CHAPTER V

DISCUSSION AND CONCLUSION

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

5.1.1 *Aureobasidium* isolates

Even though samples from different kinds of habitat such as soil, airborne, water, painted wall, wood, small plants including grass, weeds, crops, and vegetables were collected and screened in attempt to obtain various *A. pullulans* isolates (data not shown), most isolates of *A. pullulans* in this study were collected from leaves (43 isolates), indicating that *A. pullulans* was commonly found on leaves as an epiphyte. All hosts in this study were perennial plants, particularly mango, consistent with the report by Jager *et al.* (2001) that *Aureobasidium* sp. was one of the prevalent yeasts on the mango phylloplane. In this study, there were no significant correlations between host plant species and phylogenetic groupings in each clade. Interestingly, both *A. pullulans* isolates in clade 3 were evidently found in bathrooms. However, it might be too early to draw a conclusion on this finding since only a few isolates were included in this study. Moreover, there were no apparent correlations between the geography of different collection sites and the phylogeny of individual clades.

5.1.2 Phylogenetic analyses

An extensive literature exists on *A. pullulans*, emphasizing its morphological traits and production of polysaccharides and enzymes (Leathers, 2002). Overall, this literature suggests a great deal of phenotypic strain variability. *A. pullulans* has been classified using a number of molecular techniques (Chapter 2, 2.1.4). These reports proposed various groupings of strains. Since molecular taxonomy relies on phylogenetic trees, it would be more precise to classify this fungus using multilocus sequence analysis than other methods.

Regarding phylogenetic analyses, the most desirable outgroup species would be a member of the sister group to the ingroup. *A. pullulans* is a member of the

Dothideales, but other members of that clade were not available from ARS culture collection for use as an outgroup in this study. Some DNA sequences, but not for all 5 loci, are available from GenBank for *Dothidia insculpta,* a species closely related to *A. pullulans* (Schoch *et al.*, 2006) that are homologous to the *EF-1α* (DQ471081), *RPB2* (DQ247792) and ITS regions (AF027764), and in analyses of those loci (data not shown) substitution of *D. insculpta* for *A. nidulans* as the outgroup species did not affect the topology of the trees. Therefore *A. nidulans* was used as the outgroup species.

The ITS region seems to be useful to distinguish *A. pullulans* from other species, and it was previously used to distinguish species in the order *Dothideales* (de Hoog *et al.*, 1999). The ITS region is useless when it comes to subspecies differentiation. Other loci tested were found to be more informative for classification of *A. pullulans* strains into distinct clades. As described in Chapter 2 (2.1.4), the IGS1 was used because it is the most variable region of rDNA. The *BT2*, *RPB2*, and *EF-1α* loci were often used to differentiate fungi in the phylum *Ascomycota* (Hibbett *et al.*, 2007).

Concordance analysis of the DNA sequence data identified 11 genetically isolated groups among the *A. pullulans* isolates studied. Physiological and genetic information are largely concordant (Table 4.3). Under phylogenetic species theory (Taylor *et al.*, 2000) these groups could be formalized as species. However, reclassification is beyond the scope of this study. Furthermore, Isolate CU 9 occurred in clade 1 (IGS1 tree) or clade 2 (RPB2 tree). This suggested either that these two clades were not genetically isolated or that lineage sorting between the clades was incomplete.

Clades 1 through 11 appear in the multilocus tree as descendants of sequences from an *ex-neotype* isolate of *A. pullulans*. Under the requirements of monophyly these clades can rightly be placed in a broad concept species of *A. pullulans*, or perhaps a species complex. On the other hand, isolates CU 26 and CU 30 form a distinct clade that is not part of the monophyletic *A. pullulans* and as such is viewed as a distinct species. The bootstrap and posterior probabilities supporting the clade 12 branch in each of the trees is very high. As mentioned earlier, the polysaccharide formed by these two isolates is not pullulan. Isolates CU 26 and CU 30 both have a ca 500 nt insert in the 18S region amplified with primers ITS5 and ITS4, an insert found in no other isolates from this study. GenBank searches found no homology to these inserts, but

perhaps they are group I introns as have been found in 18S rDNA studies of other Ascomycetes (e.g., Adams *et al.*, 2002).

