Comparison of shrimp waste-derived chitosan produced through conventional and microwave-assisted extraction processes: physicochemical properties and antibacterial activity assessment

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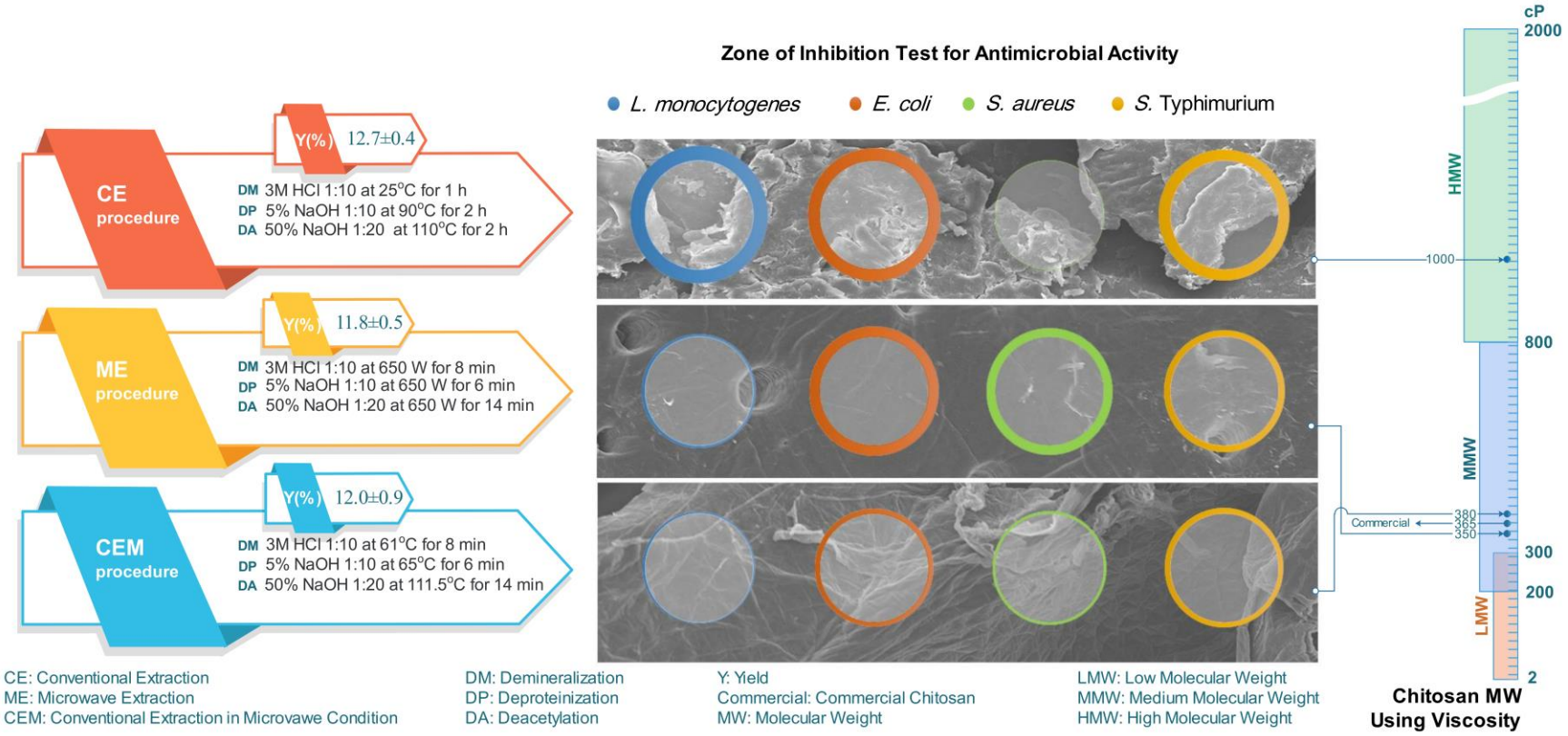
Graphical abstract



Highlights

- Shrimp waste-derived chitosan was extracted by three different heating procedures.
- Conventional & microwave-assisted heating yielded 12.7 & 11.8% chitosan, respectively.
- Conventional method extracted high molecular weight ashless, protein-less chitosan.
- Microwave method extracted medium molecular weight semi-porous structured chitosan.
- Conventionally-extracted chitosan showed the best antimicrobial activity.

Abstract

Depending on its physicochemical properties and antibacterial activities, chitosan can have a wide range of applications in food, pharmaceutical, medicine, cosmetics, agriculture, and aquaculture. In this experimental study, chitosan was extracted from shrimp waste through conventional extraction, microwave-assisted extraction, and conventional extraction under microwave process conditions. The effects of the heating source on the physicochemical properties and antibacterial activity were investigated. The results showed that the heating process parameters affected the physicochemical properties considerably. The conventional procedure yielded high molecular weight chitosan with a 12.7% yield, while the microwave extraction procedure yielded a porous medium molecular weight chitosan at 11.8%. The conventional extraction under microwave process conditions led to medium molecular weight chitosan with the lowest yield (10.8%) and crystallinity index (79%). Antibacterial assessment findings revealed that the chitosan extracted using the conventional method had the best antibacterial activity in the agar disk diffusion assay against *Listeria monocytogenes* (9.48 mm), *Escherichia coli*. (8.79 mm), and *Salmonella* Typhimurium (8.57 mm). While the chitosan obtained by microwave-assisted extraction possessed the highest activity against *E. coli*. (8.37 mm), and *Staphylococcus aureus* (8.05 mm), with comparable antibacterial activity against *S.* Typhimurium (7.34 mm) and *L. monocytogenes* (6.52 mm). Moreover, the minimal inhibitory concentration and minimal bactericidal concentration assays demonstrated that among the chitosan samples investigated, the conventionally-extracted chitosan, followed by the chitosan extracted by microwave, had the best antibacterial activity against the target bacteria.

Keywords: Shrimp waste valorization, Chitosan conventional extraction, Microwave-assisted technique, Physicochemical properties, Antibacterial activity, Circular economy

1. Introduction

The 2030 agenda for sustainable development calls for support for the seafood industries to ensure food security due to the high nutritional value and associated health benefits of seafood products [1]. Among different kinds of seafood, shrimp and prawn are healthy preferences and desirable food worldwide, with a global production of 8.25 million metric tons in 2015 and 9.66 million metric tons in 2019, with an annual growth rate of 2-3% [2]. Since about 45 wt% of the total body weight of shrimp is meat, around 55 wt% end up as waste, which is highly perishable and is decomposed rapidly under microbial spoilage, resulting in public health hazards [2]. However, this waste stream also contains valuable natural compounds, most importantly chitin, a valuable source for producing chitosan [3].

Chitosan is the most well-known derivative of chitin, with free amino groups that could be obtained through chitin deacetylation [4]. Chitosan exhibits antimicrobial activity against most bacteria, molds, and yeasts [5]. Moreover, chitosan is a biocompatible, biodegradable, and safe biopolymer. These properties allow chitosan and its derivatives to be used in various industries such as food, agriculture, pharmaceuticals, and many other aspects [6]. **Table 1** presents the factors (i.e., concentration, molecular weight, positive charge density, hydrophilic characteristics, pH, and storage condition of chitosan, as well as microbial species) affecting the antimicrobial properties of chitosan [7].

Table 1. Factors affecting the antibacterial activity of chitosan*.

Factor	Effects/Reasons
Concentration of chitosan	At lower values- when chitosan binds to the negatively charged target surface, it disturbs the cell membranes, resulting in intracellular components leakage and cell death. At higher values- reversely, the protonated chitosan coats the target surface and prevents intracellular component leakage.
Molecular weight (MW)	Although the bactericidal activity of low MW chitosan varies with bacterial strains, conditions of biological examination, and chitosan MW, there is no consensus among the results reported in the literature.
Positive charge density	At higher densities, the positive charge leads to a strong electrostatic interaction. The positive charge density is associated with the DD of chitosan or its derivatives. The positively charged bacterial cells repel each other preventing agglutination.
Hydrophilic characteristics	Dry samples cannot initiate interactions with antibacterial agents because they require water for activity. Poor chitosan solubility in water limits its application. Hence, efficient chemical modifications extend chitosan applications by improving water solubility and developing derivatives.
pH	Chitosan exhibits a stronger inhibitory action at lower pHs and becomes less effective as the pH rises. The failure of chitosan to remain bactericidal at neutral pH could be attributed to either the presence of positively uncharged amino groups or the poor solubility of chitosan.
Storage condition	Specific characteristics of chitosan, such as viscosity and MW, could be changed during storage. The stability of chitosan solutions and their antibacterial activity against bacteria are reduced by increasing storage time and temperature.
Microbial species	Because of different cell surface characteristics, chitosan shows different inhibitory activity against target Gram-positive and Gram-negative bacteria.

* Source: [7]

Chitosan's properties are largely affected by the extraction procedure used for its production [8]. The most common process to extract chitin from crustacean shells and production of chitosan involves three successive chemical reactions: demineralization (DM), deproteinization (DP), and deacetylation (DA) to remove minerals, proteins and pigments, and acetyl groups, respectively [9]. DM removes minerals from the crustacean shells by reacting with various acids, most preferentially hydrochloric acid (HCl) [10]. DP is performed to eliminate proteins and most pigments from crustacean shells by reacting with DP reagents such as sodium hydroxide (NaOH). This step is performed under various temperatures, up to 160°C, with different reaction times from a few min to a few days [11]. Excess solvent extraction is employed to bleach the remaining

pigments when a colorless product is desired [12]. The product of these two consecutive steps is called chitin, which is converted to chitosan through DA in saturated NaOH solutions at high temperatures for long durations [13]. This common process (conventional extraction method) has unacceptable environmental footprints [14] alongside high energy and time consumption [15]. To overcome this, microwave-assisted heating instead of conventional heating can reduce the chitosan extraction time leading to lower energy consumption [16]. Hence, the microwave-assisted extraction technique is a more efficient and environment-friendly procedure for extracting chitosan [15, 17].

