Screening of significant factors affecting pravastatin production by *Penicillium* sp. ESF21P

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Screening of significant factors affecting pravastatin production by *Penicillium* sp. ESF21P

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Abstract. Pravastatin is a clinically useful cholesterol-lowering agent. The development of a one-step fermentation process using pravastatin-producing microfungi may be an attractive approach from an economic point of view. To facilitate this, previously 54 fungal cultures were isolated from soil samples. Among them, *Penicillium* sp. ESF21P was the most active pravastatin producer (196.83 mg/L). The objective of the present study is to determine significant factors affecting pravastatin production by *Penicillium* sp. ESF21P. The method of the 2^{7-3} fractional factorial design with seven variables was performed using Design-Expert 6.0.8 software package. The seven factors studied were slant age, spore concentration, inoculum volume, fermentation time, temperature, initial pH of the medium, and agitation rate. The results obtained confirmed that the factorial model was significant. Amongst the tested factors, only four were important: agitation rate, slant age, initial pH of the medium, and fermentation time with a percentage contribution of 25.66%, 11.56%, 9.72%, and 7.69%, respectively. These significant factors will be optimized further using response surface methodology.

1. Introduction

According to the World Health Organization (WHO), cardiovascular diseases are considered as a leading cause of death both in developed and some developing countries. Pravastatin selectively inhibits 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the regulatory enzyme in cholesterol biosynthesis. Currently, large-scale production of this statin is based on a two-step fermentation process: the initial production of compactin and its microbial hydroxylation to pravastatin [1, 2, 3]. The development of a one-step fermentation process for production of pravastatin could be a more attractive approach.

Previously, our research group examined different fungal strains for lovastatin production [4, 5]. Later, to facilitate the development of a fermentation process for direct production of pravastatin, a number of fungal cultures isolated from both uncultivated and cultivated soils in Pahang State (Malaysia) were investigated for pravastatin production [6, 7]. In the present study, among 54 fungal isolates screened earlier, *Penicillium* sp. ESF21P was the most active pravastatin producer (196.83 mg/L). In order for a potential biotechnological product to become a commercial reality, screening of significant factors affecting its production process is very important. The objective of this study is to determine the significant factors affecting pravastatin production by *Penicillium* sp. ESF21P using a

two-level factorial method. The seven factors investigated in this study are slant age, spore concentration, inoculum volume, fermentation time, temperature, initial pH of the medium, and agitation rate.

2. Materials and Methods

2.1 Fungal culture

The fungal strain, *Penicillium* sp. ESF21P, isolated from a soil sample collected from oil palm plantation in Yayasan Pahang Plantation Holding Sdn Bhd (Gambang, Pahang State, Malaysia), was used in this study. This strain was selected during screening as the best pravastatin producer amongst the fungal isolates tested.

2.2 Experimental setup for two-level factorial analysis

For the first step of the optimization study, the method of the 2^{7-3} fractional factorial experiments with seven variables, involving a total of 16 experiments, was performed. The seven factors 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). These variables and their lower and upper levels were chosen based on the previous literature data [8-10]. All runs were performed in a random order (overall randomization). The response variable in these screening experiments was the concentration of pravastatin produced by *Penicillium* sp. ESF21P (in mg/L). The Design-Expert 6.0.8 software package (Stat-Ease Inc., USA) was used to analyze the experimental data.

2.3 Fermentation procedure

A spore suspension was prepared by suspending spores from the slant of appropriate age (6-12 d) in 10 mL of sterile distilled water containing 0.01% (v/v) Tween-80. Then, a spore suspension of appropriate value (10^4-10^8 spores/mL) was added as inoculum, in accordance with the specification in experimental design, into sterile medium reported by Konya et al. [11]: 3% (w/v) glucose, 3% (w/v) glycerol, 0.4% (w/v) peptone, 0.2% (w/v) $NaNO_3$, 0.1% (w/v) $MgSO_4 \cdot 7H_2O$. During the cultivation, pH was not controlled but the initial pH of the medium was set at different values (6.0-8.0) using 10% HCl or 10% NaOH before sterilization. The temperature and agitation rate were set using the control panel of the Ecotron microbiological incubator (Infors HT, Switzerland). The fermentations with medium total volumes of 50 mL were carried out in 250-mL conical flasks at conditions as specified in the experimental design.

