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In vitro bioactive properties of protein hydrolysates from giant squid (*Dosidicus gigas*) by *Bacillus subtilis*

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Abstract

The aim of this work was the obtention and characterization of protein hydrolysates from squid muscle frozen stored as an alternative of fermentation substrate. Hydrolysates' antioxidant, antimicrobial and functional properties were obtained by fermentation via *Bacillus subtilis* ATCC 6633, using *Dosidicus gigas* mantle, stored for 20 months at -20° C. Culture media with different proportions of collagen: muscle (C0 = 100% muscle, C25 = 75% muscle + 25% collagen, C50 = 50% muscle + 25% collagen, C75 = 25% muscle + 75% collagen, C100 = 100% collagen) of giant squid, were subjected to fermentation, from 0 to 8 hrs. Free radical scavenging activity was determined via the $\text{ABTS}^{\bullet+}$ methodology (maximum value of 3.99±0.02 mg L-ascorbic acid equivalents for 8 h-C25) and DPPH• (maximum value of 750.29±13.57 µg L-ascorbic acid equivalents for 8 h-C75). Inhibition zones (between 10 mm and 14.9 mm) were found in hydrolysates, with 8 hrs of fermentation for Gram-negative bacteria. Regarding infrared spectroscopy, after 8 hrs of fermentation, several peaks were detected, which suggest the presence of aromatic rings (1582 cm^{-1}, 856 cm^{-1} and 756 cm^{-1}), in addition to peaks that suggest the presence of surfactant from *B. subtilis* (1510 cm^{-1}, 1392 cm^{-1} and 1198 cm^{-1}). A maximum of 150% (v/v) in the foaming capacity of 2 h-C100 and 87.5% (v/v) of 8 h-C50 was obtained; for foam stability, 77.5% (v/v) with 2 h-C100 and 22.5% (v/v) with 0 h-C0. The maximum value for the emulsifying activity index was 1778.06±30.85 m^2/g of protein for 8 h-C0, while the highest index of emulsion stability was 82.04±2.81 mins for 8 h-C25. The protease activity present in the hydrolysates decreased the hardness of the gelatin to 29.6%. Results showed that the use of the giant squid stored for extended periods in freezing conditions impedes its spoiling and harmful effects on the environment, and allows the obtention of hydrolysates with antioxidant, antimicrobial and functional properties, for which the submerged fermentation with *B. subtilis* ATCC 6633 is suitable for the acquisition of bioactive peptides, which can be considered for their use in the food and pharmaceutical industries.

1. Introduction

Currently, a continuous increase in chronic diseases exists, which has impelled research and exploitation of protein hydrolysates, containing peptides formed by 3-20 amino acid residues and associated with antimicrobial, anticarcinogenic, antioxidant, antihypertensive, anticoagulant, opioid, immune system-stimulating and antiviral properties (Jemil *et al*., 2014; Idowu *et al*., 2020). The composition and bioactivity of this type of hydrolysates depend on the substrate employed; among these, the by-products of the industries related to fishing, have acquired relevance due to availability and low cost, the by-products mentioned above are generally disposed of in bodies of water, generating environmental contamination (Morachis-Valdez *et al*., 2015; Saucedo-Vence *et al*., 2015), buried or used in products which are less valued, such as fertilizers, animal and aquaculture feed (Klomklao and Benjakul 2017; Marti-Quijal *et al*., 2020). One of the species used as a substrate for the obtention of these compounds is the giant squid, whose fishing is of interest in countries such as Mexico, Chile, Peru, China, and Japan (Ezquerra-Brauer and Aubourg,

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2019). Up until now, few studies address the feasibility of the use of squid stored in freezing conditions during prolonged periods, for direct use (Raman and Mathew, 2015), or as a substrate, for hydrolysates obtention, due to the loss of functional and structural properties, because of ice crystals formation and enzymatic activity (Jia *et al*., 2019; Nakazawa and Okazaki, 2020), in comparison to fresh giant squid (Alemán *et al*., 2011; Suárez-Jiménez *et al*., 2018), for which it is necessary to carry out studies, employing species stored for prolonged periods, to provide an alternative for its utilization (Raman and Mathew, 2015) and, in this approach, avoid disposal and possible environmental contamination.

