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Review

Advanced technologies for chitin recovery from crustacean waste

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Abstract: Chitin is the second most plentiful natural biomass after cellulose, with a yearly production of about 1×10^{10}–1×10^{12} tonnes. It can be obtained mainly from sea crustaceans' shells, containing 15–40% chitin. Full or partial deacetylation of chitin generates chitosan. Chitin and chitosan are used in several industrial sectors, as they exhibit high biocompatibility, biodegradability and several biological functions (e.g., antioxidant, antimicrobial and antitumoral activities). These biopolymers' market trends are destined to grow in the coming years, confirming their relevance. As a result, low-cost and industrial-scale production is the main challenge. Scientific literature reports two major technologies for chitin and chitosan recovery from crustacean waste: chemical and biological methods. The chemical treatment can be performed using conventional solvents, typically strong acid and alkaline solutions, or alternative green solvents, such as deep eutectic solvents (DESs) and natural deep eutectic solvents (NADESs). Biological methods use enzymatic or fermentation processes. For each route, this paper reviews the advantages and drawbacks in terms of environmental and economic sustainability. The conventional chemical method is still the most used but results in high environmental impacts. Green chemical methods by DESs and NADESs use low-toxic and biodegradable solvents but require high temperatures and long reaction times. Biological methods are eco-friendly but have limitations in the upscaling process, and are affected by high costs and long reaction times. This review focuses on the methodologies available to isolate chitin from crustaceans, providing a comprehensive overview. At the same time, it examines the chemical, biological and functional properties of chitin and its derivative, along with their most common applications. Consequently, this work represents a valuable knowledge tool for selecting and developing the most suitable and effective technologies to produce chitin and its derivatives.

Keywords: chitin; biopolymer; recovery technology; deep eutectic solvents; fermentation; proteases

1. Introduction

Chitin and chitosan are polysaccharides of great commercial interest, with an annual production of about 1×10^{10}–1×10^{12} tonnes [1]. The global market for chitin and chitosan derivatives, valued at USD 3.8 billion in 2020, is estimated to reach USD 12.3 billion by 2027, growing at a compounded annual growth rate (CAGR) of 18.4% over the forecast period 2020–2027 [2].

Chitin and chitosan industrial applications will grow further, thanks to their unique properties. They have high biodegradability and biocompatibility, and low levels of toxicity and allergenicity [3]. Furthermore, these polymers perform many biological activities, including anti-inflammatory, antioxidant, antimicrobial, anticancer and hypocholesterolemic [4].

Chitin and chitosan are copolymers of N-acetyl-D-glucosamine units and D-glucosamine, respectively. Their properties are influenced by their degree of acetylation (DA), degree of deacetylation (DDA) and molecular weight [5].

Chitin is the second most plentiful macromolecule after cellulose. It was first isolated in 1811 from some of the higher fungi by Henri Braconnot, and successively in 1823 from insects' cuticles (May bug) by Antoine Odier, who called it chitin, derived from the Greek word *chiton* meaning tunic or envelope [6].

Naturally produced chitin is found in numerous species belonging to different phyla, most of which are aquatic (Cnidaria, Entoprocta, Phoronida, Ectoprocta, Brachiopoda, Bryozoa, Algae, Porifera and Mollusca), subaerial (fungi and yeasts, Onychophora) and ubiquitous (protozoa, arthropods). Of all these, the most easily accessible chitin source is the exoskeletons of Arthropods, which includes insects, arachnids (spiders and scorpions), myriapods (millipedes) and crustaceans (shrimp, krill, crabs and lobsters) [7].

Chitosan, a derivative of chitin, occurs naturally only in some mushrooms possessing the deacetylase enzyme capable of converting the chitin synthesized in their cell walls. A similar mechanism has been observed in some bacteria [8].

Chitin exists in nature in three different allomorphs (alfa, beta and gamma). In crustaceans and arthropods with rigid exoskeletons, as well as other aquatic invertebrates, chitin occurs in the alpha form. The isolation capacity of chitin from different natural sources depends on the source and the percentage of chitin present. In the same way, crystallinity, purity and polymer chain arrangement vary consistently, depending on the chitin origin [9,10].

For these reasons, commercial recovery of chitin is mostly accomplished from crustaceans, which grant the best traits mentioned above, although new alternatives, such as fungi and insects, continue to gain attention [11,12].

According to data provided by FAO, crustaceans from fisheries and aquaculture in 2018 were equal to 6.0 and 9.4 million tonnes, respectively [13]. Only about 40% of the crustaceans caught consist of edible parts, while the remaining 60% are not edible. This latter consists of 15–40% chitin [14]. Thus, it is easy to understand the importance of valorizing this large biomass, and recovering as much as possible from it [7]. Underusing crustacean by-products leads to loss of potential profits and requires payment of disposal costs. The industry has started to develop

technologies to use this waste to produce compounds with high-added value, mainly chitin, from which to obtain chitosan and other chitin derivatives [15].

Chitin and chitosan find applications in various sectors, from the primary production sector to the pharmaceutical and medical ones, thanks to their remarkable properties and multi-functionality. Figure 1 illustrates the main areas of industrial application of chitin and chitosan.

Figure 1. Main areas of industrial application of chitin and chitosan.

All these applications explain why these polysaccharides' global market size is increasingly growing [2]. Chitin is present in many biological matrices, but marine crustacean waste is still the predominant source for producing chitin [16,17].

Chitin extraction can be obtained according to a chemical or biological route. The chemical route generally involves the use of strong acids and alkaline solutions, while the biological one can follow fermentative or enzymatic processes [1,18]. Several studies have attempted to find more performing and sustainable alternative chemical treatments [19]. The use of deep eutectic solvents (DESs) and natural deep eutectic solvents (NADESs) is among them [20]. Among the biological methods, those that use fermentation processes, with lactic acid-producing or non-lactic acid-producing bacteria, are growing in importance. The enzymatic approach using proteases is a biological alternative to alkaline treatment for crustacean shell deproteinization. Both chemical and biological approaches have advantages and disadvantages [21]. Combining both methods can make the chitin extraction process more efficient [1].

This review focuses on advanced technologies to recover chitin from crustacean waste, analyzing the strengths and weaknesses of different recovery approaches. This review also explores the physicochemical and biological properties and industrial applications of chitin and chitosan. Thus, the simultaneous analysis of the properties, potential use and recovery technologies of chitin provides a comprehensive and helpful all-around overview of this topic of growing interest in different industrial sectors.

2. Chitin and chitosan properties

2.1. Physical and chemical properties

Chitin is the second most abundant polysaccharide in nature after cellulose. It is a primary component of the arthropod exoskeleton, but chitin also occurs in fungi and algae cell walls, and the cartilage of mollusks [16,17,22]. This complex polysaccharide is present primarily as a scaffold interconnected with minerals, such as calcium carbonate and phosphate, and organic matter, such as proteins, lipids and pigments. Chitin consists of N-acetyl-D-glucosamine (GlcNAc) monomers linked together via β-(1-4)-glycosidic bonds. Each sugar unit in the chain is rotated 180° with respect to its adjacent unit. The structure of chitin is similar to cellulose. The difference is due to the replacement of a hydroxyl group for an acetamido group on each monomer [3]. This substitution leads to increased intermolecular hydrogen bonding between adjacent sugar units, thus conferring higher strength to the polymer chains (Figure 2).

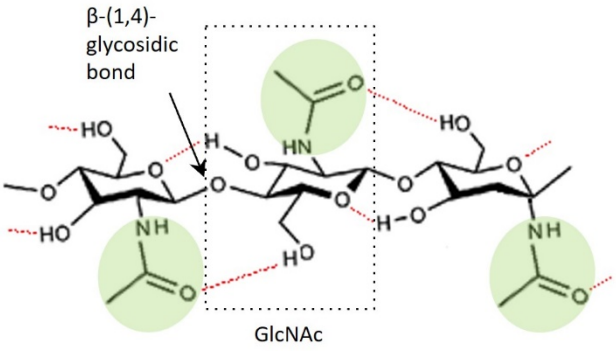

Figure 2. Chitin structure. GlcNAc monomers are linked via β-(1-4)-glycosidic bonds. Acetamido groups (in the green clouds) give rise to strong inter-chains hydrogen bonds (red dotted lines).

Chitin is insoluble in aqueous solutions due to its rigid crystalline structure. Acetamido groups are also responsible for strong intramolecular hydrogen bonds between parallel polymer chains, which, in turn, are arranged into microfibrils [23]. Chitin can exist in its α-, β- and γ-chitin allomorphs, which differ in the polymer chain orientation within the structure of the micro-fibrils. The polymer chains are antiparallel in α-chitin, whereas they are parallel in β-chitin. A mixture of parallel and antiparallel sugar chains is present in γ-chitin [9,24]. α-chitin allomorph is the most abundant native chitin, and is mainly present in the arthropod cuticles [9]. β-Chitin is present in the extracellular filaments of diatoms, in the skeletal structures of cephalopods and in tubular structures

of marine annelid worms of the family Siboglinidae [10]. The parallel polymeric chains of β-chitin and, to a lesser extent, γ-chitin have weaker intramolecular interactions. This chemical-physical characteristic leads to structural flexibility, and allows greater accessibility for enzymes [23]. In contrast, α-chitin is typical of hard and rigid structures [25]. Figure 3 shows intra-chains hydrogen bonds that link strongly the α-chitin layers.

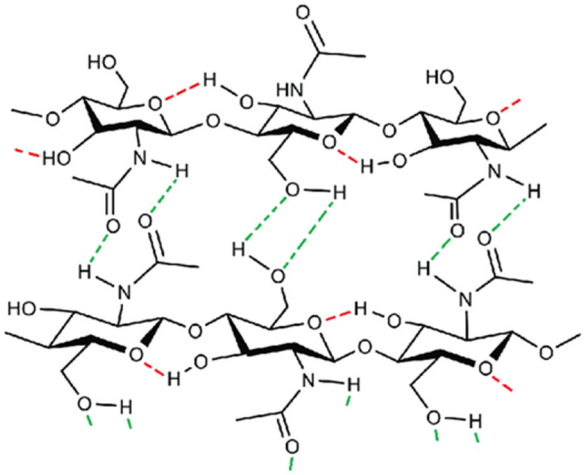

Figure 3. Layers of α-chitin connected strongly via intra-chain hydrogen bonds (green dotted lines). Inter-chain hydrogen bonds are also present (red dotted lines). Adapted from [26].

