Optimization of Medium Compositions Using Statistical Experimental Design to Produce Lipase by *Bacillus subtilis*

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Statistical experimental design was employed to optimize medium compositions to enhance the activity of lipase from *Bacillus subtilis*. Plackett–Burman design was applied to evaluate the effect of different medium compositions obtained by one-factor-at-a-time experiments, and ammonium chloride, ammonium sulfate and dipotassium hydrogen phosphate were found to influence the activity of lipase significantly. Steepest ascent method was employed to approach the experimental design space, followed by an application of central composite design and response surface methodology for further optimization. A quadratic model was found to fit the lipase activity, and the optimal medium compositions were determined as included 45 g L^{-1} NH_4Cl, 20.4 g L^{-1} $(NH_4)_2SO_4$ and 12.6 g L^{-1} K_2HPO_4. The corresponding lipase activity was 91.39 U mL^{-1}. Validation experiments were also carried out to prove the adequacy and the accuracy of the model obtained. The lipase activity reached 91.46 U mL^{-1}, which was almost equal to the actual predicted value, about 4.94-fold increase compared with that using the original medium.

Key words:
Bacillus subtilis, central composite design, lipase, Plackett–Burman design, response surface methodology

Introduction

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) constitute a group of enzymes that have a wide array of industrial applications in the manufacture of pharmaceuticals, food processing industries, detergents and wastewater treatment.[1] Lipases can catalyze the bioconversion reactions of hydrolysis, esterification, transesterification, alcoholysis, acidolysis and aminolysis.[2] Especially, they act at the lipid–water interface and have high chemo-, region- and enantio-specificity.[3] For all these reasons, lipases are among the most widely used biocatalysts in the field of organic chemistry.

Microbial lipases have attracted more attention in the last decades because of easy extraction and potential for unlimited supply. Commercial preparations of microbial lipases are produced by fermentation of different fungi, yeast and bacteria such as *Rhizopus delemar*, *Aspergillus niger*, *Geotrichum candidum*, *Candida rugosa* and *Chromobacterium viscosum*.[4,5] Recently, lipase from *Bacillus subtilis* has attracted more attention due to the classification of *Bacillus subtilis* as a generally regarded safe (GRAS) organism by the Food and Drug Administration of USA (FDA),[6] and due to its potentials used in food and chemistry industries. But lipase production by *Bacillus subtilis* has not been extensively studied compared with other commercial lipase as referenced above, which mainly focused on the research of recombinant,[7] structure,[8] purification[9] and characterization,[10] relatively few reports concerning culture optimization to enhance the lipase activity are present in the literature.[11] The purpose of this paper was to study the effect of medium compositions on the activity of lipase produced by *Bacillus subtilis* using statistical experimental design.

The greatest difficulty in optimization of culture conditions is the presence of interactive effects of medium compositions and culture condition factors. The "one-factor-at-a-time" method in which one independent variable is studied while maintaining all the other factors at a fixed level, is laborious and time consuming and cannot provide the information about mutual interactions of the parameters on the desired outcome.[12] Statistical experimental design, which is a collection of statistical techniques applicable to experimental design, model building, evaluating the effect of factors, and screening optimum conditions of factors for desirable responses, can overcome these shortages.[13] Statistical experimental design methods such as re-

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sponse surface methodology can provide a systematic and efficient plan for experimentation to achieve certain goals, so that several control factors are simultaneously studied.

In this paper, an effective and statistical experimental design tool, i.e. response surface methodology, was used to determine the optimal medium compositions to enhance the activity of lipase from *Bacillus subtilis*.

Materials and methods

Microorganism

Bacillus subtilis strain CICC 20034 (China Center of Industrial Culture Collection) was used to produce lipase, which was maintained on agar slants containing: 5 g L^{-1} peptone, 3 g L^{-1} yeast extract, 3 g L^{-1} NaCl and 18 g L^{-1} agar, cultured for 30 h at 30 °C, and then stored at 4 °C and subcultured every two months.

Inoculum preparation and flask culture

The strain was transferred from a slant culture into an Erlenmeyer flask (250 mL) containing 50 mL seed medium, which was the same as the above slant medium without agar. The pH of the medium was adjusted to 7.0 before autoclaving. The seed cultures were grown at 30 °C on a rotary shaker incubator at 200 rpm for 18 h.

