CHAPTER 3

MATERIALS AND METHODS

3.1 Microorganisms

Nineteen strains of *Bacillus* sp. used in this study were obtained from the Culture Collection Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand.

3.2 Instruments, chemicals and media

3.2.1 Instruments

Analytical balance: Mettler Toledo model AG204, Urdorf, Switzerland.

Autoclave: Tomy model SS-325, Tokyo, Japan.

Beaker heater: Gerhardt type EV 26, Germany.

Blender: Osterizer, USA.

Block Digestion Unit: Kjeldatherm, Gerhardt type TR, Germany.

Centrifuges: Beckman Superspeed Centrifuge model Avanti J25, U.S.A Eppendorf model 5430, Germany, and Sorvall: Superspeed Centrifuges model RC-5C Plus and tabletop Centrifuges model RC-5C Plus, Newtown, USA.

Circulating Water Bath: Techre model TE8 A, Cambridge, UK.

Colony counter: Funke Gerber Labortechnik, Berlin, Germany.

Condenser tube: W. Krannich, Gottingen, Germany.

Cryotube: Cryovial® Simported Ltd., Quebac, Cannada.

Freezer Dryer: Savant model Super Modulya 233, NewYork, USA.

Flask heater: Gerhardt type EV 26, Germany.

Hot plate and stirrer: Thermolyne Crimarec2, Iowa, USA.

Heat Sealer: Audion Elektro, model 235SA, Netherlands.

Incubator: Memmert model BE500 (30°C, 37°C, 50°C), Germany.

Hot plate and stirrer: Thermolyne Crimarec2, Iowa, USA.

Heat Sealer: Audion Elektro, model 235SA, Netherlands.

Incubator: Memmert model BE500 (30°C, 37°C, 50°C), Germany.

Incubator shaker: New Brunswick Scientific model innova4300, U.S.A and Thermolyne model Rosi1000, USA.

Magnetic stirrer: Ika model RO-10, Selangor, Malaysia.

Microwave: Sanyo model EM-815FW, Japan.

Moisture balance: Sortorius model MA 30, Germany.

Muffle furnace: Heraeus type MR 260 E, Germany.

Oven: Contherm Digital Series incubator, Lower Hutt, New Zealand.

Petridishes Sterile 90 mm: Millionant, SA.54, Paris, France.

pH Meter: Mettler Toledo model CH-8603, Switzerland.

Pipetteman: Gilson, Villiers-Le-Bel, France.

Precision balance: Mettler Toledo model PB3002, Urdorf, Switzerland.

Refrigerator: Sharp model FC27 (-20°C), Japan and Deep Freezer REVCO model ULT1790-7-V12 (-80°C), USA.

Sealer: Audion Elektro, Sealboy Impulse, model 235 SA, The Netherlands.

Shaking Water Bath: Memmert, model WB22 +SV1422, Germany.

Soxhlet extractor: W. Krannich, Gottingen, Germany.

Spectrophotometer: Sherwood Scientific model259, Cambridge, UK.

Vacuum sealer: Audion Elektro, model VM50, The Netherlands.

Vortex mixer: Barnstead/Thermolyne model M37610-26, Iowa, USA.

Water purification System: Branstead model MP-11A, USA.

3.2.2 Chemicals

Chemicals	Company	Grade
Ammonia solution	Merck	Analytical
Ammonium heptamolybdate	Merck	Analytical
Ammonium vanadate	Merck	Analytical
Boric acid	Merck	Analytical
Diacetylmonoxime	Merck	Analytical
Dimethyl sulfoxide	Merck	Analytical
di-Potassium hydrogen phosphate	Merck	Analytical

