Manual for chitosan production from locally sourced red prawn residues

Extraction procedures

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Fondo Europeo di Sviluppo Regionale
European Regional Development Fund

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Abstract

Bythos has produced a manual for the extraction of chitosan from red prawn heads and shells – residues sourced from a local sea food processing plant

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1.Preface

This manual describes the experimental method of extracting chitosan from 10 prawn heads of the *Aristaeomorpha foliacea* species as a typical industrial waste product.

First of all, the heads obtained as a waste product must be frozen at -20 °C.

Before starting the extraction, 10 heads must be placed to thaw at room temperature. Normally 10 heads with organic part have a weight of 20.11g ± 3,36.

When the heads are thawed, they will have to be cleaned of organic elements to obtain only the exoskeleton from which the chitosan will be obtained.

To properly clean the exoskeleton, the head must be cut into two parts in the ventral area.
See Figure 1.

Then, the organic material will be scraped off with the help of a scalpel and then the exoskeleton will be cleaned using running water.

At the end of the cleaning, the exoskeletons will be rinsed in distilled water once and left to dry in a stove for 2 nights at 60 ° C.

During these phases continue scraping to remove all the organic parts.

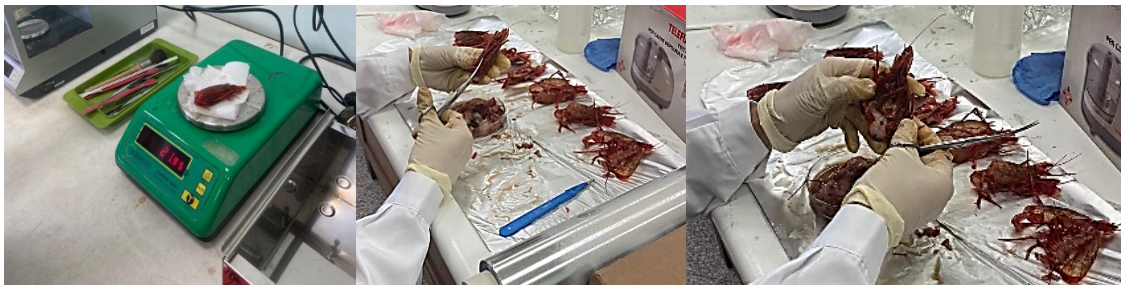


Figure 1. Weighing, head cutting and organic substances removal.

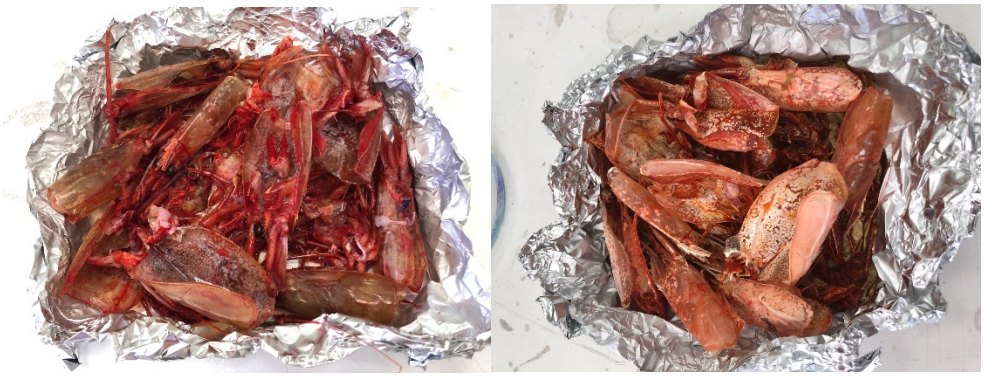


Figure 2. On the left the exoskeletons cleaned before drying and on the right the dried exoskeletons.

