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Antimicrobial Properties of Fungal Chitosan

[1]Keikhosro Karimi, [1]Azam Jeihanipour and [2]Mohammad J. Taherzadeh
[1]Department of Chemical Engineering, Isfahan University of Technology, Isfahan, Iran
[2]School of Engineering, University College of Boras, S-501 90, Boras, Sweden

Abstract: Cell wall of zygomycetes fungus is an alternative source for chitosan production. In this study chitosan was extracted from cell wall of filamentous fungus *Rhizopus oryzae* and its antimicrobial properties was studied against three typical human pathogenic microorganisms, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. The viability of these bacteria reduced by more than 60%, when 200 ppm of the fungal chitosan was present in the solution. However, the Minimum Bactericidal Concentration (MBC) of the fungal chitosan was 300, 500 and 700 ppm for *S. aureus*, *E. coli* and *K. pneumoniae*, respectively. The antimicrobial activity of fungal chitosan was lower than that of crustacean shells chitosan, which had MBC of less than 100 ppm for the above mentioned bacteria. Furthermore, fungal chitosan similar to crustacean shells chitosan exhibited better inhibitory effects against gram-positive compared to gram-negative bacteria. The possible mechanism for antimicrobial activity of fungal chitosan could be the disruption of the outer membrane of cells but not preventing the nutrients from entering into the cell.

Key words: Fungal chitosan, crustacean shells chitosan, antimicrobial properties, *Rhizopus oryzae*

INTRODUCTION

Chitosan (poly β-(1-4) d-glucosamine), deacetylated form of chitin, is industrially produced from crustacean shells (crabs, shrimp and crayfishes). Cell wall of zygomycetes is an alternative source of chitosan. Chitosan is traditionally extracted from the biomass of fungi (e.g., *Mucor rouxii* and *Rhizopus oryzae*) by alkali-acid treatment (Araki and Ito, 1975). Chitosan from cell wall of fungi has recently received increased attention due to some advantages compared to crustacean shells chitosan. Crustacean waste supplies are limited by seasons and fishing industry locations, whereas fungal mycelium can be obtainedby simple fermentation regardless of geographical location or season (Yoshihara *et al*., 2003; Chatterjee *et al*., 2005).

The crustacean shells chitosan shows antimicrobial properties against a wide variety of microorganisms including fungi, algae and some bacteria (Zheng and Zhu, 2003; Chien and Chou, 2006) and is considered as one of the most important properties linked directly to its possible biological applications in many fields including agriculture, medicine, environment, food, etc. Protonation of amine groups of chitosan chain in acidic solutions makes chitosan positively charged. Protonated chitosan is able to attract metal anions, dyes and organic compounds (Strand *et al*., 2002; Hoven *et al*., 2007). In this case diluted chitosan solutions in acidic solutions is very efficient for the coagulation-flocculation of organic suspensions (Hoven *et al*., 2007).

It has been reported that antimicrobial properties of chitosan is depend on it's molecular weight and degree of deacetylation (Zheng and Zhu, 2003; Liu *et al*., 2006). These factors influence chitosan solubility and consequently interaction with the cell walls of target microorganisms. Therefore antimicrobial properties of various kinds of chitosan are different. While antimicrobial properties of chitosan obtained from crustacean shells have been well studied, there are just a few reports on antimicrobial properties of fungal chitosan (Wu *et al*., 2005).

The goal of this study was to evaluate antimicrobial properties of fungal chitosan, extracted from cell wall of filamentous fungus *R. oryzae*, without effect of coagulation-flocculation. In this research, effect of concentration of fungal chitosan on viability of three typical human pathogenic microorganisms, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* was studied and their minimum bactericidal concentrations were measured.

MATERIALS AND METHODS

The microorganisms strain and cultivation: The chitosan was extracted from cell wall of filamentous fungus *R. oryzae* CCUG 28958, obtained from Culture Collection of University of Göteborg (Sweden). This fungus was maintained on agar slants prepared with 10 g^{-1} peptone, 20 g^{-1} agar and 40 g^{-1} D-glucose as the additional carbon source by incubation for 4 days at 30°C. Spore

Corresponding Author: [1]Keikhosro Karimi, Department of Chemical Engineering, Isfahan University of Technology, Isfahan, Iran

suspensions were prepared by addition of 10 mL of sterile distilled water to the slant and shaking it vigorously.

