Trichoderma Asperellum as a Novel Source to Prepare Chitosan Oligosaccharides by Enzymatic Hydrolysis and Its Antimicrobial Activity

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Abstract

Chitosan oligosaccharides (COS), as an important biological functional component, are mainly produced from marine resources, while their source face great challenges, such as seasonal variation, discontinuity and demineralization. In this study, the chitosan was extracted by alkaline method from *Trichoderma asperellum*, and then it was degraded with enzymatic hydrolysis to obtain the COS. The physicochemical characterization of chitosan from *Trichoderma asperellum* was compared with commercial chitosan, including chitosan content, deacetylation degree, chemical group, and molecular weight. Then, the enzymatic hydrolysis rates of *Trichoderma asperellum* chitosan and commercial chitosan were similar, 89.37 and 91.56%, respectively. Moreover, the composition of COS was tested by TLC, HPLC and MALDI-TOF, which results shown that *Trichoderma asperellum* COS was composed of chitotriose, chitopentaose and chitohexaose, and commercial COS was composed of chitotriose, chitotetraose, chitopentaose, chitohexaose and four acetylated chitohexaose. The antibacterial activity for *Trichoderma asperellum* COS was greatly stronger commercial COS. In conclusion, *Trichoderma asperellum* proved to be a novel potential source for COS production.

Introduction

COS are the degradation of chitosan composed of glucosamine linked by β -(1 $\rightarrow$ 4)-glycosidic bonds with lower than 20% degree of polymerization, and shorter chain lengths (Muanprasat et al., 2015), which possess a great potential in pharmacological and industrial applications (Benchamas et al., 2021; Yuan et al., 2019). Currently, the main commercial sources of chitosan are shrimp and crab shells (Oladzadabbasabadi et al., 2021; Sadiq et al., 2021). However, due to the discontinuity and seasonal variation of marine resources and the need for demineralization of seafood processing (Ghormade et al., 2017), finding a viable alternative raw material being one of the most interesting. The fungal cell wall is composed of a polysaccharide-based three-dimensional network, and contains mainly chitosan (Ban et al., 2018). Moreover, the fungi can be readily grown on cheap nutrients, and cell wall can be recovered by simple chemical procedures and constant quality and supply of the raw materials are possible (Pochanavanich et al., 2002). Therefore, fungi as a viable alternative material can be used to product chitosan and then degrade it to obtain COS.

Trichoderma species are recognized as biocontrol agents that control several plant diseases by producing secondary metabolites to inhibit the growth of pathogens, including bacterial (Nuankeaw et al., 2020). Some studies indicated that *Trichoderma* could produce some enzymes, such as β -1,3-glucanase, chitinase, peroxidase and polyphenol oxidase, which can be used for inhibiting the growth of bacterial in plants (Ishii et al., 2013; Vizcaino et al., 2005; Yue et al., 2021). As we all know, COS also have great potential in antibacterial activity (Yue et al., 2020). Huang et al. (2018) observed that chitosan oligosaccharides, isolated from shrimp shell waste, were more effective than chitosan in inhibiting growth of bacteria. Bi et al. (2021) also reported that a new geraniol conjugated chitosan oligosaccharide, which presented a significant inhibition effect on *E. coli* and *S. aureus*. However, antibacterial activity of COS produced from *Trichoderma* species is rarely reported.

The preparation of COS from chitosan employs physical methods, chemical methods, and enzymatic methods (Benchamas et al., 2021). Physical methods, including microwave irradiation, ultraviolet irradiation and ultrasonic treatment (Liang et al., 2018), which are highly purified and simple in structure. But these methods gain the low yield of COS. Unlike physical methods, chemical methods produce COS in a high yield at a much lower cost. One of the most commonly used methods is acid hydrolysis, but this method may produce large amounts of toxic by-products that are harmful to the environment (Lodhi et al., 2014). Enzymatic hydrolysis has become an attractive and widely used method due to its high yield and high specificity. However, this method is still not feasible in mass production because of its high cost (Hoell et al., 2010). Hence, more researches focusing on the optimization of COS preparations via enzymatic hydrolysis are required.

