Review

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Current state of chitin purification and chitosan production from insects

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Abstract

Chitin, and especially its deacetylated variant chitosan, has many applications, e.g. as carrier material for pharmaceutical drugs or as a flocculant in wastewater treatment. Despite its versatility and accessibility, chitin, the second most abundant polysaccharide on Earth, has so far been commercially extracted only from crustaceans and to a minor extent from fungi. Insects are a viable alternative source of chitin, but they have not been exploited in the past due to limited availability. Today however, for the sustainable production of animal feed, insect farming is being developed substantially. The availability of large quantities of insect biomass and chitin-rich side products such as exuviae and exoskeletons has been increasing. This review provides an overview of recently published studies of chitin extraction from insects, its subsequent conversion into chitosan and the primary analytical methods used to characterize insect-based chitin and chitosan. We have discovered a large number of research articles published over the past 20 years, confirming the increased attention being received by chitin and chitosan production from insects. Despite numerous publications, we identified several knowledge gaps, such as a lack of data concerning chitin purification degree and chitosan yield. Furthermore, analytical methods used to obtain physicochemical characteristics, structural information and chemical composition meet basic qualitative requirements but do not satisfy the need for a more quantitative evaluation. Despite the current shortcomings that need to be overcome, this review presents encouraging data on the use of insects as an alternative source of chitin and chitosan in the future.
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Keywords: chitin; chitosan; insects; purification; analysis

INTRODUCTION

Chitin is an important structural component of the cell wall of fungi and yeasts and the main carbohydrate component of arthropod exoskeletons.[1] After cellulose, it is the second most abundant biopolymer present in nature.[2] The production of chitin in the biosphere is estimated to be around 1000 billion (10^{11}) tons per year.[3] Chitin was isolated for the first time in 1799 from the shells of mollusks.[4] Chitosan, the deacetylated derivative of chitin, was obtained by Rouget in 1859 by heating chitin in alkaline medium; however, its chemical structure was determined only in 1950.[5] Despite this early discovery, the industrial production and commercialization of chitin and chitosan initially started in the 1970s.[6]

Chitin is a hard, inelastic, *N*-acetylated aminopolysaccharide (Fig. 1(a)) with high hydrophobicity, making it insoluble in water and most organic solvents.[7, 8] Fungi and invertebrates use these properties and incorporate chitin microfibrils to protect and strengthen their cell matrix or as components of mechanically resilient structures such as shells, cuticles, bones (in cuttlefish) and scaffolds (in sponges).[9, 10]

Based on various orientations of its microfibrils, chitin exists in nature in three crystalline allomorphic forms: α-, β- and γ-chitin (Fig. 1(b)). α-Chitin has antiparallel chains. It is responsible for the rigidity of the polymer and is the most abundant form.[11] β-Chitin consists of parallel chains, producing monoclinic crystals with intramolecular interactions (hydrogen bonds) in addition to intermolecular ones.[12] β-Chitin is found in the spines of diatoms, squid pens and pogonophoran tubes. γ-Chitin is a mixture of parallel and antiparallel chains combining the properties of both α-form and β-form[13]; it is present in fungi, yeasts and insect cocoons.[14–18]

After being isolated from natural sources, direct application of chitin is limited to a few applications, such as the production of scaffolds to support tissue regeneration[19] or for biological control of plant pathogens in agriculture,[20] which is due to its insolubility.

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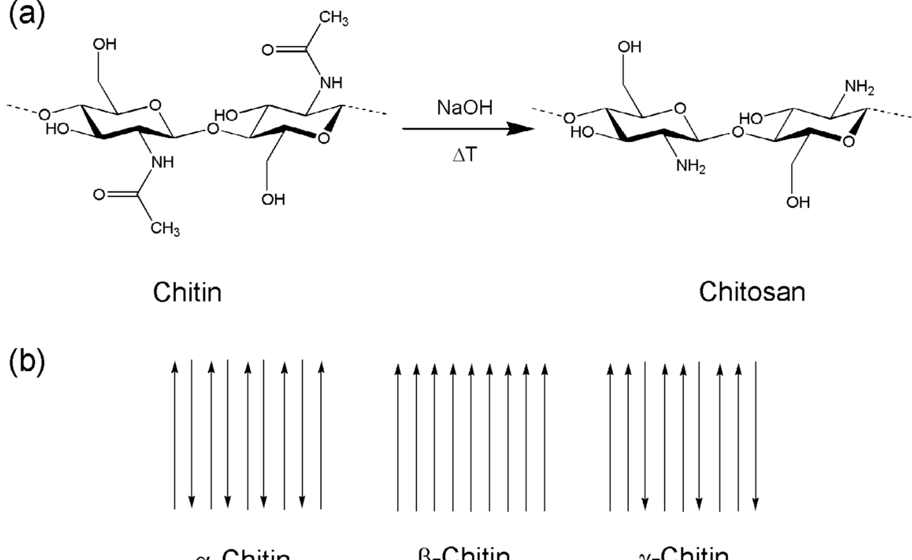

Figure 1. (a) Chitin and chitosan molecular structure. Chitin consists of *N*-acetylated-D-glucosamine (GlcNAc) and 2-amino-D-glucose (D-glucosamine, GlcN) linked by *β*-1,4 glycosidic bonds. Chitosan is the main deacetylated derivative of chitin. (b) Three crystalline allomorphic forms of chitin, with different microfibril orientations.

To widen its range of applications, chitin needs to be converted to more water-soluble derivatives with useful properties, primarily chitosan. Chitosan (Fig. 1(a)) is a cationic polysaccharide obtained from chitin by alkaline hydrolysis of the acetamido groups (deacetylation process). Due to an increased number of free primary amine groups, and partially due to a lower molecular weight, chitosan is soluble in slightly acidic solutions. To define and distinguish between chitin and chitosan, several researchers have set the threshold value of degree of acetylation to 50%: when the *N*-acetyl group content of the polysaccharide exceeds 50%, the polymer is defined as chitin; for values below 50%, it is called chitosan.[21, 22]

The physicochemical properties of chitin and chitosan may vary among samples, being affected by many factors such as the source of chitin and parameters of the purification process. Molecular weight is an important characteristic to consider as it affects chitosan viscosity[23] that is crucial to many of its applications and other important features such as antibacterial activity.[24] Native chitin from crustacean sources has a molecular weight exceeding 1000 kDa, while the molecular weight of crustacean-based chitosan ranges from 100 to 1000 kDa.[25, 26]

Due to its useful properties, such as biodegradability, biocompatibility, nontoxicity, adsorption capacity and antimicrobial activity,[1, 17] chitosan is attracting great attention for many applications within the agricultural, industrial, biotechnological and biomedical fields and in wastewater treatment.[1, 8, 27–31]

The antimicrobial activity of chitosan is one of its most exploited properties. Chitosan can inhibit the growth of human pathogens, such as *Escherichia coli*,[32, 33] *Staphylococcus aureus*,[34, 35] *Pseudomonas aeruginosa*[36] and *Aspergillus niger*.[37] Its antimicrobial activity makes chitosan suitable to be used not only in biomedical applications but also as a natural biopolymer coating material to preserve the quality and to extend the shelf-life of fresh food.[38] Chitosan-based active films against contamination and microbial spoilage have been successfully used in fruit, vegetable, egg and meat packaging.[39, 40]

Chitosan has also shown excellent potential for wound dressing. The potential of chitosan as a hemostatic topical dressing for animal tissues has previously been demonstrated: chitosan adheres to red blood cells, thus retrieving platelets for hemagglutination.[41] In cosmetics, chitosan finds application in the production of creams and lotions. It is used as a moisturizing and UV-protective agent for the skin.[42] A further application for chitosan is in wastewater treatment, where it is used as a flocculating agent owing to its ability to chelate cations and adsorb waste molecules from water, such as heavy metals.[31, 43]

Recently, chitosan has been proposed as an ecological finishing agent in the textile industry. It is used in working fabrics for hospitals or biological laboratories and for making sutures, threads and fibers in medical textiles.[44, 45] Chitosan is also used for antistatic finishing in work wear for employees of the electronic sector.[46]

Three main chitin sources are available

Currently, the main commercial source of chitin and chitosan comprises waste streams from the marine food industry – mainly exoskeletons of crustaceans.[47] Annual world production of crustaceans for human consumption was an estimated 8 million tons in 2016,[48] of which 40% comprised waste exoskeletons[49] with a chitin content of 15–40%.[50] However, the availability of fishery waste is highly seasonal, as commercial crustacean fishing starts in spring, after the spawning season.[51] Moreover, the sustainability of crustacean farming is currently under debate.[49]

The global market for chitin and chitosan is expected to reach a volume of $4.2 billion by 2021, with a compound annual growth rate of 15.4%,[52] intensifying the need for a search of other sources to satisfy the growing market.

Fungi are the second main source of chitin after crustaceans. Several research activities have focused on fungi and their commercial value as they have attracted attention as an alternative and vegan source of chitin and chitosan.[53] Chitin makes up between 1 and 15% of fungal cell wall mass[54] and its structure is comparable to that in crustaceans.[55] Even though not all fungi

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contain chitin, it is widely distributed in various fungal phyla such as Basidiomycota, Ascomycota and Zygomycota.[55] Similar to crustacean chitin, severe conditions are required to obtain chitosan from fungal chitin. In contrast, chitosan can be directly isolated from the cell wall of some fungal species without requiring the cleavage of the acetyl groups. Among them, the most investigated species for direct chitosan production include *Absidia* spp. (zygomycetes), *A. niger* (ascomycetes), *Mucor rouxii* (zygomycetes), *Rhizophus oryzae* (zygomycetes) and *Lentinus edodes* (basidiomycetes).[56–59] Nevertheless, the production of fungal chitin and chitosan has not yet been scaled up to the industrial level.[53]

In addition to crustaceans and fungi, insects are another promising and sustainable source of chitin and chitosan, although they have not received much attention previously. Insects present some advantages compared to crustaceans, as they are not subject to seasonality, and can be easily bred owing to their high fertility and reproductive rate; moreover, insect rearing facilities are being launched worldwide.[60] Notably, as bioconverters – reared for organic waste management and animal feed production – insects can be exploited as a valid alternative to crustaceans as a source for chitin and chitosan for greater ecological and economic sustainability.[61]

Arthropods, including centipedes[62, 63] and woodlice,[64, 65] have been investigated as sources of chitin. Notably, the body segments of large centipedes are suitable for the production of three-dimensional chitin rings.[62] In addition, chitin has been extracted from poriferans,[66] bryozoans[67] and tardigrades[68] and from guano of insectivorous bats.[69]

Quo vadis insect chitin?

