Induction and Optimization of Chitosanase Production by *Aspergillus fumigatus* YT-1 Using Response Surface Methodology

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With the use of a mixture of shrimp shell powder and wheat bran as carbon sources in the medium, statistical methods including Plackett-Burman design (PBD), steepest ascent method and Central composite design (CCD) were applied to optimize chitosanase production by a newly isolated *Aspergillus fumigatus* YT-1 under submerged fermentation. Significant factors for chitosanase production including $(NH_4)_2SO_4$, shrimp shell powder and $MgSO_4 \cdot 7H_2O$ were screened. Maximum chitosanase activity (21.85 U mL^{-1}) was achieved under the optimized conditions [$(NH_4)_2SO_4$ 5.05 g L^{-1}, shrimp shell powder 23.40 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.88 g L^{-1}]. According to maximum chitosanase activity (5.29 U mL^{-1}) which was obtained under unoptimized conditions, optimization resulted in an overall 3.13–fold enhancement in chitosanase activity. The optimal temperature and pH for chitosan hydrolysis were 60 °C and pH 4.8, respectively.

Key words:
Aspergillus fumigatus, chitosanase, optimization, shrimp shell powder, response surface methodology

Introduction

In recent years, much attention has been paid to chitosan oligosaccharides (COS) because COS have shown physiological activities such as antitumor effect and antimicrobial activity.[1] Compared with chitosan, chitosan oligosaccharides (COS) have some advantages such as water solubility, lower viscosities, lower molecular weights and shorter chain lengths. COS could be obtained from chitosan hydrolysis by chitosanase and this approach is popular by right of low production cost, low environmental impact and high reproducibility.[2–3] Chitosanases (EC 3.2.1.132) are glycosyl hydrolases that catalyze the hydrolysis of β-(1→4) glycosidic bonds of chitosan to produce glucosamine oligosaccharides and the enzymes could be produced by bacteria, actinomycetes, and fungi.[4–9]

It is reported that most chitosanases are induced by substrates including powder chitosan and colloidal chitosan.[10] Despite all of this, chitosanase is still unavailable in bulk quantities for commercial applications because of low enzymes productivity by chitosanolytic strains.[11] Therefore, this problem could be resolved by isolating chitosanolytic strains with high chitosanase productivity, optimizing fermentation conditions for chitosanase production and reducing chitosanase production cost. Consequently, using cheaper natural substrates for chitosanase production is popular, as this approach not only improves chitosanase productivity, but also reduces chitosanase production cost.

Shrimp shell is a kind of byproduct in seafood industries and it mainly contains chitin, chitosan and calcium carbonate. It can act as inducer and carbon source in the medium for chitosanase production. In the present study, with the use of response surface methodology (RSM), efforts were carried out to optimize chitosanase production by a newly isolated *Aspergillus fumigatus* YT-1 under submerged fermentation, while the mixture of shrimp shell powder and wheat bran was adopted in the medium. Furthermore, the optimal basic conditions for chitosan hydrolysis were preliminarily investigated.

Materials and methods

Microorganism

Aspergillus fumigatus YT-1 (GenBank Accession number JF907011) was isolated from shrimp shell-enriched marine soil in Yantai city of China and was maintained at 4 °C on potato dextrose agar (PDA) slants in microbiology laboratory, School of life Sciences, Liaocheng University.

Chitosanase production

Spore suspension (5.0×10^6 spores mL^{-1}) and mycelial suspension were prepared by the methods described in our previous report.[12] Chitosanase production was performed by inoculating mycelial sus-

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pension into the basic nutrient medium [$(NH_4)_2SO_4$ 1.5 g L^{-1}, KH_2PO_4 2.0 g L^{-1}, Tween80 1.0 mL L^{-1}, shrimp shell powder 10.0 g L^{-1} , $MgSO_4 \cdot 7H_2O$ 2.0 g L^{-1} , pH 5.0] with inoculum size 10.0 % (v/v) and cultivated at 30 °C, 200 rpm for 120 h. The crude chitosanase solution was also obtained by the methods described in our previous report.[12] Shrimp shell was purchased from Qingdao Hisea Feedstuff Science and Technology Co., Ltd. and was powdered with high speed disintegrator (QE-100, China).

Enzyme assay

Chitosanase activity was assayed using chitosan (deacetylation degree (DD) 90 %, molecular mass 200 kDa) as the substrate. The reaction mixture contained 4.5 mL chitosan solution (1.0 %, w/v) prepared by acetate buffer solution (200 mmol L^{-1}, pH 4.8) and 0.5 mL enzyme solution. After incubation at 60 °C for 15 minutes, the released reducing sugars in the supernatant of reaction mixture were estimated according to the modified 3, 5– Dinitrosalicyclic acid (DNS) method.[13] One unit (1 U) of chitosanase activity was expressed as the amount of enzyme capable of releasing 1 μmol of reducing sugar as D-glucosamine per minute under the conditions described above.

Screening of the optimal carbon and nitrogen source for chitosanase production

A preliminary screening investigation was carried out to explore the optimal carbon source and nitrogen source. Based on the basic nutrient medium described above, with the use of ammonium sulphate (AS) as nitrogen source, screening of the optimal carbon source was performed among different carbon sources, which included D-glucosamine (DG) (10.0 g L^{-1}), soluble starch (SS) (10.0 g L^{-1}), wheat straw powder (WSP) (10.0 g L^{-1}), corn stover powder (CSP) (10.0 g L^{-1}), wheat bran (WB) (10.0 g L^{-1}), shrimp shell powder (SSP) (10.0 g L^{-1}), mixture of shrimp shell powder (10.0 g L^{-1}) and wheat bran (2.0 g L^{-1}) (SSP/WB) and mixture of shrimp shell powder (10.0 g L^{-1}) and soluble starch (2.0 g L^{-1}) (SSP/SS). With the use of the optimal carbon source, screening of the optimal nitrogen source was carried out among different nitrogen sources including ammonium chloride (AC) (1.5 g L^{-1}), ammonium sulphate (AS) (1.5 g L^{-1}), potassium nitrate (PN) (1.5 g L^{-1}), urea (U) (1.5 g L^{-1}), yeast extract (YE) (1.5 g L^{-1}), peptone (P) (1.5 g L^{-1}), mixture of ammonium sulphate (1.5 g L^{-1}) and yeast extract (1.5 g L^{-1}) (AS/YE) and mixture of ammonium sulphate (1.5 g L^{-1}) and peptone (1.5 g L^{-1}) (AS/P). In addition, WSP and CSP were prepared using high speed disintegrator (QE-100, China).

