Ann Microbiol (2012) 62:193–201
DOI 10.1007/s13213-011-0246-1

ORIGINAL ARTICLE

Statistical optimization of chitosanase production by *Aspergillus* sp. QD-2 in submerged fermentation

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Received: 9 October 2010 / Accepted: 3 March 2011 / Published online: 27 March 2011

Abstract Cultivation of chitosanase production by *Aspergillus* sp. QD-2 under submerged fermentation was optimized. Factors including $(NH_4)_2SO_4$, inoculum size and initial pH were identified by two-level Plackett-Burman design (PBD) as significant for chitosanase production. The path of steepest ascent was undertaken to determine the optimal region of three significant factors. To determine the optimal values of the significant variables, Box-Behnken design (BBD) and response surface analysis were employed. The quadratic regression model of producing chitosanase showed that the optimal cultivation conditions ($(NH_4)_2SO_4$ 5.164 g/l, inoculum size 8.204% (v/v) and initial pH 4.074) resulted in the improvement of chitosanase activity (85.816 U/ml) as compared to the initial level (26.515 U/ml) after 72 h of fermentation. The optimal pH and temperature of the crude chitosanase were determined to be 5.6 and 55°C, respectively.

Keywords Chitosanase · Submerged fermentation · Optimization · *Aspergillus* · Response surface methodology

Introduction

Chitosan is a polysaccharide of β-(1→4)-linked D-glucosamine units with various degrees of N-acetylation. Chitosan and its derivatives have shown various functional properties which make it possible to use them in many fields including food, cosmetics, biomedicine, agriculture, environmental protection, and wastewater management (Kim and Rajapakse 2005). However, the application of the natural polysaccharides in medicine and food industry is limited since their high molecular weight results in a poor solubility at neutral pH values and high viscosity aqueous solutions (Wang et al. 2009). In recent years, attention has been paid to water-soluble chitosan oligosaccharides (COS) because they perform various biological activities such as inhibiting the growth of microorganisms (Moon et al. 2007; Xu et al. 2007), exerting antitumor activity (Wang et al. 2008d, antioxidant and radical scavenging (Harish Prashanth and Tharanathan 2007), anti-inflammatory (Yang et al. 2010), stimulating the immune system and exert fat lowering and hypocholesteromic effects (Zaharoff et al. 2007).

Chitosan oligosaccharides may be produced by chemical and enzymatic methods. However, there are many problems existing in chemical processes, such as harsh conditions of hydrolysis, production of a large amount of short-chain oligosaccharides, low yields of oligosaccharides, high cost in separation, and environmental pollution. Alternatively, enzymatic processes lack these defects and permit the breakdown of chitosan under mild conditions (Wang et al. 2008b).

Chitosanases (EC 3.2.1.132) are hydrolytic enzymes acting on glycosidic bonds throughout chitosan chains resulting in low molecular weight chitosan oligomers. There is a variety of chitosanase-producing microorganisms including *Bacillus* (Chiang et al. 2003; Wang et al. 2009), *Pseudomonas* (Wang et al. 2008b), *Paenibacillus* (Isogawa et al. 2009), *Serratia* (Wang et al. 2010), *Sphingomonas* (Zhu et al. 2007), *Streptomyces* (Kim and Ji 2001),

Electronic supplementary material The online version of this article (doi:10.1007/s13213-011-0246-1) contains supplementary material, which is available to authorized users.

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Table 1 Codes and levels of variables for Plackett-Burman design (PBD)

Variables	Codes	Levels	
		−1	1
Colloidal chitosan (g/l)	A	10	20
$(NH_4)_2SO_4$ (g/l)	B	2.0	4.0
KH_2PO_4 (g/l)	C	0.2	0.4
NaCl (g/l)	D	0.2	0.4
$MgSO_4{\cdot}7H_2O$ (g/l)	E	0.1	0.2
Tween 80 (ml/l)	F	10	15
Inoculum size (%, v/v)	G	2.0	4.0
Agitation rate (rpm)	H	150	220
Initial pH	I	5.0	7.0
Cultivation temperature (°C)	J	30	35

Microbacterium (Zhang and Sun 2007), *Aspergillus* (Chen et al. 2005; Eom and Lee 2003), *Gongronella* (Wang et al. 2008a), *Fusarium* (Shimosaka et al. 1996), *Mucor* (Struszczyk et al. 2009) and *Penicillium* (Zeng and Zheng 2002) genus. Most chitosanases were inducible in medium containing colloidal chitosan or chitosan as major carbon source. The bioprocesses of producing chitosanase can be affected by some cultivation parameters such as carbon sources, nitrogen sources, inorganic salt, initial pH, cultivation temperature, inoculum size and cultivation time.

