FROM CHITIN TO CHITOSAN – A POTENTIAL NATURAL ANTIMICROBIAL AGENT

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Abstract

Chitin is a naturally occurring polymer. Together with its derivatives such as chitosan, it has a wide spectrum of application possibilities, and many properties not yet exploited. Chitosan possesses many features desirable in an ideal antimicrobial polymer. It shows activity against multidrug-resistant bacterial and fungal strains that pose a challenge to modern medicine. Chitosan also shows activity against certain viruses, such as SARS-CoV-2. It might be used as a drug or a vaccine delivery system, is biodegradable, bioavailable and considered safe for medical use.

It is important to continue exploring the potential of chitosan, as well as to investigate its sources. Indeed, many sources of this polymer are still not or have been poorly described. In this paper, we compile the current state of knowledge on the antimicrobial properties of chitosan, list alternative sources of chitin to highlight the potential of these two polymers and encourage further research.

***Keywords**: chitin extraction, chitosan, antimicrobial activity, sources of chitin*

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1. Introduction

Chitin (β-1-4-linked *N*-acetylglucosamine) is the second most abundant biopolymer on the planet. It has existed since the first fungi evolved, which was about 810-715 million years ago [1]. Its first extractions were performed independently by Hachett, Braconnot and Odier, at the turn of the nineteenth century [2], and it was first deacetylated in 1859 by Rouget. Since then, chitin and its derivatives have been of great interest to scientists. They are desirable biomaterials due to their properties like biocompatibility, biodegradability, low toxicity and high reactivity [3, 4]. Chitosan, one of the chitin derivatives, is already used in beverage production for microbial control, sulphite reduction and heavy metal trapping; as a diet supplement for weight loss; in cosmetics; and in wound dressings. However, there are many more possible applications that have not been implemented yet [5]. Since the beginning of the twenty-first century, the global population has experiences pandemics caused by SARS (2003), H5N1 (2003), H1N1 (2009), Cholera in Haiti (2010), MERS-CoV (2012) and Ebola in West Africa (2014). Currently, the world is struggling not only with a novel coronavirus, SARS-CoV-2 [6], but also with drug-resistant bacterial and fungal strains that can cause severe and dangerous infections [7, 8]. The development of new diseases might induce an increase in the number of medicaments in the environment. Chitosan constitutes a promising source for pharmaceuticals based on natural products. It is considered the most promising, new generation antimicrobial polymer [9]. Its antifungal, antibacterial and antiviral activities have attracted the attention of many scientists [10-13]. It shows inhibition against some multidrug-resistant fungi and bacteria, and might be useful to prevent biofilm development [14, 15]. It might also help in developing solutions for some currently challenging pathogenic viruses like SARS-CoV-2, MERS-CoV and HIV-1, for which there are limited or no effective medicines [16, 17].

In 1999, Muzzarelli [18] estimated chitin production in the biosphere to be 10^{10} gigatons per year; however, we are still learning about new, alternative sources of this polymer that, in some cases, have not been taken into account [19, 20]. Its source determines its properties and is an important criterion in choosing the chitin extraction method [21-25]. Although seafood waste and fungi constitute the main sources of chitin, and thus chitosan, there are many alternative sources of this polymer [26].

This review summarises the current state of knowledge of the chitosan manufacturing process, the characteristics of this polymer and its potential as an antimicrobial agent. It also highlights alternative sources of chitin, from which chitosan is derived. The aim of this review is to highlight the potential of chitosan as a natural polymer with antimicrobial activity, and to encourage continued research on this polymer, its preparation and application.

2. Chitin

Chitin is a high-molecular-weight polysaccharide that forms light, rigid and hardly soluble structures [27]. It contains reactive amide groups that define its cationic nature, high adsorption capacities and biological activity [28, 29]. It is made of chains that range from 1000 to 3000 units [30], arranged into sheets linked by N-H···O=C hydrogen bonds between amide and carbonyl groups. It has three polymorphic crystalline structures: α, β and γ.

α-chitin is characterised by antiparallel chains with strong inter-sheet and intra-sheet hydrogen bonding, alongside highest molecular weight and decomposition temperature compared with the other allomorphs. It is considered the most stable chitin form. On the contrary, β-chitin has parallel chains with only intra-sheet bonding and weak intermolecular forces. It is more flexible and reactive and has greater affinity for solvents than α-chitin.

