World Journal of Microbiology and Biotechnology (2023) 39:28
https://doi.org/10.1007/s11274-022-03468-1

REVIEW

Recent advances in extraction of chitin and chitosan

Nafisa Islam[1] · Mizanul Hoque[1] · Salman Farsi Taharat[1]

Received: 10 September 2022 / Accepted: 10 November 2022 / Published online: 28 November 2022

Abstract

Chitosan is a versatile biopolymer due to its biocompatibility, biodegradability, antimicrobial, non-toxic, mucoadhesive, and highly adsorptive properties. Chitosan and its derivatives have been used for many biomedical applications. Currently, crustacean shells and other marine organisms are the significant sources of chitin/chitosan production worldwide. However, extraction from marine sources presents several challenges, including an unstable supply of raw materials. Large-scale chitosan extraction from crustacean sources harms the environment by involving harsh processing steps such as alkali deproteinization. Recently many studies have been carried out focusing on alternative sources or eco-friendlier routes for production of chitosan. This paper briefly overviews recent studies on fungi and insect cuticles as alternative chitosan sources. Milder extraction processes for fungal chitosan and the superior quality of the resultant polymer make it highly desirable for biological applications. Biological techniques involving fermentation and enzymatic processing of the raw materials are looked at in detail. In the concluding remarks, the paper highlights the potential of using a combination of "green" technologies and briefly looks at potential biological/biomedical applications of extracted chitinous materials.

Keywords Enzymatic · Fermentation · Fungi · Green · Insect · Mycotech

Abbreviations

COS	chitooligosaccharides
DA	degree of acetylation
DD	degree of deacetylation
DM	demineralization, percentage of demineralization
DP	deproteination/deproteinization, percentage of deproteination
LPMO	lytic polysaccharide monooxygenases
paCOS	partially acetylated chitooligosaccharides

Introduction

Whether from exoskeletons of insects and crustaceans, the radulae of mollusks or fungal species, the sources of chitin and chitosan are abundant and global. Chitin is one of the most abundant biopolymers in nature. It comprises repeating units of N-acetyl glucosamine (GlcNAc) connected by β-(1 → 4) linkages (Vicente et al. 2021). Chitin is a homopolymer of N-acetyl-0-glucosamine (GlcNAc), typically with large molecular weights, and has structural similarity with cellulose. Naturally occurring chitin has O-glucosamine (GlcN) units, randomly interspersed in the polymeric chain. The chitin molecules are abundant in crustacean and insect exoskeletons. They are closely associated with proteins, calcium carbonate, lipids and pigments, all of which are to be separated by chemical or biological methods to obtain pure chitin. Extraction of crustacean or insect chitin is often the forerunner step of chitosan extraction, and thus recent advances in both processes have been discussed in the subsequent sections. In nature, the cell walls of certain fungal species contain chitosan and can be used for direct commercial production of chitosan. Chitosan is the partially or fully deacetylated form of chitin. The copolymer is termed chitosan if the number of deacetylated units (GlcN) are more than the number of N-acetyl-glucosamine (GlcNAc) units, i.e. degree of deacetylation is higher than 50%. *Chitooligosachharides (COS)* and *partially acetylated chitooligosaccharides* (paCOS) are the low molecular weight versions of chitin and chitosan (Tabassum et al. 2021). The polymers and oligopolymers are represented in Fig. 1.

The source of chitosan, as well as the method of extraction, is a significant research field because these parameters heavily influence the properties and effectiveness of the

✉ Nafisa Islam
nafisaislam@che.buet.ac.bd

[1] Department of Chemical Engineering, Bangladesh University of Engineering and Technology, Dhaka 1000, Bangladesh

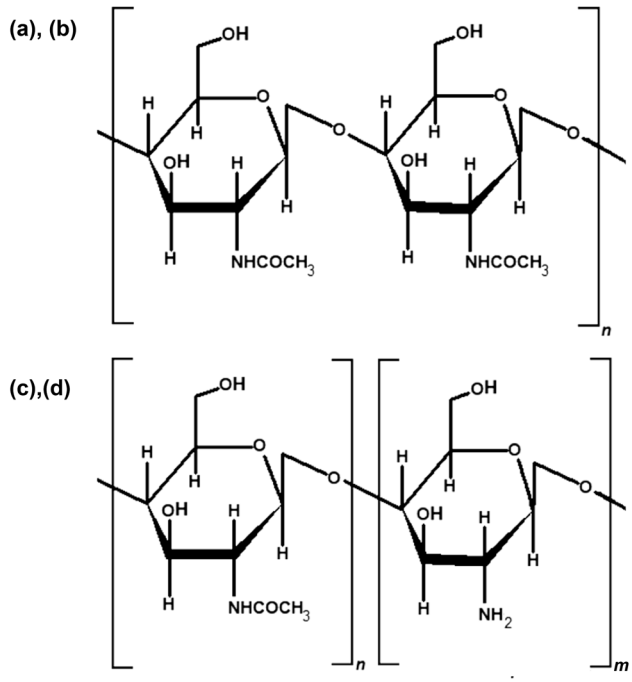

Fig. 1 Schematic representations of the polymers, **a** chitin (n > 10), **b** chitooligosachharide, COS (n < 10), **c** chitosan (m > n, m + n > 10), **d** partially acetylated chitooligosachharides, paCOS (m + n < 10). Adapted from (Afroz et al. 2021) with permission for reuse and modification

biopolymers. The molecular weight, degree of deacetylation (DD%), degree of polymerization (DP%), and polydispersity index are the major factors affecting properties such as solubility, crystallinity and hydrophilicity/hydrophobicity. Conventional (or chemical) extraction from crustacean shells involves three primary steps, (1) demineralization of the shells to remove calcium carbonate and calcium phosphate, usually carried out by HCl or other strong acids; (2) deproteination to remove the proteins, adhering to the chitosan, by incubating in strong base like NaOH, at high temperatures (Dhillon et al. 2013); and (3) deacetylation, to convert chitin to chitosan, using 50%w/w NaOH, at high temperatures. An additional decolorization step is sometimes carried out to remove carotenoid pigments using different solvents (acetone, hydrogen peroxide, sodium hypochlorite) or potassium permanganate. Further steps can be used to convert the chitosan to more biologically active COS. Reducing the use or strength of these chemicals and/or reduction of energy consumed for production could produce chitosan and associated chemicals, not only is an eco-friendlier manner, but in some cases can also give products with higher bioactivity (Mathew et al. 2021).

This review focuses on recent methods of chitin and chitosan/COS/paCOS production, which are more sustainable either by being 'greener' or more economic or both. The methods are divided into two broad topics. The first topic looks at alternative sources to shrimp shells, to utilize these readily available waste biomasses into viable sources of chitosan. The second topic looks at using biological or biochemical methods, either using the enzyme producing microorganisms directly, (fermentation mediated extraction), or by using enzymes extracted from various organisms like microbes, fishes, shrimps, plants, and animals (enzyme assisted extraction). In a concluding section, recent studies incorporating a combination of any of the methods to make extraction of chitosan greener and/or more economically favourable, are discussed.