At the end of the drying (See Figure 2), the exoskeletons were weighed before grinding and then they must be pulverized. Normally the dried samples have a weight of 10.86g ± 0.70.
A high-quality powder can be obtained crush them with a mortar and subsequently using electric coffee grinders. The powder obtained using 10 exoskeletons are 16 gr. This allows to obtain powders that pass through the sieves of the flour of 300µ. This allows to evaluate if the obtained powder is suitable for the subsequent extraction.

Again, in this step the sample must be weighed to use only 10 g of powder for the subsequent extraction of chitosan.

Now the extraction phases begin and are divided into different spets: deproteinization, demineralization, decoloration and deacetilation (No et al., 1989; Hadi et al., 2013; Taher et al., 2019). During each phase is important to work under the hood, using appropriate masks and protections.

2.Deproteinization

For this phase it is necessary to prepare a solution of 3% NaOH (w/v) at 65-70°C (Küçükgülmez, 2018; Taher et al., 2019). It is advisable to use sodium hydrate as solid drops for greater safety in the preparation of solution at high temperature.

Suggestion: for this phase it is better to prepare the solution at 80 °C before putting the sample in it. This is because when the sample is placed in the solution the temperature will drop by a few degrees.

Subsequently the sample will be placed in this solution for 2h at 65-70 °C with ratio of 1:10, w/v.

Suggestion: A solution of 3% NaOH means 3 gr of NaOH in100ml of solution. However, the solution which must be in a 1:10 ratio with the sample (10gr), and for this reason we have to prepare only 90 ml of solution. Then it will be mixed 2.7 gr NaOH with 90 ml of DW. The sodium hydroxide solution must be prepared using a glass cylinder. First the sodium hydroxide tablets will be inserted in the cylinder and then the volume will

be brought to 90 ml with DW. The solution will then be placed on a heating plate under stirring until the temperature is reached.

Then when sodium hydrate will be dissolved it will be possible to put 10 gr of sample inside the solution. See Figure 3.

It is important to monitor the temperature constantly at least every 15 minutes to keep it at 65-70 ° C. You can do this with a normal thermometer.

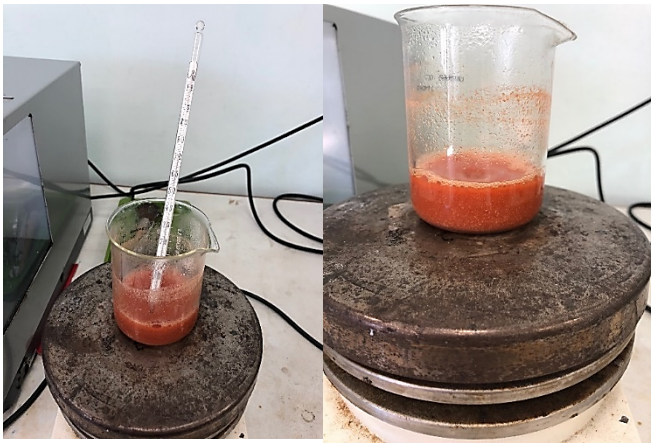


Figure 3. Phase of Deproteinization

Be careful not to reach too high temperatures. It is always advisable to work under a chemical hood with the suction turned on.

Suggestion: It is always important to recover the sample residue that is deposited in the walls of the Becker for homogeneous mixing. If working with Becker, use a magnet that involves all the bottom of this for a homogeneous mixing of the solution.

At the end of the 2h let the solution cool to room temperature for 30 minutes (Hadi et al., 2013).

Subsequently, wash the sample 3 times in distilled water by centrifuging at 800g for 10 min. With the sample quantities in this manual, it is advisable to use two 50 ml falcons to centrifuge. Tap water can be used to replace distilled water. The experimental procedure to wash the sample in detail is: transfer the sample into the falcon by balancing before using the centrifuge; centrifuge at 800g for 10 minutes; remove the supernatant and resuspend the pellet in water; centrifuge and repeat as above.

Collect the pellet and put it in the stove overnight at 60 ° C. See Figure 4.