Previous studies demonstrate that hydrolysates obtained from giant squid, employing commercial enzymes, such as pepsin, trypsin and chymotrypsin, present bioactive properties (Rajapakse *et al.*, 2005), however, microbial biodegradation has been recommended as a low-cost form, which also increases the quality of the final product, compared to acid and enzymatic hydrolysis (Idowu *et al*., 2020; Mhina *et al*., 2020). *Bacillus subtilis* has recently been used to obtain products of interest, because of its rapid growth and use of affordable substates in submerged fermentation (Mohapatra *et al*., 2017). The resulting products (bioactive peptides) are potentially applicable in the food, aquaculture, agriculture, and medical industries (Jung and Kim, 2016; Idowu *et al*., 2020; Mhina *et al*., 2020). Thus, the objective of the present work was to evaluate the *in vitro* properties (antioxidant, antimicrobial and functional) of hydrolysates obtained via fermentation in liquid media, using *B. subtilis* ATCC 6633 and culture media prepared with squid mantle mill (muscle and collagen), which is stored in freezing conditions (-20°C) for 20 months.

2. Materials and methods

2.1 Raw materials

Frozen giant squid mantle was purchased fourteen days post-capture, washed, and sliced in portions of 200 g, packed in high-density polyethylene bags, and stored at -20°C for 20 months.

Stored frozen mantle was treated according to Jemil *et al.* (2014). Briefly, collagen (inner and outer tunics) was manually separated from the muscle, and 500 g of each fraction was immersed separately in 1000 mL of water at 90°C for 20 mins. Afterwards, it was dried at 50°C for 40 hrs and ground into a fine powder (Mesh 30).

2.2 Growth medium

Powder from collagen (C) and muscle (M) was used as a nitrogen source, using the following ratios and codifications: 0%C and 100%M as C0, 25%C and 75% M as C25, 50%C and 50%M as C50, 75%C and 20%M as C75, and 100%C with 0%M as C100.

The growth medium was prepared as follows: 30 g of nitrogen source (mix of the C and M powders) (Jemil *et al*., 2014), dissolved in 1 L of phosphate-citrate-bicarbonate buffer (50 mM - 150 mM-150 mM, pH 7.5; NaCl 200 mM) and autoclaved at 121°C for 20 mins.

2.3 Culture conditions

The strain *B. subtilis* ATCC 6633 was incubated in nutritive agar for 22 hrs at 20°C, then inoculated in a nutritive broth and incubated at 20°C for 18 hrs with an agitation speed of 120 rpm. The final concentration of inoculum was determined as 4×10^6 CFU/mL.

2.4 Fermentation

The inoculation was carried out using 40 mL of inoculum in 1 L of sterilized growth medium. Then, the medium was incubated for 0, 2, 4, 6 and 8 hrs at 20 °C with agitation (120 rpm). The fermentation products were separated by centrifugation at 8500x*g* for 30 mins at 4 °C. A mixture of cryoprotectants (44% sucrose, 44% glycerol, 12% water) was added in a 1:10 ratio (cryoprotectants:sample) before frozen storage (-20 °C).

In consideration of the yields of the fermentative processes, precipitation was carried out with ethanol (96°) in a 1:1 ratio with the hydrolyzed liquid. The precipitated fractions were separated by decantation and were dried at 30 °C for 72 hrs. Afterwards, the fractions were lyophilized (Elumalai *et al*., 2020; Saallah, *et al.*, 2020).

2.5 Antioxidant activity

The samples were diluted in a 9:91 ratio (hydrolysate: water) and 10 µL of this dilution was used to carry out the measurements.

The determination of antioxidant activity was performed using the $ABTS•^+$ y DPPH• methods described by Agrawal *et al.* (2016), with some modifications. A volume of 10 µL of the hydrolysate dilution was employed, with 1 mL of the free radical solutions, and the mixture was stirred, via vortex, for 30 s. The determinations were carried out every 10 mins, for 60 mins, at a wavelength of 734 nm for $ABTS•^+$ and 515 nm for DPPH•. The percentage of inhibition of the free radicals as compared to a standard curve of L-ascorbic acid for each radical, reporting the results in mg L-ascorbic acid equivalents for the $ABTS•^+$ methodology and mg equivalents for the DPPH• methodology.

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2.6 Disk diffusion method

The negative effects of microorganisms on foods are deterioration effects or illness transmitted via foods, which represent, besides economic losses, a risk to public health worldwide (Abdelhamid *et al.*, 2020). Among the BPM indicator microorganisms, *Enterobacteria* and *Staphylococcus* are found.

The methodology described by Syahirah and Rabeta (2019), with some modifications, was used to determine the antimicrobial activity of the hydrolysates obtained on the following microorganisms: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, as well as microorganisms from the Department of Microbiological Analysis, at the School of Chemistry at UAEMex, such as *Salmonella enterica* serovar Typhimurium, *Proteus mirabilis*, *Proteus vulgaris* and *Klebsiella aerogenes*. A suspension of each microorganism (100 µL with 1.5×10^8 CFU/mL) was extended in dishes (90mm diameter) of nutritive agar. The five-millimetre sterile cellulose disks were placed over the surface of the inoculated dishes and 10 µL of the hydrolysates were added. The dishes were incubated at 37°C for 24 hrs. The codification of the inhibition diameters was carried out according to Gómez -Guillén *et al.* (2010): [-] = 6 mm, [±] 6 a 7.4 mm, [+] 7.5 a 9.9 mm, [++] 10 a 14.9 mm and [+++] >15 mm. As a positive control, amoxicillin-clavulanic acid (50 µg-12.5 µg/mL) was employed.

