CHAPTER III

MATERIALS AND METHODS

3.1 Multilocus phylogenetic analyses and phenotypic characterization of tropical isolates of *Aureobasidium pullulans*

3.1.1 *Aureobasidium* isolates

Aureobasidium pullulans was isolated from many provinces in Thailand using half strength corn meal agar (CMA; Difco, Detroit, MI, USA) containing 0.01% (w/v) Rose Bengal (Fischer Scientific, Pittsburgh, PA, USA) as previously described by Prasongsuk *et al.* (2005). All isolates were freeze-dried and deposited at the Agricultural Research Service (ARS) Culture Collection, US Department of Agriculture (USDA), Peoria, IL, USA and Fungal Section, Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

3.1.2 Phylogenetic analyses

3.1.2.1 Total DNA isolation

A total DNA isolation protocol was kindly recommended by C. D. Skory (ARS, USDA, Peoria, IL, USA). Each isolate was grown in 2 ml of yeast malt (YM) broth overnight, and cells were precipitated by centrifugation and resuspended in 1 ml of TE buffer, pH 8.0. About 0.5 ml of glass beads (0.5 mM Zirconia/Silica beads, BioSpec Products, Bartlesville, OK, USA) were added and cells were then disrupted using a FastPrep instrument (Qbiogene, Carlsbad, CA, USA), set at number 4, for 30 sec. The total DNA was isolated and purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA).

3.1.2.2 DNA amplification and sequencing

The 5 different loci (ITS, IGS1, *EF-1α, BT2, and RPB2*) were amplified by using different primers and standard PCR conditions, as shown in Table 3.1. The 50 µl reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.1 mM $MgCl_2$,

0.01% gelatin, 75 pmol of each primer, 0.2 mM of each dNTP, 2.5 U of REDTaq DNA polymerase (Sigma, St Louis, MO, USA) and 100 – 500 ng of genomic DNA. The amplified fragments were purified with the Millipore MultiScreen PCR system (Millipore, Billerica, MA, USA) and sequenced using the amplification primers and BigDye v3.1 Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and a Hitachi ABI 3730 (capillary) DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

3.1.2.3 Phylogenetic analyses

All sequences were determined from bidirectional sequencing (EMBL-EBI website, 2008). Corrected sequences were aligned again with ClustalW (Thompson *et al.*, 1994) and trimmed to equal length. The alignments were deposited in TreeBASE under study number SN4236. To generate phylograms, PAUP* (Swofford, 2003) version 4.0b10 was used for maximum-parsimony analysis and bootstrap analysis. For maximum parsimony, data sets were initially subjected to a heuristic search with random sequence addition (500 repetitions), NNI (nearest neighbor interchange) branch swapping, and maximum trees set to 5000. Subsequently, the random addition trees were used as the starting point for a heuristic search using "as-is" sequence addition, TBR (tree bisection-reconnection) branch swapping, and maximum trees set at 5000.

Congruence of the loci was assessed using the partition homogeneity test in PAUP*. Only phylogenetically informative sites were included. In each case, the null hypothesis of congruence could not be disproved at $P<0.001$.

Combined data sets were analyzed using weighted parsimony with the weighting inversely proportional to the number of parsimony informative characters. Weighting allowed each locus to contribute equally to the combined data tree. Conditions of analysis were the same as for single locus data sets. Bootstrap analysis was conducted using maximum parsimony criterion, TBR branch swapping and maximum trees set to 100 with 1000 repetitions.

MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) was used to calculate posterior probabilities of branches with the Bayesian inference method. The *EF-1α* and *RPB2* data sets included only protein

coding sequences and each data set was partitioned into codon positions 1, 2, and 3. The *BT2* locus included protein coding and intron regions and the data were partitioned accordingly into intron and exon data. The ITS data were partitioned into three regions, ITS1, 5.8s rRNA, and ITS2. IGS1 data were not partitioned. A GTR (general time-reversible) model was used with a proportion of invariant sites and a gamma shaped distribution of rates across the sites. Markov chain Monte Carlo (MCMC) analysis was conducted for up to 5×10^6 generations until the chains converged.