Alternative sources of chitosan

Fungal cell walls and arthropod exoskeletons are rich sources of naturally available chitinous substances and of particular interest is fungal source because there is no requirement to convert chitin to chitosan. The benefits of an alternative to crustacean species as sources of chitin/chitosan include the fact that there is no seasonal dependence for the supply of the biomass, and waste biomass of many pharmaceutical and biotechnological industries are potential sources. In addition to this, the extraction process is usually simpler, and uses lower amounts of or less harsh chemicals. For instance, the demineralization step can be eliminated (as compared to extraction from crustacean sources) since the fungal chitosan is not associated with $CaCO_3$ present in crustacean shells (Jones et al. 2020). The comparison of steps of chitosan extraction from various sources is shown in Fig. 2. Additionally, fungal chitosan has some desirable properties, including lower molecular weights and higher degrees of deacetylation, especially suitable for biomedical applications. The properties are easier to control by controlling the biomass growth conditions, as compared to crustacean chitosan.

Fungal species as alternative sources for chitosan production

Researchers have studied these fungal genera/species for chitosan extraction: *Candida* spp., *Saccharomyces* spp. (Afroz et al. 2021), *Rhizopus* spp. (Chatterjee et al. 2008, 2019; Kleekayai and Suntornsuk 2011; Cardoso et al. 2012), *Absidia* spp., (Jaworska and Konieczna 2001; Jiang et al. 2011), *Mucor* spp., *Mortierella isabelina*, *Lentinus edodes, Penicillium* spp, (Ebrahimzadeh et al. 2013; Namboodiri and Pakshirajan 2019; Aili et al. 2019), *Cunninghamella* spp. (de Oliveira et al. 2014; Berger et al. 2014a, b), *Gongronella* spp. (Nwe and Stevens 2004), *Mucor* spp. (Tajdini et al. 2010; Karimi and Zamani 2013; Mondala et al. 2015; Safaei et al. 2016; Zininga et al. 2019; Abasian et al. 2020), *Rhizomucor* spp. (Zamani et al. 2007; Tajdini et al. 2010)

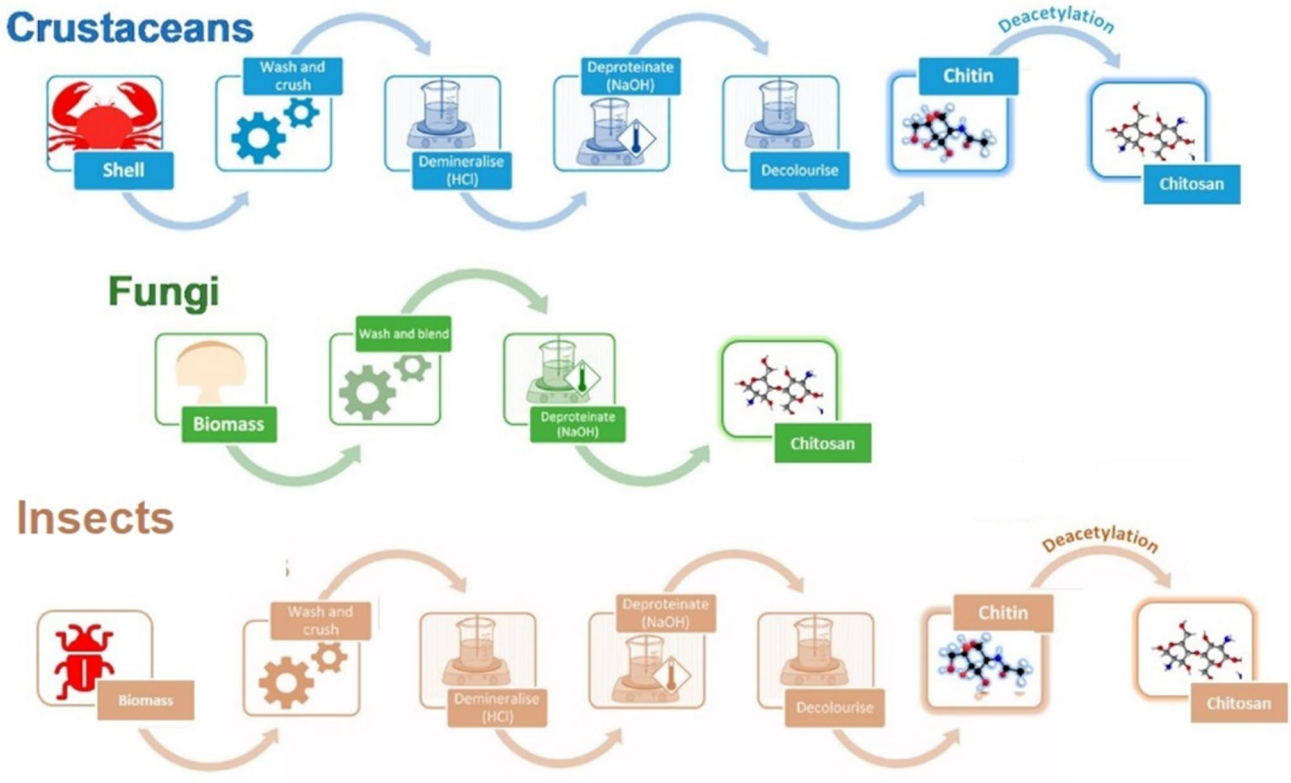

Fig. 2 Comparison of steps for chitin/chitosan extraction from crustacean shells, fungal and insect biomass. Adapted under Creative Commons Attribution License from (Jones et al. 2020)

and *Rhizopus* spp. (Chatterjee et al. 2008, 2019; Liao et al. 2008; Cardoso et al. 2012; Tasar et al. 2016) and other species have been studied. The *Zygomycetes* fungal class has higher chitosan to chitin ratio in their cell walls compared to other classes. The chitinous content in these fungal cell walls typically ranges from 6 to 45%. In a study by Campos-Takaki et al. (2014), the cell wall of fungal species, *Cunninghamella blakesleeana, Gongronella butleri, Rhizopus arrhizus, Mucor javanicus, Cunninghamella elegans* and *Syncephalastrum racemosum* were all analyzed and 10–16% of cell walls were found to have chitin, while chitosan was in the range 26–28%. The differences in yield of chitosan arise from difference of species, conditions of fermentation and process of extraction. A detailed review by Crognale et al. (2022) takes a detailed look at the sources available for, and parameters of production of fungal chitosan. Since there are advantages and disadvantages of various genera/species of fungi for chitosan production, and it is to be noted that some high yielding species may be pathogenic, it is important to assess the most suitable organisms for chitosan production according to the desired properties, applications and/or scale up possibilities The species from which chitin and chitosan contents are cultivated are largely affected by the growth medium, fermentation methods and fermentation conditions. Fungal biomass can be produced by solid-state fermentation (SSF) and submerged fermentation (SmF) (Dhillon et al. 2013). Fungal chitosan production requires various ingredients such as, yeast extract, D-glucose and peptone. Also, inexpensive nutrient sources have been studied for culturing fungi for production of chitinous substances (Kannan et al. 2010).

