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Utilization of Waste: Extraction and Characterization of Chitosan from Shrimp Byproducts

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Abstract
Chitin and Chitosan have been produced from shrimp byproduct by chemical method. Shrimp byproducts were collected from fish processing point. The process of chitin extraction from shrimp waste was involving demineralization with 2% (v/v) HCl solution (10:1 v/w, 30ºC, 12h), then de-proteinization with 4% (w/v) NaOH solution (10:1 v/w, 90ºC, 12h). Chitosan was produced by deacetylation step by treating extracted chitin under conditions of 15 psi/121ºC with 50% NaOH for 20 min and a solid/solvent ratio of 1:10 (w/v). Results show that the yield of the extracted chitosan was 54.65% and the degree of deacetylation was 70.9% with pH 8.4 and solubility of 98.15% compared to commercial chitosan 99.0%. The higher yield was due to repeating demineralization and deprotonated steps twice. Viscosity of extracted chitosan was 12.23 cPs which was higher than commercial chitosan 8.06 cPs. Other chitosan properties have been investigated including water binding capacity, oil binding capacity, emulsion capacity, emulsion stability and antioxidant activity of extracted chitosan.
Keywords: Chitosan, extraction, deacetylation, water binding capacity.

1. Introduction

Fish industrial processing plants and fish markets produce crustaceans (shrimp, crabs, prawns, lobster, and krill) as byproducts. Every year, the shellfish processing industry produces huge waste that could be an environmental hazard. About 75% of the total weight of discarded as by-products (Kuddus & Ahmad, 2013). In Saudi Arabia, the total production of shrimp is 40 000 tons in 2010 with 95 percent exported. Exported shrimp is processed, packed and frozen and sold both heads-on and head-less (FAO, 2015). Utilizing these wastes could develop added-value products that possesses physico-chemical and biological properties which can be applied in many fields. Chitin is a natural polysaccharide and the second most abundant organic compound in nature after cellulose, it is widely distributed in marine invertebrates, insects, fungi, and yeast (Knorr, 1982). Chitosan is the deacetylations process of chitin a polysaccharide (b-(1-4)-N-acetyl-D-glucosamine). Chitin is the constituent of the crustacean such as shrimps, and crabs, cartilage of the squid, and outer cover of insects, it also occur as ordered crystalline microfibrils forming structural components (Rinaudo, 2006). chitosan is normally insoluble in aqueous solutions above pH 7.0; In its crystalline form however, in dilute acids (lower than pH 6.0) such as acetic acid the protonated free amino groups on glucosamine make the molecule soluble (Martino et al., 2005).

Chemical or enzymatic method during the course of deacetylation of chitin results in the production of chitosan by breaking down the polymer N-acetyl links forming D-glucosamine units that contains a free amine group making the polymer soluble in dilute acids (Kalut, 2008). Chitosan usually is produced from shrimp processing waste (shell) using conventional chemical process through converting chitin to chitosan by alkaline deacetylation. Samar et al. (2012) reported that chitin particle size determine the yield percentage of chitosan where the smaller the particle size higher chitosan yield in the occurrence of concentrated NaOH solution. In addition, fungal chitosan was also extracted from the fungus Aspergillus niger (Muñoz et al. 2015).

The physicochemical properties, molecular weight, degree of deacetylation, ash content as well as yield of extracted chitosan depends on the source of chitin and time, temperature and alkaline concentration and the process used (Muñoz et al., 2015; Khan et al., 2002; Kumari et al., 2015; Hossain and Iqbal, 2014). Chitin, chitosan, and their derivatives have been used in technological applications in different fields (Aranaz et al., 2009). Chitosan has been used in the environmental control such as waste management to food processing, medicine and biotechnology (Kalut, 2008). Kim, (2010) mentioned that the used of chitosan was including agriculture use to improve the yield of rice and orchid production.

The aim of the present work was to utilize shrimp byproducts through extracting and characterizing crustacean chitosan and to evaluate its physiochemical and biological properties. novel methodology, such as HMS, into the state of the art of workforce sizing.

2. Materials and Methods

2.1 Preparation of shrimp waste

Shrimp waste was collected from local fish market of Buraidah city, Qassim, Kingdom of Saudi Arabia. Shrimp wastes were washed, dried at 50 ºC overnight, grinded and stored in dry place until extraction. Sodium hydroxide (NaOH) and Hydrochloric acid (HCl, 36.5g/mol). All chemical were of analytical grades.

