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Kinetic study of solid phase demineralization by weak acids in one-step enzymatic bio-refinery of shrimp cuticles

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ABSTRACT

We describe a one-step bio-refinery process for shrimp composites by-products. Its originality lies in a simple rapid (6 h) biotechnological cuticle fragmentation process that recovers all major compounds (chitins, peptides and minerals in particular calcium). The process consists of a controlled exogenous enzymatic proteolysis in a food-grade acidic medium allowing chitin purification (solid phase), and recovery of peptides and minerals (liquid phase). At a pH of between 3.5 and 4, protease activity is effective, and peptides are preserved. Solid phase demineralization kinetics were followed for phosphoric, hydrochloric, acetic, formic and citric acids with pK_a ranging from 2.1 to 4.76. Formic acid met the initial aim of (i) 99% of demineralization yield and (ii) 95% deproteinization yield at a pH close to 3.5 and a molar ratio of 1.5. The proposed one-step process is proven to be efficient. To formalize the necessary elements for the future optimization of the process, two models to predict shell demineralization kinetics were studied, one based on simplified physical considerations and a second empirical one. The first model did not accurately describe the kinetics for times exceeding 30 min, the empirical one performed adequately.

1. Introduction

World consumption of crustaceans, especially shrimps, has soared in the last decades. Annual world production of shrimps in 2012 was estimated by the F.A.O. [1] at more than 6 Mt with *Litopenaeus vannamei* accounting for some 60% of the total. In recent decades the consumption of peeled shrimps has experienced exceptional growth, generating a large amount of waste, about 50% by weight of whole shrimps [2], meat recovery accounting for only about 50% (w/w). Shrimp cuticle (shell) represents almost 30% (w/w) of this waste. Cuticle dry matter is 25–30%. Shrimp cuticle is a natural composite with a complex spatial organization in which proteins are interconnected with chitin macromolecules and with minerals [3–5]. Proteins amount to about 40% of the shrimp cuticle dry weight [6], minerals and chitin representing the other major fractions [7]. The economic balance of this process can be improved by fully utilizing chitin (around 28% of the dry matter). Peptides and pigments such as astaxanthin [8–16] could be also developed. Chitin and its derivatives, especially chitosan, its deacetylated derivative, have specific properties (highly basic polysaccharides, polyelectrolyte behavior, ability to form films, solubility in various media, metal chelation, etc). These properties make them useful for many applications [17–20] in various sectors such as food [21], cosmetics and biomaterials [22]. Chitin is a natural polysaccharide synthesized by a great number of living organisms [23]: the amount produced annually throughout the world shows it to be the most abundant renewable natural resource after cellulose [24].

Several techniques to purify chitin from various crustaceans have been described [17,18,25,26]. In all cases, the compounds associated with chitin have to be separated. A solid-phase demineralization step for chitin purification is needed, possibly together with the elimination of other components. This step is usually performed in an acid medium. Kinetic reactions associated with mineral solubilization are described in the literature mainly for pure powders and strong acids [27], but reactions for more complex, structured solid matrices are under researched [28]. Influences related to the type of acid and the reaction environment are not yet adequately formalized. Yet prediction of kinetics is essential for good process optimization [29]. Generally, demineralization is followed by water rinsing, and in a second step by deproteinization

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with strong bases [30]. All these extraction steps may damage chitin through uncontrolled depolymerization and deacetylation [31].

Proteins or protein derivatives (peptides) are strongly denatured at low pH [32,33]. For this reason the classical chemical purification of chitin cannot be envisaged as a bio-refinery process.

As an alternative efforts have been made towards reducing chemical treatments in more eco-friendly processes [34–36]. Enzymatic hydrolysis of crustacean shells was used in an alternative bio-refinery approach in order to recover simultaneously from edible proteins, peptides and chitin from whole biomass. Enzymatic reactions linked to the deproteinization step can be induced by bacterial fermentation [25,35,37–39] or by exogenous proteolytic enzyme treatment [11,15,36]. A growing number of studies show that the extraction of shell compounds can be accelerated by the joint use of sonication [40–43].

All these bio-refinery treatments involve either substantial consumption of water in a two-step bio-refinery process in the case of exogenous enzymatic hydrolysis, or too long process times, resulting generally in insufficient chitin purification rates and a need for further fermentation treatment. These constraints form a major obstacle to industrial development.

We have proposed a novel method based on exogenous enzymatic proteolysis in acid conditions in a one-step demineralization and deproteinization process taking less than 6 h [2,6,44]. Here, we call "one-step process" the simultaneous implementation of two different reactions, namely demineralization and deproteinization, in the same chemical environment, enabling proteolysis in acidic media, and without intermediate rinsing. In previous work we investigated a pepsin/phosphoric-acid mixture (40 °C, pH after 6 h = 1.9, ratio of 10% of enzyme versus dry matter, 2.7 M acid solution, 50 mL of reacting volume) in solution under excess acid conditions [2]. Deproteinization yields exceeding 95%, combined with demineralization yields exceeding 99% were obtained in 6 h, indicating a satisfactory degree of purity of chitin (>92%) for an enzymatic process. Intermediate filtrations and separations are avoided. Reaction times and water volumes are reduced.

