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Production and physicochemical characterization of chitosan for the harvesting of wild microalgae consortia

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ABSTRACT

The use of chitosan to harvest microalgae is a strategic step that seeks to reach an economically competitive price to recover lipids, proteins, and pigments. The aim of the present work was to design low-molecular-weight chitosan from shrimp shells and its physicochemical characterization, to be used for the harvesting of wild microalgae consortia. The chitosan was obtained by chemical deacetylation of shrimp shells, and physicochemical characterization was made using the instrumental methods DSC, TGA, X-ray, FTIR, and SEM. The harvesting of wild microalgae consortia was performed by the jar test method. The obtained chitosan had a low molecular weight (169 KDa), a deacetylation degree of 83 %, a decomposition temperature (T_D) of 280 °C, and a crystallinity of 38.2 %. The microalgae genera found in the consortium were *Scenedesmus* sp., *Chlorella* sp., *Schroderia* sp., and *Chlamydomonas* sp. The microalgae removal efficiency of the chitosan was 99.2 % with 20 mg L^{-1}.

1. Introduction

Biodiesel production from microalgae has been investigated for more than 20 years and continues to be economically non-feasible due to the low lipid productivity in a conventional photobioreactor, the expensive nutrients required, the harvesting, and the extraction of intracellular lipids [1]. Mathimani and Mallick [2] mentioned that 1 barrel of algae biofuel costs of US$ 300–2,600, which is considerably much more expensive than 1 barrel of petrol (US$ 40–60).

The two major challenges to the implementation of an integrated microalgae system are the large-scale production of microalgae and the harvesting process [3]. The harvesting process represents nearly 30 % of total capital investment to industrial-scale processing because the small size of microalgae cells (3–30 μm) hinders their sedimentation. It is therefore a strategic step towards lowering production cost and striving to achieve an economically competitive price [2].

The use of natural coagulants, such as chitosan, has been promoted as the so-called "modified local soil-induced ecological restoration" (MLS-IER) technology [4]. Chitosan is a polycationic polymer with more than 5000 D-glucosamine units. This biopolymer is obtained from the chitin (*N*-acetyl-D-glucosamine) recovery of shrimp or crab shells using alkaline deacetylation (NaOH or KOH 40–50 %) at high temperatures (100–120 °C) which influences the molecular weight (Mw) and the degree of deacetylation (DDA) of the obtained chitosan [5]. Chitosan is non-toxic, biodegradable, and biocompatible; under acidic solutions the amine groups ($-NH_2$) are protonated and thus acquire a positive charge ($-NH_3^+$) behaving like as cationic polyelectrolyte [6]. The protonated amino groups of chitosan allow electrostatic interactions with the negatively charged microalgae, forming bridges that can entrap particles causing the settling [4].

In this sense, Mohd et al. [7] reported the harvesting of *Chlorella sp* with 30 mgL^{-1} of chitosan at pH of 7–8, achieving 98 % of microalgae removal; Ahmad et al. [8] obtained 99 % of microalgae removal using 150 mg L^{-1} of chitosan in a pH range of 4–9. However, the efficacy of chitosan is related to the type of microalga, and de Magalhães et al. [9] mentioned that chitosan is ineffective in flocking and settling the cyanobacteria *Mycrocystis auruginosa*. Most research in this area uses chitosan as a coagulant in

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microalgae monocultures and applies commercial chitosan. The aim of the present work was the production of low-molecular-weight chitosan from shrimp shells wastes and its physicochemical characterization, with the aim to use it for harvesting wild microalgae consortia.

2. Materials and methods

2.1. Chemical reagents

Acetic acid (Clemente Jacques®, Mexico), hydrochloric acid (JT Baker®, USA), sodium hydroxide (NaOH) (Meyer®, Mexico), potassium bromide (KBr) (Merck®, Germany), aluminum sulfate (Meyer®, Mexico), Bold's Basal-3 N synthetic minerals (JT Baker®, USA), N-acetylglucosamine, and D-(+)-glucosamine hydrochloride monomers (Sigma-Aldrich®, USA) were used. All chemicals were reactive grade and used as received.

