Korean J. Chem. Eng., **27**(4), 1233-1238 (2010)
DOI: 10.1007/s11814-010-0195-6

Modeling and optimization of cutinase production by recombinant *Escherichia coli* based on statistical experimental designs

Jianghua Li*,, Long Liu*,**, Jing Wu***, Jun Sun****, Guocheng Du*,**, Jian Chen*,***, and Wenyi Tao*,**,†**

*School of Biotechnology, Jiangnan University, Wuxi 214122, China
**Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China
***State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China
****Institute of Information Technology, Jiangnan University, Wuxi 214122, China
(*Received 3 September 2009 • accepted 30 November 2009*)

Abstract−Statistics-based experiment designs were used to optimize the culture medium (glucose, yeast extract, IPTG, tween-60, and $CaCl_2$) for cutinase production by recombinant *Escherichia coli*. A 2^{5-1} fractional factorial design augmented with center points revealed that glucose, yeast extract, and IPTG were the most significant factors, whereas the other factors were not important within the levels tested. The method of steepest ascent was used to approach the proximity of optimum, followed by a central composite design to develop a response surface for culture condition optimization. The optimum culture medium for cutinase production was found to be: glucose 33. 92 g/L, yeast extract 30.92 g/L, and IPTG 0.76 g/L. A cutinase production of 145.27±1.5 U/mL, which was in agreement with the prediction, was observed in triplicate verification experiments. The results obtained here verified the effectiveness of the applied methodology and may be helpful for cutinase production on an industrial scale.

Key words: Cutinase, Recombinant *Escherichia coli*, Optimization, Statistical-based Experimental Design

INTRODUCTION

Cutinases are extracellular enzymes that hydrolyze ester bonds in cutin [1] and belong to the family of serine hydrolases containing the so-called α/β hydrolaes fold [2]. Cutinases display hydrolytic activity on soluble synthetic esters and insoluble long-chain triacylglycerols, and perform transesterification of fats and oils and (stereo) selective esterification of alcohols [3]. Therefore, cutinases have great potential applications in the dairy industry for the hydrolysis of milk fat, in household detergents, in the oleochemical industry, in the synthesis of structured triglycerides, polymers and surfactants, ingredients for personal-care products, and pharmaceuticals [4,5]. Recently, applications of cutinase in the textile industry have also gained considerable attention [1,6]. Compared with traditional techniques in cotton desizing, cutinase improves cotton wettability and fabric hydrophilicity in addition to its ability to simplify processes, reduce working time, save production cost, and alleviate environmental pollution [4,5].

With expanding cutinase applications, microbial cutinase production has attracted increasing attention [7-11]. Cutinase can be produced by both fungi and bacteria. Up to now, most studies have focused on the selection of cutinase-producing strains [12], process optimization of microbial cutinase production [13-16], and cutinase extraction and characterization [17-19]. Calado et al. developed a fed-batch culture strategy for enhanced production and secretion of cutinase by a recombinant S*accharomyces cerevisiae* SU50 strain [14]. Ferreira et al. developed a cost-effective strategy for cutinase production by a recombinant *Saccharomyces cerevisiae* [10]. Fett et al. systematically studied the microbial cutinase production by different strains [7,12,15,20]. Pio and Macedo optimized the production of cutinase by *Fusarium oxysporum* using response surface methodology [11], and Rispoli and Shah optimized the culture medium for the cutinase production from *Colletotrichum lindemuthianum* [16].

