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Two Routes to Produce Chitosan from *Agaricus bisporus*

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Abstract: Two methods were used to produce chitosan by deacetylation of chitin which was extracted from *Agaricus bisporus* stipes. The first one gives chitosan 1 with low yield of 2.5%, degree of acetylation (DA) of 4%, molecular weight (MW) of 2.973×10^5 (g/mol). The second route produces chitosan 2 with higher yield of 41%, degree of acetylation (DA) of 17.23%, molecular weight (MW) of 2.939×10^5 (g/mol). Both chitosans were characterized by XRD, FTIR, ^{1}H-NMR spectroscopy nuclear magnetic resonance of proton. The molecular weight (M_W) was determined by size exclusion chromatography (SEC). Thermal analysis shows that both chitosans have moisture content lower than 10%. However chitosan 2 has the less ash % which is the quality grade for chitosan medical applications. Accordingly, fungal chitosan 2 could have potential medical and agricultural applications.

Keywords: Biobased materials; chitosan; chitin; *Agaricus bisporus*; characterization of biomass

1 Introduction

Chitosan (CS), a polysaccharide biopolymer derived from naturally occurring chitin, has a great economic value because of its excellent biological and antimicrobial activities, film-forming character, high chelating capacity.

Among the different chitosan applications we can cite: antitumor agent, antimicrobial healing and immuno-potentialisator, as well as in slow-release drugs, water treatments (especially for the removal of heavy metal ions), active packaging films, coatings, dietary fiber, cosmetic industries …. This biopolymer derives from chitin, one of the most abundant biopolymer after cellulose, which is present in the exoskeleton of crustaceans such as shrimps, lobsters, crabs, insects and fungi [1-6].

Commercial CS is traditionally obtained by deacetylation of chitin extracted from crustaceans. Unfortunately this process has several disadvantages. First, the concentrated alkaline solution used in this process inevitably degrades chitosan and contributes to environmental pollution issues caused by liquid discharges. In addition, chitin extracted from crustaceans still contains a fraction of protein residues likely to cause allergies [7-9].

Currently, mushrooms are considered as an interesting alternative source for producing chitosan with higher purity and better quality [10, 11]. In fact, the use of biomass from fungi has several advantages, such as the simultaneous extraction of chitin and chitosan on a large scale, the availability along the year,

exemption from any protein contamination. Moreover, the extraction process is cost-effective, easy to implement and time-saving compared to crustaceans extraction processes [12, 13].

To our knowledge, only a limited work has been reported concerning the extraction of chitin and native chitosan from *A. bisporus* mushroom. [13-16]. Moreover, the production of chitosan from fungs has not been reported so far.

In our previous study [14], chitin with high purity and low content in protein was extracted from three body part of *A. bisporus* mushroom. In the present work, chitin was converted in chitosan by deacetylation of chitin extracted from the stipes of *A. bisporus*. The structure and thermal properties of the produced chitosan were investigated.

2 Materials and Methods

2.1 Organisms

1 kg of fresh *A. bisporus* mushrooms were purchased from Carrefour Villeurbanne, Lyon, France, as 2 trays of 500 g, with the following characteristics: Origin: Loire Valley; Category: 1; Caliber: Medium; N0: 790 I 29; N0: 790 I 27.They were washed, disintegrated and separated into three parts (caps, gills, stipes) as shown in Fig. 1, then only stipes were 24 hours lyophilized.

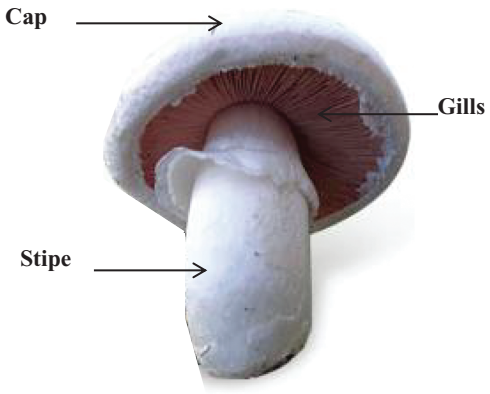

Figure 1: Three body parts of *A. bisporus*

2.2 Reagents

All of the reagents used were of a highly pure grade, the deionized water was used for all reagent solutions. The standard samples of chitin and chitosan, were supplied by Mahtani Chitosan Pvt.Ltd. India (batch N°52, batchN°ch222 for chitin and chitosan respectively). Sodium hydroxide pellets, ammonium hydroxide solution at 28–30% (w/w) and acetic acid, were purchased from Acros organics or Sigma Aldrich.