In nature, chitin exists as a copolymer consisting of GlcNAc and GlcN subunits with a DA higher than 50% [1]. Full or partial deacetylation of chitin gives rise to chitosan (Figure 4). This biopolymer is characterized by a DDA ranging between 13% and 40%, and a molecular weight of 10^5–10^6 Da [27]. Chitosan is also present in nature, for example, in fungal cell walls [17].

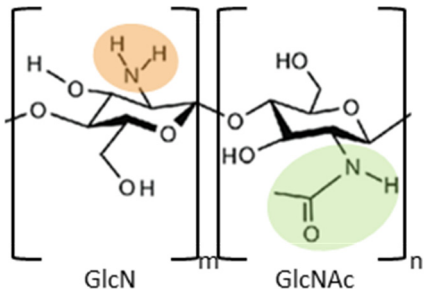

Figure 4. Chitosan structure. Amino groups of the GlcN monomers are in the orange clouds, and acetamido groups of the GlcNAc monomers are in the green clouds.

Acetyl groups enhance inter-fibril connection in the fibril bundle of chitin and chitosan. DA affects the flexibility of the fibril bundles and, consequently, the mechanical strength of chitin or chitosan-based products [28].

Chitosan is soluble in protonated water (pH < 6), becoming a natural cationic biopolymer. The positive charge gives it characteristics which explain its versatile uses based on its antimicrobial, coating and gelling properties [29]. Chitosan cationic form can make complexes with negatively charged molecules, including proteins, enzymes, cancer cells, cell wall proteins of bacteria, DNA and RNA. At alkaline pH, hydroxyl groups of chitosan's D-glucosamine units are negatively charged, and are, thus, able to bind various metal ions [29]. In addition, chitosan's primary amino and hydroxyl groups provide it with crucial reactive sites to easily convert it into other derivatives, including quaternary ammonium chitosan salts, carboxymethyl-chitosans, carboxyalkyl-chitosans, hydroxyalkyl-chitosans, phosphorylated chitosan and succinyl-chitosan [25].

Molecular weight, DDA and degree of polymerization are the principal factors affecting the physical properties of chitosan and, consequently, its biological functions [30]. Chitosan with higher DDA is more efficient as an antimicrobial agent [31], whereas mixed chitosan with a wide molecular weight range and adequate DDA can have good hemostatic properties [32].

Chitosan oligomers, also known as chitooligosaccharides (COSs), deserve particular attention, due to their numerous potentials in the pharmaceutical and medical sectors. COSs are characterized by a low degree of polymerization (<20) and a low molecular weight (<3.9 kDa). These physical characteristics make COSs highly soluble, biocompatible and capable of promoting numerous positive biological effects [33].

2.2. Biological properties and effects

Because of their chemical and physical characteristics, chitin and chitosan have considerable and promising biological effects (Figure 5) [34].

Figure 5. Biological effects of chitin and chitosan.

Few studies focus on the biological effects of chitin alone. Most scientific articles also concern the multifunctionality of chitosan and COSs. Chitin derivatives have recognized positive effects on human health, and have a crucial role in various biological mechanisms. For example, chitin has a high antimicrobial capacity, mainly carried out through cell lysis resulting in disruption of the cell membrane of the target organism. On the other hand, chitosan can chelate heavy metals, even when present in trace amounts [35].

The mammalian immune system readily recognizes chitin molecules since mammals are unable to synthesize such polymers. Recognition occurs through specific membrane receptors, thus allowing the immune system to recognize chitin-expressing pathogens, inducing the secretion of IL-17A and TNF-α by macrophages [35]. In addition, COSs have an immunomodulatory effect that depends on DDA. Consequently, COSs can promote the proliferation of immune cells that induce cytokine secretion and enhance the phagocytic activity of macrophages [36].

Because of its specific structure, chitin molecules can resist attack by pancreatic juice, bile and urine. In addition, chitin derivatives have good drug-carrying ability. All this makes chitin very useful in clinical practice [35].

Chitin derivatives, but especially COSs, inhibit angiotensin-converting enzymes, playing a relevant role in regulating hypertension [37].

As reported, chitosan has considerable biological effects, among which the ability to affect lipid absorption in the gastrointestinal tract, decrease C-reactive protein and increase serum leptin concentrations [35].

The antimicrobial activity of chitin derivatives against yeasts, filamentous fungi and bacteria is well known [38]. Chitosan is active against many Gram-positive and Gram-negative bacteria, and some pathogenic bacteria, such as *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Streptococcus* sp., S*taphylococcus aureus* and *Listeria monocytogenes* [39]. The mechanism of antimicrobial action of chitosan is still unclear, although this activity appears to depend on the degree of acetylation. The two most likely, and therefore most studied, mechanisms are the reduction of the permeability of the microorganism's cell membrane to nutrients (chitosan would then "starve" its target) and the blockade of bacterial RNA transcription [4]. In the first case, chitosan's free amino groups, which have a high ability to chelate metal ions present on the bacteria's cell surface, encase them in complexes, thus disrupting nutrient supply and preventing bacterial cell growth [39]. In the second case, due to its low molecular weight, chitosan can enter the bacterial cell, bind to DNA and interfere with its replication and expression, greatly altering the bacteria's intracellular metabolism. These mechanisms also explain the antifungal activity of chitosan and COSs [39]. Chitosan is poorly soluble at a pH greater than 6.5, which forces the creation of acid conditions that, in some cases, can be a strict limitation. Some studies involve synthetic derivatives of chitosan that instead act at neutral pH and are water soluble [4].

Several attempts have been made to combine chitosan with other polymers, such as hyaluronic acid, pectin, and carrageenan, and this seems to increase its antimicrobial activity under certain pH conditions [40]. In dealing with antimicrobial activity, it is important to emphasize what happens in the human intestine where there are trillions of microorganisms, most of which are bacteria. Many studies show that COSs act as prebiotics against such bacteria, although this effect decreases with DA and increases with the degree of polymerization [39].

In addition to antibacterial and antifungal effects, chitosan and COSs also show antiviral capacity, especially as adjuvant to HIV drugs [41]. A recent study shows *in vitro* antiviral effects of low molecular weight COS against SARS-COV-2 infected cells in a dose-dependent manner [42].

The antimicrobial activity of chitosan and COSs is lower than that of traditional antibiotics and antifungals. However, these bio-compounds remain promising and noteworthy molecules as they are highly biocompatible, and can be used in various fields [39,40,43,44].

The antimicrobial activity of these polymers occurs mainly through the membrane disruption of the cell bacteria. This mechanism of action makes them attractive as an alternative or adjunctive option to current antibiotics. Using chitin derivatives makes less likely the emergence of resistance occurring after prolonged use of conventional antibacterial, which are usually specific to a single target organism [40]. On the other hand, some studies state that bacteria can grow after the removal of chitosan from the culture medium; this suggests the emergence of a resistance mechanism like that which develops for antibiotics [4]. Further studies are needed to clarify this possibility, but certainly, bioactive polymers like chitosan are a promising alternative.

A very interesting activity of chitosan and its derivatives is antioxidant activity. It has been shown that the antioxidant activity of chitosan is most effective on low molecular weight molecules and appears inversely proportional to the degree of acetylation of chitosan [4]. The antioxidant activity of chitosan and COSs, moreover, depends, at least in part, on the degree of polymerization or molecular weight [39]. For human health, molecules with antioxidant activity are crucial. There is a strong correlation between oxidative stress and many diseases, including cancer, amyotrophic lateral sclerosis and neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, Huntington's disease) [4]. These pathologies are closely related to oxidative damage caused by free radicals. Although free radicals have beneficial effects on the body if present in small amounts, their excessive presence causes denaturation in biological macromolecules with serious effects on the integrity of cells that become apoptotic. Since the body's ability to produce antioxidant molecules decreases with age, it is essential to find and administer molecules with antioxidant activity from the outside. Chitin and its derivatives (chitosan and COSs) are excellent natural antioxidants [39].

COSs also appear to have effective neuroprotective activity. The main neuropathological features of Alzheimer's disease are the presence of intracellular neurofibrillary tangles and extracellular amyloid plaques, the latter due to the accumulation of β-amyloid (Aβ). Some studies report that COSs can significantly reduce Aβ-induced cell apoptosis and destabilize Aβ fibrils [45]. Specifically, COSs would suppress the expression and enzymatic activity of the β-site enzyme 1 (BACE1) of the amyloid precursor protein. COSs would also exert their role as neuroprotectors through inhibition of oxidative stress and neuroinflammation, nerve regeneration and promoting nerve regeneration [36].

When tissue damage occurs in the human body, the organism responds physiologically with inflammation, strongly linked to the generation of free radicals. Plasma proteins and circulating leukocytes are directed to the damaged site to initiate the healing process. If this defense process fails or the inflammatory process is directed against the organism itself, the damage can be severe, ranging from metabolic disorders (e.g., atherosclerosis), tissue damages (e.g., rheumatoid arthritis), and even tumor formation [39]. Various studies show that chitosan and its derivatives promote the inflammatory response both *in vivo* and *in vitro*. The process may vary depending on molecular weight and DA [4]. COSs appear to reduce neutrophil infiltration into organs and TNF-α and IL-1β in serum, as well as pro-inflammatory cytokines. Their marked anti-inflammatory activity makes

COS effective nutraceuticals against diabetes and obesity. They are also particularly effective in preventing and treating colitis-associated colorectal cancer and probably other cancer diseases [39].

The glycoprotein YKL-40 (also called HC-gp39) is a biomarker of inflammation. Biochemically it is an inactive chitinase of the chitin proteinase 18 family. It has been shown that it stimulates chondrocyte proliferation when it binds partially acetylated N-acetylglucosamine. For this reason, it is used in the treatment of inflammatory rheumatoid diseases. The highly deacetylated chitosan reduces the secretion of YKL-40, and, therefore, promotes the inflammatory response [46,47].

