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Media Optimization For Lovastatin Production by Statistical Approach Using *Aspergillus terreus* by Submerged Fermentation

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ABSTRACT

Lovastatin is a secondary metabolite produced by Aspergillus terreus which is used as cholesterol-lowering drugs called HMG-CoA reductase inhibitors. In the present study to increase the yield of lovastatin, production media components were optimized. The impact of media components which includes carbon, nitrogen source, organic and inorganic salts were studied with response surface methodology.

A "Plackett-Burman Design" was employed to evaluate the effects of different components in the media. The concentration of Casein, Citric acid and Sodium acetate significantly influence the Lovastatin production. The Pareto chart was employed to determine the optimal regions of these three significant factors. These three factors were optimized using central composite design (CCD) of "response surface methodology." The optimized fermentation medium composition was as follows (g/L): Casein 25.0, Citric acid 12.0, Sodium acetate 18.0, Dextrose 40.0, Cotton seed meal 5.50, Soya flour 20.5, Magnesium sulphate (MgSO4.7H2O) 1.0, Calcium carbonate 7.5, Glycerol 5.0, PEG-400 3.85, Soya oil 5.0. The validated model can precisely predict the Lovastatin production.

Keywords-*Lovastatin, Fermentation, Plackett Burman Design, Central Composite Design, Response Surface Methodology.*

1. INTRODUCTION

In filamentous fungi, many secondary metabolites were synthesized with complex chemical structure by polyketide pathway. Lovastatin, Monacolin J, Monacolin L, and Mevastatin can be produced by *Aspergillus terreus.*

Lovastatin is a potent hypercholesterolemic drug used for lowering blood cholesterol. It acts by competitively inhibiting the enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase involved in the biosynthesis of cholesterol. Commercially lovastatin is produced by a variety of filamentous fungi including *Penicillium* species, *Monascus ruber* and *Aspergillus terreus* as a secondary metabolite. Production of lovastatin by fermentation decreases the production cost compared to costs of chemical synthesis. In recent years, lovastatin has also been reported as a potential therapeutic agent for the treatment of various types of tumors and also play a tremendous role in the regulation of the inflammatory and immune response, coagulation process, bone turnover, neovascularization, vascular tone, and arterial pressure.

Lovastatin is an effective inhibitor of the enzyme hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase (mevalonate: NADP1 oxidoreductase, EC 1.1.1.34) that catalyzes the reduction of HMG-CoA to mevalonate during synthesis of cholesterol [1]. When the lactone ring of lovastatin is in its open form, as it would be in the human liver, the structure bears a strong similarity to HMG-CoA. It has been shown that lovastatin and the other monacolins are very specific competitive inhibitors of the reductase [2-5], which reduce serum cholesterol levels by blocking cholesterol biosynthesis. Lovastatin also inhibits tumor growth through the inhibition of nonsterol isoprenoid synthesis [6,7].

Lovastatin is a precursor for simvastatin, a powerful semi-synthetic statin commercially available as Zocor. Simvastatin is obtained via a selective enzymatic deacylation or by semisynthetic route [8].

Lovastatin is produced as a secondary metabolite by a variety of filamentous fungi including *Aspergillus terreus*[9]. Commercial production of lovastatin is based on *A terreus* batch fermentation and most of the literature deals with this species [10-15]. *Aspergillus terreus* fermentations are typically carried out at ∼28◦C and pH 5.8–6.3 [13]. Pelleted growth of *A terreus* has yielded higher titers of lovastatin than obtained with filamentous growth.The rapid increase in viscosity accompanied by filamentous growth greatly impedes oxygen transfer and this is said to explain the low titers of lovastatin [14]. A batch fermentation cycle runs for less than 240 hrs.

The nutrient source of a fermentation medium influences the metabolism of cells and, therefore, the productivity of a fermentation process also depends on the media composition. Carbon and nitrogen sources normally play a crucial role in fermentation productivity because these nutrients are directly linked with the product formation. Lovastatin production has been found to depend on the carbon and nitrogen sources [12,13,14]. It is understood that designing of culture conditions is a prerequisite in the production of metabolites [16].

