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Fermentative Production, Characterization and Antimicrobial Activity of Chitosan from Some Zygomycetes Fungi

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Abstract

Modern trends in natural techniques research have been elevated recently to avoid the utilization of chemical materials in agriculture which let to explore and use the biopolymers-reliant materials instead. Biopolymer chitosan which exhibited high activity against plant pathogens is one of these certified bio-substances. The mycelia of several fungi including Zygomycetes were used as an alternative source of chitin in large quantities which transformed to chitosan by deactylation. The present work aimed to extract chitosan from fungal mycelium by submerged fermentation and to study its antimicrobial activity. Six fungal strains were used for economical production of chitosan. Firstly, the different fungal strains were screened for its growth rate and chitosan yield. The obtained chitosan was characterized using the methods of vibrational spectroscopy in the infrared region and molecular weight determination. *Rhyzopus oryzea* was found to produce the highest biomass (11.6 g l^{-1}) and chitosan yield (670.0 mg l^{-1}) among the tested fungi on Potato Dextrose Broth (PDB). The suitable medium for mycelia growth and chitosan production by *R. oryzae* in shake-flask submerged culture was Cheng broth medium with the highest chitosan yield (749.7 mg l^{-1}). Eight days of incubation in Cheng broth was the optimum time for chitosan production (73.94 mg g^{-1} biomass). Among the tested carbon/nitrogen (C/N) ratios during submerged fermentation (SMF) of *R. oryzae* 9.0 C/N ratios was efficiently the best regarding to the fungal biomass (13.8 g l^{-1}) and chitosan yield (1008.17 mg l^{-1}). Antimicrobial activity was carried out against five pathogenic bacteria and six plant pathogenic fungi. At 5 mg ml^{-1} of fungal chitosan, the highest antibacterial activity was observed against *Escherichia coli* with 10.7 mm inhibition zone, whereas the highest antifungal activity was 12.8 mm for *Penicillium verrucosum*. The minimum inhibitory concentration (MIC) of chitosan extract for both bacteria and fungi strains ranged from 0.067- 2.0 mg ml^{-1}. The results revealed that fugal chitosan has multiple advantages as plant protection against the infection with pathogens, and as a bio-preservative by extension the shelf life of food.

Keywords: chitin/chitosan; mycelia biomass, Zygomycetous fungi, fermentation, antimicrobial.

1. Introduction

Recently, chitosan has become one of the best assuring biotech products which varied according to the chemical shape and functional group. Chitosan, a β.1, 4-linked glucosamine is formed by deacetylation of chitin. It has a broad variety of functions from farming to pharmaceutical products **[1, 2]**. Chitosan commonly extracted from shrimp waste, shellfish, crab, and lobster using strong alkali at excessive heat for long period **[3, 4]**.This procedure was found to possess an adverse ecological balance influence because of using huge amounts of strong base solutions in the transformation process at high temperatures. This causes variation in product properties, affects chitosan quality, and increases production costs. Industrial-scale isolation faces a challenge of limitation the chitin supplies at same season in many countries **[5]**. Some of these difficulties can be eliminated by culturing chitosan-producing fungi as novel fermentation technique **[6, 7]**. So, the culture of certain fungi can be considered as an alternate source of chitin and chitosan. The obtainment of chitin and chitosan from microbial origins has attained enhanced recognition up-to-date. Such chitosan is characterized by homogenous polymer, high degree of deacetylation, length and solubility over the current marine source. Choosing the suitable fermentation condition by microorganisms facilitate the culturing on inexpensive nutrients. More over cell wall component can be extracted by simple chemical method **[8]**. The mycelia of various fungi including Ascomycetes, Zygomycetes, Basidiomycetes and Deutermoycetes are alternative sources of chitin and

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chitosan **[9]**. The advantages of using fungi are easy comfortable harvesting, handling and controlling to produce high-quality Chitosan **[1]**. Obtaining high biomass of fungi, based on exploitation of residues from the agro-industrial sector to produce microbial chitosan gives advantages over that produced by convenient crustaceous source leading to produce it commercially. Chitosan becomes a beneficial compound that acts as a fungicide and inducer of plant defense mechanisms to control post-harvest diseases of fruits and vegetables **[10]**. In addition to its antibacterial and antifungal activities, chitosan is effectively applied in plant as growth promoter, enhancing the production of secondary metabolites and in soil correction. So, it is used as a bionematoeide, biocontrol agent, and potent fertilizer **[11, 12]**. Multiple mechanisms of action have been proposed; however, this process is not fully understood. The different response of phytopathogens and antagonists to chitosan presence suggests the involvement of specific characteristics which are not easily defined. In this regard, **Bautista-Baños [13]** reported that the strain, molecular weight, concentration, degree of deacetylation and type of chitosan are among the factors that affect the antimicrobial activity of the chitosan.

This study, aimed to produce chitosan from different zygomycetes fungal strains and examine its physio-chemical characters and its antimicrobial effect compared with commercial chitosan.

Materials and Methods

All chemicals and media used in the present study, commercial and/or analytical grade, were procured from local companies. Standards of chitosan were obtained from Sigma Company.

1. Screening and characterization of fungal Cultures

Six strains belonging to Zygomycetes species were examined for chitosan production including *Pencillium expansum, Aspergillus nigr, Aspergillus terreus, Rhizopus oryaze, Mucor rouxii and Mucor raceam*. These strains were generously offered by the department of Micro. SWERI/ARC. Giza, Egypt. All strain was maintained on potato dextrose agar (PDA) medium **[14]** and stored in a refrigerator at 4°C.

2. Medium and conditions for submerged fermentation (SMF)

In triplicates, the concentrations of carbon and nitrogen from synthetic media were measured according to the experimental design described below. The tested strains were grown, for chitosan production, in the following culture media: Potato dextrose broth (PDB) **[14]**, Handerson and Anderson medium (HA medium), Yeast Malt Extract Broth medium Merck (YMB), Cheng media **[15]** and Glucose peptone yeast (GPY) medium **[16]**.

3. Utilization of agro-industrial by-products molasses and clarified corn steep liquor (CSL) as cheap carbon and nitrogen source

To obtain an economic medium for the propagation of the selected fungal strains (as the source for fungal chitosan production) molasses and CSL were used as a replacement of the expensive carbon and nitrogen amounts in the synthetic media. A replacement was generally carried out at a concentration equivalent in carbon and nitrogen content, respectively.

Sugar cane molasses was diluted with distilled water (1:1), pH was adjusted to 3.5- 4.0 using H_2SO_4, then heated at 90ºC for 60 minutes and left overnight at 4o C for precipitation. The supernatant was decanted and total sugar content was determined (55.0 %) and kept refrigerated until use **[17]**. Total sugar content of sugarcane molasses was determined using phenol sulfuric acid method **[18]**.

