African Journal of Biotechnology Vol. 10(1), pp. 75-84, 3 January, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1006
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Medium optimization for endochitinase production by recombinant *Pichia pastoris* ZJGSU02 using response surface methodology

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Accepted 10 December, 2010

Plackett-Burman design and response surface methodology were employed to optimize the medium components for endochitinase production by *Pichia pastoris*. A Plackett- Burman design of seven factors with 12 runs was applied to evaluate the effects of different medium components. Yeast extract, oleic acid and Tween-80 were found to have significant influence on endochitinase production. The optimal concentrations of three factors were investigated by the response surface methodology using Box- Behnken design. The optimal medium components obtained for achieving the maximum activity of the endochitinase were as follows: Yeast extract 24.36 g/l, tryptone 20 g/l, YNB 5.0 g/l, potassium phosphate 100 mM, methanol 5 ml/l, oleic acid 1.758 ml/l, Tween-80 6.2 ml/l, Pichia trace metals (PTM_1) 4.0 ml/ l and biotin 4.00 × 10^{-4} g/l. Under these conditions, endochitinase activity was up to 88.26 µ/ml, which was about 1.14-fold higher than using the original medium (77.62 µ/ml). This work will be very helpful for large-scale production of endochitinase for future industrial application.

Key words: Recombinant endochitinase, Plackett-Burman design, response surface methodology, fermentation.

INTRODUCTION

Endochitinase (EC 3.2.1.14) is an important enzyme which can randomly hydrolyze internal β-1,4-glycoside bonds of linear chitooligosaccharide, which is the major building block of chitin (Fan et al., 2007; Yan et al., 2007; Yu and Li, 2008). This enzyme is of extremely great significance in present day biotechnology with applications ranging from medicine, chemical engineering, agriculture to environmental protection (Perez-Martinez et al., 2007; Yu and Li, 2008). Furthermore, there has been an increasing interest in the use of endochitinase for the control of moulds, insects and nematodes in recent years (Zhang et al., 2006) and the ability of endochitinase to hydrolyze chitin also makes it very useful for the production of value added products such as sweeteners, growth factors and single cell protein (Felse and Panda, 2000). However, the main drawback of endochitinase production is low yield and high cost (Hirano, 1996) and this enhances the necessity to improve its yield for large scale industrial requirement.

An efficient strategy for enhancing yield of the desired metabolic product in a microbial system is the optimization of medium components (Pankaj et al., 2007). There are two ways by which the problem of fermentation parameters may be addressed; classical and statistical. The classical method is based on the "one-factor-at-a-time" method, in which one independent variable is studied while maintaining all the other factors at a fixed level. This method may lead to unreliable results and inaccurate conclusions. Moreover, it does not guarantee the determination of optimal conditions, and is unable to detect the

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Abbreviations: RSM, Response surface methodology; **4-MU,** 4-methylumbe–lliferyl; **4-MUChT,** 4-methylumbelliferyl β-D-N, N', N''-triacetyl-chitotrioside; PTM_1, pichia trace metals; **YNB,** yeast nitrogen base.

Table 1. Coded and real values of the factors tested in the Plackett-Burman experimental design.

Factor	Levels of factor	
	-1	+1
Yeast extract(X_1, g/l)	5	15
Tryptone (X_2, g/l)	15	45
YNB (X_3, g/l)	5	15
Methanol (X_4, ml/l)	3	10
oleic acid (X_5, ml/l)	0.3	0.9
Tween-80 (X_6, ml/l)	2	6
PTM_1 (X_7, ml/l)	5	15

frequent interactions occurring between two or more factors. Response surface methodology (RSM), this statistical method uses quantitative data from appropriate experiments to determine and simultaneously solve multivariate equations (Gorret et al., 2004; Tokcaer et al., 2006). It is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and analyzing optimum conditions of factors for desirable responses (Coninck et al., 2000; Tatiana and Gabriela, 2007).

Pichia pastoris has been developed as an excellent expression system for enhancing the yield of heterologous protein in the past 20 years (Inan et al., 1999). However, most of the studies on this system are mainly focused on enhancing the yield of the desired protein by DNA manipulation (Cereghino and Cregg, 2006); very little information could be obtained on medium components optimization using statistical approach for higher yield of the desired protein. In the previous study, we cloned the mature endochitinase cDNA from *Trichoderma viride*, integrated it into the genome of *P. pastoris* and screened the strain that could express endochitinase effectively. The purpose of this work was to employ a Plackett-Burman design followed by response surface methodology using a Box-Behnken design to optimize the medium components for obtaining higher endochitinase activity, which has never been reported before.

