Malays. Appl. Biol. (2019) 48(5): 39–48

COMPARATIVE STUDIES ON PHYSICOCHEMICAL CHARACTERISATION, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF CHITOSAN EXTRACTED FROM *Scylla paramamosain* AND *Penaeus monodon* SHELLS

WAN ROSLINA WAN YUSOF[1*], MUHD AMIR ASYRAF NOH[2], NOR AIMUNI ABD AZIZ[2], NOORASMIN MOKHTAR AHMAD[2] and AWANG AHMAD SALLEHIN AWANG HUSAINI[2]

[1]*Centre for Pre-University Studies, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia*
[2]*Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia*
[*]*E-mail: wywroslina@unimas.my*

Accepted 20 October 2019, Published online 31 December 2019

ABSTRACT

Marine shells are commonly discarded as waste. However, they could be used to extract a multi versatile polymer, chitosan. As one of the marine by-products, chitosan can be found in the exoskeleton of crustaceans via the deacetylation process of chitin. This project aimed to investigate and compare the physicochemical and biological properties of chitosan extracted from two marine organisms because the properties depend upon the chitin source. In brief, chitosan was extracted through chemical processes from mud crab, *Scylla paramamosain* and prawn, *Penaeus monodon* shells. The percentage yield, moisture, solubility, water binding capacity and fat binding capacity of the extracted chitosan were determined. The degree of deacetylation and SEM images of the extracted chitosan were obtained. The antioxidant and antibacterial properties in both chitosan were evaluated. The results showed that chitosan from *S. paramamosain* shells has a higher percentage yield, moisture content, water binding capacity, fat binding capacity and degree of deacetylation compared to chitosan from *P. monodon* shells. In antioxidant assays, chitosan from *S. paramamosain* shells showed higher scavenging activity (22.2%) than chitosan from *P. monodon* shells (6.7%). In disk diffusion assay, chitosan from *S. paramamosain* shells displayed antibacterial activity against *E. coli* and *S. aureus*, while chitosan from *P. monodon* shells showed no activity. Thus, the study showed that *S. paramamosain* shells could be used as a starting material to produce valuable chitosan with high potential of its biological activities.

Key words: Chitosan, marine by-products, mud crab, prawn, antioxidants, antibacterial

INTRODUCTION

Recently, there has been increasing interest regarding chitosan-based materials due to its natural characteristic and multifunctional polysaccharide (Romainor *et al*., 2014; Cheung *et al*., 2015). This biodegradable copolymer is a weak base and non-toxic material. It consists of repeating units of D-glucosamine (GlcN) and N-acetyl-D-glucosamine (GlcNAc). The ratio of GlcN and GlcNAc determined the percentage of deacetylation (DD) for chitosan, which can be analysed via infra-red (IR) spectroscopy, potentiometric titration, nuclear magnetic resonance (NMR) spectroscopy and X-Ray diffraction pattern analysis (Lago *et al*., 2011). Interestingly, chitosan is available in wide range of DD and molecular weight. Based on the literature, the lowest DD values ranges between 40 to 60% and the highest ranges from 85 to 98% (Hussain *et al*., 2014). The presence of primary amino groups making it displays a cationic character. Due to this unique property, this natural copolymer has been applied in the development of bioactive materials such as in food packaging (Lago *et al*., 2011), in drug delivery systems (Bernkop-Schnürch & Dünnhaupt, 2012) and in wastewater treatment (Vidal & Moraes, 2018). Chitosan acts as an antibacterial agent due to interaction between cationic NH_3^+ group in chitosan with a negative charge of bacterial membrane (Li *et al*., 2015). This

* To whom correspondence should be addressed.

versatile copolymer can be obtained from many resources such as fungi, marine shells and plants via deacetylation process of chitin using 30% to 60% of NaOH (Hossain & Iqbal, 2014; Paul *et al*., 2014; Sarbon *et al*., 2014). Moreover, chitosan from different sources exhibit different bioactivities (Chien *et al*., 2016). Sarbon *et al*. (2014) reported that chitosan from other species of mud crabs, *Scylla olivacea* at 10 mg/mL has a reducing power ability which due to the presence of amine group. On another note, chitosan from blue crab, *Callinectes sapidus,* showed a moderate antibacterial activity against *S. aureus* and *E. coli* (Metin *et al*., 2019). Chitosan from prawn, *Penaeus monodon* with a concentration of 10 µg mL^{-1} was reported to exhibit cytotoxic activity against ovarian cancer cell line, PA-1, compared to its chitin, showing that chitosan has potential as a natural anti-cancer agent (Srinivasan *et al*., 2018). In another study done by Kae *et al*. (2019) stated that chitosan extracted from fungi, *Auricularia* sp. displayed a greater antibacterial activity against *S. aureus* and *E. coli*. Shells of crabs and prawns are natural resources that enriched with chitosan. Since marine shells are only dumped in landfills and under-utilised, a recovery process of the shells is required to obtain a valuable product such as chitosan. Recovery of chitosan from crustacean's shell is a sustainable way, beneficial to the environment as well as eco-friendly method since the shells are plenty and inexpensive (Ongkiko *et al*., 2018). Moreover, the importance and awareness of green chemistry have led many researches on the extraction of chitosan from marine shells (Dhillon *et al*., 2013). Furthermore, the chitosan obtained can be used as a health-promoting material or any potential agent in diverse fields such as in food, medical and pharmaceutical industries. Therefore, the purpose of this study was to reveal and compare the biological activities of the chitosan from the shells of mud crab, *Scylla paramamosain* and prawn, *Penaeus monodon*. The physicochemical characteristics and biological properties were evaluated to achieve the objectives of this study.