2.3 Chitin Isolation

The process used to isolate chitin was a modified version of previously reported ones [15, 17].It involved the following steps: a) Deproteinization: 4 g of lyophilized biomass of *A. bisporus* body part (stipes) were subjected to an alkali treatment with sodium hydroxide solution (NaOH) 1 M at a ratio of 1:30 (w/v) at a temperature of 80°C for 2 hours. The AIR (alkali insoluble residue) obtained was washed with deionized water and centrifuged at 10000 G for 10 min. This operation was repeated several times until neutral pH. After the final centrifugation, the alkali insoluble residue was freeze-dried. b) Separation of chitin: to obtain chitin, AIR was refluxed in 2% acetic acid CH_3COOH at a ratio of 1:100 (w:v) at 95°C for 6 h and then centrifuged (10000 g, 10 min). The precipitate chitin was then washed several times till neutral pH and then lyophilized.

The chitin determinations were done triplicate and contents were given as the arithmetical means in percent of DW (Dry Weight) with standard deviations (± SD).

2.4 Chitin Deacetylation Procedures

Chitosan from chitin deacetylation was performed in two different routes: 1) A direct deacetylation was performed from the lyophilized fungus (*A. bisporus* stipes): 4 g of the fungus stipes were dissolved in 100 mL of 60% NaOH solution, heated 3 h at 120°C, then centrifuged (10000 G for 10 min), washed with water until neutral pH, freeze-dried and weighed at the end. 2) Deacetylation of chitin after deproteinization and acid hydrolysis:1g of the chitin extracted from stipes, has been contacted with a sodium hydroxide solution (20 g of sodium hydroxide in pellet with 30 mL of water), then the mixture was heated for 1 h at 100°C. The precipitate was washed with water to neutral pH, freeze dried and weighed.

2.5 Scanning Electron Microscopy (SEM)

Samples of chitosans obtained via the two methods, were observed on a field emission gun SEM (Hitachi S800), on low vacuum mode, at a tension ranging from 5 to 15 kV. A large field detector (LFD) was used and the working distance was ranging from 5 to 10 mm.

2.6 Fourier Transform Infrared Spectroscopy (FTIR) Measurements

The IR spectra of chitins and chitosans extracted from *A. bisporus*, were acquired using a Nicolet iS10 FT-IR spectrometer-ThermoScientific over the frequency range of 4000–500 cm^{-1}. A Gemini sampling accessory, Smart iTR, was used to collect horizontal attenuated total reflectance (ATR) spectra using a standard ZnSe crystal. Samples were pressed with a Mini grip device to assure uniform contact between there and the ATR crystal. In all cases, IR spectra were recorded by accumulation of at least 64 scans, with a resolution of 4 cm^{-1}, using the OMNIC software of the instrument.

2.7 X-Ray Diffractometry (XRD)

The XRD measurements on samples were performed from 5° to 50° using a model Bruker D8 advance diffractometer without Ni-filtered, Cu Kα radiation(λ = 1.5406 Å). The diffractometer was operated with 1° diverging and receiving slits at 40 kV and 40 mA and a continuous scan was carried out with a step size of 0.020° and a step time of 0.2 s.

2.8 Thermogravimetric Analysis TGA

TGA assay was conducted with a DuPont Instrument TGA Q500 thermogravimetric analyzer (TGA). The samples (about 10 mg) were heated in open alumina pans, under helium atmosphere, from 30°C up to about 800°C, at a heating rate of 10°C/min.

Ash and Moisture contents (%) were determined with TGA thermogram using TA Universal Analysis software.

