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Influence of varying nitrogen sources on polysaccharide production by *Aureobasidium pullulans* in batch culture

Desmond P. F. Auer and Robert J. Seviour

Biotechnology Research Centre, Department of Biological and Chemical Sciences, Bendigo College of Advanced Education, Bendigo, Victoria, 3550, Australia

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Summary. Polysaccharide formation by *Aureobasidium pullulans* was affected by the nitrogen source in the medium, and its yield fell when excess ammonium ions were present, even under conditions which otherwise supported its synthesis. Experiments carried out with pH control gave results which question some of the earlier published data, so it is possible to obtain high polysaccharide yields at low pH (2.5), and mycelial growth at high pH (6.5) with certain nitrogen sources. There appeared to be no causal relationship between morphology and polysaccharide-producing capacity of the cultures.

Introduction

Pullulan is an α glucan which is thought to consist of maltotriose and maltotetraose units linked with both α (1→6) and α (1→4) linkages (Taguchi et al. 1973), although this structure has recently been questioned (Catley et al. 1986). It is produced by most strains of the polymorphic fungus *Aureobasidium pullulans*, and a recent review (LeDuy et al. 1988) suggests that it may have a wide range of both medical and industrial uses. This potential is as yet largely unrealized, but it has provoked several studies designed to optimize yields and understand the physiology of its overproduction. These studies have revealed that pullulan synthesis is affected by a range of both chemical and physical parameters, among which are carbon source (Catley 1971a), impeller speed (McNeil and Kristiansen 1987) and oxygen availability (Rho et al. 1988).

Much of the work has pursued the early idea of Catley (1971b) and clearly demonstrated that ammonium ion (NH_4^+) limitation is essential for its elaboration (Seviour and Kristiansen 1983; Bulmer et al. 1987). Such studies have also supported the view that pH, which will often change with a change in nitrogen status of the medium, is also a crucial factor in pullulan synthesis (Ono et al. 1977; Lacroix et al. 1985), but may affect its production indirectly by influencing cell morphology, as pullulan synthesis has been claimed to be produced predominantly, if not exclusively (McNeil et al. 1989) by unicellular blastospores (Catley 1983; Heald and Kristiansen 1985).

The influence of nitrogen sources other than NH_4^+ on pullulan production has not been extensively reported in the literature (Chul Shin et al. 1989), nor has the relationship between polysaccharide production and concentration of various nitrogen sources independent of pH. This note describes experiments carried out in controlled batch culture which attempted to resolve the contribution of these variables to pullulan overproduction, and the relationship, if any, between pullulan production and cell morphology as affected by these variables.

Materials and methods

Culture maintenance and inoculum preparation. Aureobasidium pullulans (QM strain 3092) was maintained on slopes of Czapek Dox agar (Oxoid, Melbourne, Australia) stored at 4° C. For preparation of inoculum for shake-flask experiments, cells were removed by scraping in sterile water, and transferred to 250-ml erlenmeyer flasks containing 50 ml Czapek Dox broth. After 48 h incubation with shaking at 28° C, the almost exclusively unicellular population was harvested aseptically by centrifugation, washed three times and finally resuspended in sterile Ringers solution. Then 1 ml aliquots were used to inoculate the various media outlined below, used in shake-flask experiments.

For inoculum preparation for the fermentor experiments (see below) the organism was grown for 48 h with shaking at 28° C in erlenmeyer flasks containing 50 ml aliquots of the same medium used in the fermentor (see Results), and added as a 1% inoculum.

Culture conditions. For shake-flask experiments, 50 ml aliquots in 250-ml erlenmeyer flasks of the mineral salts medium of Pitt and Bull (1982) containing 30 g l^{-1} glucose and the different nitrogen sources at stated concentrations (see Results) were used. Cultures

Offprint requests to: R. J. Seviour

Table 1. Effect of different nitrogen sources (all at 0.13 g l^{-1} total N) on biomass and polysaccharide production by *Aureobasidium pullulans* after 48 h

Nitrogen source	Biomass (g l^{-1})	Polysaccharide (g l^{-1})	pH	Residual glucose (g l^{-1})
Phenylalanine	3.0	4.1	4.5	16.8
Histidine	2.7	5.6	4.4	6.6
Glycine	3.4	3.1	4.5	18.0
Alanine	6.6	6.5	2.9	4.0
Arginine	4.3	3.2	4.0	16.1
Monosodium glutamate	5.4	5.4	4.9	10.8
$NaNO_2$	1.7	2.8	6.0	8.6
$NaNO_3$	5.6	4.0	5.7	9.9
KNO_3	5.7	3.8	6.2	7.8
NH_4NO_3	6.5	7.6	4.4	6.6
$(NH_4)_2SO_4$	4.3	8.7	2.9	14.1
Ammonium tartrate	11.0	3.5	3.6	5.7

For details, see text

were grown at 28° C with shaking for the required time periods before being harvested and analysed as detailed below.

