American Academic Scientific Research Journal for Engineering, Technology, and Sciences
ISSN (Print) 2313-4410, ISSN (Online) 2313-4402
http://asrjetsjournal.org/

Biological, Functional and Physico-Chemical Characterization of Chitosan Extracted From Blue Crab (Portunus segnis) Shell Waste by Chemical Method

Imen Zaghbib[a*], Soumaya Arafa[b], Mnasser Hassouna[c]

[a,b,c]*High Graduate School of Food Industry of Tunisia, Research Unit "Biopreservation and Valorisation of Agro-Food Products", 58 Avenue Alain Savary, Tunis El Khadra 1003, Tunisia*

[a]*Email: zaghbibimen@gmail.com* , [b]*Email: arafa_soumaya@yahoo.fr*, [c]*Email: Mnasser.Hassouna@isbb.rnu.tn*

Abstract

The aim of this work was to extract a bioactive material « chitosan » from the by-products of blue crab (P. segnis) by chemical extraction method. The physico-chemical, functional and biological properties of the chitosan obtained were evaluated. The yield of the extracted chitosan was calculated as 9.2 ± 2.03%. The proximate analysis showed 2 ± 0.03% moisture, 0.57 ± 0.02% ash, 0.3 ± 0.01% lipid and 0,2 ± 0,03% protein contents in chitosan. Degree of deacetylation (DD) value was calculated using potentiometric titration (78.97 ± 0.38%) and FTIR (81,47 ± 0.51%) resulting in a high DD. Whiteness value, water and fat binding capacities of the chitosan were high and are suitable for many functional food applications. Structural characterization was performed using FTIR spectroscopy which confirms the presence of amino group of chitosan synthesized. Antioxidant activity assay showed that chitosan exhibited notable antioxidant activity against DPPH (20.13 ± 1.41% to 70.63 ± 1.26%), but lower than that of ascorbic acid (49.17 ± 1.13% to 87.46 ± 1.22%) at the same concentrations (0,5 to 10 mg/mL), in a dose dependent manner. Its required IC_{50} to inhibit 50% of radical DPPH was 1.86 ± 0.31 mg/mL. Extracted chitosan showed a significant antimicrobial activity against bacteria and fungi. In conclusion, the extracted chitosan is an effective natural biopolymer possessing potential properties.

Keywords: Blue crab; chitosan; characterization; physico-chemical properties; functional properties; biological activities.

1. Introduction

During the past few decades, a remarkable increase in the frequency of alien species introductions and/reports was noted. Introducing species in the sea can be beneficial in the newly invaded environments, acting as key species playing several important ecological roles, providing additional commercially-important resources, and offering numerous ecosystem services [1].

---

* Corresponding author.

Blue crab (Portunus segnis) belong to the order of Decapoda and is classified in family Portunidae. P. segnis species proliferates easily and is widespread in the west of the Atlantic Ocean. Recently, it is found extensively in the Mediterranean causing various damages regarding fishing because of its invasiveness and destructiveness. It reached Tunisian waters in 2014 and being captured and commercialized for human consumption [2]. Blue crab processing industries generate each year a great amount of shell wastes. It was considered as a major polluter, as 80-90% of its biomass is waste. The odor emitting from this waste and also its sheer volume, have been ground to label the blue crab waste as a pollution stream [3]. About 40% of the waste amount is chitin, which has been deemed as a nuisance by environmental regulators due to its slow degradation [4]. Therefore, finding alternatives to valorize the crab shells waste into value-added products, like chitin, chitosan, antioxidants and recovery of minerals and proteins became inevitable. Chitin and its deacetylated form « chitosan » have recently become the focus of researchers and industrialists as they exhibited biological and technological properties (film-forming properties, antimicrobial activity, water retention ability, chelating/adsorption capacities, etc.). However, these properties are tightly related to the physicochemical properties of the polymers, mainly molecular weight and acetylation degree [5, 6]. Chitin is a natural biopolymer of β-(1 →4) N-acetyl D-glucosamine units. Chitin, found in the exoskeleton of crustacea such as crabs and shrimp, insect's cuticles, algae and in the cell wall of fungi, is the second most abundant natural resource next to cellulose [5]. This biopolymer is present in three different forms : α, β and γ. α-chitin is the most stable and abundant form with macromolecules arranged antiparallel, β-chitin is the form with a parallel alignment and γ-chitin regroup both parallel and antiparallel forms [5]. Conventionally, chitin extraction from crustacean shells involves strong acid and alkali treatment, under elevated temperature, for demineralization (removal of mineral substances) and deproteinization (removal of proteins). Chitosan is a natural and linear polysaccharide, consisting of β-(1→4) linked D-glucosamine and N-acetyl-D-glucosamine units [7]. It presents several important advantages such as biodegradability, non-toxicity, biocompatibility and good antimicrobial activity [2]. Due to these properties, chitosan has become a useful and highly appreciated polymer compound. Chitosan is considered as bioactive polymer and offers a wide range of applications in the food industry, including the preservation of foods from microbial deterioration and formation of biodegradable films and utilisation as a dietary fibre and as a functional food ingredient [8]. The present study was aimed to produce a value added product from blue crab shell (P. segnis), which is not used and discarded as waste product in Tunisia. With this aim, extracted chitosan was subjected the physicochemical, functional as well as antioxidant and antibacterial properties

2. Materials and Methods

2.1 Extraction of blue crab chitosan

2.1.1 Portunus segnis shells preparation

Blue crabs (P. segnis) were obtained in fresh conditions from the shrimp processing plant and fishery market, located at Mahdia, Tunisia. Samples were kept chilled in ice during transportation to the laboratory. The crab shells were completely separated from the crab waste, washed with water to remove adherent and soluble materials and then dried at 40°C in an oven. Dried shells were powdered by a grinder (Retsch PM 100).