Fungal strain improvement and metabolic engineering for chitosan production

Efforts are being made to increase the relative proportion of chitosan in the fungal cell walls. Maw et al. (2002) carried out UV mutation of *G. butleri* spores for increased chitin deacetylase (CDA) activity. The mutants were screened in addition to high CDA activity, for different hyphal morphologies. Three mutant strains were found to produce twice the extractable chitosan and double CDA activity as compared to the wild strain. However, more efforts are necessary to isolate commercially feasible fungal strain. There are several patents available on metabolic engineering on enhanced production of chitosan in fungi (Deng et al. 2005; Carr and Hammer 2006). Chitin synthase, glutamine-fructose-6-phosphate aminotransferase (GFA), and CDA—these genes were

mutated to achieve chitin production within a cell, and to improve the catalytic potential and substrate specificity of the enzymes. For example, *R. oryzae* or *A. niger* strains utilized for lactic acid and citric acid production are an attractive source of spent fungal biomass. Similarly, *S. cerevisiae*, wine yeast can be engineered to produce chitosan. One of the main concerns of mutating these organisms would be that during the yield of the primary fermentation products should not be affected (Ghormade et al. 2017).

Characteristics of fungal chitosan for potential biological applications

The advantages of fungal approaches to chitosan production include uniform physicochemical properties of fungal chitosan, made possible by accurate bioprocess control. A variety of studies shows that MW, polydispersity, and %DD of fungal chitosan can be manipulated by variations in growth media and process conditions. Obtaining reproducible values of these parameters is fundamental to guaranteeing the acceptance of fungal chitosan in sensitive applications. The allergic concern of chitosan that is extracted from crustaceans is very significant. The major allergen present in crustaceans is the muscle protein tropomyosin, which can cause mild to severe allergic reactions. As a result, the demand to produce chitosan from fungal sources is gaining traction. It is easier to make products of different MW as well as viscosity grades from fungal chitosan compared with crustacean chitosan. Chitosan from fungal sources have 3–5 times lower viscosity and MW, but higher %DD, making them appropriate for usage in food, healthcare, and pharmaceutical industries. In parallel with crustacean chitosan and its derivatives, fungal chitosan can also be a topic of interest as wound dressing material, natural preservative in food, cosmetics and pharmaceutical industries. Fungal chitosan is soluble in physiological pH ranges with its poly-cationic characteristics, lower antigen effect and lesser inorganic materials than crustacean chitosan. So, it can be used as a potential drug carrier and non-viral gene delivery system (Moussa et al. 2013; Khan et al. 2015; Duan et al. 2019; Wang et al. 2019; Morin-Crini et al. 2019; Tang et al. 2020; Joseph et al. 2021).

Use of waste biomass as alternative sources or alternative carbon sources

Waste fungal biomass from biotech industries

In different bioprocesses such as fungi originated food, antibiotics and pharmaceuticals, organic acid and enzyme production and in brewing and baking, a very large portion of biotech industries use fungi. Authors have suggested that organic agro-industrial residues such as waste fungal biomass can be used as inexpensive medium to produce chitosan (Leite et al. 2015). Furthermore, many yeasts species are used for wine production, and some are used for isolation of β-glucan. The spent species are all disposed into landfills or sent for incineration; however these wastes have the potential to generate chitinous products (Ghormade et al. 2017). Solely from citric acid production more than 80,000 ton of waste *Aspergillus niger* biomass per year is generated. Most of the enzymes are produced in the growth medium leaving huge amounts of waste biomass which could be a good source for chitin and chitosan isolation. Production of an edible mushroom *Agaricus bisporus* generates more than 50,000 ton of waste materials per year consisting of stalks and fruit bodies and it is reported that the extraction of chitin from these waste biomasses could be 1000 ton per year (Wu et al. 2004). Easily available local waste biomass sources were used by Afroz et al. (2021). To produce fungal chitosan from *Aspergillus niger* obtained from moldy onions, and locally purchased yeast (*Saccharomyces cerevisiae*).

Value addition to existing mycotech products

Mycotech products are products that are synthesized commercially from fungal sources. Fungal residues, byproducts and other organic wastes are very common in the large scale myco-products' production processes. It was reported that *Melanobatrachus indicus* and *R. oryzae* were used to produce proteins, oil and chitosan on organic wastes (Satari et al. 2016). *Metarhizium anisopliae* (Entomopathogenic fungus) has been used commercially for the control of insect pests in the agriculture field. The mycelial biomass can be used to isolate cell wall polymers after removing the conidia. It was observed that *M. anisopliae* was able produce equal % of chitin and chitosan in the cell wall (100 mg chitosan/10 g of dry biomass) (Nahar et al. 2004). Chitosan extraction from the mycelia of *M. anisopliae* can be one of the approaches for value addition. *M. anisopliae* also produced CDA enzyme which is useful for the enzymatic deacetylation of chitosan (Kulkarni et al. 2008; Ghormade et al. 2017).

Commercial perspectives of fungal chitosan: present and future

Fungal-based chitosan products are still limited at commercial scale. In the past decades, fungal chitosan applications are focused on biomedical and food technologies, such as carriers for drug/gene delivery, wound dressing, haemocompatible biomaterials, and preservatives as antimicrobial agents in food and antibacterial food packaging. Fungal-based chitosan products are still limited in the market but are slowly gaining popularity. First fungal based chitosan was patented by an US based company named Cargill in 2005.

Cargill's chitosan was highly deacetylated and obtained from microbials biomass. In Europe, a Belgium based company, KitoZyme first brought fungal based product (KitoZyme) to market. In June 2011, KitoZyme obtained Generally Recognized as Safe (GRAS) status approval from Food and Drug Administration (FDA) for their chitosan product named KiOnutrime-CsG® to be used in beverage applications. The KiOnutrime-CsG® is also registered in the European Union (EU) under the novel food regulations and is approved by the European Food Safety Authority (EFSA). Kitozyme's non-animal source chitosan products on market are: 1)KiOfine®-B, a new tool for winemakers that helps to prevent and cure contamination by *Brettanomyces bruxellensis*; 2)KiOfine®-CsG, an antioxidant used in different stages of the winemaking process (clarification and fining) and 3) Slim MED® ADVANCED, a dietary fiber for weight loss. KitoZyme has a spin-off company named KiOmed Pharma (previously Synolyne Pharma) with University of Liège to develop a chitosan-based microbeads hydrogel for the treatment of Osteoarthritis. In Canada, it was Mycodev (in 2013) who brought fungal based chitosan to public attention to commercialize a new fermentation-based product in the Canadian market, with the focus on biomedical and pharmaceutical applications. Another Canadian based company Chinova Bioworks started to commercialize their first mushroom-based chitosan in 2016. Chinova has been developing mushroom chitosan-based formulations to replace synthetic preservatives from food and beverage applications. Chinova Bioworks' mushroom chitosan 'Chiber' has already entered the marketplace. China-based biotech company Chibio has non-animal source chitosan for food and pharmaceutical applications. Chitosan wound dressing commercial products currently available in the market are mostly obtained from crustaceans' source chitosan. The production cost for the traditional crustacean- based chitosan is cheap compared with fungal based chitosan. Crustacean raw materials are readily available and cheap whereas the cost of raw materials is the main bottleneck for fungal chitosan production. Crustacean chitosan can be found from 10 US dollar per kg to 1000 US dollar per kg depending on product quality and application. On the other hand, about 5 out of 100 persons get affected by crustacean allergy, which means that about 300 million persons need non-crustacean ingredients for food and biotechnological products. This is the reason that people are interested in replacing the animal source products with non-animal source products (Huq et al. 2022).

