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BIOTECHNOLOGICAL PRODUCTION OF CHITOSAN: EXTRACTION AND CHARACTERIZATION FROM *TRICHODERMA SP*.

Aim. *The study aimed at extraction and physicochemical characterization of fungal chitosan from Trichoderma reesei (MTCC 4876).* ***Methods.*** *Fungal chitosan was isolated using submerged fermentation over 4 days in three different growth media: Potato Dextrose Broth (PDB), Yeast Potato Dextrose Broth (YPDB), and Czapek Dox Broth (CDB). Growth kinetic parameters, including specific growth rate (μ) and doubling time (td), were determined for each medium. Chitosan extraction from the harvested biomass was performed using an alkaline treatment method. Physicochemical characterization of extracted chitosan using FTIR & DSC, UV-Vis spectroscopy for optical absorption properties and Photoluminescence (PL) spectroscopy to assess fluorescence behavior.* ***Results.*** *Among the tested media, PDB demonstrated the highest biomass yield of 116 mg/g. FTIR analysis confirmed the presence of functional groups analogous to commercial chitosan, indicating structural similarity. Thermal analysis via DSC validated the thermal stability of fungal chitosan. UV-Vis spectroscopy revealed significant optical absorption, while PL spectra exhibited notable fluorescence behavior, suggesting potential use in advanced material applications.* ***Conclusions.*** *In this study chitosan was successfully isolated from Trichoderma reesei (MTCC 4876), demonstrating distinct physicochemical properties comparable to the commercial chitosan. These findings highlight the potential of fungal chitosan for diverse industrial and commercial applications, particularly where optical and thermal properties are crucial.*

Keywords: *Trichoderma reesei, fungal chitosan, chitin, FTIR, DSC.*

Introduction

Chitosan, the second most abundant biopolymer in nature, has garnered significant attention due to its diverse applications and economic potential [1]. The global chitosan market was valued at USD 18.61 billion in 2024 and is projected to grow to USD 23.11 billion in 2025, eventually reaching USD 130.87 billion by 2033 with a CAGR of 24.2% [2]. This growth is driven by increasing demand in

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pharmaceuticals, food packaging, agriculture, water treatment, and biomedicine industries [3].

Chitosan is derived from chitin, which is a linear glycan made up of randomly distributed N-acetyl-D-glucosamine (GlcNAc) units linked by glycosidic β [1—4] bonds [4—6].

Chitosan has gained significant attention due to its wide range of applications in the biomedical, pharmaceutical, agricultural, and industrial fields [7]. Although chitosan is primarily extracted from crustacean shells, fungal chitosan offers a promising alternative due to its sustainability, lower allergenic potential, and more controlled production parameters [8]. Among different fungal sources, *Trichoderma reesei*, a well-known filamentous fungus, has emerged as an excellent candidate for the chitosan extraction [9].

This study focuses on the extraction and characterization of chitosan from *Trichoderma reesei* (MTCC 4876) using submerged fermentation. Chitosan is a deacetylated derivative of chitin, primarily composed of glucosamine and N-acetylglucosamine units [10]. It is a natural polysaccharide with remarkable biological properties, including biocompatibility, biodegradability, antimicrobial activity, and film-forming abilities [11]. These properties make chitosan suitable for various applications, such as wound healing, drug delivery, wastewater treatment, and food preservation [12].

Conventionally, chitosan is extracted from the exoskeleton of crustaceans, particularly shrimp and crab shells [13]. However, the seasonal availability, environmental concerns, and allergenic potential of crustacean-derived chitosan have prompted researchers to explore alternative sources [14]. Fungal chitosan has emerged as a sustainable and efficient substitute for crustacean-derived chitosan [15]. Unlike crustaceans, fungi can be cultivated under controlled conditions, ensuring consistent chitosan yield and quality [16]. Additionally, fungal chitosan exhibits unique physicochemical properties that can be tailored for specific applications [17]. Several fungal species, including *Aspergillus niger*, *Mucor rouxii*, and *Trichoderma reesei*, have been explored for chitosan production [18]. Among these, *Trichoderma reesei* is particularly promising due to its high biomass yield, rapid growth rate, and ability to produce chitosan-rich cell walls [19].

