CHAPTER III

MATERIALS AND METHODS

Isolation of fungi

Fresh leaves from a range of plant species (Table 4) were collected and disks (0.6 mm) were aseptically cut and placed on culture media plates-Corn Meal Agar (CMA) and Malt Extract Agar (MEA)-half strength (Appendix I) plus antibacterial chloramphenicol at 0.005% (w/v). Other fungal habitats sampled included bathroom cement walls and latex painted surfaces. Sterile cotton swabs were used for collection and these smeared swabs used to inoculate onto culture media plates in triplicate. All cultures were incubated at room temperature (on average about 30°C). The initial yeast colonies were purified by restreaking on Potato Dextrose Agar (PDA) or Yeast Malt Agar (YMA) (Appendix I) and repeated until pure cultures were obtained. CMA, MEA, PDA and YMA were from Difco (Detroit, MI, USA).

Table 4 Leaves from plant species collected from five Thai provinces

Location	Species of plant
Bangkok province (Central)	*Bougainvillea spectabilis* Linn. *Hibiscus rosa-sinensis* Linn. (Hibiscus) *Mangifera indica* Linn. (Mango) *Ochna kirkii* Oliv. *Polyalthia longifolia* *Saraca indica* Linn. (Asoka) *Tamarindus indica* Linn.(Tamarind)
Lobburi province (Central)	*Annona squamosa* Linn.(Custard apple) *Cassia fistula* Linn.(Golden shower) *Eugenia uniflora* Linn.(Cayenne cherry) *Manilkara zopota* Linn.(Naseberry) *Saraca indica* Linn.(Asoka) *Tamarindus indica* Linn.(Tamarind)
Nakornratchasrima province (North East)	*Albizia saman* (Jacg.) F. Muell.(Rain tree) *Artocarpus heterophyllus* Lam.(Jack fruit) *Mangifera indica* Linn.(Mango) *Phyllanthus acidus* Linn. Skerls.(Star gooseberry) *Psidium guajava* Linn. (Guava) *Sandoricum koetjape* (Burm.f.) Merr. *Tamarindus indica* Linn.(Tamarind)

Table 4 (cont.) Species of plant leaves collected from five Thai provinces

Location	Species of plant
Pitsanulok province (Central)	*Annona squamosa* Linn. (Custard apple) *Artocarpus heterophyllus* Lam.(Jack fruit) *Bougainvillea spectabilis* Linn. *Mangifera indica* Linn.(Mango) *Phyllanthus acidus* Linn. Skeels. (Star gooseberry) *Psidium guajava* Linn. (Guava) *Tamarindus indica* Linn.(Tamarind)
Songkhla province (South)	*Averrhoa bilimbi* Linn. (cucumber tree) *Bambusa multiplex* Lour. *Bauhinia tomentosa* Linn. *Desmos chinensis* Lour. *Ochna kirkii* Oliv. *Saraca indica* Linn.(Asoka) *Syzygium malaccense* Linn. (Rose apple) *Tamarindus indica* Linn.(Tamarind) *Thuja orientalis*

Fungal identification

Morphological observation

Slide cultures were made using PDA, which were stained with lacto phenol-cotton blue solution (Fluka, Bunch, Switzerland), wet mounted and observed using bright field microscopy. Observation of colony characteristics was performed daily. The *Aureobasidium* isolates were compared with standard strains, *A. pullulans* ATCC 42023 and NRRL 6992, and with the descriptions of *Aureobasidium* by Barnett & Barry (1998), Domsch et al. (1993), and Hermanides-Nijhof (1977).

