# CHAPTER V

# DISCUSSION

## 1. Isolation of fungi

Fourteen isolates of *Aureobasidium* were obtained from a rage of habitats in several locations around Thailand. Habitats included bathroom cement walls, painted surfaces, and plant leaves. Locations were in Central, Northern and Southern Thailand (Table 5). This is a first record regarding the occurrence of *Aureobasidium* on bathroom cement walls, latex painted surfaces, and diverse plant leaves in Thailand. *A. pullulans* were previously isolated from pine needle leaf litter and collection of air samples in some Northern provinces and Bangkok Thailand (Punnapayak et al., 2003; Tokomasu et al., 1997). In some other tropical countries such as Brazil, India, Jamaica, and Malaysia, *A. pullulans* has been isolated (Deshpande et al., 1992). The diverse habitats from which *A. pullulans* were recovered supports the notion that *A. pullulans* is ubiquitous, saprophytic and epiphytic. Hot, humid environments facilitate the development of *Aureobasidium* spp.

The isolates were obtained from several kinds of plant leaves including asoka, mango, *Ochna kirkii* Oliv., rain tree, *Syzygium malaccense Linn.*, and tamarind. From my collections, *Aureobasidium* spp. (four isolates) were most frequently isolated from surfaces of tamarind leaf and three isolates were from asoka leaf surface while *Aureobasidium* could not be recovered from some plant species (Tables 4 and 5). It is possible that leaf of those plant species contains some antifungal substances which could against colonization of *Aureobasidium* (Tables 4 and 5).

Isolation of *Aureobasidium* spp. from habitats in Thailand has been achieved by using CMA and MEA half strength as culture media plus the use of an antibacterial compound - chloramphenicol. More yeast isolates were recovered from MEA half strength (9 isolates) than from CMA half strength (6 isolates) (Table 5). Both diluted solid media were useful for *Aureobasidium* isolation by restricting some microbes which were grew poorly on these media. chloramphenicol was effective in inhibiting the bacterial growth. The use of diluted media plus chloramphenicol does not circumvent the problem of the rapid growth of several filamentous fungi such as *Aspergillus*, *Penicillium*, *Rhizopus*, and *Trichoderma* over growing the yeast colonies. The presence of these fast-growing filamentous fungi is a major problem for isolation of this black yeast. Thus, use of some fungal inhibitors such as Rose Bengal or sorbitol incorporate the culture medium to restrict the growth of filamentous fungi should be considered.

## 2. Fungal identification

### 2.1 Morphological observation

The cell morphology of all yeast isolates was polymorphic including blastospores, chlamydospores, hyphae, and swollen cells (Figures 17-31). Some isolates contained blastoconidia and endoconidia. The colony grew rapidly and became black with time. The colonies of some isolates (NRM2 and LB3) were pink while those of isolates LB2 and SK3 were red and yellow, respectively, characteristic of so-called "color variant" strains (Wickerham and Kurtzman, 1975). The colony sizes ranged between 2.86 and 4.75 cm on the MEA after 7 days. Both morphological and colony characteristics corresponded well with the *A. pullulans* descriptions by

Barnett and Barry (1998), Domsch et al. (1993), and Hermanides-Nijhof (1997) and to the features of the standard strains, ATCC 42023 and NRRL 6992.

2.2 Nuclear Ribosomal DNA Internal Transcribed Spacer (ITS) sequencing

Since *A. pullulans* is polymorphic, morphological observation alone is insufficient to define the isolates. The ITS sequencing was used to help taxonomically assess the isolates. The sequences of all isolates were very similar to each other and only isolate SK3 differed slightly from the other four. Alignment using BLAST program showed that all sequences strongly correlated with those of *A. pullulans* in GenBank yielding greater than 95% similarity (Figure 33). The phylogenetic tree of *Aureobasidium* isolates based on ITS sequences showed that all isolates and *A. pullulans* in GenBank were in the same cluster as indicated by low bootstrap value within the tree (Figure 34). These results were similar to that of Sudhadham (2001) and Yurlovar and De Hoog (1997) who also used ITS sequencing to confirm the *Aureobasidium* identification. Thus the ITS technique while very useful to confirm the identification of *A. pullulans,* could not be used to distinguish between the isolates due to the high homology of ITS sequences within the same species.

