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Characterization and Antimicrobial Activity of Chitosan Extracted from Squid Pen Wastes

Nur Nadhirah Adibah Mohd Nor Naser, Maryam Mohamed Rehan*, Muhamad Arif Mohamad Jamali and Salina Mat Radzi
Faculty of Science and Technology, Universiti Sains Islam Malaysia (USIM),
Bandar Baru Nilai, 71800 Nilai, Negeri Sembilan, Malaysia
*Corresponding author (e-mail: maryam@usim.edu.my)

Chitosan is a versatile polysaccharide with applications across various industries due to its characteristics and antimicrobial properties. The physicochemical features and biological properties of chitosan are closely associated with its degree of deacetylation (DDA), which can be influenced by the selection of raw materials and the extraction process. This study aimed to evaluate the characteristics and antimicrobial potential of chitosan obtained from squid pen cartilage originating from different waste sources, namely restaurant waste (S) and market waste (SS), using chemical extraction methods, with and without demineralization step. Analyses using Ultraviolet-visible and Fourier-transform infrared spectroscopy spectrophotometers showed the presence of chitosan in all samples and identified the functional groups within the chitosan molecules. Chitosan yields ranged from 55-82.4%, while the DDA values were between 73.67-76.64%. All chitosan samples demonstrated antimicrobial activity against the tested Gram-positive and Gram-negative bacteria, with inhibition zones ranging from 9.5 ± 0.7 mm to 13.5 ± 2.1 mm using the agar well diffusion method. The MIC values for all chitosan samples varied from 25 to 100 μg/mL, while MBC values were in the range of 50 to 100 μg/mL, and MFC values against *A. niger* ranged from 25 to 100 μg/mL. The results indicate that chitosan derived from squid pen waste sources exhibits potential as a natural antimicrobial agent, and its DDA remains unaffected by the omission of the demineralization step.

Keywords: Chitosan; squid pen; seafood wastes; antimicrobial activity; demineralization

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Chitosan is a derivative compound of chitin, which is composed of a copolymer of D-glucosamine and N-acetyl-D-glucosamine residues. Chitosan is known for its non-toxic, non-immunogenic, biocompatible, biodegradable, and cost-effective properties [1] and can form various shapes such as particles, films, sponges, membranes, gels, and fibres due to its solubility in aqueous acid solutions [2]. This versatility has led to its utilization in various applications such as wound treatment, food packaging, chelation, dietary supplements, drug delivery, agricultural, and medical applications [1].

Chitosan's biochemical characteristics and overall quality play a vital role in determining its applicability in diverse fields. Its quality, stability, and suitability for various applications can be influenced by important parameters like the degree of deacetylation (DDA), molecular weight, and structural properties that can be assessed through techniques like infrared spectroscopy [3]. Moreover, chitosan exhibits anti-microbial properties against a range of bacteria [4] and fungi [5], making it a promising natural treatment option to address antibiotic resistance issues associated with foodborne and pathogenic bacteria. This approach may offer a cost-effective alternative to conventional drug-based antibiotics [6, 4].

Chitosan can be extracted from various sources containing chitin, including invertebrates such as insects, shrimps, squids, and fungi. Chitosan derived from seafood waste has gained attention due to its potential to address food waste issues arising from households, restaurants, and seafood processing facilities that can contribute to environmental pollution and pose health hazards [7]. Squid pens offer a valuable source of β-form chitosan, characterized by their superior reactivity and affinity during various physical and chemical treatments, compared to the α-form chitosan found in crab and shrimp shells [3].

The extraction process of chitosan from crustaceans or seashells can be accomplished using chemical or enzymatic methods [8], and chemical extraction is usually the preferred method commercially [7] since they are more cost-effective with short processing time [8]. Common steps in chitosan production using the chemical method involve demineralization, which removes most of the ash and minerals, primarily calcium carbonate, from the raw material [9]. This is followed by deproteinization, where protein contents are filtered out after hot alkaline treatment, yielding chitin. Finally, DDA is the crucial step in obtaining chitosan, where the acetyl

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group is removed. Various DDA methods result in different DDA [10].

Chitosan extracted from seafood wastes can exhibit variations in characteristics depending on the source or the marine species. For instance, squid pen chitosan has been found to exhibit a larger molecular weight, lower acetylation degree, higher purity, and lower residual inorganic material content compared to cuttlebone chitosan [11]. Moreover, [12] reported that chitosan extracted from two different species of squid pens, *Loligo lessoniana*, and *Loligo formosana*, displayed differences in properties, including acetylation degree, molecular weight, ash content, intrinsic viscosity, and gelation properties. Therefore, investigating the variations in chitosan properties among seafood waste sources would potentially provide information into optimizing its utilization for chitosan extraction. Furthermore, the production of chitosan can be expensive, making it crucial to explore methods for simplifying extraction processes to reduce costs and processing time and minimize chemical and power usage [13]. Unlike chitosan derived from other shells and molluscs, chitosan from squid pens contains low levels of ash and minerals, suggesting the potential to omit the demineralization step [10, 14, 15]. This may have several advantages, including reduced expenses for waste disposal of raw materials and a decreased reliance on extensive chemical usage such as acid treatment [16].