Switching from conventional to fungi-based processes requires economic and environmental factors to be carefully evaluated. Utilizing agro-industrial residues (rice and wheat straws, hardwood sawdust, cottonseed hulls, soybean residues, wine lees, grape marc, apple pomace, corn steep liquor, sweet potato distillery waste, cassava wastewater, sugarcane syrup and date waste syrup) in the fermentation process and applying milder acid-alkaline treatment conditions in the extraction process—are some of the economic and environmental advantages over the crustacean chitosan production process. Due to the increased environmental regulations fungi-based biotech industries are associated with raised pollution abatement costs and lowering the disposal cost of fungal waste can be achieved by implementing integrated bioprocess focused on fungal chitosan production. In addition to the primary product, yielding chitosan as a co-product from waste mycelia, might boost and increase the diffusion of this technology. For processing crustacean materials, new methods such as hot water, mechanochemical and glycerol treatments have been demonstrated to be greener compared with traditional methods in terms of chemical consumption and wastewater production—these novel greener methods may be used for fungal chitin/chitosan extraction and processing too. Fungal species which are pathogenic to animals and other plants are subjects to further research for safe large scale chitosan production and improvised waste management technology. As green technology, sustainable circular economy and health safety have been getting more priority in recent years, fungal chitosan-based products will gain marketability over the next decades.

Potential of insect chitosan

Insects are a viable source of chitin and chitosan, although little attention has been given to them so far. Two billion people worldwide eat 1900 various species of insects for nutrition as a reliable food source containing 30–45% protein, 25–40% fat, and 10–15% chitin in total. Southeast Asia, the Pacific, Sub-Saharan Africa and Latin America represent major insect consumers (van Huis 2013; Spranghers et al. 2017). According to Huet et al. (2020), chitin of various purity grades (45, 89.7, and 93.3%) were extracted and physicochemically characterised from *Bombyx eri* larvae revealing that insect chitins had identical crystallographic structures, thermal stability, and degree of acetylation (> 87%) to commercially accessible and isolated chitin from shrimp shell. The residues from the insect's breeding for human consumption can be used to successfully obtain chitin/chitosan. Having a low amount of minerals, insects as raw material for chitosan production have made it possible to obtain chitosan only with the deproteinization step. Utilization of enzymes has been proven efficient in removing the proteins contained in the matrix. It has shown a sufficient reduction in harsh treatment to justify the non-use of solvents harmful to the environment (Silva et al., 2021).

Insects have certain advantages over crustaceans in that they are not seasonal and can be readily bred due to their high fertility rate (Luo et al. 2019). As insect breeding centers are springing up all over the world, insects can be used as a viable source of chitin and chitosan for larger

ecological and economic sustainability as bioconverters which are reared for organic waste management and animal feed processing. It is noteworthy that insect chitin contains less calcium carbonate (6%) than crustacean chitin (30–50%), allowing for easier extraction and a more eco-sustainable process (Sajomsang and Gonil 2010). In the last few years, numbers of scientific papers have been published on chitin processing and subsequent conversions into bioproducts from various insect species(Badariotti et al. 2007; Sajomsang and Gonil 2010; Liu et al. 2012; Kaya et al. 2014, 2015a, b; Ibitoye et al. 2018; Marei et al. 2019). Insect chitosan has been further formulated into nanoparticles and showed antimicrobial propreties and also showed wound healing characteristics (Al-Saggaf 2021). Insect chitosan from *Tenebrio molitor* and *Brachystola magna* were formulated into films for food packaging purposes (Saenz-Mendoza et al. 2020). Insect farm side streams show promise as a source of chtin (Brigode et al. 2020). The various insect species biomass can thus be used by commercial chitosan producers as a profitable alternative source (Iber et al. 2022).

Biological methods of extraction

Using microorganisms or their enzymes has been explored in the last few decades as an environmentally friendly method of chitinous material extraction. The biological methods are broadly categorized into two sections as follows: (1) fermentation mediated extraction, (the microorganisms are incubated along with the raw material) and (2) enzyme mediate extraction, (extracted or commercially available enzymes are added to the raw material). The comparison of biological to chemical methods of chitin extraction is summarized in Fig. 3.

Fermentation mediated chitin/chitosan extraction

Deproteination and demineralization during fermentation

For processing shrimp and other waste, microbes can be present within the chitosan source (autofermentation) or inoculated into the source for deproteination and/or demineralization. During these fermentation steps, deproteination is by proteolytic enzymes and demineralization is by the organic acids produced by the microorganisms. Ghorbel-Bellaaj showed chitin extraction using six protease producing bacteria (*Bacillus pumilus* A1, *Bacillus mojavencis* A21, *Bacillus licheniformis* RP1, *Bacillus cereus* SV1, *Bacillus amyloliquefaciens* An6 and *Bacillus subtilis* A26), all of which showed DP > 80%. However *B. subtilis* A26 showed a high degree of demineralization (DM) as well, when supplemented with glucose (Ghorbel-bellaaj et al. 2012). Fermentation of shrimp shells, producing chitin using microorganisms such as *Bacillus Licheniformis* (Liu et al. 2014a), and *Pseudomonas aeruginosa* (Sedaghat et al. 2017), all showed high DP.

Halophilic bacteria grow in saline conditions and secrete extracellular proteolytic enzymes. Two such bacterial species *Halobacterium salinarum* and *Halobacterium dombrowskii* showed DP as high as 98.99% when producing chitin (Dayakar et al. 2021). The microbes in the species *Paenibacillus mucilaginosus* TKU032 are interesting because the typical nutrient source of this bacteria is from the marine industry waste including waste shrimp shells (Doan et al. 2020). Microorganisms isolated from the shellfish wastes (and other habitats) can also be used for the breakdown of the shellfish wastes. Protease producing bacteria *Alcaligens faecalis* were isolated from soil samples of shrimp culture ponds (Rakshit et al. 2021), while *B. pumilus* A1 was isolated from slaughterhouse polluted water for chitin production (Ghorbel-Bellaaj et al. 2013). In the latter case, the same microbe also carried out demineralization, and further steps to demineralize the product were not carried out.