Inoculum (4 %, v/v) was transferred into an Erlenmeyer flask (250 mL) containing 50 mL of fermentation medium in which the composition was varied based on the experimental designs, and the initial pH = 7.0. The fermentation cultures in shake flask were carried out at 30 °C under shaking speed of 200 rpm for 24 h.

Determination of lipase activity

Cell culture samples (10 mL) were centrifuged at 4 °C and 10 000×g for 5 min, and the supernatant was assayed for lipase activity. Lipase activity in the culture broth was measured by titrating fatty acid liberated from olive oil, with 50 mmol L^{-1} NaOH and used phenolphthalein as an indicator.[14] The reaction mixture, consisting of 5 mL substrate (25 % olive oil emulsified with a 3 % polyvinyl alcohol solution), 4 mL of 50 mmol L^{-1} phosphate buffer (pH = 8.0), and 1 mL of enzyme solution, was incubated at 40 °C for 10 min under rocking conditions. The reaction was stopped by the addition of 10 mL of anhydrous ethanol. Triplicate experiments were carried out and the mean value was calculated. One unit of lipase was defined as the lipase quantity that liberated 1 μmol of fatty acid per minute under the assay conditions.

Experimental design

Plackett–Burman (PB) design

PB experimental design is a powerful tool used to screen N-1 variables in only N experiments (N must be a multiple of four).[15] The PB experimental design assumes that there are no interactions among the different media constituents. The following first-order model is considered sufficient for screening

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i \qquad (1)$$

where Y represents the estimated target function (lipase activity), and b_0 is the intercept of the model, b_i is the linear coefficient, and k is the number of involved variables. X_i is the level of the independent variable under consideration,[16] which can be used to screen for factors significantly affecting the measured response.

Eight independent medium compositions (glycerol, NH_4Cl, $(NH_4)_2SO_4$, $MgSO_4$, $MnSO_4$, $CaCl_2$, $Na_2HPO_4 \cdot 12H_2O$ and K_2HPO_4) were investigated using PB design to identify the compositions that affected significantly lipase activity. Two concentrations, "high" and "low", were evaluated for each medium composition and designated as level +1 and level –1 respectively (Table 1). For the selection of these factors, Design Expert 7.1.6 (Stat-Ease, Inc., Minneapolis, USA) was used to generate and analyze the experimental design of Plackett–Burman.

Table 1 – *Minimum and maximum ranges for the parameters selected in Plackett–Burman design*

Variables	Factors	Concentration (g L^{-1})	
		–1 level	+1 level
X_1	Glycerol	8	16
X_2	NH_4Cl	6	12
X_3	$(NH_4)_2SO_4$	2	4
X_4	$MgSO_4$	0.4	0.8
X_5	$MnSO_4$	0.6	1.2
X_6	$CaCl_2$	0.4	0.8
X_7	$Na_2HPO_4 \cdot 12H_2O$	7.2	14.4
X_8	K_2HPO_4	1.8	3.6

Path of steepest ascent method

The direction of steepest ascent was parallel to the normal contour line of the model response curve and passed through the center point of PB design experiment. Variables that significantly influenced lipase activity were optimized with respect to enzyme activity by applying a single steepest ascent experiment. Experiments were performed along the steepest ascent path until the response increased no more. This point would be near the optimal point and could be used as center point to optimize.[17]

Central composite design and response surface methodology

After the critical medium compositions were identified by PB design and path of steepest ascent method, response surface methodology was employed to optimize the component concentration to maximize lipase activity. Three independent factors i.e. NH_4Cl, $(NH_4)_2SO_4$, K_2HPO_4 were studied at five different levels (–1.68, –1, 0, +1 and +1.68) and the factors were coded. Twenty experiments were conducted containing six replications at the center point for estimating the purely experimental uncertainty variance in triplicates. The statistical software package Design Expert® 7.1.6 was used to analyze the results of the experimental design.

In order to correlate the response variable (i.e., lipase activity) to the independent variables, the relationships and interrelationships of the variables were determined by fitting the second-degree polynomial equation to data obtained by mean values of the triplicates of each experiment at different occasions:

$$Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} b_{ij} x_i x_j \,,\ i<j \quad (2)$$

where Y represents the response variable, b_0 is the interception coefficient, b_i is the coefficient of the linear effect, b_{ii} is the coefficient of quadratic effect, b_{ij} is the coefficient of interaction effect when $i < j$, and k is the numbers of involved variables.