2.1.2 Extraction of chitin and preparation of chitosan

Chitin was extracted from P. segnis carapace according to the method of Norhidayah and his colleagues [9] with some modifications. The demineralization process begins by treating the crab shell powder in 1 M HCl solution (1:15, w/v) with constant stirring for 1 h at 25°C to remove all minerals from the sample. HCl solution was filtered with Whatman filter paper N°1 and the resulting solid was washed with distilled water until it was completely free of acid. The demineralized samples were dried in an oven at 60°C for 24 h and weighed. Deprotenization of chitin was carried out by heating the demineralised powder in 1 N NaOH solution (1:15, w/v) under constant stirring at 80°C for 6 h. Shells were filtered using Whatmann filter paper N°1 and washed with distilled water for 5-10 minutes. To remove the colour of the sample, the deproteinized powder was mixed with hydrogen peroxide solution (1:10, w/v) at room temperature for 1 h with moderate stirring. The chitin was filtered and washed with distilled water until neutrality. Finally, the alkaline deacetylation was accomplished by mixing the sample in a 50% (w/v) NaOH solution (1:20, w/v) under constant stirring at 110°C for 6 h, followed by plentiful water washings and drying was accomplished at 60°C for 24 h.

2.2 Chitosan characterization

2.2.1 Yield and proximate composition

The yield of chitosan was obtained by comparing the weight of the raw material to the weight of chitosan obtained after the treatment. The moisture, ash and protein contents were determined according to the AOAC [10] methods, while the lipid content was determined according to the method of Folch and his colleagues [11].

2.2.2 Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) was used to determine the vibration of functional groups spectra of chitosan according to the method of Norhidayah and his colleagues [9]. The sample was mixed with potassium bromide (KBr), and the dried mixture was then pressed to result in a homogeneous sample disk. Infrared spectra of KBr chitosan mixture were obtained over the frequency range of 400-4000 cm^{-1} at a resolution of 4 cm^{-1} using Perkin Elmer FTIR Spectrometer.

2.2.3 Degree of deacetylation

The degree of deacetylation (DA) of the chitosan was determined by two methods : Fourier transform infrared spectroscopy and direct titration method.

2.2.3.1 Direct titration method

The direct titration method was used to determine the degree of deacetylation of extracted chitosan as described by Kumari and Rath [12]. Approximately, 0.2 g of chitosan was dissolved in 20 mL of 0.1 M hydrochloric acid and 25 mL of deionized water under constant stirring for 30 minutes. 25 mL of deionized water was added again and stirring was continued for next 30 minutes. When chitosan was completely dissolved, titrant solution (0.1 M sodium hydroxide) was added gradually. The volume of NaOH added and pH values of the solution were

recorded using digital pH-meter. Degree of deacetylation was calculated using the following formula:

$$DDA\% = 100 - (2{,}03 \times (V_2 - V_1) / m + 0{,}0042\ (V_2 - V_1)) \quad (1)$$

where, $\mathbf{V_1}$ and $\mathbf{V_2}$: volumes of NaOH solution used (mL), **m :** mass of chitosan (g), **2,03 :** coefficient of the molecular weight of acetylated monomeric residu, **0,0042 :** coefficient resultant from difference between the molecular weight of acetylated and deacetylated monomeric residus.

2.2.3.2 Fourier transform infrared spectroscopy (FTIR)

As described by Hussain and his colleagues [13], the degree of deacetylation (DD) of the chitosan was determined by recording the absorbance of the amide-I to that of the OH group in chitosan at the absorbance ratio A_{1655}/A_{3450}. Briefly, 20 mg of chitosan powder and 120 mg of KBr was blended and pestle for approximately 10 min. The mixture was compacted using a press Perkin-Elmer. Finally, the pellet was conditioned in a desiccator placed in an oven at 80°C for 24 h before analysis. The degree of deacetylation (DD) was calculated using the equation given below :

$$DD\ (\%) = (1 - (A_{1655} / A_{3450}) / 1.33 \times 100) \quad (2)$$

The factor 1.33 represents the ratio of A_{1655}/A_{3450} for fully N-acetylated chitosan.

2.2.4 Colour measurements

The colour of obtained chitosan was measured using a Minolta (CR 400/410) colorimeter. The chitosan sample was placed in a transparent petri dish. The results were recorded as L*, a* and b* value. The whiteness was calculated based on the following equation [14]:

$$Whiteness = 100 - ((100 - L^*)^2 + a^{*2} + b^{*2})^{1/2} \quad (3)$$

2.2.5 Water binding capacity

The water binding capacity (WBC) of extracted chitosan was measured as referred to Ocloo and his colleagues [15]. Chitosan (0.5 g) was weighed in a centrifuge tube and 10 mL of distilled water were added. The mixture was then vortexed for 1 min in order to dissolve the chitosan before left at an ambient temperature for 30 min and the tube was shaken for 5 s every 10 min before being centrifuged at 3,200 rpm for 25 min. The tube was weighed again after the supernatant was decanted. The water binding capacity was calculated as the following equation:

$$WBC\ (\%) = (\text{water bound (g)} / \text{sample weight (g)}) \times 100 \quad (4)$$

2.2.6 Fat binding capacity

Fat binding capacity (FBC) was determined as the method described for WHC except that the distilled water

was replaced by corn oil [15].

The FBC was calculated as the following equation:

$$\text{FBC (\%)} = (\text{fat bound (g)} / \text{sample weight (g)}) \times 100 \quad (5)$$

2.2.7 Antioxidant activity

The free radical scavenging effect of chitosan was estimated using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay according to the method of Sarbon and his colleagues [8] with slight modifications. Approximately, 1 mL of the chitosan sample at different concentrations (0.5 to 10 mg/mL) in 1% acetic acid solution (v/v) was mixed with 3 mL of methanolic solution of DPPH (0.2 M). The mixture was vortexed and incubated for 30 min at room temperature in the dark. The absorbance of the solution was measured at 517 nm against a blank. Ascorbic acid was used as standard. The inhibitory percentage of DPPH was calculated using the following equation :

$$\text{DPPH scavenging effect (\%)} = ((Abs_{control} - Abs_{sample})/Abs_{control}) \times 100 \quad (6)$$

The IC_{50} value (mg/mL) corresponding to the concentration at which hydroxyl radical is scavenged by 50 % was also measured.

2.2.8 Antimicrobial activity

2.2.8.1 Microorganisms

Antimicrobial activities of deacetylated chitosan were examined as the inhibitory effects against the growth of four bacteria (Staphylococcus aureus ATCC25923, Listeria monocytogenes ATCC 070101121, Escherichia coli ATCC2124 and Salmonella typhimurium ATCC 25922) and two fungi (Geotrichum candidum ATCC, Aspergillus niger ATCC). All bacteria and fungi were obtained from culture collection of the Research Unity "Bio-Preservation and Valorization of Agro-Food Products" of the High Graduate School of Food Industry of Tunuisia, in nutrient agar and stored at 4 °C.