Concordance analysis was based on the exclusionary principle of Baum and Shaw (1995) and genealogical concordance phylogenetic species recognition concepts of Taylor *et al.*, (2000). Non-congruence in the apical branches was interpreted as species boundaries (Baum and Shaw, 1995). Lineages were based on strongly supported branches at one or more loci that were not contradicted strongly at other loci (Dettman *et al.*, 2006).

Aspergillus nidulans strain NRRL 187 was used to root the tree.

Table 3.1 Primers used for PCR and sequencing reactions in this study

Target DNA region	Primer[a]	Sequence 5'-3'[b]	Cycling reaction (35 cycles)	Approximately PCR product (bp)	Source
ITS	ITS5 (F)	GGAAGTAAAAGTCGTAACAAGG	95°C, 20 Sec	550	White *et al*., 1990
	ITS4 (R)	TCCTCCGCTTATTGATATGC	56°C, 30 Sec		White *et al*., 1990
			72°C, 1 min		
IGS1	ETS2-1AF (F)[c]	CGATCTGCTGAGATTAAGCC	95°C, 30 Sec	600	This study
	APIGS-5 (F)	TGCYGGTTRTTGAAGGGGWAG	58°C, 1 min		This study
	APIGS-1 (R)	CCACGTGTGACCCTGACC	72°C, 1 min		This study
	APIGS-6 (R)	GAGACATCTCTGCAACACACT			This study
	APIGS-7 (R)	ACCTCTAAGTGSGCTTCTGAG			This study
	APIGS-8 (R)	CCAGGTACGCTTTTGCGACAT			This study
EF-1α	YTEF-1A (F)[c]	TGGAAGTTCGAAACTCCAAAGTAC	95°C, 30 Sec	750	This study
	YTEF-6G (R)[c]	CGTTCTTGGAGTCACCACAGACGTTACCTC	54°C, 1 min		This study
	YTEF-CF (F)[c]	TCCAAAGTACATGGTCACCG	72°C, 1 min		This study
	YTEF-CR (R)[c]	CAGACGTTACCTCGACGGATC			This study
	YTEF-PF (F)	GGTCACCGTGATTTCATCAAG			This study
	YTEF-PR (R)	CTTGACGGAGACGTTCTTGAC			This study

Table 3.1 (continued)

Target DNA region	Primer[a]	Sequence 5'-3'[b]	Cycling reaction (35 cycles)	Approximately PCR product (bp)	Source
BT2	BT-2A (F)	GGTAACCAAATCGGTGCTGCTTTC	95°C, 30 Sec	450	Glass & Donaldson 1995
	BT-2B (R)	ACCCTCAGTGTAGTGACCCTTGGC	58°C, 1 min		Glass & Donaldson 1995
			72°C, 1 min		
RPB2	RPB-5F (F)	GAYGAYMGWGATCAYTTYGG	95°C, 30 Sec	1100	Liu *et al.*, 1999
	RPB-7R (R)	CCCATWGCYTGCTTMCCCAT	54°C, 1 min		Liu *et al.*, 1999
	RPB-PenR1 (F)[d]	GTTCACTCAACTYGTGCGYGA	72°C, 1 min		This study
	RPB-PenR2 (R)[d]	GGCAGGGTGAATYTCGCAATG			This study
	RPB-PenR3 (F)	GACTTCAAYCTYACYCTTGCTG			This study
	RPB-PenR4 (R)	GAGACATGACCATCATGGCAG			This study

[a] F and R in the parentheses mean forward and reverse primers, respectively.

[b] Degeneracy codes: R = A or G, Y = C or T, M = A or C, K = G or T, S = G or C, W = A or T.

[c,d] Primers designed by [c] C. P. Kurtzman and [d] S. W. Peterson (ARS, USDA, Peoria, IL, USA).

3.1.3 Phenotypic analyses

3.1.3.1 Morphological characteristics

Colonial morphology was observed after culturing strains on both yeast malt agar (YMA) and malt extract agar (MEA) at 28°C, for 7 days. Microscopic morphology was observed after growing cells in YM broth at 28°C, for 1-5 days and on MEA at 28°C, for 7 days.

