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Effects of melanin on the accumulation of exopolysaccharides by *Aureobasidium pullulans* grown on nitrate

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Abstract

Aureobasidium pullulans produced pullulan and melanin when grown in medium containing low nitrate levels. With high nitrate concentrations, however, this fungus produced a mixture of exopolysaccharides (EPS) without melanin synthesis. At 0.78 g l^{-1} N as nitrate, where no melanin synthesis occurred, maximum EPS yields reached 6.92 g l^{-1} and then decreased to the final yield of 2.36 g l^{-1}. Following melanin addition (0.1 g l^{-1}), yields reached 7.02 g l^{-1} at 48 h and fell to a final yield of 5.21 g l^{-1}. The EPS produced in high nitrate medium contained both pullulan and (1 → 3)-β-glucan, but only pullulan was produced with melanin-supplementation. With melanin addition a doubling of (1 → 3)-β-glucanase activity was observed in high nitrate medium compared to that without supplementation. On the other hand amylolytic activities disappeared in medium with melanin production or addition. Culture filtrates sustained a higher reducing capacity (*RC*) when melanin was present. Low *RC* appeared to reduce (1 → 3)-β-glucanase activity and increase amylolytic activities. Thus, higher *RC* appears to inhibit production/activity of amylose-degrading enzymes capable of degrading pullulan, and stimulates (1 → 3)-β-glucanase synthesis/activity, leading to a preferential accumulation of pullulan.

Keywords: *Aureobasidium pullulans*; Pullulan; EPS production; Melanin; Sodium nitrate

1. Introduction

The neutral and water-soluble polysaccharide pullulan is the predominant exopolysaccharide (EPS) produced by the polymorphic fungus *Aureobasidium pullulans*, and this polysaccharide has great potential (Shingel, 2004) in food (Ikewaki et al., 2007) and pharmaceutical industries (Shin et al., 2007). Considerable interest has been focused on the factors affecting its production (Gibbs and Seviour, 1996; Reeslev et al., 1991) and the relationship between EPS synthesis and population morphology (Reeslev et al., 1996; Simon et al., 1995). It is now clear that the EPS released by *A. pullulans* under certain culture conditions consists not only of pullulan, a linear (1 → 4:1 → 6)-α-glucan, but also other polysaccharides including 1 → 3:1 → 6)-β-glucans (Kimura et al., 2006). Factors known to influence EPS yields in *A. pullulans* include the fungal strain used (Augustin et al., 1997; Radulovic et al., 2008; Silman et al., 1990), nature of the carbon (C) source in the medium (West and Reed-Hamer, 1991; Youssef et al., 1998), culture pH (Ono et al., 1977), incubation temperature (McNeil and Kristiansen, 1990), dissolved oxygen levels (Gibbs and Seviour, 1996), as well as fermenter configuration (Gibbs and Seviour, 1992). Another important factor affecting EPS yields is the nitrogen source (N) and its level. In medium containing ammonia (NH_4^+), for example, low initial levels of N lead to increased EPS accumulation; while at high levels EPS yields decrease at the expense of increased C flow into biomass formation (Seviour and Kristiansen, 1983).

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With nitrate, increasing N concentrations result in a considerable loss of EPS during the course of the fermentation, an event coinciding with exhaustion of the carbon source (glucose) from the medium (Campbell et al., 2003). Several studies showed that EPS yields may decrease in older cultures of *A. pullulans* (Pollock et al., 1992), but not always (Silman et al., 1990). As *A. pullulans* has never been shown to produce any pullulanase activity, reasons for such decreases are still unclear (Campbell et al., 2003). Other studies have described the production of an α-amylase (1 → 4)-α-D-glucan glucanohydrolase, EC3.2.1.1) and two forms of glucoamylase (1 → 4)-α-D-glucan glucohydrolase, EC3.2.1.3) by *A. pullulans* (Saha and Bothast, 1993; Saha et al., 1993; West and Strohfus, 1996). Only one of these (glucoamylase) was active against pullulan (Campbell et al., 2003). Possible roles for these glucoamylases in affecting EPS yields during fermentation have not yet been elucidated. During batch cultures of *A. pullulans* in media containing low initial N levels, cell melanins are also produced concomitant with EPS elaboration, reaching about 0.5–0.7 g l^{-1} by the end of culture period (Gadd, 1980). Melanins are powerful antioxidant agents capable of protecting cells from damage caused by free radicals (Goncalves and Pombeiro-Sponchiado, 2005; Huang et al., 2006). In *A. pullulans* nearly half the melanins are deposited on cell walls of chlamydospores, and the rest are released into the medium as black granulates (Huang et al., 2006).

