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Functional and bioactive properties of chitosan produced from *Acheta domesticus* with fermentation, enzymatic and microwave-assisted extraction

Marios Psarianos, *[a] Nader Marzban,[a] Shikha Ojha,[b] Roland Schneider[c] and Oliver K. Schlüter*[ad]

Edible insects are an important source of chitin and chitosan. Different methods, including the use of proteases, fermentation and microwave treatment, have been proposed to replace the conventional chitin isolation methods. House crickets are among the most commonly used insects for food applications. Chitosan was produced from house crickets from chitinous materials that were isolated *via* the conventional method, a biological process that combines fermentation with *Lactococcus lactis* and digestion with bromelain and a microwave-assisted chemical method. All chitosans were evaluated for their purity, functional properties and bioactive properties, namely their antioxidant and antimicrobial activity. All three methods generated chitosan with a purity higher than 85% and an exceptionally high oil binding capacity with a maximum of 1078.62 g oil per g chitosan for the chitosan produced with the conventional method. Furthermore, all cricket-derived chitosans showed a significant level of antioxidant activity with an effective concentration of 5 mg mL^{-1} or lower and antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella enterica* ssp. *Enterica Serovar Typhimurium*. It was concluded that the biological chitin extraction method could lead to the generation of a chitosan material with high potential for application in different sectors.

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Sustainability spotlight

The projected increase of global population will lead to a high protein demand. Edible insects constitute a variety of underexplored biomass that can address this demand with a lower environmental impact than conventional livestock. Insects have a rich nutritional profile with many valuable ingredients, including chitin and its derivative, chitosan. The present work aimed to valorize the house crickets, a commonly consumed species, as a source of chitosan. Therefore, green chitin extraction methods were applied, replacing alkaline and acidic treatments with fermentation and enzymatic treatments, followed by an evaluation of the produced chitosan. The use of edible insects and the reduction in chemical use contribute to SDG 2 (Zero Hunger) and partially 13 (climate action).

1. Introduction

Edible insects have attracted considerable popularity in recent years due to their rich nutritional profiles and low environmental impact.[1] They have lower feed[1] and land requirements[2] and produce lower gas emissions compared to conventional livestock.[1] The house crickets (*Acheta domesticus*) are particularly interesting, because of their easy rearing process,[3] high reproduction rate[4] and rich nutritional profile.[5] Furthermore, house crickets are being already consumed as food and feed in different parts of the world[6] and they have been recently accepted as novel food under Regulation (EU) 2015/2283.[7]

Apart from proteins and lipids, insects contain also chitin.[8] Chitin is a polysaccharide that is abundant in fungal species and invertebrates[9] and consists of a chain of β-(1-4)-*N*-acetyl-D-glucosamine.[10] It can be found on the exoskeleton of insects[9] and its content on insects varies, based on the insect species and life stage.[8] The range of the chitin content of edible insects has been reported to be 43–108 mg per kg dry matter.[11] The substitution of the *N*-acetyl group of chitin with an amine group is called deacetylation and describes the reaction of the production of chitosan from chitin.[12]

Chitosan offers potential for a variety of applications in the medicine, cosmetic, food and agricultural sectors.[13] Chitosan

[a]*Department Systems Process Engineering, Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB), Max-Eyth-Allee 100, 14469 Potsdam, Germany. E-mail: mpsarianos@atb-potsdam.de; oschlueter@atb-potsdam.de*

[b]*Department of Land Sciences, School of Science and Computing, South East Technological University, Cork Road, X91 K0EK, Waterford, Ireland*

[c]*Department of of Microbiom Biotechnology, Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB), Max-Eyth-Allee 100, 14469 Potsdam, Germany*

[d]*Department of Agricultural and Food Sciences, University of Bologna, Piazza Goidanich 60, 47521 Cesena, Italy*

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has been characterized by antioxidant activity,[14] antitumor activity[15] and antimicrobial activity.[16] A variety of edible insect species has been tested for chitin extraction and chitosan production.[17] The insect-derived chitosan has exhibited some of the bioactive properties that are commonly reported for crustacean-derived chitosan, such as antioxidant activity[18] and antimicrobial activity.[19] Chitin is usually isolated from insects *via* processing pathways that include sequential delipidation, deproteinization, demineralization and decolorization, which often require the use of hazardous chemicals, such as HCl and NaOH at elevated temperatures, long durations[17] and are not considered environmentally friendly and that generate pollution.[20] Furthermore, the proteins and minerals that are dissolved in these reagents can be upcycled as food and feed easily because they are damaged and less biocompatible.[21]

