Economic and Non-Seasonal Source for Production of Chitin and Chitosan

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ABSTRACT

Fungal mycelia and higher edible fungi are considered as an attractive alternative for chitin and chitosan biopolymers production due to different reasons. They have a variety of unique physicochemical and biological characteristics. Despite their many benefits, commercial products now available rely on marine sources rather than fungal cell walls. Recently, the investment of fungal chitinous polymers into commercial products is encouraging and will increase soon because of their proven properties. Many studies continue to be reported on the development and optimization of both production processes and extraction procedures. As a consequence, once their economic feasibility has been proved, similar procedures will be easily scaled up to industrial levels. Furthermore, when the appropriateness of these biopolymers for specific applications is established, interest in their commercialization will grow, resulting in a greater range of commercial goods based on fungal chitinous polymers. Through the following titles, this chapter aims to highlight the chitin and chitosan importance from fungal origin, as alternatives of synthetic chemicals and those from crustacean origin, for usage in various fields due to their useful traits and environmentally-compatible characteristics.

List of content

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1. Introduction

The world faces two main challenges that threaten the existence of the human on earth, Viz. 1) the challenge of reduction/depletion of nutritional sources and 2) health risk challenges due to the wide-spread of human pathogens and toxic substances. The extensive use of chemically synthesized substances (CSS) i.e., soil fertilizers, antimicrobials, and unbalanced production systems are the major factors affecting climate change, soil fertility, emergence of antibiotic-resistant microbial pathogens, and represent risk-hazards to human. Realizing this fact, scientists urged the major industrial cities to find alternatives for the synthetic materials using green chemistry, natural products, and wasteless production systems. However, this call is facing a great resistance from the industrial sectors for economic and profit aspects, ignoring the human health and challenges facing the humanity. Scientists are working hard through different technologies and methods to eliminate the hazard emissions from the industrial sectors [1, 2] and find alternatives of chemically synthesized substances (CSS) [3, 4] to minimum specially in pharmaceuticals, fodders, and food preparations. Many natural substances have found applications in different sectors i.e., polysaccharides, proteins, enzymes, and lipids. These substances are produced during the life cycles of animals, plants, and microorganisms in all kinds of niches [5]. They are naturally produced for structural or functional purposes [6]. For example, polysaccharides represent the most abundant natural biopolymer on earth, produced as a cellular structural component and for energy storage, protection, adhesion to surfaces (i.e., biofilm formation), communication with other living cells, and interaction with the surrounding environment [7]. Polysaccharides are composed of tens to thousands of monosaccharides linked to each other by glycosidic bonds. Polysaccharides can be differentiated into two groups **(Figure 1)**: Homopolymers; polymer of a single type of monosaccharide such as: cellulose, starch, glycogen, and chitin and Heteropolymers; polysaccharides that contains two or more different monosaccharides such as Hyaluronic acid and chitosan. The presence of functional groups in the monosaccharides provides advanced and improved features of the produced polysaccharide.

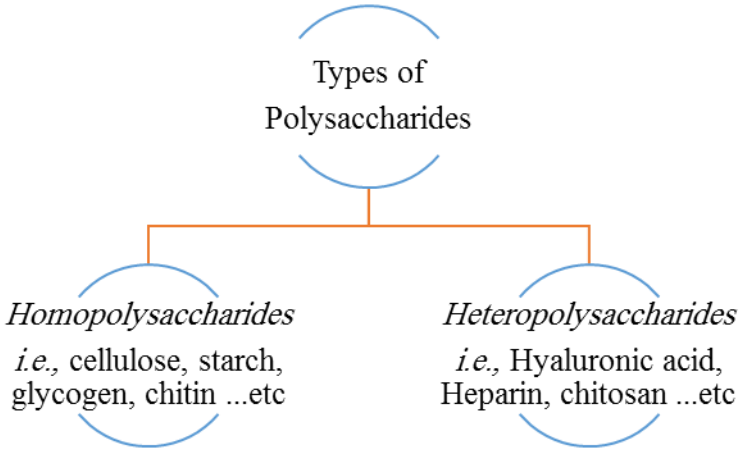

Figure 1. Types of polysaccharides based on their building blocks

Beneficial Role of Microorganisms

Long ago, microorganisms were only associated with diseases and may be with death of human, animals, and plants; however, during the years they emphasized their beneficial role in all life sectors. Starting from the discovery fungal production of penicillin by Alexander Fleming [8] to date on, microorganisms found countless biotechnological applications in environmental cleansing and protection i.e., bioremediation, bioaugmentation and wastewater treatment [8] as well as the production of many add-value metabolites i.e. amino acids, vitamins, antibiotics, enzymes, and polysaccharides.

Microorganisms and Economy

These facts paved the way for new era for human well-being and application of sustainable development goals (SDG). Nowadays, we can say that we have developed a lot of industries, health, and economic entities based on the fungal and bacterial activities using the microbial biotechnology. The size of the global sector of biotechnology lacks an official statistical figure, however, in 2007 it was estimated to be 84.8 billion dollars compared with 9.1 billion dollars in 1996 [9] which means a long leap progress in this field. In addition, the biotechnology methods were simplified which allowed its use by the public for some applications such as production of milk, food products, and biofuel. The use of biotechnology is of particular importance for the developing countries due to limitations facing their people.

