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Antimicrobial potential of a hydrolyzed protein extract of the microalgae *Nannochloropsis* sp.

Potencial antimicrobiano de un extracto de proteína hidrolizado de la microalga *Nannochloropsis* sp.

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Abstract: Microalgae are photosynthetic microorganisms widely used at the industrial level because of their high content of compounds with bioactive properties such as antimicrobial peptides. These compounds have a wide range of action mechanisms, do not produce the resistance of conventional antibiotics and could be effective against bacteria, fungi, viruses, parasites, and even tumor cells. In this work, by mechanical cell lysis treatment, a protein extract of dry biomass of *Nannochloropsis* sp was obtained, subsequently, it was hydrolyzed with an enzyme complex of proteases, papain, and pancreatin, for 6 hours, at 44 ° C and pH 7. Finally, the antimicrobial activity of the fraction of hydrolyzed extract with a size less than 3 kDa was evaluated against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*, obtaining a percentage of growth inhibition of 93.1%, 96.6%, and 14.4% respectively, which represents a peptic extract with bacteriostatic and fungistatic properties.

Keywords: microalgal protein, enzymatic hydrolysate, *Nannochloropsis* sp., antimicrobial activity.

Resumen: Las microalgas son microorganismos fotosintéticos ampliamente utilizados a nivel industrial gracias a su alto contenido de compuestos con propiedades bioactivas como lo son los péptidos antimicrobianos. Estos compuestos son eficaces contra bacterias, hongos, virus, parásitos e incluso células tumorales, poseen una amplia variedad de mecanismos de acción y no generan la resistencia de los antibióticos convencionales. En este trabajo, a través de lisis celular mecánica, se obtuvo un extracto de proteínas a partir de biomasa seca de *Nannochloropsis* sp., posteriormente fue hidrolizado usando un complejo enzimático de proteasas, papaína y pancreatina, durante 6 horas, a 44°C y pH 7. Finalmente, se evaluó la actividad antimicrobiana de la fracción de extracto hidrolizado que poseía un tamaño menor a 3 kDa contra *Staphylococcus aureus*, *Escherichia coli* y *Candida albicans*, obteniendo un porcentaje de inhibición de crecimiento de los microorganismos de 93,1%, 96,6%, y 14,4% respectivamente, lo cual representa un extracto peptídico con propiedades bacteriostáticas y fungistáticas.

Palabras clave: proteína microalgal, hidrolizado enzimático, *Nannochloropsis* sp., actividad antimicrobiana.

1. Introduction

Microalgae are photosynthetic microorganisms [1], producers of a wide variety of metabolites of interest such as lipids, carbohydrates, proteins, pigments, vitamins, among others [2-6], playing an important role in diverse applications as cosmetic, pharmaceutical industries, biofuels, biofertilizers, wastewater treatment [7].

The proteins, protein hydrolysates and peptides from an important variety of strains of microalgae have received special interest for their antioxidant, anticancer, antihypertensive and antimicrobial activity, etc. [1-4]

The mechanism of action of conventional antibiotics coupled with the excessive and improper use of these compounds has led to the increase of resistant and multiresistant microorganisms, hindering the treatment and control of infectious diseases. For this reason, a promising solution to this problem is to find compounds with different mechanisms of action such as antimicrobial peptides (AMP). AMPs are found in various sources, such as microorganisms, plants, vertebrates or invertebrates [8]. These compounds are part of the innate immune system of a large variety of individuals, playing a role against the attack of invading organisms [9]. Their broad spectrum of action gives them an important effect against bacteria, fungi, viruses, and parasites. Their structural diversity allows them to act against several biological targets such as cell membranes, proteins, nucleic acids, and enzymes [8-10].

Specifically, the potential of different peptides and protein hydrolysates of microalgal origin has been reported by various authors as possible therapeutic agents from an unconventional source, due to its antimicrobial [11], antiviral [12], immunoregulatory function [13,14], with a wide range of applications.

In this context, this work focuses on obtaining a hydrolyzed protein extract from the microalgae *Nannochloropsis* sp., and to determine its antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*.

2. Materials y methods

2.1. Biomass obtaining

The concentrated biomass of *Nannochloropsis* sp. was obtained from a culture carried out in 250L photobioreactors, under continuous light conditions with white fluorescent lamps ($135 \mu\text{molm}^{-2}\text{s}^{-1}$) and a temperature of $23 \pm 1^\circ\text{C}$. The culture medium used was agricultural fertilizer (NPK 13:40:13). After 11 days of cultivation, the biomass was washed between 3 and 5 times to remove the excess of salts and subsequently dried in a WTC BINDER oven at 50°C . It was manually macerated in a mortar to obtain a fine powder and stored in a dry place until its use.

