CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Chemical	Company
Acetic acid	Merck, USA
AFB_1-HRP conjugate	DOA Test Kit
Aflatoxin B_1 standard	DOA Test Kit
Ammonium molybdate	Carlo Erba, Italy
Ammonium sulphate, $(NH_4)_2SO_4$	Ajax, Australia
Beef extract	Himedia, India
Bovine serum albumin	Fluka, Switzerland
Calcium chloride, $CaCl_2$	Ajax, Australia
Calcium Chloride Dihydrate, $CaCl_2 \cdot 2H_2O$	Merck, USA
Casein	BDH, UK
Chitin from crab shell	Sigma, USA
Congo red	Sigma, USA
Coomassie Brilliant Blue G-250	Sigma, USA

Chemical	Company
Copper sulphate penta hydrate, $CuSO_4 \cdot 5H_2O$	Scharlau, EU
3, 5-dinitrosalicylic acid	Sigma, USA
Dibasic sodium phosphate, $Na_2HPO_4 \cdot 2H_20$	BDH, UK
Di-potassium hydrogen phosphate (K_2HPO_4)	Merck, USA
D-(+)-glucose	QRec, Singapore
Ethanol (95%)	Merck, USA
Hydrochloric acid (37%)	Carlo Erba, Italy
Iodine	Carlo Erba, Itary
Laminarin	Sigma, USA
Magnesium sulphate ($MgSO_7 \cdot 7H_2O$)	Merck, USA
Malt extract	BD, USA
Methanol	Union Science, Thailand
Microwell of MicroELISA	DOA Test Kit
Monobasic sodium phosphate, $NaH_2PO_4 \cdot 2H_2O$	Sigma, USA
Nutrient agar	Scharlau, EU
Nutrient broth	Himedia, India
N-acetyl-D-glucosamine	Sigma, USA

Chemical	Company
Peptone	Merck, USA
Phosphoric acid (85%)	Carlo Erba, Italy
Potassium Chloride, KCl	Scharlau, Spain
Potassium dihydrogen phosphate (KH_2PO_4)	Merck, USA
Potassium iodide	Carlo Erba, Italy
Potassium nitrate (KNO_3)	Rankem, India
Potato dextrose broth (PDB medium)	Lab-scan, Thailand
sodium acetate (tri-hydrate), $CH_3COONa•3H_2O$	Fisher, UK
Sodium bicarbonate, $NaHCO_3$	Merck, USA
Sodium carbonate, Na_2CO_3	Univar, Newzealand
Sodium carboxymethyl cellulose	Merck, USA
Sodium chloride	Carlo Erba, Italy
Sodium hydroxide, NaOH	Carlo Erba, Italy
Sodium nitrate, $NaNO_3$	Merck, USA
Sodium potassium tartrate	Fisher, UK
Sodium sulfate anhydrous, Na_2SO_4	Fisher, UK
Soluble starch	APS, Australia

Chemical	Company
Stop solution (0.5 M phosphoric acid)	DOA Test Kit
Sucrose	Fisher, UK
Substrate (TMB)	DOA Test Kit
Trichloro acetic acid (TCA)	BDH, UK
Tween-80	BDH, UK
Washing buffer (PBS-T)	DOA Test Kit
Yeast extract	Lab-scan, Thailand

2.2 Instruments

Name	Model	Company
Analytical balance	PB 1502-S	Mettler Toledo, Switzerland
Autoclave oven	MLS-3780	Sanyo, Japan
Centrifuge, refrigerated	6800	Kubota, Japan
Centrifuge tube		
Counting chamber	-	Hausser Scientific, USA

Name	Model	Company
Homogenizer	Terre haute	Glas-Col, USA
Hot plate and magnetic stirrer	Clifton Cerastir	Torrey Pines Scientific, USA
Incubator	WB M15	Falc, Italy
Laminar flow	Telstar Bio-II-A	Terrassa, Spain
Light microscope	CH 30 RF-200	Olympus, Japan
Micropipette	-	Gilson, France
MicroELISA reader	Ges.m.b.H	Anthous labtec, Austria
Oven 400	Memmert,	Germany
Pipette tip	-	Axygen, USA
pH meter	713	Metrohm, Switzerland
Shaker	-	Gallenkamp, XX
Spectrophotometer	Thermo-spectronic	Thermo Fisher scientific, USA
Vortex mixer	Genie-2 G-560E	Bohemia, USA

2.3 Bird chili powder samples and microorganism

2.3.1 Bird chili powder

Three samples of bird chili powder of *Capsicum frutescens* Linn. were collected randomly from local market in Chiangmai, Thailand. After purchase, each sample of chili powder was stored in a sterile glass container in the laboratory at room temperature (25-30°C) for 6 months until used. (**Figure 2.1**)

Figure 2.1 Three chili powder samples

2.3.2 *Bacillus subtilis*

Bacterial antagonist, *Bacillus subtilis* BCC 6327 strain was obtained from National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The strain was stored on nutrient agar (NA). The stock culture was grown at 30 °C and maintained at 4 °C for further experimental use.