There are two ways by which the cultivation conditions can be optimized: classical and statistical. The classical method is based on the "one-factor-at-a-time" method, in which one independent variable is studied while fixing all other factors at a specific level. This method may lead to unreliable results and less accurate conclusions. It is unable to detect the frequent interactions occurring between two or more factors. Response surface methodology (RSM) uses an efficient statistical technique for optimization of multiple variables with a minimum number of experiments. It can reduce the numbers of experimental runs needed to provide sufficient information for a statistically acceptable result. This method has been successfully applied for the optimization of multiple variables in many fermentation processes and showed satisfactory results.

The aim of this study was to apply statistical methods including Plackett-Burman design (PBD), the method of steepest ascent and Box-Behnken design (BBD) to optimize submerged fermentation parameters for enhancing chitosanase production by *Aspergillus* sp. QD-2.

Materials and methods

Microorganism

Aspergillus sp. QD-2, isolated from shrimp shell-enriched marine soil in Qingdao City, China, by using powder chitosan (deacetylation degree (DD) 90%, molecular mass 200 kDa) as a sole carbon source. The strain was maintained at 4°C on potato dextrose agar (PDA: potato leachate, 1.0 l; glucose, 20.0 g; agar, 20.0 g and nature pH).

Seed inoculum preparation

Suspension of spores was made from 7-day-old cultures that had been grown on PDA slopes at 30°C. Sterile Tween 80-water (0.02%) was aseptically added to each slope, and

Table 2 Plackett-Burman design (PBD) for ten variables with coded values along with the experimental and predicted values of chitosanase activity

Trial	A	B	C	D	E	F	G	H	I	J	Response (chitosanase activity, U/ml)	
											Experimental	Predicted
1	1	1	−1	1	1	−1	1	−1	−1	−1	61.890	61.785
2	1	1	−1	1	−1	−1	−1	1	1	1	50.336	50.441
3	−1	1	−1	−1	−1	1	1	1	−1	1	65.465	65.360
4	−1	1	1	−1	1	−1	−1	−1	1	1	52.233	52.128
5	1	1	1	−1	1	1	−1	1	−1	−1	56.070	56.175
6	−1	−1	−1	1	1	1	−1	1	1	−1	44.472	44.367
7	1	−1	1	1	−1	1	−1	−1	−1	1	50.075	49.970
8	−1	−1	1	1	1	−1	1	1	−1	1	54.718	54.823
9	1	−1	−1	−1	1	1	1	−1	1	1	50.663	50.768
10	1	−1	1	−1	−1	−1	1	1	1	−1	46.238	46.133
11	−1	1	1	1	−1	1	1	−1	1	−1	57.290	57.395
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	50.031	50.136

Table 3 Statistical analysis of Plackett-Burman design (PBD)

Term	Effect	Coefficient estimate	Standard error coefficient	T	*P*
Constant		53.290	0.1054	505.52	0.001
Colloidal chitosan (A)	−1.490	−0.745	0.1054	−7.06	0.090
$(NH_4)_2SO_4$ (B)	7.848	3.924	0.1054	37.22	0.017*
KH_2PO_4 (C)	−1.039	−0.519	0.1054	−4.93	0.127
NaCl (D)	−0.320	−0.160	0.1054	−1.52	0.371
$MgSO_4{\cdot}7H_2O$ (E)	0.102	0.051	0.1054	0.48	0.714
Tween 80 (F)	1.432	0.716	0.1054	6.79	0.093
Inoculum size (G)	5.508	2.754	0.1054	26.12	0.024*
Agitation rate (H)	−0.814	−0.407	0.1054	−3.86	0.161
Initial pH (I)	−6.170	−3.085	0.1054	−29.26	0.022*
Cultivation temperature (J)	1.250	0.625	0.1054	5.93	0.106

Outline criterion: 0.05

*Significant at 5% level

suspension of spores was made by lightly brushing the mycelium with a sterile wire loop. Then, the suspension was diluted with sterile Tween 80-water (0.02%) to give a final spore count of 5.0×10^8 spores/ml.

For inoculum preparation, the glucose pre-cultured medium (%; w/v) (glucose, 1.5; yeast extract, 0.4; K_2HPO_4, 0.2; $MgSO_4{\cdot}7H_2O$, 0.1; pH 7.0) was used (Soni et al. 2010). The cultivation was done in a 250-ml Erlenmeyer flask, containing 50 ml nutrient medium and inoculated with 2.5 ml spore suspension. The cultivation was performed at 30°C on a shaker, at 180 rpm for 24 h.