In this study, the data identified 11 clades from 51 isolates of *A. pullulans* (Table 4.4). *A. pullulans* has been divided into 3 varieties as described in Chapter 2 (2.1.1). Recently, Zalar *et al.* (2008) reported 4 variety groups of *A. pullulans* using multilocus molecular analysis of 4 loci, ITS and partial 28 S rDNA, β-tubulin (*TUB*), translation elongation factor (*EF1α*), and elongase (*ELO*). Using the same primers (Bt2a and Bt2b) of β-tubulin, the sequences of this locus were aligned for comparison with the sequences in this study (courtesy of S.W. Peterson, USDA, Peoria, IL, USA, data not shown). The tree based on the β-tubulin locus showed that *A. pullulans* var. *pullulans*, *A. pullulan* var. *subglaciale*, and *A. pullulans* var. *namibiae* were located out of all clades in this study. However, strains of *A. pullulans* var. *melanogenum* were spread all over the tree, including clades 3, 4, 5, 7, and 10. Furthermore, the geography of this variety was diverse, such as from the deep sea in Japan, Thailand, and ice in Norway. This study has strongly supported clades that do not correspond to the groups of Zalar *et al.* (2008) and are not represented in their study. In addition, sequences of 4 loci showed that *A. pullulans* var. *aubasidani* is in the same group of *A. pullulans* var. *pullulans*. This suggests that the taxonomic status of this variety should be revised.

5.1.3 Phenotypic analyses

Most isolates in this study were collected from Thailand, therefore, the growth condition was obtained from the previous studies of Prasongsuk *et al.* (2007). The pullulan yield was determined using the optimal condition of strain NRM2, the best pullulan producer, as a standard pullulan production medium (containing 5.0% sucrose and 0.1% N-sources) initial pH 6.5 and cultures were grown at 28°C, 150 rpm, for 7 days.

Since *A. pullulans* is well known for production of pullulan and many enzymes, especially, xylanases which have potential for commercials of biofuels and chemicals, phenotypic analyses should be covered to the applications of this fungus besides classical morphology. Isolates within each of the 11 clades shared many similarities in morphology, pullulan yield, and xylanase activity. Most clades showed low levels of

pullulan yields and/or low xylanase activities. In particular, isolates in clade 1 produced high levels of pullulan without melanin contamination but low xylanase activity, whereas isolates in clade 8 produced high xylanase activity but low pullulan yield. Interestingly, clade 10, includes only strain NRRL Y-12974, produced both high pullulan yield and high xylanase activity. However, there was no relationship between the level of pullulan production and xylanase activity in each isolate (data not shown). This information could be very useful for developing detection kit or analysis system, especially using IGS1, *RPB2* and *BT2* sequences, to screen for new *A. pullulans* strains with high yield and colorless pullulan or high xylanase activity from the environment.

According to the description of color variants of *A. pullulans* by Wickerham and Kurtzman (1975) that they exhibit brilliant pigments of red, yellow, orange, or purple instead of the off-white to black appearance of typically pigmented strains. In this study, it appear that there was a great diversity among the color variants since there were separated into at least 3 clades. Clade 5 exhibited purple pigment on both YMA and MEA. Clade 8, which includes reference color variant strain NRRL Y-2311-1, showed color rings on YMA and yellow pigment on MEA. The strain NRRL Y-2311-1 was colorless derivative of strain NRRL Y-2311 (Wickerham and Kurtzman, 1975) which pigments showed on a colony as many sectors. They reported that pigment formation in color variant strains was affected by pH and temperature. In this study, colonies exhibited color rings when the culture plate was left outside the incubator and placed near the window. Therefore, it can be concluded that the light also had an effect on pigment production. The dark pigment was produced when the colony was grown in the dark while no dark pigment was produced when the colony was grown in light. This indicated that light may inhibit the synthetic pathway of this pigment. Moreover, the color rings were only found in clade 8, therefore the light may play a role in gene regulation of pigment biosynthesis unique to this clade. Finally, clade 10 includes only strain NRRL Y-12974, which was previously classified as a color variant (Leathers, 1986).