MATERIALS AND METHODS

Materials

Mud crab, *S. paramamosain* and prawn, *P. monodon* shells were collected from restaurant. Culture of *E. coli* (ATCC 25922), *S. aureus* (ATCC 29213) and agar were purchased from ATCC (Malaysia). The medium molecular weight of commercial chitosan and 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl, DPPH, were purchased from Sigma Aldrich (Malaysia). Sodium hydroxide and acetic acid were purchased from Merck (Malaysia). All chemical reagents in this study were of analytical grade.

Sample preparation

The preparation of samples was conducted based on Sarbon *et al*. (2014) with slight modification. Exoskeletons (shell) of mud crab, *Scylla paramamosain* and prawn, *Penaeus monodon* were removed separately and washed properly with distilled water to remove the extraneous matter. The shells were put in an oven at 70°C for a day until dried. The dried shells were grounded using a mortar and sieved into a smaller size of about 0.5 to 5.0 mm.

Extraction of chitin by chemical method

Deproteinization

The process was done based on Kumari *et al*. (2017) with slight modification. Three percent of NaOH was used to treat the shells for 30 min at temperature. Then, NaOH was drained completely from the shells and washed with distilled water until the pH is neutral. This process is needed to remove the protein in shells. Later, the shells were dried in an oven for one day and crushed further into small pieces.

Demineralization

The demineralization process was done based on Kumari *et al*. (2017) with slight modification. This step is crucial to yield chitin. Removal of minerals in the deproteinised shells was conducted using 3% of HCl for 6 hr at 25°C, with a ratio of solid to a solution was 1:20. Removal of excess HCl in the treated samples was done by filtering and washing with distilled water until neutrality was achieved. The demineralized samples were dried in an oven at 60°C for 1 day.

Decolouration

Samples were treated in acetone for 10 min for discolouration based on Sarbon *et al*. (2014). Then, the samples were dried for 2 hr at 25°C. The residues were removed. The decolourized shells were washed and dried at 60°C for 1 day in an oven to obtain the desired chitin.

Deacetylation of chitin

The deacetylation of chitin was done according to the method of Yen *et al*. (2009) with slight modification. The chitin obtained was treated with 50% NaOH at 105°C for 2 hr with a ratio of solid chitin to a solution of 1:15. Later, the chitin was filtered and washed with distilled water until its neutral pH to obtain chitosan. The chitosan obtained was finally dried at 80°C for 24 hr in an oven.

Physicochemical properties

Percentage yield and moisture content

Percentage yield and moisture content were conducted based on the method of Analysis for Nutritional Labelling, AOAC (Sullivan & Carpenter, 1993).

Solubility

Solubility was tested according to Kumari *et al*. (2017) with a slight modification. The solubility of chitosan was determined by dissolving 0.1 g chitosan in 10 mL of 1% of acetic acid. The mixture was stirred for 30 min at room temperature and centrifuged at 10 000 r.p.m. for 10 min. The supernatant was discarded and the undissolved solid was washed and centrifuged for 10 000 r.p.m. for 10 min. After drying the solid in an oven at 60°C for 1 day, it was weighed and the percentage of the chitosan solubility was determined according to the equation:

$$\frac{(\text{Initial weight of tube + chitosan}) - (\text{Final weight of tube + chitosan})}{(\text{Initial weight of tube + chitosan}) - (\text{Initial weight of tube})} \times 100$$

Water binding capacity, WBC

Water binding capacity of chitosan was determined according to method by Wang and Kinsella (1976). Approximately, 0.5 g chitosan was placed in a centrifuge tube and then weighed. 10 mL of water was added into the tube and vortexed for 1 min. The mixture was incubated for 30 min at 25°C and shaken for 5 s in every 10 min. The supernatant was discarded after centrifugation of samples at 3500 r.p.m. for 25 min. The tube was weighed again. The percentage of WBC was obtained using the formula:

$$\frac{(\text{water bound in gram})}{(\text{Initial sample weight in gram})} \times 100$$

Fat binding capacity, FBC

Fat binding capacity of chitosan was determined according to the method by Wang and Kinsella (1976). A sample of 0.5 g chitosan was placed in a centrifuge tube and weighed. Ten mL of soy oil was mixed using a vortex mixer for 1 min so that the sample was dispersed. The sample was incubated at 25°C for 30 min and shaken for 5 s in every 10 min. The supernatant was discarded after centrifugation of samples at 3500 r.p.m. for 25 min. Finally, the tube was weighed and the percentage of FBC was calculated using the formula:

$$\frac{(\text{fat bound in gram})}{(\text{Initial sample weight in gram})} \times 100$$

Characterisation of the extracted chitosan

Degree of deacetylation, DD

DD of the extracted chitosan was confirmed by FTIR spectroscopy according to the method of Hajji *et al*. (2014). This technique is a quick method in DD analysis. The absorption ratios were obtained and calculated to evaluate the percentage of DD. To get DD, the degree of acetylation, DA, must be obtained through in determination of the ratio AM/AR, where AM is the intensity of probe band which measures of N-acetyl or amine content and AR is the intensity of a reference band, that does not change with the DA. Infra-red spectra were recorded in the range of 1200–4000 cm^{-1}. A thin film was prepared from a mixture of 150 mg potassium bromide and 10 mg chitosan. Percentage of DD was calculated using the formula:

$$\text{Percentage of degree of deacetylation, \% DD} = 100 - \left[\left(\frac{Abs\ 1655}{Abs\ 3450}\right) \times \frac{100}{1.33}\right]$$

where, absorbance at 1655 cm^{-1} is the absorbance of amide I band while the absorbance at 3450 cm^{-1} is the O-H stretching band. The ratio of A1655/A3450 is equal to 1.33 for fully N-acetylated chitin and zero for fully de-acetylated chitosan.

