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Production, Characterization and Antibacterial Activity of *Mucor rouxii* DSM-119 Chitosan

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Abstract

The production, purification and antibacterial effect of chitosan from *Mucor rouxii* strain DSM-1191 were investigated throughout current study. Chitosan was characterized by infrared spectroscopy, titration and average molecular weight. Furthermore, the antibacterial activity of chitosan produced from *Mucor rouxii* DSM-1191, against *Escherichia coli* and *Micrococcus leutus* was determined. Scanning electron micrographs of treated *Micrococcus leutus* with fungal chitosan are also presented.

Keywords: *M. rouxii*; Chitosan polymer; Antibacterial activity

Introduction

Chitosan is a cationic biopolymer consisting of β-(1→4) linked N-acetyl-D-glucosamine prepared from chitin by deacetylation with alkaline solution as shown in (Figure 1).

Over the last several years, chitinous polymers, especially chitosan, have received increased attention as one of the promising renewable polymeric materials for their extensive applications in the food industries (preservative, antimicrobial, coating, antioxidant) [1], cosmetology (hair additives, lotions, facial and body creams) [2,3], biotechnology (emulsifier, chelator, flocculent) [4], agriculture (fungicide, films, soil modifier, elicitor) [5, 6] in addition to its pharmacology and medicine uses (fabrics, fibers, artificial organs, drugs, membranes) [7,8].

Chitosan can also be found in the cell wall of certain groups of fungi, particularly zygomycetes. It is a straight chain natural hydrophilic polysaccharide having a three dimensional α-helical configuration stabilized by intramolecular hydrogen bonding [9]. The production of chitin and chitosan from fungal sources has gained increased attention in recent years due to their potential advantages such as: independence of seasonal factor, wide scale production, simultaneous extraction, extraction process is simple and cheap which resulting in reduction in time and cost required for production [10] and also absence of proteins contamination, mainly proteins that could cause allergy reaction in individuals with shellfish allergies [11, 12]. In addition, fungi can be grown easily on any simple medium or industrial by products therefore liberating the production of chitosan from the dependence on the seasonal shellfish industry [13].

The object of the current study is to produce chitosan using *Mucor rouxii* DSM- 1191as a source with an attempt to study physochemical properties of the prepared chitosan as a natural antibacterial agent against a gram-negative bacterium *Escherichia coli* and a gram–positive *Micrococcus lutues* strains.

Experimental Section

Materials

Fungal strain, *M. rouxii* DSM-1191 was grown in Yeast Peptone Glucose (YPG) Broth (YPG; Merck, Darmstadt, Germany) at 28°C for 72 h under shake incubation condition. Mycelial growth was harvested by centrifugation, washed twice with distilled water and then homogenized with 2% 1mol. NaOH at 90°C for 2 h. The alkali insoluble fraction was separated, washed with distilled water and neutralized with 10% acetic acid.

Characterization of produced chitosan

The physico-chemical characteristics of produced chitosan, after different treatments in their preparation, were determined according to [14], whereas the molecular weights of prepared chitosan were determined by gel permeation chromatography (GPC) using refractive index detector (PN-1000, Postnova Analytics, Eresing, Germany). The method of [15] was applied for determination of the degree of deacetylation of produced chitosan.

Microbial strains

Escherichia coli DSMZ-498 and *Micrococcus lutues* ATCC-9341 were examined for their susceptibility and sensitivity toward the treatment with produced chitosan.

Antimicrobial activity of chitosan

Disk diffusion method: In our study, the antibacterial activity of the series of chitosan against gram negative bacterium *E. coli* DSMZ 498 and gram positive bacterium *Micrococcus lutues* ATCC 9341 were evaluated by zone of inhibition test.