Batch fermentations were carried out using an LH 500 series system (Enztech Sydney, NSW, Australia) with a working volume of 1.8 l of the above medium of Pitt and Bull (1982), with the N source and its concentration as specified in the Results, in a 2-l vessel. Aeration, temperature and agitation were maintained at 1 vol air/vol medium/min, at 28° C and 500 rpm, unless stated otherwise. The pH was controlled at the required value with an Ingold (Enztech) electrode and an LH 505 (Enztech) pH controller controlling the feed of either 2 *M* NaOH or 2 *M* HCl as appropriate. Samples as required were obtained aseptically, and over the course of the experiment, no more than 250 ml medium was removed from the vessel.

Analytical methods. Cells removed by centrifugation were separated in some experiments into unicellular and mycelial fractions using 45 μm nylon mesh (Heald and Kristiansen 1985). Biomass, exopolysaccharide, residual NH_4^+, nitrate and glucose in the medium were measured as described previously (Seviour and Kristiansen 1983; Stasinopoulos and Seviour 1989) and glutamate determined using a colorimetric method (Boehringer Mannheim Australia Proprietory, Melbourne, Australia, Catalogue no. 139092).

Results

Shake-flask experiments

Changing N source. Variations in yields of biomass and polysaccharide produced by *A. pullulans* with a range of both organic and inorganic nitrogen sources, all at an initial concentration of 0.13 g l^{-1} total N are shown in Table 1. Where the final pH was high (>6) then the population was predominantly unicellular, and at low pH (3 or below), almost exclusively mycelial.

Increasing initial N levels. Increasing the initial $(NH_4)_2SO_4$ concentration in the medium gave results (Table 2) similar to those reported elsewhere, namely that above 0.15–0.2 g l^{-1} N, polysaccharide levels dropped, possibly as pH decreased and the population became mycelial, but biomass production increased. With other N sources, different trends were seen. For example, with $NaNO_3$ (Table 2) a drop in polysaccharide yields was only seen above 0.25–0.3 g l^{-1} N, coinciding with glucose exhaustion, even though the population was still exclusively unicellular. With monosodium glutamate, polysaccharide levels only fell slightly as its concentration in the medium increased (Table 2), and again the morphology became increasingly unicellular, consistent possibly with the pH values recorded.

These shake-flask experiments, while supporting the general view that increasing N levels can shift the pattern of carbon efflux within the cells of this fungus, are not appropriate culture systems for determining whether this is a direct effect or a consequence of changing medium pH, which may then affect substrate uptake or change culture morphology. Attempts to control pH with a range of buffers were not always suc-

Table 2. Effect of increasing levels of $(NH_4)_2SO_4$, $NaNO_3$ and monosodium glutamate on polysaccharide production by *A. pullulans* in shake flasks

Nitrogen source	$(NH_4)_2SO_4$				$NaNO_3$				Glutamate			
Initial concentration (gN l^{-1})	Biomass (g l^{-1})	Polysaccharide (g l^{-1})	Residual glucose (g l^{-1})	Final pH	Biomass (g l^{-1})	Polysaccharide (g l^{-1})	Residual glucose (g l^{-1})	Final pH	Biomass (g l^{-1})	Polysaccharide (g l^{-1})	Residual glucose (g l^{-1})	Final pH
0.05	1.63	5.25	19.33	4.19	2.72	4.03	13.09	4.95	2.50	2.81	21.96	5.63
0.10	4.00	7.69	15.05	3.25	3.66	6.83	10.08	4.70	4.13	4.38	15.53	4.81
0.15	4.31	8.75	14.07	2.81	5.82	8.87	2.78	4.55	5.44	5.44	10.76	4.88
0.20	5.31	6.44	13.97	2.31	7.48	10.50	1.24	4.63	6.94	6.13	8.75	4.88
0.25	6.38	4.44	13.53	2.06	8.36	11.17	0.15	5.09	7.88	6.25	7.39	5.19
0.30	6.91	3.00	11.52	1.81	9.34	10.27	0.15	5.88	8.13	6.25	6.03	5.50
0.35	7.50	2.94	10.05	1.63	9.32	9.60	0.12	5.93	7.88	6.25	3.26	5.44
0.40	8.75	2.62	6.03	1.63	10.20	7.80	0.12	6.25	9.06	5.62	1.61	5.19
0.45	8.75	2.62	5.18	1.50	NT	NT	NT	NT	9.25	5.50	0.63	5.00
0.50	9.31	2.37	3.84	1.50	NT	NT	NT	NT	9.88	3.94	0.00	5.13