Color variant strains of *A. pullulans* have been reported to overproduce xylanase (Leathers *et al.*, 1984; Leathers, 1986). In this study, almost all color variant isolates, especially in clade 8, produced high xylanase activity. However, an exception is strain

CU 36 in clade 5, which expressed low xylanase. In addition, it may be noted that strain CU 38 in clade 8 did not exhibit the color rings characteristic of its clade, although it did exhibit high xylanase production. To date, color variant strains have been isolated only from tropical or subtropical latitudes (Wickerham and Kurtzman 1975; Leathers, 1986). However, it cannot be concluded for certain that color variants do not exist in temperate zones since only a small number of studies have been reported. In this study, 9 of 45 new isolates from Thailand, which is entirely tropical, were apparent color variants, and they were grouped separately in clades 5 and 8. Results from this study also suggested that Thailand is an apparent source of genetically diverse strains of *A. pullulans,* especially the color variant strains.

An orange-red pigment was observed in the supernatants of some isolates after polysaccharides were precipitated with ethyl alcohol (CU 9, CU 22, and isolates in clade 5, including CU 6, CU 19, and CU 36). Isolates in clade 5 (CU 6, CU 19, and CU 36) produced a purple-red pigment in culture media whereas isolate CU 9 produced orange-pink cultures in PM, and some of this pigment remained in the aqueous culture supernatant. However, it was surprising that isolate CU 22 produced an orange-red pigment, since cultures were light olivaceous in PM. The nature of this pigment is unknown.

It is not unusual to observe changes in the colonial morphology of *A. pullulans*, particularly between initial isolations and subsequent "domesticated" laboratory cultivation. Pigmented colonies often show sectoring, as it was previously reported that the less pigmented strain NRRL Y-2311-1 was a spontaneous derivative of color variant strain NRRL Y-2311 (Wickerham and Kurtzman, 1975). As noted above, strains in clade 9 were black on initial isolation but produced less melanin over time. On initial isolation, isolate CU 9 produced cream-colored cultures in liquid PM. Later, the strain changed to produce orange-pink cultures with an orange-red color in aqueous supernatants. Similarly, some isolates in clade 9 (CU 5, CU 7, CU 22, and CU41) initially produced a dark olivaceous culture in PM, which later became light olivaceous with the concomitant production of a heavy oil. This phenomenon of altered pigmentation associated with heavy oil production was also observed by Kurosawa *et al.* (1994). The extracellular lipid was found to be produced from a light color mutant of *A. pullulans* and the

structure of extracellular lipid was proven to include a mixture of mannitol or arabitol and massoilactone (Kurosawa *et al.*, 1994). Several isolates also appeared to have genetic variations in their levels of pullulan production which may relate to heavy oil production. Some isolates produced lower pullulan yields than in preliminary studies (data not shown). Unlike the report of Kurosawa *et al.* (1994), some isolates in this study were observed that could produce high levels of bright yellow lipid, the structure of which would be interesting and beneficial to be analyzed further.

It is conceivable that unstable traits are related to extrachromosomal DNA elements. As noted, an unidentified 4 kb element was found in DNA from strain CU 9. Linear mitochondrial plasmids are present in many fungi, and cytoplasmic plasmids have been reported from some yeasts and a filamentous fungi (*Alternaria alternata*) (Jabaji-Hare *et al.*, 1994). Linear plasmid DNA may be associated with the killer phenotype, e.g., in *Kluyveromyces lactis* (Fukuhara, 1995). For these reasons, the extrachromosomal DNA in CU 9 should be further characterized.

Analysis of xylanase activity on SDS-PAGE showed visible bands of about 20 kD. However, many isolates exhibited bands of about 21 kD. These two patterns were described by Leathers (1986) as monomeric proteins possibly resulting from differential glycosylation during xylanase production. Moreover, isolates in clade 4 produced a slightly smaller xylanase (about 19 kD), and also isolates in clade 5 showed an additional band with high molecular weight (about 250 kD). This indicates that different isolates have variation in characteristic protein patterns.

In conclusion, 53 isolates, including 46 new tropical isolates, were determined to belong to 12 distinct clades by multilocus phylogenetic analyses. Two new isolates were identified as a distinct species closely related to *A. pullulans* that did not produce pullulan. Specific clades were associated with high pullulan yields, low melanin contamination, or high xylanase activities. Some clades included the so-called "color variant" strains of *A. pullulans*.

