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Statistical Approach To Optimize The Nutrient Components For Pneumocandin B0 Production By *Zelerion Arboricola* Under Submerged Fermentation

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ABSTRACT : *The evaluation of medium components for Pneumocandin B0 production by Zelerion arboricolain submerged fermentation was studied with statistical design. Seven medium components as independent variables were studied in this design of experiment. The most significant variables affecting Pneumocandin B0 production found was Mannitol , L-Proline and Coconut oil. A statistical approach, response Surface Methodology (RSM) through Central Composite Design (CCD) was used to optimize the medium components for better Pneumocandin B0 activity. From the response surface plot and analysis, it was found that the highest Pneumocandin B0 activity was achieved by using a combination of Mannitol , L-Proline and Coconut oil at concentrations of 82.23 g/l, 30.0 g/l and 6.0 g/l respectively . The statistical model was validated for Pneumocandin B0 production under the combinations predicted by the model .The production of Pneumocandin B0 with the optimized medium was correlated with the predicted value of RSM regression study. Thus, after sequential statistical media optimization strategy, a six-fold enhancement in Pneumocandin B0 production was achieved. This was evidenced by the higher value of coefficient of determination (R2=0.9638).*

KEYWORDS*:Response surface methodology, Submerged fermentation, Plackett-Burman design, Central composite design*

I. INTRODUCTION

The antifungal therapy caspofungin is a semi-synthetic derivative of Pneumocandin B0, produced by the fungus Glarea lozoyensis, was the first member of the Echinocandin class approved for human therapy. *Glarea lozoyensis* is an anamorphic species of the helotiaceae of medical relevance because it produces a family of lipohexapeptides named as Pneumocandins, natural antifungal which act by inhibiting fungal ß-(1,3) –glucan synthesis. *Glarea lozoyensis* was first isolated from a water sample from Madrid, Spain in 1985 and its antifungal activity in a yeast cell wall inhibition was detected in 1986. The earliest report of the Pneumocandins misidentified the fungus as *Zalerion arboricola* [5][6]. It produces Pneumocandin B0, the starting molecule for the synthesis of the antifungal drug caspofungin (CANCIDASTM). A semi-synthetic derivative Pneumocandin B0 of Caspofungin acetate, marketed as CANCIDASTM since 2001, the first echinocandin antibiotic brought to the market and has provided invaluable contribution to antifungal therapy to treat serious life threatening fungal infection [3][4]. Echinocandins are antifungal drugs which inhibit the synthesis of glucan in the cell wall via non competitive inhibition of the enzyme 1,3-ß-glucan synthase [1]and are thus called penicillin of antifungals (a property shared with papulacandins) as penicillin has the similar mechanism against bacteria but not fungi. Echinocandins are semi-synthetic Pneumocandins which are chemically lipopeptide in nature, consisting of large cyclic hexapeptide linked to a long chain fatty acid. Discovery of ehinocandins is stemed from the studies on papulacandins isolated from a strain of Papularia sphaerosperma (Pers.) which were liposaccharide ,i.e. fatty acid derivative of a disaccharide that also block the same target 1,3-ß-glucan synthase-and had action only on Candida species (narrow spectrum). Screening of natural products of fungal fermentation in 1970s lead to the discovery of echinocandins, a new group of antifungals with broad range of activity against Candida species. One of the first echinocandins of the Pneumocandin B0 type, discovered in 1974, echinocandin B, could not be used clinically due to risk of high degree of haemolysis. Screening semi-synthetic analog of the echinocandins gave rise to Cilofungin, the first echinofungin to enter clinical trials, in 1980, which it is presumed was later withdrawn for toxicity due to solvent system needed for systemic administration. The semi synthetic pneumocandins analog of echinocandin were later found to have same kind of antifungal activity, but low toxicity. The first approved of these newer echinocandins was caspofungin and later micafungin and anidulafungin were also approved. All these preparation so far have low oral bioavailability, so must be given intravenously only. Echinocandins have now become one of the first line treatments for Candida before the species are identified, and even as antifungal prophylaxis in hematopoetic stem cell transplant patient.

