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Successive inoculation of *Lactobacillus brevis* and *Rhizopus oligosporus* on shrimp wastes for recovery of chitin and added-value products

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ABSTRACT

The deproteinization is a major constraint for chitin recovery from crustacean wastes. In this work, high protein removal was achieved through the combination of two-stage solid state culture by *Lactobacillus brevis* and *Rhizopus oligosporus*. The first stage was carried out employing a heterofermentative starter (*L. brevis*) using glucose as sole carbon source. For the second stage, the inoculum levels of the food grade fungi were 10^3, 10^5 or 10^7 spores/ml. The highest deproteinization (96% ± 0.43%) and demineralization (66.45 ± 2.14%) were achieved with 10^7 spores/ml. The protein removal was attributed to acid and neutral proteolytic activities, which highest activity during culture was determined at pH 5. Lactic acid was the main organic acid produced along with acetic, succinic and oxalic acids. The released protein hydrolysates (120.56 mg protein/g) displayed M_W range between 25×10^3 and 11×10^3 Da. The highest concentration of astaxanthin extracted from liquid was 8.78 μg/g. Protein hydrolysates and astaxanthin showed radical scavenging activity with I_{C50} of 1.13 ± 0.03 mg/g and 2.02 ± 0.01 μg/g, respectively. The purified chitin presented molecular weight of 1313×10^3 Da, preserving high crystalline index (I_{CR} of 87.5%) and 93.67% degree of acetylation.

1. Introduction

Chitin (CH), poly(β(1 → 4))-2-acetamido-2-deoxy-D-glucan, and its deacetylat ed derivative, chitosan, found applications in drug delivery, water treatment, tissue engineering, textiles or in cosmetics, among others [1]. Lactic acid fermentations (LAF) proved successful for CH recovery, as well as protein hydrolysates and pigments from crustacean wastes. The pH and acid production have been clearly established as crucial factors in LAF for inhibition of pathogen, spoilage microorganisms and demineralization (DM) of these wastes [2–6]. DM of shrimp (*Litopenaeus vannamei)* wastes (SW) is attained by solubilization of minerals by the produced organic acid from lactobacilli, whereas deproteinization (DP) is ascribed to digestive and microbial proteases produced during fermentation. According to Pacheco et al. [4] maximum protein removal was determined at the optimum lactic acid bacteria (LAB) growth, which evidenced the key role of LAB proteases for chitin extraction during LAF of SW employing the homofermentative *Lactobacillus plantarum* as the starter. The contribution of the lactobacilli to the proteolysis has been extensively studied in milk products, which is initiated by the cell envelope proteinase that releases peptides which in turn are degraded by peptidases [7]. The role of proteolytic enzymes from LAB was corroborated by Flores-Albino et al. [6] in a *Lactobacillus plantarum* fermentation of sterile medium based on crab wastes and molasses for simultaneous chitin and lactic acid production. Therein, the proteolytic activity was more efficient at neutral pH where the highest DP was achieved.

In another related work, the presence of active digestive enzymes in shrimp hepatopancreas exerted a clear contribution in DP when SW was fermented at low temperature (15 °C) far from the optimum temperature for LAB growth [4]. Noteworthy, most of the digestive proteolytic enzymes are active at neutral and alkaline pH; they have been identified as trypsin, cathepsin, collagenase chymotrypsin, elastase, and carboxypeptidases [8–10]. The contribution of these endogenous proteases is well established because the highest DP was determined during the initial 24 h when pH of fermentation varied from 7 to 6 [4]. In spite of digestive acid, proteases have been also reported [8], the digestive enzyme composition might vary with the diet, body size and farm conditions and low pH is a constraint for DP of SW. This work is first to study, to the best of our knowledge, the use of successive inoculation of *Lactobacillus brevis* and the GRAS fungi *Rhizopus oligos-*

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porus to produce acid proteases for total DP of SW for CH recovery and other added value products. This work also demonstrates that the heterolactic *Lactobacillus brevis* offers the advantage of the production of wide variety of chemical compounds such as lactic acid, ethanol, acetic acid, and CO_2 as products of glucose fermentation [11].

2. Materials and methods

2.1. Materials

2.1.1. Shrimp wastes and chitin

SW was obtained from Mexico City's central seafood market and consisted of head and thorax. Waste was minced through a 0.3 cm sieve using a meat mincer (Torrey Mexico) and stored at −20 °C. Chitin and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were purchased to Sigma-Aldrich (USA).

2.1.2. Microorganisms and inoculum preparation

Lactobacillus brevis (isolated from SW) was maintained in Man Rogosa Sharpe (MRS) agar at 8 °C. The inoculum was prepared by deposition from the bacterial slants in MRS broth incubated at 30 °C for 24 h. Later on, 1 ml of MRS broth was employed for inoculation of 100 ml and incubated at 30 °C up to a count of 10^9 colony forming units (CFU) per ml.

Rhizopus oligosporus was cultured on potato dextrose agar (PDA) slants and kept 8 °C until needed. The spore suspension was obtained by mechanical agitation with a solution of 0.1% (v/v) of tween 80 up to a conidia count of 10^9 spores/ml.

2.2. Lactic acid fermentation of shrimp wastes

Solid state cultures (SSC), in which the substrate bed was left static, unmixed and without force aeration were carried out in two stages: (i) Flask level in a Koji style system and (ii) Laboratory-scale reactor level. The first stage allowed fungal inoculum size determination and the second, the standardization of the procedures of organic acids production, protein, mineral removal from the SW and the downstream processing.

