Antimicrobial activity of chitosan isolated from fungus *Rhizopusoryzae*

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KawtharTumaKhalaf*

***University Of Basarah , College of Pharmacy**
Email: emad_yousif2000@yahoo.com

Abstract

In this study chitosan was isolated from the fungus *Rhizopusorayzae* by using yeast peptone glucose broth medium (YPG) at the late stationary phase. Chitosan production from mycelium reached to 15 mg/g as dry weight . 25% was the N-acetylation of chitosan while 96% for the D-glucosamine. Chitosan showed antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* and reach to 20 , 15 and n 15 mm as inhibition zone respectively .

Key words : Chitosan , *Rhizopusoryzae* , Antimicrobial activity

Microbiology **Classification QD 241-441**

Introduction

Chitosan is a polycationic polymer (figure -1) consisting of β - 1,4 - linked 2 - acetamido - D -glucose and β - 1,4 - linked 2 - amino- D - glucose units (1). This molecule is rarely found in living organism and is abundant in the cell wall of certain fungi such as zygomycetes ,Fungal mycelium wastesfrom biotechnological plants can become free and rich alternative sources of chitin-chitosan materials, beside the traditional industrial source-shellfish waste materials . As well as chitosan can have unique properties compared with those derived from crustacean (2).Chitosan has great potential in agriculture, medicine, biotechnology and pharmaceutical industries (3,4) .

The development of applications for chitosan in drug delivery has expanded rapidly, because chitosan consist of functional groups such as free amine and hydroxyl groups which used in various chemical modification such as polymer electrolyte membrane for the separation of metal ions and ultrafiltaration (5,6,7). There are many application of chitosan as antibacterial agents , gene delivery rectors for protein release and drugs , it has been proved to prevent infection in wounds and made on wound healing process by enhancing the growth of skin cells (8) . In the last year many advance have been suggest in fermentation technology that provide an alternative source of chitosan for fungal cultures. Fungal cell walls and septa of *Ascomycetes, Zygomycetes, Basidiomycetes* and *Deuteromycetes* contain mainly chitin, which is responsible for maintaining their shape, strength and integrity of cell structure (9) .

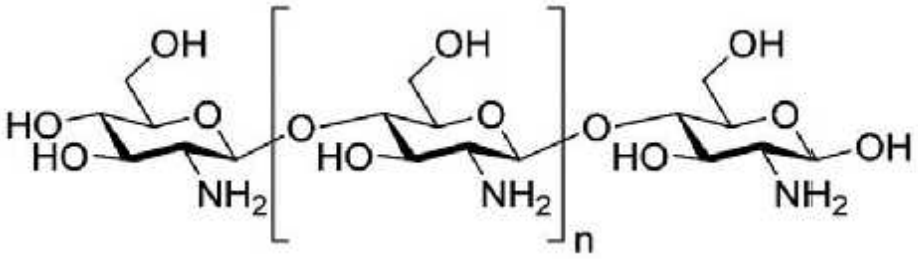

Figure (1): Chemical structure of Chitosan

Material and Methods

1- Fungal cultivation

The fungus *Rhizopusoryzae* wasisolated from the air on Potato dextrose agar (PDA) medium at 25 ±2 c°. The submerged culture was carried out in 500 ml Erlenmeyer conical flasks containing 100ml of YPG broth medium (Glucose 5 % , peptone 1 % , yeast extract 0.1 % ,$(NH4)2SO4$ 5 %, NaCl0.1 % and $MgSO4$ .7H2O inoculated with 1ml suspension (10^5 spores/ml) grown on rotary shaker (100 rpm) at 28 C° for 9 days (10). The experiments were made in duplicate.

2- Chitosan Extraction

Fungal mycelia washed twice after filtration (Whatman No .1) and then dried at 65 C°. The method of (11) and (12) was depended to Chitosan extraction, then were ground and suspended with 1M NaOH and autoclaved at 121 C° for 15 min. Alkali-insoluble fraction were collected after centrifugation at 8000 rpm for 20min, washed with distilled water and recentrifuged to a neutral PH (PH7). 2% acetic acid at 95 C° were used for residues extracted , then centrifuged at 8000 rpm for 20min and the supernatant fluid was adjusted to 10 with 2M NaOH, the solution centrifuged at 8000 rpm for 20 min , then washing three time with D.W , 95 % ethanol and acetone respectively . anddried at 60 C° .