2.2.8.2 Antimicrobial activity

Antimicrobial activity evaluation was carried out following the method described by Hamdi and his colleagues [5, 7] with slight modifications. To carry out the assay, a culture suspension of the indicator microorganism (10^6 cfu/mL) was spread on a Mueller–Hinton agar and Potato Dextrose agar, for antibacterial and antifungal activities, respectively. 6 mm diameter wells were punched in the inoculated agar medium with sterile Pasteur pipettes, and thereafter loaded with 25 mg/mL and 50 mg/mL of chitosan solution (in 0.1% acetic acid). A 0.1% acetic acid solution was included as a negative control for the antimicrobial activity assay. The plates were then incubated for 24 h at 37°C and 30°C for bactericidal and fungicidal activities, respectively. The antimicrobial activity was evaluated by measuring the inhibition zone diameters (clear zone around the well), in triplicate, against the test organisms.

2.3 Statistical analysis

One-way analysis of variance (ANOVA) was performed, and the significance of each mean property valuewas determined ($p < 0.05$) with the Duncan's multiple range testusing the SPSS statistical analysis computer program for Windows (ver. 12.0, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1 Yield and composition

The yield and proximate composition of extracted chitosan from blue crab (Portunus segnis) were compared to those of dry shells and chitin as presented in Table 1.

The blue crab shell (carapace) had a chitin and chitosan yields of 11.68 ± 1.14% and 9.2 ± 2.03%, respectively (Table 1). The yield of chitosan is higher than that found by Webester and his colleagues [3] when extracting chitosan from blue crab shell (Callinectes sapidus Rathbun). However, it is low compared to 20-30% [16] and 44.57 ± 3.44% [8] as reported in the literature. During chitosan preparation, excessive removal of acetyl groups from the polymer may cause the loss of sample mass/weight which affect the yield obtained [8]. In addition, this low yield may be due to the amount and source of chitin. The legs of crab had the highest amount of chitin compared to the carapace, which contains less chitin [16]. Biochemical composition analyses showed that chitosan, chitin and shells consisted of 2 ± 0.03% ; 3.5 ± 0.01% and 13.5 ± 0.02% water, respectively ($p<0.05$). Hamdi and his colleagues [5] calculated the moisture content of the blue crab shells waste as 10%. The moisture content of the chitosan extracted from blue crab found in the present study (2 ± 0.03%) was significantly lower from the commercial chitosan (14.15 ± 0.75%) and as reported by Sarbon and his colleagues [8] (9.48±0.59 %) ($p<0.05$). According to Ocloo and his colleagues [15], a lower moisture content of chitosan indicates better shelf stability and enhances the quality The ash content in chitosan is an indicator of the effectiveness of the demineralization step for the removal of calcium carbonate. The present study showed that the ash content of the chitosan was 0.57 ± 0.02% which was significantly lower ($p<0.05$) than ash content of shells (26,83 ± 0,02%) and chitin (0,73 ± 0,01%) (Table 1). Moreover, there was a significant difference ($p<0.05$) in the ash content between blue crab (0.57 ± 0.02%), mud crab (5.97 ± 0.90%) and commercial chitosan (7.55 ± 0.05%) [8]. However, Kucukgulmez and his colleagues [17] also found that the ash content of snow crab chitosan was 0.59–0.61 %. These results agree with those found by No and Meyers [18] who reported that a high quality grade of chitosan should have an ash content of less than 1 %. In fact, some ash residual of chitosan may affect important characteristics of the final product such as solubility and viscosity [8]. This study reveals that protein content of the dry shells, chitin and chitosan were 10,63 ± 0,01%, 0,9 ± 0,02% and 0,2 ± 0,03%, respectively ($p<0.05$) (Table 1). Hamdi and his colleagues [5] reported that crab (P. segnis) shells, chitin and chitosan had a protein content of 11.25 ± 0.72%, 1.17 ± 0.11% and 0%, respectively. Tan and his colleagues [19] suggested that low protein content of chitin strongly indicates the good quality of extracted chitins. Results of lipid content were recorded as 5.45 ±0.58%, 1.02 ± 0.01% and 0.3 ± 0.01% for dry shells, chitin and chitosan, repectively (Table

1). These findings are higher than those found by Hamdi and his colleagues [5] (1.07 ± 0.03% for dry shells, 0% for chitin and chitosan).

Table 1: The yield (%), proximate composition (%), water binding capacity (%) and fat binding capacity (%) of shell, extracted chitin and chitosan from blue crab (P. segnis) carapace.

Characteristics (%)	Shell	Chitin	Chitosan
Yield	-	11.68 ± 1.14	9.2 ± 2.03
Moisture	13.5 ± 0.02^{a}	3.5 ± 0.01^{b}	2 ± 0.03^{c}
Lipid	5.45 ± 0.58^{a}	1.02 ± 0.01^{b}	0.3 ± 0.01^{c}
Protein	10,63 ± 0,01^{a}	0,9 ± 0,02^{b}	0,2 ± 0,03^{c}
Ash	26,83 ± 0,02^{a}	0,73 ± 0,01^{b}	0.57 ± 0.02^{b}
Water binding capacity	128.67 ± 0,03^{a}	189.33 ± 0,04^{b}	652 ± 0,02^{c}
Fat binding capacity	256.57 ± 0.78^{a}	414 ± 0,24^{b}	598,67 ± 0,09^{c}

Values are means ± SD of three determinations. Means with different letters within a line indicate significant difference ($p<0.05$).

