Enzymatic Hydrolysis of Blue Crab (*Callinectes Sapidus*) Waste Processing to Obtain Chitin, Protein, and Astaxanthin-Enriched Extract

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Abstract - *Blue crab' waste is a good source of valuable substances although only few studies are related to its use, especially concerning the enzymatic hydrolysis and recovery of compounds such as astaxanthin. Besides, the reuse of crab waste may reduce environmental pollution, add value to this residue and promote a social responsibility in several small fishery communities. Therefore, this study aimed to recover protein, chitin, and astaxanthin from blue crab waste by means of enzymatic hydrolysis with alcalase and bromelain. High hydrolysis efficiency, defined by hydrolysis degree (DH), was achieved with 3% alcalase (E/S), recovering 30% of protein in 120 minutes reaction. The highest extraction yield (3.1 ± 0.4% - w/w) and astaxanthin content (97.7 ± 14.3%* $\mu g_{astaxanthin}/g_{residue}$*) were from demineralized sample under acid process (DERS), before carotenoid recovery. Thermogravimetric analysis of the sample with enzymatic deproteinization presented higher thermal stability and mass loss. The enzymatic hydrolysis of the blue crab processing waste proved to be efficient for the production of protein hydrolysates, mostly using 3% of alcalase enzyme related to the substrate (E/S). Additionally, it was possible to obtain chitin and astaxanthin-enriched extract from the hydrolyzed residue with enzymes, similar to what obtained through an alkaline deproteinization process and, consequently, promote improvements in the blue crab waste environmental management.*

Keywords - Blue crab waste, Alcalase, Astaxanthin, Chitin, Protein.

I. INTRODUCTION

Crustaceans are among the most profitable marine products. In 2012, were processed approximately 6,446,818 t of crustaceans in the world [1] and their industrialization generates a large amount of highly perishable waste, which most often is irregularly discarded.

The blue crab waste is basically composed by chitin, protein, minerals, fatty acids, and carotenoids, primarily astaxanthin [2–4]. Chitin and carotenoids have particular biological activity and can be used as additives for pharmaceutical and food industries.

Therefore, the use of crustaceans wastes is an interesting research topic, in particular concerning blue crab waste, which is very scarce in scientific literature and is one of the most important commercial seafood from the Brazilian South coast. The reuse of this residue could have a positive impact, environmentally and economically, for several small Brazilian fishery communities.

Chitosan, derived from the chitin deacetylation, has been intensively applied in different areas: agriculture (pesticides and fertilizers), water treatment (flocculants for clarification and removal of metal ions), food industry (dietary fibers, cholesterol reducers, preservatives, fungicides, bactericides, fruit coatings), cosmetic industry (skin exfoliators, acne treatments, hair moisturizers, toothpaste), and pharmaceutical industry (antitumors, hemostatics, and anticoagulants). However, its extensive application is in biomedical area (surgical sutures, dental implants, bone reconstitution, controlled release of drugs in animals and humans, materials encapsulation) [5,6].

The recovery of chitin from crustacean waste usually consists of three steps: demineralization, deproteinization, and discoloration. Deproteinization is often performed with the use of chemical products and heating with temperatures above 90°C, which can cause degradation of some amino acids [7], and leads to waste disposal problems, because neutralization of

the wastewater are necessary [8]. Therefore, the use of commercial proteases has been proposed as an environmentally friendly method to deproteinize crustacean wastes in order to obtain protein hydrolysates of high nutritional value [9–11], that can be used as a source of protein in the diet of humans and in animal nutrition [12–14].

After the enzymatic hydrolysis process is possible to use insoluble part to recover pigments in the form of a lipid–carotenoid extract, astaxanthin is the main carotenoid present, and solvents or supercritical CO_2 can be used to recover it [11,15]. Astaxanthin presents important applications in nutraceutical, cosmetic, food, and animal feed industries. The most important astaxanthin attributes are the pigmentation capacity and the antioxidant potential. The diverse biological functions of astaxanthin has attracted academic interest due to its benefits to human health (Guerin *et al.*, 2003).

Therefore, considering the environmental, economical, and process aspects discussed above, the aim of this study was to optimize chitin, protein, and astaxanthin extract recovery from blue crab (*Callinectes sapidus*) waste processing through enzymatic hydrolysis processes using alcalase and bromelain enzymes.

II. MATERIAL AND METHOD

2.1 Raw material

Blue crab (*Callinectes sapidus*) waste composed essentially of crab carapaces and legs was kindly provided by small processing crab meat industries from the city of Laguna, Santa Catarina State, Brazil. The residues, collected once in November 2014, were treated based on methods described by Holanda and Netto [15] and Mezzomo *et al.* [3].

The blue crab waste was washed in running water and dried in air-circulated oven (DL, DeLeo, Brazil) for 8 hours at 60°C. Then it was crushed in a blender (LiqFaz, Walitta, Brazil) and ground in a Willey grinder (Moinho Willey, DeLeo, Brazil). Small sample portions of 250g were conditioned in plastic bags and stored at -20°C (domestic freezer) until the processing.

2.2 Blue crab waste characterization

The waste sample characterization was performed according to methods described by the Association of Official Analytical Chemists (AOAC), determining: moisture and volatile content (method 926.12 – AOAC, 1996); total nitrogen (method 991.20 – AOAC, 2005); ash (method 900.02 – AOAC, 1996). Total lipid analysis was performed according to methods described by Bligh and Dyer [19]. Raw protein content was obtained from the total nitrogen values multiplied by a conversion factor of 6.25 [18].

