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Optimization of proteins and minerals removal from shrimp shells to produce highly acetylated chitin

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ABSTRACT

Chitin and derivatives used for biomedical or pharmaceutical applications require a high level of purity and quality that are difficult to achieve. In this study, we propose to optimize the extraction of chitin in order to obtain pure product keeping a structure as close as possible to the native form. Thus, demineralization step was firstly optimized using response surface methodology. In the optimized conditions predicted by the model, the obtained chitin has an acetylation degree (DA) and a demineralization degree (DM) equal to 99% and 100%, respectively.

Then, different microbial and fish crude alkaline proteases were tested for their efficiency in deproteinization. Crude alkaline proteases giving the highest deproteinization degrees (DP), *Bacillus mojavensis* A21 and *Scorpaena scrofa*, were selected for chitin extraction. The obtained DP was 88 ± 2% and 83 ± 1%, respectively.

At the end, effect of the use of mixed enzymatic treatment with the two selected crude enzymes and the order of demineralization/deproteinization steps were tested. The results demonstrated that two separated steps in enzymatic treatments realized on demineralized sample give the best DP (96%) preserving the DA (99%).

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1. Introduction

Chitin, a homopolymer of N-acetyl-D-glucosamine residues linked by β-1,4 bonds, is the most abundant renewable natural resource after cellulose [1]. The main sources of raw material for the production of chitin are cuticles of various crustaceans, principally crabs and shrimps. Chitin in crustaceous is closely associated with proteins, inorganic compounds, lipids and pigments. They all have to be quantitatively removed to achieve the high purity of chitins necessary for biomedical and pharmaceutical applications [2].

Conventionally, to extract chitin from crustacean shells, chemicals processing for demineralization and deproteinization have been applied. Raw materials are treated with dilute hydrochloric acid at room temperature to remove mineral salts, particularly calcium carbonate, and with strong bases to remove proteins [2]. However, the use of these chemicals may cause a partial deacetylation of chitin and hydrolysis of the polymer, resulting in final variability in their physicochemical properties and inconsistent physiological properties [3].

An alternative approach to the harsh chemical deproteinization is the use of proteolytic enzymes to remove proteins. Bustos and Healy [4] demonstrated that chitin obtained by the deproteinization of shrimp shell waste with various proteolytic microorganisms had higher MW compared to chemically prepared shellfish chitin.

Enzymatic deproteinization requires the use of proteases which are mainly derived from animal, plant, and microbial sources. Among the various proteases, bacterial proteases are the most used. Marine animals possess the same functional classes of enzymes as other living organisms, which enable them to carry out virtually the same metabolic activities. In several of the major fish producing countries, the by-products of seafood harvesting comprise about 50% of the entire harvest [5]. These materials are largely underutilized and discarded as waste. However, this abundant material also includes the enzyme-rich digestive organs which may be recovered and used for a range of commercial applications. Furthermore, fishes are poikilothermic, so their survival in cold waters required adaptation of their enzyme activities to low temperatures. Enzymes from cold adapted fish species often showed higher activities at low

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temperatures than their counterparts from warm-blooded animals [6–8]. High activity of fish enzymes at low temperatures may be interesting for the deproteinization process. For that purpose, in the present paper, deproteinization efficiency was compared using microbial and fish alkaline crude proteases. Then, experimental conditions were optimized in order to achieve high deproteinization rate.

Concerning the demineralization process, although many methods can be found in the literature for the removal of minerals, effects on the molecular weight (MW) and acetylation degree (DA) cannot be avoided with any of these extraction processes [9]. Hydrochloric acid is the most commonly used chemical in the demineralization of crustacean waste. Many HCl concentrations, incubation times and temperatures were used, but no standard process was established. It was reported that the use of high acid concentration provokes the reduction of solution viscosity of the obtained chitin [10]. Lusena and Rose [11] reported the effect of high temperature and the extended incubation on the final physicochemical properties of chitin. Only temperatures equal or higher to the ambient temperature (25 °C) were tested. It was reported that the use of high temperatures accelerates the demineralization reaction by promoting the penetration of the solvent in the matrix of chitin [12]. Furthermore, according to Marquis-Duval [13], the penetration of the solvent in the matrix of chitin strongly depends on the size of particles.

All the demineralization processes reported in literature are based on continuous treatments using relatively high concentrations and prolonged incubations going from 1 to 10 M and from 1 to 72 h [14–16]. Lower concentrations (<1 M) were rarely applied but required long incubations (24 h and even many days). Recently, a new approach by the use of successive baths of lower HCl concentration (0.55 M) was introduced by Tolaimate et al. [17] and proves good efficacy on the reduction of minerals.

