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Does the agitation rate and/or oxygen saturation influence exopolysaccharide production by *Aureobasidium pullulans* in batch culture?

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Abstract When *Aureobasidium pullulans* was grown at a number of agitation rates under batch conditions, exopolysaccharide yields were dramatically reduced at high rates i.e. at least 750 rpm. Investigations with gas blending, which allowed pO_2 manipulation and control independently of the agitation rate, showed that this yield reduction was due solely to the high pO_2 levels that occurred at these agitation rates. Thus, polysaccharide production at 1000 rpm could be elevated by maintaining the pO_2 at a low level during the initial phase of the fermentation. However, both the timing of the pO_2 decrease and the level at which it was maintained were crucial for obtaining yields at 1000 rpm, similar to those observed at low agitation rates.

Introduction

Aureobasidium pullulans is a polymorphic fungus that is capable of elaborating several exopolysaccharides in submerged culture, including pullulan (Gibbs and Seviour 1996), which is a neutral homopolymer consisting of α-(1→6)-linked maltotriosyl residues with occasional random maltotetriosyl substitution (Catley et al. 1986). The production of this polysaccharide by *A. pullulans* has attracted considerable interest because its physical and chemical properties make it suitable for a number of industrial and medical applications (Yuen 1974; Sugimoto 1979; LeDuy et al. 1988). Thus many factors have been described that influence polysaccharide production by this fungus, and these have recently been reviewed (Seviour et al. 1992; Gibbs and Seviour 1996).

The possible influence of the physical culture environment on polysaccharide production by *A. pullulans* has received far less attention than studies on the role of assorted chemical factors, which have included the type and/or concentration of the carbon or nitrogen source and the influence of various trace elements (e.g. Catley 1971a,b; Auer and Seviour 1990; West and Reed-Hamer 1992; Reeslev and Jensen 1995). For example, the effects of fermentation operating conditions such as the agitation rate are still unresolved. Most laboratory-scale stirred-tank fermentation systems are fitted with Rushton or similar radial-flow turbines, which impart a high shear stress on the medium (Lawford and Rousseau 1991), and the use of these impellers at high speeds is known to reduce metabolite yields in *Penicillium chrysogenum* (Smith et al. 1990; Makagiansar et al. 1993) and a number of *Aspergillus* species (Camposano et al. 1959; Wase et al. 1985; Manolov 1992; Park et al. 1993). The situation with exopolysaccharide, especially pullulan, production by *A. pullulans* is still very confused. For example, Ono et al. (1977) and McNeil and Kristiansen (1987) both reported an increase in polysaccharide yields at high agitation rates, yet a later investigation showed that optimum production occurred under the combined conditions of low shear stress and dissolved oxygen concentration (Wecker and Onken 1991).

Changes in the agitation or shear rate are inevitably associated with variations in both pO_2 and O_2 mass transfer rates. It is often difficult to discriminate between the influence of shear and O_2 mass transfer, as pO_2 changes alone are known to affect metabolite production in filamentous fungi such as *P. chrysogenum* (Vardar and Lilly 1982), *Aspergillus niger* (Gómez et al. 1988) and *Cephalosporium acremonium* (Zhou et al. 1992). Unfortunately this fact has not always been allowed for by the experimental designs used in most investigations into the sensitivity of metabolite production by filamentous fungi, including exopolysaccharide production by *A. pullulans*, to variations in agitation rate. Consequently the possible influence of pO_2 levels on these organisms has not often been considered. This is particularly relevant here since the effect of pO_2 alone on polysaccharide production by *A. pullulans* is disputed,

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and there have been claims of yield enhancement under conditions of both high and low oxygen transfer (Imshentskii et al. 1981; Rho et al. 1988; Wecker and Onken 1991). Therefore this study set out to attempt to resolve these conflicts by determining the sensitivity of exopolysaccharide production in *A. pullulans* to changes in agitation rate under batch conditions, and then, through the use of gas blending, assessing the effects of variations in pO_2 levels independently of the agitation rate on polysaccharide yields in this fungus.

Materials and methods

Organism and media

Aureobasidium pullulans (ATTC 9348) was maintained in brain heart infusion broth (Oxoid) supplemented with 20% glycerol, stored at –80 °C. Cells were revived by transfer to slopes of malt extract agar (Oxoid), which were incubated at 25 °C for 5 days before use or storage at 4 °C. The chemically defined mineral salts medium of Pitt and Bull (1982), suplemented with glucose and $(NH_4)_2SO_4$ at a concentration of 30 g l^{-1} and 0.6 g l^{-1} respectively, was used throughout this study.

Inoculum preparation

This was strictly standardised. Cell suspensions were prepared by flooding slopes of *A. pullulans* with 5 ml sterile distilled water followed by gentle agitation. These suspensions were used to inoculate by 300 ml conical flasks containing 50 ml experimental medium, which were subsequently incubated at 25 °C and 180 rpm for 30 h. Biomass was harvested by centrifugation, washed twice and finally resuspended in sterile quarter-strength Ringer solution. The biomass concentration of the inoculum was determined and adjusted, if necessary, to provide an initial biomass concentration in the fermentation vessel in all these experiments of approximately 0.1 g l^{-1}.