2.2.1. Koji style solid state cultures

SSC for small scale was conducted with a mixture of 100 g of minced SW (*Litopenaeus* sp), glucose 10% (wt/wt) and 5% (v/wt) of *Lactobacillus brevis* inoculum incubated at 30 °C for 120 h, followed by addition of 10% (wt/wt) of glucose and inoculated with spore suspension of *Rhizopus oligosporus* and then, incubated for additional 72 h. Fungal inoculum level was varied 10^3, 10^5 and 10^7 spores/g SW. Samples were taken every 24 h and filtered (177 μm) in darkness. Filtrates were stored at −20 °C and solids were dried at 40 °C during 24 h for subsequent analyses.

2.2.2. Laboratory-scale reactor

The selected fungal inoculum level from the flask fermentation was employed in the column reactor. In a typical procedure, a mixture consisting of SW, 10% (wt/wt) glucose and 5% (v/wt) of *L. brevis* was placed in a stainless steel column reactor, as reported elsewhere [4,5]. The column reactor had 10 kg of nominal capacity and it was filled up to 8 kg of the mixture as working capacity. Reactor dimensions were 42 cm length and 20 cm in internal diameter. Reactor contents were incubated up to 192 h at 30 °C. Sample aliquots were withdrawn every 24 h for further analyses.

2.3. Analyses of samples

2.3.1. Determination of pH, organic acids, glucose, and astaxanthin

The pH of samples was measured using a potentiometer (pH 210 HANNA, Italy). Liquid samples were diluted (1:10 wt/v) and total titratable acidity (TTA) was determined by potentiometric titration with 0.1 N NaOH to a final pH 8 and expressed as mmol of lactic acid per gram [2]. Liquid samples from fermentation were diluted (1:10) twice and centrifuged at 10,000 rpm for 20 min in the first dilution for glucose and organic acids determination by HPLC. Concentrations of lactic, oxalic, acetic, succinic, acids, as well as glucose, were determined with an HPLC system Agilent 1260 infinity equipped with an autosampler/injector, Refractive Index (RI) as a detector. A column Aminex HPX-87H 300 mm × 7.8 mm was used with a mobile phase of 5 mM H_2SO_4 with a flow of 0.6 ml/min at 40 °C.

Astaxanthin (Ast) was extracted from liquid samples from fermentation by means of dilution 1:10 (wt/v) in acetone for 1 h with agitation and centrifuged at 10,000 rpm for 10 min. All samples were maintained at the darkness until analysis. Ast was measured at a wavelength of 470 nm with a column Agilent Eclipse XD 18 (C18) with a mixture of methanol/acetonitrile/ethyl acetate/water (80:10:5:5), with a flow of 1 ml/min at 25 °C.

Samples for Ast determination were diluted 1:10 (wt/v) in acetone and centrifuged at 10,000 rpm for 10 min. Later on, 1 ml of diluted supernatant was filtered through 45 mm membrane before injection. The peak identities of organic acids and *trans*-astaxanthin were confirmed by their retention times and characteristic spectra of standard chromatograms. They were quantified from their peak areas in relation to the organic acids and *trans*-astaxanthin reference standard (Sigma-Aldrich USA) [12].

2.3.2. Chemical composition analysis

Moisture and ash from solids and CH were determined using standard methods [13]. Total nitrogen contents were measured by Kjeldahl in automated equipment (Büchi, Switzerland). Corrected protein contents were calculated by the subtraction of the chitin nitrogen from the total nitrogen content and multiplied by 6.25. DM and DP Percentages were obtained using Eq. (1).

$$Y(\%) = \frac{[(X_0 \times S_0) - (X_R \times S_R)]}{(X_0 \times S_0)} \times 100 \quad (1)$$

where Y is DP% or DM% and X_0 and X_R are the protein or ash content percentages in raw and fermented samples, respectively. S_0 and S_R are raw and fermented samples weights (g), respectively [5]. Protein hydrolysate concentrations were determined as the soluble protein in the liquid fractions by Lowry-Peterson [14].

2.3.3. Physicochemical characterization

Solid fraction from SSC was treated with HCl 0.4 N (1:15 wt/v) for 1 h to remove minerals and the obtained pure CH was fully characterized. Viscosimetric average molecular weight (M_v) was conducted in an Oswald viscosimeter using *N, N*-dimethylacetamide containing lithium chloride (5% wt/v) and calculated using Mark Houwink Kuhn Sakurada (Eq. (2)).

$$[\eta] = KM_W{}^{\alpha} \quad (2)$$

where $\alpha = 0.69$ and $K = 2.4\text{x}\,10^{-4}$ l/g.

The degree of acetylation (DA) was obtained using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy in Bruker AVANCE-III 500 (Germany) spectrometer at 200 MHz in DCl/D_2O with 3-(trimethylsilyl) propionic acid as the internal reference. DA was calculated by integration of assigned signals on the NMR spectra. I_{CR} was determined by X-ray diffraction measurements in a diffractometer (Bruker D8 Advance) with an incident radiation Cu Kα and wavelength of λ = 1.5418 Å in the range of $2\theta = 4.5$ to 70 °C with steps of 0.02° according to Eq. (3) [5,15].

$$I_{CR} = \frac{I_{110} - I_{am}}{I_{110}} \times 100 \quad (3)$$

Where I_{110} is the intensity of (110) peak at around 2θ = 20° corresponding to the maximal intensity and I_{am} is that at 2θ = 16°