II. MATERIALS AND METHODS

Culture Source:Fungal strain of *Zelerion arboricola*ATCC No.74030 .Growth media- Initially the strain was cultured in Potato Dextrose Broth (dehydrated media).30.0 ml of PDB broth was dispensed in 250ml of Erlenmeyer flask and sterilized for 15 mins at 121^{o}C. The autoclaved media was inoculated with the 2.0ml of grown slant suspension (grown slant was harvested in 9.0 ml of saline and suspension was used for inoculation). Inoculated media was incubated at 25^{o}C for 72 hrs in a shaking incubator at 180 rpm.10% of grown PDB was transferred in 250 ml Erlenmeyer flask containing 30.0 ml of seed media comprises of (g/L): Mannitol -20, KH_2PO_4- 7.0, Cotton seed meal-7.5, Dextrose-8.0, and Corn steep liquor- 3.5 and trace salt solution 1.0 ml. Composition of the trace salt solution in (g/l) : $FeSO_4.7H_2O$-1.2, $MnSO_4.4H_2O$-1.3,$CuCl_2.2H_2O$-0.015, $CaCl_2.2H_2O$-0.15, H3BO3-0.054, $(NH_4)2MoO_4$.-0.012, $ZnSO_4.7H_2O$-0.3 .The pH of the media was adjusted to 6.0 and autoclaved for 30 min . The flasks were incubated at 25^{o}C for 96 hrs in a shaking incubator at 180 rpm.10 % of grown seed was further transfer to 250 ml flasks containing 35.0 ml of production media. Basal production media was used for the media optimization. Basal media composition :Mannitol -150 g/l, KH_2PO_4 -8.0 g/l, Casein enzyme hydrolysate-9.0 g/l , L-Proline-16.0 g/l and trace salt Solution 8.0ml and pH adjusted to 5.5 .Composition of trace element solution in (gm/l) was: $FeSO_4.7H_2O$-1.2,$MnSO_4.4H_2O$-1.3,$CuCl_2.2H_2O$-0.015, $CaCl_2.2H_2O$-0.15, H3BO3-0.054, $(NH_4)2MoO_4.H_2O$-0.012, $ZnSO_4.7H_2O$-0.3 in 100ml of HCl solution. Flasks were incubated at 25ºC and 240 rpm. The yield was assessed through HPLC.Quantification of Pneumocandin B0 by HPLC Pneumocandin B0 produced in the production broth was determined by HPLC. The culture broth of 5.0 gm was taken in 25.0 ml volumetric flask 20.0 ml acetone and sonicated for 20 minutes further the volume was made up with water. The resulting extracted solution was filtered through 0.22u nylon filter paper then injected into the HPLC (Waters 2496) having C-18 column (Hypersil BDS, 5u C18 (100 mm X 4.6 mm) for the estimation of Pneumocandin B0.Concentration of Pneumocandin B0 was calculated by comparison of peak areas with those standard Pneumocandin B0 and subsequently Pneumocandin B0 activity was calculated.

Statistical Screening of Significant Variables by Plackett-Burman Design

Table 1: Range and levels of the variables in coded units at different levels for the production of Pneumocandin B0 by using Plackett-Burman design

Code	Variables	Low level (-)	High level (+)
A	Glycerol	5.0	10
B	Coconut oil	5.0	10
C	Casein Enzyme Hydrolysate	5.0	8.0
D	L-Proline	18	28
E	Dextrose	5.0	10
F	Mannitol	100	150
G	PEG-400	5.0	10

Table 2: Twelve run Plackett-Burman design matrix for 7 variables coded values along with the Pneumocandin B0 yield.