Fermentation studies

All fermentations were carried out using a B.Braun Biostat ED (Crown Scientific, Melbourne, Australia) stirred-tank fermenter with an operational volume of 10 l. The system was fitted with three 7.5-cm six-bladed Rushton turbines, which were evenly spaced on the drive shaft. The aeration rate was 0.4 l min^{-1} and a wide range of agitation speeds were used as detailed in Results. For each agitation rate, static gassing-out was performed prior to inoculation to estimate the initial oxygen transfer ability (K_La) of the system. Briefly, the procedure involved reducing the pO_2 in the bulk liquid to zero by sparging with nitrogen and then following the increase in pO_2 over time after the resumption of aeration until maximum O_2 saturation was reached [see Hesselink (1992) for full details].

Fermentations were run at a constant pH of 4.5 and a temperature of 28 °C, as these have been reported to be close to the optimal conditions for exopolysaccharide production by *A. pullulans* (McNeil et al. 1989; McNeil and Kristiansen 1990). Culture pH was measured with an Ingold pH probe and controlled by automatic addition of either 2 M NaOH or HCl as needed. The pO_2 was measured with an Ingold polarographic dissolved oxygen probe and controlled in some experiments through the use of a gas mixing station (B. Braun, Crown Scientific, Melbourne, Australia), which allowed precise pO_2 control (± 1% saturation) independently of the agitation rate, through the addition of pulses of nitrogen or oxygen as required. The redox potential was measured with an Ingold redox probe, and foaming, which occurred sporadically, was controlled by manual addition of polypropylene glycol 2025 on demand.

Analysis of samples

Measurement of the mycelial component of the biomass was achieved by passing the sample drawn from the fermenter through a nylon-mesh filter (45 μm porosity) (Heald and Kristiansen 1985). The filtrate was then passed through a 1μm Whatman GMF 150 filter to collect the unicellular forms; thus, after drying and reweighing of both filters, the mycelial percentage and total biomass concentration were determined. Quantification of the exopolysaccharide concentration and the measurement of residual glucose and ammonia levels were performed as previously described (Stasinopoulos and Seviour 1989; Auer and Seviour 1990).

Reproducibility of results

All fermentation experiments were performed at least in duplicate, to ensure the trends observed were real and reproducible. Typical data are presented in the figures while averaged data are presented in the tables.

Results

Influence of agitation rate on exopolysaccharide production by *A. pullulans*

A total of six different agitation rates were used, ranging from 125 rpm to 1250 rpm. High agitation rates (750 rpm and above) clearly resulted in substantially diminished exopolysaccharide yields compared to those seen at low speeds (Table 1), while biomass yields increased, if only

Table 1 Effect of increasing agitation rate on exopolysaccharide and biomass production by *Aureobasidium pullulans* in batch culture. Values are expressed as mean ± standard deviation. The initial growth rate was calculated for the first 24 h of the fermentation to eliminate the possible influence of NH_4^+ exhaustion. *EPS* exopolysaccharide. $Y_{p/x}$ The ratio of exopolysaccharide to biomass yields, μ growth rate

Agitation rate (rpm)	Initial K_La (h^{-1})	Final [EPS] (g l^{-1})	Final [biomass] (g l^{-1})	Final $Y_{p/x}$	Initial μ (h^{-1})	Final yield of mycelia (%)	Conversion of glucose to EPS (%)	pO_2 at onset of EPS production (% saturation)
125	10.1 ± 0.2	11.11 ± 0.43	7.21 ± 0.92	1.57 ± 0.21	0.11 ± 0.01	66.3 ± 3.6	37.6 ± 2.6	0.0 ± 0.0
250	41.4 ± 0.8	11.27 ± 0.79	9.24 ± 1.06	1.24 ± 0.17	0.14 ± 0.00	78.3 ± 7.7	37.0 ± 1.4	12.8 ± 9.1
500	133.5 ± 9.8	7.43 ± 2.00	11.64 ± 0.44	0.64 ± 0.20	0.14 ± 0.01	77.5 ± 4.4	26.1 ± 7.2	79.9 ± 13.5
750	171.0 ± 2.5	4.57 ± 0.53	12.75 ± 1.31	0.37 ± 0.08	0.15 ± 0.01	80.9 ± 6.4	15.2 ± 1.8	87.7 ± 3.0
1000	177.3 ± 1.3	5.64 ± 1.07	9.69 ± 0.92	0.60 ± 0.16	0.14 ± 0.00	87.8 ± 6.4	18.8 ± 3.6	82.3 ± 8.7
1250	174.3 ± 0.7	5.61 ± 0.56	11.30 ± 0.60	0.50 ± 0.06	0.13 ± 0.01	77.6 ± 5.5	18.7 ± 1.9	95.5 ± 1.9

slightly. In all these experiments, detectable polysaccharide formation did not commence until the NH_4^+ was exhausted from the medium, which is consistent with previous observations (Seviour and Kristiansen 1983; Auer and Seviour 1990) and all cultures, at all agitation rates examined, became predominantly mycelial on prolonged incubation.