Optimization of media ingredients play an signifycant role to improve the productivity. Designing of any appropriate production medium and conditions is of crucial importance to enhance the efficiency and productivity of secondary metabolites, as it can remarkably affect the product concentration, activity and cost benefit. Statistically, it has been proved that experimental design is more efficient than traditional method especially on multi-variables screening. As in tradition method the complex interactions among different variables were overlooked. On the other hand, statistical experimental designs provide a systematic and efficient design to achieve targets. Currently, statistical techniques were used to find out the key factors rapidly from a multi-variables *i.e.*, Plackett–Burman design and response surface methodology [17-22].

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques useful for experiment design,building models, evaluating the effects of factors and searching optimum conditions of factors for desirable responses[19]. Statistical experimental design minimizes the error in determining the effect of parameters and it shows the simultaneous, systematic, and efficient variation of all parameters. Response Surface Methodology (RSM) is an effective tool for optimizing the process condition that uses quantitative data from an appropriate experimental design to determine and simultaneously solve multivariate equations [17]. It usually involves an experimental design such as Central Composite Design (CCD) to fit a second-order polynomial by a least squares technique. An equation is used to describe the test variables, and describe the combined effect of all the test variables in the response.

The mathematical models play an important role in rational design and optimization of biochemical process [23,24]. It is difficult to obtain an accurate model for biochemical process such as Lovastatin production.

The objectives of the present investigation includes optimization of Lovastatin production by RSM and to study the interrelationship among the media ingredients on Lovastatin yield using response surface plots. Ratio of the Carbon and Nitrogen is important for production of secondary metabolite. The media ingredients were screened by Plackett-Burman (PB) Design and obtained significant factors were further optimized by RSM via Central Composite Design (CCD). Remarkable increase was found in Lovastatin yield in this study.

2. MATERIALS AND METHODS

2.1 Micro organism and preparation of spore suspension:

Stock culture of *A.terreus* was maintained on Yeast Malt Agar (YMA). The culture was incubated in BOD incubator at 28°C for 8 - 10 days. 0.5% Tween 80 solution was used to harvest the spores. After calculating the count, spore suspension was used for shake flask study.

2.2 Shake Flask fermentation process:

2.2.1 Culture Source:

Filamentous fungalstrain; *Aspergillus terreus.*

2.2.2 Growth media and culture conditions:

Spore suspension was used for the inoculation of seed media containing (g/L) : Dextrose-10.30, Skim milk-11.50, Sucrose -10.50, Soya flour-6.00, Yeast extract-1.50, Citric acid-2.5, Sodium acetate-1.25, Potassium-0.046, Magnesiumsulphate ($MgSO_4.7H_2O$)-0.12,Calcium carbonate-1.90, Glycerol-5.00 , PEG 400-0.60 and pH 7.20. The flasks were incubated at 28°C for 38±2 hrs in a shaking incubator at 200 rpm. Grown seed was transferred in production media comprises (g/L):Dextrose-40.00, Skim milk-30.0, Cotton seed meal-5.50, Soya flour-20.5, Citric acid-14.50, Sodium acetate-8.75, Magnesium sulphate ($MgSO_4.7H_2O$)-1.00, Calcium carbonate-7.5, Glycerol-5.0 , PEG 400-3.85, Soya oil-5.0, pH 7.40. Flasks were incubated in shaking incubator at 28°C and 240 rpm for 240 hrs. The yield was assessed through HPLC .

2.2.3 Quantification of Lovastatin by HPLC: The productivity of Lovastatin in the fermentation broth was analyzed by HPLC method. Methanol was used to extract the fermentation broth. 150 X 4.6mm, 5µ column was used to estimate Lovastatin concentration. 0.1% Orthophosphoric acid and Acetonitrile was used as a mobile phase in gradient system. The flow rate was set at 0.8 ml/min.Lovastatin concentration was calculated by comparing the obtained peak area with standard area.

