Synthesis, characterization, and the antifungal activity of chitosan derivatives containing urea groups

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As an abundant and renewable polysaccharide, chitosan has been drawing broad attention due to its natural properties. For the further utilization of chitosan, chemical modification can be applied in improving its water solubility and the bioactivities. In this study, four chitosan derivatives were synthesized by the reaction of chloracetyl chitosan derivative with quaternary ammonium salt (CTCS) and urea groups bearing 4-amino-pyridine, including BTCX, 2CBCTCS, 3CBCTCS, and 4CBCTCS. The structure characteristics of synthesized products were established based on FT-IR spectroscopy, \textsuperscript{1}H NMR, and elemental analysis. Their antifungal activities against \textit{Fusarium oxysporum} f. sp. niveum, \textit{Phomopsis asparagus}, \textit{Fusarium oxysporum} f. sp. cucumebrium Owen, and \textit{Botrytis cinerea} were estimated by hyphal measurement in vitro. Generally, the inhibition ratio of most products was higher than 80% at 1.0 mg/mL. Their inhibitory activity decreased roughly in the order: 4CBCTCS > 3CBCTCS > 2CBCTCS > BTCX > CTCX > chitosan, resulted from the different degrees of substitution of the effectively active groups--urea groups. Meanwhile, the effects on beneficial soil microbes, including \textit{Bacillus cereus}, \textit{Bacillus subtilis}, and \textit{Sinorhizobium saheliLMG7837}, of these synthesized chitosan derivatives were evaluated by disk diffusion method. The results showed that these substances do not inhibit the growth of beneficial bacteria and some of them could be employed as green antifungal biomaterials.

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1. Introduction

Plant diseases resulting from pathogenic fungi can cause leaf spots, stem lesions, and fruit rot and then cause the death of crops on a massive scale, which limits crop yield and can lead to great financial losses to farmers. Currently, the common method for inhibiting these plant diseases and ensuring high crop production is a liberal use of chemical fungicides. However, the increased use of chemical fungicides often causes environmental pollution and has a fatal effect on beneficial soil microbes, which is harmful to environment and human health. Therefore, the development of new green alternative fungicides is a research hotspot.

Chitosan, composing of D-glucosamine and N-acetyl-D-glucosamine residues, is generally obtained from the deacetylation of chitin, which can be extracted from crustacean, fungi, and insects [1–3]. As an abundant and renewable natural resources, chitosan exhibits excellent bioactivities, including biocompatibility, low toxicity, hemostatic activity, and wound-healing property [4–7] and it has been attracting scientific and industrial interest in fields such as biotechnology, pharmaceuticals, wastewater, cosmetics, agriculture, food science, and textiles [8–12]. However, further industrial applications of chitosan are limited for its poor solubility in neutral and alkaline condition, resulted from the linear aggregation of chain molecules as well as the rigid crystalline structure caused by the intermolecular hydrogen bonding of chitosan [13,14]. Meanwhile, comparing with commercial drugs, the finite biological activity of chitosan is not ideal and can not meet the standards of commercial development. Therefore, it is necessary to modify the structure of pristine chitosan to improve its solubility and other properties. Actually, chemical modification [15,16] can satisfy it and massive efforts have been devoted to the synthesis of functional chitosan derivatives by introducing active group onto the chitosan polymer chain. Facts proved that chemical modification could affect the solubility and the biological activity...
of chitosan exactly [17–19]. And the use of chemically modified chitosan derivatives could be a promising handling as antifungal agents to partially substitute for the harmful synthetic fungicides.

The urea group, R1R2NCONR3R4, is extensively found in natural products and is an attractive functional moiety that exhibits a wide range of bioactivities [20]. Especially, the urea derivatives have attracted considerable attention in applications of anti-proliferative, anticancer (renal cancer, colon cancer, lungs cancer, prostate cancer and breast cancer), anticonvulsant, antifungal, and antibacterial agents [21–23]. They can also serve as urea herbicides, plant growth regulators, agroprotectives, tranquillizers and as anticonvulsants [24]. Among these, the components of urea groups–nitrogen and oxygen donor atoms can offer multitude of bonding potentials, which plays a key role in improving bioactivities [25]. Inspired by the important application of urea, several unsymmetrically substituted ureas were synthesized and grafted onto chitosan, which were expected to be helpful for the improvement of the antifungal activity of chitosan.

