CHAPTER II

LITERATURE REVIEW

2.1 *Aureobasidium pullulans*

2.1.1 Taxonomy

The genus *Aureobasidium* commonly is referred to as a yeast-like fungus and often is considered to be one of many genera of black yeasts because many cultures produce fungal melanin. Fourteen species were classified using the identification key (Hermanides-Nijhof, 1977), the most well-known being *Aureobasidium pullulans*.

Aureobasidium pullulans (de Bary) Arnaud was first known as *Dematium pullulans* by de Bary in 1866 (Cooke, 1959) and was subsequently named *Pullularia pullulans* (de Bary) Berkhout, *Hormonema pullulans* (de Bary) Robak, *Aureobasidium vitis* Viala and Boyer and *Pullularia fermentans* Wynne and Gott (Domsch *et al.*, 1993).

A. pullulans genetically has been considered to be an imperfect fungus among the Fungi Imperfecti or Deuteromycetes (Cooke, 1962; Hermanides-Nijhof, 1977). *Aureobasidium* has been described as a filamentous ascomycete classified in phylum Ascomycota, class Euascomycetes, order Dothideales, family Dothideaceae (de Hoog and Yurlova, 1994; de Hoog, 1998). Recently, *A. pullulans* has been classified in class Dothideomycetes, subclass Dothideomycetidae (Hibbett *et al.*, 2007; Schoch *et al.*, 2006).

Wickerham and Kurtzman (1975) reported color variant strains, isolated from tropical and subtropical zones, that exhibit brilliant pigments of red, yellow, orange, or purple instead of the off-white to black appearance of typically pigmented strains.

Hermanides-Nijhof (1977) described two varieties of *A. pullulans* by color differences on cultures. *A. pullulans* var. *pullulans* was indicated when cultures remained pink, light brown or yellow for at least 3 weeks while *A. pullulans* var. *melanigenum* (Hermanides-Nijhof, 1977) referred to cultures that rapidly become black or dark olivaceous-green. Subsequently, a new variety, *A. pullulans* var. *aubasidani* Yurlova, was characterized by the additional production of an aubasidan-like

exopolysaccharide and could be distinguished from *A. pullulans* var. *pullulans* by absence of assimilation of *methyl*-α-D-glucoside and lactose (Yurlova and de Hoog, 1997). More recently, Zalar *et al.* (2008) reported two new varieties of *A. pullulans* using multilocus sequence analysis, *A. pullulans* var. *subglaciale* which was isolated from psychrotolerant habitats in Arctic glaciers and *A. pullulans* var. *namibiae* which refers to a single strain from Namibia, Africa.

2.1.2 Morphology

A. pullulans has a complex life cycle and exhibits polymorphic forms (Figure 2.1) ranging from blastospores, swollen cells, chlamydospores, to hypha and pseudohypha, depending on strain differences, age, media, and culture conditions (Cooke, 1959; Ramos and Garcia Acha, 1975). *A. pullulans* grows rather rapidly on malt, potato, or glucose agar, often obtaining a colony diameter of about 40 mm within 7 days at room temperature. Young colonies are flat, smooth, slimy, and shiny, and composed of blastoconidia. Color ranges from creamy to pale pink. Mature colonies develop a velvety texture and dark brown or black color with a greyish fringe. Septate hypha initially appear hyaline, smooth, thin wall, up to 20 μm in diameter but develop dark brown pigmentation, and thick walls with age. Blastoconidia are produced synchronously in dense groups from undifferentiated hyphae. These conidia may multiply by budding, giving rise to secondary conidia. Conidia are hyaline, smooth-walled, one-celled, and variable in shape and size. *A. pullulans* also occasionally develops chlamydoconidia and fragment-like arthroconidia (Cooke, 1959, Hermanides-Nijhof, 1977, and Domsch *et al.*, 1993).

The life cycle of *A. pullulans* was previously described by Ramos and Garcia Acha (1975). These authors illustrated the vegetative cycle of *Pullularia pullulans* (previous name of *A. pullulans*) which is divided into 6 subcycles (Figure 2.2).

Subcycle 1 By budding, blastospores produce new blastospores. Many buds appear simultaneously, mostly formed in polar zones.

Subcycle 2 Blastospores produce a pseudomycelium in which the daughter cells do not separate from the mother cells.