2.2. Extraction and protein quantification

60 g of dry biomass was suspended in 600 mL of 50 mM phosphate buffer at pH 7, the mixture was reserved at 4°C for 15 minutes. Subsequently, a mechanical process corresponding to homogenization was performed in an ULTRA TURRAX IKA T25 DIGITAL unit, at 6000 rpm, during 20 cycles of 5-minute on and 2-minute intervals. During homogenization, the mixture was kept in an ice bath, to prevent its heating. The extract was centrifuged at 10,000 rpm for 10 minutes and vacuum filtered with a cellulose membrane filter of 0.2 µm pore diameter. Finally, the supernatant was subjected to dialysis for 24 h, using a membrane with a pore size of 3.5 kDa of Spectra/Por (Spectrum laboratories) obtaining the crude extract.

The crude protein present in the biomass was analyzed by the percentage of total nitrogen by the Kjendhal method, using 5.2 as a conversion Nitrogen-to-protein factor [15].

The soluble protein was determined by the Lowry method [16], using a calibration curve with BSA (bovine serum albumin) between 0.1 and 1 mg/mL. The sample was analyzed in a UV-Vis Spectronic Genesys 2PC equipment, at a wavelength of 750 nm.

2.3. Enzymatic hydrolysis

The protein extract was hydrolyzed with a mixture of Papain (PROENZIMAS) and Pancreatin (Abbott Laboratories), at 44 °C and pH 7, for 6 hours, at an enzyme-substrate ratio of 10 U/g of protein for each enzyme [17]. Subsequently, the hydrolyzed extract was passed through a 3 kDa AMICON filter.

The degree of hydrolysis (DH) was quantified by equation 1, which is presented below:

$$\% DH = \frac{PS_0 - PS_t}{P} * 100 \quad (1)$$

% DH: Degree of hydrolysis (%).

PS₀: Amount of soluble protein in 6.25%(w/v) TCA (Trichloroacetic acid), before the addition of enzyme.

PS_t: Amount of soluble protein in 6.25%(w/v) TCA after the addition of enzyme, in a t time.

P: Amount of initial protein.

2.4. Polyacrylamide gel electrophoresis (SDS-PAGE)

The samples were concentrated by adding to 10 mL of them, 10 mL of 20% (w/v) TCA [18], left at 4°C overnight, centrifuged at 15,000 rpm for 15 minutes at 4°C and the obtained pellet was washed with 300 µL of cold acetone. They were centrifuged again at 15,000 rpm for 15 minutes at

4°C and the supernatant was removed. The resulting pellet was suspended in 60 µL of solubilization Buffer (100 mM Tris, 100 mM EDTA and 8M Urea) pH 8.5.

To the previous mixture was added 20 µL of a solution composed of 10 µL of 2-mercaptoethanol and 90 µL of coloring solution (SDS, Coomassie Blue R-250, glycerol) and left in incubation for 5 minutes at 80°C. Then, 15 µL were seeded in the SDS Tris-tricine gel at 14% concentration and the electrophoretic run was carried out in a Bio-Rad Mini-PROTEAN® equipment at 150 V, for 1 h and 15 minutes.

The resulting gel was stained in a Coomassie Blue R-250 solution for 12 hours under constant agitation. The molecular marker used was the Blue Prestained Protein Standard from Biolabs® Inc., with a range between 11-190 kDa.

2.5. Fourier transform infrared spectroscopy - FTIR

The extracts were analyzed in a Bruker Tensor II FT-IR with diamond ATR (Bruker Optics, Germany). The equipment consists of a photovoltaic MCT detector cooled with liquid nitrogen. The wavenumber range was from 4,000 to 700 cm⁻¹ with a resolution of 4 cm⁻¹. The curve fitting was analyzed using the OPUS Software (Bruker Optics, Germany). The baseline of the spectra was corrected and analyzed in each selected region.

2.6. Antimicrobial activity

The broth dilution method [19,20] was implemented, using the following microorganisms: *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Candida albicans* (ATCC 90028). The fraction less than 3 kDa of the hydrolyzed extract was passed through a 0.2 µm cellulose membrane filter to eliminate possible contaminants.