Screening of significant factors for chitosanase production using Plackett-Burman design (PBD)

With the use of PBD, significant factors for chitosanase production were screened among ten independent factors including $(NH_4)_2SO_4$, yeast extract, KH_2PO_4, Tween80, shrimp shell powder (SSP), wheat bran (WB), $MgSO_4 \cdot 7H_2O$, cultivation temperature, initial pH and medium volume. Each factor was examined at low level (–1) and high level (+1), respectively. Twelve experiments were generated by PBD and the matrix along with the corresponding chitosanase activity of each trial is presented in Table 1. All experiments were carried out in triplicate and the average chitosanase activity

Table 1 – *Plackett-Burman design (PBD) for ten variables with coded values along with the experimental and predicted values of chitosanase activity*

Trials	A	B	C	D	E	F	G	H	I	J	Y (Chitosanase activity, U mL^{-1})	
											experimental	predicted
1	1	1	–1	1	1	–1	1	–1	–1	–1	8.16	8.19
2	1	1	–1	1	–1	–1	–1	1	1	1	7.55	7.52
3	–1	1	–1	–1	–1	1	1	1	–1	1	3.81	3.84
4	–1	1	1	–1	1	–1	–1	–1	1	1	6.23	6.26
5	1	1	1	–1	1	1	–1	1	–1	–1	9.07	9.04
6	–1	–1	–1	1	1	1	–1	1	1	–1	5.68	5.71
7	1	–1	1	1	–1	1	–1	–1	–1	1	6.73	6.76
8	–1	–1	1	1	1	–1	1	1	–1	1	5.04	5.01
9	1	–1	–1	–1	1	1	1	–1	1	1	7.76	7.73
10	1	–1	1	–1	–1	–1	1	1	1	–1	7.53	7.56
11	–1	1	1	1	–1	1	1	–1	1	–1	3.29	3.26
12	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	5.83	5.80

Table 2 – *Codes and levels of variables and statistical analysis of Plackett-Burman design (PBD)*

Terms	Codes	Levels		*T-value*	*P-value*
		–1	1		
$(NH_4)_2SO_4$ (g L^{-1})	A	1.0	2.0	42.30	0.015[a]
Yeast extract (g L^{-1})	B	1.5	3.0	–1.15	0.456
KH_2PO_4 (g L^{-1})	C	2.0	4.0	–2.25	0.266
Tween80 (mL L^{-1})	D	1.0	2.0	–9.45	0.067
Shrimp shell powder (g L^{-1})	E	10.0	15.0	18.00	0.035[a]
Wheat bran (g L^{-1})	F	1.5	3.0	–10.00	0.063
$MgSO_4 \cdot 7H_2O$ (g L^{-1})	G	1.5	2.5	–13.75	0.046[a]
Cultivation temperature (°C)	H	25	35	1.70	0.339
Initial pH	I	4.5	5.5	–1.50	0.374
Medium volume (mL)	J	50	70	–6.10	0.103

Outline criterion: 0.05, [a] Significant at 5 % level.

was taken as the response. Codes and levels of variables and statistical analysis of PBD are shown in Table 2.

Steepest ascent method

Based on the results of PBD, the optimal regions of significant variables for chitosanase production were investigated using the steepest ascent method. During experiments, values of positive significant variables and negative significant variables were enhanced and decreased, respectively, and the results are shown in Table 3.

Central composite design (CCD)

Using 3–factor-5–level central composite design (CCD) with twenty experiments, efforts were carried out to determine the optimal values of $(NH_4)_2SO_4$ (X_1), shrimp shell powder (X_2) and $MgSO_4 \cdot 7H_2O$ (X_3) and to develop a mathematical correlation between the three significant variables and chitosanase activity (Y). All three variables were investigated at low level (-1), zero level (0) and high level (+ 1), respectively. Codes and levels of variables and matrix of CCD along with chitosanase activity of each trial are shown in Table 4. Statistical analysis of CCD is shown in Table 5. 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 was the predicted response, β_0 intercept, β_1, β_2 and β_3 linear coefficients, β_{11}, β_{22} and β_{33} quadratic coefficients and β_{12}, β_{13} and β_{23} interactive coefficients.

Table 3 – *Experimental design of steepest ascent with corresponding values of chitosanase activity*

Steps	$(NH_4)_2SO_4$ (g L^{-1})	Shrimp shell powder (g L^{-1})	$MgSO_4 \cdot 7H_2O$ (g L^{-1})	Chitosanase activity (U mL^{-1})
1	2.0	14.0	1.8	8.82
2	3.0	17.0	1.5	13.13
3	4.0	20.0	1.2	17.32
4	5.0	23.0	0.9	21.31
5	6.0	26.0	0.6	18.51

Statistical analysis

Minitab (14.12) statistical software package and Statistical Analysis System (SAS, 8.0) were used for the experimental design and analysis of the experimental data.

Investigation of the optimal temperature and pH for chitosan hydrolysis

The optimal temperature and pH for chitosan hydrolysis were evaluated by incubating different reaction mixtures at different levels of temperatures (40 °C – 70 °C) and pH (sodium acetate buffer solution, 200 mmol L^{-1}, pH 3.6 – pH 5.6; sodium phosphate buffer solution, 200 mmol L^{-1}, pH 6.0 – pH 6.8), respectively. Effects of temperature and pH on chitosan hydrolysis were expressed as relative activity, which was the percentage ratio of chitosanase activity under each level of temperature and pH to that obtained under the optimal reaction conditions, respectively.

