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Straightforward Extraction and Selective Bioconversion of High Purity Chitin from *Bombyx eri* Larva: Toward an Integrated Insect Biorefinery

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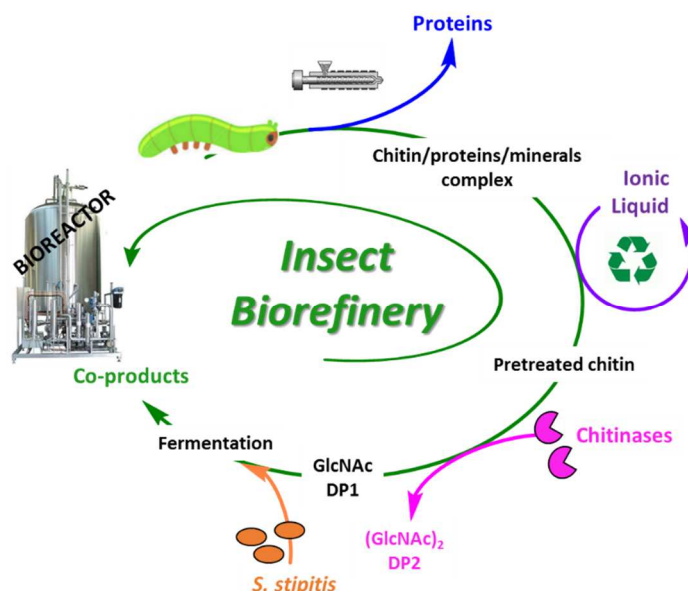
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ABSTRACT

Chitins of different purity grades (45%, 89.7% and 93.3%) were efficiently extracted from *Bombyx eri* larva and fully physico-chemically characterized. Compared to commercially available and extracted α -chitin from shrimp shell, the collected data showed that insect chitins had similar characteristics in terms of crystallographic structures (α -chitin), thermal stability and degree of acetylation (>87%). The major differences lay in the crystallinity indexes (66% vs 75% for shrimp chitin) and in the morphological structures. Furthermore, low ash contents were determined for the insect chitins (1.90% vs 21.73% for shrimp chitin), making this chitin extraction and purification easier, which is highly valuable for an industrial application. Indeed, after only one step (deproteinization), the obtained chitin from *Bombyx eri* showed higher purity grade than the one extracted from shrimp shells under the same conditions. Insect chitins were then subjected to room temperature ionic liquid (RTIL) pretreatment prior to enzymatic degradation and presented a higher enzymatic digestibility compared to commercial one whatever their purity grade and would be thus a more relevant source for the selective production of *N*-acetyl-D-glucosamine (899.2 mg/g of **chitin-2 steps** vs 760 mg/g of **chitin com**). Moreover, for

the first time, the fermentescibility of chitin hydrolysates was demonstrated with *Scheffersomyces stipitis* used as ethanologenic microorganism.

GRAPHICAL ABSTRACT



KEYWORDS

Chitin, insect biorefinery, room temperature ionic liquid, enzymatic digestibility, fermentescibility.

INTRODUCTION

Insects are a valuable protein source directly available for human and animal feed and thus a very promising alternative or a complement to feed the growing world population. In that way, Food and Agriculture Organization of the United Nations and particularly European Community authorized recently the use of insects as animal biomass for food and feed (i.e. farm fish) and therefore promoted the breeding of insects as a new source of proteins (Henry, Gasco, Piccolo & Fountoulaki, 2015; van Huis, 2013). Indeed, insects are consumed and are part of the diets of at least 2 billion people with more than 1900 insect species reported and used as human food (FAO/WUR, 2012; van Huis et al, 2013) in particularly in Southeast Asia and the Pacific, sub-Saharan Africa, and Latin America (Hanboonsong, Jamjanya & Durst, 2013). From an industrial point of view, the “insect biorefinery” is not anymore at its early stage and can be divided into three business subjects: proteins, lipids, which could be used directly for the agribusiness, and the by-product chitin, which is until now poorly exploited in industry contrary to chitosan. In addition, it has been demonstrated that insects provide a very promising alternative to other livestock. They can be farmed using less resources (land, water, energy, and feed) than livestock while providing more protein and contributing less to climate change due to generating lower greenhouse gas emissions and lower levels of pollutants (ammonia, methane)

(Gahukar, 2016; van Huis, 2013, van Huis et al, 2013). Another important feature remains in the production control and homogeneity on farms compared to seasonal dependence of seafood making it possible to satisfy the traceability criterion for food/feed applications as well as medical application for insect chitin derivatives for example.

Nowadays, the market demand for chitin is more important than the supply chain mainly coming from the seafood waste. It is important to underline that chitin from insects contains lower amount of calcium carbonate (<6%) compared to crustaceans (30-50%), and as a consequence, it can be more easily extracted resulting in a more environmentally friendly process. In addition, higher degrees of polymerization (DP) of the polymer are preserved with different and sometimes improved mechanical, gelling, biological and flexibility properties (Díaz-Rojas et al., 2006; Finke, 2007). In the last five years, a number of publications have been dedicated to the extraction of chitin as well as the subsequent transformations into bioproducts or biomaterials from different species of insects and different parts of their body (Kaya et al., 2014, 2015a, b, 2017; Liu et al., 2012; Marei et al., 2016; Rumpold & Schlüter, 2013; Sajomsang & Gonil, 2010).

Despite excellent properties such as biocompatibility, biodegradability, adsorption and chelating ability (Rinaudo, 2006; Younes & Rinaudo, 2015), chitin has been underutilized mainly due to the cost of the extraction process and the poor solubility in most organic and aqueous solvents. However, chitin can be converted into *N*-acetyl-D-glucosamine (DP₁) or *N,N'*-diacetylchitobiose (DP₂), providing a very promising application of chitin for mono- or chitooligosaccharides (COS) production. In addition to their interesting antitumoral and antimicrobial properties, *N*-acetyl-D-glucosamine and *N,N'*-diacetylchitobiose are also considered as promising platform molecules for osteoarthritis treatment, drug development and for the production of bioactive oligomers (Cao et al., 2016; Wang et al., 2008). It is worth to point out that *N*-acetyl-D-glucosamine can also be used as a carbon source by few fermentative microorganisms (Álvarez-Añorve, Calcagno & Plumbbridge, 2005; Evvyernie et al., 2001; Inokuma, Takano & Hoshino, 2013; Kumar, Jamaluddin, Natarajan, Kaur & Datta, 2000). Recently, we have demonstrated the proof of concept to produce selectively DP₁ and DP₂ with very competitive yields from commercial shrimp chitin in sequential or simultaneous strategies, including room temperature ionic liquid (RTIL) and commercial chitinases (Husson et al., 2017). Some RTILs are also considered as alternative solvents to extract chitin fibers (Qin, Lu, Sun & Rogers, 2010; Kato, Yamamoto & Kadokawa, 2012; Shamshina, Barber, Gurau, Griggs & Rogers, 2016;) or for chitin extraction to facilitate chemical and physical modifications, to form hydrogel/film or reversible sorbent for CO₂ (Aspras, Kamińska, Karzyński, Kawka & Jaworska, 2016; Jaworska, Kozlecki & Gorak, 2012; Jaworska & Gorak, 2016; Silva, Duarte, Carvalho, Mano & Reis, 2011; Silva, Mano & Reis, 2017; Shen et al., 2016; Wang, Zhu, Wang, Huang & Wang, 2010; Wu, Sasaki, Irie, & Sakurai, 2008; Xie, Zhang & Li, 2006).

In the first part of this study, we focused on the chitin extraction with different purity degree from an original insect chitin source, the *Bombyx eri*, as described in the Chart 1, used intensively in India for

the production of silk and for nutrition as the moth is an edible insect. Moreover, the protein content of *Bombyx eri* larva (unpublished data) clearly indicated that amino acid compositions are suitable for food/feed applications with >96% digestibility following the Boisen method. Because of the apparent homogenous larva cuticle, we believed that this insect is a suitable candidate for chitin extraction (Chaudhari et al., 2011; Kaya et al., 2014, 2015a, b, 2017; Liu et al., 2012; Marei et al., 2016; Rumpold & Schlüter, 2013; Sajomsang & Gonil, 2010; Vincent & Wegst, 2004). The chemical composition together with the morphological structure were compared to the commercially available chitin and to the extracted chitins from shrimp under the same conditions with the aim to highlight the particularities of chitin from the insect source. In order to have a complete comparison of this renewable bioresource with those of previous work, we continued with the study on the RTIL pretreatment of insect chitins before their enzymatic degradation. Finally, the chito-oligosaccharide hydrolysates generated were used as fermentation substrates by the yeast strain *Scheffersomyces stipitis* to illustrate the feasibility of ethanol production.