The strains were activated in broth, TSB for bacteria and Sabouraud for yeasts, at a temperature of 36°C for 24 h. The inoculum was adjusted with a 0.5 McFarland pattern.

In tubes of 5 mL capacity, were added 3 different concentrations of hydrolyzed extract, 33.35% (v/v), 20% (v/v) and 10% (v/v), 1233 µL of broth (TSB or Sabouraud), and finally, 100 µL of the inoculum, respectively. The tests were carried out in triplicate. Additionally, a feasibility control was made for each microorganism, which did not contain the extract. The samples were placed in incubation for 24 h at 36°C. Serial dilutions were made in triplicate in 0.9% (w/v) saline solution from each sample and 10 µL of each one was seeded in TSA agar (bacteria) or PDA (yeast) according to the micro drop technique [21]. The samples were left in incubation at 36°C, 24 h for bacteria and 48 h for yeast. Finally, it was made a recount of the colonies present in each sowed dilution.

The percentage of inhibition of the strains was calculated [22], according to equation 2:

$$\text{Inhibition (\%)} = \frac{MC_{\text{control}} - MC_{\text{evaluated}}}{MC_{\text{control}}} * 100 \quad (2)$$

MC_{control}: Number of colonies forming units per milliliter of the control treatment. (CFU/mL)

MC_{evaluated}: Number of colonies forming units per milliliter of the evaluated treatment. (CFU/mL)

2.7. Statistical analysis

The comparison of the different treatments was carried out by analysis of variance (ANOVA), selecting as statistically significant variables those with a *p-value* < 0.05.

3. Results and discussion

3.1. Cellular lysis and protein release

Nannochloropsis is a genus widely used at the industrial level because of their high nutritional quality and additionally being a source of value-added compounds such as pigments, polyunsaturated fatty acids [23] and, of course, proteins and peptides with biofunctionality [24] applicable to the field of animal and human health. Even though the quantity and quality of proteins is not a problem, the method of extracting them is the principal complication in the release of these compounds, due to the presence of the cell wall composed of two layers, of which the cellulose is one of the main components [25].

Figure 1 shows the beginning and end of mechanical cell disruption treatment. Samples from the upper side of the figure were visualized with an Olympus CX21 microscope with a 40X objective and those from the bottom, with a scanning electron microscope (SEM) at 8000X.

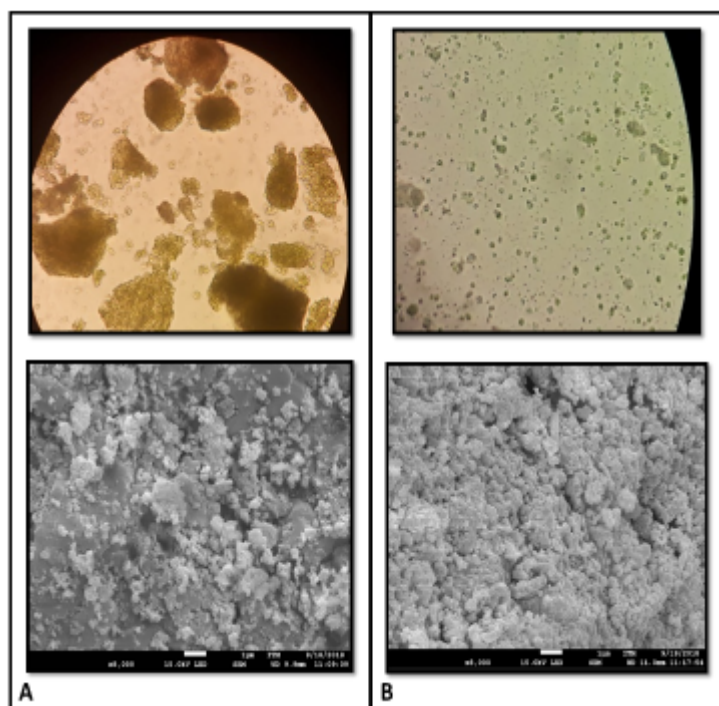


Figure 1
Cells of *Nannochloropsis* sp. before and after homogenization treatment. A) Before treatment. B) Cycle 20.

Source: The Authors.

Because of the previous drying process, the biomass before starting the treatment shows agglomerations of cells, which obstruct the lysis of the cell wall. In cycle 20 of the treatment, these accumulations are eliminated, allowing the cell to be more vulnerable to mechanical stresses and improve the probabilities of disruption. The increase in surface area and the heterogeneity in cell sizes are evidence of the effect of the mechanical action of lysis on the cell surface.

