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Research Article

Comparative study on the composition and functional properties of chitin/chitosan extracted by new combined bioprocess

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Abstract: Bio-extraction of chitin is a greener and eco-friendly process than the conventional chemical method. Taking shrimp waste of *Pandalus borealis* recovered from plant as a model, two methods were applied for chitin extraction; a chemical and a yeast-based processes (Ch-C and Ch-B respectively).

The recovered chitins and chitosan were tested for their biochemical and functional properties. Compared to commercial chitin, both lots, have similar chitin recovery (~26%) and physiochemical properties with ash<1%, protein <10%, with degrees of acetylation (DA) varying 70-80%. However, chitosan obtained from Ch-B lot showed significantly lower molecular weight (MW) and DA (MW: 6.34 KDa, DA: 23.4%) than that found in Ch-C chitosan (MW: 11.36 KDa, DA: 33.4%). The new method proved to be efficient for the production of chitosan of good quality which allowed the elaboration of membrane.

Keywords: Shrimp shell waste; Bioprocess; Chitin; Chitosan; biofilm.

1. Introduction

Chitin is the most abundant biopolymer widely found in the shells of animals and the cell wall of microorganisms. It is an insoluble linear polymer composed of β-(1-4) linked N-acetyl-D-glucosamine units, found in crustacean shells (Kumari, 2017; Haripriya, 2018), insect exoskeletons (Waśko, 2016; Kim, 2017; Ibitoye, 2018), fungi (Ospina Álvarez, 2014; Kaya, 2015; Hassainia, 2017; Triunfo, 2022), mollusk shells (Abdulkarim, 2013;Rasti, 2017), and fish scales (Rumengan, 2013; Alabaraoye, 2018). Interest in the extraction and use of chitin is increasing worldwide as it represents a suitable functional material due to its excellent properties such as biocompatibility,non-toxicit biodegradability, and its adsorption capacity. Such biomaterial is then used in many fields including medicine, cosmetics, agriculture, textile, and wastewater treatment (Hirano, 1996; El Knidri, 2018; Kulawik, 2019; Santos 2020).Chitin-rich raw material has a naturally resilient structure resulting from the high density of hydrogen bonds between the glucosamine units

embedded in a matrix containing inorganic salts, protein, lipids and pigments. Thus, difficulties are encountered during shell processing. Currently, mineral acids and bases dominate the extraction and processing of raw material including a demineralization step followed by a deproteinization phase with heating, the order can be reversed depending on the processing objective. However, such process affects the chitin functional properties such as molecular weight; viscosity and acetylation degree (Younes, 2012; Dhanabalan,2020) beside they are harmful to human health and the environment.

Bio-extraction of chitin using enzymatic or/fermentation method is a greener and eco-friendly process, that are currently more and more required to ensure sustainability of the process at industrial scale (Kim, 2015; Pellis, 2022).However, they are more expensive and more laborious than the conventional chemical method. Therefore, the development of a simple, cheaper and effective method is needed especially in developing countries (Pohling et al., 2022).

In the present work, we rely on a cheap bio-extraction process for chitin recovery using the cheap commercial powered yeast. The aim of the study is to compare the composition and functional proprieties of chitin and chitosan from *Pandalus borealis* shell waste using both yeast-based extraction and the conventional method.

2. Materials and Methods

2.1. Sampling

Coproduct of Pandalus borealis, collected form a plant in la Goulette port (Tunisia) were transferred to the laboratory (INSTM-La Goulette center) for analysis. After removal of the remaining flesh, the shells were cleaned, dried at room temperature and then stored in hermetically sealed containers sheltered from moisture and light for further characterization and processing.

2.2. Chitin extraction

The chitin extraction was carried out in two steps: deproteinization followed by demineralization. In this work, the deproteinization was handled using two different techniques (Figure 1): (i) chemical which consists in boiling the shrimp shells in NaOH (0.75M) at 100°C for 1h (Gopalakannan et al., 2000), (ii) biological in which shrimp shells were incubated in water containing commercial powdered yeasts (0.5% w/w) for 16 hours at room temperature (24±2°C). The time and concentration of yeast used in this study were based on previous work (unpublished data).

