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Shrimp biowaste fermentation with *Pediococcus acidolactici* CFR2182: Optimization of fermentation conditions by response surface methodology and effect of optimized conditions on deproteination/demineralization and carotenoid recovery

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Abstract

Fermentation of shrimp biowaste was conducted using different lactic acid bacteria (LAB) to select the efficient starter culture based on pH reduction and acid production. *Pediococcus acidolactici* CFR2182 was found to be the efficient ($P \leq 0.05$) among the five starter cultures tested. Fermentation conditions *viz*., inoculum level (X1), sugar level (X2) and incubation time (X3) were optimized using response surface methodology (RSM) to obtain the desirable pH of 4.3 ± 0.1. The optimized conditions were found to be 5% (v/w) inoculum (with 8.28 log cfu ml^{-1}), 15% (w/w) glucose and 72 h of incubation time at 37 ± 1 °C to attain a pH of 4.30. The usefulness of the predicted model was further validated by considering random combinations of the independent factors. The high correlation (with regression coefficient close to 1.0) between the predicted and observed values during validation indicated the validity of the model. The effect of fermentation, by *P. acidolactici* CFR2182, on the production of chitin (as indicated by deproteination and demineralization efficiency) and recovery of carotenoids was also studied. Deproteination of 97.9 ± 0.3% and demineralization of 72.5 ± 1.5% was achieved by fermentation of shrimp biowaste with *P. acidolactici*. The carotenoid recovery in fermented shrimp biowaste, as compared to the wet waste, varied between 72.4 and 78.5% during fermentation.

Keywords: Shrimp biowaste; Pediococcus; Fermentation; RSM; Deproteinization; Demineralization; Carotenoid

1. Introduction

Indian shrimp processing industry produces more than 100,000 tonnes of industrial wastes that makes it the single largest industrial fish waste in the country [1]. Shrimp biowaste being alkaline (pH 7.5–8.0) in its characteristic supports the growth of undesirable putrefying microflora resulting in spoilage [2,3]. Although shrimp biowaste is usually dried on the beaches it encourages not only environmental pollution but also reduces the recoverable components in these biowastes. For instance, fresh shrimp biowaste allows better pigment retention [4] while its dried counterpart loses majority of its pigmentation due to the strong affinity of the constituent pigments to oxygen [5] thus making drying unsuitable for a complete recovery of the components present in such biowastes. Any process that eliminates the drying stage prevents degradation of some proteins, allowing the full potential of protein recovery to be realized [5]. Conversion of shrimp waste into ensilage advantageously upgrades the biowaste with this approach being eco-friendly, safe, technologically flexible and economically viable [1]. Ensilation of shrimp biowaste can be accomplished either by direct addition of mineral or organic acids (acid silage) or biologically by fermentation with lactic acid bacteria (biological or fermented silage). The usefulness of fermentation preservation is mainly due to its eco-friendly nature compared to the more ecologically aggressive and economically unviable preservation methods like acid/alkali preservation or drying [6]. Traditionally chitin preparation from shrimp waste involves the use of alkalis (usually 4% sodium hydroxide) for deproteination and acids (e.g. 4% hydrochloric acid) for demineralization, making this process ecologically aggressive and a source of pollution [7]. While the chemical process isolates chitin efficiently, the protein and carotenoid components are rendered useless during protein removal and demineralization stages [7,8]. Fermentation of shrimp biowaste

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using lactic acid bacteria for the preservation of shrimp waste [8–11], production of chitin [5,7,12,13], carotenoids [14], etc. have been reported. Also, fermentation of shrimp biowaste to recover chitin considerably replaces the expensive and non-environmentally friendly chemical process [7,8,15,16]. This biotechnological process results in a liquor fraction rich in proteins, minerals and carotenids (especially astaxanthin) along with a solid fraction rich in chitin. The liquor fraction can be utilized as a protein-mineral supplement for human consumption or as an animal feed [7]. Apart from protein recovery for feed purposes, partial purification of chitin during fermentation is also feasible [16].

Researchers working on shrimp waste fermentation have reported varying levels of fermentation conditions [1,5,7,8, 12,13]. This includes sugar levels varying from 5 to 20%, inoculum levels varying from 5 to 20% and incubation time ranging between 48 and 96 h. Most of the researchers working on shrimp biowaste fermentation standardize conditions by varying one variable at a time [1]. Although a wealth of information is available on biological ensilation using lactic acid bacteria (LAB) cultures, reports on optimized conditions by considering the significant variables (*viz*., type of LAB, inoculum level, fermentation time and sugar level) in total are scarce. Response surface methodology (RSM) is a statistical method that uses quantitative data to simultaneously determine and solve multivariable equations, graphically represented as response surfaces [17].

The first objective of this work was ascertain the best lactic acid bacteria (LAB) culture, which significantly acidified the shrimp biowaste on fermentation (as determined by pH reduction and total titrable acidity or TTA), from among the five cultures (*viz*., *Lactobacillus plantarum*, *L. acidophilus*, *Lactococcus case*, *Pediococcus acidolactici* and a combination of all these four cultures). Secondly, the LAB that was efficient in acidifying the shrimp biowaste was further taken for optimizing the fermentation conditions (i.e., incubation time, glucose level and inoculum level) by employing response surface methodology (RSM). Finally, the effect of lactic acid fermentation on carotenoid recovery, de-proteination and de-mineralization of the shrimp biowaste for chitin preparation also evaluated. This was achieved by assessing several batches of shrimp biowaste fermented under optimized conditions.