2.3 Enzymatic deproteinization

Fig. 1 shows the process flowchart to retrieve chitin, hydrolyzed protein, and astaxanthin using enzymatic (alcalase and bromelain) and alkaline (KOH) deproteinization. Two proteolytic enzymes were used for enzymatic deproteinization: ProteMax 750 L (alcalase), an alkaline protease of bacterial origin; and Brauzyn BCM (bromelain), an enzyme extracted from pineapple which is able to hydrolyze proteins into low molecular weight polypeptides (both provided by Prozyn, São Paulo, Brazil). The milled blue crab waste was blended in distilled water at a ratio of 1:4 (w/v) and hydrolysis reaction conditions used were in the proportion of 1% and 3% enzyme/substrate (E/S), and following the optimal conditions for the enzyme activity, according to the manufacturers, 53°C and pH of 9.0 and 6.0 for alcalase and bromelain, respectively. As the pH for blue crab waste is proximal to 9.0, it was not necessary to adjust pH for alcalase hydrolysis. However, for the bromelain reaction, the pH was adjusted to 6.0 with HCl 0.1 M. The two enzymes studied were used previously in studies of protein hydrolysis in marine products [15,20]. The reactions were performed in a jacketed reactor coupled to a thermostatic bath for temperature control (MQBTZ 99-20, Microquímica, Brazil). The reaction time were defined based on the hydrolysis degree. After the reaction was complete, the hydrolyzed sample was heated to 90°C for 5 min for enzyme inactivation. The insoluble fraction was separated from the supernatant by centrifugation (Q222T, Quimis, Brazil) at 16,000 x g at room temperature for 15 minutes. The protein hydrolysate supernatant was frozen at -20°C and the insoluble fraction was dried in a lyophilizer (L101, LIOTOP, Brazil) for 48 hours and later stored in a freezer (Freezer 280, Brastemp, Brazil) at -20°C until submitted to carotenoid recovery.

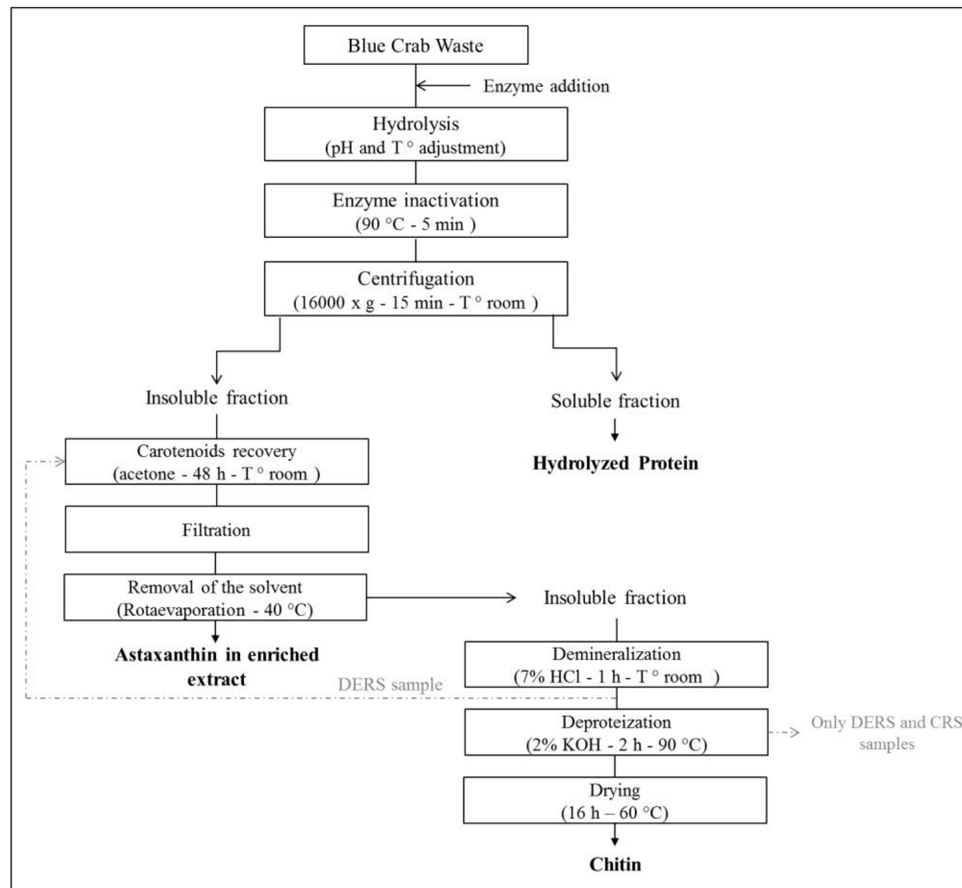

FIG.1: FLOWCHART OF THE ENZYMATIC HYDROLYSIS PROCESS FOR BLUE CRAB (*CALLINECTES SAPIDUS*) PROCESSING WASTE TO OBTAIN CHITIN, PROTEIN, AND ASTAXANTHIN.

2.3.1 Degree of hydrolysis (DH) determination

During hydrolysis reactions, 1 mL aliquots of the supernatant were added to the reaction in predetermined times of 0 (before enzyme addition), 5, 15, 30, 45, 60, 90, 120, 180 and 240 minutes. The reaction was inactivated with 9 mL of trichloroacetic acid (TCA) 6.25%, and samples were filtered after 10 minutes in Whatman nº 40 filter paper in order to remove the insoluble material precipitated by TCA. Soluble proteins were determined by spectrophotometer and expressed in mg of albumin/mL according to Lowry *et al.* [21] method. The degree of hydrolysis (DH) in proteins was determined based on Hoyle and Merrit [22] method, expressed as the percentage of TCA-soluble proteins in relation to the amount of initial total protein in the sample. Calculations were performed according to Equation (1):

$$DH\% = \frac{(PS_t - PS_{t_0})}{P_{total}} x\ 100 \quad (1)$$

In which, PS_{t0} is the amount of soluble protein at time 0; PS_t is amount of soluble protein at time *t*; e P_{total} is the total amount of protein present in the blue crab sample.

