# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Crab shell powder

All crab shells obtained from Viya Inter Crabs 2003 Co., Ltd., the pasteurized crab factory in Suratthani province were washed thoroughly with tap water and then dried at 60°C overnight. The dried materials obtained were milled to powder according to Wang *et al.* (2009) for using in further study.

### 3.1.2 Soil samples

Soil samples were collected from 4 areas in Thailand;

3.1.2.1 Soil around pasteurized crab factory in Suratthani province

3.1.2.2 Soil from the garden in Chiang Mai province that full of snails

3.1.2.3 Seaside soils in Songkhla province

3.1.2.4 Some pickled fish from Lampoon province

### 3.1.3 Media

Media used in this study were chitin medium, enrichment medium, nutrient agar (NA), LB agar medium, malt extract yeast extract glucose agar (MYG), crab shell powder agar and crab shell powder broth (Tongsiri, 1998) (see Appendix).

## 3.2 Methods

### 3.2.1 Isolation and screening of protease-producing microorganisms

Each soil sample (1 g) was suspended in 100 ml sterilized water. Then, the suspension was spreaded on LB agar medium containing 1% (w/v) skim milk and incubated at 37°C for 3 days (Jo et al., 2008).

Colonies showing clear zones were re streaked on agar medium containing 3% crabs shell powder, 0.1% $K_2HPO_4$, 0.05% $MgSO_4 \cdot 7H_2O$ and 2% agar (pH 7.0) at 37°C for 3 days. Isolates that grown in such medium were subcultured in 100 ml of minimal synthetic medium containing 3% crab shell, 0.1% $K_2HPO_4$, 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7.0) on a shaker at 180 rpm 37°C for 2 days (Wang and Chio, 1998; Wang *et al.*, 2006; Wang *et al.*, 2009; Yang *et al.*, 2000). After incubation, the culture broth was centrifuged (4°C and 12,000×g for 20 min), and the supernatant was used for further protease measurement.

### 3.2.2 Microscopic Morphology

The characteristic of bacterial species were identified by gram's straining method (Wesley and Margaret, 1988). Preparation by smears bacteria on a slide and heat-fixed. Then, the slides were stained as follows: the smear was flooded with the crystal violet for one minute. Excess dye was poured off and washed gently in tap water. The smear was exposed to iodine solution for one minute and washed with tap water and drained carefully. After that, the smear was washed with 95% alcohol for 30 seconds and washed with tap water to stop the decolorization. Finally, the slide was counterstained with safranin for 30 seconds, washed off and examined under oil.

Endospore was investigated by endospore strain protocol (Schaeffer and Fulton, 1933) air dry and heat fix the organism on a glass slide. The blotting slide was saturated with malachite green stain solution and steam for 5 minutes, keeping the slide moist and adding more dye as required. Alternatively, the slide may be steamed over a container of boiling water. The slide was washed in tap water, then counterstain with safranin for 30 seconds. It was washed with tap water; blot dry. The slide was examined under the oil immersion lens (1,000X) for the presence of endospores. Endospores are bright green and vegetative cells are brownish red to pink.

### 3.2.3 16s rDNA sequence analysis

The molecular taxonomic position of the isolate ECM04 was determined by 16S rDNA sequence comparison. Genomic DNA was extracted as described by

Genomic DNA extraction kit (RBC Bioscience, Taiwan). This method was analyzed by Institute of Product and Standardization (Biotechnology Center, Maejo University, Chiang Mai). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal primer (First Base Laboratories, Malaysia) as follow.

Phylogenetic tree of ECM04 and other species based on Neighbor Joining analysis of 16S rDNA nucleotide sequences. Numbers above lines are percentage bootstrap values of 1,000 replications of that branch of the tree; values greater than 50% were considered significant (Using ClustralX 1.86, MEGA 5.02).

1.27F (5′ – TGTGCTCCAAAGTTGCATGT – 3′)

2.357F (5′ – CTACGGGAGGCAGCAG – 3′)

3.920F (5′ – AAAGAAAAGAATGAATGAACTT – 3′)

4.520R (5′ – ACGCCATCTTTCGATACTGC – 3′)

5.1080R (5′ – CC (G/A) AA (T/C) TC (T/C) TC (A/G) CAICCCCA – 3′)

6.1522R (5′ – AAGGAGGTGATCCA (AG) CCGCA – 3′)

The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) was used to search for homologous sequences in Genbank. Multiple sequence alignment was performed using CLUSTALX (Thompson *et al.*, 1997) and the phylogenetic tree was displayed using the TREEVIEW program.

