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Research article

Screening for *Aspergillus fumigatus* strain-2T-2 with high chitosanase production activity and its application in chitosan degradation ☆

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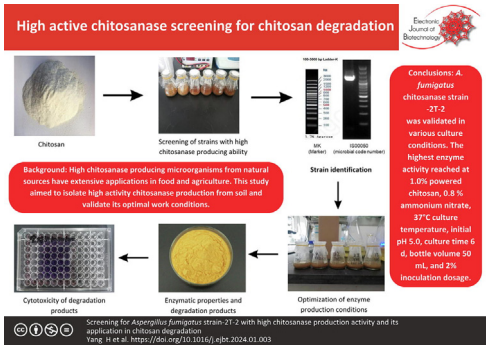
GRAPHICAL ABSTRACT



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ABSTRACT

Background: High chitosanase-producing microorganisms from natural sources have extensive applications in food and agriculture. This study aimed to optimal conditions for high-activity chitosanase production. A named CGMCC21422 chitosanase-producing strain -2T-2 was isolated from soil and identified named as *Aspergillus fumigatus* chitosanase (*A. fumigatus* chitosanase). This enzymatic activity was validated in various culture conditions. It is stored in the China General Microbiological Culture Collection Center. The efficacy of *A. fumigatus* chitosanase in the degradation of chitosan was validated. *Results:* In this study, we determined that the optimal fermentation conditions of stain-2T-2 were 1.0% powered chitosan, 0.8% ammonium nitrate, 37°C culture temperature, initial pH 5.0, culture time 6 d, bottle volume 50 mL, and 2% inoculation dosage. Under these culture conditions, the highest enzyme activity of fermentation broth in the shaker flask reached 827.53 U/mL. The optimal reactive conditions of *A. fumigatus*-produced chitosanase are 55–60°C and pH 4.5. When the reactive temperature was over 60°C, the *A. fumigatus* chitosanase was easily inactivated. The chitosanase catalyzed substrate chitosan to produced ≈20% chito-oligosaccharide and ≥80% glucosamine salt samples in a variety of acidic solutions. These reactive products are not cytotoxic or mild to MH7A cells.

☆ Audio abstract available in Supplementary material.

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Conclusions: A. fumigatus chitosanase strain -2T-2 is a strain with high chitosanase and can catalyze chitosan into chito-oligosaccharide in acidic solutions.

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1. Introduction

Extensive studies in glycobiology have led to the discovery and development of different chitosan and chitooligosaccharide [1,2]. Owing to their enormous potential value in agriculture, food, healthcare, and medicine, the demand for such materials is unmet. Chitooligosaccharide is an oligosaccharide after the degradation of chitosan with polymerization degree of 2–20 and is readily soluble in water with good bioavailability [2]. They have been widely used in the food industry and for biomedical applications, particularly for chitooligosaccharide [3,4]. In addition, Chitooligosaccharide is also involved in antitumor, antibacterial, sterilizing, antioxidation effects, enhances immunity, lowers blood pressure and blood sugar, and absorbing heavy metals [5,7,8] .

Traditional preparation method for chitooligosaccharide generally uses shrimp or crab shells as the raw material and process them with acid, alkali, and oxidant to obtain the target products. The specific process is as follows: shrimp or crab shell raw materials → crushing → acid leaching and decalcification → washing filtration → dilute alkali deproteinization → oxidation decolorization → washing → concentrated alkali deacylation → washing to neutral → drying → chitosan → dilute acid/oxidation degradation → dialysis → freeze drying → chitooligosaccharide. Traditional acid–base degradation has the following disadvantages: difficulty in controlling reaction conditions, serious environmental pollution, too expensive cost, low product yield, and low-purity chitooligosaccharide. Therefore, the development of less environmental pollution, economical and high-purity chitooligosaccharide technology is a critical issue to break the bottleneck of chitooligosaccharide manufacturers.

In the past 20 years, the enzymatic hydrolysis of chitosan by microbial fermentation has been extensively explored [3,9–12]. Enzymatic degradation methods have many advantages over the traditional methods: the degradation process and the relative molecular weight of the degradation product are easier to control, the degradation conditions are milder, the reaction process does not need many reagents, and the method is less polluting [3,4,13]. In the enzymatic hydrolysis method, chitosanase is very popular because chitosanase is a specific glycosidase in the degradation of chitosan [6,13,14]. Chitosanase-producing microorganisms have been identified in China and other countries, including *Aspergillus*, *Penicillium*, *Pseudomonas*, *Beauveria bassiana*, *Bacillus*, and *Microbacillus* [6,7,15,16]. Among these microorganisms, there are two kinds of *Aspergillus fumigatus*, which produce either chitosanase *(A. fumigatus chitosanase)* categorized in exo-beta-glucosaminidase that randomly cleave chitosan and directly generate monosaccharide glucosamine (GlcN) [17] or endo-type chitosanase that indirectly generate GlcN [18]. Although many chitosanase-producing microorganism strains have been identified through screening, products, degradation methods, and suitable enzymatic hydrolysis conditions of chitosan degradation by different chitosanase-producing microorganisms are diverse [14,19]. Moreover, the chitosanase activity produced by the strains is generally too low to achieve large-scale industrial production. Owing to limited available strains, the price of commercial chitosanase is too high to reduce production costs. Consequently, the high cost associated with the enzymatic production of chitooligosaccharide has limited the popularity and applications of this method. Therefore, it is necessary to develop more types of chitosan-degrading microorganisms with a wider range of adaptation conditions to meet the requirements for industrialization. Hence, chitosan-degrading microorganisms are conducive to the development and application of chitooligosaccharide.

This study aimed to evaluate optimal conditions for high-quality chitosanase production. We isolated and identified microbial chitosanase from natural sources, which improved their enzymatic activity and explored their application in chitooligosaccharide preparation. The entire produce and efficacy validation of the strain-2T-2 *A. fumigatus* chitosanase in chitosan degradation are summarized in graphical abstract.

