J Biochem Tech (2012) 3(4):419-425
ISSN: 0974-2328

Optimization of process parameters for tacrolimus (FK 506) production by new isolate of *Streptomyces* sp. using response surface methodology

Abha Mishra*, Sharad Verma

Received: 05 January 2012 / Received in revised form: 14 February 2012, Accepted: 14 February 2012, Published online: 30 October 2012

Abstract

An isolate of *Streptomyces* was found to be positive for the production of tacrolimus. HPLC analysis on C-18 column at flow rate of 0.5 ml/min samples gave retention time of 4.26 min as standard. Soyoil, soybean meal and L-lysine were selected as significant model factors by plackett burman (PB) design. Response surface methodology was employed to optimize these factors. A second-order quadratic model and response surface method showed that the optimum conditions (soyoil: 23.40 gm/L; soybean meal gm/L: 18.40; L-lysine: 1.00 gm/L) resulted in the improvement of tacrolimus production (235.5± 1.96 mg/L) as compared to the initial level (135.6± 2.56 mg/L). It was about 1.73 fold increase as compared with that using the original medium. The value predicted by the quadratic model was (236.3 mg/L). Analysis of variance showed a high coefficient of determination (R^2) value of 0.9978 ensuring a satisfactory adjustment of the quadratic model with the experimental data. This is first report on tacrolimus production using plackett burman design and response surface methodology in submerged fermentation.

Key words: *Streptomyces* sp., FK 506, Methylmalonyl CoA, Plackett-Burman (PB) design, Central composite design (CCD) Introduction

Introduction

For prevention of graft rejection in organ transplantation and in treatment of various auto immune diseases, a variety of macrolide compound has been used as immunosuppressive agents. One widely used immunosuppressive for these uses is cyclosporin A but side effects like nephrotoxicity, central nervous system disorders and hepatotoxicity are associated with its use (Kino et al. 1989; Shafiee et al. 1994). Tacrolimus (fk 506) is 23 membered macrocyclic polyketide with very high immunosuppressive activity. Tacrolimus have superior potency relative to cyclosporin A as a drug for preventing graft rejection. In 1984, scientists of Fujisawa healthcare co. isolated tacrolimus from fermentation broth of *Streptomyces tsukubaensis* for the first time (Akashi et al. 1996; Weber et al., 2003).

After several clinical trials, tacrolimus became commercially available in 1994 by Fujisawa healthcare co., Japan (Kim and Park 2008). For effective tacrolimus production, it is highly essential to optimize all culture condition and composition for production media, which further facilitates economic design of full scale fermentation operation system. Medium optimization by the traditional 'one-factor-at-a-time' technique is not only laborious and time consuming but also often leads to an incomplete understanding of the system behaviour, resulting in confusion and a lack of predictive ability (Gokhade et al. 1991; Rodrigues et al. 1998). Response surface methodology (RSM) is a powerful and efficient mathematical approach widely applied in the optimization of fermentation processes. It can give information about the interaction between variables necessary for design and process optimization, and give multiple responses at the same time. Response surface methodology is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching optimum conditions of factors for desirable responses. By this technique, fewer Experimental trials are needed as compared with studying one factor at a time. Also, significant interactions between the factors can be identified and quantified (AdinaraSyana and Ellaiah 2002; Li et al. 2002; Shih et al. 2008). Response surface methodology performs statistically designed experiments, estimate cofficients in a mathematical model and predict response and checks the adequacy of model. The aim of present study was to evaluate the effect of various parameters on tacrolimus production in submerged condition using Plakett-Burman (PB) and central composite design (CCD) (Puri et al. 2002; Rahulana et al. 2009; Yazgi and Degirmencioglu 2007).

Material and methods

Isolation of Streptomyces strain

Strain of *Streptomyces* sp. was isolated from local soil sample of Banaras Hindu University, Varanasi, India. The sample was given pretreatment by drying at 45^0C for 24 hrs. Culture Isolation was

Abha Mishra, Sharad Verma

School of Biochemical Engineering, Institute of Technology, Banaras Hindu University, Varanasi 221005, India

*Tel.: +915422307070, Email: abham.bce@itbhu.ac.in

done on on YMA medium and maintained at 4^0C. Isolates were tested by gram and acid fast staining. For further confimation of genus degradative property against casien, xantine and tyrosine were used (Taddei et al. 2006).

Production of tacrolimus

Seed culture was prepared by adding loopful culture of *Streptomyces* sp. in 50 ml of media (soy oil: 10 gm/L; soybean meal: 8 gm/L; $CaCO_3$: 1.8 gm/L; $(NH_4)_2SO_4$: 0.2 gm/L and pH 7) and incubated at 28^0C for 24 hrs at 120 rpm . Seed culture was transferred to production media (soy oil: 15 gm/L; soybean meal: 12 gm/L; $CaCO_3$: 3.5 gm/L; $(NH_4)_2SO_4$: 0.5 gm/L and pH 7). Culture was incubated for 9 days at 28^0C at 200 rpm.