Trichoderma reesei is a mesophilic filamentous fungus known for its ability to produce cellulolytic enzymes [20]. While this fungus has been extensively studied for its industrial enzyme applications, recent research indicates that it can also serve as a valuable source of fungal chitosan [21]. The cell wall of *T. reesei* contains chitin and chitosan, which can be extracted and purified for various uses [22]. Specifically, the strain MTCC 4876 has been identified as a potent producer of high-quality chitosan under submerged fermentation conditions [23].

To the best of our understating, *Trichoderma reesei* MTCC 4876 has not been explored for extraction of chitosan. This makes this strain a suitable choice for production of fungal chitosan.

Fermentation techniques are essential for optimizing the production of fungal chitosan [24]. Submerged fermentation (SmF) is a commonly used method for cultivating fungi in liquid media, which ensures optimal nutrient availability and controlled environmental conditions [25]. This study focuses on the extraction and characterization of chitosan from *T. reesei* (MTCC 4876) and provides valuable ideas for optimizing fungal chitosan production for industrial and biomedical advancements.

Materials and Methods

Procurement of fungal strain and its revival

The fungus used in this study is *Trichoderma reesei* (MTCC 4876), classified under the phylum Ascomycete, purchased from the Microbial Type Culture Collection and Gene bank (MTCC), IMTECH, Chandigarh, India. Potato dextrose agar was used for fungal growth & cultivation.

Cultivation condition for submerged fermentation

In this study, 500 mL Erlenmeyer flasks were used, each containing 200 mL of each of the following

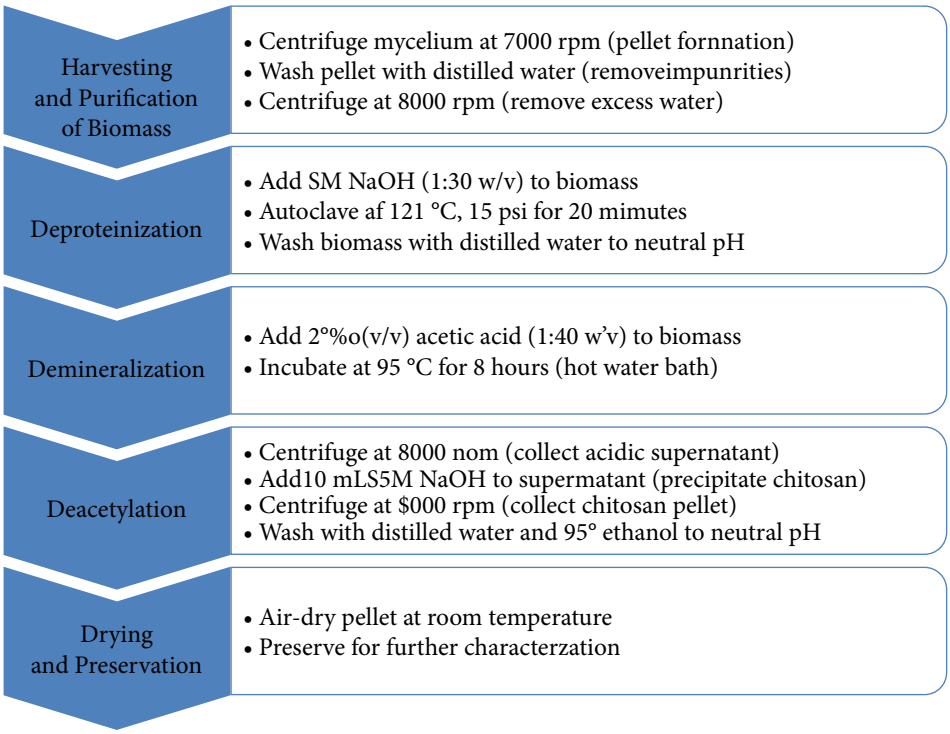

Fig. 1. Flow chart of extraction of fungal chitosan

growth media: Potato Dextrose Broth (PDB), Yeast Extract-Peptone-Dextrose Broth (YPDB), and Czapek Dox Broth (CDB). The flasks were sterilized by autoclaving at 121 °C for 15 minutes. After sterilization, they were inoculated with a fungal spore suspension at a concentration of 1.5×10^5 spores/mL (3 mL) and were incubated at 150 rpm at 30 °C for 4 days.

