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STATISTICAL OPTIMIZATION OF MEDIUM COMPOSITIONS FOR CHITOSANASE PRODUCTION BY A NEWLY ISOLATED *Streptomyces albus*

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Abstract - The nutritional medium compositions required for chitosanase production by *Streptomyces albus* YT2 were optimized, and the strain was isolated from the coastal soil of Bohai Sea. Plackett–Burman design was applied to evaluate the effect of different medium compositions obtained by one-factor-at-a-time experiments. Glucose, peptone and $MgSO_4$ were found to influence the activity of chitosanase significantly. The steepest ascent method was employed to approach the experimental design space, followed by the use of Box–Behnken design and response surface methodology for further optimization of the chitosanase production. The medium composition for optimal production of chitosanase was as follows: 0.5 g/L chitosan, 55 g/L glucose, 1 g/L peptone, 2 g/L $(NH_4)_2SO_4$, 8 g/L urea, 1 g/L NH_4Cl, 3 g/L KH_2PO_4, 1 g/L $FeSO_4{\cdot}7H_2O$, 2 g/L $ZnSO_4{\cdot}7H_2O$, 2 g/L $CaCl_2{\cdot}6H_2O$, 2 g/L $MnSO_4{\cdot}H_2O$ and 2 g/L NaCl. The chitosanase production was found to increase from 11.56 U/mL to 39.87 U/mL, a 3.47-fold increase compared with that using the original medium.

Keywords: Chitosanase; Fermentation optimization; Medium compositions; Response surface methodology; *Streptomyces albus*.

INTRODUCTION

Chitooligosaccharides, which are depolymerization products of chitosan and with 2 to 10 glucosamines united by a *β*-1,4-glycoside linkage (Ming *et al*., 2006), have a variety of functional properties, such as antitumor (Jeon *et al*., 2001) and antimicrobial activity (Mengíbar *et al*., 2011), and have several potential applications in the food, agriculture and pharmaceutical industry (Molloy *et al*., 2004; Palma-Guerrero *et al*., 2010; Wang *et al*., 2011). Chitooligosaccharides are considered to be superior to chitosan due to their low molecular weights and viscosities. In particular, chitooligosaccharides are generally soluble in neutral aqueous solution (Kima and Rajapakse, 2005). Thus, the production of chitooligosaccharides from chitosan has received increased attention.

Chitosanases (E.C. 3.2.1.132) hydrolyze the *β*-1,4-glycosidic linkage of chitosan, which is a polysaccharide consisting mainly of D-glucosamine with a variable content of N-acetyl-D-glucosamine (Piza *et al*., 1999). Preparation of chitooligosaccharides with chitosanase is preferred to chemical methods because it allows production of a specific type of chitooligosaccharides (Hsiao *et al*., 2008), but the catalytic features of chitosanase from different

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microorganisms lead to different polymerization degrees (Chiang *et al*., 2003). For example, chitotriose was obtained as the major product by hydrolyzing chitosan with chitosanase from *Gongronella* sp. JG (Wang *et al*., 2008), while heptamers and oligomers with higher polymerization degrees were obtained by chitosanase CBCIII from *Bacillus cereus* (Lin *et al*., 2009). So application of novel and potential chitosanases has recently gained importance as an advanced alternative approach for producing chitooligosaccharides (Wee *et al*., 2009).

The present study optimized the chitosanase production in shake-flask culture by *Streptomyces albus* YT2, which is a new isolated strain from our laboratory. Effective and statistical optimization steps were carried out as follows: (1) by screening medium compositions that affect chitosanase production significantly using a Plackett–Burman design; (2) by accessing the optimal region of the significant variables using the steepest ascent method; (3) by optimizing these significant variables by response surface methodology; (4) by validating the model developed under the optimized conditions.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

Strain YT2 was screened from the coastal soil of Bohai Sea, isolated with 2% (w/v) colloidal chitosan as the sole carbon source, and identified as *Streptomyces albus* YT2 based on the biochemical properties. The cells from the stock cultures were transferred to 50 mL aliquots of pre-cultivation medium dispensed into 500 mL Erlenmeyer flasks, and afterwards incubated for 24 h at 32 °C and 220 rpm. The pre-cultivation medium consisted of: 5 g/L chitosan, 20 g/L glucose, 10 g/L peptone, 5 g/L yeast extraction, 1 g/L KH_2PO_4, 1 g/L $K_2HPO_4{\cdot}3H_2O$, 3 g/L $MgSO_4$, 2 g/L NaCl, pH = 7.5.