In order to study the effect of urea groups on the bioactivity of chitosan, the synthesis, antifungal property, and the influences on beneficial soil bacteria of a group of chitosan derivatives with urea groups as substituent including BCTCS, 2CBCTCS, 3CBCTCS, and 4CBCTCS were reported in this paper. Many reports have demonstrated that polysaccharides with chloride acetyl group could easily attack pyridine to give N-alkylypyridinium salts [26,27]. Accordingly, the urea groups containing pyridine can be grafted onto chitosan in this way. On the basis of this design idea, the C2–NH2 of chitosan was modified as quaternary ammonium salt firstly, which was a strong polyelectrolyte that was able to increase the water solubility efficiently [17]. Subsequently, the chloracetyl chitosan derivative with quaternary ammonium salt (CTCS) was synthesized by the reaction between the C-6 hydroxyl of the quaternary ammonium salt of chitosan and chloracetyl chloride. Then, four isocyanates, the products of the reaction of triphosgene (BTC) with aniline, o-chloroaniline, m-chloroaniline, and p-chloroaniline, were reacted with 4-amino-pyridine to obtain several unsymmetrically substituted urea groups. Eventually, these substituted urea groups, which are helpful to improve the antifungal property of chitosan, were introduced into chloracetyl chitosan derivative with quaternary ammonium salt (CTCS) and a series of chitosan derivatives including BCTCS, 2CBCTCS, 3CBCTCS, and 4CBCTCS were designed and synthesized. These target chitosan derivatives were expected to possess the advantages of good water solubility and high antifungal activity, which can be used as potential antifungal agents that can reduce pesticide use while ensuring the healthy development of plants and sustainable agriculture. The chemical structures of the derivatives were characterized by FT-IR, 1H NMR, and elemental analyses. Meanwhile, four common phytopathogenic fungi, Fusarium oxysporum f. sp. niveum (F. oxysporum f. sp. niveum), Phomopsis asparagi (P. asparagi), Fusarium oxysporum f. sp. cucumerinum Owen (F. oxysporum f. sp. cucumerinum Owen), and Botrytis cinerea (B. cinerea) were selected to evaluate the antifungal property of the chitosan derivatives by hypha measurement in vitro. Furthermore, we check the synthesized derivatives with other beneficial soil bacteria, including Bacillus cereus (B. cereus), Bacillus subtilis (B. subtilis), and Sinorhizobium saheli LMG7837 (S. saheli LMG7837). Moreover, the relationship between the structure and antifungal activity of chitosan derivatives was discussed.

2. Materials and methods

2.1. Materials

Chitosan with molecular weight of 200kDa was supplied by Qingdao Baicheng Biochemical Corp. (China). Its deacetylation degree (DD), determined by elemental analysis, is 83% (C: 43.42%, N: 7.98%, H: 6.30%, C/N: 5.44). Iodomethane, chloroacetyl chloride, aniline, o-chloroaniline, m-chloroaniline, p-chloroaniline, and 4-amino-pyridine were purchased from the Sigma-Aldrich Chemical Corp. The other reagents such as N-Methyl pyrrolidone (NMP), dimethyl sulfoxide (DMSO), etc., were supplied by Sinopharm Chemical Reagent Co., Ltd., Shanghai, China and used without more purification.

2.2. Analytical methods

2.2.1. Fourier transform infrared (FT-IR) spectroscopy

Fourier transform infrared (FT-IR) spectra, in the range of 4000–400 cm⁻¹ with resolution of 4.0 cm⁻¹, were used on a Jasco-4100 FT-IR spectrometer (Japan, provided by JASCO Co., Ltd., Shanghai, China) and the samples were grinded and mixed with KBr disks for testing.

2.2.2. Nuclear magnetic resonance (NMR) spectroscopy

1H Nuclear Magnetic Resonance spectrometer (1H NMR) spectra were measured using a Bruker AVIII-500 Spectrometer (500 MHz, Switzerland, provided by Bruker Tech. and Serv. Co., Ltd., Beijing, China) at 25 °C and using D2O as solvents.