2.3.2 Soluble fraction characterization

Soluble fraction was characterized as the content of crude protein (method 991.20 – AOAC, 2005), ash (method 900.02 - AOAC, 1996), and lipids (method 1443 ISO, 1973).

2.4 Carotenoid recovery

Following the enzymatic hydrolysis, the recovery of the carotenoid fraction from deproteinized sample (insoluble lyophilized fraction) was performed according to Jeddi, Khaniki, and Sadighara [24]. In order to demonstrate that the demineralization stage may influence in the carotenoids recovery, one of the samples of raw blue crab waste has been demineralized prior to carotenoids recovery, this sample was encoded as DERS (represented in Fig. 1). Acetone was selected as solvent in accordance to carotenoid recovery from shrimp waste by Mezzomo *et al.* [3]. All extractions were performed in duplicate.

The dried blue crab residue was used as control sample (CRS). Extractions were performed using 1:4 (w/v) of blue crab residue in acetone. The mixture remained 48 hours at room temperature, protected from light. In sequence, the mixture was filtered to separate the insoluble fraction, and solvent removal was performed in a rotary evaporator with reduced pressure (550, Fisatom, Brazil).

2.4.1 Determination of total carotenoids as astaxanthin

The amount of astaxanthin in enriched extracts was determined through spectrophotometric methods according to Tolasa, Cakli, and Ostermeyer [25] as modified by Sanchéz-Camargo *et al.* [26]. A standard curve was later built using standard HPLC astaxanthin (≥ 97%-HPLC, Sigma Aldrich Co.) in acetone solvent, and absorbance was measured at the wavelength of 475 nm. Equation 2 was obtained from the standard curve for astaxanthin and used to calculate its concentration.

$$C_{AST} = (9.1255\ x\ Abs_{475nm}) - 0.5013 \qquad r^2 = 0.95 \qquad (2)$$

In which, C_{AST} is the concentration of astaxanthin in the extract expressed in µg/mL and Abs_{475nm} is its absorbance value at 475 nm.

2.4.2 Antioxidant activity

The antioxidant activity was evaluated by spectrophotometric methods of β-carotene bleaching as described by Matthäus [27] and by Kang *et al.* [28]. Briefly, the system formed by β-carotene and linoleic acid induces rapid discoloration in the absence of antioxidant compound. Free radicals are formed by linoleic acid and a hydrogen atom from the methyl groups in the β-carotene molecule. Decoloring rate of the β-carotene solution is determined by the difference between the initial spectrophotometric measurement at 470 nm and measurements at predetermined times until reaching 120 minutes. All extracts were dissolved in acetone at concentration of 5 mg/mL. Antioxidant activity (AA%) was calculated through Equation 3:

$$AA\% = 1 - \left\{\frac{[Abs_{120} - Abs_0]}{[AbsC_{120} - AbsC_0]}\right\} x100 \qquad (3)$$

In which, Abs_{120} and Abs_0 are the absorbance values of the sample at 120 and 0 minutes, respectively, and $AbsC_{120}$ and $AbsC_0$ are the absorbance values of the control sample at 120 and 0 minutes, respectively. Commercial standard astaxanthin (AST ≥ 97%-HPLC, Sigma Aldrich Co.) and butylated hydroxytoluene (BHT ≥ 99%-FCC, FG, Sigma Aldrich Co.) were dissolved in ethanol in the same concentration of extracts: 5 mg/mL.

2.5 Chitin recovery

Chitin was obtained from the insoluble fraction of astaxanthin recovery as described by Shahidi and Synowiecki [4]. Initially, samples were demineralized in a 7% HCl solution, in a ratio of 1:10 (w/v), for 1 hour, at room temperature. In sequence, they were washed with distilled water until the washing residue became neutral.

Samples not deproteinized by enzymatic process, coded as CRS and DERS, were deproteinized by an alkaline medium with 2% KOH solution, in a ratio of 1:20 (w/v) at 90°C for 2 hours. In sequence, samples were washed with distilled water until the washing residue became neutral. Finally, samples were dried in an air circulation oven (DL, DeLeo, Brazil) for 16 hours at 60°C. All extractions were performed in duplicate.

2.6 Chitin characterization

2.6.1 Fourier-Transform Infrared Spectroscopy (FTIR)

Fourier-Transform Infrared Spectroscopy (FTIR) analyses were performed by spectroscope (IR Prestige-21, Shimadzu Corporation, Japan) equipped with a DTGS (deuterated triglycine sulfate) detector. Chitin samples were prepared with potassium bromide pellets. Spectra were recorded in transmittance values ranging from 300 to 3,000 cm^{-1}. The enhanced resolution (k-factor of 2.0) was applied by means of Fourier self-deconvolution using the software IR solution 1.60 (Shimadzu Corporation, 2011).

2.6.2 Thermogravimetric analysis (TG)

Thermogravimetric analyses were performed using a thermal analysis device (STA 449F3, NETZSCH, Germany) with heating rate of 10°C/min (from 30 to 900°C) in a nitrogen atmosphere.

2.6.3 Scanning Electronic Microscopy (SEM)

Morphological analyses were performed through scanning electron microscopy (TM 3030, Hitachi, Japan). Chitin samples were covered with a thin layer of gold, under vacuum, and the views were zoomed in 50 to 5,000 times, with a voltage of 10kV.

2.7 Statistical analyses

Results were statistically evaluated through one-way analysis of variance (ANOVA) using statistical software (Statistica 7, Statsoft Inc., USA). Statistical differences ($p < 0.05$) were analyzed through Tukey's test.