Results and discussion

Plackett–Burman design

The optimization of culture medium was carried out by a combination of non-statistical methodology and statistical methodology based experimental designs. The selection of medium compositions was carried out through the non-statistical methodology (one-factor-at-a-time experiments). One-factor-at-a-time experiments revealed that glycerol, NH_4Cl, $(NH_4)_2SO_4$, $MgSO_4$, $MnSO_4$, $CaCl_2$, $Na_2HPO_4 \cdot 12H_2O$ and K_2HPO_4 were supposed to have more effect on lipase activity, and 18.5 U mL^{-1} of the lipase activity was observed (data not shown).

Compared with conventional factorial design that is labor-intensive and time-consuming, PB design decreases significantly the number of experiments needed to effectively achieve experimental goals.[18] The matrix developed by the PB design and the results (lipase activity) are presented in Table 2, and the regression analysis shown in Table 3. Values of "Prob > F" less than 0.05 indicate model terms are significant. An adequate precision of 32.15 indicates an adequate signal as it measures the signal-to-noise ratio, and this model could be used to navigate the design space. In this case X_2, X_3, X_8 are significant model terms. Values greater than 0.05 indicate the model terms are not significant. Regression analysis determined that the compositions of NH_4Cl (X_2), $(NH_4)_2SO_4$ (X_3,) and K_2HPO_4 (X_8) had a significant effect ($P < 0.05$) on the enzyme activity, so these compositions were evaluated in the further optimization experiments. After the neglect of insignificant terms (on the basis of P-values higher than 0.05), a modified first-order equation was developed to describe enzyme activity:

$$Y = 24.08 + 4.72\ X_2 + 1.57X_3 + 0.77\ X_8. \quad (3)$$

Table 2 – *PB design with coded values along with the predicted and observed results*

Trial	Coded variable level								Lipase activity (U mL^{-1})	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Observed	Predicted
1	1	1	–1	1	1	1	–1	–1	26.00	26.46
2	–1	1	1	–1	1	1	1	–1	30.38	29.60
3	1	–1	1	1	–1	1	1	1	21.38	21.70
4	–1	1	–1	1	1	–1	1	1	28.50	28.00
5	–1	–1	1	–1	1	1	–1	1	21.75	21.70
6	–1	–1	–1	1	–1	1	1	–1	17.38	17.02
7	1	–1	–1	–1	1	–1	1	1	17.75	18.56
8	1	1	–1	–1	–1	1	–1	1	28.38	28.00
9	1	1	1	–1	–1	–1	1	–1	28.20	29.60
10	–1	1	1	1	–1	–1	–1	1	31.35	31.14
11	1	–1	1	1	1	–1	–1	–1	20.88	20.17
12	–1	–1	–1	–1	–1	–1	–1	–1	17.05	17.02

Table 3 – *Regression analysis of the Plackett–Burman design for lipase activity*

Sources	Stdized effects	Contribution (%)	Prob > *F*
Model			<0.0001
Glycerol	–0.64	0.39	0.1036
NH_4Cl	9.44	86.58	<0.0001
$(NH_4)_2SO_4$	3.15	9.63	<0.0001
$MgSO_4$	0.33	0.11	0.2658
$MnSO_4$	0.25	0.062	0.4203
$CaCl_2$	0.26	0.064	0.3752
$Na_2HPO_4 \cdot 2H_2O$	–0.30	0.089	0.2908
K_2HPO_4	1.54	2.30	0.0081

According to the Plackett–Burman design, the optimum medium compositions were as follows: 8 g L^{-1} glycerol, 12 g L^{-1} NH_4Cl, 4 g L^{-1} $(NH_4)_2SO_4$, 0.8 g L^{-1} $MgSO_4$, 1.2 g L^{-1} $MnSO_4$, 0.8 g L^{-1} $CaCl_2$, 7.2 g L^{-1} $Na_2HPO_4 \cdot 12H_2O$ and 3.5 g L^{-1} K_2HPO_4.