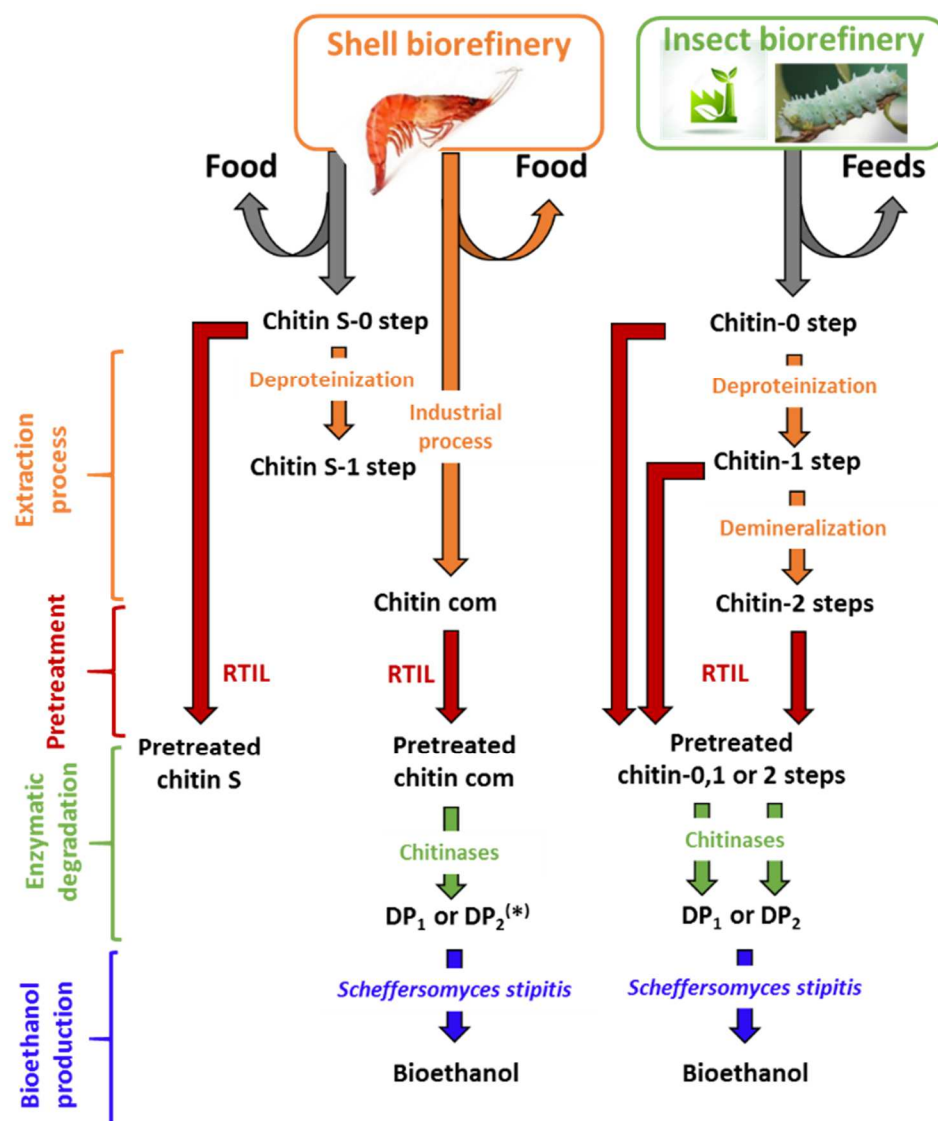


Chart 1 Extracted chitins from *Bombyx eri* larva and shrimp shells sources (*Husson et al., 2017).

2. MATERIALS AND METHODS

2.1 Chitin sources

Two animal sources have been used for this study: (a) a commercially available chitin from shrimp shells supplied by Sigma–Aldrich (Steinheim, Germany) so-called **chitin com**, or shrimp shells from the local market of Amiens (France) so called **chitin S**; (b) extracted chitins from *Bombyx eri* larva supplied by LepidUP company so-called **chitin** (Amiens, France).

2.2 Chemicals, enzymes and strain

Mineral acids and bases were used in their highest concentration commercially available, from Sigma–Aldrich (Steinheim, Germany). Kjeldahl catalyst (Cu) (9% CuSO₄·5 H₂O) was supplied by Applichem panreac ITW reagents. Tashiro indicator was made before use by mixing a solution of red methyl and green bromocresol with boric acid from Carlo Erba reagent. Chitinase from *Trichoderma viride* and chitinase from *Streptomyces griseus* were supplied by Sigma–Aldrich (Steinheim, Germany) with specific activity ≥ 600 U/g solid and ≥ 200 U/g solid, respectively. One unit will liberate 1.0 mg of *N*-acetyl-D-glucosamine from chitin per hour at pH 6.0 at 25 °C in a 2 hours assay. 1-ethyl-3-methylimidazolium acetate ([C₂mim][OAc], > 98%) was purchased from Solvionic SA (Verniolle, France). *N*-acetylglucosamine purchased from Sigma–Aldrich (Germany) and *N,N'*-diacetylchitobiose from Elicityl, (France). The strain used in this study was *Scheffersomyces stipitis* CLIB 187 from the Institut Pasteur collection.

2.3 Instrumental analysis

Scanning electron microscopy (SEM) was used to examine the microstructure of untreated and pretreated chitins. A small amount of dried samples (powder or flakes) was placed on double-sided carbon tape adhered to aluminum SEM stubs. They were then coated with a 20-nm layer of gold and examined using a Quanta 200 FEG scanning electron microscope (FEI Co., USA). The micrographs were obtained with a secondary electron detector at an accelerating voltage of 5 or 2 kV and saved as TIFF files. Infrared spectra were obtained by using a Fourier-transform infrared (FT-IR) spectrometer (IRaffinity-1S, Shimadzu) and ATR method with a germanium prism (MIRacle 10, Shimadzu). X-Ray Powder Diffraction patterns (XRPD) were acquired using a Bruker D4 Endeavor diffractometer equipped with a Cu anti-cathode (K α radiation, operating at 40 kV–40 mA). Patterns were collected in the 2 θ range of 10–60° with a step size of 0.03° and using a low background silicon holder (Bruker AXS, C79298A3244B261). The experimental conditions used for the measurement did not allow us to

observe correctly the band bellow 10°. Crystallinity index (CrI) were calculated from the XDR diffractograms using the amorphous subtraction region method (Ioelevich, 2014). Solid-state CP-MAS ¹³C NMR spectra were obtained at 125 MHz using a 500 MHz Bruker Avance spectrometer. The spinning rate was 5 kHz with 2 ms contact times and 5 s delay between scans (2048 scans). DAs were calculated from the intensity of N-acetyl carbon atom divided by the summation intensities of carbon atoms of the D-glucopyranosyl ring (Heux, Brugnerotto, Desbrière, Versali, & Rinaudo, 2000; Duarte, Ferreira, Marvao & Rocha, 2002; Kasaai, 2009). TGA was recorded on a Netzsch STA 449C Jupiter thermal analyser instrument equipped with a differential analysis microbalance coupled with a QMS 403 Aëolos mass spectrometer with a stainless steel capillary and a Secondary Electron Multiplier (SEV) detector (Channeltron). The counting time for the mass spectrometer was 20 ms per (m/z) value (scanning width: m/z = 10-150 amu) with a resting time of 1 s. The samples (approximately 20 mg of each compound) were heated in an alumina crucible under argon, by equilibrating at 25 °C, and following a ramp at 5 K/min up to 800 °C and an isotherm under air atmosphere for 30 minutes (Flow rate: 50mL/min). Kjeldahl digestion was made in a Büchi digestion unit K-435 and the distillation with a Büchi distillation Unit B-324. Liquid chromatography high performance Dionex Ultimate 3000 (Thermo scientific, Sunnyvale, CA, USA) consisting of an automatic sample changer, a pump and a detector type refractometer (RefractoMax 520), the column was a HyperRez XP Carbohydrate H+ (300 x 7.7 mm) equipped with a pre-column, kept at 75 °C. The isocratic mode is used, with ultra-pure water as mobile phase; flow rate was 0.6 ml/min. The retention time of *N*-acetylglucosamine was 14.6 min (± 0.31%) and *N,N'*-diacetylchitobiose was 12.0 min (± 0.18%). Quantification was based on calibration curves established using standard *N*-acetylglucosamine and *N, N'*-diacetylchitobiose.