To monitor the fungal growth and growth kinetics studies, 5 mL sample was taken from each flask every 24 hours. PDB yielded maximum biomass and was used for further biomass production for maximum chitosan yield.

Growth kinetics of T. reesei

The specific growth rate period refers to the rate at which the biomass of a cell population increases relative to the concentration of the biomass [26, 27]. The specific growth rate (μ) is a measure of how quickly the cells are growing and can be calculated using (1).

Additionally, an important aspect of microbial growth is the doubling time (t_d), which is the time required for the microbial population to double in size [28], which provides insights into the reproductive rate of the microbes under given conditions, calculated using (2) [29].

$$\frac{dX}{dt} = \mu X; \quad (1)$$

$$\frac{t_d}{\mu} = 0.693. \quad (2)$$

Whereas dX is $(X_2 - X_1)$ and dt is $(t_2 - t_1)$.

Chitosan isolation

Fig. 1 represent extraction process of chitosan from fungal mycelium was done with slight modification [30]. The fungal mycelium was centrifuged at 7000 rpm to obtain pellet, followed by washing with distilled water to remove impurities and centrifuged again at 8000 rpm to eliminate excess water and was weighed.

To initiate the extraction process, 5M NaOH was added to the biomass (1:30 (w/v)), and the mixture was autoclaved at 121 °C with 15 psi pressure for 20 minutes. This step helped in breaking down the fungal cell walls, followed by centrifuge to remove the alkali, and the biomass was washed multiple times with distilled water until a neutral pH is achieved. Next, 2% (v/v) acetic acid was added to the neutralized biomass (1:40 (w/v)), followed by incubation in a hot water bath at 95 °C for 8 hours. Next day, the mixture was centrifuged at 8000 rpm to collect the acidic supernatant, which contains the chitosan. To recover the chitosan, 10 ml of 5M NaOH is added to the acidic supernatant, and the mixture is centrifuged again at 8000 rpm, pellet was washed with distilled water and 95% (v/v) ethanol until a neutral pH is achieved. Finally, the chitosan-containing pellet is air-dried at room temperature and preserved for further characterization.

Determination of degree of deacetylation (%DD)

The degree of deacetylation (%DD) of chitosan is an important parameter that was determined by analyzing specific infrared absorption bands [31]. This analysis involves comparing the intensity of the amide I band, which corresponds to the acetyl groups, located at 1655 cm^{-1} (denoted as A_{1655}), to the intensity of the hydroxyl groups' band at 3450 cm^{-1} (denoted as A_{3450}). The ratio of these absorbance values provides insight into the extent of deacetylation in the chitosan sample [32].

To calculate this ratio, an empirical equation, referred to as Equation 3, is employed. This equation quantifies the relationship between the two absorbance values, A_{1655} and A_{3450}, allowing for the precise determination of the % DD where 115 is a constant. This constant arises from calibration against standard methods (e.g., NMR or chemical titration) and corrects for differences in baseline absorbance, path length, and molar absorptive of functional groups involved [33].

$$\%DD = 100 - [(A_{1655} / A_{3450}) \times 115] \qquad (3)$$

Differential scanning calorimetry (DSC)

The thermal properties of the extracted fungal chitosan were evaluated using a differential scanning calorimeter (DSC), specifically the Hitachi DSC 7000X, (JIIT Noida). For the thermal analysis, 7 mg sample of the fungal chitosan was placed in a sealed aluminum pan. The sample was then heated from 30 to 250 °C at a scanning rate of 2 °C per minute, under a nitrogen gas (N_2) atmosphere. An empty aluminum pan served as a reference. These measurements are crucial for understanding the thermal behavior of chitosan, which influences its potential applications in various industries, including biomedicine, food packaging, and water treatment [34].

