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Evaluation of Five Chitosan Production Routes with Astaxanthin Recovery from Shrimp Exoskeletons

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Shrimp waste can be potentially used as source of high value products, from biopolymers to bioactive compounds. In this work, was defined a methodology for chitosan production via of depigmentation, demineralization, deproteinization and deacetylation of chitin. Five chitosan production processes were performed from the chitin present in shrimp exoskeletons using chemical agents such as hydrochloric acid (HCl) and sodium hydroxide (NaOH). Concentrations used were 1.5 M for acid, 1.0 M and 50 % w/v for base, 85 % for ethanol and 10 % v/v for acetone, and chitosan samples obtained were compared with commercial chitosan. Results shows that percentages of deacetylation obtained varied between (80 and 81.8 %) near the degree of deacetylation of the commercial chitosan (82.1 %). The extraction process with prior depigmentation using ethanol before demineralization registered the highest value of deacetylation, and the lowest value obtained was from the product obtained via ethanol followed by a second depigmentation with acetone after deproteinization of chitin. The characteristic bands of chitosan and similarity in each sample were confirmed by FTIR are NH bonds (between 3,350 – 3,500 cm^{-1}), NH bonds of the secondary amide (between 1,420 – 1,590 cm^{-1}), the tertiary amide band (between 1,360 – 1,380 cm^{-1}) and C = O bonds of the primary amide (between 1,618 – 1,630 cm^{-1}).

1. Introduction

The shrimp is a shellfish of high consumption for its pleasant flavor, but it causes environmental problems due to the amount of waste coming from the shrimp industries (Nouri et al., 2016). As a solution to this problem is indispensable to transform this residue into a high value added product as chitosan and a byproduct called Astaxanthin (Higuera et al., 2006). Shrimp shells contain (1 – 14 %) of pigments, (30 – 40 %) of protein, (30 – 50 %) of calcium carbonate and (20 – 30 %) of chitin and carotenoids of astaxanthin. Chitin is a natural polysaccharide and the main component of the exoskeleton of crustaceans, shrimps, insects, among others (Gbenebor et al., 2017). The biological functions and chemical structure of chitin are similar to cellulose as structural polysaccharide, but it is differentiated by an acetamide group instead of the hydroxyl group at the C-2 position inside the glucose unit (Hajji et al., 2014). Chitosan is a cationic amino polysaccharide and its applicability is wide in medicine, biomedical and pharmaceutical industry (fibers, membranes, artificial organs), in the biological, agricultural, environmental sector, in the area of cosmetology (body creams, lotions, additives for the hair) and in the food industry (binder, gelling agent, thickener, antimicrobial agents and antioxidants), among others (Razmi et al 2017). Diverse techniques have been reported to extract chitin and chitosan, but the most common is chemical treatment (Al Sagheer et al., 2009). Conventional extraction methods use alkaline solutions at high concentrations and temperatures, without performing depigmentation processes to take advantage of a byproduct, called Astaxanthin (Nouri et al., 2016), is a strong orange compound in most crustaceans and is classified as carotenoid. This organic pigment has a long polyunsaturated hydrocarbon chain

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(Di Caprio et al., 2015). The search for renewable sources to obtain astaxanthin from crustaceans has a growing economic interest as an alternative to the synthetic production of pigments (Pacheco et al., 2009). The objective of this work was to extract chitosan including the depigmentation process of the shrimp exoskeleton to obtain astaxanthin as a byproduct, using ethanol and acetone as depigmenting agents and compare the functional groups and degrees of deacetylation of the chitosan. Synthesized by FTIR, and perform gel tests by dissolving the chitosans obtained in a 4 % v / v acetic acid solution.

