CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Chemicals	Company
Absolute ethanol	Merck
Acrylamide	Sigma
Adenosine triphosphate	Sigma
Allylcyanide	Sigma
Allylisothiocyanate	Sigma
Ammonium chloride	Merck
Ammonium persulfate	Sigma
Ammonium sulfate	Merck
Agar	Difco
Bacto agar	Difco
Barium chloride	Merck
Bis-acrylamide	Sigma
Boric acid	BDH

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Chemicals	**Company**
Bovine serum albumin	Sigma
Bromophenol blue	Sigma
Brown mustard seeds meals (*Brassica juncea*)	Lanna Products Co., Ltd.
Calcium chloride	Merck
Chloroform	Merck
Coomassie blue R-250	Sigma
Dithiothreitol	Sigma
D-glucose	Merck
Dinitrosalicylic acid	Merck
Dipotassium hydrogenphosphate	BDH
Disodium hydrogenphosphate	BDH
EDTA	Merck
Ethanol 95%	Vitdhayasom
Ethyl methanesulfonate	Sigma
Ferric chloride	Merck
Ferrous sulfate	Merck
Glacial acetic acid	Merck
Glycerol	Merck

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Chemicals	**Company**
Hexane (commercial grade)	Merck
Hexane (GC grade)	Merck
HK/G-6-PD mixtures	Sigma
Hydrochloric acid	Merck
Low molecular weight protein marker	Pharmacia
Malt extracts	Difco
N-methyl-N'-nitro-N-nitrosoguanidine	Sigma
Mercaptoethanol	Sigma
Myrosinase (*Sinapis alba*)	Sigma
NADP	Sigma
Nutrient broth	Difco
Peptone	Difco
Phenol	Merck
Potassium dihydrogenphosphate	BDH
Potato dextrose agar	Difco
Reagent kit for glucose assay (glucose oxidase)	Human
Sinigrin monohydrate	Sigma
Sodium azide	Sigma

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Chemicals	Company
Sodium chloride	Merck
Sodium dihydrogenphosphate	BDH
Sodium dodecylsulfate	Merck
Sodium hydroxide	BDH
Sodium potassium tartrate	Merck
Sodium thiosulfate	Merck
Sulfuric acid	Merck
Starch	Merck
TEMED	Sigma
Tris(hydroxymethyl)aminomethane	Sigma
Tryptone	Difco
Yeast nitrogen base	Difco

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2.1.2 Instruments

Instruments	Company
Cetus thermal cycler	Perkin-Elmer
Hewlett-Pack gas chromatograph 5890 series II	Hewlett-Packard

Instruments	Company
Shimadsu 14A gas chromatograph	Shimadsu
U-2000 Spectrophotometer	Hitachi
UV-265 Spectrophotometer	Shimadsu
Peristatic pump	Eyela
Haemocytometer	Newbauer
Beckman J2-MC centrifuge	Beckman
Mini VE electrophoresis apparatus	Pharmacia Biotech
Flexi-Dry^{TM} Freeze-dryer	FTS System

2.2 General methods

2.2.1 Preparation of sinigrin agar plate

A 100 ml agar medium comprising of 5 mM sinigrin, 5 mM ammonium chloride and 1.5 g agar was melt by heating in boiling water for 10 min. It was then autoclaved at 121˚C for 10 min. Then, 15 ml of the medium were poured into each Petri disk.

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2.2.2 Preparation of sinigrin-barium agar plate

A 100 ml agar medium comprising of 5 mM sinigrin, 2.5 mM barium chloride, 5 mM ammonium chloride and 1.5 g agar was melt by heating in boiling water for 10

min. It was then autoclaved at 121°C for 10 min. Then, 15 ml of the medium were poured into each Petri disk.

2.2.3 Preparation of mustard extract medium

Two hundred grams of brown mustard seed meals (*Brassica juncea* var. Forge), obtained after fixed-oil expellation, were ground finely by a mortar and defatted three times with one liter hexane each. After the defatted mustard powder has been allowed to air-dry, it was then heated at 110°C for 30 min, prior to being suspended in boiling water. During this time, it was vigorously stirred and heated further for 15 min. The slurry was stirred for an additional 3 h at room temperature and then filtered. The yellow-brown mustard extract obtained after centrifugation (10,000xg, 30 min) was assayed for glucose and glucosinolate contents.

