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The production of exopolysaccharides by *Aureobasidium pullulans* in fermenters with low-shear configurations

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Abstract No increases in exopolysaccharide (EPS) yields in *Aureobasidium pullulans* were observed when grown with reduced-shear impellers instead of standard Rushton turbines in the same vessel. However, yields were dramatically reduced when the organism was grown in an airlift reactor. This fall in production could be counteracted by improving fluid circulation through the placement of impellers within the draught tube, a strategy that resulted in the highest EPS concentration (approx. 13 g l^{-1}) of all the fermenter configurations tested.

Introduction

Aureobasidium pullulans elaborates pullulan, a neutral homopolymer consisting essentially of (1→6)-α-linked maltotriosyl residues with random maltotetraosyl substitution occurring throughout the molecule (Catley et al. 1986). Its physical and chemical properties make it potentially attractive for several industrial and medical applications (Yuen 1974; Sugimoto 1979; LeDuy et al. 1988), hence considerable attention has been focused on investigating factors that influence its production (Gibbs and Seviour 1996a), especially the role of medium composition (e.g. Catley 1971; Auer and Seviour 1990; Reeslev and Jensen 1995). Any influence of the physical culture environment on production has received comparatively little attention.

Most studies with *A. pullulans* have used stirred-tank fermenters fitted with Rushton impellers. Whether alternative impeller designs or fermenter configurations offer any advantages for pullulan production is not known. Impeller designs that achieve better bulk mixing and mass transfer in viscous filamentous fermentation fluids and also impart less shear stress have been identified (Buckland et al. 1988; Nienow 1990). However, few reports exist of their use for fungal exopolysaccharide (EPS) production, although greater β-glucan productivity was achieved from *Schizophyllum commune* with axial-flow impellers than with Rushton turbines (Rau et al. 1992; Gura and Rau 1993).

More information is available on fungal behaviour in bioreactors other than stirred tanks, especially airlift fermenters. These rely on differences in fluid density and hydrostatic pressure between the sparged and unsparged regions of the vessels to provide mixing (Merchuk 1990). High β-glucan yields, which were comparable or better than those returned in stirred tanks, were achieved in such systems from *Acremonium persicinum* (Stasinopoulos and Seviour 1992) and *Sclerotium glucanicum* (Wang and McNeil 1995). Similar results have been reported for *A. pullulans* (Wecker and Onken 1991), although Gibbs and Seviour (1992) showed that the EPS yields depended on the nitrogen source used.

The above studies, comparing stirred-tank and airlift reactors, have all used two or more different fermenters, where variations in vessel geometry complicate the interpretation of the data. To address this and investigate the suggestion that changing shear stress has a negligible effect on EPS production by *A. pullulans* (Gibbs and Seviour 1996b), a range of configurations using a single fermentation vessel were examined here.

Materials and methods

Organism and media

Aureobasidium pullulans (ATCC 9348) was maintained, prepared and cultured as previously described (Gibbs and Seviour 1996b). The chemically defined mineral salts medium of Pitt and Bull (1982), supplemented with glucose and $(NH_4)_2SO_4$ at a

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concentration of 30 g l^{-1} and 0.6 g l^{-1} respectively, was used throughout the study.

Fermentation studies

All fermentations were carried out in a B. Braun Biostat ED (Crown Scientific, Melbourne, Australia), thus allowing strict comparisons to be drawn between the various mixing systems. The vessel was fitted with several impeller systems as detailed in Results, and yields were compared to those achieved with Rushton turbines (Gibbs and Seviour 1996b). It could also be converted into an airlift reactor by removing the baffles and impellers and inserting the draught tube supplied by B. Braun. An operational volume of 10 l was used throughout, except for the airlift configurations where it was increased to 12 l to allow sufficient draught tube clearance. Air was supplied at 0.4 l l^{-1} min^{-1} in all cases and, where possible (see Results), static gassing-out was performed prior to inoculation to estimate the initial O_2 transfer ability (k_La) of the system (Gibbs and Seviour 1996b). For all other fermentation conditions refer to Gibbs and Seviour (1996b).

