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Extraction, Characterization, and *In Vivo* Antimicrobial Activity of Chitosan Derived from the Egyptian Shrimp (*Metapenaeopsis stridulans*) Wastes

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ABSTRACT

In the present study, extraction of chitin from shrimp (*Metapenaeopsis stridulans*) wastes was performed chemically or biologically (using *Bacillus subtilis* (B) and *Lactobacillus plantarum* (L) via single-step (L or B) or successive co-fermentation techniques (L/B or B/L)). The proximate composition, physicochemical properties, and antibacterial activity of the prepared chitosan samples were determined. Although the bio-extracted chitosan samples (3.59-6.64%) yields were lower than the chemically extracted samples (8.44%), the bio-extracted samples showed better quality. The DDA and CrI % of the biologically and chemically extracted chitosan samples were (79.5-80.35%) and (71.0-77.7%), respectively. The intrinsic viscosity and molecular weights of the bio-extracted chitosan (B, L, B/L, and L/B) samples (η= 0.0779, 0.0847, 0.0943 and 0.0909 dl/g, Mw=26.37, 30.17, 35.09 and 33.32 kDa, respectively) were lower than the chemically extracted samples (η=0.1903 dl/g, Mw=94.34 kDa). XRD revealed the crystalline nature of all the polymers. The bio-extracted chitosan samples showed greater inhibition zones (IZ) against *B. cereus*. Chitosan sample (B) exhibited the highest antimicrobial activity against *B. cereus*, *Salmonella* sp. and *Vibrio* sp. The present findings proved the potential use of bio-extracted chitosan as an effective, safer, and more natural preservative in the food industry to combat foodborne pathogens.

1. INTRODUCTION

Shrimp has recently been identified as one of Egypt's most significant fishing resources. Although Egypt produced 7,235 tonnes of cultured shrimp in 2013, an additional 54,937 tonnes of shrimp and prawns were imported to meet the domestic consumer demand **(GAFRD, 2014; FishStatJ, 2016)**. The marine shrimp sector in Egypt is expected to grow in the coming years, especially with the expansion of fish farming through mega-national projects that include the processing of this farmed shrimp. Accordingly, shrimp waste that is expected to increase should be utilized and converted into value-added products. Both the economy and the ecology will gain from such a waste-to-wealth plan. One of these value-added products is chitin and its deacetylated counterpart, chitosan. Crustacean shells' composition varies with species and seasons. Their shells are composed of 30-40 % proteins, 30-50 % calcium carbonate, 20-30 % chitin, pigments, and other minor components (**Aranaz *et al.*, 2009**).

Chitin is the world's second most abundant biopolymer after cellulose (**Satam *et al.*, 2018**). It can be chemically extracted by dissolving in strong acids and bases to dissolve calcium carbonates and proteins, respectively. This method is costly and causes aggressive pollution to the environment (**El Knidri *et al.*, 2018**). An alternative eco-friendly and economical method that has been used for chitin extraction from crustaceans' shells is the biological method (**Zhang *et al.*, 2017**). The biological method involves using microorganisms such as lactic acid and proteases producing bacteria for the demineralization and deproteinization steps (**Liu *et al.*, 2014; El Knidri *et al.*, 2018**).

Chitosan is a linear polymer comprising a (1-4)-linked 2-amino-2-deoxy-b-D-glucopyranose (**Anand *et al.*, 2018**). Chitosan is made by removing enough acetyl groups (CH_3-CO) from the molecule to make it soluble in most diluted acids. The acetyl component of the polymer is what distinguishes chitin from chitosan. Chitosan, which has a free amino group, is the most valuable chitin derivative (**Pillai *et al.*, 2009**).

Chitosan has positive ionic charges allowing it to bind chemically to negatively charged fats, lipids, cholesterol, metal ions, proteins and macromolecules (**Li *et al.*, 1992**). Chitin and Chitosan have good characteristics that make them two of the most popular biopolymers with many applications. These characteristics include biocompatibility, biodegradability, adsorption, antibacterial properties, gel-forming properties, and the ability to form films and chelate metal ions (**Shukla *et al.*, 2013**). Chitosan has been used as a functional biopolymer in food preservation, medicine, agriculture, wastewater treatment, and so on (**Al Hoqani *et al.*, 2020**).

Chitosan, as a natural polyaminosaccharide possesses many characteristics that attract food scientists and food industries. Food biopreservation is one of the possible applications of chitosan in food. Several studies have reported the significant antibacterial effect of chitosan or its conjugates against many foodborne and spoilage microorganisms (**Huang *et al.*, 2004; Möller *et al.*, 2004; Wu *et al.*, 2004; Yang *et al.*, 2005; Huang *et al.*, 2007; Li *et al.*, 2007; Ganan *et al.*, 2009; Alfaifi *et al.*, 2020; Wrońska *et al.*, 2021**). The antibacterial activity of chitosan may be attributed to the linkage between the electronegative charges of the bacterial cell surface and the amino groups of chitosan, leading to the leakage of intracellular components (**Li & Zhuang, 2020**).

The aim of this research was to optimize the production of chitin and its deacetylated products chitosan from shrimp (*Metapenaeopsis stridulans*) wastes, using different biological treatments and the traditional chemical method, and then characterize the products using FTIR, XRD, and SEM in addition to chemical methods. The extracted chitosan was further evaluated for antibacterial activity against Gram-positive and Gram-negative foodborne pathogens.

2. MATERIALS AND METHODS

2.1. Materials

2.1. 1 Collection of shrimp wastes

Shrimp wastes used in this study were those of a single species *Metapenaeopsis Stridulans* purchased from Ataka Port, Suez in Egypt during the fishing season of 2021. It is a common commercial species fished mainly in the Red Sea. Shrimps (250 Kg) were maintained on ice in ice bins and transported to the fish processing unit of the Faculty of Fish Resources, Suez University, Egypt. Peeling was manually performed, and the collected wastes (back and tail) were thoroughly washed with tap water. After rinsing with deionized water, they were dried in a drying oven at 65˚C for 14h. The dry shrimp wastes were ground into powder by a home-use blender and stored at ambient temperature until further procedures (Sh).

2.1.2. Bacterial strains

Protease-producing bacteria, *Bacillus subtilis* subsp. DSM 1088, *Lactobacillus plantarum* DSM 20174 ATCC 14917, *Bacillus cereus, Salmonella* sp., and *vibrio* sp. were obtained from the Microbiological Resources Center (MIRCEN), the Faculty of Agriculture, Ain Shams University, Egypt.

2.1.3. Chemicals

Sodium hydroxide (NaOH) was purchased from TopChem Pharmaceuticals Ltd, Ireland. Hydrochloride acid (HCl) was purchased from Diachem Chemicals, Egypt. Oxalic acid and other chemicals were purchased from Nice Chemicals Pvt Ltd, India. Mueller Hinton Agar, MRS broth, and nutrient broth were purchased from Oxoid Ltd, England.

2.2. Extraction of chitin

Chitin extraction was achieved using two methods, either chemically or biologically (Fig. 1).

