CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Isolation and screening of microorganisms

It was found that forty-five isolates formed clear zone on LB agar medium after 2 days of cultivation (Table 4.1). Twenty-nine isolates could grow on crab shell agar and twenty strains showed protease activity in liquid medium.

It can assume that these isolates can produce some enzyme for digested crab shell. All twenty isolates will further use for determination protease activity.

The measuring protease activity by using casein as substrate and tyrosine as the reference compound, isolate number EST03, SST03, ECM04 and SCM03 produced protease activity 2.16, 2.06, 2.64, and 2.40 unit/ml, respectively. The ECM04 isolate produced the highest protease activity of 2.64 unit/ml (Table 4.1).

After the comparison of protease activity the result showed in Table 4.1 that two isolates obtained from soil in Suratthani province and two isolates obtained from soil in Chiang Mai province can produced higher protease activity than others isolates.

Table 4.1 Forty-five isolates from different areas obtained from first screening.

Source of microorganisms	**Number of isolates on LB+1%+ skim milk**	**Number of isolates on crab shell agar**	**[a]Protease activity of some strains (U/ml)**
Soil around Suratthani pasteurized crab factory	8	8	EST01 = 0.94 EST02 = 0 EST03 = 2.16 EST04 = 1.54 SST01 = 0 SST02 = 1.78 SST03 = 2.06 SST04 = 1.99
Pickled fish from Lampoon province	10	4	LPF102 = 0.03 LPF104 = 0 LPF203 = 0 LPF205 = 0.06
Soil from the garden in Chiang Mai province that full of snails	12	7	ECM01 = 0 ECM02 = 0 ECM03 = 0 **ECM04 = 2.64** ECM05 = 1.64 SCM03 = 2.4 SCM06 = 0

Table 4.1 (cont.) Forty-five isolates from different areas obtained from first screening.

Source of microorganisms	Number of isolates on LB+1%+ skim milk	Number of isolates on crab shell agar	[a]Protease activity of some strains (U/ml)
Seaside soils in Songkhla province	15	10	SKS102 = 0 SKS103 = 1.99 SKS201 = 0.88 SKS202 = 0.82 SKS203 = 0.15 SKS204 = 0.41 SKS205 = 0.59 SKS206 = 1.00 SKS207 = 1.29 SKS208 = 0.05
Total	**45**	**29**	

[a]Protease activity obtain from minimal synthetic medium after 2 days incubation.

4.2 Morphological characteristic

In the studied of morphological characteristic as shown in Figure 4.1, it was found that (A) EST03, and (D) SCM03 are gram-negative bacilli in chain. (C) ECM04 is a gram-positive single cell-bacilli with endospore-former. (B) SST03, is a club-shaped gram-negative. In size it is approximately 1 to 2 μm.

Isolate ECM04 was rough edge cream colony and under the oil immersion lens (1,000×) for the presence of endospores. Endospores are green and vegetative cells are brownish red to pink (Figure 4.2).

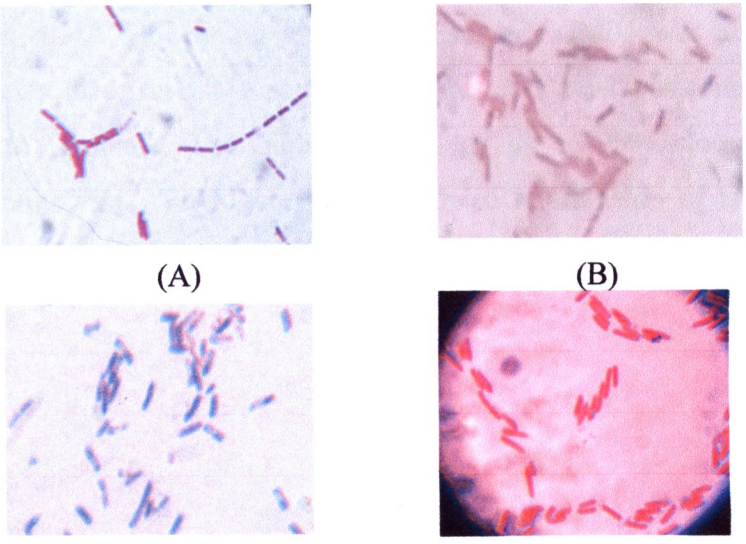

Figure 4.1 The morphology characteristic of 4 isolates which could produce higher protease enzyme; (A) EST03, (B) SST03, (C) ECM04, and (D) SCM03.

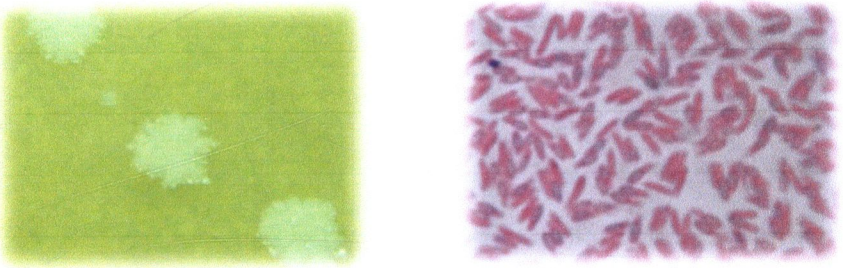

Figure 4.2 The morphology characteristic of strains ECM04. (A) colonies morphology and (B) single rod cell showing green endospore.

