Influence of Zn^{2+} on yeast–mycelium dimorphism and exopolysaccharide production by the fungus *Aureobasidium pullulans* grown in a defined medium in continuous culture

MORTEN REESLEV,* BIRGITTE B. JØRGENSEN and O. B. JØRGENSEN

Department of Biotechnology, The Technical University of Denmark, Lyngby, Denmark

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The yeast–mycelium dimorphism of *Aureobasidium pullulans* was studied in continuous culture in a defined medium. At a constant dilution rate (0.08 h^{-1}) the morphological status of the culture could be controlled by the input concentration of Zn^{2+} . As the input concentration of Zn^{2+} was increased (in intervals from 0 to $7.6\text{ }\mu\text{M}$) the culture shifted from a zinc-limited to a carbon-limited state. In this interval the culture gradually passed through three growth regimes based on morphology and concentration of exopolysaccharide and biomass. The first growth regime was found when the input concentration of Zn^{2+} was kept below $0.45\text{ }\mu\text{M}$. Growth in this regime was zinc-limited and more than 90% of the biomass was in the yeast growth form. An increase in the input concentration of Zn^{2+} in this growth regime led to a proportional increase in both the biomass and the concentration of exopolysaccharide. When the input concentration of Zn^{2+} was varied between $0.45\text{ }\mu\text{M}$ and $0.80\text{ }\mu\text{M}$ a second growth regime could be detected where simultaneous limitations in two nutrients were recognized. Although the carbon source (glucose) was exhausted an increase in the input concentration of Zn^{2+} led to a proportional increase in the steady-state biomass concentration. The increase in biomass concentration was at the expense of exopolysaccharide production, which gradually decreased. The culture, still being primarily limited by Zn^{2+} , remained in the yeast growth form. In a third growth regime (input concentration of Zn^{2+} above $0.80\text{ }\mu\text{M}$) no increase in the steady-state biomass was seen when the input concentration of Zn^{2+} was increased. The concentration of residual glucose and exopolysaccharide was close to zero, and no further carbon could be diverted to an increase in the biomass. Glucose was the primary limiting nutrient. Increasing the input concentration of Zn^{2+} in this growth regime led to a gradual increase in the mycelial growth form at the expense of the yeast growth form. More than 90% of the biomass was in the mycelial growth form when an input concentration of Zn^{2+} of $7.6\text{ }\mu\text{M}$ was used.

Introduction

Aureobasidium pullulans is capable of growing by budding as a yeast as well as by hyphal extension. It produces the exopolysaccharide pullulan, the production of which depends on several parameters (reviewed by Catley, 1979, and by Seviour *et al.*, 1992). The most important parameters appear to be pH, temperature, nitrogen availability and morphology. The connection between morphology and exopolysaccharide production was first recognized by Catley (1973, 1980), who found that the yeast cells were the main producers of exopolysaccharide. Later, it was shown (Reeslev *et al.*,

1991) that there was a relationship between the initiation of exopolysaccharide production and the emergence of yeast cells on the mycelium (M–Y transition). These events occurred at the same time as a significant decrease in the growth rate caused by limitation in some nutrient(s) or growth factor(s) in the yeast extract. The connection between morphology and exopolysaccharide production has complicated the interpretation of the effect of environmental parameters on exopolysaccharide production.

It is necessary to distinguish between direct effects on exopolysaccharide production and indirect effects caused by a change in the morphology of the cells. McNeil *et al.* (1989) showed that changes in the pH of the growth medium in continuous culture affected exopolysaccharide production as well as the relative proportions of the morphological growth forms. Assuming that exopoly-

* Author for correspondence. Present address: Department of General Microbiology, University of Copenhagen, Sølvgade 83H 1307, Copenhagen K, Denmark. Tel. +45 35 322057; fax +45 35 322040.

saccharide was produced only by the yeast cells would lead to the conclusion that the specific exopolysaccharide production would be highest at low pH (2.5). However, if the specific rate of exopolysaccharide production by the yeast cells was independent of pH as suggested by Heald & Kristiansen (1985), then the filamentous form of *A. pullulans* may make a significant contribution to exopolysaccharide biosynthesis.

