CHAPTER 3
MATERIALS AND METHODS

3.1 Intruments, chemicals and media

3.1.1 Instruments

24 Well Cell Culture Cluster: Coaster® Corning Incorporated, U.S.A.

Aflatoxin (Total) Quantitative Test Kit Ser: 15056 Veratox®, U.S.A.

Analytical balance: Mettler Toledo model AG204, Urdorf, Switzerland

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

Beaker heater: Gerhardt type EV 26, Germany.

Blender: Osterizer, U.S.A.

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

Boiling Sterilizer: Applied Medic Ltd., partnership, Bangkok, Thailand.

Centrifuge: Beckman Superspeed Centrifuge model Avanti J125,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.

3.1.2 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
di-Potassium hydrogen phosphate	Merck	Analytical

Chemicals	Company	Grade
Folin-Ciocalteu's pshenol	Merck	Analytical
Glucose	Merck	Analytical
di-Sodium hydrogen phosphate	Merck	Analytical
Hydrochloric acid	Merck	Analytical
Selenium reagent mixture	Merck	Analytical
Sodium carbonate	Merck	Analytical
Sodium citrate	Merck	Analytical
Sodium hydroxide	Merck	Analytical
Sodium potassium tartate	Merck	Analytical
Sulfuric acid	Merck	Analytical
Sulphosalicylic 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 barley	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

Chemicals	Company	Grade
Phenol red	Sigma	Analytical
Sodium phytate	Sigma	Analytical
Pepsin	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	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
Rice flour	TWFP CO.,LTD	Food
Glutinous rice flour	TWFP CO.,LTD	Food
Tapioca flour	TWFP CO.,LTD	Food

3.1.3 Media

Media	Company
Agar powder	Purified Agar Ltd.
Rice bran	Bangkok Agro-industrial products Public Co.,Ltd.
Broken rice	Bangkok Agro-industrial products Public Co.,Ltd.
Wheat bran	Bangkok Agro-industrial products Public Co.,Ltd.
Soybean meal	Bangkok Agro-industrial products Public Co.,Ltd.
Rice bran, solvent extract	Bangkok Agro-industrial products Public Co.,Ltd.
Corn	Bangkok Agro-industrial products Public Co.,Ltd.

3.2 Microorganisms

The fungal strains used in this study were obtained from BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency (BIOTEC), Thailand.

Table 3.1 Fungal strains used in this study

No.	Original code	BCC	Scientific name	Group/Substrate
1	KPFC 17	103	*Penicillium* sp.	Soil fungi
2	KPFC 175	210	*Dihetrospora* sp.	Soil fungi
3	KPFC 277	274	*Aspergillus* sp.	Soil fungi
4	KPFC 614	4435	*Aspergillus* sp.	Soil fungi
5	KPFC 672	497	*Nodulisporium gregarium*	Soil fungi
6	KPFC 678	4441	*Penicillium* sp.	Soil fungi
7	KPFC 770	4487	*Nectria* sp.	Soil fungi
8	KPFC 919	4607	*Aspergillus* sp.	Soil fungi
9	KPFC 947	4605	*Aspergillus* sp.	Soil fungi
10	KPFCN 16	5772	*Aspergillus niger*	Soil fungi
11	RFD 412	1171	*Xylaria badia*	Xylariaceae

3.3 Screening of β-glucanase producing strains

3.3.1 Culture maintenance

Fungi was firstly grown on the patato dextrose agar (PDA) plate at a temperature of 30°C for 5 days. The spore suspensions were collected aseptically by adding sterile distilled water containing 0.05% Tween 80 to each plate and brushing lightly with a sterile spatula. Mycelium was separated from spore suspension by filtering through a pad of sterile cotton wool. The spore was harvested aseptically by centrifugation and resuspended in fresh and sterilized distilled water. The resuspended spore (0.5 ml) was dispensed in 1.5 ml sterile cryotubes. Sterilized glycerol was added to the final concentration of 50% (v/v) level. The labelled tubes were vortexed to ensure an even dispersion. Spore concentration was determined by direct counting on a haemocytometer. Finally, the tubes were kept at -80°C.

3.3.2 Inoculum culture

Fungi was firstly grown on the patato dextrose agar (PDA) plate at a tempareture of 30°C for 5 days. The inoculum culture from plate was cut by a sterile needle to make about 3 pieces of 0.5x0.5x0.5 cm and then the fungi inoculum were inoculated in to 250 ml Erlenmeyer flask containing 50 ml of wheat bran-soybean medium (WS) containing 3% (w/v) wheat bran and 2% (w/v) soybean meal in 250 ml shaking flask, incubated at 30°C and 200 rpm. After incubation for 5 days, the culture broth was centrifuged at 4°C and 13,000 rpm for 15 min, and the supernatant as collected for screening.

3.3.3 β-glucanase activity assay

β-Glucanase activity was determined according to the modified method of Mandels *et al*., (1976). The β-glucanase activity of the enzyme was determined using β-glucan from barley (Sigma) as a substrate. One unit of enzyme activity was defined as the amount of the enzyme resulting in the release of 1 μg of glucose per min at 40 °C under the reaction conditions.

