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Review

Chitin isolation from crustaceans and mushrooms: The need for quantitative assessment

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ABSTRACT

This review examines key journal articles on the isolation of chitin from mushroom biomass comparing these findings to those related to crustacean chitin. It highlights the need for standardizing chitin characterization, emphasizing that chitin comprises a family of polymers with variations in molecular weight (Mw), degree of acetylation (%DA), and acetylation patterns (P_A), leading to diverse physicochemical properties and biological activities. The review positions fungi and mushrooms as emerging sources of 'vegan' chitin, being non-animal and free from allergenic proteins. Their ability to be cultivated year-round, along with rapid growth and low-cost biowaste substrates, makes them attractive alternatives to crustacean chitin. Market adoption of mushroom chitin will depend on its potential applications in high-value products. Traditionally, chitin characterization has been semi-qualitative, but there is now a growing recognition of how sample inconsistencies impact research quality. This review underscores the importance of quantitative analysis for achieving practical, repeatable, and reproducible results while addressing the challenges in characterizing fungal chitin. We argue that accurately determining the properties of fungal chitin is essential and should be a fundamental aspect of every study, as these properties significantly influence the polymer's characteristics and biological activity.

1. Introduction

Chitin is the second most abundant biopolymer made of monomeric *N*-acetyl-D-glucosamine units linked by glycosidic β-(1,4) linkages. The chemical structure of chitin is similar to that of cellulose, given that a 2-acetamide group replaces one hydroxy group on the C-2 carbon atom in each monomeric unit (Fig. 1(a) and (b)). Chitin is a major structural component of crustaceans, insects, helminths, and fungi, and provides rigidity to their bodies (Reese et al., 2007). Chitin is a starting material for chitosan (Fig. 1(c)), which is formed by the *N*-deacetylation of chitin and is getting attention as a functional biopolymer with a wide range of applications in industries such as biotechnology, cosmetics, personal care, agriculture, etc. The chitin and chitosan market is growing fast and is expected to reach a combined revenue of US$29 billion by the end of 2031 (Verified Market Research, 2024).

Strictly speaking, chitin is a family of compounds, and the polymer's properties and eventual applications vary depending on the chitin source, its allomorph, and the isolation process. There are three allomorphs of chitin: α-chitin, β-chitin, and γ-chitin. When the polymeric chains are arranged in an anti-parallel direction to each other, the polymer is called α-chitin; due to the type of arrangement, α-chitin has strong intermolecular and intramolecular hydrogen bonding (Liao et al., 2022). In contrast, β-chitin chains are parallel in direction and this type of chitin has strong intermolecular but weak intramolecular hydrogen bonding. The β-chitin is considered a monohydrate in the dry state with the chemical formula $[C_8H_{13}O_5N{\cdot}H_2O]_n$ (Almeida et al., 2019). For γ-chitin, every third polymeric chain follows the opposite direction to the previous chains (Seenuvasan et al., 2020; Usman et al., 2016).

Among all chitin sources, the major one is zooplankton (Jeuniaux & Voss-Foucart, 1991). However, collecting and processing ocean-based zooplankton cuticles is not viable for commercial production, leading to a focus on the waste from the seafood industry. Currently, the largest industrial source of chitin is crustacean biomass such as crab, shrimp, and lobster shells, which contain around 8–40 % chitin; the chitin in crustaceans is α-chitin in nature (Amelia et al., 2020). β-Chitin is found in squid pens, *Aphrodite chaetae*, lorica (shell) of sessile ciliates, and pogonophore tubes, although the total amount of β-chitin is lower than that of α-chitin (Jones et al., 2020). γ-Chitin exists in the cocoon fibers of the *Ptinus* beetle and the stomach of *Loligo* (Jang et al., 2004). It was reported that both α- and γ-chitin are present in fungi cell walls such as

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Ganoderma lucidum (reishi) mushrooms (Álvarez et al., 2014). There are less conventional chitin sources such as yeast (Cabib & Bowers, 1971), mold (Synowiecki & Al-Khateeb, 2003), and marine algae (Venkatachalam et al., 2015).

Crustacean and insect sources may cause allergies – conditions caused by hypersensitivity of the immune system to certain environmental compounds, due to the presence of allergenic proteins (Bose et al., 2022). The literature claims that an allergic reaction to proteins contained in crustaceans is developed in ~2 % of the US population (Sicherer et al., 2004). The immune reaction to crustaceans is usually triggered by the presence of residual proteins, and the immunogenic properties of chitin depend on the size and shape of chitin particles (Alvarez, 2014). The amount of residual protein in biomass depends on the means of its collection (i.e., the peeling process). In most peeling processes, the crustacean shells contain proteinaceous tissue remnants after deshelling, resulting in elevated protein content in raw biomass which may remain in isolated chitin. A few allergenic proteins have been identified as the cause of allergies, particularly, muscle proteins found in crustacean species (Lopata et al., 2010). Tropomyosin, sarcoplasmic calcium-binding protein, troponin C, and enzymes such as arginine kinase, triosephosphate isomerase, and α-amylase are possible allergenic sources (Barre et al., 2018; Lyon et al., 2022). The major shrimp allergen, tropomyosin, is present not only in crustaceans but also in insects (e.g., cockroaches and house dust mites) (Muzzarelli, 2010). This protein is recognized by the immune system (through activating macrophages, and T and B lymphocytes) and stimulates the production of protein-specific IgE antibodies, which consequently promote the allergy symptoms. It is unclear whether crosslinking between chitin and proteins in crustacean- and insect-derived chitin is covalent (Kramer et al., 1995), although the presence of residual proteins (Venkatachalam et al., 2015) after extraction with the ionic liquid (King et al., 2017; Venkatachalam et al., 2015) suggests there is an existing covalent bonding (Rudall, 1963). The presence of proteins is unavoidable when working with crustacean or insect biomasses.

Fungi and mushroom sources have recently increased in importance for the extraction of 'vegan' chitin because they can be derived from non-animal sources and be free from potential allergenic proteins. In addition, mushrooms can be cultured specifically for chitin production and provide a year-round supply. Fungal chitin is not limited by seasonal and regional variation, contrary to crustacean chitin. In addition, biowastes can be considered inexpensive as they can be reused to cultivate the fungi (Huq et al., 2022). Their growth is conducted under controlled conditions with no need for sunlight. Mushrooms can be cultured by stacking (vertical growth) on a minimal land area. They grow in 2–3 weeks, whereas crustaceans require ~12 months (growth model of *Litopenaeus vannamei*) (Radulovich & Fuentes-Quesada, 2019). This fast growth rate can balance out low chitin yield per wet weight of fruit body compared with that for crustaceans. It is also suggested that fungi/mushrooms better accommodate the milder chitin recovery process (Tan et al., 1996). Mushrooms such as *Lentinus edodes* (shiitake), *Agaricus bisporus* (button mushrooms), *Plerotus ostreatus* (oyster mushroom), *Ganoderma lucidum* (reishi), *Inonotus obliquus* (chaga), *Hericium erinaceus* (lions mane), *Cordyceps sinensis* (caterpillar fungus), *Flammulina velutipes* (winter mushroom), *Auricularia auricula-judae* (jelly ear), *Grifola frondose* (hen-of-the-woods), etc. are used as chitin sources.

Potential market adoption of mushroom chitin not only depends on its availability but also on the polymer's potential use in high-value products. Different sources and isolation methods impact the physical properties of the polymer and its potential applications in various end markets. If the properties of mushroom chitin are properly elucidated following each preparation method, many new opportunities would arise for highly engineered biomaterials. Just like for synthetic polymers where their characteristics define many of their physical and chemical properties, such as solubility, gelling potential, surface and interfacial properties, and structure-forming properties, mushroom-chitin characteristics can be indicative of the appropriateness of this polymer for particular uses. However, while in the case of crustacean biomass, most of the chitin properties are determined, the characterization of chitin isolated from fungal sources is frequently missing in scientific reports. Despite the large number of papers about the fungal chitin production, the reproducibility of the results often presents an issue, since basic characterization of the polymer is neglected in many papers and makes it challenging to critically compare conflicting experimental results.

This review takes a strategic approach by surveying key journal articles on the isolation of chitin from mushroom biomass and comparing it to the extraction process from crustacean biomass. As mentioned above, chitin polymers differ in their purity, molecular weight (Mw), degree of acetylation (%DA), and acetylation pattern (P_A). These different characteristics result in a diversity of physicochemical properties and different biological activities of the polymer. Hence, a special emphasis will be given to the characterization of chitin (or absence of such) with a discussion of the challenges still to be met in the characterization of chitin from fungal sources. We advocate in this review that the accurate determination of properties of fungal chitin is essential and should be presented in every paper, as these properties influence the polymer properties and determine the polymer's biological activity.

2. The structure of biomass

2.1. Crustacean

The crustacean species do not grow continually, – the exoskeleton limits their development. To grow, shrimp must periodically shed their exoskeleton ('molt') and deposit a new one. Such replacement determines both the biological aspects of crustacean species (which we, as chemists, will not cover here) and the crustacean exoskeleton structure. Data on chitin distribution throughout the cuticle is insufficient, but the studies on chitin localization specify that the crustacean cuticle contains epi-, exo-, and endocuticle portions, as well as the membrane layer (Travis, 1963; Vittori, 2024). The membrane layer consists of chitin and proteins, whereas exo- and endocuticles contain chitin, proteins, and minerals.

From a bottom-up perspective, the cuticle composition is as follows. Chitin chains (18–25 in number) are kept together via hydrogen bonding, forming a nanofiber, that possesses both crystalline and amorphous regions. The assembly of these chitin chains is wrapped

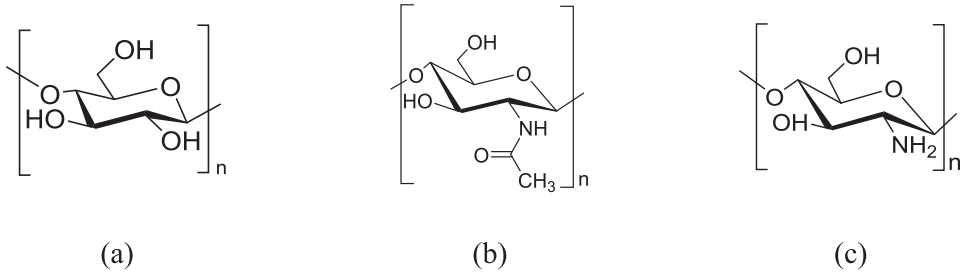


Fig. 1. Chemical structure of a) cellulose, b) chitin, and c) chitosan.

around by proteins. Several of these chitin-protein associations form a chitin-protein microfiber. In turn, several microfibrils produce chitin macrofiber. These macrofibers are arranged into a honeycomb-like planar arrangement, where honeycomb voids uptake the minerals from water during the biomineralization process (Fig. 2a) (Nikolov et al., 2010).