Chemicals	Company	Grade
di-Potassium hydrogen phosphate	Merck	Analytical
di-Sodium hydrogen phosphate	Merck	Analytical
Folin-Ciocalteu's pshenol	Merck	Analytical
Gucose	Merck	Analytical
Hydrochloric acid	Merck	Analytical
Selenium reagent mixture	Merck	Analytical
Sodium carbonate	Merck	Analytical
Sodium citrate	Merck	Analytical
Sodium hydroxide	Merck	Analytical
Sodium postassium tartate	Merck	Analytical
Sulfuric acid	Merck	Analytical
Sulphoaslicylic acid	Merck	Analytical
Thiosemicabazide	Merck	Analytical
Trichloroacetic acid	Merck	Analytical
tri-sodium citrate dihydrate	Merck	Analytical
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-formazan	Sigma	Analytical
dinitro-salicylic acid	Sigma	Analytical
β-glucan from baley	Sigma	Analytical
Calcium chloride	Sigma	Analytical
Choramphenicol	Sigma	Analytical
Copper (II) sulfate pentahydrate	Sigma	Analytical
L-*trans*-3-carboxyoxiran-2-carbonyl-L-leucylag-matine (E-64)	Sigma	Analytical
Ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetra-acetic acid (EGTA)	Sigma	Analytical
Ethylene diamine tetraacetic acid (EDTA)	Sigma	Analytical
Oat spelt xylan	Sigma	Analytical
Pancreatin	Sigma	Analytical
Pepsin	Sigma	Analytical

Chemicals	Company	Grade
Phenol red	Sigma	Analytical
Trypsin inhibitor from *Glycine max,*	Sigma	Analytical
Sodium phytate	Sigma	Analytical
Soluble starch	Sigma	Analytical
Tyrosine	Sigma	Analytical
Urea	Sigma	Analytical
Nitric acid (65%)	Fluka	Analytical
Ortho-phosphoric	Fluka	Analytical
Tarshiro indicator	Fluka	Analytical
Xylose	Fluka	Analytical
Soy protein concentrate	Mighty International	Analytical
Carboxymethyl Cellulose	BDH	Analytical
Pumice stone	BDH	Analytical
Citric acid	Carlo Erba	Analytical
Ferrous sulfate	Carlo Erba	Analytical
Magnesium chloride	Carlo Erba	Analytical
Magnesium sulfate heptahydrate	Carlo Erba	Analytical
Sodium chloride	Carlo Erba	Analytical
Phenylmethanesulfonyl fluoride (PMSF)	Calbiochem-Novabiochem	Analytical

3.2.3 Media

Media	Company
Agar powder	Purified Agar Ltd.
Bacto peptone	Difco
Yeast extract	Difco
Corn flour	Maizena
Rice bran	Local market
Rice flour	Local market

Media	Company
Corn steep liquor	Sigma
D-Manitol	Sigma
Dulbecco' s Modified Eagle' s Medium (DMEM)	Gibco
L-glutamine	Gibco
Fetal bovine serum	Hyclone
Meat extract	Merck
Soybean meal (SBM)	Thai Feedmill Public Co Ltd.

3.3 Media Culture and inoculum preparation

Freeze dried cultures were streaked onto nutrient agar (NA) plate and incubated at 37°C for 15 h. To maintain culture throughout the study, a single colony was restreaked, subcultured onto NA slants, and stored in refrigerator. The culture was transferred using the same procedure every 2 weeks.

Inoculum was prepared by transferring a loopfull of microorganisms from a slant culture into 5 ml of sterile nutrient broth (NB) (Appendix A-1.1). The culture was then incubated for 15 h at 37 °C with shaking at 200 rpm in an orbital shaker. This would provide the bacterial suspension having an approximate cell concentration of 2×10^8 CFU/ml.

3.4 Screening of protease producing strains

3.4.1 Culture condition

A 0.2 ml volume of cell suspensions was used to inoculate a 125-ml Erlenmeyer flask containing 20 ml of SBM medium containing 3% (w/v) soybean meal, 0.1% (w/v) K_2HPO_4 and 0.05% (w/v) $MgSO_4$, pH 7.0. After 24 h of incubation at 37°C on a rotary shaker operated at 150 rpm, the number of cell was estimated by plate count on modified manitol salt agar (Appendix A-1.2). The cell-free supernatant was recovered by centrifugation at the speed of 10,000 rpm at 4°C for 20 min and used for determining extracellular protease activity. Total protein in the supernatant was measured according to the method of Lowry *et al*. (1951). The detail of the analysis is described clearly in Appendix B-1.