2.4 Extraction of chitin from insect and shrimp sources

Bombyx eri larvae were sacrificed by freezing overnight. The insects were then boiled into water for 2 min. After filtration, cooling and removal of water, the insects were passed through an extrusion apparatus to separate unpurified chitin and the viscous liquid containing mainly the proteins and the lipids. The residue was washed several times with water followed by ethanol and acetone and dried under vacuum to obtain unpurified chitin so-called **chitin-0 step**. The deproteinization step was performed on the unpurified chitin-0 step by adding a NaOH aqueous solution (1 M) and heating at 80 °C for 24 h under stirring. After cooling down the dispersion, the solid was filtered, washed thoroughly with water MQ until neutral pH followed by ethanol and acetone and dried under vacuum to give the deproteinized **chitin-1 step**. The resulting solid was then poured into an HCl aqueous solution 1 M for 35 min at 80 °C. After this time, the solid was filtered, washed with water MQ until neutral pH, ethanol and acetone and dried under vacuum to give the partially demineralized **chitin-2 steps**. **Chitin-0 step**, **chitin-1 step** and **chitin-2 steps** were obtained with a yield of 3.3%, 31.1% and 73.2% respectively. Shrimp shells from local market were separated manually by removing the meat

and the head part of shrimp. The raw shell was washed several times with water followed by ethanol and acetone and dried under vacuum to obtain unpurified chitin so-called **chitin S-0 step**. **Chitin S-1 step** was obtained in a similar way than **chitin-1 step**. **Chitin S-0 step** and **chitin S-1 step** were obtained with a yield of 51.0% and 17.1% respectively.

2.5 Nitrogen content using Kjeldahl method

Nitrogen contents of the chitin samples before and after [C₂mim][OAc]-pretreatment were determined using the Kjeldahl method adapted from the literature (Kirk, 1950). In brief, 100 mg of chitin was dissolved in 10mL of concentrated sulfuric acid in presence of Kjeldahl catalyst (9% CuSO₄.5H₂O). After heating at high temperature (>350 °C) until the solution became transparent without white smoke, the solution was cooled at room temperature. 70 mL of distilled water and 70mL of a solution of NaOH 40% (w/w) were added and distilled. The distillate was trapped in a 30 mL of Tashiro indicator solution and finally titrated with a solution of HCl 0.1 M until the solution became violet. Chitin and protein contents were obtained using the methodology described by Diaz-Rojas et al. (2006) described in the supplementary data.

2.6 RTIL-pretreatment of chitin

Chitin (2% w/v) was added to 10 mL of RTIL, and incubated in an oil bath at 110 °C with stirring for 40 min. This combination of biomass loading, temperature and incubation time was selected according literature data about RTIL-pretreatment of (ligno)cellulosic polymers (Auxenfans et al., 2012a, b, 2014). Each chitin suspension was cooled in an ice bath. The pretreated chitin was precipitated by adding 20 mL of ultra-pure water (deionized water with a resistivity of 18.3 MΩ cm, Barnsted Easy Pure RF) to the mixture (v/v) with vigorous stirring for 30 min in order to increase the polarity of the medium. After a centrifugation step (10.733 g, 20 min, 4 °C with an Allegra 64R Beckman Coulter Rotor: F0850), the supernatant containing RTIL and water was kept for future recycling. The chitin powder was collected by vacuum filtration thoroughly and washed with ultra-pure water. The resulting solid was then poured into ultra-pure water, sonicated for 5 min and collected again by vacuum filtration. This step was repeated 5 times before freeze drying the product. This regeneration step was undertaken to prevent eventually chitinase deactivation by high RTIL concentrations in the reaction media as finely described in a previous study for other hydrolases (Auxenfans, Husson, & Sarazin, 2017).

2.7 Enzymatic hydrolysis

The procedure of implementation was adapted from literature data (Auxenfans et al., 2012a, b, 2014). Chitinase-catalyzed hydrolyses of the different chitin substrates (untreated or RTIL-pretreated) were carried out in stirred flasks. In a typical hydrolysis reaction, 10 mg of chitin or pretreated chitins were added to 0.9 mL of phosphate buffer (10 mM, pH 6.0) and incubated for 30 min (40 °C; 1000 rpm). After this pre-incubation step, hydrolysis was initiated by addition of 100 µL of 1 mg/mL chitinase preparation. The final concentrations of chitin and enzyme in the reaction medium (1 mL) were 1% w/v and 0.1 mg/mL, respectively. Enzymatic reaction was stopped by incubating at 90 °C for 20 min to deactivate the enzyme. Control experiments with enzyme after thermal deactivation and without were also carried out. Supernatant was diluted in ultra-pure water and filtered (0.2 µm) prior to analysis by high performance liquid chromatography (HPLC). The conversion yields into chito-mono/oligosaccharides were expressed as mean values with standard deviations (±) in mg of chito-mono/oligosaccharides obtained per gram of chitin (mg / g chitin).

2.8 Yeast fermentation

The growth medium was the yeast-malt (YM) medium: yeast extract 3 g/L, malt extract 3 g/L, peptone 3 g/L, pH 4.8, containing no ethanologenic source (Mehmood et al., 2015). The YM medium was supplemented with GlcNAc commercial substrate at 20 g/L and sterilized at 121 °C for 20 min. For yeast fermentations on chitin hydrolysates, the YM medium components were added to the supernatant issued from enzymatic reactions in quantity necessary to reach the final YM concentrations described above, i.e. yeast extract 3 g/L, malt extract 3 g/L, peptone 3 g/L, pH 4.8, and then the whole medium was sterilized at 121 °C for 20 min. All the fermentations were conducted in Erlenmeyer flasks with cotton plugs at 30°C and 150 rpm after inoculation of a mid-log phase preculture of *S. stipitis* cells. The yeast growth was routinely followed by measuring optical density at 600 nm (OD_{600nm}) values. The biomass was determined as cell dry weight (g/L) after filtration through dried and pre-weighed membrane filter with 0.22-µm pore size (Millipore, Ireland) with subsequent washing in physiological water. The samples were freeze dried and weighted. All the experiments were realized at least twice. Substrates and ethanol concentrations were determined by high performance liquid chromatography (HPLC) Dionex Ultimate 3000 as described above. The theoretical maximum ethanol yield on GlcNAc is 0.417 g of ethanol per gram of substrate (Inokuma, Hasunuma, & Kondo, 2016; Inokuma, Matsuda, Sasaki, & Hasunuma, 2018).

3. RESULTS AND DISCUSSION

3.1 Insect chitin versus Shrimp chitin

3.1.1 Extraction process, composition and purity

Chitin is always present as a protein/CaCO₃/chitin complex in the shells of crustaceans and insects. The calcium carbonate deposits provide the rigidity (Rinaudo, 2006) on the structure. Thus, to obtain purified chitin, or in other word, to eliminate the two other major constituents (proteins and CaCO₃), deproteinization by alkali treatment and a demineralization by acidic treatment, are the two steps classically used. It is noteworthy that demineralization is generally performed using HCl (a costly process, causing environmental problems i.e. harmful effluent wastewater) and results in the degradation of the native chitin, such as depolymerization (Foster & Webber, 1961; Percot, Viton & Domard, 2003). The lower amount of CaCO₃ present into the insect exoskeleton (<6%), compared to the one of the crustaceans (30-50%), is of great importance to preserve the physicochemical properties of chitin during the extraction process (Finke, 2013; Finke, 2007; Kaya et al., 2017). Therefore, we decided to extract three types of chitin from *Bombyx eri* larva source, with different purity grades **chitin-0 step**, **-1 step** and **-2steps** respectively. The first one, so called **chitin-0 step** was obtained by separating the solid (raw chitin) and the juice (lipids and proteins) with an extruder. After washing with water, ethanol and acetone, the resulting protein/CaCO₃/chitin complex was therefore obtained. **Chitin-1 step** was then obtained by deproteinization step from the **chitin-0 step** and the corresponding **chitin-2 steps** by demineralization of the **chitin-1 step** (see material and method part). For comparison, chitins from shrimp sources were obtained under the same extraction conditions and deproteinization step to get the **chitin S-0 step** and **chitin S-1 step**, respectively (Chart 1).

We first determined the composition of our extracted chitins and so their purity. Indeed, the nitrogen contents of the commercial and extracted chitins were measured and calculated using the Kjeldahl method (Kirk, 1950) (Table 1). As expected, the nitrogen contents of the extracted chitins from insect source decreased consistently after deproteinization step, from 10.30 to 6.03%, thereby confirming the effectiveness of the alkali treatment. After acid treatment, no change was noticed (6.03%). By comparing the values obtained for the **chitin S-0 step** and the **chitin S-1 step**, 9.23 and 2.91% respectively, same conclusions could be drawn.

Using the methodology described by Diaz-Rojas et al. (2006) and assuming that crustacean and insect shells are mainly composed only of chitin, proteins, minerals, lipids and water, we can estimate the percentages of chitin and protein for each sample, Q and P respectively (Table 1) and then, the purity of our extracted chitins. The procedure and equations used for this purpose are described in the supporting information and Table S1. First, the values obtained for the **chitin-0 step** (45.8%) and the **chitin S-0 step** (49.7%) meant that the percentage of total proteins was slightly higher in the case of the shrimp source. In addition, one important information, which could be highlighted from this study, was the larger ratio of chitin in the crude insect skeleton (**chitin-0 step**, 45.0%) compared to the one of shrimp (**chitin S-0 step**, 19.3%). This was mainly due to the lower mineral content (1.90% vs 21.73%) determined by thermogravimetric analysis under air at 800 °C and described in the following part. In addition, after only one step of extraction (deproteinization step), the purity of the **chitin-1 step** was well above the one of the **chitin S-1 step** (89.7 vs 65%). The two extraction steps performed on the

insect skeleton resulted in a chitin with a slightly lower degree of purity than the commercial one (93.3% vs 98.8%). We also assumed that the deproteinization step removed some minerals, which explained the values of 0.75 and 30.31% found for the **chitin-1 step** and **chitin S-1 step** respectively. In the case of samples containing a large amount of minerals, such as the **chitin S-1 step**, the protein content found using this method was not reliable. Then, the percentage of chitin in this sample has been therefore over-evaluated since we have only considered the presence of minerals, water and lipids.

