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Extracellular polysaccharide (EPS) production by a novel strain of yeast-like fungus *Aureobasidium pullulans*

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ABSTRACT

A yeast-like fungus producing a large amount of extracellular polysaccharide was isolated from the biogas reactors at North Wyke Research, UK (latitude, 50°46′29″N; longitude, 3°55′23″W). This strain was identified as *Aureobasidium pullulans* based on nucleotide sequence of the D1/D2 domain of the large-subunit (LSU) rRNA gene. Extracellular polysaccharide or pullulan, produced from this new strain of *A. pullulans* in shake flasks and a stirred tank fermenter was investigated. A maximum polysaccharide concentration of 40.1 g l^{-1}, and productivity of 12.5 g l^{-1} per day was obtained in a batch culture from stirred tank fermenter. The optimal cultivation conditions for pullulan production for this strain in 250 ml shake flask containing 100 ml of sucrose medium were observed at 28 °C and with 200 rpm. The effect of different sugars and nitrogen sources on pullulan production was investigated. It was found that more pullulan was produced when the strain was grown in the medium supplemented with $NaNO_3$ than when it was cultivated in the medium supplemented with C5 and C6 sugars and other nitrogen sources.

1. Introduction

Microbial extracellular polysaccharides (EPS) are polymers produced by microorganisms. In recent years, a major emphasis has been put on the search for novel microbial EPS as well as understanding their role in ecosystems (Singh, Saini, & Kennedy, 2008). Generally in a more complex ecosystem, microbes produce different EPS. A number of yeasts and yeast-like fungi have been isolated from livestock manures and co digestion experiments (Rao et al., 2010), out of these, some were novel species and others had potential to produce EPS.

Biogas reactors contain a large diversity of microorganisms, including many novel microbes (e.g. *Methanobrevibacter acididurans* sp. nov.) (Chouari et al., 2005; Savant, Shouche, Prakash, & Ranade, 2002) and yeasts (e.g. *Cryptococcus shivajii* sp. nov.) (Rao et al., 2010). Some microorganisms in the biogas reactors play a vital role in biomass hydrolysis, VFA (volatile fatty acids) production, EPS production and biomethane production (Klocke, Maehnert, Mundt, Souidi, & Linke, 2007; Klocke et al., 2008). Microbial aggregates form biofilms by creating a network of cells and extracellular polymeric substances (EPS), which include any substances of biological origin. The abbreviation "EPS" has often been expanded to extracellular polysaccharides or exopolysaccharides. However, EPS have been shown to be a rich matrix of polymers, including polysaccharides, proteins, glycoproteins, nucleic acids, phospholipids, and humic acids. Carbohydrate was identified as the predominant constituent in the EPS. EPS are typically reported to aid in the formation of a gel-like network that keeps microorganisms together in biofilms, cause the adherence of biofilms to surfaces, and protect microorganisms against noxious environmental conditions (Frolund, Palmgren, Keiding, & Nielsen, 1996). From a microbiological standpoint, bio-aggregates such as activated sludge flocs or biofilms are subdivided into cells and organic matter exterior to cells and the latter has been traditionally characterized as extracellular polymeric substances (EPS) or exocellular polymers. The importance of EPS in the biogas reactor is due to its critical role in bioflocculation. EPS are also thought to promote cell–cell recognition/communication and protect cells against harsh environmental conditions. Different carbon sources and the carbon/nitrogen ratio (C/N) can also influence the production and composition of EPS (Miqueletoa, Dolosica, Pozzia, Forestia, & Zaiat, 2010).

The polymers synthesized by microorganisms have received increasing interest, mainly because of their useful physiochemical features and uncomplicated biodegradability in the natural environment. Pullulan is one such polymer that is a water-soluble

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glucan gum produced aerobically by growing a yeast-like fungus *Aureobasidium pullulans* (Leathers, 2002). Pullulan is composed of α-1,6-linked maltotriose subunits polymerized with α-1,4 and α-1,6 linkages in a ratio of 2:1 to produce a linear glucan. Pullulan has a wide range of commercial and industrial applications in many fields such as food science, health care, pharmaceuticals and even lithography. Due to its strictly linear structure, pullulan is also very valuable in basic research as well as being a well-defined model substance (Duan, Chi, Li, & Gao, 2007; Kandemir et al., 2005; Singh et al., 2008). *A. pullulans* also produces many hydrolytic enzymes including amylases, proteases, esterases, pectinases, and the hemicellulases, xylanase and mannanase (Chi et al., 2009; Leathers, 2003; Yoshikawa et al., 2006, 2008).