Myco-Industrial Applications

Before the discovery of the living nature of fungi, the man felt their effect and used their activity. Pharaohs in ancient Egypt, the Babylonians, and the Sumerians used the yeast fermentation for baking, beer, and winemaking [10]. Today, fungi found many applications as an edible biomass (i.e. mushrooms), production of fermented food (i.e. beer, wine, bread, and cheese), biocides (i.e. antimicrobials, insecticidal, and nematicidal), organic acids (i.e. gluconic acid, citric acid, malic acid, lactic acid, and others), Immunomodulators (i.e., cyclosporine, mycophenolic acid, mizoribine, lectins and terpenoids), Vitamins (i.e. riboflavin), enzymes (i.e. lipases, pectinases, catalases, and others), polyunsaturated fatty acids (i.e. linoleic acid), fungal flavors (i.e. Amyl vinyl carbinol) [11]. Furthermore, fungal polysaccharides were reported to possess a positive effect on human health. According to Misaki [12], the polysaccharide isolated from *A. auricula* (commonly as wood ear) have antibiotic, antiulcer, antidiabetic, and anti-tumor effects and acts as immunomodulatory, and lower blood cholesterol levels. Polysaccharides have both structural and functional roles in the fungal cells. They can be extra- (EPS) or intra-cellular (IPS), or involved in fungal cell wall structure (FCW) [13]. Polysaccharides (mainly glucans, chitins, and glycoproteins) represent the main component

in the fungal cell wall and were estimated to be (90%). Chitin represents about 1 and 15% of the fungal dry cell mass [14] depending on the fungal species and culture conditions.

2. Chitin and Chitosan

In 1811, chitin was initially isolated by Henry Braconnot from mushroom cells under the name of "Fungine". The same compound has been isolated from the cuticle of insects in 1823 by Auguste Odier where it was renamed as "chitin". Naming arises from the Greek word "Khiton" which means "protection". The chemical structure of chitin was first determined in 1929 by Albert Hofmann using chitinase enzymes. Chitin (β-(1-4)-poly-N-acetyl-d-glucosamine), a long-chain of mono-polysaccharide of N-acetyl glucosamine subunits (Fig. 2), connected to each other's by β (1→4) glycosidic linkages. It has been considered that cellulose is the most abundant biopolymer in nature followed by chitin. Physically, chitin is a highly insoluble and hard compound giving strength and protection of the fungal cells and forms the exoskeleton of insects [15]. Chitin presents in the cell walls of fungi specially in the members of Ascomycetes, Basidiomycetes, and Phycomycetes, exoskeleton of insects, crustaceans, and marine invertebrates [16, 17]. Although first isolated from fungi, more interest has been focused on their production from crustacean sources. Chitin comes in three different crystalline allomorphs, depending on the source—α, β, and γ, classified by the orientation of the microfibrils. α-chitin: The most abundant and stable form was composed of two units of N-N'-diacetyl-glucosamine that form two anti-parallel and stable polymer chains. Likewise, it is mostly found in arthropods, crustaceans, and fungi cell-walls. β-chitin: These are obtained from sea diatoms and have a more flexible or loose crystalline structure made of a single unit of poly-N-acetylglucosamine organized in polymer chains in parallel to each other with no inter-sheet hydrogen bonds. γ-chitin: is a combination of α- and β-chitin, composed of mixed parallel and anti-parallel arrangements, and obtained from yeast and fungi. The crystalline structures of the chitin polymorphs have various physicochemical properties due to their different molecular packing arrangements. Due to the presence of extremely crystalline structure and intramolecular and intermolecular hydrogen bonding, chitin has limited applicability due to its low reactivity and solubility in most solvents. Many attempts were achieved to develop more reactive and soluble chitin derivatives (chitosan) through several chemical approaches.

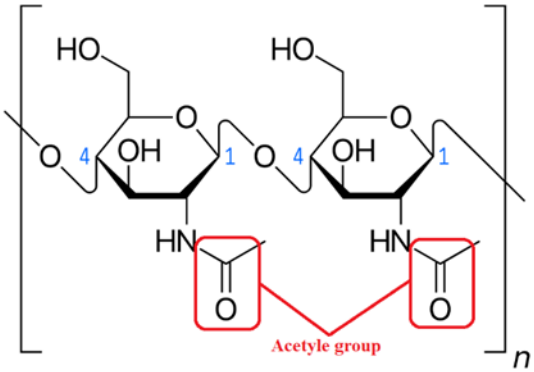

Figure 2. The building block of chitin showing the β (1→4) linkage

Chitosan, first discovered by Rouget in 1859, a heteropolysaccharide composed of acetylated unit (β-(1→4)-linked N-acetyl-D-glucosamine) and deacetylated unit (D-glucosamine). Chitosan is a derivative of chitin obtained by its deacetylation (removing of acetyl group (**Figure 2**), usually using alkaline substances (i.e. sodium hydroxide). The degree of deacetylation specifies how much chitosan is transformed from chitin, and when the degree of deacetylation exceeds 50%, chitin is categorized as chitosan. Chitin is N-deacetylated to create chitosan, which increases its versatility by allowing it to

dissolve in diluted acetic and formic acids. Chitosan present naturally in many fungal species (such *Absidia* sp., *Aspergillus* sp, *Gongronella* sp., and *Rhizopus* sp.), however, it's usually made from crustacean chitin deacetylation, which is performed by enzymatic hydrolysis, or under strong alkaline conditions, and high temperature. These methods can alter the DA and Mw of the final product, affecting its final qualities. Chitosan has three functional groups: an amino group (at positions: C2) as well as primary (at positions: C3) and secondary (at positions: C6) hydroxyl groups. Chitosan has been discovered to have a higher reactivity than chitin.

2.1 Sources of Chitin and Chitosan

Chitin have been discovered, isolated, and defined from three distinctive forms of life **(Figure 3)**.

Figure 3. Sources of natural chitin and chitosan

2.1.1 Marine Source

Crustacean shells (e.g., lobsters, shrimp, crabs, krill, barnacles, and crayfish) constitute the conventional and current commercial source of chitin [18]. Dima *et al*. [19] reported that the crustacean waste contains about 10–25% (based on the dry weight) of Chitosan. The crustacean shells of crabs, shrimps, and crawfish consist of 20-30% chitin, 30- 50% calcium carbonate, and calcium phosphate, as well as 30-40% protein [7]. Studies conducted by Ashford *et. al*. [20] revealed that chitin represents 13-15% and 14-27% of dry weight of crab and shrimp processing wastes, respectively [20]. Crustacean chitin is a rigid composite of highly mineralized chitin protein, requires harsh demineralization processes. Crustacean shells are amongst the most significant chitin and chitosan sources owing to the abundance of biomaterial, and also well economic extraction processes. The human consumes hundreds to thousands of tons of crustacean sea food [21] resulting in wastes of about 40% of these amounts [21]. Although, the bulk amount of this waste may be considered as an advantage for chitin production, many other disadvantages are associated with chitin production from this source (Table 1).

