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Use of response surface methodology to optimize culture medium for production of lipase with *Candida* sp. 99-125

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Abstract

Response surface methodology (RSM) was employed to optimize culture medium for production of lipase with *Candida* sp. 99-125. In the first step, a Plackett–Burmen design was used to evaluate the effects of different components in the culture medium. Soybean oil, soybean powder and K_2HPO_4 have significant influences on the lipase production. The concentrations of three factors were optimized subsequently using central composite designs and response surface analysis. The optimized condition allowed the production of lipase to be increased from 5000 to 6230 IU/ml in shake flask system. The lipase fermentation in 5 l fermenter reached 9600 IU/ml.

Keywords: Lipase; Fermentation; Medium optimization; Response surface methodology

1. Introduction

Lipases (EC3.1.1.3) which catalyze the hydrolysis of triglycerides to fatty acids and glycerol, have wide uses in the modification of fats and oils. In recent decades, it has been shown that lipases could be used in non-aqueous enzymatic synthesis for optical pure compound production [1]. Microbial lipases have considerable industrial potential as catalysts for hydrolysis, synthesis and *trans*-esterification of tri-acyglycerols owing to advantages such as high levels of production and diversity of stero-specific properties [2]. Lipase from *Candida* sp. is one of the most important commercially available lipases [3]. It is useful in a variety of biotransformations. Recent interest stems from its ability not only to hydrolyse ester bonds, *trans*-esterify triglycerides and resolve racemic mixtures, but also, to synthesise ester and peptide [4–7].

Lipase activity and production depend upon the composition of the fermentation medium. The general optimization of the medium is by varying one parameter while keeping the other at constant level. The disadvantage of this single variable optimization is that it does not reflect the interaction effects among these variables employed and it does not depict the net effect of the various medium constituents on the enzyme activity. In order to overcome this major problem, optimization studies are done using response surface methodology (RSM) which is a mathematical and statistical technique widely used to determine the effects of several variables and to optimize different biotechnological processes [8,9]. Plackett–Burmen (P–B) designs as a two-level experimental design require fewer runs than a comparable fractional design and can be used to identify the more important independent variables from a long list of Candidate factors and select them to realize a complete factorial design. The method of steepest ascent (descent) is a procedure for moving sequentially along the path of steepest ascent (descent), that is, in the direction of the maximum increase (decrease) in the response. The central composite design (CCD) and response surface analysis could find out the relations between the variables and response, moreover, the optimum of every variable would be obtained by differential approximation. This technique has been used to study the optimization of physiochemical parameters and factors of many fermentation medium and process with various microorganism [10–12]. There are some study on RSM for the production of lipase [13,14]. But the methodology was used in the experiments with only the carbon source being varied.

Tan et al. had made a preliminary study in the production of lipase with *Candida* sp. 99-125. The maximum lipase yield reached 8300 IU/ml in 30 l fermenter [15]. In the present work, a response surface approach including a Plackett–Burmen design, path of steepest accent and central composite design was used for

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comparison and advanced optimization of medium constituents included carbon source, nitrogen source and inorganic compound for lipase production.

2. Materials and methods

2.1. Microorganism

Candida sp. 99-125 was preserved on an agar slant at 4 °C in our laboratory. The organism was grown on agar slants containing (w/v) 0.2% yeast extract, 0.5% peptone, 1% glucose and 2% agar. Slants were incubated at 26 °C for 72 h.

2.2. Chemicals

Olive oil used was of chemical grade. All other chemicals were of analytical grade. Soybean oil and soybean powder were obtained from local market.

2.3. Inoculum

The strain was cultured in the medium containing soybean oil (4%), soybean powder (4%), K_2HPO_4 (0.1%), KH_2PO_4 (0.1%). After cultivating at 26 °C in 220 rpm for 48 h, 2 ml of this suspension (3×10^8 cells/ml) was used as inoculum to a 250 ml shake flask containing 50 ml of production medium.

2.4. Production of lipase

2.4.1. Shake flask system

The medium was sterilized at 121 °C for 25 min. The composition of the medium and the quantities of constituents used in the composition of the medium varied according to design of the matrix. The initial pH was maintained in the range 6–7. The culture was incubated at 26 °C for 120 h on a rotatory shaker maintained at 220 rpm.

2.4.2. Five litres fermenter

The 5 l fermenter loading the optimum medium was sterilized at 121 °C for 25 min. After cooling to scheduled temperature 26 °C, the fermentation was initialized by injecting 7% (v/v) inoculum. The culture was incubated at 26 °C and 220 rpm.