Corn steep liquor kindly provided by Egyptian starch and Glucose Manufacturing Company, 6 October Street, Mostord, Cairo, Egypt. It was boiled for 10 min., cooled to room temperature, kept refrigerated at 4ºC overnight and centrifuged at 4000 rpm to remove precipitates. The clarified CSL was kept at 4ºC until use after determination of its nitrogen content (3.3% w/v). Determination of CSL total nitrogen content of clarified CSL was carried out using microkjeldahl procedure **[19]**.

4. Characterization of Chitosan

The obtained chitosan was characterized using the methods of Vibrational Spectroscopy in the infrared region to determine the deacetylation degree and molecular weight.

Fourier – Transform Infrared (FT-IR) spectroscopy

The structure of extracted fungal chitosan was confirmed by infrared spectroscopy using KBr pellet method in FTIR (Perkin – Elmer Instruments, Norwalk, CT). FTIR spectra were recorded in the middle infrared (4000 cm^{-1} to 400 cm^{-1}) with a resolution of 4 cm^{-1} in the absorbance mode for 16 scans at room temperature. Analyses were performed by Micro Analytical Center, Faculty of Science, Cairo University.

The mycelial chitosan samples were prepared by grinding the dry mycelial chitosan powder with powdered potassium bromide (KBr), in the ratio of 1:5 (sample: KBr), and then compressed to form KBr pellet and subjected to FTIR analysis **[20]**.

Deacetylation degree

According to Stamford **[21]**, Vibrational Spectroscopy in the Infrared Region is a technique

used to determine the degree of chitosan deacetylation by the ratio between the absorbance at wavelengths 1655 and 3450 cm. To measure the absorbance in a spectrum of transmittance versus wavelength, it is necessary to: 1) draw the baselines; 2) calculate the difference in transmittance; 3) convert the transmittance values for absorbance (A = log T1/T2), apply the values in the equation proposed by Domzy and Roberts **[22]**. The equation aims to calculate the degree of chitosan deacetylation characteristics relating to the peaks of the amine group and the acetamide heteropolymer.

DD % = 100 – [(A1655/A3450)] x 100/115 ---------

baseline

A1655 is the absorbance of wavelength 1655 cm^{-1} obtained using the baseline proposal for **Domszy and Roberts [22]**. The A3450 is the absorption band at wavelength 3450 cm^{-1}. The number 115 equals the value of (A1655/A3450) found in pure chitosan.

5. Antimicrobial activity of extracted and commercial chitosan.

Antimicrobial activity was carried out according to the methods described by **[23, 24]**.

Tested microorganisms:

Antibacterial assay was done against two gram-positive pathogenic bacteria (*Bacillus cereus* EMCC1080 and *Staphylococcus aureus* ATCC13565) and three Gram-negative bacteria *Escherichia coli* O157-H7ATCC51659, *Salmonella typhi* ATCC15566, and *Pseudomonas auruginosa* NRRLB- 272). The strains were grown on nutrient agar plates at 37° C for 24 hr and kept in a refrigerator at 4°C until use.

The antifungal assay was done against six phytopathogenic fungi species including *Aspergillus flavus* NRRL 3357, *Aspergillus parasiticus* SSWT2999, *Aspergillus niger*, *Aspergillus ochraceous* ITAL14, *Pencillim verrecosum* ITEM10027, and *Fusarium proliferatum* MPVP328. They were grown on potato dextrose agar (PDA) dishes for 7 days at 28°C

Antibacterial assay

The assay was conducted using the disc diffusion method on nutrient agar. The tested microorganisms were inoculated to Tryptic soya broth tubes and incubated at 37°C for 4 hr. The turbidity of these cultures was adjusted by using 0.5 Mc-Farland. A uniform bacterial lawn was developed by sterile cotton swabs on the surface of solid nutrient agar plates. Whatman filter paper no.1 was used to prepare discs 6 mm which impregnated with different concentrations of each extracted and commercial chitosan (5 and 10 mg ml^{-1}). The impregnated discs were applied on the surface of streaked nutrient agar plates. HCl 0.25 M was used as negative control while 1 mg ml^{-1} Ceftriaxone was used as a positive control. The triplicates plates were inverted and incubated at 35°C for 16-18hr **[23, 24]**.

Antifungal assay

The antifungal assay was conducted using the disc diffusion technique on Potato Dextrose Agar (PDA) media **[24]**. The spore suspension was prepared by transferring a loopful of grown tested fungi to a test tube containing 10 ml of 0.01% tween 80 solutions. From spore suspension, 100 µl was spread on the solidified Potato Dextrose Agar plates using a glass rod, and the plates left to dry for half an hour. The impregnated discs were applied to the surface of the dry plates. HCL 0.25 M was used as negative control while Miconazole (Sigma- Aldrich) with a concentration of 1.0 mg ml^{-1} was used as a positive control. The plates were inverted and incubated at 28°C for 24- 48hr. After incubation, the inhibition zones were measured including the diameter of the disc. Zones are measured to the nearest millimeter using a ruler which is held on the back of the inverted petri plates, three replicates were averaged and the results were expressed as mean ±SE according to **[23]**.

Estimation of minimum inhibitory concentration (MIC)

The determination of MIC was conducted using the tube dilution method **[25]**. A 24 h culture of the tested bacterial species was diluted in 10 ml of tryptic soy broth (TSB) with reference to the 0.5 McFarland standards to achieve inoculum of 10^8 CFU ml^{-1}. In culture tube containing eleven concentrations of chitosan extract and commercial one (5.0, 4.0, 3.0, 2.0, 1.5, 1.0, 0.75 0.5, 0.25, 0.1 and 0.05 mg ml^{-1} in HCl 0.25 M) were prepared. Each tube was inoculated with 100 µl of bacterial cell suspension and incubated at 37 °C for 24h. The growth of the inoculum in broth was measure by turbidity of the broth and the lowest concentration which inhibited the growth of the tested organism was taken as the minimum inhibitory concentration (MIC).

MIC against fungi was performed by using the technique of **Sokmen [26]**. The chitosan at different concentrations was separately dissolved in 0.5 ml of 0.1% Tween 80 (Merck, Darmstadt, Germany), then mixed with 9.5 ml of melting, 45 °C, PDA and poured into Petri dish (6 cm). The prepared plates were centrally inoculated with 3 µl of fungal suspension (10^8 CFU ml^{-1}; 0.5 McFarland standards). In triplicates, plates were incubated at 25 °C for 48h. At the end of the incubation period, mycelial growth was monitored and MIC was determined

Statistical analysis

Results were subjected to one-way analysis of variance (ANOVA) of the general liner model

(GLM) using SAS **[27]** statistical package. The results were the average of three replicates ($p \leq 0.05$).