In our previous work, significant enhancement of cutinase production by *Thermobifida fusca* was achieved with two-stage pH control [5] and two-stage batch and fed-batch cultivation [21]. After optimization of culture conditions, the maximal cutinase activity reached 51.0 U/mL. However, problems remain with regard to cutinase production by *Thermobifida fusca*, such as the low cutinase activity and high production cost due to the need of cutin as an inducer. Therefore, to further increase cutinase activity and productivity, we constructed a recombinant *E. coli* BL(21)DE3 [22]. The over-expression of a recombinant cell product is often the primary goal in a biotechnological process. In this regard, several strategies such as strain development, culture condition optimization, and mathematical modeling have been widely used. For a certain biotechnological process, developing an appropriate culture medium is of crucial importance because the culture medium can significantly affect product yield [23]. The traditional method of optimization involves varying one factor at a time, while keeping the other constant. Though the "one-at-a-time" strategy is simple and easy to implement, this approach is not only time consuming, but also ignores the possible interactions of all the factors involved in a bioprocess. The deficiency can be overcome by applying more efficient, statistically based experimental design. In this respect, fractional factorial design and response surface analysis are important tools to determine optimal process conditions. The statistics-based experimental designs for optimization have been used in many areas of biotechnology such

†To whom correspondence should be addressed.
E-mail: wytao@jiangnan.edu.cn

as optimization of a culture medium [23], enzyme production [11], ethanol production [24], and biomass production [25].

The aim of this work was to optimize the culture medium to achieve maximum cutinase production by recombinant *E. coli BL(21)DE3 based on* statistics-based methodologies. First, a 2^{5-1} fractional factorial design identifies the most significant factors affecting cutinase production; subsequently, a steepest ascent method locates the neighborhood of an optimum, and finally a central composite design builds a response surface for culture medium optimization.

MATERIALS AND METHODS

1. Microorganism and Media

The recombinant strain *E. coli BL(21)DE3* was used in this study. The plasmid had PELB signal peptide and ampicillin resistance, and IPTG was the inducer of cutinase production.

The seed culture medium consisted of (g/L): Peptone 10, NaCl 10, yeast extract 5, and ampicillin 0.1. Non-optimized fermentation medium was composed of (g/L): glucose: 15, yeast extract 10, $CaCl_2$ 0.08, Tween-60 (T-60) 0.07, IPTG 0.4. The initial pH of the culture medium was adjusted to 7.2.

2. Microbial Cutinase Production by Shaker Flask Cultivation

100 μL of strain solution in glycerol tube was transferred to 25 mL seed culture medium in 250 mL flasks and grown in a rotary shaker at 200 rpm and 37 °C for 6 h. The seed culture was inoculated into the flasks with an inoculum of 1% when the optical density (OD) of the seed culture at 600 nm reached 4.0. The shaker was operated at 200 rpm and 37 °C. The sample was taken for analysis after cultivating for 32 h.

3. Cutinase Activity Analysis

Cutinase esterolytic activity was determined by using the colorimetric procedure with *p*-nitrophenyl butyrate (PNB) as the substrate as described by Calado et al. [8]. The cutinase activity here referred to the total cutinase activity including intracellular and extracellular cutinase activity. Ultrasonication was applied to disrupt the cell for the measurement of intracellular cutinase activity. One unit of cutinolytic activity was defined as the amount of cutinase that catalyzes PNB and generates one micromole of *p*-nitrophenol in 1 min at 20 °C.

EXPERIMENTAL DESIGN AND DATA ANALYSIS

A three-step experimental design was used in developing a model for optimal cutinase production in this work. The variables were coded according to the following equation:

$$x_i=(X_i-X_{i,0})/DX_i \quad (1)$$

where x_i is the coded value of an independent variable, X_i is the independent variable's real value, $X_{i,0}$ is the independent variable's real value at the center point, and ΔX_i is the step change value. The cutinase concentration was taken as the dependent variable or response.

1. Fractional Factorial Design

The first task consisted of a fractional factorial design (FFD) to identify medium components that had a significant effect on the recombinant cutinase production. The major benefit of applying a fractional design is the reduced number of experiments that need to be carried out to obtain maximum information. Statistical optimization not only allows a quick search of a large experimental domain with considerably fewer trials, but also reflects a factor's role in the medium. Moreover, the interaction between different variables can be estimated. Finally, the factorial design allows the effect of a given factor to be determined at several levels of the other factors, so the conclusions are valid over a range of experimental conditions. There are five independent variables in this work and each one was regarded as a factor in our optimization procedures. For a moderately large number of factors, smaller fractions of the 2^k design are frequently useful. A first-order model was then fitted to the data obtained from the fractional factorial design experiments. For regions remote from the maximum, this first-order approximation is sufficient. The response surface is hence represented locally by a sloping plane.