To sum up, the low mineral contents found in the extracted chitins from *Bombyx eri* larva source (< 2%), regardless to the purity grade is of great importance. Indeed, it would firstly facilitate the extraction process in terms of cost, time saving and waste water and secondly, higher degrees of polymerization (DP) of the polymer should be preserved as already mentioned previously.

3.1.2. Thermal stability and structural characterizations

Significant information concerning these complexes may be obtained by TGA and differential thermal analysis: (i) The first mass loss (<10 %) is observed below 100 °C for all samples which corresponded to the water content. (ii) By comparing the DTG_{max} (Fig. 1b, Table 1) of the commercial and extracted chitins ranging from 369 to 378 °C, we can conclude that there is no significant thermal stability difference between shrimp and insect sources. (iii) The effectiveness of the deproteinization step under the same conditions, independently from the chitin sources can be also noticed. In fact, when alkali treatment was done on insect or shrimp sources to obtain the **chitin-1 step** or the **chitin S-1 step** (Fig 1b), we observed a clear disappearance of the derivative corresponding to the proteins (from 200 °C to 250 °C). These observations were validated by the results obtained after Kjeldahl analysis (Table 1) (Kirk, 1950). (iv) The last issue of particular importance is the percentage of impurities depending on the sources. These percentages of impurities (mainly minerals) were determined by thermogravimetric analysis at 800 °C under air atmosphere and are reported in Table 1. As expected, values were notably lower for the extracted chitins from insect sources even without a demineralization step (Table 1). Under the same experimental conditions, percentages of impurities were found to be 21.7 and 30.3 % for **chitin S-0 step** and **chitin S-1 step** versus 1.90 and 0.75 % for **chitin-0** and **-1 step**, respectively.

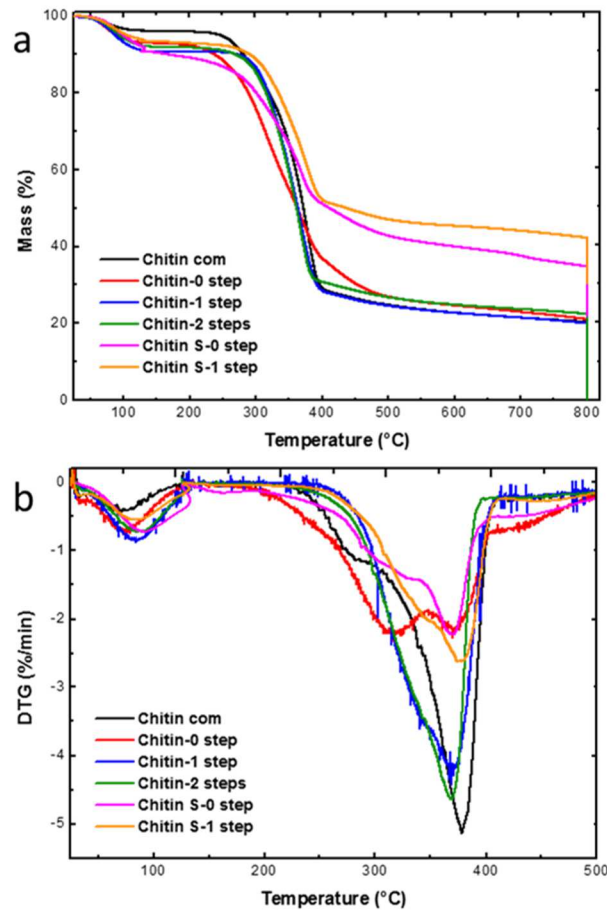


Fig. 1 Thermogravimetric (a) and differential thermal analysis (b) of the commercially available and extracted chitins from shrimp and insect sources under argon atmosphere from 50 to 800 °C following by an isotherm at 800 °C under air for 30 minutes.

Table 1.

Characterization data of the commercially available and extracted chitins from *Bombyx eri* larva and shrimp shells sources.

Chitin	CrI ^a (%)	DA ^b (%)	DTG ^c (°C)	Nitrogen content ^d (N, %)	H ₂ O ^c (%)	Ash content ^e (%)	Protein ^e (P, %)	Chitin purity ^e (Q, %)
Extracted chitins								
from <i>Bombyx eri</i> larva								
Chitin -0 step	41	ND	370	10.3	7.02	1.90	45.8	45.0
Chitin-1 step	61	ND	369	6.03	9.16	0.75	0.4	89.7
Chitin-2 steps	66	93	369	6.03	7.92	0.32	0	93.3
from shrimp shells								
Chitin S-0 step	ND	ND	370	9.23	9.23	21.73	49.7	19.3
Chitin S-1 step	42	100	375	2.91	6.53	30.31	ND	<65% ^g
Commercially available chitin from shrimp shells								
Chitin com	75 ^f	87 ^f	378	6.26	3.84	0	0	98.9

(a) calculated using XRD diffractograms and the amorphous subtraction region method; (b) evaluated by solid-state CP-MAS ¹³C NMR; (c) determined using thermogravimetric analysis under nitrogen atmosphere from 50 to 800 °C following by an isotherm at 800 °C under air for 30 minutes; (d) evaluated using the Kjeldahl method; (e) evaluated using the methodology described by Diaz-Rojas et al. (2006) ; (f) Husson et al., 2017; ND: Not determined.

ATR-FTIR is the most widely used technique for the determination of the crystallographic structure of chitin. Indeed, the amide I band of the α -chitin should have 2 peaks at around 1650 cm⁻¹ corresponding to inter- and intra-molecular hydrogen bonding (Rinaudo, 2006; Younes & Rinaudo, 2015). ATR-FTIR spectra of extracted chitins and commercial one is represented in Fig. 2 and in the SI Fig. S1-S6. The infrared spectrum of **chitin-0 step** showed barely intense band at around 1050 cm⁻¹ characteristic of the carbon skeleton of chitin compared to commercial chitin. This is due to the presence of a large amount of proteins represented by one intense amide band at around 1640 cm⁻¹ (same observation was noticed for the **chitin S-0 step**, fig. S5, SI), whereas the IR spectra of the **chitin-1** and **-2 steps** displayed 2 bands at 1630 and 1655 cm⁻¹ respectively. These two characteristic bands were also present in the IR spectra of the commercial chitin and represent the crystalline α form.

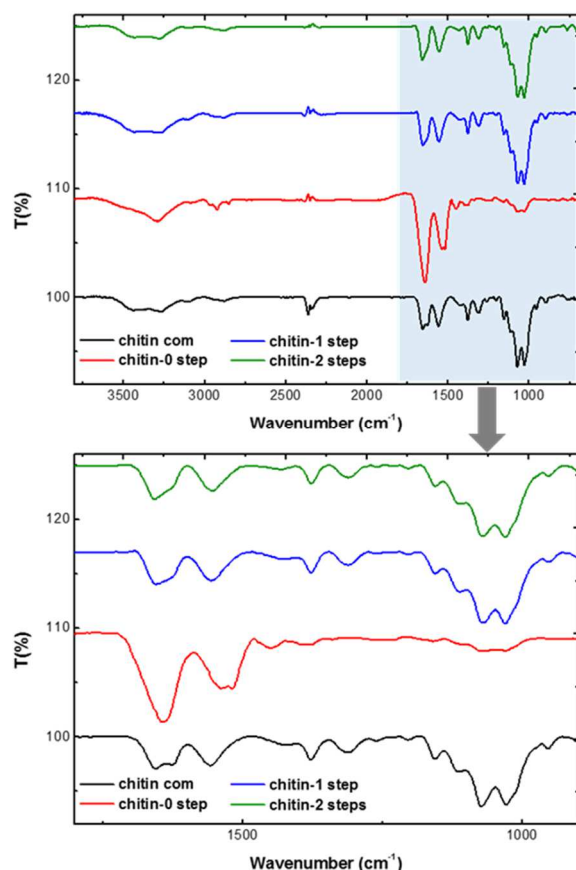


Fig. 2 Top: ATR-FTIR spectra of **chitin com** (black line), extracted **chitin-0 step** (red line), **chitin-1 step** (blue line) and **chitin-2 steps** (green line). Bottom: Zoom of the 1800-700 cm^{-1} range.