The microwave-assisted process leads to chitosan extraction with molecular weights of 300–360 kDa and a deacetylation degree (DD) of 80-95% after 10 min of reaction [18]. This technology could save massive energy when implemented on an industrial scale and has been proven to be an economical extraction method [19]. **Table 2** compares the experimental studies reported on different chitosan extraction procedures, i.e., conventional extraction method (CE) and microwave-assisted extraction method (ME), in terms of physicochemical and antibacterial properties of the end product, chitosan (i.e., yield (Y), molecular weight (MW), ash, protein content (PC), crystallinity index (CI), DD, and minimum inhibitory concentration (MIC) corresponding to bacteria tested (B)). Among these studies, only a few have compared the two procedures simultaneously [15, 16, 20]; however, these investigations have failed to look into the various attributes of the end product chitosan and have presented limited analyses. For instance, Kinderi *et al.* (2016) only compared CE and ME extraction methods from the physicochemical point of view. Another study investigated the effect of autoclave and ME extraction methods on limited physicochemical properties and antibacterial activity against two agents (i.e., *Salmonella* Typhimurium and *Staphylococcus aureus*). The study by Cheng *et al.* (2020) compared the

physicochemical properties of chitosan extracted through CE and ME methods for the deacetylation of commercial chitin only.

138 Table 2. The physicochemical properties and antibacterial activity of chitosan extracted from different procedures reported in the
139 literature.

Extraction & *microbial* condition	**Method**	**Y (%)**	**MW (Da)**	**Ash (%)**	**PC (%)**	**CI (%)**	**DD (%)**	**B**	**MIC**	**Ref.**
DP: 3% NaOH for 30 min at 80°C. DM: 3% HCl for 30 min at 25°C. DA: 40% KOH for 6 h at 90°C	CE	-	6273	0.03	8	82	78	-	-	[21]
DM: 1.5 N HCl, for 30 min at 25°C DP: 2 N NaOH at >55°C for 2h DA: 50% NaOH for 3-5 h at 90-100°C *0.1 g of sterile chitosan was added in 100 mL of cultured bacteria suspension in a flask and incubated with shaking at 37°C. MIC was defined as the lowest concentration of chitosan required to completely inhibit bacterial growth after incubation at 37°C for 72 h*	CE	-	12.00	0.18	1.1	-	80	EC PA[5] SA ST BS[6] BC VC[7] SD[8] EA[9] PM[10] BF[11]	0.01 0.05 0.03 >0.1 0.01 0.01 0.01 0.01 0.01 0.01 0.006%	[22]
DM: 2% HCl (10:1, 30°C, 12 h). DP: 4% NaOH (10:1, 90°C, 12 h). DA: NaOH 50% microwave oven for 10 min at 1400 W. *Inhibition zones of visible growth in broth media*	ME	-	1267	0.31	2.8	-	78.8	EC[1] ST[2] SA[3] BC[4]	0.4 0.8 0.05 0.05%	[17]
DM: 2% HCl (10:1), at 30°C for 12 h. DP: 4% NaOH (10:1) at 90°C for 12 h. DA: 45% NaOH (15:1), microwave oven at 600w for 15 min. *MIC was evaluated by microplate serial dilution technique, according to the supplementary standard M100-S16 of the Manual Clinical and Laboratory Standards Institute (CLSI).*	ME	44.8	-	-	-	-	81	SA EC SE[12] KP[13] CA[14] CP[15] CT[16] CG[17]	0.46 30 3.75 60 0.23 0.23 0.23 30 ppm	[23]
DM: 3M HCl (10:1), at 75°C for 2 h. DP: 10% NaOH (10:1) at 80°C for 2 h. DA: 50% NaOH (20:1) for 2 h at 100°C	CE	-	1410	-	-	65	81.5	-	-	[15]
DM: 3M HCl (10:1), microwave oven at 500w for 8 min. DP: 10% NaOH (10:1), microwave oven at 160-350w for 8 min DA: 50% NaOH (20:1), microwave oven at 500w for 8 min.	ME	-	123	-	-	56	82.7	-	-	
DM: 1% HCl (20:1, 25°C) DP: 1% NaOH (20:1, 70°C) using an autoclave DA: 1 N NaOH (1:50, 121°C, 15 min) using an autoclave *25 µL bacterium inoculated into 5 mL of 1% CE chitosan in AA* 1% dispersed in broth*	-	6.7	109	-	-	-	90.6	ST SA	1.6×10^{7} 1.7×10^{4} CFU/mL	[16]

DM: 1% HCl (20:1, 25°C) DP: 1% NaOH (20:1, 70°C) DA: 300 W, 170°C, 22 min *25 µL bacterium inoculated into 5 mL of 1% ME chitosan in AA 1% dispersed in broth*	ME	13.4	127	-	-	-	94.6	ST SA	1.7×10^6 3162 CFU/mL	
DA: 1 g chitin in 30 mL 50% NaOH at 90°C for 3 h using a water bath.	CE	-	3.9×105	-	-	27.4	74.5	-	-	[20]
DA: 1 g chitin in 30 mL 50% NaOH at 90°C for 1 h.	ME	-	3.6×105	-	-	27.71	73.9	-	-	

*Acetic acid, 1. *Escherichia coli*, 2. *Salmonella* Typhimurium, 3. *Staphylococcus aureus*, 4. *Bacillus cereus*, 5. *Seudomonas aeruginosa*, *6. Bacillus subtilis*, *7. Vibrio choler*, 8. *Shigella dysenteria*, *9. Enterobacter agglomeran*, *10. Prevotella melaninogenica*, *11. Bacteroides fragile*, *12. Salmonella* Enterica, *13. Klebsiella pneumoniae*, *14. Candida albicans*, *15. Candida pelliculosa*, *16. Candida tropicalis*, *17. Candida guilliermondii.*

It should be highlighted that the physicochemical parameters of chitosan do not necessarily indicate better quality or properties. Principally, the properties of chitosan are affected by a range of factors, including parameters revealing its mechanical attributes, e.g., stiffness, tensile strength, and thermal stability, as well as those determining its chemical and biological attributes, e.g., solubility, water uptake, and biodegradability. It is also crucial to note that these attributes can limit chitosan applicability in certain applications. Therefore, depending on the intended application and the desired properties of the final product, chitosan with particular physicochemical and biological properties should be selected.

In light of the above, the present study was set to extract chitosan using CE and ME procedures. Then, the physicochemical (DD, CI, surface morphology, viscosity, ash content, and protein content) and antimicrobial (agar disk diffusion, minimal inhibitory concentration (MIC), and minimal bactericidal concentration (MBC) assays) properties of produced chitosans were thoroughly compared. The outcomes of the present study are expected to contribute to the industrial processes aimed at producing chitosan from shrimp wastes for use in various industries, ranging from feed and food to pharmaceutical and medical applications.

2. Materials and methods

2.1. Chemicals

The wastes of the shrimp *Penaeus monodon*, including shell and head, were obtained from a shrimp processing company (Emperor Marine Marketing Sdn. Bhd.) located in Setiawan, Perak, Malaysia. The HCl (37%) and NaOH pellet (99%) were purchased from R&M Chemicals (Malaysia). The commercial chitosan was purchased from Sigma-Aldrich (USA) as a reference to compare the experimental results.

2.2. Chitin and chitosan extraction

Three chitin and chitosan extraction methods, i.e., the CE, ME, and conventional extraction procedure in the ME reaction conditions (CEM), were investigated to compare the final products' physicochemical and antibacterial properties. Principally, these methods were performed under the same production process steps, i.e., DM, DP, and DA.

2.2.1. Pretreatment of the feedstock

Shrimp wastes, including shell and head, were grinned to a smaller size (~15 mm) by a commercial blender and then were dried overnight in an oven at 90°C. A grinding mill further reduced dried shrimp wastes' particle size to mesh 40. The shrimp waste powder was stored in a sealed bag before being subjected to chitin and chitosan extraction.

2.2.2. Chitosan extraction procedures

In this study, chitosan samples were extracted through the chemical reactions, i.e., DM, DP, and DA, and using three different heating procedures, i.e., CE, ME, and CEM. After each reaction (i.e., DM, DP, and DA), through a post-process, the resulting slurry was filtered and washed using distilled water to reach neutral pH. The resulting solid was dried in an oven at 80°C for 12 h to obtain the process product and calculate each step yield. The specific process parameters controlled for all extraction methods were the reaction temperature and time using different heating sources at constant reaction conditions (i.e., solution concentration and solid-to-liquid ratio). The details of the extraction procedures are explained as follows;

- ***Conventional extraction procedure***

The CE procedure was carried out based on the optimum reaction condition in the literature [24]. DM was conducted using 3 M HCl solution in a solid-to-liquid ratio of 1:10 at room temperature for 1 h. Afterward, chitin was produced through DP of the post-processed demineralized material at 90°C for 2 h using 5 wt% NaOH solution in a solid-to-liquid ratio of 1:10. The extracted chitin after post-process (CE-Chitin) went under DA using 50 wt% NaOH solution and the solid-to-liquid ratio of 1:20 at 110°C for 2 h. This process's final product was labeled CE-Chitosan.

- ***Microwave-assisted extraction procedure***

The ME procedure was carried out according to the reaction condition of the best results reported by Knidri *et al.* [18]. The pre-processed sample was stirred in HCl solution at 350 rpm for 10 min to prepare a semi-homogenous emulsion while CO_2 gas was released as a fume. DM in a 3 M HCl solution with a solid-to-liquid ratio of 1:10 was performed using a 2.45 GHz microwave oven (ME711K, 800W, Samsung) at a microwave power of 650 W for 8 min. The resulting material underwent DP in 5 wt% NaOH solution with a solid-to-liquid ratio of 1:10, which was heated using a microwave oven at 650 W for 6 min. The product labeled ME-chitin was deacetylated through microwave heating under similar DP conditions reported in the previous section for a reaction duration of 14 min. This final product was labeled as ME-chitosan.