There is limited research on the properties of chitosan extracted from different types of squids obtained from different waste sources and the impact of demineralization on its properties. Therefore, this study aimed to characterize chitosan extracted from squid pen waste obtained from both restaurant (mixed types of squids) and market (same type) sources using chemical extraction methods, with and without demineralization. Characterization involved analysing the DDA and utilising UV-visible and FT-IR spectroscopy. Additionally, the antimicrobial activity of the extracted chitosan was assessed against selected Gram-positive (*Bacillus subtilis, Salmonella epidermidis,* and *Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa*, *Salmonella typhimurium,* and *Pseudomonas vulgaris*), as well as *A. niger*.

EXPERIMENTAL

Sample Collection and Pre-treatment

Two distinct sources of squid pen cartilage samples were used in this study. One was collected from restaurant wastes consisting of mixed squid types (labelled as 'S'), while another sample was purchased from the market comprising squid of the same types (labelled as 'SS' for the same species). The restaurant waste samples (S) specifically contain the cartilage portions and were gathered from four local restaurants in Nilai, Negeri Sembilan, Malaysia. These cartilage segments are inedible and typically discarded as waste after being separated from the edible parts used in cooking. The second set of samples (SS) was obtained from whole squids purchased from a market in Nilai, Negeri Sembilan, Malaysia, which were subsequently separated from the flesh to isolate the cartilage portions. All squid pen samples were washed thoroughly using tap water and then dried on absorbent paper. The samples were then stored in sealed plastic bags in a -40°C freezer until further use. The pens' length, width, and thickness were measured according to the study [17].

Extraction of Squid Pen Chitosan Using Chemical Method

Extraction of chitosan from squid pen cartilage samples was conducted according to [11] with some adaptation. The cartilage samples were first subjected to pre-treatment with boiling water for 1 h using a hot plate stirrer (Favorit, Germany). Before the chitosan extraction process, the pre-treatment method was employed to remove foreign matter and any remaining flesh that might have adhered to the pen cartilage, even after thorough washing (Figure 1). The squid pen cartilages were then dried in a drying oven (BINDER, United States) at 50°C overnight. After drying, the samples were ground and sieved to obtain squid pen powder with particle sizes less than 1 cm. Then, the weight of the dried squid pen was recorded for each sample. The squid pen samples from different waste origins (S and SS) were further divided into two separate flasks, each for evaluating different extraction protocols, one that includes the demineralization step (S1 and SS1) and another method of extraction without the demineralization step (S2 and SS2) (Table 1).

Table 1. Squid pen cartilage samples of different sources and chitosan extraction protocol.

Sample	Source	Extraction protocol
S1	Restaurant waste (mixed squid types)	With demineralization step
S2	Restaurant waste (mixed squid types)	Without demineralization step
SS1	Market waste (same squid type)	With demineralization step
SS2	Market waste (same squid type)	Without demineralization step

Figure 1. Dried squid pen after washing (left) and squid pen cartilage pre-treated by boiling (right).

The demineralization process was carried out by acid hydrolysis, in which the squid pen powder was mixed with a 28% (w/v) hydrochloric acid (HCl) at a ratio of 1:8. This mixture was stirred continuously at room temperature for 2 h using an orbital shaker (PROTECH, Malaysia). The pH of the solutions was regularly monitored throughout the acid hydrolysis process to maintain the pH below 5 using a pH meter (Mettler Toledo, Malaysia). After 2 h, the samples were then filtered and washed with distilled water to neutralize the pH. The samples were then collected and allowed to dry in a drying oven at 45°C overnight.

The next step was deproteinization, which is the first step for S2 and SS2 following pre-treatment. Firstly, the dried samples were mixed with sodium hydroxide (NaOH) (w/v) in a 1:10 ratio and incubated at 80°C for 2 h in a shaking water bath (MEMMERT, China). After that, the deproteinized samples were washed with distilled water until the pH reached a neutral level. This step was carried out to remove dissolved proteins and excess NaOH. The samples were then allowed to dry in a drying oven at a temperature of 45°C. After deproteinization, the dried samples obtained at this stage were expected to consist primarily of β-chitin, as described in [11]. The isolated β-chitin powder was weighted for subsequent calculation of the isolated yield.

Following deproteinization, the DDA process was performed to obtain chitosan using the NaOH DDA method. Chitin obtained in the previous step was treated with 70% NaOH (w/v) at a 1:10 ratio and incubated for 4 h at 90°C with agitation. The samples were then washed with distilled water until the washing water reached a neutral pH and then left to dry overnight at 50°C in a drying oven. The chitosan yield was determined using equation (1) according to [18]:

$$\text{Chitosan Yield (\%)} = \frac{\text{Weight of chitosan (g)}}{\text{Weight of chitin (g)}} \times 100 \quad (1)$$

Ultraviolet-Visible (UV-VIS) Spectroscopy Analysis

UV-visible absorption spectra of the chitosan samples were obtained using a UV-Visible spectrophotometer (Agilent, Cary 50 spectrophotometer) to analyse the spectral properties of the samples. Firstly, 0.01 g of dried chitosan was dissolved in 10 mL of 0.1 M acetic acid solution and subsequently diluted with 100 mL of distilled water. The mixture was vortexed twice and left for 48 h before the spectrophotometric evaluation [3]. The spectra were recorded at room temperature in the wavelength range of 180-500 nm and were read in triplicate. For each sample, blank 0.1 M acetic acid was used as blank.