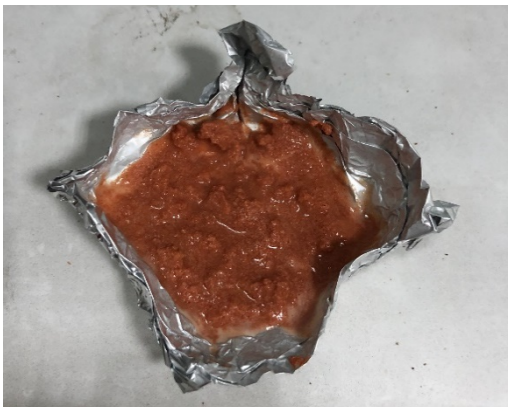


Figure 4. The pellet obtained after deproteinization step.

2.Demineralization

Remove the sample from the stove, it will be like a dry powder. See Figure 5.

Figure 5. Dried powder obtained after deproteinization.

For demineralization phase it will be necessary to prepare a solution 1N HCl. Mix the sample with this solution for 60 min at 25 °C in ratio 1:15 w/v.

It is advisable to use 37% HCl. To prepare 100 ml of this solution mix 8.30 ml of HCl with distilled water.

Given by:
37%, d: 1.19 gr / ml and M: 36.46.
1 gr equiv. = 36.46 gr
37 gr: 100 gr = 36.46 gr: X
X = 98.54 gr (containing 36.46 gr or 1 grequiv. Of HCl)
convert grams to ml
V = 98.54 / 1.19 = 82.80 ml (per L of sol.)
that adjust to 100 ml will be 8.28 ml.

However, it is necessary to prepare 140 ml of solution since the ratio with the sample must be 1:15 w/v.

So, in the end 11.60 ml of HCl with 128.4 ml of distilled water.

After mixing the solution under the hood, I insert the powder keeping the temperature at 25 ° C.

After 60 min under stirring, wash the samples 3 times in distilled water by repeating the procedures as above by centrifuging at 800 g 10 min.

At the end of the washes, put the sample in an oven at 60 °C for one day.

3.Decolorization

Remove the sample from the stove and prepare a solution of 0.315% NaOCl (v/v) to treat the sample 5 min at 25 °C in ratio 1:10 w/v.

For this phase it is possible to use bleach which has a sodium hypochlorite content of 10%.

10%:100ml= 0.315%:x
x=0.135*100/10
quantity of bleach for 100ml of final solution is 3.5 ml.

However, the ratio with the sample must be 1: 10 and therefore 90 ml of solution must be prepared.

3.5:100=x:90
x= 90*3.5/100= 3.15 ml
For 90 ml of solution we have to use 86.85ml distilled water and 3.15 ml of bleach.

Put the sample in the solution 5 min at 25 °C under a stirring hood.

Wash the samples 3 times in distilled water by repeating the above procedures by centrifuging at 800 g for 10 minutes.

Put the pellet in the stove until dry at 60 °C overnight.

After this phase the sample is lighter, light yellow. This sample is the chitin.

After removing it from the stove, it will be necessary to weigh it. See Figure 6.

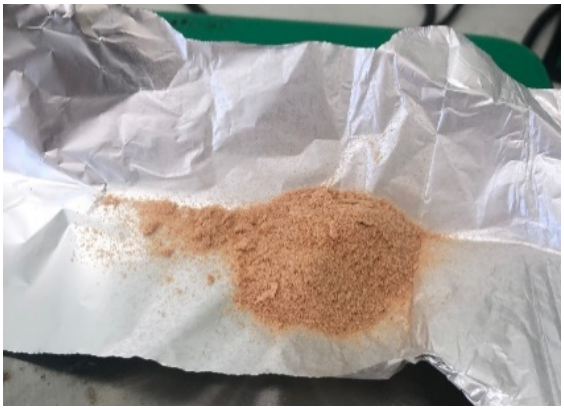


Figure 6. Powder obtained after decoloration.