2.1.2 Terrestrial Source

Insects (i.e. arthropod) are third source of chitin with ecological and economic sustainability [22] and present some advantages over crustacean sources, as their growth is not seasonal and are highly fertile and reproductive [23]. However, few studies on insect chitin have been reported by Hahn *et al*. [24]. Mosquitoes, silkworms, cockroaches, honeybees, *Extatosoma tiaratum*, *Sipyloidea sipylus*, and *Drosophila melanogaster* could all be often used for obtaining chitin from their cuticles and wings. Cuticles of insects represents chitin complex with melanin, a matrix of cuticular proteins, lipids, and other compounds [25]. Similarly, the chitin content of insects such as *Hydrophilus piceus* and *Agabus bipustulatus,* varies between 10 and 20% [26].

2.1.3 Microbial Source

Chitinous polymers are found in different microbial sources as bacteria, algae, lower and higher fungi, and yeast [27]. Different fungal sources such as filamentous fungi, yeasts, and higher fungi have been examined as an alternative chitosan and chitin sources [28].

Fungal mycelia are considered as the second source of chitin and produced through the controlled fungal fermentation processes. Members of some fungal divisions are known for their capability to produce chitin i.e. Deuteromycetes, Ascomycota (i.e. *A. niger*), Basidiomycota (i.e. *Lentinus edodes*), and Zygomycota (i.e. *Absidia spp.*, *Rhizophusoryzae,* and *Mucor rouxii*), while others loss this capability [29]. Due to the high chitin and chitosan content in its mycelia, the fungus species *Mucor circinelloides* of the Zygomycetes class has attracted attention from researchers. So, fungi have attracted the attentions as an alternative source chitin [30], mainly for its environmental and economic reasons. In addition to chitin, chitosan can simply isolate from the fungal cell wall without deacetylation process. Yeasts are further a chitinous polymers source, including *Candida albicans*, *Komagataella pastoris*, *Schizophyllum commune,* and *Saccharomyces cerevisiae*. In fungi and yeasts, it ranges from 2 to 42 percent on a dry basis. The amount of chitin/chitosan in cell walls varies depending on the age, species, and physical conditions.

Further, many higher fungi have also been reported as chitinous sources, such as: *Agaricus bisporus, Gonoderma lucidum,* and *Armillariella mellea.* The chitin percentage in cell walls of *Agaricus* sp., for instance, varied among 13.3 and 43 independently verified during the life cycle of the organism, and it also varies during post-harvest storage. There is a direct relation between chitin and chitosan content, structure, and degree of polymerization in fungal cell wall and the species, cultivation conditions (temperature, pH, and dissolved oxygen), media composition (carbon and nitrogen sources and micronutrients), and fermentation systems (batch, fed-batch, solid state, sub-merged, or continuous processes).

The fungi chitin possesses principally the same structure as that of the crustacean chitin [31]. However, the difference arises from the fact that fungal chitin is, naturally, connected with other types of biopolymers such as glucans and mannans which are absent in the arthropods' exoskeleton [32].

2.1.3.1 Advantages of Fungal Chitinous Polymers over Crustacean Chitin

Nowadays, the fermentative fungi are used as alternatives for chitin and/or chitosan production on the industrial scale, for the following reasons: Microbial chitin and/or chitosan became, industrially, popular due to the advantages of achieving pure and uniform products with consistent characteristics. Fungal chitin contains less contaminants, subsequently, requires less purification steps than crustacean chitin that is associated with the presence of lipids, minerals, proteins, and pigments [33]. The ease of cultivation of fungi in the laboratory and extraction in eco-friendly processes and cheap substrates support the feasibility of fungi use as a major chitosan source in the commercial market [34]. Furthermore, environmental and seasonal fluctuations limit the availability of marine chitin, which explains the rising demand for this product. Chitin and chitosan from fungi and yeast are valued for their chelating activity, biocompatibility, adsorption properties, and potential antimicrobial activity [35]. Chitosan from fungal sources distinguishes it from other sources in terms of the absence of heavy metals like nickel and copper, DA, its unique molecular weight homogeneity, and charge and viscosity distribution [36]. Chitin and chitosan could be produced directly from fermentation-based fungal growth. Chitin could further be recovered from fungal waste biomass produced in bioprocessing facilities. Fungi-based fermentation of different substrates for chitin and/or chitosan production is a commercially viable technique, and the extracted chitosan can offer Mw ranging from 2.7 × 104 - 1.9 × 105 Da, around 84–90% DDA and with a viscosity of 3.1–6.2 cp [34]. Because fungal chitin contains fewer inorganic elements than crustaceans, it is used as a pharmaceutical carrier. Ghormade *et*

al. [34] reported that chitosan from fungal sources have higher % DDA, but 3–5 times lower viscosity and MW, making them appropriate for usage in pharmaceutical industries, healthcare, and food.

