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Antibacterial Activity of Chitosan Against the Asian Pear Pathogenic Bacterium *Bacillus pumilus*

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In this study, the antibacterial activity of chitosan against the Asian pear pathogenic bacterium *Bacillus pumilus* was investigated. Results showed that the bacterial cell numbers increased with the increase of incubation time and the strength of Luria-Bertani broth in the absence of chitosan. However, chitosan solution at 0.01, 0.05 and 0.10 mg/mL markedly inhibited the growth of *B. pumilus* strains B0703 and B0706, while the antibacterial activity of chitosan solution increased with the increase of chitosan concentration, incubation time and the strength of Luria-Bertani broth regardless of the tested bacterial strains. Overall, the results indicated that chitosan may be a promising bactericide in fruit production.

Key Words: Antibacterial activity, *Bacillus pumilus*, Chitosan, Concentration, Growth broth.

INTRODUCTION

Chitosan is a modified, natural nontoxic carbohydrate polymer derived by deacetylation of chitin, the second most abundant natural polymer in the world[1-3]. During the past several decades, chitosan and its derivatives have been receiving increased attention for its commercial applications in the fields of nutrition, food, environmental protection, biotechnology, chemical engineering as well as pharmaceutical and biomedical research due to its unique polycationic nature[4-9]. In addition, chitosan's toxicity profile and allergenicity is relatively low and its biocompatibility, biodegradability and bioactivity make it a promising substance for the new applications as a biocide in agriculture field[10,11].

The rapidly growing interest in the new applications of chitosan and its derivatives for agriculture has focused on its potential effects in its reported antifungal properties[10,11]. In addition, chitosan displays a broad spectrum of antibacterial activity against both gram-positive and gram-negative bacteria[1-11]. Indeed, chitosan has several advantages over other types of bactericide because it possesses higher antibacterial activity, broader spectrum of activity, higher killing rate and lower toxicity toward mammalian cells[10,11]. However, little information is available about antibacterial activity of chitosan against bacterial pathogen, in particular *B. pumilus*, from Asian pear, which ranks third after apple and orange in China[12].

The objective of this research was to evaluate the antibacterial activity of chitosan against the Asian pear pathogenic bacterium *B. pumilus*.

EXPERIMENTAL

Chitosan (degree of *N*-deacetylation not less than 85 %, practical grade, from crab shells) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solution of chitosan (5 mg/mL) was prepared in 1 % acetic acid with pH being adjusted to 6.0 with NaOH[1]. After stirring (160 rpm) for 24 h at room temperature, the stock solution was autoclaved at 121 ºC for 20 min. Sterile deionized water of pH 6.0 was used as a control.

Cultivation of the microorganism: Two strains of *B. pumilus* (B0703 and B0706) were isolated from naturally infected twigs of field-grown pears in previous study[12]. All bacterial strains involved in this study were deposited in the culture collection of the Institute of Biotechnology, Zhejiang University, China. The bacterial strains were cultured for 48 h on nutrient agar medium[1] at 28 ºC. After incubation, each bacterial suspension was prepared in sterilized water and the initial concentration of bacteria was adjusted to approximately 10^9 colony forming units (CFU)/mL.

Counting surviving cells: Bacterial suspensions were ten -fold serially diluted and 10 µL samples were inoculated on nutrient agar medium in hexaplicate for each dilution and were

incubated for 48 h at 28 ºC. After incubation, the surviving cells on the agar were counted based on the colony forming units and then the mean value of the cells at the lowest dilution was calculated. Each experiment was carried out in duplicate and was replicated twice.

Effect of chitosan concentration on the antibacterial activity: Several chitosan solutions (5 mL) were prepared by adding chitosan stock to deionized water to give a final chitosan concentration of 0.01, 0.05 and 0.10 mg/mL. Bacterial solution was added to 5 mL of chitosan solution to give a final bacterial concentration of 10^8 CFU/mL and then the mixture was incubated at 28 ºC on a rotary shaker (Hualida Company, Taicang, China) at 160 rpm. In the control treatment chitosan stock was replaced with sterile deionized water of pH 6.0 in order to obtain the same pH. After 6 h, samples were collected from each cell suspension and bacterial counting was followed as indicated above.