Cultures grown at agitation rates of 125 rpm (Table 1) and 250 rpm (Fig. 1) both returned similar high final exopolysaccharide yields of approximately 11 g l^{-1}, with almost 40% of the glucose initially provided converted into exopolysaccharide (Table 1). However, the organism's growth rate (μ) was slower at 125 rpm, which may be a reflection of a reduced initial K_La at that agitation rate. The pO_2 profiles were also similar at these two agitation rates since, in both, pO_2 decreased sharply and O_2 remained undetectable for the majority of the fermentation. At 125 rpm, the initial redox potential of the broth fell sharply, closely paralleling the pO_2 profile, but then began to rise after 24 h, corresponding to NH^+_4 exhaustion and therefore the onset of polysaccharide and melanin production. A similar trend was observed in cultures grown at 250 rpm, but the initial decrease in redox was much smaller. The reasons for these changes are not known.

Increasing the agitation rate to 500 rpm (Table 1) led to an increase in biomass yield and a clear reduction in exopolysaccharide production to about 7.5 g l^{-1} and therefore a substantially lower exopolysaccharide/biomass ratio ($Y_{p/x}$; i.e. 0.64 compared with more than 1.2 at 125 rpm and 250 rpm). The proportion of glucose converted into polysaccharide was also reduced. The redox potential rose throughout the fermentation, despite the pO_2 decreasing for the first 24–32 h to a minimum of around 70% saturation. Additional increases in the agitation rate to 750, 1000 and 1250 rpm led to further marked and reproducible reductions in polysaccharide yields, to levels approximately half those achieved at 125 rpm and 250 rpm (Table 1). The fermentation profiles at these three higher speeds were all very similar, possibly because the initial K_La was around the same value of 170 h^{-1}, so only the results with 1000 rpm (Fig. 2) will be discussed in detail here.

Biomass yields were similar to those at 500 rpm, despite a further marked reduction in polysaccharide production. Hence the proportion of glucose converted to polysaccharide now fell below 20% of the initial amount provided. The pO_2 did not drop below 80% saturation at 750 rpm or 90% at 1000 rpm and 1250 rpm, and the redox profiles were similar to those seen at 500

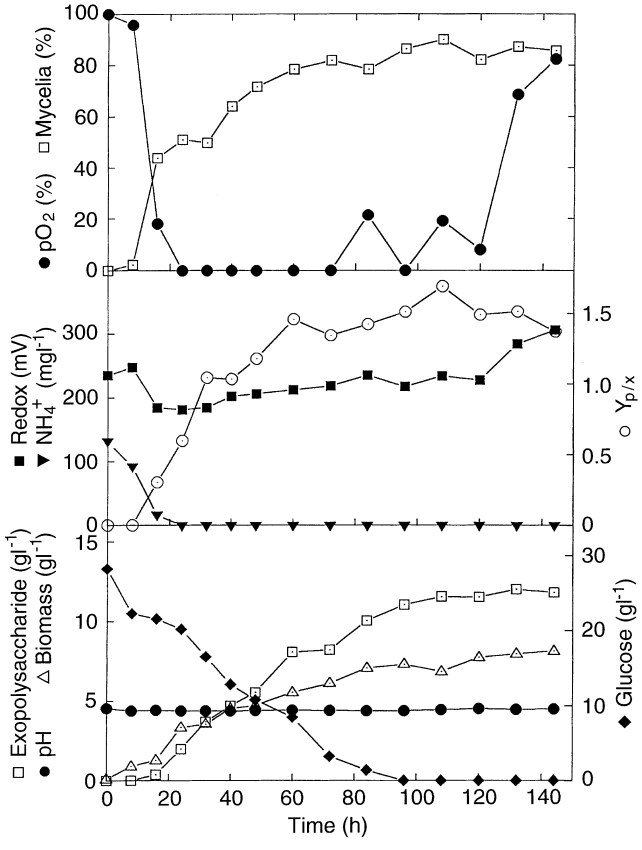

Fig. 1 Exopolysaccharide production by *Aureobasidium pullulans* growth in a stirred-tank fermenter at 250 rpm and with no pO_2 control. $Y_{p/x}$ ratio of exopolysaccharide to biomass yields

