

Effect of Temperature and pH on the Antibacterial Activity of Crab Chitosan

G. Sugumar, A. Selvan and U. Ramesh

Fisheries College and Research Institute,
Thoothukudi - 628 008, India

The antibacterial activity of chitosan against *Staphylococcus aureus* and *Escherichia coli* at 5, 20 and 30°C and at pH 5.0, 5.5, 6.0 and 6.5 was investigated by maintaining the cultures in phosphate buffered saline (PBS) and tryptic soy broth (TSB) with 0.05% chitosan. Temperature had a direct influence on the bactericidal activity of chitosan against both bacteria. Inhibition against *S. aureus* and *E. coli* was seen at all temperatures tested with greatest activity at 30°C. The rate of reduction of viable cells of *E. coli* was slow and complete inhibition could not be obtained at 5°C. The antibacterial activity of chitosan was inversely proportional to pH, and there was stronger inhibition at pH <6.0. *S. aureus* lost their viability more rapidly than *E. coli*. Unlike *S. aureus*, *E. coli* was not completely inhibited at pH 6.5. Results indicated that the antibacterial activity of chitosan was strong at 30°C and at pH <6.0.

Key words: Chitosan, antibacterial activity, *Staphylococcus aureus*, *Escherichia coli*, pH, temperature

Chitosan has antimicrobial and antifungal properties and consequently studied as a potential natural antimicrobial agent in cosmetics (Chang *et al.* 1989). Chitosan was found bactericidal against gram-positive and gram-negative organisms, indicating non-specific action and has been reported against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Listeria monocytogenes* and *Saccharomyces cerevisiae* in buffers and laboratory media (Papineau *et al.*, 1991, Wang, 1992; Sugumar *et al.*, 2003; Rakesh *et al.*, 2006). A reduction of one to two log cycles of total bacteria, pseudomonads, staphylococci, coliforms, gram-negative bacteria and micrococci was reported in the presence of 1% chitosan (Darmadji and Izumimoto, 1994). Liu *et al.* (2001) observed that *E. coli*, *S. aureus*, *Bacillus subtilis*, *Sarcina* and *Actinomyces* could be inhibited by chitosan. Chitosan was shown to have good antibacterial

activity against *Salmonella enterica*, which was comparable to the standard antibiotics (Yadav & Bhise, 2004). The studies conducted by Sugumar *et al.* (2004) revealed that chitosan was very effective against *S. aureus*. The aim of the present study is to investigate the antibacterial activity of chitosan against *S. aureus* and *E. coli* under various temperatures and pH levels.

Materials and methods

Chitosan was prepared from crab shell waste according to Madhavan and Nair (1974) with slight modifications. The dried crab shells were deproteinised with 3% NaOH at the ratio of 1:10 (w/v) at 120-130°C for 3 h. The deproteinised shells were washed until NaOH was removed and dried overnight in an oven at 60°C. The shells were then demineralised with 5% HCl (1:10 w/v) overnight by shaking at ambient

temperature. The shells were washed with tap water until neutral and then dried at 60°C to obtain chitin. Deacetylation of chitin was carried out by hydrolyzing with 47% NaOH (w/v) at the solid to solvent ratio of 1:10 (w/v) at 120-130°C for 1-2 h and the end product was tested for deacetylation by introducing the samples into 1% acetic acid for complete dissolution. The chitin thus prepared had a viscosity of 290 centipoise (cP) and deacetylation of 80%.

Laboratory stock cultures of *S. aureus* (ATCC 12598) and *E. coli* isolated from marine fish were used as experimental organisms. Cells were grown in tryptic soy broth (TSB) (HiMedia, Mumbai) at 37°C for 18-24 h. They were centrifuged at 5000 rpm for 15 min and washed twice with phosphate buffered saline (PBS) (0.01M, pH 7.0). Washed cells were suspended in PBS and used for susceptibility studies.

One g of chitosan was dissolved in 100ml of 1% acetic acid and this chitosan solution was added into the experimental flasks so as to give a final concentration of 0.05%. Selected concentration of chitosan (0.05%) was added separately into 250ml Erlenmeyer flasks containing 100ml of PBS (0.01 M) and TSB medium. The pH of the experimental solution was adjusted to 6.0 aseptically and the flasks were separately inoculated with *S. aureus* and *E. coli* to a cell density of approximately 10^7 ml⁻¹ and incubated at 5, 20 and 30°C. The cell density was estimated at intervals of 0, 1, 3, 6, 12, 24 and 48 h by spread plate technique and the plates were incubated at 37°C for 48h and the reduction in counts recorded. Similarly the cells were maintained in PBS and TSB with varying pH levels of 5.0, 5.5, 6.0 and 6.5 and the cell density was estimated at regular intervals as mentioned above. All experiments were carried out in duplicates and mean values recorded.