The possibility that COSs reduce cancer cell growth is not new. This ability has been attributed to the cationic nature and molecular weight of COSs. More recent studies show that antitumor activity occurs with highly charged COSs (regardless of whether the charge is positive or negative). Other studies show that some chitosan polymers are more effective than others in antitumor activity (for example, chitohexose against A549 cells). Chitin and chitosan, at concentrations of 1500 μg/mL or higher, can inhibit 100% of THP-1 cancer cell growth. Again, low molecular weight chitin at the concentration of 250 μg/mL shows the same effect, suggesting that the inhibitory effect for chitin is inversely proportional to molecular weight. Finally, modified COSs, synthesized by replacing the hydroxyl groups with the aminoethyl group at the C-6 position, appear to prevent cell invasion associated with metastasis [47].

Studies of the effects of chitin on cardiovascular disease have been conducted mainly by binding chitin to other substances, and the results have been very promising. For example, chitin with glucan and pomegranate polyphenols seems to be able to counteract endothelial dysfunction, instead combined with graphene oxide as aerogel beads effectively absorb excess bilirubin in the blood [48].

On the other hand, chitosan, taken orally to the extent of 5% in the diet, can reduce serum cholesterol and inhibit atherogenesis. Specifically, chitosan reduces serum cholesterol by promoting its accumulation in the liver, bile, and faeces through the reverse cholesterol transport pathway [49].

The chemical-physical characteristics and biological effects of chitin, chitosan, and COSs makes these compounds usable in various fields, as will be extensively discussed in the next section.

3. Industrial application of chitin and its derivatives

Chitin and, especially, chitosan are biopolymers widely used in various sectors, such as medicine, agriculture, cosmetics and industrial and non-industrial applications. Their growing interest is also in other more innovative fields, such as the packaging sector, aquaculture, functional textiles, water purification, paper industry, textile industry, cosmetotextiles, beverage industry and more. All these are thanks to their remarkable biological versatility, biocompatibility, biodegradability and low toxicity [22].

Chitosan, for example, can be used as a non-toxic, biodegradable alternative to plastic. Some researchers have successfully developed a plastic-like material made of chitosan, making products such as bottles, toys and components for consumer electronic devices [50]. Another application of chitin and chitosan is in self-healing protective coatings and varnishes. Atay et al. demonstrated a chitosan-reinforced epoxy dye composite coating with self-healing properties [51]. Table 1 reports some of the applications of chitosan in the various areas of interest.

Table 1. Main applications of chitosan. Adapted from [52].

Area	Applications	Form
Food industry	Additives for human and animal	Solution
	Antibacterial, antifungal, antioxidants	Film
	Edible films	Blend
	Diet foods and dietary fibres	Coating
	Hypolipidemic and hypocholesterolemic activities	Bead
	Astringency	
	Prebiotics	
	Infant feed ingredient	
Beverage industry	Filtration and clarification of fruit juices and beverages	Solution
	Natural flocculant	Particle
	Acidity-adjusting agent	Bead
	Colour stabiliser	
	Antimicrobial agent	
	Natural flavour extender	
	Bioactive compounds encapsulation	
	Preservatives and active packaging	
	Complexing agents in wine industry	
	Removal of dead yeast, excess tannin, particulates	
	Water purification	
Environment	Adsorbent/biosorbent	Powder
	Coagulant/flocculant	Gel/hydrogel
	Antifouling agent	Bead
	Interactions with proteins and amino acids	Nanoparticle
	Odours-adsorbent	Microsphere
	Polymer for ultrafiltration	Sponge
	Material for treatment of contaminant water	Fibre and hollow fibre
	Antibacterial material	Solution
	Coating material	Membrane
	Papermaking wastewater treatments	
Paper and pulp	Wet strength agent	Nanoparticle
industry	Reduction in paper water vapour permeability	Powder
	Antimicrobial protective coating for paper packaging	Coating
	Anti-termite in paper making	
	Retention and drainage agents	
	Modification of cellulose fibres	
	Biodegradable packaging	
	Wrapping and toilet paper	
	Cardboard	
	Chromatography paper	
	Photochromic paper	

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Area	Applications	Form
Textile industry	Dye-binder for textiles	Microcapsule
	Impregnated textile materials	Fibre
	Binding agent for non-woven	Gel and gelatinous dispersion
	Surface modification of textiles	Coating
	Textiles with anti-bacterial properties	
	Textile antimicrobial finishing	
	Sanitary fibrous products	
	Surgical threads	
	Textile preservative and deodorant agent	
	Non-allergenic fibres	
Cosmetics	Encapsulating agent	Solution
	Skin delivery formulations	Film
	Functional additives	Powder
	Thickening agent	
	Hydrating and film-forming agent	
	Antistatic effect	
	Bacteriostatic	
	Products: shampoos, creams, lotions, nail polish, etc.	
	Products for oral/dental care	
Pharmaceutics	Drug delivery systems (e.g., spray-drying, ionic gelation, reverse micellar method, matrix coating, freeze drying, reactions in supercritical fluids)	Microspheres, nanoparticles Tablets Sponges/foams
	Medical/biomedical applications (e.g., hemostatic and wound healing dressings, tissue engineering)	Bandages, films, membranes, nanofibers Scaffold
Agriculture	Booster of plant growth and plant production	Solution
	Stimulator of crop yield	Film
	Pesticide formulations	Powder
	Soil conditioner	Spray
	Modification of plant-microbial interactions	Coating
	Stimulator of secondary metabolites	Gel
	Frost protection	Powder
	Controlled agrochemical release	Nanoparticle
	Biofertilizers and biocontrol agent	
Aquaculture	Removal of organic/inorganic compounds	Microsphere
	Removal of bacteria	Bead
	Removal of ammonia	Powder
	Functional food	
	Micro-carrier for bioactive compounds	
	Probiotics	
	Drugs microencapsulation	
	Drug delivery	
	Oral delivery (vaccination)	
	Antimicrobial and antioxidant	

3.1. Food and beverage industry

The food industry can derive many advantages from the properties of chitin and chitosan. Chitosan is used as an alternative, inexpensive, natural thickening and stabilizing agent in processed foods. Chitosan finds applications in the food sector as a nutritional ingredient, as an antimicrobial and antioxidant agent. All this is possible due to its bioactive nature and cationic form [22].

Several studies highlight the applications of chitosan and its derivatives in preserving food from microbial spoilage, extending the shelf-life of food products, forming biodegradable films and integrating food packaging. Due to the film-forming properties of chitin and chitosan, chitin derivatives edible films as food wraps and coatings have become widespread in recent years to extend the shelf life of food products. Chitosan can be used for fruit, decreasing the loss of transpiration and delaying fruit ripening by replacing non-biodegradable and more toxic polymers This application is also due to the selective gas permeability of chitin and chitosan films [53–55].

Chitosan and its derivatives are also widely used in functional food packaging, such as paper-based packaging and novel functional packaging films integrated with several plant extracts and oils, including prickly pear extract, lemon essential oil and Eucalyptus globulus essential oil [44,56–58].

Chitosan can also be used in the beverage industry, especially in wine production, as an agent in clarification, deacidification and wine stabilization. Chitin and chitosan can find applications as amendment agents in winemaking [59]. These polymers have proven effective in removing a mycotoxin called ochratoxin produced during winemaking by some species of fungi. Ochratoxin can cause acute toxicity in the kidneys of mammals and be cancerogenic to humans [60]. In addition, chitin and chitosan are used in brewing to clarify the beverage as a natural flocculant [61]. Widely used for clarification is chitosan of fungal origin [22].

3.2. Agriculture and aquaculture

In agriculture, chitin and chitosan has been used since the 1990s as a bactericide and a bacteriostatic. They protect plants against pathogenic bacteria that cause adverse effects on crops during the growth and post-harvest phase. This application is possible because these biopolymers can activate defense mechanisms in plants and, therefore, are often used in plant disease control as powerful elicitors by causing the accumulation of secondary metabolites in the plant, thereby increasing its defenses [62]. Due to its chelating properties, chitosan also finds application in some sprays for removing pesticides and is also an excellent antifungal [22]. Incorporating chitinous biomass into the soil enhances the protection of some crops by stimulating certain natural microbes, especially bacterial strains of the genus *Bacillus* sp. [63]. Chitin derivatives can also be helpful for the controlled release of pesticides and herbicides [64].

Due to the high number of carotenoids, crustacean shells are used as additives in fish feed in aquaculture, especially salmon. Abdel-Ghany and Salem (2019) reviewed the use of chitosan and its derivatives in aquaculture, its use as a functional food, a nutritional supplement, for its ability to transport bioactive compounds, for encapsulation of pathogen or nucleic acids, and the removal of pollutants from wastewater [65]. Chitosan has attracted increasing attention in the seafood industry for its non-toxicity, biodegradability, and biocompatibility, and for its antibacterial, antioxidant, and gel-enhancing properties [66]. For this reason, chitosan is successfully incorporated into seafood products to improve food quality, stability and human nutrition [22].

3.3. Wastewater treatment

Increased environmental awareness and urgency related to water pollution by heavy metals, pesticides and more are increasingly driving the search for new methods of purifying water before reaching the environment. Chitin derivatives are widely used for the clarification of wastewater and effluents due to the ability of their NH_2 group to form coordinated covalent bonds with metal ions. Furthermore, chitosan is much more effective than activated carbon in removing polychlorinated biphenyls from polluted water. Chitosan, carboxymethyl chitosan and cross-linked chitosan have proven effective in removing heavy metal ions, including Ni^{2+}, Co^{2+}, Cu^{2+}, from drinking water [67–69]. Similarly, chitin-derived biopolymers are excellent adsorbents for removing textile dyes from water. Chitin polymers with long chains and high molecular weight can be spun into fibers and films for water treatment [70].

