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# Screening of protease-producing *Serratia marcescens* FS-3 and its application to deproteinization of crab shell waste for chitin extraction

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ABSTRACT

*Serratia marcescens* FS-3, isolated from a soil sample in the southwestern area of Korea, exhibited a strong protease activity of 60.7 U/ml after 3 days of incubation. The protein removal from natural crab shell wastes with 10% *S. marcescens* FS-3 inoculum was 84% after 7 days of fermentation. At the same time, 47% demineralization occurred. When the shell waste was treated with 1% Delvolase® as a reference, deproteinization rate was 90%. In combination of 10% *S. marcescens* FS-3 culture supernatant and 1% Delvolase®, deproteinization rate of the shell waste was 85%, while the rate was 81% in 10% FS-3 culture supernatant only. These results suggest that bio-deproteinization of the crab shell wastes using *S. marcescens* FS-3 could be applicable to the chitin production process.



## 1. Introduction

Chitin and its derivatives hold economic value because of their versatile biological activities and agrochemical applications (Cosio, Fisher, & Carroad, 1982; Flach, Pilet, & Jolles, 1992; Knorr, 1991). The main sources of raw material for the production of chitin are cuticles of various crustaceans, principally crabs and shrimps. Chitin in biomass is closely associated with proteins, minerals, lipids, and pigments. They all have to be quantitatively removed to achieve the high purity necessary for biological applications (Roberts, 1992).

Conventionally, preparation of chitin from various crustacean shells involves demineralization and deproteinization with the use of strong acids or bases (Brine & Austin, 1981; Knorr, 1991). Typically, raw shells are treated with approximately 1 M aqueous solutions of NaOH at temperatures ranging from 65 to 100 °C (Roberts, 1992).

For alternative approaches to overcome the shortage of the chemical treatments, microorganisms (Hall & Silva, 1992; Kungsuwan, Kiatkungwalkrai, Mukka, Chandrkrachang, & Stevens, 1996; Teng, Khor, Tan, & Tan, 2001), and proteolytic enzymes (Oh, Shih, Tzeng, & Wang, 2000; Wang & Chio, 1998) for the deproteinization of crustacean wastes has been applied. For example, Yang, Shih, Tzeng, and Wang (2000) reported that shrimp shells was fermented with *Bacillus subtilis* and recorded 88% of protein removal.

A few studies on the use of protease-producing bacteria for the deproteinization of shrimp wastes have been reported, but little has been done with crab shell wastes. Therefore, we attempted to deproteinize and demineralize crab shell wastes using a bacterium *Serratia marcescens* FS-3 which was isolated from environmental samples and exhibited strong protease activity.

## 2. Materials and methods

### 2.1. Materials

Snow crabs (*Chionoecetes opilio*) were purchased from crab market, boiled, and broken into pieces (2–3 cm) with scissors. Flesh was removed with fork and the shells were applied for further experiments. Commercial enzymes such as Delvolase®, Cytolase PCL5®, Econase CEPi®, Econase MP 1000®, Maxazme™ NNP®, and Ccllupulin MG® were purchased from Bision Ltd., Korea.

### 2.2. Screening and identification of protease-producing bacterium

The soil samples from seaside soils in the southwestern area of Korea were extracted in 1% NaCl solution with shaking (200 rpm) and inoculated on LB agar medium containing 1% (w/v) skim milk at 30 °C for 3 days. Colonies showing clear zones were replated on 1% skim milk agar medium. The isolates were subcultured in liquid media containing 5% crab shell on a shaker at 180 rpm at 30 °C. Among them, isolate FS-3 showed the highest protease activity in the culture broth.

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The isolate FS-3 was subjected to taxonomical classification as described in Bergy's Manual of Systematic Bacteriology (Sneath, Mair, Sharpe, & Holt, 1986). Carbon source assimilation was examined by the Biolog GP test kit (Biolog Inc., Hayward, Co.) according to the manufacturer's specifications. To identify the bacterium at the gene level, polymerase chain reaction (PCR) was performed to amplify a part of the 16S rRNA gene of the bacterium. The forward primer Y1 (5′-TGG CTC AGA ACG AAC GCT GGC GGC-3′) and the reverse primer Y2 (5′-CCC ACT GCC TCC CGT AGG AGT-3′) were synthesized and used for the PCR reactions (Weisbug, Barns, Pelletier, & Lane, 1991). The PCR product was cloned using pGEM-T Easy vector (Promega, Inc., Madison, USA). The nucleotide sequence of 16S rRNA gene of FS-3 was determined by an ABI Prism 377 DNA Sequencer (PE Applied Biosystems, USA), and compared with published 16S rRNA sequences using a Blast search at NCBI.

