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Characterization of physico-chemical properties of chitin extracted from *Coprinopsis cinerea*, a coprophilous fungus

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ABSTRACT

Chitin has great applicability in biomedical and biotechnological fields because of its non-toxic, biodegradable and biocompatible nature, and having antimicrobial and antioxidant properties. The most common source of chitin is the crustaceous shell; however, mushrooms are an alternative source for isolating these biopolymers because their cell wall has a high content of chitin. The main objective of this study was to isolate chitin from the coprophilous mushroom *Coprinopsis cinerea* and to determine its physico-chemical properties. The material characterization was done using X-ray diffraction (XRD), Fourier transform infrared (FTIR)spectroscopy, Scanning electron microscopy (SEM) and Thermogravimetric analysis (TGA). We extracted 42.8 grams of chitin per 100 grams of mycelial biomass of *C. cinerea*. The extracted chitin had a significant similarity with commercial chitin, including diffractogram peaks, characteristic infrared analysis bands. The crystallinity index (CrI) value of chitin was calculated as 57.48%. The scanning electron micrograph (SEM) indicated the presence of nanofibre on the surface of the chitin. The maximum degradation temperature of *C. cinerea* chitin was found to be 378°C by Thermogravimetric analysis (TGA). This is the first report of extraction of chitin from *C. cinerea*, a coprophilous fungus and determination of its physico-chemical properties. This data gives us basic information on the possibility of introducing this biomaterial in the field of biomedicine.

Keywords : Coprophilous mushroom, *Coprinopsis cinerea* ,Chitin, XRD, FTIR, TGA

INTRODUCTION

Chitin is a structural amino polysaccharide found in the cell wall of yeast, fungi, protists and diatoms as well which also forms the exoskeletons of broad variety of invertebrates including sponges, worms, mollusks and, especially, arthropods species. It has a wide range of uses in fields such as cosmetics, pharmacy, medicine, bioengineering, biological material science, agriculture, textiles and environmental engineering based upon its nontoxic, ecofriendly, biocompatibility and biodegradability characteristics (Ospina *et al*., 2014; Kaya *et al.,* 2015; Ospina *et al*., 2015; Hoque *et al.,* 2018). Commercially, chitin is obtained by processing the outer skeleton of crustaceans such as shrimp, crab, prawn and crayfish after they have been consumed as food. However, the availability of crustacean shell waste is limited and is subject to season and supply. In recent years, chitin extracted from fungal mycelia is gaining importance. Fungal mycelia can be cultivated throughout the year and can be performed in bioreactor with all automated and controlled conditions, therefore, mycelium biomass produced in each batch is homogeneous in quality and quantity (Yen and Mau, 2007; Abdou *et al.,* 2008).

Coprinopsis cinerea is a coprophilous fungus, which in nature grows on herbivores dung (Buller, 1931; Uljé and Noordeloos, 1999). It is commonly called as inky cap mushroom and serves as a model oraganism for fruit body development in basidiomycetes (Kues and Liu, 2000). Till date there is no report on the extraction of chitin from *C. cinerea*. This study describes the extraction of chitin from *C. cinerea* cultivated under submerged culture conditions and analysis of its physico-chemical properties by using various advanced analytical techniques.

MATERIAL AND METHODS

Chemicals: The chemicals and reagents (analytical grade) used in the media and reagent preparation were purchased from Sisco Research Laboratory (SRL) and Himedia, Mumbai, India.

Fungal culture: The pure culture of *C. cinerea* (KX468975) is a lab isolate, isolated by us from horse dung. The culture used for the present study was maintained in 2% wheat flour agar (WFA) medium at 4°C (Mohankumar and Savitha, 2017).

Growth of mushroom mycelium (*C. cinerea*): Submerged fermentation was carried out to cultivate *C. cinerea* (KX468975) in 2% wheat flour broth (WFB) medium incubated for 7 days at 30°C and pH 6 under dark (Mohankumar and Savitha, 2017). After growth, the mycelia was separated from culture filtrate and used for the extraction of chitin.