2.3 Nutritional physiology

The carbon and nitrogen assimilation patterns of all isolates correlated well with the control strains *A. pullulans* NRRL Y-2311-1, and NRRL Y-7469. *Aureobasidium* isolates assimilated a diverse range of carbon sources such as cellobiose, galactose, glucose, glycerol, sucrose, and xylose but could not assimilate cellobiose, chitin, p-coumaric acid, and sodium succinate (Tables 6 and 7). This carbon assimilation pattern is in agreement with other previous reports. Zimbabwean isolates of *A. pullulans* (de Bary) Arnaud utilized a broad range of substrates including cellobiose, glucose, glycerol, sucrose, xylan, and xylose (Okagbue et al.,

2001). Other workers mentioned that *A. pullulans* could utilize cellobiose but not cellulose (Dennis and Buhagiar, 1973; De Hoog and Yurlova, 1994). This is also supported by the lack of cellulase activity from strains of *A. pullulans* (Federici, 1982) although β-glucosidase has been detected from this fungus (Hayashi et al., 1993). That the isolates could not utilize chitin correlated well to the reports which showed no chitinase activity from other *A. pullulans* strains (Federici, 1982; De Hoog and Yurlova, 1994). The ability to utilize galactose and glycerol of all isolates were in agreement with a previous report in which *A. pullulans* differed from *A. prunorum* and *Trichosporon pullulans* by its ability to utilize these substrates (Dennis and Buhagiar, 1973).

The assimilation of α-methyl-D-glucoside and lactose by all isolates are similar to the data of *A. pullulans var. pullulans* (Yurlova and De Hoog, 1997). However, there is an intra-specific variation of *Aureobasidium* isolates and standard strains in assimilation of dulcitol, glucosamine, glycerol, rhamnose, and sodium citrate.

For nitrogen assimilation, L-arginine, creatinine HCl, L-isoleucine, L-lysine, L-serine, sodium nitrate, sodium nitrite, and L-tryptophane were utilized by all isolates but not creatine monohydrate or L-threonine. General utilization of amino acids is clear. *A. pullulans* P-66 assimilated a range of nitrogen sources including amino acid but it could not utilize L-lysine (Cooke and Matsuura, 1963). On the other hand, several tested strains of *A. pullulans* were able to utilize L-lysine (Cernakova et al., 1980; De Hoog and Yurlova, 1994) and also I found this so for the Thai isolates (Table 7).

The intra-specific variation for carbon and nitrogen assimilation for the isolates indicated that the ability of utilizing substrates is unique for each isolate. It is

possible to use the nutritional physiology as criteria to help define the *Aureobasidium* isolates.

### 3. Exopolysaccharide (EPS) production

*A. pullulans* produces the biopolymer gum pullulan with some possible reasons including: 1. to adhere to habitat surfaces such as plant leaves or painted surfaces, 2. it protects the yeast against desiccation, and 3. pullulan is an energy reserve (Pace, 1987). In this thesis, all isolates when cultured on the production medium produced EPSs (Table 8) with five strains showing higher yields-BK4 (7.9 g.l$^{-1}$), BK6 (6.1 g.l$^{-1}$), LB3 (4.6 g.l$^{-1}$), NRM2 (7.7 g.l$^{-1}$), and SK3 (5.8 g.l$^{-1}$).