HPLC analysis

After the removal of cell mass acetonitrile extract of culture broth was analysed by HPLC. C18 column was used. 50% methanol was used as mobile phase at the flow rate of 0.5 ml/min. Detection wavelength was 220 nm and total sample run time was 10 min (Wang et al., 2009).

HPLC analysis of purified samples

Purification was done according to the method as described by Cabri et al. (2008) . HPLC analysis was used to confirm the presence of tacrolimus against refrence under the same conditions as mentioned elsewhere.

Kinetic study of tacrolimus production

The level of cell growth was measured by collecting duplicate 20 ml samples of fermentation broth at different intervals, starting at 0 hr. The mycelia were collected on preweighed Whatman filter paper no. 1. The samples were washed twice with distilled water, and filters containing mycelia were dried at 65°C and weighed. The level of FK506 production was determined by HPLC.

Effect of different carbon sources on tacrolimus

Several carbon sources were used to study the effect on tacrolimus production. The quantity of carbon shared by these sources kept same (11.25 gm/L) in production media and rest of the physicochemical parameters were constant.

Effect of different nitrogenous sources on tacrolimus

Different nitrogenous sources (both organic and inorganic) were also studied for higher production of tacrolimus. The quantity of nitrogen shared by these sources kept same (organic nitrogen 0.72 gm/L and inorganic nitrogen 0.105 gm/L) in production media and rest of the physicochemical parameters were constant.

Effect of Amino acids on tacrolimus production

Various amino acids (0.5 gm/L) used were filter-sterilized by membrane filter (0.22 µm, MilliPore, Mumbai, India) and added after 40 h of inoculation (Yoon and Choi 1997).

Effect of methylmalonyl-CoA

Methylmalonyl CoA is an one of the precursor of the tacrolimus which contributes in its structure and is provided by methyl oleate. To study the influence of methylmalonyl-CoA on tacrolimus, methyl oleate was used in different concentration in production media. The concentration of methyl oleate used were 4mM, 8mM, 12mM, 16mM and 20 mM (Mo et al., 2009) .

Experimental designs and data analysis

Plackett-Burman design

In the first optimization step, a Plackett-Burman (PB) design was used to determine the likely effects of medium components on tacrolimus production. PB design is an efficient screening design when main effects are to be considered. This is a very economical design with the run number a multiple of four and comprises of two level screening designs. Eight assigned variables were screened in PB design of 12 experiments. Eight factors consisting of medium components and operating conditions prepared at two levels –1 for low level and +1 for high level (Rajendran et al. 2007). The factors (gm/L) such as soy oil, soybean meal, ammonium sulphate, L-lysine, calcium carbonate, methyl oleate (mM), pH and temperature at same level were studied. The actual values of the variables at low level (−1) and high level (+1) is given in (Table 1).

PB experimental design is based on the first order model as given in equation 1.

$$Y= \beta_0 +\Sigma \beta_i X_i \quad \text{.....(1)}$$

where, Y is the response, β_0 is the interception coefficient, β_i is coefficient of the linear effect and X_i is independent variable.

Table 1. Actual value of process variables (A: soy oil; B: soybean meal; C: L-lysine; D: ammonium sulphate; E: methyl oleate; F: calcium carbonate; G: pH; H: temperature)

Process variables	A	B	C	D	E	F	G	H
Low level(−)	10	5	.2	.2	8	2.5	6.5	26
High level(+)	20	15	.8	.8	16	4.5	7.5	32

Central composite design (CCD)

After the identification of components affecting the production by PB experimental design three variables (soy oil, soybean meal and L-lysine) were selected for response surface methodology of CCD (Chakravarti and Sahai 2002). CCD was based on second degree polynomials which include all significant interaction terms, and were used to calculate the predicted response as given in equation (2).

$$Y= \beta_0 +\Sigma \beta_i X_i + \Sigma \beta_{ii} X_i^2 + \Sigma \beta_{ij} X_i X_j \quad \text{……(2)}$$

where Y represents response variable, β_0 is the interception coefficient, β_i is coefficient of the linear effect, β_{ii}, the coefficient of quadratic effect and β_{ij}, the coefficient of interaction effect. F-test was employed to evaluate the statistical significance of the quadratic polynomial. The multiple coefficients of correlation R and the determination coefficient of correlation R^2 were calculated to evaluate the performance of the regression equation. The optimum levels of the selected variables were obtained by solving the regression equation using a multi-stage optimization program and also by analysing the contour plots (Sakamoto et al. 1995; Sunitha et al. 1998) .