3.1.3.2 Xylanase activity

Preinocula were grown in 10 ml of basal medium consisting of 0.67% (w/v) yeast nitrogen base (Difco Laboratories, Detroit, MI, USA), 0.2% (w/v) asparagine, 0.5% (w/v) KH_2PO_4, amended with 1% (w/v) glucose, in 50-ml flasks. Preinocula were incubated at 200 rpm, and 28°C for 3 days. Ten milliliter of basal medium containing 1% (w/v) purified xylan from oat spelts (Sigma, St. Louis, MO, USA) in 50-ml flasks was inoculated with preinocula at 1% (v/v) cultures, and incubated for 3 days as above. Xylanase was assayed using a modification of the dinitrosalicylic acid method (Miller, 1959) as previously described (Leathers *et al.*, 1984, 1986). The experiments were performed in triplicate.

Supernatant from each culture was applied to SDS-PAGE. Individual supernatants were mixed with an equal volume of Laemmli sample buffer (25% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue in 62.5 mM Tris-HCl (pH 6.8) Bio-Rad Laboratories, Inc., Hercules, CA) and boiled for 10 min. Fifteen microliters of samples were loaded on precast 8 – 16% gels (Criterion, Bio-Rad Laboratories, Hercules, CA). After electrophoresis at 180 V for 1 h, the SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad Laboratories, Hercules, CA) for 1 h and destained with double distilled water for 30 min.

3.1.3.3 EPS production

A fresh colony growing on YMA solid medium (2-3 days cultivation) was used to inoculate a 25-ml flask containing 10 ml of YM, which was then incubated overnight at 28°C with shaking at 150 rpm. The cell density in this preinoculum was determined using a haemacytometer, and 5 x 10^7 cells were used to inoculate a 300-ml flask containing 100 ml of pullulan production medium (PM). PM contained 5% (w/v) sucrose, 0.06% (w/v) peptone, 0.04% (w/v) yeast extract, 0.5% (w/v) K_2HPO_4, 0.04% (w/v) $MgSO_4.7H_2O$, and 0.1% (w/v) NaCl (Prasongsuk *et al.*, 2007). These flasks were incubated at 28°C with 150 rpm shaking for 7 days. The color change of each culture was observed at day 7. Cells were removed by centrifugation (10,000 x g, 20 min) and EPS in the supernatant was precipitated using 2 volumes of 95% ethyl alcohol (Prasongsuk *et al.*, 2007). Cell pellets and EPS were dried at 60°C. Cell and EPS dry weights were measured and yields were determined. The experiments were performed in triplicate.

3.1.3.4 NMR spectra

EPS or pullulan structure was analyzed by ^{1}H-NMR (nuclear magnetic resonance spectroscopy). Individual extracted EPS samples were lyophilized and then dissolved in D_2O to a final concentration of 2 mg/ml. All NMR experiments were performed on a Bruker Avance 500 spectrometer (Bruker Biospin Corp., Billerica, MA, USA) equipped with a 5-mm broadband inverse (BBI) probe with *z*-gradient. All experiments were performed at 27°C. The data were processed with Topspin v1.3 software and the chemical shifts were reported as parts per million from external tetramethylsilane (TMS) based on the lock signal.

3.2 Study of the relationship between α-amylase activity and pullulan profiles

Five fungal isolates (CU 3, CU 20, CU 36, NRM2 and NRRL Y-12974) were selected for a more detailed study on pullulan production and the effects of α-amylase activity on pullulan profiles. All five strains were first cultured on 1% (w/v) starch agar