2.2. Microalgae consortia and culture conditions

The consortium of microalgae used in this research was collected from the San Miguel Allende dam (20° 52′27.1′′ N, 100°48′37.0′′ W), in the Bajío region of Mexico. The genera of microalgae identified morphologically in the consortium were *Scenedesmus sp*., *Chlorella sp*., *Schroderia sp*., and *Chlamydomonas sp*. The consortium was initially adapted for 4 months to grow in Bold's Basal-3 N synthetic mineral medium under controlled conditions of temperature (25 °C) and aeration (0.3 vvm), with a luminance of 2,220 lx (TRACEABLE, SN:150056159) or luminous intensity and photoperiod (16 light hours-8 darkness) within a growth chamber. The microalgae were cultivated in 6 L flat plate photobioreactors with a working volume of 4 L at pH 8 (under the operating conditions described above), for 8 days under photoautotrophic conditions. In culture, the inorganic carbon source was environmental CO_2, which was provided through aeration. The biomass concentration was determined by UV/Vis spectrophotometry (HACH, DR3900) at 680 nm, which was correlated with biomass production (gL^{-1}) by means of a calibration curve (range of 0.01–1.03 $g{\cdot}L^{-1}$ and $R^2 = 0.9507$) of the microalgae consortium used in the present work.

2.3. Preparation of chitosan from shrimp shell waste

Shrimp wastes (cephalothorax and exoskeleton) were obtained from a local restaurant of Celaya, México. The shrimp shells were sun-dried for 24 h and then ground in an industrial blender (TAPISA, T12 L, Cd. México, México). The conversion to chitosan was made according to the method of Rodríguez-Núñez et al. [10], with some modifications. During the first step, the chitin was demineralized with 3.6 % (w/w) HCl solution at room temperature for 4 h, after this, the recuperated chitin was immersed in 4.5 % (w/v) NaOH solution at 60 °C for 4 h to remove the proteins. The final step consisted of alkaline deacetylation with 45 % (w/v) NaOH solution at 120 °C for 2 h, which was followed by repeated washes with tap water and drying at 45 °C for 12 h. Prior to use, the chitosan was ground in an industrial blender to decrease particle size.

2.4. Determination of Mw and DDA of chitosan

The degree of deacetylation (DDA) of chitosan was determined by UV/Vis-spectrophotometry; *N*-acetylglucosamine and D-(+)-glucosamine hydrochloride were used as monomers to obtain the standard curve (R^2 = 0.9880), according to the procedure described by Liu et al. [11] with some modifications. Specifically, 8.7 mg of chitosan were dissolved in 50 mL of 0.1 N HCl, and absorption intensity was recorded on a UV/Vis spectrophotometer (Thermo Scientific, Multiskan Go, USA) at 201 nm.

The viscosity molecular weight (Mw) was investigated by using an Ubbelohde viscometer at 30 ± 1 °C. To obtain the standard curve ($R^2 = 0.9613$), five chitosan concentrations (0.0014, 0.0012, 0.0010, 0.008, and 0.006 g mL^{-1}) were dissolved in CH_3COOH 0.3 M/CH_3COONa 0.2 M solution and filtered through membranes with a pore size of 0.45 μm. The viscosity Mw was calculated based on the Mark-Houwink equation:

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

where [η] is the intrinsic viscosity, *K* and *a* are constant values that depend on the nature of the polymer and solvent as well as on the temperature, and M is the relative molecular weight. In this study, K =0.074 mL g^{-1} and $a = 0.76$ are the empirical constants that depend on the polymer nature, the solvent, and the temperature [10].

2.5. Structural properties

2.5.1. FTIR spectroscopy

The chitosan obtained was analyzed using Fourier transform infrared spectrometer (Thermo Nicolet spectrometer, Nexus 670-FTIR, Madison, WI). Briefly, the chitosan was dried in a vacuum oven 60 °C for an overnight and then ground with potassium bromide (KBr) spectroscopic grade (1 mg of dried sample and 100 mg of dried KBr). The accessory diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) for analysis of the powdered sample was used to determine spectra in the absorbance mode at room temperature (mid-infrared region, 4000-600 cm^{-1}), and the OMNIC software was used to obtain the results. In total, 100 scans were measured with a resolution of 4 cm^{-1} [12].

