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Pullulan

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Introduction

Pullulan is a linear homopolysaccharide of glucose that often is described as an $\alpha-(1\rightarrow6)$ linked polymer of maltotriose subunits. This unique linkage pattern endows pullulan with distinctive physical traits. Pullulan has adhesive properties and can be used to form fibers, compression moldings, and strong, oxygen-impermeable films. Pullulan is derivatized easily to control its solubility or provide reactive groups. Consequently, pullulan and its derivatives have numerous potential food, pharmaceutical, and industrial applications. Pullulan is produced as a water-soluble, extracellular polysaccharide by certain strains of the polymorphic fungus *Aureobasidium pullulans* (De Bary) Arnaud (formerly known as *Pullularia pullulans* (De Bary) Berkhout or *Dematium pullulans* De Bary). A number of reviews on pullulan have appeared, including those by Yuen (1974), Jeanes (1977), Zajic and LeDuy (1977), Slodki and Cadmus (1978), Catley (1979), Kondrat’eva (1981), Sandford (1982), LeDuy et al. (1988), Deshpande et al. (1992), Pollock (1992), Robyt (1992), Seviour et al. (1992), Tsujisaka and Mitsuhashi (1993), Dillon and Martin (1994), Côté and Ahlgren (1995), Lachke and Rale (1995), De Baets and Vandamme (1999), and Israilides et al. (1999).

Historical Outline

*Aureobasidium pullulans* was first described (as *Dematium pullulans*) by De Bary in 1866 (Cooke, 1959). Bauer (1938) made early observations on polysaccharide formation by *A. pullulans*, and Bernier (1958) isolated and began to characterize the polymer. Bender et al. (1959) studied the novel polysaccharide and named it “pullulan”. During the 1960s, the basic structure of pullulan was resolved (Bender and Wallenfels, 1961; Wallenfels et al., 1961, 1965; Bouveng et al., 1962, 1963a; Sowa et al., 1963; Ueda et al., 1963). Bender and Wallenfels (1961) discovered the enzyme pullulanase, which specifically hydrolyzes the $\alpha-(1\rightarrow6)$ linkages in pullulan and converts the polysaccharide almost quantitatively to maltotriose. Thus, pullulan is commonly viewed as an $\alpha-(1\rightarrow6)$ linked polymer of maltotriose subunits. Catley and coworkers subsequently established the occurrence of a minor percentage of randomly distributed maltotetraose subunits in pullulan (Catley et al., 1966; Catley, 1970; Catley and Whelan, 1971; Carolan et al., 1983). Commercial production of pullulan began in 1976 by the Hayashibara Company, Ltd., in Okayama, Japan (Tsujisaka and Mitsuhashi, 1993).
Chemical Structure

Bernier (1958) isolated water-soluble polysaccharides from cultures of *A. pullulans* and reported that glucose is the major product of acid hydrolysis. Based on the positive optical rotation and infrared (IR) spectrum of pullulan, Bender et al. (1959) concluded that the polymer is an α-glucan in which α-(1→4) linkages predominate. Subsequent studies using IR, periodate oxidation, and methylation analysis established that pullulan is essentially a linear glucan containing α-(1→4) and α-(1→6) linkages in a ratio of 2:1 (Wallenfels et al., 1961, 1965; Bouveng et al., 1962; Sowa et al., 1963). Partial acid hydrolysates of pullulan include isomaltose, maltose, panose, and isopanose (Bender et al., 1959; Bouveng et al., 1963a; Sowa et al., 1963). The discovery of the enzyme pullulanase provided a critical tool for the analysis of the structure of pullulan (Bender and Wallenfels, 1961). Pullulanase specifically hydrolyzes the α-(1→6) linkages of pullulan and converts the polymer almost quantitatively to maltotriose (Bender and Wallenfels, 1961; Wallenfels et al., 1961, 1965). Based on this result, pullulan frequently is described as a polymer of α-(1→6) linked maltotriose subunits (Figure 1). However, pullulan also can be viewed as a polymer of panose or

Fig. 1 Chemical structure of a representative portion of pullulan, illustrating the primary structure of repeating linkages. Figure courtesy of Dr. Gregory L. Côté.
isopanose subunits, which may reflect more accurately the biosynthetic origins of the molecule. Indeed, a number of enzymes have been described since that produce panose or isopanose from pullulan (see Section 9).

