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Optimization of Nutrient Components for Tacrolimus Production by Streptomyces tsukubaensis using Plackett-Burman followed by Response Surface Methodology

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ABSTRACT: *Tacrolimus belongs to macrolide lactone groups isolated from Streptomyces tsukubaensis, is used to prevent organ transplantation rejection. The aim of this study was to enhance the production of Tacrolimus by optimizing the nutrient components in fermentation media. Nutrient components play a significant role in the production of secondary metabolites. Many optimization techniques are available for this study and each optimization techniques has its own advantages. Plackett-Burman multifactorial design was employed to evaluate the significant nutrient factor in the medium using high and low levels of the factors where Dextrine white, Cotton seed meal (CSM) and Polyethylene glycol (PEG)-400 were found most important components among eight variables. These screened components were further optimized by central composite design with response surface methodology. A central composite design was based on second order polynomial used to determine the optimal levels of screened variables. The optimal level of each variable obtained from polynomial model was 173.94 g/l dextrine white, 19.83 g/l Cotton seed meal (CSM) and 24.72 g/l Polyethylene glycol (PEG)-400 respectively. Tacrolimus yield i.e., 1.08 mg/g was achieved with the combination of these optimal values. Validation experiments were performed to verify the adequacy and accuracy of the model.*

KEYWORDS: *Plackett-Burman design, Response surface methodology, Streptomyces tsukubaensis, submerged fermentation, Tacrolimus.*

I. INTRODUCTION

Tacrolimus is an immunosuppressive drug, inhibit the activity of patient's immune system. It is produced biosynthetically as secondary metabolites [1][2]. It is 23-membered macrocyclic lactone.. It belongs to the group of polyketide and synthesized by type I polyketide synthases (PKSs) [3]. Tacrolimus was first isolated from the fermentation broth of Japanese soil sample and reported as a macrolide antibiotic by Kino et al. in 1984 [4].

It is also known as FK-506 (Fermentek catalogue number 506) and fujimycin [5][6]. This drug is suppress immune system and used to prevent the rejection of transplanted organs. The immunosuppressive activities are due to its effect to reduce the activity of enzyme peptidyl-propyl isomerase and to interact the protein immunophilin FKBP12 (FK 506 binding protein). The FK 506-FKBP12 complex then acts on a target protein calcineurin by inhibiting its phosphatase activity [7].

In 1994, FDA (Food and Drug Administration) approved Tacrolimus for liver transplantation. It also has been used in patients for heart, kidney and bone marrow transplantation. It is also useful in the treatment of various autoimmune disease. Tacrolimus has been shown to be effective in treating a number of disease such as asthma,(PCT Application No WO90/14826) inflammatory and hyperproliferative skin disease and cutaneous manifestations of immunologically induced illness (Euoropean Patent No 315, 978). As an immunosuppressive agent, tacrolimus is 100 times more potent than cyclosporin [8].

It is produced by a type of soil bacterium *Streptomyces tsukubaensis* [9]. The name is derived from **Tsukuba** macrolide immunosuppressant [10]. Tacrolimus was discovered by Fujisawa Pharmaceuticals Co., which merged with Astellas Pharma in 2004. The commercial product of tacrolimus are Prograf, for the prevention of graft rejection in bone marrow and organ transplantation and Protopic, for the topical treatment.

To enhance the production of Tacrolimus it is necessary to optimize the culture conditions and media ingredients. Medium optimization by the traditional method is not only time consuming but also expensive due to large number of variables. Plackett-Burman design was used to screen the significant variables from the large number of variables. The screened variables were further employed for the optimization of nutrient components. Response surface methodology is an effective statistical technique to verify the screened variables.

II. MATERIALS AND METHODS

Microorganisms : In this study, *Streptomyces tsukubaensis* was used for the production of Tacrolimus by submerged fermentation. The culture was maintained on the growth medium (YMA) containing yeast extract 4.0g/l. Malt extract 10.0g/l, dextrose 4.0g/l and Agar 15g/l with pH 7.0. The culture media was incubated at 28^{o}C for 7 days.