Result and Discussion

1. Screening of Different Fungal Strains

Screening for the efficiency of six fungal strains for chitosan production in chitosan production under submerged fermentation conditions on PDB are presented in **Table (1)**. At the same factors of fermentation parameters, biomass of the strains ranged from7.8 g l^{-1} for *Mucor rouxii* to 12.0 g l^{-1} for *Aspergillus niger*. *R. oryzae* gave maximum production of chitosan (670.0 mg l^{-1}) followed by *A. terreus* (554.6 mg l^{-1}) and *P. expansum* (450.0 mgl^{-1}). According to **[28, 29** and **30]**, any common fungi constituting the phylum Zygomycota, includes chitosan as original parts of their cell wall. **New and stevens [31]** reported that chitosan biosynthesis starts with the reproduction of chitin by the conversion of glucose into N-acetylglucosamine-1-phosphate. Consequently, the enzyme chitin syntheses transfer the sugar dimer of N-acetyl glucose amine section into the developed chitin polymer chain. Besides that Zygomycetes, deacetylation of the rising chitin chain happens through the activity of the enzyme chitin deacetylase which occurs in the production of mycelial chitosan. As shown above, *Rhizopus oryzae* may be considered good candidates for use in chitosan production and therefore used for further optimization study.

Table 1. Screening of six different fungal strains for mycelial growth production of chitosan in shake flask submerged culture using potato dextrose broth (PDB) for 7 days.

Fungi	Biomass (g/L)	Chitosan	
		(mg/L)	(mg/g biomass)
Rhizopus oryzae	11.6 ± 0.44^{a}	670.0± 5.44^{a}	57.8± 0.20^{a}
P. expansum	9.0± 0.46^{b}	450.0± 0.34^{c}	50.0± 0.17^{b}
A. niger	12.0± 0.25^{a}	393.6± 3.94^{d}	32.8± 0.34^{d}
A. terreus	11.8± 0.61^{a}	554.6± 3.18^{b}	47.0± 0.40^{c}
Mucor rouxii	7.8± 0.44^{b}	210.6± 1.88^{f}	27.0± 0.25^{f}
Mucor raceama	8.1± 0.31^{b}	230.9± 3.01^{e}	28.5± 0.36^{e}

Means with the same letter in the same column are not significantly different (Mean ± SE)

2. Examination of different media with submerged fermentation for chitosan production

To select the most favorable medium for chitosan production using submerged conditions, the selected fungus, *Rhizopus oryzae* was cultured on five media in shake flask submerged culture. The results of mycelial dry weight and chitosan biomass production are shown in **Table (2)**. The growth of *R. oryzae* on five different media Potato dextrose broth (PDB), Handerson and Anderson medium (HA), Yeast Malt Extract Broth medium Merck(YMB), Cheng media (Cheng) and Glucose peptone yeast (GPY) media gl^{-1} were observed for 7 days at 35^{o}C. Cheng, YMB, and GPY salt broth media gave the highest mycelial biomass (12.2, 11.4, and 11.0 g l^{-1}, respectively). This is in agreement with **[15, 16]**. On the other hand, Cheng (CH) broth media exhibited also the highest production of chitosan (749.7 mg l^{-1}). These findings are in consistence with those reported by Cheng, **[15]**. Contrary to the expected, the growth of *R. oryzae* in Handerson and Anderson (HA) was very slow with minimal biomass.

Table 2. The effect of different media on mycelial growth and chitosan production by *Rhizopus oryzae*.

Tested medium	Biomass (g/L)	Chitosan	
		(mg/L)	(mg/g biomass)
PDB	10.5± 0.21^{a}	610.5± 2.86^{c}	55.5± 1.85^{c}
HA	7.8± 0.49 ^{b}	377.5± 5.62^{d}	48.4± 1.09^{d}
YMB	11.4± 0.56^{a}	684.0± 4.96^{b}	60,0± 1.21^{bc}
Cheng	12.2± 0.62^{a}	749.7± 2.47^{a}	73.5± 2.13^{a}
GPY	11.0± 0.87^{a}	682.0± 2.91^{b}	62.0± 2.40^{b}

Means with the same letter in the same column are not significantly different (Mean ± SE)

3. Selection of the optimum incubation period

At this point, the study was carried out to select the optimum incubation period under submerged cultivation **(Table 3)**. The maximum value of fungal chitosan was 861.9 mg l^{-1} in fermented medium after 8 days of inoculation. After the edge of the exponential stage, the mass of mycelial chitosan decreased gently. Such products were comparable to those obtained by other research taking into consideration the difference between the condition and the used fungi **[8, 32]**. In contrast, the production of fungal biomass started to increase from 4th day. **Viccini [33]** attributed that to the consuming chitin and chitosan biopolymers by the microorganism as nutrients and an increment in biomass, followed by an increase in enzymatic activity, hydrolysis of polymers by hydrolytic enzymes, and the presence of the hydrolyzed components. Obviously, at the edge of the exponential stage, the cell walls accumulated extra chitin than chitosan, as indicated by the yield of chitin resumed in biomass. In solid-state cultures, the rate of alkali-insoluble material can be realized as a fungal growth factor because it has been reviewed to be mainly constituted by the maximum concentration of chitosan was obtained after 8 days the cell wall and the rate of insoluble alkaline substances constant in the early stages of growth. After 9 days it gradually decreased and this finding is in agreement with that observed by **Maghsoodi [34].**

Table 3. The effect of incubation period on mycelial growth and chitosan production by *R. oryza* on the suitable selected medium (CH).

Incubation Period (days)	Biomass (g/L)	Chitosan	
		(mg/L)	biomass (mg/g)
2	3.5± 0.23[d]	119.8± 3.2[e]	34.23± 1.26[e]
4	5.5± 0.51[c]	312.7± 2.0[d]	56.85± 0.99[c]
6	9.5± 0.64[b]	689.3± 1.47[b]	70.34± 0.97[b]
8	11.8± 0.26[a]	861.9± 1.32[a]	73.94± 1.47[a]
10	13.2± 0.45[a]	620.0± 7.4[c]	47.00± 0.54[d]

Means with the same letter in the same column are not significantly different (Mean ± SE)

4. Utilization of agro-industrial byproducts as a source of carbon and nitrogen

Submerged fermentation (SMF)

Agriculture-based industries produce remarkable amounts and varieties of agro-industrial by-product like glucose syrup, sugarcane molasses, sugar beet molasses and corn steep liquor. Carbon and nitrogen obtained from Agro- industrial by-products (AIP) can be reprocessed as raw materials in the agro-based industries, which can decrease the total production cost.

As shown in **Table (4)**, molasses and corn steep liquor gave the highest value of mycelial chitosan. These findings are commensurate with the findings of **Cardoso [35]** who used molasses and Corn steep liquor as a cheap agro-industrial waste that contained high amount of carbon and nitrogen. So, there is a modern trend for replacement of highly cost nitrogen and carbon source with another cheap one **[36]**. According to **Abasian [37]**, a nitrogen source existing in the production medium was the most significant factor for the growth of microorganisms. However, the carbon source doesn't only acts as a chief element for the formation of cellular material, but it is also a significant source of energy **[38]**. Chitosan production from *R. oryzae* in sugarcane molasses (940.0 mg l^{-1}) and corn steep liquor (943.8 mg l^{-1}) at moisture 35% and pH 5.0 is shown in **Table (4).**

Nitrogen and carbon content and the type of microorganism played an important role in the biomass yield and fungal chitosan production. . In SMF, the C: N ratio was a primary parameter to control the production because the influence of carbon concentration and C: N ratio on fungal growth and sporulation are contingent; therefore, consideration of nutritional requirements is essential to improve yields of fungal growth.