For other details, see text: NT = not tested

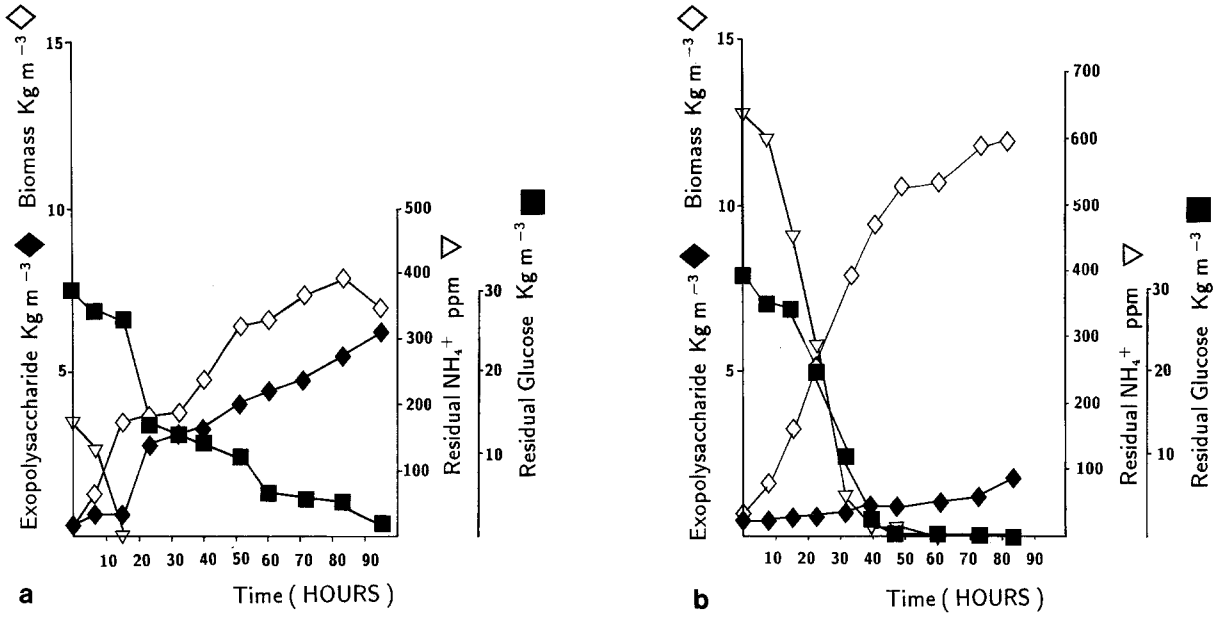

Fig. 1a, b. Production of polysaccharide by *Aureobasidium pullulans* at constant pH 4.5 with different $(NH_4)_2SO_4$ concentrations. **a** 0.13 g l^{-1} N $(NH_4)_2SO_4$ as N source. **b** 0,52 g l^{-1} N $(NH_4)_2SO_4$ as N source. For all other details see text

cessful, so fermentor experiments were carried out with the purpose of resolving these effects of changing N concentration and culture pH, using $(NH_4)_2SO_4$ and glutamate as the N sources tested, chosen from these shake-flask experiments.

Fermentor studies with pH control

Effects of increasing initial N levels on polysaccharide formation at pH 4.5. Increasing the initial $(NH_4)_2SO_4$ concentration from 0.13 to 0.52 g l^{-1} N at a constant