Song et al. analyzed the use of chitin and chitosan in the paper industry. This industry generates a considerable number of pollutants due to the large number of chemicals used during papermaking, so much so that it is among the most polluting production activities in the world [71]. Kaur et al. highlighted chitosan's remarkable flocculating and coagulating power due to its high cationic charge density and long polymer chains that bridge aggregates and precipitation [72]. Thanks to these properties, chitosan can be used as an innovative and environmentally friendly treatment method in paper mill wastewater treatment. It is one of the most promising bioflocculants for wastewater purification [14,73]. Chitosan can absorb dyes, humic acids, metal ions and bacterial and xenobiotic cells from wastewater from paper production and other industries [71]. Chitosan has many advantages as a bioflocculant, as it is environmentally friendly, widely distributed in nature and biodegradable [74].

3.4. Paper and textile industry

Chitosan is also used to improve papermaking [71]. Indeed, due to its antibacterial properties and the film-forming ability of chitosan, possibilities have opened up to create functional papers, such as antibacterial paper and oiled paper [75]. Furthermore, using it as an additive in papermaking has improved paper characteristics such as dry and wet strength [76,77], color fixing capacity in paper and its ability to function as a retention and drainage additive [71]. The remarkable capabilities of chitosan, such as antimicrobial activity, non-toxicity, biodegradability and biocompatibility, have prompted the application of chitosan also in the textile industry with excellent results. Indeed, the way is opening for the development of bioactive textiles [78–80]. The results of application studies have shown that chitosan acts as a bioactive compound in textiles, e.g., as an antimicrobial finish for textiles and cosmetotextiles. Other notable characteristics of chitosan in textiles, include its antistatic activity, deodorising property, cost-effectiveness, non-toxicity, chelating property, film-creating ability, chemical reactivity and improvement in dyeing [22].

3.5. Cosmetics

Chitosan and its derivatives have many applications in cosmetics, for the body, skincare, hair care, and dental care. Chitin is a moisturizing agent and a film-forming tensor [22]. Furthermore, chitosan and chitin are chelators of metals responsible for many contact allergies. Some

carboxymethyl chitosan products are multifunctional ingredients in cosmetic formulations and are non-cytotoxic [81]. They are used as antioxidant, moisture-absorbing, antimicrobial and stabilizer agents in emulsions. Lately, there has also been talking of cosmeceuticals, i.e., cosmetic products that affect beauty and health. For example, cosmetics with pharmaceutical/medicinal properties based on chitosan are already on the market. They contain essential oils and active ingredients, such as enzymes, antioxidants, vitamins and phytochemicals [82]. These products can be applied as cream lotions and ointments [83].

3.6. Medical and pharmaceutical industry

One of the most relevant biomedical applications of chitosan is wound healing. Several chitosan-based products are on the market as topical dressings in wound management. Chitosan fortification makes bandages extremely effective due to the chitosan antimicrobial action and biocompatibility. Chitosan and its derivatives also have a stimulatory effect on cells, thus accelerating wound healing and dermal regeneration. In wound dressing products, the intrinsic antimicrobial and hemostatic action of chitosan is combined with its ability to deliver drugs, thereby enhancing tissue growth and regeneration [43].

In the field of tissue engineering, i.e., the discipline that aims to restore, improve or replace different types of biological tissues, chitosan can create scaffolds with excellent controlled delivery capabilities of loaded therapies and growth factors [84]. Main areas of tissue engineering using chitosan include the fabrication of biomaterials for cartilage tissue, bone tissue, blood vessel tissue, skin tissue, periodontal tissue and corneal regeneration [85]. The mechanical strength and structural integrity of chitosan-based biomaterials can be improved by adding biopolymers, such as chitin, alginate, polylactic acid, hydroxyapatite and bioactive nanoceramics [86–89].

Due to their non-toxicity, biocompatibility, biodegradability, stability and sterilizability, chitosan and its derivatives have been employed to formulate various drug delivery systems for oral, parenteral and topical administration, and targeted drug delivery [90,91].

In addition, chitosan has an ideal drug release rate, easy modifiability, cross-linking ability with other polymers, antimicrobial properties, gel-forming ability, bioadhesion, immunostimulant, macrophage activation and gas permeability [92].

4. Chitin recovery technologies

Crustacean shells are primarily made up of chitin, proteins, calcium carbonate and calcium phosphate whose content differs according to the species and position within a body [21]. Additionally, various minor components including proteoglycans, lipids and other inorganic materials contribute to shell structure [21]. Extraction of chitin involves separating the polymer from the other components, and requires two main steps: demineralization (also known as decalcification) for the removal of calcium, phosphorus and other minerals, and deproteinization, for the removal of protein and other toxic compounds [1]. The order in which these two steps are executed is not important. In many cases, the demineralization is performed first to increase the surface area available for deproteinization [21]. Additionally, phases of decolorization and deodorization can be carried out as needed [20].

Chitin extraction can be achieved chemically, typically with strong acids and alkali solutions, or biologically, using fermentation or enzymatic methods [1,18]. Currently, alternative and eco-friendly chemical treatments, such as the use of deep eutectic solvents (DESs) and natural deep eutectic solvents (NADESs), are being investigated for chitin recovery [20,34].

Chemical and biological approaches each have their own advantages and disadvantages [21]. It is also possible to combine both methods to achieve an even more efficient process [1,93].

Figure 6 shows an overview of the main chitin extraction methods.

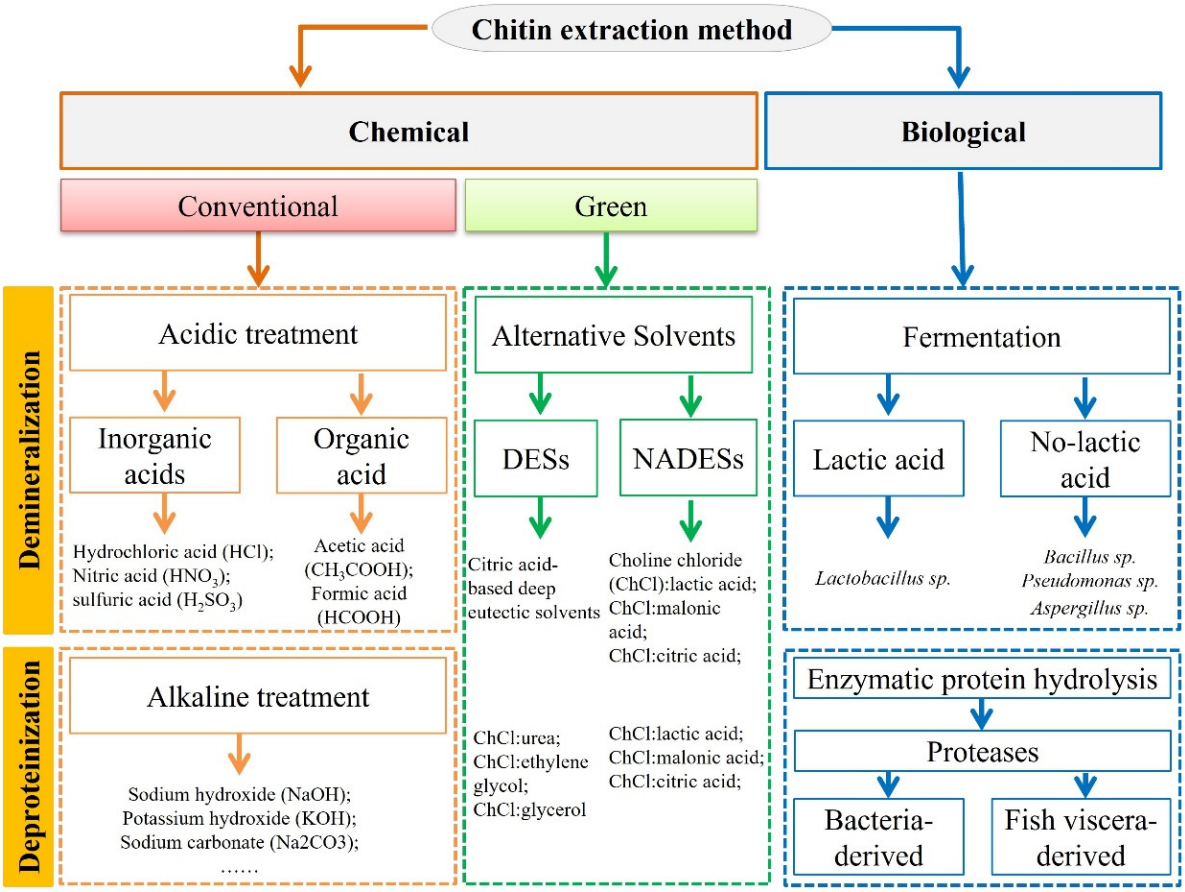

Figure 6. Chitin recovery technologies.

4.1. Chemical methods

The following subsections examine conventional methods for extracting chitin under acidic and alkaline conditions, as well as alternative methods using DESs and NADESs as green solvents.

4.1.1. Conventional methods

The acid-alkaline chemical method for chitin extraction is widely used in both industrial and laboratory production because of its purity, yield and cost-effectiveness [94]. It does not require specialized equipment or expensive reagents [95].

In the demineralization step, crustacean shell minerals are typically dissolved in diluted hydrochloric acid (HCl), which efficiently converts carbonate salts into chloride salts and carbon dioxide (CO_2) [96]. Single or double demineralization steps can also be performed using other inorganic acids (e.g., nitric-, sulfuric acid) or organic acids (e.g., acetic-, formic acid) [97,98]. Then, minerals are separated from residual shell solids by filtration and washing [99,100]. The demineralization reaction is carried out commonly at room temperature, taking two to three hours [21]. However, it can be performed even at temperatures up to 100 ℃ with shorter or longer reaction times [34].

The deproteinization step makes proteins water-soluble, allowing them to be separated from chitin easily. As a result of this reaction, part of the exoskeleton's lipids can also be extracted as well [101]. Deproteinization is achieved by using alkaline reagents, mainly sodium hydroxide because of its low cost, high temperatures typically ranging from 65 to 100 ℃, and reaction times from 21 min to 96 h [34,97]. Nevertheless, deproteinization reaction can also be conducted at room temperature or above 100 ℃ [34,95,97,99].