A number of alternative and more environmentally friendly methods including fermentation, proteolysis and deep eutectic solvents have been proposed to replace the conventional treatments for chitin extraction.[22,23] As mentioned before, the conventional method for extracting chitin through a sequential chemical process has several drawbacks, such as long durations that can last many hours or days, high energy requirements and the use of hazardous chemicals that can cause pollution and degrade the proteins and minerals in the by-products.[24,25] Bioprocesses such as fermentation and enzymatic treatment can reduce the risk of using hazardous chemicals.[26] Furthermore, microwave processing has been shown to facilitate the conventional method of chitin isolation by significantly reducing the treatment time.[27]

The treatment of a biomass with hydrolases is particularly interesting. Chitin and chitosan have been observed to undergo partial hydrolysis due to the treatment of proteases, lipases and glycosyl hydrolases.[28] The hydrolysis of chitin and chitosan *ca.* lead to a chitosan with a lower molecular weight, which has been linked to improved bioactive properties.[15] At the same time, the treatment of a chitin-rich biomass with proteases can be applied as a method for deproteinization.[29] For instance, an enzymatic treatment with bromelain has been applied to house crickets as an alternative process to conventional chemical deproteinization in order to recover chitosan. It was found that the produced chitosan had a lower molecular weight when the enzymatic treatment was applied in comparison to chitosan that was recovered *via* the conventional process and to chitosan produced from standard chitin. Additionally, this chitosan exhibited higher antioxidant activity than the chitosan that was produced from standard chitin.[30]

These methods have also been underlined for the potential when applied to edible insects for chitin extraction.[31,32] A chitinous material can be isolated from mealworms with fermentation,[33] while proteolysis has been successfully applied to mealworms for chitin isolation.[34] Moreover, a combination of fermentation with *Lactococcus lactis* and digestion with bromelain has been proposed to isolate a chitinous material from house crickets that can be used for chitosan production.[30]

The present study aims to compare the biological processing pathway that was applied by Psarianos *et al.*[30] for the extraction of chitin from house crickets with the conventional method and a microwave-assisted method. After deacetylating this chitin into chitosan, the study aims to evaluate the properties of the produced chitosans in terms of functionality and bioactivity.

2. Materials and methods

2.1 Sample preparation

Living crickets (*Acheta domestica*) were purchased from Tropic Shop (Nordhorn, Germany) and were inactivated by freezing at −20 °C. They were thawed at 4 °C, separated from frass, washed with water and dried at 60 °C until constant weight. The dried insects were milled for 10 s with a laboratory Retsch mill (Haan, Germany) and were defatted using hexane (solid/liquid ratio 1 : 20) for 1 h at room temperature. The hexane was recycled using a rotary evaporator (Büchi R-100, Flawil, Switzerland) and the defatted cricket biomass was stored for further use. Standard chitosan was used as a reference for all experiments. All chemicals were purchased from Carl Roth Gmbh & Co. KG (Karlsruhe, Germany), apart from 2,2-diphenyl-1-picrylhydrazyl (Alfa Aesar, Massachusetts, United States).

2.2 Chitin extraction

2.2.1 Chemical method. The samples were deproteinized using a 1 M NaOH solution (solid/liquid ratio 1 : 50) for 24 h at 80 °C. Afterwards, they were demineralized with a 1 M HCl solution (solid/liquid ratio 1 : 30) for 2 h at 98 °C. After both deproteinization and demineralization, the sample was washed with warm distilled water through a 0.063 mm filter, until the water reached a neutral pH. Afterwards, it was dried at 60 °C until constant weight.[35,36]

2.2.2 Biological method. The biological method was adapted from the work of Psarianos *et al.*[30] Briefly, the cricket biomass was fermented with *Lactococcus lactis* (Library of the Department of Microbiome Biotechnology of the Leibniz Institute for Agricultural Engineering and Bioeconomy) for 7 days at 30 °C, 150 rpm, using sterile MRS Bouillon Lactobacillus broth acc. with an inoculant of <1% v/v and a 10% w/v of cricket biomass. Afterwards, the samples were digested with bromelain, after being mixed with water (solid/liquid ratio 1 : 20), for 5 h at 60 °C, pH = 5.5 (as per supplier's specifications) and an enzyme/substrate ratio of 2% w/w. Between both steps, the samples were washed and afterwards dried, as described in Section 2.2.1.[37,38]

2.2.3 Microwave-assisted method. The microwave-assisted method was adapted from the work of Knidri *et al.* (2019).[27] The samples were deproteinized with a 1 M NaOH solution (solid/liquid ratio 1 : 50) with a microwave treatment for 8 min at 500 W. Afterwards the samples were demineralized with a 1 M HCl solution (solid/liquid ratio 1 : 30) with a microwave treatment for 8 min at 500 W. Between each steps the samples were washed and in the end, they were dried, as described in Section 2.2.1. The final temperature after the treatment was always 95–100 °C.

