Optimization of Single-step Pravastatin Production by *Penicillium brefeldianum* ESF21P through Response Surface Methodology

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Abstract

The aim of the present study was to optimize the fermentation process for enhanced production of pravastatin by a wild-type Penicillium brefeldianum ESF21P strain using statistical approaches. Initial screening of significant variables influencing pravastatin production was carried out using 2^{7-3} fractional factorial design. The seven variables involved in this study were slant age, spore concentration, inoculum volume, fermentation time, temperature, initial pH of the medium and agitation rate. Amongst these, slant age, fermentation time, initial pH of the medium and agitation rate had significant influences on pravastatin accumulation. These four variables were further optimized using the central composite rotatable design of response surface methodology.

The analysis revealed that the optimal values of the selected variables were a slant age of 5.95 days, fermentation time of 11.87 days, initial pH of the medium of 6.13 and agitation rate of 211.31 rpm (rounded to 210 rpm). These optimized conditions resulted in a maximum level of pravastatin accumulation (234.36 mg/L), approaching the value predicted by the model of 251.19 mg/L. This study confirmed that statistical approaches can be successfully applied as practical tools in improving single-step production of pravastatin by Penicillium brefeldianum ESF21P.

Keywords: Central composite rotatable design, Fractional factorial design, Lipid-lowering agent, Submerged fermentation.

Introduction

Nature produces an amazing variety of natural products. A large number of pharmaceutically active secondary metabolites produced by microorganisms have already been characterized[7]. Nevertheless, new bioactive products from bacteria, actinomycetes and filamentous fungi continue to be discovered at an accelerating rate. Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, which is the regulatory and rate-limiting enzyme of the cholesterol biosynthetic pathway. They are considered to be a class of extremely successful drugs that lower the cholesterol level in the blood, decreasing the risk of heart attack or stroke[13].

As highlighted in various excellent reviews, in addition to their important hypocholesterolemic effects, statins have many other potentially useful biological features including antithrombotic properties, neuroprotective effects, antihypertensive properties, immunomodulatory effects, antioxidant effects, anti-cancer properties, beneficial effects on bone formation and mineral density, positive effects both on glucose metabolism and insulin sensitivity and suppressive effects on the formation of cholesterol gallstones and crystals in the human gallbladder[2, 11, 19, 24]. Therefore, research on the production of statins has accelerated in recent decades.

Based on their origin, currently-known statins can be divided into natural, semi-synthetic and synthetic groups. Pravastatin is one of the statins of well-proven effectiveness and safety, which was approved by the U.S. Food and Drug Administration under the marketing name Pravachol[24]. As reported by Koga et al[9], amongst the natural statins produced by microorganisms, a significant advantage of pravastatin is that it exhibits stronger and highly tissue-selective inhibition of cholesterol biosynthesis in the liver. Another advantage is that, amongst the commercially available statins, it has the lowest potential for drug interactions, for example, with antibiotics[15].

In the biotechnological industry, pravastatin is currently manufactured in a two-step production process[19, 24]. First, compactin (also called ML-236B or mevastatin) is produced by *Penicillium* strains followed by biotransformation to pravastatin. This bioconversion can be achieved using a number of microorganisms from different genera such as *Streptomyces*, *Actinomadura*, *Nocardia*, *Bacillus* and others. Although this two-step production process of pravastatin is currently considered efficient, it faces a number of limitations.

The development of a one-step fermentation process for the production of this natural hypocholesterolemic agent is based on an active microbial culture that can synthesize it directly and could therefore provide a more attractive biotechnological approach from both engineering and

economic points of view. Therefore, the search for new strains of microorganisms which are able to accumulate this natural statin directly, has gained considerable momentum.

Previously, using a bioprospecting approach, various pravastatin-producing strains of *Penicillium* were isolated by our research group from little-explored tropical ecosystems in Pahang State, Peninsular Malaysia[20, 22, 23]. Amongst them, the wild-type strain *Penicillium brefeldianum* ESF21P, isolated from oil palm plantation soil, was distinguished by its relatively high capacity to produce pravastatin directly under submerged fermentation in a shake-flask system[22]. However, wild-type microbial isolates generally produce biotechnologically important metabolites in very low concentrations and therefore, further work is required to increase their productivity.