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medium to determine the presence of α-amylase activity (assay described below). Liquid shake-flask cultures were subsequently used to study the relationship between pullulan profiles and α-amylase activity. All five strains were cultured in standard pullulan production medium (PM), containing 5.0% (w/v) sucrose and 0.1% (w/v) N-sources (the ingredients is described in 3.1.3.3). Reference strain NRRL Y-12974 was also grown in three modified PM media containing 5.0% (w/v) sucrose and 0.3% (w/v) N-sources; 5.0% (w/v) soluble starch and 0.1% (w/v) N-sources; or 5.0% (w/v) soluble starch and 0.3% (w/v) N-sources. The 0.3% (w/v) N-sources contained 0.18% (w/v) peptone and 0.12% (w/v) yeast extract, In all cases, a fresh colony growing on YMA (2-3 days cultivation) was used to inoculate a 25-ml flask containing 10 ml of YM, which was incubated overnight at 28°C with shaking at 150 rpm. The concentration of cells in this preinoculum was determined using a haemacytometer, and 4×10^7 cells were used to inoculate a 250-ml flask containing 80 ml of PM or modified PM media. Cultures were incubated at 28°C with 150 rpm shaking, and sampled at days 2, 4, 6, and 8 of cultivation. The experiment was divided into 2 parts (Figure 3.1). Each part was done in triplicate. In the first part of the experiment, a 1-ml sample was taken from each culture. These samples were centrifuged and the supernatants were used to determine the activities of α-amylase and pullulanase. These samples were also applied to SDS-PAGE for zymogram analysis. The pH and OD_{600} was measured in the remaining culture, and then cells were removed by centrifugation at 8,000 rpm (10,000 x g) for 20 min. EPS in supernatant was precipitated using 2 volumes of 95% ethyl alcohol (Prasongsuk *et al.*, 2007) followed by centrifugation at 10,000 rpm (16,900 x g) for 30 min. Precipitated EPS was lyophilized, and dried EPS was characterized for α-amylase sensitivity, pullulanase sensitivity, molecular weight, and viscosity. In the second part of the experiment, culture broths were centrifuged at 8,000 rpm (10,000 x g) for 20 min, and EPS in the supernatants were precipitated using 2 volumes of 95% ethyl alcohol followed by centrifugation at 10,000 rpm (16,900 x g) for 30 min. Cells and EPS were dried in an incubator at 60°C and dry weights were determined.

The experiments were performed in triplicate. The results were statistically analyzed and standard errors calculated using SigmaPlot Version 10.0.

