

# Isolation and Characterization of Chitin Nanofibers from *Calocybe indica* and its Applications

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**Abstract:** In this study, chitin nanofibers are isolated from *Calocybe indica* (Milky White Mushroom), and they are further characterized using various analytical techniques such as UV-VIS, FTIR, TGA, Fe - SEM, and XRD. Antioxidant activity of the chitin nanofibers was studied by performing various assays such as DPPH, ABTS, H<sub>2</sub>O<sub>5</sub> Scavenging Activity, Hydroxyl Radical Activity, Superoxide Assay, and Reduced Glutathione Activity. Isolated Chitin nanofibers were then chemically treated to form hydrogels. The hydrogels formed were further characterized.

**Keywords:** *Calocybe indica*; chitin nanofibers; antioxidant activity; hydrogels.

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## 1. Introduction

*Calocybe indica* (milky white mushroom) is a native edible mushroom species that appears white in color and has a firm consistency [1, 2]. It belongs to the genus *Calocybe* and the family *Lyophyllaceae*. *C. india* is commercially cultivated in agricultural fields or along the roadside in tropical and subtropical parts of India [3]. The Pileu or the cap of this species measures 10 - 14 cm wide and is found to be convex in shape, which further flattens, as the mushroom ages. The stem is generally 10 cm high with neither the ring nor volva [4]. These can be grown in a warm, humid climate (30~38 °C; 80 % to 85 % humidity) and hold a longer shelf life without the need for refrigeration. They are less prone to contamination and discoloration when kept under controlled conditions. Additionally, the cost of production of these species is low, suggesting that the industrial production could be a short crop cycle (approx. 7-8 weeks) [3].

Chitin is a fibrous substance generally found in the exoskeletons of shellfish and the cell walls of fungus [5]. Due to the small size of the chitin nanofibers, they show high mechanical and physical properties [6]. The chitin nanofibers extracted from the cell walls of edible mushrooms have more scope as novel functional food [7-9].

Hydrogels are polymer networks that consist of hydrophilic polymer units which tend to swell when added to water [9, 10]. These can be natural or artificial based on their origin. Chitin hydrogels are naturally originated polymers that have been reported to be non-toxic,

biocompatible, and biodegradable and hence have potential use in the field of biomedical sciences [11-13].

## 2. Materials and Methods

### 2.1. Chemicals used.

All chemicals used in this study were of analytical grade. Sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium hypochlorite (NaClO), acetic acid (CH<sub>3</sub>COOH), ethanol (C<sub>2</sub>H<sub>5</sub>OH), calcium chloride dihydrate (CaCl<sub>2</sub> · 2H<sub>2</sub>O), methanol (CH<sub>3</sub>OH) were bought from SISCON India Pvt Ltd.

### 2.2. Collection of milky white mushroom.

Milky white mushrooms were collected from Mushroom Incubation Center, Center for Ocean Research, Dr. Jeppiaar Research Park, Sathyabama Institute of Science and Technology, Chennai. The mushrooms were washed, dried, and then cut into uniform size.

### 2.3. Isolation of chitin nanofibers.

Extraction of chitin nanofibers from *Calocybe indica* was carried out as described earlier [6, 14]. Following the chemical treatment, the sample was filtered and washed several times with distilled water to neutralize the residue. The wet fibers were then dispersed in water to obtain 1 wt % concentration, to which acetic acid was added to adjust the pH to 3. The wet fibers were then ultra-sonicated for 1 hour to obtain well-dispersed chitin nanofibers [15]. The nanofiber slurry was further diluted with ethanol and lyophilized to form a sheet of chitin nanofibers.

### 2.4. Characterization of chitin nanofibers.

Chitin nanofibers were characterized to determine the presence of various functional groups, find the changes in the chemical compound after chemical treatment, confirm thermal stability, elucidate its size and surface morphology and find its crystallinity using the techniques such as UV-Visible Spectroscopy, FTIR, TGA, Fe-SEM and XRD.