2.3 Deacetylation

Deacetylation of all materials was performed with a 50% NaOH solution at 130 °C for 2 h. Afterwards, the samples were washed

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with water through a 0.063 mm filter until the neutrality of the water and dried at 60 °C until constant weight.[39]

2.4 Properties of chitosan

2.4.1 Chitosan content (g chitosan per 100 g). The chitosan content of all chitosan samples was estimated by measuring glucosamine content.[40] Standard chitosan was used for the calibration curve at a concentration range of 2.93–11.72 μg mL^{-1} and the results were expressed as a percentage of chitosan (%).

2.4.2 Solubility (%). Solubility was determined after homogenizing 0.1 g of chitosan with 10 mL of an acetic acid 1% solution for 1 h at room temperature inside a falcon tube. Afterwards, the mixture was centrifuged at 3407×*g* for 10 min and the supernatant was decanted. The pellet was dried at 60 °C until constant weight. Solubility was estimated as:

$$\text{Solubility (\%)} = (M_1 - M_2)/(M_1 - M_0) \times 100 \quad (1)$$

where M_0, M_1 and M_2 are the weight of the empty falcon and the weight of the falcon together with the sample before and after the process, respectively.[41]

2.4.3 Oil binding capacity (g oil per g chitosan). The oil binding capacity (OBC) was determined after homogenizing 0.5 g of chitosan with 10 mL of commercial rapeseed oil and the mixture was vortexed for 60 s and then centrifuged at 3407×*g* for 10 min. The supernatant was decanted and the OBC was estimated as:

$$\text{OBC (g oil per g chitosan)} = (m_f - m_0)/m_0 \quad (2)$$

where m_f and m_0 are the final and initial weight of the sample, respectively.[41,42]

2.4.4 pH. A chitosan stock solution was prepared at a concentration of 5 mg mL^{-1} by mixing 500 mg of chitosan with 100 mL of acetic acid 1% for 24 h at room temperature.[30] Afterwards, the solution was stored in at 4 °C for a maximum of a week to avoid destabilization.[43] The pH of the solution was measured directly with a pH meter (Lab 850, SI Analytics GmbH, Rhineland-Palatinate, Germany).

2.4.5 Particle size distribution, polydispersity index (PDI) and zeta potential (mV). For determination of the particle size distribution, PDI and the zeta potential, the stock solution that is mentioned in Section 2.4.4 was diluted to a concentration of 1 mg mL^{-1} and was placed inside a folded capillary cuvette and analyzed directly with a zetasizer (Malvern Panalytical, Malvern, UK).

2.5 Bioactivity

2.5.1 Antioxidant activity. For the determination of the antioxidant activity, the stock solution (Section 2.4.4) was diluted to a concentration range of 0.5–5 mg mL^{-1}. An ascorbic acid solution at the same concentration levels was used as a positive control.

2.5.1.1 Free radical scavenging activity (2,2-diphenyl-1-picrylhydrazyl). The free radical scavenging activity was determined by mixing 1 mL of the chitosan solution at different concentrations with 3 mL of a DPPH solution of 6×10^{-5} M and incubating the mixture at room temperature in the dark for 30 min. Then the absorbance of the mixture was measured with a UV/Vis spectrometer at 515 nm. 1% acetic acid was used as a blank. Scavenging activity was expressed as:

$$\text{Scavenging activity (\%)} = (A_0 - A_1)/A_0 \times 100 \quad (3)$$

The concentration of each solution that can result in 50% of scavenging activity (IC50) was determined from a linear regression of the concentration of the solution and the scavenging activity.[44]

2.5.1.2 Ferric iron reducing power (FRAP). The ferric iron reducing power was estimated as follows: 0.5 mL of each solution at different concentrations was mixed with 0.5 mL of sodium phosphate buffer (0.2 M, pH = 6.6) and 0.5 mL of potassium ferricyanide 1%. The mixture was incubated at 50 °C for 20 min and then was mixed with 0.5 mL of trichloroacetic acid 10%. Then 2 mL of water was added to the mixture and finally, 0.4 mL of ferric chloride 0.1%. The mixture was vortexed and the absorbance was measured with a UV/Vis spectrometer at 700 nm. A higher absorbance indicates a higher antioxidant activity. The effective concentration (EC50), which is the concentration that can lead to an absorbance of 0.5, was estimated with linear regression of the concentration of the solution and the absorbance.[45]

2.5.2 Antimicrobial activity. The antimicrobial activity of chitosan was tested using the stock solution (Section 2.4.4) at different concentrations against four bacterial strains: *Escherichia coli*, *Staphylococcus aureus* and *Salmonella enterica* ssp. *Enterica Serovar Typhimurium*. The strains were stored at −80 °C before use. Each strain was streaked onto a plate count agar plate and incubated for 18 h at 37 °C. A single colony was removed from each plate, inoculated in tubes containing 25 mL of sterile Mueller–Hinton Broth (MHB), and incubated for 22 h at 37 °C. The overnight cultures were shaken and aliquots (10^7–10^8 cells per mL) were diluted in sterile MHB to produce solutions of approximately 10^6 cells per mL. All solutions (100 μL) were subjected to a minimum inhibitory concentration (MIC) antimicrobial test at serial dilutions of 1 : 2 by mixing with 50 μL of the bacterial culture of all strains for 24 h at 37 °C and then adding 50 μL of iodonitrotetrazolium chloride (INT) solution and incubating for 1 h using a 96-well microplate. The MIC of each solution was determined as the lowest concentration at which the pink color did not occur. MHB was used as a blank and gentamycin sulfate 0.2 mg mL^{-1} was used as a positive control.[46]