Fermentation of Tacrolimus: Seed culture was prepared by inoculating 50 ml seed media (starch 5.0 g/l, peptone 5.0 g/l, glycerol 5.0 g/l, yeast extract 2.0 g/l and malt extract 2.0 g/l) with loopful of grown culture and incubated at 28^{o}C for 24 hrs. The grown seed was transferred to production media (Dextrine white 50 g/l, soya peptone 5.0 g/l, cotton seed meal 5.0 g/l, glycerol 5.0 g/l, polyethylene glycol (PEG)-400 20.0 g/l, Soya flour 7.0 g/l, dextrose 12.5 g/l , soluble starch7.0 g/l) and incubated at 28^{o}C and 200 rpm for 10 days.

HPLC Analysis : The productivity of Tacrolimus in the fermentation broth was analyzed by HPLC method. Acetone was used to extract the fermentation broth. Ascentis express 100 X 4.6mm, 2.6µ column was used to estimate Tacrolimus concentration. 0.1% triflouro acetic acid and acetonitrile was used as a mobile phase. The flow rate was set at 0.8 ml/min. Tacrolimus concentration was calculated by comparing the obtained peak area with standard area.

III. Experimental Design and Data Analysis

Identification of suitable variables using Plackett-Burman (PB) Design: Robin L. Plackett and J.P. Burman were presented this experimental design in 1946 [11]. It is a powerful screening method to recognize the most important factors using as few number of experiments. This design is valuable in the initial phase of experiments to identify the active factors when full philosophy about the process is missing. In the first optimization step, Plackett-Burman design was used to identify the critical factors of media having significant effect on the Tacrolimus production. The medium components were dextrine white, soy-flour, polyethylene glycol (PEG)-400, glycerol, cotton seed meal (CSM), dextrose, soy peptone and soluble starch. Twelve experiments were developed with eight assigned and three unassigned variables. The factors were examined at two levels -1 for low level and +1 for high level [12]. The name of variables, its code and actual value was demonstrated in “Table 1”.

Plackett-Burman design experiment is based on the first order model as shown in equation (1).

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

Where, Y is the response, β_0 is the intercept coefficient, β_i is the variable estimates and Xi is the independent variable.

All the experiments were carried out in triplicate and its average were taken as response.

Table 1: Experimental codes and level of factors in the Plackett-Burman design

Code	Variables	Low level (-)	High level (+)
A	Glycerol	5	15
B	Dextrine white	80	150
C	CSM	7	15
D	PEG	10	20
E	Soy flour	7	15
F	Dextrose	10	15
G	Soya Peptone	5	10
H	Soluble Starch	5	10

Central Composite Design (CCD)

Response optimization method was used to enhance the production of Tacrolimus by using RSM. Three critical variables for Tacrolimus production were screened on the basis of previous knowledge. The screened three factors (Dextrine white, PEG-400 and CSM) were further employed for optimization *via* CCD. Seventeen experiments were generated with three factors at five different levels ($-\alpha$, -1, 0, +1, $+\alpha$) that include 8 trials of factorial design, 6 trials of axial points (2 for each variables) and 3 trials of centre points [13]. "Table 2" show the variables and their levels. CCD was based on second degree polynomials which include all significant interaction terms. The relationship of three factors was elucidated by quadratic model.

$$Y = \beta_0 + \Sigma \beta_i Xi + \Sigma \beta_{ii} Xi^2 + \Sigma \beta_{ij} Xi Xj \quad \text{.....(2)}$$

Where, Y is response variables, β_0 is the interception coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient and X is coded independent variables.

Obtained data from CCD was subjected to statistical analysis. Design-Expert 8.0 Software was used to determine the regression analysis.

