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Extraction of Chitosan from Iraqi Marine and Freshwater Crustaceans' Shells

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ABSTRACT

Aim: The aims of this study are to prepare chitosan from Iraqi freshwater crabs and marine shrimps in addition to estimation of its physicochemical properties.

Materials and Methods: The procedure of chitosan isolation involved the following steps: **Demineralization step:** Baths of 10% acetic acid solutions mixed with 1% hydrochloric acid (HCl) solutions were used for removal of calcium carbonate from grounded shell at ambient temperature with a solution to solid ratio of 10ml/g. **Deproteinization step:** This process was achieved by using reflux apparatus for refluxing of demineralized samples in 4% sodium hydroxide solutions at 100^{o}C with a solution to solid ratio of 10 ml/g. **Deacetylation of chitin to chitosan:** The isolated chitin powder was deacetylated by treatment with 50% NaOH solution under autoclaving conditions (15 psi/121 °C) for 30 minuteswith a solution to solid ratio of 10 ml/g and finally the Viscosity-average molecular weight of chitosan, Degree of De-acetylation (DDA), Percentage yield of chitosan and surface morphology of chitosanwere all determined.

Results: The chitosan samples prepared from two different sources are dissolved in 1% acetic acid solution with the Viscosity-average molecular weights of the chitosan derived from marine shrimp and freshwater crab are 152055 g/mol and 110408 g/mol at 25^{o}C respectively. The DDA of shrimp source chitosan and crab source chitosan are 52% and 54% respectively. Chitosan yield of about 82.2% from the total extracted chitin and 37% out of total 100g of dry shrimp shell. Chitosan yield of about 84.6% from the total dry chitin content and 11% chitosan yield out of total 100grams of dry crab shell.

Conclusion: The freshwater crab and marine shrimp were good sources of chitin (the raw material of chitosan) with higher yield of chitin in marine shrimp source than freshwater crab source. The use of autoclave in this study resulted in decrease of deacetylation time to 30 minutes instead of several hours when using the boiling method but not helped in high increase in DDA.

Key words: Chitosan, Marine, Crustaceans' Shells, Freshwater

INTRODUCTION

Today, naturally derived biomaterials have been attracting scientist's interest all over the world. Recently a special attention has been made toward using the materials which are derived from nature. Such materials would have some advantages over synthetic ones.[1, 2.]

Chitosan is a chitin derived polymer which is produced by deacetylation of chitin. Chitin is mainly found in exoskeleton of crustaceans such as crabs and shrimps and also in some fungi. [3.] Many biomedical applications have been identified for chitosan including wound healing, bandage, skin grafting, homeostasis, stitch materials, drug delivery, preventing dental plaque, hypertension control, and cholesterol control. [4, 5, 6.]

Several desirable properties have been reported for chitosan including high osteoinductivity, biocompatibility, easy application and gradual biodegradability that makes it a good candidate for bone regeneration. [7.]

This study was an attempt to prepare chitosan locally from two different sources; one source was the exoskeleton of fresh water crabs collected from banks of Tigris River running through Mosul city in the north of Iraq while the other source was

from exoskeleton of marine shrimps brought from Basra city in the south of Iraq in addition to estimation of some of the physicochemical properties of the prepared chitosan samples.

MATERIALS AND METHODS

Collection of Specimens and Outer shells isolation

Freshwater crabs were collected from the bank of Tigris River running through Mosul city while marine shrimp samples were brought from Basra city. The outer shells were scraped free of loose tissue, cleaned thoroughly with hot water from sand, then the shells dried in electric hot oven for 24 hours at 80 ^{o}C and crushed into small particles and grinded by using an electric miller into powder which was then passed through a sieve to get particles size of approximately 1mm **.**About 100 gram of each powder sample was taken and placed in containers to be ready for extraction of chitin and chitosan preparation.