Analysis of samples

Quantification of the biomass and total EPS concentrations, and the measurement of residual glucose and ammonia levels were performed as previously described (Gibbs and Seviour 1996b).

The percentage of pullulan in the total EPS produced was estimated according to Leathers et al. (1988). Analyses were performed on the EPS recovered at the end of the fermentation and from only one of the replicate runs under each set of operating conditions. The nature of the contaminating polysaccharide was investigated by fractionating the crude EPS with cetyltrimethylammonium bromide (Scott 1965). Two fractions, neutral (pullulan) and acidic, were isolated. The acidic fraction was subjected to complete acid hydrolysis (Adams 1965) and digestion with an exo-(1→3)-β-glucanase isolated from *A. persicinum* (Pitson et al. 1995). As a result the contaminating polysaccharide was determined to be a (1→3)-β-glucan with single glucose units attached to the main chain via (1→6)-β linkages. The viscosities of 0.5% pullulan solutions in deionised water were also measured with a Brookfield viscometer (Model RVT, Selby Scientific, Melbourne, Australia), using a number 3 spindle rotating at a shear rate of 85 s^{-1}.

Reproducibility of results

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

Results

Influence of impeller design on EPS production by *A. pullulans*

The fermenter was fitted with either two 70-mm-diameter variable-pitch axial-flow impellers (Fig. 1) or a

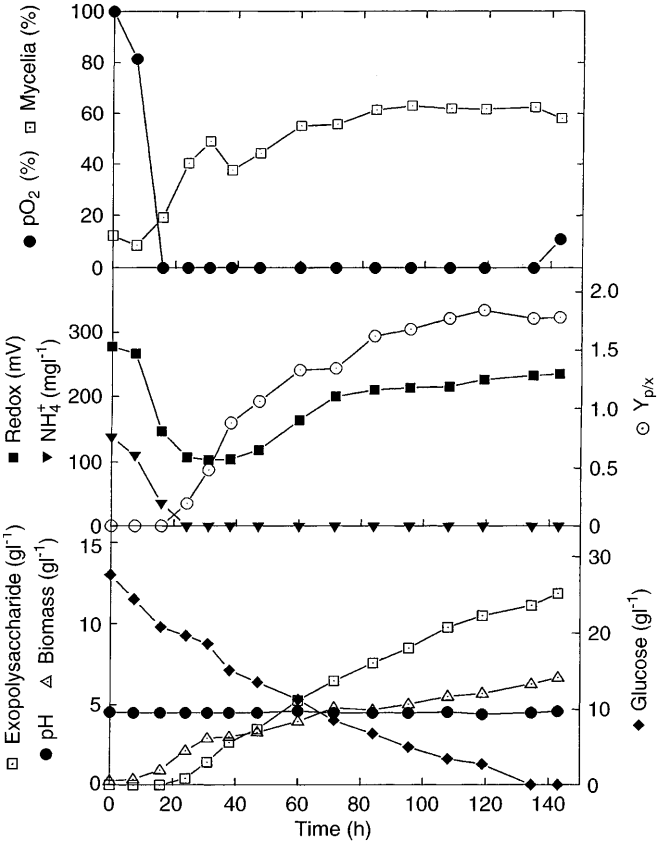

Fig. 1 Exopolysaccharide production by *Aureobasidium pullulans* grown in a stirred-tank fermenter fitted with axial-flow impellers at an agitation rate of 250 rpm. $Y_{p/x}$ the ratio of exopolysaccharide to biomass yield