The results show that the initial crude protein of the biomass was 8.84%. The mechanical extraction process achieved a percentage of cell lysis of $60.1 \pm 3.3\%$ and a soluble protein concentration in the extract of $0.88 \pm 0.03\%$, getting an extraction yield of 9.9 %.

The extraction yield found in this study was very similar to the one got by Zhang et al. [26], who obtained a yield of 12.1% when submitting 1 g of *Chlorella pyrenoidosa* biomass to a homogenization system at 8,000 rpm for 10 minutes and 2-minute intervals. However, it should be noted that the amount of biomass used in this study was greater. On the other hand, some authors have reported higher extraction yields when implementing other mechanical methods such as high- pressure homogenization and grinding. Safi et al. [15] reported a 95% percent lysis and 50% (w/w) protein release in *Nannochloropsis gaditana* cells, under a high-pressure homogenizer; while Pan et al. [27] achieved a percentage of cell lysis superior to 98% on *Nannochloropsis* sp. using a turbine bead mill.

3.2. Enzymatic Hydrolysis, Electrophoresis, and FTIR

After 6 hours of the enzymatic reaction, the degree of hydrolysis obtained on the protein extract was $84.84 \pm 3.52\%$. While the biomass solution that was used as control (sample in phosphate buffer at the same reaction conditions without cell lysis) presented a degree of hydrolysis of $24.66 \pm 1.05\%$.

The use of enzymatic complexes permits achieve more complete hydrolysis by overlaying the specific effects of each, as corroborate Morris et al. [17], who found degrees of hydrolysis greater than 50% in the enzymatic hydrolysis of *Chlorella vulgaris* biomass in suspension at 10 % (w/v) in water, with mixtures of papain-trypsin and papain-pancreatin.

Polyacrylamide gel electrophoresis (SDS-PAGE) evidences the change in the molecular size of the proteins present in the extract before and after the enzymatic treatment as Figure 2 shows.

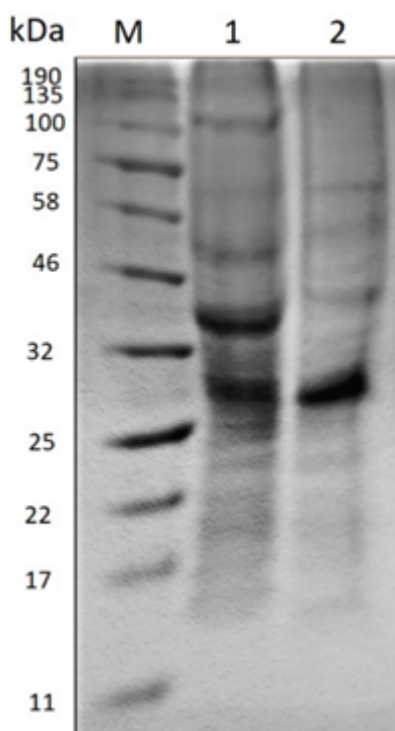


Figure 2

Polyacrylamide gel electrophoresis (SDS-PAGE). M) Molecular marker. 1) Crude extract before hydrolysis. 2) Hydrolyzed extract.

Source: The Authors.

In the crude extract before hydrolysis, the amount of bands existent suggests the presence of a large variety of proteins, the most prominent being between 25-32 kDa and 32-46 kDa. On the other hand, in the hydrolyzed extract the enzymatic action becomes evident when the molecular size of some proteins decreases, due to the diminution of the thickness of some bands such as that between 32-46 kDa and 46-58 kDa. Moreover, the band of approximately 100 kDa disappears and a new band stands out between 58-75 kDa.

Concerning the results of Fourier transform infrared spectroscopy (FTIR) analysis in Figure 3, the original biomass of *Nannochloropsis* sp. has an important peak between 2954-2850 cm^{-1} corresponding to the aliphatic chains of lipids and another one between 1710-1740 cm^{-1} related to esters of fatty acids and chlorophyll ketones [28].