2.9 ^{1}H NMR Spectroscopic Analysis

Samples of chitosan were prepared by stirring at room temperature during 12 hours 8 mg of chitosan in a solution composed of 1 ml of D_2O and 5 µl of Hydrochloric acid (37%).After dissolution, approximately 1 ml of the chitosan solution was transferred to a 5 mm NMR tube.^{1}H NMR spectra were obtained with a Bruker ALS 300 (300MHZ) spectrometer. The number of acquired transients was 64 corresponding to approximately 8.5 min of signal acquisition.

2.10 Size Exclusion Chromatography for Determination of Average Molecular Weights of Chitosans

Chitosan samples (0.1% w/v) were dissolved in $AcOH/AcNH_4$ buffer (pH 4.5) and then filtered on 0.45 um pore size membrane (Millipore). Size-exclusion chromatography was performed by means of an LC pump (Agilent Technologies 1260 Infinity) on two serially connected columns (TSK G2500-PW and

TSK G6000-PW Tosoh Bioscience). The detection was operated by a differential refractometer (Wyatt Optilab T-rex) coupled on-line with a MALLS detector (Wyatt Dawn Heleos II). A degassed AcOH/$AcONH_4$ buffer (pH 4.5) was used as eluent after two filtrations on 0.1 um pore size membrane (Millipore). The flow rate was maintained at 0.5 mL/min, and the amount of sample injected was 100 µL. Chromatograms were analyzed by the Wyatt Astra software (version 6.1.2).

3 Results and Discussion

3.1 Extraction and Characterization of Chitin from A. bisporus *Stipes*

The yield of chitin in dry weight of *A. bisporus* stipes was (7.4% ± 1.2) which is very close to that reported by Vetter as 7.2% [16]. Similar chitin content (8.5%) was found by F. Di Mario who isolated chitin from the mycelium of *A. bisporus* [13].

3.2 Characterization of Chitin

The FTIR spectrum of chitin from *A. bisporus* stipes (Fig. 2A), is similar to that of a commercial α chitin Fig. 2B [17]. The α and ß chitins can be distinguished by FTIR through the vibration modes of amide I in the spectral region 1660–1620 cm^{-1}. In α-chitin, two absorptions are observed at about 1660 and 1627 cm^{-1} and in the ß-chitin only one band is present at 1656 cm^{-1} [17]. So, the two peaks around 1652 and 1622 cm^{-1} observed in FT-IR spectrum Fig. 2 (arrows) evidenced the α crystalline form of chitin extracted from stipes [17]. The lack of the absorption peaks at 1540 cm^{-1} assigned to the stretching vibrations in protein, confirms that the extracted chitin is protein free [1] and thus the chosen process of deproteinization was effective to remove all impurities. The main bands observed by FTIR spectroscopy were assigned according to literature [17].

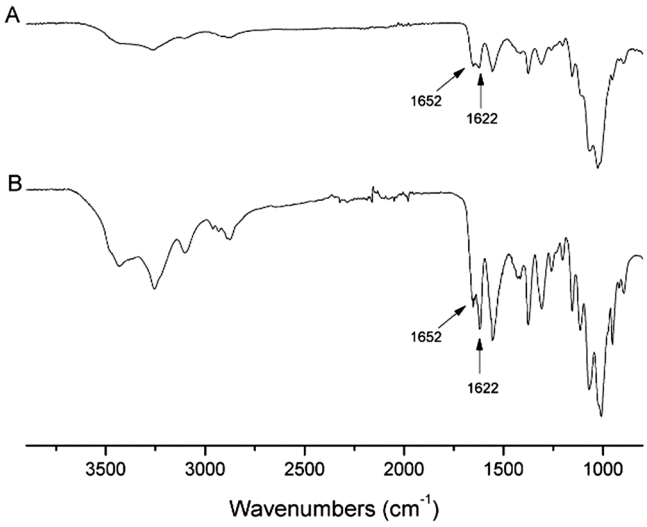

Figure 2: FT-IR spectra of (A) Chitin from stipes of *A. bisporus*, (B) Commercial alpha-Chitin

3.3 Chitin Deacetylation

The difference between the obtained chitosans from the two chitin deacetylation routes (as described in materials and methods) was discussed. Tab. 1 shows that deacetylation of chitin with the first route (directly from the lyophilized fungus *A. bisporus* stipes) produced chitosan 1 with lower content of 2, 50%, DA of 4% and Mw of 2.9×10^5 (g/mol). However, deacetylation of chitin following the second route (after two stages: deproteinization and acid hydrolysis) gives chitosan 2 with higher content of 41%, DA of 17,23% and Mw of 2.9×10^5 (g/mol). Both chitosans have moisture content lower than 10%, which is the quality grade for chitosan intended for medical applications [18].

