The Assessment of Chitosan Solutions Effects on Bacterial Strains

MARIOARA NICOLETA FILIMON[1,2], ROXANA POPESCU[3], ADRIAN SINITEAN[1,2], PAULA MANIU[1,2], GABI DUMITRESCU[4], DOINA VERDES[3]*, CRISTIAN SEBASTIAN VLAD[3]

[1]West University of Timisoara, Faculty of Chemistry, Biology, Geography, Department of Biology-Chemistry, 16 Pestalozzi Str., 300115, Timisoara, Romania

[2]West University of Timisoara, Laboratory of Advanced Researches in Environmental Protection, 4 Oituz Str., 300115, Timisoara, Romania

[3]University of Medicine and Pharmacy Victor Babes, 2 Eftimie Murgu Str., 300041, Timisoara, Romania

[4]Banat's University of Agricultural Sciences and Veterinary Medicine from Timisoara, 119 Calea Aradului Str., 300645, Timisoara, Romania

The interest in the antimicrobial actions of chitosan is due to its multiple properties and effects. The aim of the study was to assess the potential antibacterial effects of chitosan applied on 7 bacterial strains: Escherichia coli, Streptococcus pyogenes, Pseudomonas aeruginosa, Enterococcus faecalis, Clostridium perfringens, Legionella pneumophila and Staphylococcus aureus. Six different concentrations of chitosan were dissolved in 1% acetic acid, following two working protocols (Kirby-Bauer method and testing for bacterial cell viability). The sensitivity of tested bacterial strains following the effect of exposure to chitosan decreased as follows: E. coli > L. pneumophila > S. aureus > S. pyogenes > C. perfringens > P. aeruginosa > E. faecalis. The inhibition rates for the bacterial strains E. faecalis, S. pyogenes and S. aureus highlighted again the strong antibacterial properties of this product. Conclude that the chitosan presents a different antibacterial effect against several bacterial strains of interest directly with the employed concentrations.

Keywords: antibacterial effect, chitosan

Chitosan is a cationic polysaccharide comprising many monosaccharide units of β- (1,4) bound 2-amino-2-deoxy-D-glucopyranose. It is the most important derivative of chitin, the main source being marine crustaceans. When the degree of deacetylation of chitin reaches 50%, it becomes soluble in aqueous acids and is called chitosan [1]. Chitosan can also be extracted from the cell walls of fungi, mainly from *Zygomycetes sp.* The chitosan obtained from fungi cells has many advantages, such as seasonal factor independence and the potential for widespread production [2].

Due to its multifaceted beneficial properties: biocompatible, biodegradable, regenerative, non-toxic, wound-healing, antiviral, antimicrobial, antifungal. The chitosan is applicable in many fields of interest: medical, pharmaceutical, cosmetics, agriculture and so forth. It is useful in food industry as preservative, reduces cholesterol, thickens and stabilize sauces. In cosmetic industry, it is used for moisturizing products, toothpaste, shampoos and anti-acne treatments [1,3]. Perhaps the most studied aspects of chitosan applicability are in the field of biomedicine. The applications of this product cover surgical sutures, dental implants, artificial skins, bones reconstruction, contact lenses and encapsulating materials drugs. The biodegradable and the effective properties highly recommends its use in many areas of biotechnology, agriculture and pharmacology due to anticoagulant, haemostatic, antitumoral and bacteriostatic effects [1,4-6].

The antimicrobial activity of chitosan was extensively investigated and there were several studies that analysed its effects and derivatives [7-9]. This interest arises because this polymer has a strong antimicrobial component, an efficient antibacterial activity even at low concentrations and is equally non-toxic towards mammalian cells [10,11].

One of the methods used in this study was the diffusion method on solid culture mediums [12,13]. This method is one of the most frequently used and represents a standardized method for detecting bacteriostatic activities of natural antimicrobial agents. However, this method is considered very time-consuming (18-24 h), and requires many bacteria in order to be relevant (~$1.5x10^8$ cells / mL) [14,15]. Molecular methods to assess the antimicrobial activity of various natural compounds taking less than 12 hours to obtain the results and a smaller number of bacteria to test.

In what concerns the mechanisms of action of chitosan at cellular level, it was stated that this compound degrades the outer layer of bacterial cell walls, by weakening of the inner layers first and releasing afterwards the cytoplasmic matrix from the cell. The induced modifications in both Gram-negative and Gram-positive bacteria may result in deterioration of cell integrity and mitotic processes, causing in the end the cell deaths [16,17].

