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Physicochemical and antibacterial properties of chitosan extracted from swimming crab shells and wooden grasshoppers using different extraction methods

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Abstract

Marine by-products and insects are among the sources of chitin used in chitosan production and increase the value of the product that may be used in the food industry. The conversion of chitin to chitosan requires proper extraction methods in order to minimise energy use and waste while also producing good-quality chitosan. This study aimed to evaluate different methods of extracting chitosan from two sources and to characterise its physicochemical and antibacterial properties. The study utilised two distinct chitosan sources, i.e. crab shells and wooden grasshoppers, as well as two distinct extraction methods, i.e. conventional and green chemistry methods. The yield, water-ash content, solubility, physicochemical properties as determined by infrared spectroscopy (FTIR), degree of deacetylation (DD), crystallinity (XRD), microstructure (SEM) and antibacterial activity were all evaluated for chitosan quality. The results indicated that the green chemistry extraction of crab shells (M2P1 treatment) produced the highest yield, solubility and crystallinity index of all treatments, with a DD of 60.9%. The functional groups and microstructure of chitosan were remarkably similar across all treatments. Antibacterial activity was determined using the microdilution method against Gram-positive (*Clostridium acetobutylicum*) and Gram-negative (*Escherichia coli*) bacteria and the minimum inhibitory concentrations were identified, notably 2000 ppm for the green chemistry method. The green chemical extraction method using crab shells (M2P1) demonstrated that the extracted chitosan possessed beneficial physicochemical properties, especially on yield and solubility, and antimicrobial properties against both Gram-positive and Gram-negative bacteria. As such, based on the DD percentage and antibacterial activity, this implies that the extracted chitosan may be used as an alternative for the preservation of food in the food industry.

1. Introduction

Living organisms in the ocean produce approximately 10^{12}–10^{14} tonnes of chitin every year (Dhillon *et al*., 2013). The development of commercial extraction processes for commercially viable polymers would help to release this massive amount of chitin for use as a raw material. Typically, seafood by-products are burned, sent to landfill, dumped at sea or left to spoil (Yadav *et al*., 2019). If not treated appropriately, they may also harm human health, biodiversity and the environment. As a result, there is significant interest in seafood recycling, especially crustacean biowaste (Philibert *et al*., 2017). Meanwhile, insects are found all over the earth (Kaya, Lelešius, Nagrockaite *et al*., 2015). Grasshoppers, particularly *Valanga nigricornis* (Burm.), have become a typical daily menu item in Thailand, Uganda, Mexico, China and some regions of Indonesia. Nevertheless, in other regions, insects are viewed as plant pests. In contrast, the increased demand for animal-derived goods implies a rise in the need for raw materials for use as feed; sustainable future raw materials are necessary to meet the needs of a market that is becoming

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increasingly concerned about environmental issues (Allegretti *et al*., 2018; Dicke, 2018). Therefore, these marine by-products and insects may be investigated for their potential use as chitin sources for chitosan manufacturing.

Chitosan is commonly obtained using conventional chemical extraction as it produces higher yields than other procedures and is simple to perform (Beaney *et al*., 2005). However, it also requires more time, is relatively energy and water-intensive, and generates significant levels of caustic waste (Beaney *et al*., 2005; El Knidri *et al*., 2016). Furthermore, it is a relatively costly method and is harmful to the environment (Tan *et al*., 2020). Various chitosan extraction methods have been developed to be both more energy- and water-efficient at the same time as ecologically benign. Microbial activities, enzymatic techniques, microwave irradiation and ultrasonic chambers (sonicators) are among the innovative extraction methods that have been widely developed to replace traditional chemical extraction methods (Arbia *et al*., 2013; Anwar *et al*., 2017).

Chitin, chitosan and their derivatives have properties that make them attractive for use in a variety of industries, including biomedical, food, agriculture, genetic engineering, environmental pollution control, water treatment, paper manufacturing and photography (Younes and Rinaudo, 2015). Since chitosan is non-toxic, biodegradable and biocompatible, it is widely used as a component and encapsulant in the culinary, biomedical and agricultural industries (Yadav *et al*., 2019; Casadidio *et al*., 2019). In the food industry, chitosan can be used as an emulsifier, fining agent, thickening and stabilising agent, antioxidant and in low-calorie food mimetics (Harkin *et al*., 2019), as well as in food preservation due to its antibacterial and antifungal properties (Raafat and Sahl, 2009; Manigandan *et al*., 2018). Chitosan's antimicrobial activity against a variety of foodborne filamentous fungi, yeast and bacteria has sparked interest in its use as a natural food preservative (No *et al*., 2007). Chitosan is biocompatible, non-allergenic, non-toxic and has bio-functional properties, with the ability to remove specific elements, molecules and materials from foods, such as colours and fats (Manigandan *et al*., 2018). Thus, chitosan has been approved for use as a food additive in Korea and Japan since 1995 and 1983, respectively (No *et al*., 2007). The antioxidant, antibacterial and antifungal properties of chitosan-based films make them excellent substitutes for synthetic chemicals. The use of chitosan-based derivatives could also offer a viable means of extending the shelf life of foods without compromising their sensory qualities (Sridhar *et al*., 2021).

