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MINI-REVIEW

Bioproducts from *Aureobasidium pullulans*, a biotechnologically important yeast

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Abstract It has been well documented that *Aureobasidium pullulans* is widely distributed in different environments. Different strains of *A. pullulans* can produce amylase, proteinase, lipase, cellulase, xylanase, mannanase, transferases, pullulan, siderophore, and single-cell protein, and the genes encoding proteinase, lipase, cellulase, xylanase, and siderophore have been cloned and characterized. Therefore, like *Aspergillus* spp., it is a biotechnologically important yeast that can be used in different fields. So it is very important to sequence the whole genomic DNA of the yeast cells in order to find new more bioproducts and novel genes from this yeast.

Keywords *A. pullulans* · Pullulan · Extracellular enzymes · Functional genes · Biotechnology

Introduction

Aureobasidium pullulans is a cosmopolitan yeast-like fungus and popularly known as black yeast due to its melanin production (de Hoog 1993). Its three distinctive forms are elongated branched septate filaments, large chlamydospores, and smaller elliptical yeast-like cells. Beginning as yellow, cream, light pink, or light brown, the colonies become blackish due to chlamydospore production at a later stage. The formation of dark-colored chlamydospores is a characteristic feature. An undesirable characteristic feature of *A. pullulans* is the production of a dark pigment, which is a melanin-like compound. In the fourth revised and enlarged edition of "The Yeasts, A Taxonomic Study", it is regarded as the yeast by Kurtzman and Fell (2000). *A. pullulans* has three varieties: *A. pullulans* var. *pullulans*, *A. pullulans* var. *melanogenum*, and *A. pullulans* var. *aubasidani* Yurlova (Yurlova and Hoog 1997).

From an ecological point of view, *A. pullulans* strains are ubiquitous species found mainly in soil, including Antarctic soils, water, the phylloplane, wood, and many other plant materials, rocks, monuments, and limestone (Urz'ı et al. 1995). *A. pullulans* has also been reported as a slime-producing contaminant of paper mills and can colonize optical lenses (Webb et al. 2000). In recent years, it also has been found to be widely distributed in hypersaline habitats and coastal water and deep sea (Nagahama 2006; Gunde-Cimerman et al. 2000; Li et al. 2007a).

In the last two decades, it has been found that different strains of *A. pullulans* have many uses in different fields because of their capacity of producing pullulan, extracellular enzymes, siderophore, and single-cell protein (Table 1). Many extracellular enzymes, such as protease, lipase, and amylase, from *A. pullulans* have been purified and characterized. At the same time, many genes encoding protease, lipase, and xylanase have also been cloned and characterized from this organism. Some strains of *A. pullulans* were found to be able to control growth of the unwanted microorganisms. Therefore, it is thought to be a biotechnologically important yeast.

Although Deshpande et al (1992) made a thorough review on enzymes from *A. pullulans* and single-cell protein production using *A. pullulans*, the present review article is mainly focused on recent progress in extracellular enzymes and their genes, pullulan and its biosynthesis,

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Table 1 Bioproducts from *A. pullulans* and their potential applications

Bioproducts	Applications	References
Pullulan	Oxygen-impermeable films and fibers, thickening or extending agents or adhesives or encapsulating agents, low-calorie food formulation, anticoagulant, antithrombotic and antiviral activities, materials for chemical industries	Duan et al. 2008
Amylases	Starch liquefaction and saccharification, textile desizing, detergent additives, ethanol production, analysis in medical and clinical chemistry, production of high fructose syrup, production of yeast cells and other microorganisms	Chi et al. 2001, Gupta et al. 2003
Cellulases	Improvement of cellulosic fibres, stone washing, detergent additives, production of single-cell protein and biofuels, waste treatment	Zhang and Chi 2007
Lipases	Catalysis of a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis, Production of biodiesel	Hasan et al. 2006
Alkaline protease	Detergents additive, leather processing, silver recovery, medical purposes, food processing, feeds, chemical industry, waste treatment, digestion of protein	Ni et al. 2008a, b
Xylanase	Paper, fermentation and food industries as well as in waste treatment	Li et al. 1993
β-Fructofuranosidase and maltosyltransferase	Relief of constipation, improvement of blood lipid composition in hyperlipidemia, enhancement of calcium and magnesium absorption and suppression of the production of intestinal putrefactive substances in both humans and animals and a growth factor for bifidobacteria	Yoshikawa et al. 2007, Yun et al. 1997
Mannanase	Biobleaching of pulp in paper industry, bioconversion of biomass wastes to fermentable sugars, upgrading of animal feed stuff and reducing the viscosity of coffee extracts, production of manno-oligosaccharides	Lin and Chen 2004
Single-cell proteins	Animal feed and human food, protein sources for production of bioactive peptide	Gao et al. 2007, Chi et al. 2008
Biocontrol	Inhibition of the unwanted microorganisms on fruits, grains and vegetables	Mounir et al. 2007
Siderophore	Medicine, metal recovery and remediation of waste sites	Wang et al. 2008

siderophore and its genes in *A. pullulans*, and biocontrol using *A. pullulans*.