Although the literature provides details on the antibacterial effects of chitosan in various concentrations, further studies are required to confirm its efficiency on other bacterial strains of interest. The aim of this study was, therefore, to highlight the effects of chitosan on standard bacterial strains by employing two different experimental protocols. The novelty of this study resides therefore in the comparison of the efficiency of two different methods on the following standard strains: *S. pyogenes*, *E. faecalis* and *C. perfringens*.

Experimental part

The chitosan used in this experiment - Deacetylated chitin, Poly (D-glucosamine) commercial name (Sigma-Aldrich) had the following physicochemical properties: CAS Number: 9012-76-4, viscosity: 200-800 cP, 1 wt. % in

**email: dverdes@umft.ro; Phone: 0721591220*

1% acetic acid (25°C, Brookfield), degree of deacetylation 75-85%, solubility: aqueous solutions of diluted acids. Six solutions with different concentrations of chitosan in acetic acid 1% were prepared: c1-1.5 mg/mL, c2-1.3 mg/mL, c3-1 mg/mL, c4-0.7 mg/ mL, c5-0.5 mg/mL and c6-0.3 mg/ mL. The acetic acid 1% was obtained from 1 mL of a standard solution of glacial acetic acid (CH_3CO_2H, Sigma-Aldrich) and 50 mL distilled water. To obtain the various sought concentrations of chitosan, six masses of 0.015g, 0.013g, 0.01g, 0.007g, 0.005g and 0.003g of chitosan, respectively, were weighed with an analytic balance; 1 mL of acetic acid 1% was poured over the chitosan.

Tested bacteria

The bacteria tested are Gram-positive: *Streptococcus pyogenes* (ATCC 196415), *Enterococcus faecalis* (ATCC 29212), *Clostridium perfringens* (ATCC 13124), *Staphylococcus aureus* (ATCC 25923) and Gram-negative: *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 10145), *Legionella pneumophila* (ATCC 33152).

Assessment of minimum inhibitory concentration

The assessment of antibacterial activity of chitosan was undertaken by employing the agar diffusion method (Kirby-Bauer method) that allows one to estimate the MIC based on inhibition distances (mm) around the culture media. The solid culture medium employed was Plate-Count-Agar (Sigma-Aldrich). 50µL of the liquid culture medium with the bacteria tested was transferred on a Petri dish. The discs were applied and on each disc a volume of 10 µL of different concentrations of chitosan were applied. The bacterial plates were incubated at 37°C for 48 h. The test was carried out in triplicates. The zones of inhibition were measured afterwards by using a scale and the antimicrobial activity was calculated [18]. Two antibiotics were used as control: ampicillin (100 mg/mL) and chloramphenicol (25 mg/mL).

The cell viability assays with TTC (2,3,5 triphenyltetrazolium chloride)

Following the presence of bacteria, the TTC is reduced to formazan dye, of red colour, that indicates the activity and viability of cells [19]. The microbial cells from the liquid growing media had a concentration of 10^7 UFC/mL. On each replicate, a microbial culture of 100 µL was applied, along with 50µL of chitosan solution. The ELISA plates were incubated for 6 h at 37°C and 100 rpm speed to facilitate the development of bacteria. Following the incubation period, a 10 µL of TTC solution (0.5% concentration) was applied in 96 wells. The plates were again incubated for 2 h at 37°C to allow the interaction of bacterial colonies with the TTC. Tecan's Sunrise absorbance microplate reader was used to read the concentrations at 460 nm. The assessment of inhibition rate to chitosan was based on the following formula:

Inhibition rate (%)=[(Absorbance control-Absorbance treatment)/Absorbance treatment]x100

Results and discussions

Following incubation period, the MIC (mm) was estimated for each bacterial strain, as a function of employed chitosan concentrations. The graphs show the MIC (mm) as mean ± standard deviation (SD) for the Gram-positive and Gram-negative bacterial strains, respectively antibiotics: ampicillin (am) and chloramphenicol (ch) (fig. 1, fig. 2).

As far as the efficiency of chitosan on *S. pyogenes* strain is concerned, only c1 (1.5 mg/mL) indicated a significant