The pH of the medium eternally impacts the physiology of a microorganism by influencing nutrient value, enzyme activity, oxidative-reductive reactions, and the morphology of the cell membrane. The initial pH range of 3 - 6 was the most common **[39]**. In the present study the mycelia was grown on the SMF medium, in the initial pH of 5.0.These are in agreement with **[40]** who stated that this might be because the pH ranging from 4.5 to 5.5 favored the production of enzyme chitin deacetylase, required to convert chitin to chitosan in the fungal cell wall. As shown in Table (4), corn steep liquor and molasses were in numerous amounts to the medium to evaluate the effect of the nitrogen and carbon concentration on the production of the amount of fungal chitosan. A replacement was generally carried out at a concentration equivalent in carbon and nitrogen content respectively. Data obtained in **Table (5)** illustrate that the lowest ratio gave the lowest yield of fungal biomass of R. orayzae strain (4.53gl^{-1}).

Although, the fungal strain *R. oryzae* grown on the medium at C: N ratio of 9 and 12, had no difference regarding to the fungal biomass (13.80 and 13.0 g l^{-1}, respectively).

C: N ratio 9 gave the highest yield of fungal Chitosan (1008.17 mg l^{-1}) followed by C: N ratio 12 which was very close to control (930.73 and 989.82 mg l^{-1}, respectively). So, the nine C: N ratio was the more convenient for submerged fermentation for fungal chitosan production. While 3, 6 and 15 C: N ratios gave the lowest value (218.34, 708.00 and 658.04 mg l^{-1}, respectively).

Table 4. Utilization of some agro-industrial by-products for mycellial growth and chitosan production by *R. oryzae* in shake flask submerged culture

Agro-industrial by-products(AIP)		Biomass (g/l)	Chitosan Yield	
			(mg/L)	(mg/g biomass)
Carbon source	Glucose Syrup	9.0± 0.35[c]	606.6± 2.01[d]	67.4± 0.26[c]
	Sugar Cane Molasses	12.2± 0.35[a]	940.0± 2.71[a]	75.2± 0.28[a]
	Sugar beet Molasses	11.0± 0.40[b]	712.8± 1.84[b]	64.8± 0.21[d]
Nitrogen source	Soybean Cake	10.0± 0.50[bc]	625.0± 2.82[c]	62.5± 0.17[e]
	Corn Steep Liquor	13.0± 0.32[a]	943.8± 3.21[a]	72.6± 0.30[b]
	Gluten	9.2± 0.40[c]	510.0± 1.68[f]	60.0± 0.23[f]
Control*		9.5± 0.17[c]	558.6± 2.14[e]	58.6± 0.35[g]

*Medium containing glucose (275 gl^{-1}) as carbon source and $(NH_4)_2 HPO_4$(40 gl^{-1}) as nitrogen source; Means with the same letter in the same column are not significantly different (Mean ± SE)

Table 5. Mycelial chitosan production during growth *R. oryzea* on selective media with different C: N ratios.

C: N (ratio)	Biomass (g/L)	Chitosan		Final pH
		(mg/L)	(mg/g biomass)	
3	4.53± 0.42[c]	218.34± 2.90[f]	48.20± 0.28[e]	5.5
6	11.00± 0.44[b]	708.00± 4.71[d]	64.36± 0.57[c]	5.9
9	13.80± 0.55[a]	1008.17± 0.74[a]	73.06± 1.04[ab]	7.8
12	13.00± 0.40[a]	930.73± 1.48[c]	71.60± 0.58[b]	8.1
15	10.80± 0.50[b]	658.04± 3.28[e]	60.93± 0.35[d]	7.3
Control*	13.40± 0.76[a]	989.82± 2.60[b]	73.87± 0.38[a]	7.9

* Selected medium Chang, pH = 5.0, C: N ratio = 12.9; Means with the same letter in the same column are not significantly different (Mean ± SE).

Finally, our results revealed that carbon and nitrogen source was a significant factor used to optimize for mycelial chitosan production. Chitosan is a nitrogen-containing biopolymer, which is deacetylated from chitin. Fungi need organic or inorganic N source as a nutrient to synthesize the chitin and chitosan for their cell wall. Therefore, the N source is one of the most important factors for the production of chitosan by fungi **[31]**. This information had been confirmed by **Vaingankar and Juvekar [41]**.

5. Chitosan characterization:

Fourier – Transform Infrared (FT-IR) spectroscopy

The FTIR spectrum of mycelial chitosan from *R. oryzea* recovered from submerged fermentation compared with standard chitosan (Sigma) is presented in **Fig. (1).** Infrared spectroscopy has been used by scientists for many years for the gross characterization of extracted chitosan from widely differing origins. It can provide information concerning the presence of specific entities within the molecule mainly their functional groups, the degree of acetylation/ deacetylation, and impurities **[12, 42]**. The optical density of some functional groups of the extracted fungal chitosan samples were illustrated in Fig. 1. Occurrence of many bands had been suggested as internal reference bands of chitosan: the OH stretching band at 3450 cm^{-1}; the C-H stretching bands within 2870 - 2880 cm^{-1}; the skeletal vibrations involving the C-O-C stretching band at 1030 - 1070 cm^{-1}; the -CH_2 bending centered at 1420 cm^{-1}; the anti-symmetric stretching of the C-O-C bridge around 1160 cm^{-1}; 1315 - 1320 cm^{-1} (amide III bands); 1620 - 1630 cm^{-1} (-NH bending of NH_2); and 890 – 900 cm^{-1} (C-O-C bridge as well as glucosidic linkage) **[43]**. In the current study, our results were similar to those stated by **Khan [44]** and similar to that of mycelial chitosan obtained from *M. rouxii* **[39]**. The most significant parts of these spectra were those showing functional groups including hydroxyl stretching band at 3445cm^{-1}, amide I band at 1641, primary amine band at 1638 - 1561 cm^{-1} amide II band at 1561 cm^{-1}. The band at 897 cm^{-1} was referenced as a glycosidic linkage of β-anomer. These finding are in agreement with **[15, 32 and 45].**

Quality of the extracted fungal chitosan prepared from different species of fungi

The quality parameters of chitosan which extracted from R. oryzea strain were evaluated in comparison with standard chitosan **(Table 6)**. The percentage of those parameters in purified fungal chitosan was very close to that of the standard one. Moreover, it contained lower ash as an indication of the good quality. The color was developed from brownish white in chitin to bright off-white in chitosan. The pH was 7.8 referring to well solubility in 1% acetic acid.