Chitin extraction: The extraction of chitin from the mycelium of *C. cinerea* was carried out according to the method described by Ospina *et al.,* (2015). Forty grams dried mycelia biomass of *C. cinerea* was homogenized in 100 ml of deionized water. The suspension obtained was centrifuged at 7000 rpm for 15 min, and the precipitate was submitted to deproteinization with NaOH (4 M) at a ratio of 1:20 (p/v) by constantly stirring at 100°C for 2 hrs. Later, it was washed repeatedly with deionized water, centrifuged and supernatant was discarded. The deproteinization treatment was conducted twice and the pellet was dried at 50°C until it reached a constant weight.

X-ray diffraction (XRD) studies of chitin: X-ray diffraction analysis was performed on extracted chitin (in triplicate) using X´Pert PRO MPD equipment with a 1.8 kW (40 mA y45 kV) ceramic Cu tube, K-alpha radiation at 1.5406 A°. The crystallinity index (CrI) was calculated according to the following formula described by Erdogan *et al.* (2017).

$$CrI = [(I_o - Iam)/I_o] \times 100$$

Where, I_o = Maximum intensity at 2θ 20°

Iam = Intensity of amorphous diffraction at 2θ 16°

Fourier transform infrared spectroscopy (FTIR) of chitin: Fourier transform infrared spectroscopy (FTIR)

analysis for chitin was done using an Agilant spectrophotometer that had aDTGS PerkinElmerdetector. The samples were dispersed in KBr under anhydrous condition to form pellets. The analysis was conducted at a temperature of 24°C, with 4000450 cm^{-1}wavenumber range and 4 cm^{-1}resolution (Ospina *et al.,* 2014).

Scanning electron microscopy of chitin: Scanning electron microscopy was used to examine the surface morphology of the chitin extracted from *C. cinerea*. Examination was carried out on samples coated with gold using a TESCAN VEGA 3 scanning electron microscope.

Thermogravimetric analysis (TGA) of chitin: A thermogravimetric analysis was performed for chitin according to the method described by Ospina *et al.* (2015). Test was performed in an air atmosphere with a TGA Q500 device at a warming speed of about 10°C/min. The extracted sample was heated up at a temperature ranging from 0 to 900°C to check the thermostability.

RESULTS AND DISCUSSION

Yield of chitin: The chitin flakes extracted from *C. cinerea* by the method given by Ospina *et al*. (2015) was small in size, more homogeneous and light brown in color (**Fig. 1**). The color is possibly due to the fact that there is a presence of lignin. The dry weight of chitin in *C. cinerea* was found to be 42.8%. Ospina *et al.,* (2015) reported that the dry weight of chitin in crustaceans varied between 13% and 42% and in some insect species it varied between 2.59% and 36%. Our results clearly indicate that, the chitin content of *C. cinerea* is higher than that of crustaceans and insects. Vetter (2007) reported the chitin contents in the pileus and stipe of some cultivated mushroom species such as *Agaricus bisporus* (pileus-6.67%, stipe-3.71%), *Pleurotus ostreatus* (pileus-3.78%, stipe-2.8%) and *Lentinula edodes* (pileus-8.07%, stipe-6.5%). Similarly, Kaya *et al.* (2015) reported the dry weight of chitin in *Fomitopsis pinicola* to be 30.11%. As it is seen above, there are significant differences even between mushroom species in terms of their chitin content.

Scanning electron microscopy (SEM): The surface morphology of the chitin of *C. cinerea* was examined with scanning electron microscopy (SEM). The results indicated that there are nanofibres on the surface of the chitin of *C. Cinerea* (**Fig. 2**). This is in agreement with the results documented by Ifuku *et al.* (2011) who reported the presence of uniformity in structure of nanofibrils present on the surface of chitin in five mushroom species , namely *Pleurotus eryngii*, *Agaricus bisporus*, *Lentinula edodes*, *Grifola frondosa* and *Hypsizygus marmorcus*. On the contrary, Erdogan *et al.* (2017) reported that the chitins from *Lactarius vellereus* and *Phyllophora ribis* did not possess any nanofibres on their surface. A similar study reported that chitin of *Xanthoria parietina*, a lichen species, also did not show any nanofibres on the surface of the chitin (Kaya *et al.,* 2015). Surface morphology is reported to be one of the important factors in the characterization of chitin which accounts for their application in various biomedical fields. In this regard Muzarelli (2011) documented that nanofibre chitin of fungal origin can be used in anti-tumor applications and immune-modulating activity.

