in several experimental models of organ transplantation (Reynolds and Demain, 1997).

On the production level of rapamycin, only few publications reporting optimization of culture conditions including carbon, nitrogen and trace elements nutrition (Cheng *et al*., 1995a, b; Kojima *et al*., 1995; Lee *et al*., 1997) for production of rapamycin by *S. hygroscopicus*. The possible metabolic precursor for rapamycin biosynthesis in the same bacteria was previously studied (Fang and Demain, 1995).

One of the most advantageous methods in optimizing bioprocesses is the application of statistically designed experiments which allows more extensive and deeper study of variables and the possible cross interactions among them. The efficiency and time-saving properties of this application elected it for implementation in industrial biotechnology, where it has been applied to improve production of many enzymes (Abdel-Fattah, 2002; Abdel-Fattah *et al*., 2005), biopolymers (Soliman *et al*., 2005), antibiotics as well as biotechnological products (Abdel-Fattah and Gaballa, 2006).

To the best of knowledge, optimization of rapamycin production has not been carried out before by implementing factorial designs and numerical modelling based experiments. In the present work, the significance of medium components commonly used for the production of rapamycin by *S. hygroscopicus* DSMZ 41530 is addressed through Plackett-Burman screening design. To find out the optimum level of the most significant variables as well as studying possible interactions created among studied variables, Central Composite Design (CCD) was applied.

MATERIALS AND METHODS

Microorganism

Streptomyces hygroscopicus DSMZ 41530 was maintained on GYM agar of the following composition (g L^{-1}): glucose, 4; yeast extract, 4; malt extract, 10; $CaCO_3$, 2 and agar, 12. It was used to prepare a spore suspension as described by Kojima *et al*. (1995), where it was stored at -80°C.

Seed Cultivation and Production

A seed culture was initiated by adding 0.4 mL of a thawed spore suspension to a 250 mL Erlenmeyer flask containing 25 mL of a seed medium consisting of (g L^{-1}): Glucose, 10; Bactopeptone, 4; yeast extract, 4; casamino acids, 1.5; $MgSO_4 \cdot 7H_2O$, 0.5 and K_2HPO_4 1.0, pH 7.0-7.3. Incubation was at 28°C for 2 days on a rotary shaker (200 rpm). The resulting culture was centrifuged at 4°C for 15 min (5000 rpm) and the cells were washed once with 100 mM 2-(N-morpholino) ethanesulfonic acid monohydrate (MES) buffer (pH 6.0) containing 0.5% NaCl and 0.05% $MgSO_4 \cdot 7H_2O$. The washed cells were resuspended in the same buffer to make a 10 mL suspension and 0.5 mL portions of the suspension were inoculated into 250 mL Erlenmyer flasks containing 25 mL of chemically-defined fermentation medium of different compositions according to the experimental design for the production of rapamycin. Fermentations were normally conducted at 28°C for 7 days on a rotary shaker (200 rpm). Cell growth was measured by optical means and then converted to dry cell weight (DCW: g L^{-1}) as described by Kojima *et al*. (1995).

Preparation of Culture Extracts

Culture broth (10 mL) was centrifuged at 4°C for 15 min (5000 rpm) to separate supernatant fluid from cells. The supernatant was put aside and the precipitated pellet was extracted with 10 mL methanol by shaking at 30°C for 1 h. The methanol extract was centrifuged at 4°C for 15 min (5000 rpm) and the resulting methanol supernatant was combined with the culture supernatant,

followed by extraction with 20 mL ethyl acetate. The separated organic layer was dried by the addition of anhydrous sodium sulfate, allowed to stand for about 10 h and concentrated to dryness under reduced pressure. The resulting residue was re-dissolved in 0.5 mL methanol.