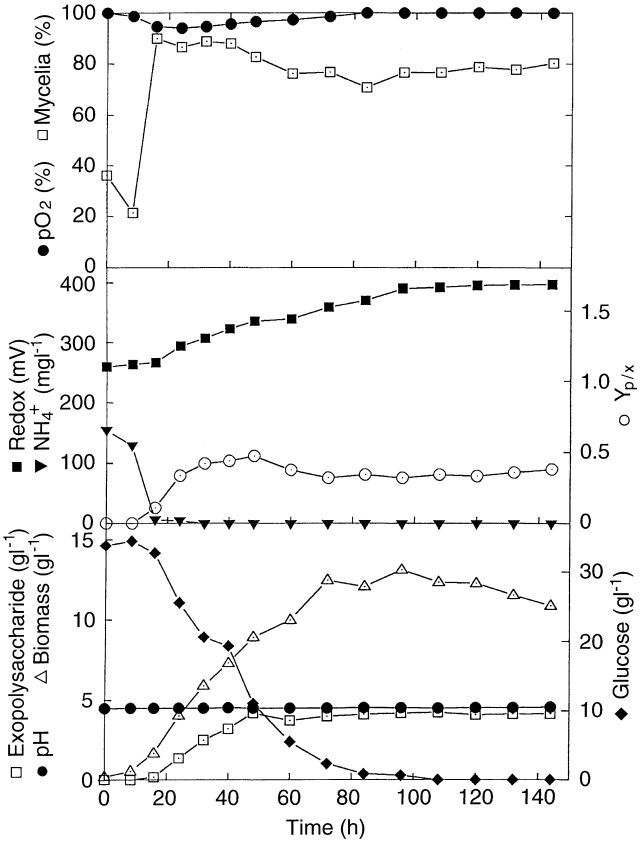

Fig. 2 Exopolysaccharide production by *A. pullulans* grown in a stirred-tank fermenter at 1000 rpm and with no pO_2 control.

rpm. To verify that the reduced exopolysaccharide yields returned at these high agitation rates were not due to some negative effect of high shear on the structure of existing polysaccharide, which might influence its subsequent recovery by ethanol precipitation, the fungus was grown at 250 rpm for 144 h and then at 1250 rpm for a further 48 h (data not shown). No reduction in polysaccharide recovery and values obtained after ethanol precipitation was observed when the agitation rate was increased in this way.

The role of pO_2 in exopolysaccharide production by *A. pullulans* under conditions of high shear

The experiments described above demonstrate that, at high agitation rates, the pO_2 always remained above 80% saturation during the course of the fermentation, and polysaccharide yields fell. To investigate whether a possible relationship might exist between exopolysaccharide yields and pO_2 levels, further batch cultures, where the pO_2 was varied and controlled independently of the agitation rate, were carried out. The agitation rate used in all these experiments was 1000 rpm.

Early experiments attempted to mimic at 1000 rpm the pO_2 profile seen at 125 rpm and 250 rpm, i.e. where the highest polysaccharide yields were returned. Thus pO_2 was left uncontrolled for the first 16 h of the fermentation and then set to either 5% or 10% saturation for the rest of the run (data not shown). However, the results obtained were inconsistent, as some runs returned increased polysaccharide yields (approx. 8 g l^{-1} compared with approx. 5.5 g l^{-1} when no pO_2 control was exercised), while others showed no such stimulation. Inspection of the data suggested that polysaccharide production was only stimulated at the low pO_2 levels if NH_4^+ was still present in the medium when the pO_2 control was initiated. Thus the timing of the decrease in pO_2 levels appeared to be crucial in batch culture. Since there was little or no difference in terms of polysaccharide yields between the two pO_2 levels used in the initial experiments, an intermediate value of 7% was selected for further investigations into the importance of the timing of pO_2 control in exopolysaccharide production by *A. pullulans*.

Controlling the pO_2 at 7% saturation after 32 h incubation (Fig. 3), i.e. when NH_4^+ was clearly exhausted from the medium, resulted in no stimulation of exopolysaccharide production compared to when no pO_2 control was exercised. However, when the pO_2 was maintained at 7% for the entire fermentation (Fig. 4) or pO_2 control was initiated and maintained 8 h into the

