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Antimicrobial and Antibiofilm Properties for Chitosan Extracted by Biological Methods

Running title: *Antimicrobial and Antibiofilm for Chitosan Extract*

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ABSTRACT

Background: The use of biological methods using biological materials for extraction is a substitute way to solve chemical extraction problems. **Objective**: to use chitosan that extracted from biological source as considerable tool in medical fields such as antibacterial, antifungal and antibiofilm. **Materials and Methods**: Basically, chitosan have extracted by using biological treatment consisting of two-steps: de-mineralization with aiding of *lactobacillus plantarum* and de-proteinization with aiding of *Pseudomonas aeruginosa* protease. **Results:** Chitosan displayed a more potential antibacterial effect toward gram-positive bacteria than gram-negative. Since the rates of inhibition were 47 and 50% toward *Staphylococcus aureus* and *Bacillus cereus*, separately, in addition, a weak growth inhibition was recorded by chitosan on fungi as compared with bacteria. A strong anti-biofilm influence was also recorded by Chitosan on *Pseudomonas aeruginosa* as the inhibition rate reached to 62% while 59% was recorded on *Klebsiella pneumoniae* and 52% was recorded on *Enterococcus faecalis*. **Conclusions:** the fungal derived chitosan has benefit as encouraging anti-bacterial and anti-biofilm factor for many diseases treatment especially those related with biofilm forming fungi and bacteria especially multi drug resistant bacteria isolated that concerning with wide spread nosocomial infections.

Key words: chitosan, antimicrobial and antibiofilm formation

Introduction

Aspergillus flavus

Aspergillus is a fungal genus belonged to Ascomycota. *A. flavus*, and it is known as saprotrophic and fungal cause of many serious human diseases that attracted attention globally due to its use in industrial purposes and potential toxigenecity. *A. flavus* has an ability to survive in hard ecosystems allows it to overcomes on many substrates such as living organisms, soil and plant [(1)]. The identification of this fungus has depended on morphological characters i.e. the color of conidia and mycelia, diameter of colony, production of exudates and soluble pigments, presence of both sclerotia and cleistothecia. micromorphology characterization is basically dependent on seriation, shape and size of vesicle, morphology of stipe and conidia as well as morphology of ascospores and cleistothecia [(2, 3)]. Toxigenic strains of *Aspergillus flavus* produced a potential toxic metabolites, named aflatoxins, these metabolites are responsible for carcinogenic, mutagenic and teratogenic influences and threaten human and animal health [(4)].

Lactobacillus plantarum

Lactic acid bacteria (LAB) have been used for centuries for feed, as well as for food fermentation [(5)]. From the populous *Lactobacillus*, *Lactobacillus plantarum* is a LAB member belongs to Gram positive group, commonly located in fermented food as well as in the gastro - intestinal tract, furthermore, it is usually used in the food industry as a considerable starter probiotic [(6)].

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Pseudomonas aeruginosa

Pseudomonas aeruginosa is a bacilli grouped as Gram-negative bacteria, and characterized as obligated aerobes, non-fermented, saprophytic, spread widely in natural environment for instance, soil, vegetables, plant surfaces, sewage , moist environment, and fresh water. *P aeruginosa* mainly characterized with its minimal nutritional needs, and tolerance to different physical conditions, this explains the ability of this bacterium to persist on inert materials, in addition, its minimal nutritional requirement "growing in distilled water" as we as its significant resistance to several unrelated microbial inhibitor agents, sponsored at great deal to its ecological success and its property as an active opportunistic pathogenic microbe [(7)].

As reported by [(8)], *P aeruginosa* displays a significant rate of nosocomial infection especially for patients with prolonged admission in hospital and affinity of nosocomial pathogens to acquire traits with a resistance toward even new commercial antibiotic, this may leads to a great difficulties in their dealing and control.

Over view on chitosan

Chitosan is defined depending on the chemical structure as a copolymer of α-(1,4) glucosamine [(9)]. This substance is basically comprised of 1, 4 D-glucosamine joined to residues of N-acetyl-D-glucosamine (figure-1), derived from de-Nacetylation process of chemical material termed chitin [(10)] mentioned that chitosan known as less realized in organisms than the former chitin and usually found as a component in the cell walls of fungal group called ; Zygomycetes which including: *Absidia, Gongronella, Rhizopus and Mucor*.