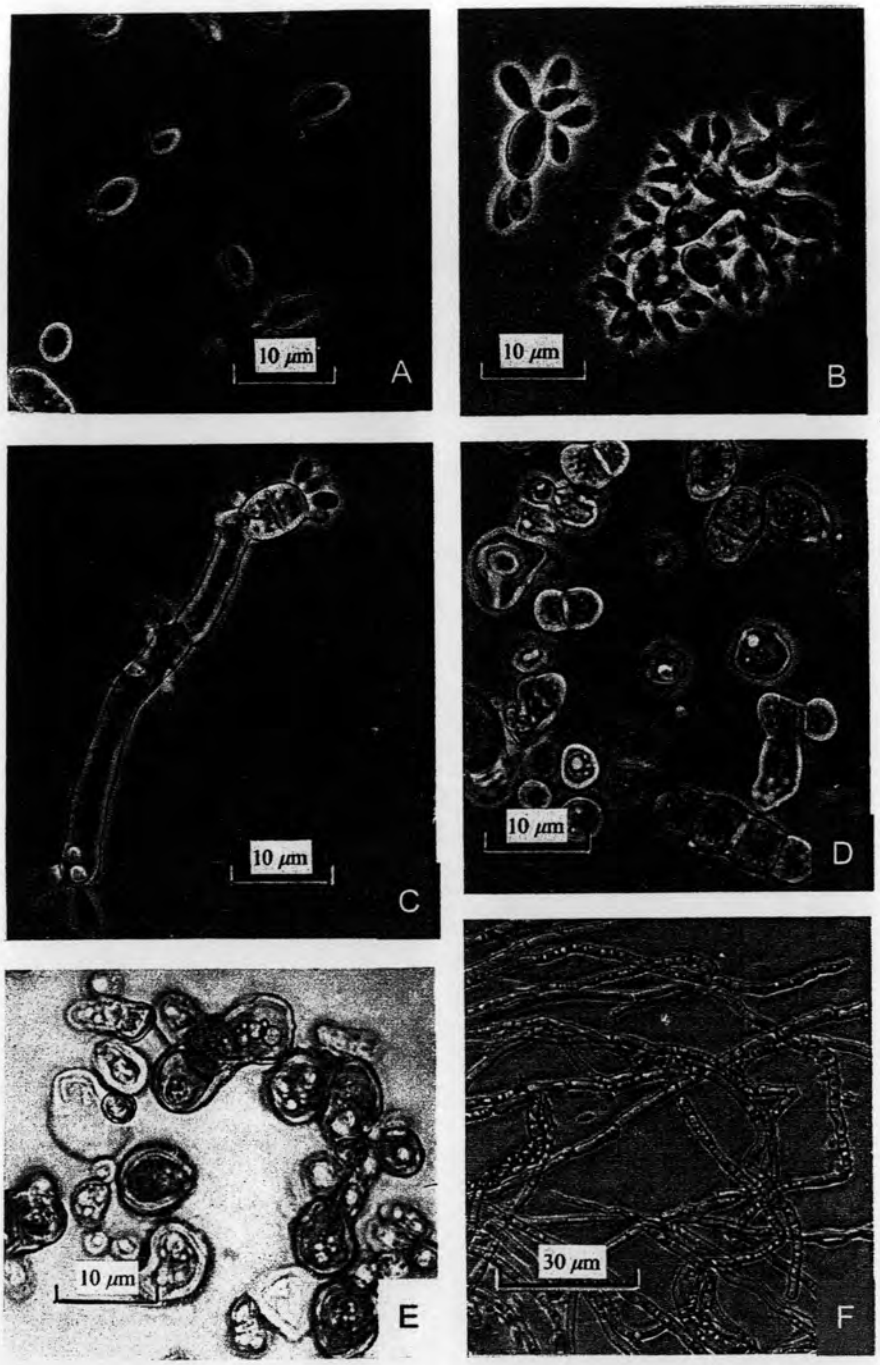

Figure 2.1 Polymorphic forms of *Pullularia pullulans* (*Aureobasidium pullulans*) (A) Blastospore (B) Pseudomycelium (C) Septate swollen cells with buds and germ tubes (D) Septate swollen cells with thickened walls developing to chlamydospores (E) Mature chlamydospores (F) Mycelium (Ramos and Garcia Acha, 1975)

Subcycle 3 Blastospores differentiate to become swollen cells. These swollen cells may develop in two different pathways. Some cells bud off blastospores, others produce germ tubes and develop to be a true mycelium.

Subcycle 4 Swollen cells differentiate to become septate cells. These septate swollen cells may develop differently. Some produce blastospores, or germ tubes which develope further to become a septate mycelium. If the septate swollen cells remain in the same medium for more than 48 h, their walls become thicker and darker and the cells become chlamydospores.

Subcycle 5 Chlamydospores produce germ tubes and then develop to become a true mycelium, or occasionally they produce blastospores.

Subcycle 6 The developed mycelium produces dark pigmented cells and converts into chlamydospores.

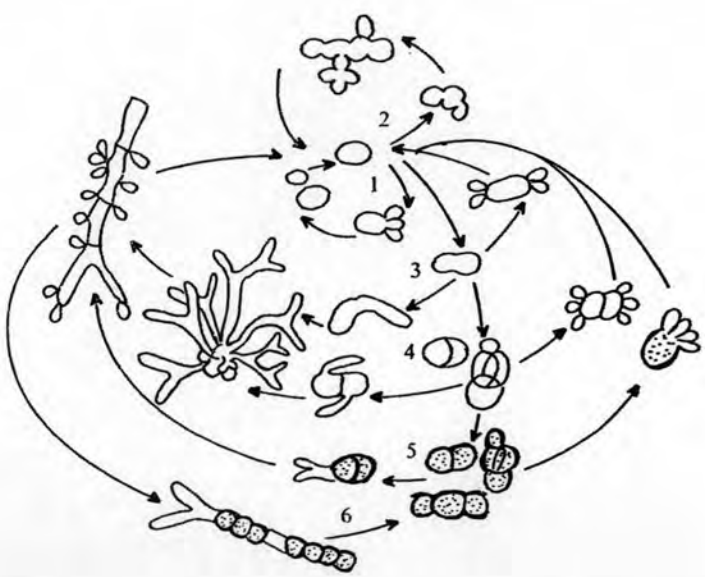

Figure 2.2 The life cycle of *Pullularia pullulans* (*Aureobasidium pullulans*) (Ramos and Garcia Acha, 1975)

2.1.3 Ecology

A. pullulans has been isolated from various types of habitats. It is described as a ubiquitous fungus and cosmopolitan saprophyte (Cooke, 1959; Domsch *et al.*, 1993). It is often the dominant fungus found on leaves (phylloplane) and the aerial parts of plants as a plant pathogen. Andrew *et al.* (2002) studied the colonization of *A. pullulans* on apple leaves using fluorescence *in situ* hybridization (FISH). They reported that certain sites are relatively conducive (veins; wounds) or nonconducive (unwounded interveinal areas) to colonization, and that morphotypes involved in colonization are blastospores, swollen cells, and chlamydospores. McGrath and Andrew (2006, 2007) subsequently reported that *A. pullulans* primarily colonizes veins, and that growth-promoting substances occur locally in the veinal areas. Moreover, this species has been isolated from soil, wood, fresh water, marine estuary sediments, fruit, leather, cotton fabrics, plastics, concrete surfaces, painted walls, and indoor environments where moisture accumulates, especially bathroom walls, shower curtains, kitchens, tile grout, window sills, and liquid wastes. It is also important in the post-harvest decay of fruits and other crops (Schena *et al.*, 2003). It can colonize optical lenses and electronic equipment and has been implicated in the biodeterioration of plasticized polyvinyl chloride (Webb *et al.*, 2000). *A. pullulans* was reported to be the prominent endophyte isolated from several cactus species in Arizona (Suryanarayanan *et al.*, 2005). It also can be found in stress environments such as hypersaline water (Gunde-Cimermana *et al.*, 2000), marine environments (Chi *et al.*, 2009) and glacial or subglacial ice (Zalar *et al.*, 2008). 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 also has been reported as an allergen or opportunistic fungus in many cases, such as a rare case of disseminated nosocomial fungal infection due to *A. pullulans* var. *melanigenum* (Bolignano and Criseo, 2003), allergic diseases (Taylor *et al.*, 2006), corneal ulcers (Panda *et al.*, 2006), severe asthma (Niedoszytko *et al.*, 2007), and hypersensitivity pneumonitis (Temprano *et al.*, 2007).

2.1.4 Classification

Historically, morphological and nutritional characteristics have been used to distinguish strains of *A. pullulans* from other similar yeasts (Dennis and Buhagiar, 1973). The identification key of the genus *Aureobasidium* was constructed based on colonial and microscopic characteristics (Hermanides-Nijhof, 1977). Using this key, two varieties, *A. pullulans* var. *pullulans* and *A. pullulans* var. *melanigenum*, were identified. Subsequently, Cernakova *et al.* (1980) classified 43 strains of *A. pullulans* into three groups based on biochemical characteristics. Yurlova and de Hoog (1997) established a new variety, *A. pullulans* var. *aubasidani* Yurlova, based on production of an aubasidan-like polysaccharide and the inability to assimilate *methyl*-α-D-glucoside and lactose.

Conventional taxonomy based on morphological and physiological characters is essential for species recognition but it is limited and often can lead to misidentification (Valente *et al.*, 1999). In many cases, molecular techniques help solve these problems. Various methods have been used to identify *A. pullulans* species. Yurlova *et al.* (1996) differentiated the genera *Aureobasidium*, *Hormonema* and *Kabatialla* by using PCR-ribotyping and restriction analysis. Li *et al.* (1996) developed an oligonucleotide probe which hybridized specifically to the 18S rDNA of *A. pullulans* strains. Comparisons of ITS1, 5.8S and ITS2 rDNA sequences were able to distinguish species among fungi in the order *Dothideales* (de Hoog *et al.*, 1999). Prasongsuk *et al.* (2005) reported that the ITS sequences of *A. pullulans* among different isolates from Thailand were similar. Recently, Loncaric *et al.* (2009) distinguished 200 strains of *A. pullulans* from *Kabatiella lini* and *Hormonema prunorum* using enterobacterial repetitive intergenic consensus (ERIC)-, repetitive extragenic palindromic (REP) and BOX-PCR techniques (abbreviated as Rep-PCR). This technique was also useful for investigations of intra-specific diversity. Based on rep-PCR patterns, macromorphological studies, and protein patterns from SDS-PAGE, *A. pullulans* was divided into two groups. The first group shared similar characteristics whereas the second group was complex, consisting of strains with few similarities within the group.