2.5.2. X-ray diffraction (XRD) and crystallinity index

To determine the crystalline structure of the dried chitosan, a sample powder was placed in a sample holder for X-ray diffractometry. The X-ray diffraction patterns were recorded in the reflection mode in an angular range of 5–60° (2θ) at room temperature, using a diffractometer Siemens model D5000 (Karlsruhe, Germany) with a Bragg Brentano geometry and monochromatic CuK_α radiation (λ =1.5418 Å) at 34 kV and 20 mA [6]. Furthermore, the crystallinity index was calculated according to Segal et al. [13]. Specifically, the maximum height of (020) peak represents both crystalline and the minimum between (020) and (110) peaks is representing the amorphous part:

$$CrI = \frac{I_{(020)} - I_{(am)}}{I_{(020)}} \; x \; 100$$

where *CrI* is the crystalline index (%), $I_{(020)}$ is the intensity at (020) peak ($2\theta = 10.07°$), and $I_{(am)}$ is the intensity at the minimum between (110) and (020) peaks and represents the intensity of the amorphous diffraction region.

2.6. Thermal properties

2.6.1. Thermogravimetric analysis (TGA/DTGA)

Thermogravimetric analysis of the chitosan was performed on equipment TGA (TA Instruments, Discovery, New Castle, DE). A sample of approximately 10 mg was heated from 50 to 800 °C at a heating rate of 10 °C/min, under an inert atmosphere of N_2 with a flow rate of 60 mL/min [6].

2.6.2. Differential scanning calorimetry (DSC)

Differential scanning calorimetry analysis was performed on a TA Instruments DSC model Discovery (New Castle, DE) under a

nitrogen atmosphere. Around 5–8 mg of chitosan were sealed in aluminum pans and heated at 10 °C/min from room temperature (25 °C) to 300 °C.

2.7. Morphological analysis

2.7.1. Scanning electron micrographs (SEM) and elemental analysis

Morphological analysis of the powder chitosan surface was performed at magnifications of 500X and 2000X, using a scanning electron microscope (JEOL, JSM-7600 F, Japan). The chitosan particles were coated with a thin layer of Au-Pd using a sputter coater (Quorum model QI5OR-ES, Sussex, UK) before scanning. Morphological analysis of the chitosan powder was performed particles to investigate the size, shape, and form of the particles [12]. In addition, the samples were analyzed to determine the elemental composition using an Oxford X-Max 20 energy dispersive X-ray spectroscopy (EDS) detector and an Oxford wavelength-dispersive X-ray spectroscopy (WDS) detector.

2.8. Harvesting test

The analyses of harvesting were evaluated through jars tests at room temperature (25 °C) according to Rodríguez-Núñez et al. [10]. The dosages of chitosan and aluminum sulfate investigated were 12, 16, 20, 24, and 28 mg L^{-1}. The chitosan solution was prepared using chitosan powder in acetic acid 1% (pH ~ 4.0), while aluminum sulfate was dissolved in distilled water. The chitosan and aluminum sulfate were evaluated as primary coagulants. Specifically, 500 mL of microalgae cultures were poured into beakers of 1 L and placed on jars equipment (TEMSA, MOD. JTR1020, México). The pH of the microalgae cultures was 8.0 and was adjusted to 6.5 (using 1 M hydrochloric acid) for each jar test. Unity was started and adjusted to a quick mix (200 rpm during 30 s), followed by slow mix (100 rpm during 10 min) and resting for 10 min (settling time). The conditions were similar for both treatments (chitosan solution and aluminum sulfate). Finally, the percentage of microalgae removal was calculated as the difference between the initial and final turbidity, registered with a portable turbidimeter (HANNA Instrument, HI93703, USA).

2.9. Statistical analysis

Batch experiments were conducted in triplicate (n = 3) and data represent the mean value. ANOVA and LSD tests using the software package Statgraphic plus 4.0 estimated the statistical significance of differences between means ($p \leq 0.05$) (StatPoint Technologies, Inc, Warrenton, VA, USA).