MATERIALS AND METHODS

Microorganism, medium and reagents

The recombinant *P. pastoris* strain containing the mature endochitinase cDNA sequence, was constructed and maintained on bouillon agar slants containing (g/l); glucose 10, peptone 10, beef extract 10, $NaCl_3$ and agar 20, cultured for 2 days at 30°C, then stored at 4°C and subcultures every two months. The seed medium contained: Yeast extract 10 g/l, tryptone 20 g/l, 100 mM potassium phosphate with pH 6.0, yeast nitrogen base with ammonium sulphate and without amino acids (YNB) 13.4 g/l, biotin 4×10^{-4} g/l and glycerol 10 g/l. All media were autoclaved for 20 min at 121°C. 4-Methylumbe -lliferyl (4-MU) and 4-Methylumbelliferyl β-D-N, N', N''-triacetyl-chitotrioside (4-MUChT) were purchased from Sigma Co. Ltd. Pichia trace metals (PTM_1) solution consisted of the following components: $CuSO_4 \cdot 5H_2O$ 6.0 g/l; $MgSO_4 \cdot 7H_2O$ 3.0 g/l; H_3BO_3 0.02 ml/l; $ZnCl_2$ 20.0 g/l; NaI 0.08 g/l; Na_2MoO_4 0.2 g/l; $CoCl_2$ 0.5 g/l; $FeSO_4 \cdot 2H_2O$ 65 g/l and H_2SO_4 5.0 ml/l. All other reagents were analytical graded and were used as supplied.

Inoculum preparation and flask culture

The strain was transferred from a slant culture into an Erlenmeyer flask (250 ml) containing 25 ml seed medium, followed by incubation at 28°C with vigorous agitation in a shaking incubator at 250 rpm until the culture reached an OD_{600} = 3.0. The cells were harvested by centrifugation at 12,000 rpm for 10 min at 4°C and transferred into an Erlenmeyer flask (250 ml) containing 25 ml induced medium whose components varied, based on the experimental design. Triplicate experiments were carried out and the mean value was calculated.

Endochitinase activity analysis

After the cells were cultured for 48 h, aliquots of the culture were taken out and centrifuged at 10,000 rpm for 10 min and the supernatants were used for the determination of endochitinase activity according to the method of Perez-Martinez et al. (2007) with 4-MUChT as the substrate. The reaction mixture consisted of 50 µl supernatant and 50 µl 0.02 mg/ml of 4-MUChT dissolved in citric acid-disodium hydrogen phosphate buffer solution with pH 5.6. The reaction mixture was incubated for 5 min at 40°C and stopped by adding 1.9 ml of 0.5 M sodium carbonate. Fluorescence was quantified on a fluorometer at 361 nm excitation and 447 nm emission wavelengths. One unit of endochitinase activity was defined as one milliliter of enzyme which produced 1ng of 4-MU/ min at pH 5.6 and 40°C.

Plackett-Burman design

The preliminary experiments revealed that seven factors, including yeast extract, tryptone, yeast nitrogen base (YNB), methanol, oleic acid, Tween-80 and PTM_1, were supposed to have effects on endochitinase production. It was known that the Plackett-Burman design could evaluate the main effects of factors. The factors having significant effects on endochitinase production were identified using this experimental design. As shown in Table 2, the design matrix covered seven factors to evaluate their effects on endochitinase production and endochitinase activity was given as response value. Each factor was investigated at a high (+1) and a low (-1) level (Table 1). The factors, which were significant at 95% of confidence level ($P < 0.05$) from the regression analysis were considered to have greater effects on the endochitinase activity and further optimized by response surface methodology using Box-Behnken design. The first-order model used to fit the results of Plackett-Burman design was represented as

$$Y = \beta_0 + \sum \beta_i x_i \quad (1)$$

Where, Y was the predicted response; β_0 was the intercept; β_i was the linear coefficient and χ_i was the coded independent factor.

Box-Behnken design and response surface methodology

Box-Behnken design and response surface methodology were applied to optimize the three most significant factors (yeast extract, oleic acid and Tween-80) for improving the endochitinase activity, screened by Plackett-Burman design. The three independent

Table 2. Experimental design and results of the N = 12 Plackett-Burman design.

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Endochitinase activity (μ/ml)
1	1	-1	1	-1	-1	-1	1	85.35
2	1	1	-1	1	-1	-1	-1	81.40
3	- 1	1	1	-1	1	-1	-1	83.83
4	1	-1	1	1	-1	1	-1	83.55
5	1	1	-1	1	1	-1	1	83.06
6	1	1	1	-1	1	1	-1	89.62
7	-1	1	1	1	-1	1	1	79.84
8	-1	-1	1	1	1	-1	1	79.33
9	-1	-1	-1	1	1	1	-1	82.58
10	1	-1	-1	-1	1	1	1	90.45
11	-1	1	-1	-1	-1	1	1	82.34
12	-1	-1	-1	-1	-1	-1	-1	77.62

factors were studied at three different levels and a set of 15 experiments was carried out. For statistical calculations, coding of the factors was done according to the following equation

$$\chi_i = \frac{X_i - X_0}{\triangle X} \quad i = 1,2,3,....k \tag{2}$$

Where, χ_i was the coded value of an independent factor, X_i was the actual value of an independent factor, X_0 was the actual value of an independent factor at the center point, and $_{\triangle}X$ was the step change. For predicting the optimal point, a second-order polynomial equation was fitted to correlate the relationship between factor and response. The model equation used for the analysis was given as

$$Y = \beta_0 + \sum \beta_i \chi_i + \sum \beta_{ij} \chi_i \chi_j + \sum \beta_{ii} \chi_i^2 \quad i = 1,2,3,.....k \tag{3}$$

Where, Y was the predicted response, β_0 was the intercept term, βi was the linear coefficient, βii was the squared coefficient, and βij was the interaction coefficient. χi, χj represented the independent factors (medium component) in the form of coded values. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2. Each experimental design was carried out in duplicate, and the mean values were given.