2. Methods

2.1. Materials

Experimental soil samples were collected from the Southern district of Qingdao, mainly from areas where shrimp and crab shells accumulate such as seasides and lakesides. In total, 10 soil samples were used for microorganism strain screening. Chitosan (deacetylation degree 90–95%, average molecular weight 1,600,000 Da) and glucosamine hydrochloride (BR) were purchased from Sinopharm Chemical Reagents Co., Ltd. Colloidal chitosan was prepared in our laboratory as detailed below. Glucosamine hydrochloride and glucosamine sulfate were purchased from Shanghai Maclin Biochemical Technology Co., LTD. MH7A rheumatoid arthritis synovial fibroblasts were purchased from Guangzhou Genio Biological Technology Co., LTD. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Shanghai Beyotime Biotechnology Co., LTD. The remaining reagents were of analytical grade purity or higher.

The primary screening medium (plate separation medium) comprised the following: $(NH_4)_2SO_4$ 0.5%, K_2HPO_4 0.2%, NaCl 0.5%, $MgSO_4{\cdot}7H_2O$ 0.1%, powdered chitosan 1.0%, and Agar 2.0%. The initial fermentation medium (w/v) comprised the following: $(NH_4)_2SO_4$ 0.5%, yeast extract 0.5%, K_2HPO_4 0.07%, KH_2PO4 0.03%, NaCl 0.5%, $MgSO_4{\cdot}7H_2O$ 0.05%, and colloidal chitosan 1.0%.

Preparation of MTT solution: MTT 100 mg was accurately weighed using a precision balance and dissolved in 20 mL of phosphate buffered saline (PBS), then filtered with a 0.22 μm filter (5 mg/mL MTT solution). The entity preparation process needs to avoid light. After preparation, it was stored in a −20°C refrigerator and shielded from light, then diluted at 0.5 mg/mL final concentration before use.

2.2. Preparation of colloidal chitosan

Following description in the literature [20], 3 g of powdered chitosan was dissolved in a beaker filled with 200 mL of 0.2 M HCl. The solution was placed on a magnetic mixer and stirred until it became clear, and then adjusted to pH 9.0 by the addition of NaOH. The chitosan solution was centrifuged at 3,000 rpm/min speed for 5 min and washed with deionized water until the

supernatant was pH neutral. The washed precipitate was suspended in deionized water, and its pH was adjusted to 6.2 with 0.2 M NaAc and 0.3 M HAc (the pH of colloidal chitosan used for enzyme activity determination was adjusted to 5.6 in the same way). The final concentration of chitosan was adjusted to 1.0% (w/v) by adding deionized water, and the solution was stored at 4°C for further use.

2.3. Screening for chitosanase-producing microorganism strains

For the preliminary screening, the soil samples were diluted 10^{-7} times with sterile water, spread on a preliminary screening medium plate, and cultured at 28°C. After 3–5 d culture, a transparent circle was observed around the colony and a diameter slightly larger than the transparent circle was picked up. Single colonies were inoculated again on a primary screening medium plate and cultured at 28°C for 1–3 d. The colonies with a larger ratio of transparent circle to colony diameter (D/D) were selected as the starting strains for shaking-flask re-screening fermentation.

For the re-screening, the strains obtained from the preliminary screening were inoculated in the initial fermentation medium, cultured for 72 h in a 120 rpm/min shaker at 28°C, and centrifuged at 4,000 rpm/min for 10 min. Then, the enzyme activities of chitosanase in the supernatant were directly measured, and the strain with the highest enzyme production capacity was selected.

2.4. Strain identification

The 18S rDNA/ (internal transcribed spacer, ITS) sequence of the selected strain was detected by Sangon Biotechnology (Shanghai City, China). The sequencing results were input into NCBI to compare the existed strains.

2.5. Determination of chitosanase activity

The standard curve of glucosamine was prepared as described method in the literature [21]. Briefly, colloidal chitosan was used as the substrate in the following reaction system: 1.0 mL of 0.2 mol/L acetic acid buffer (pH 5.5), 0.5 mL of 1% colloidal chitosan, and 0.5 mL of supernatant (taken from the fermentation broth after centrifugation at 6,000 rpm/min for 5 min, containing chitosanase). The solution was reacted in a 50°C water-bath for 30 min and then immersed in a boiling water bath for 10 min to terminate the reaction. After centrifugation at 4,000 rpm/min for 5 min, a sample of the supernatant was taken for chromogenic reaction with the DNS reagent to determine chitosanase activity at OD_{540}. The control group comprised boiled supernatant solution. The fermentation enzymes of all strains were grouped and treated in the same way, and the concentration of enzyme required to produce one micromole (μM) of glucosamine (reducing sugar) per minute was one enzyme activity unit (U).

2.6. Determination of molecular weight and degree of polymerization (DP) in the degraded chitosan

The average molecular weight (MW) of the degraded chitosan was determined by an Agilent 1260 gel permeation chromatograph (Agilent Technologies, USA) equipped with a refractive index (RI) detector. Chromatography was performed on TSK 5000-PWXL, G4000-PWXL, and TSK G3000-PWXL columns, using 0.2 M CH_3-COOH/0.1 M CH_3COONa aqueous solution as the mobile phases. The flow rate was 0.8 mL/min for TSK 4000-PWXL and TSK 3000-PWXL or 0.6 mL/min for TSK 5000-PWXL. The column temperature was kept at 30°C.

DP of the degraded chitosan was analyzed by hydrophilic interaction liquid chromatography using an LC-2030C 3D Plus HPLC system (SHIMADZU, Kyoto, Japan) with an evaporative light scattering detector (Essentia ELSD-16). Chromatography was performed on a Click Maltose column (4.6 mm × 150 mm, 5 μm), using a binary mobile phase (acetonitrile and ammonium formate buffer) stepwise at a flow rate of 1.0 mL/min and with a column temperature of 30°C [22].

2.7. Optimization of culture conditions for the maximal chitosanse activity production

To optimal culture conditions for the maximal chitosanse activities, we utilized different carbon sources, including powdered chitosan, colloidal chitosan, soluble starch, glucose, and sucrose as carbon sources; nitrogen source, including ammonium nitrate, urea, peptone, potassium, and ammonium sulfate; various temperature, medium pH value, fermentation time, liquid loading amount, and inoculation amount in the fermentation medium. The chitosanse enzyme activity of the fermentation liquid was measured. An L18(3^7) orthogonal experiment with three factors and seven levels was designed based on a single-factor experiment.

