

## Article

# Green Extraction of Chitin from Deep-Water Red Shrimp (*Aristeus antennatus*) By-Products via Lactic Acid or Non-Lactic Acid Fermentation, Recovery Optimization, and Chitin Conversion into Chitosan to Produce Chitosan-Based Biofilms

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## Abstract

The increasing demand for seafood over the years has led to an increase in by-products produced by the seafood processing sector. These by-products, which can represent up to 70% of processed products, are rich in nutrients and bioactive compounds, allowing them to be used in several sectors, such as food, packaging, cosmetics and pharmaceuticals, provided that recovery occurs in an eco-friendly manner. In the present work, two lactic acid bacteria (*Lactobacillus lactis* and *L. brevis*) and the yeast *Yarrowia lipolytica*, which are organisms capable of producing organic acids and proteases during the fermentation process, were used separately to extract chitin from deep-water shrimp (*Aristeus antennatus*) by-products. The results showed that *L. lactis* was the most effective microorganism in removing both the mineral and protein fractions; therefore, it was chosen to optimize the chitin extraction technique, after which chitin was eventually converted into chitosan. The obtained chitosan showed a deacetylation degree (DDA%) of 82%, indicating good film-forming capacity. The developed biological technique allowed the valorization of shrimp by-products by recovering chitin and eventually chitosan, allowing us to produce biofilms that could help prolong seafood shelf life and, from a circular economy point of view, contributing further to promoting the sustainability of the production sector.

**Keywords:** chitin; chitosan-based film; green extraction method; shrimp by-products; sustainability; lactic acid bacteria; yeast; fermentation; *Aristeus antennatus*



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## 1. Introduction

Over the last few decades, global fishery and aquaculture production has been increasing, reaching 223.2 million tons in 2022 [1], to satisfy the rising demand for seafood. Indeed, human seafood consumption was equivalent to an estimated 20.7 kg per capita in 2022 and is anticipated to grow further in the future. In high-income countries, seafood is mostly processed, causing by-product production to also increase; however, instead of being discarded as waste, these by-products are increasingly used in food or non-food products, accounting for up to 34 and 53 percent of the total production of fish meal and oil, respectively [1]. Despite this, the Marine Ingredients Organization (IFFO) reported that there is still a large proportion of seafood by-products, almost 12 million tons, wasted instead of being turned into high-value products. In this context, it has to be highlighted

that disposal can be a serious problem for enterprises, as it results in increased costs; moreover, if not conducted properly, it can cause pollution, which negatively impacts the environment. Therefore, to improve the seafood industry's revenue and sustainability, it is important that seafood by-products are valorized, as they represent a large portion of the processed product. For instance, in the fish processing sector, by-products, such as heads, skins, bones, scales, and viscera, can represent up to 70 percent of the whole fish, while the by-products from shrimp processing account for up to 60 percent of the whole shrimp. Therefore, given the importance of valorizing seafood by-products, to increase the economic and environmental sustainability of the production sector, researchers and enterprises have been paying increasing attention to sustainable extraction processes to obtain bioactive compounds such as n-3-series polyunsaturated fatty acids, fish enzymes, collagen, carotenoids, chitin, chitosan, glucosamine, functional foods, nutraceuticals, fish protein hydrolysates, and fish protein concentrates to be used in the food industry and for human nutrition and health [2]. Moreover, these compounds could help improve seafood shelf life by means of their bioactive properties, which in turn can help reduce fish loss and waste, which are estimated annually to be up to 35% of the global fishery and aquaculture production industry [1]. Thus, chitin and chitosan are applied as antioxidants or antibacterial and antifungal agents in the food industry.

Chitin is the second most available polysaccharide after cellulose, as it is a fairly ubiquitous compound found in fungal and algal cell walls, insect exoskeletons, the endoskeletons of cephalopods and crustacean shells. However, commercial chitin is mainly obtained from the by-products produced by the crustacean processing industry, as more than 6 million tons of crustacean waste is produced every year [3]. It is obtained from the exoskeletons of crustaceans using demineralization and deproteinization processes to remove the mineral and protein fractions, respectively, from the shell material, followed by a decolouration step if a colourless product is wanted. To obtain chitin, two types of methods are used: chemical and biological (microbial) methods. The former shows several disadvantages; for instance, it is eco-unfriendly and negatively affects the physicochemical properties of chitin. Moreover, the removed proteins and minerals, although potentially valuable supplements for human food and animal feed, are sufficiently damaged that they are no longer appropriate for these applications [4,5]. Therefore, it is increasingly important to use biological methods as they are a safer treatment option for chitin recovery. Lactic acid- and protease-producing microorganisms have been used for the demineralization and deproteinization steps because of the proteases and organic acids produced by these microorganisms, which contribute to mineral and protein removal from crustacean shell waste. The biological treatment consists of fermenting the crustacean biowaste using different species of lactic or non-lactic microorganisms [4,5]. Bacteria of the genus *Lactobacillus* were used either alone or in combination with other microorganisms to achieve high deproteinization and demineralization degrees of shrimp wastes [6–8].

