ORIGINAL PAPER

CAPITALIZING OF MARINE RESOURCES FROM THE BLACK SEA BY PREPARATION AND CHARACTERIZATION OF CHITOSAN CRAB *PACHYGRAPSUS MORMORATUS*

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Abstract. *Every year in our country, especially in the coastal zone of the Black Sea due to the intensive food processing and canning of fish and seafood from fisheries and markets, a large amount of raw material is produced, of which more than half consists from crab shells. Crab shells are a rich source of chitin, chitosan and cellulose. In order to obtain chitosan the procedure was started with the chemical extraction of chitin from the stone crab shells of the species Pachygrapsus mormoratus, from the Black Sea, because it is a species of crustaceans which are abundant on the Black Sea coast. In obtaining the chitin raw material were followed certain steps, in a chronological order, namely: the step of deproteinization, demineralization, decolorization and then converted the chitin into chitosan by a strong alkaline deacetylation method. For the characterization of chitosan physico-chemical studies were conducted in order to determine the parameter values. FTIR analyzes were performed to confirm the presence of the extracted chitosan. The results confirm and support the use of chitosan extracted from crab stone Pachygrapsus mormoratus for obtaining pharmaceutical biomaterials.*

Keywords: *Chitosan, chemical extraction, Pachygrapsus mormoratus*

1. INTRODUCTION

The Black Sea offers multiple possibilities for capitalizing on the pharmaceutical field [1]. For the exploitation of marine resources for pharmaceutical purposes, the marine habitats along the Romanian seaside [2, 3], which are free of possible contaminants with coastal pollutants [4, 5] have been studied. The possibilities of obtaining nutritional principles from marine organisms have been studied [6]. One of the directions for capitalizing on marine resources was the capitalization of marine algae for pharmaceutical purposes [7-9]. Multiple studies have been conducted in characterizing macrophages that can be used in the preparation of topical pharmaceutical formulations [10-12]. Another area of exploiting marine resources was the use of algal biomass in agriculture [13].

Another area of research is the study of macromolecular compounds such as proteins, polyphenols and polysaccharides from marine resources and their use for pharmaceutical purposes. Among proteins, collagen extracted from marine fish has been studied to obtain pharmaceutical formulations [14-17]. The study of polyphenols from natural resources is also a preoccupation in experimental studies [18-28]. Regarding the functional characteristics of some polysaccharides from marine resources, the medical applications of some

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chitooligosaccharide class compounds were studied [29]. Clinical studies [30-33] on the therapeutic effects of bioactive compounds are found in the literature. In this respect, chitosan is a suitable biopolymer for use in the medical and pharmaceutical field due to its remarkable biocompatibility, biodegradability and bioavailability properties [34, 35], resulting in the effects it exerts: antimicrobial, fungistatic, hemostatic and tissue regeneration effect, fabrication of resorbable suture bandages and sutures, anti-tumor effect, antihypertensive, antidiabetic, antioxidant, anti-allergic effects and carrier system for target drugs [36, 37]. Chitosan is obtained by partial deacetylation of chitin and is a 2-amino-deoxy-D-glycosy polymer containing acetyl groups in its monosaccharide structure.

Chitin and chitosan are part of a family of natural biopolymers found in nature in the external structure of several invertebrate species, such as crustaceans, fungi, insects and some algae [38, 39]. The purpose of this paper was to extract chitosan from stone crab shells, *Pachygrapsus mormoratus* from the Black Sea, through a biotechnological extraction process that allowed to obtain a polymeric product that can be used to make pharmaceutical forms with various applications.

2. MATERIALS AND METHODS

2.1. OBTAINING CHITOSAN FROM STONE CRAB SHELLS

The raw material from which the study was started is the shell of crustaceans on the Romanian seashore, namely stone crabs, *Pachygrapsus mormoratus* from the Black Sea. To obtain chitosan from this resource was studied an extraction method to establish a biotechnological process with optimum yield.

Preparation of raw material

Stone crabs (*Pachygrapsus mormoratus*) were harvested from the Black Sea coast in the coastal area of Năvodari-Vadu beach, Constanța County, between June and July 2018 (Fig. 1). The shells were cleaned and eviscerated using a knife, then washed and oven-dried at a temperature of 65 °C to constant mass (Fig. 2) and crushed at a size between 0.5-5.0 mm using a crusher and the pestle of a large mortar (Fig. 3). The weight of dry samples was 1523 g.

Figure 1. The stone crab (*Pachygrapsus mormoratus*).