2.7 Infrared spectroscopy

The infrared spectra of the lyophilized hydrolysates were obtained, according to Djellouli *et al.* (2019), using 1 mg of the sample at room temperature, with a Jasco FT/IR 400 spectrometer at a range of 600-4000 cm^{-1}, with a resolution of 4 cm^{-1}.

2.8 Foaming capacity

One-hundred microliters of each hydrolysate were placed in tubes with 10 mL of phosphate buffer (50 mM, pH 7.0), and a Tissue-Tearor (Biospec Products Inc., USA) was used at 20000 rpm for 1 min at 20°C, for the generation of foam. The foaming capacity was established as the increase in volume, about the initial volume in percentage (e.g., 100% indicates that 10 mL of foam were generated from the original 10 mL of hydrolysate).

2.9 Foam stability

The foams obtained with each hydrolysate were maintained at 20°C for 30 mins. The stability was determined as the percentage in the volume of foam that remained once it had formed (e.g., with an initial foam volume of 10 mL, stability of 40% indicates that, after 30 mins, only 4 mL of foam remained).

2.10 Emulsifying properties

The emulsifying activity index (EAI) allows the comparison of the efficiency of emulsion formation between different types of proteins, under the same work conditions inside the laboratory, as a point of comparison, for which egg albumin was employed, finding a value of 35 m^2g^{-1}.

It was determined according to the methodology described by Jemil *et al.* (2014), with modifications. 100 mL of hydrolysate were homogenized with 5 mL of water and 5 mL of canola oil, employing a Tissue-Tearor at 30000 rpm for 1 min, at 20°C. 50 mL were taken from the bottom portion of the emulsion and were diluted at 1:100 with a 0.1% SDS solution (p/v).

The absorbance of the dilutions was measured, immediately (A_0) and 10 mins after emulsion formation (A_{10}), at 500 nm using a spectrophotometer (UV-Visible Spectrophotometer; Thermo Scientific GENESYS 10S Series) and were utilized for the determination of the emulsifying activity index (EAI) and emulsifying stability index (ESI), via the following formula:

$$EAI\ [m^2g^{-1}] = \frac{2 \times 2.303 \times Abs}{0.5 \times P}$$

$$ESI[min] = \frac{A_0}{\Delta A} \times t$$

Where $\Delta A = A_0 - A_{10}$ and P = Concentration of protein contained in 100 µL of the hydrolysate employed, expressed in grams.

2.11 Effect on the gel hardness of gelatin

A solution of 5% gelatin was prepared, which was added to cylindrical containers (3.5 cm in diameter), with a proportion of 100 µL of hydrolysate and 30 mL of gelatin solution. The containers were refrigerated for 20 hrs and were subjected to a texture profile analysis (TAX -T2), to evaluate the force required to break the gels. The methodology described by Okita *et al.* (2020) was followed.

3. Results and discussion

3.1 Yield

The overall yield of each fermentation was 3.2±0.3 g of hydrolysis products per 100 mL, independently of the agitation time (2, 4, 6 and 8 hrs) or the composition of the culture media. The result was higher than that reported by Jemil *et al.* (2014) for *Sardinella aurita*, *Salaria basilisca*, *Zosterizessor ophiocephalus* and *Dasyatis pastinaca*, with yields of 9 g/L at 24 hrs; this could be due to the presence of an appropriate content of

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carbon source (Orhan *et al*., 2005; Elumalai *et al.,* 2020). In the present study, citrate could stimulate the expression of hydrolytic enzymes and the generation of bioactive compounds. On the other hand, for the substrate employed for fermentation, by t = 0, a yield of 120±7 mg per each 100 mL was obtained, probably due to the hydrolysis over the nitrogen source, generated with an intermediate thermal treatment (Korczek *et al*., 2020; Siewe *et al*., 2020) and the production of *B. subtilis* metabolites during the generation process of the inoculum in the nutritive agar (Arima, 1968; Hassan and Ibrahim, 2017; Uddin *et al*., 2017).