III. RESULTS AND DISCUSSION

The chemical composition of blue crab waste is presented in Table 1. The moisture content in the dried blue crab waste was 35.8 ± 0.1%, and high levels of proteins and minerals (ash) were found, 33 ± 1% and 56.2 ± 1.2% respectively, which are characteristic elements of crustacean shells, while the lipid content was 1.93 ± 0.07%. Félix-Valenzuela, Higuera-Ciapara, and Goycoolea-Valencia [29] found similar values of proteins (33.8 ± 2.3%) and lower results for lipids (0.41 ± 0.01%) from demineralized blue crab (*Callinectes sapidus*) waste. No results were found in the specialized literature regarding other compounds of blue crab waste.

TABLE 1
CHARACTERIZATION OF BLUE CRAB PROCESSING WASTE (CALLINECTES SAPIDUS)

Components (%)	Blue crab *(Callinectes sapidus)*
Moisture and volatile content[1]	35.8 ± 0.1
Lipids (db)[1,2]	1.93 ± 0.07
Raw protein (db)[1,2]	33 ± 1
Ash (db)[1,2]	56.2 ± 1.2

[1]Results are expressed in mean values from two replications ± standard deviation. [2] db: dry basis.

Values found in this study were larger than those from Jo *et al.* [30] for snow crab (*Chionoecetes opilio*), namely 22 ± 2% of protein and 42 ± 1% of ash. Holanda and Netto [15] obtained values of 39.42 ± 0.49% of protein, 3.79 ± 0.08% of lipids, and 31.98 ± 1.37% of ash, for shrimp waste (*Xiphopenaeus kroyeri*), with all values in dry basis (db).

3.1 Enzymatic hydrolysis

The application of proteolytic enzymes for deproteinization of marine crustaceans waste was studied with the aim of converting waste in useful biomass [31]. The alcalase enzyme was successfully applied for protein hydrolysis of marine products and by-products, forming hydrolysates with high values of essential amino acids [22,32]. Bromelain, is a proteolytic enzyme found in the tissues of plant, as pineapple, and has been widely used in food, medical–pharmaceutical, cosmetic and other industries [33,34]. However, no data was found in the literature regarding to the use of bromelain enzyme in crustacean residues. Due to safe character, bromelain was tested in order to verify their efficiency in blue crab waste protein hydrolysis.

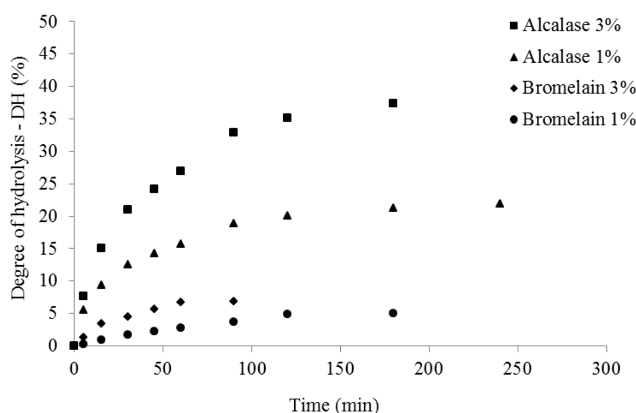

FIG. 2: DEGREE OF PROTEIN HYDROLYSIS FOR BLUE CRAB (*CALLINECTES SAPIDUS*) WASTE, USING ALCALASE 3% (■); ALCALASE 1% (▲); BROMELAIN 3% (♦); BROMELAIN 1% (●).

The kinetic curves of protein hydrolysis for blue crab waste performed for both enzymes (alcalase and bromelain) as presented in Fig. 2. Two concentrations of enzymes were evaluated (1 and 3% of ratio E/S). The curves show a usual trend in enzyme activity, with an initial stage of high reaction rate followed by a slower rate and finally a stationary stage where practically no hydrolysis occurs. This pattern may be associated with product inhibition by compounds formed during hydrolysis, decreased concentration of peptide bonds available, and the action of soluble peptides competing for substrate with the hydrolyzed protein [15,35].

The use of alcalase 3% (E/S) presented the highest performance, hydrolyzing about 30% of the protein material present in the initial sample within 120 minutes of reaction. The same enzyme at 1% (E/S) reached 20% in the same reaction time. The increase in enzyme concentration (E/S) significantly increases enzyme activity in the substrate; however, both curves obtained exhibit similar behavior, starting their stationary step at 120 minutes. For bromelain, the increase in enzyme concentration provided small increase of 2% in protein hydrolysis. In addition, the stationary phase of bromelain 3% (E/S) started at 60 minutes, whereas with 1% (E/S) of the same enzyme the stationary phase occurred only with 120 minutes of reaction. These results suggest that the alcalase enzyme has more affinity with the substrate and process conditions.

The alcalase enzyme show higher DH (Degree of Hydrolysis) than values described in the specialized literature for shrimp waste (*Xiphopenaeus kroyeri*), where a DH value of about 15% with E/S of 3% [15], and similar values were found by Klompong *et al.* [36], obtaining 30% of DH with alcalase at 2.5% (E/S) in yellowstripe fish meat (*Selaroides leptolepis*).

From a technological and economic perspective, enzymes from microbial sources such as alcalase show greater efficiency on protein hydrolysis of marine products when operating under alkaline pH [37].

Mu, Zhao, and Li [38] reported that the increase in DH can increase the antioxidant activity of peptides obtained from protein hydrolysis. Therefore, the high DH value from alcalase enzyme in the blue crab waste suggest the importance of studies to identify the amino acids from the raw material responsible for biological activities related to blue crab waste processing.

The approximate composition of protein hydrolysis (soluble fraction) is presented in Table 2. Corroborating with data from Fig. 2, the soluble fraction of blue crab deproteinization with alcalase 3% (E/S) coded as SFA3 showed highest values of soluble protein, approximately 3.5 times higher than the soluble fraction without enzymes (SFC). SFA3 also provided the highest mineral content (0.83 ± 0.03%), when compared with other samples. The hydrolyzed sample with 1% (E/S) of alcalase (SFA1) also showed significant increase of protein and mineral contents in the soluble fraction when compared with the standard sample.