Extraction of Chitin and Chitosan

The procedure of chitin isolation involved two main steps, demineralization and deproteinization:

Demineralization step: Baths of 10% acetic acid solutions mixed with 1% hydrochloric acid (HCl) solutions were used for removal of calcium carbonate from grounded shell at ambient temperature with a solution to solid ratio of 10ml/g. The process was repeated several times until emission of Co_2 gas ceased.The solution was filtered through a clean piece of clothes and the precipitate was washed with water for several times to get rid of residues of the acid followed by drying in electric hot oven at 60^{o}C for 24 hours.

Deproteinization step: This process was achieved by using reflux apparatus for refluxing of demineralized samples in 4% sodium hydroxide solutions at 100^{o}C with a solution to solid ratio of 10 ml/g. The treatment was repeated several times. The number of bathes depends on clarity of the solution**.** Absence of proteins was indicated by the absence of color of the medium. The purified chitin samples were washed to neutral pH and dried in hot oven at 60^{o}C for 24 hours and weighed.

Deacetylation of chitin to chitosan: The isolated chitin powder was deacetylated by treatment with 50% NaOH solution under autoclaving conditions (15 psi/121 °C) for 30 minuteswith a solution to solid ratio of 10 ml/g. Samples were then washed with distilled water to reach neutrality, dried in electric hot oven at 60^{o}C for 24 hours and weighed.

Determination of physicochemical properties of samples

Each sample prepared was then submitted to group of investigations to test degree of deacetylation (DDA), intrinsic viscosity (ɳ), viscosity-average molecular weight (Mv), surface morphology, and percentage yield of obtained chitosan.

Determination of the Viscosity-average molecular weight of chitosan from intrinsic viscosity

Ubbelohdeviscometer was used for determination of the molecular weight of the polymers.The viscometric average molecular weight (Mv) of the polymer samples were studied depending on the intrinsic viscosity [ɳ] measurements of chitosan and at a suitable temperature and in a specific solvent. The viscosity-average molecular weight of the samples was carried out from the[ɳ] values, depending on Mark-Houwink equation

$$[\eta]=K\,\overline{Mv^{a}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where, K and a are constants for a certain solute-solvent and temperature, where K= 1.81×10^{-3} cm^3/g and a = 0.93 for the acetic acid used in this study. However, 1% acetic acid solution was used as a suitable solvent for chitosan and the temperature of the measurement was fixed at 25.0±0.1 ^{o}C. The kinematic viscosity can be calculated as follows.

1. Five different concentrations of chitosan (0.20, 0.10, 0.05, 0.025 and 0.125%) were prepared using 1% acetic acid solution.
2. The flow time of both the solvent (t_o) and each specific concentration of prepared chitosan solution (t) was measured at 25.0±0.1 ^{o}C. The value was recorded as an average of about three repeated readings for each solution.
3. The following calculations were used for determination of the average molecular weight of chitosan:

Relative viscosity (ɳr)

Relative viscosity is the ratio of solution viscosity to solvent viscosity, which is proportional to a first approximation for dilute solutions to the ratio of the solution flow time to solvent flow time.

$$\eta_r = \frac{\eta}{\eta_0} = \frac{t}{t_0} \quad (1)$$

Where η and η0 are the viscosities of the solution and solvent respectively; t and t0 are the respective flow time of the solution and the solvent respectively.

Specific viscosity (ηsp)

Specific viscosity is the fractional increase in viscosity, defined as

$$\eta_{sp} = \frac{\eta - \eta_0}{\eta_0} = \frac{t - t_0}{t_0} = \eta_r - 1 \quad (2)$$

Both ηr and ηsp are dimensionless (i.e. without a unit). As concentration increases, so does viscosity. Hence, to account for the concentration effects, the specific viscosity is divided by concentration to give the reduced viscosity (ηred).

$$\eta_{red} = \frac{\eta_{sp}}{c} \quad (3)$$

Where concentration (C) is commonly expressed as g per 100 ml (dl).Finally plotting of the reduced viscosity (ηsp/c) versus concentration (C) graphically to determine the intrinsic viscosity of chitosan.