3.2 Antioxidant activity

The antioxidant activity of the hydrolysates was determined via the ABTS•$^{+}$ and DPPH• techniques. The results obtained between the different methodologies present a difference in magnitude; with the ABTS•$^{+}$ methodology, concentrations in the range of mg equivalents of L-ascorbic acid were found, while for the DPPH• methodology, concentrations in the range of microgram equivalents of L-ascorbic acid were found. These results suggest that the components of the hydrolysates responsible for scavenging free radicals are found, with greater availability, in aqueous media (ABTS•$^{+}$), while those in organic media (DPPH•) present a lower availability; this could be due to the conformation changes that occur once the components of the hydrolysate interact with the methanol (DPPH•), exposing the hydrophobic groups of the peptides, presumably responsible for the antioxidant activity of the hydrolysates (Mhina *et al*., 2020).

Table 1 shows the results of the antioxidant activity of the ABTS•$^{+}$ and DPPH• radicals. It is possible to observe that the mixtures do not present significant differences (p<0.05) between 0, 2 and 4 hrs; however, in C0, C25 and C50, there is a tendency in the decrease of antioxidant activity at 2 hrs. This is possibly due to the use of existing protein fractions, as a nitrogen source, by *B. subtilis,* during the growth stage (Yuliani *et al*., 2018; Kai, 2020). By 4 hrs, and until the end of fermentation, an increase in antioxidant activity was observed, possibly related to the increase in the production of the enzyme during the growth stage of *B. subtilis* (Ling Ho, 2015)

Table 1. Antioxidant Activity of hydrolysates obtained via fermentation with *B. subtilis* ATCC 6633 at 0, 2, 4, 6 and 8 hrs, from growth mediums C0, C25, C50, C75 and C100.

Fermentation time (h)-Growth medium	Antioxidant activity ABTS•+ (mg eq AA/mL)	Antioxidant activity DPPH• (µg eq AA/mL)
0-C0	2.609±0.043^{abcd}	365.906±60.696^{abc}
0-C100	2.582±0.031^{abcd}	333.924±38.225^{abc}
0-C25	2.689±0.050^{abcde}	385.374±63.863^{abcd}
0-C50	2.567±0.073^{abc}	439.604±29.813^{abcde}
0-C75	2.597±0.022^{abcd}	353.392±28.486^{abc}
2-C0	2.536±0.016^{ab}	243.541±10.529^{a}
2-C100	2.425±0.012^{a}	326.972±22.074^{abc}
2-C25	2.557±0.026^{ab}	354.782±17.952^{abc}
2-C50	2.467±0.016^{a}	301.942±27.437^{abc}
2-C75	2.525±0.029^{ab}	386.764±31.248^{abcd}
4-C0	2.616±0.022^{abcde}	288.037±16.763^{abc}
4-C100	2.452±0.086^{a}	339.486±12.515^{abc}
4-C25	2.721±0.043^{abcde}	393.717±17.952^{abcd}
4-C50	2.584±0.052^{abcd}	283.866±9.465^{ab}
4-C75	2.601±0.033^{abcd}	356.173±21.467^{abc}
6-C0	2.947±0.015^{de}	336.705±57.820^{abc}
6-C100	2.870±0.093^{bcde}	482.710±27.671^{cdef}
6-C25	2.988±0.008^{e}	443.775±30.167^{bcde}
6-C50	2.942±0.017^{cde}	382.593±41.777^{abcd}
6-C75	2.870±0.052^{bcde}	482.710±36.225^{cdef}
8-C0	3.836±0.166^{f}	630.097±43.931^{efg}
8-C100	3.950±0.117^{f}	679.743±42.536^{fg}
8-C25	3.996±0.177^{f}	648.388±48.721^{fg}
8-C50	3.666±0.075^{f}	564.773±62.475^{defg}
8-C75	3.865±0.083^{f}	750.293±27.155^{g}

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different (p<0.05).

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and, as a consequence, a higher concentration of protein fragments of low molecular weight, capable of scavenging free radicals (Mhina *et al.*, 2020), since, in agreement with Saallah *et al.* (2020), the scavenging capacity of the DPPH radical increases with the decrease of the molecular weight in the protein hydrolysates; this could be due to the exposure of side-chain amino acid residues, facilitating the interaction between peptides and free radicals (Saallah *et al.*, 2020).