Nuclear ribosomal DNA Internal Transcribed Spacer (ITS) Sequencing

Aureobasidium cultures were grown for 3 days on cellulose acetate membranes which was placed onto the surface of PDA plates. These fresh *Aureobasidium* cultures were ground in liquid nitrogen (roughly about 0.5 mg of cell/ml of liquid nitrogen) and genomic DNA extracted using the Dneasy Plant Protocol (Quiagen, Valencia, CA, USA). The 5.8S rDNA and flanking internal transcribed spacer regions (ITS1 and ITS2) were amplified using 2 µl of undiluted genomic DNA in a 100 µl reaction using the primers ITS5 and ITS4 (White et al., 1990). Each reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 12.5 pmol each dNTP, 50 pmol each primer, and 2 U Taq polymerase (Desai and Pfaffle, 1995). PCR (25 cycles) was carried out using a GeneAmp 9600 thermocycler (Perkin Elmer, Foster City, CA, USA) set to 95°C for 10 s, 56°C for 30 s, and 72°C for 1 min. Initial denaturation was conducted at 95°C for 1 min with a final extension for 10 min at 72°C. Successful PCR products were cleaned of primers

and salts, using the QIAquick PCR Purification Kit (Quiagen, Valencia, CA, USA). ABI PRISM® BigDye Terminators v3.0 Cycle Sequencing reactions (Applied Biosystems, Foster City, CA, USA) were prepared according to the manufacturer's protocol, using primers ITS5 and ITS4, and the PCR product as template (White et al., 1990). Reactions were analyzed on an ABI PRISM® 3100 Automated DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

The ITS sequences of all yeast isolates were compared to those of other *Aureobasidium* spp. in the GenBank (http://www.ncbi.nlm.nih.gov). These sequences were submitted to GenBank. Analysis of the sequences was performed using online ClustalW program. Phylogenetic tree of the yeast isolates based on their ITS sequences was constructed using PAUP program version 4.0 beta 10 (Sinauer and Associates, Sunderland, Mass, USA).

Nutritional physiology

Carbon and nitrogen assimilation were investigated according to Barnett et al. (2000). Inocula were prepared using Yeast Malt Broth (YMB) in shake-flasks (100 ml/ 250-ml flask, 150 rpm, 25°C) (Difco, Detroit, MI, USA) (Appendix I).

The carbon (0.5 M, 0.5 ml) or nitrogen source (0.5 M, 0.5 ml) was added to 10X yeast nitrogen base (Difco, Detroit, MI, USA) (0.5 ml) (Difco yeast carbon base for nitrogen assimilation) plus 4 ml of sterile distilled water. The carbon, nitrogen, yeast nitrogen base and yeast carbon base were filtered sterilized while distilled water was sterilized by autoclaving. An inoculum (100 µl) of yeast culture ($2.5x10^7$ cell/ml) was added. Cultures were incubated at 25°C. Distilled water was used as a control

culture medium. Cultures were microscopically observed. Growth was assessed by cell turbidity (OD 600 nm) of the dispersed mycelium/yeast cells.

Exopolysaccharide (EPS) production

EPS was prepared by growing cultures in a production medium (PM) in shake-flasks (100 ml/ 250-ml flask, 150 rpm, room temperature). The production medium was based on the original studies of Ueda et al. (1963) and Punnapayak et al. (2003). PM contained glucose (5%); $(NH_4)_2SO_4$ (0.06%); K_2HPO_4 (0.5%); $MgSO_4.7H_2O$ (0.04%); NaCl (0.1%); and yeast extract (0.04%), with the pH adjusted to 6.5. EPS was recovered after 5 days by removing the yeast mycelium by centrifugation (10,000 g, 15 min), and precipitating the EPS from the culture supernatant with 95% ethanol (1:2, supernatant: ethanol). EPS was dried at 60°C.

In order to optimize the EPS production, a range of nutritional parameters were evaluated. The parameters included carbon (glucose and sucrose), nitrogen ($NaNO_3$, $(NH_4)_2SO_4$, and peptone) were varied. Data on the EPS production were averaged from triplicate cultures. Relative yields were assessed through statistical analyses including Analysis of Variance (ANOVA) and Duncan's multiple range test (DMRT).

Analysis of EPS

Hexose content analysis

Total hexose of EPS was determined using the anthrone assay (Leal-Serrano et al., 1980). Samples (20-200 μg) in 1 ml of distilled water were placed in 18x150 mm pyrex tubes. Cold anthrone reagent (5 ml)(Appendix I) was added to each sample and the contents mixed rigorously. Tubes were then capped with glass marbles and the reaction mixtures boiled for 15 min. After cooling for 20 min, absorbance was determined at 620 nm. Glucose was used as the control carbohydrate.