Functional Group Detection by Fourier Transform Infrared (FT-IR) Spectral Analysis

The extracted chitosan samples were characterised using Fourier Transform Infrared (FT-IR) spectrophotometer (ThermoFisher, NICOLET, iS50 ATR) in the frequency range of 500 to 4,000 cm^{-1} [11]. The infrared spectrum of all chitosan samples was measured and analysed for the presence or absence of specific functional groups, as well as the chemical structure of chitosan.

Determination of Degree of Deacetylation (DDA)

The degree of DDA of the chitosan samples was determined using FT-IR spectra according to the method used by [19] and [10]. The DDA was calculated as follows:

$$\text{\% DA} = \frac{(A1320 / A1420) - 0.3822}{0.03133}, \quad (2)$$

$$\text{\% DDA} = 100 - \text{\% DA}, \quad (3)$$

where
DDA = degree of deacetylation (%)

DA = degree of acetylation (%)
A1320 = Absorbance at 1320 cm^{-1} representing the peak for the amide group
A1420 = Absorbance at 1420 cm^{-1} representing the peak for the amine group

The absorbance at 1,320 cm^{-1} measures the extent of N-acetylation, while the absorption band at 1,420 cm^{-1} compares D-glucosamine and N-acetyl-glucosamine. The ratio A1320/A1420 is used as an indicator for calculating the degree of DDA in the extracted chitosan. The ratio was reported to provide minimal errors and demonstrate sensitivity to the chemical composition of both chitin and chitosan [19].

Antimicrobial Assay

The chitosan samples were evaluated for their antimicrobial activity against six bacteria, which are three Gram-positive bacteria (*Bacillus subtilis, Salmonella epidermidis,* and *Staphylococcus aureus*) and three Gram-negative bacteria (*Pseudomonas aeruginosa, Salmonella typhimurium,* and *Pseudomonas vulgaris*). These bacteria were selected to assess the antimicrobial activity of the chitosan extract against a diverse range of bacteria commonly associated with infections. The antimicrobial activity analysis of chitosan samples includes the agar well diffusion method, as well as the determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). In addition, the antifungal activity of chitosan was also examined against *Aspergillus niger* by determining the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). These microbial strains were collected from the Microbiology Laboratory in the Faculty of Science and Technology at the Universiti Sains Islam Malaysia. Before the experiments, the bacteria were sub-cultured in Mueller Hinton broth (MHB) and incubated at 37°C for 24 h [17], while *A. niger* was cultured on potato dextrose agar (PDA, Merck) medium and incubated at 25°C for five days.

Agar Well Diffusion Method

Antimicrobial assays of chitosan extracts were conducted using the agar well diffusion method on Mueller Hinton Agar (MHA) plates. The test organisms were initially inoculated in Mueller Hinton Broth (MHB) and incubated overnight at 37°C until the turbidity reached a final inoculum of 10^6 cells/mL. Using an aseptic technique, 100 µL of the culture suspension was pipetted onto sterile Mueller Hinton Agar plates and spread evenly, allowing it to disperse into the agar for 5 min. Four wells, each with a diameter of 6 mm, were created in the inoculated media. These wells were separately filled with 100 µL of a chitosan sample (10 mg/mL prepared in 1% acetic acid), streptomycin (10 mg/mL, used as a positive control), or 100 µL of 1% acetic acid (used as a negative control). After filling, the plates were left to allow diffusion for approximately 30 min at room temperature and then incubated for 18-24 h at 37°C. Following incubation, the plates were examined for the formation of clear zones around the wells. These zones of inhibition represented the antimicrobial activity of the tested compounds and were subsequently measured. Each test was carried out in triplicates.

Minimum Inhibitory Concentration (MIC) Analysis

The determination of the Minimum Inhibitory Concentration (MIC) was conducted using the micro-dilution method according to the technique described by [6] and [15]. Selected test microorganisms, including the Gram-positive bacterium *Staphylococcus aureus*, the Gram-negative bacterium *Bacillus subtilis*, and the fungus *Aspergillus niger*, were used for Minimum Inhibitory Concentration (MIC) evaluation of the chitosan samples. First, a chitosan solution was prepared by dissolving 100 µg/mL of the chitosan sample in 1% (v/v) acetic acid. Next, 100 µL of Mueller Hinton (MH) broth was added to each well of a 96-well microtiter plate. Then, serial dilutions were performed to achieve concentrations of 100, 50, 25, 12.5, and 6.35 µg/mL, except for the control well.

After that, 10 µL of test microorganisms (*Staphylococcus aureus, Bacillus subtilis,* and *Aspergillus niger*) with a concentration of 10^5 cells/mL for bacteria and 10^5 spore/mL for *A. niger* in Mueller Hinton broth were introduced into the wells. The negative control well consisted of 100 µL of broth. The microtiter plate containing the test organisms in chitosan solution and the control was then incubated at 37°C for 18 h. After the initial 18 h incubation, 20 µL of 10 mg/mL of Triphenyl Tetrazolium Chloride (TTC) (Bio Basic, Canada) was added to each well. The plate was further incubated for an additional 2 h to allow for colour development. The wells were then examined for visible signs of bacterial growth (pink colour) or turbidity. The MIC was determined to have the lowest concentration of chitosan, which effectively inhibited bacterial growth. All experiments were conducted in triplicate.

Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) Analysis

Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) of chitosan against selected bacteria and fungus, respectively, were determined following MIC determination. For MBC evaluation, after 24 h of incubation at 37°C, aliquots from wells with visible bacterial growth were streaked using an inoculation loop onto Mueller Hinton agar plates and incubated for 24 h at 37°C. The plates were observed for the presence or absence of bacterial growth. MBC is defined as the lowest concentration of chitosan that kills 99.9% of the bacterial population and is considered the concentration of the sample that produced fewer than ten colonies. The MFC assay followed a similar method as MBC, but *A. niger* was streaked onto a PDA medium and incubated at 35°C for 48 h. All experiments were conducted in triplicates.

Statistical Analysis

The data from the antimicrobial assays were subjected to statistical analysis using Minitab version 19, which involved one-way ANOVA and included mean and standard deviation. A significance level of $p < 0.05$ was used to evaluate the statistical significance of differences among data groups.

RESULTS AND DISCUSSION

Physical Analysis of Squid Pen Cartilage and Chitosan Samples

Squid pen cartilage samples from both the restaurant waste (S) and market waste (SS) sources were measured for their length, width, and thickness following washing and drying. The squid pen cartilage from restaurant waste (S) ranged from 8.0 to 17.5 cm in length (average length of 12.63 ± 4.1 cm), 1.1 to 2.1 cm in width (average of 1.68 ± 0.5 cm) and 0.40 to 0.43 mm in thickness (average of 0.41 ± 0.02 mm), while those collected from the market (SS) exhibited lengths ranging from 20.5 to 21.0 cm (average length of 20.65 ± 0.2 cm), width between 2.0 to 3.5 cm (2.7 ± 0.6 cm) and thickness ranging from 0.58 to 0.63 mm (average of 0.6 ± 0.03 mm). The wider range of cartilage sizes observed in the restaurant wastes may result from the mixture of squid species used by the four restaurants where the waste was collected. In contrast, the samples obtained from the market were deliberately selected from the same type of squid, which could reflect less variation in the size of squid pen cartilage.

Chitosan was extracted from the squid pen cartilages using a chemical extraction method via two different approaches: i) involving a demineralization step following pre-treatment, and ii) excluding the demineralization step and proceeding to deproteinization. After the deproteinization step, the resulting chitin was subjected to DDA to produce chitosan. Visual examination of the chitosan powder observed colour variations (Figure 2). Chitosan from the market waste was white when extracted without the demineralization step (SS2), while it appeared yellowish with demineralization (S2). Chitosan from mixed types of squid from the restaurant waste exhibited brownish colours. The range of colour of chitosan in our study was similar to findings by [20], where it ranged from yellowish to tanned. The authors also reported that the colours depended on the DDA reaction time and temperature [20].

Yield of Chitosan Extracted from Squid Pen

The yield of chitosan extracted from squid pen cartilage ranged from 55-82.4% (Figure 3). The lowest yield of 55% was observed for squid pen chitosan derived from restaurant waste sources extracted with the demineralization step (S1). In comparison, the highest yield of 82.4% was obtained without demineralization from the same waste (S2). This difference in yield could be attributed to potential losses during the demineralization step (S1 = 55.00% and SS1 = 66.67%), which includes additional washing steps and may result in a lower chitosan yield compared to extraction without demineralization.

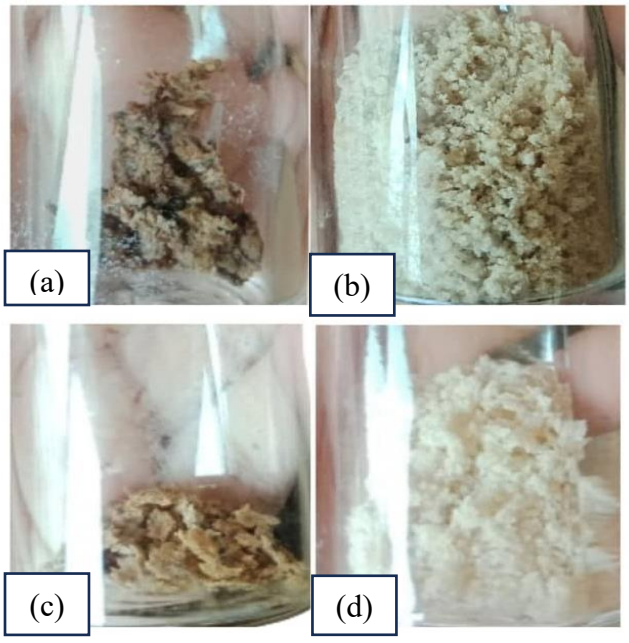

Figure 2. Chitosan samples extracted from the squid pen cartilage of different sources: (a) S1 (b) S2 (c) SS1 (d) SS2.