Rapamycin HPLC Analysis

HPLC analysis was conducted for quantitative analysis of rapamycin as described previously (Kojima and Demain, 1998). The analysis was carried out on a Waters™LC Module I (Millipore Corp, Milford, MA, USA). The sample (10 μL) was loaded onto a C18 reversed phase column (Nova-Pak C18, 3.9×150 mm, Millipore) and eluted isocratically with the mobile phase (1,4-dioxane/water (55/45)) at a flow rate of 1.0 mL min^{-1} for 40 min. Rapamycin was monitored at 287 nm and eluted after 27 min Retention Time (RT). Since RT of rapamycin extracted from fermentation broth was somewhat variable from one run to another, a Relative Retention Time (RRT) was calculated of the individual eluted peaks as compared to the RT of authentic rapamycin which was given an RRT of 1.00. RRT values remained constant as long as the same mobile phase was used. Concentrations of rapamycin were calculated by measuring peak areas.

Experimental Design

For improvement of rapamycin production in this study, a sequential optimization strategy based on statistical experimental design was implemented. First, categorical factors are studied to determine the significance of medium contents on rapamycin production. Once the most crucial factors have been identified, central composite design based on response surface methodology was applied to address the optimum medium formula. Finally, the predicted optimum is verified experimentally.

Plackett-Burman Screening Design

Plackett and Burman (1946) experimental design was applied to investigate the significance of various medium components on rapamycin production. Seven culture variables were tested in 8-trials experiment. Two levels of each variable were used: -1 for low and +1 for high level based on Plackett-Burman matrix design, which is a fraction of a two-level factorial design and allows the investigation of n-1 variables in at least n-experiments. Table 1 shows the lower and higher levels of each variable. In this study the independent variables were screened in 8 combinations according to the matrix shown in Table 2. The main effect of each variable was calculated simply as the difference between the average of measurements made at high setting (+1) and the average of measurements observed at low setting (-1) of that factor.

Plackett-Burman experimental design is based on the first order model (Eq. 1):

$$Y = \beta_0 + \Sigma \beta_i X_i \quad (1)$$

Table 1: Variables and their levels employed in Plackett–Burman experimental design for evaluation of medium constituents' significance on rapamycin production by *S. hygroscopicus* DSMZ 41530

	Values	
Variables	-1	1
Fructose (g L^{-1})	5.0 g	40.0 g
Mannose (g L^{-1})	1.0 g	10.0 g
Lysine-HCl (g L^{-1})	1.0 g	10.0 g
$(NH_4)_2SO_4$ (g L^{-1})	0.7 g	6.5 g
K_2HPO_4 (g L^{-1})	0.4 g	5.0 g
$MgSO_4.7H_2O$ (mg L^{-1})	0.0	25.0 mg
$FeSO_4.7H_2O$ (mg L^{-1})	0.0	140.0 mg

Table 2: Plackett-Burman experimental design for evaluation of factors affecting rapamycin production

Trial	Fructose (X_1)	Mannose (X_2)	Lysin-HCl (X_3)	$(NH_4)_2SO_4$ (X_4)	K_2HPO_4 (X_5)	$MgSO_4$ (X_6)	$FeSO_4$ (X_7)	Rapamycin (mg L^{-1})
1	-1	1	1	-1	1	1	-1	60.3
2	-1	-1	1	1	-1	1	1	40.2
3	1	-1	-1	1	1	-1	1	55.1
4	1	1	-1	-1	1	1	-1	61.8
5	-1	1	1	-1	-1	1	1	70.8
6	1	-1	1	1	-1	-1	1	57.9
7	1	1	-1	1	1	-1	-1	53.6
8	-1	-1	-1	-1	-1	-1	-1	62.4

where, Y is the predicted response (rapamycin yield), β_0, β_i are constant coefficients and x_i is the coded independent variables estimates or factors.

Central Composite Optimization Design

In order to describe the nature of the response surface in the experimental region and find out where the optimum condition is likely to be located, a Central Composite Design (CCD) was applied (Biles and Swain, 1980). Factors of highest confidence levels were prescribed into three levels, coded -1, 0 and +1 for low, middle and high concentrations (or values), respectively. Two external values for each variable are also included within the CCD with coded values, -1.48 and +1.48, that act as probes for the experimental design. Table 5 shows the design matrix of a 26 trials experiment. For predicting the optimal point, a second order polynomial function was fitted to correlate relationship between independent variables and response (rapamycin mg L^{-1}). For the three factors this equation is:

$$Y = \beta_0+\beta_1 X_1+\beta_2 X_2+\beta_3 X_3+\beta_4 X_4+\beta_{12} X_1X_2+\beta_{13} X_1X_3+\beta_{14} X_1X_4+\beta_{23} X_2X_3+\beta_{24} X_2X_4+\beta_{34} X_3 X_4 + \beta_{11} X_1^2+\beta_{22} X_2^2+\beta_{33} X_3^2+\beta_{44} X_4^2 \quad (2)$$

where, Y is the predicted response, β_0 model constant, X_1, X_2 and X_3 independent variables, β_1, β_2 and β_3 are linear coefficients, β_{12}, β_{13} and β_{23} are cross product coefficients and β_{11}, β_{22} and β_{33} are the quadratic coefficients. Microsoft Excel 97 was used for the regression analysis of the experimental data obtained. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. Experiments were performed in triplicate and mean values are given.

Statistical Analysis of Data

The results obtained from the statistically designed experiments were subjected to standard regression analysis. Essential Experimental Design free software (Steppan *et al*., 1999) was used for data analysis, determination of coefficients, as well as polynomial model reduction. Factors having highest t-value and confidence level over 95% were considered to be highly significant on rapamycin production.

RESULTS AND DISCUSSION

Evaluation of Culture Conditions Affecting Production of Rapamycin by *Streptomyces hygroscopicus*

In order to evaluate the significance of 7 variables, chosen based on literature, on rapamycin production by the type strain *S. hygroscopicus*, an experimental statistical design approach has been implemented and the design matrix with 8 different trials is shown in Table 2. Each variable has

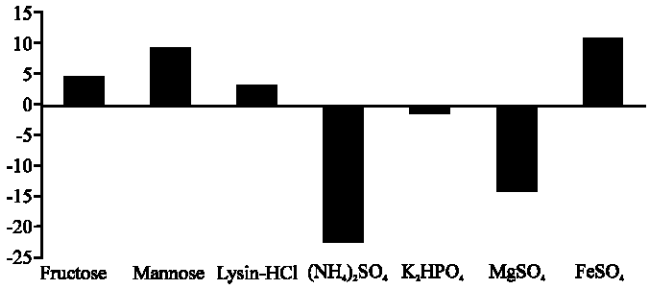

Fig. 1: Effect of some nutritional factors on rapamycin production produced by *S. hygroscopicus* DSMZ 41530

been tested in two levels, as Plackett-Burman design is 2-level design, where -1 is the low level of variable, while +1 is the high level. The actual values of the low and high levels of variables are shown in Table 1.

From the design matrix the main effect, which is the mean difference between the response corresponding to high levels and low levels of a variable, is shown in Fig. 1. Figure 1 showing the significant positive effect of mannose, fructose, ferrous sulfate and lysine-HCl; while ammonium sulfate, potassium phosphate and magnesium sulfate showed inhibitory effect on rapamycin production. These results are in agreement with the previously published works, where it was reported that the best combination for optimum rapamycin production by *S. hygroscopicus* was 2% fructose and 0.5% mannose (Kojima *et al*., 1995). This could be attributed to the fact that secondary metabolites are up regulated when secondary carbon source is added to a chemically defined medium. In addition, some studies on antibiotics production revealed that mannose rather than glucose is the more immediate precursor of the 6-deoxy-5-keto-D-arabino-furanose derived moiety in hygromycin produced from *S. hygroscopicus* (Habib *et al*., 2003). The positive lysine effect was previously reported by investigators (Cheng *et al*., 1995a), where they stated that addition of lysine in a chemically defined medium stimulated rapamycin production by 150%. In another work, they studied the effect of phosphate, ammonium, magnesium and iron nutrition of *S. hygroscopicus* with respect to rapamycin biosynthesis; where they find that phosphate, ammonium and magnesium salts interfered with rapamycin production in concentrations optimal for growth (Cheng *et al*., 1995b). These observations point to the existence of phosphorus, magnesium and nitrogen-negative regulation mechanisms for rapamycin biosynthesis. On the other hand, they observed that ferrous ion stimulated rapamycin production at concentrations greater than the required for growth.