Plate count agar: Different concentrations 0.1, 0.2, 0.4 and 0.5% of *M. rouxii* chitosan, were separately added into 200mL flasks containing 40mL, Yeast peptone glucose (YPG) broth. The sterilized flasks were cooled at 45°C then inoculated with 0.5 ml from cells of each test microbe (2×10^5 cell/ml). The inoculated flasks were incubated at 30°C for 24h. Surviving culture cells were counted by spreading on

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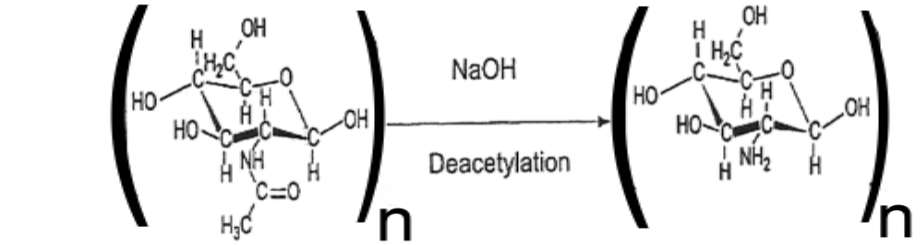


Chitin
2-acetamido-2-deoxy-β-D-glucose-(N-acetylglucan)

Chitosan
2-acetamido-2-deoxy-β-D-glucose-(N-acetylglucosamine)

Figure 1: The deacetylation process of chitin.

nutrient agar (NA) plates. The inhibition ratios were calculated with the following formula:

Inhibition ratio (%) = (C – E) / C × 100

Where C is the average number of the surviving cells of the control groups (zero chitosan concentration), E is the average number of the surviving cells of the chitosan concentrations.

Tetrazolium/formazan-test method: In the presence of bacteria, TTC is reduced to red formazan. The red formazan obtained indicates the activity and viability of the cells [16]. Therefore, the TTC- test method is considered a fast method for evaluating the antibacterial activity of chitosan. To do this test 100µl of dissolved chitosan in 1% acetic acid was poured in 40ml nutrient broth medium containing 20 µl of (10^8 cfu/ml) challenge microorganisms *E. coli* and *Micrococcus lutues*) as an inoculums volume. Chitosan-free solution was used as a blank control., then all flasks were incubated with shaking at 37 °C/200 rpm for 3 h, then 1 ml from each flask containing the treated and the control was added to sterilized test tubes containing 100 µl TTC (0.5 % w/v). All tubes were incubated at 37 °C for 20 min. The resulted formazan was centrifuged at 4000 rpm for 3 min followed by decantation of the supernatants. The pellets obtained were resuspended and centrifuged again in ethanol. The red formazan solution obtained at the end which indicated the activity and viability of the cells was measured by photometer at 480 nm.

Micrograph capture: Toward the explanation of chitosan antimicrobial action, micrographs of treated *Micrococcus lutues* with fungal chitosan was captured using scanning electron microscope (Topcon-Microscope (ATB-55), Hitachi, Japan), after 1 and 5h from the treatment of *Micrococcus leutus* with chitosan , as well as control culture. Samples were prepared for scan electron microscope (SEM) as described by [3].

Results and Discussion

Fungal chitosan production and analysis

The growth of *M. rouxii* as well as chitosan production was dependent on the nutrients used and chemico-physical environment of the fermentation medium [17]**.** In this study the maximum value of the dry cell biomass of *M. rouxii* DSM-1191 strain under the fermentation condition with YBG culture medium at 28°C, 200 rpm was 24.7 g (of dry mycelia per litre of medium) after 48 h of incubation the amount of the biomass decreased as the amount of nutrients decreased.

Extracted chitosan from the fungal mycelia after 48 h of fermentation reached 0.78 g/l of dry mycelia weight. The decline of the extractable chitosan after 48 h of fermentation could be due to physiological changes in the fungal cell wall during the incubation period.