2.8. The optimal conditions of chitosan degradation by A. fumigates chitosanase

For the preparation of *A. fumigatus* chitosanase in crude enzyme liquid (hereafter referred to "enzyme solution"), *A. fumigatus* fermentation liquid rich was obtained under the optimized conditions for the orthogonal experiment. After centrifugation at 6,000 rpm for 10 min, the supernatant was collected for subsequent experiments. Chitosanase activity in the supernatant was measured as above description.

To discover the optimal work conditions of *A. fumigatus* chitosanase, the solution containing chitosanase was used to degrade chitosan at different temperatures, chitosanase: chitosan ratios (4%, w/v), pH values, and reactive time. The molecular weight of the chitooligosaccharide was determined according to the above method.

2.9. Measurement of glucosamine (monosaccharide) salts in different chitosan degraded conditions

The 3% and 10% chitosan were dissolved in different acid solutions and degraded according to the above-optimized conditions, and the molecular weight and DP were detected every 24 h according to previous methods until the chitosan was degraded to the sample with monosaccharide content ≥80%. The presence of glucosamine was determined by high-performance liquid chromatography (HPLC) method. Briefly, alone solvent, various saccharide standard samples and tested samples were loaded into HPLC columns. Readout peak is identical to only solvent, monosaccharide, disaccharide, trisaccharide, tetrasaccharide, five sugars, six sugars, and seven sugars at 5.0 min, 7.5 min, 12.5 min, 15.0 min, 17.5 min, 18.5 min, 20 min, and 21 min of retention time.

Acid solutions included adipic acid, glutamic acid, succinic acid, malic acid, acetylsalicylic acid, lactic acid, hydrochloric acid, acetic acid, and aspartic acid. MW (Da) and degree of polymerization (DP) of chitosan degradation products in different acid solutions were measured at pH 4.5, 55°C, 3% powdered chitosan, chitosanase solution:chitosan volume at 1:5 ratio (v/v) for 24 h and 48 h culture.

2.10. Cytotoxicity assay of degradation products

MH7A cells in the logarithmic growth phase were harvested and the cell concentration was adjusted to 1×10^5 cells /mL. Then, 100 μL cell suspension was transferred into experimental wells of 96-well plate. After 24 h culture in 5% CO_2 incubator, 100 μL

different degradation products were added to each well (3parallelproductsineachgroup). The 96-well plates were cultured for 24 h; then, the old medium was discarded, and 100 μL of 0.5 mg/mL MTT solution was added to each well (note that MTT should be protected from light throughout the operation). After 4 h of culture, 100 μL dimethyl sulfoxide solution (DMSO) was added to each well, and the absorbance was measured at 490 nm with a microplate reader.

2.11. Statistical analysis

Data are expressed as the mean ± standard deviation (sd). Single-factor analysis of variables between multiple groups was performed with Duncan's tests. Differences between the means of ≥3 independent experiments were considered significant for P values of <0.05. Statistical analyses were performed using SPSS 19.0 software.

3. Results

3.1. Screening and identification of chitosanase-producing strain

In total, 3 out of 20 cultured strains produced chitosanase (Table 1). These data indicated that strain -2T-2 can produce the highest activity of chitosanase (116.96 U/mL). To confirm the -2T-2 strain gene-specific sequence, 1400 bp of the DNA fragment by polymerase chain reaction (PCR) implication was determined (data not shown) by Sangon Biotechnology Co., Ltd (Shanghai). This DNA sequence was identified as *A. fumigates* by GenBank database search. The -2T-2 strain has 100% identity with reported stains such as MT102247.1, LC485158.1, MN326853.1, and MF563964.1. The result was stored in the Center for General Microbiology, China Microbial Species Conservation and Administration Commission on December 31, 2020. The collection number was CGMCC 21422. The later experiment always used -2T-2 strain for our experiments.

3.2. Effects of carbon source, carbon source addition amount, nitrogen source, nitrogen source addition amount, pH, temperature, bottling amount, inoculation amount, and fermentation time on chitosanase activity

To obtain the maximal chitosanase activity produced in -2T-2 strain, we performed different combinations using various carbon source, nitrogen source, pH, and fermentation time. The results showed that 1.0% powdered chitosan and 0.1% glucose (G) as the carbon source resulted in higher enzyme activity in the fermentation liquid (Fig. 1A). Of the various parameters tested, 1.0% powdered chitosan and 0.1% glucose as the carbon source had the best enzyme production effect, and the enzyme activity in the fermentation liquid was 132.07 U/mL. In contrast, when 1.0% colloidal chitosan and 0.1% glucose were used as the carbon source, the chitosanase activity was the second best (45.08 U/mL). *A. fumigatus* did not synthesize chitosanase when chitosan was not present in the culture medium. In addition, only powdered or colloidal chitosan without glucose in fermentation media just got ∼20 U/mL chitosanase activity. The effect of carbon source amount level on the chitosanase production is shown in Fig. 1B. When the concentration of powdered chitosan was 1.0 %, the -2T-2 strain had the highest chitosanase activity, which was 142.97 U/mL. When the amount of powdered chitosan was increased to 1.2%, the chitosanase activity in the fermentation broth was 137.70 U/mL. However, there was no significant difference between 1.0% and 1.2%. If we used < 1.0% powdered chitosan, the chitosanase activity was significantly decreased.

Table 1
Chitosanase activity of different strains.

Strain number	-1T-2	-1T-3	-2T-2
Enzyme activity (U/mL)	50.80 ± 2.55[b]	62.65 ± 6.75[b]	116.96 ± 9.03[a]

The result is reported as means ± SD. Single-factor analysis of variance between multiple groups was performed using Duncan methods. According to Duncan analysis, the letter "a" represents the groups whose mean values are relatively larger. From "a" to "b", the mean values decrease in turn. There is no significant difference between groups with the same letter. There is a significant difference between groups with different letters, $p \leq 0.05$.