F-test was used to determine the significance of the model [14][15]. The optimum values of screened variables were achieved by solving regression equation and by analyzing the 3D surface plots [16].

Table 2: Experimental code and levels of factors in CCD

Code	Variables	-α	-1	0	1	+α
A	Dextrine white	11.59	120	150	180	200.45
B	PEG	9.27	12	16	20	22.73
C	CSM	11.59	15	20	25	28.41

IV. RESULT AND DISCUSSION

Statistical evaluation of factors by Plackett-Burman design: The design for twelve runs with two levels of concentration for each variable with the response were shown in table 3. The result of statistical analysis were shown in table 4. Plackett-Burman design is a powerful tool for screening of media components. The Plackett-Burman result showed variation in the activity of Tacrolimus in its twelve runs ranging from 0.213 to 0.742 mg/g. Dextrine white, Polyethylene glycol (PEG)-400 and Cotton seed meal (CSM) showed significant effect on the Tacrolimus production as represented in Pareto chart, "Fig.1". The obtained significant factors were further optimized by central composite design.

Table 3: Plackett-Burman experimental design for evaluation of medium components for Tacrolimus production by *S. tsukubeansis* and its response

Run	A	B	C	D	E	F	G	H	Tacrolinus mg/g
1	+	+	-	+	+	+	-	-	0.304
2	-	+	+	-	+	+	+	-	0.388
3	+	-	+	+	-	+	+	+	0.229
4	-	+	-	+	+	-	+	+	0.331
5	-	-	+	-	+	+	-	+	0.465
6	-	-	-	+	-	+	+	-	0.524
7	+	-	-	-	+	-	+	+	0.742
8	+	+	-	-	-	+	-	+	0.598
9	+	+	+	-	-	-	+	-	0.416
10	-	+	+	+	-	-	-	+	0.213
11	+	-	+	+	+	-	-	-	0.305
12	-	-	-	-	-	-	-	-	0.685

Table 4: Statistical analysis of Plackett-Burman:

[SS= sum square, df= degree of freedom, MS= mean square, *= significant]

Factors	SS	df	MS	F-value	*p-value*
Model	0.320	8	0.040	10.42	0.0399
A	1.200E-005	1	1.200E-005	3.129E-003	0.989
B	0.041	1	0.041	10.65	0.0470*
C	0.11	1	0.11	29.64	0.0122*
D	0.16	1	0.16	41.86	0.0075*
E	1.408E-003	1	1.408E-003	0.37	0.5873
F	2.821E-003	1	2.821E-003	0.74	0.4541
G	3.00E-004	1	3.00E-004	0.078	0.7979
H	1.613E-004	1	1.613E-004	0.042	0.8506