Based on the facts and circumstances, it is critical to determine the extraction method and source of chitosan that yields the best results in terms of physicochemical and antibacterial properties, as well as the lowest energy usage and waste during the extraction process for use in the food industry. This study aimed to evaluate different methods of extracting chitosan from two sources and to characterise its physicochemical and antibacterial properties.

2. Materials and methods

2.1 Preparation of raw materials

Two sources of chitin/chitosan were used in the present experiment, Javanese grasshoppers and crab shell by-product. The Wooden grasshoppers were obtained from a traditional market in Gunungkidul, Yogyakarta, Indonesia, while the crab shell by-product was obtained from fish auction sites on the north coast of Java, Indonesia. The crab shells were washed, air-dried and refrigerated overnight. They were then boiled in water at 80°C for 15 mins, soaked in 5% H_3PO_4 for 15 mins, oven -dried at 50°C for two days, and ground to pass through a 2 mm sieve (Suprapto *et al*., 2012; Setiyawan *et al*., 2021). The grasshoppers, meanwhile, were washed with water and killed by freezing, then thawed at room temperature, oven-dried at 50°C for two days and then ground as above (Liu *et al*., 2012).

2.2 Microorganisms

The bacteria *Clostridium acetobutylicum* (FNCC 0085) representing Gram-positive bacteria and *Escherichia coli* (FNCC 0091) representing Gram-negative bacteria were supplied from the Food and Nutrition Culture Collection (FNCC), Gadjah Mada University, Indonesia. These microorganisms were used in the tests for evaluating minimum inhibitory concentrations (MICs). MICs are the lowest antimicrobial doses that prevent the visible growth of a microbe after overnight incubation and are typically expressed in mg/mL or mg/L (Balouiri *et al.*, 2016; Oliveira *et al.*, 2022). MICs are most commonly used in research to measure the in vitro activity of novel antimicrobials. To quantify *in vitro* antimicrobial activity against bacteria and fungi, either the broth or agar dilution method can be used (Balouiri *et al.*, 2016). The microorganisms were maintained at 37°C in a nutrient agar medium. They were then cultured into Brain Heart Infusion (BHI) media at 37°C for 24 hrs under an anaerobic condition for the *C. acetobutylicum* and an aerobic condition for the *Escherichia coli* with nutrient broth (NB) as their media.

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2.3 Minimal inhibitory concentrations

The MIC were determined using a microdilution method in 96-well bottom plates based on Asli *et al.* (2017) and Orellano *et al*. (2019). Briefly, bacteria suspension was adjusted to 5×10^6 colony-forming units (CFU)/mL, cultured in a 96-well bottom plate with BHI in the presence of a control medium (Orellano *et al*., 2019). Different concentrations of chitosan were dissolved in 1% acetic acid (2000, 1000, 500, 250 and 125 ppm) (Meera and Venkataraman, 2017). The plate was incubated at 37°C for 24 hrs in an anaerobic condition for the *C. acetobutylicum* and an aerobic condition for the *Escherichia coli*. Bacterial growth was quantified by measuring the optical density at 550 nm (OD 550 nm) using a microplate reader (Asli *et al*., 2017). The MIC values were defined as the lowest concentration at which no visible growth was observed.

2.4 Chitosan extraction

Chitosan was prepared using two methods, i.e. conventional acid-base extraction based on Hajji *et al*. (2014) and Kumari and Rath (2014), and the green chemistry extraction method from Kaya, Baran, Erdołan *et al*. (2014) and Anwar *et al*. (2017) with a slight modification.

2.4.1 Acid-base conventional extraction method

The demineralisation step was carried out by treatment with 2 N HCl solution at ambient temperature with a solution-to-solid ratio of 1:15 mL/mg for 12 to 24 hrs. The resulting solid fraction was washed with distilled water until a neutral pH was achieved. Deproteinisation was carried out with a solution of 2 N NaOH under maceration using 20 mL of solution per gram of demineralised shells for 12 to 24 hrs. The resulting solid fraction was then washed with distilled water until a neutral pH was reached, then chopped and sieved on 10 mesh. The chitin content was determined based on the weight differences of the raw materials and the chitin obtained after the acid and alkaline treatments. The chitin extracted from different species was treated with 50% NaOH (15 mL/g) at ambient temperature for five days. Every 48 hrs, the mixture was stirred and replaced with a new NaOH solution. After filtration, the residue was washed to neutrality with water several times and then dried.