3.3 Antimicrobial activity

In Table 2, the results corresponding to antimicrobial activity at 0 and 8 hrs of fermentation, are shown, in which an increase in the inhibition zone can be observed for *E. coli, P. aeruginosa, S. enterica* ser. Typhimurium, *P. mirabilis* and *P. vulgaris*. However, for *S. aureus,* despite not exhibiting a tendency, it presented inhibition zones at both fermentation times. This could be because, during the hydrolysis process, peptides with antimicrobial properties were generated, which possess inhibitory effects towards microorganisms related to the invasion and deterioration of foods, including a wide range of *in vivo* pathogens, such as bacteria, fungi, and viruses, as well as parasites (Abdelmoteleb *et al.*, 2017; Hussain *et al.*, 2020). The action and effectivity of these biologically-active peptides vary, depending on structural characteristics, such as peptide size, the composition of amino acids, the charge, hydrophobicity and secondary structure. Thus, this exhibits a varied selectivity and sensibility over the target microorganisms (Patrzykat and Douglas, 2005; Kannan *et al.*, 2012). The majority of the antimicrobial peptides possess characteristics in common, regardless of the source of origin. Usually, these are composed of less than 50 amino acids, of which approximately 50% are hydrophobic amino acids, and are spatially confirmed as an amphipathic 3D structure (Rydlo *et al.*, 2006). The antimicrobial peptides possess a net positive charge with an excess of basic amino acids (lysine and arginine). A cationic and amphipathic nature are two important structural characteristics of antimicrobial activity. The hydrophobic character allows the peptide to enter the membrane, while the positive charge initiates the interaction with the surface of bacteria, which is negatively charged (Wieprecht *et al.*, 1997; Gómez-Guillén *et al.,* 2010), for which the antimicrobial activity of the hydrolysates is related, at first instance, with protein fractions and peptides, resulting from the action of enzymes generated by *B. subtilis*, during the process, which tends to increase its activity when the molecular weight decreases (Gómez-Guillén *et al.*, 2010). Related to the secondary metabolites produced by *B. subtilis,* which, in agreement, with Kaspar *et al.* (2019) and Wang *et al.* (2014), have a wide spectrum of antimicrobial activity, among which peptides belonging to the iturins (cyclic lipoheptapeptides), fengycins (cyclic decapeptides), surfactin (cyclic lipopeptide) and additional variants (Wang *et al.*, 2014; Caulier *et al.*, 2019; Olishevska *et al.*, 2019).

3.4 Infrared spectroscopy

Infrared spectroscopy has been used for the acquisition of information about the chemical composition and structural conformation of diverse compounds (Gómez-Guillén *et al.*, 2010). The infrared absorption spectrum (4000 to 650 cm^{-1}) for the C0, C50 and C100 hydrolysate samples obtained in this study, after 8 hrs of fermentation, are presented in Figure 1. The samples were selected for the detection of variations, according to the change of composition during the increase in the quantity of collagen as a nitrogen source and the decrease in the quantity of muscle.

The characteristic absorption peaks of proteins were identified (Djellouli *et al.*, 2019; Ling *et al.*, 2020), corresponding to the Amide A peak (3262 cm^{-1}), which

Table 2. Antimicrobial activity of growth medium and hydrolysates obtained via fermentation with *B. subtilis* ATCC 6633 at 0 and 8 hrs from growth mediums C0, C25, C50, C75 and C100

	S. aureus ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. enterica* ser. Typhimurium	*P. mirabilis*	*P. vulgaris*	*E. aerogenes*
0h-C0	++	+	++	-	+	++	+
0h-C25	±	+	+	-	+	±	+
0h-C50	++	+	+	-	+	+	+
0h-C75	+	+	+	+	+	+	+
0h-C100	+	+	+	-	++	+	+
8h-C0	+	++	++	-	++	+	+
8h-C25	++	++	++	±	++	+	+
8h-C50	±	++	++	+	++	++	+
8h-C75	++	++	++	±	++	++	-
8h-C100	+	++	+	+	++	+	+
Positive control	+++	+++	+++	+++	+++	+++	+++

The codifications of the diameters of the inhibition zones: [-] =6 mm, [±] 6 to 7.4 mm, [+] 7.5 to 9.9 mm, [++] 10 to 14.9 mm and [+++] >15 mm.

indicates the presence of N-H/O-H stretching and bending, Amide I peak (1650 cm^{-1}), corresponding to C = O stretching, Amide II peak (1546 cm^{-1}), corresponding to N-H bending and C-N stretching, as well as the Amide III peak (1276 cm^{-1}), which represents the complex peak, resulting from various shifts (Ling *et al*., 2020), as well as the absorption peaks, which suggest the presence of aromatic rings at 1582 cm^{-1} (corresponding to C = N stretching and C = C bending vibrations) and at 756 cm^{-1} and 856 cm^{-1} (related to aromatic C-H bending vibrations) (Barth,2000; Djellouli *et al*., 2019). On the other hand, peaks present at 1510 cm^{-1}, 1392 cm^{-1}, 1198 cm^{-1}, coincide with that reported by Verma *et al*. (2020), corresponding to the surfactant produced by *B. subtilis,* using molasses as a sole nutrient. With this, it could be said that the products found in the present study, are considered aromatic compounds and can contribute to the antioxidant capacity demonstrated in the analysis carried out.