The efficiency of demineralization and deproteinization reactions is affected by reagent concentration, solid-to-solvent ratio, temperature and reaction time [34]. However, the chemical method results in toxic acid and alkaline waste that can be emitted into the environment and harm it. It also requires the consumption of large volumes of water for neutralization steps between acid and alkali treatments, high temperatures and long reaction times. All these factors limit the sustainability of the whole process [20,34]. Over the past few years, scientists have become increasingly interested in low-impact chitin recovery methods.

A recent study by Pohling et al. employed citric acid as a green solvent to demineralize ground shrimp shells in two steps, achieving similar results to those obtained by one-step hydrochloric acid demineralization [97].

Greene et al. investigated lactic acid at various concentrations and temperatures to perform the green crab shells demineralization [102]. The lactic acid reacts with the calcium carbonate contained within the chitin fraction, leading to a calcium lactate precipitate that can be removed by washing [18]. Therefore, lactic acid is a potential alternative to HCl strong acid. Nevertheless, results from Greene et al. do not support the use of lactic acid in the demineralization process. In fact, a high ratio of lactic acid and calcium carbonate is needed, which compromises the process's commercial viability. Mixed acid systems (lactic acid and HCl) could overcome this problem [102].

Yang et al. introduced a new fractionation method, termed as HOW-CA (hot water-carbonic acid) process, for high value chitin extraction from crustacean shell. HOW-CA process uses pure water at elevated temperatures for deproteinization and pressurized carbonic acid (CO_2) in an aqueous solution at room temperature for demineralization. This fractionation method exhibited high deproteinization and demineralization efficiencies (>90%) [103].

Boric' et al. developed a hybrid process comprising one step of demineralization with organic acids, such as lactic acid, and two cycles of dielectric barrier discharge plasma (DBD plasma), to remove minerals and proteins from shrimp shell wastes. In contrast to Greene's findings, lactic acid was proven to be highly efficient in the optimization of the demineralization step. The hybrid demineralization/DBD plasma process removed 90% of proteins and 100% of minerals from the shrimp shells [104].

Table 2 summarizes non-conventional chemical methods discussed so far.

Table 2. Non-conventional chemical methods for chitin recovery.

Step	Method	Source species	Concentration	Temperature/ duration	Results	Reference
Demineralization	Citric acid (×2)	Northern shrimp (*Pandalus borealis*)	50%	21 °C/35 min–1 h	Residual ash (%): 0.34 ± 0.12	[97]
	Lactic acid	Green crab (*Carcinus maenas*)	1.14 M	Room temperature/3h	Body mass loss (%): 60.6	[102]
	CO_2 (aq)	Gray shrimp (*Crangon crangon*)	10 atm	Room temperature/2h	Residual ash (%): <1	[103]
Deproteinization	Pressurized hot water	Gray shrimp (*Crangon crangon*)	-	180 °C/1h	Residual protein (%): 4.7	[103]
	DBD plasma	Northern shrimp (*Pandalus borealis*)	-	-/2 × 6 min	Chitin yield (%): 17; residual protein (%): <10%	[104]

4.1.2. Green alternative methods

Abbott et al. discovered the deep eutectic phenomenon in 2003 by mixing choline chloride (ChCl) and urea at a 1:2 molar ratio. ChCl and urea have melting points of 302 and 133 °C, respectively. This combination of solid starting materials led to a liquid eutectic mixture at room temperature, having a melting point of 12 °C and unusual solvent properties [105].

Since the introduction of deep eutectic solvents (DESs) by Abbott et al. the scientific community has become increasingly interested in these mixtures, due to their outstanding characteristics, such as thermal and chemical stability, low vapor pressure and design ability [106]. These solvents can be considered sustainable as they are cost-effective (obtainable from low-cost components), non-toxic, low flammable, biodegradable and ease of preparation [20,34,106,107]. As they are capable of dissolving and extracting materials from natural sources, they are widely used in recovery procedures for valuable products [20].

DESs are a subclass of ionic liquids (ILs), consisting of hydrogen bond donors (HBDs) and hydrogen bond acceptors (HBAs), linked by hydrogen bonds. HBDs and HBAs, combined at the appropriate molar ratios, exhibit a lower melting point than that of each component alone [106]. Usually, HBA is a choline-based compound: the most common one is ChCl, a low-cost, biodegradable, biocompatible and harmless salt [108]. ChCl can be produced commercially on an industrial scale and extracted from biomass or synthesized from fossil reserves [105,109].

Other HBAs reported for choline-based DESs, include choline acetate, choline bromide, choline dihydrogen citrate, choline hexafluorophosphate, choline iodide, choline nitrate, choline tetrafluoroborate and choline perchlorate, that can combine with urea or polybasic alcohol to make DESs with comparable characteristics and functions to ChCl-based DESs [108].

Natural zwitterionic betaine, an analog of ChCl, was used as an HBA. It exhibits biodegradability, nontoxicity and eco-friendliness, and can be used in food additives and

pharmaceuticals [21,106]. Nevertheless, producing DES with betaine proved relatively harder than that with ChCl [108]. Moreover, several inorganic salts, for instance quaternary ammonium salts (QASs), $ZnCl_2$ and $FeCl_3$, are used as HBAs for the preparation of DESs [108].

The most used HBDs in the preparation of DESs include urea, glycerol, citric acid (CA), ethylene glycol (EG), levulinic acid (LA), malonate, oxalic acid (OA), acetamide, sucrose and phenol [108].

Metal ions were present in early DESs [105]. The goal of reducing toxicity led to the development of natural deep eutectic solvents (NADESs). The NADESs are made up of more compounds that are primary metabolites in plants, such as sugars, organic acids, amino acids, alcohols and amines [20]. In general, NADESs can be classified into five groups: (1) ionic liquid NADESs comprised of an acid and a base, (2) neutral NADESs, consisting of sugar and polyalcohol, (3) neutral NADESs with bases that include sugar/polyalcohol and organic bases, (4) neutral NADESs with acids, made from sugar/polyalcohol and organic acids and (5) NADESs based on amino acids, containing amino acids and organic acids [106]. NADESs stand out as a green alternative to conventional solvents because of their following characteristics: (1) their constituent components have a relatively simple structure and are easily found on the market from bulk chemicals; (2) during extraction, they are not volatile, and can be reused and recycled; (3) their synthesis requires minimal energy; (4) they are safe to use; (5) they decompose easily and without releasing toxic substances; (6) they have comparable extraction properties to conventional volatile organic solvents; (7) they are extremely stable at high temperatures and (8) they are not flammable.

Several research studies have been conducted on the ability of DESs and NADESs to extract chitin. Saravana et al. extracted chitin was from shrimp shells (*Marsupenaeus japonicas*) using various types of DESs composed of ChCl and other compounds, such as organic acids and alcohols. The physicochemical properties of the DES-produced chitin were compared to those of conventionally produced chitin. As a result of using ChCl-malonic acid in a 1:2 ratio as DES, high purity chitin was obtained with a yield of 19.41 ± 1.35% [110]. According to a study by Zhao et al., the deproteinization of shrimp shell chitin was achieved using several DESs (betaine HCl-urea; ChCl-urea; ChCl-ethylene glycol; ChCl-Glycerol) and microwaves. Deproteinization efficiency exceeded 88%. The quality of DES-prepared chitin equaled that of traditional acid/alkali-prepared chitin [111]. Huang et al. used a NADES composed of choline chloride and malic acid to produce chitin from crab shells. Results showed that most proteins and minerals were removed from crab shells, and that chitin was 76% crystallinity. The quality of chitin obtained by using NADES was similar to that obtained by acid-alkali methods [112]. Bradić et al. evaluated the efficiency of numerous NADESs consisting of choline chloride-lactic acid, choline chloride-malonic acid, choline chloride-urea, and choline chloride and citric acid, for the extraction of chitin from shrimp shell biomass [113].

Table 3 summarizes some chitin extraction results by using DESs and NADESs.

Table 3. Chitin extraction by methods based on DES and NADES.

Method	Source species	Reagent	Temperature/duration	Results	Reference
DES	Shrimp shells (*Marsupenaeus japonicas*)		80 ℃/2 h	Chitin yield	[110]
		ChCl-lactic acid (1:2)		29.20 ± 1.97%	
		ChCl-ethylene glycol (1:2)		52.54 ± 2.01%	
		ChCl-urea (1:2)		50.54 ± 1.07%	
		ChCl-malonic acid (1:2)		28.86 ± 0.07%	
DES	Shrimp shells			Deproteinization	[111]
		Betaine HCl-urea (1:2)	50 ℃/-	92.20 ± 0.80%	
		ChCl-urea (1:2)	60 ℃/-	95.10 ± 1.20%	
		ChCl-ethylene glycol (1:2)	60 ℃/-	93.40 ± 0.60%	
		ChCl-glycerol (1:2)	90 ℃/-	91.30 ± 1.40%	
NADES	Crab shells	ChCl-malic acid	-/-	Demineralization/ deprotenization 99.8%/92.3%	[112]
NADES	Shrimp shells (species unspecified)	ChCl-lactic acid (1:1)	60 ℃/2h	Chitin yield	[113]
		ChCl-malonic acid (1:1)	60 ℃/2h	15–22.50% (1:50, shrimp shell powder: NADES)	
		ChCl-urea (1:2)	80 ℃/2h		
		ChCl-citric acid (1:1)	80 ℃/2h		

4.2. Biological methods

The biological approach to chitin extraction from crustaceans involves both fermentation-based methods and enzyme-based methods. The fermentation employs microorganisms producing organic acids and/or proteases for the digestion of both minerals and proteins, whereas the second method relies only on the use of protease enzymes for protein digestion.

Microorganism-based fermentation was found to be a more efficient and cost-effective method than enzymatic one. While demineralization and deproteinization rates can exceed 90% in the fermentation process, deproteination performed with crude enzymes rarely exceeds 90%, regardless of reaction time. Additionally, microbial fermentation residues contain high protein contents, which can be used as culture media to reduce biological treatment costs. Conversely, the cost of proteases is significantly high, mainly when compared to the cost of bases used in chemical deproteination. The use of recombinant enzymes and/or identification of new proteolytic enzymes from efficient secretors of proteases could increase deproteinization efficacy and reduce process costs [6].

