

# **Microbiology**

**Journal**

ISSN 2153-0696



Academic  
Journals Inc.

[www.academicjournals.com](http://www.academicjournals.com)

## Antimicrobial Effects of Native Chitosan against Opportunistic Gram-negative Bacteria

<sup>1,4</sup>A.C.L. Batista, <sup>2</sup>G.C. Dantas, <sup>3</sup>J. Santos and <sup>1</sup>R.V.S. Amorim

<sup>1</sup>Departamento de Histologia e Embriologia-CCB/Universidade Federal de Pernambuco, Cidade Universitária, 50670-901, Recife-PE, Brazil

<sup>2</sup>Departamento de Biologia Molecular-CCEN/Universidade Federal da Paraíba, Campus I-Cidade Universitária, 58051-900, João Pessoa, PB, Brazil

<sup>3</sup>Departamento de Estatística-CCEN/Universidade Federal da Paraíba, Campus I-Cidade Universitária, 58051-900, João Pessoa, PB, Brazil

<sup>4</sup>RENORBIO, NPCIAMB, Universidade Católica de Pernambuco, Boa Vista 50 050-590 Recife, PE, Brazil

*Corresponding Author: Rosa Valéria S. Amorim, Departamento de Histologia e Embriologia-CCB/Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, 1235, Cidade Universitária, 50670-901, Recife-PE, Brazil  
Tel: +55 81 2126.8515 Fax: +55 81 2126.8516*

### ABSTRACT

Recently, the emergence of new strains of bacteria resistant to most antibiotics, like Enterobacteriaceae, has killed dozens of people in different parts of the world. Here, two types of non-modified chitosan (CS), nominated CSA (High Molecular Weight, 85% deacetylation degree or Dd) and CSB (Medium Molecular Weight, 90.43% Dd) were tested for their antimicrobial activity against potentially pathogenic bacteria. The microorganisms tested were *Staphylococcus aureus*, *Escherichia coli* and *Lactobacillus casei*. The inhibitory effects on growth were determined by the agar-diffusion test; among the strains analyzed, CSB showed an inhibitory effect against *E. coli* and *L. casei*. The statistical analysis of antimicrobial activity show that it was more evident with *E. coli* during the first five hours of incubation with CSB ranging from 0.5 to 2.0% but CSB 1.0% showed the maximum inhibitory effect until 72 h. For *L. casei*, CSB concentrations ranging from 0.5 to 2.0%, showed inhibitory effects in the 72 h study but 1.5% give the greatest inhibitory effects. The antimicrobial activity to *E. coli* was confirmed by Transmission Electron Microscopy (TEM) which indicates that damage to cell wall and membranes of *E. coli* with loss of cell contents was the main inhibitory action of CSB.

**Key words:** Antimicrobial effect, chitosan, *Escherichia coli*, *Lactobacillus casei*, *Staphylococcus aureus*

### INTRODUCTION

The treatment of infections caused by microorganisms, especially bacteria, has been a common challenge in clinical practice, especially under the jurisdiction of genetic-biochemical pathogens to develop resistance to antibiotics (Shakyawar *et al.*, 2011). The specific drug resistance in bacteria causing human infection was rare at the beginning of the era of antibiotic. Today, there are still many cases of death due to infection with multiresistant bacteria, worldwide and a common concern for many scientists. Besides, there is increasing evidence on the association of chronic bacterial infections with development of various types of cancers (Pitout, 2010; Ghorashi *et al.*, 2010; Ibrahim and Makkiya, 2011; Frederick, 2011; Raja *et al.*, 2011).

Recently, a new bacterial strain that is spreading rapidly and has proven resistant to most antibiotics is leaving the worldwide medical community on alert. The mutant bacteria which would be more resistant form of *E.coli*, can lead to death due to pneumonia and urinary tract infections. The first cases emerged in India and Pakistan and arrived in the United Kingdom by the English who went to India to undergo cosmetic surgery and medical treatments (Pitout, 2010; Manikandan *et al.*, 2011).