3.2 Fourier transform infrared spectroscopy (FTIR)

Infrared spectroscopy was used to characterize the structure of chitosan (Figure 1). FTIR spectra showed different stretching vibration bands around 3401, 3259, 2926, 2887, 1670 and 1620, 1390, 1103, 1008 and 614 cm^{-1}. Concentrated band detected at approximately 3401 cm^{-1} corresponds to the intramolecular hydrogen bond which showed the alcohol group O-H in the chitosan. According to Sarbon and his colleagues [8], the band identified as the alcohol group (O-H band) was at 3695.36 cm^{-1}. The vibrational modes involved in intermolecular hydrogen bonding CO–HN and the intramolecular bonds -NH groups in the extracted chitosan were detected in the bands due to NH of the amide group at 3259 cm^{-1} [2]. As mentioned by Sarbon and his colleagues [8], the stretching band of N-H in the extracted chitosan from mud crab (Scylla olivicea) shells was in the range of 3458.73 cm^{-1}–3,440.48 cm^{-1}, while for the commercial chitosan the stretching band for N-H was in the range of 3369.11–3413.07 cm^{-1}. Stretching vibration bands around 2926 and 2887cm^{-1} proved the presence of methyl group in NH-$COCH_3$ and methylene group in CH_2OH, respectively [20]. The peaks at 1620 cm^{-1} and 1670 cm^{-1} (amide I) were assigned for stretching vibration of C=O bonds of the acetamide groups and symmetric deformation of CH_3 [4]. When the absorption band observed at 1670 cm^{-1} decrease, while band at 1620 cm^{-1} growth and has a larger intensity, indicating the prevalence of NH_2 groups and the occurrence of effective deacetylation [12]. Sarbon and his colleagues [8] reported the bending band of N-H in the extracted chitosan was in the range of 1622.76–1623.92 cm^{-1}, while the bending band for N-H was in the range of 1639.59–1655.16 cm^{-1}, for the commercial chitosan. Moreover, the band detected at 1390 cm^{-1}corresponds to a C—NH deformation (amide II). The absorption band at 1150 cm^{-1} was assigned to the asymmetric stretching of

the C—O—C bridge [4]. Results of the present study compared to those reported in the literature showed different IR spectra because different sources of chitosan, the method of isolation of chitin and the deacetylation process (different concentrations and reaction time) showed different peak on the spectrum [8].

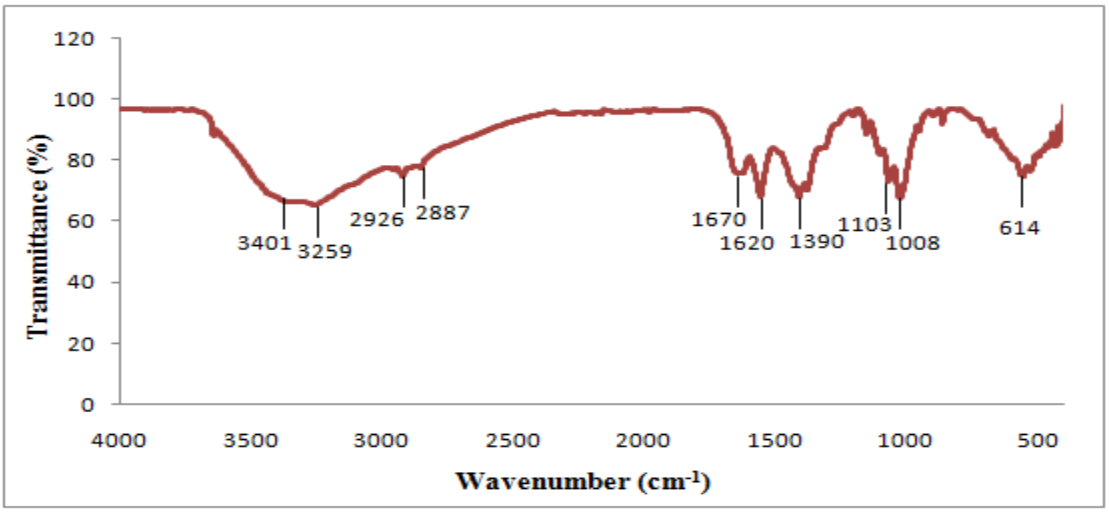

Figure 1: FTIR spectra of extracted chitosan.

3.3 Degree of deacetylation

The degree of deacetylation (DD) is one of the most important parameter that affects performance and various properties of chitin and chitosan such as biological, physicochemical and mechanical properties [12]. The DD of chitosane calculated using direct titration method as proposed by Kumari and Rath [12] was 78.97 ± 0.38% indicating a high degree of deacetylation. Analysis of the titration plot showed two inflection points (V_1 and V_2) and the difference of the values of these two points correspond to the acid consumed by the amine groups. On the other hand, the DD of chitosane calculated using infrared spectroscopy was 81,47 ± 0.51%. In the two methods used, the calculated values of DD were high and approximately similar. From these results, blue crab shells from P. segnis can be considered a good source of chitosan. Hafsa and his colleagues [4] found a DD of 73.68% for shrimp shells from (P. longirostris). According to Sarbon and his colleagues [8] the DD of commercial chitosan was 58.4%, while for the mud crab chitosan was 53.4 %. As mentioned by Kucukgulmez and his colleagues [17], the DD of the extracted chitosan from snow crabs was about 92.19%. Different results of DD observed between the present study and litterature were due to the analytical methods performed. Rasweefali and his colleagues [21] reported that sources, species, isolation method of chitin, deacetylation process and method (alkaline concentration, reaction time, and temperature ; potentiometric, titration method, IR spectroscopy…) affects the DD of chitosan. Kumari and his colleagues [22] indicated that the DD values are highly dependent on the type of method employed. According to [23], FTIR spectroscopy method is mostly used for a qualitative evaluation and comparison studies. It provides a number of advantages as it does not need long-term procedures for sample preparation and it gives information about the chemical structure. However, conventional methods like potentiometry, conductometry, titration, ninhydrin assay and adsorption of free amino groups of chitosan by pictric acid) are not applicable for highly acetylated chitin. In addition, several

parameters (ionic strength of the solvent, pH and temperature of solution) severely affect the results being obtained using conventional methods.

3.4 Colour

Chitin and chitosan samples were visually off white (Figure 2) and had whiteness values, respectively, 57.89 ± 0.12 and 62.6 ± 0.03. According to Sarbon and his colleagues [8] the chitosan extracted from mud crabs (62.10 ± 7.02) showed lowest degree of whiteness ($p<0.05$) as compared to the commercial chitosan (77.82 ± 0.47). Fernandez-Kim [24] repotred that the whiteness obtained from different processing protocols ranged from 24.0 to 47.3. Metin and his colleagues [25] found that whiteness index of chitosan sample from blue crab (Callinectes sapidus) was 90.23 ± 0.27.

