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Optimization of fermentation conditions for P450 BM-3 monooxygenase production by hybrid design methodology*

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Abstract: Factorial design and response surface techniques were used to design and optimize increasing P450 BM-3 expression in *E. coli*. Operational conditions for maximum production were determined with twelve parameters under consideration: the concentration of $FeCl_3$, induction at OD_{578} (optical density measured at 578 nm), induction time and inoculum concentration. Initially, Plackett-Burman (PB) design was used to evaluate the process variables relevant in relation to P450 BM-3 production. Four statistically significant parameters for response were selected and utilized in order to optimize the process. With the 416C model of hybrid design, response surfaces were generated, and P450 BM-3 production was improved to 57.90×10^{-3} U/ml by the best combinations of the physicochemical parameters at optimum levels of 0.12 mg/L $FeCl_3$, inoculum concentration of 2.10%, induction at OD_{578} equal to 1.07, and with 6.05 h of induction.

Key words: Optimization, P450 BM-3, Plackett-Burman (PB) design, Hybrid design
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INTRODUCTION

P450 enzymes are found throughout nature and can catalyze the oxidation of various chemicals (Ortiz de Montellano, 1995). Because of their important roles in these processes, numerous systems have been developed for the expression of P450s in vitro (Helvig and Capdevila, 2000; Nakamura *et al*., 2001; Deeni, 2001). But P450 enzymes fermentation levels in the microbial system are too low to catalyze the reactions for industrial preparations. Therefore, it is necessary to improve fermentation conditions to obtain maximum P450 levels.

Optimization of enzyme production and activity requires consideration of a large number of potentially influential factors, so it is essential to select the more important ones prior to optimization.

The classical method—studying one variable at a time while holding all others constant—is extremely inefficient in many cases. In recent years, a number of statistical experimental designs, which include regression analysis, factorial design and EVOP (evolutionary operation), have been employed for studying interaction and selecting optimal conditions (Kar *et al*., 2002). However, even these either involve a large number of experiments or are too restricted in the number of variables which can be studied.

Here, the methodology of Plackett-Burman was used as a tool for the initial screening, and is based on balanced incomplete blocks (Plackett and Burman, 1946). Instead of using more extensive factorial design, the statistical procedure would furnish more complete information. Each design of N experiments can be used to study up to $N-1$ variables.

After initial screening, response surface analysis can be limited to the most critical variables for determining their optimum operation conditions. Recently, many statistical experimental designs with response surface methodology have been employed for optimizing enzyme production from microorgan-

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ism (de Coninck *et al*., 2000; Murthy *et al*., 2000; Kalil *et al*., 2000; Uyar and Baysal, 2004; Dahiya *et al*., 2005). Hybrid design is a superior and more accurate modelling technique when compared to the response surface methodology (Roquemore, 1976). Response surfaces plotted by 3D plots can provide a good way for visualizing the parameter interaction. Therefore, both techniques can be used together to predict optimum process conditions for microbial enzyme production.

The aim of this work was to increase the production of P450 BM-3 by optimizing the fermentation conditions. Therefore, Plackett-Burman design based on screening design and factorial design was used to determine the relevant factors, and then the factors having more significant effect were further optimized by hybrid design methodology and response surface analysis.

The most important influential parameters were selected by Plackett-Burman (PB) design and optimized by hybrid design to maximize the P450 BM-3 production.

MATERIALS AND METHODS

Microorganism

The basis system involving the construction of plasmid and the expression of P450 BM-3 is described elsewhere (Li *et al*., 2000; 2005). pET28a(+) expresses P450 BM-3 with three sites mutagenesis (D168N, A225V, K440N) in *E. coli* BL21.

Chemicals

All chemicals used were of analytical grade. The yeast extract, tryptone, kanamycin, NADH (reduced nicotinamide adenine dinucleotide) and IPTG (isopropyl-β-D-thiogalactoside) were purchased from Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd. (Shanghai, China).

Medium and culture conditions

The strain was grown aerobically on LB medium containing 30 μg/ml kanamycin overnight at 37 °C in tube. Aliquots of the cell culture from the tube were transferred to 50 ml LB medium supplemented with 30 μg/ml kanamycin and 0.1 mg/L $FeCl_3$ in a 250 ml flask. The shaking flask was incubated at 180 r/min and 37 °C. After reaching an optical density at 578 nm (OD_{578}) of 0.8, P450 BM-3 expression was induced by adding 0.5 mmol/L IPTG for 5 h. *E. coli* cells containing P450 BM-3 were harvested.

