Extraction, characterization and bioactivity of chitosan from farms shrimps of Basra province by chemical method

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Abstract. This paper include the extraction of chitin and transformation it to chitosan from the shrimps shells (waste) that grow up at sea farm in Al-Basra Province, the extraction done according to the literatures by two methods the difference between them was in the first step, but other steps were same methods, the results show sample A was higher than sample B in good yield and other physicochemical properties ( moisture, intrinsic viscosity, deacetylation degree, solubility, and fat binding capacity) which were measured to both samples, chitosan samples were characterization with FT-IR spectrum, in addition the biological activity for chitosan was tested against E.coli, P. asparagi, S. aureus, and B. cerus, the results show that sample A has inhibited these bacteria more than sample B.

Keywords. Chemical method, deacetylation degree, chitin, physicochemical properties, glucosamine.

1. Introduction
In recent years, many researchers have concentrated on chitosan as one of the source of bioactive material [1, 2] it’s a liner amino-polysaccharide polymer consisting of two monomers 2-deoxy-D-glucosamine, 2-deoxy-N-acetyl-D-glucosamine units which bonded with glycosides’ α(1-4) linked figure (1) [3], chitosan that derived from chitin by N-deacetylation step is abundant natural polymer like cellulose, the nitrogen content in it is (89.6%) as a free amino groups [4, 5, 6], the importance of chitosan due to its biological, pharmaceutical, and medical properties, chitosan have many applications such as artificial skin, cosmetic, photography, ophthalmology, food and nutrients, wound healing, and water treatment [7, 8, 9, 10]. Chitin (C8H13O5N)n is a long chin polymer from N-acetyl-d-glucosamine β(1-4) linked figure (2), it’s homopolymer [11, 12], first isolation of chitin was in 1811 by Henry Braconnnot from mushroom [13]. Both compounds are polysaccharides and they occurring naturally in exoskeleton on marine and insect, and they extracted from their sources by two methods, (i) chemical methods that depend on the treatment of raw-materials with alkaline solution and then with acidic solution [14, 15], (ii) biological method, which used application of microorganism and enzymes for chitin isolation [3, 16, 17], in this work we use the chemical method.
The aim of this paper is to extract and purify the chitosan from Prawn shrimp shells, which are recently found in southern Iraq/ Basra [18], for future use in synthesis some derivatives of it.

1.1. Experimental part
The chemicals that used in this research were analytical grade, which used without more purification, and it supplied by sigma chemical, the shrimps were obtained from the fishes market in Basra city, two kilo were brought and peeled washed, all the shrimp were from the same species (prawn shrimp), FT-IR spectrum was measured by FT-IR shimadzu spectrum 8400S by using KBr disk.

1.2. Extraction of Chitin from the Shells
The shrimp were washed well with running tap water, and then peel the shells of, washed again to remove any soluble organic matter or impurities, then the shells divided into two parts to make compared in the way that used in the first step, part A: the shells put in a boiled water 1h. to remove any tissues reminded, then drying in an oven at(160°C) for 2h, next dry shells are ground until it become fine powder with standard grinder. Part B: the shells that washed well, dry in an oven at 160°C for 2h then grounded it well to make fine powder by using standard grinder. After grounded the shells and make fine powder to increase the extraction ratio the following process will do on the two samples A&B:

1. Demineralization: this step do to remove calcium phosphate, calcium carbonate, salts and other minerals that found, using Islama, et al. [19] method with modulation, by dispersed the samples with the acid (2-3M) of hydrochloric acid at room temperature in the ratio of 1:15 (w/v) for 24h., the samples quite squashy and rinsed with tap water to remove the acid, calcium chloride and any impurities, then washed with distill water.

2. The demineralized samples were treated with 10% NaOH at room temperature for 24h. in ratio of 1:15 (w/v). after that the samples were collected and washed to neutrality in running tap water firstly then in distilled water, finally dry the samples, the product was chitin, the result chitin go to characterization.
1.3. *Decolourization (bleaching)*

Chitin which extracted from sample a need to decolorization first it mixed with acetone at ratio (1:10 w/v) a solid/ solvent for (10 min.), then filtered and dried to 2h at room temperature, second step was bleached with (0.315%) sodium hypochlorite for (5 min.) in same ratio [20], product discolored chitin filtered, washed and dried.

1.4. *Transmutation of chitin to chitosan*

By using the method of Rigby [21] with modulation, which include simple deactylation of chitin to preparation chitosan in an alkaline medium, by remove the actyl groups from chitin with 50% NaOH solution with (1:15) (w/v) ratio solid to solution at 50°C for 24h, after those samples were filtered and washed with distil water until they neutrality and deactylation step repeated three times, then washed with ethanol, finally drying in an oven at 60°C for 3h. to make the characterization.

