CHAPTER II

LITERATURE REVIEW

Aureobasidium pullulans

Taxonomy

The genus *Aureobasidium* was established in 1891 by Viala and Boyer who proposed that this fungus appeared to be the cause of a scald of grape leaves (Cooke, 1959). *A. pullulans* is recognized as a member of yeast-like fungus and black yeast due to the yeast phase in the life cycle and melanin pigment production, respectively. Fourteen species including *A. puliulans* are known (Hermanides-Nijhof, 1977). However, the color variant strains of *A. pullulans* are known (Wickerham and Kurtzman, 1975).

A. pullulans, initially classified in the Deuteromycetes (fungi imperfecti), order Moniliales, family Dematiaceae (Hermanides-Nijhof, 1977; Ramos and Garcia Acha, 1975), was recently reclassified as an ascomycetous yeast-like fungus in the family Dothideaceae, although the perfect stage has not been yet reported (De Hoog and Yurlova, 1994). Synonyms of *A. pullulans* include *A. vitis, Dematium pullulans, Hormonema pullulans, Pullularia fermentans* and *P. pullulans* (Domsch et al., 1993). *A. pullulans* has two varieties: *A. pullulans* var. *pullulans* and *A. pullulans* var. *melanigenum* (Hermanides-Nijhof, 1977). A recent report includes a new variety, *A. pullulans* var. *aubasidani*, established by Yurlova and De Hoog (1997).

Taxonomic outline of *A. pullulans*:

Division Ascomycota

Class Euascomycetes

Order Dothideales

Family Dothideaceae

Genus *Aureobasidium*

Species *Aureobasidium pullulans*

Variety *Aureobasidium pullulans* var. *pullulans*

Aureobasidium pullulans var. *aubasidani*

The taxonomy of *A. pullulans* includes the use of classical methods: colony and morphological characteristics which are fundamental for classification. An identification key based on these criteria has been constructed (Hermanides-Nijhof, 1977). Morphological examination and nutritional physiology were used to differentiate *A. pullulans*, *Trichosporon pullulans* and *A. prunorum* (Dennis and Buhagiar, 1973). The type of conidiogenesis, nutritional physiology, and optimal conditions for polysaccharide production are also useful criteria to identify species. A variety of *A. pullulans* based on these criteria was proposed (De Hoog and Yurlova, 1994). Molecular biological methods are now being used. Thus differences in morphology, ecology and the DNA relatedness has been used to compare between typical pigmented and color variant strains (Leathers et al., 1988). The color variant character exhibited only 37% to 44% DNA relatedness to the typical pigmented strains. However, these *A. pullulans* color variants still belong to this species as judged by their very high homology of nuclear ribosomal DNA Internal transcribed spacer (ITS) sequences compared to the wild type (Sudhadham, 2001).

Molecular biological approaches are being increasingly employed. The ITS sequences were used to distinguish the genera *Aureobasidium*, *Hormonema*, and *Kabatiella* and a phylogenetic tree of *A. pullulans* and allied fungi based on the ITS sequences was constructed (Yurlova et al., 1999).

A. pullulans is distinguishable from other black yeasts and any filamentous fungi via classical or molecular biological assessments, even if strain variants look morphologically similar.

Ecology

A. pullulans is commonly found in a wide variety of environments including the phyllosphere of several plants and fruits (Domsch et al., 1993), and diverse moist and decaying environments (De Hoog et al., 1999). It is a ubiquitous saprophyte that occurs in the temperate, Mediterranean, arid and tropical zones (Deshpande et al., 1992). In Thailand, *A. pullulans* has been found as a fungus isolated from pine needles (Tokomasu et al., 1997). Recently, several strains of *A. pullulans* var. *pullulans* were successfully isolated from several locations around Thailand (Punnapayak et al., 2003).

Plant surfaces are a characteristic habitat of *A. pullulans,* it occurring both saprophytically and epiphytically (assumed no pathogenic status). Diverse plants are colonized (Cooke, 1959). *A. pullulans* was found as a dominant yeast in the phyllosphere of mango (De Jager et al., 2001). This yeast was more frequently isolated from juvenile and mature leaves than from foliol and flush leaves. The fungus occurs on pine needles in Thailand (Tokomasu et al., 1997) and in Germany (Tokomasu et al., 1994). From population biology considerations of *A. pullulans* on apple leaves, it was proposed for post harvest biocontrol of apples (Andrews et al.,

2002). The aquatic Eurasian Water Milfoil (*Myriophyllum spicatum*) is colonized by *A. pullulans* (Smith et al., 1989). Moreover, *A. pullulans* can occur as an endophyte in some plants. Several isolates of *A. pullulans* were obtained from surface sterilized twigs, buds, leaves, and seeds of sycamore (*Acer pseudoplatanus* L.), and from twigs of horse-chestnut and lime (Pugh and Buckley, 1971). Endophytic fungi (163 isolates), including *Aureobasidium* spp., were recovered from the leaf samples of *Musa acuminata* after sterilizing the surface with formaldehyde (37%) (Cao et al., 2002). *A. pullulans* grows on weathered wood surfaces and was assumed to utilize the lignocellulosic photodegradation products, this depending on the ability of the fungus to use lignin breakdown products as a sole carbon and energy source (Schoeman and Dickinson, 1997). However, Eveleigh (1961) believes that surface growth in such instances also includes surface detritus as a nutrient source.

There are numerous reports on the colonization of *A. pullulans* on painted and unpainted surfaces (Crang and Pechak, 1978). It can be dominant and causing a major blackening disfiguration. As noted it could use surface detritus as a nutritional source (Eveleigh, 1961). However, after observing growth on painted and unpainted wood surfaces of *A. pullulans*. It was suggested that it probably used wood components as carbon source (Horvath et al., 1976). Although cellulose in the wood failed to support growth, wood also contains simple aromatics, polyphenols, lignans, and other aromatic extractives which can be used as carbon and energy for fungal growth (Horvath et al., 1976). This of course is a transient resource as during weathering these materials will be washed away. This fungus colonizes indoor habitats such as ceilings and painted walls (Lachke and Rale, 1994). The nutrient source could be materials that leach through from the surface that the paint is covering, while it would be difficult to attack the polymerized paint component. The disfigurer could be using

the plasticizers in paint. The black disfiguration of painted surfaces continues as a major concern to the paint industry (English et al., 2003).

Diverse habitats of *A. pullulans* include forest soil (Deshpande et al., 1992), historical church window glass (Schbereiter-Gurtner et al., 2001), and plasticized polyvinyl chloride (pPVC) (Webb et al., 2000), etc. The isolation of *A. pullulans* from air has been already noted above, where collection of *A. pullulans* by plate exposure in Thailand was successful (Punnapayak et al., 2003). *A. pullulans* was also found as the most abundant fungus collected by airborne exposure in some working areas in Finland (Kiviranta et al., 1999).

These diverse ecological niches support the notion that *A. pullulans* is ubiquitous fungus which commonly occurs from plant leaves to damp indoor surfaces.