2.3 Experimental Methods and Analysis:

2.3.1 Screening of the nutrient medium by change one variable per time method for Lovastatin production:

Standard composition of production medium in g/L was Dextrose-40.0, Skim milk-30.0, Cotton seed meal-5.50, Soya flour-20.50, Citric acid-14.50, Sodium acetate-8.75, Magnesium sulphate ($MgSO_4.7H_2O$)-1.00, Calcium carbonate-7.50, Glycerol-5.0 , PEG400-3.85, Soya oil-5.0, pH 7.40. Flasks were incubated in shaking incubator at 28°C and 240 rpm for 240 hrs. The effect of the one factor studied by replacing skim milk with casein and its impact on Lovastatin production using COVT method.Dextrose-40.0, Casein-33.0, Cotton seed meal-5.50, Soya flour-20.50, Citric acid - 14.50, Sodium acetate-8.75, Magnesium sulphate ($MgSO_4.7H_2O$)-1.00, Calcium carbonate-7.50, Glycerol-5.0 , PEG400-3.85, Soya oil-5.0, pH 7.40. The optimum Lovastatin production was determined to be 10.0 g/L, which is higher than the standard media composition.

2.3.2 Plackett-Burman Design for Screening Medium Components

The experimental design with the name, symbol

code, and level of the variables is shown in Table 1. Each independent variable is represented in two levels, high and low, which are denoted by (+) and (-) respectively. A set of nine medium components were screened by Plackett Burman design [25]. Plackett-Burman Design was introduced as a first optimization step to identify the factors that have significant effects on the Lovastatin production. The ingredients studied by Placket Burman design were carbon sources (Dextrose, Casein, Glycerol), nitrogen sources (Soya flour, Cotton seed meal, Soya oil), Surfactant (PEG-400) minerals and salts (Citric acid and Sodium acetate). Based on Plackett-Burman factorial design, each variable was examined in two levels: −1 for low level and +1 for high level. This design was used to screen and evaluate the important factor(s) that influence the response of nine assigned factors and two dummy variable to estimate experimental error in 12 experimental designs. Plackett-Burman experiments were designed by using Design Expert Version 8.0 USA. All experiments were performed in duplicates and the average of it was taken as the response. The factors included in the screening experiment and their settings are given in Table 1.

Table 1: Two levels of the factors used in Plackett-Burman Design

Code	Factors	Low level (-1)	High level (+1)
A	Dextrose	30	60
B	Casein	20	40
C	Soya Flour	15	30
D	Cotton seed meal	5.0	10
E	Citric acid	8.0	16
F	Sodium acetate	7.50	10
G	Glycerol	4.0	10
H	PEG-400	1.50	5.0
I	Soya oil	4.0	8.0

Table 2: Twelve runs Plackett-Burman design matrix for nine variables with coded values along with theyield.

Run	A	B	C	D	E	F	G	Yield
1	+	+	-	+	+	+	-	9.23
2	-	+	+	-	+	+	+	10.76
3	+	-	+	+	-	+	+	9.91
4	-	+	-	+	+	-	+	11.2
5	-	-	+	-	+	+	-	9.005
6	-	-	-	+	-	+	+	10.09
7	+	-	-	-	+	-	+	10.12
8	+	+	-	-	-	+	-	10.37
9	+	+	+	-	-	-	+	8.59
10	-	+	+	+	-	-	-	10.29
11	+	-	+	+	+	-	-	11.21
12	-	-	-	-	-	-	-	10.64

Table 3 : Coded values for each factor of the Central Composite Design

Code	Factors	-α	-1	0	+1	+α
A	*Sod.acetate*	12.64	14	16	18	19.36
B	*Citric acid*	9.95	12	15	18	20.05
C	*Casein*	19.89	25	32.50	40	45.11

Table 4: Experimental code and levels of factors in Central Composite Design

Run	A	B	C	Activity (mg/g)
1	-	-	-	12.98
2	+	-	-	11.86
3	-	+	-	12.21
4	+	+	-	14.03
5	-	-	+	13.36
6	+	-	+	15.18
7	-	+	+	13.56
8	+	+	+	12.58
9	-1.68	0	0	13.29
10	1.68	0	0	15.83
11	0	-1.68	0	14.57
12	0	1.68	0	14.26
13	0	0	-1.68	13.69
14	0	0	1.68	13.41
15	0	0	0	11.59
16	0	0	0	11.36
17	0	0	0	11.85