We further investigated the effects of the nitrogen source on the chitosanase activity of the fermentation broth (Fig. 1C). When 2.0% ammonium nitrate was used as the nitrogen source, the chitosanase activity of the fermentation liquid was 192.10 U/mL, which was significantly higher than that of the other groups ($P < 0.05$). When the amount of ammonium nitrate was added into the culture media from 1.0% to 2.0%, the chitosanase activity of the fermentation liquid doubled from 103.07 U/mL to 205.99 U/mL (Fig. 1D). However, when we added more ammonium nitrate from 2% to 2.5% or 3%, the chitosanase activity was significantly decreased with increasing ammonium nitrate concentration ($P < 0.05$). Therefore, the concentration of ammonium nitrate in the medium was selected as 2.0% for later experiment.

The optimal initial pH value of the culture media showed that the -2T-2 strain had the highest chitosanase activity when the initial pH value of the culture medium was 4.5 (Fig. 2A), which the chitosanase activity of the fermentation liquid was 305.22 U/mL. The chitosanase activity was significantly decreased when the initial pH value was below 4.5 or above 5.0 ($P < 0.05$). In addition, we also evaluated the impacts of the culture media temperature on the chitosanase production of the strain (Fig. 2B). When the strain was cultured at 37°C, the chitosanase activity of the fermentation liquid was high up to 346.06 U/mL. When the temperature was lower than or higher than 37°C, the chitosanase activity of fermentation broth decreased significantly ($P < 0.05$). The influence of the culture media bottling volume on the chitosanase activity of fermentation liquid is shown in Fig. 2C. When 50 mL of culture medium was contained in a 100-mL conical flask, the chitosanase activity of the fermentation liquid was high up to 373.01 U/mL. When the bottling amount was 25 mL, the chitosanase activity of the fermentation liquid was 337.89 U/mL, which was not significantly different to that of the 50-mL group ($P > 0.05$). When the volume of bottling media was 75 mL and 100 mL, the chitosanase activity of the strain was significantly decreased. The experimental results of the optimal inoculation amount are shown in Fig. 2D. When the inoculation amount was 5% (v/v), the strain had the highest enzyme production capacity (301.95 U/mL), but there was no significant difference between each group ($P > 0.05$), indicating that the inoculation amount had little effect on the enzyme production capacity of the strain. In addition, we also evaluated the effects of culture time on the chitosanase activity of fermentation liquid and found that the chitosanase activity had the highest value at day 5 (424.46 U/mL) (data not shown).

3.3. Orthogonal experiment

Based on the above single-factor experiment, an L18(3^7) type orthogonal experiment with seven factors and three levels was designed. The experimental results and the relationship between the levels of each factor and Ki value are shown in Table 2. The analysis of the orthogonal experiment results showed that the order of influence on chitosanase activity was as follows: temperature > amount of carbon source > amount of inoculation > pH value > amount of bottling > amount of nitrogen source > fermentation time. The optimal modification conditions of *A. fumigatus* chitosanase produce were as follows: 1% chitosan

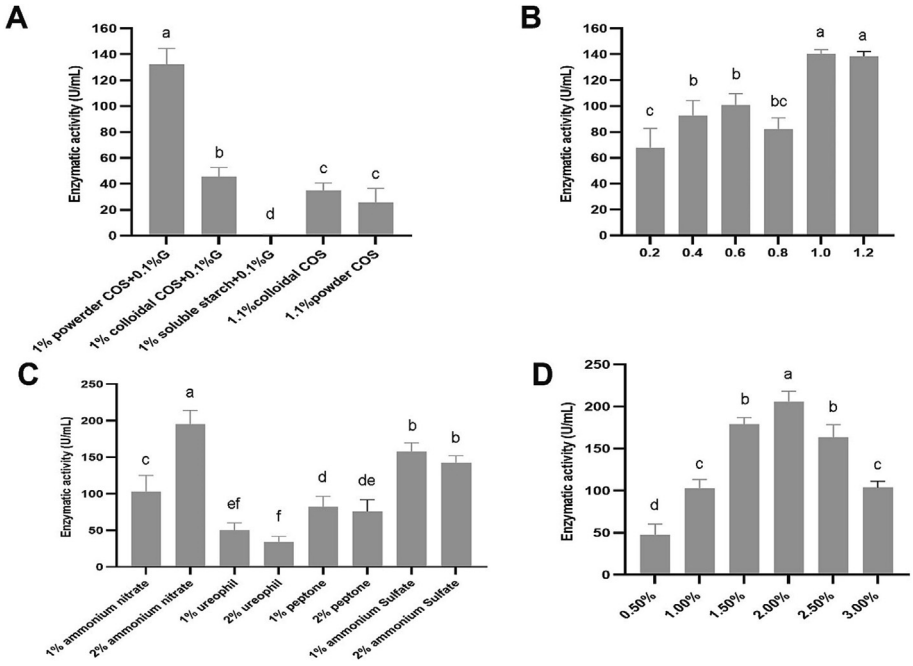

Fig. 1. The effects of different carbon sources or nitrogen sources on chitosanase activity for four days culture. (A) carbon sources; (B) different powdered chitosan proportion; (C) nitrogen sources; (D) different concentration ammonium nitrate addition. COS: chitosan; G: glucose.