- ***Conventional extraction procedure under ME reaction conditions***

The chitin and chitosan extraction in CEM was performed based on the conventional procedure under microwave extraction reaction conditions. The maximum temperatures achieved for ME process steps were 61, 65, and 111.5°C for DM, DP, and DA, after 8-, 6-, and 14-min

reaction time in the microwave oven, respectively. At the beginning of each process, the samples were heated to achieve the specified temperature and kept almost constant during the reaction time. DM was conducted using 3 M HCl solution in a solid-to-liquid ratio of 1:10 at 61°C for 8 min. Afterward, chitin was produced through DP of the demineralized material at 65°C for 6 min using 5 wt% NaOH in a solid-to-liquid ratio of 1:10. The extracted chitin was labeled as CEM-Chitin and was further deacetylated using 50 wt% NaOH solution and the solid-to-liquid ratio of 1:20 at 111.5°C for 14 min. The product was labeled as CEM-Chitosan.

2.3. Physicochemical characterization

2.3.1. FT-IR analysis

Fourier-transform infrared (FT-IR) spectra of chitin and chitosan samples extracted under different conditions, as well as commercial chitosan, were recorded with an FT-IR spectrophotometer (IRTracer-100, Shimazu, Japan) using the ATR mode of operation. The spectra were recorded over 400-4000 cm^{-1}, and 64 scans accumulated at a resolution of 16 cm^{-1}. The intensity of the absorption of the reference band at 1420 cm^{-1} and the amide III band at 1320 cm^{-1} were used to calculate the chitosan DD% (**Eq. 1**) [18]:

$$(A_{1320}/A_{1420}) = 0.3822 + 0.03133\ DA\% \quad (1)$$

where DA% is the acetylation degree calculated as 100 - DD%.

2.3.2. XRD analysis

XRD analyses were carried out to calculate the CI (I_{CR}) of chitin and chitosans extracted under different conditions as well as commercial chitosan using an X-ray diffractometer (miniFlexII, Rigaku, Japan) in the 2θ angle range of 5 to 50 degrees at 30 kV and 15 mA with Cu

kα radiation at λ=1.5406 A°. **Equation 2**, documented in the literature [25], was used to determine the I_{CR}.

$$I_{CR}=[(I_{110}-I_{am})/I_{110}]\times 100 \quad (2)$$

where I_{110} and I_{am} stand for intensity of the maximum intensity detected at 20° and amorphous diffraction at 16°, respectively.

2.3.3. SEM analysis

The surface morphology of the extracted chitin and chitosan samples alongside commercial chitosan as reference was studied by a scanning electron microscope (SEM) (JSM-6360LA, JEOL Ltd., Japan). The samples were first coated with an ultrathin layer of gold using an auto-fine coater (JFC-1600, JEOL Ltd., Japan) to increase the electrical conductivity and ease the observation of the sample surface under the SEM microscope. The SEM's working distance and accelerated voltage were set at 17 mm and 10 kV, respectively.

2.3.4. Viscosity

The chitosan samples' viscosity was determined using a Rheometer (Thermo Scientific HAAKE RheoStress 1) at 20°C, using a Z34 DIN Ti sensor with a 34 mm diameter. A HAAKE RheoWin JobManager ver. 3.50.0012 was employed for creating and executing measurement and evaluation routines (Thermo Fisher Scientific, Karlsruhe, Germany).

2.3.5. Inductively coupled plasma optical emission spectrometry

The ash content was measured to analyze the mineral content of the raw material and products, as described by Knidri et al. (2019) [18]. The samples (1 g) were burned at 600°C for 6 h. After cooling them in desiccators, the remaining products were weighted to determine each

sample's ash content. The ash was dissolved in 4 mL of distilled water. The calcium contents were analyzed using an inductively coupled plasma optical emission spectrometry (ICP-OES) (Avio 200, PerkinElmer, USA). The spectrometry was carried out to measure the remaining minerals in the products. The most sensitive wavelength, 317.933 nm, for Ca was used, and each measurement was replicated three times.

2.3.6. UV-spectroscopy

The protein contents of the samples were determined by measuring the UV absorbance (SHIMADZU UV-Vis 1800) at 280 nm and 260 nm wavelengths [26]. Protein solutions were prepared by soaking samples in NaOH solution (1 wt%) for 24h; afterward, the mixtures were filtered, and separated solutions were used for analysis. **Equation** 3 was used for the determination of residual proteins in each sample [18]:

$$[\text{protein}]_{\text{mg/mL}} = (1.31 * A_{280}) - (0.57 * A_{260}) \quad (3)$$

2.4. Antibacterial properties assessment

2.4.1. Strains and chemicals

Selected pathogenes for this study were the common ones found in food, including *Salmonella* Typhimurium ATCC 14028 (*S.* Typhimurium), *Escherichia coli* ATCC25922 (*E. coli*.), *Staphylococcus aureus* ATCC 25923 (*S. aureus*), and *Listeria monocytogenes* ATCC 14028 (*L. monocytogenes*) purchased from American Type Culture Collection (ATCC). All bacterial strains were stored at -20°C in glycerol-containing media (12.5v/v%) to prevent bacteria from being damaged during freezing, storage, and thawing [27]. The culture medium was tryptone soy agar, kept at 37°C overnight. The preparation of the inocolumn culture followed the procedure

described by Taghavi et al. (2021) [28]. Chlorhexidine diacetate (Sigma Aldrich) and 99.5% pure acetic acid, Muller-Hinton broth (MHB), Muller-Hinton agar (MHA), and Tryptic soy agar were purchased from Merck (Germany).

2.4.2. Antibacterial activity assessment

Three antibacterial activity assays, i.e., agar disk diffusion, MIC, and MBC, were experimentally evaluated to describe the effect of chitosan production procedures on chitosan antibacterial activity.

- ***Agar disk diffusion***

The five chitosan samples, including the commercial medium MW chitosan (CH-MMW), commercial low MW chitosan (CH-LMW), CE-Chitosan, ME-Chitosan, and CEM-Chitosan, were tested *via* the agar disk diffusion assay. The protocols described by the Clinical and Laboratory Standards Institute (CLSI) [29] and the method reported by Choo et al. [30] were used in this study with minor modifications. In brief, the MHA test plate's surface was inoculated by spreading 100 µL of the bacterial suspension inoculum. Then, 6 mm diameter sterile paper disks were transferred to the plates, and 40 µL chitosan samples were loaded onto the disks. Broth with no inoculum (MHB) and chlorhexidine diacetate (CHX) were employed as negative and positive controls, respectively. Also, 1 wt% acetic acid solution was considered a blank control. The cell density was estimated to be approximately 10^8 cells.mL^{-1} (0.5 McFarland standard) through UV-Vis measurements at 600 nm. Then, the plates were incubated at 32°C for 24 h [28]. Each experiment was carried out in triplicates.

- ***MIC assay***

Testing for MIC of samples was performed according to the CLSI standard M7-A6 [29] and the method reported by Rukayadi et al. [31], with minor modifications. Assays were performed using standard broth microdilution with an inoculum of 10^6 CFU.ml^{-1} (0.5 McFarland standard diluted 1:100 with MHB) in U-shaped 96-well microtiter plates. A mixture of chitosan solution (1 wt% of chitosan dissolved in 1 wt% acetic acid) and the target bacteria (10^6 CFU.mL^{-1}) in the MHB medium was prepared. The final concentration of target bacteria was 5×10^5 CFU.mL^{-1}. Columns 1 (only medium) and 2 (medium and inoculum) were considered negative and positive controls, respectively. Columns 3 to 12 of the U-shape microtiter plate contained the lowest to the highest concentrations of the antibacterial compounds. The microtiter plates underwent a 24-h aerobic incubation at 32°C.

Moreover, a solution of acetic acid with a concentration of 1 wt% was separately assessed to find the probable acetic acid's effect on microbial growth. CHX, which has been proven a safe and effective medical antibacterial agent, was considered positive control. The MIC assays were performed in triplicates.

- ***MBC assay***

MBC is defined as the lowest concentration of an antibacterial agent (i.e., chitosan solution) in which ≥99.9% of the test microorganism is killed [32]. The MBC assays of all chitosan samples per strain were carried out according to the method reported by Rukayadi et al. [31] and Özogul et al. [33]. This procedure involves spreading 100 µL medium from each well onto the MHA test plates. Columns 1 and 2, i.e., control-negative (no growth) and control-positive (growth), respectively, were cultured onto MHA. The plates were incubated at 32°C for 24 h. The MBC assays were also accomplished in triplicates.

3. Results and Discussion

3.1. Chitosan extraction process yield

The overall process flow and reaction conditions of the three extraction procedures are demonstrated in **Figure 1**. In the chitin extraction and chitosan production processes, including CE, ME, and CEM, after each step, the process yields were calculated (**Fig. 1**). The yields of the DM step at CE, ME, and CEM procedures were measured at 30.4 ± 1.6, 24.5 ± 0.8, and 28.1 ± 0.3%, respectively. The lower DM yields for the ME and CEM procedures could be attributed to their higher reaction temperature leading to smaller particle sizes. The smaller particles would, in turn, cause more weight loss during filtration and washing after each process step, resulting in lower yields [23]. The DP yields of the CE, ME, and CEM procedures were calculated at 53.7 ± 1.9, 58.2 ± 0.8, and 49.1 ± 4.1%, respectively. Contrary to the DM step, the DP yield for the ME procedure was the highest, and it could be justified that heat treatment by microwave power during the previous step (DM step) caused some protein removal [34]. The yields of the DA step of the CE, ME, and CEM processes were recorded at 77.7 ± 1.7, 82.9 ± 1.5, and 87.2 ± 0.4%, respectively. These values were in the same range reported by previous studies, such as Samar et al. (2013), who determined the DA yield of chitosan samples extracted using the ME procedure was 85.39 ± 0.62% [17]. Overall, the total yields of CE, ME, and CEM processes were calculated at 12.7 ± 0.4, 11.8 ± 0.5, and 10.8 ± 1.4%, respectively.