2.2 Extraction of chitin from shrimp waste

Extraction steps of chitin from shrimp shell were following Synowiecki (1997) method with modification. The process was modified to extract chitin from shrimp byproducts as demineralization and deprotonated steps were repeated twice. The process was involving demineralization with 2% (v/v) HCl solution (10:1 v/w, 30ºC, 12h) to remove minerals, separation of insoluble fraction by centrifugation (4000 rpm, 15min) then washing with distilled water until it was completely free of acid. Followed by deproteinization with 4% (w/v) sodium hydroxide solution (10:1 v/w, 90ºC, 12h) to remove protein, separation of alkali-insoluble fraction by centrifugation (4000 rpm, 15min) then washing of alkali-insoluble fraction with distilled water, then drying at 40 ºC overnight. The product obtained was designated as purified shrimp waste chitin.

2.3 Transformation of chitin to chitosan

The different extraction steps of chitosan from extracted chitin were done according to Youn et al. (2007). The deacetylation step was achieved by treating extracted chitin under conditions of 15psi/121ºC with 50% NaOH for 20 min and solid/solvent ratio of 1:10 (w/v). After deacetylation, the chitosan was washed until neutralization and dried at 40 ºC overnight then stored until further analysis.

2.4 Characteristics of the extracted chitosan

2.4.1 Ash content

Ash content of the extracted chitosan was determined according to the method of AOAC (2007).

2.4.2 Degree of deacetylation

The degree of deacetylation (DD) of extracted chitosan was determined according to the method of Qin et al. (2004). Chitosan (0.3 g) was dissolved in 0.1 M HCl (20 ml). From the titration of this solution with 0.1 M NaOH solution, a curve with two inflexion points was obtained. The difference between the volumes at these two points corresponded to the acid consumed for the esterification of amine groups allowed the determination of DD of the chitosan. The titration was performed with a pH meter.

2.4.3 FTIR-ATR

Infrared spectra for Chitosan samples were measured by FTIR-ATR using ALPHA-Eco FT-IR Spectrometer with Eco-ZnSe-sampling module; Bruker Optics, Germany. The spectra were obtained and recorded in the region of 4000–600 cm-1.

2.4.4 Solubility

Solubility of the extracted Chitosan was determined according to the method of Fernandez-Kim (2004). Chitosan powder samples (0.1 g) was placed into a centrifuge tube then dissolved in 10 ml of 1% acetic acid for 30 min using an incubator shaker operating at 240 rpm at 25ºC. The solution was then immersed in a boiling water bath for 10 min, then cooled to room temperature (25ºC), and centrifuged at 10,000 rpm for 10 min. The supernatant was decanted. The undissolved particles were washed in distilled water (25ml) then centrifuged at 10,000 rpm. The supernatant was removed and undissolved pellets were dried at 60ºC for 24hr. Finally, the particles were weighed and the solubility percentage was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{(initial weight of tube + chitosan) – (Final weight of tube + chitosan)X 100}}{\text{(Initial weight of tube + chitosan) – (initial weight of tube)}}$$

2.4.5 Water binding capacity

Water binding capacity (WBC) of the extracted chitosan was measured using a method of Wang and Kinsella (1976). WBC was initially carried out by weighing a centrifuge tube containing 0.5 g of chitosan sample, adding 10 ml of water, and mixing on a vortex mixer for 1 min to disperse the sample. The contents were left at ambient temperature for 30 min with intermittent shaking for 5 s every 10 min and then centrifuged at 3500 rpm for 25 min. After the supernatant was decanted, the tube was weighed again. WBC was calculated as follows:

WBC (%) = [water bound (g)/ initial sample weight (g)] x 100

2.4.6 Oil binding capacity

Oil binding capacity (OBC) of the extracted chitosan was measured using a method of Wang and Kinsella (1976). OBC was initially carried out by weighing a centrifuge tube containing 0.5 g of sample, adding 10 ml of corn 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 for 5 s every 10 min and then centrifuged at 3500 rpm for 25 min. After the supernatant was decanted, the tube was weighed again. OBC was calculated as follows:

OBC (%) = [fat bound (g)/ initial sample weight (g)] x 100

2.4.7 Viscosity

Viscosity of the extracted chitosan was determined according to El-hefian *et al.* (2010), with small modification. A Brookfield digital viscometer, model DV-II + Pro, Brookfield, UK; with an attached UL adapter was used. Solutions (10g/L) were kept at room temperature (23±2 °c) in glass bottles at dark place until analysis. Shear rates range from 8.56 to 33.02 s^{-1} was applied. Viscosity was reported in centipoises (cP). Each measurement was recorded as an average value of 5 readings.