Catley and coworkers established that pullulan contains maltotetraose subunits (Figure 2) in addition to the predominant maltotriose subunits (Catley et al., 1966; Catley, 1970; Catley and Whelan, 1971). The frequency of maltotetraose subunits appears to vary on a strain-specific basis, from about 1% to 7% of total residues (Taguchi et al., 1973a; Catley et al., 1986). Evidence suggests that maltotetraose subunits are distributed randomly throughout the molecule (Carolan et al., 1983). Unlike the maltotriose subunits in pullulan, maltotetraose residues are substrates for many α-amylases, and it has been proposed that hydrolysis of pullulan at these sites accounts for the decrease in molecular weight commonly observed in late cultures (see Section 9).

It is possible that pullulan contains additional minor structural features, perhaps depending on culture conditions and strain differences. Sowa et al. (1963) reported that pullulan from one source contains 6% α-(1 → 3) linkages, and Fujii et al. (1984) indicated that pullulan from A. pullulans strain FERM-P4257 includes some α-(1 → 3) linkages. Fungal glucoamylases generally attack pullulan in a sequential, exo-fashion from the non-reducing end, hydrolyzing both α-(1 → 4) and α-(1 → 6) linkages. Marshall (1975) found that a glucoamylase from Cladosporium resinae digests pullulan rapidly but incompletely and concluded that the molecule may contain anomalous linkages. Similarly, Catley et al. (1986) reported that pullulan from A. pullulans strain P-50 is partially resistant to digestion by glucoamylase from Aspergillus niger and suggested that the molecule may contain randomly distributed branches or alternative subunit residues. Zemek et al. (1980) proposed that pullulan from six strains of A. pullulans contains glycogen-like stretches of 52–135 glucosyl residues at the non-reducing termini.

![Fig. 2](image-url)  
**Fig. 2** Chemical structure of the secondary (minor) repeating structure of pullulan, occurring in about 1% to 7% of total linkage subunits. Figure courtesy of Dr. Gregory L. Côté.
Many reports describe additional (non-pullulan) polysaccharides from cultures of A. pullulans. Bernier (1958) noted the production of a distinct insoluble “jelly” associated with mycelial mats, and Wallenfels et al. (1965) developed a method to better isolate pullulan (termed “restpullulan”) from this material. Bouveng et al. (1962) reported that cultures grown on sucrose produce both pullulan and an acidic glucan that contains uronic acid, while cultures grown on maltose produce a heteropolysaccharide that contains glucose, galactose, and mannose. On xylose, cultures made three polysaccharides: (1) pullulan; (2) an acidic heteropolysaccharide that contained glucose, galactose, mannose, and glucuronic acid; and (3) a β-linked glucan containing (1→3) and (1→6) linkages (Bouveng et al., 1963b). Taguchi et al. (1973a) reported that the production of acidic extracellular polysaccharide varies among three strains of A. pullulans. Cell walls of A. pullulans contain both heteropolysaccharides and β-glucan with (1→3) and (1→6) linkages (Brown and Lindberg, 1967a,b; Brown et al., 1973). Kikuchi et al. (1973) described an insoluble heteropolysaccharide from A. pullulans strain S-1 that contains glucose, mannose, and galactose in ratios similar to polysaccharides from cell-wall extracts, and they suggested that this material is released by autolysis, possibly during cell morphogenesis. Elinov and Matveeva (1972) and Elinov et al. (1974, 1975) reported that A. pullulans strain VKPM F-448 produces a homoglucan with a β-(1→3) linked backbone and α-(1→4) linked side chains attached by β-(1→6) linkages. This potentially useful polysaccharide was later termed “aubasidan” (Elinov et al., 1987). Yurlova and de Hoog (1997) subsequently identified a distinct taxonomic group of A. pullulans strains that make both pullulan and aubasidan-like polysaccharides. Fujii et al. (1984) reported that strain FERM-P4257 secretes a β-(1→3)-linked glucan as an alternative to pullulan, depending on the medium used. Leal-Serrano et al. (1980) reported that A. pullulans strain CBS 105.22 produces an acid β-glucan with 68% (1→3) and 32% (1→6) linkages and containing malic acid. Hamada and Tsujisaka (1983) found that Aureobasidium sp. strain K-1 forms an acidic glucan containing sulfotiacetic acid, with a β-(1→3) linked backbone and single β-(1→6) branch linkages on three of four backbone residues. Promma et al. (1997) described a marine isolate of A. pullulans that makes an acidic α-glucan containing malic acid, with alternating (1→6) and (1→4) backbone linkages and regular (1→3) branch linkages.