### 2.5. Antioxidant activity of chitin nanofibers.

The antioxidant property of chitin nanofiber was evaluated using different assays to ensure correlation between different assays and identify antioxidant properties against various substances. DPPH Assay was carried out following the method described earlier [16, 17]. ABTS assay was performed as described earlier [18]. The IC<sub>50</sub> value of the sample was calculated, and the percentage inhibition was calculated [19]. Hydrogen peroxide assay was performed with slight modifications [20]. 40 mM H<sub>2</sub>O<sub>2</sub> was dissolved in 0.1 M PBS solution. 3.4 mL of PBS solution was added to each concentration of chitin sample followed by 0.6 mL of 40 mM H<sub>2</sub>O<sub>2</sub> solution. The absorbance was read at 230nm. Percentage inhibition was calculated. Hydroxyl radical scavenging activity was performed with the method described earlier with slight modifications [21]. The reaction mixture contained 1 mL of different concentrations of sample solution; 1 mL of Iron-EDTA solution, 0.5 mL of 0.018% EDTA solution and 0.5 mL of 0.22% Ascorbic acid solution. 3 mL of NASH reagent was added and

incubated at room temperature for 15min. The intensity of yellow color developed was read at 412nm. Further, the Percentage Inhibition was also calculated. Superoxide assay was executed according to the method described earlier with slight modifications [22]. The reaction mixture contained 100 $\mu$ l of different concentrations of the sample, 100  $\mu$ L of 1.5M NBT, 200  $\mu$ L of 0.1M EDTA, 50  $\mu$ L of riboflavin, and 2.55 mL of 0.067 M PBS solution. The reaction tubes were illuminated for 30 minutes. The absorbance was read at 560 nm. Percentage inhibition was calculated.

### 2.6. Preparation of hydrogel.

Calcium solvent was prepared by heating 225g of Calcium Chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) with 300 mL of methanol and refluxed at 70°C for 4 hr [12]. Chitin nanofiber was then slowly added to the calcium solvent. The suspension was heated at 70°C with continuous stirring for 4 hr. The chitin solution was obtained and carefully washed with water to prepare a solid gel. The addition of Chitin solution into an excessive amount of water facilitated the formation of chitin hydrogels [11]. Excess Calcium chloride in the chitin hydrogel was then removed by dialysis against water for 3 days with a change of freshwater 4 times per day.

## 3. Results and Discussion

### 3.1. UV-visible spectroscopy.

Chitin nanofibers exhibited maximum absorbance at 230nm (Figure 1). Dhananasekaran *et al.* [23] have reported absorbance maxima at 201nm for chitin nanoparticles in 0.1M HCl. Peaks between 300nm to 400nm might be due to small amounts of impurities.

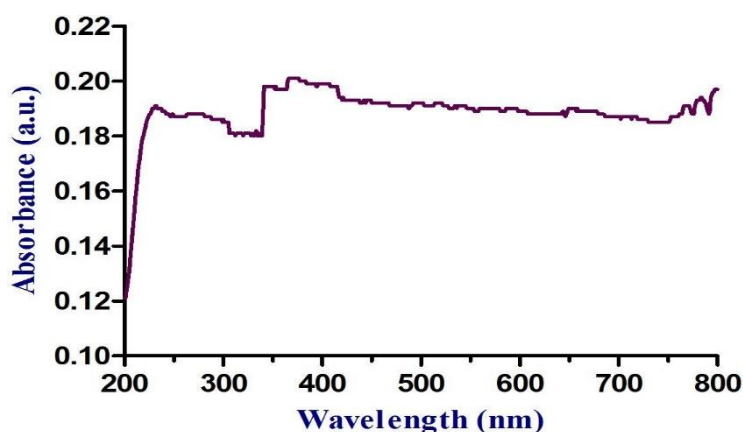
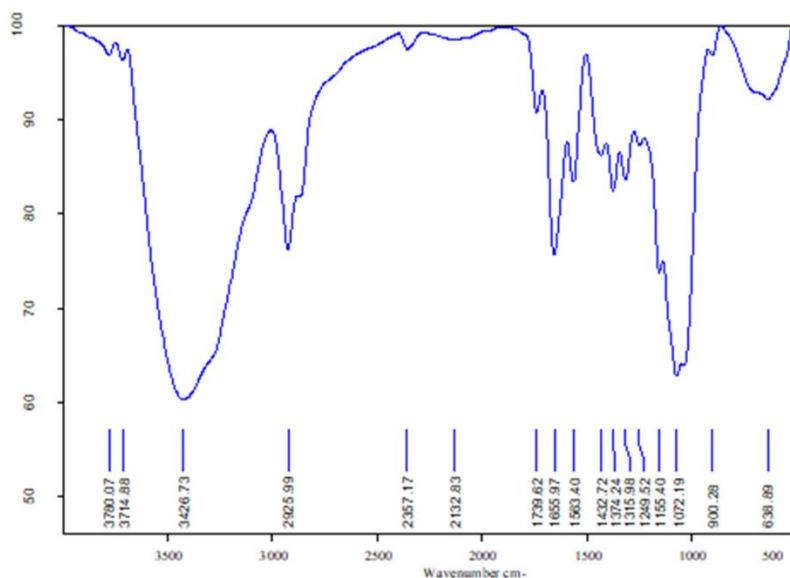