The different authors pointed out the importance of optimization of the extraction process parameters (time, temperature, acid concentration, numbers of baths, granulometry) in order to minimize chitin degradation and bring the impurity levels down to the satisfactory level for specific applications.

In the present paper, firstly, chemical demineralization was optimized using the response surface methodology. For this, a fractional factorial design was applied to study the effect of experimental factors on the DM and on the DA of the resulting chitin. Five factors were studied: HCl concentration, temperature, reaction time, number of successive baths and the particles size.

Secondly, enzymatic deproteinization was studied. Six microbial and nine fish alkaline crude proteases were compared for their efficiency on shrimp waste deproteinization. Then, the use of combine crude proteases for the deproteinization, and the influence of the order of chitin extraction steps (demineralization/deproteinization) were tested.

2. Materials and methods

2.1. Raw material

The shrimp (*Metapenaeus monoceros*) shells were obtained in fresh condition from the shrimp processing plant located in Sfax, Tunisia. Shell wastes were washed thoroughly with tap water, desiccated at room temperature and milled. Then, they were sieved and kept at room temperature until used.

2.2. Chemical demineralization

2.2.1. Reactor design for demineralization

For each experiment, 10 g of shrimp waste homogenate was placed in a reactor, in which HCl solution was added at a ratio of 1:10 (*w/v*) under heating and stirring. The contents of the reactors were stirred at 30 rpm. Selected reaction temperature was maintained constant (at ±0.5 °C) and controlled by a Fluke data logger system (model 2640A, Everett, WA). For reactions carried out at 4 °C, an ice bath was placed around the reactor.

At the end of the reaction, the product is filtered through four layers of gauze with the aid of a vacuum pump and washed to neutrality with deionized water and then dried at room temperature.

Table 1
Variables and experimental domains used for the optimization of the demineralization step.

Variables	Level (−)	Level (+)
X_1: HCl concentration (mol/L)	0.5	2
X_2: Temperature (°C)	4	50
X_3: Reaction time (min)	30	90
X_4: Number of successive baths	1	3
X_5: Granulometry (mm)	2–3	3–4

2.2.2. Statistical design for demineralization

A two-level fractional factorial design 2^{5-2}, with five factors and two variation levels, was used for the study of the demineralization reaction, being analyzed by the ANOVA test. The studied factors were: HCl concentration (X_1), temperature (X_2), reaction time (X_3), number of successive baths (X_4) and granulometry (X_5). The variation levels in the codified form (X_n), based on literature data [10–17], are presented in Table 1.

In a fractional factorial design, we assume that interaction effects between three or more factors are negligible and thus useful information on the main effects and second order interactions may be obtained by running only a fraction of the complete factorial design. In a 2^{5-2} fractional factorial design, five variables (factors) are studied each of them at two levels. The effects of two variables (extra variables) are confounded with high order interactions between the three other variables (basic variables).

In this work, the 2^{5-2} fractional factorial design is obtained by writing down the complete 2^3 factorial as the basic design (with the three variables X_1, X_2 and X_3) and then equating factors X_4 and X_5 to the $X_1X_2X_3$ and X_1X_2 interactions, respectively.

The last conditions allow us to identify the independent generators (1) and the corresponding complete defining relation (2) which is the set of all columns that correspond to the identity column I:

$$I = X_1X_2X_3X_4 = X_1X_2X_5 \quad (1)$$

$$I = X_1X_2X_3X_4 = X_1X_2X_5 = X_3X_4X_5 \quad (2)$$

From this set of generators, it is possible to develop the complete alias structure for this design as indicated in the following:

$$I_0 = b_0 + b_{1234} + b_{125} + b_{345} \qquad I_4 = b_4 + b_{35} + b_{123} + b_{1245}$$

$$I_1 = b_1 + b_{25} + b_{234} + b_{1345} \qquad I_5 = b_5 + b_{12} + b_{34} + b_{12345}$$

$$I_2 = b_2 + b_{15} + b_{134} + b_{2345} \qquad I_6 = b_{13} + b_{24} + b_{235} + b_{145}$$

$$I_3 = b_3 + b_{45} + b_{124} + b_{1235} \qquad I_7 = b_{23} + b_{14} + b_{135} + b_{245}$$

It is to be noticed that three and four-factor interactions are considered as negligible.

To simplify the calculations, coded variables X_j are used instead of natural variables U_j. The range of variation of each U_j ($U_{j,low}$ and $U_{j,high}$) is transformed into a variation of X_j between −1 and +1 [18–20] according to the following equation:

$$X_j = \frac{(U_j - U_{0j})}{\Delta U_j} \quad (3)$$

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