Economic value linked to beneficial insects has been known to humans for a long time. The commercial production of silk from *Bombyx mori* originated in China during the Neolithic period.[70] Humans have learned to farm several insect species and exploit them for specific applications with the course of time. Production of biocontrol insects started in the middle of the 20th century. For example, mass production of *Cochliomyia hominivorax* for biocontrol started in Florida during the late 1950s.[71] Organized, large-scale production of insects for human and animal nutrition has been more recent: Protix, a Dutch company, launched the first facility in 2015.

Some industries in the domain of beneficial insect breeding are witnessing rapid growth. For example, the market for edible insects is estimated to exceed $522 million in 2023.[72] According to a report published in 2016, globally, more than 120 registered companies are involved in the business of farming and processing and/or marketing insects for animal and human nutrition.[73] In 2019, approximately 6kt of insect protein meal was produced in Europe[74] to be used for animal nutrition, from the black soldier fly (*Hermetia illucens*), the yellow mealworm (*Tenebrio molitor*) and, to a smaller extent, the lesser mealworm (*Alphitobius diaperinus*).[75] In particular, the black soldier fly is processed by around 80% of all EU insect-producing companies.[75] *H. illucens* could be grown on a wide range of organic side streams and contributes to a circular economy.[76, 77] Exoskeletons from *H. illucens* larvae contain up to 35% chitin,[78] which means that it is one of the main compounds that could be isolated from the byproducts of the insect farming industry.[73]

Thus, chitin-rich byproducts from insect farming present a new and sustainable source of commercial chitin. Given the sustainability aspect and the expected rise in insect production, byproducts from insect farming present a very interesting source of chitin for the future.

The inner soft tissues of insects are covered by a hard, protective layer called the exoskeleton. The exoskeleton has several functions in insect bodies, including but not limited to: (i) as a protective covering and (ii) as a facilitator of metamorphosis. The exoskeleton is rich in chitin and is shed from the body during metamorphosis.[79] Chitin is contained in the procuticle, the innermost layer of the cuticle, which is in turn the outermost layer of the arthropod exoskeleton.[80, 81] In its native form, chitin is arranged into microfibers embedded in a protein matrix.[82] To extract chitin from the arthropod cuticle, the purification process remove proteins, lipids, minerals, pigments and catechols contained therein.[83] Whole insects generally contain 30–60% protein,[84] 10–25% lipid,[84] 5–25% chitin,[50, 78, 85] 5–10% catechols[85] and 2–10% minerals such as calcium, phosphorus, potassium and magnesium salts.[84, 86]

To date, little has been reported on the extraction methods for insect chitin and its physicochemical properties. Here, a total of 52 papers reporting chitin purification and chitosan production from 58 insect species were collected, summarized and analyzed (Tables 1 and 2).

CHITIN PURIFICATION

Various types of chitin purification processes can be performed, such as physical, biotechnological and chemical methods and a combination of these. Physical techniques such as crushing and stirring are used in parallel with chemicals or catalysts. Biotechnological extraction and deacetylation of chitin has been gaining interest as an environmentally friendly alternative to chemical processes. The biotechnological methods are mainly based on the use of microbial proteases or whole microorganisms for the removal of proteins and the application of deacetylases for the deacetylation of chitin. However, the biotechnological processes developed so far produce lower yields, are time-consuming and result in products of lower purity.[83, 134] Notably, enzymatic deacetylation of chitin using deacetylases has proven to be unsuitable for chitosan production.[135, 136]

Chemical processes, utilizing acidic and alkaline solutions, are currently the most applied on an industrial scale to produce large amounts of chitin and chitosan from crustacean shells[134, 137] (Fig. 2). Typically, chemical treatment provides pure chitin and chitosan, although it produces large volumes of waste due to the high concentrations of chemicals used.[134, 137, 138] The literature reviewed here describes only chemical methods for chitin and chitosan production from insects.

Chemical methods for extracting chitin from insect exoskeletons are similar to those used for marine sources and consist of two steps: demineralization and removal of proteins. In the first step, minerals contained in the cuticle are removed with diluted acids. Various mineral acids (e.g. hydrochloric acid) or organic acids (e.g. acetic acid) can be used for this purpose. The removal of proteins is carried out with alkaline treatments, mainly applying diluted sodium hydroxide solution. Deproteinization treatment can simultaneously extract part of the dyes and soluble lipids contained in the exoskeleton. For prawn shells, two-step purification can be performed in reverse order without affecting the properties of chitin.[83]

An additional step of bleaching can be performed to remove residual pigments and improve the color of purified chitin, using

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Table 1. Methods for chitin purification from insects and respective yields

Raw material		Demineralization			Deproteinization				
Insect species	Stage/ body part	Reagent and conc.	Temp. (°C)	Duration (h)	Reagent and conc.	Temp. (°C)	Duration (h)	Chitin yield (dry chitin weight/dry insect weight) (%)	Ref.
Musca domestica	Larvae	—	—	—	1 M NaOH	95	6	—	87
Shistocerca gregarea	Adults							—	
Nezara viridula	Adults							—	
Periplaneta americana	Adults	0.9 M CH_3COOH	—	—	1.8 M KOH	40	—	—	88
Blatella germanica	Adults							—	
Vespa orientalis	Adults							—	
Gryllus bimaculatus	Adults	—	—	—	1 M NaOH	95	3	5.1	89
Apis mellifera	Adults	1 M HCl	Room temp.	1	1 M NaOH	80	6–64	51.0–77.2	90
Dociostaurus maroccanus	Adults	2 M HCl	55	1	2 M NaOH	50	18	12.0, 14.0	91
Hermetia illucens	Larval exoskeleton	0.5 M CH_2O_2	Room temp.	1	2 M NaOH	80	2	31.0–35.0	92
Bombyx eri	Larvae	1 M HCl	80	0.6	1 M NaOH	80	24	3.3	93
Acheta domesticus	Adults	0.1 M $C_2H_2O_4$	Room temp.	3	1 M NaOH	95	—	4.3–7.1	94
Colophon sp.	Adults	2 M HCl	Room temp.	6	1.5 M NaOH	10	24	—	18
Melolontha melolontha	Adults	4 M HCl	75	—	1 M NaOH	150	18	—	64
Agabus bipustulatus	Adults							14.0–15.0	
Anax imperator	Adults							11.0–12.0	
Hydrophilus piceus	Adults	1 M HCl	90	1	1 M NaOH	110	18	19.0–20.0	95
Notonecta glauca	Adults							10.0–11.0	
Ranatra linearis	Adults							15.0–16.0	
Leptinotarsa decemlineata	Larvae, adults	2 M HCl	65–75	2	2 M NaOH	80–90	16	7.0, 20.0	96
Vespa crabro	Adults							—	
Vespa orientalis	Adults	2 M HCl	75	2	4 M NaOH	150	18	—	97
Vespula germanica	Adults							—	
Ailopus simulatrix	Adults							5.3	
Ailopus strepens	Adults							7.4	
Duroniella fracta	Adults							5.7	
Duroniella laticornis	Adults							6.5	
Oedipoda miniata	Adults	4 M HCl	75	1	2 M NaOH	175	18	8.1	98
Oedipoda caerulescens	Adults							8.9	
Pyrgomorpha cognata	Adults							6.6	
Celes variabilis	Adults							6.6–9.9	
Decticus verrucivorus	Adults	4 M HCl	75	2	4 M NaOH	150	20	10.0–11.8	13
Melanogryllus desertus	Adults							4.7–7.3	