2.4.2 Green chemistry extraction method

The sample powder (20 g) was treated with 2 N HCl solution (100 mL) at 70°C for 2 hrs to remove minerals and catechols. The demineralisation step was followed by rinsing with distilled water until neutrality was reached. Deproteinisation was performed using alkaline treatment with 2 M NaOH (50 mL) solution at 85°C for 16 hrs, and the product was washed with distilled water until the pH became neutral (Kaya, Baran, Erdołan *et al*., 2014). Chitin deacetylation used the sonication method in which 20 g dry chitin was suspended in 100 mL of 60% NaOH aqueous solution. The solution was heated in an ultrasonic bath at 60°C for 30 mins. The product was then filtered and washed using distilled water until neutral and allowed to dry (Anwar *et al*., 2017). The weight of the chitosan produced was measured and the yield was calculated as shown in Table 1.

2.5 Chitosan characterisation

2.5.1 Degree of deacetylation

The degree of deacetylation (DD) was determined using an infrared spectroscopy method based on Kaya, Baran, Erdołan *et al*. (2014). The DD of the chitosan samples was calculated according to the following equation:

$$DD\ (\%) = 100 - [(A1658\ /A3450) \times 115].$$

Where DD: degree of deacetylation, A1658: absorbance value at 1658 cm^{-1} (amide) and A3450: absorbance value at 3450 cm^{-1} (hydroxyl band)

2.5.2 Composition analysis

Samples were ground and screened with a 1-mm screen. Dry matter (method 950.15) and ash (method 942.5) content were analysed based on the methods by the Association of Official Analytical Chemists (1990).

2.5.3 Solubility of chitosan

An amount of 0.1 g chitosan was placed into a pre-weighed centrifuge tube, then dissolved in 10 mL of 1% aqueous acetic acid at 30°C under constant stirring for one hr and centrifuged. The supernatant was removed, and the pellet was dried at 60°C for two days. The solubility of the sample was calculated using the following equation:

$$Solubility\ (\%) = (M1\text{-}M2)/(M1\text{-}M0) \times 100$$

where M0 is the initial weight of the tube, and M1 and M2 are the initial weight of the tube plus sample and the final weight of the tube sample, respectively. All physicochemical experiments were repeated in triplicate and the mean was taken. The solubility result is shown in Table 1 (Luo *et al*., 2019).

2.5.4 Functional group

FTIR spectra acquisition was performed using an FTIR spectrophotometer (Bruker Vertex 80, Germany) equipped with attenuated total reflectance (ATR) technique and DTGS (deuterated triglycine sulfate)

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detector. The sample was placed directly above the ATR crystal and measurement was performed using the absorbance mode employing a resolution of 8 cm^{-1} and a total of 32 scans. The sample was scanned in the mid-infrared region with a wavenumber range of 4000–650 cm^{-1}. Background spectra were assessed by measuring the spectra of air before each sample measurement. The spectra were then analysed using OPUS software 8.5 (Bruker, Germany) to identify the functional groups in the different chitosan samples. The spectra of this sample are shown in Figure 1.

2.5.5 Crystallinity

X-ray diffraction spectrometer (XRD) analysis of chitosan was used to detect its crystallinity. A Shimadzu-7000 Advance diffractometer was used at 40 kV, 30 mA and 2θ with a scan angle from 5° to 45°, with a scan speed of 2°/min (Kaya, Baran, Erdoŧan *et al*., 2014). Crystalline index values (CrI) were measured using the following equation:

$$CrI_{110} = [(I_{110}-I_{am})/ I_{110}] \times 100$$

Where I_{110}: maximum intensity at 2θ ≅ 20° and I_{am}: intensity of amorphous diffraction at 2θ ≅ 16°.

Sigma-Aldrich commercial shrimp chitin was used as a comparison for the chitosan from grasshoppers and crab shells with different extraction methods.

2.5.6 Microstructure

The microstructure of chitosan was examined using a scanning electron microscope (SEM). The samples were coated with gold for SEM analysis using an MC1000 ion sputter (Hitachi Corp). The Hitachi SU3500 SEM instrument settings were accelerating voltage (Vacc) 3kV and 5kV, spot intensity 30%, working distance 6 mm, and magnification 5K and 10K (Karimy *et al*., 2020).