Results and discussion

Screening of the optimal carbon and nitrogen source for chitosanase production

As shown in Fig. 1(a), the mixture of shrimp shell powder and wheat bran (SSP/WB) was the most effective carbon source for chitosanase production. Wheat bran is a type of nutrient-richer by-product of the wheat processing industry which could supply microorganisms with protein, hemicellulose, iron, manganese, zinc and copper, and so on.[14–15] Wheat bran was also added in the medium for chitosanase production by *Aspergillus* sp. CJ22–326–14 and *Trichoderma koningii*, respectively.[16–17] Furthermore, using a certain amount of wheat bran in the medium could reduce chitosanase production cost. It was shown that chitosanase could be produced by the strain YT-1 while D-glucosamine was adopted as carbon source. And the consequent chitosanase activity was lower than those obtained while using SSP, SSP/WB and SSP/

Table 4 – *Codes and levels of variables and matrix of Central composite design (CCD) along with experimental and predicted values of chitosanase activity*

Trials	$(NH_4)_2SO_4$ (X_1, g L^{-1})	Shrimp shell powder (X_2, g L^{-1})	$MgSO_4 \cdot 7H_2O$ (X_3, g L^{-1})	Chitosanase activity (Y, U mL^{-1})	
	levels (values)	levels (values)	levels (values)	experimental	predicted
1	–1 (4.0)	–1 (18.0)	–1 (0.6)	17.15	17.17
2	–1 (4.0)	–1 (18.0)	1 (1.2)	16.89	16.82
3	–1 (4.0)	1 (28.0)	–1 (0.6)	17.75	17.76
4	–1 (4.0)	1 (28.0)	1 (1.2)	17.48	17.20
5	1 (6.0)	–1 (18.0)	–1 (0.6)	17.26	17.55
6	1 (6.0)	–1 (18.0)	1 (1.2)	17.51	17.51
7	1 (6.0)	1 (28.0)	–1 (0.6)	17.81	17.90
8	1 (6.0)	1 (28.0)	1 (1.2)	17.66	17.65
9	–1.682 (3.32)	0 (23.0)	0 (0.9)	15.71	15.90
10	1.682 (6.68)	0 (23.0)	0 (0.9)	16.81	16.60
11	0 (5.0)	–1.682 (14.59)	0 (0.9)	18.03	17.89
12	0 (5.0)	1.682 (31.41)	0 (0.9)	18.38	18.50
13	0 (5.0)	0 (23.0)	–1.682 (0.4)	19.08	18.84
14	0 (5.0)	0 (23.0)	1.682 (1.4)	18.12	18.34
15	0 (5.0)	0 (23.0)	0 (0.9)	21.31	21.45
16	0 (5.0)	0 (23.0)	0 (0.9)	21.62	21.45
17	0 (5.0)	0 (23.0)	0 (0.9)	21.56	21.45
18	0 (5.0)	0 (23.0)	0 (0.9)	21.28	21.45
19	0 (5.0)	0 (23.0)	0 (0.9)	21.35	21.45
20	0 (5.0)	0 (23.0)	0 (0.9)	21.58	21.45

Table 5 – *Regression analysis of central composite design (CCD)*

Terms	Coefficient estimate	Standard error coefficient	T-value	P-value
Constant	21.4507	0.09237	232.225	0.000
X_1	0.2065	0.06129	3.369	0.007[b]
X_2	0.1815	0.06129	2.961	0.014[a]
X_3	–0.1497	0.06129	–2.443	0.035[a]
X_1^2	–1.8395	0.05966	–30.832	0.000[b]
X_2^2	–1.1518	0.05966	–19.306	0.000[b]
X_3^2	–1.0121	0.05966	–16.965	0.000[b]
X_1X_2	–0.0613	0.08007	–0.765	0.462
X_1X_3	0.0787	0.08007	0.983	0.349
X_2X_3	–0.0513	0.08007	–0.640	0.537

Outline criterion: 0.05, [a] Significant at 5 % level, [b] Significant at 1 % level; $R^2 = 99.29$ %, Adj-$R^2 = 98.66$ %.

SS as carbon sources, respectively. It indicated that substrates abundant in chitosan could induce chitosanase production by the strain YT-1 more efficiently than glucosamine. In some previous reports, it was mentioned that simple sugars such as glucose and glucosamine could induce chitosanase production by *Bacillus* sp. S65 and *Bacillus Alvei* Nrc-14, whereas they could not induce chitosanase production by *Acinetobacter* sp. C-17.[5, 18–19] Therefore, the abilities to utilize different carbon sources for chitosanase production by chitosanolytic strains varied with species differences.