A. pullulans is one of the so-called black yeasts. It is ubiquitous and a cosmopolitan saprophyte, routinely found on leaves and various surfaces such as concrete, wood, and forest barks (Bhadra et al., 2008). Isolates of *A. pullulans* exhibit polymorphic forms ranging from blastic conidia and swollen cells to pseudohyphae, hyphae, and chlamydospores, depending on isolate differences, age, medium and culture conditions (Leathers, 2003). The so-called 'color variant' isolates of *A. pullulans* are prominent producers of endoxylanase (EC 3.2.1.8) and have been isolated only from tropical or subtropical zones (Bhadra et al., 2008).

Many, but not all, strains of *A. pullulans* are capable of producing pullulan. Pullulan production is a variable characteristic in both color variant and normal isolates (Singh & Saini, 2008). *A. pullulans* is a biotechnologically important yeast for pullulan and other bioproducts (Abdel-Lateff, Elkhayat, Fouad, & Okino, 2009; Anastassiadis & Rehm, 2006; Chi et al., 2009). Therefore, the present study was conducted to explore the potential of *A. pullulans* isolated from the biogas reactor for EPS production.

2. Materials and methods

2.1. Isolation of the organism, media and maintenance

Samples were collected from laboratory scale biogas reactors operating at North Wyke Research, South West England, UK (latitude, 50°46′29″N; longitude, 3°55′23″W). A small aliquot of the sample (50 μl) was plated on yeast-malt agar medium (YM) containing ($g\,l^{-1}$) peptone, 5.0; yeast extract, 3.0; malt extract, 3.0; dextrose, 10; and agar, 15.0 supplemented with chloramphenicol 0.1. The plates were incubated at 28 °C for 5 days. The yeast like colonies that subsequently appeared on the plates were grouped initially based on their colony morphology, with between 30 and 40 colonies of each morphotype isolated. Three to five representatives of each morphotype were purified by repeated sub-culturing on Rose Bengal–chloramphenicol plates containing ($g\,l^{-1}$) peptone, 5.0; dextrose, 10.0; mono-potassium phosphate, 1.0; magnesium sulphate, 0.5; Rose Bengal, 0.05; chloramphenicol, 0.1; and agar, 15.0. For routine sub-culturing and maintenance, the strains were grown either on YM agar or in YM broth at 28 °C.

2.2. Examination of growth characteristics

The morphological, physiological and biochemical characteristics of yeasts and yeast-like fungus were determined according to the standard methods as described by Yarrow (1998). Growth temperature was determined by cultivation on YM agar and incubating at different temperatures.

2.3. Isolation and purification of nuclear DNA

Nuclear DNA was isolated from stationary phase grown cultures according to the method of Makimura, Murayama, and Yamaguchi (1994). For this purpose, cells were harvested by centrifugation and the cell pellet suspended in lysis buffer [100 mM Tris–HCl (pH 8.0) containing 2% Triton X-100, 1% SDS and 1 mM EDTA] and then lysed by vortexing with 0.3 g glass beads (0.45–0.52 mm in diameter, Sigma, Poole, UK) (Rao et al., 2010). The cell lysate was then used to prepare genomic DNA.

2.4. DNA sequence analysis

The D1/D2 domain of the 26S rRNA gene region was PCR-amplified from DNA isolated from yeasts. The D1/D2 domain was amplified using primers NL1 and NL4 (O'Donnell, 1993). Amplified fragments were analysed by 1.0% agarose gel electrophoresis, purified with a QIAquick PCR purification kit (QIAGEN) as per the manufacturer's instructions, and cycle sequenced directly using an ABI BigDye terminator cycle sequencing kit, version 3.1 (Applied Biosystems). The D1/D2 fragment was sequenced using the external amplification primers NL1 and NL4. All purified sequencing reaction mixes were sequenced using an ABI PRISM 3730 capillary sequencer (Applied Biosystems).

Sequence similarity searches were conducted using EMBL FASTA Sequences. Closely related taxa were retrieved, aligned and manually corrected using CLUSTAL_X (Thompson et al., 1997). The alignment files were saved with "phy" extension. Dendrograms were constructed using the PhyML program (Guindon & Gascuel, 2003) from 100 replicates of non-parametric bootstrap analysis, using the GTR model of nucleotide substitution and 4 substitution rate categories.