3. RESULTS AND DISCUSSIONS

3.1 Plackett-Burman Design for Screening Medium Components

Plackett-Burman Design was used as a screening method to determine which of the nine components of the fermentation medium significantly effect on the product yield. The significant variables screened via Plackett-Burman design were shown in Pareto chart (Fig.1).The experimental results were interpreted on the basis of partition of the overall effect of all the factors to the response into individual factor effect. This partition has been made statistically. When the value of the concentration effect of the tested variable is positive, the conclusion is that the influence of the concerning variable is greater at a high concentration tested, and when negative, this means that the influence of the given variable is greater at a low concentration [26]. Screened variables of Plackett-Burman was further proceeded with CCD to optimize the actual required concentration of selected variables.

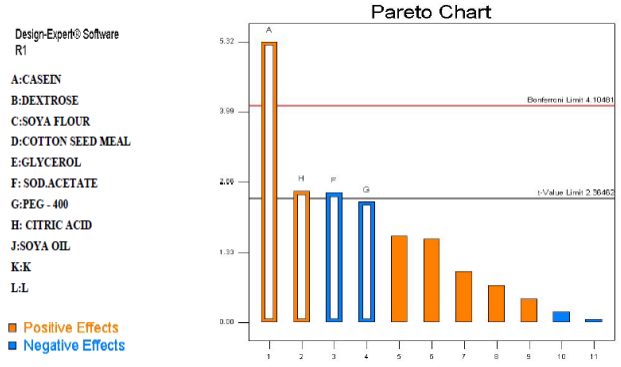


Figure 1: Pareto Chart of Main Effects for Plackett-Burman Design variables

Optimization of the independent variables by RSM via CCD:

Casein (A), Sodium acetate (B) and Citric acid (C) was screened via Plackett-Burman were further proceeded with (response surface) a Central Composite Design consisting of a set of 17 runs of experiment with three replicates at central point was conducted. Table 3 shown variables and their levels for central composite design (CCD). The CCD matrix of the independent variables in coded units (experimental design) and experimental values of response is given in Table 4. All the experiments were performed in 250 ml Erlenmeyer flask containing 30 ml of media.

Multiple regression analysis was used to analyze the data and polynomial equation derived from regression analysis for Lovastatin production was shown in equation (2).

$$Y = 14.01 - 0.62\ A - 0.41\ B + 0.091\ C - 0.24\ AB - 0.84\ AC - 0.79\ BC + 0.024\ A^2 - 0.60\ B^2 - 0.34\ C^2 \quad \text{.....(3)}$$

Where, Y is response of Lovastatin production, A is Casein , B is Sodium acetate and C is Citric acid.

Table 5: ANNOVA for Central composite design

Source	SS	df	MS	F value	p value Prob > F
Model	24.01	9	2.67	9.87	0.0032
A-Sod.actetate	5.27	1	5.27	19.51	0.0031
B-Citric acid	2.34	1	2.34	8.68	0.0215
C-Casein	0.11	1	0.11	0.42	0.5395
AB	0.47	1	0.47	1.74	0.2285
AC	5.68	1	5.68	21.02	0.0025
BC	4.99	1	4.99	18.48	0.0036
A^2	6.625E-003	1	6.625E-003	0.025	0.8800
B^2	*4.06*	1	*4.06*	15.01	0.0061
C^2	1.32	1	1.32	4.87	0.0631
Residual	1.89	7	0.27		
Cor Total	25.90	16			

[SS= sum square, df= degree of freedom, MS= mean square, *= significant] R2 = 97.20%, Adj R2 = 83.31%