Statistical analysis

Design expert, version 7.0 (Statease Inc., Minneapolis, USA) was used for the experimental designs and regression analysis of the experimental data. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equation was judged statistically 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.

Experimental validation of the optimized condition

In order to validate the optimization of medium composition, three tests were carried out using the optimized condition to confirm the result from the analysis of the response surface.

RESULTS AND DISCUSSION

Screening of the important factors by Plackett-Burman design

Plackett-Burman design offered an effective screening procedure and evaluated the significance of a large number of factors in one experiment, which was time-saving and maintained convincing information on each factor. Table 1 shows the high and low levels of factors chosen for trials in Plackett-Burman design. Table 2 represents the seven independent factors and their concentrations at different coded levels and the experimental responses for 12 runs. The endochitinase activity showed considerable variation depending on the seven independent factors in the medium, by applying the regression analysis on the experimental data. The corresponding first-order model equation fitted to the data obtained from the Plackett-Burman design experiment has the formula

$$Y = 83.2 + 2.32x_1 + 0.101x_2 + 0.339x_3 - 0.62x_4 + 1.56x_5 + 1.48x_6 + 0.148x_7 \tag{4}$$

The regression coefficients and determination coefficient (R^2) for the linear regression model of endochitinase activity are presented in Table 3. The model was significant ($P < 0.05$) and $R^2 = 0.9570$, indicating that 95.7% of the variability in the response could be explained by the model. Statistical analysis of the data showed that yeast extract, oleic acid and Tween-80 had a significant effect on endochitinase activity, with the confidence level above 98% ($P < 0.05$). The other factors had confidence levels below 95% were considered insignificant.

Yeast extract was found to be a significant influencing factor, probably because it served as an important carbon source and provided energy for the *P. pastoris* growth. Compared with other commonly used carbon sources, yeast extract made *P. pastoris* culture to a relatively higher

Table 3. Regression results of the Plackett-Burman design.

Model term	Factors	Coefficient	Effect	*t*-value	*P*-value
Intercept		83.2475		220.57	0.000[a]
X_1	Yeast extract	2.3242	4.65	6.16	0.004[a]
X_2	Tryptone	0.1008	0.20	0.27	0.803
X_3	YNB	0.3392	0.68	0.90	0.420
X_4	Methanol	-0.6208	-3.24	-4.29	0.013 [a]
X_5	Oleic acid	1.5642	3.13	4.14	0.014[a]
X_6	Tween-80	1.4825	2.97	3.93	0.017[a]
X_7	PTM_1	0.1475	0.30	0.39	0.716

[a] Statistically significant at 95% of confidence level ($P < .05$).

Table 4. Coded and real values of factors in Box-Behnken experimental design.

Factor	Level of factor		
	-1	0	1
Yeast extract (A, g/l)	15	23	31
Oleic acid (B, ml/l)	0.5	1.3	2.1
Tween-80 (C, ml/l)	5	10	15

cell density easier (Li and Yu, 2006) and hence enhancing the expression level of endochitinase. Oleic acid could induce the production of peroxisome, help to oxidize the peroxide, reduce the damage to the *P. pastoris* cells and enhance the stability and activity of AOX_1, a very strong promoter responsible for the transcription and translation of endochitinase gene integrated into the genome of *P. pastoris* (Snyder et al., 1999). Therefore, the addition of oleic acid was likely to play an important role, in cooperation with gene manipulation and achieving high expression levels of endochitinase. Tween-80 was a kind of important surfactant, used in microbial culture for enhancing the permeability of cell member to make the intracellular endochitinase secreted into the culture medium more easy (Zhang et al., 2006). This result is in good agreement with the result reported by Quyen et al. (2003), that addition of Tween-80 significantly enhanced the expression of a lipase from *Bacillus thermocatenulatus* BTL2 in *P. pastoris*. The concentrations of yeast extract, oleic acid and Tween-80 were selected for further optimization to achieve a maximum endochitinase activity.

Box-Behnken design and response surface methodology

Following screening, response surface methodology using Box-Behnken design was employed to determine the optimal levels of the three selected factors that significantly affected endochitinase activity. The respective low, zero and high levels with the coded levels for the factors were defined in Table 4. The concentrations of the other factors were selected, low level for the factors with negative effect and high level for the factors with positive effect in Table 1. The experimental design and results are shown in Table 5. Based on a regression analysis of the data from Table 5, the effects of three factors on endochitinase activity were predicted by a second-order polynomial function, as

$$Y = 89 + 1.19A + 0.48B + 0.95C + 0.61AB + 0.011AC - 0.047BC - 3.91A^2 - 0.72B^2 + 0.61C^2 \quad (5)$$

Where, Y was the predicted response and A, B, C were the concentration of yeast extract, oleic acid and Tween-80, respectively.