Chitosanase Production

For chitosanase production by *S. albus* YT2, 4 % (v/v) of inoculum (1.1×10^7 cfu/mL) was transferred into an Erlenmeyer flask (500 mL) containing 50 mL of fermentation medium at an initial pH 7.5, in which the compositions were varied based on the experimental designs. The fermentation culture in shake flasks was carried out at 32 °C and 220 rpm for 48 h. After the fermentations designed for each set of experiments, the culture broth was centrifuged for 10 min at 8 000×g using a bench-top centrifuge (Hermle Z323K, Germany) and the chitosanase activity in the cell-free supernatant was determined.

Determination of Chitosanase Activity

Chitosanase activity was determined by quantitative estimation of the reducing sugars produced from chitosan, and 85% deacetylated chitosan was used as substrate in the chitosanase assay. The reaction mixture contained 0.9 mL of 1% (w/v) soluble chitosan, 0.1 mL of diluted enzyme solution and 1 mL of 0.1 mol/L sodium acetate buffer (pH 5.8). The reaction tubes were incubated at 50 °C for 15 min. The reducing sugars formed in the supernatant were estimated spectrophotometrically at 540 nm using the dinitrosalicyclic acid method (Miller, 1959), with glucosamine as the calibration standard. Data are the means of duplicates, and standard errors were less than 5% of the means. One unit of chitosanase was defined as the amount of enzyme that could liberate 1 μmol of reducing glucosamine per min under the conditions described above.

Experimental Design and Data Analysis

Plackett–Burman Design

The variables that significantly influence the chitosanase production were screened using a fractional factorial Plackett–Burman (PB) design (Plackett and Burman, 1946). Thirteen independent medium compositions were evaluated at two levels (high and low) which were designated as level + 1 and level -1 respectively (Table 1).

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

Variables	Factors	Concentration (g/L)	
		-1 level	1 level
X_1	Chitosan	0.25	0.50
X_2	Glucose	15	25
X_3	Peptone	3	6
X_4	$(NH_4)_2SO_4$	2	4
X_5	Urea	4	8
X_6	NH_4Cl	1	2
X_7	$MgSO_4$	1	2
X_8	KH_2PO_4	3	6
X_9	$FeSO_4{\cdot}7H_2O$	1	2
X_{10}	$ZnSO_4{\cdot}7H_2O$	1	2
X_{11}	$CaCl_2{\cdot}6H_2O$	1	2
X_{12}	$MnSO_4{\cdot}H_2O$	1	2
X_{13}	NaCl	1	2

The significant variables were screened in 20 combinations according to the design matrix, and the responses were measured as chitosanase activity. 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.

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 the PB design experiment. The significant variables screened with the Plackett–Burman design experiments were roughly optimized using the steepest ascent method to determine the center points of each variable for the next optimization step. Experiments were performed along the steepest ascent path until no improvement in the response could be observed. This point should be near to the optimal point and could be used as the center point to optimize (Sun *et al*., 2007) in the following steps.

Box–Behnken Design and Response Surface Methodology

As screened through the Plackett–Burman design, three independent variables, i.e., glucose, peptone and $MgSO_4$, were studied at three different levels (-1, 0, +1) and the factors were coded. Seventeen experiments were conducted containing five replications at the center point for estimating the purely experimental uncertainty variance. The statistical software package Design Expert® 7.1.6 was used to analyze the results. The behavior of each variable, their interactions, and statistical analysis to obtain predicted responses were explained by the following second-order polynomial equation (Coelho *et al*., 2011, Liu *et al*., 2006):

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

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 the interaction effect when $i < j$, and k is the numbers of variables involved.

Validation of the Quadratic Model

In order to validate the optimization of medium compositions, fermentations were conducted using optimal medium compositions for the enzyme production at 32 ºC and 220 rpm for 48 h in 500 mL Erlenmeyer flasks containing 50 mL aliquots of the optimized medium.