2.4.8 Emulsifying properties

Emulsifying properties of the extracted chitosan samples were determined according to Sciarini *et al.* (2009). A 60 ml suspension of 0.5% w/v concentration of the extracted chitosan sample was mixed with 6ml of commercial corn oil and homogenized for 1 min, the suspension was then centrifuged at 800 xg for 10 min. The emulsifying capacity (EC) was calculated as follows:-

$$EC = (e_v / t_v)x100$$

Where: $\mathbf{e_v}$**:** the emulsion volume and $\mathbf{t_v}$ **:** the total volume.

Emulsion stability (Es) of extracted chitosan emulsions at 0.5% concentration against high temperatures was determined in the emulsions that were heated in a water bath at 80°C for 30min according to Sciarini *et al.,* (2009). The emulsion stability Es was calculated as follows:-

$E_s = (f_{ev}/i_{ev})x100$ Where:- $\mathbf{f_{ev}}$: is the final emulsion volume and $\mathbf{i_{ev}}$: is the initial emulsion volume.

2.4.9 Antioxidant properties of chitosan

The scavenging ability of the extracted chitosan on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals were determined according to Shimada *et al.* (1992). A chitosan sample (4 mL) in 0.2% acetic acid solution was mixed with 1 mL of methanolic solution containing DPPH radicals, resulting in a final concentration of 10 mM DPPH. the mixture was shaked vigorously and left to rest for 30 min in the dark. The absorbance was then measured at 517 nm against a blank. The scavenging ability was calculated as follows:

$$\text{Scavenging ability (\%)} = \frac{(A_{517} \text{ of control} - A_{517} \text{ of sample})}{A_{517} \text{ of control}} \text{ X } 100$$

2.5 Statistical analysis

SPSS 18.0 was used in this study to analyze the data. One-way of variance analysis was carried out using Duncan's test with a confidence level as $p < 0.05$.

3. Results and Discussion

3.1 Extraction of chitosan

Extracted chitosan from shrimp waste is shown in Table 1. The chitosan yield was 54.65% of the shrimp waste. In this study, the higher yield of chitosan was due to repeating the process of demineralization and deprotonation for three times which resulted in removal of minerals and proteins. Chitosan yield depends on the length and acid and alkaline concentrations during the process of demineralization and deprotonation (Samar *et al.,* 2012). It could be noticed that the yields of chitosan increased significantly with decreasing of chitin particle size at each concentration of NaOH solution used in deacetylation, it was 72.09% 85.72% and 88.15% for chitosan at 20, 40 and 60 mesh extracted by 30% NaOH. Time length of the deacetylation process can results in depolymerization of the chitosan polymer that leads to loss of sample mass/weight from excessive removal of acetyl groups from the polymer during deacetylation and loss of chitosan particles during washing (Hossain and Iqbal, 2014). Puvvada *et al,* (2012) reported that chitosan yield was found to be 34% after purification of the total exoskeleton. Extraction of chitosan from crab shell reported yield in the range 30-36.7% and the difference in yield was due to the reaction time (Divya *et al.*, 2014; Yen *et al.,* 2009).

3.2 *Degree of deacetylation*

The degree of deacetylation (DD) of the extracted chitosan in this study was 70.9±3.78 as in Table 1. As the most important characteristic of chitin; (DD) value depends on the raw material and the processes used for the deproteinization and the demineralization (Younes *et al.,* 2014). Based on this different values have reported in the literature. Puvvada *et al.* (2012) reported that DD was 89.79% and concluded that higher DD values were due to higher amount of protein which affects the chemical, physical and biological properties of chitosan, such as adsorption, covalent linking, encapsulation. Muñoz *et al.* (2015) reported the deacetylation degree was 73.6% and stated that the process of DD depends on the source of chitin and time, temperature and alkaline concentration used. The DD values are highly dependent on the process used (Khan *et al.,* 2002; Kumari *et al.,* 2015). Hossain and Iqbal, (2014) concluded that the degree of deacetylation (DD) is influenced by NaOH concentration.

3.3 Physico-chemical properties of extracted chitosan

3.3.1 Ash content

The ash content of the extracted chitosan was found to be 1.4% (Table 1). The purity of chitosan is measured by the presence of ash content in the material, which depends on the effectiveness method used to removing inorganic materials. In shrimp shells, calcium carbonate that presence in large amount can result in higher ash content. Chitosan, unlike chitin has high content of highly protonated free amino group that attracts ionic compounds giving a soluble mater in an inorganic acid (Mohammed, *et al.,* 2012; Divya *et al.,* 2014).