2.5. Cell cultivation in shake flasks and fermenter

EPS yield was determined by growing each culture in 100 ml of EPS production medium (EPM) containing ($g\,l^{-1}$) sucrose, 50.0; peptone, 0.6; yeast extract, 0.4; K_2HPO_4, 5.0; $MgSO_4{\cdot}7H_2O$, 0.4 and NaCl, 1.0 in 250 ml flasks at 28 °C, on a rotary shaker (Thermo Scientific Max^{Q} 4000 shaker) under agitation (200 rpm) for 4 days. Cells also cultivated in Electrolab FerMac 200 Fermenter (Electrolab Limited, Gloucester, UK) with 1.5 L EPS production medium at 25 °C and agitation (300 rpm) with stirrer.

2.6. Isolation and purification of EPS

The fermented broth was clarified using a refrigerated centrifuge (SORVALL RC 5B Plus) and the cell-free supernatant was used as the source of polysaccharide. Cells were separated from the broth by centrifugation at 3000 rpm for 5 min at 20 °C and EPS precipitation was carried out from the culture supernatant with two volumes of isopropyl alcohol.

2.7. Effect of other carbon and supplementation of nitrogen sources

Cells were cultivated using EPS production medium containing ($g\,l^{-1}$) peptone, 0.6; yeast extract, 0.4; K_2HPO_4, 5.0; $MgSO_4{\cdot}7H_2O$, 0.4; and NaCl, 1.0 in 250 ml flasks at 28 °C, on a rotary shaker under agitation (200 rpm) for 4 days. Different carbon sources ($50\,g\,l^{-1}$) were added to the above production medium. To test the effect of extra nitrogen availability on pullulan production, for the above medium containing sucrose as carbon source we added extra $3.0\,g\,l^{-1}$ of Corn Steep Liquor (CSL), urea, NH_4SO_4, and $NaNO_3$ in four different flasks.

2.8. HPLC analysis

Extracted and precipitated EPS was dissolved in water and measured by high performance liquid chromatography (HPLC), (Thermo Spectra System, Waltham, MA, USA) equipped with an

Agilent ZORBAX Bio Series GF-450 Column to determine the molecular weight (MW) of the polysaccharide. The samples were eluted with HPLC grade water at a flow rate of 0.5 ml min^{-1} at 80 °C and detected with a differential refractive index detector (Perkin Elmer Series 200, Waltham, MA, USA). The data was processed with Chromquest software.

3. Results and discussion

3.1. Molecular characterization of isolated strains

Isolation on YM agar plates yielded more than 20 yeast colonies on each plate which could be distinguished into five different morphotypes based on colony morphology. Most colonies were smooth, moist, yeast like and pale pink. These five strains were identified as *Hanseniaspora guilleiermondii*, *Pichia kluyveri*, *Issatchenkia orientalis*, *Candida orthopsilosis* and *A. pullulans* based on the nucleotide sequence of the D1/D2 domain of the large-subunit (LSU) rRNA gene.

The fifth strain and the closest phylogenetic neighbours with respect to the nucleotide sequence of the D1/D2 domain of the large-subunit (LSU) rRNA gene are shown in Fig. 1. Based on the pylogenetic analysis this strain was identified as *A. pullulans.* This strain was phylogenetically very close to other *Aureobasidium* sp. and in the same clade of *A. pullulans* YS67, previously reported as a strong xylanase producer isolated from forest tree bark sample (Bhadra et al., 2008).

3.2. EPS production and analysis

EPS yield was determined by growing the culture in 100 ml of EPS production medium (EPM) in flasks. The flasks were incubated at 28 °C and shaken at 200 rpm for 4 days in a Thermo Scientific Max^{Q} 4000 orbital shaker (UK). EPS in the supernatant was precipitated using two volumes of 95% ethyl alcohol and dried at 60 °C. Individual precipitated EPS samples were dissolved in water. HPLC analysis (Fig. 2) has shown that the sample peak was matched with a standard pullulan 50 kDa (25 mg/ml; Sigma).

Table 1
Effect of carbon and nitrogen source on EPS production.

	Wet biomass (g 100 ml^{-1})	EPS (g 100 ml^{-1})
Sucrose	2.37	5.09
Xylose	1.65	0.10
Glucose	1.66	0.31
Cellobiose	2.25	1.74
Fructose	2.56	3.65
CSL	3.00	2.25
NH_4SO_4	3.79	3.60
$NaNO_3$	3.33	5.47
Urea	2.25	2.02

3.3. Effect of carbon sources

Several fermentation parameters are known to influence the production of pullulan, including pH, medium composition, age of culture, and strain of *A. pullulans*. Duan, Chi, Wang, and Wang (2008) demonstrated that pullulan yield was the highest when *A. pullulans* Y68 was grown in a glucose containing medium. To study the effect of different sugars other than sucrose on EPS production we used xylose, glucose, fructose and cellobiose containing medium. We noticed the maximum yield in the control sucrose production medium than medium containing other sugars. In sucrose medium the new isolate produced 5.09 g 100 ml^{-1} of EPS. The production was comparable and higher than recently reported EPS producing strains. After fermentation cell biomass was also low for other sugar substrates compared to the control production medium (Table 1). We compared the new isolate with *A. pullulans* NRRL 58533 and *A. pullulans* NRRL 58530 both strains produced 4.37 g 100 ml^{-1} and 3.55 g 100 ml^{-1} of pullulan respectively.