Research on the antimicrobial activity has been focused on the study of biomacromolecules and new biocompatible materials for clinical applications. Chitosan has become increasingly important biomaterials used for this purpose, as N-carboxybutyl chitosan (Muzzarelli *et al.*, 1990); chitosan-Ag-nanoparticle composite (Sanpui *et al.*, 2008); hydroxylbenzenesulfonamides derivatives of chitosan (Zhong *et al.*, 2009).

Chitosan (CS) is a highly basic polysaccharide, consisting of  $\beta(1\rightarrow4)$ -linked 2-acetamido-2-deoxy-b-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-b-Dglucopyranose (GlcN) which occurs in fungal cell walls, particularly in members of the Order Zygomycetes (Amorim *et al.*, 2005, 2006; Rinaudo, 2006; Chatterjee *et al.*, 2006). It has considerable antimicrobial activity, especially with the site of action as the microbial cell wall (Muzzarelli *et al.*, 1990). The antimicrobial activity of CS is suggested to be mediated through the interaction between the  $\text{NH}_3^+$  groups of CS which bind to the anionic groups of the cell walls of several microorganisms (Liu *et al.*, 2004, 2006). However, this activity is influenced by different physicochemical properties, such as Molecular Weight (MW) (Falcon *et al.*, 2008; No *et al.*, 2002), degree of Deacetylation (Dd) and the substituent groups (Wang *et al.*, 2008; Zhong *et al.*, 2008).

Most of the antimicrobial activities have been described with modified CS derivatives; however, a few studies have been conducted with nonmodified CS. The present researched was aimed to study two types of nonmodified CS as alternative antimicrobial agents against two gram-positive and one gram-negative bacteria. Factors such as Molecular Weight MW, concentration and pH of CSs and the duration of exposure of the bacteria to CS were investigated.

## MATERIALS AND METHODS

**Isolates and chemicals:** The present study was conducted at Laboratory of Biopolymers (LABIPOL), Federal University of Paraiba, João Pessoa-Brazil, during 2008-2009. Two different types of commercial CS from marine crustaceans were used: CSA (High Molecular Weight, 85% Dd) from Sigma Chemical Co. (USA) and CSB (Medium Molecular Weight, 90.43% Dd) from SPfarma (São Paulo, Brazil). Acetic acid, sodium hydroxide, peptone, agar and other reagents were of analytical grade and supplied by Sigma Chemical Co. (USA).

The microorganisms tested were a multiresistant *Staphylococcus aureus* 319U (isolated from cases of bovine mastitis), *Escherichia coli* American-type Culture Collection (ATCC) 25922 and *Lactobacillus casei* ATCC 7469. The strains were maintained on Nutrient Agar (NA) slants at 4°C.

**Antibacterial testing:** The CSA and CSB samples were dissolved in acetic acid (0.25%), at concentrations ranging from 0.05 to 2.0% (pH 3.5-6.0). The CSA solution was autoclaved due to its high molecular weight. CSB and acetic acid solutions were filtered through a 0.22  $\mu\text{m}$  membrane (Millipore). The inhibitory effects were determined by the agar-well diffusion test (adapted from Lachica *et al.*, 1972) at a final optical density  $A_{600}$  of 2.0/100 mL of NA. The cultures were homogenized and plated; after solidification, twenty microliters of the inhibitory agent (CSA or CSB) were added into holes of 6-mm diameter punched in the agar. Acetic acid was used as the

control; concentrations of acetic acid below 0.50% did not show inhibitory effects against all the microorganisms tested. After incubation at 37°C, at 5, 10, 24, 48 and 72 h, the activity of each CS preparation was estimated by measuring the diameter of the growth-inhibition zone for the corresponding microorganism.

**Transmission electron microscopy TEM analysis:** *E. coli* was prepared for electron microscopy as follows: One milliliter of *E. coli* culture, at a final optical density  $A_{600}$  of 2.0/100 mL of nutrient broth was added into CSB solution, to give a CSB final concentration of 0.5 and 1.0% (w/v). After incubation on a rotary shaker (120 rpm) at 37°C for 24 h, the suspension was centrifuged. The cells were washed twice with 5 mmol Phosphate Buffered Saline (PBS) pH 7.2 and then fixed with 2.0% (v/v) glutaraldehyde in same buffer PBS. The samples were postfixed with 1% (w/v) OsO<sub>4</sub> in 5 mmol L<sup>-1</sup> PBS for 1 h at room temperature and washed three times with the same buffer, dehydrated in graded ethanol, then embedded in Epon a low-viscosity embedding medium. Thin sections of the specimens were cut with a diamond knife on an Ultracut Ultramicrotome and the sections were double-stained with saturated uranyl acetate and lead citrate. The grids were examined with a transmission electron microscope JOEL (JOEL-Hitachi, Tokyo, Japan) at an operating voltage of 75 kV.