It is well known that the fermentation medium plays a vital role in carrying out the successful biotechnological process. Therefore, after our initial screening program, we investigated single-step pravastatin production by *P. brefeldianum* ESF21P in media of different compositions[21]. The highest level of pravastatin produced (198.65 mg/L) was achieved using the fermentation medium reported by Kónya et al.[10]

From the literature, it is known that increased yields of target microbial metabolites may also be achieved by optimizing the fermentation variables[6]. The well-known one-factor-at-a-time approach for the optimization of fermentation variables involves assessing one variable at a time while keeping the others at fixed levels. Being single-dimensional, it is a laborious and time-consuming method, which often does not guarantee the determination of optimal conditions because it is unable to predict the response under untested values of variables and does not include the possibility of two-way interactions amongst the factors.

Hence, statistical methods provide a better platform for optimization purposes as they offer the possibility of the simultaneous study of many factors. Response surface methodology (RSM) is a collection of both statistical and mathematical techniques that is helpful in defining the effects and interactions of many pre-cultural and fermentation factors together that play significant roles in biotechnological processes. This facilitates the prediction of responses beyond the tested ranges of these factors.

Previously, the RSM approach has been successfully utilized to optimize different components of the medium and fermentation variables for enhanced production of natural statins such as lovastatin and compactin under submerged and solid-state fermentation processes[3-5,16-18]. However, to date, similar studies on the optimization of direct pravastatin production have not been reported. The efficiency of single-step pravastatin production through microbial fermentation, therefore, has clear potential for improvement. To provide conditions that would fully exploit the increased potential of the pravastatin-producing *P. brefeldianum* ESF21P strain selected during our initial screening program, the fermentation variables require optimization.

Previously, the method of 2^{7-3} fractional factorial design (FFD) was successfully applied by our research group to evaluate the effects of seven variables on pravastatin production by this fungal strain in a shake-flask system[25]. In the present study, the fermentation parameters that had significant effects, were optimized further using central composite rotatable design (CCRD) which is one of the response surface methodologies.

Material and Methods

Fungal producer of pravastatin: The fungal strain, *P. brefeldianum* ESF21P, was isolated from peat soil collected from a 15-year-old oil palm plantation in Gambang (Pahang State, Peninsular Malaysia). This microbial strain was selected during our initial screening program as the best pravastatin producer amongst the fungal isolates tested[22].

Inoculum preparation: A spore suspension was prepared by suspending fungal spores from the appropriate PDA slant in 10 mL of sterilized distilled water containing 0.01% (v/v) tween-80. The number of fungal spores was counted using a hemocytometer after vortexing and appropriate dilution.

Submerged fermentation procedure: A spore suspension of appropriate density was added as inoculum in accordance with the specifications of the experiments. The selected fermentation medium used for direct pravastatin production was composed of 3% (w/v) glucose, 3% (w/v) glycerol, 0.4% (w/v) peptone, 0.2% (w/v) $NaNO_3$ and 0.1% (w/v) $MgSO_4 \cdot 7H_2O$[10, 21]. Before sterilization using a Hiclave HVE-50 autoclave (Hirayama, Japan), the initial pH of the fermentation medium was adjusted to the desired value using 10% HCl or 10% NaOH. The controlled parameters of temperature and agitation rate were set using the control panel of the Ecotron microbiological incubator (Infors HT, Switzerland). The shake-flask fermentations with medium final total volumes of 50 mL were carried out in 250-mL conical flasks at conditions as specified in the design of the experiments.

Pravastatin determination: Ethyl acetate extracts from the whole-cell broths were obtained according to the slightly modified extraction procedure described by Manzoni et al[12] and then used for HPLC analysis of pravastatin (Agilent 1200, Agilent Technologies, USA). The protocol applied was as follows: first, the pH of the whole-cell broth was adjusted to 3±0.2 with 1N trifluoroacetic acid and then an equal volume of ethyl acetate was added. The extraction procedure was performed in an Ecotron incubator shaker (Infors HT, Switzerland) at 200 rpm and 30°C for 1 h. After extraction, the fermentation samples were filtered through a Whatmann quantitative filter paper (grade 41) and the organic phase of each sample was collected. The ethyl acetate filtrates were then dried over anhydrous Na_2SO_4

followed again by filtration from the drying agent and concentrated using a rotary evaporator to give a final volume of 4 mL.