Table 1. Comparison between fungal and other chitin natural sources

Basis for comparison	Fungal chitin	Other chitin sources
Availability	Does not have seasonal or geographic limitations.	Limited by sites of fishing industry and seasons.
Inorganic materials	Low levels	High levels
Natural chitosan	Presence	Absence
Extraction process	Simpler	Requires harsh solvents
Cost of waste management	Low	High
Demineralization treatment	Not required	Required
Physico-chemical properties	Consistent	Vary
Contribution in industrial waste management	Yes	No
Induction of the plant immune response	More effective	Less effective

3. Fungal Cell Wall Structure and Function

The fungal cell wall is a dynamic structure that surrounds and lies directly beneath the cell membrane. A rigid inner layer and a relatively mobile outer layer made up the fungal cell wall. The inner layer consists of a hydrophobic core of tightly packed β -1,3-glucans and fibrils of chitin that are embedded in an amorphous matrix consists of β -1,3-, β -1,4, and β -1,6-glucan amorphous matrix. On the other hand, the outer layer is made up of an amorphous matrix with β -1,3-glucans and glycoproteins surrounding the cell surface (**Figure 4**) though most fungi, especially for Ascomycetes showed this model.

Generally, fungal cell wall is composed of polysaccharides, mainly glucan, mannans, glycoproteins, and chitin. Glucan layer in the fungal cell wall may account for (50–60 %) of the dry weight [37] and is composed of β-(1,3)-linked glucose, and in some cases, β-(1,6)-linkages can be found [38]. Even though the fact that chitin is an important structural component of the fungal cell wall, it only accounts for 10–20% of filamentous fungus cell walls and 1–2% of the dry mass of yeast cell walls. In the fungal cell wall, chitin/chitosan occurs in two forms, as free amino-glucoside and covalently bound to β-glucan, thus forming the copolymers chitin–glucan complex (CGC), and chitosan–glucan complex (ChGC), respectively [39].

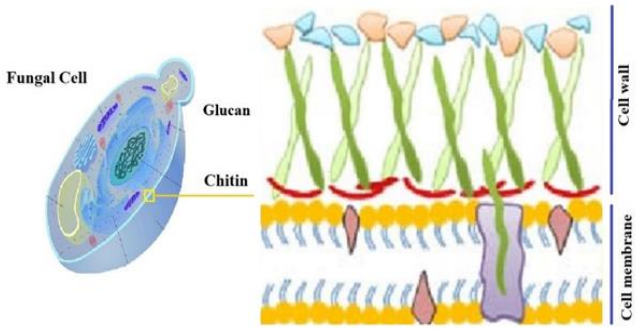

Figure 4. Simplified shape showing chitin site in the fungal cell wall

4 Microbial synthesis of chitin

Chitin de novo synthesis in the fungal cell wall is catalyzed by the membrane associated synthases [40]. The number of chitin synthase genes in fungi varies from one in *Schizosaccharomyces pombe* to eight in mycelial ascomycetes *A. nidulans, Aspergillus fumigatus,* or in basidiomycetes *Ustilago maydis,* and *Cryptococcus neoformans* [41]. Many chitin synthases are zymogens located in the plasma membrane in the areas of apical growth, where they are further transformed into their active forms [42-44]. Membrane bound chitin synthases (an integral membrane enzyme) catalyze synthesis of chitin chains from the activated uridine 5'-diphosphate-GlcNAc (UDP-Glc-NAc) monomers and release the formed polymer into the extra cellular space. Individual chitin chains in the extracellular space associate with each other via intra-molecular interactions and form chitin microfibrils which are then linked by covalent binding to the cell wall glycan [45]. This crosslinking is catalyzed by transglycosylases or inherent transglycosylase activity of chitinases [46]. This process primarily takes place at the site of active fungal growth Viz., budding tips (in yeast cells), and hyphal apex or growing tips (in filamentous fungi). In addition, zygomycetes such as *Mucor indicus,* can synthesize chitosan. The presence of chitin and chitosan in significant amounts in the cell walls of zygomycetes, allows the use of this species for the biotechnological production of chitin derivative [46].

4.1 Isolation and Purification of Chitin and Chitosan

The annual chitin biosynthesis was estimated to be 1000 billion tons [47]. The purification process of chitinaceous products differs according to the source producer (Crustacean and Fungal). This is due to differences in the types and natures of contaminants. The crustacean chitin is naturally closely associated with lipids, minerals, proteins, and pigments, while fungal chitin contains less contaminants, subsequently, requires less purification steps [33]. Generally, commercial industrial purification of chitin involves the following steps concerning contaminant (**Figure 5**):

Demineralization/decalcification: minerals (especially CaCO3) are found in crude chitin from shell wastes. Minerals may reach about 50% in crab and shrimp shells (Kurita 2006). For effective removal shells should be washed, dried, and ground to smaller sizes, and then they should be mixed with dilute solution of hydrochloric acid or organic acid (i.e. acetic acid) [47].

Discoloration: for effective removal of residual pigments, bleaching process should be performed using sodium hypochlorite, hydrogen peroxide, and acetone resulting in enhanced color properties [48]. Deproteinization: Many substances have been evaluated for their deproteinization efficiency; however, dilute solution of sodium hydroxide is the most widely used [48].Sodium hydroxide is capable of removal of proteins, glycoproteins, and branched polysaccharides. Separation of chitin and chitosan: in contrast to crustacean and insect chitin, chitosan can be found naturally in fungal crude chitin. Due to its acid solubility chitosan can removed by acid

treatment for (3-14 hours) at 95 °C using (2- 10%) hydrochloric or acetic acid.