**Statistical analysis:** Inhibition studies were carried out in triplicate and the results were analyzed using the statistical package SPSS- version 13.0. Two-way Analysis of Variance (ANOVA) was used to test for differences between treatments (time and CS concentrations). In cases where the differences between the mean values were significant, posthoc pairwise comparisons were conducted using the Tukey's multiple-comparison test. A p-value<0.05 was considered significant.

## RESULTS AND DISCUSSION

**Influence of CSs concentration on antibacterial activity:** The antimicrobial effect of nonmodified CSs on two gram-positive and one gram-negative bacterium were investigated. Among the CSs analyzed, antimicrobial activity was observed only when CSB (medium molecular weight, 90.43% Dd) was used against *E. coli* and *L. casei*. However, CSA and CSB were not effective in inhibiting *S. aureus*, in the conditions of test used here; suggesting that the multiresistant strain of *S. aureus* was also resistant to the effect of the CSs used which should acts by a different mechanism of action, compared with *L.casei*, also gram-positive bacterium.

The antibacterial activities assessed in this work using different concentration of CSB against *E. coli* and *L. casei* are shown in Fig. 1. In *E. coli*, a gram-negative bacterium, were founded ANOVA resulted in values of  $p<0.01$  for all concentrations of CSB used. Subsequently, by application of Tukey's test, the maximum inhibitory effect of CSB was observed with 1.0% CSB ( $p<0.01$ ); other concentrations of CSB were less effective, after 24 h. In the case of *L. casei*, were founded ANOVA resulted in values of  $0.01<p<0.05$  for the concentrations of CSB studied. The Tukey's test showed two homogeneous subgroups ( $p<0.01$ ) for the concentrations; the first group (0.05-0.30%) resulted in lower inhibitory effect and the second group (0.5-2.0%) resulted in greater inhibitory effect. Concentrations of 1.5% showed superior inhibitory effects. Increase in the CSB concentration resulted in an enhanced level of antimicrobial activity, only in 72 h of cultivation to *L. casei*. Zheng and Zhu (2003), in their study of the effects of CS with different molecular weights, observed that increasing the concentration of CS, especially CSs of medium molecular weight, until 1.0% resulted in an increased antimicrobial activity against *E. coli* and *S. aureus*; this concentration

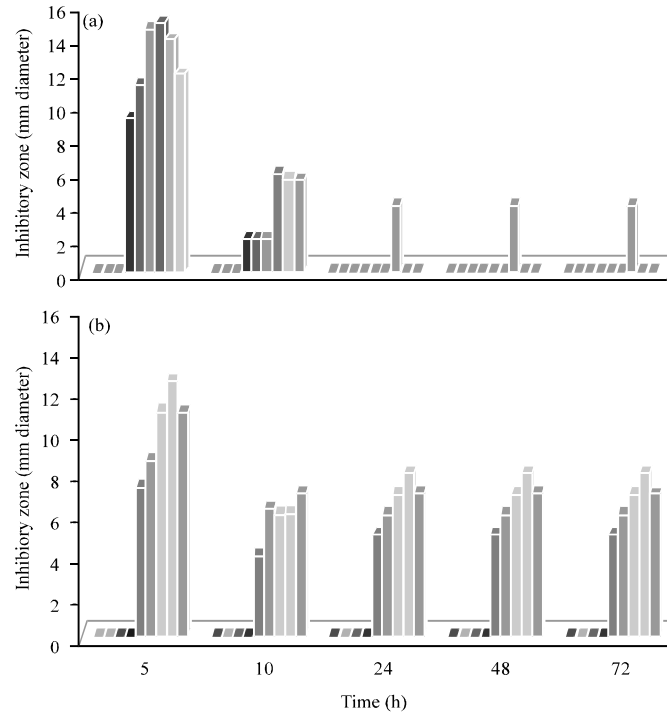


Fig. 1 (a-b): The effect of concentration of CSB and time of incubation on the antimicrobial activity against (a) *E. coli* and (b) *L. casei*. The different columns represent the different concentrations of CSB in the order of left to right: 0.05, 0.10, 0.15, 0.30, 0.50, 0.80, 1.00, 1.50 and 2.00%