Chitosanase production

Chitosanase production was performed in 250-ml flasks, containing 50 ml nutrient medium with the following composition: chitosan (deacetylation degree (DD) 90%, molecular mass 200 kDa) 15.0 g/l, $(NH_4)_2SO_4$ 2.0 g/l, KH_2PO_4 0.4 g/l, NaCl 0.4 g/l, $MgSO_4{\cdot}7H_2O$ 0.1 g/l, Tween 80 10.0 ml/l. Prior to sterilization, the initial pH was adjusted to 6.0. After sterilized at 121°C for 20 min, the culture medium was inoculated with 2% (v/v) seed inoculum and cultivated at 30°C, 200 rpm for 72 h in a rotary shaking incubator. At the end of fermentation, the mycelium was separated from the enzyme- containing broth by centrifuging at 10,000*g* for 15 min (4°C) and the supernatant was treated as the crude chitosanase for activity measurements.

Table 4 Experimental design of steepest ascent and corresponding values of chitosanase activity

Steps	$(NH_4)_2SO_4$ (g/l)	Inoculum size (%, v/v)	pH	Chitosanase activity (U/ml)
1	3.5	2.0	6.0	54.522
2	4.0	4.0	5.0	65.435
3	4.5	6.0	4.5	76.067
4	5.0	8.0	4.0	85.549
5	5.5	10.0	3.5	72.132

Analytical assay

Chitosanase activity was assayed using chitosan [deacetylation degree (DD) 90%, molecular mass 200 kDa] as the substrate. The reaction mixture contained 2.5 ml of 1.0% (w/v) chitosan dissolved in 200 mM acetate buffer (pH 5.6) and 1.0 ml of the enzyme solution. The reaction mixture was incubated at 55°C for 15 min. The amount of reducing sugar in the supernatant was measured using the modified 3,5-Dinitrosalicyclic acid (DNS) method (Miller 1959). One unit of chitosanase was expressed as the amount of enzyme that could liberate 1 μmol of reducing sugar as D-glucosamine per min under the conditions described above.

Evaluation of carbon sources and nitrogen sources for chitosanase production

Different carbon sources including powder chitosan [deacetylation degree (DD) 90%, molecular mass 200 kDa], colloidal chitosan which was prepared according to the

Table 5 Codes and levels of variables for Box-Behnken design (BBD)

Variables	Codes	Levels		
		−1	0	1
$(NH_4)_2SO_4$ (g/l)	X_1	3.5	5.0	6.5
Inoculum size (%, v/v)	X_2	6.0	8.0	10.0
pH	X_3	3.0	4.0	5.0

Table 6 Box-Behnken design (BBD) along with the experimental and predicted values of chitosanase activity

Run	X_1	X_2	X_3	Response (chitosanase activity, U/ml)	
				Experimental	Predicted
1	0	1	1	72.734	72.409
2	0	1	−1	68.805	67.980
3	−1	0	−1	60.061	60.202
4	0	−1	1	67.632	68.457
5	−1	0	1	62.928	62.569
6	1	−1	0	68.358	67.674
7	−1	1	0	64.380	65.064
8	1	1	0	71.526	71.992
9	1	0	1	67.951	67.810
10	1	0	−1	64.343	64.702
11	−1	−1	0	65.326	64.860
12	0	−1	−1	67.085	67.410
13	0	0	0	85.809	85.451
14	0	0	0	84.815	85.451
15	0	0	0	85.729	85.451

methods described by Roberts (1992), sodium carboxymethyl cellulose, microcrystalline cellulose, starch, chitin, D-glucosamine and lactose and different nitrogen sources including ammonium sulphate, ammonium chloride, potassium nitrate, urea, yeast extract and peptone were individually evaluated for their performances in chitosanase production.

Plackett-Burman design (PBD)

Plackett-Burman design was used to screen for and evaluate the factors which had significant impacts on chitosanase production. The design of experiments was formulated for ten factors using the software Minitab 14.12. The ten factors tested were: colloidal chitosan (A), $(NH_4)_2SO_4$ (B), KH_2PO_4 (C), NaCl (D), $MgSO_4 \cdot 7H_2O$ (E), Tween 80 (F), inoculum size (G), agitation rate (H), initial pH (I) and cultivation temperature (J). Each factor was tested at two levels, high (+1) and low (−1) (Table 1). A total of 12 experiments are shown in Table 2. All the trials were carried out in triplicate, and the average chitosanase activity for each trial was used as the response. Results from analysis of variance (ANOVA) and parameter estimates are summarized in Table 3.

Steepest ascent method

The method of steepest ascent is a procedure for moving along the direction of the maximum increase in the response (Li et al. 2009). The significant variables which were screened by Plackett-Burman design can be further optimized by this method. The direction of the method is the direction in which chitosanase production increased rapidly by increasing or decreasing the values of the significant factors. The experimental design and results of the steepest ascent method are shown in Table 4.