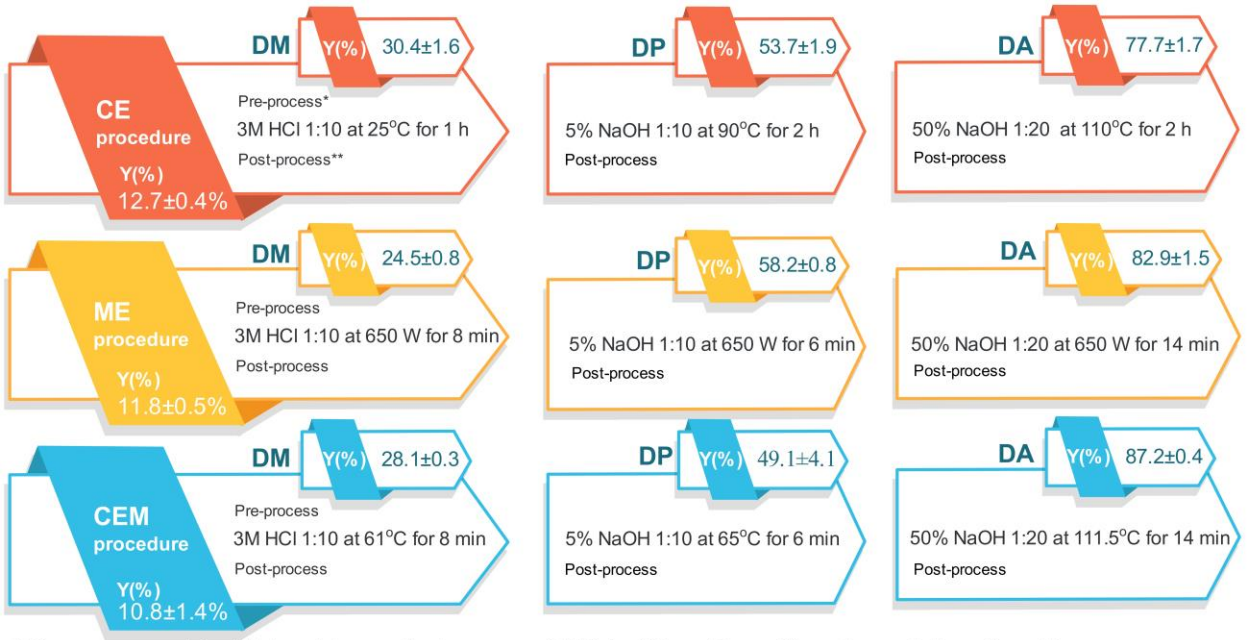


Figure 1. The experimental procedures and yields obtained for the different extraction approaches employed. *Abbreviations:* CE: conventional extraction, ME: microwave-assisted extraction, CEM: conventional extraction procedure in the ME reaction conditions, DM: demineralization, DP: deproteinization, DA: deacetylation, Y: yield.

As shown in **Figure 1**, microwave heating in the process of chitin extraction and chitosan production led to a 7% lower yield than conventional heating. Yield upgrading is considered one of the major advantages of conducting reactions under microwave heating [35]. In the case of chitin DM under acidic conditions, however, the yield was slightly lower, with the microwave heating showing lower solid removal in the process. On the other hand, the waste treated by microwave heating contained lower ash content (*Section 3.2.5*). Therefore, the higher solid removal through the DM stage by conventional heating might be attributed to the higher removal of other constituents [36]. Furthermore, apart from the heating mode, the minerals and protein contents of the waste were completely removed after the whole process based on the mineral content (*Section 3.2.5*) and protein content (*Section 3.2.6*) analysis of the wastes. Therefore,

considering the critical importance of the chitosan quality [36], a bit lower yield of its recovery by microwave heating could be neglected, considering the better grade of chitosan obtained by this heating mode, as reflected by its anti-microbial properties (*Section 3.3*). Moreover, it should be noted that performing the process by microwave heating for a shorter time would also be advantageous from an environmental footprint perspective [35].

3.2. Physicochemical properties assessment

3.2.1. FT-IR analysis

Figure 2 presents the FT-IR spectra for different samples; ME-Chitosan, CE-Chitosan, CEM-Chitosan, and commercial chitosan. The characteristic peaks observed at 3600-3800 cm^{-1} and 2800-3000 cm^{-1} could be related to the stretching vibrations of the chitosan structure's hydroxyl group (-OH) and -CH. The peaks at approximately 1500-1800 cm^{-1} could correspond to the amide-II's bending vibration and the amide-I's stretching vibration. These characteristic peaks confirm the sample's saccharide structure [18]. The DD% of samples was calculated using **Equation 1**. The absorbance of the bands at 1320 and 1420 cm^{-1} and the calculated DD% for four analyzed samples are reported in **Table 3**.

The DD values of all samples were just above 80%, confirming that the heating methods and the reaction durations did not affect the DD%. Similar results were also reported by Samar et al. (2013), who produced chitosan from mesh 40 chitin through the ME procedure and recorded a DD value of 83.05%, against 85% for a commercial chitosan sample [17]. It should be noted that the reported DDs, determined based on FT-IR bands, represent the average values but not the distribution of DDs of randomly formed chiton and chitosan molecules. Even though the experimental determination of the DD distribution of chitin or chitosan molecules is currently

impractical, reliable reports show that chitin and chitosan with narrowly distributed DD have sharper biological functions [37]. In this context, microwave heating which reportedly provides quicker and cleaner reactions both rationally and empirically [35], seems to be a better heating mode for delimiting the randomness of the reactions and probably achieving a narrower DD distribution.

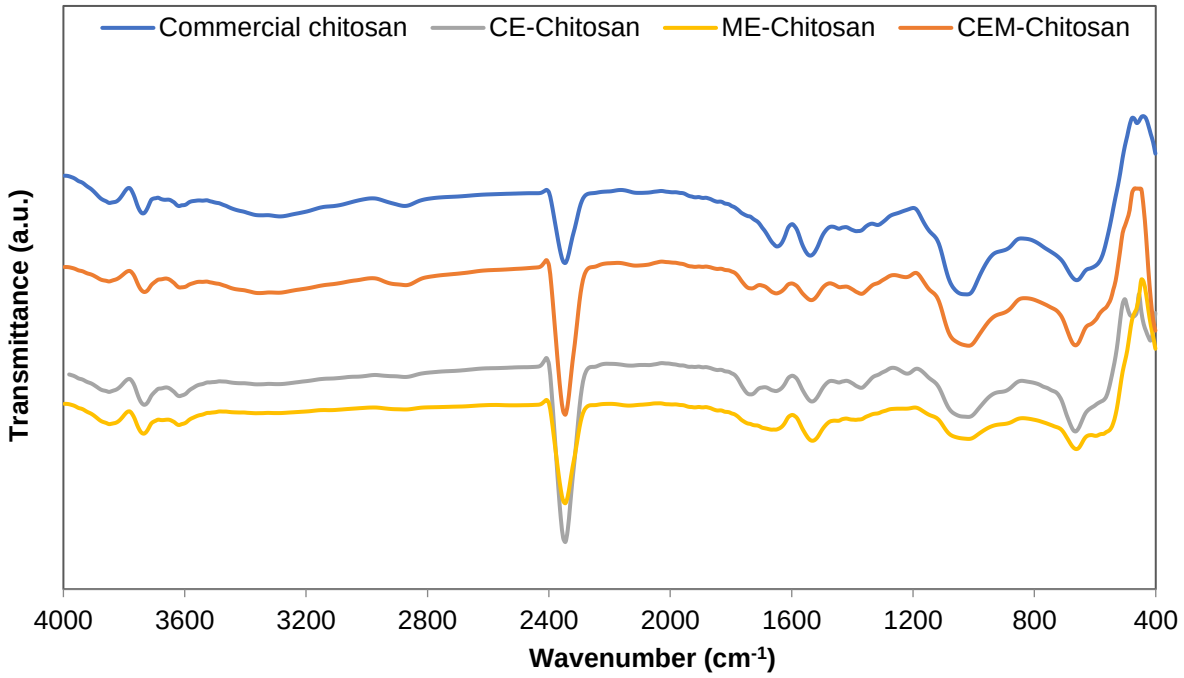


Figure 2. FT-IR spectra of CE-Chitosan, ME-Chitosan, CEM-Chitosan, and commercial chitosan samples.

Table 3. DD for different chitosan samples, including CE-Chitosan, ME-Chitosan, CEM-Chitosan, and commercial chitosan samples.

Sample	**A_{1320}**	**A_{1420}**	**DD (%)**
Commercial chitosan	97.470	97.241	80.21
CE-Chitosan	96.998	96.434	80.09
ME-Chitosan	98.064	97.547	80.11
CEM-Chitosan	96.446	96.126	80.17

3.2.2. XRD analysis

The XRD diffractograms obtained from the analysis of chitosan samples are presented in **Figure 3**. The two characteristic chitin and chitosan peaks around $2\theta=10^{o}$ and $2\theta=20^{o}$ were observed in the diffractograms of all analyzed samples [18, 38, 39]. The intensity of peaks recorded at $2\theta=16^{o}$ and the peaks recorded at $2\theta= \sim 20^{o}$ for all chitin and chitosan samples were used to calculate the CI according to **Equation 2**. As reported in **Table 4**, while the CI of commercial chitosan was 85%, the CI of ME-Chitosan, CE- Chitosan, and CEM-Chitosan samples stood at 83, 81, and 79%, respectively. The highest CI was obtained for the chitosan prepared using ME, the closest to commercial chitosan. The higher CI of the chitosan prepared by microwave heating revealed the higher reactivity of the catalysts under microwave irradiation [36].

The CI typically increases with DD% due to the fewer large acetyl side groups in the chitosan molecule resulting in a more efficient and regular packing of the polymer chains [40, 41]. The low CI of the CEM-Chitosan (79%) could be ascribed to the less efficient reaction conditions for chitin deacetylation.