of CS could inhibit both bacteria completely. Liu *et al.* (2006) observed the same results while testing various concentrations of CS above 0.002% against *E. coli* corroborate the results presented in this study.

Throughout the previous thirty years, CS have aroused the interest of the scientific community in the world, mainly due to the large feasibility of applications in many fields. In the medical field, CS has been tested against numerous bacteria of medical interest (Muzzarelli *et al.*, 1990; Zheng and Zhu, 2003; Liu *et al.*, 2004; Zhong *et al.*, 2008, 2009). Moon *et al.* (2007) found an inhibitory effect of water-soluble CS against *S. aureus* isolated from cases of bovine mastitis. Liu *et al.* (2006) observed a marked effect of the MW of CSs on the antimicrobial activity toward various strains of *E. coli* and thereby showed a relation between the inhibitory effect and low molecular weight; similar to the studies of Falcon *et al.* (2008), No *et al.* (2002), Wang *et al.* (2008) and Zhong *et al.* (2008) and corroborate the results presented in this work.

**Influence of the time exposure of CSs on antibacterial activity:** The influence of the time of exposure on the antimicrobial action of CSB against *L. casei* and *E. coli* shown that CS was more effective against *L. casei* for all times of exposure. However, the greatest inhibitory effect was found in *E. coli*, during the first five hours of exposure time (Fig. 1), with ANOVA results in values of  $p < 0.01$  for the exposure time. The results from the Tukey's test showed the inhibitory effect of CSB on *E. coli*, with three homogeneous subgroups comprising 5-, 10- and 24 to 72 h durations which significantly differed among themselves; the maximum inhibitory effect occurred in the subgroup of 5 h. In the case of *L. casei*, were founded ANOVA resulted in values of  $p < 0.01$  for the

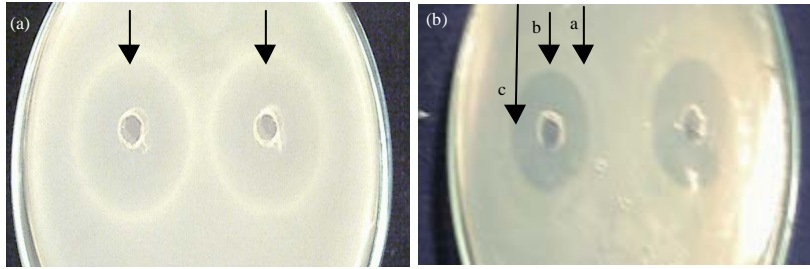


Fig. 2 (a, b): (a) Inhibitory action of 1.5% CSB against *L. casei* in 5 h time exposition (duplicate). (b) Inhibitory action against *E.coli*: inhibition disc at left: 1.0% CSB, a: inhibition disc at 5 h, b: inhibition disc at 10 h, c: inhibition disc at 24 h

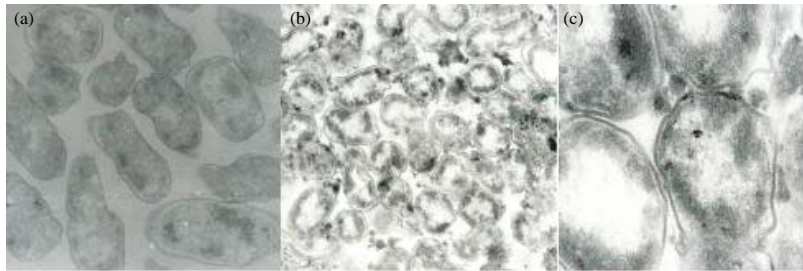


Fig. 3(a-c): Electron micrographs of *E. coli* untreated amplified (a) 12.000, treated with CSB 0.5% amplified, (b) 12.000 and (c) 50.000