Figure 1. UV- Visible Spectroscopy of Chitin Nanofibers.

### 3.2. FT-IR spectroscopy of chitin nanofiber.

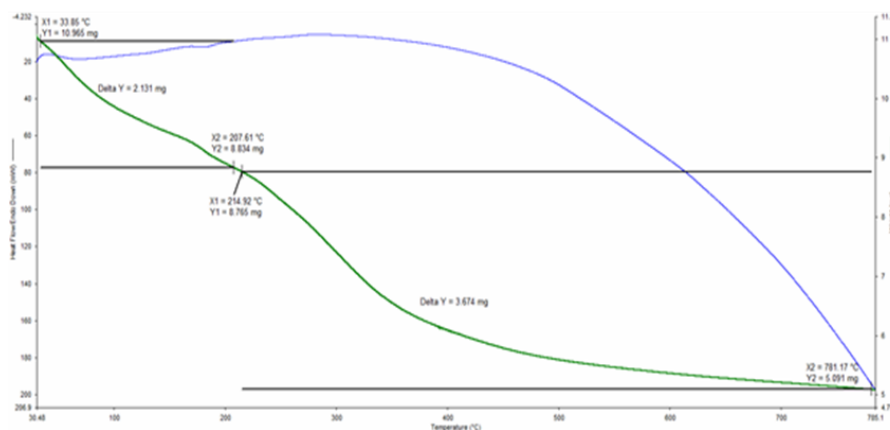
FTIR analysis was carried out in the three regions ( $500\text{cm}^{-1}$ – $1500\text{cm}^{-1}$ ,  $1500\text{cm}^{-1}$ – $2500\text{cm}^{-1}$  and  $2500\text{cm}^{-1}$ – $3500\text{cm}^{-1}$ ) for the pure chitin nanofibers (Figure 2). Significant troughs were observed at  $1655\text{cm}^{-1}$  and  $1563\text{cm}^{-1}$ , confirming the presence of amide I and amide II groups, respectively. Ifuku *et al.* [6] showed similar troughs between  $1660\text{cm}^{-1}$  and  $1620\text{cm}^{-1}$  for the amide I group and  $1560\text{cm}^{-1}$  for the amide II group. Troughs at  $2925\text{cm}^{-1}$  and  $3426\text{cm}^{-1}$  are due to the C-H stretching and O-H stretching, respectively, in chitin. Trung *et al.* 2015 illustrated IR bands in a similar range.



