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Biopolymers isolation (chitin and chitosan) from mushroom biomass of *Pleurotus ostreatus* (Jacq: Fries) Kummer

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ABSTRACT/RESUME

Abstract: *Chitin and chitosan, polysaccharides similar to cellulose, have shown their importance in many fields: biology, medicine, environment, pharmacy, agribusiness, etc. Published research over the last 5 years have proposed fungal biomasses development as an alternative source to marine biomasses, particularly Basidiomycetes that are widely used for biomass production due to cell wall polysaccharides. The aim of this study is to extract chitin and chitosan from mycelium of a Local edible Mushroom strain, Pleurotus ostreatus (Jacq: Fries) Kummer (LPO). The approach consist of using condensed mycelium recovered during harvesting period of LPO grown on an original mixture of olive pomace (OP), coffee grounds (CG) and wheat straw (WS) under solid state fermentation. Chemical treatments based on 1M NaOH at 121°C for 15 min and 0.35M CH_3COOH at 95°C for 8 hours enable to extract chitin and chitosan with optimal yields of, respectively, 175.92 mg and 3.7 mg per g of dry biomass. Compared to commercial marine chitin (Sigma Aldrich), Fourier Transform Infra-Red spectroscopy (FTIR) analyzes highlighted strong similarity in peaks but with low intensities with the condensed mycelium of LPO. Scanning electron microscopy (SEM) analysis showed a crystalline nanofibrous structure for chitin and a smooth and firm surface without nanofibers and nanopores for chitosan. Our findings suggest that LPO is a potential candidate to produce chitin and chitosan by bioconversion of an agro-industrial wastes mixture.*

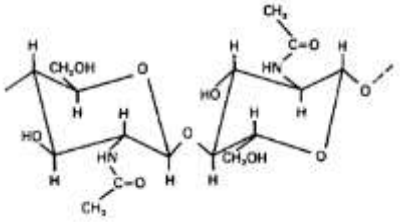
I. Introduction

Chitin and chitosan are two biopolymers that are increasing interest from researchers and scientists because of their many applications, in agriculture [1], environment [2], medicine [3, 4], food industry [5], papermaking and textile industry [6]. Chitin (figure 01) is poly [β-(1→4)-2-acetamido-2-deoxy-D-glucopyranose] [7]. It is the second most abundant polysaccharide on earth after cellulose [8]. It is found mainly in marine litter, shrimps, crabs, and in squid backbone. It is associated with proteins, minerals and calcium carbonate [9]. It is also present in arthropods exoskeleton and in fungi cell wall, in particular, Basidiomycota, Ascomycota and Chytridiomycota. Indeed, it is one of the main components of the mushrooms cell wall [10]. Chitosan (figure 01) is poly [β-(1→4)-2-amino-2-deoxy-D-glucopyranose] [11] and naturally present in fungi of the order Mucorales [12, 13]. Commercially, chitosan is efficiently obtained from deacetylation of

shellfish chitin [13]. However, industrial isolation of chitin and chitosan from marine products is limited, due to seasonal problems, limited supply in some countries and environmental pollution related to large quantities of shell wastes. In addition, the conversion of chitin to chitosan at high temperature causes variability in the properties of chitosan, such as molecular weight [14]. Research has been conducted on the use of fungi mycelium as an alternative source for chitin and chitosan production. In addition, fungi contain small amounts of mineral impurities associated with chitin. Fungal phyla the most studied for this purpose are Mucoromycota with the genus *Absidia, Mucor, Rhizopus* [15 - 18], Ascomycota with the genus *Aspergillus* [14, 18, 19], *Penicillium* [20] and Basidiomycota [21-29] whit *Agaricus bisporus* (button mushroom) [21 - 24], *Ganoderma lucidum* (reishi) [25], *Lentinula edodes* (shiitake) [22-24, 26-28], and oyster mushroom species, *Pleurotus eryngii* [22, 24], *Pleurotus sajor-caju* [29], and many others mushrooms species [30-33]. *P. ostreatus* is the third most cultivated edible mushroom in the world [34]. Its growth time is shorter than others edible mushrooms; it has less demanding conditions development; it can be grown in a simple and inexpensive way using raw agricultural and agro-industrial wastes for its nutrition and growth under a solid state fermentation [34 - 36]. It can be, therefore, a good alternative source for chitin and chitosan extractions.