3.4.2 Growth determination

Bacillus sp. was enumerated by total plate count method. The dilution plate technique was carried out by a series of 10-fold dilution of sterile 0.1% peptone water diluent. An aliquot of 0.1 ml of each dilution was plated on modified manitol salt agar plates in duplicate. The petridishes were incubated in incubator at 37°C for 15 h. The numbers of bacterial colonies were counted by using a colony counter.

3.4.3 Protease activity assay

Protease activity was determined at the physiological conditions in the animal' s digestive tract according to the method of An *et al*. (1994) with a slight modification using soy protein isolate (SPI) as a substrate. The assay mixture consisted of 200 μl of substrate containing of SPI 1 mg/ml, 325 μl of McIlvaine's buffer pH 6.8, 625 μl of distilled water and 100 μl of enzyme solution. The reaction mixture was incubated at 39.5 °C for 30 min and the reaction was terminated by the addition of 200 μl of 50% trichloroacetic acid (TCA), and then centrifuged at 2,500 rpm for 10 min to remove the resulting precipitate. The soluble peptide in the supernatant was measured by method of Lowry *et al.* (1951) using tyrosine as a standard. One unit of activity was defined as 1 nmole of tyrosine released per min. A blank was run in the same manner except the enzyme was added after the addition of TCA solution.

3.4.4 Resistance to pH and heat

To test resistance to pH, crude protease of selected strains of *Bacillus* sp. was incubated in McIlvaine's buffer pH 3.0 at the ratio of 1:1 at room temperature (25°C). The remaining protease activity at pH 6.8 was determined under standard assay condition after pre-incubation for 10, 20, 30 and 60 min. To test resistance to heat, crude protease was exposed to heat treatment at various temperatures ranging from 55 to 85°C for 10 min. The reaction was immediately cooled down in iced-water and the remaining activity was measured under standard assay condition. Particular at pelleting temperature (75°C), the remaining activity of crude protease was measured at pH 6.8, 39.5°C after heating in water bath at 75°C for 2, 5 and 10 min, comparing with the crude protease without incubation.

3.4.5 Resistance to protease inhibitors

The crude protease was incubated for 15 min at room temperature with an equal volume of different inhibitors including 0.2 mM E-64, 2 mM EDTA, 20 mM EGTA, 2 mM PMSF and 0.2 g/l SBTI. Residual activities at the standard assay condition were compared with the control.

3.5 Optimization of crude protease production

3.5.1 Liquid phase fermentation

Optimization of crude protease production was carried out in a minimal synthetic medium containing varying levels of SBM and supplemented with various ingredients to be investigated. Conditions optimized included initial pH, incubation temperature, ratio of medium to air content, agitation rate, cultivation volume, inoculum size and cultivation time. Usually an effective prior condition was used as the basis for the latter experimental until the optimal condition was obtained.

3.5.2 Production of crude protease powder

Crude protease production was carried out at the optimum condition. The crude protease was mixed with various carriers (corn flour, rice bran, rice flour, SBM, and soy protein isolate) at the ratio 1:2 (v/w) and dried at 50°C for 6 hours in incubator. After drying, the mixture was ground with blender. To determine the protease activity, the crude protease powder was extracted with cold distilled water at the ratio 1:5 (w/v), vortex for 2 min at room temperature. The supernatant was recovered by centrifugation at 10,000 rpm at 4°C for 20 min. The sediment was re-extracted with the same procedure. The supernatant of both extraction was pooled and determined the protease activity.

3.6 Shelf-life of crude protease powder

3.6.1 Effect of temperature, time, and air exposure during storage

After grinding, the product was packed in aluminum bag, sealed tightly, and kept at various temperatures (3-5°C, 30°C and 45°C). The protease activity of product was determined at 0, 2, 4, 6, 8, 10 and 12 weeks during storage. The effect of air exposure was carried out by comparing protease activity of products which were between the freshly opened product and opened product. Similar study was carried out to compare shelf-life of products obtained with commercial feed enzymes.