Cultured medium was made by dilution the mustard extract to a final glucosinolate concentration of 10 mM in 50 mM potassium phosphate buffer, pH 6.5. It was then autoclaved at 121°C for 15 min.

2.2.4 Determination of protein content

Reagent A was 2% sodium carbonate in 0.1 N NaOH. Reagent B_1 was 2% sodium potassium tartrate and regent B_2 was 1% copper sulfate pentahydrate. Reagent C was freshly prepared by mixing reagent $A:B_1:B_2$ by the ratio 50:1:1 (v/v/v). Stock bovine serum albumin (1000 μg/ml) was diluted with distilled water to final concentrations of 100, 200, 300, 400, 500, 600 and 700 μg/ml, used as standards. One

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hundred μl of standard (or sample) and 1.0 ml of reagent C were mixed gently and incubated at room temperature for 10 min. Then, 100 μl of 50% Folin-phenol reagent in distilled water were added and mixed gently. After incubating at room temperature for 30 min, an absorbance at 500 nm was then measured. Protein concentration of every sample was determined directly from the calibration curve (shown in appendix).

2.2.5 Glucosinolate assay

2.2.5.1 Determination of pure sinigrin content (Palmieri *et al.*, 1982)

Absorbance values at 227 nm of different sinigrin concentrations in distilled water (25, 50, 100, 125 and 150 μM) were measured. Calibration curve was constructed by plotting between absorbance values and sinigrin concentrations. The determination of sinigrin content in liquid samples was done by diluting the sample to appropriate concentration and then monitoring an absorbance value. Sinigrin concentration was determined directly from the calibration curve (shown in appendix).

2.2.5.2 Determination of total glucosinolate content (Smiths *et al.*, 1993)

A sample or standard (50 μl) was added to 1.0 ml reaction reagent (33mM sodium phosphate buffer, pH 6.5 containing 3 mM $MgCl_2$, 1 mM L-ascorbic acid, 0.55 mM ATP and 0.72 mM NADP). After mixing, an absorbance at 340 nm was measured (A_1). Then, 5 μl of HK/G-6-PD mixtures (activity of 700 and 350 U/ml) was added and incubated for 15 min at 30˚C. The second absorbance (A_2) was then measured. In order to quantify glucosinolates, 10 μl of the *Sinapis alba* myrosinase in

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33 mM sodium phosphate buffer, pH 6.5 (14 U/ml) was added into the reaction mixtures and incubated for 30 min at 30˚C before the third absorbance (A_3) was read. Glucose content could be calculated from A_2-A_1 by using a calibration curve of glucose (shown in appendix). Glucose level was determined from the absorbance difference, A_3-A_2, using the calibration curve for sinigrin (shown in appendix).

2.2.6 Preparation of spore suspension (Hawkworth, 1991)

Spores from the culture grown in potato dextrose agar slants were aseptically transferred into a screw-capped vial containing sterile distilled water. It was vigorously shaken for 3-5 min or until no cluster of spores was observed. The suspension was adjusted to desired concentration using Newbauer haemocytometer. Normally, inoculum was made from 2 week old culture and the concentration was $1x10^6$ spores/ml, unless stated otherwise.

2.2.7 Measurement of fungal cell biomass (Hawkwort, 1991)

Fungal cell pellet was collected by filtering through filtered paper (Whatman filter paper No. 93). It was then washed with distilled water (normally 4-5 volumes of cultured medium) until yellow-brown color was disappeared. The mycelium was transferred to a pre-weighed watch glass and then heated at 70˚C until constant weight was determined.

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2.2.8 Disruption of fungal cell pellets (Hawkwort, 1991)

Fungal mycelium was collected and washed as described in 2.2.6. Then, the mycelium was cooled at 4°C in the presence of 0.1 M sodium phosphate buffer, pH 7.4 for 30 min. Disruption was carried out by a mortar in cold room (4°C) Cell lysate was maintained at 4°C until to being analyzed.