4 Physiological Function

The physiological function of pullulan is uncertain. Since Aureobasidium strains cannot break down pullulan significantly to metabolizable sugars, pullulan presumably does not serve as a storage material. It is generally supposed that pullulan and similar polysaccharides serve to protect cells from dessication or help them adhere to environmental substrates. Andrews et al. (1994) reported that pullulan helps A. pullulans adhere to leaf surfaces. Bardage and Bjurman (1998) concluded that pullulan is important in the adhesion of A. pullulans blastospores to painted wood.

5 Chemical Analyses

General methods applicable to polysaccharides can be used to detect and quantitate pullulan. As described above, the basic structure of pullulan was deduced in part
from its specific optical rotation and IR absorption characteristics. Periodate analysis, methylation analysis, and partial acid hydrolysis were also important. However, studies using pullulanase, which specifically hydrolyzes the $\alpha-(1 \rightarrow 6)$ linkages in pullulan, were definitive in resolving the fundamental structure of the polymer (Bender and Wallenfels, 1961). Assays based on pullulanase remain the principal method for the specific detection and measurement of pullulan. Catley (1971a) developed a sensitive radioassay for pullulan. A. pullulans cultures were fed $^{14}$C-glucose to produce labeled pullulan. After pullulanase digestion, maltotriose and maltotetraose were separated by paper chromatography and measured by liquid scintillation counting. Finkelman and Vardanis (1982b) described a simplified radioassay based on the solubilization by pullulanase of radioactive counts into ethanol. Leathers et al. (1988) described a quantitative pullulan assay based on reducing sugars (maltotriose equivalents) released by pullulanase digestion. Israelides et al. (1994) suggested the complete hydrolysis of pullulan by means of pullulanase and glucoamylase, followed by the specific measurement of glucose using glucose oxidase. Imshenetski and Kondrat’eva (1978) devised a rapid assay based on the turbidity of pullulan suspensions in water–ethanol mixtures. An enzyme sensor for measuring pullulan or pullulanase also has been reported, utilizing immobilized glucoamylase and glucose oxidase (Renneberg et al., 1985). The molecular weight of pullulan has been estimated by various methods, including light scattering (Ueda et al., 1963), sedimentation behavior (Wallenfels et al., 1965), viscosity (Wallenfels et al., 1965), and chromatography (Catley, 1970).

6 Occurrence

A. pullulans is a ubiquitous fungus, commonly isolated from environmental samples (Cooke, 1959; Hermanides-Nijhof, 1977). It is found in soil and water, particularly as an early-colonizing saprophyte on decaying leaf litter, wood, and many other plant materials. A. pullulans often is reported as a (usually mild) plant pathogen, notably of grapes. It is important in the post-harvest decay of fruits and other crops. A. pullulans has been studied for its role in the deterioration of house paints (Brand and Kemp, 1973) and in the rotting of military equipment in the tropics (Marsh and Bollenbacher, 1949). It also has been reported as a troublesome slime-producing contaminant of paper mills. It can colonize optical lenses and electronic equipment and has been implicated in the biodeterioration of plasticized polyvinyl chloride (Webb et al., 2000). Clinical isolates are known, and insect associations have been reported. The fungus has been described as omnivorous, and isolates produce an impressive array of degradative enzymes, including amylases (Federici, 1982; Leathers, 1987, 1993; Linardi and Machado, 1990; Saha et al., 1993) proteases, (Ahearn et al., 1968; Federici, 1982), esterases (Federici, 1982), pectinases (Dennis and Buhagiar, 1973; Finkelman and Zajic, 1978; Federici, 1982), and hemicellulases, including xylanase (Flannigan, 1970; Biely et al., 1978, 1979; Saha and Bothast, 1998) and mannanase (Kremnicky et al., 1996; Kremnicky and Biely, 1997). So-called “color-variant” strains of Aureobasidium, some of which produce pullulan, are natural overproducers of endoxylanase (Leathers et al., 1984; Leathers, 1986; 1989). A. pullulans utilizes cellobiose, but not cellulose (White et al., 1948; Marsh et al., 1949; Flannigan, 1970; Dennis, 1972; de Hoog and Yurlova,
1994; Saha et al., 1994). Surveys suggest that many, but not all, strains are capable of producing pullulan (Ueda et al., 1963; Leathers et al., 1988; Augustin et al., 1997). However, optimal culture conditions for pullulan production appear to vary considerably on a strain-specific basis (Silman et al., 1990; Yurlova et al., 1995).

