Demineralization of Crab Shells by Chemical and Biological Treatments

Woo-Jin Jung, Gyung-Hyun Jo, Ju-Hee Kuk, Kil-Yong Kim, and Ro-Dong Park*

Glucosamine Saccharide Materials-National Research Laboratory (GSM-NRL), Division of Applied Bioscience and Biotechnology, Institute of Agricultural Science and Technology, Chonnam National University, Gwangju 500-757, Korea

Abstract To achieve demineralization of crab shell waste by chemical and biological treatments, lactic acid and lactic acid bacterium were applied. In 5.0 and 10% lactic acid, pH rapidly decreased from 6.8 to 4.2 and from 4.5 to 2.4 at day 3, respectively, and thereafter the pH remained at an almost constant level. In a 10% lactic acid bacterium inoculum, pH lowered to 4.6 at day 5. Relative residual ash content rapidly decreased to 49.1 and 16.4% in 5 and 10% lactic acid treatments, respectively, for the initial 12 h. In 2.5, 5 and 10% lactic acid bacterium inoculums, relative residual ash content rapidly decreased to 55.2, 40.9 and 44.7%, respectively, on the first day. Residual dry masses were 76.4, 67.8 and 46.6% in 2.5, 5 and 10% lactic acid treatments, respectively, for the initial 12 h. After a one-time exchange of the lactic acid solution, in the 5.0% lactic acid treatment, residual dry mass rapidly decreased from 66.0 to 41.4%. In 2.5, 5 and 10% lactic acid bacterium inoculums, residual dry masses decreased to 67.6, 57.4 and 59.6% respectively, on the first day. Protein contents after demineralization ranged from 51.3~54.7% in the chemical treatments and decreased to 32.3% in the lactic acid fermentation process. A negative relationship was shown between pH and demineralization rate in lactic acid and lactic acid bacterium treatments. These results suggest that lactic acid fermentation can be an alternative for demineralization of crab shells, even though the rate and efficiency of the demineralization is lower than the chemical treatment.

Keywords: demineralization, biological treatment, crab shell waste, lactic acid fermentation, chitin

INTRODUCTION

Chitin, a linear copolymer of β(1→4)-2-acetamido-2-deoxy-D-glucan, occurs widely in nature as a principal structural polymer in the integument of insects and crustaceans such as shrimp and crab shells and in the cell walls of many fungi. For chitin preparation, harsh chemical treatments are usually required to remove calcium carbonate and protein from raw chitinous material. This raw material has been most abundantly available in crab shells, shrimp shells, cuttle fish and prawn shells. The raw material has been conventionally treated with a combination of HCl and NaOH [1,2].

Demineralization of crustacean cuticles is performed by acids such as HCl, HNO_3, H_2SO_4, CH_3COOH, and HCOOH. Especially, hydrochloride seems to be the preferred reagent in the case of chitin-containing waste materials including shrimp, krill, crab, prawn, and lobster [3]. However, the aggressive nature of the harsh chemical treatments results in anomerization and hydrolytic effects on the chitin structure [4]. It also causes the serious problem of waste treatment.

These days, milder and environment-friendly protocols have been introduced to overcome the short-comes of a chemical process. Myint *et al.* [5] suggested a reduction of chemical input to prevent the possible decrease of molecular weight of chitin and chitosan. Pretreated shrimp in combinations with crushing, drying, grinding, washing with acidified water, boiling and fermentation can be deproteinized and decalcified at ambient temperature using only 2.5% NaOH and HCl, respectively [6].

In this paper, we investigated the demineralization of crab shell wastes by lactic acid bacterium fermentation and compared these results with the efficiencies of the chemical treatments.

MATERIALS AND METHODS

Crab Shell Waste

Red crab (*Chionoecetes japonicus*) shell waste was obtained from Shinyoung Chitosan Ltd. (Yeongdeck, Korea). The dried shell waste was roughly milled with an electric mixer and samples (particle size : 0.6~1.7 mm) were collected and used for treatments.