In this context, the aim of this study was to extract chitin and chitosan from condensed mycelium of a local strain of edible Agaricomycete, *P. ostreatus*. This strain was, first, grown on an original mixture of agricultural and agro-industrial residues (olive pomace, coffee grounds and wheat straw) [36], followed by the physicochemical analysis of isolated fungal chitin and chitosan.

Characterization of these biopolymers was performed by Fourier Transform Infra-Red spectroscopy and Scanning Electron Microscopy.



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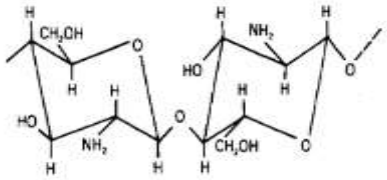

b/

Figure 01. *Structure of chitin (a) and chitosan (b) [37]*

II. Materials and methods

II.1. Materials

II.1.1. Mushroom

The mushroom of interest is a Local strain of *Pleurotus ostreatus* (Jacq: Fries) Kummer (LPO) isolated in Oued-Aissi by Mansour-Benamar in 1993 [38]. It has been maintained since then by successive transplanting and re-isolation from freshly harvested fruit bodies, at our laboratory "Laboratoire de Production, Amélioration et Protection des Végétaux" of Biological and Agricultural Sciences Faculty of Mouloud Mammeri University of Tizi-Ouzou (Algeria).

II.1.2. Cultivation substrate

Pleurotus strain used was grown on an original mixture of agricultural and agro-industrial residues: olive pomace (OP) - coffee grounds (CG) - wheat straw (WS) supplemented with $CaCO_3$. OP was recovered in a three-phase oil mill located on the road that connects Tizi-Ouzou to Boghni; CG came from our own daily consumption; WS was bought at the market of Oued-Aissi (Tizi-Ouzou prefecture, Algeria).

II.1.3. Origin of commercial chitin and chitosan

Commercial chitin and commercial chitosan used as controls were from Sigma Aldrich.

II.2. Methods

II.2.1. Mushroom cultivation

LPO cultivation was carried out by Amrane and Belkacemi in 2017 [36], on the humidified mixture (OP (44%) / CG (44%) / WS (10%)), supplemented with 2% $CaCO_3$ and inoculated with 7% LPO spawn. Incubation lasted 3 weeks at 25-28°C and 70% - 80% ambient humidity. LPO fruiting lasted 4 weeks at 18 to 22°C and 80% - 85% ambient humidity.

II.2.2. Recovery and preparation of the condensed mycelium

We called condensed mycelium, LPO mycelium developed into and around substratum mixture. It is constituted by the stroma (abundant and dense

mycelium) formed (figure 02a), fruit bodies and primordia whose growth has stopped, long feet and small mushrooms (figure 02b) whose weight does not exceed 2g. This mycelium was picked up manually at the incubation time end and throughout the fruiting period (figure 02). It was washed with distilled water and then dried at 60°C to constant weight. Then, it was weighed and powdered using a Rettsch/Rheinische Strab 36-D-42781 Haan mechanical grinder (Germany type SM1).

Figure 02. *Dense mycelium (a) and long feet and small mushrooms of LPO (b)*

II.2.3. Extraction of chitin and free chitosan

Chitin and free chitosan extraction from LPO was carried out by a modified method of Rane & Hoover [15] and Crestini et al. [26]. Briefly, some condensed mycelium powder mass was treated with 1M NaOH at 121°C for 15 min. The resulting dispersion was centrifuged at 3000 rpm (SIGMA D-37520, type 4-16K model) to separate the phases. The solid phase representing the insoluble alkaline fraction symbolized by AIM (alkaline insoluble material) was washed with distilled water until neutral pH then dried at 60°C and weighed. Some of AIM fraction was acid treated with CH_3COOH (0.35M) at 1g / 100ml at 95°C for 5h. The dispersion was centrifuged for recovery both solid and liquid phases. Solid phase represents the insoluble acidic fraction symbolized by AAIM (Acetic Acid Insoluble Material). It was washed with distilled water to neutral pH, dried at 60°C and weighed. Substances of liquid phase were precipitated by adding small volumes of 2M NaOH until pH 9-10. Formed precipitate was recovered by centrifugation, washed with distilled water to neutral pH, dried and weighed.

