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Biosafety of Handling of Some Pathogenic Bacteria

Sahira Nsayef Muslim; Alaa Naseer Mohammed Ali; Rana Sabah Jawad; Israa M.S. AL_Kadmy; Ahmed Sahi Dwaish; Nadheema Hammood Hussein

Department of Biology, College of Science, AL-Mustansiryia University

Emails: s2008mk@yahoo.com, alaan29775@gmail.com, dr.ranajawad79@yahoo.com, israaalkadmy@gmail.com, ahmedsahi33@gmail.com, nadheema_a@yahoo.com

Abstract:

Yeast extract peptone glucose medium (YPG) was the best medium for higher fungal biomass production and chitosan production. The chitosan was extracted by using a two-steps_biological treatment process: demineralization by *lactobacillus plantarum* and deproteinization by *Pseudomonas aeruginosa*. Chitosan exhibited a strong antibacterial effect on the gram-positive bacteria than gram-negative since the inhibition rates were 47 and 50% against *Staphylococcus aureus* and *Bacillus cereus*, respectively, also the growth inhibition effect by chitosan against fungi was not strong as in the case of bacteria. So that *Pseudomonas aeruginosa,* that has frequently multi drug resistant and contribution to the high morbidity and mortality of patients, may be used as useful tool in biological treatment for deproteinization and extraction of chitosan that has a promising applications in the medical field.

Keywords: biosafety, chitosan, pathogenic bacteria

Introduction

Aspergillus is a member of the phylum ascomycota. *Aspergillus flavus* is a saprotrophic and pathogenic fungus that attracted worldwide attention for its industrial use and toxigenic potential. *Aspergillus flavus* has an ability to survive in difficult environments allows it to easily overcomes on other organisms for substrates in the soil or in the plant(١). Macromorphological features which are considered include conidial and mycelial colour, colony diameter, colony reverse colour, production of exudates and soluble pigments, presence of sclerotia and cleistothecia. Micromorphology characterization is mainly dependent on seriation, shape and size of

vesicle, conidia and stipe morphology and morphology of cleistothecia and ascospores (2,3). Aflatoxins are produced by toxigenic strains of *Aspergillus flavus*. Such mycotoxins are toxic metabolites, difuran coumarin derivates, responsible for carcinogenic, mutagenic and teratogenic effects and represent a risk to human and animal health(4).

Pseudomonas aeruginosa belongs to a vast genus of obligate aerobic, non-fermenting, saprophytic, Gram-negative bacilli widespread in natural environment such as soil, plant surfaces, fresh vegetables, sewage, waste water, sink, moist environment, and river water. Its profound ability to survive on inert materials, minimal nutritional requirement "growing in distilled water", which is evidence of its minimal nutritional needs[2], tolerance to a wide variety of physical conditions and its relative resistance to several unrelated antimicrobial agents and antiseptics, contributes enormously to its ecological success and its role as an effective opportunistic pathogen(5).

Pseudomonas aeruginosa exhibits considerable rate of nosocomial infection in prolonged admission of patients in hospital and tendency of nosocomial pathogenic to acquire new antibiotic resistance traits poses a great problem in their treatment and control. Clinical isolates were highly resistance to the antibiotic when compared to the environmental isolates; this may be due to the constant exposure to the antibiotic in the hospitals environment(6).

Chitosan is chemically defined as a copolymer of α-(1,4) glucosamine (C6H11O4N)n (7). Chitosan is essentially composed of --1, 4 D-glucosamine (GlcNAc) linked to N-acetyl-D-glucosamine residues, derived from de-Nacetylation of chitin. chitosan is less commonly found in living organisms than chitin and can be found in the cell walls of certain groups of fungi, particularly Zygomycetes fungi including *Absidia*, *Gongronella*, *Mucor* and *Rhizopus*(8). chitosan shows many unique properties, such as biocompatibility, biodegradation, biological activity and low-toxicity(9). Chitosan has recently many fields of applications such as in cosmetics, pharmaceuticals, food additives and agriculture. It is used as a component of toothpastes, hand and body creams, shampoos, body creams, lowering of serum cholesterol, cell and enzyme immobilizer, as a drug carrier, material for production of contact lenses, or eye bandages, etc. Chitosan have the important food related applications which include purification of drinking water, recovering protein from fish wash water and meat processing plants, using in animal feeds, clarifying wine etc. Chitosan is used in the thin layer chromatography for the separation of nucleic acid(7).The aim of this research to extract chitosan from *Aspergillus flavus* by using biological methods and detection of antimicrobial activity.

