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Research Article

ELUCIDATION OF ANTIBACTERIAL PROPERTIES OF CHITOSAN AGAINST BACTERIAL CONTAMINANTS OF WATER

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ABSTRACT

The *in vitro* antimicrobial activity of chitosan was investigated against the bacterial isolates of *E. coli*, *K. pneumoniae*, *E. faecalis*, *P. aeruginosa*, *Salmonella enterica* subsp. *enterica* serovar *typhi* and *S. dysenteriae* using disc diffusion method. The highest tested concentration of 400 µg/ml of chitosan had a good potential of antibacterial activities (inhibition diameter ranged from 07-13 mm) against all the tested organisms. Highest antibacterial activity was found against *E. coli* and the lowest activity was found against *E. faecalis*. The results revealed that, the inhibitory effects of chitosan differed depending on the types of the tested bacteria. The MIC range observed for chitosan was from 8 mg/ml to 32 mg/ml with the lowest MIC value was observed against *E. coli* and *K. pneumoniae.* The MBC values observed were in the range from 32 to 128 mg/ml and the lowest MBC value with highest antibacterial activity was observed against *E. coli* and *Salmonella enterica* subsp. *enterica* serovar *typhi*. In conclusion, chitosan could be promising natural antimicrobial agent with potential applications in controlling bacterial contaminants of water.

Keywords: Chitosan, antimicrobial activity, disc diffusion, minimum inhibitory concentration (MIC).

INTRODUCTION

Chitosan is a linear, semi-crystalline polysaccharide composed of (1→4)-2-acetamido-2-deoxy-b-D-glucan (N-acetyl D-glucosamine) and (1→4)-2-amino-2-deoxyb- D-glucan (D-glucosamine) units. Chitosan is primarily produced from alkaline deacetylation (40-50% NaOH) of chitin. This N-deacetylation is almost never complete, chitosan is considered as a partially N-deacetylated derivative of chitin[1].

Chitosan has three types of reactive functional groups, an amino group as well as primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions, respectively. The varied proportion of the two monosaccharides in chitosan contributes to different properties, such as Degree of Deacetylation (DD), molecular weight, viscosity, pKa values and so on[2]. However, these properties can greatly influence its physicochemical characteristics and directly affect its application[3]. In contrast to chitin's insolubility, the presence of free amine groups along the chitosan chain allows it to dissolve in diluted acids such as acetic acid, lactic acid and formic acid due to the protonation of these groups, rendering the corresponding chitosan salt soluble[4].

The properties that make chitosan commercially important are its biodegradability, biocompatibility in animal tissues, non-toxicity, and the ability to transform into gels, beads, fibres, colloids, films, flakes, powders and capsules. Due to its unique biological characteristics, many applications have been found either alone or blended with other natural polymers (starch, gelatin, alginates) in the food, pharmaceutical, textile, agriculture, water treatment and cosmetic industries. Additionally, other properties such as analgesic, antitumor, haemostatic, hypocholesterolemic, antimicrobial and antioxidant properties of chitosan have also been reported[5].

Chitosan is not only a polymer bearing amino groups, but also a polysaccharide, which consequently contains breakable glycosidic bonds. Chitosan is actually degraded *in vivo* by several proteases, and mainly lysozyme. Till now, eight human chitinases have been identified, three of them possessing enzymatic

activity on chitosan. The biodegradation of chitosan leads to the formation of non-toxic oligosaccharides of variable length. These oligosaccharides can be incorporated in metabolic pathways or be further excreted. The degradation rate of chitosan is mainly related to its degree of deacetylation, but also to the distribution of N-acetyl D-glucosamine residues and the molecular mass of chitosan[6].

The function of chitosan is not affected by the host and it does not elicit any undesirable local or systemic effects. Chitosan is well tolerated by live tissues, including the skin, ocular membranes, as well as the nasal epithelium[7]. Non toxicity is another attractive feature of chitosan compared with other natural polysaccharides. Chitosan has an LD_{50} of around 16 g/kg, very similar to that for salt and glucose by *in vivo* toxicity assays carried out on mice. It is reported that the toxicity of chitosan is dependent on the DD value[8].

Chitosan is known for its antimicrobial activity towards Gram-positive bacteria and effective control of fungi and viruses. The degree of activity is determined by the type of microorganisms, pH, molecular weight, degree of acetylation and the presence of lipids and proteins. Two main mechanisms have been reported about antibacterial and antifungal activities of chitosan. In the first proposed mechanism, positively charged chitosan can interact with negatively charged groups at the surface of cells, and as a consequence, alter its permeability. This would prevent essential materials entering the cells and lead to the leaking of fundamental solutes out of the cell. The second mechanism involves the binding of chitosan with the cellular DNA (via protonated amino groups), which would lead to the inhibition of the microbial RNA synthesis. The chitosan antimicrobial property might in fact result from a combination of both mechanisms[9]. The chitosan nanoparticles show potential in drinking water disinfection applications such as antimicrobial agents in membranes, sponges and surface coatings of water storage tanks[10].