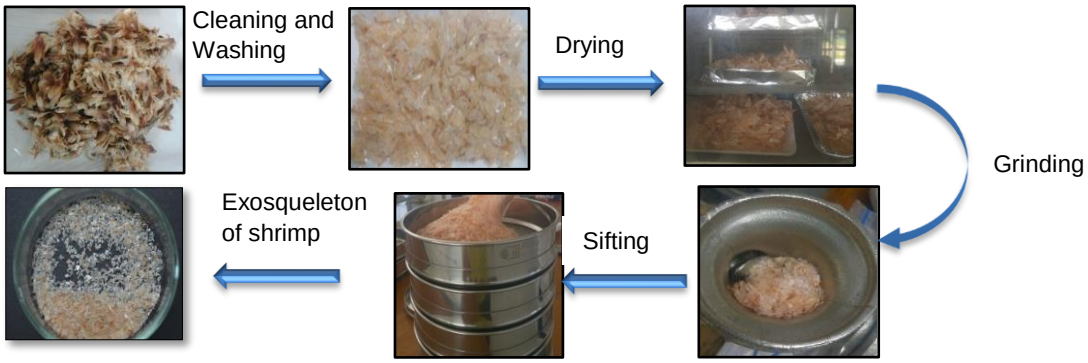

Figure 1: a). Processes of shrimp exoskeleton pretreatment

2. Materials and methods

2.1 Materials

All reagents were analytical grade and bidistilled water was used to prepare the solutions. Hydrochloric acid (37 %), glacial acetic acid, ethanol, acetone, and sodium hydroxide pellets (97 %) were purchased from Panreac AppliChem and acetic acid was purchased from Chemi Reagents Ltda. Commercial chitosan, with 82 % deacetylation was acquired at Sigma-Aldrich. The exoskeletons of the culture shrimp (*Litopenaeus Vannamei*) were supplied by the company Aqua Panama Overseas Inc., Cartagena (Colombia).

2.2 Methods

Five methods of chitosan extraction were used to compare the purity and quality of the final products, in each method a modified extraction process was employed. The exoskeletons of the shrimp were initially subjected to a cleaning process where all organic matter was separated, then washed with distilled water and dried in an oven for 360 min at 65 °C. Subsequently, they were ground and sieved until obtaining a particle size of 0.5 mm (Gbenebor et al., 2017).

2.3 Extraction of chitosan by the conventional chemical method

The demineralization stage 1 was perfomed using a 1:10 ratio of shrimp exoskeleton in a 1.5 M hydrochloric acid solution, at 500 rpm, at room temperature for 180 min to guarantee complete demineralization of the shells, then vacuum filtering was carried out, washed with abundant water until reaching the neutral pH and dried in the oven at 60 °C for 180 min (Kumari et al., 2015).
For deproteinization stage 2, a ratio of 1:10 w / v was used in a 1.0 M sodium hydroxide solution at 80 ° C and 500 rpm for 120 min. It was then vacuum filtered, washed with abundant of water until neutral pH and oven dried at 60 °C for 180 min (Nouri et al., 2016).
The deacetylation of the chitin stage 3 from the deproteinization was done using a ratio of 1:10 w / v, in an alkaline medium at 50 % w / v sodium hydroxide, 100 °C temperature and 500 rpm for 180 min (Al Sagheer et al., 2009).

2.4 Extraction of the five types of chitosan by chemical methods

Chitosan without depigmentation (Q0): the normal chitosan extraction process was carried out without depigmentation process; it was started directly with the demineralization, deproteinization and deacetylation of the shrimp exoskeleton (Al Sagheer et al., 2009).
Chitosan depigmentation with ethanol (Q Ethanol 1): the chitosan extraction process was carried out starting with depigmentation with ethanol, using a 1:10 p / v ratio, in an 85 % v / v ethanol solution, at room temperature, 500 rpm for 120 min It was filtered and washed until excess ethanol was removed and dried in the oven at 60 °C for 180 min (Gbenebor et al., 2017).

Chitosan depigmentation with ethanol, followed by a double depigmentation with acetone (Q Ethanol Acetone 1): the chitosan extraction process was carried out starting with depigmentation with 85 % ethanol and then with 10 % acetone under the same conditions (Nouri et al., 2016).
Chitosan depigmentation with ethanol before the deacetylation process (Q Ethanol 2): the chitosan extraction process was carried out starting with demineralization, deproteinization followed by depigmentation with 85 % ethanol, and finally the deacetylation process of the exoskeleton.
Chitosan depigmentation with ethanol, followed by a double depigmentation with acetone before deacetylation process (Q Ethanol Acetone 2): the chitosan extraction process was carried out starting with demineralization, deproteinization followed by depigmentation with 85 % ethanol and a double depigmentation with 10% acetone under the same conditions and finally the deacetylation process.
The ethanol and acetone exit streams containing the pigment were subjected to evaporation at a temperature not higher than 45 °C. The astaxanthin was separated from ethanol by centrifugation at 4,500 rpm for 15 min.