2.2. Fungal

Different fungi contain different levels of chitin; however, the amount of chitin is significantly lower than that found in crustacean waste biomass. In fungal sources, the synthesis of chitin and glucans takes place in the phospholipid bilayer of the cell membrane; the primary biological function of fungal chitin is to provide structural support for the fungal cell wall. Thus, the fungal classes of Basidiomycetes, Ascomycetes, Zygomycetes, and Deuteromycetes are known to contain chitin and chitosan in their cell walls (Elsoud & El Kady, 2019). Contrarily to chitin in crustaceans, chitin in the fungal cell wall exists as chitin–glucan complex (CGC), a natural copolymer of chitin and glucan, where chitin and glucans are covalently cross-linked (Fleet & Phaff, 1981; Heux et al., 2000; Kollár et al., 1997; Sietsma & Wessels, 1979). Different fungi have different proportions of chitin to glucan at the cellular level; often the amount of glucans exceeds that of chitin. The fungal cell wall also contains mannoprotein(s) (Cabib et al., 1982). Chitin and glucan polysaccharides form the structure of the cell wall of the fungi and the mannoprotein acts as a filling material (De Nobel et al., 1990; Zlotnik et al., 1984). The initial model of fungal cell wall structure suggested that the most commonly present glucans were β-glucans that possessed (1 → 3) backbone and (1 → 6) backbone (e.g., β-(1 → 3)-D-glucan, β-(1 → 6)-D-glucan), covalently associated with chitin. It was thought that individual biosynthesized chitin chains form chitin microfibrils via hydrogen bonding and are deposited next to the cell membrane. These microfibrils are linked to β-1,3-glucans by covalent bonds (Kollár et al., 1995). These β-1,3-glucans form branched networks with β-1,6-linked glucans, lengthen through the cell wall, and tie to mannoproteins on the cell wall surface (Kollár et al., 1997). The reason for this spatial packing model has been demonstrated by data obtained from both chemical and enzymatic hydrolysis (Kollár et al., 1997; Sietsma & Wessels, 1979), gene disruption (Hartland et al., 1994), fractional solubilization, separation of cell wall components followed by carbohydrate analysis (Fesel & Zuccaro, 2016; Kollár et al., 1995), and solid-state (SS) nuclear magnetic resonance (NMR) experiments (Heux et al., 2000). The amount and ratio between glucans, and the branching of glucans depend on both the mushroom species and the extraction

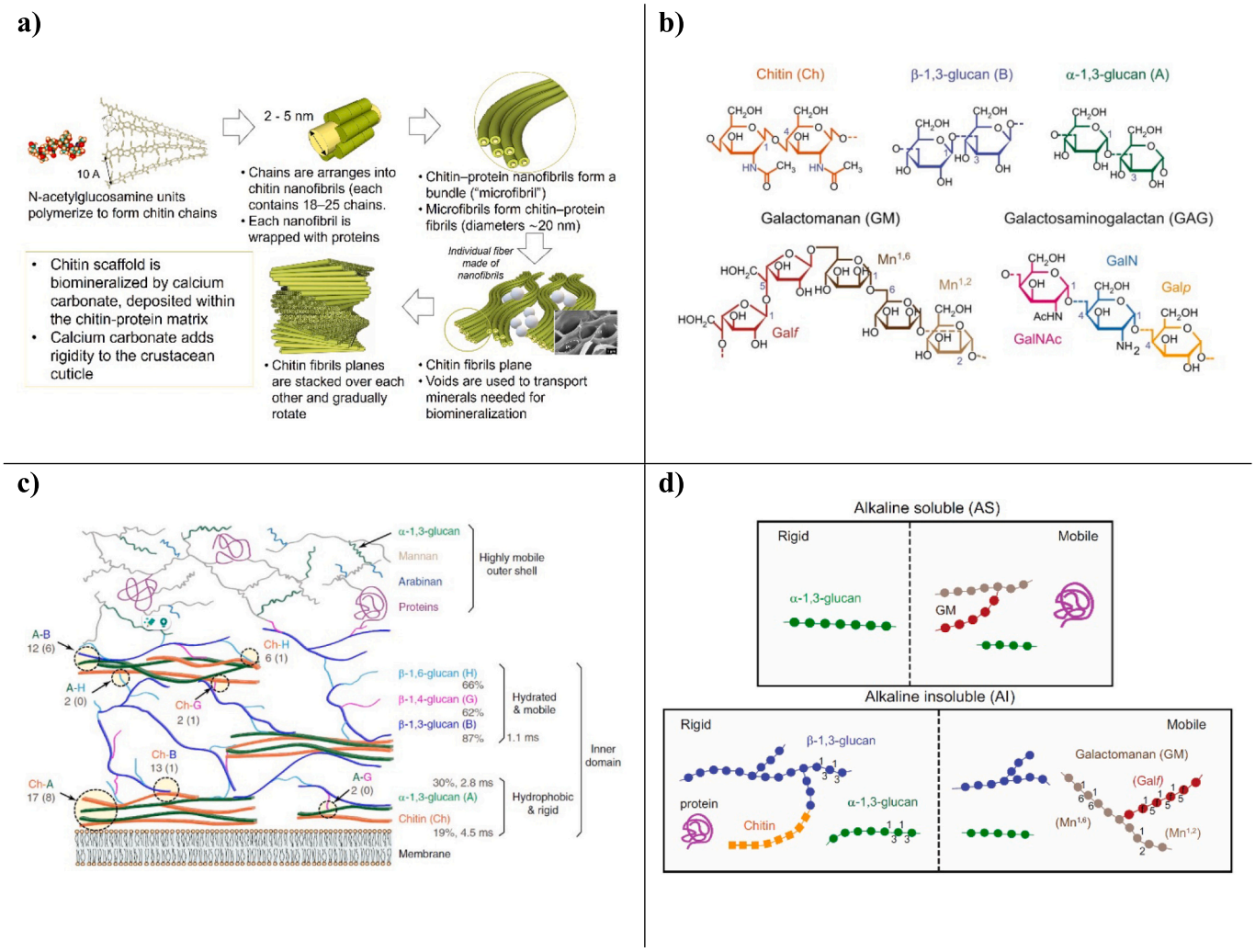


Fig. 2. Overview of the composition and structural features of crustacean cuticle and fungal cell walls, highlighting key components and their organization. a) Composition of crustacean cuticle (adapted (Nikolov et al., 2011), Copyright 2011 Elsevier); b) Representation of the components of fungal cell wall (adapted (Chakraborty et al., 2021), Copyright 2021 Springer Nature); c) Illustration of a model of the supramolecular structure of fungal cell wall (*Aspergillus fumigatus*) (adapted (Kang et al., 2018), Copyright 2018 Springer Nature); d) An overview of the proteins and polysaccharides found in the alkali-insoluble and soluble fractions' stiff and mobile parts (adapted (Chakraborty et al., 2021), Copyright 2021 Springer Nature).

process used (Sietsma & Wessels, 1977). In addition, between two major polysaccharides, chitin corresponds to a fibrillar rigid structure, and glucans represent a matrix-resembling structure (Leong et al., 2021). Most of these methods are, however, destructive and fall short of uncovering the polymer assembly in its natural state.

The three-dimensional structure of the carbohydrate-rich cell wall structure in the pathogenic fungus *Aspergillus fumigatus* was recently investigated using SS Magic Angle Spinning Dynamic Nuclear Polarization (MAS-DNP) NMR spectroscopy, to determine mutual special arrangement of carbohydrates and proteins inside the fungal cell wall (Kang et al., 2018). Because of the analysis of the intact structure, the information of the cell wall composition and polysaccharides distribution was directly obtained, without the need for isolation of specific cell wall components. Ultra-high NMR resolution with a 30-fold sensitivity enhancement allowed for the understanding of the hydrophobicity and dynamics of the constituent carbohydrates, differentiation between the rigid and flexible components, and elucidation of the spatial proximities between polysaccharides, providing information on intermolecular packing, site-specific molecular hydration, etc.

This MAS-DNP NMR coupled with glycosyl linkage analysis led to a new structural model of the fungal cell wall that differed from the earlier (simplified) idea of fungal cell wall structure; particularly it was found that there is a two-domain distribution of α-1,3-glucans. SS MAS-DNP NMR revealed the presence of multiple (as many as 65) intermolecular and inter-allomorph interactions and indicated a distribution of molecules in two domains: rigid one and dynamic one. In the poorly hydrated and mechanically rigid inner portion of the cell wall core ("rigid domain"), chitin and α-1,3-glucans closely interact and form a relatively stiff "hydrophobic scaffold." This scaffold is embedded in a soft and well-hydrated flexible matrix made of β-1,3-, β-1,4-, and β-1,6-glucans (Fig. 2b, c) (dynamic domain). The whole assembly is then capped by a highly dynamic shell coating layer that contains glycoproteins (galactomannan (GM) and galactosaminogalactan (GAG)) covalently connected to structural proteins through amino acid residues-linkers and a minor fraction of α-1,3-glucans (Chakraborty et al., 2021; Fernando et al., 2023).

This interweaving between chitin glucans has major significance in the methods of extraction, yield, and functional properties of the polymer. The associations between branched β-1,3 and 1,6-glucans which possess β-1,4-linkages to chitins were found to be alkali-insoluble (Fig. 2d) (Kang et al., 2018). Based on the high degree of hydration and mobility of these β-1,3/−1,6-glucans, the new model questions the earlier postulated role of these glucans to provide the structural support and rigidity to fungal cell wall network (Bowman & Free, 2006; Latgé, 2007). Alternatively, it was suggested that the rigidity is provided by chitin/alkali-soluble α-1,3-glucan scaffold, whereas β-1,3-glucans link together the rigid and mobile portions, the observation evident from a great number of interactions of β-1,3-glucans with other macromolecules (e.g., α-1,3-glucan). Finally, contrarily to previous findings, α-1,3-glucan was observed in both the outer surface and the inner rigid core. The glucan also exhibits high rigidity and covalently and physically interacts with β-1,3-glucans (although it is unclear whether covalent interactions exist between α-1,3-glucans and chitin) (Kang et al., 2018).

3. Chitin content and yield

A large amount of chitinous waste mainly contains chitin, proteins, and minerals, mostly calcium carbonate (Table 1) (Attwood & Zola, 1967; Hackman, 1960; Kramer et al., 1995; Percot et al., 2003a; Shimahara & Takiguchi, 1988). Table 1 shows the amount of chitin in crustaceans, insects, cepahlopods, and fungal sources.

The chitin content in the crustacean biomass is typically assessed using the 'modified' Black and Schwartz method. As originally proposed by Black & Schwartz (Black & Schwartz, 1950), dried crude biomass is first demineralized using 1 M hydrochloric acid (HCl) at 100 °C for 2 h, and filtered through a sintered glass crucible or a piece of linen, to determine acid soluble fraction (minerals). After that, deproteinization is conducted in 1.25 M sodium hydroxide (NaOH) solution, also at 100 °C for 2 h, to determine base soluble fraction (proteins). The undissolved solids (chitin and silica) are then recovered by filtration through an alundum or a Gooch crucible followed by oven-drying. The obtained material is further incinerated in a muffle furnace, burning out chitin and leaving out silica. It was indicated that the filtration may be laborious and challenging to carry out, due to the presence of fat in raw biomass. Hence, Horowitz et al. (Horowitz et al., 1957) demonstrated that acetone washing of crude biomass before demineralization/deproteinization allows for getting rid of residual fat, which makes it possible to substitute filtration with centrifugation. It was also shown that formic or acetic acids (FA or AA, respectively) could be used in place of HCl, to minimize depolymerization and, hence, potential chitin loss brought about by the formation of the water-soluble oligomeric fraction. The 'modified' procedure thus includes boiling the crustacean in acetone for 5 min, demineralization with FA or AA, and deproteinization with NaOH (Brown, 1959).

Concerning the amount of chitin in fungal biomass, for direct chitin content measurement, the polymer is isolated and its yield is determined gravimetrically, although it may be not pure chitin but CGC (Irbe et al., 2023). This, however, does not provide the actual content of chitin in biomass before the isolation process. In indirect quantification of chitin content, concentrated KOH is used for the conversion of chitin to chitosan, which is further hydrolyzed to glucosamine (Ride & Drysdale, 1972) or *N*-acetylglucosamine (Vetter, 2007), which is then quantified using colorimetry with 3-methyl-2-benzothiazolone-hydrazone-hydrochloride (MBTH) (Tsuji et al., 1969). This method was shown to be useful for quantification of the chitin content of numerous mushrooms (*Lentinus edodes*, *Lycophyllum shimeji*, *Pleurotus sajorcaju*, *Volvariella volvacea*, *A. bisporus*, *P. ostreatus*, etc.) (Alimi et al., 2023).

Another indirect method is based on the formation of an insoluble colored polyiodide–chitosan complex formed by the interaction between potassium triiodide and chitosan, followed by measurements of the solution's optical density (Nitschke et al., 2011). Once the chitosan is produced from isolated chitin and dissolved in an acidic solution (usually 1 % in AA), a small volume of this solution is applied onto a thin-layer chromatography plate and stained by Lugol's solution, which contains iodine and potassium iodide. The intensity of the color is quantitatively measured using spectrophotometry. Another technique involves capturing an image of the substrate and then analyzing the image with software (Bio-Rad Quantity One quantitation software v.4.2).

Table 1
Percentage of chitin in crustaceans, insects, cephalopods, and fungal sources.

Sources	Chitin (%)	Proteins (%)	Minerals (%)	Glucans (%)	Reference
Crustacean Biomass					
Shrimp	15–40	20–40	30–60	NA	(Díaz-Rojas et al., 2006; Hu et al., 2020; Tan et al., 2020)
Crab	25–30	15-20	30–50	NA	(Amelia et al., 2020)
Krill	2	33–55	–	NA	(Jeuniaux & Voss-Foucart, 1991; Peng et al., 2019)
Fly larvae	35	50	–	NA	(Shumo et al., 2019; Xiong et al., 2023)
Squid pen	25–49	43–75	–	NA	(Messerli et al., 2019)
Mushroom	1–7	14-38	–	20–57	(Bak et al., 2014; Dimopoulou et al., 2022; Ofenbeher-Miletić et al., 1984)

The amount of chitin in biomass can also be elucidated via nitrogen content which can be measured with techniques like combustion analysis and the Kjeldahl method (Ming et al., 2015). Assuming a known nitrogen-to-chitin conversion factor, the chitin content can be determined by measuring the amount of nitrogen in the extracted chitin. However, the obtained number includes not only chitin nitrogen but also protein impurities (if present) (Brown, 1959), being suitable only for pure polymer without proteins (i.e., the Kjeldahl procedure shall be conducted only after the deproteinization step).