4.3 16s rDNA sequence analysis

The 16s rRNA sequencing analysis of isolate ECM04 was also studied to confirm its species. The 16s rDNA sequence of isolate ECM04 was compared to other bacteria gene in Genbank. The highest homology up to 99% closed to *Bacillus amyloliquefaciens DSM7*. Phylogenetic tree showed relationship of isolate ECM04 and its close species as Figure 4.3 and 4.4.

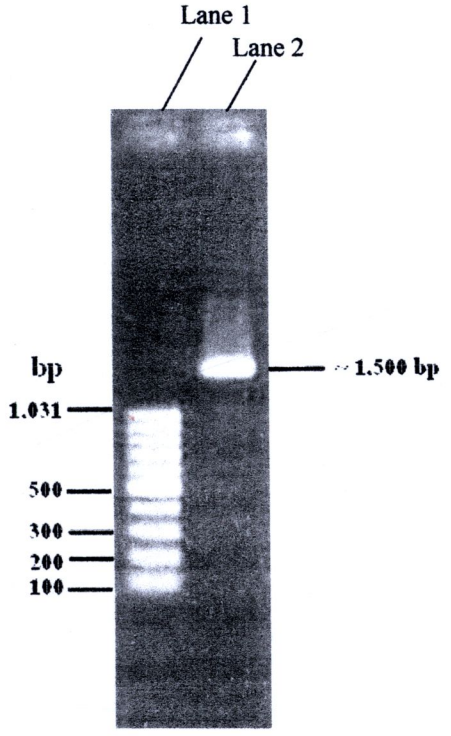

Figure 4.3 PCR product of the isolate ECM04.
Lane 1 (from left), standard DNA O' GeneRuler^{TM} 100 bp DNA Ladder.
Lane 2, PCR product of 16s rRNA of isolate ECM04.

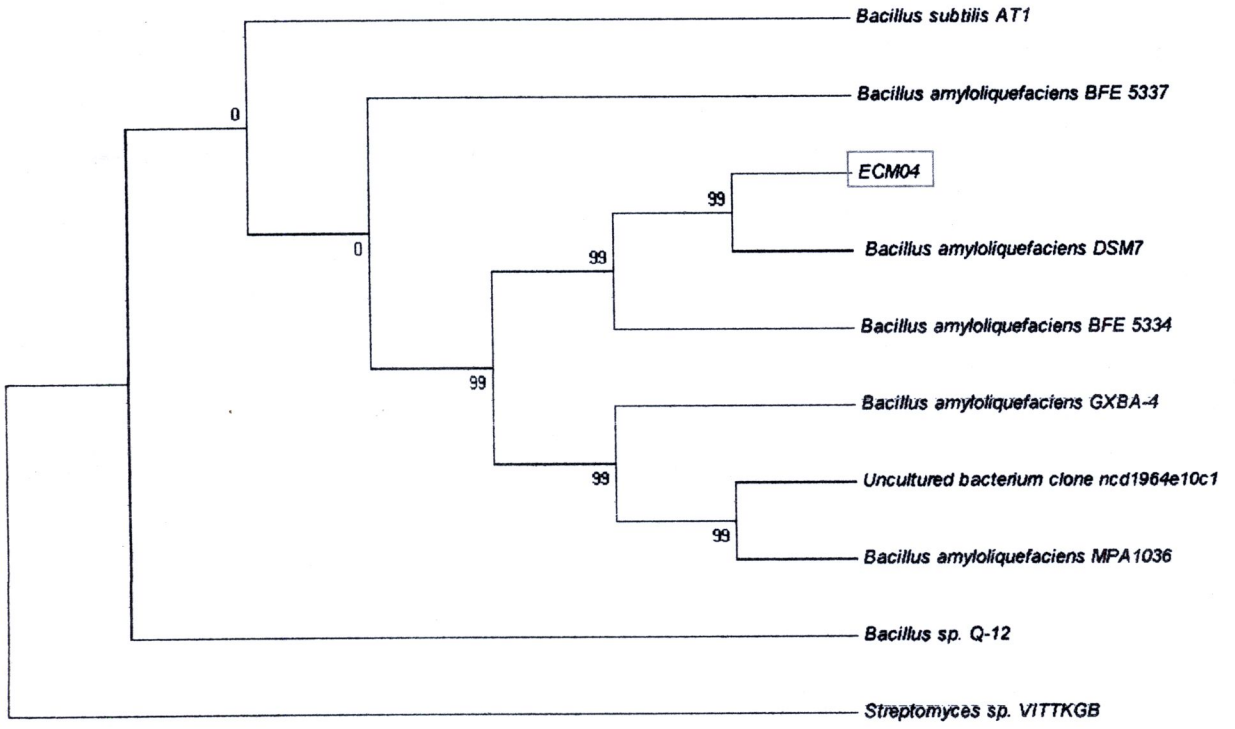

Figure 4.4 Phylogenetic tree of bacteria isolate ECM04.