Degree of deactylation

Infrared spectroscopy of fungal chitosan obtained from R. oryzae is presented in **table (6)**. It had been performed to investigate the degree of deacetylation (DD). The degree of deacetylation is considered one of the principal parameters in the chitosan characterization. It is defined as the number of amines related to the number of amide groups in the polymer chain. The degree of deacetylation of fungal chitosan was around 87%, observed in a table (6), where the presence of the amide band (1423 cm^{-1}) and amide I (1642 cm^{-1}) bands which characterized the chitosan chemical structure.

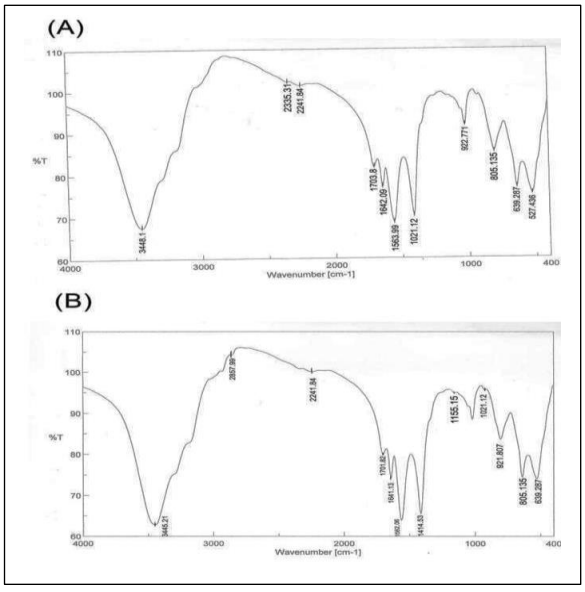

Fig. 1. The FTIR spectrum of standard chitosan (sigma) (A) was compared with mycelial chitosan from *R. oryzea* obtained from submerged fermentation (B).

The degree of deacetylation for mycelial chitosan extracted from *R. oryzae* gave slightly less value 86.9% than the commercial chitosan (Sigma) which extracted from crap shell give (87.6%). These results suggested that this variation was certainly due to the difference of chitosan source and the method of extraction. This is in agreement with **[46, 47]** who confirmed that fungal chitosan with a magnificent degree of deacetylation has large positive charges, and it is more conventional for food application as coagulation or chelating agent and a clarifying agent or antimicrobial agent. The products were kept airtight at ambient temperature for 12 months and no changes were found in the quality in terms of solubility, pH, and reabsorption capability.

Table 6. Characteristic of Purified Extracted Fungal Chitosan in the Laboratory

Composition(%) on a dry weight basis	*R. oryzae*	*Sigma*
Moisture	7.9	7.6
Ash	0.5	0.9
Protein	Null	Null
Lipid	Null	Null
Color	Off white	Off white
Solubility in 1% acetic acid	99.8	98.8
PH	7.8	8.0
Degree of Deacetylation (%)	86.917	87.6

6) Antimicrobial activity of fungal and commercial chitosan:

The biological activity of chitosan has been well documented both in vitro and in-situ studies. The level of inhibition of pathogenic microorganisms is highly correlated with chitosan concentration. The polycationic nature of chitosan is the key to its antimicrobial properties and the length of the polymer chain improves its activity. As a natural elicitor and antifungal agent, chitosan is a talented alternative for the management of postharvest plant diseases. The two concentration of extracted and commercial of chitosan 5 and 10 mg ml^{-1} were screened against different strains of foodborne pathogenic bacteria **(Table 7)**. The two concentration of different chitosan showed the antibacterial activity against all tested bacteria. Extracted chitosan showed degree of inhibition closed to that developed by commercial one (sigma). It recorded maximum antibacterial activity against gram negative bacteria from 8.0 to 10.67 mm at 5 mg $ml^{-1}$and from 8.0 to 12.0 mm at 10 mg ml^{-1}. E. coli showed more sensitivity to the extracted chitosan (10.67 and 12.0 mm) than commercial chitosan (9.7 and 11.3 mm) using 5, 10 mg ml^{-1}, respectively. However, commercial chitosan has more effective on *S. typhi* (10.3 and 12.33 mm) than extracted chitosan. Whereas, extracted chitosan had a lower effect on gram positive bacteria *B. cereus* and *S. aureus* (8.33 and 8.0 mm) than commercial chitosan (12.33 mm) at 10 mg ml^{-1}. The negative control (HCl 0.25 M) showed no inhibition whereas, positive control (antibiotics ceftriaxone at concentration 1mg ml^{-1} showed an inhibition of 14.67 mm in *B. cereus*, 15.33 mm in *S. aureus*, 24.33 mm in *E. coli*, 16.33 mm in *S. typhi* and 17.67 mm in *P. areugnosa*.

Table (8) shows the antifungal activity of fungal chitosan and commercial one against different strains of mycotoxigenic fungi. These concentrations can reduce the in vitro growth of several fungi except which have chitosan as a component of their cell walls **[48]**. Generally, these findings are harmonious with those reported by many investigators **[49, 50]**. Extracted chitosan had an effect on all tested fungi ranged from 9.3 to 12.8 mm at 5 mg ml^{-1} and 10 to 14.5 mm at 10 mg ml^{-1}. These values were very close to those of commercial chitosan (10.3 to 14 .8 mm at 5 mg ml^{-1} and 10.7 to 16.3 mm at 10 mg ml^{-1}). The highest inhibition zone was shown against *P. verecossum* at both concentrations recording 12.8 and 14.5 mm for fungal chitosan and 14.8 and 16.3mm for commercial chitosan. The lowest activity was observed against *A. ochraceaus* at 5 mg ml^{-1} of fungal and commercial chitosan (9.3 and 10.3 mm, respectively).

Table 7. Antibacterial activity of extracted and commercial Chitosan.

Sample ID	Inhibition zone diameter in mm (Mean ± SE)				
	B. cereus	***Staph. aureus***	***E. coli***	***S. typhi***	***Ps. areugnosa***
1	8.33 ± 0.33^{d}	8.00 ± 0.0^{c}	10.67 ± 0.67^{bc}	9.33 ± 0.67^{c}	8.00 ± 0.0^{d}
2	8.33 ± 0.33^{d}	8.00 ± 0.0^{c}	12.00 ± 0.58^{b}	9.33 ± 0.67^{c}	8.00 ± 0.0^{d}
3	10.00 ± 0.0^{c}	9.0 ± 0.58^{c}	9.7 ± 0.33^{c}	$10.3\pm1.2b^{c}$	9.3 ± 0.67^{c}
4	12.33 ± 0.33^{b}	12.33 ± 0.67^{b}	11.33 ± 0.88^{bc}	12.33 ± 0.67^{b}	11.67 ± 0.33^{b}
Negative control	0.00 ± 0.0^{e}	0.00 ± 0.0^{d}	0.00 ± 0.0^{d}	0.00 ± 0.0^{d}	0.00 ± 0.0^{e}
Positive control	14.67 ± 0.88^{a}	15.33 ± 0.33^{a}	24.33 ± 0.67^{a}	16.33 ± 0.33^{a}	17.67 ± 0.33^{a}