Morphology and life cycle

A. pullulans is a black yeast with dark chlamydospores (Yurlova and De Hoog, 1997); however, naturally color variants as red, yellow or purple strains occur (Wickerham and Kurtzman, 1975) *A. pullulans* is polymorphic being comprised of blastospores, swollen cells, chlamydospores, hyphae and pseudohyphae (Andrews et al., 1994) (Figure 1) . *A. pullulans* is distinguishable from other species by conidial and hyphal size and also colony characteristics (Hermanides-Nijhof, 1977). The colonies of *A. pullulans* appear smooth, soon covered with slime, and are yellow, cream, light pink or light brown and finally changing to black. Hyphae are hyaline, smooth, thin-walled, 2-16μm wide. Conidia are hyaline, smooth, ellipsoidal, 7.5-16 x 3.5-7 μm. Cultural characteristics distinguish between species varieties. Cultures that remain pink, light brown or yellow for three weeks are the characteristic of *A.*

pullulans var. *pullulans*. In contrast cultures of *A. pullulans* var. *melanigenum* become black or dark olivacious green rapidly.

Morphological changes during the life cycle of *A. pullulans* can be controlled by the nitrogen source (Ramon and Garcia Acha, 1975), and this led to suggestion of a 6 subcycles of vegetative cycle (Figure 1):

- Subcycle 1: Blastospores produced new blastospores by budding

- Subcycle 2: (presence of nitrate as nitrogen source): Blastospores continued budding to produce as aseptate chain of blastospores (pseudomycelium).

- Subcycle 3: (presence of ammonium nitrate as nitrogen source): Blastospores differentiated into swollen cells (larger blastospores). Swollen cells can produce either one or more germ-tubes and give rise to a septate mycelium or the septa.

- Subcycle 4: Septate swollen cells gave rise to mycelium and blastospore or converted to thick wall chlamydospores (chlamydospores were not formed when ammonium ion was used as nitrogen source in the culture medium)

- Subcycle 5: Chlamydospores produced germ-tubes which developed to mycelium or blastospores. The mycelium and blastospores were capable of budding and transferred to sub-cycle 1, 2 or 3 depending on the environmental conditions.

- Subcycle 6: The cells of mycelium produced dark pigment and thick wall converted to chlamydospores. The chlamydospores did not germinate unless transferred to a fresh culture medium.

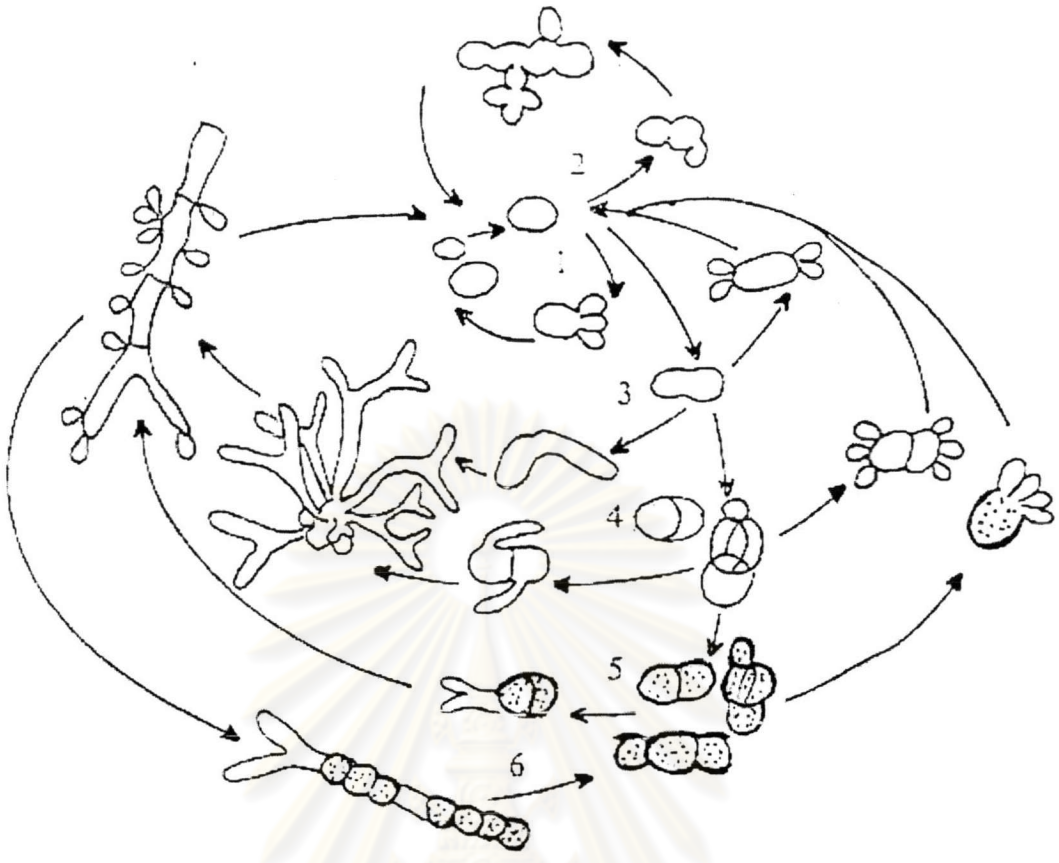

Figure 1 Relationship of different morphological forms of *A. pullulans*, subcycles 1, 2, 3, 4, 5, and 6 are involved. (Ramon and Garcia Acha, 1975)

Developmental stages of *A. pullulans* have been examined using scanning electron microscopy (SEM) (Pechak and Crang, 1975). In the developmental stage (Figure 2), blastospores initiated the budding of new blastospores (generated blastospores) and developed hyphae at the same time. The hyphae elongated and produced a number of blastospores which became septate and dark. Blastospores also produced swollen cells and converted to dark thick-walled chlamydospores. The chlamydospores can germinate and pass through the stage of blastospores.

