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Review

Aureobasidium pullulans, an economically important polymorphic yeast with special reference to pullulan

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***Aureobasidium pullulans*, popularly known as black yeast, is one of the most widespread saprophyte fungus associated with wide range of terrestrial and aquatic habitats, in temperate and tropical environment. It is a polymorphic fungus that is able to grow in single yeast-like cells or as septate, polykaryotic hyphae showing synchronous conditions, with budding cells. This fungus has been exploited potentially for commercial production of various enzymes (amylase, xylanase, pectinase, etc). Single cell protein, alkaloids and polysaccharide, especially pullulan, an exopolysaccharide, is a linear α-d-glucan connected with α-1,4 glycosidic bond mainly of maltotriose repeating units interconnected by α-1,6 linkages. Pullulan has been considered as one of the important polysaccharide for production of biodegradable plastics. More than 300 patents for applications have been developed. It is the only fungus which produce higher amount of pullulan and has been exploited all over the world. The fungus has excellent genetic make-up to produce various important metabolites at commercial production with limited species. Some of the *A. pullulans* have potential antagonistic activity against a number of phytopathogenic fungi used as bio-control agents of post-harvest diseases. It has been found to be tolerant to many metal ions which are common pollutants of soil and water. Several strains of this fungus have ability to degrade xenobiotic compounds. In the light of the above facts, this review article has emphasized on the orientation, morphology, biochemical characteristics, habitats and its economic potentials with special reference to pullulan.**

Key words: *Aureobasidium pullulans*, single cell protein, xenobiotic compounds, pullulan.

INTRODUCTION

Aureobasidium pullulans (De Bary) G. Arnoud is a ubiquitous, polymorphic and oligotrophe black yeast like microfungus that occurs frequently in wide range of tropical and temperate environment with fluctuating moisture content in phyllospere, and also isolated from damp indoor surfaces, food and feed substances (Samson et al., 2004). It has also been found in the osmotically stressed environments like hyper saline water in salterns (Gunde-Cimerman et al., 2000) and the rocks (Urzi et al., 1999). This fungus disperses easily due to production of yeast-like propagules in large quantity and found globally but rarely reported in the intense cold environment, as investigations on fungal diversity are limited to frozen Antarctic soils and Siberian permafrost where basidiomycetous yeasts were found (Babjeva and Reshetova, 1998). Several *Aureobasidium* like black yeasts were isolated from glacial and sub-glacial ice in the coastal Arctic habitats and the adjacent sea water (Zalar et al., 2008).

From the available records, it is apparent that this fungus is most common in temperate zone with numerous records both from the British Isles and the United

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Abbreviations: SCP, Single cell protein; **FOS,** fructo-oligosaccharides; **UDP,** uridine diphosphate; **UDPG,** uridine diphosphate glucose.

States including Alaska, Antarctica, Denmark, Germany, Netherlands, Poland, Austria, Czech Republic, Russia and Japan. There are several reports of its occurrence in Mediterranean and arid zones including Italy, France Egypt, Iraq, Pakistan and South Africa. It has also been extensively isolated from tropical and subtropical region namely, Brazil, India, China, Thailand, Malaysia, Jamaica (West Indies), etc.

An extensive list of the habitats and geography from which strains of *A. pullulans* have been isolated is well documented by Leathers (1993) and Zalar et al. (2008). The fungus has been frequently isolated from moorland, peat bogs or peat podzol and forest soils. Other noteworthy habitats include fresh water, estuarine and marine sediments and sea water. Lis and Andrews (1997) studied the occurrence and distribution of *A. pullulans* by fluorescence dye and found it frequently on the phylloplane of several plants. Kuter (1986) isolated *A. pullulans* from green and senescent sugar maples, but it did not seem to be inducer and biodeteriorator. Slavikova and Vadkertiova (1997) reported seasonal occurrence of *A. pullulans* from March to April in river Danube. Punnapayak et al. (2003) have isolated this fungus from the air in several locations of Thailand while Prasongsuk et al. (2005, 2007) have reported strain of this taxon from different habitats including plant leaves to painted surfaces in Thailand. Singh and Saini (2007) isolated pullulan producing novel color variant strain of *A. pullulans* from the phylloplane of Ficus sp in India. Similarly, Haifeng et al. (2007) isolated *A. pullulans* strain N13D from deep sea sediments of the Pacific Ocean. Zamora et al. (2008) isolated *A. pullulans* from needles and twigs of pine plantation from northern Spain.