1.5. *Physicochemical measurements*

The physicochemical properties are measure by standard methods as following:

- **Moisture Content**: By gravimetric method moisture content determined (Blacket et.al. 1965).

- **Deactylation degree**: DAD deactylation degree was determined with acid/base titration method [22], with modification. (0.1G) chitosan in (30ml) aqueous hydrochloric solution at room temperature was dissolved, with adding some drops from methyl-orange indicator, coloring solution (red-chitosan solution) that result tittered with (0.1mol/l) sodium hydroxide solution, even solution color change be orange solution. The DAD computed by this equation:

\[
\text{DAD} \% = \frac{C_1 V_1 - C_2 V_2}{W_{\text{ch}} \times 0.994 \times 0.016}
\]

Where:

- **C1**: Hydrochloric acid concentration (standard).
- **C2**: sodium hydroxide concentration (standard).
- **V1**: volume of hydrochloric acid concentration (standard), which chitosan was dissolved in.
- **V2**: volume of sodium hydroxide concentration (standard) that used in titration.
- **Wch**: chitosan weight.
- **0.016**: this number is gram equivalent weight of (-NH₂) group in (1ml) standard (1mol/l) HCl aqueous solution.
- **0.0994**: it’s the ratio (-NH₂) groups with chitosan weight. The degree of acetylation was calculated by subtracting the value of degree of deacetylation from 100%.

- **Solubility**: In the organic solvent chitin is insoluble, while chitosan is soluble in acidic medium with PH below 6, it dissolved in (1-2%) acidic acid, formic acid, and lactic acid (Batista & Roberts 1990).

- **Intrinsic viscosity**: The intrinsic viscosity was determined for chitosan at acetate buffer (0.5 M acetic acid- 0.2 M sodium acetate) at room temperature and it calculated with Huggins equation (Wang et.al. 2004).

\[
\eta_0 / C = [\eta] + K [\eta]^2 C
\]

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

Where:

- **M**: average molecular weight viscosity, K and a: are constant, and their values depending with the solvent that used, and polymer type. Chitosan buffer solution (0.5M acetic acid- 0.2M
sodium acetate) the constant are 3.5*10^{-4} and 0.76 respectively, to make this experimental some vary dilute solutions were used.

- **Fat binding capacity**: To measured FBC for chitosan the method of Knorr (1982) was used, with weighted the centrifuge tube which contain 0.5g chitosan, then added 10 ml of oil such as (soybean oil) and mixed it on vertex to 2min., for dispersing the sample, the pro ducts left at room temperature to 30min., with shaking( 5sc) each 10 min., then at (3000rpm) centrifuged 25nim, when it finished the tube must weight again, and the F.B.C. calculated as bellow:

FBC % = (fat linked / sample weight) *100

2. **Bioactivity Test**

To test the biological activity for the extracted samples we used the disk diffusion method, this method is simple, easy, and don’t take more time with good results. The agar that used was Muller Hinton agar, the disks were 5mm and loaded with 100μl of solutions samples, then put in plates and incubated for 24h the activity against microorganisms detected by measured the zone which surrounded with disks [23].

3. **Results and Discussion**

Chitosan was extracted from raw material (shrimps shells) with treatment chitin in alkalin aqueous media (high concentration of sodium hydroxide) by hydrolysis of (-NH-CO-CH3) groups to amine groups of the repeating units. Two methods in preparing the shrimps shells powders as in scheme (1) A in this method the shrimp sells boiled 2h, drying at 160C⁰ then grounded, while in B method the shrimps shells was, drying at 160C⁰ and then grounded, we do this to compared between to method in the yield of the product. The color was different in the two samples the figure (1) plays this, and then the two samples were treated with the same steps for extraction by demineralization the powder (shrimps shells) with (2-3M) hydrochloric acid, after this step treatment the result product with 10% NaOH to produce chitin which then converted to chitosan as explain in figure (3), sample A need another step to decolorizing the color of it while sample B don’t need as we show in figure (4).