**Figure 2.** FTIR Spectroscopy of chitin Nanofibers.

### 3.3. Thermogravimetric analysis of chitin nanofiber.

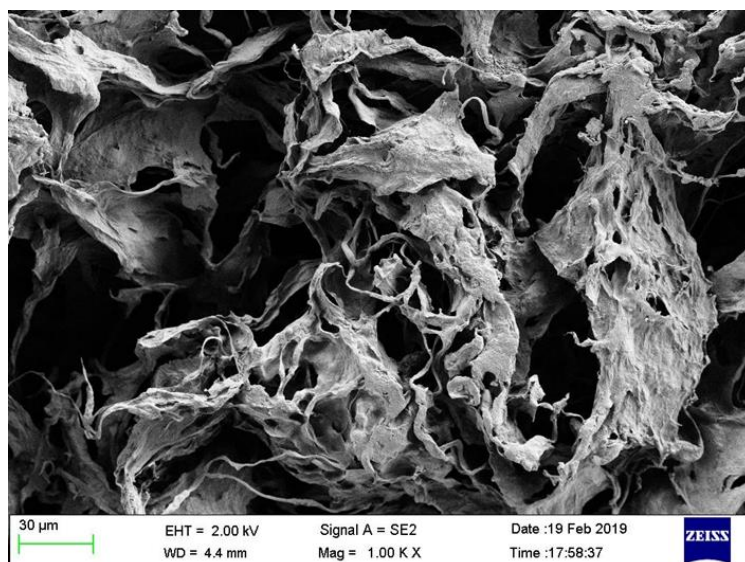
The thermogravimetric analysis of chitin nanofibers was carried out to determine the thermal stability and degradation profile. From the TGA curve (Figure 3), it can be inferred that the nanofiber starts to decompose at 207°C, characterized by a slight loss of mass. This might be due to the release of moisture content from the sample. Further, a dramatic weight loss is observed between 213°C to 402°C, indicating pyrolysis and decomposition of nanofibers. At 407°C, the loss in the material is slowed down. It is thus inferred that the chitin nanofiber is being thermostable and further suggests that it can be considered an ideal material for fabrication and drug delivery applications.



**Figure 3.** TGA Curve of Chitin Nanofiber.

### 3.4. Scanning electron microscopy analysis of chitin nanofiber.

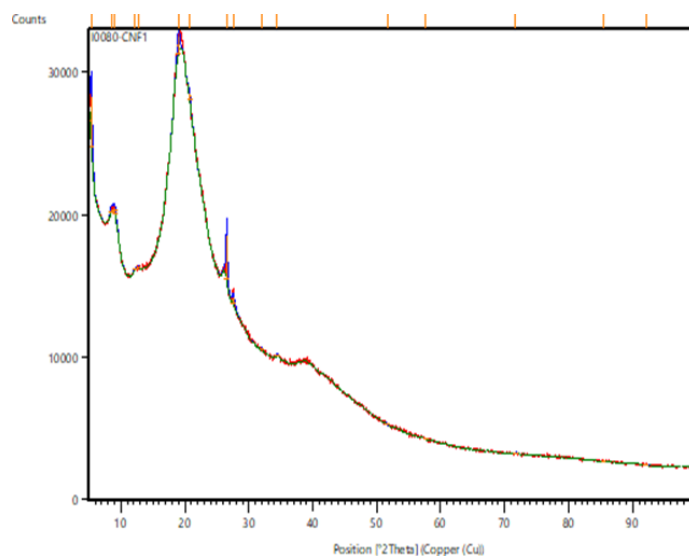
SEM results showed non-uniformly shaped long and aggregated chitin nanofibers with lengths ranging from 20µm to 100µm (Figure 4). However, Ifuku *et al.* [6] has reported chitin nanofibers with uniform shape and size on a nanometer scale.



**Figure 4.** SEM image of Chitin nanofiber.

### 3.5. X-ray diffraction crystallography.

The XRD profile showed sharp peaks at  $2\theta = 9.03^\circ$  and  $18.99^\circ$ , indicating that the chitin nanofibers are highly crystalline (Figure 5). The peaks confirm a stable  $\alpha$ -chitin structure [24].



**Figure 5.** XRD spectra of Chitin Nanofiber.

### 3.6. Antioxidant property.

Antioxidant activity of the chitin nanofibers from *Calocybe indica* was studied by comparing and correlating the scavenging activity of different assays.

