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Factors ınfluencing antibacterial activity of chitosan against *Aeromonas hydrophila* and *Staphylococcus aureus*

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ABSTRACT
In this study, the effect of the incubation time, incubation temperature, pH, metal and sodium ions on the antibacterial activity of chitosan against food borne spoilage bacteria focused. *Aeromonas hydrophila* and *Staphylococcus aureus,* were used as two food borne bacteria. Acetic acid was used dissolving of chitosan. Results showed that chitosan solution at 5 mg/mL significantly inhibited the growth of *A. hydrophila* and *S. aureus* in presence of time, pH and metal ions ($p < 0.01$). However, temperature did not significantly affect the antibacterial activity (4°C, 25°C and 37°C) ($p > 0.01$). Chitosan activity increased with increasing incubation time. The influence of below pH 6 on the antibacterial activity of chitosan was significantly influenced ($p<0.01$). The addition of Ba^{+2} reduced chitosan activity against *A. hydrophila* while the addition of Ca^{+2} have increased chitosan activity against *S. aureus*. Sodium ions at a concentration of 25 mM reduced chitosan activity against *S. aureus.* These results show that chitosan can be used food industry to prevent the development of spoilage bacteria.

***Key Words*:** Chitosan, antibacterial activity, incubation time, incubation temperature, pH, metal and sodium ions.

INTRODUCTION

Among food-borne risks microbial hazards are the top when the number of microorganisms exceeds a definite level. The food becomes exhaustless and becomes risky for human health. Especially, the foods may be infected by toxic and pathogenic bacteria such as *Escherichia coli, Staphylococcus aureus, Aeromonas hydrophila* and *Pseudomonas aeruginosa*. Currently, artificial food additives are used in order to prevent contamination of foods. However, they may cause impairment of human health. Hence, amount of use of these additives have been reduced and some of them have been prohibited. Moreover, additives which are obtained from natural resources have been started to be used instead of these artificial additives.

Although Chitin is a polymer of N-acetyl-D-glucosamine, chitosan is N-acetyl-D-glucosamine and D-glucosamine copolymer (Rinaudo, 2006; Shahidi, 2007). Chitosan is considered a compatible, non-antigenic, nontoxic, and biofunctional food additive (Novack *et al.*, 2003). The pharmaceutical and food industry have received preservative and an important new material (Illum, 1998).

The use of chitosan as an antimicrobial material for food has been widely reported, for example, in bread (Lee *et al.*, 2002), seafood (Tsai *et al.*, 2002; López-Caballero *et al.*, 2005), fruit and vegetables (Chien *et al.*, 2007; Badawy and Rabea, 2009), meat (Sagoo *et al.*, 2002; Rao *et al.*, 2005), sausage (Lin and Chao, 2001; Soultos *et al.*, 2008) and dairy products (Suman *et al.*, 2010).

Chitosan has antimicrobial activity against food-borne bacteria, yeast and filamentous fungi (Sagoo *et al.*, 2002). Therefore, chitosan has been shown to inhibit food spoilage microorganisms such as *Escherichia coli*, *Staphylococcus aureus* and *Candida* sp. (Rhoades and Roller, 2000; Chung *et al.*, 2005; Yang *et al.*, 2005).

Furthermore, the chitosan showed antibacterial activity on dental caries connected *Klebsiella pneumoniae* and *Bacillus subtilis* (Fujiwara *et al.*, 2004). Bacterial cell binding and DNA binding mechanisms arises antibacterial activity of chitosan (Chung and Chen, 2008). Another reported mechanism involves the interaction of diffused hydrolysis products with microbial DNA, which leads to an inhibition of the mRNA and protein synthesis (Hadwiger and Loschke, 1986).

The antimicrobial activity of chitosan has been shown to be affected by pH, with higher activity observed at lower pH values, and to increase with rising temperature (No *et al.*, 2002; Taha and Swailam, 2002). However, the presence of sodium ions has been reported to reduce chitosan's activity; a similar but more dramatic effect was obtained through the addition of other metal ions, probably due to formation of complexes between chitosan and metal ions (Taha and Swailam, 2002; Tsai and Su, 1999).

In this study is impacted the antibacterial activity of chitosan against *Aeromonas hydrophila* and *Staphylococcus aureus* as in vitro including pH, incubation time, incubation temperature and metal ions.