R^2 = 96.53%, Adj R^2 = 87.27%

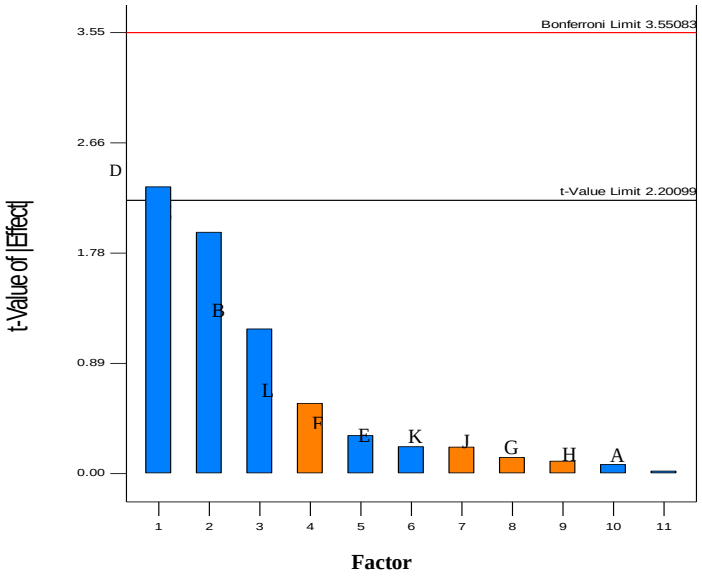

Figure 1**:** Pareto chart of main effects

Central Composite Design Result

Three variables were screened on the basis of Plackett-Burman design experiment. These screened variables were further employed for optimization via central composite design. Seventeen experiments were generated using different combinations of these three factors- Dextrine white, Cotton seed meal (CSM) and Polyethylene glycol (PEG-400) to determine the optimal levels as in “Tabl 5”.

Table 5: CCD experimental design and its responses

Run	A	B	C	Tacrolimus (mg/g)
1	-	-	-	0.687
2	+	-	-	0.968
3	-	+	-	0.754
4	+	+	-	0.720
5	-	-	+	0.654
6	+	-	+	0.859
7	-	+	+	0.985
8	+	+	+	1.014
9	-1.68	0	0	0.686
10	1.68	0	0	0.982
11	0	-1.68	0	0.727
12	0	1.68	0	0.955
13	0	0	-1.68	0.748
14	0	0	1.68	0.985
15	0	0	0	0.768
16	0	0	0	0.795
17	0	0	0	0.745

Multiple regression analysis was used to analyze the data and polynomial equation derived from regression analysis for Tacrolimus production was shown in equation (3).

$$Y = 0.77 + 0.072A + 0.050B + 0.057C - 0.061AB - 0.00163AC + 0.083\ BC + 0.018A^2 + 0.021B^2 + 0.030C^2 \quad (3)$$

Where, Y is response of Tacrolimus production, A is dextrine white, B is CSM and C is PEG-400.

Analysis of variance (ANOVA) was used to check the adequacy of the model "Table 6". The F-value 15.58 of the model was and *p-value* 0.0008 was represents the model was significant. The smaller *p-value* indicates the significance of the level. The determination coefficient (R^2) was used to check the goodness of the model [17]. The R^2 values always lies between 0 to 1. The R^2 values closer to 1 denotes better correlation between observed and predicted values. The multiple correlation coefficient (R^2 = 0.9524) and adjusted coefficient (adjusted R^2 = 0.8913) were high which indicates the significance of the model. The coefficient of variation (CV) show the degree of precision to which the experiments are compared. The lower value of CV (5.09) shows higher reliability of the experiments. The optimum level of variables and interaction effects were found out by 3D surface plots.

Table 6: Analysis of variance (ANOVA) for quadratic polynomial model

[SS= sum square, df= degree of freedom, MS= mean square, *= significant]

Variables	SS	df	MS	F-value	*p-value*
Model	0.2500	9	0.02700	15.5800	8.0E-04
A	0.070	1	0.07000	39.7400	4.0E-04
B	0.035	1	0.03500	19.6600	3.0E-03
C	0.045	1	0.04500	25.3400	1.5E-03
AB	0.030	1	0.030	17.0700	4.4E-03
AC	2.11E-05	1	2.11E-05	0.0120	9.2E-01
BC	0.056	1	0.056	31.5000	8.0E-04
A2	0.004	1	0.004	2.08	0.1928
B2	0.005	1	0.005	2.69	0.1453
C2	0.010	1	0.010	5.57	0.0504

R^2 = 95.24%, Adj R^2 = 89.13%

The 3D surface plots and their corresponding contour curves were shown in "Fig.2-4". It explained the interaction between variables and the optimum concentration of each factor involved in Tacrolimus production.