The aim of this study was to product COS by enzymatic hydrolysis from *Trichoderma asperellum*, and proper enzymatic hydrolysis process was optimized via single factor experiments and orthogonal design. Furthermore, the composition and antimicrobial activity were studied and compared with commercial COS. This study would provide an investigation basis for the preparation of COS from fungus and as a potential antibacterial agent in the food field.

Materials And Methods

Materials and reagents

Trichoderma asperellum ZZY was isolated from contaminated soil and has been preserved in the China General Microbiological Culture Collection Center (CGMCC). The deposit number of ZZY is CGMCC No.12071. The commercial chitosan derived from shrimp shells were purchased from Sigma-Aldrich Co. (USA), which was named as Chitosan-2. The T-series dextran standards (T-10, 40, 70, 500, 2000) were purchased from Solarbio Co. (Beijing, China). All other chemicals used in this paper were of analytical grade. *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Bacillus subtilis* (*B. subtilis*) and *Salmonella bacilli* (*S. subtilis*) were obtained from Tianjin University of Science and Technology.

Culture conditions

The strain was stored in -4 °C and need to be activated for 3 generations on PDA solid medium. And inoculated in 100 mL of seed medium (2% glucose, 20% potato) and fermented with shaking 160 rpm for 3 day at 25 °C. Then the seed medium was inoculated into 1000 mL of liquid-submerged fermentation medium and fermented for 7 days. After the mycelia under filtered and washed with distilled water were obtained, dried and grinded.

Basic ingredient analysis of *Trichoderma asperellum*

The total sugar content, protein content, fat content, cellulose content and ash content of *Trichoderma asperellum* were determined. Total sugar content was measured by the phenol-sulfuric acid colorimetric method (Dubois et al., 1956) using glucose as the standard. The protein content was determined by the

Bradford method (Bradford, 1976) using bovineserum albumin as the standard. The fat content was determined by continuous Soxhlet extraction according to Kouřimská et al. (2021). The cellulose content was measured by anthrone-H₂SO₄ colorimetry according to Cheng et al. (2019). The ash content was measured according to the procedure of Oellig et al. (2020).

Extraction of chitosan from *Trichoderma asperellum*

Based on the references reported (Kaya et al., 2015; Santos-Moriano et al., 2018), the alkali method was used for the preparation of chitosan in this article. The *Trichoderma asperellum* powder was extracted with NaOH (6%, w/v) at 90°C for 2 h. The insoluble parts, containing chitin, were filtered and washed to neutral for remove the protein and other small molecules. The deacetylation of chitin was reacted in NaOH (50%, w/v) at 110°C for 1 h. Chitin will be turned to chitosan, included in the insoluble parts. After filtered and washed to neutral again, these parts would be extracted three times by acetic acid solution (10%, w/v) at 90°C for 2 h and solid-liquid ratio was 1:30. The extract solutions were collected together and were adjusted to pH=9 with NaOH (1 mol/L) and set quietly for 6 h to precipitate chitosan. And the *Trichoderma asperellum* chitosan (Chitosan-1) was obtained after centrifugation, washed to neutral and dried.

Physicochemical characterization of chitosan

FT-IR analysis

The functional groups of Chitosan-1 and Chitosan-2 were measured with FT-IR. The sample of 1 mg and dried KBr of 150 mg were mixed and measured by FT-IR spectrometer (PerkinElmer Corp., USA) within the range of 4000–400 cm⁻¹.

Molecular weight distribution

The chitosan samples were detected using HPLC (Agilent-1200, Agilent Technologies Co. Ltd., USA) with TSK-GEL G4000PWxl column (7.8 mm × 300 mm, Tokyo, Japan). Other related parameters: column temperature of 30°C, volume of 20 uL, flow rate of 0.6 mL/min, mobile phase of distilled water. The glucans were used to make standard curve ($y = -0.35x + 8.9799$, $R^2 = 0.9835$).