The morphological forms of this fungus are governed by many factors like temperature, pH, and oxygen concentration. Nutritional factors mainly the source and concentration of carbon, nitrogen and mineral affect the morphology mainly yeast like cells which are responsible for the production of pullulan, an important polysaccharide. Morphological studies have been well reviewed by Deshpande et al. 1992. This review has concluded that *A. pullulans*, is an omnipresent fungus which can survive in different type of habitat: some of the recent studies on the behavior of this fungus clearly show two important characteristics of anti-fungal and anti-bacterial activity along with pullulan production. This approach makes such strain more economical for industrial use.

Earlier, *A. pullulans* was identified as *Dematium pullulans* De Bary (1884) and *Pullularia pullulans* (De Bary) Berkhout (1866). Using the criteria of conidiogenesis that is, synchronous holoblastic conidial production, *A. pullulans* was placed previously under Fungi Imperfecti, Order Moniliales, and Family Dematiaceae. However, further reports included it under Ascomycota though; perfect stage has not yet been reported (de Hoog and Mc Ginnis, 1987). In recent classification, *A. pullulans* is regarded as Ascomycetous yeast and is placed in the Order Dothideales, Family Dothideaceae (Yurlova et al., 1999). The fungus was taxonomically characterized by de Hoog and Yurlova (1994) on the basis of its morphological and nutritional characteristics comprising single species with three different varieties such as *A. pullulans* var. *pullulans*, *A. pullulans* var. *melanogenum* and *A. pullulans* var. *aubasidani* Yurlova. Zalar et al. (2008) have found four strains of arctic *Aureobasidium*, that is, *A. pullulans* var. *pullulans*, *A. pullulans* var. *melanogenum*, *A. pullulans* var. *subglaciale* and *A. pullulans* var. *namibiae.*

A. pullulans exhibits polymorphism, for it can grow as budding yeast or as mycelia depending upon environmental conditions. The life cycle of *A. pullulans* was thoroughly reviewed by de Hoog and Yurlova (1994). The formation of dark-coloured chlamydospores is the characteristic feature of this fungus (Ramos and García-Acha, 1975). Some workers have described the vegetative cycle of *A. pullulans* as well as colony characteristics on solid nutrient medium. Colonies are initially smooth and eventually become covered with slime. Starting as yellow cream, light pink or light brown, the colonies finally become blackish due to production of a specific melanin at chlamydospore production stage. When observed under light microscope, the hyphae look hyaline, smooth and thin-walled, 2 - 16 µm wide with cells often wider than long forming rough and compact mycelia. *A. pullulans* can be recognized by straight conidia and by the presence of lobed chains of thick-walled chlamydospores.

A review regarding the application of *A. pullulans* had been published in 1992 by Deshpande et al., emphasing the production of various enzymes, single cell proteins and polysaccharide by this fungus. The use of this fungus in environmental pollution control and biodeterioration of xenobiotic compounds has also been added in this review. Although the author has covered almost all the potential areas in brief, but pullulan is little covered. However, after a long gap, Leathers wrote a review entitled “Pullulan” in 2002, emphasing on historical outline, chemical structures various analytical and assay methods of pullulan including biosynthesis of pullulan in different nutrient sources. The genetics of *A. pullulans* has been extensively covered in this review. Since then several recent reports have come regarding novel method of immobilization, use of cheaper carbon sources and use of continuous fermentation for better yield and productivity. A novel fungus *Eurotium chevalieri* has also been reported to produce pullulan which was at par to *A. pullulans*, commenced the possibility of the isolation of newer strains from natural ecosystem (Gaur et al., 2010). Moreover, Shingel in 2004 wrote a review on pullulan emphasing on various factors namely; pH, various substrates, molecular weight distribution of native pullulan along with molecular and hydrodynamic property of pullulan and also mentioned, a novel area that is

biomedical application of pullulan. A recent review entitled "Bioproducts from *A. pullulans*, a biotechnologically important yeast" has been written by Chi et al. 2009 elaborating some new metabolites like Siderophores production by *A. pullulans* and biocontrol efficiency against post-harvest diseases. However, they have also shown similar beneficial metabolites like various enzymes namely; protease, amylase, lipase, cellulase, xylanase and single cell protein (SCP) producing capabilities of *A. pullulans*.