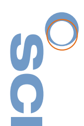

Table 1. Continued

Raw material		Demineralization			Deproteinization				
Insect species	Stage/ body part	Reagent and conc.	Temp. (°C)	Duration (h)	Reagent and conc.	Temp. (°C)	Duration (h)	Chitin yield (dry chitin weight/dry insect weight) (%)	Ref.
	Legs							13.2	
	Thorax							6.8	
Apis mellifera	Head	2 M HCl	80	6	2 M NaOH	100	20	8.9	99
	Abdomen							8.6	
	Wings							7.6	
Argynnis pandora	Wings	2 M HCl	50	24	2 M NaOH	50	24	22.0	100
	Other body parts							8.0	
Calliptamus barbarus	Adults	1 M HCl	100	0.5	1 M NaOH	80–90	21	20.5	101
Oedaleus decorus	Adults							16.5	
Palomena prasina	Adults	2 M HCl	100	2	2 M NaOH	140	20	10.8	67
Omophlus sp.	Adults	2 M HCl	50	4	2 M NaOH	100	20	—	102
Vespa crabro	Larvae, pupae, adults	1 M HCl	50	6	1 M NaOH	60	16	2.2, 6.2, 10.3	103
Drosophila melanogaster	Adults	2 M HCl	40	3	3 M NaOH	70	20	7.8	104
	Whole body (f, m)							15.7 f, 16.6 m	
	Antennae (f, m)							5.9 f, 10.5 m	
	Head (f, m)							16.0 f, 15.9 m	
	Eyes (f, m)							8.9 f, 8.9 m	
Melontha sp.	Thorax (f, m)	2 M HCl	60	20	2 M NaOH	100	20	13.8 f, 17.5 m	105
	Abdomen (f, m)							7.1 f, 6.5 m	
	Elytra (f, m)							37.9 f, 40.1 m	
	Hindwings (f, m)							17.9 f, 17.3 m	
	Legs (f, m)							17.8 f, 16.1 m	
Blaberus giganteus	Wings	1 M HCl	Room temp.	24	2 M NaOH	90	9	26.9	106
	dorsal pronotum							21.2	
Hylobius abietis	Adults	2 M HCl	Room temp.	—	2 M NaOH	—	2	27.9	107
Periplaneta americana	Wings	4 M HCl	75	2	4 M NaOH	150	20	18.0	108
	other body parts							13.0	
Hermetia illucens	Larvae	0.5 M HCl	Room temp.	2	1.9 M NaOH	50	2	46.0 (chitin), 7.0 (amorphous chitin)	109
Musca domestica	Adults	2 M HCl	Room temp.	3	1.25 M NaOH	95	3	7.7–8.5	110
Gryllus bimaculatus	Adults	2 M HCl	Room temp.	3	1.25 M NaOH	95	3	2.4	111
Holotrichia parallela	Adults	1 M HCl	100	—	1 M NaOH	80	—	15.0	112
Cicada	Sloughs							—	
Bombyx mori	Chrysalis	1 M HCl	30	2	1 M NaOH	90	2	—	113
Tenebrio molitor	Larvae							—	
Grasshopper	Adults							—	
Catharsius molossus	Adults	1.3 M HCl	80 + room temp.	0.5 + 12	4 M NaOH	90 + room temp.	6 + 12	24.0	114

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Table 1. Continued

Raw material		Demineralization			Deproteinization				
Insect species	Stage/ body part	Reagent and conc.	Temp. (°C)	Duration (h)	Reagent and conc.	Temp. (°C)	Duration (h)	Chitin yield (dry chitin weight/dry insect weight) (%)	Ref.
Bombus terrestris	Adults	1 M HCl	100	0.3	1 M NaOH	85	24	—	115
Schistocerca gregaria	Adults							12.2	
Apis mellifera	Adults	1 M HCl	Room temp.	—	1 M NaOH	100	8	2.5	116
Calosoma rugosa	Adults							5.0	
Ephestia kuehniella	Adults	1 M HCl	100	0.3	1 M NaOH	85	1	9.5–10.5	117
Cicada lodosi	Adults							4.8	
Cicada mordoganensis	Adults							6.5	
Cicadatra platyptera	Adults	2 M HCl	100	2	2 M NaOH	100	20	8.8	118
Cicadatra atra	Adults							6.7	
Cicadatra hyalina	Adults							5.5	
Cicadivetta tibialis	Adults							5.9	
Brachystola magna	Adults	1 M HCl	97	0.5	1 M NaOH	82	24	10.4	119
Apis mellifera	Adults	—	—	—	15 M NaOH	—	—	11.4–36.8	120
Calliphora erythrocephala	Larvae	—	—	—	1 M NaOH	50	2	12.2	121
Bombyx mori	Larvae	1 M HCl	100	—	1 M NaOH	80	—	2.6–4.3	122
Cicada	Sloughs	1 M HCl	100	0.3	1 M NaOH	80	36	36.0	11
Tenebrio molitor	Larvae, superworm, adults	2 M HCl	Room temp.	24	3.7 M NaOH	80	24	4.6, 3.9, 8.4	123
Allomyrina dichotoma	Larvae, pupae, adults							10.5, 12.7, 14.2	
Chrysomya megacephala	Larvae	0.1 M $C_2H_2O_4$	—	3	1 M NaOH	95	6	—	124
Hermetia illucens	Larvae, prepupae, pupae	1 M HCl	100	0.5	1 M NaOH	80	24	3.8, 4.7, 6.3	125
Zophobas morio	Larvae	1 M HCl	35	0.5	0.5–2 M NaOH	80	20	4.7–5.2	126
Mayfly	Adults	2 M HCl	50	—	2 M NaOH	100	—	10.2	127
Hermetia illucens	Pupal exuviae, adults	1 M HCl	—	—	1 M NaOH	80	24	—	128
Clanis bilineata	Larval skins	2 M HCl	Room temp.	24	3.7 M NaOH	60	24	—	129
Cryptotympana atrata	Sloughs	2 M HCl	Room temp.	24	3.7 M NaOH	60	24	—	130
Musca domestica	Larvae	—	—	—	1 M NaOH	100	3	—	131
Bombyx mori	Pupal exuviae	1 M HCl	100	—	1 M NaOH	80	3	15.0–20.0	132
Beetle	Larvae							15.0–20.0	
Hermetia illucens	Prepupae	NADESs	50–80	2	NADESs	50–80	2	6.0–26.0	133

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reagents such as sodium hypochlorite, acetone and hydrogen peroxide.

Prior to the chitin purification process, insect samples are generally pretreated. Pretreatments include cleaning by washing with water and detergent, drying in an oven or at ambient temperature and grinding into powder to increase accessibility to the chemical agents. Solvent penetration into the particles and thus purification efficiency are greatly affected by corn size. Kim *et al.*[110] obtained a higher demineralization efficiency and chitosan with a higher degree of deacetylation using ground insect samples instead of non-ground ones. A particle size lower than 200–250 μm is suitable for chitin purification.[90, 116, 117, 132] A fat removal step may be required, especially for those insect samples rich in lipids, such as whole larvae. Larvae can be boiled and passed through an extruder or an oil press to separate unpurified chitin from the liquid fraction, containing mainly lipids and some proteins.[93, 109]

Demineralization

Crustacean shells contain a substantial amount of minerals, which can be up to 50% in crab and shrimp shells.[50] In contrast, insects have a much lower mineral content, generally ranging between 2 and 10% for whole insects. However, this value varies depending on the species and the stage of development.[84, 86]

The demineralization of insect samples comprises the decomposition of minerals into their respective water-soluble salts. The solubilized salts can be separated from chitin by filtration and washing of the solid phase. Acidic treatment also releases catechol compounds and leads to a slight discoloration of biomass.[115] The demineralization process can be affected by the type and concentration of acid used, treatment time and temperature, particle size of the sample and the solute-to-solvent ratio.[139]

Hydrochloric acid is the most preferred reagent used for the demineralization of insect exoskeletons (Table 1). Hydrochloric acid has also been one of the most widely used acids for the demineralization of crustacean shells on an industrial scale.[134] Because of its high environmental impact, hydrochloric acid has been substituted in many cases by organic acids. Furthermore, hydrochloric acid can have detrimental effects on the structure and chemical composition of chitin,[140] as has been confirmed by investigations of Percot *et al.*,[141] who reported a lower molecular weight and a lower degree of acetylation of chitin after demineralization. As a rule of thumb, the harsher the demineralization treatment – in terms of pH, duration and temperature – the higher is the degree of hydrolysis and the worse are the aforementioned effects on chitin.[142]

In contrast to the frequently used hydrochloric acid, only four investigations have reported the use of organic acids for the demineralization of insect biomass. Ibitoye *et al.*[94] and Song *et al.*[124] used oxalic acid and Badawy and Mohamed[88] and Hahn *et al.*[92] used acetic acid and formic acid, respectively.

In most cases, the concentration of the acidic solution used for demineralization is 1–2 mol L^{-1}. Few papers have reported the application of a higher concentration of 4 mol L^{-1}.[13, 64, 98] The solute-to-solvent ratio depends on the acid concentration, as it needs two molecules of hydrochloric acid to convert one molecule of calcium carbonate, the main mineral component of the insect exoskeleton, into calcium chloride, carbon dioxide and water.[143]

The time taken for demineralization treatment is usually short. Most protocols have reported an incubation time between 30 min and 3 h. In a few cases, the treatment lasted for up to 6 h,[99, 103] 12 h[114] and 24 h.[101, 123, 144] The range of temperature used for demineralization varies widely from room temperature to 100 °C. High temperatures are used for very short periods (20–30 min), as reported by Kaya *et al.*[100] and Monter-Miranda *et al.*[119] In contrast, longer treatment periods (12–24 h) are applied for incubations done at room temperature.[106, 114, 123] High temperatures promote the penetration of the solvent into the chitin matrix[145]; however, they can cause polymer degradation.[25]

The efficiency of demineralization (DME) can be evaluated by assessing the mineral content of insect samples before (MC_{BT}) and after treatment (MC_{AT}) according to the following equation:

$$\mathrm{DME}\,(\%) = \frac{\mathrm{MC_{BT}}(\%) - \mathrm{MC_{AT}}(\%)}{\mathrm{MC_{BT}}(\%)} \times 100 \tag{1}$$

From the scanty data available in the literature on the efficiency of the demineralization of insect biomass, we can observe that the highest efficiency (86–98%) was achieved by Zhou *et al.*,[133] using natural deep eutectic solvents on *H. illucens* prepupae (Table 1). Of the organic acids used, oxalic acid, used by Ibitoye *et al.*,[94] resulted in a higher degree of demineralization compared to that reported by Kim *et al.*[110,111] with hydrochloric acid, although a lower concentration of oxalic acid had been used. Demineralization efficiency for the *H. illucens* larval exoskeleton, reported by Hahn *et al.*,[92] was similar to that obtained by Ibitoye *et al.*,[94] using formic acid. However, additional data on the efficiency of insect demineralization are not available. Thus, future studies should focus on assessing the suitability and optimization of the current methods. Precise evaluation of the efficiency of various acids will enable choosing acids with a lower environmental impact but guarantee good demineralization. For instance, Mahmoud *et al.*[140] and Ameh *et al.*[146] have reported that the efficiency of demineralization of shrimp shells using lactic or acetic acids is comparable to that obtained using hydrochloric acid. Values of demineralization efficiency for shrimp shells, using either hydrochloric acid or acetic and lactic acid, as reported by Mahmoud *et al.*,[140] are similar to those obtained by Ibitoye *et al.*[94] with oxalic acid using insect samples. Organic acids, namely lactic, acetic and oxalic acids, can therefore be a valid alternative to hydrochloric acid for the demineralization of insect biomass. The utilization of organic acids also provides other benefits as they are less harmful to the environment, can preserve the characteristics of purified chitin, can be produced from low-cost biomass and the extracted organic salts can be used for other applications.[140]

Once the minerals have been solubilized and removed, the insect biomass is washed with distilled water until its pH is restored to neutral. After the neutralization step, insect samples are subjected to deproteinization.