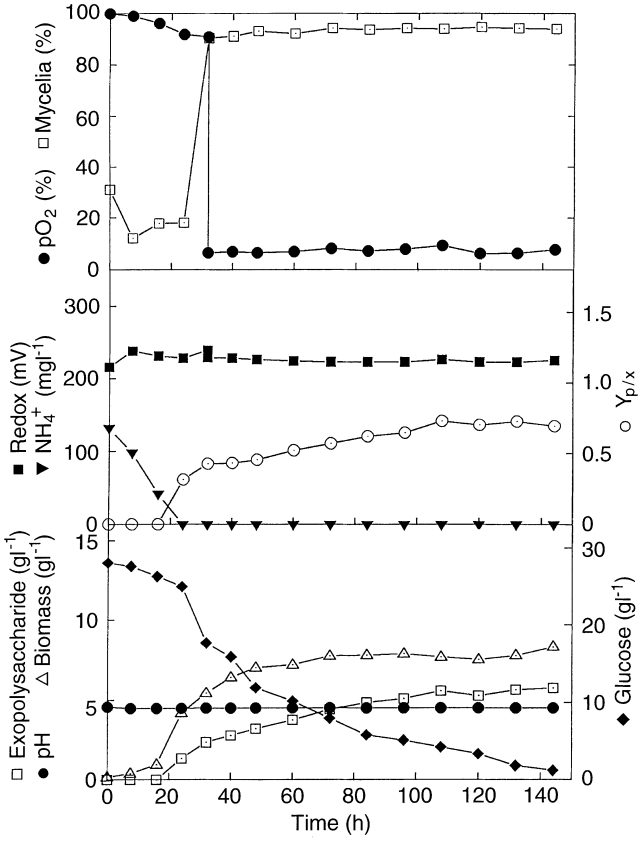

Fig. 3 Exopolysaccharide production by *A. pullulans* grown in a stirred-tank fermenter at 1000 rpm and with the pO_2 maintained at 7% saturation after 32 h incubation

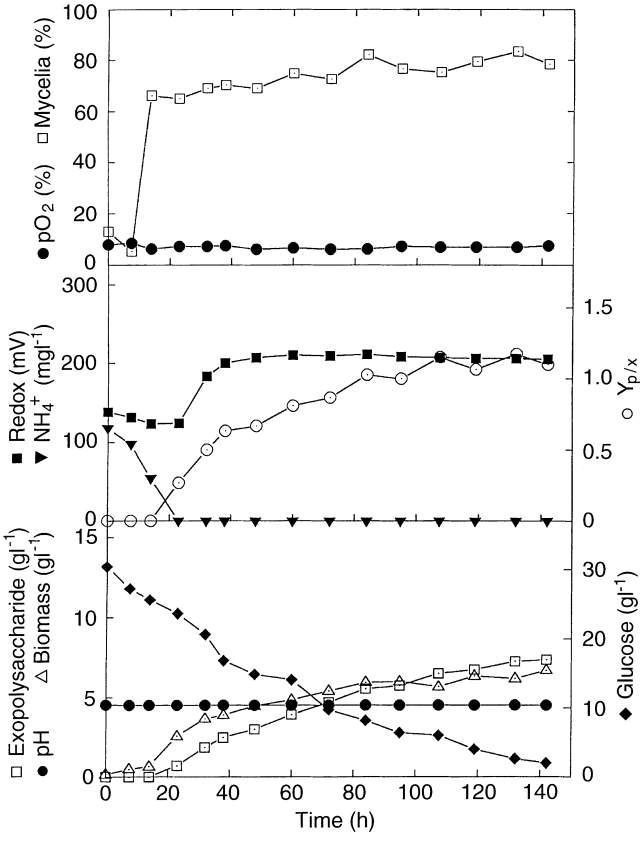

Fig. 4 Exopolysaccharide production by *A. pullulans* grown in a stirred-tank fermenter at 1000 rpm and with the pO_2 maintained at 7% saturation throughout the entire fermentation.

Table 2 Effect of different pO_2 control strategies on exopolysaccharide and biomass production by *A. pullulans* in batch culture. Values are expressed as mean ± standard deviation. The initial growth rate was calculated for the first 24 h of the fermentation to eliminate the possible influence of NH_4^+ exhaustion. *EPS* exopolysaccharide, $Y_{p/x}$ the ratio of exopolysaccharide to biomass yields

Fermentation conditions	Final [EPS] (g l^{-1})	Final [biomass] (g l^{-1})	Final $Y_{p/x}$	Initial μ (h^{-1})	Final yield of mycelia (%)	Conversion of glucose to EPS
1000 rpm						
No pO_2 control	5.64 ± 1.07	9.69 ± 0.92	0.60 ± 0.16	0.14 ± 0.00	87.8 ± 6.4	18.8 ± 3.6
pO_2 7% 0 h→	7.77 ± 0.42	6.48 ± 0.22	1.23 ± 0.13	0.11 ± 0.00	62.9 ± 15.6	27.2 ± 1.0
pO_2 7% 8 h→	8.17 ± 1.01	7.46 ± 0.33	1.10 ± 0.17	0.10 ± 0.07	77.1 ± 10.6	27.2 ± 3.5
pO_2 7% 32 h →	5.53 ± 0.23	8.37 ± 0.04	0.66 ± 0.03	0.14 ± 0.00	92.8 ± 1.1	19.4 ± 0.5
pO_2 7% 0 →32 h	7.30 ± 0.49	9.47 ± 0.24	0.75 ± 0.05	0.12 ± 0.01	67.3 ± 6.3	24.3 ± 1.6
pO_2 7% 0→16 h	9.09 ± 1.20	9.24 ± 0.85	1.01 ± 0.23	0.13 ± 0.00	69.5 ± 9.5	30.3 ± 4.0
pO_2 15% 0→16 h	10.25 ± 1.07	9.04 ± 0.62	1.15 ± 0.19	0.13 ± 0.01	64.9 ± 7.1	34.2 ± 3.6
pO_2 30% 0→16 h	6.67 ± 1.07	9.37 ± 0.66	0.72 ± 0.16	0.13 ± 0.01	77.0 ± 4.1	22.3 ± 3.6
pO_2 50% 0→16 h	5.98 ± 0.16	8.04 ± 0.52	0.75 ± 0.07	0.14 ± 0.01	95.2 ± 1.7	20.0 ± 0.6
250 rpm						
No pO_2 control	11.27 ± 0.79	9.24 ± 1.06	1.24 ± 0.17	0.14 ± 0.00	78.3 ± 7.7	37.0 ± 1.4
pO_2 ≥90% 0 h→	6.13 ± 0.13	11.25 ± 0.01	0.54 ± 0.01	0.13 ± 0.01	80.0 ± 0.7	20.4 ± 0.4