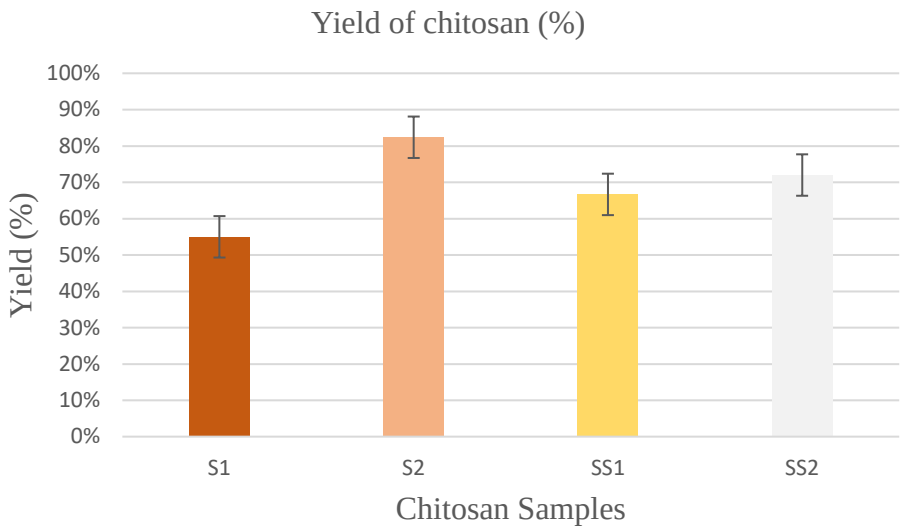

Figure 3. Yield of chitosan (%) extracted from restaurant wastes (S1: with demineralization; S2: without demineralization) and market wastes (SS1: with demineralization; SS2: without demineralization).

This may be caused by depolymerization of chitosan molecules, potentially resulting in the loss of fragmented molecules during the thorough washing process [10]. The yield obtained in this study is higher than that reported by [20], with 28.45% using a different extraction method. Furthermore, [10] reported maximum yields of 65% and 54% for chitosan extracted from squid pen, depending on DDA time and temperature. The variations in yields could be due to factors such as the origin of the squid pen and the specific extraction methods employed [21].

UV-Visible Spectrophotometer Analysis

UV-Visible spectra of chitosan extracted from squid pens from restaurant and market waste sources with different extraction procedures were investigated from 180 to 500 nm, as shown in Figure 4. The optical characteristics of all four chitosan samples revealed the highest absorbance peak at a wavelength of 225 nm for S1, SS1, and S2, and 221 nm for SS2, all consistent with the range of wavelength of the chitosan compound. Compared to the observation by [22],

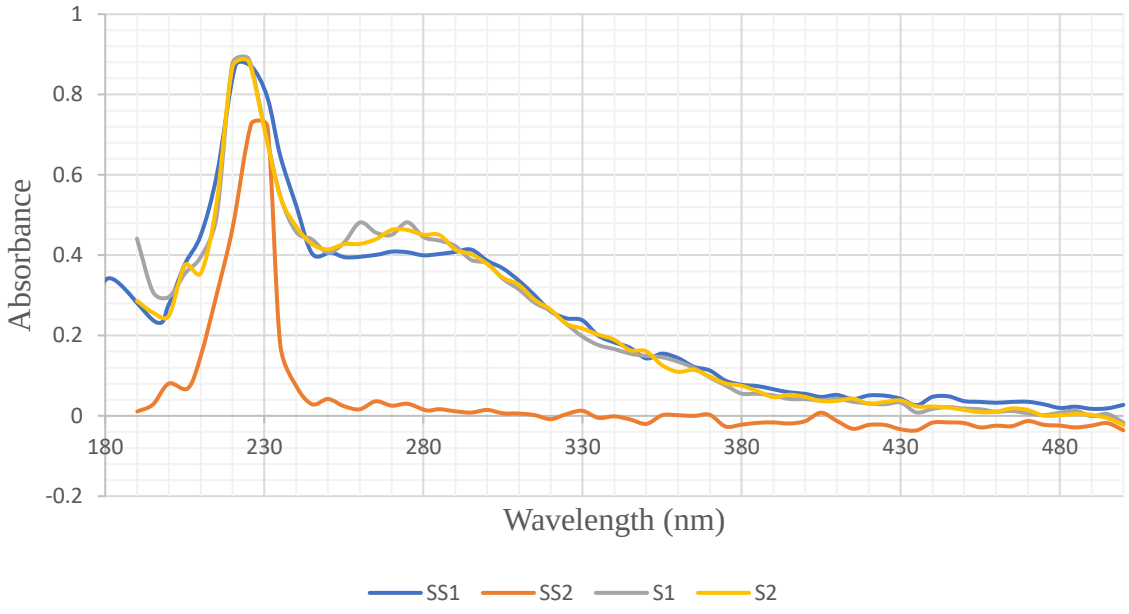

Figure 4. UV-Visible Absorption Spectra of chitosan samples from restaurant wastes (S1: with demineralization; S2: without demineralization) and market wastes (SS1: with demineralization; SS2: without demineralization) when scanning from 180 to 500 nm.

the spectrum of pure chitosan displayed two absorption bands at 214 nm, resulting from the amide linkages of partially deacetylated chitin and a shoulder at 311 nm, which is in line with this study, indicating the samples to be chitosan.