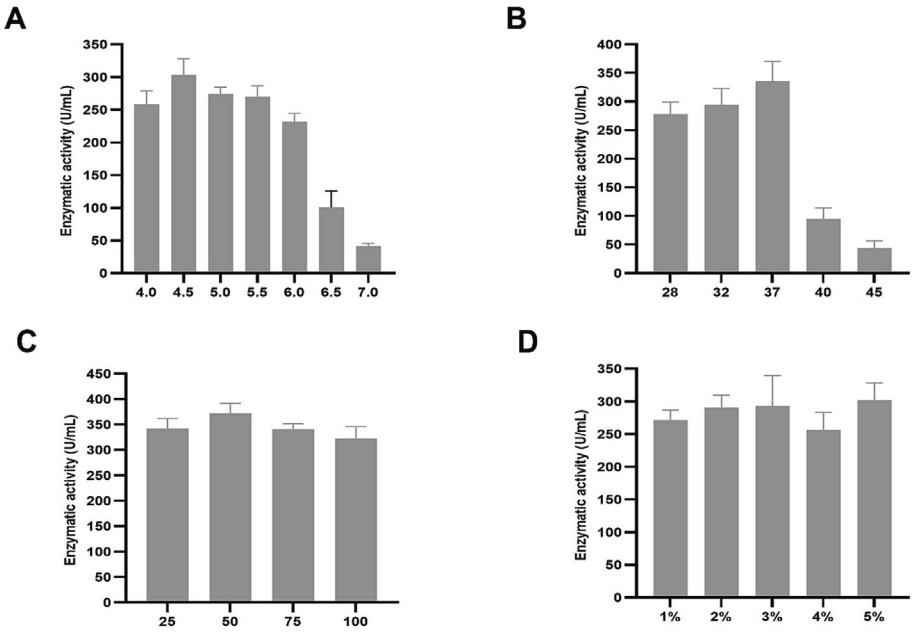

Fig. 2. The effects of different pH value, temperatures, medium bottling volume, and inoculation amount on chitosanase activity. (A) pH value; (B) temperatures; (C) medium bottling volume (mL); (D) inoculation (v/v).

dosage, 2% ammonium nitrate, 37°C culture temperature, pH 5.0; culture time for 6 d; 50 mL bottle volume, and 2% inoculation dosage (Table 2, condition 5). The results of three repeated experiments under optimized conditions are shown in Table 3. As shown in Table 3, the average value of chitosanase activity in *A. fumigatus* culture medium obtained from three repeated experiments under the optimized process was 827.53 U/mL, which was significantly higher than that obtained under non-optimized culture conditions, indicating an obvious improvement in chitosanase activity in *A. fumigatus* culture medium under the optimized conditions.

3.4. Optimization of chitosan degradation conditions by A. fumigates chitosanase

The chitosan degradation ability of the *A. fumigatus* fermentation broth was measured at different pH values and temperatures. The results are shown in Fig. 3. The degradation effect was strongest when the pH value was < 4.5. With pH increased (e.g., to pH >5.0), chitosan changed from colloidal to flocculent or even precipitated, affecting the activity of chitosanase and resulting in an inability to degrade chitosan (Fig. 3A). We also evaluated the

Table 2
Orthogonal experiment and results of chitosan degradation production by *A. fumigates* chitosanase.

	Carbon source amount (%)	Nitrogen source amount (%)	T (°C)	pH value	Fermentation time (d)	Bottling amount (mL)	Inoculation amount (%)	chitosanase activity (U/mL)
1	0.8	1.5	32	4	4	50	2	791.6
2	0.8	2	37	4.5	5	75	4	524.11
3	0.8	2.5	40	5	6	100	6	235.8
4	1	1.5	32	4.5	5	100	6	477.96
5	1	2	37	5	6	50	2	795.68
6	1	2.5	40	4	4	75	4	434.27
7	1.2	1.5	37	4	6	75	6	678.89
8	1.2	2	40	4.5	4	100	2	274.18
9	1.2	2.5	32	5	5	50	4	92.46
10	0.8	1.5	40	5	5	75	2	327.27
11	0.8	2	32	4	6	100	4	308.49
12	0.8	2.5	37	4.5	4	50	6	533.91
13	1	1.5	37	5	4	100	4	170.05
14	1	2	40	4	5	50	6	289.29
15	1	2.5	32	4.5	6	75	2	259.89
16	1.2	1.5	40	4.5	6	50	4	181.89
17	1.2	2	32	5	4	75	6	181.89
18	1.2	2.5	37	4	5	100	2	261.93
(1) k_1	2,721.18	2,627.66	2,112.29	2,764.47	2,385.9	2,684.83	2,710.55	
(1) k_2	2,427.14	2,373.64	2,964.57	2,251.94	1,973.02	2,406.32	1,711.27	
(1) k_3	1,671.24	1,818.26	1,742.7	1,803.15	2,460.64	1,728.41	2,397.74	
(2) K_1	453.53	437.94	352.05	460.75	397.65	447.47	451.76	
(2) K_2	404.52	395.61	494.1	375.32	328.84	401.05	285.21	
(2) K_3	278.54	303.04	290.45	300.53	410.11	288.07	399.62	
(3) R	174.99	134.9	203.65	160.22	81.27	159.4	166.55	

(1) k_1, k_2, and k_3 are the chitosanase activity sum of the six experimental conditions, respectively; (2) $K_1 = k_1/6$, $K_2 = k_2/6$, $K_3 = k_3/6$; (3) R, the range is the k_{max}–k_{min} among the three levels of a factor. T, temperature.

Table 3
Results of three repeated experiments in an orthogonal experiment under optimized condition.

Number	1	2	3	Average
Enzyme activity (U/mL)	838.97	836.52	807.12	827.53

The optimal condition of chitosanase production by *A. fumigatus* was as follows: 1% chitosan dosage, 2% ammonium nitrate, 37°C culture temperature, pH 5.0; culture time for 6 d; 50 mL bottle volume, and 2% inoculation dosage.

effects of temperature on chitosan degradation of chitosanase. When the temperature increased, the degradation effect was significantly improved. At a temperature of 55–65°C, the strongest degradation was observed (Fig. 3B), and when the temperature reached 70°C, chitosanase activity decreased sharply.

3.5. Analysis of chitosan degradation products in different acid solutions

The *A. fumigatus* chitosanase can degrade chitosan in a variety of acid solutions, including adipic acid, glutamic acid, succinic acid, malic acid, acetylsalicylic acid, lactic acid, hydrochloric acid, acetic acid, and aspartic acid. The molecular weight (MW) and degree of polymerization (DP) of the hydrolysates are shown in Table 4 and Table 5, respectively. *A. fumigatus* chitosanase could not degrade chitosan dissolved in tartaric acid and citric acid (Table 4). With