4.4 Effect of culture conditions on enzyme production

4.4.1 Effect of cultivation time

The isolate ECM04 was cultured in a flask for protease production in a medium containing 7% crab shell wastes at 37°C. As shown in Figure 4.5, the highest level of protease activity was 2.0 U/ml in the cultural supernatant after 36 hours culture and decreased slightly thereafter.

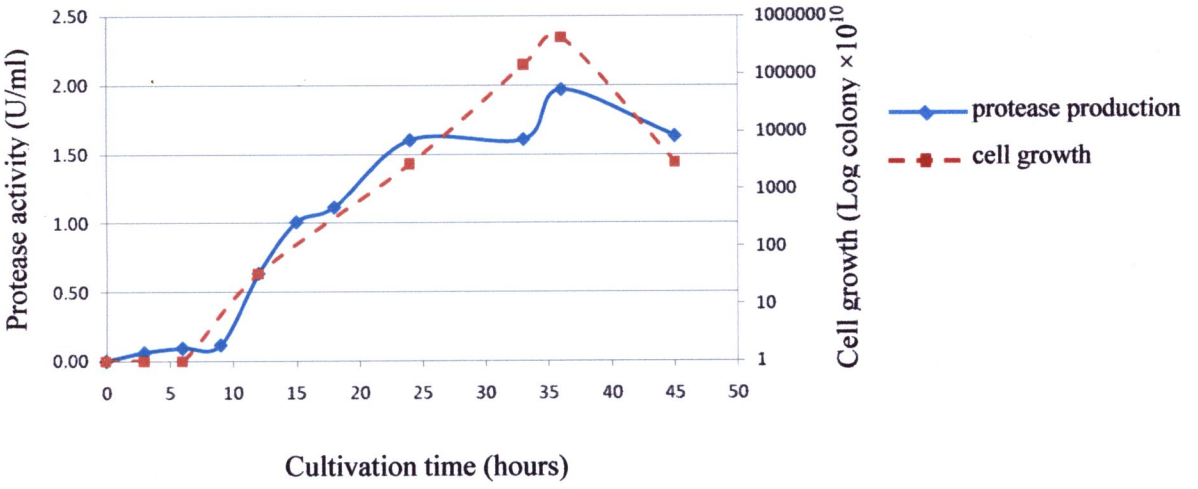

Figure 4.5 Effect of cultivation time on protease production and cell growth.

4.4.2 Effect of crab shell powder concentration on protease production

The effect of different initial crab shell powder concentration on the protease production by isolate ECM04 was investigated. The results showed in Figure 4.6. The maximal protease activity was significantly higher than others concentration when the crab shell powder was 7%. This is related to previous report by Yang and his worker (2000) which have been improve protease production by *B. subtilis* when 7% shrimp and crab shell were applied.

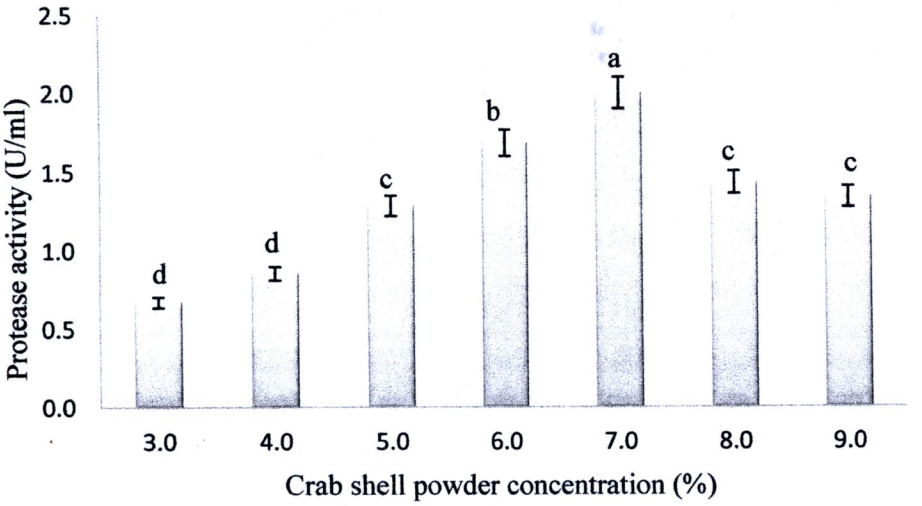

Figure 4.6 Effect of crab shell concentration on enzyme activity. Different letters refer to the significantly different.

4.4.3 Effect of carbon source on protease production

To study the effect of carbon sources on the production of protease, the growth of ECM04 isolate was carried out in minimal synthetic medium containing 7% crab shell powder and 0.5% either no additional carbon sources of D(-)arabinose, carboxymethyl cellulose (CMC), cellulose, glucose, lactose, D(+)xylose, respectively. The production of protease by isolate ECM04 was greatly enhanced and significantly different with other carbon sources and control by the addition of CMC into the medium (Figure 4.7).

The secretion of protease was slightly enhanced by addition of the carbon sources tested but different with adding D(-)arabinose and D(+)xylose, which repressed protease production to some degree.