Effect of incubation time on the antibacterial activity of chitosan: Chitosan solutions of 5 mL in volume were prepared by adding 100 µL chitosan stock to 4.90 mL sterile deionized water to give a final chitosan concentration of 0.10 mg/mL. *B. pumilus* strains were inoculated into chitosan solution as indicated above. In the control treatment, chitosan stock was replaced with sterile deionized water of pH 6.0 in order to obtain the same pH. Antibacterial activity of chitosan solution on the growth of *B. pumilus* was determined after 0, 2, 4 and 6 h of incubation, respectively.

Effect of the growth broth on the antibacterial activity of chitosan: In order to clarify the nutritional factor in the antibacterial activity of chitosan solution, chitosan solutions of 5 mL in volume were prepared by adding 100 µL chitosan stock to 4.9 mL Luria-Bertani broth with different strength nutrients to give a final chitosan concentration of 0.10 mg/mL. The Luria-Bertani growth broths were prepared at 10 %, 50 % and full strength concentrations. The mixture was incubated at 28 ºC on a rotary shaker at 160 rpm. In the control treatment chitosan stock was replaced with sterile deionized water of pH 6.0 in order to obtain the same pH. After 6 h, samples were collected from each cell suspension and bacterial counting was followed as indicated above.

Statistical analysis: The software STATGRAPHICS Plus, version 4.0 (Copyright Manugistics Inc., Rockville, Md., USA) was used to perform the statistical analysis. Levels of significance ($P < 0.05$) of main treatments and their interactions were calculated by analysis of variance after testing for normality and variance homogeneity.

RESULTS AND DISCUSSION

Chitosan solution at three different concentrations showed effective antibacterial activity against *B. pumilus* strains B0703 and B0706 compared to the control after 6 h of incubation (Table-1). In addition, the antibacterial activity of chitosan solution increased with the increase of chitosan concentration regardless of the tested bacterial strains. The surviving cell numbers of strain B0703 in chitosan solution of 0.01 mg/mL decreased 0.58 $\log_{10}$ CFU/mL, while the surviving cell numbers in chitosan solution of 0.10 mg/mL decreased 2.41 $\log_{10}$ CFU/mL compared to the control (Table-1). Similarly, the surviving cell numbers of strain B0706 in chitosan solution of 0.01 mg/mL decreased 0.66 $\log_{10}$ CFU/mL, while the surviving cell numbers in chitosan solution of 0.10 mg/mL decreased 3.23 $\log_{10}$ CFU/mL compared to the control (Table-1). These results are consistent with the result of Li *et al.*[1,6,7], who found that the antibacterial activity of chitosan was influenced by its concentration in the solution.

TABLE-1
EFFECT OF CHITOSAN CONCENTRATION ON THE ANTIBACTERIAL ACTIVITY OF *B. pumilus* STRAINS B0703 AND B0706

Chitosan concentration (mg/mL)	Cell numbers (log10 CFU/mL)	
	B0703	B0706
0.00	7.89 ± 0.04d	7.99 ± 0.21d
0.01	7.31 ± 0.06c	7.33 ± 0.08c
0.05	6.35 ± 0.05b	5.36 ± 0.15b
0.10	5.48 ± 0.07a	4.76 ± 0.51a

Columns with the same letters are not significantly different ($P < 0.05$). Error bars represent the standard error of the mean. Data are from a representative experiment repeated twice with similar results

This result also indicated that in the absence of chitosan, the surviving cell numbers in sterile deionized water increased with the increase of incubation time regardless of the tested bacterial strains. The surviving cell numbers of strains B0703 and B0706 were increased by 0.63 and 0.31 log CFU/mL, respectively, after 2 h of incubation, while the surviving cell numbers of strains B0703 and B0706 were increased by 1.88 and 2.21 log CFU/mL, respectively, after 6 h of incubation compared to the initial value (Table-2). In the presence of chitosan, the surviving cell numbers were significantly decreased compared to the initial value regardless of the tested bacterial strains (Table-2). After 2 h of incubation, the surviving cell numbers of strains B0703 and B0706 decreased 2.29 and 1.56 log CFU/mL, respectively, while the surviving cell numbers of strain B0703 and B0706 decreased 3.22 and 2.86 log CFU/mL compared to the initial value after 6 h of incubation (Table-2). This result is consistent with the result of Li *et al.*[1], who found that a certain incubation time is required for the chitosan solution to inhibit the bacterial growth.