Demineralization during fermentation

For biochemical demineralization of chitosan sources, fermentation with lactic acid producing bacteria (LAB) is used. These bacteria can also perform deproteination in the correct conditions. Authors have used LAB such as, *Lactobacillus plantarum* isolated from sausages (Neves et al. 2017), salmon (Castro et al. 2018), or from fermented food (Francisco et al. 2015). *Serratia marcescens* B742 and *Lactobacillus plantarum* ATCC 801 were used to produce chitin, and the DP and DM rates were 94.5 and 93.%, respectively (Zhang et al. 2012). Another study used *Lactobacillus brevis* for demineralization and *Rhizopus oligosporus* as deproteinizing fungi (Aranday-García et al. 2019). Arbia et al. carried out several studies on demineralization and deproteination of shrimp shell *Parapenaeus longirostris* using *Lactobacillus helveticus* (Arbia et al. 2013, 2017, 2019).

Various authors have attempted to get maximum deproteinization and demineralization rates using the same microbial species, however this requires careful optimization of parameters of fermentation: nutrients (C/N ratio, in particular), temperature and duration of fermentation. Alternatively, two (or more) microbial species are simultaneously or separately inoculated into the source, to perform deproteination and demineralization, not necessarily in that order, in a process termed as cofermentation. A detailed study on cofermentation using *Bacillus licheniformis* 21886 and *Gluconobacter oxydans* DSM-2003 showed the best DM and DP results were obtained when the waste was first inoculated with *Bacillus* followed by *Gluconobacter*, rather than simultaneous inoculation of the two species (Liu et al. 2014a). A study was carried out by Zhang et al. (2021) for successive

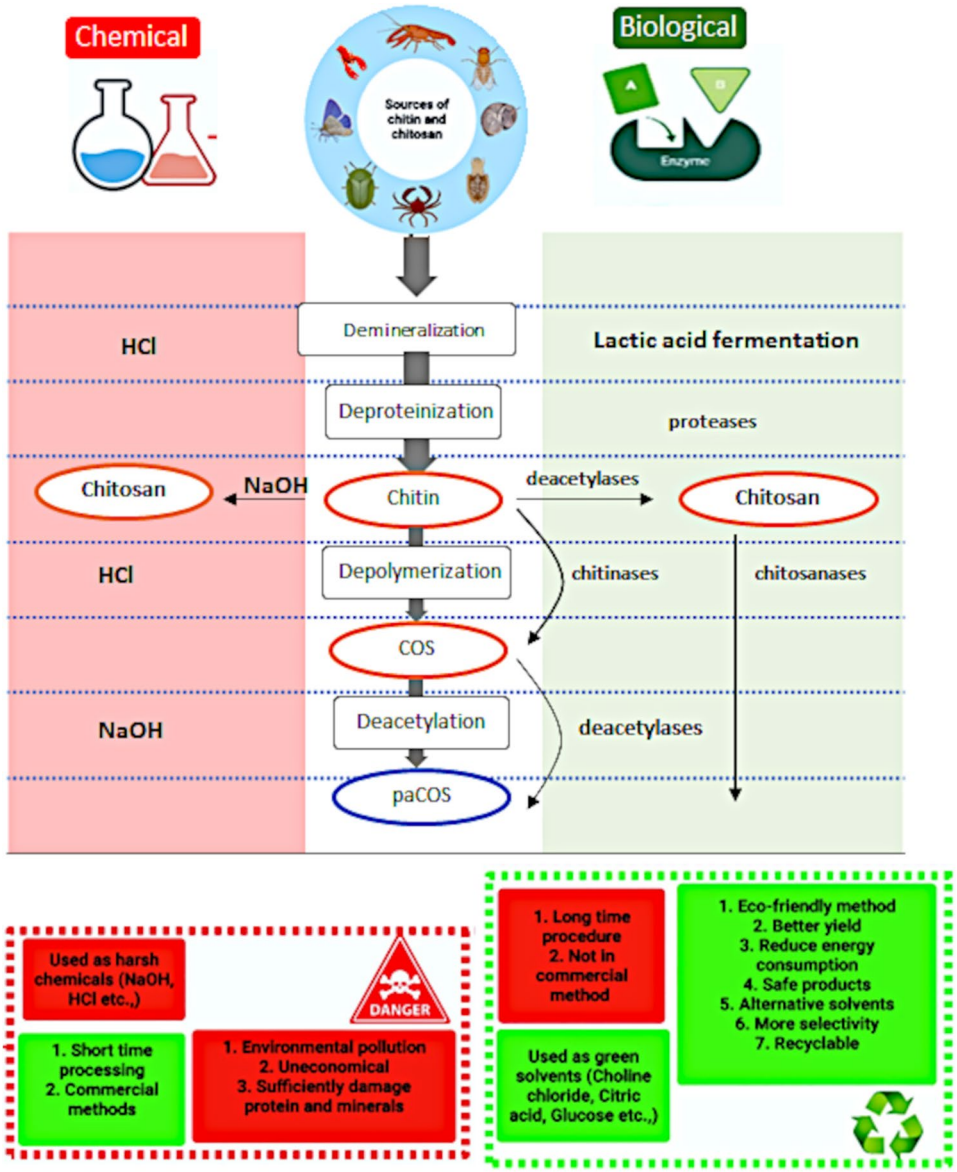

Fig. 3 Comparison of chemical and biotechnological extraction. Advantages (green boxes) and disadvantages (red boxes) compared for chemical extraction (bottom left, large red dotted box) and using green extraction methods (bottom right, large green dotted box) Adapted with permission from (Arnold et al. 2020; Mohan et al. 2022)

co-fermentation with *B. subtilis* and *A. pasteurianus* for extracting chitin from shrimp shells, showing efficiencies of DP of 94.5% and DM of 92.0%. Bahasan et al. (2017) isolated demineralization microbe *Kurthia gibsonii* from fermented milk and deproteination microbe, *Aspergillus spp.* from bread, and inoculated two shrimp species, *Fenneropenaeus semisulcatus* and *Fenneropenaeus indicus* to extract chitin. Liu et al. (2020) used successive fermentation with *Lactobacillus rhamnoides* and *Bacillus amyloliquefaciens* (BA01) strain. Some recent work has summarized in the Table 1.

Both successive fermentation and cofermentation approaches can lead to > 95% DP and DM degrees, and do not produce environmentally harmful by-products. Previous studies illustrate the importance of optimizing the nutrient source and other parameters and the need for considering the compatibility of the cofermentable species. It is also advisable to carry out the demineralization prior to deproteination since the minerals present in the crustacean shells can affect the enzymatic proteolytic accessibility and thus DP efficiencies (Younes et al. 2015b). Thus if carried out properly cofermentation can reduce expensive double sterilization, replacing the medium, and collecting residues between DP and DM processes (Zhang et al. 2021).