2.6 Statistical analysis

This study used a completely randomised factorial design where the first factor was the extraction methods, which consisted of M1 (conventional acid-base extraction method) and M2 (green chemistry extraction method), and the second factor was the chitin sources, i.e. P1 (crab shells) and P2 (wooden grasshopper), conducted in five replicates. The data obtained were analysed using analysis of variance (ANOVA) and continued to a post hoc test using Least Significant Differences (LSD). All of the statistical analyses were performed using COSTAT software.

3. Results and discussion

Table 1 illustrates the significant interactions between the extraction methods and chitin/chitosan sources (P<0.05) for the yield, moisture, DD and ash content parameters. However, no significant interaction was found for the solubility parameter.

3.1 Ash content

The ash concentration of chitosan indicates the efficacy of the procedure used to remove inorganic contaminants. As indicated in Table 1, M2P1 had the highest ash content among other treatments (P<0.05). The demineralisation process using 2 N HCl heating at 70°C for two hrs with a sample-to-HCl solution ratio of 1:5 was seemingly incapable of fully removing the inorganic materials from the swimming crab shells. Demineralisation entails the breaking down of calcium carbonate into calcium chloride as well as the release of carbon dioxide (Yadav *et al*., 2019). The reason for the suboptimal removal is that crustacean shells (20–40%) contain high levels of inorganic materials compared to insects (less than 10%) (Liu *et al*., 2012). The ash content varies primarily due to the source and material composition (Martín-lópez *et al*., 2020), which could explain why the ash content of chitosan from the wooden

Table 1. Physicochemical characteristics of chitosan.

	P1	P2	Mean
Moisture[#] (%)			
M1	9.99±1.53	9.64±1.23	9.82±1.38[a]
M2	7.41±0.88[b]	9.96±1.24[a]	8.69±1.06[b]
Mean	8.70±1.2[b]	9.80±1.24[a]	
Ash[#] (%)			
M1	0.13±0.07[c]	1.17±0.19[b]	0.65±0.13[a]
M2	26.6±1.17[a]	0.26±0.08[bc]	13.4±0.63[b]
Mean	13.4±0.62[a]	0.72±0.14[b]	
Yield[#] (%)			
M1	15.6±3.02[b]	10.3±0.07[c]	12.9±1.54[b]
M2	21.2±0.74[a]	9.50±0.97[c]	15.3±0.86[a]
Mean	18.4±1.88[a]	9.90±0.52[b]	
DD[#] (%)			
M1	62.5±0.46[a]	54.0±0.04[d]	58.3±0.25[a]
M2	60.9±0.16[b]	59.9±0.08[c]	60.4±0.12[b]
Mean	61.7±0.31[a]	56.9±0.06[b]	
Solubility (%)			
M1	7.54±2.89	12.1±5.45	9.8±4.17[b]
M2	18.5±8.03	17.8±5.73	18.2±6.88[a]
Mean	13.0±5.46	14.9±5.59	

P1: Swimming crabs shell, P2: Wooden grasshopper, M1: conventional chemistry extraction, M2: Green chemistry extraction, DD: degree of deacetylation.
[#]Significant interaction between isolation method and materials resource.

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grasshopper (M2P2) was lower than that of crab shell (M2P1) even when using the same extraction method. Due to the difficulty of removing all minerals due to the heterogeneity of the matrix, a higher volume or more concentrated acid solution is needed (Younes and Rinaudo, 2015). Furthermore, the ash content of chitosan affects solubility. The presence of residual ash will consequently lower the viscosity and solubility (Marei *et al*., 2016).

3.2 Chitosan yield

The M2P1 treatment produced the highest yield among the other treatments ($P<0.05$). This may indicate that 30 minutes of sonication at 60°C is the appropriate time and temperature. Longer times may reduce the chitosan yield because it will dissolve. This condition reflected the study by Anwar *et al*. (2017), which demonstrated that the longer the ultrasonic irradiation period, the greater the DD, but the lower the percentage yield of the product. Long-term ultrasonic irradiation at high power altered the depolymerisation of chitosan into tiny molecules that are both easier to dissolve in water and lose during the washing process. In contrast, the wooden grasshopper sources yielded less chitosan than the crab shells in both of the extraction methods used. The primary reasons for the lower insect chitosan content compared to crustacea were the high protein and fat content, as also reported by Luo *et al*. (2019).

