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SHORT COMMUNICATION

Efficient decomposition of shrimp shell waste using *Bacillus cereus* and *Exiguobacterium acetylicum*

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Abstract Two bacterial cultures were isolated and tested for degradation of shrimp shell waste. According to morphological examination, physiological tests, and applied molecular techniques, isolates were identified as *Bacillus cereus* and *Exiguobacterium acetylicum*. Both strains were cultivated separately in flasks with 100 mL of shrimp shell waste broth (3% of washed, dried and ground shrimp shell waste in tap water, pH 7.0) at 37°C. At determined periods of time, deproteinization and demineralization of residuals were measured. Fermentation of 3% shell waste with *B. cereus* indicated 97.1% deproteinization and 95% demineralization. For *E. acetylicum*, the level of deproteinization and demineralization was 92.8 and 92%, respectively. Protein content was reduced from 18.7 to 5.3% with *B. cereus* and to 7.3% with *E. acetylicum*. No additional supplements were used during the fermentation of shell waste. *B. cereus* strain showed higher efficacy in decomposition of shell waste and was used for large-scale fermentation in 12 L of 10% shrimp shell waste broth. Incubation of bacteria with shell waste during 14 days at 37°C resulted in 78.6% deproteinization and 73% demineralization. High activity of isolated cultures in decomposition of shrimp shell waste suggests broad potential for application of these bacteria in environmentally friendly approaches to chitin extraction from chitin-rich wastes.

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Introduction

Chitin is the second most abundant natural polymer on the earth after cellulose [12]. In nature, it is usually found attached to other polysaccharides and proteins. The amount of chitin in dried processed waste is approximately 14–27% and 13–15% for shrimp and crab, respectively [16]. Currently applied methods to isolate chitin from such waste and to transform it to useful carbohydrate products involve harsh chemical treatments with bases for deproteinization and strong acids for demineralization that eventually result in the formation of undesired by-products such as irregularly deacetylated polymers [21]. To overcome the drawbacks of chemical treatments, studies have been conducted for alternative disposal methods. Bioconversion of waste is probably the most cost-effective and environment friendly procedure for waste utilization [4]. It has been shown that chitin can be prepared through microbiological and fungal treatments [12]. Biological processes using microorganisms have been evaluated for demineralization [3] and deproteinization [14] of shellfish waste. Lactic acid bacterial fermentation of shrimp biowaste for demineralization was studied with added carbohydrate sources such as cassava or molasses [3], organic acids [10], and salt supply [11]. Deproteinization of crustacean shell wastes was reported using protease-producing microorganisms such as *Pseudomonas aeruginosa* K-187 [20], *Pseudomonas maltophilia* LC-102 [13], *Candida parapsilosis* [2], and *Bacillus subtilis* [21]. However for demineralization, additional treatment was needed.

The main goal of this work was to isolate and characterize the effective bacterial strains for deproteinization and demineralization of shellfish waste.

Materials and methods

Shrimp shell waste

Shrimp shell waste was obtained from a shrimp processing plant (Bayou La Batre, AL) and kept frozen until experiments. Before the experiments, shell waste was washed with tap water, dried and ground to a final particle size <100 μm.

Isolation and identification of microbial cultures

Samples of soil, sewage water, crab, and shrimp shell waste were collected from rendering plants (Bayou La Batre). Soil, crab, and shrimp shell waste samples (1 g) were placed in tubes with 9 mL of sterile phosphate buffered saline (PBS) (Fisher Scientific, Hampton, NH). Serial dilutions of every sample were made and placed separately onto shrimp shell waste agar plates (3% prepared shrimp shell waste in tap water and 1.5% agar, pH 7.0). Plates were incubated at 37°C for 3 days. All types of colonies were transferred to new shrimp shell waste agar plates for isolation of pure cultures. Isolated cultures were tested for their proteolytic activity on casein agar plates (Edge Biologicals, Memphis, TN). Morphological characterization of the cultures was done with high resolution CitoViva microscope system [17]. Gram staining and 3% H_2O_2 solution were used for primary characterization of the cultures. Bacterial cultures were identified using API 20 E V4.0 (bioMerieux, Marcy-l'Etoile, France) tests. Gram-positive spore-forming rods were plated on *Bacillus cereus* agar (Edge Biologicals, Memphis, TN) as well as tested for susceptibility to specific gamma bacteriophages for further identification. Fatty acid methyl ester (FAME) analysis was performed with an Agilent 6850 gas chromatograph by RCLN50 method [15].