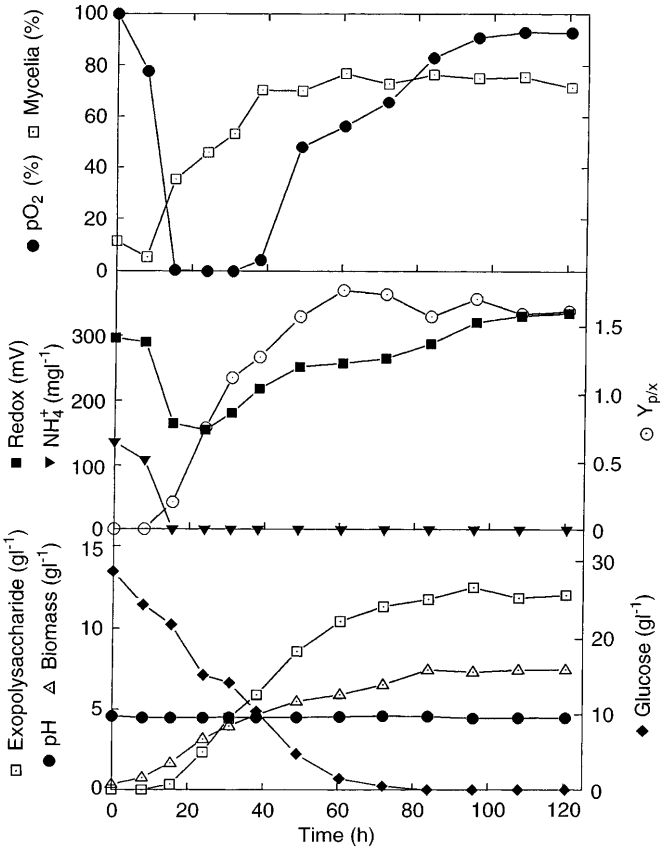

Fig. 2 Exopolysaccharide production by *A. pullulans* grown in a stirred-tank fermenter fitted with a helical-ribbon impeller at an agitation rate of 250 rpm

single 140-mm-diameter helical ribbon (Fig. 2). An agitation rate of 250 rpm was selected for both, since high EPS yields were achieved at this rate with Rushton turbines (Gibbs and Seviour 1996b). Final EPS yields varied little between the three impeller systems. Pullulanase hydrolysis revealed that at least 75% of the total EPS in all cases was pullulan, with the helical-ribbon impeller returning the highest value of 95%. With all three systems, EPS was never detected until NH_4^+ was exhausted from the medium.

The variation in pO_2 profiles between the axial-flow and helical-ribbon impellers (Figs. 1, 2) may reflect differences in the k_La of the two mixing systems (Table 1) where, at 250 rpm, the value for the axial-flow impellers was more than half that for the helical ribbon. Rushton turbines gave an initial k_La value approximately double that with the helical ribbon. Therefore to compare these alternative impellers to the Rushton turbines it was necessary to increase the agitation rate to achieve similar k_La values for each.

To reach an initial k_La of about 40 h^{-1} (equivalent to the Rushton turbines at 250 rpm), the agitation rate used with the axial-flow impellers had to be increased to 700 rpm, which led to a decrease in both total EPS (7.7 g l^{-1} cf. 11.6 g l^{-1}) and pullulan yield (53% of the total cf. 74.5%). An increase of about 2.5 g l^{-1} in the final biomass concentration at the higher rate also contributed to the reduction in $Y_{p/x}$. Interestingly, the fermentation finished more quickly at 700 rpm (measured by there being no further increase in biomass production), and was terminated after 120 h. However, no increase in the initial specific growth rate under these conditions, compared with that obtained at 250 rpm, was found.

A similar increase in agitation rate (250 rpm to 750 rpm) with the Rushton turbines in an earlier study (Gibbs and Seviour 1996b) saw reductions in both the pullulan content and total concentration of EPS of around twice that recorded here. Since increased O_2 saturation levels were considered responsible for lowered yields from *A. pullulans* at such high agitation rates (Gibbs and Seviour 1996b), the smaller decrease is possibly a reflection of a reduced O_2 mass-transfer rate of the axial-flow impellers compared to the Rushton turbines, a view supported by the pO_2 profile. Here pO_2 levels decreased rapidly after inoculation, and O_2 became briefly undetectable after approximately 24 h. Thus the pO_2 at the beginning of the EPS production phase was low, although it increased slowly to almost 100% saturation by the end of the run.