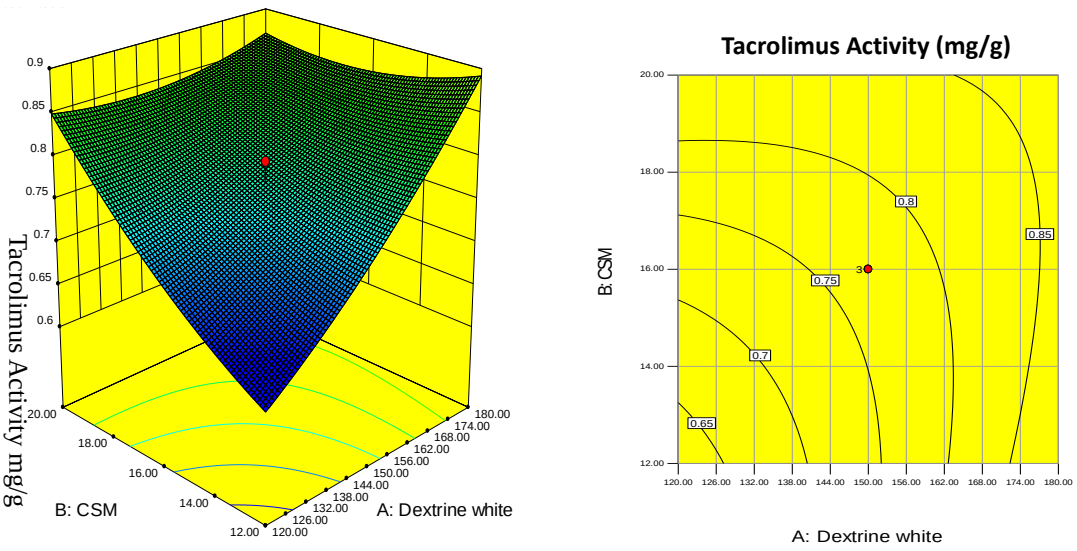

Figure 2: 3D surface plot and 2D contour graph of Tacrolimus production showing interaction between dextrine white and CSM.

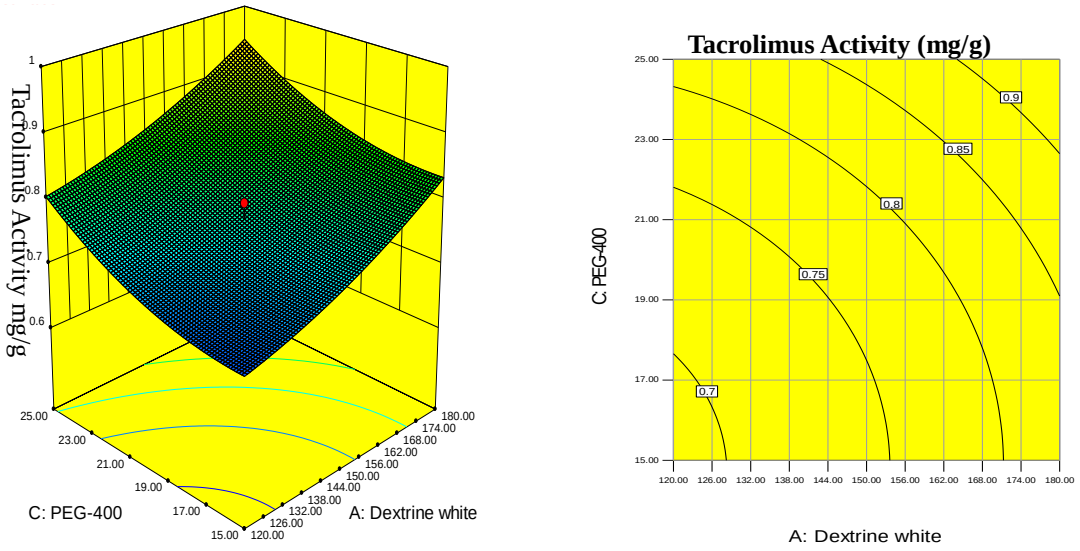

Figure 3: 3D surface plot and contour fraph of Tacrolimus production showing interaction between dextrine white and PEG-400.

