Research Article

Optimization of Statin Production from *Aspergillus tamarii*

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ABSTRACT

The present investigation was focused on screening of Lovastatin production using different agro-based wastes and augment the lovastatin production by isolating *Aspergillus tamarii* using response surface methodology (RSM) conducting statistical design such as Plackett - Burman design (PB) and Box - Behnken design (BB). Substrates such as rice husk, wheat bran, corn and sorghum bran were initially screened for evaluation of effective lovastatin production using *Aspergillus tamarii* under solid state fermentation (SSF). Among different substrates, wheat bran was the best substrate for lovastatin production proving highest yield of statin was 100 µg/ml. Further with submerged fermentation, the yield of statin was 96 µg/ml when glucose and lactose were used as a substrate. Thus, *Aspergillus tamarii* has the potential to be the source of statin production and can be used in different therapeutic applications.

Key words: *Aspergillus tamarii*, Optimization, Response surface methodology (RSM), Box-Behnken (BB), Statin.

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INTRODUCTION

Statins are class of molecules having polyketide structure produced by secondary fungal metabolism.[1] Statins play a major role in inhibiting HMG - COA reductase, which involve in limiting of cholesterol biosynthesis.[2,3] Treatment with statins are considered safe and viable for avoidance of cardiovascular illness.[4] Lovastatin are categorized as fungal secondary metabolites which are evidenced to be produced by submerged fermentation (SmF) and solid state fermentation.[2] Lovastatin are acquired from different genera and types of filamentous fungi. Few fungal genera including *Aspergillus*, *Penicillium*, *Monascus*, *Paecilomyces*, *Trichoderma*, *Scopolariopsis*, *Doratomyces*, *Phoma*, *Phythium*, *Gymnoascus*, *Hypomyces* and *Pleurotus* are accounted as lovastatin producers.[5,6] Solid state fermentation is to process wide range of agricultural wastes, that could be used for growing fungal species and to minimize the overall value of the product.[7] The application of statistical experimental design techniques in fermentation process development can result in improvement of product yield, reduce process variability, give closer confirmation of the output response to nominal and reduce overall costs. The variables that are found significant in this initial screening can be further optimized using Response Surface Methodology (RSM). RSM has been used extensively in media optimization and is a collection of statistical techniques that uses Design of Experiments (DoE) for building models, evaluating the effect of factors and predicting optimum conditions and is extensively

Applied in the optimization of medium composition, conditions of enzymatic hydrolysis, fermentation and food manufacturing processes. Several experimental design models can be employed to reduce the number of experiments under different conditions. Plackett-Burman and Box - Behnken designs are among the

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most widely used statistical techniques for optimization of biological process.[8] Thus, optimization parameters involves in signifying lower cost of media, accessing the stability of the product, increasing yield and substrate porosity.[9,10] The present study aims to optimize and demonstrate the effect of different factors followed by factor-factor interactions on Lovastatin production by isolated *Aspergillus tamarii* using response surface methodology (RSM) under solid state fermentation and submerged state fermentation.

MATERIALS AND METHODS

Optimization of statin production from *Aspergillus tamarii* using surface response methodology: The spore suspension (1x 10^8 spores/ml) of *Aspergillus tamarii* used as inoculum. Agro-industrial residues such as rice husk, wheat bran, sorghum bran were evaluated for their potential as substrates in solid state fermentation for production of statin. 5g of each substrate was taken and to this trace ion solution supplemented with K_2HPO_4 (1g/l) is added. The spore suspension was inoculated and incubated for 5 days at 35°C. Optimum statin production by various parameters including incubation temperature (25°C- 35°), incubation period (10-18days), pH(3-7), moisture content (25-75ml), inoculum concentration (v/v), carbon sources (w/v), nitrogen sources (w/v) and trace salt supplement (K_2HPO_4).