2.6 Statistical analysis

Each method used to produce chitosan was replicated three times. Every analytical test was performed for each replicate of production of each material at least twice. Data were analyzed with an analysis of variance (ANOVA) and Tukey's post hoc test to separate significantly different means ($p \leq 0.05$). The Shapiro–Wilk test and the Levene test were performed prior to the analysis to verify if the data follow normal distribution and homogeneity of variance, respectively. The analysis was

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performed with IBM SPSS Statistics 23 (IBM Corp., Armonk, N.Y., USA).

3. Results and discussion

3.1 Characterization of chitosan

Table 1 summarizes the properties of the chitosans that were produced from the crickets and of commercial chitosan. All chitosans produced from the insect biomass showed a high level of purity with a chitosan content higher than 87% for all samples, without significant differences ($p > 0.05$). A high chitosan content (%) was expected for all samples since the biological method has been successfully applied to house crickets to produce chitosan.[30] Furthermore, the positive effect of microwave treatment has also been reported for shrimp chitosan.[27] The process to extract chitin from insects involves the sequential removal of lipids, proteins, minerals and when necessary also pigments.[17] The purity of chitosan is important, since it is one of the factors that determine its functionality and quality, together with the molecular weight, degree of deacetylation, crystallinity and viscocity.[47]

The resulting chitosans from the crickets that have been produced with the methods described in the present study have exhibited insignificant ash residues, while their molecular analysis has revealed a notable presence of amino groups.[30] It was therefore concluded that the remaining impurities are of protein origin. Chitin is mainly found in the procuticle of edible insects, where the cuticle is mainly composed of proteins.[48] Additionally, insect chitin has been reported to form complexes with melanin, which is difficult to remove during the chitin extraction and chitosan production.[49] The harsher processing conditions that led to the formation of Chitosan_C might have been more efficient in disrupting that complex in comparison to Chitosan_B and Chitosan_M, even if the level of purity is comparable.

Considering the low solubility of cricket proteins[50] and the chitin–melanin complex in insects, it was hypothesized that the remaining impurities in the produced chitosan were mainly protein residues and melanin, while traces of minerals might also be present. This hypothesis would apply to all chitosans, since microwave heating has been reported to be applicable for the production of chitosan with similar structural properties in compare to conventional heating. Moreover, the biological processing pathway has been reported to lead to the production of chitosan with comparable structure to the one produced from chitin that is extracted with the conventional method.[30,51]

The solubility of the chitosans showed significant differences ($p < 0.05$). The commercial one had the highest solubility (97.34 ± 0.48) and the ones that were generated from the crickets showed a low solubility (70.73 ± 6.69 for Chitosan_C, 55.07 ± 4.40 for Chitosan_B and 42.56 ± 0.52 for Chitosan_M). Chitosan solubility is affected by several factors, including pH, temperature, solvent, ionic strength and degree of acetylation (DA%).[52] The low solubility of the chitosans obtained from the crickets could be explained by reduced hydrophilicity due to a high DA% within the range of 20% and 50%.[53] The chitosans produced from house crickets with the methods described in the present study have been reported to have a DA% of 30–40%.[30]

The pH is a significant factor that affects the solubility of chitosan, with chitosan being reported to be soluble at acidic pH values and precipitate at 6 < pH < 7.5.[54] The aqueous acetic acid used in the present study has been considered among the solvents traditionally used for chitosan solubilization,[55] while the low pH values would be expected to facilitate its solubility.[56] Temperature affects chitosan solubility as well, with thermal treatments at temperatures >50 °C leading to the formation of hydrogels.[55] In the present study, solubilization was performed at room temperature to avoid the formation of gels.