Determination of Degree of Deacetylation (DDA)

The degree of deacetylation is defined as the ratio of the number of amino groups in chitosan to the sum of the amino and acetyl groups[8.] .The absorbance spectra of chitosan powder samples were obtained using FT-IR spectrometer (Bruker) with a frequency range of 4000-400 cm^{-1}.The degree of deacetylation (DDA) was calculated by using a baseline proposed by Domszy and Roberts in 1985[9.].The computation equation for this baseline is:

DDA (%) = 100 - [(A_{1655} /A_{3450}) X 100 / 1.33]

Where A_{1655} and A_{3450} are the absorbance at 1655 cm^{-1} of the amide-I band as a measure of the N-acetyl group content and 3450 cm^{-1} of the hydroxyl band as an internal standard to correct for film thickness or for differences in chitosan concentration powder form. The factor `1.33' denoted the value of the ratio of A_{1655} /A_{3450} for fully N-acetylated chitosan. It was assumed that the value of this ratio was zero for fully deacetylated chitosan and there was a rectilinear relationship between the N-acetyl group content and the absorbance of the amide-I band (Domszy and Roberts, 1985. [9.]

Determination of the Percentage yield of chitosan

The yield of chitosan is calculated by weighing each grinded shell sample before and after chitosan preparation (demineralization and deproteinization of the sample) and the percentage is calculated as follow:
[Weight of the sample after preparation (in gram) / weight of sample before preparation (in gram)] × 100.

The surface morphology of chitosan

The surface morphology of chitosan was examined with scanning electron microscopy (Vega3Tescan) in Department of Production Engineering and Metallurgy in University of Technology in Iraq**.**

RESULTS

The intrinsic viscosity of chitosan samples

Ubbelohode viscometer was used for determination of the intrinsic viscosity of chitosan. The chitosan powder samples were dissolved in 1% acetic acid solution and five different concentrations of chitosan solution were prepared from each

sample solution (0.20, 0.10, 0.05, 0.025 and 0.0125%). The average measurements of the flow time of acetic acid (the solvent) at 25 ^{o}C was 40 second (t_o=40 sec.). The average flow times for chitosan solution concentrations were measured and used beside the flow time of solvent in the calculation of the relative viscosity, specific viscosity, and reduced viscosity and for the two type of chitosan samples used in the study and the results were scheduled as shown in the tables (1) and (2), then the reduced viscosities for each chitosan sample were plotted versus their concentration. The measured intercept being considered the intrinsic viscosity as shown in figures (1) and (2).

Table (1): Determination of flow time (t), relative viscosity (ηr), specific viscosity (ηsp) , and reduced viscosity (ηred) of five shrimp derived chitosan concentration solutions.

*C (g/dl)	t (sec)	t/t_0 (η_r)	$(t/t_0)-1$ (η_{sp})	η_{sp}/c (η_{red})
0.20	2840	71	70	350
0.10	1000	25	24	240
0.05	400	10	9	180
0.025	190	4.75	3.75	150
0.0125	100	2.5	1.5	120

*C concentrations of chitosan solution

Table (2): Determination of flow time (t), relative viscosity (η_r), specific viscosity (η_{sp}) , and reduced viscosity (η_{red}) of five crab derived chitosan concentration solutions.

*C (g/dl)	T (sec)	t/t_0 (η_r)	$(t/t_0)-1$ (η_{sp})	η_{sp}/c (η_{red})
0.20	2440	61	60	300
0.10	840	21	20	200
0.05	340	8.5	7.5	150
0.025	165	4.125	3.125	125
0.0125	90	2.25	1.25	100