2.2.1. Chemical method

Demineralization was achieved by adding the shell powder to 2N hydrochloric acid with a ratio of 1: 20 (W/V) and constant stirring for 2h at an ambient temperature. The residue was then collected, washed with tap water, then washed again with distilled water. Finally, the residue was collected and dried overnight in a drying oven at 60- 80˚C. The demineralized powder was weighted and then used to perform the deproteinization step, according to **Varun *et al.* (2017)**, with slight modifications . The demineralized powder was treated with 2N NaOH with a ratio of 1: 20 (W/V) at 50˚C and a continuous stirring on a magnetic stirrer for 2h. After that, the residue was collected, washed, and dried as previously described.

The produced powder was decolorized according to **Divya *et al.* (2014)**, with some modifications. The powder was soaked in oxalic acid 1% with a solid to a solvent ratio of 1: 20 (W/V) at ambient temperature, with stirring over a magnetic stirrer (140 rpm) for 2h. This step was repeated, and the dried powder produced after drying at 60-80˚C was determinedas a chemically produced chitin (Ch).

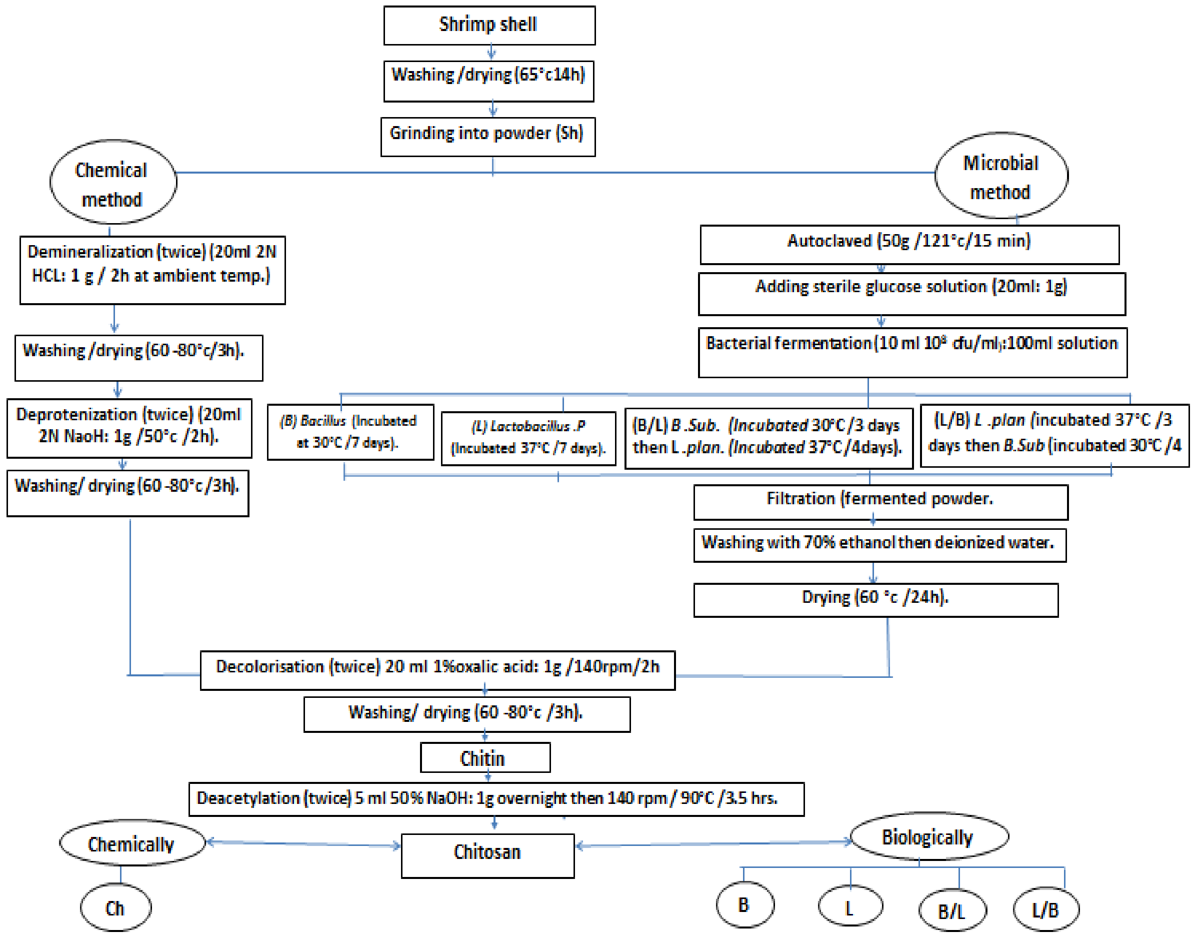

Fig. 1. Scheme showing the steps for chemical and biological extraction of chitin and chitosan from shrimp (*Metapenaeopsis Stridulans*) shell waste

2.2.2. Biological process

It is a fermentation process carried out using bacteria following the method of **Zhang *et al.* (2022)**, with minor modifications to perform deproteinization and demineralization.

2.2.2.1. Preparation of inoculums

L. plantarum and *B. subtilis* were activated by transferring them into 5ml of MRS and nutrient broth, and the cultures were incubated at 37˚C or 30˚C for 48h, respectively. Then, 2ml of each of the previously incubated cultures were transferred into 100ml of sterile MRS broth or nutrient broth and incubated as stated earlier to get the inoculums ready for use in the following fermentation process (10^8 CFU/ ml).

2.2.2.2. Fermentation process

According to **Chakravarty *et al.* (2018)**, 50g of the shells' powder was added to each of the four conical flasks (1 L), then covered with a cap and autoclaved at 121˚C for 15min. This step was followed by the addition of 20% sterile glucose solution (1:20 w/v; ratio) to each autoclaved shells' powder flasks. One of the prepared inoculums was added to each shell's powder flask with a ratio of 10% (v/v) and incubated at the desired fermentation temperature for each bacterium strain (37˚C or 30˚C) for 7 days with manual shaking.

The four inoculum treatments applied during the fermentation were as follows:

1) Single-step fermentation: i) using *B. subtilis* at 30˚C (B); or ii) *L. plantarum* at 37˚C (L).

2) Successive co-fermentation: i) adding *B. subtilis* (30˚C for 3 days), then *L. plantarum* (37˚C for 4 days) (B/L); or ii) adding *L. plantarum* (37˚C for 3 days), then *B. subtilis* (30˚C for 4 days) (L/B).

Afterward, the fermented supernatants were filtered off, and the fermented shell powder produced from each fermentation treatment was washed with deionized water and sterilized with 70% (v/v) ethanol, and then dried in a drying oven (Hi-Temp Vacuum Oven, Thermo Scientific) at 60˚C for 24h. The produced powder was decolorized twice using 1% oxalic acid, as described previously in the chemical method. The dried powder produced after drying at 60- 80˚C was determined as biologically produced chitin (B, L, B/L, or L/B).

2.3. Chitosan production

The chitin that was extracted either chemically or biologically as previously described was deacetylated using a 50% NaOH solution with a solid to solvent ratio of 1: 5. After soaking overnight, heating with shaking (140 rpm) at 90˚C for 3.5h was conducted, followed by filtering and washing with tap water and deionized water. This step was repeated and then dried in the oven. The final dried product obtained according to the preparation process was designated as chitosan produced chemically (Ch) or biologically (B, L, B/L, or L/B).

