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Bio-clarification of water from heavy metals and microbial effluence using fungal chitosan

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ABSTRACT

Water pollution is among the most hazardous problems that threaten human health worldwide. Chitosan is a marvelous bioactive polymer that could be produced from fungal mycelia. This study was conducted to produce chitosan from *Cunninghamella elegans* and to use it for water pollutants elimination, e.g. heavy metals and waterborne microorganisms, and to investigate its antibacterial mode of action against *Escherichia coli*. The produced fungal chitosan had a deacetylation degree of 81%, a molecular weight of 92.73 kDa and a matched FT-IR spectrum with standard shrimp chitosan. Fungal chitosan exhibited remarkable antimicrobial activity against *E. coli*, *Staphylococcus aureus* and *Candida albicans*. Chitosan was proved as an effective metal adsorbent, toward the examined metal ions, Cu^{2+}, Zn^{2+} and Pb^{2+}, and its adsorption capacity greatly increased with the increasing of metal concentration, especially for Cu and Zn. The scanning electron micrographs, of treated *E. coli* cells with fungal chitosan, indicated that the cells began to lyse and combine after 3 h of exposure and chitosan particles attached to the combined cells and, after 12 h from exposure, the entire bacterial cell walls were fully disrupted and lysed.

Therefore, fungal chitosan could be recommended, as a bioactive, renewable, ecofriendly and cost effective material, for overcoming water pollution problems, from chemical and microbial origins.

1. Introduction

Water is the most essential and vital component of our life through its basic role in the majority of biological functions in the Earth's ecosystems. Ominously, it is not available to obtain a safe drinking water in many parts across the world because of their contamination from various sources. Today, water pollution is actually a serious matter that affects our lives [1–3].

More than 700 agent/compounds have been reported as water pollutants alongside with the waterborne microbial pathogens. Many pollutants, from organic and inorganic sources, were reported as risk factors for human because of their high toxicity and carcinogenicity nature [4]. Furthermore, most metal ions and inorganic compounds are not bio-transformable or biodegradable and they will persist and accumulate in the environment or in the body.

The assessment and ensuring microbial water quality and control of pathogenic microorganisms continue to be serious challenges for public health protection, eco-toxicology, and bioterrorism prevention [5]. The recurrent waterborne outbreaks of *Escherichia coli* O157 proposed it as a dangerous threaten to health via water contamination [6].

Thus, polluted wastewater recycling and water treatment were repeatedly advised to have safe water for human routine activities [3].

Chitosan is a marvelous biodegradable, non-toxic, and biocompatible polymer, produced principally from chitin after alkaline deacetylation (removing the acetyl groups, CH_3—CO). Since the complete deacetylation is not achievable, chitosan is commonly considered as a partially *N*-deacetylated chitin derivative [7,8].

The foremost commercial chitosan sources are shells of crab and shrimp, but the advances in fermentation and microbiological sciences promoted the use of fungi as alternative sources to produce chitosan from their cell walls [9]. Different fungal species were evaluated and employed for the production of chitosan including *Aspergillus niger*, *Mucor rouxii*, *Penicillium notatum, Rhizopus arrhizus, Absidia orchidis* and *Cunninghamella elegans* [10–14].

Chitosan regularly contains three types from reactive functional groups, at C-2, C-3, and C-6 positions, i.e. amino group, primary

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hydroxyl groups and secondary hydroxyl groups, respectively [15]. The varied physico-chemical characteristics of chitosan contribute to its bioactive properties, e.g. molecular weights (50–2000 kDa), deacetylation degrees (70–95%), p*K*a values and viscosity [16]; these physiognomies could directly influence its targeted application [17].

From the successful applications of chitosan biopolymers, especially from fungal sources, are their use as powerful antimicrobial agent, coagulation factor, chelating agent for heavy metals adsorption [9,18–22].

However, the current study was designed to produce fungal chitosan from *C. elegans* and to use it for the elimination of water pollutants, e.g. heavy metals and waterborne microorganisms, and to investigate its antibacterial mode of action against *E. coli*.

2. Materials and methods

2.1. Microorganisms

The used fungi for the production of chitosan in this study was *Cunninghamella echinulata* var. *elegans*, deposited as *C. elegans* (RCMB-012002), and was obtained from the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt.

E. coli (ATCC-25922), *Staphylococcus aureus* (ATCC-25923), *Candida albicans* (ATCC-10231) and *Penicillium expansum* (RCMB-1006) were used for the antimicrobial experiments.

2.2. Propagation conditions

Spores of 7 days old *C. elegans* culture on Czapek Dox agar (HiMedia Lab. Pvt. Ltd., Mumbai, India) were scrapped and diluted in 20% Tween 80 solution to prepare the stock spores suspension. The suspension was inoculated into 500 mL of Czapek Dox broth in 2 L Erlenmeyer flasks, to have a final concentration of 10^4 spores/mL, and the pH was adjusted to 4 ± 0.2 using 1 N HCl. The flasks were shaking incubated at 25 °C and agitated at 75× g for 96 h.