Means with the same letter in the same column are not significantly different; Sample ID (1): 5mg/ml of extracted chitosan; (2): 10 mg/ml of extracted chitosan, (3): 5mg/ml of commercial chitosan (sigma); (4): 10mg/ml of commercial chitosan (sigma)

The positive control meconazole at 1 mg ml^{-1} showed an inhibition zone of 16.7, 20.3, 16.0, 18.0, 15.2 and 21.8 mm against *A. flavus, A. parasiticus, A. niger, A.ochraceous, F. proliferitum* and *P. verecossum*, respectively. **Benhamou, [51]** reported that chitosan as a biomaterial reduced of soil-borne pathogens. Many possible elucidations have been suggested for antimicrobial activity. Polycationic chitosan molecule interacts with the lipopolysaccharides and proteins which consider main anionic cell wall components of the microorganism. These results in the escape of intracellular components due to disruption in permeability barrier; preventing nutrients from entering the cell. Upon entry into the cell, it can bind to DNA, and thus inhibits RNA and protein synthesis; binds through hydrophobic interactions, etc. Chitosan shows wide spectrum of antimicrobial activity against both gram-positive and gram-negative bacteria and fungi **[12, 38, 46, and 47].**

Minimum inhibitory concentration (MIC) values

Figure (2) represents the minimum inhibitory concentration of extracted and commercial chitosan against of pathogenic bacteria. The highest activity was recorded against *S. aureus* by either fungal chitosan (0.067 mg ml^{-1}) or commercial (0.042 mg ml^{-1}) chitosan, followed by *B. cereus* and *E. coli* with MIC values with 0.2 by fungal chitosan for each or with 0.07 and 0.15 mg ml^{-1} by commercial one, respectively. Whereas, the lowest activity with MIC of extracted chitosan was showed against *S. typhi* (0.33mg ml^{-1}), and against *P. aeruginosa* with MIC of commercial chitosan was 0.2 mg ml^{-1}. These findings are harmonious with those reported by **Hassan [12]** who found that chitosan had a highly effect on gram positive more than gram negative bacteria

Regarding to the tested fungi **(Fig. 3)**, the lowest MIC of extracted chitosan was recorded against *A. parasitcus* with MIC of 0.6 mg ml^{-1} and 0.67 mg ml^{-1} for *P. verrucosum* , whereas the commercial chitosan had more activity against *P. verrucosum* (0.33 mg ml^{-1}) followed by *A. ochraceous* (0.58 mg ml^{-1}) and *F.*

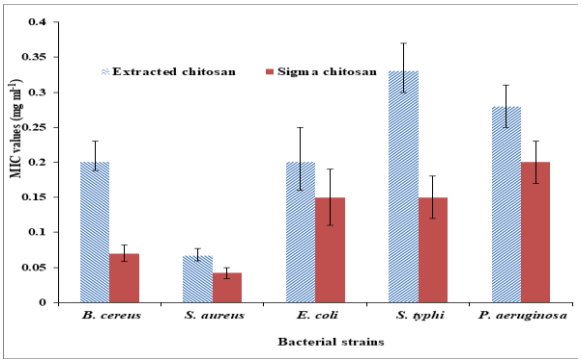

Fig. 2. Minimum inhibitory concentration of extracted and commercial chitosan for tested bacteria.

proliferatum (0.6 mg ml^{-1}). The highest MIC of extracted chitosan was recorded against *A. flavus* (2.0 mg ml^{-1}) followed by *A. niger* (1.17 mg ml^{-1}) and *A. ochraceous* (1.15 mg ml^{-1}). Whereas, lower MIC for commercial chitosan was required against *A. flavus* (1.7 mg ml^{-1}) followed by *A. parasiticus* (1.08 mg ml^{-1}). Fungal chitosan had an inhibition activity against both Gram positive and Gram negative bacteria **[12, 52, 53** and **54]** and different pathogenic fungi as reported by **Nguyen [55]**. This property is useful in food preservation, food protection and plant diseases control. Those investigations confirmed the data related to the antifungal influence of chitosan on the mycelial growth of *R. solani* **[56]** and several plant pathogenic fungi, such as *Fusarium solani* **[57]**. The use of chitosan in agricultural products can be recommended as a suitable substitutional to fungicides to control post-harvest diseases, moreover to maintain the quality of the fruits.

Table 8. Antifungal activity of extracted and commercial Chitosan (Mean± SE)

	Inhibition zone diameter in mm (Mean ±SE)					
Sample ID	*A. Flavus*	*A. Parasiticus*	*A. niger*	*A. Ochracious*	*F. Proliferitum*	*P. verecossum*
1	10.8±0.16^{b}	9.5±0.29^{c}	10.2±0.16^{b}	9.3±0.73^{b}	10.7±0.16^{b}	12.8±1.0^{d}
2	10.8±0.44^{b}	10.0±0.58^{c}	11.2±0.44^{b}	10.8±0.16^{b}	10.7±0.33^{b}	14.5±0.29^{cd}
3	11.5±0.5^{b}	11.8±0.44^{b}	11.5±0.50^{b}	10.3±0.73^{b}	10.5±0.76^{b}	14.8±0.44^{bc}
4	11.8±0.73^{b}	12±0.29^{b}	11.8±0.60^{b}	11.2±1.0^{b}	10.7±0.67^{b}	16.3±0.6^{b}
Negative control	0.0±0.0^{c}	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{e}
Positive control	16.7±1.0^{a}	20.3±0.88^{a}	16.0±1.0^{a}	18.0±0.29^{a}	15.2±0.73^{a}	21.8±0.44^{a}

Means with the same letter in the same column are not significantly different, 1: 5mg/ml of extracted chitosan; 2: 10 mg/ml of extracted chitosan, 3: 5mg/ml of commercial chitosan (sigma); 4: 10mg/ml of commercial chitosan (sigma)

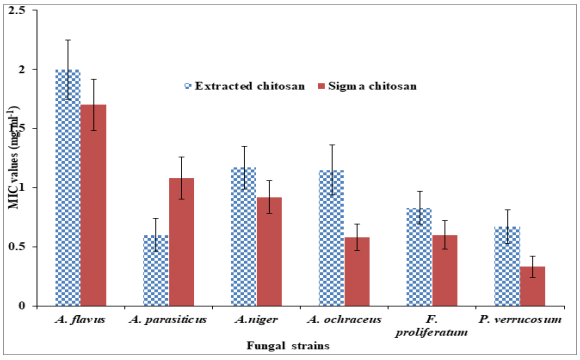

Fig 3. Minimum inhibitory concentration of extracted and commercial chitosan for tested fungi.

Conclusion

The use of submerged fermentation gave high-quality fungal chitosan by *Rhizopus oryzae pencillium expanisum*, and *Aspergillus terreus*. This technique provides an efficient and economical production which can be used in large-scale to get high-quality chitosan with high antimicrobial activity against several phyto pathogenic microorganisms. The complexity of nutrient demands is crucial for raising yields of fungal growth. As a non-toxic biodegradable material, as well as an elicitor, chitosan has the potential to suit a new class of plant protection and support towards the aim of sustainable agriculture and has shown notable activity against pathogenic bacteria and fungi strains.