2.2.9 Activity assay of enzyme myrosinase (Wilkinson *et al.*, 1984)

Enzyme activity of myrosinase was routinely determined by the coupled enzyme assay with a slight modification. The assay system was carried out on a double beam Shimadsu UV-265 spectrophotometer. Cuvette holders were connected to a circulating waterbath, equipped with a temperature control apparatus, which was adjusted to a desired temperature of 25°C. One ml of buffered substrate (33 mM sodium phosphate buffer, pH 7.4 containing 5 mM sinigrin, 0.55 mM ATP, 0.72 mM NADP, 3.5 unit of hexokinase and 1.75 unit of glucose-6-phosphate dehydrogenase) was pre-incubated for a sufficient time at 25°C (3-5 min). Then, appropriate amount of the enzyme was added. After mixing, the reaction rate was monitored at 340 nm. Myrosinase activity could be calculated from the rate of absorbance change, which was due to the formation of NADPH (ε_{340nm} = 620 $M^{-1}cm^{-1}$). One unit of myrosinase activity was defined as the amount of enzyme catalyzed the liberation of 1 μmol of glucose per minute from sinigrin under the conditions which were described above.

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2.2.10 Determination of reducing sugar (Miller, 1959)

The amounts of 0.5 ml of sample were added in 1 ml of DNS reagent (1% dinitrosalicylic acid in 1 N NaOH) containing in a screw-capped tube. After mixing and capping, it was heated in boiling water for 10 min. An absorbance at 492 nm was measured. The concentration of reducing sugar was determined from a calibration curve (shown in appendix), using glucose at concentration of 1, 2.5, 5 and 10 mM as standards.

2.2.11 SDS polyacrylamide gel electrophoresis

a. Gel polymerization

A mini-VE vertical slab-gel unit was assembled in the casting stands according to the manufacturer's manual, using a 0.75 mm thick spacer. The 10 % total acrylamide (%T) separating gel solution was made by mixing 10.0 ml of monomer solution (30.8%T and 2.7% cross link, C), 7.5 ml of separating gel buffer (1.5 M Tris HCl buffer, pH 8.8), 0.3 ml of 10% SDS and 12.1 ml of deionized water to give a final volume of 30 ml. Then, 150 µl of 10% ammonium persulfate and 10 µl of TEMED were added and mixed gently. The solution was poured into the slab compartment approximately 1 cm, being below the upper level. The gel was overlaid carefully with deionized water and it was allowed to polymerization was confirmed by observing a meniscus between the gel layer and deionized water.

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Stacking gel solution comprised with 4 %T was made by mixing 1.33 ml of monomer solution, 2.5 ml of stacking gel buffer (0.5 M Tris-HCl buffer, pH 6.8), 0.1 ml of 10% SDS and 6.0 ml of deionized water. The amounts of 50 μl of 10% ammonium persulfate and 5 μl of TEMED were added and mixed gently. Subsequently, the overlaid water was poured off from the gel unit and the about 2 ml of stacking gel solution were carefully filled up instead. Then, a comb was inserted between the sandwich glasses. This had to be done carefully without an air bubble formed below the teeth of the comb. It was allowed to set for about 1 h for polymerization completion.

b. Preparation of protein samples

Interesting protein fractions were concentrated by ultrafiltration, which were contained in low ionic strength buffer. Appropriate concentrations of crude protein samples were about 10-20 mg per ml, whereas, those of the partially purified samples were about 5-10 mg per ml. One part of protein samples was mixed with one part of the sample buffer (0.125 M Tris-HCl, 4% SDS, 20% (v/v) glycerol, 0.2 M dithiothreitol and 0.02 % bromophenol blue, pH 6.8), prior to being heated in boiling water for 3 min.

c. Sample application

The comb was slowly removed from the gel and rinsed with running buffer. The gel was installed properly in the electrophoretic apparatus according to the

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manufacturer's instruction. A constant current of 20 mA was circulated to pre-equilibrate the buffer system for 15 min. Then, the amounts of 5-10 μl of samples and molecular weight protein markers were loaded carefully into each lane by using a suitable micro syringe.

d. Separating procedure

During separation within the stacking gel, a constant current of 1.5 mA per lane was supplied until the bromophenol blue tracking-dye entered into the separating gel. For protein separation in the separating gel, the current was supplied constantly at 2 mA per lane. When the tracking-dye front reached the gel bottom, the power supply turned off. After pouring out the running buffer (0.025 M Tris, 0.192 M glycine and 0.1 % SDS, pH 8.3), the gel was de-assembled and was removed from the sandwich glasses.

e. Staining and destaining

Staining was performed at room temperature by placing the gel in staining solution (0.025 % coomassie brilliant blue R-250, 40% methanol and 7% acetic acid). It was shaken on a rotary shaker, operating at 30-40 rpm for 4 h. Destaining protocol was done as follows. The gel was shaken with destaining solution I (7% acetic acid and 40% methanol) for 30 min, prior to being replaced with destaining solution II (7% acetic acid and 5% methanol). The destaining solution II was changed every 6-12 h until the gel background was clear.