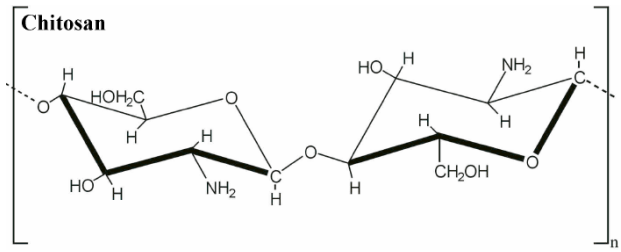

Figure 1: Chemical structure chitosan

Many distinctive properties have shown in chitosan, for instance, biocompatibility, biodegradation, low-toxicity and biological activity [(11)]. Chitosan involves in many application fields such as in cosmetics like hand, body creams and shampoos, drug industries as a drug carrier,. In addition, it is used as ingredient of toothpastes, immobilizer for enzymes and cells, matter for manufacturing of pharma industrial eye products such as contact lenses and bandages, etc. Chitosan also enter in diet related claims which include drinking water purification, dietary additives, meat processing purposes, wine purification, etc. Chitosan used in separation of nucleic acid during thin layer chromatography technique [(9)]. For all above, this research aimed to explain the biological extraction of chitosan from *Aspergillus flavus* and elucidation of its antimicrobial activity.

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Fungal Growth and chitosan production

According to data viewed in [10], YPG medium yielded a high biomass of *Aspergillus flavus* (Figure-2), this cell weight is more than that obtained after fungal culturing in other two traditional media (BG and TVB). The displayed data was clearly revealed that there is an exponential association between the fungal biomass and chitosan production. Andrade *et al.* [11] discussed the previous results through the presence of high nitrogen content within the chemical formula in YPG medium as compared with the two tested media; because that nitrogen is play a major role during chitosan biosynthesis.

The increasing in the incubation period led to increase in yielded mycelial biomass and chitosan until the 60th or the 72nd hour of culturing whereby the fungi is in the late exponential phase of growth and the highest amount of chitosan is extracted and after this time chitosan extraction exhibited a gradual decrease although fungal biomass continued to increase [12]. As alternative source to chitosan production , chitosan has produced from endophytic fungi, this source of chitosan is more appropriate than shells of crustaceans ,regarding its recovery and can be scaled up for large scale production [13].

Lactic acid is yielded from the breakdown of glucose leads to form lactic acid formation and increasing the acidity, which enhances the ensilation that encourage the growth of unwanted microorganisms. Lactic acid may react with the $CaCO_3$ ingredient in the fraction of chitin, this reaction yielded a calcium lactate, which precipitates and can be separated via washing. As a result of demineralization process, organic salts formed, theses salts could be act as anti-freezing or preservative factors [14]. The combination of two processes includes Lactic acid fermentation and chemical treatments have been regarded as a substitute technique for chemical extraction of chitin by which decreasing the amount of acid and alkali needed [15]. It was considered as a pretreatment of shrimp waste as followed by de-mineralization and de-proteinization using small concentrations of hydrogen chloride (0.5 M) and sodium hydroxide (0.4 M) [16].

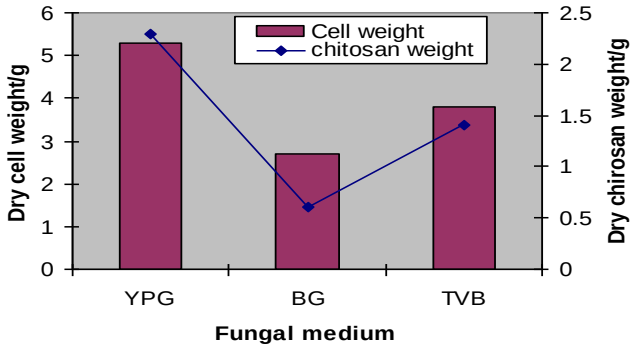

Figure-2: report of chitosan and biomass production from *A. flavus* cultivate on different media.

Overview on antimicrobial properties of fungal derived chitosan

A potential antibacterial effect have been authenticated using chitosan extracted from fungal source, the data displayed in figure (3) adopted by [17] as shown in this table, chitosan exhibited inhibition percentage ranged between 50-47 % toward experimented gram-positive bacteria

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genera, this effect has more than which detected toward experimented gram-negative bacteria genera. On the other hand, as no inhibition was noticed by chitosan against tested *Enterococcus faecalis*.