Enzyme preparation

For enzyme preparation, the cells were centrifuged at 8000 r/min for 15 min, washed once with 0.1 mol/L potassium phosphate buffer (pH 7.4), then resuspended in the same buffer (5 ml per 50 ml medium), and broken up under ice-cooling with Sonifier (output level 200 W, three 2 min duty cycles of 20% each). The suspension was centrifuged for 20 min at 8000 r/min. The crude extracts were used directly for the activity assay.

Activity assay of P450 BM-3

The activity assay was carried out as modified according to Schwaneberg *et al*.(1999). For all assay procedures, after addition of 25 μl of 10-*p*-NCA (*p*-nitrophenoxydecanoic acid) solution in DMSO (dimethyl sulfoxide) (25 mmol/L), 1725 μl Tris/HCl buffer (pH 8.2, 0.1 mol/L), and 500 μl of P450 BM-3 extract solution, the samples were preincubated for 5 min. Then the reaction was started by adding 250 μl aqueous NADH solution (6 mmol/L). The formation of *p*-nitrophenolate was determined from the absorption at 410 nm [with an extinction coefficient=13.2 L/(mmol·cm)]. The enzyme activity was defined as the initial rate of *p*-nitrophenolate formation, with the unit of activity being the amount of enzyme that produces 1 μmol of *p*-nitrophenolate per minute under the assay conditions.

Experimental design

1. Plackett-Burman (PB) design

PB designs are very useful for screening process variables from a large number of factors, and require fewer runs than other factorial designs (Stowe and Mayer, 1966).

The influence of twelve variables on P450 BM-3 activity was investigated using the methodology of PB design. Each of the independent variables was tested at two levels, a high level (+) and a low level (−), as shown in Table 1.

2. Hybrid design

After the four relevant variables were selected by screening, hybrid design was used to obtain a

Table 1 Range of variables for PB design

	Level	
	−1	+1
Yeast extract (g/L)	5.0	7.5
Tryptone (g/L)	10	15
NaCl (g/L)	10	15
Original pH	7.0	7.5
Kanamycin (μg/ml)	30	45
$FeCl_3$ (mg/L)	0.10	0.15
Induction at OD_{578}	0.8	1.2
IPTG (mmol/L)	0.50	0.75
Induction time (h)	5	7
Induction temperature (°C)	30	35
Inoculum concentration (%)	1	2
Medium volume in 250 ml flask	50	75

quadratic model with the P450 BM-3 activity as response. The empirical polynomial model for four factors was in the following form:

$$y=\beta_0+\sum_{i=1}^{k}\beta_i X_i+\sum_{i=1}^{j-1}\sum_{j=1}^{k}\beta_{ij}X_iX_j+\sum_{i=1}^{k}\beta_{ii}X_i^2, \quad (1)$$

where y was the predicted response as the dependent variable; X_i (i=1, 2, 3 and 4) were the controlling variables or input predictors; and β_0, β_i (i=1, 2, 3 and 4), β_{ij} (i=1, 2, 3 and 4; j=i, …, 4) and β_{ii} were the regression coefficients. They were estimated by multiple non-linear regression analysis. Surfaces were then built using the quadratic model for the statistically significant variables.

Table 2 presents the values in actual and coded form of the variables used to optimize the P450 BM-3 activity. The first three variables were studied at five different levels, and the last variable at four different levels. All the variables were taken with the central coded value considered as zero.

Table 2 The values of variables for the P450 BM-3 activity determination for hybrid design

Coded values (X_i)	Variables (x_i)			
	$FeCl_3$ (mg/L)	Induction at OD_{578}	Induction time (h)	Inoculum concentration (%)
−1.4697	0.088	0.71	4.530	−
−1.0509	−	−	−	0.975
−1.0000	0.100	0.80	5.000	1.000
0.0000	0.125	1.00	6.000	1.500
0.5675	−	−	−	1.784
1.0000	0.150	1.20	7.000	2.000
1.4697	0.162	1.29	7.470	−
1.7654	−	−	−	2.383

RESULTS AND DISCUSSION

Screening design

Table 3 presents the PB design matrix and the results obtained for P450 BM-3 activity from experiments.

Dummy variables were used as the measure of variability for PB design. They gave a direct estimate of the standard error of a factor effect.