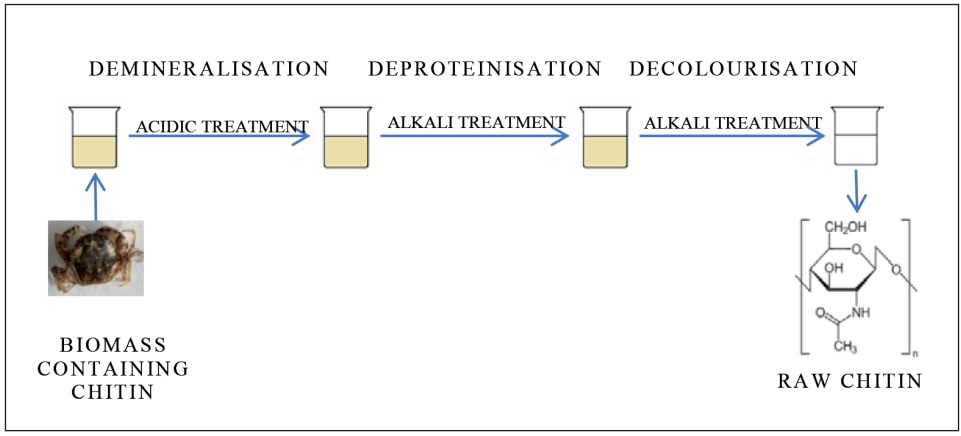

Figure 1. The process of chitin extraction

γ-chitin is the rarest form, characterised by one antiparallel chain for every three chains [31]. Chitin is mainly obtained from crustaceans [28, 21]. It usually occurs combined with proteins, minerals, lipids and pigments [32]. The process of chitin extraction usually consists of three steps: demineralisation, deproteinisation and, optionally, decolourisation (Figure 1).

Methods used to obtain chitin can be divided into chemical and biological [26]. In chemical extraction, to remove mineral salts like calcium carbonate or calcium phosphate, the sample is treated with a strong acid: hydrochloric acid (HCl), nitric acid (HNO_3), sulphuric acid (H_2SO_3), acetic acid (CH_3COOH) or formic acid (HCOOH). The next step, protein separation, is performed with a strong alkaline treatment, usually sodium hydroxide (NaOH) [2]. The most common chemical method of chitin extraction, used for industrial production, is performed with HCl, NaOH and a hydrogen peroxide (H_2O_2) treatment for purification [32, 33]. According to studies performed by Hoqani and colleagues [34] on shrimp waste, the optimal conditions for this method are: 3% HCl at 25°C for 1 h, 50% NaOH at 110°C for 3 h and then 30% H_2O_2 treatment for 3 h, for its decolourisation [34]. This method is considered economically viable and efficient, but on a commercial scale, it is not neutral for the environment. It consumes a lot of energy, and a huge amount of water, resulting in high carbon dioxide (CO_2) and wastewater production [26]. Moreover, the harsh reagents used in this method can negatively affect the properties of the extracted chitin [2]. These harsh reagents might be replaced with ionic liquids (ILs, organic salts with melting points below 100°C), like [C2mim]OAc (1-ethyl-methylimidazolium acetate). The use of ILs enables obtaining chitin with higher purity and molecular weight than that obtained during the industrial production [35, 36]. Other alternative reagents are natural deep eutectic solvents, called NADES [37-39]. NADES are a mixture of two or more primary metabolites that becomes liquid at room temperature [40]. They are considered safe for the environment and non-toxic [41]. The advantages of using NADES, like malic acid or choline chloride, is the short extraction time, efficiency and possibility to retrieve and to reuse them [39]. Another potential way to obtain chitin is electrochemically assisted extraction. This procedure, performed on black coral *Cirrhipathes* sp., lasts 12 h, requires fewer chemicals than other methods and results in pure, colourless chitin scaffolds in its original membranous formation [42]. Meanwhile, hybrid chitin extraction with use of dielectric barrier discharge (DBD) for deproteinisation

and lactic acid for demineralisation, performed on *Pandalus borealis* shells, allowed the removal of about 90% of protein, and almost all mineral content. The deproteinisation process lasted about 6 min, and the mineral content removal was performed for 15 or 30 min. The only waste produced in this process was gaseous products (CO_2N_2 and H_2CO) [43, 44].

The biological method of chitin extraction involves bacterial fermentation and allows reuse of the obtained proteins and minerals as nutrients for humans or animals [2, 32, 45]. Fermentation performed on *Penaeus vannamei* waste with *Lactobacillus rhamnoides* and *Bacillus amyloliquefaciens* yielded chitin comparable to commercially produced chitin. The demineralisation and deproteinisation efficiency were 97.5% and 96.8%, respectively. Furthermore, the broth resulting from fermentation is suggested to have a high nutritional value could be used as food for animals [45].