Response surface methodology (RSM)

The Box-Behnken design (BBD) was employed to optimize three significant factors including $(NH_4)_2SO_4$, inoculum size and initial pH to increase chitosanase production. The independent factors were investigated at three levels as −1, 0 and +1 (coded values), respectively (Table 5). The experimental design and results were shown in Table 6. The behavior of the system was explained by the following quadratic model equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

where Y is the predicted response, X_1, X_2 and X_3 are coded independent variables, β_0 is the intercept, β_1, β_2 and β_3 are linear coefficients, β_{11}, β_{22} and β_{33} are quadratic coef-

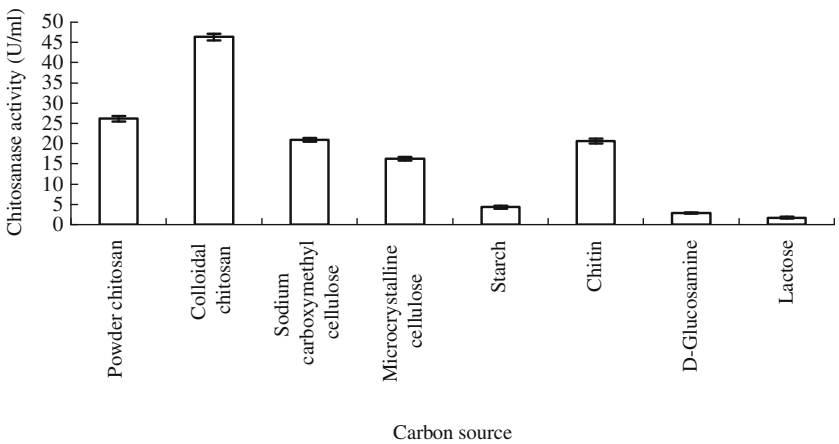

Fig. 1 Effects of different carbon sources on chitosanase production by *Aspergillus* sp. QD-2. *Data points*: mean values from three independent experiments. *Error bars*: standard deviations of triplicate independent experiments

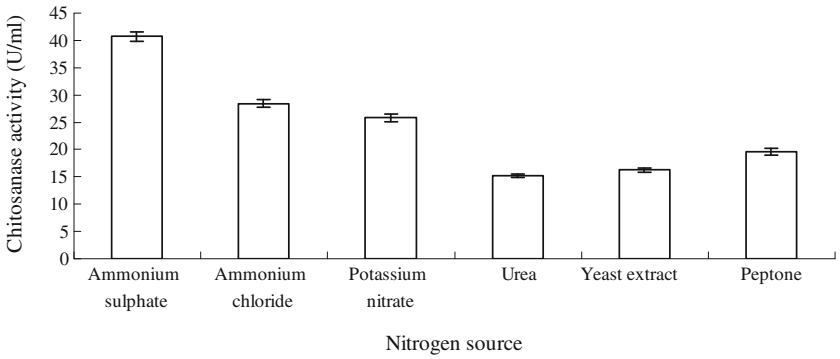

Fig. 2 Effects of different nitrogen sources on chitosanase production by *Aspergillus* sp. QD-2. *Data points*: mean values from three independent experiments. *Error bars*: standard deviations of triplicate independent experiments

ficients and β_{12}, β_{13} and β_{23} are interactive coefficients. The software Minitab 14.12 and Statistical Analysis System (SAS, 8.0) were used for the experimental design and analysis of the experimental data.

Effects of temperature and pH on chitosanase activity

The optimal temperature of the crude chitosanase was evaluated by incubating the reaction mixtures for 15 min at different temperatures ranging from 30 to 80°C in 200 mM sodium acetate (pH 5.6). The optimal pH of the enzyme was evaluated by incubating the reaction mixtures in the present of appropriate buffers using 200 mM sodium acetate (pH 3.6–5.6) and 200 mM sodium phosphate (pH 6.0–8.0), respectively, at 55°C and for 15 min.

Results and discussion

Effects of different carbon and nitrogen sources

As shown in Fig. 1, among these carbon sources, maximum chitosanase production was attained when colloidal chitosan was taken as the carbon source followed by powder chitosan. D-glucosamine as the sole carbon source also induced chitosanase but the enzyme activity reached 6.13% of the activity when the medium contained colloidal chitosan as the sole carbon source. Therefore, colloidal chitosan was adopted as the carbon source in the subsequent experiments.

As shown in Fig. 2, the levels of chitosanase activity were relatively lower when organic nitrogen sources including urea, yeast extract and peptone were employed. However, ammonium sulphate could stimulate the highest chitosanase production. Similarly, ammonium sulphate was reported to be the optimal nitrogen source for *Microbacterium* sp. OU01 to produce chitosanase (Sun et al. 2007).