#### 3.6.1. DPPH assay.

DPPH assay is a rapid technique for screening the radical scavenging activity of specific compounds. The results showed that the percentage inhibition increased gradually as the concentration increased (Table 1). The  $IC_{50}$  value of the chitin nanofiber sample was found to be at  $500\mu\text{g}/\text{mL}$ . The Scavenging activity may be primarily due to the Hydrogen donating ability of the chitin nanofibers [18].

### 3.6.2. ABTS assay.

Unlike DPPH assay, the ABTS assay is considerably fast as it involves electron transfer among the molecules. Percentage inhibition of ABTS radical scavenging assay increased more significantly than DPPH assay, and the IC<sub>50</sub> value was found to be 3µg/ mL (Table 1), which was less than that obtained by DPPH assay.

### 3.6.3. H<sub>2</sub>O<sub>2</sub> scavenging activity.

Unlike the DPPH Assay and ABTS assay, H<sub>2</sub>O<sub>2</sub> scavenging assay did not show any inhibition as the concentration of the sample increased. The major principle behind this assay is the significant decrease in absorbance of H<sub>2</sub>O<sub>2</sub> as it gets oxidized [20].

### 3.6.4. Hydroxyl radical scavenging activity.

The percentage inhibition pattern of the Hydroxyl scavenging assay was like the ABTS assay. There was a steady increase in the scavenging activity, and the IC<sub>50</sub> value was found to be at 30µg/ mL (Table 1).

### 3.7. SOD assay.

Superoxide assay is characterized by the ability of chitin to inhibit the photochemical reduction of NBT [22]. The NBT molecules are reduced to blue formazan [20]. Unlike the other assays, scavenging activity by SOD assay increased gradually and peaked after 100µg/ mL concentration. The IC<sub>50</sub> value for the chitin sample was calculated to be 80µg/ mL (Table 1).

**Table 1.** Percentage inhibition of different Antioxidant assays.

Concentration (µg/ mL)	%Inhibition of DPPH Assay	% Inhibition of ABTS Assay	% Inhibition of H <sub>2</sub> O <sub>2</sub> Assay	%Inhibition of Hydroxyl Scavenging Assay	% Inhibition of SOD Assay
10	3.33	60.7	-	46.9	12.50
30	8.33	69.8	-	50	18.75
100	18.33	75.8	-	53.1	68.75
300	43.33	86.6	-	56.4	75.00
500	51.66	88	-	57.9	81.25
800	55.00	89	-	57.9	87.50
1000	60.01	91.1	-	60.7	93.75

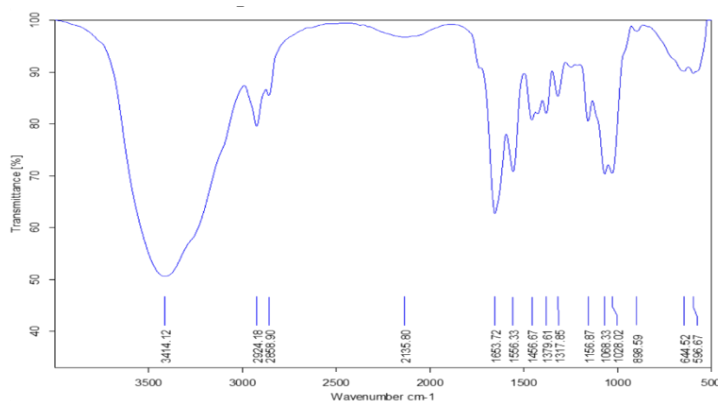
### 3.8. FTIR spectroscopy of chitin hydrogel.

FTIR spectra of Chitin Hydrogel showed a narrow trough at 3414cm<sup>-1</sup> that confirmed the presence of the hydroxyl group (Figure 6). IR bands in the region between 1156cm<sup>-1</sup> and 896cm<sup>-1</sup> correspond to the C-O-C stretching and C-O stretching [25]. Troughs at 1653cm<sup>-1</sup> and 1556cm<sup>-1</sup> represent the presence of amide groups. Tang *et al.* [26] illustrated IR bands for Chitin/CMC hydrogels at 3446 cm<sup>-1</sup> for OH stretching and bands at 1660 cm<sup>-1</sup>, 1623 cm<sup>-1</sup>, and 1557 cm<sup>-1</sup> for amide groups.