The use of glucose and sucrose to optimize EPS yields was investigated. Sucrose yielded greater EPS than glucose for all isolates. Sucrose was previously found as the best carbon source for pullulan production for other strains (Leathers, 2002; 2003). Among fructose, glucose, maltose, and sucrose, *P. pullulans* gave the highest pullulan yields (14.8 g.l$^{-1}$) using sucrose (Catley, 1971). Sucrose also resulted in greatest yields by *A. pullulans* strain P56 (0.19 g.l$^{-1}$ per hour) with glucose, fructose, xylose, lactose and sucrose being evaluated (Schuster et al., 1993). A color variant *A. pullulans* Y-2311-1 also preferred sucrose more than glucose with highest yields of 26.2 g.l$^{-1}$ (Gibson and Coughlin, 2002). A typical pigment strain *A. pullulans* ATCC 42023, gave greater pullulan yield on sucrose (7 g.l$^{-1}$) (West and Reed-Hamer, 1991) and remarkably sucrose was the most suitable substrate for pullulan production with a doubling of EPS production when *A. pullulans* QM 3092 (3.1 g.l$^{-1}$ to 6.9 g.l$^{-1}$) was switched from glucose to sucrose (Reeslev et al., 1997). Sucrose is better carbon source than glucose because clearly *A. pullulans* produces sucrase (invertase, EC.

3.2.1.26) (Reese and Maguire, 1971). In contrast to all of these studies, glucose yielded better production than sucrose with Thai isolates (Punnapayak et al., 2003).

In commercial production, the carbon source is starch hydrolysate (Leathers, 2002; 2003), a less-expensive substrate. In this thesis, I have compared only glucose and the disaccharide sucrose. Even though sucrose was the best carbon and cheaper than glucose, this substrate is still so far more expensive than starch hydrolysate. However, the using starch as substrate I could get low-molecular weight pullulan resulted from the action of some hydrolytic enzymes such as amylase and pullulanase (Table 15).

At the optimal carbon sucrose, nitrogen sources were optimized for these isolates. $(NH_4)_2SO_4$ was optimal nitrogen for isolates BK4 (23.1 g.l$^{-1}$), BK6 (17.0 g.l$^{-1}$) and SK3 (10.4 g.l$^{-1}$) while peptone was better for the other two strains giving 25.1 g.l$^{-1}$ for NRM2 and 15.0 g.l$^{-1}$ for LB3. A. pullulans NRRL 6220 gave EPS yield (32.7 g.l$^{-1}$) on $(NH_4)_2SO_4$ higher than on other nitrogen substrates including ammonium citrate, $NaNO_3$, and urea (Badr-Eldin et al., 1994). On the other hand, A. pullulans ATCC 42023 gave greater pullulan yields on complex nitrogen sources including peptone than that on ammonium sulfate (Reed-Hamer and West, 1994). In commercial production, peptone has been used as nitrogen source (Leathers, 2002; 2003).

Peptone is useful for EPS production. Overall the use of nitrogen sources for pullulan production appears strain dependent.

Through optimization, the maximal EPS yield (25 g.l$^{-1}$) was obtained from strain NRM2 (Figure 44) this yield being relatively high compared literature values: 6.9 g.l$^{-1}$ (Reeslev et al., 1997), 10.2 g.l$^{-1}$ (Leathers et al., 1988), 14.8 g.l$^{-1}$ (Catley, 1971), 23.01 g.l$^{-1}$ (Vijayendra et al., 2001), 26 g.l$^{-1}$ (Gibson and Coughlin, 2002).

From observation during the production period, only blastospores and hyphae appeared responsible for EPS production. Blastospores were the most common morphotype all cultures in the production medium. Catley (1980) reported similar results with *A. pullulans* ATCC 9348, where blastospore and mycelium produced pullulan, but blastospore was more efficient than mycelium producing pullulan yields 4-5 times higher than that from mycelium. On the other hand, two recent reports indicate that only swollen cells and chlamydospores can produce pullulan, but neither blastospores nor hyphae (Campbell et al., 2004; Simon et al., 1993). These data require further assessment.

Pullulan is a non toxic gum (Leathers, 2002; 2003, Yuen et al., 1974), but care in production should be taken as a recent report described *A. pullulans* to cause invasive pulmonary infection in human (Hawkes et al., 2005).