Despite the costs, the supernatant obtained from enzymatic chitin extraction contains amino acids useful as a valuable nutritional resource [21].

4.2.1. Fermentation-based methods

A variety of microorganisms have been used to extract chitin from crustacean waste, including *Lactobacillus* spp., *Pseudomonas* spp. and *Bacillus* spp. [18]. It is possible to use one microorganism that produces both organic acids and proteases, or two different ones, i.e., an organic acid producer and a protease producer microorganism. However, using a single microorganism in fermentation can result in poor chitin extraction yields. In this type of fermentation, the efficiency

of demineralization or deproteinization is low depending on the microbial species; hence, chemical treatments are needed in addition to microbial fermentation to achieve high-purity chitin. In the process of chitin production, co-fermentation or two-step fermentation with two different cultures of acid-producing and proteolytic microorganisms could increase the efficiency of deproteinization and demineralization without using acidic or alkaline chemical treatments [21,93].

Methods of fermentation can be classified into two major categories: lactic acid fermentation and non-lactic acid fermentation (Table 4) [21,93,114].

Table 4. Fermentation methods for crustaceans' waste demineralization and deproteinization.

Method	Source	Microorganism	Demineralization (%)	Deproteinization (%)	Reference
Lactic acid fermentation with single microorganism	Prawn heads	*Lactobacillus plantarum* 541	88	83	[115]
	Shrimp shell		63	66	
	Shrimp shells (*Parapenaeus longirostris*)	*Lactobacillus helveticus*	98	78	[18]
	Shrimp shell	*Lactobacillus plantarum*	87	99	[116]
	Crab biomass (*Allopetrolisthes punctatus*)	*Lactobacillus plantarum*	99.6	95.3	[117]
Lactic acid fermentation with two microorganisms	Red crab shell	*Lactobacillus paracasei* KCTC-307 + *Serratia marcescens* FS-3	97.2	52.6	[118]
	Prawn waste	*Lactococcus lactis* + *Teredinobacter turnirae*	-	-	[119]
	Shrimp shell powders	*Serratia marcescens* + *Lactobacillus plantarum*	94.5	93	[120]
		Exiguobacterium profundum + *Lactobacillus acidophilus*	95 ± 3	85.9 ± 1.2	[121]
Non-lactic acid fermentation	Shrimp shell	*Bacillus pumilus A*1	88	94	[122]
	Shrimp shell	*Balistes capriscus*	-	78 ± 2	[123]
	Crab shell	*Bacillus licheniformis*	83 ± 0.5	90 ± 1.5	[124]
	Crab shell	*Bacillus pumilus*	80 ± 0.6	94 ± 1	[124]
	Shrimp and crab shell powder	*Pseudomonas aeruginosa* K-187	72	48	[125]
	Natural shrimp shell	*Pseudomonas aeruginosa* K-187	78	55	[125]
	Crab shell	*Pseudomonas aeruginosa* F722	92	63	[126]
	Shrimp shell	*Aspergillus niger* strains (0576, 0307, and 0474)	-	95	[127]

4.2.1.1. Lactic acid fermentation

Fermentation with lactic acid-producing bacteria could be sufficient to extract chitin completely, and it is commonly used for demineralization and deproteinization processes [128]. During fermentation, these bacteria produce acid capable of dissolving a lot of minerals, mainly $CaCO_3$, thus improving biowaste storage, and synthesize proteases to release proteins from the solid chitin complex by partial hydrolysis [115]. Rao et al. recovered chitin by fermenting shrimp heads and shells with *Lactobacillus plantarum* 541 in a drum reactor. According to this study, raw chitin yields from heads and shells were 4.5 and 13%, respectively, which are comparable to those obtained by chemical methods. Prawn heads were demineralized at 88% and deproteinized at 83%, while shrimp shells were demineralized at 63% and deproteinized at 66% [115].

Arbia et al. carried out fermenting shrimp shell waste of *Parapenaeus longirostris* using *Lactobacillus helveticus*, achieving 98% of demineralization and 78% of deproteinization [18].

Neves et al. investigated the biological extraction of chitin from freshwater shrimp shells, cultivated in tanks, by fermentation with *Lactobacillus plantarum* isolated from meat products. The deproteinization rate was approximately 99% and the demineralization rate was approximately 87% using batch fermentations up to 72 h and simple strategies such as pH adjustment and reinoculation. The yield of chitin was about 40% greater than that obtained by chemical methods [116].

Castro et al. performed chitin extraction from *Allopetrolisthes punctatus*, a crab species proliferating in Chile and Peru seashores, inoculating *Lactobacillus plantarum* sp. 47 in crab biomass. Extracted and purified chitin, after 60 h fermentation, showed 99.6% and 95.3% demineralization and deproteinization, respectively. Comparatively, chitin extraction by chemical methods produced lower yields and lower-quality products [117].

Based on the findings discussed above, fermentation with *Lactobacillus* spp. appears to be an effective alternative to chemical treatment. Despite this, some species of lactic acid-producing bacteria have poor deproteinization capabilities; chitin extraction may then be facilitated by adding protease-producing cultures to the fermentation process. However, protein degradation may remain unsatisfactory when lactic acid-producing bacteria and protease-producing bacteria are combined in a co-fermentation process. Then additional treatments may be required [93]. Jung et al. performed one-step extraction of chitin from red crab shell waste by co-fermentation with *Lactobacillus paracasei* KCTC-3074, a lactic-acid-producing bacterium, and *Serratia marcescens* FS-3, a protease-producing bacterium. Based on the results of this study, a demineralization rate of 97.2% was achieved. Nevertheless, the deproteinization level of 52.6% in co-fermentation was lower than in fermentation with the protease-producing bacterium alone (83.8%) but higher than in fermentation with the lactic acid-producing bacterium alone (19.5%). In co-fermentation, *Serratia marcescens* FS-3 is less contributing to deproteinization, probably due to the lower pH than the optimum neutral pH for the proteolytic activity in the culture supernatant. In fact, the organic acids produced by the cocultured *Lactobacillus paracasei* KCTC-3074 cause the pH to decrease [118]. In a co-fermentation process, this issue is always going to arise when at least two bacterial strains are co-cultured in a single batch [93]. Therefore, in order to improve the co-removal of proteins and minerals from crustacean shell wastes, it is necessary to identify and characterize novel microorganisms as well as protocols that employ proliferating organisms capable of secreting organic acids and proteases.

Aytekin and Elibol assessed different protocols to extract chitin from prawn waste by the cocultivation of the lactic acid-producing bacterium *Lactococcus lactis* and protease-producer marine bacterium *Teredinobacter turnirae*. The bacteria were first cultivated separately, and then co-fermented. The highest process yield of 95.5% was obtained when *T. turnirae* was inoculated first, followed by *L. lactis* inoculation after four days, in a medium containing 5% glucose [119]. Zhang et al. obtained chitin deproteinization and demineralization rates of 94.5% and 93.0%, respectively, by successive two-step fermentation of shrimp shell powders with *Serratia marcescens* B742 and *Lactobacillus plantarum* ATCC 8014 [120]. A recent study by Xie et al. used two-step fermentation to extract chitin from shrimp shells with protease-producing strains, *Exiguobacterium profundum*, and lactic acid-producing strains, *Lactobacillus acidophilus*, both of which were isolated from traditional fermented shrimp paste. A total of 85.9 ± 1.2% of protein and 95 ± 3% of minerals were removed. Chitin recovery and yield were 47.82% and 16.32%, respectively [121]. These results suggest that two-step fermentation might be more efficient than the co-removal of minerals and proteins when two different microorganisms are used, one of which is an organic acid producer and the other is a protease producer.

4.2.1.2. Non-lactic acid fermentation

In non-lactic acid fermentation, bacteria and fungi were used, including *Bacillus* sp., *Pseudomonas* sp. and *Aspergillus* sp. [114].

Six protease-producing *Bacillus* sp. (*B. pumilus* A1, *B. mojavencis* A21, *B. licheniformis* RP1, *B. cereus* SV1, *B. amyloliquefaciens* An6 and *B. subtilis* A26) were tested for their ability to ferment media containing shrimp shell waste for chitin extraction. The highest levels of protein removal of about 77 ± 3% and 78 ± 2% were achieved with *Bacillus mojavensis* A21 and *Balistes capriscus* proteases, respectively, after 3 h of hydrolysis at 45 °C using an enzyme/substrate ratio of 20 U/mg. However, the demineralization rate did not exceed 67%. In cultures containing shrimp shell waste supplemented with 5% (w/v) glucose, demineralization efficiency was remarkably improved without compromising deproteinization [122]. In a successive study by Hajji et al., crab shells waste was fermented for chitin production using several protease-producing *Bacillus* sp., including *B. subtilis* A26, *B. mojavensis* A21, *B. pumilus* A1, *B. amyloliquefaciens* An6, *B. licheniformis* NH1 and *B. cereus* BG1). The highest demineralization DM was achieved with *B. licheniformis* NH1 (83 ± 0.5%) and *B. pumilus* A1 (80 ± 0.6%), while the highest deproteinization was obtained with A1 (94 ± 1%), followed by NH1 (90 ± 1.5%) strains. Adding 5% (w/v) glucose to crab shell waste cultures promoted demineralization efficiency and slightly enhanced deproteinization, as demonstrated in the previous study [124].

Pseudomonas aeruginosa is able to secrete many proteins, including alkaline proteases [18,83]. *P. aeruginosa* K-187, isolated from the soil of northern Taiwan, was used by Oh et al. for the deproteinization of shrimp and crab shell wastes. In cultures with shrimp or crab shell wastes as the sole carbon source, *P. aeruginosa* K-187 produces proteases and chitinases/lysozymes, and deproteinizes shrimp and crab shell waste either solid-, liquid-solid or liquid fermentation [125]. In optimal reaction conditions, protein removal was 72% for shrimp and crab shell powder (SCSP) after 7-day incubation, compared to 78% and 45% for natural shrimp shell (NSS) and acid-treated SCSP. In contrast, with the protease produced under pre-optimization conditions, the percent of protein removal for SCSP, NSS and acid-treated SCSP was 48%, 55% and 40%, respectively. As a

comparison, three other protease-producing bacteria (*Euarotium repens* CCRC 30508, *Monascus purprens* CCRC 31530 and *Bacillus subtilis* CCRC 10029) were tested for crustacean waste deproteinization, but showed less effective at deproteinizing proteins than *P. aeruginosa* K-187 [125].