The 16S rRNA gene was PCR amplified using universal 16S primers that correspond to positions 0005F and 0531R. Products of sequencing reactions were analyzed with an ABI 3100-AVANT Genetic Analyzer in MIDI Labs (Newark, DE). Sequence analysis was performed using BLAST and Sherlock DNA microbial analysis software and database.

Bacterial fermentation of shrimp shell waste

Prepared shell waste (300 mg) was placed in 250-mL flasks and tap water was added to 100 mL. pH of the shell waste broth was adjusted to 7.0. After sterilization, inoculum (10% v/v) of overnight culture grown in 3% shell waste broth was added to each flask and incubated at 37°C on a rotary shaker (200 rpm/min). At determined periods of time, residuals were washed and dried at 50°C for further analysis. Fermentation experiments were conducted in duplicate.

In large-scale fermentation of shrimp shell waste, only *B. cereus* 8-1 strain was tested. Bacterial culture was incubated overnight in 10% (w/v) shell waste broth (10% prepared shell waste in tap water, pH 7.0) at 37°C on a rotary shaker (200 rpm/min). Then, 1.2 L of overnight culture with a cell density of 1.3×10^9 CFU mL^{-1} and 1,200 g of sterile dried ground shrimp shell waste were placed in a 20-L sterile bottle. Sterile tap water was added to yield the final volume of 12 L. A magnetic stir bar was added, and the bottle was placed on a magnetic stirring plate located in a 37°C incubator. Fermented residues were filtered through a cloth to separate the solid materials, washed with distilled water 10 times, oven dried, and analyzed for deproteinization and ash content. The fermentation was carried out in triplicate.

Analytical methods

All samples were analyzed in triplicate. Moisture content was calculated after drying the samples in the oven at 105°C for 24 h. To determine the ash content, samples were treated at 600°C for 4 h in a muffle furnace (Thermoline 1300 Furnace, Barnstead International, Dubuque, IA). Protein content was measured using Lowry method in samples before and after fermentation with bacteria. Deproteination (% DP) was calculated according to [11] using the following equation:

$$\%\,\mathrm{DP} = [(P_\mathrm{O} \cdot O) - (P_\mathrm{R} \cdot R)] \times 100/(P_\mathrm{O} \cdot O)$$

where P_O and P_R are protein mass fractions in g/g before and after fermentation and O and R are mass (g) of original sample and residue after fermentation, respectively.

Demineralization (% DM) was calculated using the equation above but replacing P_O and P_R in the equation by A_O and A_R, which represent ash concentrations in the original and fermented residue, respectively [11].

All used chemicals were obtained from Fisher Scientific (Hampton, NH).

Results

Isolation and characterization of shellfish-degrading bacterial cultures

A total of 15 cultures were isolated from different sources. All cultures were tested for their proteolytic activity. Only

five isolates showed zones of proteolysis on casein plates. Two of these isolates (strains 8-1 and 3) forming the biggest clearing halos were used for further experiments as promising candidates for deproteinization of shrimp shell waste. Both cultures had smooth colonies on the nutrient agar, but differed in color: strain 8-1 formed colonies of white color, while the colonies of strain 3 were yellowish. Microscopic examination showed the two cultures to be Gram-positive rods. Strain 8-1 produced spores in the stationary phase of growth. Strain 3 appeared to be irregular rods after 18-h growth and cocci in old culture.

Bacterial strains were analyzed for morphological characteristics and were identified using API 20 E V4.0 tests. Strain 3 was additionally characterized by FAME analysis. Results showed that the major fatty acids for strain 3 are iso-C13:0; anteiso-C13:0; iso-C15:0; C16:0 and iso-C17:0. Obtained data indicated strain 8-1 to be *B. cereus*, and strain 3 to be *Exiguobacterium acetylicum*. The confirmation of this identification came from sequencing the 16S rRNA gene. Strain 3 showed 98% identity with *E. acetylicum* strain DSM 20416 (DQ019167) and 8-1 strain showed 100% identity with *B. cereus* MZ-30 (EU586795).

Shell waste decomposition with isolated bacteria

Two bacterial cultures *B. cereus* 8-1 and *E. acetylicum* 3 were studied for their ability to decompose shell waste. Strains were cultivated in 100 mL of 3% shell waste broth at 37°C in a shaker incubator. The results in Fig. 1a show that after 7 days of incubation the protein content in shell waste decreased from 18.7 to 5.3% with *B. cereus* 8-1 and to 7.3% with *E. acetylicum* 3. Further incubation of the bacterial cultures resulted in decrease of protein to 4.7% (*B. cereus* 8-1) and 6.7% (*E. acetylicum* 3) on day 14 of fermentation. Analysis of fermented residuals indicated 92.8 and 97.1% deproteinization and 92 and 95% demineralization with *E. acetylicum* 3 and *B. cereus* 8-1, respectively.