2.4 Isolation and characterization of aflatoxigenic fungi from bird chili powders

2.4.1 Preparation of dilution series and pour plate method

One gram of each chili sample was added into sterile peptone (1%) solution and prepared dilution series up to 10^{-6}. One ml of each serial dilution was introduced into five replicate sterile petri dishes and molten potato dextrose agar (PDA) was poured over inoculum. Plates were manually rotated and incubated for one week at 30±2°C (Elliot, 1988; Rajasinghe *et al*., 2009).

2.4.2 Identification of aflatoxigenic and non-aflatoxigenic fungal species

The single of isolated fungal colonies that grew out after incubation were transferred to fresh PDA plates under aseptic condition and to PDA slant for storage. The isolated fungal colonies in fresh PDA plates were incubated for 7 days at 30±2 °C and their morphological features were studied and recorded.

Slide cultures, freshly prepared slides under aseptic condition and culture on PDA, both vegetative and reproductive characters, were observed under the microscope (40X). The color photographs were used in identifying fungi and identification of fungi was confirmed by comparing with published data or descriptive key (Barnett and Hunter, 1987). The isolated aflatoxigenic fungi were used in further experiment.

Aflatoxigenic and non-aflatoxigenic fungi grown on replicate dilution plates were counted and recorded. The total number of fungal colonies and number of colonies of each fungal species grown on the same plate were recorded. Frequency of occurrence of each fungal species was calculated per replication dilution plate using the following equation.

$$\text{Frequency of occurrence of a fungal species} = \frac{\text{Number of colonies of a fungal species}}{\text{Total number of fungal colonies}} * 100$$

2.4.3 Molecular identification of the fungal species

The most frequently isolated fungi from bird chili powder (white fungal colonies) was identified for its species by using 18S rRNA sequence. (Bunyard *et al*., 1944; White *et al*., 1990; Thomson *et al*., 1994; Landvik, 1996; Hall, 1999)

i) Culture selection

Fungal culture was grown on PDA for 4-16 weeks at 25 °C. Actively growing mycelium was scraped off the surface of a culture and transferred to 2 ml of microcentrifuge tubes and the biomass was lyophilized at -80 °C for 24 hours.

ii) DNA extraction

Extraction buffer (1% CTAB, 0.7 M NaCl, 50 mM Tris-HCl, 10 mM EDTA, pH 8) was added to fungal samples. The samples were ground in a 2 ml microcentrifuge tube and the volume adjusted by adding 700 µl extraction buffer and mixed by inverting the tubes and incubated at 65 °C for 1 hour. Samples were centrifuge at 12,000 xg for 10 min at 25 °C. The aqueous supernatant was transferred into a new microcentrifuge tube with phenol-chloroform-isoamyl alcohol by mixing gently and by centrifugation at 12,000 xg for 10 min at 25 °C. The upper liquid phase was transferred to a new microcenfuge tube containing 7.5 M of ammonium acetate. The DNA was precipitated by ethanol (-20 °C overnight) and centrifugation at 12,000

xg for 10 min at 15 °C. The DNA-pellet was washed with ice-cold 70% ethanol and dried at 25 °C. The pellet was redissolved in 50 µl of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA pH 8.0)

iii) PCR amplification

Primers used for PCR amplification and for sequencing of the internal transcribed spacer region (ITS) were ITS4 and ITS5 (White *et al*., 1990; Bunyard *et al*., 1994; Landvik 1996). Amplification was performed in a 50 µl reaction mix : 10 mM of each dNTP (1 µl), 10 µM of each primer (1 µl), 10% of dilution buffer (5 µl), 25 mM of $MgCl_2$ (5 µl), 4 M of enhancer (5 µl) and 60-62% of sterile distilled water (30.8 µl), 0.2 µl of Taq DNA polymerase kit from FERMENTAS and 10-50 ng of genomic DNA template (1 µl) carried out using a PCR Model MJ Research DYAD ALD in 200 µl reaction tubes. (95°C, 0.5 min; 52°C, 1 min; 72°C, 1.5 min; 35 cycles). PCR products (7 µl aliquots) were checked by electrophoresis in 1% agarose gels with 0.003% ethidium bromide in 0.5xTBE buffer (0.044 M Boric acid, 1.1 mM EDTA, 0.045 M Tris, pH 8) for purity.

iv) DNA purification and sequencing

PCR products were purified using NucleoSpin® Extract Kit (Macherey-Nagel, Germany). The PCR products were sequenced by Macrogen. Inc. (Korea) with the same primers as in the PCR amplification.

v) Phylogenetic analyses

Each sequence was checked for ambiguous bases and refined visually, assembled using BioEdit 7.0.9.1 (Hall, 1999). The consensus sequences for each

DNA region were multiply aligned by Clustal W 1.6 (Thompson *et al*., 1994) and checked manually with all sequences derived from the GenBank database and the accession numbers that are included in phylogenetic trees. The alignment included the most similar sequence identified through BLAST search.

2.5 Aflatoxin analysis of chili powder sample and *Aspergillus flavus* production

The aflatoxin in dried bird chili powder and production capability of *A. flavus* that isolated from bird chili powder were determinated by MicroELISA assay (Shinphuti *et al*., 2009; DOA-Aflatoxin ELISA Test Kit).