Antimicrobial activity of fungal chitosan was tested against 3 strains. They were two gram-negative bacteria (*Escherichia coli* CCUG 17620 and *Klebsiella pneumoniae* CCUG 225) and one gram-positive bacterium (*Staphylococcus aureus* CCUG 15195). The microorganisms were maintained on nutrient agar slants (peptone 5%, beef extract 3%, NaCl 8%, agar 12%) at 4°C and activated at 37°C for 24 h before use in nutrient broth (peptone 5%, meet extract 3%, NaCl 8%).

Fungal cultivation: Cultivations were performed in 200 mL volumes in 500 mL cotton-plugged-Erlenmeyer flasks in a shaker incubator at 32°C for 2 days. The synthetic media contained in g $^{-1}$: glucose 50, $(NH_4)_2SO_4$ 7.5, KH_2PO_4 3.5, $MgSO_4.7H_2O$ 0.75, [0] $CaCl_2.2H_2O$ 1.0, yeast extract 5 and 10 mL l trace metal solution.

Extraction of chitosan: After cultivation, fungal mycelia was recovered by filtration, washed with distilled water until a clear filtrate was obtained and dried at 65°C. Chitosan extraction was carried out according to the method presented by Synowiecki and Al-Khateeb (1997) with modification. Dried fungal mycelia were finely grounded and suspended in 0.5 molar NaOH solution (1:30 w v^{-1}) and autoclaved at 90°C for 120 min. Alkali-insoluble fractions were collected after centrifugation at 4000 rpm for 15 min and washed 10 times to a neutral pH (pH = 7). The residues were further extracted using 2% acetic acid (1: 200 w v^{-1}) at 65°C for 8 h. The extracted slurry was centrifuged at 4000 rpm for 15 min and the acid insoluble material was discarded. The pH of the supernatant fluids was adjusted to 10 by addition of 2 molar NaOH, chitosan suspension was centrifuged at 4000 rpm for 15 min and the precipitated chitosan was washed with distilled water and dried at 65°C until constant weight achieved.

Assays for antibacterial activity: Chitosan solution (1%w/v) was prepared in 1% (v/v) acetic acid solution. Then, the chitosan solution was diluted by physiologic serum (0.9% NaCl solution) to get a final concentration of 0.02, 0.04, 0.06, 0.08 and 0.1% (w v^{-1}). Two references were applied in the assays for antibacterial activity: A reference with acetic acid excluding chitosan and another reference with neither acetic acid nor chitosan. pH of the solutions were adjusted to 5.5 by addition of 2 M NaOH and the solutions were autoclaved at 121°C for 20 min. Samples (10 mL) of the prepared chitosan solution were inoculated under aseptic conditions with 0.05 mL of the freshly prepared suspension of the bacteria to reach final bacterial concentration of 10^6 CFU mL^{-1} by incubation at 37°C for 24 h. Viable cells (log CFU mL^{-1}) were enumerated on nutrient agar by spread plating 1 mL of serial dilutions of physiologic serum followed by incubation at 37°C for 48 h.

For determination of the Minimum Bactericidal Concentration (MBC) of chitosan, chitosan solution (1 in 1% acetic acid) was added to physiologic serum for final chitosan concentrations of 0.01, 0.02, 0.0, 0.06, 0.08 and 0.1% (w v^{-1}). The MBC was defined as the lowest concentration of chitosan that reduce 99.9% of the active bacteria at 37°C for 24 h (Avadi *et al*., 2004). The antimicrobial properties of fungal chitosan were also compared by crustacean shells chitosan purchased from sigma (28191).

All experiments were carried out at least in 2 duplicates and the average standard deviation was less than 5%.