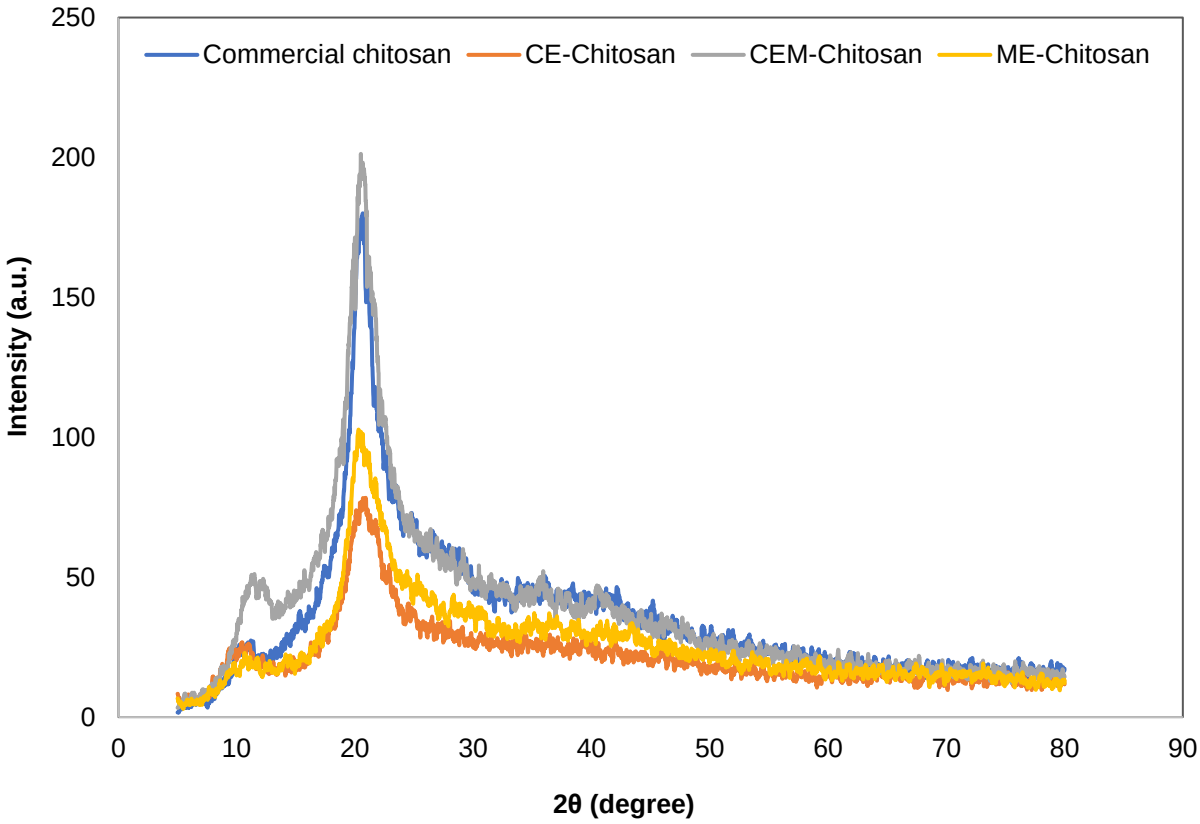


Figure 3. XRD diffractograms of the commercial chitosan, CE-chitosan, ME-chitosan, and CEM-chitosan samples.

Table 4. Characteristic crystalline peaks and crystallinity index for different chitosan samples, including commercial chitosan, CE-Chitosan, ME-Chitosan, and CEM-Chitosan samples.

Sample	Peak characteristic	Crystallinity index (%)
Commercial chitosan	2θ=20.56	85
CE-Chitosan	2θ=20.4	81
ME-Chitosan	2θ=20.42	83
CEM-Chitosan	2θ=20.2	79

3.2.3. SEM analysis

The morphology of chitosan samples was analyzed using SEM, and photographs are shown in **Figure 4**. All four samples were observed with layers of flakes with a lamellar organization and compact structure. The same results were reported previously by Knidri et al. [18] and Zheng et

al. [42]. Interestingly, only the ME-Chitosan showed a semi-porous structure, while other samples were non-porous. These pores would accelerate the DA rate, obtaining DDs similar to those of CE-Chitosan and CEM-Chitosan despite having shorter reaction times [20]. The semi-porous structure of ME-Chitosan makes it a desirable candidate for preparing adsorbents for different applications [43].

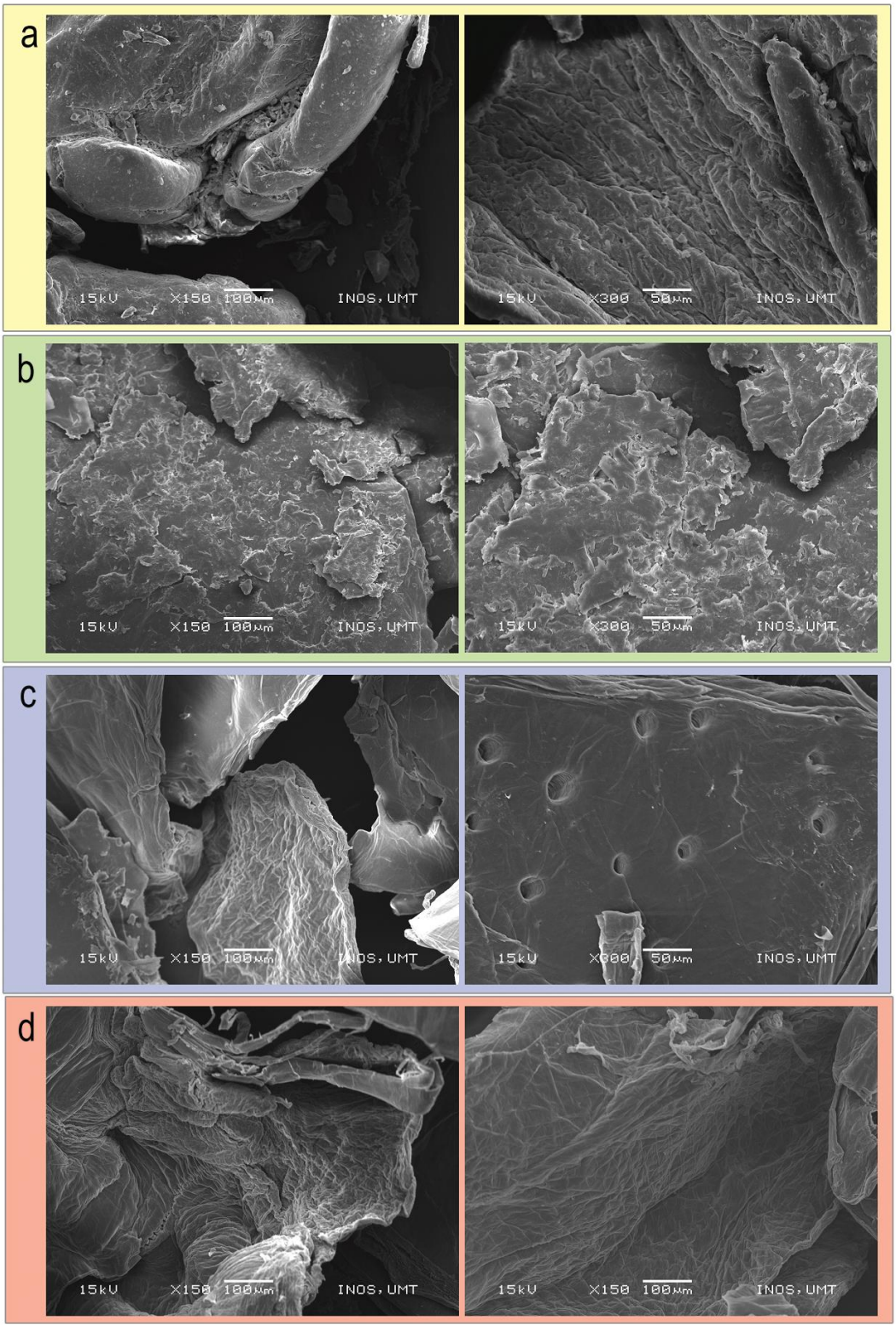


Figure 4. SEM photographs of (a) commercial chitosan, (b) CE-Chitosan, (c) ME-Chitosan, and (d) CEM-Chitosan.

3.2.4. Molecular weight determination

The functional properties of chitosan are affected by its molecular weight [44]. Heating in a highly concentrated solution of NaOH and irradiation affects the molecular weight of polysaccharide compounds like chitosan [20, 45]. The effects of heating methods on the molecular weight of chitosan samples were investigated through viscosity measurements. **Figure 5** provides the viscosity results of three samples, CE-Chitosan, ME-Chitosan, and CEM-Chitosan, alongside commercial chitosan, on a scale to find the molecular weight of the samples. The scale was prepared based on the specification sheet data of chitosan produced by Sigma [46-48]. The most interesting finding is that microwave-assisted heating resulted in lower viscosities and molecular weights. The results showed that the microwave irradiation power of 650 W was sufficient to produce free radicals, and consequently, more oxidative reactions occurred, reducing the molecular weights. It has been reported that water molecules would make the process of molecular weight reduction faster due to the oxidative chain scission at glycosidic bonds and the disruption of hydrogen bonding [45]. Although microwave irradiation could decrease the chitosan molecular weight, the functionality of chitosan would also be negatively affected, limiting its applications in bio-scaffolds and for biomedical purposes [49].

The viscosity of the CEM-Chitosan samples was in the range of reported values for medium molecular weight chitosan [47]. However, the viscosity of the CE-Chitosan sample was in the range of reported values for high molecular weight samples [46]. These differences could be due to the different reaction conditions, especially the heating source and the reaction duration. Using microwave power as a heating source resulted in obtaining lower molecular weights at shorter reaction times, while conventional heating resulted in higher molecular weights after longer

reaction durations. Consequently, it could be concluded that different chitosan samples can be synthesized with different functionalities and applications using different heating methods.

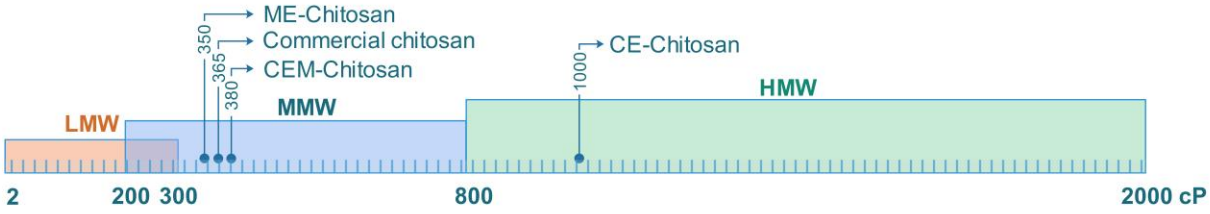


Figure 5. Obtained MW categories based on the measured viscosities for different chitosan samples.