Table 3 PB design matrix for determining the effects with P450 BM-3 activity as responses

Run	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P450 BM-3 activity ($\times10^{-3}$ U/ml)
1	+	+	+	+	−	+	−	+	+	−	−	+	−	−	−	37.88
2	+	+	+	−	+	−	+	+	−	−	+	−	−	−	+	35.35
3	+	+	−	+	−	+	+	−	−	+	−	−	−	+	+	27.78
4	+	−	+	−	+	+	−	−	+	−	−	−	+	+	+	29.85
5	−	+	−	+	+	−	−	+	−	−	−	+	+	+	+	41.34
6	+	−	+	+	−	−	+	−	−	−	+	+	+	+	−	45.76
7	−	+	+	−	−	+	−	−	−	+	+	+	+	−	+	37.60
8	+	+	−	−	+	−	−	−	+	+	+	+	−	+	−	40.53
9	+	−	−	+	−	−	−	+	+	+	+	−	+	−	+	32.30
10	−	−	+	−	−	−	+	+	+	+	−	+	−	+	+	29.55
11	−	+	−	−	−	+	+	+	+	−	+	−	+	+	−	27.61
12	+	−	−	−	+	+	+	+	−	+	−	+	+	−	−	39.47
13	−	−	−	+	+	+	+	−	+	−	+	+	−	−	+	25.25
14	−	−	+	+	+	+	−	+	−	+	+	−	−	+	−	49.24
15	−	+	+	+	+	−	+	−	+	+	−	−	+	−	−	25.83
16	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	23.67

A, B, C, E, F, G, I, J, K, M, N, O are yeast extract, tryptone, NaCl, original pH, kanamycin, $FeCl_3$, induction at OD_{578}, IPTG, induction time, induction temperature, inoculum concentration, and medium volume in 250 ml flask, respectively; D, H, L are dummy variables

The effects of the variables on each response are presented in Table 4. P450 BM-3 production was affected by $FeCl_3$ concentration, inoculum concentration, induction at OD_{578}, and induction time.

As shown in Table 4, an increase in $FeCl_3$ concentration led to a decrease in P450 BM-3 activity. Increasing inoculum concentration and induction time, however, led to an increase in P450 BM-3 activity. In relation to the compositions of medium, increasing the concentrations of yeast extract, tryptone and NaCl resulted in an increase in P450 BM-3 activity, although this effect did not reach statistical significance.

Table 4 Effect estimates on P450 BM-3 activity from PB design

Variable		Effect	Relative significance	
Code	Factor	(+) to (−)	*t*-test	Confidence level (%)
A	Yeast extract (g/L)	3.60	0.799	78.16
B	Tryptone (g/L)	0.15	0.033	51.29
C	NaCl (g/L)	4.14	0.919	81.37
D	Dummy	2.72	–	–
E	Original pH	3.09	0.686	74.84
F	Kanamycin (μg/ml)	0.04	0.009	50.35
G	$FeCl_3$ (mg/L)	−4.48	−0.994	83.20
H	Dummy	4.56	–	–
I	Induction at OD_{578}	−6.43	−1.427	91.30
J	IPTG (mmol/L)	1.95	0.432	66.41
K	Induction time (h)	4.79	1.063	84.77
L	Dummy	5.72	–	–
M	Induction temperature (°C)	1.31	0.292	61.29
N	Inoculum concentration (%)	4.29	0.952	82.19
O	Medium volume in 250 ml flask	−3.87	−0.860	79.83

Table 4 also shows that original pH, the concentrations of kanamycin and IPTG, and induction temperature did not exert statistically significant effects on P450 BM-3 activity.

These results determine which at the variables have more significant effect on the responses. So the variables selected for further optimization were the following: $FeCl_3$ concentration, inoculum concentration, induction at OD_{578}, and induction time.

Hybrid design

Table 5 shows the experimental design and the results for P450 BM-3 activity from experiments and predicted responses. Based on the principle of saving costs, yeast extract, tryptone, NaCl, kanamycin and IPTG were fixed at 5 g/L, 10 g/L, 10 g/L, 30 μg/ml and 0.5 mmol/L, respectively, and medium volume was 50 ml in 250 ml flask. The original pH of medium and induction temperature were fixed at 7.5 and 35 °C according to their effects of PB design.