3.4. Effect of nitrogen sources

We studied the fermentation production when the medium was supplemented with extra nitrogen source such as Corn Steep Liquor (CSL), urea, NH_4SO_4, and $NaNO_3$. More EPS was produced with $NaNO_3$ than without nitrogen addition. $NaNO_3$ addition

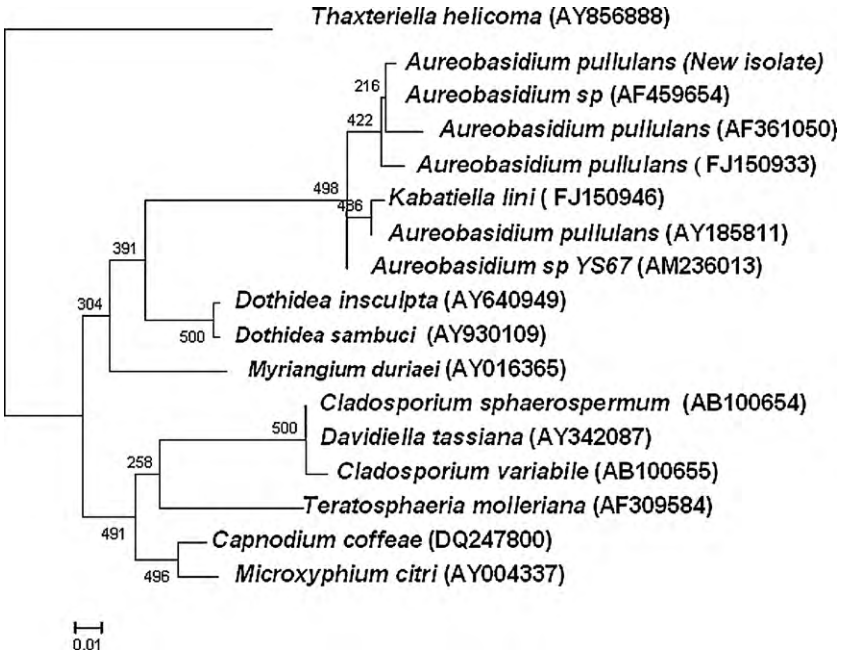

Fig. 1. Neighbour-joining phylogenetic tree based on sequences of the D1/D2 domain of the 26S rRNA gene showing the relationship of the new isolate *Aureobasidium pullulans* with other member species. The tree was constructed using the PhyML program. Bootstrap values ≥50%, determined from 100 replicates, are shown at branch nodes. Scale bar, two base substitutions per 100 nucleotides.

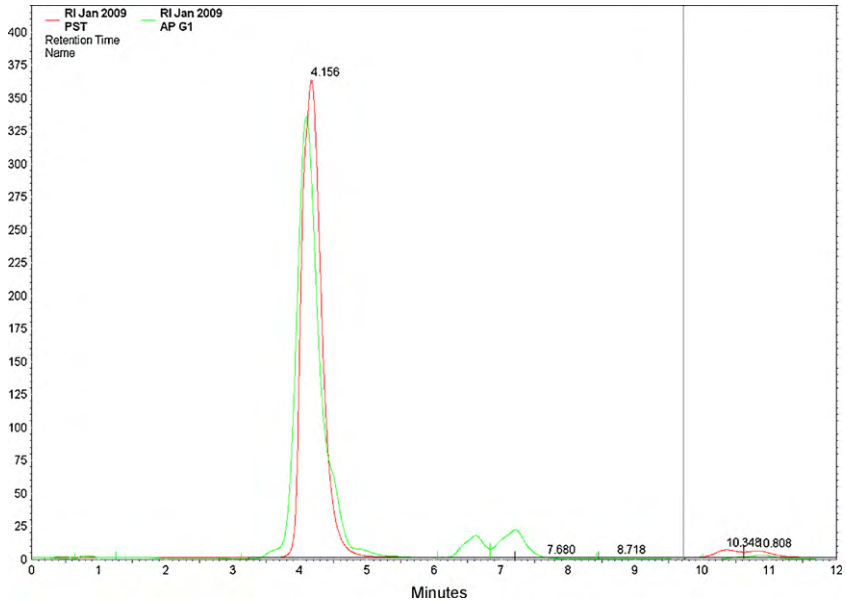

Fig. 2. HPLC chromatogram of standard pullulan (PST in red colour) (Sigma Aldrich, UK) and pullulan (APG1 in green colour) produced by new isolate of *Aureobasidium pullulans*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