Samar et al. (2013) reported that the production process parameters, including temperature, alkali concentration, reaction time, chitin extraction procedure, and particle size, might influence the MW of chitosan. Hence, it can be deduced that the main factor in increasing the CE-chitosan MW was the longer DA process. Moreover, the same MW class of chitosan was obtained through ME and CEM, showing that the heating source did not significantly affect the MW of the produced chitosan. On the contrary, in an experimental study, the increasing reaction temperature was reported to reduce the MW of chitosan [16]. From chitin extracted *via* CE, the authors produced chitosan through ME at 400 W within 5 to 20 min and steeped it in a strong NaOH solution at room temperature for 1 d. They reported that the product viscosity was decreased from 1500 to 400 cP (HMW to MMW) by increasing reaction time from 5 to 20 min, respectively.

3.2.5. Ash and calcium content determination

To assess the effect of heating type on the mineral removal from shrimp waste, the amount of ash and the remained calcium in the initial shrimp waste sample, as well as CE-Chitosan, ME-Chitosan, and CEM-Chitosan samples obtained after each consecutive step, i.e., DM, DP, and DA were measured. The initial shrimp waste sample contained 24.89 ± 0.84% ash and 5.74 ± 0.20

mg.mL^{-1} calcium concentration. As demonstrated in **Figure 6**, the mean values of the ash contents of all samples, except for the DM sample obtained *via* the CE heating method, were lower than the commercial chitosan, i.e., 0.63%. Then, the ash contents of all the DP process products decreased to less than 0.21%; and finally, all DA process products were ashless.

The calcium content of the commercial chitosan was 1.98 mg.mL^{-1}, the highest amount among the analyzed samples. For all the DM samples, the remaining Ca concentrations were lower than 0.2 mg.mL^{-1}. These results prove the high efficiency of the DM process of shrimp waste for all three procedures studied.

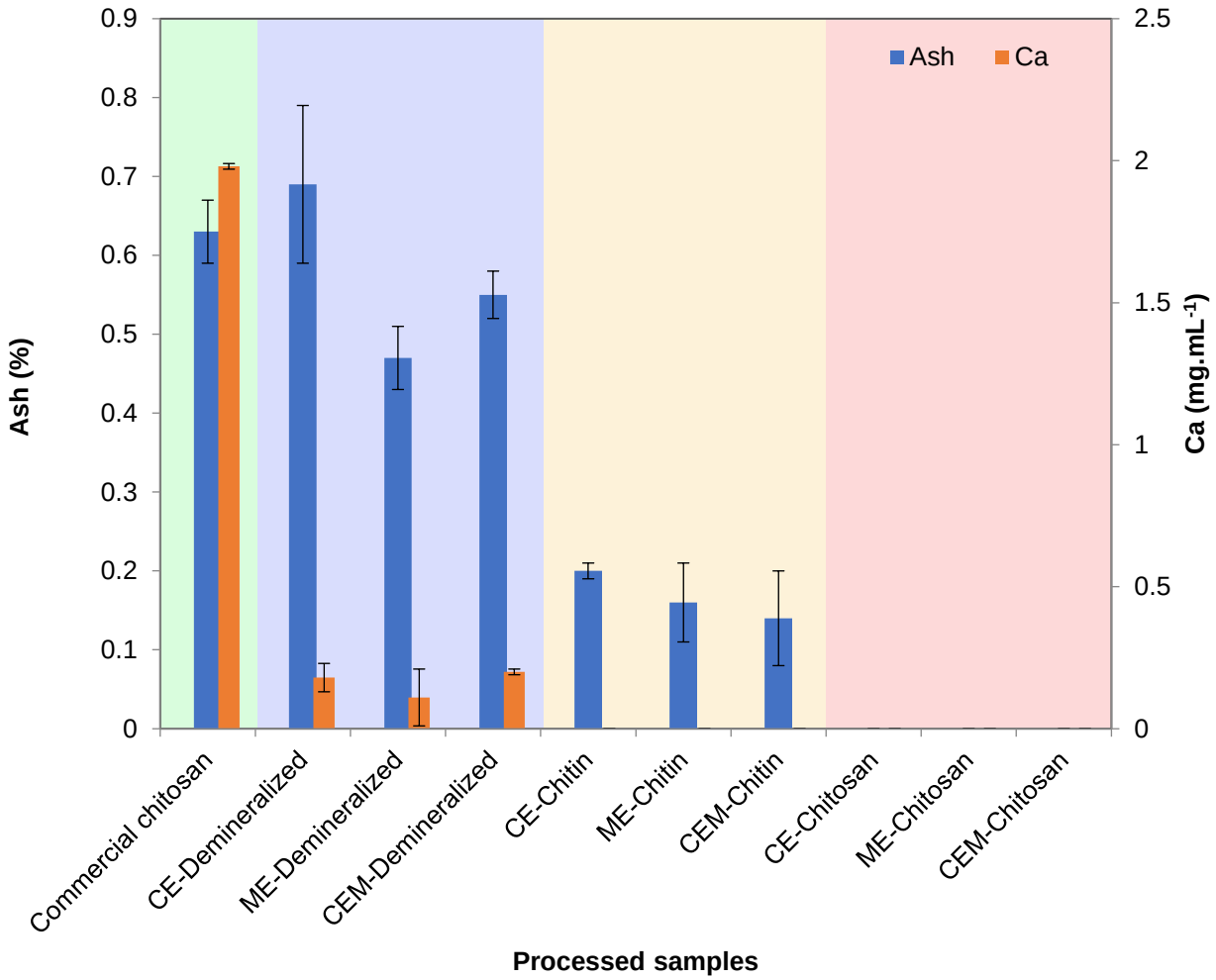


Figure 6. Ash and calcium content of different samples after the DM, DP, and DA processes of chitosan extraction through the CE, ME, and CEM methods

3.2.6. Remaining proteins determination

The protein concentration in the shrimp waste sample was 0.64±0.03 $mg.mL^{-1}$ and further reduced to 0.52, 0.32, and 0.22 $mg.mL^{-1}$ after DM, through CE, ME, and CEM extraction procedures, respectively. The protein contents of the samples further dropped to lower than 0.03 $mg.mL^{-1}$ in all samples after DP, revealing the high efficiency of this reaction in removing the total proteins from the samples to produce chitin. The protein contents of all the chitosan samples except ME-Chitosan were lower than the UV-Spectrophotometry detection limit, confirming the 100% efficiency of CE and CEM procedures. The efficiency of the ME procedure was obtained at 97%. These values were higher than those obtained by previous studies. For instance, the maximum amount of protein reportedly removed by Knidri et al. (2019) using a microwave-assisted procedure at 650 W in a 5% NaOH solution for 6 min was 95% [18]. Samar et al. (2013), Marei et al. (2016), and Ma et al. (2015) also reported protein removal rates above 90% using conventional alkaline treatment with NaOH solution of 1-4M at 90-100°C; however, the times taken to achieve high protein removal rates were substantially higher at 8-12 h [17, 50, 51] against 2 h taken in the present study.

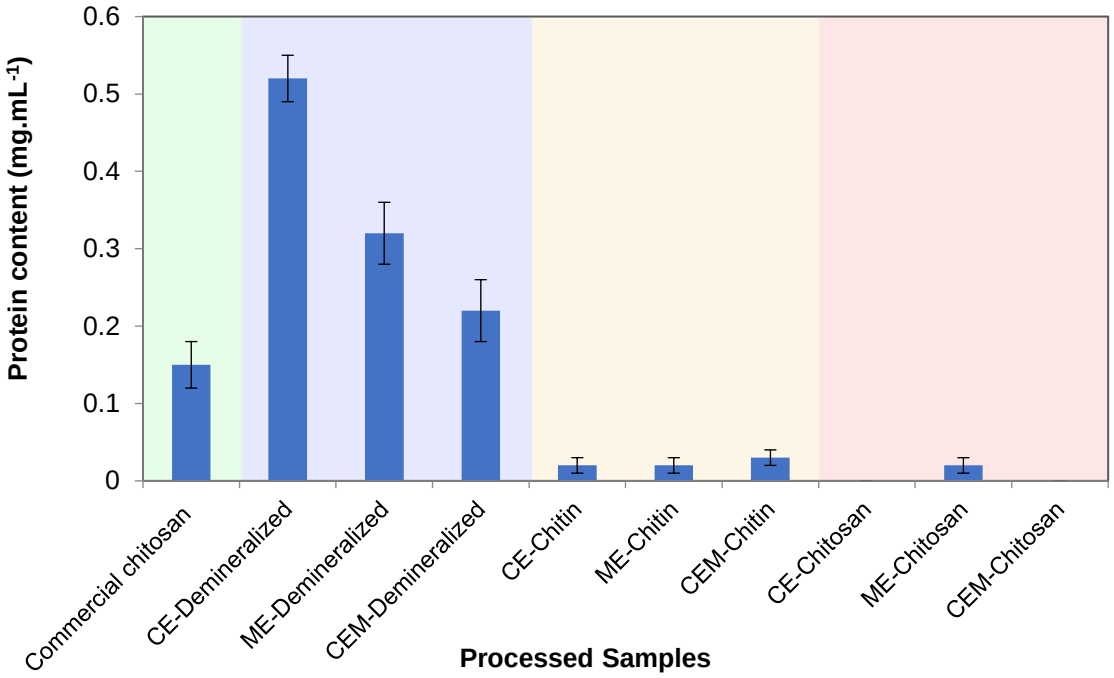


Figure 7. The protein content of different samples, i.e., demineralized shrimp waste, chitin, and chitosan, extracted through CE, ME, and CEM procedures alongside the commercial chitosan as control.