*Aristeus antennatus* is a commercially significant deep-sea shrimp in the Mediterranean and Atlantic waters [9–11]. Based on previously reported data, *A. antennatus* catches in Italy were approximately 1000 tons in previous years, thus representing a fishery resource of great value [9]. As far as the Italian catches were concerned, a great part of these catches was performed by the Sicilian trawl fleet, which increased from 182 tons in 2004 to 402 tons in 2016, indicating an increase in production volume of 121%, which is higher than that recorded for other shrimp species [10]. Moreover, this crustacean is also distributed in the Mediterranean and Atlantic south of the Iberian peninsula, reaching as far as the Portuguese coasts. Additionally, this species is the main target of a monospecific fishery, due to its high commercial value (50% of the landings by value in some ports) [11].

Nevertheless, its by-products, which are rich in bioactive compounds such as chitin/chitosan, are often discarded, remaining largely unexploited compared to those of the other species [12]. Thus, there is a substantial opportunity to recover underutilized biomolecules such as chitin/chitosan via a circular economy approach, which can then be employed in other production sectors, such as the food one. To date, few studies [13,14] have addressed the challenge of valorizing *Aristeus antennatus* by-products, for instance by recovering carotenoids using environmentally friendly GRAS solvents [13] or microwave-assisted extraction (MAE) [14]. In this context, considering the commercial relevance of this fishery species, the main objective of the present study was to valorize *Aristeus antennatus* by-products. Thus, the present study aimed to recover chitin using biological methods based on lactic acid or non-lactic acid fermentation to produce a chitosan-based biofilm that can be used in the packaging sector, thereby achieving a circular economy. In particular, the lactic acid fermentation processes were based on using two new strains of lactic acid bacteria only, i.e., *Lactobacillus lactis* or *Lactobacillus brevis*. These microorganisms were chosen after reviewing the literature, as lactic acid bacteria, especially those belonging to the *Lactobacillus* genera, were proven to be very effective in extracting chitin from crustacean by-products. Moreover, they are safe organisms commonly used in food processing, and to the authors' knowledge, the strains considered in the present work have not been used in previous studies aimed at recovering chitin via lactic acid fermentation.

In contrast, for the non-lactic acid fermentation method, the yeast *Yarrowia lipolytica* was used to overcome the knowledge gap regarding this microorganism. Indeed, in a previous study [15], it was showed that this yeast species contributed to a very high deproteinization degree but a low demineralization degree, which could be enhanced in the future, as suggested by authors, by adding carbon sources to the fermentation broth. So, in the present study, *Yarrowia lipolytica*-based non-lactic acid fermentation was performed by adding a carbon source in the culture medium to evaluate the effectiveness of the added carbon source in improving the yeast's demineralization efficiency.

The three extraction processes were compared in terms of fermentation efficacy, and the most efficient microorganism was utilized to optimize the extraction method. Eventually, the chitin obtained was converted into chitosan to produce a biofilm, thus allowing its antioxidant and antimicrobial properties to be studied in the future to evaluate its potential for prolonging seafood shelf life. Overall, the present study provided useful information about using novel or partially studied microorganisms to obtain high-purity chitin (with less than 1% of minerals) from largely unexploited shrimp by-products in an eco-friendly approach, i.e., without or limiting the use of harmful chemicals. In this context, chitin of a higher purity will yield higher-quality chitosan with better solubility and functional properties.

## 2. Materials and Methods

### 2.1. Shrimp Shell Waste

The shrimp shell waste (heads and shells with tails) of deep-water red shrimp (*Aristeus antennatus*) was supplied by local restaurants in Campania (Italy), immediately transported to the laboratory under refrigerated conditions, and frozen at  $-18\text{ }^{\circ}\text{C}$  until use.

To extract chitin from *A. antennatus* by-products, the samples were thawed and washed several times with distilled water to remove impurities, dried in an oven at  $55\text{ }^{\circ}\text{C}$  for 12 h, and then homogenized using a mixer (Bosch VitaPower Serie 4, Robert Bosch S.p.A., Milan, Italy).

## 2.2. Microorganisms and Inoculation

Two different strains of Lactic Acid Bacteria (LAB), namely *Lactobacillus lactis* ATCC 11454 and *Lactobacillus brevis* ATCC 8287, and a yeast strain, *Yarrowia lipolytica* Y6, were obtained from the microorganism collection of the Food Safety Division of the Experimental Station for the Food Preserving Industry (PR, Italy), isolated, and identified from food products. To prepare the starting culture, the yeast was cultured in the Malt Extract Broth (MEB) medium (Thermo Fisher Diagnostics S.p.A., Segrate, Italy), while the LAB were cultured in the De Man, Rogosa e Sharpe (MRS) medium (Thermo Fisher Diagnostics S.p.A., Segrate, Italy).