Results in (Table 1) show that growth curve and biomass yield of *M. rouxii* DSM-1191 increased rapidly up after 48 h of incubation; the maximum biomass yield during this time was 24.7 g of dry mycelia per litre after that the growth slowed down. In the case of chitosan, the yield increased with incubation time, the maximum yield of chitosan reached during this time was 0.78 g/l dry mycelia weight after 48h followed by decline when the culture was incubated after this point.

The main components of the mycelia are water, protein and alkali insoluble fraction containing chitin, chitosan and acidic polysaccharides [18]. During the incubation time the analysis of purified *M. rouxii* mycelia revealed that the protein contents gradually decreased from 6.35 to 5.85% on a dry weight basis of the mycelia (Table 2).

The degree of deacetylation has been found to influence the physical and chemical properties and biological activity of chitosan. The degree of deacetylation value for chitosan isolated from *M. rouxii* was 86%, this result slightly different from the reported percentage degree of deacetylation of chitosan obtained from other fungal mycelia ranged from 65-95% [19,20].

Growth time (H)	Mycelial yield (g/l)	Chitosan yield (g/l)
12	4.8	0.22
24	10.1	0.51
48	12.9	0.78
72	24.7	0.74
84	16.3	0.61

Table 1: Mycelial biomass and chitosan production from *M. rouxii* DSM-1191 grown in YPG medium at 28°C.

Component	Incubation Time (hours)			
	12	24	48	72
Water (%)*	81.1	82.4	85.1	80.9
Proteins (%)*	6.35	6.26	6.12	5.85

* % on a dry basis of mycelia

Table 2. Changes in the mycelial component (water, protein content) during the incubation time of *M. rouxii* DSM-1191

Figure 2 showed the FTIR spectrum of chitosan from *M. rouxii* in compare with standard chitosan from Sigma. IR spectrum of chitosan was carried out using the KBr disc method. The main characteristic peaks of chitosan are at 3455 (-OH stretch), 2867 (C-H stretch), 1589 (N-H bend), 1154 (bridge O stretch), and 1094 cm^{-1} (C-O stretch). From the spectra there is no qualitative difference in peak locations between the standard chitosan and the fungal chitosan produced from *M. rouxii* strain especially in the amino characteristic peak at about 1589 cm^{-1}.

The molecular weight of *M. rouxii* chitosan was 2.1×10^4 (Da) and this may be lower than that of crab chitosan.

Surface topography of cotton fabrics

The Surface of chitosan coated cotton fabrics were morphologically observed by scanning electron microscope (SEM) to investigate changes in the topography of the cotton fabrics Figure 3 shows the SEM micrograph of a sample of treated cotton, where the surface with no agglomerated particles are visible on the surface which indicates a homogeneous distribution of the chitosan in the coating layer and the absence of unwanted agglomeration during formation of the resulting coatings.