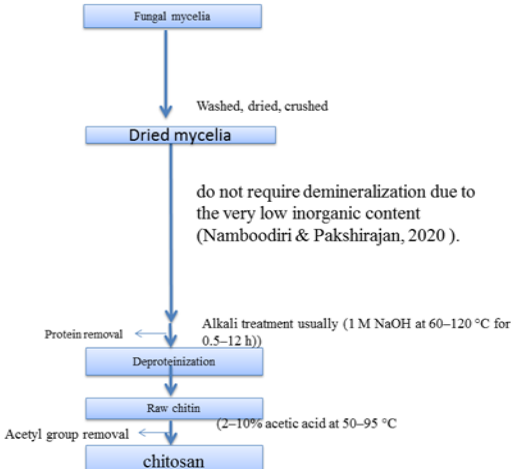

Figure 5. Isolation and purification of chitin and chitosan from fungal mycelia

4.2 Purification of Chitin by Biological Method

The chitin chemical treatment procedure has several disadvantages for both isolated chitin and the environment. Concerning the product, the removal of proteins and associated minerals normally needs aggressive reagents as acids and alkali under high temperature for a prolonged time that can cause deacetylation and depolymerization of chitin and often affect the physicochemical properties [49]. For the environment, these chemicals are threatening to the environment, energy consuming, and extremely hazardous [14]. Due to the detrimental effects of chemical method, an alternative to these harsh procedures is the switch to a biological means of extraction (**Figure 6**). In the enzymatic deproteinization process, enzymes obtained from microbes, plants, and animal sources such as extracellular proteases secreted from fungi, trypsin, pepsin, papain, and pancreatin. The residual protein level of chitin extracted by enzymatic deproteinization is higher than that of chitin isolated by alkali treatment. Organic acid, such as lactic acid-mediated demineralization, or various microorganisms are involved in enzymatic demineralization. The high cost of enzymes can be reduced by utilizing a fermentation technique to deproteinize them. In fermentation process, microorganisms including *Pseudomonas aeruginosa, Lactobacillus* sp., *Bacillus subtilis, Bacillus licheniformis, and Bacillus cereus*, mediate the process of demineralization and deproteinization, and may reduce the chemicals quantities required for the extraction of chitin, thereby curtailing the toxic amount of the produced effluent. The degree of deproteinization and demineralization based on the biological methods used to perform the stages can range between 60-95% and 70-95%, respectively. It has been reported that the use of lactic acid bacteria for fermentation of crustacean shells, resulted in lowering the medium pH to approximately pH 4 which facilitates protein hydrolysis, while leaving the associated chitin intact [50]. The chitin obtained by the deproteinization of shrimp shell waste with various proteolytic microorganisms including *Aspergillus oryzae*, *Pediococcus pentosaseus, Streptococcus faecium, Bacillus subtilis, and Pseudomonas maltophilia* had higher molecular weights compared to chemically chitin obtained from shellfish [51]. The biological approach of extraction is more effective and environmentally friendlier than chemical extraction methods. As a result, there is a need to exploit these new approaches for chitin synthesis, because microorganism-mediated fermentation processes are incredibly considered as fast, simple, and the possibility to be optimized by controlling the parameters of the process, while the use of negligible solvent consumption and the ambient temperature would result in cost reduction and less negative impact on the environment.

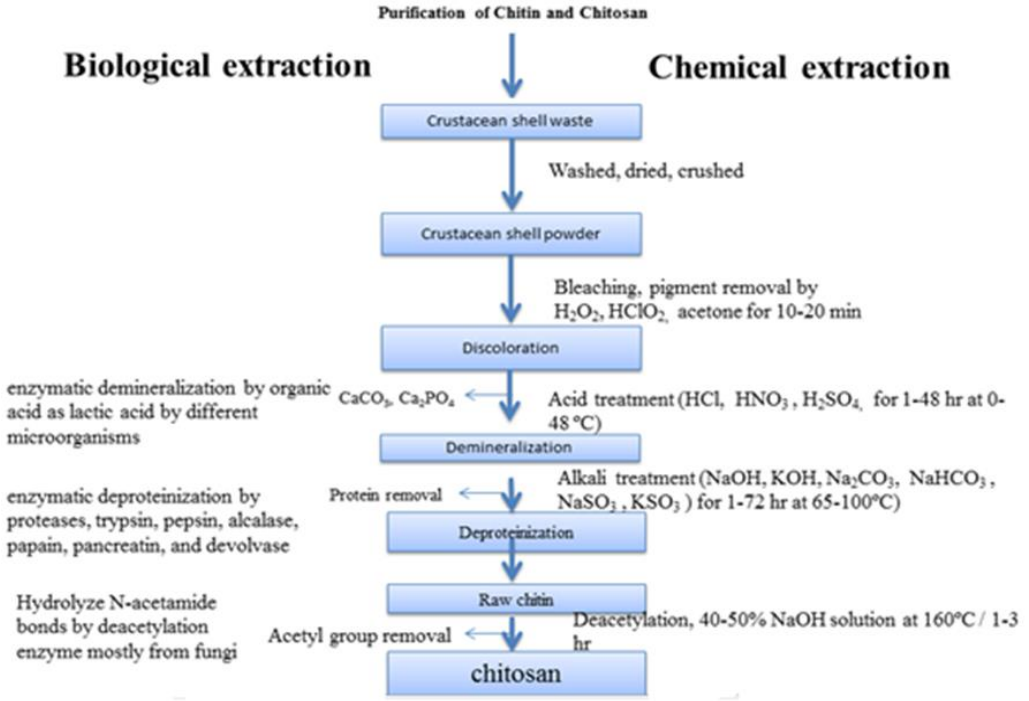

Figure 6. Comparison between biological and chemical methods of chitin extraction

4.3 Microwave-Assisted Extraction

Chemical extraction has been revolutionized by the advent of microwave-assisted extraction. Microwave technology is a convenient alternative to traditional heating methods. Microwave heating could minimize extraction time; therefore, the use of microwave radiation to extract substances is an energy-saving method, environmentally friendly, and more efficient. The reactants slowly rise in temperature during conventional heating, resulting in a uniform significant rise in temperature. Furthermore, using microwave heating instead of conventional heating during the demineralization and deproteinization stages was reported to reduce the time from a day to few hours. Moreoer, the application of microwave-assisted extraction resulted in chitosan with a greater crystallinity and molecular weight. The shift from the traditionally used heating methods to a more efficient extraction technique may lower requirement energy and reduce the amounts of chemicals used, thereby leading to a greener synthesis [52, 53].