Results and discussion

Isolation of Streptomyces strain

Presence of LL-Diaminopimelic acid in the cell wall confirmed that isolated strain belongs to *Streptomyces species.* Colonies characteristics showed noncapsulated filamentous mycelial

structure. *Streptomyces* species was further confirmed on the basis of results of gram staining, acid fast staining and degradation of xantine, tyrosine and casein (Table 2).

Table 2. Response of the new isolates of *Streptomyces* for different tests

Test	Result
Gram staining	Positive
Acid fast staining	Negative
Xantine degradation	Positive
Tyrosine degradation	Positive
Casein degradation	Positive

HPLC analysis of culture broth

HPLC analysis of acetonitrile extract of culture broth showed nine prominant peaks at retention time of 3.27, 3.55, 4.20, 4.26, 4.44, 4.55, 6.27, 6.44 and 9.06 (Fig. 1A).

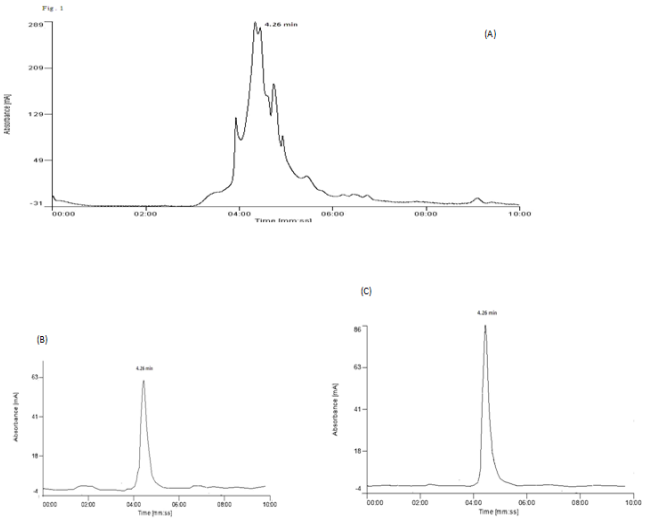

Fig. 1 (A). HPLC chromatogram of acetonitrile extract of culture broth of *Streptomyces* sp., (B) HPLC chromatogram of purified culture broth of *Streptomyces* sp., (C) HPLC chromatogram of authentic acrolimus.

HPLC analysis of purified samples

Purified samples were examined through HPLC analysis. A distinct peak appears at the retention time of 4.26 min. Both crude and purified samples had similar peaks with reference under identical HPLC conditions (Fig. 1B & 1C). Antifungal test was also found positive for purified samples.

Kinetic study of tacrolimus production

Production of tacrolimus by *Streptomyces* sp. was found to be started on 3rd day of 9 days fermentation and reached to maximum on 8th day (Fig 2A).

Effect of different Carbon source on tacrolimus production

Glucose, fructose, sucrose, maltose, lactose, soy oil, peanut oil, cotton seed oil, sun flower oil and rape seed oil were used to study the effect of carbon source on tacrolimus production. There were a remarkable dfference in concentrations of tacrolimus produced by *Streptomyces* sp. by using different carbon sources. Among these carbon sources, soy oil was found most appropiate carbon source for growth and tacrolimus production by new isolate of *Streptomyces* sp. (Table 3).

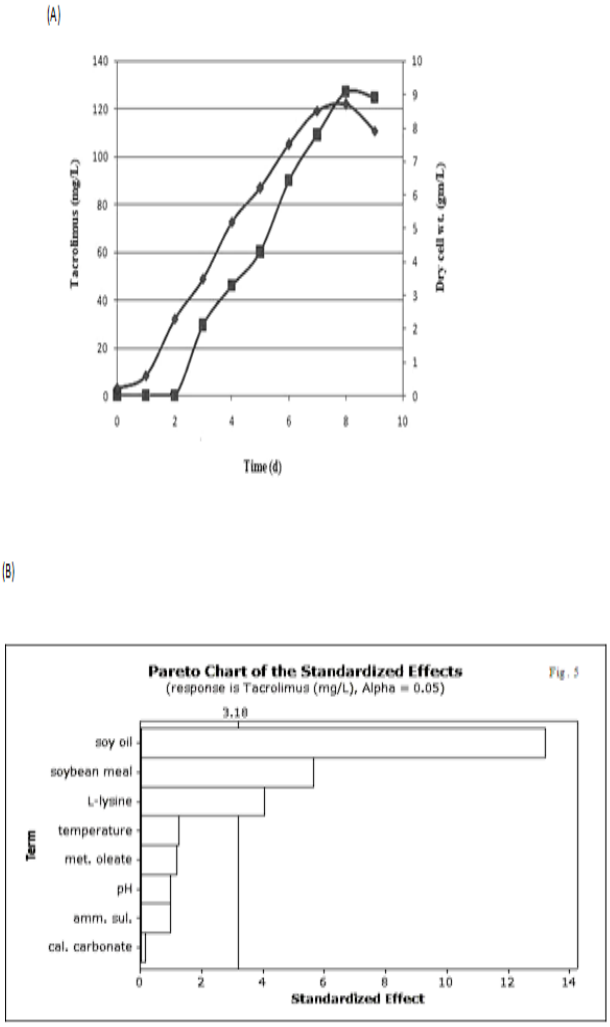

Fig. 2. (A) Time cource of tacrolimus fermentation by *Streptomyces* sp. (diamond- dry cell weight and square- tacrolimus concentration), (B) Pareto chart for standard effect of different variables.

