SIMPLE AND ECOLOGICAL OBTENTION OF CHITIN FROM CRUSTACEAN WASTE

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***Abstract*— Crustacean farming generates large quantities of residue, posing environmental and economic problems. These residues contain chitin, proteins and a carotenoid pigment, all of high commercial value, and wide applicability. These compounds are associated with each other in a complex biological counterfoil, rich in calcium carbonate. The available extraction methods for shrimp residues are slow and burdensome, relying upon thermal treatments with concentrated acids and bases, or fermentative methods with high costs in energy, water volume and chemical products. We present here different assays, which were performed to develop an economical process which is environmentally friendly, with acceptable yield, comprising two brief and consecutive steps. The best condition tested was: demineralization with citric acid (ratio weight of residue: citric acid =5.5) for 20 min. at 40ºC, followed by deproteinization with pancreatic enzymes in 0.5 M ammonium citrate buffer at 40ºC. More than 80% of the raw chitin was recovered (4% minerals, 8% protein and 21% carotenoids remnant content), while 70% of the total proteins and 60% of the total carotenoids remained in the final filtrate. Discoloration of the raw chitin was easily performed with acetone.**

***Keywords*—chitin, biological matrix, crustaceans.**

I. INTRODUCTION

Approximately 40-50% of crustaceans' weight (shrimps, prawns and crabs) is discarded, even though some countries have begun to use this waste to obtain two important by-products: chitin and astaxanthin. Crustacean fishing in Argentina totals about 50,000 tons annually. These results in the need to eliminate several tons of bio-waste, mostly dumped into the sea, together with non-targeted species and juvenile individuals. This waste represents an important residue that is dragged by the current, concentrated, and sunk in such a way that it has become a major environmental concern (Arbia *et al.*, 2013).

The biological matrix of these residues is formed by a honey-comb-like mineralized chitin-protein structure in which calcium salts are mainly amorphous calcium carbonate. This biological matrix is a complex integration of several layers in which chitin, proteins, pigments and salty minerals are finely ordered, overlapping and associated among themselves, thereby hampering the residue's extraction.

Chitin accounts for 20-60% of this wastes dry mass (more in crabs than in shrimps) (Le Roux and Berge, 2014). It is a glucosamide polymer and the most abundant organic substance in nature, after cellulose. More than 65% of Glucosamine monomers in chitin are acetylated, being insoluble in water. Chitin is a precursor of other products of great technological interest and high commercial value: Glucosamine and Chitosan.

Proteins account for another 20-30% of this waste, dry mass. Mainly bonded to chitin, they form a twisted ply-wood structure. A region known as R & R has been identified in shell proteins of insects and crustaceans, whose folding conformation determines the helical association of chitin - protein. In this structure, there is a domain preserved in all these species, rich in tyrosine and phenylalanine which forms a hydrophobic pocket that could harbor the carbohydrates (Ferrari *et al.*, 2012).

In the outer layers, hexamerins, (cryptocyanin, crustocryns and crustocyanins) are associated to astaxanthin, a carotenoid pigment. They contain high frequency of phenolic amino acids, which act as chromophores, giving a blue color to the complex. This is its natural form, but it turns red when this bonding to proteins is broken, - for example, when cooked (Le Roux and Berge, 2014).

Calcium salts, mainly amorphous calcium carbonate are laid progressively after the mold in a variety of polymorphic forms in their shells. Its content depends on the animal age and season accounting for 20-30% of dry weight. (Belandria and Morillo, 2013; Arbia *et al.*, 2013; Fabritius *et al.*, 2013; Kunkel, 2013).

Current methods for chitin extraction are either chemical or fermentative. In the first case, they include an initial demineralization with a concentrated mineral acid followed by alkaline protein removal with sodium hydroxide (Kock *et al.*, 2010; Belandria and Morillo, 2013) or both steps inverted (Escobar Sierra *et al.*, 2013). Pretreatment with NaClO has recently been described (Kaya *et al.*, 2015). These methods not only apply chemicals that are environmentally harmful, but they can affect chitin integrity thereby causing de-amination, de-acetylation and de-polymerization (Rheza Kasaai *et al.*, 2013) while no carotenoid recovery is described.