3- Antimicrobial activity

Antimicrobial activity of chitosan was measured by the paper disc diffusion method . Filter paper (Whatman No.1) disc (6mm) in diameter were impregnated with 2 mg / ml of chitosan , which were then aseptically applied to the surface of an agar plate at well – spaced intervals.disc impregnated with 5% of acetic acidwere used as the control , the plates were incubated at 37 c° for 24 h , and growth inhibition zone , including the diameter of disc were measured .

4- Diazonium test

Dissolve 50 mg of chitosan in 3 ml of HCL and add 5 ml distal water , put in ice - bath . Crystals of $NaNO3$are dissolve in 5 ml of distal water and add to solution of chitosan .The position result appear as clear solution depended as :

$$RNH_2 + HNO_2 \xrightarrow{(HCL + Na2No_3\ cold)} RN_2CL + N_2\ evaluation$$

5- Chitosan analyses

The Fourier transfer infrared (FT-IR) spectra of chitosan were carried out using the KBr disc method. depend on the infra-red spectrum the degree of acetylation (DA) was determined according to (13) using following equation:

A(%) = (A1641/A3014) x 100/1.33

A: Absorbance of chitosan

D-glucosamine in chitosan extracted were measurement by using Spectrophotometric method . Sample 10mg of chitosan was hydrolyzed with hydrochloric acid (HCL) 4M for 12h at 90 C° and the content of D-

glucosamine estimated at 530nm as described (14) .

Result and Discussion

Results showed the chitosan was yield from mycelia submerged culture of the fungus *Rhizopusoryzae*after 9 days cultivation reach to 15 mg/g dry weight mycelia at end of growth phase and the biomass of *Rhizopusoryzae*is 2g/100ml as dry weight .This result agree with result of (15) who studied different species of fungi and observed that highest biomass of *Rizopusoryzae* was 12.7 g/kg after 12 days of cultivation and study of (3) which their foundtwo mucoraleanstrain*Mucorracemosus*and *Cumninghamella* sp. At the end of growth , The biomass reach to 1.5 g / 100 ml and 2.5 g /100 ml respectively.In additional several studies showed that chitosan accumulation of each fungus has been previously reported that the late exponential phase produce the most extractable chitosan (10).Ninety-six percentage (96%) of D-glucosamine from hydrolysis of chitosan of fungus *R. oryzae.* was 96% compared with other studies the yield varied from approximately 50 to 90% of fungi fraction according to the age of the culture (3) and(16) found that chitosan from *Mucorrouxii* (zygomycetes) contained 81.3%D-glucosamine . The degree of acetylation of cell wall chitosan was estimated based on FT-IR spectra. The infra-red spectrum of insoluble fraction extracted was similar to the commercial chitosan (Sigma) .The peak at 2300 cm were due to CO2 interference . The degree of N-acetylation, estimated based on the ratio between the absorbance of amide II (N-H) approximately 1641 $CM^{-1}$and the C-H at 3014CM^{-1} was found to be around 40% Table - 1 , Figure-2 .This result is agree with the result of (3) who reported49% degree of N-acetylationfor*Mucorracemosus*(zygomycetes).Chitosan appear antimicrobial activity against all microorganisms used in this study . Aclear zones inhibition were 20, 15 and 15 mm for *E. coli, S. aureus and C. albicans* respectively , (Table – 2 and figure 3) . Chitosan have been to have antimicrobial activity by attacking the microbial cell wall . This activity may be dependent on type of chitosan ,its molecular weight and degree of decetlyation (17). The *E. coli* was more sensitive to chitosan .this effect may be return to the interaction of chitosan with endotoxins which are major component of the outer membrane of gram negative bacteria (1).