*C concentrations of chitosan solution

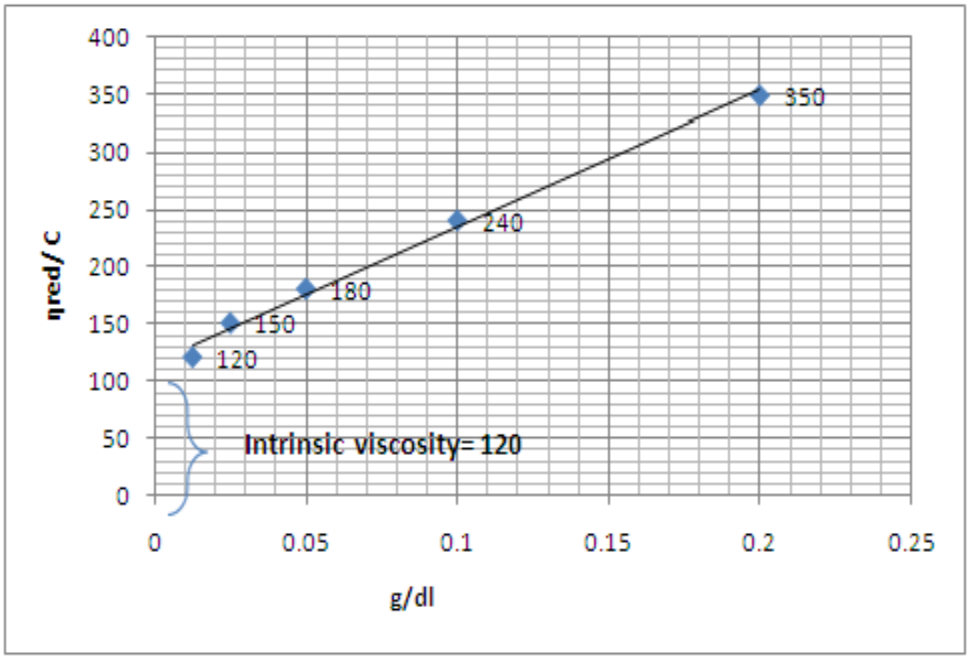

Figure (1): Plotting of reduced viscosity (η_{red})versus concentrations of chitosan solution (c) in graph to extrapolate the intrinsic viscosity value of shrimp derived chitosan solution.

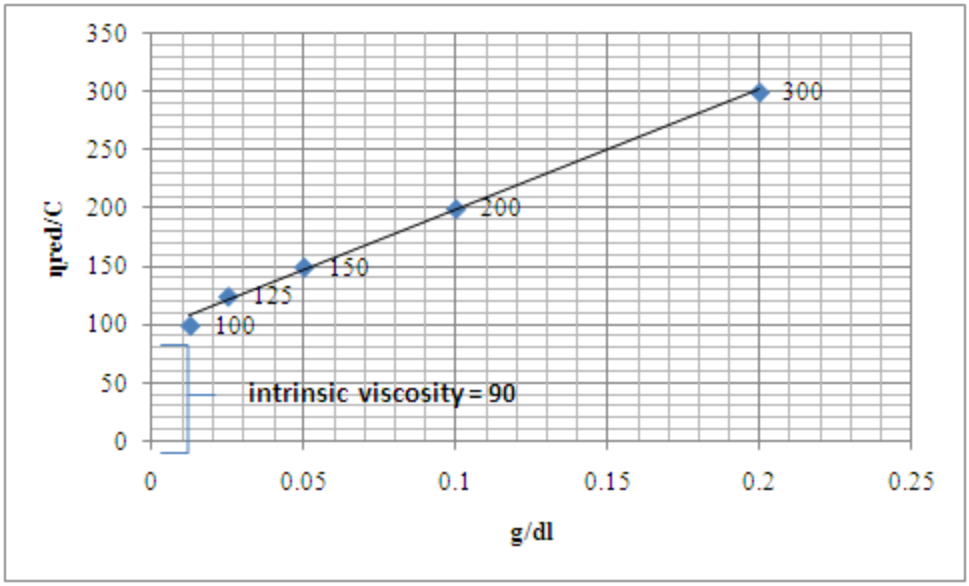

Figure (2): Plotting of reduced viscosity (η_{red})versus concentrations of chitosan solution (c) in graph to extrapolate the intrinsic viscosity value of crab derived chitosan solution.