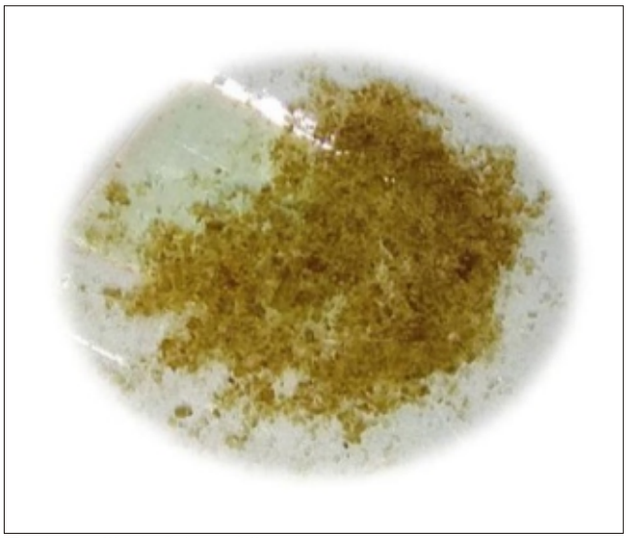

Fig 1: Chitin flakes of *Coprinopsis cinerea*

X-ray diffraction (XRD): Crystallinity of chitin extracted from *Coprinopsis cinerea* was determined by X- ray diffraction analysis. Diffractogram shows three sharp crystalline peaks at 2θ= 6.02°, 20° and 29.5°. These peaks are similar to the ones reported by Ospina *et al.* (2014), in case of

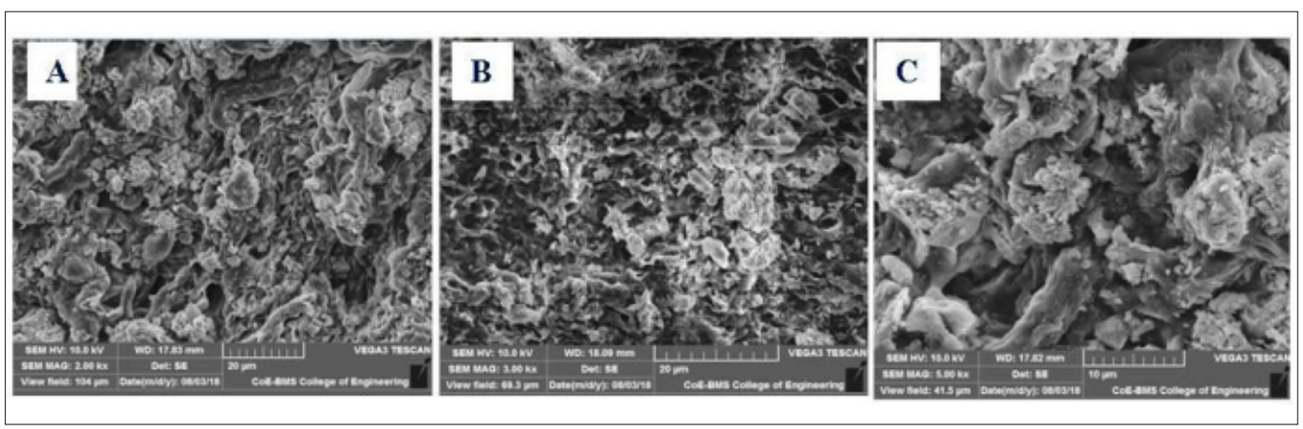

Fig 2: Scanning electron micrographs of chitin of *Coprinopsis cinerea*: A) 2000X, B) 3000X and C) 5000X.