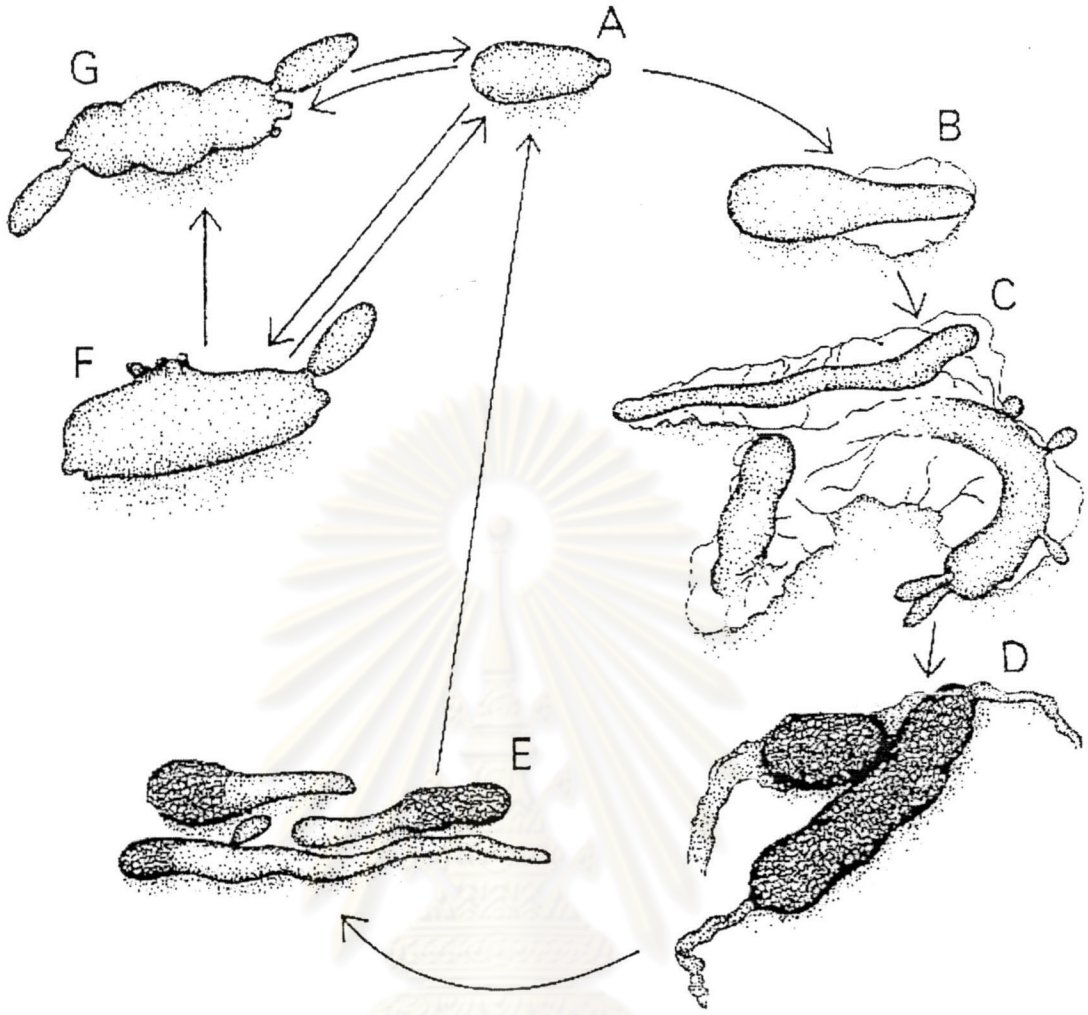

Figure 2 Developmental stages of *A. pullulans* (Pechak and Crang, 1975)

A: blastospore

B: germinating blastospores

C: hyphae

D: chlamydospores

E: germinating chlamydospores

F: swollen cell

G: septate swollen cell

Physiology

A. pullulans assimilates diverse carbon and nitrogen sources and generally uses amino acids (Cooke and Matsuura, 1963). The carbon spectrum of several isolates is broad and includes cellobiose, fructose, glucose, lactose, raffinose, starch and xylose (Cernakova et al., 1980). In comparing the carbon assimilation of *A. pullulans* with that of *Trichosporon pullulans* and *A. prunorum,* possible substrates that can be used to distinguish these fungi included D-arabinose, D-galactose, glycerol, and myo-inositol (Dennis and Buhagiar, 1973). All 117 strains tested of *A. pullulans* utilized glycerol and D-arabinose while none of 15 *T. pullulans* strains utilized these substrates. *A. prunorum* failed to grow on D-arabinose, D-galactose and myo-inositol even with 2 months incubation. *A. pullulans* var. *pullulans* can utilize α-methyl-D-glucoside and lactose while *A. pullulans* var. *aubasidani* cannot (Yurlova and De Hoog, 1997). Though the assimilation patterns vary to some degree for each *A. pullulans* strain, the patterns have been useful to aid in the identification of *A. pullulans* and its variants (Cernakova et al., 1980; Yurlova and De Hoog, 1997).

The temperature range for growth is between 2 to 35°C with an optimal temperature around 25°C. However, *A. pullulans* has even been found as a dominant psychrophile in arctic ice (-5°C) along with *Cladosporium* and *Penicillium* species (Gunde-Cimerman et al., 2003).

The halotolerant forms of *A. pullulans* were recorded (Gunde-Cimerman et al., 2000) with isolates from hypersaline water (3-30% NaCl). Torzilli (1997) found *A. pullulans* on the salt marsh, where a salt range can be from 0-28% salt according to tides, drying and rain.

Application aspects of *A. pullulans*

A. pullulans has a range of potential applications including a source of the exopolysaccharide gum pullulan, industrial enzymes (amylase, pectinase and xylanase), and single cell protein (Deshpande et al., 1992; Leathers, 2002; 2003).

1. Exopolysaccharide Pullulan

A. pullulans is intensively studied as it produces pullulan, a commercial biopolymer (Desphande et al., 1992; Leathers, 2002; 2003). In addition to *A. pullulans*, *Tremella mesenterica* and *Cystaria* spp. produce pullulan (Lachke and Rale, 1994). Pullulan is an extracellular linear homopolysaccharide composed of repeating maltotriose subunits linked through α-1-6-glucosidic bonds (summarized in Leathers, 1993). The maltotetraose oligomers can be randomly located in the pullulan structure (Leathers, 2003, Figure 6). *A. pullulans* also produces the exopolysaccharide, aubasidan, a polymer of glucan linked with α-1,4-D-, β-1,6-D- and β-1,3-glucosidic bonds (Yurlova and De Hoog, 1997). The capability to produce aubasidan has been considered characteristic enough to define a species variant *A. pullulans* var. *aubasidani*.

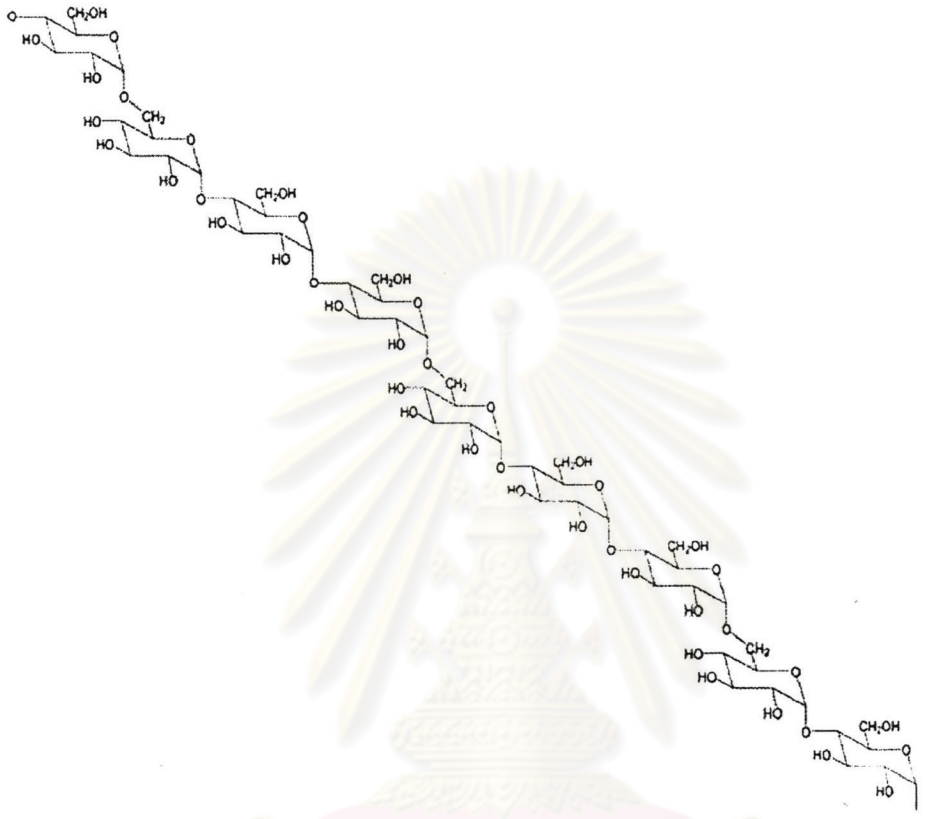

Figure 3 Chemical structure of pullulan (Leathers, 2003)

1.1 Pullulan biosynthesis

The pullulan biosynthetic pathway has not been definitively defined, but synthesis via UDP-glucose and a lipid intermediate moiety has been proposed (Berry, 1988). Glucose units are carried by UDP and then linked via the intermediary action of the lipid via energy from the pyrophosphate bond. Continued polymerization yielded pullulan. The synthesized pullulan was considered to be secreted through the cell wall. The hypothetical scheme of pullulan biosynthesis is outlined in Figure 4.