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2.2.12 GC analysis of sinigrin or glucosinolate breakdown product

(Sakorn *et al.*, 1999)

The analysis of glucosinolate breakdown products was conducted using a GC analysis, Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard) or a Shimadsu 14A gas chromatograph (Shimadsu), equipped with a flame ionization detector (FID) and a chrompack columm (50x0.3 mm ID), connected to the injector port. The carried gas (helium) was operated at a constant flow rate of 0.5 $cm^3 min^{-1}$ and a pressure of 50 kPa. Temperature of the column was initially maintained at 80°C for 6 min and then programmed to increase at a rate of 10°C min^{-1} for additional 6 min. Temperature of the injector and detector was maintained at 230 and 280°C respectively. Standard allylcyanide and allylisothiocyanate were prepared at concentrations of 1, 2.5, 5 and 10 mM in distilled water. Samples or standards (normally 1.0 ml) were extracted with an equal volume of chilled hexane (GC grade). Samples of standards (normally 1.0 ml) were extracted with an equal volume of chilled hexane (GC grade). After adding approximately 2 g of solid ammonium sulfate, capping, shaking and centrifuging (13,000xg, 15 min), 1 μl of hexane layer was injected. Concentrations of any glucosinolate breakdown products in the sample were determined directly from such calibration curve (shown in appendix). In this assay, allylcyanide and allylisothiocyanate at concentration of 5 mM each were used as internal standards.

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2.3 Screening and characterization of glucosinolate degrading microorganisms

2.3.1 Sample collection

Samples suspected glucosinolate degrading microorganism were collected from decayed mustard seed meals (*Brassica juncea*), which were obtained from three collection sites in Lamphun, Thailand. Solid and semi solid meal samples were bottled in sterile vials, whereas, liquid samples were in sterile tubes.

2.3.2 Screening and characterization of glucosinolate degrading microorganisms

The fungal strains were obtained from soil screening. One hundred fifty soil samples potentially containing glucosinolate-degrading microorganisms were collected from Lamphun province, Thailand. Soil samples were bottled in sterile vials. One gram of each sample was suspended in 10 ml sterile distilled water. After shaking, it was allowed to settle at room temperature for 2-3 hr. Then, 100 μl of supernatant was spreaded onto nutrient agar plates (2 g beef extract, 2 g tryptone and 1.5 g agar in 100 ml distilled water) and potato dextrose agar plates (3.9 g potato dextrose agar in 100 ml distilled water). Re-plating was made until pure isolate was obtained. The selected strains were inoculated onto sinigrin agar plates (as described in 2.2.1) and

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sinigrin-barium agar plates (as described in 2.2.2). Incubation was taken at 30°C and growths were observed daily. The strains that grown on sinigrin barium agar plates were re-plated onto mustard extract agar (as described in 2.2.3). Incubation was carried out at 30°C and growths were observed daily. Each distinct growing colony was picked and then plated onto nutrient agar plates or potato dextrose agar plates.

2.3.3 Preliminary examination of sinigrin degradation potential and myrosinase producing ability by glucosinolate degrading microorganism in liquid medium

The overnight-grown in nutrient broth (2 g beaf extract and 2 g tryptone in 100 ml of 0.1 M sodium phosphate buffer, pH 7.0) glucosinolate degrading bacteria, isolated from decayed mustard seed meal samples was harvested by centrifugation (10,000xg, 15 min). After washing two times with 0.1 M sodium phosphate buffer, pH 7.0, bacterial cell were suspended in the same buffer. The turbidity of the bacterial suspension was adjusted to be related for the MacFarland standard No.2. Quantities of 100 μl of the suspension were inoculated in 1 ml of sinigrin glucose medium (5.5 mM sinigrin, 11 mM glucose, 6.7 g/l yeast nitrogen base in 0.1 M sodium phosphate buffer, pH 7.0) and incubated at 30°C. Growth was monitored by measuring an absorbance at 660 nm. Samples were taken every 12 h. Supernatants obtained after centrifugation were assayed for glucose and sinigrin contents. In this case, sinigrin was determined

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by the spectrophotometric method while glucose was determined by glucose-oxidase method.