Optimization by steepest ascent path

Plackett–Burman design experiment proved to be a valuable tool for screening significant variables that affected the lipase activity, but it was unable to predict the optimum levels of the compositions.[19] Based on the modified first-order equation obtained and regression results, it was predicted that NH_4Cl, $(NH_4)_2SO_4$ and K_2HPO_4 were significant factors, which meant that increasing the concentration of X_2, X_3 and X_8 had positive effect on the lipase activity. The path of the steepest ascent was determined to find the proper direction of changing factors above to improve lipase activity. The experimental design of the steepest ascent and corresponding results are shown in Table 4. Regarding the results from the steepest ascent path, it was apparent that the yield profile showed a maximum 71.75 U mL^{-1} at run 7. Consequently, this point was near to the region of maximum lipase activity response. So these variables i.e. 40 g L^{-1} NH_4Cl, 18 g L^{-1} $(NH_4)_2SO_4$, 10.6 g L^{-1} K_2HPO_4 were chosen for further optimization.

Response surface methodology

Once the ranges of relevant variables were selected through the PB screening and experiment of steepest ascent path, significant gross curvature had been detected in the design space, central composite design (CCD) and response surface methodology (RSM) were employed to make a quadratic model, consisting of trials plus a star configuration to appraise quadratic results and central points to estimate the pure process variability and reassess gross curvature,[20] with lipase activity as response. RSM is an effective statistical modeling technique that performs multiple regression analysis of quantitative data to solve multivariable problems simultaneously.[21] Three compositions i.e. NH_4Cl, $(NH_4)_2SO_4$ and K_2HPO_4 that significantly affected lipase activity were optimized by RSM using a 3-factor-5-level CCD. The CCD matrix included six central points and six axial points, with an axial distance of ±1.68 to make the design orthogonal.[22] Therefore, a group of 20 treatment combinations with different combinations of NH_4Cl, $(NH_4)_2SO_4$ and K_2HPO_4 were performed (Tables 5 and 6). The results were analyzed by standard analysis of ANOVA and following quadratic regression an equation was obtained in terms of lipase activity.

Table 4 – *Experimental design and results of the steepest ascent path*

Trial	NH_4Cl (g L^{-1})	$(NH_4)_2SO_4$ (g L^{-1})	K_2HPO_4 (g L^{-1})	Lipase activity (U mL^{-1})
Origin	12	4	3.6	30.03
1	16	6	4.6	35.63
2	20	8	5.6	38.53
3	24	10	6.6	43.70
4	28	12	7.6	50.52
5	32	14	8.6	56.55
6	36	16	9.6	59.88
7	40	18	10.6	71.75
8	44	20	11.6	66.8
9	48	22	12.6	53.25

Table 5 – *Coded and real values of variables in central composite design*

Factors	Levels of factors				
	–1.68	–1	0	1	1.68
A: NH_4Cl (g L^{-1})	31.59	35	40	45	48.41
B: $(NH_4)_2SO_4$ (g L^{-1})	12.95	15	18	21	23.05
C: K_2HPO_4 (g L^{-1})	7.24	8.6	10.6	12.6	13.97

The highest activity of the lipase observed was 91.17 U mL^{-1} at run 8 (Table 6). The *F*-value of 1117.29 implies the model is significant, because there is only a 0.01 % chance that the "Model *F*-Value" could occur due to noise (Table 7). Regression equation obtained after ANOVA indicated that the *R*-Squared value of 0.999 (a value of

Table 6 – *Observed and predicted values of lipase activity for the CCD matrix*

Trial	Coded variable level			Enzyme activity (U mL^{-1})	
	A	*B*	*C*	Observed	Predicted
1	–1	–1	–1	71.91	71.71
2	1	–1	–1	75.58	75.77
3	–1	1	–1	78.31	78.50
4	1	1	–1	87.35	87.38
5	–1	–1	1	78.08	78.03
6	1	–1	1	88.17	87.95
7	–1	1	1	76.82	76.60
8	1	1	1	91.17	91.34
9	–1.68	0	0	69.60	69.75
10	1.68	0	0	85.67	85.55
11	0	–1.68	0	74.96	75.11
12	0	1.68	0	83.79	83.67
13	0	0	–1.68	81.77	81.63
14	0	0	1.68	90.10	90.27
15	0	0	0	82.55	82.43
16	0	0	0	82.75	82.43
17	0	0	0	82.08	82.43
18	0	0	0	82.36	82.43
19	0	0	0	82.51	82.43
20	0	0	0	82.32	82.43

Table 7 – *Analysis of variance for the second-order polynomial model for optimization of lipase activity*

Source	SS	DF	MS	*F*-value	Prob>*F*
Model	628.63	9	69.85	1117.29	< 0.0001
Residual	0.63	10	0.063		
Lack of fit	0.26	5	0.072	1.38	0.3662
Pure error	0.36	5	0.053		
Total	629.26	19			

R^2 = 0.9990; Pred R^2 = 0.9946; *CV* % = 0.31; Adeq Precision = 122.138; Adj R^2 = 0.9981; SS, sum of squares; DF, degrees of freedom and MS, mean square.