Enzymes and other non-fermentative means of biochemical extraction

Demineralization

For demineralization commercially available organic acids such as lactic acid and citric acid are viable alternatives to

Table 1 Fermentation mediated extraction of chitinous products (*DP* deproteinization, *DM* demineralization)

Step	Source	Source of enzyme/bacterial species	Product	DDA, DP, DM, other parameters	Ref
DP	Shrimp shell and parts (*P. vannamei*)	*H. salinarum* and H. *dombrowski*	Chitin produced by chemical demineralization	DP 98.99%	(Dayakar et al. 2021)
Partial DP	Shrimp head powder	*Paenibacillus mucilaginosus*	Partially deproteinated hydrolysate fiurther deproteinted and demineralized	DP 45%	(Doan et al. 2020)
DP and DM	Shrimp head waste Litopenaeus vannamei)	*Bacillus Licheniformis (B)*	Chitin	In combination with *G oxydans* (*G*), tried but DM and DP low. *B* followed by *G* gives best DP and 87 ± 0.2 and DM of 93.5 ± 0.1	(Liu et al. 2014b)
	Fresh shrimp (*Pandalus borealis*) heads	*Bacillus licheniformis*	Chitin, no further conversion	In combination with Endogenous enzymes for autolysis	(Guo et al. 2019)
DP and DM	Shrimp shell powder	*Pseudomonas aeruginosa*	Chitin which was further processed chemically to get chitosan	DP 94% DM 82%	(Sedaghat et al. 2017)
DP and DM	Shrimp shell (*Metapeneaus monoceros*)	*Bacillus pumilus* A1, *Bacillus mojavencis* A21, *Bacillus licheniformis* RP1, *Bacillus cereus* SV1, *Bacillus amyloliquefaciens* An6 and *Bacillus subtilis* A26	Chitin (Shrimp waste hydrolysates) and antioxidants were prepared	6 bacterial strains were used, all gave 80% + but B subtilis gave 91% DP and 79% DM when glucose was added	(Ghorbel-bellaaj et al. 2012)
DM	Shrimp (*Macrobrachium rosenbergii*)	*Lactobacillus plantarum*	Chitin is chemically converted to chitosan using 15 M NaOH for 168 h	DP 99% DM 87% (but fermentation was for three days and reinoculation was required)	(Neves et al. 2017)
DM	Shrimp (*Penaeus merguiensis*)	*Lactobacillus plantarum* from tuna and milkfish, co-culture of T1 and L137	Chitin is chemically converted to chitosan using 55% NaOH overnight	DP 84.4%, DM 86.6%	(Francisco et al. 2015)
DP and DM	Shrimp waste	*Lactobacillus brevis* and *Rhizopus oligosporu*	Chitin produced was fibrillated to produce chitin nanofibrils	DP was by lactobacillus at 96% DM by lactobacillus was 66% and further DM was by fungus rhizopus	(Aranday-García et al. 2019)
DP –> DM	Shrimp shell powders	*Serratia marcescens* B742 and *Lactobacillus plantarum* ATCC 801	Chitin		(Zhang et al. 2012)
DM	*Allopetrolisthes punctatus* crab	*Lactobacillus plantarum*	Chitin	99.6% demineralization 95.3% deproteinization	(Castro et al. 2018)
DM –> DP	Shrimp shell waste of *Parapenaeus longirostris*	*Lactobacillus helveticus*	Chitin	98% demineralization and 78% deproteinization	(Arbia et al. 2017)
DM–> DP	*Fenneropenaeus semisulcatus* and *Fenneropenaeus indicus*	*Kurthia gibsonii* for demineralization and *Aspergillus flavus*., for deproteination	Chitin	Not measured	Bahasan et al. 2017)

Table 1 (continued)

Step	Source	Source of enzyme/bacterial species	Product	DDA, DP, DM, other parameters	Ref
DM	Pacific white leg shrimp *Litopenaeus vannamei* heads and tails	Four different strains of LAB namely *L. plantarum* FTDC0350, *L. acidophilus* FTDC3871, *L. casei* ATCC0442, and *Lactococcus lactis* Gh1	Chitin was converted to chitosan with 50% NaOH for 720 min	*L acidophilus* gave highest: 90.8% of demineralization and 76% of deproteinization	(Tan et al. 2020)
DP − > DM		*B. subtilis* and *A. pasteurianus*	Chitin	DP 94.5%, DM 92.0%, chitin yield 18.0%,	(Zhang et al. 2021)
DM + DP or DM− > DP	Shrimp shell	*Lactobacillus rhamnoides* and *Bacillus amyloliquefaciens*	Chitin	DM of 97.5%	(Yu et al. 2020)
DM + DP or DM− > DP	*Shrimp shell and* Mucor circinelloides	*Mucor circinelloides* for shrimp shells	Crustacean species produced chitin and fungal species produced chitosan	Not measured	(Yun Nian Tan et al. 2021)
		Lactobacillus plantarum and *Bacillus subtilis for fungus mucor circinelloides*	Chitosan		

conventionally used harsh mineral acids. Lactic acid has proved to be effective as the calcium carbonate of the crustacean shells can react with the lactic acid to form calcium lactate, which is easily removed and lowers environmental pollution (Greene et al. 2016; Marzieh et al. 2019). Formic acid has been used for demineralization in a recent study (Baron et al. 2017). (The fermentation of sources with lactic acid producing bacteria, LAB, has been discussed in "Fermentation Mediated Extraction" section).

Deproteination using enzymes

For deproteination, enzymes are available by extraction from various organisms, and can be used with chitinous sources without fermentation (organisms added to chitinous sources). Crude proteases can be extracted from the natural sources mentioned; alternatively, commercially available proteases can also be used for the deproteination step. It is to be noted however that commercial enzymes are usually more expensive and the crude proteases can sometimes give more effective results owing to the fact that a mixture of proteases is available when extracted from the source. On the other hand, use of commercial enzymes can give a more reliable product and can be easily scaled up for industrial extraction of chitosan (Mathew et al., 2021). Proteases typically used are alcalases, pepsin, papain, pancreatin, delvolase, trypsin, chemotrypsin, proteinase K and pectinase among others (Mathew et al. 2021). A few works have been summarized in Table 2.

Deproteination using extracted proteases

Microbial sources which are used for protease extraction include *Paenibacillus woosongensis TKB2* (Paul et al. 2015a), *Micromonospora chaiyaphumensis* s103 (Mhamdi et al. 2017) and *Bacillus licheniformis* can produce Alcalase, a serine endopeptidase. A surfactant and oxidant resistant protease was isolated from *Bacillus cereus* SV1 giving DP efficiency of 88.8% ± 0.42 (Manni et al. 2010b). In a related work, the chitin produced in this manner was further processed to give chitosan, which in turn showed remarkable antibacterial activity of against different bacteria (Manni et al. 2010a). A recent study was carried out to isolate proteolytic enzymes from halophilic bacteria, since these enzymes can withstand high salt conditions, and are extracted from marine wastes (Maruthiah et al. 2015). Thus, these halophilic naturally occurring proteases are an economically attractive option for biological extraction of chitinous substances. Marine sources like fish and invertebrate aquatic species can also be a source of crude proteases. Mukhin and Novikov (2001) showed that proteolytic enzymes for digestion of crustacean shell components can be isolated from crustacean wastes; this work showed

