CHAPTER VI

CONCLUSION

A. pullulans was successfully isolated from distinct habitats and locations in Thailand. This furthers our knowledge of the occurrence of this fungus in tropical climates. The *A. pullulans* isolates were from distinctive and diverse sites: from leaves to painted surfaces. On the basis of morphology, nutritional physiology, ribosomal DNA ITS sequencing, and the types of EPS, all isolates were identified as *A. pullulans var. pullulans*. Isolates included typical black colonies and color variants. Although *A. pullulans* is ubiquitous, color variant strains have thus far only been isolated from tropical and subtropical sites. Since this fungus is polymorphic, morphological criteria alone are not sufficient for identification; however, the molecular technique (ITS sequencing) employed, though giving a firm genetic base to the identification, did not resolve the various strains. I look forward to when fuller analyses using further molecular biological approaches, will result in definitive identification even at the strain/morphological level. Nutritional physiology and the type of EPS were helpful parameters in the characterization of the isolates.

Fourteen *A. pullulans* strains were isolated, and all found to produce exopolysaccharides (EPSs). It was significant that the five higher-yielding strains (BK4, BK6, LB3, NRM2, and SK3) which were from different locations in Thailand. For EPS production, all isolates were more productive with sucrose compared to glucose.

The production of the EPSs was dependent on the nitrogen source and differed between strains. $(NH_4)_2SO_4$ resulted in higher yields from strains BK4, BK6, and

SK3, while peptone was optimal nitrogen source for strains NRM2 and LB3. Under the optimal conditions (sucrose and peptone), the maximum EPS yield of 25.1 $g.l^{-1}$ was obtained from strain NRM2 after 7 days. The pH decreased from 6.5 to around 3 within 24 hr and remained acidic for 7 days. Only blastospores and hyphae were responsible for EPS production (Table 9). Blastospores were the most common morphotype of all cultures when grown in the production medium. Strain SK3 also produced a fair amount of hyphae (50%) observed only after 5 days.

All EPSs showed to be pullulan through a series of tests including anthrone carbohydrate analysis, pullulanase sensitivity test, infrared analysis, and ^{13}C-NMR spectroscopy. The higher-molecular weight EPSs were from strain BK6 (2,450,000 Da) and NRM2 (1,770,000 Da) after 3 days culture. The molecular weight of EPSs from all cultures decreased in late culture presumably as a result of *A. pullulans* producing extracellular hydrolytic enzymes such as alpha-amylase and pullulanase. In assays for alpha-amylase and pullulanase, all strains of *A. pullulans* positive in both solid and liquid starch-based medium. Culture on sucrose still resulted in constitutive synthesis of alpha-amylase and pullulanase.

In order to obtain high-molecular weight pullulan, two approaches were considered. First was the preparation of an amylase-negative mutant. Unfortunately, degradation of late culture EPSs from the mutant was still similar to that of the wild type.

In another approach, an amylase inhibitor (acarbose) was incorporated the culture medium. The EPSs from strain investigated (strain NRM2) in late culture were of higher molecular weight if cultured in the presence of acarbose. As the basis of this experiment was used of an amylase inhibitor, it was suggested that in the routine

reduction in the molecular weight of EPS, alpha-amylase would be in part responsible.

A. pullulans was found to diverse habitats in Thailand, there being considerable differences between strains. All isolates produced pullulan. However it appeared that *A. pullulans* produced amylases constitutively and even if cultured on sucrose, the molecular weights of the EPSs were gradually reduced by enzyme action. An initial attempt to prevent this enzymatic degradation by use of a specific amylase inhibitor, showed an incipiently positive result.