Assays for antibacterial activity

Antibacterial activity of *M. rouxii* DSM-1191 chitosan was

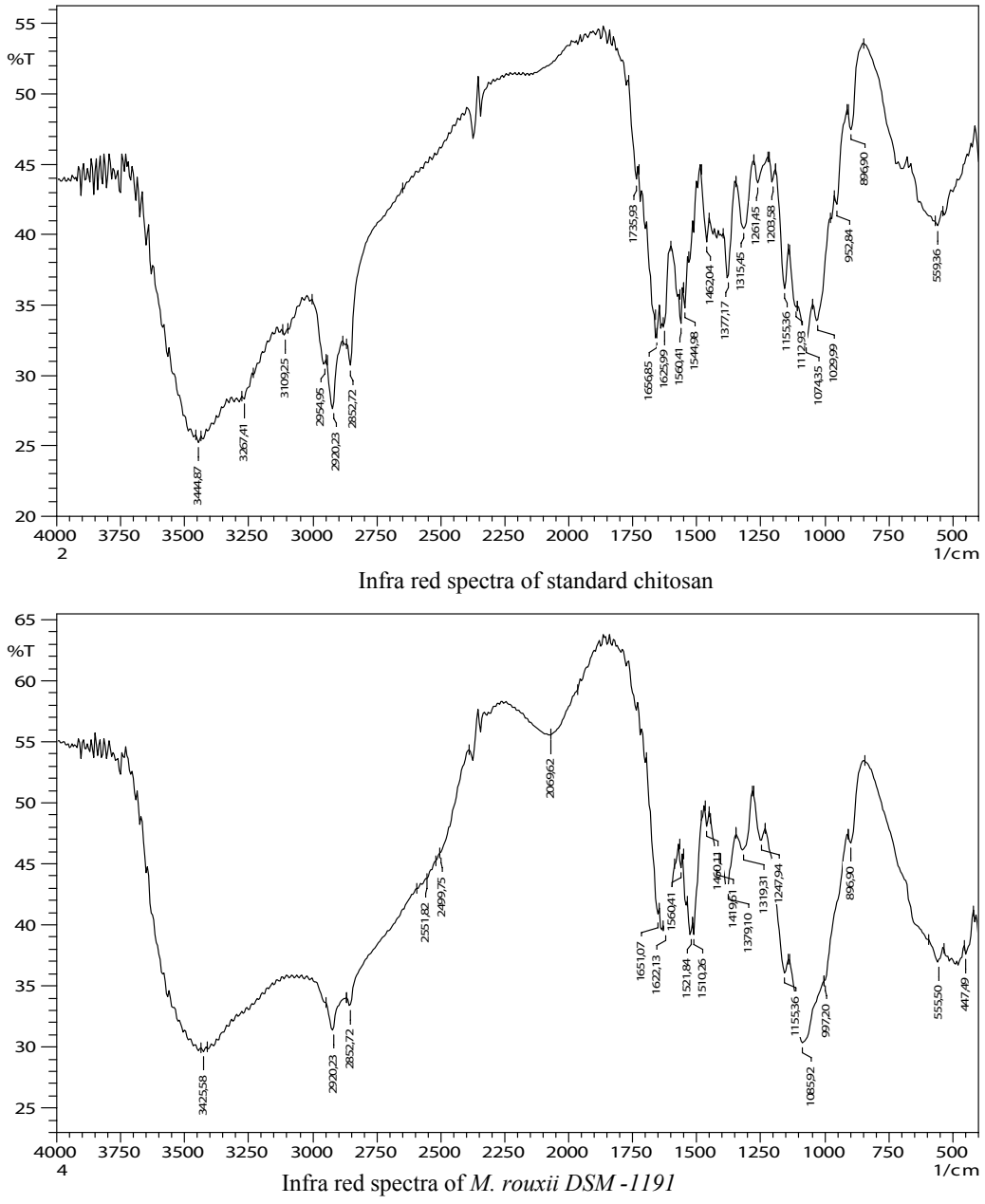


Figure 2: Infra red spectra of fungal chitosan from *Mucor rouxii* DSM-1191 (A) and standard chitosan (B).

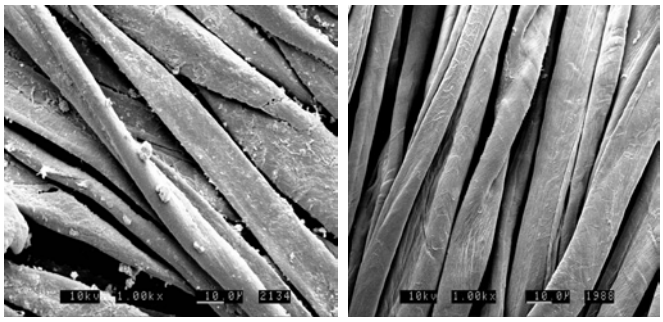
examined against the *E. coli* DSMZ-498 and *Micrococcus lutues* ATCC-9341 in the form of inhibition zones, evaluated by the disc diffusion assay, as shown in (Figure 4). The bactericidal activity against *E. coli* and *M. lutues* resulted in a clear zone of inhibition within and around the samples impregnated with different concentrations of chitosan. The antibacterial activity and inhibitory levels of chitosan have been shown to be significantly dependent on the degree of deacetylation (DD), and molecular weight (MW) [21].