2. The Path of Steepest Ascent

The next task was to move rapidly towards the neighborhood of the optimum response based on the method of steepest ascent, that is, the direction along which the response increases rapidly. If one imagines a normal path from the fitted first-order model resulting from the fractional factorial design towards the proximity of optima, then the direction will be parallel to this normal vector. One usually considers the center of region as the origin for the path of steepest ascent. Experiments may be conducted along this path until no further increase in the response is observed. The maximum point along the path leads to the vicinity of an optimum.

3. Central Composite Design

The objective of the third experiment is to develop an empirical model of the process and to obtain a more precise estimate of an optimum operating condition for the factors involved. This approach to process optimization is called response surface methodology and the second design is a central composite design, one of the most important experimental designs used in process optimization studies. For predicting the optimal point, a second-order polynomial function was fitted to the experimental results.

$$y=b_0+\sum b_i x_i+\sum b_j x_j^2+\sum b_{ij} x_i x_j+e \quad (2)$$

where y is the predicted response, x_i and x_j stand for the independent variables, b_0 is the intercept, b_i and b_j are regression coefficients, and e is a random error component.

4. Data Analysis

Design-Expert 7.1.6 Trial version was used for the regression analysis of the experimental data obtained. The quality of fit of the polynomial model equation is expressed by the coefficient of determination R^2, and its statistical significance is checked by the F-test. The significance of the regression coefficient was tested by the *t*-test. The level of significance was given as values of Prob>F less than 0.05. A differential calculation was then employed for predicting the optimum point.

RESULTS AND DISCUSSION

1. Fractional Factorial Design

The purpose of the first optimization step was to identify the components of the medium that have a significant effect on cutinase production. Five medium ingredients were assessed, and the range and levels of the variables are given in Table 1.

A full factorial design would need 32 experiments, which is a

Table 1. Applied levels of independent variables in the fractional factorial design

Variable	Component	Applied levels (g/L)	
		−1 (Low)	+1 (High)
X_1	Glucose	10	30
X_2	Yeast extract	5	25
X_3	IPTG	0.2	0.8
X_4	Tween-60	0.03	0.20
X_5	$CaCl_2$	0.05	0.15

Table 2. Fractional factorial experimental design and results

Run	Factor					Cutinase (U/mL)	
	x_1	x_2	x_3	x_4	x_5	Observed[a]	Predicted[b]
1	−1	−1	−1	−1	+1	61.12	59.54
2	+1	−1	−1	−1	−1	70.25	64.66
3	−1	+1	−1	−1	−1	95.18	90.63
4	+1	+1	−1	−1	+1	93.27	102.85
5	−1	−1	+1	−1	−1	75.19	73.99
6	+1	−1	+1	−1	+1	83.45	84.24
7	−1	+1	+1	−1	+1	104.27	105.58
8	+1	+1	+1	−1	−1	120.69	115.81
9	−1	−1	−1	+1	−1	60.35	61.21
10	+1	−1	−1	+1	+1	78.13	73.44
11	−1	+1	−1	+1	+1	102.89	93.79
12	+1	+1	−1	+1	−1	95.07	104.02
13	−1	−1	+1	+1	+1	67.03	76.16
14	+1	−1	+1	+1	−1	90.25	86.40
15	−1	+1	+1	+1	−1	101.13	106.75
16	+1	+1	+1	+1	+1	125.90	118.97
17	0	0	0	0	0	100.56	89.01
18	0	0	0	0	0	98.01	89.01
19	0	0	0	0	0	102.36	89.01
20	0	0	0	0	0	101.78	89.01
21	0	0	0	0	0	97.09	89.01