Shrimp by-product fermentation was carried out by inoculating 15 g of by-products with the aforementioned microorganisms ( $10^8$  cfu/g) and 5 mL of glucose (20%) and the batch cultures were incubated for 3 days at 30 °C at 150 rpm. In the second batch cultures, where a selected microorganism was used for shrimp by-product fermentation, 15 g of by-products was inoculated with a suspension of *L. lactis* ( $10^9$  cfu/g) and 15 mL of glucose (20%) and incubated for 7 days at 30 °C at 150 rpm to obtain pure chitin that was then converted into chitosan. For each batch, at the end of the incubation period, the samples were centrifuged, washed several times with distilled water, and dried at 55 °C.

## 2.3. Chemical Extraction of Chitin

For chemical deproteinization, shrimp shell waste was treated with 0.75 M NaOH (Carlo Erba Reagents S.A.S, Val-de-Reuil, France) at a ratio of 1:6 (g/mL) for 24 h at room temperature under constant agitation (80 rpm).

In contrast, for chemical demineralization, the samples were treated with 1.25 M HCl (TITOLCHIMICA S.p.A., Villamarzana, Italy) at a ratio of 1:10 (g/mL) for 1 h at room temperature under constant stirring (200 rpm).

After the reactions, the samples were drained, washed several times with distilled water until a neutral pH was achieved, and oven-dried at 55 °C.

## 2.4. Deacetylation of Chitin to Obtain Chitosan

To obtain colourless powders, biological and chemical chitin samples were treated with 90% ethanol (TITOLCHIMICA S.p.A., Villamarzana, Italy) (1:60 g/mL) at 70 °C for 30 min at 200 rpm. Then, the pure chitin was converted into chitosan through a deacetylation process that was carried out with 19 N NaOH (Carlo Erba Reagents S.A.S, Val-de-Reuil, France) (1:45 g/mL) at 90 °C for 6 h at 200 rpm. After each reaction, the samples were centrifuged, rinsed with distilled water until a neutral pH was achieved, and placed in an oven at 55 °C.

## 2.5. Physicochemical Analyses

The shrimp shell waste and chitin extracted were analyzed to determine total nitrogen, chitin content, total lipids, proteins, and ash and moisture contents.

The analyses were carried out in triplicate using the following methods: Moisture levels were determined using AOAC 90.15:1993. Lipid content was determined using the Soxhlet method (AOAC 920.39). Total nitrogen and ash contents were evaluated using AOAC 954.01/988.05 and 938.08, respectively. Chitin and protein contents were evaluated by determining total nitrogen (Nt) and non-nitrogen compounds (i.e., ash, moisture and lipids), as described above, and applying the following equations [16]:

$$\text{Chitin \%} = (\text{Nt} \cdot \text{Cp} + \text{K} - 100) \cdot \text{Cq} / (\text{Cp} - \text{Cq}) \quad (1)$$

$$\text{Protein \%} = (\text{Nt} \cdot \text{Cq} + \text{K} - 100) \cdot \text{Cp} / (\text{Cq} - \text{Cp}), \quad (2)$$

where K is the sum of the non-nitrogen compounds, and Cp (6.25) and Cq (14.5) are the conversion coefficients that relate the mass fraction of nitrogen with protein and chitin, respectively [16].

#### 2.5.1. pH Measurements

The pH was determined using a pH metre (CRISON–GLP21, HACH LANGE S.R.L., Lainate, Milan, Italy). Before the analyses, the system is usually calibrated using two certified buffer solutions at pH 4.01 and 7.00 (CHEMIFARM S.R.L., Parma, Italy).

#### 2.5.2. Lactic Acid Determination

D- and L-Lactic acid contents were determined using the Enzytec™ kit (R-Biopharm Italia S.R.L., Melegnano, Italy). Briefly, the assay is based on the reaction of both lactic acids in the presence of NAD and D- or L-Lactate dehydrogenase to pyruvate and NADH, and the NADH formed is equivalent to the amount of D- and L-lactic acids converted.

#### 2.6. Determination of Fermentation Efficacy

The demineralization and deproteinization degrees (DDM% and DDP%, respectively) were calculated using the following equations:

$$\text{DDM \%} = [(\text{Initial ash} - \text{final ash}) / \text{Initial ash}] \cdot 100 \quad (3)$$

$$\text{DDP \%} = [(\text{Initial total protein} - \text{final total protein}) / \text{Initial total protein}] \cdot 100 \quad (4)$$

#### 2.7. ATR–FTIR Spectroscopy

All chitosan samples were analyzed via Fourier transform (FTIR) infrared spectroscopy using an infrared spectrophotometer, FT-IR Microscope Spotlight 200i (PerkinElmer, Inc., Shelton, CT, USA), equipped with Spectrum v10.7.2 software.

The FTIR spectra were measured between 650 and 4000  $\text{cm}^{-1}$  with a resolution of 4  $\text{cm}^{-1}$  and acquired in attenuated total reflectance (ATR) using a special accessory (micro-ATR), which allows even opaque samples to be characterized by placing the germanium crystal directly on the sample. The crystal's penetration depth is generally less than 6  $\mu\text{m}$ .