Figure 3. Two methods to drying shrimp shells
The moisture results for the shrimps shells contents for samples in table (1), the moisture was 70.44%, 69.66% for sample A and B respectively, those results are closely related to (71.6%) which obtained by Ushaumari and Ramanujan [24], and M.S. Hossain and Iqbal [25], chitin moisture lies from (10.30%-11.90%) and it closeley to what found by Abdulkarim et. al. [26], while for chitosan samples the moisture were from (9.05%-11.21%), the commercial chitosan products moisure is less than (10%) according to Li [27], because in nature chitosan have hygroscopic propertise [28], and it affected by water in the stage of storege. The difference in results in our study with other studies due to the sources of chitin and the prossing that related to extraction. Yield of chitin and chitosan have been calculated for shrimps shells, the shells (waste) from the shrimps was (46%) and this result is closly to the scope that registered by Lertushiwony (et.al., 2002), who found the waste contents from fresh shrimps almost different at (45-55%) from the wieght of fresh shrimps, while the yield of extracted chitin from the waste varied (14-18 %) and this results depending on concentration of hydrochloric acid during extracted proces. The yield of chitosan were (18-19%) which is closely related th what found before Alimuniar and Zainuddin [29], who scored (18%) yiled, but lower than those reported by No and Meyers [30] who found approximated (23%), the yiled affected by procssing extraction steps and loss of chitosan during the washing of samples. Solubility of chitosan affected by some factors, DDA deacetylation degree, the concentration of NaOH that used in extraction, temp erature, partical size, time treatment applied to chitin in extraction. Chitosan solubilty was controlled with deacetylation degree, and it must be at least (85%) to ensure the complete solubility [31], it founf to be in from (76.45-95.36%), lower solubility values of chitosan due to incomplete removal of acetyl groups and protien [32]. Table (1) show some study properties of chitosan samples.

**Figure 4.** The difference in color between A&B

**Figure 5.** The extraction process for chitosan shells shrimps

**Deacetylation degree DAD,** acetyl groups are difficult to remove in chitin, therefore they need for high concentration of sodium hydroxide solution and heat, deacetylation degree in flounced by concentration of NaOH [33]. 70% NaOH concentration was used for deacetylation step and this will
repeated three times to ensure more removed to acetyl groups, the DD of chitosan samples ranged (A:90.97%-B:69.45%), No and Meyer [20] found that DAD of chitosan ranges from (56%- 99%).

**Intrinsic-viscosity**, chitosan viscosity concedes as an important factor to determined M.W. molecular weight, for it, chitosan have higher viscous solution provide highly molecular weight, which is unacceptable in industrial, chitosan viscosity affected by the demineralization time, it decrease by increased the demineralization time [34]. Viscosity of chitosan in acitic acid trend to be increased by decreased in the PH of acid. Intrinsic viscosity was used to characterized hydrodynamic properties to the polymer and to determined its average molecular weight so it concedes an important factor, but using Mark-Houwink equation, intrinsic viscosity of chitosan samples were (16.31dl/g- 13.30dl/g).

**Molecular weight**, according to Fernandez-kim (1991) chitosan was a bio-polymer, which had high molecular weight, so it varies with sources varies, and with the preparation method. The MW for commercial chitosan product fall in the range (100000-1200000 Dalton), [27], No and Meyers found that average molecular weight for chitosan was (0.12- 1.5 x 10^6 Dalton), Hosain and Iqbal [25] found MW (1.05 x10^6 Dalton), chitosan samples molecular weight were (1.03 x 10^6 – 0.18 x 10^6Dalton), and these results don’t agreement with Bouhenna et.al. [35], which found chitosan MW is (20050Dalton).

In the production process there are several factors that effected on the MW, including NaOH concentration, particles size, high temperature, reaction time, chitin concentration dissolving oxygen, previous treatment of chitin, and shear stress [27].

**Fat binding capacity**, chitosan capacity to binding to fat is measure by used soybean oil, its samples show high binding ratio which were ( 489.87-455.76%) and these results are in agreement with those (314-535%) which registered by No [36], according to Rout [37], the change in the series steps of extraction will affected on the FBC, an increasing in the FBC was noticed when deminreaztion was successive period to de-protenization , then followed by deacetylation, while decrease in F.B.C was noticed when de-protrinzation perform prior to demineralization was observed when deproteinization was performed prior to demineralization, then follow by deacetylation.

| Table 1. Some study properties of chitosan samples A&B |
|-------------------------------------------------------|
| chitosan | Moisture% | DDA% | η dl/g | M.W. Dalton | Fat binding% |
| A | 70.44% | 90.97 | 16.31dl/g | 1.03 x 10^6 | 489.87 |
| B | 70.02% | 69.45 | 13.30dl/g | 0.18 x 10^6 | 455.76 |