Materials and methods

Microorganisms

1- Aspergillus flavus

Isolation of *Aspergillus flavus* isolated from stem and leaf segments taken from medicinal plants and identified according to Nelson (10).

2-Bacterial and fungal isolates

Bacterial isolates such as *Lactobacillus plantarum*, *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus*, *Escherichia coli, Shigella dysenteriae* , *Enterococcus faecalis* and *Salmonella typhimurium*. Also the fungal isolates such as *Aspergillus niger, Aspergillus terreus* , *Cladosporium* sp. , *Penicillium* sp. and *Candida albicans* were collected from biology department laboratories.

Fungus and culture medium

Three growth media that was described by (11) including: YPG (yeast extract 3gm/l, peptone 10gm/l, glucose 10gm/l and 1 ml/l trace elements), BG (nutrient broth 8gm/l; yeast extract 0.1gm/l; glucose 5gm/l; KCl 0.1gm/l; $MgSO_4.7H_2O$ 0.25gm/l; $MnCl_2.4H_2O$ 0.002gm/l and $FeSO_4.7H_2O$ 0.00029 gm/l), and TVB (glucose 20 gm/l; $(NH)_2SO_4$ 1.4 gm/l; KH_2PO_4 2 gm/l; $CaCl_2$ 0.3gm/l; $MgSO_4$.7H2O 0.3 gm/l; molybdic acid 85% 0.01 gm/l and 1ml/l trace elements (per 500 mL: $FeSO_4$.7H2O [5 gm], $ZnCl_2$ [1.66 gm], $CoCl_2.6H_2O$ [2 gm], $MnSO_4.7H_2O$ [1.96 gm] and hydrochloric acid 12 M [10 mL]) were prepared.. Inoculum was prepared aseptically by adding sterilized distilled water onto the fungal mycelia grown on potato dextrose agar plates (PDA) and then carefully scraping the spores from the mycelia using a stab wire. Spore suspension was filtered into a sterilized flask and spore count was performed using a haemacytometer. The spore suspension was prepared as $1x10^7$ spores/ml. 5 mL of spore suspension was inoculated into each medium. The fungus was grown as submerged batch cultures at 30 °C and with agitation of 150 rpm for 96 hours. After cultivation, fungal mycelia were recovered by filtration (no.1; Whatman), washed twice with distilled water until a clear filtrate was obtained and then dried at 65 ºC to a constant weight.

Extraction of Chitosan

The chitosan was extracted by modification the method that described by(12) as follow:

1-Preparation of inoculum

Lactobacillus plantarum was transferred in 5 mL MRS broth and incubated at 30^oC for 24 hrs. Then 2 mL of starter culture was transferred in 100 mL sterile MRS broth and incubated at 30°C for a further 24 hrs.

2-Fermentation

Ten grams of dried fungal mycelium and grind was added to 10% of glucose as carbon source followed by 10% culture inoculum. Then incubated for 7 days at 30°C, in the presence of 5%CO_2. The fermentative culture medium was centrifuged at 3000 rpm for 5 min. The recovered solids were washed thoroughly several times using deionized-distilled water followed by drying hot air oven.

3-Calcium carbonate separation

Demineralization

Demineralization is carried out by lactic acid producing bacteria through the conversion of an added carbon source.

Protein separation

Separation of protein is carried out by proteases secreted into the fermentation medium. The deproteinization can be made by y proteolytic bacteria. 10%(v/v) of 24-hour culture of *Pseudomonas aeruginosa* in brain heart infusion medium was added to a sterilized mineral
solution (0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$) containing 3% (w/v) dried decalcified fungal mycelium. Then the mixtures was filtered and the residue was washed with distilled water until neutralization, then dried in a hot air oven at 40°C until constant dry weight and stored
until further analysis.

4-Verification of the produced chitosan

Quality of the produced chitosan was checked by a solubility test with 1% Acetic Acid. Chitosan dissolves completely in 1% Acetic Acid. For the estimation of chitosan produced the sample was put inside a clean beaker and 10 to 20 mL of 1% acetic acid was added to it. The solution was kept in BOD shaker for 30 to 40 minutes. Then the sample was taken out and weighed, carefully.

Antimicrobial activity of extracted chitosan

1-Antibacterial activity

The Agar cup diffusion method was used for detecting the antibacterial activity for chitosan. In this method, nutrient agar was prepared, poured into culture plates and left overnight for solidification. The six bacterial species *Staphylococcus aureus, Bacillus cereus*, *Escherichia coli, Shigella dysenteriae*, *Enterococcus faecalis* and *Salmonella typhimurium* were taken as test strains and inoculated in liquid culture and incubated for 24hrs at 35°C. These were to be used as the source for the antibacterial assay. The bacterial strains were spreaded over the media. Two wells of 6mm diameter were dug into each plate and 12 µl of chitosan dissolved in 1% acetic acid were poured in each well. The plates were then incubated at 35°C for 24 hours.. If there is any antimicrobial activity in the extract then a zone of clearance will be formed around the corresponding well, which occurs due to diffusion of the extract through the agar. All the results were compared with the standard antibacterial antibiotic ampicillin (20 µg/disc)(13).