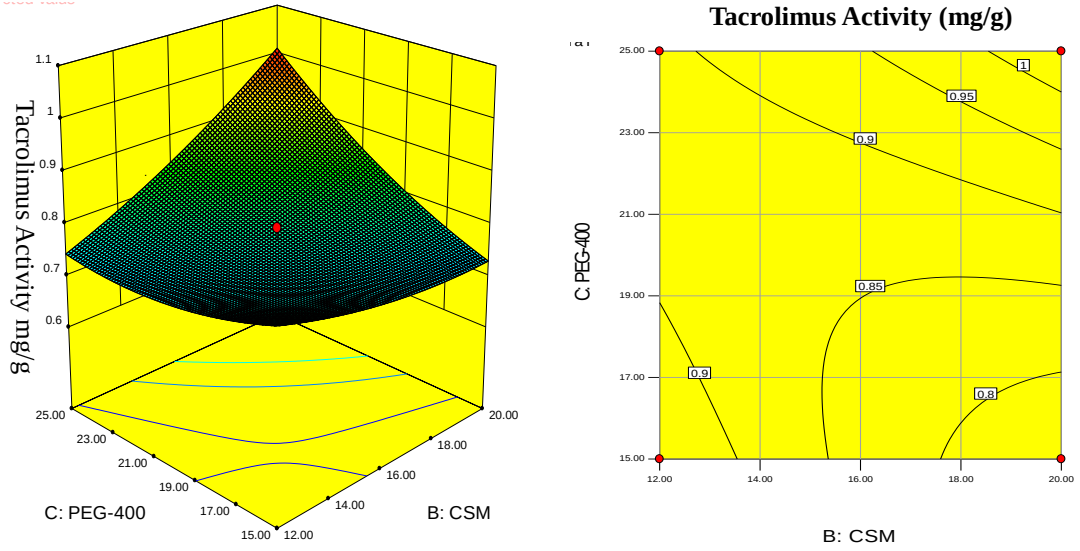

Figure 4: 3D surface plot and 2D contour graph of Tacrolimus production showing interaction between CSM and PEG-400.

Each figure represents the effect of two factors on Tacrolimus production while the third factor was held at zero level. The interaction between Cotton seed meal (CSM), Polyethylene glycol (PEG)-400 and dextrine white, Cotton seed meal (CSM) was significant for Tacrolimus production. Synergetic effect of dextrine white, Cotton seed meal (CSM) and Polyethylene glycol (PEG)-400 showed enhancement in the production of Tacrolimus. The optimal level of each variable obtained from polynomial model was 173.94 g/l dextrine white, 19.83 g/l CSM and 24.72 g/l PEG-400 respectively.

Validation of Model

To verify the adequacy of the model obtained from CCD for tacrolimus production, three sets of experiments were performed. The optimum level of media components were 173.94 g/l dextrine white, 19.83 g/l CSM and 24.72 g/l PEG-400 respectively. The mean value for tacrolimus production was seen 1.08 mg/g, which was in agreement with the predicted value.

Conclusion

Statistical technique is a major tool for the enhancement of metabolite production. In this study, Plackett Burman and central composite design with response surface methodology were employed for the optimization of nutrient components in a medium for the production of tacrolimus by S. tsukubeansis. Dextrine white, CSM and PEG-400 were chosen as a critical factor in Plackett-Burman design. These components played a major role for the enhancement of Tacrolimus production. The screened factors were further optimized by central composite design. The obtained optimal concentration for maximum production of tacrolimus were 173.94 g/l dextrine white, 19.83 g/l CSM and 24.72 g/l PEG-400 respectively. Under such conditions, the highest Tacrolimus production was achieved 1.08 mg/g. Thus it is concluded that the statistical technique is an effective tool for improving the microbial fermentation technology. This technique will also be useful for developing the fermentation process by reducing the cost of expensive raw materials and maximizing the yield of products.

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