2.4. Characterization of chitin and chitosan

Dried shell powder (Sh) and the extracted chitin and chitosan (Ch, B, L, B/L, or L/B) are the samples included in the following analytical methods.

2.4.1. Proximate analysis of shrimp wastes and chitosan

Moisture, protein, fat, and ash contents of samples were determined according to **AOAC (2007)**. This procedure was done in triplicates, and the mean content was computed. Moisture contents were determined by drying the sample (3 g) in a vacuum oven at 105˚C for 24h, and the decrease in the weight was measured, which corresponds to the loss of water molecules as shown in the following equation:

The moisture content % = ((wet sample – dried sample)/ wet sample) x 100

The ash content was performed by putting the sample (2 g) in a muffle furnace at 650˚C for 5h. The percentage of ash content was calculated according to the following equation:

% Ash = (Weight after ashing / Weight before ashing) X 100

The protein content in samples was determined using the micro-biuret method (**Boyer, 1993**). The Soxhlet extraction method was carried out with hexane at 60°C for 9h to determine fat content in samples (**Hajji *et al*., 2014**).

2.4.2. The solubility and pH of chitosan samples

The solubility of chitosan samples was measured according to the method of Fernandez-Kim with modifications (**Fernandez-Kim, 2004**). A 1% solution of chitosan sample was constituted by adding 0.1 g (W1) of each chitosan sample previously dried at 105˚C for 24h into 10ml of 1% acetic acid in a 15ml falcon tube. The tubes were sealed and placed in a vortex for 2h after that, the samples were transferred into the water bath at

80˚C for 24h. The solution was centrifuged at 10, 000 rpm for 15min (Model CM50, s\N: 1510001E, 24V VDC 5A, USA). After the liquid phase was poured out, the residue was washed with 10ml of distilled water and centrifuged again at 10,000 rpm for 15min. The supernatant was decanted, and the residue was dried at 105˚C for 24h (W2) and then weighed. The percentage of solubility was determined using the following formula:

$$\% \text{ solubility} = ((W1 - W2) / W1) \times 100$$

The pH of chitosan samples' solutions was carried out in triplicates using a pH meter (OHAUS STARTER 2100 Bench pH meter, OHAUS Instruments, USA).

2.4.3. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of chitin and chitosan samples were recorded using FTIR spectrophotometer (Bruker, OPPTIK GmbH Rudolf-Plank-str.27 D-76275 Ettlingen), Model ALPHA II in the range of 400–4000 cm^{-1}, in the Physical Lab, the Faculty of Science, Suez University, Egypt. ATR mode of operation was used, and 24 scans were accumulated at a resolution of $4cm^{-1}$.

2.4.4. Degree of deacetylation

The FTIR instrument was used to determine the degree of acetylation (DA) and deacetylation (DDA) of the samples (**Mohammed *et al.*, 2013; El Knidri *et al.*, 2016**). The absorption band at 1320 cm^{-1} and 1420 cm^{-1} was chosen to measure the DA. The DDA was expressed in percentage according to the succeeding equation:

$$A_{1320}/A_{1420} = 0.3822 + 0.03133\ DA$$

$$DDA = 100 - DA$$

2.4.5. X-ray diffraction (XRD)

The crystallinity of samples in the powder form was evaluated by a wide-angle X-ray diffraction analysis, using an X-ray diffractometer XRD Siemens D5000 powder diffractometer Cu Kα radiation (wavelength l = 0.15406 nm), with a nickel filter at 40kV and 30mA. The diffractometer was operated within the range of 5˚<2q< 100˚ using a step size of 0.05 deg. About 20mg of the sample was spread on a sample stage, and the relative intensity was recorded in the scattering range (2 h) of 5–40 in steps of 0.1. The crystalline index (CrI; %) was determined based on the following equation:

$$CrI_{020} = (I_{020} - I_{am}) \times 100 / I_{020}$$

Where, I_{020} is the maximum intensity, and I_{am} is the intensity of amorphous (I_{16}) (**Zhang *et al.*, 2005**).

2.4.6. Field emission scanning electron microscope (FE-SEM)

Morphology of chitosan samples was inspected using the field emission scanning electron microscope (FE-SEM) model Quanta FEG 250 FEI co, Netherlands. The SEM has a magnification range of 5,000 and an accelerating voltage of 20KV. It was used to characterize the prepared chitosan samples.

2.4.7. Intrinsic viscosity and molecular weight (Mw)

Using an Ostwald capillary viscometer, the viscosity of the chitosan samples was determined. To estimate the molecular weight, the intrinsic viscosity of the chitosan polymer, which was dissolved in 1% acetic acid and 1M NaCl, was calculated based on the relative viscosity. In order to estimate the intrinsic viscosity (equation 1), the viscosity of chitosan was evaluated at various concentrations (0.1 %, 0.2 %, 0.3 %, 0.4 % and 0.5%) (**Fernandez-Kim, 2004**).

$$[\eta] = \lim_{C \to 0} \frac{(\eta - \eta_s)}{\eta_s C} \quad \text{...... (1)}$$

Where, η is the chitosan solution viscosity; η_s is the solvent viscosity, and C is the chitosan concentration. The Mark-Houwink equation was used to determine the molecular weight (**Rinaudo, 2006; Vallejo-Domínguez *et al*., 2021**).

$$[\eta] = \mathbf{K\ Mw}^{\alpha} \quad \text{...... (2)}$$

Where, $^{\alpha}$ (0.71) and (K) ($5.59x10^{-5}$) are empirical viscometric constants.

2.5. *In vitro* antimicrobial testing

The antibacterial activity of chitosan solutions (Ch, B, L, B/L, or L/B) against the Gram-positive (*Bacillus cereus*) and Gram-negative foodborne bacteria (*Salmonella* sp. and *vibrio* sp.) was determined using the disc diffusion assay as described by **Arancibia *et al*. (2014)**, with slight modifications. Chitosan samples (Ch, B, L, B/L, or L/B) were prepared for the antimicrobial effect by dissolving 0.5g of chitosan sample in a 100ml of acetic acid solution (1%). Petri plates were prepared by pouring 20ml of Muller Hinton Agar medium (MH) and were allowed to solidify. Bacterial inoculums were prepared, adjusted to 0.5 of McFarland, and swabbed on MH agar plates. Sterile filter paper discs (6mm diameter, Whatman No. 1) impregnated with 20µl of each chitosan solution were placed on the surface of agar plates. The agar plates were incubated for 24h at 37˚C. Antibacterial activity was evaluated by measuring the diameter of inhibition zones against the tested bacteria, and results were expressed in mm. Each test was performed in duplicate.

2.6. Statistical Analysis

The average and standard deviation data for the experiments, which were done in triplicate, were given. Using the SPSS statistical package program (SPSS 22.0. for windows, SPSS Inc., Chicago, IL, USA), the statistical significance of differences between data groups was checked by one-way ANOVA, with a significance level of $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Yields of chitin and chitosan after the chemical and biological extraction methods

The yielding proportions of various products obtained at different extraction stages using chemical and biological methods were calculated based on the dry weight of the shrimp wastes, as shown in Table (1). Lower yields were shown after demineralization and deproteinization (15.76±0.25%) and decolorization (11.8±0.43%) through the chemical method in comparison with the biological process. Several previous studies have used the chemical method to extract chitin from shrimp wastes. These studies demonstrated a massive difference in the yields of chitin and chitosan (**Lertsutthiwong *et al*., 2002; Puvvada *et al*., 2012; Samar *et al*., 2013; Hossain & Iqbal, 2014; Srinivasan *et al*., 2018; Al Hoqani *et al*., 2020**). **Al Hoqani *et al*. (2020)** suggested that, the variability in yields resulting from the chemical extraction methods in different studies may be attributed to the variability in acid and base concentrations, temperature and methods of deacetylation. Furthermore, the parent source of chitin contributes as a factor affecting the yield of chitin (7-40%) from crustaceans' wastes (**Tolaimate *et al*., 2003; Liu *et al*., 2012**).