Yeast extract has been included in most media used for growth and exopolysaccharide production of *A. pullulans*. Defined media, lacking complex components such as yeast extract, are, however, desirable for quantitative growth analyses. In shake-flask cultures using a defined medium (Reeslev *et al.*, 1990), the trace elements Zn^{2+} and Fe^{3+} were found to have a profound effect on growth, morphological development and production of exopolysaccharide. No effects of Mn^{2+} , Ca^{2+} or Cu^{2+} were seen.

The objective of this study was to examine further the effects of zinc and iron on the dimorphism of and exopolysaccharide production by *A. pullulans* in continuous culture in a defined growth medium, and to study the relationship between exopolysaccharide production and morphology.

Methods

Microbial strain and culture media. The fungus *Aureobasidium pullulans* QM 3092 was supplied by Dr B. Kristiansen, University of Strathclyde, UK. The medium referred to as b-medium (basic medium) contained (mM) $(\text{NH}_4)_2\text{SO}_4$ (18.20), KH_2PO_4 (21.90), NaCl (25.67), MgSO_4 (0.81), glucose (55.51), citric acid (1.63) together with (μM): CaCl_2 (126.2), MnCl_2 (6.8), CuCl_2 (1.6), Na_2MoO_4 (0.9), CoCl_2 (1.2), H_3BO_3 (6.5), KI (0.4). Synperonic PE/L61 (antifoaming agent) was added to a concentration of 0.07 g l^{-1} . All components were dissolved in distilled water. The distilled water was (by distillation in an all-glass apparatus) prepared from water that had previously been demineralized by passage through an ion-exchange column. The pH of the medium was adjusted to 2.5 using HCl. This acidification prevented any precipitation [e.g. of $\text{Fe}(\text{OH})_3$] and reduced contamination. All chemicals used (except Synperonic) were of analytical grade.

When investigating the effect of adding Zn^{2+} or Fe^{3+} or both to the b-medium, FeCl_3 and/or ZnCl_2 were added to concentrations of $17.8 \mu\text{M}$ and $7.6 \mu\text{M}$ respectively.

The vz-medium (variable zinc medium) was b-medium plus FeCl_3 ($17.8 \mu\text{M}$) with various concentrations of ZnCl_2 with 83.26 mM-glucose.

In all experiments the glassware was rinsed in dilute HCl followed by a rinsing in glass-distilled water.

Inoculum preparation and cultural conditions. A 1 ml frozen culture sample (-80°C) was allowed to thaw. The sample was then suspended in 12 ml 1% (w/v) sterile aqueous NaCl. The suspended cells were then transferred aseptically into four Erlenmeyer flasks (500 ml) containing 125 ml sterile inoculum medium, as described by Reeslev *et al.* (1991). The shake-flasks were incubated at 27°C for about 72 h yielding a heterogeneous culture consisting of both yeast cells and mycelium. Cultures inoculated with yeast cells only were much less prone to growing on the wall of the fermenter in the early stages of culturing. When wall growth was avoided in the early stages it seldom became a problem at later stages. Therefore, all experiments described were

inoculated with yeast cells only. Mycelial cells were separated from yeast cells by filtration using sterile Whatman no. 54 papers ($20 \mu\text{m}$ pore size). The filtrate was centrifuged at 9800 g (r_{av} , 8.8 cm) for 10 min and the pellet suspended in 1% (w/v) sterile, aqueous NaCl.

Continuous culture experiments employed a 1 litre Porton-type fermenter with two four-bladed impellers and no baffles. The dilution rate was $0.080 \text{ h}^{-1} \pm 0.003 \text{ h}^{-1}$; temperature $27^\circ\text{C} \pm 1^\circ\text{C}$; working volume was typically around 800 ml (depending on the viscosity of the broth); aeration 600 ml min^{-1} ; rate of agitation 1000 r.p.m. The pH of the culture was maintained at 5.0 ± 0.2 by automatic titration with 2.0 M aqueous NaOH.

