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Production of chitin from red crab shell waste by successive fermentation with *Lactobacillus paracasei* KCTC-3074 and *Serratia marcescens* FS-3

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Abstract

Successive two-step fermentation was carried out from red crab shell wastes for biological extraction of chitin in combination of the 1st step with a lactic acid bacterium *Lactobacillus paracasei* subsp. *tolerans* KCTC-3074 and the 2nd step with a protease producing bacterium *Serratia marcescens* FS-3, and vice versa. In the 1st step fermentation with KCTC-3074, the pH decreased rapidly from pH 6.90 to 3.31 and TTA increased rapidly to 10.99 for 5 days. At day 7 in the 2nd step fermentation with FS-3, pH further dropped to 2.82 and TTA also dropped to 1.71. In the 1st step fermentation using FS-3, the pH decreased slightly from pH 6.90 to 5.89, and TTA was low as indicated by 1.50 at 5 days. At day 7 in the 2nd step fermentation with KCTC-3074, the pH value was 3.62, and TTA increased to 8.95. The successive fermentation in the combination of FS-3 and KCTC-3074 gave the best result in co-removal of $CaCO_3$ and proteins from crab shells. In this combination, the rates of demineralization and deproteinization were 94.3% and 68.9%, respectively, at the end of fermentation. To date, this is the 1st report on successive fermentation for biological extraction of chitin from crustacean shells.

Keywords: Biological extraction; Successive fermentation; *Lactobacillus paracasei* subsp. *tolerans* KCTC-3074; *Serratia marcescens* FS-3; Chitin

1. Introduction

Chitin, N-acetyl-D-glucosamine polyresidues linked by β-1-4 bonds, is an important natural resource. Chitin and its deacetylated form, chitosan, are widely used in food, cosmetic industries, medical products, agriculture, wastewater treatment, and membranes because of its biodegradability, biocompatibility and non-toxicity. Generally, chitin is obtained mainly from a variety of crustaceans, especially shrimps and crabs by chemical processes using strong acids and bases. However, these processes may cause hydrolysis of the polymer (Brine & Austin, 1981; Healy, Romo, & Bustos, 1994; Simpson, Gagne, & Simpson, 1994), inconsistent physical properties in chitin (Gagne & Simpson, 1993), and source of pollution (Allan, Fox, & Kong, 1978).

Recently, studies of biological process for chitin production have reported using organic acids producing bacteria and enzymes for the demineralization and deproteinization of crustacean shells. Deproteinization processes have been reported for chitin production mainly from shrimp using mechanical (Rao, Tuyen, Stevens, & Chandrkrachang, 2001) and enzymatic processes (Synowiecki & Al-Khateeb, 2000), and microbes such as *Lactobacillus* (Rao, Munoz, & Stevens, 2000; Rao et al., 2001; Rao, Guyot, Pintado, & Stevens, 2002), *Pseudomonas aeruginosa* K-187 (Oh, Shih, Tzeng, & Wang, 2000), and *Bacillus subtilis* (Yang, Shih, Tzeng, & Wang, 2000). Also, demineralization processes have been reported for crayfish using *Lactobacillus pentosus* 4023 (Bautista et al., 2001). In these biological processes, demineralization and deproteinization occur simultaneously but incompletely.

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In our earlier co-fermentation study using lactic acid bacterium *L. paracasei* KCTC-3074 and protease producing bacterium *Serratia marcescens* FS-3, it was found that the two strains were applicable for extraction of chitin by bio-demineralization and bio-deproteinization from crab shells, but deproteinization was not completely achieved (Jung, Jo, Kuk, Kim, & Park, 2006). We supposed that these results came from the drastic pH drop from neutral to 3.5 for 7 days in the co-fermentation of KCTC-3073 and FS-3 mainly due to the acid production by KCTC-3074. In the fermentation of FS-3 alone, the pH values in the culture medium maintained around 6.0, which is the optimum pH for the FS-3 neutral protease. In the co-fermentation system, the proliferation of FS-3 should be affected by the environmental pH disparity, resulting in less production of protease and therefore low deproteinization. In this work, for the efficient processing of chitin recovery from red crab shell waste, we studied successive two-step fermentations with the same two bacteria, especially to improve the deproteinization efficiency, by maintaining the optimum culture conditions for the two species.