exposure time (h). From the Tukey's test, 5 h and (24-72 h), 10 h and (24-72 h) were homogeneous subgroups, with significant difference only between the 5- and 10-h subgroups were observed, with the peak inhibitory effect occurring in the first 5 hours (Fig. 1, 2a). The results showed that the effects of CS (medium molecular weight) were different for the two types of bacteria, with low CSB concentration being more effective against *L. casei* until 72 h (Fig. 1). However, in *E. coli*, these concentrations were effective in the first hours of incubation. Subsequently, it reduced gradually until it stabilized toward the end of the 24-h period (Fig. 2b), with only 1.0% CSB remaining effective until 72 h (Fig. 1).

**Transmission electron microscopy:** With respect to the level of ultrastructural changes the CSB used at 0.5% concentration shown similar effect in *E. coli* compared with CBS 0.1%, caused morphological extra- and intracellular modifications, there was irregularly shaped and without membranes or cell wall on one side (Fig. 3b, c).

Here, many cells were markedly degraded from bacilliform to a spherical shape and irregularly endocellular structures were densely packed and we can observed a disruption of the cell envelope, with the loss of cell contents, compared with intact cell envelope of non-treated cells (Fig. 3a).

The possible mechanisms of the antimicrobial activity of CS proposed in literature are as follows; the CS on the surface of the cell forms a polymer membrane which prevents nutrients from entering the cell. Low-MW CS enters the cell through cell membrane, because CS can absorb the

electronegative substances in the cell and flocculate them, it disturbs the physiological activities of the bacteria and consequently kills them (Zheng and Zhu, 2003). Moon *et al.* (2007) also observed the inhibitory effects of modified CS against *S. aureus*, confirmed by the cell-membrane damage.

**Influence of the pH of CSs on antibacterial activity:** The influence of pH CSB solution was also verified in this study with *L. casei* and *E. coli* with 72 h of incubation (Fig. 4, 5). To *L. casei* CSB with final pH 5.7 showed the greatest inhibitory effects (Fig. 4), with *E. coli* CSB final pH 5.5 showed the greatest inhibitory effects (Fig. 5), probably by the highest degree of protonated CSB.

The important property of CS is its degree of Deacetylation (Dd), the deacetylation confers positive charges on the surface of CS when dissolved in dilute aqueous acidic solutions (pH<6.5) that can convert the glucosamine units of CS to their soluble form, R-NH<sub>3</sub><sup>+</sup>. The antimicrobial activity of CS is suggested to occur through the interaction between the NH<sub>3</sub><sup>+</sup> groups of CS which bind to the anionic groups in the cell walls of microorganisms (Liu *et al.*, 2004, 2006). Thus, CS can interact by electrostatic interaction with molecules negatively charged on bacterial cell surface of Gram-negative bacteria, such as Lipopolysaccharide and teichoic acids (Yang *et al.*, 2000) and carbonyl and phosphoryl groups of the phospholipid, as phosphatidylcholine (Li *et al.*, 2010). The

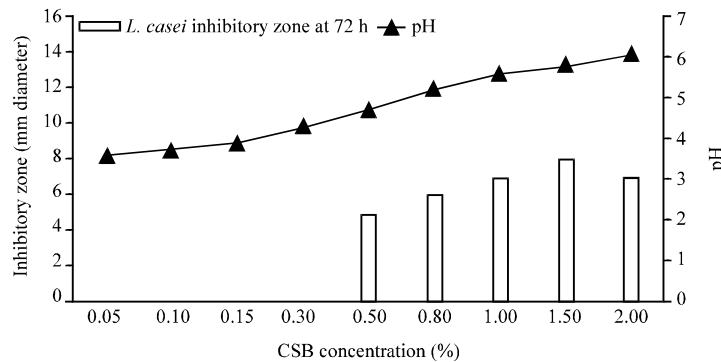


Fig. 4: The inhibitory effect of CSB with different concentrations and influence of final pH of solution on the growth of *L. casei* during 72 h of incubation