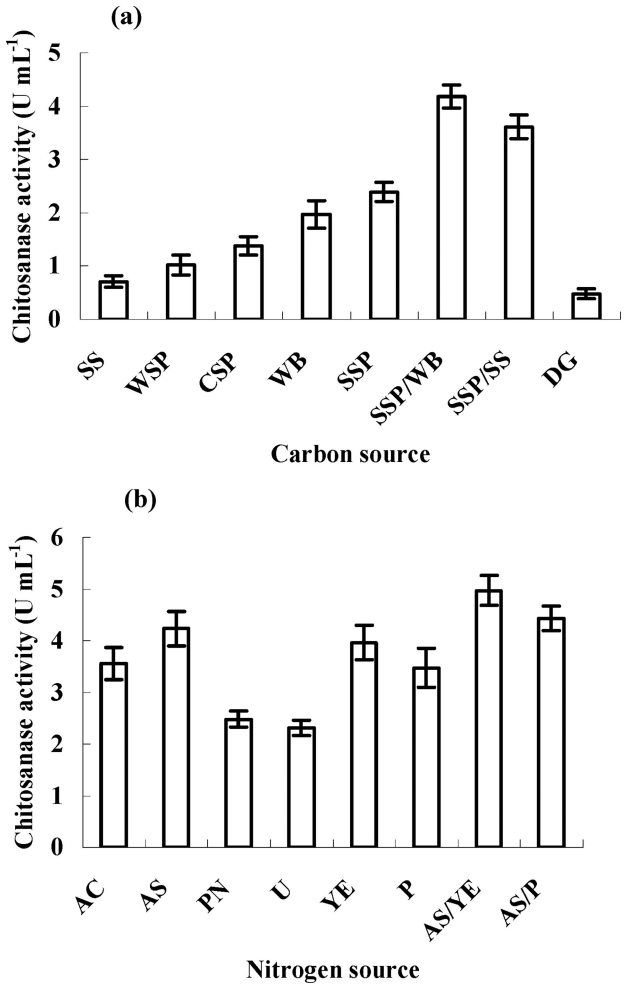

Fig. 1 – *Effects of carbon sources (a) and nitrogen sources (b) on chitosanase production. SS: Soluble starch (10.0 g L^{-1}); WSP: Wheat straw powder (10.0 g L^{-1}); CSP: Corn stover powder (10.0 g L^{-1}); WB: Wheat bran (10.0 g L^{-1}); SSP: Shrimp shell powder (10.0 g L^{-1}); SSP/WB: Mixture of shrimp shell powder (10.0 g L^{-1}) and wheat bran (2.0 g L^{-1}); SSP/SS: Mixture of shrimp shell powder (10.0 g L^{-1}) and soluble starch (2.0 g L^{-1}); DG: D-glucosamine (1.5 g L^{-1}); AC: ammonium chloride (1.5 g L^{-1}); AS: ammonium sulphate (1.5 g L^{-1}); PN: potassium nitrate (1.5 g L^{-1}); U: urea (1.5 g L^{-1}); YE: yeast extract (1.5 g L^{-1}); P: peptone (1.5 g L^{-1}); AS/YE: mixture of ammonium sulphate (1.5 g L^{-1}) and yeast extract (1.5 g L^{-1}); AS/P: mixture of ammonium sulphate (1.5 g L^{-1}) and peptone (1.5 g L^{-1}). Data points: mean values from three independent experiments. Error bars: standard deviations of triplicate independent experiments.*

While using the mixture of shrimp shell powder and wheat bran (SSP/WB) as carbon source in the medium, efforts were carried out to investigate the effects of different nitrogen sources on chitosanase production by the strain YT-1. As shown in Fig. 1(b), the mixture of ammonium sulphate and yeast extract (AS/YE) was the most effective nitrogen source for chitosanase production. While ammonium sulphate existed in the medium, addition of organic nitrogen sources such as yeast extract and peptone could induce chitosanase production by the strain YT-1 more effectively. In some previous reports, it was mentioned that the mixture of ammonium sulphate and yeast extract (AS/YE) was also used as nitrogen source for chitosanase production by *Aeromonas* sp. HG08 and organic nitrogen sources such as peptone and yeast extract were also used for chitosanase production by other strains such as *Bacillus* sp. strain KCTC 0377BP and *Bacillus Alvei* Nrc-14.[18, 20–21] Therefore, SSP/WB and AS/YE were adopted for chitosanase production in the subsequent experiments.

Screening of significant factors for chitosanase production using Plackett-Burman design (PBD)

As shown in Table 2, it was obvious that $(NH_4)_2SO_4$, shrimp shell powder and cultivation temperature exerted positive effects on chitosanase production, whereas yeast extract, KH_2PO_4, Tween80, wheat bran, $MgSO_4 \cdot 7H_2O$, initial pH and medium volume exerted negative effects. It indicated that factors including $(NH_4)_2SO_4$ ($P = 0.015$), shrimp shell powder ($P = 0.035$) and $MgSO_4 \cdot 7H_2O$ ($P = 0.046$) had significant effects on chitosanase production. Therefore, control of levels of $(NH_4)_2SO_4$, shrimp shell powder and $MgSO_4 \cdot 7H_2O$ were essential for chitosanase production. In some previous reports, it was mentioned that $(NH_4)_2SO_4$ and $MgSO_4 \cdot 7H_2O$ were also significant factors for chitosanase production by other chitosanolytic strains such as *Microbacterium* sp. OU01 and *Bacillus* sp. RKY3.[10,22] In addition, it was shown that smaller volume of medium (50 mL) could promote chitosanase production more efficiently than higher volume (70 mL), which indicated that concentration of dissolved oxygen had a positive effect on chitosanase production by the strain YT-1.

Steepest ascent method

Based on statistical analysis of the Plackett-Burman design (PBD) (Table 2), the optimal regions of three significant factors were investigated using the steepest ascent method. Values of yeast extract, KH_2PO_4, Tween80, wheat bran, temperature, initial pH and medium volume were 1.5 g L^{-1}, 2.0 g L^{-1}, 1.0 mL L^{-1}, 1.5 g L^{-1}, 35 °C, pH 4.5 and 50 mL, respectively. Data in Table 3 indicate that chitosanase activity reached a plateau on the fourth step. Therefore, this condition was selected for further optimization.

Optimization of significant variables using response surface methodology

Results in Tables 5 and 6 indicate that both linear terms (X_1, X_2 and X_3) and quadric terms (X_1^2, X_2^2 and X_3^2) had significant effect on chitosanase activity, whereas interaction terms (X_1X_2, X_1X_3 and X_2X_3) had insignificant effect. The resulting regression model is given below:

Table 6 – *Analysis of variance (ANOVA) for the fitted quadratic polynomial model*

Source	Degrees of freedom	Sum of squares	Mean square	*F-value*	*P-value*
Model	9	71.9499	7.9944	155.85	0.000[b]
Linear	3	1.3382	0.4461	8.70	0.004[b]
Square	3	70.5110	23.5037	458.21	0.000[b]
Interaction	3	0.1006	0.0335	0.65	0.599
Residual Error	10	0.5129	0.0513		
Lack of fit	5	0.3965	0.0793	3.41	0.102
Pure error	5	0.1164	0.0233		
Total	19	72.4628			

[b] Significant at 1 % level.

$$Y = 21.4507 + 0.2065X_1 + 0.1815X_2 - 0.1497X_3 - \\ - 1.8395X_1^2 - 1.1518X_2^2 - 1.0121X_3^2 - \\ - 0.0613X_1X_2 + 0.0787X_1X_3 - 0.0513X_2X_3$$

where Y was the predicted response (chitosanase activity), X_1, X_2 and X_3 the codes of $(NH_4)_2SO_4$, shrimp shell powder and $MgSO_4 \cdot 7H_2O$, respectively.