Pullulan also has been isolated from the saprophytic (and sometimes mycoparasitic) fungus *Tremella mesenterica* (Fraser and Jennings, 1971), from the obligate tree parasitic fungi *Cyttaria harioti* and *C. darwinii* (Waksman et al., 1977; Oliva et al., 1986), and from the fungal agent of chestnut blight, *Cryphonectria parasitica* (Corsaro et al., 1998). Interestingly, Corsaro et al. (1998) reported that pullulan from *C. parasitica* exhibits mild phytotoxic effects against both host and non-host (tomato) tissues.

7 Biosynthesis

7.1 Culture Conditions and Cell Morphology

Much of the published research on pullulan concerns the control of pullulan synthesis by culture conditions and the relationship between pullulan production and cell morphology. Overall, this literature is confusing and seemingly contradictory. In part, this may be because multiple factors interact in the regulation of pullulan biosynthesis. Perhaps more important may be the role of strain variability. In comparative studies, *A. pullulans* strains have been observed to differ considerably with respect to growth, pullulan yields, and cell morphology (Ueda et al., 1963; Cernakova et al., 1980; Kockova-Kratochvilova et al., 1980; Park, 1982b; Gadd and Cooper, 1984; Leathers et al., 1988; Silman et al., 1990; Augustin et al., 1997). Optimal conditions for pullulan production may depend on the particular isolate of *A. pullulans* employed.

*A. pullulans* strains typically produce pullulan when cultured on sucrose, glucose, fructose, maltose, starch, or maltooligosaccharides (Bender et al., 1959; Ueda et al., 1963; Catley, 1971b; Behrens and Lohse, 1977; Imshenetskii et al., 1981a, 1985b; Leathers et al., 1988; West and Reed-Hamer, 1991; Badr-Eldin et al., 1994). Sucrose often has been described as the optimal substrate. Less frequently, sugars such as xylose, arabinose, mannose, galactose, rhamnose, and lactose have been reported to support pullulan production, usually in reduced yields (Bouveng et al., 1962, 1963b; Ueda et al., 1963; Imshenetskii et al., 1981a; Le-Duy et al., 1983). Perhaps the best-characterized pullulan-producing strain is *A. pullulans* QM3090 (also known as NRRL Y-2567 or ATCC 9348; in some publications, this strain is referred to as QM 3092). Using this strain, Catley (1972) found that pullulan synthesis is inducible by glucose, fructose, or saccharides that can be broken down to these sugars. Induction of pullulan synthesis was inhibited by cycloheximide, indicating that new protein synthesis is required (Catley, 1972). Catley (1971b) reported that glycerol can contribute to pullulan synthesis, but only in the presence of the inducer glucose. This result also suggests that potential contamination by glucose is a concern in substrate-utilization studies.

*A. pullulans* has been exploited for some time as a model organism for studies of fungal morphogenesis. Although the organism is actually polymorphic, studies usually have focused on dimorphic transitions between yeast-like cells and mycelia, or between yeast-like cells and chlamydospores (large resting cells). Again, much of this literature appears to be inconsistent, possibly owing to differences among strains of *A.*
Pullulan. Brown et al. (1973) described a strain of A. pullulans that is mainly yeast-like during growth on nitrate as a nitrogen source and is more filamentous during growth on ammonium. On the other hand, Park (1982b) found under different conditions that ammonium is not required for morphogenesis from yeast-like to filamentous growth, although it does favor maintenance of mycelial growth. Sevilla et al. (1977) described a yeast-like strain of A. pullulans that grows in a filamentous form only with cysteine as a nitrogen source. However, Park (1982a) characterized a different strain in which mycelial germination is supported by a number of amino acids. Vinroot and Torzilli (1988) found that high pH (7.5) inhibits yeast-like growth. In contrast, Park (1982a) concluded that pH is not correlated with cell morphology. Low inoculum densities appear to favor mycelial development in some strains (Ramos and Garcia Acha, 1975; Park, 1984), while high inoculum densities seem to favor mycelial growth in other strains (Catley, 1980; Vinroot and Torzilli, 1988). Similarly, high agitation rates may favor either yeast-like (Leal-Serrano et al., 1980) or mycelial (Vinroot and Torzilli, 1988) growth. A number of other factors have been found to affect the morphology of A. pullulans. Moragues et al. (1988) observed that n-alkanols promote hyphal development of strain CECT 2660 (ATCC 48433). Copper and yeast extract were reported to promote filamentous growth of strain IMI 45533 (Gadd and Griffiths, 1980; Cooper and Gadd, 1984). Conditions of limiting nitrogen and low buffering capacity (allowing a drop in culture pH) promoted a shift in A. pullulans strain CECT 2660 from yeast-like cells to chlamydospores (Domínguez et al., 1978; Bermejo et al., 1981a,b).

