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# Critical factors in chitin production by fermentation of shrimp biowaste

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**Abstract** Factors affecting *Lactobacillus* fermentation of shrimp waste for chitin and protein liquor production were determined. The objective of the fermentation is medium conditioning by *Lactobacillus* through production of proteases and lowering of the pH. The efficiency was tested by conducting fermentation of biowaste in 1-l beakers with or without pH adjustment using different acids. Addition of 5% glucose to the biowaste supported the growth of lactic acid bacteria and led to better fermentation. Among four acids tested to control pH at the start and during fermentation, acetic acid and citric acid proved to be the most effective. In biowaste fermented with 6.7% *L. plantarum* inoculum, 5% glucose, and pH 6.0 adjusted with acetic acid, 75% deproteination and 86% demineralization was achieved. Replacement of acetic acid by citric acid gave 88% deproteination and 90% demineralization. The fermentation carried out in the presence of acetic acid resulted in a protein fraction that smelled good and a clean chitin fraction.

## Introduction

Shrimp waste in the tropical region contains 10–20% calcium, 30–40% protein, and 8–10% chitin (Legarreta et al. 1996). Chitin and its derivative chitosan have many applications in the pharmaceutical, cosmetic, food, and textile industries (Hirano 1995). To isolate chitin from shrimp waste chemically, 4% NaOH is used for deproteination and 4% HCl for demineralization. An alternative method using lactic acid fermentation has emerged, which can to a considerable extent replace the expensive and non-environmentally friendly chemical process (Hall and DeSilva 1992; Rao and Stevens 1997). This biotechnological approach leads to a liquor fraction rich in proteins, minerals, and asthaxanthin and to a solid chitin fraction. The liquor fraction could be utilized either as a protein-mineral supplement for human consumption or as an animal feed.

Fermentation of shrimp biowaste in the presence of a selected *L. plantarum* strain results in medium conditioning, supposedly by production of lactic acid and various proteases. Lactic acid is produced by breakdown of glucose, creating the low pH condition of ensilation that suppresses the growth of spoilage microorganisms (Legarreta et al. 1996). The lactic acid reacts with the calcium carbonate component in the chitin fraction, leading to the formation of calcium lactate, which precipitates and can be removed by washing. Deproteination of the biowaste and simultaneous liquefaction of the shrimp proteins occurs mainly by proteolytic enzymes produced by the added *Lactobacillus*, by gut bacteria present in the intestinal system of the shrimp, or by proteases present in the biowaste (B. Woods, personal communication, 1997). This results in a fairly clean chitin fraction and in liquor with a high content of soluble peptides and free amino acids (Fagbenro 1996). Ensilation can also be conducted by addition of (organic) acids combined with *Lactobacillus* treatment (Dapkevicius et al. 1998).

The efficiency of fermentation using lactic acid bacteria depends on factors such as the quantity of inoculum, glucose, initial pH and pH during fermentation, the amount and type of acid used, and fermentation time. This research aimed to study the effect of these fermentation parameters individually and in combination, on deproteination, demineralization, and the prevention of spoilage during fermentation.

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## Materials and methods

### Micro-organism and cultivation methods

*Lactobacillus plantarum* 541 was selected (Kungsuwan et al. 1996) from the strain collection in the Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand. Strains were stored at 4 °C in MRS-agar slants (De Man et al. 1960) and routinely cultivated in *Lactobacillus* MRS medium. Overnight cultures in Erlenmeyer flasks were used as inoculum (5% v/v). Media were sterilized for 20 min at 115 °C. The density of the bacterial population was assayed after serial dilution by counting colony forming units (cfu/ml) on MRS-agar plates.

### Fermentation of shrimp biowaste under different conditions

The frozen shrimp waste including head and carapace (moisture 72–78%, ash 18–23%, pH 8.2–8.6) was obtained from Surapong Sea Foods Co. Ltd, Samutsakorn, Thailand, and chopped at the start of the experiment using a thermomixer (Vorwerk, Model 3300, France). Static fermentation of 200–300 g shrimp waste in duplicate was conducted in 1-l beakers, covered with aluminum foil, at 30 °C in a controlled temperature incubator. Stirring was provided regularly at 1-h intervals with a glass rod. The fermented slurry was filtered through a coarse cloth to separate the solid materials containing chitin. This crude chitin was washed with distilled water, oven dried, weighed, and further analyzed for protein, moisture, ash, and total nitrogen content.