Fermentative methods with lactic bacteria manage to solubilize partially the calcium salts and hydrolyze the proteins, but require large volumes of water and long reaction periods (6 days at 45-55ºC) while they yield a product that still contains significant amounts of proteins (Waldeck *et al.*, 2006; Shahidi and Janak Kamil, 2001). The separation of the protein fraction is achieved later by treatment with NaOH 1-10% at 65-100° C.

(Kock, 2010). The success of the process relies on the complexing and solubilizing action of lactic acid, either added, or generated "on-site" by fermentation. It is a weak acid, forming weak complexes with calcium (pK=0.82), which efficiency increases at pH values <3.5 (Xu *et al.*, 2013).

Citric acid was used in the assays for demineralization, as it is a stronger, tricarboxylic acid (pK_a 3.13; 4.77 and 6.40) that forms more stable complexes with calcium, (pK_a=3.5) and has an affordable cost. It is also a food additive, authorized for use in all countries. In this paper, optimal conditions (time and temperature) for this step are described. Ammonium citrate was also used as a buffer in the deproteinization step in order to further reduce the mineral content of the final product.

Taking into account that both the union of chitin to proteins and of proteins to astaxanthin rely on the presence of aromatic amino acids in specific sites (Ferrari *et al.*, 2012), it seems convenient to focus on a specific enzymatic process for these amino-acids, for example chymotrypsin. The pancreatic extract, a mixture of endo- and exopeptidases (trypsin, chymotrypsin, and carboxypeptidase) is an affordable and accepted food supplement.

Although various enzymatic methods have been described for the recovery of crustacean waste components, the reported degree of hydrolysis achieved is lower than that for the alkaline treatment. Meanwhile a high mineral content in chitin is reported (Gortari and Hours, 2013; Younes *et al.*, 2014). Ammonium citrate was used in these assays, as buffer for the deproteinization step in order to further reduce the mineral content of the matrix, as well as to ease the enzymatic action, where optimal conditions are described.

Our aim was to develop an environmentally-friendly method for chitin and astaxanthin production and to obtain amino acid from waste, thereby avoiding the use of strong acids or bases, large volumes of water and high temperatures. The method is also easy to implement and appropriate for developing countries.

II. METHODS

a) Shrimp (*Pleoticus muelleri*) waste was obtained from a retailer (without previous washing) and was ground to an average size of 1 cm^2. Shrimp waste composition was assessed by AOAC (1990) methods: moisture by drying at 100 °C (AOAC 925.10 and 950.46); nitrogen content by Kjeldahl method (AOAC 2.051(ISO 3188), ash at 600 ° (AOAC 923.03) and lipids by the Soxhlet method (AOAC 920.39) using petroleum ether: acetone: water (15:75:10). The carotenoid content in this extraction medium was established at neutral pH, at 474 nm using a Perkin Elmer Lambda 25 device, while the reported extinction coefficient was used (Belandria and Morillo, 2013).

b) De-mineralization with citric acid (Biopack) was followed by acidimetric titration: the evolution of pH towards neutrality corresponds to acid consumption and the persistence of acidity in the medium indicates the end of the reaction.

c) Ammonium hidroxide (Biopack) and pig pancreatin (Bago) were used for deproteinization.

d) Protein/peptides in solution were determined by the Bradford method using Comassie Blue G-250 (Sigma) according to Kruger (2009) using Bovine Serum Albumin (BSA) as a reference (Sigma), while peptides/amino acid content in solution was measured at 280nm, using a Perkin Elmer Lambda 25 device. The extinction coefficient for 280 nm was calculated using the Edelhoch formula (Pace *et al.*, 1995), according to reported crustacyanin amino-acid composition (Cianci, 2002).

e) The carotenoid content in the filtrates, after each step and in the acetone used for the final discoloration step was assessed as previously described.

f) The de-acetylation degree was estimated by recording the pH while titration of a 0,1M HCl suspension (1g) with NaOH 0,1M. The difference between the volumes required for neutralization (pH 7) and the 2nd inflexion point of the curve is proportional to the fraction of free amino groups, or the degree of deacetylation (Hernandez Cocoletzi *et al.*, 2009).

g) An Infrared Spectrum was obtained for the ethanolic suspension in a FT-IR HATR Perkin–Elmer 100 Series device.