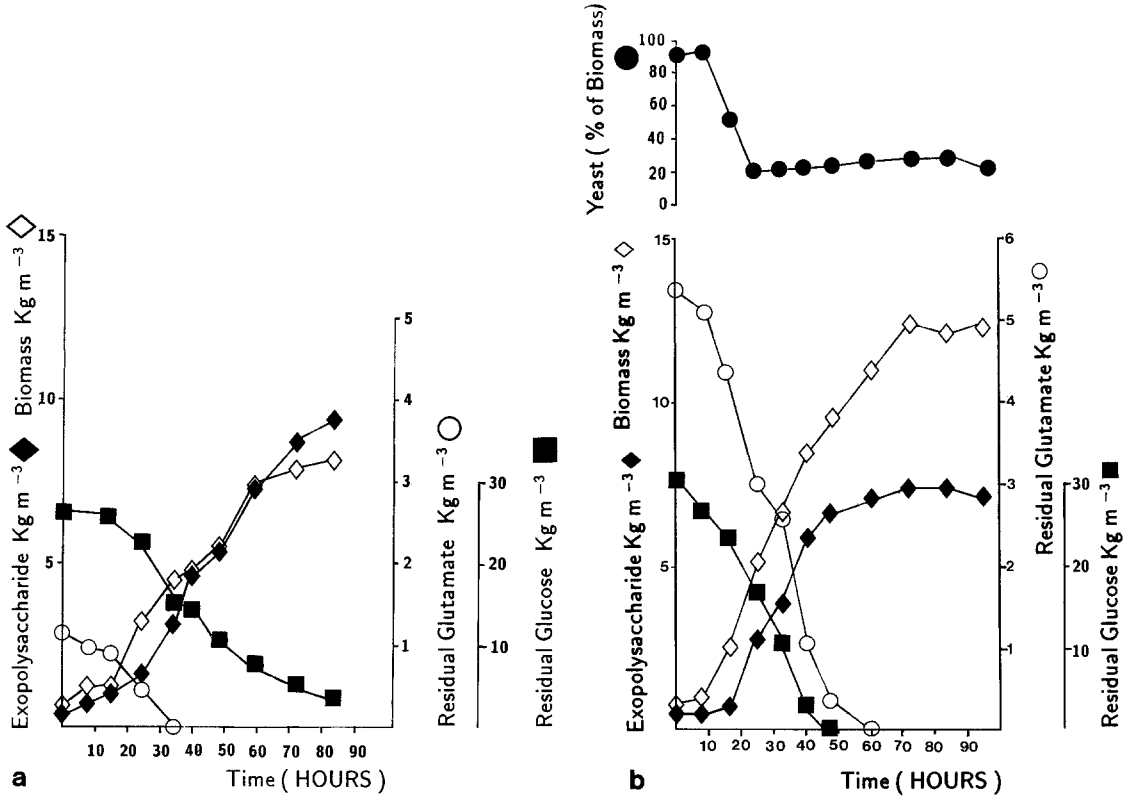

Fig. 2a, b. Production of polysaccharide by *A. pullulans* at constant pH 4.5 with different glutamate concentrations. **a** 0.13 g l^{-1} N as glutamate as N source. **b** 0.52 g l^{-1} N as glutamate as N source. For all other details, see text

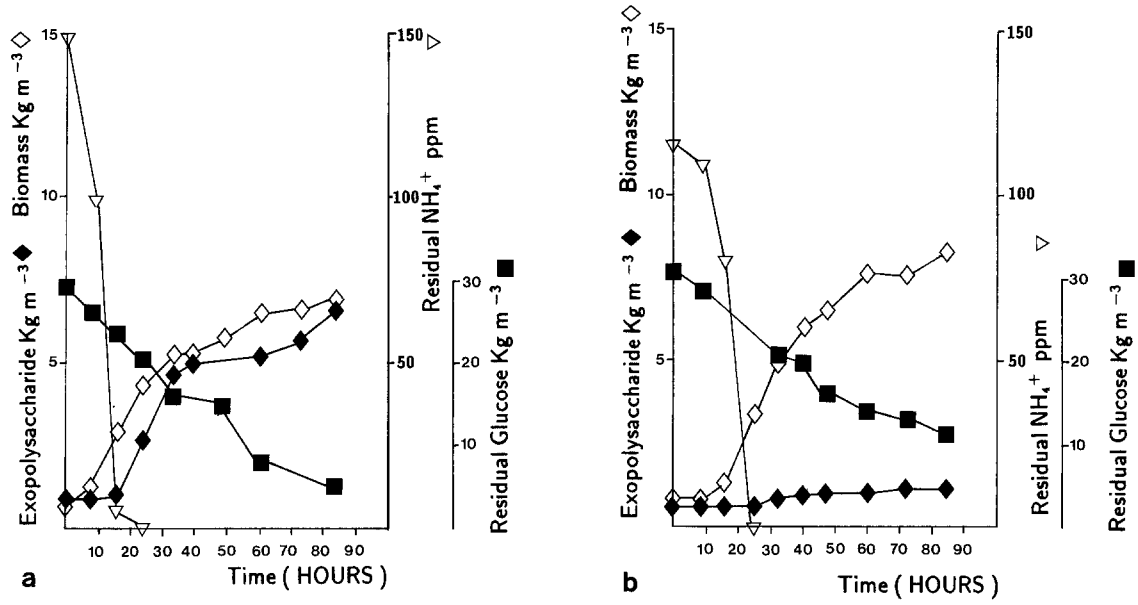

Fig. 3 a, b. Production of polysaccharide by *A. pullulans* grown on 0.13 g l^{-1} N as $(NH_4)_2SO_4$ at different constant pH values. **a** pH 6.5. **b** pH 2.5. For all other details, see text

pH of 4.5, selected because other studies (McNeil et al. 1989) have shown it to be optimal for polysaccharide synthesis, again led to a drastic reduction in polysaccharide yields and a stimulation in biomass production, as seen by comparing Fig. 1a and 1b. However, a similar fourfold increase in glutamate levels at pH 4.5, although stimulating biomass formation and substrate uptake, led to a much lower reduction in polysaccharide yields (Fig. 2a, b), and exhaustion of glutamate from the medium was not an absolute requirement for onset of its synthesis (e.g. Fig. 2b). Morphology also changed, as the level of N increased, with a heteroge-