Table 3. Effect of different carbon sources and correspondng concentration of tacrolimus in culture broth when *Streptomyces* strain incubated for 9 days at 28^0C temperature and pH 7 under shaking condition

Carbon source	Dry cell wt. (gm/L)	Tacrolimus production (mg/L)
Glucose	10.4 ± 1.32	90.5 ± 1.50
Fructose	9.2 ± 1.43	86.4 ± 2.10
Sucrose	7.9 ± 2.10	84.2 ± 2.40
Maltose	7.7 ± 1.80	83.6 ± 2.10
Lactose	8.6 ± 1.50	83.2 ± 2.50
Soy oil	7.9 ± 1.50	116.4 ± 2.30
Peanut oil	8.2 ± 1.90	106.2 ± 2.40
Cotton seed oil	9.4 ± 1.60	110.4 ± 2.70
Sun flower oil	9.9 ± 2.10	109.7 ± 2.56
Rape seed oil	10.1 ± 1.50	112.9 ± 1.89

Effect of different nitrogenous source on tacrolimus production

Soybean meal was found to be most supportive complex nitrogenous source for the production although there was no significant difference in the concentration of tacrolimus (Table 4).

Table 4. Effect of different organic nitrogenous sources and correspondng concentration of tacrolimus in culture broth when *Streptomyces* strain incubated for 9 days at 28^{0}C temperature and pH 7 under shaking condition.

Nitrogenous source	Dry cell wt. (gm/L)	Tacrolimus production (mg/L)
Peptone	11.3 ± 2.10	112.5 ± 1.7
Yeast extract	11.9 ± 1.90	110.8 ± 1.6
Soybean meal	10.9 ± 1.50	113.4 ± 2.8
Cotton seed meal	10.6 ± 1.50	108.6 ± 2.1

As an inorganic nitrogen source ammonium sulphate was found most appropiate (Table 5).

Table 5. Effect of different inorganic nitrogenous sources and correspondng concentration of tacrolimus in culture broth when *Streptomyces* strain incubated for 9 days at 28^{0}C temperature and pH 7 under shaking condition

Nitrogen source	Dry cell wt. (gm/L)	Tacrolimus production (mg/L)
Ammonium sulphate	7.9 ± 1.5	124.4 ± 2.8
Ammonium nitrate	9.8 ± 1.2	120.5 ± 1.5
Ammonium chloride	8.6 ± 1.8	112.9 ± 2.1
Ammonium citrate	10.6 ± 1.9	118.4 ± 1.9
Urea	11.8 ± 2.1	121.2 ± 2.3
Potassium nitrate	10.7 ± 2.4	117.1 ± 1.8

Effect of Amino acids on Tacrolimus production

Addition of different amino acids (0.5 gm/L) to production media showed remarkable increase in production of tacrolimus in case of L-lysine was found to enhance more production as compare to other (Table 6).

Table 6. Effect of amino acids and correspondng concentration of tacrolimus in culture broth when *Streptomyces* strain incubated for 9 days at 28^{0}C temperature and pH 7 under shaking condition

Amino acid	Dry cell wt. (gm/L)	Tacrolimus production (mg/L)
DL-alanine	11.4 ± 1.50	128.2 ± 1.34
L-arginine HCl	11.6 ± 1.67	127.7 ± 1.52
DL-aspartic acid	11.9 ± 2.10	127.9 ± 2.11
L-cysteine HCl	12.1 ± 1.86	128.2 ± 1.67
L-glutamic acid	11.2 ± 1.34	128.8 ± 1.23
Glycine	10.23 ± 1.45	129.2 ± 1.87
L-histidine HCl	12.7 ± 2.10	127.5 ± 1.65
DL-isoleucine	11.45 ± 2.41	129.9 ± 1.67
L-leucine	10.45 ± 1.67	129.1 ± 1.34
L-lysine	12.71 ± 2.60	135.6 ± 2.56
DL-methionine	11.72 ± 2.1	110.5 ± 1.67
L-ornithine HCl	11.12 ± 1.71	129.1 ± 2.21
DL-phenyl analine	9.34 ± 1.56	116.4 ± 2.91
L-proline	12.1 ± 2.31	129.2 ± 1.67
DL-serine	11.71 ± 1.82	129.2 ±1. 45
DL-threonine	11.50 ± 1.73	125.6 ± 2.1
DL-tryptophan	12.23 ± 1.40	121.4 ± 1.32
L-tyrosine	11.32 ± 1.30	108.4 ± 1.56
DL-valine	11.80 ± 2.22	122.7 ± 1.23

Effect of methyloleate on tacolimus production

Addition of methyl oleate to production media 4mM to the 12 mM concentration showed significant increase in production of tacrolimus. But after 12 mM of methyloleate decreasing profile was found (Table. 7).