TABLE 2
PROXIMATE COMPOSITION OF THE PROTEIN HYDROLYSATES IN SOLUBLE FRACTION FOR BLUE CRAB (*CALLINECTES SAPIDUS*) WASTE

Sample[1]	Proteins (%)[2]	Lipids (%)	Ash (%)[2]
SFC	1.13 ± 0.02^{a}	< 0.5	0.19 ± 0.01^{a}
SFA1	3.62 ± 0.01^{c}	< 0.5	0.3 ± 0.0^{b}
SFA3	4.12 ± 0.12^{d}	< 0.5	0.83 ± 0.03^{f}
SFB1	1.23 ± 0.06^{ab}	< 0.5	0.46 ± 0.01^{c}
SFB3	1.48 ± 0.02^{b}	< 0.5	0.58 ± 0.01^{d}

[1]SFC – control sample (soluble fraction without enzymes, only blue crab waste in distilled water); SFA1 and SFA3 – soluble fraction of blue crab waste deproteinization with alcalase 1% and 3% (E/S), respectively; SFB1 and SFB3 – soluble fraction of blue crab waste deproteinization with bromelain 1% and 3% (E/S), respectively. [2] The same letter in the same column indicates that there was no significant difference between values ($p < 0.05$).

The soluble fraction obtained from hydrolysis with the bromelain 1% (E/S), SFB1, did not differ statistically in protein amount (1.23 ± 0.06%) from the standard sample, although the mineral content was higher (0.46 ± 0.01%). With 3% (E/S) of bromelain (SFB3) an increase in the amounts of protein and minerals was observed, 1.48 ± 0.02% and 0.58 ± 0.01%, respectively, when compared to the standard sample. Increasing the concentration of ratio E/S had a positive influence for hydrolysis with alcalase but it did not differ statistically from hydrolyzed samples with bromelain. The amount of lipids from hydrolysates in the soluble fraction was less than 0.5% for all samples evaluated.

3.2 Astaxanthin recovery and antioxidant activity

Table 3 shows the extraction yield, the amount of astaxanthin, and antioxidant activity of extracts obtained from alkaline and enzyme deproteinization.

TABLE 3
EXTRACTION YIELD, AMOUNT OF ASTAXANTHIN, AND ANTIOXIDANT ACTIVITY FROM EXTRACTS OF BLUE CRAB WASTE (*CALLINECTES SAPIDUS*), OBTAINED FROM ALKALINE AND ENZYME DEPROTEINIZATION.

Sample[1]	Extraction yield (w/w%, b.w.)[2]	Astaxanthin ($\mu g/g_{residue}$)[2]	Antioxidant activity (%)
CRS	$0.38 \pm 0.01^{A,a}$	15.3 ± 0.7^{a}	86.9 ± 2.2^{b}
DERS	3.1 ± 0.4^{A}	97.7 ± 14.3^{b}	88.6 ± 1.9^{b}
HEA1	0.38 ± 0.08^{a}	12.0 ± 2.5^{a}	92 ± 2^{bc}
HEA3	0.62 ± 0.03^{b}	20.0 ± 1.3^{a}	88.6 ± 2^{b}
HEB1	0.34 ± 0.07^{a}	14.2 ± 2.9^{a}	96.9 ± 4.5^{cd}
HEB3	0.40 ± 0.06^{a}	17.0 ± 2.3^{a}	69 ± 1^{a}
AST	-	-	74.0 ± 0.4^{a}
BHT	-	-	99.4 ± 0.6^{d}

[1]CRS – control sample (raw blue crab waste); DERS – demineralized blue crab waste; HEA1 and HEA3 – blue crab waste deproteinized with alcalase 1% and 3% (E/S), respectively; HEB1 and HEB3 – blue crab waste deproteinized with bromelain 1% and 3% (E/S), respectively; AST – Commercial standard astaxanthin; BHT - butylated hydroxytoluene.
[2]The same letter in the same column indicates that there was no significant difference between values ($p < 0.05$).

The sample demineralized under acid process (DERS) before the carotenoid recovery obtained the highest extraction yield, at 3.1 ± 0.4% (w/w), about 8 times more than the control sample (CRS) and with more astaxanthin than other samples at 97.7 ± 14.3% $\mu g_{astaxanthin}/g_{residue}$ (almost 5 times higher than HEA3 result). This indicates that the acid demineralization process before the astaxanthin recovery increased the availability of carotenoids and enables their extraction with organic solvent. Samples submitted to protein hydrolysis did not present significant differences in the extraction yield among one another and compared with the control sample, indicating that the highest yield obtained with 3% (E/S) using the enzyme alcalase is due to compounds (amino acids, minerals, etc.) being solubilized during the astaxanthin extraction process.

Holanda and Netto [15] obtained higher values of 121 $\mu g_{astaxanthin}/g_{residue}$ using alcalase 3% (E/S) in enzymatic hydrolyses of shrimp waste (*Xiphopenaeus kroyeri*), this value was 10% higher than their standard sample and 6 times higher that obtained in this study in the same conditions. The amount of carotenoids present in certain raw materials is due to the amount of carotenoids available in animal feed, environmental conditions, and its species [4].

Except from the HEB3 sample, the other extracts showed antioxidant activity with statistically higher values than commercial astaxanthin. Astaxanthin is found in nature conjugated with proteins or with one or two esterified fatty acids that provide stability to the molecule, as free astaxanthin is more susceptible to oxidation. Thus, astaxanthin obtained from natural sources tends to be more stable [16].