RESULTS AND DISCUSSION

Selection of Significant Variables Affecting Chitosanase Activity

The optimization of the culture medium was carried out by a combination of non-statistical methodology and statistical methodology based experimental designs. Medium compositions used in the PB design were selected through non-statistical methodology (one-factor-at-a-time experiments). One-factor-at-a-time experiments revealed that chitosan, glucose, peptone, $(NH_4)_2SO_4$, urea, NH_4Cl, $MgSO_4$, KH_2PO_4, $FeSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}6H_2O$, $MnSO_4{\cdot}H_2O$ and NaCl could potentially have more effect on the chitosanase activity, and 11.56 U/mL of enzyme activity was observed (data not shown).

The matrix developed by the PB design and the results (chitosanase activity) are presented in Table 2, and the regression analysis is shown in Table 3. An adequate precision of 11.17 indicated an adequate signal as it measures the signal-to-noise ratio, and this model could be used to navigate the design space. Values of "Prob > *F*" less than 0.05 indicated that model terms were significant, on the basis of statistical analysis, Regression analysis determined that the compositions of glucose (P <0.0001), peptone (P = 0.0204,) and $MgSO_4$ (P = 0.0258) had a significant effect on the enzyme activity, so the compositions of these compounds were evaluated in the further optimization experiments. After the neglect of insignificant terms (with *P*-values higher than 0.05), a modified first-order equation was developed to describe enzyme activity:

$$Y = -4.08 + 0.66X_2 - 0.65X_3 - 1.86X_7 \quad (2)$$

According to the Plackett–Burman design, the optimum medium composition was as follows: 0.5 g/L chitosan, 25 g/L glucose, 3 g/L peptone, 2 g/L $(NH_4)_2SO_4$, 8 g/L urea, 1 g/L NH_4Cl, 1 g/L $MgSO_4$, 3 g/L KH_2PO_4, 1 g/L $FeSO_4{\cdot}7H_2O$, 2 g/L $ZnSO_4{\cdot}7H_2O$, 2 g/L $CaCl_2{\cdot}6H_2O$, 2 g/L $MnSO_4{\cdot}H_2O$ and 2 g/L NaCl.

Table 2: Plackett–Burman design with coded values along with the observed results

Trial	Coded variable level													Chitosanase Activity (U/mL)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	
1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	14.75
2	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	11.57
3	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	5.78
4	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	15.95
5	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	16.39
6	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	5.77
7	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	7.82
8	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	5.74
9	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	6.35
10	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	12.66
11	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	6.98
12	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	16.97
13	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	9.35
14	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	15.72
15	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	14.13
16	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	10.88
17	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	6.17
18	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	5.54
19	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	8.02
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	8.07

Table 3: Regression analysis of the PB design for chitosanase production

Sources	Sum of Squares	Contribution (%)	Coefficient Estimate	Prob > *F*
Model				<0.0001
Chitosan	6.28	1.90	0.56	0.1292
Glucose	216.28	65.53	3.29	<0.0001
Peptone	18.99	5.75	-0.97	0.0204
$(NH_4)_2SO_4$	2.72	0.82	-0.37	0.1606
Urea	0.48	0.15	0.16	0.3878
NH_4Cl	0.50	0.15	-0.16	0.3610
$MgSO_4$	17.24	5.22	-0.93	0.0258
KH_2PO_4	4.30	1.30	-0.46	0.1071
$FeSO_4{\cdot}7H_2O$	1.14	0.34	-0.24	0.2242
$ZnSO_4{\cdot}7H_2O$	10.38	3.15	0.72	0.0719
$CaCl_2{\cdot}6H_2O$	12.56	3.81	0.79	0.0507
$MnSO_4{\cdot}H_2O$	12.00	3.63	0.77	0.0553
NaCl	0.99	0.30	0.22	0.2159

Steepest Ascent Method for Determination of Center Point

Plackett–Burman design experiments proved to be a valuable tool for screening significant variables that affected the chitosanase activity, but it was unable to predict the optimum levels of the compositions (Chen *et al*., 2009). Based on the modified first-order equation obtained and the regression results, glucose, peptone and $MgSO_4$ were found to be significant factors, which meant that increasing the concentration of X_2 while decreasing X_3 and X_7 had a positive effect on the chitosanase activity. The path of steepest ascent was determined to find the proper direction of changing the factors above in order to improve enzyme activity. The experimental design of the steepest ascent and the corresponding results are shown in Table 4.