Plackett-Burman design

The data listed in Table 2 indicated that there was a wide variation in chitosanase activity from 44.472 to 65.465 U/ml in the 12 trials. This variation suggested that the optimization of fermentation parameters was important for improving productivity. It showed that factors including $(NH_4)_2SO_4$ (B), $MgSO_4{\cdot}7H_2O$ (E), Tween 80 (F), inoculum size (G) and cultivation temperature (J) had positive effects on chitosanase production, whereas factors including colloidal chitosan (A), KH_2PO_4 (C), NaCl (D), agitation rate (H) and initial pH (I) had negative effects (Table 3). Factors that had significant effects (confidence levels>95%, $P<0.05$) on chitosanase production were $(NH_4)_2SO_4$ (B) ($P=0.017$), inoculum size (G) ($P=0.024$) and initial pH (I) ($P=0.022$).

Table 7 Results of regression analysis of Box-Behnken design (BBD)

Term	Coefficient estimate	Standard error coefficient	T	P
Constant	85.4510	0.5066	168.674	0.000
X_1	2.4354	0.3102	7.850	0.001**
X_2	1.1305	0.3102	3.644	0.015*
X_3	1.3689	0.3102	4.412	0.007**
X_1^2	−11.6484	0.4566	−25.509	0.000**
X_2^2	−6.4051	0.4566	−14.026	0.000**
X_3^2	−9.9819	0.4566	−21.859	0.000**
X_1X_2	1.0285	0.4387	2.344	0.066
X_1X_3	0.1852	0.4387	0.422	0.690
X_2X_3	0.8455	0.4387	1.927	0.112

R^2 =0.9961, Adj-R^2 =0.9890

*Significant at 5% level,
**significant at 1% level

Table 8 Analysis of variance (ANOVA) for the fitted quadratic polynomial model

Source	Degrees of freedom	Sum of squares	Mean square	*F*	*P*
Model	9	975.688	108.410	140.80	0.000**
Linear	3	72.663	24.221	31.46	0.001**
Square	3	895.797	298.599	387.82	0.000**
Interaction	3	7.228	2.409	3.13	0.126
Residual error	5	3.850	0.770		
Lack of fit	3	3.240	1.080	3.54	0.228
Pure error	2	0.610	0.305		
Total	14	979.538			

**Significant at 1% level

The path of steepest ascent

Based on the above regression analysis of Plackett-Burman design, the path of steepest ascent was employed to find the proper region of three significant factors by increasing concentration of $(NH_4)_2SO_4$ and inoculum size, while decreasing initial pH. Table 4 lists the directions of changes for the significant variables. It was obvious that chitosanase activity reached a plateau on the fourth step; therefore, this condition was chosen for further optimization.

Optimization of significant variables using response surface methodology

The significant independent variables including $(NH_4)_2SO_4$, inoculum size and initial pH were further optimized using the Box-Behnken design. As indicated in Table 5, three factorial points, denoted as high (+1), middle (0), and low (−1) level, were assigned for each variable. The design matrix and the corresponding results of the experiments are shown in Table 6. With the use of the statistical software Minitab 14.12 and Statistical Analysis System (SAS, 8.0), the regression analysis results and analysis of variance (ANOVA) for the fitted quadratic polynomial model are summarized in Tables 7 and 8, respectively. The resulting regression model was given below:

$$Y = 85.4510 + 2.4354X_1 + 1.1305X_2 + 1.3689X_3 - 11.6484{X_1}^2 - 6.4051{X_2}^2 - 9.9819{X_3}^2 + 1.0285X_1X_2 + 0.1852X_1X_3 + 0.8455X_2X_3$$

where Y is the predicted response (chitosanase production), X_1, X_2 and X_3 are the coded values of the concentration of $(NH_4)_2SO_4$, inoculum size and initial pH, respectively.

Results of regression analysis (Table 7) showed that linear terms (X_1, X_2 and X_3) and quadric terms (${X_1}^2$, ${X_2}^2$ and ${X_3}^2$) had significant effects on the response (chitosanase production). However, no interactions between each variable's pair were found to contribute to the response at a significant level. The value of determination coefficient (R^2=0.9961) indicated only 0.39% of the total variations was not explained by the model. The adjusted determination coefficient (Adj-R^2=0.9890) was also very high to confirm the significance of the model.