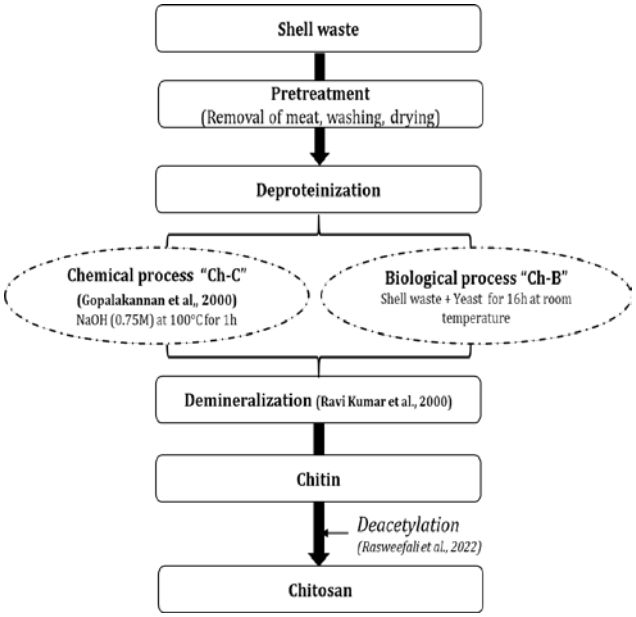

Figure 1. Process of chitin/chitosan extraction from *P.borealis* shell waste

The demineralization was carried out using Gopalakannan et al. 2000 method. Both lots of deproteinized shells were soaked in a HCl solution (1.25M) for 1 hour at room temperature, then thoroughly washed. The chitin obtained biologically (Ch-B) and chemically (Ch-C) are dried at 30°C then stored for characterization and further processing. Chitin solubilization was performed as described by Ravi-Kumar

(2000). Chitin yield (%) was calculated using the following equation (Rasweefali et al., 2022):

$$yyyeld(\%) = \frac{wwe\gamma ghtofch\gamma t\gamma n}{wwe\gamma ghtofdryyedshr\gamma mpshell} * 111111$$

2.3. Chitin deacetylation

Chitin deacetylation was carried out according to the method of Galed et al. (2005). The chitin obtained by both techniques (Ch-B and Ch-C) was incubated in a solution of NaOH (12.5M) overnight at room temperature then for 4 hours at 100°C.

2.4. Biochemical characterization

2.4.1. Ash and moisture content:

The mineral (ash) content of the samples is determined by incineration of organic biomass according to the official AOAC (1995) method.

2.4.2. *Carbohydrate content:*

Carbohydrate was determined according to Dubois's method (Dubois et al.,1956) with some modifications. Thus, to 20 mg of powdered material, 20 ml of the hydrogen chloride solution HCl (2M) are added to tubes and placed in ultrasound bath for 5 minutes. The tubes are then incubated in a water bath at 100°C for 30 min with a vortex for 1 minute every 10 minutes. After centrifugation, sulfuric acid and phenol were added to the supernatant. The carbohydrate determination is carried out using a spectrophotometer at a wavelength λ = 490 nm.

2.4.3. Lipid content:

The extraction is carried out according to the method of Folch (1957) with some modifications. 20 ml of Folch's solution (methylene chloride: methanol (2: 1) and 0.01% BHT) were added to 1g of sample and homogenized for one minute. Afterwards, 5 ml of NaCl (0.73%) is added in order to separate both phases. Cold centrifugation is then carried out at 4°C for 10 min with a rotation speed of 4000 rpm. Finally, the lower phase containing the lipids is recovered in previously weighed tubes which are then dried using a rotavapor.

2.4.4. Protein content:

The protein content was based on the determination of total nitrogen as described by Lourenço (2004) with some modifications. Each sample of dried shrimp shell was weighed (30 mg) in a screw tube, to which 2ml of sulfuric acid (H_2SO_4) were added then incubated for 60min at 100°C. After cooling, 3ml of H_2O_2were added. The tubes were again incubated for 30 min at 100°C. This step is repeated three times. The digestion product is cooled at room temperature and then injected into the Flow Injection Analysis system (FIA). The quantityof nitrogen was than determined.