Table 4: Experimental design and results of the steepest ascent path

Trial	Glucose (g/L)	Peptone (g/L)	$MgSO_4$ (g/L)	Chitosanase activity (U/mL)
Origin	25	3.0	1.0	13.63
1	30	2.5	0.8	20.40
2	35	2	0.6	22.74
3	40	1.5	0.4	26.81
4	45	1.0	0.2	28.99
5	50	0.5	0.1	32.37
6	55	0.25	0	25.28

Regarding the results from the steepest ascent path, it was apparent that the yield profile showed a maximum of 32.37 U/mL at run 5. Consequently, this point was near to the region of maximum chitosanase activity response. Thus, an appropriate center point for the further optimization step was chosen: 50 g/L glucose, 0.5 g/L peptone and 0.1 g/L $MgSO_4$.

Box–Behnken Design and Response Surface Methodology

Once the ranges of the relevant variables were selected through the PB screening and steepest ascent experiment, Box–Behnken design (BBD) and response surface methodology (RSM) were used to obtain a quadratic model, and then appraise the quadratic results and central points to estimate the pure process variability (Wang *et al*., 2008, Ottoni *et al*., 2012), with chitosanase activity as response. Three compositions, i.e., glucose, peptone and $MgSO_4$, that significantly affected chitosanase activity were optimized by RSM using a 3-factor-3-level BBD. Therefore, 17 experiments with different combinations of glucose, peptone and $MgSO_4$ were conducted (Tables 5 and 6). The results were analyzed by ANOVA and, following quadratic regression, an equation was obtained in terms of chitosanase activity.

Table 5: Coded and real values of variables in the Box-Behnken design

Factors	Levels of factors		
	-1	0	1
A: Glucose (g/L)	45	50	55
B: Peptone (g/L)	0	0.5	1
C: $MgSO_4$ (g/L)	0	0.1	0.2

Table 6: Observed and predicted values of chitosanase activity for the BBD matrix

Trial	Coded variable level			Chitosanase activity (U/mL)	
	A	*B*	*C*	Observed	Predicted
1	-1	-1	0	33.05	31.92
2	1	-1	0	35.90	36.37
3	-1	1	0	31.96	31.49
4	1	1	0	32.93	34.06
5	-1	0	-1	32.93	34.31
6	1	0	-1	38.34	38.12
7	-1	0	1	32.37	32.59
8	1	0	1	37.19	35.81
9	0	-1	-1	33.05	32.80
10	0	1	-1	37.20	36.29
11	0	-1	1	34.74	35.64
12	0	1	1	29.17	29.42
13	0	0	0	29.80	29.11
14	0	0	0	29.72	29.11
15	0	0	0	27.41	29.11
16	0	0	0	30.20	29.11
17	0	0	0	28.44	29.11

The highest activity of chitosanase observed was 38.34 U/mL in run 6 (Table 6). The model *F*-value of 8.63 implied that the model was significant, because there was only a 0.48% chance that the "Model *F*-Value" could occur due to noise (Table 7). The regression equation obtained after ANOVA indicated that the *R*-Squared value of 0.9173 (a value of *R*-Squared > 0.75 indicated the aptness of the model) 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 8.283 indicated an adequate signal. This model could be used to navigate the design space, and a low coefficient of variation (CV = 4.34 %) demonstrated that the experiments were precise and reliable. The "Lack of Fit *F*-value" of 2.15 implied that the Lack of Fit was not significant relative to the pure error. A second-order polynomial function was fitted to the experimental chitosanase activity, resulting in the following regression equation in terms of actual factors:

$$\text{Chitosanase activity} = 309.74 - 11.55A + 7.53B - 32.75C - 0.19AB - 0.30AC - 48.60BC + 0.12A^2 + 5.36B^2 + 308.68C^2 \quad (3)$$

Table 7: Analysis of variance for the second-order polynomial model for optimization of chitosanase activity

Source	SS	DF	MS	*F*-value	Prob>*F*
Model	155.60	9	17.29	8.63	0.0048
Residual	14.02	7	2.00		
Lack of Fit	8.66	3	2.89	2.15	0.2365
Pure Error	5.37	4	1.34		
Total	169.62	16			

R^2 =0.9173; CV% = 4.34; SS, sum of squares; DF, degrees of freedom and MS, mean square.