As shown in Table 8, the *F* values of the model and lack of fit were 140.80 and 3.54, respectively. The high *F* value of the model and non-significant lack of fit

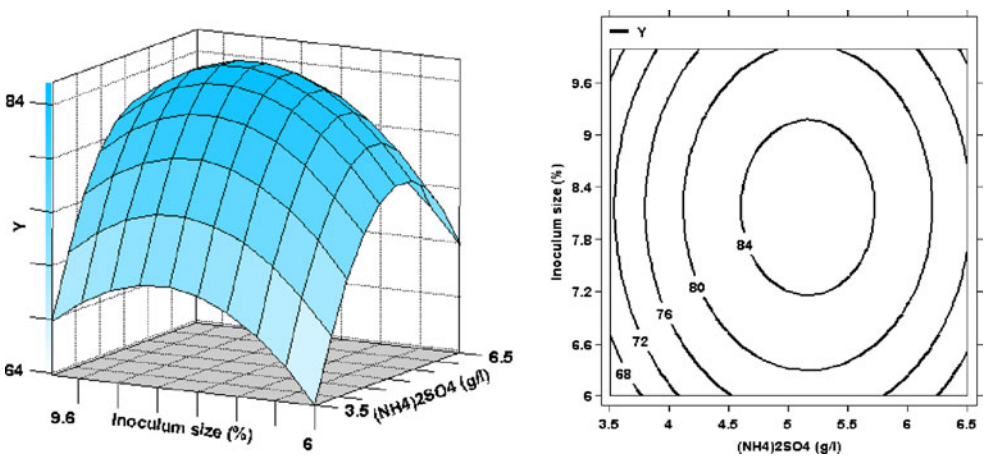

Fig. 3 Response surface plot and contour plot of the combined effects of $(NH_4)_2SO_4$ and inoculum size on chitosanase production (*Y*) with constant initial pH (pH 4.0)

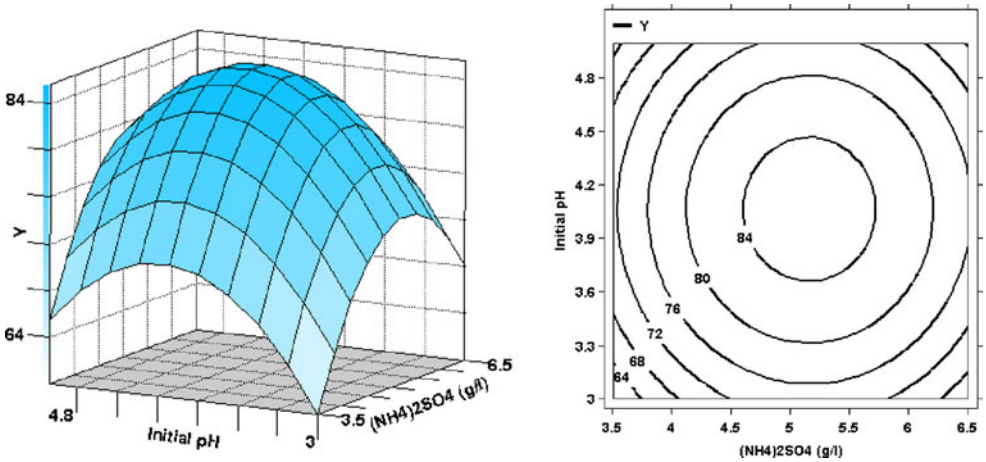

Fig. 4 Response surface plot and contour plot of the combined effects of $(NH_4)_2SO_4$ and initial pH on chitosanase production (*Y*) with constant inoculum size (8.0%, v/v)

indicated that the model was a good fit. The *P* values of the model (0.000) and the lack of fit (0.228) also suggested that the obtained experimental data was a good fit with the model.

The response surface plots and their corresponding counter plots of the combined effects of each independent variable's pair on chitosanase production are given in Figs. 3, 4 and 5. Since no interactions between the three variables were observed from Table 7, no elliptical contour plots were found from Figs. 3, 4, and 5. Figure 3 shows the combined effects of $(NH_4)_2SO_4$ and inoculum size on chitosanase production while initial pH was fixed at its middle level (pH 4.0). It reveals that the optimal ranges of $(NH_4)_2SO_4$ concentration and inoculum size for chitosanase production were 5.0–5.5 g/l and 7.8–8.4% (v/v), respectively. Figure 4 indicates the combined effects of $(NH_4)_2SO_4$ and initial pH on chitosanase production while inoculum size was fixed at its middle level (8%, v/v). It shows that the optimal ranges of $(NH_4)_2SO_4$ concentration and initial pH were 5.0–5.5 g/l and pH 3.9–4.2. Figure 5 shows the combined effects of inoculum size and initial pH on chitosanase production, while $(NH_4)_2SO_4$ concentration was fixed at its middle level (5.0 g/l). It indicates that the optimal ranges of inoculum size and initial pH were 7.8–8.4% and 3.9–4.2, respectively.