Figure 2. Drying the cleaned crab shells.

Figure 3. Grinding the crab shells.

The process for chitosan preparation

The method involved the design of a biotechnological process consisting of several stages of work, starting with the collection and preparation of the raw material and continuing with the extraction process described in Fig. 4.

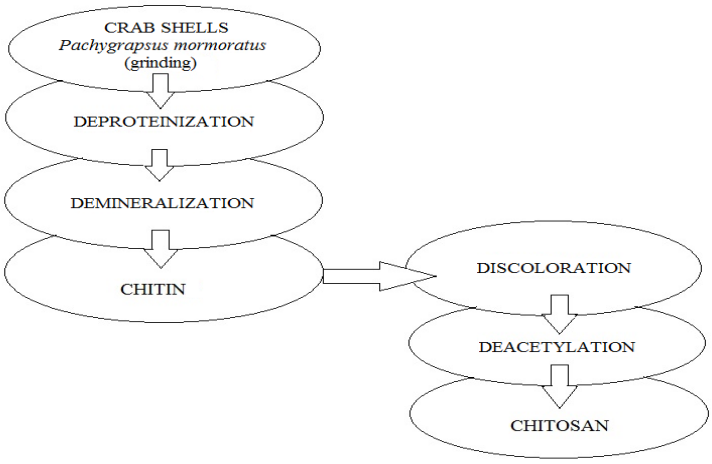

Figure 4. The chitosan extraction process from the shells of *Pachygrapsus mormoratus* crab from the Black Sea.

2.2. *CHARACTERIZATION OF THE PHYSICOCHEMICAL PARAMETERS OF THE OBTAINED CHITOSAN*

Chitosan exhibits major variability in chemical and physical properties [40]. Among the main parameters of physicochemical analysis are the degree of deacetylation (DD), solubility, ash content (or purity indicator) and pH value. Also, the obtained chitosan was characterized by FTIR spectrophotometric assays [41].

Determination of deacetylation degree by potentiometric titration

The degree of deacetylation of a sample of chitosan was determined by potentiometric titration of an acidic solution of chitosan with a standardized aqueous NaOH solution. For the pH measurements a Consort C861 Multi-parameter analyzer (pH meter) was used, to which a PH10B pH electrode was connected. The formula for calculating the degree of deacetylation of chitosan is:

$$DD[\%] = 2.03\frac{V_2 - V_1}{m + 0.0042(V_2 - V_1)} \quad (1)$$

where: m is the mass of the sample, V_1, V_2 are the volumes of 0.1M NaOH corresponding to the inflection points, 2.03 is the coefficient resulting from the molecular weight of the monomer unit of chitin, 0.0042 is the coefficient resulting from the difference between the molecular masses of chitin monomer units and chitosan [42, 43].

Ash content

The ash content of chitosan is a characteristic which relates to the purity of the obtained product and can influence the solubility of the polymer. The method used to determine the ash value is to keep the samples in a preheated furnace at 650 °C for 4 hours. After cooling the samples to 200 °C, allow to stand in a desiccator until the samples are completely cooled. The percentage of ash can be calculated using the following calculation formula [44]:

$$\%Ash = \frac{m_{residue}}{m_{sample}} \times 100 \quad (2)$$

Moisture content

This characteristic is determined by the gravimetric method. The moisture content was determined by drying the samples at constant mass and weighing the samples before and after drying and using the equation [45]:

$$\%Moisture = \frac{m_{wet.sample} - m_{dry.sample}}{m_{wet.sample}} \times 100 \quad (3)$$

pH value

The pH measurements were made using a pH meter to which a standard electrode was connected.

Determination of the solubility of chitosan samples

The sample of chitosan powder was dissolved in a 1% acetic acid solution. The sample was centrifuged for 30 minutes at room temperature with a rotational speed of 250 rpm. This solution was maintained in a hot water bath for 10 minutes, cooled to 25 °C and centrifuged for 10 minutes at a rotational speed of 10,000 rpm. The supernatant was decanted and the undissolved particles were washed with distilled water and centrifuged again. The undissolved residue remaining after removal of the supernatant was dried in an oven at 60 °C for 24 hours. The sample thus obtained was weighed and the solubility percent of the chitosan sample was determined using the formula below [38]:

$$\%\text{Solubility} = \frac{\left(m_{initial\text{-}tub} + CT\right) - \left(m_{final\text{-}tub} + CT\right)}{\left(m_{initial\text{-}tub} + CT\right) - \left(m_{initial\text{-}tub}\right)} \times 100 \quad (4)$$

Fourrier transform infrared spectrometry

For the spectrometric analysis in IR the sample of chitosan powder was tested using a JASCO FTIR 4200 spectrophotometer with a ATR crystal accessory. The ATR provides extremely high-pressure contact on the powder sample [46]. The spectra was recorded at a wavelength range of 400-4000 cm^{-1}.