In a subsequent study by Oh et al., crab shell waste samples were inoculated with *P. aeruginosa* F722 (isolated from soil samples of Yeosu, Korea), to study the effectiveness of microbial demineralization and deproteinization for chitin extraction. Inoculated waste was incubated at 25, 30 and 35 ℃ for 7 days. Carbon sources were supplemented with different concentrations of glucose. Demineralization and deproteinization were 92% and 63%, respectively, at the optimal temperature of 30 ℃ [126].

The deproteinization and demineralization efficiency of crustacean shells was also tested by proteolytic enzymes released from the fungus *Aspergillus niger*. Lin Teng et al. investigated the concurrent chitin production from shrimp shells and fungi in a one-pot fermentation process, where fungi proteases hydrolyze proteins into amino acids that in turn are used as a nitrogen source for fungal growth. Three proteolytic *A. niger* strains (0576, 0307, and 0474) were selected among 34 zygomycete and deuteromycete strains screened for protease activity. A lower percentage of residual proteins (5%) was observed in shrimp chitin isolates, whereas a higher protein percentage was found in fungal chitin (10–15%). The combining of fungi and shrimp shells in a single reactor, with glucose supplementation, promoted the release of protease by the fungi, facilitating the deproteinization of shrimp shell powder. In turn, obtained hydrolyzed proteins were utilized for fungal growth, resulting in a decrease in the pH of the medium and further demineralization of shrimp shells [127].

4.2.1.3. Factors affecting fermentation efficiency

The optimization of fermentation process of crustacean wastes is crucial for improving deproteinization and demineralization and producing high-quality chitin. Specifically, the fermentation process requires an optimal preparation of media with a sufficient carbon and energy source, such as glucose, to support bacterial growth [21]. Tan et al. explored a way to reduce costs and utilize multiple waste streams. The authors co-fermented shrimp waste with *Lactobacillus plantarum* ATCC 14917 and *Bacillus subtilis* ATCC 6051, and replaced the glucose needed for fermentation with waste substrates (sugarcane molasses, light corn syrup, red grape pomace, white grape pomace, apple peel, pineapple peel and core, potato peel, mango peel, banana peel and sweet potato peel) as a cost-effective and resource-efficient strategy. The chitin was successfully extracted after deproteinization and demineralization of the prawn shells. Based on the results of the experiments, waste substrates are generally suitable as glucose substitutes in the fermentation process [129].

Beyond the availability of carbon sources, several other factors significantly affect fermentation efficiency, including pretreatment of raw material, inoculum levels, shell content in medium, shell size, pH values at start-up and during fermentation, the fermentation state (solid, or liquid) and oxygen conditions (aerobic or anaerobic process), among others [93].

Several statistical procedures, including the Box–Behnken design, Plackett and Burman design [122,123,130,131], Taguchi experimental design [120,132] and response surface methodology [18], have been used to optimize the medium components, and, in general,

fermentation process. These procedures represent effective and powerful tools for analyzing interactions between parameters using a limited number of experiments.

In accordance with the Plackett-Burman factorial design, Ghorbel-Bellaaj et al. selected the main factors influencing fermentation efficiency. Only four variables were found to influence proteins and minerals removal degree: Shrimp shell concentration, glucose concentration, incubation time and inoculum size. The authors used a Box–Bhenken design with these four variables and three levels for the determination of the deproteinization and demineralization efficiencies in fermented shrimp by *P. aeruginosa* A2. Variables, such as the initial pH of the medium, temperature, speed of agitation and culture volume, did not affect fermentation efficiency. Then, from response surface methodology, maximum demineralization and deproteinization were 96% and 89%, respectively, under optimal conditions (shrimp shell concentration 50 g/L, glucose 50 g/L, 5 days and inoculum of 0.05 optical density) [133]. The statistical methods used in this study proved useful to identify significant variables influencing the fermentation that can be fixed or eliminated in further optimization, and determine deproteinization and demineralization efficiencies.

4.2.2. Enzymatic-based methods for deproteinizing crustacean waste

Proteases hydrolyze proteins into amino acids and peptides at relatively low concentrations and over a wide pH and temperature range [134–136]. Their use for deproteinizing crustacean shells has been proposed as a biological alternative to alkaline treatment [137]. Protease-based methods take the advantage of the specificity of the enzymes and mild reaction conditions (usually 4–59 °C). Therefore, proteins can be removed with the least amount of deacetylation and damage to chitin chains [21]. In addition, using enzyme deproteinization, the previous demineralization is more convenient since it increases enzyme permeability and reduces enzyme inhibitors [137]. In general, reaction times are similar to those used in chemical deproteination: typically, 2–8 h, but some reports allow as much as 24 h [21]

Proteases come from a variety of sources, such as plants, animals, fungi and microbes [136,138,139], and those used for the deproteinization of crustacean wastes are commonly obtained from bacteria or fishes [21,93,136] (Table 5).

Table 5. Some proteases isolated from bacteria and fish.

Organism source	Enzyme	Crustacean source	Temperature/time	Deproteinization (%)	Reference
Bacteria	*Bacillus mojavensis* A21 protease	Shrimp processing by-products	45 °C/3h	77 ± 3%	[115]
	Balistes capriscus protease	Shrimp processing by-products	45 °C/3h	78 ± 2%	[18]
	Micromonospora Chaiyaphumensis S103 protease	Shrimp shell (*Penaeus kerathurus*)	45 °C/3h	93	[116]
Fish (viscera)	*Zosterisessor ophiocephalus* protease *Raja clavate* protease *Scorpaena scrofa* protease	Shrimp waste	45 °C/3h	76–80	[118]
	Portunus segnis protease	Blue crab shells	50 °C/3h	84.69 ± 0.65	[140]
	Portunus segnis protease	Shrimp shells	50 °C/3h	91.06 ± 1.40	[140]

Proteases from bacteria are the most extensively studied enzymes, as they offer more advantages than those from plants and animals: they can be cultured at an industrial scale, have a short fermentation period, are high yielding, cost-effective and easily obtainable, occupy a small space and can be subjected to lofty genetic manipulation [134,141]. As a result of their properties, these bacterial enzymes are ideal for biotechnological applications, and are widely used in industry [141].

Bacillus sp. is one of the most prominent sources of proteases. It produces high yields of neutral and alkaline proteolytic enzymes with exceptional properties, including high stability at extreme temperatures, pH, organic solvents, detergents and oxidizing agents [139,142]. The genus *Bacillus* is probably the most important bacterial source of proteases, and is capable of producing high yields of neutral and alkaline proteolytic enzymes with remarkable properties, such as high stability towards extreme temperatures, pH, organic solvents, detergents and oxidizing compounds Younes et al. tested different crude microbial proteases from *B. mojavensis* A21, *B. subtilis* A26, *B. licheniformis* NH1, *B. licheniformis* MP1, *Vibrio metschnikovii* J1 and *Aspergillus clavatus* ES1, and crude alkaline protease extracts from Sardinelle (*Sardinella aurita*), Goby (*Zosterisessor ophiocephalus*) and Grey triggerfish (*Balistes capriscus*) for enzymatic deproteinization of the shrimp processing by-products. The highest levels of protein removal of about 77 ± 3% and 78 ± 2% were achieved with *Bacillus mojavensis* A21 and *Balistes capriscus* proteases, respectively, after 3 h of hydrolysis at 45 °C using an enzyme/substrate ratio of 20 U/mg [123].

Proteases isolated from *Micromonospora Chaiyaphumensis* S103 were used for *Penaeus kerathurus* waste shells deproteinization in the process of chitin preparation. The removal rate of protein was 93% after 3 h of hydrolysis at 45 °C and pH 8.0 using a 20 U/mg enzyme/substrate

ratio [143]. Additionally, the S103 crude enzyme exhibited excellent stability in several organic solvents, retaining 100% of its original activity after 90 days of incubation in methanol, hexane, acetone, and DMSO. The properties of S103 proteases make them ideal for chitin recovery. The S103 crude enzyme exhibited excellent stability in several organic solvents, retaining 100% of its original activity after 90 days of incubation in methanol, hexane, acetone and DMSO. The properties of S103 proteases make them ideal for chitin recovery [137].

Bacteria can produce proteases by using a wide range of carbon and nitrogen sources, including various types of fishery by-products and agro-industrial wastes [52,134]. In this way, the cost of protease production would be reduced, and their application would become more attractive. Doan et al. investigated several types of marine by-products, including demineralized crab shell, demineralized shrimp shell, shrimp head and squid pen, as sources of carbon and nitrogen nutrition for *Paenibacillus* sp. TKU047, a protease-producing bacterium. Strain TKU047 displayed the highest protease productivity (2.98 U/mL) when cultured for two days on a medium supplemented with 0.5% shrimp head powder. TKU047 protease displayed optimal activity at 70–80 °C and pH 9, with a pH range of stability from 6 to 11 [134].

Fish viscera, a valuable and low-cost by-product of the fishing industry, contains a wide range of proteases, including alkaline proteases that are highly stable between pH 6.0 and 12.0 [137,140,144].

A study by Nasri et al. tested crude alkaline proteases from the viscera of goby (*Zosterisessor ophiocephalus*), thornback ray (*Raja clavata*) and scorpionfish (*Scorpaena scrofa*) for the deproteinization of shrimp wastes. The protein removal rates after 3 h of hydrolysis at 45 °C with an enzyme/substrate ratio of 10 were about 76–80% for all three crude proteases. These results suggested that enzymatic deproteinization of shrimp waste by fish endogenous alkaline proteases could have been applied to the chitin production process [135].