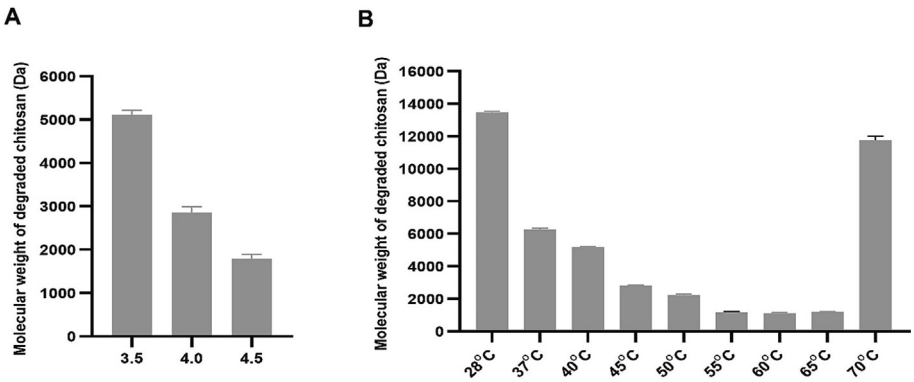

Fig. 3. The effects of different pH value and temperatures on chitosan degradation by *A. fumigatus* chitosanase. (A) MW of chitosan degradation in 55°C, 4% powdered chitosan, chitosanase:chitosan volume at 1:5 (v/v) ratio for 24 h culture at different pH value; (B) MW of chitosan degradation in pH 4.5, 4% powdered chitosan, chitosanase:chitosan volume at 1:5 (v/v) ratio for 24 h culture at different temperatures.

longer time culture, more monosaccharide and disaccharide with low MW (Table 4 at 48 h) were obtained in adipic acid, malic acid, acetylsalicylic acid, acetic acid and aspartic acid solution (Table 5).

3.6. Degradation of high-concentration chitosan solutions by chitosanase

As shown by the experimental results in Table 6 and Table 7, *A. fumigatus* chitosanase can effectively degrade high-concentration chitosan solution. As the time increased, the molecular weight of chitosan gradually decreased. Monosaccharide and disaccharide degradation products were not found when the degradation was performed for 1–4 d. After 3 d of degradation, the degradation products were chitosanase.

3.7. Generation of glucosamine (monosaccharide, GlcN) salt

Aspergillus fumigates produced chitosanase can effectively degrade chitosan into monosaccharide or disaccharide in lactic acid, aspartic acid and glutamic acid solution because there were lower MW at 50 d culture (data not shown). Among these degradation products, the content of GlcN can reach more than 80%. After degradation of 3% chitosan in aspartic acid and glutamine solution for 20 d, the monosaccharide content in the solution could reach 80.511% (Fig. 4A) and 93.016% (Fig. 4B), respectively. In addition, if 10% chitosan in glutamate solution for 40 d, the monosaccharide content in the solution was 100% (Fig. 4C). After degradation of 3% chitosan in lactic acid solution for 20 d, the monosaccharide content in the solution reached 82.317% (Fig. 4D). We observed that there are noticeable differences in retention time for the peaks of glucosamine in different culture conditions due to different culture times. In addition, Fig. 4C shows two peaks at 5.0 min and 7.5 min retention time, which respectively stand for solvent and glucosamine because these retention times are identical to solvent and monosaccharide by HPLC assay.

3.8. Cytotoxicity of degradation products chitooligosaccharide (molecular weight about 3000 Da) and glucosamine on MH7A cells

To evaluate the effects of chitosan degradation products on MH7A cells growth, we tested MH7A cell survival rates in different concentrations of chitooligosaccharide and glucosamine. Results are shown in Table 8 and Table 9. Chitooligosaccharide in glutamic acid, lactic acid, acetic acid, hydrochloric acid, butyric acid and caproic acid almost do not affect MH7A cell growth (cell survival rate $\geq$ 90%). However, $\geq$500 μg/mL chitooligosaccharide in acetylsalicylic acid and $\geq$100 μg/mL aspartic acid exhibited toxicity to MH7A cells (cell viability <90%) (Table 8). In contrast, the cytotoxicity of glucosamine samples obtained by *A. fumigatus* chitosanase degrading chitosan to MH7A cells was not different from that of commercially available glucosamine hydrochloride, and was lower than that of commercially available glucosamine sulfate. Glucosamine obtained after degradation of chitosan dissolved with glutamic acid, lactic acid and aspartic acid was slightly cytotoxic to MH7A cells at concentrations $\geq$1000 μg/mL (90% > cytotoxicity >80%) (Table 9).

4. Discussion

Chitosanase is an enzyme that hydrolyzes partially acetylated chitosan into chitooligosaccharide [23]. The generation of chitooligosaccharide is the main application of chitosanase. To improve the production efficiency of chitooligosaccharide by *A. fumigates* stain-2T-2 produced chitosanase and expand the appli-

Table 4
Molecular weight (Da) of chitosan degradation products in different acid solutions.

Degradation time	Adipic acid	Glutamic acid	Succinic acid	Malic acid	Acetylsalicylic acid	Lactic acid	Hydrochloric acid	Acetic acid	Aspartic acid	Tartaric acid	Citric acid
12 h	1,103.3 ± 40.45c	605.67 ± 19.86f	551.33 ± 34.59f	781.67 ± 17.95e	1,825.00 ± 30.79a	1,168 ± 29.21c	––	891.67 ± 77.89d	1,242.33 ± 37.90b	––	––
48 h	810.33 ± 13.20bc	744.33 ± 22.73c	464.00 ± 29.82e	547.00 ± 25.24d	536 ± 28.84de	197 ± 37.51f	1952.33 ± 93.07a	525.00 ± 24.98de	862.33 ± 45.54b	––	––

a, b, c, d, e, f: Stand for comparison of average molecular weight in each group using Duncan test. a > b > c > d > e > f. If there is only letter like a,or b,c,d,e,f, this means no significant differences among groups ($P > 0.05$). If there are two letters like bc, or de, this means that there were significant differences among groups ($P < 0.05$).

Table 5
The degree of polymerization (DP) of chitosan products degraded by chitosanase in different acid solutions (degradation time: 48 h).