Many molecular methods have been applied for differentiation of *A. pullulans* at a subspecific level. Mokrousov and Bulat (1992) divided 46 isolates of *A. pullulans* and 2 isolates of *A. microstictum* into 6 groups according to the cross-hybridization of amplified DNAs from UP-PCR and rDNA restriction polymorphisms. By using PCR-ribotyping and UP-PCR/hybridization, 42 strains of *A. pullulans* were organized into four groups (Yurlova *et al.*, 1995). Using similar techniques, Mokrousov (1995a, 1995b) proposed that 40 strains classified as *A. pullulans* could be divided into three separate species. Using random amplified polymorphic DNA (RAPD) as well as assessment of morphological, biochemical and physiological characters, Urzi *et al.* (1999) classified *A. pullulans* isolated from stones and other environmental sources. They reported 13 different electrophoretic profiles using 3 different primers. Schena *et al.* (1999, 2003) differentiated *A. pullulans* using the same technique in order to test *A. pullulans* as a biocontrol against plant pathogens. The RAPD patterns exhibited very high genetic variability.

Concerning molecular taxonomy, multilocus sequence analysis provides more precise phylogenetic trees than earlier single locus studies because increasing the number of nucleotides provides improved statistical support in the combined data (Peterson, 2008). Concordance analysis of DNA sequences provides a useful framework to identify and detect important species and strains in industrial, agricultural, and medical research (Peterson, 2008). A higher-level classification of the fungi based on recent molecular phylogenetic studies was reported by Hibbett *et al.* (2007). Fungi in the order *Dothideales* were classified by DNA analyses of gene sequences from nuclear large subunit (LSU) rRNA, nuclear small subunit (SSU) rRNA, RNA polymerase II (*RPB2*), and translation elongation factor-1 alpha (*TEF1* or *EF-1α*). *A. pullulans* was within the same clade as *Columnosphaeria fagi* (previously *Discosphaerina fagi*; Schoch *et al.*, 2006) based on rRNA gene sequences (Lumbsch and Lindemuth, 2001). DNA sequences (SSU, LSU, *RPB1*, *RPB2*, and *EF-1α*) from ex-neotype strain CBS 584.75 of *A. pullulans* var. *pullulans* (assembling the Fungal Tree of Life) were analyzed and deposited in GenBank (DQ471004, DQ470956, DQ471148, DQ470906, and DQ471075, respectively). Zalar *et al.* (2008) recently reported new varieties of *A.*

pullulans isolated from an Arctic environment using DNA sequence analysis of rDNA internal transcribed spacers (ITS) and partial 28 S rDNA, β-tubulin (*TUB*), translation elongation factor (*EF1α*), and elongase (*ELO*). Two new varieties, *A. pullulans* var. *subglaciale* and *A. pullulans* var. *namibiae*, were defined from 4 groups of *A. pullulans* strains.

The rDNA ITS region is one of the most widely analyzed DNA regions of this fungus. However, this region is rather conserved within *A. pullulans* (de Hoog *et al.*, 1999; Prasongsuk *et al.*, 2005), and is probably most useful for the identification of *A. pullulans* at the specific level. For subspecific differentiation, analysis of a more variable DNA locus is necessary. The intergenic spacer region (IGS) shows a high level of sequence variation, providing useful information to distinguish closely related fungal species (Valente *et al.*, 1999). Sugita *et al.* (2002) described the application of IGS sequences to identify *Trichosporon* species. Sutar *et al.* (2004) reported that the IGS1 region is a more discriminatory tool in the typing of *Wickerhamomyces anomala* (*Pichia anomala)* than the ITS region, and Nagarajan *et al.* (2004) used the IGS region to identify *Fusarium oxysporum* isolates.

DNA sequence analyses of fungal SSU and LSU in the phylum *Ascomycota* often were supported by using protein coding regions such as *β-tub* (beta tubulin or *BT2*), *RPB1*, *RPB2*, and *tef1* (or *EF-1α*) (Hibbett *et al.*, 2007). *Penicillium* species were successfully distinguished and new species were identified using 3 loci, the ID region (ITS-LSU), *CF* (calmodulin fragments), and *EF-1α* (Peterson, 2004; Peterson *et al.*, 2004, 2005), while 460 *Aspergillus* isolates were classified using 4 loci, *BT2*, *CF*, the ID region, and *RPB2* (Peterson, 2008). New species of *Blastobotrys* sp. (Kurtzman, 2007a) and *Candida* (Kurtzman, 2007b, 2007c) were identified using 4 loci, LSU, ITS, MtSm (mitochondrial small subunit rRNA), and *COXII* (cytochrome oxidase II).

2.1.5 Applications

Although *A. pullulans* is well-known for production of the exopolysaccharide pullulan, other polymers have been reported. With different strains and media, many studies also report additional non-pullulan polysaccharides from cultures of *A. pullulans*.

Kikuchi *et al.* (1973) described an insoluble heteropolysaccharide from *A. pullulans* that contains glucose, mannose, and galactose in ratios similar to polysaccharides from cell wall extracts. Cell walls of *A. pullulan* contain both heteropolysaccharides and β–glucan with β-(1,3) and β-(1,6) linkages (Brown *et al.*, 1973). Elinov *et al.* (1987) reported that *A. pulllulans* produced a glucan with a β-(1,3) linked backbone and α-(1,4) linked side chains attached by β-(1,6) linkages which was later named "aubasidan". Simon *et al.* (1993) studied the cell walls of *A. pullulans* using electron microscopy and revealed that both pullulan and the insoluble heteropolysaccharide are localized on the outer surface of the chlamydospores. They described a highly dense peripheral layer composed of chains of pullulan arranged in a network covering the inner layer of β-(1,3)-glucan composed of glucose and mannose (Figure 2.3). β-glucan from *A. pullulans* was recently reported that exhibits some biological activities including antitumor and antiosteoporotic effects and prevents food allergies (Tada *et al.*, 2008).

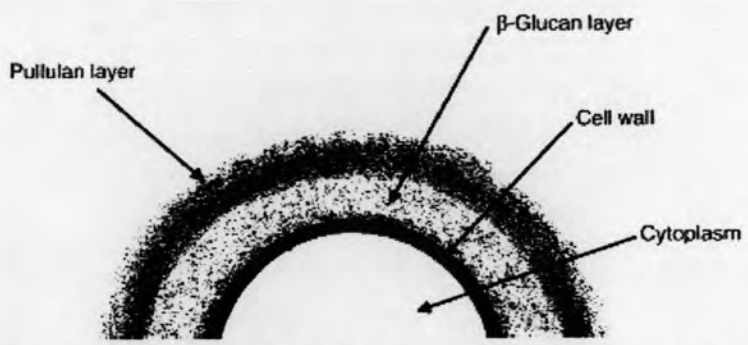

Figure 2.3 Schematic illustration of polysaccharide layers on the outer surface of *Aureobasidium pullulans* cell wall (Shingel, 2004).