Table 1. FT- IR spectra of chitosan

Stretching vibration of bands cm^{-1}	Types of functional groups
3425-3477cm-1	Stretching vibration of OH
3286.7cm-1 2300 cm-1 2931-3014 cm-1 1641	Stretching vibration of NH interference CO2 Stretching vibration of CH2/CH3 Stretching vibration of CO

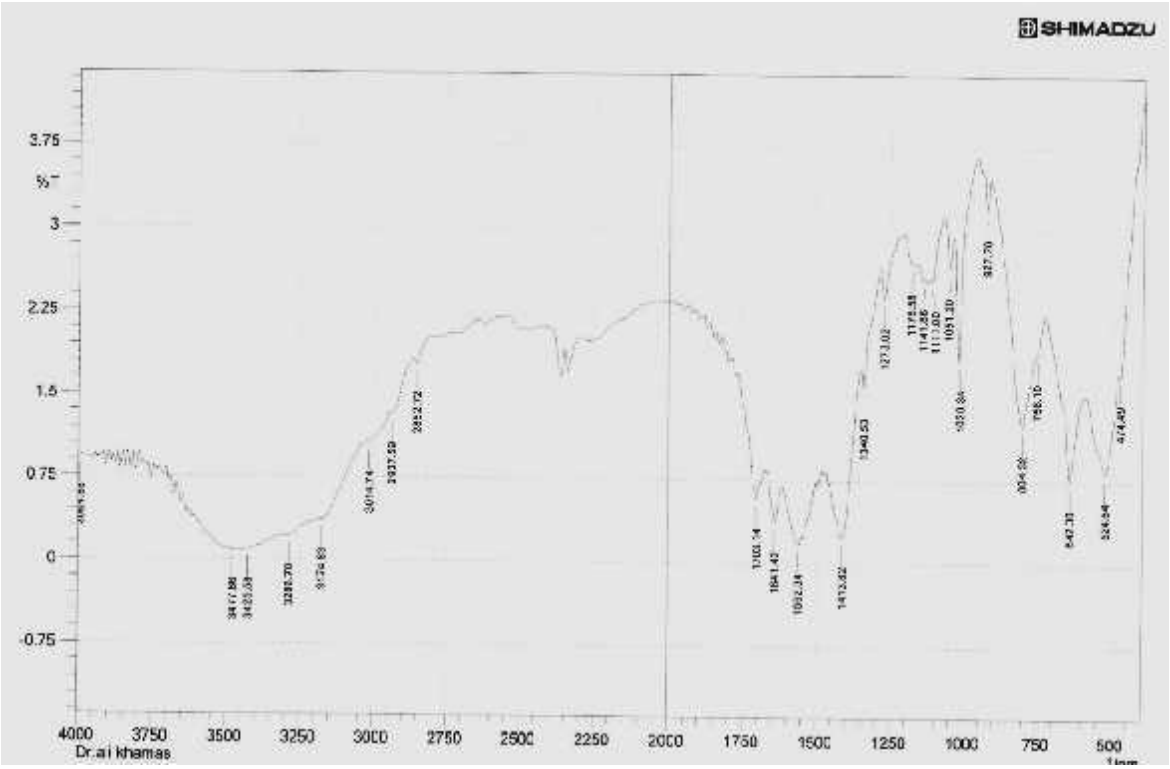

Figure (2) : FT- IR spectra of chitosan isolated from fungus *Rhizopusoryza*

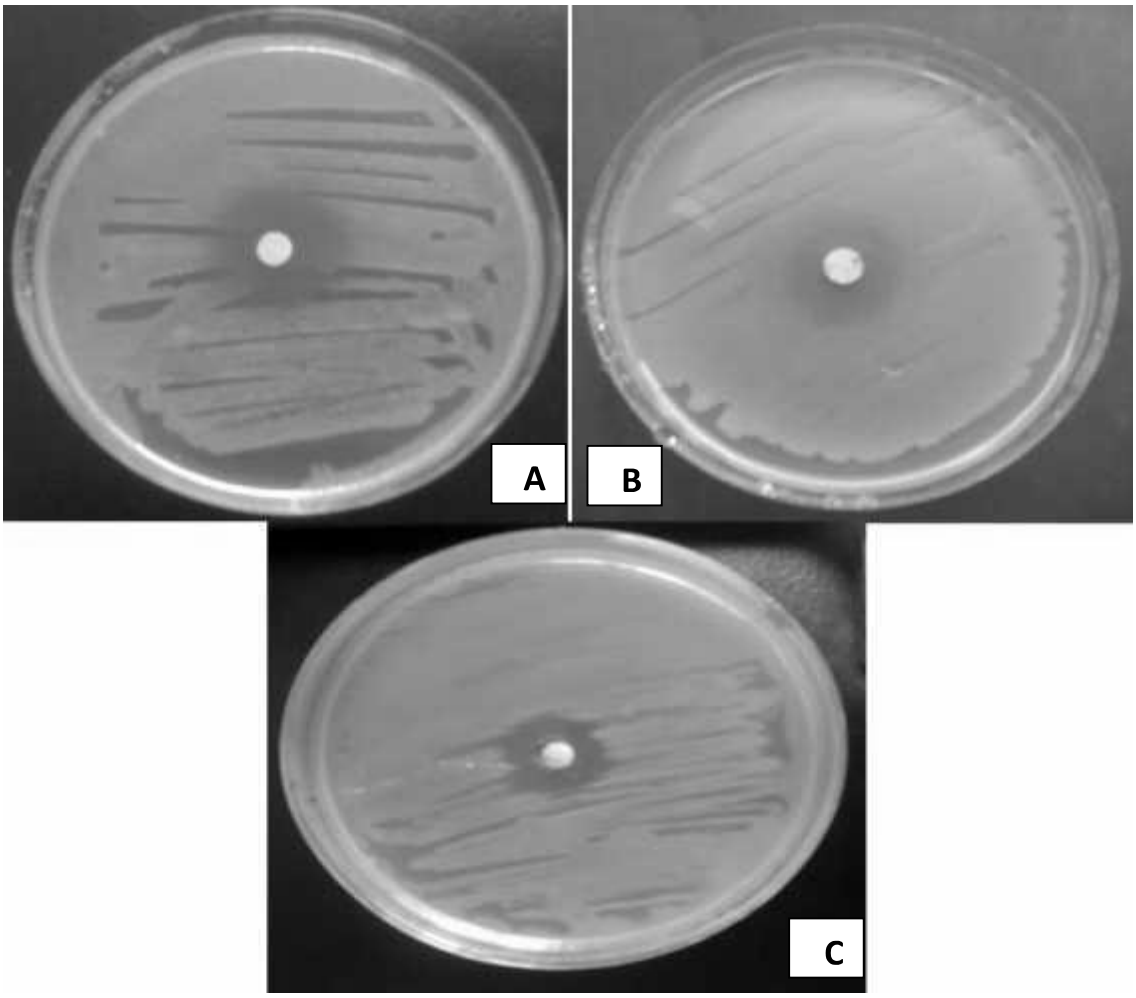

Figure (3) : Inhibition zone diameter (mm)

A: *E. coli* B: *S. aureus* , C: *C. Albicans*

Table -2: Inhibition zone of chitosan against bacteria

Bacterial species	Inhibition zone (mm)
E. coli	20
S. aureus	15
C. Albicans	15

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الفعالية الضد مايكروبيـةللكيتـوسان المعـزول مـن الفـطـر *Rhizopusoryzae*

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كوثر طعمة خلف

جامعة البصرة / كلية الصيدلة

Email: emad_yousif2000@yahoo.com

الخلاصة:

عزل في الدراسة الحالية الكيتوسانChitosan من الفطر *Rhizopusoryzae* باستخدام الوسط الزرعي (YGP) Pepton glucose broth medium في نهاية طور الأستقرار . بلغ انتاج الكيتوسان من الخيوط الفطرية حوالي 15 مليغرام / غرام من الوزن الجاف وان نسبة 25% تمثلت بالمركب N-acetylationللكيتوسان بينما بلغت نسبة D-glucoseamine حوالي 96% . أظهر الكيتوسان فعالية ضد ميكروبية تجاه بكتريا *Escherichia coli* و*Staphyllococcusaureus* و *Candida albicans* بلغت اقطارالتثبيط لها 20 ، 15 ، 15 مليميتر على الترتيب .

الكلمات المفتاحية : الكيتوسان ، الفطر *Rhizopusoryzae* ، الفعالية الضد ميكروبية