Table 3 shows the statistical data analysis based on standard regression technique for calculation of coefficients values according to the polynomial equation model (Eq. 1 in the materials and methods part), along with the p-value that determines the significance of variables. The lower the p-value is the higher the significance and this supports the choice of the four variables namely: fructose, mannose, lysine-HCl and $FeSO_4$ for further optimization study as they were the most significant positive affecting factors on rapamycin production.

Based on the Plackett-Burman experimental design results, it is worthy to further optimize the four significant medium components to find out where the optimum level should be located. Other medium constituents will be added to the medium content in its low level as designed in the screening experiment.

Optimization of Rapamycin Production by Type Strain *Streptomyces hygroscopicus*: Application of Central Composite Design (CCD)

In order to approach the optimum response region of the rapamycin production, significant independent variables (Fructose, X_1; Mannose, X_2; Lysin-HCl, X_3 and $FeSO_4$, X_4) were further

Table 3: Standard regression analysis of Plackett-Burman experimental design matrix

Variables	Coefficient	t-stat	p-value
Fructose	2.07	35.07	0.018
Mannose	4.42	74.95	0.008
Lysin-HCl	1.36	23.05	0.028
$(NH_4)_2SO_4$	-10.88	-184.70	0.003
K_2HPO_4	-0.51	-0.40	0.756
$MgSO_4$	-6.78	-115.12	0.006
$FeSO_4$	5.21	88.39	0.007

$R^2 = 0.999$; Adjust $R^2 = 0.999$

Table 4: Variables and their settings employed in central composite design for optimization of rapamycin production by Type strain *S. hygroscopicus* DSMZ 41530

Variables	-1.5	-1	0	1	1.5
Fructose (g L^{-1})	30.0	40	60	80	90.0
Mannose (g L^{-1})	7.5	10	15	20	22.5
Lysin-HCl (g L^{-1})	7.5	10	15	20	22.5
$FeSO_4$ (mg L^{-1})	100.0	120	160	180	200.0

Table 5: Central Composite Design (CCD) matrix representing the effect of different significant variables on rapamycin production by *S. hygroscopicus* DSMZ 41530

X_1 Fructose	X_2 Mannose	X_3 Lysin-HCl	X_4 $FeSO_4$	X_1X_2	X_1X_3	X_1X_4	X_2X_3	X_2X_4	X_3X_4	X_1^2	X_2^2	X_3^2	X_4^2	Rapamycin (mg L^{-1})
-1	-1.00	-1.00	-1.00	1	1	1	1	1	1	1.000	1.000	1.000	1.000	34.2
1	-1.00	-1.00	-1.00	-1	-1	-1	1	1	1	1.000	1.000	1.000	1.000	45.7
1	-1.00	1.00	1.00	-1	1	1	-1	-1	1	1.000	1.000	1.000	1.000	55.2
1	1.00	-1.00	-1.00	1	-1	-1	-1	-1	1	1.000	1.000	1.000	1.000	52.4
-1	-1.00	1.00	-1.00	1	-1	1	-1	1	-1	1.000	1.000	1.000	1.000	32.3
0	0.00	0.00	-1.48	0	0	0	0	0	0	0.000	0.000	0.000	2.198	67.9
1	1.00	-1.00	1.00	1	-1	1	-1	1	-1	1.000	1.000	1.000	1.000	62.8
1	1.00	1.00	-1.00	1	1	-1	1	-1	-1	1.000	1.000	1.000	1.000	87.1
0	0.00	0.00	0.00	0	0	0	0	0	0	0.000	0.000	0.000	0.000	44.2
0	-1.48	0.00	0.00	0	0	0	0	0	0	0.000	2.198	0.000	0.000	48.6
-1	1.00	1.00	1.00	-1	-1	-1	1	1	1	1.000	1.000	1.000	1.000	82.3
0	0.00	0.00	0.00	0	0	0	0	0	0	0.000	0.000	0.000	0.000	78.6
1	1.00	1.00	1.00	1	1	1	1	1	1	1.000	1.000	1.000	1.000	30.9
-1	1.00	-1.00	1.00	-1	1	-1	-1	1	-1	1.000	1.000	1.000	1.000	27.4
-1	-1.00	-1.00	1.00	1	1	-1	1	-1	-1	1.000	1.000	1.000	1.000	66.6
-1	1.00	-1.00	-1.00	-1	1	1	-1	-1	1	1.000	1.000	1.000	1.000	88.5
1.48	0.00	0.00	0.00	0	0	0	0	0	0	2.198	0.000	0.000	0.000	74.1
1	-1.00	1.00	-1.00	-1	1	-1	-1	1	-1	1.000	1.000	1.000	1.000	69.6
0	0.00	0.00	1.48	0	0	0	0	0	0	0.000	0.000	0.000	2.198	55.2
0	1.48	0.00	0.00	0	0	0	0	0	0	0.000	2.198	0.000	0.000	57.8
0	0.00	1.48	0.00	0	0	0	0	0	0	0.000	0.000	2.198	0.000	49.1
-1.48	0.00	0.00	0.00	0	0	0	0	0	0	2.198	0.000	0.000	0.000	72.9
-1	1.00	1.00	-1.00	-1	-1	1	1	-1	-1	1.000	1.000	1.000	1.000	34.9
0	0.00	-1.48	0.00	0	0	0	0	0	0	0.000	0.000	2.198	0.000	56.7
-1	-1.00	1.00	1.00	1	-1	-1	-1	-1	1	1.000	1.000	1.000	1.000	45.7
1	-1.00	-1.00	1.00	-1	-1	1	1	-1	-1	1.000	1.000	1.000	1.000	25.9