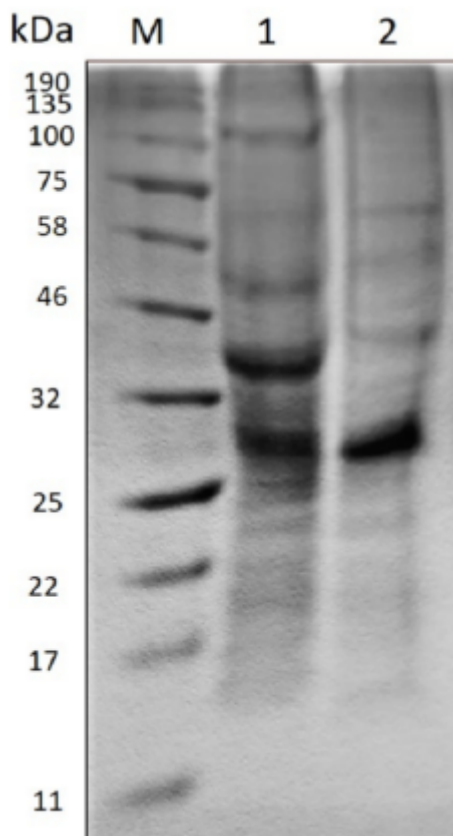


Figure 3

FTIR spectra of biomass and protein extracts of *Nannochloropsis* sp.

Source: The Authors.

Comparing the sample of the crude and hydrolyzed extract with the original biomass, a band of lower intensity between 3000 and 2850 cm^{-1} is presented and the band related to chlorophyll disappears, suggesting that the mechanical extraction process implemented removes very few amounts of compounds linked to lipids and pigments such as chlorophyll, being selective to proteins and carbohydrates.

The analysis by Fourier transform infrared spectroscopy, allows to relate the vibrational frequencies of the C = O and N-H bonds corresponding to Amida I and Amida II, with the secondary structures of the proteins, as a method to deduce the structural conformation of the proteins and the changes that could occur when some type of treatment is undergoing [29-31].

In the samples of crude and hydrolyzed extract, the typical peaks of protein secondary structures are evident [32,33]: β -sheets (1632 and 1637 cm^{-1}), α -helices (1659, 1661 cm^{-1}), β -turns (1683, 1681 cm^{-1}), respectively.

Although the changes in the reported wavelengths and the intensities of the peaks attributed to the secondary structures were minimal, these could be because of the hydrolysis process is responsible for increasing the number of hydrogen bonds due to interactions between released species NH_3^+ and COO^- with water, as Fang et al. deduced [29].

Apparently, this fact demonstrates that both the extraction protocol and the handling of the extracts during the different processes did not affect the folded structure of the proteins, maintaining their biological function [32,34].

3.3. *Antimicrobial activity*

Figure 4 shows the growth on the logarithmic basis of the microorganisms evaluated in the presence of three concentrations of extract in addition to an extract-free control sample.

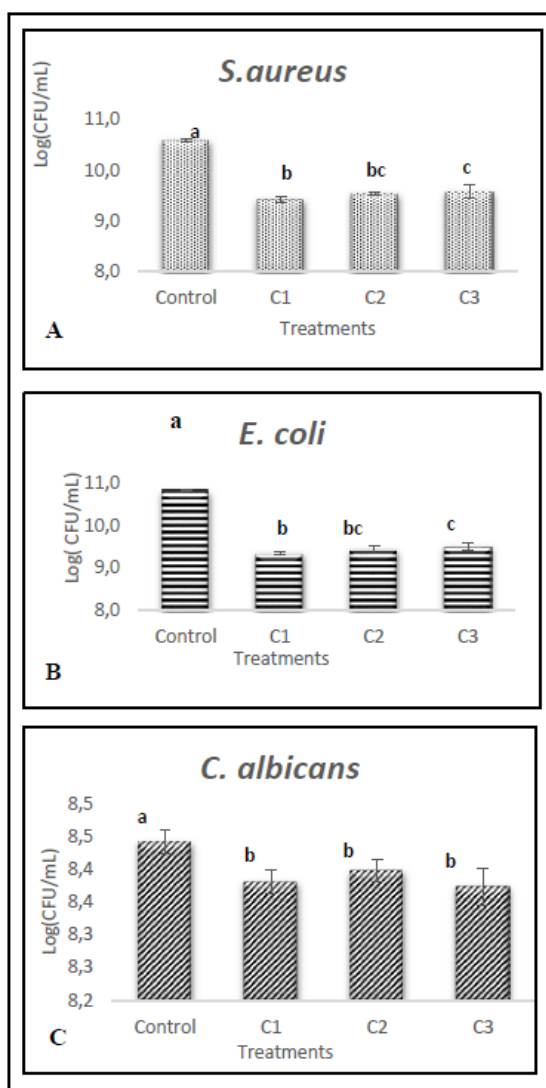


Figure 4
Growth of A) *S. aureus*, B) *E. coli* and C) *C. albicans* with different concentrations of hydrolyzed extract (C1:33.35%(v/v); C2: 20%(v/v) y C3:10%(v/v)).