Fourier transform infra-red spectroscopy (FT-IR)

Fourier Transform Infrared (FT-IR) spectroscopy assesses the physiochemical characteristics of biopolymers, providing detailed information on their molecular structure. Extracted chitosan was characterized using FT-IR spectrophotometer (Spectrum BX-II model) at JIIT Noida, in transmission mode over 4000 cm^{-1} to 400 cm^{-1}, with a resolution of 4 cm^{-1} [35].

Spectroscopic analysis of extracted fungal chitosan

The UV-Vis and Photoluminescence spectral analysis are vital for understanding optical properties and structural modifications of compounds [36]. Samples were prepared by dissolving chitosan and fungal chitosan in a dilute acetic acid solution (1% acetic acid) and were filtered and final concentration adjusted to 2 mg/mL. UV-Vis measurements were conducted within a wavelength range of 200—600 nm using a quartz cuvette while PL measurements were performed using a spectrofluorometer with an excitation wavelength of 320 nm, and the emission spectra were recorded in the range of 220—600 nm.

In this study, the isolated fungal chitosan was characterized using an UV-Vis spectrophotometer (Shimadzu UV-1800 model), at JIIT Noida & Photoluminescence study on Perkin Elmer LS 55 located at PMSE, JIIT Noida.

Results and Discussion

Fig. 2 and Fig. 3 shows the wet cell biomass of *Trichoderma reesei*. The maximum value of biomass was obtained 2 days after inoculation under submerged fermentation with a specific growth rate and doubling time of three media as shown in Table 1. The specific growth rate of PDB is maximum while the doubling time is minimum among all of three selected media. Wet biomass of PDB media is maximum, so extraction is carried out from PDB only. Extraction of chitosan was done by alkali and acid treatment and after four days of fermentation, 116 mg/g of chitosan was obtained. DD% plays an important role in the physicochemical properties of fungal chitosan. In the present research the degree of deacetylation of *Trichoderma reesei* is 72% (from Equation 3), similar to the study of other industrially important fungus like *Rhizopus oryze* degree of deacetylation is 72.51% reported by Dhanashree *et al.* (2018) [37].

UV-Vis and Photoluminescence spectra of commercial chitosan and fungal chitosan

In Fig. 4*a* and Fig. 4*b*, chitosan displays a prominent adsorption band at 200—250 nm regions. This peak is often associated with the $\pi \rightarrow \pi^*$ transitions of any carbonyl (C=O) groups present, especially if the fungal chitosan is not entirely deacetylated. Residual acetyl groups can contribute to this signal.

Table 1. **Specific growth rate and doubling time of *T. reesei* in three different culture media**

Growth kinetics	PDB	YPDB	CZB
Specific growth rate μ (day^{-1})	1.02	0.21	0.63
Doubling time t_d (day)	0.68	3.31	1.11

In Fig. 4*c*, The photoluminescence (PL) spectrum displayed by the fungal chitosan features a broad peak of emission between 430—460 nm, signifying intense blue light emission resulting from electron transitions that are controlled by functional groups like –OH, $–NH_2$, and carbonyl groups as shown in Fig. 3. The peak broadness is indicative of the existence of a number of emissive sites, a reflection of the heterogeneous molecular weight and structural heterogeneity of the fungal chitosan [38]. There is peak shift which may be due to residual proteins, pigments, or polyphenolic compounds, causing stronger and broader absorbance features. Bio-molecule residues and fungal-source-specific pigments would also create trap states accountable for the emission intensity. The commercial chitosan, however, has a slightly blue-shifted emission peak located between 400—430 nm with diminished intensity and peak width, which can be attributed to its enhanced purity and homogeneous molecular topology [39].