2.4 Analytical determination of pravastatin

Ethyl acetate extracts from the whole cell broths obtained according to the extraction procedure of Manzoni et al. [12] were used for HPLC analysis of pravastatin (Agilent 1200; Agilent Technologies, USA). HPLC grade pravastatin (≥ 98% purity, Sigma, USA) was used as a standard.

2.5 Statistical analysis

All the experiments were conducted in triplicate to ensure reproducibility of the results. An analysis of variance (ANOVA) was performed using Design-Expert 6.0.8 software. The percentage contribution was considered in determining the main variables that have a significant influence on pravastatin production (defined as the percentage contribution of a given variable's effect being ≥ 5%).

3. Results and Discussion

3.1 Effects of factors on pravastatin accumulation

The goals of the current screening experiments were the investigations of the variables under study that influencing pravastatin production and selection of the important ones on the basis of their effects. Based on literature data, seven factors were chosen as most likely to influence fungal growth and statin production [8-10]. The design comprised of 16 runs without center points, which is acceptable. The levels of variables corresponding to the design of the 2^{7-3} fractional factorial experiments together

with the observed response are listed in Table 1. The maximum pravastatin production was found in experimental run 5 and the minimum in experimental run 12.

Table 1. Experimental design table for a two-level factorial method.

Run	A: SA (d)	B: SC (spores/ mL)	C: IV (%)	D: FT (d)	E: T (°C)	F: pH	G: AR (rpm)	Pravastatin concentration (mg/L)	
								Actual	Predicted
1	12(+1)	10^4(-1)	3(-1)	16(+1)	29(+1)	8(+1)	210(-1)	4.92	4.91
2	12(+1)	10^4(-1)	5(+1)	16(+1)	25(-1)	6(-1)	260(+1)	8.55	9.45
3	6(-1)	10^8(+1)	3(-1)	16(+1)	29(+1)	6(-1)	260(+1)	5.59	4.69
4	12(+1)	10^8(+1)	3(-1)	16(+1)	25(-1)	6(-1)	210(-1)	22.31	22.02
5	6(-1)	10^4(-1)	5(+1)	16(+1)	29(+1)	6(-1)	210(-1)	25.58	25.87
6	6(-1)	10^4(-1)	3(-1)	16(+1)	25(-1)	8(+1)	260(+1)	3.20	3.80
7	6(-1)	10^8(+1)	3(-1)	8(-1)	29(+1)	8(+1)	210(-1)	16.12	15.94
8	6(-1)	10^4(-1)	5(+1)	8(-1)	29(+1)	8(+1)	260(+1)	9.02	8.59
9	6(-1)	10^4(-1)	3(-1)	8(-1)	25(-1)	6(-1)	210(-1)	13.30	13.17
10	6(-1)	10^8(+1)	5(+1)	16(+1)	25(-1)	8(+1)	210(-1)	13.51	13.52
11	6(-1)	10^8(+1)	5(+1)	8(-1)	25(-1)	6(-1)	260(+1)	5.24	5.97
12	12(+1)	10^4(-1)	5(+1)	8(-1)	25(-1)	8(+1)	210(-1)	1.53	1.71
13	12(+1)	10^8(+1)	3(-1)	8(-1)	25(-1)	8(+1)	260(+1)	2.55	2.98
14	12(+1)	10^4(-1)	3(-1)	8(-1)	29(+1)	6(-1)	260(+1)	5.85	5.12
15	12(+1)	10^8(+1)	5(+1)	8(-1)	29(+1)	6(-1)	210(-1)	3.57	3.69
16	12(+1)	10^8(+1)	5(+1)	16(+1)	29(+1)	8(+1)	260(+1)	4.41	3.81

- Note: SA, slant age; SC, spore concentration; IV, inoculum volume; FT, fermentation time; T, temperature; pH, initial pH of the medium; AR, agitation rate.
- Coded levels of variables are given in the brackets.
- Results of pravastatin concentration are presented as mean from three replicates.

Table 2. The percentage contributions of the main effect of each variable and of the interactive effects between variables on pravastatin accumulation.