Table 1. Yields of chitin and chitosan after each step of chemical (Ch) and biological (B, L, B/L, and L/B) extraction of chitosan from shrimp wastes

Extraction step / Sample	Yield (%)*		
	Demineralization and deproteinization	**Decolorization**	**Deacetylation**
Ch	15.8 ± 0.25^{d}	11.8 ± 0.43^{e}	8.44 ±0.32^{a}
B	19.7 ± 0.31^{c}	18.7 ± 0.17^{a}	6.30 ± 0.41^{c}
L	21.9 ± 0.22^{a}	16.4 ± 0.33^{b}	6.64 ± 0.23^{b}
B/L	20.2 ± 0.41^{b}	15.3 ± 0.25^{d}	6.36 ± 0.45^{bc}
L/B	21.9 ± 0.29^{a}	15.8 ± 0.14^{c}	3.59 ± 0.35^{d}

Data are presented as mean ± SD, *n*=3.
Different superscript letters (a, b, c, d, e) following values in the same column indicate significant differences ($P \leq 0.05$).
* On a dry weight basis.

The yields of both the demineralization and the deproteinization steps after the single-step fermentation (B or L) or the successive co-fermentation (B/L or L/B) were nearly close and were higher than the yield resulting from the chemical extraction method. **Ahmed *et al*. (2021)** successfully produced chemically 57.88gm dry chitin from 275gm dry shrimp shells with ratio of 21.05%. **Tan *et al*. (2020)** found that, a single- step fermentation of shrimp wastes using *Lactobacillus acidophilus* FTDC3871 produced a high percentage of demineralization (90.8%) and deproteinization (76%) of chitin after 3 days. Another study **(Zhang *et al*., 2022)** used the same strains (*B. subtilis* and *L. plantarum*) applied in the current study for a one-step successive co-fermentation of shrimp shells. They assessed that the final percentage of deproteinization and demineralization were 94.1% and 96.3%, respectively.

The highest yields of chitosan after the deacetylation step were obtained by using the chemical process (8.44±0.32%) followed by samples treated biologically with L, B/L, and B (6.64±0.23%, 6.36 ± 0.45%, and 6.30±0.41%), respectively, nevertheless, there was no statistical difference between the yields of B/L and B. However, the yield (3.59±0.35%) produced after the deacetylation of the (L/B) extracted sample was significantly lower than all other yields produced by the chemical and other biological methods (B, L, or B/L). The use of bacteria as a biological method for extraction may cause reductions in particle size which leads to a reduction in the yields after the deacetylation step.

Generally, chitosan yields in this research were relatively low, mainly when the biological method was used to extract chitosan. This might be attributed to the depolymerization of the chitosan polymer, loss of sample mass/weight from excessive removal of acetyl groups from the polymer during deacetylation and loss of chitosan particles during washing.

3.2. Proximate composition of shrimp wastes and chitosan

The chemical composition of the dried shrimp wastes and the chitosan extracted by chemical and biological methods are shown in Table (2). Dried shrimp wastes contained 10.13%, 8.6%, 36.51%, and 6.65% from moisture, total nitrogen, ash, and lipid, respectively. Ibrahim *et al.* (2019) reported relatively close results; the wastes of shrimp purchased from Al-Fayoum, Egypt contained 5.28% moisture, 7.09% total nitrogen (TN), 36.15% ash, and 7.77% lipid. The values of the proximate composition of the chitosan extracted either chemically or biologically by bacteria (B, L, B/L or L/b) were significantly ($P \leq 0.05$) reduced compared to the dried shrimp wastes. The moisture, TN, ash, and lipid contents ranged from 5.70-7.00%, 5.72-6.72.0%, 2.83-7.75%, and 0.0%, respectively. Moreover, the biological treatment B recorded the lowest effect on ash and total nitrogen reductions. Notably, the proximate composition of chitosan differs with the type of raw material, extraction method, and temperature used (**Ghorbel-Bellaaj *et al.*, 2012**). The better the quality of chitin and chitosan, the lower the ash and moisture contents (**Ilyas *et al.*, 2021**).

Table 2. Mean values of moisture, total nitrogen, ash, and lipid of shrimp wastes (Sh) and chitosan extracted by chemical (Ch) and biological (B, L; B/L, L/B) processes

Parameter (%)	Shrimp waste (Sh)	Chitosan				
		Ch	B	L	B/L	L/B
Moisture	10.13±0.24^{a}	6.40±0.03^{c}	6.30±0.08^{c}	7.00±0.06^{b}	6.99±0.22^{b}	5.70±0.14^{d}
	On a dry basis*					
Total nitrogen (TN)	8.60± 0.33^{a}	5.72±0.13^{c}	6.27±0.26^{b}	5.72±0.41^{c}	5.82±0.34^{c}	5.96±0.27^{c}
Ash	36.51±0.51^{a}	2.83±0.14^{d}	7.75±0.43^{b}	3.99±0.31^{c}	3.80±0.59^{c}	3.82±0.43^{c}
Lipid	6.65± 0.25^{a}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}

Data are presented as mean ± SD, n=3.

Different superscript letters (a, b, c, d) following values in the same raw indicate significant differences ($P \leq 0.05$).

* On a dry weight basis.

3.3. Physicochemical properties of shrimp chitosan

3.3.1. Solubility

Solubility is one of the characteristics used to determine the quality of chitosan. **Rinaudo *et al.* (2006)** reported that, chitosan is soluble in aqueous solutions of organic acids with pH< 6 but insoluble in water, aqueous bases, and organic solvents. As shown in Table (3), the chitosan samples extracted chemically had a high solubility value (81.63%) in 1% acetic acid. However, all the biologically extracted samples had low values ranging from 50% to 57.5%. These low solubility values may be due to the formation of organic materials during the fermentation process, which may cause impeding dissolution in acetic acid. According to **Hossain and Iqbal (2014)**, the alkaline concentration, temperature, time of reaction, the product's size and the rate of deacetylation affect the solubility of chitosan.

3.3.2. Degree of deacetylation (DDA)

The DDA process refers to the degree of removing acetyl groups from the molecular chain of chitin and the replacement of amino groups (NH_2). **Shirvan *et al.* (2019)** stated that, the reactive amino groups within the structure of chitosan influence its applications. The production of a DDA > 50% confirms the nature of the resulting product that it is chitosan and becomes soluble in acidic aqueous solution (**Younes *et al.*, 2014**). For chitosan to be applicable, the DDA should be more than 70% (**Rasweefali *et al.*, 2021**). The DDA is an essential factor affecting the antibacterial and many other applicable properties (**Kumari *et al.*, 2017; Varun *et al.*, 2017**). The DDA of chemically and biologically extracted chitosan obtained from the FTIR analysis had close proportions ranging from 79.5- 80.35% (Table 3). **Rasweefali *et al.* (2021)** reported that the DDA of commercial chitosan varied from 70 to 85%. The DDA of chitosan is affected by the chitin extraction method, raw material species and sources, alkaline concentration, reaction time and temperature (**El Knidri *et al.*, 2018; Shirvan *et al.*, 2019**).