4. Chitin quality

The use of strong alkaline and acidic media traditionally used in pulping of either crustacean or fungal biomass can lead to deacetylation or depolymerization of the isolated chitin (Schmitz et al., 2019). Without a doubt, this results in the necessity of determining not only chitin yield but also the two most important properties of chitin – its Mw and %DA as *minimal information* on the product. Otherwise, results are not only incomparable with each other but could not be translated into commercial applications. Such realization that material quality has to be somewhat "standardized" has received growing attention in the past decade. In his book "Chitin: Fulfilling the Biomaterial Promise" (Khor, 2014) (by far one of the best books on chitin until now and still relevant), Khor outlined the following requirements for chitin characterization:

> "While purity is focused on the identity of chitin, quality […] is concerned with the properties a particular sample possesses. The two main characteristics of chitin of interest are the Mw and DA or DD. Their determination is a **recurrent bone of contention** within the chitin community."

While these data (Mw, %DA) are mostly available for chitin isolated from crustacean biomass, characterization data for chitin from fungal sources is largely missing, as will be shown in the critical assessment section. The following subsections will illustrate the importance of these pieces of characterization for material preparation, and quickly mention methods that could be suitable for obtaining these data.

4.1. The %DA

4.1.1. Effect of %DA on the polymers' properties

The %DA refers to the percentage of *N*-acetylglucosamine units in the polymer chain relative to the sum of *N*-acetylglucosamine units and glucosamine units (Kasaai, 2009). %DA influences different properties of chitin including solubility (Franca et al., 2008; Lamarque et al., 2005; Schatz et al., 2003; Sorlier et al., 2001; Yamaguchi et al., 2003), elongation, and tensile strength (Ren et al., 2005), thermal stability (Nam et al., 2010), electrostatic behavior (de Alvarenga, 2011), and hydrophilic-lipophilic balance (Dey & Ghosh, 2020). It also impacts the polymer's biodegradability and modes of interaction with other molecules.

In water and other common solvents, chitin with a high %DA is not soluble. This is because the acetyl groups help to increase hydrophobic interactions and strengthen intermolecular hydrogen bonding, which makes the polymer less soluble and more crystalline. Low %DA polymer (%DA < 50 %) known as chitosan, is soluble in acidic aqueous solutions. Even higher solubility can be achieved by converting chitosan into salts, and by reacting free amino groups on D-glucosamine units with acids (Bonferoni et al., 2009). It's important to note that chitosans, which are water-soluble at acidic pH, have distinct properties from chitins, as noted in numerous publications (Kumar, 2000; Rinaudo, 2006). This review focuses on chitin, mentioning chitosan only when it formed during chitin isolation under highly basic conditions. The mechanical strength of chitin with a high %DA is generally higher (Kasaai, 2009; Mushi, 2021). The acetyl groups promote strong intermolecular hydrogen bonding (amide-amide type) and hydrophobic interactions, which result in a more crystalline and structured polymer. The material's stiffness and tensile strength are enhanced by the crystallinity. On the other hand, low %DA chitosan typically shows low mechanical strength. A less stiff and more amorphous material arises from the decrease in a number of acetyl groups, which interrupts the ordered crystalline structure. Although the presence of free amino groups decreases tensile strength, it increases the polymer's flexibility. With a decrease in %DA, thermal stability also declines, and the thermogravimetric analysis (TGA) curves' peak temperature for thermal decomposition moves downward (Brown, 1959).

Both chitin and chitosan materials can be engineered using various fabrication techniques to achieve degradation times ranging from days to weeks or even months, depending on the intended application of the device (Di Martino et al., 2005). The degradation rate appears to increase with higher %DA – for instance, the initial degradation rates for chitin with %DA values of 7, 19, and 29 were measured at 0.2 % per day, with remaining Mws of 98 %, 82 %, 60 %, and 50 % of initial Mw, respectively (Ren et al., 2005). Moreover, the rates rose to 0.8 %, 3.0 %, and 7.8 % per day for chitin with %DA values of 38, 44, and 48 %, respectively (Zhang & Neau, 2001). This aligns with findings from Tomihata and Ikada, which indicated a significant rise in the in vivo biodegradation rate of chitin films when the %DA reached 27 % (Tomihata & Ikada, 1997). Degradation of 50 % DA chitosan was found to be maximal compared to higher or lower % DA values (Freier et al., 2005; Schmitz et al., 2019; Zhang & Neau, 2001).

Chitosanases, chitinases, and lytic polysaccharide monooxygenases (LPMOs) have been used to degrade chitin and chitosan in soil (Ilyina et al., 2000; Izume & Ohtakara, 1987; Ribeiro et al., 2014). Zhang & Neau utilized a commercial enzyme formulation containing almond emulsin, achieving a 50 % reduction in the specific viscosity of chitosan (Zhang & Neau, 2002). Li et al. also demonstrated effective chitosan degradation using a commercial neutral protease derived from *Bacillus subtilis* (Li et al., 2007). A chitinolytic enzyme from *Streptomyces kurssanovii* showed high activity towards chitin, facilitating large-scale production of low-Mw, 10 kDa, chitosan (Ilyina et al., 2000). The recent review covers processes controlling bacterial chitin degradation (Beier & Bertilsson, 2013).

4.1.2. The methods for %DA determination

A critical review with pros, cons, and sources of error for most of the methods for %DA was given by Alvarenga in 2011 (de Alvarenga, 2011). The authors review titration methods including the linear potentiometric technique, hydrolysis/ high-performance liquid chromatography (HPLC) methods, ^{1}H NMR, SS NMR techniques (Cárdenas et al., 2004), elemental analysis, and pyrolysis/ gas chromatography (GC) method.

Fourier-transform infrared (FTIR) spectroscopy has been used by many (Kasaai, 2008; Kumirska et al., 2010), and in some cases combined with titration (Varan, 2017). Unlike transmission FTIR, where the chitin powder sample is mixed with KBr, in Attenuated Total Reflectance (ATR-) FTIR, the sample is placed on the device's stage over the ZnSe or diamond crystal. This results in challenges for chitin measured by ATR- vs. transmission FTIR. Thus, ATR-FTIR's limited penetration depth, usually only a few microns into the sample, limits its ability to analyze the sample's bulk properties, especially in cases where the sample is not homogeneous and %DA varies throughout the sample. In addition, ATR-FTIR is often less sensitive or quantitative for low-concentration analytes when compared to transmission FTIR. According to Kumirska's work (Kumirska et al., 2010), ATR-FTIR tends to exhibit lower resolution in the amide I bands ($\sim$1650 cm^{-1}), which are crucial for analyzing chitin's acetylation level. This lower resolution makes it harder to accurately distinguish between acetylated and deacetylated regions when %DA is low. Hence, FTIR on chitosan requires the use of KBr pellets; ATR-equipped instruments do not deliver sufficient detail at low %DA for the analysis.

Fungal chitin presents a specific challenge due to the presence of glucans, covalently bonded to the chitin structure. Overall increased

hydroxyl group content increases the intensity of the reference hydroxyl bands typically used for %DA calculations in FTIR spectra. Since hydroxyl groups are often used as a baseline or comparison against the amide bands for determining %DA, this extra hydroxyl content from glucan complicates accurate acetylation measurements.

There remain questions about the effectiveness of FTIR at low %DA since the method relies, in part, on quantifying rapidly shrinking acetyl carbonyl peaks. In addition, the method requires very high purification of samples before accurate measurement of chitosan carbonyls can be acquired. According to Kurita et al., FTIR is only useful in the 45–100 % DA range (Kurita et al., 2000). While ATR-FTIR is a convenient, non-destructive technique, its limitations make it less ideal for detailed quantitative studies, especially when working with low %DA chitin samples. For such cases, transmission FTIR remains more reliable for quantitative acetylation measurements due to its better penetration depth, higher sensitivity, and clearer spectral resolution.

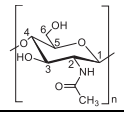
Several analytical approaches are used to determine the %DA in chitin and chitosan via FTIR spectroscopy. One common method is the absorption ratio technique (Kasaai, 2008; Kumirska et al., 2010), which involves constructing a calibration line based on the absorbance ratios of a probe band (PB)—which varies with %DA—relative to a reference band (RB)—which remains constant with %DA. Absorbances for PB and RB were determined using the baseline method. However, constructing a reliable calibration line is challenging, as selecting the proper baseline is not straightforward, and finding the optimal combinations of probe band PB and RB is challenging, as evidenced by the numerous PB/RB ratios reported in the literature.

Common absorption bands for *N*-acetylation include Amide I (1655 cm^{-1}) and Amide II (1560 cm^{-1}). Reference bands for normalization are different and include O—H stretching (3450 cm^{-1}), C—H stretching (2870–2880 cm^{-1}), etc. The extensive list of PB and RB bands is provided by Duarte et al. (Duarte et al., 2002).

A method by Beil et al. uses the first derivative of ATR-FTIR spectra (Beil et al., 2012). The derivative values of the Amide III band at 1327 cm^{-1} and the C—H deformation band at 1383 cm^{-1} are measured against the bridge oxygen vibration at 1163 cm^{-1} as an internal standard. This method shows a linear correlation with the first derivative ultraviolet (UV) spectroscopy, confirmed by elemental analysis and Raman spectroscopy. It enables the %DA determination of chitosan and chitin in the presence of water, eliminating the need for drying procedures.

Calculations of %DA by NMR differ among various techniques such as ^{1}H NMR, ^{13}C NMR, and ^{15}N NMR. ^{1}H liquid-state NMR is taken in D_2O/CH_3COOD or D_2O/DCl solvent, and is only suitable for a limited lower %DA range (i.e., chitosans). The %DA is calculated using integrals of the protons of the acetylated unit vs. the total number of protons, and different types of protons can be used for calculation, depending on spectra resolution (Table 2).

Table 2
Summary of NMR techniques for %DA calculation: formulas, advantages, and limitations.

Types of NMR	The formula used (numeration of atoms is given in the structure below): 	%DA	Advantages	Limitations	Ref
^{1}H NMR	$\%DA = \frac{I_{CH3}}{3} \Big/ \left(\frac{I_{\Sigma(H2,H3,H4,H5,H6a,H6b)}}{6}\right) \times 100$[a] $\%DA = \frac{I_{CH3}}{3I_{H1\ total}} \times 100$ $\%DA = \frac{I_{H1\ (acetylated)}}{I_{H1\ (acetylated)} + I_{H1\ (deacetylated)}} \times 100$ $\%DA = 1 - \left(\frac{I_{H1\ (deacetylated)}}{I_{H1\ (deacetylated)} + \frac{I_{CH3}}{3}}\right) \times 100$	0–60	High sensitivity; data precision; and detailed analysis of co-unit distribution.	Sample preparation required; suitable only for a limited %DA range where the sample where the sample is soluble in the solvent.	(Hirai et al., 1991; Lavertu et al., 2003; Tan et al., 1998) (Lavertu et al., 2003; Rinaudo et al., 1993; Tolaimate et al., 2000) (Hirai et al., 1991; Lavertu et al., 2003; S. C. Tan et al., 1998) (Giraudeau et al., 2014; Lavertu et al., 2003)
^{13}C CP-MAS NMR	$DA = 100 \times \frac{I_{CH3}}{\frac{1}{6}\sum I_{C1,C2,C3,C4,C5,C6}}$	0–100	No solution preparation required; suitable for the full range of DA; allows the use of wet sample; enhanced sensitivity at stronger magnetic field. Techniques like cross-polarization enhance precision, accuracy, and detection limits.	Lower resolution and sensitivity than ^{1}H NMR; high equipment cost; specialized requirements and complexity, particularly for newer models with stronger magnetic fields. The detection limit >5 %.	(Duarte et al., 2002; Giraudeau et al., 2014; Guinesi & Cavalheiro, 2006; King et al., 2017; Ottøy et al., 1996; Raymond et al., 1993; Tolaimate et al., 2000; Vårum et al., 1991)
^{15}N CP NMR	$DA = \frac{I_{N-acetamide}}{I_{N-acetamide} + I_{N-amine}}$	0–100	No solution preparation required; suitable for the full range of DA; allows the use of wet sample; enhanced sensitivity at stronger magnetic field. Techniques like cross-polarization enhance precision, accuracy, and detection limits. Ideal for chitin/chitosan blends with other polysaccharides.	Limitations mentioned for ^{13}C NMR apply here. Potential errors when protein impurities present; low sensitivity.	(Heux et al., 2000; Yu et al., 1998)

[a] I – integrals that represent the area under the peaks in the respective spectrum, proportional to the number of nuclei (protons in ^{1}H NMR, carbons in ^{13}C CP NMR, and nitrogens in carbons in ^{15}N CP NMR) contributing to each signal.