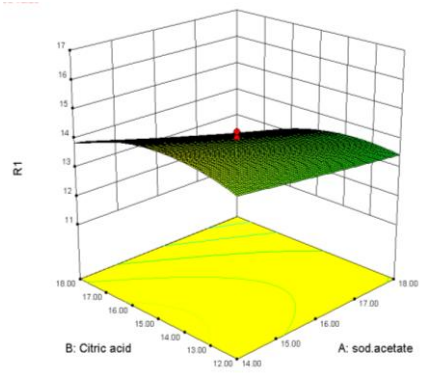
3.2 ANOVA for Response Surface Quadratic Model

Analysis of variance (ANOVA) was used to check the adequacy of the model "Table 5". The F-value 9.87 of the model was and p-value 0.0032 was represents the model was significant. There is only a 0.32 % chance that a "Model F-Value" this large could occur due to noise.

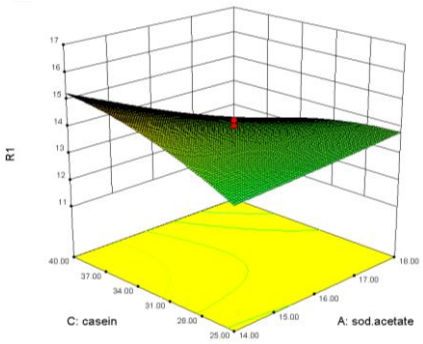
Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case C, AC,

BC are significant model terms.Values greater than 0.1000 indicate the model terms are not significant.The "Lack of Fit F-value" of 4.20 implies the Lack of Fit is not significant relative to the pure error. There is a 20.34% chance that a "Lack of Fit F-value" this large could occur due to noise.The R-Squared value is 0.9720 is and "Adj R-Squared" value is 0.8331. Adequate precision measures the signal to noise ratio. This model can be used to navigate the design space. The optimum level of variables and interaction effects were found out by 3D surface plots.

(A)



(B)



(C)

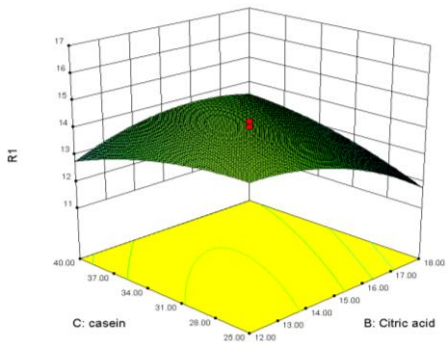


Figure 2: 3D surface plot for Lovastatin production showing interaction between - (A) Citric acid and Sod. acetate (B) Casien and Sod. acetate (C) Casien and Citric acid.

Each graph of figure 2 represents the effect of two factors on Lovastatin production while the third factor was held at zero level. The interaction between Sodium acetate, Casein and Citric acid, Casein was significant for Lovastatin production. Synergetic effect of Sodium acetate, Citric acid and Casein showed enhancement in the production of Lovastatin. The optimal level of each variable obtained from polynomial model was 18.0 g/l Sodium acetate, 12.0 g/l Citric acid and 25.0 g/l Casein respectively.

4. CONCLUSION

This work has demonstrated the use of central composite design by determining the conditions which are required to get optimum yield of Lovastatin production.

This methodology could therefore be successfully employed to process development where an analysis of effects and interactions of many experimental factors are required. Central composite experimental design maximizes the amount of information that can be obtained, while limiting the numbers of individual experiments required. Response curves are very helpful in visualizing the main effects and interaction of factors. Thus smaller and less time consuming experimental designs could generally suffice for the optimization of many processes. From the above Pareto chart of standardized effects,

it can be seen that nitrogen sources like Casein, Citric acid and Sodium acetate have significant effect on Lovastatin production. Hence, to optimize Lovastatin production, it is necessary to optimize the concentrations of Casein, Citric acid and Sodium acetate whereas other components of the medium can be kept constant. Thus, for the fermentation of Lovastatin, the response surface methodology was found to be a favourable strategy to optimize fermentation medium for Lovastatin production. Being convenient and effective, this method might be useful in optimization of the overproduction of other metabolites as well.

Hence, statistical experimental designs are powerful tools for the rapid search of key factors from a multi-variable system and minimizing the error in determining the effect of parameters and the results are achieved in an economical manner.

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