Therefore, the overall low solubility has been attributed mainly to the high degree of acetylation. However, the remains of the complex between insect chitosan with melanin and insoluble proteins is expected to lead to an overall reduced solubility and different physicochemical properties in compare to a chitosan produced by crustaceans.[49]

The extraction of cricket-derived chitin with the chemical method has been reported to be more efficient in removing the impurities and generate a purer chitin. On the contrary, for example the biological processing pathway was reported to generate a chitin-rich fraction, where chitin is the main component but the chitin content is <60% w/w.[30] Due to their low solubility, the proteins from house crickets have been identified as the main impurity.[50] During the deacetylation some of the protein impurities can be extracted to the alkaline medium, leading to a chitosan with a higher purity.[57] This case was exhibited for the cricket-derived chitosan, which undergoes

Table 1 Properties of chitosan that was commercially purchased or generated from the crickets from chitin isolated through the chemical (Chitosan_C), biological (Chitosan_B) and microwave-assisted method (Chitosan_M)[a]

	Commercial chitosan	Chitosan_C	Chitosan_B	Chitosan_M
Chitosan content (%)	—	89.18 ± 3.12^{a}	87.96 ± 5.33^{a}	87.11 ± 6.82^{a}
Solubility (%)	97.34 ± 0.48^{a}	70.73 ± 6.69^{b}	55.07 ± 4.40^{c}	42.56 ± 0.52^{d}
OBC (g oil per g chitosan)	411.70 ± 13.98^{a}	1078.62 ± 184.68^{b}	885.30 ± 163.03^{b}	860.92 ± 280.29^{b}
pH	3.32 ± 0.00^{a}	3.50 ± 0.12^{a}	3.78 ± 0.04^{b}	3.43 ± 0.15^{a}
PDI	0.43 ± 0.07^{a}	0.73 ± 0.21^{ab}	0.84 ± 0.22^{ab}	1.15 ± 0.28^{b}
Zeta potential (mV)	27.56 ± 2.20^{a}	38.86 ± 3.41^{ab}	50.58 ± 10.94^{b}	52.35 ± 5.64^{b}

[a] Data are expressed as mean ± SD. Different superscript letters (a,b…) indicate significant differences ($p < 0.05$) among the means of the properties of samples that were generated with different methods.

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deacetylation and purification simultaneously during the treatment with the saturated NaOH.[30]

The insufficient deproteinization of chitin prior to deacetylation has been reported to have lower solubility and higher DA%.[57] The difference in the chitin and protein content among the starting insect materials prior to deacetylation influenced the solubility of the resulting chitosan. Similarly, the DA% of the cricket derived-chitosans has been reported to be similar and high, with Chitosan_C being the least acetylated.[30]

One further parameter that can influence the solubility is particle size, with smaller particles exhibiting higher solubility.[58] Chitosan is a molecule than can aggregate or form chitosan–protein aggregates generating larger complexes. The application of bromelain in the production process of Chitosan_B could generate oligopeptides that can form aggregates with chitosan at an acidic pH.[59,60] In the case of the microwave treatment, it has been reported that microwave processing can form irreversible aggregates after degrading chitosan.[61] Aggregation of chitosan is a further explanation of the low solubility, since the commercial chitosan shows a significantly lower particle size (Fig. 1) than the cricket-derived ones, which is consistent with its higher solubility, while Chitosan_B has the highest particle size and a low solubility.

The chitosans showed a significantly different OBC ($p < 0.05$), with the chitosan being produced from the crickets demonstrating over twice the OBC, compared to commercial chitosan. The OBC was high (>800 g oil per g chitosan) for all samples that were generated from the insects. The OBC of cricket-derived chitosans was not affected by the chitin isolation method ($p > 0.05$). A high value of OBC has also been reported for chitosan isolated from other insect species including cicadas, silkworms and mealworms (574–795%, 412–635%, 408–643% respectively).[17,41] The differences in the OBC between commercial and insect-derived chitosans can be attributed to possible differences in crystallinity, salt-forming groups and protein residues in the samples.[62]

Cricket-derived chitosans exhibited a similar particle size distribution, according to Fig. 1, with a main fraction of higher particle size and a smaller one with a lower particle size, when isolated from chitin with chemical treatments. In particular, the larger fraction showed a particle size of 482.20 and 494.70 nm for Chitosan_C and Chitosan_M, respectively. The smaller fraction showed a particle size of 81.74 and 52.92 nm for Chitosan_C and Chitosan_M, respectively. On the contrary, Chitosan_B exhibited only one large fraction with 584.23 nm, similarly to the commercial chitosan that exhibited one fraction of 253.6 nm. Chitin can be partially hydrolyzed during the demineralization process due to the harsh acidic conditions, long treatment times and amount of solvent, resulting in some chitin losses.[63] These losses could explain the larger particle size of Chitosan_B and the low particle size fraction of Chitosan_C and Chitosan_M. Additionally, the presence of residual impurities could influence the particle size distribution of the chitosans is expected to affect the particle size. It is also important to consider the values of the PDI since the chitosans that are produced from the insects show values higher than 0.5, which indicates a higher heterogeneity.[64] Furthermore, Chitosan_B and Chitosan_C showed significantly higher ($p < 0.05$) zeta potential values than the commercial one (Table 1 and Fig. 1), which is an indication of higher colloidal stability and resistance to aggregation.[65]