Under certain conditions, SS ^{13}C cross-polarization (CP) MAS (Giraudeau et al., 2014) and multi CP-MAS (King et al., 2017) NMR spectra can accurately represent the actual populations of carbon atoms in a sample and allow for converting SS ^{13}C CP-MAS integrals into absolute component fractions. This approach has been used to determine the %DA by integration, with results validated against other methods. The evaluation of acetyl content can be effectively achieved using ^{15}N NMR, including ^{15}N CP-MAS. Since the ^{15}N nucleus is present only in chitin and chitosan among polysaccharides, this technique shows great promise for assessing acetyl content without the need for extensive purification processes. The article by Heux et al. systematically compares %DA evaluated from ^{1}H liquid-state NMR and ^{13}C and ^{15}N SS CP-MAS NMR in the whole range of acetyl content from 0 to 100 %, demonstrating a good agreement between the methods (Heux et al., 2000). Table 2 presents various formulas from the literature used for calculating the %DA, along with a discussion of their respective advantages and limitations.

Conspicuous for its absence in the Alvarenga review is the use of colorimetry. Amines are very reactive, and acid dyes readily attach to them. Noel et al. used Orange II and Coomassie Brilliant Blue to characterize amine-functionalized polystyrene (Noel et al., 2011). Chitosan has been characterized colorimetrically using Bromocresol Purple (Chauhan et al., 2010). There is no particular accuracy advantage or disadvantage compared to titration methods. As it was mentioned above, colorimetry with 3-methyl-2-benzothiazolone-hydrazone-hydrochloride (MBTH) (Bak et al., 2014) can also be used.

4.2. Mw

4.2.1. Effect of Mw on the polymers' properties

Chitin is typically polydisperse and exists as a mixture of molecules with different Mws. Chitin's Mw varies widely depending on its source, species age, and processing method. Even when belonging to the same class, such as crustaceans, different sources (i.e., crab vs. shrimp) may have varying degrees of polymerization. The Mw of chitin has been shown to correlate with the age and size of the species from which it is isolated. Processing methods—mechanical degradation, chemical treatments (pulping vs. extraction), or enzymatic actions—significantly impact chitin's Mw. Extraction conditions (temperature, duration, and chemical concentration) are also important.

The proper characterization of the isolated product is necessary as higher Mw polymer exhibits different properties from those of traditionally 'pulped' chitin (Wineinger, Kelly, et al., 2020; Wineinger, Shamshina, et al., 2020). The variability in Mw has important implications for the application of chitin. The Mw of chitin affects its viscosity, solubility, and mechanical strength (Kumirska et al., 2011). For example, polymeric materials of high Mw polymer will demonstrate much improved mechanical properties (stress-strain properties, impact, fracture, fatigue, creep, stress relaxation, etc.) (Khatami et al., 2024).

A higher Mw of chitin also decreases its degradation rate (Lamarque et al., 2005), and lower Mw chitin may be more desirable for applications where biodegradability is critical, such as in drug delivery systems or applications like biomaterials where quicker degradation is beneficial (Park & Kim, 2010). The Mw also determines how chitin behaves during chemical processing, such as deacetylation to form chitosan or during dissolution in specific solvents used in making composites or films (Rathke & Hudson, 1994).

4.2.2. The methods for Mw determination

While numerous methods exist for determining the Mw of the soluble deacetylated chitin derivative, chitosan, as summarized by Knaul et al., the determination of chitin's Mw is primarily limited to three main techniques: gel permeation chromatography (GPC, also known as size exclusion chromatography, SEC) (Sugita et al., 2008), viscosity measurements using the Mark–Houwink–Sakurada (MHS) equation (Chauhan et al., 2010), and dynamic light scattering (DLS) (Noel et al., 2011). A critical requirement for all these methods is the complete dissolution of chitin in the chosen solvent, without degradation or aggregation. The solvents utilized for MHS, DLS, and GPC are generally restricted to alkaline solutions, *N*,*N*-dimethylacetamide/lithium chloride (DMAc/LiCl), and sodium hydroxide/urea aqueous solutions (NaOH/urea at 8 wt%/4 wt%). The pros, cons, and sources of error for most of the methods for Mw determination are given in references (Khatami et al., 2024). Particularly, intrinsic viscosity and use of Mark–Houwink coefficients' values require consistent polymer – and as we already mentioned above, chitin is a family of compounds, with different allomorphs and %DA.

In regard to GPC, it was shown that chitin solubility is an issue for the analysis, where the primary solvent for chitin is DMAc/5 % LiCl unsuitable for the dissolution of high Mw polymer, and may lead to partial precipitation of biopolymer inside the GPC column. Hence, for the >1500 kDa range, it is hard to access the polymer's Mw (Vetter, 2007; Wineinger, Kelly, et al., 2020; Wineinger, Shamshina, et al., 2020). Our recent publication (Wineinger, Shamshina, et al., 2020) demonstrates that DLS effectively determines the Mw of high Mw biopolymers dissolved in ILs, where other solvents fail. It also provides the first quantitative proof that IL-extracted biopolymers have much higher Mws.

Literature shows crustacean chitin Mw values ranging from 150 kDa to approximately 2000 kDa (Table 3, **Entries 1–11**). This broad variability is largely attributed to the extraction conditions employed. ILs stand out in chitin extraction by dissolving it in its native state, preserving the highest Mw values (3900–23000 kDa, Table 3, **Entry 12**), unlike traditional pulping methods, where harsh chemicals hydrolyze the polymer chains and reduce Mw. For fungal chitin, although fewer studies are available (only 3 examples), Mw values reported in the literature ranged from 50 to 1200 kDa (Table 3, **Entries 13–15**). This may suggest that fungal chitin generally shows lower Mw than crustacean chitin, likely due to biological differences, though this remains speculative.

Fungal chitosan has a similar Mw range to fungal chitin, with reported values from 24 to 1400 kDa, aligning closely with fungal chitin (Table 3, **Entries 16–24**). This suggests that despite the partial deacetylation of chitin into chitosan, the overall Mw remains consistent.

4.3. Crystallinity index

The crystallinity Index (CrI) of chitin is another essential characterization to understand the supramolecular structure (Ioelovich, 2014). Crystallinity defines the degree of long-range order in the polymer and affects its hardness, density, and solubility, important for its processing. Powder X-ray Diffraction (pXRD) also called Wide-Angle X-ray Scattering (WAXS) (Noel et al., 2011) is a common method to provide different aspects of crystallinity information. The term "powder XRD" (or pXRD) is commonly used for powdered crystalline materials, while the term "WAXS" is applied to both crystalline and semi-crystalline materials, as well as complex fluids and soft matter. In chitin characterization, these terms are often used interchangeably.

The technique involves measuring X-ray scattering at wide angles, typically between 10° and 80°, to analyze materials with short-range order. It provides insights into the size, shape, and arrangement of atoms, molecules, or nanoparticles (Lamba, 2016). XRD offers complementary data to other methods, such as phase identification and crystallite size, using the two-phase model to differentiate between amorphous and crystalline regions. The CrI represents the fraction of crystalline material in the pattern. However, XRD assumes uniform composition in each sample particle, making it less accurate for heterogeneous samples with a detection limit of around 2 %. It also requires particles smaller than 10 μm for optimal analysis, limiting the range of samples that can be effectively studied.

α-Chitin diffractogram typically shows peaks at 9.2, 12.7, 19.2, and 26.3° 2Θ (Abdou et al., 2008; Sagheer et al., 2009) whereas for some mushrooms (i.e., *Lentinula edodes*) additional peak at 5.4°–5.6° 2Θ can

Table 3
Mw values for crustacean and fungal chitin and chitosan.

#	Chitin type	Solvent system	Technique to determine Mw	Mark–Houwink parameters		MW, kDa	Ref
				K	α		
Pulped Crustacean Chitin (Deproteinization using NaOH, demineralization using HCl)							
1	Chitin produced from shrimp shells	5 % DMAc/LiCl[a]	MHS[b]	0.024	0.69	150	(Percot et al., 2003b)
2	Chitin produced from crab shells	5 % DMAc/LiCl	MHS[b]	7.6×10^{-5}	0.95	70–220	(Setoguchi et al., 2012)
3	Commercial chitin obtained from Bioshell, Inc. (USA)	5 % DMAc/LiCl	DLS[c]	2.4×10^{-3}	0.69	510	(Terbojevich et al., 1988)
4	Decalcified chitin	5 % DMAc/LiCl	GPC[d]	None Provided	0.60	540	(Striegel, 1997)
5	Chitin produced from northern shrimp shells (*Pandalus Borealis*)	5 % DMAc/LiCl	GPC with RI and MALLS detectors	7.6×10^{-5}	0.95	80–710	(Poirier & Charlet, 2002)
6	Chitin produced from shrimp shells	NaOH/Urea/Water = 8/4/88 w/w/w	MHS[b]	0.26	0.56	922	(Li et al., 2010)
7	Chitin produced from northern shrimp shells (*Pandalus borealis*)	2.77 M $NaOH_{aq}$	MHS[b]	0.100	0.68	960–1060	(Rødde et al., 2008)
8	Chitin produced from White Shrimp Shells	2.77 M $NaOH_{aq}$	MHS[b]	0.100	0.68	1110–1356	(Thắng, 2013)
9	Chitin produced from Black Tiger Shells	2.77 M $NaOH_{aq}$	MHS[b]	0.100	0.68	1112–1149	(Thắng, 2013)
10	Chitin produced from shrimp shells using the pulping method	2.77 M $NaOH_{aq}$	DLS[c]	0.100	0.68	1200–1600	(Einbu et al., 2004)
11	Chitin produced from crab shells	NaOH/Urea/Water = 8/4/88 w/w/w	DLS[c]	0.26	0.56	2156	(Li et al., 2010)
Extracted Crustacean Chitin (Dissolved in 1-ethyl-3-methylimidazolium acetate [C_2mim][OAc] IL then precipitated in antisolvent)							
12	Chitin extracted from white-leg shrimp (*Litopenaeus vannamei*)	[C_2mim][OAc]	DLS[c]	NA	NA	4000–23,000	(Wineinger, Kelly, et al., 2020; Wineinger, Shamshina, et al., 2020) (Wineinger, Kelly, et al., 2020; Wineinger, Shamshina, et al., 2020)
Fungal Chitin							
Pulped Fungal Chitin (Deproteinization using NaOH, demineralization using HCl (the 2nd step may be omitted))							
13	Chitin produced from *Hericium erinaceus*	NaOH/Urea/Water = 11/4/85 w/w/w	MHS[b]	0.26	0.56	50–200	(Liao & Huang, 2020)
14	Chitin produced from *Komagataella pastoris*	5 % DMAc/LiCl	GPC[d]	NA		490	(Farinha et al., 2015)
15	Chitin produced from Shiitake stipes	Hexafluoro-isopropanol	MALDI-TOF-MS	NA		1176–1227	(Zhang et al., 2022)
Fungal Chitosan							
16	Chitosan produced from *P. ostreatus*	0.20 M HOAc/ 0.10 M NaOAc/ water	MHS[b]	1.81×10^{-4}	0.78	24	(Ban et al., 2018)
17	Chitosan produced from white *A. bisporus*	0.20 M HOAc/ 0.10 M NaOAc/ water	MHS[b]	1.81×10^{-4}	0.78	56	(Ban et al., 2018)
18	Chitosan produced from Shiitake stipes	0.20 M HOAc/ 0.10 M NaOAc/ water	GPC[d]	NA	NA	54 - 437	(Yen & Mau, 2007)
19	Chitosan produced from Ugandan edible mushrooms	0.25 M HOAc / 0.25 M NaOAc/ water	MHS[b]	1.40×10^{-4}	0.83	291–348	(Ssekatawa et al., 2021)
20	Chitosan produced from *Rhizopus oryzae*	0.50 M HOAc/ 0.50 M NaOAc/ water	GPC[d]	NA	NA	500	(Muzzarelli et al., 1994)
21	Chitosan produced from *Brown A. bisporus*	0.20 M HOAc/ 0.10 M NaOAc/ water	MHS[b]	1.81×10^{-4}	0.78	600	(Ban et al., 2018)
22	Chitosan produced from *A. bisporus*	0.25 M HOAc/ 0.25 M NaOAc/ water	MHS[b]	1.81×10^{-4}	0.78	605	(Fadhil & Mous, 2021)
23	Chitosan produced from *T. titanicus*	1 wt% HOAc/ water	MHS[b]	4.74×10^{-5}	0.72	986	(Kasongo et al., 2020)
24	Chitosan produced from *Mucor rouxii*	0.8 wt% NaOAc, 0.05 wt% NaN_3, and 21 wt% HOAc	GPC[d]	NA	NA	200–1400	(Arcidiacono & Kaplan, 1992)

[a] Dimethylacetamide with 5 % LiCl salt.
[b] Determined by Mark-Houwink-Sakurada (MHS) intrinsic viscosity method.
[c] Determined by Dynamic Light Scattering.
[d] Determined by Gel Permeation Chromatography.

be identified (Álvarez et al., 2014). These peaks correspond to the crystalline planes of (020), (021), (110), and (013), for a typical α-chitin crystalline structure (Fan et al., 2008; Minke & Blackwell, 1978). Determination of CrI by Segal Method is conducted through comparison of intensities of the highest crystalline peak and amorphous contribution at $2\theta \sim = 12.6$ or baseline height at 16°, according to the following equation (Kaya et al., 2014):

$$CrI = \frac{I_{1\,1\,0} - I_{amorphous}}{I_{1\,1\,0}} \times 100$$

where $I_{1\ 1\ 0}$ = the maximum intensity at $2\theta \sim = 19°$, and I_{am} = the intensity of amorphous diffraction at $2\theta \sim = 12.6$ or baseline height at 16°.