Table 1: Chitin deacetylation

	Content % of dry weight	DA% by IR	Moisture %	Ash %	DA% by ^{1}H RMN	Molecular weight Mw (g/mol)
Chitosan 1	2,50 ± 1,4	5,9 ± 0,6	6,76 ± 0,3	2,77 ± 0,1	4 ± 0,2	$2.973 \times 10^5 \pm 2,6$
Chitosan 2	41 ± 1,2	20,34 ± 0,4	7,11 ± 0,5	0,31 ± 0,1	17.23 ± 0,1	$2.938 \times 10^5 \pm 2,8$

Ash content of chitosan 2 (0,31%) is lower than that of chitosan 1 (2,77%). A high quality grade chitosan should have less than 1% of ash content since chitosan solubility is reduced by the presence of residual ash which will, consequently, contribute to lower viscosity [19].

Degree of acetylation (DA %) is an important parameter associated with the physical–chemical properties of chitosan [20]. In the present work, DA values of both chitosans are in accordance with those reported by da Silva Amorim et al. [21], Pochanavanich and Suntornsuk [20], Chatterjee et al. [22] who reported that the deacetylation degree of chitosan from fungi occurred between 80 to 90% DD corresponding to (20 to 10% DA). For medical applications chitosan should have a DA less than 30% [18].

The molecular weight (Mw) of both chitosans obtained in this work, is lower than that of crab shells (having a Mw between 1.0×10^4 to 9.0×10^5) [20]. The lower molecular weight of chitosan from *A. bisporus* is expected to reduce the tensile strength and elongation of the chitosan films.

3.4 Characterization of Chitosan

3.4.1 FT-IR Spectroscopic Analysis

The FTIR spectra of chitosan 1, chitosan 2 and commercial chitosan sample with DA 10, are shown in Fig. 3. The spectra of the different samples are quite similar with the characteristic absorption bands of at 1655 cm^{-1} (arrow) and 1597 cm^{-1} (full arrow), relative to amide I band (carbonyl ν(C=O)) and amide II (amine ν(NH_2) tensions), respectively [23]. The difference in the intensity of the amide II band in chitosan samples is explained by the difference in the deacetylation degree, with the highest level observed for chitosan 2 [24].

All samples exhibited wide bands at around 3200–3374 cm^{-1}corresponding to stretching vibration of OH and NH groups. Characteristics bands of their polysaccharide structures are proven by asymmetric stretching of (C–O–C bridge) and 1080 cm^{-1} for (C–O stretching) [23].

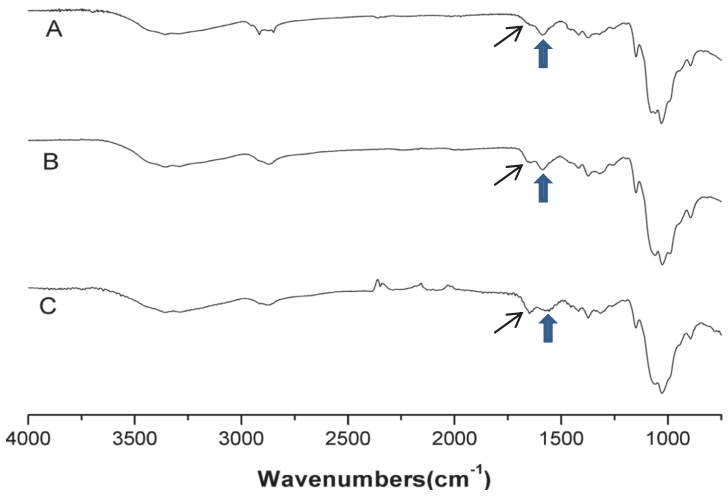

Figure 3: FT-IR spectra of (A) Chitosan 1, (B) Commercial Chitosan (DA = 10), (C) Chitosan 2