It is possible to know the % of chitin obtained using the formula according to Küçükgülmez, (2018)

Yield of chitin (%) = (Extracted chitin (g)/Crayfish shells (g)) x100

In our case for example:

Yield of chitin (%) = (1,55 g / 20,11g) x100 =7.70%

Suggestion: It is possible to make this phase even more efficient by carrying out an acetone treatment and subsequently the bleach treatment as described above. The acetone treatment allows to break the strong bonds of the carotenoids to bleach and subsequently whiten with bleach. If you want to use only the bleach treatment, it is advisable to leave the sample in solution for 1h and not 5 minutes.

So, for decolorization using also acetone, the chitin residue was mixed with acetone at a solid/ solvent ratio of 1:10 (w/v) for 10 min, centrifuged at 800g for 10 minutes three times, washed with distilled water and the pellet dried at 60°C overnight. Then is necessary bleaching with 0.315% NaOCl (v/v) for 5 min as described above (Nadarajah, 2006; Fernandez-Kim, 2004).
In this case for example, to obtain a ratio of 1:10 solid/solvent with acetone a final solution of 15.5 ml was necessary (for 1,55 gr of sample). So, 13,95 ml of acetone was mixed with 1,55 of sample.

To obtain chitosan it will be necessary to deacetylate the sample with a 50% NaOH solution in a ratio of 1:15 with the sample. This treatment must be carried out at 90 °C under stirring for 5h. The exact quantities will

depend on the amount of chitin obtained. In our case we obtained 1.53 gr of chitin. The time of incubation depends on the degree of deacetylation that is desired. The solution will be constantly monitored to keep the temperature constant and recover the sample on the beaker walls to ensure homogeneous mixing. See Figure 7.

Figure 7. Deacetilation phase using NaOH 50% solution in ratio 1:15 with sample.

Work under the hood, using appropriate masks and protections.

At the end of this phase the solution will be almost completely evaporated and the pH of the sample must be neutralized using distilled water and carrying out the three washes by repeating the above procedures by centrifuging the samples at 800 g for 10 minutes.

Suggestion: Check the solution to prevent it from completely evaporating and becoming pasty. At the end of the incubation time, insert distilled water to neutralize the pH.

Then, dry the sample at 60 ° C overnight.

At the end of this phase a whitish powder will be obtained which will be chitosan (in this case 0,95 g).

It is possible to know the deacetylation of chitosan with Fourier transform infrared Spectroscopy (FT-IR) with frequency of 4000-400 cm^{-1}. Then the DD of the chitosan was calculated using the baseline of Khan et. al., (2002). The computation equation is:

$$DD = 100 - [(A_{1655} / A_{3450}) X 100 / 1.33]$$

Where A_{1655} and A_{3450} were the absorbance at 1655 cm^{-1} of the amide-I band as a measure of the N-acetyl group content and 3450 cm^{-1} of the hydroxyl band as an internal standard to correct for disc thickness. The factor 1.33, denoted the value of the ratio of A_{1655}/A_{3450} for fully N-acetylated chitosan.

Figure 8 showed the powders in several representative phases. From left to right: the flour obtained by grinding the exoskeletons, the flour after the first deproteinization treatment; chitin; chitosan.
It is evident that the final product must have a colour tending towards white.