4.4 Preparation of Chitosan

Chitosan can be found naturally in fungal crude chitin, however, chitin from crustacean shell and insects need aggressive treatment for its deacetylation. There are two different processes for chitosan preparation: chemical and enzymatic process. Chitosan is produced for large-scale production with chemical process. In chemical process, chitin is treated with sodium hydroxide solution (40–45%) at 160 °C for 1–3 hours. The degree of deacetylation (DDA%) depends on various factors including temperature, particle size, alkali concentration, solid-to-solvent ratio, and time [48]. Deacetylation process is to remove acetyl group from chitin resulting in two types of monomers; N-acetylglucosamine and N-glucosamine (**Figure 7**). Deacetylation process can be repeated for obtaining higher deacetylation degree. The degree of deacetylation is a ration between the deacetylated to acetylated monomers in the molecule. As mentioned above, many disadvantages are linked to the use of chemical deacetylation such as: environmental pollution problems and high energy consumption [54]. So, enzymatic process has been developed to overcome these drawbacks. The enzymes use for the deacetylation of chitin catalyze the

hydrolyze N-acetamide bonds [55]. Deacetylase enzyme was extracted from two strains of *Celletotrichum lindemuthianum, Absidia coerulea, Mucor rouxii, and Aspergillus hidulans*. This enzyme has a high affinity for β-(1, 4)-linked N-acetyl-D-glucosomine polymers. The enzyme process is usually carried out in both batch and continuous culture. The molecular weight of chitosan is lower in the batch process in terms of time. Furthermore, even though the yield is low, higher Mw chitosan can be obtained in a specific culture.

Figure 7. Deacetylation of chitin to chitosan

5 Chemical Analysis and Detection

To determine chitin and chitosan in the hydrolyzates of alkali-resistant fractions, GlcN was, frequently, quantified by colorimetric methods [7]. On the other hand, in acid hydrolyzates, GlcN was determined using high-performance liquid chromatography (HPLC) of 9- fluroenyl methoxy carbonyl (FMOC), phenyl isothiocyanate (PITC)-GlcN [56], or by gas chromatography-mass spectrometry (GC-MS) [57]. Using dyes that intercalate with polysaccharides, chitin can be located in cell walls or spores of fungi. Calcofluor white shows enhanced fluorescence when binding to b (1,4) glucans such as chitin, chitosan, and cellulose, whereas b (1,3) glucans are selectively stained with aniline blue [7]. Various wheat germ agglutinin (WGA) labeling techniques in a combination with fluorochromes or gold labeling are also described for the detection of chitin in fungi [58]. Penman et al. [56] reported the use of Fourier transform (FT) Raman spectroscopy to discriminate between different mixed fungal species in culture media based on their cell walls.

6. Characterization of Chitin and Chitosan

The successful use of chitosan and chitin in different fields such as agriculture, biomedicine, and food, among others, depends on their physicochemical properties. These properties are related to the isolation method of the samples and their origin [59]. For the commercial production purposes of chitosan, chitin is deacetylated using strong alkali [60]. The natural chitin and chitosan varies in their physicochemical properties. This variability is attributed to their origin, the nature of the chemicals used for isolation and the deacetylation processes, and variability in the degree of deacetylation and the amount and nature of the contaminant protein [61]. The physicochemical properties are the determinant factors for their final possible utilization. The MW and DDA of biomolecules are two essential characteristics that govern most structure-function relations. The (DA), a relation between the structural units of 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose, has a significant impact on chitin solubility and solution characteristics [62]. The degree of *N-acetylation* (DA) of chitin is generally 0.90, which indicates the presence of certain types of amino groups. Chitin may contain roughly 5–15 percent amino groups as a result of deacetylation reaction which may take place during extraction process. Sajomsang and Gonil [63] reported that the DA of chitin (> 100%) indicates the presence of some inorganic materials in the polymer structure. According to Han *et al*. [64], the degree of deacetylation (DD) is considered instead for chitin/chitosan characterization. The DD of chitosan ranges from 56% to 99% and varies depending on the temperature, concentration of the utilized alkali, and contact time [65]. It has been noticed that the DD of chitosan increases with the

extended reaction time, reaching 90.18 percent at 120 minutes. It affects chitosan's solubility, chelating, flocculation, and capacity with metal ions, selective acylation, and other physicochemical aspects. It has been widely employed in various industries, including food, agriculture, medical, engineering, and the environment, due to its biodegradable, biocompatible, and non-toxic qualities. Based on DDA, CS can be divided into two categories: low degree of deacetylation (55–70%) and high degree of deacetylation (70–99%). Various methods for determining DDA in chitin and chitosan have been proposed. The DA/DD values can be calculated using various analysis techniques for the chitin/chitosan samples determination. The methods include Fourier transform infrared spectroscopy (FTIR), 13C solid-state nuclear magnetic resonance (NMR), (CP/MAS) NMR [66], elemental analysis [67], ultraviolet (UV) spectrophotometry [30], and titration methods [68]. The most commonly used method of DA/DD values is infrared (IR) spectroscopy. Chitin/chitosan samples are pelleted by blended with KBr, and then analyzed using a FTIR spectroscopy [69]. The DA/DD is then calculated using the equation of Baxter *et al*. [70] which represent the ratio of the absorbance values (A) at 1655 and at 3450 cm−1 as follows:

$$DA\% = (A_{1655}/A_{3450}) \times 115$$

The comparison conducted by Kasaai [71] between the various methods for determination of the degree of deacetylation of chitin and chitosan revealed that NMR spectroscopy and FTIR have many advantages over the other methods as they need shorter-term for the preparation of the samples and provide more information on their chemical structure. UV and ^{1}HNMR methods are more sensitive than ^{13}C NMR, FTIR, and ^{15}N-NMR spectroscopy. The FTIR method is mostly utilized in qualitative evaluation and comparative research. For highly acetylated chitin, traditional procedures (adsorption of free amino groups of chitosan by picric acid, conductometry, titration, ninhydrin test, and potentiometry) are ineffective. The findings produced using traditional methods are substantially influenced by factors including the solvent's ionic strength, pH, and temperature of the solution. The molecular mass distribution is another essential feature that has a significant impact on the properties of polymers. It has been reported that the antimicrobial activity of chitosans is highly related to their low molecular weight [33]. Antimicrobial activity is an important criteria for the application of chitosan in food sectors (e.g. as fruit coatings) [72]. The molecular mass distribution of chitin and chitosan are mostly determined using Mark–Houwink equation by measuring the intrinsic viscosity [66] although gel permeation chromatography (GPC) [73] and size exclusion chromatography (SEC) [68] have also been documented. Capillary viscometry is the most extensively and simplest method used for the determination of chitosan molecular weight.