To obtain the same initial k_La with the helical ribbon achievable with the Rushton turbines at 250 rpm, it was calculated that the agitation rate had to increase to 550 rpm. However, we decided against performing this experiment because of the risk of damaging probes from the vibrational instability of this stirrer system at this rate. Instead, EPS yields in the airlift configuration were investigated.

Table 1 Effect of fermenter configuration on exopolysaccharide and biomass production by *Aureobasidium pullulans* in batch culture. Values expressed as means ± 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. *ND* not determined, $Y_{p/x}$ the ratio of exopolysaccharide to biomass yields, μ specific growth rate, *EPS* exopolysaccharide

Fermenter configuration	Initial k_La (h^{-1})	Final [EPS] (g l^{-1})	Final [biomass] (g l^{-1})	Final $Y_{p/x}$	Initial μ (h^{-1})	Final [mycelia] (%)	Conversion of glucose to EPS (%)	pO_2 at onset of EPS production (% saturation)
Airlift	ND	4.25 ± 0.03	5.92 ± 0.10	0.73 ± 0.01	0.10 ± 0.00	87.6 ± 0.3	15.0 ± 0.1	0.0 ± 0.0
Assisted airlift	ND	13.12 ± 0.55	7.30 ± 0.09	1.80 ± 0.10	0.11 ± 0.00	61.6 ± 1.8	43.8 ± 1.9	0.0 ± 0.0
Axial; 250 rpm	9.5 ± 0.0	11.56 ± 0.27	6.88 ± 0.22	1.69 ± 0.10	0.10 ± 0.01	68.2 ± 10.3	38.5 ± 0.9	0.0 ± 0.0
Axial; 700 rpm	41.1 ± 0.5	7.73 ± 0.09	9.30 ± 0.36	0.83 ± 0.04	0.10 ± 0.00	81.8 ± 1.1	25.8 ± 0.3	14.0 ± 8.4
Helical ribbon	24.0 ± 0.2	11.25 ± 0.46	7.98 ± 0.26	1.42 ± 0.11	0.09 ± 0.00	79.4 ± 7.0	37.5 ± 1.5	0.2 ± 0.2

EPS production by *A. pullulans* in airlift fermenters

Depressed EPS yields were obtained with the airlift configuration (Fig. 3), where only about 4 g l^{-1} was detected after 144 h incubation, despite the fermentation being performed under otherwise identical conditions in the same vessel as that used earlier. The main polysaccharide released (approx. 70%) was the (1→3)(1→6)-β-glucan described earlier (Table 2). Exhaustion of NH_4^+ did not occur until 32 h, so release of detectable EPS levels was delayed until then. The pO_2 level fell rapidly after inoculation to zero, where it remained. However, the probe was situated at the base of the downcomer section, a physical zone that would have been the most O_2-starved region in the fermenter (Merchuk 1990). Such a location also made k_La determinations of questionable validity.

One possible reason for the poor yields in the airlift vessel may have been that the cells were too O_2-limited. Thus, an assisted-airlift configuration, based on the design of Solomons (1980), was devised, as shown in Fig. 4. A single Rushton turbine was fitted onto the drive shaft half the vessel diameter from the tank bottom, placing it just above the base of the draught tube. The axial-flow impeller was also placed one vessel diameter further up the same drive shaft. The purpose of the Rushton turbine was to achieve efficient bubble break-up and hence better O_2 transfer, while the axial-flow impeller assisted in fluid circulation by lifting the broth up the draught tube. When this system was run at an agitation rate of 250 rpm, a dramatic increase in EPS production to about 13 g l^{-1} occurred (Fig. 5). This was the highest recorded for any fermenter configuration or operating condition tested in this study, and the biomass yield approximately doubled. Almost half of the glucose supplied initially was converted into the EPS and 90% was pullulan.