3. RESULTS AND DISCUSSION

3.1. EXTRACTION PROCESS

Table 1 show the results obtained for each stage of the pre-established working procedure.

Table 1. The results obtained during the extraction process of chitosan

Stage No.	Type of operational process	Process of extracting chitosan from marine crab shells
Stage 1	Deproteinization	This stage involves removing proteins and decomposing sugars by isolating raw chitin. In the extraction process, the shredded shell sample was treated with 2M NaOH 30 (w/v) at 90 °C for 2h to achieve primary protein decomposition. After washing the sample with water, it was subjected to another alkaline treatment with 50 mL of 2% NaOH solution for 1h to decompose albumin into water-soluble amino acids.
Stage 2	Demineralization	This process is necessary to remove calcium carbonate and other minerals from the crab shells. This step was performed using a 1% HCl solution, taking into account 4 times the shell mass. Samples were left to soak for 24 hours to remove calcium carbonate from the shells. The suspension formed was centrifuged, the supernatant removed and the decanted powder collected.
Stage 3	Discoloration	The discoloration stage involves the removal of carotenoids and dyes present in crab shells naturally. The discoloration was carried out using 1% potassium permanganate solution and a reaction time of 1h, and then the sample was treated with 1% oxalic acid for another hour. The suspension thus formed was centrifuged with distilled water to neutral pH. The suspension was decanted and the supernatant stained with dyes was removed. This decolourising extraction was repeated 10 times until the colorants were removed from the chitin powder.
Stage 4	Deacetylation	Chitosan is obtained from isolated chitin by a strong alkaline hydrolysis reaction using high temperatures. Deacetylation of chitin was made by adding a 50% NaOH solution and boiling at 100 ^{0}C for 2 hours under the niche. After cooling the sample for 30 minutes at room temperature, it was washed continuously with 50% NaOH solution and filtered. The obtained chitosan was dried in the oven for 6 hours at 110 °C, triturated and sifted.

The deacetylation step is decisive in the production of chitosan and involves the hydrolysis of chitin acetamide groups. Due to the resistance of these groups imposed by the trans-linkages between C_2 and C_3 substituents in the polysaccharide ring, the process requires a strong alkaline treatment. Fig. 5 shows the scheme for the conversion of chitin into chitosan via the deacetylation process.

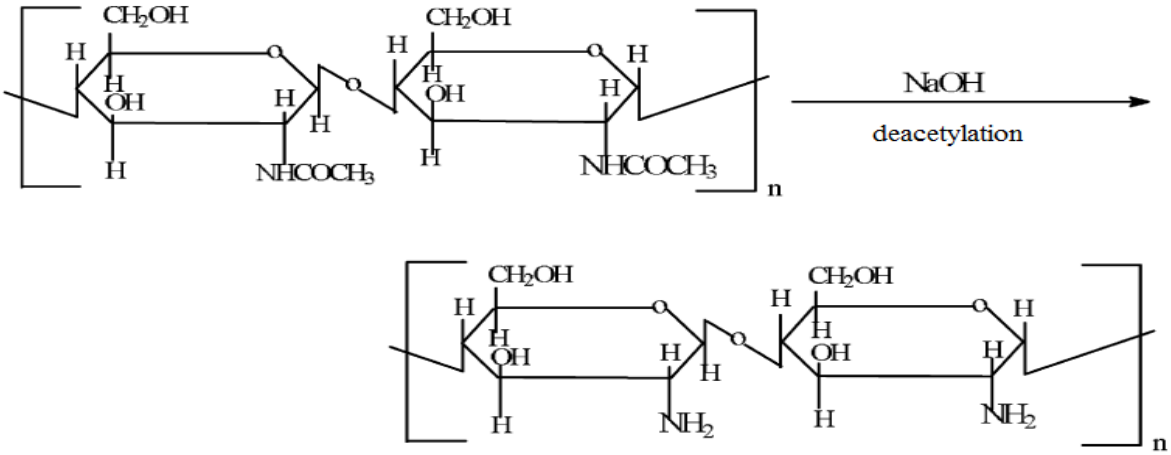

Figure 5. The conversion scheme of chitin into chitosan via the deacetylation process.