The deacetylation degree (DDA%) of the samples was calculated using the following formula:

$$\text{DDA \%} = 100 - [(A_{1560} / A_{1027}) \cdot 100], \quad (5)$$

where  $A_{1560}$  is the absorbance at 1560  $\text{cm}^{-1}$  and  $A_{1027}$  is the absorbance at 1027  $\text{cm}^{-1}$ , as determined by Duarte et al. [17].

#### 2.8. Film-Forming Capacity Assays

Solubility tests were carried out by dissolving chitosan samples in a 1% acetic acid (Carlo Erba Reagents S.A.S, Val-de-Reuil, France) solution. Then, their film-forming capacity was evaluated by dissolving chitosan (1%  $w/v$ ) in a 1% acetic acid solution with glycerol (Sigma Aldrich, Saint-Louis, MO, USA) in a proportion of 30% ( $w/w$ ) relative to the chitosan mass. This formulation was developed and optimized in previous research projects by the Packaging Division of SSICA [18] based on chitosan samples obtained from other sources.

The solution was constantly stirred at room temperature for at least 6 h, then overnight at 4 °C, until the chitosan was completely dissolved [19]. This solution was then deposited on a 12 cm in diameter Petri dish (LABOINDUSTRIA S.p.A., Padova, Italy) and allowed to dry in air at room temperature until the solvent was completely evaporated.

### 2.9. Scanning Electron Microscope (SEM) Micrographs

The microstructures of the edible chitosan-based films were evaluated via a scanning electron microscope and qualitative X-ray microanalysis (JSM-IT 300, Jeol Ltd., Tokyo, Japan) at the Packaging Division of SSICA. The samples were mounted on stubs and coated with gold and palladium under high-vacuum conditions. The SEM micrographs were taken at 100× magnification.

### 2.10. Statistical Analysis

Experiments and analyses were carried out in triplicate, and one-way analysis of variance (ANOVA) and Tukey's HSD multiple comparisons were performed using RStudio software (2023.12.1+402) to analyze significant differences ( $p < 0.05$ ).

## 3. Results

### 3.1. Proximate Composition of Shrimp By-Products

The proximate composition of *A. antennatus* by-products is shown in Table 1, revealing similar findings to those obtained by Liu et al. [20], who analyzed the meat and by-product composition of five shrimp species. After analyzing the composition of *A. antennatus* by-products, we found that they represent a rich source of bioactive compounds that could be employed in the nutraceutical, food, and packaging sectors.

**Table 1.** Proximate composition of shrimp by-products (heads and shells with tails).

| Proximate Composition | g/100 g of By-Products |
|-----------------------|------------------------|
| Protein               | 6.10 ± 0.12            |
| Fat                   | 1.42 ± 0.03            |
| Ash                   | 15.30 ± 0.10           |
| Chitin                | 12.48 ± 0.34           |
| Moisture              | 64.70 ± 0.07           |

### 3.2. Physicochemical Characterization of Extracted Chitin and Fermentation Efficacy

The results showed that shrimp waste fermentation with *L. lactis* led to a drastic reduction in ash and protein contents, with a DDM and DDP of 81.8 and 85.8%, respectively (Table 2). Moreover, pH drastically decreased during the 3-day treatment period (Table 1), and significant differences in pH were observed among treatments ( $p < 0.05$ ). In particular, *L. lactis* was the microorganism that led to the lowest pH after three days of incubation ( $p < 0.05$ ), as well as to the highest percentage of demineralization.

**Table 2.** Changes in total nitrogen, chitin, protein, and ash contents (on a dry weight basis); DDP (%); DDM (%); and pH after three days of fermentation with LAB and *Y. lipolytica* and after chemical extraction.

|                      | Total Nitrogen (%)       | Proteins (%)              | Ash (%)                   | Chitin (%)                | DDM (%)                   | DDP (%)                   | pH                       |
|----------------------|--------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|--------------------------|
| Shrimp waste         | 5.16 ± 0.02 <sup>A</sup> | 17.00 ± 0.24 <sup>A</sup> | 43.34 ± 0.15 <sup>A</sup> | 35.60 ± 0.48 <sup>A</sup> | n.d.                      | n.d.                      | 7.72 ± 0.01 <sup>A</sup> |
| <i>L. lactis</i>     | 6.53 ± 0.05 <sup>B</sup> | 2.41 ± 0.35 <sup>B</sup>  | 8.49 ± 0.24 <sup>B</sup>  | 89.10 ± 0.20 <sup>B</sup> | 81.80 ± 0.25 <sup>A</sup> | 85.80 ± 0.55 <sup>A</sup> | 4.73 ± 0.02 <sup>B</sup> |
| <i>L. brevis</i>     | 4.89 ± 0.14 <sup>C</sup> | 2.86 ± 0.42 <sup>B</sup>  | 15.93 ± 0.44 <sup>C</sup> | 81.21 ± 0.18 <sup>C</sup> | 63.10 ± 0.05 <sup>B</sup> | 83.20 ± 0.87 <sup>A</sup> | 4.86 ± 0.01 <sup>C</sup> |
| <i>Y. lipolytica</i> | 5.28 ± 0.07 <sup>A</sup> | 1.13 ± 0.02 <sup>C</sup>  | 24.92 ± 0.16 <sup>D</sup> | 73.95 ± 0.09 <sup>D</sup> | 43.20 ± 0.08 <sup>C</sup> | 93.40 ± 0.52 <sup>B</sup> | 5.01 ± 0.03 <sup>D</sup> |
| Chemical             | 6.80 ± 0.21 <sup>B</sup> | 1.01 ± 0.05 <sup>C</sup>  | 0.00 ± 0.00 <sup>E</sup>  | 96.00 ± 0.11 <sup>E</sup> | 100 ± 0.00 <sup>D</sup>   | 96 ± 0.75 <sup>B</sup>    | n.d.                     |