high number. Consequently, a 2^{5-1} fractional factorial design consisting of 16 factorial runs along with 5 other experiments at the center of the design for analysis of variance was performed. The experimental design and the results of the fractional factorial design are summarized in Table 2 with the levels in coded units. The cutinase concentrations shown are the mean values of triplicate experiments. The values of the regression coefficient were calculated and the first-order model could be written from the coefficients:

$$y_{cutinase}=89.01+5.62x_1+15.79x_2+6.98x_3+1.08x_4+0.50x_5 \quad (3)$$

Based on these experimental results, statistical analysis was carried out using Fisher's statistical test. The model F-value of 27.86 in Table 3 implies that the model is significant. There is only a 0.01% chance that a "Model F-value" this large could occur due to noise. Values of "P>F" less than 0.05 indicate model terms are significant. In this case the variables X_1 (glucose), X_2 (yeast extract), X_3 (IPTG) have significant effects, while the variables X_4 (Tween-60) and X_5 ($CaCl_2$) have no significant effects on the cutinase production.

Table 3. Analysis of variance for the first-order model from fractional factorial design

Source of variance	SS_i	*Degree of freedom*	MS_i	F	P>F
Model	5295.26	5	1059.05	27.86	<0.0001
X_1	504.56	1	504.56	13.27	0.0027
X_2	3988.87	1	3988.87	104.93	<0.0001
X_3	779.11	1	779.11	20.50	0.0005
X_4	18.77	1	18.77	0.49	0.4938
X_5	3.95	1	3.95	0.10	0.7519
Curvature	456.72	1	456.72	12.01	0.0038
Residuals	532.18	14	38.01		
Pure error	21.47	4	5.37		
Total	6284.17	20			

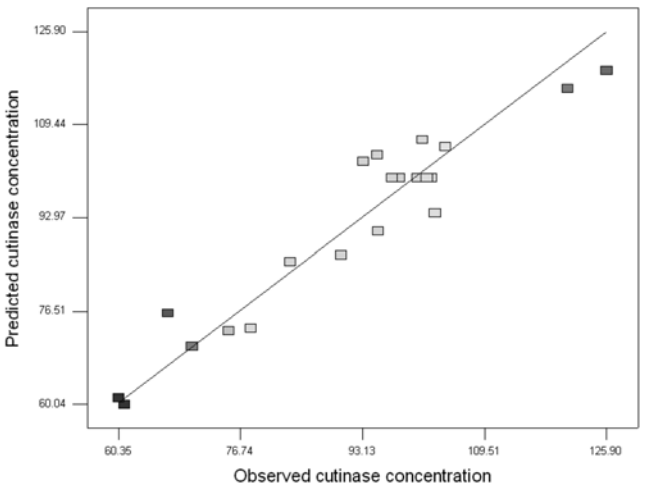

Fig. 1. Plots of observed vs. predicted cutinase concentration.

The coefficient of determination R^2 of the model was calculated to be 90.87%. This indicates that the model could explain 90.87% of the variability for cutinase production. This ensured a satisfactory adjustment of the first-order model to the experimental data. The adequate precision value measures signal-to-noise ratio and a ratio greater than 4 is desirable. The adequate precision value of 16.56 indicated the adequate signals, and the model can be used to navigate the design space for further optimization. Fig. 1 represents the relationship between the observed cutinase concentration values and the predicted values determined by the model Eq. (3). It can be seen that most points are nearby the line adjustment, which means that the values determined experimentally are similar to those determined by the model.