Table 3. Physicochemical characteristics of chitosan samples extracted from shrimp wastes using chemical (Ch) and biological (B, L; B/L, L/B) methods

Property	Chitosan				
	Ch	**B**	**L**	**B/L**	**L/B**
pH	4.8±0.1	4.4±0.2	4.3± 0.2	4.6±0.1	4.5±0.2
Solubility (%)	81.63 ± 0.14^{a}	50.0 ± 0.71^{d}	53.47 ± 0.23^{c}	57.50 ± 0.42^{b}	50.67 ± 0.54^{d}
DDA (%)	80.35±0.31	79.79±0.53	80.17±0.33	80.17±0.25	79.50±0.71
I_{am}	156	132	135.9	123	146
I_{20}	621.9	470	526	551.9	336.6
CrI (%)	74.9	71.0	74.1	77.7	56.6
Intrinsic viscosity (dl/g)	0.1903±0.02	0.0779±0.01	0.0847±0.01	0.0943±0.01	0.0909±0.01
Mw (kDa)	94.34 ± 0.57^{a}	26.37 ± 0.43^{e}	30.17 ± 0.52^{d}	35.09 ± 0.76^{b}	33.32 ± 0.64^{c}

Data are presented as mean ± SD, *n*=3.

Different superscript letters (a, b, c, d) following values in the same raw indicate significant differences ($P \leq 0.05$).

N.B: I_{am} : The intensity of amorphous and had been identified as $I_{16,}$ I_{020} : The maximum intensity, CrI : The Crystallinity Index value, DDA: Degree of deacetylation, and Mw: Molecular weight.

3.3.3. Viscosity and molecular weight

Table (3) demonstrates the intrinsic viscosity of chemically (Ch) and biologically (B, L, B/L, and L/B) extracted chitosan samples. The intrinsic viscosity values of chitosan samples extracted biologically were slightly lower than those of the chemically extracted samples. The intrinsic viscosity values of chitosan samples extracted by Ch, B, L, B/L or L/B, were 0.1903, 0.0779, 0.0847, 0.0943 and 0.0909 dl/g, respectively. **Shirvan *et al.* (2019)** reported that, the viscosity of chitosan influences its efficiency as an antimicrobial additive. The biodegradation of chitosan molecules and the hydrolysis of polymer molecules in solutions cause a reduction in the viscosity of chitosan (**Chattopadhyay & Inamdar, 2010**). **Aranaz *et al.* (2021)** stated that, the primary constraint, especially in the food industries, is the high viscosity in solutions as they are difficult to manage.

The viscosity of the chitosan is affected by its molecular weight and the degree of deacetylation, a lower molecular weight results in lower viscosity. The molecular weight (Mw) of chitosan was determined to ensure its quality and applications (Table 3). The molecular weights of chitosan samples extracted by Ch, B, L, B/L, and L/B were 94.34, 26.37, 30.17, 35.09, and 33.32 kDa, respectively. All chitosan samples in this study were classified in the low molecular weight class. **Santoso *et al.* (2020)** grouped chitosan into three groups, high molecular weight (≥1000 kDa), medium molecular weight (100: 1000 kDa), and low molecular weight (≤100 kDa). The biologically extracted chitosan samples (B, L, B/L, and L/B) had lower molecular weights (<50 kDa) in comparison to the Mw of commercial chitosan (50-2000 kDa) (**Mourya *et al.*, 2011**).

Kumari *et al.* (2017) found that chitosan extracted from shrimp wastes (*Crangon crangon*) with a 78% DDA exhibited a Mw of 6.27 kDa. However, **Kucukgulmez *et al.* (2011)** reported that chitosan from shrimp (*Metapenaeus stebbingi*) shells produced after the deacetylation (50% NaOH at 90˚C for 2 h) a low Mw (2.20 kDa) with 92.19% DDA.

The Mw values of chitosan samples are affected by many factors like the source of raw material, extraction process, concentration of alkali used during the deacetylation, insoluble matters in the solution, DDA, and particle size (**Younes *et al.*, 2014; Kucukgulmez *et al.*, 2011; Yen *et al.*, 2009**). All chitosan samples in the present study had low Mw, pH ranging between 4.3 to 4.8, and slightly higher DDA; these mean that they possess large positive charges. According to **Chung *et al*. (2004) and Chung (2006)** these samples may have good antibacterial properties.

3.3.4. The Fourier Transform Infrared Spectroscopy (FTIR) spectrum of chitosan

The FTIR analysis of chitosan samples (Ch, B, L, B/L, and L/B) depended on identifying bands and their vibrations (**Pawlak and Mucha, 2003**). The bands' wave numbers in Fig. 2 are illustrated in Table 4. The FTIR spectrum of chitosan samples revealed the symmetric stretching vibration of hydrogen-bonded OH and amines (NH_2) bands ranging from 3416 to 3434 cm^{-1}, amino peak bands ranging from 3096- 3360 cm^{-1}, and bands ranging 2852- 2921 cm^{-1} represents the C-H groups stretching. All the chitosan samples had no bands in the 1900- 1660 cm^{-1} range, which means the absence of (-C-O) containing carbonyl and carboxyl groups, which agrees with the results reported by **Shavandi *et al*. (2015)**.

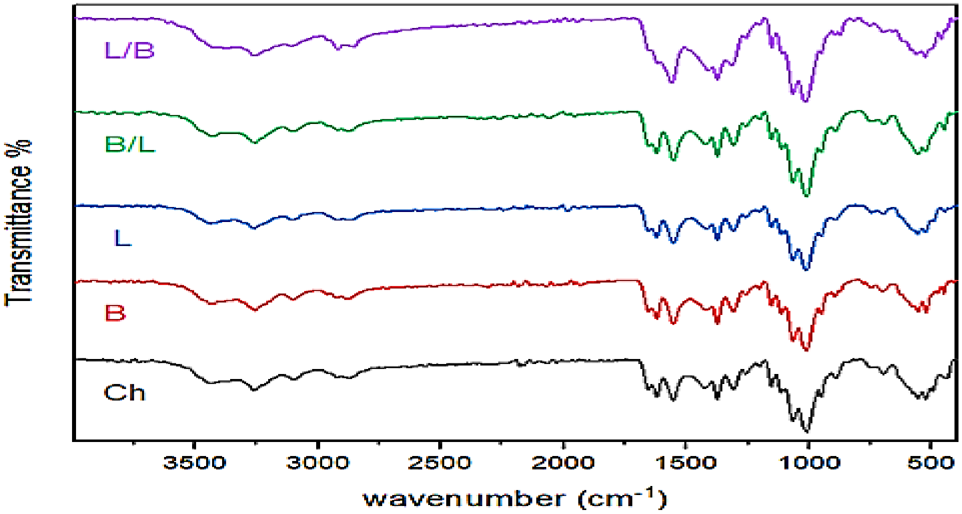

Figure 2. FTIR spectra of chitosan extracted from shrimp wastes by chemical (Ch) and biological (B, L, B/L, and L/B) methods.