Both photographs show the clear zone of inhibition around the discs impregnated with different concentrations of chitosan compared with untreated discs.

Using total count agar method, chitosan showed an excellent antimicrobial activity for both gram–negative bacterium *E. coli* and gram-positive bacterium *Micrococcus luteus*. Chitosan showed more effective suppression against Gram-positive bacterium *Micrococcus luteus* comparing to Gram-negative bacterium *E. coli*.

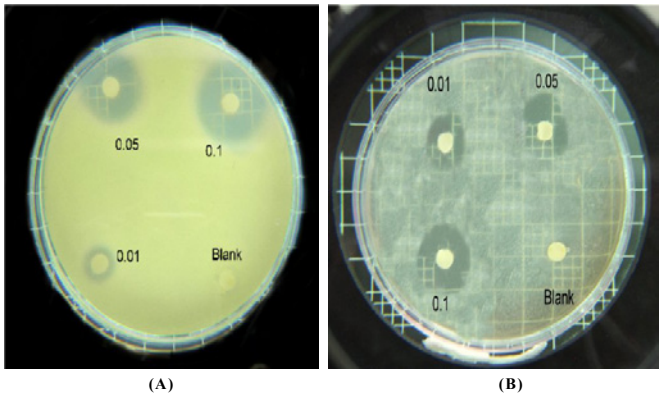
As shown in (Figure 5), treatment for 5 h with chitosan leads to 95% and 98% inhibition of *E. coli* and *M. luteus* growth respectively.

In the TTC test method the antibacterial activity of chitosan was estimated for the *E. coli* and *Micrococcus luteus,* This test serves as indicating system for the determination of the viability of microorganisms, since absorbance of formazane is directly proportional to the amount of living bacteria as shown in (Figure 6)**.**



(A) (B)

Figure 3: SEM micrographs of: (A) blank cotton fabric, (B) cotton fabric treated with chitosan.



(A) **(B)**

Figure 4: Disc diffusion test of different concentrations of chitosan (%) for the growth inhibition of: *Micrococcus luteus* (A) and *E.coli* (B).

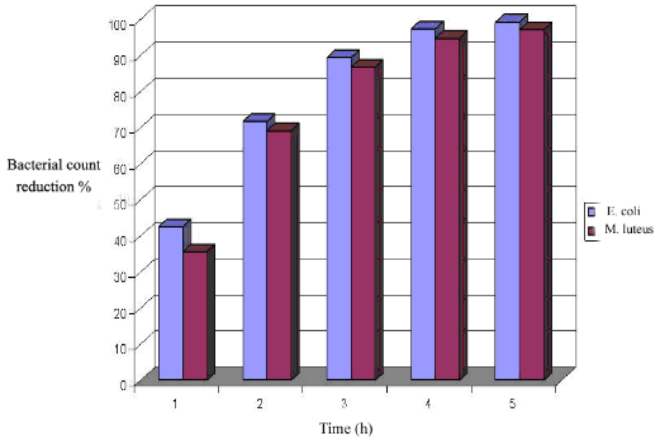


Figure 5: Antibacterial activity of *M. rouxii* chitosan against Gram-negative bacterium *E.coli* and Gram-positive bacterium *Micrococcus luteus* determined by plate count agar.

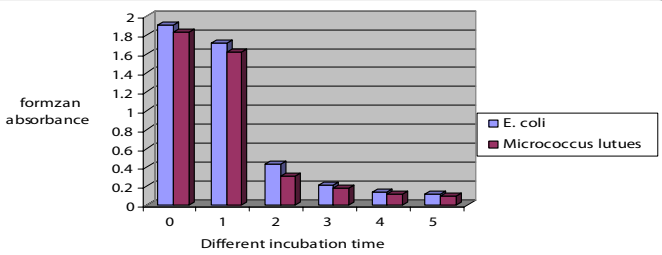


Figure 6: Absorbance of formazan for chitosan at different incubation time.