3. Chitosan

Chitin can be dissolved in some anhydrous carboxylic acids and concentrated mineral acids, like phosphoric acid. A research team led by Biniaś [46] developed a method for the fabrication of biomaterials from chitin with use of concentrated phosphoric acid; it yields chitin fibres and spheres. However, chitin deacetylated derivatives like chitosan (Figure 2) have more applications than chitin by itself [20]. Chitosan is more reactive and more soluble than chitin [30]. It might be modified in various ways and is considered a promising antitumour, antifungal, antibacterial, antioxidant and antiviral agent [10-13]. Chitosan is considered a relatively non-toxic and safe polymer [3]. To deacetylate chitin, it is treated with NaOH at a high temperature (90-140°C). The duration and applied temperature in this procedure affect the chitosan properties. However, there is also an enzymatic method that is more stable and does not affect the structure of the chitosan obtained [30].

Chitosan refers to a group of polymers comprising long chains of *N*-acetylglucosamine. It can be divided into four groups based on its deacetylation degree: low (55%-70%), medium (70%-85%), high (85%-95%) and ultrahigh (95%-100%) [47]. Its molecular weight varies between 50 and 2000 kDa. It is insoluble in water and alkaline solutions but it can be dissolved in almost all aqueous acids [11, 48]. Its activity depends on the position

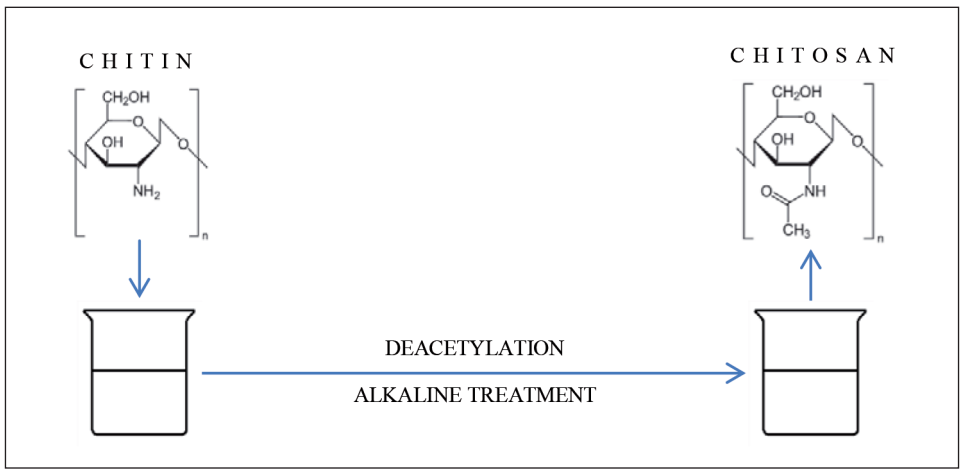

Figure 2. The process used toobtain chitosan

of sulphate groups in glucosamine residues [49]. The presence of polar groups provides good hygroscopicity, moisture retention [50] and electrostatic attraction that provides its mucoadhesion [51]. The positively charged amino groups allow chitosan to bind negatively charged surfaces. It can bind to heavy metals, and its sorption ability depends on its form and temperature [52]. Chitosan also reacts with aldehydes and acyl chlorides, and it can be also modified via hydrolysis or sulfation [11]. Its degradation products also show biological activities [53]. These features present the possibility to create chitosan derivatives with a broad spectrum of biological activities.

4. Antimicrobial Activity of Chitosan

There are many factors that can affect chitosan's antimicrobial activity, like conditions of the chitin extraction method, which impact its molecular weight and deacetylation degree, conditions and time of chitosan storage, experimental conditions and, finally, the type of the target [9, 12, 54, 55].