### 3.2.4 Measurement of protease activity

For measuring protease activity, a diluted enzyme solution (0.25 mL) was mixed with 1.25 ml of 0.65% casein in pH 8 phosphate buffer and incubated for 10 min at 37 °C. The reaction was terminated by adding 5 ml of 0.1 M trichloroacetic acid (TCA) (Wang *et al.*, 2006; Yang *et al.*, 2000). The reaction mixture was centrifuged and the soluble peptide in the supernatant fraction was measured by the method of Sigma (1999) with tyrosine as the reference compound. One unit of protease activity was defined as the amount of enzyme required to release 1 μmol of

tyrosine/min. Determination diagram of preparation and measurement of protease are show in Figure 3.1.

### 3.2.5 Protein determination

Protein content was determined by the method of Bradford (Bradford, 1976) using Bio-Rad dye reagent concentrate and bovine serum albumin as the standard. The protein concentration was estimated by measuring the absorbance at 595 nm by using spectrophotometer.

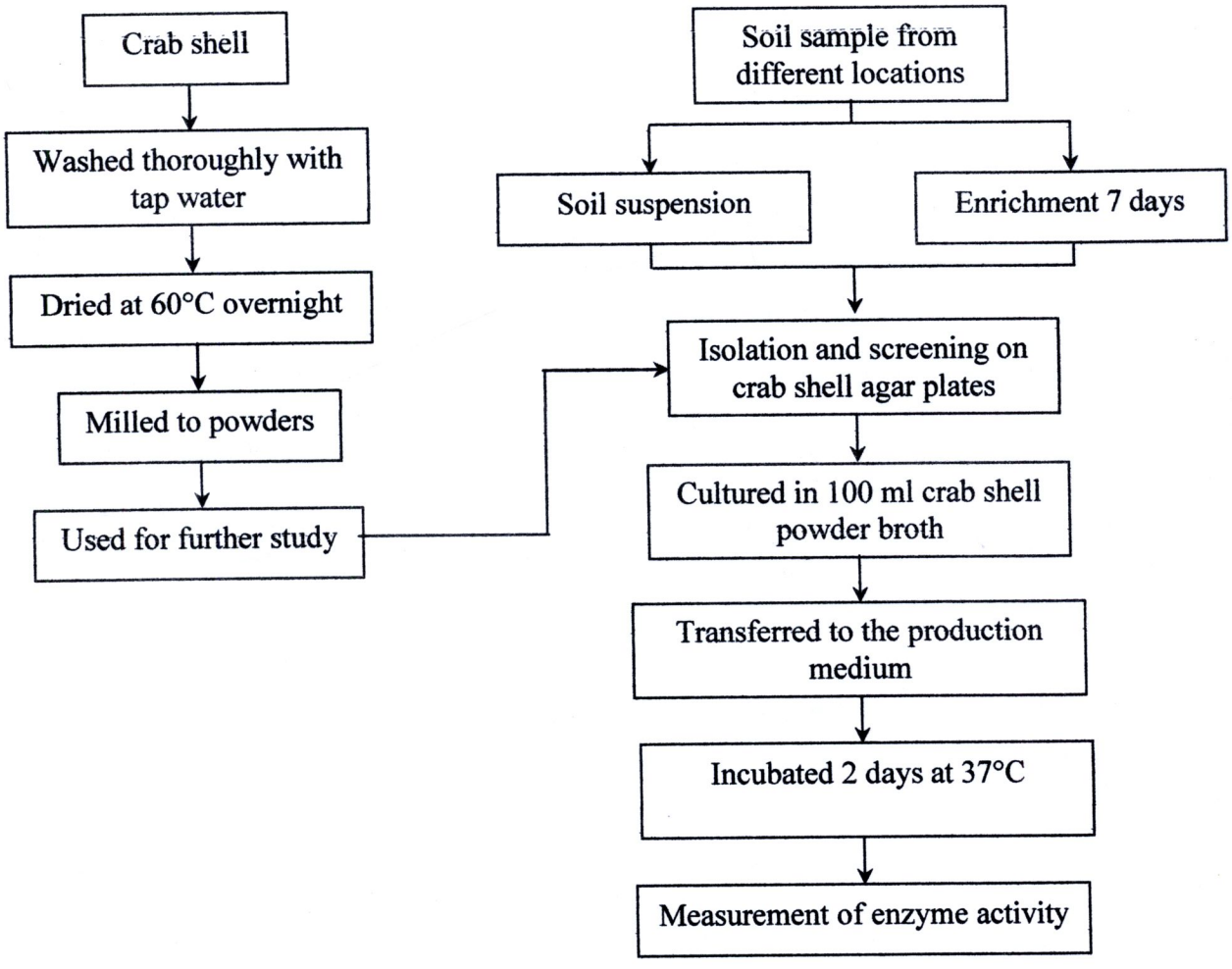


**Figure 3.1** Diagram of material preparation and measurement of protease activity.

### 3.2.6 Measurement of total protein in crab shell (Bradford, 1976)

#### 3.2.6.1 Acid treatment of crab shell powder

Crab shell powder was mixed with a 2 N HCl at a ratio of 1:8 (w/v). The mixture was stored at room temperature for 2 days. The mixture was filtered and the solid remains were washed with deionized water until a neutral pH was obtained (Wang and Chio, 1998).