Source: The Authors.

The results were analyzed using an analysis of variance (ANOVA) and Fisher's minimum significant difference test (LSD). The means of each treatment for the same microorganism with one letter in common do not differ significantly at 5%.

In the case of bacteria, there are statistically significant differences with a confidence level of 95% between the control sample and the samples containing the protein extract, as well as the extract samples with the highest concentration (C1: 33.35% (v/v)) and the samples with the lowest concentration (C3: 10% (v/v)).

In contrast, the growth of *Candida albicans* although there are statistically significant differences between the control sample and those where the protein extract was added, there are no differences between the samples with different concentrations of the extract.

Regarding the percentages of growth inhibition obtained for each treatment with different extract concentrations, the bacteria showed very similar inhibitions, nevertheless, *E. coli* was the microorganism most susceptible to its action, as shown in Figure 5.

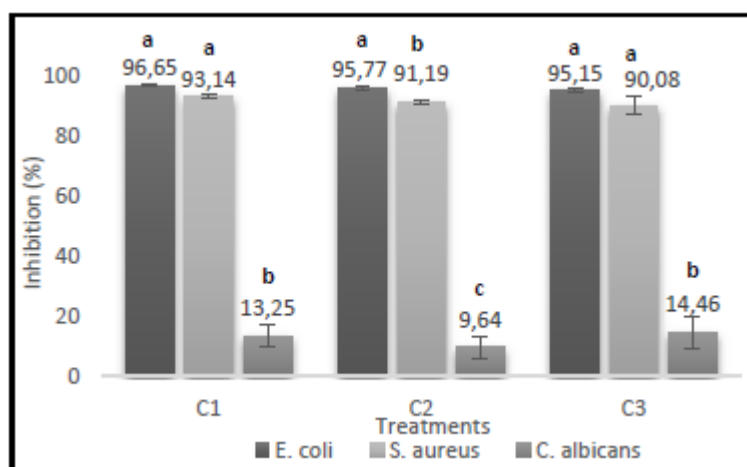


Figure 5

Percentage of inhibition of *E. coli*, *S. aureus* and *C. albicans* in different concentrations of enzymatically hydrolyzed extract (C1:33.35%(v/v); C2: 20%(v/v) y C3:10%(v/v)).

Source: The Authors.

The antimicrobial activity assay demonstrates that the hydrolyzed protein extract exhibits bacteriostatic activity against *E. coli* and *S. aureus* and fungistatic activity against *Candida albicans*.

The analysis of variance performed and Fisher's minimum difference test (LSD) shows the statistical differences between the microorganisms studied. The letters in common for each treatment do not differ significantly from 5%.

In all the treatments applied there were significant differences between bacteria (*E. coli* and *S. aureus*) and yeast (*C. albicans*). In the case of the extreme treatments, referring to the highest (C1) and lowest (C3) extract concentration, there were no statistically significant differences between the bacteria. On the contrary, in the treatment where the C2 extract concentration was applied, there were statistical differences among all microorganisms, being *E. coli* the most susceptible to its effect. This fact could be explained in part due to the production of proteases by *S. aureus*, which would block the action of the peptides present in the extract [35]. Sun et al. [11] presented similar results using a peptide fraction of the protein isolated from *Spirulina platensis* against *E. coli* and *S. aureus*, obtaining inhibition halos of 16 mm and 12 mm, respectively.

Other authors such as El-Baz et al. [12] evaluated a protein extract of *Spirulina platensis* that presented antimicrobial action against the microorganisms *Enterococcus faecalis* and *Candida albicans* but did not reveal any effect against the bacteria *E. coli* and *Staphylococcus aureus*.

4. Conclusions

The microalgae *Nannochloropsis* sp. is an important source of high-quality proteins. Due to enzymatic hydrolysis with a complex of papain-pancreatin proteases on the protein extract obtain this study, was achieved a degree of hydrolysis greater than 80%, reaching a considerable percentage of low molecular weight peptides (<3 kDa). These molecules showed significant percentages of inhibition on *S. aureus*, *E. coli*, and *C. albicans*, cataloging the extract as bacteriostatic and fungistatic, respectively, against the microorganisms evaluated.

Based on the results of the FTIR, apparently, the mechanical treatment implemented on the proteins of *Nannochloropsis* sp. did not affect the folding of the proteins and kept their biological function.

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