2. Materials and methods

2.1. Red crab shell

Red crab (*Chionoecetes japonicus*) was purchased from Yeongdeok crab market, Korea. The leg shell part was separated after steaming and washed with tap water. The shells were cut (2–3 cm length) with scissors and used for fermentation.

2.2. Microorganisms

The lactic acid bacterium *Lactobacillus paracasei* subsp. *tolerans* KCTC-3074 was obtained from the Korean Collection for Type Cultures (KCTC). The protease producing bacterium *S. marcescens* FS-3 was isolated and identified from a disposal site for crab shells on the west coast of Korea as described elsewhere (Jung, Kuk, Kim, & Park, 2005). The strains were stored in a deep freezer at −70 °C.

2.3. Preparation of inocula

In order to prepare a starter culture, the cells were transferred into 100 ml of sterile Man Rogosa Sharpe (MRS) broth for *L. paracasei* KCTC-3074 and LB broth for *S. marcescens* FS-3, and incubated at 30 °C for 2 days. To prepare an inoculum for fermentations, 2.0 ml of the starter culture were transferred into 100 ml of sterile MRS broth (2% inoculation) or LB broth, and incubated with shaking (180 rpm) at 30 °C for 2 days. The inoculum yielded a cell concentration of approximately 10^8 colony-forming units (cfu)/ml.

2.4. Successive two step fermentations with L. paracasei KCTC-3074 and S. marcescens FS-3

To fresh crab leg shells (2.5 g) in 50 ml of 10% glucose solution, 10% *L. paracasei* KCTC-3074 or *S. marcescens* FS-3 was inoculated and incubated in a shaking incubator (180 rpm) at 30 °C for 5 days. After the 1st fermentation, samples were filtered and washed briefly with distilled water. To the sample, 50 ml of 10% glucose solution was added and 10% *L. paracasei* KCTC-3074 or *S. marcescens* FS-3 was inoculated. The 2nd fermentation was carried out at the same condition as in 1st fermentation but for 7 days. To investigate the effect of nitrogen supply, the first fermentation was done as described above with 10% *L. paracasei* KCTC-3074. After 1st fermentation and brief filtering, samples were incubated with 10% *S. marcescens* FS-3 at 30 °C in a shaking incubator (180 rpm) for 7 days with and without 0.5% ammonium sulfate [$(NH_4)_2SO_4$].

2.5. Analysis

Residual dry weight and chitin yield after fermentation was measured after drying at 60 °C for 48 h in an oven. Ash content was determined after combustion at 500 °C for 3 h in an electric furnace (A.O.A.C., 1990). The pH was measured with a pH meter (PH*i* 34, Beckman, USA). Total titratable acidity (TTA) was determined in the diluted samples by titration with 0.1 N NaOH to pH 8.4 and expressed as percent lactic acid (Pearson, 1976). Protein content was determined by the modified method of Shimahara and Takiguchi (1988) as described by Jung et al. (2005).

3. Results

3.1. Successive fermentation with KCTC-3074 and FS-3

The changes in pH, total titratable acidity (TTA), protein, ash contents and chitin yield were chased during the successive treatment of KCTC-3074 and FS-3 (Table 1). The pH decreased rapidly from pH 6.90 to 3.31 for the 5 days in 1st step fermentation with KCTC-3074, but showed little change in the 2nd step with KCTC-3074 or FS-3. TTA increased rapidly to 10.99 for the 5 days in the 1st step with KCTC-3074. TTA in the 2nd step with KCTC-3074 showed little change, however with FS-3 it drastically dropped down to 1% level during fermentation. Ash contents decreased to 3.66% at the end of the 1st fermentation with KCTC-3074, and further decreased to 1.30% and 0.80%, respectively, in 2nd step with KCTC-3074 and FS-3 at day 7, suggesting almost complete demineralization. Protein contents decreased to 18.50% from 22.36% in the 1st step with KCTC-3074 for the 5 days, and further decreased to 15.90% and 10.84%, respectively, in the 2nd step with KCTC-3074 and FS-3 at day 7.