The UV-visible spectra pattern for the chitosan SS2 sample extracted from market squid waste without demineralization exhibits slight deviations compared to the other three samples. There is a minor shift in the highest absorbance peak, which occurs at 221 nm instead of 225 nm, and the absence of a shoulder peak at 255-280 nm, which is present in the other samples. These discrepancies may be attributed to the presence of impurities [23] or alterations in the molecular weight of the extracted chitosan [24]. Nonetheless, all samples showed the characteristic absorption spectrum of chitosan, with the highest absorbance at around 220 nm [25].

Functional Group Analysis using Fourier Transform Infrared (FT-IR)

FT-IR spectroscopy is sensitive to polar groups, making it a valuable tool for characterizing the biochemical characteristics of polymers like chitosan [3]. The specific composition of molecular groups in chitosan can be analysed based on the distinctive absorption peaks observed in its FT-IR spectrum. The FT-IR spectra of the chitosan samples are presented in Figure 5 and exhibit characteristic features consistent with chitosan in previous studies [3,11, 21].

The FT-IR spectra revealed several key absorption bands that characterize the chitosan samples. A broad peak spectrum ranging from 3,070 to 3,450 cm^{-1} was observed, corresponding to the symmetric stretching vibration of hydrogen-bonded -OH groups that overlap with amines (NH_2). Hydroxyl and amino groups are the main active functional groups in chitosan [3]. The O-H absorption strength of all chitosan samples is similar, within the range of 3,356 to 3,359 cm^{-1}. The fundamental frequency of the alcohol hydroxyl group is found to be between 3,220 and 3,550 cm^{-1}, and the absorption peaks for stretching vibrations of hydrogen bonds (O-H and N-H) have shifted to lower frequencies. This could be attributed to the influence of hydrogen bonds within the chitosan molecular chain. In addition, a band between 2,872 and 2,879 cm^{-1} was observed in all chitosan samples, indicating aliphatic C-H stretching.

Another significant region of the absorption spectrum, ranging from 1,576 to 1,590 cm^{-1}, corresponded to the vibration of NH in primary amine groups (R-NH_2), indicating the DDA of chitin [26]. The absence of bands within the range of 1,660 to 1,900 cm^{-1} suggests the lack of -C-O containing carbonyl and carboxyl group in the chitosan [27]. Another peak appeared at 1,371 to 1,374 cm^{-1}, corresponding to the amide III bands and C–N stretching, which are characteristics of chitosan. A strong absorption region between 800 and 1,200 cm^{-1} was also observed, reflecting the chitosan saccharide structure. Furthermore, the asymmetrical vibration stretch of the C-O-C bridge was observed at 1,149 to 1,150 cm^{-1}, which is likely due to chitosan DDA. The consistency in the FT-IR observations indicates that chitosan was successfully extracted, with the respective functional groups present and consistent in all samples.

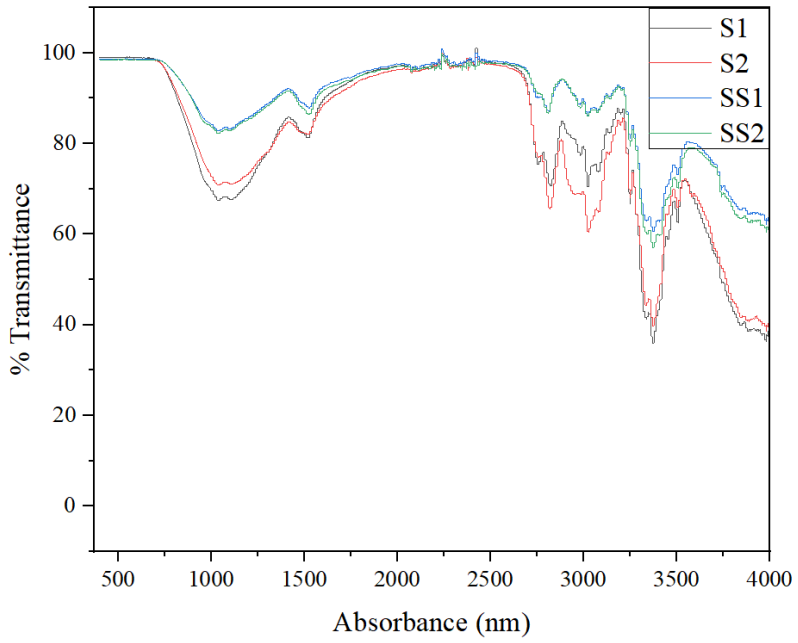

Figure 5. FT-IR spectra of chitosan samples from restaurant wastes (S1: with demineralization; S2: without demineralization) and market wastes (SS1: with demineralization; SS2: without demineralization).