In-spite of all the information up to 2009, the commercial production of pullulan with a proper fermentation technique regarding the better continuous production in solid state and submerged fermentation have not yet been taken up properly because commercial production technology with specific design of fermentor technology is still lacking behind and must be developed for better pullulan production. Moreover, immobilization in solid state as well as submerged fermentation using suitable solid matrix must be discussed. However, in present review an approach has been incorporated in the light of these aspects.

ECONOMIC IMPORTANCE OF *A. PULLULANS*

A. pullulans has been employed for different useful purposes as it produces a variety of important metabolites, enzymes, antibiotics, single cell protein (SCP) and polysaccharides (Chi et al., 2009). Tests have shown it to be safe for the use as SCP (Chi et al., 2008). *A. pullulans* has shown the potential in controlling and monitoring environmental pollution. Due to ubiquitous nature of this fungus on phylloplane, any change in its occurrence might prove to be an indicator of environmental perturbations generated by chemicals or other biological organisms (including genetically engineered organisms) landing on the leaf surfaces. Some isolates of *A. pullulans* showed antagonistic activity against a number of phytopathogenic fungi and were considered as possible biocontrol agents of post harvest diseases (Mounir et al*.,* 2007). The strain L47 of *A. pullulans* was isolated in South Italy from corpospere of table grape berries and was successfully applied to control post harvest diseases of different fruits and vegetables (Schena et al., 2002).

This strain provided high protection levels against *P. digitatum* on grape fruit, *Botrytis cinerea*, *Aspergillus niger* and *Rhizopus stolonifer* on grape, *B. cinerea* and *Monilia lax a* on sweet cherry and *B. cinerea* on kiwi fruit, cherry, tomato, apples and strawberries (Schena et al., 1999).

Enzymes from *A. pullulans*

Different strains of *A. pullulans* isolated from different environments can produce amylase, protease, lipase, cellulose, xylanase, etc, which have great potential applications in industry.

Proteases have been shown to have many applications in detergents, leather processing, silver recovery, medical purposes, food processing, feeds, chemical industry as well as waste treatment. Proteases also contribute to the development of high-added applications or products using the enzyme-aided digestion of protein from different sources (Kurmar and Tagaki, 1999). However, little has been known about protease from marine-derived yeasts (Chi et al*.,* 2007). Amylases have many applications in bread and baking industry, starch liquefaction and saccharification, textile desiging, paper industry, detergent industry, analysis in medical and clinical chemistry, and food and pharmaceutical industries. Because most yeasts from environments are safe (GRAS, generally regarded as safe) compared to bacteria, interest in amylolytic yeasts has been increased in recent years as their potential value for conversion of starchy biomass to single cell protein and ethanol has been recognized (Gupta et al*.,* 2003). Recently, some amylases from terrestrial yeasts also have been found to have the ability to digest raw starch. However, very few studies exist on the amylase-producing marine yeasts (Li et al., 2007a). Although amylase activity produced by bacteria is much higher than that produced by *A. pullulans*, the bacteria only can produce α-amylase (Nidhi et al*.,* 2005).

Lipases catalyze a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis. Therefore, lipases, especially microbial lipases, have many industrial applications (Hasan et al*.,* 2006). Although lipases from *Candida rugosa* and *Candida antartica* have been extensively used in different fields, very few studies exist on the lipase produced by yeasts isolated from marine environments (Chi et al*.,* 2006). Lipases from *A. pullulans* are extracellular. It was also found that the crude lipase produced by A. pullulans HN2.3 has high hydrolytic activity towards olive oil, peanut oil, soybean oil and lard (Liu et al., 2008a). Cellulose is the most abundant organic material on the earth consisting of glucose units linked together by β-1,4-glycosidic bonds. It has been observed that most of the cultures of *A. pullulans* have usually failed to show any cellulolytic activity (Buzzini and Martini, 2002). However, Kudanga and Mwenje (2005) reported that some isolates of *A. pullulans* of the tropical origin produced CMCase (endo-glucanase) and alpha-cellulase (exoglucanase) activity. Zhang and Chi (2007) reported the ability to produce cellulose by different strains of *A. pullulans* isolated from different marine environments. Unfortunately, it is still completely unknown about cellobiohydrolases in *A. pullulans*, and the gene encoding β-glucosidase in *A. pullulans* has not been cloned. Xylanases have many applications in paper, fermentation and food industries, as well as in waste treatment. The fungus *A. pullulans* Y-2311-1 was shown to be among the most proficient of the xylan-degrading fungi, secreting extremely high levels of xylanolytic enzymes into culture media.