Although the sample DERS obtained the highest carotenoid extraction values, its antioxidant activity of 88.6 ± 1.9% did not differ significantly with values obtained for the standard sample (86.9 ± 2.2%). Extracts from the samples submitted to enzymatic hydrolysis, except HEB3, show antioxidant activity statistically higher or equal the standard sample. The increase in [E/S] influenced negatively the antioxidant activity of enzymatic extracts. Carotenoid recovery from the sample hydrolyzed with bromelain 1% (E/S) presented the best values of antioxidant activity (96.9 ± 4.5%) with no statistical differences with the commercial antioxidant BHT (99.4 ± 0.6%).

From the data in Table 3 it was not possible to relate the antioxidant activity of the extracts with the amount of astaxanthin extracted, because the extraction method affects directly the type of carotenoid extracted.

Crustacean waste has large amounts of astaxanthin both esterified and free [3], with low amounts of astaxanthin combined with proteins – these are mostly found in the meat of crustaceans and salmonid fish [16]. This is confirmed in this study because no significant increase in astaxanthin content and antioxidant activity was detected using protein enzymatic hydrolysis in raw blue crab waste before the recovery astaxanthin process.

3.3 Chitin characterization

3.3.1 Fourier-transform infrared spectroscopy (FTIR)

Infrared spectroscopy can be used to characterize samples of chitin and chitosan, because both have characteristic bands of functional groups inherent to this kind of sample [39,40].

The FTIR spectra presented in Fig. 3 refer to chitin samples obtained by: alkaline deproteinization, with demineralization prior and after the astaxanthin recovery (respectively, CRS and DERS); deproteinization with alcalase at 1% and 3% (respectively, HEA1 and HEA3); and deproteinization with bromelain at 1% and 3% (HEB1 and HEB3, respectively).

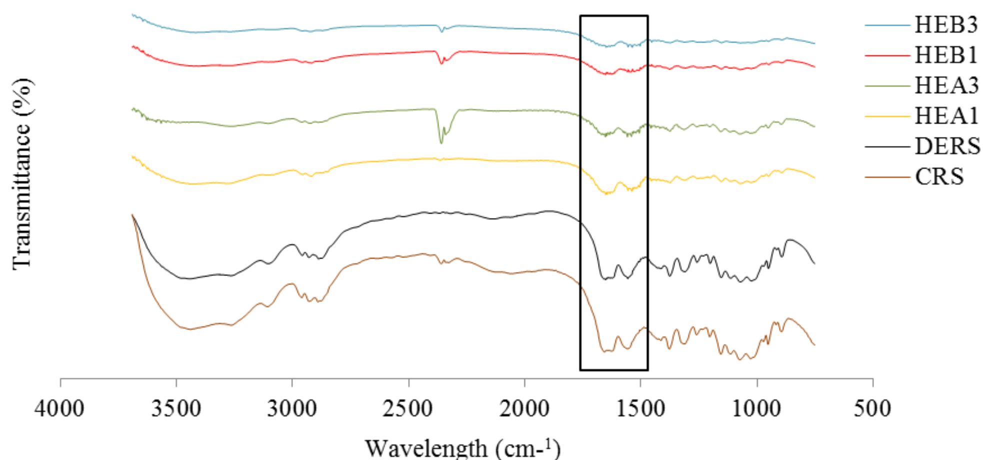

FIG. 3: FTIR SPECTRA OF CHITIN OBTAINED FROM BLUE CRAB WASTE (*CALLINECTES SAPIDUS*) THROUGH ENZYMATIC AND ALKALINE DEPROTEINIZATION. CRS – ALKALINE DEPROTEINIZATION, WITH DEMINERALIZATION AFTER ASTAXANTHIN RECOVERY; DERS – ALKALINE DEPROTEINIZATION, WITH DEMINERALIZATION PRIOR TO ASTAXANTHIN RECOVERY; HEA1 AND HEA3 – ENZYMATIC DEPROTEINIZATION WITH ALCALASE 1% AND 3% (E/S), RESPECTIVELY; HEB1 AND HEB3 – ENZYMATIC DEPROTEINIZATION WITH BROMELAIN 1% AND 3% (E/S), RESPECTIVELY.

Characteristics chitin bands were found for all samples and the FTIR spectra of this work present spectra similar to those described in the specialized literature [11,40,41]. Bands in the region between 890-1150 cm^{-1} are polysaccharide bonds. Usually, samples of chitin have four feature bands between 1300-1700 cm^{-1}, as found in the present study. The band at 1365 cm^{-1}, called amide III, corresponds to the CH_2 group and CO-NH deformation. The band at 1380 cm^{-1} that is less intense in HEA and HEB is attributed to angular deformation of CH_3 symmetric group. The band at 1540 cm^{-1}, called amide II, represents two vibrational modes: plane N-H and stretching C-H. The band at 1610 cm^{-1} is called amide I and can be attributed to axial deformation of C=O. The bump in amide I that is most clearly observed in samples CRS and DERS, indicates that the chitin extracted from the blue crab waste adopts polymorphic structure of α-chitin, which is the most abundant and stable form of this compound [41,42], as expected according to the literature.

Finally, the intense and wide bands identified at 3400 cm^{-1} are characteristic of axial stretching vibrations of the OH group.

Due the prevalence of NH_2 groups, during the N-deacetylation of chitin the band at 1655 cm^{-1} gradually decreased, while that at 1590 cm^{-1} increased. The analysis of these two peaks (highlighted in Fig. 3) can indicate efficiency of chitin production [43].

From Fig. 3 is possible to verify that alkaline deproteinization and the acid demineralization (CRS and DERS) provide very similar spectra, indicating that the carotenoid recovery, before or after demineralization, did not influence the chitin chemical composition. The samples CRS, DERS, HEA3 and HEB3 present similar bands at 1655 cm^{-1} and 1590 cm^{-1}, this suggests that enzymatic deproteinization, with 3% [E/S], was as effective as chemical deproteinization in the production of chitin from crab blue waste.