Result and Discussion

Effect of temperature on the antibacterial activity of chitosan

Increasing temperature had an influence on the bactericidal activity of chitosan against both *S. aureus* and *E. coli* (Figs 1 & 2). Among the temperatures of 5, 20 and 30°C studied, the antibacterial activity of chitosan was greatest at 30°C. Complete inhibition of *S. aureus* cells was observed in PBS with 0.05% chitosan at all the three experimental temperatures viz. 5, 20 and 30°C within 6 h of incubation (Fig. 1 A). However, in experimental conditions in growth medium, complete inhibition of *S. aureus* cells was achieved within 6 h only at 30°C. At incubation temperatures of 20 and 5°C, the same effect was achieved in 12 and 48 h respectively (Fig. 1 B). The nutrients and the salts in TSB medium seem to have conferred a little protection upon the cells of *S. aureus*.

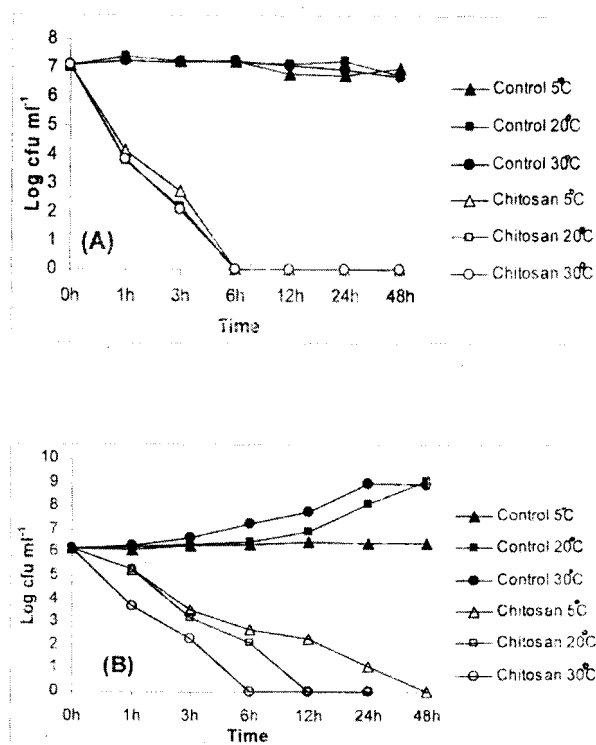


Fig. 1. Effect of temperature on the inhibitory activity of chitosan against *Staphylococcus aureus* in (A) Phosphate Buffered Saline and (B) Tryptic Soy Broth.

On exposure of *E. coli* cells to chitosan in PBS, the cell density got reduced from the initial level of 10^7 to less than 10 in 24 h at 30°C and the same effect was achieved after 48 h at 20°C (Fig. 2A). At 5°C , complete inhibition could not be observed; however, there was considerable reduction in the cell density. When maintained in growth medium with 0.05% chitosan, the inhibition was relatively low (Fig. 2B). Complete inhibition of *E. coli* was observed at 30°C in TSB, but it could not be achieved at 5 and 20°C . However, Rakesh Kumar *et al.* (2006) reported that complete inhibition of *E. coli* was not achieved at 0.05 and 0.1% of chitosan in PBS, although there was reduction in cell density by 3 to 4 log units by 24h. At 20°C , cell density decreased to 60 cfu ml^{-1} while at 5°C it reduced to 10^3 cfu ml^{-1} by 48 h implying that at low temperature the inhibitory effect of chitosan was relatively low. These differential responses to temperature may be due to changes in the reaction

rate between chitosan and cells and changes in the number of available binding sites on the cell surface. Tsai and Su (1999) reported that the stress of low temperature might change cell surface structures so as to decrease the number of surface binding sites or electro negativity of chitosan. Therefore, higher temperature might have increased the binding of chitosan on bacterial cell surface with increased chemical reaction rate, thus causing leakage of the intracellular materials that eventually causes death of bacterial cells. In all the experiments, the inhibitory effect of chitosan was more against *S. aureus* than *E. coli*. A stronger bactericidal effect of chitosan for gram-positive bacteria than gram-negative bacteria has already been reported (Jeon *et al.*, 2001; No *et al.*, 2002; Sugumar *et al.*, 2003).