Plackett- Burman (PB) design: The Plackett-Burman experimental design identifies the critical physio-chemical parameters required for elevated secondary metabolite production by screening 'n' variables in (n+1) experiments. Nine factors such as pH, incubation temperature (°C), fermentation period (days), moisture substrates (g), inoculum concentration (ml), carbon sources (g/l), nitrogen sources (g/l) in culture medium were evaluated along with two dummy factors as shown in (Table 1). Each factor was screened at two levels of high (+1) and low (-1).

Box- Behnken (BB) design: The Box- Behnken statistical screening design was used to statistically optimize the formulation factors, evaluate main effects and interaction of effects. This design was specifically selected since it requires fewer runs than a central composite design in case of three or four variables. A design matrix comprising of 17 experimental runs was constructed. The non-linear computer generated quadratic model is given as

$$Y=b0 + b1x1 + b2x2 + b3x3 + b12x1x2 + b13x1x3 + b23x2x3 + b11x12 + b22x22 + b33x32$$

where,

Y is the measured response associated with each factor level combination;

b0 is an intercept; b1 to b33 are regression coefficients computed from the observed experimental values of Y;

x1, x2 and x3 are the coded levels of independent variables.

The terms x1, x2 and xi2 (i=1, 2, or 3) represent the interaction and quadratic terms, respectively.[11]

A Box- Behnken factorial design was used in further optimization of statin production. The range and the levels of the variables investigated in this study are given in Table 2.[12] A 17 trial design was further used for evaluating the cumulative effect of the positive variables as given in Table 3.

Optimization of media components for submerged fermentation

Medium used for submerged fermentation

A cumulative factors of sugars, nitrogen source, salts and trace elements were mixed to form a universal medium for optimizing the factors of submerged fermentation. The components of the universal media includes glucose 20g; lactose 20g; sodium glutamate

Table 1: Experimental values for the Plackett-Burman design.

Factor	Variable	Units	Experimental Values	
			Low (-1)	High (+1)
1	Temperature	°C	25	35
2	Time	Days	10	18
3	pH		3	7
4	Moisture	%	25	75
5	Trace element	mg/ml	0.5	1.5
6	Carbon Source	G	2	6
7	Nitrogen Source	G	0.25	1
8	Solid Substrate	G	5	15
9	Inoculum Volume	ml	3	7
10	Dummy(1)	-	-1	+1
11	Dummy(2)	-	-1	+1

Table 2: Box - Behnken Variables.

Factor	Variable	Units	Experimental Value		
			-1	0	+1
A	Solid substrate	G	5	10	15
B	Moisture	ml	25	50	75
C	pH	-	3	5	7

12.5g; $KH_2PO_4$5g; K_2HPO_4 5g; $FeSO_4$ 0.2g; $MnSO_4$ 0.1g, $ZnSO_4$ 0.1g; $CaCl_2$ 20mg; $CuCl_2$ 5mg; H_3BO_3 11mg; $(NH_4)_6MO_7O_{24}$ 5mg; distilled water –1000ml;pH 6.5.

Plackett - Burman design

All the factors involved in the components of media with physical factors were involved in the statistical evaluation of each factorial composite in the Plackett - Burman design. Factors such as glucose, lactose, glucose+lactose, temperature, pH, time in days, dipotassium hydrogen phosphate, sodium glutamate, trace element composition and inoculum volume with two dummy

Variables were taken for analysis. Each factor was screened at two levels of high (+1) and low (-1) and given in Table 4.

Variables that impact the yield positively were taken further for Box-Behnken design. The variables are included in Table 5. A 29 trial design was further used for evaluating the cumulative effect of the positive variables as given in Table 6.

Table 4: Experimental variables for PB design for submerged fermentation.

Factor	Variable	Units	Experimental Values	
			Low (-1)	High (+1)
1	Glucose	g	1	3
2	Lactose	g	1	3
3	Glucose+Lactose	g	0.5	1.5
4	Temperature	°C	25	35
5	pH	-	3	7
6	Time	Days	10	18
7	Dipotassium hydrogen phosphate	g	0.2	0.3
8	Sodium glutamate	g	1	1.5
9	Trace element	mg	2	7
10	Inoculum Volume	ml	0.5	1.5
11	Dummy(1)	-	-1	+1
12	Dummy(2)	-	-1	+1

Table 5: Box - Behnken variables for submerged fermentation.