The protein content was determined by multiplying nitrogen content with 6.25 as the nitrogento-protein conversion factor.

2.5. Determination of functional properties

2.5.1. *Viscosity and molecular weight*:

The viscosity was measured by an RM180 Rheomatrheometer. Molecular weight is determined according to the Mark Howink equation (Kanatt et al., 2004).

The intrinsic viscosity is expressed in dl/g and expressed by Terbojevich (1996):

Chitin $[\eta]=K*M^{\alpha}$ ($K=7.6.10^{-5}$; $\alpha= 0.95$)

Chitosan $[\eta]=K*M^{\alpha}$ ($K=1.81.10^{-3}$; $\alpha= 0.93$)

2.5.2. *Water and Oil holding capacity*:

The water and oil retention capacity were determined according to the method described by Wang and Kinsella (1976) and according to the following formulas:

OHC (%)=((Mf-Mi)/Ms)*100

WHC (%)=((Mf-Mi)/Ms)*100

With: Mi=initial weight (g), Mf=final weight (g) and Ms= weight of sample

2.5.3. Acetylation degree:

The analyzes were carried out by FTIR (Shimadzu) on potassium bromide (KBr) pellets containing chitin/chitosan to determine the degree of acetylation (DA).The studied spectra were 400-4000 cm^{-1}.For chitin, DA was determined using the following formula (Khan 2000).

$$DA(\%)=((A1655\ )/A3450)*100/1.33$$

A1655 cm^{-1}: absorbance at 1655 cm^{-1}
A3450 cm^{-1}: absorbance at 3450 cm^{-1}

Regarding chitosan, the calculation of AD is based on the method of Brugnerotto (2001) by determining the ratio of the absorption bands respectively at 1320cm^{-1} and 1420cm^{-1}.

2.6. Biofilm elaboration

0.1g of chitosan was dissolved in 20ml of acetic acid (0.35M). Dissolution was ensured by magnetic stirring for 6h. The solution was then poured into Petri dishes and dried until a pure chitosan membrane was obtained.

2.7. Statistical analysis

For all the studied parameter, data were subjected to 5% variance analyzes using SPSS 24.0 software and the Tuckey test was performed to identify differences between the means.

3. Results & Discussion

3.1. Composition and chitin recovery and properties

The proximate composition of shrimp shell waste varies with species including its physiological stage, the geographical location, and the harvesting season (Rødde et al., 2008). The proximate compositions of *P. borealis* raw material and the recovered chitin using both methods are summarized in Table 1. Published data on *P. Borealis* monthly sampled and analyzed showed mean values of protein, and mineral contents in shell of 36.5 and 22% respectively with no statistically significant difference among months for both parameters (Rødde et al., 2008). In the present study, much lower protein content (12.5%) and higher mineral levels (43.3%)were found in imported *P. borealis* shell. Such discrepancy may be related to the effect of shrimp processing that were used both onboard of the fishing vessel and in the plant including salt adding and cooking. Substantial amount of lipid (1.5%) and low level of carbohydrate (~0.5%) were found in *P. borealis*shell (Table 1). The published study did not report these data to allow comparison.

Table1: Biochemical composition of raw and chitin extracted with biological (Ch-B) and chemical (Ch-C) pathway from *P. borealis*(n=6 for each strain in each parameter; letter, symbol for statistical analysis ($p<0.05$).

	P. borealis			Chitin
	Raw	Ch-B	Ch-C	Commercial
Moisture	11,68±0,12$^{\alpha}$	5,42±0,16	5,97±0,31	5,55±0,09
Ash	48,26±0,50*	0,87±0,05	0,85±0,03	0,43±0,11**
Lipid	1,5±0,2	1,52±0,17^{A}	2,60±0,16^{B}	0,76±0,17
Protein	12,49±0,15	4,49±0,14	4,25±0,27	3,59±0,17^{a}
Carbohydrate	0,51±0,07^{X}	0,07±0,03	0,16±0,02^{Y}	0,03±0,01
Chitin %		26,32±0,47	26,04±0,63	

Chitin production from crustacean shell waste using green solvent and biotechnological means is rapidly expanding (McReynolds et al., 2022). In the present trial, the substitution of strong base and heating with commercial powdered yeast inoculated in acidified solution ensured the deproteinization of the shrimp shell (Table 1). Thus, a significant decrease of protein contents wasfound in both lots Ch-B/Ch-Cwhen compared to raw shrimp shell. The present study revealed the effective deproteinization of raw shrimp shell via yeast fermentation. However further investigation is needed to establish the biochemical pathway of this process.