After that, 5 µL from the organic phase was injected for HPLC analysis on a 250×4.6 mm ID Zorbax Eclipse Plus C18 column, 5 µm particle size (Agilent Technologies, USA). The mobile phase used for HPLC analysis consisted of acetonitrile and water (60:40, v/v) with pH adjusted to pH 3±0.2 by the addition of 1N H_3PO_4 [10, 12]. The flow rate was maintained at 0.8 mL/min and absorbance was measured at 238 nm. HPLC grade pravastatin (≥ 98% purity) was used as an external standard.

Design of the experiments: For the first step of the optimization study, the method of 2^{7-3} fractional factorial experiments with seven variables was performed to screen the significant variables affecting pravastatin production as described previously[25]. This method was chosen in order to reduce the number of experimental runs required. The number of variables involved is seven and the method of the full 2^7 factorial experiments would involve 128 experiments. A total of 16 sets of experiments were employed in this study. The variables studied were: slant age (*A*), spore concentration (*B*), inoculum volume (*C*), fermentation time (*D*), temperature (*E*), initial pH of the medium (*F*) and agitation rate (*G*). The range for each variable was selected from the data available in the literature based on the maximum production of compactin, which is the biochemical precursor of pravastatin, by different *Penicillium* strains[3,4,18].

To avoid bias, all experimental runs were performed in a random order (overall randomization). The response variable in these screening experiments was the concentration of pravastatin produced by *P. brefeldianum* ESF21P (in mg/L). The Design-Expert 6.0.8 statistical software package (Stat-Ease Inc., Minneapolis, USA) was used to analyze the experimental data. Based on the results obtained from 2^{7-3} fractional factorial experiments, the correct experimental range in terms of the significant variables was selected for the second step of optimization using CCRD.

In this second part of the study, the production of pravastatin by *P. brefeldianum* ESF21P was studied under the influence of the four variables identified as having significant effects: slant age (*A*), fermentation time (*D*), initial pH of the medium (*F*) and agitation rate (*G*). A total of 30 experimental runs were established based on computer simulation. The data obtained were then fitted by regression to a quadratic model that describes the pravastatin yield (*Y*) as the dependent variable in terms of the selected significant variables. The relative effects of the variables on pravastatin production were identified using response surface plots. The optimal values of the variables giving maximum production of pravastatin were determined based on the software suggested solution.

Statistical analysis: All experiments were conducted in triplicate to ensure the reproducibility of the results. The mean value of the pravastatin concentrations from each set of triplicates is given as the yield (*Y*), which is the dependent variable or response. For CCRD, the statistical software package Design-Expert 6.0.8 (Stat-Ease Inc., Minneapolis, USA) was used for the design of the experiments and regression analyses of the experimental data as well as to plot three-dimensional (3D) response surface plots.

Results and Discussion

Screening of important variables using factorial analysis: Natural statins, including pravastatin, are produced commercially by microbial fermentation. The synthesis of these compounds by wild-type fungal cultures is such that their production levels are usually quite low. Therefore, microbiologists and biotechnologists are challenged to identify suitable cultivation conditions in order to improve the productivity of the microbial producers of statins. From the literature, it is known that factorial designs are widely used in experiments involving several variables where it is necessary to investigate the joint effects of the variables on a response. The well-known FFD is a subset of full factorial design. A major application of fractional factorials is in screening experiments in which many variables are considered with the aim of identifying those that have significant effects[14].