TABLE-2
EFFECT OF INCUBATION TIME ON THE ANTIBACTERIAL ACTIVITY OF CHITOSAN SOLUTION AT 0.10 mg/mL AGAINST *B. pumilus* STRAINS B0703 AND B0706

Incubation time	Cell numbers (log10 CFU/mL)			
	Control		Chitosan solution	
	B0703	B0706	B0703	B0706
0 h	7.19 ± 0.02a	7.15 ± 0.02a	7.58 ± 0.01d	7.71 ± 0.03d
2 h	7.82 ± 0.05b	7.46 ± 0.06b	5.29 ± 0.01c	6.15 ± 0.07c
4 h	8.85 ± 0.05c	8.54 ± 0.11c	4.51 ± 0.06b	5.15 ± 0.07b
6 h	9.07 ± 0.03d	9.36 ± 0.04d	4.36 ± 0.06a	4.85 ± 0.07a

Columns with the same letters are not significantly different ($P < 0.05$). Error bars represent the standard error of the mean. Data are from a representative experiment repeated twice with similar results.

The effects of Luria-Bertani broth and its dilutions on the antibacterial activity of chitosan against strains B0703 and B0706 are shown in Table-3. In the absence of chitosan, the bacterial population increased with the increase in concen-

tration of Luria-Bertani broth regardless of the tested bacterial strains. However, in the presence of chitosan, bacterial growth was significantly inhibited compared to the initial value regardless of the tested bacterial strains. The surviving cell numbers of strain B0703 decreased 0.31 $\log_{10}$ CFU/mL in 10 % strength LB, decreased 0.55 $\log_{10}$ CFU/mL in 50 % strength LB and decreased 1.51 $\log_{10}$ CFU/mL in full strength LB, while the surviving cell numbers of strain B0706 decreased 0.45 $\log_{10}$ CFU/mL in 10 % strength LB, decreased 1.33 $\log_{10}$ CFU/mL in 50 % strength LB and decreased 1.87 $\log_{10}$ CFU/mL in full strength LB, compared to the initial value (Table-3). The antibacterial activity of chitosan increased with the increase in concentration of LB may be attributed to the increase in the ionic strength of the solution, which contained NaCl. This result is consistent with the result of Chung *et al*.[13], who revealed that a higher ionic strength may enhance the solubility of chitosan and thus increases its antibacterial activity. However, in contrast, Devlieghere *et al*.[9] found that NaCl had a negative effect on the antimicrobial activity of chitosan. The difference maybe due to a number of factors, such as characteristics of the chitosan, the tested microorganism and NaCl concentration used in these studies.

TABLE-3
EFFECT OF STRENGTH OF LURIA-BERTANI BROTH ON THE ANTIBACTERIAL ACTIVITY OF CHITOSAN SOLUTION AT 0.10 mg/mL AGAINST *B. pumilus* STRAINS B0703 AND B0706

LB broth (%)	Cell numbers (log10 CFU/mL)			
	Control		Chitosan solution	
	B0703	B0706	B0703	B0706
0	7.82 ± 0.05a	7.75 ± 0.05a	6.06 ± 0.05d	6.60 ± 0.05d
10	8.04 ± 0.05b	8.47 ± 0.04b	5.75 ± 0.04c	6.15 ± 0.07c
50	8.23 ± 0.04c	8.91 ± 0.05c	5.51 ± 0.06b	5.27 ± 0.05b
100	8.99 ± 0.05d	9.35 ± 0.05d	4.55 ± 0.06a	4.73 ± 0.06a

Columns with the same letters are not significantly different ($P < 0.05$). Error bars represent the standard error of the mean. Data are from a representative experiment repeated twice with similar results

In summary, our data clearly demonstrated that chitosan was able to inhibit the growth of the Asian pear pathogenic bacterium *B. pumilus*. To the best of our knowledge, this is the first report about antibacterial activities of chitosan against bacterial pathogen *B. pumilus*. In addition, the antibacterial activity of chitosan solution againstf *B. pumilus* increased with the increase of chitosan concentration, incubation time and the strength of growth broth regardless of the tested strains. Overall, this result showed chitosan has potential as a bactericide against bacterial pathogen in fruit production.

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