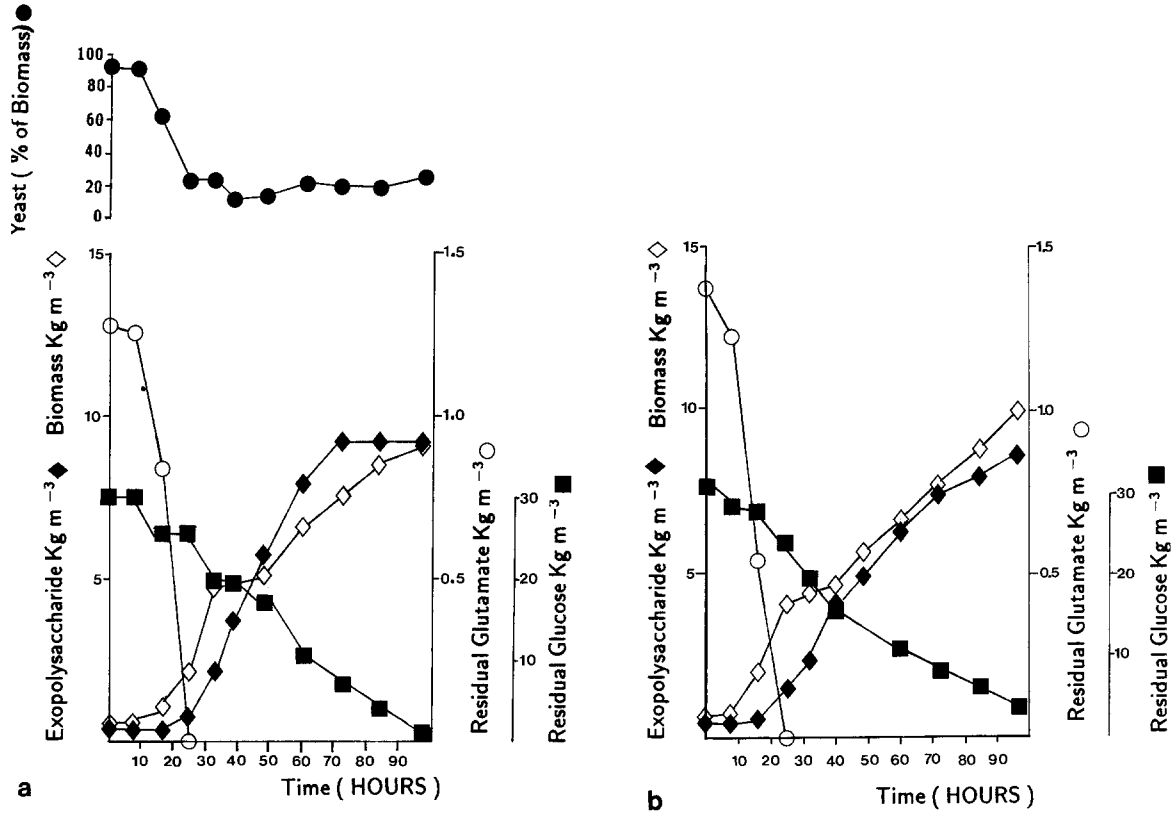

Fig. 4 a, b. Production of polysaccharide by *A. pullulans* grown on 0.13 g l^{-1} N as glutamate at different constant pH values. **a** pH 2.5. **b** pH 6.5. For all other details, see text

nous, almost equal mixture of unicells, mycelium and chlamydospores at 0.13 g l^{-1} N as $(NH_4)_2SO_4$ giving way to large mycelial clumps or aggregates with few or no visible unicells at 0.52 g l^{-1} N. Mycelial forms also increased at the higher glutamate level, increasing from c. 10% of the population at 0.13 g l^{-1} N to c. 80% of the total biomass at 0.52 g l^{-1} N (Fig. 2b).

Influence of pH on polysaccharide production with varying nitrogen levels. A series of experiments was carried out where *A. pullulans* was grown at several constant pH values with varying concentrations of $(NH_4)_2SO_4$ and glutamate in attempts to determine separately their effects on polysaccharide synthesis in this fungus.

A comparison of fermentation patterns obtained with *A. pullulans* grown with 0.13 g l^{-1} N as $(NH_4)_2SO_4$ at constant pH of 2.5, 4.5 and 6.5 (Figs. 1a, 3a, b) showed pH had little effect on biomass yields. However, decreasing the culture pH to 2.5 almost completely inhibited polysaccharide formation (Fig. 3b) and although increasing the pH from 4.5 to 6.5 did not seem to affect polysaccharide yields, the rate of production was higher at pH 6.5 (Figs. 1a, 3a). The influence of culture pH on morphology was clearly apparent, with large mycelial clumps at 2.5 (Fig. 7a) and a predominantly unicellular population at pH 6.5, with profuse blastospore production by budding from small hyphal fragments common.

Repeating the experiments with 0.13 g l^{-1} N as glutamate however gave a different pattern. With this N source, changing culture pH had little effect, not only on biomass production, but also on polysaccharide yields (Figs. 2a, 4a, b). Also the morphology was different to that expected from previous studies. At pH 6.5, the culture was totally unicellular, as found with $(NH_4)_2SO_4$, but at pH 2.5, as much as 25% of the biomass was also unicellular, and blastospores were seen budding from swollen cells (Fig. 7b), a process previously thought (Seviour et al. 1984) to be inhibited at low pH.