This study will be beneficial for the taxonomic revision of this fungus and could be used as a guideline for the identification and selection of new commercial strains producing high amounts of pullulan and xylanase. Moreover, *A. pullulans* produces numerous valuable bioproducts, including a broad range of enzymes, antibiotics,

gluconic acids, polymalic acid, heavy oil, siderphores, and non-pullulan exopolysaccharides. It will be very useful to study such other phenotypic characters in each clade. Moreover, it has been suggested that sequencing the whole genome of *A. pullulans* would facilitate an understanding of bioproducts formation and lead to the discovery of yet more bioproducts and novel genes (Chi *et al.*, 2009). A phylogeny of the species will inform the selection of representative strains for genome analyses.

5.2 Relationship between α-amylase activity and pullulan profiles

5.2.1 Growth and extracellular polysaccharide production

Bars in Figure 4.15 – 4.27 represented standard error. Standard error was considered more appropriate than standard deviation because of the small sample size.

As detailed in Chapter 2, it has long been proposed that α-amylase negatively affects the molecular weight of pullulan in late cultures, by attacking rare maltotetraose residues (Cately, 1970). However, this hypothesis has not been clearly proven. Levels of α-amylase have been reported to be low in pullulan-producing cultures, and not clearly correlated with pullulan molecular weight (Leathers, 1987; 1993; Saha *et al.*, 1993; Prasongsuk *et al.*, 2007). Furthermore, it has been suggested that glucoamylases (EC 3.2.1.3) or other unknown amylolytic enzymes may be involved in the degradation of pullulan (Saha *et al,* 1993; West and Strophus, 1996). In addition, thus far it has proven difficult to obtain amylase mutants of *A. pullulans* (unpublished observations by T. D. Leathers, USDA, Peoria, IL, USA). To further test the relationship between α-amylase and pullulan molecular weight, five strains of *A. pullulans* were selected from different clades based on different colors of pigment in PM (CU 3, CU 20, and CU 36) and two comparative strains (NRRL Y-12974 and NRM2). They were grown in standard pullulan production medium over an eight-day period and both enzyme activities and pullulan profiles were determined during the cultivation. Moreover, NRRL Y-12974 was selected to be grown in modified PM to study the effect of carbon and nitrogen sources on α-amylase activity and pullulan profiles because this strain was previously studied and could serve as a reference strain (Leathers *et al.*, 1988; Leathers, 1993; Leathers and Gupta, 1994; Saha *et al.*, 1993).

Some previous studies have suggested that pullulan production takes place mainly in stationary growth phase (McNeil and Kristiansen, 1987, 1990), while other studies indicated that pullulan production is associated with active growth (Klimek and Ollis, 1980). Under conditions tested here, the five representative strains accumulated EPS during growth (measured as OD_{600}) on standard pullulan production medium (containing 5.0% sucrose and 0.1% nitrogen). Strain NRRL Y-12974, the best pullulan producer, continued to accumulate EPS from day 4 – 8 while OD_{600} values declined. Thus, in most cases pullulan production appears to be growth associated. In the exceptional case of strain NRRL Y-12974, this may still be the case. The drop in OD_{600} may be associated with morphogenesis to, e.g., a more mycelial form, rather than cell lysis. It may be noted that pullulan yields were somewhat different from those obtained in the preceding phylogenetic section, possibly because culture conditions were slightly different, i.e., 80 ml of medium in 250-ml flasks instead of 100 ml in 300-ml flasks. The difference in aeration might affect cell growth and pullulan production.

When strain NRRL Y-12974 was grown in a modified PM containing 5.0% sucrose and 0.3% nitrogen, the drop in OD_{600} was eliminated, suggesting that the additional nitrogen supported continued cell growth (i.e. yeast-like cells). At the same time, the EPS yield from medium with 0.3% nitrogen (C:N = 50:3) was lower than from medium with 0.1% nitrogen (C:N = 50:1), presumably because the yeast cells used more carbon source for growth and less to produce the EPS.

Strain NRRL Y-12974 was also grown in modified PM containing 5.0% starch and either 0.1% or 0.3% nitrogen. Although the strain grew well in starch media, total extracellular EPS was initially high but later fell during cultivation. The high yield at earlier stage might be caused by the starch in the medium that was precipitated by ethyl alcohol, along with any newly synthesized pullulan. Based on C/N ratio, total EPS fell more rapidly in starch medium containing 0.3% nitrogen, suggesting that the additional nitrogen supported more rapid utilization of starch and/or reduced production of pullulan.