The statistical significance of Equation (5) was checked by *f*-test, and the analysis of variance (ANOVA) for the second-order polynomial model is shown in Table 6. It was evident that the model was highly significant, as suggested by the model F value and a low probability value ($P = 0.0081$). The analysis of factor (*f*-test) showed that, the second-order polynomial model was well adjusted to the experimental data and the coefficient of variation (CV) indicated the degree of precision with which the treatments were compared. Usually, the higher the value of CV, the lower the reliability of the experiment. Here, a lower value of CV (1.78) indicated a better precision and reliability of the experiments (Box and Hunter, 1978). The precision of a model can be checked by the determination coefficient (R^2) and correlation coefficient (R). The determination coefficient (R^2) was calculated to be 0.9526, indicating that 95.26% of the variability in the

Table 5. Box-Behnken experimental design matrix with experimental values of endochitinase activity by the recombinant *P. pastoris*.

Runs	Yeast extract (A)	Oleic acid (B)	Tween-80 (C)	Endochitinase activity (μ/ml) (Y)
1	-1	-1	0	82.43
2	1	-1	0	84.49
3	-1	1	0	83.03
4	1	1	0	87.54
5	-1	0	-1	83.91
6	1	0	-1	85.37
7	-1	0	1	86.01
8	1	0	1	87.52
9	0	-1	-1	87.96
10	0	1	-1	88.14
11	0	-1	1	89.72
12	0	1	1	89.72
13	0	0	0	88.42
14	0	0	0	89.72
15	0	0	0	88.85

Table 6. Analysis of variance (ANOVA) for the second-order polynomial model.

Source	DF	MS	F-value	P > F
Model	9	82.21	11.17	0.0081[a]
Residual	5	4.09		
Lack of fit	3	3.21	2.42	0.3057
Pure Error	2	0.88		
Total	14			
R^2				0.9526

Coefficient of variation (CV) = 1.78; correlation coefficient (R) = 0.9432; DF, degrees of freedom and MS, mean square. [a]statistically significant at 95% of confidence level (P < 0.05).

response could be explained by this model. Normally, a regression model having an R^2 value that is higher than 0.9 was considered to have a very high correlation (Haaland, 1989). The closer the value of R to 1, the better the correlation between the experimental and predicted values. Here, the value of R (0.9432) for Equation (5) indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. Linear and quadratic terms were both significant at the 1% level. Therefore, the quadratic model was selected in this optimization study.

The significance of the regression coefficients was tested by a *t*-test. The regression coefficients and corresponding *P*-values for the model are given in Table 7. The *P*-values were used as a tool to check the significance of each coefficient, which was necessary to understand the pattern of the mutual interactions between the best factors. The smaller the *P*-value, the bigger the significance of the corresponding coefficient (Li et al., 2007; Liu et al., 2003; Li and Lu, 2005). The results showed that, among the independent factors, A (yeast extract) and C (Tween-80) had more significant effects on endochitinase activity. The positive coefficient of them showed a linear effect to increase endochitinase activity.

Comparison of observed and predicted endochitinase activity

A regression model could be used to predict future observations on the response Y (endochitinase activity) corresponding to particular values of the regressor variables. In predicting new observations and in estimating the mean response at a given point, one must be careful about extrapolating beyond the region containing the original observations. It was very possible that a model that fitted well in the region of the original data would no longer fit well outside the region. Figure 1 shows observed endochitinase activity (the response) versus those from the empirical model Equation (5). The figure proved that, the predicted data of the response from the empirical model was in good agreement with the observed ones in the range of the operating variables.

Localization of the optimum condition

The 3D response surface plots described by the regression model were drawn to illustrate the effects of the independent factors and the interactive effects of each independent factor on the response factors. It also show the optimum concentration of each component required for the endochitinase production (Figures 2 to 4). Each figure presented the effect of two factors while the other factor was held at zero level. These 3D plots provided a

Table 7. Regression result of Box-Behnken design.

Model term	Degree of freedom	Estimate	P-value
Intercept	1	89	
A	1	1.19	0.0136[a]
B	1	0.48	0.1949
C	1	0.95	0.0312[a]
AB	1	0.61	0.2335
AC	1	0.011	0.9815
BC	1	-0.047	0.9213
A^2	1	-3.91	0.0004[a]
B^2	1	-0.72	0.1851
C^2	1	0.61	0.2510

[a]Statistically significant at 95% confidence level (P < 0.05).