RESULTS

Antibacterial properties of chitosan extracted from the cell wall of the fungus *R. oryzae* were studied in this work and compared with a crustacean shells chitosan. Three pathogenic bacteria, *E. coli*, *K. pneumoniae* and *S. aureus*, were grown and diluted in physiologic serum. Since the chitosan is insoluble in water, it was dissolved in acetic acid solution. Since acetic acid was reported to have antimicrobial properties, its solution without chitosan was applied as reference. The results are summarized in Fig. 1 and Table 1. The control experiment in Fig. 1, which contains only acetic acid show that acetic acid at concentration below 1000 ppm and in absence of any nutrient, causes some minor growths for all of the three microorganisms.

The effect of fungal chitosan concentration and its antimicrobial properties on *E. coli*, *K. pneumoniae* and *S. aureus*, was studied in the range of 100 to 1000 ppm and results are presented in Fig. 1. For all of these bacteria, 200 ppm of fungal chitosan was enough to reduce about 60% of the viable bacteria. Zero at 600 ppm of the fungal chitosan, a sharp rise of antibacterial activity on these bacteria was observed. No viable cell of *E. coli*

Table 1: Minimum Bactericidal Concentrations (MBC)* of fungal-compared to crustacean chitosan against three bacterial strains

	Chitosan (ppm)	
Bacterial strain	Fungal	Crustaceanshells
E. coli	500±100	<100
K. pneumoniae	700±100	<100
S. aureus	300±100	<100

*MBC is the lowest concentration of chitosan that reduces 99.9% of the viability of bacteria

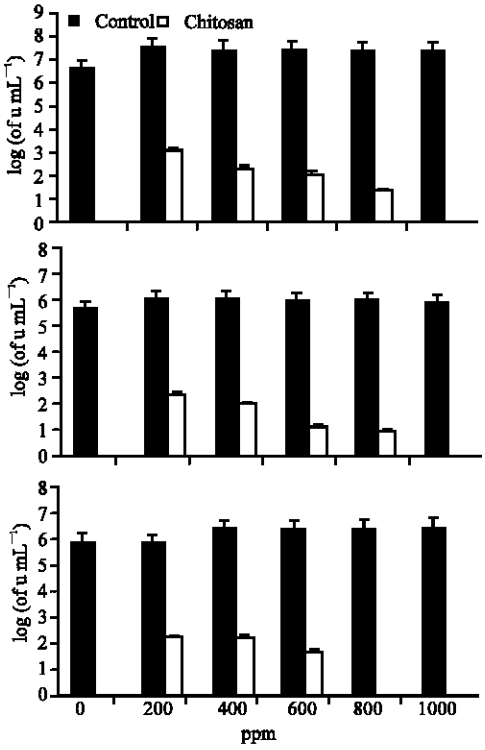

Fig. 1: Reduction in viability of (a) *E. coli*, (b) *S. aureus* and (c) *K. pneumoniae* at presence of different concentration of fungal chitosan compared to the control conditions with identical conditions but without chitosan

and *K. pneumoniae* was observed, when 1000 ppm of the fungal chitosan concentration was available in the solution. Similar trend were also observed for *S. aureus*. However, lower concentration of the chitosan, 800 ppm, was enough to completely stop the growth of this bacterium.

Minimum Bactericidal Concentration (MBC) of fungal chitosan measured and compared with the results of a crustacean shells chitosan. The most important results are shown in Table 1. Less than 100 ppm of the crustacean shells chitosan could reduce more than 99% of the initial bacteria, where as MBC of fungal chitosan for these bacteria was more than 200 ppm. The results show that *S. aureus* which is a gram-positive bacterium is more sensitive to the fungal chitosan compare to the gram-negative bacteria. As shown in Table 1, MBC of fungal chitosan for *S. aureus* and *E. coli* are 300 and 500 ppm, respectively. However, relatively high concentration of the fungal chitosan, 700 ppm, was necessary to deactivate *K. pneumoniae*.

DISCUSSION

Antimicrobial activity of chitosan has been reported against many strains of bacteria, filamentous fungi and yeasts. However, it was shown that the biological activity of chitosan significantly depends on its physico-chemical properties of this polymer such as molecular weight and molecular fraction of glucosamine units in the polymer chain (usually referred as the degree of chitosan N-deacetylation), pH of chitosan solution and, of course, the target microorganism (Chung *et al*., 2003; Tsai and Hwang, 2004; Liu *et al*., 2006; No *et al*., 2006).