α -crystallographic structure has been also confirmed by solid state ^{13}C NMR since the C_3 and C_5 signals at around 75 and 73 ppm respectively in the extracted chitins from insect are separated which is not the case in the crystalline β -form (Fig. S10, S12 and S14 in the SI) (Cortizo, Berghoff & Alessandrini, 2008; Jang, Kong, Jeong, Lee & Nah, 2003). Solid state ^{13}C NMR is one of the most accurate to determine the degree of acetylation (DA) in chitin. In the insect cuticle, chitin is always covalently bound to catechol and sclerotin-like proteins (Majtán et al., 2007), and due to the overlapping of the methyl signal and signals coming from the catechol molecule, we were unable to calculate the DA of **chitin 0-step** and **chitin 1-step** (See the SI Fig. S10 and S12). For the extracted **chitin 2-steps** and **chitin S 1-step** the DA were found to be respectively 93 and 100% (See the SI Fig. S14 and S16) close to the 87% value of the commercial chitin (Fig. S7, in the SI).

It is well known that chitin possess crystalline and amorphous regions where the crystallinity is due to an inter- and intra-molecular hydrogen-bonding network. These crystallinity indexes (CrI) were calculated on the basis of X-ray diffractograms shown on Figure 3. **Chitin 2-steps** showed similar X-ray pattern (obtained in the 2θ range of $10\text{-}60^\circ$) to the one of commercial chitin with four characteristic

crystalline reflections at 19.3 (strongest one), 20.8, 23.3 and 26.4°. The CrI was found to be significantly higher for the **chitin com**, 75% versus 61% and 66% for the **chitin-1** and **-2 steps** respectively. Broad signals were obtained in the case of the **chitin 0-step** confirming that further purification is necessary such as the removal of proteins (same observation was noted for the **chitin S-0 step**, Fig. S18, SI) (Paul et al., 2015).

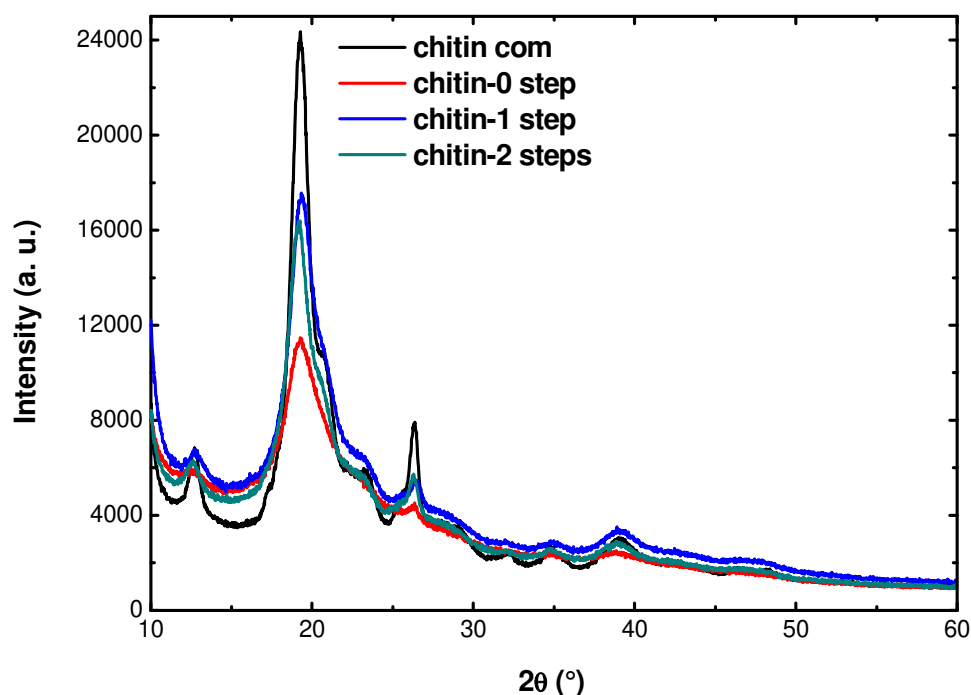


Fig. 3 Diffractograms of **chitin com** (black line), **chitin-0 step** (red line), **chitin-1 step** (blue line) and **chitin-2 steps** (green line).

In summary, in our study which are supported by ^{13}C CP-MAS NMR, XRD, FT-IR and TGA measurements we can conclude that extracted chitin from insect present the same crystalline allomorph (α -form) but lower crystallinity index (61% for the **chitin-1step** vs 75% for the commercially available chitin). Indeed, since strong acidic hydrolysis can be employed to remove amorphous regions and isolate crystalline regions (Pereira, Muniz, & Hsieh, 2014), we assume that this difference in crystallinity indexes is related to higher DP for the **chitin-1 step**. This could also explain the slightly lower DTG_{max} (369% for the **chitin-1step** vs 378% for the commercially available chitin) since crystalline regions are thermodynamically more stable than amorphous regions.

Finally, SEM images of extracted chitins from insect source are represented in Fig. 4. The morphology of the extracted **chitin S-0** or **-1 step** from shrimp (see SEM images Fig. S23 and S24, SI) showed nanofibers and pores together in agreement with the literature (El Knidri, 2017; Ifuku, Shervani &

Saimoto, 2013). The extracted chitins from insect, however, presented a smooth surface morphology where pores were not detected (Fig. 4, A-C).

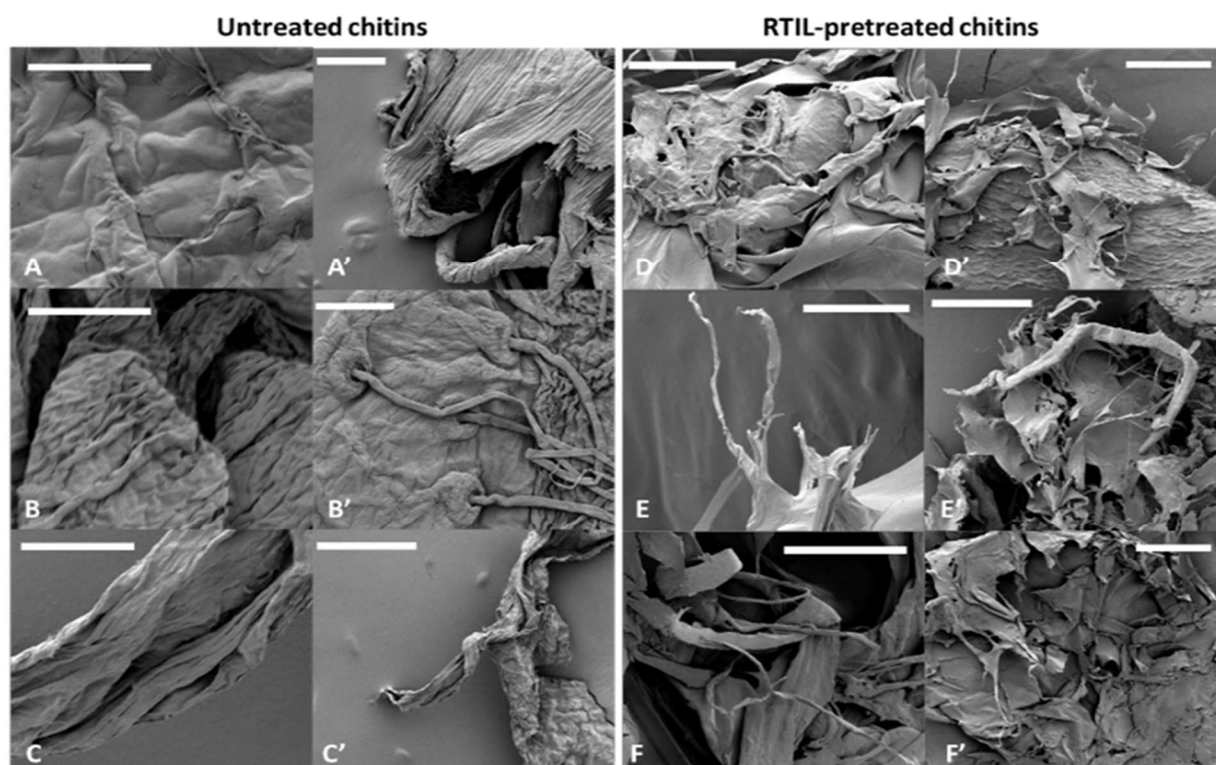


Fig. 4 SEM images of **chitin-0 step** (A and A') and **[C₂mim][OAc]-pretreated chitin-0 step** (D and D'); **chitin-1 step** (B and B') and **[C₂mim][OAc]-pretreated chitin-1 step** (E and E'); and **chitin-2 steps** (C and C') and **[C₂mim][OAc]-pretreated chitin-2 steps** (F and F'). Pretreatment: 40 minutes at 110°C. Bars represent 20 μ m (A-F) and 100 μ m (A'-F').