2.5.1 Aflatoxins analysis of bird chili powder

Ten gram each of bird chili powder sample were transferred to 250 mL Erlenmeyer flasks and 50 mL of 70% methanol were added. Then the samples were placed on a rotary shaker at 160 rpm for 30 min and allowed to settle on whatman No.1 filter paper. 1 mL filtrated solution was added to 3 ml 0.01 M phosphate buffer saline 0.05% Tween 20% (PBS-T; washing buffer) to dilute the solution. 50 µl of 0, 0.2, 0.5, 1, 2 and 5 ng/ml (ppb) aflatoxin B_1 standard solution and diluted sample solution were added into microwell of MicroELISA plate that was covered by aflatoxin B_1 antibody. 50 µl AFB_1-HRP conjugate enzyme were added and shaked. The mixture on microplate was incubated in dark at room temperature (30 °C) for 30 min. After incubation, the mixture solution on microplate was discarded and washed by washing buffer (PBS-T) for 3 times. The solution was discarded until dryness microplate. And then, 10 µl substrate (TMB) was added into microplate well and

incubated in dark at room temperature (30 °C) for 10 min. The mixture appeared as blue solution and the light blue indicated that the solution was very toxic. After that, 100 µl 0.5 M phosphoric acid was added for stop reaction, the color change to yellow. The absorbance value was measured by MicroELISA reader at 450 nm and the amount of aflatoxin B_1 was calculated from standard curve of aflatoxin B_1 (Shinphuti *et al.*, 2009).

2.5.2 Aflatoxin production capability of *A. flavus*

A. flavus isolated from bird chili powder sample was maintained on PDA as a pure culture. The slant culture of *A. flavus* was incubated at 30±2 °C for 7 days. The spore suspension was prepared by adding 10 mL sterile distilled water containing a drop of Tween-80 into *A. flavus* slant culture and counted fungal spores by using a haemocytometer (Paranagama *et al*., 2003). A semi-defined medium (SMKY) (20 g sucrose, 7 g yeast extract, 3 g KNO_3, 0.5 g $MgSO_7 \bullet 7H_2O$ per 1 L of distilled water) was prepared as culture medium. 50 ml of SMKY was dispended into 250 ml Erlemeyer flasks and sterilized by autoclaving. After cooling, the flasks were inoculated with 5 ml spore suspensions of *A. flavus* and incubated for 8 days at 30±2 °C (Parangama *et al*., 2003). The liquid portion was separated from the mycelia mats by using whatman no.1 filter paper. 1 ml of liquid medium was transferred into test tube that contained 5 ml of 70% methanol. The procedure of extraction and determination of aflatoxin were the same as the analysis of aflatoxin in bird chili powder (**2.5.1**; Shinphuti *et al*., 2009).

2.6 Growth pattern of *B. subttilis* on nutrient broth medium

The single colony of the 3-days old *B. subtilis* strain on nutrient agar was inoculated in nutrient broth (NB) medium (1% peptone, 0.3% beef extract, 0.5% NaCl, pH 7.0) for using as preculture. The preculture was incubated on a constant temperature shaker at 30 °C with continuous shaking at 160 rpm for 12 h. The preculture broth was transferred to 300 ml fresh NB in 2 replicates and incubated at 30 °C, 160 rpm. One ml of the culture broth was harvested every 4 to 8 h until 56 h. The suspension was pipetted into semimicro cuvettes and optical density (OD) of the suspension was measured at 610 nm with a spectrophotometer. The OD 610 nm values from 3 replicates of the culture flasks were averaged and plotted against time on a linear coordinate. (Tempelaars *et al*., 2011)

2.7 Testing for the antagonistic activity of *B. subtilis* against *A. flavus*

B. subtilis was tested for antagonism against the isolated aflatoxigenic fungi, *A. flavus* by dual culture of antagonist (*B. subtilis*) and pathogen (*A. flavus*) in broth medium and on agar plate.

2.7.1 The antagonistic activity of *B. subtilis* against *A. flavus* in broth

B. subtilis was tested for their *in vitro* antagonism against *A. flavus* from bird chili powder samples. According to the modified method of Jong-Gyu-Kim (2007), The spore suspension of *B. subtilis* was cultured in NB medium for 24 h (10^6 spores/mL counted by pour plate technique). The spore suspension of *A. flavus* was prepared by adding a mycelial plug of 0.6 mm diameter into sterilized 10 ml distilled

water and mixed by vortex ($8.62x10^6$ spores/ml counted cell by heamacytometer). The treatment test tube was inoculated with 500 µL suspension of *B. subtilis* and the same volume of *A. flavus* spore suspension into 5 ml potato dextrose broth (PDB) in 5 replicates and incubated at 30 °C for 7 days. *A. flavus* fungal culture grown in the absence of the *B. subtilis* strain was used as a control. Mycelial mass from culture was harvested after 7 days and dried.

The mycelial mass was separated from culture broth by filtration through Whatman No.1 filter paper and washed three times with 10 ml of hot distilled water. Fungal growth was determined gravimetrically after drying in an oven at 75 °C overnight, cooled at room temperature in a desiccator and weighed (Yonni *et al*., 2004). The dry weight of mycelial mass was used as a measurement of the fungal growth.