## 4. Exopolysaccharide (EPS) analysis

### 4.1 Total hexose content

EPSs from all isolates contained high hexose content ranging from 97 to 99% analyzed using the anthrone assay (Table 10). The hexose content varied for EPSs from each isolate (Table 10) ranging from 97 to 99%. Pullulan from *A. pullulans* (de Bary) Arnaud strain 105-22, was in the similar range of 90-93% (Leal-Serrano et al., 1980). Other components are sometimes reported. Thus EPSs from *A. pullulans* (de Bary) Arnaud strain 105-22 also contained such substances as malic acid (8.5-9.0%), protein (2.5-3.0%), and phosphate (0.4-0.5%) (Leal-Serrano et al., 1980)

Elemental analysis of pullulan from *A. pullulans* (De Bary) Arnaud (IMI 145194) showed all carbohydrate, and EPS also contained trace amount of N, P, and S

with the resultant conclusion little protein contamination was present (Madi et al., 1997). The slight variations in content can reflect the method of pullulan recovery.

I found the EPSs from isolates BK6 and NRM2 gave the lowest hexose content (97%) (Table 10) perhaps with protein as a possible contaminant from fungal cells.

4.2 Sensitivity to Pullulanase

Pullulanase sensitivity of EPSs (Leathers et al., 1988) for all isolates ranged between 56-97% (Table 10). The sensitivities of EPSs from isolates BK6 and NRM2 were lower than the other three (Table 10). One possibility is that these EPSs are less susceptible to pullulanase. Another possibility is that melanin, which contaminated EPSs, could be inhibitory to pullulanase (Leathers et al., 1988). From my observation, EPS from isolate BK6 contained the highest melanin content and showed lowest sensitivity to pullulanase. This possibility was previously reported (Punnapayak et al., 2003; West and Reed-Hamer, 1993c).

4.3 Infrared (IR) analysis

The IR spectra of all EPSs were similar to that of Sigma pullulan standard (Figure 47, Table 11). Specific functional groups including alkane, carbonyl, ether, hydroxyl, hydroxyl bonding in alcohol, primary alcohol, and $\alpha$-configuration (Table 11) were correlated to those of pullulan structure. The typical pullulan at around $\lambda = 850$ cm$^{-1}$ indicates the $\alpha$-configuration (Yurlova and De Hoog, 1997). Madi et al. (1997) noted the $\alpha$-configuration at $\lambda = 856$ cm$^{-1}$ of EPS from *A. pullulans* de Bary Arnaud (IMI 145194).

The IR has been used to distinguish two EPS from *A. pullulans* namely pullulan and aubasidan (Yurlova and De Hoog, 1997). Pullulan has the $\alpha$-configuration peak at $\lambda = 850$ cm$^{-1}$ while for aubasidan $\beta$-configuration yields a peak

at $\lambda = 890$ cm$^{-1}$. Leal-Serrano et al. (1980) recorded an $\lambda = 890$ cm$^{-1}$ characteristic of β-configuration for a polysaccharide from *A. pullulans* de Bary Arnaud (105-22).

### 4.4 $^{13}$C-NMR Spectroscopy

$^{13}$C-NMR spectra of all EPSs (Figures 48-53) were very similar to those of the pullulan standard. Lack of other major signals indicated the absence of contaminating glucans. The spectra indicate the bond ratios of 2:1 for α-1,4 and , α-1, 6, and this correlates well to the general pullulan structure. Early studies of pullulan showed (1→4) and (1→6) linkages in a 2:1 molar ratio (Bernier, 1958). The more recent NMR data support this structure (Gorin, 1971), including the very recent analysis of pullulan from *A. pullulans* P-56 grown on beet molasses (Lazaridou et al., 2002).

### 4.5 Molecular weight analysis using High performance size exclusion chromatography (HPSEC) and viscosity measurement

The molecular weight and viscosity of EPSs during the production period decreased late in culture. Molecular weights and viscosities decreased from 547,000-2,450,000 Da (17.41-3.49 cP) to 10,200-167,000 Da (2.63-1.53) at day 7 (Tables 13 and 14). Smaller polymers were observed (Table 13) which could be the result of hydrolase action. Pullulan contains maltotetraose subunits (up to 5%) randomly located in the polymer (Leathers, 1993), and these units are susceptible to attack by alpha-amylase (Catley, 1970). Thus these enzymes have been proposed to be responsible for reduction of pullulan molecular mass in late culture (Catley, 1970; Leathers, 1993), and the falling of pullulan yield (Campbell et al, 2003).Thus I thought that alpha-amylase and pullulanase should be assessed in my *A. pullulans* isolates.