Fructo-oligosaccharides (FOS, a popular prebiotic) can be produced from molasses sugar by *A. pullulans* KCCM 12017 (Shin *et al.*, 2004). *A. pullulans* LP23 isolated from soy sauce mash converts L-fructose to L-sorbitol by addition of erythritol to the reaction mixture as an accelerator (Sasahara and Izumori, 2005).

Since it has been described as omnivorous, different strains can produce various extracellular enzymes such as amylases, proteases, lipases, esterases, pectinases, and hemicellulases, including xylanase and mannanase (Leathers, 2002; Chi *et al.*, 2009). More recently, other enzymes from *A. pullulans* were studied such as

tannase (Banerjee and Pati, 2007), β-fructofuranosidases (Yoshikawa *et al.*, 2008), and interestingly, cellulases (Kudanga and Mwenje, 2005; Zhang and Chi, 2007; Leite *et al.*, 2007). It usually has been reported that cellulolytic activity could not be detected from *A. pullulans* cultures (Deshpande *et al.*, 1992; Leathers, 1986; Buzzini and Martini, 2002). In the more recent studies, exoglucanase and CMCase (endoglucanase) were produced by *A. pullulans* isolated from tropical areas (Kudanga and Mwenje, 2005) and marine environments (Zhang and Chi, 2007). In addition, endoglucanase and thermostable β-glucosidase could be produced from wheat bran cultures (Leite *et al.*, 2007).

Other bioproducts of *A. pullulans* have been reported. A cyclic depsipeptide antifungal antibiotic, aureobasidin, was isolated from *A. pullulans* R106 by Takesako *et al.* (1991). Aureobasidin is active against fungi including *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Candida glabrata, Aspergillus nidulans* and *A. niger.* The aureobasidin A (AbA) inhibits a fungal enzyme, inositol phosphorylceramide (IPC) synthase, expressed from the *AUR1* gene (Takesako *et al.*, 1993; Hashida-Okado *et al.*, 1996). More recently, the gene (*aba1*) encoding the AbA biosynthesis complex was cloned and characterized (Slightom *et al.*, 2009). Continuous gluconic acid fermention by *A. pullulans* DSM 7085 has been studied and optimized by Anastassiadis *et al.* (2003, 2005, 2006). It is used as a food additive and in cleaning products. Poly(β-L-malic acid) (PMA) was produced by *Aureobasidium* sp. strain A-91 in glucose cultures (Nakata *et al.*,1993). Subsequently, Nakajima-Kambe *et al.* (1996) improved the production yield of the same strain using non-growing cells, addition of $CuSO_4$ and optimization of conditions. PMA was also produced by additional strains of *A. pullulans* and *A. belleyi* (Liu and Steinbuchel, 1996). PMA is used in pharmaceutical applications as a pro-drug or for drug-delivery systems, and its derivative polyesters might be used for the production of detergents, biodegradable plastics and other substances.

A. pullulans HN6.2 isolated from a marine environment was found to produce a high level of siderophore, an iron-chelating ligand with applications as an antimicrobial agent and in metal recovery and remediation of waste sites (Wang *et al.*, 2009). Marine

A. pullulans 4#2 is also used as single-cell protein with nutritive components for animal feed (Chi *et al.*, 2008).

A. pullulans has been utilized as a biocontrol agent against post-harvest disease. *A. pullulans* strain Ach1-1 was applied to control *Penicillium expansum*, a blue mold grew on harvested apples (Bencheqroun *et al.*, 2006). Strain L47 was proven to have biocontrol activity against rots of sweet cherries and table grapes (Schena *et al.*, 2003).

Radulovi *et al.* (2008) demonstrated that the content of metals (Cu, Fe, Zn, Mn, Pb, Cd, Ni and Cr) decreased when *A. pullulans* strain CH-1 was grown on the acid hydrolysate of peat from the Vlasina Lake, due to biosorption. During this process, the metals were accumulated in the biomass. However, the metals were not bound to pullulan.

In addition, Dos Santos *et al.* (2009) demonstrated that immobilized cells of *A. pullulans* FE13 isolated from stainless steel effluents have potential applications in the biodegradation of phenol.

Xylanases

Xylan is a heteropolymer of β-1,4-linked xylose with arabinosyl and/or 4-*o*-methylglucurosyl side chains (Leathers, 1986; Chi *et al.*, 2009). As the major component of hemicellulose, xylan may comprise up to 30% of the dry weight of agricultural residues (Leathers, 1989). Xylanases hydrolyze biomass and have potential for conversion to fuels and chemicals (Leathers, 1989) and also have many applications in paper, fermentation and food industries, as well as in waste treatment (Chi *et al.*, 2009). Color variant *A. pullulans* strain NRRL Y-2311-1 was reported to produce remarkable xylanase (endo-β-1,4-xylanase, EC 3.2.1.8) yields with high specific activity (Leathers, 1986). Two extracellular xylanases with similar molecular weights (20 and 21 kD) were detected in many strains (Leathers, 1986), and it was suggested to be the result of differential glycosylation during xylanase production. The more prominent xylanase (20 kD) was subsequently purified and characterized (Leathers, 1989).

Purified enzyme exhibited a pI of 8.5 and specific activity of 2100 IU/mg under optimal conditions (pH 4.5 and 45°C) and was specific for polymeric xylan.

Li *et al.* (1993) purified another xylanase (APX-II) from the same strain. The enzyme has a mass of about 25 kD with a pI of 9.4 and has the highest activity at pH 4.8 and 54°C. Subsequently, Li and Ljungdahl (1994) characterized a gene encoding xylanase (*xynA*) from *A. pullulans* NRRL 2311-1. The cDNA consisted of 895 bp. It has an open reading frame encoding a polypeptide of 221 amino acids with a calculated mass of 23,531 Da and contains a putative 34-amino-acid signal peptide. The data presented suggested that the highly active xylanases, APX-I and APX-II, secreted by *A. pullulans* were encoded by the same gene. The *xynA* gene was then expressed in *Saccharomyces cerevisiae* under the GAL1 promoter in the pYES2 vector. Cloned pCE4 had xylanase activity levels of 6.7 U ml^{-1} in the cell-associated fraction and 26.2 U ml-1 in the culture medium 4 h after galactose induction. Two xylanases were observed with sizes of 25 and 27 kD and N-terminal amino acid sequences identical to that of APX-II (Li and Ljungdahl, 1996).

Christov *et al.* (1999) purified β-xylanase and β-xylosidase of *A. pullulans* strain NRRL Y-2311-1 from a xylose culture. The β-xylanase exhibited very high specificity for xylan extracted from *Eucalyptus grandis* dissolving pulp, whereas the β-xylosidase was only active on p-nitrophenyl xyloside and xylobiose. Both enzymes altered the carbohydrate composition of sulfite pulp by increasing the relative cellulose content at the expense of reduced hemicellulose content of pulp.