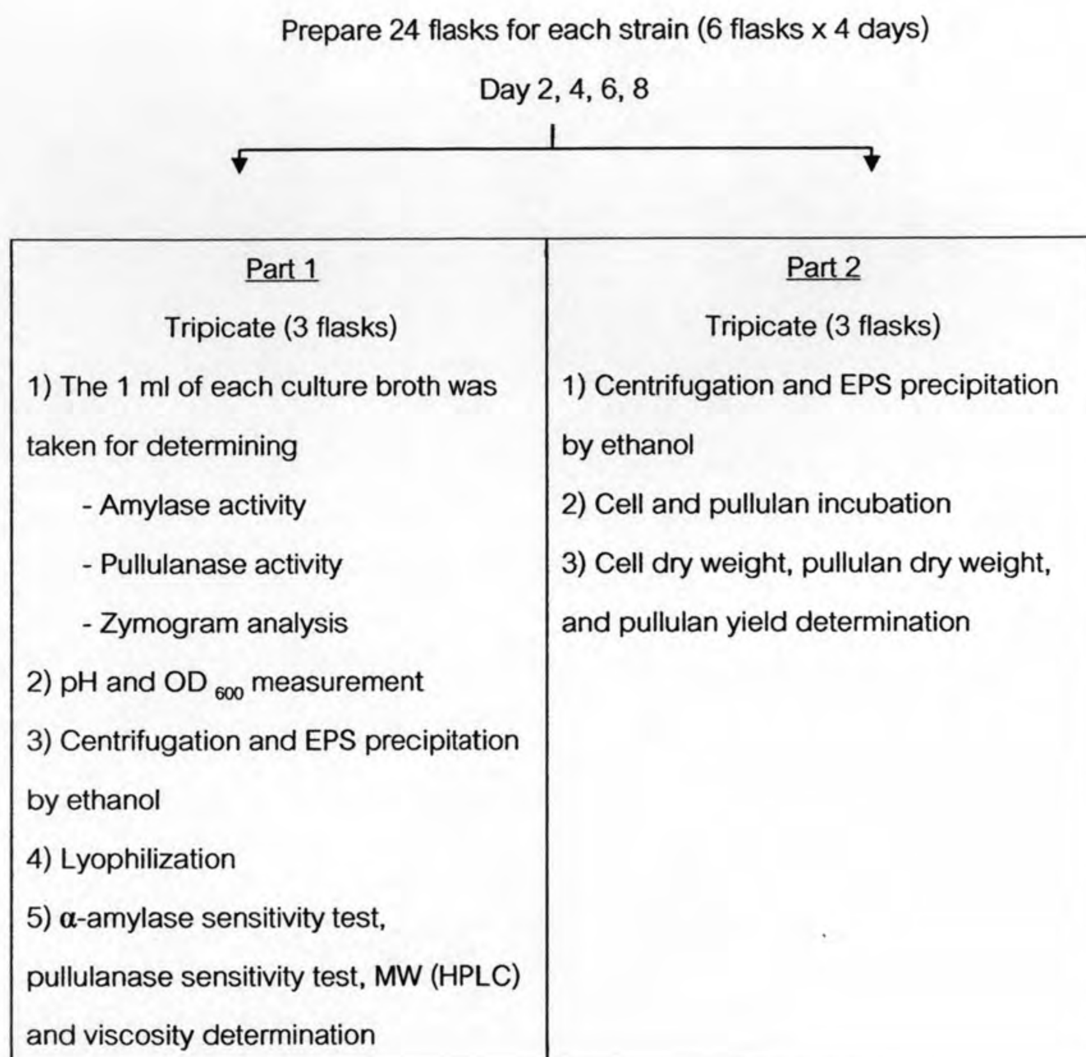

Figure 3.1 Flow chart of the experiments performed in the study of relationship between α-amylase activity and pullulan profiles

3.2.1 EPS analyses

3.2.1.1 α-amylase sensitivity test and pullulanase sensitivity test

Sensitivity tests were based on the pullulanase sensitivity assay described by Leathers *et al.* (1988). Lyophilized EPS from each sample at day 2 was resuspended at 0.1% (w/v) in 50 mM sodium acetate buffer, pH 5.0. α-amylase (type II-A: *Bacillus* sp., Sigma, St. Louis, MO, USA) or pullulanase (*Klebsiella pneumonia,* Sigma, St. Louis, MO, USA) was added to 0.1 U/ml and samples were incubated at

$28^{o}C$ for 20 h. Reducing sugar equivalents were determined by the dinitrosalicylic acid method (Miller, 1959).

3.2.1.2 Molecular weight determination

Determination of the molecular weight of EPS using high performance size-exclusion chromatography (HPSEC) was modified from Prasongsuk *et al.* (2007). Freeze-dried EPS (1% (w/v)) was dissolved in distilled water and filtered using Nanosep MF 0.45 µm microcentrifuge filter tubes (Pall corporation, East Hills, NY, USA). Ten microliter of filtrate were applied to a Shodex SB 806M HQ HPSEC column (300 x 8 mm, Showa Denko, Tokyo, Japan) equilibrated with 50 mM $NaNO_3$ using a flow rate of 0.5 ml/min. Eluate from the column was analyzed by optical rotation using a Shodex OR-1 detector. Molecular weight estimates based on elution positions were made using pullulan molecular weight standards (Shodex 5,800 to 1.66 million).

3.2.1.3 Viscosity measurement

Freeze-dried EPS was dissolved in distilled water at 1% (w/v). Viscosity was measured using a Brookfield Digital Rheometer model DV-III+ (Brookfield, Middleboro, MA, USA) at $25^{o}C$ with a rotation of 30 rpm (shear rate of 39.6 1/S) using spindle SC4-18 and chamber 13R.

3.2.2 Assay for α-amylase and pullulanase activities

3.2.2.1 α-amylase (E.C. 3.2.1.1) screening

The α-amylase screening method was described by Prasongsuk *et al.* (2007). Each strain was cultured on 1% (w/v) starch agar medium (Saha *et al.*, 1993) containing 1% (w/v) soluble starch, 0.2% (w/v) $NaNO_3$, 0.05% (w/v) $MgSO_4.7H_2O$, 0.05% (w/v) NaCl, 0.001% (w/v) $FeSO_4$, and 0.04% (w/v) yeast extract, and incubated at $28^{o}C$ for 5 days. The plates were flooded with iodine solution, and halo zones surrounding colonies indicated the presence of α-amylase activity.