Run	A	B	C	D	E	F	G	Yield
1	+	+	-	+	+	+	-	0.105
2	-	+	+	-	+	+	+	0.095
3	+	-	+	+	-	+	+	0.059
4	-	+	-	+	+	-	+	0.131
5	-	-	+	-	+	+	-	0.086
6	-	-	-	+	-	+	+	0.124
7	+	-	-	-	+	-	+	0.261
8	+	+	-	-	-	+	-	0.129
9	+	+	+	-	-	-	+	0.106
10	-	+	+	+	-	-	-	0.083
11	+	-	+	+	+	-	-	0.105
12	-	-	-	-	-	-	-	0.198

Optimization of Significant variables by Response Surface Methodology

The three selected variables, i.e. Coconut oil , L-Proline , and Mannitol each with 5 coded levels i.e. (--α , -1, 0, +1, +α) were optimized by Response surface model, a multiple regression analysis technique [9]. The design was explained with 17 runs with replicates at the Centre values as given in tables 3.

Table 3: Experimental code and levels of factors in CCD

Code	Variables	-α	-1	0	1	+α
A	Coconut oil	5.61	8	11.5	15	17.39
B	L-Proline	25.27	28	30	36	39.27
C	Mannitol	66.48	75	87.50	100	108.52

Table 4: Experimental code and levels of factors in CCD

Run	A	B	C	Activity (mg/g)
1	-	-	-	0.111
2	+	-	-	0.211
3	-	+	-	0.285
4	+	+	-	0.295
5	-	-	+	0.287
6	+	-	+	0.298
7	-	+	+	0.296
8	+	+	+	0.304
9	-1.68	0	0	0.258
10	1.68	0	0	0.295
11	0	-1.68	0	0.188
12	0	1.68	0	0.298
13	0	0	-1.68	0.197
14	0	0	1.68	0.284
15	0	0	0	0.268
16	0	0	0	0.258
17	0	0	0	0.260

Statistical data analysis and modeling

The three variables were coded according to the following equation;

$Z=(X-Xo)\ \Delta X$.....*(1)*

'Z' is the coded value of independent variable, 'X' the corresponding real value of an independent variable and 'ΔX' the step change of real value at the variable for the value 'Z'. The relationship between the response and the independent variables was explained by using 2nd order polynomial equation,

$Y= \beta 0+\sum \beta ixi+\sum \beta iixii+\sum \beta ijxixj$..... *(2)*

'Y' is the predicted response, 'ß0' is the interception coefficient, ßi is the linear coefficient, ßii is the quadratic coefficient and ßij is the interaction coefficient. The multiple regression analysis of the model and construction of response surface graphs were performed by using Design-Expert 8.0 Software to determine the regression analysis and analysis of variance(ANOVA). The quality of regression equation was determined by the coefficient of determination R^2 and its significance was judged by F-test [10][11].The fitted 2nd order polynomial equation was explained in the form of 3D graphs to show the relationship between the response and experimental variables. The point optimization method was used to optimize the maximum response of each variable.

III. RESULT AND DISCUSSION

In the present study *Zelerion arboricola* fungal strain was used for Pneumocandin B0 production by employing Plackett Burman and response surface methodology. Six fold productivity of Pneumocandin B0 was acheived by applying central composite experimental design in response surface methodology. Eight variables viz Mannitol , Dextrose, Casein Enzyme Hydrolysate, L-Proline ,PEG-400,Glycerol and Coconut oil, were analyzed through placket burman statistical analysis. Coconut oil, Mannitol and L-Proline were found as important factors, hence these three important independent factors were further analyzed . Among them Coconut

oil was found best for higher yield of Pneumocandin B0.Concentration of all three significant factors were optimized by using Response surface methodology.Pneumocandins are acylated cyclic hexapeptides belonging to the echinocandin group of antifungal antibiotics. Pneumocandin A0 is the most prominent product in comparison to Pneumocandin B0 in the fermentation. It contains threonine together with five unusual, hydroxylated, amino acid residues and a 10, 12 – dimethylmyristoyl side chain. Pneumocandin B0 differ from Pneumocandin A0 only in one aspect that it has a 3- hydroxyproline residue at the position occupied by 3 hydroxy-4-methylproline in Pneumocandin.A0.In the present study, various statistical approaches applied to improve the Pneumocandin B0 yield . Different factors were studied simultaneously for the better yield. In order to verify the above fact ,various experiments were performed by using statistical approach using different components of media as discrete variables. The conventional method of single factor optimization by maintaining other factors involved at an unspecified constant level is not only tedious , but also can lead to misinterpretation of results, especially because the interactions between different factors is overlooked [7].In the present study, the significant increment in Pneumocandin B0 production from 0.059 mg/gm to.0.304 mg/gm was achieved by applying the statistical approach.