In the previous studies, antibacterial properties have been measured in different media containing nutrient including (0) proteins (No *et al*., 2006; Chung *et al*., 2003; Tsai and Hwang, 2004; Liu *et al*., 2006a,b; Qin *et al*., 2006). All of these studies showed the antibacterial properties for different type of crustacean shells chitosan. On the other hands, chitosan is a well known flocculating agent for coagulation of proteins (Jean Roussy *et al*., 2005). Therefore, deactivation of bacteria and coagulation of proteins take place simultaneously and antibacterial property of chitosan might be affected by un-availability of nutrient. On the other hand, flocculation process reduces the concentration of chitosan in the solution which can lead to significant error in determination of MIC (Minimum Inhibitory Concentration) and MBC. Therefore, it is difficult to come to an obvious conclusion that the inhibitory effects of chitosan reflect the eliminating of the nutrients by flocculation or possibly separation of bacteria from the media, or chitosan is a real inhibitor which reduces the viability of the bacteria. For this reason, in this study antibacterial activity of fungal chitosan has been considered in the absence of any nutrient. During the experiments, 2 references were applied in the assays for antibacterial activity, reference with acetic acid excluding chitosan and another reference with neither acetic acid nor chitosan. The former reference, the acetic acid without chitosan, showed that acetic acid did not inhibit the growth of all the three microorganisms and even minor growth was observed (Fig. 1). Acetic acid is known as an effective inhibitor for growth of many bacteria (No *et al*., 2006). However, what was observed in the current work at acetic acid concentration below 1000 ppm are in agreement with some other investigation which show the consumption and growth of different microorganisms on acetic acid (Taherzadeh *et al*., 1997).

Effect of concentration of fungal chitosan as well as crustacean shells chitosan on viability of *E. coli*,

K. pneumoniae and *S. aureus* has been investigated in this research. As expected, antimicrobial properties of fungal chitosan were increased by increasing the concentration, which is in agreement with previous investigations. However, much higher concentration of fungal chitosan is necessary to efficiently reduce the viability of the bacteria, when compared to crustacean shells chitosan. Comparison of minimum bactericidal concentration of fungal chitosan and the crustacean shells chitosan (Table 1) shows that antibacterial activity of crustacean shells chitosan is higher than fungal chitosan and the MBC of fungal chitosan for the same bacteria is at least two times more than MBC of the crustacean shells chitosan in the same conditions. The physico-chemical properties or presence of some impurities in fungal chitosan could be responsible for the lower antimicrobial properties of fungal chitosan compare to crustacean shells chitosan. Crustacean shells chitosan showed a stronger bactericidal effects for gram-positive bacteria rather than gram-negative bacteria (No *et al*., 2006; Liu *et al*., 2004; Je and Kim, 2006) and the results of this study showed the same property for fungal chitosan as well.

Different mechanisms have been proposed for antimicrobial properties of chitin, chitosan and their derivatives, however, the exact mechanism is still unknown (Entsar *et al*., 2003). The mostly accepted mechanism explains that interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents that finally leads to death of bacteria (Helander *et al*., 2001; Liu *et al*., 2004; Je and Kim, 2006). Also oligomeric chitosan may penetrate into the cells of microorganisms and prevents the growth of cells by preventing the transformation of DNA into RNA. In addition, deprivation of metals, trace elements or essential nutrients by chelating action of chitosan and to form a polymer membrane of chitosan which prevents nutrients from entering the cell has also been proposed as factors that limit the growth of bacteria. However, in this study because of the absence of any nutrients in experiments, all of the later mechanisms may not be possible. Therefore, base on the method and results of the current work, one may concluded that between the suggested mechanisms for antimicrobial properties of chitosan, the leakage of proteinaceous and other intracellular constituents is an acceptable mechanism for the antimicrobial property of the chitosan.

CONCLUSION

Results of this study showed that fungal chitosan has antimicrobial properties against *E. coli*, *K. pneumoniae* and *S. aureus*, but not as effective as the antimicrobial activity of crustacean shells chitosan. Accordingly, the possible mechanism for antimicrobial activity of fungal chitosan could be the disruption of the outer membrane of cells but not preventing the nutrients from entering into the cell.

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