3.2.2.2 Assay for α-amylase and pullulanase activities

Activities of α-amylase and pullulanase were determined using Blue-starch (Starch Azure, Sigma, St. Louis, MO, USA) and Red-pullulan (Procion Red MX-5B, Megazyme, Co. Wicklow, Ireland) as substrates, respectively. These substrates were depolymerized by endo-acting enzymes to produce low molecular weight dyed fragments which remain in solution on addition of ethyl alcohol to the reaction mixture. High molecular weight material was removed by centrifugation, and the color of the supernatant was measured at 595 nm (for α-amylase assay) or 510 nm (for pullulanase assay). The reaction mixture containing 200 μl of 1% (w/v) substrate in 50 mM sodium acetate buffer (pH 5.0), 50 μl of supernatant from each culture broth, and 2.5 μl of 2% (w/v) sodium azide was incubated at 28°C for 20 h. Five hundred microliters of precipitation buffer (4% (w/v) sodium acetate trihydrate, 0.4% (w/v) zinc acetate, 800 ml of 200 proof ethyl alcohol, and 200 ml of deionized water per L) was added and mixed by vortex briefly. The reaction mixture was incubated at room temperature for 10 min and then centrifuged at 10,000 rpm for 10 min. Two hundred microliters of the supernatant was applied to a microtiter plate and the absorbance measured as described above. The assays were calibrated using commercial enzymes of known activities. One unit of enzyme activity is defined as the amount necessary to release 1 μmole of maltose equivalents per min.

3.2.2.3 Zymogram analysis

The method of zymogram analysis was kindly recommended by K. M. Bischoff (ARS, USDA, Peoria, IL, USA). Twenty-five μl samples of individual supernatants from both starch and sucrose PM culture broths were mixed with 25 μl of Laemmli sample buffer (25% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue in 62.5 mM Tris-HCl, pH 6.8, Bio-Rad Laboratories, Hercules, CA) and boiled for 5 min. The samples were centrifuged at 13,000 rpm for 5 min and loaded on both a 10% SDS-PAGE gel (Sambrook *et al.*, 1989) and a 10% SDS-PAGE gel containing 0.5% (w/v) soluble starch. After electrophoresis at 150 V. for 55 min, the SDS-PAGE gels were stained with

Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad Laboratories, Hercules, CA, USA) for 1 h and repeatedly destained in a solution of 40% (v/v) methanol and 10% (v/v) acetic acid in water to visualize the protein bands. The SDS-PAGE gel containing 0.5% (w/v) soluble starch was incubated twice for 30 min each in assay buffer (50 mM sodium acetate buffer, pH 5.0) containing 20% (v/v) isopropanol to renature proteins. The gel was further washed twice for 15 min each with assay buffer and then soaked with assay buffer containing 8 M urea for 1.5 h. The gel was washed for 5 x 25 min with assay buffer and then soaked with Gram's iodine solution for 20 min. After rinsing with deionized water, the amylase profile was visualized on the gel as a clear zone resulting from the hydrolysis of the starch. All steps were performed at room temperature with agitation.

3.2.3 α-amylase gene analyses

3.2.3.1 Genomic DNA sequence analysis

Aureobasidium pullulans reference strain NRRL Y-1294 was employed to determine the genomic DNA sequence of the α-amylase gene. Collected mycelia were immediately frozen in liquid nitrogen and disrupted using the Beat Mill MM 301 (Retsch, Newtown, PA, USA) followed by DNA purification with the DNeasy Plant Mini kit (QIAGEN, Valencia, CA, USA). Initial degenerate primers (APamylF1 and APamylR2.2, Table 3.2) for the α-amylase gene were designed using the amino acid sequence alignment of fungal α-amylase from the database in GenBank. The PCR products were purified and sequenced. The putative gDNA sequence was analyzed using the homology search with Blastx (National Center for Biotechnology Information, 2008) to the α-amylase amino acid sequences in GenBank. Flanking regions (both 3' and 5' ends) were further determined using the GenomeWalker™ Universal kit (Clontech, Mountain View, CA, USA). The isolated gDNA was digested with four blunt-end restriction enzymes (*Dra*I, *Stu*I, *Pvu*II, and *Eco*RV) to construct four different libraries. Each library was then ligated with the GenomeWalker Adaptor. New gene-specific primers (GSP) were designed by using initial data sequences (Table 3.2). PCR-based DNA walking in GenomeWalker libraries was performed using the adaptor primer (AP1) and GSP (AP-GSP3f, AP-GSP1r, AP-