Ohta *et al.* (2001) purified acidophilic endo-1,4-beta-xylanase (XynI) from the culture supernatant of *A. pullulans* var. *melanigenum* (ATCC 20524) grown on oat-spelt xylan. The purified enzyme had a molecular weight of 24 kD and an isoelectric point of 6.7. Xylanase activity was optimal at pH 2.0 and 50°C. The xylanase gene (*xynI*) consisted of 663 bp, encoding a presumed prepropeptide of 34 amino acids and a mature protein of 187 amino acids. The deduced amino acid sequence showed 94% identity with that of a previously reported equivalent gene (*xynA*) encoding a xylanase with an optimal pH of 4.8 from the color variant strain, NRRL Y-2311-1. Sequence alignment and phylogenetic analysis showed that the enzymes were closely related to

the family-11 xylanases. Using the same strain, Tanaka *et al.* (2006) subsequently purified a new β-xylanase (XynII). The purified enzyme had a molecular weight of 39 kD and a pI of 8.9. Xylanase activity was optimal at pH 6.0 and 70°C. The xylanase gene (*xyn*II) encoded a 26 amino acid signal peptide and a 335 amino acid mature protein. DNA regions encoding the signal sequence and the mature protein were interrupted by introns of 56 and 73 bp, respectively. Sequence alignment and phylogenetic analysis suggested that the XynII belongs to the family-10 xylanases.

2.2 Pullulan

2.2.1 Chemical structure

Historical studies of pullulan structure and analyses were described by Leathers (2002). Studies of pullulan structure began with Bernier (1958), who reported that glucose is the major component of pullulan. Bender (1959) concluded from the infrared (IR) spectrum and the positive optical rotation that pullulan is a polymer composed of α–1,4 linked glucose. Subsequently, using IR, periodate oxidation, and methylation analysis, it was established that pullulan is a linear glucan containing α–1,4 and α–1,6 linkages in a ratio of 2:1 (Bouveng *et al.*, 1962; Sowa *et al.*, 1963). Bender and Wallenfels (1961) discovered the enzyme pullulanase which hydrolyzed the α–1,6 linkages of pullulan and provided maltotriose. From all results, pullulan is often described as a polymer of α-1,6 linked maltotriose subunits (Figure 2.4).

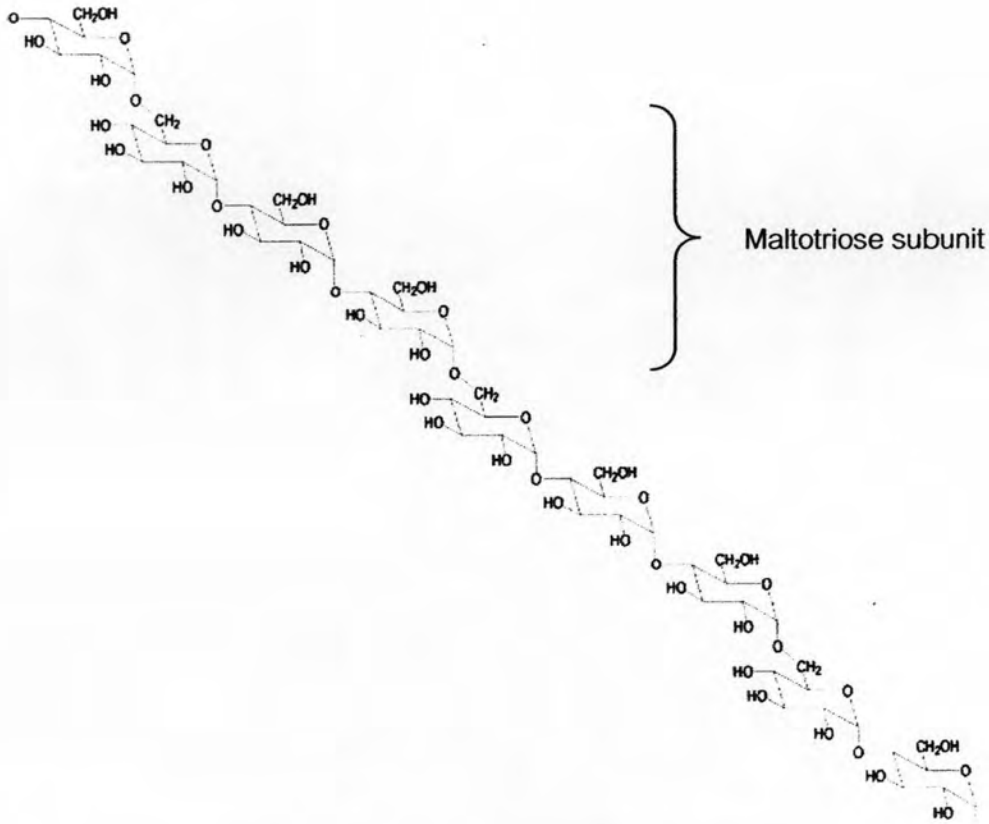

Figure 2.4 The primary (major) structure of pullulan (Leathers, 2002)

Subsequently, Catley and Whelan (1971) found that pullulan contained not only maltotriose subunits but also maltotetraose subunits (Figure 2.5) as a minor structure which varies from about 1 – 7% of total residues (Taguchi *et al.*, 1973; Catley *et al.*, 1986) on a strain specific basis. Maltotetraose subunits are distributed randomly throughout the molecule (Carolan *et al.*, 1983). The maltotetraose residues were proposed to be substrates for hydrolysis by α-amylase and the cause of the decrease in molecular weight and viscosity observed in late cultures (Catley, 1970).

Furthermore, Catley *et al.* (1986) found that pullulan was partially resistant to digestion by glucoamylase from *Aspergillus niger* and suggested that the molecule might contain randomly distributed branches or alternative subunit residues.

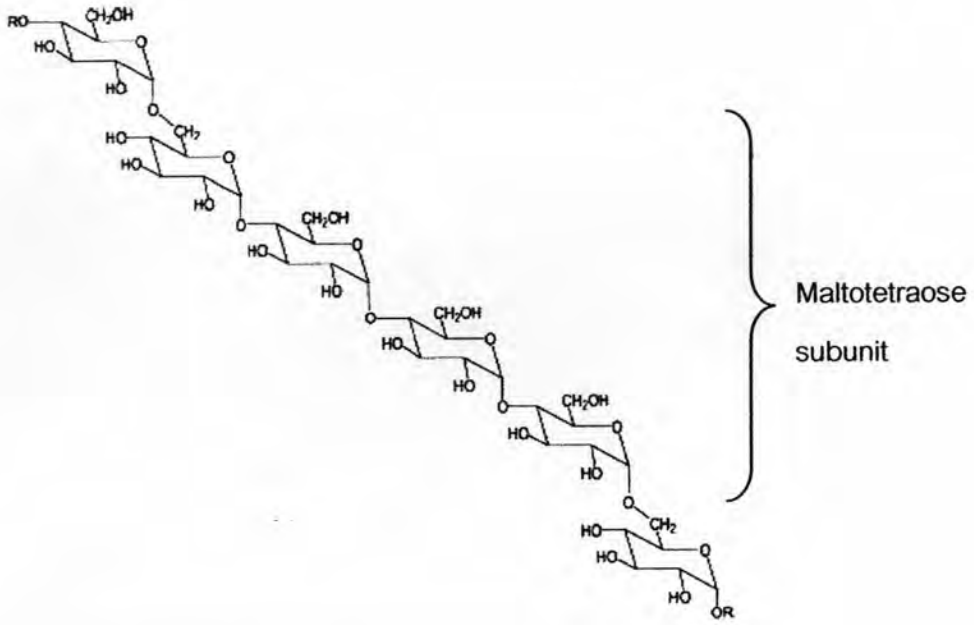

Figure 2.5 The secondary (minor) structure of pullulan (Leathers, 2002)