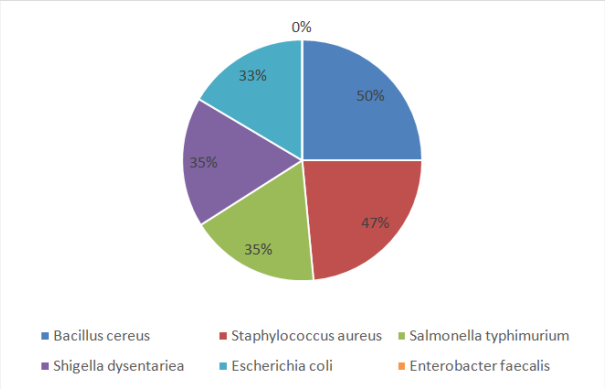

Figure 3: inhibition percentages of the fungal derived chitosan against examined bacterial isolates adopted by [17]

The former study was also tested chitosan against different fungal isolates including four plant pathogenic molds as well as one yeast. The strongest inhibition by chitosan was calculated against *Penicillium* sp., furthermore, less inhibition efficacy was caused by commercial antifungal Nystatin toward the same mold. On the other hand, chitosan was affected the growth of other tested fungal isolates at different ranges as shown in (figure 4).

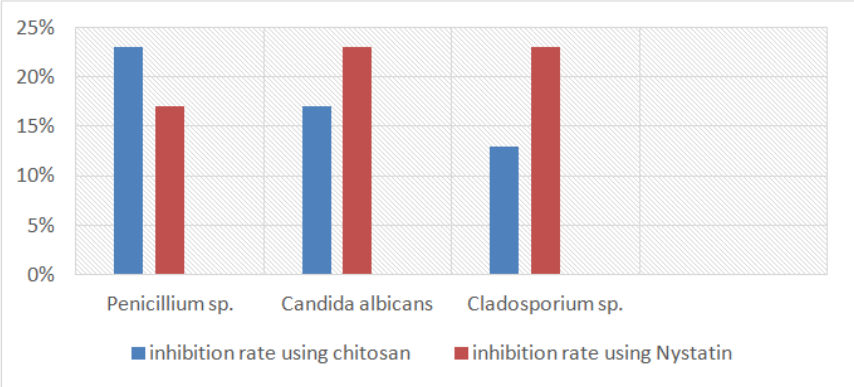

Figure 4: the data resulted from using chitosan and nystatin (100 µg/mL PDA) as antifungal agent, adopted by [17]

Two main mechanisms reported in [18] and [19], by which the ability of chitosan to inhibit the microbial growth have explained, one of them suggested that chitosan characterized with poly cationic nature. Thus, it can interact with anionic groups on the microbial cell surface, this interaction block the transmission of essential solutes through microbial cellular membrane. The second explanation included the ability of chitosan to facilitate permeability of microbial membrane when it is protonated under acidic conditions.

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Biofilm inhibition by the extracted chitosan

Seven bacterial and fungal genera were assayed for detection biofilm production by [20] and the results revealed that these strains exhibited different abilities to form biofilm. The highest biofilm level was formed by *Pseudomonas aeruginosa and Klebsiella pneumoniae,* followed by *Enterococcus faecalis* thus those isolates were considered as potent biofilm producers. While, *Escherichia coli*, *Kingella kingae* and *Staphylococcus aureus* produced lower level of biofilm so that these isolates were considered as a poor biofilm producers. In contrast, *Candida albicans* produced middle level of biofilm as shown in table (1).

The pathogenicity of bacteria improved by biofilm formation, this evidence was explained in [21], by the colonization ability by bacteria on polymeric surface and by bacterial formation of cellular clusters with multi layers embedded in extra cellular material biofilm extracellular matrix is mainly consists of Exopolysaccharide (EPS), each biofilm-forming bacterial individual produces a unique EPS component. This component acts as protection agent from attack by the human innate immune system [22].

The resulted data in [20], revealed that the extracted chitosan in microtiter plate method showed different ratios in inhibition the biofilm formed by the tested bacteria. The higher inhibition rate by chitosan against *Pseudomonas aeruginosa* with 62% followed by *Klebsiella pneumoniae* with 59% and *Enterococcus faecalis* with 52%. In contrast, the lower inhibition rates by chitosan against *Candida albicans* and *Staphylococcus aureus* with 21 and 18%, respectively (table 3). According to the above results, *Aspergillus flavus* yielded a chitosan with ability to inhibit biofilm formed by pathogens. The previous study mentioned three modes of action related to anti-biofilm activity of polysaccharides, the first one is that act as surfactant molecules that modify the physical properties of bacteria and abiotic surfaces. And the second one is they performed as signaling molecules that modified the expression of gene concerns with biofilm formation in bacteria.