The viscosity-average molecular weight of chitosan samples

The viscosity-average molecular weight (Mv) of the samples was carried out from the[η] values, depending on Mark-Houwink equation[10.]

$$[\eta]=K\,\overline{Mv}^{a} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where $K = 1.81 \times 10^{-3} cm^3/g$ and $a = 0.93$

So the Viscosity-average molecular weights of chitosan derived from marine shrimp and freshwater crab are 152055 g/mol and 110408 g/mol at 25°C respectively

Degree of deacetylation in chitosan samples (DDA)

Fourier transform infra-red spectroscopy (FT-IR) spectra of the studied chitosan samples were depended in the calculation of their degree of deacetylation (DDA) by using a baseline proposed by Domszy and Roberts in 1985 [9.] Figure (3) and (4) .The computation equation for this baseline is:

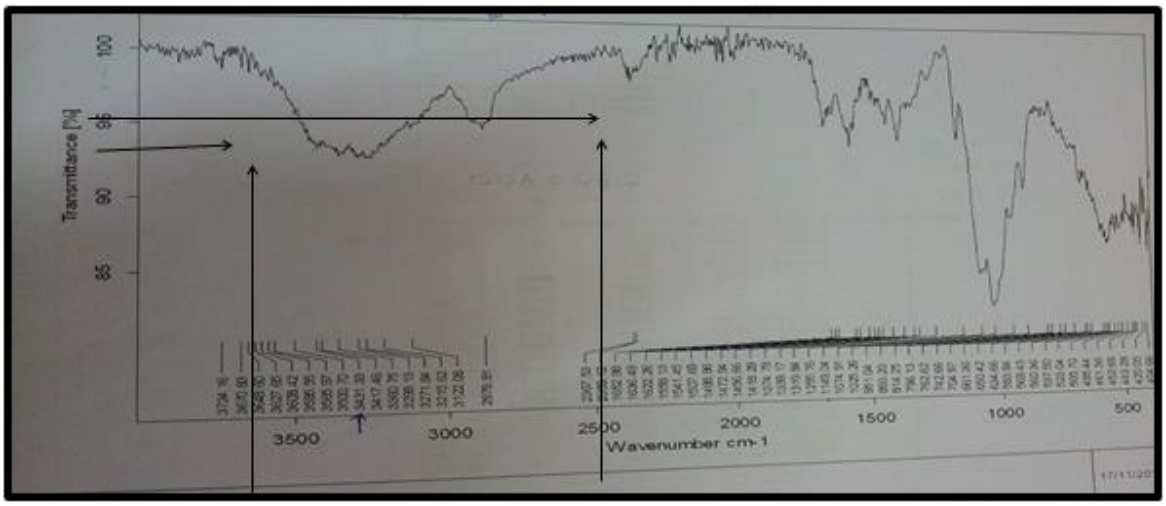

Figure 3: FTIR spectra of shrimp derived chitosan

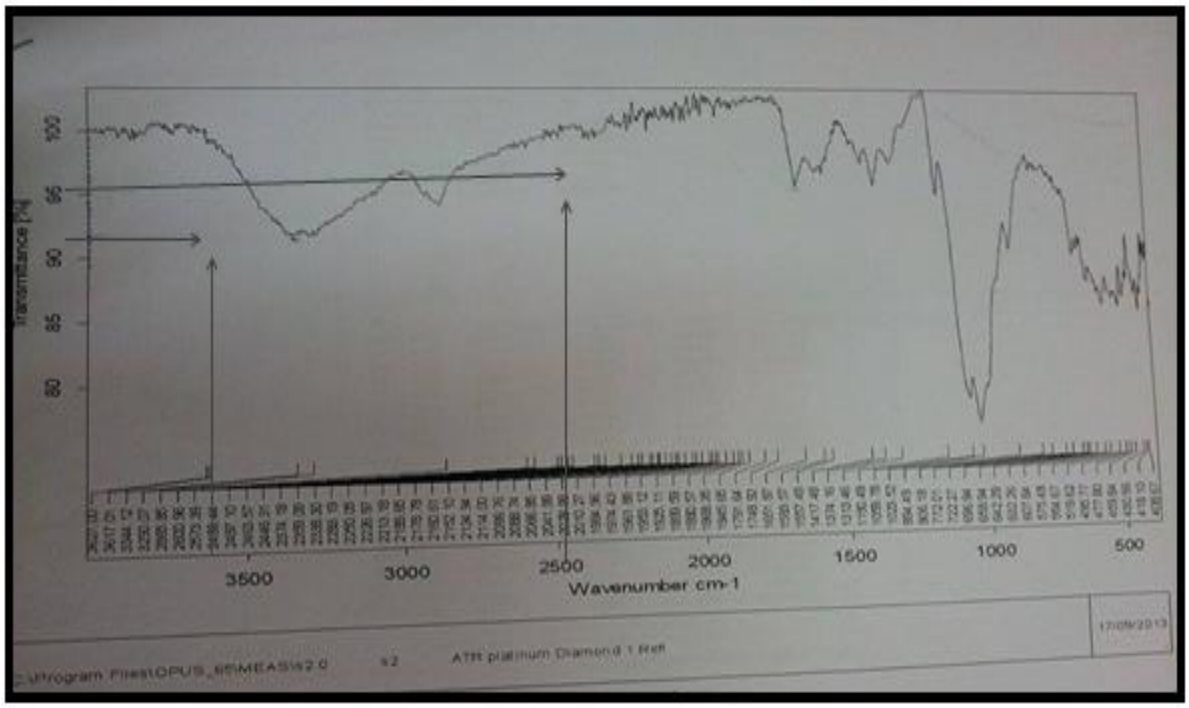

Figure (4): FTIR spectra of crab derived chitosan

DDA (%) = 100 - [(A_{1655} /A_{3450}) X 100 / 1.33]

And to convert the absorbance values from percent transmittance (%T), the following equation were used :
Absorbance = 2 – log (%T)
So the DDA of shrimp source chitosan and crab source chitosan are 52% and 54% respectively Table (3)

Table (3): Determination of transmittance, absorbance and degree of deacetylation(DDA) of standard, shrimp andcrab source chitosan.

Chitosan Sample	% T	A_{1655}	%T	A_{3450}	DDA%
Shrimp Source	95.5	0.0200	93.0	0.0315	52%
Crab Source	95.0	0.0223	92.0	0.0362	54%

The Percentage yield of chitosan

Each 100 g of dry shells waste isolated from each freshwater crab and marine shrimp were processed for the chitosan production yielded about 13g and 45 g of chitin respectively and their further processing with deacetylation process yielded 11 g and 37 g of chitosan respectively.

Scanning Electron Microscopic (SEM) Study of Surface Morphology of Chitosan Samples

The SEM micrographs of chitosan derived from shrimp shell were investigated and the SEM image of the sample surface was appeared brittle with very dry and cracked flakes and have disarranged and cracked thin species .The surface morphology of the sample was smooth and compact and with low elastic or gel properties as shown in figure (5). SEM image of chitosan derived from crab shell has shown irregular and folded surface and has rough structure and its morphological surface has shown uneven folds, with some caves and porous elastic surfaces as shown in figure (6).