Table 7. Effect of methylmalonyl-CoA on the production of tacrolimus by *Streptomyces* sp. in different concentration of methyl oleate

Methyl oleate (mM)	Dry cell wt. (gm/L)	Tacrolimus (mg/L)
0 mM	7.9 ± 1.50	124.4 ± 2.8
4 mM	9.9 ± 1.23	135.6 ± 3.1
8 mM	10.3 ± 1.25	137.5 ± 2.6
12 mM	10.7 ± 1.89	140.4 ± 3.3
16 mM	10.4 ± 1.34	136.4 ± 2.5
20 mM	10.5 ± 1.45	133.6 ± 2.2

Experimental designs and data analysis results

Plackett-Burman design result

Eight variables were studied by Plackett-Burman design in different combinations of high level (+) and low levels (−) by total 12 run of experiments. Effect of these variables came as different concentrations of tacrolimus for different combinations (Table 8). Out of these eight variables, soy oil, soybean meal and L-lysine showed significant positive effect on the tacrolimus production. The effect of medium components and operating conditions on tacrolimus production was also studied using pareto chart (Fig 2B).

Table 8. The experimental design using the PB method for screening of medium components (A: soy oil; B: soybean meal; C: L-lysine; D: ammonium sulphate; E: methyl oleate; F: calcium carbonate; G: pH; H: temperature)

RUN	A	B	C	D	E	F	G	H	Tacrolimus (mg/L)
1.	+	+	−	+	+	−	+	−	190.0
2.	+	−	+	−	−	−	+	+	186.0
3.	−	−	−	+	+	+	−	+	125.0
4.	+	+	+	−	+	+	−	+	192.0
5.	−	−	+	+	+	−	+	+	132.0
6.	+	+	−	+	−	−	−	+	187.0
7.	−	+	−	−	−	+	+	+	140.0
8.	−	+	+	+	−	+	+	−	167.0
9.	−	−	−	−	−	−	−	−	123.0
10.	−	+	+	−	+	−	−	−	155.0
11.	+	−	−	−	+	+	+	−	169.0
12.	+	−	+	+	−	+	−	−	183.0

Central composite design (CCD) results

Multiple regression analysis of the experimental data obtained. *F*-test was employed to evaluate the statistical significance of the quadratic polynomial. The multiple coefficients of correlation (R) and the determination coefficient of correlation (R^2) were calculated to evaluate the performance of the regression equation. By using central composite design t-value and p-value were also estimated (Table 9).

Multiple regression analysis was used to analyze the data and thus a polynomial equation was derived from regression analysis as follows:

$$Y=143.421+24.835A+6.926B + 4.619C +10.769A*A+ 1.545C*C -1.450A*C \quad \ldots\ldots\ldots\ldots (3)$$

Analysis of Variance for different models on effect of independent variables on tacrolimus production also estimated (Table 10).

The fitted response for the above regression model was plotted. These contour plots (Fig 3) and their respective 3D surface plots (Fig 4) provided a visual interpretation of the interaction between two factors and facilitate the location of optimum experimental

Table 9. Coefficients, t values and p value calculated from the tacrolimus obtained in the screening experiments (A-Soy oil; B- Soybean meal; C- L-lysine; coef- cofficient; SE coef-Standard error for estimated cofficient; T- t value; P- p value; S- Predictor; PRESS- prediction sum of square)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	11064.2	11064.24	1229.36	509.44	0.000
Linear	3	9369.5	9369.55	3123.18	1294.2	0.000
Square	3	1677.3	1677.31	559.10	231.69	0.000
Interaction	3	17.4	17.48	5.79	2.40	0.129
Residual error	10	24.1	24.13	2.41		
Lack of fit	5	16.6	16.62	3.32	2.21	0.202
Pure error	5	7.5	7.51	1.50		
Total	19	11088.4				

conditions. The 3D response surface curves were explained the interactions of medium components and the optimum concentration of each component required for the tacrolimus production. Each figure presents the effect of two factors while the other factor was held at zero level.