In the spectrum of chitosan samples, special absorption features were obtained at 1654 (Ch), 1654 (B), 1656 (L), 1655 (B/L), and 1655 cm^{-1} (L/B) correspond to the bending vibration of (NH of R-NH_2) primary amine groups (amide I) (Fig. 2). **Abdel-Rahman *et al*. (2015)** and **Radhakumary *et al*. (2003)** indicated that the presence of amide I confirms the occurrence of deacetylation of chitin. NH bending (amide Π) group was observed in all chitosan samples in the region between 1548- 1622 cm^{-1}. The bands of CH_2 bending and CH_2 deformation were revealed at 1420 cm^{-1} (Ch), 1418 cm^{-1} (B), 1419 cm^{-1} (L), 1421 cm^{-1} (B/L), and 1415 cm^{-1} (L/B). In an amide III band, the C–N stretch band appeared around 1374 cm^{-1}, and the C-H group in the pyranose ring peaked at 1306- 1310 cm^{-1} in all chitosan samples. Peaks in the 1200- 1259 cm^{-1} range appeared in all chitosan samples extracted biologically (B, L, B/L, and L/B) except chemically extracted chitosan (Ch), confirming C-N stretching.

Table 4. Assignments of the relevant bands of FTIR spectra (cm^{-1}) of chitosan extracted from shrimp wastes after deacetylation.

Functional group and vibration modes	Ch	B	L	B/L	L/B
Number of active groups	19	24	23	23	21
-NH_2 and -OH groups stretching vibration and intermolecular hydrogen bonding.	3434	3416.24	3424.59	3430.29	3432.03
Amino peak	3277.24	3257.09 3096.53	3262.18 3100.27	3358.73 3273.84	3251.90 3102.10
CH_2 stretch in CH_2OH group	2912.60 2869.46	2914.62 2868.19	2920.63 2854.11	2917.38 2856.03	2919.45 2852.38
-NH_2 deformation of primary amines (Amide I)	1654.29	1653.88	1656.21	1654.92	1655.08
NH bending (Amide Π)	1622.17 1548.35	1622.14 1550.63	1621.48 1548.97	1621.65 1550.62	1620.65 1550.77
CH_2 bending and CH_2 deformation	1420.12	1418.29	1418.89	1421.46	1415.07
CH_3 stretch in $NHCOH_3$ group	1374.02	1373.68	1374.14	1374.13	1373.56
C-H in pyranose ring	1310.24	1310.83	1306.02	1307.65	1307.55
Complex vibrations of NHCO group (Amide III band)	-	1257.56 12.04.05	1258.63 1200.52	1257.54 1203.68	1257.61
Asymmetric bridge oxygen stretching	1153.40	1154.62	115488	1154.64	1154.92
C-O-C glycosidic linkage	1065.01	1071.97	1069.54	1070.97	1071.51
C-O in primary OH group	1021.32	1007.45	1007.15	1011.20	1011.51
Pyranose ring skeletal vibration	890.81	952.75 894.75 874.61	951.41 893.7	893.71	952.46 874.28
Amide VI	733.56	779.39 705.86	745.21 706.33	739.84 707.25	737.79 710.62
C-X group, where X is a halogen	573.44 554.94 527.59 441.39	575.18 553.39 522.74	574.71 571.47 501.10	572.67 531.84 476.92 445.39	578.43 573.03

The bands of asymmetric bridge oxygen (C-O-C) stretching were produced because the deacetylation was noted in chitosan samples in the 1153- 1155 cm^{-1} range. Asymmetric stretching of the C-O-C bridge and C-O appeared at 1065- 1072 cm^{-1} and 1007- 1021 cm^{-1}, respectively, and according to **Abdel-Rahman *et al.* (2015)** these groups characterize chitosan. Absorbance bands between 890 cm^{-1} and 952 cm^{-1} illustrated the presence of the pyranose ring and saccharide structure of chitosan (**Khan *et al.*, 2013; Islam *et al.*, 2014**). As mentioned by Mohammed et al., those bands 1798, 1430 and 876 cm^{-1} are related to the presence of mineral (CaCO3), while the band 1540 cm^{-1} reflected the presence of protein, and all these bands should not appear in the spectrum of chitosan extracted by any methods (**Mohammed *et al.*, 2013**). Peaks that appeared under 800 cm^{-1} may be related to the groups that hinder the chitosan solubility, especially those that are extracted biologically (B, L, B/L, and L/B).

3.3.5. X-ray Diffractometry Analysis (XRD)

The comparison of the XRD patterns among shrimp wastes (Sh) and all chitosan samples extracted chemically (Ch) and biologically (B, L, B/L, L/B) are shown in Fig. 3.

The analogy of the XRD patterns of the shrimp shells and the extracted chitosan samples indicates the characteristics of the crystal. Amorphous crystal characteristics of the pattern are relatively flat. All the samples showed four peaks at about 9.3° (020), 19.5° (110), 20.8° (120), and 26.3° (130) that corresponded to the six typical crystalline planes, while original shrimp wastes powder revealed only a peak for calcite at 28°.

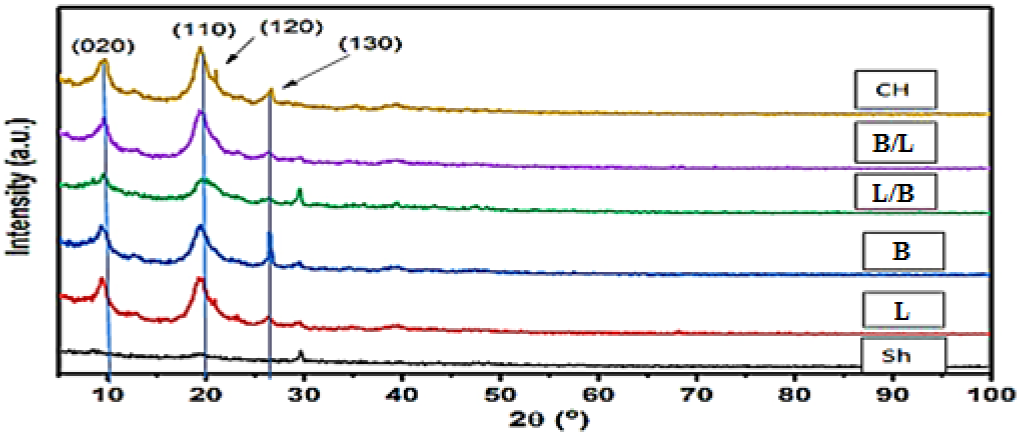

Figure 3. XRD patterns of shrimp shells (Sh) and chitosan extracted by chemical (Ch) and biological (B, L, B/L, and L/B) process.