Degree of Deacetylation (DDA) of Chitosan Samples

The degree of DDA for the chitosan samples was determined using FT-IR spectra by calculating the ratio of absorbance bands at 1,320 and 1,420 cm^{-1}, according to the baseline equation proposed by [19]. The DDA values for all the chitosan samples extracted from squid pen waste in this study ranged from 73.67-76.64%. The highest DDA was observed in chitosan extracted from restaurant waste without the demineralization step (S2). Meanwhile, the lowest DDA was found in chitosan from market waste without the demineralization step (SS2). The results suggested that the demineralization step in the chitosan extraction method from the squid pen did not affect its DDA. The DDA value is consistent with the study by [28], which reported a DDA of 73% when chitosan was extracted from squid pen waste using 60% NaOH with a demineralization step.

A similar range of DDA values for chitosan extracted from squid pen waste has been reported by [20], with the highest DDA of 83.94% achieved when a longer DDA time (8 h) was applied at 90°C. Similarly, [10] demonstrated that chitosan derived from squid pen could possess a DDA of up to 90% when DDA was carried out at 130°C for 8 h, indicating that increasing DDA temperature and time can lead to higher DDA values. Furthermore, [29] reported a DDA of 97.63% for chitosan extracted from shrimp shell waste when the DDA process was repeated twice at a temperature of 80°C. Therefore, optimization of DDA time and temperature, including more extended DDA periods, could potentially enhance DDA values in future studies, as higher DDA is associated with improved chitosan quality [20]. However, the DDA values obtained in this study exceed 70%, which falls within the medium range for chitosan DDA [30], and most commercial chitosan typically exhibit an average DDA in the range of 50–100% (commonly 80–90%) [31, 32].

Antimicrobial Activity of Chitosan Extracted from Squid Pen

The antimicrobial activity of the four chitosan samples against different bacteria, including Gram-positive (*Bacillus subtilis*, *Salmonella epidermidis*, and *Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Pseudomonas vulgaris*) bacteria was evaluated using the agar well diffusion assay. All chitosan samples exhibited antimicrobial potential against the tested bacteria (Table 2), as indicated by the presence of inhibition zones. The diameters of the inhibition zones ranged from 9.5 ± 0.7 mm to 13.5 ± 2.1 mm, suggesting that chitosan extracted from squid pen waste possesses antimicrobial activity against the bacteria.

The antibacterial activity was considered moderate (inhibition zone 9–12 mm) to high (inhibition zone > 12 mm) following the scale proposed by [33] and [34]. The largest inhibition zone was observed for chitosan extracted from squid pen of restaurant waste (S1) against *Salmonella typhimurium*, with a diameter of 13.5 ± 2.1 mm. At the same time, *Pseudomonas aeruginosa* showed the least sensitivity to chitosan sample SS2, with a diameter of 9.5 ± 0.7 mm. However, no significant differences in diameter values were

Table 2. Diameter of inhibition zones in the antimicrobial testing of chitosan samples against test bacteria using the agar well diffusion method.

Test Bacteria	Diameter of inhibition zone (mm) for different chitosan samples				Positive control (Streptomycin)	Negative control (Acetic acid)
	S1	S2	SS1	SS2		
Staphylococcus aureus	10.0 ± 0.0	9.5 ± 0.7	11.5 ± 2.1	10.5 ± 0.7	9.5 ± 0.7	6.3 ± 0.7
Pseudomonas aeruginosa	11.0 ± 2.8	10.5 ± 0.5	9.5 ± 1.0	9.0 ± 1.0	12.0 ± 0.0	-
Pseudomonas vulgaris	11.0 ± 1.4	12.5± 0.7	11.5 ± 0.7	9.5 ± 0.7	11.5 ± 0.7	-
Salmonella typhimurium	13.5 ± 2.1	12.5 ± 1.5	11.0 ± 2.0	11.5 ± 2.5	12.0 ± 1.41	6.1 ± 0.0
Bacillus subtilis	11.0 ± 0.0	11.0 ± 0.0	11.5 ± 2.8	11.0 ± 2.5	12.5 ± 0.7	-
Staphylococcus epidermidis	12.5 ± 2.1	10.5 ± 0.7	12.5 ± 0.7	10.5 ± 0.7	11.5 ± 0.7	-

*Results are expressed as mean ± standard deviation of diameters measured with agar diffusion test of three independent biological replicates. S1: chitosan from restaurant wastes with demineralization; S2: chitosan from restaurant wastes without demineralization; SS1: chitosan from market wastes with demineralization; SS2: chitosan from market wastes without demineralization.

observed for all chitosan samples against the tested bacteria ($p > 0.05$). This suggests similar inhibition activities were observed for chitosan extracted from squid pen waste from different sources, with or without demineralization.

The effectiveness of chitosan extracts against *S. aureus*, *B. subtilis*, and *A. niger* was further evaluated through MIC, MBC, and MFC analyses. MIC values of 50 µg/mL were observed for all chitosan samples against *B. subtilis* and *S. aureus* (Table 3). For squid pen chitosan from a market source extracted with or without demineralization step (SS1 and SS2, respectively), this concentration was insufficient to kill *B. subtilis*, as indicated by an MBC value of 100 µg/mL (Table 4). *B. subtilis* is a Gram-positive bacterium with a thick cell wall comprising long glycan strands [35], which may contribute to the reduced sensitivity to chitosan's bactericidal effect. The lowest MIC value against *A. niger* was observed at 25 µg/mL for squid pen chitosan from restaurant waste extracted with demineralization (S1), which corresponded to the lowest fungicidal concentration (MFC) against the fungus. Other chitosan samples displayed an inhibition on *A. niger* with a MIC value of 50 µg/mL, consistent with their MFC value, except for the squid pen chitosan from restaurant waste without demineralization (S2) with MFC of 100 µg/mL.