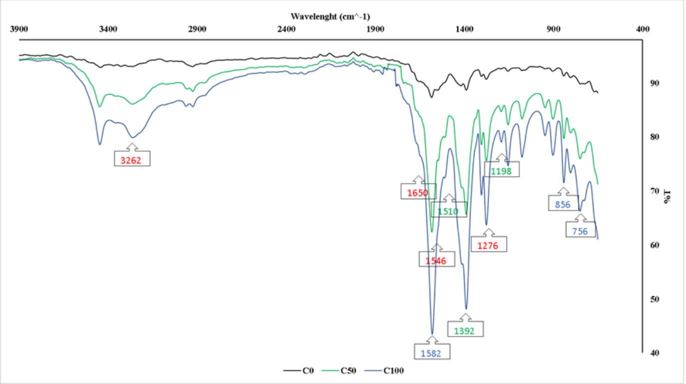

Figure 1. IR spectra of the hydrolysates obtained from CO, C50 and C100 after 8 hrs of fermentation.

3.5 Foaming capacity and foam stability

During the fermentation process, an initial increase in the volume of foam generated was observed (Figure 2-A) at t = 0, and after a decrease, possibly attributed to the shortening in the length of the protein fractions present, derived from the activity of the enzymes produced by *B. subtilis*. Jia *et al*. (2020) reported a similar behaviour in ovalbumin at 9 hrs of fermentation with lactobacilli. The foam formation capacity in the hydrolysates obtained during the fermentation process is found to be closely related to the presence of high molecular-weight protein fractions, while Zamorano-Apodaca *et al*. (2020) mention a decrease in the foaming capacity upon the decrease of molecular weight in the hydrolysates obtained in the mixture of fish by-products, using the enzyme alcalase. According to Saallah *et al.* (2020), upon the decrease in the peptide chain size, the capacity of stabilizing the air bubbles also decreases; these bubbles are necessary for the generation of foam. Also, the smallest peptides absorb water faster, which decreases the superficial tension. Likewise, the presence of metabolites of *B. subtilis,* because of fermentation, could impact the capacity of foam formation (Coutte *et al*., 2017).

The stability of the foam generated by the hydrolysates exhibits similar behaviour in the culture media, regardless of composition (C0, C25, C50, C75 and C100), as is shown in Figure 2-B, presenting a higher percentage of stability at 2 and 6 hrs. According to Ding *et al*. (2020), the increase in foam stability could be due to the increase in the hydrophobic groups and the decrease in the surface tension of the solution, which allows the diffusion of the proteins towards the gas-liquid interphase, resulting in the formation of stronger films. On the other hand, Jia *et al*. (2020) indicate that the decrease in the size of the peptide, due to the action of proteases, weakens proteins' adsorption capacity, which decreases the ability to maintain the bubbles and thus, these collapse with ease. The results of our study have a similar tendency to those reported by Klompong *et al*. (2007), for hydrolysates of *Selaroides leptolepis,* prepared using alcalase and Flavourzyme$^{\text{Ò}}$, which suggests that this could be related to the presence of greater-size protein fractions, which could form more flexible layers around the air bubbles, necessary to stabilize the foam generated.

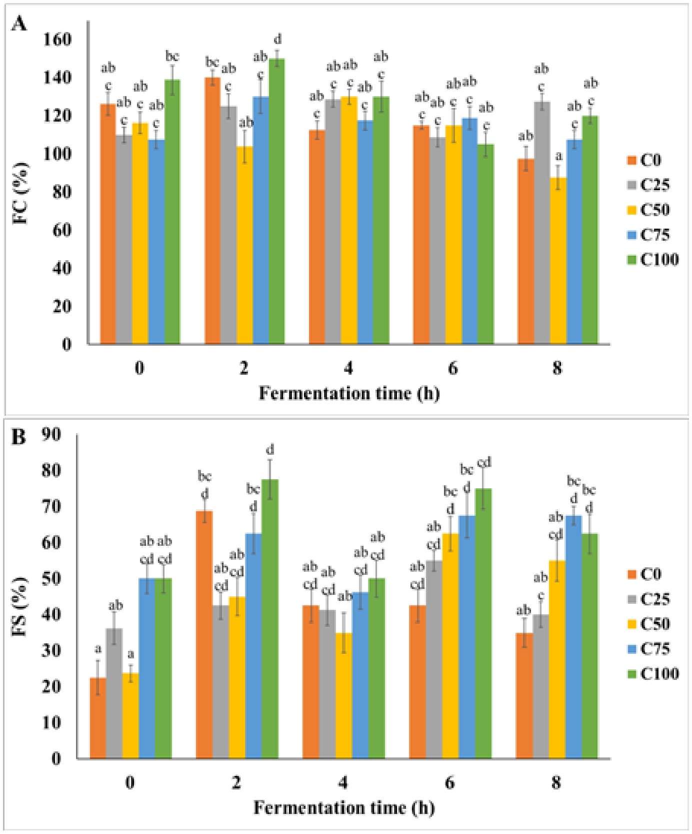

Figure 2. A) Foaming capacity (FC) and B) foaming stability at 30 mins (FS), of hydrolysates obtained via fermentation with *B. subtilis* ATCC 6633 at 0, 2, 4, 6 and 8 hrs from growth mediums C0, C25, C50, C75 and C100. Values are presented as mean±SE. Bars with different notations are statistically significantly different ($p<0.05$).