EPS sensitivity to pullulanase (EC 3.2.1.41)

Pullulanase sensitivity of EPS was determined according to Leathers et al. (1988). Dried EPS samples were dissolved at 0.1% (w/v) in 0.05 M sodium acetate buffer pH 5.0. Pullulanase (EC 3.2.1.41) from *Klebsiella pneumoniae* (Sigma, St. Louis, MO, USA) was added up to 0.1 U/ml and the mixture incubated for 15 hr at 25°C. Maltotriose reducing sugar equivalents were detected by the dinitrosalicylic acid (DNS) method (Miller, 1959)

Infrared (IR) analysis

Each EPS sample was prepared as a mull for analysis. Mull was prepared by grinding 2-5 mg of EPS in an agate mortar. The mull was examined as a thin film between flat salt plates. The pellet (pressed disk, 0.5-1 mg) was mixed with KBr powder (100 ng). The mixture was pressed (10,000-15,000 psi) into a transparent

disk. The prepared samples were analyzed using a Fourier Transform Infrared Spectrometer, FTIR (Perkin-Elmer, Norwalk, CT) model 160X.

^{13}C-Nuclear Magnetic Resonance (NMR) spectroscopy

^{13}C-NMR spectroscopy was carried out using a 500 MHz Bruker Advance Spectrometer (Figure 9) equipped with 5 mm inverse broadband probe operating at 125.7 MHz at 70°C. The EPS sample was dissolved in 50% (v/v) D6-dimethylsulfoxide (D6-DMSO)(Cambridge Isotope Labolatory, Inc., Andover, MA)/H_2O at a final concentration of 2% (w/v). Samples (0.7 ml) in NMR tube (Wilmad, Buena, NJ) were subjected to NMR analysis. Chemical shifts were expressed in part per million downfield from internal DMSO standard.

Molecular weight determination

Molecular weight of EPS was determined by using high performance size-exclusion chromatography (HPSEC) (Figure 10). Samples (15 mg/ml) were prepared by dissolving dry EPS in $NaNO_3$ (50 mM) with sodium azide (0.02%). Samples (10 µl) were applied to a Shodex KB-806 M HPSEC column (8x330 mm.) (Showa Denko, Tokyo, Japan) equilibrated with $NaNO_3$ (50 mM) with sodium azide (0.02%) using a flow rate of 0.5 ml/min. The column eluate was analyzed on-line by multi-angle light scattering at 690 nm. (Dawn EOS; Wyatt Technology Corporation, Santa Babara, CA, USA) with refractive index detection (Optilap DSP; Wyatt Technology Corporation, Santa Barbara, CA, USA). A value of 0.147 g/ml was used for the dn/dc of the EPS. Molar mass and size were calculated from the light

scattering and refractive index (RI) signals using Astra for Windows (version 4.73, Wyatt Technology Corporation, Santa Barbara, CA, USA).