Fourier Transform Infra-Red Spectroscopy (FTIR)

The extracted fungal chitosan was characterized using the FTIR and, it was compared to the commercial chitosan that was utilized as the industry standard. Fig. 5 shows the FTIR spectra of fungal chitosan and commercial chitosan in the wave number range of 4000—400 cm^{-1} in transmittance mode. These peaks appear as a result of the molecular vibrations associated with specific functional groups characteristic of the chitosan structure. FTIR spectrum of the fungal chitosan showed the presence of signature peaks at 3354, 2928, 1590, 1380, 1420, 1057, 1124 and 520 cm^{-1}, corresponding to the characteristic functional groups found in chitosan. The N=H bond at 3000—3500 cm^{-1} and the C=O bond at 1400—1650 cm^{-1} displayed unique vibrational band in commercial

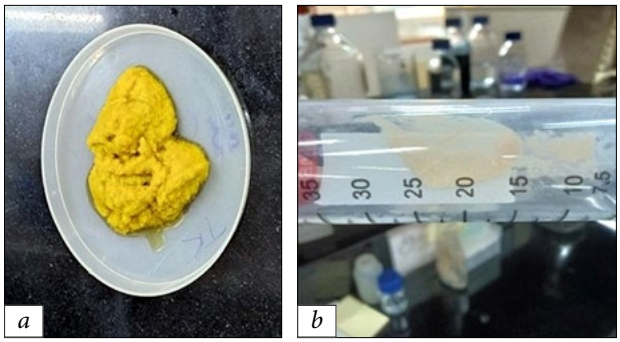

Fig. 2. Extraction of fungal chitosan. *a* — Wet biomass. *b* — Extracted fungal chitosan

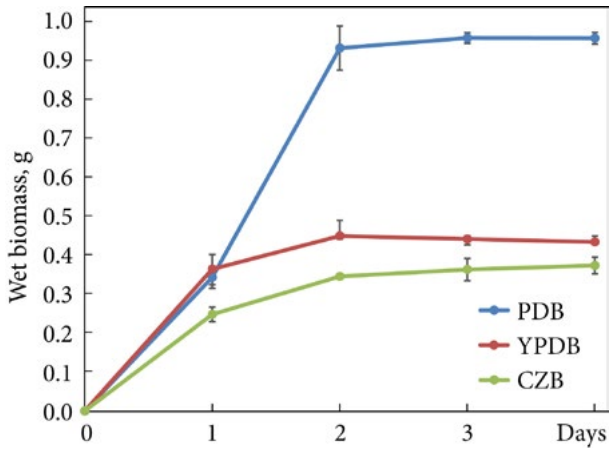

Fig. 3. Wet cell weight of *Trichoderma reesei* recovered from each 100 mL in PDB, YPDB, and CZB

chitosan. The (N=H), (O=H), and (NH_2) groups, which are found in chitosan in varying levels with the NH_2 groups present in the least quantity, could be attributed to the band at 3428—3423 cm^{-1}. The presence of a methyl group in $NHCOCH_3$, a methylene group in CH_2OH, and a methane group in the pyranose ring was demonstrated by their corresponding stretching vibrations in the range of 2926—2923 cm^{-1}. It was determined that C=O vibrations in the amide 1 band were responsible for the absorption band at 1644—1628 cm^{-1}. The trans-secondary amides identified as the amide II band correspond to the characteristic absorption band at 1576—1574 cm^{-1}. The absorption band at