In 2014, Sila et al. extracted chitin from shrimp (*Penaeus longirostris*) shells using alkaline proteases obtained from viscera of the Tunisian barbel (*Barbus callensis*), a small carp-like fish widespread throughout Tunisia belonging to the family *Cyprinidae*. A high shells deproteinization level (80 %) was recorded [145].

Younes et al. selected crude alkaline proteases from *Scorpaena scrofa*, a marine species in the family *Scorpaenidae*. The deproteinization rate was 83 ± 1% [138]. Hamdi et al. used digestive alkaline proteases from *Portunus segnis* for chitin extraction from blue crab (*Portunus segnis*) and shrimp (*Penaeus kerathurus)* shells. The enzymatic treatment, conducted at pH 8.0 and 60 °C, led to very high levels of deproteinization (approximately 85% for *P. segnis*, and 91% for *P. kerathurus*), facilitating chitin release [140].

Among alkaline proteases found in fish viscera, trypsin, secreted from the pancreas, pyloric caeca, and intestine, has gained interest over the years due to its high stability and activity at high pH and temperature [144]. It is a serine protease able of hydrolyzing peptide bonds at carboxyl groups of arginine and lysine residues and is highly active between pH 8.0 and 10.0 [144]. In the past, trypsin and trypsin-like enzymes have been isolated and characterized from the viscera of a variety of cold- and warm-water fish, including the spleen of yellowfin tuna (*Thunnus albacores*) and skipjack tuna; the viscera of mandarin fish (*Siniperca chuatsi*), silver mojarra (*Diapterus rhombeus*), grey triggerfish (*Balistes capriscus*) and striped seabream (*Lithognathus mormyrus*); the pyloric caeca of bluefish (*Pomatomus saltatrix*), chinook salmon, tambaqui (*Colossoma macropomum*) and Monterey sardine (*Sardinops sagax caerulea*); viscera of *Tunisian barbel* (*Barbus callensis*); the intestine of Nile tilapia (*Oreochromis niloticus*); and the entire viscera from

the true sardine (*Sardinops melanostictus*) and Japanese anchovy (*Engraulis japonica*) [146,147]. Recently, Batav and Gothalwal (2020) obtained trypsin from the visceral waste of Catla, an economically important freshwater fish in the *Cyprinidae* family, known as the major South Asian carp [148].

4.2.2.1. Commercial enzymes

Several commercial enzymes have been utilized in crustacean waste degradation (Table 6). Globally, microbial proteases are the most commercially exploited enzymes; most of them are produced from different species of the *Bacillus* genus [141].

Table 6. Some commercial enzymes in crustaceans waste deproteinization.

Commercial enzyme	Crustacean source	Deproteinization (%)	Reference
Alcalase 2.4 L	Shrimp (*Xiphopenaeus kroyeri*)	93.41%	[115]
Alcalase 2.4 T	White-leg shrimp (*Litopenaeus vannamei*)	59	[149]
Alcalase 2.4 T	Black tiger shrimp (*Penaeus monodon*)	57	[149]
Swine pancreatin	Shrimp (*Xiphopenaeus kroyeri*)	92.23	[18]
Pepsin	White shrimp shell	92	[150]
Protease	White-leg shrimp shells (*Litopenaeus vannamei*)	91.10	[151]
Crude protease from *Bacillus cereus* SV1	Shrimp shell (*Metapenaeus monoceros*)	88.8 ± 0.4	[152]
Bluefish trypsin	*Penaeus monodon*	-	[146]

The commercial crude protease from *Bacillus cereus* SV1 was applied for chitin extraction from shrimp waste material of *Metapenaeus monoceros*. A high level of deproteinization 88.8 ± 0.4% was achieved at room temperature for 6 h with an enzyme/substrate ratio of 20 [152]. A high protein removal efficiency, equal to 91.10%, was also achieved by using a commercial protease from *Streptomyces griseus* in the deproteinization of white-leg shrimp shells (*Litopenaeus vannamei*) [151]. Recently, a commercial Alcalase enzyme 2.4 T (Novazyme, Denmark) was used for deproteinizing shrimp shells in the chitin extraction process from white-leg shrimp (*Litopenaeus vannamei*) and black tiger shrimp (*Penaeus monodon*). The reaction was carried out at 40 ℃ for 36 h. For white-leg shrimp shells and black tiger shrimp shells, deproteinization rates were about 59% and 57%, respectively [149].

4.2.2.2. Novel proteases

One of the main disadvantages of enzymatic deproteinization is the high cost of enzymes and the low extraction efficiency. High proteolytic activity is essential to recover high-quality chitin. The use of novel proteases obtained using recombinant DNA technology could increase deproteinization efficacy, and reduce chitin production costs [93]. Recombinant aspartic proteases P6281 (protease A) and saccharopepsin (protease B) were used to hydrolyze *Litopenaeus vannamei* shrimp shell proteins. The process was completed within hours and resulted in high recovery rates of protein (91.4%) and chitin recover (88.9%) [153]. Moreover, discovering novel highly efficient proteolytic enzymes isolated from organisms with very high organic acid and protease secretion

capabilities is crucial with a view to improving crustaceans' waste deproteinization efficiency. Ormane-Benmrad et al. performed the purification and biochemical characterization of a novel thermostable protease, with high proteolytic activity, from the oyster mushroom *Pleurotus sajor-caju* strain CTM10057 [154]. Mahmoud et al. purified and characterized the AKD9 protease from *Bacillus subtilis* D9. This alkaline serine protease with a molecular mass of 48 kDa exhibited optimal activity at 50 °C with pH 9.5, and showed high stability till 65 °C and pH 8–11 for 1 h [154]. In a study by Foophow et al. a novel extracellular haloprotease Vpr was purified and characterized from *Bacillus licheniformis* strain KB111 isolated from mangrove forest sediments in Chanthaburi, Thailand. This serine protease of ~70 kDa (KB-SP) can work in harsh conditions, with a broad alkaline pH and salinity range. Its activity increases in the presence of metal ions K^+, Ca^{2+} and Mg^{2+} [155]. Due to their properties, such as stability and enhanced thermostability, these novel enzymes have the potential to deproteinize crustaceans waste efficiently.

4.3. Cleaner technologies for increasing the sustainability of the chitin recovery process

Currently, the conventional chemical methods are the most used for large-scale chitin extraction from crustaceans' waste. However, these commercialized chemical processes have several disadvantages limiting their sustainability in terms of economic viability, environmental protection and social equity.

In the first place, they generate wastewater containing toxic chemicals that must be properly managed and disposed of. Furthermore, they require high temperatures and large volumes of water to neutralize acid and alkaline treatments, resulting in a high environmental impact. In addition to these environmental disadvantages, there are also technical-economic ones. In fact, conventional chemical methods require materials resistant to corrosion caused by strong acids and bases, which increases equipment costs and, therefore, process costs. Additionally, they negatively affect the physicochemical properties of chitin, resulting in a decrease in MW and DA, which impairs chitin's quality [18,20,34,93,114,136].

In conclusion, the environmental footprint of conventional chemical methods is unacceptable, and the resulting chitin derivatives do not meet the requirements of high-tech applications [156].

A feasible alternative to conventional chemical methods is eco-friendly extraction by green solvent mixtures, such as DESs and NADESs, which have easy preparation and low price, are non-toxic, low flammable and biodegradable. However, there is a lack of scientific literature on green chitin extraction methods. Moreover, the existing research works are carried out on a laboratory scale. It is therefore clear that further specific studies are needed [20,34].

The biological route for chitin recovery has many economic and environmental benefits, since it is relatively inexpensive and less environmentally damaging than conventional chemical treatment. The biological method enables the extraction of chitin with higher molecular weight and better-preserved structure than chitin obtained through the conventional chemical route [114]. Biological methods are carried out under mild reaction conditions. For this reason, they are a viable alternative, not only to conventional, but also to green chemical processes [34].

The biological extraction method, compared to the chemical one, offers several advantages. It has higher reproducibility and needs a simpler manipulation and a smaller solvent consumption. Finally, it requires lower energy. Nevertheless, one of its major disadvantages is the lower demineralization and deproteinization efficiency [18,21,93]. As described in section 4.2, one of the

possible ways to improve the efficiency of the biological chitin extraction process is co-fermentation or two successive-step fermentation (using different cultures of acid-producing and proteolytic microorganisms). In order to improve deproteinization efficiency, it is possible to isolate new proteolytic enzymes from different matrices (e.g., mushroom or bacteria strains), or produce engineered enzymes using recombinant DNA technology [93]. The use of these technological solutions could result in lower enzyme costs and faster reaction times [34,93].

However, the biological chitin extraction process is still limited to studies at the laboratory scale [34,93,114]. Therefore, improving the efficiency of biological methods for large-scale chitin extraction remains the greatest challenge.

5. Conclusions

The market demand for chitin and chitosan is growing enormously. It is driven and oriented by the higher knowledge of the properties of these polymers and the growing development of suitable extraction processes, as well as reactions capable of modulating their characteristics by chemically modifying the macromolecules. It is now known that factors, such as DA or DDA and molecular weight, can strongly influence the mechanical, chemical-physical and functional properties of chitin and chitosan. The source from which these polysaccharides are extracted is relevant. Marine crustacean wastes are still the predominant source of chitin and chitosan, due to large quantities deriving from the food industry. However, fungi and insects are becoming increasingly promising as sources of chitin. Further research will be needed to determine how the characteristics of these sources affect the properties of chitin and chitosan.

The type of extraction treatment pursued, whether chemical or biological, is decisive for the chitin-derivatives yields, quality and the process sustainability (environmental and economic).

Although commercial processes for chitin recovery are mostly based on conventional chemical methods, recovery by biological methods leads to higher-quality chitin and its derivatives, with lower environmental impacts. Biological extraction processes are currently more expensive than chemical ones. Therefore, fully sustainable processes are not possible at an industrial scale. The economic sustainability of the entire process can, however, be improved by optimizing fermentation or enzymatic hydrolysis. In a future perspective, advanced biotechnologies could lead to new, genetically modified microorganisms and engineered enzymes that are more performing and efficient in the demineralization and deproteinization phases for the purpose of recovering chitin from different matrices.

Conflict of interest

The authors declare no conflicts of interest.

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