The present research work aimed to optimize the direct production of pravastatin by a recently reported wild-type fungal strain isolated from oil palm plantation soil and identified as *P. brefeldianum* ESF21P[22]. For this, two statistical approaches were applied. For the first step of the optimization study, to evaluate the influence of the seven tested variables on single-step pravastatin production, the method of 2^{7-3} fractional factorial experiments was used. The goal of these screening experiments was the selection of the key variables influencing pravastatin production by this strain on the basis of their main and interactive effects. The seven variables studied were slant age (*A*), spore concentration (*B*), inoculum volume (*C*), fermentation time (*D*), temperature (*E*), initial pH of the medium (*F*) and agitation rate (*G*).

The matrix of the levels of variables corresponding to the experimental design together with the observed responses was reported by our research group earlier[25]. The pravastatin concentration data were fitted as a function of the experimental variables using Design-Expert 6.0.8 software in a reduced 3-factor interaction (3FI) factorial model by regression. Based on the experimental data obtained from 2^{7-3} fractional factorial experiments, the following equation for pravastatin production was derived:

$$\text{Pravastatin concentration (mg/L)} = 9.08 - 2.37A + 1.93D - 2.17F - 3.53G + 1.41AB - 2.04AC + 1.41AD - 2.33AE - 1.19AF + 2.16AG + 1.45ABD \quad (1)$$

where *A* is slant age, *B* is spore concentration, *C* is inoculum volume, *D* is fermentation time, *E* is temperature, *F* is initial pH of the medium and *G* is agitation rate.

From the experimental data obtained, the percentage contribution of the main effect of each variable, as well as the percentage contribution of the interactive effects between variables, were calculated by the Design-Expert 6.0.8 software. It is noteworthy that the influence of the tested parameters on the direct production of pravastatin by *P. brefeldianum* ESF21P based on the percentage contribution of the main effects declined in the following order: agitation rate (25.66 %), slant age (11.56 %), initial pH of the medium (9.72 %), fermentation time (7.69 %), temperature (0.19 %), inoculum volume (0.048 %) and spore concentration (0.015 %). The results of the initial screening demonstrated that among the seven tested variables, only agitation rate (*G*), slant age (*A*), initial pH of the medium (*F*) and fermentation time (*D*) contributed significantly to direct pravastatin production and therefore were selected for further optimization.

Moreover, based on their percentage contribution, it was found that the interactions between slant age and temperature (11.18%), slant age and agitation rate (9.59 %), as well as slant age and inoculum volume (8.62 %), also had a considerable influence on direct pravastatin biosynthesis by *P. brefeldianum* ESF21P under submerged fermentation. It should be noted here that amongst the analyzed interactive effects, the interaction between slant age and initial pH of the medium had the lowest percentage contribution (2.91 %).

Although identifying the significant variables is important, these variables need to be incorporated for optimal production of pravastatin at the correct levels.

Table 1
Results of the central composite rotatable design after square root transformation

