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Demineralization of crab shell waste by *Pseudomonas aeruginosa* F722

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Abstract

Crab shell (CS) waste samples (particle size 3–10 and 20–35 mm) were inoculated with the newly isolated *Pseudomonas aeruginosa* F722 to study the efficiency of microbial demineralization (DM) and deproteinization (DP) in the process of extracting chitin. The inoculated waste was incubated for 7 days at 25, 30 and 35 °C. Various concentrations of glucose were supplemented as carbon source. At the optimal temperature of 30 °C, DM was 92% and DP was 63% DP, whereas the pH dropped from initial pH 8.0 to 4.1. In comparative experiments with different amounts of CS waste, 5% CS waste treatment was shown to be the optimal amount for efficient DM. A positive relationship is correlated between DM and glucose concentration ($r^2 = 0.821$), whereas a negative relationship is correlated between DM and pH ($r^2 = 0.793$). DP and protease activity were little affected by different crab shell sizes.

Keywords: *Pseudomonas aeruginosa* F722; Crab shell waste; Demineralization; Deproteinization; Glucose supplement

1. Introduction

Chitin and chitosan are being developed for application in many products including functional food ingredients, medicines, pharmaceuticals, cosmetics, textile, fine chemicals for water treatment and biodegradable packaging films [1–3]. The major sources of chitin are the shells of crustaceans such as shrimp, crab and krill that are wastes from the processing of marine food products and contain chitin, protein and calcium carbonate [4–7].

To extract chitin from crustacean shells, chemical processing for demineralization (DM) and deproteinization (DP) has been applied by treatment with acid and alkali to remove calcium carbonate and proteins, respectively [3,8]. To isolate chitin from shrimp waste chemically, 4% NaOH is used for deproteinization and 4% HCl for demineralization. The traditional chemical method creates a disposal problem due to the large amounts of toxic waste that without further treatment would pollute the environment. This processing is expensive due to enforced environmental controls and disposal measures [2,6,9].

To overcome these problems, an alternative method using lactic acid fermentation has been emerged, which can to a considerable extent replace the expensive and non-environment friendly chemical process [4,10–12]. The alternative processes of DM and DP have been reported for chitin extraction mainly from shrimp biowaste using microorganisms that produce acid and proteolytic enzymes. Microorganisms studied include *Lactobacillus plantarum* [8], *Pseudomonas aeruginosa* [4], *P. maltophilia* [4], *Bacillus subtilis* [3,13], *Lactobacillus paracasei* [14], *Lecanicillium fungicola* [15] and *Penicillium chrysogenum* [16]. Fermentation of crustacean shell biowaste using selected *Lactobacillus* sp. strain as inoculum resulted in medium conditioning, supposedly by production of lactic acid and proteases [8,14]. Lactic acid produced by breakdown of glucose creates the low pH condition of ensilation that suppresses the growth of the spoilage microorganisms [14,17,18]. The calcium carbonate component in the shell fraction is solubilized by the low pH condition, leading to the formation of precipitable calcium lactate separable from the chitin fraction. Deproteinization of the biowaste occurs mainly by proteolytic enzymes produced by the added microorganisms and by proteases present endogenously in the biowaste [2,14,17].

The efficiency of fermentation using microorganisms depends on the quantity of inoculation, the glucose concentration, the initial pH and the pH during culture and the

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fermentation time [5]. This research aimed to study the effect of glucose supplement and the amount and size of the crab shell waste on the efficiency of DM and DP using *P. aeruginosa* F722, a newly isolated bacterium.

2. Materials and methods

2.1. Crab shell samples

Crab shell (CS) waste was obtained from Taehoon Chemical Co., Korea. Two kinds of dried shell powder samples (particle size 3–10 and 20–35 mm) were prepared by electric grinding. Most experiments were carried out using the large particle size (20–35 mm) sample.

2.2. Microorganism and preparation of inoculation

P. aeruginosa F722 was isolated from a soil sample of Yeosu, Korea [19]. The strain was stored in 50% glycerol as cryoprotectant at −70 °C. In order to prepare a starter culture, cells were transferred into 100 ml of sterile LB broth and incubated at 30 °C in a rotary shaker at 150 rpm for 24 h. To prepare an inoculum for fermentation, 1.0 ml of the starter culture was transferred into 100 ml of sterile LB broth (1% inoculation) and incubated with shaking (150 rpm) at 30 °C for 24 h. The cell concentration in the inoculum was approximately 1×10^8 cfu/ml.

2.3. Treatments

Crab shell waste particles in a relative amount of 5%, 10%, 20% and 30% were mixed with various amounts of the inoculum (1.0%, 5.0% and 10.0%) in a 500 ml flask containing 200 ml glucose (0%, 1.0%, 2.5%, 5.0% and 10.0%). The fermentation was carried out at 25, 30 and 35 °C with shaking (180 rpm) for 7 days. Every experiment was done in triplicate.

