CHAPTER I

INTRODUCTION

1.1 Rationale

The yeast-like fungus *Aureobasidium pullulans* is one of the so-called black yeasts, an anamorphic species complex associated with the order Dothideales. *A. pullulans* is difficult to identify due to its polymorphic forms ranging from blastic conidia and swollen cells to pseudohyphae, hyphae, and chlamydospores, depending on strain differences, age, media, and culture conditions (Cooke, 1959). *A. pullulans* is a ubiquitous and cosmopolitan saprophyte (Cooke, 1959; Domsch *et al.*, 1993) routinely found on leaves and various surfaces such as concrete, wood, painted walls, and indoor environments. In Thailand, *A. pullulans* was isolated as airborne spores (Punnapayak *et al.*, 2003), and from leaves, bathroom cement walls, and latex-painted surfaces (Prasongsuk *et al.*, 2005).

A. pullulans is well-known for producing the exopolysaccharide (EPS) pullulan. Pullulan is tasteless, odorless, non-toxic, water soluble, and forms oxygen-impermeable films (Leathers, 2003). Pullulan has been used for making biofilms and adhesives utilized in food, drug, and cosmetic industries. The major pullulan structure is a polymer of α-1,6- linked maltotriose subunits, or a linear glucan containing α-1,4 and α-1,6 linkages in a ratio of 2:1 (Sowa *et al.*, 1963). Pullulan also contains a minor structural feature which is a low percentage of α-1,6-linked maltotetraose subunits (Leathers, 2002). These maltotetraose subunits are distributed randomly, about 1% to 7% of total residues, throughout the pullulan molecule. It was reported that pullulan helps the fungus adhere to environmental habitats and also protects the cells from desiccation (Andrews *et al.*, 1994). In addition, *A. pullulans* produces a range of hydrolytic enzymes including amylases, proteases, esterases, pectinases, and hemicellulases, including xylanase and mannanase (Leathers, 2002).

In particular, "color variant" strains of *A. pullulans* are prominent producers of endoxylanase (EC 3.2.1.8) (Leathers *et al.*, 1984). Color variant strains, isolated to date only from tropical or subtropical zones, exhibit brilliant pigments of red, yellow, orange,

or purple instead of the off-white to black appearance of typically pigmented strains (Wickerham and Kurtzman, 1975). Strain NRRL Y-2311-1, a colorless derivative of red strain NRRL Y-2311, overproduces xylanase with a high specific activity (Leathers *et al.*, 1984; Leathers, 1986). Xylanases are important for bioconversions of hemicellulose to fermentable sugars (Saha, 2003).

Besides remarkable xylanase production, color variants also produce pullulan in yields comparable with those from typically pigmented strains (Leathers *et al.*, 1988). Pullulan production is a variable characteristic in both types of strains. In a comparative study, NRRL Y-12974 was a good producer of pullulan among color variant strains, while NRRL Y-6220, previously described as a pullulan producer, had the highest pullulan production among typically pigmented strains (Leathers *et al.*, 1988). More recently, Prasongsuk *et al.* (2007) studied pullulan production from tropical isolates of *A. pullulans*, identifying apparent color variant strain NRM2 as the best pullulan producer among 15 new isolates.

Concerning the classification of *A. pullulans*, morphological and nutritional characteristics are conventional methods to distinguish strains of *A. pullulans* from other similar yeasts (Dennis and Buhagiar, 1973). However, conventional taxonomy is limited and often can lead to misidentification (Valente *et al.*, 1999). In many cases, molecular taxonomy helps solving these problems through a phylogenetic approach.

In spite of the phenotypic diversity of this commercially important organism, a molecular phylogenetic taxonomy comparable to phenotypic analyses has not been reported. In this study, 5 loci (internal transcribed spacer, intergenic spacer 1, translation elongation factor-1 alpha, beta tubulin, and RNA polymerase II) were sequenced from new tropical isolates (Thailand) and comparative strains. Phenotypic characteristics were determined for all isolates, including colony characteristics, pullulan production, and xylanase activity in an attempt to identify specific characteristics of each clade.