2. The Path of Steepest Ascent

The path of steepest ascent was determined by Eq. (3). Tween-60 and $CaCl_2$ concentrations were fixed at the center of the fractional factorial design, because they were not significant at the probability level of 95%. Since the signs of the three significant factors in the first-order model (Eq. (3)) are positive, they are expected to have a positive impact on cutinase production if their concentration in the medium is increased from the concentration variable space already covered in the factorial design. Table 4 illustrates the results

Table 4. Experimental design of the steepest ascent and corresponding results

	Coded values			Real variables (g/L)			Cutinase (U/mL)
Run	x_1	x	x	X_1	X_2	X_3	y
Origin	0.0	0.0	0.0	20	15	0.50	99.96
1	0.4	0.5	0.3	24	20	0.59	108.65
2	0.8	1.0	0.6	28	25	0.68	115.76
3	1.2	1.5	0.9	32	30	0.77	121.67
4	1.6	2.0	1.2	36	35	0.86	117.34
5	2.0	2.5	1.5	40	40	0.95	113.25
6	2.4	3.0	1.8	44	45	1.04	109.12

of the experiment as well as the directions the variables were changed, starting from the center point of the fractional factorial design. The response for this point was determined as the average of Runs 17 to 21 that appear in Table 2. Regarding the results from the path of steepest ascent, it is clearly seen that the yield profile shows a maximum cutinase production of 121.67 U/mL for Run 3 in Table 4. Subsequently, the composition of this run became the starting point for further optimization in the next section.

3. Central Composite Design

As seen above, a neighborhood of an optimum was approached along the path of steepest ascent. To explain the nature of the response surface in the optimum region, a central composite design was performed, and the levels of the three significant variables, glucose (X_1), yeast extract (X_2), and IPTG (X_3) were further optimized. For the three factors, this design comprised a full 2^3 factorial design with its 8 cubic corner points, augmented with 6 replicates of the center point, and the 6 axial points. The level of the two non-significant factors (Tween-60 and $CaCl_2$) was kept at the central point of the fractional factorial design. The range of the variables investigated in this step is given in Table 5, and the experimental design and the results are presented in Table 6. The experimental results of the central composite design were fitted with a second-order polynomial expression. The regression coefficients were calculated and the fitted equation (in terms of coded values) for predicting cutinase production was:

$$y=147.97+7.04x_1+3.35x_2+1.21x_3+4.55x_1x_2 -1.80x_1x_3-4.63x_2x_3-7.30x_1^2-7.32x_2^2-9.05x_3^2 \quad (4)$$

This equation includes all terms regardless of their significance. The coefficient of determination R^2 of the model was calculated to be 87.92%. This indicates that the model could explain 87.92% of the variability for cutinase production and the full quadratic model was a good fit. As shown in Table 7, the model *P*-value of 0.0015 implies that the model is a significant fit. The model *F*-value of 8.09 implies the model is significant and there is only a 0.15% chance that a model *F*-value this large could occur due to noise. The "lack of fit *F*-value" of 0.32 implies the lack of fit is not significant relative to the pure error.

The maximum point of the model Eq. (4) is at x_1=0.64 (glucose 33.92 g/L), x_2=0.46 (yeast extract 30.92 g/L), and x_3=−0.12 (IPTG 0.76 g/L). The model predicted a maximum response for cutinase concentration of 150.93 U/mL at this point. To confirm the predicted results of the model, triplicate experiments at the optimal condition were carried out and a value of 145.27±1.50 U/mL was ob-

Table 5. Levels of the factors for central composite design

	Applied levels (g/L)				
Variables	−1.68	−1	0	1	1.68
X_1	26.96	29	32	35	37.04
X_2	26.64	28	30	32	33.36
X_3	0.72	0.74	0.77	0.80	0.82

Table 6. Experimental design and results of the central composite design

	Coded levels			Cutinase (U/mg)	
Run	x_1	x_2	x_3	Experimental	Predicted
1	−1	−1	−1	110.13	110.83
2	+1	−1	−1	121.25	119.39
3	−1	+1	−1	116.76	117.69
4	+1	+1	−1	145.81	144.45
5	−1	−1	+1	122.17	126.10
6	+1	−1	+1	125.83	127.47
7	−1	+1	+1	110.02	114.44
8	+1	+1	+1	132.15	134.02
9	−1.68	0	0	120.18	115.55
10	+1.68	0	0	138.09	139.18
11	0	−1.68	0	123.02	121.69
12	0	+1.68	0	135.16	132.95
13	0	0	−1.68	118.14	120.38
14	0	0	+1.68	130.21	124.45
15	0	0	0	132.35	147.97
16	0	0	0	154.23	147.97
17	0	0	0	150.47	147.97
18	0	0	0	148.72	147.97
19	0	0	0	156.09	147.97
20	0	0	0	145.32	147.97