	Adipic acid	Glutamic acid	Succinic acid	Malic acid	Acetylsalicylic acid	Lactic acid	Hydrochloric acid	Acetic acid	Aspartic acid
^{1}GlcN	7.918	——	——	57.797	12.343	——	——	9.626	8.856
$(GlcN)_2$	5.23	——	——	8.112	9.923	——	7.595	5.758	65.501
$(GlcN)_3$	17.319	21.049	40.37	4.781	22.416	51.151	11.668	17.431	11.03
$(GlcN)_4$	33.118	37.178	32.547	4.591	30.265	27.476	34.196	34.116	4.989
$(GlcN)_5$	24.778	27.566	18.435	6.404	18.054	15.847	28.09	23.706	6.982
$(GlcN)_6$	11.636	14.207	7.305	5.950	7.000	5.525	18.451	9.364	2.642
$(GlcN)_7$	——	——	1.344	4.204	——	——	——	——	——
$(GlcN)_8$	——	——	——	3.059	——	——	——	——	——
$(GlcN)_9$	——	——	——	2.043	——	——	——	——	——
$(GlcN)_{10}$	——	——	——	1.436	——	——	——	——	——
$(GlcN)_{11}$	——	——	——	0.955	——	——	——	——	——
$(GlcN)_{12}$	——	——	——	0.670	——	——	——	——	——

GlcN: glucosamine; 1, 2, 3, 4, 5…12 stand for 1,2—12 GlcN.

Table 6
Molecular weight of chitosan products (Da) degraded by chitosanase.

Incubation time	1 d	2 d	3 d	4 d
Chitosan concentration, 10 %	4,900	2,439	1,389	912
Chitosan concentration, 20 %	10,485	5,520	2,240	1,501

Table 7
Polymerization degree of chitosan products degraded by chitosanase.

Polymerization degree		$GlcN_1$	(GlcN)2	(GlcN)3	(GlcN)4	(GlcN)5	(GlcN)6	(GlcN)7	(GlcN)8	(GlcN)9	(GlcN)10	(GlcN)11 and above
Chitosan concentration, 10%	1 d	——	——	2.79	7.71	10.02	6.01	6.25	5.24	3.26	2.56	56.15
	2 d	——	——	9.19	20.45	21.89	14.04	10.11	5.03	2.42	2.53	14.33
	3 d	——	——	13.664	35.455	30.583	18.676	1.602	——	——	——	——
	4 d	——	——	14.955	36.309	28.885	17.968	1.882	——	——	——	——
Chitosan concentration, 20%	1 d	——	——	——	——	——	——	——	——	——	——	——
	2 d	——	——	——	——	——	——	——	——	——	——	——
	3 d	——	——	10.941	22.57	28.467	23.316	10.635	4.071	——	——	——
	4 d	——	——	13.677	26.139	29.15	21.546	7.695	1.792	——	——	——

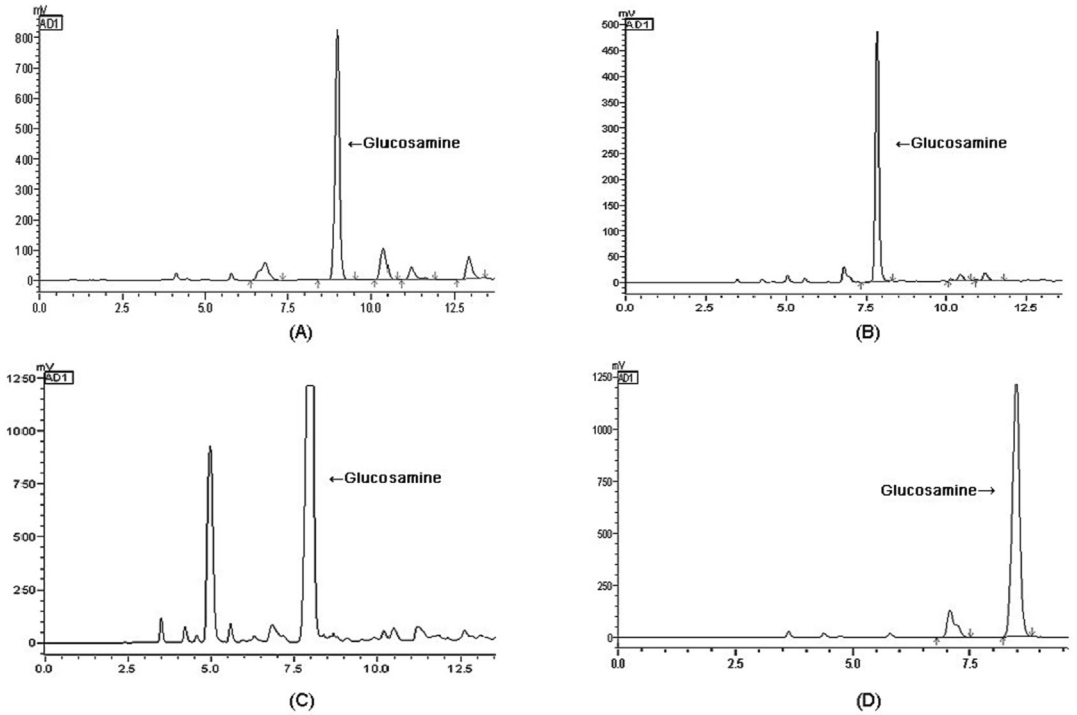

Fig. 4. Liquid phase diagram of glucosamine generation by *A. fumigatus* chitosanase. (A) 3% chitosan in aspartic acid solution for 20 d; (B) 3% chitosan in glutamate solution for 20 d; (C) 10% chitosan in glutamate solution for 40 d; peak at retention time 5.0 min stand for solvent for dissolving samples; (D) 10% chitosan in lactic acid solution for 20 d.

Table 8
Effects of chitooligosaccharide (molecular weight ≈3000 Da), degradation products in different acid solutions, on the survival rate (%) of MH7A cells.

Chitooligosaccharide	Glutamic acid	Lactic acid	Acetic acid	Hydrochloric acid	Butyric acid	Caproic acid	Acetylsalicylic acid	Aspartic acid
50 μg/mL	109.75 ± 15.37	112.97 ± 18.26	100.89 ± 4.86	108.53 ± 9.10	150.07 ± 12.24	102.83 ± 3.84	102.89 ± 8.99	97.69 ± 18.47
100 μg/mL	113.17 ± 17.00	104.72 ± 13.50	101.92 ± 17.08	113.76 ± 22.99	106.90 ± 6.91	102.83 ± 7.16	90.67 ± 9.49	82.63 ± 7.16
250 μg/mL	114.14 ± 22.06	104.82 ± 18.13	98.82 ± 7.97	112.78 ± 18.63	101.39 ± 2.21	89.01 ± 2.16	100.22 ± 3.64	86.58 ± 2.41
500 μg/mL	94.09 ± 12.06	96.85 ± 14.36	90.84 ± 19.87	112.20 ± 4.70	120.92 ± 15.80	90.53 ± 4.82	86.27 ± 6.72	88.77 ± 6.89
1000 μg/mL	94.82 ± 5.50	102.14 ± 5.25	89.75 ± 13.87	113.48 ± 12.10	106.57 ± 21.86	112.80 ± 3.46	48.11 ± 3.66	74.36 ± 4.88

Effects of chitooligosaccharide on the survival rate (%) of MH7A cells were calculated based on 0 μg/mL chitooligosaccharide culture conditions.