Then the effect of CMC concentration was further studied. It was found that 0.75% CMC was the most effective concentration for protease production (Figure 4.8).

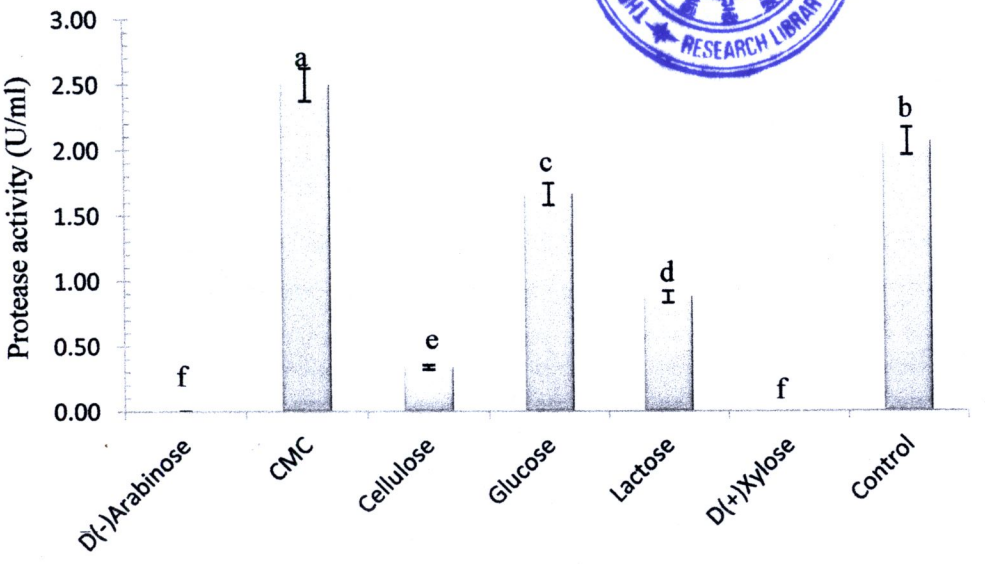

Figure 4.7 Enzyme activity in each carbon source supplement (0.5%). Different letters refer to the significantly different.

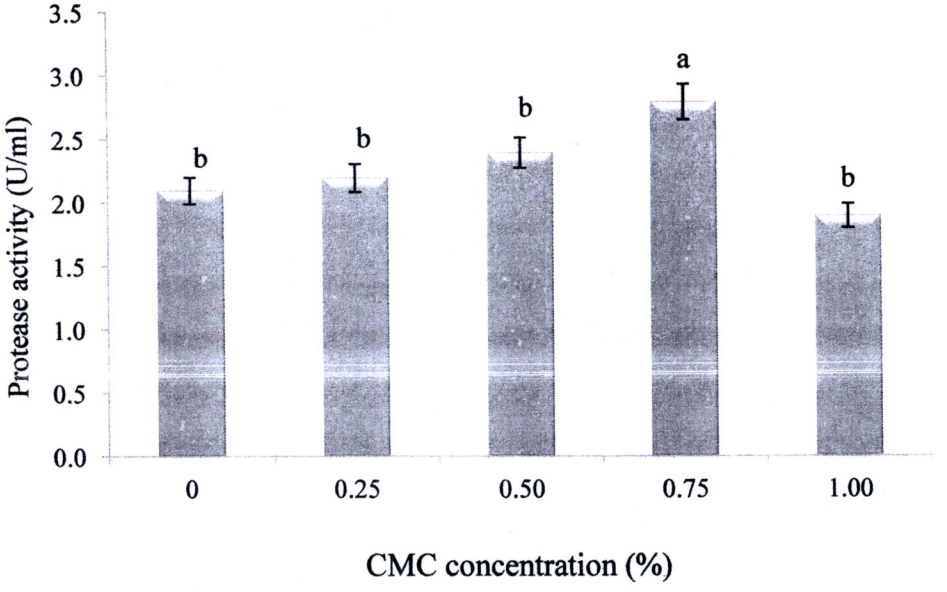

Figure 4.8 Enzyme activity in each CMC concentration. Different letters refer to the significantly different.

The previous studied of Yang and his worker (2000) with the same carbon source in protease production of *B. subtilis*, they found that 1% arabinose was the most effective substrate and concentration for protease production. In addition to the research of Oh and his worker (2000) the protease production by *P. aeruginosa* K-187 was slightly enhanced by the addition of CMC, lactose or rice bran into the medium similar with isolate ECM04 which used in this study.

4.4.4 Effect of nitrogen source on enzyme production

Isolate ECM04 was cultured in minimal synthetic medium containing 7% crab shell powder and 0.75% CMC. The effect of adding nitrogen on protease production by isolate ECM04 was significantly lower than control.

Then the nitrogen sources are not effective on protease production (Figure 4.9) different with *P. aeruginosa* K-187 which Oh and his worker (2000) studied. They reported that the secretion of protease was slightly enhanced by most of the nitrogen sources tested with the exception of polypeptone and beef extract, which repressed protease production to some degree. When the concentration effects of nitrogen sources were further studied, it was found that 0.5% ammonium nitrate was most effective for protease production.