A vast rural Indian population is dependent on the supply of untreated water, despite of spending billions of rupees for water purification. Thus the rural population is thriving on the contaminated water supply which is the root cause of their ailments. Rural India has more than 700 million people residing in about 1.42 million habitations spread over 15 diverse ecological regions. Meeting the drinking water needs of such a large population can be a daunting task. The non-uniformity in level of awareness, socio-economic development, education, poverty, practices, rituals and water availability add to the complexity of the task. In order to promote the general health and disinfect the water several materials are being employed as antimicrobial agents. Keeping this in mind, the present study was performed to investigate the antibacterial characteristics of chitosan against the waterborne pathogens.

MATERIALS AND METHODS

Chitosan with the deacetylation degree of 95% and molecular weight of 360 kDa was purchased from India Sea Foods, Cochin. The six bacterial isolates used in this study includes, *Escherichia coli, Klebsiella pneumoniae, Enterococcus faecalis, Pseudomonas aeruginosa, Salmonella enterica* subsp. *enterica* serovar *typhi* and *Shigella dysenteriae* were obtained from Aharam Mineral Waters Private Limited, Erode. The reference strains of bacteria were maintained on nutrient agar (HiMedia, Mumbai, India) slants at 4°C with a subculture period of 30 days.

Assessment of antibacterial activity of chitosan[11]

Sterile Mueller Hinton agar media was prepared and dispensed into Petri dishes. The inoculum suspension of all the bacterial isolates equivalent to 0.5 McFarland standard was spread over the agar plates using a sterile cotton swab, for uniform distribution of bacteria. Chitosan concentrations of 100 µg/ml, 200 µg/ml, 300 µg/ml and 400 µg/ml were prepared by dissolving it in 1% acetic acid and stirred in a magnetic stirrer at 60°C till a fine homogenous suspension was obtained. The filter paper discs of 6 mm in diameter were impregnated with different concentrations of chitosan and were gently placed on the inoculated agar surface. Controls were prepared using ampicillin. The plates were incubated for 24 h at 37°C. The results were recorded by measuring the diameter of inhibition zone at the end of 24 h.

Determination of MIC and MBC of chitosan[12]

The chitosan was serially diluted (twofold) in 1 ml of Mueller Hinton broth to obtain a concentration range of 512 mg/ml to 0.5 mg/ml. To this, 1 ml of 0.5 McFarland standard adjusted inoculum of bacterial isolates was added. Ampicillin was used as a control. The test tubes were incubated at 37°C for 24 h and evaluated for the presence or absence of visible turbidity in the broth after the incubation period. The lowest concentration (highest dilution) of chitosan preventing the appearance of turbidity was recorded as the MIC. After the MIC determination, the tubes without growth were subcultured on Mueller Hinton agar plates at 37°C for 24 h. The incubated plates were

examined for the growth of microorganisms and the MBC was determined as the lowest concentration of the chitosan which did not show any visible growth in the plates.

RESULTS AND DISCUSSION

Assessment of antibacterial activity of chitosan

Antimicrobial activity of chitosan was evaluated at concentrations of 100 µg/ml, 200 µg/ml, 300 µg/ml and 400 µg/ml against the bacterial isolates using disc diffusion method. The evaluation was based on the diameter of clear inhibition zone surrounding the filter paper discs loaded with different chitosan concentrations. The antibacterial activities of the chitosan at different tested concentrations against bacterial isolates from water are shown in table 1.

The highest tested concentration of 400 µg/ml of chitosan resulted in a zone of inhibition of 13 mm against *E. coli*. This was followed by 12 mm zone of inhibition against *S. dysenteriae*; while at the same concentration it showed an inhibition zone of 11 mm against *K. pneumoniae*. *Salmonella enterica* subsp. *enterica* serovar *typhi* and *P. aeruginosa* were inhibited by 10 mm zone of growth inhibition. The lowest activity was found against *E. faecalis* with the zone of growth inhibition of 9 mm. The results revealed that, the inhibitory effects of chitosan differed depending on the types of the tested bacteria.

According to the reports, chitosan exhibited significant antibacterial activity against different bacterial strains *viz*. *Salmonella enterica* subsp. *enterica* serovar *typhi, P. aeruginosa, S. aureus, E. coli, K. pneumoniae* and *B. subtilis*[13]. The low molecular weight chitosan inhibits the growth of *B. cereus, E. coli, S. aureus, P. aeruginosa, S. enterica, B. subtilis, L. monocytogenes* and *K. pneumoniae* and also proved that low molecular weight chitosan have greater effect on the growth and multiplication of microorganisms[14].