The samples HEA1 and HEB1 present the band 1590 cm^{-1} lower than 1655 cm^{-1}, this behavior indicates that use of 3% [E/S] is more effective than 1% [E/S] to produce chitin by enzymatic deproteinization.

3.3.2 Thermogravimetric analysis (TG)

Thermogravimetric analysis (TG) measures the mass changes as a function of temperature, in inert (nitrogen) or oxidative (air or similar) atmosphere. In polymeric compounds such as chitin, TG is used to assess the loss of moisture and compounds, as well as intermolecular interactions and thermal stability of analyzed materials [44].

Fig. 4 shows the TG curves for chitin obtained from blue crab waste through enzymatic and alkaline deproteinization. The first degradation stage, due to water loss, occurs in a similar way for all samples, between 50 and 115 °C, where all TG

curves are superimposed, indicating that samples have a similar initial moisture content, and the weight loss in this stage was of approximately 5%.

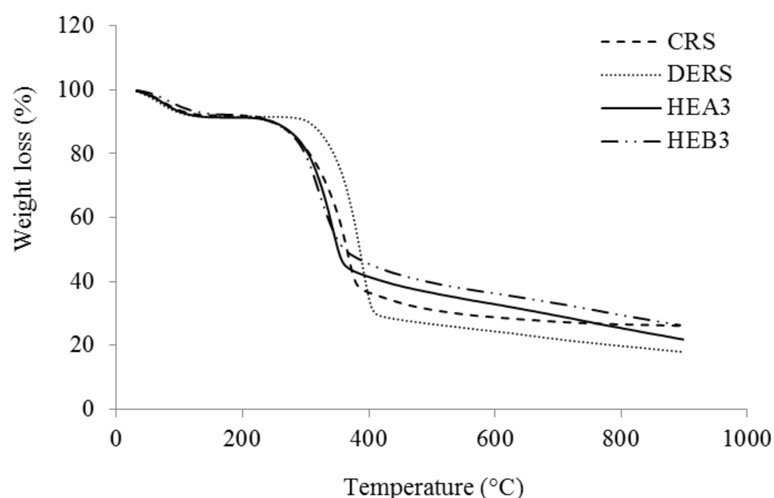

FIG. 4: TG CURVES FOR CHITIN OBTAINED FROM BLUE CRAB WASTE (*CALLINECTES SAPIDUS*) THROUGH ENZYMATIC AND ALKALINE DEPROTEINIZATION. CRS – ALKALINE DEPROTEINIZATION, WITH DEMINERALIZATION AFTER ASTAXANTHIN RECOVERY; DERS – ALKALINE DEPROTEINIZATION, WITH DEMINERALIZATION PRIOR TO ASTAXANTHIN RECOVERY; HEA3 – ENZYMATIC DEPROTEINIZATION WITH ALCALASE 3% (E/S); HEB3 – ENZYMATIC DEPROTEINIZATION WITH BROMELAIN 3% (E/S).

The second degradation stage refers to thermal decomposition of chitin and of organic and inorganic materials present in the samples [42]. The DERS sample showed higher thermal stability compared to other samples, since its degradation (organic compounds) started under 286°C. On the other hand, degradation for other samples began under 222°C. The weight loss in this step for DERS sample was higher than for other samples, i.e., 59%, followed by CRS (52%), HEA3 (46%), and HEB3 (40%). The greater the weight loss in this step, the higher is the purity degree of the sample because it indicates a larger amount of chitin [45].

Residual mass was observed in all samples; that is, the weight loss of samples did not reach zero, which indicates that not all chitin content was decomposed until reaching 900°C. The same behavior was reported by Pereira *et al.* [46] in chitin-chitosan polymers, and Wysokowski *et al.* [47] in standard chitin.

In a study with chitin obtained from shrimp shells (*Litopenaeus vannamei*), Antonino [48] obtained results similar to the present study. In the first degradation stage, relative to sample moisture, the chitin from gray shrimp lost 8.0% of mass under a peak temperature of 73°C. The second decomposition occurred under a peak temperature of 314°C, with 61.6% of weight loss.

Sagheer *et al.* [42] obtained for chitin samples from the flower crab (*Portunus pelagicus*) the first peak under about 60°C, with average weight loss of 5%, and a second stage of degradation under 326°C with weight loss between 65% and 73%. Other authors reported similar chitin TG analyses obtained from shrimp shells from the Brazilian Atlantic coast [45] and from chitin-chitosan polymers [46].

3.3.3 Scanning electronic microscopy (SEM)

Fig. 5 represents a SEM of chitins obtained from blue crab waste through enzymatic processing with alcalase and bromelain, as well as with alkaline deproteinization as control sample, zoomed in 30x and 150x, respectively. SEM shows that samples with alkaline and enzymatic deproteinization have a similar structure. It is possible to observe that the morphologies present compaction and non-homogeneity with irregular particles of various shapes and sizes.

Similar morphology with non-homogeneous structure and the presence of irregular particles with various shapes and sizes was found by Wysokowski *et al.* [49] in commercial α-chitin obtained from crab shells.

Through image analysis it is possible to note that there are no significant differences in the morphology of chitin deproteinized with enzymes alcalase and bromelain, when compared to the sample deproteinized through traditional methods with an alkaline reagent. The SEM results presented in Fig. 5 show that the deproteinization process (neither using enzymes nor alkaline reagents) has no influence in size, shape, or porosity.