GSP7f) for both 3' and 5' ends (see Appendix B). The map of genome walking of the α-amylase gDNA is shown in Figure 3.2. The complete sequence of the α-amylase gene was analyzed using the homology search with Blastx and the mRNA sequence, amino acid sequence, initial start codon, stop codon, and number of exons were predicted by FGENESH+ (Softberry, Inc, 2008) using the *Aspergillus terreus* alpha-amylase (XP_001209405) as a similar protein sequence. The alignment of the putative mRNA sequence and gDNA sequence was performed using ClustalW (The European Molecular Biology Laboratory, 2008) to determine the introns and exons sequences. The α-amylase amino acid sequences of *A. pullulans* and other species was analyzed using DNA Star program (DNASTAR, Inc, Madison, WI, USA).

The first putative PCR fragment was cloned in the pCR-XL-TOPO vector using the TOPO XL PCR cloning kit (Invitrogen, Carlsbad, CA, USA). The other PCR fragments were purified using QIAquick PCR Purification kit (QIAGEN, Valencia, CA, USA), cloned in the pCR 4Blunt-TOPO vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA, USA), and sequenced using the amplification primers and BigDye v3.1 Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and a Hitachi ABI 3730 (capillary) DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Table 3.2 Primers used for amplification of α-amylase gDNA and mRNA of *A. pullulans*

Primer	Sequence 5'-3'	Source
APamylF1 (F)[a]	GGNTTYACNGCNATHTGGATH[b]	This study
APamylR2.2 (R)	RTADATDATYGGDATYCCRTC[b]	This study
AP-GSP3f (F)	CTACTGGATCACACAAGCCTTCCAGTC	This study
AP-GSP1r (R)	CTGCGAGTTGAACGGGTTGTATATGG	This study
AP-GSP5r (R)	GTAATACGACTCACTATAGGGCACGC	This study
AP-GSP7f (F)[c]	CAAGG CCTATCCCGTCTACTCTGA	This study
AP-LS1f (F)	GAAACCAACAGGGATTGCCCTAG	This study
AP-LS3r (R)	GACGGGTCGCTTACAACCATTAC	This study
AP1 (F or R)	GTAATACGACTCACTATAGGGC	GenomeWalker Universal Kit

[a] F and R in the parentheses mean forward and reverse primers, respectively.

[b] Degeneracy codes: R = A or G, Y = C or T, H = A or T or C, D = G or A or T, N = A or C or G or T.

[c] Primer designed by C.D. Skory (ARS, USDA, Peoria, IL, USA).

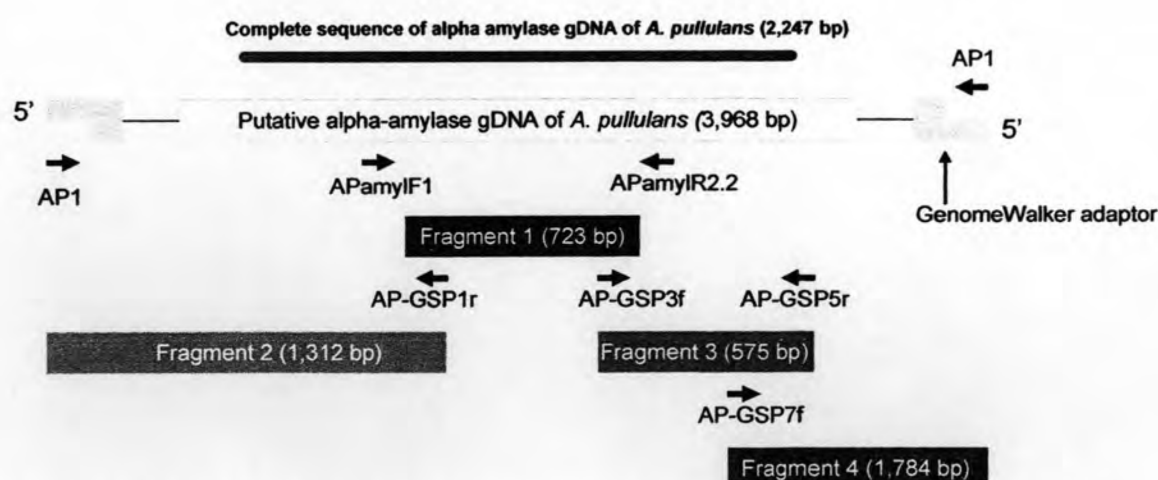

Figure 3.2 Map of alpha-amylase genome walking of *Aureobasidium pullulans* NRRL Y-12974