3.2 Ionic Liquid Pretreatment of chitins from *Bombyx eri* larva and effects on the morphological structure and the composition

Chitin is a polymer even more difficult to dissolve than cellulose in most solvents. Pretreatment consisting in solubilizing chitin into ionic liquids can overcome this weak solubility and hence increase the chitin process ability by creating new materials (sponges, films, microspheres, and aerogels) or to enhance efficiency of subsequent saccharification process. From 2002 with the work proposed on cellulose by Swatoski et al. RTILs have been intensively used as potential solvents for some polysaccharides, vegetal biomasses (lignocellulosic biomass) (van Osch et al., 2017; Yoo & Ragauskas, 2017; Zhang, Hu, & Lee, 2017) and more recently animal biomass (Husson et al., 2017; Silva, Mano & Reis, 2017, Shen et al., 2016). Keeping in mind large-scale applications, the

ecotoxicity as well as biodegradability are key criteria in selecting the IL for this study. It is well-known that 1-ethyl-3-methylimidazolium acetate [C₂mim][OAc] is considered as reasonably non-toxic and non-corrosive compared to 1-butyl-3-methylimidazolium chloride or 1-ethyl-3-methylimidazolium chloride (Liu, Wang, Buyondo, Wang & Garver, 2012). Moreover, the feasibility of scale-up with this ionic liquid (IL) was demonstrated on lignocellulosic biomass deconstruction pointing out as well challenges still existing prior to pilot scale demonstration (Liang et al., 2018). Previous studies also reported that the dissolution of chitin in ILs depends on different factors including: (i) DA and MW: LMW chitins are more soluble in IL; (ii) the crystallinity *i.e.* β -chitin has a parallel arrangement with weak intermolecular forces leading to higher reactivity and affinity for solvent compared to α -chitin; (iii) the nature of the cation of the ILs: a short alkyl chain on the IL enhance the solubility of chitin; and (iv) the nature of the anion of the IL since chitin requires a more basic anion such as acetate due to the increased number of hydrogen bond donors and acceptors compared to cellulose (Remsing, Swatoski, Rogers, & Moyna, 2006; Wang, Zhu, Wang, Huang, & Wang 2010; Barber, Griggs, Bonner, & Rogers, 2013; Kadokawa, 2015 ; Jaworska, Górak, & Zdunek, 2017).

Therefore, extracted chitins (**chitin-0**, **-1** and **-2 steps**) were pretreated under mild conditions with [C₂mim][OAc] as previously described (Husson et al., 2017). The effect of RTIL pretreatment on chitin structures were investigated, characterized (Table 2 and SI) and correlated with the data obtained in the enzymatic hydrolysis part.

SEM observation revealed a significant effect of RTIL-pretreatment on morphology and microstructure of all the extracted chitins. Such pretreatment created delaminated structures, detached thin sheets and, occasionally, separated microfibrils, from a denser and smoother untreated chitin material (Fig. 4, A-C vs D-F).

The α -chitin structure remained for the pretreated **chitin-1** and **-2 steps** as shown in the FT-IR spectra (SI, Fig. S2 C/D) with the presence of the amine I and II bands before and after pretreatment, in agreement with our previous work (Husson et al, 2017). X-ray diffraction was also used to follow the change of chitin crystalline structure after pretreatment. For untreated and pretreated samples, the 19.3° (strongest one) and 26.4 °peak positions remains essentially the same. However, crystallinity of regenerated **chitin-1** and **-2 steps** decreased if RTIL pretreatments were applied, (Fig. 6 and Table 2). These changes in crystallinity could be caused either by disruption of the crystal structure itself or changes in particle structure (surface corrugation) connected with defects in crystal structure of particles (Jaworska & Górak, Zdunek, 2017).

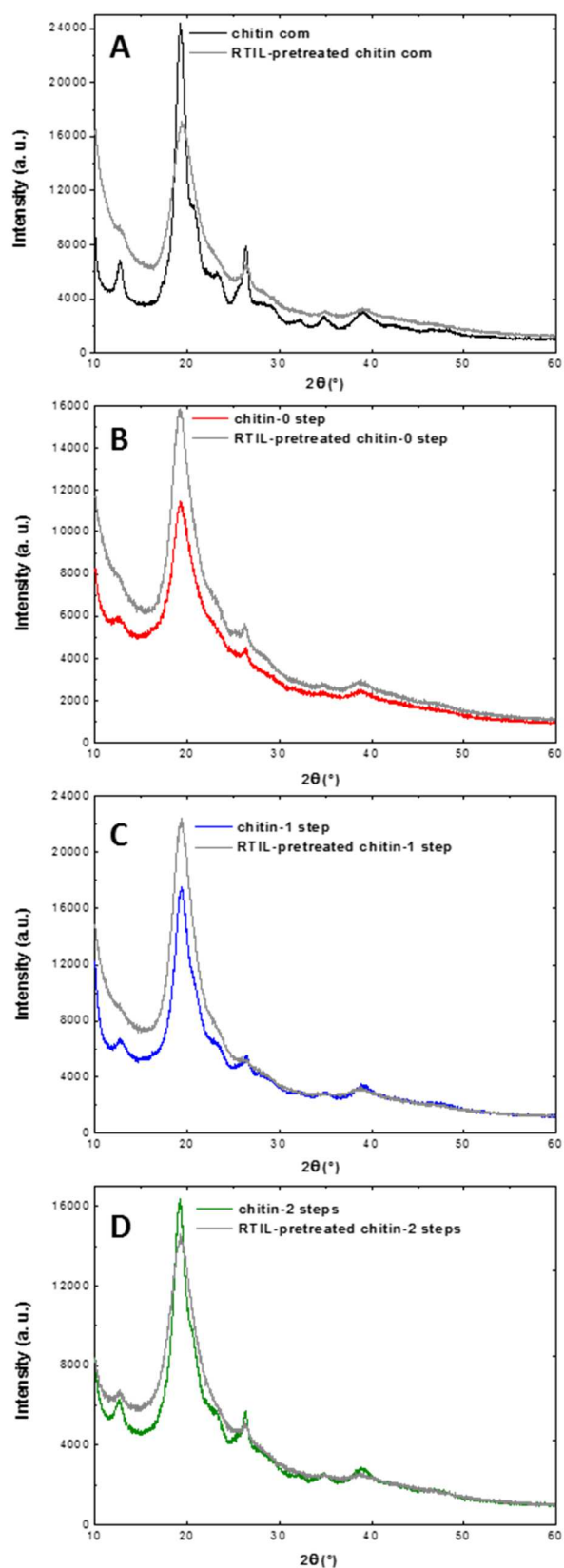


Fig. 6 Diffractograms of untreated and [C₂mim][OAc]-pretreated chitin com (A), of untreated and [C₂mim][OAc]-pretreated chitin-0 step (B), of untreated and [C₂mim][OAc]-pretreated chitin-1 step (C) and of untreated and [C₂mim][OAc]-pretreated chitin-2 steps (D).

The decrease of CrI would be consistent with the above SEM observations. In addition, DAs were not affected by the pretreatment (see SI, fig. S8, S11, S13 and S15). On the contrary, a significant decrease in the DTG_{max} after RTIL-pretreatment was observed for the **chitin com** and the extracted **chitin-1** and **2 steps** (Fig. 5 and Table 1) indicating the depolymerization of the polymer or a change from crystalline to amorphous form as observed for lignocellulosic biomass.

In addition, we assume that RTIL-pretreatment for 40 minutes led to a higher chitin purity since, in all cases, the disappearance of the band corresponding to the proteins was observed by TGA analysis (Fig. 1). This observation was confirmed with the loss of the band corresponding to the proteins at around 1640 cm^{-1} in the IR spectra especially in the case of the pretreated **chitin-0 step** (Fig. 5B). From that perspective and with the aim of determining the nitrogen content in the extracted chitins, **chitin-0**, **-1** and **-2 steps** but also **chitin S-0 step** were pretreated for 24h following the procedure described previously. After 24 h of RTIL-pretreatment, chitin solubility increased suggesting that RTIL disrupted intra-chain bonds improving solubility and the removal of some residual proteins.