Substrate : 0.5% β-glucan from barley (Sigma) in 0.2 M citrate phosphate buffer,pH 3.0 and 7.0

Procedure

A 1.5 ml of reaciton mixture contained 0.5 ml of appropriate diluted enzyme solution and 1.0 ml of β-glucan substrate in 0.2 M citrate phosphate buffer, pH 3.0 and 7.0. The mixture was incubated at 40 °C for 30 min. The reaction was stopped by the addition 3.0 ml of dinitrosalicylic acid solution (DNS). After mixing on a vortex mixer, the tubes were boil for 5 min and cool in cold water. Then add 10 ml of distilled water and mix. Measure the color formed against the spectro zero at 540 nm (OD_{540}). A blank was done in the same manner except that the enzyme was added after the addition of dinitrosalicylic acid solution.

3.3.4 Evolution of safety

3.3.4.1 Cytotoxicity test

Cytotoxicity of crude β-glucanase powder was determined by the 3-(4,5-dimethylthiazoyl-2-yl)2,5diphenyltetrazolium bromium (MTT) ASSAY according to the method of Plumb *et al*., (1989). (Appendix B-1)

3.3.4.2 Aflatoxin test

To test the aflatoxin in the enzyme extract and in the feed trated with the enzyme of the selected strains, the ELISA test kit was used due to the method of the Aflatoxin (Total) Quantitative Test Kit Ser: 15056 Veratox®. (Appendix B-2)

3.3.4.3 Ochratoxin test

The ochratoxin in the enzyme extract and in the feed treated with the enzyme of selected strains were tested by using the ELISA test kit according to the method of the Ochratoxin Quantitative test Kit Serial: 2516 Veratox®.(Appendix B-3)

3.3.5 Proteolytic stability

For *in vitro* evaluation the β-glucanase preparation was incubated at low and neutral with or without proteolytic enzymes (pepsin and pancreatin).

Incubate at low pH and pepsin

A solution (0.2 ml) was added to 5 ml each of 0.1 N glycine-HCl buffer (pH 3.2) with or without 4000 U/ml of pepsin (1:10,000 pfs, Sigma Chemical Co., St.

Louis, MO). After preincubation of the mixtures at 40°C for 60 min. This dilution was assayed for β-glucanase activity.

Incubation at neutral pH and pancreatin

A solution (0.4 ml) was added to 5 ml of 0.1 M phosphate buffer (KH_2PO_4-NaOH) at pH 7 containing 2 mg/ml pancreatin. After preincubation the mixture at 40°C for 30 min. This dilution was assayed for β-glucanase activity.

3.3.6 Resistance to heat

To test resistance to heat, crede β-glucanase was exposed to heat treatment at pelleting temperature (75°C), the remaining activity of crude β-glucanase was measured at pH 7.0, 40 °C after heating in water bath at 75 °C for 2 and 5 min, the reaction was immediately cooled down in iced-water and the remaining activity was measured under standard assay condition, comparing with the crude β-glucanase without incubation.

3.4 Optimization of crude β-glucanase production

Crude β-glucanase production by selected microorganisms was carried out in medium using solid-state fermentation, in which cultured conditions such as carbon sources, ratio of carbon sources : nitrogen source (soybean meal), media additive, moisture ratio, inoculum size and cultivation time were evaluated. According to the method of Tapingkae (2003), the medium were mixed with distilled water at ratio of 1:1.5, the cultured were inoculated with 10^5 spores/g medium and incubated 30°C for 24 h.

3.4.1 Effect of carbon sources

This medium consisted of 40% soybean meal and 60 % Carbon sources such as defatted rice bran, corn, broken rice and rice bran was used for wheat bran substitution in Wheat bran Soybean medium (WS).

3.4.2 Effect of carbon source and nitrogen source ratio

For selected carbon sources was mixed with nitrogen source (soybean meal) at the ratio of 50: 50, 60: 40, 70: 30, 80: 20, 90: 10 and 100: 0.

3.4.3 Effect of media additive

Study of media additive such as mineral (K_2HPO_4 2 %, $MgSO_4.7\ H_2O$ 0.4 %, $CaCl_2.2H_2O$ 0.2% (w/v)) 0.5 ml: 1g medium, Urea (1 g/kg medium) and Ami (10 ml/ kg medium).

3.4.4 Effect of moisture ratio

The medium and distilled water were mixed at the ratio 1:1.0, 1:1.5, 1:2.0, 1:2.5 and 1:3.0.

3.4.5 Effect of inoculum size

To achieve the optimum inoculum size of microorganism the inoculum was added to medium from 10^3, 10^4, 10^5 and 10^6 spores/g medium.

3.4.6 Effect of time course profiles

This study was to investigate the influence of time course profiles on the β-glucanase production in solid state fermentation (SSF) for 24, 48, 72, 96, 120 and 144 hours.