## 5. Enzyme assays

### 5.1 Alpha amylase screening

All *Aureobasidium* cultures were positive for alpha-amylase activity detected using starch agar plate assay (Figure 54). Several yeasts produce amylases including *Candida*, *Cryptococcus*, *Debaryomyces*, *Rhodotorula*, and *Trichosporon* (Linardi and Machado, 1990). *A. pullulans* strains specifically can produce alpha-amylase and glucoamylase (Linardi and Machado, 1990). Indeed all 198 strains of *A. pulluans* tested showed amylase activities on starch (0.2%) agar plates (Federici, 1982). Similarly, classical strains of *A. pullulans* - CBS 105.22, CBS 626.85, CBS 123.37, CBS 298.56, CBS 584.75, CBS 146.30 and CBS 701.76 - were all positive for amylase activity by starch agar plate assay with iodine staining (De Hoog and Yurlova, 1994), yet 12 strains of 46 *A. pullulans* strains tested showed starch-degrading activity when tested on starch (2%) agar plate assay (Buzzini and Martini, 2002). Thus the ability to produce amylases was strain dependent. In this thesis, all five tentative pullulan production isolates were positive on starch agar plates (Figure 54).

### 5.2 Alpha-amylase and pullulanase

All cultures exhibited both alpha-amylase and pullulanase activities when grown on starch (1%) or sucrose (1%) production media. The data (Tables 15 and 16) indicate that all strains constitutively produced alpha-amylase and pullulanase as the enzymes were produced in culture on sucrose. *Aureobasidium* strain NRRL Y-12974, constitutively produced alpha-amylase and pullulanase when grown on glucose besides producing them when induced on such substrates as corn starch, maltose, and soluble starch (Leathers, 1993). Amylases produced from *A. pullulans* are less active against pullulan than against starch (Leathers, 2002) which is similar to my results. It

would be best to have specific activities of the two enzymes to be able to make a definitive response.

Alpha-amylase or endoamylase (1,4 $\alpha$-D-glucan glucanohydrolase) hydrolyzes starch and related polysaccharides by randomly cleaving internal $\alpha$-1,4 glucosidic linkages. These enzymes are widely distributed in bacteria, fungi, plants and animals (Fogarty and Kelly, 1980). A few yeast are recognized as potential sources of alpha-amylases including *A. pullulans* (Fogarty and Kelly, 1980; Linardi and Machado, 1990), though of course the classical yeasts in beer production cannot attack starch and plant amylases hydrolyze the starch to glucose. However, *A. pullulans* NRRL Y-12974 is a distinctive yeast and can grow on amylopectin (0.46 $U.ml^{-1}$), amylose (0.29 $U.mi^{-1}$), soluble starch (0.37 $U.ml^{-1}$) and also sucrose (0.12 $U.ml^{-1}$) (Saha et al., 1993), and even glucose and maltose (Leathers, 1993). Yields reported were extremely low with the highest amylase activity (0.03 $U.ml^{-1}$) was observed on starch-grown cultures.

I also found alpha-amylase production by all strains tested in this thesis (Figure 54, Table 15)

*Aureobasidium* NRM2 produced the highest activities of alpha-amylase (0.738 $U.ml^{-1}$) from starch-grown cultures and 0.408 $U.ml^{-1}$ from sucrose-grown cultures (Table 15) which relatively higher than the previous reports which gave results of starch-grown cultures as 0.03 $U.ml^{-1}$(Leathers, 1993) or 0.37 $U.ml^{-1}$ and sucrose-grown cultures as 0.12 $U.ml^{-1}$ (Leathers, 1993; Saha et al., 1993).

Pullulanase hydrolyzes $\alpha$-1,6 glycosidic bonds in pullulan, and is produced by several specific bacteria including *Escherichia intermedia*, *Klebsiella pneumoniae*, and *Streptococcus mitis* and by plants for example rices (*Oryza sativa*) (Doman-Pytka and Bardowski, 2004). In assessment of pullulanase production, all strains produced

very low activity of pullulanase (Table 16) with the maximum of 0.40 U.ml$^{-1}$ by strain BK6 on starch. *Aureobasidium* NRRL Y-12974 cultures produced maximal activity of 0.39 U.ml$^{-1}$ when grown on corn starch (Leathers, 1993). This strain grew well ($A_{660}$ = 4.18) on pullulan (1%) and released pullulan-degrading enzymes (Saha et al., 1993).