2.3. Chitosan extraction and characterization

After the growth of *C. elegans*, fungal mycelia were harvested by filtration through Whatman filter paper No. 2, dried and weighted. The chitosan extraction was performed according to Tayel et al. [12]. Briefly, fungal mycelia were deproteinized by homogenizing with 1 M NaOH at 90 °C for 2 h. The alkali-insoluble materials were separated by centrifugation (at $4000 \times g$ for 15 min). The precipitate was repeatedly washed and centrifuged until reaching to a pH of 7 ± 0.2. Residues were then extracted for 6 h at 60 °C using 10% v/v acetic acid on a rotary shaking water bath, then the slurry was recentrifuged, and acid insoluble material was discarded. The supernatant fluids pH was adjusted to pH 9.0 using 4 M NaOH solution, then solution was centrifuged and the precipitated chitosan was washed with deionized water, 95% ethanol and acetone, respectively, then dried until reaching to a constant weight.

The molecular weight of fungal chitosan was determined by gel permeation chromatography (GPC) with the following specifications:

GPC, a laser light scattering device PN-3000 (15 °C and 90 °C) together with a refractive index detector PN-1000, were from Postnova analytics, Eresing, Germany. Columns Nucleogel GFC 1000-8, (Macherey-Nagel GmbH & Co. KG, Düren, Germany) as well as Gral 300 by (Polymer Standards Service GmbH, Mainz, Germany).

Standard pullulans (with molecular weight of 11,800, 47,300, 112,000, and 780,000) were used for calibration.

The deacetylation degree of chitosan (DD) was determined according to Niamsa and Baimark [23], from their infra-red spectra recorded on FTIR (Biorad, model FTS 45, Germany). The absorbance ratio was measured at A1655/A3450.

2.4. Evaluation of chitosan antimicrobial activity

The antimicrobial activity of fungal chitosan was determined against microbial strains using two antimicrobial assays

2.4.1. Well diffusion method

The produced fungal chitosan was dissolved in 1% acetic acid solution to prepare a concentration of 1% (w/v), then the solution was autoclaved. 25 μL from chitosan solution were pipetted into 6 mm diameter wells in the appropriate agar media plates, e.g. Nutrient agar for *E. coli*, *S. aureus*, Yeast Malt Extract agar for *C. albicans* (ATCC-10231) and Czapek Dox agar for *P. expansum*. Plates were streaked with proper microbial strains and incubated for 24–48 h. The appeared clear zones surrounding wells were measured and their mean diameters were calculated.

2.4.2. Determination of the minimal inhibitory concentration (MIC)

According to the previously described methods [11,24], determination of chitosan MIC toward each microbial strain was conducted, using ρ-iodonitrotetrazolium violet (INT) as an indicator of microbial growth or inhibition.

2.5. Heavy metal absorption by fungal chitosan

Chitosan was evaluated for the absorption of copper (Cu^{2+}), zinc (Zn^{2+}), and lead (Pb^{2+}), in aqueous solution. Metals were added to deionised water in the form of copper sulfate, zinc chloride and lead nitrate. Different amounts from each individual metal, e.g. 100, 200 and 300 ppm, were added, vortxed well and press filtered. 0.25% (w/v) from chitosan was added to metal solutions and kept for a total contact time of 450 min, at 25 °C under shaking conditions ($120 \times g$).

The basic and treated solutions were filtered and the dispersed metallic ion content was determined using the inductively coupled plasma – atomic emission spectroscopy (ICP-AES, Perkin-Elmer 4300 DV, Waltham, MA).

The adsorption capacity of fungal chitosan (q_e) was calculated using the following equation:

$$q_e = \frac{(C_0 - C_e)V}{m}$$

where "C_0" and "C_e" are the initial and the equilibrium metals concentration (mg/L), respectively, "V" is the volume of solution (L) and "m" is the amount of adsorbent used (g).

2.6. Elucidation of the antimicrobial mode of action from chitosan

The antimicrobial action of fungal chitosan against potential contaminating microorganisms was elucidated by scanning electron microscope imaging (SEM-Joel JSM-6510LV, Peabody, MA) working at 20 kV, and magnification of 15,000×. *E. coli* cells were served as the model of contaminating microorganisms. Micrographs of treated *E. coli*, with fungal chitosan, were captured after 3, 6 and 12 h from the exposure to chitosan MIC, as well as control culture.

Samples were prepared for SEM according to the method of Marrie and Costerton [25].

3. Results

The productivity of *C. elegans* from mycelial biomass dry weight, after the fermentation period of 96 h at pH 4 and incubation