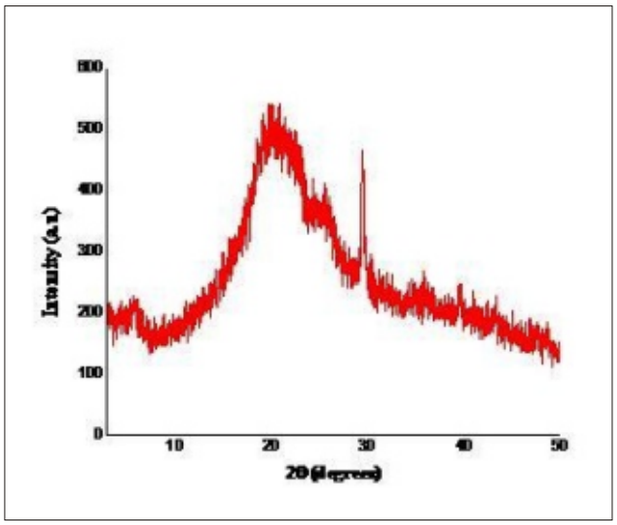

Fig 3: X-ray diffractogram of chitin of *C. cinerea*

chitin from *Ganoderma lucidum* which is reported to give crystalline peaks at 5.6°, 21.7° and 30.1°. On the contrary, crustacean chitin is reported to have two sharp peaks around 9° and 19° (Wang *et al.,* 2013). Similarly, Erdogan *et al* . (2017) reported sharp crystalline peaks at 9.20° and 19.64° in case of chitin extracted from *Lactarius vellereus* and in case of *Phyllophora ribis* at 9.38° and 19.60°.

Crystallinity index (CrI) of the chitin extracted from *Coprinopsis cinerea* was calculated as 57.48%. In literature, the CrI values reported are around 67.8% and 64.1% for crab and shrimp chitins (Hajji *et al.,* 2014). In case of chitin from *Lactarius vellereus* and *Phyllophora ribis*, Erdogan *et al.* (2017) reported CrI values of 64% and 49%, respectively. Ifuku *et al.* (2011) documented that CrI values of five mushroom species varied between 47.6% and 88.5%. Crystallinity index (CrI) values are reported to be the indicators of the purity of chitin (Ifuku *et al.,* 2011). Higher the crystallinity index higher the purity.

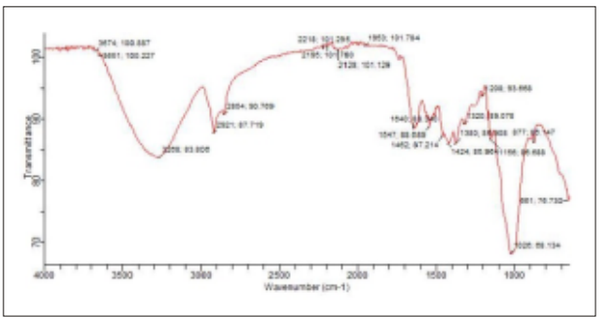

Fig 4: FTIR spectrum of chitin of *C. cinerea*

Fourier transform infrared spectroscopy (FTIR): FTIR analysis of chitin extracted from *C. cinerea* is shown in **Fig. 4**. The FTIR shows chitin representing bands at 3674, 3268, 2854, 1640, 1547, 1380, 1320, 1156 and 1020 cm^{-1} (**Table 1**). This is in agreement with the FTIR analysis of chitin extracted from *Ganoderma lucidum* (Ospina *et al*., 2014). The band at 3268 cm^{-1} represents the N-H stretching and the band at 2854 cm^{-1} represents the C-H stretching. Teng *et al.* (2001) reported that bands near to 2900 cm^{-1} are representative bands for chitin. The band at 1640 cm^{-1} corresponds to amide I stretching C=O, while the band at 1547 cm^{-1} to the stretching or N-H deformation of amide II.

Thermogravimetric analysis (TGA): The result of the TGA thermal details of chitin extracted from *Coprinopsis cinerea* is shown in **Fig. 5** and **Table 2**. The curve in the **Fig. 5** shows an evidence of a loss of weight in three stages. The first stage of 11.87% weight loss occurs in the range between 24 and 103° C which could be a result of the loss of absorbed water. This process is reported to be an endothermic reaction, which resulted from the existence of adsorbed and bound water in the samples (Yang *et al.,* 2015). The second stage starts at approximately 103° C and continues to 378° C and during this interval there is 55.06% weight loss. This weight loss is attributed to saccharide degradation in the molecular structure (Ospina *et al*., 2015). In the third stage there is 13%