The solubility of chitosan is related to its ionic strength, as well, due to the chitosan's ability to form pseudo colloidal dispersions when solubility is low. Fig. 2 presents the zeta potential of the commercial chitosan and the ones produced from the crickets. Chitosan has been reported to exhibit a large particle size >1000 nm and a high zeta potential (approx. 50 mV).[55] Chitosans exhibit a positive charging at pH < 7 due to the presence of $-NH_3^+$. When the pH decreases and a solution becomes more acidic, the positive charge of chitosan increases and it becomes more soluble. Chitosan with higher molecular weight tend to aggregate and lose their positive charging as the pH increases due to the formation of hydrogen bonds. This aggregation causes chitosan to function as a micelle and was reported to lead to higher absolute values of the zeta potential.[66] Chitosans with particularly high values of the zeta potential, as in the case of the cricket-derived chitosans, have been reported to have good rheological properties (because the viscosity of

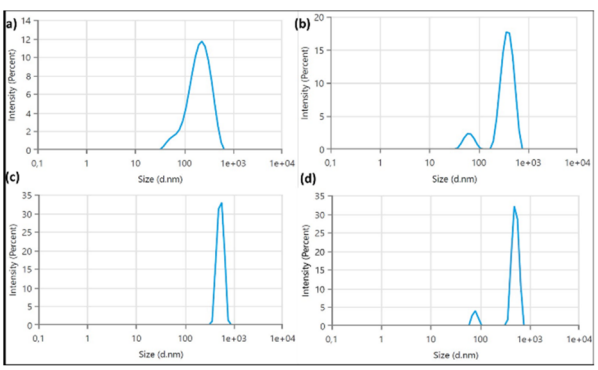

Fig. 1 Particle size distribution of chitosan that was commercially purchased (a) or generated from the crickets from chitin isolated through the chemical (b), biological (c) and microwave-assisted method (d).

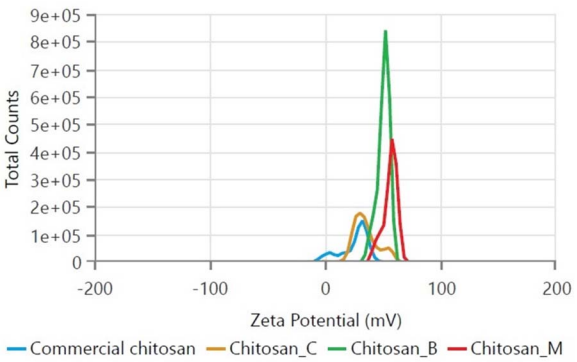

Fig. 2 Zeta potential (mV) of chitosan that was commercially purchased or generated from the crickets from chitin isolated through the chemical (Chitosan_C), biological (Chitosan_B) and microwave-assisted method (Chitosan_M).

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a chitosan-dispersion would decrease when the dispersion is stirred faster), as well as good colloidal stability.[55] Additionally, a high positive charging of chitosan would indicate a stronger antimicrobial activity.[55,66]

The cricket-derived chitosans contains impurities and have a lower chitosan content in comparison to the commercial one that is of analytical grade. Additionally, the cricket-derived chitosans are more acetylated than the commercial one[30] and therefore has a lower solubility. Considering that the residual impurities on the cricket-derived chitosan are expected to be insoluble proteins and melanin, which also contain amino groups and have colloidal properties, the cricket-based chitosans could show a higher zeta potential. Based on these properties, the cricket-derived chitosans show higher potential for more applications compared to the commercial one.

Based on the results presented in Table 1, the application of the treatment with *L. lactis* followed by the digestion with bromelain was considered appropriate alternative to the conventional method for chitin extraction. This alternative would address the disadvantages of the conventional method, such as the generation of large amounts of liquid waste and loss of nutrients,[26] since the side streams can be more easily repurposed. The long treatment time of Chitosan_B is the main disadvantage of its production method. Therefore, it is important to optimize this process to reduce the duration. However, the application of microwaves is also applicable for the production of cricket-derived chitosan, since it can reduce the treatment time and the energy consumption of the process.

3.2 Bioactivity of chitosan

3.2.1 Antioxidant activity. Fig. 3 presents the antioxidant activity of the chitosans. All chitosans were found to have both free radical scavenging activity and ferric iron reducing power. The free radical scavenging activity of all chitosans ranged between 10% and 60%, with ascorbic acid showing approximately 90% of scavenging activity. The absorbance at 700 nm indicates FRAP was found for all chitosan to range between 0.1 and 1. The absorbance obtained for ascorbic acid was approximately 1.7. A higher concentration of chitosan in the solution led to a higher antioxidant activity in the solution.