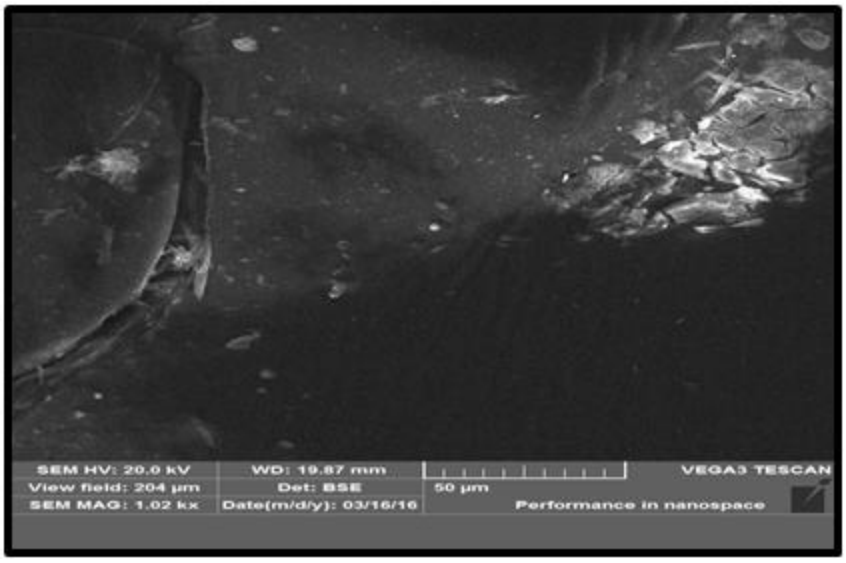

Figure (5): SEM micrograph of shrimp derived chitosan

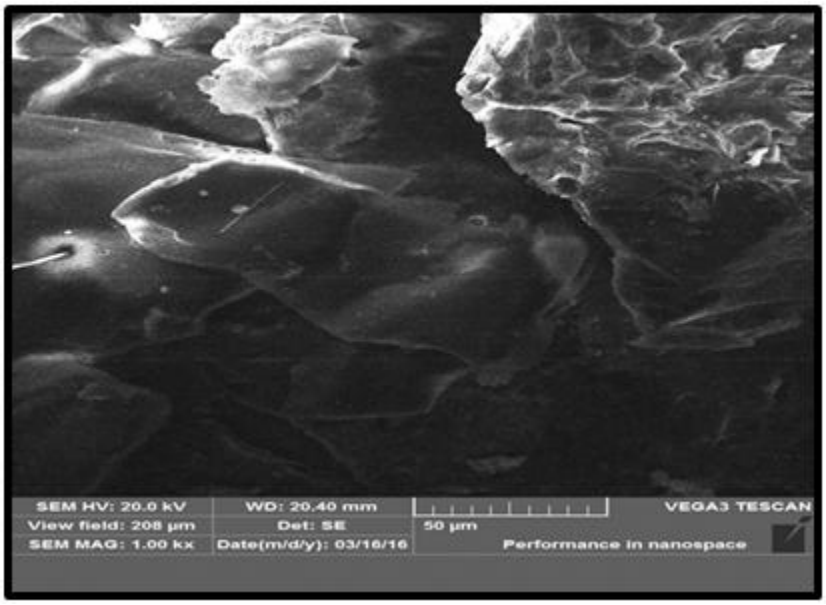

Figure (6): SEM micrograph of crab derived chitosan

DISCUSSION

The current study was an attempt to prepare chitosan from locally available freshwater crab collected from the shores of Tigris River in Al-Rashidiya area in Mosul City in the north of Iraq, while shrimp gathered from Shatt-al –Arab in Basra city in the south of Iraq during fishing. The present study was the first attempt in Iraq to extract chitosan from Crab shell and to investigate its physicochemical properties.

The intrinsic viscosity and viscosity-average molecular weight of chitosan samples

The viscosity of chitosan is affected with the change of concentration of solution and temperature [11]. Therefore, in this study the temperature was constant at 25 ^{o}C and the chitosan solutions of all samples were at the same concentrations. In the current study, the intrinsic viscosity and the viscosity-average molecular weight values of the marine shrimp derived chitosan were larger than that of freshwater crab derived chitosan and the most important cause for this viscosity value difference was probably due to the difference in the sources from which chitosan samples were prepared[12, 13]. The molecular weight of chitosan samples can be determined by various methods but viscometry is relatively simple and rapid

method and by using the Mark-Houwink Equation, the viscosity-average molecular weights of chitosan samples were determined [14]. In our study, the obtained viscosity- average molecular weight of crab derived chitosan and shrimp derived chitosan was (110408 g/mol), (152055 g/mol) respectively which located in the range reported by Struszczyk (2002) [15]. Higher molecular weight chitosan often render highly viscous solutions [16]. The Physical and biological properties of chitosan are governed by two factors; the molecular weight and the degree of deacetylation[17].

Degree of De-acetylation (DDA)