Peak deconvolution is another technique to calculate CrI (Gupta & Singhal, 1983; Nansé et al., 1997). This process involves separating crystalline peaks from amorphous regions by fitting Gaussian or Lorentzian functions to the diffraction pattern. The crystalline content is then quantified by integrating the areas under the crystalline peaks and comparing them to the total area, which includes both the crystalline and amorphous contributions (Podgorbunskikh et al., 2022). While Segal's method is recognized for its rapid execution, the peak deconvolution method offers greater accuracy.

5. A brief overview of chitin isolation practices

5.1. Crustaceans

Because crustaceans contain both minerals and proteins in addition to chitin, demineralization using HCl and deproteination processes using NaOH are required to extract the polymer from the crustacean shells (Abidin et al., 2020; El Knidri et al., 2018; Hahn et al., 2020; Iber et al., 2022; Kozma et al., 2022; No & Meyers, 1995; Pakizeh et al., 2021; Synowiecki & Al-Khateeb, 2003; Yang et al., 2020). Once most of the proteins are removed, some proteins in the form of amino acids (e.g., aspartic acid, histidine) remain in the chitin structure even after the hydrolysis of glycoproteins (Foster & Hackman, 1957; Radulovich & Fuentes-Quesada, 2019). High temperatures (80–90 °C) and high concentrations of used chemicals that are standard practice during pulping are detrimental to the quality of produced chitin (Pillai et al., 2009). As a result, the use of milder conditions such as decreased temperature (≤ 60 °C), less concentrated chemicals (NaOH and HCl) (Percot et al., 2003b), or weaker organic acids for demineralization (e.g., acetic acid, propionic acid) (Mohan et al., 2022; Morgan et al., 2021; Rogers et al., 2018; Younes et al., 2014) are becoming a more widespread practice.

The recent comprehensive review paper has been dedicated to the pulping of chitin from crustacean biomass using Deep Eutectic Solvents (DESs), such as cholinium chloride/thiourea, choline chloride/urea, cholinium bromide/urea, and betainium chloride/urea (Ferreira et al., 2020; Khajavian et al., 2022; Li et al., 2022; Morgan et al., 2021; Özel & Elibol, 2021; Sulthan et al., 2023; Wang et al., 2022). In essence, this is also a pulping process, but instead of using an acid and a base consecutively, the demineralization and deproteinization steps under DES-treatment proceed simultaneously, leaving purified chitin. 'Pulping ILs' are also known (Rogers et al., 2018).

The enzymatic isolation of chitin (Mohan et al., 2022) typically involves the use of enzymes such as lipases, chitinases (Brown, 1959), pepsin (Duong & Nghia, 2014), or proteases (Kim & Je, 2010; Younes et al., 2012, 2014) to break down proteins and lipids attached to the chitin. The overall protocols include biomass pre-treatment, enzymatic hydrolysis, and polymer separation and purification. The enzymatic extraction approach is considered as a sustainable approach due to its decreased dependence on harsh chemical reagents. The use of enzymes also prevents unwanted depolymerization and deacetylation unlike harsh chemicals (Rass-Hansen et al., 2007; Vázquez et al., 2013), however, purity of chitin is reduced due to its contamination with a higher amount of residual proteins (Abidin et al., 2020; Lopes et al., 2018).

A drastically different approach is the use of ILs for the extraction of chitin from crustaceans, by chitin dissolution in the ILs. Some common ILs such as 1-butyl-3-methylimidazolium chloride ($[C_4mim]Cl$), 1-butyl-3-methylimidazolium acetate ($[C_4mim][OAc]$), 1-allyl-3-methylimidazolium chloride ([Amim]Cl), 1-ethyl-3-methylimidazolium chloride ($[C_2mim]Cl$), 1-ethyl-3-methyl-imidazolium bromide ($[C_2mim]Br$), 1-allyl-3-methylimidazolium acetate ([Amim][OAc]) have been reported effective for chitin extraction (Jaworska et al., 2012; Mahmood & Moniruzzaman, 2019; Shamshina, 2019; Shamshina & Berton, 2020; Silva et al., 2017). For the purpose, the IL should be suitable for chitin dissolution. When crustacean biomass is heated with ILs either using thermal heating or with a constant impulse of microwave heat, chitin gets dissolved in the ILs, while minerals remain undissolved and are separated from IL/chitin solution by centrifugation. The supernatant after centrifugation is poured into water and chitin precipitates out. The IL is removed by subsequent washing.

5.2. Fungal biomass

Harnessing fungal chitin is not without some challenges. The chitin yield per wet weight of mycelium or fruit body is relatively low compared with the animal-based counterpart. This can be mitigated to a large degree by the rapid growth rate of many fungi on a diversity of simple substrates or "waste" organic matter and relatively simple extraction protocols. However, the major perceived obstacle discouraging researchers from working with fungal chitin has been the presence of glucans (Cabib et al., 1982; De Nobel et al., 1990; Fleet & Phaff, 1981; Zlotnik et al., 1984), and the isolation of CGC, with glucans being covalently attached to the polymer. Isolation of fungal chitin (or CGC) is conducted mostly by deproteinization-demineralization sequence. Table 4 below summarizes the methods reported for the isolation of chitin from mushrooms, with respect to the species the polymer was isolated from, conditions for its isolation, and characterization conducted.

5.2.1. Types of mushrooms

Data listed in Table 4 demonstrates the significant research conducted on the chitin composition of various well-known fungal species. *Lentinula edodes* (Table 4, **Entries 1–4**, **13**, **41**, **53**, **78**), *Agaricus bisporus* (Table 4, **Entries 14**, **17–19**, **34**, **39**, **46**, **49–51**, **66**, **74**, **76**, **89**), *Ganoderma lucidum* (Table 4, **Entries 35**, **69–73**, **79**), *Pleurotus ostreatus* (Table 4, **Entries 11**, **56**, **67**, **80**), *Fomes formentarius* (Table 4, **Entries 7**, **61**), *Armillariella mellea* (Table 4, **Entries 30**, **55**), *Absidia coerulea* (Table 4, **Entries 33**, **38**), and *Aspergillus niger* (Table 4, **Entries 47, 81–88**), are mostly common for this purpose. In addition, there are some other species like *Boletus boyinus* (Table 4, **Entry 5**), *Laccaria laccata* (Table 4, **Entry 6**), *Pholiota gummosa* (Table 4, **Entry 62**), *Fomitopsis pinicola* (Table 4, **Entry 8**), *Termitomyces albuminosus* (Table 4, **Entry 10**), *Termitomyces titanicus* (Table 4, **Entry 32**), *Komagataella pastoris* (Table 4, **Entry 12**), *Hericium erinaceus* (Table 4, **Entries 20–29**), *Morchella esculenta* (Table 4, **Entry 31**), *Phyllophora Ribis* (Table 4, **Entry 37**), *Lactarius vellereus* (Table 4, **Entry 36**), *Grifola frondosa* (Table 4, **Entry 42**), *Hypsizygus marmoreus* (Table 4, **Entry 43**), *Mucor indicus* (Table 4, **Entry 44**), *Agaricus Sp* (Table 4, **Entry 45**), *Mucor rouxii* (**Table 4**, **Entry 48**), *Auricularia auriculajudae* (Table 4, **Entry 52**), *Trametes versicolor* (Table 4, **Entry 54**), *Pleurotus eryngii* (Table 4, **Entry 56**), *Amanita muscaria* (Table 4, **Entry 58**), *Amanita pantherine* (Table 4, **Entry 59**), *Cantharellus cibarius* (Table 4, **Entry 60**), *Pholiota gummosa* (Table 4, **Entry 62**), *Russula nigricans* (Table 4, **Entry 63**), *Russula vinosa* (Table 4, **Entry 64**), *Tricholoma terreum* (Table 4, **Entry 65**), *D. confragosa* (Table 4, **Entry 75**), *Mucor circinelloides* (Table 4, **Entry 77**), *Agaricus campestris* (Table 4, **Entry 90**).

Most articles do not provide details on the specific parts of mushrooms used for extraction, leading to the assumption that whole fruit bodies were utilized without fractionation. While some fungi, such as yeast and certain species of *Aspergillus*, naturally lack parts like pileus, stipes, and gills, it is still important for studies involving other mushroom species to specify which parts were used. The absence of data on chitin content in these specific parts limits our understanding of their potential contributions to overall chitin yield. A few studies mentioned the particular parts of the mushrooms, and the chitin yield from each part (Table 4, **Entries 15–19**). Stipes appear to have the largest chitin content, followed by pileus, and the gills.

Table 4
The reported methods for the isolation of chitin from mushrooms, isolation conditions, and characterization.