Protein removal

Deproteinization of insect biomass is commonly achieved using alkaline solutions. A wide range of chemicals has been tested as deproteinization reagents with crustacean samples, including sodium hydroxide, sodium carbonate, sodium bicarbonate, potassium hydroxide, potassium carbonate, calcium hydroxide, sodium sulfite, sodium bisulfite, trisodium phosphate and sodium sulfide.[139] As well as for demineralization, the efficiency of deproteinization depends on the concentration of the alkali, solid-to-solvent ratio and time and temperature of the treatment.[147] Although high temperatures are crucial for deproteinization efficiency, they can cause undesirable side reactions if combined

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Table 2. Methods for chitin deacetylation and characteristics of respective chitosan

Raw material		Deacetylation						
Insect species	Stage/body part	Reagent and concentration	Temperature (°C)	Duration (h)	Chitosan yield (%)	Deacetylation degree (%)	Molecular weight (kDa)	Ref.
Musca domestica	Larvae	15 M NaOH	70	8	—	90	—	87
Gryllus bimaculatus	Adults	19–25 M NaOH	—	15	41.7 (from chitin)	56–85	50–190	89
Dociostaurus maroccanus	Adults	22 M NaOH	150	4	81.7 (from chitin)	64	7	91
Hermetia illucens	Larval exoskeleton	12 M NaOH	120–140	3–6	8.0–16.0 (from initial biomass)	43–72	—	92
		10 M NaOH	4 °C	12	4.0 (from initial biomass)	34	—	
Acheta domesticus	Adults	—	—	—	2.3–5.8 (from initial biomass)	80	—	94
Agabus bipustulatus	Adults				71.0 (from chitin)		—	
Anax imperator	Adults				67.0 (from chitin)		—	
Hydrophilus piceus	Adults	22 M NaOH	120	2	74.0 (from chitin)	74–78	—	95
Notonecta glauca	Adults				69.0 (from chitin)		—	
Ranatra linearis	Adults				70.0 (from chitin)		-	
Leptinotarsa decemlineata	Larvae	19 M NaOH	100	3	67.0 (from chitin)	76	3	96
	Adults				72.0 (from chitin)	82	3	
Calliptamus barbarus	Adults	19 M NaOH	130	2	74.0–75.0 (from chitin)	70–75	—	101
Oedaleus decorus	Adults				75.0–76.0 (from chitin)		—	
Drosophila melanogaster	Adults	22 M NaOH	150	48	70.9 (from chitin)	—	—	104
Hylobius abietis	Adults	22 M NaOH	100	4	86.2 (from chitin)	81	7	107
Hermetia illucens	Larvae	19 M NaOH	100	2	80.0 (from chitin), 32.0 (from amorphous chitin)	90	160	109
Musca domestica	Adults	19 M NaOH	95–105	3–5	6.8 (from initial biomass)	33–97	—	110
Gryllus bimaculatus	Adults	19 M NaOH	100	3	1.8 (from initial biomass)	—	308	111
Cicada	Sloughs	22 M NaOH	100	8	28.2 (from initial biomass)	84	38	113
Bombyx mori	Chrysalis				3.1 (from initial biomass)	85	4	
Tenebrio molitor	Larvae				2.5 (from initial biomass)	86	40	
Grasshopper	Adults				5.7 (from initial biomass)	90	39	
Catharsius molossus	Adults	18 M NaOH	25–90	247	—	95	450	114
Schistocerca gregaria	Adults	19 M NaOH	100	8	—	98	—	116
Apis mellifera	Adults				—	96	—	
Calosoma rugosa	Adults				—	95	—	
Brachystola magna	Adults	15 M NaOH + 0.25 g $NaBH_4$	105–110	—	8.1 (from initial biomass)	57	26	119
Apis mellifera	Adults	19 M NaOH	150	1	20.0–30.0 (from chitin); 16.0–25.0 (from bleached chitin)	75–84	200–250	120
Calliphora erythrocephala	Larvae	19 M NaOH	100–120	1–4	66.7 (from chitin)	63–81	263–413	121
Bombyx mori	Larvae	15 M NaOH +1 g/L $NaBH_4$	100	—	—	78–97	3290–5900	122

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Table 2. Continued

Raw material		Deacetylation						
Insect species	Stage/body part	Reagent and concentration	Temperature (°C)	Duration (h)	Chitosan yield (%)	Deacetylation degree (%)	Molecular weight (kDa)	Ref.
Tenebrio molitor	Larvae, superworm, adults	21 M NaOH	90	9	80, 83.3, 78.3 (from chitin)	75, 76, 76	—	123
Allomyrina dichotoma	Larvae, pupae, adults				83.4, 83.4, 75.0 (from chitin)	76, 76, 75	—	
Chrysomya megacephala	Larvae	25 M NaOH	90	9	26.2 (from initial biomass)	88–90	501	124
Zophobas morio	Larvae	19 M NaOH	90	30	65.0–75.0 (from chitin)	64–81	—	126
Mayfly	Adults	22 M NaOH	150	6	78.4 (from chitin)	84	4	127
Clanis bilineata	Larval skins	21 M NaOH	110	4	—	—	—	129
Cryptotympana atrata	Sloughs	21 M NaOH	110	4	—	—	—	130
Musca domestica	Larvae	15 M NaOH + $NaBH_4$ 0.75 g/L	110	4	60.0–70.0 (from chitin)	83	—	132

with very long incubation times. These include partial deacetylation of chitin, hydrolysis of the biopolymer (lowering its molecular weight) and change of characteristics.[139, 148]

Sodium hydroxide is the most widely used base for deproteinization of crustacean biomass for the industrial production of chitin.[149] It has also been widely used for chitin purification from insects (Table 1). Badawy and Mohamed[88] are the only researchers to have used potassium hydroxide to deproteinize insect samples. The use of potassium hydroxide has also been suggested by Fu *et al.*[150] and Castillo *et al.*[151] as a more eco-friendly alternative to sodium hydroxide for shrimp deproteinization, as the liquid waste generated using potassium hydroxide is suitable for use as a fertilizer, owing to its high phosphorus, potassium and nitrogen content.[150] However, major deproteinization processes of insect samples have been performed using sodium hydroxide in low concentrations (0.5–2 mol L^{-1}). In a few cases, a concentration of 4 mol L^{-1} has been used.[13, 64, 97] The incubation time required for the deproteinization of insect biomass varies greatly from a few hours to a few days; however, the treatment typically lasts for 16–20 h (Table 1). Using the same working conditions, long deproteinization times applied to crustacean biomass have been reported to lead to a higher loss of proteins compared to shorter treatments.[152] The deproteinization reaction is generally performed at 80–100 °C, with a few exceptions being 40 °C[88] or 175 °C.[98]

As well as for demineralization, the efficiency of deproteinization (DPE) can be evaluated by measuring the protein content of insect samples before (PC_{BD}) and after deproteinization (PC_{AD}), according to the following equation:

$$\mathrm{DPE}\,(\%) = \frac{\mathrm{PC_{BD}}(\%) - \mathrm{PC_{AD}}(\%)}{\mathrm{PC_{BD}}(\%)} \times 100 \qquad (2)$$

Deproteinization efficiency of insect biomass has been mentioned only by Kim *et al.*[110,111] In both cases a maximum efficiency of 86–87% was achieved by applying 1.25 mol L^{-1} sodium hydroxide for 3 h at 95 °C on adult specimens of *M. domestica*[110] and *G. bimaculatus*.[111] These values are similar to those obtained by Zhou *et al.*,[133] who used natural deep eutectic solvents to remove proteins from *H. illucens* prepupae. The deproteinization efficiency of sodium hydroxide on crustacean shells at both high and room temperature was 71–76%.[149]

Results from chitin purification

Information is lacking on the results obtained from chemical demineralization and deproteinization of insect samples. Most papers have reported only chitin yield as the end result of the purification process (Table 1). Chitin yield is measured as the percentage ratio of dry weight of chitin and dry weight of the source material. Chitin yield from insect biomass varied from a minimum of approximately 2% – obtained from *Vespa crabro* larvae,[103] *G. bimaculatus* adults,[111] *Apis mellifera* adults[116] and *B. mori* larvae[122] – to a maximum of 36% obtained from *A. mellifera* adults[120] and cicada sloughs.[11]

However, most authors have reported a chitin yield of between 5 and 15% (Table 1). Yields of chitin extracted from shells of crustaceans, mainly shrimps, prawns and crabs, varied from 5 to 32%.[26, 64, 121, 147, 153–156] Chitin content can vary widely depending on the species, developmental stage and body part of the crustacean.[91, 103, 153, 156] For example, Thirunavukkarasu and Shanmugam[156] reported that the yield of chitin was higher from