Factor	Variable	Units	Experimental Value		
			-1	0	+1
A	Inoculum Volume	ml	0.5	1	1.5
B	Glucose+Lactose	ml	1.5	2	2.5
C	pH	-	3	5	7
D	Dipotassium hydrogen phosphate	G	0.2	0.25	0.3

Statistical analysis

The data was analyzed using Statistical Package for Social Sciences (SPSS) 17.0. All the data are shown as Mean ± Standard Error Mean (SEM). Mann Whitney U test was performed to find the significance of the test results obtained as the variables were independent and the sample size was less than 10 (n=3). The significance value was considered at two measure of 95% (p<0.05) and 99% (p<0.01) were determined to be statistically significant.

RESULTS

Table 6: Box-Behnken experimental run.

Run	Inoculum Volume	Glucose + Lactose	pH	Dipotassium hydrogen phosphate
1	0	-1	0	1
2	-1	0	0	1
3	0	0	0	0
4	-1	1	0	0
5	1	0	0	1
6	-1	0	1	0
7	0	0	0	0
8	-1	0	0	-1
9	0	0	-1	-1
10	0	-1	0	-1
11	0	1	1	0
12	0	0	1	-1
13	-1	0	-1	0
14	1	-1	0	0
15	0	1	0	1
16	0	1	0	-1
17	0	-1	1	0
18	1	0	1	0
19	0	-1	-1	0
20	0	0	-1	1
21	-1	0	0	-1
22	0	0	0	0
23	0	0	0	0
24	1	1	0	0
25	0	0	0	0
26	0	1	-1	0
27	1	0	-1	0
28	0	0	1	1
29	-1	-1	0	-1

Optimization of fungal statin by solid state fermentation using response surface methodology: To select the best natural substrate for statin production, the substrates such as rice husk, wheat bran, corn and sorghum bran were evaluated and zone of inhibition was calculated from statin obtained from each substrate and compared with standard graph to obtain the yield of statins (Table 7). Rice husk showed no production of statin, Corn and sorghum showed evident production of statin but performed poorly when compared to wheat bran.

The levels of the variables for the PB design were selected according to the previous single-factor experiments. Based on the selection, a 12 - run PB experiment was chosen to pick up the main factors in the fermentative process for the production of statin. According to t-test results obtained (Table 1) solid substrate, pH, moisture content were considered as the three major factors affecting the production and the rest of factor level were below 90% and therefore considered to be insignificant. The BB experiment results were analysed which indicated that the experimental results of BB could be fitted into the final equation of factors as second order regression

$$R1=1.205+0.117 * A + 0.0564 * B + 0.126 * C - 0.00261 * AB - 0.0078 * AC + 0.00232 * BC + 0.00467 * A2 - 0.000032 * B2 - 0.01113 * C2$$

where R1 is the yield of statin obtained and A,B,C are the coded values for solid substrates, moisture and pH respectively (Figure 1).

All the relationships among the three variables are non-linear, although they exhibit a nearly linear relationship of factor B with factors A and C, in the form of almost straight lines. At higher concentrations of statin, these become curvilinear or non-linear. Factors B and C have curvilinear relationship at all levels of the two variables on the response R1. Response surface plots show the relationship between these factors even more clearly.

Optimization of fungal statin by submerged fermentation using response surface methodology

The variables selected for PB design were based on the media components of universal medium that constituted all the essential nutritional factors such as carbon and nitrogen sources, trace elements and other salts. The 10-factors based 12-run design of PB based statistical approach aided in deriving the variables that affect the yield of extracted fungal statin positively. This run was completed using 2 dummy variables to maintain the statistical strength of the PB.