2.7.2 The antagonistic activity of a single streak of *B. subtilis* against *A. flavus* on agar plate

B. subtilis was used for antagonism against *A. flavus* by dual culture in PDA plate. The culture plate was prepared by swabbing of 7 days old *A. flavus* mycelia onto PDA plate, and then the single colony of 7 days old *B. subtilis* on NA was single streaked onto middle about 2 cm of the culture plate. For control, the swabbed *A. flavus* on PDA plate without a single streak of *B. subtilis* was incubated for 7 days at 30°C. Fungal growth inhibition was observed for the clear zone around single streak of *B. subtilis* and compared with control plate.

2.7.3 The antagonistic activity of *B. subtilis* against a mycelial plug of *A. flavus* on agar plate

B. subtilis was used for *in vitro* antagonism against the isolated aflatoxigenic fungi (*A. flavus*). The antifungal activity was determined by dual culture in NA plate. *B. subtilis* culture was incubated in nutrient broth at 30 °C, 160 rpm for 54 h. The culture plates were prepared by adding 1 ml spore suspension of *B. subtilis* (10^8 spores/ml) in 10 ml NA and mixing by vortex. The spore suspension in NA was introduced in autoclaved petri dish. After solidifying, a mycelial plug of 0.6 cm diameter from 3 days old *A. flavus* was cut and transferred onto the middle of *B. subtilis* NA plate. The fungal plug placed on NA plates without spore suspension of *B. subtilis* was used as control. The diameter fungal growth in both the control and dual culture plates was measured on 3rd day after incubation. The levels of inhibition were calculated by using the equation as previously mentioned by Yuan and Crawford (1995). The level of inhibition was defined as the subtraction of the fungal growth radius (γ_0 in centrimeters) of the control plate from the distance of the growth in the dual culture plate (γ in centrimeters), where $\Delta\gamma = \gamma_0 - \gamma$. And the percentage of inhibition was calculated by using the following equation.

$$\% \text{ inhibition of fungal growth} = \frac{(\gamma_0 - \gamma)}{\gamma_0} \times 100$$

After 3 days of incubation, mycelial fungi from both control and dual culture plates were transferred to fresh potato dextrose agar medium to check for the viability of the mycelial in that area.

2.8 Inhibitory activity of extracellular metabolites in cell free supernatant of *B. subtilis*

Whether the mycelial fungal growth of *A. flavus* was inhibited by using extracellular metabolites in cell free supernatant of *B. subtilis* was determined in broth and agar plate medium. The fungal morphology affected after inoculated with cell free supernatant of *B. subtilis* by slide culture, was observed under the microscope.

2.8.1 Inhibition of fungal growth mycelium by cell free supernatant of *B. subtilis* in broth medium

The preculture of *B. subtilis* was inoculated in 300 ml NB medium and incubated on a constant temperature shaker (30 °C, 160 rpm). 30 ml culture medium was collected during 12, 24 and 36 h of incubation. Cells were removed by centrifugation at 14,000 g for 20 min at 4 °C. The inhibition of fungal growth by the extracellular metabolites from *B. subtilis* culture broth was estimated by the dried mycelial weight. Cell free supernatant was added in 100 ml flasks at concentrations of 25% v/v cell free supernatant at 12, 24, 36 h of incubation and adjusted to a final volume of 30 ml with PDB. The control flask was without cell free supernatant.

Each treatment flask was inoculated with 100 µl of aflatoxigenic fungi spore suspension containing 8.62×10^6 spores/ml and incubated at 30 °C in a shaking incubator at 160 rpm. *A. flavus* Mycelium was harvested after 5 days, filtered, dried, and weighted the dried mycelial was recorded as same in **2.7.1**. The inhibition percentage of mycelial mass was calculated using the following equation.

$$\% \text{ inhibition of mycelial mass} = \frac{(\text{dried wt. of control} - \text{dried wt. of treatment})}{\text{dried wt. of control}} \times 100$$

2.8.2 Inhibition of fungal mycelium by cell free supernatant of *B. subtilis* on agar plate

The cell free supernatant was prepared from nutrient broth culture, and incubated on a shaking incubator at 160 rpm, 30 °C. The culture broth was collected at 54 h of incubation which supernatant was removed by centrifugation at 14,000 rpm for 20 min at 4 °C. The growth inhibitory effect was estimated by using the radial growth inhibition assay as described in **2.7.3**. One ml cell free culture was added into 10 ml of the soft PDA. The test plates were prepared by pouring about 15 ml of PDA as the base layer and kept at room temperature until solidifying. Then each test plate was overlaid with cell free supernatant in soft PDA as the seed layer. A 3 days-old mycelial plug of 0.6 cm diameter was cut using a sterile cork borer and was transferred to the test plates with cell free supernatant seed layer in 6 replicates. Control contained only PDA without *B. subtilis* cell free supernatant. Fungal growth was measured every day for 5 days, after 3 days of inoculation (3, 4, 5, 6 and 7 days). Fungal growth inhibition was expressed as percentage of radial growth inhibition in comparison with the control, as same in the equation in **2.7.3.**