4.1. Antibacterial Activity of Chitosan

There are a few proposed mechanisms of chitosan's antibacterial activity. One of them relies on the electrostatic interaction of chitosan with bacterial cell wall. The positively charged chitosan interacts with negatively charged bacterial cell wall components, causing disruption of the cell wall and its leakage. This mechanism has been observed during assays performed with chitosan acetate solutions against *Escherichia coli* and *Staphylococcus aureus* [56], and during studies on cationic chitosan derivatives such as N-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (HTCC) performed on drug resistant *Klebsiella pneumoniae*, *S. aureus* and *Enterococcus faecium* [57]. The other mechanism concerns the ability of low-molecular-weight chitosan to penetrate the bacterial membrane, combine with bacterial DNA and inhibit messenger RNA (mRNA) synthesis. Meanwhile, high-molecular-weight chitosan inhibits the growth of aerobic bacteria. It forms a dense film on the cell surface and prevents the uptake of nutrients and oxygen [9, 54]. Chitosan chelates ions like Mg^{2+} and Ca^{2+}, which maintain enzymatic functions and integrity of the cell membrane [9, 58]. The chitosan antibacterial activity has also been observed against *K. pneumoniae* and *S. aureus*, which are drug resistant and cause severe infections [8, 59, 60]. There are also studies showing that chitosan ascorbate exhibits antibacterial activity, most notably in terms of inhibition of *Corynebacterium matruchotii* and *Aggregatibacter actinomycetemcomitans* [61]. Furthermore, studies on graphene/chitosan nanocomposites have shown its inhibitory activity against *Pseudomonas aeruginosa* and *K. pneumoniae* biofilm development, which is a favourable factor involved in bacterial drug resistance [14, 62]. Chitosan might also be useful for wound healing. Besides its antimicrobial activity, it helps in collagen deposition, induces hyaluronic acid synthesis at the wound site and activates macrophages to prevent tissue overgrowth [63]. Nanosystems based on hyaluronic acid and chitosan are considered a promising therapeutic agents in wound healing [58]. Chitosan is already used in various forms as a wound dressing [9].

4.2. Antifungal Activity of Chitosan

The fungal cell wall is composed mainly of glucan, mannan and chitin. The structure of the cell wall is unique for each species [64]. The majority of studies on fungal chitosan have focused on its activity against fungi considered to be pests, because they cause substantial damages to crops, and the pesticides currently in use are a threat to the environment, especially when their use is poorly managed [65]. Chitosan can stimulate the chitinase activity of plants and is considered to be very effective against spore

germination, germ tube elongation and radial growth of fungi [66]. There have also been a few studies concerning the activity of chitosan against fungal strains that are pathogenic to humans. Some fungal infections might cause systemic infections and are becoming a major problem [7]. Furthermore, some of them are resistant to drugs [67]. It is difficult to fight pathogenic fungi because the same drug used to fight fungal pathogens might be toxic to the host. Chitosan nanoparticles can cause morphological, structural and molecular changes in fungal cells [68, 69]. The assays performed on fungal strains of *Candida* sp. and *Cryptococcus* sp. with HTCC showed its inhibitory activity, with low cytotoxicity against human erythrocytes and mammalian cells. It disrupted the integrity of fungal cell membrane, causing its death [56].

4.3. Antiviral Activity of Chitosan

Viral particles consist of nucleic acid genome (RNA or DNA), a capsid (a protein coat) and in some cases an envelope (lipid membrane). Due to the lack of the elements crucial for growth and replication, viruses rely on living cells to provide all of the enzyme systems, energy, ribosomes and molecular building blocks needed for replication [70]. Chitosan can cause structural changes in viral particles and damage their integrity or bind to viral receptor proteins [71]. Some chitosan sulfonic acid derivatives show inhibitory activity against the replication of retroviruses and members of the *Herpesviridae* family [72]. Sulphated chitosan derivatives – (1-4)-2-deoxy-2-sulfoamido-3-O-sulfo-β-d-glucopyranan – show potent and specific inhibition against HIV-1 infection. They block the interaction between gp120 viral protein and its cellular receptor [16]. Modified chitosan is also considered a potential agent against SARS-CoV-2, the novel coronavirus responsible for the current global COVID-19 pandemic. The HTCC and hydrophobically modified HTCC showed activity against HCoV-NL-63 and murine hepatitis virus (both from coronavirus family) [73]. It interacts with the coronaviral spike protein (S) of HCoV-NL63, blocking its interaction with its cellular receptor angiotensin-converting enzyme 2 (ACE-2) [73, 74]. Experiments performed *in vitro* and *in vivo* with HTCC showed successful inhibition of SARS-CoV-2 and MERS-CoV replication [17]. Moreover, chitosan nanoparticles can synergise curcumin activity against HCV-4 replication and entry into a hepatic cells [75]. Chitosan might also be used for pulmonary delivery of drugs and for vaccine delivery system [49, 76, 77]. It is degraded by lysozymes into non-toxic products and releases drug at a slow rate. Chitosan can absorb proteins, amino acids and nucleic acids via complexation and ion exchange [50]. It addition, all positively charged chitosan species shows mucoadhesive properties, and their hydrophobic derivatives aggregate between cells in a pattern that allows for local drug release and improves its biodegradation [78].