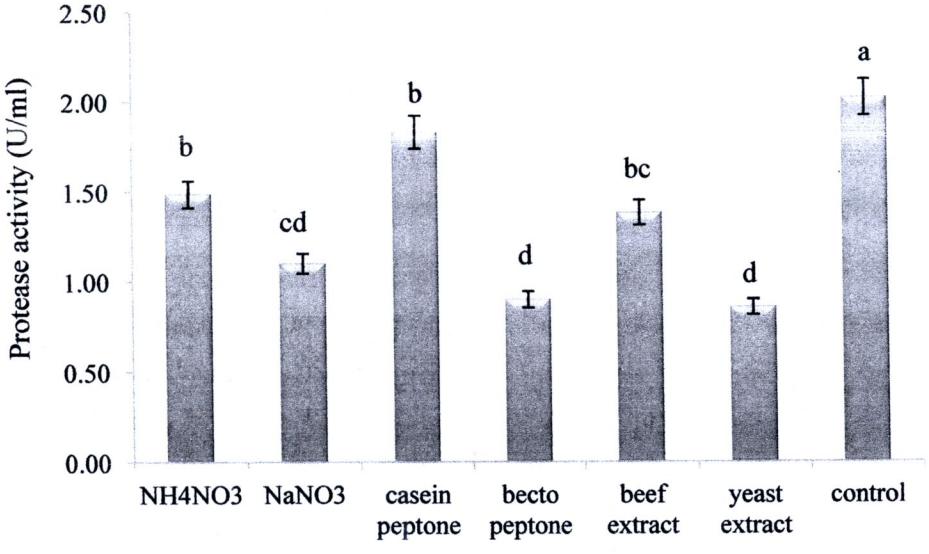

Figure 4.9 Enzyme activity in each nitrogen source supplement (1.0%). Different letters refer to the significantly different.

4.4.5 Effect of pH and temperature on enzyme production

Protease activity of isolate ECM04 cultured in optimal medium was increased significantly in pH 9 and 10 but not significantly different during pH 9 and 10 (Figure 4.10). In commonly of this medium with not adjusted pH was among pH 9-10 that mean pH adjustment was not important for this medium.

The most effective temperature for cultivation was 37°C. Protease activity of isolate ECM04 cultured at this temperature was more than others temperature significantly (Figure 4.11). This pH range was quite high when compared with the strain *B. subtilis* Y-108 produced higher protease activity when the initial pH and temperature were 6.0 and 30°C, respectively (Yang *et al.*, 2000).

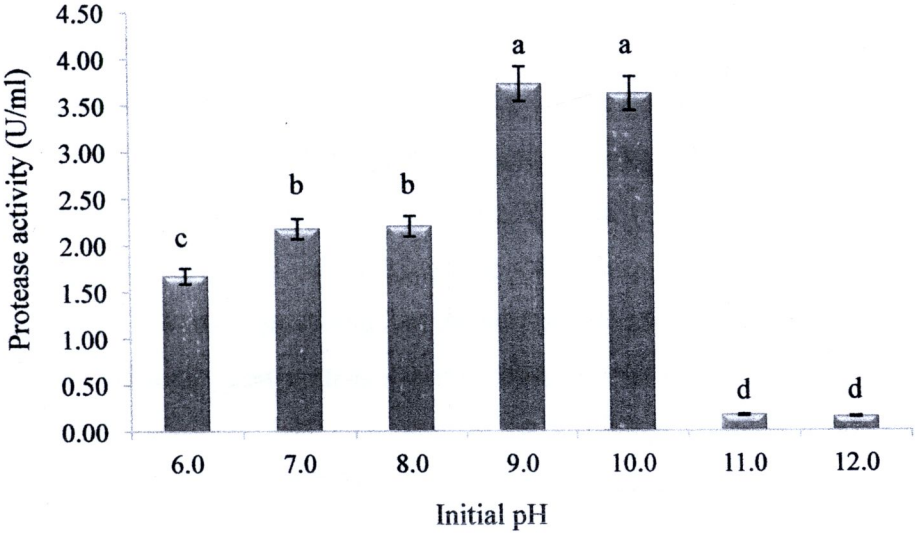

Figure 4.10 Effect of cultivation condition on protease production in various initial pH. Different letters refer to the significantly different.

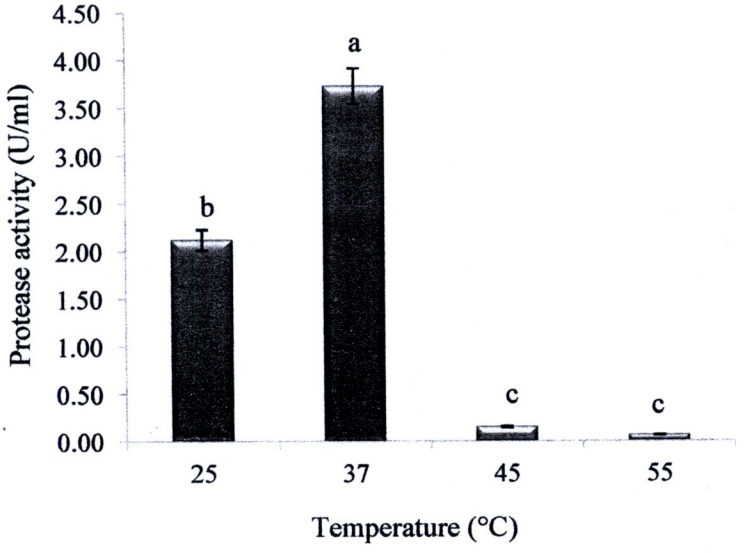

Figure 4.11 Effect of cultivation temperature on protease production in different temperature. Different letters refer to the significantly different.