The 25 µl of spore suspensions of each fungal isolate (as described in 2.2.6) were inoculated in sinigrin glucose medium (5.5 mM sinigrin, 11 mM glucose, 6.7 g per liter yeast nitrogen base in 0.1 M sodium phosphate buffer, pH 6.5). Incubation was taken at 30˚C under reciprocal shaking, operated at 150 rpm. Samples were taken periodically to assay for glucose and glucosinolate contents.

2.3.4 Sinigrin degradation by *Aspergillus* sp. NR463 in liquid culture

Twenty five µl of inoculums (as described in 2.2.6) were placed in 2.5 ml of both induction medium (10 mM glucose, 1 mM sinigrin and 6.7 g per liter yeast nitrogen base in 0.1 M potassium phosphate buffer pH 6.5) and growth medium (11 mM glucose and 6.7 g per liter yeast nitrogen base in 0.1 M potassium phosphate buffer, pH 6.5) which were contained in 25 ml Erlenmeyer flasks. Incubation was performed at 30˚C in waterbath shaking at 150 rpm. Cultured filtrates were taken periodically to assay for glucose and sinigrin contents.

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Fungal cell pellet harvested at late log-phase was transferred to incubate further in singrin medium (5.5 mM sinigrin in 0.1 M potassium phosphate buffer, pH 6.5) or sinigrin glucose medium (5.5 mM sinigrin, 5.5 mM glucose and 6.7 g per liter yeast nitrogen base in 0.1 M potassium phosphate buffer, pH 6.5). Cultured flasks were incubated at 30˚C in a waterbath shaker, operating at 150 rpm. Cultured filtrated

were taken periodically to assay for glucose, sinigrin, myrosinase activity and sinigrin hydrolytic product contents. Fungal cell pellet was collected by filtering through filter paper (Whatman filter paper no. 93) and washed with distilled water, before subjecting for fungal cell disruption (as described in 2.2.8). Cell free extracts obtained after centrifugation (10,000xg, 10 min, 4°C) were determined for myrosinase activity.

2.4 Production of intracellular myrosinase from *Aspergillus* sp. NR463

Myrosinase production by *Aspergillus* sp. NR463 was carried out in mustard extract medium. Cultured condition such as pH of cultured medium, glucosinolate concentration temperature, inoculum size and spore age were studied. In addition, stabilities of myrosinase were also studied.

2.4.1 Effect of pH of cultured medium on myrosinase production

Quantities of 400 μl inoculum (as described in 2.2.6) were added in a 250 ml Erlenmeyer flask containing 50 ml mustard extract medium at different pH values (10 mM glucosinolates contained in 50 mM sodium phosphate buffer, pH 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5). Incubation was carried out at 30°C in a waterbath, shaken at 150 rpm. At regular intervals of incubation, cultured filtrates were taken to assay for the contents of glucose and glucosinolates. At that time, two mycelial samples were also harvested and washed (as described in 2.2.7). One mycelial sample was placed on a pre-weighed

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watch glass and dried at 70°C until a constant weight was obtained. The other sample was maintained in 50 mM sodium phosphate buffer, pH 7.2 (30 ml) at 4°C for 30 min, prior to being disrupted by a mortar (as described in 2.2.8). Cell free extract obtained after centrifugation (10,000xg, 30 min, 4°C) was assayed for myrosinase activity content.

2.4.2 Effect of glucosinolate concentration on myrosinase production

Optimum pH value of culture medium, obtain from 2.4.1, was introduced to use in this experiment. Enzyme production in mustard extract medium was performed by varying glucosinolate concentration to 2.8, 5.5, 8.3 and 10 mM. During growth of the *Aspergillus* in mustard extract medium, such parameters as glucose and glucosinolate contents in cultured filtrates, myrosinase activity and mycelial dry mass were analyzed regularly as described in 2.4.1.

2.4.3 Effect of incubation temperature on myrosinase production

Optimal pH and glucosinolate concentration obtained from the proceeding experiments were used in this study. Incubation was done at 25 to 37°C. All procedures were similar to those described in 2.4.1.

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2.4.4 Effect of inoculum size and spore age on myrosinase production

To evaluate the effect of inoculum size on myrosinase production, 400 μl of inoculum at varying concentrations of $5x10^5$, $1x10^6$, $5x10^6$ and $1x10^7$ spores per ml were studied. Whereas, spore age of 1, 2, 3 and 4 weeks grown in PDA slants were also investigated for the effect on enzyme production.