Different letters (A–E) indicate significant differences between treatments ( $p < 0.05$ ). Not determined: n.d.

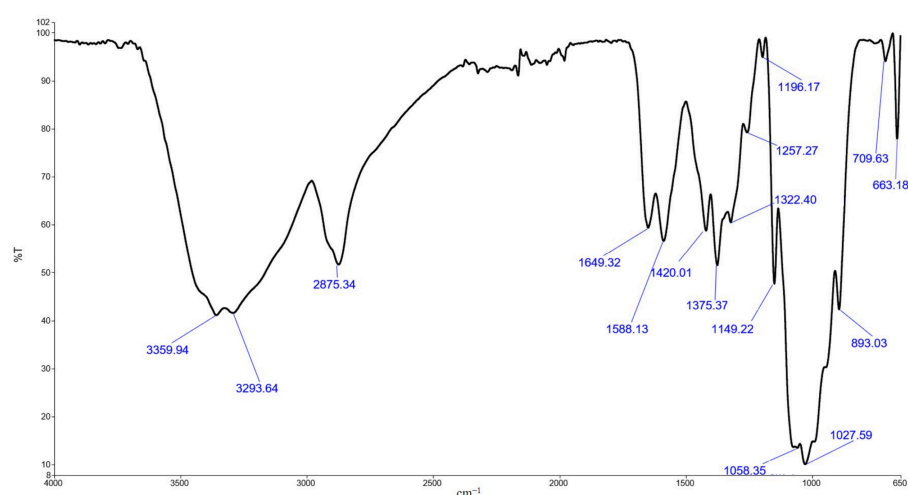
After 3 days of incubation with the selected microorganisms, their viability was evaluated. The results showed that the number of both *L. lactis* and *L. brevis* increased slightly after three days of fermentative treatment, with no significant differences compared to the initial inoculum size ( $10^8$  cfu/g). These findings revealed that both LAB were not

inhibited in the acidic environment and that they had not yet entered the autolysis stage, suggesting that prolonging the treatment time could have helped reduce the mineral and protein contents. Indeed, the duration of the fermentation process, along with the inoculum size and sugar concentration, can affect fermentation efficacy.

Taking this into account and considering that *L. lactis* was the most effective microorganism in terms of removing both the mineral and protein fractions, this lactic acid bacterium was selected to optimize the chitin extraction technique to obtain a high-purity product (less than 1% of proteins and minerals). In fact, it is necessary to remove most of these components during the chitin extraction process to obtain chitosan with good film-forming properties. In particular, after 7 days of treatment, *L. lactis* contributed to lowering the pH, from 4.73 to 4.29, and decreasing the ash content to its lowest at 5.2% with a DDM of 88%. Significant differences were observed between the 3-day and 7-day fermentative treatment periods regarding the ash content and the demineralization percentage ( $p < 0.05$ ), but no significant differences ( $p > 0.05$ ) were observed in terms of protein content and the deproteinization percentage. After 7 days, the number of *L. lactis* decreased slightly without significant differences compared to the initial inoculum size ( $p > 0.05$ ). The D,L-lactic acid analysis showed a concentration of 11.2 g/kg after 7 days of incubation, which is higher than what was observed in other studies aimed at extracting chitin from shrimp by-products using other lactic acid bacteria [21]. The chitin obtained from *A. antennatus* by-products incubated with *L. lactis* for 7 days was converted into chitosan, which was subsequently characterized and used to produce a biofilm.

### 3.3. ATR-FTIR Spectroscopy

ATR-FTIR spectroscopy was used to assess the functional groups present in chitosan. The infrared spectrum of chitosan extracted using the optimized microbial method is shown in Figure 1. The strong bands in the  $3293\text{--}3359\text{ cm}^{-1}$  region correspond to N-H and O-H stretching, as well as intramolecular hydrogen bonds. The absorption bands at  $2875\text{ cm}^{-1}$  can be attributed to C-H symmetric stretching and is also found in other polysaccharide spectra [22]. The presence of residual *N*-acetyl groups was confirmed by the bands at approximately  $1649\text{ cm}^{-1}$  (C=O stretching of amide I) and  $1322\text{ cm}^{-1}$  (C-N stretching of amide III).