### General analytical procedures

The moisture content was measured by drying the samples in an oven at 105 °C for 24 h. Ash content was determined by burning the samples in a crucible at 600 °C in a muffle furnace (Sanyo Gallenkamp, UK). The pH values and pH drop (–dpH/d$t$, pH/h) were measured using a benchtop pH meter (Jenway 6051). Protein content was measured by using a standard biuret protein assay in samples before and after fermentation. Total nitrogen content was determined by Kjeldahl (Kjeltech, Gallenkamp, UK) for the initial biowaste as well as the fermented residues, the difference reported as protein nitrogen, and protein content was calculated by multiplying with a factor of 6.25. Samples for analysis were collected in triplicate from each fermentation vessel. Deproteination (%DP) was calculated using the following equation, where $P_O$, $P_R$ are protein concentrations (g/g) before and after fermentation and $O$, $R$ are mass (g) of original sample and fermented residue, respectively.

$$\%DP = [(P_O \times O) - (P_R \times R)] \times 100/(P_O \times O).$$

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

## Results

### Fermentation with *Lactobacillus*

#### *Optimization of glucose concentration for pH reduction*

Shrimp biowaste was fermented with a fixed amount of inoculum (10% v/w) and variable concentrations of glucose (0, 2.8, 4.2, 5.6, 7.0, and 8.4% w/w). The initial pH was not adjusted. In the absence of glucose, pH was 7.0 after 20 h. In the presence of 4.2% glucose, pH 5.5 was achieved within 12 h (Fig. 1), corresponding to a pH drop (–dpH/d$t$) of 0.25 pH/h.

### Control of pH of *Lactobacillus* fermentation by acids

#### *Optimization of glucose, with initial lowering of pH*

The experiment reported in Fig. 1 was repeated, but this time the initial pH was reduced to about pH 5.5 by addition of 1% acetic acid (Kungsuwan et al. 1996). Supposedly due to proteolysis and liberation of ammonia, the pH bounced back to pH 6.5 in all the samples during the first 2 h, and subsequently varying pH profiles were observed (Fig. 2). A glucose concentration as low as 3.5% in combination with the initial amount of

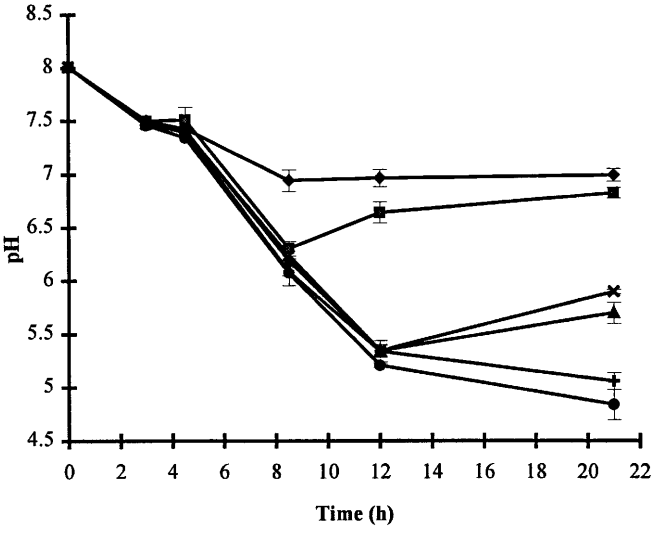


**Fig. 1** pH trends during fermentation of shrimp biowaste with different quantities of glucose and 10% inoculum (◆ 0.0%, ■ 2.8%, ▲ 4.2%, × 5.6%, + 7.0%, ● 8.4%). Data in the figures represent means of duplicate experiments and *bars* represent standard deviations