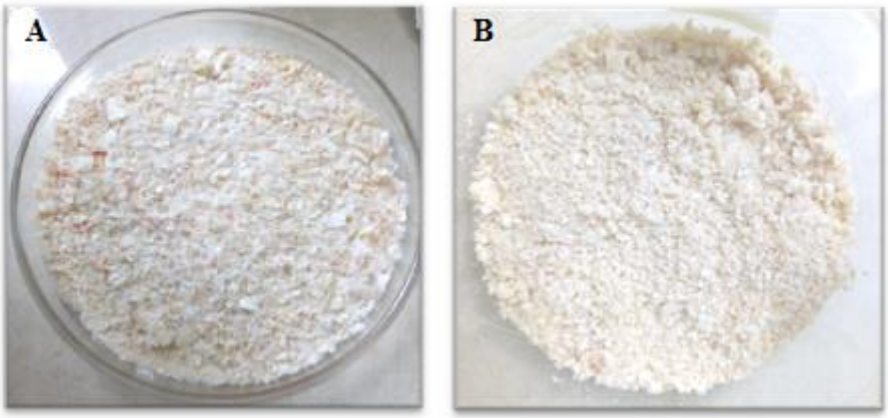

Figure 2: Chitin (A) and chitosan (B) powder.

The colour of chitosan powder varies from pale yellow to white and is reported to be associated with the content of the carotenoid pigment astaxanthin [8]. The off white colour of chitosan produced and the lower whiteness compared to the commercial chitosan and other extracted chitosan may be caused by the degradation of the pigments present in the chitin during the deacetylation step. Therefore, additioanl treatment is required to remove pigments and prepare attractive white-colored chitosan, such as organic solvents or bleaching agents [21].

3.5 Water binding capacity

Results of water binding capacity (WBC) were depicted in Table 1. There was a significant difference ($p<0.05$) in the WBC of the shell (128.67 ± 0.03%), chitin (189.33 ± 0.04%) and chitosan (652 ± 0.02 %) (Table 1). This is in line with that reported by Hamdi and his colleagues [5] who recorded a WBC of about 640.46 ± 5.52% for the same crab specie (P. segnis). The extracted chitosan from the blue crab (P. segnis) shells showed higher WBC compared to the commercial chitosan (327 ± 9.99%), extracted chitosan from the mud crab shells (180 ± 0.00%) [8] and the shrimp chitosan (582.40%) [15]. However, the WBC value of extracted chitosan from the blue crab (P. segnis) shells was lower than the value reported by Kucukgulmez and his colleagues [17] for chitosan extracted from M. sstebbingi shells (712.99%) but agrees with crawfish chitosan (660.6%) [24].

Rasweefali and his colleagues [21] reported that WBC depends on deproteinization, demineralization, and deacetylation reactions. Difference observed between WBC recorded in the present study and WBC of extracted chitosan from the mud crab shells [8] was because, in this research, the isolation process of chitin starts with demineralization followed by deproteinization. According to Sarbon and his colleagues [8] and Fernandez-kim [24], the WBC increased when the demineralization process was conducted before the deproteinization process. Moreover, a decrease in the water binding capacity of chitosan may be due to the decolouration step when it followed deacetylation [24].

3.6 Fat binding capacity

Fat binding capacity (FBC) of chitosan is one of the most important properties that determine its suitability to be used for biological functions like anti-cholesterolemic agents and as a functional food and dietary ingredient [21]. The fat binding capacity analysis was conducted using corn oil and showed a significant difference ($p<0.05$) between the shell (256.57 ± 0.78%), chitin (414 ± 0,24%) and chitosan (598,67 ± 0,09%) results (Table 1). From these findings, it can be concluted that the chitosan can easily bind or absorb fat (598,67 ± 0,09%). This is in line with that reported by Hamdi and his colleagues [5] who recorded a FBC of about 516.90 ± 6.49% for the same crab specie (P. segnis). Lower results were reported by Sarbon and his colleagues [8] for chitosan extracted from mud crabs (260±0.00%) and commercial chitosan (329 ± 7.07%). Metin and his colleagues [25] studied the properties of chitosan from blue crab shells (Callinectes sapidus) and found the fat binding capacity as 437.93 %. Kumari and his colleagues [26] reported that FBC of the chitosan synthesized from fish scales, crab and shrimp shells were 226%, 246% and 104%, respectively. Different results observed may due to several factors : sequence steps in which demineralization is conducted prior to deproteinization and vice vesra, type of oil used, the size of the chitosan particles and chitosan's molecular weight [8, 21].

3.7 Antioxidant activity

The DPPH is a stable free radical at room temperature, which has been extensively used as a tool to evaluate the free radical-scavenging activities of antioxidants based on electron and H atom transfer [4]. Figure 3 shows the scavenging ability on DPPH of extracted chitosan ranging from 20.13 ± 1.41% to 70.63 ± 1.26% and ascorbic acid ranging from 49.17 ± 1.13% to 87.46 ± 1.22% at varying concentrations (0,5 to 10 mg/mL). These results suggest that extracted chitosan exhibited notable antioxidant activity against DPPH, in a dose dependent manner, but lower than that of ascorbic acid at the same concentrations. In addition, these findings suggest that the free radicals of DPPH could react with the amine groups in the extracted chitosan to form a more stable molecules. At the same concentration (10 mg/mL), lower scavenging abilitities were observed by Metin and his colleagues [25] for chitosan extracted from blue crab shells (Callinectes sapidus) (55.3 ± 5.05%) and by Sarbon and his colleagues [8] for the commercial chitosan (28.67%) and chitosan extracted from mud crab (Scylla olivacea) 30 ± 0.001%. Hafsa and his colleagues [4] reported that the scavenging ability shrimp shell (Parapenaeus longirostris) chitosan on DPPH radicals ranged from 11.45% to 32.78% for concentrations varying from 0.25 to 1 mg/mL.