***Corresponding author**
Tel: +82-62-530-2133 Fax: +82-62-530-0876
e-mail: rdpark@chonnam.ac.kr

Demineralization with Hydrochloric Acid, Ethyenediaminetetraacetic Acid (EDTA) and Lactic Acid

Crab shell waste (Dry weight, DW 20 g) was thoroughly mixed with 1,000 mL of 2 N HCl. This treatment was carried out at room temperature. Crab shell waste (DW 20 g) was immersed in 2 liters of 0.1 M EDTA solution, previously adjusted to pH 7.5 with aqueous ammonia, at room temperature. Crab shell waste (DW 20 g) was thoroughly mixed with 200 mL of various concentrations [0, 2.5, 5 and 10% (v/w)] of lactic acid, at room temperature. On the 2nd day, the exhausted lactic acid solution was exchanged for a fresh one.

Demineralization with Lactic Acid Bacterium Fermentation

Microorganism and Preparation of the Inoculum

Lactic acid bacteria, *Lactobacillus paracasei* subsp. *tolerans* KCTC-3074, was obtained from Korean Collection Type Cultures. The strains in 50% glycerol as a cryoprotectant were stored in a deep freezer at -70°C. In order to prepare a starter culture, the initial cell was transferred into 250 mL of sterile MRS broth and incubated at 30°C for 2 days. To prepare an inoculum for fermentations, 2.0 mL of the starter culture was subsequently transferred to 100 mL of sterile MRS broth (2% inoculation), and incubated statically at 30°C for 2 days. The prepared inoculum yielded a cell concentration of approximately 10^8 cfu/mL.

Flask Fermentation

Crab shell waste (DW 20 g) was thoroughly mixed with 200 mL of 10% glucose and inoculated with various amounts of the inoculum [0, 2.5, 5 and 10% (v/v)]. Each fermentation was carried out at 30°C with rotary shaking (180 rpm) for 5 days.

Sample Analysis

Dry weight was measured after drying at 60°C for 48 h in an oven. The pH was measured with a pH meter (Beckman, PHi 34, USA). Ash content was determined after combustion at 500°C for 3 h in an electric furnace [7]. Protein content was determined by the modified method of Takiguchi *et al.* [8]. That is, 150 mg of dried material was added to 25 mL of 10 N NaOH in a 100-mL flask. The flask was covered with aluminum foil and heated at 121°C for 60 min in an autoclave. The reaction mixture was then cooled rapidly, neutralized with HCl in an ice bath, and filtered. In a test tube, 0.25 mL of sample solution, 2.5 mL of 0.5 M acetate buffer (pH 5.1), and 2.5 mL of ninhydrin-hydrindantin solution was added and mixed. After incubation in boiling water for 10 min, absorbance was measured at 564 nm. The protein content P was calculated from Eq. (1), where A_{564} stands for absorbance at 564 nm and W for sample mass.