Fructo-oligosaccharides (FOS) are a class of prebiotics widely used as a functional food material. A transfructosylating reaction by β-fructofuranosidases (FFases; EC 3.2.1.26) from *A. pullulans* has typically been used to produce FOS. *A. pullulans* DSM 2404 was found to form at least five kinds of FFases in a sucrose medium. The fructosyltransferase (FTase) produced by *A. pullulans* CCY-27-1-94 is stable in a broad range of pH and temperature up to 65°C, with an optimum pH 4.4 and temperature of 65°C (Onderkova et al., 2007). In addition, it has been found that β-fructofuranosidase and maltosyltransferase from *A. pullulans* have many advantages over those from the bacterial strains and *Aspergillus* sp. due to no repression of its expression by glucose and high transfructosylating activity (Yoshikawa et al*.,* 2007).

Mannan and heteromannans are widely distributed in nature as part of the hemicellulose fraction in plant cell walls. Mannan consists of β-1,4-linked d-mannopyranose residues. Mannanases are useful in many fields including bio-bleaching of pulp in paper industry, bioconversion of biomass wastes to fermentable sugars, upgrading of animal feed stuff, and reducing the viscosity of coffee extracts. They also have potential applications in the production of manno-oligosaccharides, which are utilized selectively by intestinal *Bifibacterium* species and used as valuable food sweetener or additive (Lin and Cheng, 2004). In screening for producers of extracellular β-1,4-mannanase among yeasts and yeast-like micro-organisms, the best producers were found among strains of *A. pullulans* (Kremnicky and Biely, 1997). However, the gene encoding mannanase in *A. pullulans* have not been cloned yet.

A. pullulans as single cell protein (SCP)

A variety of microalgae such as *Spirulina* and *Chlorella* and brown algae are extensively used as feed for cultured marine animals (Chi et al*.,* 2006; Ravindra, 2000). However, they have some limitations for animal consumption. Some yeasts such as *Saccharomyces cerevisiae*, *Candida utilis* and *Candida tropicalis* also have been used for their single-cell protein (Ravindra, 2000). They have many advantages over algae and bacteria (Ravindra, 2000; Gao et al., 2007). Unfortunately, little is known about the marine yeasts that have high protein content and can be used as aquafeed. A total of 327 yeast strains from seawater, sediments, mud of salterns, guts of the marine fish, and marine algae were obtained. Chi et al. (2009) estimated the crude protein of the yeast and found that eight strains of the marine yeasts grown in the medium with 20 g/l glucose contain more than 30.4 g protein per 100 g of cell dry weight. They belong to *Metschnikowa reukaui* (2E00001), *Cryptococcus aureus* (2E00002), *A. pullulans* (2E00060), *Y. lipolytica* (2E00004), and *Hanseniaspora uvarum* (2E00007), respectively. With the exception of *A. pullulans* 4#2 (2E00003) with nucleic acid of 7.7% (w/w), all other yeast strains contain less than 5% (w/w) of nucleic acid. Analysis of fatty acids shows that all the yeast strains tested have a large amount of $C_{18:0}$ and $C_{18:1}$ fatty acids, while analysis of amino acids indicates that the yeast strains tested have a large amount of essential amino acids, especially lysine and leucine which are very important nutritive components for marine animals (Chi et al*.,* 2008). Therefore, *A. pullulans* that contains high content of protein may be especially important in single-cell protein production by transforming the waste products such as starch, protein, cellulose and xylan into cell protein in *A. pullulans.*

Siderophore from *A. pullulans*

Siderophores are low-molecular-weight, iron-chelating ligands produced by nearly all the microorganisms. Siderophores can affect microorganisms in the environments in several ways as result of their role as iron-scavenging compounds, especially marine micro-organisms because iron is an essential nutrient for virtually all forms of life and is difficult to obtain due to its low solubility in marine environments. It has been confirmed that yeasts produce only hydroxamate-type compound, while bacteria produce hydroxamate as well as catecholate siderophores (Riquelme, 1996).