MATERIALS AND METHODS

Preparation of chitosan stock

Chitosan (degree of N-deacetylation 80-85%, from crab shells) was obtained from Sigma-Aldrich (USA). The tests were conducted set with chitosan and a control set without chitosan. Chitosan (0.5 g) was dissolved in 100 mL 1% acetic acid with pH adjusted to 5.2 with 1N NaOH (Li *et al.*, 2008). After stirring at 160 rpm for 24 h at room temperature, this resulting stock solution was autoclaved

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at 121°C for 20 min. Sterile nutrient broth of pH 7.2 was used as a control.

Bacterial strains

To obtain inocula for the examination, two strains of food spoilage bacteria, *A. hydrophila* (ATCC 7966) and *S. aureus* (ATCC 29213) were obtained from the the American Type Culture Collection (ATCC). Cultures were maintained by the transfer on nutrient agar medium. After cultivation and washing, cells were suspended and diluted in sterile saline (0.85% NaCl) to an approximate concentration of $6.4x10^7$ CFU/mL for *A. hydrophila* and $5.6x10^7$ CFU/mL for *S. aureus.* These suspensions were used as the inocula for the time, temperature, pH, chitosan-metal and chitosan-sodium ions experiments.

Influence of environmental conditions on antibacterial activity of chitosan

The experiment studies were carried out with different environmental conditions for time (4h, 8h and 24h), temperature (4°C, 25°C and 37°C), pH (5-8), metal and sodium ions. Incubation time for antibacterial activity, 0.5 ml of bacterial cell suspension was transferred into an aseptic test tube containing 4.5 ml of chitosan solution (final concentration: 5mg/mL) at pH 7 and incubated at 37°C for variable times (4h, 8h and 24h). The incubated solution was then plated on TSB agar and incubated at 37°C for 24 h to obtain viable cell counts. After incubation, the colonies were counted to analyze the bactericidal activity. All experiments were performed in triplicate.

Incubation temperature for antibacterial activity, 0.5 ml bacterial cells were transferred an aseptic test tube containing 4.5 ml of chitosan solution (final concentration: 5mg/mL) at pH 7 and incubated for 24 h at variable temperatures (4°C, 25°C and 37°C). Following incubation with the chitosan, the solution was plated to TSB agar in order to obtain viable cell counts. All experiments were performed in triplicate.

Effect of pH on the antibacterial activity of chitosan, nutrient broth was adjusted to 5.0, 6.0, 7.0 or 8.0 with a pH-meter (Metler Toledo 320, Halstead, UK) by adding dilute HCl or NaOH aqueous solution. Bacterial cell suspensions were incubated with chitosan at the different pH conditions (5.0–8.0), plated on TSB agar, and then incubated at 37°C for 24 h. After incubation, the colonies were counted.

Effect of Metal Ions and NaCl, chitosan (5 mg/mL) was added to bacteria cell suspension (50 mL, 10^7 cfu/mL) in nutrient broth and phosphate buffer (5 mM, pH 7.0) containing 0 or 25 mM $MgCl_2$, $BaCl_2$, $CaCl_2$ and NaCl. The reaction mixtures were incubated for 24 h at variable temperatures (4°C, 25°C and 37°C) and incubated at 37°C for variable times (4h, 8h and 24h). Surviving cells were counted by spreading on nutrient agar plates .

Statistical analysis

Repeated Measures Analysis of variance was used to evaluate the data. Analyses were conducted with chitosan, temperature and pH applications as between subject factors and time as the within subject factor. As a result of the Repeated Measures Analysis of variance, DUNCAN multiple comparison test was used for grouping of between subject factor intervals that were significantly different. Bonferroni multiple comparison test was used for grouping within subject factors. SPSS 15.0 software was used in the statistical analyses. All analyses were run in triplicate for each replication (SAS, 2001).

RESULTS AND DISCUSSION

Effect of incubation time on the antibacterial activity of chitosan

The antibacterial activity of chitosan against *A. hydrophila* and S. *aureus* with respect to incubation time are shown in **table 1.**

After 4 h of incubation in chitosan, viable cell counts of *A. hydrophila* decreased by 0.10 log CFU/mL while viable cell counts of *S. aureus* decreased by 0.20 log CFU/mL compared to the control. After 24 h of incubation in 5 mg/mL chitosan, *A. hydrophila* viable cell counts decreased by 2.30 log CFU/mL compared to the control. Similarly, viable *S. aureus* decreased by 1.90 log

CFU/mL was compared to the control and the large increase in the reduction of viability between the 4 and 24 h incubation times showed that a certain incubation time is required for the chitosan to exert its antibacterial effects.

The results of this study indicated that the activity of chitosan was affected by the incubation time, which is consistent with the result of Li *et al.* (2008), who found that a certain incubation time is required for chitosan to begin to inhibit bacterial growth.