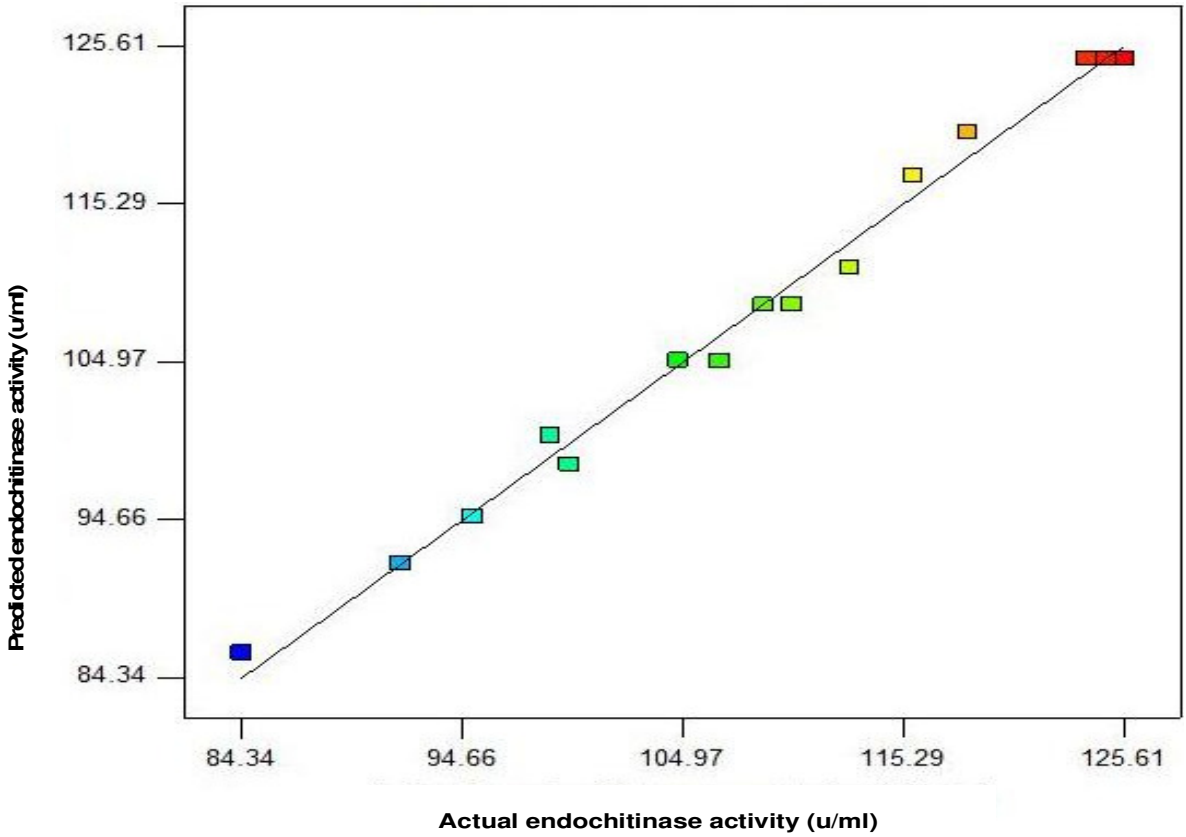

Figure 1. Comparison of the observed endochitinase activity (µ/ml) and the predicted endochitinase activity (µ/ml).

visual interpretation of the interaction between two factors and facilitated the location of optimum experimental conditions. The model predicted that the optimal values of the tested factors in the coded units were A = 0.1856, B = 0.4343 and C = -0.762. At these values, the corresponding concentrations of yeast extract, oleic acid and Tween-80 were 24.36 g/l, 1.758 and 6.2 ml/l, respectively. The maximum predicted value of endochitinase activity obtained was 88.85 µ/ml.

Model adequacy checking

Usually, it was necessary to check the fitted model to ensure that it provide an adequate approximation to the real system. Unless the model showed an adequate fit, proceedings with the investigation and optimization of the fitted response surface likely gave poor or misleading results. The residuals from the least squares fit played an important role in judging model adequacy. By constructing