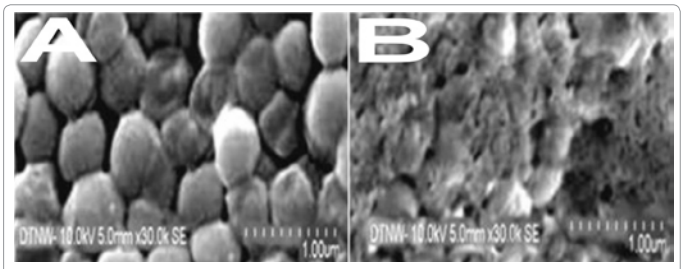


Figure 7: Scanning electron micrograph of *Micrococcus lutues* DSM-1191 treated with 1% of fungal chitosan. (A) Control (untreated) cells; (B) after 5h from the treatment with chitosan.

Scanning electron microscopic analysis

Scanning electron micrographs of *Micrococcus lutues* treated with fungal chitosan, as well as control, are illustrated in (Figure 7)**.** The micrographs pronounced that typical (untreated) cells had a normal smooth surface (Figure 7A)**,** whereas after 6 h from the treatment of *M. lutues* with chitosan, cell wall was fully disrupted (Figure7B)**;** cellular debris from cell lysis of *M. lutues* was observed as a mixture with cell wall residues.

The mode of action of chitosan biocides can be interpreted as follows: The bacterial cell surfaces are known to be negatively charged while chitosan is positively charged because of the presence of protonated amino groups, therefore the bacteria cell wall will be absorbed on the cationic surface of the chitosan membrane, which binds and disrupt the cytoplasmic membrane of the bacteria. The

interaction between chitosan and microbial cells could be on the cell surface, which leads to increase permeability of cell wall and leakage of intracellular components, or inside the cell, which could inhibits DNA and RNA synthesis and directs cells into death [22; 23]. Rabea et al.[24] reported that the exact mechanisms for the antimicrobial effect of chitosan, chitin, and any other derivatives were unknown, although several mechanisms have been proposed. Several proposed mechanisms all involve some kind of damage or interaction with the cell membrane. One such proposed action is the positively charged chitosan interacting with the negative cell membrane which in turn alters its permeability, allowing leakage of intracellular material to the media [25, 26]. This causes the release of cytoplasmic constituents, such as the DNA and the RNA, to take place continuously and eventually leading to death of the bacteria. Activity considerably varies with the type of chitosan, the target organism and the environment in which it is applied. Consequently, literature reports somewhat vary and are, occasionally, contradictory. But generally speaking, yeasts and moulds are the most sensitive group, followed by Gram-positive bacteria and finally Gram-negative bacteria [1]**.** Chitosans also could act as chelating agents that selectively can bind certain trace metals and thus inhibit microbial growth. Chitosan can activate several defense processes in the host issue, act as a water binding agent and inhibit various enzymes. Even binding of chitosan with DNA and inhibition of mRNA synthesis has been shown to occur through chitosan penetration towards the nuclei of the microorganisms and interfering with the synthesis of mRNA [24].

Conclusion

Chitosan could be considered as a biocidal agent against a wide range of target organisms. *M. rouxii* DSM-1191 was a good candidate for chitosan production by fermentation which seems to be economical. The mycelia of *M. rouxii* DSM-1191 may serve as a source of chitosan obtained with yields after 48 h of fermentation was 0.78 g/l. *M. rouxii* DSM-1191 chitosan showed stronger bactericidal effects for both gram-positive *Micrococcus luteus* and Gram-negative bacteria *E. coli*.

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