**Figure 1.** FTIR spectrum of chitosan obtained from chitin extracted from *A. antennatus* by-products using *L. lactis*.

A band at  $1588\text{ cm}^{-1}$  corresponds to the N-H bending of the primary amine [23], while the  $\text{CH}_2$  bending and  $\text{CH}_3$  symmetric deformations were confirmed by the presence

of bands at approximately  $1420$  and  $1375\text{ cm}^{-1}$ , respectively. The absorption band at  $1149\text{ cm}^{-1}$  can be attributed to the asymmetrical stretching of the C-O-C bridge, while the bands at  $1058$  and  $1027\text{ cm}^{-1}$  correspond to C-O stretching. Additionally, all bands observed in the spectra of the chitosan samples extracted in this study were also reported in other studies [24].

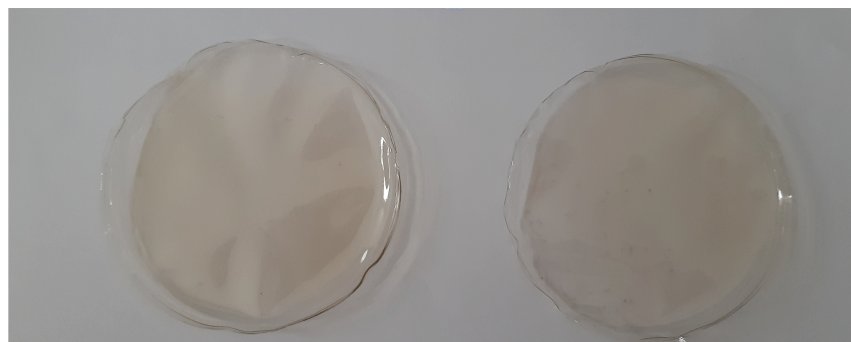
The spectra of chitosan samples extracted using the biological method were similar to both the spectra of the chitosan samples extracted via the chemical process and the spectra of the commercial chitosan, which exhibited the same bands as those mentioned above. It has to be highlighted that all the chitosan samples exhibited a prominent characteristic band at  $1588\text{ cm}^{-1}$ , which revealed the success of N-deacetylation in all extracted chitosan samples [25]. The spectra of the chitosan samples obtained in the present work were similar to those exhibited by the chitosan samples extracted from other sources, such as house crickets and the white shrimp *Penaeus vannamei* [25,26].

The similarity of the spectra obtained via ATR-FTIR spectroscopy between chemical and biological chitosan samples were confirmed by the results obtained in terms of deacetylation degree. Indeed, the chitosan samples obtained from chitin extracted using the microbial method showed a deacetylation degree of 81.89%, with no significant differences ( $p > 0.05$ ) with the chitosan obtained from chitin extracted using the chemical method (82.01%).

### 3.4. Film-Forming Capacity Assays

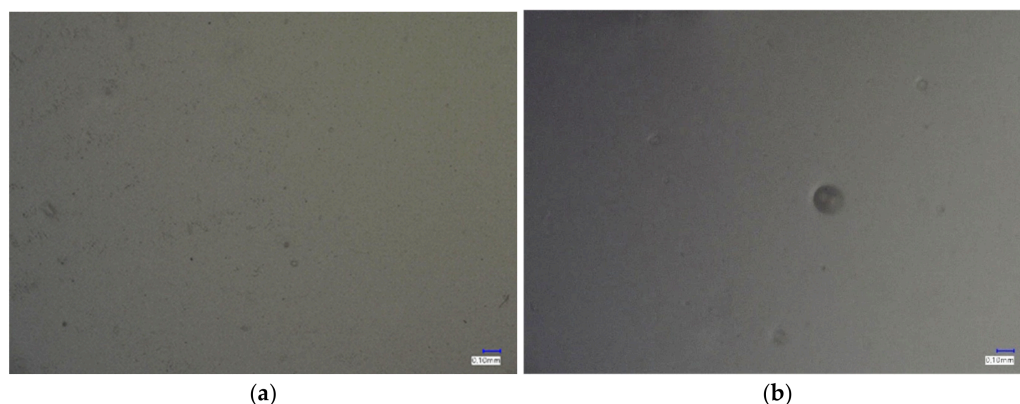
In a preliminary study, the film-forming capacity assays were carried out on all the chitosan samples, i.e., the chitosan samples obtained from all chitin samples extracted using the three microorganisms alone. Nevertheless, the low purity of chitin due to the incomplete removal of native proteins and minerals compromised the chitosan-based biofilms' structural integrity, which were too fragile due to reduced cross-linking and the presence of residual impurities and particles that were not completely solubilized. In this context, the higher the purity of chitin, the higher the chitosan quality in terms of better solubility and functional properties. Moreover, the deacetylation degree of the chitosan samples ranged from 49 to 51%.