incubation period (Table 2) a reproducible and marked increase in polysaccharide yield was then observed at 1000 rpm, even though the final yields (approx. 8 g l^{-1}) were less than those that were obtained at 125 rpm and 250 rpm.

The initial μ values and biomass yields for the cultures when pO_2 control was initiated very early in the fermentation were lower than when no pO_2 control was imposed at 1000 rpm (Table 2). These observations, together with the relatively high $Y_{p/x}$ values obtained, suggested that exopolysaccharide yields might be increased if biomass production could be raised. In an effort to increase the biomass yield and, hopefully, polysaccharide production it was decided to control the pO_2 for the first 32 h of incubation only, having already shown that pO_2 control after this time had no effect on exopolysaccharide yields. As shown in Table 2, this strategy succeeded in raising the biomass concentration slightly but had no additional stimulatory effect on exopolysaccharide yields. A further restriction in the pO_2 control period to just the first 16 h of the fermentation (Fig. 5), however, led to a small increase in polysaccharide production to a final level of approximately 9 g l^{-1}.

Having established that the first 16 h was the critical period of the incubation for pO_2 control, the pO_2 was then increased to 15% saturation for that period (Fig. 6), a strategy that resulted in a subsequent increase in exopolysaccharide yields almost to levels achieved at 125 rpm and 250 rpm with no pO_2 control, but where dissolved oxygen was undetectable during the polysaccharide production phase. However a further increase in the pO_2 to 30% and 50% saturation (Table 2) for the first 16 h of incubation gave no stimulation in polysaccharide production compared to when no pO_2 control was exercised at 1000 rpm.

The role of pO_2 in exopolysaccharide production by *A. pullulans* under conditions of low shear

The data given above convincingly show that it is possible to increase polysaccharide yields at high agitation rates by decreasing the pO_2 during the initial phase of

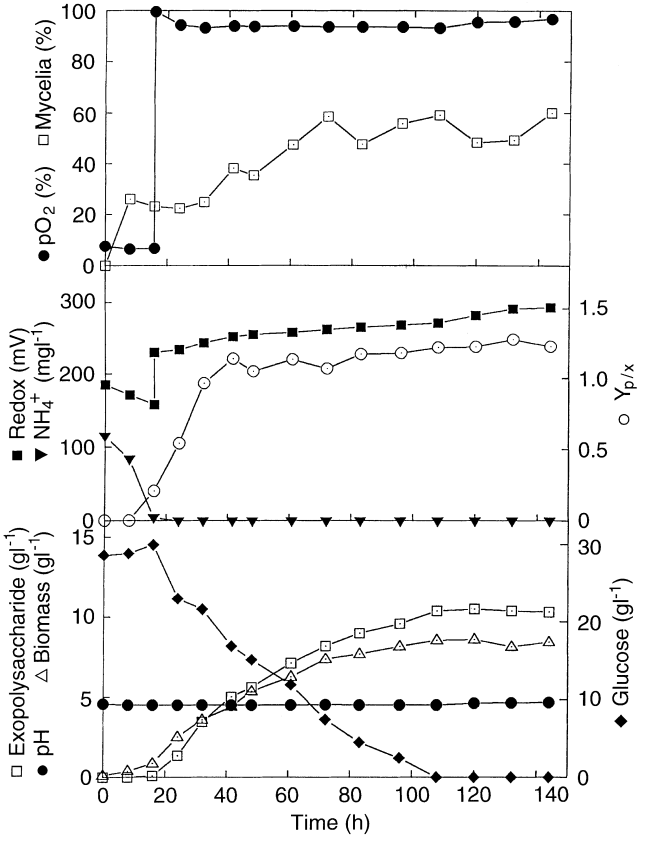

Fig. 5 Expolysaccharide production by *A. pullulans* grown in a stirred-tank fermenter at 1000 rpm and with the pO_2 maintained at 7% saturation for the first 16 h of incubation