3.3 Degree of deacetylation

The green chemistry extraction method using ultrasonic irradiation gave a higher DD result than conventional extraction using the maceration method ($P<0.05$). The efficient deacetylation of chitosan was observed at 60°C, with a NaOH concentration of 60% (w/v) and an ultrasonic irradiation time of up to 30 minutes (Anwar *et al*., 2017). Ultrasound-assisted extraction proved to be an efficient method for chitin deacetylation, requiring a shorter reaction time and consuming less energy than the conventional process (Martín-López *et al*., 2020). The DD in this research was around 54–62%, which falls within the low DD category. Chitosan can be classified based on its DD as having either a high degree of deacetylation (HDD) (70–99%) or a low degree of deacetylation (LDD) (55–70%) (Joseph *et al*., 2021). An LDD is suitable for an emulsifying agent, antimicrobial activity, pharmaceutics, polymer nanocomposite and food formulations. Species and preparation methods have been found to influence the DD (Luo *et al*., 2019). The concentration of NaOH, temperature, reaction duration and recurrence of alkaline treatment steps all have a significant impact on the molecular weight and deacetylation of chitosan (Yadav *et al*., 2019).

3.4 Solubility

The solubility values in this study were generally lower than those reported in other studies (Kumari *et al*., 2017). Solubility depends on the operating temperature of the deacetylation process. Higher temperatures during the deacetylation process lead to a reduction in the solubility of chitosan. The quality of chitosan depends on the DD and the acetyl group distribution along the chains, which in turn affect its solubility. Cheng *et al*. (2020) found that chitosan with a higher DD (microwave extraction method) was more soluble than that with a lower DD (water bath extraction method). Furthermore, a lack of solubility may be due to incomplete acetyl group removal during the extraction, especially during the deacetylation stage (Sani *et al*., 2017). Nevertheless, M2P1, which is the green extraction method using ultrasonic irradiation, had the highest solubility among other treatments. This is probably due to the lower molecular weight caused by the ultrasonic irradiation process. The ultrasonic process can degrade the large molecular weight of chitosan into chitosan with a moderate molecular weight so that it becomes more soluble than before (Yuliana *et al*., 2012).

3.5 Antibacterial activity of chitosan

Table 2 shows the antibacterial test for chitosan against certain bacteria species, i.e. *C. acetobutylicum* and *Escherichia coli.* The MICs show that the green chemistry extraction method (M2) with a 2,000-ppm concentration significantly inhibited the growth of *C. acetobutylicum* ($P<0.05$), while *E. coli* was not significant. This is probably due to the greater bactericidal effect of chitosan on Gram-positive bacteria (such as *Bacillus megaterium, Bacillus cereus, Listeria monocytogenes, Lactobacillus plantarum, Staphylococcus aureus, Lactobacillus brevis, Clostridium* and *Lactobacillus bulgaricus*) (Kyoon *et al*., 2002) than on Gram-negative bacteria (such as *E. coli, Pseudomonas fluorescens* and *Salmonella*) (Kyoon *et al*., 2002; Raafat and Sahl, 2009). Gram-positive and Gram-negative bacteria are both susceptible to the antibacterial activity of chitosan, the former more so due to differences in the molecular architectural characteristics in the cell walls (negative charge distribution and availability) (Duan *et al*., 2019). Chitosan is effective in suppressing the growth of both Gram-positive and Gram-negative bacteria, although there are certain discrepancies in the effectiveness of chitosan against the two types of bacteria (Rajasekaran and Santra, 2015; Yu *et al*., 2020). MICs ranged from 125 to 2,000 ppm and could inhibit the growth of the two bacteria tested. A chitosan concentration of 250 ppm had a bactericidal effect against *Clostridium perfringens* (Chang *et al*.,

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Table 2. Antibacterial test of chitosan.

	2000 ppm	1000 ppm	500 ppm	250 ppm	125 ppm	Mean
C. asetobutyricum[#]						
M1	115±31.6^{cd}	124±13.7^{bc}	111±11.2^{cde}	115±13.1^{cd}	123±15.0^{c}	118±16.9^{b}
M2	223±41.9^{a}	144±16.5^{b}	104±12.3^{cde}	91.6±16.8^{e}	98.4±17.1^{de}	132±20.9^{a}
Mean	169±36.7^{a}	134±15.1^{b}	107±11.8^{c}	104±15.0^{c}	111±16.0^{c}	
E. coli						
M1	155±167	39.7±72.7	139±80.0	172±14.4	80.0±15.2	120±111
M2	70.1±115	114±132	77.0±59.1	93.2±17.3	184±16.3	95.4±107
Mean	113±141	76.8±102	108±69.6	133±15.9	132±15.7	

[#]Significant interaction between isolation method and dosage of chitosan.