3. Results and discussion

3.1. Determination of mw and DDA of chitosan

The chitosan preparation conditions from chitin recovered from shrimp shell are important due to their influence on structural parameters such as molecular weight (Mw), degree of deacetylation (DDA), and acetyl group distribution [6]. In this sense, the chitosan obtained had a low molecular weight (169 kDa) with 83 % of DDA, which is related to the chemical treatment used which caused the depolymerization of the chitosan chains, manly during the deacetylation process under alkaline conditions [6]. The chitosan obtained fell into the range of the commercial low-molecular-weight chitosan and DDA supplied by Sigma-Aldrich (50–190 kDa and 75–85% DDA, CAS Number 9012–76-4). Muley et al. [14] reported the obtaining of chitosan from shrimp shells by chemical treatment (deacetylation with 50 % NaOH) reaching 78.04 % of DDA and 173 kDa of Mw. Salazar and Valderrama [15] mentioned that the chitosan obtained with sodium nitrate showed 133.33 kDa of Mw and 81.78 % of DDA. The methodology used in this work proved to be efficient to produce quality chitosan of low molecular weight, in comparable even with trademarks.

3.2. Structural properties

3.2.1. FTIR spectroscopy

Fig. 1 shows the FTIR spectra of chitosan powder. The chitosan powder presented an FTIR spectrum similar to those reported by various authors [6,16,17]. The broadband around 3290 cm^{-1} was attributed to the axial stretching of hydroxyl groups (O—H) overlapped to the asymmetric/symmetric stretching of the amine bonds (N—H) and the band observed at 2867 cm^{-1} was determined by the signal of axial stretching of -C-H in the polymer chain [6]. The band situated at 1656 cm^{-1} represented the —C=O stretching of the acetamide groups of the chitosan (amide I) and the band at 1568 cm^{-1} agreed to the trans-secondary amides (amide II). Moreover, the amino group bending (N—H) was spliced with the length of the amide (II) [17]. The bands at 1419 cm^{-1} belonged to CH_2 deformation vibrations in the CH_2OH groups and the signals situated at 1375 cm^{-1} agreed with the symmetrical angular deformation of $—CH_3$ in $NHCOCH_3$ groups. The bands in the regions of 1259 cm^{-1} to 1330 cm^{-1} belonged to —CO, NH_2 of the amide III and —OH deformation in the plane [6,16]. The bands situated at 1152 and 1148 cm^{-1} corresponded to symmetric and asymmetric stretching signals of glycosidic linkage (C—O—C). Besides, the bands at 1060 cm^{-1} and 1081 cm^{-1} represented the C—O in secondary and primary OH groups, and the band at 892 cm^{-1} belonged to CH groups from pyranose ring skeletal vibrations [18].

3.2.2. X-ray diffraction (XRD)

The X-ray diffraction patterns of the chitosan powder are shown in Fig. 2. The characteristic peaks of chitosan were centered at 2θ = 10.7° and 2θ = 20.07° corresponding to the crystallographic planes (020) and (110), respectively. The results were similar to those reported by various authors [17–19]. Furthermore, the chitosan powder obtained showed 38.206 % of the crystallinity index, proving that as in the case of chitin, solid chitosan molecules are also organized into ordered and crystalline regions and coexist with amorphous regions. The crystallinity index obtained was similar to the 40 and 42 % reported by Mogilevskaya et al. [20] and Loelovich [21], respectively; however, these values are related to the degree of deacetylation and the treatment used for the

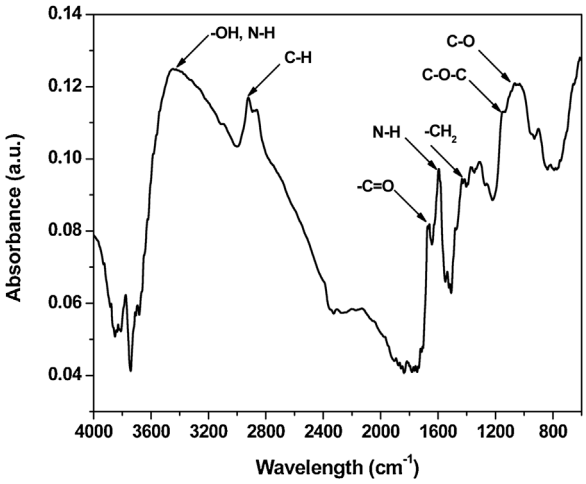

Fig. 1. FTIR spectra of homemade chitosan powder.