Morphology and particle sizes

The sample was characterized by particle sizes and morphology using a Scanning Electron Microscope (SEM) under different magnifications.

Biological properties of extracted chitosan

DPPH scavenging activity

The DPPH radical scavenging activity of the extracted chitosan was evaluated by DPPH scavenging assay according to Shen *et al*. (2010) with a slight modification. Initially, 1 mL of chitosan sample was prepared by dissolving 3.0 mg chitosan in 0.5% of acetic acid solution. Later, a reaction mixture containing 1 mL chitosan, 1 mL ethanol and 1 mL of 0.1 mM solution of DPPH in absolute ethanol was prepared. The reaction mixture was raised to a final volume of 4 mL with the addition of 0.5% of acetic acid solution. The mixtures were shaken thoroughly and incubated at 25°C for 30 min. The absorbance was measured at 517 nm against blank (without DPPH solution) using a Jasco UV-Vis spectrophotometer. Ascorbic acid was used as a standard. The higher absorbance value of the sample indicated lower scavenging

activity of radicals. The ability of scavenging activity was calculated using the following formula:

$$\text{DPPH scavenging activity (\% inhibition)} = \frac{A_0 - A_1}{A_0} \times 100$$

where, A_0 is the absorbance of the blank, and A_1 is the absorbance in the presence of all of the extract samples and reference.

Disk diffusion assay

Antibacterial activity of the chitosan was determined according to Shanmugam *et al*. (2009). The antibacterial activity of extracted chitosan against two bacteria strains was tested using the disk diffusion method. Two species of bacteria (Gram-positive: *S. aureus* and Gram-negative: *E. coli*) were used as the test organisms. Individual species of bacteria was inoculated in nutrient broth and incubated at 37°C for 24 hr. 0.5 McFarland standard was used as a standard to compare turbidities of the inoculum. Mueller Hinton agar, MHA, medium was poured into sterile petri dishes and incubated at 37°C for 24 hr. Discs of 6 mm diameter were used. The concentration of chitosan at 25 mg/mL was loaded onto the discs. Positive control (tetracycline, 1 mg/mL) and negative control (0.2% acetic acid) were also loaded into the discs, respectively. The petri dish was incubated at 37°C for 24 hr in an upright position. The antibacterial activity was evaluated based on the diameter zone of inhibition.

Data analysis

All experiments were carried out in triplicate and the data obtained are expressed as mean ± standard deviation, SD. Data were analyzed using independent t-test in Microsoft Excel 2016. The level of significant was set at $p < 0.05$.

RESULTS AND DISCUSSIONS

Physicochemical properties

Chitosan from both sources was successfully deproteinised, demineralised and deacetylated via chemical treatment. Deacetylation is a vital process to achieve a good quality of chitosan (Lago *et al*., 2011). The same chemical treatments were used to extract chitosan from both sources. Therefore, the quality of both extracted chitosan was evaluated based on physicochemical properties as shown in Table 1. Percentage yield of the extracted chitosan from *S. paramamosain*, 6.2% was higher than the yield obtained from prawn, *P. monodon*, 2.4%. The sample mass loss occurs during chemical treatment could decrease the percentage yield of extracted chitosan. Moisture content in chitosan from *S. paramamosain* was higher (8.5%) compared to chitosan from *P. monodon* (2.7%). This may be due to larger size particles of chitosan from *S. paramamosain* which has the ability to retain more water molecules. However, the lower moisture content represents a better quality of chitosan because moisture absorption affects its shelf stability and flake form (Ocloo *et al*., 2011). Thus, chitosan is recommended to be stored in a place with a temperature below 25°C and has a low humidity. The present study also showed that chitosan from both sources was highly soluble in 1% of acetic acid. When the DD of chitosan is more than 50%, protonation of amino group at C-2 position of glucosamine units make the chitosan soluble in acidic solution (Roy *et al*., 2017). Water binding capacity of chitosan from *S. paramamosain* was found to have higher percentage (444%) compared to chitosan from *P. monodon* (118%). Also, chitosan from *S. paramamosain* was found to have higher fat binding capacity (412%) compared to chitosan from *P. monodon* (117%). WBC and FBC properties could be related to bulk density of chitosan and preparation methods. Higher FBC could attributed to high ash content in the chitosan. Meanwhile, the degree of deacetylation of both chitosan from mud crab and prawn shells were analysed via FTIR and the values were calculated and found to be 57% and 52%, respectively. According to Ali *et al*. (2019), chitosan from mud crabs with 92% of DD can be obtained when 55% NaOH was used in the deacetylation process for

Table 1. Physicochemical properties of the extracted chitosan from mud crab, *S. paramamosain* and prawn, *P. monodon* shells

Percentage (%)	*S. paramamosain*	*P. monodon*
Yield	6.2 ± 0.1[a]	2.4 ± 0.1[b]
Moisture	8.5 ± 1.3[a]	2.7 ± 1.0[b]
Solubility	100 ± 0.1	100 ± 0.0
Water binding capacity	444 ± 44[a]	118 ± 0.2[b]
Fat binding capacity	412 ± 37[a]	117 ± 2.5[b]
Degree of deacetylation	57 ± 0.1	52 ± 0.1

Different alphabetical superscripts indicate the difference between the measured values in each row ($p < 0.05$).