3.2. Successive fermentation with FS-3 and KCTC-3074

During the successive treatment of FS-3 and KCTC-3074, the pH decreased slightly from pH 6.90 to 5.89 for the 5 days in the step fermentation with FS-3, and showed a gradual decline to 4.05 and 3.62 in the 2nd step with FS-3 or KCTC-3074, respectively (Table 1). TTA content showed

Table 1
Changes in pH, total titratable acidity (TTA), ash and protein contents during successive fermentation in the combinations of *L. paracasei* KCTC-3074 and *S. marcescens* FS-3

Treatment		pH	TTA (%)	Ash (%)	Protein (%)	Chitin yield (%)
Fermentation	Days					
Raw material	0	6.90 ± 0.10^{a}	–	41.16 ± 2.50^{a}	22.36 ± 1.82^{a}	–
1st Step L. paracasei KCTC-3074						
	5	3.31 ± 0.15^{h}	10.99 ± 1.00^{b}	3.66 ± 0.21^{fgh}	18.50 ± 1.10^{b}	–
2nd Step *L. paracasei* KCTC-3074						
	1	3.30 ± 0.17^{h}	11.00 ± 1.05^{b}	2.50 ± 0.25^{ghi}	18.30 ± 1.01^{b}	–
	3	3.27 ± 0.23^{h}	11.50 ± 1.20^{ab}	1.70 ± 0.29^{hi}	18.01 ± 1.30^{b}	–
	5	3.26 ± 0.20^{h}	11.59 ± 1.05^{ab}	1.50 ± 0.23^{i}	17.50 ± 1.26^{bc}	–
	7	3.10 ± 0.05^{h}	11.90 ± 0.09^{a}	1.30 ± 0.20^{i}	15.90 ± 1.03^{cd}	38.67 ± 1.35^{a}
2nd Step *S. marcescens* FS-3						
	1	3.19 ± 0.01^{h}	1.26 ± 0.18^{h}	1.05 ± 0.08^{i}	18.13 ± 1.08^{b}	–
	3	2.85 ± 0.03^{i}	1.37 ± 0.45^{h}	0.98 ± 0.27^{i}	17.21 ± 1.34^{bc}	–
	5	2.83 ± 0.02^{i}	1.53 ± 0.09^{h}	0.93 ± 0.23^{i}	14.34 ± 1.06^{d}	–
	7	2.82 ± 0.05^{i}	1.71 ± 0.09^{gh}	0.80 ± 0.12^{i}	10.84 ± 1.03^{e}	36.67 ± 1.33^{ab}
1st Step S. marcescens FS-3						
	5	5.89 ± 0.04^{b}	1.50 ± 0.07^{h}	23.70 ± 1.80^{b}	7.42 ± 0.50^{f}	–
2nd Step *S. marcescens* FS-3						
	1	5.46 ± 0.02^{c}	1.10 ± 0.10^{h}	20.71 ± 0.64^{c}	7.37 ± 0.23^{f}	–
	3	5.12 ± 0.09^{d}	1.43 ± 0.14^{h}	19.15 ± 1.00^{c}	7.01 ± 0.24^{f}	–
	5	5.11 ± 0.03^{d}	2.45 ± 0.01^{fg}	7.92 ± 0.02^{e}	6.52 ± 0.34^{f}	–
	7	4.05 ± 0.04^{ef}	3.91 ± 0.09^{e}	4.05 ± 0.54^{fg}	6.06 ± 0.25^{f}	30.00 ± 1.10^{c}
2nd Step *L. paracasei* KCTC-3074						
	1	4.19 ± 0.01^{e}	2.91 ± 0.04^{f}	14.45 ± 1.16^{d}	7.39 ± 0.44^{f}	–
	3	3.92 ± 0.14^{f}	4.50 ± 0.29^{e}	5.59 ± 0.87^{f}	7.27 ± 0.62^{f}	–
	5	3.68 ± 0.08^{g}	5.52 ± 0.56^{d}	3.62 ± 0.43^{fgh}	7.09 ± 0.52^{f}	–
	7	3.62 ± 0.05^{g}	8.95 ± 0.22^{c}	2.36 ± 0.18^{ghi}	6.96 ± 0.39^{f}	33.50 ± 0.90^{b}

Values in a vertical column followed by different superscripted letters are significantly different at $P \leqslant 0.05$ by Tukey's studentized range (HSD) test.