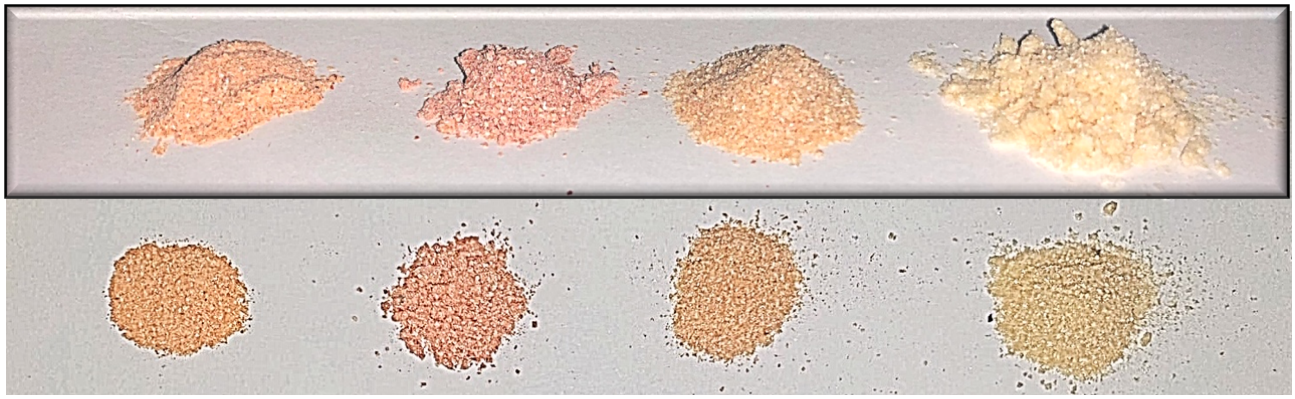


Figure 8. Powders obtained in several representative phases showed in two perspectives.

Details about Fourier transform infrared (FT-IR) Spectroscopy FT-IR JASCO 4100

1) The sample is mixed with potassium bromide in a ratio of 1:10 and pounded in a mortar (Figure 9).

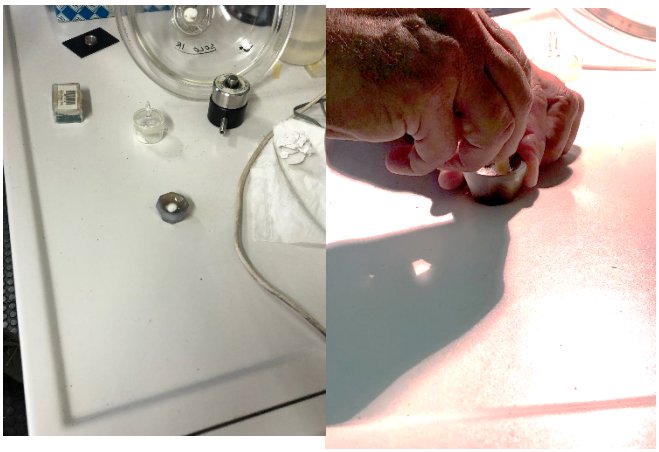


Figure 9. Sample mixed with Potassium Bromide

2) After being pounded (no granules should be felt) the sample is transferred in the instrument to make the tablets. The sample is inserted gradually by rotating the instrument to evenly distribute the sample on the bottom (Figure 10).

Figure 10. Sample transferred in the instrument to make the tablets

3) The instrument is closed and transferred into the pressure machine to make the tablets by keeping the sample at 10 atm for 10 minutes (Figure 11).

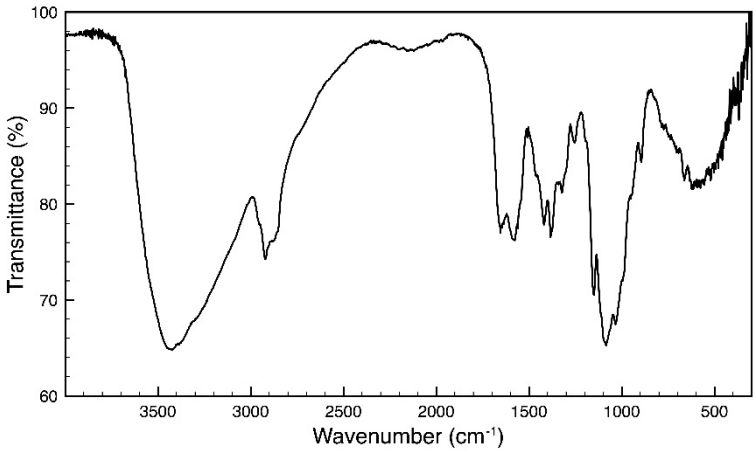
Figure 11. Pressure machine to make the tablets

3) The tablet obtained must be thin and homogeneous and inserted in the reader for the spectroscope (Figure 12 and 13).