2.4.5 Preservation of mycelium containing myrosinase

Fungal mycelium grown in mustard extract medium at optimal condition for 48 h were harvested and washed (as described in 2.2.8). Mycelial samples were maintained in 20 ml of 40 mM sodium phosphate buffer, pH 7.0 at 4°C. These mycelial samples were disrupted after 1, 12, 24 and 48 h, respectively. While the other mycelial sample was kept at -40°C for 24 or 48 h, before thawing at room temperature and disrupting with the same procedure. Each cell free extract was determined for myrosinase activity content.

2.4.6 Stability of crude myrosinase extract

Crude enzyme extracts of the *Aspergillus* were maintained at 0, 4, 30°C and room temperature. Anti-microbial growth agents such as sodium azide and thimerosal were added into enzyme solution at a final concentration of 0.02% each. At regular interval of time, myrosinase activity in each sample was determined (as described in 2.2.9).

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2.5 Mutagenesis of *Aspergillus* sp. NR463

2.5.1 UV mutagenesis of *Aspergillus* sp. NR463 (Petruccioli *et al*., 1995).

The fungal culture was obtained from soil screening. Conidiospores from 7-day-old cultures, grown on potato dextrose agar at 30°C, were suspended in sterile water containing 0.1 % (v/v) Tween 20. Ten milliliters of this suspension (approx 1×10^6 conidiospores ml^{-1}) were placed into a sterile petri dish (diameter 9 cm) and irradiated with UV light at a distance of 30 cm away from the light source (254 nm, 60 W) for various times ranging from 60s to 20 min. Conidial survival was determined on potato dextrose agar plates at 28°C. Five replicates were carried out for each interval. After treatment with UV, the plates were incubated in the dark at 30°C for 3 days. Strains showing different growth rates and different colony morphology were selected. Slow growing colonies were selected subsequently for up to 7 days. Selected strains were grown individually on fresh potato dextrose agar plates and incubated at 30°C. All strains were maintained at 4°C in the dark.

2.5.1.1 Production of intracellular myrosinase from *Aspergillus* sp. NR463 UV mutant strains

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Myrosinase production by *Aspergillus* sp. NR463 UV mutant strains was accessed by using sinigrin agar plates, potatodextrose agar plates, mustard extract agar plates and sinigrin barium agar plates method. They were carried out in mustard

extract medium. Cultured condition such as pH of cultured medium, glucosinolate concentration, temperature, inoculum size and spore age were studied. In addition, stabilities of myrosinase were also studied. Methods were used as described in 2.4.1 to 2.4.6.

2.5.2 EMS mutagenesis of *Aspergillus* sp. NR463 (Al-Aidross and Seifert, 1980; Chadha *et al.*, 2004)

The alkylating reagent ethyl methanesulphonate (EMS) was used in a procedure adapted from that of Al-Aidross and Seifert (1980). About 10^9 spores ml^{-1} of *Aspergillus* sp. NR463 were collected from one-week-old slant and 300 μl ethyl methanesulphonate (EMS) was added. They were exposed for 30, 60, 90, 120, 150, 180 and 210 min. The spores of each exposure time were diluted, spread on PDA and incubated for 48-72 h at 30 °C. The survival percentage was calculated from the number of colonies obtained at each time interval.

2.5.2.1 Production of intracellular myrosinase from *Aspergillus* sp. NR463 EMS mutant strains

Myrosinase production by *Aspergillus* sp. NR463 EMS mutant strains were selected by using sinigrin agar plates, potato dextrose agar plates, mustard extract agar plates and sinigrin barium agar plates method. They were carried out in mustard extract medium. Cultured condition such as pH of cultured medium, glucosinolate concentration, temperature, inoculum size and spore age were studied. In addition,

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stabilities of myrosinase were also studied. Methods were use as described in 2.4.1 to 2.4.6.

2.5.3 MNNG mutagenesis of *Aspergillus* sp. NR463 (Roy and Das, 1978)

Working under a safety hood, a small amount of MNNG (a few milligrams) was poured into a preweighed tube. After weighing the tube again, distilled water was added to a concentration of 1 mg/ml. The tightly closed tube was shaken to accelerate solution formation. Aliquots of about 0.5 ml are distributed to small vials and kept frozen in the dark until used. Unfrozen samples should not be reused. To check for accidental unfreezing and refreezing, a small thumbtack may be placed on ice in a similar vial. The yellow color of the solution serves as a quality guarantee; if any doubt arises, the absorbance at 400 nm of a 1 mg/ml solution through a 1-cm light path should be 1.0.