2.2.3. Elemental analysis

The elemental analyses by combustion were used to evaluate the degree of substitution in chitosan derivatives. And the elemental analyses (carbon, hydrogen, and nitrogen) were performed on a Vario Micro Elemental Analyzer (Elementar, Germany). The degrees of substitution (DS) of chitosan derivatives were calculated on the basis of the percentages of carbon and nitrogen according to the following equations [28]:

\[
DS_1 = \frac{n_1 \times M_C - M_N \times W_{C/N}}{n_2 \times M_C}
\]

\[
DS_2 = \frac{M_N \times W_{C/N} + n_2 \times M_C \times DS_1 - n_1 \times M_C}{n_3 \times M_C}
\]

\[
DS_3 = \frac{M_N \times W_{C/N} + n_2 \times M_C \times DS_1 - n_1 \times M_C - n_3 \times M_C \times DS_2}{n_4 \times M_C}
\]

\[
DS_4 = \frac{n_1 \times M_C - M_N \times W_{C/N} + n_2 \times M_C \times DS_1 - n_1 \times M_C - n_3 \times M_C \times DS_2 - n_4 \times M_C \times DS_3}{n_5 \times M_C - n_3 \times M_C}
\]

where DS1, DS2, DS3, and DS4 represent the deacetylation degree of chitosan, the degrees of substitution of N,N,N-trimethyl in chitosan derivative, chloroacetyl in chitosan derivative-CTCS, and urea groups in chitosan derivatives-BCTCS, 2CBCTCS, 3CBCTCS, or 4CBCTCS; M_C and M_N are the molar mass of carbon and nitrogen, M_C = 12, M_N = 14; n_1, n_2, n_3, n_4, and n_5 are the number of carbon of chitin, acetylamido group, trimethyl chloroacetyl group, and urea group, n_1 = 8, n_2 = 2, n_3 = 3, n_4 = 2, n_5 = 12; n_1 and n_2 are the number of nitrogen of trimethyl and urea group, n_1 = 1, n_2 = 3; W_{C/N} represents the mass ratio between carbon and nitrogen in chitosan derivatives.

2.3. Synthesis of chitosan derivatives

2.3.1. Synthesis of the amines of 4-amino-pyridine

As shown in Scheme 1, the mixture of 5 mmol triphosgene (BTC) and 10 mmol aniline, o-chloroaniline, m-chloroaniline, or p-chloroaniline was stirred in 15 mL of acetic ether at room temperature (r.t.) for 1 h. The reaction mixture was then refluxed to clarify at 60 °C and four different isocyanates were obtained under the condition of reduced pressure at 50 °C. Subsequently, 10 mmol
4-aminopyridine dissolving in 15 mL of acetone was added dropwise into the flask that containing isocyanate. The mixture was stirred at room temperature (r.t.) for 2 h and refluxed at 60 °C for an additional 0.5 h. Upon reaction completion, several unsymmetrically substituted ureas were synthesized after distilling solvents. And the products were purified by crystallization from the solvent that the ratio of water and ethanol was 1:1.

2.3.2. Synthesis of chitosan derivative CTCS
Chitosan (2 mmol) was dispersed in 20 mL of N-methyl-2-pyrrolidone (NMP) and stirred at room temperature for 1 h. Then, NaI (0.9 g), 15% NaOH aqueous solution (3 mL), and CH3I (3 mL) were added, subsequently. The mixture was refluxed for an additional 2 h at 60 °C. After reflux reaction, the solution was poured into ethanol to afford some flavescent precipitate, which is chitosan derivative with quaternary ammonium salt (Elemental analysis: C: 31.43%, N: 4.52%, H: 5.49%, C/N: 6.95, DS: 0.59). The precipitate collected by filtration and chloroacetyl chloride (1.5 mL) were then dissolved in 30 mL of N,N-dimethylformamide (DMF) and stirred at room temperature for 12 h. Next, the solution was precipitated and filtered with ethanol and the precipitate was washed with excess ethanol for three times. At last, the resultant product CTCS was obtained by freeze-drying overnight in vacuum. Chitosan derivative CTCS: Yield: 73.35%; Elemental analysis: C: 35.23%, N: 4.42%, H: 6.14%, C/N: 7.97, DS: 0.59.