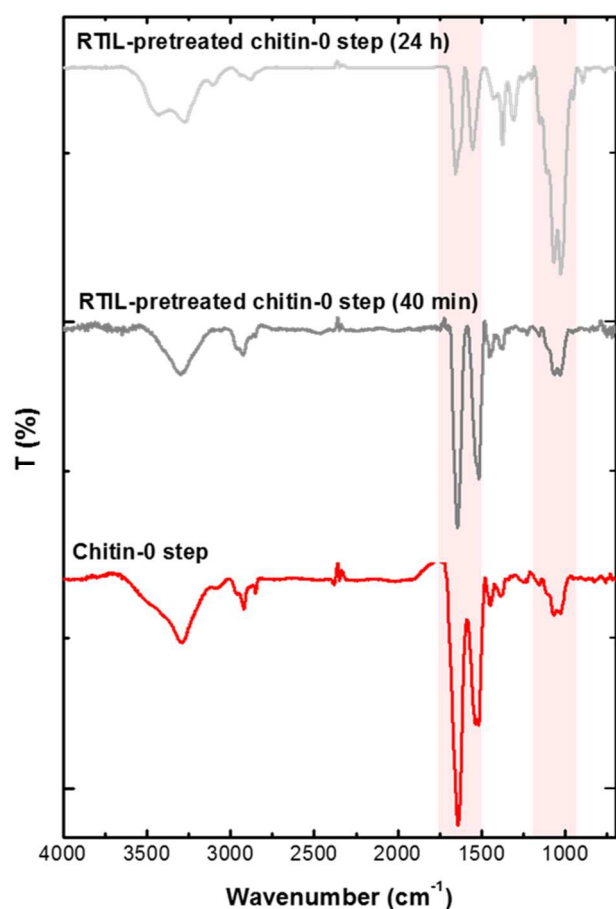


Fig. 7 ATR-FTIR spectra of untreated and $[C_2mim][OAc]$ -pretreated **chitin-0 step** for 40 minutes and 24h.

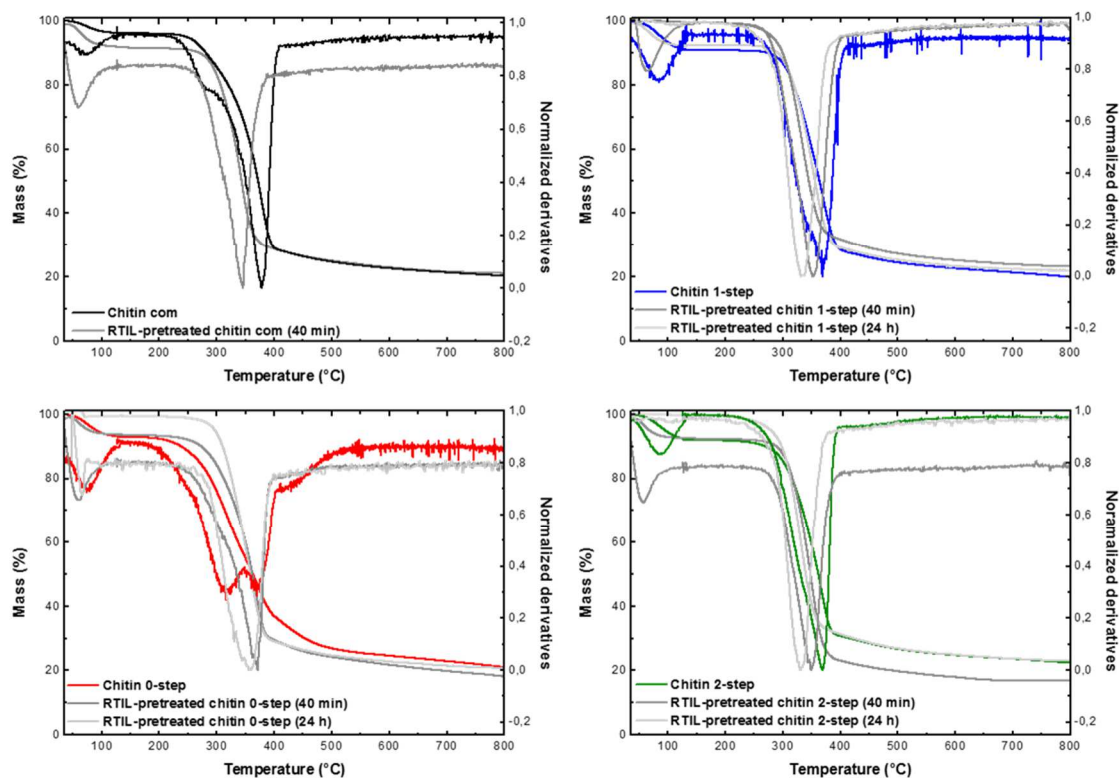


Fig. 5 Thermogravimetric and differential thermal analysis of untreated and [C₂mim][OAc]-pretreated chitin com (A), chitin-0 step (B), chitin-1 step (C) and chitin-2 steps (D), under argon atmosphere.

Decreases of the CrIs, calculated from the X-ray diffractograms, were observed in the case of the **chitin-1** and **-2 steps** after 24 h of pretreatment (Table 1, see also SI Fig. S21 and S22). Depolymerization of the polymer and/or change from crystalline to amorphous form were also confirmed with the decrease of the DTG_{max}. The DTG_{max} values obtained after 24h of pretreatment were significantly lower than those obtained after 40 minutes (Table 2, Fig. 5 and see also the SI, Fig. S29, S31-S33). These observations are consistent with the SEM micrographs, where thin layers of chitin were obtained (see the SI, Fig. S25-28).

The RTIL-pretreatment purification of the **chitin-0 step** has been also highlighted thanks to the determination of the nitrogen content (See SI, Table S1). Indeed, after 40 minutes and 24 h of RTIL-pretreatment of the extracted **chitin-0 step**, this percentage decreased from 10.3 to 9.2 and 7.5% respectively. More recently, it was noticed after the 24 h RTIL-pretreatment of the **chitin S-0 step**, the nitrogen content dropped from 9.23 to 5.76%. These data were consistent with the IR spectra of the pretreated chitins depicted in Figure 7. In agreement with literature data, the [C₂mim][OAc] was not only a good solvent to pretreat chitin but could also remove proteins as well as minerals residues (Qin, Lu, Sun, & Rogers, 2010).

Table 2. Characterization data of the untreated and RTIL-pretreated chitins.

Chitin	CrI ^a (%)	DA ^b (%)	N content ^c (%)	Ash content ^d (%)	DTG _{max} ^d (°C)
Extracted chitins from <i>Bombyx eri</i> larva					
Chitin-0 step	41	ND	10.3	1.90	370
RTIL-pretreated Chitin-0 step (40min)	38	100	9.2	0.36	371
RTIL-pretreated Chitin-0 step (24h)	63	ND	7.5	0.57	354
Chitin-1 step	61	ND	6.03	0.75	369
RTIL-pretreated Chitin-1 step (40min)	46	93	ND	<0.25	352
RTIL-pretreated Chitin-1 step (24h)	41	ND	5.85	<0.25	331
Chitin-2 steps	66	93	6.03	0.32	369
RTIL-pretreated Chitin-2 steps (40min)	42	95	ND	1.03	349
RTIL-pretreated Chitin-2 steps (24h)	34	ND	ND	ND	330
Extracted chitins from shrimp shells					
Chitin S-0 step	ND	ND	9.23	21.73	370
RTIL-pretreated Chitin S-0 steps (24h)	58	ND	5.79	14.87	358
Chitin S-1 step	42	100	2.91	30.31	375
Commercially available chitin from shrimp shells					
Chitin com	75 ^g	87 ^g	6.26	<0.25	378
Purified chitin com	82	92	6.11	ND	382
RTIL-pretreated Chitin com (40min)	50 ^g	91 ^g	ND	0.26	347

(a) calculated using **XRD** diffractograms and the amorphous subtraction region method; (b) evaluated by solid-state CP-MAS ¹³C NMR; (c) evaluated using the Kjeldahl method; (d) determined using thermogravimetric analysis under nitrogen atmosphere from 50 to 800 °C following by an isotherm at 800 °C under air for 30 minutes; (g) Husson et al., 2017; ND : Not determined.

3.3 Enzymatic hydrolysis of chitins: impact of [C₂mim][OAc]-pretreatment.

In this part, we propose to investigate the enzymatic hydrolysis of the chitins extracted from *Bombyx eri* larva (**chitin-0 step** and **chitin-2 steps**) at different purity grades and the impact of [C₂mim][OAc]-pretreatment on the performances. For this, the pathway consisting in the chitinase-catalyzed hydrolysis of [C₂mim][OAc]-pretreated chitins in an aqueous medium was selected. Indeed, we demonstrated recently the relevance of this route in the case of commercially available chitin from shrimp (**chitin com**) (Husson et al, 2017).

Hydrolysates obtained with the chitinase from *Trichoderma viride* contained mainly the *N*-acetylglucosamine monomer while chitinase from *Streptomyces griseus* led to a hydrolysate enriched in *N,N'*-diacetylchitobiose dimer (**HPLC chromatograms obtain after enzymatic hydrolysis and ¹H/¹³C NMR charaterization of *N*-acetylglucosamine and *N,N'*-diacetylchitobiose are presented into the supplementary material, Figs. 34 to 39**). This selectivity was maintained whatever the purity of chitins and the implementation of [C₂mim][OAc]-pretreatment. An opposite result was recently reported by Berton et al. (2018) who suggested that the selectivity of the chitinase from *S. griseus* could be influenced by the purity grade of chitin (Berton, Shamshina, Ostadjoo, King & Rogers, 2018). In this context, we thus suggested that the source of chitin would be also a potential influencing parameter.