The results of crystallinity index values (CrI) of chitosan samples are shown in Table 3. The highest CrI value was demonstrated by the chitosan samples extracted biologically by the co-culture bacteria (B/L) (77.7%), followed by (Ch) (74.9%), (L) (74.1%), and (B) (71.0%), respectively. On the other hand, the lowest CrI value was revealed by the chitosan samples extracted by the co-culture bacteria (L/B) (56.6%). **Gbenebor *et al*. (2017)** explained that the CrI is a vital property affecting chitosan's physical and biological properties, including biodegradability. The structural stability of the chain that formed after fermentation with co-culture bacteria (L/B) is more vulnerable to deacetylation and declines as N-acetyl groups are gradually removed, eventually reducing crystallinity. The steady decrease in Crl is a result of crystal structural distortion caused by increasing reagent concentrations (50% NaOH) that allow the breaking of intra and intermolecular hydrogen bonds (**Gbenebor *et al*., 2017**). Similar results of CrI values were obtained from the previous studies on chitosan extracted from shrimp wastes by different methods. **Rasweefali *et al*. (2021)** reported that the CrI value of commercial chitosan was 64.1%. Also, **Kumari *et al*. (2017)** found a higher CrI value (82%) for chitosan extracted from shrimp wastes when chitin was deacetylated for up to 6 h. However, **Ma *et al*. (2015)** found that the CrI value was 46% after 7 h of deacetylation time. Many factors affect the CrI of chitosan, such as the resource of species used to extract chitin, conditions adapted during chitin conversation to chitosan and polymorphism (**Abdel-Rahman *et al*., 2015; Noishiki *et al*., 2003; Baskar and Kumar, 2009**).

3.3.6. Field Emission scanning electron microscope (FE-SEM)

The surface morphology of all chitosan samples extracted chemically (Ch) and biologically (B, L, B/L, and L/B) using the FE-SEM at 1000x magnification are illustrated in Fig. 4. The morphology structure of the chitosan sample (Ch) showed a long, smooth and porous fibrillar structure. The surface of sample (B) showed lamellar organization but had much crystal, while sample (L) had a smooth and agglomerated fibrillar structure. The morphology structure of samples (B/L and L/B) revealed some of the lamellar particles on the surface, which gave that organization a contrast of dark lamellar straps and a contraction in the surface.

Kucukgulmez *et al*. (2011) examined the surface morphology of chitosan extracted from shrimp wastes at different magnifications and observed porous, crumbling flakes with a fibrillar structure. Also, **Marei *et al*. (2016)** indicated that the surface morphologies of shrimp's chitosan were to some extent dense, smooth, long, and wide regular nanofibers without porous surface structure. **Yen and Mau (2007)** attributed the differences in the morphology structure to their different intra-sheet/ inter-sheet or hydrogen-bonding systems.

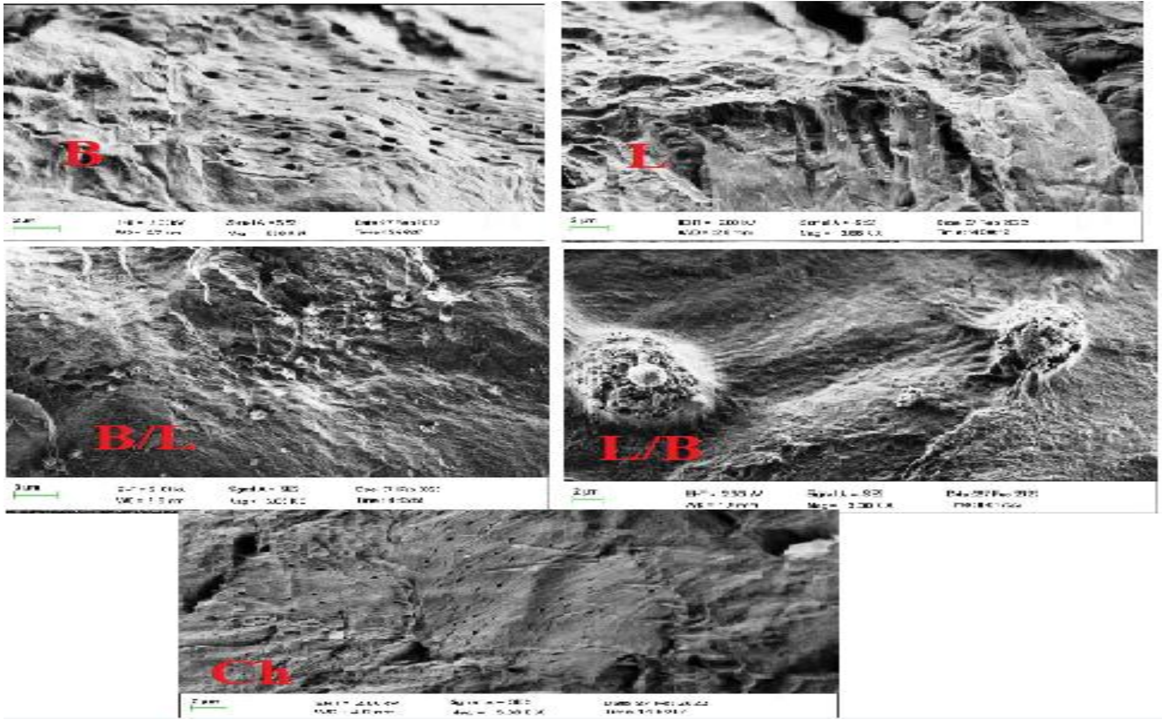

Figure 4. FE-SEM micrograph of chitosan extracted from shrimp wastes by chemical (Ch) and biological (B, L, B/L, and L/B) methods.

3.4. The *in vitro* antibacterial activity of chitosan

Fig. 5 shows photographs of the antibacterial activity of chitosan (0.5 g chitosan/ 100 ml acetic acid solution (1%)) against the Gram-positive (*Bacillus cereus*) and Gram-negative (*Salmonella* sp. and *vibrio* sp.) bacteria using the disc diffusion assay. As shown in Fig. 6, the presence of inhibition zones (mm) produced by any of the chitosan samples (Ch, B, L, B/L, and L/B) against the tested foodborne bacteria indicates a good antibacterial potential of all the tested samples. Chitosan sample (B) exhibited the highest antimicrobial activity with inhibition zone diameters of 20±0.2 mm, 14±0.5 mm, and 17±0.6 mm against *B. cereus*, *Salmonella* sp., and *vibrio* sp., respectively. All the biologically extracted chitosan samples (B, L, B/L, and L/B) had higher values of inhibition zone diameter (20±0.2, 20±0.8, 18±1.0, 20±0.4 mm) against *B. cereus* in comparison to the chemically extracted chitosan (Ch) (17±0.7 mm). Chitosan samples extracted by B, B/L, and L, respectively, had higher values of inhibition zone diameters against *Vibrio* sp. than Ch and L/B extracted samples. All the tested chitosan samples exhibited the minimum inhibition activity against *Salmonella* sp., especially L/B extracted chitosan sample.

