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Chitosan Production from *Aspergillus oryzae* SU-B2 by submerged fermentation and studying some of its Physiochemical and antibacterial Characteristics

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Abstract

The present study was focused on production of chitosan from local isolated fungi by submerged fermentation. Ten fungal isolates were screened and A2 isolate was found to produce maximum yield of chitosan (352 mg/L) with dry biomass (9.48 g/L). Fungal chitosan production was confirmed by structure analysis according to FTIR spectra. A2 isolate has been identified and characterized according to genetic analysis using PCR technique to amplify internal transcript spacer ITS1 and ITS4 in 5.8S rRNA gene. PCR amplicons were analyzed by DNA sequencer and the alignment of nucleotides sequencing were achieved. It was found that the isolate has similarity 99 % in comparison to the sequence of Aspergillus oryzae HQ285580 in data base of NCBI gene bank. According to IR spectroscopic analysis , the degree of deacetylation of produced chitosan from Aspergillus oryzae SU-B2 by submerged fermentation was 55.23% , the viscosity of chitosan was 3.7 cP and Molecular weight was 3.6×10^5 Da. This study has been conducted to assess antimicrobial activity of fungal chitosan. Result shown that maximum inhibition zone against *Salmonella typhimurium* was (20mm) followed by *Staphylococcus aureus* (8mm), *Candida albicans* (6mm), the lowest inhibition zone gave by *Escherichia coli* and *Bacillus subtilus* (2mm)

Keywords : chitosan , Aspergillus oryzae SU-B2, Submerged fermentation , FTIR spectra , ITS

INTRODUCTION

Chitosan, is a deacetylated derivative form of chitin, is a natural amino polysaccharide formed from copolymers of D-glucosamine and N-acetyl D-glucosamine [1, 2]. Chitosan is considering the second natural polymer after cellulose, nontoxic, polycationic and biodegradable, exhibiting various applications properties in food, waste water treatment, biomedical and cosmetics industry [3,4].

Chitosan production from crustacean shell waste needs deacetylation of chitin which is require strong alkali treatment, highly temperature and prolonged processing time, that's make chitosan process costly [5]. Recent researches have been carried out that many of these problems can be overcome by mycelial chitosan, predominately *Zygomycetes* species which contain chitin and chitosan as a natural polymer in the cell wall component [6,7]. Mycelia of various fungi are also conducted for chitin isolation: *Allomyces*, *Aspergillus*, *Penicillium*, *Fusarium*, *Mucor*, *Rhizopus*, *Choanephora*, *Phycomyces*, *Zygorrhynchus*, and *Thamnidium* [8]. Fungal chitosan produced through enzymatic deacetylation of chitin chain during common step in the modification of sugar chains by action of N-deacetylation [9]. The advantages of using fungi for chitosan production are the easy obtained of fungal mycelium, cultured on cheap nutrients, cell wall recovered by simple chemical steps, demineralization treatment not required and controlling to produce consistent properties of chitosan [10, 11].

According to the above advantages, the objective of present work is mainly focused to screen different fungal isolates for the highest production of chitosan by submerged fermentation method. Furthermore, characterization of physiochemical properties and antimicrobial activity of produced fungal chitosan.

MATERIALS AND METHODS

Materials

All chemical solutions were obtained from Fluka (Switzerland).primers were purchased from IDT (Integrated DNA Technology company (Canada). All microbial culture media were procured from HIMEDIA (India).

Microorganism isolation and its maintenance

Ten isolates of fungi, namely (A1, A2, A3, A4, A5, A6, A7, A8, A9 and A10) were isolated from different sources (tomato, watermelon, peaches, zucchini, spoilage bread, yoghurt and soil). All isolates were cultured on sterilized PDA plates supplemented with 50 ppm chloramphenicol, grown at 30˚C for 5 1o 10 days and then subcultured on sterilized SDA slants as pure isolate. Isolates stored at 4˚C and maintain monthly basis for further experiments.