3.4.2 X-ray Diffraction

The Diffraction pattern of chitosan 1 and chitosan 2 Fig. 4 showed the two typical peaks of chitosan at around 10° and 20° corresponding to the (0 2 0) and (1 1 0) planes of the crystalline lattice [25-29]. From the diffraction patterns, it can be seen that chitosan 2 exhibited sharper peaks indicative of higher crystalline degree than chitosan 1. This is confirmed from the crystalline index (CrI%) attaining about 3.6% and 15.6% for chitosan 1 and chitosan 2 respectively. The CrI was evaluated according to Eq. (1) [30].

$$CrI = \left(\frac{(I_{110} - I_{am})}{I_{110}}\right) \times 100 \quad (1)$$

Where I_{110} is the maximum intensity at $2\theta \cong 20°$ and I_{am} is the intensity of amorphous diffraction at $2\theta \cong 16°$.

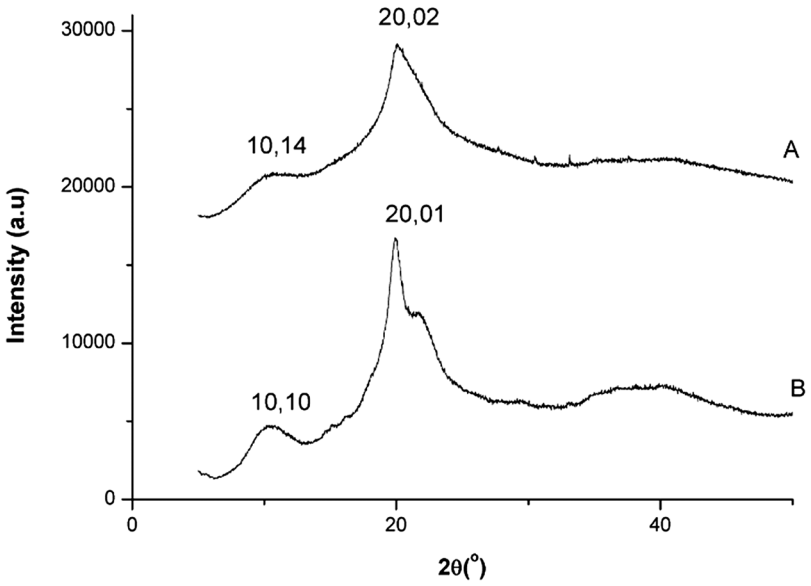

Figure 4: XRD patterns of (A) Chitosan 1, (B) Chitosan 2

3.4.3 TGA

The TGA curve in Fig. 5 shows that both chitosan thermograms are quite similar and presented three steps of degradation. The first one, between 0 and 100°C, is due to evaporation of water absorbed by chitosan. The second step, starting at a temperature of 220°C and extending to 350°C, is attributed to the a random splitting of the glycosidic bonds and depolymerization of the polysaccharide [31]. The third weight loss, occurring in the interval 402°C–600°C, is attributed to the oxidation of the carbon degradation residue under oxygen atmosphere. This degradation profile is in agreement with literature data [32].

It was also found that Chitosan 2 exhibited a higher thermal stability than that of chitosan 1. This result is due to the difference in their degree of acetylation DA (17.23% and 4%, respectively) and crystalline index. Kittur et al. [33] reported that there is an inverse relationship between thermal stability, deacetylation degree and crystalline index. At last, when comparing ash content of both chitosans, (see Tab. 1), it can be seen that chitosan 2 contains the lower yield of ash (0.31%).