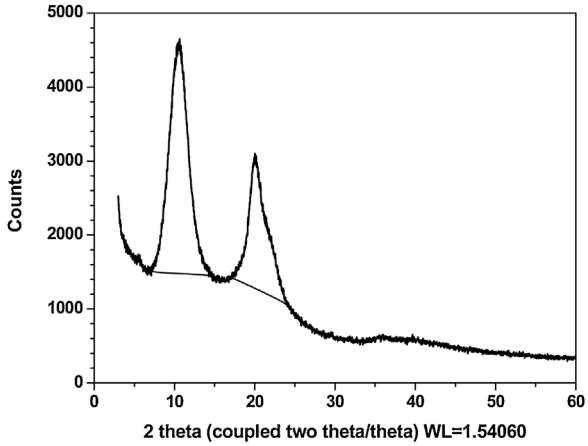

Fig. 2. X-ray diffraction spectra of homemade chitosan powder.

production of chitosan, being able to change according in the different research works.

3.3. Thermal properties

3.3.1. Thermogravimetric analysis (TGA/DTGA)

Fig. 3 shows the TGA/DTGA thermograms obtained from the chitosan powder. The decomposition temperature (T_D) of chitosan powder occurred at 280 °C, related to the depolymerization and decomposition of the acetyl and amine groups of chitosan [22]. Mass loss was around 50 % with 32 % of residue ashes. The DTGA thermogram showed that the first thermal event occurred as temperature range of 50–155 °C, which is related to water evaporation (mass loss from 5 to 7%). This was followed by a second event, related to the T_D of chitosan powder (220–490 °C) produced by thermal and oxide decomposition of chitosan; this process also involves the vaporization and elimination of volatile compounds, also known as products of combustion (CO, CO_2, solvents, low-molecular-weight molecules, and functional groups (Nieto et al. [23]). Cardenas et al. [24] reported that the T_D decreased with decreasing acetyl units; thus, the chitosan obtained presented a high degree of deacetylation due to its T_D (280 °C) being lower than that indicated by various authors, above of 300 °C [24,25,23].

3.3.2. Differential scanning calorimetry (DSC)

Fig. 4 shows the DSC thermogram of chitosan powder. The results show a broad endothermic peak around 91.28 °C, which is related to the evaporation of residual water or solvent from the chitosan [6]; the enthalpy required to release these components was 353.18 J/g. The endothermic peak started at 42.6 °C and ended at 165.8 °C. Stable heat flow in the chitosan sample was observed; afterward, the exothermic peak began at 270 °C. This indicates the thermal decomposition process of chitosan (depolymerization, saccharide ring dehydration, decomposition of deacetylated and acetylated chitosan units), being similar to the T_D obtained in the TGA/DTGA thermogram in the present work (Fig. 3); the DSC thermogram obtained from chitosan was similar to that reported by Pendekal and Tegginamat [26]. Another important point to note is that the melting point (Tm) not was observed due to amorphous regions in the chitosan that cause the dispersion of the energy points. Moreover, chitosan has the Tm close to the T_D, hindering its Tm [27].

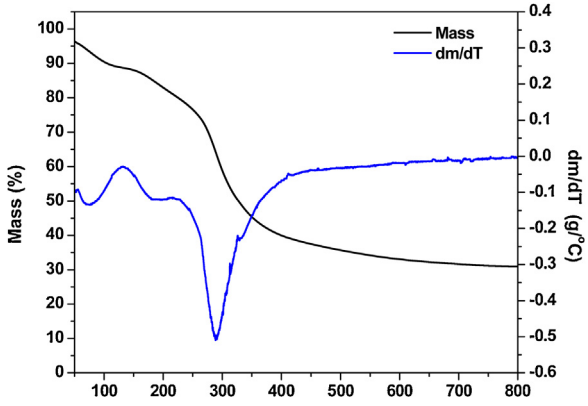

Fig. 3. TGA and DTG thermogram of homemade chitosan powder.