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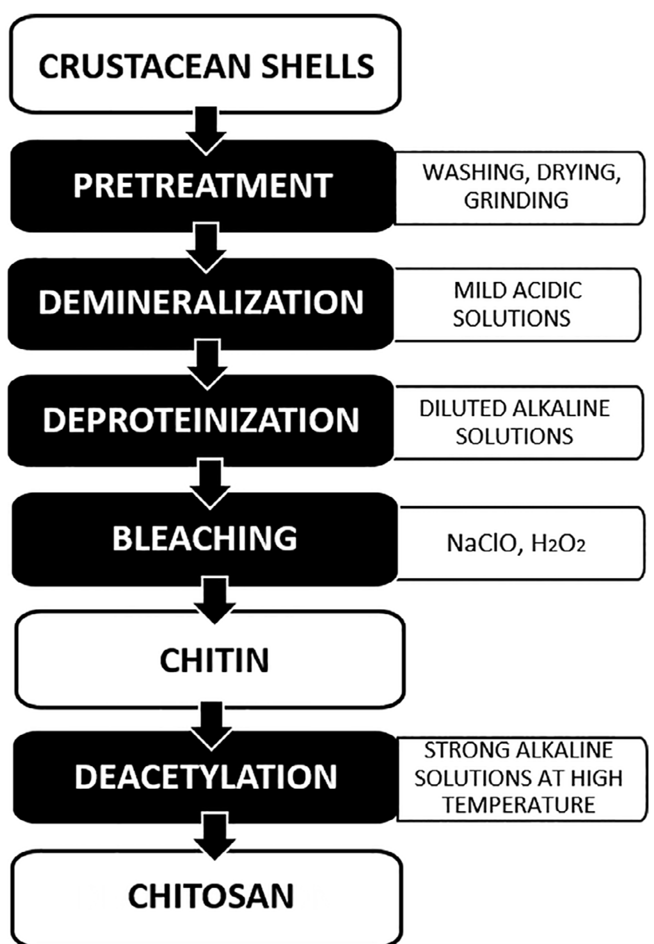

Figure 2. Industrial process for chitin purification and chitosan production from crustacean shells.

the cuticle of crab legs than from its carapace and claws. These findings are in accordance with the results of Kaya *et al.*[99] who found a higher chitin content in the legs than in other body parts of honeybees. Thus, the chitin content of a body part correlates with the mechanical load on that body part.

Only Huet *et al.*[93] and Kaya *et al.*[103] have measured the degree of purification for chitin extracted from *Bombyx eri* larvae and three developmental stages of *V. crabro*, respectively (Table 1). Using various times and temperatures for both demineralization and deproteinization treatments, they obtained similar results, that is, between 93 and 97%. Using natural deep eutectic solvents, Zhou *et al.*[133] achieved a slightly lower degree of purification (74–91%) from *H. illucens* prepupae. The lack of quantitative assessment of the purity of insect-based chitin makes it difficult to evaluate the suitability of the methods.

After demineralization and deproteinization, chitin can be directly deacetylated to chitosan or can be bleached to improve its color and remove the residual lipids.

Bleaching as the final step

Many kinds of pigments and structural colors are involved in the coloration of insect cuticle. They originate from the tyrosine-mediated cuticle-tanning pathway, such as melanins, or originate from 3,4-dihydroxyphenylalanine and dopamine during the process of cuticular tanning and sclerotization.[157] During demineralization and deproteinization treatments, a small amount of pigment and lipid is removed; however, chitin retains a brownish appearance. For commercial purposes, the color of chitin and chitosan is required to be as white as possible. Thus, an additional step of bleaching is used to remove residual pigments and improve chitin, and thus chitosan, color.

Industrial methods for purifying chitin from crustacean waste include the use of sodium hypochlorite or hydrogen peroxide as bleaching agents.[158, 159] A few papers have reported the use of hydrogen peroxide combined with hydrochloric acid for bleaching insect samples.[115, 120] Sometimes sodium hypochlorite[11, 94, 106, 124] or ammonium peroxidisulfate[89] are used, but insect decolorization is performed using a mixture of methanol–chloroform[91, 108, 109, 118, 127] or alcohol–chloroform.[64, 97] Treatment with potassium permanganate and oxalic acid is also done frequently.[87, 90, 112–114, 117, 128]

Even though they are often used, the approaches using organic solvents have no or marginal bleaching efficiency, as they do not break the bonds between chitin and tannins or catecholamines.

Bleaching treatment of insect samples is performed at room temperature for a short duration (40–90 min).[99, 103, 106, 118] Higher temperatures were applied by Chae *et al.*[89] (50 °C for 30 min with ammonium peroxidisulfate) and Nemtsev *et al.*[120] (75 °C for 1 h with hydrogen peroxide). A combination of potassium permanganate and oxalic acid has also been used, where the bleaching step was split into two steps: treatment with potassium permanganate at room temperature and with oxalic acid at high temperatures (60–70 °C).[90, 114]

Evaluation of the success of the bleaching step using various reagents has not been provided. The bleaching effect of a decolorization treatment can be quantitatively evaluated by measuring the L^*, a^* and b^* values of a sample using a colorimeter, according to the CIELab color system. CIELab is a color space that expresses colors as three values: L^* for lightness, a^* from green to red and b^* from blue to yellow. From these values, the whiteness index can also be calculated.[160]

Information on the effect of chitin bleaching on the yield and characteristics of chitosan has been given only by Nemtsev *et al.*[120] Part of the chitin extracted from honeybee corpses was bleached using 3% hydrogen peroxide for 1 h at 75 °C. The yield of chitosan derived from non-bleached chitin (20–30%) was slightly higher than that of chitosan produced from bleached chitin (16–25%); the degree of deacetylation of the two types of chitosan was similar.[120] Furthermore, chitin bleaching can greatly affect the viscosity of solubilized chitosan.[120, 159]

After the bleaching treatment is complete, chitin is dried, and characterized to investigate its suitability for a desired application. Otherwise, chitin can be deacetylated to afford chitosan.

CONVERSION OF CHITIN INTO CHITOSAN

Chitosan is obtained from the deacetylation of chitin (i.e. the removal of acetyl groups from the chitin polymer). The resultant chitosan has a high degree of free amino groups (—NH_2) that provides active sites for many chemical reactions, thus making chitosan a versatile polymer that is suitable for several modifications and applications.[159] Furthermore, chitosan has a much higher solubility than chitin and can be dissolved in slightly acidic solutions, while chitin solubilization requires strong and highly concentrated mineral acids[159] or solutions of lithium chloride and organic solvents such as dimethylformamide, dimethylacetamide and *N*-methyl-2-pyrrolidone.[161]

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Chitin can be converted to chitosan by chemical deacetylation. Alternatively, enzymatic deacetylation using deacetylases has also been attempted. The enzymatic activity of various deacetylases towards chitin has been established; however, they are not efficient in converting chitin to chitosan because of the crystallinity of chitin.[135, 136, 162] Pretreatment of chitin, such as heating, grinding and treating with an alkaline solution, can lead to a higher, yet insufficient, enzymatic activity.[136] Due to this limitation, on an industrial scale, chemical deacetylation is the most commonly used method for chitosan preparation from crustacean waste because of the low cost and suitability for mass production.[139] Deacetylation is performed by incubating chitin in a concentrated solution of sodium hydroxide. Chitin deacetylation can be performed either heterogeneously or homogeneously. In the heterogeneous method, chitin is usually treated with a hot concentrated solution of sodium hydroxide for a few hours. Within the frame of the homogeneous method, chitin is incubated in a concentrated sodium hydroxide solution at room temperature for a few hours, followed by dissolution in crushed ice at 0 °C.[92, 139] Heterogeneous conditions result in an irregular distribution of *N*-acetyl-D-glucosamine and D-glucosamine units and a blockwise distribution of acetyl groups along the chitosan chain. In contrast, chitosan obtained under homogeneous conditions has a random distribution of acetyl groups along the chain. Hence, chitosans produced using these two methods can have different physicochemical properties.[163]

Almost solely chemical heterogeneous deacetylation has been reported for chitosan production from insects with sodium hydroxide being used as the deacetylating agent. In a few cases, sodium hydroxide was combined with sodium borohydride as a 'protecting reagent'.[119, 122, 132] For heterogeneous deacetylation of insect biomass, the sodium hydroxide concentration ranges from 40 to 60% (i.e. about 15–22 mol L^{-1}) (Table 2). In most cases, the deacetylation step lasts from 1 to 9 h, with a few exceptions of longer incubation times of up to 2 days.[104, 126] Temperatures of heterogeneous deacetylation of insect samples range from 90 to 150 °C (Table 2). Ideally, deacetylation should result in non-degraded chitosan with a high degree of deacetylation, enabling its solubilization in dilute acidic solutions.[159] The degree of deacetylation is defined as the proportion of glucosamine monomer residues in the chitosan chain and it can affect the solubility and performance of chitosan in many of its applications.[164] Deacetylation can be optimized and adjusted according to need by adjusting various factors, including temperature, time, alkali concentration, solid-to-solvent ratio and particle size.

Assessment of chitin conversion into chitosan

Results obtained for chemical heterogeneous deacetylation of insect samples, in terms of chitosan yield, degree of deacetylation and molecular weight, are reported in Table 2. Chitosan yield was calculated from the dry biomass of the original insect biomass or the dry weight of chitin. Chitosan yields measured from the original biomass ranged from 2 to 8%. The highest values (26–28%) were obtained by Song *et al.*[124] from *Chrysomya megacephala* larvae and by Luo *et al.*[113] from cicada sloughs. Chitosan yield calculated from the respective chitin dry weight ranged from 60 to 83% (Table 2). Only Hahn *et al.*[92] have performed both heterogeneous and homogeneous deacetylation of the *H. illucens* larval exoskeleton, obtaining more than double the yield of chitosan with the heterogeneous method compared to the homogeneous method. Yields of chitosan produced from crustaceans with heterogeneous deacetylation varied from 4 to 15% (related to the initial dry biomass).[26, 113, 121, 153, 156, 165] These values are slightly higher than those obtained from insects. The primary reason for that is the presence of larger amounts of protein and fat in insect samples.[166] However, as with chitin, chitosan yield can be affected not only by the purification process, but also by the species and harvest time.[163] Considering the reported data, a unique definition of 'yield' for both chitin and chitosan is needed to make the methods of measurement uniform.