In this regard, the complete solubilization of the chitosan samples obtained via the optimized extraction method in an acetic acid solution was achieved without using ultrasound. The high degree of deacetylation observed in the chitosan samples could have contributed to the significant increase in solubility in the acetic acid solution, thus confirming the correlation between the deacetylation degree and solubility in acidic solutions [27]. Moreover, the chitosan samples showed excellent film-forming properties, and the films obtained were both uniform (Figure 2).



**Figure 2.** Chitosan-based biofilm obtained from shrimp chitin extracted using the microbial method.

The chitosan-based biofilms showed some clusters of particles in certain areas of the film (Figure 3b), but overall, the surface was fairly homogeneous and smooth (Figure 3a), which indicated good dispersion of the chitosan in the used solvents and the structural integrity of the chitosan.



**Figure 3.** (a) SEM image of the chitosan-based biofilm from shrimp chitin; (b) clusters in the chitosan-based biofilm from shrimp chitin.

#### 4. Discussion

Microbial methods for obtaining chitin with good physicochemical properties as well as proteins and minerals, which could potentially be employed as supplements for animal and human consumption, should be explored to study innovative green chitin extraction methods from crustacean by-products. In this case, homolactic and heterolactic bacteria have been employed in the lactic acid fermentation processes to extract chitin from crustacean biomass [28]. Indeed, during the shell demineralization process, the lactic acid generated through bacteria metabolism will dissolve calcium carbonate, thus facilitating the formation of calcium lactate, which can be easily separated by washing. In particular, the lactic acid solubilizes calcium carbonate and other minerals from shrimp waste; in addition, during lactic fermentation, calcium carbonate is converted to calcium lactate via lactic acid interactions [29]. Moreover, the low-pH condition improves the ensilation process by suppressing the growth of spoilage microorganisms. In this process, lactic acid is produced through glucose decomposition during bacterial catabolism. Taking this into account and considering that shrimp by-products have low amounts of fermentable sugars, the addition of carbon sources, based on organic acid production, to the fermentation broth is needed to improve the demineralization process.

Mineral removal from shrimp by-products facilitates the deproteinization process, making proteins much more accessible and easier to remove via the joint action of the proteolytic enzymes produced by the inoculated lactic acid bacteria and the enzymes present in the by-products themselves, which are activated by the medium's low pH [28,29]. These enzymes carry out protein hydrolysis, thus driving the breakdown of the proteins into soluble peptides and free amino acids, which can be easily washed away, and contributing to their successfully separation from insoluble chitin and to the deproteinization efficiency of the biological process [28]. The liquid fraction was proven to be a rich source of nutraceutical constituents with pharmaceutical application, as well as essential nutrients, such as proteins, carbohydrates, and n-3-series polyunsaturated fatty acids [29].

Several factors, such as the inoculum size, sugar concentration, initial pH, pH changes throughout fermentation, incubation temperature, and the duration of the fermentation process, can affect fermentation efficiency [28–30]. In a previous study [30], media containing date syrup, glucose, and sucrose yielded demineralization degrees of 82%, 75%, and 71%,

respectively. In the present study, glucose was added as a energy source, yielding a higher demineralization degree (88%) when shrimp by-products were incubated with *L. lactis* at an inoculum size of  $10^9$  ufc/mL for seven days at 30 °C. In Khorrami et al.'s study [30], the inoculation of shrimp by-products with *L. plantarum* using glucose as an energy source led to a sharp decrease in pH after seven days of incubation, which is comparable to what was obtained in the present study. The reduction in pH led to mineral removal from shrimp by-products, which occurred due to the formation of carbon lactate. In the present work, a sugar concentration of 20% (*w/w*) was used, in accordance with what was reported in previous studies, which used a concentration of at least 7.5% of supplemented sugars (*w/w*), to ensure that the pH drops below 5.6 and that the fermentation process is not contaminated when using unpasteurized shrimp by-products [31]. Incubating shrimp by-products for three days with the other lactic acid bacteria, i.e., *L. brevis*, yielded a DDM% value lower than that obtained using other LAB [30,31]. In addition to bacteria, fungi, such as *Yarrowia lipolytica*, *Candida tropicalis*, and *Pichia kudriavzevii*, were also investigated for chitin extraction from shrimp by-products through fermentation. These studies demonstrated that yeast species could effectively reduce the concentrations of protein and minerals in shrimp heads [15,28]. In particular, Ta et al. [15] observed that *Y. lipolytica* had the highest protease activity, with a 1.5-fold increase compared to *Bacillus subtilis* and 5.5-fold increase compared to *C. tropicalis* and *P. kudriavzevii*. In the present study, *Y. lipolytica* was the microorganism that contributed to the highest deproteinization degree (93.40%), compared to what was obtained using batch cultures of inoculated *Litopenaeus vannamei* heads by Ta et al. [15], i.e., a deproteinization degree of 85.8% after 5 days of incubation. In terms of demineralization, a demineralization degree of 43.20% was obtained in the present study, which is comparable to that obtained by Ta et al. [15], i.e., a DDM% of 49.4% after 5 days of incubation. Mineral removal driven by *Y. lipolytica* fermentation of shrimp by-products is due to the production of organic acids, such as citric acid, which reacts with calcium carbonate, the principal mineral in shrimp waste, to convert it to its soluble form, which is then removed via washing [15,32]. In the present study, carbohydrate addition to stimulate acid accumulation in *Y. lipolytica* did not enhance the efficiency of the process. Thus, to achieve higher deproteinization and demineralization degrees in the future, chitin extraction from shrimp by-products may be divided into two separate stages, namely demineralization and deproteinization, considering the best-performing microorganisms for each stage evaluated in the present work.