2.2.2 Chemical analyses

The basic structure and quantity of pullulan are detected by general assay methods for polysaccharides i.e. specific optical rotation, IR absorption, periodate oxidation, methylation analysis, and acid hydrolysis (Leathers, 2002). Moreover, assays based on pullulanase hydrolysis are specific for the detection and measurement of pullulan. Leathers *et al.* (1988) described a quantitative pullulan assay based on reducing sugar (maltotriose equivalents) released by pullulan digestion. Israilides *et al.* (1994) suggested the complete hydrolysis of pullulan by pullulanase and glucoamylase, followed by the specific measurement of glucose using glucose oxidase. Gas chromatography (GC) and GC-mass spectrometry (MS) have been used to determine the carbohydrate composition of pullulan from *A. pullulans* cultures grown with different carbon sources (Lee *et al.*, 1999). Pullulan has been used in many ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy studies (Benesi and Brant, 1985; Glinel *et al.*, 2000; Dais *et al.*, 2001). Benesi and Brant (1985) completely assigned the NMR signals for all carbon atoms in the repeating unit of pullulan, and Dais *et al.* (2001) investigated the temperature dependence of the spin–lattice relaxation times of pullulan ring

carbons. More recently, diffusion-ordered NMR spectroscopy (DOSY) was used to estimate the molecular weight of pullulan in diluted aqueous solution (Veil *et al.*, 2003).

The molecular weight of pullulan has been estimated by various methods, including light scattering, sedimentation behavior, viscosity, and chromatography (Leathers, 2002). Pullulan is known to exhibit non-Newtonian (pseudoplastic) behavior (Catley, 1979). Whole-broth viscosity was used to compare the pullulan properties among different strains (Silman *et al.*, 1990). Toda *et al.* (2000) measured viscosity of the culture broths with different initial pHs of 6 and 7. They reported that higher molecular weight EPS was produced from the condition of pH 7. Gel permeation chromatography was used to compare the molecular weights among new exopolymers from cultures grown on glucose, mannose, and glucosamine (Lee *et al.*, 1999). Pullulan was used as a reference molecular mass standard to determine the molecular weight of sodium alginate based on the use of high performance size-exclusion chromatography (HPSEC) (Ci *et al.*, 1999). Lazaridou *et al.* (2002) used the same technique to characterize the molecular weight of pullulan produced from beet molasses by *A. pullulans* P56 in a stirred tank fermentor under varying agitation conditions. HPSEC and viscosity measurements were used to study the relationship between the molecular weight of pullulan and α-amylase activity in production medium (Prasongsuk *et al.*, 2007).

2.2.3 Physiological function

There are some reports mentioned that pullulan helps cells adhere to environmental surfaces, such as leaf surface (Andrew *et al.*, 1994) and painted wood (Bardage and Bjurman, 1998).

2.2.4 Biosynthesis

A hypothetical scheme of pullulan biosynthesis was proposed by Berry (1988) (Figure 2.6 A). Taguchi *et al.* (1973) found that cell extracts produced pullulan from uridine 5'-diphosphate-glucose (UDPG) in the presence of adenosine 5'-triphosphate (ATP). Cell extracts were not able to produce pullulan from sucrose, but acetone-dried

cells were ^{14}C-labeled sucrose became incorporated in lipid intermediates (Taguchi *et al.*, 1973). This lipid contained glucose and pyrophosphate. Catley and McDowell (1982) found that ^{14}C-glucose was found to be part of lipid-linked glucose, isomaltose, panose, and isopanose. For this reason, they proposed that pullulan actually was made from either panose or isopanose subunits (Catley and McDowell, 1982) (Figure 2.6 B). In addition, they suggested that the minor maltotetraose subunits were formed from occasional direct linkage of panose and isopanose. On the other hand, Hayashi *et al.* (1994) described a glucosyltransferase that produced isomaltose and panose from maltose. Finkelman and Vardanis (1982) prepared protoplasts of *A. pullulans* that could produce pullulan and indicated that pullulan synthesis was not associated with the cell wall, cell membrane, or periplasmic space. In addition, pullulan was suggested to be synthesized intracellularly and secreted by *A. pullulans* (Leathers, 2002).

Since the pullulan biosynthetic pathway is similar to glycogenesis, the effect of different sugars on pullulan production and enzymes involved has been investigated (Duan *et al.*, 2008). In a glucose medium, *A. pullulans* strain Y68 produced more pullulan than in fructose and xylose media. They also proposed the pathway of pullulan biosynthesis involving many carbon sources (Figure 2.7). Chi *et al.* (2009) reported that pullulan biosynthesis was associated with low intracellular levels of UDP-glucose and very high activities of UDPG-pyrophosphorylase, phosphoglucomutase, and glucosyltransferase. In glucose culture, UDP-glucose is synthesized continuously to supply the precursors for pullulan synthesis. In contrast, levels of UDP-glucose are higher in fructose and xylose cultures, due to the low level of glucosyltransferase activity. The proposed pathway of pullulan biosynthesis will be useful to further enhance pullulan yields (Chi *et al.*, 2009).