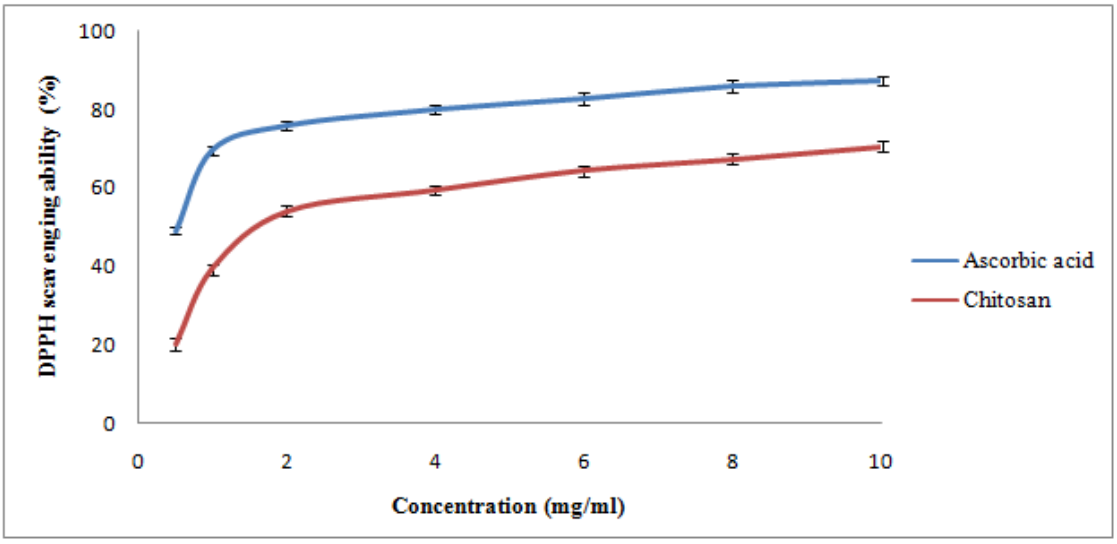

Figure 3: Scavenging ability (%) of extracted chitosan from blue crab (P. segnis) at different concentrations (0.5, 1, 2, 4, 6, 8, 10 mg/mL) on DPPH. Each value is presented as mean ± SD (n = 3).

According to Avelelas and his colleagues [27] and Aranaz and his colleagues [6], reaction mechanism between the DPPH radical and the antioxidant depends on the structural component of the antioxidant, the degree of deacetylation and the molecular weight (MW). Antioxidant activity is better when a high degree of deacetylation was recorded. Regarding the effect of the molecular weight, antioxidant properties are more remarkable for low MW samples rather than for high molecular weight ones since shorter chains form fewer intramolecular hydrogen bonds and therefore the reactive groups are more accessible, contributing to the radical scavenging activity. The half-maximal inhibitory concentration (IC_{50}) for scavenging activity on the DPPH value of chitosan extracted from blue crab was presented as 1.86 ± 0.31 mg/mL. This result is in agreement with that found by Hamdi and his colleagues [5] for extracted chitosan from blue crab (P. segnis) (1.76 ± 0.25 mg/mL). Sarbon and his colleagues [8] reported higher IC_{50} value of chitosan obtained from mud crab of 11.37 mg/mL. In the other hand, lower IC_{50} values of chitosan extracted from shrimp Metapenaeus monoceros (1.62 mg/mL) [28] was depicted. The results obtained in the present study suggest that the blue crab shells can be considered as good raw material for the manufacturing of chitosan products towards antioxidant applications.

3.8 Antimicrobial activity

The antimicrobial activity of extracted chitosan from blue crab shells was assessed using well agar diffusion method at two different concentrations of extracted chitosan (25 mg/mL (C25) and 50 mg/mL (C50)) (Figure 4).

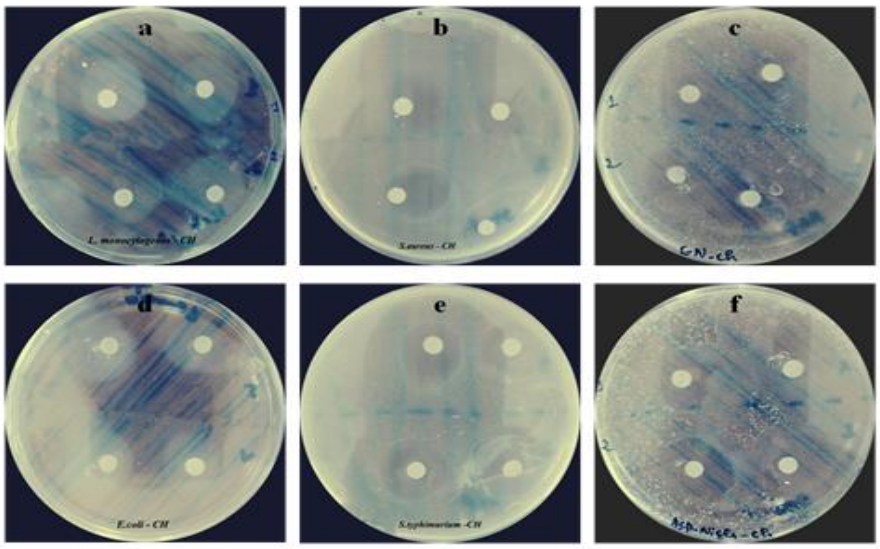

Figure 4: Antimicrobila potential of blue crab chitosan. a : L. monocytogenes ; b : S. aureus ; c : Geotrichum candidum; d : E. coli ; e : S. typhimurium ; f : Aspergillus niger.

Antibacterial activity of chitosan was evaluated against four common pathogenic bacteria, the Gram negative strains Escherichia coli and Salmonella typhimurium and the Gram positive strains Listeria monocytogenes and Staphylococcus aureus (Table 2). Results revealed that chitosan was effective against all tested bacteria. Inhibition zone diameters, in the range of 9,53 ± 0,01 mm to 16,04 ± 0,05 mm and 10,52 ± 0,03 mm to 17,65 ± 0,53 mm were reached at 25 mg/mL and 50 mg/mL of chitosan, respectively. The highest potency of chitosan against bacteria was detected toward L. monocytogenes (17.65 ± 0.53 mm) and S. typhimurium (11,20 ± 0,28 mm), for Gram+ and Gram- strains, respectively, at 50 mg/mL of chitosan.