$$P\ (\%) = 2.37\ (A_{564}/W) \quad (1)$$

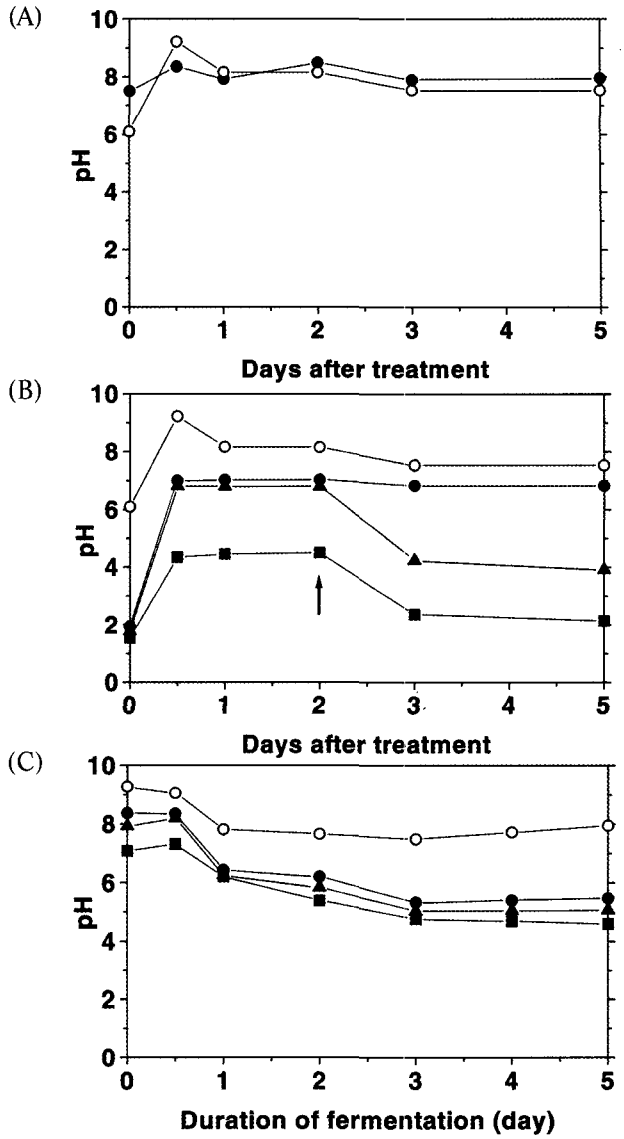

Fig. 1. Changes of pH in each medium during fermentation with (A) EDTA [Control (-○-), 0.1 M EDTA (-●-)], (B) lactic acid [Concentration 0% (-○-), 2.5% (-●-), 5% (-▲-), and 10% (-■-)] and (C) lactic acid bacterium [inoculum 0% (-○-) 2.5% (-●-), 5% (-▲-), and 10% (-■-)]. The arrow in B indicates the exchange of the exhausted lactic acid solution for a fresh one.

RESULTS

The pH in each was measured during EDTA and lactic acid treatments, and lactic acid fermentation (Fig. 1). The pH in the EDTA treatment was maintained continuously above pH 7 and little changed throughout the experimental period. In the 2.5% lactic acid treatment, pH maintained at about 7.0 for 5 days. In 5.0 and 10% lactic acid, pH rapidly decreased from 6.8 to 4.2 and from 4.5 to 2.4 after the exchange of the exhausted acid solution for a fresh one, respectively. Thereafter, pH maintained

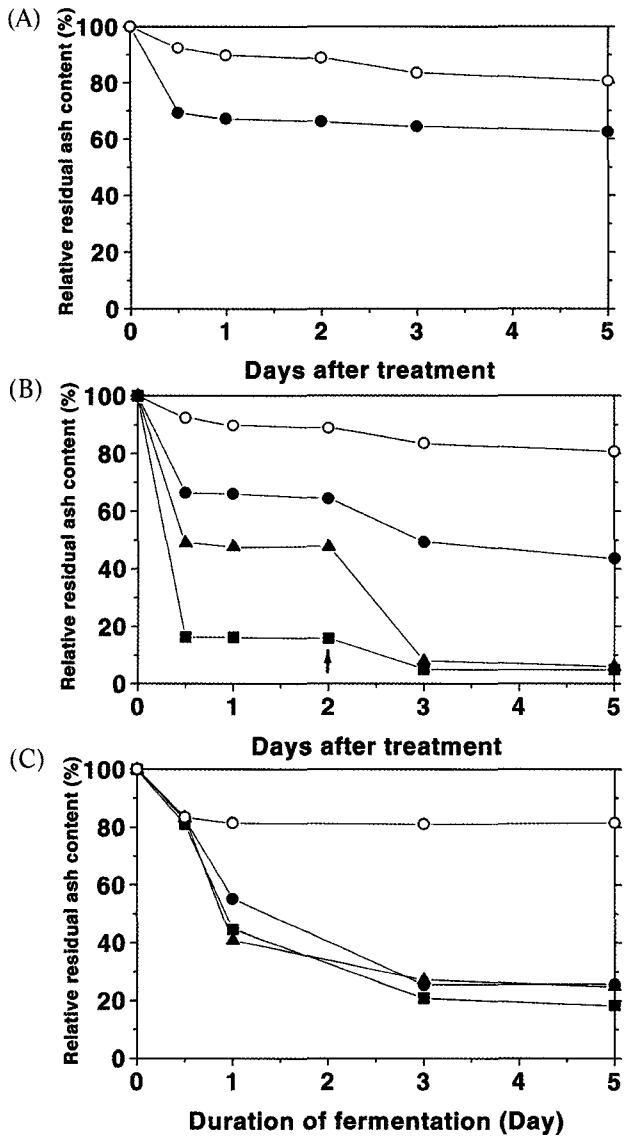

Fig. 2. Changes of relative residual ash content in crab shell wastes during demineralization with (A) EDTA [Control (-○-), 0.1 M EDTA (-●-)], (B) lactic acid [Concentration 0% (-○-), 2.5% (-●-), 5% (-▲-), and 10% (-■-)] and (C) lactic acid bacterium [inoculum 0% (-○-), 2.5% (-●-), 5% (-▲-), and 10% (-■-)]. The arrow in (B) indicates the exchange of the exhausted lactic acid solution for a fresh one.