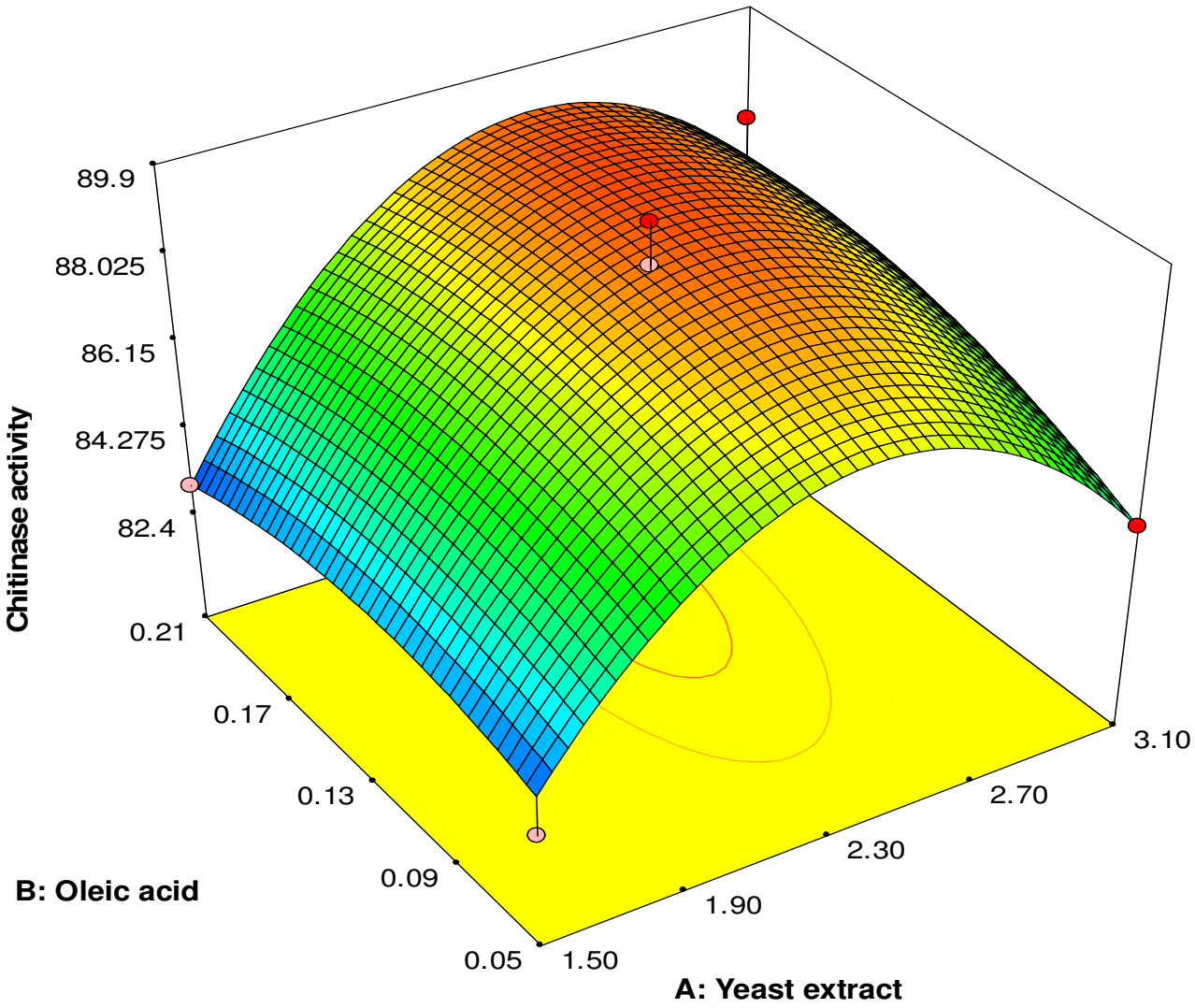

Figure 2. Response surface curve for endochitinase activity (μ/ml) by the recombinant *P. pastoris* as a function of yeast extract (g/l) and oleic acid (ml/l) concentration when Tween-80 concentration was maintained at 11 ml/l.

a normal probability plot of the residuals, a check was made for the normality assumption, as given in Figure 5. The normality assumption was satisfied as the residual plot was approximated along a straight line. Figure 6 presents a plot of residuals versus the predicted response. The general impression was that the residuals scattered randomly on the display, suggesting that the variance of the original observation was constant for all values of *Y*. Both plots (Figures 5 and 6) were satisfactory, so we concluded that the empirical model was adequate to describe the endochitinase activity by response surface.

Verification of the predicted activity in the optimal medium

Three additional experiments in shake flasks were performed in order to verify the predicted activity under the optimal medium compositions. The mean value of endochitinase activity was 88.26 μ/ml, which was in excellent agreement with the predicted value. The final medium composition optimized was: yeast extracts 24.36 g/l, tryptone 20 g/l, YNB 5.0 g/l, potassium phosphate 100 mM, methanol 5 ml/l, oleic acid 1.758 ml/l, Tween-80 6.2 ml/l, PTM_1 4.0 ml/l and biotin 4.00×10^{-4} g/l.

Conclusion

In this study, we demonstrated that Plackett-Burman design and response surface methodology using Box-Behnken design were effective on optimizing endochitinase production by the recombinant *P. pastoris*. The final medium composition optimized was: yeast extract 24.36 g/l, tryptone 20 g/l, YNB 5.0 g/l, potassium phosphate 100 mM, methanol 5 ml/l, oleic acid 1.758 ml/l, Tween-80 6.2