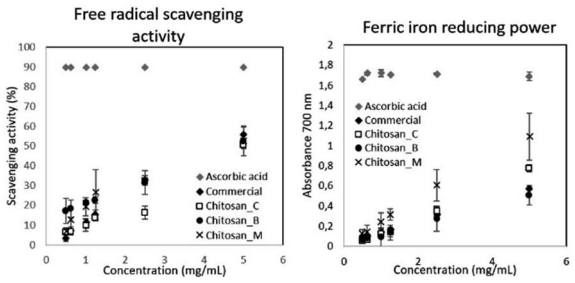

Fig. 3 Free radical scavenging activity (left) and ferric iron reducing power (right) of chitosan that was commercially purchased or generated from the crickets from chitin isolated through the chemical (Chitosan_C), biological (Chitosan_B) and microwave-assisted method (Chitosan_M). Error bars represent the standard deviation of each value based on multiple replications of the measurement.

Both free radical scavenging activity and ferric iron reducing power were observed for all chitosan samples at a concentration range of 0.5–5 mg mL^{-1}. This concentration range has been reported also for insect-derived chitosan from different species, including *C. barbarous*, *O. decorus*, *Musca domestica* and *Chrysomya megacephala*.[18,44,67]

Regarding the scavenging activity, Chitosan_B and commercial chitosan showed no significant differences ($p > 0.05$) of the IC50, while Chitosan_C was significantly higher ($p < 0.05$). On the contrary, regarding the EC50, the solution of Chitosan_C showed a significantly lower EC50 ($p < 0.05$). Commercial chitosan and Chitosan_B solutions showed no significant differences ($p > 0.05$), meaning that regarding both the scavenging activity and the reducing power, Chitosan_B could replace the commercial one.

Table 2 shows the IC50 and EC50 of all chitosans. The IC50 of chitosan refers to the chitosan concentration in a dispersion that is sufficient to achieve a 50% antioxidant activity in compare to a dispersion without chitosan. Consequently, a lower IC50 means that the amount of chitosan that is required to achieve a 50% antioxidant activity is lower and that this chitosan exhibits a stronger effect. The effective concentration of solutions of chitosan of crab[14] or insect origin[44,68] has been reported to be much higher than the one reported by the present study. The lower effective concentration indicates a stronger antioxidant activity and is attributed to the lower molecular weight that has been reported for chitosan produced from house crickets with the methods described in Section 2.2.[30] It has been shown, that a chitosan with lower molecular weight exhibits stronger bioactive properties.[15] The antioxidant activity of chitosan has been attributed to its amino group and its hydroxyl groups that react with free radicals.[56] Chitosans with lower molecular weight exhibit a lower amount of intramolecular bonds and a higher molarity for the same respective mass and consequently a higher amount of amino and hydroxyl groups are available for reacting with free radicals.[56,69,70]

Additional factors affecting the antioxidant activity of chitosan are the degree of acetylation, whereas more acetylated chitosans exhibit lower antioxidant activity,[69] and solubility since chitosan needs to be solubilized to participate in oxidative reactions.[71] Therefore, processing pathways and fractionation processes that generate chitosan with higher molecular weight

Table 2 IC50 and EC50 of chitosan that was commercially purchased or generated from the crickets from chitin isolated through the chemical (Chitosan_C), biological (Chitosan_B) and microwave-assisted method (Chitosan_M)[a]

	IC50 (mg mL^{-1})	EC50 (mg mL^{-1})
Commercial chitosan	4.36 ± 0.28^{a}	4.26 ± 0.11^{ab}
Chitosan_C	5.31 ± 0.47^{b}	3.37 ± 0.25^{b}
Chitosan_B	4.62 ± 0.55^{a}	5.06 ± 1.13^{a}
Chitosan_M	4.46 ± 0.37^{a}	2.27 ± 0.54^{c}

[a] Data are expressed as mean ± SD. Different superscript letters (a,b…) indicate significant differences ($p < 0.05$) among the means obtained for samples that were generated with different methods.

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and degree of acetylation will lead to chitosan with reduced antioxidant activity. The cricket-derived chitosans have been reported to have low molecular weight with Chitosan_B exhibiting particularly low molecular weight due to partial depolymerisation of chitosan from bromelain.[30] Since, the processes that lead to depolymerisation of chitin and chitosan are known to generate chitosan with stronger bioactive properties,[15] the cricket-based chitosans with the low molecular weight were expected to exhibit strong antioxidant activity.

The antioxidant activity of a biologically active molecule can be estimated with various methods that correspond to different reaction mechanisms. The DPPH method describes the free radical scavenging activity that is based on the transfer of electrons and H atoms, while the FRAP method describes the reduction of ferric ions due to the electron transfer reaction.[72] The examination of several protocols for the *in vitro* antioxidant activity of a bioactive molecule can provide a more comprehensive argument of its activity and highlight the mechanism of the antioxidant activity of that molecule. A statistical correlation of these methods has been reported in the case of lignin.[73]

Insect-derived chitosan has been shown to require higher effective concentrations for reducing power than free radical scavenging.[44] This was not the case for the chitosans produced from the house crickets, which showed a stronger free radical scavenging activity. Therefore, the antioxidant activity of chitosan from insects depends on both the production method and the species of origin. The lack of significant differences of the effective concentration of the cricket-derived chitosans and the commercial one highlights the importance of house crickets as a chitosan source alternative to the crustacean sources.