3.2. CHARACTERIZATION OF THE OBTAINED CHITOSAN

The results obtained for the physicochemical parameters of the extracted chitosan are shown in Table 2.

Table 2. The results of the main parameters of the chitosan sample.

Physico-chemical parameter	Extracted Chitosan (CT)
Degree of deacetylation (DD) [%]	71.50
Ash content [%]	2.23
pH value	7.30
Solubility [%]	74.75
Moisture [%]	8.54

Degree of deacetylation (DD)

Fig. 6 shows the titration curves (black color) and the corresponding derivative curves (blue color) for the titration of chitosan (CT) with an aqueous solution of NaOH. Applying the calculation formula, a value of 71.5% for CT was obtained.

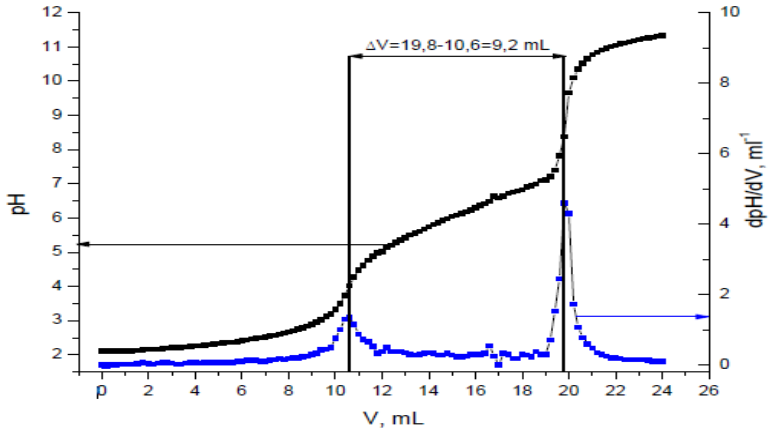

Figure 6. The titration curve (black color) and the corresponding derived curve (blue color) for potentiometric titration of the CT solution with NaOH sol.

The degree of deacetylation is influenced by the concentration of NaOH solution, since the bonds between acetyl groups are difficult to remove and require high temperatures [48]. The degree of deacetylation of standard chitosan ranges between 30% and 95% depending on the raw material source and the work procedures applied [49], but also the purification method and other conditions that may influence the analysis of this parameter [50]. Also, the reaction times in the demineralization step influence the DD value, which means that a more efficient removal of calcium carbonate from the initial samples will result in a chitosan that will deacetylate more efficiently.

Ash content

Applying the calculation formula given by equation (2) it was obtained an ash content of 2.23% for the sample of chitosan (CT); the result presented in Table 1. A longer demineralization time leads to a higher degradation of the $CaCO_3$ ions, which leads to a low amount of ash content. A small percentage of ash indicates the degree of efficiency of the demineralization step in the removal of calcium carbonate, as Puvvada Y.S. and collaborators also confirms [50].

Moisture content

By using equation (3), it was determined the moisture content of the investigated chitosan, being obtained a value of 8.54%. It has been noticed that the initial moisture level and the strong hygroscopic character may limit the applicability of chitosan, therefore the water content of the chitosan samples must be measured and optimized prior to analysis and controlled during storage.

pH value

According with the measurements, the chitosan sample showed a pH value of 7.3. At a small pH, the positive ions of the NH_3^+ group turn chitosan into a water-soluble cationic polyelectrolyte. When the pH value increases above 6.0, the positive charge of amino groups is lost and thus chitosan becomes insoluble in water. The transformation from the soluble to insoluble state of chitosan is carried out around a pH value of 6.0-6.5. The solubility of chitosan depends on the degree of deacetylation and the method used for the deacetylation of the chitin from which it originates.

Solubility of the chitosan samples

Using the calculation formula presented in equation (4) was obtained a solubility percentage of 74.75% for the CT sample. Of all the factors influencing the solubility of chitosan, the degree of deacetylation is the most important one. A low solubility value indicates an incomplete removal of the proteins and acetyl groups from the polymer chain.

Fourier transform infrared spectroscopy

From the FTIR spectrum of the main absorption bands obtained for the studied chitosan sample, it noticed that the highest absorbance is recorded between the wavelengths 1020 cm^{-1} and 1220 cm^{-1}, which confirms the presence of the free amino groups ($-NH_2$) in the C_2 position of the glucosamine chain. The results are consistent with the data obtained by Maragoni et al. [51].