4 hr at 110°C. Longer duration and higher concentration of NaOH during deacetylation treatment might enhance the formation of free amino groups, leads to high DD in chitosan. The degree of deacetylation influences the physicochemical and bioactivities of chitosan (Kumari *et al*., 2017).

Characterisation of the extracted chitosan

The degree of deacetylation, and absorption peaks of extracted chitosan were analysed via FTIR while the morphology of the extracted chitosan from both sources was conducted using SEM. Figure 1 displays the stack of FTIR spectra for the extracted chitosan from different sources compared to commercial chitosan and chitin. The nature of cellulose in all extracted chitosan and chitin of this study was confirmed based on the pattern of bands that below 1250 cm^{-1}. The spectra obtained in this study were similar to previous report by Kasaai (2010). The presence of hydroxyl group was detected in all extracted chitosan similar to the commercial chitosan by the peak at 3429.15 cm^{-1}. Meanwhile, the alkane in chemical structure of chitosan in all extracted chitosan was supported by the presence of a peak from 3000-2840 cm^{-1}. The difference between chitin and chitosan IR spectra in the interval peak 3450–3280 cm^{-1} is due to the larger N-H and O-H groups as shown in Figure 1. There are several internal reference bands in DD determination which are the O-H stretching band at 3550-3200cm^{-1}, the C-H stretching bands within 2870–3000 cm^{-1}, the skeletal vibrations involving the C-O-C stretching band at 1030 or 1070 cm^{-1}, the amide III band at 1315–1320 cm^{-1}, the N–H bending of amide I at 1620–1630 cm^{-1}, and the C-O-C bridge as well as glucosidic linkage at 890–900 cm^{-1} (Kasaai, 2010). However, various factor can affect the determination of DD in chitosan including impurities and humidity by interfering the position and intensities of peaks in IR spectra. Chitosan is a hygroscopic material, thus the OH groups in chitosan are sensitive to humidity. An increase in water molecules in the chitosan causes an increase in the intensity of the absorption of O-H stretching band at 3450 cm^{-1} or causes broadening effect (Beil *et al*., 2012). Drying the sample before DD determination could minimize the error. The reduction of degree of acetylation in chitosan in this study was followed with a reduction in absorbance of amide I band at 1655 cm^{-1}. The C-H stretching band at 2870–2880 cm^{-1} can be used as a reference band because its position and intensity do not alter with water content or hydrogen bond. The SEM images of the extracted chitosan from *S. paramamosain* and *P. monodon* are depicted in Figure 2. The structure of chitosan from both sources appears as a finely thin layer leave and fibrous. Fractures were observed on the surface of extracted chitosan, which might have produced during crushing and extracting processes. Muley *et al*. (2018) have reported the same findings on structures of chitosan from prawn shells.

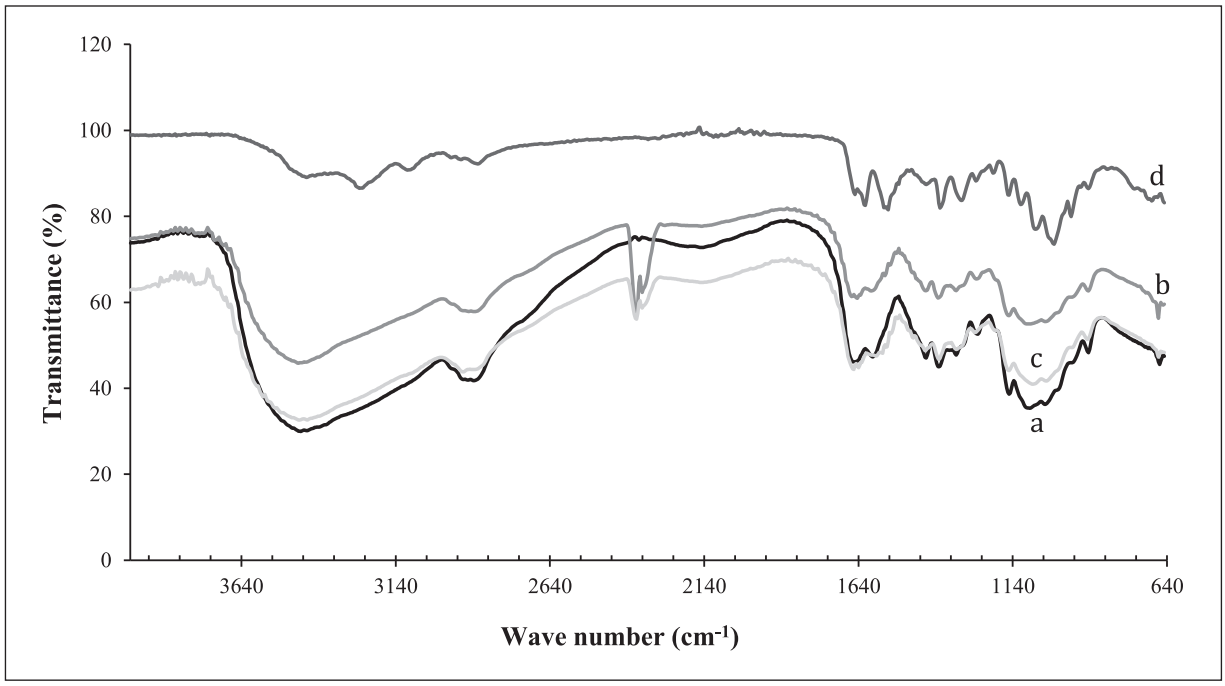

Fig. 1. Stack spectra of extracted chitosan from *P. monodon* (a), extracted chitosan from *S. paramamosain* (b), commercial chitosan (c) and chitin (d).