Correlations between A. pullulans morphology and pullulan production often have been reported, although it is not clear that these are cause-and-effect relationships. Catley (1980) concluded that the yeast-like form of strain QM 3090 is the primary producer of pullulan. During growth on a suitable carbon source, batch cultures exhibited a drop in pH and a shift from mycelial to yeast-like morphology upon limitation of ammonium nitrogen (Catley, 1971a, 1973). Pullulan was produced in late exponential and early stationary-phase cultures, and thus primarily by yeast-like cells. Ono et al. (1977b) made similar observations using A. pullulans strain S-1 and concluded likewise that yeast-like cells are the source of pullulan. Kelly and Catley (1977) isolated mutants of QM 3090 that exhibit an increased proportion of yeast-like cells and increased pullulan yields. Consistent with the notion that ammonium inhibits pullulan synthesis, Imshenetskii et al. (1981a) found that derivatives of A. pullulans strain 1125 produce more pullulan on nitrate than on ammonium nitrogen sources. Seviour and Kristiansen (1983) also reported that free ammonium ions inhibit pullulan formation by strain QM 3092 (ATCC 201428). Heald and Kristiansen (1985) decided that the yeast-like form of QM 3092 is the primary source of pullulan and found that the rate of pullulan synthesis is independent of pH. Using this strain, McNeil and Kristiansen (1987) reported that pullulan yield and molecular weight, as well as the percentage of yeast-like cells, increase as a function of fermentor impeller speed. Using strain QM 3090, Bulmer et al. (1987) concluded that pullulan is a secondary metabolite produced by yeast-like cells during ammonium limitation.

In contrast, McNeil et al. (1989) found that pullulan production by strain QM 3092 under chemostat conditions is optimal at pH 4.5, which favors a 50% mixture of yeast-like and hyphal cells. Auer and Seviour (1990) confirmed that ammonium ions
repress pullulan formation by strain QM 3092. Nevertheless, they found no clear correlations among morphology, pH, and pullulan yields. Reeslev et al. (1991, 1993) and Reeslev and Jensen (1995) reported that pullulan production by batch cultures of QM 3092 is correlated with the formation of yeast-like cells, promoted by low levels of Zn$^{2+}$, Fe$^{3+}$, or yeast extract. However, under chemostat conditions that favored yeast-like growth from pH 3 to 7, pullulan production was found to be optimal only at pH 4.0 (Reeslev et al., 1997). Gibbs and Seviour (1992) concluded that bioreactor design and nitrogen source affect pullulan formation by strain QM 3092, but not its morphology. LeDuy and Boa (1983) reported that strains 2552 and 140B grow with different morphologies on peat hydrolysate medium but make equivalent amounts of pullulan. Studies with A. pullulans strain ATCC 42023 (ppKM-3) found that ammonium and complex nitrogen sources are superior to nitrate for pullulan production (West and Reed-Hamer, 1991; Reed-Hamer and West, 1994).

Lacroix et al. (1985) developed a two-stage fermentation process for pullulan, based on the finding that strains 2552 and 140B grow best at pH 2.0 but make pullulan only at pH 5.5. Lee and Yoo (1993) reported that A. pullulans strain IFO 4464 provides optimal yields of pullulan at an initial pH of 6.0, although pullulan molecular weights are highest at an initial pH of 3.0. Imshenetskii et al. (1981b) determined that an initial pH of 8.0 is optimal for pullulan synthesis by strain 11254 and its derivatives. Using A. pullulans strain IMI 145194, Madi et al. (1996) found that pH 4.5 is optimal for pullulan production, while yeast-like growth is favored at pH 6.5. Yamasaki et al. (1993a,b) observed that strain S-2 is primarily yeast-like during active growth, associated with pullulan accumulation, and becomes partially mycelial in late cultures. On the other hand, Simon et al. (1993, 1995) concluded that pullulan formation by A. pullulans strain CBS 70076 is associated mainly with swollen cells and chlamydo-spores. Similarly, Andrews et al. (1994) reported that strains Y-117 (ATCC 90393) and FS 43–262 (ATCC 90394) make pullulan on conversion to swollen cells and chlamydo-spores, mediated by conditions of limiting nitrogen or medium acidification.