Sampling and determination of steady state. After inoculation or after changes in growth conditions had been introduced, at least 5 to 6 mean residence times were allowed to pass before sampling was initiated. Samples were collected on three consecutive days after which the constancy of the data was evaluated. Changes in growth conditions were only performed for cultures at steady state.

In order to minimize the disturbance of the system the sample volume withdrawn from the fermenter was kept low and was usually in the range 5–7% of the working volume. In the experiments where the transient response to a change in the medium composition was followed, time zero was the time where the first sample was withdrawn from a newly started continuous culture.

Quantitative determination of biomass, morphology and exopolysaccharide. Samples were centrifuged at 9800 g (r_{av} , 8.8 cm) for 10 min. The supernatant was decanted and used for the assay of exopolysaccharide and glucose. The pellet was resuspended in 1% aqueous NaCl to the original sample volume and a 5 ml sample was withdrawn for the determination of yeast cell biomass. The remaining part was used for determination of the total dry weight of the biomass by filtration through a pre-weighed membrane filter (cellulose nitrate filter, $1.2 \mu\text{m}$ pore size, Sartorius), and drying at 85°C to constant weight.

The yeast cells were recovered quantitatively from the 5 ml culture sample by gravity filtration through a nylon mesh of $41 \mu\text{m}$ square porosity (Hydro-Bios, Kiel, Germany) and washing the filter with 10 ml 1% (w/v) aqueous NaCl. Determinations of the dry weight were made from measurements of the optical density at 550 nm in a Shimadzu UV-VIS recording spectrophotometer UV-260 (glass cuvettes, 1 cm light path). Turbid samples were diluted with 1% (w/v) aqueous NaCl in order to measure the OD_{550} at values below 0.3. The dry weight was determined from linear calibration curves of OD_{550} against dry weight of yeast cells (determined as described above for total biomass). The mycelial biomass was determined indirectly by subtracting the yeast biomass dry weight from the total biomass dry weight. Determinations of the exopolysaccharide concentration were performed by ethanol precipitation by adding 1 vol. 96% (v/v) aqueous ethanol. The precipitate was collected on a pre-weighed membrane filter (cellulose nitrate filter, $1.2 \mu\text{m}$ pore size, Sartorius) and dried to constant weight at 85°C . No attempts were made to determine the pullulan content of the exopolysaccharide. In order to keep the sample size low, only single determinations of biomass, morphology and exopolysaccharide were made. The reproducibility of sampling and of determinations of biomass, morphology and exopolysaccharide was tested. Eight samples were withdrawn immediately after each other from a continuous culture at steady state. The results of these determinations (mean dry wt, $\text{g l}^{-1} \pm \text{SD}$) were: biomass 7.09 ± 0.13 , exopolysaccharide 1.32 ± 0.13 , yeast biomass 3.37 ± 0.03 .

Determination of glucose and zinc. The concentration of glucose in the culture supernatant was determined in a glucose and L-lactate analyser (YSI model 2000). The concentration of zinc in the 'zinc-free' vz-medium was determined by atomic absorption spectrophotometry (Perkin-Elmer 5000, deuterium background correction, HGA400 graphite furnace, AS-1 automatic sample injection system).

Results and Discussion

Medium without added Zn^{2+} and Fe^{3+}

Steady-state growth of *A. pullulans* in b-medium was obtained and the values of biomass, yeast biomass and exopolysaccharide determined (Table 1). The steady state could be maintained for at least 380 h. Zinc and iron are known to be essential nutrients (Vallee, 1983), and the fact that a steady state was obtainable in the absence of these metals probably reflects their presence as impurities. In this growth medium, steady-state cultures comprised only yeast cells. This is in agreement with earlier findings in shake-flask experiments (Reeslev *et al.*, 1990), which showed that the presence of both Zn^{2+} and Fe^{3+} reduced significantly the percentage of biomass in the yeast form.