4.5 Effect of pH and temperature on enzyme activity

The effect of pH on the protease activity was studied by using casein as a substrate under the standard assay conditions (Wang *et al.*, 2006; Yang *et al.*, 2000). The protease was with maximum values at pH 8 (Figure 4.12). The effect of pH on protease activity was determined by the measurement of activity at various pH at 37°C for 10 minutes. The protease activity was highest at pH 8 similar with previous studied of *Bacillus* sp. TKU004 (Wang *et al.*, 2006) the protease activity was determined by the measurement with the similar condition. The protease activity was increase at pH range 5–8.

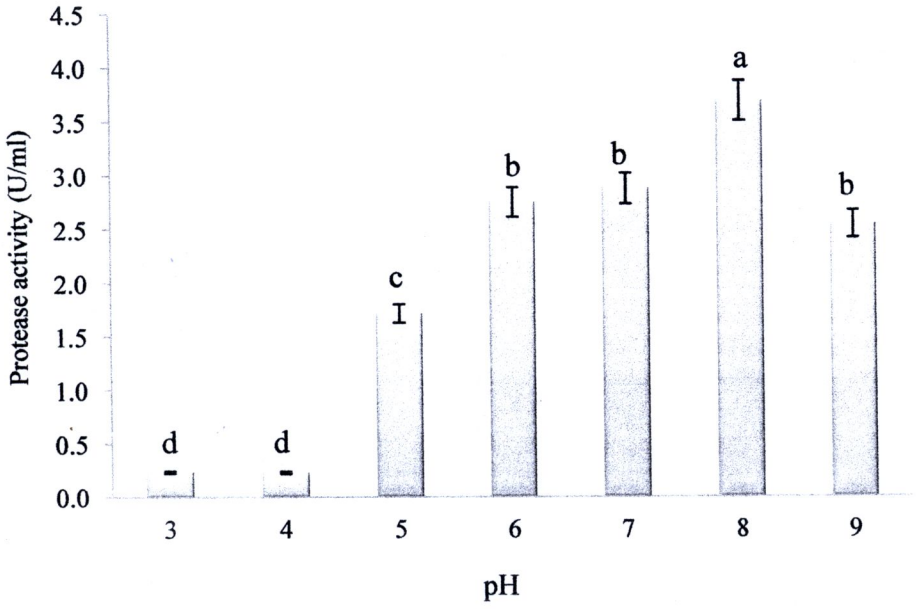

Figure 4.12 Effect of pH on enzyme activity.
Different letter refer to the significantly different.

The effect of temperature on the activity of crude protease was studied with casein as a substrate. The optimum temperature ECM04 protease was 37°C (Figure 4.13). To examine the optimum temperature of ECM04 crude protease, the enzyme solution in 50 mM phosphate buffer (pH 8) was allowed to stand for 30 min at various temperatures, and then the residual activity was measured. ECM04 protease maintained its initial activity from 3.65 to 3.7 U/ml.

There was difference comparing with *Bacillus* proteases according to the research of Wang and his team (2006), who reported that the optimum temperature of that strain was 60°C.

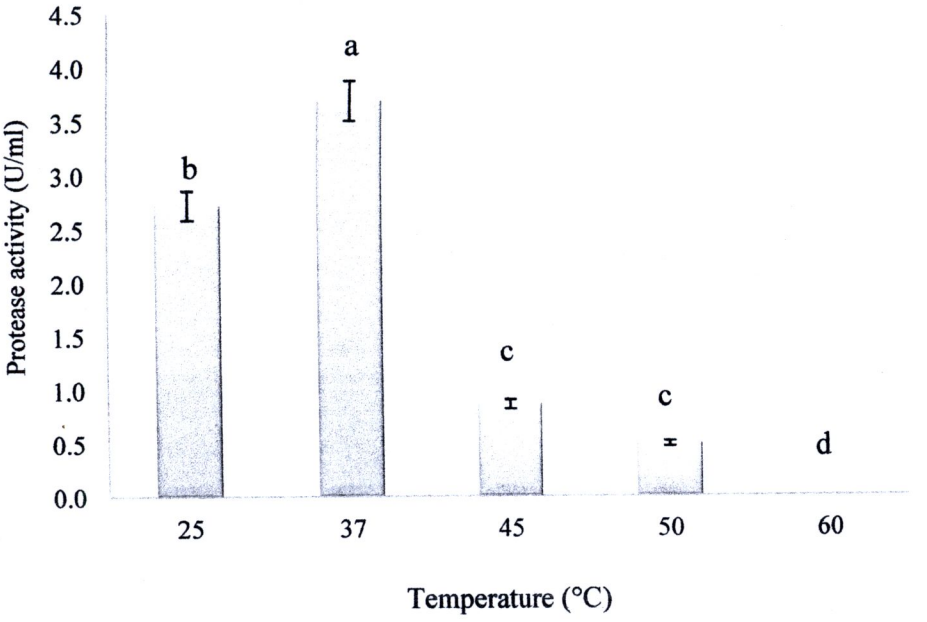

Figure 4.13 Effect of temperature on enzyme activity.
Different letter refer to the significantly different.