Effect of pH on the antibacterial activity of chitosan

As shown in Fig. 3A cell counts of *S. aureus* in PBS without chitosan at varying pH levels remained almost unchanged for up to 24 h and decreased slightly at pH levels of 5.0 and 5.5 on further incubation. In the case of *S. aureus* in growth medium, control samples showed an increase and the increase was relatively low at pH 5.0 compared to higher pH levels, thus revealing a mild inhibitory effect of low pH on the bacteria even in conditions of starvation. In samples treated with chitosan, complete inhibition could be seen by 6 h at pH 5.0 and 6.0 and within 24 h at pH 6.5. When *S. aureus* cells were maintained in growth medium (Fig. 3B), the inhibitory effect of chitosan was almost the same in all pH levels except at pH 6.5 which could bring about complete inhibition only after 24 h. In control samples, cell counts increased by over 2 log units in less than 24 h in growth medium especially when the pH was above 5.5, whereas the cells in PBS reduced up to 2 log units in 48 h when the pH was 5.5 or below.

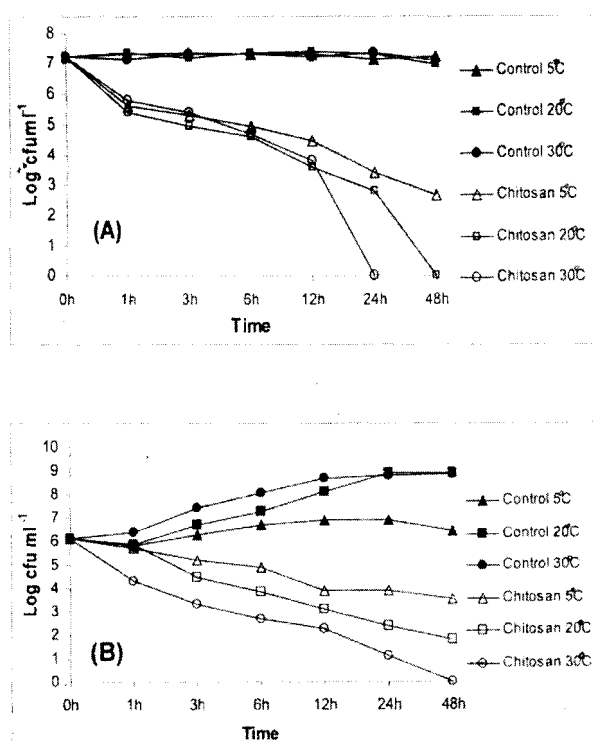


Fig. 2. Effect of temperature on the inhibitory activity of chitosan against *E. coli* in (A) Phosphate Buffered Saline and (B) Tryptic Soy Broth.

The inhibitory effect of chitosan against *E. coli* was quite different from that of *S. aureus*. No marked effect of pH could be observed against *E. coli* in control samples indicating that *E. coli* tolerate low pH conditions better than *S. aureus* (Fig. 4A). The inhibitory effect of pH on *E. coli* was gradual in PBS and complete elimination was recorded in 24h at pH 5, 5.5 and 6. At pH 6.5, complete inhibition was not recorded even after 48h. In growth medium also, the pattern of inhibition was similar to that in PBS, where complete inhibition was seen at pH levels up to 6.0 (Fig. 4B). Though the cell counts decreased by 3 log units at PH 6.5 in about 6h, an increase in cell numbers were noticed afterwards. Tsai & Su (1999) reported higher antibacterial effect of chitosan at acidic pH. When the pH is between 5.0 and 6.0, the amino groups of the glucosamine residues will be protonated which influences interactions with negative residues at cell surface. In more acidic condi-