A. Description of the assays

First set of demineralization experiments: The demineralization of 100g residue was performed with different citric acid: calcium molar ratios (8:1; 4:1 and 2:1) at 20°C, 40°C and 60°C, while stirring (2000 rpm) – Taking into account partial ionization of citric acid (a weak acid), an excess of acid was initially used, in order to set the best conditions for further assays. Finally, the suspensions were filtered through gauze, and the residues were dried and weighted.

A second set of demineralization experiments were performed at 40°C for 40 min. The best condition in the previous assays (the 2:1 molar ratio citric acid: calcium) was repeated using 200g of residue. In order to make the process more economical and avoid the formation of the fine precipitate observed in the initial assays, citric acid was reduced in two other assays with molar ratios: 1.66:1 and 1.25:1 (citric acid: calcium), until no precipitate was observed. The final mineral content in residues and the efficacy of the method were established.

Deproteinization: A sample of de-mineralized shrimp waste was treated with 300 mg pancreatic enzymes (1000 USP) at 40ºC in a water bath at pH 8 (ammonium hydroxide/citric acid 0,5M) for up to 3 hours with gentle stirring (100 rpm). At the end of the incubation period, the product was filtered through gauze and dried. This filtered solution was used to determine protein and the pigment content, as well as to estimate the degree of hydrolysis. The infrared Spectrum and mineral content for the dried residue is informed.

Finally, a 3g sample of the obtained chitin was extracted with 100 mL acetone (Anedra) in order to assess the final content of carotenoids, while another 3g sam-

ple was used to establish protein content. For this purpose, hydrolysis in 100 mL 4% NaOH was performed and the amino acid content in solution was assessed by Kjeldahl determination and 280 nm absorption.

III.RESULTS

Shrimp waste composition turned out to be: 70.3% water, 6.1% minerals, 14.5% chitin, 5.7% lipids and 3.4% proteins (net protein content without taking into account chitin nitrogen content which is 5.8%). Initial carotenoid content in the waste was 3.3 %.

A. De-mineralization process optimization

In the first set of experiments, for all three assays, the pH was initially 1.8-2.0 and increased quickly in the first 20 minutes, with a trend to stabilize. The solution turned orange in all experiments. A rapid formation of bubbles showed the release of carbon dioxide, while a fine powder was formed. These first molar relationships were chosen supposing that in order to have a complete reaction, the citric acid intake by the citric-calcium complex should be greater than the stoichiometric amount of minerals, while the pH should remain circa 3.

Working with a molar relationship of 8:1 (citric acid: calcium), the pH rose to 2.5. Working at 60°C and 40°C, the demineralized residue was less than nominal (4-6%), while a fine precipitate hindered the process of filtration. Working at 20°C, a residue 24% higher than the nominal value was obtained, indicating incomplete calcium removal.

Using a 4:1 (citric acid: calcium) molar ratio, the pH evolved to values close to 3. Again, working at 60°C and 40°C, the demineralized residue was slightly smaller than nominal and at 20°C, it was 21% higher (incomplete extraction). The fine precipitate was also observed.

When a molar relationship 2:1 (citric acid: calcium) was assayed, the final pH value was 3.5. Working at 60ºC and 40ºC, the residue was 8-9% lower than the expected value, while at 20ºC it was 21% higher. Again, a fine precipitate was observed, although in a lesser extent. In a second stage, triplicate assays on 20g waste were made, obtaining very similar values with a high degree of repeatability (CV% = 6%) with a 40minute treatment.

The intermediate temperature was chosen for the following working conditions, as higher temperature seemed to produce more fine powder and 20ºC did not seem to be sufficiently effective.

In the second set of experiments, when 2:1 molar ratio (citric acid: calcium) was used, the residue obtained was 89% of the nominal value and it mineral content was 14%. Furthermore, 17 g of a fine powder was separated from the filtered suspension. This powder, once dried, showed a 21% mineral content, which is similar to the calcium content in a di-citric tricalcium complex. Yield was good, but an excess of citric acid was used.

For the 1.66:1 ratio yield was 92% of nominal value, retaining 8% of minerals. The net content of organic matter was then 85%. In the filtrate 6.3 g of fine dust was recovered, which once dried and analyzed, turned out to contain 21% minerals (congruent with calcium tri-citrate). This would account for a stoichiometric excess of 4 g of citric acid. The ideal relationship in this case appears to be 200 g residue: 36 g citric acid.