Microbial production of chitosan

Inoculum preparation

The cultures were allowed to grow for 5 to 10 days and the spores were collected by flooding with physiological saline solution (0.85 %), and harvested the spore suspension in a sterile tube. The spores were implemented to rinse twice with sterile distilled water by interchange centrifuging at 1500g for 5 minutes and gentle vortexing. Spores were counted by heamocytometer and the number was adjusted to 1×10^7 spores/ ml.

Fermentation

Submerged fermentation was followed by inoculated 10 ml of spore suspension (1×10^7 spores/ ml) in Erlenmeyer flasks (500ml) containing 300ml of Yeast-Peptone Glucose (YPG) broth (glucose 3g, yeast extract 0.3g, peptone 3g, $(NH_4)_2SO_4$ 1.5g, NaCl 0.3g, $CaCl_2$ 0.03g, $MgSO_4.7H_2O$ 1.5g in distilled water at pH 5.0). Each submerged flasks were incubated in shaking incubator (150rpm) at 30˚C for

96 hrs. Mycelium cell mass obtained was separated by vacuum filter using Whatman No.1 filter paper and the separated mass was dried in an oven at 65˚C until constant weight, biomass dry weight was calculated as the fallowing [12].

$$biomass\ dry\ weight(g\ /l\ ) = \frac{mass\ dry\ weight}{size\ of\ medium\ (ml)} \times 1000.$$

Chitosan extraction

The fungal mycelia were collected and treated (alkali treatment) with 1N NaOH **(**1: 40 w/v) of mycelium dry weight and homogenized, and then content was sterilized at 121˚C for 20 minutes. The alkali insoluble materials (AIMs) were harvested by centrifugation at 6000 Xg for 20 min. and then washed repeated times with distilled water to neutralize them (pH 7). AIMs were dried in an oven at 40˚C, the dried content were treated with acetic acid 3% **(**1: 40 w/v) in a reflux condenser for 12 h at 95˚C., the acid insoluble fraction was separated by centrifugation at 6000 Xg for 15 min., the chitosan represented supernatant was isolated. The pH was adjusted to 10 with a 2N NaOH solution in order to precipitate the mycelium chitosan. The flocculated chitosan was centrifuged at 6000 Xg, for 15 minutes. Separated chitosan was washed several times with distilled water to neutralize it. Also, ethanol (96%) and acetone were implemented to rinse the chitosan and then dried in a vacuum oven dryer at 60˚C to constant weight. production efficiency was estimated according to quantity of chitosan production [13].

Infra-Red Spectroscopy

Fungal chitosan structure was confirmed by IR spectroscopy using KBr pellet method in FTIR (FTIR 600, Biotech .Eng . Mangement Co.Ltd). In FTIR spectra were recorded in the middle infrared (4000 cm^{-1} to 400 cm^{-1}). The fungal chitosan sample was prepared by grinding the chitosan powder with powdered KBr, in the ratio of 1:5 (sample: KBr) and then compressed the mixture using a hydraulic device stressful of FTIR with 8 bar pressure for 60 seconds and subjected to FTIR analysis[Kumirska et al. 2010].

Identification of isolate

Selected isolate was morphological identified under microscopic examination, used microscopical properties such as conidial heads, degree of sporulation, fruiting bodies and identity of conidiogenous cells by light microscope after staining by lactophenol blue solution [14]. Fungal genomic identification was done by using 5.8S rRNA gene sequencing, DNA extraction was performed based on manufacturer's instructions of DNA purification Kit (ZYMO RESEARCH).

Sample was amplified with polymerize chain reaction (PCR) Multi Gene Optimax Gradient by using Master Mix (Intron, Korea). The internal transcribed spacer (ITS) primer sets: (Forward primer ITS1 5'-TCCGTAGGTGAACCTGCGG-3') and (Revere primer ITS4
5'-TCCTCCGCTTATTGATATGC-3').

The product was purified using PCR product purification kit (PCRquick-spin™ CAT. 17202). The PCR product (amplicons) was sequenced commercially by Macrogen-Korea forward and reverse primers. Sequence data were aligned with the public database available Gen Bank resources using National Center for Biotechnology Information (NCBI-BLAST) [15].