In additional experiments, *B. cereus* 8-1 was cultivated in 10% shell waste broth in 20-L bottles at 37°C. After 14 days of incubation with bacterial culture, the protein content of shell waste decreased to 6% (Fig. 1b). Bacterial fermentation resulted in a high level of deproteinization and demineralization (78.6 and 73%, respectively) of shell waste (Table 1).

Discussion

Various microorganisms have been reported to be isolated and used for efficient decomposition of shell waste. Usually for isolation of prospective bacteria for decomposition of shell waste, yeast extract [19], ammonium salts [8], and other additives are used. Two bacterial strains described in this article were isolated using shell waste as the only source of carbon and nitrogen and without any other supplements. Based on morphological examination, physiological tests, and molecular techniques, applied isolated strains were identified as *B. cereus* and *E. acetylicum*. *B. cereus* is usually known as a soil bacteria [7], capable of growing on shell waste [1]. *Exiguobacterium* bacteria are also found in diverse environments [18]. It was reported that *Exiguobacterium* strains have been isolated from shrimp [6]. The cultures belonging to *Bacillus* and *Exiguobacterium* genus are known as highly effective producers of proteolytic enzymes [5, 9]. There is no reported data about deproteinization and demineralization of shell waste with these bacteria.

Our data show a high level of deproteinization (92.8–97.1%) and demineralization (92–95%) of shrimp waste with isolated bacterial cultures in 3% shell waste broth without any additional supplements. Fermentation of 3% shrimp biowaste by *L. plantarum* 541 with 5% glucose and citric acid led only to 88% deproteinization and 90% demineralization [10]. High efficacy of strain *B. cereus* 8-1 was demonstrated in large-scale fermentation of 10% shell

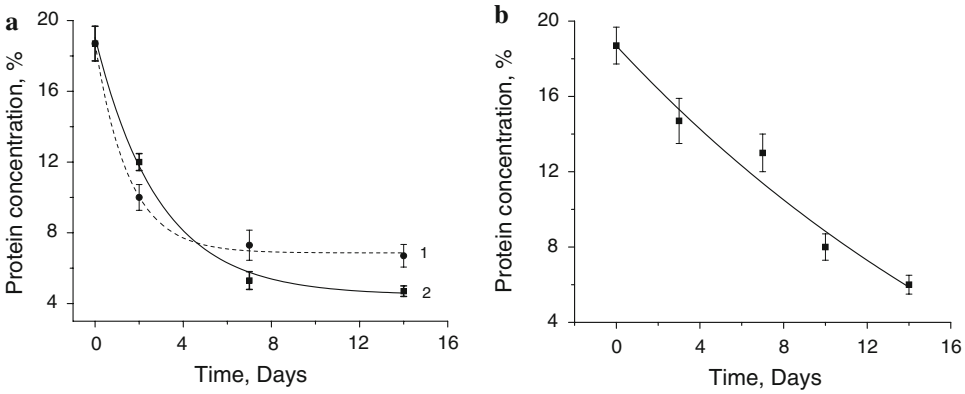

Fig. 1 Decrease in protein content during bacterial fermentation. **a** Fermentation of 3% shell waste in 100 mL of shell broth with *E. acetylicum* 3 (*1*) and *B. cereus* 8-1 (*2*). **b** Fermentation of 10% shell waste in 12 L of shell broth with *B. cereus* 8-1. Values are the mean ± SD of three experiments

Table 1 Characterization of shell waste before and after bacterial fermentation

Sample	Protein (%)	Ash (%)	DP (%)	DM (%)
Raw material	18.7 ± 0.98	42.0 ± 0.58	–	–
Fermented residuals	6.0 ± 0.5	17.0 ± 0.34	78.6 ± 2.6	73.0 ± 1.5

Fermentation of 10% shell waste in 12 L of shell broth at 37°C for 14 days with

B. cereus 8-1

Values are the mean ± SD of three experiments

DP Deproteinization, *DM* demineralization

waste. To our knowledge, high levels of deproteinization together with high demineralization of shell waste without additional procedures or supplements have never been reported. This suggests broad potential for application of these bacteria in environmentally friendly approaches to chitin extraction from chitin-rich wastes.

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