Table 2 Application of enzymes for chitin/chitosan extraction and further applications

Source of chitin/chitosan	Source of enzyme	DM DP, other parameters	Ref	Product and its applications, if any
Extracted proteases				
Shrimp shell (*Metapenaeus monoceros*)	*Bacillus mojavensis* A21 and *Balistes capriscus*	77 ± 3% and 78 ± 2% deproteinization compared to 94% for chemical	(Younes et al. 2015b)	Chitin which was further processed chemically to produce chitosan Chitosan samples inhibited the growth of most Gram-negative, Gram-positive bacteria and fungi tested, exhibited antioxidant and antitumor activities which was dependent on the molecular weight
Black tiger shrimp shell wastes (BTSHWs	*Paenibacillus woosongensis TKB2* (compared to commercially available protease from Sigma Aldrich, USA)	DP 80 ± 0.4%. and DM satisfactory	(Paul et al. 2015b)	Chitin
Shrimp (M. monoceros) shells	*B. cereus* SV1	DP 88.8% ± 0.42	(Manni et al. 2010a)	Chitin which was further processed chemically to produce chitosan using 50% (w/v) NaOH at 80 °C for 4 h, which were found to exhibit antibacterial activities
Other Extracted Enzymes				
Shrimp based chitosan	Bacterial chitosanase *Bacillus licheniformis*		(Affes et al. 2020)	Low molecular weight chitosan, showing antioxidant, antibacterial and antifungal activity
Commercially Available Proteases				
Shrimp shell (*L. vannamei*)	Trypsin and Ficin (Sigma Aldrich)	DP 92%	(Marzieh et al. 2019)	Chitin
Shrimp shell	*Streptomyces griseus* (EC no. 232-909-5, Sigma Aldrich)	DP 91.1%	(Hongkulsup et al. 2016)	Chitin
Crayfish (*Procambarus clarkii*) shell waste	Proteinase K (Sinopharm)	DP 91%	(Dun et al. 2019)	Trypsase, papain, alkaline protease, proteinase K, neutral protease, Protamax were all tested and the enzyme with highest DP was further optimized to produce chitin
Squid pens from *I. argentinus*	Alcalase 2.4L, Neutrase 0.8L and Esperase 8L		(Wang et al. 2006)	Chitin was converted to chitosan using NaOH ranging from 30 to 70%

isolation of a potent protease from digestive system of king crab, which was used for digesting the proteins in king crabs and prawn shells. In a comprehensive study by Younes et al. (2015b) proteinases isolated from 6 species of microbes and 3 species of fish, were used to deproteinize shrimp shell wastes. Proteolytic preparations from *Bacillus mojavensis* A21 *Bacillus subtilis* A26, *Bacillus licheniformis* NH1, B. licheniformisMP1, *Vibrio metschnikovii* J1, *Aspergillus clavatus* ES1 and crude alkaline protease extracts from *Sardinelle* (Sardinellaaurita), *Goby* (Zosterisessor ophiocephalus) and *Grey triggerfish* (Balistes capriscus) were all optimized for chitin extraction and the highest DP efficiency of 77% was with proteases from *B. mojavensis* and 78% with those from *B. capriscus.* The enzyme to solid (E/S) ratio is major governing factor to increase deproteination. In another similar work, highly stable alkaline proteases extracted from red scorpionfish (*Scorpaena scrofa*) were used for chitin extraction and a DP efficiency (Younes et al. 2015a).

Deproteination using commercially obtainable proteases

Commercially enzymes have been used by many authors for replacing the harsh chemicals used during chitin and chitosan extraction, as well as further processing of these biopolymers. Commercial proteases like papain, alcalase 2.4L, pepsin, trypsin, pectinase, Proteinase K, and endogenous enzymes have been used traditionally for shrimp shell waste processing (Mathew et al. 2021) but these enzymatic processing steps can be applied for chitin and chitosan extraction. The work by Marzieh et al. (2019) showed an interesting insight into DP efficiency by use of trypsin and ficin with/without sodium metabisulfite. The results showed that sodium metabisulfite has an effect on the enzyme activity, enzymatic chitins (especially chitin produced by trypsin) exhibited higher degrees of acetylation, crystalline index as well as a smooth microfibrillar crystalline. The action of commercial protease from *Streptomyces griseus* was optimized for different parameters (incubation time, pH, enzyme–substrate ratio and particle size), and showed DP of 91.10% (Hongkulsup et al. 2016) A recent study optimized several commercial proteases tryptase, papain, alkaline protease, proteinase K, neutral protease, and Protemax and obtained a maximum DP of 91% using proteinase K (Dun et al. 2019). An interesting application of protease enzymes is the use of those present in shrimp heads, which are rich in various endogenous enzymes. These enzymes can be activated by incubation or UV irradiation, and autolysis occurs on the substrates present in the shrimp heads/wastes. Use of these enzymes can facilitate in economical shrimp waste treatment for deproteinization and chitin production (Cao et al. 2020). Studies on the optimum conditions for autolysis by endogenous enzymes showed that pH, autolysis time and temperatures have an influence on the DP percentage (Cao et al. 2008, 2009; da Silva et al. 2017). Shrimp head was processed by a combination of autolysis and fermentation, and the endogenous enzymes were responsible for autolysis gave a DP of 88.35%. A pilot study of 20 kg of shrimp head autofermentation was carried out, giving insights into the future methods of green extraction of chitinaceous products (Guo et al. 2019).

Both crude or commercial proteases are useful of deproteinization step, but commercially purified enzymes are expensive and sometimes less efficient compared to crude proteases, which usually get co-extracted with useful enzymes. Some studies add an additional chemical treatment to increase the DP efficiency, however the overall decrease in harsh chemicals still provide a greener alternative to the overall process. Chemical deproteination is more thorough than enzymatic deproteination, (Kaur and Dhillon 2014), which is why it is a worthwhile research to look into a combination of chemical and enzymatic processes, or a combination of different proteases, preferably with one of the enzymes being acid or alkaline stable.

Enzymatic modification of chitin (or chitosan)

Commercially obtainable enzymes are available for mediating the different steps of chitosan extraction including deacetylation and molecular weight degradation. Traditionally, chitin and chitosan were converted to oligosachharides using harsh chemicals such as hydrochlocric acid, nitrous acid, phosphorus acid, lactic acid, formic acid and hydrogen peroxide. The chemical processing not only is harmful to the environment, but also produces a range of products, including secondary compounds, and undesired low molecular weight products. Enzymatic modification of native chitin to chitosan, chitin oligosaccharide, chitosan oligosachharides, are gaining interest to obtain more soluble and bioactive chitin derivatives. Previously nonspecific enzymes like cellulases, hydrolases and even proteases were used to degrade the polymers into their oligopolymeric counterparts. However, the recent trends are to use specific regio-selective enzymes to produce COS and paCOS with desired degrees of polymerizations. Enzymes such as chitinases, chitosanases, chitin deacetylases, and the recently discovered lytic polysaccharide monooxygenases (LPMO) are reviewed in detail (Kaczmarek et al. 2019). Chitinases all hydrolyze glycosidic β-(2.4)-links of acetylated D-glucosamine units and produce chitobioases (disachharides of β-1,4-linked glucosamine units). Some chitinases are also able to create new links between small polysachharide fragments, making these an excellent candidate for producing COS (Arnold et al. 2020). Chitosanases (EC 3.2.1.132) constitute a family of enzymes capable of performing endohydrolysis in partially acetylated chitosan, from the reducing end and exo-β-D-glucosaminidase attack chitosan from its non-reducing end.