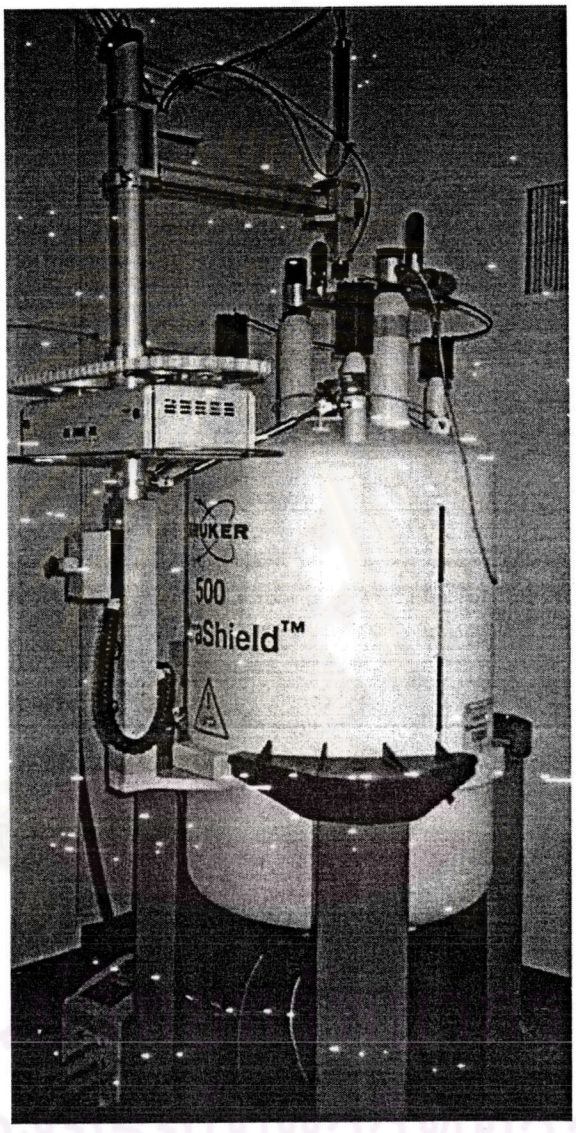

Figure 9 NMR spectrophotometer (500 MHz Bruker Advance Spectrometer)

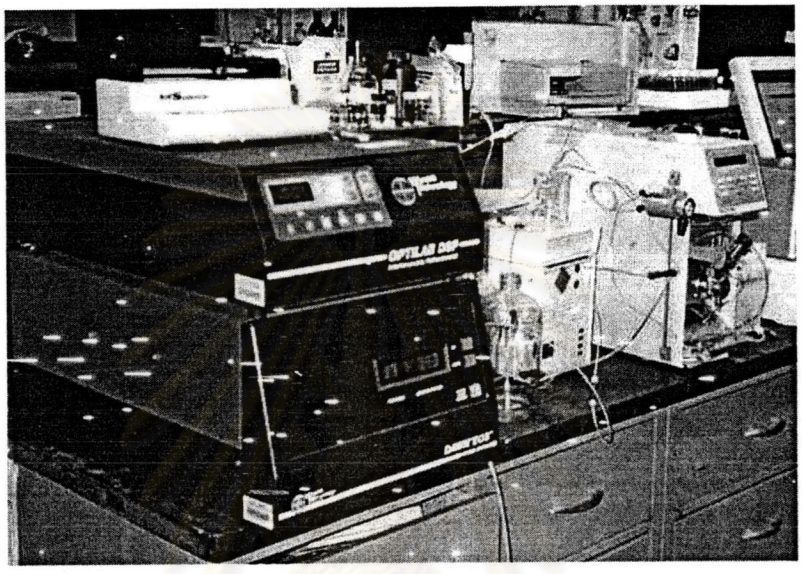

Figure 10 High performance size-exclusion chromatography (HPSEC)

Viscosity measurement

The EPS samples were dissolved in sterile distilled water at a final concentration of 10 mg/ml. Viscosity was measured using Brookfield Digital Rheometer model DV-III+ (Brookfield, Middleboro, MA) (Figure 11) with rotation at 125 rpm, a shear rate at 264 sec^{-1}, at 25°C.

Figure 11 Brookfield Digital Rheometer model DV-III+

Enzyme assays

Alpha-amylase (E.C. 3.2.1.1) screening

Cultures were screened for ability to hydrolyze starch using solid culture medium containing ($g.l^{-1}$) soluble starch (10 g), $NaNO_3$ (2 g), $MgSO_4.7H_2O$ (0.5 g), NaCl (0.5 g), $FeSO_4$ (0.01 g), yeast extract (0.4 g). After 5 days growth, the plates

were flooded with iodine solution. A halo or pale yellow zone around colony indicated amylase activity.

