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*preliminary communication*

# Fermentation of Shrimp Biowaste under Different Salt Concentrations with Amylolytic and Non-Amylolytic *Lactobacillus* Strains for Chitin Production

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**Summary**

Fermentation of shrimp biowaste was conducted with two *Lactobacillus plantarum* strains at pH=6.0 under various salt concentrations. The non-amylolytic strain *L. plantarum* 541 and amylolytic strain *L. plantarum* A6 showed to possess reasonable growth in biowaste with the addition of salt as high as 6 %. Fermentation carried with 10 % inoculum, 5 % glucose, with initial reduction of pH to 6.0 using acetic acid, showed that there was no spoilage of the biowaste. Growth trends in shrimp waste, in terms of CFU/mL developed on MRS-agar plates, changes in pH and lactic acid production (in g/L) were determined. The deproteination and demineralization efficiency with strain 541 was better than with strain A6. A maximum of 81.4 % demineralization and 59.8 % deproteination under 2 % salt conditions occurred with strain 541, whereas with strain A6, a demineralization of 65.5 % and deproteination of 52.2 % was observed.

*Key words:* chitin, chitosan, biopolymers

## Introduction

Fermentation of crustaceans, fish products and vegetables under high concentrations of salt has been a common practice for a long time (*1*). Shrimp is a high value aquacultural product and is processed for the meat, leaving the carapace and the head as waste products (*2*). Partial fermentation of this biowaste using lactic acid bacteria for the production of chitin has been studied and reported (*3*). The fermentation results in the removal of protein and minerals and in addition to crude chitin, a liquor fraction rich in protein, minerals and asthaxanthin is obtained. The pH of the biowaste is in the range of 7.5–8.0 and this high pH leads to the growth of undesirable putrefying microflora resulting in spoilage (*4*,*5*). It is reported that during fermentation with *L. plantarum* 541, high amounts of glucose (5–20 %) are added to stimulate rapid acidification (*6*).

In commercial scale, glucose addition is an expensive alternative. The use of a cheaper source of carbon, such as cassava flour, which is abundant in many tropical countries, in combination with amylolytic lactic acid bacteria such as *L. plantarum* A6 may help to decrease the cost of the overall process (*7*,*8*). Amylolytic lactic acid bacteria could convert the starch directly into lactic acid (*8*). Combinations of low initial pH and high salt concentration may also present advantages during fermentation of shrimp waste using salt resistant strains 541 and A6 (*9*). Considering these possibilities of exploration, lactic acid fermentation of shrimp biowaste was conducted at optimum pH of 6.0–6.5 under mass fraction of salt between 2–6 % with one amylolytic (A6) and the other non-amylolytic (541) *Lactobacillus* strain. The aim of this research was to study the effect of these salt concentrations on the shelf life, deproteination, demineralization and overall fermentation efficiency of shrimp

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biowaste. Growth trends in terms of CFU/mL developed on MRS-agar plates, changes in pH and lactic acid production have also been reported.

## Material and Methods

### *Microorganisms and cultivation methods*

The non-amylolitic strain *L. plantarum* 541 was selected from the strain collection of the Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand. The amylolytic strain *L. plantarum* A6 was isolated from the process of cassava retting (LMG 18053, BCCM, Gent, Belgium). Strains were stored at 4 °C in MRS-agar slants (*10*) and routinely cultivated in *Lactobacilli* MRS medium. Overnight cultures in Erlenmeyer flasks were used as inoculum (100 mL of inoculum/1000 g of shrimp waste). Media were sterilized for 20 min at 115 °C. 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 Surapon Sea Foods Co. Ltd, Samutsakorn, Thailand, and crushed at the start of the experiment using a thermo mixer (Vorwerk, Model 3300, France). Fermentation of 200 g of shrimp waste was conducted in duplicate in 1-litre beakers, covered with aluminum foil, at 30 °C in a controlled temperature incubator. Glacial acetic acid (approx. 5 mL) was added to bring down the pH of shrimp waste below 6.0, a value that inhibits spoilage and optimizes deproteination and demineralization (*3*). A well grown culture of 20 mL (100 mL of inoculum/1000 g of shrimp waste) was used as inoculum. Salt was added to shrimp waste in the predetermined concentration. Stirring was provided regularly at 1-hour 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 were measured using a bench top pH meter (Jenway 6051). Protein content was measured by using standard biuret protein assay in samples before and after fermentation. Samples for analysis were collected in triplicate from each fermentation vessel. Deproteination (% DP) was calculated using the following equation:

$$\% \text{ DP} = [(P_O \cdot O) - (P_R \cdot R)] \cdot 100/(P_O \cdot O)$$

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 fermented residue, respectively. Demineralization efficiency (% DM) was calculated using the same equation 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.

## Results

### *Fermentation of biowaste with L. plantarum 541*

The growth trends (CFU/mL) with all salt concentrations did not indicate significant differences (Fig. 1). No lag phase was observed in ferments under 0 and 2 % salt, whereas a short lag phase of 3–4 h was observed in the ferments growing under 4 and 6 % salt. After 4 h, the trends were similar for all the samples, confirming the adaptability of the strain to high salt concentration as well as shrimp waste conditions. The growth was highest under conditions with 0 % salt followed by those with 2 % salt, but under other conditions the growth was low. The differences are less visible in the figures because they are represented in logarithmic scale. The highest values of colony formations were reached in 12–13 h.

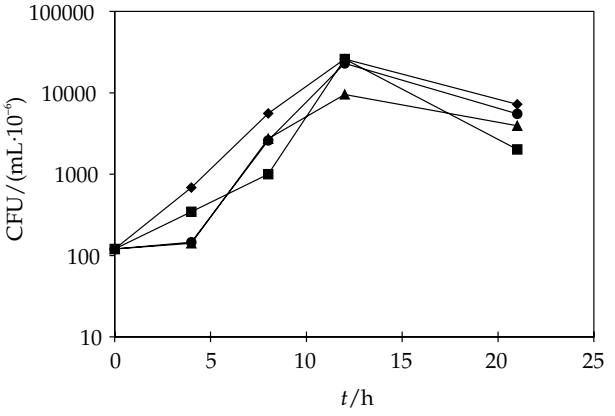


**Fig. 1.** Growth trends of *L. plantarum* 541 during fermentation of shrimp waste under salt conditions (◆ 0 %, ■ 2 %, ▲ 4 %, ● 6 %)

The lowest values in pH were observed in 8 h, indicating rapid acidification in the samples without salt and with 2 % salt (Fig. 2). The pH value in 8 h was 5.5 for 0 % salt, 5.8 for 2 % salt, but it remained between 6.0–6.2 under 4 and 6 % salt conditions. The lowest value of pH in 6 % salt was achieved in 12 h. Salt contributes to minute lowering of pH, and the amount of acetic acid needed to maintain the pH below 6.0 was very low (*11*)**.** The pH was adjusted only in the beginning. In fermentation with controlled pH, the pH needed to be controlled only during the initial 5–6 h, after which it decreased automatically, depending on the fermentation efficiency. In this experiment, after reaching the lowest values, the pH started to increase. This increase is attributed to an inefficient fermentation. Presumably, if acetic acid is not added as a supplement, all the lactic acid produced during fermentation initially contributes to the lowering of pH, but after 10–12 h it gets converted to salts due to its reaction with calcium carbonate. This indicates that the increased salt concentrations are unable to complement the use of acid.