### 2.3. Cultivation

To prepare inoculum for fermentation, 1.0 ml of the starter culture in LB broth was subsequently transferred at a ratio of 2% (v/v) into 100 ml of LB broth containing 5% crab shell wastes, and cultured in a shaking incubator (180 rpm) at 30 °C for 3 days ($A_{660}$ = 3.5).

### 2.4. Deproteinization of crab shell wastes

Four treatments have been done on the crab shells; (1) FS-3 inoculum, (2) Delvolase®, (3) FS-3 culture supernatant, and (4) FS-3 culture supernatant plus Delvolase®. Five milliliters of FS-3 was inoculated into 50 ml of media composed of 5% crab shell and 10% (w/v) glucose (pH 6.9), and incubated in a shaker at 180 rpm at 30 °C. For commercial enzyme, 1% Delvolase® was added to 50 ml of the buffer as recommended in the supplier's instruction containing 5% (w/v) crab shell. FS-3 supernatant (5 ml) was added to 50 ml of liquid media (pH 6.9) containing 5% (w/v) crab shell. FS-3 cultural supernatant (5 ml) and Delvolase® (0.5 ml) were mixed together into 50 ml of liquid media (pH 6.9) containing 5% (w/v) crab shell. After 7 days fermentation at 30 °C, the deproteinized crab shell was obtained.

### 2.5. Sample analysis

Dry weight was measured after drying in a dry oven at 80 °C for 24 h (AOAC, 1990). The pH was measured with a pH meter (PH*i* 34, USA). Ash content was determined by combusting crab shell in an electric furnace at 500 °C for 3 h, and measuring ash weight (AOAC, 1990). The moisture content was measured by drying the crab shell in an oven at 105 °C for 24 h (AOAC, 1990). Protease activity was measured using azocasein as the substrate as described by Chun, Kang, and Kim (2002). One unit of the enzyme activity was defined as the amount of protein causing an increase of 0.01 in $\Delta A_{440}$ at the assay conditions. Protein content was determined by modified method of Takiguchi, Ohkouchi, Yamashita, and Shimahara (1987). That is, 150 mg of dried material was added to 50 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. Final volume of filtrate was adjusted to 150 ml with distilled water. In a test tube, 0.50 ml of sample solution, 5 ml of 0.5 M acetate buffer (pH 5.1), and 5 ml of ninhydrin-hydrindantin solution are 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.

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

### 2.6. Statistical analysis

Treatment effects were determined by analysis of variance (one-way ANOVA) according to the general linear model procedure of the Statistical Analysis System 8.1 (SAS Institute, 2000). Means were separated with Tukey's Studentized Range Test at $P$ = 0.05.

## 3. Results

### 3.1. Screening of protease-producing bacterium

Most of the suspensions which were washed out of the soil samples with 1% NaCl solution produced protease activity, when inoculated into the medium supplemented with 1% skim milk as the protein source. During the subsequent screening experiments, various kinds of microbial colonies formed large and clear halos on the LB-skim milk agar were observed (Fig. 1). Several bacterial cultures, in which the colonies formed large and clear halos, were tested for protease activity after grown in the LB-skim milk liquid medium. Most of these isolates exhibited significant levels of protease activity in the liquid medium. Among them, bacterial isolate FS-3 showed the highest protease activity.

### 3.2. Identification of strain FS-3

Bacterial isolate FS-3 was subjected to a taxonomic analysis as described in Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986), and identified as a member of the genus *S. marcescens*. This taxonomic identification was based mainly on the following criteria. This was confirmed by morphological characteristics of the isolate observed using SEM (Fig. 2). The strain was Gram-negative, aerobic, motile, catalase-positive, and rod-shape (0.47–0.60 μm with × 1.15–1.36 μm length), as shown in Table 1 and Fig. 1. The isolate FS-3 grows in 7% NaCl, at pH 11.0, and at 50 °C (Table 1). Assimilation tests using the Biolog system identified the isolate FS-3 as *S. marcescens* (with similarity index of 0.65). On the basis of the nucleotide sequence of the 16S rRNA gene, GS-3 was confirmed as *S. marcescens*. The phylogenetic tree determined by the Clustal method showed that FS-3 is most similar *S. marcescens* AU1209, with more than 99% similarity (Fig. 3). From