explored, each at three main levels and two external surface probes, 1.48 and -1.48 (Table 4). Table 5 shows the design matrix of the variables in coded units together with the experimental results of rapamycin produced (mg L^{-1}). All cultures were performed in triplicate and the average of the observations was used.

Presenting experimental results in the form of surface plots Fig. 2 showed that higher levels of fructose and middle levels of mannose and lysine-HCl support high rapamycin levels. On the other hand, $FeSO_4$ was not significant affecting the rapamycin production by the type strain, although higher levels of production were attained with decreasing the concentration $FeSO_4$ in the medium. For predicting the optimal point, within experimental constrains, a second-order polynomial function was fitted to the experimental results (non-linear optimization algorithm) of rapamycin production (mg L^{-1}):

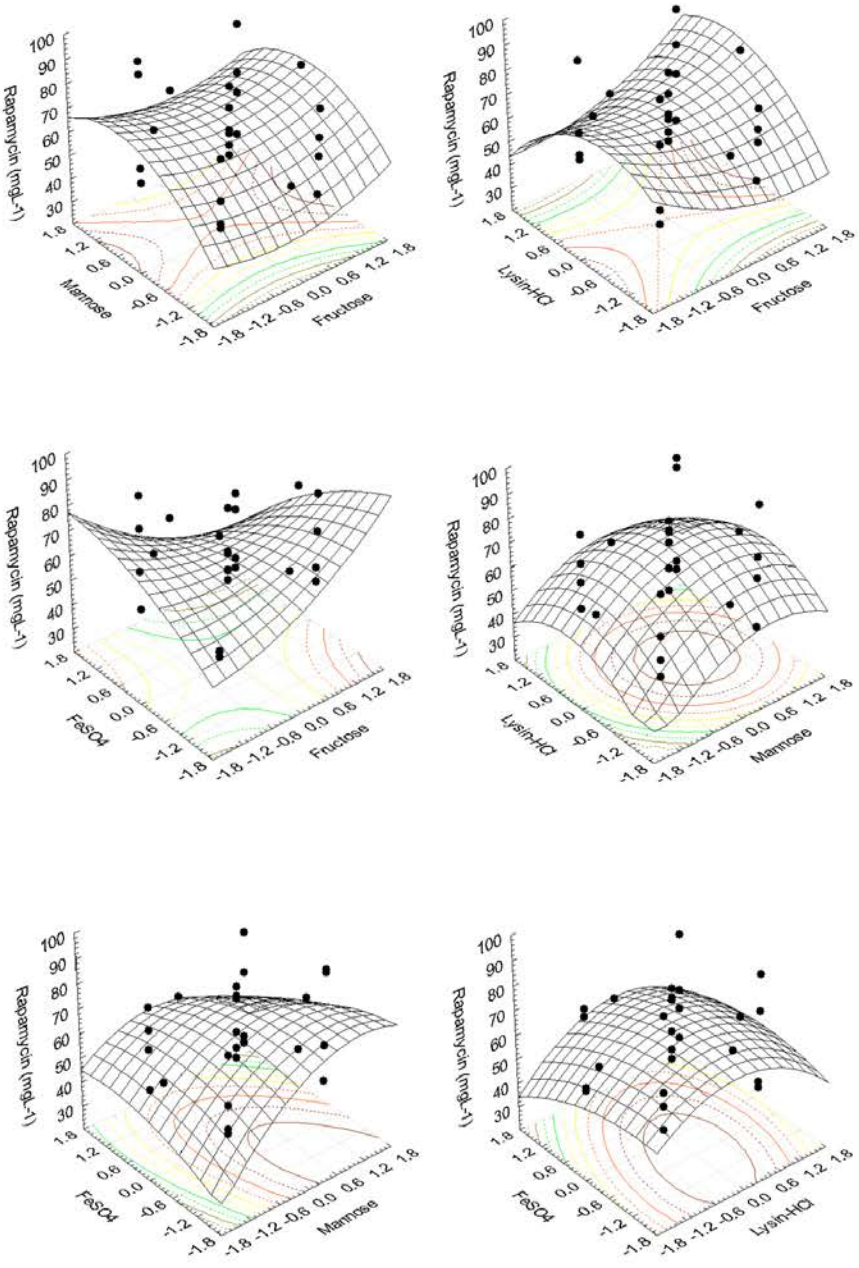

Fig. 2: Three dimensional response surface graphs showing the behaviour of rapamycin response as affected by different culture conditions in CCD

$$Y_{rapamycin} = 64.82+0.96\ X_1+5.13\ X_2+1.13\ X_3-3.27\ X_4-1.09\ X_1X_2+4.84\ X_1X_3-7\ X_1X_4-1.64\ X_2X_3-4.44\ X_2X_4+1.77\ X_3X_4+3.08\ X_1^2-6.15\ X_2^2-6.29\ X_3^2-2.35\ X_4^2$$

where, X_1, X_2, X_3 and X_4 are Fructose, Mannose, Lysin-HCl and $FeSO_4$, respectively. At the model level, the correlation measures for the estimation of the regression equation are the multiple correlation coefficient R and the determination coefficient R^2. The closer the value of R is to 1; the better is the correlation between the measured and the predicted values. In this experiment, the value of R was 0.88 for produced rapamycin. This value indicates a high degree of correlation between the experimental and the predicted values.