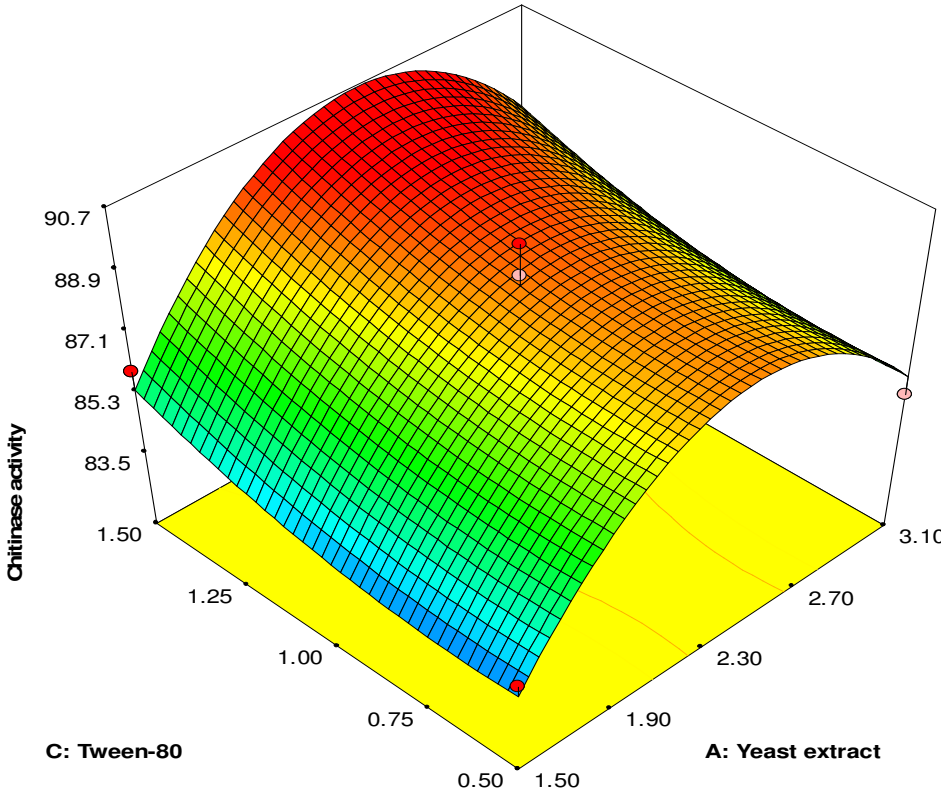

Figure 3. Response surface curve for endochitinase activity (µ/ml) by the recombinant *P. pastoris* as a function of yeast extract (g/l) and Tween-80 (ml/l) concentration when oleic acid concentration was maintained at 1.5 ml/l.

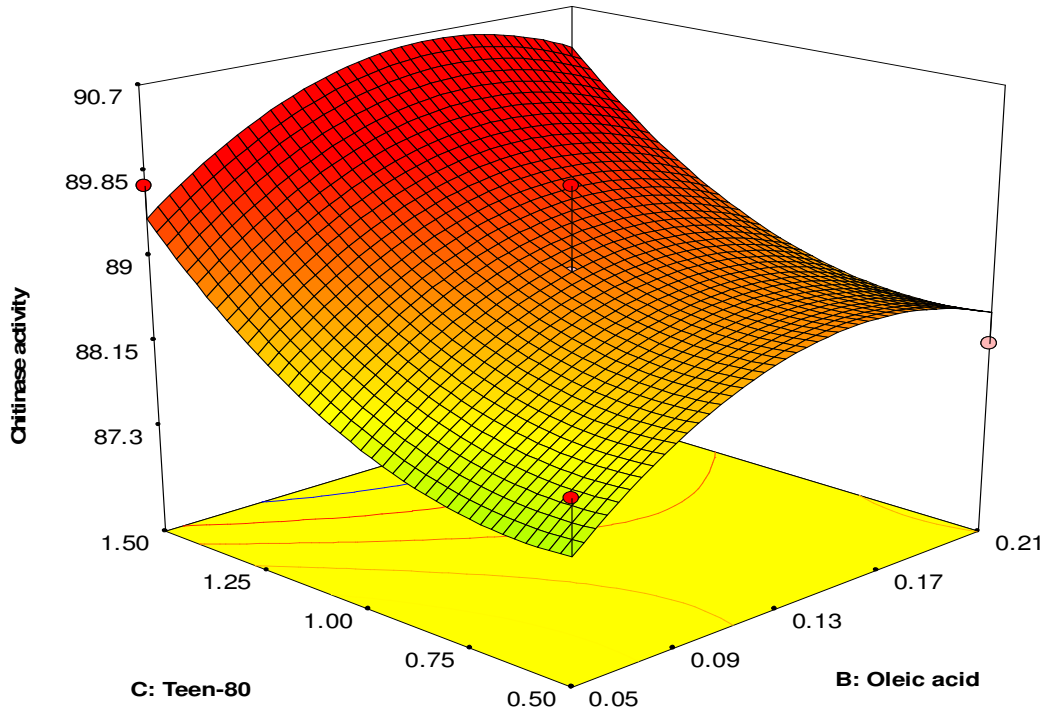

Figure 4. Response surface curve for endochitinase activity (µ/ml) by the recombinant *P. pastoris* as a function of oleic acid (ml/l) and Tween-80 (ml/l) concentration when yeast extract concentration was maintained at 23 g/l.