3.2.3.2 Southern blot hybridization

The copy number of the α-amylase gene in the genome was determined by Southern blot hybridization. Cloned fragment 3 (Figure 3.2) in the pCR4 Blunt-TOPO vector was used as a template and AP-GSP3f and AP-GSP5r (Table 3.2) were used as primers for amplifying DIG (digoxygenin)-labeled probe (Roche, Indianapolis, IN, USA) (see Appendix B). Genomic DNA of strain NRRL Y-12974 was completely cut with 5 different restriction enzymes (*Xba*I, *Kpn*I, *Pst*I, *Pvu*II, and *Eco*RI) and loaded on a 0.7% agarose gel. After electrophoresis at 350 V overnight, the gel was incubated twice for 30 min each in denaturing buffer (17.55% (w/v) NaCl and 1.6% (w/v) NaOH). The gel was then incubated in transfer buffer (17.55% (w/v) NaCl and 0.032% (w/v) NaOH) for 15 min. DNA was transferred to a positively charged nylon membrane using transfer buffer. After 2 h, the membrane was rinsed with neutralization buffer (0.2 M sodium phosphate, pH 6.8), dried, and crosslinked at 120,000 joules/cm^2 by Stratalinker 1800 (Stratagene, Cedar Creek, TX, USA).

For hybridization, the membrane was incubated in a hybridization chamber using a roll tube with 20 ml of UltraHyb buffer (Ambion, Foster City, CA, USA) at 42°C, for 1 h. One µl of probe stock was diluted in 5 ml of UltraHyb and denatured at 68°C for 15 min. The denatured probe was added to the 20 ml of UltraHyb buffer and hybridization continued overnight at 42°C. After hybridization, the membrane was washed twice for 5 min each with low stringency wash buffer (2xSSC and 0.1% (w/v) SDS) and then washed twice for 15 min each at 65°C with high stringency wash buffer (0.5xSSC and 0.1% (w/v) SDS).

For detection, the membrane was equilibrated in 100 ml of 0.1 M maleic acid buffer, pH 7.5, for 5 min and incubated in 100 ml maleic acid buffer containing 1% (v/v) blocking solution (Roche, Indianapolis, IN, USA) for 30 min. The membrane was then incubated in antibody solution (100 ml maleic acid buffer containing 1% (v/v) blocking solution and 10 µl of Anti-DIG-AP) for 30 min. After that the membrane was washed twice for 15 min each in 100 ml of maleic acid buffer and incubated in detection solution (0.1 M Tris-HCl (pH 9.5) and 0.1 M NaCl) for 5 min. Finally, the membrane was

placed between two acetate sheets and wet with substrate (10 µl of CDP star (Roche, Indianapolis, IN, USA) in 1 ml of detection solution). The Kodak Image Station 1000 was used for chemiluminescent detection.

3.2.3.3 Detection of α -amylase mRNA

Expression of the α-amylase gene during cultivation was verified by detection of the presence of mRNA that remained or accumulated in the cell using reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated using the RNeasy Plant Mini kit (QIAGEN, Valencia, CA, USA). The first-strand cDNA was synthesized from isolated mRNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Contaminating genomic DNA was removed from total RNA using the DNA-free kit (Applied Biosystems/Ambion, Austin, TX, USA). Both first-strand cDNA and DNase-treated RNA were used as the templates, and APamylF1 and APamylR2.2 were used as primers to amplify the PCR fragment of the α-amylase gene (see Appendix B). The large subunit (26S) of rDNA was used as an internal control. New primers (AP-LS1f and AP-LS3r, Table 3.2) for 26S rDNA of *A. pullulans* were designed using the sequence alignment from the GenBank database. The PCR reaction is described in the Appendix B.