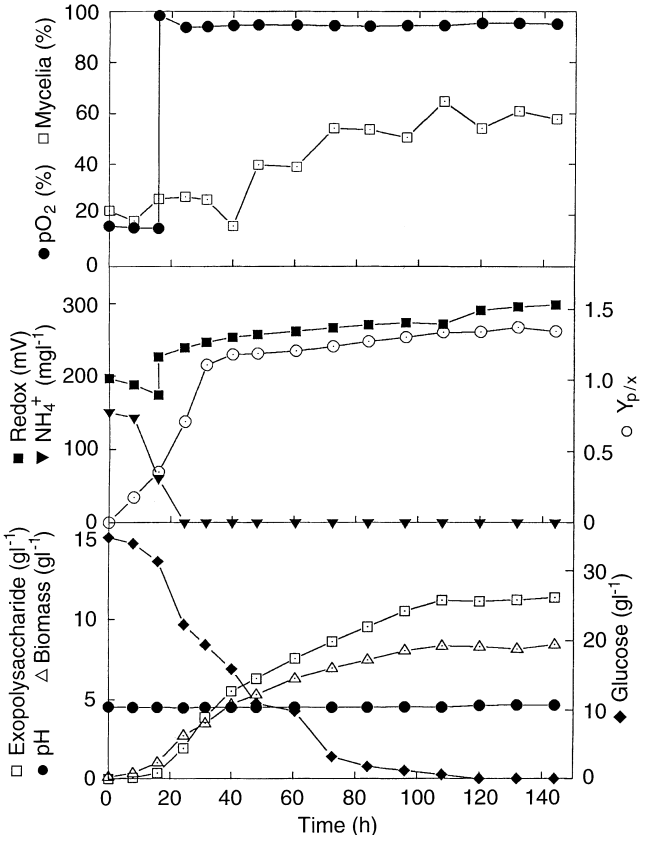

Fig. 6 Exopolysaccharide production by *A. pullulans* grown in a stirred-tank fermenter at 1000 rpm and with the pO_2 maintained at 15% saturation for the first 16 h of incubation

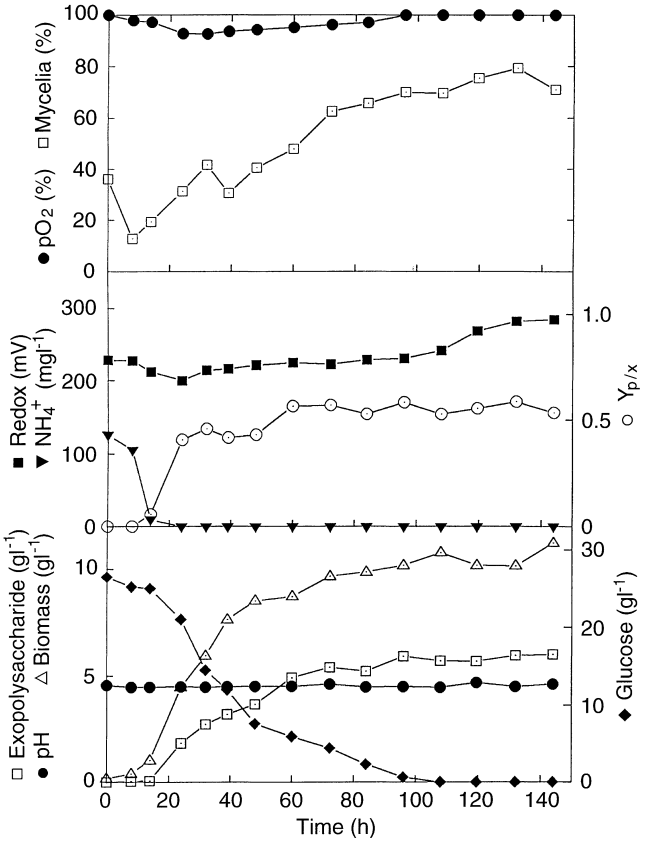

Fig. 7 Exopolysaccharide production by *A. pullulans* grown in a stirred-tank fermenter at 250 rpm and with the pO_2 maintained at greater than 90% saturation for the entire fermentation.

the fermentation with *A. pullulans*. Therefore it was decided to see whether the high polysaccharide yields observed at the low agitation rates with no pO_2 control would be affected by maintaining the pO_2 at a constant high level throughout the fermentation. Thus *A. pullulans* was grown at 250 rpm where the pO_2 was held above 90% for the entire fermentation (Fig. 7) and, under these conditions, a polysaccharide yield approximately half that achieved at 250 rpm without pO_2 control was obtained. This result again confirms the negative influence of high pO_2 levels on polysaccharide production by *A. pullulans*.

Influence of fermentation conditions on the morphology of *A. pullulans*

Agitation rate and pO_2 appeared to have little or no obvious effect on the morphology of *A. pullulans*. Although the inoculum in all these experiments contained mainly blastospores, these quickly differentiated into the diverse morphological forms exhibited by this fungus, resulting in all cases in heterogeneous cultures, which were ultimately predominantly mycelial. The proportions of mycelia varied between experiments (Tables 1,2) but there was no clear relationship between the percentage of mycelia present and the level of polysaccharide production. The individual hyphae of *A. pullulans* also seem to be insensitive to the different agitation rates investigated, as little alteration in hyphal length, hyphal diameter or branching frequency was observed at the different agitation rates examined. Some hyphal fragmentation was apparent but this occurred at all agitation rates and only towards the end of the fermentations, probably as a consequence of autolysis.