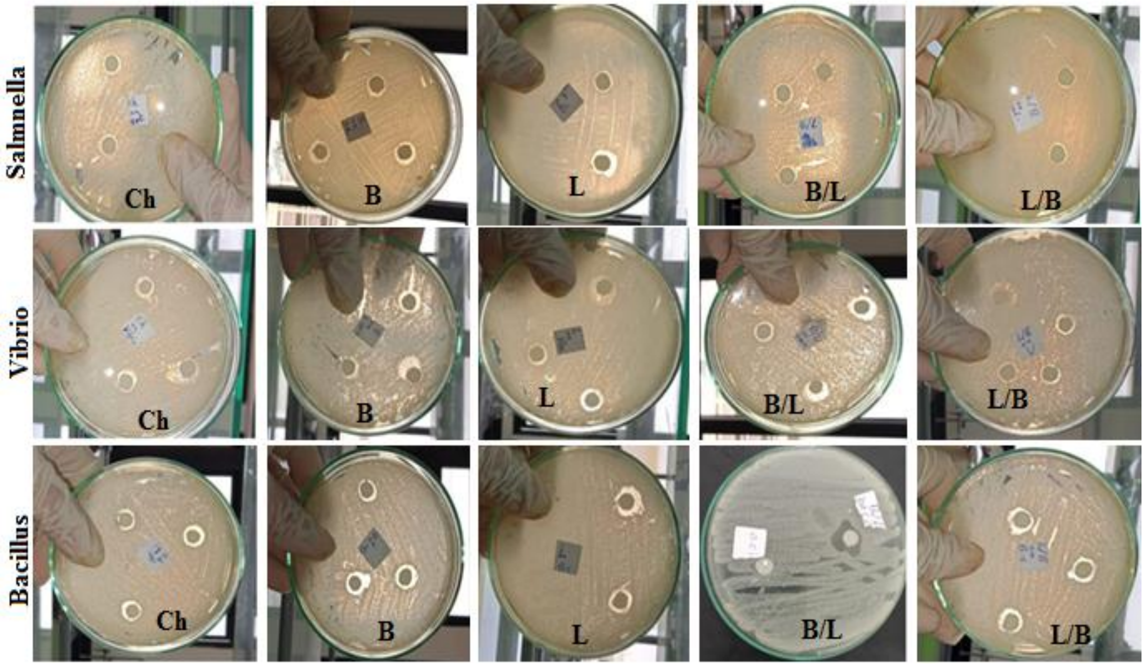

Figure 5. Antibacterial effects of chitosan extracted from shrimp wastes by chemical (Ch) and biological (B, L, B/L, and L/B) techniques against *Salmonella* sp., *Vibrio* sp., *and Bacillus cereus.*

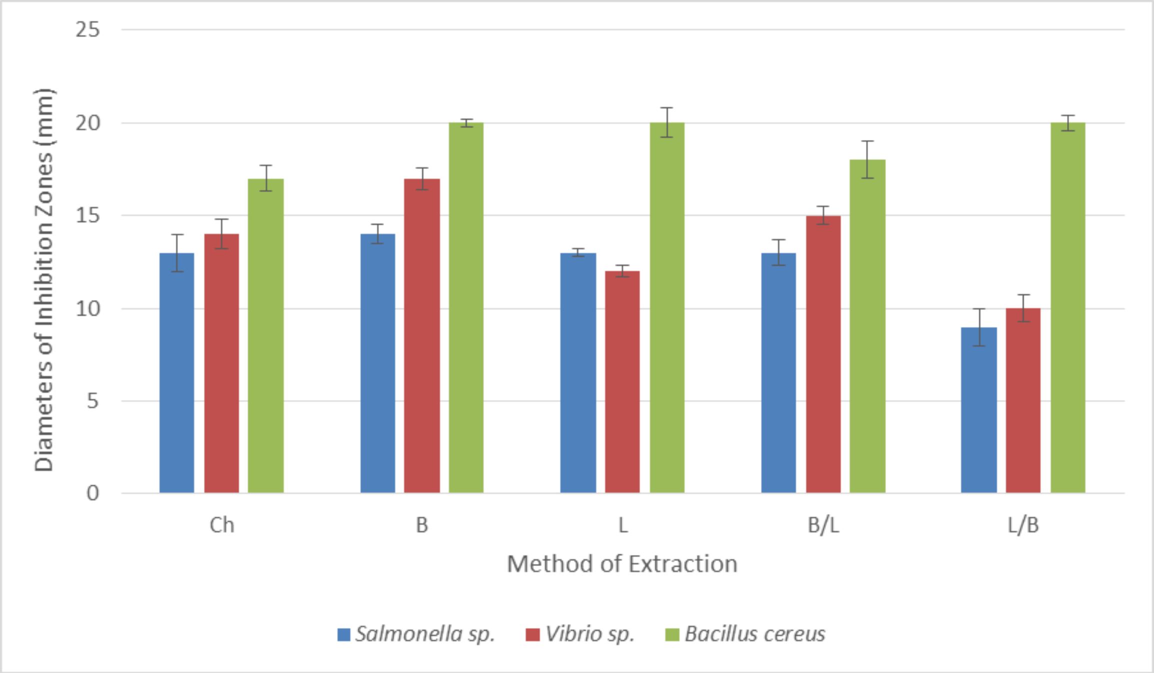

Figure 6. Effect of the chitosan samples against the tested bacteria expressed as zone inhibition in mm diameter.

Results of *B. cereus* demonstrated that it is more susceptible to all chitosan samples used in this study, which is consistent with the results of **Raafat and sahl (2009)**. Earlier studies indicated higher antibacterial activity of chitosan against Gram-positive bacteria than Gram-negative bacteria, possibly due to the differences in the cell surface structure (**Sudatta *et al.*, 2020; Chung *et al.*, 2004; No *et al.*, 2002**). Chitosan's antibacterial activity varies according to its physicochemical properties and the type of bacterium. Furthermore, the chitosan's antibacterial properties are pH-dependent (**Ke *et al.*, 2021**). The low pH causes chitosan to have more positive charges, which strengthens its antibacterial capabilities (**Varlamov and Mysyakina, 2018**). The chitosan's structure and its molecular weight (MW) dictate whether it has extracellular, intracellular, or both extracellular and intracellular antibacterial action (**Ke *et al.*, 2021**).

CONCLUSION

The present study demonstrates that the biological extraction method of chitosan from shrimp wastes is an excellent alternative to chemical extraction. As one of the recent trends, the biological extraction method used in this study was performed using two techniques. The first technique was single-step fermentation using either *B. subtilis* subsp. DSM 1088 or *L. plantarum* DSM 20174 ATCC 14917, while the second technique was the successive co-fermentation of the two previous strains alternatively. Using the biological method will solve the environmental and economic problems of shrimp wastes in a friendly way and will result in low-cost chitosan.

The difference in structure and surface morphology of chitosan extracted chemically and biologically was illustrated using XRD, FTIR and SEM. The FTIR confirmed the occurrence of chitin deacetylation. XRD analyzed the crystallinity index values of the extracted chitosan samples. Although all the biologically extracted shrimp wastes resulted in low yields (3.59% - 6.64%) with low solubility (50.0- 57.5%), they possessed a low Mw (26.7- 35.09 kDa), besides good values of DDA (79.5- 80.17%) and CrI (71.0- 77.7%). These physicochemical characteristics augmented the antibacterial potency of the biologically extracted chitosan at a concentration of 5 mg/ml against foodborne Gram-positive (*B. cereus*) and Gram-negative (*Vibrio sp*. and *Salmonella sp*.) bacteria. Thus, biologically extracted chitosan can be applied as a biopreservative in food which in turn help in dealing with problems that result from chemical preservatives. Further investigations are needed to study the biological extraction methods under different conditions, besides evaluating the antibacterial effect of the biologically extracted chitosan samples against other pathogens.

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