3.4. Morphological analysis

3.4.1. Scanning electron micrographs (SEM) and elemental analysis

The micrographs obtained by SEM of the chitosan powder are shown in Fig. 5. The chitosan obtained was a fine and homogeneous powder of small particles (average = 0.34 ± 0.046 mm of length) with a rough surface and without pores. These irregularities should occur in the chitosan particles due to the processes such as the milling, drying, and the use of chemicals. The chitosan produced in the present work was similar to that reported by Agarwal et al. [28] and Muhammed et al. [29].

Table 1 shows the results of the elemental analysis. For N and N, the values were similar to the 44.02 % C and 7.96 % of N reported by Hussein et al. [30] in samples of chitosan obtained from shrimp shell by the chemical method. Liu et al. [31] reported values of % C = 44.63 and % N = 8.71 in commercial chitosan. Gupta et al. [32] mentioned that the results of elemental analysis change according to the deacetylation degree of chitosan, due to the loss of acetyl groups and reported ranges of 5.13–8.03% for N and 28.56–48.36% for C. In this sense, the chitosan obtained in the present work is of good quality and within the ranges previously reported.

3.5. Harvesting microalgae test

The effect of chitosan as a coagulation-flocculation agent in microalgae harvesting is presented in Figs. 6 and 7. The best results were obtained with 20 mg L^{-1} of chitosan, reaching 99.2 % of microalgae removal, while the aluminum sulfate reached 12.54 % as maximum removal. Fig. 6 shows that at a chitosan concentration of 28 mg L^{-1}, the removal of microalgae cells decreased. Ahmad et al. [8] explained that when using a dosage greater than the optimal dose of chitosan (greater than 30 mg L^{-1}), the excess of cationic charges allows the re-stabilization of the microalgae cells, decreasing the efficiency of the process and interfering with floc formation.

Moreno et al. [33] reported the flocculation of *Scenedesmus* sp. and *Chlorella* sp. with chitosan, reaching 94–99 % removal using 200 mg L^{-1}. Mohd et al. [7] mentioned 98 % of microalgae removal (*Chlorella* sp.) with 30 mg L^{-1} of chitosan. In this sense, Yin et al. [34] pointed out, in a review, that 20–150 mg L^{-1} of chitosan were required to harvesting microalgae. The results showed that similar or even lower doses of chitosan were required when compared to previous works. The parameters that influence the coagulation-flocculation capacity of chitosan are the degree of acetylation and the molecular weight because the chitosan in acidic conditions has a high cationic charge density due to the amine functional groups protonated (-NH_3^+), which interact with the negative charge of the microalgae cells. This situation induces the flocculation process by the neutralization of charges (weakening of the electrostatic repulsion), reducing interparticle repulsion (Mohd et al. [7]; Ahmad et al. [8]). In this sense, the degree of deacetylation is an

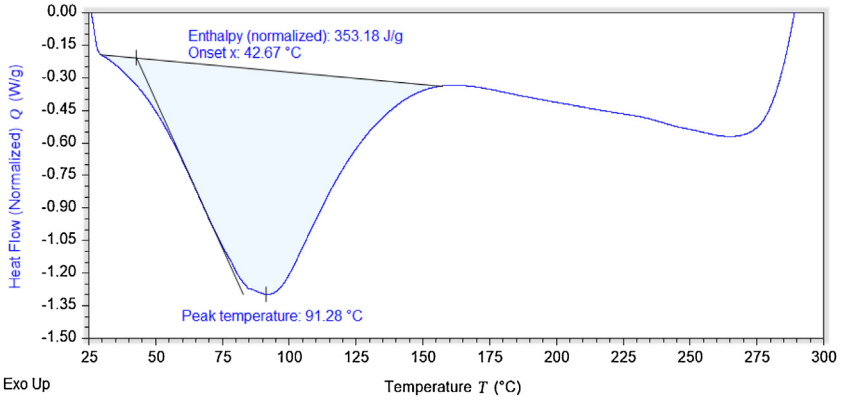

Fig. 4. DSC thermogram of homemade chitosan powder.