Siderophores are also found to have many applications in medical and environmental sciences. They can be used to control growth of the pathogenic bacteria in marine fish and the complexing ability of siderophores can be used to develop the processes for metal recovery or remediation of waste sites, including radioactive waste as they are extremely effective at solubilizing actinides and other metals from polluted environments (Li et al., 2008). Most bacterial infections in marine animals are found to be caused by *Vibrio parahaemolyticus*, *Vibrio anguillarum* and *Vibrio harveyi*. Therefore, it is very important to find some antibacterial agents against these pathogens. Although many antibacterial peptides and killer toxins have been found to be active against some pathogens in marine animals, they are not stable in marine environments and easily attacked by proteases produced by marine micro-organisms (Li et al., 2007d; Wang et al., 2007). Over 300 yeast strains isolated from different marine environments were screened for their ability to produce siderophore. Among them, one yeast strain HN6.2 (2E00149) which was identified to be *A. pullulans* was found to produce high level of siderophore. Under the optimal conditions, this yeast strain could produce 1.1 mg/ml of the siderophore. L-Ornithine was found to enhance the siderophore production, while Fe^{3+} could greatly inhibit the siderophore production. The crude siderophore produced by the yeast strain HN6.2 is able to inhibit cell growth of *V. anguillarum* and *V. parahaemolyticus*, the common pathogenic bacteria isolated from diseased marine animals. This is the first time to report that the crude siderophore produced by the

marine-derived yeast can inhibit growth of the pathogenic bacteria isolated from marine animals (Wang et al., 2008). The first step in siderophore biosynthesis is the N^5-hydroxylation of ornithine catalyzed by orinithine N^5-oxygenase. The further reactions of siderophore biosynthesis are catalyzed by non-ribosomal peptide synthetases (Haas, 2003). Chi et al. (2009) reported that the presence of Fe^{3+} in the medium can greatly repress the expression of the gene encoding ornithine N^5-oxygenase, while Wang et al. (2008) reported that the presence of L-ornithine can enhance the exression of the gene encoding ornithine N^5-oxygenase in *A. pullulans* HN6.2.

Biocontrol with *A. pullulans*

Currently, fungicide treatments represent the primary method for the control of post-harvest diseases of fruits and vegetables. However, public concern about fungicide residue and development of fungicide resistant isolates of post-harvest pathogens have promoted the search for alternative means, less harmful to human health and to the environment. In recent years, considerable success has been achieved utilizing microbial antagonists to control post-harvest diseases. Because the infection of fruits by post-harvest pathogens often occurs in the field prior to harvest, it may be advantageous to apply antagonists before harvest. For this approach to be successful, putative biocontrol strains must be able to tolerate low nutrient availability, UV-B radiation, low temperatures, and climatic changes. The yeast-like fungus *A. pullulans* is one of the most widespread and well-adapted saprophytes, both in the phyllosphere and in the carposphere. *A. pullulans* has a high tolerance to dessication and irradiation and has been considered as an effective biocontrol agent against post-harvest diseases (Mounir et al., 2007). It was found that two of *A. pullulans* (SL250 and SL236), plus a proven antagonist (isolate L47), are able to control *Penicillium digitatum* on grapefruit, *Botrytis cinerea*, *Rhizopus stolonifer*, and *A. niger* on table grape and *B. cinerea* and *R. stolonifer* on cherry tomato.

Bencheqroun et al. (2007) applied the yeast-like fungus *A. pullulans* strain Ach1-1 to control mold growth on apple caused by *Penicillium expansum*. The competition for apple nutrients, most particularly amino acids, may be a main mechanism of the biocontrol activity of *A. pullulans* strain Ach1-1 against blue mold caused by *P. expansum* on harvested apple fruit. Lugauskas *et al*. (2008) identified micromycetes capable of developing on lubricants of various origin and nature, used in various industrial applications to investigate the reaction of micromycetes on different oil products and to discuss the possibilities to use the obtained species of micromycetes for environment protection from intensive pollution with lubricants and their wastes. Different micromycetes reacted differently to the impact of various lubricant components. They found that *A. pullulans* responded somewhat poorly as compared to other fungus.