Alpha-amylase (E.C. 3.2.1.1) activity assay

Cultures were grown in 1% starch medium-see above, and the culture supernatant assayed for amylase. The amylase assay was based on activity towards borohydride-reduced starch prepared as substrate. The borohydride-reduced starch was prepared according to Strumeyer (1967). The reaction mixture containing 1% borohydride-reduced starch (0.5 ml) and appropriately diluted enzyme solution (0.5 ml) in 50 mM sodium acetate buffer pH 5.0. After 30 min of incubation at 50°C, the reducing sugar liberated in the reaction mixture was measured by the Somogyi-Nelson method (Nelson, 1944). One unit (U) of alpha-amylase activity is defined as the amount of enzyme that produced 1 μmole reducing sugar as glucose in the reaction mixture per minute under the specified conditions.

Pullulanase (EC 3.2.1.41) activity assay

Pullulanase was assayed in the supernatant of starch grown cultures-see above. Pullulanase activity was determined using borohydride-reduced pullulan as substrate. The borohydride-reduced pullulan was prepared according to Strumeyer (1967). The reaction mixture containing 1% borohydride-reduced pullulan (0.5 ml) and appropriately diluted enzyme solution (0.5 ml) in 50 mM sodium acetate buffer pH 5.0. After 30 min of incubation at 50°C, the reducing sugar liberated in the reaction mixture was measured by the Somogyi-Nelson method (Nelson, 1944). One unit (U)

of pullulanase activity is defined as the amount of enzyme that produced 1 μmole reducing sugar as glucose in the reaction mixture per minute under the specified conditions.

Alpha-amylase (E.C. 3.2.1.1) zymogram of native electrophoresis gel

Enzymes from starch and sucrose cultures were separated using native electrophoresis (Davis, 1986). The Tris-glycine gels and Tris-glycine native buffer were purchased from Invitrogen (Carlsbad, CA, USA). The enzyme samples were run on the gel at a constant 125 V for 2 hr. The gel was then soaked in a 2% starch solution (in 50 mM acetate buffer pH 5.0) for 3 hr at 50°C. Activity was revealed by staining with iodine solution (clearing of the starch).

Enhancement of the molecular weight of EPS

Two approaches were considered to obtain greater molecular weight EPS. As alpha-amylase synthesized by the *Aureobasidium* cultures was considered to attack the pullulan and thus fragment it and reduce its size, consideration was given to:

A. Preparation of amylase-negative mutants

B. Use of amylase inhibitors during the pullulan fermentation

Preparation of amylase-negative mutants using N-methyl-N'-nitro-N-nitrosoguanidine (NTG)

A. pullulans in yeast form was prepared in Yeast Malt Broth (YMB) (100 ml/250-ml flask) incubated at 28°C, 150 rpm. These yeast cells were subjected to

mutagenesis using NTG (Miller, 1992). NTG was used over a range of concentrations (50, 100, 200, 400 μg/ml), and cells incubated with NTG at 28°C for 30 min. Treated cells were plated onto YMA and incubated at 28°C. Surviving yeast colonies were counted and a kill curve prepared. Treated cells (less than 10% survival) were screened on starch agar plates. By flooding with iodine solution, and observing any amylase negative colonies (no clearing zone), rapid subculture allowed recovery of such strains before the iodine diffused completely through the colony killing all cells. The excess carcinogenic NTG was destroyed using 1 N NaOH. All materials coming in contact with NTG were immersed in a large beaker of 1 N NaOH or laboratory bleach prior to recycling or dispersal (Miller, 1992).

Strain stability and EPS production

An amylase-negative mutant was subcultured for five generations and then was re-tested for its amylase producing ability using starch agar plate assay. The EPS produced by the mutant was compared to that of the wild type strain.

Inhibition of amylase activity by use of an amylase inhibitor (acarbose) during the EPS fermentation

The filter-sterilized amylase inhibitor, acarbose (50 mg/ml) (Bayer, Leverkusen, Germany), was added to the EPS sucrose production medium at 0.05 % (w/v). The EPSs were ethanol precipitated (2:1) from the culture supernatant, and dried at 60°C. The molecular weight of the EPSs from the 3, 5 and 7 day cultures were determined and compared to those of the wild type culture.