2020). Chen *et al*. (2012) found that after 48 hrs of incubation, nanoparticles of carboxymethyl-chitosan at a concentration of 2,000 g/mL inhibited 100% of *S. aureus* and *E. coli* biofilms. Chitosan activity is generated by interactions between the biopolymer chitosan and the cell's permeability (pH less than 6.5) and when negatively charged microbial cell walls release proteinaceous and other intracellular components (Rabea *et al*., 2003; Goy *et al*., 2016; Vilar Junior *et al*., 2016). Additionally, chitosan serves as a chelating agent, binding trace metals selectively and inhibiting the formation of toxins and microbial growth (Rabea *et al*., 2003). According to Goy *et al*. (2016), the decrease in antibacterial activity with increasing polymer concentrations can be explained by the spatial configuration of the polymer chains: lower polymer concentrations result in a more homogeneous molecular distribution in the solvent, with a low number of contacts between nearby chains, thus increasing the charged sites available for external coupling. Additionally, the interaction of diffused hydrolysis products with microbial DNA inhibits mRNA and protein synthesis (No *et al*., 2007). Due to its antibacterial activity against both Gram-positive and Gram-negative bacteria, the extracted chitosan using green extraction chemistry at a dosage of 2,000 ppm can be used as a food preservative in the food industry.

3.6 FTIR

FTIR spectroscopy is a fingerprint analytical technique that is widely used for qualitative analysis to identify functional groups of different samples. The use of the FTIR technique in sample measurement provides advantages such as minimising the sample preparation step as the sample can be placed directly above the crystal, thus enabling a rapid analysis time. Figure 1 depicts the FTIR spectra of standard chitosan and chitosan extracted from swimming crabs and wooden grasshoppers obtained using both conventional and green extraction techniques. The FTIR spectra of the chitosan obtained from swimming crabs and wooden grasshoppers using the conventional extraction technique had a very similar FTIR spectra pattern to the commercial chitosan standard. Additionally, all the peaks showed the same wavenumber as the commercial

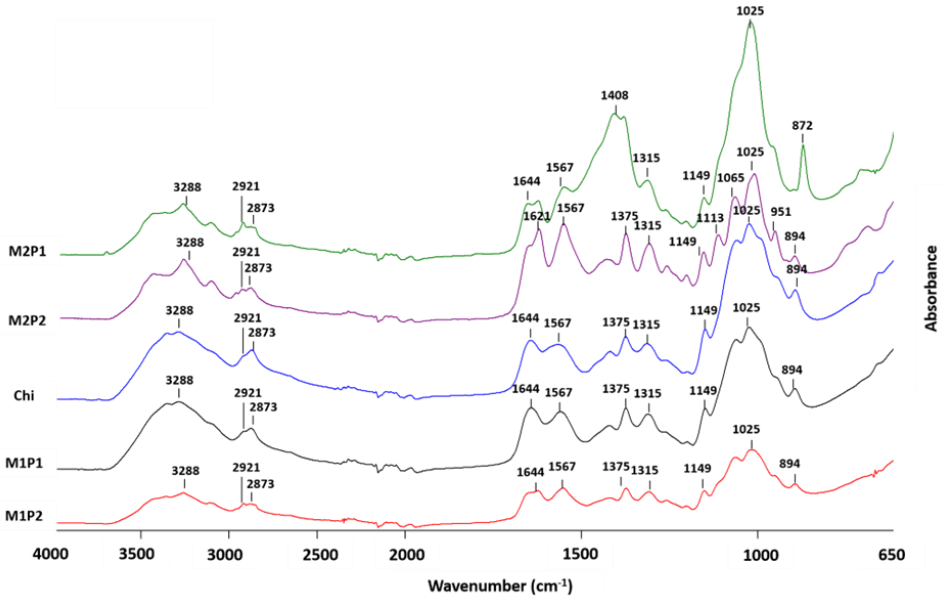

Figure 1. FTIR spectra of commercial chitosan standard (chi), chitosan extracted using conventional technique from swimming crabs (M1P1) and wooden grasshoppers (M1P2), and chitosan extracted using green extraction technique from swimming crabs (M2P1) and wooden grasshoppers (M2P2).

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chitosan standard. Meanwhile, the chitosan obtained from the swimming crabs and wooden grasshoppers using the green extraction technique displayed a slightly different FTIR spectra pattern from the commercial chitosan, especially in the swimming crabs. The wavenumber of 3288 cm^{-1} is associated with the stretching vibration of the -OH group. The wavenumber range of 3100–3500 cm^{-1} corresponds to the stretching vibration of the -NH and -OH groups (Paulino *et al*., 2006; Hajji *et al*., 2014); however, the vibration of -NH overlapped with -OH due to hydrogen bonding that resulted in peak broadening. Meanwhile, peaks at 2921 and 2873 cm^{-1} were associated with the asymmetric and symmetric stretching vibration from -CH, respectively. Two peaks were observed around 1650/1620 and 1550 cm^{-1}, indicating that the isolated chitosan is in the alpha chitin form (Kaya, Lelešius, Nagrockaite *et al*., 2015).