little change for the 5 days in the 1st step, and increased in the 2nd step to 3.91 and 8.95, respectively, with FS-3 and KCTC-3074 for 7 days. Ash contents decreased to 23.70% at the end of the 1st step fermentation with FS-3, and further decreased to 4.05% and 2.36%, respectively, in 2nd step with FS-3 and KCTC-3074 at day 7. Protein contents decreased to 7.42% from 22.36% in 1st step with FS-3 for 5 days, and further decreased to 6.06% and 6.96%, respectively, in the 2nd step with FS-3 and KCTC-3074 at day 7.

3.3. Demineralization and deproteinization

The demineralization rate was 91.11% and 42.42%, after initial 5 days fermentation, with *L. paracasei* KCTC-3074 and *S. marcescens* FS-3, respectively (Fig. 1A). After 1st fermentation with KCTC-3074, demineralization rate increased to 97.62% in the 2nd step with FS-3 at day 3 but showed little difference between KCTC-3073 and FS-3. After the 1st fermentation with FS-3, demineralization increased to 86.42% in the 2nd step with KCTC-3074 at day 3. At day 7 of the 2nd fermentation, demineralization rates in all treatments reached over 94%.

During the first fermentation, the deproteinization rate was 17.26% and 66.82% with KCTC-3074 and FS-3, respectively, at day 5 (Fig. 1B). After fermentation with FS-3, deproteinization rate increased to 67.49% for 3 days in 2nd step with KCTC-3074. However, after the 1st fermentation with KCTC-3074, deproteinization was 23.03% in the 2nd

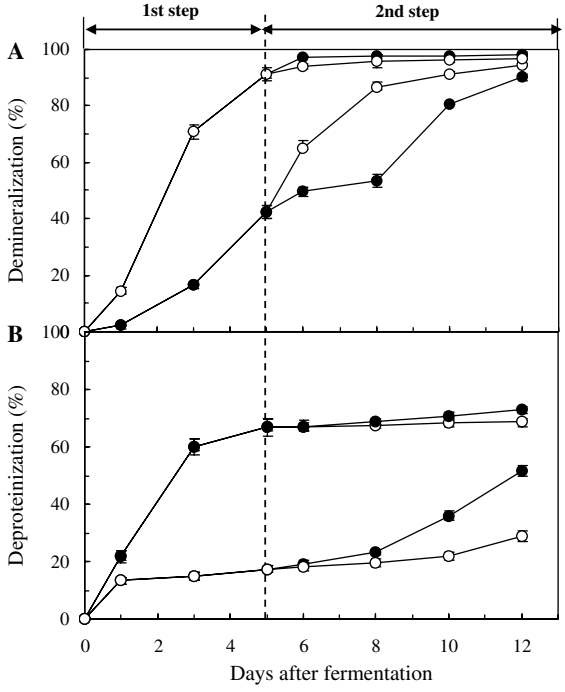

Fig. 1. Rates of demineralization (A) and deproteinization (B) from crab shells during the 1st and the 2nd step fermentations in the combinations with *L. paracasei* KCTC-3074 (○) and *S. marcescens* FS-3 (●).

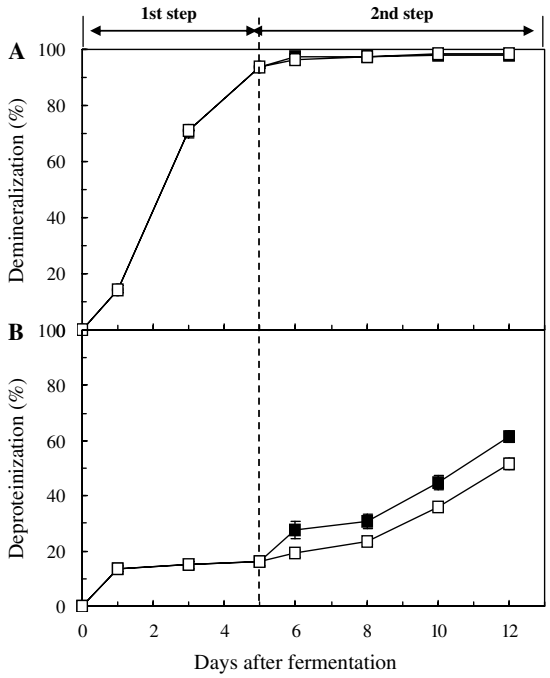

Fig. 2. Effect of $(NH_4)_2SO_4$ addition on demineralization (A) and deproteinization (B) from crab shells during the 1st step fermentation with *L. paracasei* KCTC-3074 and the 2nd step with *S. marcescens* FS-3. 0.5% $(NH_4)_2SO_4$ was added (■) or not (□) at the 2nd step fermentation.