The chitosan molecular weight can be used as a standard parameter for quality as it greatly influences the polymers' properties. Low Mw chitosan is suitable for applications in the food market as an anti-tumor, antioxidant, and antibacterial substance. The low molecular weight chitosan can easily penetrate the microbial cell membrane and binds to RNA preventing its transcription, and finally cell death [72]. On the other hand, chitosan with medium Mw was reported to have higher anti-cholesterol activity compared with that of high Mw [15]. The CS molecular weight is approximately from 200 to 1000 kDa. CS with MW (>300 kDa) is classified as high molecular weight and low molecular weight chitosan (< 300 kDa). The Mw of chitosan is also based on its origin which in turn affects its physicochemical properties such as bio-functionalities, adsorption on solids, viscosity, and solubility [74]. Temperature characteristics of polymers are especially important for characterization as they provide data regarding their thermal stability which is a determinant parameter for their ability for specific applications. Furthermore, chitin and chitosan thermal stability is dependent on other characteristics such as the CI, Mw, and DA/DD [75]. The thermal properties are determined by differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) [68]. Carbon,

nitrogen, and hydrogen content of the biopolymer from different sources were conducted by an elemental analyzer. The value of nitrogen content indicated the residual protein contaminant remaining in the polymer samples and consequently the effectiveness of the deproteinization process [76]. In addition, the percentage N of chitin and chitosan is critical because it reveals the product's purity. The completely acetylated chitin has a N-content value of 6.89% [77]. N-content value (> 6.89%) indicates protein remnants, while (< 6.89%) indicates remnants of inorganic materials [63]. To explain this, the extracted chitin from the house crickets is not completely pure, however it is considered as purer compared with the commercial chitin due to the presence of inorganic materials. This consideration is suggested by the lower percentage N of 4.79% obtained by Kaya *et al*. [78]. In contrast, fungi chitin contains residual chitin-glucan that cannot be removed completely by chemical methods [79] resulting in increased N-content. X-ray diffraction (XRD) examination can be fruther used for the determination of the crystalline structure of chitosan and chitin, as well as the crystallinity index (CI), which shows the samples' degree of purity [31]. The CI values of chitin and chitosan play a major role in assigning their application fields and effectiveness [59] and are dependent on the fungal source, as well as the growth conditions and the extraction procedure [53]. The surface morphology of the chitin and chitosan was also investigated by scanning electron microscopy (SEM) to reveal the details of their microstructures. Chitin has been described as having five different surface structures, which can be more used to define chitosan. These surface structures include the following: A rough and hard surface without pores or fibers, with only pores, a combination of fibers and pores, with two types of pores with fibers and only fibers [80]. Fungal chitin was found to have a microfibrillar crystalline structure in SEM by Chan, Chen, and Yuan (2001). On the contrary, the fungal chitin obtained from shiitake stipes by Yen and Mau, [81] showed aggregation of flakes with firm and dense structure without porosity and the fungal chitosan did not reveal microfibrillar structure under SEM. Previously, shiitake stripes was made up of tight cell walls made up of combinations of polysaccharides and chitin. Due to the significant amount of glycans in crude chitin, fungal chitin could not have a fibrillar or filamentous structure. The differences in crystallinity structure reported in cran and fungal chitins could be due to their differing intra-sheet or inter-sheet hydrogen-bonding systems. Ibitoye *et al*. [82] reported different surface morphologies and possible varying utilization of chitin and chitosan extracted from the same species of the organism (house crickets). Solubility is also affected by the deacetylation process's operating temperature. Solubility is reduced at higher temperatures during the deacetylation process. One of the most essential aspects in determining the quality of chitosan is its solubility. The degree of deacetylation influences the solubility of chitosan, which impacts its quality. Particle size, alkali concentration, ratio of chitin to alkali solution, temperature, and deacetylation reaction duration are all important factors impacting chitosan solubility, according to Hossain and Iqbal [83]. Lower solubility values, according to Brine and Austin [84], indicate inadequate deacetylation process. Chitosan solubility is determined by removing of the acetyl group during the deacetylation, and a lower value of DD may skew the results. The order of the demineralization and deproteinization processes affects fat binding capacity (FBC). When the deproteinization step was done first, the chitosan's fat binding ability was lowered. According to Hossain and Iqbal [83], varying the sequence of purification process may result in different fat binding capacity. They found out that chitosan with more fat binding capacity results from conducting demineralization step before deproteinization. According to No *et al*. [85], the average chitosan's fat binding capacity is about 417%. The water-binding capacity (WBC) is also affected by the deproteinization and demineralization processes [83]. According to Cho *et al*. [86], the WBC for commercial chitosan from shrimp and crab shells is between 458 and 805 percent. Water-binding

capacity and fat binding capacity are functional qualities that differ with the technique of manufacture, according to Mohan *et al*. [87].

7 Chitin and Chitosan Applications

Chitin's poor solubility is a significant impediment to its popular use [49]. Chitosan is a desirable polysaccharide that is soluble in dilute acetic acid and contributes chelating, polycationic, and dispersion-forming capabilities. Chitosan has exceptional biological and chemical characteristics which can be used in a wide variety of medical, pharmaceutical, and industrial applications [7].