3.2.2 Antimicrobial activity. According to the results presented in Table 3, all chitosans showed an antimicrobial activity that is comparable but lower to the one of the commercial chitosan. Chitosan_C and Chitosan_B have been produced from house crickets and reported to have a low molecular weight.[30] This leads to a stronger antimicrobial activity for Gram-negative strains, while the opposite is observed for Gram-positive strains.[15] This would explain, why the MIC of the chitosans against *Staphylococcus aureus* is higher, compared to the other strains. Specifically for *Staphylococcus aureus*, it has been suggested that chitosan forms a membrane around the cell surface, not allowing nutrients to enter the cell, while for *Escherichia coli*, the antimicrobial activity of chitosan is pervasion-based.[16]

Table 3 Minimum inhibitory concentration (mg mL^{-1}) of chitosan that was commercially purchased or generated from the crickets from chitin isolated through the chemical (Chitosan_C), biological (Chitosan_B) and microwave-assisted method (Chitosan_M)

Minimum inhibitory concentration for antimicrobial activity (mg mL^{-1})			
	Escherichia coli	*Staphylococcus aureus*	*Salmonella enterica*
Commercial chitosan	0.14	0.56	0.28
Chitosan_C	0.14	1.13	0.28
Chitosan_B	0.56	1.13	1.13
Chitosan_M	0.28	0.56	1.13

Similar to the antioxidant activity, the antimicrobial activity of chitosan has been mainly attributed to the presence of $-NH_3^+$.[74] Regarding Gram-negative bacteria, chitosan interacts with anionic structures that are found on their surface, such as lipopolysaccharides and proteins.[75] Regarding Gram-positive bacteria, chitosan interacts with their cell wall layer, consisting of negatively charges of peptidoglycans and teichoic acids.[76] Chitosans with lower molecular weight and lower degree of acetylation would be expected to exhibit a higher amount of available reactive and positively charged amino groups.[77] Additionally, chitosan with higher molecular weight was reported to form a barrier across Gram-positive bacteria blocking the transfer of nutrients, while chitosan with lower molecular weight can cross the cell of Gram-negative bacteria more easily,[16] making chitosan with high molecular weight more effective against Gram-positive bacteria and *vice versa*.[78]

The antimicrobial properties of the chitosans produced from house crickets confirmed and were similar to the ones reported by Malm and Liceaga (2021), who suggested a minimum inhibitory concentration of higher than 0.5 mg mL^{-1} for chitosan produced from house crickets,[19] while in the present study the respective concentration was 0.14–1.13 depending on the sample. The minimum inhibitory concentration of the cricket-derived chitosans against the examined bacteria is within the range that is reported for chitosan from marine sources (100–1000 ppm that would correspond to 0.1–1 mg mL^{-1}).[79] This finding highlights the potential of the cricket-derived chitosan as an antimicrobial agent comparable to crustacean-derived chitosan, which further underscores their potential in the agri-food chain, considering also their rich nutritional profile.[48]

4. Conclusions

A variety of processing pathways used for chitosan production from other resources can be applied to the house cricket biomass. Microwave processing, as well as, the combination of biological processes, such as fermentation and digestion, are efficient in replacing the conventional process for chitosan production based on its properties. Chitosans that were produced from the crickets were found to have significant bioactive properties. In specific, they showed high antioxidant activity and were quite efficient as antimicrobial agents against some commonly encountered pathogenic bacterial strains. The findings reported by the present study underline the applicability of alternative and more environmentally friendly methods for chitin isolation to house crickets, to produce a chitosan. The chitosan produced with these methods showed the highest zeta potential (52.35 mV for Chitosan_M), higher antioxidant activity with the lowest IC50 (4.46 mg mL^{-1} for Chitosan_M) and comparable EC50 (lowest 2.27 mg mL^{-1} for Chitosan_M), better antimicrobial activity (lowest minimum inhibitory concentration 0.28 mg mL^{-1} for Chitosan_M) and higher OBC (885.30 g oil per g chitosan for Chitosan_B). However, the isolating of high-value insect-derived chitin with environmentally friendly methods is still a challenging matter.

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Data availability

The data supporting the findings of this study are available within the article.

Author contributions

MP: conceptualization, data curation, formal analysis, investigation, methodology, validation, writing, review – editing; NM: conceptualization, data curation, formal analysis, investigation, methodology, validation, writing, review – editing; SO: conceptualization, investigation, methodology, validation, writing, review – editing; RS: methodology, writing, review – editing; OS: fund acquisition, supervision, writing, review – editing.

Conflicts of interest

There are no conflicts to declare.

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