Effect of temperature on the antibacterial activity of chitosan

The antibacterial activities of chitosan against strains of *A. hydrophila* and *S. aureus* at different temperatures are shown in **table 2.**

Temperature also has an effect on the antimicrobial activity of chitosan. Higher temperature (37°C) has been shown to enhance its antimicrobial activity compared to refrigeration temperatures.

Effect of pH on the antibacterial activity of chitosan

The antibacterial activities of chitosan against *A. hydrophila* and *S. aureus* at different incubation pH levels are shown in **table 3.** For both strains, antibacterial activity was more pronounced at lower pH levels ($p < 0.01$). Viable cells of *A. hydrophila* were reduced from their initial populations by 1.80–0.57 log cfu/mL. Similarly, *S. aureus* were reduced from their initial populations by 1.70– 1.00 log cfu/mL.

The antimicrobial activity of chitosan becomes notable when pH decreases to 5-6. This is likely due to the fact that the amino groups of chitosan become positively ionized below pH 6. Unmodified chitosan is not antimicrobially active at pH 7, likely due to lack dissolution and lack of positive charges on the amino groups (Chung *et al.*, 2005). In this study, *A. hydrophila* and *S. aureus* were found to be less susceptible to chitosan at pH 8.0, with a population reduction of 0.57- 1.00 log cfu/mL, respectively. These results are slightly different from those reported by Chung *et al.*, (2003), who found that the inhibition percentage of chitosan solution decreased with increasing of pH but dropped dramatically when the pH was higher than 6.0.

Effect of metal and sodium ions on the antibacterial activity of chitosan

The effects of metal ions on the antibacterial activity of chitosan towards *A. hydrophila* and *S. aureus* are shown in **tables 4** and **5.**

The effect of chitosan on *A. hydrophila* and *S. aureus* with regard to metal and sodium ions decreased along with a rise in incubation temperature and incubation time.

Table 1: Inhibitory effect of chitosans on growth of *A. hydrophila and S. aureus* after different time (pH: 7, temperature: 37°C).

Bacteria	Bacteria cell (cfu/mL)	Chitosan conc. (mg/ml)	Time (h)		
			4h	8h	24h
A. hydrophila	$6.4x10^7$	Control	6.70±0.11	6.40±0.19	6.70±0.16
		5	6.30±0.04	5.50±0.28	4.10±0.27
			6.50±0.20[b]	5.95±0.45[a]	5.40±1.30[a]
S. aureus	$5.6x10^7$	Control	5.40±0.07	5.80±0.15	5.40±0.07
		5	5.40±0.05	3.50±0.21	3.70±0.28
			5.40±0.00[b]	4.65±1.15[a]	4.55±0.85[a]

[a,b] means followed by different superscripts differ ($p<0.05$).

Table 2: Inhibitory effect of chitosans on growth of *A. hydrophila* and *S. aureus* after different temperature (pH: 7, time: 24 h).

Bacteria	Bacteria cell (cfu/mL)	Chitosan conc. (mg/ml)	Temperature (°C)		
			4°C	25°C	37°C
A. hydrophila	$6.4x10^7$	Control	6.60±0.09	6.60±0.09	6.50±0.25
		5	5.30±0.37	5.40±0.32	5.20±0.35
			5.95±0.65[a]	6.00±0.60[a]	5.85±0.65[a]
S. aureus	$5.6x10^7$	Control	5.50±0.10	5.50±0.12	5.50±0.12
		5	4.20±0.34	4.28±0.31	4.20±0.32
			4.85±0.65[a]	4.89±0.61[a]	4.85±0.65[a]

[a] means followed by different superscripts differ ($p<0.05$).

Table 3: Inhibitory effect of chitosans on growth of *A. hydrophila*and *S. aureus* after different pH (temperature: 37°C, time: 24 h).

Bacteria	Bacteria cell (cfu/mL)	Chitosan conc. (mg/ml)	pH			
			5	6	7	8
A. hydrophila	$6.4x10^7$	Control	6.50±0.00	6.60±0.09	6.50±0.14	6.60±0.28
		5	4.60±0.44	5.00±0.50	5.73±0.30	5.83±0.15
			5.55±0.95[a]	5.80±0.80[a,b]	6.12±0.38[a,b]	6.22±0.38[b]
S. aureus	$5.6x10^7$	Control	5.40±0.10	5.50±0.07	5.50±0.12	5.70±0.19
		5	3.90±0.43	4.00±0.44	4.30±0.33	4.60±0.23
			4.65±0.75[a]	4.75±0.75[a]	4.90±0.60[a,b]	5.15±0.55[b]

[a,b] means followed by different superscripts differ ($p<0.05$).