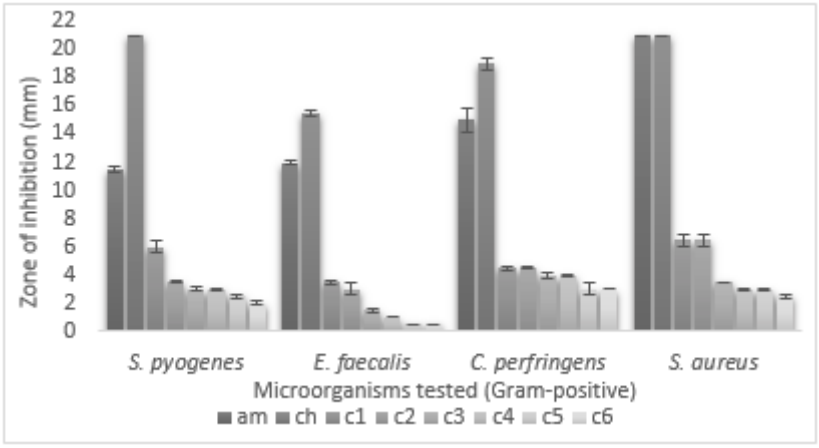

Fig. 1. The mean±SD of Gram-positive bacterial strains MIC following exposure chitosan

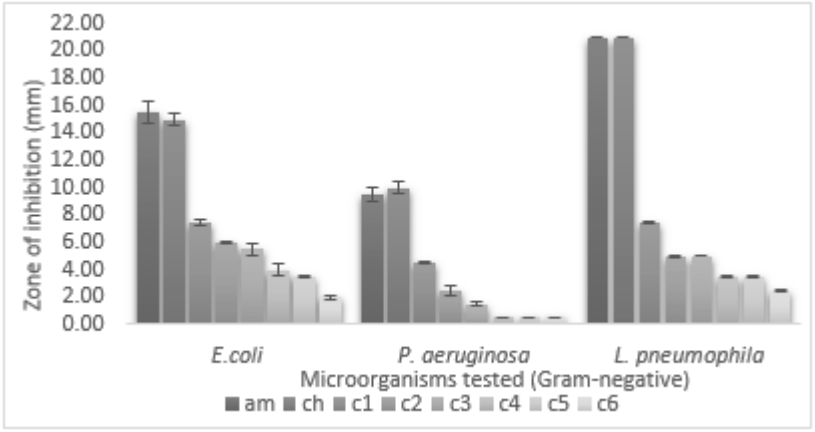

Fig. 2. The mean±SD of Gram-negative bacterial strains MIC following exposure chitosan

sensitivity, with a corresponding MIC of 6±0.41mm. The other chitosan concentrations induced a somehow intermediary sensitivity, with MIC varying between 3.5±0.07 and 2±0.13 mm (fig. 1). The usage of various chitosan concentrations (c1-c3) on *E. faecalis* induced an intermediary sensitivity as well, with MIC ranging between 3.50±0.12 mm and 1.50±0.10 mm. At lower dosage of chitosan, the antibacterial effects were not noticeable (c4 - c6) (fig. 1). As for *C. perfringens* strain, it was observed an intermediary efficiency for all employed chitosan concentrations. The values of MIC recorded decreased from 4.50±0.17 mm down to 3±0.41 mm (fig. 1). The *S. aureus* strain recorded a high sensitivity to c1 (1.5 mg/mL) and c2 (1.3 mg/mL), with MIC of 6.5±0.41 mm. Following exposure to c3 (1 mg/mL), c4 (0.7 mg/mL), c5 (0.5 mg/mL) and c6 (0.3 mg/mL), the *S. aureus* strain showed an intermediary sensitivity, with corresponding MIC values decreasing directly with the employed chitosan concentrations, from 3.5±0.43 to 2.5±0.14 mm (fig. 1). The values of MIC were thus compared with the values recorded by testing exposure to other concentrations of chitosan for *S. typhimurium* and *S. aureus*. The diameter of inhibition zones was 16 mm for the former and 24 mm for the latter bacterial strain [20].

Other previous studies showed that the antibacterial property of chitosan is directly related to its own molecular mass, degree of deacetylation, employed concentration and *p*H [2]. The MIC for chitosan of *E. coli* and *S. aureus* strains were 20 ppm, despite previous findings who showed that much higher concentrations (10-1000 ppm) are needed to induce a similar response [21]. Moreover, in other studies that focused on these two bacterial strains, it was noticed an MIC varying between 0.005% - 1.5% [2, 22]. All these findings strongly suggest that a solution of chitosan of 0.2 % concentration has significant antibacterial effects against *E. coli* and *S. aureus* strains. The effect of pH on the antibacterial efficiency of chitosan was thus assessed, the conclusion being that the highest inhibition efficiency is reached (66 and 52%) for *E. coli* and *S. aureus* strains at a *p*H of 3 [23].