2.3.3. Synthesis of chitosan derivative BCTCS, 2CBCTCS, 3CBCTCS, and 4CBCTCS
1 mmol CTCS was stirred for 24 h at 60 °C in 20 mL of N,N-dimethylformamide (DMF) with four kinds of synthesized urea groups (3 mmol), respectively. The solutions were precipitated in acetone. Then the precipitates were filtered and washed with ethanol. Thus, the unreacted ureas and other outgrowth were all extracted. Finally, the chitosan derivatives were obtained after drying at 60 °C for 6 h. (BCTCS: Yield: 80.35%; Elemental analysis: C: 42.11%, N: 8.37%, H: 5.78%, C/N: 5.03, DS: 0.61. 2CBCTCS: Yield: 70.56%; Elemental analysis: C: 42.96%, N: 7.54%, H: 5.84%, C/N: 5.70, DS: 0.39. 3CBCTCS: Yield: 70.89%; Elemental analysis: C: 42.56%, N: 7.87%, H: 5.64%, C/N: 5.41, DS: 0.43. 4CBCTCS: Yield: 74.27%; Elemental analysis: C: 41.97%, N: 8.04%, H: 5.98%, C/N: 5.22, DS: 0.51.)

2.4. Antifungal assay
The antifungal ability was assessed by the model of Guo’s methods [29]. Firstly, all samples (chitosan and chitosan derivatives) were dispersed in distilled water at a concentration of 5 mg/mL as stock solutions. Then, each sample solution was added to Fungi Medium to give final concentrations of 0.1, 0.5 and 1.0 mg/mL. The final solutions were poured into sterilized Petri dishes (9 cm). After the mixture was cooled, the fungi mycelium of 5.0 mm diameter was transferred to the test plate and incubated at 27 °C for 2–3 days. When the mycelia of fungi reached the edges of the control plate (without the presence of samples), the growth inhibition was calculated by the formula:

\[
\text{Antifungal index (％)} = \left(1 - \frac{D_a}{D_b}\right) \times 100
\]

where \(D_a\) is the diameter of the growth zone in the test plates and \(D_b\) is the diameter of the growth zone in the control plate. All the experiments were performed in triplicate and the data were expressed as mean ± the standard deviation (SD, n = 3). Significant difference analysis was determined using Scheffe’s multiple range test. A level of \(P<0.05\) was considered statistically significant.

2.5. The activity on inhibiting beneficial bacteria
The disk diffusion method was chosen to evaluate the influences of chitosan derivatives on beneficial bacteria presenting in the soil, including Bacillus cereus (B. cereus), Bacillus subtilis (B. subtilis), and Sinorhizobium saheli LMG7837 (S. saheli LMG7837). First, 0.1 mL B. cereus, B. subtilis, and S. saheli LMG7837 (10^5 CFU/mL) were inoculated on the beef-protein medium respectively, and then spreadon the entire surface of the medium by sterile spatula. Next, four different concentrations (0.5 mg/mL, 1.0 mg/mL, 2.0 mg/mL, and 4.0 mg/mL) of test solutions were prepared using water as solvent. After that, the sample discs were placed onto the beef-protein medium plates and the dishes were then kept in an incubator at 37 °C for 24 h. The plates were subsequently examined for presence of inhibition zones indicative of the effects of chitosan derivatives on beneficial bacteria presenting in the soil. The positive control (PC) of B. cereus and B. subtilis is chloramphenicol and the positive control of S. saheli LMG7837 is gentamicin sulfate.
3. Results and discussion

3.1. Structure of chitosan and chitosan derivatives

The structures of chitosan and chitosan derivatives are characterized by FT-IR and $^1$H NMR. The FT-IR and $^1$H NMR spectra of chitosan and chitosan derivatives are shown in Figs. 1 and 2 respectively.