Finally, for the 1.25:1 molar ratio, the yield was 88% with 8.8% minerals content. No precipitate was recovered in the filtrate, but mineral content in the residue was higher than in the previous assay

It was concluded that the optimal working molar relationship must be close to 1,5:1, i.e. for 100 g of crustacean waste (containing 60 mmol of calcium), 90 mmol of acid citric (18 g) in 300 mL water, that is a relationship residue: citric acid in weight of 5.5.

B. Deproteinization

Demineralized waste was obtained in the selected condition and was dried. A sample of 30g of the demineralized waste was deproteinized as described above, for different lapses. At the end of each incubation period, the product was filtered through gauze and dried. The peptide/amino acid and pigment content were assessed in the filtrate by spectrophotometry, in order to estimate the progression of hydrolysis. The results are summarized in Table 1.

After 3 hours of enzymatic treatment, the residue still exceeded the nominal value of chitin and contained 5% of material to be extracted. The mineral content showed a progressive decrease, but after 3 hours, the chitin still retained significant mineral content. The 280nm absorbance in the filtered solution increased, but the protein extracted (as calculated using the Edelhoch formula), did not reach the nominal value to be extracted, (4g). Pigment extraction apparently occurred in the initial phases of the process, showing a similar value for all assayed conditions (0.8mg). This content should not be compared to the initial content as the demineralized waste was dried before deproteinization causing pigment losses.

After 3 hours of the process, the chitin contained 5.6% nitrogen, a value similar to that of the chitin (5.8%) and significantly lower than that of the proteins (6.25%) or Chitosan (7.7%), indicating that the process was efficient.

Based on these results, a 3-hour process did not seem to be sufficient to extract the total protein content. The initial enzymatic rate was estimated on at 0.9g protein/hour. Accordingly, a 5 hours process would be needed for the amount of this enzyme and amount of the residue. Alternatively, a greater quantity of pancreatin could be used, for example: 5000 USP for 100 g waste in a 1-hour process.

Table 1: Deproteinization process

Process time	Residual weight (g)	Mineral content (%)	Extracted Protein (g)
30 min	27.9	17.1	0,3
1 hour	25.9	18.8	0,6
2 hours	20.2	18.9	2.2
3 hours	17.9	14.0	3.1