Date were analyzed by non-linear multiple regression using SAS software (SAS Institute Inc., USA). The equation with the optimized coefficients is given by:

Table 5 Matrix for hybrid design with the experimental and predicted values of P450 BM-3 activity

Run	$FeCl_3$	Induction at OD_{578}	Induction time	Inoculum concentration	Observed response ($\times10^{-3}$ U/ml)	Predicted response ($\times10^{-3}$ U/ml)
1	0	0	0	1.7654	56.44	56.44
2	−1	−1	−1	0.5675	33.33	34.37
3	1	−1	−1	0.5675	44.92	43.88
4	−1	1	−1	0.5675	41.93	40.89
5	1	1	−1	0.5675	41.29	42.33
6	−1	−1	1	0.5675	39.92	38.88
7	1	−1	1	0.5675	35.53	36.57
8	−1	1	1	0.5675	48.79	49.83
9	1	1	1	0.5675	40.49	39.45
10	1.4697	0	0	−1.0509	46.29	46.29
11	−1.4697	0	0	−1.0509	40.27	40.27
12	0	1.4697	0	−1.0509	36.59	36.59
13	0	−1.4697	0	−1.0509	48.14	48.14
14	0	0	1.4697	−1.0509	59.39	59.39
15	0	0	−1.4697	−1.0509	50.45	50.45
16	0	0	0	0	57.20	57.20

$$y = 57.2 + 0.5769X_1 + 0.1481X_2 + 1.3311X_3 - 0.8670X_4 - 6.9926X_1^2 - 2.0175X_1X_2 - 2.9550X_1X_3 - 1.3999X_1X_4 - 7.4162X_2^2 + 1.1075X_2X_3 + 3.880X_2X_4 - 1.6038X_3^2 - 1.6274X_3X_4 + 0.2472X_4^2, \quad (2)$$

where y is the output response (i.e. P450 BM-3 activity), X_1, X_2, X_3 and X_4 are the coded values of $FeCl_3$ concentration, induction at OD_{578}, induction time and inoculum concentration, respectively.

Table 6 shows the analysis of variance (ANOVA) for P450 BM-3 activity. The response has a very high correlation coefficient of 0.995.

Table 6 ANOVA and regression analysis for P450 BM-3 activity

Source	Sum of squares	Mean square	Degree of freedom	F-value
Model	921.98	65.86	14	7.61
Residual	8.65	8.65	1	–
Total	930.63	–	15	–
Correlation coefficient	0.995	–	–	–

To study the interactions of variables and analyze the P450 BM-3 activity, three-dimensional response surfaces are plotted in Fig.1.

The response surfaces were plotted to define the optimal conditions for P450 BM-3 activity. All possible combinations of the four variables were studied.