The degree of deacetylation of chitosan produced from insects with heterogeneous deacetylation varied between 62 and 98% (Table 2). A lower value has been reported only by Monter-Miranda *et al.*[119] who obtained 57% deacetylated chitosan from *Brachystola magna* adults. The degree of deacetylation of chitosan extracted from crustaceans with the heterogeneous method normally ranges from 56 to 98%,[167] while the average degree of deacetylation obtained with homogeneous treatment is 48–55%.[139] At least 80–85% deacetylation is necessary for the good solubility of chitosan.[167] The degree of deacetylation can be increased or decreased by changing temperature, time and sodium hydroxide concentration.[159] Moreover, there is a correlation between temperature and rate of deacetylation: high temperatures can increase deacetylation, whereas long residence times can improve deacetylation but only up to a certain point. For instance, alkali treatment using 50% sodium hydroxide at 100 °C beyond 2 h does not deacetylate crustacean-based chitin further significantly; rather it can degrade the polymer chain.[159]

Chitosan is a biopolymer of high molecular weight, which varies depending on the source and deacetylation treatment applied. The molecular weight of crustacean-based chitosan ranges from 100 to 1000 kDa.[25, 26] Chitosan produced from insects has a molecular weight ranging from 26 to 300 kDa (Table 2). Very low values (3 and 7 kDa) have been reported, too.[91,96,127] High reaction temperatures (150 °C) combined with long incubation times (4–6 h) may have caused polymer degradation. The use of standard chitosans with known molecular weights can be useful to assess the validity of the applied analysis method. However, differences in chitosan molecular weights can also be related to the insect species, as shown by Kim *et al.*,[111] who applied the same deacetylation conditions as Kaya *et al.*[96] and obtained a chitosan with a much higher molecular weight (308 kDa from adult crickets *versus* approximately 3 kDa from both adults and larvae of the Colorado potato beetle). A very high molecular weight (3290–5900 kDa) has been reported by Paulino *et al.*[122] for chitosan produced from silkworm using 40% sodium hydroxide and sodium borohydride at 100 °C. This could be because sodium borohydride prevents oxidative cleavage of glycosidic bonds during deacetylation.

Deacetylation treatment conditions can affect chitosan molecular weight and thus its physicochemical properties or bioactivity. Notably, molecular weight has a great influence on the biological activity of chitosan: chitosan with low molecular weight (i.e. lower than 150 kDa) has good antibacterial properties.[168, 169] Studies carried out on bacteria potentially pathogenic to humans, such as *Salmonella typhimurium*, *Listeria monocytogenes*, *Bacillus cereus* and *E. coli*, have confirmed that low-molecular-weight chitosan has a greater effect on reducing microorganism growth and multiplication.[168, 169] Small chitosan chains have higher mobility, attraction and ionic interaction than long chains, facilitating an effective binding of chitosan to the membrane surfaces of bacteria.[168]

To evaluate the suitability of chitin and chitosan for desired applications, they need to be characterized. Several metrics can

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be used to assess chitin and chitosan characteristics and properties, such as degree of deacetylation, molecular weight, viscosity, morphology and solubility. Analysis methods and the investigated characteristics vary according to the final purpose.

ANALYTICAL METHODS

As mentioned in the previous sections, multiple studies have described the extraction of insect-based chitin and its subsequent conversion to chitosan. Although the processing conditions have been stated in detail in the relevant articles, discrete and quantitative values regarding yield and degree of purification are missing. This hampers the assessment of the economic potential of chitin and chitosan derived from insects and the side streams of their cultivation, especially in comparison to production from crabs, shrimps or fungi. However, a general comparison, in terms of structure, chemical composition and purity, with commercialized sources can be made with the help of physicochemical and/or spectroscopic data. Table 3 summarizes the main methods applied for the characterization of insect-based chitin and chitosan. Additionally, we include NMR spectroscopic data in the table. NMR, not being a method currently applied for the analysis of insect-based chitin, is promising towards validity and significance of the data.

The resulting data can support performance assessment of insect-based material in prospective applications. Furthermore, the data contribute to focusing and identifying new application fields.

Infrared spectroscopy

Infrared spectroscopy is the most frequently applied method to examine insect-based chitin and chitosan; especially to determine the degree of deacetylation of the polysaccharides. The technique, which exploits infrared light to obtain data from excited vibrational states of the functional groups in a sample, enables rapid analysis. According to the functional groups contained in the molecules, the most significant bands of insect-based chitin and chitosan occur at wavenumbers of 1310–1320 cm^{-1} (CN stretching, amide III), 1550–1560 cm^{-1} (NH bending, amide II), 1590–1600 cm^{-1} (NH_2 bending), 1650–1655 cm^{-1} (CO stretching, amide I), 3100–3110 cm^{-1} (NH symmetric stretching), 3255–3270 cm^{-1} (NH asymmetric stretching) and 3430–3450 cm^{-1} (OH stretching)[170, 171] (Fig. 3). The same is true for chitin and chitosan derived from crustaceans, where the wavenumbers of the peaks can vary slightly among different natural sources.[88]

For insect-based chitin and chitosan, infrared spectra were used to confirm the homogeneity of chitin and chitosan isolated from several insect species,[113] to compare the spectral bands with those of commercially available chitin and chitosan[94, 123] or to confirm the purity of insect-based chitin after the isolation process[106] by considering the strength and position of characteristic bands.

The most practical application of this method has been for determining the degree of acetylation of investigated samples by calculating and comparing the absorption values measured at specific wavelengths.[89] Accurate quantification is challenging[172]; comparability and significance are minor due to the different bands applied for calculating the degree of acetylation by different researchers. Further sources of errors can be individual-specific or due to inappropriate baseline settings within the spectra and impurities in the sample, e.g. the presence of proteins can lead to an overlap of the characteristic chitin and chitosan peaks in the infrared spectrum and thus to wrong values.[22, 173] In the authors' opinion, infrared spectra used to obtain valid information or even quantitative values from insect-based chitin and chitosan are of limited conclusiveness. It is a useful auxiliary for qualitatively determining the presence of functional groups, but not to provide quantitative data subject to strong variation based on, for example, impurities or water content in samples.

Tools like partial least squares regression are hence mandatory for increasing the validity of the data and will also provide an added value to the infrared spectra of insect material,[174] although extensive and time-consuming calibration work is required. Since manual evaluation concerning the determination of the degree of acetylation is error-prone and less comparable, an automated software-assisted extraction of relevant data would thus be beneficial. Nevertheless, the identification of chitin- and chitosan-related functional groups, such as acetamido or amino groups, present in insect-based material is feasible using infrared spectroscopy.

X-ray diffraction

X-ray diffraction is the second most common analysis method used to characterize chitin from insect-based materials. Although X-ray diffraction is a powerful analytical technique for obtaining structural information, in the literature reviewed it has been exclusively used to determine the polymorphic form of chitin crystallites and the crystal structure and crystal content of chitin and chitosan isolated from insects.

The X-ray pattern provides information on the periodic arrangement of atoms within a sample. The resulting diffractogram shows intensity as a function of 2θ, which is defined as the angle between the incident and diffracted beams.

X-ray diffraction measurements of insect-based chitin revealed strong significant peaks at 9–11° and 19–20°, in addition to minor peaks at 12–13°, ~21°, ~23° and ~26°.[91, 112, 126, 128, 131] These peaks are valid for the highly symmetric orthorhombic crystal structure of insect chitin, representing the α-polymorphic form. However, there are exceptions: Chitin from the cocoons of a moth (*Orgyia dubia*) is in the γ-form, exhibiting an X-ray diffraction pattern with high homology to the diffractogram obtained for α-chitin, differing from each other mainly in the peak at 12.9°.[144] γ-Chitin and β-chitin are polymorphs with a lower degree of order than α-chitin, making these polymorphs more reactive. For example, β-chitin is more accessible to swelling and for enzymatic and chemical reactions than the α-form,[14] and therefore more readily undergoes crystal disintegration. Thus, the conversion of β-chitin results in chitosan with lower crystallinity compared to chitosan obtained by deacetylation of α-chitin.[175]

Previous studies have confirmed that chitosan exists as two crystalline polymorphs, either as a hydrated polysaccharide ('tendon' form)[176] or as an anhydrous form ('annealed' chitosan).[177] The presence of crystallites or crystalline regions in the amorphous regions of chitosan could be due to the unreacted chitin.[178] The position of the X-ray peaks in the diffractogram is similar for insect-based chitosan and chitin and comparable to peak positions determined for commercially available chitosan.[89] Main peaks for the chitosan crystal structure can be identified at 2θ of ~10° and ~20°.[91, 113, 116]

The crystallinity index for chitin and chitosan is commonly calculated using the relation of the peak intensities measured at 16° (I_{am}), which is attributable to the amorphous content of the

Table 3. Overview of major analytical methods applied for investigation of insect-based chitin and chitosan. Due to its importance, validity and sensitivity, NMR spectroscopy is listed as prospective powerful tool to analyze insect-based chitin and chitosan although it is not discussed here