In *Aspergillus phoenics* and *Sclerotium rolfsii*, melanin-covered hyphal cell walls show strong resistance to attack by several depolymerase enzymes including chitinase and (1 → 3)-β-glucanase (Bloomfield and Alexander, 1967). However, relatively little is known about any possible roles for melanin in EPS accumulation by *A. pullulans*. A series of fermentations were carried out to determine whether melanin affected the yields and chemical composition of the EPS produced by *A. pullulans* and hence its susceptibility to enzymatic degradation. The effects of melanin on the activity of EPS-degrading enzymes produced by *A. pullulans* ATCC 9348 were also investigated.

2. Methods

2.1. Organism, inoculum preparation and culture conditions for CSTR studies

A. pullulans de Bary Arnaud (ATCC 9348) was maintained as previously described (Gibbs and Seviour, 1996). Methods for preparing a standardized inoculum of 0.1 g l^{-1} (dry weight) for constant stirring tank reactor (CSTR) fermentations are also described in Gibbs and Seviour (1996). The media used contained either 0.13 or 0.78 g l^{-1} N in the form of $NaNO_3$, and 30 g l^{-1} glucose as sole N and C sources, respectively, as detailed later. Melanin from *A. pullulans* (see below) was added to the medium containing 0.78 g l^{-1} N at a final concentration of 0.1 g l^{-1} before fermentation. Cells harvested from malt extract agar (Oxoid) slopes were used to inoculate 500 ml Erlenmeyer flasks containing 150 ml of medium of the same composition used in the subsequent fermentations, and grown by shaking for 36 h at 26 °C.

Fermentations were carried out with a Braun Biostat MD fermentation system (Sartorius) consisting of a 5 l CSTR, fitted with three 6.5 cm single-bladed Rushton turbines, evenly spaced on the drive shaft, and no baffles. Culture conditions used were those described previously (Gibbs and Seviour, 1996). Cells were aerated at a rate of 0.3 vvm and agitated at 500 rpm. Any foaming was controlled by the manual addition of polypropylene glycol 2025 (BDH) (Stasinopoulos and Seviour, 1989). A constant pH of 4.5 and temperature of 26 °C were used (McNeil and Kristiansen, 1990; McNeil et al., 1989). Culture pO_2 and pH were monitored with Ingold polarographic pO_2 and pH probes (Mettler Toledo), respectively. Control of culture parameters, where necessary, was achieved with the Braun DCU, and data recorded using the Braun Multi-Fermenter Computer System for Windows (MFCS/win) software version 2.0 (Sartorius). All fermentations were repeated at least once.

2.2. Production of EPS by A. pullulans

Samples from the CSTRs were collected at 8 h intervals up to 56 h and then every 12 h. Methods for analysis of biomass and EPS levels were those described previously (Gibbs and Seviour, 1996). Residual glucose concentrations in culture filtrates were determined with a glucose hexokinase kit (p/n Tr 15003) from Trace Diagnostics (Melbourne, Australia), following the manufacturers' instructions. Residual NO_3^{-1} concentration was determined in culture filtrates with a Shimadzu LC-10Ai ion chromatography system (Shimadzu Oceania Pty. Ltd.). This system was fitted with an anion exchange column (number 302 IC), quaternary amine (Vydac) at a temperature of 30 °C. Detector (CDD-6A, Shimadzu Oceania Pty. Ltd.) and 3 mM phthalic acid adjusted to pH 4.9 with 1 M sodium tetraborate as the mobile phase at a flow rate of 1.5 ml/min.

2.3. Chemical characterization of EPS material and enzyme assays

Samples were centrifuged at 15,000*g*, 4 °C for 10 min, and filtered if necessary, to produce crude filtrates. These were added to 2 vol. 95% ethanol to precipitate polysaccharide material, and dialyzed extensively against distilled water at 4 °C before being freeze dried, reconstituted with 20 mM sodium acetate buffer (pH 4.5) and used in subsequent enzymatic assays.

Activities for (1 → 3)-β-glucanase activities in culture filtrates were determined using laminarin from *Laminaria digitata* (Sigma) as substrate, and amylolytic activity assays used amylose (type III, linear (1 → 4)-β-glucan) from potato (Sigma). Culture filtrates (50 μl) and substrates