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 et al. 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 adhesives 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.

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.

Yeast strain Y68 (2E00055), producing a large amount of pullulan, was isolated from the leaves collected in the south of China. This strain was re-identified to be *A. pullulans* by BIOLOG analysis, routine yeast identification, and molecular methods (Duan et al. 2007). Under optimal conditions, 5.9% (*w*/*v*) pullulan is produced within 60 h. This is the highest pullulan yield produced by yeasts obtained so far. No pigment in the medium was observed during the fermentation, suggesting that the yeast strain Y68 is a non-pigmented yeast strain (Chi and Zhao 2003). Recently, a maximum pullulan concentration of 4.9% (*w*/*v*) in the fermented medium of *A. pullulans* also has been achieved at initial sugar concentration of 10% (*w*/*v*; Lazaridon et al. 2002). Furthermore, melanin is produced by the black yeast strain during fermentation. This will make it more difficult to purify the exopolysaccharide at the end of fermentation (Deshpande et al. 1992). Therefore, *A. pullulans* Y68 may be more suitable for industrial pullulan production according to its high yield and no pigment production.

Although many investigations on biochemical mechanisms of exopolysaccharide biosynthesis in bacteria have been carried out (Grobben et al. 1996; 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 ATP and UDP-glucose are added to a reaction mixture (Shingel 2004). It is regarded that the pullulan chains or pullulan precursors originate from UDP-glucose (Shingel 2004). Catley and McDowell (1982) have proposed the order of the biochemical events preceding pullulan formation. We thought that the size of UDP-glucose pool and glucosyltransferase activity in the cells of *A. pullulans* Y68 obtained in our laboratory may be correlated with high pullulan production. Therefore, effects of different sugars on pullulan production, UDP-glucose 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, our results demonstrate that when more pullulan is synthesized, less UDP-glucose is left in the cells of *A. pullulans* Y68, demonstrating that pullulan biosynthesis in *A. pullulans* Y68 is different from that in bacteria (Grobben et al. 1996; Degeest and Vuyst 2000). However, it was observed that more pullulan is synthesized and the cells have higher activities of α-phosphoglucose mutase, UDPG-pyrophosphorylase, and glycosyltransferase. Therefore, 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. pullulan* Y68 was proposed based on the results of our studies and those from other researchers (Duan et al. 2008). 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 according to the pathway. We think 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-pyrophosphorylase 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, we believe that the proposed pathway of pullulan biosynthesis will be helpful to metabolism-engineer the yeast strain to further enhance pullulan yield.

Enzymes and their genes from *A. pullulans*

Different strains of *A. pullulans* isolated from different environments can produce amylase, proteinase, lipase, cellulase, xylanase, mannanase, and transferases, which have greatly potential applications in biotechnology.

Protease

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 (Kurmar and Tagaki 1999). Proteases also contribute to the development of high-added applications or products using the enzyme-aided digestion of proteins from different sources (Kurmar and Tagaki 1999). However, little has been known about protease from marine-derived yeasts (Chi et al. 2007).

All the purified alkaline proteases from the marine yeasts *A. pullulans* strain 10 (2E00061), HN2-3 (2E00059), and N13d (2E00056) which were isolated from different marine environments have molecular weights of about 32 kDa. It was found that the optimal pHs for them are 9.0 and all of them are serine alkaline protease. However, the optimal temperature for the purified protease from *A. pullulans* HN2-3 is 52°C and that for the purified protease from *A. pullulans* 10 is 45°C, while that for the purified protease from *A. pullulans* N13d is 48°C. The purified protease from *A. pullulans* 10 is activated by Cu^{2+} (at a concentration of 1.0 mM) and Mn^{2+} and the purified protease from *A. pullulans* HN2-3 is activated by Zn^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, and Na^{+}, while the purified protease from *A. pullulans* N13d is activated by Mn^{2+}, Mg^{2+}, and Na^{+} (Ma et al. 2007). All the purified proteases from them are metalloenzyme (Ma et al., 2007). The results also show that Ser residues are essential for the enzyme active sites and Cys residues are important for active sites of the enzyme (Ma et al. 2007).