2.8.3 Fungal hyphal morphology as affected by the crude extracellular metabolites

Fungal hyphal morphology was observed by slide culture. The exponential cell free culture supernatant of *B. subtilis* was used as crude extracellar metabolite. The culture of fermentation broth was incubated at 30 °C, 160 rpm with shaking incubator for 9 h. The cell free supernatant was prepared by centrifugation at 14,000

rpm, 4 °C for 20 min. One ml of cell free supernatant was mixed with 10 ml soft PDA (10% v/v) and poured into sterilized petri dish. The control was without cell free supernatant. Both of test plate and control plate were cut in square size 1x1 cm by sterile knife. The square of cut agar was transferred in sterilized glass slide and inoculated mycelial fungi by a loop on 4 sides of square agar. The slide culture was incubated on a petri dish at 30 °C for 3 days. Morphology of test and control agar were investigated under the microscope with the magnifications of 10 X and 40 X (Postgate *et al*., 1961).

2.9 Plate screening of hydrolytic enzymes produced from *B. subtilis*

B. subtilis was screened for its capacity to produce hydrolytic enzymes by plate agar screening. The *B. subtilis* was grown on NA supplemented with different substrates for each enzyme production. The different substrates, *i.e.* soluble starch, colloidal chitin, casein, Na-carboxymethyl cellulose and Tween 20 were used for assessment of amylase, chitinase, protease, cellulase and lipase, respectively. The hydrolytic enzyme production from *B. subtilis* was determined as follows and each experiment was performed in three replicates.

2.9.1 Amylase screening plate

The screening plate for amylase enzyme production was prepared as agar medium that contained 20.0 g soluble starch, 0.5 g yeast extract, 1.0 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4.7H_2O$, 1.36 g KH_2PO_4 and 15.0 g agar per 1,000 ml distilled water and adjusted pH to 7.0. The *B. subtilis* agar plug with a diameter of 0.6 cm was placed in the middle of the prepared agar plate and incubated at 30 °C for 2 days. After 2 days

of incubation, the plates were dropped with 1% iodine in 2% potassium iodide. The plate considered positive for amylase production was appeared as a clear zone surrounding the colony.

2.9.2 Chitinase screening plate

The screening plate, a colloidal chitin agar medium, was consisted of 20.0 g colloidal chitin, 0.5 g yeast extract, 1.0 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4.7H_2O$, 1.36 g KH_2PO_4, 15.0 g agar per 1,000 ml distilled water and adjusted pH to 7.0. The 0.6 cm plug of *B. subtilis* was inoculated in the screening plate and incubated at 30 ℃ for 3 days. A clear zone formed around the colony was considered positive for chitinase production.

2.9.3 Protease screening plate

The 0.6 cm plug of *B. subtilis* was incubated on the NA plates containing casein (1%w/v) and incubated at 30 ℃ for 2 days. The plates were flooded with 25% TCA (trichloro acetic acid) for 15 min. The clear zone formed around the colony indicated the casein was digested in area around the colony, while the undigested casein was precipitated with TCA.

2.9.4 Cellulase screening plate

The CMC agar plate for cellulase enzyme screening was prepared using 0.2% Na-carboxymethyl cellulose as substrate and contained 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.5% KCl, 0.2% peptone and 1.7% agar. The 0.6 cm plug of *B. subtilis* was inoculated on CMC agar. Plates incubated at 30 °C for 2 days were

flooded with 0.25%w/v aqueous Congo red for 0.5-1 hour and destained with 1 M NaCl for 15 min. The clear zone was revealed surrounding the colony, indicating the production of cellulase enzyme.

2.9.5 Lipase screening plate

The 0.6 cm plug of *B. subtilis* was grown on peptone agar medium that consisted of 10.0 g peptone, 5.0 g NaCl, 0.1 g $CaCl_2.2H_2O$ and 16 g agar per 1,000 ml distilled water and adjusted pH to 6.0. Peptone agar medium was supplemented with Tween 20 (1 ml of Tween 20 was added to 100 ml medium) as a substrate. The plates were incubated at 30 °C for 2 days. A clear zone formed surrounding the colony was shown for positive lipase production.

2.10 Determination of enzyme activity

The *B. subtilis* was assayed quantitatively for enzyme activity, i.e. chitinase, protease and cellulase, respectively. The reaction mixtures for each enzyme activity assay were divided into 4 treatments :

Treatment 1 : blank (distilled water)

Treatment 2 : control 1 (substrate of each enzyme with distilled water)

Treatment 3 : control 2 (supernatant of each enzyme with distilled water)

Treatment 4 : sample (substrate of each enzyme with supernatant of each enzyme and distilled water).