The pH of all culture supernatants gradually fell during cultivation, possibly because of the production of such acids as gluconic acid (Anastassiadis *et al.*, 2003; 2005) polymalic acid (Liu and Steinbuchel, 1996; Nakajima-Kambe *et al.*, 1996), and

uronic acid (Pouliot *et al.*, 2005). In standard PM, the pH of cultures of NRRL Y-12974 fell to pH 4.2, while cultures in modified PM media dropped only to about 5.5. This indicated that different carbon and nitrogen sources might affect the amount of acid produced.

5.2.2 Characteristics of extracellular polysaccharides

NMR spectra of EPS from all five strains grown in standard PM were similar to the spectrum of pullulan standards (See Appendix F). However, the NMR spectra of EPS from 8 day starch-grown cultures of strain NRRL Y-12974 suggested a mixture of pullulan and residual starch (see Appendix F). This was found even in medium containing 0.3% nitrogen sources, which supported better growth than medium containing 0.1% nitrogen. It could be concluded that the strain was unable to completely utilize starch under the conditions tested, and that residual starch precipitated with ethyl alcohol in the EPS analysis.

A further measure of pullulan authenticity and purity is the sensitivity of the EPS to pullulanase and amylase. EPS from sucrose-grown cultures exhibited pullulanase sensitivities ranging from 41% to 92% of commercial pullulan standards, with EPS from strain NRRL Y-12974 showing the highest sensitivity. This result indicated that all strains produced authentic pullulan, and establishes minimal levels of pullulan purity. Partial sensitivity to pullulanase might indicate the presence of non-pullulan polysaccharides or other material precipitated by ethyl alcohol, or may reflect the presence of pullulanase inhibitors, possibly including fungal melanin. By this measure, 2 days starch-grown cultures contained a minimum of only 13-16% pullulan. The amylase sensitivity assay measures total reducing sugar and is thus primarily a measure of starch contamination. This assay confirmed the presence of residual starch in starch-grown cultures.

As reviewed in Chapter 2, pullulan molecular weight varies depending on strain characteristics and culture conditions. Furthermore, molecular weight generally falls with culture time, generally believed to be the result of hydrolytic enzymes in the culture supernatant. Consistent with these observations, the five representative strains produced EPS on standard PM that varied in initial molecular weight and generally fell to lower molecular weight forms during cultivation. Isolate CU 20 from clade 1, which

makes relatively high levels of pullulan with low melanin contamination, exhibited the highest initial molecular weight pullulan (day 2). Strain NRRL Y-12974, which produced the highest yields of pullulan, produced the lowest initial molecular weight product. Interestingly, EPS from isolate CU 3 appeared to suffer only a partial loss in molecular weight, and will be further discussed below. Pullulan from strain NRRL Y-12974 grown in medium containing 5.0% sucrose and 0.3% nitrogen showed an enhanced initial molecular weight, demonstrating that culture conditions can have a dramatic effect on molecular weight. EPS from starch-grown cultures of NRRL Y-12974 were of extremely low molecular weight.

The solution viscosities of the polysaccharides are expected to fall as a function of molecular weight, and this was observed with two notable exceptions. The solution viscosity of EPS from isolate CU 3 gradually increased over time while the solution viscosity of EPS from isolate CU 36 increased dramatically. The reason for this increase in viscosity is unclear. It is possible that these isolates produce polysaccharides other than pullulan, particularly in late cultures, although this is not evident from NMR spectra. Strain CU 36 is a member of clade 5, and it should be noted that isolates CU 6 and CU 19 in clade 5 also made highly viscous EPS. In addition, the pullulanase sensitivity test of CU 36 showed that the purity of pullulan was only 41%. As reviewed in Chapter 2 (2.1.5), *A. pullulans* has been reported to produce a variety of polysaccharides besides pullulans.

In some cases there seems to be an inverse relationship between pullulan yields and molecular weights (Leathers, 1987; Pollock *et al.*, 1992; Audet *et al.*, 1996). The molecular weight of pullulan depends on the specific strain (Pollock *et al.*, 1992; Thorne *et al.*, 1993, 2000), and culture conditions used (Wiley *et al.*, 1993; Gibson and Coughlin, 2002; Seo *et al.*, 2004). From these reports, it is possible that not only endoglucanases can affect the reduction of molecular weight but also variation of strains and conditions. The biosynthesis of pullulan depends on many enzymes (as described in chapter 2, 2.2.4). Different strains may produce different levels of enzyme activities. Furthermore, culture conditions such as pH, carbon sources and nitrogen sources may affect enzyme production and function. Since it has been reported that pH often dropped during cultivation (Bermejo *et al.*, 1981a, 1981b, Tsujisaka and