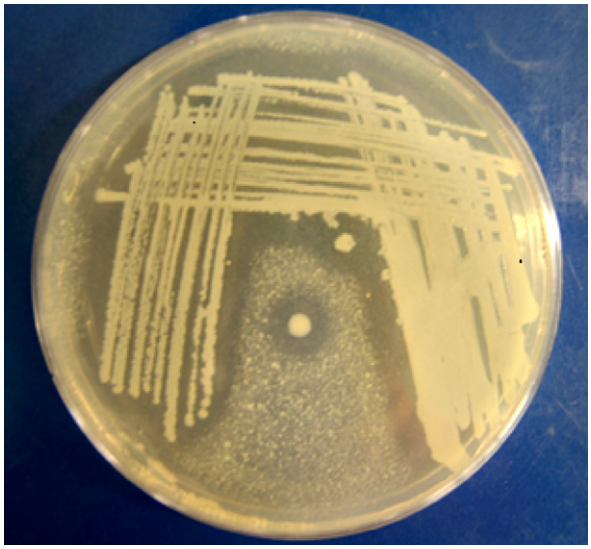

**Fig. 1.** Clear zone of FS-3 on the LB medium containing 1% skim milk.

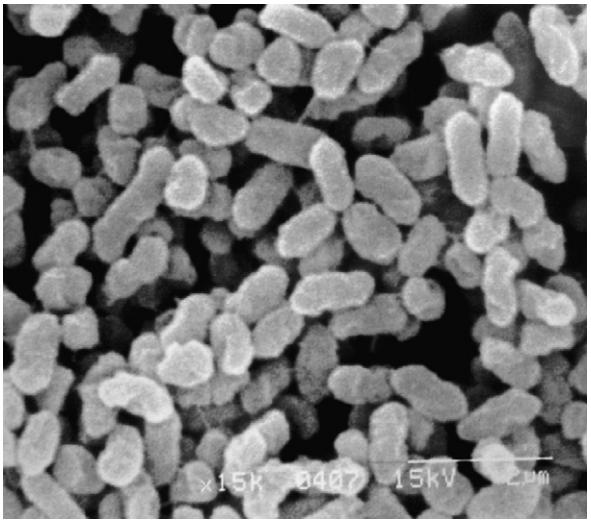


**Fig. 2.** Scanning electron micrograph of FS-3.

**Table 1**
Morphological, biological and physiological characteristics of *Serratia carcescens* FS-3

| Characteristics result | |
|---|---|
| Size | 0.47–0.60 μm width × 1.15–1.36 μm length |
| Gram staining | Gram-negative |
| Mobility | + |
| Sporangium | Not swollen |
| Spore | + |
| Catalase | + |
| Urease | + |
| Voges–Proskauer test | + |
| Acid from | |
| D-Glucose | + |
| D-Mannitol | – |
| D-Xylose | – |
| L-Arabinose | – |
| Hydrolysis of casein | + |
| Hydrolysis of starch | + |
| Utilization of citrate | – |
| Nitrate reduction | – |
| Formation of indole | – |
| Growth in NaCl 2% | + |
| 5% | + |
| 7% | + |
| 10% | – |
| Growth at pH 4.5 | + |
| 9.0 | + |
| 11.0 | + |
| Growth at 5 °C | – |
| 10 °C | + |
| 42 °C | + |
| 50 °C | + |

these results, isolate FS-3 was identified as a member of *S. marcescens* and designated as *S. marcescens* FS-3.

### 3.3. Cell growth and protease production of S. marcescens FS-3

The isolate *S. marcescens* FS-3 was cultured in a flask for protease production in a medium containing 5% crab shell wastes at 30 °C. Cell growth and enzyme production were best at 30 °C among various temperatures (20–42 °C) tested in shaking incubator (180 rpm). As shown in Fig. 4, the highest level of protease activity was 60 U/ml in the cultural supernatant after 3 days culture and decreased slightly thereafter. The cultural supernatants were used for deproteinization of crab shell wastes.