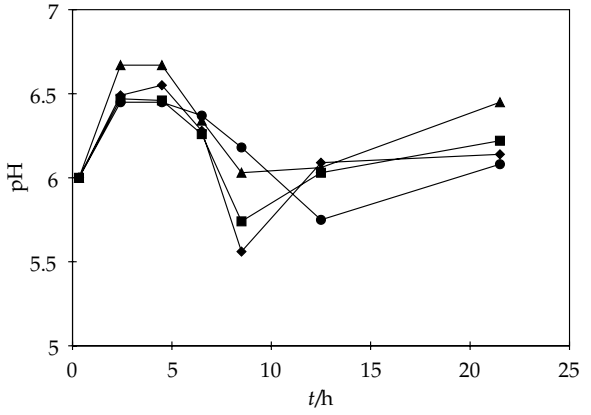


**Fig. 2.** Changes in pH during fermentation of shrimp biowaste with *L. plantarum* 541 under salt conditions (◆ 0 %, ■ 2 %, ▲ 4 %, ● 6 %)

The trends in lactic acid (g/L) production are presented in Fig. 3. In all conditions, maximum lactic acid was produced between 12–13 h, parallel to the biomass production (Fig. 1). The maximum lactic acid production was observed in fermentation with 0 and 2 % salt.

The deproteination and demineralization efficiency of fermentation using *L. plantarum* 541 is presented in Table 1. Demineralization (DM) varied between 67.2–81.4 %, which is considered low for an efficient fermentation. Highest DM was 81.4 %, observed in fermentation under 2 % salt, followed by that in 0 % salt. DM was lowest for samples with 4 % salt. Since the lactic acid production was also lowest in 4 % salt conditions, low DM was expected. Similarly, deproteination (DP) was low and ranged between 45.6–59.8 %. This DP should be higher than 75 %. The highest DP was observed under 2 % salt conditions and the lowest in 4 % salt.

### *Fermentation of biowaste with L. plantarum A6*

Growth characteristics of *L. plantarum* A6 during shrimp waste fermentation under 0, 2, 4 and 6 % mass fractions of salt are presented in Fig. 4. The highest growth was observed in conditions of control (*i.e.* without any salt addition). The growth in the first few hours was low under 0 and 2 % salt, but in ferments with 4 and 6 % salt, a negative growth was observed with a lag phase between 4–5 h. But this lag was recovered with signs of high growth under 4 and 6 % salt indicating adaptability similar to the trends of strain 541. The highest biomass production was reached in 12 h, after which a decline was observed.

The changes in pH were similar in all conditions of salt concentration (Fig. 5). The rates of pH increase, after initial lowering with acetic acid and the convex curves, were similar. The lowest values in pH were observed between 12–14 h, unlike those in fermentation with strain 541 (8 h). The values for 0–4 % salt were below 6.0, except for ferments with 6 % salt. After this drop in 12–14 h, the pH started to rise, indicating the consumption of lactic acid.

The lactic acid production (in g/L) was highest for 0 and 2 % salt conditions (Fig. 6). DM efficiency was not significantly different and ranged between 60.2–65.5 % (Table 2). This DM value is very low for an efficient fermentation, as were the DP values which ranged between 51.2–54.0 %.

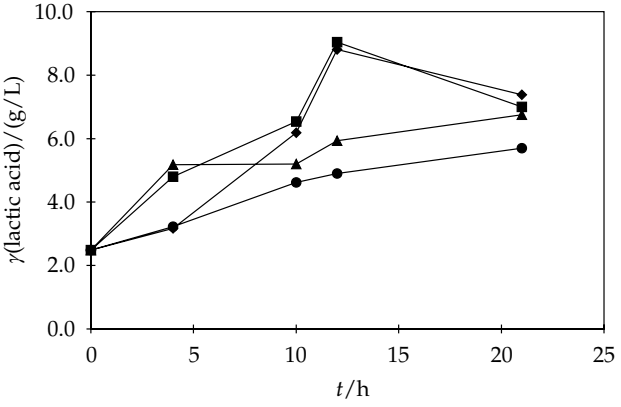


**Fig. 3.** Lactic acid production during fermentation of shrimp waste with *L. plantarum* 541 under salt conditions (◆ 0 %, ■ 2 %, ▲ 4 %, ● 6 %)