1.2 Pullulan production

Pullulan production has been studied to optimize yields and/or high quality by using a range of *Aureobasidium* strains, fermentation conditions, and fermentation processes

1.2.1 Pullulan and morphology of *A. pullulans*

Poor pullulan formation was observed by the mycelial form of *Dermatium pullulans* while blastospores were more effective (Imshenetskii and Kondrat'eva, 1978). Later, Catley (1980) noted that blastospores and mycelium both produced pullulan but the blastospores were more efficient than mycelium with 4-5 times yields. He used ATCC 9348. In contrast Campbell et al., (2004) and Simon et al., (1995) stated that only swollen cells and chlamydospores can produce pullulan, but neither blastospores nor hyphae. The morphological form of *A. pullulans* responsible for pullulan production thus remains unclear. However, in this thesis, examination of *A. pullulans* morphology during the pullulan production will be carried out in order to clarify which morphological form is responsible for pullulan production.

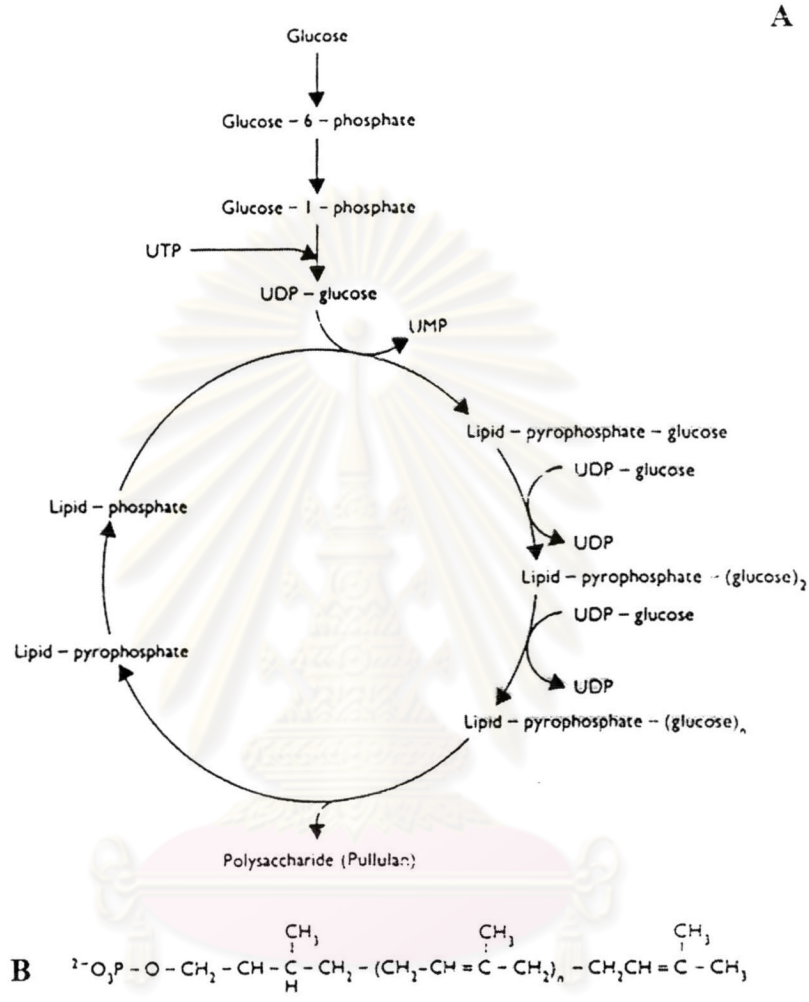

Figure 4 (A) Model of pullulan synthesis and transport of pullulan across the cell membrane, (B) structure of probable lipid carrier molecule, dolichol phosphate. (Berry, 1988)

1.2.2 Fermentation condition

P. pullulans QM 3092 uses mono- and disaccharides including glucose, fructose, maltose and sucrose in pullulan production with sucrose giving the highest yields (14.8 $g.l^{-1}$) (Catley, 1971). Sucrose also gives the highest yields by a mutant

strain of *A. pullulans* P56 in comparison to glucose, fructose, xylose, and lactose (Schuster et al., 1993). Sucrose was also found to be the most suitable substrate for pullulan production with a doubling of EPS production *A. pullulans* QM 3092 (3.1 $g.l^{-1}$ to 6.9 $g.l^{-1}$) was switched from glucose to sucrose (Reeslev et al., 1997). A color variant *A. pullulans* Y-2311-1 also used sucrose more efficiently than glucose with highest yields of 26.2 $g.l^{-1}$ (Gibson and Coughlin, 2002). A typical pigmented strain *A. pullulans* ATCC 42023, gave the greater yield on sucrose (7 $g.l^{-1}$) while maltose gave lowest yields (1.95 g.l-1) (West and Reed-Hamer, 1991). *A. pullulans* NRRL 6220 produced high pullulan yields with sucrose (41.9 $g.l^{-1}$), fructose (33.5 $g.l^{-1}$) or maltose (32.5 $g.l^{-1}$) (Badr-Eldin et al., 1994). The highest EPS yield of 10.2 $g.l^{-1}$ was gained using *A. pullulans* Y-12,996 grown on corn starch (Leathers et al., 1988). In contrast to all of these studies, glucose yielded better production that sucrose with Thai isolates (Punnapayak et al., 2003).

Xylan and lactose were not suitable for pullulan production with the color variant strains (Leathers et al., 1988). With alternative less-expensive substrates, *A. pullulans* P56 cultured using spent grain liquor yielded 11 $g.l^{-1}$ (Roukas, 1999). *A. pullulans* NRRL Y-12,974 grown on fuel ethanol byproducts and produced EPS, including corn fiber (0.9 $g.l^{-1}$) and corn condensed distiller's solubles (4.5 $g.l^{-1}$) (Leathers and Gupta, 1994). *A. pullulans* CFR-77, a local Indian isolate, grown on a concentrated sugar cane juice (jaggery) yielded a maximum of 23.01 $g.l^{-1}$ from 5%-jaggery medium (Vijayendra et al., 2001). Hydrolyzed starch waste with *A. pullulans* NRRL Y-6992 yielded a maximal EPS of 69 $g.l^{-1}$ using 20 % (w/v) of substrate (Barnett et al., 1999). This substrate was DE 42 obtained through a hydrolysis with alpla-amylase for 2 hr followed by hydrolysis with pullulanase and amyloglucosidase for 70 h. (Barnett et al., 1999).