Method applied	General principle	Main applications for (insect-based) chitin and chitosan
Infrared spectroscopy	Excitation of vibrations by irradiation with infrared beams	Determination of deacetylation degree
X-ray spectroscopy	Detection of elastic scattered X-rays	Crystallinity determination Determination of chitin polymorph
Thermogravimetric analysis	Mass loss or heat flow determination during heating	Degradation temperature Moisture content Determination of chitin polymorph
Elemental analysis	Combustion and content determination of different elements	Determination of deacetylation degree for chitin and chitosan Purification degree of chitin
Viscometry	Measuring the viscosity of the polysaccharide-containing solutions	Measuring viscosity-average molecular weight
Scanning electron microscopy	Reflection of electrons interacting with atoms	Determination of chitin surface morphology
NMR spectroscopy	Investigation of the electronic environment of single atoms and the interaction with neighboring atoms	Determination of deacetylation degree Distribution of acetyl groups Determination of impurities

sample, and at 20° (I_{110}) according to the equation of Segal *et al.*[179]:

$$\text{Crystallinity index}\,(\%) = \frac{I_{110} - I_{am}}{I_{110}} \times 100 \qquad (3)$$

The reported crystallinity values for chitin vary greatly and cover a wide range (40–90%) but are mainly between 60 and 80%.[91, 93, 94, 126, 132] The broad range of values reported is due to the varied sources, different purification methods used and is a function of the drying, storage or preprocessing conditions.[128] Grinding of chitin is, for example, an effective method for decreasing its crystallinity, disturbing the overall structure of the polysaccharide.[178]

Hence, there is a clear need to independently determine all significant factors that affect the crystallinity of insect-based chitin and chitosan using X-ray-based investigations. The authors believe that low crystallinity values achieved for insect-based chitin and chitosan are of major importance and are highly relevant for most applications. For instance, lower crystallinity of chitosan facilitates its solubility in acidic solutions, increases its sorption ability and increases the accessibility of the primary free amino groups of chitosan.[180, 181]

Thermogravimetric analysis

Similar to X-ray diffraction analysis, thermogravimetric analysis has also been used to determine the polymorphic form of insect-based chitin. Additionally, the temperature at which chitin and chitosan completely decompose can be measured by thermogravimetric analysis. The method records mass loss of a sample over time as temperature increases and is visualized in a thermogram. For insect chitin, thermograms exhibit two decomposition steps. One is a result of water evaporation between 50 and 110 °C, leading to a low mass loss of the sample (1–7%). A second peak at 300–400 °C is caused by the dehydration of the saccharide backbone, polymerization of the degradation products and decomposition of the acetyl function.[122] The amount of mass lost during this second decomposition step ranges from 50 to 95%.[102, 103, 105, 128] The maximal thermal degradation temperature (DTG_{max}), which corresponds to the temperature at which the highest mass loss is determined, is in a more narrow temperature range: DTG_{max} values for chitin from different body parts of *Z. morio*, *Melolontha* sp., *A. pandora* or *H. illucens* have been evaluated to be 350–390 °C, indicating the presence of the α-form.[98, 100, 103, 126] DTG_{max} values obtained for α-chitin isolated from marine fishery waste are in the same range.[11, 93] Typically, α-chitin has a higher second decomposition temperature than β-chitin,[102] which is attributed to the lower crystallinity of the latter.[182] Sometimes, a third decomposition peak at temperatures above 700 °C has been reported which is attributed to residual minerals not removed during demineralization.[11]

The thermograms of insect-based chitin do not reveal large deviations to the chitosan produced. The main difference here is

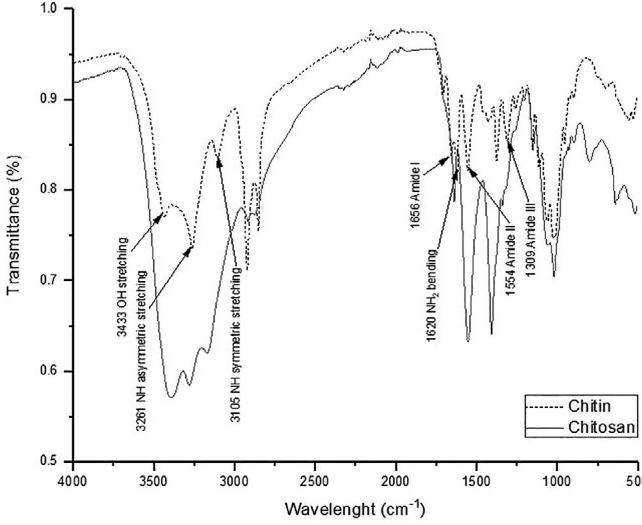

Figure 3. Exemplary infrared spectra of chitin and chitosan obtained from *H. illucens* pupal exuviae (own measurements).

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a shift of the decomposition peak to slightly lower temperatures during chitosan heating (<320 °C), which means that chitin is more stable.[95] This is due to the increased number of *N*-acetyl groups in the chitin providing a higher stability than the primary amino groups of the chitosan.[181, 183]

Although several thermogravimetric analysis studies and data for insect chitin and chitosan are available, valid or useful characteristic values, such as the activation energy of degradation, have not been calculated and published yet. Hence, there is a need for substantial discussion and evaluation rather than stating superficial qualitative expressions. The DTG_{max} values obtained reveal significant differences for chitin or chitosan, but these are not application-relevant. Processing chitin and chitosan at elevated temperatures does not significantly improve processing properties, as phase transition from crystalline to amorphous form does not occur until decomposition.

The information obtained using thermogravimetric analysis is thus limited, especially as the polymorphic form of chitin can also be identified with X-ray spectroscopy. Another option is to perform a more comprehensive thermal analysis. Newer instruments can perform calorimetric and thermogravimetric measurements simultaneously, providing additional information about the type and enthalpy values of conversion occurring during heating and purity of chitin and chitosan.[126, 131]

Elemental analysis

Thermogravimetric analysis enables the determination of sample degradation temperature. In contrast, elemental analysis utilizes degradation and combustion of insect-based chitin and chitosan and the subsequent detection of carbon, hydrogen, nitrogen and oxygen to determine its molecular composition and acetyl content. Although complementary to several other methods, the determination of degree of acetylation (DA) of insect-based chitin and degree of deacetylation (DD) of insect-based chitosan via elemental analysis is of high practical relevance,[184, 185] and is calculated using the following equations[186, 187]:

$$\mathrm{DA}\,(\%)=\left(\frac{\frac{\mathrm{C}(\%)}{\mathrm{N}(\%)}-5.14}{1.72}\right)\times 100 \quad (4)$$

$$\mathrm{DD}\,(\%)=\left(\frac{6.89-\frac{\mathrm{C}(\%)}{\mathrm{N}(\%)}}{1.72}\right)\times 100 \quad (5)$$

Theoretically, fully acetylated chitin contains 6.9% nitrogen and fully deacetylated chitosan contains 8.7% nitrogen.[188] Assuming 100% purity of samples, the higher the nitrogen content the lower is the degree of acetylation and vice versa. Typically, the measured nitrogen content for insect-based chitin is lower than the theoretical value, resulting in an overestimation – according to Eqn (4) – of the degree of acetylation. For example, Erdogan and Kaya[91] used elemental analysis to determine the nitrogen content of chitin from *D. maroccanus* adults and nymphs at 4.6 and 5.7%, resulting in degrees of acetylation of 232 and 187%, respectively. Degrees of acetylation exceeding 100% have been determined for chitin extracted from *D. maroccanus* and species such as *O. asellus* (169%) and *V. crabro* (127%).[64, 103, 109, 115] The validity of the conclusions is hence severely limited. It can be assumed that these overestimations result from nitrogen-free impurities, such as lipids and sugars, in samples, which make up to 43% of edible insects such as *A. domesticus* and *T. molitor*.[189]

The overestimation of the degree of acetylation pertains to chitin from insects. It is different for insect-based chitosan and the determination of its degree of deacetylation via elemental analysis. The degree of deacetylation for chitosan obtained from the conversion of chitin from *Z. morio* and *B. mori* ranges from 52 to 95%,[126, 132] which are reasonable values. It can be assumed that the validity of these values is due to a higher degree of purification of chitosan, in contrast to the insect-based chitin samples containing a significant amount of impurities. The conversion steps from chitin to chitosan involve severe conditions such as use of high temperature and sodium hydroxide concentration, followed by selective precipitation of chitosan from solution by pH neutralization. These steps provide additional purification, resulting in valid calculations of degrees of deacetylation for insect-based chitosan using elemental analysis.

However, the presence of impurities in both chitin and chitosan cannot be excluded. As already mentioned in the chitin purification and deacetylation section of this review, purity can be determined only for a minority of chitins and chitosans produced from insects. For accurate degree of acetylation of chitin or degree of deacetylation of chitosan, pre-quantification of impurities is mandatory. Nevertheless, a thorough and effective purification is crucial to provide unimpeded characterization of insect-based chitin and chitosan using various methods.

Molecular weight measurement via viscometry

The so-called viscosity-average molecular weight of insect-based chitosan can be determined by measuring the intrinsic viscosity of a solution using an Ubbelohde viscometer. Molecular weight determination is based on the fact that the viscosity of the solution, in addition to the degree of deacetylation, depends on the molar mass of chitosan. Previous studies have reported a value for insect chitosan viscosity-average molecular weight in the range of 426–450 kDa.[87, 114] Other authors have determined much lower values (<10 kDa) for *D. maroccanus* and *L. decemlineata* chitosan.[91, 96] Odote *et al.*[121] investigated the viscosity-average molecular weight of lobster, prawn and crab chitosan in comparison to that of chitosan from blowfly larvae and found similar values for all chitosans, which is potentially due to their similar chitin conversion conditions. The same authors also stated that the viscosity-average molecular weight of insect chitosan decreases with higher deacetylation temperature and incubation time, confirming that the applied conversion parameters are more significant than the origin of chitin used for deacetylation. Another application for viscometry is to record the progress of chitosan hydrolysis: Nemtsev *et al.*[120] reported a decrease of honeybee-derived chitosan viscosity-average molecular weight during enzymatic hydrolysis in slightly acidic solution from 257 to 21 kDa.