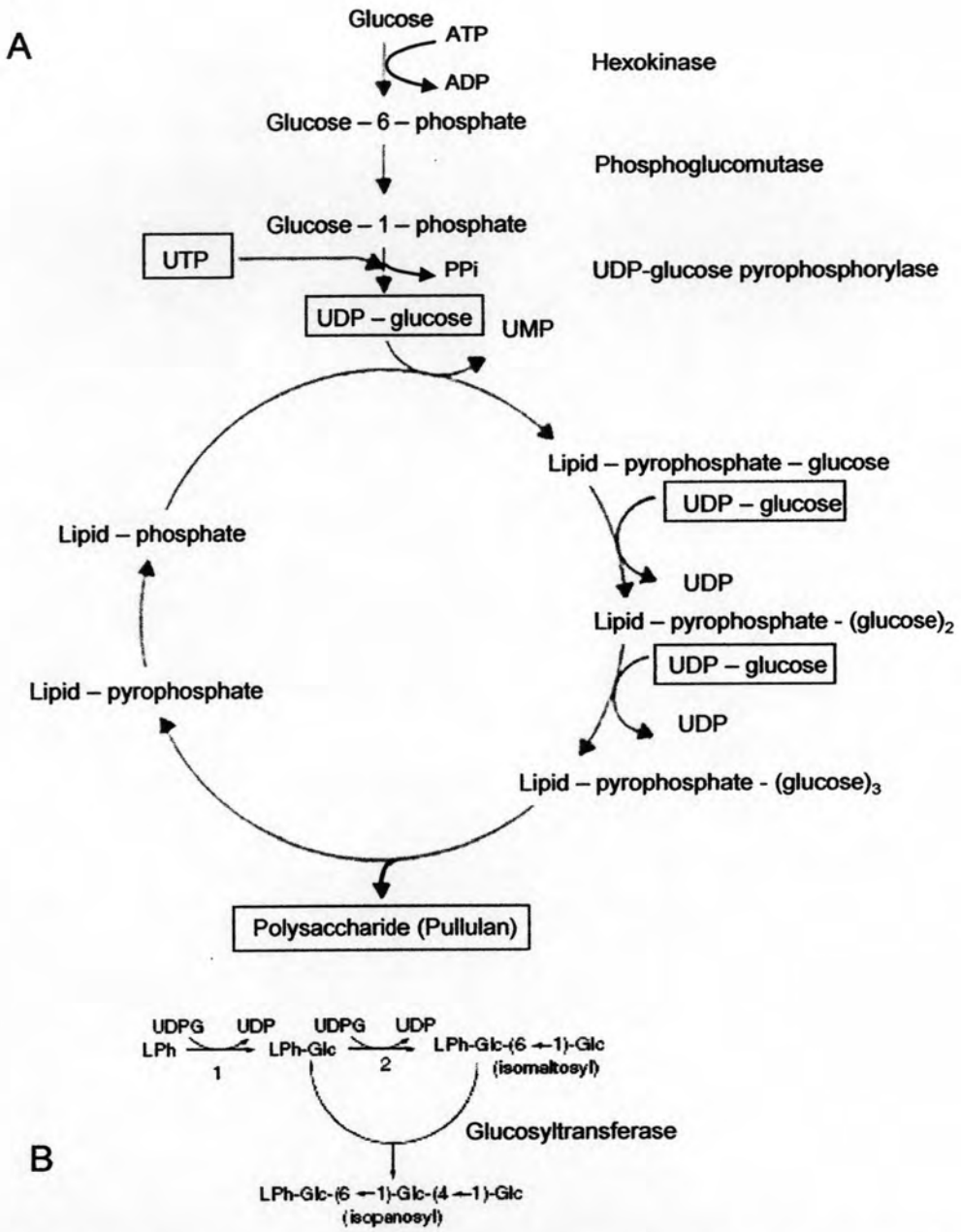

Figure 2.6 (A) Possible pathway of pullulan biosynthesis and enzymes involved (A) modified from Berry (1988) (B) proposed by Catley and McDowell (1982), LPh = phospholipid intermediate, LPh-Glc = glucose conjugates (Shingel, 2004).

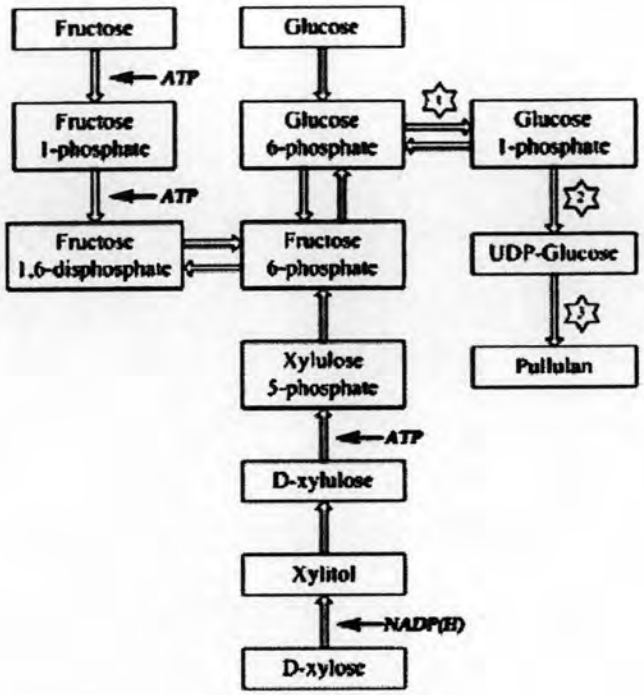

Figure 2.7 Proposed pathway of pullulan biosynthesis. 1, α-phosphoglucomutase, 2, UDPG-pyrophosphorylase, and 3, glucosyltransferase (Duan *et. al.*, 2008).

2.2.5 Biodegradation

Pullulan molecular weight generally decreases with culture time, presumably because of the hydrolysis by enzymes such as α-amylase (EC 3.2.1.1), an endoglucanase which recognizes α-(1,4) linkages, and glucoamylase (amyloglucosidase, EC 3.2.1.3), an exoglucanase that attack from the non-reducing end to produce glucose.

Historically, the maltotriose subunit, making up the major structure of pullulan, is resistant to α-amylases (Wallenfels *et al.*, 1965). However, Catley (1970) identified minor maltotetraose subunits in pullulan, and demonstrated that these residues were substrates for hydrolysis by α-amylase. Catley (1970) also reported that *A. pullulans* produced amylase and suggested that this activity was results in the degradation of pullulan molecular weight and viscosity in late cultures. Later, Miura *et al.* (1978) added amylase inhibitor to cultures and reported that the molecular weight of pullulan was enhanced. The minor maltotetraose residues make up about 1 – 7% of total residues

(Taguchi *et al.*, 1973; Catley *et al.*, 1986) and randomly distributed in pullulan (Carolan *et al.*, 1983). Thus, hydrolysis at these points would result in a large decrease in viscosity with relatively little saccharification (breakdown to glucose). Such a system might be important for the production of biofilms.

However, amylase levels have been reported to be extremely low on a variety of non-starch substrates, and only slightly higher on starch (Leathers, 1987; 1993; Saha *et al.*, 1993). Leathers (1987) suggested that low amylase levels might be necessary but not sufficient for production of high molecular weight pullulan. The α-amylase activity was studied from *A. pullulans* strain NRRL Y-12974 during growth of cultures in different carbon sources including glucose, maltose, soluble starch, and cornstarch (Leathers, 1993). The activity was lower in glucose-grown cultures than maltose- and starch-grown cultures. All cultures showed both α-amylase activity and activity against pullulan. Using the same strain, Saha *et al.* (1993) studied the amylolytic enzymes produced from a variety of carbon sources. The α-amylase, glucoamylase A, and glucoamylase B were collected from different fractions. Comparative pullulan hydrolysis showed that glucoamylase B had much higher relative rate than α-amylase and glucoamylase A. West and Strohfus (1996) also reported a pullulan-degrading enzyme activity in the culture medium using corn syrup as a carbon source, which was likely glucoamylase B, while α-amylase activity was not detectable. Punnapayak *et al.* (2003) reported that using glucose as an optimal carbon source, amylase activity gradually decreased, while EPS yield increased during the EPS production period. More recently, Prasongsuk *et al.* (2007) determined the α-amylase and pullulan-degrading activities in cultures grown on sucrose and starch media. They found that cultures grown on starch produced higher activities than cultures grown on sucrose while the molecular weight and viscosity of EPS from all cultures gradually decreased. Thus, it could be said that the α-amylase was expressed constitutively at lower level in sucrose-grown cultures and induced to a higher level in starch-grown cultures. Moreover, when the α-amylase inhibitor, acarbose, was added to the EPS production medium containing sucrose, the molecular weight of EPS was slightly higher than in cultures without acarbose. Since α-amylase perhaps responsible for the reduction of molecular weight of pullulan, thus

characterization of *A. pullulans* α-amylase gene may help to understand the function and role of this enzyme in pullulan production.

Pullulanase is a distinct enzyme that attacks the α-(1,6) linkages of pullulan. Thus, pullulanase converts pullulan to maltotriose, with minor amounts of maltotetraose. Bender and Wallenfels (1961) first isolated this enzyme from *Klebsiella planticola*. *A. pullulans* generally is not believed to produce pullulanase, but numerous bacteria produce this enzyme, such as *Micrococcus* sp. (Kimura and Horikoshi, 1990) and *Ruminobacter amylophilus* (Anderson, 1995). This enzyme is commercially important in starch saccharification, because it attacks α-(1,6) branch points in amylopectin and thus enhances the activity of amylases.

2.2.6 Production

The control of pullulan production has been described in many reports that referred to various culture conditions such as carbon sources, nitrogen sources, pH, temperature, vitamins and minerals. Cell morphology has often been observed during pullulan production as well. However, previous reports on this matter seem to be inconsistent and contradictory.