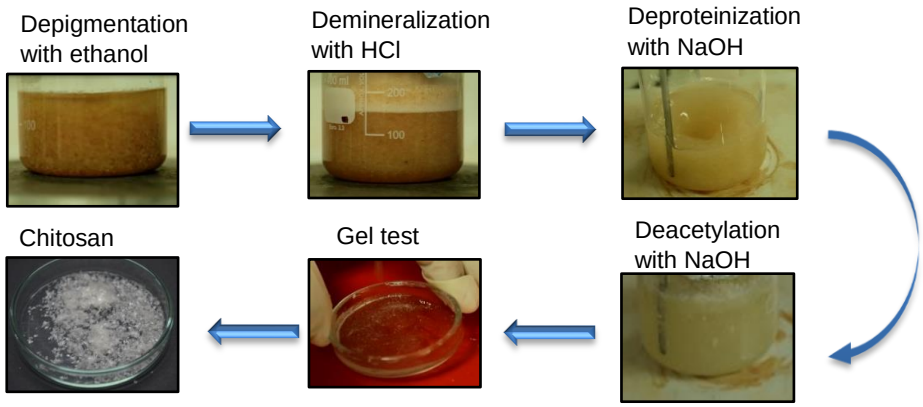

Figure 2: Chemical extraction of chitosan

2.5 Infrared spectroscopy by Fourier transform (FTIR)

Fourier infrared analysis of Fourier infrastructure (FTIR) was used to determine the functional groups of shrimp shells, the five types of chitosan and commercial chitosan, with an IR Affinity-S / N A213749 equipment using the method of KBr pellets (500 mg), followed by compression at 22 - 30 MPa to form the pellets. The transmittance measurements were carried out in the range of 400-4000 cm $^{-1}$ at a resolution of 4 cm $^{-1}$. This analysis was carried out in the Hydrocarbons and Derivatives Analysis Laboratory of the Center for the Petrochemical Industry - Sena (Regional Bolívar), Cartagena, Colombia.

2.6 Determination of the degree of acetylation (DA)

The absorption band at 1,320 cm^{-1} and 1,420 cm^{-1} was chosen to measure the degree of acetylation DA %, knowing that, as presented in Eq(1):

$$DA\ \% = 31.92\frac{A_{1320}}{A_{1420}} - 12.20 \quad (1)$$

The DA % was determined by the method proposed by (Habiba et al., 2017), which is based on the relationship between a reference band at 1,420 cm^{-1} and the amide band III at 1,320 cm^{-1} applying the following Eq(2) (Takara et al., 2015):

$$DD\ \% = 100 - DA\ \% \quad (2)$$

3. Results and discussion

3.1 FTIR results

The structures of the five types of chitosan extracted were confirmed by the FTIR analysis and similar absorption bands were observed (Figures 3 and 4). In the spectra of the five types of chitosan, absorption bands were detected in the range (3,750-3,565 cm^{-1}), which corresponded to vibrations of the OH group due to the elimination of the acetyl group (Ghannam et al., 2016). Other absorption bands were found in the region (2,850-2,900 cm^{-1}) due to stretching vibrations of the CH group of alkane groups. Bands were observed between (1,470 – 1,580 cm^{-1}) which are characteristic of the NH_2 group which shows the conversion of chitin to chitosan (El Knidri et al., 2016), the CN group corresponds to the bands of the amines (1,450-1,420 cm^{-1}), while the bands

between (1,039 – 1,160 cm^{-1}) are due to COC stretch vibrations (Ruiz et al., 2013), the asymmetric stretch of the CH_2 group was in the range (1,300 – 1,450 cm^{-1}), and the stretch of CO of the structure is observed at (1,060 – 1,110 cm^{-1}) (Osuna et al., 2012).