Results in Table 6 show that lack of fit ($P = 0.102$) was not significant and the model ($P = 0.000$) was appropriate for predicting chitosanase activity. Determination coefficient ($R^2 = 99.29$ %) and adjusted R^2 (Adj-$R^2 = 98.66$ %) also indicated that the model was accurate and the experimental values were in good agreement with the predicted values. As shown in Fig. 2, there were no elliptical contour plots, which indicated that no variable pairs had significant effect on chitosanase activity. According to the obtained regression equation, the optimal values of the significant factors were calculated using Statistical Analysis System (SAS, 8.0). According to the canonical analysis, the predicted maximum chitosanase activity (21.47 U mL^{-1}) could be obtained while the concentrations of $(NH_4)_2SO_4$, shrimp shell powder and $MgSO_4 \cdot 7H_2O$ were 5.05 g L^{-1} ($X_1 = 0.053231$), 23.40 g L^{-1} ($X_2 = 0.079016$) and 0.88 g L^{-1} ($X_3 = -0.073884$), respectively. While the strain YT-1 was cultivated under the above predicted conditions for chitosanase production in triplicate, values of maximum chitosanase activity including 21.29 U mL^{-1}, 21.93 U mL^{-1} and 22.32 U mL^{-1} were obtained. The corresponding discrepancy between the predicted value and experimental values were 0.84 %, 2.14 % and 3.96 %, respectively. The discrepancy might be due to the slight variation in experimental conditions. Relatively low discrepancy between the predicted value and experimental values indicated that RSM used in this work was effective for experimental design and prediction of chitosanase production by the strain YT-1. Consequently, based on the above results, average value of maximum chitosanase activity was 21.85 U mL^{-1} (Fig. 3). Furthermore, results in Fig. 3 also indicate that maximal chitosanase activity was 5.29 U mL^{-1} under unoptimized conditions [$(NH_4)_2SO_4$ 1.5 g L^{-1}, shrimp shell powder 10.0 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 2.0 g L^{-1}]. It was obvious that optimization resulted in 3.13–fold increase of chitosanase activity.

Comparison of carbon sources for chitosanase production, chitosanase activity and enzyme assay conditions by several chitosanolytic strains is shown Table 7. It was obvious that chitosanase assay conditions such as reaction pH, temperature and time differed from each other. Therefore, there were no standard assay methods for chitosanase activity which had been adopted and it was impossible to compare

Table 7 – *Comparison of carbon sources, chitosanase activity and enzyme assay conditions by several chitosanolytic strains*

Strains	Carbon Sources	Enzyme assay conditions			Chitosanase activity (U mL^{-1})	References
		Temperature (°C)	pH	Time (minute)		
YT-1	SSP/WB	60	4.8	15	21.85	This study
Microbacterium sp. OU01	CC	50	5.8	15	118	10
Bacillus sp. KCTC 0377BP	CC	50	-	10	100	20
Serratia sp. TKU016	SSP	37	7.0	30	0.022	23
Pseudomonas sp. TKU015	SSP	37	4.0	30	0.025	24
Bacillus thuringiensis ZJOU-010	SSP	40	5.0	15	4.25	25
Bacillus sp. TKU004	SPP	37	7.0	30	0.16	26

SSP: Shrimp shell powder; SPP: Squid pen powder; CC: Colloidal chitosan; SSP/WB: Mixture of shrimp shell powder and wheat bran.