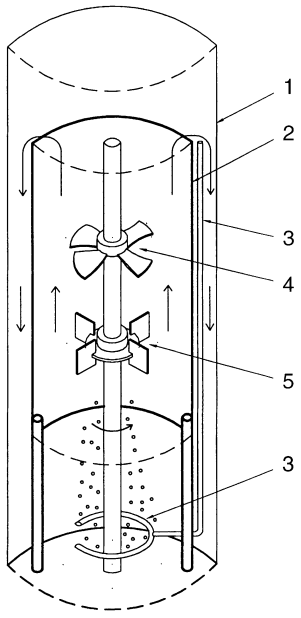

Fig. 4 A schematic diagram of the assisted-airlift configuration, a design based on the work of Solomons (1980). *1* Fermenter vessel, *2* draught tube, *3* sparger, *4* axial-flow impeller, *5* Rushton turbine

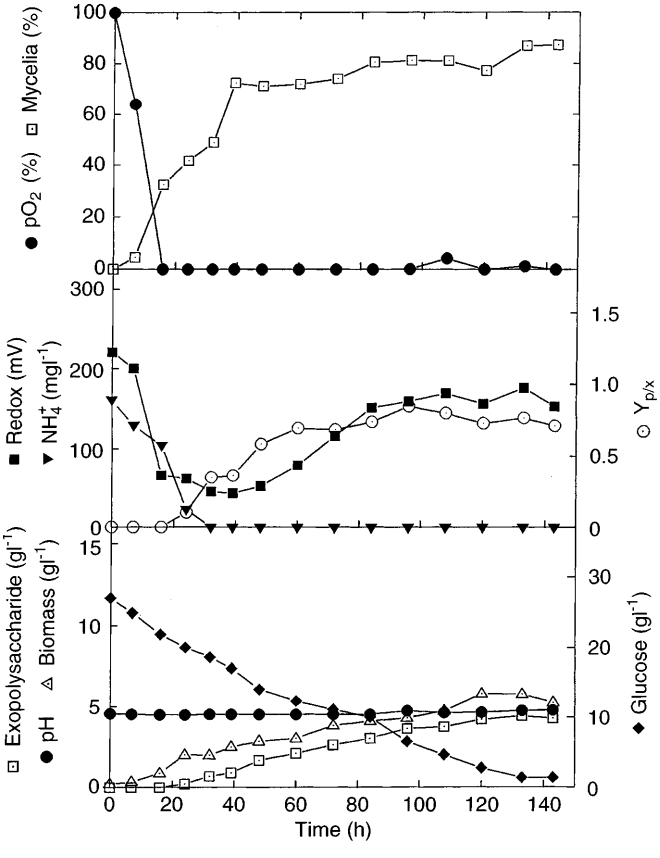

Fig. 3 Exopolysaccharide production by *A. pullulans* grown in an airlift fermenter

To investigate whether fermenter configuration influenced the molecular mass and therefore the solution properties of the pullulan produced, the viscosity of solutions of pullulan elaborated under the fermentation conditions supporting high total EPS yields, was measured. However, its apparent viscosity varied little (Table 2) with changing culture conditions.

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

Fermenter configuration had no obvious effects on the morphology of *A. pullulans* as determined microscopically. Blastospores in the inocula quickly differentiated into the diverse morphological forms seen in this fungus (Gibbs and Seviour 1996a), giving rise ultimately to predominantly mycelial cultures in all the experiments performed. The final proportion of mycelial forms varied from around 60% to 90%, but no correlation between these percentages and EPS yields was noted.