The stretching vibration of carbonyl (C=O), which derives from amide I, was observed at 1644 cm^{-1} but shifted slightly in M2P2 (1621 cm^{-1}). The vibration of amide II was observed from the stretching vibration of -NH at 1567 cm^{-1} (Song *et al*., 2013). Additionally, the vibration of amide III, which comes from C-N stretching, was observed at 1315 cm^{-1}. The vibration of amide I and amide III confirmed the presence of residual N-acetyl groups in chitosan. Three major bands are used to identify α-chitin at around 1660 cm^{-1} (C=O secondary amide stretch, Amide I), around 1620 cm^{-1} (C=O secondary amide stretch) and around 1550 cm^{-1} (N-H bend, C-N stretch, Amide II) (Kaya, Baran and Karaarslan, 2015). Studies by Kaya, Bitim, Mujtaba *et al*. (2015) and Song *et al*. (2013) explained that amide I, II and III are responsible for 1660, 1550 and 1310 cm^{-1} chitin characteristics bands, respectively. The band at 1149 cm^{-1} was associated with the stretching vibration of the C-O-C bridge whereas the peaks at 1113, 1065 and 1025 cm^{-1} correspond to the stretching vibration of C-O. Bending vibration of -CH was observed at the peaks of 1408, 1375, 951, 894 and 872 cm^{-1}. Although the chitosan samples obtained using the green extraction technique showed a slightly different FTIR spectra pattern than the commercial chitosan standard, the main vibrations of the functional groups for chitosan were still observed. The differences may therefore be caused by the presence of other compounds extracted using the green extraction technique that affects the FTIR spectra of chitosan. A previous study reported the possibility of contamination with glycosaminoglycans (a type of polysaccharides) in chitosan obtained from animal origin because it can be found in most animals including insects and crustacea (Yamada *et al*., 2011).

3.7 X-ray diffractogram and crystallinity

The XRD pattern for chitosan from crabs and grasshoppers using different extraction methods, is represented in Figure 2. From the results, the chitosan samples showed similar XRD patterns, with three strong reflections as shown in Table 3, with the strongest peak of around 19 found for all treatments. Some chitosan XRD patterns in the literature showed two distinct peaks, usually around 2 θ 10 and 20 (Kaya, Bitim, Mujtaba *et al*., 2015; Antonino *et al*., 2017; Martín-lópez *et al*., 2020; Hao *et al*., 2021). Diffraction peaks for chitosan have been observed at theta values of 10° and 20° (Ibitoye *et al*., 2018). The intensity of the crystalline and amorphous regions was used to calculate the degree of crystallinity (Luo *et al*., 2019).

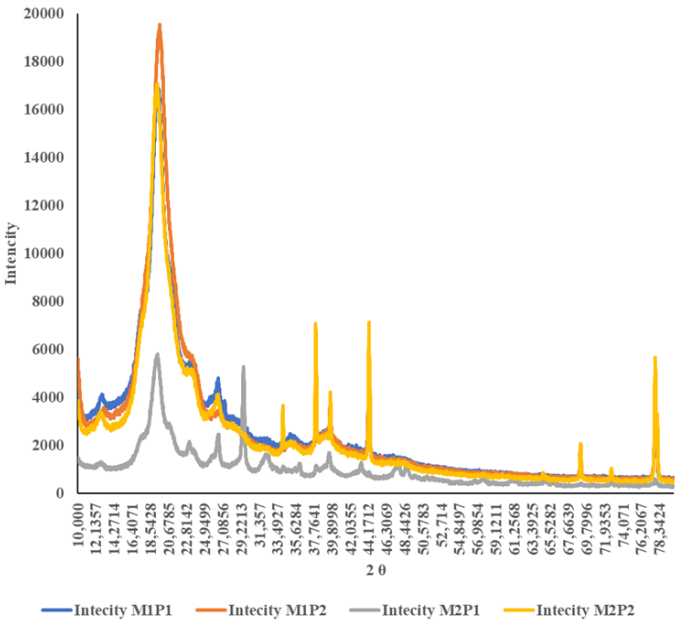

Figure 2. X-ray diffractograms of chitosan extracted using a conventional technique from swimming crabs (M1P1) and wooden grasshoppers (M1P2), and chitosan extracted using a green extraction technique from swimming crabs (M2P1) and wooden grasshoppers (M2P2).

Table 3. Peaks and crystalline of chitosan from treatments.