2.10.1 Proteolytic activity

i) Preparation of pre-culture for protease activity assay

B. subtilis was grown in 100 ml of NB in 250 ml Erlenmeyer flask for 20 h at 30˚C with rotary shaking at 160 rpm. 3 ml ($1.0x10^8$ spores/ml) of pre-culture was inoculated into 300 ml protease production liquid culture in 500 ml Erlenmeyer flask. The protease production medium consisted of 1.0 g glucose, 10.0 g peptone, 0.2 g yeast extract, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$ and 0.5 g K_2HPO_4 per 1,000 ml distilled water. The culture flasks were incubated on rotary shaker at 30˚C, with 160 rpm shaking incubator. The culture filtrate containing protease was collected by centrifugation at 6000 rpm, 4 ˚C for 20 min at 4, 8, 12, 24, 28, 32, 36, 48, 54 and 60 h of incubation time, the cell free culture filtrate were kept at -20 ˚C until used to assay proteolytic enzyme. In addition, the growth rate was measured at OD 610 nm with spectrometer as described in **2.5**. The experiments were performed in duplicates.

ii) Determination of protease activity

The protease activity using 1.5%w/v casein as a substrate. The reaction mixture of hydrolytic assay containing 500 µl of crude extract with 1,000 µl of 1.5%w/v casein in Na-phosphate buffer, pH 7.0 and adjusted volume to 2,000 µl with distilled water and then incubated in water bath at 40˚C for 10 min. 2 ml of 0.4 M TCA (trichloroacetic acid) was added to stop reaction and precipitate protein, the mixture was centrifuged at 3,000 rpm for 20 min to remove protein precipitate. 0.25 ml of clear mixture was added into a new tube, added 1.25 ml of 0.4 M Na_2CO_3 then shaken to well mix. 0.25 mL Folin reagent was added into each reaction tube and

incubated at room temperature for 10 minutes. The absorbance was measured at 660 nm in a spectrophotometer. One unit (U) of proteolytic activity was defined as the amount of enzyme required to produce 1 µmol of L-tyrosine in one minute, at 40˚C. Proteolytic activity was calculated as shown in **Appendix D.1** Specific activity of protease was calculated as protease activity (U/mg protein).

2.10.2 Chitinolytic activity

i) Preparation of pre-culture for chitinase activity assay

The single colony of *B. subtilis* was grown in 100 ml NB in 250 ml Erlenmeyer flask for 20 h at 30°C and inoculated into 300 ml colloidal chitin medium broth in 500 ml Erlenmeyer flask. The chitinase production medium consisted of 2% w/v colloidal chitin as substrate, 0.05% w/v yeast extract, 0.1% w/v $(NH_4)_2SO_4$, 0.03% w/v $MgSO_4 \cdot 7H_2O$ and 0.136% w/v KH_2PO_4 with adjusted pH to 7.0. The culture flask were incubated on a rotary shaker with 160 rpm, at 30 °C. The culture filtrate containing chitinase were harvested by centrifugation at 6000 rpm at 4 °C for 20 min at 4, 8, 12, 16, 20, 24, 28, 32, 36, 45, 48, 52 and 56 h of incubation time. The cell free supernatant was kept at -20°C until determination of chitinase activity and protein content. The growth rate was measured at OD 610 nm with a spectrophotometer. The experiments were performed in duplicate.

ii) Determination of chitinase activity

The chitinase activity was assayed using the method of Somogyi-Nelson (Green et al., 1989). The reaction mixture containing 1000 µl of 2% w/v colloidal chitin in 0.1 M potassium phosphate buffer, pH 7.0 as a substrate with 600 µl of crude extract was incubated in a water bath at 40 °C for 3 h. Then 1 ml of Somogyi's reagent was added. The mixture was boiled at 100°C for 10 min and cooled. 1 ml of Nelson's reagent was added and incubated at room temperature for 20 min and 1 ml distilled water was added. After centrifugation at 3000 rpm, at 4°C, for 20°C, the amount of N-acetyl glucosamine (GlcNAc) released in the supernatant and measured at absorbance for 520 nm of a colored complex formed between a copper-oxidized sugar and arsenomolybdate. One unit (U) of chitinase activity was defined as the amount of enzyme required to produce 1 µmol of GlcNAc per minute under the conditions of study. Chitinase activity was calculated as shown in **Appendix D3**. Specific activity of chitinase was calculated from chitinase activity (U/mg protein).

2.10.3 Cellulolytic activity

i) Preparation of preculture for assaying cellulase activity.

The single colony of *B. subtilis* was inoculated into 100 ml nutrient broth in 250 mL Erlenmeyer flask. The inoculated flask was incubated at 30 °C and 160 rpm for 20 h and used as preculture flask. 3 ml ($1.0x10^8$ spores/ml) of preculture was inoculated into 300 ml cellulase production liquid culture in 500 ml Erlenmeyer flask. The cellulase production medium consisted of 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.5% KCl, 0.2% peptone and 0.2% Na-Carboxymethyl cellulose as substrate. The culture flasks were incubated on a rotary shaker with 160 rpm, at 30°C.