step with FS-3. In case of ammonium sulfate addition as a nitrogen source, the level of deproteinization was elevated from 51.0% to 61.8% (Fig. 2). Yields of crude chitin ranged between 30.0 and 38.67% after fermentation with four combinations.

4. Discussion

In order to improve the extraction efficiency of chitin from crab shell waste, successive fermentation was conducted using both lactic acid bacterium *L. paracasei* KCTC-3074 and protease producing bacterium *S. marcescens* FS-3. As shown in Table 1, single fermentation with *L. paracasei* KCTC-3074 alone achieved higher level of demineralization but was less efficient for deproteinization; while single fermentation with *S. marcescens* FS-3 alone achieved higher level of deproteinization but was less efficient for demineralization. These results are mainly due to the ability of *L. paracasei* KCTC-3074 to produce sufficient amount of organic acids to solubilize $CaCO_3$ but less protease, and *S. marcescens* FS-3 to secrete mainly proteases but not sufficient amount of organic acids.

In our earlier study, co-fermentation together with *L. paracasei* KCTC-3074 and *S. marcescens* FS-3 was exploited to improve the extraction efficiency of chitin from crab shell waste and the highest values of 97.2% demineralization and 52.6% deproteinization were achieved at 7 days of fermentation (Jung et al., 2006). These results warrant the necessity of other protocols in order to improve the bio-deproteinization of crab shells. Thus, the successive fermentation was tried with combination of the 1st step with *L. paracasei* KCTC-3074 and the 2nd step with *S. marcescens* FS-3, and vice versa, as a candidate protocol for the biological extraction of chitin from crab shells.

For demineralization, single cultivation with KCTC-3074 achieved high removal rate, and successive cultivation with FS-3 and KCTC-3074 also achieved the same high rate. For deproteinization, single cultivation with FS-3 achieved high removal rate, and successive cultivation with KCTC-3074 and FS-3 also achieved a high rate. Altogether, the successive fermentation with the combination of FS-3 and KCTC-3074 gave the best results in the co-removal of $CaCO_3$ and proteins from crab shells (Table 1 and Fig. 1). In this combination, the levels of demineralization and deproteinization were 94.3% and 68.9%, respectively, at the end of fermentation. But in other combination of KCTC-3074 and FS-3, the levels were 98.1% and 51.5%, respectively.

These results suggest two important considerations. First, bio-deproteinization is less efficient than bio-demineralization in every treatment. Second, the sequential order of processing is an important consideration. For example, for achieving higher level of deproteinization the fermentation with protease producing bacterium *S. marcescens* FS-3 should come first, and for achieving higher demineralization the fermentation with organic acid producing bacterium *L. paracasei* KCTC-3074 should come first.

These values are comparable to other trials. Demineralization was about 90% during the fermentation of crayfish in 4% lactose and 0.1–0.5% nitrogen source using immobilized cells of *L. pentosus* 4023 (Bautista et al., 2001). Demineralization and deproteinization was 86% and 75% under pH 6.0 adjusted with acetic acid, and 90% and 88% in case of citric acid, respectively, from shrimp bio-waste (Rao et al., 2000). When a 2% salt as N-source was used, demineralization and deproteinization from shrimp waste was 81.4% and 59.8% under 5.5% *Lactobacillus plantarum* 541 (Rao et al., 2002). In this study, supplementation of N-source in culture media also improved the extraction efficiency of chitin from crustacean shell. In case of ammonium sulfate addition, deproteinization was improved (Fig. 2). This may be due to proliferation of the microorganisms and the production of protease by the addition of the inorganic N-source. To date, the successive fermentation is the first case for biological extraction of chitin.

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