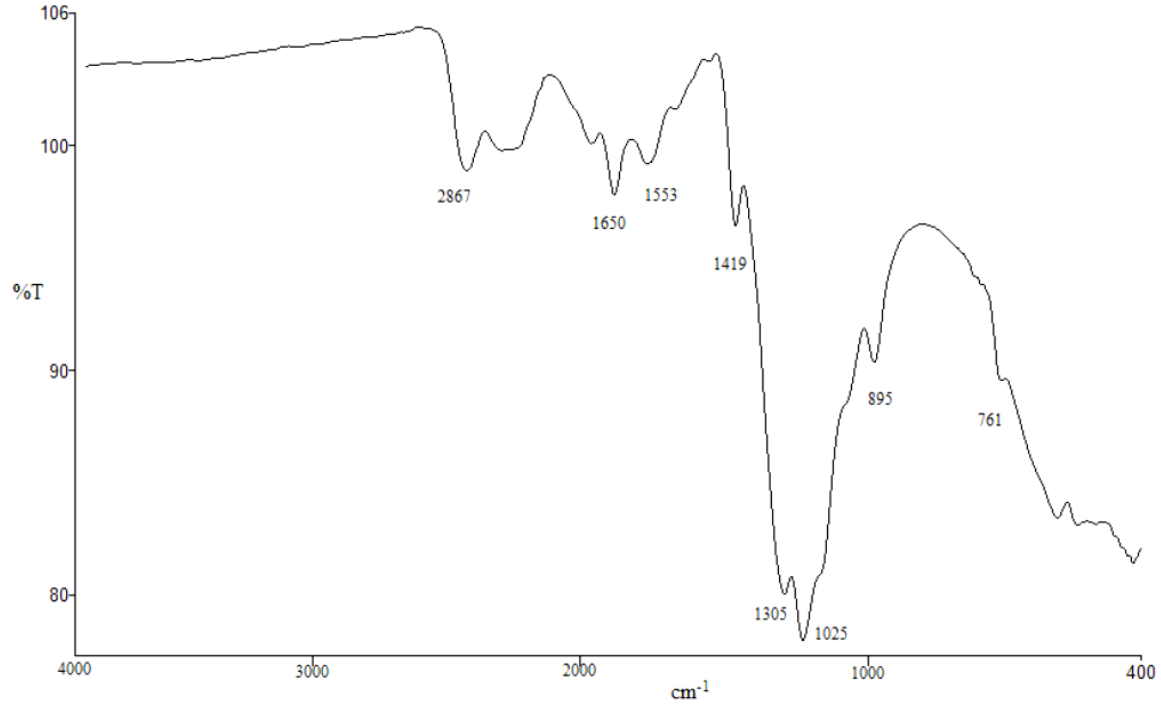

Figure 7. FTIR spectrum for chitosan sample.

The peak at 1305 cm^{-1} represents the C-O linkage of the primary alcoholic group (CH_2-OH). The absorbance bands corresponding to the peaks at 2867 cm^{-1} indicate the CH_3 symmetric stretch and the CH_2 asymmetric stretch, at 2576 cm^{-1}, the CH stretch, at 1650 cm^{-1} represents the extent of C=O in the amide group (amide I), at 1553 cm^{-1} – C-N stretching - into the secondary amide group (amide II), at 1419 cm^{-1} – bending of the CH_2 group and deformation of CH_3. These values are close to those obtained from Puvvada et al. [50]. The other absorbance peaks obtained corresponding to the wavelengths of 895, 761 cm^{-1} are similar to the spectrum of chitosan present on the pharmaceutical market, taken as a reference.

4. CONCLUSIONS

The following conclusions can be drawn from this research: *i)* chitosan was obtained from the *Pachygrapsus mormoratus* stone crab shells from the Black Sea through a biotechnological extraction process and characterized in terms of physicochemical parameters; *ii)* chitosan with a degree of deacetylation of 71.5% was obtained; *iii)* FTIR investigation performed for the chitosan sample revealed values and peaks similar to those of standard chitosan, thus confirming the structure of the polysaccharide in the analyzed sample; *iv)* chitosan is a natural polymer that can be used for its remarkable properties and represents a biological, ecological and economic alternative, making it a promising candidate for use in medicine and topical pharmaceutical preparations; *v)* the results was obtained from the physico-chemical parameters determination, which fell within the limits and intervals imposed by the literature for biomedical applications, together with the very safe profile of toxicity, include the chitosan extracted from *Pachygrapsus mormoratus* from the Black Sea in the field of materials with high potentials for use in the pharmaceutical industry for current and future applications.

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