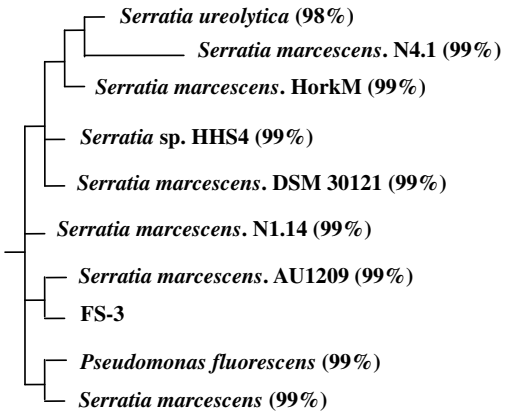


**Fig. 3.** Phylogenetic location of bacterial strain FS-3 based on partial 16S rRNA sequences.

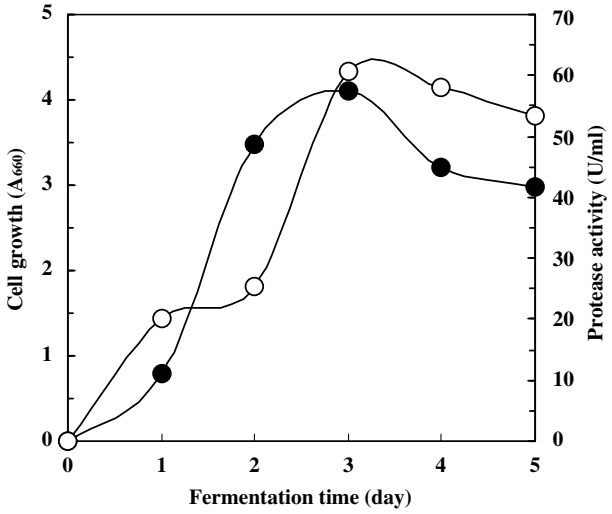


**Fig. 4.** Cell growth and protease activity of protease-producing *Serratia marcescens* FS-3. -●- , Cell growth; -○- , protease activity.

### 3.4. Deproteinization rate of crab shells

To compare the deproteinization efficiency of crab shells, four treatments have been done on the crab shells; (1) FS-3 inoculum, (2) Delvolase®, (3) FS-3 culture supernatant, and (4) FS-3 culture supernatant plus Delvolase®. As shown in Fig. 5, when treated with *S. marcescens* FS-3 only, the deproteinization rate gradually increased up to 84% on day 7. When treated with 1% Delvolase® only, the rate reached to about 85% after 1 day and maintained a 90% plateau thereafter. When the shells was treated in the combination of 1% Delvolase® and FS-3 culture supernatant, the rate was about 60% after 1 day and increased to about 85% after 7 days. In case of the culture supernatant only, the deproteinization rate was about 76% after 7 days of fermentation.

### 3.5. Properties of crab shell residues after various treatments

The pH, moisture, dry weight, ash amount, protein amount, and the rates of deproteinization and demineralization of crab shell residues after treatments are shown in Table 2. Deproteinization rates ranged 81–90% and demineralization rates ranged 0.01–47%. When treated with *S. marcescens* FS-3, the demineralization occurred with deproteinization. But, little demineralization occurred in the treatments with enzymes only, such as Delvolase®

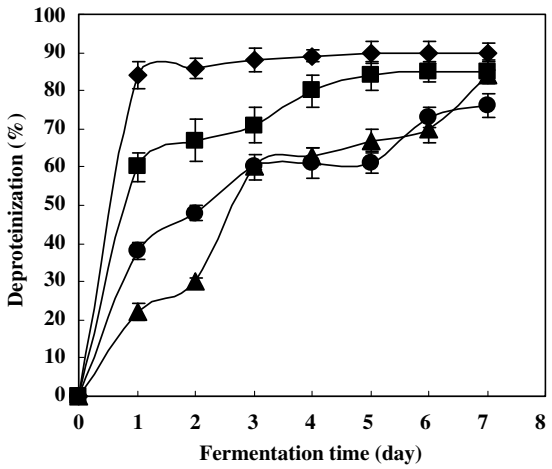


**Fig. 5.** Deproteinization of natural crab shell wastes 7 days after biological treatments. -▲- , *Serratia marcescens* FS-3; -♦- , Delvolase®; -●- , *Serratia marcescens* FS-3 supernatant; -■- , combination of *Serratia marcescens* FS-3 culture supernatant and Delvolase®.

and FS-3 culture supernatant plus Delvolase®. Fermentation solution, cultured with *S. marcescens* FS-3 contained acetic acid analyzed by gas chromatography. The yield of acetic acid was 68 μmol/ml and demineralization of crab shell wastes by *S. marcescens* FS-3 was 47% after treatment 7 days (data not shown). Thus, the ash content in the residues was lowest in 10% FS-3 inoculum but highest in Delvolase® only.