The culture supernatant containing cellulase production was collected by centrifugation at 6000 rpm at 4 °C for 20 min at 4, 8, 12, 16, 24, 28, 32, 36, 40, 48, 52 and 56 h of incubation time, the cell free culture filtrate were kept at -20°C until used to assay cellulose activity. And the *B. subtilis* growth rate on cellulase production liquid medium was measured at OD 610 nm with a spectrophotometer. The experiments were performed in duplicates.

ii) Determination of cellulase activity

The cellulase activity in supernatant was determined according by Miller (1959). The reaction mixture containing 400 µl of crude extract with 500 µl of 1% w/v Na-carboxymethyl cellulose in 0.1 M sodium acetate buffer, pH 5.0 and adjusted volume to 1000 µl with 0.1 M sodium acetate buffer, pH 5.0 was incubated in water bath at 50°C for 10 min. 3 ml DNS (dinitrosalicylic acid) reagent was added, and the reaction mixture was boiled at 100°C for 5 min and immediately cooled on ice bath for 5 min. The absorbance was measured at 540 nm with a spectrophotometer. One unit (U) of cellulolytic activity was defined as the amount of enzyme required to produce 1 µmol of glucose in one minute, at 50 °C.

2.10.4 Protein content determination

Protein content was determined by dye binding method of Bradford (Bradford, 1976), using bovine serum albumin (BSA) as a standard. Bradford reagent was prepared by dissolving 0.1 g Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol and adding 100 ml 85% (w/v) phosphoric acid. After the dye has completely dissolved, the solution was diluted to 1.0 liter and filtered through the Whatman No. 1

filter paper before use. 1 ml of 1 mg/ml BSA standard solution in each dilution and protein samples from crude extracts were added with 5 ml of Coomassie Brilliant Blue reagent. The mixture was shaken and incubated at room temperature for 10 minutes. The absorbance was measured at 595 nm in a spectrophotometer. The amount of protein in each sample was determined from the standard curve that plotting the absorbance units versus micrograms of protein.

2.11 Effect of dried mycelia on production of lytic enzymes

The lytic enzyme production of *B. subtilis* was tested by culturing the spore suspension of *B. subtilis* in NB and supplemented with dry fungal cell wall mycelia of *A. flavus.* The lytic enzymes; chitinase, β-1, 3- glucanase and protease, were induced by the aflatoxigenic cell wall mycelium.

2.11.1 Preparation of dried mycelium

Erlenmeyer flasks (250 ml) containing 100 ml of PDB were incubated with 6 mm diameter discs of PDA of actively growing mycelium of *A. flavus*. The culture was incubated at 30°C for 7 days. The mycelium was collected by filtration through Whatman No.1 filter paper, washed with distilled water and homogenized in distilled water using a laboratory homogenizer. The suspension was centrifuged three times (6,000 rpm for 10 min), after washed with distilled water and air dried for 7 days. The mycelium was stored at 4°C until used as C-source (El-Katany *et al.*, 2000).

2.11.2 Qualitative determination of lytic enzyme activity

To test the lytic enzyme of *B. subtilis* in the *in vitro* antagonism study, the spore suspension of *B. subtilis* was cultured in NB supplemented with 0.5% w/v dry fungal cell wall mycelia of *A. flavus*. Erlenmeyer flasks (250 ml) containing 100 ml of NB was inoculated with single colony of *B. subtilis* and incubated at 30°C for 20 h then used as pre-culture. Spore suspension inoculum of *B. subtilis* ($1.0x10^8$ spore/ml of culture medium) was inoculated into duplicated 100 ml Erlenmeyer flasks containing 20 ml of NB supplemented with dried fungal mycelium as the sole carbon source (5 gL^{-1}). And the control flasks were NB without dried fungal mycelium. The culture was grown at 30 °C with rotary shaking at 120 rpm for 5 days. The culture was centrifuged at 4 °C for 10 min at 5000 xg and supernatants were tested for enzyme activity (El-Katany *et al*., 2000). The cell free supernatant was determined for the release of glucose by DNS method (Miller, 1959) and measured for GlcNAc using Somogyi's and Nelson's reagents (Green *et al*., 1989).

2.11.3 Enzyme activity assays

i) Chitinase activity

Chitinase activity was assayed using the colorimetric method described by Taechowisan *et al*. (2003). The assay mixture contained 1000 µl of 2% w/v colloidal chitin in 0.1 M potassium phosphate buffer, pH 7.0 as a substrate and 600 µl of crude extract. The reaction mixture was incubated for 3 h at 40 °C and added 1 ml of Somogyi's reagent. The mixture reaction was boiled for 10 min and immediately

cooled. 1 ml of Nelson's reagent was added and incubated at room temperature for 20 minute and 1 ml of distilled water was added. The mixture was centrifuged at 3000 rpm, 4°C for 20 minute. The supernatant were measured at absorbance 520 nm. The amount of GlcNAc released was calculated from standard curves and the activities of chitinase were expressed in specific activity (U/mg protein).

ii) β-1, 3- glucanase activity

β-1, 3- glucanase was assayed by incubating 500 µl of 2.0%w/v laminarin in 50 mM acetate buffer (pH 4.8) with 200 µl crude extract at 45 °C for 30 minute. The reaction was added 2 ml of dinitrosalicylate (DNS) reagent and boiled at 100 °C for 15 min and immediately cooled. 4 ml distilled water was added and the absorbance at 540 nm was measured. The amount of reducing sugars released was calculated from standard curves of glucose and specific activity (U/mg protein) (Miller, 1959).

iii) Protease activity

Protease was assayed by incubating 1000 µl of 1.5% (w/v) casein in 0.05 M Na-phosphate buffer (pH 7.0) with 500 µl crude extract at 40°C for 10 minute. The reaction was stopped by adding 2 ml of 0.4 M trichloracetic acid (TCA) and centrifuge at 3000 rpm, 4°C for 20 minute. 250 µl of clear supernatant were added 1.25 ml of 0.4 M Na_2CO_3 and shaken to mix well. 0.25 ml of Folin reagent was added and incubated at room temperature for 10 minute. The absorbance was measured at 660 nm. Specific activity of protease was calculated as protease activity (U/mg protein).