Table 2: Antibacterial and antifungal activities of blue crab chitosan**.**

Microorganisms tested	**Diameter of inhibition zone (mm)**	
	C25	**C50**
Bacteria		
Escherichia coli	$9,53 \pm 0,01^{a}$	$10,80 \pm 0,11^{b}$
Salmonella typhimurium	$11,20 \pm 0,28^{b}$	$10,52 \pm 0,03^{a}$
Staphylococcus aureus	$13,06 \pm 0,08^{c}$	$15,04 \pm 0,05^{d}$
Listeria monocytogenes	$16,04 \pm 0,05^{d}$	$17,65 \pm 0,53^{f}$
Fungi		
Aspergillus niger	$13,03 \pm 0,04^{c}$	$16,03 \pm 0,04^{e}$
Geotrichum candidum	$11,07 \pm 0,09^{b}$	$14,13 \pm 0,04^{c}$

C25 : Concentration of chitosan solution tested (25 mg/mL) ; **C50 :** Concentration of chitosan solution tested (50 mg/mL). Values are means±SD of three determinations. Means with different letters within a column indicate significant difference ($p < 0.05$).

The antifungal activity of chitosan against two different pathogenic fungi species, Aspergillus niger and Geotrichium candidum, was evaluated (Table 2). Chitosan synthesized inhibited effectively the growth of A. niger and G. candidum as evidenced by the inhibition zone measured. The highest potency of chitosan against fungi was detected toward A. niger (13,03 ± 0,04 mm (C25) and 16,03 ± 0,04 mm (C50)) then G. candidum (11,07 ± 0,09 mm (C25) and 14,13 ± 0,04 mm (C50)). Several mechanisms have been proposed for the antimicrobial action of chitosan. It has been suggested that chitosan seems to have a growth-inhibitory activity. This could be caused by the formation of an external barrier, chelating metals and provoking which reduce the membrane permeability and blocks cell access to nutrients [6]. Another hypothesis explains the antibacterial mechanism of chitosan is the ionic surface interaction of the protonated amino groups with the negatively charged cell wall surface of microorganisms resulting in wall cell leakage [4]. Also, It has also been hypothesized that chitosan's antibacterial activityis caused by the inhibition of the mRNA and protein synthesis via the penetration of chitosan into the nuclei of the microorganisms [4] According to Zouhour and his colleagues [29], chitin and chitosan have a significant effect against pathogenic Candida species. Their activities against fungus are assumed to be fungi-static rather than fungicidal. Similar to the effects observed in bacteria cells, chitin and chitosan interfer directly with fungal growth. The antifungic mechanism of these biopolymers involves cell wall morphogenesis. Antimicrobial activity mechanisms of chitosan remained debatable and several hypothesis were proposed, depending on the lack of appropriate polymer characterization, purity issues, the lack of methodological uniformity and the use of different microorganisms. Therefore, further studies are required to explain the specific mechanism of chitosan [6].

4. Conclusion

In the present study, chitin and chitosan were extracted from blue crab (P. segnis) shells using chemical method. Demineralization and deproteinzation processes were used for chitin extraction followed by deacetylation step to obtain chitin derivatives that known as chitosan. The adapted method was effective for obtaining highly purified chitosan with significant outcomes. The degree of deacetylation calculated by potentiometric titration and FTIR method was 78.97 ± 0.38 % and 81,47 ± 0.51 %, respectively. From these results, blue crab shells from P. segnis can be considered a good source of chitosan. Whiteness value, water binding and fat binding capacities of the extracted chitosan were high and are suitable for many functional food applications. Furthermore, chitosan was found to possess interesting antioxidant proprieties using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. Moreover, extracted chitosan showed a significant antimicrobial activity against two Gram-positive (Listeria monocytogenes and Staphylococcus aureus) and two Gram-negative bacteria (Escherichia coli and Salmonella typhimurium), as well as against two fungi (Geotrichum candidum and Aspergillus niger). According to these findings, it can be concluded that chitosan extracted from blue crab shells is a potentially effective biopolymer which can be used as a natural additive in different fields such as medicinal and food industries. Valorization of blue crab shell wastes will minimize the discard of this potential natural resource, thus will reduce the negative environmental impacts and pollution.

5. Recommendations

The chitosan extracted and its active components could be potential candidates to be used as natural alternatives

for further application in food preservation to limit the oxidation phenomenon, inhibit the bacterial growth and to extend the shelf life of the food products. However, the confirmation of antioxidant and antimicrobial efficiencies and organoleptic impact of the chitosan in food stuffs need to be evaluated.

Acknowledgement

Authors are grateful to the financial support provided by the High Graduate School of Food Industry of Tunisia for carrying out this research.

References

[1] M. Shaiek, R. El Zrelli, F. Crocetta, L. Mansour and L. Rabaoui, L. ‘‘On the occurrence of three exotic decapods, Callinectes sapidus (Portunidae), Portunus segnis (Portunidae), and Trachysalambria palaestinensis (Penaeidae), in northern Tunisia, with updates on the distribution of the two invasive portunids in the Mediterranean Sea,’’ BioInvasions Records, vol. 10, Article in press, 2021.

[2] M. Hamdi, A. Hammami, S. Hajji, M. Jridi, M. Nasri and R. Nasri. ‘‘Chitin extraction from blue crab (Portunus segnis) and shrimp (Penaeus kerathurus) shells using digestive alkaline proteases from P. segnis viscera,’’ International Journal of Biological Macromolecules, vol. 101, pp. 455-463, 2017.

[3] C. Webster, O. Onokpise, M. Abazinge, J. Muchovej, E. Johnson and C. Louime. ‘‘Turning waste into usable products : A case study of extracting chitosan from blue crab,’’ American Journal of Environmental Sciences, vol. 10(4), pp. 357-362, 2014.

[4] J. Hafsa, M. A. Smach, B. Charfeddine, K. Limem, H. Majdoub and S. Rouatbi. ‘‘Antioxidant and antimicrobial proprieties of chitin and chitosan extracted from Parapenaeus Longirostris shrimp shell waste,’’ Annales Pharmaceutiques Françaises, vol. 74(1), pp. 27-33, 2015.

[5] M. Hamdi, S. Hajji, S. Affes, W. Taktak, H. Maâlej, M. Nasri and R. Nasri. ‘‘Development of a controlled bioconversion process for the recovery of chitosan from blue crab (Portunus segnis) exoskeleton,’’ Food Hydrocolloids, vol. 77, pp. 534-548, 2018.

[6] I. Aranaz, A. R. Alcántara, M. C. Civera, C. Arias, B. Elorza, A. H. Caballero and N. Acosta. ‘‘Chitosan: An Overview of Its Properties and Applications,’’ Polymers, vol. 13, pp. 3256, 2021.