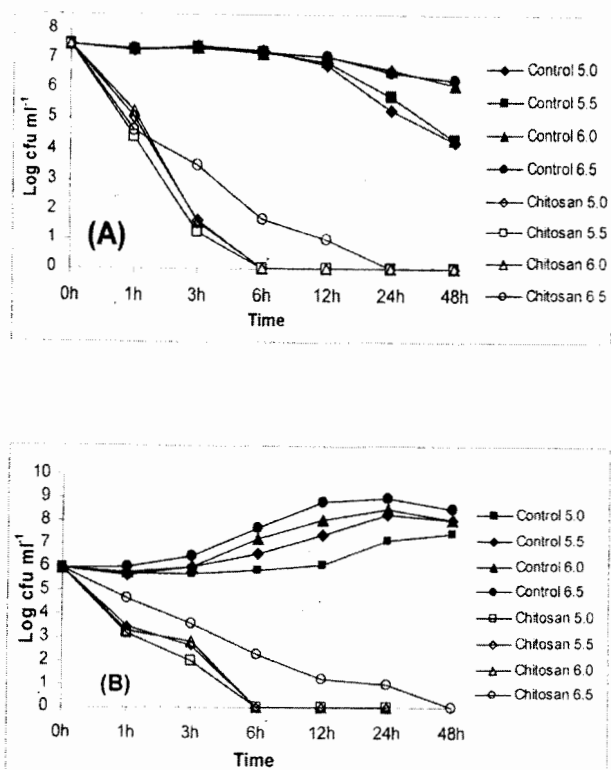


Fig. 3. Effect of pH on the inhibitory activity of chitosan against *Staphylococcus aureus* in (A) Phosphate Buffered Saline and (B) Tryptic Soy Broth.

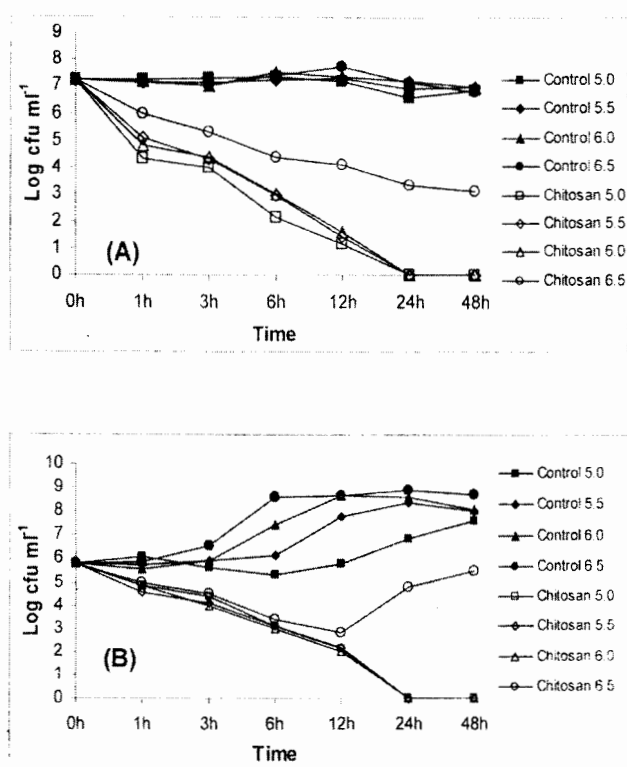


Fig. 4. Effect of pH on the inhibitory activity of chitosan against *E. coli* in (A) Phosphate Buffered Saline and (B) Tryptic Soy Broth.

tions, chitosan carries more positively charged amino groups resulting in greater adsorption, which has also been verified by electron microscopy. Wang (1992) demonstrated that antibacterial activity of chitosan at pH 6.5 was weaker than at pH 5.5 and that the *S. aureus* cells were more susceptible to chitosan than the cells of *Salmonella*, *E. coli* and *Yersinia enterocolitica*. The antibacterial activity of chitosan was observed to be limited at pH above 6.0, but greater activity was found at lower pH against several gram-positive and negative bacteria (No *et al.*, 2002). In gram negative bacteria, the differences in the distribution of negative charges on the surface of cell wall were attributed for the differences in the susceptibility of different bacteria against chitosan. At low pH, chitosan was shown to be better adsorbed by bacterial cells. Thus the increased adsorption would result in greater changes in the structure of the cell wall and in the

permeability of the cell membrane. Further, with more positive charges, the intermolecular electron repulsion is increased leading to a longer persistence, which is known to prevent chitosan from penetrating bacterial cell walls of both gram-negative and positive bacteria (Chung *et al.*, 2003; 2004).

The results of the study showed that antibacterial activity of chitosan was inversely proportional to pH and directly influenced by temperature. The effect of chitosan was more pronounced against *S. aureus* than against *E. coli* at all the tested conditions.

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