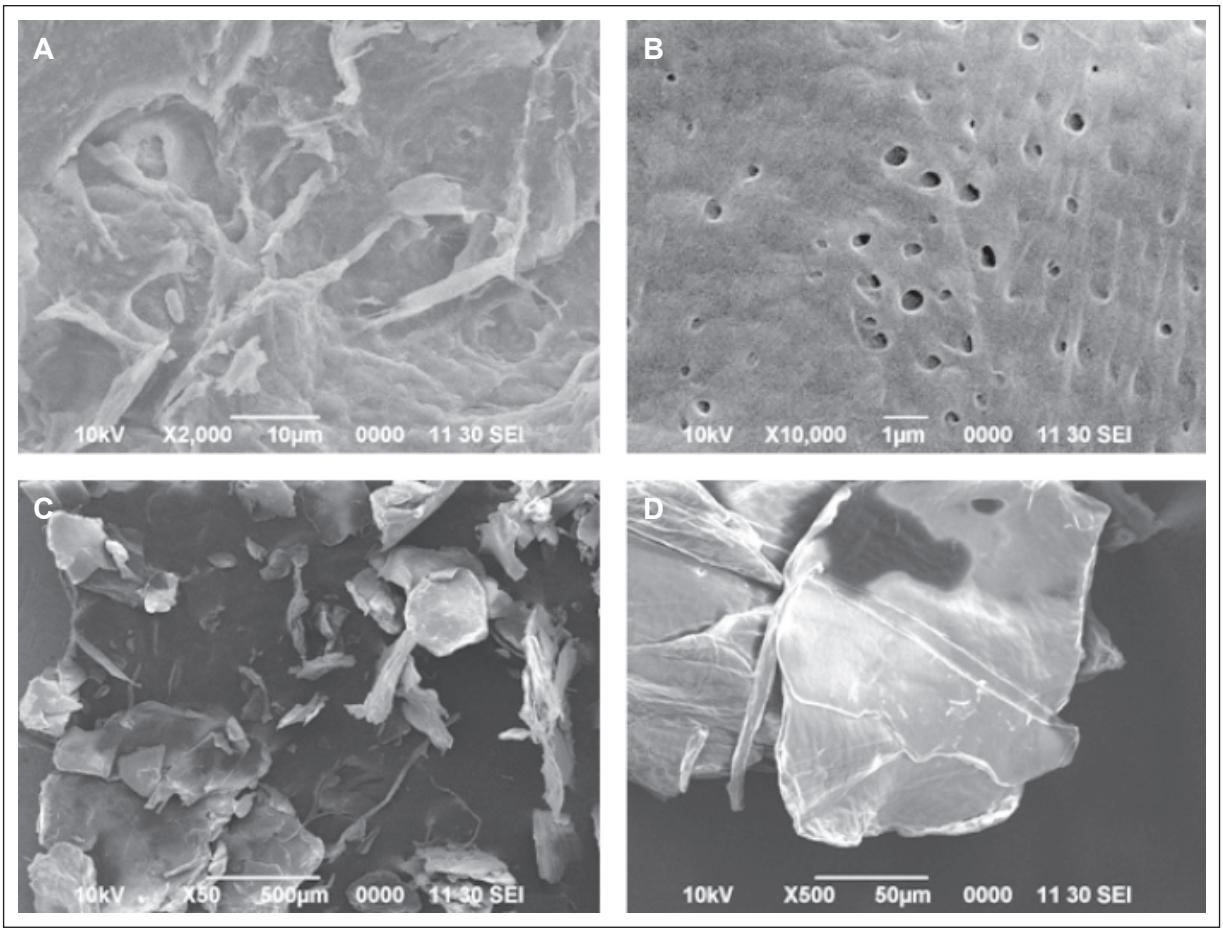

Fig. 2. Scanning electron microscopy images of extracted chitosan from *S. paramamosain* (A, B) and *P. monodon* (C, D) at different magnifications.

Biological properties of extracted chitosan

Figure 3 represents the DPPH scavenging activity of both extracted chitosan compared to ascorbic acid and commercial chitosan, at the same concentration, 3.0 mg/mL. The results showed that chitosan from the *S. paramamosain* has 22.2±1.0% of scavenging activity, compared to commercial chitosan standard, 43.0±1.8%. Chitosan from *P. monodon* showed the lowest activity among all, 6.7±1.5%. Meanwhile, ascorbic acid showed the highest DPPH scavenging activity which was 96±4.0%. A study was done by Sarbon *et al*. (2014) showed that 10 mg/mL of chitosan extracted from *S. paramamosain* had 30% DPPH scavenging activity. It showed that concentration of chitosan may affect the DPPH scavenging activity. The ability of chitosan to scavenge DPPH radicals is due to the presence of free amine group through either indirect or direct transfer of hydrogen atom to the radical (Avelelas *et al*., 2019). Chitosan with high number of free amino groups obtained through deacetylation process has better quality with high antioxidant properties. Thus, the anti-oxidative action of chitosan is greatly dependable on the degree of deacetylation and its molecular weight. Table 2 shows the zone of inhibition of extracted chitosan from both organisms against *E. coli* and *S. aureu*s in disk diffusion method. The results showed that chitosan from *S. paramamosain* exhibited anti-microbial activity at a concentration of 25 mg/mL against *E. coli* and *S. aureus* while no activity for chitosan from *P. monodon* and commercial chitosan as shown in Figure 4, respectively. The high viscosity of chitosan solution might affect the results of commercial chitosan in disk diffusion assay as it was highly viscous. Therefore, the chitosan was ensured to completely dissolve in acetic acid before the assay. Ahamed *et al*. (2018) have reported that chitosan from crab shells could be a bactericidal agent to *S. aureus* when the synthesized chitosan with molecular weight of 600±10 kDa was able to kill 95% of the bacteria in liquid culture test. A study was done by Chien *et al*. (2016) reported that chitosan from crab shells at 0.5 mg/mL showed an inhibitory effect on various pathogenic bacteria with the inhibition zone of 12