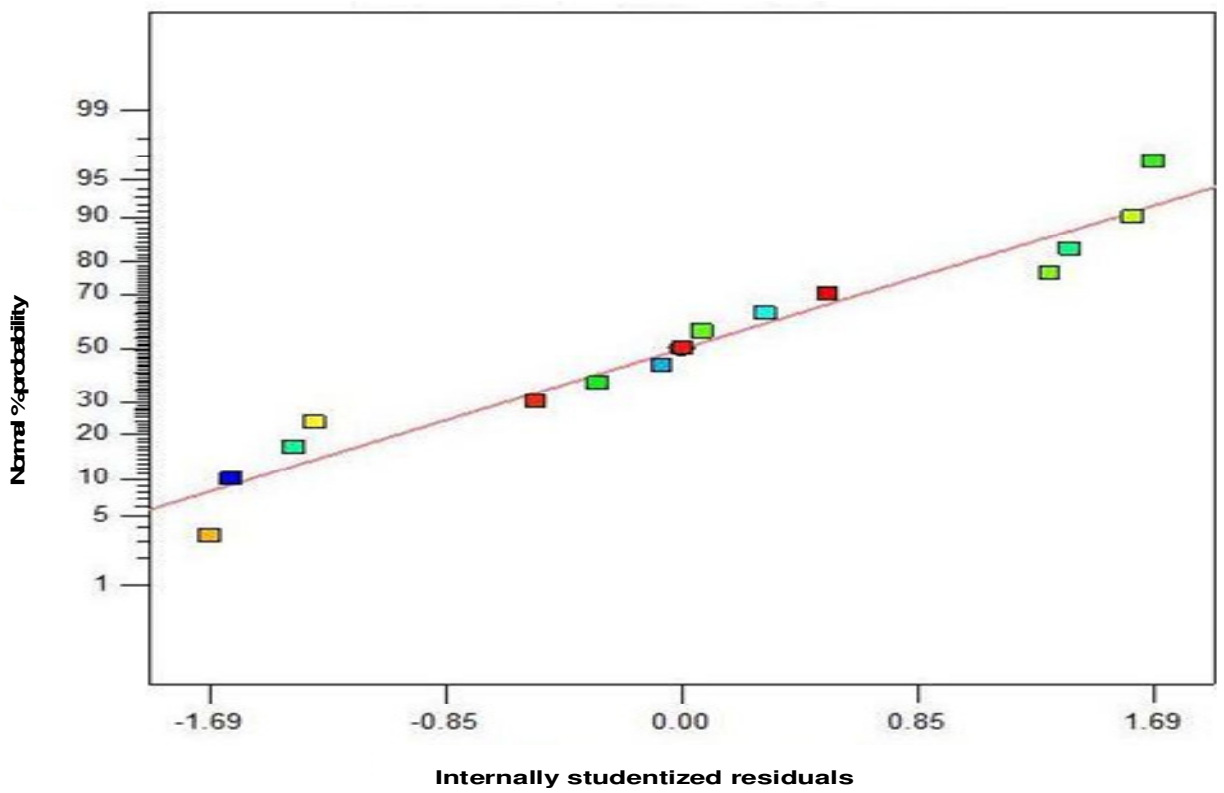

Figure 5. Normal probability of internally studentized residuals.

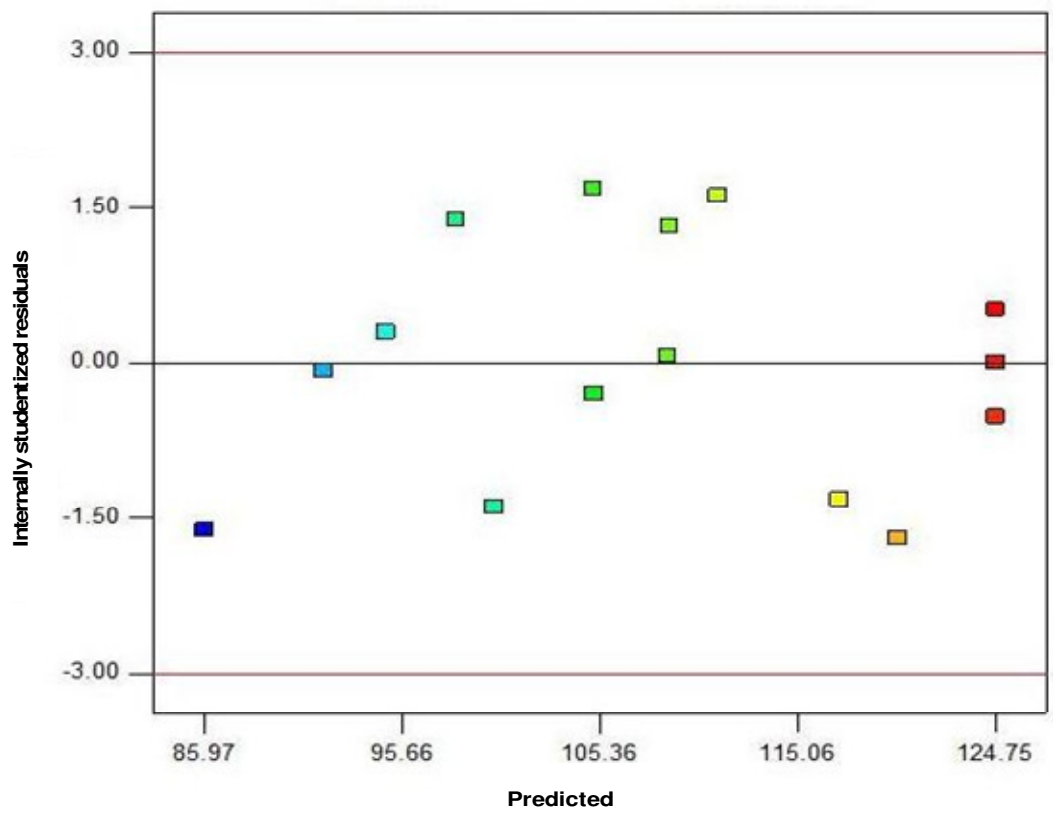

Figure 6. Plot of internally studentized residuals versus the predicted response.

ml/l, PTM_1 4.0 ml/l and biotin 4.00×10^{-4} g/l, which resulted in an overall 1.14-fold increase compared with that using the original medium. Validation experiments were also carried out to verify the adequacy and the accuracy of the model, and the results showed that the predicted value agreed with the experimental values accurately. The optimum culture medium obtained in this experiment gave a basis for further study with large scale fermentation in a fermenter for the production of endo-chitinase from this strain.

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