In searching for a low cost substrate, beet molasses pretreated with sulfuric acid and using a colorless strain (*A. pullulans* P56) gave 24 $g.l^{-1}$ (Lazaridou et al., 2002). Pullulan yield in relation to strain and carbon source was presented (Table 1).

Table 1 Pullulan yield in relation to *A. pullulans* strain and carbon source

A. pullulans strain	Carbon source	Pullulan yield	Reference
A. pullulans NRRL 6220	Sucrose (5 % w/v)	41.9 $g.l^{-1}$	Badr-Eldin et al. (1994)
A. pullulans NRRL 6220	Hydrolyzed potato starch waste (20% w/v)	69 $g.l^{-1}$	Barnett et al. (1999)
P. pullulans QM 3092	Sucrose (5 % w/v)	14.8 $g.l^{-1}$	Catley (1971)
A. pullulans Y-2311-1	Sucrose (5% w/v)	26 $g.l^{-1}$	Gibson and Coughlin (2002)
A. pullulans P56	Pretreated beet molasses (5% w/v initial sugar)	24 $g.l^{-1}$	Lazaridou et al., 2002
A. pullulans Y-12, 996	Corn starch (2 % w/v)	10.2 $g.l^{-1}$	Leathers et al. (1988)
A. pullulans NRRL Y-12,974	Corn fiber (3% dry w/v)	0.9 $g.l^{-1}$	Leathers and Gupta (1994)
A. pullulans NRRL Y-12,974	Corn condensed distilled's solubled (10% wet w/v)	4.5 $g.l^{-1}$	Leathers and Gupta (1994)

Table 1 (con't) Pullulan yield in relation to *A. pullulans* strain and carbon source

A. pullulans strain	Carbon source	Pullulan yield	Reference
A. pullulans QM 3092	Sucrose (1.5% w/v)	6.9 $g.l^{-1}$	Reeslev et al. (1997)
A. pullulans P56	spent grain liquor (2.3% w/v initial sugar)	11 $g.l^{-1}$	Roukas (1999)
A mutant strain of *A. pullulans* P56	Sucrose (5 % w/v)	0.16 $g.l^{-1}$ per hour	Schuster et al. (1993)
A. pullulans CFR-77	Concentrated sugar cane juice (jaggerry) (5% w/v)	23.01 $g.l^{-1}$	Vijayendra et al. (2001)

Various nitrogen sources including organic and inorganic nitrogen including corn gluten, soybean, peptone, nitrate and ammonium salts have been evaluated for pullulan production. Of these Lancke and Rale (1994) found ammonium salts as the best nitrogen source. However, in complete contrast, complex nitrogen sources (tryptone, peptone, soytone, casamino acids and corn steep liquor) with *A. pullulans* ATCC 42023 resulted in greater yields than with ammonium sulphate (Reed-Hamer and West, 1994). The EPS is often not pure pullulan and the proportion of pullulan in the EPS was found to depend on the nitrogen source. Casamino acids, a very expensive laboratory substrate, gave lowest pullulan content of the EPS, compared to

ammonium sulphate. Also, the pullulan content was varied with the nitrogen sources. The casamino acids gave lowest pullulan content of polysaccharide while the high pullulan-content polysaccharide was liberated on other nitrogen sources.

Not only carbon and nitrogen source, pH is also a factor affecting to pullulan yield. West and Reed-Hamer (1993a) testing a range of 2.0 to 7.5, and using a sucrose-yeast extract medium with *A. pullulans* ATCC 42023 obtained optimal pullulan yields at pH 6.5 and lowest ones at pH 2. Similarly, highest yields of pullulan from *A. pullulans* IFO 4464 were obtained when the initial pH was 6 (Lee and Yoo, 1993). Protoplast of *A. pullulans* ATCC 42023 yielded pullulan at an initial pH 5.3 (Finkelman and Vardanis, 1982). Confounding these data of optimum between 5.0 and 7.0 are Reeslev's results with acidic pH being best pH 3-6 with maximal yields at pH 4.0 - *A. pullulans* QM 3092 (Reeslev et al., 1997), while Lazaridou et al. (2002) using *A. pullulans* P 56 grown on beet molasses gained maximal yields at an initial pH of 7.5 (24 $g.l^{-1}$ from 50 $g.l^{-1}$ in shake flasks). Thai isolates responded maximally in the more neutral range *A. pullulans* SU (0.158 $g.g^{-1}$) with greatest EPS at pH 6.5, for *A. pullulans* CU and PR the optimal for both was pH 7.5 (0.185 $g.g^{-1}$ and 0.225 $g.g^{-1}$) (Punnapayak et al., 2003). Overall an initial pH for optimal pullulan production appears in the range of 6 or 6.5 but it is clearly strain dependent.

The temperature of cultivation can affect pullulan yield. With *A. pullulans* ATCC 42023 cultured from 23 to 33°C the highest pullulan yield (16.3 $g.l^{-1}$) was at 26°C while the lowest (8.63 $g.l^{-1}$) was at 33°C (West and Reed-Hamer, 1993b). For two strains of *A. pullulans* isolated in Thailand (CU and PR), 30°C was optimal for EPS production giving respectively 0.186 $g.g^{-1}$ and 0.225 $g.g^{-1}$ for strain CU and PR while for strain SU the optimal temperature was 25°C (0.238 $g.g^{-1}$) (Punnapayak et

al., 2003). The yields in a range of studies in relation to temperature are presented in the Table 2.

Table 2 Pullulan yield in relation to *A. pullulans* strain and temperature

A. pullulans strain	Temperature (°C)	Pullulan yield	Reference
A. pullulans SU	25°C	0.238 $g.g^{-1}$	Punnapayak et al. (2003)
A. pullulans ATCC 42023	26°C	16.3 $g.l^{-1}$	West and Reed-Hamer (1993b)
A. pullulans Y-2311-1	26°C	26 $g.l^{-1}$	Gibson and Coughlin (2002)
P. pullulans F-1125	28°C	6.2 $g.l^{-1}$	Imshenetskii and Kondrat'eva (1977)
A. pullulans Y-12, 996	28°C	10.2 $g.l^{-1}$	Leathers et al. (1988)
A. pullulans IFO 4464	28°C	22 $g.l^{-1}$	Lee and Yoo (1993)
A. pullulans CU	30°C	0.186 $g.g^{-1}$	Punnapayak et al. (2003)
A. pullulans PR	30°C	0.225 $g.g^{-1}$	Punnapayak et al. (2003)

It can be concluded that optimal temperature for pullulan production by *A. pullulans* is in the range of 25-30°C. However, the effect of temperature is strain dependent, with those strains from Thailand perhaps being better at higher temperature.

The effect of vitamins and mineral salts on pullulan production by *A. pullulans* ATCC 42023 showed that supplementation with biotin, ferric chloride, manganese

chloride, and myo-inositol with sucrose increased yield from 2.33 $g.l^{-1}$ (non vitamins or mineral salts) up to 3.52 $g.l^{-1}$ (biotin), 4.68 $g.l^{-1}$ (ferric chloride), 4.22 $g.l^{-1}$ (manganese chloride), and 2.75 $g.l^{-1}$(myo-inositol) (West and Reed-Hamer, 1992) Pullulan production might respond to multiple vitamins or growth factors as yeast extract increases pullulan yield from 2.33 $g.l^{-1}$ up to 7 $g.l^{-1}$ (West and Reed-Hamer, 1992).