The process of deacetylation involves the removal of acetyl groups from the molecular chain of chitin, leaving behind a compound (chitosan) with a high degree chemical reactive amino group (-NH2). In this study, Fourier transform infra-red spectroscopy (FT-IR) spectrometer was used to estimate the DDA of chitosan samplesand by this method we can distinguish our end product by comparing the FT-IR spectra of the resulting product with the spectra of the standard known product. FT-IR spectra gave characteristics absorption bands of $–NH_2$ and –OH groups stretching vibration at frequency wavenumber of 3450cm^{-1} and the band for amide I at 1650 cm^{-1} was also seen in the infrared spectrum of chitosan. This method has a number of advantages and disadvantages. First, it is relatively fast, non-destructive to the samples and unlike other spectroscopic methods, does not require purity of the sample to be tested nor require dissolving of the chitosan sample in an aqueous solvent[18]. In the present study, DDA of the obtained shrimp derived and crab derived chitosan were 52% and 54% respectively and this result was comparable to that reported by Sarbon et al., (2015) [19] who extracted chitosan from mud crabs (S.olivacea) with DDA of 53.42%. In this study, the both extracted chitosan types (crab and shrimp source) dissolved in diluted 1% acetic acid and this was comparable with that reported by Rinaudo, 2006[16]; Islam et al., 2011[20]; Zakaria et al., 2012[21] who declared that when the degree of deacetylation of chitin reaches about 50% and above it will dissolve in aqueous acidic media and in this case it was called chitosan.

However, in order to prepare chitosan with a high DDA, the deacetylation process needs to be repeated several times with fresh NaOH solution, increasing the NaOH solution concentration in addition to increasing the time or temperature of process but chain degradation could occur for that high DDA chitosan [22, 23]. The use of autoclave in this study resulted in decrease of deacetylation time to 30 minutes instead of several hours when using the boiling method but not helped in high increase in DDA.

Percentage Yield of chitosan

In thisstudy,13g and 45 g of chitin was isolated from 100 g of dry shell waste of freshwater crab and marine shrimp respectively and were processed with same method into chitosan. About 37 gramof white and odorless chitosan powder has been produced by deacetylation process in autoclave out of 45 g of chitin which was isolated from dry shrimp shell, with a chitosan yield of about 82.2% from the total extracted chitin and 37% out of total 100g of dry shrimp shell. About 11 g of chitosan powder has been produced from 13 g of dry chitin which was isolated from crab shell, with chitosan yield of about 84.6% from the total dry chitin content and 11% chitosan yield out of total 100grams of dry crab shell.This wide variation in chitin content could be due to different species, nutrition and habitat of animal from which chitin (raw material of chitosan) was extracted [24, 25]. This observed wide difference in yield percentage when compared with reported values could also be probably attributed to the mineral composition of the retrieved chitosan source which would have affected the yields. The shrimp shells had the higher chitin and lower mineral content in comparing with crab shell which had the least chitin and higher mineral content. Our results was close to the range reported by shahidi and Synowiecki, (1991) [26] and Synowiecki and Al-Khateeb (2000) [27] who declared that the chitin content on a dry basis of crab processing waste (13 to 26%) and lower than that in case of shrimp (14 to 42%).Also our result was nearly comparable to that reported byPuvvada et al., (2012) [28] who reported a 34% chitosan yield while Divya et al., (2014) [29] prepared chitosan from shrimp shell waste with a yield of about 46% .

In concerning of chitin yield from crab shell, our result was consistent with that range reported by other researcher including Hertrampf and Piedal-Pascual (2000) [30] who isolated 10.6% chitin from crab shell ; Odote, et al (2005) [31] reported chitin yield of about 12% from crab shell while Tharanathan and Kittur, (2003) [32] extracted 14% of chitin from blue crab.

Scanning electron microscopy

The surface morphology of chitosan was reported to be affected by molecular weight and degree of deacetylation[33].The lower the degree of deacetylation, the higher was the surface smoothness as revealed by SEM study[34].in the present study the morphological investigation of chitosan samples was done by scanning electron microscope which revealed smooth and compact surface of shrimp derived chitosan while the morphological surface of crab derived chitosan appeared irregular

with uneven folds. The surface morphology of imported chitosan was more homogenous and elastic with compact folded surface and whitening spots which may indicate longer polymer chain with higher molecular weight and higher degree of deacetylation.

CONCLUSION

The freshwater crab and marine shrimp were good sources of chitin (the raw material of chitosan) with higher yield of chitin in marine shrimp source than freshwater crab source. The use of autoclave in this study resulted in decrease of deacetylation time to 30 minutes instead of several hours when using the boiling method but not helped in high increase in DDA.

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