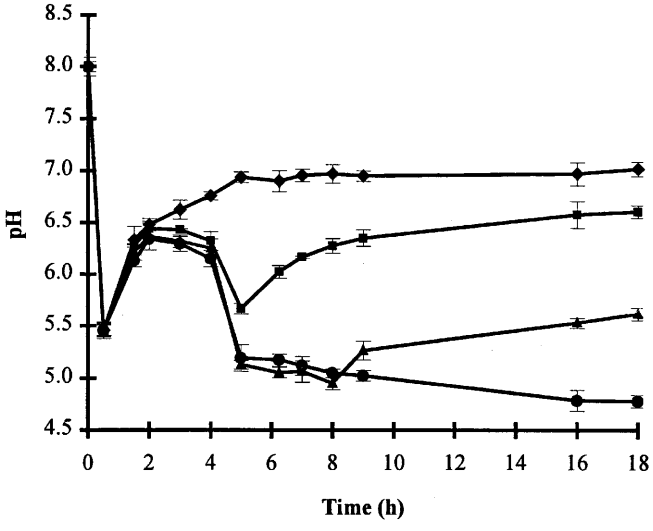


**Fig. 2** pH trends during fermentation of shrimp biowaste with different quantities of glucose, 10% inoculum and 1%(v/w) acetic acid added initially (◆ 0.0%, ■ 1.75%, ▲ 3.5%, ● 7.0%)

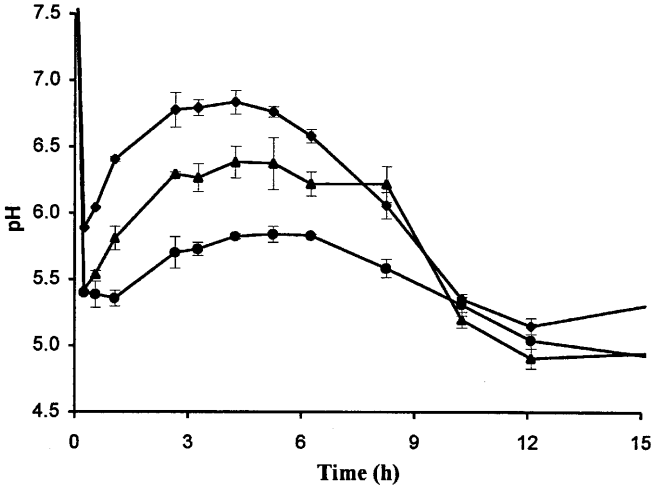


**Fig. 3** Changes in pH during fermentation in shrimp biowaste with different initial amounts of acetic acid with 10% *Lactobacillus* inoculum (◆ 1%, ▲ 2%, × 3%)

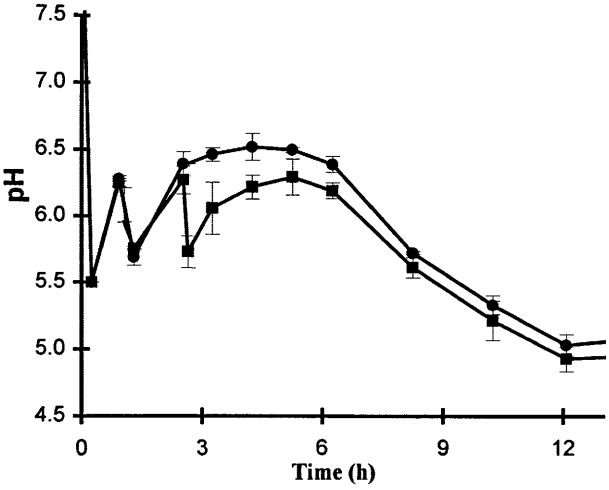


**Fig. 4** Changes in pH during *Lactobacillus* fermentation in shrimp biowaste with acetic acid added intermittently [● 2% (1% acetic acid at 0 h and 1 h), ■ 3% (1% acetic acid at 0 h, 1 h, and 3 h)]

acetic acid was enough to lower the pH to about 5.0 and to prevent spoilage ($-dpH/dt = 0.6$ pH/h).

*Reduction of initial pH by various quantities of acetic acid*

Shrimp biowaste was treated at the start of the fermentation with 1%, 2%, and 3% (v/w) glacial acetic acid in the presence of 10% (v/w) inoculum (Fig. 3). After an initial drop in pH from about pH 7.0 to pH 5.5, due to addition of acid, the pH started to increase in the case of the 1% or 2% acetic acid additions. With 3% acetic acid, the pH rise was delayed and was small. After 12 h, all pH values were below 5.0.