Screening of medium components by using Plackett Burman: Different components like .Mannitol , Dextrose, Casein Enzyme Hydrolysate, L-Proline ,PEG-400,Glycerol and Coconut oil were used as a variables in present study. The effect of different medium components on Pneumocandin B0 production was studied with placket Burman design. Remarkable difference was obtained in yield of Pneumocandin B0 with different media as per placket Burman.

ANOVA for Plackett Burman

Source	Sum of squares	df	Mean square	F value	p value Prob > F
Model	0.031	7	4.442E-003	7.23	0.0373*
A	1.920E-004	1	1.920E-004	0.31	0.6060
B	2.821E-003	1	2.821E-003	4.59	0.0085*
C	0.014	1	0.014	23.24	0.0988
D	5.985E-003	1	5.985E	9.74	0.0355*
E	5.880E-004	1	5.880E	0.96	0.3834
F	6.816E-003	1	6.816E-003	11.03	0.0291*
G	4.083E-004	1	4.083E-004	0.66	0.4608
Residual	2459E-003	1	6147E-004		
Cor Total	0.034	11			

R-Squared - 0.9627, Adj R-Squared - 0.7985

The Model F-value of 7.23 implies the model is significant. There is only a 3.73 % chance that a "Model F-Value" this large could occur due to noise. .The "Pred R-Squared" of 0.9627 is in reasonable agreement with the "Adj R-Squared" of 0.7985. This model can be used to navigate the design space.The determination coefficient and correlation coefficient is the major tool to determine the goodness of model.P value less than 0.0500 indicates that the model terms are significant. In this case B (Cocconut oil), D (L-Proline), F(Mannitol) are significant model terms. Values greater than 0.1000 indicate the model terms are not significant

Central Composite Design (CCD) Result

CCD was used to identify the critical factor. It consists of factorial axial to recognize the quadratic effects and central trials to calculate the process variability. The P-Value was used as a tool to validate the significance level of each coefficients and also to recognize the model of the mutual interactions between the variables. The smaller p- value (less than 0.0001) indicates model terms are significant. Here Coconut oil ,L-proline and Mannitol was found as a significant model term. The regression model's goodness of fit was checked by multiple correlation coefficients (R^2) [8] The R^2 values lies between 0 and 1. The R^2 value closer to 1 denotes better correlation between observed and predicted values. A response surface plot was employed to understand the main variables and the interaction among two factors while other factors were held at middle level. Interacting effect of coconut oil, L-Proline and Mannitol illustrated maximum yield of Pneumocandin B0. The optimum levels of media constituent's (Coconut oil ,L-Proline and mannitol) was required for higher production of Pneumocandin B0. Under such optimum conditions, the maximum production of Pneumocandin B0 was achieved i.e 0.304 mg/gm.

ANOVA for Response Surface Quadratic Model

Source	Sum of squares	df	Mean square	F value	p value Prob > F
Model	0.043	9	4.74E-03	20.71	0.0003*
A-Cocconut oil	2.68E-03	1	2.68E-03	11.69	0.0111*
B-L-Proline	0.015	1	0.015	67.07	< 0.0001*
C-Mannitol	0.013	1	0.013	58.93	0.0001*
AB	1.08E-03	1	1.08E-03	4.72	0.0663
AC	1.04E-03	1	1.04E-03	4.52	0.0711
BC	7.38E-03	1	7.38E-03	32.23	0.0008*
A2	5.40E-04	1	5.40E-04	2.36	0.1687
B2	2.74E-04	1	2.74E-04	1.19	0.3105
Residual	0.96	6	0.16		
Cor Total	12.82	11			