III. Chitin and free chitosan characterization

III.1. Fourier Transform Infra-Red spectroscopy (FTIR)

Structure of chitin and chitosan extracted from LPO condensed mycelium was confirmed by infrared spectroscopy using preparation method of KBr pellets at Mouloud Mammeri University of Tizi-Ouzou. FTIR spectra were recorded between 4000 cm^{-1} and 400 cm^{-1} using a JASCOFTIR-4200 Type A Fourier Transform Spectrophotometer at room temperature.

III.2. Scanning Electron Microscopy (SEM)

Samples, extracted from the chitin and the chitosan of LPO, were examined using PHILIPS ESEM XL 30 Scanning Electron Microscope (SEM) at a voltage of 10 to 25 KV at Mouloud Mammeri University of Tizi-Ouzou.

IV. Results and discussion

IV.1. Extraction of chitin

Chitin was extracted from condensed mycelium of local strain of *Pleurotus ostreatus* grown on an original substrate made of a mixture of agricultural and agro-industrial wastes, olive pomace, coffee grounds and wheat straw. To our knowledge, this is the first time this type of wastes mixture had been used for the cultivation of mushrooms.

Chitin was recovered after basic and acidic treatment of dry condensed mycelium. The basic treatment removes proteins and lipids. The acidic treatment separates chitosan from chitin. Chitin yield isolated from condensed mycelium of LPO is about 175.92 mg/g of dry weight biomass (DW), which equals a yield of 17.59% DW.

The chitin amount extracted from local *P. ostreatus* condensed mycelium is higher compared to that extracted by Di Mario et al. [24] from *P. ostreatus* SMR684 (15.30% DW) and by Vetter [23] from *P. ostreatus* (6.58% DW), *A. bisporus* (13.98% DW) and *L. edodes* (14.62% DW). However, LPO chitin yield is less than that extracted by Wu et al. [13] from *A. bisporus* stipes during postharvest storage (19.02% DW), by Di Mario et al. [24] from *Auricularia auricula-judae* (19.6% DW) and by Ospina Álvarez [25] from the medicinal mushroom *Ganoderma lucidum* (41% of mycelial biomass).

Some of the cultures were performed by SSF [23], but most of them were conducted in submerged fermentation (SMF) [24, 25, 39] with different physicochemical conditions leading to different results.

The chitin yields isolated from cuttlefish bones, crab shells and shrimp shells are, respectively, 5%, 10% and 20% [40]. A study conducted by Wang et al. [41] showed that krill's dry weight provides 27.8% chitin.

IV.2. Extraction of free chitosan

Chitosan extraction was performed as described previously by Rane & Hoover [15] and Crestini et al. [26]. Several authors have shown that the maximum yield of fungal chitosan is generally produced during the late exponential growth phase [13, 17]. Condensed mycelium of LPO, recovered

from the culture carried out on the mixture (OP - CG - WS), had a very low content of free chitosan, 0.37% of condensed mycelium dry weight. However, most of the studies carried out had shown that fungi belonging to Basidiomycota like POL had a very little or no free chitosan. Indeed Wu et al. [13] had not yielded any chitosan from stipes of *A. bisporus* after acidic treatment. Crestini et al. [26] had extracted chitosan from mycelium of *L. edodes*, produced on wheat straw. With SSF, chitosane amount was 0.62% of the substrate weight (SW) and with SMF; it was up to 50 times less than the chitosan produced under SSF culture. Thus, the cultivation method is an important factor for fungal chitosan production.

Chitosan maximum yield obtained in Malt-Yeast-Peptone-Glucose with *Pleurotus eous* was 14.56 mg/100ml after 12 incubation days (ID), it was low at the 15th ID and negligible at the beginning of the cultivation (3rd and 9th ID) [39]. Mushrooms are known to grow slowly [35]. Di Mario et al. [24] had isolated only 1% chitosan DW, from 7 species of Basidiomycota.