2.11.4 Determination of the released glucose and GlcNAc

The released glucose from NB supplemented with dried mycelial fungi and NB as control, were measured by DNS method (Miller, 1959) 100 µl of crude enzyme was diluted with 900 µl distilled water and added 2 ml with dinitrosalicylate (DNS) reagent. The mixture was boiled for 15 minute and cooled to room temperature. 4 ml of distilled water was added into the mixture tubes and measured absorbance for 540 nm. The amount of glucose released was calculated from standard curves of glucose.

GlcNAc was released in cell free culture of NB supplemented with dried fungal mycelium of *A. flavus* and NB after 5 days incubation. The cell free supernatant was measured for the amount of GlcNAc by Somogyi's and Nelson's method. 100 µl of crude extract was diluted with distilled water to adjust total volume to 1 ml. 1 ml of Somogyi reagent was added and boiled at 100°C for 10 min. After boiling, the mixture was immediately cooled and 1 ml of Nelson's reagent was added then shaken its well and kept at room temperature for 20 min. 2 ml of distilled water was added and shaken to mix well. The absorbance at 520 nm was measured and the amount of GlcNAc was calculated from standard curve of GlcNAc.

2.12 Inhibition of fungal growth by cell free supernatant from each enzyme production of *B. subtilis*

The single colony of *B. subtilis* was inoculated into 100 ml NB medium in 250 ml Erlenmeyer flask and incubated at 30 °C with rotary shaker 160 rpm for 20 h ($1x10^8$spores/ml) as preculture. 3 ml of preculture were inoculated into 300 ml NB, protease and chitinase production liquid culture media. The protease production broth consisted of 1.0 g glucose, 10.0 g. peptone, 0.2 g yeast extract, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$ and 0.5 g K_2HPO_4 per 1000 ml distilled water. And, the chitinase production broth consisted of 2% w/v colloidal chitin as substrate, 0.05% w/v yeast extract, 0.1% w/v $(NH_4)_2SO_4$, 0.03% w/v $MgSO_4 \cdot 7H_20$, 0.136% w/v KH_2PO_4 with adjusted pH to 7.0. After inoculated into NB, protease and chitinase production broth, the cultures broth was incubated on shaking incubator 160 rpm, at 30 °C. The cell free culture filtrates of NB, protease and chitinase production were collected during exponential and stationary phase of bacterial growth rate. The cell of *B. subtilis* were removed by centrifugation at 14,000 rpm for 20 min at 4 °C and the cell free culture filtrate was kept at -20°C until used in antifungal activity on culture plate. The fungal growth inhibitory effect was estimated by using the radial growth inhibition assay. One ml of cell free cultures from each enzyme induce production was added onto 10 ml of the soft PDA. The test plates were prepared by pouring about 15 ml of PDA as the base layer and kept at room until solidifying. Then each test plate was overlaid with cell free culture in soft potato dextrose agar as the seed layer. A 3 days old mycelia plug of 0.6 cm diameter was cut using a sterile cork borer and was transferred to the test plates will cell free culture seed layer in 3 replicates. Control plate contained only PDA. Fungal growth was measured after 5 days of inoculation. Fungal

growth inhibition was expressed as percentage of diameter growth inhibition in comparison with the control, as in the equation in **2.7.3**.

2.13 Antifungal activity of the cell free supernatant on bird chili powder

Three milliliters of *B. subtilis* preculture ($1x10^8$ spores/ml) in NB was inoculated into 300 ml fleshly NB in 500 ml Erlenmeyer flask. The inoculated flask was incubated with a rotary shaker at 160 rpm, 30 °C for 12 hour. The cell free supernatant was collected by centrifugation at 6000 rpm for 20 min. Fifteen grams of dried bird chili powders were distributed in each of 250 ml Erlenmeyer flask and sterilized by autoclave. 2 ml of *B. subtilis* cell free supernatant were added into each flask and the flasks were kept for 24 h with thorough shaking so that all the supernatant could be absorbed by chili powder. The flasks were inoculated with 100 µl of *A. flavus* spore suspension ($1x10^4$spore/ml) that was prepared by adding 10 ml of sterile distilled water onto the 7 days old of *A. flavus* slant culture. The flasks without cell free supernant of *B. subtilis* and spore suspension of *A. flavus*, with only cell free supernatant of *B. subtilis* and with only spore suspension of *A. flavus* on chili powder were used as control. After incubation at 30°C for 7 days with vigorous shaking for 5 min daily, the growth of *A. flavus* was evaluated macroscopically and pour plate as same as **2.4.1** with 10^{-2} dilution.