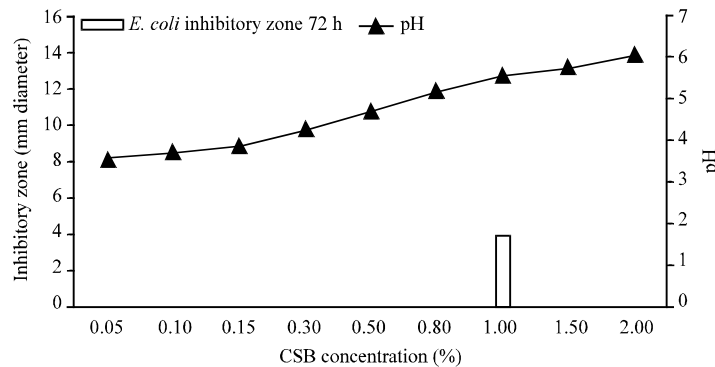


Fig. 5: The inhibitory effect of CSB with different concentrations and influence of final pH of solution on the growth of *E. coli* during 72 h of incubation

carbonyl and phosphoryl groups of lipid components and the inner core of the LPS molecules contribute to the stability of the LPS layer through electrostatic interactions with divalent cations such as Mg<sup>2+</sup> (Kong *et al.*, 2008).

## CONCLUSIONS

In conclusion, although a wide variety of substituent for CS has been described which supposedly increase its antimicrobial activity, the present work suggests that CS without any substitute group is an important antimicrobial agent against *E. coli*. The stability of the antibacterial activity of CBS until 72 h of exposure to the bacteria showed that CS, a natural and low-cost biopolymer, can be applied as a viable growth inhibitory agent against potential pathogenic bacteria such as multi-resistant strain of *E. coli*.

## ACKNOWLEDGMENT

The authors acknowledge Dr. Maria Socorro V. Pereira (CCEN/DBM-UFPB), for kindly providing the strain of *Staphylococcus aureus* 319U.

## REFERENCES

- Amorim, R.V., W.M. Ledingham, K. Fukushima and G.M. Campos-Takaki, 2005. Screening of chitin deacetylase from Mucoralean strains (Zygomycetes) and its relationship to cell growth rate. *J. Ind. Microbiol. Biotech.*, 32: 19-23.
- Amorim, R.V.S., W.M. Ledingham, J.F. Kennedy and G.M. Campos-Takaki, 2006. Chitosan from *Syncephalastrum racemosum* using sugar cane substrates as inexpensive carbon sources. *Food Biotechnol.*, 20: 43-53.
- Chatterjee, S., B.P. Chatterjee and A.K. Guha, 2006. Kinetics of *Mucor rouxii* fermentation in relation to chitosan production. *Res. J. Microbiol.*, 1: 90-94.
- Falcon, A.B., J.C. Cabrera, D. Costales, M.A. Ramírez, G. Cabrera, V. Toledo and M.A. Martínez-Tellez, 2008. The effect of size and acetylation degree of chitosan derivatives on tobacco plant protection against *Phytophthora parasitica* nicotianae. *World J. Microbiol. Biotechnol.*, 24: 103-112.
- Frederick, A., 2011. *Escherichia coli*, its prevalence and antibiotic resistance in Malaysia: A mini review. *Microbiol. J.*, 1: 47-53.
- Ghorashi, Z., N. Nezami, R. Ghotaslou and S. Ghorashi, 2010. Pattern of *Pseudomonas aeruginosa* drug resistance in Tabriz children hospital. *Pak. J. Biol. Sci.*, 13: 400-404.
- Ibrahim, M.A. and A. Makkiya, 2011. Consequences of chronic bacterial infections on development of cancer. *Res. J. Microbiol.*, 6: 310-321.
- Kong, M., X.G. Chen, C.S. Liu, C.G. Liu, X.H. Meng and L. Yu, 2008. Antibacterial mechanism of chitosan microspheres in a solid dispersing system against *E. coli*. *Colloids Surf, B. Biointerfaces*, 65: 197-202.
- Lachica, R.V.F., P.D. Hoepflich and C.E. Franti, 1972. Convenient assay for Staphylococcal Nuclease by the metachromatic well-agar-diffusion technique. *Applied Microbiol.*, 24: 920-923.
- Li, X.F., X.Q. Feng, S. Yang, G.Q. Fu, T.P. Wang and Z.X. Su, 2010. Chitosan kills *Escherichia coli* through damage to be of cell membrane mechanism. *Carbohydr. Polym.*, 79: 493-499.
- Liu, H., Y. Du, X. Wang and L. Sun, 2004. Chitosan kills bacteria through cell membrane damage. *Int. J. Food Microbiol.*, 95: 147-155.
- Liu, N., X.G. Chen, H.J. Park, C.G. Liu, C.S. Liu, X.H. Meng and L.J. Yu, 2006. Effect of MW and concentration of chitosan on antibacterial activity of *Escherichia coli*. *Carbohydr. Polymers*, 64: 60-65.