Characterization of fungal chitosan

Degree of deacetylation (DD)

The DD was estimated according to the IR spectrum by measuring the absorbance ration of A_{1655} cm^{-1} which represent the amide I band and A_{3450} cm^{-1} which represent the hydroxyl group band, and used as internal standard reference. The equation proposed for determination of degree of deacetylation is as follow [13].

DDA % = 100 – [A_{1655}/A_{3450}] x 100/1.33

Determination of Viscosity and Molecular Weight

Viscosity of chitosan was estimated using Brookfield viscometer (Model DV-II + Brookfield Engineering Laboratories, Inc., Stonghton, MA.).Chitosan solution was prepared in 1% acetic acid at a 1% concentration, stirred until be soluble. Measurement was made using a No. 5 spindle at 50 rpm on solution at 25^{o}C with values reported in centipoises (cPs) units [2]. The viscosity- average molecular weight of fungal chitosan solution was calculated using the Mark Houwink equation as follow [Akila 2014]:

$\eta = k\ [m_w]^a$

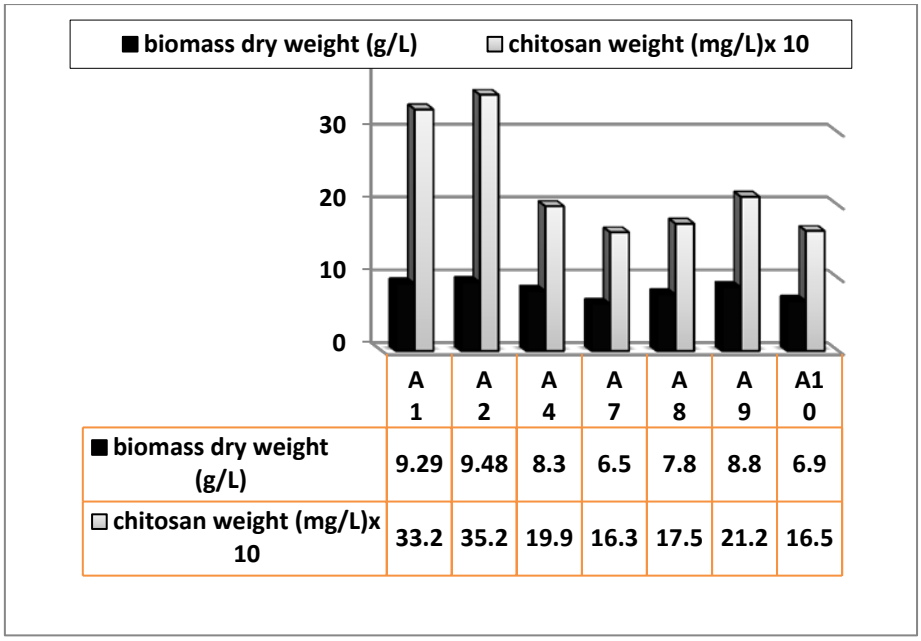
a = 0.79, k = 1.49× 10^{-4} (dl/gm), ŋ = (cPs), M_w = Molecular weight (Dalton)

Antimicrobial Activity of chitosan

Antimicrobial activity of fungal chitosan was analyzed by disc diffusion method, against pathogenic microorganisms obtained from biotechnology research center at Al-Nahrain University. *Escherichia coli* subcultured on (MAC agar), *Salmonella typhimurium* subcultured on (SS agar) *Staphylococcus aureus* subcultured on (NA), *Bucillus subtilus* subcultuerd on (NA), *Candida albicans* subcultuerd on (SDA), each strain was uniformly spread on plate using sterile cotton swab. A Chitosan solution was prepared in 1% acetic acid at a concentration of 100 mg/ml. Discs soaked in chitosan solution for 24h and were added to plates. Each plate was incubated for 24h at 37°C for bacterial growth and 72h at 25°C for yeast, the zone inhibition of growth was measured in mm [16].