Design. On PB design the factors based on ≥95% confidence level were chosen for further interactive effects by Box-Behnken design. According to the t-test analysis, inoculum volume, pH, glucose+lactose and dipotassium hydrogen phosphate were derived as the variable affecting the production of statin under submerged conditions of fermentation. As the confidence level was below 95% other variables were termed insignificant.

The Model F-value of 2.66 implies the model is significant. There is only a 3.89% chance that a "Model

Table 7: Yield of statin using various solid substrates.

S.No	Substrates	Yield (µg/ml)
1	Rice Husk	--
2	Wheat Bran	85µg/ml
3	Corn	59µg/ml
4	Sorghum Bran	55µg/ml

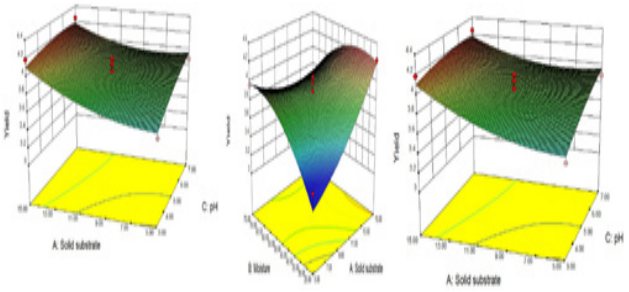

Figure 1: The surface plots of response surface methodology with the effect of solid substrate, moisture and pH for production of statin.

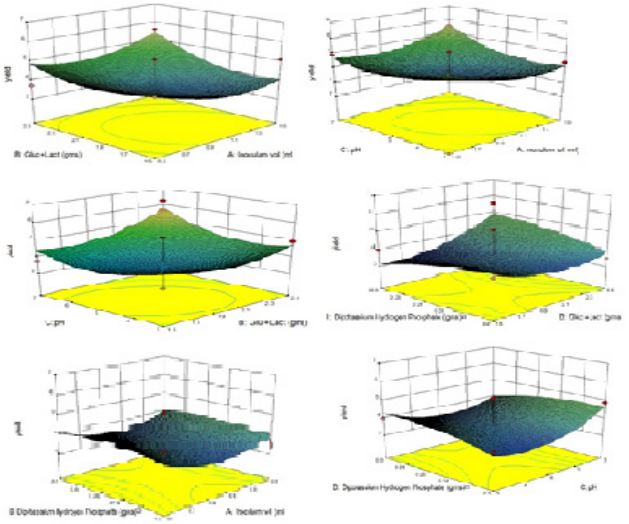

Figure 2: The surface plots of response surface methodology with the effect of inoculum volume, glucose + lactose, pH and dipotassium hydrogen phosphate for production of statin.

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 AC, BC is a significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 1.16 implies the Lack of Fit is not significant relative to the pure error. There is a 48.16% chance that a "Lack of Fit F-value" this large could occur due to noise.

Yield= 4.75-0.059 A+0.25B + 0.123C+ 0.18D -0.26AB+0.666AC+0.5775BC+0.52BD+0.15CD+0.39A^2-0.048B^2-0.377C^2-0.35D^2

All the relationships among the four variables are non-linear, although they exhibit a nearly linear relationship of factor B with factors A, C and D in the form of almost straight lines. At higher concentrations of statin, these become curvilinear or non-linear. Factors A, B, C and D have curvilinear relationship at all levels of the two variables on the response R1. Response surface plots show the relationship between these factors even more clearly (Figure 2).

DISCUSSION

In the present study, statin production was evaluated by natural substrates such as Rice Husk, Wheat Bran, Corn and Sorghum Bran. Further, zone of inhibition was calculated from the