Reducing the acidity of the medium should preserve the solubilized protein components. Neutralization in the aqueous phase of the step could even be omitted if the pH was close to 3.5–4.0. This would both reduce the environmental impact of the proposed bio-refining process, and increase its profitability. Demineralization and deproteinization performance obtained within 6 h in the study of Le Roux et al. [2,6,44] were taken as targets to be reached or exceeded during our study, while seeking to raise the pH of the solution. According to [2,44], the initial size of the solid pieces of crushed shells affects demineralization and deproteinization kinetics: these processes are faster as size is smaller, but the rate increase is negligible below 1 mm. We analyzed conditions and parameters (acid and water quantities, type of acid, temperature, pH, and solution corresponding to the enzyme pH activity range), needed to collect information to optimize the new alternative solution. Appropriate information for choosing an acid in a non-exhaustive list of five acids was collected, and the best experimental conditions defined for a solid particle size range between 0.5 and 1 mm. To corroborate the decoupling observed by [2] between the kinetics of demineralization and the kinetics of protein removal, an analysis in the presence and absence of the enzyme, in conditions favorable to deproteinization, is proposed for two other acids.

Our earlier work [2] focused on the use of phosphoric acid, which we compared with hydrochloric acid typically used in conventional chemical methods. We then studied the behavior of the process when a weak acid conventionally used in food was added: acetic acid and formic acid. Another organic acid with a low pK_a was also studied: citric acid. We compared the kinetics of demineralization for five acids under conditions close to stoichiometric. The effect of molar ratio and reaction volume were then analyzed for acetic acid and formic acid.

Based on the reaction with acetic acid, we formalized the kinetics obtained. First, the models were based on knowledge reported in the literature (main reaction stoichiometric equations and simplified knowledge model described by Fusi et al. [27]). Secondly, the results from the knowledge models were compared with an empirical model proposed by the authors. This formalization aimed to offer a suitable tool for later digital process optimization. We made a parametric adaptation of the empirical model using all the results obtained in this work.

2. Material and methods

2.1. Raw materials and sample preparation

Shrimps (*L. vannamei*), farmed in Ecuador (Omarsa sa) and frozen HOSO (head on shell on) in packs of 2.5 kg, were used as raw material. Mean weight per shrimp was 13.3 ± 0.8 g (70–80 shrimps per kilogram). The shrimps were thawed (for 24 h at ambient temperature) and manually peeled. The cuticles were washed, dried for 24 h in a controlled oven at 30 °C (Thirode), with an air velocity around the cuticles of 0.5 m s^{-1}, and relative humidity 50%, ground to powder for 1 min in a blender (model 80106 Waring), sized using a sieve shaker (AS200 Basic, Retsch) and stored in 200 ± 20 g lots at −20 °C under vacuum. The ground size distribution range was between 0.5–1 mm. Identical assays made at different times yielded similar results, indicating that content was not damaged in time.

The composition of the 0.5–1 mm ground cuticle [2], after mild drying, was: 11.2 ± 2.0 % water, 23.3 ± 3.6% minerals (∼1.17 g for 5 g of raw material), 35.6 ± 2.0% proteins (∼1.78 g for 5 g of raw material), 25.0 ± 3.0% chitin (∼1.25 g for 5 g of raw material), and ∼5% others (fatty acids, glycosides, pigments).

All the acids were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). The enzyme acid stable protease (ASP; 3000 SAPU/g; conditions:. 30–60 ° C and pH [2.0–4.0], Bio-Cat Inc.) was used for most of the experiments, with an enzyme/dry raw material ratio of 1:10.

Solution pH was measured with an analytical pHmeter (CyberScan pH/Ion 510, Eutech Instruments) and with an electrolytic pH electrode (InLab Expert Pro, Mettler Toledo).

2.2. Characterization of materials

Ash content was measured gravimetrically after incinerating the sample in an aluminum container (12 h at 550 °C [45]) to quantify minerals. Filtered solutions were first freeze-dried (Pilote, Cryotec) before applying the gravimetric method. According to Raabe et al. [3], calcium carbonate is the main form of the minerals from shrimp shells (>90% of total inorganic matter). Verification was carried out by scanning electron microscopy coupled with energy dispersive X-ray (EVO-40 EP, SEM- Zeiss).

Dry weight was measured by weighing samples after drying in an oven at 105 °C for 24 h [45].

Percentages of residual minerals (RM) were given by:

$$\mathrm{RM} = 100 \times \frac{W_{\mathrm{ash}}}{W_0} \quad (1)$$

With W_{ash} the ash quantity remaining in residual solids found in 5 g of cuticle and W_0 the initial ash quantity found in 5 g of cuticle before acid treatment (W_0 = 1.17 g). Demineralization yields (DY) were:

$$\mathrm{DY} = 100 - \mathrm{RM} \quad (2)$$

The protein content is obtained by summing the concentrations of the various amino acids present. This method is the most con-