## 4. Discussion

The application of microorganisms or proteolytic enzymes for deproteinization of marine crustacean wastes is a current research trend in conversion of wastes into useful biomass. It is a simple and environment-friendly alternative to chemical methods employed in the preparation of chitin. In this paper, the microbial fermentation process has been applied using a protease-producing bacterium *S. marcescens* FS-3, which was isolated from environmental samples. To our knowledge, the use of *Serratia* spp. for this purpose has never been demonstrated before. Fermentation of the crab shells with FS-3 deproteinized up to 84% of the shell proteins on 7 days fermentation, suggesting efficiency of the bio-deproteinization process (Fig. 5). This value is comparable with other similar trials. Oh et al. (2000) reported that fermentation of shrimp shell powder with *Pseudomonas aeruginosa* K-187 recorded 72% of deproteinization after 7 days treatment. Yang et al. (2000) reported that after 7 days treatment of natural and HCl-treated crab shell wastes with *B. subtilis*, deproteinization rates were 67% and 62%, respectively. Shimahara and Takiguchi (1988) have used *P. maltophilia* for deproteinization of demineralized shell chips from various sources and found protein content of the demineralized shell chips from prawn diminishes to approximately 1%. As for demineralized crab and lobster, the residual protein was 5% and 8%, respectively, after 8 days of deproteinization.

The deproteinization rate was affected by crab shell content, when fermented with *S. marcescens* FS-3 (Fig. 6). Around 80% deproteinization was recorded in 5–10% shell content where all the shell was submerged in the culture medium (solid to liquid ratios, 1:5–10 w/v), but less than 55% deproteinization in 20–30% shell content where submerged fermentation occurred (solid to liquid ratios, 1:1.7–2.5 w/v). Demineralization also declined from 52.1% to 18.9% according to the content, showing an efficiency of submerge culture over the submerged fermentation and a limitation of increasing shell content in the culture medium. This result was different from others that submerged fermentation removed proteins better than the liquid fermentation from shrimp shells (Wang & Chio, 1998).

A few studies on use of proteolytic enzymes for the deproteinization of crustacean wastes have been reported. Tuna proteinase, papain, and a bacterial proteinase have also been used for the deproteinization (Broussignac, 1968). The residual protein associated with the chitin after enzyme treatment was about 5% (Takeda & Abe, 1962; Takeda & Katsuura, 1964). Gagne and Simpson (1993) also showed that the residual protein levels in the shrimp waste after deproteinization were 1.3% and 2.8% for chymotrypsin and papain-treated samples. High enzyme to waste ratio (E/W) was needed for maximum deproteinization. The E/W ratios were 0.7% and 1.0 (w/w) for chymotrypsin and papain, respectively (Gagne & Simpson, 1993).

In this study, we also compared the deproteinization efficiency of commercial enzymes such as Delvolase®, Cytolase PCL5®, Econase CEPi®, Econase MP 1000®, Maxazme™ NNP®, and Ccllupulin MG®, and found out that Delvolase® obtained by submerged culturing of a selected strain of *Bacillus licheniformis* showed the high-

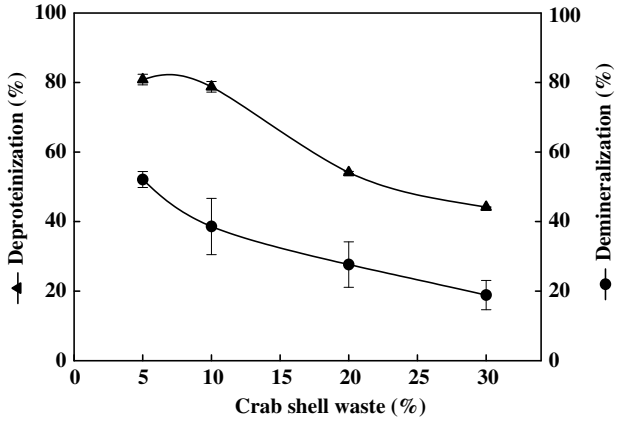


**Fig. 6.** Effect of crab shell waste content on deproteinization of the shell wastes. Five milliliters of *Serratia marcescens* FS-3 was inoculated into 50 ml of media composed of 10% (w/v) glucose and 5–30% crab shells (pH 6.9), and incubated in a shaker at 180 rpm at 30 °C for 7 days.