1.2.3 Fermentation method

A range of methods have been evaluated for pullulan production in order to obtain higher pullulan yields. Comparison of pullulan production between batch and continuous fermentation showed that that batch fermentation with *A. pullulans* P56 yielded 0.16 $g.l^{-1}$ per hour, while continuous fermentation increased pullulan yield up to 0.35 $g.l^{-1}$ per hour using a dilution rate at 0.05 $l.h^{-1}$ (Schuster et al., 1993). Using the same strain, maximum pullulan yield in batch fermentation as 31.3 $g.l^{-1}$ with sugar utilization (100%) while the highest pullulan yield of 24.5 $g.l^{-1}$ was obtained from fed-batch fermentation (Youssef et al., 1999). Fed-batch fermentation for pullulan production by *A. pullulans* IFO 4464 was conducted and compared to batch fermentation (Shin, et al. 1987). Using sucrose (10% w/v) as carbon source with this strain, maximal pullulan yield for fed-batch was 58 $g.l^{-1}$ while 36 $g.l^{-1}$ pulluian was obtained from batch fermentation. By fed-batch fermentation, *A. pullulans* ICCF-68 gave the highest pullulan yield of 101 $g.l^{-1}$ using glucose (19% w/v) (Moscovici et al., 1996). A reciprocating plate bioreactor for pullulan fermentation using *A. pullulans* strain 2552 was used to assess the effects of flow rate and dissolved oxygen (Audet et al., 1996). The highest EPS yield (28.2 $g.l^{-1}$) and biomass yield (8.7 $g.l^{-1}$) were obtained while using the highest aeration level as 0.8 vvm and dissolve oxygen level at 1.5 $mg.l^{-1}$. From the various studies of pullulan production, batch fermentation

appears as the generally used method, but perhaps simply because it is easiest to carry out. Continuous and fed-batch fermentation appears to be more effective, due to the higher yield obtained, than batch fermentation. Relation of pullulan yield to fermentation method for pullulan production was presented (Table 3).

Table 3 Pullulan yield in relation to *A. pullulans* strain and fermentation method

A. pullulans strain	Fermentation method	Pullulan yield	Reference
A. pullulans ICCF-68	Fed-batch	101 $g.l^{-1}$	Moscovici et al. (1996)
A. pullulans P56	Batch	0.16 $g.l^{-1}$ per hour	Schuster et al. (1993)
	Continuous	0.35 $g.l^{-1}$ per hour	
A. pullulans IFO 4464	Batch	36 $g.l^{-1}$	Shin et al. (1987)
	Fed-batch	58 $g.l^{-1}$	
A. pullulans P56	Batch	31.3 $g.l^{-1}$	Youssef et al. (1999)
	Fed-batch	24.5 $g.l^{-1}$	

1.2.4 Strain selection

Because of the melanin contamination during pullulan production, several attempts have been to use color variants or reduced pigmentation mutants. Pullulan production by color variant strains of *A. pullulans* was compared to several typical black pigmented strains (Leathers et al., 1988). The highest pullulan yield was from NRRL Y-12,974 and YB-4026 which were able to use cornstarch with 10%

conversion. Less melanin in the pullulan was obtained from *Aureobasidium* strain NRRL Y-12,974 (Leathers et al., 1988). A typical melanin pigmented strain, *A. pullulans* ATCC 42023, mutated using nitrous acid to obtain a reduced pigmentation strain (*A. pullulans* RP-1), which produced pullulan on glucose or sucrose in essentially the same yields as that of wild type though with colorless. Another reduced pigmentation mutant, *A. pullulans* NYSRP-1 selected by being resistant to nystatin, produced up to 66% less melanin than those from typical pigment strains on various carbon sources (West and Strohfus, 2001). Using a non-pigmented strain *A. pullulans* P-56 on sucrose, yield via batch fermentation gave maximal EPS of 31.3 $g.l^{-1}$ fed-batch fermentation maximally yielded 24.5 $g.l^{-1}$ (Youssef et al., 1999).

High-yield, pure pullulan was produced from a *P. pullulans* UV-mutant gave greater yields (10 $g.l^{-1}$) than that of wild type (3 $g.l^{-1}$) (Tarabasz-Szymanska and Galas, 1993). The EPS from the mutant also had lower melanin content.

Immobilized *A. pullulans* cells have been employed for pullulan production. Thus *A. pullulans* immobilized by adsorption on solid supports or entrapment in open polyurethane foam have been used for pullulan production (Mulchandani et al., 1989). Pullulan (18 $g.l^{-1}$) was obtained from both types of immobilized cells. *A. pullulans* strain ATCC 201253 entrapped in agarose and carrageenan was applied for pullulan production (West, 2000). The agarose-entrapped cells produced maximal pullulan yield (5.7 $g.l^{-1}$) higher than that from carrageenan-entrapped cells (4.9 $g.l^{-1}$).

Non-pigmentation strains or reduced-pigmentation mutants yield cleaner pullulan.

1.3 Chemical analysis of pullulan

Analysis of EPS was carried out using several methods in order to clarify the type, purity and components of the EPSs. The IR technique is useful for identification

of basic structure of polysaccharide including specific functional groups such as alkane, carbonyl, hydroxyl, hydroxyl in alcohol, and methyl groups (Amornsit and Petsom, 1992). Infrared (IR) analysis of two physically distinct *A. pullulans* (IMI 145194) EPSs (molecular weight and polymer composition) gave identical IR spectra which agreed well to a pullulan standard (Sigma), so that both were confirmed to be pullulan (Madi et al., 1997). A polysaccharide containing malic acid from *A. pullulans* had an IR spectrum with a characteristic 890 cm^{-1} of the β-configuration (Leal-Serrano et al., 1980). This same peak occurred in the spectra of a new EPS "aubasidan" from a new variety *A. pullulans* var. *aubasidani* (Yurlova and de Hoog, 1997). In the case of EPS from *A. pullulans*, IR technique could be used to confirm the type of EPS through α- or β- configuration. However, other methods must be used in combination of the IR results in order to obtain confirmation of the type of EPS.

Bonds between the glucose moieties in pullulan can be elucidated by ^{13}C-NMR spectroscopy (Gorin, 1981) or methylation analysis combined with Gas chromatography-Mass spectroscopy (GC-MS) (Madi et al., 1997). These elegant and definitive methods do not appear to be used very often by biologists may be due their cost. However, lower cost analyses based on enzymatic or acid hydrolyses followed by chromatographic analysis (paper or gas chromatograph), can be used although the results are not so definitive. Thus alpha-amylase and pullulanase was used to analyze pullulan from *A. pullulans* CH-1 and the digested fragments characterized by paper chromatography (Jakovljevic et al., 2001). This pullulan was susceptible to alpha-amylase yielding heterogenous fragments of different molecular weights. It could be that alpha-amylase attack to maltotetraose units which randomly exist in pullulan molecule (Catley, 1970). Therefore, they somehow interpreted this to mean that tetrasaccharide units must locate randomly in pullulan structure. However, this

method of analysis does not show any analysis of tetrasaccharide presence. The purity of pullulan from mutant of *P. pullulans* was determined by acid hydrolysis using HCl and found that the final products of hydrolysis were only glucose indicated that this EPS only has one sugar (Tarabasz-Szymanska and Galas, 1993). Commercial pullulans show over 95% sensitivity to pullulanase by these methods (Leathers et al., 1988). Pullulanase sensitivity is widely used to determine the pullulan content of EPS from *A. pullulans*. After pullulanase treatment, the reducing sugars are assessed as maltotriose equivalents by one of several methods such as DNS (Miller, 1959).