All the proteases from the three *A. pullulans* strains can be used to hydrolyze proteins from different sources to produce bioactive peptides that have angiotensin-converting enzyme (ACE) inhibitory activity and antioxidant activity. For example, ACE inhibitory activity of the crude bioactive peptides from shrimp (*Trachypenaeus curvirostris*) muscle under the catalysis of the purified protease from strain HN2-3 is the highest (88.3%), while antioxidant activity of the crude bioactive peptides from spirulina (*Arthrospira platensis*) powder is the highest (82.8%) under the catalysis of the purified protease from strain N13d. Therefore, the

alkaline proteases from the three marine yeasts have potential uses in production of the bioactive peptides from shrimp muscle and spirulina (*A. platensis*) powder (Ni et al. 2008a).

Both the *ALP1* gene encoding alkaline protease from *A. pullulans* 10 and the *ALP2* gene encoding alkaline protease from *A. pullulans* HN2-3 have an open reading frame of 1,248 bp encoding a 415-amino-acid protein. The *ALP2* gene cloned from *A. pullulans* HN2-3 contained two introns which had 54 and 52 bp, respectively. In contrast, *ALP1* gene from *A. pullulans* 10 contains two introns with 54 and 50 bp, respectively (Ni et al. 2008b). It was found that the sequence of *ALP2* gene has very high similarity (85.01%) to that of *ALP1* gene isolated from *A. pullulans* strain 10. It has been confirmed that the amino acid sequences deduced from both the cDNA*ALP1* gene cloned from cDNA of *A. pullulans* strain 10 and the cDNA*ALP2* gene cloned from cDNA of *A. pullulans* HN2-3 are closely related to that of *Aspergillus fumigatus* alkaline protease. This may imply that *A. pullulans* from different environments is closely related to *Aspergillus* spp.

As mentioned above, the alkaline protease produced by *A. pullulans* HN2-3 is more suitable for the production of bioactive peptides than that produced by other yeasts (Ni et al. 2008a). Therefore, the cDNA*ALP2* gene obtained above was cloned into the multiple cloning sites of the surface display vector pINA1317-YlCWP110 and expressed in cells of *Yarrowia lipolytica*; the protease displaying cells can form clear zone on the double plate containing milk protein and have high protease activity (Ni et al. 2009). The yeast cells displaying alkaline protease can be used for bioactive peptide production from different sources of proteins. We found that although all the bioactive peptides produced by the yeast cells displaying alkaline protease have ACE inhibitory activity and antioxidant activity, ACE inhibitory activity of the crude bioactive peptides from the digest of single-cell protein of the marine yeast strain G7a is the highest (80.8%), while antioxidant activity of the crude bioactive peptides from the digest of spirulina (*A. platensis*) powder is the highest (74.0%).

Although the commercial alkaline proteases mainly come from *Bacillus* spp. due to their high activity and tolerance to organic compounds, some properties of the alkaline protease and its gene from *A. pullulans* are similar to those of the alkaline proteases and their genes from *Bacillus* spp. (Table 2). Because *A. pullulans* is generally regarded as safe, the alkaline protease and its gene from *A. pullulans* can be more easily accepted than those from bacteria.

Amylase

Amylases have many applications in bread and baking industry, starch liquefaction and saccharification, textile desizing, paper industry, detergent industry, analysis in medical and clinical chemistry, and food and pharmaceutical industries (Gupta et al. 2003). Starch is the best substrate for production of yeast cells on a large scale due to its low price and easily available raw material in most regions of the world (Chi et al. 2001). Because most of 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. The marine yeast *A. pullulans* N13d (2E00021) isolated from the deep sea sediments of the Pacific Ocean was found to be able to produce an extracellular glucoamylase. Under optimal conditions, 10 U/ml

Table 2 Some properties of alkaline proteases from *A. pullulans* and *Bacillus* spp