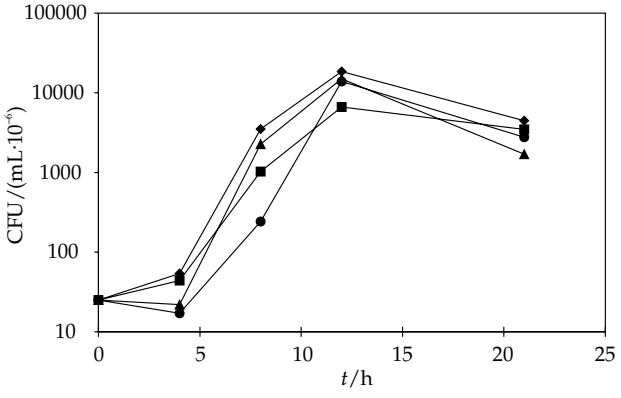


**Fig. 4.** Growth trends during fermentation of shrimp waste with *L. plantarum* A6 under salt conditions (◆ 0 %, ■ 2 %, ▲ 4 %, ● 6 %)

Table 1. Protein and ash content in residues fermented under salt concentrations with *L. plantarum* 541

| *w*(salt)/% | *m*(protein)*/g | *m*(ash)*/g | DP/% | DM/% |
|---|---|---|---|---|
| 0 | 5.54 | 3.62 | 54.2 | 76.4 |
| 2 | 4.86 | 2.86 | 59.8 | 81.4 |
| 4 | 6.58 | 5.04 | 45.6 | 67.2 |
| 6 | 5.66 | 3.33 | 53.2 | 78.3 |
| Original | 12.10 | 15.37 | | |

*Values are from 200 g of shrimp waste, DP = deproteination, DM = demineralization

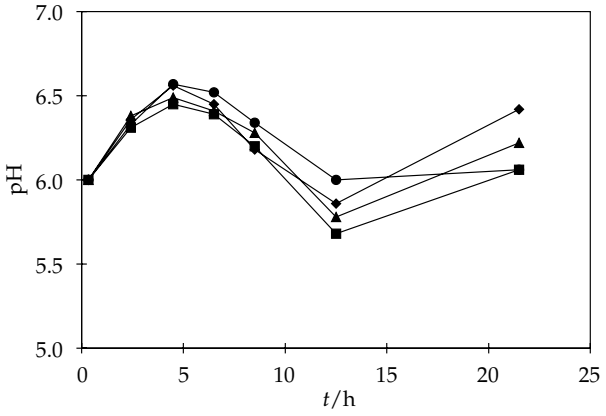


**Fig. 5.** Changes in pH during fermentation of shrimp waste with *L. plantarum* A6 under salt conditions (◆ 0 %, ■ 2 %, ▲ 4 %, ● 6 %)

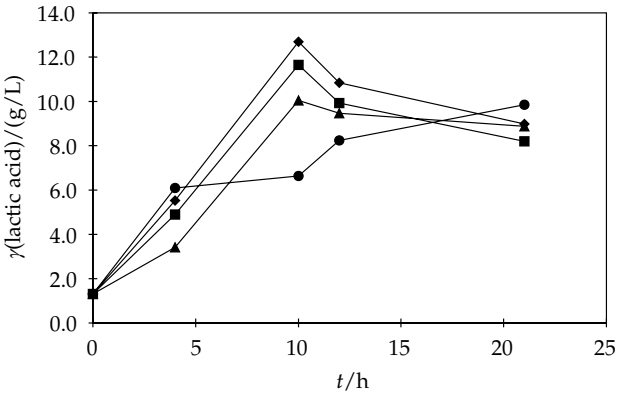


**Fig. 6.** Lactic acid production during fermentation with *L. plantarum* A6 under salt conditions (◆ 0 %, ■ 2 %, ▲ 4 %, ● 6 %)