Run	Block	*A*: SA	*D*: FT	*F*: pH	*G*: AR	Square root (pravastatin, mg/L)	
						Actual	Predicted
1	1	7(+1)	10(-1)	5.5(-1)	195(-1)	3.66	3.00
2	1	7(+1)	14(+1)	5.5(-1)	195(-1)	2.21	3.25
3	1	7(+1)	10(-1)	5.5(-1)	225(+1)	2.50	3.41
4	1	7(+1)	14(+1)	5.5(-1)	225(+1)	4.23	4.97
5	1	5(-1)	10(-1)	5.5(-1)	225(+1)	3.92	3.56
6	1	5(-1)	14(+1)	6.5(+1)	225(+1)	5.82	6.62
7	1	6(0)	12(0)	6.0(0)	210(0)	16.99	14.34
8	1	6(0)	12(0)	6.0(0)	210(0)	15.55	14.34
9	1	5(-1)	14(+1)	5.5(-1)	225(+1)	6.72	5.97
10	1	7(+1)	10(-1)	6.5(+1)	195(-1)	5.33	6.72
11	1	7(+1)	14(+1)	6.5(+1)	225(+1)	5.95	5.74
12	1	6(0)	12(0)	6.0(0)	210(0)	15.15	14.34
13	1	6(0)	12(0)	6.0(0)	210(0)	12.76	14.34
14	1	5(-1)	10(-1)	6.5(+1)	195(-1)	7.20	6.60
15	1	5(-1)	14(+1)	6.5(+1)	195(-1)	4.91	4.64
16	1	5(-1)	10(-1)	6.5(+1)	225(+1)	7.68	7.28
17	1	7(+1)	14(+1)	6.5(+1)	195(-1)	3.42	3.92
18	1	7(+1)	10(-1)	6.5(+1)	225(+1)	8.08	7.24
19	1	5(-1)	10(-1)	5.5(-1)	195(-1)	2.15	2.99
20	1	5(-1)	14(+1)	5.5(-1)	195(-1)	3.12	4.10
21	2	6(0)	12(0)	6.0(0)	210(0)	15.59	17.01
22	2	6(0)	12(0)	7.0(+2)	210(0)	10.13	10.34
23	2	8(+2)	12(0)	6.0(0)	210(0)	10.07	9.02
24	2	6(0)	12(0)	6.0(0)	210(0)	15.32	17.01
25	2	4(-2)	12(0)	6.0(0)	210(0)	9.63	9.90
26	2	6(0)	8(-2)	6.0(0)	210(0)	9.33	9.57
27	2	6(0)	16(+2)	6.0(0)	210(0)	10.20	9.18
28	2	6(0)	12(0)	6.0(0)	180(-2)	3.71	2.50
29	2	6(0)	12(0)	5.0(-2)	210(0)	6.95	5.97
30	2	6(0)	12(0)	6.0(0)	240(+2)	4.46	4.90

[a]Coded levels of the independent variables are given in the brackets.
[b]SA - slant age; FT - fermentation time; pH - initial pH of the medium; AR - agitation rate.
[c]Results of pravastatin concentrations are presented as the mean of three replicates.

Therefore, before further optimization using RSM, it is a general practice to revise the range for each significant variable to locate the curvature. From the factorial model derived (Eq. 1) as well as from ramps of pre-optimization (data not shown), it was found that lower values of slant age, initial pH of the medium and agitation rate together with a higher value of fermentation time were positive influences on direct pravastatin production by *P. brefeldianum* ESF21P. Therefore, the new slant age range chosen was 4-8 days with an initial pH of the medium of 5.0-7.0 while the agitation rate was in the range of 180-240 rpm.

However, according to our preliminary experimental results on the time course of pravastatin accumulation (data not shown), the fermentation time should be retained in its initial range of 8-16 days as pravastatin production considerably decreased after 16 days of fermentation.

Improvement of pravastatin production using central composite rotatable design: The 2^{7-3} FFD used in this study is a preliminary technique for a rapid illustration of the main and interactive effects of various variables on single-step production of pravastatin by *P. brefeldianum* ESF21P. The main limitation of this statistical approach is that it tests each factor at two levels only and cannot give a precise estimate of the optimum level of the factor. Therefore, further optimization of the selected variables for direct pravastatin production is necessary. In most cases, the variables screened by FFD are optimized further by RSM. It is known that RSM has several advantages that include suitability for multiple factor experiments, reduction in experiment numbers required, identification of interactions between factors, finding the most suitable conditions and forecasting response[14]. Therefore, in our study, after conducting fractional factorial analysis, the design was further expanded to a CCRD, which is one of the designs in RSM. To develop a second-order approximation to the response surface, a CCRD with five coded levels was applied.

To find the optimum conditions for all significant variables, a full CCRD with a total of 30 sets of experiments was used. However, the numerical analysis of the obtained responses and the diagnosis of residuals revealed some deviation from normality (data not shown). From the literature, it is known that data transformations can successfully be applied when residual analysis indicates problems with the underlying model assumptions[14]. Therefore, to improve the fit of the model to the data, square root transformation was suggested by the Design-Expert 6.0.8 software. Experimental and simulated data after square root transformation are shown in table 1.