produced the highest yield of EPS at 5.47 g 100 ml^{-1}. In contrast NH_4SO_4 addition yielded more cell biomass than $NaNO_3$. All nitrogen supplemented medium except urea shown more cell biomass than the control production medium (Table 1). Lin, Zhang, and Thibault (2007) demonstrated that different strains had an influence on EPS production, and the nitrogen source on the molecular mass of the EPS. This study also concluded that EPS production kinetics by *A. pullulans* was dependent on the specific microorganism–medium–environment system. Campbell, McDougall, and Seviour (2003); Orr et al. (2009) have shown that various concentrations of $NaNO_3$ affected the EPS formation by *A. Pullulans*.

Pullulan was produced in late exponential and early stationary phase of cultures. An undesirable characteristic feature of most of the strains of *A. pullulans* is the production of dark pigment, which is a melanin-like compound and appears dark green to black in colour (Prasongsuk et al., 2007; Zheng, Campbell, McDougall, & Seviour 2008; Singh & Saini, 2008). Melanin is one of the major problems in pullulan production and is responsible for dark green to black colour of the broth. Thus, an appropriate downstream processing of the fermentation broth is required to alleviate the pigmentation problem for melanin producing strains (Singh, Saini & Kennedy, 2009). With this new strain the fermentation broth turned to dark green only after 7 days of incubation or a medium addition of CSL. This new isolate was able to produce the maximum amount of EPS within 4 days of incubation without turning the fermentation broth to a green colour. Generally from strains producing melanin-free pullulan, recovery and purification is accomplished with one precipitation step using a suitable organic solvent.

Gniewosz and Duszkiewicz-Reinhard (2008) reported a white mutant of *A. pullulans* that produce less melanin compared to the parent strain, mutageneisis may help to generate *A. pullulans* that produce less melanin in the fermentation medium.

3.5. Pullulan production in the FerMac 200 Fermenter

Cells were also cultivated in an Electrolab FerMac 200 Fermenter (Electrolab Limited, Gloucester, UK) with 1.5 L of EPS production medium at 25 °C and agitation (at 300 rpm) with a stirrer for 4 days. The maximum EPS concentration was 40.1 g l^{-1} and an EPS productivity of 12.5 g l^{-1} per day was obtained in a batch culture.

Lazaridou and Roukas (2002) reported a maximum pullulan concentration of 49 g l^{-1}, from beet molasses with 100 g l^{-1} sugar concentration in a stirred tank reactor with impeller speed of 700 rpm. In another study Roukas and Liakopoulou-Kyriakides (1999) cultivated *A. pullulans* in a fermenter and reported a maximum EPS concentration of 23 g l^{-1} with a, 40% yield and a maximum sugar utilization of 96%. Fermentation medium optimization may further improve EPS production with the new isolate of *A. pullulans*.

In a recent study, Manitchotpisit et al. (2009) isolated 45 strains of *A. pullulans* from leaves. Based on the phylogenetic analyses, these isolates were classified into 12 clades. The strains in these clades showed different colors on different culture media including two clades with 'color variants' and some clades exhibited high levels of pullulan production or xylanase activity. Clade 12 isolates also make an EPS different from pullulan and have a distinct colony appearance. Most EPS producing *A. pullulans* strains were reported from tropical countries and EPS production depends on strain performance within the *A. pullulans* these range from 1.3 g l^{-1} to 29 g l^{-1}. Although the literature on pullulan biosynthesis is contradictory because of difference among the strains of *A. pullulans* (Loncaric, Oberlerchner, Heissenberger, & Moosbeckhofer, 2009). The concentration of EPS produced by *A. pullulans* was dependent on the carbon source.

4. Conclusions

We were able to isolate a biotechnologically important yeast-like fungus from a biogas reactor. This new strain of *A. pullulans* was able to produce a high level of EPS in comparison with other tropical *A. pullulans* isolates. The concentration of the nitrogen in the fermentation medium affects microbial biomass accumulation and the yield of pullulan produced by *A. pullulans*. The study has also confirmed that the concentration of EPS produced by *A. pullulans* was dependent on the carbon and nitrogen sources. Further work on medium and process optimization for EPS production is in progress.

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