R-Squared - 0.9638 , Adj R-Squared- 0.9173

The Model F-value of 20.71 implies the model is significant. There is only a 0.03% chance that a "Model F-Value" this large could occur due to noise. .The "Pred R-Squared" of 0.9638 is in reasonable agreement with the "Adj R-Squared" of 0.9173. This model can be used to navigate the design space.The determination coefficient and correlation coefficient is the major tool to determine the goodness of model.P value less than 0.0500 indicates that the model terms are significant. In this case A (Cocconut oil), B (L-Proline), C (Mannitol) and BC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

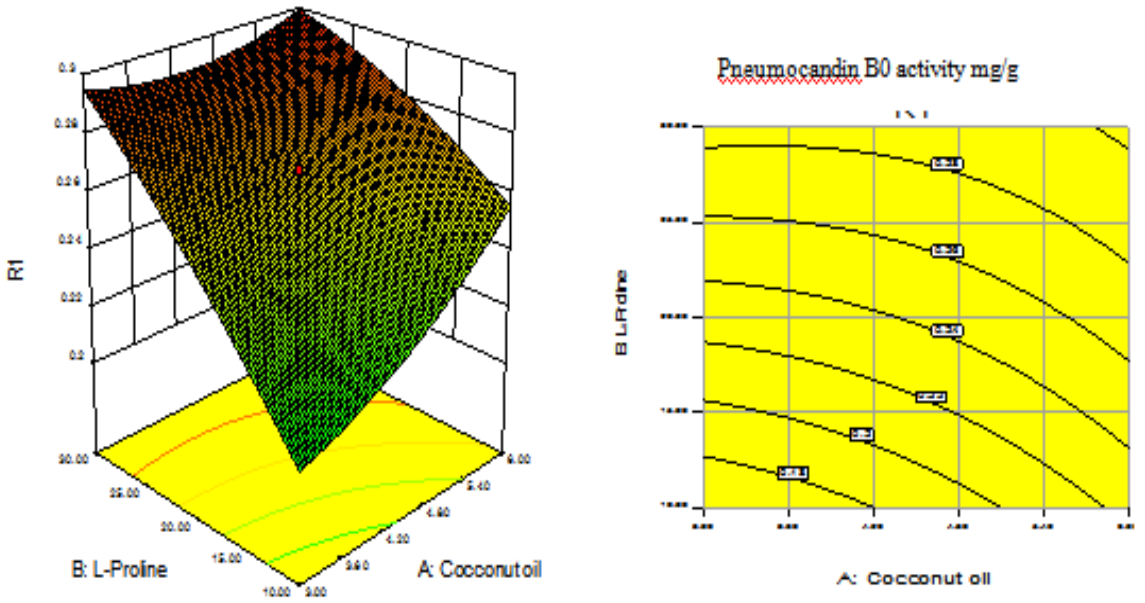

Fig.1 Response surface and contour plot of L-Proline concentration vs. Coconut oil concentration on Pneumocandin B0.production

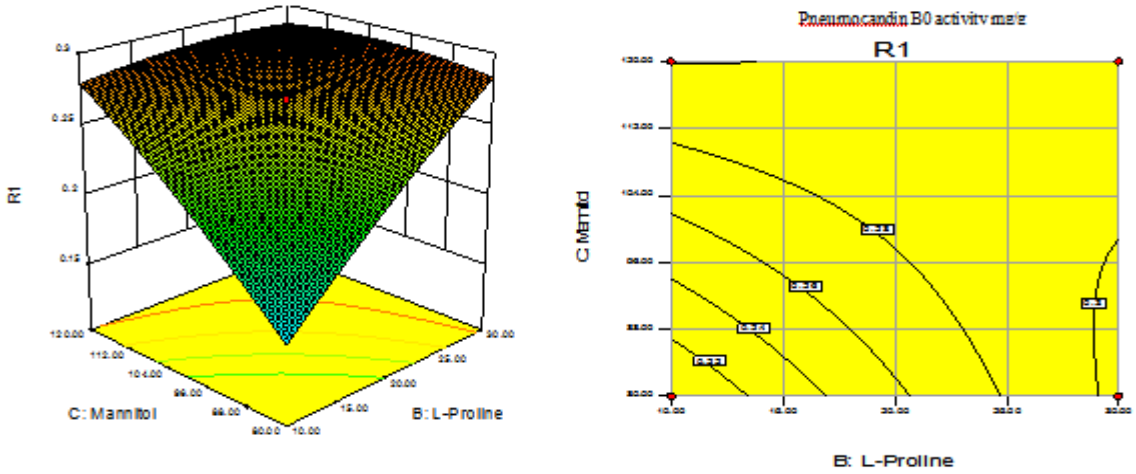

Fig.2 Response surface and contour plot of Mannitol concentration vs. L-Proline concentration on Pneumocandin B0.production