By applying multiple regression analysis to the obtained experimental data after square root transformation, the experimental results of the CCRD were fitted with a second-order polynomial model. The following polynomial equation was derived to represent the square root (Sqrt) of pravastatin concentration (Y) as a function of the variables tested:

$$\text{Sqrt}\ (Y) = 15.67-0.22A-0.10D+1.09F+0.60G-0.21AD+0.03AF-0.04AG-0.76DF+0.33DG+0.03FG-1.89A^2-1.91D^2-2.21F^2-3.33G^2 \quad (2)$$

where A is slant age, D is fermentation time, F is initial pH of the medium and G is agitation rate.

The goodness of fit of the model and the significance of each variable were estimated by ANOVA (Table 2). ANOVA confirmed that the quadratic model was significant. Moreover, the high R^2 value (94.33%) obtained here supports the applicability of the model using the range of variables included.

Table 2
Analysis of variance (ANOVA) for central composite rotatable design

Source	Mean square	*F*-value	*p*-value (Probability > *F*)
Model	36.65	16.63	<0.0001
Slant age (*A*)	1.15	0.52	0.4822
Fermentation time (*D*)	0.24	0.11	0.7474
Initial pH of the medium (*F*)	28.69	13.02	0.0029
Agitation rate (*G*)	8.62	3.91	0.0680
AD	0.72	0.32	0.5780
AF	0.015	6.772E-003	0.9356
AG	0.024	0.011	0.9177
DF	9.36	4.25	0.0584
DG	1.70	0.77	0.3951
FG	0.012	5.510E-003	0.9419
A^2	97.68	44.32	<0.0001
D^2	99.86	45.30	<0.0001
F^2	134.43	60.99	<0.0001
G^2	303.56	137.73	<0.0001
Lack of fit	2.16	0.93	0.5793

The model recorded an adequate precision of 13.379 which indicated an adequate signal (response), meaning that this model can be used to navigate the design space.

Response surface plots: To investigate the relative effects of the important variables on single-step pravastatin production by *P. brefeldianum* ESF21P, three-dimensional (3D) response surface curves were constructed (Fig. 1) based on eq. 2. The response surface plots represent the interactions between pairs of variables while keeping the other two variables constant. From these plots, the trend of direct pravastatin production under the experimental conditions could be clearly observed.

In fig. 1A, the response showed a non-linear effect with increasing slant age and fermentation time and pravastatin concentration reached maximum values at 6 days and 12 days respectively. Bailey and Bhatia[1] reported that the physiological state of the inoculum when it is transferred to the production stage can have a major effect on the performance of the fermentation. In terms of fermentation time, Manzoni et al[12] indicated that the highest yield of pravastatin (53 mg/L) achieved by *Monascus paxii* AM12M mutant strain reached in 21 days. In the case of *Aspergillus terreus* BST strain, pravastatin production showed the same kinetic profile, the yield at 14 days being 118 mg/L and increasing to 127 mg/L at 21 days. In our study, the optimal fermentation time required to reach maximum accumulation of pravastatin by *P. brefeldianum* ESF21P was shorter (12 days) which is beneficial in terms of process cost.

From fig. 1B, it can be seen that increase in the value of the initial pH of the fermentation medium led to enhanced pravastatin production, but at a pH higher than 6.0, the production decreased. From the literature, it is known that many microbial producers react to a change in the pH of the external medium in a manner directed to the maintenance of a relatively constant intracellular pH[1].

Referring to fig. 1C, the agitation rate should be maintained at 210 rpm, while slant age should be retained at 6 days to obtain the highest level of pravastatin. The results obtained showed that agitation rates lower or greater than 210 rpm led to a decrease in product accumulation. This may be related to the influence of the agitation speed on the morphological form of the fungal producer in submerged culture and in turn, by the consequential effect on maintaining adequate oxygen supply during the fermentation process. These observations are consistent with previous studies[1, 8].

Figure 1D shows that the optimum region for pravastatin accumulation was achieved at an initial medium pH value of 6.0 and with a fermentation time of 12 days. As illustrated in fig. 1E, an increase in fermentation time along with agitation rate enhanced pravastatin production which reached the highest level at 12 days and 210 rpm respectively. From fig. 1F, it can be seen that the response showed a non-linear effect with the maximum amount of pravastatin produced at an initial medium pH of 6.0 and agitation rate of 210 rpm.