Pullulan from *A. pullulans*

Pullulan, which is a linear α-D-glucan made mainly of maltotriose repeating units interconnected by α (1→6) linkages, is a water-soluble homopolysaccharide produced extracellularly by the polymorphic micromycete *A. pullulans* (Sutherland, 1998). The regular alternation of α-1,4 and α-1,6 bonds results in two distinctive properties of structural flexibility and enhanced solubility (Leathers, 1986).

This polysaccharide is of economic importance with increased application in food, pharmaceutical, agricultural and chemical industries (Deshpande et al., 1992; Sutherland, 1998; Shingel, 2004; Singh et al., 2008). Pullulan produces a high viscosity solution at a relatively low concentration and can be used for oxygen-impermeable films and fibers, thickening or extending agents, or adhesive or encapsulating agents (Singh et al., 2008). Despite being a α-D-glucan, pullulan is resistant to α-D-amylolysis and may be used in low-calorie food formulation. The chemical formula of pullulan is $(C_6H_{10}O_5).H_2O$.

In recent years, many authors (Alban et al., 2002; Shibata et al., 2001) have reported that sulfated pullulan and phosphorylated pullulan have an anticoagulant, antithrombotic and antiviral activities, and chlorinated, sulphinylethylated, etherified, carboxylated, acetylated and esterified pullulan can be used as important materials for chemical industries. So it becomes very important to search for better pullulan-producing yeast strains.It is now widely accepted that pullulan is a linear polysaccharide with maltotriosyl repeating units joined by α-(1→6)-linkages. Alternatively, the structural formula of pullulan may be presented as a regular sequence of panoses bonded by α-(1→4)-linkages. Minor structural abnormalities are reported in pullulan. A careful hydrolysis of pullulan by exo-and endoenzymes showed chain fragments resistant to the action of enzymes. Such resistance was attributed to the presence of maltotetraose residues distributed randomly along the pullulan chain. However, these structural abnormalities should not affect the overall physico-chemical properties of pullulan (Catley et al., 1986).

The producer of pullulan, *A. pullulans*, is a black yeast widely spread in all ecological niches including forest soils, fresh and sea water, plant and animal tissues, etc. Generally, the culture of *A. pullulans* is classified as non pathogenic; however, some strains may cause health problems. Pullulan has been commercially produced since 1976 by the Hayashibara Company Ltd (Okayama, Japan), which remains the principal supplier. Recent arrangements with Pfizer for production of oral care strips may result in expanded markets for pullulan.

BIOSYNTHESIS OF PULLULAN

Exopolysaccharide produced by *A. pullulans*

Even in the first work on pullulan biosynthesis, researchers observed that the culture produced two different exopolysaccharides. One of these polymers corresponds to pullulan, and the second is frequently described as a water-insoluble jelly-like material. An electron microscopy study revealed that both pullulan and the insoluble polysaccharide are localized on an outer surface of the chlamydospores, the cells that were considered as the main polysaccharide producer on non growth media. The highly dense peripheral layer was ascribed to the chain of pullulan arranged in a network covering the inner layer of β-(1→3)-glucan composed of glucose and mannose (Simon et al., 1993).

Very little is known up to now explaining how the mechanism of biosynthesis of these jelly-like glucans is associated with the pullulan elaboration, though there were indications that the elaboration of insoluble exopolysaccharide is dependent on genetic type of *A. pullulans*. In particular, it is not clear yet whether environmental conditions, for example, pH or morphological changes of the cells are responsible for its extracellular elaboration (Imshenetskii et al., 1983).