The tested chitosan concentrations on *E. coli* strain showed a certain antibacterial response for c1 and c2 and intermediary for other employed concentrations. The MIC for c1 and c2 were 7.5±0.21 mm and 6±0.65 mm. The inhibition distances for the other employed concentrations showed a decrease directly with the chitosan concentrations from 5.5±0.41 mm to 2±0.14 mm (fig. 2). The antibacterial response for *P. aeruginosa* to chitosan is intermediary for the first three employed concentrations (c1, c2 and c3), with inhibition distances varying between 4.5±0.14 mm and 1.5±0.07 mm. As far as the latter three concentrations are concerned (c4, c5 and c6), based on the recorded MIC, we can conclude that the chitosan is inefficient (fig. 2). A previous laboratory experiment that tested the efficiency of chitosan extracted from two species of crustaceans from Tunis on the very same bacterial strains as above showed that a concentration of 5 mg/mL of chitosan is as well inefficient [24]. Therefore, it can be concluded that the employed chitosan concentrations from this experiment can have antibacterial and fungicide properties, including the possibility of employing the existent natural reserves of this compound. *L. pneumophila* strain was sensitive to c1, with an inhibition distance of 7.5±0.05 mm. As for the other applied chitosan concentrations, it was proved that this strain showed an intermediary sensitivity, with decreasing inhibition distances from 5±0.04 mm to 2.5±0.07 mm, directly with the employed chitosan concentrations (fig. 2). The results of this experiment are in line with those of Fujimoto et al. (2006), who showed that at low pH, the antibacterial effects of chitosan are enhanced, following exposure of two strains of *L. pneumophila* (SG1 and SG6). Nevertheless, the conclusion was that supplementary tests are needed to establish if the pH is indeed vital in enhancing the efficiency of chitosan on both strains of *L. pneumophila*. The same study revealed that the maximum efficiency of 10^2 diluted chitosan solution inhibited the growth of both strains of *L. pneumophila* [25].

The second part of the experiment testing the viability of bacterial cells in culture media. The testing by measuring the inhibition rate was undertaken on the following bacterial strains: *S. pyogenes, E. faecalis* and *S. aureus*. This method measured the inhibition rate of bacterial cells in liquid growing media with the aid of a spectrophotometer, following exposure to various concentrations of chitosan. The inhibition rate for employed chitosan concentrations of *S. pyogenes* varied between 65% - 92.75% following exposure to six concentrations of chitosan (fig. 3). The inhibition rate for *E. faecalis* strain varied between 74.85 - 58.25%. These values suggest a moderate antibacterial efficiency of tested chitosan concentrations. The results confirm the findings of the classic Kirby-Bauer method. In the case of *S. aureus*, the inhibition rate varied between 95.35 - 70.82%. There were noticed similar high values of inhibition rates following exposure to all six concentrations (fig. 3).

Previous studies indicated that the chitosan has a strong antibacterial efficiency against two strains of bacteria of much interest for biomedical studies: *S. typhimurium* and *S aureus*. It was proved that the chitosan was more efficient against *S. aureus* compared to *S. typhimurium*. The study showed that following exposure to a concentration of 0.5 % chitosan triggers 98.7% decrease in *S. aureus* growth and 97.2% of *S. typhimurium*, after an incubation period of

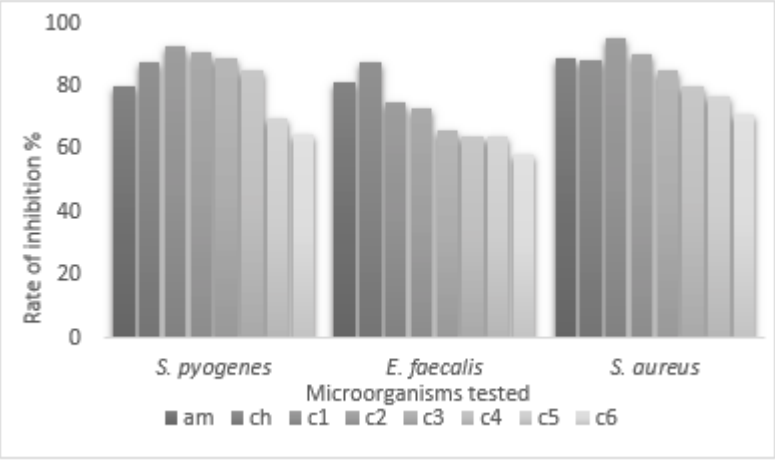

Fig. 3. Inhibition rate to various chitosan concentrations (c1-c6)

5 hours [20]. The values obtained in this study are concurrent and comparable with the aforementioned experiments.