2.5. Assay of lipase activity

Lipase activity was determined according to an olive emulsion method [16]. The fatty acids released were determined by titration with 0.05 mol/l NaOH solution. One unit of lipase was defined as the enzyme required to release 1 μmol fatty acid per minute under 40 °C

2.6. Experimental design

In preliminary experiments, we evaluated various carbon and nitrogen sources, inorganic salt for their suitability to sustain good production of lipase by *Candida* sp. 99-125. Preliminary data indicated that the major variables affecting the performance of the culture in terms of lipase yields are soybean oil, soybean powder, K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$ and Span 60. Therefore, these seven medium ingredients were chosen for further optimization through RSM.

2.6.1. Plackett–Burmen design

The influences of seven variables on lipase yield were investigated using the methodology of Plackett–Burmen. Each independent variable was tested at two levels, a high (+1) and a low (−1) level. The seven factors' two level are soybean oil (X_1): 3% and 4%; $(NH_4)_2SO_4$ (X_2): 0.1% and 0.125%; K_2HPO_4 (X_3): 0.3% and 0.4%; KH_2PO_4 (X_4): 0.1% and 0.125%; soybean powder (X_5): 5.5% and 6.5%; $MgSO_4$ (X_6): 0.05% and 0.06%; Span 60 (X_7): 0.1% and 0.125%. Twelve experimental runs were carried in this experiment. Three dummy variables were used to estimate the experimental error and check the adequacy of the first-order model. The calculational software SAS (version 8.0) was used for the regression analysis of the experimental data obtained. The quality of fit of the first-order model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by an F-test. The significance of the regression coefficients was tested by a t-test.

2.6.2. Path of steepest accent (descent)

Based on the results obtained from the P–B design, the fitted first-order model is

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i$$

Y is the predicted response, β_0, β_i, are constant coefficients, and x_i is the coded independent variables or factors.

The direction of steepest ascent (descent) is the direction in which Y increases (decreases) most rapidly. This direction is parallel to the normal to the fitted response surface. One usually takes as the path of steepest ascent (descent) the line through the center of the region of interest and normal to the fitted surface. Thus, the steps along the path are proportional to the regression coefficients β_i. The path of steepest ascent (descent) started from the center of the first design. To move away from the first design center along the path of steepest ascent (descent), we moved 1.0575, −0.6925, −0.037 in soybean oil, soybean powder, K_2HPO_4 directions, respectively. These new units were determined according to concentration range of unity level from first design and estimated coefficient ratio from the first-order model Eq. (1).

2.6.3. Central composite design (CCD) and response surface analysis

A CCD with five coded levels was used for exploring the sub-region of the response surface in the neighborhood of the optimum. For the three factors, this trial was essentially a full 2^3 factorial design has six axial points (or called star points) and six replication of center points, resulting in a total number of 20 experiments. The experimental results of the CCD were fitted with a second-order polynomial equation by a multiple

Table 1
The matrix of the Plackett–Burmen design experiments, together with the observed experimental data

Run	X_1 (%)	X_2 (%)	Dummy variable I	X_3 (%)	X_4 (%)	Dummy variable II	X_5 (%)	X_6 (%)	Dummy variable III	X_7 (%)	Lipase activity (IU/ml)
1	4	0.100	1	0.3	0.100	−1	6.5	0.06	1	0.100	5238
2	4	0.125	−1	0.4	0.100	−1	5.5	0.06	1	0.125	5450
3	3	0.125	1	0.3	0.125	−1	5.5	0.05	1	0.125	5050
4	4	0.100	1	0.4	0.100	1	5.5	0.05	−1	0.125	5050
5	4	0.125	−1	0.4	0.125	−1	6.5	0.05	−1	0.100	4800
6	4	0.125	1	0.3	0.125	1	5.5	0.06	−1	0.100	5100
7	3	0.125	1	0.4	0.100	1	6.5	0.05	1	0.100	4425
8	3	0.100	1	0.4	0.125	−1	6.5	0.06	−1	0.125	4550
9	3	0.100	−1	0.4	0.125	1	5.5	0.06	1	0.100	4875
10	4	0.100	−1	0.3	0.125	1	6.5	0.05	1	0.125	5225
11	3	0.125	−1	0.3	0.100	1	6.5	0.06	−1	0.125	4525
12	3	0.100	−1	0.3	0.100	−1	5.5	0.05	−1	0.100	4900

regression technique

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i x_j + \sum_{i<j} \beta_{ij} x_i x_j$$

Y is the predicted response, β_0, β_i, β_{ii}, β_{ij} are constant coefficients, and x_i, x_j are the coded independent variables or factors.

The quality of fit of the second-order model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by an F-test. The significance of the regression coefficients was tested by a t-test.