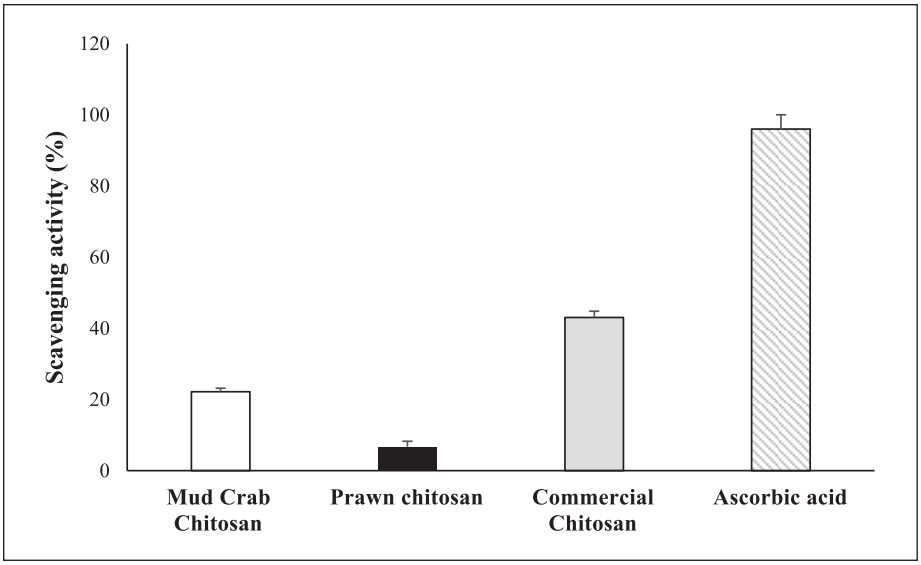

Fig. 3. DPPH scavenging activity of the extracted chitosan from mud crab, and prawn, as compared to commercial chitosan and ascorbic acid.

Table 2. Zone inhibition of both extracted chitosan against *E. coli* and *S. aureus*

Inhibition zone (mm)	*E. coli*	*S. aureus*
Chitosan of *S. paramamosain*	8.8±1.0	8.5±1.0
Chitosan of *P. monodon*	–	–
Commercial chitosan	–	–
Tetracycline (positive control)	31.5±0.2	33.8±0.2
1% Acetic acid (negative control)	–	–

(–) shows no inhibition zone in disk diffusion assay.

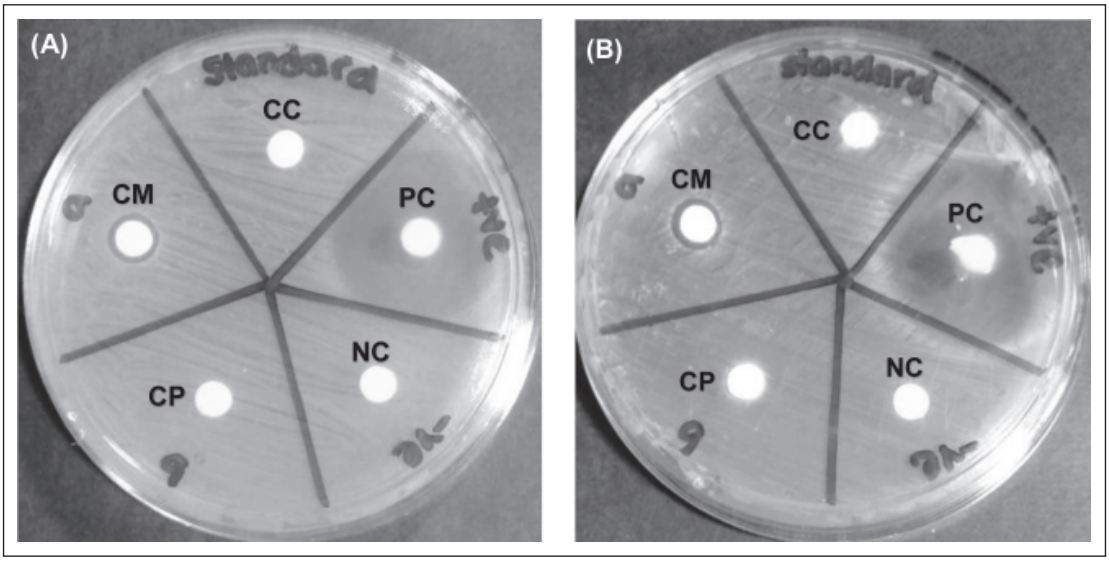

Fig. 4. Disk diffusion assay of chitosan extracted from mud crab, *S. paramamosain* and prawn, *P. monodon* against *E. coli* (A) and *S. aureus* (B). CC: commercial chitosan; CM: chitosan from mud crab; CP: chitosan from prawn; PC: tetracycline as positive control; NC: acetic acid as negative control.

