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ORIGINAL PAPER

Enhancement and Characterization of Chitosan Extraction from the Wastes of Shrimp Packaging Plants

A. Alishahi · A. Mirvaghefi · M. R. Tehrani · H. Farahmand · S. A. Shojaosadati · F. A. Dorkoosh · Maher Z. Elsabee

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Abstract Chitin is the second most abundant carbohydrate polymer in nature next to cellulose. The main source of chitin is crustacean shells. Chitosan, derived from chitin by alkaline deacetylation, characteristically plays an important role in applications. Several parameters determine the chitosan specifications, namely, chitosan sources, incubation time, pretreatment conditions, temperature and acid and alkaline treatment. Chitosan extraction has been achieved by microwave heating and compared with that of conducting the deacetylation in an autoclave. High molecular weight chitosan (as determined by viscosity measurements of chitosan in dilute acetic acid solution), white color, high water binding capacity (WBC) and fat binding capacity (FBC) has been obtained by microwave treatment. In addition, microwave treatment saves a large amount of energy (due to the shorter time of heating) which is a very important factor for commercial productions. The data extracted from X-ray diffraction, nuclear magnetic resonance and Fourier transforms infrared spectroscopy have shown more crystalline and higher DDA of chitosan produced in microwave than the one in autoclave. Moreover, the antibacterial activity of chitosan prepared by microwaves was higher than that produced using the autoclave.

Keywords Chitosan · Color characteristics · Deacetylation · Viscosity · Crystallinity · Antibacterial properties

A. Alishahi (✉) · A. Mirvaghefi · H. Farahmand
Department of Fisheries, The University of Agriculture and Natural Sciences of Gorgan, Gorgan, Iran
e-mail: aalishahi@ut.ac.ir

M. R. Tehrani · F. A. Dorkoosh
Department of Pharmaceutics, Tehran University of Medical Sciences, Tehran, Iran

S. A. Shojaosadati
Department of Chemistry and Engineering, The University of Tarbirt Modares, Tehran, Iran

M. Z. Elsabee
Department of Chemistry, Faculty of Science, Cairo University, Cairo 12613, Egypt

Introduction

Chitin is a linear polysaccharide of β-(1–4)-2-acetamido-2-deoxy-D-glucopyranose. Chitosan is the most important derivative of chitin, a linear polymer of β-(1–4)-2-amino-2 deoxy-D-glucopyranose. The major sources of chitin and chitosan are the exoskeletons of invertebrates, such as arthropods, mollusks, annelids, fungi and algae. They are associated with other constituents, such as lipids, calcium carbonate, proteins and pigments. Great deal of crustacean and mollusk skeletons and shells are discarded annually by processing plants as processing leftovers. The estimation of chitin present in the sea amounts to 1,560 million tons [1]. Fungal chitin and chitosan have some advantages as compared with animal ones, such as a greater uniformity in composition, continuous availability and the absence of inorganic salts in their matrices. However, fungal chitin is associated with other polysaccharides, namely, cellulose, glucan, mannan and polygalactosamine, which make its isolation difficult [2]. Therefore, exploitation of chitin and chitosan from the shrimp processing factories has commercial privileges due to low cost of production and high price of the products. On the other hand, shrimp shell contains the other commercial compounds such as

astaxanthin, carotenoid that has so far not been synthesized, and is marked as a fish food additive in aquaculture.

There are three methods of chitosan extraction:

1. biological extraction process [3–5]
2. enzymatic hydrolysis [6] and
3. chemical process [7]

The objective of the present study is to enhance the extraction of chitin from the shrimp processing wastes and ultimately to produce chitosan with a high deacetylation degree, high viscosity in solution and bright color. Two processes have been tried and compared to produce chitosan using an autoclave and a microwave for heating.

Methods and Materials

Isolation of Chitin

Chitin was isolated from the Black Tiger shrimp (*Metapeneus monodon*) processing leftovers. The species is native to Southeastern Asia. Black Tiger shrimp is profusely farmed in Asia and the other tropical and subtropical waters due to high growth rate, high resistance to the changes of temperature, salinity and oxygen and low mortality. Black Tiger shells are a source of a hazardous pollution in shrimp farming, since they flocculate on the pond bottom and cause water contamination and disease. Further, they are also wastes in the shrimp packaging plants due to a very low decomposition rate.