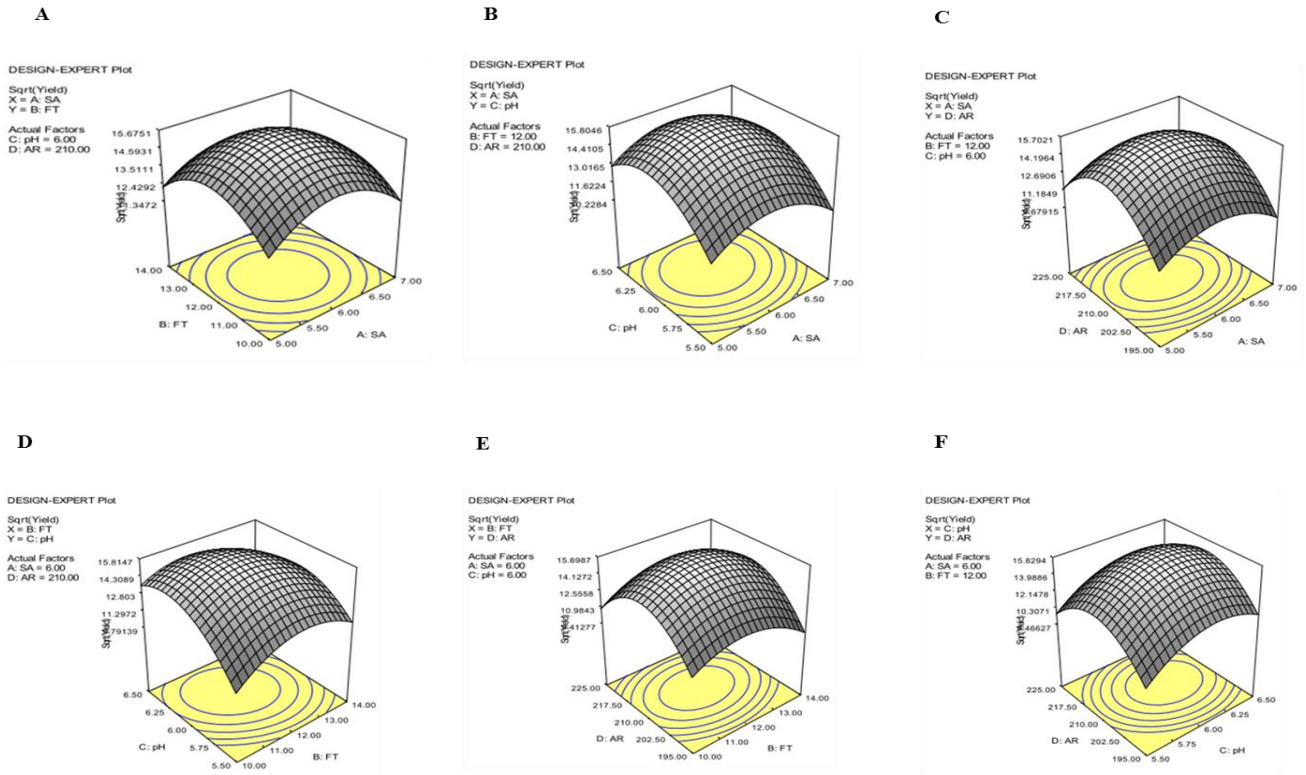

Figure 1: Response surface plots from model equation showing relative effects of significant variables on single-step production of pravastatin by *Penicillium brefeldianum* ESF21P (SA - slant age; FT - fermentation time; pH - initial pH of the medium; AR - agitation rate)