**Table 2**
Properties of crab shell wastes residue 7 days after biological treatments

| Treatment | pH | Dry weight (g) | Ash (%) | Protein (%) | Deproteinization (%) | Demineralization (%) | Cell count (CFU/ml) |
|---|---|---|---|---|---|---|---|
| No | $6.9 \pm 0.1^{a,*}$ | $1.50 \pm 0.03$a | $41.6 \pm 1.0^{a}$ | $22.4 \pm 2.0^{a}$ | – | – | – |
| A | $5.9 \pm 0.1^{c}$ | $0.83 \pm 0.05^{cd}$ | $22.0 \pm 0.8^{c}$ | $3.6 \pm 0.02^{b}$ | $84 \pm 1.6^{ab}$ | $47.00 \pm 1.20^{a}$ | $3.0 \times 10^{6}$ |
| B | $6.5 \pm 0.1^{b}$ | $1.10 \pm 0.06^{b}$ | $41.3 \pm 1.4^{a}$ | $2.3 \pm 0.01^{c}$ | $90 \pm 2.9^{a}$ | $0.01 \pm 0.01^{c}$ | – |
| C | $6.9 \pm 0.2^{a}$ | $0.70 \pm 0.05^{d}$ | $36.0 \pm 0.9^{b}$ | $4.4 \pm 0.04^{b}$ | $81 \pm 2.9^{b}$ | $12.0 \pm 0.05^{b}$ | – |
| D | $5.9 \pm 0.2^{c}$ | $0.95 \pm 0.07^{c}$ | $40.5 \pm 1.2^{a}$ | $3.4 \pm 0.01^{bc}$ | $85 \pm 3.2^{ab}$ | $0.03 \pm 0.01^{c}$ | – |

A, *Serratia marcescens* FS-3; B, Delvolase®; C, *Serratia marcescens* FS-3 supernatant; D, Combination of *Serratia marcescens* FS-3 culture supernatant and Delvolase®.
* Values in a column followed by different superscripted letters are significantly different at $P < 0.05$ by Tukey's Studentized Range Test.

est deproteinization activity for crab shell waste. When treated the crab shells with 1% Delvolase®, the deproteinization rate reached to about 85% within 1 day and maintained 90% of plateau thereafter. The commercial enzyme Delvolase® was most rapid and effective in deproteinization of the crab shells, but no complete removal of the residual protein associated with the chitin. The E/W ratios between 0.5% and 1.0% (w/w) for Delvolase® showed little difference in deproteinization efficiency of the shells but the ratio less than 0.25% for Delvolase® resulted in lower efficiency (data not shown).

There were few reports comparing the deproteinization effects between microbes and enzymes. Bustos and Michael (1994) have compared the effects of microbial and enzymatic deproteinization. A maximum value of 82% in deproteinization was achieved with *P. maltophilia* after 6 days of incubation, but no more than 64% in deproteinization was achieved by using a purified microbial protease under the same condition. Rao, Tuyen, Stevens, and Chandrkrachang (2001) applied the combination of papain and GBW protease as commercial enzymes on the deproteinization of shrimp wastes and found that removal rate of protein in the waste with enzymes were low. As shown in Fig. 5, the deproteinization rate was about 76% after 7 days of incubation of the shells with FS-3 culture supernatant, exhibiting about 10% lower efficiency than microbial deproteinization. Beside the high deproteinization efficiency, microbial fermentation is another advantage of demineralization (Table 2). About 50% of demineralization occurred in concordance with deproteinization during microbial fermentation. This was considered that organic acids produced by the bacteria dissolved the calcium carbonate from the crab shell waste. Finally, the combination of Devolase® and FS-3 culture supernatant was also effective in deproteinization. The rate was about 60% after 1 day and increased to about 85% after 7 days. The removal rate in combination of Delvolase® and FS-3 culture supernatant was lower than that of Delvolase®, mainly due to pH drop from FS-3 culture supernatant (pH 5.6–5.8).

From above results, it has been demonstrated that the protease produced by *S. marcescens* FS-3 can be effectively deproteinization of crab shell wastes. Further more, the utilization of the processing waste of crab shells by bio-convertion should solve the environmental problem associated with crustacean processing.

## Acknowledgements

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