3.7 Characterization of crude protease powder from *Bacillus* sp. FAS001

3.7.1 Optimum pH

The pH-activity profile of crude protease from *Bacillus* sp. FAS001 was studied as method as described in 3.4.3. In this study, pH values of citrate phosphate buffer in substrate mixture were varied from 4.0-8.0.

3.7.2 Optimum temperature

The temperature-activity profile of crude protease was assessed by the method described in 3.4.3. Temperature of the assay system were varied from 25 to 60°C.

3.7.3 Activity profiles

In addition to protease activity, the activity of amylase, β-glucanase, cellulase, pentosanase, phytase were determined. Amylase activity was measured according to the method of Rick and Stegbauer (1974). β-glucanase and cellulase were measured by method of Mandels *et al*. (1976) with slight modification. The activity of pentosanase was measured according to the method of Bailey *et al*. (1992). Phytase activity was determined by method of Engelen (1994) with a slight modification. All enzyme activity was measured at pH 3.0 and pH 6.8. (Appendix B-2)

3.7.4 Cytotoxicity test

Cytotoxicity of *Bacillus* sp. FAS001 and crude protease powder was determined by the 3-(4,5-dimethylthiazoyl-2-yl)2,5diphenyltetrazolium bromide (MTT) assay according to the method of Plumb *et al*. (1989). *Bacillus* sp. FAS001 was cultured in both NB and SBM medium. After 24 h of incubation at 37°C on a rotary shaker operated at 150 rpm, cultured broth with cell was harvested and freeze-dried. For crude protease powder, *Bacillus* sp. FAS001 was cultivated in optimized media under optimal cultivation conditions and dried with a condition previously described in 3.5.2. Each sample was weighed and dissolved in dimethylsulfoxide (DMSO) at 37°C and then adjusted to give a stock concentration of 20 mg/ml. The samples were diluted in the growth medium to give the final concentration of 200 and 100 μg/ ml. The mouse lung cell lines (L929) were seeded in a 96-well plate with 500 cells/well, and incubated for

24 h. All the samples at 200 and 100 µg/ ml were added to the cells and incubated at 37°C in a fully humidified, 5% CO_2: air atmosphere for 24 h. The tested samples were removed from the cell cultures and the cells were incubated for a further 24 h in fresh medium. At 4 h before the end of the incubation 50 µl of MTT solution (5 mg/ml) was added to each well containing cells and incubated in a CO_2 incubator at 37°C for 4 h. Media was removed by needle and syringe and 200 µl of DMSO was added to dissolve crystals. Finally, 25 ml of Sorenzen's glycine buffer, pH 10.5 was added to stabilizer the color. The plate was transferred to plate reader and measured absorbance at 570 nm. A result was derived from the percentage of cells survival compared to controls

3.8 Testing of enzyme quality by *in vitro* digestibility

3.8.1 Experimental design

The experimental design was the randomized complete block design with 4 different diets including piglet, growing, finishing and pregnant. The diet composition and chemical analysis are shown in Table 3.1-3.2. and 5 treatments listed as follows:

i. Diet with no enzyme added **(Con.)**
ii. Diet supplemented with imported enzyme 1 **(IE1)**
iii. Diet supplemented with imported enzyme 2 at the same protease activity as IE1 **(IE2)**
iv. Diet supplemented with crude protease at the same protease activity as IE1 **(FAS001 1.0X)**
v. Diet supplemented with crude protease at 1.5 times of protease activity to IE1 **(FAS001 1.5X)**

3.8.2 Procedure

To compare the effect of enzyme supplement, the protease activity of imported enzymes were measured with the same procedure as described in 3.4.3. The addition level was based on the level recommended by manufactures. *In vitro* digestibility of all diets were measured according to the method of Boisen (1993) as described in Appendix B-3. Parameters including dry matter, crude protein, crude fiber, ether extract and ash were analyzed according to the method of AOAC (1995) as described in Appendix B-4.