The molecular weight of pullulan can be measured by such techniques as gel permeation chromatography and size exclusion chromatography, or assessed via viscosity measurements. With methods available to me, I decided to use high performance size-exclusion chromatography to determine the molecular weight and the rheological property of EPS was examined by viscosity measurement.

1.4 Chemical modification of pullulan

Pullulan has been chemically modified to enhance its physical properties. Isocyanate derivatives showed greater water resistance and good solubility in general organic solvents (Shibata et al., 2001). Modified pullulans include chloroalkylated pullulan (Mocanu et al., 1999), pullulan sulfate (Mihai et al., 2001) and etherified pullulan (Shibata et al., 2002). The chloroalkylated pullulans are more hydrophobic (Mocanu et al., 1999), while propyl- and butyl- esterified pullulans have a clear glass transition, water resistant and have good solubility in general organic solvents (Shibata et al., 2002).

1.5 Biodegradation of pullulan

Pullulan is degraded by "pullulan-degrading enzymes" (Doman-Pytka and Bardowski, 2004) which are produced by a variety of micro-organisms. The enzymes

are grouped into: pullulanases (such as isopullulanase, neopullulanase, pullulanase type I and II, etc.) alpha-amylase and glucoamylase (Doman-Pytka and Bardowski, 2004). The mode of action of these enzyme groups is different (Figure 5). Pullulanase (EC 3.2.1.41) degrades pullulan by hydrolysis of the α-1,6 bond of the pullulan molecule. However, α-amylase and glucoamylase are also capable of degrading pullulan and only alpha-amylase affect pullulan molecular weight as they attack maltotetraose which randomly located in pullulan molecule (Leathers, 1993). Similar to this observation, Catley (1970) stated that alpha-amylase could attack pullulan at the maltotetraose molecules (Figure 6) resulting in reduction of pullulan molecular weight in late culture of pullulan production.

Pullulanase producers include such bacteria as *Bacillus stearothermophilus*, *Clostridium* sp., *Klebsiella pneumoniae*, *Thermus* sp., besides others (Doman-Pytka and Bardowski, 2004).

1.6 Properties and applications

The properties and applications of pullulan have been reviewed (Leathers, 2003 and Yuen et al., 1974). Pullulan is edible, non-toxic, odorless, and tasteless. It can be formed as film with high-oxygen impermeability, a useful property in food packaging and in high-fat food products to prevent oxidation. Derivatives of pullulan have been used as a binder and stabilizer in the food industry. Because of its resistance to mammalian amylase, pullulan can be incorporated into diet foods. In different manner, pullulan can be made into fibers that resemble nylon and rayon, and these are being considered for manufacture. Pullulans have been used in cosmetics, shampoos, and lotions. In agriculture, pullulan is employed as coating material for plant seed and fertilizer. A novel application of this polysaccharide is that it is a growth promoter for probiotic bacteria *Bifidobacterium* sp. (Leathers, 2003).

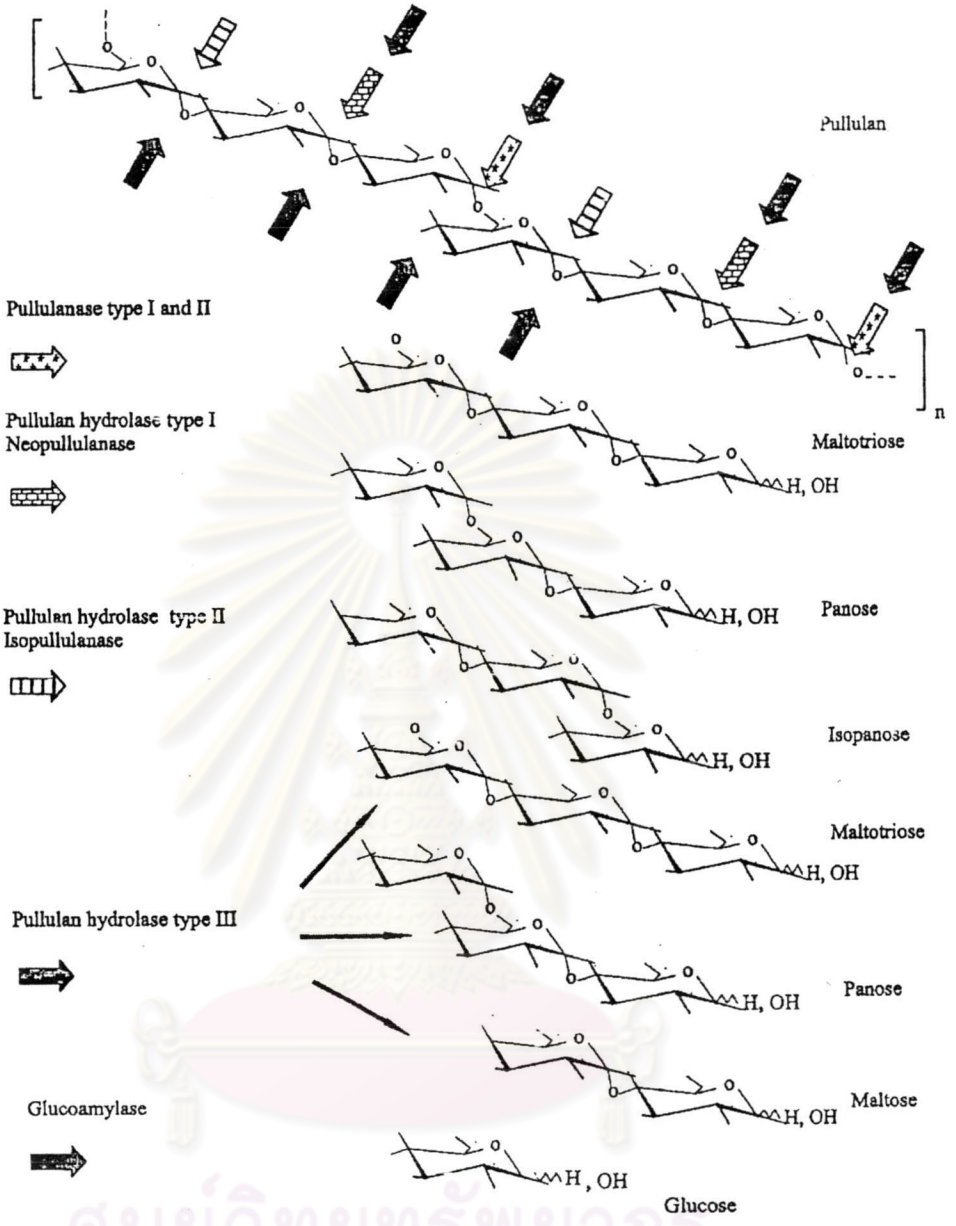

Figure 5 Enzymatic degradation of pullulan. (Doman-Pytka and Bardowski, 2004)