mm. In the same study, chitosan from crab shells displayed the most effective inhibitory growth towards *L. monocytogenes* and the least effective towards *B. cereus* with the inhibition zones of 18.8–25.6 mm and 10.8–18.6 mm, respectively. According to Chung and Chen (2008), the destruction of cell walls and membrane of *E. coli* could be attributed to the antibacterial action of chitosan at a concentration of 1.5 g/L, thus supporting that chitosan could be a natural bactericidal agent. On the same note, chitosan gel at 1 g/L had displayed an inhibitory effect towards Gram-positive bacteria, *S. aureus* after 24 hr of interaction (Goy *et al*., 2016). However, chitosan from *P. monodon* in this study might have antibacterial activities if the DD is higher because previous studies had reported a significant antibacterial property of chitosan from shrimp and prawn (Samar *et al*., 2013; Paul *et al*., 2014).

CONCLUSIONS

This study reveals the potential of marine shells that could be utilised as a source of natural chitosan and its derivatives. Despite being dumped as a waste, the marine shells, especially chitosan from *S. paramamosain* could be employed as an attractive formulation in biotechnology. Chitosan extracted from *S. paramamosain* showed promising potential as an antioxidant and antibacterial agent compared to chitosan from *P. monodon*. However, the quality of the chitosan from both sources could be increased by enhancing their degree of deacetylation via different techniques such as microwave heating or enzyme treatment. Malaysia, in particular in Sarawak is a mega-biodiversity region. Therefore it is recommended to investigate the biological potential of chitosan from other sources such as plants, fungi or marine organisms.

ACKNOWLEDGEMENTS

The authors would like to thank the laboratory staff in Centre for Pre-University Studies and Molecular Genetic Laboratory for their technical assistance. This study was supported by the Department of Higher Education (Malaysia) under Fundamental Research Grant Scheme (FRGS/1/2017/STG04/UNIMAS/03/01).

REFERENCES

Ahamed, M.I.N., Gomathi, N., Ragul, V., Priya, M., Priya, R., Rumaan, R.S., Thariq, M.V. & Ahmed, N.A. 2018. Novel preparation of chitosan from crab shell using probe sonicator and its antibacterial activity. *Journal of Academia and Industrial Resear*ch, **6(8):** 133-136.

Ali, M., Shakeel, M. & Mehmood, K. 2019. Extraction and characterization of high purity chitosan by rapid and simple techniques from mud crabs taken from Abbottabad. *Pakistan Journal of Pharmaceutical Sciences*, **32(1):** 171-175.

Avelelas, F., Horta, A., Pinto, F.V. & Marques, C. 2019. Antifungal and antioxidant properties of chitosan polymers obtained from nontraditional *Polybius henslowii* sources. *Marine Drugs*, **17(4):** 1-15.

Beil, S., Schamberger, A., Naumann, W., MacHill, S. & Van Pée, K.H. 2012. Determination of the degree of N-acetylation (DA) of chitin and chitosan in the presence of water by first derivative ATR FTIR spectroscopy. *Carbohydrate Polymers*, **87(1):** 117-122.

Bernkop-Schnürch, A. & Dünnhaupt, S. 2012. Chitosan-based drug delivery systems. *European Journal of Pharmaceutics and Biopharmaceutics*, **81(3):** 463-469.

Cheung, R.C.F., Ng, T.B., Wong, J.H. & Chan, W.Y. 2015. Chitosan: An update on potential biomedical and pharmaceutical applications. *Marine Drugs*, **13(8):** 5156-5186.

Chien, R.C., Yen, M.T. & Mau, J.L. 2016. Antimicrobial and antitumor activities of chitosan from shiitake stipes, compared to commercial chitosan from crab shells. *Carbohydrate Polymers*, **138(2016):** 259-264.

Chung, Y.C. & Chen, C.Y. 2008. Antibacterial characteristics and activity of acid-soluble chitosan. *Bioresource Technology*, **99(8):** 2806-2814.

Dhillon, G.S., Kaur, S., Brar, S.K. & Verma, M. 2013. Green synthesis approach: extraction of chitosan from fungus mycelia. *Critical Reviews in Biotechnology*, **33(4):** 379-403.

Goy, R.C., Morais, S.T.B. & Assis, O.B.G. 2016. Evaluation of the antimicrobial activity of chitosan and its quaternized derivative on *E. coli* and *S. aureus* growth. Brazilian *Journal of Pharmacognocy*, **26(1):** 122-127.

Hajji, S., Younes, I., Ghorbel-Bellaaj, O., Hajji, R., Rinaudo, M., Nasri, M. & Jellouli, K. 2014. Structural differences between chitin and chitosan extracted from three different marine sources. *International Journal of Biological Macromolecules*, **65:** 298-306.

Hossain, M.S. & Iqbal, A. 2014. Production and characterization of chitosan from shrimp waste. *Journal of the Bangladesh Agricultural University*, **12(2014):** 153-160.

Hussain, R., Iman, M. & Maji, T.K. 2014. Determination of degree of deacetylation of chitosan and their effect on the release behavior of essential oil from chitosan and chitosan-gelatin complex microcapsules. *International Journal of Advance Engineering Applications*, **37:** 69-77.