Variable	Code	Sum of squares	% Contribution of effect
Slant age	A	89.63	11.56
Spore concentration	B	0.11	0.015
Inoculum volume	C	0.37	0.048
Fermentation time	D	59.64	7.69
Temperature	E	1.48	0.19
Initial pH of the medium	F	75.39	9.72
Agitation rate	G	199.02	25.66
Interaction AB	AB	32.01	4.13
Interaction AC	AC	66.87	8.62
Interaction AD	AD	31.61	4.08
Interaction AE	AE	86.72	11.18
Interaction AF	AF	22.59	2.91
Interaction AG	AG	74.35	9.59
Interaction ABD	ABD	33.73	4.35

From the experimental data presented in Table 1, 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 using Yates' method [13] and are presented in Table 2. As shown in Table 2, among the analyzed variables four were found to be significant: agitation rate, slant age, initial pH of the medium, and fermentation time. Among these four factors, the agitation rate had the highest percentage contribution (25.66%), while the fermentation time had the lowest one (7.69%). The interaction between slant age and temperature (11.18%) also had a considerable influence on pravastatin accumulation.

3.2 Statistical modeling and ANOVA

The pravastatin concentration data (Table 1) as the dependent variable 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 the 2^{7-3} fractional factorial experiments, the following equation (1) in terms of the coded variables for pravastatin production was derived:

$$\text{Pravastatin concentration (mg/L)} = 9.08 - 2.37 \times A + 1.93 \times D - 2.17 \times F - 3.53 \times G + 1.41 \times A \times B - 2.04 \times A \times C + 1.41 \times A \times D - 2.33 \times A \times E - 1.19 \times A \times F + 2.16 \times A \times G + 1.45 \times A \times B \times D \quad (1),$$

where: A - slant age; B - spore concentration; C - inoculum volume; D - fermentation time; E - temperature; F - initial pH of the medium; G - agitation rate.

The results of the evaluation of the factorial model by ANOVA are presented in Table 3. The F-value of the model (69.10) confirms that it is significant. The data presented in Table 3 justify that amongst the tested variables only four were significant having $p < 0.05$: slant age (A), fermentation time (D), initial pH of the medium (F), and agitation rate (G). The predicted R^2 of 0.9162 was in reasonable agreement with the adjusted R^2 of 0.9804. The adequate precision of 27.695 confirms that the model could be used to navigate the design space. The results of the analysis demonstrated that all model statistics and diagnostic plots are acceptable.

Table 3. Evaluation of the factorial model using ANOVA.

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value (PROB>F)
Model	771.55	11	70.14	69.10	0.0005
A	89.63	1	89.63	88.30	0.0007
D	59.64	1	59.64	58.75	0.0016
F	75.39	1	75.39	74.26	0.0010
G	199.02	1	199.02	196.05	0.0002
AB	32.01	1	32.01	31.53	0.0049
AC	66.87	1	66.87	65.87	0.0013
AE	86.72	1	86.72	85.43	0.0008
AG	74.35	1	74.35	73.24	0.0010
ABD	33.73	1	33.73	33.22	0.0045
Residual	4.06	4	1.02		
Total	775.61	15			

Standard deviation	1.01	*R*-squared	0.9948
Mean	9.08	Adjusted *R*-squared	0.9804
Adequate precision	27.695	Predicted *R*-squared	0.9162

[a]Note: A, slant age; B, spore concentration; C, inoculum volume; D, fermentation time; E, temperature; F, initial pH of the medium; G, agitation rate.

The obtained results were in a good agreement with literature data reported by other researchers. The speed of agitation is one of the main factors affecting the performance of microbial cells and their productivity [14]. Moreover, according to Atalla *et al*. [15], the agitation rate is a key factor for statin production affecting oxygen supply during submerged fermentation of microbial producer.

From the factorial model derived here (equation 1), it was predicted that decreasing the slant age, initial pH of the medium and agitation rate, and increasing the fermentation time, should enhance pravastatin production. Therefore, the new slant age range chosen was 4-8 d with a new initial pH of 5.0-7.0, while the new agitation rate was in a range of 180-240 rpm. However, the fermentation time should be kept in an initial range of 8-16 d as our preliminary experimental results on the time course of pravastatin production (data not shown) demonstrated that pravastatin production considerably decreased by 16 d of fermentation.

4. Conclusion

The two-level factorial analysis was able to determine significant variables influencing pravastatin production by *Penicillium* sp. ESF21P. It was found that the agitation rate had the highest level of significance for pravastatin accumulation (25.66%) followed by slant age (11.56%), initial pH of the medium (9.72%) and fermentation time (7.69%). These four important factors will be optimized further using response surface methodology.

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