3.6 Emulsifying activity index

In Figure 3-A, a significant increase ($p<0.05$) in the emulsifying activity index was observed (which is dependent on time), according to that reported by Zamorano-Apodaca *et al.* (2020), who observe an increase in the EAI upon a decrease of molecular weight of the collagen hydrolysates obtained in subproduct mixtures of various species (different sharks, mullet, guitarfish, weakfish, snapper, ray, squid, seabass, and pompano dolphinfish).

Saallah *et al.* (2020) mentioned that a decrease in the molecular weight of the protein fractions present increases the emulsifying activity index value (EAI), which could be a result of the hydrolysate alteration, due to the partial hydrolysis, which allows the diffusion and interaction at the water-oil interphase, originated by a decrease in molecular weight and higher solubility. Additionally, partial hydrolysis allows the unfolding of the proteins present, thus exposing hydrophobic groups and increasing the interaction between lipids and proteins (Karami *et al.*, 2019). Furthermore, Klompong *et al.* (2007) and Liu *et al.* (2014) observed a decrease in the EAI upon the increase of the hydrolysis degree in the samples; however, Hajfathalian *et al.* (2017) mentioned that there is no tendency in the results reported for the capacity and emulsifying stability, due to the degree of hydrolysis or enzymes employed, for which they recommend that, in order to maintain or improve the emulsification properties in hydrolysates, the hydrolysis process must be controlled, since excessive hydrolysis can lead to a loss in the emulsifying properties.

3.7 Emulsifying stability index

In Figure 3-B, a time-dependent increase is observed for emulsifying stability index (ESI). An opposite trend was found by Klompong *et al.* (2007) and Liu *et al.* (2014), who reported a decrease in the ESI value when increasing the hydrolysis degree and Zamorano-Apodaca *et al.* (2020) when using hydrolysate fractions with lower molecular weight. This could be due to the presence of metabolites derived from the fermentation with *B. subtilis*, which present emulsifying capacity; among these, the surfactin 1 lipopeptide, which is composed of linear fatty acid and a cyclic peptide moiety, has demonstrated such capacity, independent of the by-products used for its obtention, as cashew apple (Felix *et al.*, 2018), waste frying oil (Valenzuela-Ávila *et al.*, 2019) and sugarcane molasses (Rocha *et al.*, 2020); it is possible to suggest the presence of these compounds, due to the peaks formed at 1510 cm^{-1}, 1392 cm^{-1} and 1198 cm^{-1} in FTIR (Figure 2), which match with those reported by Verma *et al.* (2020). Hoffmann *et al.* (2021) suggest that surfactin presents stabilizing characteristics, due to electrostatic interactions, thus representing a promising candidate as an emulsifier and stabilizer in food formulations, with greater performance than lectins.

3.8 Effect on gel hardness

The use of hydrolysates as a source of bioactive peptides, with antioxidant or emulsifying properties, can be directed toward fish gels (Lu *et al.*, 2013). In the present work, the effect of the hydrolysates on the hardness of the gelatin gels was evaluated (Figure 4), finding a decrease in the hardness of the gels (70.4%; C50; 8 hrs). No significant differences ($p<0.05$) were found between the fermentation times of 0 and 8 hrs for each culture media, finding differences between the C50 culture media with 8 hrs of fermentation and the C25 and C100 media at 0 and 8 hrs. This difference could be due to the composition of the culture media, with the capacity to reach an optimal amino acid ratio for the proteases generation in the C50 culture media, according to Contesini *et al.* (2017), Bacillus proteases are produced mainly during the stationary phase, therefore, are regulated by stress due to the presence of carbon and nitrogen, thus the nutritional effect and environmental conditions on the synthesis of proteases play an important role in the repression or expression of the enzyme (Da Silva, 2017; Sharma *et al.*, 2017).