Deacetylation degree and content of chitosan

The degree of deacetylation was determined by the two-abrupt-potentiometric titration method (Y. Zhang et al., 2011). In brief, accurately disperse 0.2 g chitosan into 30 mL HCl (0.1 mol/L) and added into 50 mL distilled water. Then it was titrated with 0.1 mol/L NaOH (alkaline titration), after the two abrupt pH changes, both the pH value and the consumption of NaOH (or HCl) were recorded. The deacetylation degree was calculated using the volume curve of the acid-base titrant.

The content of the chitosan samples was measured with ninhydrin assay (Leane et al., 2004). Chitosan standard solution and 1% ninhydrin were mixed in sodium acetate buffer (pH=5.5), then was heated to

boil and diluted with 60% ethanol. The curve was made according to the linear relationship between the concentration of chitosan standard and absorbance value at 570 nm ($y=7.264x-0.0097$, $R^2=0.9981$).

Preparation of COS

The COS were obtained by the enzymatic hydrolysis of chitosan. The chitosanase was added into different concentrations of chitosan solution at proper temperature to degrade the chitosan. After centrifugation at 4000 r/min for 10 min, COS were obtained by drying the supernatant. The degradation products of Chitosan-1 and Chitosan-2 were named as COS-1 and COS-2, respectively. In addition, the precipitate were washed to pH 7 and dried to calculate the rate of enzymatic hydrolysis. The effect of enzymatic temperature (A, 30 - 50°C), enzymatic time (B, 2 - 10 h), enzymatic dosage (C, 20 - 50 U/g) and substrate concentration (D, 2 - 10%, w/v) on enzymatic hydrolysis rate was also investigated. Then based on the results of the single factor experiment, all factors were combined according to orthogonal experiments. The $L_9 (3^4)$ orthogonal table was used for orthogonal design. The enzymatic hydrolysis rate was calculated using follow equation:

$$\text{Enzymatic hydrolysis rate (\%)} = \frac{m_1 - m_2}{m_1} \times 100$$

Where m_1 and m_2 stand for the dry weight of chitosan sample, and residual weight of chitosan sample after enzymatic hydrolysis, respectively.

Composition analysis

Thin layer chromatography (TLC) analysis

COS samples (2mg) were added to 0.5 mL distilled water. The expansion agent was n-butyl alcohol: water: acetic acid: ammonia = 10:7:7:1 (v:v:v:v), and the colorants were 2.5 g diphenylamine, 5 mL phenylamin, 25 mL 85% phosphoric acid, 1 mL drochloric acid and 250 mL acetone (Zhu et al., 2012).

HPLC analysis

The COS-1 and COS-2 were dissolved with mobile phase (acetonitrile: water=3:1, v/v) to a concentration of 5 mg/mL and filtered. Chromatographic column: APS-2 HYPERSIL. And the remaining operations follow step 2.5.2.

MALDI-TOF analysis of COS

The COS-1 and COS-2 (400 µg) were dissolved in 100 µL methanol-water (1:1, v/v) and mixed with 2, 5-dihydroxy-benzoic acid (DHB) in equal proportions. Then the mixed solution was spotted on the sample target, natural dried and put into the BRUKER to access mass spectrometry data. The samples were detected with the detection mode of linear positive ions.

Antibacterial activity

The antimicrobial activity was measured using a disc diffusion test (L. Zhang et al., 2021). The *E. coli*, *S. aureus*, *B. subtilis* and *S. subtilis* were used to test the effect on the COS in inhibiting the growth of bacteria. COS-1 and COS-2 were dissolved with distilled water to different concentrations (2, 4, 8, 16 and 32 mg/mL) and filtrated. Then the nutrient agar was added into each plate and solidified. And 100 uL bacterial suspension (10^7 cfu/mL) was spread on the surface of plates and a filter paper disk (diameter: 6 mm, thickness: 1 mm) containing COS-1 or COS-2 sample was placed in the plate. 2 mg/mL penicillin sodium was used as a positive control. Then these plates were incubated at 37 °C for 24 h. The antimicrobial activity of COS-1 and COS-2 was evaluated by determining the diameters of inhibitory zones with a vernier calipe. Determining the minimum inhibitory concentration (MIC) of COS were evaluated by double broth dilution method according to No et al. (2002) .