RESULTS

Screening of fungal isolates for chitosan producers are presented in Fig. 1. In our screening among ten fungal isolates, we established that A2 isolate gave maximum chitosan production (352 mg/L) with dry biomass (9.48 g/L) followed by A2 isolate (332 mg/L) with dry biomass (9.29 g/L). The FTIR spectrum of fungal chitosan from A2 isolate obtained from submerged fermentation was shown in Fig.2. The most important and significant bands of these spectra are the amide bonds at approximately 1655cm-1 and hydroxyl stretching band at 3440 cm-1. The band at 862 cm-1 was represented as glycosidic linkage of β-anomer. These bands were proposed as internal standard reference of chitosan



	A1	A2	A4	A7	A8	A9	A10
biomass dry weight (g/L)	9.29	9.48	8.3	6.5	7.8	8.8	6.9
chitosan weight (mg/L)x 10	33.2	35.2	19.9	16.3	17.5	21.2	16.5

Fig. 1 screening of fungal isolates for chitosan and biomass dry weight production.

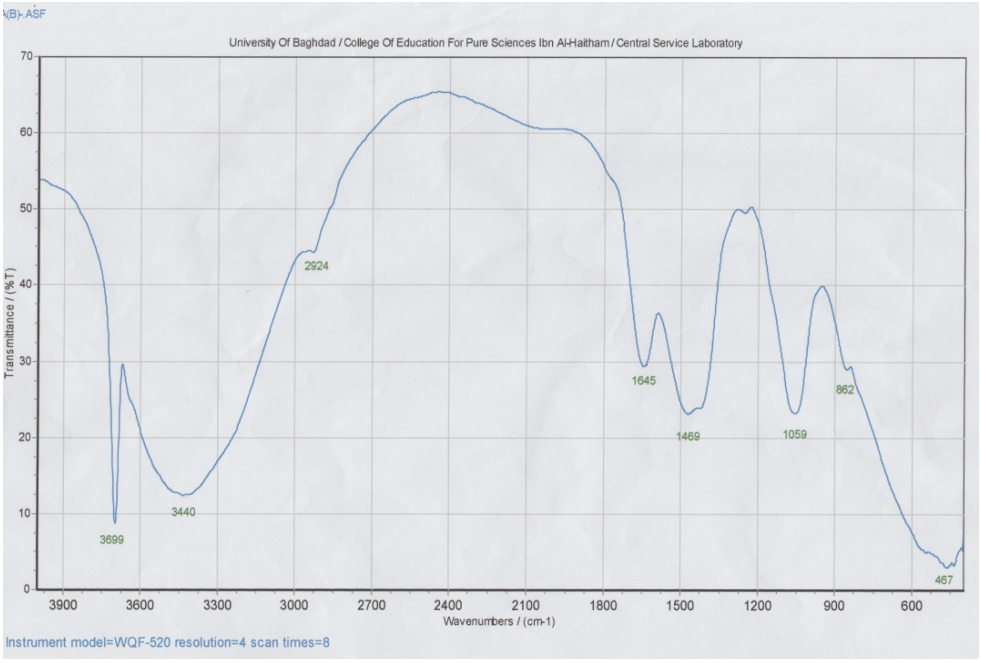


Fig.2 FTIR spectra of fungal chitosan obtained from *Aspergillus oryza* SU-B2 by submerged culture fermentation.

The colony and cell morphology features of the A2 isolate was observed, after 7 days colony spreading quickly on PDA culture with incubation at 30°C, floccose, grayish yellow to olive. Vesicles pyriform to subglobose, conidia was globose and smooth, colony texture was wet. Molecular identification by 5.8S rRNA gene sequencing. The homology of the partial 5.8S rRNA gene sequences of the isolate was performed using the BLAST algorithm in NCBI. Alignment comparison of the 5.8S rRNA sequence with those of the other fungal isolates Fig.3 confirmed the high similarity 99% to *Aspergillus oryza* SU-B2.