#	Sources	Deproteinization with NaOH			Demineralization with HCl/Acetic acid (AA)[a]			Yield, %	C or CGC[b]	Characterization used in the paper	DA, %	CrI, %	MW, kDa	T_{dec}	Ref
		Conc., M	Time, h	Temp., °C	Conc., M	Time, h	Temp., °C								
1	*Lentinula edodes*	1.25	48	RT	2	24	RT	15.8	CGC	FTIR; pXRD; TGA; SS CP-MAS	94.3	71	1175.7	315	(Kaya, Akata, et al., 2015)
2		2.50	48	RT	2	24	RT	13.1	CGC	NMR; MALDI-TOF	86.9	72	1212.5	315	(Zhang et al., 2022)
3		1.25	120	RT	2	24	RT	10.2	CGC		86.6	73	1226.7	316	
4		2.50	120	RT	2	24	RT	9.4	CGC		75.1	79	1209	321	
5	*Boletus bovinus*	2	24	85	2	17	60	8.5	C	FTIR; pXRD; TGA; SS CP-MAS	94.0	78–85	ND	320	(Oberemko et al., 2019)
6	*Laccaria laccata*	2	24	85	2	17	60	6.2	C	NMR	92.7	78–85	ND	363	
7	*Fomes fomentarius*	2	20	140	2	2	100	2.4	CGC	SEM; FTIR; pXRD; TGA	62.3	59	ND	334	(Kaya, Baublys, et al., 2015) (Kaya, Akata, et al., 2015)
8	*Fomitopsis pinicola*	2	17	80–90	2	2	65–75	30	CGC	SEM; FTIR; pXRD; TGA	72.5	52	ND	341	(Kaya, Baublys, et al., 2015)
9	Edible Mushroom (no name provided)	1	8	80	1	24	50	9.9	CS[c]	FTIR; pXRD; Viscosity for M_W[d]	21.9 (CS)[c]	48	348 (CS)[c]	ND	(Ssekatawa et al., 2021)
10	*Termitomyces albuminosus*	0.5	2	85	0.27	2	60	13.5	CGC	FTIR; pXRD; TGA; SS CP-MAS NMR	65.4	65	ND	315	(Hong & Ying, 2019)
11	*P. ostreatus*	0.5	2	65	No demineralization			4.43	CS[c]	FTIR; pXRD; TGA; NMR, GPC[d]	44.1	56	600 (CS)[c]	220	(Ban et al., 2018)
12	*Komagataella pastoris*	5	2	65	No demineralization			13.4	CGC	FTIR; pXRD; TGA; SS CP-MAS NMR	ND	50	490	315	(Farinha et al., 2015)
13	*Lentinula edodes*	1	3	100	No demineralization			25.08	CGC	Color measurement, SEM, DSC, elemental analysis	9.8	NS	54–61 (CS)[c]	310	(Yen & Mau, 2007) (Yen & Mau, 2007)
14	*Agaricus bisporus*	1	2	80	0.33	6	95	7	CGC	SEM; FTIR; pXRD; TGA	70.8	65	ND	300	(Boureghda et al., 2021)
15	*Pleurotus ostreatus*	0.5	2	80	No demineralization			Cap – 27 Stalk – 49	CGC	SEM; FTIR; pXRD; TGA	ND	63	ND	375	
16	*Pleurotus ostreatus*	1	2	80	0.33	6	95	Cap – 21 Stalk – 41	CGC	SEM; FTIR; pXRD; TGA	ND	58	ND	289	
17	White *A. bisporus Stipe*	1	2	80	0.33	6	95	7.4	C	FTIR; pXRD; TGA	69.8	63	ND	309	(Hassainia et al., 2018)
18	White *A. bisporus Pileus*	1	2	80	0.33	6	95	6.4	C		66.8	ND	ND	ND	
19	White *A. bisporus Gills*	1	2	80	0.33	6	95	5.9	C		63.4	ND	ND	ND	
20	*Hericium erinaceus*	0.5	3	85	1.1	12	RT	16[f]	C	SEM; FTIR; pXRD; TGA; SS CP-MAS NMR	90.0	ND	201	318	(Liao & Huang, 2020)
21	*Hericium erinaceus*	1	3	85	1.1	12	RT	10[f]	C		85.0	ND	175	ND	
22	*Hericium erinaceus*	1.5	3	85	1.1	12	RT	9[f]	C		80.0	ND	150	ND	
23	*Hericium erinaceus*	2	3	85	1.1	12	RT	8.5[f]	C		78.0	ND	140	ND	
24	*Hericium erinaceus*	1	3	65	1.1	12	RT	12.5[f]	C		86.0	ND	180	ND	
25	*Hericium erinaceus*	1	3	75	1.1	12	RT	10[f]	C		88.0	ND	190	ND	
26	*Hericium erinaceus*	1	3	95	1.1	12	RT	8.5[f]	C		72.0	ND	112	ND	
27	*Hericium erinaceus*	1	2	85	1.1	12	RT	11[f]	C		85.0	ND	180	ND	
28	*Hericium erinaceus*	1	4	85	1.1	12	RT	8[f]	C		85.0	ND	180	ND	
29	*Hericium erinaceus*	1	5	85	1.1	12	RT	8[f]	C		85.0	ND	180	ND	
30	*Armillariella mellea*	0.5	2	80	0.274–0.822	1–3	55–60	70	CGC	FTIR; pXRD	80.1	61	ND	ND	(Ivshina et al., 2009)
31	*Morchella esculenta*	0.5	2	80	0.274–0.822	1–3	55–60	50	CGC	FTIR; pXRD	40.6 (CS)	61	ND	ND	
32	*Termitomyces titanicus*	3	2	100	No demineralization			38.0	CGC	Conductimetric titration; Viscometry	30.5 (CS)[c]	ND	986 (CS)[c]	ND	(Kasongo et al., 2020)

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Table 4 (*continued*)

#	Sources	Deproteinization with NaOH			Demineralization with HCl/Acetic acid (AA)[a]			Yield, %	C or CGC[b]	Characterization used in the paper	DA, %	CrI, %	MW, kDa	T_{dec}	Ref
		Conc., M	Time, h	Temp., °C	Conc., M	Time, h	Temp., °C								
33	*Absidia coerulea*	6.25	3	100	No demineralization			0.187	CS[c]	FTIR; pXRD[d]	5.2 (CS)[c]	ND	500 (CS)[c]	ND	(Muzzarelli et al., 1994)
34	*Agaricus bisporus*	1	30	95	0.33	6	95	16.0 (C)	CS[c]	FTIR[d]	26.7 (CS)[c]	ND	604 (CS)[c]	ND	(Fadhil & Mous, 2021)
35	*Ganoderma lucidum*	4	2	100	No demineralization			83.23	CS[c]	FTIR; pXRD[d]	19.9 (CS)[c]	NS	ND	350–450	(Ospina et al., 2015)
36	*Lactarius vellereus*	2	24	85	2	15	60	11.4	CGC	SEM; pXRD; TGA	ND	64	ND	354	(Erdogan et al., 2017)
37	*Phyllophora ribis*	2	24	85	2	15	60	7.9	CGC		ND	49	ND	275	
38	*Absidia coerulea*	0.5	0.33	121	1.67 AA	6	60	0.612	CS[c]	FTIR; pXRD; SS NMR[d]	15.0 (CS)[c]	ND	6 (CS)[c]	ND	(Wang et al., 2008)
39	*Agaricus bisporus*	0.5	24	100	2	48	RT	3.5	CGC	FTIR; pXRD	>95.0	77	ND	ND	(Ifuku et al., 2011)
40	*Pleurotus eryngii*	0.5	24	100	2	48	RT	3.5	CGC	FTIR; pXRD	>95.0	80	ND	ND	
41	*Lentinula edodes*	0.5	24	100	2	48	RT	3.5	CGC	FTIR; pXRD	>95.0	65	ND	ND	
42	*Grifola frondosa*	0.5	24	100	2	48	RT	3.5	CGC	FTIR; pXRD	>95.0	65	ND	ND	
43	*Hypsizygus marmoreus*	0.5	24	100	2	48	RT	3.5	CGC	FTIR; pXRD	>95.0	48	ND	ND	
44	*Mucor indicus*	0.5	0.33	120	No demineralization			26	CGC	SEM; FTIR; pXRD	>10.0	82	ND	ND	(Burballa et al., 2018)
45	*Agaricus Sp*	1	0.5	121	2.2	12	95	19.7	CS[c]	UV Spectrophotometry[d]	11.0 (CS)[c]	ND	ND	ND	(Kannan et al., 2010)
46	*Agaricus bisporus*	1	0.5	95	0.3	6	95	27	CGC	FTIR	79.7	ND	ND	ND	(Wu et al., 2004)
47	*Aspergillus niger*	1	0.5	95	0.3	6	95	24.0	CGC	FTIR	76.5	ND	ND	ND	(Wu et al., 2005)
48	*Mucor rouxii*	1	0.5	95	0.3	6	95	13.3	CGC	FTIR	50.1	ND	ND	ND	
49	*Agaricus bisporus*	1	2	90	0.8	3	90	5.7	CGC	FTIR; pXRD; DSC	ND	ND	ND	310	(Wu et al., 2019)
50	White *Agaricus bisporus*	0.5	48	100	2	48	RT	NS	CGC	FTIR; pXRD	>95.0	77	ND	ND	(Abidin et al., 2020)
51	*A. bisporus*	1	24	40	0.83 AA	3	90	8.5	CGC	FTIR	91.0	ND	ND	ND	(Di Mario et al., 2008)
52	*Auricularia auriculajudae*	1	24	40	0.83 AA	3	90	19.6	CGC	FTIR	95.1	ND	ND	ND	
53	*Lentinula edodes*	1	24	40	0.83 AA	3	90	10.1	CGC	FTIR	92.0	ND	ND	ND	
54	*Trametes versicolor*	1	24	40	0.83 AA	3	90	13.1	CGC	FTIR	97.0	ND	ND	ND	
55	*Armillaria mellea*	1	24	40	0.83 AA	3	90	11.1	CGC	FTIR	92.7	ND	ND	ND	
56	*Pleurotus ostreatus*	1	24	40	0.83 AA	3	90	15.3	CGC	FTIR	98.7	ND	ND	ND	
57	*Pleurotus eryngii*	1	24	40	0.83 AA	3	90	8.7	CGC	FTIR	94.2	ND	ND	ND	
58	*Amanita muscaria*	2	1	NS	2	NS	NS	13.1	ND	TEM; FTIR	58.9	ND	ND	ND	(Baumgartner et al., 2019)
59	*Amanita pantherina*	2	1	NS	2	NS	NS	10.9	ND	TEM; FTIR	57.2	ND	ND	ND	
60	*Cantharellus cibarius*	2	1	NS	2	NS	NS	7.9	ND	TEM; FTIR	46.4	ND	ND	ND	
61	*Fomes fomentarius*	1	1	NS	2	NS	NS	43.1	ND	TEM; FTIR	52.5	ND	ND	ND	
62	*Pholiota gummosa*	2	1	NS	2	NS	NS	16.4	ND	TEM; FTIR	52.8	ND	ND	ND	
63	*Russula nigricans*	2	1	NS	2	NS	NS	18.5	ND	TEM; FTIR	57.7	ND	ND	ND	
64	*Russula vinosa*	2	1	NS	2	NS	NS	28.2	ND	TEM; FTIR	76.1	ND	ND	ND	
65	*Tricholoma terreum*	2	1	NS	2	NS	NS	16.8	CS[e]	TEM; FTIR	33.2 (CS)[e]	ND	ND	ND	
66	*Agaricus bisporus*	0.25, 0.5–1, 6.2–7.5	10, 10, 5	95	1 AA	2	50	12	CGC	SEM; FTIR; pXRD	ND	56	ND	ND	(Singh & Dutta, 2017)
67	*Pleurotus ostreatus*	2	2	NS	No demineralization			8.3	CGC	FTIR; pXRD	ND	71	ND	ND	(Ayser et al., 2024)
68	*D. confragosa*	1	3	65	No demineralization			69.8	CGC	FTIR; pXRD	ND	41	ND	ND	(Nawawi, Jones, et al., 2020)
69	*Ganoderma lucidum*	1	2	40	No demineralization			41.3	ND	pXRD; TGA	ND	ND	ND	314	(Álvarez et al., 2014)
70		1; 2	2	90	No demineralization			34	ND	pXRD; TGA	ND	ND	ND	314	
71		4	3	100	No demineralization			14	ND	FTIR; pXRD; TGA	ND	ND	ND	314	
72		2; 4	2	90	No demineralization			9	ND	pXRD; TGA	ND	ND	ND	ND	

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Table 4 (*continued*)

#	Sources	Deproteinization with NaOH			Demineralization with HCl/Acetic acid (AA)[a]			Yield, %	C or CGC[b]	Characterization used in the paper	DA, %	CrI, %	MW, kDa	T_{dec}	Ref
		Conc., M	Time, h	Temp., °C	Conc., M	Time, h	Temp., °C								
73		2; 4; 6; 8	3	100	No demineralization			8	ND	pXRD; TGA	ND	ND	ND	ND	
74	*Agaricus bisporus*	1	3	65	No demineralization			15.0	CGC	FTIR	ND	ND	ND	ND	(Nawawi, Lee, et al., 2020)
75	*D. confragosa*	1	3	65	No demineralization			15.0	CGC	FTIR; pXRD	ND	ND	ND	ND	(Hong & Ying, 2019)
76	*Agaricus bisporus*	1	2	80	0.33	6	95	7.4	ND	FTIR; pXRD; H-NMR (for chitosan)	ND	ND	ND	ND	(Hassainia et al., 2020)
77	*Mucor circinelloides*	0.5	2	90	1.75	6	60	50.0	CGC	FTIR; pXRD; TGA	ND	ND	ND	ND	(Fai et al., 2011)
78	*Lentinula edodes*	1	3	100	1 AA	3	100	25–36[g]	CGC	SEM; DSC; pXRD	ND	NS	ND	NS	(Rogers et al., 2018)
79	*Ganoderma Lucidum*	4	2	90	0.27	3	65	1.125	CGC	FTIR; NMR	ND	ND	ND	ND	(Savin et al., 2020)
80	*Pleurotus ostreatus*	1	0.25	121	0.35	5	95	17.6	ND	FTIR	ND	ND	ND	ND	(Benbelkacem et al., 2022)
81	*Aspergillus niger*	7.5	4	118	No demineralization			56	CGC	FTIR; pXRD	ND	ND	ND	ND	(Muzzarelli et al., 1980)
82		7.5	6	118	No demineralization			55	CGC	FTIR; pXRD	ND	ND	ND	ND	
83		10	4	128	No demineralization			44	CGC	FTIR; pXRD	ND	ND	ND	ND	
84		10	6	128	No demineralization			43	CGC	FTIR; pXRD	ND	ND	ND	ND	
85		7.5	4	95	No demineralization			71	CGC	FTIR; pXRD	ND	ND	ND	ND	
86		7.5	6	95	No demineralization			69	CGC	FTIR; pXRD	ND	ND	ND	ND	
87		10	4	95	No demineralization			70	CGC	FTIR; pXRD	ND	ND	ND	ND	
88		10	6	95	No demineralization			70	CGC	FTIR; pXRD	ND	ND	ND	ND	
89	*Agaricus bisporus*	1	0.5	60	No demineralization			35.8 3.7 (CS)	CGC	FTIR; pXRD	ND	ND	ND	ND	(Boureghda et al., 2021)
90	*Agaricus campestris*	1	0.5	60	No demineralization			36.0 4.0 (CS)	CGC	FTIR; pXRD	ND	ND	ND	ND	

[a] Demineralization is conducted with HCl, when it is undertaken with acetic acid, the concentration entries are supplied with AA denotation.
[b] CGC = Chitin Glucan Complex.
[c] Chitosan (CS) was prepared by deacetylation of chitin isolated from mushrooms.
[d] No characterization of the intermediate chitin was provided rather was done for chitosan.
[e] The protocol results in the formation of chitosan.
[f] Data were extracted from the graphs of the corresponding reference.
[g] The yield depends on decoloration method sodium hypochlorite (yield = 28 %); ethanol (yield = 36 %); and potassium permanganate (yield = 25 %).