[7] M. Hamdi, R. Nasri, S. Li and M. Nasri. ‘‘Bioactive composite films with chitosan and carotenoproteins extract from blue crab shells: Biological potential and structural, thermal, and mechanical characterization,’’ Food Hydrocolloids, vol. 89, pp. 82-812, 2019.

[8] N. M. Sarbon, S. Sandanamsamy, S. F. S. Kamaruzaman and F. Ahmad. ‘‘Chitosan extracted from mud crab (Scylla olivicea) shells: physicochemical and antioxidant properties,’’ Journal of Food Science and Technology, vol. 52(7), pp. 4266-4275, 2014.

[9] M. A. Norhidayah, M. Faridah, A. Azrilawani and A. A. Alyza. ‘‘Chitin and chitosan extraction from Portunus pelagicus,’’ Malaysian Journal of Analytical Sciences, vol. 21(4), pp. 770-777, 2017.

[10] AOAC. 1990. Official methods of analysis of the Association of Official Analytical Chemistry, 15th edition. The Association of Official Analytical Chemistry: Washington, DC, 1990 Inc.

[11] J. Folch, M. Lees and G. H. S. Stanley. ‘‘A simple method for the isolation and purification of total lipides from animal tissues,’’ The Journal of Biological Chemistry, vol. 226, pp. 497-509, 1957.

[12] S. Kumari and P. K. Rath. ‘‘Extraction and Characterization of Chitin and Chitosan from (Labeo rohit) Fish Scales,’’ Procedia Materials Science, vol. 6, pp. 482-489, 2014.

[13] Md. R. Hussain, M. Iman and T. K. Maji. ‘‘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 Advanced Engineering Applications, vol. 2(4), pp. 4-12, 2013.

[14] S. Seo, J. M. King and W. Prinyawiwatkul. ‘‘Simultaneous depolymerisation and decolorization of chitosan by ozone treatment,’’ Journal of Food Science, vol. 72(9), pp. 522–526, 2007.

[15] F. C. K. Ocloo, E. T. Quayson, A. Adu-Gyamfi, E. A. Quarcoo, D. Asare, Y. Serfor-Armah et al. ‘‘Physicochemical and functional characteristics of radiation-processed shrimp chitosan. Journal for Radiaiont’’, Physics and Chemistry, vol. 80, pp. 837–841, 2011.

[16] S. Hirano. ‘‘Production and Application of Chitin and Chitosan in Japan’’ in Chitin and Chitosan: Sources, Chemistry, Biochemistry, Physical Properties and Applications’’ G. Skjak-Braek, T. Anthonsen and P. Sanford, Ed. Elsevier Science Publishers, Essex, 1989, pp. 37-43.

[17] A. Kucukgulmez, M. Celik and Y. Yanar. ‘‘Physicochemical characterization of chitosan extracted from Metapenaeus stebbingi shells,’’ Journal of Food Chemistry, vol. 126(3), pp. 1144-1148, 2011.

[18] H. K. No and S. P. Meyers. ‘‘Preparation and Characterization of Chitin and Chitosan-A Review,’’ Journal of Aquatic Food Product Technology, vol. 4(2), pp. 27-52, 1995.

[19] W. Tan, Q. Li, F. Dong, L. Wei and Z. Guo. ‘‘Synthesis, characterization, and antifungal property of chitosan ammonium salts with halogens,’’ International Journal of Biological Macromolecules, vol. 92, pp. 293-298, 2016.

[20] H. Martín-López, S. C. Pech-Cohuo, E. Herrera-Pool, N. Medina-Torres, J. C. Cuevas-Bernardino, T. Ayora-Talavera et al. ‘‘Structural and Physicochemical Characterization of Chitosan Obtained by UAE and Its Effect on the Growth Inhibition of Pythium ultimum,’’ Agriculture, vol. 10(10), pp. 464, 2020.

[21] M. K. Rasweefali, S. Sabu, K. V. Sunooj, A. Sasidharan and K. A. Martin Xavier. ‘‘Consequences of

chemical deacetylation on physicochemical, structural and functional characteristics of chitosan extracted from deep-sea mud shrimp,'' Carbohydrate Polymer Technologies and Applications, vol. 2, pp. 100032, 2021.

[22] S. Kumari, P. Rath, A. S. H. Kumarb and T. N. Tiwari. ''Extraction and characterization of chitin and chitosan from fishery waste by chemical method,'' Environmental Technology & Innovation, vol. 3, pp. 77-85, 2015.

[23] M. R. Kasaai. ''Various methods for determination of the degree of N-acetylation of chitin and chitosan: a review,'' Journal of Agricultural and Food Chemistry, vol. 57(5), pp. 1667-76, 2009.

[24] S. O. Fernandez-kim. ''Physicochemical and functional properties of crawfish chitosan as affected by different processing protocols''. Dissertation of MSc. Graduate Faculty of Seoul National University, 2004, pp. 107

[25] C. Metin, Y. Alparslan, T. Baygar and T. Baygar. ''Physicochemical, Microstructural and Thermal Characterization of Chitosan from Blue Crab Shell Waste and Its Bioactivity Characteristics,'' Journal of Polymers and the Environment, vol. 27, pp. 2552-2561, 2019.

[26] S. Kumari, S. H. K. Annamareddy, S. Abanti and P. K. Rath. ''Physicochemical properties and characterization of chitosan synthesized from fish scales, crab and shrimp shells,'' International Journal of Biological Macromolecules, vol. 104, pp. 1697-1705, 2017.

[27] F. Avelelas, A. Horta, L. F. V. Pinto, S. C. Marques, P. M. Nunes, R. Pedrosa et al. ''Antifungal and Antioxidant Properties of Chitosan Polymers Obtained from Nontraditional Polybius henslowii Sources,'' Marine Drugs, vol. 17, pp. 239, 2019.

[28] I. Younes, S. Hajji, V. Frachet, M. Rinaudo, K. Jellouli and M. Nasri. ''Chitin extraction using enzymatic treatment. Antitumor, antioxidant and antimicrobial activities of chitosan,'' International Journal of Biological Macromolecules, vol. 69, pp. 489-498, 2014.

[29] L. Zouhour, S. Salah, S. Saloua and E. A. Amor. ''Extraction and characterization of chitin and chitosan from crustacean by-products:biological and physicochemical properties,'' African Journal of Biotechnology, vol. 10(4), pp. 640-7, 2011.