4.6 Protein removal of crab shell wastes

To compare the deproteinization efficiency of crab shells, four treatments have been done on the crab shells; (1) liquid phase fermentation with isolate ECM04, (2) soaked in water, (3) incubated in 2 N NaOH, and (4) incubated in crude enzyme. As shown in Figure 4.14, when treated with 2 N NaOH, the deproteinization gradually increased up to 79.65% on day 3. When cultured in liquid phase fermentation with isolate ECM04, the rate reached to 63.78% at 3 days and maintained about 63% thereafter. When crab shell was treated in crude enzyme, the deproteinization was about 53.49% at 4 days and maintained about 47% thereafter. In case of incubated in water, the deproteinization rate is 50.22% on day 4 (Figure 4.14).

These results indicate that treated in crude enzyme was not as efficient as deproteinization in liquid phase fermentation because crude protease was decreased by the time caused the deproteinization of crab shell to been lowered. A lot of literatures fairly agree that high concentrations of protein compounds stimulate production of microbial proteases. Often definite kinds of proteins have to be incorporated into the medium for protease production.

The observed efficiency of fermentation with crude enzyme at the first day of cultivation, it was found that incubation with crude enzyme was a little higher than soaked in water but after that percentage of deproteinization was not different. It was expected that because crude protease was decreased by the time then after first day it was out of protease.

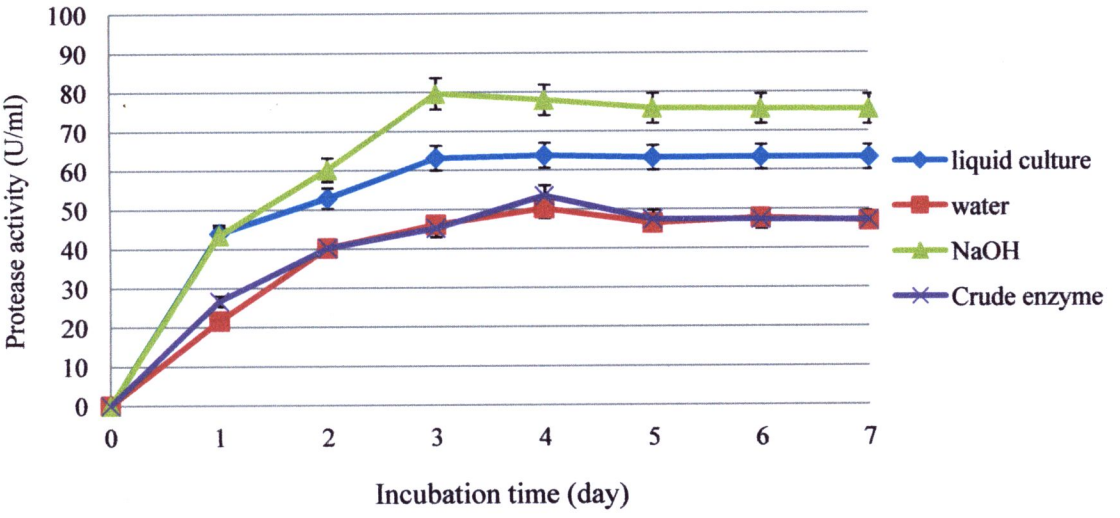

Fig 4.14 Deproteinization of crab shell powder in liquid phase fermentation with isolate ECM04, soaked in water, soaked in 2 N NaOH, and incubated with crude enzyme.

4.7 Protein removal of crab shell in liquid phase fermentation

Since the media containing crab shell, 0.75% CMC and small amounts of minerals was more suitable for the production of protease by isolate ECM04, the following was investigated. To seek the feasibility of isolate ECM04 chitin preparation from crab shell, isolate ECM04 was tested for its deproteinization performance in liquid phase fermentation. Deproteinization of crab shell was studied after the substrate was fermented with isolate ECM04 for 1–7 days. The percents of protein removal were shown in Figure 4.15. However, the percentage of protein removal reached the peak 63.78% on the 3rd day. On this day, maximum protease activity appeared also (3.74 U/ml as shown in Figure 4.15).