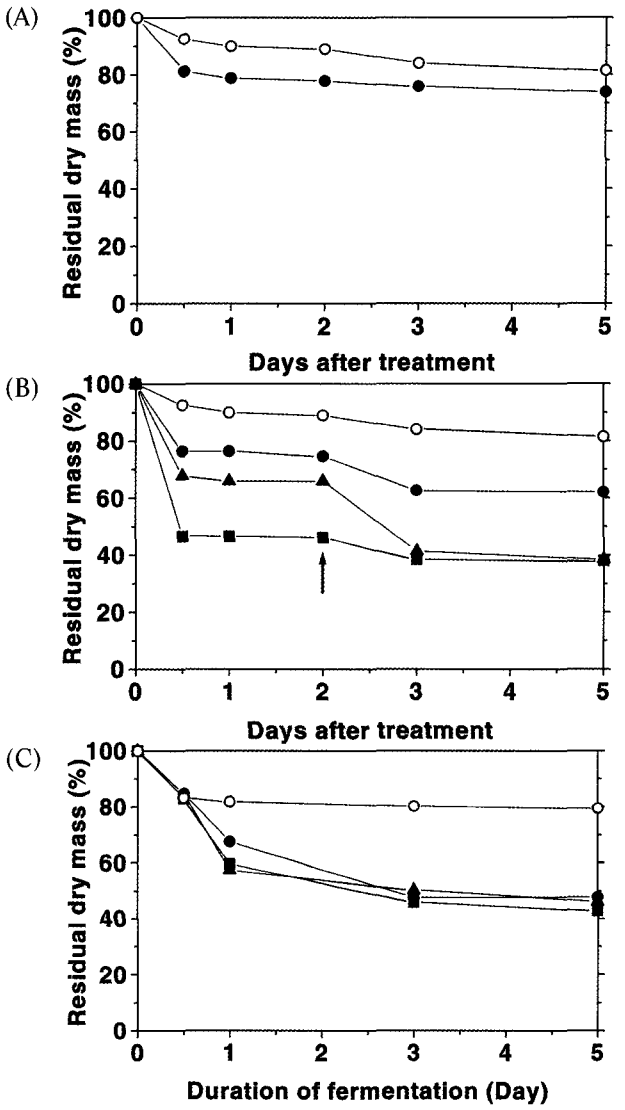

Fig. 3. Changes of residual dry mass in crab shell wastes during demineralization with (A) EDTA [Control (-○-), 0.1 M EDTA (-●-)], (B) lactic acid [Concentration 0% (-○-), 2.5% (-●-), 5% (-▲-), and 10% (-■-)] and (C) lactic acid bacterium [inoculum 0% (-○-), 2.5% (-●-), 5% (-▲-), and 10% (-■-)]. The arrow in B indicates the exchange of the exhausted lactic acid solution for a fresh one.

at almost constant levels. In the 2.5 and 5.0% lactic acid bacterium inoculums, pH rapidly decreased from 8.2 to about 6.3 and from 7.3 to about 6.2 in the first day, respectively. Thereafter, the pH levels slowly decreased. In the 10% inoculum, the pH lowered down to 4.6 at day 5.

Changes of relative residual ash content in crab shell wastes during demineralization with EDTA, lactic acid and microorganism treatments were measured (Fig. 2). The residual ash content when treated with 0.1 M EDTA decreased to 70.0% for the initial 12 h and then maintained a constant level. In 5 and 10% lactic acid treatments, the values rapidly decreased to 49.1 and 16.4%, respectively, for the initial 12 h, and decreased from 47.7 to 8.0% and from 15.9 to 5.2%, respectively, after the exchange of the exhausted acid solution for a fresh one. In lactic acid fermentation with 2.5, 5 and 10% inoculums, the relative residual ash content rapidly decreased to 55.2, 40.9 and 44.7%, respectively, on the first day, and thereafter slowly decreased.