2.4. Dry weight, ash, pH, TTA, glucose, protein and organic acids

Dry weight was measured after drying at 80 °C for 24 h in an oven [5,18]. Ash content was determined after combustion at 500 °C for 3 h in an electric furnace [5,18]. The pH was measured with a pH meter (PHi 34; Beckman, USA). Total titratable acidity (TTA) was determined in diluted samples by titration with 0.1 M NaOH to pH 8.0 and expressed as percent lactic acid [5,18]. Glucose content was determined as reducing sugar with dinitrosalicylic acid reagent, according to the method of Miller [20,21]. Organic acid in the fermentation broth was analyzed by HPLC (LC-10AD; Shimazu, Japan) with a UV detector (210 nm), using an organic acid column (Pak C18 MG, 4.6 mm × 250 mm, Shiseido, Japan), with 0.1% H_3PO_4/CH_3CN (97.5:2.5, v/v) as the mobile phase [5]. Protein content in the shell sample was determined by the modified method of Takiguchi and Shimahara [22]. Briefly, dried material (150 mg) was added to 50 ml of 10N NaOH in a 250-ml flask. The flask was covered with aluminum foil and heated in an autoclave at 121 °C for 50 min. The reaction mixture was cooled rapidly, neutralized with 12N HCl and filtered. In a test tube, 0.25 ml of the sample solution, 2.5 ml of 0.5 M acetate buffer (pH 5.1) and 2.5 ml of ninhydrin–hydrindantin solution were added and mixed. After incubation in boiling water for 10 min, absorbance was measured at 564 nm. The protein content P was calculated from the following equation, where A_{564} represents absorbance at 564 nm and W dry sample mass:

$$P\,(\%) = 2.37\left(\frac{A_{564}}{W}\right)$$

2.5. Demineralization and deproteinization rates

Demineralization (DM, %) was calculated using the following equation, where A_O and A_R are the ash concentrations (g/g) before and after fermentation, and O and R are the mass (g) of original sample and fermented residue, respectively [8]:

$$\mathrm{DM}\,(\%) = \frac{A_O O - A_R R}{A_O O} \times 100$$

Deproteinization (DP, %) was calculated using the same equation, where P_O and P_R are the protein concentrations (g/g) before and after fermentation, and O and R are the mass (g) of original sample and fermented residue, respectively [8]:

$$\mathrm{DP}\,(\%) = \frac{P_O O - P_R R}{P_O O} \times 100$$

2.6. Protease, chitinase and chitosanase activities

For protease activity, 50 μl of culture supernatant was mixed with 450 μl of 1% azocasein in phosphate buffer (pH 7.0) and incubated for 1 h at 50 °C. The reaction was stopped by adding 250 μl of 25% trichloroacetic acid (TCA). After settling for 15 min, the precipitated protein was removed by centrifugation at 10,000 rpm for 10 min and the soluble peptide in the supernatant fraction (500 μl) was mixed with the 500 μl of 1N NaOH. The absorbance of the mixture was measured at 440 nm with bovine serum albumin (BSA, Sigma) as reference compound. One unit of protease activity was defined as the amount of protein which resulted in an increase of 0.01 absorbance unit per minute [23,24]. Chitinase and chitosanase were estimated by reducing sugar groups with swollen chitin and colloidal chitosan as the substrate, respectively [25,26]. The reaction mixture containing 900 μl of 1% swollen chitin (DD 8%) or 1% soluble chitosan (DD 99%) and 100 μl of crude enzyme (pH 5) was incubated at 37 °C for 1 h or 30 min, then stopped by addition of 200 μl of 1N NaOH. Reaction mixture was centrifuged at 10,000 rpm for 5 min and then supernatant was used to measure reducing sugar groups. One unit of chitinase and chitosanase activities was defined as the amount of enzyme which produced 1 μmol of reducing sugar per hour and per minute, respectively.

2.7. Statistical analysis

The effects of treatments were evaluated by analysis of variance (one-way ANOVA) according to the general linear model procedure of the Statistical Analysis System 9.1 [24]. Means were obtained with Tukey's Studentized range test at $p = 0.05$. Regression analysis was also undertaken to determine the closeness of relationship of either DM or DP with the measured variables glucose concentration, pH, TTA and protein in the medium, using Sigma plot version 8.0. The level of significance of each independent variable and squared multiple correlation coefficients (r^2) were obtained.

3. Results and discussion

3.1. Effect of temperature and the solid to liquid ratio on DM and DP

Application of microorganisms and enzymes to extract chitin from marine crustacean wastes is a current research trend for bio-conversion of wastes into useful biomass [3]. Some species of *Lactobacillus* and *Bacillus* have been successfully applied for production of chitin mainly from crustaceans shells [3–5,13,14,18,22].

In this study, the strain *P. aeruginosa* F722 has been applied for chitin extraction. The strain produced a high protease activity (~50 U/ml) in the culture medium and produced organic acids mainly of lactic, succinic and citric acids (roughly in a ratio 1:1:1, w/w/w, 0.3 mg/ml each) [5,25,26]. The strain also secreted substantial amounts of chitinase (0.5 U/ml) but little chitosanase.