Table 7. Analysis of variance for quadratic response surface model

Source	Sum of squares	*Degree of freedom*	Mean square	*F*-value	*p*-value (Prob>F)
Model	3489.93	9	387.77	8.09	0.0015
x_1	675.96	1	675.96	14.10	0.0038
x_2	153.44	1	153.44	3.20	0.1039
x_3	19.98	1	19.98	0.42	0.5331
x_1x_2	165.62	1	165.62	3.45	0.0927
x_2x_3	25.85	1	25.85	0.54	0.4797
x_1x_3	171.31	1	171.31	3.57	0.088
x_1^2	767.93	1	767.93	16.02	0.0025
x_2^2	771.28	1	771.28	16.09	0.0025
x_3^2	1181.21	1	1181.21	24.64	0.0006
Residual	479.45	10	47.95		
Lack of fit	116.58	5	23.32	0.32	0.8809
Pure error	362.87	5	72.57		
Total	3969.39	19			

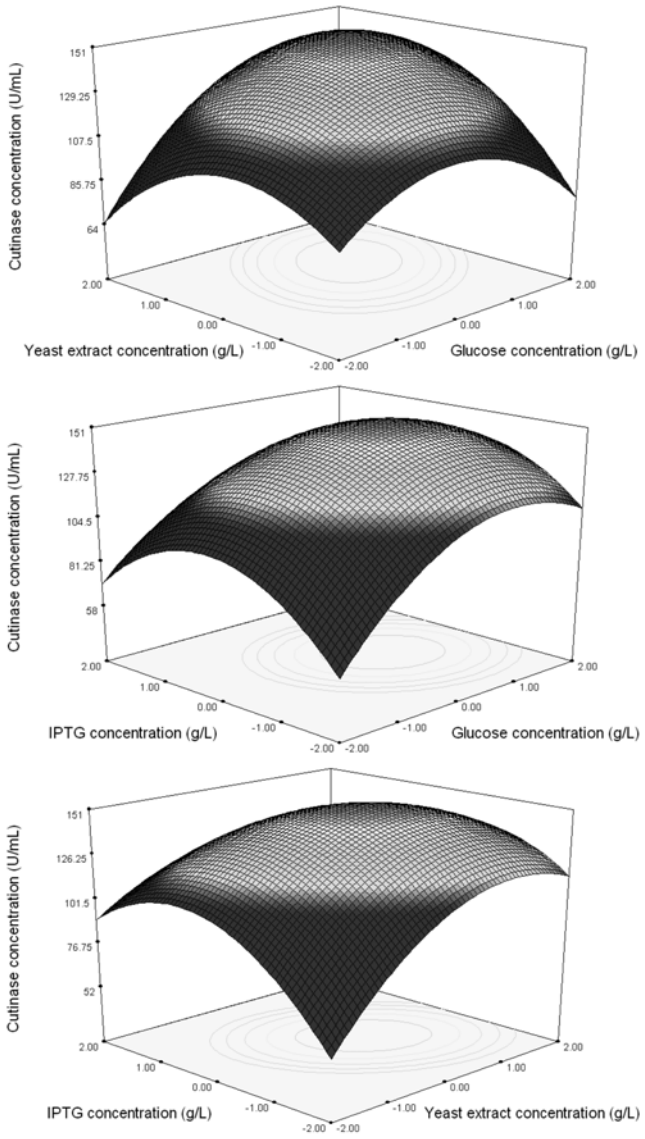

Fig. 2. Response surface of cutinase concentration: (a), (b), and (c) fixed IPTG (0.76 g/L), yeast extract (30.92 g/L), and glucose (33.92 g/L) concentrations at their optimum points, respectively.