Obtained statin from each substrates and compared with standard graph to obtain the yield of statin. Wheat Bran showed very good yield of about 85 µg/ml; while Corn and Sorghum showed evident production of statin yielding 59 µg/ml and Rice Husk showed no production at all. Using different cheap waste products for lovastatin production. Among the tested substrates, baggase was found to be the most suitable yielding 50 µg/ml lovastatin, followed by strawberry waste yielding 40 µg/ml lovastatin. In our study we quantified the fungal statin by the similar assay and found the highest production of statin (85 µg/ml) was obtained when wheat bran was used as a substrate followed by corn (59 µg/l) and sorghum (55 µg/l).[13] All screened fermented substrate with various fungal strains, wheat bran had the maximum lovastatin yield having 2.84; 2.69; 2.53 and 1.84 mg/g of dried fermented matter (DFM) by *A. fumigatus*; *A. niger*; *Penicillium citrinum* and *Rhizopus oligosporus*.[14] Wheat bran was a suitable susbtrate for lovastatin production. Consequently, sugar cane baggase; olive cake and potato peel also yielded a good amount of lovastatin by all different fungal strains of 2.41, 2.28 and 2.25 mg/g dry fermented matter, by *A. niger* respectively. While, groundnut shells; beet peel; rice hulls and pea pods showed low production of lovastatin 1.94; 1.75; 1.75 and1.13 mg/g dry fermented matter, respectively by *Rhizopus oligosporus*.[15] Studies with the production of lovastatin from *Pleurotusostreatus* and quantification of lovastatin was assessed by performing a bioassay against *Candida albicans* and determining the concentration based on the zone diameter plotted graphically. They observed a maximum yield of 113µg/ml when wheat bran was used as a solid substrate followed by rice

Bran (63µg/ml), rice straw (41µg/ml) and sugarcane (31.1µg/ml).In our study we quantified the production of fungal statin by the similar assay and found the highest production of statin (85µg/ml) was obtained when wheat bran was used as a substrate followed by corn (59µg/ml) and sorghum (55 µg/ml).[16]

Fifteen isolates were screened from soil sample for lovastatin production and got a yield ranging between 16.66mg/L to 133.33mg/L by SmF and between 4.166mg/g by SSF. In our study fungal statin yield was determined to be 100 µg/ml using submerged fermentation.[17] Lovastatin production in mutant strains of *Aspergillus terreus* and tested them against *Candida albicans*. They observed that the second mutagenesis stage gave lovastatin titres (60.3mg/L) higher than that of the parent culture ATCC20543.[18] Further optimization were carried out using surface response methodology conducting statistical design such as Plackett - Burman design (PB) and Box - Behnken design (BB). The media used for the production for the fungal statin using submerged fermentation in our study which included (g/l): glucose-45; mono-hydrate sodium glutamate-12.5; KH_2PO_4-5; K_2HPO_4-5; $FeSO_4 \cdot 7H_2O$-0.2; $MnSO_4 \cdot 4H_2O$-0.1; $ZnSO_4$,$7H_2O$-0.2; $MgSO_4 \cdot 7H_2O$-0.1;$CaCl_2.2H_2O$-0.02;$CuCl_2.2H_2O$-0.005; H_3BO_3-0.011and $(NH4)_6MO_7O_{24}4H_2O$-0.005.[19] The optimum temperature for statin production was determined to be 30°C and the optimum pH was found to be 7 in our study. The same media along with three other different media was also used for statin production in their study using *Aspergillus terreus* isolated from soil samples. They found an optimum production at a temperature of 30°C and at pH 6 and obtained a yield of 471.91mg/L and 409.56mg/L in fermentation broth and mycelial extract respectively.[20]

CONCLUSION

This report predominantly focused on screening of different agro-based wastes under solid state fermentation using isolated fungus *Aspergillus tamarii*. Of all different substrates, wheat bran showed highest yield of statin. Further with submerged fermentation, the yield of statin were highest having glucose and lactose

as substrate. Thus, *Aspergillus tamarii* can used as source of statin production and further used in the applications of therapeutics.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

SSF: Solid state fermentation; **SmF:** Submerged fermentation; **RSM:** Response surface methodology; **PB:** Placket Burmann; **BB:** Box - Behnken.

SUMMARY

The isolate *Aspergillus tamarii* being a natural statin producer can be used as an alternative for the chemically synthesized statin, thereby reducing risk of side effects and production cost.

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