Table 9
Effects of glucosamines, degradation products in different acid solutions, on the survival rate (%) of MH7A cells.

	Glucosamine hydrochloride (commercially available)	Glucosamine sulfate (commercially available)	Glucosamine aspartate salt	Glucosamine lactate	Glucosamine glutamate
50 μg/mL	94.68 ± 11.90	85.77 ± 6.21	90.15 ± 7.48	95.42 ± 4.69	100.95 ± 10.26
100 μg/mL	96.21 ± 13.54	76.73 ± 1.23	96.06 ± 4.07	90.30 ± 9.46	93.78 ± 4.08
250 μg/mL	105.00 ± 18.15	77.72 ± 11.50	98.36 ± 18.23	89.16 ± 9.36	90.92 ± 15.79
500 μg/mL	87.80 ± 3.86	74.33 ± 9.24	94.50 ± 10.56	94.57 ± 19.00	89.44 ± 8.63
1000 μg/mL	89.66 ± 2.90	75.24 ± 8.59	86.71 ± 5.76	88.64 ± 4.53	80.91 ± 8.03

Effects of glucosamines on the survival rate (%) of MH7A cells were calculated based on 0 μg/mL glucosamine culture conditions.

cations of *A. fumigatus*, the chitosanase from *A. fumigatus* was further studied.

Chitosanase can be divided into inducible enzymes and structural enzymes by different manufacturer methods. So far, most of the reported chitosanase are inducible enzymes, only some are structural enzymes [5,24]. The present results showed that *A. fumigatus* did not synthesize chitosanase when there was no chitosan in the culture medium. Therefore, *A. fumigatus* chitosanase is an inducible enzyme. It can be divided into exonuclease and endonuclease by different ways of degradation. Exonucleases start from the non-reducing end of the polysaccharide to form monosaccharide. Endonucleases degrade the polysaccharide into oligosaccharides with different degrees of polymerization (DP) by random cleavage at the intermediate position [25]. According to the results of this experiment, monosaccharides were not produced in the solution in the initial stage of degradation (Table 7), which confirmed that *A. fumigatus* chitosanase is an endonuclease.

The active center and spatial structure of chitosanase are affected by environmental pH, which changes the dissociation state, or change the conformation and activity. When the appropriate pH exists in culture conditions, chitosanase can play the best catalytic function. The study from Hirano et al. [25] indicated that *A. fumigatus* ATCC13073 chitosanase had the best activity at pH 6.0. However, with the increase in pH value, chitosan changes from colloidal to flocculent or can precipitate, which may affect enzyme activity and its determination. Here, our results show that when the pH value was <4.5, chitosan could be fully dissolved, and *A. fumigatus* chitosanase can play its hydrolyzing role in degradation. When the pH value was ≥5.0, chitosan was not completely dissolved, and the undissolved chitosan could not be degraded by *A. fumigatus* chitosanase, but remained in powder form. Therefore, when using *A. fumigatus* chitosanase to degrade chitosan, it is necessary to control the pH value between 4.0 and 4.5 to start the degradation of chitosan after full dissolution.

The spatial structure of chitosanase will be affected by temperature, and only at the appropriate temperature can it combine with the substrate to achieve maximum catalytic function. The optimal temperature for microbial chitosanase is generally within 50–60°C [26]. The results of this experiment show that the best temperature of *A. fumigatus chitosanase* is 55–65°C. When the temperature reaches 70°C, the enzyme activity is greatly reduced.

Chitooligosaccharide are oligosaccharides with a degree of polymerization between 2 and 20 and molecular weight ≤3,200 Da [27]. *A. fumigatus* chitosanase obtained in this study could degrade chitosan (1,600,000 Da) at a concentration of 20% (w/v) into chitooligosaccharides (Table 7) and most of the degradation products had very low or no cytotoxicity (Table 8), which has great prospects for development in the industrial production of chitooligosaccharide.

A. fumigatus chitosanase can also be used in the production of glucosamine, in addition to the preparation of chitosaccharides. Glucosamine is a monosaccharide containing an amino group after the hydrolysis of chitosan and generally exists mostly in the form of glucosamine hydrochloride [28]. Glucosamine is a special compound that can be used both in medicine and marine healthcare products. It has anti-inflammatory effects and is mainly used for the treatment and prevention of osteoarthritis, including in the neck, knee, shoulder, hip, wrist, spine, and ankle joints. At present, most glucosamine products on the market are glucosamine hydrochloride and glucosamine sulfate.

The chloride ions in glucosamine hydrochloride have negative effects in people with hypertension [29], glucosamine sulfate has strong cytotoxicity (Table 9), and *A. fumigatus* chitosanase can degrade chitosan into different types of glucosamine salts in different acid solutions. It has a positive effect on eliminating the negative effect of chloride ions in glucosamine hydrochloride and the toxic effect of glucosamine sulfate, opening up the potential applications of glucosamine.

5. Conclusions

In this study, the conditions for the production of chitosanase by *A. fumigatus* fermentation were optimized, and the enzymatic characteristics of chitosanase by *A. fumigatus* were studied as presentation in graphical abstract. It was found that *A. fumigatus* chitosanase could be used to apply the preparation of chitosaccharides and different types of glucosamine salt.

Author contributions

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- Draft manuscript preparation: HY Yang, LS Wang, RG Xing, PC Li.
- Revision of the results and approval of the final version of the manuscript: all authors.

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Conflict of interest

The authors declare no conflicts of interest.

Supplementary material

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