Figures 1 to 3 show the contour plots for chitosanase activity. Evidently, the chitosanase activity varied significantly with the concentration of glucose, peptone and $MgSO_4$. The optimum 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 glucose to 55 g/L, peptone to 1 g/L, and $MgSO_4$ to 0 g/L would favor maximum chitosanase activity, giving 40.73 U mL^{-1}.

Glucose was the best carbon source for the production of chitosanase from *S. albus* YT2 while

not catabolically repressed, which was different from that in *Acinetobacter* sp. C-17 (Zhu *et al*., 2003) and *Bacillus cereus* D-11 (Gao *et al*., 2008). Peptone as a better nutrient source is needed by several microorganisms (Chen *et al*., 2009), but did not favor chitosanase production from *S. albus* YT2 in higher concentration. The effect of ions on the chitosanase from *S. albus* YT2 was studied (data not shown) and it was found that Mg^{2+} was an inhibitor of the chitosanase from *S. albus* YT2, only 62% activity was obtained at 20 μmol/L of $MgSO_4$, which proved the optimized result that no added $MgSO_4$ was correct.

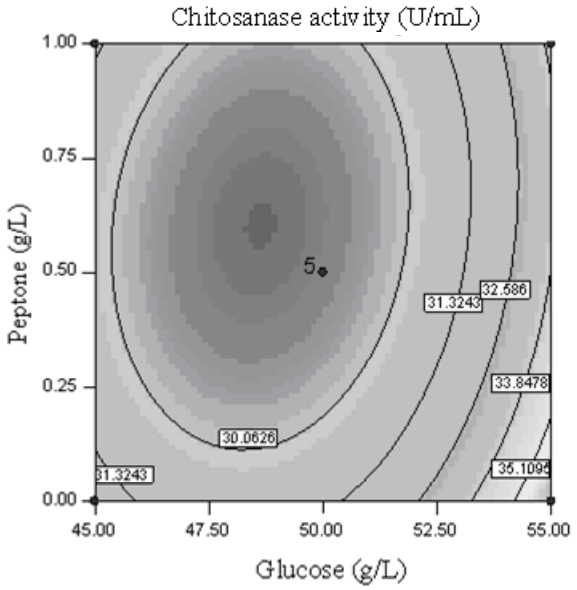

Figure 1: Contour plot of chitosanase activity showing the interaction between glucose concentration and peptone concentration at $C = 0$

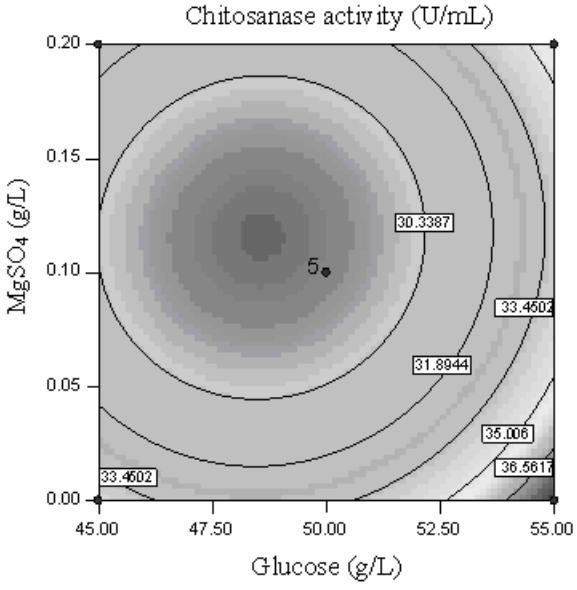

Figure 2: Contour plot of chitosanase activity showing the interaction between glucose concentration and $MgSO_4$ concentration at $B = 0$