A. pullulans is a strict aerobe, and oxygen is required for both its growth and pullulan production (Ono et al., 1977b; Rho et al., 1988; Dufresne et al., 1990; Madi et al., 1996). However, Wecker and Onken (1991) reported that optimal pullulan yields from strain QM 3090 are obtained at decreased dissolved oxygen conditions, and Gibbs and Seviour (1996) observed that high oxygen levels dramatically reduce yields. Audet et al. (1996) concluded that intermediate dissolved oxygen concentrations are optimal for pullulan synthesis by strain 2552, as high concentrations enhance yields at the expense of molecular weight. It also has been suggested that shear stress associated with fermentor agitation may influence both cell morphology and pullulan formation (McNeil and Kristiansen, 1987).

Optimal temperatures for pullulan production appear to vary slightly on a strain-specific basis, usually in the range of 24°C to 30°C (Zajic, 1967; Imshenetskii et al., 1981b; McNeil and Kristiansen, 1990; Tsujisaka and Mitsuhashi, 1993; West and Reed-Hamer, 1993a). Vitamins and minerals also may influence pullulan synthesis. Bender et al. (1959) found that thiamine increases pullulan yields. Biotin, ferric chloride, manganese chloride, and zinc chloride were reported to enhance pullulan formation by A. pullulans strain ATCC 42023 (West and Reed-Hamer, 1992; West and Strophus, 1997a). However, while using strain QM 3092, Reeslev et al. (1993) and Reeslev and...
Jensen (1995) determined that Fe³⁺ and Zn²⁺ inhibit the development of both yeast-like cells and polysaccharide.

Although the growth of *A. pullulans* is not always easy to measure reliably, many studies support the idea that pullulan production occurs primarily during the stationary-growth phase (Ueda et al., 1963; Ono et al., 1977b; McNeil and Kristiansen, 1987, 1990). In a survey of 18 strains of *A. pullulans*, Leathers et al. (1988) reported an inverse relationship between biomass and pullulan yields, consistent with the notion that pullulan is a secondary metabolite generated during growth limitation in excess carbon. On the contrary, a survey of 31 different strains under different conditions found that biomass and pullulan yields vary independently (Augustin et al., 1997). Kristiansen et al. (1982) determined that pullulan production follows intermediate kinetics. Other studies show that pullulan formation is associated principally with active cell growth. A kinetic analysis by Klimek and Ollis (1980) concluded that pullulan synthesis is wholly growth-associated.

Some studies have suggested an inverse relationship between pullulan yields and apparent molecular weights (Leathers, 1987; Pollock et al., 1992; Audet et al., 1996). The molecular weight of pullulan may depend not only on the synthetic characteristics of the culture but also on endogenous glucanases that partially degrade pullulan. Pullulan molecular weight generally falls with culture time, presumably because of the action of these enzymes (see Section 9). Interestingly, Madi et al. (1997) reported that strain IMI 145195 produces two distinct pullulan fractions that differ only in molecular weight (2 x 10⁶ and 4.3 x 10⁵). Similarly, McNeil and Kristiansen (1987) observed two molecular weight species in some pullulan preparations. Strains have been isolated that favor production of high-molecular-weight pullulan (Pollock et al., 1992; Thorne et al., 1993, 2000), and molecular weight can be influenced by control of culture conditions (Yuen, 1974; Kato and Shiosaka, 1975b; Kaplan et al., 1987; Wiley et al., 1987, 1993). Low initial phosphate and pH levels have been reported to favor the formation of high-molecular-weight pullulan (Sugimoto, 1978; Tsujisaka and Mitsuhashi, 1993). Apparent pullulan molecular weights as high as approximately 10⁶ to 10⁷ have been reported.