R-Squared > 0.75 indicated the aptness of the model) was in reasonable agreement with the adjusted *R*-Squared of 0.9981, and this ensured a satisfactory adjustment of the quadratic model to the experimental data. "Adeq. Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable, and the ratio of 122.138 indicates an adequate signal. This model can be used to navigate the design space (Table 7), and a low coefficient of variation (CV = 0.31 %) demonstrated the experiments were precise and reliable. A second-order polynomial function was fitted to the experimental lipase activity, resulting in the following regression equation:

$$\text{Lipase activity} = 82.43 + 4.70\,A + 2.55\,B + \\ + 2.57\,C + 1.20\,AB + 1.47\,AC - \\ - 2.05\,BC - 1.69\,A^2 - 1.02\,B^2 + 1.25\,C^2 \quad (4)$$

Figs. 1–3 show the response surface plots and their contour plots of lipase activity. Evidently, lipase activity varied significantly with the concentration of NH_4Cl, $(NH_4)_2SO_4$ and K_2HPO_4. The op-

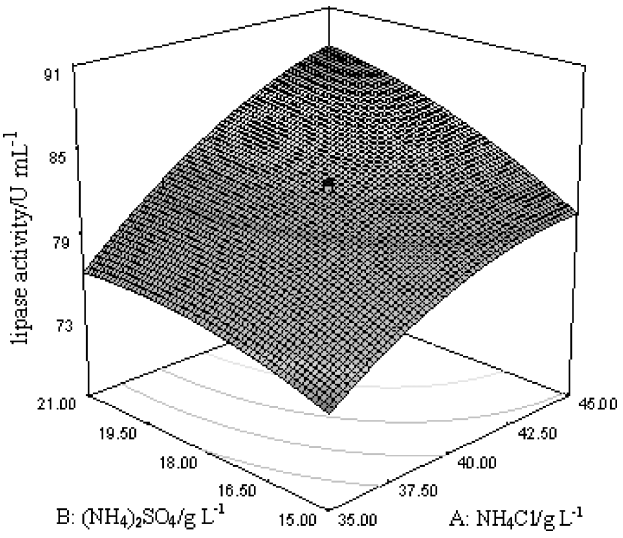

Fig. 1 – *Response surface plot and its contour plot of lipase activity showing the interaction between $(NH_4)_2SO_4$ concentration and NH_4Cl concentration at C = 0*

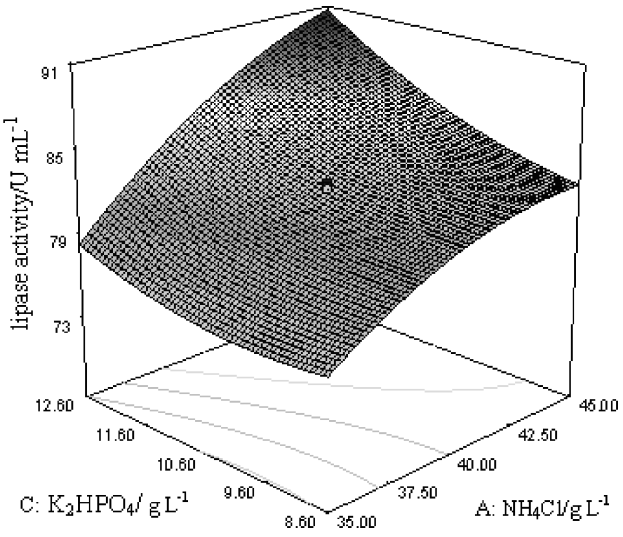

Fig. 2 – *Response surface plot and its contour plot of lipase activity showing the interaction between K_2HPO_4 concentration and NH_4Cl concentration at B = 0*