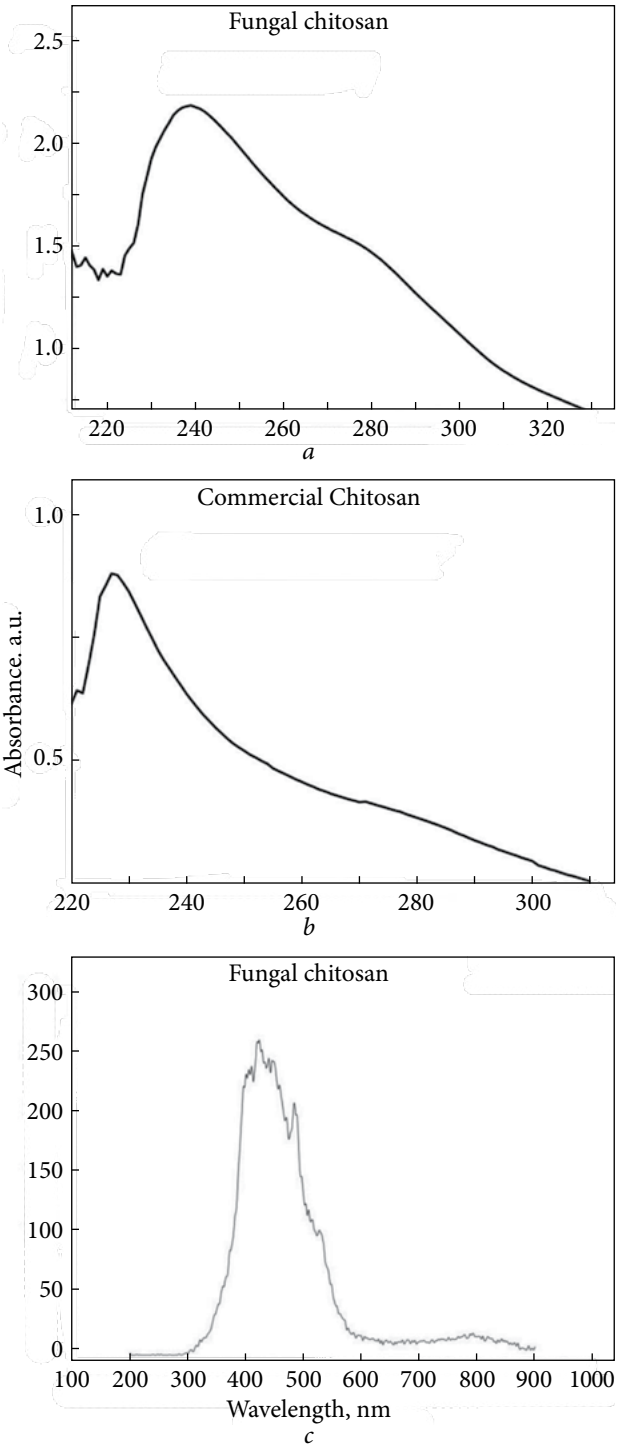

Fig. 4. UV-Vis and Photoluminescence spectra of chitosan and fungal chitosan: *a* — UV-Vis spectra of fungal chitosan; *b* — UV-Vis spectra of commercial chitosan; *c* — Photoluminescence spectra of fungal chitosan

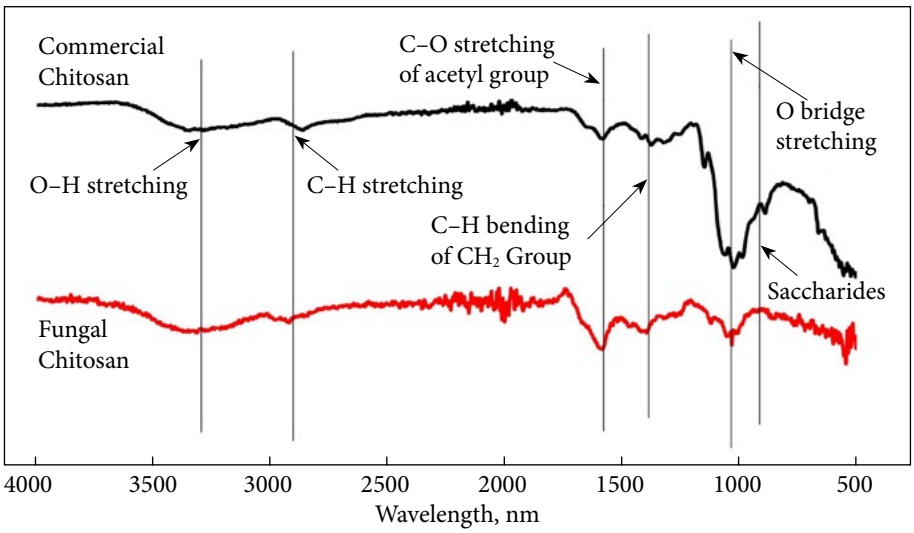

Fig. 5. FTIR spectra of commercial chitosan and fungal chitosan obtained from *Trichoderma reesei*