Effect of high N levels on polysaccharide production at different pH values. When the initial concentration of $(NH_4)_2SO_4$ was increased fourfold to 0.52 g l^{-1} N and *A. pullulans* was grown at pH 6.5, the results shown in Fig. 5 were obtained. Although there was very little change in either biomass or final polysaccharide yields compared to the run at pH 4.5 (Fig. 1b), culture morphology had changed from the almost exclusively unicellular form encountered in all previous experiments at 6.5 to a progressively increasing mycelial population (Figs. 5, 7c). This time, repeating the experiment with 0.52 g l^{-1} N as glutamate gave similar results, with very little difference in polysaccharide yields at 6.5 compared to 4.5 (Fig. 2b), but again with a predominantly mycelial morphology (Fig. 6b). If the culture pH was maintained at 2.5, then very few unicells were seen at all, and polysaccharide was elaborated to levels only slightly lower than those found at 4.5 and 6.5 (Fig. 6a). Also capsular material could be seen around these hyphae, although it is not known if this was pullulan (Fig. 7d).

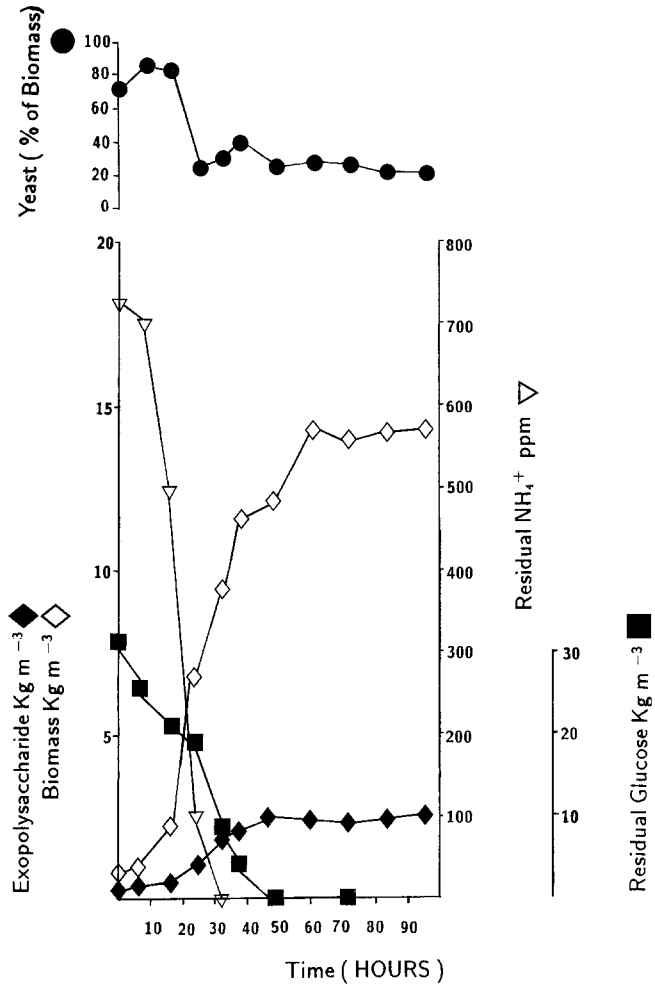

Fig. 5. Production of polysaccharide by *A. pullulans* grown on 0.52 g l^{-1} N as $(NH_4)_2SO_4$ at constant pH 6.5. For all other details, see text

Melanization of cells

In all these experiments, melanin deposition of hyphal cells and production of chlamydospores only occurred following exhaustion of the nitrogen source from the medium.

Discussion

This investigation attempted to distinguish between the effects on exopolysaccharide production in *A. pullulans* of increasing nitrogen levels in the medium and inadvertently changing other culture variables such as pH, since both have been suggested as important factors in its elaboration (e.g. Ono et al. 1977; Bulmer et al. 1987). The results suggest a complex pattern of interactions between this organism and its environment, but as well as substantiating many of the previous studies on polysaccharide synthesis, where pH control was not always used, they also question some of the conclusions reached in them. For example, unlike some reports (e.g. Lacroix et al. 1985), the present data show that *A. pullulans* will synthesize expolysaccharides almost as well at