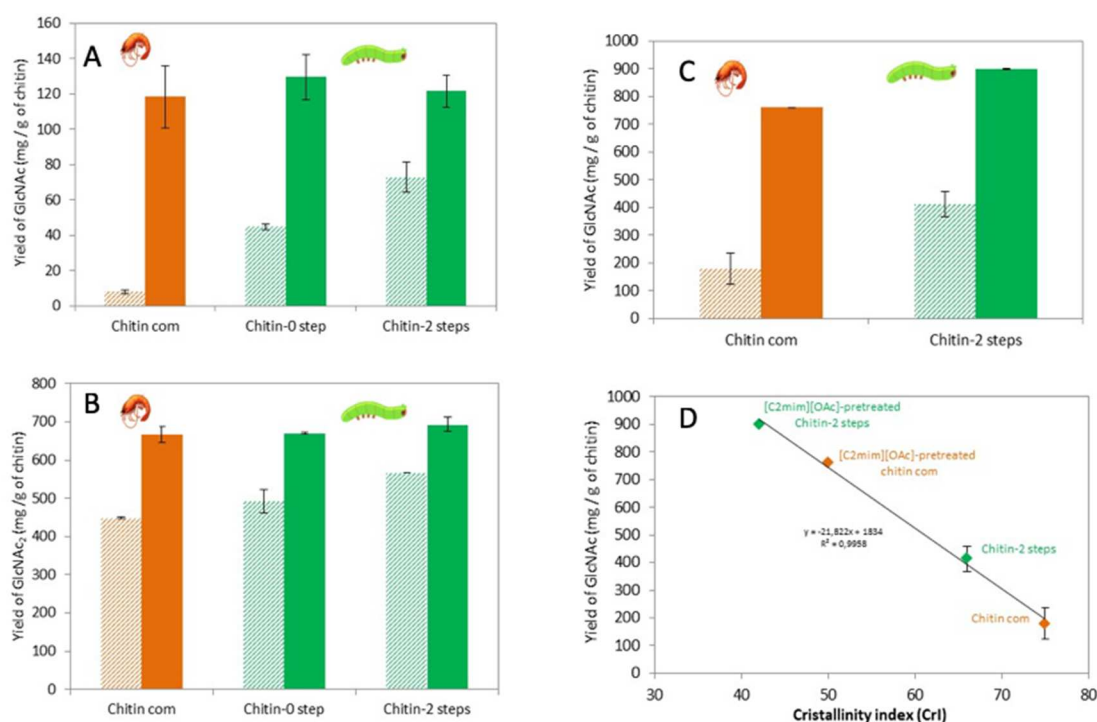


Fig. 8 (A) *N*-Acetylglucosamine or (B) *N,N'*-diacetylchitobiose yield (mg per g of chitin) at 24 h of enzymatic hydrolysis of chitins, before (stripped bars) and after (full bars) RTIL-pretreatments, catalyzed by the respective chitinase from *T. viride* (top) or *S. griseus* (bottom); (C) *N*-Acetylglucosamine yields obtained by the enzymatic hydrolysis of extracted and commercial chitins in aqueous buffer before (stripped bars) and after (full bars) [C₂mim][OAc]-pretreatment (these reactions were catalyzed by the chitinase from *S. griseus* for 24 h then supplemented with the chitinase from *T. viride* for 12 h) and (D) correlation of *N*-acetylglucosamine yields and initial CrI of chitins before enzymatic hydrolysis.

Concerning the chitinase from *T. viride*, the data reported in the Figure 8 (A) clearly showed that the untreated chitins (**chitin-0 step** and **chitin-2 steps**), regardless their purity, exhibited a higher enzymatic digestibility (44.8 and 72.9 mg/g, respectively) than **chitin com** (7.9 mg/g). After [C₂mim][OAc]-pretreatment, enzymatic hydrolysis was significantly improved and the yields obtained for the three distinct substrates were similar (123 mg/g in average). Likewise, Figure 8 (B) showed that hydrolyses of untreated chitins catalyzed by the chitinase from *S. griseus* led to yields of GlcNAc₂ superior to 400 mg/g whatever the sources and the purity grade. It could be noticed that insect chitins present again a better digestibility with this enzyme in comparison to **chitin com**. After [C₂mim][OAc]-pretreatment, an increase of the performances by a factor 1.4 was observed and very competitive yields of GlcNAc₂ can be achieved for the three chitins. Interestingly, the yield obtained for **chitin com** (667.8 mg/g) was similar to those obtained for the **chitin-0 step** (669.5 mg/g).

Then, the combination of the two chitinases in two subsequent hydrolysis steps were applied to **chitin-2 steps** and **chitin com** before and after [C₂mim][OAc]-pretreatment (Fig. 8C). The untreated **chitin-2 steps** exhibited again a higher digestibility (413.3 mg/g) than the untreated **chitin com** (179.1 mg/g). After [C₂mim][OAc]-pretreatment, the production of GlcNAc from the **chitin-2 steps** was significantly improved (by a factor 2.2) and achieved a very promising value of 899.2 mg/g of chitin (vs 760 mg/g for **chitin com**). Reporting yield of GlcNAc as a function of CrI (Fig. 8D) evidenced a linear relationship between the structural parameter and the enzymatic digestibility suggesting that the performances of enzymatic depolymerisation could be counter-balanced by the decrease of the CrI. In this way, [C₂mim][OAc]-pretreatment remains thus a promising pretreatment to disrupt the highly ordered intra- and inter-chain hydrogen bond network of chitin allowing also to efficiently produce COS by enzymatic route.

3.4 Fermentescibility of enzymatic hydrolysates.

The enzymatic hydrolysates were then tested for their ability to be valorized by microbial fermentation. The molecule of interest targeted here was the ethanol which could be obtained from the yeast *Scheffersomyces stipitis* on GlcNAc (Inokuma, Hasunuma, & Kondo, 2016; Inokuma, Matsuda, Sasaki, Hasunuma & Kondo, 2018). Thus, we experimented this strain fermentation in aerobic conditions in classical shake flasks with cotton plugs in a culture medium containing no ethanologenic substrate (Mehmood et al., 2015, 2018) supplemented either with commercial GlcNAc or with the enzymatic hydrolysates of [C₂mim][OAc]-pretreated chitins from shrimp shells or *Bombyx eri* larva. *S. stipitis* could grow and consume totally the substrates in all the conditions tested (see SI, Fig. S40). An ethanol production was observed on all the samples containing the monomer GlcNAc, whatever their origin whereas no ethanol could be detected on chitin hydrolysates rich in dimer GlcNAc₂ (see Table S2, SI).

Without optimization of the parameters, the higher yield was obtained on commercial GlcNAc, equal to 0.19 g ethanol/g GlcNAc. The *S. stipitis* fermentations on chitin hydrolysates led to ethanol yields of 0.14 g/g for shrimp chitin and 0.09 g/g for *Bombyx eri* larva chitin, corresponding to maximum theoretical yields of 32.5% and 22.3% respectively. These values are lower compared to commercial substrates (44.7%), but show undeniably the feasibility of the valorization process of chitin issued from shellfish or insect in ethanol. These results also imply that all the products used in the first steps of chitin extraction (or co-products formed simultaneously) like deproteinization, demineralization or RTIL-pretreatment were not a radical hindrance to yeast fermentation, even if a lot of work remains to accomplish in order to optimize the ethanolic production. One can suppose that the fermentation process could be realized with another fermentative microorganism in order to produce other molecules of interest with higher added value than ethanol.

4. CONCLUSIONS

Herein, we reported for the first time the chitin extraction from a new species of insect by demonstrating that *Bombyx eri* larva is a source of chitin with great potential since the skeleton contains 45% of chitin versus 19.3% for the shrimp shells. Moreover, after only one step of extraction, we obtained a chitin with the high purity grade of almost 90%, mainly because of the low mineral contents. In addition, untreated chitins extracted from *Bombyx eri* larva, exhibit a better enzymatic digestibility in comparison to the commercially available chitin from shrimp shells. After [C₂mim][OAc]-pretreatment, a very competitive yields of GlcNAc can be achieved by chitinase-catalyzed hydrolysis, suggesting that *Bombyx eri* larva would be thus a more relevant source of chitin for selective enzymatic production of COS. Finally, our fermentation results showed for the first time that *S. stipitis* produced ethanol on commercial GlcNAc in aerobic conditions, but also on enzymatic hydrolysates of chitins derived from shrimp and from the new *Bombyx eri* larva insect source after a [C₂mim][OAc]-pretreatment. This is the first report of a natural wild-type yeast strain producing ethanol on chitin derivatives, highlighting the great potentialities of this substrate. Some further optimization would nevertheless be necessary for a cost-effective production of high added value molecules in a concept of insect biorefinery. Nevertheless, we believe that chitin extracted from insects can be a complementary and competitive resource for the chitin market.

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