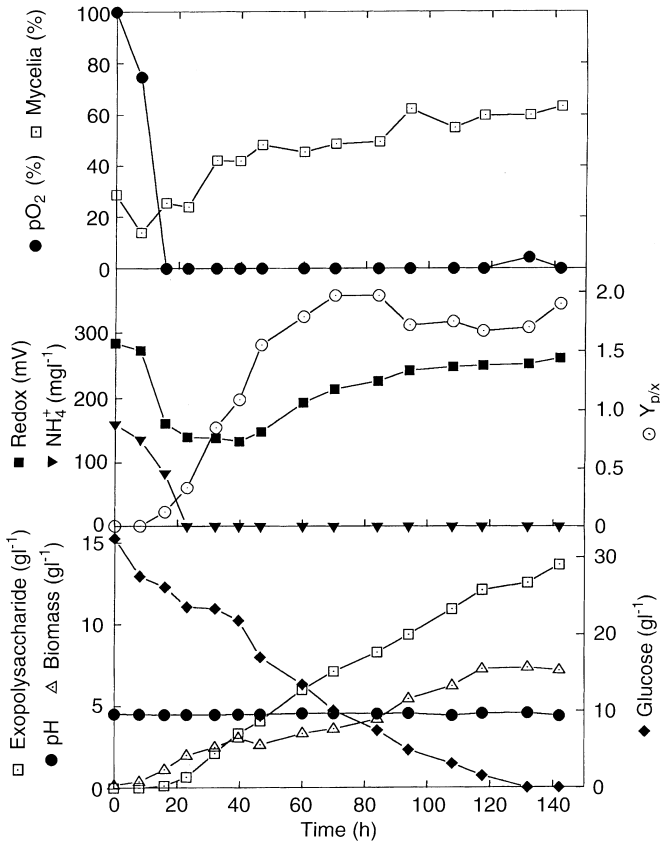

Fig. 5 Exopolysaccharide production by *A. pullulans* grown in an assisted-airlift fermenter

Table 2 The pullulan content and viscosity of the exopolysaccharide produced by *Aureobasidium pullulans* under various fermentation conditions. *ND* not determined, 1 cP = 1 mPa s

Fermenter configuration and operating conditions	Pullulan in elaborated EPS (%)	Viscosity of purified pullulan (cP)
Airlift	31.4	ND
Assisted airlift	88.2	140
Axial; 250 rpm	74.5	150
Axial; 700 rpm	53.4	ND
Helical ribbon	95.0	140

Discussion

The axial-flow impellers, helical-ribbon impeller and airlift configuration can all be considered as reduced-shear systems because they induce different patterns of fluid movement from the standard Rushton turbine. Unlike the Rushton turbine, the pumping action of the axial-flow and helical-ribbon impellers generates fluid flow from the top to the bottom of the vessel, resulting in increased in bulk mixing and a reduction in shear stress (Guérin et al. 1984; Buckland et al. 1988). Airlift reactors also have a gentle axial-flow circulation loop, generated by the difference in hydrostatic pressure between the aerated and non-aerated compartments of the vessel (Merchuk 1990). None of these low-shear systems increased EPS yields compared to the Rushton turbines in *A. pullulans*, which adds confidence to earlier work suggesting that shear stress has a little influence on EPS production in this fungus (Gibbs and Seviour 1996b). However, slight increases in production rate, together with the apparently reduced power drawn by these impellers (as judged by motor current outputs), suggest they may offer an attractive alternatives to Rushton turbines for the commercial exploitation of EPS production by *A. pullulans*.

Impeller types other than Rushton turbines have been used sparingly for metabolite, including EPS, production by filamentous fungi. Schizophyllan production by *S. commune* has been examined in vessels equipped with axial-flow impellers by Rau et al. (1992) and Gura and Rau (1993). Final β-glucan yields were slightly higher than those with Rushton turbines and more significantly the EPS production rate was greater with axial-flow impellers (i.e. 3.6 compared to 1.4 g l^{-1} day^{-1}). Only low aeration rates could be used with *S. commune* because the axial-flow impellers employed were prone to flooding. Hence reduced O_2 availability, and not a decrease in the shear rate, was considered to be the likely reason for this increase in glucan synthesis (Gura and Rau 1993).