Table 3. Minimum Inhibitory Concentration (MIC) of chitosan samples against *S. aureus*, *B. subtilis* and *A. niger*

Chitosan Sample	Bacteria	Concentration of chitosan (µg/mL)					Positive control	MIC (µg/mL)
		100	50	25	12.5	6.25		
S1	*S. aureus*	-	-	+	+	+	-	50
	B. subtilis	-	-	+	+	+	-	50
	A. niger	-	-	-	+	+	-	25
S2	*S. aureus*	-	-	+	+	+	-	50
	B. subtilis	-	-	+	+	+	-	50
	A. niger	-	-	+	+	+	-	50
SS1	*S. aureus*	-	-	+	+	+	-	50
	B. subtilis	-	-	+	+	+	-	50
	A. niger	-	-	+	+	+	-	50
SS2	*S. aureus*	-	-	+	+	+	-	50
	B. subtilis	-	-	+	+	+	-	50
	A. niger	-	-	+	+	+	-	50

+: Presence of growth; - No growth; S1: chitosan from restaurant wastes with demineralization; S2: chitosan from restaurant wastes without demineralization; SS1: chitosan from market wastes with demineralization; SS2: chitosan from market wastes without demineralization.

Table 4. Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) of chitosan samples on selected bacteria and fungus, respectively

Chitosan Sample	Bacteria	Concentration of chitosan (µg/mL)					MBC / MFC (µg/mL)
		100	50	25	12.5	6.25	
S1	*S. aureus*	-	-	+	+	+	50
	B. subtilis	-	-	+	+	+	50
	A. niger	-	-	-	+	+	25
S2	*S. aureus*	-	-	+	+	+	50
	B. subtilis	-	-	+	+	+	50
	A. niger	-	-	+	+	+	100
SS1	*S. aureus*	-	-	+	+	+	50
	B. subtilis	-	+	+	+	+	100
	A. niger	-	-	+	+	+	50
SS2	*S. aureus*	-	-	+	+	+	50
	B. subtilis	-	+	+	+	+	100
	A. niger	-	-	+	+	+	50

+: Presence of growth; - No growth. S1: chitosan from restaurant wastes with demineralization; S2: chitosan from restaurant wastes without demineralization; SS1: chitosan from market wastes with demineralization; SS2: chitosan from market wastes without demineralization.

The observed difference in the bactericidal and fungicidal effects of squid pen chitosan derived from restaurant and market waste sources against *B. subtilis* and *A. niger*, respectively, could be attributed to differences in their antimicrobial properties. This could arise from variations in the composition of the squid pen material [36]. Squid pen waste from the market source predominantly consists of a specific type of squid, which could exhibit different antimicrobial properties compared to the mixed types obtained from restaurant sources. Furthermore, our results suggested that demineralization of squid pen chitosan from restaurant waste may enhance its fungicidal activity against *A. niger* compared to undemineralized chitosan. The antibacterial and antifungal activity of chitosan can be influenced by various factors such as molecular weight, degree of DDA, chitosan concentration, and pH [37, 38]. Spectrophotometric assays may provide a more precise verification of the antimicrobial results.

Consistent with the findings of this study, previous research has reported the antimicrobial activity of chitosan against various bacteria, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus, Pseudomonas aeruginosa* and *Salmonella typhimurium* [39]. Chitosan extracted from squid pen was also shown to be antibacterial against the pathogenic bacterium *Porphyromonas gingivalis* [40]. The mechanisms underlying chitosan's antibacterial activities could include disruption of bacterial cell membranes or cell walls due to the positively charged nature of chitosan, the formation of a protective film on the porins of the cell surface, blocking nutrient exchange and leading to microbial cell death, chitosan penetration into bacterial cell walls, affecting DNA/RNA and protein synthesis, and chelation of metal ions by unprotonated amino groups on the chitosan surface, disrupting cell walls or membranes [37]. Chitosan's antifungal activity against *A. niger* has also been previously documented. In particular, [5] reported strong dose-dependent antifungal activity of chitosan extracted from shrimp shells against *A. niger* mycelial growth. Further studies are needed to determine the mechanism of antimicrobial activity of squid pen chitosan against the test bacteria and *A. niger*.

CONCLUSION

In this study, chitosan was extracted from squid pen waste obtained from different sources, including from restaurants with mixed squids and from the market comprising the same squid types, with and without the demineralization step. Spectroscopy analyses revealed that chitosan was effectively extracted and exhibited the characteristic functional groups of chitosan molecules. The extracted chitosan exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria, as well as antifungal activity against *A. niger*. These findings suggest that the demineralization step for chitosan extracted from squid pen cartilage may be omitted under the chemical extraction method while maintaining the DDA and antimicrobial properties against known pathogenic microorganisms. Further studies are needed to optimize the extraction procedure for achieving higher DDA values and to explore other commercial characteristics and properties of the extracted chitosan.

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