Producers	Molecular mass (kDa)	Optimal pH and emperature	Activated by	Inhibited by	Hydrolysis	Size of alkaline protease genes (bp)	References
The marine-derived *A. pullulans* HN2-3	33.0	9.0, 52°C	Zn^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Na^{+}	PMSF, EDTA, and iodoacetic acid	Production of bioactive peptides from marine proteins	1,248 (two introns: 54 and 52 bp)	Ni et al. 2008a, b
The marine-derived *A. pullulans* strain 10	32.0	9.0, 45 °C	Cu^{2+} and Mn^{2+}	Hg^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, and Co^{2+}, PMSF, EDTA and iodoacetic acid	Production of bioactive peptides from marine proteins	1,248 (two introns: 54 and 50 bp)	Ma et al. 2007
Bacillus spp.	20–42	8–11.5, 40–75°C	Ca^{2+}, Cu^{2+}, and other metal ions	PMSF	Production of bioactive peptides from marine proteins	1,122–1,868 (no intron)	Gupta et al. 2003

of glucoamylase is produced by the marine yeast strain (Li et al. 2007a). It was noticed that the crude glucoamylase actively hydrolyzes potato starch granules, but poorly digests raw corn starch and sweet potato starch, resulting in conversion of 68.5%, 19%, and 22% of them into glucose within 6 h of incubation in the presence of 40 g/l of potato starch granules and 20 U/ml of the crude glucoamylase. Only glucose is detected during the hydrolysis, indicating that the crude enzyme can hydrolyze both α-1,4 and α-1,6 linkages of starch molecule in the potato starch (Li et al. 2007b). 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).

The molecular weight of the purified enzyme from *A. pullulans* N13d is 98 kDa. The optimal pH and temperature of the purified enzyme are 4.5 and 60°C, respectively. Among raw potato starch, corn starch, and sweet potato starch tested, all of them are absorbed by the purified enzyme, but only raw potato starch is digested by the purified enzyme. 15.8% of raw potato starch (1.0%, *w*/*v*) and 21.1% of cooked potato starch (1.0%, *w*/*v*) are hydrolyzed within 30 min by 0.5 U/mg starch of the purified enzyme and only glucose is detected in the hydrolysate, indicating that the enzyme is glucoamylase with debranching activity (Li et al. 2007c). Such glucoamylase has wide uses in fermentation and food industries.

Lipase

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 antarctica* 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). A total 427 yeast strains from seawater, sediments, mud of salterns, guts of the marine fish, and marine algae were obtained. After lipase activity of the yeast cultures was estimated, we found that nine yeast strains grown in the medium with olive oil could produce lipase (Wang et al. 2007). They belong to *Candida intermedia* YA01a (2E00162), *Pichia guilliermondii* N12c (2E00159), *Candida parapsilosis* 3eA2 (2E00087), *Loddermyces elongisporus* YF12c (2E00372), *Candida quercitrusa* JHSb (2E00033), *C. rugosa* wl8 (2E00221), *Y. lipolytica* N9a (2E00152), *Rhodotorula mucilaginosa* L10-2 (2E00168), and *A. pullulans* HN2.3 (2E00148), respectively. Only lipase from *A. pullulans* HN2.3 is extracellular, while other yeast strains tested produce cell-bound lipase. The crude lipase produced by *A. pullulans* HN2.3 works best at pH 8.5 and 35°C. We found that lipase production by *A. pullulans* HN2.3 is dependent on the time when olive oil is added to the medium. When olive oil is added to 6-h-old culture with 0.4% (*w*/*v*) glucose, the highest lipase activity is achieved. Under optimal conditions, over 8.02±0.24 U/ml of lipase is produced by *A. pullulans* HN2.3 within 96 h. 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).

The molecular mass of the purified lipase from *A. pullulans* HN2.3 was estimated to be 63.5 kDa. The optimal pH and temperature of the purified enzyme are 8.5 and 35°C, respectively. It was also found that the purified lipase has the highest hydrolytic activity towards peanut oil (Liu et al. 2008a).

The extracellular lipase structural gene (*LIP1* gene) isolated from cDNA of *A. pullulans* HN2-3 has an open reading frame of 1,245 bp long encoding a lipase. The coding region of the gene is separated by only one intron (55 bp). It encodes 414 amino acid residues of a protein with a putative signal peptide of 26 amino acids. The protein sequence deduced from the *LIP1* gene contains the lipase consensus sequence (G-X-S-X-G) and three conserved putative *N*-glycosylation sites. According to the phylogenetic tree of the lipases, the lipase from *A. pullulans* HN2-3 is genetically closely related to the lipases of *A. fumigatus* (XP_750543) and *Neosartorya fischeri* (XP_001257768), and the identities are 50% and 52%, respectively (Liu et al. 2008b). This may imply that *A. pullulans* is phylogenetically close to *Aspergillus* spp.