Kae, A., Chang, T., Frias, R.R., Alvarez, L.V., Bigol, U.G., Paul, J. & Guzman, M.D. 2019. Biocatalysis and Agricultural Biotechnology Comparative antibacterial activity of commercial chitosan and chitosan extracted from *Auricularia* sp. *Biocatalysis and Agricultural Biotechnology*, **17(July 2018):** 189-195.

Kasaai, M.R. 2010. Determination of the degree of N-acetylation for chitin and chitosan by various NMR spectroscopy techniques: A review. *Carbohydrate Polymers*, **79(4):** 801-810.

Kumari, S., Hari, S., Annamareddy, K., Abanti, S. & Kumar, P. 2017. Physicochemical properties and characterization of chitosan synthesized from fish scales, crab and shrimp shells. *International Journal of Biological Macromolecules*, **104(2017):** 1697-1705.

Lago, M.A., De Quirós, A.R.B., Sendón, R., Sanches-Silva, A., Costa, H.S., Sánchez-Machado, D.I., Lopez-Cervantez, J. & Valdez, H. 2011. Compilation of analytical methods to characterize and determine chitosan, and main applications of the polymer in food active packaging. *CYTA – Journal of Food*, **9(4):** 319-328.

Li, Z., Yang, F. & Yang, R. 2015. Synthesis and characterization of chitosan derivatives with dual-antibacterial functional groups. *International Journal of Biological Macromolecules*, **75:** 378-387.

Metin, C., Alparslan, Y., Baygar, T. & Baygar, T. 2019. Physicochemical, Microstructural and Thermal Characterization of Chitosan from Blue Crab Shell Waste and Its Bioactivity Characteristics. *Journal of Polymers and the Environment*, **27(11):** 2552-2561.

Muley, A.B., Chaudhari, S.A., Mulchandani, K.H. & Singhal, R.S. 2018. Extraction and characterization of chitosan from prawn shell waste and its conjugation with cutinase for enhanced thermo-stability. *International Journal of Biological Macromolecules*, **111:** 1047-1058.

Ocloo, F.C.K., Quayson, E.T., Adu-Gyamfi, A., Quarcoo, E.A., Asare, D., Serfor-Armah, Y. & Woode, B.K. 2011. Physicochemical and functional characteristics of radiation-processed shrimp chitosan. *Radiation Physics and Chemistry*, **80(7):** 837-841.

Ongkiko, A.G.M., Fernando, L.A.T. & Diaz, L.J.L. 2018. Continuous extraction process of chitin from discarded shells of philippine blue swimming crab (*Portunus pelagicus*). *Philippine Engineering Journal*, **37(2):** 37-52.

Paul, S., Jayan, A., Sasikumar, C.S. & Cherian, S.M. 2014. Extraction and purification of chitosan from chitin isolated from sea prawn (*Fenneropenaeus indicus*). *Asian Journal of Pharmaceutical and Clinical Research*, **7(4):** 201-204.

Romainor, A.N.B., Chin, S.F., Pang, S.C. & Bilung, L.M. 2014. Preparation and characterization of chitosan nanoparticles-doped cellulose films with antimicrobial property. *Journal of Nanomaterials*, **2014:** 1-10.

Roy, J.C., Salaün, F., Giraud, S., Ferri, A., Chen, G. & Guan, J. 2017. Solubility of chitin: solvents, solution behaviors and their related mechanisms. In: *Solubility of Polysaccharides*. Z. Xu (Ed.). IntechOpen.

Samar, M.M., Khalaf, M.M. & El-razik, M.M.A. 2013. Physicochemical, functional, antioxidant and antibacterial properties of chitosan extracted from shrimp wastes by microwave technique. *Annals of Agricultural Sciences*, **58(1):** 33-41.

Sarbon, N.M., Sandanamsamy, S., Kamaruzaman, S.F.S. & Ahmad, F. 2014. Chitosan extracted from mud crab (*Scylla olivacea*) shells: Physicochemical and antioxidant properties. *Journal of Food Science and Technology*, **52(7):** 4266-4275.

Shanmugam, A. & Thirunavukkarasu, N. 2009. Extraction of chitin and chitosan from mud crab *scylla tranquebarica* (Fabricius, 1798). *International Journal of Applied Bioengineering*, **4(2):** 31-33.

Shen, Q., Zhang, B., Xu, R., Wang, Y., Ding, X. & Li, P. 2010. Antioxidant activity *in vitro* of the selenium-contained protein from the Se-enriched Bifidobacterium animalis 01. *Anaerobe*, **16(4):** 380-386.

Sullivan, D.M. & Carpenter, D.E. 1993. *Methods of Analysis for Nutrition Labeling*. AOAC International.

Srinivasan, H., Kanayairam, V. & Ravichandran, R. 2018. Chitin and chitosan preparation from shrimp shells *Penaeus monodon* and its human ovarian cancer cell line, PA-1. *International Journal of Biological Macromolecules*, **107:** 662-667.

Vidal, R.R.L. & Moraes, J.S. 2018. Removal of organic pollutants from wastewater using chitosan: a literature review. *International Journal of Environmental Science and Technology*, **16:** 1741-1754.

Wang, J.C. & Kinsella, J.E. 1976. Functional properties of novel proteins: alfalfa leaf protein. *Journal of Food Science*, **41(2):** 286-292.

Yen, M.T., Yang, J.H. & Mau, J.L. 2009. Physicochemical characterization of chitin and chitosan from crab shells. *Carbohydrate Polymers*, **75(1):** 15-21.