The chitosan amount determined in *Aspergillus niger* (phylum Ascomycota), grown 12 days on soybean residue was 1.71% DW [42]. Aili et al. [20] had studied the temperature effect on production of chitin and chitosan from *Penicillium Camembertii* inoculated on synthetic solid medium. They had found 0.85, 0.87 and 0.6% chitosan DW at 20°C, 25°C and 28°C, respectively, after 6 days incubation.

Chitosan is rare in nature; it is only present in the cell wall of Mucoromycota fungi [13, 16, 43]. However, chitosan amount may be high in some Mucorales species and weak in others.

Rane & Hoover [16] had extracted chitosan from five Mucorales species, cultivated in SMF conditions: *Absidia. coerulea* ATCC 14076 yielded the most chitosan, 51 mg/100 mL of medium at pH 5.5 in 48 h, and cobalt supplementation showed an increase of approximately 20% in chitosan yield. They found that *Mucor rouxii* ATCC 24905 and *Gongronella butleri* NRRL 1308 had presented the best chitosan percentages of with around 20% each.

Always with *A. coerulea* ATCC 14076, New et al. [40] had extracted lesser chitosan (6.5% DW) under same experimental conditions used by Rane & Hoover [16], but at 30°C for 7 incubation days.

Chitosan usually originates from the chitin deacetylation of crustaceans. Sawssen Hajji et *al*. [1] had yielded 1.2%, 5.3% and 14.9%, from respectively cuttlefish, shrimp and crab. According to Anshar Patria (2013) [44], chitosan yield from Shrimp shells was 67.42 %.

IV.3. Chitin characterization by infrared spectroscopy

Chitin infra-red spectrum (figure 03) extracted from LPO mycelium is similar to that of commercial chitin (figure 04) of crustaceans but with a very low characteristic peak intensity.

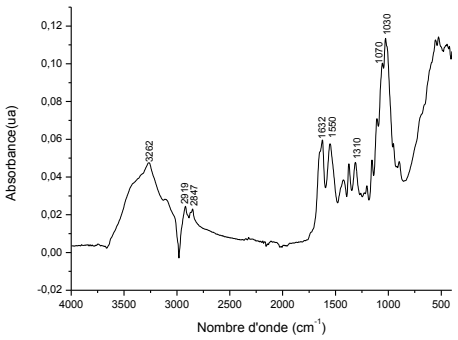

Figure 03. *FTIR spectrum of chitin isolated from condensed mycelium of local Pleurotus ostreatus grown on OP/CG/WS substrate*

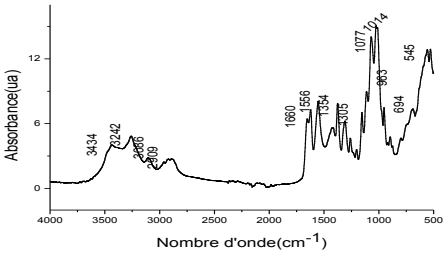

Figure 04. *Commercial chitin FTIR spectrum*

The infra-Red spectrum shows that characteristic bands of chitin isolated from mycelium of LPO are in agreement with that of commercial chitin of Crustacea. Indeed, the spectra are similar. This indicate that mycelium of LPO contains chitin.

In our study, infrared spectrum of chitin extracted from LPO mycelium is characterized by appearance of 1632, 1560 and 1310 cm^{-1} bands corresponding to CO elongation band I, (NH, CN) of amide II and CN of amide III, respectively. Absorption band at 1070 cm^{-1} corresponds to C-O-C bond. Absorbance bands at 3262, 2919 and 2847 cm^{-1} correspond to the vibration mode of symmetrical N-H, C-H(CH_3) and asymmetric C-H(CH_2) bonds, respectively [45].

IV.4. Free chitosan characterization by FTIR

Chitosan FTIR spectrum extracted from condensed mycelium of LPO (figure 05) shows for the same absorption bands as in commercial chitosan but their intensities are less important. There is a considerable reduction in amide I peak intensity (1630 cm^{-1}).