- Manikandan, S., S. Ganesapandian, M. Singh and A.K. Kumaraguru, 2011. Emerging of multidrug resistance human pathogens from urinary tract infections. *Curr. Res. Bacteriol.*, 4: 9-15.
- Moon, J.S., H.K. Kim, H.C. Koo, Y.S. Joo, H.M. Nam, Y.H. Park and M.I. Kang, 2007. The antibacterial and immunostimulative effect of chitosan-oligosaccharides against infection by *Staphylococcus aureus* isolated from bovine mastitis. *Applied Microbiol. Biot.*, 75: 989-998.
- Muzzarelli, R., R. Tarsi, O. Filippini, E. Giovanetti, G. Biagini and P.E. Varaldo, 1990. Antimicrobial properties of N-carboxybutyl chitosan. *Antimicrob. Agents Ch.*, 34: 2019-2023.
- No, H.K., N.Y. Park, S.H. Lee and S.P. Meyers, 2002. Antimicrobial chitosan and chitosan oligomers with different molecular weights. *Int. J. Food Microbiol.*, 74: 65-72.
- Pitout, J.D.D., 2010. The latest threat in the war on antimicrobial resistance. *Lancet Infect. Dis.*, 10: 578-579.
- Raja, A., P. Prabakaran and P. Gajalakshmi, 2011. Drug resistant tuberculosis and its surveillance. *Curr. Res. Tuberculosis*, 3: 1-8.
- Rinaudo, M., 2006. Chitin and chitosan: Properties and applications. *Progress Polym. Sci.*, 31: 603-632.
- Sanpui, P., A. Murugadoss, P.V.D. Prasad, S.S. Ghosh and A. Chattopadhyay, 2008. The antibacterial properties of a novel chitosan-Ag-nanoparticle composite. *Int. J. Food Microbiol.*, 124: 142-146.
- Shakyawar, S.K., A. Goyal and V.K. Dubey, 2011. Database of *in silico* predicted potential drug target proteins in common bacterial human pathogens. *Am. J. Drug Discovery Dev.*, 1: 70-74.
- Wang X., Y. Du, J. Yang, Y. Tang and J. Luo, 2008. Preparation, characterization and antimicrobial activity of quaternized chitosan/organic montmorillonite nanocomposites. *J. Biomed. Mater. Res.*, 84: 384-390.
- Yang, D.Z., X.F. Liu, Z. Li, H.Y. Xu, Y.L. Guan and K.D. Yao, 2000. On the factors influencing the antibacterial activities of chitosan. *Chinese J. Applied Chem.*, 17: 598-602.
- Zheng, L.Y. and J.F. Zhu, 2003. Study on antimicrobial activity of chitosan with different molecular weights. *Carbohydrate Polymers*, 54: 527-530.
- Zhong, Z., P. Li, R. Xing and S. Liu, 2009. Antimicrobial activity of hydroxybenzenesulfonilides derivatives of chitosan, chitosan sulfates and carboxymethyl chitosan. *Int. J. Biol. Macromol.*, 45: 163-168.
- Zhong, Z., R. Xing, S. Liu, L. Wang, S. Cai and P. Li, 2008. Synthesis of acyl thiourea derivatives of chitosan and their antimicrobial activities *in vitro*. *Carbohydr. Res.*, 343: 566-570.