Analysis of fermented residues revealed that the more the acid being added, the less was protein removed. Ferments with single addition of 2% acid had no significant difference with those with intermittent addition at 0 h and 1 h. Ferments with 3% acetic acid added in installments (0 h, 1 h, and 3 h) resulted in a significantly lower protein content (1.40 g vs. 2.19 g) than those with a single addition. Demineralization was highest at 87% with 3% acetic acid, though the liquor had a strong acidic smell. A single addition of acid was observed to produce a greater pH drop (Fig. 4) and better demineralization efficiency than intermittent addition of the same quantity of acetic acid (Table 1).

*Fermentation at various fixed pH values*

In the next series of experiments, fermentations were conducted using glacial acetic acid to maintain pH at 6.5, 6.0, and 5.5. pH was controlled for the first 12 h, after which it declined. The lower the pH maintained, the more protein was left in the residue (Table 2, A). By contrast, the maximum demineralization (84%) occurred in samples maintained at pH 5.5. The samples maintained at pH 6.5 showed signs of spoilage, whereas the fermentation products at pH 5.0 were of good quality.

*Fermentation at pH 6.0 using various acids*

Different acids were used to maintain the ferment at pH 6.0. Citric acid achieved the highest deproteination (88%), followed by hydrochloric acid, acetic acid, and – the lowest – lactic acid. The protein remaining in the

**Table 1** Protein and ash content of residues of chopped shrimp waste (200 g) after fermentation with 10% *L. plantarum* inoculum and 5% glucose with varying quantities of acetic acid. Values having the same superscripts within a column are not significantly different ($P > 0.05$) Spoilage of the biowaste as observed by sensory evaluation is represented using a combination of "+" and "–", where "+ + + + +" refers to maximum spoilage and "–––––" represents the best quality of liquor and chitin

| Acetic acid | Fermented residue (g) | Protein content (g) | Ash content (g) | Deproteination (%) | Demineralization (%) | Spoilage |
|---|---|---|---|---|---|---|
| 1% | 14.43 | $1.48^{b}$ | $2.53^{d}$ | 84.47 | 70.02 | – |
| 2% | 12.72 | $1.58^{b}$ | $1.68^{abc}$ | 83.42 | 80.09 | –– |
| 3% | 12.29 | $2.19^{c}$ | $1.10^{a}$ | 77.02 | 86.97 | ––––– |
| 1% + 1% | 13.77 | $1.33^{ab}$ | $2.14^{cd}$ | 86.04 | 74.64 | –– |
| 1% + 1% + 1% | 12.46 | $1.40^{ab}$ | $1.78^{cd}$ | 85.31 | 78.91 | –– |
| Fresh | 44.67 | $9.53^{d}$ | $8.44^{e}$ | | | |

**Table 2** Protein and ash content of residues after fermentation with 10% *Lactobacillus* inoculum and 5% glucose in different conditions. All experiments used 300 g fresh material. Values having same superscripts within a column in each section (A and B) are not significantly different ($p > 0.05$). Spoilage of the biowaste as observed by sensory evaluation is represented using a combination of "+" and "−", where "++++" refers to maximum spoilage and "−−−−" represents the best quality of liquor and chitin. Sections are as follows: A: Fermentation with *Lactobacillus* at pH 5.5, 6.0 and 6.5 maintained with acetic acid; B: fermentation with *Lactobacillus* at pH 6.0 maintained using different acids

| | Condition | Quantity (mmol) | Fermented residue (g) | Protein content (g) | Ash content (g) | Deproteinization (%) | Demineralization (%) | Spoilage |
|---|---|---|---|---|---|---|---|---|
| A | pH 6.5 | | 20.03 | $3.94^{a,b}$ | $3.33^{c}$ | 78.15 | 71.51 | + |
| | pH 6.0 | | 20.64 | $4.33^{b,c}$ | $2.66^{b}$ | 75.98 | 77.25 | −− |
| | pH 5.5 | | 18.86 | $4.96^{c}$ | $1.88^{a}$ | 72.49 | 83.92 | −−− |
| | Original | | 64.78 | $18.03^{d}$ | $11.79^{d}$ | | | |
| B | Acetic | 91.6 | 18.05 | $3.85^{b}$ | $1.61^{b}$ | 75.37 | 86.23 | −− |
| | Lactic | 130.5 | 18.77 | $6.0^{d}$ | $1.52^{b}$ | 61.61 | 87.00 | − |
| | Citric | 72 | 17.02 | $1.88^{a}$ | $1.08^{a}$ | 87.97 | 90.76 | + |
| | HCl | 49.91 | 17.81 | $3.68^{b}$ | $1.54^{b}$ | 76.46 | 86.83 | + |
| | No acid | | 19.90 | $4.98^{c}$ | $4.20^{c}$ | 68.14 | 64.07 | +++ |
| | Original | | 69.12 | $15.63^{e}$ | $11.99^{d}$ | | | |