Isolated microorganism was confirmed as genus *Streptomyces* by acid fastness and gram staining. Isolated strain was found positive for gram stain and negative for acid fast test. Casein, tyrosine and xantine degradation ability was also used for confirmation of genus *Streptomyces*. *Streptomyces* sp. was found to be the producer of tacrolimus (fk 506) on the basis of results came out during the study. Crude culture broth of *Streptomyces* sp. showed several major peaks at retention time 3.27, 3.55, 4.20, 4.26, 4.44, 4.55, 6.27, 6.44 and 9.06. On purification number of peaks was reduced and single peak appeared in HPLC analysis. Identical retention time was 4.26 min during HPLC analysis and absorption peaks at 220 nm when campared with standard macrolide lead to conclusion that fermentation broth of *Streptomyces* sp. contain significant amount of tacrolimus. Inhibition activity of tacrolimus against *A. niger* was also studied. Earlier, sakamoto et al. (1995) also reported antifungal activity of tacrolimus and present study is in confirmity with these findings. Response surface methadology (RSM) was used to optimize fermentation media.

Table 10. Analysis of variance (ANOVA) for the fitted quadratic polynomial model (DF-degree of freedom; Seq SS- sequential sum of square; MS- mean square; P- p value)

Term	Coef	SE Coef	T	P
Constant	143.421	0.6336	226.371	0.000
A	24.835	0.4204	59.081	0.000
B	6.926	0.4204	16.475	0.000
C	4.619	0.4204	10.988	0.000
A*A	10.769	0.4092	26.316	0.000
B*B	0.675	0.4092	1.649	0.130
C*C	1.545	0.4092	3.776	0.004
A*B	−0.175	0.5492	−0.319	0.757
A*C	−1.450	0.5492	−2.640	0.025
B*C	−0.200	0.5492	−0.364	0.723
RSq=99.78%; RSq(pre)=98.76%; RSq(adj)=99.59%				

Plackett-burman design showed soy oil, soybean meal and L-lysine as significant media component. Since methylmalonyl CoA is the precursor for tacrolimus synthesis and the fatty acid content of soy oil might have enhanced the biosynthesis of methylmalonyl CoA. One of the metabolic pathways for the synthesis of methylmalonyl CoA is goverened by the enzyme methylmalonyl CoA mutase (MCM). This enzyme was found to enhance the biosynthesis of methylmalonyl CoA in oil based fermentationand decreases in carbohydrate based fermentation. A possible source for methylmalonyl-CoA is carboxylation of propionyl-CoA which is produced in fatty acid metabolism (Botella et al., 2009). High amino acid and mineral contents of soybean meal supported higher

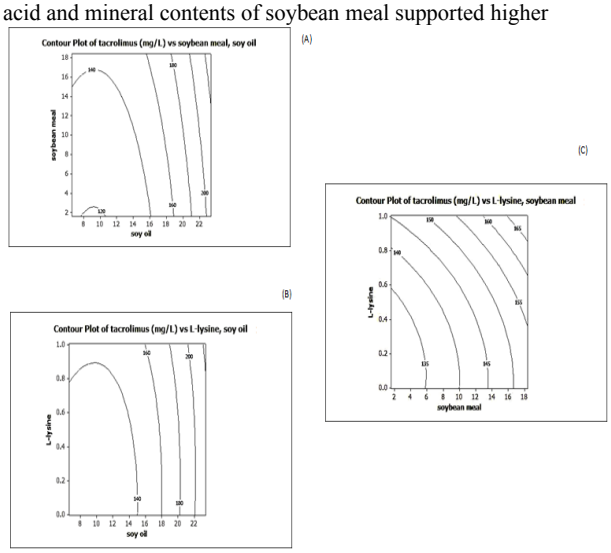

Fig. 3(A). Contour plot of tacrolimus production showing interaction of soy oil and soybean meal, (B) Contour plot of tacrolimus production showing interaction of soy oil and L-lysine, (C) Contour plot of tacrolimus production showing interaction of soybean meal and L-lysine.

production of tacrolimus by strenghening the biosynthesis of enzyme which involved in the tacrolimus synthesis. The addition of L-lysine appreciably affected the production of tacrolimus. This could have been because of the metabolic support of L-lysine derivatives in termination and cyclization of carbon-chain backbone biosynthesis of macrolide. Some amino acids had negative effect on tacrolimus production. This was probably because of the feedback inhibition and decreased availability of precursors derived from shikimic acid pathway (Singh and Behera 2009).Temperature and pH were found to be insignificant. this might be due to the biosynthetic enzymes of organism have no remarkable change in activity within the range of temperature and pH used in the study. The regression model's goodness of fit was checked by determination coefficient (R^2) (Park et al. 2002). The determination of coefficient (R^2) was, calculated as 0.9978 for tacrolimus production (a value of $R^2 > 0.75$ indicated the aptness of the model) which indicates the statistical model can explain 99.78% of variability in the response, which is in reasonable agreement with the adjusted R^2 of 0.9959. The goodness of a model can be checked by the determination of coefficient (R^2) and correlation coefficient (R) (Almeida e Silva et al. 2003; Cui et al. 2006; Nikerel et al. 2005]).