The shells were collected, washed, and grounded to final 300-μm sieve size, then subjected to demineralization and deproteinization.

Demineralization

Demineralization was carried out in dilute HCL solution [7]. All samples were treated with 1 M HCl solution at ambient temperature with a solution-to-solid ratio of 20 mL/g.

Deproteinization

Deproteinization was implemented using alkaline treatments with 1 M sodium hydroxide solution at 70 °C. Solution-to-solid ratio was 20 mL/g [7]. At last, to remove any impurities, pigments and other unpleasant materials, it was soaked in ethanol (10 mL/g) for 3 h and later boiled in acetone (10 mL/g) for another 3 h. The purified chitin was then dried and weighed. Ash content of purified chitin was evaluated by burning the samples at 600 °C in a kiln.

Deacetylation

After being steeped in strong sodium hydroxide at room temperature [8] for 1 day, the samples were heated in an autoclave for 0.3, 1, 1.5, 2 and 2.5 h at pressure of 2 Pa. In the microwave, the treatments were 5, 10, 15 and 20 min at 400 W.

After deacetylation, the samples were washed several time with distilled water to neutral pH and then freeze-dried.

Determination of Yield, Moisture, Ash of Chitosan

Chitosan yield was determined as the weight differences between the raw materials and the chitosan obtained after ultimate treatment. Moisture content of the samples was determined by drying the samples at 60 °C for 24 h or until constant weight. The ash content was determined by burning the sample (1 g), at 600 °C in a kiln. The difference between the initial and final weights gives the ash content.

Measurement of Viscosity and Color

Viscosity was assessed using a Brookfield viscometer, model LVDV-II + (Brookfield Engineering Labs, Stoughton, MA, USA). In brief, 1% chitosan solution was prepared using 1% acetic acid. Measurement was made using a No. 5 spindle at 50 rpm at 25 °C with values reported in centipoises (cPs) units. Intrinsic viscosity was used to calculate the viscosity average molecular weight for the prepared samples from the Mark-Houwink-Sakurada equation:

$$[\eta] = KM^a$$

where K and a are 0.078 and 0.76, respectively [9].

Color measurements were evaluated with a portable Minolta Chroma Meter CR-200 (Minolta Camera Co. Ltd, Osaka, Japan) using standard illuminant C or D_{65}, and reported as L^* (lightness), a^* (+for redness—for greenness)and b^* (yellowness). The measurements were implemented at different locations on each sample.

Water Binding Capacity and Fat Binding Capacity

Water Binding Capacity (WBC) and Fat Binding Capacity (FBC) of chitosan were measured using the method of No et al. [10].

The method was carried out by weighing a centrifuge tube containing 0.5 g sample, adding 10 mL of water or olive oil, and mixing on a vortex mixer for 1 min to disperse the sample. The contents were left at ambient temperature for 30 min with shaking every 5 s for 10 min and

centrifuged at 3,200 rpm for 25 min. The supernatant was decanted and the tube was weighed again. WBC and FBC were evaluated using the following formula:

$$\text{WBC}\,(\%) = [\text{water bound (g)}/\text{sample weight (g)}] \times 100$$

$$\text{FBC}\,(\%) = [\text{fat bound (g)}/\text{sample weight (g)}] \times 100$$

Determination of the Deacetylation Percent

Titration

The deacetylation (DDA %) percent was determined by the titration method in which chitosan was dissolved in 0.1% acetic acid to form a 0.01% solution. This was followed by titration with 0.0025 N potassium polyvinyl sulfate with 1% toluidine blue (TBO) as an indicator. The acetyl content of chitosan was measured from the amount of titrant used [11].

Elemental Analysis

The DDA% of chitosan samples was calculated using the following formula [12]:

$$\text{DDA\%} = \frac{6.857 - \text{C/N}}{1.7143} \times 100$$

where C/N is the ratio carbon/nitrogen as determined using elemental analysis.