5.2.2. Effect of deproteinization conditions on %DA

Isolation of fungal chitin is typically conducted by deproteinization-demineralization sequence ("pulping"). While most of the studies used a deproteinization-demineralization sequence, others did not attempt the removal of minerals (Table 4, **Entries 11**–**13**, **15**, **32**, **33**, **35**, **44**, **67–75**, **81–90**). The reason for utilizing NaOH is that among diverse fungal β-glucans, few are alkali-soluble. For example, β-glucan from *L. edodes* (shiitake mushroom), lentinan, is soluble in alkali solution, so are schizophyllan from *S. commune*, pleuran from *P. ostreatous* (oyster mushroom), grifolan from *G. frondosa* (maitake mushroom), and ganoderan from *G. lucidum* (reishii mushroom) (Shimahara & Takiguchi, 1988).

For deproteinization, the weak to medium strength alkali (NaOH) solution is typically used, in an attempt to prevent deacetylation. Alkali concentration varied from 0.5 M (Table 4, **Entries 10**, **11**, **20**, **30**, **31**, **38–44**, **50**, **66**, **77**) to 1–1.5 M (Table 4, **Entries 1**, **9**, **13–19**, **21**, **22**, **24–29**, **34**, **45–49**, **51–57**, **61**, **66**, **68–70**, **74–76**, **78**, **80**, **89**, **90**), 2–2.5 M (Table 4, **Entries 2**, **4–8**, **23**, **36**, **37**, **58–60**, **62–65**, **67**, **70**, **72**, **73**), and 3 M (Table 4, **Entry 32**). The most common concentration of NaOH employed was 1–1.5 M (accounts for ~45 % of cases). Few studies report using strong alkali solution, such as 4 M or higher (Table 4, **Entries 12**, **35**, **66**, **71–73**, **79**, **81–88**). The study (Muzzarelli et al., 1980) stands out due to the use of highly concentrated solutions of NaOH (7.5–10 M) (Table 4, **Entries 66**, **73**, **81–88**).

At the same time, it is known that chitin deacetylation takes place by maintaining a stirred slurry of chitin in a NaOH solution of high pH, at an elevated temperature. The caustic concentration of 10–60 wt% (2.5–15 M), the temperature range between 70 and 95 °C, and reaction times from as little as five minutes to as long as 48 h are usually used for the intentional deacetylation of chitin polymer and are reasonably common across many studies. If stainless steel autoclaves are used, temperatures as high as 150 °C are reported for the purpose (Ahlafi et al., 2013; Boureghda et al., 2021; Chang et al., 1997; No et al., 2000; C. Zhang et al., 2009). The use of NaOH at high temperatures (80–90 °C) that are standard practice during the deproteinization stage of fungal chitin is detrimental to the quality of the produced polymer (Pillai et al., 2009). Some researchers report Mw declines as deacetylation proceeds (Ospina et al., 2015) and others claim low %DA values with no Mw loss (Chang et al., 1997). Different mushroom biomasses likely differ in levels of crystallinity, particle sizes, and associated glucans. These differences can lead to differences in water uptake and swelling properties when biomass is immersed in an aqueous solution. This, in turn, will impact the rates of diffusion and the kinetics of the deacetylation reaction (Erdogan et al., 2017; Ospina et al., 2015; Wang et al., 2008).

Thus, not surprisingly, the extent of deacetylation depended on the combination of NaOH concentration, temperature, and time, and we would like to emphasize that all three variables interrelatedly affect the reaction result. The effect of NaOH concentration is better illustrated by examples where it was changed gradually, on the same type of mushrooms, at otherwise identical conditions. For instance, %DA of chitin from *Lentinula edodes* dropped from 94.3 to 86.9 % during 48 h treatment, and from 86.6 to 75.1 % during 120 h treatment, when NaOH concentration increased from 1.25 to 2.5 M (Table 4, **Entry 1** vs. **2**; **Entry 3** vs. **4**). Likewise, for *Hericium erinaceus* mushroom, a gradual increase of NaOH concentration from 0.5 M to 2 M (in 0.5 M step) produced chitin with %DA decreasing from 90 % (for 0.5 M NaOH solution) to 78 % (for 2 M NaOH solution), see Table 4, **Entries 20**–**23**. Use of as concentrated as 6.25 M solution of NaOH resulted in the formation of chitosan (Table 4, **Entry 33**) with %DA as low as 5.2 %. Whereas a highly concentrated alkaline solution was used in the studies (Table 4, **Entries 33**, **66**, **73**, and **81**–**88**) as well, the characterization was not sufficient to ascertain what type of polymer (chitin vs. chitosan) was obtained.

In respect to time, it varied from as little as 0.5 h to as high as 120 h (5 days), whereas the temperature at which deproteinization was conducted varied from room temperature (RT) to 140 °C. Here, we also will look at the effect of temperature and time comparing examples with the same type of mushrooms, at otherwise identical conditions. Thus, %DA of chitin from *Lentinula edodes* dropped from 94.3 to 86.6 % and from 86.9 to 75.1 % upon treatment with 1.25 and 2.5 M NaOH, respectively, when the time of treatment increased from 48 to 120 h (Table 4, **Entry 1** vs. **3**; **Entry 2** vs. **4**). The longevity of treatment appeared to have less effect at shorter reaction times – when the same type of mushrooms underwent treatment with 1 M NaOH solution for 2, 4, and 5 h duration, the %DA remained the same, 85 % (Table 4, **Entries 27–29**). Extremely long reaction times such as 30 h or above at elevated temperatures result in the formation of chitosan (Table 4, **Entry 34**).

Concerning the temperature, when *Hericium erinaceus* mushroom was treated with 1 M NaOH at 65 and 95 °C (Table 4, **Entries 24–26**), its %DA dropped from 85 % to 72 %. In light of the above, a few examples are quite surprising. For instance, the treatment of *Fomitopsis pinicola* with relatively weakly concentrated NaOH (2 M) at 90 °C, for 17 h period (Table 4, **Entry 8**) did not cause significant deacetylation – the product with %DA of 72 % was formed. Likewise, handling *Fomes fomentarius* with 2 M NaOH at a temperature as high as 140 °C, for 20 h period (Table 4, **Entry 7**) resulted in a product with a %DA of 62 %. The same NaOH solution was used for the deproteinization of *Boletus bovinus* and *Laccaria laccata* (Table 4, **Entries 5** and **6**) for 24 h at 85 °C. While the manuscript title claimed chitosan formation, the study reported % DA of 94 % for the product from *B. bovinus* and 92 % for the product from *Laccaria laccata* with no *N*-deacetylation happening during the isolation process, confirmed by ^{13}C NMR spectra.

5.2.3. Effect of deproteinization conditions on mw

To a lesser extent, the conditions of the deproteinization stage affect the Mw of the polymer. Unfortunately, our discussion will be limited due to the scarcity of data (i.e., Mw was reported in only 10 studies). It appears that Mw of chitin from *Lentinula edodes* stayed at approximately the same value of ~1200 kDa when NaOH concentration increased from 1.25 to 2.5 M, independent of the reaction time (48 h or 120 h, Table 4, **Entries 1–4**). However, for *Hericium erinaceus* mushroom, a gradual increase of NaOH concentration from 0.5 M to 2 M (in 0.5 M step) produced chitin which Mw dropped ~30 %, from 200 kDa to 140 kDa, see Table 4, **Entries 20**–**23**. Use of as concentrated as 6.25 M solution of NaOH resulted in the formation of chitosan (Table 4, **Entry 33**) with Mw as low as 500 kDa, although we do not know the starting Mw value. When a highly concentrated alkaline solution was used in some studies (Table 4, **Entries 35**, **66**, **73**, and **81**–**88**), the characterization did not include Mw of the polymer.

If different mushroom species are considered (a speculative presumption as we do not know Mw of raw polymer in the species), Mw changes according to the time and temperature difference. It is however unclear why a relatively low Mw value of 6.4 kDa was obtained for *Absidia coerulea* (Table 4, **Entry 38**) at the low NaOH concentration of 0.5 M; this is likely attributed to prolonged use of AA during demineralization. The same thing was observed for *Boletus bovinus,* and *Laccaria laccata* (Table 4, **Entries 5** and **6**) where Mw was in the 5–5.8 kDa range.

Temperature effect can be described when analyzing Mw of chitin obtained from the same mushroom (Table 4, **Entries 24–26**), where an increase in temperature from 65 °C to 95 °C at otherwise identical conditions resulted in a ~ 40 % drop in Mw value, from 180 to 112 kDa. Reaction time appears to have little effect on Mw (Table 4, **Entries 27–29**).

5.2.4. Demineralization

In contrasting of chitin extraction from crustaceans and insects, the removal of minerals is not required in the case of mushrooms, due to their low inorganic content, and often only deproteinization is conducted to remove proteins contained in the cell walls; many studies (~25 %) did not employ demineralization (Table 4, **Entries 11**–**13**, **15**, **32**, **33**, **35**, **44**, **67–75**, **81–90**). When used, demineralization is usually conducted with either HCl or AA. AA was used for the demineralization

of several types of mushrooms (Table 4, **Entries 38**, **51**–**57**, **66**, and **78**), in ~10 % of all cases; in other cases (65 % of all cases) HCl was employed. Acid concentration varied from 0.83 M to 1.67 M for AA, and 0.27–2.2 M for HCl.

Based on numerous works with crustacean chitin, it is known that the prolonged treatment of the polymer with acid at high temperatures generally causes acid-catalyzed depolymerization (Rojas et al., 2015). Depolymerization involves the breakdown of the β-1,4-glycosidic bonds in chitin, forming smaller oligomers or monomers. Deacetylation also occurs, but it has also been reported that Mw degradation occurs faster in acidic media than deacetylation does (Einbu & Vårum, 2007). Unfortunately, the invariability of experimental conditions for the deproteinization step are obstacles that hinder the proper analysis and prediction. The only study in this regard is the report by Ivshina et al. (Table 4, **Entries 31**, **32**), where the fungal biomass was treated with HCl solutions (0.274–0.822 M) at 55–60 °C for 1–3 h. The only outcome of the comparison between the use of different concentrations of acid was the conclusion that the concentration of 0.274 M of HCl was sufficient for demineralization. While the study stated that an increase in the acid concentration and treatment temperature destroy acetamide and glycosidic bonds in the chitin-containing material, experimental data to support this point was not provided.

6. Critical assessment of characterization

To set our review apart from other publications on similar topics, we aim to create a comprehensive overview of the existing literature highlighting key findings, gaps, and challenges. By offering a critical appraisal of the relevant studies, this review provides readers with an all-encompassing understanding of the current state of knowledge. It underscores the essential factors in chitin (or CGC) characterization particularly important given the increasing recognition of how inconsistencies in chitin samples can impact the quality of research outcomes, especially in the biomedical field.

Up to now, the analysis of properties and structural investigations of chitin or CGC has been primarily semiqualitative, with just a few exceptions, even though complete characterization is necessary to comprehend the underlying chemistry of chitin. We believe that looking ahead, characterization has to emphasize *quantitative* rather than *qualitative* aspects, so that the isolation methods produce the polymer of certain known characteristics (i.e., Mw and %DA), repeatedly and reproducibly, while reducing ambiguities between data across different studies.

Among the reviewed literature examples (Table 4), only few presented sufficient characterization. Thus, the CGC-nanofibers from Shiitake mushroom stipes were comprehensively characterized (Table 4, **Entries 1**–**4**) (Zhang et al., 2022) including surface morphology and nanofibers' dimensions (via scanning and transmission electron microscopy (SEM and TEM), respectively). Chemical characterization of a heterogeneous chitin-glucan material was conducted using FTIR spectroscopy and carbohydrate units of the present glucans were thoroughly elucidated by high-performance anion exchange chromatography (HPAEC). Reliable %DA values were obtained from Solid-State Cross-Polarization Magic-Angle-Spinning Nuclear Magnetic Resonance spectrum (SS ^{13}C CP-MAS NMR). Finally, the molecular weight (Mw) of the product dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) was assessed using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). The authors also accessed the thermal properties, surface properties and crystallinity.