The R^2 value is always between 0 and 1. The R^2 value close to 1, signifies stronger the model and the better it predicts the response. Here the value of R (0.9978) for Eq. (3) being close to 1 indicated a close agreement between the experimental results and the theoretical values predicted by the model equation (Kılıc et al. 2002, Muralidhar et al. 2001; Zhang and Gao 2006) . The P values denotes the significance of the coefficients and also important in understanding the pattern of the mutual interactions between the variables. Value of P less than 0.05 indicate model terms are significant. Here soy oil (A), soybean meal (B), L-lysine (C), soy oil (A) * soy oil (A), L-lysine (C) * L-lysine (C) and soy oil (A) * L-lysine (C) are significant model terms. Synergetic effect of soya oil,

soybean meal and L-lysine showed dramatic increase in tacrolimus production. The optimum operating condition obtained from the

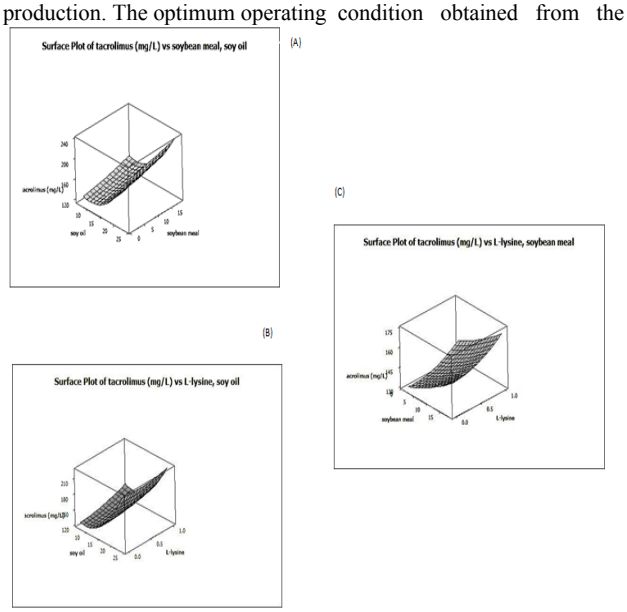

Fig. 4 (A). Surface plot of tacrolimus production showing interaction of soy oil and soybean meal, (B) Surface plot of tacrolimus production showing interaction of soy oil and L-lysine, (C) Surface plot of tacrolimus production showing interaction of soybean meal and L-lysine

polynomial model were 23.40 gm/L soy oil, 18.40 gm/L soybean meal and 1.00 gm/L L-lysine Along with other component and parameters in optimal condition. To confirm the model adequacy for predicting the maximum tacrolimus production, three additional experiments under this optimum medium composition were performed. The mean value of tacrolimus concentration was 235.5± 1.96, which was in good agreement with the predicted value (236.3 mg/L). The model was proved to be adequate. These optimized media component can enhances the production of tacrolimus from 135.6± 2.56 mg/L to 235.5± 1.96 mg/L.

Conclusion

RSM study was performed for the first time to optimize the medium components for tacrolimus production by *Streptomyces* sp. A highly significant quadratic polynomial obtained by the CCD. The more economic production of this expensive drug can be achieved in near future by optimization of media component by statistical tools. In combination with statistical approach it can be regarded as economically attractive in process application develop novel technology using unexpensive raw materials.

Acknowledgment

One of the authors (Sharad Verma) is thankful to Council of Scientific and Industrial Research (CSIR), India for providing financial assistance as Junior Research fellow.

References

AdinaraSyana K, Ellaiah P (2002) Response surface optimization of the critical medium components for this production of alkaline protease by a newly isolated *Bacillus* sp. J Pharm Pharmaceut Sci 5:272–278.

Akashi T, Nefuji T, Yoshida M et al (1996) Quantitative determination of tautomeric fk 506 by reversed phase liquid chromatography. J pham Biomed Anal 14:339– 346.

Almeidae-Silva JB, Lima UA, Taqueda MES et al (2003) Use of response surface methodology for selection of nutrient levels for culturing *Paecilomyces variotii* in eucalyptus hemicellulosic hydrolyzate. Bioresour Technol 87:45–50.

Botella L, Lindley ND, Eggeling L (2009) Formation and Metabolism of Methylmalonyl CoA in *Corynebacterium glutamicum*. J Bacteriol 191:2899–2901.

Cabri W, Paissoni P, Roletto J et al (2008) Process of purification of tactolimus. U.S. patent application 20080160586.

Chakravarti R, Sahai V (2002) Optimization of compactin production in chemically defined production medium by *Penicillium citrinum* using statistical methods. Process Biochem 38:481–486.

Cui FJ, Li Y, Xu ZH et al (2006) Optimization of the medium composition for production of mycelial biomass and exo-polymer by *Grifola frondosa* GF9801 using response surface methodology. Bioresour Technol 97:1209–1216.