Reference

[1] Malerba, M. and Cerana, R. (2018). Recent Advances of Chitosan Applications in Plants.polymer.10 (2): 118; https://doi.org/10.3390/polym10020118

[2] Omer, A. M.; Ziora, Z. M.; Tamer, T. M.; Khalifa, R. E.; Hassan, M. A.; Mohy-Eldin, M. S. and Blaskovich, M. A. (2021). Formulation of quaternized aminated chitosan nanoparticles for efficient encapsulation and slow release of curcumin. Molecules. 26(2): 449.

[3] Knorr, D. (1991). Recovery and Utilization of Chitin and Chitosan in Food Processing Waste Management. Food Technology. (45): 114-122.

[4] Tharanathan, R.N. and Kittur, F.S. (2003). Chitin - the undisputed biomolecule of great potential. Crit. Rev. Food Sci. Nutr. (43): 61-87.

[5] New, N.S.; Taylor W.F. (2004). Effect of Urea on Fungal Chitosan Production in Solid Substrate Fermentation. Process Biochemistry. (39): 1639-1642.

[6] Chiang, C.L.; Chang, C.T. and Sung, H.Y. (2003). Production and properties of chitosanase from a mutant of Bacillus subtilis IMR–NKI. Enzyme and Microb. Technol. (32): 260–7.

[7] Kučera, J. (2004). Fungal mycelium—the source of chitosan for chromatography. Journal of Chromatography B. 808(1): 69-73.

[8] Salwa, A. and Khala, F. (2004). Production and Characterization of Fungal Chitosan UnderSolid-State Fermentation Conditions. Int. J. Agri. Biol. 6 (6).

[9] Pochanavanich, P. and Suntornsuk, W. (2002). Fungal chitosan production and its characterization. Letters in Applied Microbiology. (35):17-21.

[10] Romanazzi ,G.; Feliziani, E.; Bautista-Banos. S. and Sivakumar, D. (2017). Shelf life extension of fresh fruit and vegetables by chitosan treatment. Critical Reviews in Food Science and Nutrition. 57(3):579-601.

[11] Chien, P. and Chou, C.(2006).Antifungal activity of chitosan and its application to control post-harvest quality and fungal rotting of Tankan citrus fruit (Citrus tankan Hayata). journal of the science of Food and Agriculture. 86 (12): 1964-1969.

[12] Hassan, M. A.; Omer, A. M.; Abbas, E.; Baset, W. and Tamer, T. M. (2018). Preparation, physicochemical characterization and antimicrobial activities of novel two phenolic chitosan Schiff base derivatives. Scientific reports. 8(1): 1-14.

[13] Bautista-Baños, S.; Ventura-Aguilar, R. I.; Correa- Pacheco, Z. and Corona-Rangel, M. L. (2017). Chitosan: a versatile antimicrobial polysaccharide for fruit and vegetables in postharvest – a review. Revista Chapingo Serie Horticultura. 23(2): 103-121. doi: http://dx.doi.org/10.5154/r.rchsh.2016.11.030

[14] Booth, C. (1971). Methods in Microbiology. Acad. press, London and New York. (4):795.

[15] Cheng, L.C.;Shian, T. W.; Wang, J.W.; Szu, H.W; Chung, M.H. and Yi-Ming Kuo, Cheng-H. T. (2014). Production and Isolation of Chitosan from Aspergillus terreus and Application in Tin (II) Adsorption. J. APPL. POLYMR. SCI. DOI: 10.1002/APP.40436.

[16] Nadarajah, K.; kader, j and Masri, M.M.M. (2001). Production of chitosan by fungi. pakistan journal of biological sciences.(4):263-265.

[17] Pyke, M. (1958). The technology of yeast. In "Chemistry and Biology of yeast" A. H. Cook (Ed.) 353-586. Academic press, New York.

[18] Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. T. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Analytical chemistry, 28.(3): 350-356.

[19] Egan, H.; Kirk, R.S. and Sawyer, R. (1981). Person's Chemical Analysis of Food, Eighth

Edition, Churchill Living ston, Edinburgh, London, Melbourne and New York.15-18.

[20] Kumirska, J.; Czerwicka, M.; Kaczyński, Z.; Bychowska, A.; Brzozowski, K.; Thöming, J. and Stepnowski, P. (2010). Application of Spectroscopic Methods for Structural Analysis of Chitin and Chitosan. Marine Drugs. (8): 1567-1636.

[21] Stamford, T.; Stamford ,T.L.M.; Stamford, N.P.; Barros Neto B. and Campos-TakakI, G.M. (2007). Growth of Cunninghamella elegans UCP 542 and production of chitin and chitosan using yam bean medium. Electronic Journal Biotechnology. (10):61-68.

[22] Domzy, J.G. and Roberts, A.F. (1985). Evaluation of infrared spectroscopic techniques for analyzing chitosan. Macromol. Chem. (186):1671-1677.

[23] Bauer, A.; Kirby, W.; Sheriss, J. and Turck, M. (1966). Antibiotic susceptibility testing by standardized single method. Am. J. Clin. Pathol. (45):493-496.

[24] EUCAST, (2015). Disk Diffusion Method for Antimicrobial Susceptibility Testing.European Committee on Antimicrobial Susceptibility Testing (Version 5.0). www.eucast.org.

[25] Irith, W.; Kai, H. and Robert, E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protocols. 3(2):163-175.

[26] Sokmen, A.; Gulluce, M.; Akpulat, H. A.; Daferera, D.; Tepe, B.; Polissiou, M. and Sahin, F. (2004). The in vitro antimicrobial and antioxidant activities of the essential oils and methanol extracts of endemic Thymus spathulifolius. Food control. 15(8): 627-634.

[27] SAS (1999). Statistical Analysis System, SAS / STAT User's Guide. Release 6.03 Edn. SAS Institute, Cary, NC, 1028 PP.

[28] Teng, W.L.; Khor, E.; Tan, T.K..; Lim, L.Y.and Tan, S.L. (2001). Concurrent production of chitin from shrimp shells and fungi. Carbohydr. Res. 332:305–316.

[29] Guha, A.K.; Chatterjee, B.P.; Chatterjee, S. and Adhya, M., (2005) Chitosan from Mucor Rouxii: Production and Physico-Chemical Characterization. Process Biochemistry. (40): 395-400.

[30] Habibi, A.; Karami, S.; Varmira, K. and Hadadi1, M. (2020). Key parameters optimization of chitosan production from Aspergillus terreus using apple waste extract as sole carbon source. Bioprocess and Biosystems Engineering. (44):283–295.

[31] New, N. and Stevens, W.F. (2002) Chitosan Isolation from the Chitosan-Glucan Complex of Fungal Cell Wall Using Amylolytic Enzymes. Biotechnology Letters. (24): 1461-1464.