Results And Discussion

Main ingredient analysis of *Trichoderma asperellum*

The total sugar content, protein content, fat content, cellulose content and ash content of *Trichoderma asperellum* were determined, respectively. As shown in Table 1, the total sugar content of *Trichoderma asperellum* was very high, so it was suitable as a raw material for preparing COS. In addition, the *Trichoderma asperellum* contains a lot of cellulose and small account of protein, fat and ash.

Table 1
Basic composition of raw materials

Basic ingredient content (%)	Total sugar	Protein	Fat	cellulose	Ash
<i>Trichoderma asperellum</i>	25.30±1.27	17.82±0.53	12.44±0.76	24.7±0.22	12.35±0.42

Physicochemical characterization of chitosan

Chitosan content, molecular weight and degree of deacetylation

The extraction yield for *Trichoderma asperellum* chitosan was 9.53±0.02% and its chitosan content was 42.15 ± 0.11%. The molecular weight were shown in Fig. 1 (A), the main retention time of Chitosan-1 was 13.796 min and the molecular weight distribution concentrated at 18 kDa, which calculated according to the retention times of standards. Affes et al. (2019) reported that the molecular weight from 142.19 to 1244.70 kDa of chitosan in several shrimp shell materials was demonstrated. Generally, low molecular weight chitosan exhibits better solubility and has more prominent antibacterial, immuno-enhancing activity than and high molecular weight chitosan (X. Chen et al., 2017).

In addition, the chitosan of deacetylation extracted from Chitosan-1 (87.59±0.42%) was significantly higher than that of Chitosan-2 (82.40±0.57%). It can be concluded that the *Trichoderma asperellum* is more easily in deacetylating chitin. Generally, chitosan with a higher degree of deacetylation would

expose more active groups (Ma et al., 2022). Therefore, the chitosan in *Trichoderma asperellum* may have more potential in medicine than that in shrimp shells.

FTIR spectrum analysis

The data in Fig. 1 (B) presents the FT-IR spectrum of Chitosan-1 and Chitosan-2. FT-IR spectrum of *Trichoderma asperellum* chitosan was not different with commercial chitosan. Some prominent band 1661 cm^{-1} – 1559 cm^{-1} – 1315 cm^{-1} corresponded to the stretching vibration of C=O (amide band I), N-H (amide band II) and N-H (amide band II), and the signal near at 1118 cm^{-1} and 1072 cm^{-1} were due to C-O-C and C-O stretching, which were characteristic band of chitosan (Khajavian et al., 2022). Besides the absorption peak at near 3356 cm^{-1} was attributed to the stretching vibration of -OH. At the peaks around 2925 cm^{-1} assigned to $-\text{CH}_2$, and the peak intensity of Chitosan-1 was higher than Chitosan-2.

Preparation of COS by enzymatic hydrolysis

Single factor optimization results

The effects of enzymatic temperature, enzymatic time, enzymatic dosage and substrate concentration on Chitosan-1 and Chitosan-2 enzymatic hydrolysis rate were determined using single factor experiments, and the results were presented in Fig. 2. As shown in Fig. 2(A), Chitosan-1 and Chitosan-2 enzymatic hydrolysis rate were remarkably increased with the temperature increased from 30 to 40 °C, and reached to a maximum value of $86.34\% \pm 0.86\%$ and $88.92 \pm 1.34\%$, respectively. Subsequently, the enzymatic hydrolysis rate was descended with extraction temperature of 50 °C, indicating that excessive temperature could degrade the chitoanase and leading to the hydrolysis rate reduction.

Figure 2(B) exhibited the effects of time on enzymatic hydrolysis rate with other conditions set to fixed values. Results showed that Chitosan-1 and Chitosan-2 enzymatic hydrolysis rate were rapidly increased to $79.43 \pm 0.18\%$ and $74.92 \pm 0.45\%$ as the extraction time ascended to 6 h, and then no significantly change. The hydrolysis rate has not changed due to the enzymatic hydrolysis reaction reached saturation.