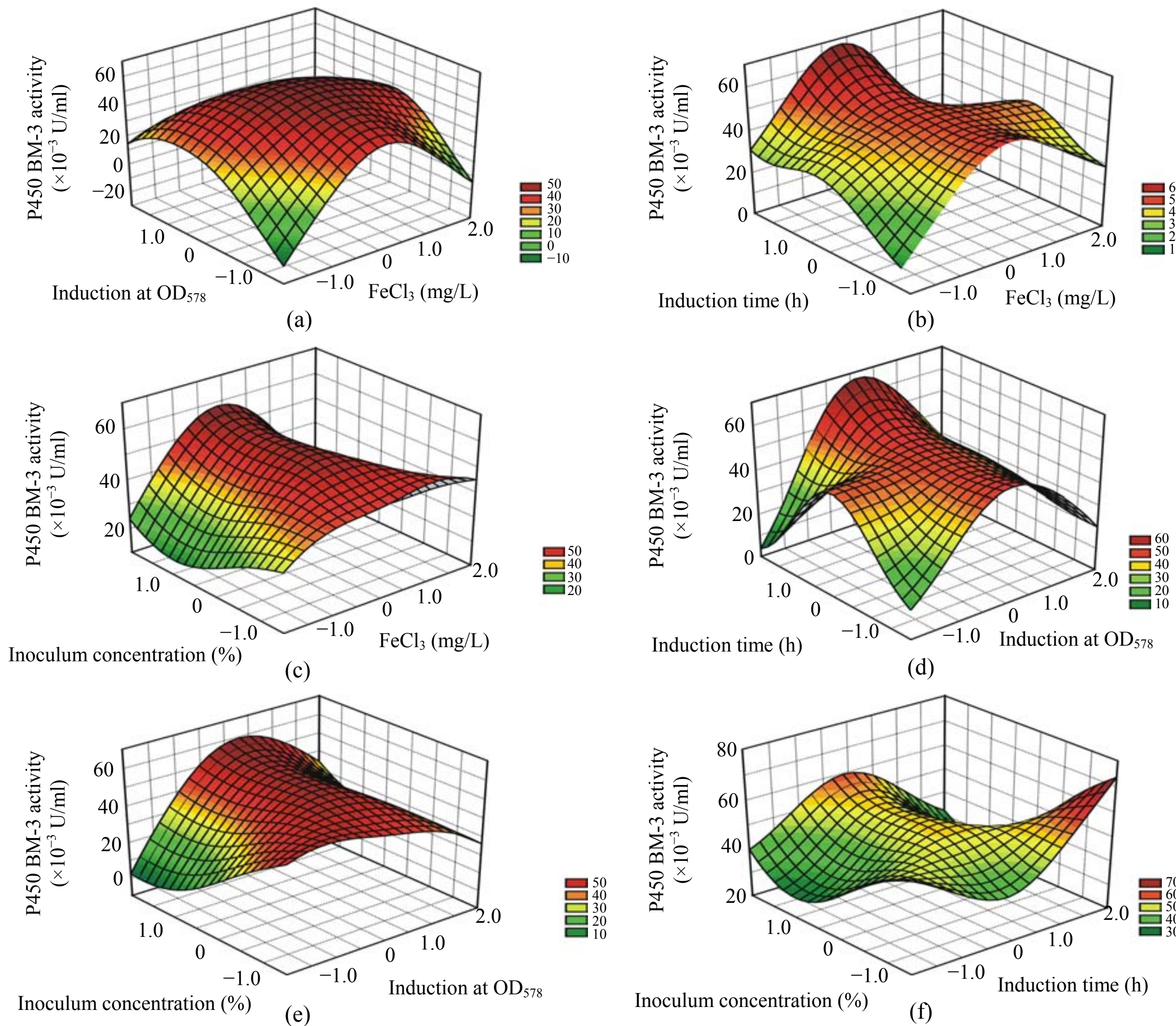

Fig.1 Response surface curves of P450 BM-3 activity expressed as a function of (a) $FeCl_3$ concentration and induction at OD_{578}, (b) $FeCl_3$ concentration and induction time, (c) $FeCl_3$ concentration and inoculum concentration, (d) induction at OD_{578} and induction time, (e) induction at OD_{578} and inoculum concentration, and (f) induction time and inoculum concentration

Figs.1a, 1d and 1e revealed that P450 BM-3 activity was maximal when the coded value of induction at OD_{578} was at the central point, which corresponded to the actual value of 1.0. And Figs.1a, 1b and 1c showed that when the coded value of $FeCl_3$ was at the central point, which was equal to the actual value of 0.125 mg/L, high P450 BM-3 activity was obtained. Figs.1c, 1e and 1f revealed that P450 BM-3 activity increased greatly when inoculum concentration was increased.

The optimal operating conditions obtained from the polynomial model were $FeCl_3$ of 0.12 mg/L, inoculum volume of 2.10 ml in 100 ml medium, when OD_{578} equal to 1.07, IPTG added to the cultures, and in 6.05 h of induction with 57.41×10^{-3} U/ml of predicted P450 BM-3 activity. Under the conditions, the P450 BM-3 production in the experimental result was 57.90×10^{-3} U/ml. While when the initial conditions were adopted, which corresponded to $FeCl_3$ of 0.10 mg/L, inoculum volume of 1.0 ml in 100 ml medium, when OD_{578} equals 0.8, IPTG was added to the cultures, and in 5 h of induction, P450 BM-3 activity was at a lower level of 37.62×10^{-3} U/ml. Thus by optimizing the fermentation process parameters using hybrid design, P450 BM-3 production was increased from 37.62×10^{-3} to 57.90×10^{-3} U/ml.

CONCLUSION

The methodology of Plackett-Burman is very useful for selecting relevant variables for further optimization. This makes it efficient to consider a large number of variables and can provide much more useful information than other factorial designs. Four more important factors were picked out using this technique. The main parameters were $FeCl_3$ concentration, inoculum concentration, induction at OD_{578}, and induction time.

High P450 BM-3 activity was obtained by using hybrid design and response surface analysis. Thus hybrid design is a powerful tool well suited for modelling the fermentation process.

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