Table 4: Effects of chitosan-metal and chitosan-sodium ions on the growth of *A. hydrophila*and *S.aureus* at different times (pH: 7, temperature: 37°C).

Bacteria	Bacteria cell (cfu/mL)	Metal ions Conc. (25mM)	Time (h)		
			4h	8h	24h
A. hydrophila	$6.4x10^7$	Control	7.22±0.03	7.25±0.10	7.12±0.19
		$MgCl_2$	6.60±0.05	4.93±0.31	4.07±0.47
		$BaCl_2$	5.97±0.39	4.83±0.67	3.97±0.23
		$CaCl_2$	5.83±0.66	3.47±0.21	2.27±0.08
		NaCl	5.20±0.55	4.57±0.31	3.70±0.26
			6.16±0.34[c]	5.01±0.16[b]	4.23±0.79[a]
S. aureus	$5.6x10^7$	Control	6.27±0.11	6.46±0.09	6.34±0.08
		$MgCl_2$	5.33±0.12	5.03±0.31	4.07±0.59
		$BaCl_2$	5.13±0.08	4.40±0.55	3.27±0.96
		$CaCl_2$	4.97±0.39	3.50±0.91	3.23±0.08
		NaCl	3.30±0.49	3.13±0.54	2.83±0.46
			5.00±0.48[c]	4.50±0.59[b]	3.95±0.63[a]

Initial citosan concentration was used as 5 mg/ml. [a,b,c] means followed by different superscripts differ ($p<0.05$).

Table 5: Inhibitory effect of chitosans on growth of *A. hydrophila* and *S.aureus* after different temperature (pH: 7, time; 24 h).

Bacteria	Bacteria cell (cfu/mL)	Metal ions Conc.(25mM)	Temperature (°C)		
			4°C	25°C	37°C
A. hydrophila	$6.4x10^7$	Control	7.15±0.12	7.31±0.04	7.13±0.17
		$MgCl_2$	5.17±0.54	5.50±0.47	5.70±1.00
		$BaCl_2$	3.87±1.21	4.63±0.76	4.00±1.27
		$CaCl_2$	3.73±0.39	3.83±0.80	5.50±0.70
		NaCl	3.90±0.35	5.03±0.66	4.53±0.38
			4.76±0.65[a]	5.26±0.58[b]	5.37±0.53[b]
S. aureus	$5.6x10^7$	Control	6.32±0.06	6.25±0.09	6.50±0.11
		$MgCl_2$	4.6±0.75	5.37±0.08	4.20±0.52
		$BaCl_2$	3.93±0.58	4.60±0.60	5.33±0.08
		$CaCl_2$	3.23±0.98	3.17±0.53	4.50±0.47
		NaCl	3.00±0.15	2.27±0.12	4.00±0.15
			4.22±0.59[a]	4.33±0.72[a]	4.91±0.45[b]

Initial chitosan concentration was used as 5 mg/ml. [a,b] means followed by different superscripts differ ($p<0.05$).

Divalent cations at concentrations of 25 mM reduced the bactericidal effect of chitosan on *A. hydrophila* and *S. aureus*, the most effective being Ba^{+2}. Na^{+} ion at 25 mM also reduced the activity of chitosan against *A. hydrophila* and *S. aureus*.

The addition of metal ions decreased the antimicrobial activity of 5 mg/mL chitosan was enhanced by sodium ions. The surviving cell numbers *A. hydrophila* decreased by 2.91 log CFU/mL after incubation with chitosan in the presence of sodium ions for 24h. Under these conditions, the surviving cell numbers *S. aureus* decreased by 3.61 log CFU/mL.

CONCLUSION

Consequently, chitosan is a potential bactericidal against food spoilage bacteria, under various environmental conditions.

We have found that 0.50% chitosan is sufficient to inhibit growth and to inactivate the production of *A. hydrophila* and *S. aureus*. Furthermore, the bactericidal activities of chitosan against *A. hydrophila* and *S. aureus* are affected by factors including temperature, time, pH and certain salts. In this context, chitosan can be employed as a preventing or remedial agent to avoid bacterial food spoilage. In particular, the results of this study showed that chitosan has a strong antibacterial activity against *A. hydrophila* and *S. aureus*, which will be helpful in the control of fruit and vegetable contamination. This study demonstrates the potential of chitosan an alternative natural product, to use for synthetic food additive replacement. While this study clearly shows that chitosan can be used as a natural preservative for the food industry, there exist many opportunities for further study and refinement.

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