*A. pullulans* is considered to be one of the “black yeasts”, and pullulan contamination by fungal melanin is a common problem. Melanin production appears to vary considerably, depending on strain characteristics and culture conditions (Kockova-Kratochvilova et al., 1980; Leathers et al., 1988; Silman et al., 1990). Pigment accumulation typically occurs late in culture growth, possibly associated with the formation of chlamydospores. Naturally occurring and mutant strains have been isolated that produce reduced levels of melanin (Leathers et al., 1988; Silman et al., 1990; Bock et al., 1991; Pollock et al., 1992; Tarabasz-Szymanska and Galas, 1993; West and Reed-Hamer, 1993b).

### 7.2 Biosynthetic Mechanism

Relatively little is understood about the mechanism of pullulan biosynthesis. Unlike bacterial dextrans, which are synthesized extracellularly by secreted glucansucrases, pullulan is synthesized intracellularly and secreted by *A. pullulans*. Ueda and Kono (1963) found that acetone-dried cells of *A. pullulans* transform maltose to form panose, maltotriose, and maltotetraose. Taguchi et al. (1973b) reported pullulan synthesis by cell-free extracts of *A. pullulans* from uridine 5’-diphosphate-glucose (UDPG) in the presence of adenosine 5’-triphosphate.
(ATP). Extracts did not form pullulan from sucrose or adenosine 5'-diphosphate-glucose (ADPG), and ATP absolutely was required. Acetone-dried cells produced pullulan from sucrose and incorporated 14C-labeled sucrose into organic extracts that included lipid fractions, predicting the involvement of a lipid intermediate (Taguchi et al., 1973b). Further analysis indicated that the lipid is a discrete species that contains a glucose moiety and pyrophosphate. Cold sucrose quickly chased the label from this fraction, indicating a rapid turnover of this intermediate. Ono et al. (1977a) confirmed that glucolipids are formed in mycelia believed to be accumulating pullulan. Catley and McDowell (1982) found that A. pullulans incorporates 14C-labeled glucose into lipid-linked glucose, isomaltose, panose, and isopanose. Based on these results, a reaction mechanism was proposed in which pullulan is formed by the polymerization of either panosyl or isopanosyl moieties (Catley and McDowell, 1982). An occasional direct linkage of panosyl and isopanosyl moieties was postulated to form the minor maltotetraosyl elements in pullulan. Mechanisms involving polyprenyl-linked saccharides are well established in the synthesis of oligosaccharides and polysaccharides of diverse organisms, including Klebsiella (Sutherland and Norval, 1970) and Saccharomyces (Lehle and Tanner, 1978). This model contrasts with the transglucosylation mechanism invoked in the synthesis of dextran (Robyt, 1995). Nevertheless, a glucosyltransferase that catalyzes the synthesis of panose and isomaltose from maltose has been characterized from A. pullulans (Hayashi et al., 1994a,b).

Catley and Hutchison (1981) prepared spheroplasts of A. pullulans that were impaired in pullulan elaboration and concluded that the assembly or secretion of the polysaccharide may be associated with the cell wall, plasma membrane, or periplasmic space. However, Finkelman and Vardanis (1982a) prepared protoplasts of A. pullulans that were able to produce pullulan and concluded to the contrary. No enzymes involved in pullulan biosynthesis have been identified at this time, and attempts to repeat the cell-free synthesis of pullulan as described by Taguchi et al. (1973b) have proven unsuccessful (Catley and McDowell, 1982; Israilides et al., 1999).

8 Genetics and Molecular Biology

A. pullulans is genetically imperfect and traditionally has been considered to be among the Fungi Imperfecti or Deuteromycetes (Wynne and Gott, 1956; Cooke, 1962; Hermanides-Nijhof, 1977). More recently, Aureobasidium has been described as a filamentous ascomycete (Eucaryomycetes, order Dothideales, family Dothideaceae) capable of growing yeast-like in culture (de Hoog and Yurlova, 1994; de Hoog, 1998). Aureobasidium commonly is referred to as a yeast-like fungus and often is considered to be one of the “black yeasts” because many cultures produce fungal melanin. However, the fungus is more properly polymorphic and has a complex life cycle featuring a range of morphological forms (Cooke and Matsura, 1963; Ramos and Garcia Acha, 1975; Pechak and Crang, 1977; Kockova-Kratochvilova et al., 1980). Cultures commonly contain a mixture of cell morphologies, ranging from yeast-like budding cells to highly mycelial forms and including “swollen cells” and large chlamydospores. The distribution of cell morphologies varies considerably as a function of culture conditions and strain differences.

As previously discussed, strain variability appears to be an important factor in pullulan production and other characteristics of A.