The sources of these chitin modifying enzymes are various and can be isolated from different microorganisms, including bacteria, cyanobacteria, fungi, and plants (Thadathil and Velappan 2014). LPMOs are enzymes that aid in the hydrolysis of chitin by oxidation, leading to the easy accessibility of chitin by chitinases.

In a study by Deng et al. (2020), chitinase rChit46 was used to recover chitin from shrimp shells, but it was successfully done only after deproteinization, since removal of the proteins exposed the chitin to the chitinase for enzymatic action. The natural chitin oligomer recovery rate was 89.9%. In another study recombinant chitinase LlChi18A from *L. lactis ssp*. was used for production of chitooligomers (Lv et al. 2016). In a comprehensive study using fourteen chitin deacetylases from bacterial, fungal and viral sources Hembach et al. (2017) produced chitosan oligosaccharides of very specific degree and pattern of polymerization. Bacterial chitosanase from *Bacillus licheniformis* GA11 was extracted and its activity optimized for shrimp chitosan depolymerization and further tests were carried out to evaluate the COS antioxidant, antibacterial and antifungal activity (Affes et al. 2020). In another study bacterial chitosanase was extracted from *Bacillus thuringiensis* and used to depolymerize colloidal chitosan (Olicón-Hernández et al. 2017).

Chitin deacetylases (ChDa) are a group of enzymes catalyzing the hydrolysis of acetamido groups of N–acetyl–D–glucosamine residues in chitin and chitosan whereas chitooligosaccharides deacetylases (CODa) perform the same catalysis on chitooligosaccharides. These enzymes and their mode of action can be found in the review by Kaczmarek et al. (2019), and these enzymes can be utilized for future research on COS and paCOS production. One of the factors to consider when using these chitinolytic enzymes is that they are unable to penetrate the crystalline chitin or chitosan structure, and pretreatment is usually carried out chemical or mechanically to produce "colloidal chitin/chitosan" or swollen chitin/chitosan. Another factor is that chitinase/chitosonases producing species by fermentation or extracted enzymes are both viable methods to produce oligopolymers of interest for potential commercial production (Arnold et al. 2020).

Biological extraction on non-crustacean sources

Fermentative extraction could potentially be carried out on non-crustacean, specifically fungal sources of chitosan. When fungal species and shrimp shells are combined in a single reactor, the fungi produce proteases, which helps produced deproteinized shells and hydrolyzed proteins. The proteins were a nutrient source to enhance fungal growth this in turn lowers the pH of the fermentation broth helping in demineralization process (Teng et al. 2001). In a recent study, dual extraction of crustacean and fungal chitosan was carried out, the fungal species *Mucor circinelloides* acting as a protease secreting source for prawn shells, and subsequently being cofermented with *Lactobacillus plantarum* and *Bacillus subtilis* (Yun Nian Tan et al. 2021).

Concluding remarks on biological extraction and modification of chitinous material

A major hurdle in the usage of the enzymes for hydrolyzing chitinous biomass, is that same enzymes from different species show different activities and the fact that the enzymes are relatively expensive. However, techniques like fermentation, co-fermentation or harvesting a microbe for its enzyme can greatly reduce the cost and nutrient requirement for production of the enzymes. Another major hurdle is that there are not too many for scaling up to commercial or pilot-scale, making this an aspect of further research. Furthermore, much of the literature has focused on valorizing marine waste, and usually looks at chitin as end-product. Future research could be carried out for enzymatic production of chitosan, COS and paCOS for biological and medical research due to increased biocompatibility, biodegradability, low toxicity, antimicrobial, antiviral, antitumor, and antioxidant activities (Kaczmarek et al. 2019).

Concluding remarks

Other than the methods mentioned in this review, there are two major avenues of green extraction: alternative solvents and physicochemical methods of extraction, which have been discussed in other review papers (Mohan et al. 2022). Chitosan from alternative sources such as fungal, mycotech products, or insects have been applied for biological and biomedical applications such as antimicrobial agent (Tayel et al. 2010; de Oliveira et al. 2014; Abdel-Mohsen et al. 2016; Chang et al. 2019), anticancer effects (Mora-Montes et al. 2011), anioxidant effects (Zimoch-Korzycka et al. 2016; Abdel-Gawad et al. 2017; Araújo et al. 2017), wound dressing applications (Tchemtchoua et al. 2011), tissue engineering (Nwe and Tetsuya, 2010) and many others. Futhermore, green solvents can be used for dissolution of chitin/chitosan, opening the avenue for formulating fibers (Shamshina et al. 2014; Zavgorodnya et al. 2017), nanofibers(Li et al. 2018), films (Yang et al. 2019; Haghighi et al. 2020), hydrogels (Wang et al. 2016; Azadi et al. 2018), scaffolds (Wang et al. 2017), sponges (Huang et al. 2018) and other shapes, thus paving the way for biological applications as shown in Fig. 4.

Green approaches to modifying chitosan to its more soluble and more bioactive derivatives are also now coming into attention. It is evident that only very few studies have been carried out where chitin and chitosan, or its derivatives,

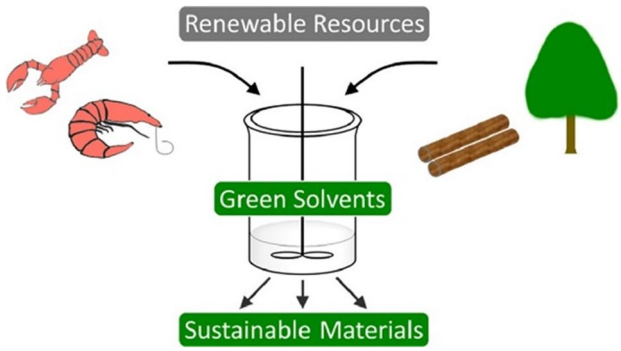

Fig. 4 Use of green solvents and natural chitosan for sustainable material formulations. Reprinted with permission from (Kostag and el Seoud 2021)

are extracted and formulated in fully ecologically friendly method, for biological applications. Thus, further research should focus on combining the alternative routes for production of chitin/chitosan along with green processes for modification for biological applications.

Author Contributions All authors contributed to the study conception and design. Data collection and analysis were performed by all the authors. The sections 'Introduction', 'Biological methods of Extraction', and 'Concluding Remarks' were drafted by NI; section; 'Alternative sources of Chitosan' by MH and SFT. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data availability Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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