Influence of pH and cell morphology

Relatively low initial pH (pH 2.5) suppresses synthesis of pullulan but stimulates elaboration of the insoluble glucan. An optimal value of pH for the pullulan production lies in the range between 5.5 and 7.5 (Lee et al., 2001). There were only a few reports where highest pullulan content was achieved by cultivating the microorganism in acidic pH. It is of interest to note that the optimal pH established for the biomass growth is 4.5 or lower. This difference in optimal values of pH for the pullulan synthesis and cell mass growth indirectly correlates with the independent character of these two processes (Kondratyeva, 1981). However, the relationship between morphology and the polysaccharide-producing capacity of the culture cannot be ignored since the polysaccharide elaboration is known to be associated with the specific cell morphology (Simon et al., 1993), though the exact cellular type responsible for pullulan synthesis is still a matter of debate. In an overwhelming number of studies, pullulan elaboration was found to occur only with the yeast-like morphology of *A. pullulans* (Campbell et al., 2004), whilst in other several papers the ability to synthesize the polysaccharide was the characteristic of the chlamydospore population (Simon et al., 1993). At least there is convincing agreement among researchers that pH provokes morphological changes of cells, which in turn may additionally differentiate biosynthesis routes. The yeast-like cells at neutral pH produce pullulan of a very high molecular weight (Lee et al., 2001), whilst combined cultivation of the mycelial and the yeast-like cellular forms can be beneficial for high pullulan concentration (Roukas and Biliaderis, 1995).

Mechanism of pullulan biosynthesis

Although many investigations on biochemical mechanisms of exopolysaccharide biosynthesis in bacteria have been carried out (Degeest and Vuyst, 2000), relatively little is understood about the mechanisms of pullulan biosynthesis in *A. pullulans.* If the pullulan biosynthesis and regulation in *A. pullulans* are elucidated, it will be very easy to enhance pullulan yield using molecular methods. Pullulan can be synthesized from sucrose by cell-free enzymes of *A. pullulans* when both adenosine triphosphate (ATP) and uridine diphosphate (UDP)-glucose are added to a reaction mixture (Shingel, 2004). Chi et al. (2009) reported that the size of UDP-glucose pool and glucosyltransferase activity in the cell of *A. pullulans* Y68 obtained in their laboratory may be correlated with high pullulan production. Therefore, effects of different sugars on pullulan production, UDP-glucose (UDPG) pool, and activities of α-phosphoglucose mutase, UDPG-pyrophosphorylase, and glucosyltransferase in the cells of *A. pullulans* Y68 were investigated (Duan et al., 2008). It was found that more pullulan is produced when the yeast strain is grown in the medium containing glucose than when it is cultivated in the medium supplementing other sugars. However, Chi et al. (2009) concluded that when more pullulan is synthesized, less UDP-glucose is left in the cells of *A. pullulans* Y68. High pullulan yield is positively related to high activities of α-phosphoglucose mutase, UDPG-pyrophosphorylase, and glucosyltransferase in *A. pullulans* Y68 grown on different sugars. A pathway of pullulan biosynthesis in *A. pullulans* Y68 was proposed based on different studies (Duan et al., 2008; Chi et al., 2009). It is thought that the lower amount of pullulan produced by *A. pullulans* Y68 from fructose and xylose may be caused by the longer biosynthetic pathway leading from fructose and xylose to UDP-glucose. It is thought that most of UDP-glucose is used to synthesize pullulan when the glucosyltransferase activity is very high, leading to very low UDP-glucose level in the yeast cells. This may imply that very high glucosyltransferase activity is the unique characteristic of *A. pullulans* Y68 which can produce high yield of pullulan. Because the phosphoglucomutase and UDPG-pyrophosphotylase activity in the yeast cells grown in the medium containing glucose is also very high, UDP-glucose is synthesized continuously to supply the precursors for high pullulan synthesis when the very high glucosyltransferase activity occurs in the cells of *A. pullulans* Y68. However, high level of UDP-glucose is left when the yeast cells are grown in the medium containing xylose and fructose, respectively, due to low glucosyltransferase activity. Therefore, it is believed that the proposed pathway of pullulan biosynthesis will be helpful for the metabolism-

engineering of the yeast strain to further enhance pullulan yield.

Substrates and efficiency of pullulan fermentation

The major attention in the fermentation studies of *A. pullulans* was devoted to developing optimal cultivation conditions while maintaining a high productivity of the cells. The main objectives were high yield, short fermentation time, low cost, and high purity of the final product to meet the stringent requirements for food, cosmetics and pharmaceutical applications (Shingel, 2004).