3.3. Antibacterial activity assessment

Various theories have been proposed to explain chitosan's antimicrobial mode of action [52] corresponding to its physicochemical properties, e.g., MW and viscosity [7]. **Figure 8** presents the clear zone diameter of each compound against four tested bacteria *via* Agar disk diffusion. Since acetic acid solution (1 wt%), as the chitosan solvent, shows no antibacterial activity against all tested bacteria except *S.* Typhimurium, the clear zones observed could be attributed to chitosan antibacterial activity.

CE-Chitosan had the largest clear zone diameter against *L. monocytogenes, E. coli*., and *S.* Typhimurium, while it had no antibacterial activity against *S. aureus*. The lower resistivity of *L. monocytogenes* as a Gram-positive bacterium can be justified based on the weaker barrier function of its outer membrane compared to Gram-negative bacteria *E. coli* and *S.* Typhimurium in

preventing the entrance of macromolecules and its lower negative charge value [53]. Even though several mechanisms, like the lethal flocculation of bacteria [54], have been previously reported, the most probable mechanism of the antibacterial action of CE-Chitosan could be the disintegration of the cell's outer membrane, which in turn led to increased permeability [37]. However, CE-Chitosan has a negligible effect on the Gram-positive bacterium *S. aureus*, for which the low rate of CE-Chitosan adsorption on the cells might be responsible. In the study by Takahashi et al. [55], chitosan with relatively high molecular weight in the range of 100 kDa and DD of 82% showed a noninhibitory effect on *S. aureus*, whereas chitosans with lower molecular weights or higher DD had a significant inhibitory effect on this bacterium [55].

On the other hand, ME-Chitosan had the highest antibacterial activity against *S. aureus*. Even though the lower molecular weight of ME-Chitosan had a substantial role in its higher inhibitory effect on *S. aureus*, there should be another factor causing the inhibition by ME-Chitosan but not by the commercial low-molecular-weight chitosan. In this case, the differences in the antimicrobial activity of chitosans might be attributed to the pattern of acetylation [37]. The antibacterial activity of ME-Chitosan and CEM-Chitosan against *L. monocytogenes* and CE-Chitosan against *S. aureus* was similar to that of the commercial chitosan samples. Whereas the commercial chitosan samples, i.e., CH-MMW and CH-LMW, showed significant antimicrobial activities against *E. coli*. and *S*. Typhimurium.

Although the MW and viscosity of CH-LMW and CH-MMW as commercial chitosan samples are various, their antibacterial activity had the same trend against Gram-positive and Gram-negative bacteria. It might be deduced that the MW and viscosity of all chitosan samples tested were not the only governing factor affecting their antibacterial activity. This finding aligns with the report by Atay et al. (2010) [7]. Compared to fully deacetylated chitosans, those with DDs

552 of nearly 51% were reportedly adsorbed on bacterial cells at a 20–30% higher rate but had a less
553 inhibitory effect on bacterial cells [56]. The acetylation pattern is the other factor affecting the
554 biological function of chitosan [57], but currently, there is no practical method for pattern analysis
555 for large chitosan molecules [37]. Moreover, higher MW chitosan (higher than 10 kDa) is an
556 effective inhibition against Gram-negative and Gram-positive bacteria [58]. Overall, chitosan
557 samples were shown better antibacterial activity against Gram-negative bacteria. This might be
558 due to chitosan interaction with the negatively charged groups of lipopolysaccharides, resulting in
559 chitosan-induced cell permeability changes. Since the outer membrane represents the first control
560 barrier to the entry or expulsion of molecules, its destabilization causes susceptibility to the entry
561 of external agents [59].

562 Also, CE-Chitosan, ME-Chitosan, and CEM-Chitosan solutions exhibited the same
563 antibacterial activity against target bacteria, except for the CE-Chitosan against *L. monocytogenes*
564 and ME-Chitosan against *S. aureus*. The former might be due to the higher viscosity of CE-
565 Chitosan solution, causing chitosan to bind to the cell surface of *L. monocytogenes* as a Gram-
566 positive bacterium. This binding alters the interaction of the membrane with the cell wall and
567 induces an osmotic imbalance of the cell even though the membrane and cell wall remain intact
568 [60]. The latter could be related to the easier passage of the higher MW chitosan through the cell
569 wall of Gram-positive bacteria compared to the Gram-negative bacteria's cell wall [61].

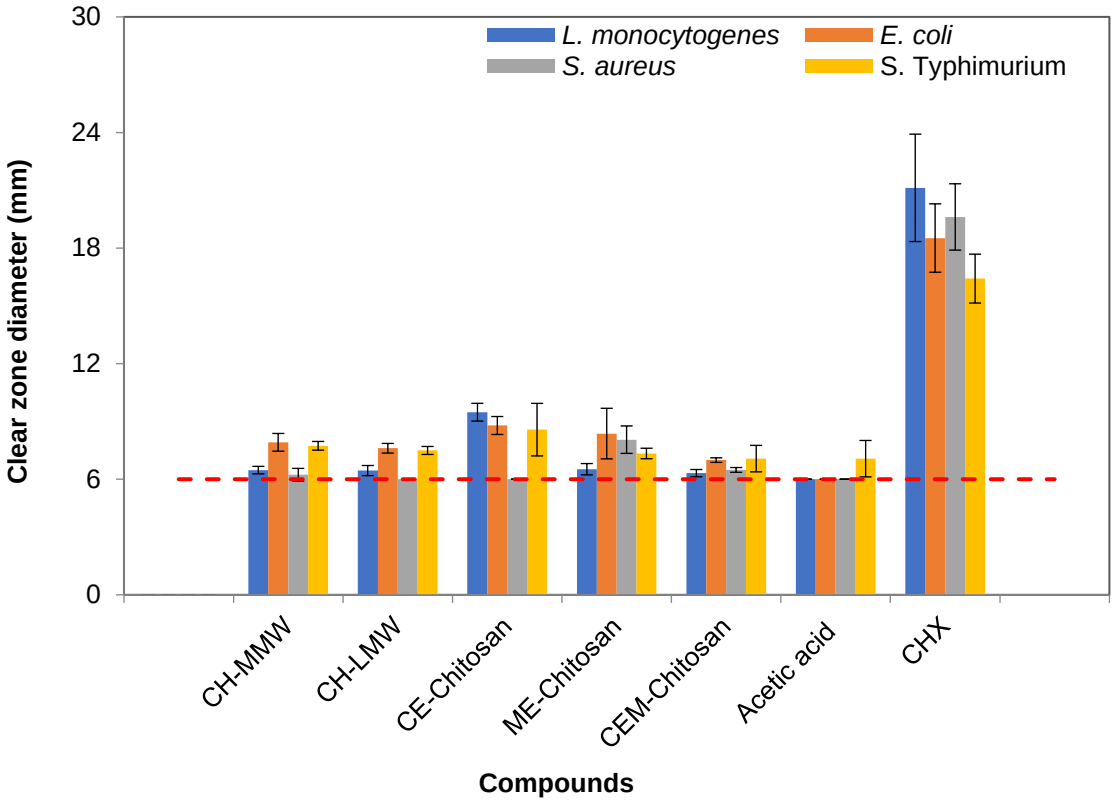


Figure 8. Clear zone diameter of the compounds assessed

The MIC of samples against target bacteria, i.e., *L. monocytogenes*, *E. coli*., *S. aureus*, and *S. Typhimurium*, was determined *via* the standards broth microdilution. **Table 5** demonstrates MIC and MBC values of the investigated compounds, including two commercial chitosan, three different chitosan samples extracted using different methods, acetic acid (used to eliminate the error related to the antibacterial activity of solvent in chitosan solution), and CHX as positive control against target bacteria.

The MIC results showed that acetic acid had an inhibitory activity at 1250 $\mu g.mL^{-1}$ concentration against all target bacteria. CEM-Chitosan was shown to exert no antibacterial activity against the target bacteria since all MIC values were similar to acetic acid. The other chitosan samples, i.e., CH-MMW, CH-LMW, CE-Chitosan, and ME-Chitosan, had the same

583 activity as CHX against *L. monocytogenes.* Moreover, against *E. coli*, CE-Chitosan, and ME-
584 Chitosan had similar MIC values to CH-MMW (625 $\mu g.mL^{-1}$), while CH-LMW had the best
585 inhibitory activity of 312.5 $\mu g.mL^{-1}$. MIC value for CE-Chitosan and ME-Chitosan samples
586 against *S. aureus* was 312.5 $\mu g.mL^{-1}$, while it stood at 156.2 $\mu g.mL^{-1}$ for commercial chitosan
587 samples. All chitosan samples were shown no inhibitory activity against *S.* Typhimurium, except
588 in the case of CE-Chitosan, which had an inhibitory activity at a concentration of 625 $\mu g.mL^{-1}$.

589 MBC assay results showed that CE-Chitosan, followed by ME-Chitosan, had the most
590 favorable bactericidal activity against *L. monocytogenes*, even better than CHX. However, CH-
591 LMW had the same MBC against *L. monocytogenes* as CHX of 156.2 $\mu g.mL^{-1}$. Moreover, *E. coli*.
592 and *S. aureus* had bactericidal activity only for CH-LMW at a concentration of 2500 and 312.5
593 $\mu g.mL^{-1}$, respectively. Meanwhile, no chitosan samples showed bactericidal activity against *S.*
594 Typhimurium.

595 CE-Chitosan sample's inhibitory activity against all target bacteria and bactericidal activity
596 against *L. monocytogenes* might be due to the higher chitosan MW and bacteria characteristics.
597 Overall, the inhibitory effect of chitosan could be related to MW and the type of bacteria [62]. The
598 results of the present study were in line with the findings by Goy *et al*. (2016), who confirmed the
599 higher activity of high MW chitosan against *S. aureus* (Gram-positive) compared to *E. coli* (Gram-
600 negative) [63]. In a study focused on a different type of chitosan composite in terms of antibacterial
601 activity, the chitosan–molybdenum disulfide nanocomposite showed high activity against *E. coli*
602 and *S. aureus*, which was similar to the present study's findings about the CE and ME chitosan's
603 antibacterial activity against these bacteria [64]. Another study evaluated the effects of chitosan's
604 DD and MW on the antibacterial activity of chitosan fibers against *E. coli*, *S. aureus*, and *C.*
605 *albicans*. The results showed that the antibacterial activity of chitosan fibers depends on a

combined effect of DD and MW. For instance, increasing MW for the same DD value led to different outcomes; a greater inhibition rate against *S. aureus* or a lower inhibition rate against *E. coli* [65]. The results of an experimental study focused on the antibacterial activity of chitosan-based nanohybrid membranes against drug-resistant bacterial isolates from burn wound infections showed high antibacterial activity against drug-resistant bacterial isolates with inhibition zones ranging from 12.1-21.8 mm [66].