Figure 2 (C) showed the effects of enzymatic dosage on enzymatic hydrolysis rate yields. As shown, there was a rapid rise of Chitosan-1 and Chitosan-2 enzymatic hydrolysis rate (maximum value of $87.78 \pm 0.29\%$ and $88.97 \pm 1.02\%$, respectively.) with the enzyme dosage increased to 30 U/g, then gradually decreased. These results indicated that appropriate enzyme dosage would promote the hydrolysis of chitosan, while the excessive enzyme dosage could cause damage of chitosan.

The effects of substrate concentration on enzymatic hydrolysis rate were presented in Fig. 2 (D). As shown, Chitosan-1 and Chitosan-2 enzymatic hydrolysis rate were firstly increased to peak value of $89.62 \pm 0.29\%$ and $90.04 \pm 0.48\%$, respectively (substrate concentration of 8 g/mL), and then decreased with substrate concentration continuously raised to 10 g/mL.

Therefore, enzymatic temperature of 50 °C, enzymatic time of 6 h, enzymatic dosage of 30 U/g and substrate concentration of 8 g/mL were selected as the intermediate values for further optimization in orthogonal design.

Orthogonal design

During the optimization experiments, 9 groups of experiments were carried out and the results of orthogonal design were shown in Table 2. The results showed that the enzymatic hydrolysis rate of chitosan-1 was 80.13–89.37% by $L_9(3^4)$ orthogonal experiment design. Base on the maximum hydrolysis rate the optimum formulation was $A_1B_2C_2D_2$ (Run 2). And the optimal enzymatic hydrolysis conditions were enzymatic temperature of 37 °C, enzymatic time of 6 h, enzymatic dosage of 30 U/g, and substrate concentration of 8 g/L. Under this condition, the maximum hydrolysis rate was 89.37%. The variance analysis were performed as shown in Table 3, it indicated that the influence degree of the four factors on hydrolysis rate of Chitosan-1 was $A>B>D>C$. In addition, the optimal resulted in a maximum Chitosan-2 hydrolysis rate of 91.56%, which formulation was $A_3B_3C_2D_1$ (Run 9). And the optimal enzymatic hydrolysis conditions were enzymatic temperature of 43 °C, enzymatic time of 7 h, enzymatic dosage of 30 U/g, and substrate concentration of 6 g/L. The influence degree of the four factors on hydrolysis rate of Chitosan-2 was $C>A>B>D$ (Table 3).

Table 2
Orthogonal design and result analysis

Run	A (°C)	B (h)	C (U/g)	D (g/mL)	Enzymatic hydrolysis rate of Chitosan-1 (%)	Enzymatic hydrolysis rate of Chitosan-2 (%)
1	1(37)	1(5)	1(28)	1(6)	80.13	70.18
2	1	2(6)	2(30)	2(8)	89.37	80.75
3	1	3(7)	3(32)	3(10)	87.41	78.24
4	2(40)	1	2	3	88.52	81.96
5	2	2	3	1	89.17	87.02
6	2	3	1	2	88.28	78.01
7	3(43)	1	3	2	88.54	82.58
8	3	2	1	3	88.87	78.37
9	3	3	2	1	88.34	91.56
Chitosan-1	k1	83.57	84.78	84.87	85.18	
	k2	88.88	89.36	88.72	88.82	
	k3	88.51	86.82	87.37	86.96	
	Range	5.31	4.58	3.85	3.64	
	Rank	A>B>D>C				
Chitosan-2	k1	76.49	77.60	76.31	82.19	
	k2	81.29	81.13	83.72	80.61	
	k3	83.31	82.36	81.06	78.29	
	Range	6.82	4.76	7.41	3.9	
	Rank	C>A>B>D				

Table 3
Chitosan-1 and Chitosan-2 variance analysis of orthogonal experiment

Samples	Source	Sum of squares	degree of freedom	mean square	F-value	significance
Chitosan-1	A	105.223	2	52.612	11.236	**
	B	63.325	2	31.663	6.762	*
	C	45.886	2	22.943	4.900	*
	D	39.754	2	19.877	4.245	
	Error	42.141	9	4.682		
	Total	296.330	17			
Chitosan-2	A	147.107	2	73.554	18.068	**
	B	73.493	2	36.747	9.027	**
	C	169.118	2	84.559	20.772	**
	D	46.246	2	23.123	5.680	*
	Error	36.638	9	4.071		
	Total	472.603	17			
Note: * stand for $p < 0.05$, ** stand for $p < 0.01$						