2. Materials and methods

2.1. Materials

Shrimp waste (*Penaeus monodon*) was procured from processing plants located at Tadri and Mangalore (west coast of India). It was transported to the lab under frozen condition and kept at −20 °C till further use. The lactic acid bacteria (LAB) cultures were obtained from the Institute (CFTRI, Mysore) culture collection. The bacterial species evaluated included *L. plantarum* B4496, *L. acidophilus* B4495, *Lactococcus lactis* B634, *P. acidolactici* CFR2182 and mixture of all four cultures at 1:1:1:1 (v/v/v/v). All the cultures were maintained on Mann–Rogosa–Sharpe (MRS) agar (Hi-Media, India) slants, stored at 4 °C and sub-cultured periodically [18]. In all the fermentation experiments D-glucose was used as the sugar source. Chemicals used for the study were of analytical grade, unless otherwise mentioned.

2.2. Screening of lactic acid cultures for starter selection

The frozen shrimp waste was thawed overnight in a refrigerator (4 °C) and minced in a Waring blender (Stephen mill, UM5 Universal, Hong Kong) for 10 min to homogenize the mass. The LAB cultures were grown in 100 ml of MRS-Broth (Hi-Media, India) for 24 h at 37 ± 1 °C in a shaking incubator (Technico Ltd., India) set at 100 rpm. The cells were harvested by centrifuging (C31 Cooling centrifuge, Remi-India, India) at 3000 × *g* for 10 min. The harvested cells were washed twice with sterile physiological saline and resuspended in physiological saline (100 ml). The biomass in the inoculum, after serial dilution, was assayed by counting colony forming units (cfu) on MRS agar (Hi-Media, India) plates.

For the initial screening experiments, shrimp biowaste (100 g) was mixed with distilled water (1:1, w/v) followed by 10% inoculum (v/w), 15% (w/w) glucose and 2% (w/w) salt in a 250 ml conical flask. The flasks were then flushed with nitrogen and sealed with a layer of parafilm (Hi-Media, India) to create anaerobic conditions. The mixture was allowed to ferment for 72 h in an orbital shaking incubator at 100 rpm and 37 ± 1 °C. pH and total titrable acidity (TTA) were recorded at 0, 24, 48 and 72 h. pH was determined using a digital pH meter (Cyberscan 1001, Eutech, Singapore). TTA was estimated as per the method described in Sachindra et al. [14] by determining the ml of 0.1N sodium hydroxide (NaOH) required for increasing the pH of one gram of fermented mass to 8.0. Possible differences among treatments were determined by statistically analyzing the data obtained by applying analysis of variance (ANOVA) and Duncan's multiple range test using STATISTICA software (Version 5.5, 1999 Edition; Statsoft Inc., Tulsa, OK, USA), to decide on the best culture that gave desirable pH of 4.3.

2.3. Optimization of fermentation conditions using P. acidolactici

P. acidolactici CFR2182 was employed, for optimizing the fermentation conditions, based on the screening experiments. The inoculum was prepared as mentioned in the previous section. The inoculum used in the optimization experiments had a cell density of 8.28 log cfu ml^{-1}.

The independent factors considered for optimization included inoculum level (X1; % v/w), glucose (X2; % w/w) and incubation time (X3; h). Box–Behneken design with 3 factors in one block encompassing 15 runs was employed for the study. The design consisted of three factors (X1, X2 and X3) at three equidistant levels (5, 10 and 15% for X1; 7.5, 12.5 and 17.5% for X2; and 24, 48 and 72 h for X3). In the optimization experiments, unlike screening experiments, only pH (Y) was recorded as the response (dependent) variable. Each run comprised 100 g shrimp biowaste mixed with distilled water (1:1, w/v) and 2% (w/w) salt along with respective levels of glucose (w/w) and inoculum (v/w). The different ingredients were mixed according to the pre-determined levels in 250 ml conical flasks, flushed with nitrogen, sealed with a layer of parafilm to create anaerobic condition and incubated at 37 ± 1 °C in an incubator for specified time. The designed model was further validated using random combinations of the independent variables. All the results were analysed by employing STATISTICA software, to determine the optimum conditions for fermentation. pH was determined as described earlier.

2.4. Effect of fermentation on recovery of chitin and carotenoids

Homogenized shrimp biowaste (100 g) was mixed with 1:1 (w/v) distilled water and fermented under optimized fermentation conditions (i.e. 5% inoculum, 15% sugar, 72 h fermentation with 2% salt) in 250 ml conical flasks. The experiment was carried out in four different batches with each batch comprising four numbers (one each for 0, 24, 48 and 72 h) of 250 ml conical flask containing the fermentation mass. The inoculum used in these experiments had a cell density of 8.36 log cfu ml^{-1}. Samples (250 ml flask representing 100 g shrimp biowaste) were drawn every 24 h up to 72 h (at which time the fermented samples recorded a pH of 4.15 ± 0.15). Each sample was filtered using cheese cloth to collect the fermentation liquor and the residue washed three times with distilled water (1:10, w/v). The surface moisture on the washed residue was drained completely and weighed. Part of the wet residue was analysed for carotenoid and the remaining mass dried overnight at 55 ± 1 °C. The dried sample was referred to as crude chitin.