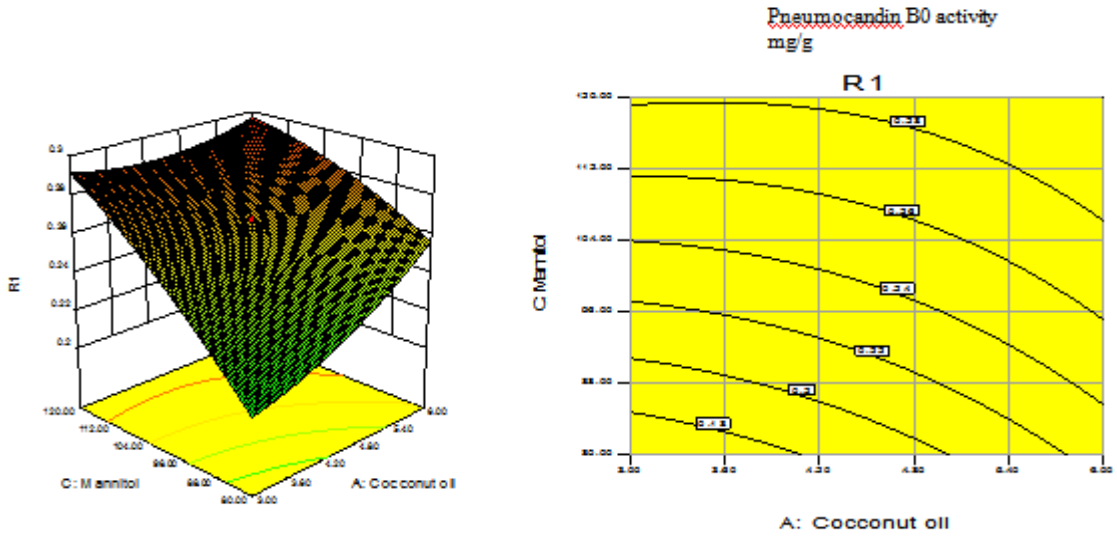

Fig.3. Response surface and contour plot of Mannitol concentration vs. Coconut oil concentration on Pneumocandin B0.production.

Validation of Final concentration: Validation of the predicted results was accomplished by performing additional experiments in triplicate with the parameters suggested by the numerical modeling (suggested solution). These three sets of experiments yield average titer value was 0.308 mg/gm.Good agreement between the predicted and experimental results confirmed the experimental adequacy of the model and the existence of the optimal point.The response surface describing the quadratic effect of Coconut oil,Mannitol and L-Proline concentration on Pneumocandin B0 production.

IV. CONCLUSION:

Plackett Burman and CCD with Response surface methodology were employed for the optimization of fermentation medium components for the production of Pneumocandin B0 by *Zelerion arboricola*. It was found that Mannitol , L-Proline and Coconut oil were the significant components to enhance the productivity. The highest productivity of Pneumocandin B0 was found when the concentration of the following components were applied i.e Mannitol , L-Proline and Coconut oil at concentration 82.23 g/l, 30.0 g/l and 6.0 g/l respectively . Overall, it can be concluded that the statistical approach is an imperative approach for the optimization of medium components. Validation experiments were also performed to verify the accuracy of the results.

Results also revealed the basis, for further study with large scale fermentation for Pneumocandin B0 production.The approach was proved fruitful for higher Pneumocandin B0 production. Statistical experiments help to understand the hidden complexity of medium ingredients role in production of Pneumocandin B0.

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