Fourier Transforms Infrared Spectroscopy (FTIR)

Infrared spectra were measured using KBr pellets in the transmission mode in the range 400–4,000 cm^{-1} by Perkin-Elmer 2000 spectrophotometer. The DDA of the samples were calculated from the IR spectra following the method of Brugnerotto et al. [13]:

$$[\text{DA\%} = 31:92 \times (A_{1320}/A_{1420}) - 12:20];\ r = 0:990$$

Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) spectra were recorded by Bruker AVANCE II 600 spectrometer in 2% deuterated acetic acid in D_2O solution.

For the determination of DDA% the method of Lavertu et al. [14] was performed. It is the following equation:

$$\text{DDA}\,(\%) = \left(\text{H}_{4.9}/\text{H}_{4.9} + \text{H}_{2.06/3}\right) \times 100$$

where $\text{H}_{4.9}$ is the peak at 4.9 ppm and $\text{H}_{2.06/3}$ the peak at 2.06 and 3 ppm.

Finally, the DDA% of the chitosan samples was measured using the average of the above methods.

X-ray Diffraction

X-ray diffraction measurements of the chitosan samples were carried out using Scintag powder diffractometer with 2θ angles of 5° and 40°. Ni-filtered Cu K α-radiation was utilized as the X-ray source. X-ray diffraction was used to determine the crystallinity of the extracted samples of chitosan. To calculate the crystallinity of the samples, the area of the crystalline peaks is divided by total area under the curve.

Scanning Electron Microscopy

The surface specifications and morphology of chitosan were detected by scanning electron microscopy (SEM). The dried sample of chitin and chitosan was ground and then coated with gold under vacuum using a sputter coater. The SEM was performed by a JEOL JSM-630 J scanning electron microscope operated at 20 kV.

Antibacterial Scanning

Chitosan solution (1% w/v) in acetic acid 1% was dispersed in brain heart infusion (BHI) broth to give final chitosan concentration of 0.1% (w/w). Subsequently, each bacterium (25 μL) was inoculated into BHI broth (5 mL) containing the chitosan solution. Following bacterial inoculation, tubes incubated at 37 °C for 24 h. A 0.1 mL dilution of the broth was spread plated onto BHI agar and incubated at 37 °C for 24 h for later colony counting.

Statistical Analysis

All experiments were carried out in triplicate and the results were expressed as mean ± SD. Analysis of variance was performed using SPSS statistical package program (SPSS 13.0 for windows, SPSS Inc., Chicago, IL).

Results and Discussion

Chemical Composition

The raw material (Black Tiger shrimp) consisted of 50.12 ± 0.01% $CaCO_3$, and 25.45 ± 0.02% protein and 24.33 ± 0.19% chitin. A considerable portion of the raw materials is composed of minerals; therefore, this could be utilized commercially as mineral additives to animal and aquatic feeds in addition to the relatively high protein content which could be also added into the animal feeds [15].

The duration of the demineralization stage depends on mineral content of chitosan sources; the larger mineral

content the greater the demineralization period required [16]. The demineralization period in this study was 3 h to produce chitosan with desirable ash content of 0.45 ± 0.02%, moisture of 2.5 ± 0.11% and a yield of 15.25 ± 0.90%. Generally, chitosan with the moisture content less than 10% is suitable [17].

Viscosity and Color of Chitosan

Color L^*, a^* and b^* values of chitosan samples prepared in autoclave and microwave are shown in Figs. 1 and 2 respectively. The L* values increases with time of treatment in the autoclave and the microwave while the a* and b* values decrease with time.

The data show that when the incubation time increases, the obtained chitosan shows lighter appearance. The latter characteristic is very important for commercial production and customer satisfaction.