3. Results and discussion

3.1. Plackett–Burmen design

The experimental results of lipase production by a Plackett–Burmen design are shown in Table 1. Analysed by SAS software, a first-order model was fitted to the data obtained from the experiment. The effects of the seven factors: soybean oil (X_1), $(NH_4)_2SO_4$ (X_2), K_2HPO_4 (X_3), KH_2PO_4 (X_4), soybean powder (X_5), $MgSO_4$ (X_6) and Span 60 (X_7) were calculated to be 211.5, −40.7, −74, 1, −138.5, 24, 42.7, respectively. We obtained the following model in the coded variables.

First-order model equation

$$Y_1\,(\mathrm{IU/ml}) = 493.3 + 211.5X_1 - 40.7X_2 - 74X_3 + X_4 - 138.5X_5 + 24X_6 + 42.7X_7 \quad (1)$$

This fit of the model was checked by the coefficient of determination R^2, which was calculated to be 0.999, indicating that 99.9% of the variability in the response could be explained by the model. The statistical significance of the second-order model equation was evaluated by the F-test analysis of variance (ANOVA) which revealed that this regression is statistically significant ($P < 0.1$) at 90% of confidence level. The t-test was used to identify the effect of every factor on lipase production, shown in Table 2. It indicated that soybean oil, soybean powder and K_2HPO_4 were the greatest important factors.

3.2. Path of steepest ascent (descent)

Based on the first-order model equation obtained and the three important effect factors above, the path of steepest ascent (descent) was determined to find proper direction of changing variables increasing or decreasing the concentration according to the sign of the main effects to improve lipase production. The path of steepest ascent started from the center of the Plackett–Burmen design and moved along the path in which the concentration of soybean oil increased, while soybean powder and K_2HPO_4 decreased. The design and results of the path of steepest ascent experiments are shown in Table 3. It was shown that the highest response is 5980 IU/ml when medium was: (w/v) soybean oil 4.56%, soybean powder 5.31% and K_2HPO_4 0.31%. It suggested that the point was near the region of maximum production response.

Table 2
Regression analysis of the effect of every factor on lipase production

Factor	t-value	$P > \|t\|$	Significant level (%)
Soybean oil	22.6607	0.02808	97.2
$(NH_4)_2SO_4$	−4.3571	0.1436	
K_2HPO_4	−7.9286	0.0799	92.0
KH_2PO_4	0.1071	0.9321	
Soybean powder	−14.8393	0.04284	95.7
$MgSO_4$	2.5714	0.2361	
Span 60	4.5714	0.1371	

Table 3
Experimental results of the path of steepest ascent (descent)

	Soybean oil (%)	Soybean powder (%)	K_2HPO_4 (%)	Lipase activity (IU/ml)
(1) Base point (zero level in the P–B design)	3.50	6.00	0.35	
(2) Origin step unit (concentration range of unity level)	0.50	0.50	0.05	
(3) Slope (estimated coefficient ratio from equation)	211.50	−138.50	−74.00	
(4) Correspondent concentration (2) × (3)	105.75	−69.25	−3.70	
(5) New step unit (4) × 0.01[a]	1.06	−0.69	−0.04	
Experiment number 1	3.50	6.00	0.35	5130
Experiment number 2	4.56	5.31	0.31	5980
Experiment number 3	5.62	4.62	0.28	5060
Experiment number 4	6.67	3.92	0.24	4530
Experiment number 5	7.73	3.23	0.20	4010
Experiment number 6	8.79	2.54	0.16	3600

[a] 0.01 is a factor determined by experimenter based on process knowledge or other practical consideration.

3.3. Central composite designs and response surface analysis

The central composite design (CCD) was conducted in the optimum vicinity to locate the true optimum concentrations of soybean oil (U_1), soybean powder (U_2) and K_2HPO_4 (U_3) for lipase production. The levels of the variables for the CCD experiments were selected according to the results of the previous experiments. The design matrix and the corresponding experimental data was shown in Table 4. The experimental results of

Table 4
The matrix of the CCD experiment, and the corresponding experimental data

Run	Soybean oil (%) U_1	Soybean powder (%) U_2	K_2HPO_4 (%) U_3	Lipase activity (IU/ml)
1	3.00	4.00	0.10	4200
2	3.00	4.00	0.30	3990
3	3.00	6.00	0.10	4695
4	3.00	6.00	0.30	4755
5	5.00	4.00	0.10	5140
6	5.00	4.00	0.30	4570
7	5.00	6.00	0.10	4815
8	5.00	6.00	0.30	5500
9	2.32	5.00	0.20	3400
10	5.68	5.00	0.20	4575
11	4.00	3.32	0.20	3750
12	4.00	6.68	0.20	5625
13	4.00	5.00	0.03	4570
14	4.00	5.00	0.37	6235
15	4.00	5.00	0.20	6070
16	4.00	5.00	0.20	6000
17	4.00	5.00	0.20	6050
18	4.00	5.00	0.20	6175
19	4.00	5.00	0.20	6020
20	4.00	5.00	0.20	6030