The optimal levels of the three components as obtained from the maximum point of the polynomial model were estimated using the Solver function of Microsoft Excel tools and found to be (g L^{-1}): fructose, 90; mannose, 18.9; lysine-HCl, 16.8 and $FeSO_4$, 0.1 with a predicted volumetric production of 93.6 mg L^{-1}. The optimal value of the rapamycin production is 4 folds the basal conditions. This reflects the necessity and value of optimization process.

Verification of Polynomial Model

Optimal conditions realized from the optimization experiment were verified experimentally and compared with the calculated data from the model. The estimated rapamycin yield was 95 mg L^{-1}, where the predicted value from the polynomial model as 93.6 mg L^{-1}. This verification revealed a high degree of accuracy of the model of more than 98%, which is an evidence for the model validation under the investigated conditions.

Applying response surface methodology, represented by Central Composite Design, to optimize the selected factors for maximal production is an efficient method that tests the effect of factors interaction. Besides, it converts the bioprocess factor correlations into a mathematical model that predicts where the optimum is likely to be located. It is worthwhile to advice the microbial industry sponsors to apply such experimental designs to maintain high efficiency and profit bioprocess. In the present investigation, the optimized medium succeeded to increase the rapamycin yield by more than four folds; where production level in basal medium was 23.2 mg L^{-1} and increased after applying RSM to 95 mg L^{-1}.

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