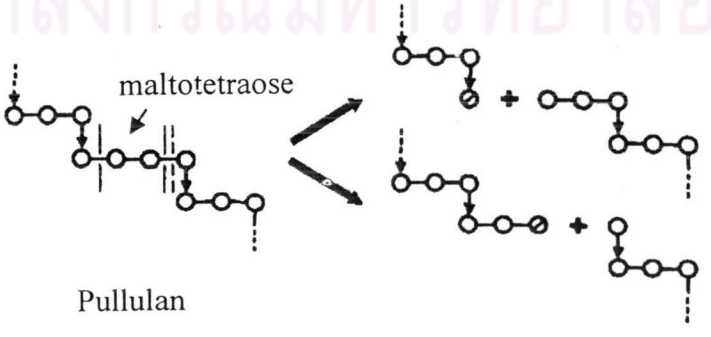

Figure 6 Pullulan degradation by alpha-amylase (Catley, 1970)

2. Amylase production

A. pullulans produces several important enzymes including alpha-amylase, DNase, β-glucosidase, laccase, pectinase, phosphatase, and xylanase (Deshpande et al, 1992). Amylase has diverse applications, especially in food industry. However, amylase produced by *A. pullulans* could be a problem in attacking its own product pullulan and reducing latter's molecular weight.

For growth on cheap starchy substrates amylases are essential but the use of these substrates and co-comitant induction of amylases can result in reduction the size of the pullulans.

A. pullulans NRRL-Y-12974, a color variant, produces α-amylase and the two forms of glucoamylase (glucoamylase A and glucoamylase B) when grow on starch at 28 °C (Saha and Bothast, 1993). This definitive study details the pH optima for these enzymes respectively, 5.0, 4.5, 4.0-4.5, with temperature optima of 55, 50-60, and 65 °C. Amylase of *Aureobasidium* NRRL Y-12974 attacked pullulans produced without induction through culture on glucose and maltose, and also with induction on soluble starch and corn starch (Leathers, 1993). The attack of the pullulan by amylases is a generic problem. I decided to address this problem by preparation of amylase negative mutants and also by considering the use of amylase inhibitors.

An enigma is that one study in which *A. pullulans* produced only glucoamylase to convert starch into biomass. The highest level of glucoamylase was obtained when using starch as carbon source (Federici, 1984).

3. Other applications

A. pullulans has a variety of interesting attributes in addition to enzyme and pullulan production. *A. pullulans* has been considered as a source of single cell protein based on culturing on waste straw hydrolysate (Han et al., 1976). It was not

toxic in rat feeding trials. Another attribute is that *A. pullulans* can utilize phenolic compounds. Thus immobilized *A. pullulans* has been proposed for phenolic waste treatment and a reactor was set up capable of removal of 40-50 mg/l/hr. of phenolics (Takahashi et al. 1981). Yet another attribute is *A. pullulans* capability to produce such biopolymers as aubasidan, poly-β-L-malic acid and fructooligosaccharides. Yurlova and De Hoog (1997) proposed a new variety of *A. pullulans* called "var. *aubasidani*" based on production of a novel biopolymer, aubasidan. Aubasidan is similar to pullulan in structure but has moieties in β-1,3 linkage.

Poly (β-L-malic acid) (PMA) (Figure 7) is a polyester exploited in medical application with use in drug-delivery systems. In general testing for PMA production, four from eight strains tested were shown to produce PMA (Liu and Steinbuchel, 1996). The highest yield of PMA using *A. pullulans* CBS 591.75 grown on glucose in a stirred-tank reactor was 9.8 $g.l^{-1}$. Another strain A-91, PMA was produced by non-growing cells of this strain yielded 80 $g.l^{-1}$ PMA (from 160 g glucose) under the optimal conditions (30°C, shake-flask at 240 rpm, the pH being controlled by adding 40 $g.l^{-1}$ of $CaCO_3$) while PMA of 25 $g.l^{-1}$ was produced from fermentation of this strain (Nakajima-Kambe et al., 1996).

Fructooligosaccharides (FOS) are used in the food processing industry. *A. pullulans* ATCC 9348 and *Gluconobacter oxydan* give remarkable yields of 160 $g.l^{-1}$ from 30% sucrose (around 53% conversion efficiency) in continuous culture (Sheu et al., 2002).

The diversity of *A. pullulans* capabilities has resulted in continued assessment of its physiology. *A. pullulans* from a Japanese sponge, yielded three new metabolites including diketopiperazines, with a D-cis-4-hydroxyproline residue and orcinotriol,

and a 1,3-dihydroxyphenol derivative (Shigemori et al., 1998). These novel metabolites are under investigation.

A. pullulans produces gluconic acid, a strain isolated from a wild flower yielding 140 $g.l^{-1}$in shake-flask and 260 $g.l^{-1}$ in continuous culture (Anastassiadis et al., 2003). These studies of gluconic acid production are important in relation to its use in the pharmaceutical, food, beverage and textile industries. A further metabolite is the antifungal "aureobasidin" produced by *A. pullulans* R106 (Takesako et al., 1991). It belongs to the family of antifungal cyclic depsipeptides (Fostel and Lartey, 2000). Aureobasidin is composed of eight α-amino acids and one hydroxyl acid (Figure 8)(Takesako et al., 1991), and with variations already eighteen aureobasidins (named A to R) have been characterized by NMR spectroscopy (Ikai et al., 1991). Aureobasidin inhibits inositol phosphoceramide (IPC) synthase which is involved in synthesis of sphingolipid an important component of fungal cell membrane (Fostel and Lartey, 2000). Aureobasidin is a developmental antifungal, with low toxicity to animal cells, but active towards such pathogens as *Candida albicans*, *Cryptococcus neoformans*, and some *Aspergillus* spp. (Takesako et al., 1991).

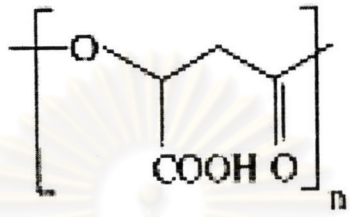

Figure 7 Chemical structure of Poly (β-L-malic acid), PMA. (Godde et al., 1999)

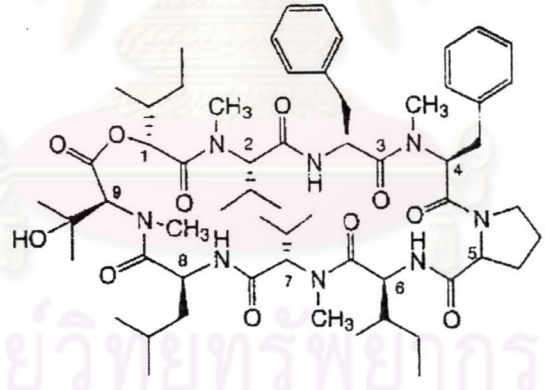

Figure 8 Chemical structure of aureobasidin A. (Fostel and Lartey, 2000)