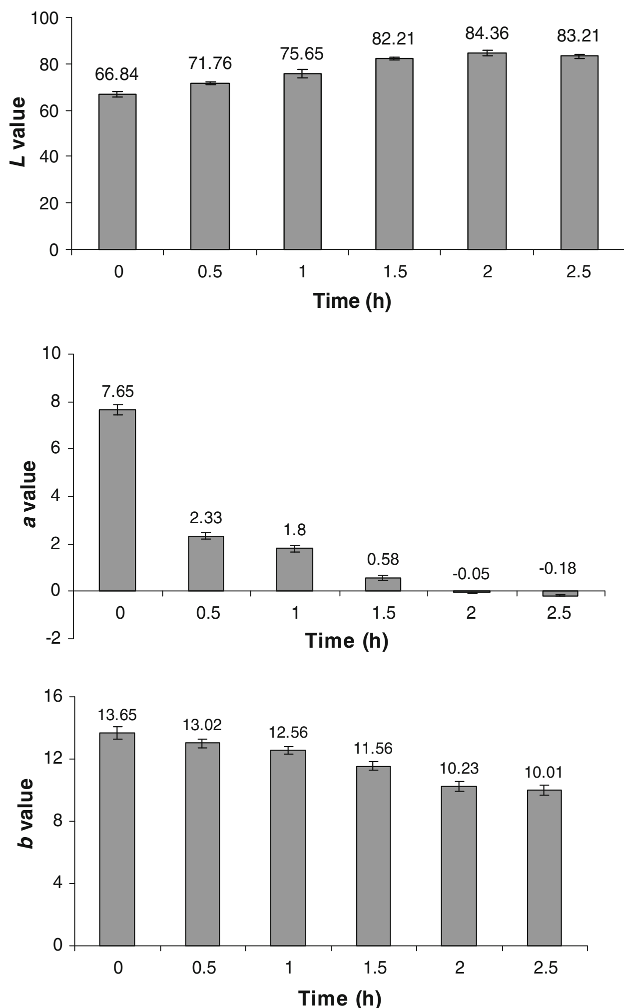

Fig. 1 Color L^*, a^* and b^* values of chitosan samples prepared in autoclave in different times

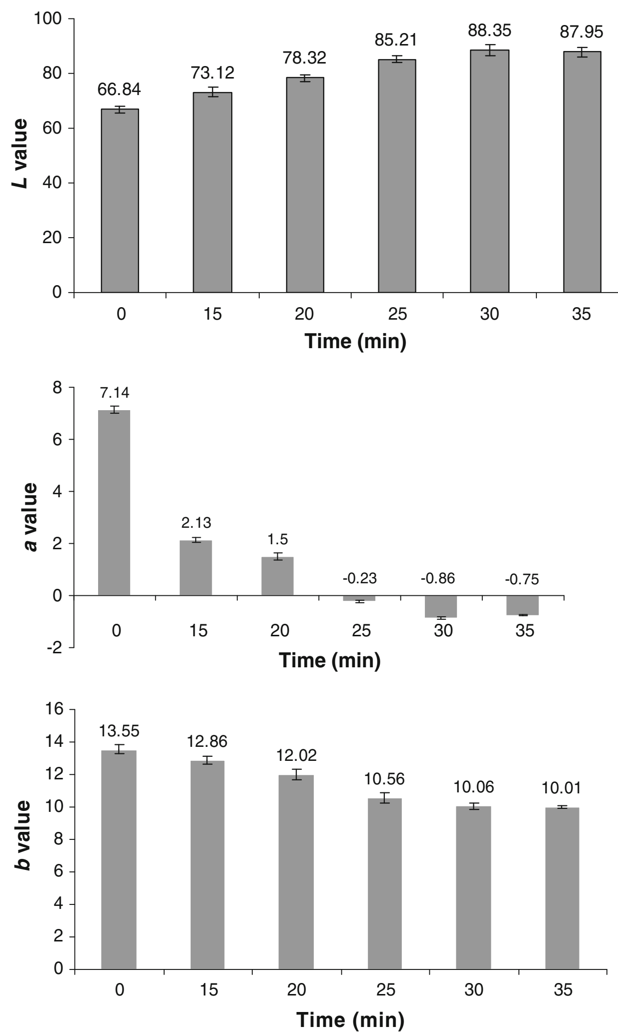

Fig. 2 Color L^*, a^* and b^* values of chitosan samples prepared in microwave in different times

The use of the microwave for the deacetylation of chitin has two advantages over that of using the autoclave: first the incubation time in microwave has diminished from 1 to 2 h to several minutes (15–30 min); which means lower energy consumption.