Aspergillus sp. spores, suspended in buffer (about 10^6 spores per milliliter of 50 mM Tris-maleate buffer, pH 7.5), were exposed to MNNG at room temperature. The spores are gently and continuously shaken during exposure to prevent sedimentation and then washed three times by centrifugation in sterile water. To maximize the number of mutants in the initial spore population, the dose was a 15 minute exposure to 20 $\mu g\ ml^{-1}$ MNNG. To attain a high mutant frequency among the survivors, a dose of 10-minute exposure to 100 $\mu g\ ml^{-1}$ MNNG, was used. The

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exposed spores are then plated out to mustard extract agar plate (as described in 2.2.3) and sinigrin agar plate (as described in 2.2.2) for selected the mutant strains.

2.5.3.1 Production of intracellular myrosinase from *Aspergillus* sp. NR463 MNNG mutant strains

Aspergillus sp. NR463 MNNG mutant strains capable of producing myrosinase were selected by using sinigrin agar plates, potatodextrose agar plates, mustard extract agar plates and sinigrin barium agar plates method. They were carried out in mustard extract medium. Cultured condition such as pH of cultured medium, glucosinolate concentration, temperature, inoculum size and spore age were studied. In addition, stabilities of myrosinase were also studied. Methods were use as described in 2.4.1 to 2.4.6.

2.6 Characterization of *Aspergillus* sp. NR463 and mutant strains.

2.6.1 Method for activity assay of the *Aspergillus* myrosinase

All methods used to determine enzyme activity of the *Aspergillus* myrosinase based on a kinetic monitoring assay, which were determined at the zero order reaction. An acceptable reaction rate ($\Delta A/\Delta t$) should be monitored constantly for at least 5 min. The assay was traditionally carried out at 25°C, comprising of 1.0 ml of reaction reagent and 10 µl of enzyme solution. Enzyme should be in a solution with low ionic

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strength. Aliquots of enzyme with appropriate dilution should be kept at the desired temperature for at least 5 min, before starting the assay.

2.6.1.1 Determination of enzyme activity by a spectrophotometric method

The assay was carried out on a double beam Shimadsu UV-265 spectrophotometer. Cuvett holders were connected to a circulating water bath equipped with a temperature control apparatus which was adjusted to a desired temperature. The buffered substrate of 1.0 ml total volume (100 μM sinigrin in 33 mM sodium phosphate buffer, pH 7.4) was maintained in quartz cuvettes (1.0 ml light path) at a desired temperature for sufficient time (3-5 min) before adding the enzyme. After mixing, the rate of sinigrin hydrolysis was monitored directly by following the decrease of absorbance by time ($\Delta A/\Delta t$) at 227 nm. A constant reaction rate should be observed for at least 5 min. One unit of β-thioglucosidase activity was expressed as the amount of enzyme that catalyzed the hydrolysis of 1 μmole of sinigrin per min at the conditions described above.

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2.6.1.2 Determination of enzyme activity by a coupled enzyme method

The assay was carried out on a double beam Shimadsu UV-265 spectrophotometer. The cuvette holders were connected to a circulationg waterbath equipped with a temperature control apparatus in which was adjusted to a desired

temperature. One ml of buffered substrate (33 mM sodium phosphate buffer, pH 7.4 containing 5 mM sinigrin, 3 mM $MgCl_2$ 0.55 mM ATP, 0.72 mM NADP, 3.5 hexokinase and 1.75 U glucose-6-phosphate dehydrogenase) was pre incubated for a sufficient time (3-5 min) at 25°C. Then, appropriate amounts of the enzyme were added. After mixing, the reaction rate was monitored at 340 nm. Myrosinase activity was calculated from absorbance increasing rate, which was due to the formation of NADPH. One unit of myrosinase activity was defined as the amount of enzyme that catalyzed the liberation 1 µmol of glucose per minute from sinigrin under the conditions describe above.

2.6.2 Effect of enzyme concentration on activity assay

Aliquots of the *Aspergillus* myrosinase in 10 mM sodium phosphate buffer, pH 7.4 were maintained at the assayed temperature for at least 5 min, before starting the assay. In this study, the reaction was carried out at 25°C. The amounts of the enzyme which exhibited the zero ordered reaction would later be used in further experiments.