tained. The good correlation between these two results verifies the validity of the response model and the existence of an optimal point. The cutinase concentration was increased by 3-fold compared to that without medium optimization. The response surfaces shown in Fig. 2 are based on the quadratic model of Eq. (4), holding one variable constant at its optimum level, while varying the other two within their experimental range. Fig. 3 shows the residuals vs. predicted response as determined from the quadratic model. It shows a nearly constant variance throughout the response range.

A significant enhancement of cutinase concentration was achieved via medium optimization based on a series of statistical experimental designs. In our previous work, the microbial cutinase by *Thermobifida fusca* was produced and the highest cutinase activity was 51 U/mL. [5] The results obtained here revealed that the recombinant *E. coli* was an ideal host for the recombinant cutinase production.

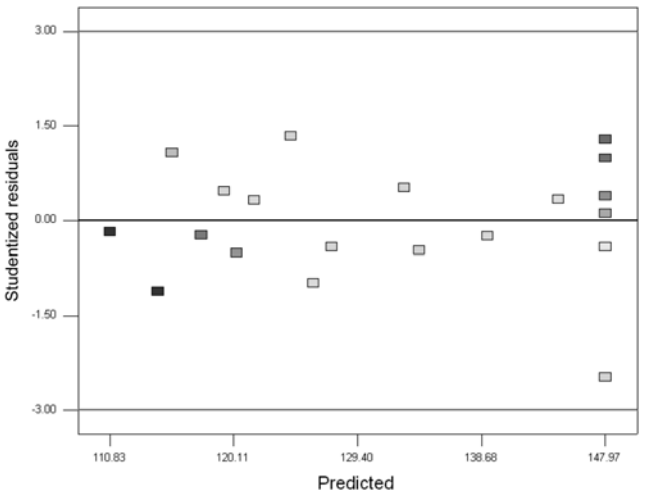

Fig. 3. Studentized residuals vs. predicted response by the obtained quadratic model.

By optimizing the culture medium, the production of cutinase could be greatly improved.

It should be noted that though the proposed approach in this work was effective for the cutinase production, the obtained results were only the local optimum. To reach the global optimization, the artificial neural network coupling quantum-behaving particle swarm optimization (QPSO) algorithm was an ideal alternative, and the related work is being conducted now.

CONCLUSION

Microbial cutinase production has attracted increased attention due to the wide applications of cutinase in the dairy and textile industries. This study proved that statistical experimental design offers an efficient and feasible approach for cutinase fermentation medium optimization. A maximum cutinase production of 150.93 U/mL was achieved with the following optimized medium: glucose 33.92 g/L, yeast extract 30.92 g/L, and IPTG 0.76 g/L. Validation experiments were also carried out to verify the adequacy and the accuracy of the model, and the results showed that the predicted value closely agreed with the experimental values. The results also provide a basis for further study with large scale fermentation for production of cutinase.

ACKNOWLEDGEMENTS

This project was financially supported by the Program for Changjiang Scholars and Innovative Research Team in University (No. IRT0532), the National Science Fund for Distinguished Young Scholars of China (No. 20625619), 973 Project (2007CB714306), the Self-determined Research Program of Jiangnan University (JUSRP30901), and Programs for New Century Excellent Talents in University (NCET-05-0488 and NCET-07-0378).