Carbon sources seem to play the most important role for pullulan yield. *A. pullulans* strains typically produce pullulan when cultured on sucrose, glucose, fructose, maltose, or starch (Leathers *et al.*, 1988; West and Reed-Hamer, 1991; Badr-Eldin *et al.*, 1994; Gibson and Coughlin, 2002; Punnapayak *et al.*, 2003; Prasongsuk *et al.*, 2007). Sucrose often has been described as the optimal substrate. In contrast, *A. pullulans* was reported to produce reduced pullulan yields when grown in xylose, arabinose, mannose, galactose, lactose, rhamnose (Imshenetskii *et al.*, 1981; LeDuy *et al.*, 1983). This could be because UDP-glucose is the main substrate for pullulan synthesis as described in 2.2.4. Other carbon sources from agricultural wastes and products have also been used such as corn fiber (Leathers and Gupta, 1994), potato starch waste (Barnett *et al.*, 1999), spent grain liquor (Roukas, 1999), beet molasses (Lazaridou *et al.*, 2002), and sweet potato (Wu *et. al.*, 2008).

A nitrogen source is required for cell growth and metabolism. However, limiting nitrogen has been suggested to maximize pullulan production, because cells can use the carbon source to produce pullulan instead of for growth. Wu *et al.* (2009) reported that pullulan production decreased, while biomass accumulation increased in medium with excess nitrogen. Various nitrogen sources have been evaluated such as nitrate, ammonium sulfate, peptone, urea, and soybean (Leathers, 1988, Reed-Hamer and West, 1994; Campbell *et al.*, 2003; Punnapayak *et al.*, 2003; Seo *et al.*, 2004; Prasongsuk *et al.*, 2007; Wu *et al.*, 2009). Limiting nitrogen also has been reported to affect the morphology of *A. pullulans*. Conditions of limiting nitrogen and low buffering capacity promoted a shift from blastospores to chlamydospores (Bermejo *et al.*, 1981a, 1981b).

It has been reported that pH often drops during cultivation (Bermejo *et al.*, 1981a, 1981b, Tsujisaka and Mitsuhashi, 1993; Wu *et al.*, 2009). Initial pH used varies widely from pH 5.0 – 7.5 whereas optimal pH for pullulan production varies from about pH 4.5 – 7.5 (McNeil *et al.*, 1989; Lee and Yoo, 1993; West and Reed-Hamer 1993; Madi *et al.*, 1996; Reeslev *et al.*, 1997; Lazaridou *et al.*, 2002; Punnapayak *et al.*, 2003; Prasongsuk *et al.*, 2007). Some studies reported that the medium pH affected the morphology. McNeil *et al.* (1989) found that chemostat conditions (optimal at pH 4.5) favored a mixture of yeas-tlike and hyphal cells, while Madi *et al.* (1996) reported that yeast-like growth is favored at pH 6.5. Reeslev *et al.* (1997) described that pH 3-7 favored blastospores. Recently, Wu *et al.* (2009) concluded that at pH lower than 3, the biomass was predominantly mycelial. However, when the pH was higher than 5, the biomass was almost entirely unicellular.

Optimal temperatures for pullulan production appear to vary slightly, usually in the range of 24-30^{o}C (Tsujisaka and Mitsuhashi, 1993; Gibson and Coughlin 2002; Punnapayak *et al.*, 2003; Prasongsuk *et al.*, 2007).

Vitamins and minerals such as biotin, ferric chloride, manganese chloride and zinc chloride may enhance pullulan formation (West and Reed-Hamer, 1992; West and Strophus, 1997). On the other hand, Reeslev *et al.* (1993) and Reeslev and Jensen (1995) reported that Fe^{3+} and Zn^{2+} inhibited pullulan production.

A. pullulans requires oxygen for growth and pullulan production (Madi *et al.*, 1996). Audet *et al.* (1996) found that intermediate dissolved oxygen gave high yields of pullulan but high dissolved oxygen gave lower pullulan yield, although with higher molecular weight. However, there are some studies which reported optimal pullulan yields at decreased oxygen conditions (Wecker and Onken, 1991; Gibbs and Seviour, 1996).

Some studies reported that pullulan was produced during the stationary growth phase and mentioned that pullulan was a secondary metabolite generated during growth limitation in excess carbon (Bulmer *et al.*, 1987; McNeil and Kristiansen, 1987, 1990; Leathers, 1988). In contrast, a number of studies showed that pullulan formation was associated with active cell growth (Klimek and Ollis, 1980).

Blastospores often have been reported as the main morphotype during pullulan production (McNeil and Kristiansen, 1987; Bulmer *et al.*, 1987; Reeslev and Jensen, 1995; Yamasaki *et al.*, 1993). However, some studies were reported that swollen cells and chlamydospores are responsible for pullulan formation (Simon *et al.*, 1993; Andrews *et al.*, 1994; Campbell *et al.*, 2004).

Melanin contamination is a common problem in commercial production. Many strains produce dark pigment in late cultures, which may be the result of the formation of chlamydospores. Certain natural strains and mutants produce less melanin (Leathers *et al.*, 1988; Silman *et al.*, 1990; Pollock *et al.*, 1992; West and Reed-Hamer, 1993b; West and Strohfus, 2001). Zheng *et al.* (2008) described that amylolytic activities disappeared in medium with melanin production, leading to accumulation of pullulan. However, with high nitrate concentrations, the fungus produced a mixture of exopolysaccharides (EPS) without melanin synthesis (Zheng *et al.*, 2008).

In summary, there seem to be various optimal culture conditions that are too confusing to be concluded. Perhaps, the most likely important factor is strain variation (Leathers, 2002). Therefore, the study of phylogeny of *A. pullulans* comparable with their pullulan production would be very useful for strain selection in which high yield without melanin contamination could be obtained.

2.2.7 Properties and applications

The properties and applications of pullulan have been described in many reviews such as Leathers (2003), Shingel (2004), Rekha and Sharma (2007), and Singh (2008).

The unique linkage pattern of pullulan is responsible for its unique properties of flexibility and solubility, resulting in film- and fiber-forming characteristics. Dry pullulan powders are white and non-hygroscopic and water-soluble. It is nontoxic, non-mutagenic, odorless, tasteless, and edible. It can be used as a partial replacement for starch in pastas or baked goods. At low concentration, pullulan solutions have low viscosity. It can be used as a low-viscosity filler in beverages, sauces, cosmetics, lotions, and shampoos. Pullulan films are formed by drying a pullulan solution. They are thin, clear, highly oxygen-impermeable, readily dissolved in water, and thus can be melted in the mouth as edible food coatings. Specialty films may include colors or flavors, and decorative pullulan chips are produced for food uses. Oral care films include popular breath strips. Pullulan has been sulfated, phosphorylated, chlorinated, sulfinethylated, chloroalkylated, etherified, carboxylated, acetylated, or esterified. Pullulan and derivatives of pullulan have many potential pharmaceutical, photographic, lithographic, and electronic applications. Pullulan derivatives are promising as non-toxic conjugates for vaccines and interferon and can facilitate liposome delivery. Sulfated pullulan and phosphorylated pullulan have an anticoagulant, antithrombotic, and antiviral activities. Pullulan-based hydrogels and nanoparticles may have a variety of uses (Leathers, 2003).