3.4.4 Liquid State ^1H Nuclear Magnetic Resonance

^{1}H NMR spectrum of chitosan 1 Fig. 6A and chitosan 2 Fig. 6B presented the typical signals of chitosans with the following peaks indexation: at 2.1 ppm from the three methyl H atoms (GlcNAc), 3.2 ppm from H2

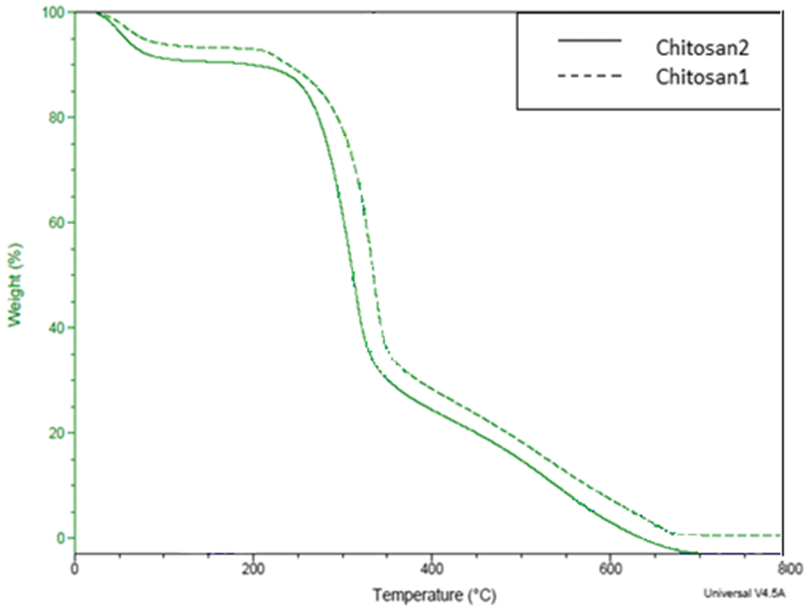

Figure 5: TGA curve of Chitosan 1 and Chitosan 2

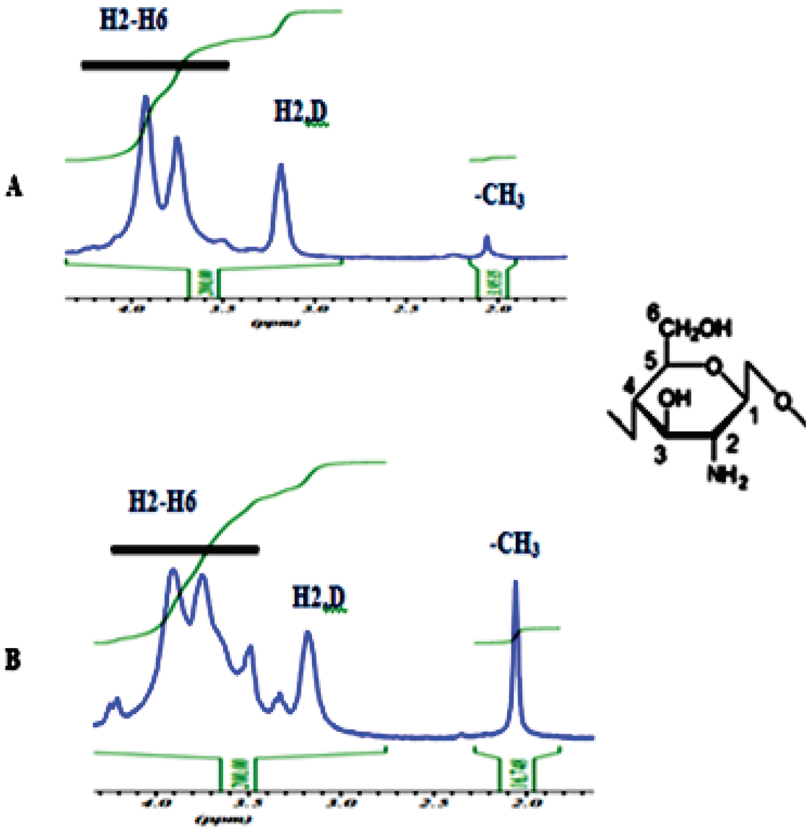

Figure 6: ^{1}H NMR spectrum of (A) Chitosan 1, (B) Chitosan 2

(GlcN), several overlapping signals from 3.4 to 4.4 ppm assigned to H2–H6 [34-37]. The signal of–OH, $–NH_2$, and NH-CO-CH_3 protons were not observed, presumably due to rapid exchange of the labile protons with D_2O [38, 39].