Changes of residual dry mass were observed during demineralization with EDTA, lactic acid and microorganism treatments (Fig. 3). The residual dry mass in the EDTA treatment maintained an 80% level without sig-

Table 1. Residual dry mass, residual ash content and protein content after demineralization of crab shell wastes for 5 days

Treatments		Residual dry mass (%)	Residual ash (%)	Protein (%)
HCl	2 N	36.5 ± 1.2	0.1 ± 0.0	54.7 ± 2.5
EDTA	0 M	81.4 ± 4.1	80.6 ± 7.5	-[b]
	0.1 M	73.8 ± 3.5	62.5 ± 5.5	-
	0.1 M[a]	34.6 ± 2.2	0.2 ± 0.1	53.6 ± 1.9
Lactic acid	0%	81.4 ± 3.1	80.6 ± 2.3	-
	2.5%	62.0 ± 2.7	43.6 ± 1.5	-
	5.0%	38.4 ± 1.3	6.0 ± 0.2	51.3 ± 2.7
	10%	38.0 ± 1.8	5.0 ± 0.4	-
L. paracasei	0%	79.4 ± 2.6	81.5 ± 3.5	-
KCTC-3074	2.5%	47.6 ± 2.1	25.6 ± 1.4	-
	5.0%	46.0 ± 1.4	24.9 ± 1.2	32.3 ± 1.5
	10%	42.8 ± 1.0	18.3 ± 1.5	-

[a] Every 2 days, the exhausted EDTA solution was exchanged for a fresh one. [b] No measurement. All results shown are the mean of three determinations.

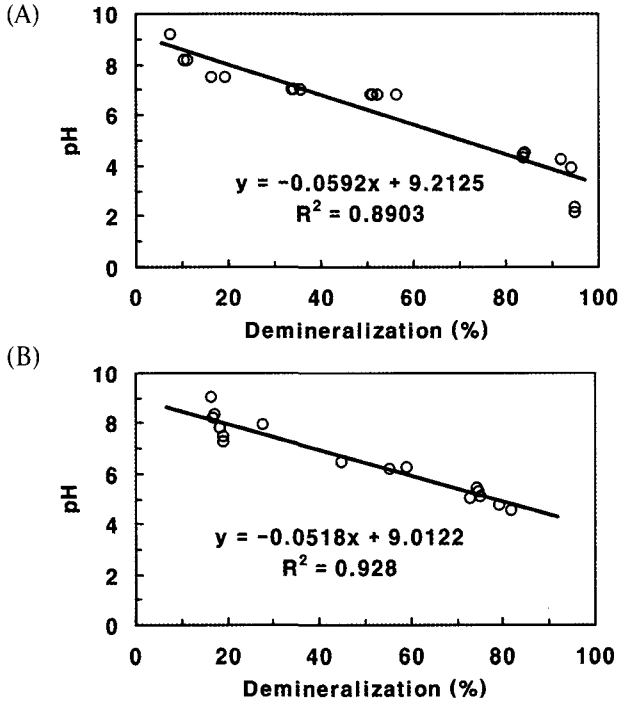

Fig. 4. Correlation between pH in each medium and demineralization in crab shell wastes with (A) lactic acid treatment and (B) lactic acid bacterium fermentation.

nificant changes throughout the experimental period. Residual dry mass rapidly decreased to 76.4, 67.8 and 46.6% in 2.5, 5 and 10% lactic acid treatments, respectively, for the initial 12 h, and then maintained almost constant levels for 2 days. After the exchange of the lactic acid solution, in 5.0% lactic acid, the dry mass rapidly decreased from 66.0 to 41.4%. The dry mass rapidly decreased to 67.6, 57.4 and 59.6% in 2.5, 5 and 10% lactic acid bacterium inoculums, respectively on the first day. Thereafter, the dry mass slowly decreased.

Residual dry mass, ash content and protein content after demineralization of crab shell waste for 5 days are given in Table 1. In the case of an exchange of the exhausted 0.1 M EDTA solution for a fresh one every 2nd day, residual dry mass and ash content were 34.6 and 0.20%, respectively, while the values were 73.8 and 62.5%, respectively, in the case of no exchange of the EDTA solution. The values were 36.5 and 0.1%, respectively, in the 2 N HCl treatment as a reference. In the 5% lactic acid treatment, residual dry mass and ash content were 38.4 and 6.0%, respectively, while the values were 46.0 and 24.9%, respectively, in lactic acid bacterium fermentation with the 5% inoculum. Protein contents ranged from 51.3~54.7% in the chemical treatments and 32.3% in lactic acid bacterium fermentation.