Although bacterial lipases have been extensively investigated, lipase and its gene from *A. pullulans* are significantly different from those from the bacteria (Table 3). Therefore, the lipase with high hydrolytic activity towards peanut oil may have highly potential applications in degradation and reuse of the wasted peanut oil for biodiesel production.

Cellulase

Cellulose is the most abundant organic material on the earth consisting of glucose units linked together by β-1,4-glycosidic bonds. Therefore, it has become of considerable economic interest to develop the processes for the effective treatment and utilization of cellulosic wastes as inexpensive carbon sources (Wen et al. 2005).

Cellulases are enzymes that degrade crystalline cellulose to glucose. Three types of cellulases, endoglucanases (EC 3.2.1.4, endo-1,4-β-D-glucanases), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21), are considered to be needed to degrade crystalline cellulose to glucose in

Table 3 Some properties of lipases from *A. pullulans* and bacteria

Producers	Molecular mass (kDa)	Optimal pH and temperature	Activated by	Inhibited by	Hydrolysis	Size of lipase gene (bp)	References
The marine-derived *A. pullulans* HN2.3	63.5	8.5, 35°C	No metal ions tested	Hg^{2+}, Fe^{2+} and Zn^{2+}, PMSF, iodoacetic acid	Prefers peanut oil and lard	1,245(one intron: 55 bp)	Liu et al. 2008a, b
Some bacterial strains	31–60	7.0–9.0, 45–55°C	Ca^{2+}, Sn^{2+}, Tween 20, Triton X-100	Mn^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, EDTA, SDS	All the lipids	891–953 (no intron)	Sharma et al. 2001

vivo, and they act synergistically (Kim et al. 2003). Cellulases have diverse applications in environmental, food, and agricultural industries. Cellulases have also found a number of industrial applications in the textile industries. For example, they are used to improve the appearance of cellulosic fibers and used as a replacement for pumice stones in the "stone washing" process. In addition, cellulases are also potentially useful for converting cellulosic plant polymeric biomass into single-cell protein and biofuels (Deshpande et al. 1992).

It has been observed that most of the cultures of *A. pullulans* have usually failed to show any cellulolytic activity (Dennis and Buhagiar 1973; Deshpande et al. 1992; Leathers 1986; Buzzini and Martini 2002). However, Kudanga and Mwenje (2005) reported that some isolates of *A. pullulans* of tropical origin produced CMCase (endoglucanase) and alpha-cellulase (exoglucanase) activity.

In our previous studies (Zhang and Chi 2007), the ability to produce cellulase by different strains of *A. pullulans* isolated from different marine environments was evaluated. We found that *A. pullulans* 98 (2E 00098) among them could produce CMCase (4.51 U/mg) and FPAase (4.75 U/mg protein). The thin-layer chromatography results indicate that the crude enzyme can hydrolyze CMC and filter paper, producing a large amount of monosaccharide and a trace amount of disaccharide (Zhang and Chi 2007).

The molecular mass of the purified CMCase is 67.0 kDa. The optimal pH and temperature of the purified enzyme are 5.6 and 40°C, respectively. Only oligosaccharides, but no monosaccharides, are released from CMC after hydrolysis for 1 h with the purified CMCase, indicating that the purified CMCase indeed has endo-1, 4-β-D-glucanase activity. The CMCase structural gene (*CMC1* gene) isolated from cDNA of *A. pullulans* 98 has an open reading frame of 1,497 bp long encoding a CMCase. It was found that the sequence of the *CMC1* gene has high similarity to that of the CMCase genes from *Aspergillus terreus* (62.12%) and *A. fumigatus* (60.92%). This again demonstrates that *A. pullulans* is closely related to *Aspergillus* spp. (Zhang et al. 2007).

The β-glucosidase produced by *A. pullulans* is most active at pH 4.0–4.5, with apparent optimum temperatures at 80°C (Leite et al. 2007). When *A. pullulans* ER-16 was cultivated in different hemicellulosic materials on solid-state cultivation for β-glucosidase production, wheat bran is found to be most appropriate for β-glucosidase production by the yeast strain. *A. pullulans* exhibits maximum enzyme production (1.3 U/ml or 13 U/g) at 120 h (Leite et al. 2008). However, in another study, β-glucosidase produced by a yeast-like *Aureobasidium* sp. exhibits optimum activities at pH 2.0–2.5. The enzyme has maximum activities at 65°C and is stable in a wide pH range and at high temperatures (Iembo et al. 2002).