Table 5
The significance of the regression coefficients of the model

Term	Estimate	Standard error	t	$P>\|t\|$	Significant level (%)
U_1	319.336	99.620	3.20554	0.00940	99
U_2	367.462	99.620	3.68864	0.00419	99
U_3	202.476	99.620	2.03249	0.06952	93
U_1^2	−711.086	96.977	−7.33249	0.00010	
U_1U_2	−81.875	130.160	−0.62904	0.54343	
U_1U_3	33.125	130.160	0.25450	0.80427	
U_2^2	−463.598	96.977	−4.78047	0.00075	
U_2U_3	190.625	130.160	1.46455	0.17376	
U_3^2	−210.807	96.977	−2.17377	0.05482	

the CCD design were fitted with the second-order polynomial Eq. (2)

$$Y_2\,(\mathrm{IU/ml}) = 6054.3 + 319.3U_1 + 367.5U_2 + 202.5U_3 - 711.1U_1^2 - 81.9U_1U_2 + 33.1U_1U_3 - 463.6U_2^2 + 190.6U_2U_3 - 210.8U_3^2 \quad (2)$$

The fit of the model was checked by the coefficient of determination R^2, which was calculated to be 0.9108, indicating that 91.08% of the variability in the response could be explained by the model. The statistical significance of the second-order model equation was evaluated by the F-test analysis of variance which revealed that this regression is statistically significant ($P < 0.0004$) at 99% of confidence level.

Table 5 shows the significance of the regression coefficients of the model. It indicated that soybean oil and soybean powder had high significance ($P = 0.0094$ and 0.0042, respectively) on lipase production, because soybean oil was carbon and energy resource for the strain and had inductive effect on lipase production. On the other hand, soybean powder provided nitrogen resource in order to form the enzyme (lipase) in the strain. The interaction effects of each factor, however, had no significant. The contour plots described by the model equation (Y_2) are represented in Figs. 1–3. It indicated that the maximum lipase yield reached 6200 IU/ml approximately. The optimal concentrations for the three components as obtained from the maximum point of the model were calculated by the SAS software to be 4.187%, 5.840% and 0.284% for soybean oil, soybean powder and K_2HPO_4, respectively. The model predicted a maximum response of 6218 IU/ml lipase yield for this point.

3.4. Verification of optimum condition

To confirm these results, lipase fermentation was done with a culture medium representing this maximum point and yielding lipase 6230 IU/ml (average of three repeats). The result improved about 20% than the single variable optimization of

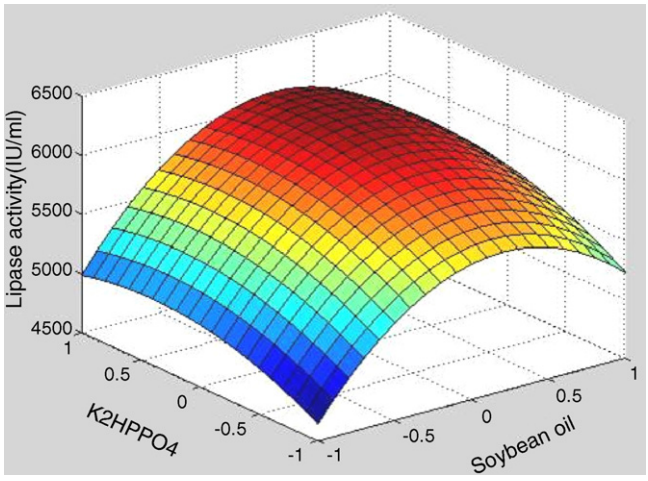

Fig. 2. Response surface plot described by the model Y_2, which represents the effect of K_2HPO_4 and soybean oil on lipase production (soybean powder = 5%, w/v).