The second advantage of using microwave is the better color of the resulting chitosan after boiling in ethanol and acetone respectively. References [18, 19] discussed the various bleaching agents on the discoloration stage of chitosan. The literature contains no report on the effectiveness of autoclave and microwave treatments on chitosan color.

The most probable reason for this outcome may be due to microwave rays oxidizing the existing pigments in crustacean shell such as astaxanthin which contain double bonds and eliminating thus the unpleasant colors [18, 19].

Figure 3a, b depict the impact of microwave and autoclave heating times on the viscosity value, respectively.

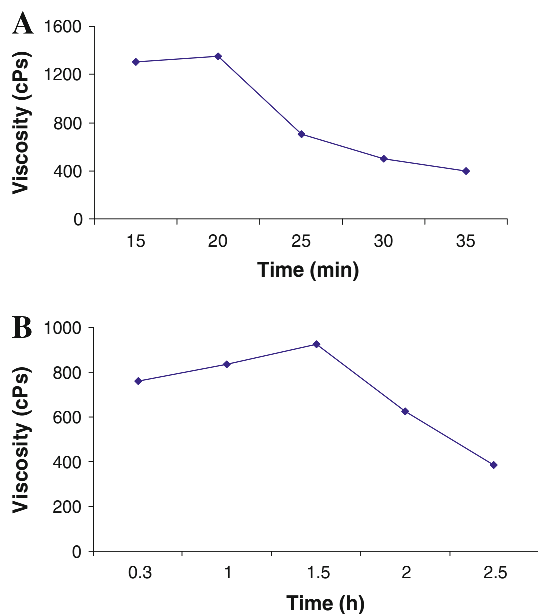

Fig. 3 Effect of microwave time (**a**) and autoclave time (**b**) on the viscosity

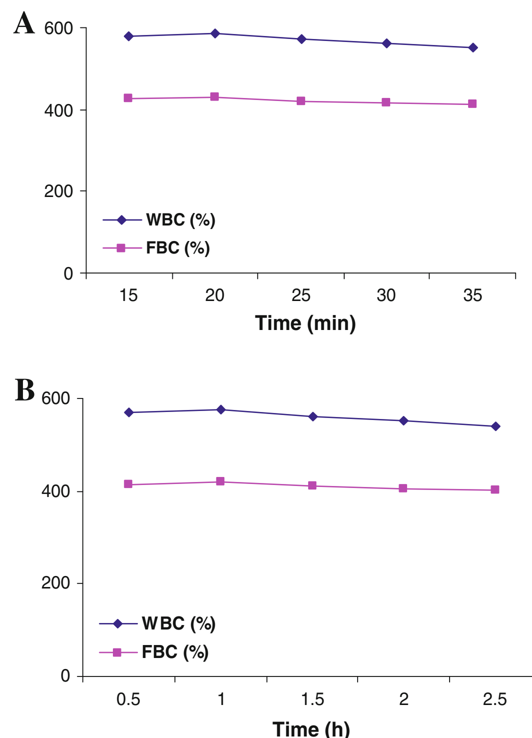

Fig. 4 Effect of microwave (**a**) and autoclave (**b**) times on WBC and FBC, respectively

As shown in Fig. 3, the viscosity of 1% chitosan solution slightly increases to some extent then declines rapidly with further heating. The maximum increase in using the microwave was more (1,520 centipoises) than that of the autoclave (925 centipoises). The latter result demonstrates that to produce chitosan with high viscosity, microwave is better than autoclave. Al-sagheer et al. [20] showed a similar result.

In many applications of chitosan, high molecular weight is preferred; for instance, in extending the shelf life of foods such as bread [21], pork [22] and fish. Jeon et al., [23] found that chitosan solutions with high viscosity have shown better preservative effect on foods. Abdou et al. [7] demonstrated that viscosity decreased during autoclave heating time and show no increase of viscosity during autoclave times. The hydrolysis and viscosity of chitosan solutions have shown direct and indirect correlations with the heating time, temperature and alkaline concentration [10, 18, 19, 23].