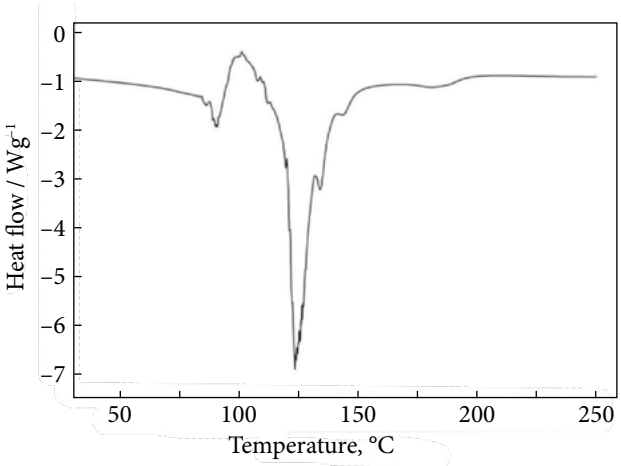

Fig. 6. DSC of fungal chitosan from *Trichoderma reesei*

Table 2. **FTIR signature peaks of chitosan with type of bonds and wavelength (40)**

Type of bonds	Wavelength range (cm^{-1})
N-H stretch	3550—3500
O-H stretch	3400—3300
C–H stretch	2800—2600
C-O stretching of acetyl group	1650—1550
C-H bending of CH_2 group	1450—1350
O bridge stretching of glucosamine residue	1100—1000
Saccharide	1100—850

1435cm^{-1} corresponds to the CH_2 bending vibrations seen in the CH_2OH group. The spectra between 1168 and 1137 cm^{-1} show the presence of three separate vibrational modes of the C–O–C, C–OH, and C–C ring vibrations. Characteristic bands were found at 1085—1077 $cm^{-1,}$ indicating that glycosidic connections exhibit C–O–C stretching vibrations. Table 2 shown different signature peaks of FTIR with different wavelength.

Differential scanning calorimetry (DSC)

Chitosan's DSC thermogram revealed common polysaccharide behavior, with two different disintegration phases (Fig. 6). In this study, the glass transition temperature of the fungal chitosan from *Trichoderma reesei* was determined to be 75 °C due to loss of water, and it was visible as a single endothermic peak. The endothermic effect of the fungal chitosan is related to positive ΔH and increased enthalpy, and a wide area under the curve suggests that the polymer requires significant heat flow to change its property within a specific temperature range. The exothermic peak (T_m) at 120 °C was correctly ascribed to the breakdown of the high amine (GlcN) content. Since autoclaving will cause the extracted chitosan (T_g 75 °C) to

lose its structural integrity, alternate procedures for sterilizing the polymer should be taken into consideration.

Conclusions

The growing demand for cost-effective and eco-friendly alternatives has recently highlighted the manufacture of chitin and chitosan using fungi. Unlike the traditional sources such as crab shells, the abundant fungal mycelium wastes from various industrial biotechnological processes present a promising source of chitosan. Extracting chitosan from fungal mycelium waste is considered a green synthesis method, offering a potential replacement for conventional techniques that have significant limitations. This study was able to recover and characterize chitosan from *Trichoderma reesei* through submerged fermentation, focusing on its structural and functional properties. *Trichoderma reesei* is a widely used fungus in industrial settings, particularly for producing cellulase and hemicellulase enzymes for bioethanol production so biomass from these industries can be used as raw material for production of the fungal chitosan. The kinetics of growth of *Trichoderma reesei* were systematically monitored to determine the optimal operating conditions for the production of chitosan, with specific focus on biomass yield and nutrient utilization within a specified time frame. The recovered chitosan was characterized through a series of spectroscopic and thermal analyses to determine its purity, chemical composition, and physicochemical properties.