An interrelationship between pH in the medium and demineralization level in crab shells was constructed (Fig. 4). Negative relationships were shown in lactic acid treatment (r^2=0.8903) and lactic acid fermentation (r^2=0.928).

DISCUSSION

Demineralization of crustacean wastes has been carried out with inorganic acids such as hydrochloric [1,2,9] and sulphuric [10], and organic acids such as formic [11], acetic [12], lactic [13], and ethylenediaminetetraacetic acid [14,15]. In this study, we tried lactic acid bacteria fermentation [16,17] in order to demineralize crab shell wastes as a lesser harsh means.

When lactic acid was added to the shell powder, pH increased during the initial 12 h from pH 2 to pH 4.4~7.0 (Fig. 1B), concurrent with a rapid decrease in residual ash content (Fig. 2B), suggesting a rapid solubilization of calcium carbonate. This process was depending

on the acid concentration; the higher the acid concentration, the more efficient was the solubilization of $CaCO_3$, and the lower the residual ash content in the treated mass.

In lactic acid bacteria fermentation with the 10% inoculum, medium pH was maintained around pH 5 after day 3, which is similar with the values of Cira *et al.* [18]. Shirai *et al.* [19] reported that lactic acid fermentation of shrimp wastes in which contained 10% (w/w) glucose and a 5% inoculum of *Latobacillus* sp. B2 lowered from pH 7.5 to pH 4.5. Medium pH likely depends on the content of the energy source such as glucose and sucrose. The pH lowered down to 4.0 when the ratio of shrimp to cassava was adjusted to 2.0 (w/w) [20].

Relative residual ash content of isolated dry material ranged from 5~50% depending on the concentration of chemicals and the inoculum volume of lactic acid bacterium (Fig. 2). Increasing the inoculum concentration resulted in improved demineralization (Fig. 2C), which is in agreement with the report of Shirai *et al.* [19]. Residual ash contents after 5 days of the treatments with 5% and 10% lactic acid were much lower than those in bacterial fermentations.

Residual dry masses in the 5 and 10% lactic acid treatments were about 38% at day 5, while those in lactic acid bacterium fermentations were 43~46%. Protein content in the bacterial fermentations was lower, suggesting utilization of protein in the shell waste during the fermentation process (Table 1).

As shown in Fig. 4, it turned out that pH in the medium is a critical factor for solubilization of $CaCO_3$ from the shells. The pH and residual ash content showed a negative correlation, in which a decrease in pH related to an increase in demineralization. Thus, productivity of organic acids by fermentation is important for efficient demineralization.

The fermentation process for chitin production is still less efficient than conventional chemical treatment. However, this technology will come to offer the advantages of less effluent production and recovery of the protein fraction as an added-value product.

Acknowledgement This study was supported by the National Research Laboratory (NRL) program from the Ministry of Science & Technology (MOST), Korea.

REFERENCES

[1] Hackman, R. H. (1954) Chitin. 1. Enzyme degradation of chitin and chitin esters. *Austr. J. Biol. Sci.* 7: 168-178.

[2] Whistler, R. S. and J. N. BeMiller (1962) Chitin. *J. Org. Chem.* 27: 1161-1163.

[3] Allan, G. G., J. R. Fox, and N. Kong (1978) Marine polymers. Part 8. A critical evaluation of the potential sources of chitin and chitosan. pp. 64-78. In: R. A. A. Muzzarelli and E. R. Pariser (eds.). *Proceeding of 1st International Conference on Chitin/Chitosan*. MIT Sea Grant Rep. MITSG, USA.