7.1 Biological Activities of chitosan

During aerobic metabolism, free radicals and reactive oxygen species are produced spontaneously in the body, causing the oxidation of proteins, carbohydrates, lipids, nucleic acids, and sterols. Antioxidant defense mechanisms deteriorate with age, resulting in an increase of free radicals and reactive oxygen species (ROS). The uncontrolled generation of these free radicals is harmful because it causes cellular damage, which can lead to many diseases such as cancer, stroke, atherosclerosis, diabetes, retinal damage, rheumatoid arthritis, and heart attacks. To combat these free radicals, the body has created natural antioxidants. However, as these systems age, their capacity reduces, resulting in redox imbalances. As a result, the body needs to be nourished with a diet rich in antioxidants. Free radical scavengers are preventive antioxidants, and their presence interrupts the oxidative chain at several levels. and molecular weight. Recently, low MW chitosan has been reported to be an effective antioxidant than high MW chitosan. Chitin and chitosan are known to adsorb metal by reducing the formation of ROS and free radicals, antioxidative nutraceuticals such as chitosan, polyphenols, carotenoids, ascorbic acid, and tocopherols can assist to reduce oxidative damage and lessen the risk of age-related illnesses. Furthermore, the degree of deacetylation and molecular weight of chitin and chitosan influence their antioxidant properties. Low molecular weight chitosan has recently been found to be a more potent antioxidant than high molecular weight chitosan. Owing to their free amino groups, chitin and chitosan achieve larger affinity to metal ions. The ability to chelate metal ions involved in oxidative reactions can be highly useful in pharmaceutical products, water treatment, health care, and food preparation and storage [7]. Cancer is currently the cause of 12% of all deaths worldwide. Antioxidative nutraceuticals and anticarcinogenic including phytochemicals (such as retinoids, polyphenols, flavonoids, etc.) can contribute to preventing the onset of some cancers by a variety of methods, involving DNA mutagenesis prevention and mortality induction. Some nutraceuticals can adsorb mutagens and hence reduce their carcinogenic action, in addition to reducing oxidative stress and subsequent DNA damage. The majority of anticarcinogenic investigations revealed that chitin and chitosan, as well as their oligomers, block heavy metal-induced geno-toxicity, and also have growth inhibitory and antimetastatic effects on a range of tumor tissue. Chitin or chitosan as a dietary supplement may protect against a variety of cancers by potentially adsorbing carcinogens and transport them out of the digestive tract [7].

7.2 Antidiabetic Activity of Chitosan

Type 1 diabetes (insulin-dependent) and type 2 diabetes (non-insulin-dependent) are the two main forms of diabetes. Obese Type 2 Diabetes Mellitus has hyperinsulinemia and non-obese Type 2 Diabetes Mellitus exhibits hypoinsulinemia. Obesity is known to produce peripheral insulin resistance, which results in hyperinsulinemia. Hypertriglyceridemia and hyperinsulinemia both are symptoms of obesity-related type 2 diabetes mellitus. Improving the abnormality of glucose metabolism and lipid metabolism are useful to prevent the obesity-related Diabetes Mellitus (type II). MIura *et al*. [88] showed for the first time, that the supplementation of chitosan as a 5% food mixture resulted in consistent level of blood glucose and lipid-lowering effects in both the normal mice and neonatal streptozotocin-induced diabetic mice. Hayashi & Ito, [89]

examined the effect of long-term administration of low-molecular weight chitosan, given as drinking water, on hypertriglyceridemia, hyperinsulinemia, and hyperglycemia of diabetes mellitus in male genetically obese type NIDDM mice. As a result, the anti-diabetic effect of low-molecular weight chitosan is unlikely to be attributed to D-glucosamine, a monosaccharide found in chitosan. Chitosan is thought to be absorbed predominantly after it has been changed into oligosaccharide, which inhibits dietary fat absorption in the intestine.

7.3 Antiulcer Activity of Chitosan

Ulcers are non-healing, open sores which can appear in a variety of places on the body. Peptic open sores or holes in the stomach or duodenal lining cause abdominal pain, bloating, nausea, and loss of appetite, among other symptoms. Ulcers are caused by pepsin imbalances, aggressive stomach acid, and a compromised protective mucosal covering caused by the bacterium *H. pylori*. Chitosan and chitin are often used for wound dressings to help cut and burn wounds heal faster. Chitin and chitosan were expected to have some protective benefits because similar compounds used to repair skin ulcers were proven as beneficial at preventing stomach mucosal ulcers and injury. This is attributed to its propensity to dissolve easily in acid; low molecular weight chitosan may have been more effective. It has been proposed that the solubilized chitosan exerts its protective effect by coating the ulcerated area and neutralizing H+ and pepsin as a result of their antioxidant capacity in the gastric juices [7].

7.4 Pharmacokinetics

Because of the acidic environment of the stomach, the enzymes found in gastric juice and saliva, and the bacterial enzymes in the large intestine, it has been suggested that chitosan is partially digested and absorbed. It has been experimentally proven that the oral intake of chitosan (1 g/day), increases the concentration of N-acetyl D-glucosamine in serum [7, 90].

7.5 Adverse Effects and Toxicity

In general, chitosan is considered as a non-toxic material. In clinical studies, very few adverse effects are mild and transient like nausea, flatulence, throat irritation, and itching [7, 91].

8. Conclusion

Chitin and chitosan found many beneficial applications, especially in medical and pharmaceutical fields due to their outstanding properties. Marine crustaceans are the main industrial source of chitin and chitosan. However, microbial sources represent a non-seasonal and efficient route for the economic production of chitin and chitosan. They have advantages that made them suitable alternatives for the replacement of crustacean sources. Microbial chitin and chitosan need fewer purification efforts and allow conserved properties of the produced chitin and chitosan. Further investigations and searching for low-cost substrates for cultivation of the microbial sources of chitin and chitosan are needed to increase the “benefit/cost” ratio

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