Table 2. Protein and ash content in residues fermented under salt concentrations with *L. plantarum* A6

| *w*(salt)/% | *m*(protein)*/g | *m*(ash)*/g | DP/% | DM/% |
|---|---|---|---|---|
| 0 | 5.90 | 5.56 | 51.2 | 63.8 |
| 2 | 5.78 | 5.30 | 52.2 | 65.5 |
| 4 | 5.57 | 5.49 | 54.0 | 64.3 |
| 6 | 5.79 | 6.12 | 52.1 | 60.2 |
| Original | 12.10 | 15.37 | | |

*Values are from 200 g of shrimp waste, DP = deproteination, DM = demineralization

## Discussion

The strains were tolerant to salt up to 6 % (*9*). They may not be considered salt resistant, but could be inferred as salt tolerant bacteria. The fermentation efficiency with strain 541 was less affected by the presence of salt than with strain A6. Overall, the DP and DM of biowaste with both strains were low, though slightly higher with 2 % salt concentration. The growth and lactic acid production were not affected to an extent that it would either increase the shelf life of the products or increase DP and DM with increased salts. The strain 541 showed higher fermentation efficiency, especially in terms of demineralization (81 %). Lowering of pH below 6.0 using acetic acid was deemed to be necessary for reduction in spoilage. Preliminary experiments only with the addition of salt and no reduction in pH led to complete spoilage of shrimp biowaste. The addition of salt did not help to maintain low pH conditions either. As a result all the lactic acid produced in these salt conditions was utilized, subsequently increasing the pH after 10–14 h (*7*). Both strains were tolerant to acidic conditions and had no significant effect on the growth of the strains and lactic acid formation (*12*).

Salt concentrations used (2–6 %) were not enough to suppress the growth of spoilage organisms and needed supplementation of acetic acid. In general, higher salt concentration is needed to suppress the spoilage of organism, as in the case of fish sauce production. In conventional technique for fish silage, more than 20 % salt is added. This amount would be too high for shrimp biowaste and lactic acid fermentation would not be possible. It can be inferred that the use of salt in the range of 2–6 % is only a limited option and further research should be done in the use of a halophillic lactic acid bacteria that could resist the salt conditions and produce lactic acid as well. In addition, if the bacteria had amylolytic activity, a cheaper source of carbon such as cassava starch, could be used leading to a very efficient, low cost and environmentally friendly biological process of chitin production.

## Conclusion

The strains *L. plantarum* 541 and A6 showed resistance to salt up to 6 %. The DM and DP were highest at 2 % salt concentrations for both strains. The shrimp waste fermented with strain 541 resulted in DM of 81.4 % and DP of 59.8 %, which was higher than in shrimp waste fermented with strain A6 when DM was 65.5 % and DP was 52.2 %. The addition of acetic acid at the beginning of fermentation was essential, contributing to the reduction in spoilage.

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# Fermentacija otpadaka škampa za proizvodnju hitina pri različitim udjelima soli s amilolitičkim i neamilolitičkim sojevima *Lactobacillus*


**Sažetak**

Fermentacija otpadaka škampa provedena je s pomoću 2 soja *Lactobacillus plantarum*, pri pH=6,0 i različitim udjelima soli. Neamilolitički soj *L. plantarum* 541 i amilolitički soj *L. plantarum* A6 dobro su rasli na otpacima s udjelom soli od čak 6 %. Nakon fermentacije provedene s 10 % inokuluma, 5 % glukoze i uz početno snizivanje pH na 6,0 dodatkom octene kiseline, utvrđeno je da nije došlo do onečišćenja otpadaka škampa. Ustanovljeni su rast sojeva uzgojenih na pločama MRS-agara (u CFU/mL) tijekom fermentacije na otpacima, promjena pH i proizvodnja mliječne kiseline (u g/L). Veća učinkovitost deproteinizacije i demineralizacije postignuta je sa sojem 541 nego sa sojem A6. Sa sojem 541 postignuta je maksimalna demineralizacija od 81,4 % i deproteinizacija od 58,9 % uz dodatak 2 % soli, dok je sa sojem A6 maksimum demineralizacije iznosio 65,5 %, a deproteinizacije 52,2 %.