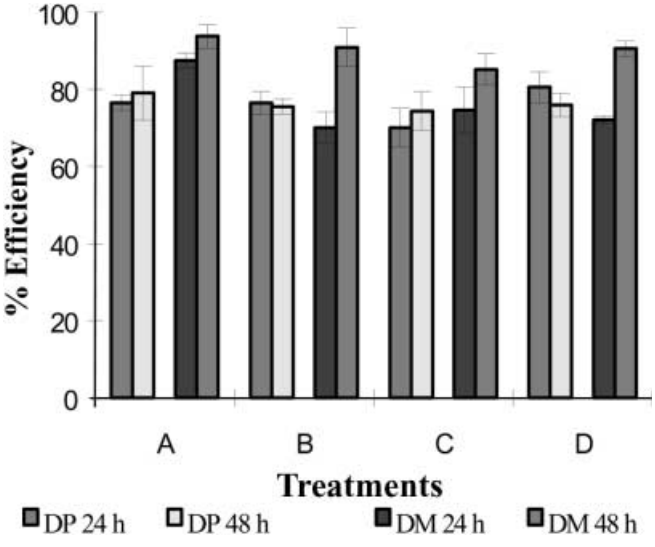


**Fig. 5** Deproteination and demineralization efficiency in shrimp biowaste treated for 24 and 48 h. The ratio *Lactobacillus* inoculum (% v/w): glucose (% v/w): acid is *A* 10%: 5%: acetic acid, *B* 0%: 5%: acetic acid, *C* 10%: 5%: citric acid, *D* 0%: 5%: citric acid. *DP* Deproteination, *DM* demineralization

residues was 1.88–6.0 g (Table 2, B). In regard to demineralization, the shrimp residue containing citric acid had the lowest ash content at 1.08 g (90.7% DM). Citric acid indicated the highest deproteination and demineralization, but the fermentation slurry became viscous, with formation of crystals in the liquor. The liquor obtained after fermentation with acetic acid had a good color and uniform texture.

*Effect of fermentation time*

Since acetic acid and citric acid proved to be the best among the acids, tests were conducted during 24 h and 48 h to optimize fermentation time with these acids. Deproteination did not increase during prolonged fermentation (Fig. 5). The demineralization increases in all cases tested were in the order of 10–20% with an increase in fermentation time by 24 h.

## Autofermentation

*Autofermentation of biowaste without and with pH control*

Data on deproteination and demineralization in the absence of added *Lactobacillus* without and with pH adjustment by initial addition of 3% acetic acid are presented in Table 3, A. Autofermentation of shrimp biowaste is efficient, presumably due to the presence of intestinal microflora in shrimp waste (Woods 1998). Data on the number of colony forming units (cfu/ml) measured at different time intervals during fermentation without added inoculum compared to fermentation with 10% inoculum indicate that inherent bacteria are present in notable quantities (Fig. 6).

In experiments with no inoculum but initial addition of 1%, 2%, and 3% acetic acid, the pH was observed to decrease steadily in proportion to the acid added (Fig. 7). Table 3, B, indicates that at 1% acetic acid, the fermented residue of shrimp waste without *Lactobacillus* inoculum had higher protein removal than the residue fermented with added *Lactobacillus* shown in Table 1 (88% vs. 84%). The highest deproteination occurred for shrimp wastes with 1% acetic acid, while the least deproteination was observed with the treatment containing 3% acid. Shrimp waste (without *Lactobacillus*) treated with 3% acetic acid had the lowest ash content at 2.23 g (82.3% DM).