3.3.2 Solubility

The solubility of extracted chitosan was 98.15% compared to commercial chitosan 99% as shown in Table 2. Chitosan solubility is considered as one of the most important parameters in its quality where higher solubility results in better chitosan. Temperature and time of deacetylation, alkali concentration and ratio to chitin, and prior treatments applied to chitin isolation and particle size are the critical factors affecting chitosan solubility (Hossain and Iqbal, 2014). It is estimated that deacetylation must be at least 85% in order to reach the desired solubility (No *et al.*, 1995). increasing deacetylation degree Proportionally increase chitosan solubility where samples treated with 50% and 60% NaOH gave solubility ranging from 96.01- 97.2% (Hossain & Iqbal, 2014). Partial removal of protein and acetyl group gives lower solubility values (Brine & Austin1981; Hossain & Iqbal, 2014). Chitosan solubility in inorganic acid, due to highly protonated free amino group that attracts ionic compounds (Mohammed*, et al.,* 2012; Divya *et al.,* 2014).

3.3.3 Viscosity

Viscosity is an important factor of chitosan that depends on the molecular weight. Higher chitosan provides highly viscous solutions. In this study the viscosity of the extracted chitosan was found as 12.23cp compared to commercial chitosan as 8.06cp (Table 2). Low viscous chitosan is desirable for industrial handling and application. The molecular weight of chitosan is very sensitive to extraction of chitin conditions including, alkali solution, concentration and ratio, temperature and reaction time (Younes *et al.,* 2014). Chitosan viscosity decreases with the longer time of demineralization and increase in weak acid such as acetic acid but decrease with strong acid such as HCl, (Moorjani *et al.,* 1975; Hossain & Iqbal, 2014). Viscosity is used to determine molecular weight where high molecular weight of chitosan yields high viscous solution (Divya *et al.,* 2014).

3.4 Functional properties of chitosan

3.4.1 Antioxidant

The Antioxidant property of the extracted chitosan to quench 1,1-diphenyl-2-picrylhydrazyl (DPPH) was found 28.68% compared to the commercial chitosan 29.54% as in Table 2. The antioxidant activity of chitosan derivatives increased with decreasing the degree of deacetylation (DD) caused by the increasing primary amino groups (Yen *et al.,* 2008) and with the decreasing of molecular weight causing the inter- and intra-molecular hydrogen bonds partly destroyed (Chien *et al.,* 2007; Xing *et al.,* 2008; Ying *et al.,* 2011). The obtained results suggest that extracted chitosan probably contained bioactive compounds such as peptides or chitooligosaccharides, which could react with free radicals as electron donors to convert them to more stable products through terminating the radical chain reaction. These bioactive compounds might present in the shrimp waste including phenolics, oligopeptides, or chitooligosaccharides; they are good electron donors making products more stable by terminate the radical chain reactions (Ghorbel-Bellaaj *et al.,* 2012).

Emulsifying properties of extracted chitosan samples were determined using commercial corn oil. The emulsion stability study showed that the emulsion properties of the extracted chitosan was 91.69% with emulsion capacity of 86.68 % compared to the commercial chitosan 83.78% with commercial chitosan 75.55%, respectively as in Table 2. Xingke and Wenshui (2011) reported that chitosan has excellent emulsifying properties where it's emulsifying activity and stability depends on the concentration, degree of deacetylation and molecular weight. They concluded that chitosan with higher DD than 60% showed superior emulsifying activity and stability. However, chitosan with low molecular weight give better emulsifying activity than those with high molecular weight. Emulsifying stability of chitosan increased with a higher Mw.

3.4.2 Water and Oil binding

Water binding capacity (WBC) of the extracted chitosan was 3.33% compared to commercial chitosan 3.12% as in Table 2. The oil binding capacity (OBC) of shrimp chitosan was measured using corn oil. The result was 2.31% for the extracted chitosan compared to commercial chitosan 1.56 % as shown in Table 2. The sequence of demineralization and deproteinization steps has a pronounced effect on WBC and OBC. Changing the sequence steps resulted in increased FBC when demineralization was first performed before deproteinization then followed by deacetylation and FBC was decreased when deproteinization is performed prior to demineralization, followed by deacetylation (Rout, 2001; Hossain & Iqbal, 2014).

Table 1: shrimp chitosan yield, ash and deacetylation degree.

Parameters	Value
Extraction Yield (%)	54.65±1.92
Ash content (%)	1.4±0.20
Deacetylation degree (%)	70.9±3.78
pH	8.4

Table 2: Shrimp chitosan properties,(viscosity, Solubility , Antioxidant).yield, ash and deacetylation degree.