According to the canonical analysis, the predicted maximal chitosanase activity of 85.693 U/ml could be achieved when concentration of $(NH_4)_2SO_4$, inoculum size and initial pH were set at 5.164 g/l (X_1=0.10962), 8.204% (v/v) (X_2=0.10193) and 4.074 (X_3=0.07390), respectively.

In order to confirm the optimization results, the suggested fermentation conditions were performed in triplicate. Under the optimized conditions (colloidal chitosan 15.0 g/l, $(NH_4)_2SO_4$ 5.164 g/l, inoculum size 8.204% (v/v) and initial pH 4.074), the maximal chitosanase activity was 85.816 U/ml (Fig. 6). The value was found to be 0.14% higher than the predicted value. This discrepancy might be due to the slight variation in experimental conditions. In addition, the maximal chitosanase activity under unoptimized conditions (powder chitosan 15.0 g/l, $(NH_4)_2SO_4$ 2.0 g/l, inoculum size 2.0% (v/v) and initial pH 6.0) was 26.515 U/ml (Fig. 6). It showed that the optimization resulted in 3.24-fold increase of chitosanase production.

The level of chitosanase activity (85.816 U/ml) of *Aspergillus* sp. QD-2 was higher when compared with the cloned *Streptomyces lividans* TK24 (pDF22) (35–40 U/ml)

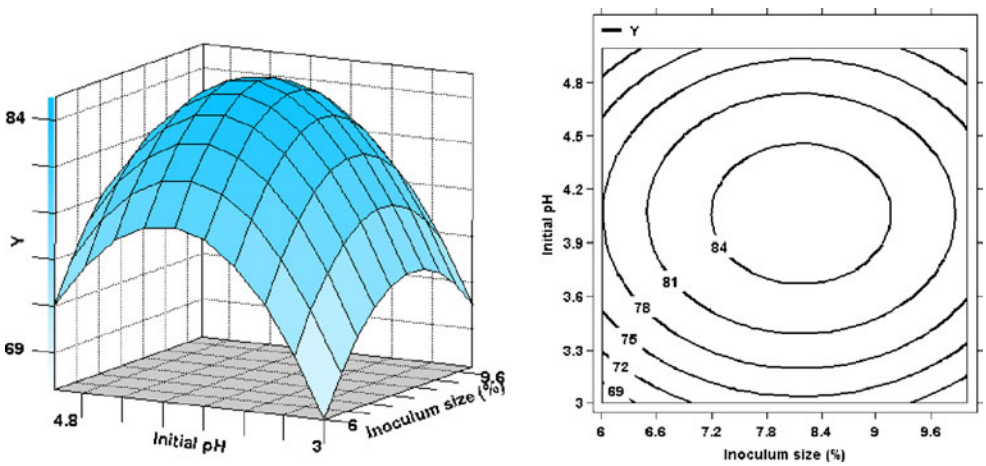

Fig. 5 Response surface plot and contour plot of the combined effects of inoculum size and initial pH on chitosanase production (*Y*) with constant concentration of $(NH_4)_2SO_4$ (5.0 g/l)

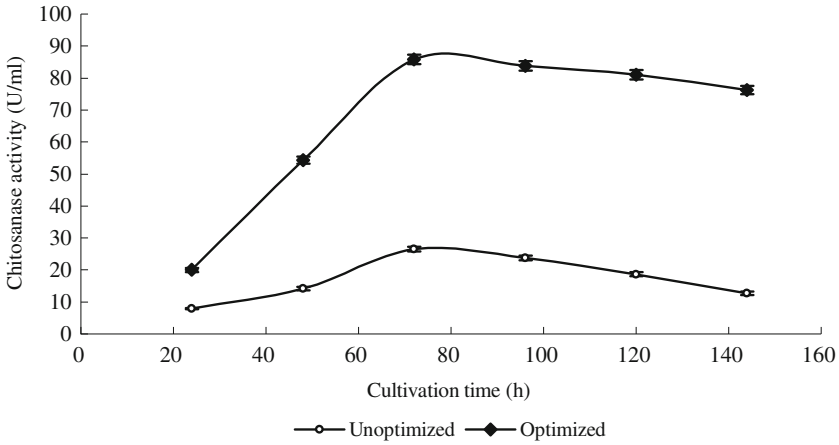

Fig. 6 The time course of chitosanase production by *Aspergillus* sp. QD-2 under both optimized and unoptimized conditions. *Data points*: mean values from three independent experiments. *Error bars*: standard deviations of triplicate independent experiments