Gokhade DV, Patil SG, Bastawde KB (1991) Optimization of cellulase production by *Aspergillus niger* NCIM 1207. *Appl.* Biochem Biotechnol 30:99–109.

Kılıc M, Bayraktar E, Ates S et al (2002) Investigation of extractive citric acid fermentation using response surface methodology. Process Biochem 37:759–767.

Kim HS, Park YI (2008) Isolation and identification of a novel microorganism producing the immunosuppressant tacrolimus. J Biosci Bioeng 105:418–421.

Kino T, Hatanaka H, Miyata S et al (1989) FK-506, a novel immuno-suppressant isolation from *Streptomyces*: immuno - suppressive effect of fk 506 *in vitro*. J antibio 40:1256–1265.

Li C, Bai J, Chai Z et al (2002) Optimization of culture medium for bacteriocin production by *Lactococcus lactis* using response surface methodology. J Biotech 93:27–34.

Mo S, Ban Y H, Park JW et al (2009) Enhanced fk506 production in *Streptomyces clavuligerus* CKD1119 by engineering the supply of methyl melonyl- CoA precursor. J Ind Microbio Biotech 36:1473–1482.

Muralidhar RV, Chirumamila RR, Marchant R et al (2001) A response surface approach for the comparison of lipase production by *Candida cylindracea* using two different carbon sources. Biochem Eng J 9:17–23.

Nikerel IE, Toksoy E, Kirdar B et al (2005) Optimizing medium composition for *Taq* I endonuclease production by recombinant *Escherichia coli* cells using response surface methodology. Process Biochem 40:1633–1639.

Park YS, Kang SW, Lee JS et al (2002) Xylanase production in solid state fermertation by *Aspergillus niger* mutant using statistical experimental designs. Appl Microbiol Biotechnol 58:761–766.

Puri S, Beg QK, Gupta R (2002) Optimization of alkaline protease from *Bacillus* sp. by response surface methology. Curr Microbiol 44:286–290.

Rahulana R, Nampoothiri KM, Szakacsb G et al (2009) Statistical optimization of l-leucine amino peptidase production from *Streptomyces gedanensis* IFO 13427 under fermentation using response surface methodology. Biochem Eng J 43: 64–71.

Rajendran A, Thirugnanam M, Thangavelu V (2007) Statistical evaluation of medium component by plackett-burman statistical design and kinetic modeling of lipase production by pseudomonas fluorescens. Ind J Biotech 6:469–478.

Rodrigues DCGA, Silva SS, Felipe MGA (1998) Using response surface methology to evaluate xylitol production by candida guilliermondii by fed batch process with exponantial feeding rate. J Biotech 62:73–77.

Sakamoto K, Izumi S, Miyauchi M et al (1995) A new assay method for immunosuppressants with a tacrolimus like mode of action. J antibio 48:727–729.

Shafiee A, Motamedi H, Chen T (1994) Enzymology of fk-506 biosynthesis:purification and characterization of 31-o-desmethyl fk506:methyl transferase from Streptomyces sp. Ma6858. Eur J Biochem 225:775–764.

Shih IL, Yu JY, Wu JY (2008) Application of response suyrface methodology to the production of polysaccharide by *Agrobacterium* sp. J Biotech 136:S53.

Singh BP, Behera BK (2009) Regulation of tacrolimus production by altering primary source of carbons and amino acids. Lett App Micro 49:254–259.

Sunitha I, SubbaRao MV, Ayyanna C (1998) Optimization of medium constituents and fermentation conditions for the production of L-glutamic acid by the co-immobilized whole cells of *Micrococcus glutamicus* and *Pseudomonas reptilivora* . Biopro Eng 18:353–359.

Taddei A, Rodriguez MJ, Vilcher EM et al (2006) Isolation and identification of *Streptomyces sp.* From venezuelan soils: Morphological and biochemical studies. Microbio Res 161:222–231.

Wang S, Magill JE, Vicente FB (2005) A Fast and Simple High-Performance Liquid Chromatography/Mass Spectrometry Method for Simultaneous Measurement of Whole Blood Tacrolimus and Sirolimus. *Archi. Patho*. Lab Med 129:661–665.

Weber T, Welzel K, Pelzer P et al (2003) Exploiting the genetic potential of polyketide producing *Streptomycetes*. J Biotech 106:221–232.

Yazgi A, Degirmencioglu A (2007) Optimisation of the seed spacing uniformity performance of a vacuum-type precision seeder using response surface methodology. Biosys Eng 97:347–356.

Yoon YJ, Choi CY (1997) Nutrient effect on fk-506, a new immunosuppressant, production by *Streptomyces sp.* In defined medium. J Fermen Bioeng 6:599–603.

Zhang J, Gao NF (2006) Application of response surface methodology in medium optimization for pyruvic acid production of *Torulopsis glabrata* TP19 in batch fermentation. J Zhejiang Univ Sci B 8:98–104.