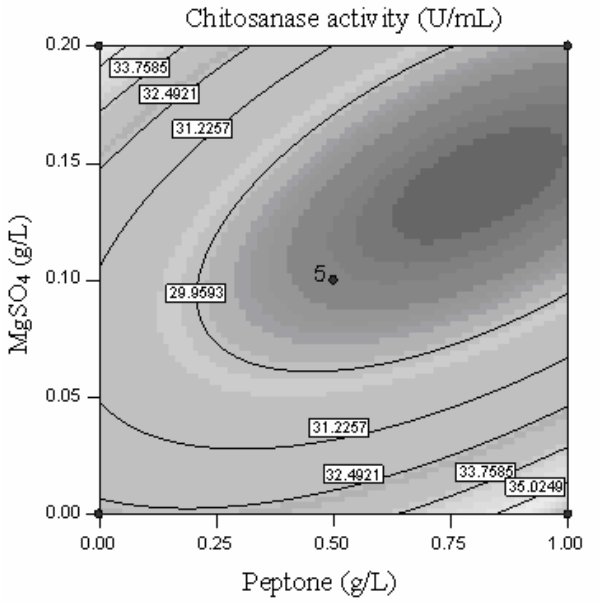

Figure 3: Contour plot of chitosanase activity showing the interaction between peptone concentration and $MgSO_4$ concentration at $A = 0$

Experimental Validation of the Optimized Condition

In order to confirm the optimized results, the culture of *S. albus* YT2 was studied using the optimal medium composition, and the results are shown in Figure 4. The maximum rate of chitosanase activity was 39.87 U/mL at 48 h, which was almost equal to the predicted value. By means of optimizing the medium composition, the activity of chitosanase was enhanced from 11.56 U/mL to 39.87 U/mL, a 3.45-fold increase. This result therefore corroborated the predicted values and the effectiveness of the model, which indicated that the optimized medium was propitious for the production of chitosanase from *S. albus* YT2. The final medium composition optimized with response surface methodology was: 0.5 g/L chitosan, 55 g/L glucose, 1 g/L peptone, 2 g/L $(NH_4)_2SO_4$, 8 g/L urea, 1 g/L NH_4Cl, 3 g/L KH_2PO_4, 1 g/L $FeSO_4 \cdot 7H_2O$, 2 g/L $ZnSO_4 \cdot 7H_2O$, 2 g/L $CaCl_2 \cdot 6H_2O$, 2 g/L $MnSO_4 \cdot H_2O$ and 2 g/L NaCl.

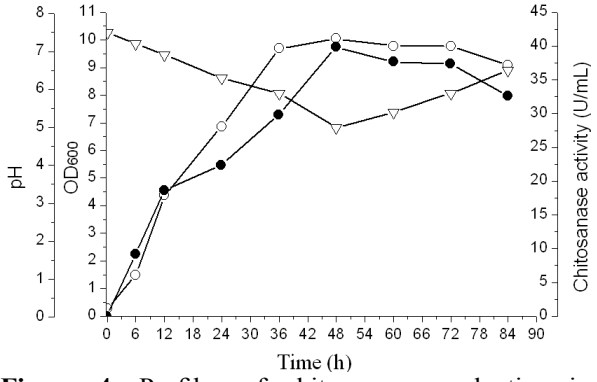

Figure 4: Profiles of chitosanase production in shake-flask experiments using the optimized medium. -●- chitosanase activity; -○- OD600; -v- pH

Microbial chitosanase production has been reported by several authors, such as *Bacillus cereus* (1.5 U/mL) (Piza *et al*., 1999), *Bacillus sp.* RKY3 (63.53 U/mL) (Wee *et al*., 2009), *Streptomyces griseus* (0.92 U/mL) (Kim *et al*., 2001), *Acinetobacter sp.* C-17 (2.8 U/mL) (Zhu *et al*., 2003), *Aspergillus sp* (1.18 U/mL) (Li *et al*., 2008) and *Matsuebacter chitosanotabidus* (15.24 U/mL) (Park *et al*., 1999). Nevertheless, as far as we know, this constitutes the first report of chitosanase production by *S. albus*. Furthermore, the maximum chitosanolytic activity (39.87 U/mL) of *S. albus* YT2 was relatively high, and this organism has potential to be a suitable chitosanase producer. At the same time, further study of the purification and catalytic characterization of chitosanase from *S. albus* YT2 is in progress.

CONCLUSIONS

Statistically based experimental designs proved to be effective for optimizing the culture medium for chitosanase activity by *S. albus* YT2, which resulted in about a 3.5-fold increase in the chitosanase activity relative to 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 work provides the basis for further studies on a large scale using bioreactors.

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