Unfortunately, it is still completely unknown about cellobiohydrolases in *A. pullulans*, and the gene encoding β-glucosidase in *A. pullulans* has not been cloned.

At present, the commercial cellulases mainly come from *Aspergills* spp. and *Trichoderma* spp. However, Table 4 shows that some properties of cellulase from *A. pullulans* are greatly different from those of cellulase from fungi.

Xylanase

The major structure of hemicellulose is xylan, which is a polymer of β-1,4-linked xyloses with arabinosyl and/or 4-*o*-methylglucurosyl side chains. The enzymatic degradation of xylan to xylose requires the catalysis of both endoxylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37). The internal glycosidic bonds of the main chain are hydrolyzed by the endo-β-1,4-xylanase releasing smaller xylo-oligosaccharides. β-xylosidase subsequently removes single unsubstituted xylose moieties from the non-reducing ends of xylo-oligosaccharides. 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. D-Xylose, xylobiose, xylan, and arabinose all induced, while glucose repressed, xylanase activity (Leathers 1986). It was shown that two xylanases with similar molecular masses are secreted into the culture supernatant by *A. pullulans* grown on xylan or xylose,

Table 4 Some properties of cellulases from *A. pullulans* and fungi

Producers	Molecular mass (kDa)	Optimal pH and temperature	Activated by	Inhibited by	Cellulase activity	Size of CMCase gene (bp)	References
The marine-derived *A. pullulans* 98	67.0	5.6, 40°C	Na^+, Mg^{2+}, Ca^{2+}, K^+, Fe^{2+}, and Cu^{2+}	Fe^{3+}, Ba^{2+}, Zn^{2+}, Mn^{2+}, and Ag^+, PMSF, SDS, DTT, iodoacetic acid, EDTA and EGTA	CMCase and FPAase	1,497	Zhang et al. 2007
Fungi	34–61	5.0–6.0, 50–70°C	Unknown	Cu^{2+}, EDTA	CMCase and FPAase	711–1,493	Wen et al. 2005, Ikeda et al. 2006

and one of these, which we designated APX-I, has extremely high specific activity towards oat spelt xylan. The enzyme with a p*I* of 9.4 was purified and the enzyme has a mass of about 25 kDa. The predominant end products of birchwood xylan or xylohexaose hydrolysis are xylobiose and xylose. The enzyme has the highest activity at pH 4.8 and 54°C (Li et al. 1993).

xynA gene encoding xylanase from *A. pullulans* has an open reading frame encoding a polypeptide of 221 amino acids with a calculated mass of 23.5 kDa and contains a putative 34-amino-acid signal peptide. One intron of 59 bp is present in the coding region. The data presented suggest that the highly active xylanases, APX-I and APX-II, secreted by *A. pullulans* are encoded by the same gene (Li and Ljungdahl 1994). The *A. pullulans xynA* gene can be expressed in *Saccharomyces cerevisiae*. A comparison of the *A. pullulans* and *S. cerevisiae* signal peptides demonstrated that the XynA signal peptide is at least three times more efficient than that of *S. cerevisiae* invertase or mating a-factor pheromone in secreting the heterologous xylanase from *S. cerevisiae* cells (Li and Ljungdahl 1996).

The yeast *A. pullulans* when grown on xylose produces hemicellulolytic enzymes with predominant xylanase and β-xylosidase activity and no cellulase activity. *A. pullulans* enzymes are able to hydrolyze selectively the hemicellulose portion of unbleached sulfite pulps directed toward the manufacture of dissolving pulp. The treated pulp by the *A. pullulans* enzymes contains less xylan (48%) and glucomannan (15%) than the untreated reference of dissolving pulp (Christov and Prior 1996).

The α-glucuronidase gene (*aguA*) of *A. pullulans* NRRL Y-2311-1 lacks introns and encodes a polypeptide of 836 amino acids that contains a putative signal peptide of 15 amino acids, resulting in a mature protein with a calculated molecular mass of 91.0 kDa. The *aguA* gene can be expressed in *S. cerevisiae* Y294. The heterologous α-glucuronidase has maximal activity at 65°C and at pH 5 and pH 6 (de Wet et al. 2006).

Table 5 shows that xylanase and its gene from *A. pullulans* are very similar to those from *Aspergillus* spp. and some bacterial strains.