Table 5. MIC ($\mu g.mL^{-1}$) and MBC ($\mu g.mL^{-1}$) of compounds obtained against bacteria.

Compound	**Bacteria**							
	L. monocytogenes		***E. coli***		***S. aureus***		***S. typhimurium***	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
CH-MMW	<9.8	312.5	625	5000	156.2	>5000	1250	>5000
CH-LMW	<9.8	156.2	312.5	2500	156.2	312.5	1250	5000
CE-Chitosan	<9.8	39.1	625	5000	312.5	>5000	625	5000
ME-Chitosan	<9.8	78.1	625	5000	312.5	5000	1250	5000
CEM-Chitosan	1250	5000	1250	5000	1250	>5000	1250	5000
Acetic acid	1250	5000	1250	5000	1250	>5000	1250	5000
CHX	<9.8	156.2	39.1	78.1	<9.8	39.1	39.1	1250

Although chitosan and its derivatives possess antimicrobial activity against bacteria, as confirmed in the literature and the present study, their mechanism of action has not yet been thoroughly scrutinized [67]. According to some experts, when chitosan amino groups come into contact with physiological fluids, they become protonated and, when bound to the anionic groups of microbes, they result in agglutination of the microbial cells and growth suppression [68]. In another theory, the bacterial tendency to absorb polysaccharides is responsible for antibacterial activity. Hence, chitosan absorption by bacteria changes the bacterial cell wall structure and consequently changes the cell membrane permeability [69].

Therefore, chitosan samples' different properties and antibacterial activity could affect their effectiveness in various applications, including food, pharmaceutical, medical, and other industries. Chitosan's effectiveness as a natural preservative in the food industry is partly determined by its MW and crystallinity. The high MW chitosan obtained by conventional extraction in this study could potentially be more effective as a preservative due to its stronger antimicrobial activity, while the porous medium MW chitosan obtained by ME could have advantages in terms of solubility and ease of application. CE under microwave conditions could also improve chitosan's solubility and antimicrobial activity due to its lower crystallinity index. On another hand, the chitosan sample's MW and DD are important factors in determining its efficacy as a drug delivery system or wound healing agent in the pharmaceutical industry. The different chitosan samples investigated in this study had varying DD, which could affect their interactions with cells and tissues. The antibacterial activity of chitosan could also be relevant in these applications to prevent or treat bacterial infections associated with drug delivery or wound healing.

Overall, the antibacterial activity observed in this study suggests that chitosan extracted through the CE method could have potential clinical relevance in various industries, particularly in preventing or treating bacterial infections. Moreover, by inhibiting bacterial growth, chitosan as a bio-preservative can extend the shelf life of high-value and strategic food products. Chitosan's antibacterial activity can also prevent or treat bacterial infections in a drug delivery system or wound healing agent in the pharmaceutical and medical industries.

3.4. Statistical analysis

The results of ANOVA analysis in evaluating the effects of the antibacterial compound, bacteria type, and their interaction on the antibacterial activity responses, i.e., clear zone diameter, MIC, and MBC, are tabulated in **Table 6**. The degree of freedom (df), the sum of squares (SS), mean square (MS), F-value (F), and significant (sig.) level are reported. It can be observed that, except for the effect of the bacteria type on the clear zone, i.e., $p>0.05$, the other differences were insignificant ($p<0.05$).

Table 7 tabulates Duncan's multiple range test results to compare the mean values of clear zone diameter (mm), MIC, and MBC *vs.* the antibacterial compound and bacteria type. In antibacterial compound assessment, CEM-Chitosan and CH-LMW, followed by CH-MMW, with the lowest clear zone mean value among chitosan samples, had significant differences with clear zone mean values of ME-Chitosan and CE-Chitosan. Although MIC mean values for CEM-Chitosan did not differ from acetic acid, the mean values for ME-Chitosan and CH-MMW, followed by CH-LMW and CE-Chitosan as the best MIC result, were significantly different. Despite the similar behavior of the MBC mean values for CEM-Chitosan and acetic acid, CH-MMW (not completely meaningful), CE-Chitosan, and ME-Chitosan, followed by CH-LMW as the best MBC result, had significant differences in MBC mean values.

In bacteria type assessment, chitosan antibacterial activity against *E. coli*. with the largest mean clear zone values, was significantly more active than *L. monocytogenes* and *S.* Typhimurium, followed by *S. aureus*. Among all bacteria, chitosan samples against *L. monocytogenes* had the best result in MIC. Moreover, MIC mean values for *S. aureus*, *E. coli*., and *S.* Typhimurium increased significantly. Although MBC mean values difference for *S. aureus* and *S.* Typhimurium was insignificant, MBC mean values decreased for *E. coli*. and then *L. monocytogenes*, the most killed bacteria exposed to chitosan, significantly.

Table 6. Results of ANOVA analysis in evaluating the effects of the antibacterial compound, bacteria type, and their interaction on the antibacterial effect clear zone diameter ($\mu g.mL^{-1}$), MIC ($\mu g.mL^{-1}$), and MBC ($\mu g.mL^{-1}$).

Parameter	Antibacterial compound (df=6)				Bactria type (df=3)				Antibacterial compound × Bactria type (df=18)			
	SS	MS	F	Sig.	SS	MS	F	Sig.	SS	MS	F	Sig.
Clear zone	1459.37	243.23	204.54	0.00	7.57	2.52	2.12	0.11	70.82	3.93	3.31	0.00
MIC	1.5e7	2.4e6	489.21	0.00	5.0e6	1.7e6	334.98	0.00	4.2e6	2.3e5	47.04	0.00
MBC	3.5e8	5.9e7	19.35	0.00	2.3e8	7.8e7	25.65	0.00	2.4e8	1.3e7	4.35	0.00

Table 7. Duncan's multiple range test results to compare the mean values of clear zone diameter (mm), MIC, and MBC versus the antibacterial compound and bacteria type.

Parameter	Antibacterial compound								Bactria type***				
	Std err	CH-MMW	CH-LMW	CE-Chitosan	ME-Chitosan	CEM-Chitosan	AA*	CHX	Std err	LM	EC	SA	ST
Clear zone	0.32	7.09^{b*}	6.89^{ab}	8.21^{c}	7.57^{bc}	6.72^{ab}	6.27^{a}	18.92^{d}	0.24	8.91^{ab}	9.17^{b}	8.34^{a}	8.81^{ab}
MIC	20.46	503.74^{bc}	445.14^{b}	445.14^{b}	549.31^{c}	1250.00^{d}	1250.00^{d}	21.97^{a}	15.47	364.12^{a}	673.83^{c}	496.19^{b}	1017.48^{d}
MBC	504.38	5266.93^{cd}	2239.58^{b}	4596.35^{c}	3977.86^{c}	6666.67^{d}	6666.67^{d}	699.87^{a}	381.27	1770.83^{a}	3839.29^{b}	6002.60^{c}	5595.24^{c}

* Different letters indicate significant differences between means at a 5% probability level by Duncan's test.

**Acetic acid

***SA: *S. aureus*, ST: *S.* Typhimurium, LM: *L. monocytogenes*, EC: *E. coli*.

The current study also suffered from some limitations: 1) the effects of heating process parameters on the physicochemical properties and antibacterial activity of chitosan were investigated, but other extraction methods, such as enzymatic extraction or exyraction systems based on chemicals, were not included herein, and hence their associated chitosan properties and activities could not be compared. 2) This study only focused on the antibacterial activity of chitosan extracted from shrimp waste against a limited number of bacterial strains. 3) Although chitosan is generally considered safe for human consumption, the potential cytotoxicity of chitosan against human cells should still be investigated.

4. Conclusions and prospects

This experimental study assessed the effects of three different extraction procedures, i.e., CE, ME, and CEM, on the physicochemical properties and antibacterial activity of shrimp waste-derived chitosan. The physicochemical properties assessment revealed that the CE resulted in high molecular weight chitosan, while the ME extraction procedure led to porous chitosan with the lowest molecular weight. The low ash content, complete protein removal, and high DD results proved the high efficiency of the DM, DP, and DA processes, respectively. Antibacterial activity was assessed through agar disk diffusion, MIC, and MBC assays. The results displayed that while it showed no activity against *S. aureus*, CE-Chitosan had the best antibacterial activity in the agar disk diffusion assay against *L. monocytogenes*, *E. coli*., and *S.* Typhimurium. On the other hand, ME-Chitosan, with the highest activity against *S. aureus*, had similar antibacterial activity against *E. coli* and *S.* Typhimurium to CE-Chitosan. Although CEM-Chitosan had the lowest antimicrobial activity among all the chitosan samples, its antibacterial activity against *S. aureus* in the agar disk diffusion assay was considerable. Moreover, MIC and MBC assays demonstrated that among the

chitosan samples assessed, CE-Chitosan, followed by ME-Chitosan, had the best antibacterial activity against the target bacteria.

The study highlights the importance of selecting the extraction process to obtain chitosan with desirable physicochemical properties and antibacterial activity. Therefore, future research should optimize the extraction conditions to obtain chitosan with specific properties and explore its potential applications in different industries. Among such applications would be the use of chitosan for food preservation which has gained considerable attention due to its natural origin, biodegradability, and safety. Standardizing chitosan extraction methods to produce reliable and reproducible products to facilitate the development of new applications and improve the efficiency of current ones is also suggested as a future research need. Finally, future studies should also investigate the environmental and economic impacts of chitosan extraction methods to improve the overall sustainability of these processes.

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