Discussion

The data presented here unequivocally show that a reduction in exopolysaccharide production by *A. pullulans* occurs with increasing agitation rates, and also demonstrate that increased pO_2 levels in the medium, and not increased shear forces, are responsible for this effect. This assertion is justified because the gas mixing system used in these experiments allowed pO_2 variation while the physical environment (i.e. mixing etc.) within the fermentation vessel was maintained essentially unchanged, therefore allowing a distinction between the influences of pO_2 and shear on this organism to be made. It should be mentioned, however, that the cells

were not exposed to identical physical environments in experiments using such pO_2 control, since the addition of pulses of nitrogen or oxygen would slightly alter the gas velocity and hence the mixing characteristics. When Wecker and Onken (1991) used gas blending in their study with *A. pullulans*, they concluded that polysaccharide production was favoured under combined conditions of low pO_2 and shear rate. However the present data suggest that the pO_2 may be the major controlling factor, because the high yields achieved at low agitation rates could be dramatically reduced by raising the pO_2 and vice versa. The reports of polysaccharide production by *A. pullulans* being enhanced under high oxygen-transfer conditions (Imshenetskii et al. 1981; Rho et al. 1988) are not convincing since the data were generated using very crude methods of pO_2 control in shake flasks. When pO_2 control was imposed in a fermenter, results similar to those presented here were achieved, although unfortunately the control methods used were not detailed (Rho et al. 1988). Significantly, the yields of β-glucans produced by some filamentous fungi also increase under oxygen-limiting conditions (Dosoretz et al. 1990; Rau et al. 1992; Stasinopoulos and Seviour 1992).

An increase in the proportion of blastospores has been but forward as an explanation of increased exopolysaccharide yields by both McNeil and Kristiansen (1987) and Wecker and Onken (1991) at high and low agitation rates respectively. This suggested relationship is relevant because it has been proposed that blastopores are the main polysaccharide producers (Cately 1980; Heald and Kristiansen 1985). However, this idea does not have universal support (Seviour et al. 1984; McNeil et al. 1989; Simon et al. 1993), and it is certainly not supported by observations here, because predominantly mycelial cultures were obtained under all experimental conditions examined. A reduction in metabolite production by other filamentous fungi at increased agitation rates has also been blamed on the mycelial deformation and damage suffered (Camposano et al. 1959; Wase et al. 1985; Smith et al. 1990; Manolov 1992; Park et al. 1993; Makagiansar et al. 1993), but again this suggestion can not be used to explain these results, because the hyphae of *A. pullulans* showed no evidence here to suggest they suffered any ill effects on exposure to high shear rates.

The present data also suggest that the period of time in the batch culture during which the cells are exposed to low pO_2 conditions is critical, although the crucial period for pO_2 control at high agitation rates identified by this work seems to contradict the pO_2 profiles seen in cultures grown at 125 rpm and 250 rpm. Here pO_2 remains high for the first 16 h and then rapidly falls to zero. Presumably, however, the actual oxygen transfer to the cells would be reduced at these low agitation rates because of the large air-bubble size and poor bubble break-up, leading to slug formation. In addition, an apparent requirement for the presence of NH_4^+ at the onset of pO_2 control is interesting because it is generally accepted that NH_4^+ represses polysaccharide production, which does not occur until NH_4^+ is exhausted from the medium (Seviour and Kristiansen 1983; Bulmer et al. 1987; Auer and Seviour 1990). This might suggest that, once the NH_4^+ is exhausted, the fungus is committed to a set level of exopolysaccharide production.

How O_2 exerts its effect on metabolite, including exopolysaccharide, production by strictly aerobic filamentous fungi has received suprisingly little attention, but it appears unlikely that there is a single regulatory mechanism. For example, Kubicek et al. (1980) suggested that pO_2 influenced citric acid production by affecting the activity of enzymes involved in the respiratory chain of *A. niger,* while the reduction is cephalosporin C production by *C. acremonium* at low pO_2 levels was reportedly due to the repression of an enzyme responsible for the conversion of penicillin N to a precursor of cephalosporin C (Zhou et al. 1992). An O_2-induced modulation of biosynthetic enzyme activity has also been proposed to explain the stimulation of β-glucan formation by *Schizophyllum commune* which occurs under oxygen limitation (Rau et al. 1992), but with no supporting experimental evidence. Clearly work is urgently required to resolve the underlying physiological and biochemical mechanisms responsible for these striking effects on polysaccharide yields in *A. pullulans*.

Finally, the results presented here suggest an alternative, and possibly complementary, approach to increasing exopolysaccharide yields to those based on manipulation of medium composition. They also illustrate the often neglected influence of the physical environment on the growth and behaviour of filamentous fungi in fermenters.

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