5. Sources of Chitin

The properties and features of chitin depend on its source; however, the ecology or even the gender of the investigated specimens also might be relevant [22]. It might differ in structure, molecular weight and deacetylation degree, which affects the solubility and chemical reactivity [79]. The main source of chitin used for industrial production is seafood waste, not only because of its availability, but also because it is important from the environmental point of view to recycle waste [80-84]. Yet, there are many other sources of this polymer in the biosphere [18, 85].

Fungi from class Zygomycetes have a cell wall mostly composed of chitin. It is considered a promising source of chitin [86, 87]. This chitin source does not require the harsh chemical treatment, does not need to be demineralised, the material for extraction is available all year long (fermenting plants produce tons of fungal mycelia waste) and it is not contaminated with heavy metals [9, 87]. Extracted chitosan polymers

might be bound to glucan and inorganic compounds, but it is possible to purify them during an additional step of acid treatment [88]. Nwe et al. [87] showed that fungal chitosan stimulates plant growth better than chitosan obtained from shrimp and might be an excellent scaffolding material for construction of a template for tissue regeneration [87]. The deacetylation degree of chitosan extracted from *Absidia coerulea*, *Rhizopus microsporus* var. *oligosporus*, and *Mucor circinelloides* is apparently higher than the deacetylation degree of standard crustacean chitosan [25].

The research performed on mushrooms has mostly focused on their nutritional values, but some researchers have confirmed the presence of chitin in their cell wall [23, 89-93]. Research on two mushroom species, *Lactarius vellereus* and *Phyllophora ribis*, showed that the chitin content in both cases is higher than in some insect species. The chitin obtained from these mushrooms might be useful as an antimicrobial and antioxidant agent. Interestingly, chitin obtained from the aforementioned mushrooms, in addition to *Lentinula edodes*, has a structure with no nanofibres or nanopores [23, 94].

The first evidence of chitin in skeletal fibres of marine sponges was published in 2007 [95]. The three-dimensional structure of this chitin makes it an promising candidate for application in biomedicine [96]. Mutsenko and colleagues [97] suggest that chitinous scaffolds derived from the demosponge *Aplysina aerophoba* might be an attractive model for further human mesenchymal stromal cell (hMSC)-based tissue engineering. The *in vitro* studies showed it supports adhesion, viability, growth and proliferation of hMSCs [97].

Chitin is also abundant in the cuticle of insects. It occurs in complexes with proteins and it is localised in the cuticle proper [98]. Chitin obtained from the dragonflies *Sympetrum fonscolombii* eye was characterised as α-chitin fibrils varying in length, and with a high crystallinity index [99]. Cockroach (*Periplaneta americana*) wings are made with α-chitin that has a nanoporous structure, yet it has no microfibres. Moreover, it has a different structure than chitin obtained from other body parts of cockroaches [100]. α-Chitin was also isolated from beetle larva cuticle and silkworm (*Bombyx mori*) pupa exuvia [101]. Whereas most studies have reported α- or β-chitin forms, chitin obtained from cocoon of the moth *Orgyia dubia* represents the γ-form [102]. Research has also been performed using honeybees (*Apis mellifera*), as their corpses constitute an apiculture waste. While the chitin content was estimated as 8.8%, honeybee bodies are also considered a potential source of proteins and lipids [103]. Researchers have also focused on a host of other insects as a source of chitin: bumblebees *Bombus terrestris* [104]; cicadas [105]; black soldier flies [106]; Coleoptera species *Lucanus cervus* and *Polyphylla fullo*; Orthoptera species *Bradyporus (Callimenus) sureyai* and *Gryllotalpa gryllotalpa* [107]; grasshoppers [108]; larvae and adults of Colorado potato beetles (*Leptinotarsa decemlineata*) [109]; superworm (*Zophobas morio*) larvae [110]; and an invasive Asian hornet *Vespa velutina*, which is suggested to be a promising alternative source of chitin [111].

Spiders like *Geolycosa vultuosa* or *Hogna radiata* might constitute another source of chitin [112]. Both species contain α-chitin. It has been estimated that spiders could produce 2-6 million tons of cuticle per year globally. Their cuticle (in most cases) does not contain mineral phases; hence, the demineralisation step could be skipped during extraction. The isolated chitin from *Caribena versicolor* is almost 'ready-to-use'. The structure is tubular and porous, and it is suggested that it might be used in tissue engineering [113].