Water Binding Capacity and Fat Binding Capacity

As shown in Fig. 4a, b, WBC and FBC of chitosan prepared in microwave were higher than that obtained in the autoclave. No significant differences in WBC [(570–595%) and FBC (428–397%)] as a function of the heating time were observed in chitosan samples prepared by microwave or by the autoclave [WBC (510–547%) and FBC (380–404%)]. Cho et al. [24], No et al. [10], and Youn et al. [18, 19] have presented the same outcomes. According to Cho et al. [24], WBC and FBC of five commercial chitosan products were in the range of 458–805 and 314–535%, respectively.

Scanning Electron Microscopy

Figure 5 shows SEM photographs of powdered chitin and chitosan obtained from the microwave and the autoclave. A very uniform concentrated structure and laminated characteristic were observed for chitin, while the surface of chitosan obtained in both methods seems homogeneous that may reflect less crystalline structure than chitin.

Determination of Chitosan Degree of Deacetylation

Figure 6a, b demonstrates the outcomes of deacetylation of chitosan obtained by the two methods, the autoclaves and the microwave.

According to Kurita [8], steeping of chitin for 1 day in strong sodium hydroxide (45%) at room temperature before implementing the heating methods applied in the present study, can highly facilitate the deacetylation of chitin. Generally, the deacetylation increases rapidly in the preliminary stages of heating in both the autoclave and the microwave, followed by a slow increase until a plateau is obtained. Longer heating times lead usually to higher percentage of DDA. A maximum 93% is reached after 3 h of refluxing in the autoclave and a 95% DDA is obtained

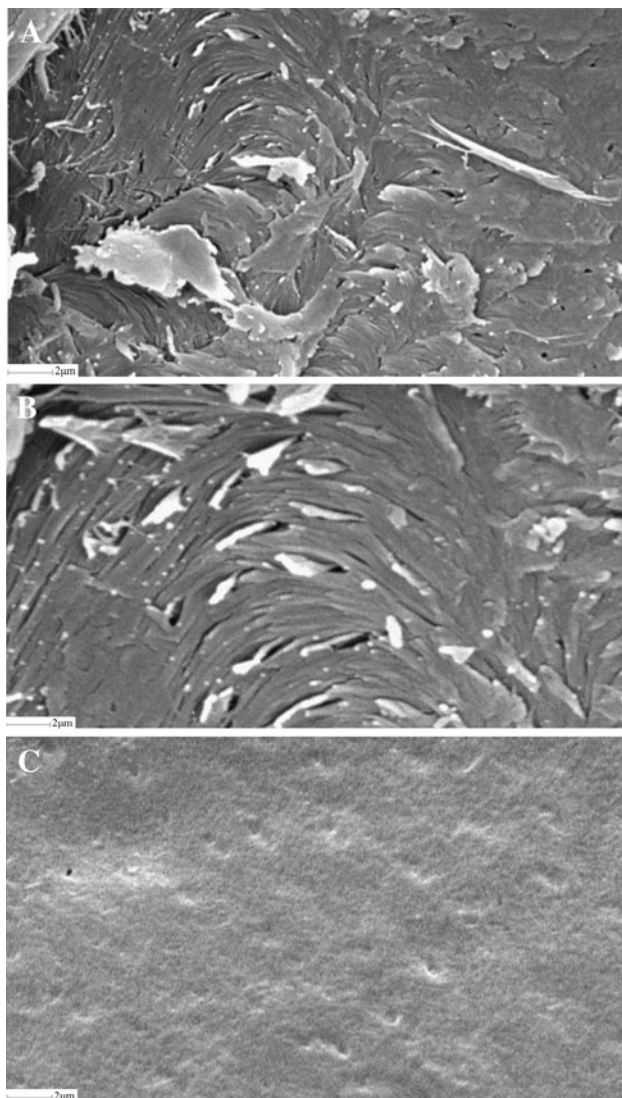

Fig. 5 SEM photograph of chitin (**a**) and chitosan obtained by microwave (**b**) and autoclave(**c**)

after about 35 min in the microwave oven. If performed on the commercial scale, microwave heating should save large amount of energy. Al-Sagheer et al., [20] demonstrated that a maximum of DDA percentage (87.5–93%) was obtained in microwave heating, in about 15 min. This could be due to the higher microwave power used in their work (600 W versus 400 W in the present work).