Figure 12. Tablet obtained and inserted in the reader for the spectroscope

Figure 13. Spectroscope

4) Subsequently the graphs obtained from the analysed samples (Figure 14)



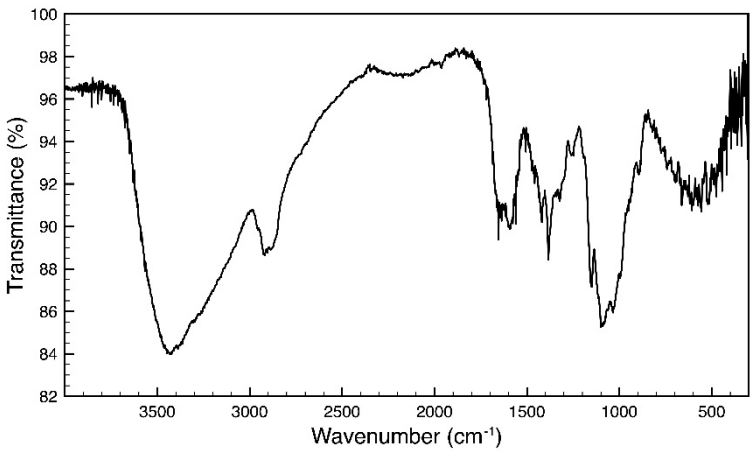


Figure 14. The two graphs regard two samples whose difference only in the decolorization phase. The top sample were treated only with bleach for 10 minutes, the bottom one with acetone and bleach (see decolorization protocol). The results are very similar, the second sample is only a little concentrated. However, the best method seems to be using bleach alone. It was found that decolorization using only bleach (without acetone) for 10 minutes gave the best result according to Taher et al., (2019)

4.Economic expense plan

On average 21 grams of heads provides 2 grams of chitosan. The costs listed below are under laboratory conditions and would obviously be different in an industrial, large-scale context.
See Table 1

Table 1. Description of economic expense plan in the lab

Materials	**Product Price €**	**Quantity of materials utilized**	**Product utilized €**
Exoskeleton	0,00	20,11g ± 3,36	0,00
HCl 1L	19,59	11,60 ml	0,22
NaOH	10,98	≃15 gr	0,16
Distilled Water 5L	2,00	2,000 ml	0,80
NaOCl 3L	3,29	3,15 ml	0,003
Acetone 500 ml	13,90	13,95 ml	0,38
Gloves	15,90	100 pz	1,28
	Consumption kWh	**Time of utilization**	
Energy 0,05 € /kWh			
Centrifuge	1650 W	≃ 2h	0,30
Coffee grinders	300W	2 minutes	0,002
Stove *	2000W	≃ 50 h	5,00
Agitator	600W	≃ 8,30 h	0,30
Chemical hood	150 W	≃ 9 h	0,067

Labour cost**	5,00 for hour	4h	20,00
Total cost			28,51

*It is possible delete this instrument because it is possible dry the sample at room temperature but it takes longer to obtain chitosan.
** If you have automatic machines that monitor the temperature, the work man only deals with the preparation of the samples and the various solutions. Otherwise the work man increases.

Regarding the table: in the "Materials" column is reported the material used for the experimental steps described in this manual. In the "Product price" column the corresponding price of the product available on the internet or in commercial market (including shipping fees). In the "Product utilized" column, the price corresponding to the quantity of product used in this manual.

However, it is worth noting that many purchases are made only once, e.g. the thermometer. Furthermore, the use of this instrument can be deleted if you have instruments that can control the temperature in automatic way.

In this economic prevision the laboratory instruments used such as beakers, thermometers and other laboratory instruments are not mentioned since they can be replaced by the industry with other suitable materials resistant to temperatures and chemical treatments.

Moreover, in the same time it is possible to produce higher quantity of chitosan using higher number of heads and more sample quantities (>10 gr). For this reason, the cost of work man can be much lower.

5. Acknowledgements
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