Mitsuhashi, 1993; Wu *et al.*, 2009), this may be one of possible causes of inconsistent pullulan synthesis, and low pullulan molecular weight. Under certain conditions, cells may produce other biopolymers such as β-glucan, insoluble heteropolysaccharides, or heavy oil, instead of pullulan. Alternatively, cells may continue to produce pullulan but at lower molecular weights. This may relate to the inverse relationship between pullulan yields and molecular weight. For example, strain NRRL Y-12974 produced high yields of lower molecular weight pullulan under nitrogen limiting conditions (0.1%), but under higher nitrogen conditions (0.3%), it produced lower yields of higher molecular weight pullulan. Perhaps the strain continues to produce pullulan during growth limitation, but it is of lower molecular weight. It is even possible that this phenomenon accounts for the drop in pullulan molecular weight over time in late cultures.

According to Madi *et al.* (1997), strain IMI 145195 produced two distinct EPS fractions that differed only in molecular weight ($2x10^6$ and $4.3x10^5$). In this study, two distinct ethanol precipitates were also observed in some isolates (see Appendix H). Moreover, sensitivity to pullulanase of each EPS fraction showed other contaminating components. This indicated that total EPS was composed of both pullulan and non-pullulan.

From this hypothesis, further studies about the relationship between the molecular weight of pullulan and its biosynthesis should be considered. The pullulanase sensitivity of EPS should be tested over time (such as day 2, 4, 6, and 8). Viscosity and molecular weight should be measured from diluted culture broths. Diffusion-ordered NMR spectroscopy (DOSY) can be used to estimate the molecular weight of pullulan specifically in diluted solution. Furthermore, this technique can prevent problems with insoluble EPS after freeze drying and help save time. Activities of enzymes involved in pullulan synthesis such as UDPG-pyrophosphorylase, phosphoglucomutase, and glucosyltransferase and UDP-glucose level should be determined during cultivation. This proposed study may lead to a better understanding of the production and molecular weight of pullulan.

In conclusion, all five representative strains produced EPS that included authentic pullulan in standard PM medium, and the molecular weight of this material

generally fell over time. Starch-grown culture supernatants contained EPS of extremely low molecular weight, however this material was primarily residual starch.

5.2.3 Production of α-amylase

In preliminary experiments, it was observed that culture supernatants exhibited high reducing backgrounds that hampered enzyme assays based on the production of reducing sugar equivalents. To circumvent this problem, α-amylase and pullulanase activities 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.

The result of the α-amylase screening on starch agar plates showed that all five strains could produce α-amylase. By this test, strains NRRL Y-12974 and CU 20 seemed to produce more amylase than the other strains. However, quantitative assays of α-amylase of all five representative strains grown on standard pullulan production medium showed no detectable activity. More accurately, any α-amylase activity was below the limits of detection of the assay (<0.02 U/ml). Starch-grown cultures of NRRL Y-12974 contained very low but detectable levels of α-amylase (<0.04 U/ml).

The α-amylase activity gel (zymogram) confirmed that the activities of α-amylase from these cultures were too low to be detected. Furthermore, extracellular protein levels were too low to be detected by Coomassie Blue staining. Interestingly, the band of standard α-amylase on 10% SDS-PAGE containing 0.5% starch appeared to be of slightly higher molecular weight than on the same gel without starch. This might be due to binding between starch and the enzyme which affected its migration on the gel.

In conclusion, α-amylase activities were below the limits of detection in cultures that showed a dramatic drop in the molecular weight of pullulan. Starch-grown cultures had low levels of α-amylase and low molecular weight EPS that was perhaps primarily starch. Concerning the hypothesis that α-amylase is responsible for the reduction in pullulan molecular weight during cultivation, these results neither supported nor refuted the hypothesis. It is conceivable that even at extreme low levels, amylase in standard PM was responsible for the reduction in pullulan molecular weight. The early studies of Catley *et al.* (1970) indicated that pullulan could be attacked by α-amylase, and these

studies indicated that the organism could produce this enzyme under the certain conditions. The reduction in pullulan molecular weight did appear to be enzymatic. In control experiments, purified EPS of the isolate CU 20 obtained at day 2 was resuspended in buffer at low pH and agitated for 8 days. The sample suffered no loss of molecular weight or viscosity in the absence of enzyme (data not shown). When either bacterial α-amylase or glucoamylase was added to these controls, the EPS showed a slight reduction in molecular weight and viscosity over 8 days. This confirms that pullulan is susceptible to these enzymes, however the reduction in molecular weight was not nearly as great as that observed in vivo. It is possible that the corresponding enzymes from *A. pullulans* have a higher specificity for pullulan. It is also possible that other, as yet uncharacterized, enzymes are involved in the degradation of pullulan in late cultures.