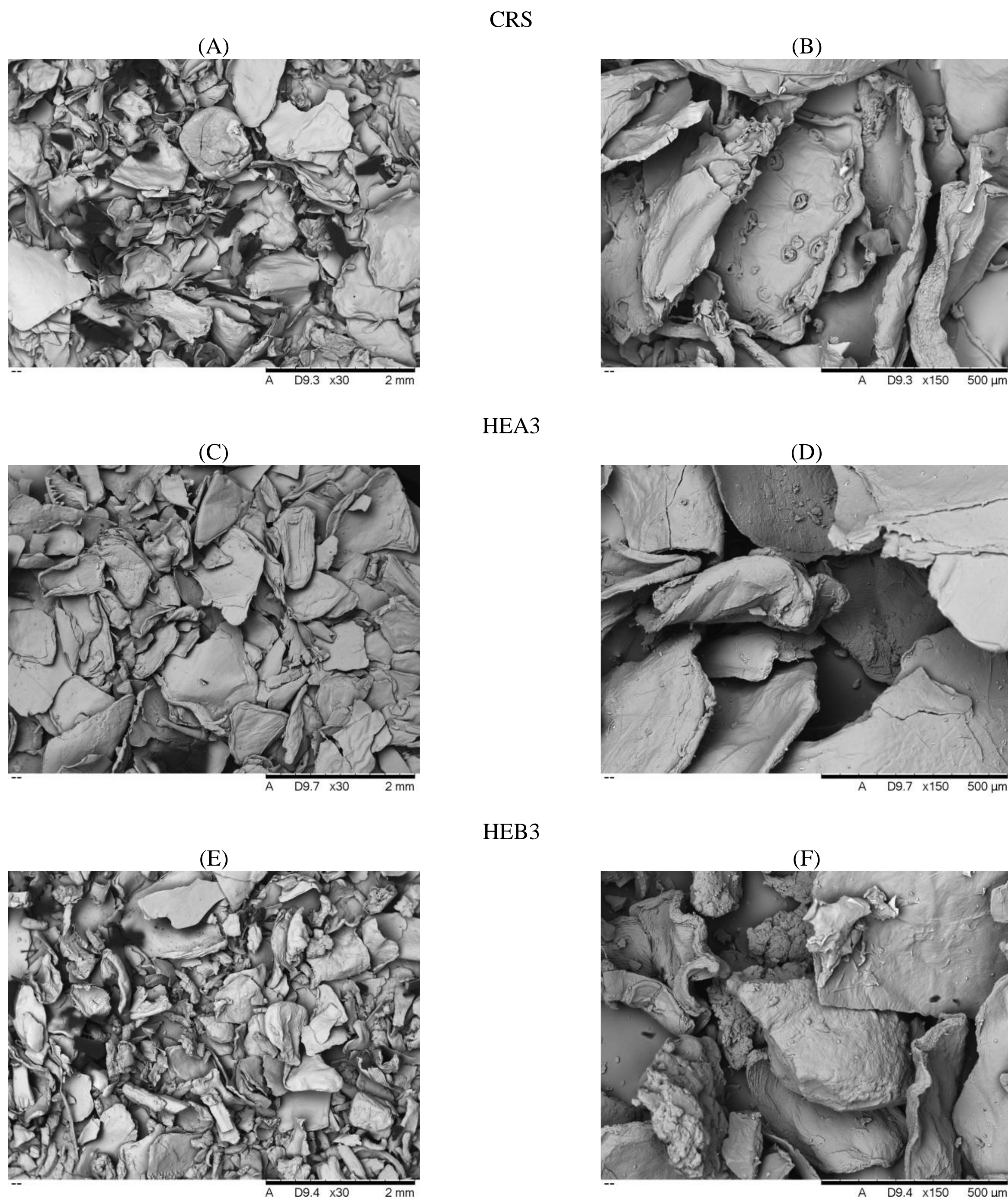

FIG. 5: SCANNING ELECTRONIC MICROSCOPY OF CHITIN OBTAINED FROM BLUE CRAB WASTE (*CALLINECTES SAPIDUS*). CRS – ALKALINE DEPROTEINIZATION AT ZOOM: (A) 30X AND (B) 150X; HEA3 – ENZYMATIC DEPROTEINIZATION WITH ALCALASE 3% (E/S) AT ZOOM: (C) 30X AND (D) 150X; HEB3 – ENZYMATIC DEPROTEINIZATION WITH BROMELAIN 3% (E/S) AT ZOOM: (E) 30X AND (F) 150X.

IV. CONCLUSION

This study revealed the practical relevance of blue crab waste processing as a proper source of proteins, chitin, and carotenoids. Protein hydrolysis using the enzyme alcalase at 3% [E/S] obtained the best degree of hydrolysis, resulting in approximately 30% of protein material after 120 minutes of reaction, as well as similar composition of blue crab deproteinization in soluble fraction. Alcalase 3% also yielded high values of soluble protein, approximately 3.5 times higher than the soluble fraction without the presence of enzymes. The enzyme bromelain had low DH efficiency in for the studied concentrations of 1% and 3% (E/S).

Extracts obtained from insoluble fraction of the enzymatic hydrolysis in this study indicate that blue crab waste presents a proper source of astaxanthin. Its antioxidant activity can be compared to that of positive controls such as standard astaxanthin and BHT, particularly in the case of the extract hydrolyzed using bromelain 1% (E/S), which indicates that they can be used in other systems as natural antioxidants.

The FTIR, TG and SEM analyses of chitins obtained from enzymatic hydrolysis showed characteristics similar to those reported in the traditional literature. It was also possible to assess that the enzymatic treatment to obtained hydrolyzed protein does not change the structure of chitin drastically. It is possible to conclude that the enzymatic hydrolysis, a deproteinization environmentally friendly method, of blue crab waste (*Callinectes sapidus*) processing can be used to produce, protein concentrated, chitin and astaxanthin enriched extract, and the use of crustacean waste, associate to enzymatic deproteinization is a promising clean technology for the disposal and management of the crustacean waste.

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