Although the literature on pullulan biosynthesis is contradictory because of differences among the numerous strains of *A. pullulans*, it was clearly demonstrated that the yield of pullulan strongly depends on the rate of substrate conversion (Kondratyeva, 1981). Moreover, the concentration of the polysaccharide produced by *A. pullulans* is dependent on the carbon source. It is important to note that the exopolymer synthesis was observed on glucose, sucrose, fructose and maltose. On a medium containing maltose as a carbon source, the wild fungus intensively grew, but had low pullulan-producing activity. The problem of low pullulan producing activity was solved later by the development of several mutant strains of *A. pullulans* with improved ability to synthesize pullulan (Pollock et al., 1992; West and Strohfus, 2001). By using these cultures, it became possible to perform large-scale fermentation processes under well-controlled conditions. Progress was particularly stimulated by the development of new fermentation reactors designed to maintain high productivity of the culture. In addition, visual inspection methods (Guterman and Shabtai, 1996) and several analytical techniques, including capillary electrophoresis and high performance liquid chromatography (Barnett et al., 1999; Wiley et al., 1993), were applied successfully to monitor changes in the cell morphology and carbohydrate composition of the cultivation broth.

In order to reduce the cost of the fermentation product, pullulan biosynthesis from the hydrolyzates of potato starch waste was studied (Barnett et al., 1999). Fermentation of *A. pullulans* on a medium containing 20% maltose-rich hydrolyzates yields 115% higher concentration of pullulan than that obtained on glucose syrup, indicating that maltose is a better substrate than glucose for pullulan production by the studied strain of *A. pullulans.* Other wastes from the agricultural and food industries such as deproteinized whey (Roukas, 1999), beet molasses (Lazaridou et al., 2002), sugar cane juice, and even peat hydrolyzate (LeDuy and Boa, 1983) are also considered as economical and efficient substrates for the pullulan production. An exhaustive literature survey devoted to the use of different industrial wastes for pullulan production and the problems associated with the recovery and characterization of the final product has been recently presented (Lazaridou et al., 2002).

Fermentation techniques

The influence of aeration on vital activity of cells producing pullulan was studied in detail (Deshpande et al., 1992). Under anaerobic conditions, the cell population neither grows nor produces pullulan. An intense aeration during fermentation leads to a significant increase of pullulan concentration. This effect is especially pronounced on a nitrogen-rich media. An inverse effect of aeration was detected upon fermentation on the media deficient in nitrogen source, where intense aeration suppressed pullulan production. An increase in oxygen transfer rate achievable by increasing a gas partial pressure may improve the polysaccharide producing activity of *A. pullulans.* High airflow rates and high working pressure is beneficial for the growth of cell mass and pullulan synthesis. In order to prevent cell disintegration, cell immobilization procedures were applied to pullulan production (Urkut et al., 2007). The elaboration of pullulan using cells of *A. pullulans* entrapped in agarose and carrageenan was studied by West in 2000. Both immobilized systems were found technically acceptable for pullulan production; however, the highest content of pullulan was obtained with the use of agarose-entrapped culture. Other researchers noticed that this method is inconvenient for pullulan production because of the several undesirable events, including the restriction of polysaccharide diffusion through microporous sorbents and the destruction of the immobilization system due to a rapid increase of the entrapped biomass (Urkut et al., 2007).

Another approach to stabilize growth conditions and thereby increase pullulan yield is the use of continuous fed-batch cultivation. An optimization of fed-batch cultivation was performed by the investigation on the effect of feed mode and composition of the feed solution on the efficiency of pullulan fermentation. The fed-batch culture gives high pullulan yields; however, the higher rates of pullulan production and substrate uptake are characteristics of the traditional batch cultivation.

In conclusion, the literature data cited here clearly indicate that the great variety of environmental conditions, as well as variability in strain characteristics, influence the metabolite pathways of the pullulan biogenesis. From the biochemical point of view, the mechanism of the pullulan biosynthesis appeared to have a very complex pattern. In this context, evident knowledge regarding the mechanism of the substrate transformation as well as the routes of pullulan genesis could help to control molecular weight, molecular weight distribution and architecture of pullulan directly in a course of fermentation.

CONCLUSION

Aureobasidium pullulans is an industrially important fungus which produces a number of by-products including several enzymes, single cell protein and an

industrially important polysaccharide namely pullulan besides playing an important role in biodeterioration and in controlling environmental pollution. More than 300 patents describing the production and use of pullulan and pullulan derivatives are known. Although a detail study has been done regarding the morphology, distribution and economic importance of this fungus still, we think that, a lot of investigations are needed for better exploitation of this economically important fungus.

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