In contrast to chitosan, chitin solubility is limited to a few solvents. Nevertheless, Draczynski reported that the solubilization of honeybee chitin in dimethylacetamide–LiCl results in a viscosity-average molecular weight of 426–738 kDa that depends on the time of deproteinization.[90]

More sophisticated methods and instruments are required, instead of the standard laboratory Ubbelohde viscometer, for measuring weight-average and number-average molecular weights and polydispersity or molecular weight distribution of chitin and chitosan. For example, gel permeation chromatography measurements with a multi-angle laser light scattering detector revealed a molecular weight for insect-based chitosan of approximately 308 kDa and a polydispersity index of 1.2.[111]

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However, viscosity-average molecular weight as an outcome is important as it affects many other physicochemical or application-specific investigations, such as bioactivity, adhesion force and gelation properties.

Scanning electron microscopy

Previously described analysis methods generated information concerning structural features, chemical composition or physicochemical properties of insect-based chitin and chitosan. Scanning electron microscopy enables visualization of the polysaccharide surface in the nanometric range using a focused beam of electrons.

Various surface morphologies have been described for insect-based chitin: rough or smooth surfaces with or without pores and/or fibrils.[116] The range of diversity of various chitin surfaces derived from insects is shown in Fig. 4. However, most chitin surfaces extracted from insects exhibit a rough fibrillary structure with pores. The diameter of the fibrils ranges from 10 to 50 nm,[64, 91, 101, 108] and the chitin fibrils are surrounded by a protein matrix.[190] The pore diameters range from 100 to 500 nm. The highly porous structure increases the accessible surface area and thus the adsorption capacity of the material.

Scanning electron microscopy imaging of insect-based chitosan has been performed in only a small number of studies. Kaya *et al.*[101] reported chitosan with a surface morphology similar to that of the chitin from which it was derived, which suggests that the chitin structure is preserved in chitosan. The surface morphology of chitin also depends on other factors. It can be influenced by characteristics inherent to the natural source, such as species,[95, 118] sex[13] or body part,[99] and by process conditions, such as the selected pretreatment procedure.[93] Moreover, chitin content, degree of purification and washing procedure can play a major role as the surrounding matrix and matrix constituents on the surface not removed during purification mask the chitin. Due to this, the validity of this method for investigating chitin and chitosan is limited. However, knowledge of the surface structure of chitin and chitosan is important for processes in which surface architecture greatly affects functionality.

Assessment of analytical investigations

The methods described are the most frequently mentioned analytical techniques applied for the investigation of insect-based chitin and chitosan. The major conclusion from screening the analysis results is that there is a high structural and chemical homology between chitin and chitosan derived from insects and marine animals. However, despite the large amount of available data, knowledge gaps exist that need to be filled in prospective studies to assess the potential of insect-based chitin and chitosan. What has already been said for chitin yield and degree of purification also holds true here: there is a lack of valid data and/or the results were not properly evaluated or discussed to accelerate research on this topic. For example, many infrared spectra from chitins and chitosans from different sources are available, which are nearly the same as each other and provide no additional information. Similarly, thermogravimetric analysis data have limited validity as the DTG_{max} values reported are in the same temperature range. In contrast, X-ray data show strongly varying crystallinity values for chitin and chitosan. Thus, it is unclear if the data presented are a result of processing conditions, life cycle stage or body part of the insect.

Hence, the authors suggest firstly performing comprehensive studies to identify parameters that contribute significantly to the properties of insect-based chitin and chitosan. Secondly, the authors strongly believe that spectroscopic and chromatographic data would be more valid if they were related to the degree of purification of the chitin and chitosan investigated. Therefore, it is necessary to first determine the purity of polysaccharides after extraction and deacetylation since contaminations can disrupt accurate measurements of degree of deacetylation. Elemental analysis is a tool which could be applied to, at least, estimate the degree of purification and to evaluate if the measurements performed are valid. Thirdly, there is a need to standardize the calculation methods and equations; for example, to agree on specific bands and peaks used for calculation when determining crystallinity via X-ray spectroscopy. Fourthly, we recommend using statistical software to evaluate the results and to increase the information content of a presented data set. In our opinion, infrared spectroscopy can reveal degrees of deacetylation and impurity content accurately if evaluated using multivariate data analysis or similar methods. Lastly, techniques such as titration-based methods can be applied for higher sensitivity in determining degree of deacetylation. Although revealing a precise determination method, titration is time-consuming. Another highly accurate and automatable tool, to determine the degree of deacetylation of chitin and chitosan in solid state or solubilized, is NMR spectroscopy. Several studies exhibited the potential of ^{1}H NMR, ^{13}C NMR and ^{15}N NMR spectroscopy to determine the degree of deacetylation, the distribution of acetyl groups and the cross-linkages of the chitin and chitosan.[191–193] Possibly due to the need for enhanced equipment and specific expertise, comprehensive studies concerning insect-based chitin and chitosan are lacking. We further recommend performing application-based investigations, such as measurement of viscosity, adhesion, film formation and adsorption capacity. For successful commercialization of insect-based chitin and chitosan, such detailed knowledge is mandatory.

CONCLUSIONS AND FUTURE PROSPECTS

Chitin and especially chitosan are natural polymers with many useful properties and are widely used in a broad range of applications. Presently, the main commercial source of chitin and chitosan comprises waste streams from the marine fishery industry; however, their availability is limited by geography and season. The recent increase in demand for chitin and chitosan in the global market has drawn attention to alternative sources independent of marine fishery waste. Insect breeding farms, which are used for waste management through insect-mediated bioconversion or for producing proteins and fats from larval stages, are being launched worldwide. In addition to the production of valuable compounds, insect breeding generates several side streams (dead adults, exuviae, exoskeletons, frass and residual feed) that have not yet been valorized. These side streams provide a cheap source of chitin, which is abundantly available and not regionally or seasonally limited. Furthermore, the chitin content of exuviae and exoskeletons exceeds 23%,[78] suggesting favorable conditions for chitin and chitosan production in the future.

In contrast, the chitin content of whole insects is generally lower.[78, 85] Due to the moderate content, insect breeding only for chitin isolation is not economically feasible without cascade usage of other compounds derived from the insect breeding.

The chitin content in insects is a function of species, type of feed and of life cycle stage. Moreover, the life cycle stage determines the complexity of the matrix in which chitin is embedded. Insects

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Figure 4. Scanning electron microscopy images of chitin extracted from (a) *Cicadatra atra*, (b) *Cicada hyalina*, (c) *Cicada lodosi*, (d) *Cicada mordoganensis*, (e) *Cicada platyptera* and (f) *Cicadivetta tibialis*. Reprinted from Mol *et al.*[118] with permission of Wiley.

undergo sclerotization during metamorphosis to the adult stage. Sclerotization comprises cross-linkage of the insect cuticle with catecholic compounds. This makes necessary a more sophisticated purification process and at least one additional bleaching step to isolate pure chitin from insect biomass. On the contrary, decolorization is not mandatory for chitin purification from marine fishery waste. The need for bleaching arises because the chitin isolated from dead adult insects and exuviae contains these sclerotized structures with dark colour. Chitin production can be more economically feasible from side streams of insects bred for a different purpose.

The purification of chitin begins earlier in the process and comprises fractionating the chitin-containing compounds from those with no or low chitin. Although insect breeding, for the isolation of protein and fat, is a highly automated and controlled operating process, a mechanical process for separating chitin-rich substances needs to be developed to eliminate the current practice of manual collection. Similarly, manual collection of chitin-rich material is opposed to the prospective application of insect-based chitosan in medicine and requires good manufacturing practice or laboratory-like controlled conditions. Furthermore, the raw materials used for insect breeding vary according to availability (e.g. vegetable waste from agri-food chain, cereal straw, distillers' grains and cereal meals). Although insect-mediated bioconversion is a flexible and robust process and leads to a high-quality fat and protein fraction almost independent of the varying quality of the side streams, it is challenging for an automated process to lead to chitin and chitosan with a consistent composition.

Currently, chitin purification from insect biomass is an area of focus for researchers and is mainly performed on a laboratory scale using the same methods applied for purification from crustacean shells. Most available literature on chitin and chitosan production from insects is limited to a description of the chitin extraction process and its subsequent deacetylation into chitosan. Information on the quantitative evaluation of extraction, purification, deacetylation and bleaching efficiency, and the degree of purification of products is missing. In the case of insects, it is

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especially important to assess the bleaching process, as insect exuviae undergo sclerotization by catecholamine linkage, which leads to dark coloration.[85, 194] These factors hinder accurate assessment of the economic potential of chitin and chitosan production from insects and comparison with the process chain of chitin isolation from crab shells.

A comparison of degree of deacetylation, molecular weight, stability, crystallinity and surface structure between insect- and crustacean-based chitin and chitosan, performed using various analytical methods, showed high similarity. This is encouraging regarding the performance of chitin and chitosan derived from insects for industrial applications and their use in new fields. However, for a comprehensive assessment, it is necessary to carry out application-relevant investigations and to exploit the full potential of the methods in use for characterization.

Based on current knowledge, it can be supposed that insects will be an important source of chitin and chitosan in the future, especially if future studies focus on filling the knowledge gaps highlighted in this review. The efficiency of each step of the purification process needs to be critically evaluated to optimize methods applied to crustaceans and adapt them to insect biomass. Alternatives to traditional chemical purification methods should also be considered to make the process more environmentally friendly. Future studies should focus on these aspects to make optimal use of the side streams of insect breeding.

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