[32] Senthilkumar, V.;Yesodha, P. and Krishnaveni, B.(2015). Extraction of chitosan from Aspergillus terreus sps, study of its antimicrobial and dye degradation ability. International Journal of Phytopharmacology. 6(4):196-202.

[33] Viccini, G. (2001). Analysis of Growth Kinetic Profiles in SSF. Food Technol. Biotechnol., 39(40): A-B.14.

[34] Maghsoudi, V.; Yaghmaei, S. and Beygi, S. (2008). Influence of different nitrogen sources on amount of chitosan production by Aspergillus niger in solid state fermentation. Iran J. Chem. Eng. (27):47–52.

[35]Cardoso, A.; Lins, C.I.M.; Santos, E.R.; Silva, M.C.F. and Campos-Takaki, G.M. (2012). Microbial enhance of chitosan production by Rhizopus arrhizus using agro- industrial substrates. Molecules. (17): 4904-4914.

[36] Leite, M. V.; Stamford, T. C. M.; Stamford-Arnaud, T. M.: Lima, J. M. N.; Silva, A. M., Okada, K. and Campos-Takaki, G. M. (2015). Conversion of agro-industrial wastes to chitosan production by Syncephalastrum racemosum UCP 1302. International Journal of Applied Research in Natural Products. 8(4): 5-11.

[37] Abasian, L.; Shafiei Alavijeh, R.; Satari, B. and Karimi, K. (2020). Sustainable and effective chitosan production by dimorphic fungus Mucor rouxii via replacing yeast extract with fungal extract. Applied biochemistry and biotechnology. 191(2): 666-678.

[38] VishuKumar, A. B.;Varadaraj, M. C.;Gowda, L. R. and Tharanathan, R. N.(2005). Characterization of chitooligosaccharides prepared by chitosanolysis with the aid of papain and pronase, and their bactericidal action. The Biochemical Journal. (391): 167-175.

[39] Synowiecki, J. and Ali-Khateeb, N.A.A.Q. (1997). Mycelia of Mucor rouxii as a Source of Chitin and Chitosan. Food Chemistry, 60, 605-610.

[40] Arcidiacono, S. and Kaplan, D.L. (1992). Molecular Weight Distribution of Chitosan Isolated from Mucor rouxii under Different Culture and Processing Conditions. Biotechnology and Bioengineering. (39): 281-286.

[41] Vaingankar, P.N. and Juvekar, A.R. (2014). Fermentative Production of Mycelial Chitosan from Zygomycetes: Media Optimization and Physico-Chemical Characterization. Advances in Bioscience and Biotechnology. (5): 94

[42]Baxter, L. R.; Schwartz, J. M.; Bergman, K. S.; Szuba, M. P.; Guze, B. H.; Mazziotta, J. C., and

Phelps, M. E. (1992). Caudate glucose metabolic rate changes with both drug and behavior therapy for obsessive-compulsive disorder. Archives of general psychiatry. 49(9): 681-689.

[43] Kasaai, M.R.; Arul, J. and Charlet, G. (2000). Intrinsic Viscosity-Molecular Weight Relationship for Chitosan. Journal of Polymer Science Part B: Polymer Physics. (38): 2591-2598.

[44] Khan, T.A.; Peh, K.K. and Ch'ng, H.S. (2002). Reporting Degree of Deacetylation Values of Chitosan: The Influenceof Analytical Methods. Journal of Pharmacy and Pharmaceutical Sciences. (5): 205-212

[45] Rial, M.; Cortizas, A. M. and Rodríguez-Lado, L. (2015). A novel approach to map soil organic carbon content using spectroscopic and environmental data. Procedia Environmental Sciences. (27): 49-52.

[46] Crestini, C.; Kovac, B. and Giovannozzi-Sermanni, G. (1996). Production and Isolation of Chitosan by Submerged and Solid-State Fermentation from Lentinusedodes. Biotechnology and Bioengineering. (50): 207-210.

[47] Islam, S.Z.; Khan, M.; Nowsad Alam, A.K.M.; Kaku, H.; Shibuya, N.; Xu, P.; Aryan, A.P. and Fincher, G.B. (1997). N-acetylchitooligo saccharide elicitor expression of a single 1 ,3-β-glucanase gene in suspension-cultured cells from barley (Hordeumvulgare). Physiol. Plant. (100): 111–118.

[48] EL Ghaouth, A.; Arul, J.;Grenier, J. and Asselin, A.(1992). Antifungal activity of chitosan on two pathogens of strawberry fruits.phytopathology. (82):398-402.

[49] El Hassni, M.; El Hadrami, A.; Daayf, F.; Chérif, M.; AitBarka, E. and El Hadrami, I. (2004). Chitosan, antifungal product against Fusarium oxysporum f. sp. Albedinis and elicitor of defense reactions in date palm roots. Phytopathol. Mediterr. (43): 195–204.

[50] Uppal, A.K.; El Hadrami, A.; Adam, L.R.; Tenuta, M.; Daayf, F. (2008). Biological control of potato Verticillium wilt under controlled and field conditions using selected bacterial antagonists and plant extracts. Biol. Control. (44): 90–100.

[51] Benhamou, N.; Lafontaine, P.J. and Nicole, M. (1994). Seed treatment with chitosan induces systemic resistance to Fusarium crown and root rot in tomato plants. Phytopathology. (84): 1432–1444.

[52] Jeihanipour, A.; Karimi, K. and Taherzadeh, M. J. (2007). Antimicrobial properties of fungal chitosan. Research journal of biological sciences. 2(3): 239.

[53] Logesh, A. R.; Thillaimaharani, K. A.; Sharmila, K.; Kalaiselvam, M. and Raffi, S. M. (2012). Production of chitosan from endolichenic fungi isolated from mangrove environment and its antagonistic activity. Asian Pacific Journal of Tropical Biomedicine. 2(2): 140-143.

[54] Joes Carlos, M. H.; Stefani, P. V. Y.; Janette, A. M.; Melani, M. S. S. and Gabriela, P. O. (2016). Assessing the effects of heavy metals in ACC deaminase and IAA production on plant growth-promoting bacteria. Microbiological research. (188): 53-61.

[55] Nguyen, V. Q.; Ishihara, M.; Mori, Y.; Nakamura, S.; Kishimoto, S.; Fujita, M. and Matsui, T. (2013). Preparation of size-controlled silver nanoparticles and chitosan-based composites and their anti-microbial activities. Bio-medical materials and engineerin. 23(6): 473-483.

[56] Struszcyk, M.H. (2002). Chitin and chitosan. part II .Application of chitosan.polymers.47(6): 396-403.

[57] Sabah R. M.; Elsayed, M. Z. and Ivan D. E. (2019). Inhibition of Mycelial Growth of RhizoctoniaSolaniby Chitosan in vitro and in vivo. The Open Agriculture Journal .Content list available at: https://openagriculturejournal.com