Table 3.1 Composition of the diets for *in vitro* digestibility experiment.

Components	Diets (kg.)			
	Piglet	**Growing**	**Finishing**	**Pregnant**
Broken rice	829.7	-	-	-
Ground corn	0.0	340.0	402.0	0.0
Cassava meal	0.0	466.9	483.7	691.4
Rice bran	22.0	342.0	300.0	800.0
Palm kernel meal	-	-	25.0	200.0
Fish liver meal	20.0	-	-	-
Fish meal (CP 65%)	100.0	60.0	20.0	20.0
Full fat soybean meal (CP 36%)	421.0	397.0	383.0	44.0
Soybean meal (44%)	217.0	233.0	215.0	132.0
M-Di calcium phosphate (21%)	18.0	-	-	11.0
Dicalcium phosphate (18%)	-	11.8	10.8	-
Rock phosphate	26.5	15.9	19.2	31.7
Salt	6.0	10.0	10.0	10.0
Tallow	80.0	90.0	90.0	0.0
Molasses	0.0	20.0	20.0	30.0
Nucospray K10	175.0	-	-	-
Veeolac	25.0	-	-	-
Enzyme[1]	- / +	- / +	- / +	- / +

[1] All of the enzymes were added to the diets.

Table 3.2 Chemical analysis (%) of the diets for *in vitro* digestibility experiment.

Chemical Compositions (%)	Diets			
	Piglet	**Growing pig**	**Finishing pig**	**Pregnant pig**
Dry matter	89.89±0.05	89.42±0.05	89.22±0.11	89.61±0.05
Crude protein	21.92±0.08	16.56±0.01	14.62±0.12	12.90±0.03
Crude fibre	5.23±0.06	4.05±0.14	4.41±0.10	5.96±0.22
Ether Extract	8.15±0.14	9.50±0.14	9.70±0.30	11.82±0.11
Ash	7.08±0.09	7.23±0.15	7.79±0.04	8.21±0.07

3.9 Testing effect of enzyme on production performance in pigs

3.9.1 Experimental design

The pigs were used in a completely randomized block design with five dietary treatments and six blocks by the times of weaning.

3.9.2 Diets

Four pig diets, code 120, 140, 160 and 200, were offered at different phase of feeding, 1 to 18, 19 to 28, 29 to 35 and 35 to 42 days of the experiment, respectively. The diet composition and chemical analysis are shown in Table 3.3 and 3.4. Each experimental diet was mixed and divided into 5 treatments as follows:

i. Diet with no enzyme added **(Con.)**
ii. Diet supplemented with imported enzyme 1 **(IE1)**
iii. Diet supplemented with imported enzyme 2 at the same protease activity as IE1 **(IE2)**
iv. Diet supplemented with crude protease at the same protease activity as IE1 **(FAS001 1.0X)**
v. Diet supplemented with crude protease at 1.5 times of protease activity to IE1 **(FAS001 1.5X)**

The addition rate was calculated to provide the same enzyme activity (base on the protease activity assay in 3.4.3) and was based on the level recommended by manufacture.

Table 3.3 Composition of the weaning diets for productive performance experiment.

Components	Diets (kg.)			
	120	**140**	**160**	**200**
Broken rice	220.7	640.3	628.0	-
Ground corn	-	-	100.0	400.0
Cassava meal	-	-	-	423.0
Palm kernel meal	-	-	-	20.0
Wheat bran	82.0	100.0	108.3	142.0
Rice bran	160.0	160.0	169.0	205.0
Fish liver meal	20.0	20.0	100.0	-
Fish meal (CP 65%)	100.0	100.0	20.0	90.0
Full fat soybean meal (CP 36%)	469.0	496.0	100.0	350.0
Soybean meal (44%)	-	85.0	515.0	184.0
Di-calcium phosphate	0.5	17.5	35.0	3.0
Rock phosphate	29.0	28.5	20.5	23.0
Salt	4.0	6.0	7.5	7.0
Tallow	20.0	80.0	60.0	90.0
Molasses	0.0	20.0	56.0	40.0
Sweet whey	400.0	25.0	20.0	-
Elacota	0.0	25.0	-	-
Nucospray K10	400.0	150.0	-	-
Veeolac	25.0	-	-	-
HP 300	25.0	-	-	-
Lysine	0.1	3.1	4.5	4.4
Methionine	1.8	1.2	1.3	1.55
Threonine	0.0	1.6	2.3	1.85
Glutamine	1.0	-	-	-
Enzyme[1]	- / +	- / +	- / +	- / +