Effect of adding Fe^{3+}

When $17.8 \mu\text{M-Fe}^{3+}$ was added to b-medium (Table 1) no significant change in the steady-state biomass concentration was found (Student's *t*-test, $P = 0.05$) and the

Table 1. Mean value and standard deviation of the steady-state concentrations of total biomass, yeast biomass and exopolysaccharide obtained using b-medium and b-medium plus Fe^{3+}

The chemostats were run at a dilution rate of 0.08 h^{-1} .

	b-medium*	b-medium plus Fe^{3+} †
Biomass (g l^{-1})	1.98 ± 0.30	2.15 ± 0.24
Yeast biomass	1.93 ± 0.32	2.09 ± 0.19
Exopolysaccharide (g l^{-1})	0.73 ± 0.26	1.26 ± 0.07

* Values are the mean of 35 determinations from nine independent chemostat experiments.

† Six determinations from two independent chemostat experiments.

Fig. 1. Transient response of adding Zn^{2+} to a steady-state continuous culture (grown initially on b-medium) of *A. pullulans* at a dilution rate of 0.08 h^{-1} . ■, Total biomass; ●, mycelial biomass; □, exopolysaccharide. The arrow indicates the time at which the medium was changed.

culture remained in the yeast form. A significant increase in the concentration of exopolysaccharide was found (Student's *t*-test, $P = 0.0005$).

Effect of adding Zn^{2+}

The effect of addition of $7.6 \mu\text{M-Zn}^{2+}$ to b-medium is shown in Fig. 1. The first three data points represent steady-state growth in b-medium. Thereafter the input medium was shifted to b-medium plus Zn^{2+} at the time indicated by the arrow resulting in the appearance of mycelium, the fraction of which increased to 74 % of the dry weight. The total biomass concentration remained unchanged when compared to the variability of the steady-state values obtained in b-medium in nine separate experiments (see Table 1), while the exopolysaccharide concentration seemed to decline after addition of Zn^{2+} .

Effect of adding both Zn^{2+} and Fe^{3+}

The effect of adding both Zn^{2+} and Fe^{3+} ($7.6 \mu\text{M}$ and $17.8 \mu\text{M}$ respectively) to a steady-state culture growing in b-medium is shown in Fig. 2. After addition of Zn^{2+} and Fe^{3+} , an increase in the concentration of the total biomass occurred, stabilizing at a biomass concentration of around 5 g l^{-1} . At this point, growth was limited by the availability of glucose. The addition of Zn^{2+} and Fe^{3+} led to a shift in morphology and the mycelial fraction of the biomass increased from $\leq 5\%$ to 75–80%. The new steady-state concentration of biomass was reached after approximately 2 residence times, while around 10 residence times were required for the completion of the morphological shift. A similar phenomenon was reported by Shepherd & Sullivan (1976) for the dimorphic response of *Candida albicans* in continuous culture. They stated that when a change in morphology occurred, more

Fig. 2. Transient response of adding Zn^{2+} and Fe^{3+} to a steady-state continuous culture (grown initially on b-medium) of *A. pullulans* at a dilution rate of 0.08 h^{-1} . ■, Total biomass; ●, mycelial biomass; ○, yeast biomass; △, residual glucose. The arrow indicates the time at which the medium was changed.

Fig. 3. Effect of varying the input concentration of Zn^{2+} on chemostat culture of *A. pullulans* at a dilution rate of 0.08 h^{-1} . The steady-state levels of total biomass (■), mycelial biomass (●), exopolysaccharide (□) and residual glucose (△) are shown. Each point represents the mean of three measurements made on three consecutive days; the bars give the highest and lowest value measured and when not shown were smaller than the dimensions of the symbols. The different growth regimes (I–III) are indicated.

than 12 'doubling times' were needed to obtain steady state while stabilization of the biomass concentration occurred within 7 'doubling times'.