#### 3.2.6.2 Deproteination by alkali

Crab shell powder was mixed with 2 N NaOH solution at a 3: 8 (w/v) ratio. The mixture was allowed to react at 100°C for 30 min and then it was centrifuged. After centrifugation, the supernatant was used for analysis of protein concentration (Oh *et al.*, 1999).

### 3.2.7 Inoculum preparation

Microorganism obtained from the first screening were subcultured in 100 ml liquid medium containing 3% crabs shell powder, 0.1% $K_2HPO_4$ and 0.05% $MgSO_4 \cdot 7H_2O$ in shaken flasks at 37°C for 24 hours, 2% of the culture broth were transferred to cultured medium.

### 3.2.8 Protease production in liquid phase fermentation

Two milliliters of inoculums was transferred into 100 ml minimal synthetic medium containing 7.0% crab shell powder, 0.75% CMC, 0.1% $K_2HPO_4$ and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7.0) in 250 ml Erlenmeyer flask and incubated for 2 days at 37°C. After incubation, the culture broth was centrifuged (4°C and 6,000 rpm for 20 min) and the supernatant was used for protease determination (Wang *et al.*, 2005).

## 3.3 Effect of culture conditions on enzyme production

### 3.3.1 Effect of crab shell powder concentration

The minimal synthetic medium containing 0.1% $K_2HPO_4$ and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7.0), was used gradually supplemented with the various crab shell powder concentration (3-7%) to be investigated.

### 3.3.2 Effect of carbon source supplement

The effect of carbon source supplement was investigated in the minimal synthetic medium containing 0.1% $K_2HPO_4$, 0.05% $MgSO_4 \cdot 7H_2O$ and effective crab shell content (pH 7.0), which was used gradually supplemented with 0.5% the various carbon source (glucose, lactose, carboxymethyl cellulose, D (-) arabinose, D (+)xylose, cellulose). The effective carbon was used for investigation of carbon content (0.25%-1.0%).

### 3.3.3 Effect of nitrogen source supplement

The effect of nitrogen source supplement was investigated in the minimal synthetic medium containing 0.1% $K_2HPO_4$, 0.05% $MgSO_4 \cdot 7H_2O$ and effective crab shell and carbon source content (pH 7.0), which was used gradually supplemented with 1.0% the various nitrogen source (sodium nitrate, ammonium nitrate, casein peptone, becto-peptone, beef extract, and yeast extract). The effective nitrogen was used for investigation of nitrogen content (0.25%-1.0%).

### 3.3.4 Effect of pH and temperature on enzyme production

With the uses of the optimal culture condition, the effect of initial pH and temperature on protease production was also investigated until the optimal condition was found.

The minimal synthetic medium with crab shell powder and carboxymethyl cellulose in optimal content was culture in various initial pH (6-12) and temperature (25, 37, 45 and 55°C)

## 3.4 Effect of pH and temperature on enzyme activity

The optimum pH of protease activity was studied by assaying the samples at different pH values. The optimum pH of protease was determined by measuring activity at pH 3-8 as described above after the sample had been diluted by a 50 mM buffer solution of various pH values (pH 3–8). The buffer systems used were sodium acetate buffer (50 mM, pH 3-5) and phosphate buffer (50 mM, pH 6–8).

To determine the optimum temperature for protease activity values of sample was measured at various temperatures (25, 37, 45, 50, and 60°C). The optimal temperature of protease was studied by incubating the samples at various temperatures for 30 min. The residual activity was measured as described above.

### 3.5 Protein removal of crab shell wastes

The culture supernatant, containing protease obtained from the liquid-phase fermentation described above was tested for crab shell waste deproteinization. After the substrates were incubated with the protease solution at 37°C for various lengths of time (1-7 days), the solid residuals were isolated and washed, followed by complete deproteinization with alkali. The protein content was analyzed for the calculation of the protein removal. For comparison, three other conditions were also studied for their crab shell waste deproteinization capabilities. The same experimental procedure was employed for these conditions with the ratio 3:8 at 37°C for (1) cultured in liquid phase fermentation with this strain, (2) incubated in water, and (3) incubated in 2 N NaOH.

### 3.6 Protein residue compare with total protein in crab shell

Crab shell was incubated in fours conditions after 3 days, the solid residuals were washed. The protein content was analyzed for the calculation of the protein residule by Kjeldahl assay (AOAC, 1990). For comparison, protein content from four conditions was studied for their crab shell waste deproteinization capabilities.

### 3.7 Statistical analysis

Effect of crab shell powder concentration, carbon source supplement, nitrogen source supplement, cultivation conditions, and pH and temperature on enzyme activity was determined by analysis of variance (one-way ANOVA) according to the general linear model procedure of the SPSS analysis version 17. Means were separated with Duncan's Multiple Range Test at $P \leq 0.05$.