### 5.3 Native gel electrophoresis of Alpha-amylase - zymograms

Native gel electrophoresis showed that all strains produced distinct alpha-amylases whether grown on starch or sucrose (Figure 55). When grown on starch, *A. pullulans* BK4, BK6 and NRM2 produced two bands of alpha-amylase activity while strains *A. pullulans* LB3 and SK3 only produced a single band (Figure 55). Only a single activity band was produced each culture was grown on sucrose, while confirming that all strains constitutively produced alpha-amylase. Strains LB3 and SK3 produced only one alpha-amylase when grown on sucrose or starch, while strains BK4, BK6 and NRM2 produced one alpha-amylase and two when grow on sucrose and starch, respectively, indicating that an extra alpha-amylase could be induced by growing cultures on starch-based medium. The induction of new (extra) alpha-amylase by starch confirmed previous reports (Leathers, 1993; Saha et al., 1993). Yet for *A. pullulans* strain NRRL Y-12,794, only single alpha-amylase enzyme was found following growth on soluble starch (Saha et al., 1993). Thus, the ability of alpha-amylase production would be strain dependent (Figure 55).

Unfortunately, using protein stain, no protein bands were detected at an equivalent position to the amylase band, even after enzyme preparation have been concentrated by ammonium sulfate precipitation. Presumably the amylases are of quite high specific activity in order to recognize only by their activity. In summary though classical saccharomyces species are amylase negative, *A. pullulans* is positive

and also produced amylases and pullulanases constitutively even when grown on sucrose. This is an impediment to producing higher molecular weight pullulan.

## 6. Enhancement of the molecular weight of EPS

Two approaches were considered for the enhancement of pullulan molecular weight:

1. The use of amylase-negative mutants.

2. Use of an amylase inhibitor incorporated in the production medium

6.1. Preparation of amylase-negative mutants using N-methyl-N'-nitro-N-nitrosoguanidine (NTG) for mutagenesis.

An amylase-negative mutant (ANM1) was prepared from a high-yielding pullulan strain *Aureobasidium* NRM2, by NTG mutagenesis (Figure 58). This mutant appeared not to grow on starch as it lacked amylase, it grew well on sucrose and produce EPSs. Unfortunately, degradation in late culture of EPSs from the mutant was still similar to that of the wild type (Figures 59 and 60). This is enigmatic. *A. pullulans* has more than one amylase and thus the mutagenesis appeared to knock out one of them, and one could survive that the lowering of the viscosity was due to a second cryptic enzyme. Leathers (1993) stated that amylase-negative mutant of *A. pullulans* has not been yet successfully isolated perhaps due to the multiple amylase genes in this organism.

6.2 Inhibition of amylase activity by incorporation of an amylase inhibitor (acarbose) into the EPS fermentation medium

A perfect alpha-amylase inhibitor was not aviable and hence acarbose was evaluated. Acarbose is produced by *Actinoplanes utahensis* - O-4,6-dideoxy-4-[[(1S,4R,5S,6S)-4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexen-1-yl] amino]-a -

D-glucopyranosyl-(1->4)-$O$-α -D-glucopyranosyl-(1->4)-D-glucose

(http://www.rxlist.com/cgi/generic/acarbose.htm). The action of acarbose results from

a competitive, reversible inhibition of alpha-amylase. Acarbose was normally used for

diabetic patients.

An alpha-amylase inhibitor acarbose was incorporated into an EPS production

medium. The degradation of the EPSs from strain NRM2 in the presence of acarbose

was less severe than those from strain NRM2 without acarbose (Figures 60 and 61).

However as degradation of EPSs molecular weight in late culture still occurred, all be

it to a lesser degree, the concentration of acarbose (0.05 % w/v) used could have been

too low to inhibit all alpha-amylases produced from *A. pullulans*.