*Autofermentation at different pH levels and different acids*

Autofermentation was conducted with glacial acetic acid at pH 5.5, 6.0, and 6.5. The fermented products spoiled at pH 6.5, although high deproteination (82%) was

**Table 3** Protein and ash content in fermented residues after fermentation in absence of *Lactobacillus* inoculum in various conditions. All experiments used 300 g of fresh material. Values having same superscripts within a column in each section (A, B, C or D) are not significantly different ($P > 0.05$). Spoilage of the biowaste as observed by sensory evaluation is represented using a combination of "+" and "−", where "++++" refers to maximum spoilage and "−−−−" represents the best quality of liquor and chitin. Sections are as follows: A: Autofermentation without and with initial adjustment with acetic acid; B: autofermentation with initial addition of 1,2 and 3% acetic acid (v/w); C: autofermentation at pH 5.5, 6.0 and 6.5 maintained using acetic acid; D: autofermentation at pH 6.0 maintained using different acids

| | Condition | Quantity (mmol) | Fermented residue (g) | Protein content (g) | Ash content (g) | DP (%) | DM (%) | Spoilage |
|---|---|---|---|---|---|---|---|---|
| A | 0% | – | 23.44 | $2.52^{a}$ | $7.85^{b}$ | 87.3 | 47.9 | ++++ |
| | 3% | – | 19.36 | $3.09^{b}$ | $4.55^{a}$ | 84.4 | 69.8 | −− |
| | Original | – | 83.6 | $19.76^{c}$ | $15.08^{c}$ | | | |
| B | 1% | | 20.15 | $1.65^{a}$ | $4.11^{b}$ | 88.46 | 67.54 | + |
| | 2% | | 18.9 | $2.03^{ab}$ | $2.82^{a}$ | 85.83 | 77.73 | − |
| | 3% | | 19.43 | $2.28^{b}$ | $2.23^{a}$ | 84.05 | 82.35 | −− |
| | Original | | 67.00 | $14.30^{c}$ | $12.66^{c}$ | | | |
| C | PH 6.5 | | 20.97 | $3.16^{b}$ | $4.76^{c,d}$ | 82.47 | 59.28 | ++ |
| | PH 6.0 | | 19.35 | $3.27^{bc}$ | $4.05^{b,c}$ | 81.86 | 65.36 | + |
| | PH 5.5 | | 21.58 | $4.35^{d}$ | $3.86^{a,b}$ | 74.87 | 66.98 | − |
| | No acid | | 22.53 | $1.95^{a}$ | $6.53^{e}$ | 89.18 | 44.14 | ++++ |
| | Original | | 64.78 | $18.03^{e}$ | $11.79^{f}$ | | | |
| D | Acetic | 149.2 | 12.48 | $1.75^{b}$ | $2.60^{a}$ | 90.94 | 74.76 | + |
| | Lactic | 175.6 | 12.79 | $1.90^{b}$ | $2.88^{a}$ | 90.17 | 72.04 | ++ |
| | Citric | 129.0 | 14.54 | $1.77^{b}$ | $2.87^{a}$ | 90.84 | 72.14 | +++ |
| | HCl | 92.3 | 15.33 | $1.97^{b}$ | $3.35^{b}$ | 89.80 | 67.48 | +++ |
| | No acid | | 15.78 | $1.26^{a}$ | $4.36^{c}$ | 93.48 | 57.67 | ++++ |
| | Original | | 63 | $19.32^{c}$ | $10.30^{d}$ | | | |

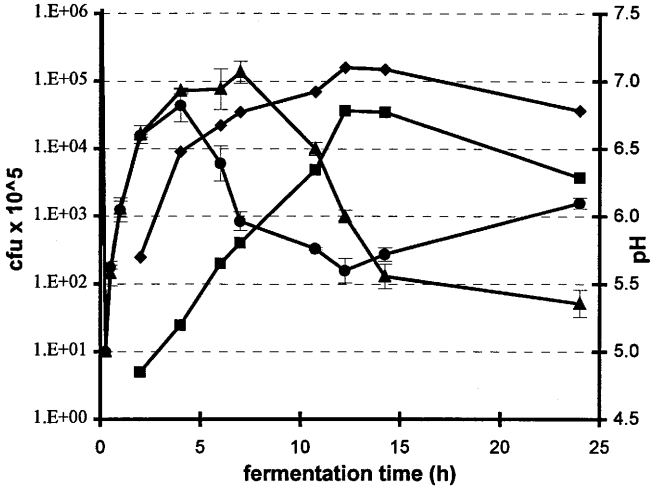


**Fig. 6** Growth of *Lactobacillus* bacteria and pH changes during fermentation with inoculum (◆ cfu/ml, ● pH) and without inoculum (■ cfu/ml, ▲ pH)