Properties	Extracted Chitosan	Commercial Chitosan
Viscosity cPs	12.23±0.67[a]	8.06±0.31[b]
Solubility %	98.15±0.47[a]	99±0.11 [a]
Antioxidant %	28.68± 1.2 [a]	29.54±0.93 [a]
Emulsion stability %	91.69±2.6[a]	83.78±.3.1[b]
Emulsion capacity %	86.68±3.1[a]	75.55±2.1[b]
Water binding %	3.33±0.28[a]	3.12±0.18 [b]
Oil binding %	2.31±0.69 [a]	1.56±0.21[b]

[a-b]Means with different superscripts within a row indicate significant difference ($p < 0.05$).

3.4.3 FTIR spectrum

FTIR spectrum analysis of chitosan is shown in figure 1. A broad band at 3480-3360 cm^{-1} is corresponding to the stretching of OH groups. Dilyana (2010) reported that the range of 3425-2881 cm^{-1} related to N-H in NH2 association in primary amines due to different stretching vibration bands. The band at 2871 cm^{1} is due to CAH stretching. Kumari *et al.* (2015) reported that stretching vibrations in the range of 2921-2879 cm^{-1} corresponding to methyl group in NHCOCH3, methylene group in CH2OH and methyne group in pyranose ring. The chitosan spectrum exhibited absorption bands of the amide I and amide II bands around 1650 and 1587 cm^{1}, respectively. The band at 1587 cm^{-1} has larger intensity than at 1650 cm^{-1}, which suggests effective deacetylation (Kumari *et al.,* 2015). The scissoring band of the ethylene group was also observed at 1418 cm^{-1}. The absorption bands around 1056 cm^{-1} and 890 cm^{-1} correspond to the vibration of CAOAC bonds and confirm the bonding of the monomers through b-glucosidic linkages however, the observation in this spectrum analysis is similar to what was observed by Muñoz (2015).

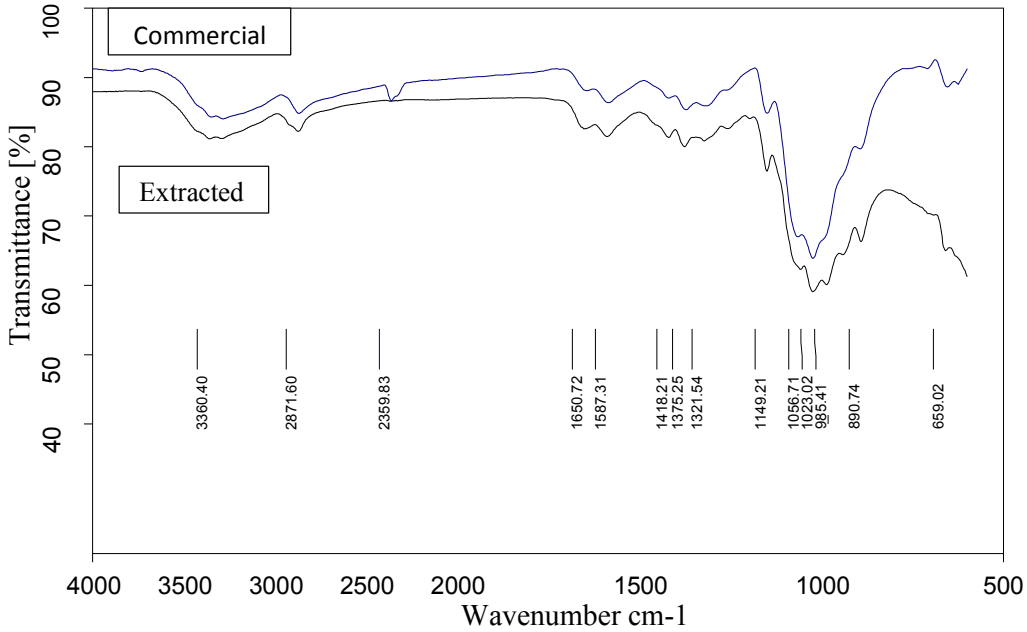

Figure 1: FTIR spectrum of extracted and commercial chitosan with scan ranging from 4000-600 cm^{-1}

4. Conclusion

Crustaceans' utilization (shrimp byproducts) from Saudi fish industry and fish markets to produce bioactive chitosan was achieved and the physico-chemical and biological properties were evaluates as well. Chitosan was produced through deacetylation step by treating extracted chitin. Results show that the yield of the extracted chitosan was higher than reported in literature due to repeating the demineralization and deprotonated steps twice.

Solubility and antioxidant activities of the extracted chitosan were similar to that of commercial chitosan. Other properties of the extracted chitosan including viscosity, water binding and oil binding capacity, emulsion capacity, emulsion stability showed higher values compared to that of commercial chitosan.

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