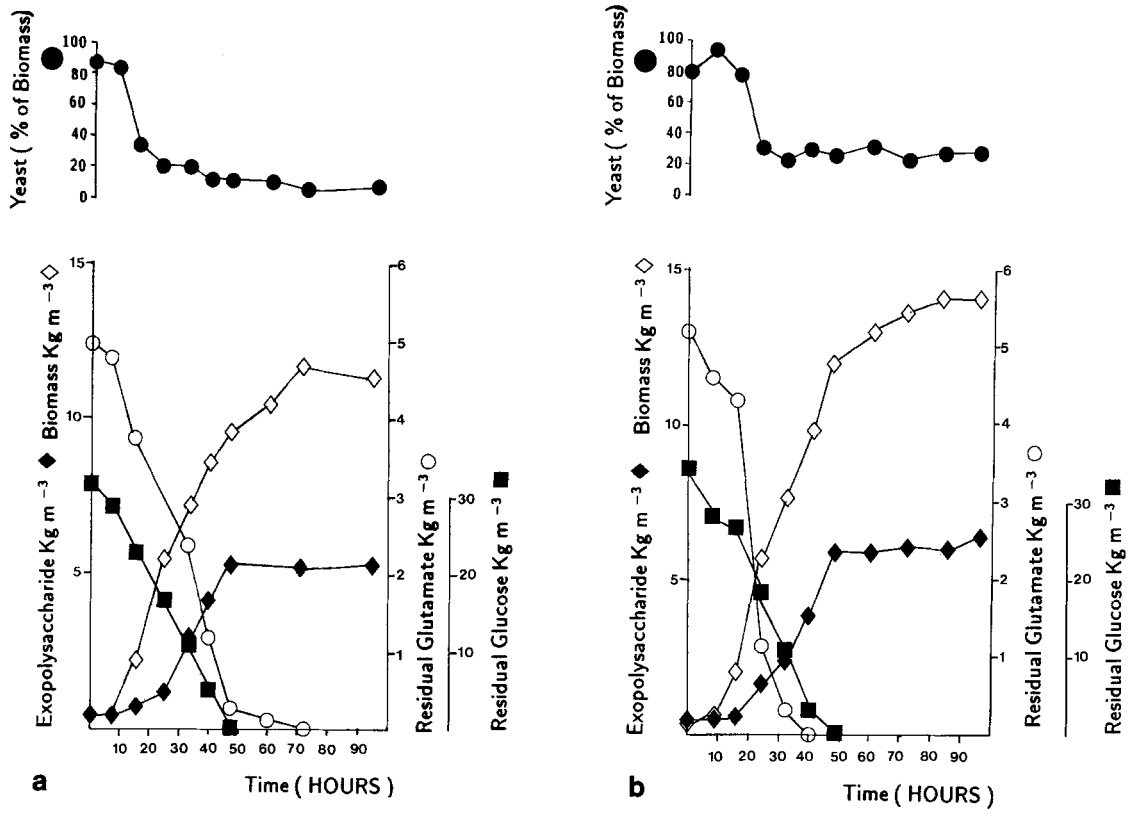

Fig. 6a, b. Production of polysaccharide by *A. pullulans* grown on 0.52 g l^{-1} N as glutamate at different constant pH values. **a** 2.5. **b** 6.5. For all other details, see text

low pH (2.5) as at higher values (4.5 and 6.5), if a nitrogen source other than $(NH_4)_2SO_4$, i.e. glutamate, is used. Therefore although polysaccharide production can be affected by pH, it would appear that its influence depends on both the nitrogen source used and its concentration.

These effects can not be explained totally in terms of changes in the pattern of substrate utilization under these different regimes, and the results obtained also question an exclusive role for pH in determining population morphology (Heald and Kristiansen 1985; Bulmer et al. 1987). Thus it was possible to achieve a predominantly mycelial population at high pH (6.5) with high initial levels of both glutamate and $(NH_4)_2SO_4$ (Figs. 5, 6b) and substantial production of blastospores at low pH (2.5) with glutamate at low, but not high concentration. In some instances, these morphological changes were not in accord with a widely held view (Catley 1980; Heald and Kristiansen 1985) that polysaccharide synthesis is directly related to cell morphology, and support recent chemostat work (McNeil et al. 1989) showing that filamentous cells of *A. pullulans* synthesize polysaccharide. The observation that an almost totally unicellular population only produced very low levels of polysaccharide (Table 2) is again inconsistent with this view. Interpretation of such cultural studies is difficult, based as it is on the unlikely assumption that a particular morphological form is necessarily committed to polysaccharide elaboration at all times during its life cycle, under all cultural conditions, and that if different cell types produce polysaccharides, then they do so all at the same rate and to the same extent. In an organism with such a complex morphology as *A. pullulans*, these assumptions are not readily tested experimentally.

This study has again emphasized the potent inhibitory effect of NH_4^+ on polysaccharide formation, independent of any pH changes, noticed in some earlier work with *A. pullulans* (Catley 1971b; Seviour and Kristiansen 1983; Bulmer et al. 1987) and in some other polysaccharide-producing fungi (e.g. Stasinopoulos and Seviour 1989) and bacteria (e.g. Souw and Demain 1979). Although $NaNO_3$ also inhibited polysaccharide production (Table 2) it did so at much higher levels, and glutamate over the same concentration range had very little inhibitory effect (Table 2). One suggestion that NH_4^+ acts by specific inhibition of protein synthesis (Bulmer et al. 1987) is based on indirect evidence only. Certainly in other systems subject to NH_4^+ regulation such as antibiotic synthesis (Aharonowitz 1980) and lipid accumulation (Evans and Ratledge 1984), both type and concentration of N source, by affecting the amounts and activities of key enzyme systems involved in N assimilation can determine the compositions of intracellular metabolite pools (Kusnan et al. 1987). Many of these, as allosteric effectors, could directly control carbon flow within the cells to either biomass or polysaccharide formation. Preliminary work here has shown that *A. pullulans* possesses both an nicotinamide adenine dinucleotide (NAD^+)-dependent