Table 1: Biofilm inhibition rates of the extracted chitosan against bacterial and fungal isolates

Bacteria	Optical density		Biofilm inhibition rate%
	Before addition of chitosan	**After addition of chitosan**	
Staphylococcus aureus	0.20	0.164	18
Pseudomonas aeruginosa	0.52	0.197	62
Escherichia coli	0.29	0.194	33
Kingella kingae	0.25	0.162	35
Enterococcus faecalis	0.43	0.206	52
Klebsiella pneumonia	0.48	0.196	59
Candida albicans	0.12	0.094	21

Since the chitosan leads to formation of an impermeable stratum around the microbial cell, this may block the passage of elemental solutes. It has been established by electron microscopy that

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the site of action in gram-negative bacteria is the outer membrane [23]. This indicated that chitosan might have the antibacterial property which could be used in pharmacological research.

The synergistic effect of chitosan and antibiotics

The combination between chitosan and different antibiotics resulted to enhancement of antimicrobial effect more than using each one alone, this evidence has shown by the study of [17], depending of previous study, the mixing between chitosan and two commercial antibiotics (ceftazidime and ceftazidime) resulted to recording an enlarge zone o inhibition on tested bacteria with less MIC value as compared with those recorded after application antibiotics and chiton only as displayed in table 2, figure 5.

Table 2: MIC values & inhibition zones after application of two commercial antibiotics for reference strains with and without chitosan

Antibiotic	Tested bacteria	Value of MIC after application of antibiotic alone (µg /ml)	Diameter of inhibition zone (mm)	Value of MIC after application of antibiotic + chitosan (µg /ml)	Diameter of inhibition zone (mm)
Ceftazidime	*P aeruginosa*	1024	21	128	28
	S aureus	512	18	64	24
Samacycline	*P aeruginosa*	1024	20	64	25
	S aureus	2500	14	512	17

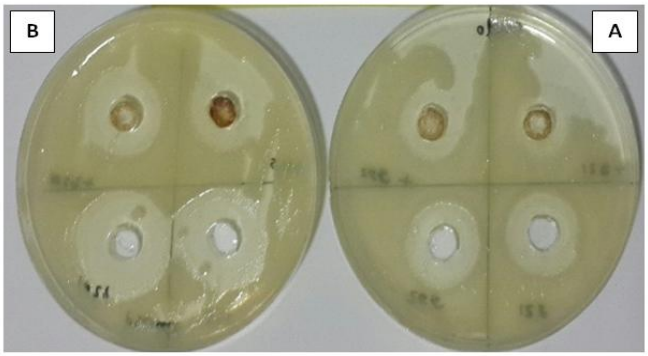

Figure-5: Zones of inhibition after application of ceftazidime on A): tested *P. aeruginosa,* B): Zones of inhibition after application of ceftazidime and chitosan on tested *P. aeruginosa*

Conclusion

Current review give an approach to use chitosan that extracted from biological source as considerable tool in medical fields such as antibacterial, antifungal and antibiofilm factor besides to enhancement of antibiotic activity. Especially chitosan highly affected on *P. aeruginosa*, that demonstrated considerable rate of nosocomial infection for patients especially those of prolonged persistence in hospital, and the affinity of nosocomial pathogens to gain antibiotic resistant new traits poses a great problem in their management.