Fig. 1 illustrates the FT-IR spectra of regular bands of chitosan and chitosan derivatives. The spectrum of unmodified chitosan shows characteristic absorption bands at approximately 3421 cm$^{-1}$, 2919 cm$^{-1}$, 2881 cm$^{-1}$, 1596 cm$^{-1}$, and 1087 cm$^{-1}$. Among these, the broad peak at 3421 cm$^{-1}$ can be attributed to O–H and N–H stretching vibrations [30,31]. The peaks at 2919 cm$^{-1}$ and 2881 cm$^{-1}$ correspond to stretching vibrations of CH$_3$ and CH$_2$ [32], respectively. The band at 1596 cm$^{-1}$ indicates the presence of the amino group of chitosan [33,34]. Additionally, the peak at 1087 cm$^{-1}$ corresponds to the absorbance of β-(1,4) glycosidic in chitosan [30]. As to CTCS, it carries trimethyl group and chloracetetyl group in C$_2$-NH$_2$ and C$_6$-OH of chitosan, which can be recognized by FT-IR. Thus, the peaks of these functional groups are expected to be found in the spectrum of CTCS. Fortunately, new peaks appear at about 1750 cm$^{-1}$, 1469 cm$^{-1}$, and 790 cm$^{-1}$. The bands at 1750 cm$^{-1}$ and 790 cm$^{-1}$ are attributed to the vibrations of C=O and C–Cl, which confirms the formation of chloracetetyl chitosan derivative [35,36]. Meanwhile, the peak found at 1469 cm$^{-1}$ is the characteristic band of trimethyl group, which could prove the existence of quaternary ammonium salt [32,37]. As to the spectra of final products, which were obtained through the reaction of CTCS and urea groups, not only are the characteristic peaks of CTCS still exist, but also the bands of urea groups can be observed, logically. As shown in the figure, the absorbance of C=O at 1749 cm$^{-1}$ and C–Cl at 790 cm$^{-1}$ of chloracetetyl group are in existence. However, these peaks get weaker, which illustrates that the band of C–Cl was destroyed and new groups were grafted onto CTCS. Furthermore, the peak at 1654 cm$^{-1}$ is strengthened, resulted from the appearance of −OH−CO−NH−, and new peaks appear at about 1600 cm$^{-1}$, 1534 cm$^{-1}$, and 809 cm$^{-1}$, which could be assigned to the typical absorption of benzene and pyridine [26,27]. Hence, it is reliable to confirm that the new groups grafted onto CTCS are the urea groups shown in Scheme 1. And the above analysis preliminarily proves that the products of CTCS, BCTCS, 2CBCTCS, 3CBCTCS, and 4CBCTCS were synthesized successfully.

The formation of the synthesized products was further demonstrated by $^1$H NMR spectra (Fig. 2). In terms of the $^1$H NMR spectrum of chitosan, the single peak at 3.0 ppm, multiple peaks at 3.6–3.9 ppm, and the peak at 4.6 ppm are assigned to the hydrogen protons on C–2, C–3–C–6, and C–1, respectively [4]. Meanwhile, the peak at 2.0 ppm reveals the presence of hydrogen protons of the N-acetyl residue [4,30]. Besides all the characteristic proton signals of chitosan, the $^1$H NMR spectrum of CTCS shows the prominent peaks of −N(CH$_3$)$_3$ at 3.1 ppm [38]. Additionally, because of the existence of chloride acetylated group, new resonance peak, which is related to the methylene protons of −COCH$_2$Cl group, appears at 4.4 ppm in the spectrum of CTCS [36]. Regarding to the $^1$H NMR spectra of the final products (BCTCS, 2CBCTCS, 3CBCTCS, and 4CBCTCS), the characteristic proton signals derived from CTCS could be observed distinctly. Furthermore, the characteristic resonance of −COCH$_2$Cl at 4.4 ppm has weakened while new signals appear at 6.8–8.7 ppm, insuring that the urea groups were grafted onto CTCS successfully. The spectra of urea groups presenting peaks at around 6.8–8.3 ppm associate with the protons on pyridine ring and benzene ring [21,27]. And the peaks at 8.39 ppm and 8.56 ppm should be related to the protons on −NH in the molecules of urea groups [39,40]. Based on the above analyses, it is enough to confirm that the chitosan derivatives were obtained.

3.2. Solubility of the chitosan derivatives

Chitosan has poor water solubility. After quaternization, the water solubility of chitosan was improved obviously. However, the introduction of chloride acetylated group reduced the water solubility of CAST. When the urea groups was grafted onto CAST, N-alkylpyridinium salts were formed, which could promote the water solubility of chitosan derivatives. Therefore, compounds BCTCS, 2CBCTCS, 3CBCTCS, and 4CBCTCS showed favourable water solubility, and their aqueous solutions could be prepared at 0.1–1.0 mg/mL at room temperature.

3.3. Antifungal activity

The potential antifungal activity of chitosan and chitosan derivatives was tested by four destructive phytopathogenic fungi, F. oxysporum f. sp. niveum, P. asparagus, F. oxysporum f. sp. cucumerium Owen, and B. cinerea. Meanwhile, the yields and the degrees of substitution of chitosan derivatives with urea groups were shown in Table 1. Their antifungal indices and rule were discussed as follows.