β-Fructofuranosidase and maltosyltransferase

Fructo-oligosaccharides (FOS) are a class of prebiotics widely used as a functional food material. Studies have shown that FOS relieves constipation, improves blood lipid composition in hyperlipidemia, enhances calcium and magnesium absorption, and suppresses the production of intestinal putrefactive substances in both humans and animals and has the ability to act as a growth factor for bifidobacteria. Its commercial production is easy, and its taste is close to that of conventional sweeteners such as sucrose, high fructose corn syrup, and starch-derived sugars.

A transfructosylating reaction by β-fructofuranosidases (FFases; EC 3.2.1.26) from *A. pullulans* and *Aspergillus niger* has typically been used to produce FOS. *A. pullulans* DSM 2404 was found to form at least five kinds of FFases (FFases I–V) in a sucrose medium. During the early stage

Table 5 Some properties of xylanases and their genes from different strains of *A. pullulans,* different species of *Aspergillus* spp., and some bacterial strains

Producers	Molecular mass (kDa)	Optimal pH and temperature	p*I*	Size of xylanase gene (bp)	References
A. pullulans	25.0	4.8, 54°C	9.4	922 (*xylA* gene)	de Wet et al. 2006
Aspergillus spp.	18.8–35	4.0–6.0, 45 −60°C	7–8	678–745	Lu et al. 2008
Some bacterial strains	11–85	6–7, 60–65°C	8.5	576–720	Ninawe et al. 2008

of sucrose culture, FFase I of the five enzymes is formed as the main FFase and large amounts of FOS and glucose are accumulated in the medium. FFase I is formed even in a medium containing glucose as a sole carbon source. Therefore, the expression of FFase I is not repressed by glucose and that the transfructosylating activity of the enzyme may play a key role in FOS accumulation in the sucrose culture. However, it is generally known that the expression of fungal FFases is repressed by glucose in the media. The purified β-fructofuranosidase I (FFase I) formed by *A. pullulans* DSM 2404 has a molecular weight of about 430 kDa and shows high transfructosylating activity. The yield of fructo-oligosaccharides production using purified FFase I is 62% (Yoshikawa et al. 2007). However, when the reaction is carried out with additional commercial glucose isomerase (GI) at an activity ratio of FFase I and GI of 1:2, the maximum FOS yield reaches 69%. This value is higher than that obtained previously using other *Aureobasidium* spp. (53–59%; Yoshikawa et al. 2008).

The fructosyltransferase (FTase) produced by *A. pullulans* CCY 27-1-94 is stable in a broad range of pHs and temperatures up to 65°C, with an optimum pH 4.4 and temperature of 65°C (Onderková et al. 2007).

It was found that the culture fluid and culture broth homogenate of *A. pullulans* CFR 77 which produces fructosyltransferase gives a conversion yield of up to 57% from sucrose at concentrations of 55% and 80% (*w*/*v*). However, the cells are not suitable for FOS production (Sangeetha et al. 2004).

It also has been shown that isomalto-oligosaccharides (IMO) have the same function as the FOS mentioned above. *A. pullulans* is found to have the ability of producing the enzyme involved in the formation of IMO by transglucosylation reaction when cultivated in maltose medium. However, the whole cells cannot be applied directly as an immobilized cell system due to poor reaction performance, which presumably results from a diffusional restriction of substrate and reaction products in the gel matrix. Therefore, continuous production of isomalto-oligosaccharides from maltose syrup by the permeabilized cells of *A. pullulans* (treated by toluene) immobilized into calcium alginate gel was performed. The immobilized cell column maintains its full activity over 45 days when the reactor is operated at a velocity of 0.1/h at 50°C using 60% (*w*/*v*) maltose syrup as a substrate, and the maximum productivity achieved is around 60 g l^{-1} h^{-1} (Yun et al. 1997).

However, the genes encoding β-fructofuranosidase and maltosyltransferase in *A. pullulans* have not been cloned. In addition, it was found that β-fructofuranosidase and maltosyltransferase from *A. pullulans* have many advantages over those from the bacterial strains and *Aspergillus* spp. due to no repression of its expression by glucose and high transfructosylating activity (Table 6; Yoshikawa et al. 2007).

Mannanase

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. β-Mannanase (EC.3.2.1.78) is the key enzyme that catalyzes the random hydrolysis of β-mannosidic linkages in mannan and heteromannan. Manno-oligosaccharides are generated when mannan is hydrolyzed by endo-β-mannanases. These oligosaccharides can be further cleaved by β-D-mannosidase, β-D-glucosidase, and β-D-galactosidase to produce mannose, glucose, and galactose (Lin and Chen 2004).