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Conflict

No conflict of interest

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References

1. Hedayati, M.T., Pasqualotto, A.C., Warn, P.A., Bowyer, P., Denning, D.W. (2007). *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. *Microbiol*, 153, 1677–1692.
2. Rodrigues, P., Soares, Z., Kozakiewicz, R., Paterson, R.M., Lima N., Venâncio A. (2007). Identification and characterization of *Aspergillus flavus* and aflatoxins. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology. A. Mendez-Vilas (Ed.)*, pp, 527-534.
3. Pitt, J.I., Samson, R.A., Frisvad, J.C. (2000). Integration of Modern Taxonomic Methods for *Penicillium* and *Aspergillus* Classification, Hardwood Academic Publishers, Reading, UK, pp, 9-50.
4. Abdel-Wahhab, M.A., Hassan, N.S., El-Kady, A.A., Khadrawy, Y.A., et al. (2010) Red ginseng extract protects against aflatoxin B_1 and fumonisins-induced hepatic pre-cancerous lesions in rats. *Food Chemi Toxicol*, 48(2), 733–742.
5. Guidone, A., Zotta, T., Ross, R.P. (2014). Functional properties of *Lactobacillus plantarum* strains: A multivariate screening study. *LWT-Food Science and Technology*, 56(1), 69–76.
6. Ahren, I.L., Jie. X., Önning, G., Olsson, C., Ahrné, S., Molin, G. (2015). Antihypertensive activity of blueberries fermented by *Lactobacillus plantarum* DSM 15313 and effects on the gut microbiota in healthy rats. *Clinical Nutrition*, 34(4), 719-726.
7. Sivaraj, S., Murugesan, P., Muthuvelu, S., Purusothaman, S., Silambarasan, A. (2012). Comparative Study Of *Pseudomonas Aeruginosa* Isolate Recovered From Clinical And Environmental Samples Against Antibiotics. *International Journal of Pharmacy and Pharmaceutical Sciences*, 4(3), 103-107.
8. Haleem, H., Tarrad, K.J., Banyan, I.A. (2011). Isolation of *Pseudomonas aeruginosa* from Clinical Cases and Environmental Samples, and Analysis of its Antibiotic Resistant Spectrum at Hilla Teaching Hospital. *Medical Journal of Babylon*, 2011, 8(4), 618-624.
9. Zvezdova, D. (2010). Synthesis and characterization of chitosan from marine sources in Black Sea. *Научни Трудове На Русенския Университет*, 49(9), 65-69.
10. Jaworska, M.M., Konieczna, Z. (2001). The Influence of Supplemental Components In Nutrient Medium on Chitosan Formation by the Fungus *Absidia orchidis*. *Applied Microbiology and Biotechnology*, 56, 220-224.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 2050 - 2057
Received 05 March 2021; Accepted 01 April 2021.

11. Andrade, V.S., Neto, B.B., Souza, W., Campos-Takaki, G.M. (200). A factorial design analysis of chitin production by *Cunnighamella elegans*. *Canadian Journal Microbiology*, 46, 1042-1045.
12. Kucera, J. (2004). Fungal Mycelium–The Source of Chitosan for Chromatography, J. Chromatogr. B. Analyt. *Technology and Biomedicine and. Life Sciences*, 808, 69–73.
13. George, T.S., Guru, K.S., Vasanthi, N.S., Kannan, K.P. (2011). Extraction, purification and characterization of chitosan from endophytic fungi isolated from medicinal plants. *World Journal of Science and Technology*, 1(4), 43-48.
14. Jung, W.J., Kuk, J.H., Kim, K.Y., Park, R.D. (2005). Demineralization of red crab shell waste by lactic acid fermentation. *Applied Microbiology and Biotechnology*, 67, 851–854.
15. Yen, M.T., Yang, J.H., Mau, J.L. (2009). Physicochemical characterization of chitin and chitosan from crab shells. *Carbohydrates and Polymers*, 75, 15–21.
16. Arbia, W., Arbia, L., Adour, L., Amrane, A. (2013). Chitin Extraction from Crustacean Shells Using Biological Methods – A Review. *Food Technology and Biotechnology*, 51(1), 12–25.
17. Muslim, S.N., Khazaa, S.S., Hussein, N.H., Taha, B.M., Mohammed, N.J. (2016). Improving of antibacterial activity for antibiotics by extracted chitosan from Aspergillus flavus. *International Advances in Science and Engineering and Technology*, 4(4), 1-3. 66th International Conference on Environment and Natural Science (ICENS) Dubai, UAE.
18. Helander, I., Nurmiaho-Lassila, E., Ahvenainen, R., Rhoades, J., Roller, S. (2001). Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. *International Journal of Food and Microbiology*, 71, 235-244.
19. Kumar, M.N. (200). A review of chitin and chitosan applications. *Reactive and Functional Polymer*, 46, 1-27.
20. Muslim, S.N., Khazaal, S.S., AL_Kadmy, I.M.S., Mohammed. M.T., Mohammed Ali, A.N. (2016). Extraction of Chitosan from Trichoderma reesei by Biological Methods and Using It as Antibiofilm Formation Agent. August 2016; Conference: 66th International Conference on Environment and Natural Science.
21. Arbia, W., Arbia, L., Adour, L., Amrane, A. (2013). Chitin Extraction from Crustacean Shells Using Biological Methods – A Review. *Food Technology and Biotechnology*, 51(1), 12–25.
22. Watnick, P., Kolter, R. (2000). Biofilm, city of microbes. *Journal of Bacteriology*, 182, 2675-2679.
23. Rendueles, O., Kaplan, J.B., Ghigo, J.G. (2012). Antibiofilm polysaccharides. Environmental Microbiology. Society for Applied Microbiology and Blackwell Publishing Ltd.