The antifungal activity of chitosan and chitosan derivatives against F. oxysporum f. sp. niveum was tested and the results are shown in Fig. 3. Obviously, several conclusions according to the graph can be gained as follows: firstly, for all tested samples,
the inhibitory rates increase with the rise of concentration. For instance, the inhibitory rates of BCTCS are 6.33%, 48.00%, and 75.98% when the corresponding concentrations are 0.1 mg/mL, 0.5 mg/mL, and 1.0 mg/mL. As it can be seen from Fig. 3, this trend is evident and is suitable for all samples. Secondly, because of the introduction of active groups–urea groups, the final products have better ability of inhibiting F. oxysporum f. sp. niveum compared with chitosan and CTCS. While chitosan and CTCS with the inhibitory rates 19.54% and 20.19%, the inhibitory rates of BCTCS, 2CBCTCS, 3CBCTCS, and 4CBCTCS are 75.98%, 77.64%, 79.80%, and 88.16% at 1.0 mg/mL, respectively. The increase is more than fifty percent, verifying the advantage of the introduction of urea groups. Thirdly, the inhibitory indices of all samples have a certain regularity, which is 4CBCTCS > 3CBCTCS > 2CBCTCS > BCTCS > CTCS > chitosan. Results above demonstrated that the difference of active groups grafted onto the synthesized chitosan derivatives contributed much to the antifungal action and consequently increased the antifungal activity of them.

Fig. 4 shows the antifungal activity of chitosan and chitosan derivatives against P. asparagus. All samples exhibit antifungal property against P. asparagus and the inhibitory indices enhance with increasing concentration. The samples that possess relatively weak inhibitory activity are chitosan and CTCS. Their inhibitory indices are only 15% or so at 1.0 mg/mL, which much lower than the other four products at the same concentration. Besides, the four final products have a remarkable antifungal activity against P. asparagus and the ability to inhibit P. asparagus is more obvious than the fungi of F. oxysporum f. sp. niveum and F. oxysporum f. sp. cucumebrium Owen. For example, when the concentration is 0.5 mg/mL, the inhibitory indice of 3CBCTCS against P. asparagus can reach to 68.9% while its inhibitory indices against F. oxysporum f. sp. niveum and F. oxysporum f. sp. cucumbrium Owen are only 39.3% and 53.7%. Both of the two kinds of measured mycelium belong to the same species–F. oxysporum, which should be the possible reason for the slighter antifungal activity compared with P. asparagus. Furthermore, the data illustrates a distinct rule of inhibitory property, which is 4CBCTCS > 3CBCTCS > 2CBCTCS > BCTCS > CTCS > chitosan. And the inhibitory indices of 4CBCTCS, 3CBCTCS, 2CBCTCS, BCTCS, CTCS, and
chitosan at 1.0 mg/mL against *P. asparagus* are 93%, 87.89%, 82.98%, 82.65%, 15.86%, and 11.8%, respectively.

Fig. 5 shows the antifungal activity of chitosan and all the derivatives against *F. oxysporum* f. sp. cucumebrium Owen. The inhibitory indices of all the samples mounted up with increasing concentration. All chitosan derivatives bearing urea groups show better antifungal activity than chitosan and CTCS, and the inhibitory indices of BCTCS, 2BCTCS, 3BCTCS, and 4BCTCS are 71.84%, 71.89%, 76.24%, and 88.57% at 1.0 mg/mL, respectively. The rule of the compounds against *F. oxysporum* f. sp. cucumebrium Owen was similar to that of them against *F. oxysporum* f. sp. niveum and *P. asparagus*. The antifungal activity is affected by both the DS and electron-withdrawing ability of different substituted groups.

As exhibited in Fig. 6, the most rules discussed above still can be appropriate for the antifungal activity of all samples against *B. cinerea*. For instance, the inhibitory indices of all samples are concentration-dependent and the inhibitory property of final products is better than chitosan and CTCS. These results above mentioned indicate that the urea groups have positive effect on the antifungal activity when they are introduced into chitosan and the relationship between the structure and antifungal activity of chitosan derivatives was discussed below.