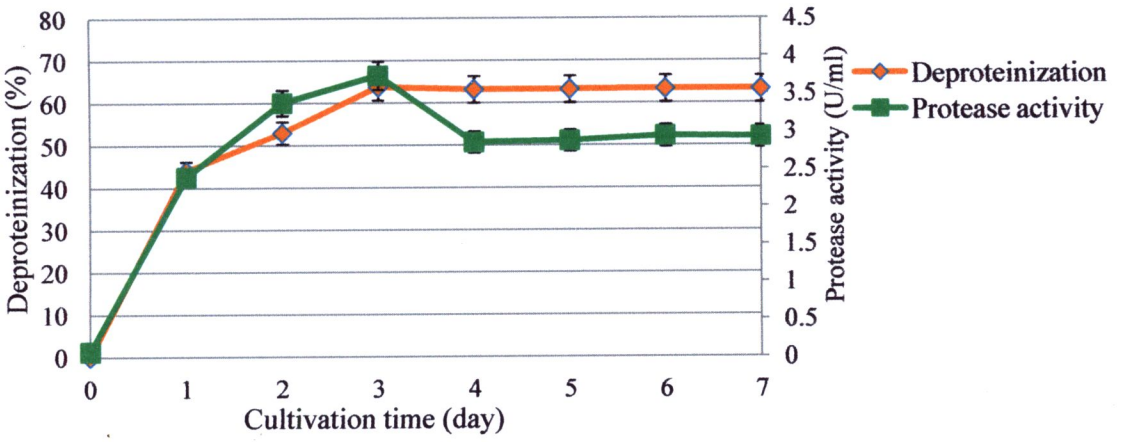

Figure 4.15 Deproteinization and protease activity of isolate ECM04.

According to the research of Wang and Chio (1998) by using *Pseudomonas aeruginosa* K-187 for deproteinization shrimp and crab shell. They found that deproteinization of shrimp and crab shell powder, untreated shrimp shell, and shrimp heads were 48, 55, and 60%, respectively. For shrimp shell and shrimp heads, protein removal reached 40 and 43%, respectively, after only 1 day of fermentation.

These results showed that percentage of protein removal of isolate ECM04 is more than *P. aeruginosa* K-187 but it was spend 3 days for protein remove longer than *P. aeruginosa* K-187.

Chemical treatments by acids and bases are used in most research on deproteinization of SCS. There were only a few studies on the use of proteolytic enzymes for the deproteinization of crustacean wastes. Broussignac (1968) demonstrated that use of papain, trypsin, or pepsin produced chitin with as little deacetylation as possible. Tuna proteinase, papain, and a bacterial proteinase have also been used for the deproteinization step. The residual protein associated with chitin after the enzyme treatments was about 5%. When a purified microbial protease was used, no more than 64% deproteinization was achieved under the same conditions. Microorganisms seem to be the best alternative to harsh chemical treatment for deproteinization of prawn shell waste (Wang and Chio, 1998).

The study of protease produced by *P. aeruginosa* K-187 and its application in the deproteinization of shrimp and crab shell wastes (Oh *et al.*, 2000). They compared protease immobilization with free protease and suggested that immobilization is

considered favorable in saving enzyme for re-use. They have demonstrated that the crude protease produced by *P. aeruginosa* K-187 can be covalently immobilized on a reversibly soluble polymeric support.

4.8 Protein residue compare with total protein in crab shell

After 3 days treated in four conditions, protein residues were studied by kjeldahl assay compare with total protein in crab shell. The results show that total protein in crab shell with no treated was 17.38% protein residue after treated with NaOH was 8.38% and liquid culture was 8.53% that mean protein removal were 51.80% and 50.88% repectively. There were not significantly different between NaOH and liquid cultured

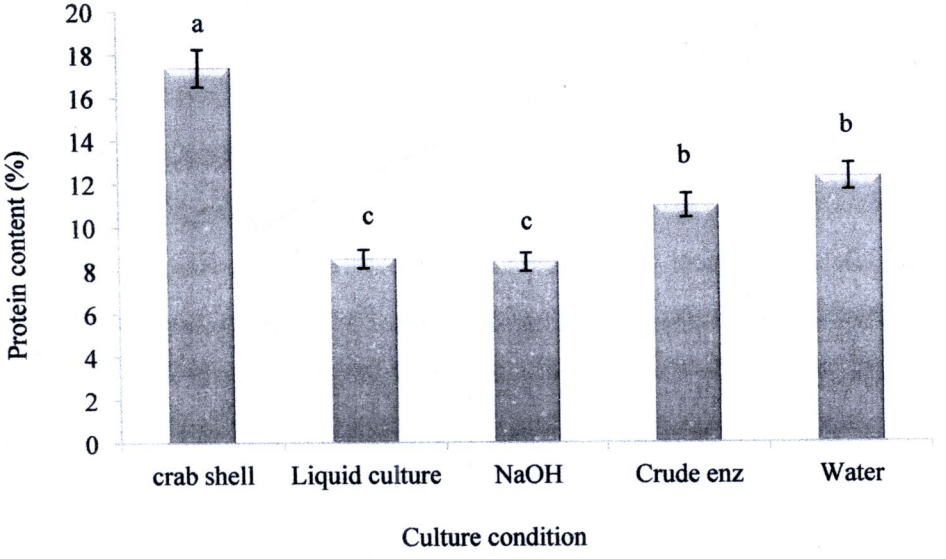

Figure 4.16 Residue protein after treated in each condition for 3 days compared with total protein in crab shell.
Different letter refer to the significantly different.