2.6.3 Optimum pH

Optimum pH for activity of the *Aspergillus* myrosinase was determined by the spectrophotometric assay (as described in 2.6.1.1). In this case, pH values of buffered substrates were varied from 4 to 9 (4-6: sodium boric acid-phosphate buffer, 6-8 : sodium phosphate buffer and 8-9 : tris buffer).

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2.6.4 Optimum temperature

Optimum temperature for activity of the *Aspergillus* myrosinase were assessed by the method described in 2.6.1.1. Temperatures of the assayed system were varied from 25 to 37°C.

2.6.5 Kinetic constants

Kinetic constants for sinigrin-hydrolyzing activity (β-thioglucosidase) of the *Aspergillus* myrosinase were determined by the coupled enzyme assay (0.1-5.0 mM sinigrin) depended on sinigrin concentrations. Apparent K_m and V_{max} values were calculated from the double reciprocal plots of Lineweaver and Burk method.

2.7 Analysis of glucosinolate breakdown product by gas chromatography

Reaction mixtures of 1.0 ml total volume were carried out in screw capped vial (10 x 50 mm) containing 0.1 M designed buffer (sodium phosphate or sodium citrate-phosphate or tris), 10 mM Glucosinolate (mustard extract medium) and 0.3 unit of the *Aspergillus* enzyme. The fungal cell pellets of the *Aspergillus* grown in mustard extract medium in optimal condition were collected after 48 h. After washing with deionized water, the myceliums were disrupted immediately at 4°C without adding any buffer. The myrosinas activity was determined by standard method.

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During incubation at 30°C, samples were taken periodically to assay glucose and sinigrin concentrations. After incubation for 1-2 h, the vials were placed in an ice bath. Samples and standards were extracted with an equal volume of chilled hexane (GC grade). After adding approximately 2 g of solid ammonium sulfate, capping, shaking and centrifuging (3,000xg, 10 min), the hexane layer was subjected for GC analysis, as described in 2.2.12.

2.8 RAPD analysis of mutant strains (Pomazi *et al.*, 1994)

Aspergillus sp. NR463 and selected mutant strains were investigated by using RAPD for checking the mutation assay. This method used random primers to amplify target DNA yielding DNA fingerprint of the fungi to prove parent strains.

Frozen mycelial mats were ground with a mortar and pestle to fine powder in liquid nitrogen. Approximately 20 mg of homogenized cell powder was mixed with 600 µl of lysis buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM EDTA and 2% SDS) and incubated at 60°C for 10 min. The DNA was extracted sequentially with an equal volume of phenol/chloroform (1:1), and chloroform/ isoamyl alcohol (24:1). Total nucleic acids were precipitated with 0.7 volume of isopropanol and vacuum dried. Finally, the pellets were re-dissolved in water and treated with RNaseA at 37°C for 1.5 h to remove RNA, and the RNase was inactivated at 60°C for 1 h. Final DNA concentrations were determined by measuring with a Model U

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spectrophotometer (Hitachi, Tokyo, Japan), and the integrity of each DNA sample was examined with an agarose gel electrophoresis.

RAPD was determined according to the procedure of Pomazi *et al.* (1994). Ten 10-mer oligonucleotides of the RAPD primer kit N produced by Operon Technologies Inc. (Alameda, CA, USA) were chosen after preliminary tests for their potential to differentiate these fungi. Nucleotide sequences of the primers were: OPN02 (5'-ACCAGGGGCA-3'), OPN04 (5'-GACCGACCCA-3'), OPN05 (5'-ACTGAACGCC-3'), OPN06 (5'-GAGACGCACA-3'), OPN07 (5'-CAGCCCAGAG-3'), OPN11 (5'-TCGCCGCAAA-3'), OPN12 (5'-CACAGACACC-3'), OPN13 (5'-AGCGTCACTC-3'), OPN14 (5'-TCGTGCGGGT-3') and OPN16 (5'-AAGCGACCTG-3'). Amplification was carried out in a Perkin-Elmer Cetus DNA thermal cycler with the following program: initial denaturation at 94°C for 4 min, 35 cycles of 1 min at 94°C, 1 min at 38°C, 2 min at 72°C and a final extension at 72°C for 7 min. Amplification products were separated by agarose gel electrophoresis and stained with ethidium bromide.

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