Generally, the data shows that urea groups could directly influence the antifungal activity of chitosan derivatives. Meanwhile, this conclusion is further confirmed by the fungistatic rule, which is 4BCTCS > 3BCTCS > 2BCTCS > BCTCS > CTCS > chitosan. The reasons for this phenomenon can be analyzed from the following three aspects: firstly, owning to the improvement of the antifungal activity of urea groups, the inhibitory indices of the final products (BCTCS, 2BCTCS, 3BCTCS, and 4BCTCS) are higher than CTCS and chitosan. Secondly, as for 4BCTCS, 3BCTCS, 2BCTCS, and BCTCS, BCTCS presents the relatively weak antifungal activity although its DS is the highest. So it is inferred from this results that the electron-withdrawing atom—Cl plays a key role in inhibiting the growth of fungi. Furthermore, it can be confirmed by the earlier reports of Guo and Tan [13,27], which verified that the stronger electron-withdrawing capacity of halogen atoms have positive effect on the antifungal activity. Exactly, strong electron-withdrawing substitution can disrupt cell walls and cytoplasmic membranes to lead to the death of fungi [41,42]. Therefore, the chitosan derivatives bearing stronger electropositive groups showed stronger antifungal activity. Finally, it seems that different chlorine atom positions could have different influences on the antifungal activity according to the fungistatic rule of 4BCTCS, 3BCTCS, and 2BCTCS. Taking 2BCTCS as an example, because the chlorine atom in the adjacent position occupies a larger space position, the steric hindrance of CI hinders urea groups attached on CTCS and affects the DS of urea groups in chitosan derivatives. However, the steric hindrance of 4BCTCS is rather lower and the DS is higher accordingly. As is known, the more functional groups chitosan derivative carries, the stronger antifungal activity it presents. Hence, the above analysis can explain the fungistatic rule of chitosan derivatives, which is 4BCTCS > 3BCTCS > 2BCTCS. In summary, it is reasonable to presume that the urea groups should be a significant factor that influenced the antifungal activity of samples and the structure–activity relationship would be investigated in the future.

### 3.4. The influences of chitosan derivatives on beneficial bacteria

The influences of chitosan derivatives on beneficial bacteria was depicted in Fig. 7. In this study, three beneficial bacteria presenting in the soil, including *B. cereus*, *B. subtilis*, and *S. saheli* LMG7837, were selected to evaluate the activity of inhibiting beneficial bacterial growth of final samples. In each plate, four synthesized products (BCTCS, 2BCTCS, 3BCTCS, and 4BCTCS) and one positive control (PC) were placed and the position was marked in the figure. Meanwhile, we designed four concentration gradients (0.5 mg/mL, 1.0 mg/mL, 2.0 mg/mL, and 4.0 mg/mL) corresponding to A, B, C, and D. As shown in Fig. 7, in addition to positive controls, other chitosan derivatives at any concentration had no effect on the growth of beneficial bacteria. In other words, the use of these products will not destroy the ecological balance. Therefore, the synthesized chitosan derivatives would be an environmentally friendly fungicides that can protect biological diversity as well as ensure the healthy development of sustainable agriculture.

### 4. Conclusions

Nowadays, plant pathogenic fungi jeopardize essential agricultural products and limit crop harvest worldwide, especially in developing countries. Although many commercially available fungicides possess good antifungal activity, the environment issues that arise from these antifungal agents cannot be ignored. Thus, the development of new chemical fungistat, which not only can restrain the growth of the fungi effectively, but also be biodegradable and environmentally friendly, is urgent. Chitosan is a good carrier of active groups in consideration of its desirable for the capability of flexible chemical modification and the urea groups are a key factor in contributing to increasing the antifungal activity of chitosan. In this study, four water-soluble chitosan derivatives containing urea groups were designed and synthesized. Moreover, little work has been done to demonstrate the restrained effect of such products against *F. oxysporum* f. sp. niveum, *P. asparagus*, *F. oxysporum* f. sp. cucumebrium Owen, and *B. cinerea* by hypha measurement in
vitro. The data illustrates that the four final products exhibit higher inhibitory indices than chitosan. Meanwhile, the important role of urea is confirmed by the regularity and the relationship between its structure and the antifungal activity of chitosan derivatives had discussed shorty. Furthermore, B. cereus, B. subtilis, and S. saheli LMG7837 were selected to assess the property of inhibiting beneficial bacterial growth of chitosan derivatives by disk diffusion method. The result indicates that these products have no lethal effect on soil beneficial microbes. In brief, this study suggests that the use of such compounds would be an ideal solution as antifungal agents to partially substitute for the harmful fungicides and further comprehensive study to ascertain this hypothesis on relations of structure and antifungal activity will be carried out.

Conflict of interest statement

The authors have declared no conflicts of interest.

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