In summary, under optimized conditions, the enzymatic hydrolysis rate of chitosan in *Trichoderma asperellum* was close to that in commercial products. Furthermore, the total sugar content of COS obtained after *Trichoderma asperellum* hydrolysis ($90.2 \pm 0.97\%$) was higher than that of commercial products ($88.5 \pm 1.14\%$). This indicated that *Trichoderma asperellum* can be used as a good source of raw materials for preparing COS.

Composition analysis of COS-1 and COS-2

TLC analysis

As shown in Fig. 3, the results showed that COS-1 and COS-2 contained a small amount of oligosaccharides. In addition, the oligosaccharides have a trailing effect, it could be inferred that COS-1 and COS-2 were mixture of oligosaccharides.

HPLC analysis

As shown in Fig. 4, the COS-1 was composed of three kinds of oligosaccharides that the retention time were 6.273, 6.930 and 11.466 min. And COS-2 was composed of five kinds of oligosaccharides that the retention times were 5.794, 6.821, 8.571, 10.245 and 11.228 min. These experimental results are consistent with the results in TLC analysis.

MALDI-TOF analysis

Enzymatic hydrolysis products were analysis by MALDI–TOF to determine the components of chitosan oligosaccharides. As shown in Fig. 5, the fragment ions (m/z 524.757, 846.860 and 1007.804) of COS-1 were clearly monitored and assigned to the quasi-molecular ion peaks $[M+Na]^+$ of chitotriose, chitopentaose, and chitohexaose (M. Chen et al., 2010). The molecular weight of glucosamine residue was m/z 161, which was also the relative molecular mass differences between main peaks (m/z 524.721, 685.799, 846.880, 1007.804). The COS-1 was constituted with three chitosan oligosaccharides, which was consistent with the analysis of HPLC and TLC about COS-1. Another the fragment ions (m/z 524.721, 685.799, 846.880, 1007.804, 1175.516) of COS-2 were assigned to the quasi-molecular ion peaks $[M+Na]^+$ of chitotriose, chitotetraose, chitopentaose, chitohexaose and four acetylated chitohexaose (Wang et al., 2012). The molecular weight of acetylyzed glucosamine residue was m/z 167, and this was the relative molecular mass differences between chitohexaose (m/z 1007.804) and four acetylated chitohexaose (m/z 1175.516). The COS-2 was constituted with five chitosan oligosaccharides, which was consistent with the analysis of HPLC and TLC about COS-2.

Antibacterial activity

The disc diffusion test method was used to evaluate antibacterial activity of COS-1 and COS-2 against *E. coli*, *S. aureus*, *B. subtilis* and *S. bacilli*. As shown in Fig. 6, antibacterial activities of COS-1 and COS-2 presented a dose-dependent manner. Inhibitory zone diameter of COS-1 and COS-2 with different concentrations was presented in Table 4. When the chitosan oligosaccharides concentration was 8 mg/mL, only inhibitory zones of COS-1 against *S. bacilli* was 10.45 mm. Then as the concentration of chitosan oligosaccharides increased, inhibitory zones of COS-1 and COS-2 against these four bacteria was increased. When the concentration increased to 32 mg/mL, inhibitory zones of COS-1 against *E. coli*, *S. aureus*, *B. subtilis* and *S. bacilli* were 10.48, 11.12, 8.46, 11.45 mm, and the corresponding inhibitory zones of COS-2 were 7.75, 8.52, 8.05 and 8.88 mm, respectively. These results indicated that COS-1 and COS-2 inhibited the growth of four bacteria in this study, inhibitory effects of COS-1 was higher than COS-2 at the same concentration, especially *S. bacilli*.