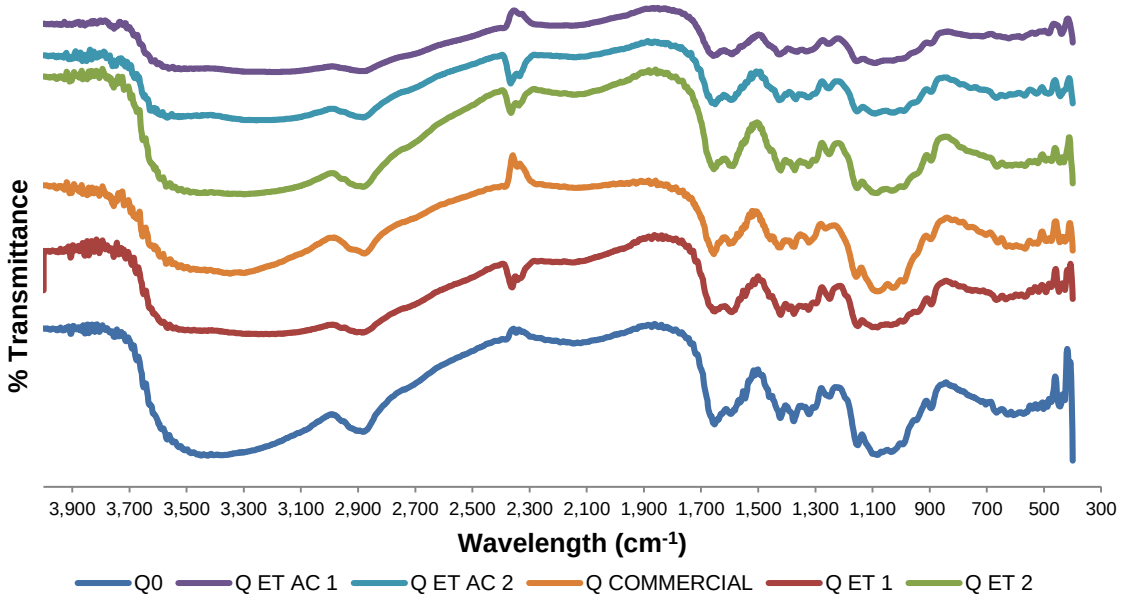

Figure 3: Fourier transform infrared (FTIR) spectrum of commercial chitosan and different samples of chitosan. From bottom to top indicated by the order of the graph

It was adopted that the relationship between the two bands A1320 / A1420 gives a small experimental error regardless of the technique and the state of the material (Brugnerotto et al., 2001).

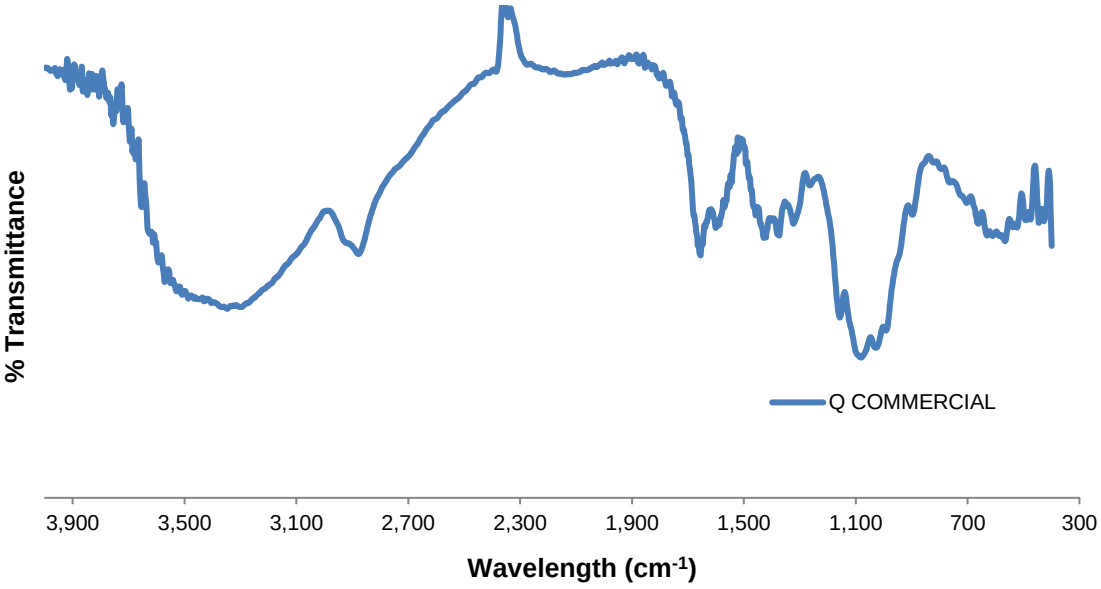

Figure 4: Fourier transform infrared (FTIR) spectrum of commercial chitosan

Table 1 shows a summary of the vibrations in the bands of commercial chitosan and of the chitosan extracted with ethanol. To calculate the degree of deacetylation (DD %) of the chitosan, Equations (1) and (2) (Habiba et al., 2017) and (Takara et al., 2015) were used respectively, the percentage of transmittance of the infrared spectroscopy of each sample was used (Figure. 3).