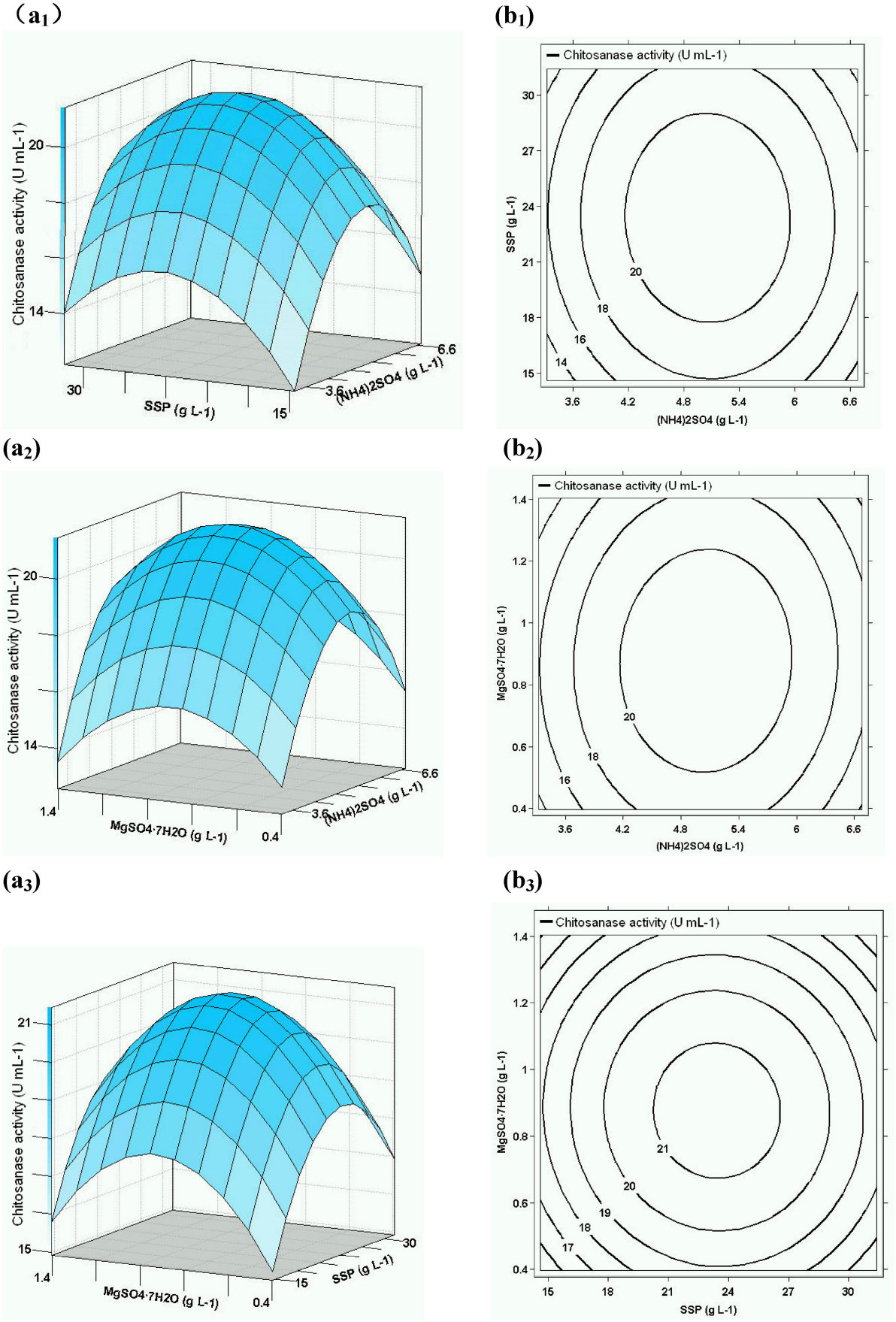

Fig. 2 – *Response surface plots (a) and contour plots (b) of the combined effects of each independent variable' s pair on chitosanase activity. (a_1, b_1): $(NH_4)_2SO_4$ and SSP; (a_2, b_2): $(NH_4)_2SO_4$ and $MgSO_4 \cdot 7H_2O$; (a_3, b_3): SSP and $MgSO_4 \cdot 7H_2O$; SSP: Shrimp shell powder*

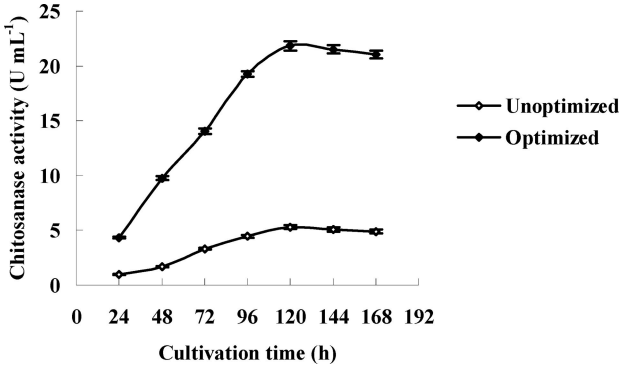

Fig. 3 – *Time courses of chitosanase production by Aspergillus fumigatus YT-1 under both optimized and unoptimized conditions. Data points: mean values from three independent experiments. Error bars: standard deviations of triplicate independent experiments.*

chitosanase activity by different chitosanolytic strains directly. In addition, it indicated that different substrates such as the mixture of shrimp shell powder and wheat bran (SSP/WB), colloidal chitosan (CC), shrimp shell powder (SSP) and squid pen powder (SPP) were used as carbon sources for chitosanase production by different chitosanolytic strains, respectively. Though it was obvious that CC could induce chitosanase production more effectively than other substrates, it was a type of relatively expensive substrate, which could result in increase of chitosanase production cost. In contrast with CC, inexpensive substrates including SSP, WB and SSP not only could induce chitosanase production by chitosanolytic strains effectively, but also could reduce chitosanase production cost. While using the inexpensive substrates as carbon sources, value of chitosanase activity by the strain YT-1 compared favorably with values of chitosanase activities reported in the literature for other chitosanolytic strains.

Effect of temperature and pH on chitosan hydrolysis

As shown in Fig. 4, it indicated that the optimal temperature and pH for chitosan hydrolysis were 60 °C and pH 4.8, respectively. Comparison of the optimal temperature and pH for chitosan hydrolysis

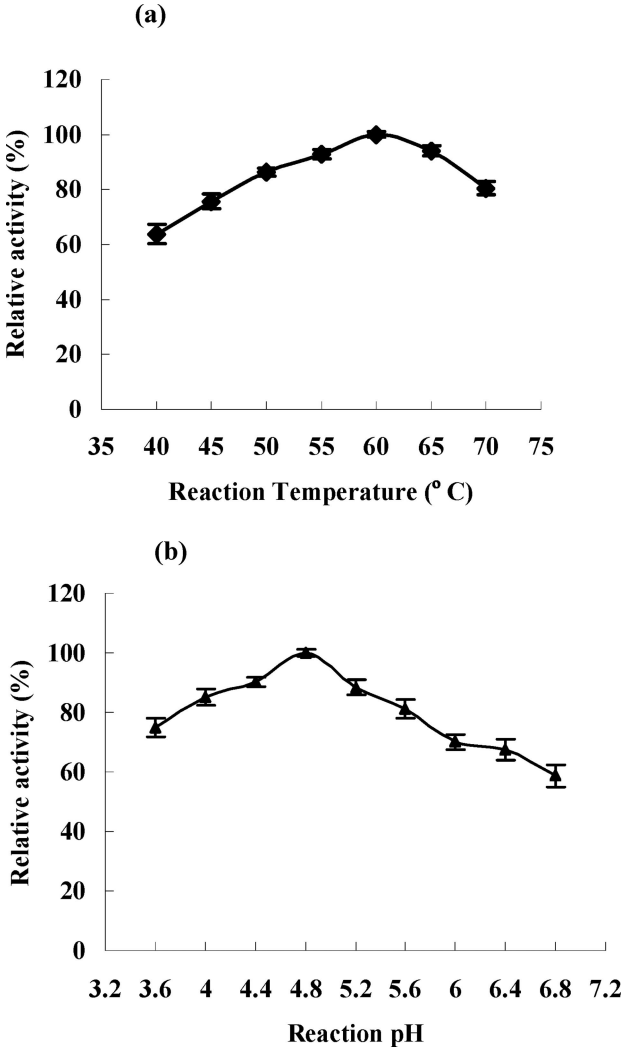

Fig. 4 – *Effects of temperature (a) and pH (b) on chitosan hydrolysis by the crude chitosanase. Data points: mean values from three independent experiments. Error bars: standard deviations of triplicate independent experiments.*

by chitosanases with different sources is shown in Table 8. The optimal reaction temperatures and pH varied with differences of chitosanase sources. It was obvious that a majority of the optimal conditions for chitosan hydrolysis were under acidic conditions, which was probably relevant to the acid dissolubility of chitosan.