Thus, further studies are needed to determine whether α-amylase is important in the reduction of pullulan molecular weight. To facilitate such studies, the α-amylase from *A. pullulans* was cloned and characterized as described below.

5.2.4 α-amylase gene analyses

The putative α-amylase gene of *A. pullulans* was cloned and sequenced for the first time. The gene from gDNA was 2,247-bp long with 7 introns and 8 exons. The mRNA was 1,878-bp long encoding 625 amino acid residues. From amino acid sequence alignment, *A. pullulans* α-amylase was similar to those of *Neosartorya fischeri, Aspergillus* sp. and *Penicillium* sp. Theses are fungi in the phylum Ascomycota, class Eurotiomycetes which is closely related to the class Dothideomycetes of *A. pullulans* (Hibbett *et al.*, 2007). Southern Blot analysis indicated that this gene is present as a single copy in the *A. pullulans* genome. This simplifies analysis of gene expression, since mRNA should be the product of a single gene. This also suggests that it should be possible to genetically engineer *A. pullulans* for improved pullulan production by deleting or modifying this gene.

Since the amount of total RNA was rather low (in total volume of about 48 µl, from NRRL Y-12974 cultured in sucrose PM were 37.12 ng/µl, 46.98 ng/µl, 82.48 ng/µl, and 84.55 ng/µl for day 2, 4, 6, and 8, respectively, from NRRL Y-12974 cultured in

starch PM were 139.94 ng/µl, 150.17 ng/µl, 206.35 ng/µl, 171.12 ng/µl for day 2, 4, 6, and 8, respectively), reverse transcription (RT)-PCR was performed instead of a Northern blot analysis. The experiment revealed that the α-amylase gene was transcribed during cultivation in both starch and sucrose PM. Although RT-PCR is semi-quantitative, the PCR products from cells grown in starch PM showed higher concentrations than those from sucrose PM, suggesting transcriptional induction by starch. However, there were no PCR products from the culture at day 2. This indicated that the gene expression began after day 2. In contrast, enzyme assays showed very low activities at days 2-8 in starch-grown cultures, and no detectable activity in sucrose-grown cultures. There are various possibilities for this result. The enzyme assay is sensitive but not highly specific, and may detect activities other than α-amylase. This might explain the appearance of activity in day 2 starch cultures. Conversely, transcribed mRNA may or may not be efficiently translated under the culture conditions used. This could explain the appearance of α-amylase mRNA in sucrose-grown cultures. On the other hand, the α-amylase gene may be constitutively expressed in sucrose medium and could be induced to a higher level of expression in starch medium.

The 26S rDNA was used as an internal control of the RT-PCR reaction. The result showed that although amylase PCR products could not be amplified from the first strand cDNA at day 2, the 26S rDNA was amplified from all cDNA templates in the same amount. This indicated that the first strand cDNA was of good quality and same quantity. Therefore, difference between concentrations of PCR products from cells grown in starch PM and sucrose PM were from difference of gene expression level. However, 26S rDNA was also amplified to a lesser extent from DNase I-treated RNA. This suggests that some contaminating 26S rDNA remained in these preparations after DNase I treatment even though these preparations showed no α-amylase contamination, probably because the levels of 26S RNA are so much greater in the cell.

The α-amylase gene sequence of *A. pullulans* will be useful for future studies on expression of this gene and characterization of its enzyme. The α-amylase cDNA could be cloned in an expression vector and transformed to an appropriate host to provide higher quantities of the enzyme. This would be useful to study the structure and properties of the α-amylase enzyme and determine its activity towards pullulan. The

cloned gene could also be used in genetic engineering studies to eliminate or modify α-amylase expression in *A. pullulans*.

In conclusion, the results obtained from this study contribute to a better understanding of pullulan production which can be applied to strain improvements in the future.