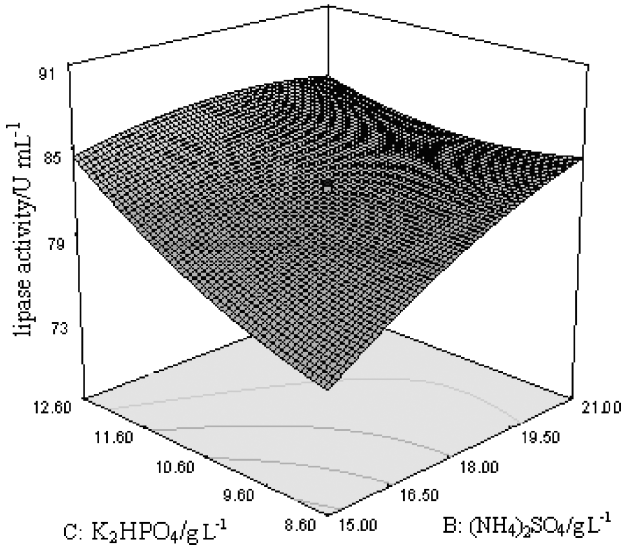

Fig. 3 – *Response surface plot and its contour plot of lipase activity showing the interaction between K_2HPO_4 concentration and $(NH_4)_2SO_4$ concentration at A = 0*

timum value of each variable was identified based on the hump in the three dimensional plot, or from the central point of the corresponding contour plot. The results predicted by the model equation from RSM indicated that a combination of adjusting the concentration of NH_4Cl to 45 g L^{-1}, $(NH_4)_2SO_4$ to 20.4 g L^{-1}, and K_2HPO_4 to 12.6 g L^{-1} would favor maximum lipase activity, giving 91.39 U mL^{-1}.

In order to confirm the optimized results, the culture of *Bacillus subtilis* CICC 20034 was studied using predicted medium compositions. Maximum rate of lipase activity was observed as 91.46 U mL^{-1} at 24 h, which was almost equal to the actual predicted value. By means of optimizing medium compositions, the activity of lipase was enhanced from 18.5 U mL^{-1} to 91.46 U mL^{-1}, and 4.94-fold increase had been obtained. This result therefore corroborated the predicted values and the effectiveness of the model, which indicated that the optimized medium was propitious to the production of lipase from *Bacillus subtilis*.

The optimized concentration of mineral nitrogen sources was very high (45 g L^{-1} NH_4Cl and 20.4 g L^{-1} $(NH_4)_2SO_4$) in this study. Furthermore, it was found that organic nitrogen sources were not favorable to the lipase activity, and the enzyme activity obtained was only about one third of used mineral nitrogen sources in the one-factor-at-a-time experiments although good growth seemed to require organic nitrogen sources (data not shown). In addition, the same conclusion was also obtained with complex (organic and mineral) nitrogen sources, and the results were agreed with the former research with *Rhodotorula glutinis*.[23] The reason was probably the characteristic of the strain, and mineral nitrogen sources could promote the lipase activity of *Bacillus subtilis* strain CICC 20034, and then obtained higher enzyme activity.

Conclusions

Statistically based experimental designs proved effective in optimizing culture medium for lipase activity by *Bacillus subtilis* strain CICC 20034. The optimized medium compositions were as follows: 8 g L^{-1} glycerol, 45 g L^{-1} NH_4Cl, 20.4 g L^{-1} $(NH_4)_2SO_4$, 0.8 g L^{-1} $MgSO_4$, 1.2 g L^{-1} $MnSO_4$, 0.8 g L^{-1} $CaCl_2$, 7.2 g L^{-1} $Na_2HPO_4 \cdot 12H_2O$ and 12.6 g L^{-1} K_2HPO_4, which resulted in about 5 times the lipase activity than that using the original medium. Validation experiments were also carried out to verify the adequacy and accuracy of the model, and the results showed that the predicted value agreed well with the experimental values. The optimum culture medium obtained in this experiment gives a basis for further study with large-scale batch fermentation in a bioreactor for lipase from this strain.

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List of symbols

Y – response variable, U mL^{-1}
b_0 – interception coefficient
b_i – linear coefficient
k – number of involved variables
b_{ii} – coefficient of quadratic effect
b_{ij} – coefficient of interaction effect ($i < j$)
R^2 – determination coefficient

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