(Fink et al. 1991), *Aspergillus* sp. CJ22-326 (3.61 U/ml) (Chen et al. 2005) and *Aspergillus oryzae* IAM2660 (0.05 U/ml) (Zhang et al. 2000). Furthermore, it was also higher than that of *Bacillus* sp. S65 (25 U/ml) (Su et al. 2006), *Bacillus cereus* D-11 (4.85 U/ml) (Gao et al. 2008), *Acinetobacter* sp. C-17 (2.8 U/ml) (Zhu et al. 2003), *Streptomyces griseus* HUT 6037 (0.92 U/ml) (Kim and Ji 2001), *Sphingomonas* sp. CJ-5 (0.9 U/ml) (Zhu et al. 2007), *Bacillus cereus* TKU018 (22 mU/ml) (Wang et al. 2009), *Pseudomonas* sp. TKU015 (25 mU/ml) (Wang et al. 2008a]) and *Serratia marcescens* TKU011 (0.024 U/ml) (Wang et al. 2008b). Though the level of chitosanase activity (85.816 U/ml) was lower than that of *Microbacterium* sp. OU01 (118U/ml) (Sun et al. 2007) and *Bacillus* sp. KCTC 0377BP (100 U/ml) (Choi et al. 2004), the value suggested that the strain was the most efficient producers of chitosanase among chitosanolytic fungi strains described so far in the literature.

Effects of temperature and pH on chitosanase activity

As shown in Fig. 7a, the crude chitosanase showed the optimal temperature of 55°C, which was comparable to Chi A (50–55°C) from *Aspergillus* sp. CJ22-326 (Chen et al. 2005) and chitosanaseII from *A. fumigatus* KH-94 (50–60°C) (Kim et al. 1998). As shown in Fig. 7b, the optimal pH for the crude chitosanase was 5.6, which was similar to chitosanase I of *A. fumigatus* KH-94 (pH 5.5) (Kim et al. 1998), chitosanase from *A. oryaze* IAM2660 (pH 5.5) (Zhang et al. 2000) and chitosanase II from *A. fumigatus* KB-1 (pH 5.5) (Eom and Lee 2003).

Conclusion

Although many reports on chitosanase production by chitosanolytic microorganisms have been published, little information on the statistical optimization of chitosanase production by *Aspergillus* genus under submerged fermentation is available. Therefore, this study will provide a new reference in this field. In this work, statistical methods were used to optimize cultivation conditions for chitosanase production by *Aspergillus* sp. QD-2 which was isolated from shrimp shell-enriched marine soil in Qingdao City, China. Factors including $(NH_4)_2SO_4$, inoculum size and initial pH were identified as significant for chitosanase production, and chitosanase activity was increased from 26.515 to 85.816 U/ml after optimization. It was demonstrated that statistical methods including PBD, the path of steepest ascent and BBD were effective in determining optimized cultivation conditions for chitosanase production. The experimental maximum chitosanase activity (85.816 U/ml) agreed very well with the predicted data (85.693 U/ml), which reflected the accuracy and applicability of the statistical

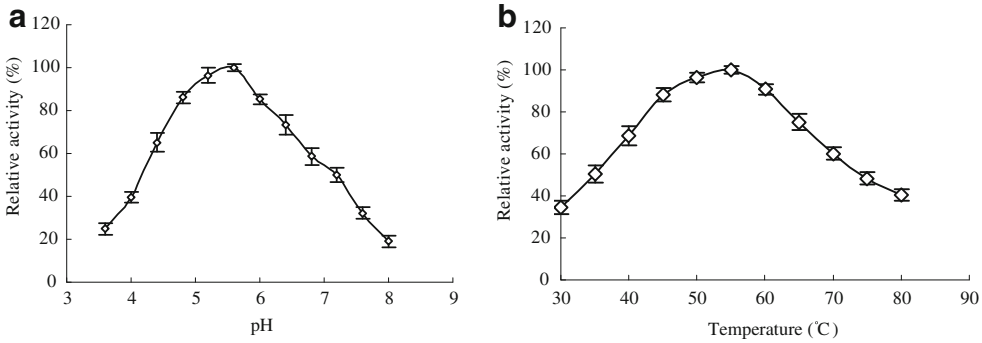

Fig. 7 Effects of temperature (**a**) and pH (**b**) on activity of chitosanase from *Aspergillus* sp. QD-2. *Data points*: mean values from three independent experiments. *Error bars*: standard deviations of triplicate independent experiments

methods. The optimal pH and temperature of the crude chitosanase were determined to be 5.6 and 55°C, respectively, which showed that chitosanase produced by the strain QD-2 could be applied to chitosan hydrolysis under acid conditions. Further studies are under way to identify the strain with molecular biological methods and to purify the chitosanase and clone the chitosanase gene from *Aspergillus* sp. QD-2.

Acknowledgement This research was supported by the encouragement foundation of Department of Science & Technology of Shandong Province of China for excellent middle-aged scientists (NO.2005gg3202084).

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