Table 1: FTIR bands of chitin samples isolated from *Coprinopsis cinerea* and commercial chitin

Functional group and vibration modes	Classification	Wavenumber (cm^{-1}) frequency	
		***C. cinerea* chitin**	**Commercial chitin**
O-H stretching	-	3674	3437
N-H stretching	-	3268	3261-3103
CH_3 sym.stretch and CH_2 asym. stretch	Aliphatic compounds	2218, 2195	2392
CH_3 sym.stretch	Aliphatic compound	2854	2862
C=O secondary amide stretch	Amide I	1640	1665
C=O secondary amide stretch	Amide I	-	1621
N-H bend, C-N stretch	Amide III	1547	1553
CH_2 ending and CH_3 deformation	-	1424,1468	1428
CH bend, CH_3 sym. deformation	-	1380	1375
CH_2 wagging	Amide III, components of protein	1320	1311
Asymmetric bridge oxygen stretching	-	1156	1154
Asymmetric in-phase ring stretching mode	-	-	1115
C-O-C asym. stretch in phase ring	Saccharide rings	-	1069
C-O aym. stretch in phase ring	-	1020	1020
CH_3 wagging	Along chain	-	951
CH ring stretching	Saccharide rings	-	897

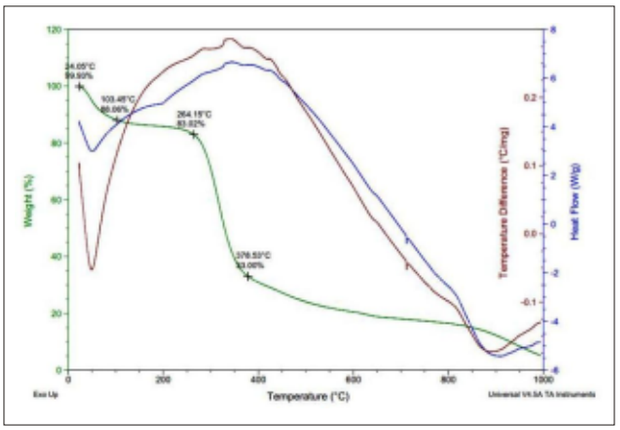

Fig 5: Thermogram of chitin of *C. cinerea*

weight loss in a temperature range of 378-800° C, which is reported to a thermal decomposition of glucosamine (Ramya *et al.,* 2012). It is noted that, chitin start degrading at 264.15° C, so it is only safe to work with this material only up to this temperature.

The maximum degradation temperature of chitin obtained from *C. cinerea* was measured at 378°C. This is in agreement with the results of Jang *et al.,* (2004) who reported 300-400°C, the degradation temperature values for α-chitin. Erdogan *et al.* (2017) reported the degradation temperature values of chitin from *Lactarius vellereus* and *Phyllophora ribis*were to be 354 and 275°C. In crustaceans and insects the maximum degradation temperature values of chitin was found to be 350° and 380° C. Considering these results, it is

Table 2: TGA thermal analysisof chitin of *Coprinopsis cinerea*

Percentage decomposition (%)	Decomposition temperature (°C)
10	80
20	280
30	300
40	320
50	330
60	350
70	420
80	700
90	980

seen that the thermal stability of the chitin obtained from *Coprinopsis cinerea* was higher than the chitin of crustaceans and few mushrooms.

CONCLUSION

Chitin is an important polysaccharide found as supporting material in the cell wall of mushrooms. Due to its high biocompatibility, it has generated an attractive interest in various fields such as biomedical, pharmaceutical, food and environmental industries for various applications. In the present study, chitin was extracted from the mycelia of *C. cinerea* and evaluated its physico-chemical properties using SEM, XRD, FTIR and TGA studies. We extracted 42.8% of chitin from mycelia of *C. cinerea*, which is the highest amount among the coprophilous mushrooms. The high thermal stability, good crystallinity index value and the nanofibres on the surface of the chitin extracted by us makes the usage of this material in the food packaging industries and as a drug delivery channel. In future, the chitin obtained from *C. cinerea* can be exploited for the synthesis of chitosan and other chitin derivatives.

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