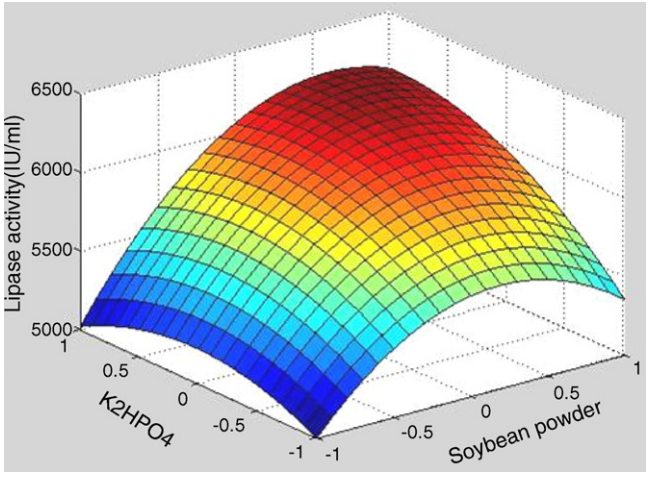

Fig. 3. Response surface plot described by the model Y_2, which represents the effect of K_2HPO_4 and soybean powder on lipase production (soybean oil = 4%, v/v).

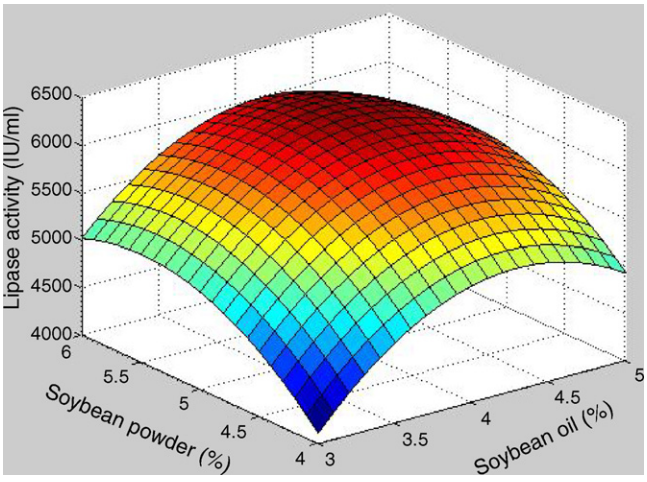

Fig. 1. Response surface plot described by the model Y_2, which represents the effect of soybean oil and soybean powder on lipase production (K_2HPO_4 = 0.2%).

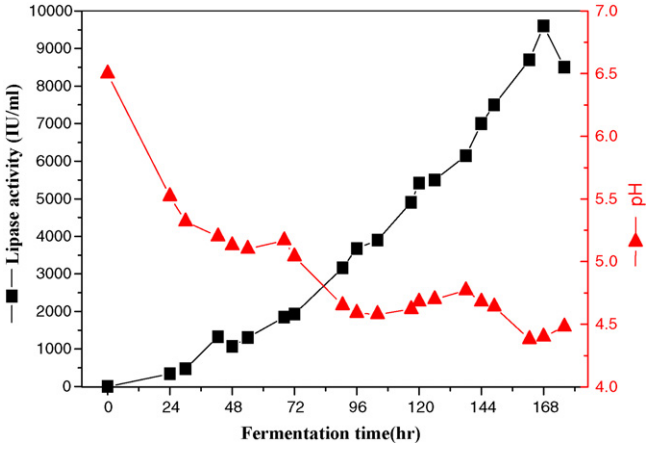

Fig. 4. The course of lipase fermentation in 5 l fermenter to verify the optimum point of culture medium. (w/v) soybean oil 4.187%, soybean powder 5.840%, K_2HPO_4 0.284%, KH_2PO_4 0.1%, $(NH_4)_2SO_4$ 0.1%, $MgSO_4$ 0.05% and Span 60 0.1%.

culture medium in which the lipase yield was 5000 IU/ml. The well correlation between predicted and measured values of these experiments justifies the validity of the response model and the existence of an optimum point. Compared with the results reported above, the lipase yield reached 9600 IU/ml in 5 l fermenter (shown in Fig. 4.) higher than the result in 30 l fermenter mentioned in Ref. 15. The fermentation time, however, prolong to about 168 h. It was believed that the lipase yield in 30 l scale fermentor in this optimum condition would be higher.

4. Conclusions

The response surface methodology allowed a rapid screening of the important influence factors and development of a polynomial model to optimize culture medium for production of lipase from *Candida* sp. 99-125. The R^2 value of 0.91 showed a good fit of the model with the experimental data. The model was predicted accurately the maximum point of lipase production. The optimum culture medium was (w/v) soybean oil 4.187%, soybean powder 5.840%, K_2HPO_4 0.284%, KH_2PO_4 0.1%, $(NH_4)_2SO_4$ 0.1%, $MgSO_4$ 0.05% and Span 60 0.1%. The lipase yield increased to 6230 and 9600 IU/ml in shake flask system and 5 l fermenter, respectively, which was obviously higher than the result reported.

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