Reports of helical-ribbon mixing systems with fungi are also scarce. Both Rho et al. (1988) and Simon et al. (1993) used these impellers with *A. pullulans*, but gave no reason for their choice. It has been stated that both EPS production and productivity by *A. pullulans* are higher with a helical-ribbon system compared to Rushton turbines (Choplin et al. 1987). This claim is not supported by the data presented here. Helical-ribbon impellers are not suitable for all EPS fermentations, as demonstrated by the low (approx. 3 g l^{-1}) schizophyllan yields from *S. commune* (Rau et al. 1992; Gura and Rau 1993).

Although Rau et al. (1992) and Gura and Rau (1993) reported that the apparent viscosity of EPS solutions from *S. commune* varied greatly with impeller design (with the axial-flow and helical-ribbon impellers recording the highest and lowest values respectively), a constant pullulan viscosity value was obtained here for all the high-yielding conditions examined. This suggests that the molecular mass of pullulan and its degree of polymerisation are not affected by the physical environment. *S. commune* is capable of enzymically degrading its own EPS (Rau et al. 1992) and so the reported differences in viscosity may have been due to the activity of these enzymes. Viscosity values can vary with fermentation conditions for *A. pullulans* (Lounes et al. 1995; Audet et al. 1996), but the viscosity of the total EPS, and not isolated and purified pullulan, was measured. This may be significant because it has been shown here and in other studies (e.g. Bouveng et al. 1963; Leal-Serrano et al. 1980; Promma et al. 1993) that *A. pullulans* is capable of secreting polysaccharides other than pullulan.

The relative proportions of pullulan and β-glucan did vary with fermentation conditions. Under conditions supporting high EPS yields the pullulan content was always about 80%–85% of the total, while values of around 20%–25% were usually recorded for conditions giving reduced EPS yields. A similar trend was observed when the agitation rate of the Rushton turbine was varied (Gibbs and Seviour 1996b). Although these data are preliminary, *A. pullulans* may always elaborate a constant amount of β-glucan and the high EPS yields could be a result of increased pullulan production.

Conversion to an airlift configuration resulted in a fall in EPS production; such results are difficult to explain, especially since Wecker and Onken (1991) achieved equivalent yields from *A. pullulans* in stirred-tank and bubble-column reactors. However, the current findings agree with an earlier study (Gibbs and Seviour 1992). The aspect ratios and general vessel geometry of the two airlift fermenters in these studies were different, so reduced EPS production by *A. pullulans* may be a general feature of airlift vessels. No difference in cell morphology or population composition was observed in the airlift reactor and the stirred-tank configurations. One explanation may lie with the aeration rate used, on the basis of the experiences of Stasinopoulos and Seviour (1992) and Wang and McNeil (1995). In both studies, increases in aeration rates were necessary to achieve equivalent or higher β-glucan yields compared with those in stirred-tank fermenters. Hence while low pO_2 conditions are required for enhanced EPS production by *A. pullulans* (Gibbs and Seviour 1996b), the aeration rate used here may have led to insufficient mixing and localised nutrient and O_2 starvation. This view is supported by the threefold increase in EPS and pullulan yields achieved when fluid circulation was assisted in the airlift reactor with a combination of impellers. The high final yield of about 13 g $l^{-1}$with this system strongly suggests that similar reactor designs are worthy of investigation with other EPS-producing fungi.

Results of this study suggest that greater emphasis should be placed on achieving sufficient O_2 transfer than on reducing shear stress for achieving high EPS and pullulan yields from *A. pullulans*. Care must be taken, however, because high O_2 saturation levels can dramatically reduce EPS production in this fungus (Gibbs and Seviour 1996b). Therefore fermenter design is a very important consideration, which should be given more attention than has previously been the case.

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