In general, the concentration of exopolysaccharide obtained in these experiments was low. However, the exopolysaccharide concentration per biomass unit before and after addition of Fe^{3+} and Zn^{2+} decreased significantly from $0.29 \text{ g exopolysaccharide (g biomass)}^{-1}$ to $0.03 \text{ g exopolysaccharide (g biomass)}^{-1}$.

Continuous cultivation of *A. pullulans* in b-medium apparently resulted in a zinc-limited culture with a concentration of iron that was close to being limiting for growth. Shifting to b-medium plus Fe^{3+} also yielded a zinc-limited culture, but with a higher availability of iron. This increase in the availability of iron may be responsible for the increase in exopolysaccharide production. When b-medium plus Zn^{2+} was used, the culture was iron-limited, and a shift in morphology was observed. The concentration of biomass did not significantly increase (Fig. 1), probably because the concentration of iron was close to being limiting in b-medium. A decrease in exopolysaccharide production was observed in these conditions. The shift to b-medium plus both Fe^{3+} and Zn^{2+} (Fig. 2) resulted in a carbon-limited culture which resembled the iron-limited culture in giving preference to the mycelial growth form and almost negligible exopolysaccharide production.

Varying the input concentration of Zn^{2+}

The effect of Zn^{2+} concentration was studied using vz-medium containing $17.8 \mu\text{M-Fe}^{3+}$. The steady-state concentrations of biomass, exopolysaccharide and residual glucose are shown in Fig. 3. At least three separate growth regimes could be recognized. The first (I) (input

concentrations of Zn^{2+} from 0.0 to $0.45 \mu\text{M}$) was characterized by a linear increase in the steady-state concentration of biomass with increasing input concentrations of Zn^{2+} , indicating zinc-limited growth. The concentration of exopolysaccharide increased proportionally with increases in the input Zn^{2+} concentration and a linear decrease in the residual glucose was measured. In this regime more than 90% of the biomass was in the yeast form. In the second growth regime (II) (input concentration of Zn^{2+} from 0.45 to $0.80 \mu\text{M}$) the residual concentration of glucose was close to zero. However, the concentration of biomass continued to increase proportionally with increasing input concentrations of Zn^{2+} until it stabilized at 7.3 g l^{-1} , which is comparable to glucose-limited cultures with an input glucose concentration of 15 g l^{-1} (Reeslev, 1992). The increase in biomass could therefore be explained by the simultaneous decrease in the production of exopolysaccharide. As in the first growth regime, more than 90% of the biomass was in the yeast form. It is interesting that the yeast cells were able to grow without substantial exopolysaccharide production, suggesting that exopolysaccharide production is not constitutive for growth of yeast cells.

In continuous culture a proportional relationship existed between the quantity of growth-limiting nutrient consumed and the steady-state concentration of biomass (Herbert *et al.*, 1956). This permits an estimation of the concentration of zinc in the 'zinc-free' medium and of the growth yield on zinc. The contaminating concentration of zinc derived from the analytical grade chemicals, distilled water and corrosion of the metal parts of the fermenter was estimated to be around $0.2 \mu\text{M}$ by the method described by Light (1972) and was found to be $0.15 \mu\text{M}$ by atomic absorption spectrophotometry. The biomass yield on Zn^{2+} was estimated assuming that as long as zinc was the limiting nutrient, the concentration (s) of Zn^{2+} in the culture vessel was small compared to the entering concentration (s_r). This assumption allows approximation of the relationship $Y = x/(s_r - s)$ to $Y = x/s_r$, where x is the steady-state concentration of biomass and Y is the growth yield on zinc. In the first and second growth regime (Fig. 3) a proportional relationship existed between the steady-state biomass and the entering concentration of Zn^{2+} , and the yield coefficient was determined by linear regression. This revealed that $1 \mu\text{mol Zn}^{2+}$ could support a biomass concentration of $7.86 \pm 0.97 \text{ g}$ corresponding to a yield on zinc around $120 \text{ kg dry wt cells (g zinc)}^{-1}$. This yield represents the minimum content of Zn^{2+} necessary for growth of *A. pullulans* under the given conditions. The cellular content of zinc of *Candida utilis* cells growing under zinc-limiting conditions ($0.8 \mu\text{M-Zn}^{2+}$) in continuous culture at a dilution rate of 0.4 h^{-1}