Table 4
Inhibitory zone diameter of COS-1 and COS-2 with different concentrations (mm)

Concentration of COS (mg/mL)	<i>E. coli</i>		<i>S. aureus</i>		<i>B. subtilis</i>		<i>S. bacilli</i>	
	COS-1	COS-2	COS-1	COS-2	COS-1	COS-2	COS-1	COS-2
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00	0.00	0.00	10.54	0.00
16	7.65	6.54	9.43	6.58	8.24	7.54	10.62	6.52
32	10.48	7.75	11.12	8.52	8.46	8.05	11.45	8.88
Penicillin sodium (2 mg/mL)	11.23	10.75	11.55	11.5	12.00	10.75	10.25	14.88

Furthermore, the lowest concentrations of COS-1 and COS-2 prevented bacteria growth were also investigated (Table 5). MICs of COS-1 against *E. coli*, *S. aureus*, *B. subtilis* and *S. bacilli* were 15.80, 15.57, 15.72 and 6.87 mg/mL, respectively. And the corresponding MICs of COS-2 were 15.93, 15.93, 15.80 and 15.90 mg/mL, respectively. In a word, *Trichoderma asperellum* COS showed stronger antibacterial activity than commercial COS.

Table 5
Minimal inhibitory concentration (MIC) of COS-1 and COS-2 (mg/mL)

Samples	<i>E. coli</i>	<i>S. aureus</i>	<i>B. subtilis</i>	<i>S. bacilli</i>
COS-1	15.80	15.57	15.72	6.87
COS-2	15.93	15.93	15.80	15.90

The antibacterial activity of chitosan oligosaccharides is greatly dependent on its physicochemical properties, especially degree of deacetylation and molecular weight (Benchamas et al., 2021). The molecular weight reduction and higher degree of deacetylation of chitosan or chitosan oligosaccharides would expose more active groups, and making which had better bioactivities (Meng et al., 2012). This might explain stronger antibacterial activity of COS-1 than COS-2. Moon et al. (2007) have reported that chitosan oligosaccharides with a higher degree of deacetylation tended to have a higher bactericidal action against *Staphylococcus aureus*. Huang et al. (2018) have reported that an increase in molecular weight led to decrease in the antibacterial activity of chitosan from squid (*Illex argentinus*).

Conclusions

A new COS was prepared by enzymatic hydrolysis from *Trichoderma asperellum*. The optimal enzymatic hydrolysis conditions for *Trichoderma asperellum* chitosan were enzymatic temperature of 37 °C, enzymatic time of 6 h, enzymatic dosage of 30 U/g, and substrate concentration of 8 g/L. Under this condition, the maximum hydrolysis rate was 89.37%. The composition of *Trichoderma trichoderma* COS was determined by MALDI-TOF, including chitotriose, chitotetraose, chitopentaose, chitohexaose and four acetylated chitohexaose. Besides, the antibacterial activity for *Trichoderma asperellum* COS against *E. coli*, *S. aureus*, *B. subtilis* and *S. subtilis* were greatly stronger compared to commercial COS. In conclusion, *Trichoderma asperellum* can be used as a good raw material for the preparation of COS and applied as a potential antibacterial agent in the food field.

Declarations

Author Contribution Statement

ZZY conceived and designed research. ZJY conducted experiments. LK and WLY contributed new reagents or analytical tools. PLC and WSQ analyzed data. WSQ wrote the manuscript. All authors read and approved the manuscript.

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Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

Figure 1

(A) The HPLC of *Trichoderma asperellum* chitosan. (B) The FT-IR spectra of Chitosan-1 and Chitosan-2.

Figure 2

Effects of enzymatic temperature (A), enzymatic time (B), enzymatic dosage (C) and substrate concentration (D) on Chitosan-1 and Chitosan-2 enzymatic hydrolysis rate.

Figure 3

Thin layer chromatograms of COS-1 and COS-2.

Figure 4

Liquid chromatography of COS-1 and COS-2.

Figure 5

MALDI-TOF/MS spectrum of COS-1 and COS-2 in positive ion mode.

Figure 6

Growth of (a) *Escherichia coli*, (b) *Staphylococcus aureus*, (c) *Bacillus subtilis*, (d) *Salmonella bacilli* in agar plate with COS-1 and COS-2.