2-Antifungal activity

The antifungal activity of chitosan was investigated by normal inoculation method. Different fungal isolates such as *Aspergillus niger, Aspergillus terreus* , *Cladosporium* sp. , *Penicillium* sp. and *Candida albicans*. Potato dextrose agar medium was prepared and the selected fungi were inoculated and left in normal condition for the growth of the fungi. This was to be used as the mother culture acting as the source for of fungal strain for corresponding applications. Five sterilized culture plates were taken, among them five were taken for the chitosan and one was taken as control. In the first five culture plates equal amount of media with 3 mL of chitosan was poured along with 25 ml of media. The control plate was composed with media and no chitosan and left for solidification of media. The culture plates were

then inoculated with fungal strains from the mother culture and incubated for 5 days at 28°C. The plates were then checked for presence of anti-fungal activity. The average of two measurements was taken as mycelial colony diameter of the fungus in mm. All the antifungal results were compared with the standard antifungal antibiotic Nystatin (100 μg/mL PDA). Lactose was used as negative control(13). The percentage inhibition of radial mycelial growth of the test fungus was calculated as follows: % Inhibition = (C-T/C) × 100

Results and Discussion

Fungal Growth and chitosan production

Aspergillus flavus exhibited higher growth on YPG medium (Figure-1), in comparison with the other media(BG and TVB). There was relationship between the biomass production and chitosan production for *Aspergillus flavus*. high biomass yields result in high chitosan production (14). Andrade *et al*. (15) reported that high nitrogen content leads to increase in the synthesized enzymes that involved in chitodan biosynthesis. So that the highest yield of chitosan in YPG due to high nitrogen content as comparison with other media. The increasing in the incubation period led to increase in mycelial biomass and chitosan production until 60 to 72 hours 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(16). The production of chitosan from endophytic fungi can be consedered as alternative source to chitosan production from the shells of crustaceans and the recovery of produced chitosan from fungi was appropriated and can be scaled up for large scale production(17).

Lactic acid is formed from breakdown of glucose to formlactic acid and decreasing the pH value, which improves the ensilation that encourage the growth of contaminated microorganisms. Lactic acid reacts with the calcium carbonate component in the chitin fraction and leading to the formation of calcium lactate, which precipitates and can be removed by washing. The resulting organic salts from the demineralization process could be used as de- and anti-icing agents and/or preservatives(18). The combination of Lactic acid fermentation with chemical treatments has been considered as an alternative method for chemical extraction of chitin decreasing the amount of alkali and acid required(19). It was considered as a pretreatment of shrimp waste followed by demineralization and deproteinization using low concentrations of HCl (0.5 M) and NaOH (0.4 M)(20).

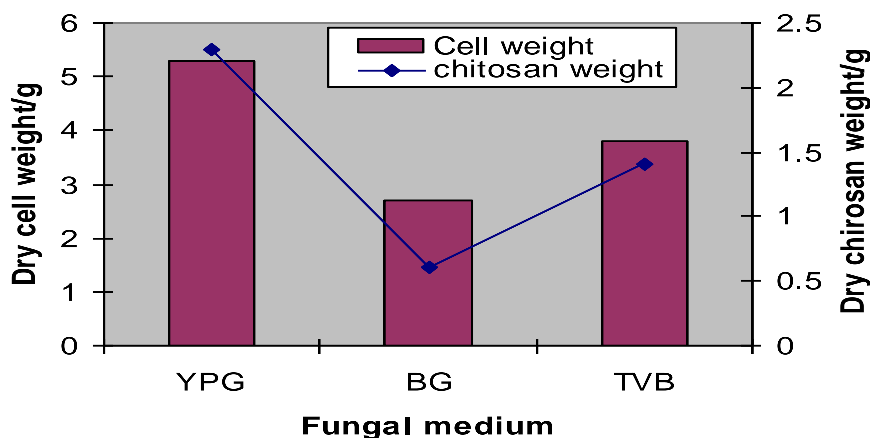

Figure.1: Profile of biomass and chitosan production from *Aspergillus flavus* grown on different media.