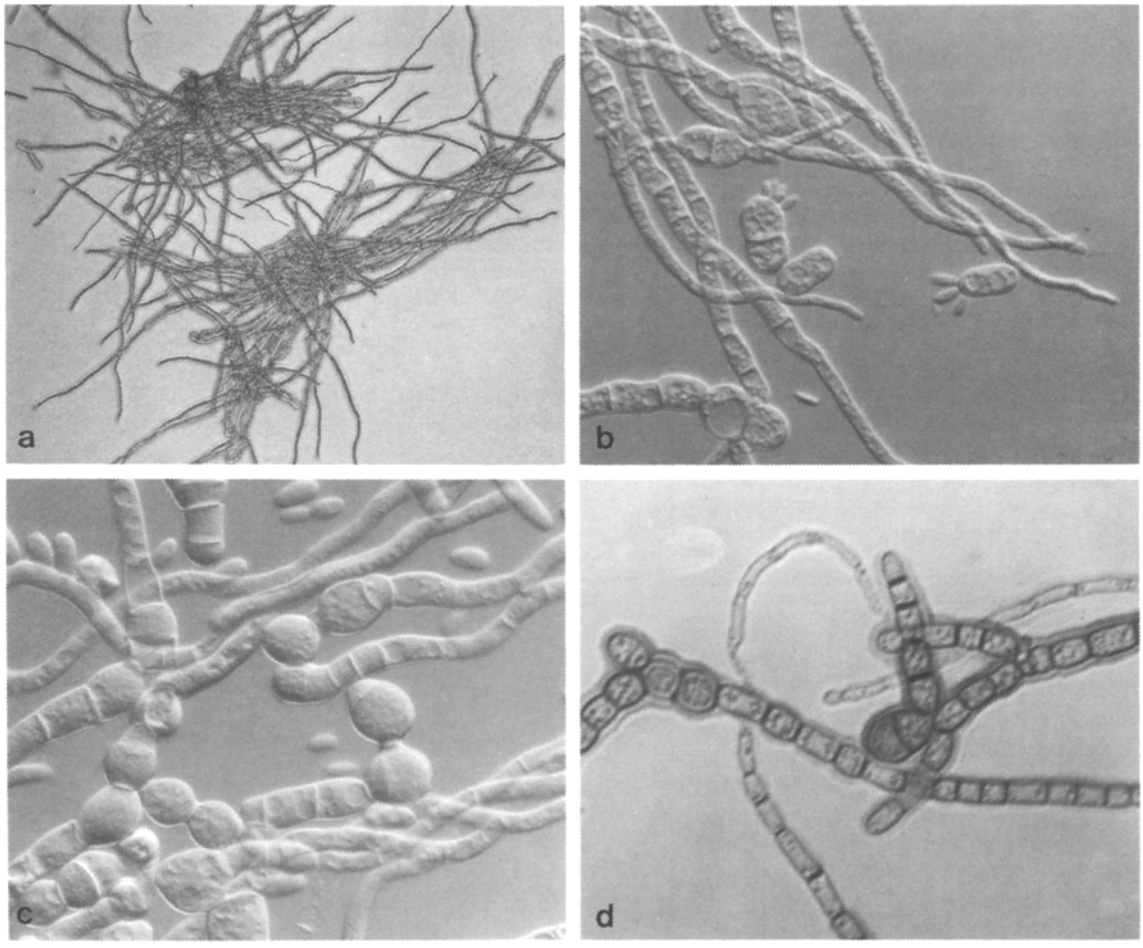

Fig. 7. **a** Mycelial clumps of *A. pullulans* grown at pH 2.5 with 0.13 g l^{-1} N as $(NH_4)_2SO_4$ (×180). **b** Budding swollen cells and blastospores at pH 2.5 with 0.13 g l^{-1} N as glutamate viewed with Nomarski optics (×1750). **c** Presence of mycelial cells at pH 6.5 with 0.52 g l^{-1} N as glutamate viewed with Nomarski optics (×1750). **d** Presence of capsular (polysaccharide) material around hyphae grown on 0.52 g l^{-1} N as glutamate at pH 2.5 (×1750)

glutamate dehydrogenase (EC 1.4.1.2) and a glutamine synthetase (EC 6.2.1.2)/glutamate synthase (GOGAT) (EC 1.4.7.1) pathway for NH_4^+ assimilation (Auer and Seviour, unpublished). However, their role if any in the regulation of polysaccharide production in this organism is not yet clear.

Finally, the suggestion that pullulan is a secondary metabolite (Bulmer et al. 1987) is not supported by this or earlier work (e.g. Kristiansen et al. 1983). Its synthesis was not initiated at the stationary phase, but usually parallelled increases in biomass, and nor was it always associated with exhaustion of nitrogen source from the medium. This needs to be considered in any attempt directed towards seeking an understanding of possible regulatory mechanisms (Martin and Demain 1978).

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