3.5 Preservation of crude β-glucanase as dry powder

The crude β-glucanase was mixed with various carriers rice flour, Glutinous rice flour and Tapioca flour at ratio 1:1. Then, the mixtures were dried at 40°C for 6 h in the incubator. After drying, the mixtures were subsequently ground with blender. The β-glucanase activity in the mixed sample was measured at not only before drying but also after grinding. The best carrier, the optimum mixing ratio and the optimum drying method were judged from the enzyme product (powder form) which expressed the highest activity after drying.

3.6 Shelf-life of crude β-glucanase powder

The effect of air exposure was carried out by comparing β-glucanase activity of products which were between the freshly opened product and opened product. After grinding, the product was packed in aluminum bag by sealer, and kept at various

temperatures (4-7°C, 30°C and 45°C). The β-glucanase activity of products were measured at 0, 4, 8, and 12 week after storage.

3.7 Testing of enzyme quality by *in vitro* digestibility

The raw materials (barley, oat and wheatbran) measured the *in vitro* digestibility (Boisen, 1991) of β-glucan. The experimental model was a completely randomized design with 5 treatments listed as follows:

i. Diet with no enzyme added (Control)
ii. Diet supplement with crude β-glucanase at the 0.5 times β-glucanase activity of imported enzyme
iii. Diet supplement with crude β-glucanase at the same β-glucanase activity of imported enzyme
iv. Diet supplement with crude β-glucanase at the 1.5 times β-glucanase activity of imported enzyme
v. Diet supplement with imported enzyme

The imported enzyme used in all studies was Roxazyme G2® which contains an enzyme complex derived from *Trichoderma longibrachiatum.* The main enzyme activities of this complex are β-glucanase 18,000 units and cellulase 8,000 units and xylanase 26,000 units per gram. It has been developed especially to complement the digestive enzymes of pigs and poultry, so that the non-starch polysaccharides (NSP) in cereals and legumes.

The addition rate was calculated to provide the same enzyme activity (based on the β-glucanase activity and the manufacture's recommended addition rate). The procedure of *in vitro* digestibility was described in Appendix B-7.

3.8 β-glucan analysis

The assay is specific for mixed linkage [(1-3) (1-4)-β-D-glucan. Kits suitable for performing 100 assays are available from Megazyme. Samples are suspended and hydrated in a buffer solution of pH 6.5 and then incubated with purified lichenase enzyme and filtered. An aliquot of the filtrate is then hydrolysed to completion with purified β-glucosidase. The D-glucose produced is assayed using a glucose

oxidase/peroxidase reagent. The detail of the analysis is described clearly in Appendix B-6.

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

Two pig diets, with different phase of feeding, 1 to 18 days and 19-42 days of the experiment. The diet compositions were calculated by using linear programming through LINDO software version 6.1 (LINDO Systems, 2003). The diet composition and chemical analysis are shown in Table 3.2 and 3.3, respectively. Each experimental diet was mixed and divided into 3 treatments as follows:

i. Diet with no enzyme added (Control)

ii. Diet supplement with imported enzyme.

iii. Diet supplement with crude β-glucanase at the same β-glucanase activity as imported enzyme.

3.9.3 Animals, housing and management

The experiment was carried out at swine farm in Department of Animal Science, Faculty of Agriculture Chiang Mai University. Eighty four weaning cross breed pigs (42 male and 42 female) at the same age, and average body weight 8.0 ± 0.82 kg (mean ± S.D.) were used. Pigs were randomly allotted into three treatments, as two male and two female piglets per pen with seven replication per treatment and housed in an opened nursery with slotted floor cage dimension of 2.0 x 2.0 m which had slated floors, two nipple drinkers and a feed trough (Figure 3.1). The piglets diet was given 2 times daily at 8:30 a.m. and 4:30 p.m.

Table 3.2 Composition of the weaning diets for productive performance experiment.

Components	**Diets (%)**	
	Formula 1 (1-18 days)	**Formula 2 (19-42 days)**
Barley	15	15
Broken rice	34.37	42.83
Soybean meal	41.94	33.66
Fish meal	1	1.08
Limestone	2.13	1.92
Salt	0.25	0.25
Premix	0.25	0.25
Methionine	0.04	0.006
Tallow	5	5
Toxin binder	0.1	0.1

Table 3.3 Chemical analysis (%) of the weaner diets for productive performance experiment

Chemical Compositions (%)	**Diets**	
	Formula 1	**Formula 2**
Dry matter	89.79	89.94
Crude protein	24.23	21.17
Crude fiber	4.73	4.62
Ether extract	6.42	6.44
Ash	5.93	5.81
Nitrogen free extract	48.48	51.90
β-glucan	0.332	0.309

Figure 3.1 Pen dimension of 2.0 x 2.0 m which had slotted floors, two nipple drinkers and a feed trough.

3.9.4 Data Collection

The pigs were fed in the three treatment diets. Daily feed intake was calculated as the different between given feed and residual feed 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 were weighed in each replicate and measured at the start and every weeks until the end of the experiment at day 42nd.