The culture conditions and strains of *A. pullulans* play significant roles in pullulan production. Carbon source is the most important factor. Sucrose has often been described as the optimal substrate (Leathers, 2002). Other factors such as nitrogen

sources, minerals and vitamins, pH, temperature, and agitation rate are also important and variable in each strain. The mechanism of pullulan biosynthesis is still not clearly known. It is synthesized in the cells and secreted at different cellular stages depending on the strain. Catley (1980) concluded that the yeast-like cells are the pullulan producers. In contrast, Campbell *et al.* (2004) reported that only swollen cells and chlamydospores can produce pullulan. They also stated that pullulan production is mediated by nitrogen limitation. As *A. pullulans* can produce dark melanin in late culture, it often contaminates in the harvested pullulan. Mutant strains and reduced pigmentation wild-type strains have been isolated in attempt to reduce melanin levels (Leathers *et al.*, 1988 and Pollock *et al.*, 1992). Many studies have been focused on attempts to obtain a higher yield of high molecular weight (10^{5} to 10^{7} Dalton) (Leathers, 2002). Pullulan without pigment contamination would be desirable for industrial applications.

It has been reported that pullulan is susceptible to degradation by pullulanase (EC 3.2.1.41), α-amylase (EC 3.2.1.1), and glucoamylase (EC 3.2.1.3). The maltotetraose subunits in pullulan are substrates for α-amylase (Catley, 1970). *A. pullulans* produces α-amylase and secretes the enzyme at a low level in cultures *grown* on various non-starch media and at a slightly higher level on starch media (Saha *et al.*, 1993). Furthermore, *A. pullulans* has been reported to produce glucoamylase (Saha *et al.*, 1993) and low activity of pullulanase was reported (Prasongsuk *et al.*, 2007). These enzymes may be the cause of pullulan biodegradation in late cultures and their action may decrease the molecular weight of pullulan, which adversely affects its desirable properties. However, addition of amylase inhibitor to the culture medium had a slightly effect on the molecular weight of pullulan (Prasongsuk *et al.*, 2007). Therefore, further study of the relationship between α-amylase activity and the molecular weight of pullulan, including an analysis of α-amylase gene, is needed for a better understanding of the role of this enzyme in pullulan production.

The range of these studies is as follows. Tropical strains of *A. pullulans* were isolated from various habitats in Thailand. These isolates were classified based on multilocus phylogenetic analyses using concordance analysis of DNA sequences from

the rRNA ITS region, the rRNA IGS1 region, *EF-1α*, *BT2*, and *RPB2*. Morphological characteristics, pullulan production, and xylanase activity were also determined for all isolates in an attempt to identify specific characteristics of each clade. Moreover, representative strains were selected to study the relationship between the activities of α-amylase and pullulan profiles during cultivation. Using different pullulan production media, the α-amylase and pullulanase activities were assayed. Pullulan profiles, including α-amylase sensitivity, pullulanase sensitivity, molecular weight, and viscosity were determined and compared with the α-amylase activity. Furthermore, the putative α-amylase gene was characterized and the copy number of the gene was analyzed using Southern blot hybridization. Finally, transcription of α-amylase mRNA during cultivation was detected using reverse transcription-PCR.

1.2 Objectives of this study

1. To characterize the Thai isolates of *A. pullulans* with multilocus sequence analysis
2. To study the relationship between α-amylase activity and pullulan profiles

1.3 Key words

Aureobasidium pullulans, Multigene phylogeny, Pullulan, α-amylase, Pullulanase, Xylanase

1.4 Anticipated benefits

1. Tropical strains of *A. pullulans* isolated from Thailand will be differentiated and classified.
2. The complete α-amylase gene of *A. pullulans* will be characterized.