The demineralization process induces the decomposition of $CaCO_3$ in the shrimp shell as follows (Al Sagheer et al., 2009):

$$HCl + CaCO_3 \rightarrow CaCl_2 + H_2O + CO_2\uparrow$$

The percentage of ash content in the final product indicates the rate of demineralization (Ben habiles et al., 2012). As mentioned, high level of mineral was found in *P. borealis* shell which may include intrinsic $CaCO_3$ but also adsorbed salt. Following the demineralization step, the ash content in both lots showed a drastic decrease in both chitins lots. Compared to commercial chitin, the values of protein and ash were higher in both extracted chitins (mean values: 4.4 vs 3.6%, and 0.8 vs 04% respectively). The higher protein content could be related to a difference in analytical method used as difference is not important though was statistically significant.

Moisture showed a significant decrease (Table 1) following shell treatment, and values were similar in all chitin lots including the commercial chitin.

It is worth noting that according to the protein and mineral contents, the obtained chitins are of good quality as their levels are < 10 and 1% respectively (Noh, 1995; No, 2000)

Crustacean shells may contain chitin up to ~ 30% of its volume (Srinivasan et al., 2018). In this study, the chitin recovery found for *P. borealis*(Table 1) using both biological and chemical deproteinization was similar with a mean value of 26.2 %. These values are within the range of reported values for chitin recovery (Pakizeh et al., 2021).

3.2. Functional proprieties of chitin

The Degree of acetylation (DA) influences the solubility of chitin and its derivatives, the interactions between the chains, their flexibility/conformation and consequently their fields of application (Kumirska et al., 2009). In this study, the raw shells of *P. borealis* has a DA of 91.26 ± 0.62 % (figure 2). Statistical analysis showed a significant decrease (69.3±0.7%) in both extracted chitins.

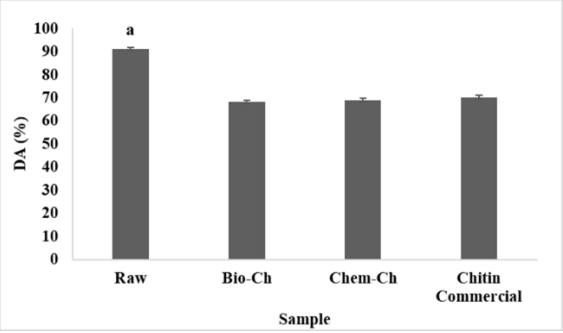

Figure 2. Degree of acetylation in raw, chitin from chemical (Chem-Ch) and biological (Bio-Ch) pathway from *P. borealis*(n=6 for each strain in each parameter; letter, symbol for statistical analysis ($p<0.05$)

Considering the others studied parameters we may conclude that yeast-based method was efficient and can substitute the conventional process.

The water holding capacity (WHC) and the oil holding capacity (OHC) are quality parameters of extreme importance in the food industry (Mohan et al., 2020). For instance, the sensory attributes as well as the processing of a product are driven by the fixation of water on the flesh (Luo et al., 2019. Chitin extracted with chemical pathway from *P. borealis* has a high WHC and OHC (Table 2).

Such values are higher than value reported for chitin from insect (Mohan et al., 2020) and carb (Luo et al., 2019). Bio-extracted chitin showed significantly lower WHC/OHC than Ch-Ch lot which may offer a different application in the industry.

Table 2. Functional properties of chitin from chemical (Ch-C) and biological (Ch-B) from *P. borealis*, (n=6 for each strain in each parameter; letter, symbol for statistical analysis ($p<0.05$)

	P. borealis		Chitin Commercial
	Ch-B	Ch-C	
WHC(%)	576,53±15,95*	747,07±27,71	761,49±47,41
OHC(%)	450,72±20,60	753,31±31,13[a]	339,23 ±58,9
MW(KDa)	403,33±17,98	434,32±31,27	241,7±6,86[A]
Viscosity (dl)	16,427±0,54	17,35± 1,08	

WHC: water holding capacity, OHC: oil holding capacity; MW: molecular weight

In general, the water holding capacity (WHC) and the oil holding capacity (OHC) of a product, vary with the amount of salt-forming groups, the degree of crystallinity, deproteinization and demineralization processes (Kumari et al., 2017).