One point must be emphasized here is that while the DDA percentage increases with the heating time the viscosity of subsequently obtained chitosan solution decreases, this can be explained by the adverse effect of heating on the polymer chains leading to more chain cession and consequently decreasing the viscosity of chitosan solutions. These two parameters are of a paramount importance characterizing the final chitosan products and will

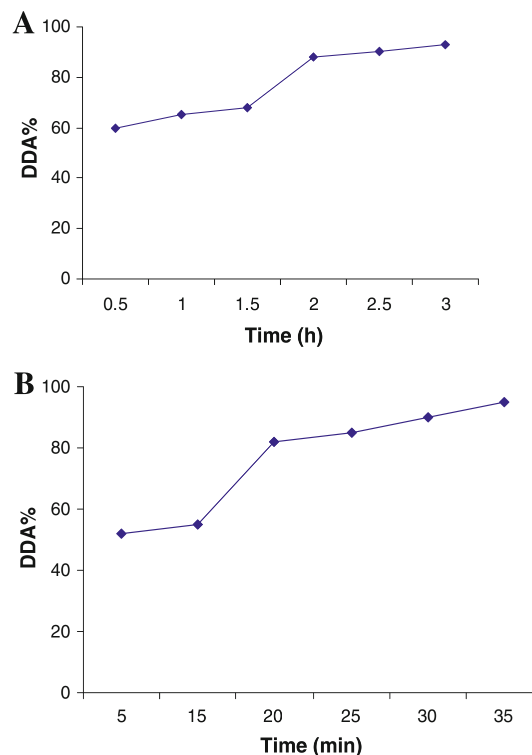

Fig. 6 DDA % of chitosan prepared in autoclave (**a**) and microwave (**b**)

determine its applicability for various purposes. Therefore, a compromise should be reached in order to optimize the physical characteristics of the prepared chitosan. [16].

XRD Spectrum

The crystallinity of chitin was reduced after the deacetylation reaction. In addition, the crystallinity of chitosan prepared in microwave was higher than the one prepared in autoclave.

The XRD data (similar to references [7 and 20]) demonstrated the sharpness of the band is higher in chitin samples than the chitosan. Moreover, the crystallinity of chitosan prepared in microwave is slightly higher than that in the autoclave. Harish Prashanth et al. [25] confirmed these results. They illustrated that the band at $2\theta = 9.9°$ for the chitosan obtained from squid pens (β-chitin) decreases dramatically after deacetylation. Kurita et al. [26] argued that the crystallinity and structure of chitin and chitosan were completely relevant to inter and intramolecular hydrogen bonding [27] and Rinaudo [28] demonstrated that whenever intermolecular (CO···HN) and intramolecular ($CO \cdots HOCH_2$) hydrogen bonding were higher, the chitosan showed higher crystallinity and more tightly ordered structure.

NMR

The NMR pattern of chitosan shown in Fig. 7 indicates that the samples prepared in the microwave have smaller bands at 2.5 ppm, a characteristic for the remaining acetyl groups of chitin, than those prepared in the autoclave. Therefore, as mentioned above, chtiosan prepared in microwave has higher level of deacetylation than the one prepared in autoclave [20, 29].

Antibacterial Activity

As illustrated in Fig. 8, the antibacterial activity of chitosan prepared in the microwave was higher than the one prepared in the autoclave. In addition, chitosan obtained from both methods had a stronger antibacterial effect against *Staphylocococcus aureus* than *Salmonella typhimurium.* As the heating time increased in both microwave and autoclave, the antibacterial activity against *S. aureus* decreased from 3.5 to 4.53 log CFU/mL and 4.23–5.35 log CFU/mL, respectively. On the other hand, the bactericidal effect of chitosan prepared in microwave and autoclave against *S. Typhimurium* has increased with the increase of heating time from 6.23 to 5.85 and 7.21–6.91 log CFU/mL, respectively. According to Yang et al. [30], *S. aureus* was most sensitive to chitosan and its derivatives in the late-exponential phase. Many authors [23, 31–33] have proposed that chitosan and its derivatives hinder the growth of most bacteria, even though their inhibitory effects depend on the molecular weight of chitosan and the species of bacteria. Generally, chitosan demonstrated stronger antibacterial effects on gram-positive bacteria than on gram-negative bacteria. The outcomes of our study advocate the findings of their study. Chitosan's antibacterial effect on