Conclusions

The recorded values for the inhibition zones revealed the antibacterial properties of chitosan. The sensibility of bacterial strains tested against this product decreased as follows: *E. coli* > *L. pneumophila* > *S. aureus* > *S. pyogenes* > *C. perfringens* > *P. aeruginosa* > *E. faecalis*. The inhibition rate for the bacterial strains *E. faecalis, S. pyogenes* and *S. aureus* confirmed the antibacterial effects of chitosan. The results based on spectrophotometric readings strongly support the results obtained by applying the classic method.

References

1. RINAUDO, M., Prog. Polym. Sci., 31, 2006, p. 603.
2. RABEA, E.I., BADAWY, M.E.-T., STEVENS, C.V., SMAGGHE, G., STEURBAUT, W., Biomacromolecules, 4, 2003, p. 1457.
3. HONARKAR, H., BARIKANI, M., Monatshefte fur Chemie, 140, 2009, p.1403.
4. LIU, H., DU, Y., WANG, X., SUN, L., International Journal of Food Microbiology, 95, nr. 2, 2004, p. 147.
5. TAYEL, A.A., MOUSSA, S., OPWIS, K., KNITTEL, D., SCHOLLMEYER, E., NICKISCH-HARTFIEL, A., International Journal of Biological Macromolecules, 47, nr. 1, 2010, p. 10.
6. MOUSSA, S., IBRAHIM, A., OKBA, A., HAMZA, H., OPWIS, K., SCHOLLMEYER, E., International Journal of Biological Macromolecules, 48, nr. 5, 2011, p. 736.
7. HUSSEIN-AL-ALI, S.H., EL ZOWALATY, M.E., HUSSEIN, M.Z., ISMAIL, M., WEBSTER, T.J., Int. J. Nanomedicine, 9, 2014, p. 549
8. KARA, F., AKSOY, E.A., YUKSEKDAG, Z., HASIRCI, N., AKSOY, S., Carbohydr. Polym., 112, 2014, p. 39.
9. ARORA, D., SHARMA, N., SHARMA, V., ABROL, V., SHANKAR, R., JAGLAN, S., Appl. Microbiol. Biotechnol., 100, 2016, p. 2603.
10. LI, Y., CHEN, X.G., LIU, N., ET AL., Carbohydrate Polymers, 67, nr. 2, 2007, p. 227.
11. KONG, M., CHEN, X.G., XING, K., PARK, H.J., International Journal of Food Microbiology, 144, nr. 1, 2010, p. 51.
12. JORGENSEN, J.H., TURNIDGE, J.D., WASHINGTON, J.A., Manual of Clinical Microbiology, p. 1526, ASM Press, Washington, DC, USA, 7th edition, 1999.
13. VALGAS, C., DE SOUZA, S.M., SMˆANIA, E.F.A., SMˆANIA JR., A., Brazilian Journal of Microbiology, 38, nr. 2, 2007, p. 369.
14. WEHR, H.M., FRANK, J.H., APHA Inc.,Washington, DC, USA, 17th edition, 2004.
15.EATON, A.D., CLESCERI, L.S., GREENBERG, A.E., ET AL., APHA, Washington, DC, USA, 21st edition, 2005.
16. RAAFAT, D., VON BARGEN, K., HAAS, A., SAHL, H.G., Applied and Environmental Microbiology, 2008, p. 7455.
17. GALVAN MARQUEZ, I., AKUAKU, J., CRUZ, I., CHEETHAM, J., GOLSHANI, A., SMITH, M.L., Int. J. Food Microbiol., 164, 2013, p. 108
18.VANDER, B.D.A., VLIETNCK, A.J., Assay for Bioactivity. (K.Hostiettman Academic Press, London), 1991, p. 47.
19. ELOFF, J.N., Planta Medica., 64, nr.8, 1998, p. 711.
20. MOUSSA, S.H., TAYEL, A.A., AL-HASSAN, A.A., FAROUK, A., Journal of Mycology, 753692, 2013, p. 1.
21. LIU, X.F., GUAN, Y.L., YANG, D.Z., LI, Z., YAO, K.D., J. Appl. Polym. Sci., 79, 2001, p. 1324.
22. NO, H.K., PARK, N.Y., LEE, S.H., MEYERS, S.P., Int.J.FoodMicrobiol., 74, 2002, p.65.
23. CHUNG, Y.C., WANG,H.L., CHEN,Y.M., LI, S.L., Bioresour.Technol., 88, 2003, p.179.
24. LIMAM, Z., SELMI, S., SADOK, S., EL ABED, A., African Journal of Biotechnology, 10, nr. 4, 2011, p. 640.
25.FUJIMOTO, T., TSUCHIYA, Y., TERAO, M., NAKAMURA, K., YAMAMOTO, M., International Journal of Food Microbiology, 112, 2006, p. 96.

Manuscript received: 08.12.2017