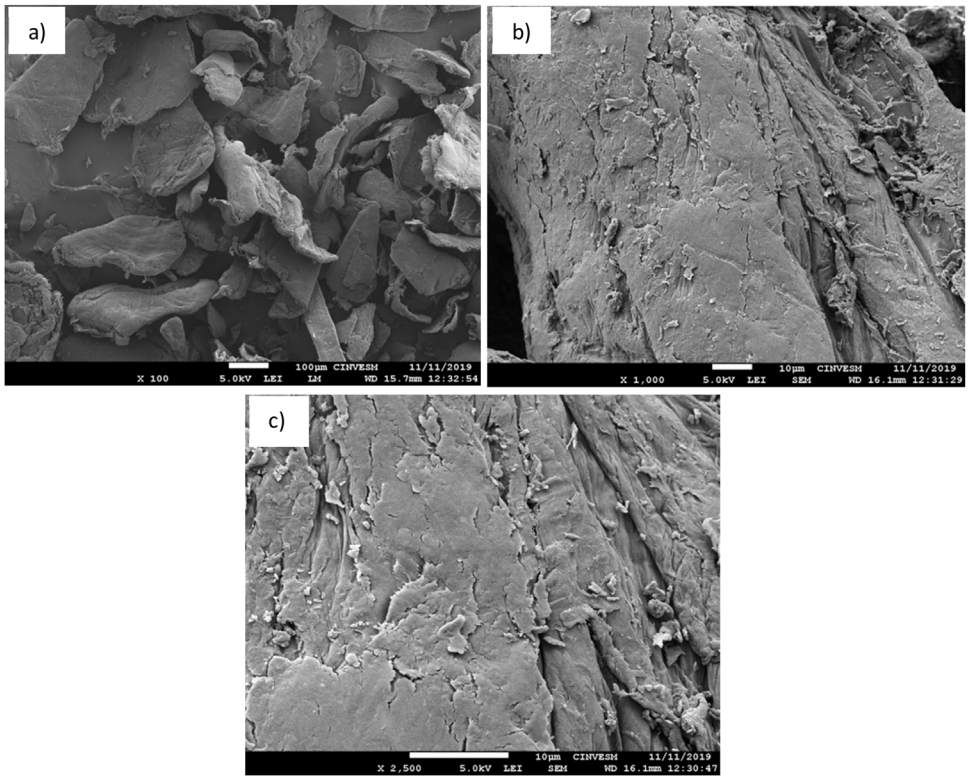

Fig. 5. SEM micrographs of homemade chitosan powder a) x100, b) x1000 and c) x2500.

Table 1
Elemental analysis composition of homemade chitosan powder.

Element	Weight%	Atomic%
C_k	46.01	52.55
N_k	9.58	9.38
O_k	44.41	38.07
Total	100	

important structural parameter of chitosan because it indicates the number of functional amino groups with the ability to protonate in an acidic aqueous medium. The influence of the molecular weight is due to the fact that in chitosan with high molecular weight, the bridging mechanisms dominate, attaching the microalgae cells that subsequently can entrap particles when settling. In contrast,

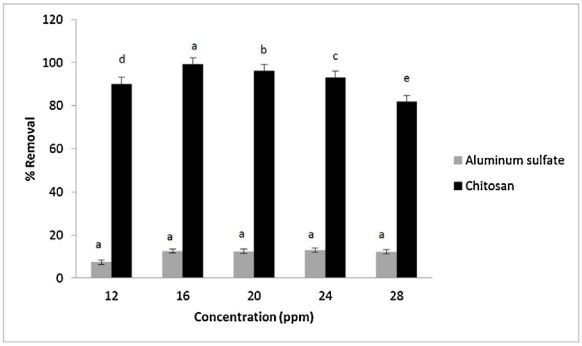

Fig. 6. Removal percentage of microalga with chitosan solution and aluminum sulfate as coagulant-flocculants agents.