Table 8 – *Comparison of temperature and pH for chitosan hydrolysis by chitosanase with different sources*

Chitosanase Sources	Temperature (°C)	pH	References
Aspergillus sp. CJ22–326	50 (Chitosanase A); 65 (Chitosanase B)	4.0 (Chitosanase A); 6.0 (Chitosanase B)	8
Pseudomonas sp. TKU015	50	4.0	24
Sphingomonas sp. CJ-5	56	6.5	27
Bacillus cereus D-11	60	6.0	1
Serratia marcescens TKU011	50	5.0	28
Aspergillus fumigatus YT-1	60	4.8	This study

Conclusions

Though it is mentioned that *Aspergillus fumigatus* strains have been adopted for chitosanase production in some previous reports, colloidal chitosan and powder chitosan are often used as inducers and carbon sources in the medium.[29] Reports about optimization of chitosanase production by chitosanolytic strains using low-cost natural substrates such as shrimp shell powder (SSP) and wheat bran (WB) are extremely few. Therefore, this work will provide a new technological reference in this field. To our best knowledge, this is the first report about optimization of chitosanase production by *A. fumigatus* strain using SSP/WB as carbon sources in the medium. Significant factors including $(NH_4)_2SO_4$, SSP and $MgSO_4 \cdot 7H_2O$ were screened and the optimization using RSM resulted in 3.13–fold increase of chitosanase activity. It indicates that RSM used in this work is accurate and effective in determining the optimal fermentation conditions for chitosanase production by the strain YT-1. It is also firstly reported that shrimp shell powder (SSP) has significant effect on chitosanase production. Purification, characterization of chitosanase from the strain YT-1 and analysis of chitosan hydrolysates are in progress in our laboratory.

ACKNOWLEDGEMENTS

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List of abbreviations and symbols

SS – soluble starch
WSP – wheat straw powder
CSP – corn stover powder
WB – wheat bran
SSP – shrimp shell powder
SSP/WB – mixture of shrimp shell powder and wheat bran
SSP/SS – mixture of shrimp shell powder and soluble starch
DG – D-glucosamine
AC – ammonium chloride
AS – ammonium sulphate
PN – potassium nitrate
U – urea
YE – yeast extract
P – peptone
AS/YE – mixture of ammonium sulphate and yeast extract
AS/P – mixture of ammonium sulphate and peptone
Y – predicted response (Chitosanase activity, U mL^{-1})
β_0 – intercept
$\beta_1, \beta_2, \beta_3$ – linear coefficients
$\beta_{11}, \beta_{22}, \beta_{33}$ – quadratic coefficients
$\beta_{12}, \beta_{13}, \beta_{23}$ – interactive coefficients
PBD – Plackett-Burman design
CCD – Central composite design
R^2 – Determination coefficient
SPP: Squid pen powder
CC: colloidal chitosan

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Supplementary materials

Supporting information
Methods for Isolation of chitosanolytic strains

Three types of shrimp shell-enriched marine soil in Yantai city of china were used as screening sources. Each sample (5.0 g) was transferred to 50 mL basic enrichment medium [$(NH_4)_2SO_4$ 1.5 g L^{-1}, KH_2PO_4 2.0 g L^{-1}, Tween80 1.0 mL L^{-1}, colloidal chitosan (deacetylation degree (DD) 90 %, molecular mass 200 kDa) 10.0 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 2.0 g L^{-1}] in a 250 mL Erlenmeyer flask and was cultivated at 30 °C for 96 h. After enrichment for three times, each sample solution (0.2 mL, successively diluted to 10^{-3}, 10^{-4} and 10^{-5} times) was spread on the selective medium agar plates [$(NH_4)_2SO_4$ 1.5 g L^{-1}, KH_2PO_4 2.0 g L^{-1}, colloidal chitosan (deacetylation degree (DD) 90 %, molecular mass 200 kDa) 10.0 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 2.0 g L^{-1}, agar powder 20.0 g L^{-1}]. Initial pH of the selective media for isolation of bacteria strains, actinomycetes strains and fungi strains were pH 7.0, pH 7.0 and pH 5.0, respectively. While preparing colloidal chitosan solution (10.0 g L^{-1}), powder chitosan (10.0 g) was dissolved in 700 mL of 0.4 mol L^{-1} muriatic acid solution and initial pH was adjusted to pH 5.0 using 2.0 M sodium hydroxide solution. Finally, the solution was set the volume to the mark in a 1000 mL measuring flask using distilled water.

Each sole colony was obtained after being streaked on selective medium agar plates and each strain was incubated in liquid selective medium (50 mL) in which equiponderant shrimp shell powder was used instead of colloidal chitosan to produce chitosanase. The inoculums sizes of the strains were 10.0 % (v/v) without exception using bacterial cell suspension (5.0×10^6 cells mL^{-1}) or mycelial suspension of actinomycete strains and fungi strains, which were prepared with spore suspension (5.0×10^6 spores mL^{-1}). The cultivation was carried out at 30 °C, 200 rpm in a rotary shaking incubator and cultivation times of bacteria strains, actinomycete strains and fungi strains were 48 h, 144 h and 120 h, respectively. The strain with maximum chitosanase activity among the obtained chitosanolytic strains was selected and further studied.

Supporting information
Figure S1 Comparison of chitosanase activity by nine major chitosanolytic strains

Comparison of chitosanase activity by nine major chitosanolytic strains including two bacteria strains (YB-2 and YB-5), three actinomycetes strains (YA-1, YA-2 and YA-7) and four fungi strains (YT-1, YT-3, YM-2 and YM-3) is shown in Fig. S1. Therefore, the stain YT-1 with maximum chitosanase activity was selected and further studied.