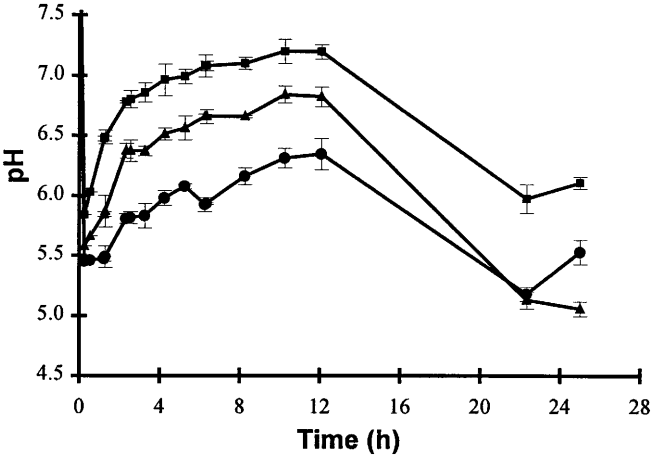


**Fig. 7** Changes in pH with different amounts of acetic acid added initially, without *Lactobacillus* inoculum (■ 1%, ▲ 2%, ● 3%)

observed. Demineralization was low at all the levels of pH (Table 3, C).

In the next experiments, biowaste was treated at pH 6.0 using different types of acids, including lactic acid. The protein contents of samples treated with various acids did not indicate significant differences ($P > 0.05$) implying that the fermentation efficiency depends more on the pH level than the type of acid being used (Table 3, D). However, shrimp wastes mixed with citric or hydrochloric acid showed signs of spoilage after 24 h fermentation. Although highly deproteinated, there was formation of white spots and sedimentation, high viscosity, and spoilage in materials treated with citric acid. The ash contents of the residues in Table 3, D, indicate that the fermentation with organic acids (acetic, lactic, and citric) had similar effects on demineralization ($P < 0.05$) and was more efficient than with hydrochloric acid.

## Discussion

The effect of glucose quantity, *Lactobacillus* inoculum, quantity and type of acid, and fermentation time, indi-

vidually or in combination, on deproteination, demineralization, and spoilage was investigated. Addition of glucose to fermenting *Lactobacillus* led to a pH reduction after 4–6 h fermentation. From the results, a glucose concentration of 5% with an initial pH reduction is inferred to be optimal. This amount is high but is required for efficient fermentation of the waste which has a high calcium carbonate content. Without pH adjustment it took more than 5 h for the bacteria to convert glucose to lactic acid, and during this delay spoilage occurs due to putrefying enzymes active at neutral or higher pH. Low pH prevents spoilage and affects the metabolism of the growing *Lactobacilli* and its fermentation products (Giraud et al. 1991).

Demineralization was 1.2 times lower in fermentations maintained at constant pH 5.5 without inoculum (67%) than in those with inoculum (84%). Higher deproteination without adjustment of pH indicates that proteolysis is more efficient at higher pH (Shirai et al. 1997). Combined treatment with *Lactobacillus* and acid produced lower deproteination and higher demineralization than did treatment with *Lactobacillus* or acid individually. Fermentation of waste with *Lactobacillus* inoculum resulted in a high-quality protein liquor output, whereas solely acid-fermented waste had a strong and pungent acidic smell in the protein fraction (Dapkevicius et al. 1998). Therefore, fermentation using only endogenous micro-organisms does not seem to be a reasonable option. The presence of lactic acid bacteria in the liquor is useful for its use as human or animal food. Using 5% glucose, 10% inoculum, at pH 6.0 with acetic acid represents the optimum conditions according to this research. In this fermentation process the demineralization efficiency and the quality of the derived products is high, and the addition of commercial proteases could increase deproteination. It is concluded that combined *Lactobacillus* and acetic acid treatment remains a cost-effective and environmentally friendly procedure for the fermentation of shrimp waste.

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