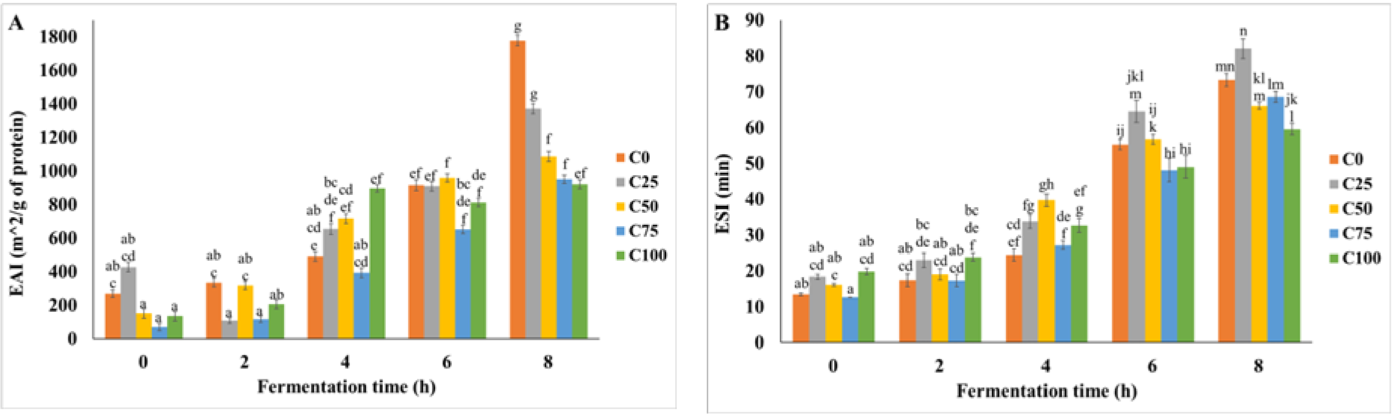

Figure 3. A) Emulsifying Activity Index (EAI) and B) Emulsifying Stability Index (ESI) of hydrolysates obtained via fermentation with *B. subtilis* ATCC 6633 at 0, 2, 4, 6 and 8 hrs, from growth mediums C0, C25, C50, C75 and C100. Values are presented as mean±SE. Bars with different notations are statistically significantly different ($p<0.05$).

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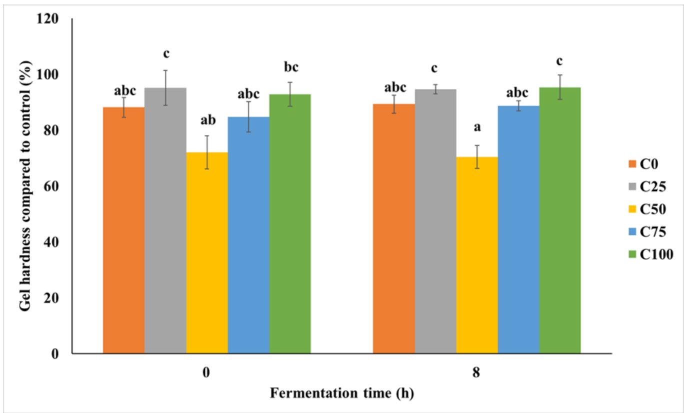

Figure 4. Gel hardness compared to control of gelatin gels added with hydrolysates obtained via fermentation with *Bacillus subtilis* ATCC 6633 at 0 and 8 h, from growth mediums C0, C25, C50, C75 and C100. Values are presented as mean±SE. Bars with different notations are statistically significantly different ($p<0.05$).

Lu *et al*. (2013) and Singh and Benjakul (2018) mention texture as one of the attributes of quality in foods, which affects acceptability and market value, particularly in fish and fish products, which suggests the need for the inactivation of mentioned proteases before their use in products of this nature. Furthermore, hydrolysates with active proteases can be directed towards specific targets, such as in the case of Okita *et al*. (2020), who used alcalase in surimi gels, to decrease hardness, adhesivity and cohesivity of gels meant for people who suffer dysphagia. Likewise, it could be used as a meat tenderizer; El-Din *et al*. (2017) observed that proteases of *Bacillus* sp. possess an auto-limiting capacity, which represents an advantage for problems control, such as over-tenderization and mushy texture of meat, compared to vegetable proteases, which frequently present these problems.

4. Conclusion

The findings of this study demonstrated that the squid, stored in freezing conditions for prolonged periods, resulted in high-quality raw material for the obtention of bioactive hydrolysates, having a yield of 3.2 g/100 mL, employing *B. subtilis* ATCC 6633. All hydrolysates obtained presented foaming capacity and resistance to bubble collapse. Additionally, the infrared analysis revealed the presence of secondary metabolites, with possible surfactant properties, related to the increase in the emulsifying activity index and stability. Proteolytic activity was also observed, with possible applications in meat tenderization. Besides demonstrating good functional properties, the present study identified an increase in antioxidant activity, this may be by the presence of aromatic rings in the protein fragments capable of scavenging free radicals. The antimicrobial activity demonstrated effective inhibition of Gram-negative bacteria. Therefore, the hydrolysates obtained under specific conditions of fermentation could be employed in the food and pharmaceutical industries.

Conflict of interest

The authors declare they have no actual or potential competing financial interests.

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