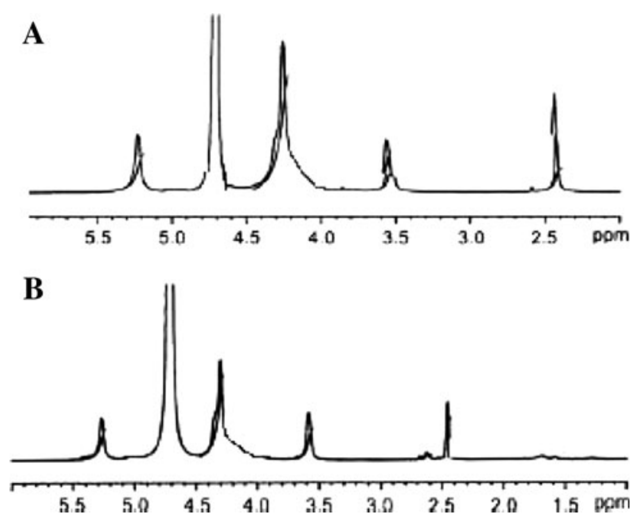

Fig. 7 H NMR spectrum measured at 60 °C for chitosan prepared in autoclave (**a**) and microwave (**b**)

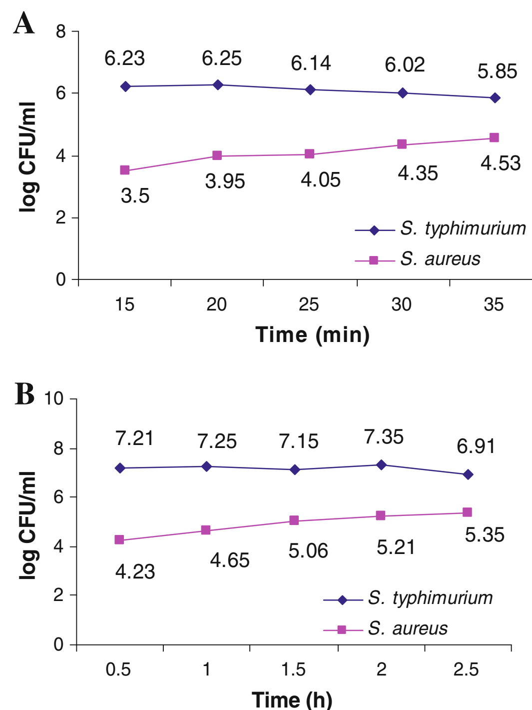

Fig. 8 Antibacterial effect of chitosan prepared in microwave (**a**) and autoclave (**b**)

gram-positive bacteria was strengthened as the molecular weight increased. On the contrary, in gram-negative bacteria the bactericidal effect of chitosan was increased as the viscosity of chitosan solution decreased [23, 31, 34].

The lower inhibitory effect of chitosan on S. *typhimurium* can likely be attributed to rather small number of charged amino groups in chitosan molecule. This may explain the reason for the better antibacterial effect of chitosan prepared in microwave than the one in autoclave since the latter has lower DDA and molecular weight and consequently relatively less number of amino groups.

Conclusions

Chitosan was extracted from the wastes of shrimp packaging plant in the north of Iran. Demineralization and deproteinization were performed by dilute HCl and NaOH solution, respectively. Deacetylation was enhanced and reinforced in the study by microwave with regard to autoclave. The XRD and SEM outcomes demonstrate that chitosan prepared in microwave is more crystalline than the one produced in autoclave.

Viscosity and DDA percentage in chitosan prepared in microwave was higher than that produced in the autoclave. The use of microwave heating has led to dramatic decrease of heating times from several hours to few minutes.

Antibacterial activity of chitosan prepared in microwave was higher than the one prepared in autoclave. In addition, chitosan produced in microwave proved to have higher molecular weight and crystallinity.

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