[4] Ng, C. H., S. Chandrkrachang, and W. F. Stevens (2000) Effect of the rate of deacetylation on the physico-chemical properties of cuttlefish chitin. *Adv. Chitin Science* 4: 50-54

[5] Myint, K. T., C. H. Ng, S. Chandrkrachang, and W. F. Stevens (2002) Optimal demineralization of crab shells wastes for chitin production. pp. 15-18. In: K. Scchiva, S. Chandrkrachang, P. Methacanon, and M. G. Peter (eds.). *Advance in Chitin Science. Vol. V. Proceeding of the 5th Asia Pacific Chitin and Chitosan Symposium & Exhibition*. Bangkok, Thailand.

[6] Stevens, W. F. (2002) Production and storage of high quality chitosan from shrimp, crab and fungus. pp. 6-11. In: K. Scchiva, S. Chandrkrachang, P. Methacanon, and M. G. Peter (eds.). *Advance in Chitin Science. Vol. V. Proceeding of the 5th Asia Pacific Chitin and Chitosan Symposium & Exhibition*. Bangkok, Thailand.

[7] A.O.A.C. (1980) *Official Methods of Analysis of the Association of Official Analytical Chemists*. Washington DC., USA.

[8] Takiguchi, Y, K. Ohkouchi, H. Yamashita, and K. Shimahara (1987) A new method for quantitative determination of protein associated with crustacean chitin. *Nippon Nogei Kagaku Kaishi*. 61: 437-441.

[9] Roberts, G. A. (1998) Chitosan production routes and their role in determining the structure and properties of the products. pp. 22-31. In: A, Domard, G. A. F. Roberts, and K. M. Varum (eds.). *Advances in Chitin Science. Vol. II*. Jacques Andres, Lyon, France.

[10] Peniston, Q. P. and E. L. Johnson (1978) Demineralization of crustacea shells. *US Patent* 4,066,735.

[11] Horowitz, S. T., S. Roseman, and H. J. Blumenthal (1957) Preparation of glucosamine oligoshaccharides: 1. Separation. *J. Am. Chem. Soc.* 79: 5046-5049.

[12] Bautisa, J., O. Cremades, R. Corpas, R. Ramos, F. Iglesias, J. Vega *et al.* (2000) Preparation of chitin by acetic acid fermenation. pp. 28-33. In: M. G. Peter, A. Domard, and A. A. Muzzaralli (eds.). *Advences in Chitin Science. Vol. 4*. University of Potsdam, Potsdam, Germany.

[13] Zakaria, Z., G. M. Hall, and G. Shama (1998) Lactic acid fermentation of scampi waste in a rotating horizontal bioreactor for chitin recovery. *Process Biochem.* 33: 1-6.

[14] Takeda, M. and E. Abe (1962) Isolation of crustacean chitin: Decalcification by disodium ethylenediaminotetraacetate and enzymic hydrosis of incidental proteins. *Norisho Suisan Koshusho Kenkyu Hokoku* 11: 339-406.

[15] Takeda, M. and H. Katsuura (1964) Purification of king crab chitin. *Suisan Daigaku Kenkyu Hokoku* 13: 109-116.

[16] Lin, J. Q., S. M. Lee, and Y. M. Koo (2004) Modeling and simulation of lactic acid fermentation with inhibition effects of lactic acid and glucose. *Biotechnol. Bioprocess Eng* 9: 52-58.

[17] Wee, Y. J., J. S. Yun, D. H. Park, and H. W. Ryu (2004) Isolation and characterization of novel lactic acid bacterium for the production of lactic acid. *Biotechnol. Bioprocess Eng.* 9: 303-308.

[18] Cira, L. A., S. Huerta, G. M. Hall, and K. Shirai (2002) Piot scale lactic acid fermentation of shrimp wastes for chitin recovery. *Process Biochem.* 37: 1359-1366.

[19] Shirai, K, I. Guerrero, S. Huerta, G Saucedo, A. Castillo, R. O. Gonzalez, and G. M. Hall (2001) Effect of initial glucose concentration and inoculation level of lactic acid

bacteria in shrimp waste ensilation. *Enzym. Microbial. Technol.* 28: 446-452.
[20] Hall, G. M. and S. Silva (1991) Lactic acid fermentation of shrimp (*Penaus monodon*) waste for chitin recovery. pp. 633-638. In: C. J. Brine, P. A. Sandford, and J. P. Zikakis (eds.). *Advance Chitin and Chitosan. 5th International Conference on Chitin & Chitosan.* Elsevier, London, UK.

[Received October 29, 2004; accepted January 6, 2005]