Crustaceans such as lobsters, krill and crayfish might also be an important source of chitin because their shells constitute a significant part of crustal wastes [32, 114, 115]. The α-chitin extracted from the lobster *Homarus americanus* has a strong, fibrous crystalline structure [116]. The data regarding crustaceans that are not commercially

fished are very limited. However, gammarids they produce α-chitin. The chitin content of *Gammarus argaeus* species is about 11%-12%. It has nanofibrils and pores [117].

In nudibranch from the eolid group, chitin occurs in three different organs: radula teeth, cuticle of the head alimentary tract and in intracellular granules. These chitinous granules are placed in epidermal cells of the skin and in gut epithelium and serve as self-defence [118]. *Conus inscriptus* has a shell consisting of α-chitin with a lower molecular weight than commercial chitin. It has regularly arranged dense pores with irregularly arranged dense nanofibres and a low molecular weight. It could perhaps be used for tissue engineering, textiles or wound dressing [24].

Chitin has also been extracted from shell and operculum of *Nerita (Dostia) crepidula* [119] and the prosmatic layer of the shell of the Japanese pearl oyster *Pinctada fucata* [120], and has also been found in shells of *Tympanotonus fusatus* and *Lissachatina filica* [121]. There were also studies performed on chitons, a group of molluscs. It is suggested, that the chiton chitosan has higher antioxidant activity than commercially available chitosan [122].

The literature about chitin in bryozoans is very limited. However, research conducted by Kaya and colleagues [123] revealed that *Plumatella repens* contains chitin composed of nanofibres with nanopores. Furthermore, the chitin content in dry mass of the bryozoan was higher than in comparatively studied species of insect (*Palomena prasina*) and fungus (*Fomes fomentarius*) [123].

Barnacles (*Austromegabalanus psittacus* and *Chelonibia patula*) produce hard shells consisting of parallel α-chitin fibres [124, 125]. Interestingly, studies conducted by Kaya and colleagues [125] showed that chitin contained in *C. patula* shells can be obtained in a shorter time compared with common chitin sources like shrimp, crayfish or crab.

Squids bone, called gladius or pen, contain β-chitin. The pen of *Loligo chinensis* has a higher chitin content than in shrimp and crab shells, and a low mineral content. Hence, demineralisation of chitin obtained from this species could likely be skipped [126]. Chitosan obtained from a commercially fished *Loligo vulgaris* is considered an effective antioxidant and antimicrobial agent [127]. β-Chitin has also obtained from a squid *Uroteuthis duvauceli*, and the cuttlefish species *Sepia prashadi* [128, 129] and *Sepia officinalis* [79]. Greven and colleagues [130] isolated α-chitin from cuticle of velvet worms, *Peripatoides novazealandia*. Its structure has no nanopores, yet shows distinct nanofibres. Chitin is also found in fish scales [20, 131]. Obtaining chitin from fish scales could solve the problem of fish waste utilisation. α-Chitin was recently isolated from a fast growing plant *Leucaena leucocephala* pods [19]. This tree was originally found in Central America and Mexico and has spread throughout the world including Asia and Malysia [132].

6. Conclusions

There are a few methods of chitin extraction. Considering the impact on the environment and time of extraction, as well as the purity of the obtained polymer, the electrochemically assisted methods and the ones performed with NADES seem to be worth further examination and, if possible, application in industrial chitin production. Selection of the method and its conditions depends on the source of chitin, and determines its quality as well as its reactivity. Chitosan is a promising biomaterial as an antimicrobial agent. It has many desirable features like reactivity and low toxicity. It can be used as antimicrobial agent alone or as an enhancer for other molecules with biological activity. It may be used in medicine to create safer drugs and more effective vaccines. It shows activity against bacterial, fungal and viral pathogens with which modern medicine is currently struggling. Chitosan constitutes an attractive replacement for current drugs not only due to its

biological activity, but also because of its biodegradability and bioavailability. It is mainly obtained from crustaceans, and it creates a great opportunity to reduce seafood wastes residing in the environment. However, there are many other organisms producing chitin, and they might constitute an attractive source of this polymer due to ease of cultivation, amount of produced chitin per year, its quality and properties, or because they may require less harsh and less time-consuming methods of its extraction. It seems that besides seafood waste and fungi, insects and spiders may also be a promising source of chitin. Hence, it might be worth studying them more thoroughly.

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