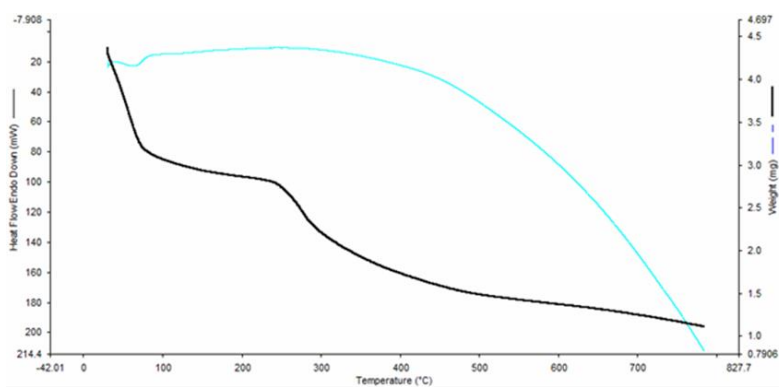
### 3.9. Thermogravimetric analysis of chitin hydrogel.

The TGA curve of hydrogel showed contrasting results from the TGA curve of chitin nanofibers (Figure 7). The hydrogel started to decompose at 95°C, accompanied by a slight loss in mass due to the release of moisture content. A dramatic weight loss was observed

between 213°C to 402°C indicating the decomposition of the hydrogel. The weight loss slowed down at 407°C. Thus, the results indicate that the hydrogel is thermostable and can be used for fabrication and drug delivery applications.



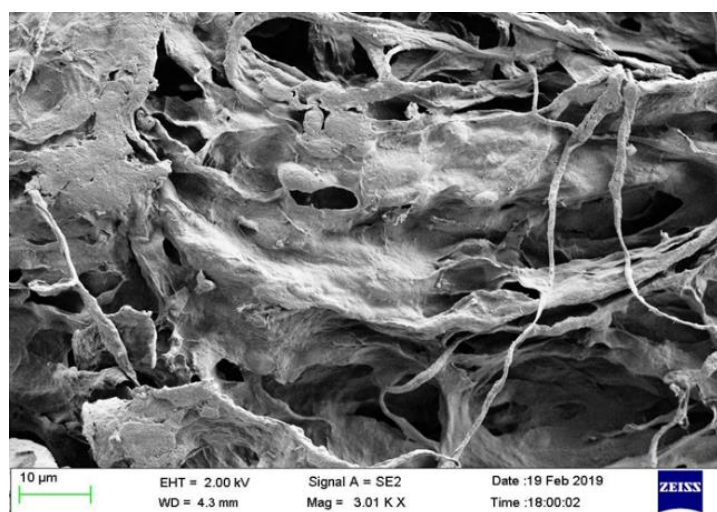
**Figure 6.** FTIR Spectroscopy of chitin Hydrogel.



**Figure 7.** TGA Curve of Chitin Hydrogel.

### 3.10. Scanning electron microscopy analysis of chitin hydrogel.

SEM image of chitin hydrogels showed a well-connected network of chitin nanofibers with a three-dimensional porous structure (Figure 8). These pores help water molecules to diffuse into the hydrogels. The obtained results were identical to those reported by Tang *et al.* [26].



**Figure 8.** SEM image of chitin Hydrogel.

## 4. Conclusions

Chitin nanofibers from *Calocybe indica* were produced and characterized. Various antioxidant assays for the chitin fibers were performed and compared with each other. Chitin hydrogel was prepared from the obtained chitin nanofibers, which were then characterized. The thermal stability of both the chitin nanofibers and the hydrogels was studied. In conclusion, these nanofibers can be used for various biomedical applications.

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This research has no acknowledgment.

## Conflicts of Interest

The authors declare no conflict of interest.

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