(Lawford *et al.*, 1980) was estimated to be $0.31 \text{ nmol } Zn^{2+} (\text{mg dry wt cells})^{-1}$, corresponding to a yield of $49 \text{ kg dry wt cells (g zinc)}^{-1}$. The discrepancy in the yield value between our value and that derived from Lawford *et al.* (1980) could be related to differences in the physiology of these micro-organisms and/or differences in the growth rates of the cells. Tempest (1970) found a twofold decrease in the biomass yield on magnesium when the dilution rate was increased from 0.1 to 0.4.

In the third growth regime (III) (input concentration of Zn^{2+} above $0.80 \mu\text{M}$) an increase in the input concentration of Zn^{2+} did not lead to a higher level of biomass, which stabilized around 7.3 g l^{-1} due to depletion of the carbon source. Growth in the third growth regime was primarily limited by glucose and production of exopolysaccharide was negligible. The ratio of the two morphological growth forms gradually changed and the biomass became almost 90% mycelial for an input Zn^{2+} concentration of $7.60 \mu\text{M}$. In this growth regime Zn^{2+} was no longer the limiting nutrient and thus Zn^{2+} may accumulate in the cells. It therefore seems probable that the increase in the proportion of the biomass that is in the mycelial growth form is a response to increasing cellular concentrations of zinc. Lawford *et al.* (1980) varied the input concentration of Zn^{2+} in continuous culture with *C. utilis* and found a transition growth regime between zinc limitation and carbon limitation. In this growth regime (input concentrations of Zn^{2+} between $4.5 \mu\text{M}$ and $20 \mu\text{M}$) the culture was carbon-limited and increases in the input concentration of Zn^{2+} led to a linear increase in the cell-associated zinc without any effect on biomass concentration. Similar transition regions between carbon and nitrogen limitations have been recorded (Egli & Quayle, 1986; Duchars & Attwood, 1989), in which a decrease in the C:N ratio of the growth medium led to a significant increase in the cellular content of nitrogen.

The increase in the proportion of biomass in the mycelial growth form could be explained if germ-tube formation is stimulated by Zn^{2+} . Gadd *et al.* (1987) found that the rate of Zn^{2+} uptake was similar for yeast and mycelium of *A. pullulans*, while Sabie & Gadd (1990) showed that the Zn^{2+} -uptake capability of *C. albicans* changed considerably during germination of yeast cells into hyphae, indicating differences in the kinetic parameters of the Zn^{2+} -uptake mechanism between the two growth forms.

Zinc has been shown to affect morphogenesis in several other dimorphic fungi. The responses have been either stimulation of mycelial growth as with *Coccidioides immitis* (Roessler *et al.*, 1946) or suppression of the mycelial phase, e.g. *Histoplasma capsulatum* (Pine & Peacock, 1958), *Mucor rouxii* (Bartnicki-Garcia & Nickerson, 1962), *Sporothrix schenckii* (Alsina &

Rodriguez-Del Valle, 1984) and *C. albicans* (Widra, 1964; Yamaguchi, 1975; Bedell & Soll, 1979; Soll *et al.*, 1981; Sabie & Gadd, 1990). These data suggest that zinc may have an important role in the yeast-mycelium dimorphism of *A. pullulans* and other dimorphic fungi.

The fact that the morphology of *A. pullulans* can be controlled in continuous culture by varying only the input zinc concentration makes this an attractive system for studying biochemical differences between the growth forms and the mechanism(s) controlling the dimorphism.

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