Chitosan samples (A&B) were charactereization by FT-IR spectrum, and then the data compared with standard chitosan from sigm-alderich figure (4), table (2) show the important absorption bands for two samples figure (5) for sample B and figure (6) for sample A, from FT-IR spectral observe stretching vibration bands at (3446.9-3421 cm-1) which due to (-NH, -OH and, -NH2) related to chitosan [38, 39, 40], vibration bands at (2922.3-2879.8, and 2920-2877.9 cm-1) assigned to (-CH3) in (-NHCO CH3), (-CH2) group in (-CH2OH), and for pyranose methylene groups. Amid band I is doublet and attributed to occurrence of inter-molecular hydrogen bonds which due to (-C=O……..HN-) and inter-molecular from (-C=O……..HOCH2) [39, 41, 42], while secondary amid band II was located at ( 1562,1595-1541 cm-1) which related to trans-secondary amides, and this band depending on the degree of inter-molecular association between (C=O…….HN-) groups, band at (1257.6, and 1259.6 cm-1) assigned to complex vibration of (-NHCO) [42], while area (1000-1260cm-1) due to (-C=O-C-), (-C=OH), and (-C-C-) ring vibration mode [43].

| Table 2. FT-IR data for standerd and experimintally chitosan samples (A&B) |
|--------------------------------------------------------------------------|
| Wave numbers in cm^-1 | Vibration groups |
|------------------------|-----------------|
| Standard | Sample A | Sample B | | |
| 3423 | 3446.9 | 3421.8 | (-NH2) for prarymary amin and (-OH) for pyranose ring |
| 2923 | 2922.3 | 2920 | (-CH3) for (-CH2OH) |
| 2880 | 2879.8 | 2877.9 | (-CH) for pyranose ring |
1667-1629 1656-1562 1653-1595 (-C=O) for (NHCOCH₂) belong to amid band I
1422 1429 1469.8 (-CH₂) for (-CH₂OH)
1380 1377 1379 (-CH₃) for (-NHCOCH₂)
1322 1315 1319 (-C-H) for pyranose ring
1262 1257.6 1259.6 Complex vibration of (-NHCO) belong to amid band III
1155 1203 1259 (C-O-C)Sym. Glycosidic linkige
1077 1157 1157 (-C-O-C)Asym. Glycosidic linkage
1074 1103 1095 (-C-O) for secondary (-OH)
1031 1012.7 1028 (-CO) for primary (-OH)
897 896.9 895 For pyranose ring skeleton
664 665.5 667 (-NH) for out of plane
616 607.6 605.7 (-OH) for out of plane

Figure 6. FT-IR spectrum of standard chitosan –sigm

Figure 7. FT-IR of chitosan sample B
4. Biological activity against bacteria

The extracted samples tested against two type of bacteria gram-negative and gram-positive the results summarized in table (3). The bioactivity of chitosan depending on some factors like, type of organism, physical state of chitosan, environmental of it (time, temperature, PH, ionic force), and some properties such as molecular weight, density, hydrophobic and hydrophilic, positive charge [44]. On acidic solution chitosan carried positive charge and it interact with the negative charge on the bacteria cell surface due to residues of carbohydrate, proteins, and lipids, thus it inhibit the growth of microorganism this explain of action of chitosan according to [45, 46]. Chitosan has more inhibition for gram-negative bacteria than gram-positive [46, 47]. Many studies reported these results in their experimental [48, 49], and we must don’t forget the affected of chitosan DAD on its activity against microorganisms’, chitosan DAD plays vary important role in inhibition microorganisms, higher inhibition rate due to higher chitosan DAD, and this was so clear in the result of this study, the inhibition of sample A was higher than inhibition of sample B, and the zone inhibition of gram-negative bacteria were higher than zone inhibition of gram-positive bacteria, the results of our work is agreement with Takahashi [50], and Jung [51] and other studies. In general when chitosan DAD was closed to 100%, it inhibited all type of tested bacteria at the (MIC) minimum inhibition concentration due to many free amino groups [52].

![Figure 8. FT-IR of chitosan sample A](image)

| No.        | Inhibition zone (mm) |
|------------|-----------------------|
|            | Staphylococcus aureus (G+) | B. cerus (G+) | P. asparagi (G-) | E. Col. (G-) |
| Sample A   | 22                     | 25             | 27              | 29           |
| Sample B   | 18                     | 20             | 22              | 24           |
| Penicillin | 35                     | 32             | 31              | 30           |

5. Conclusion

Shrimps have gained importance not only because it is a source of food rich in proteins, fats, and nutrients, but even its crusts have a medicinal significance in ancient medicine. Its shells contain bioactive compound chitin, chitin is extracted from shrimps shells, its unsoluble in organic solvents, this quality reduces its use so it is converted into chitosan by treating it with alkalin medium, chitosan dissolved in some organic acides in light concentration and because it contain free amino groups, it has bioactivity propretise more than chitin, it’s a non toxic compound, easy to absorb and get out of the body. chitosan has a high fat-binding ability, and from the results obtained the extracted samples of
chitosan affected by the steps that used in extraction process, and its physicochemical properties will affected also, for better result it important matter to be have good skill in production process to ensure best and purity product. As a result it is possible to add chitosan or packing some materials perishable by bacteria and fungi.

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