Mannanases are useful in many fields including bio-bleaching of pulp in paper industry, bioconversion of biomass wastes to fermentable sugars, upgrading of animal

Table 6 Some properties of fructofuranosidase and maltosyltransferase from *A. pullulans* and some bacterial strains

Producers	Molecular mass (kDa)	Optimal pH and temperature	Activated by	Inhibited by	Potential application	Size of the gene (bp)	References
A. pullulans DSM 2404 (fructofuranosidase)	430	4.4, 65°C	Unknown	Unknown	FOS production	Unknown	Yoshikawa et al. 2008, Onderková et al. 2007
A. pullulans (maltosyltransferase)	Unknown	5.5, 50°C	Unknown	Unknown	IMO production	Unknown	Yun et al. 1997
Bifidobacterium longum (fructofuranosidase)	59.4	6.0, 37°C	EDTA, DTT, and BSA	Cu^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, SDS, pHMB	Hydrolysis of oligofructose and inulin	1,596	Janer et al. 2004
A. globiformis IFO 3062	60.5	7.0, 37°C	Unknown	Unknown	Transfers of fructosyl residue from sucrose	1,644	Isono et al. 2004

feed stuff, and reducing the viscosity of coffee extracts. They also have potential application in the production of manno-oligosaccharides, which are utilized selectively by intestinal *Bifidobacterium* species and used as valuable food sweetener or additive (Lin and Chen 2004).

In screening for producers of extracellular β-1,4-mannanase among yeasts and yeast-like microorganisms (Kremnicky and Biely 1997), the best producers were found among strains of *A. pullulans*. A xylanolytic yeast strain *A. pullulans* NRRL Y 2311-1 was found to produce all enzymes required for complete degradation of galactomannan and galactoglucomannan. The enzymes differ in function and cellular localization: endo-β-1,4-mannanase is secreted into the culture fluid, β-mannosidase is strictly intracellular, and α-galactosidase and β-glucosidase are found both extracellularly and intracellularly. Among these enzyme components, only extracellular β-mannanase and intracellular β-mannosidase are inducible (Kremnický and Biely 1997).

However, the gene encoding mannanase in *A. pullulans* have not been cloned, although some information on mannanase and its gene from some bacterial strains have been obtained (Table 7). Therefore, it is necessary to characterize the mannanase and its gene from *A. pullulans*.

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 residues 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 desiccation 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.

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 a result of their role as iron-scavenging compounds, especially marine microorganisms 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. For example, 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).

Table 7 Some properties of mannanase from *A. pullulans* and some bacterial strains

Producers	Molecular mass (kDa)	Optimal pH and temperature	Activated by	Inhibited by	Hydrolysis	Size of mannanase gene (bp)	References
A. pullulans NRRL Y 2311-1	Unknown	4.8, 30°C	Unknown	Unknown	Degradation of mannan	Unknown	Kremnický and Biely 1997
Some bacterial strains	39–60	6.0–10.0, 45–70°C	Mg^{2+}	Ca^{2+}, Cu^{2+}, Pb^{2+}, Mn^{2+}, Hg^{2+}, and Ag^{+}	Degradation of copra mannan	1,080–1,185	He 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 microorganisms (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 committed step in siderophore biosynthesis is the N^5-hydroxylation of ornithine catalyzed by ornithine N^5-oxygenase. The further reactions of siderophore biosynthesis are catalyzed by non-ribosomal peptide synthetases (Haas 2003). We found that the presence of Fe^{3+} in the medium can greatly repress the expression of the gene encoding ornithine N^5-oxygenase, while the presence of L-ornithine can enhance the expression of the gene encoding ornithine N^5-oxygenase in *A. pullulans* HN6.2 (Wang et al. 2008).

Single-cell proteins

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 *S. 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. After crude protein of the yeasts was estimated by the method of Kjehldahl, we 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. pullulan* (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 5.0% (*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).

As mentioned above, *A. pullulans* can produce protease, amylase, cellulase, and xylanase. 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*.

Perspectives

As stated above, it can be clearly observed that the different bioproducts can be produced by different strains of *A. pullulans* isolated from different environments and have highly potential applications. The enzymes from this species of the yeast and the genes encoding the enzymes cloned from the yeasts are unique. However, the whole genome of *A. pullulans* has not been sequenced yet. Therefore, we think that it is very important to sequence the whole genomic DNA of the yeast cells in order to find new more bioproducts and novel genes from this yeast.

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