Many different models have been proposed for antimicrobial action of chitosan, the most acceptable model being the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes. In this model the interaction is mediated by the electrostatic forces between the protonated NH^{3+} groups and the negative residues, presumably by competing with Ca^{2+} for electronegative sites on the membrane surface[15]. This electrostatic interaction results in twofold interference: i) by promoting changes in the properties of membrane wall permeability, thus provoke internal osmotic imbalances and consequently inhibit the growth of microorganisms, and ii) by hydrolysis of the peptidoglycan in the microorganism wall, leading to the leakage of intracellular electrolytes such as potassium ions and other low molecular weight proteinaceous constituents (e.g. proteins, nucleic acids, glucose and lactate dehydrogenase).

Determination of MIC and MBC of chitosan

The MIC and MBC values of chitosan were determined against bacterial isolates by broth dilution technique and the results are presented in table 2. As shown in table 2, MIC values of chitosan varied depending on bacterial strains. The MIC range observed for chitosan was from 8 mg/ml to 32 mg/ml. The lowest MIC value of chitosan was observed against *E. coli* and *K. pneumoniae* with 8 mg/ml. While MIC value of 16 mg/ml was recorded against *Salmonella enterica* subsp. *enterica* serovar *typhi, S. dysenteriae* and *E. faecalis.* The highest recorded MIC value of chitosan was 32 mg/ml against *P. aeruginosa*. The MBC values observed were in the range from 32 to 128 mg/ml. The lowest MBC value of 32 mg/ml was observed against *E. coli* and *Salmonella enterica* subsp. *enterica* serovar *typhi*. This was followed by 64 mg/ml against *K. pneumoniae*, *P. aeruginosa* and *S. dysenteriae.* The highest MBC value of 128 mg/ml was observed against *E. faecalis*.

The antimicrobial activity chitosan was analysed with the molecular weight of 32 kDa and deacetylation degree of 86% and reported a MIC of 5 mg/ml against *E. coli*[16]. MIC of 4 mg/ml was reported for chitosan with 2.91 kDa molecular weight and deacetylation degree of 86.4% against *E. coli*. MIC of 10 mg/ml was reported against *Salmonella enterica* subsp. *enterica* serovar *typhi* for chitosan with a molecular weight of 1,000 kDa, and deacetylation degree of 80%[17]. The antimicrobial activity of chitosan was studied with a molecular weight of 400 kDa and deacetylation degree of 95% against two Gram-negative microorganisms, *E. coli* and *Salmonella enterica* subsp. *enterica* serovar *typhi* and two Gram-positive organisms, *S. aureus* and *B. cereus*. The MIC of tested chitosan against *E. coli* has been ranged from 1.0% to 4% (w/v), where it has been ranged from 0.8% to 0.05% (w/v) against *Salmonella enterica* subsp. *enterica* serovar *typhi*. On the other hand, MIC of chitosan ranged from 0.5% to 1.00% (w/v) against *B. cereus* and *S. aureus*. Generally it could be observed that, antibacterial activity of chitosan has been influenced by its molecular weight, degree of deacetlyation and concentration of solution[18].

High molecular weight chitosan cannot pass through cell membranes and forms a film that protects cells against nutrient transport through

the microbial cell membrane. Low molecular weight chitosan derivatives are water soluble and can better incorporate the active molecule into the cell. Gram-negative bacteria, often represented by *E. coli*, have an anionic bacterial surface on which cationic chitosan derivatives interact electrostatically. Thus, many chitosan conjugates have cationic components such as ammonium, pyridinium or piperazinium substituents introduced into their molecules to increase their positive charge. Gram-positive bacteria like *S. aureus* are inhibited by the binding of lower molecular weight chitosan derivatives to DNA or RNA[19].

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Table 1: Antibacterial activity of chitosan by disc diffusion method

S.No	Microorganism	Zone of inhibition (mm) Concentration of chitosan				Ampicillin
		400 µg/ml	300 µg/ml	200 µg/ml	100 µg/ml	
1	*E. coli*	13	11	9	7	20
2	*K. pneumoniae*	11	9	0	0	17
3	*E. faecalis*	9	6	0	0	13
4	*P. aeruginosa*	10	9	7	0	18
5	*Salmonella enterica* subsp. *enterica* serovar *typhi*	10	9	0	0	16
6	*S. dysenteriae*	12	10	8	7	18

Table 2: MIC and MBC values of chitosan by broth dilution technique

S.No	Microorganism	Chitosan concentration (mg/ml)	
		MIC	MBC
1	*E. coli*	8	32
2	*K. pneumoniae*	8	64
3	*E. faecalis*	16	128
4	*P. aeruginosa*	32	64
5	*Salmonella enterica* subsp. *enterica* serovar *typhi*	16	32
6	*S. dysenteriae*	16	64

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