Treatments	XRD peaks			Crystallinity
M1P1	19.4	21.1	17.2	17.2
M1P2	19.6	21.4	77.8	16.1
M2P1	19.3	29.4	20.6	34.8
M2P2	19.4	21.1	77.8	21.1

The crystalline index (CrI) of chitin was calculated from the XRD data. Crystallinity was calculated as the ratio of the total area under the curve to the sum of the areas under the crystalline diffraction peaks (Hajji *et al*., 2014). The results showed that the M2P1 treatment has the highest CrI among other treatments (Table 3). Chitosan's crystallinity is highly correlated with its DD. The molecular chain of non-deacetylated chitin is relatively uniform and has a high degree of regularity,

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which results in a high degree of crystallinity. Deacetylation increases the heterogeneity of the molecular chain, resulting in a decrease in crystallinity; however, as DD increases, the molecular chain tends to become homogenised, increasing crystallinity (Cheng *et al*., 2020). The results indicate that using an ultrasonicator to extract chitosan reduced depolymerisation and enabled the production of chitosan with a high molecular weight (Martín-López *et al*., 2020). The lower value of CrI in this study compared to other studies probably led to an overestimation of the impact of the amorphous phase (Antonino *et al*., 2017). The CrI values of chitins isolated from various species differ according to the hardness of their shells. Furthermore, those with soft shells had lower crystalline index values, which corresponded to their lower crystalline index values (Kaya, Baran, Mentes *et al*., 2014). The variation between the CrI values obtained and those found in the literature is ascribed to the presence of intermolecular bonds following deacetylation. One of the most significant effects of crystallinity is found in the biological activities of chitosan, such as the antimicrobial effect; depending on the source of the material, the different molecular arrangements in the structure of the biopolymer may cause electrostatic interaction with the microbial cell walls (Martín-López *et al*., 2020).

3.8 Microstructure

Microstructure analysis was conducted using an SEM. Chitosan has three distinct surface morphologies: porous with a microfibrillar structure, porous without a microfibrillar structure and porous with only a microfibrillar structure (Kaya, Baran, Mentes *et al*., 2014). Similar results can be seen in the SEM photographs of the grasshopper source with a different extraction method, which mostly shows only a microfibrillar with no porous structure. The chitosan from crab shells M1P1 and M2P1 (Figure 3) was discovered to be formed in a microfibrillar crystalline structure with porous, which was more visible than in the chitosan derived from grasshoppers. A study of chitosan morphology on five aquatic insects and one crustacea species showed only a porous surface without a microfibrillar structure (Kaya, Baran, Mentes *et al*., 2014). This result is similar to that reported by Liu *et al*. (2012), who found that crustacea have a more clearly defined microfibrillar crystalline structure than insects. Furthermore, the non-porous structure of the wooden grasshopper could be due to the high protein content of the wooden grasshopper, which keeps the exoskeleton non-porous in comparison to the swimming crab shell (Kaya, Lelešius, Nagrockaite *et al.,* 2015; Sani *et al*., 2018).

4. Conclusion

Green chemical extraction using crab shells as a source of chitosan produced the best results in terms of physicochemical and antibacterial properties. The LDD of the extracted chitosan demonstrates its suitability for application in the food industry as an emulsifier or antimicrobial agent. Furthermore, the extracted chitosan has antibacterial activity against both Gram-positive and Gram-negative bacteria, meaning it can be used as a food preservative in the food industry. Nonetheless, further research is needed into the demineralisation process in order to produce chitosan with improved physicochemical properties, notably regarding the ash content solubility and antibacterial properties. A suboptimal demineralisation procedure will lead to an increase in the ash content, indirectly affecting the viscosity and solubility of chitosan.

Conflict of interest

The authors declare no conflict of interest.

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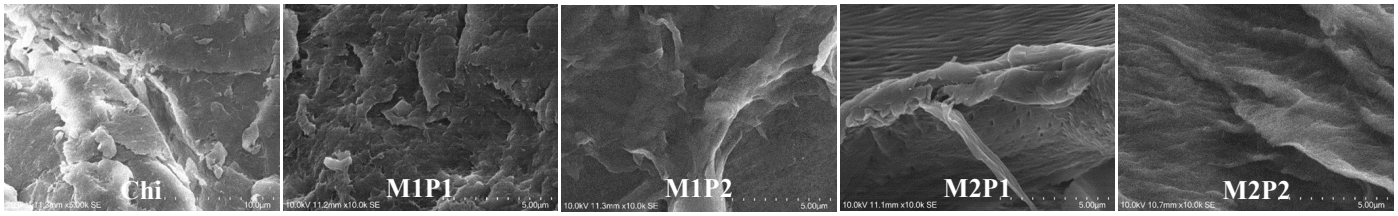

Figure 3. SEM figure of commercial chitosan standard (Chi), chitosan extracted using a conventional technique from swimming crabs (M1P1) and wooden grasshoppers (M1P2), and chitosan extracted using green extraction technique from swimming crabs (M2P1) and wooden grasshoppers (M2P2).

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