Table 1: Vibrational modes of Chitosan commercial and chitosan Ethanol 1

Vibration modes	Wave number (cm^{-1})	
	Chitosan commercial	Chitosan Ethanol 1
Ring stretching	906	907
CH_3 wagging along chain	983	972
Asymmetric in-phase ring stretching mode	1,043	1,051
Asymmetric bridge oxygen stretching	1,118	1,089
Amid III band and CH_2 wagging	1,122	1,122
CH bending and symmetric	1,278	1,276
CH_3 deformation	1,292	1,286
CH_2 bending and CH_3 deformation	1,446	1,450
Amid II band	1,479	1,477
Amid I band	1,579	1,583
Amid I band	1,620	1,620
Symmetric CH_3 stretching, asymmetric CH_2 stretching	2,372	2,377
NH stretching	2,954	2,950
OH stretching	3,516	3,522

3.2 Deacetylation test results

The results of deacetylation tests are shown in Table 2, it is observed that the extracted chitosan obtained a degree of deacetylation close to the degree of deacetylation of the commercial chitosan with values that oscillated between 80.43 - 81.81 %. In Table 3, the results obtained in the present investigation are compared with previous studies regarding the degree of deacetylation of chitosan. It is observed that the results obtained exceed the percentage in 20 % when compared with (Nouri et al., 2016), 16 % compared with (Hajji et al., 2014), 5 % compared with (Nouri et al., 2016), and 1 % compared to (Mohammed et al., 2013) but they present a lower degree of acetylation when compared with previous investigations, where they used different extraction processes and different sources of exoskeletons, (1 - 13 %) (Al Sagheer et al., 2009), (1 – 8 %) (El Knidri et al., 2016), and (12 – 14 %) (Kucukgulmez et al., 2011).

Table 2: Degree of deacetylation of commercial chitosan and different samples of chitosan calculated by FTIR

Deacetylation degree (DD %)						
	Chitosan commercial	Chitosan Ethanol 1	Chitosan Ethanol 2	Chitosan 0	Chitosan Ethanol Ac 1	Chitosan Ethanol Ac 2
Eq (1)	17.98	18.19	18.5	18.78	18.82	19.57
Eq (2)	82.02	81.81	81.50	81.22	81.18	80.43

Table 3: Comparison of degrees of deacetylation of chitosan with other authors

Extraction processes	Degree of deacetylation (DD %)	Source
Chemical (shrimp)	80.43 - 81.81	This work
Chemical (shrimp)	82.02	Kumari et al., 2015
Chemical (shrimp)	61	Nouri et al., 2016
Chemical and enzymatic	76 – 82	Hajji et al., 2014
(shrimp, crab and fish)	88, 83 and 95	Al Sagheer et al., 2009
Chemical - microwave (shrimp)	83 – 90	El Knidri et al., 2016
Chemical - Prawns shells	82.73	Mohammed et al., 2013
Chemical - Metapenaeus stebbingi shells	92.19 ± 2.56	Kucukgulmez et al., 2011

4. Conclusions

The process of depigmentation of the shrimp exoskeleton before implementing the steps of demineralization, deproteinization and deacetylation to extract chitosan allows extracting the natural colorant (Astaxanthin) that possesses the exoskeleton of the shrimp without affecting the acetyl and amino groups of the biomaterial, this provides a value added to the extraction process. In this study, the five extraction processes used show that

shrimp exoskeletons obtain a high degree of deacetylation in the range (80.43 - 81.81 %). However, the processes of demineralization, deproteinization and deacetylation of the exoskeleton provide a gradual decrease of the acetyl groups and facilitate the formation of amino groups in the chitosan, but decrease the weight of the initial exoskeleton and the production yield of the extracted chitosan, due to the amount of minerals and proteins extracted from chitin. The results conclude that the exoskeletons of the culture shrimp (*Litopenaeus Vannamei*) is a source of chitosan and astaxanthin, these byproducts could have different applications in the biomedical, pharmacological and food industries.

Acknowledgments

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