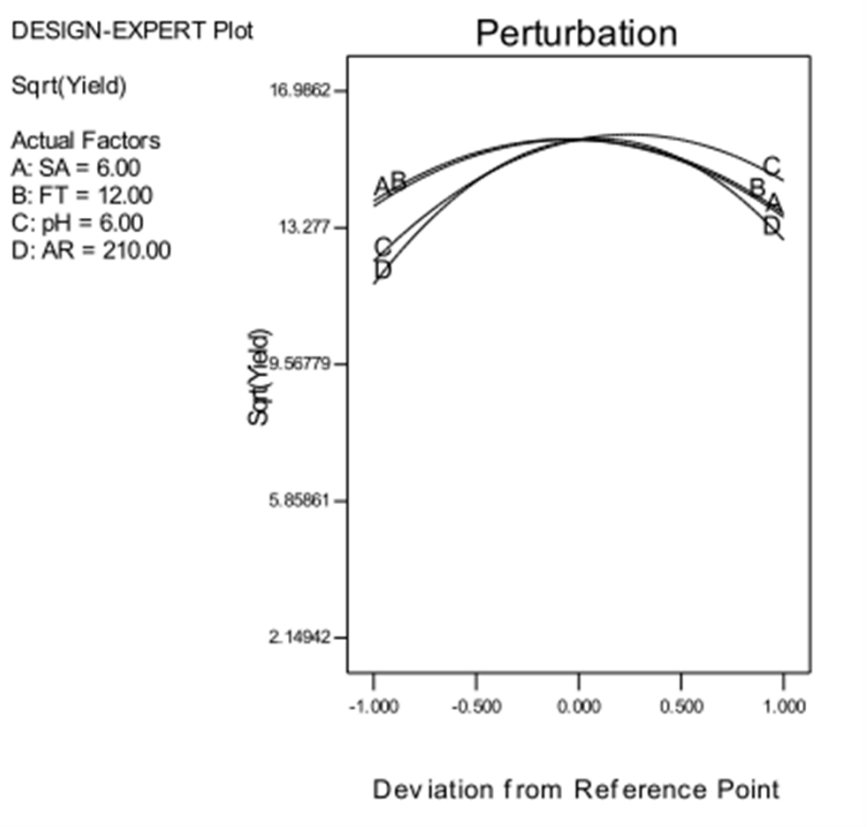

Figure 2: Perturbation plot showing the influence of the four identified significant variables on direct production of pravastatin by *Penicillium brefeldianum* ESF21P (SA - slant age; FT - fermentation time; pH - initial pH of the medium; AR - agitation rate)

The effects of all important variables on direct pravastatin production can be compared with the help of a perturbation plot (Fig. 2). The lines in this figure represent the influence and sensitivity of each variable on single-step pravastatin production by *P. brefeldianum* ESF21P. Each of the four significant variables studied had a relatively important effect on pravastatin accumulation as this variable changed from the reference point.

Model validation study: After optimization using CCRD, it is always necessary to examine the fitted model in order to ensure that it provides an adequate approximation to the true system[14]. Model validation was performed based on the software suggested optimal values: slant age of 5.95 days, fermentation time of 11.87 days, initial pH of the medium of 6.13 and agitation rate of 211.31 rpm (rounded to 210 rpm). The validation study resulted in a pravastatin concentration of 234.36 mg/L, compared with the response predicted by the software at 251.19 mg/L. The close similarity between predicted and experimental values indicated that the model had been validated successfully.

Conclusion

Pravastatin is an effective hypocholesterolemic agent in the statin family. This study used statistical optimization to improve single-step pravastatin production by the wild-type strain *P. brefeldianum* ESF21P isolated from oil palm plantation soil. Four out of seven variables studied by 2^{7-3} FFD significantly influenced the direct production of pravastatin by this fungal strain in a shake-flask system. Therefore, these key variables were optimized further using CCRD. The software determined optimum levels of the significant variables to be slant age of 5.95 days, fermentation time of 11.87 days, initial pH of the fermentation medium of 6.13 and agitation rate of 211.31 rpm (rounded to 210 rpm). A shake flask study using the optimized values of all important variables resulted in the production of 234.36 mg/L pravastatin which is 93.3 % of the model-predicted value under optimized conditions.

The combination of factorial experiments with CCRD for statistical optimization of the bioprocess variables for single-step production of pravastatin by *P. brefeldianum* ESF21P under submerged fermentation proved to be an effective and reliable tool to select the statistically significant variables and identify their optimal values. The selected experimental designs helped to limit the number of individual experiments required, saving time, materials and costs while increasing the reliability and the efficiency of the experiments.

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