The degrees of acetylation of chitosan was calculated from the areas of the signals in 2.1 ppm (methyl) and the sum of the areas from 3.2 to 4.2 ppm (H2, H3, H4, H5, and H6) in the ^1H NMR according to Eq. (2) [40].

$$DA\% = \left(\frac{2 \times A_{CH_3}}{A_{(H_2-H_6)}}\right) \times 100 \quad (2)$$

A_{CH3} is integral of $–CH_3$ signal, $A_{(H2\text{-}H6)}$ – the summation of integrals of H2, H3, H4, H5, and H6 correspond to hydrogen of deacetylated residues.

3.4.5 Scanning Electron Microscopy (SEM)

Several recent studies have shown that surface morphology is a crucial feature in effectively targeting the use of chitosan in a particular area. For example, a chitosan with a porous surface structure, is more desirable in the adsorption domain of metal ions, whereas a chitosan with a fibrillar surface structure is very much in demand in the textile field [18, 41].

The surface morphologies of chitosans obtained in our study are given in Fig. 7. The two chitosans have the same dense, smooth, fibrillar and non-porous surface. Similar results have been observed in the surface morphology of chitosan obtained from certain fungi and insects [42]. Thus, this fibrillar surface property of both chitosans predestines them for use in tissue engineering including the manufacture of suture in surgery.

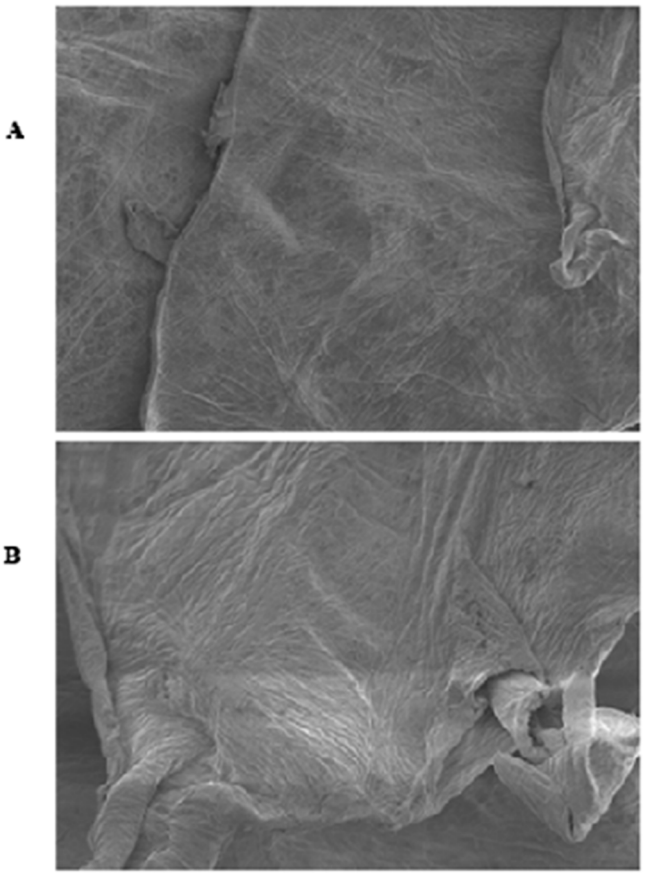

Figure 7: SEM micrographs (A) Chitosan 1, (B) Chitosan 2

4 Conclusion

Physicochemical properties of chitosan are much important for determining its application areas. In this study, two routes were tested to produce fungal chitosan by deacetylation of chitin which was extracted from *A. bisporus* stipes.

Deacetylation of chitin with the first route (directly from the lyophilized fungus *A. bisporus* stipes) produced chitosan 1 with lower content of 2,50%, DA of 4% and Mw of 2.973×10^5 (g/mol). However deacetylation of chitin with the second route (after two stages deproteinization and acid hydrolysis) gives chitosan 2 with higher content of 41%, DA of 17,23% and Mw of 2.938×10^5 (g/mol). Both chitosans have better moisture content lower than 10% which is the quality grade for chitosan medical applications. Ash content of chitosan 2 (0,31%) is better than that of chitosan 1 (2,77%).

Therefore the second method gives higher yield of Chitosan with best quality grade which could have potential medical and agricultural application.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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