[1] All of the enzymes were added to the diets (see Section 3.9.2).

Table 3.4 Chemical analysis (%) of the weaner diets for productive performance experiment.

Chemical Compositions (%)	Diets			
	120	**140**	**160**	**200**
Dry matter	94.24±0.66	89.93±0.63	89.25±0.62	88.33±0.62
Crude protein	23.50±0.16	21.96±0.15	19.22±0.13	18.49±0.13
Crude fibre	3.06±0.02	3.50±0.02	3.70±0.03	5.00±0.03
Ether Extract	9.47±0.07	11.32±0.08	11.40±0.08	10.49±0.07
Ash	8.55±0.06	7.28±0.05	6.91±0.05	7.12±0.05

3.9.3 Animals, housing and management

The experiment was carried out at Kittiwat Farm, Chiangmai, Thailand. Three hundred and twenty weaning pigs (160 male and 160 female piglets) at the same age, and 4.97± 0.82 kg weight (mean ± S.D.) were used. All pigs were obtained by 6 times of weaning. Pigs were randomly allotted into five treatments, two male and two female piglets per pen. Sixteen replications per treatments were used. Pigs were housed in an environmentally controlled nursery with a pen dimension of 1.5 m × 1.3 m which had slate floors, two nipple drinkers and a feed trough (Figure 3.1). The piglets diet was given four times daily, at 7:00 a.m., 10:00 a.m., 1:00 p.m. and 4:30 p.m.

3.9.3 Data Collection

The pigs were fed in the five experimental diets, Con., IE1, IE2, FAS001 1.0X and FAS001 1.5X. Daily feed intake was calculated as the different between given feed and residual feed at every day. Feed conversion ratio (FCR) was calculated as the ratio of weight gain to the amount of feed consumed during the experiment period. Pig weight in each replicate were measured at the start and every weeks until the end of the experiment at 42 day.

Figure 3.1 Experimental pens.

3.10 The estimation of enzyme added diets by measuring blood urea nitrogen (BUN)

At the start and every two weeks of the experiment, blood samples were collected at 6:00 a.m. via jugular veins from two pigs per replication (1 male and 1 female) and placed in tubes with 100 µl of ethylenediamine tetra-acetic acid (EDTA) as an anticoagulant. Blood sample were kept in ice box until centrifugation at 3000 rpm for 10 min. to obtain plasma samples. The plasma was kept frozen at 0°C until the time of testing. Serum urea nitrogen concentrations were determined colorimetrically by measuring the product formed in the direct reaction of urea and diacetyl monoxime as described by Wybenga *et al.* (1971). (Appendix B-5).

3.11 Statistical analysis

Data on the numbers of cell and protease production in screening, protease production, carrier selection and storage test and *in vitro* digestibility were tested and compared between experimental groups using ANOVA and the Duncan's new multiple range test with the Statistical Package for Social Science (SPSS) (SPSS, 1999). The production performance data of piglets for average daily feed intake (ADFI), average daily gain (ADG) and feed conversion ratio (FCR) were subjected to analysis of variance using the general linear model procedure (GLM) of SPSS. Least square mean was need to compare the difference in the treatment means. The initial weight was used as the covariable for analysis. Blood urea was subjected to analysis of variance using the general linear model procedure (GLM) of SPSS. Least square mean was need to compare the difference in the treatment means. The initial BUN was used as the covariable for analysis.