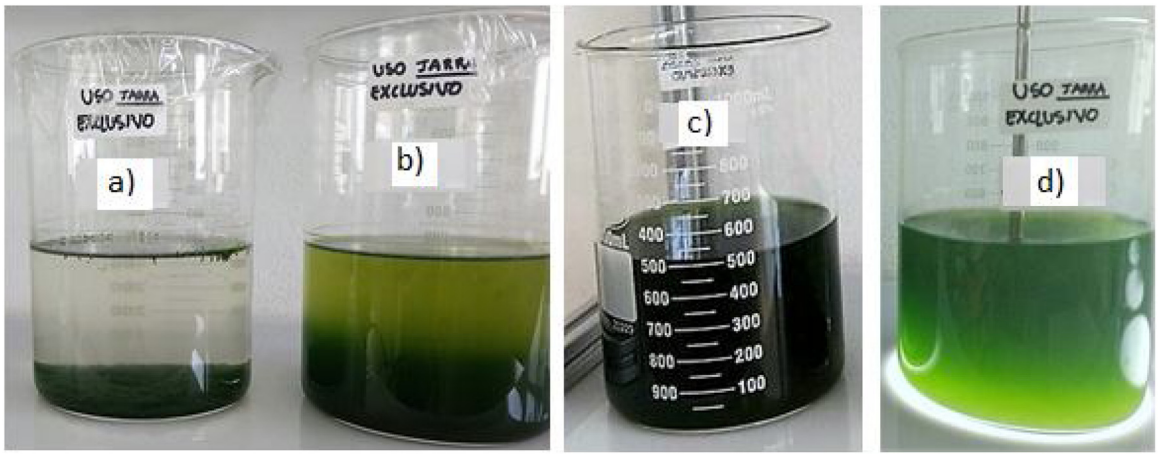

Fig. 7. a) Chitosan concentration (10 mg L^{-1}), b) Control of acetic acid (1 %) C) Aluminum sulfate concentration (10 mg L^{-1}), d) Aluminum sulfate concentration (20 mg L^{-1}).

in chitosan with low molecular weight, the flocculation process occurs by neutralization of charges, in this sense, an increase in molecular weight is directly proportional to an increment of the hydrodynamic diameter of a chitosan molecule but inversely related to the surface charge density (Lürling et al. [4]; Strand et al. [34]).

Furthermore, the use of chitosan for harvesting microalgae reduces the use of inorganic flocculants, avoiding the disadvantages of using inorganic flocculants which can contaminate the downstream process (changes in the microalga color and damage to the methanogenic activity), which limits the recycling of the culture medium [35].

In the Biotechnology Laboratory of the University of Guanajuato (Campus Celaya, Mexico), since 2018, the harvesting of microalgae has been carried out using the chitosan obtained in the same laboratory. In this sense, the estimated cost of chitosan obtained is USD$224 kg^{-1}, while a commercial brand sells it at USD $963.66 kg^{-1} (Sigma-Aldrich®) plus the expenses of the laboratory supplier. Considering that 40 mg L^{-1} are required to harvest a volume of 1000 mL of microalgae in suspension, then USD$224 is required for 25,000 L of microalgae cultures. This allows to reduce the costs and to avoid the centrifugation of huge amounts of culture, thereby decreasing the cost of inorganic coagulants and the operating costs. Finally, we do not have the disadvantages related to the use of use inorganic coagulants.

4. Conclusions

The chitosan obtained from shrimp shell wastes showed good physicochemical properties due to its low molecular weight, high acetylation degree, and decomposition temperature, as well as its low crystallinity. The harvesting of microalgae consortia using chitosan of low molecular weight is not only possible, but even more feasible than the use of chemical coagulants such as aluminum sulfate. The use of chitosan as a microalgae coagulant is therefore a viable alternative to avoid the use of chemical coagulants and to reduce the costs of this stage, which represents up to 30 % of the total cost of the process.

CRediT authorship contribution statement

Stefanie Acosta-Ferreira: Formal analysis, Investigation. **Omar S. Castillo:** Conceptualization, Supervision, Funding acquisition. **J. Tomás Madera-Santana:** Writing - review & editing, Resources. **Daniel A Mendoza-García:** Investigation, Methodology. **Carlos A. Núñez-Colín:** Data curation, Formal analysis. **Claudia Grijalva-Verdugo:** Writing - original draft, Writing - review & editing. **Alma G. Villa-Lerma:** Writing - review & editing. **Adán T. Morales-Vargas:** Visualization. **J. Rubén Rodríguez-Núñez:** Conceptualization, Project administration, Funding acquisition, Writing - original draft.

Declaration of Competing Interest

The authors report no declarations of interest.

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