Antimicrobial activity of extracted chitosan

In vitro antibacterial activity for chitosan was assayed against six pathogenic bacteria. The results that revealed in Table (1) exhibited a strong antibacterial effect on the gram-positive bacteria, as *Bacillus cereus* and *Staphylococcus aureus*. The diameter of zone inhibition by the addition of chitosan was significant effective for *Bacillus cereus* and *Staphylococcus aureus* to be 9 and 10 mm, respectively . In contrast, the chitosan has inhibited less effect for *Escherichia coli* with an inhibition rate 33%. On the other hand, chitosan did not any effect against only *Enterococcus faecalis*.

Table.1: Antibacterial activity of the extracted chitosan against gram-negative and positive bacteria

Bacteria	Diameter of inhibition zone for ampicillin(mm)	Diameter of inhibition zone forchitosan(mm)	Inhibition rate%
Staphylococcus aureus	19	10	47
Bacillus cereus	18	9	50
Escherichia coli	18	12	33
Shigella dysenteriae	17	11	35
Enterococcus faecalis	20	0	0
Salmonella typhimurium	20	13	35

In vitro antifungal Antimicrobial activity of extracted chitosan was determined against five phytopathogenic fungi with antifungal antibiotic Nystatin as positive control. Generally the growth inhibition effect by chitosan against fungi was not strong as in the case of bacteria. Chitosan showed significant inhibition (23%) of mycelial growth against *Penicillium* sp. among all tested fungi (Table 2). On the other hand, the growth of *Cladosporium* sp. and *Candida albicans* (13 and 17%,

respectively) was moderately inhibited by the chitosan. However, the growth of *Aspergillus niger* and *Aspergillus terreus* were never inhibited by chitosan, though the growth of all the five fungi was totally inhibited by antifungal antibiotic Nystatin (100 µg/mL PDA).

Table-2: Antifungal activity of the extracted chitosan against phytopathogenic fungi

Fungus	Diameter of inhibition zone for Nystatin (mm)	Diameter of inhibition zone for chitosan(mm)	Inhibition rate%
Aspergillus niger	16	0	0
Aspergillus terreus	17	0	0
***Cladosporium* sp.**	23	20	13
***Penicillium* sp.**	17	13	23
Candida albicans	23	19	17

In the recent years, there was a great attention forward the antimicrobial activity of chitin, chitosan, and their derivatives against different groups of microorganisms, such as bacteria, yeast, and fungi. There were two main mechanisms suggested as the cause of the inhibition of microbial cells by chitosan. The first included the interaction with anionic groups on the cell surface, due to its polycationic nature, causes the formation of an impermeable layer around the cell, which prevents the transport of essential solutes. The second involved the permeabilizing effect that observed at slightly acidic a condition in which chitosan is protonated, but this permeabilizing effect of chitosan is reversible(21). The antifungal and antibacterial activities of chitosan can be employed in production of biofertilizers and biopesticides of economical benefits (22).

Conclusions

Our results indicate that *P. aeruginosa,* that exhibited considerable rate of nosocomial infection in prolonged admission of patients in hospital and tendency of nosocomial pathogenic to acquire new antibiotic resistance traits poses a great problem in their treatment and control, may be used as useful tool in biological treatment for deproteinization and extraction of chitosan that has a promising applications in the medical field such as antibacterial and antifungal activity..

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السلامة الأحيائية في التعامل مع بعض انواع البكتيريا المسببة للأمراض

ساهرة نصيف مسلم؛ علاء نصير محمد علي؛ رنا صباح جواد؛ إسراء الكاظمي؛ أحمد ساهي دويش؛

نظيمة حمود حسين

قسم علوم الحياة، كلية العلوم، الجامعة المستنصرية

البريد الإلكتروني: s2008mk@yahoo.com :، alaan29775@gmail.com،
dr.ranajawad79@yahoo.com، israaalkadmy@gmail.com،
ahmedsahi33@gmail.com، nadheema_a@yahoo.com

الخلاصة:

خلاصة الخميرة ببتون الجلوكوز المتوسطة (YPG) هي أفضل وسيط لزيادة إنتاج الكتلة الحيوية الفطرية وإنتاج الشيتوزان. تم استخلاص الشيتوزان باستخدام اثنين من خطوات عملية المعالجة البيولوجية: (demineralization by lactobacillus plantarum and deproteinization by Pseudomonas aeruginosa). اظهر الشيتوزان تأثير مضاد للجراثيم قوي على البكتيريا إيجابية الجرام من غرام سلبية حيث أن معدلات تثبيط ٤٧ و ٥٠٪ ضد المكورات العنقودية الذهبية والشمعية العصية على التوالي، وكذلك تأثير الشيتوزان على تثبيط النمو ضد الفطريات لم يكن قويا كما في حالة من البكتيريا.