Fourier Transform Infrared Spectroscopy (FTIR) was employed to determine the functional groups present in the recovered chitosan, with characteristic peaks corresponding to the hydroxyl (–OH), amine (–NH), and carbonyl (C=O) functional groups, indicating its polymeric nature. Differential Scanning Calorimetry (DSC) provided an insight into the thermal stability of chitosan through a clear endothermic peak corresponding to water loss and a higher degradation temperature, confirming its crystallinity and thermal stability.

The spectroscopic characterization of the chitosan using UV-Visible (UV-Vis) spectroscopy further confirmed its purity, where its absorption maximum falls within the expected range of biopolymer materials. Photoluminescence spectra analysis was conducted to determine the optical properties of the chitosan, with the emission peaks corresponding to the electronic transitions in the chitosan, an aspect which would be of utmost significance to its potential biomedical and optoelectronic applications.

The whole process of chitosan extraction and characterization from *Trichoderma reesei* proved its excellent structural integrity and functional properties. The findings of this study contribute to the increasing pool of information on fungal chitosan as a viable substitute for crustacean-based chitosan, with future prospects in the fields of pharmaceuticals, biotechnology, and material sciences. Future research activities should aim to optimize fermentation conditions and investigate the bioactivity of fungal chitosan to enable better industrial applications. *Trichoderma reesei*, in particular, has emerged as a viable alternative source for the chitosan production. This approach not only aligns with sustainability goals but also leverages the widespread availability of fungal biomass, making it a practical and environmentally responsible choice for large-scale chitosan production.

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Competing Interests. The authors have no financial and non-financial competing interests to declare that are relevant to the content of this article.

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БІОТЕХНОЛОГІЧНЕ ВИРОБНИЦТВО ХІТОЗАНУ:
ЕКСТРАКЦІЯ З *TRICHODERMA SP.* ТА ХАРАКТЕРИЗАЦІЯ

Мета. Дослідження спрямоване на виділення грибного хітозану з *Trichoderma reesei* (MTCC 4876), визначення його фізико-хімічних властивостей і оцінку потенціалу застосування у промисловості. ***Методи.*** Грибний хітозан виділяли шляхом глибинної ферментації протягом 4 діб на рідких поживних середовищах наступного складу: картопляно-глюкозному (PDB), дріжджово-картопляно-глюкозному (YPDB) та середовищі Чапека-Докса (CDB). Для кожного середовища визначали показники росту, включаючи специфічну швидкість росту (μ) та час подвоєння (td). Хітозан отримували з біомаси методом лужної обробки. За допомогою інфрачервоної спектроскопії з перетворенням Фур'є (FTIR) визначали його структурну характеристику для ідентифікації функціональних груп і порівняння з комерційним хітозаном. Термічну стабільність аналізували методом диференціальної скануючої калориметрії (DSC), а оптичні властивості — за допомогою спектроскопії в ультрафіолетовій і видимій областях (UV-Vis). Фотолюмінісцентну характеристику визначали за допомогою спектроскопії фотолюмінесценції (PL). ***Результати.*** Серед досліджуваних середовищ найбільший вихід біомаси (116 мг/г) був отриманий на PDB. Аналіз FTIR підтвердив наявність функціональних груп, аналогічних до комерційного хітозану, що вказує на їх структурну подібність. Термічний аналіз методом DSC засвідчив термічну стабільність грибного хітозану. Спектроскопія UV-Vis показала значне оптичне поглинання, а спектри PL виявили помітну флуоресценцію, що свідчить про перспективність використання отриманого хітозану для виробництва інноваційних матеріалів. ***Висновки.*** У представленій роботі виділено хітозан із *Trichoderma reesei* (MTCC 4876), який за своїми фізико-хімічними властивостями подібний до комерційного хітозану. Отримані результати підкреслюють перспективність використання грибного хітозану в різних сферах промисловості і комерційної діяльності, особливо там, де важливими є його оптичні властивості й термостабільність.

***Ключові слова*:** *Trichoderma reesei*, грибний хітозан, хітин, FTIR, DSC.