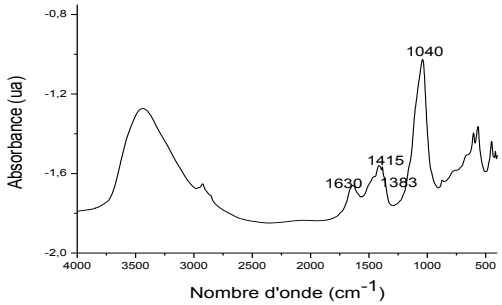

Figure 05. *FTIR spectrum of free chitosan extracted from condensed mycelium of local Pleurotus ostreatus grown on OP/CG/WS substrate.*

Compared to the standard chitosan spectrum (figure 06), the FTIR spectrum of the chitosan extracted from LPO mycelium (figure 05) does not contains of amine NH_2 band [20, 24]. It can be explained by the fact that chitosan extracted from mycelium is natural and comes in free form while the commercial chitosan is derived by deacetylation of chitin. Band at 3475 cm^{-1} correspond to the vibration band of OH hydroxyl group.

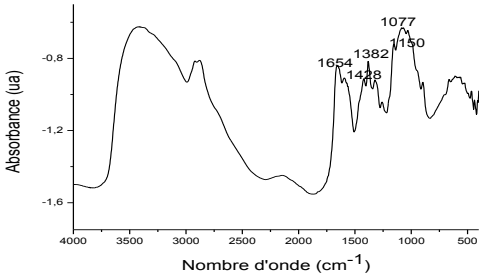

Figure 06. *Commercial chitosan FT-IR spectrum*

IV.5. Chitin and free chitosan characterization by SEM

The SEM characterization method was used to examine morphology of different fractions. Figures 07 and 08 show micrographs of chitin and free chitosan extracted from condensed mycelium of LPO. Scanning electron microscopy analysis of chitin isolated from LPO (figure 07a) shows that their surface is crystalline nanofibrous. It is compared with chitin morphology of commercial chitin (figure 07b). SEM analysis of chitosan extracted from LPO (figure 08a) shows that their surface is smooth and firm without nanofibers and nanopores. It is compared to the morphology of chitosan commercial (figure 08b).

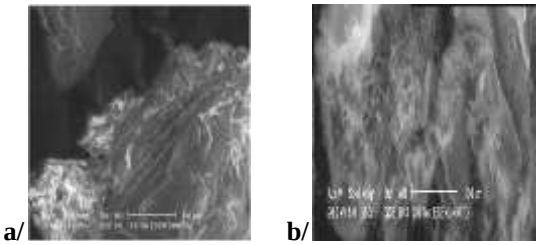

Figure 07. *Micrographs of chitin extracted from condensed mycelium of LPO grown on mixture OP/CG/WS (a) and commercial chitin (b)*

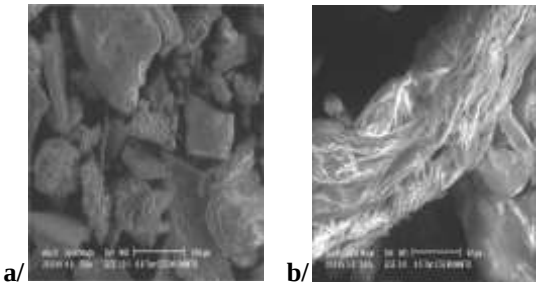

Figure 08. *Micrographs of free chitosan isolated from condensed mycelium of POL grown on mixture OP/CG/WS (a) and commercial chitosan (b)*

V. Conclusion

The alkaline and acid treatments used in our study to extract chitin and chitosan from condensed mycelium of local *Pleurotus ostreatus* had produced optimal amounts of 175.92 mg and 3.7 mg per gram of dry fungal biomass respectively. FTIR and SEM analyses show that chitin and chitosan obtained from condensed mycelium resemble standard chitin and chitosan, respectively. Compared to results obtained by other authors, it appears that chitin and chitosan exist in variable quantities according to the physicochemical and nutritional parameters, fungal species and extraction method.

Condensed mycelium of the local strain of *Pleurotus ostreatus* (Jacq: Fries) Kummer grown on solid substrate composed of a mixture of agricultural and agro-industrial residues, namely, olive pomace, coffee grounds and wheat straw , can be used to isolate raw chitin; free chitosan amount remained weak but there is the prospect to recover it from chitin.

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