REFERENCES

1. P. B. Agrawal, V. A. Nierstrasz and M. M. C. G. Warmoeskerken,

Enzyme Microbial. Technol., **42**, 473 (2008).
2. C. M. L. Carvalho, M. R. Aires-Barros and J. M. S. Cabral, *Biotechnol. Bioeng.*, **66**, 17 (1999).
3. C. M. L. Carvalho, M. L. M. Serralheiro, J. M. S. Cabral and M. R. Aires-Barros, *Enzyme Microbial. Technol.*, **21**, 117(1997).
4. J. Chen, Q. Wang, Z. Z. Hua and G. C. Du, *Enzyme Microbial. Technol.*, **40**, 1651 (2007).
5. G. C. Du, S. L. Zhang, Z. Z. Hua, Y. Zhu and J. Chen, *Biotechnol. J.*, **2**, 365 (2007).
6. C. Silva, R. Araújo, M. Casal, G. M. Gübitz and A. Cavaco-Paulo, *Enzyme Microbial. Technol.*, **40**, 1678 (2007).
7. W. F. Fett, H. C. Gerard, R. A. Moreau, S. F. Osman and L. E. Jones, *Curr. Microbiol.*, **25**, 165 (1992).
8. C. R. C. Calado, S. M. S. Monteiro, J. M. S Cabral and L. P. Fonseca, *J. Biosci. Bioeng.*, **93**, 354 (2002).
9. C. R. C. Calado, M. A. Taipa, J. M. S. Cabral and L. P. Fonseca, *Enzyme Microbial. Technol.*, **31**, 161 (2002).
10. B. S. Ferreira, C. R. C. Calado, V. F. Keulen, L.P. Fonseca, J. M. S. Cabral and M. M. R. Fonseca, *Appl. Microbiol. Biotechnol.*, **61**, 69 (2003).
11. T. F. Pio and G. A. Macedo, *Enzyme Microbial. Technol.*, **41**, 613 (2007).
12. W. F. Fett, H. C. Gerard, R. A. Moreau, S. F. Osman and L. E. Jones, *Appl. Environ. Microbiol.*, **58**, 2123 (1992).
13. C. R. C. Calado, M. Mannesse, M. Egmond and J. M. S. Cabral, *Biotechnol. Bioeng.*, **78**, 692 (2002).
14. C. R. C. Calado, C. Almeida, J. M. S. Cabral and L. P. Fonseca, *J. Biosci. Bioeng.*, **96**, 141 (2003).
15. W. F. Fett, C. Wijey and R. A. Moreau, *J. Appl. Microbiol.*, **86**, 561 (1999).
16. F. J. Rispoli and V. Shah, *J. Ind. Microbiol. Biotechnol.*, **34**, 349 (2007).
17. M. T. Cunha, M. J. L. Costa, C. R. C. Calado, L. P. Fonseca, M. R. Aires-Barros and J. M. S. Cabral, *J. Biotechnol.*, **100**, 55 (2003).
18. C. R. C. Calado, B. S. Ferreira, D. M. M. R. Fonseca, J. M. S. Cabral and L. P. Fonseca, *J. Biotechnol.*, **109**, 147 (2004).
19. C. Kepka, E. Collet, J. Persson, Å. Ståhl, T. Lagerstedt, F. Tjerneld and A. Veide, *J. Biotechnol.*, **103**, 165 (2003).
20. W. F. Fett, C. Wijey, R. A. Moreau and S. F. Osman, *Lett. Appl. Microbiol.*, **31**, 25 (2000).
21. G. Q. He, G. H. Huo, L. M. Liu, Y. Zhu, G. C. Du and J. Chen, *Biotechnol. Biopro. E.*, **14**, 46 (2009).
22. S. Chen, X. Tong, R. W. Woodard, G. C. Du, J. Wu and J. Chen, *J. Biol. Chem.*, **283**, 25854 (2008).
23. T. F. Pio and G. A. Macedo, *J. Ind. Microbiol. Biotechnol.*, **35**, 59 (2008).
24. E. Mübeccel and S. F. Mutlu, *Bioresour. Technol.*, **73**, 251 (2000).
25. X. Yu and A. K. Watson, *Appl. Microbiol. Biotechnol.*, **47**, 301 (1997).
26. L. Liu, J. Sun, D. X. Zhang, G. C. Du, J. Chen and W. B. Xu, *Enzyme Microbial. Technol.*, **44**, 24 (2009).