The chitin obtained from shrimp by-products via the *L. lactis*-based fermentative process was eventually converted into chitosan for biofilm production. Indeed, one of the most promising application areas for biodegradable natural polymers such as chitosan is the food packaging sector, in particular the fish processing sector, while also considering some of its other interesting properties, i.e., antioxidant capacity, that can help prolong seafood shelf life, thus facilitating a perfect circular economy. Moreover, chitosan derived from shrimp shell waste exhibits significant antimicrobial activity against both Gram-negative (e.g., *Escherichia coli* and *Pseudomonas aeruginosa*) and -positive bacteria (e.g., *Staphylococcus aureus* and *Streptococcus mutans*) [33]. Another important aspect is that chitosan can be obtained relatively easily from a wide range of natural sources, such as shrimp and crab shells, and exhibits good thermal and chemical stability, making it highly suitable for the development of bio-based packaging films [34].

With regard to chitosan, its complete solubility in acetic acid was achieved without using ultrasound. As reported in the literature [27,35], its solubility in acetic acid is influenced by residual ash content; indeed, the lower the ash content, the higher the solubility [35]. Moreover, a correlation between the deacetylation degree and solubility in acidic solutions was observed [27], which was confirmed by the results obtained in the

present study. Indeed, in this work, the parameters applied to the deacetylation process contributed to the extraction of high-deacetylation-degree chitosan (81.89%), which in turn led to high solubility in acetic acid solutions. Moreover, the chitosan samples showed good film-forming capacity. Indeed, films without discontinuities and with an acceptable thickness ( $83.2 \pm 14.2 \mu\text{m}$ ) were produced, and the physicochemical properties of the chitosan obtained, such as a deacetylation range between 80 and 90%, could ensure good film-forming capability. Additionally, antimicrobial properties should be guaranteed with this high degree of deacetylation, as reported in the literature [19,36].

In the future, the antimicrobial and antioxidant properties of the chitosan-based biofilms will be evaluated; moreover, the biofilms will be tested for seafood preservation to study their effectiveness in prolonging the shelf life of highly perishable food products.

## 5. Conclusions

The chemical method remains the predominant technique for extracting and preparing chitin/chitosan from shrimp shells. Nevertheless, the fermentation method offers a promising approach to sustainably recovering chitin from shrimp by-products. Indeed, environmentally friendly, efficient, and gentle extraction processes are needed to make the process more sustainable and to obtain supplements that can be used for animal and human consumption. To ameliorate chitin extraction from shrimp by-products, it is crucial to optimize the fermentation conditions and upscale the process, which is currently restricted to the laboratory scale, to be competitive with the chemical method. In this case, it is important to employ efficient bacteria and supplement them with carbon sources to obtain high-purity chitin. In the present work, the procedure used to extract chitin from *A. antennatus* by-products was based on a fermentation method using a new strain of lactic acid bacteria, i.e., *L. lactis*, and resulted in high-purity chitin. Moreover, the obtained chitin was successfully converted into chitosan with a good degree of deacetylation and suitability for the subsequent film production phase. In this study, chitin was deacetylated to obtain chitosan via the traditional method, i.e., using NaOH solutions; in the future, green deacetylation processes may be investigated and compared with the traditional method to make the whole process green and sustainable.

Moreover, future work will focus on upscaling the process and testing the properties of chitosan-based biofilms, such as their antimicrobial and antioxidant powers. Moreover, the biofilms' effectiveness in prolonging seafood shelf life will be investigated to make the seafood production sector even more sustainable and profitable.

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## Abbreviations

The following abbreviations are used in this manuscript:

|       |                                                       |
|-------|-------------------------------------------------------|
| AOAC  | Association of Official Analytical Chemists           |
| ATR   | Attenuated total reflectance                          |
| DDP%  | Degree of deproteinization                            |
| DDM%  | Degree of demineralization                            |
| DDA%  | Degree of deacetylation                               |
| FTIR  | Fourier transform infrared spectroscopy               |
| GRAS  | Generally recognized as safe                          |
| IFFO  | Marine Ingredients Organization                       |
| LAB   | Lactic acid bacteria                                  |
| MAE   | Microwave-assisted extraction                         |
| MEB   | Malt extract broth                                    |
| MRS   | De Man, Rogosa and Sharpe                             |
| NADH  | Nicotinamide adenine dinucleotide reduced form        |
| NAD   | Nicotinamide adenine dinucleotide                     |
| SEM   | Scanning electron microscope                          |
| SSICA | Experimental Station for the Food Preserving Industry |

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