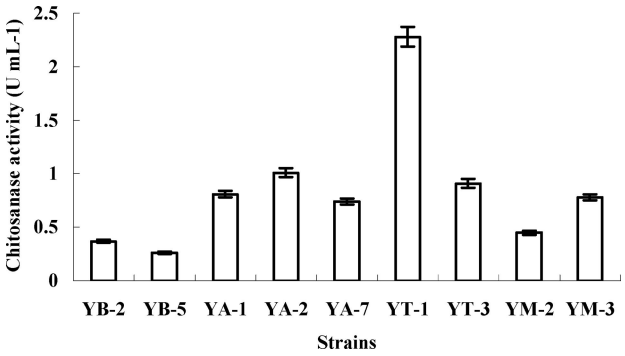

Fig. S1 – *Comparison of chitosanase activity by nine major chitosanolytic strains. Bacteria strains: YB-2 and YB-5; Actinomycetes strains: YA-1, YA-2 and YA-7; Fungi strains: YT-1, YT-3, YM-2 and YM-3. Data points: mean values from three independent experiments. Error bars: standard deviations of triplicate independent experiments.*

Supporting information
Methods for identification of chitosanolytic fungus strain YT-1

The strain YT-1 was identified by the methods described in our previous report.[1]

Supporting information
Results of identification of the chitosanolytic fungus strain YT-1

As shown in Fig. S2, it was obvious that spores catenated together and ventricosus conidiophore apex seemed almost spherical. Therefore, it indicated that the strain belonged to *Aspergillus* genus. Amplification of ITS sequence for the strain YT-1 (Accession number of ITS sequence: JF907011) resulted in a PCR product of

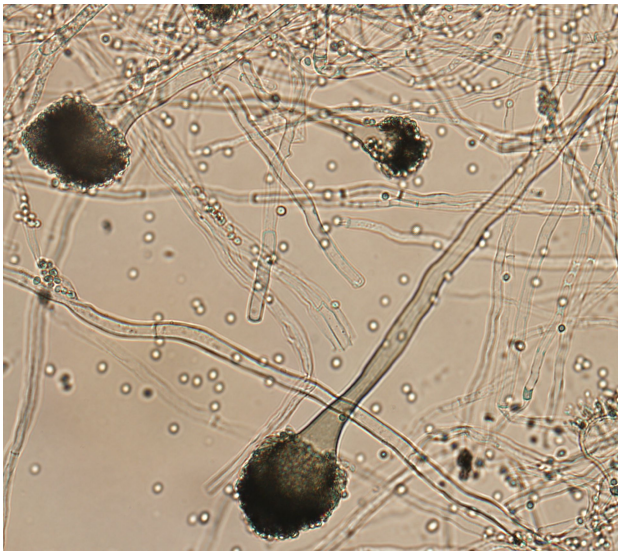

Fig. S2 – *Microphotograph (400×) of the conidial head of the strain YT-1*

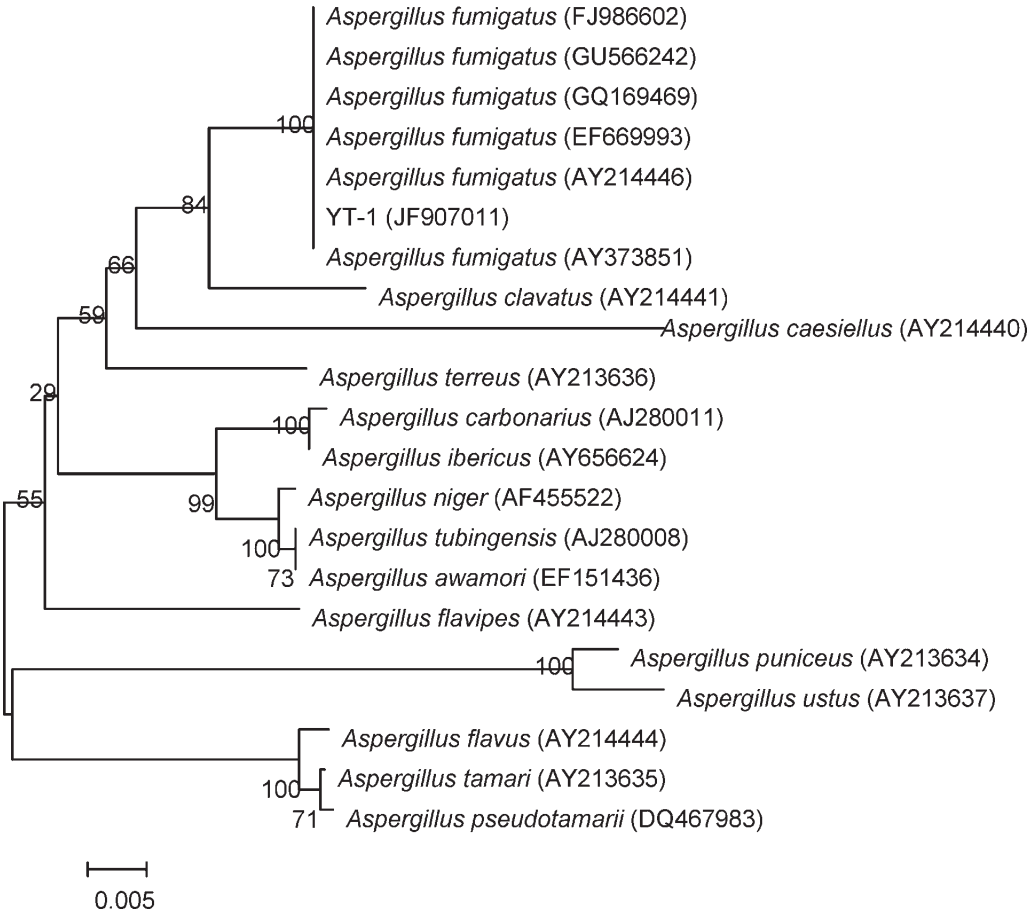

Fig. S3 – *Neighbour-joining tree based on phylogenetic analysis of the ITS gene sequence comparison of different Aspergilli strains*

527 bp in size. Sequence-similarity calculations indicated that the strain YT-1 was most closely related to *Aspergillus fumigatus* (Accession number: FJ986602), *Aspergillus fumigatus* (Accession number: GU566242), *Aspergillus fumigatus* (Accession number: GQ169469), *Aspergillus fumigatus* (Accession number: EF669993), *Aspergillus fumigatus* (Accession number: AY214446) and *Aspergillus fumigatus* (Accession number: AY373851), respectively (Fig. S3). Therefore, the chitosanolytic fungus strain YT-1 was identified as *Aspergillus fumigatus*.

Supplementary references

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