Likewise, chitin polymer from two mushroom species (*Boletus bovinus* and *Laccaria laccata*, Table 4, **Entries 5**–**6**) (Oberemko et al., 2019) was chemically characterized by ATR-FTIR and SS ^{13}C CP-MAS NMR (which was also employed to assess %DA). Thermal stability was evaluated by TGA, and crystallinity index (CrI) was calculated from pXRD diffractogram. However, Mw, a vital characteristic that influences chitin properties (Shrivastava, 2018) and defines the types of materials that could be prepared from chitin (Aravamudhan et al., 2014; Shamshina et al., 2019), was not determined. The same characterization was provided in the examples presented in Table 4, **Entries 7**, **8**, **10**, and **14**–**16**.

Hot alkaline treatment of *Komagataella pastoris* resulted in the formation of CGC (Table 4, **Entry 12**). Authors have determined a ratio of glucan:chitin as 75:25 mol/mol. The CGC was also characterized for purity (protein impurities ~3 wt%, inorganics ~0.9 wt%), crystallinity (~50 %), and Mw (~500 kDa). SS NMR provided structural insight into the glucan copolymers.

The work, aimed to investigate the physicochemical properties of chitin from *P. repens* species and to compare these properties with properties of the polymer from insects and fungi (Table 4, **Entry 7**), employed ATR-FTIR spectroscopy for chemical characterization, pXRD for crystallinity index determination, and TGA for thermal stability assessment. However, in this case, %DA was calculated by elemental analysis. While the SS ^{13}C CP-MAS NMR method to determine %DA is unaffected by the accuracy of the sample's weighing or the presence of impurities, assessment of %DA through elemental analysis necessitates rigorous purification of chitin samples and is justified only after confirming a purity of the analyte (dos Santos et al., 2009).

Often, the aim of the study was obtaining mushroom *chitosan* which was subjected to comprehensive analysis whereas characterization of intermediate chitin polymer was not conducted. Thus, fungal chitosan from brown *A. bisporus*, white *A. bisporus*, and *Pleurotus ostreatus* mushrooms was prepared using a two-step protocol, isolation of chitin followed by deacetylation (Table 4, **Entry 11**). While the study provided a comprehensive characterization of the final chitosans, it offered only a limited description of the intermediate chitin. When Shiitake stipes were used for the preparation of fungal chitosan (Table 4, **Entry 13**) using a similar approach, the intermediate chitin polymer was characterized only for composition (elemental analysis) and thermal stability. However, the majority of chitin properties prior to the deacetylation step were not assessed (Liao & Huang, 2020).

Another important question that many reports have not addressed is the presence of glucans. Often, the purity from glucans is undocumented, and if NMR spectra are provided, they lack assignment data for glucans. Additionally, details on isolation, purification, and drying methods are frequently omitted from the synthetic protocols, leading to missing critical properties. As compilers of this review, we observed that most studies have overlooked at least one essential characterization, such as %DA (Table 4, **Entries 12**, **15**, **16**, **36**, **37**, **49**, **66–90**), CrI (Table 4, **Entries 13**, **18–29**, **32–35**, **38**, **45–65**, **69–90**), Mw (Table 4, **Entries 5–8**, **10**, **14–19**, **30**, **31**, **35–37**, **39–90**), and T_{dec} (Table 4, **Entries 9**, **18**, **19**, **21–34**, **38–48**, **50–68**, **72–90**). We would like to urge scientists to provide as many details as possible. Comprehensive documentation and detailed information are crucial for ensuring the successful application and integration of this polymer in various biotech processes.

Insufficient characterization, sadly, might also be due to a lack of expertise. For example, a study aimed to determine the physicochemical properties of chitin produced from *Fomitopsis pinicola* (Table 4, **Entry 8**) presented FTIR that exhibited significant deviations from the standard chitin pattern. Specifically, for α-chitin, the amide I band is split into two components (1660 and 1620 cm^{-1}) (Brunner et al., 2009; Focher et al., 1992; Pearson et al., 1960), whereas the Amide II band is observed at 1558 cm^{-1}; all are medium-intensity sharp peaks. Infrared absorption spectra of *α*-chitin obtained from *Fomitopsis pinicola* show low-intensity broad peaks of Amide I (1660–1620 cm^{-1}) and medium-intensity sharp amide II. The band at 1560 cm^{-1} displaying a greater intensity than the one at 1660 cm^{-1} might demonstrate the effective deacetylation of chitin or the presence of glucans in addition to chitin. This raises the question of whether %DA can be accurately determined for the impure material. As in many previous studies, Mw was not accessed.

The article (Table 4, **Entries 69–72**) provided the chitin yield, pXRD results, and FTIR spectral characterization. However, peaks of Amide I (1660–1620 cm^{-1}) were absent in the FTIR spectrum. The article

claimed that "FTIR spectrum between chitin from *Ganoderma lucidum* of the final assay and standard chitin from crab shells, Sigma-Aldrich presented a correlation percentage of 79.53%." In fact, a match of 95 % or higher is generally considered to be a good match for FTIR identification purposes. Analogously, in the pXRD analysis of the same compound, the peak located at 30° 2θ was present in the diffractograms; this peak is not characteristic of chitin.

It's highly problematic when analyses are of poor quality—to the extent that they may not meet publishing standards. Thus, the results of the study of chitin isolation from Ugandan edible mushrooms (Table 4, **Entry 9**) are difficult to evaluate – the supplied FTIR spectrum of α-chitin indicates a low degree of similarity between the isolated product and a 'typical' chitin spectra. Thus, no C—H stretches (~2900 cm^{-1}) or Amide I stretch (1620 cm^{-1}) could be identified in the published spectrum of "mushroom chitin". Contrarily, a strong sharp peak at 2349 cm^{-1} in the middle of the spectrum does not belong to this polymer. In addition, C–O–C and C—O stretching vibration region, typically apparent as a great number of narrow bands between 1200 and 950 cm^{-1} are not observed in chitin from Ugandan edible mushrooms. The pXRDs were simply of unpublishable quality. We advocate that researchers shall uphold rigorous standards to ensure meaningful contributions to the field.

The work dedicated to elucidation of the best conditions for extraction of fungal chitin from *Hericium erinaceus* (Table 4, **Entries 20–29**) determined the chitin yield, purity, Mw, and %DA, and provided the best conditions for the polymer isolation (NaOH concentration of 2 %, reaction temperature of 85 °C, and reaction time of 3 h). This study investigated different parameters (concentration, temperature, reaction time) whereas other studies simply adhered to a single set of isolation parameters. Interestingly, there were a few differences noted in absorbances between obtained chitin and the spectrum of standard chitin polymer, particularly in the C—O region. Normally, numerous peaks of high intensity are seen within the region 1020–1160 cm^{-1} of FTIR spectrum, associated with vibration modes of C—OH, C—O—C and C—C bonds (Duarte et al., 2002; Wanjun et al., 2005), where peaks at 1025 cm^{-1} (C5—OH stretching) and 1068 cm^{-1} (C3—OH stretching) belong to various C—OH stretching of hydroxyl groups (Cárdenas et al., 2004; Kumirska et al., 2010; Shamshina et al., 2021), the peak at 1113 cm^{-1} represents asymmetric in phase ring stretching (Kaya, Akata, et al., 2015), and the peak at 1156 cm^{-1} is associated with C—O—C ring asymmetric bridge (Darmon & Rudall, 1950). Typically, this region contains the peaks of highest intensity in the spectra of any carbohydrate. In the published spectrum, however, the intensity of this band appeared to be significantly decreased which might indicate the breaking of glycosidic bonds in chitin chains to some extent, during the polymer isolation process.

The second issue with the report was the purity (98 %) that was reported as the purity from proteins. As it was shown above, it is the separation of chitin from glucans which is challenging, and the purity from glucans is the important value to be reported. Mw of the polymer was determined via measurements of intrinsic viscosity ([η]) of chitin solutions in NaOH-urea, using the constants of the MHS equation for this solvent. The pXRD peaks of the products appear broad, suggesting more amorphous polymer than commercial chitin although SS ^{13}C CP-MAS NMR shows quite pure isolated material.

A similar misrepresentation was repeated in another study (Table 4, **Entries 36**, **37**) where a proper comparison among the pXRD spectrum of chitin and chitosan was not considered. In addition, in some studies (Table 4, **Entry 38**), pXRD was performed to understand the crystalline nature of the product, however, no quantification, i.e. CrI, was provided.

The characterization of chitosan was primarily based on basic conductimetric titration and viscometry to determine the %DA and average Mw, respectively. These techniques provide fundamental properties but do not offer detailed structural or chemical insights. More advanced techniques such as FTIR, NMR, or XRD could provide a comprehensive understanding of the molecular structure and crystallinity of chitosan.

It also appears that a significant number of studies did not provide any characterization (Table 4, **Entries 49–90**). All these studies were mostly included in investigating yield or performing some other characterizations without any quantification or proper analysis. While yield calculation is an important part of the analysis, other characterization techniques could help to understand the isolation of fungal chitin better.

7. Conclusion

Chitin is increasingly being used in biomedical applications such as tissue engineering, stem cells, gene and drug delivery, and nanotechnology. A search for "chitin" in the SciFinder database yields over 60,000 citations, with about 30,000 of those from the last 10 years (2014 to 2024). Therefore, it is more important than ever to address the challenges and concerns associated with chitin isolation.

Chitin, a natural biopolymer, exhibits different associations in the structure of crustaceans and mushrooms. In crustaceans, chitin maintains a structure organized in a hierarchical manner (nanofibers to microfibers to and macrofibers) and is embedded into a rigid composite protein-mineral matrix. On the other hand, in fungi, chitin contributes to the formation of the cell wall of the mushrooms. Advancements in solid-state NMR spectroscopy have confirmed that mushroom cell wall is comprised of a rigid hydrophobic scaffold of chitin and α-1,3-glucan that is firmly attached to a soft and well-hydrated relatively mobile matrix made of β-glucans (β-1,3-, β-1,4-, and β-1,6-). These differences between the structure of crustaceans and mushrooms require tailored extraction processes to achieve the highest yield and best quality of chitin.

This review aims to highlight the factors necessary for standardizing chitin processing and characterization, which have not always been sufficient. Traditionally, the characterization of chitin's properties and structural studies has been semi-qualitative. However, sample inconsistencies significantly affect research quality, suggesting that quantitative analysis is crucial for defining practical, repeatable, and reproducible results. Proper characterization and structural elucidation of chitin require the material to be as pure as possible, paving the way for innovative biopolymer applications.

Today's scientific instruments make reliable quantitation achievable. Quality assessment of chitin is essential for specific applications and involves determining the three most important properties of chitin: Mw, %DA, and CrI. The %DA indicates the percentage of *N*-acetylglucosamine units compared to the total of *N*-acetylglucosamine and glucosamine units. High %DA indicates low solubility, better mechanical strength, and higher thermal stability, while low %DA increases solubility but lowers mechanical strength and thermal stability. The Mw of chitin is closely related to its solubility and the mechanical strength of materials derived from it. The crystallinity index describes the orderliness of polymer chains in chitin's molecular structure, affecting hardness, density, and solubility.

Isolation of chitin from mushrooms requires demineralization by organic or inorganic acids, deproteinization by NaOH or other alkali where the concentration of acids or alkali, temperature, and time have significant effect on the quality of chitin. Species such as *Lentinula edodes*, *Agaricus bisporus*, *Ganoderma lucidum*, *Pleurotus ostreatus*, and *Aspergillus niger* were studied the most while some other species such as *Boletus boyinus*, *Laccaria laccata*, and *Pholiota gummosa* were investigated less frequently. Most studies used the fruiting body for chitin isolation as this portion provides a higher yield than other parts like caps, stems, or gills.

The critical analysis of the literature showed that HCl is frequently used for demineralization process, followed by use of acetic acid. Most of the studies are not detailed about the effect of acid concentration and temperature on the depolymerization of chitin. The most commonly used deproteinization methods employ alkali solution at a higher concentration (up to 10 M) which leads to significant deacetylation of chitin. Higher concentrations of NaOH and temperature also affect the

Mw leading to lower value.

The analysis of literature review has shown that many studies lack characterizations like %DA, Mw, and CrI. Many studies yet either fail to include analyses such as FTIR, NMR, and TGA or provide incomplete data. Some consistent and reliable studies could be expected in this field to minimize these research gaps or to confirm the reproducibility and comparability of the prevailing results.

CRediT authorship contribution statement

Akhiri Zannat: Writing – review & editing, Writing – original draft. **Julia L. Shamshina:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Conceptualization.

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Data availability

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