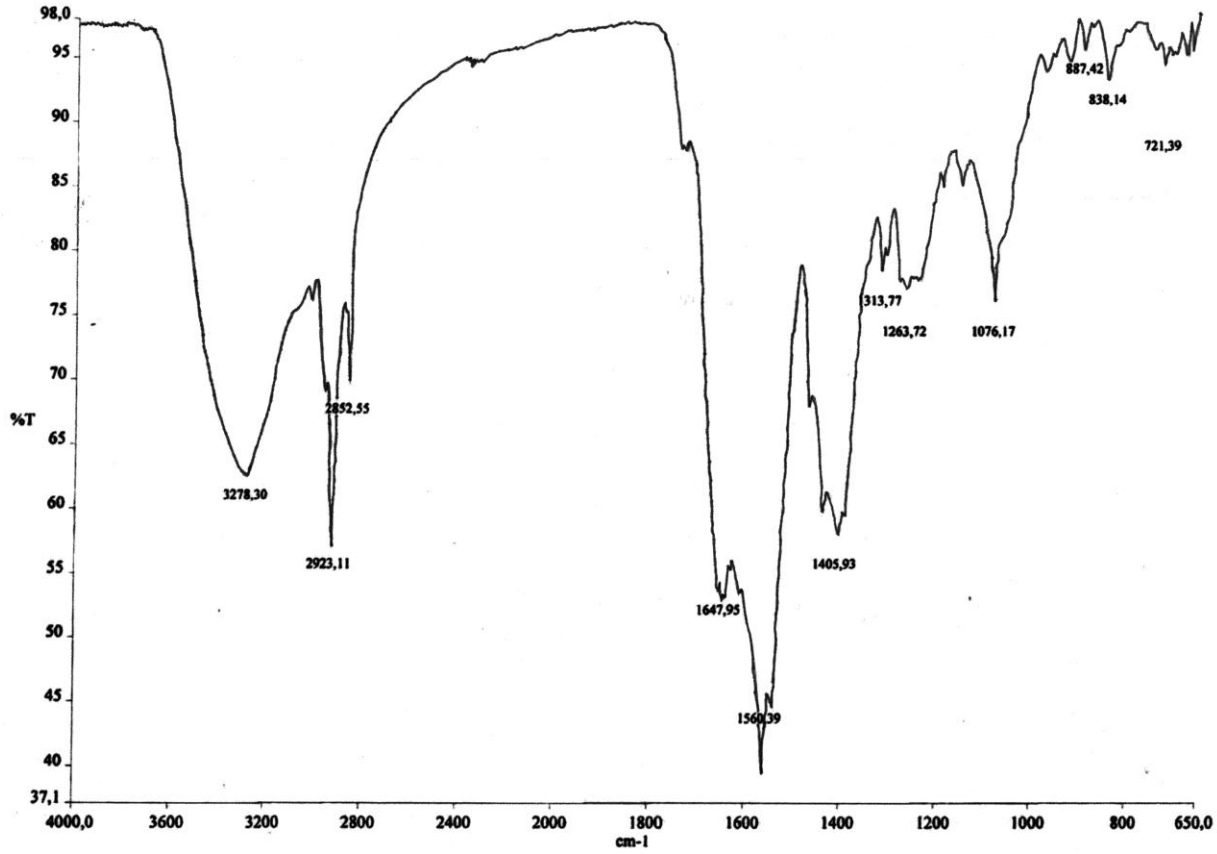

Figure 1. FTIR Chitin

A final assay was performed in order to verify our previous conclusions, eliminating the intermediate drying step and decreasing water volume. Two hundred grams shrimp waste was treated with 35 g of citric acid, dissolved in 600 mL of water for 40 min, while stirring (2000 rpm) at 40ºC. After this process, the residue was gauze filtered and washed with 50 mL water. Without drying, the demineralized product was suspended in 300 mL of 0.5M ammonium citrate buffer pH 8 and 5000 USP pancreatin was added. Gentle stirring was maintained for 1 hour while heating at 40ºC. The product was then filtered by gauze, washed with water and dried.

The process yield was 92%, and the mineral content in this chitin was 4.2%. Potentiometric titration allowed the estimation of a degree of de-acetylation lower than 20%. The FTIR for this chitin showed the characteristic peaks of Amide II (1550 cm^{-1}) and Amide I (1660 cm^{-1}), methylene and methyl bands at 2800 cm^{-1} - 2900 cm^{-1}, with a clear broad absorption band between 3300-3450 cm^{-1}. No significant absorption was observed between 1650 cm^{-1} and 2800 cm^{-1}, indicating absence of aromatic compounds, sulfides or esters.

Figure 1 shows the FTIR of chitin.

The 1st and the 2nd filtrate were analyzed for peptide/amino acid content. Initially the Bradford method was used rendering values of 13.2% and 12% of total protein content respectively. But as part of the original content of proteins could be present as small peptides or amino acids, particularly in the 2nd filtrate, which would not be detected by the method (Kruger, 2009), absorbance at 280 nm was also measured. The 1st filtrate content accounted for 15.3% of nominal value and 70.3% was estimated for the 2nd filtrate by U.V absorption.

In order to assess the protein content in the raw chitin, an alkaline hydrolysis of a 3g sample was performed. Micro-Kjeldahl determination of amino acid content in the solution yielded 6.9 % while 280nm absorption rendered 8.3%. The difference in weight after alkaline treatment was also recorded (8%). It was concluded that 8% of nominal protein content remained in the waste.

The carotenoid content in the 1st and 2nd filtrate was 12% and 61% of initial value as measured at 474 nm. Discoloration of the raw chitin with acetone was also performed and a further 21% of initial content was recovered.

IV. CONCLUSIONS

This new procedure offers the possibility of industrialization of crustacean waste, using organic chemicals, an environmentally acceptable and relatively economical process, optimized water use, and is therefore, a potential treatment of large volumes of waste. Furthermore, this procedure allows to obtain highly appreciated by-

products, providing an excellent alternative for the extraction of chitin for the pharmaceutical and food industries. We are working in the process improvement and by-products isolation.

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