According to IR spectroscopic analysis, the obtained result indicated that, degree of deacetylation of produced chitosan from *Aspergillus oryzae* SU-B2 was determined to be 55.23%. The viscosity of produced chitosan from submerged fermentation was 3.7 cP. Molecular weight is one of the key factors prevailing functional properties of chitosan, in present study the molecular weight of chitosan was 3.6×10^5 Da. This study has been conducted to assess antimicrobial activity of fungal chitosan (Table 1), result shown that maximum inhibition zone against *Salmonella typhimurium* was (20mm) followed by *Staphylococcus aureus* (8mm), *Candida albicans* (6mm), the lowest inhibition zone gave by *Escherichia coli* and *Bucillus subtilus* (2mm).

Score	Expect	Identities	Gaps	Strand
857 bits(464)	0.0	468/470(99%)	0/470(0%)	Plus/Plus

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Query 1      CAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTG 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 118    CAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTG 177

Query 61     AGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 178    AGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCG  237

Query 121    ATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGA 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 238    ATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGA 297

Query 181    GTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCAT 240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 298    GTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCAT 357

Query241   TGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjc358      TGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGGACGGG  417

Query 301    CCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 418    CCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGC  477

Query 361    TCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCCAGGTTGACCTCGG  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 478    TCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCCAGGTTGACCTCGG  537

Query 421    ATCAGGTAGGGATACCCGCTGAACTTAAGCATATCATAAGGCCGGAGGAA  470
             ||||||||||||||||||||||||||||||||||||||||||||| ||||| ||||||||||||
Sbjct 538    ATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAAAAGCCCGGAGAAA  587
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Fig. 3 Alignment of nucleotides sequence of ITS 1 of 5.8S rRNA gene of local isolate A2 (Query) in comparison with nucleotides sequence of standard isolate of *Aspergillus oryzae* SU-B2 in NCBI.

Table 1 Size of inhibition zone for chitosan produced from *Aspergillus oryzae* SU-B2 against selected Microorganisms

Microorganisms	Chitosan inhibition zone (mm)	Acetic acid 1% inhibition zone
Salmonella typhimurium	28	8
Escherichia coli	10	8
Staphylococcus aureus	29	21
Bacillus cereus	13	11
Candida albicans	8	2

DISCUSSION

Chitosan is produced in the fungal cell wall by deacetylation of its precursor, chitin, the high ratio of fungal chitosan is due to the active growth [17]. So the process of screening is necessary to determine the most efficient fungi produced chitosan. The absorbance of amide I band and hydroxyl group band represent introductory attained for product to be chitosan [18]. The DD of chitosan produced from *Aspergillus oryzae* SU-B2 was slightly lower than values of DD from other fungi [19, 20]. The degree of deacetylation is an influential parameter simulating the physicochemical properties of chitosan and high DD has high positive charge and suitable for food processing application [21]. The molecular weight of fungal chitosan depends on kind of fungi and fermentation process such as by solid state fermentation or submerged fermentation [17]. The lower viscosity and molecular weight of chitosan show efficient antimicrobial assay especially against bacterial pathogen and widely used for pharmaceutical and cosmetic products [22]. In general the molecular weight from fungi lower than from shrimp shell [23].it is clear to notes the antimicrobial activity of chitosan

produced from *Aspergillus oryzae* SU-B2 with variable effect against selected microorganisms. Antimicrobial activity of chitosan influence by several factors such as degree of deacetylation, molecular weight, pH of medium and temperature [24]. Chitosan has shown sufficient antimicrobial activity against several pathogenic and spoilage microorganisms including gram-positive and gram-negative bacteria [25].

Conclusion

In conclusion, this study was conducted for screening of fungal isolates for chitosan producers and we found ascomycetes *Aspergillus oryzae* SU-B2 produced maximum yield of chitosan. Fungal chitosan production was confirmed by structure analysis according to FTIR spectra. Some of physiochemical characterization was determined. The DD of chitosan produced from *Aspergillus oryzae* SU-B2 was slightly lower than reported values of DD from other fungal chitosan. The lower viscosity and molecular weight of produced chitosan shown effective antimicrobial assay especially against *Salmonella typhimurium* followed by *Staphylococcus aureus* and *Candida albicans.*

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