الكلمات المفتاحية: السلامة الأحيائية، الشيتوزان، البكتيريا المسببة للأمراض.

References

1- Hedayati, M. T. ; Pasqualotto, A. C.; Warn, P. A.;Bowyer, P. and Denning, D. W.(2007). *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. Microbiology, 153, 1677–1692.

2-Rodrigues, P.; Soares, Z.; Kozakiewicz, R.; Paterson, R.M.; Lima, N.and 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. and Frisvad, J.C.(2000).Integration of Modern Taxonomic Methods for *Penicillium* and *Aspergillus* Classification, Hardwood Academic Publishers, Reading, UK, 9-50.

4-Abdel-Wahhab, M. A.; Hassan, N. S.; El-Kady, A.A.; Khadrawy, Y.A.; El-Nekeety, A.A.; Mohamed, S.R.;Sharaf, H.A. and Mannaa, F.A. (2010).Red ginseng extract protects against aflatoxin B_1 and fumonisins-induced hepatic pre-cancerous lesions in rats. Food and Chemical Toxicology,48(2):733–742.

5- Sivaraj, S.; Murugesan, P.; Muthuvelu,S.; Purusothaman,S. and 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.

6- Haleem,H.; Tarrad, K.J.and 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,8(4):618-624.

7-Zvezdova,D.(2010).Synthesis and characterization of chitosan from marine sources in Black Sea. Научни Трудове На Русенския Университет ,49(9):65-69.

8-Nwe, N.;Furuike,T. and Tamura,H.().Production of Fungal Chitosan by Enzymatic Method and Applications in Plant Tissue Culture and Tissue Engineering: 11 Years of Our Progress, Present Situation and Future Prospects. In: Biopolymers,(Magdy Elnashar),pp:135-162.

9-Sabnis, S and Block, L.H.(2000). Chitosan as an enabling excipient for drug delivery systems. I. Molecular modifications. Int J Biol Macromol.;27(3):181-6.

10-Nelson, P.E.; Toussoun, T.A.; Marasas, W.F.O.(1983). *Fusarium* species. An Illustrated Manual for Identification. The Pennsylvania State University Press, 190p.

11- Paul, D.C.; Nadarajah, K. and Abdul Kader, A.J.(2004). Production and quality of chitosan extracted from local fungal isolates. The 4th Annual Seminar of National Science Fellowship.pp:110-115.

12- Pal,J.; Verma,H.O.; Munka,V.K.; Maurya,S.K.; Roy,D. and Kumar, J.(2014). Biological Method of Chitin Extraction from Shrimp Waste an Eco-friendly low Cost Technology and its Advanced Application. International Journal of Fisheries and Aquatic Studies, 1(6): 104-107.

13-Kawsar,S.M.A.; Mamun,M.A.; Rahman, M.S.; Yasumitsu,H. and Ozeki,Y.(2010). *In Vitro* Antibacterial and Antifungal Effects of a 30 kDa D-Galactoside-Specific Lectin from the Demosponge, *Halichondria okadai*. International Journal of Biological and Life Sciences 6(1):31-37.

14-Jaworska, M.M. and 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.

15-Andrade, V. S., Neto, B.B., Souza, W and Campos-Takaki, G.M.(2000). A factorial design analysis of chitin production by *Cunnighamella elegans*. Canadian Journalof Microbiology 46: 1042-1045

16-Kucera, J. (2004). Fungal Mycelium–The Source of Chitosan for Chromatography, J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci., Vol. 808, pp. 69–73.

17- George,T.S.; Guru, K.S.; Vasanthi,N.S. and 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

18- Jung, W.J.; Kuk, J.H. ; Kim, K.Y. and Park, R.D.(2005). Demineralization of red crab shell waste by lactic acid fermentation, Appl. Microbiol. Biotechnol. 67:851–854.

19- Yen, M.T. ; Yang, J.H. and Mau, J.L.(2009). Physicochemical characterization of chitin and chitosan from crab shells, Carbohydr. Polym. 75 (2009) 15–21.
20-Arbia,W.; Arbia,L.; Adour,L. and Amrane,A.(2013).Chitin Extraction from Crustacean Shells Using Biological Methods – A Review. Food Technol. Biotechnol. 51 (1) 12–25.
21-Helander I, Nurmiaho-Lassila E, Ahvenainen R, Rhoades J, Roller S. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. Int. J. Food Microbiol., 2001: 71: 235-244.
22- Kumar, M.N.(2000). A review of chitin and chitosan applications. React Funct Polym; 46: 1-27.