Generally, it is recognized that the viscosity increases with the molecular weight (MW) of the molecule (No et al., 2000). In this trial, both CH-C and CH-B chitins had similar MW values (Table 2) which are in the range of reported data for shrimp chitin (Rasweefali et al., 2022).

3.3. Chitosan properties and application

Chitosan is recovered following the deacetylated of chitin with concentrated NaOH or KOH (Aye, 2004; El Knidri, 2018). The chitin deacetylation is affected by several factors which are alkali strength, alkali concentration, reaction time, and temperature (Luo, 2018; Setiati, 2021).

In this study, the same process was applied for chitosan extraction. However, a higher deacetylation occurred in the CH-B chitin which is reflected by a lower value of DA that was similar to commercial chitosan (Table 3).

Table3: Functional proprieties of chitosan extracted with biological (Chs-B) and chemical (Chs-C) pathway from*P. borealis* (n=6 in each case in each parameter; letter, symbol for statistical analysis ($p<0.05$).

	P. borealis		Chitosan
	Chs-B	Chs-C	(Commercial)
Viscosity (dl)	6.21 ± 0.17a	9.84±0.54b	8.64 ± 0.16c
Mw (KDa)	6.34 ± 0.31β	11.36±0.91	9.04 ± 0.34λ
DA (%)	23.36 ± 0.67	33.45±0.31**	23.78 ± 0.87

MW: molecular weight; DA: degree of acetylation

Considering the viscosity and MW, Chs-B showed significantly lower values than that of Chs-C. This suggest that chitosan (Chs-B)may offer a better functionality for application as material with high MW and viscosity are harder to process (Rasweefali et al., 2022).

Because of its antimicrobial and antioxidant properties (Kulawik, 2019; Triunfo, 2022), chitosan was used as a constituent in biofilm for smart packaging (Silva-Pereira, 2015;Roy, 2022) as it has poor mechanical properties (Fathima et al., 2018).

In this work, membranes were produced (Figure 3)as an application to lock in the use of the cascade process.

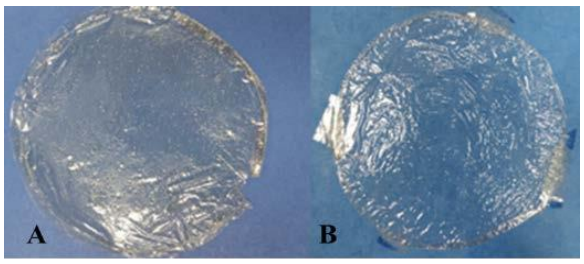

Figure 3. Membranes elaborated with pure chitosan from *P. borealis: **A** (Chs-C: 30ml) and **B** (Chs-B:20ml) dried at 70°C for 24h.*

It is worth noting that the membrane was made with pure chitosan without the addition of any agent. A clear visual difference was noticed between both membranes. The Chs-C (Fig. 3 A) was more fragile at removal from the plate.
The time and temperature of drying as well as the physical properties of the solution allowing the membrane elaboration are the parameters to be optimized in further study, followed by a characterization of the final product.

4. Conclusion

The new method proved to be efficient for the production of a good quality chitosan which allowed the elaboration of membrane. The important step of deproteinization is biological, it is less expensive, less energetic and less polluting. Besides, the byproducts of deproteinization step can be valorized for its richness in protein. This process scheme which allows the use of each component of the chain integrates perfectly the circular economy and can applied easily in processing plant as start-up.

5. Author Contributions

Ben Sadok B. and Sadok S. designed the experiment; Ben Sadok conducted the practical work**,** investigation, methodology, writing-original. Sadok S. supervision writing-review and editing. All authors have read and agreed to the published version of the manuscript."

Conflicts of Interest: "The authors declare no conflict of interest."

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