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Effect of Ammonium Ion Concentration on Polysaccharide Production by *Aureobasidium pullulans* in Batch Culture

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Summary. Experiments have been carried out to study the production of polysaccharides by *Aerobasidium pullulans* in a 6L fermenter. In batch culture, a lag in polysaccharide production but not biomass was experienced. This lag increased with increasing initial nitrogen concentration. Polysaccharide production commenced on reaching nitrogen limiting conditions. At high nitrogen levels, the production of polysaccharides was reduced considerably.

Introduction

There is a great deal of current interest in the possible medical and industrial applications of microbial polysaccharides (Berkeley et al. 1979; Sutherland and Ellwood 1979). Although the number of reported fungal exocellular polysaccharides continues to expand, their industrial potential in most cases is unknown. One exception is pullulan, a neutral α-glucan synthesised by *Aureobasidium pullulans* (Slodki and Camus 1978), with properties suitable as a possible food packaging material (Yuen 1974).

The effects of various parameters on pullulan production have been the subject of a recent review by Catley (1979), yet comparatively little is known about the control mechanisms involved. Some of the more important factors affecting pullulan elaboration would seem to include nitrogen availability (Catley 1971, 1973) and culture pH (Ono et al. 1977). Unfortunately, many of the earlier studies used shake flask cultures, where measurement or control of culture variables and repeated sampling are difficult. Therefore, this study undertook to investigate the effects of varying initial levels of NH_4^+ on total polysaccharide production in *A. pullulans* in a 6L fermenter.

Materials and Methods

The organism used in this study was *Aureobasidium pullulans* Quartermaster Strain No. 3092 (kindly supplied by Dr. B J Catley, Heriot-Watt University). It was maintained as a freeze dried culture and reclaimed by incubation on plates of PDA at 28° C.

The experiments were carried out in a 6L fermenter (Chemap) which was using a 10% inoculum. The medium composition, both in the shake flasks and the fermenter, was as follows:

(in kg/m³) sucrose 30; $(NH_4)_2SO_4$ variable; K_2HPO_4 5;

$MgSO_4 \times 7\,H_2O$ 0.2; NaCl 1; Yeast extract 0.4. The fermenter was operating at 28° C, agitation speed 1,000 rpm and air flow rate 1 vvm.

The cell dry weight was obtained by filtration using Whatman 6F/C filter papers (sample volume 10 ml), or in the case of very viscous samples by centrifugation at 16,000 *g* for 20 min at 10° C. The polysaccharide was extracted from a 10-ml sample of cell free extract by addition of an equal volume of 66% ethanol. The residual sucrose concentration was determined using the colourimetric method of Dubois et al. (1956) after the polysaccharide had been extracted from the sample. The concentration of ammonium ions in the culture filtrate was estimated with an ammonium selective electrode.

Results

Polysaccharide Production in Batch Culture

Results from a typical batch run with an initial $(NH_4)_2SO_4$ concentration of 0.6 $kg \cdot m^{-3}$ are illustrated in Fig. 1. Frequent sampling in the early stages of the fermentation revealed a very rapid uptake of NH_4^+ from the medium, so that after 12 h very little was still detectable. Only after this time was it possible to detect any increase in polysaccharide in the medium, although increase in biomass production commenced immediately after inoculation. There-

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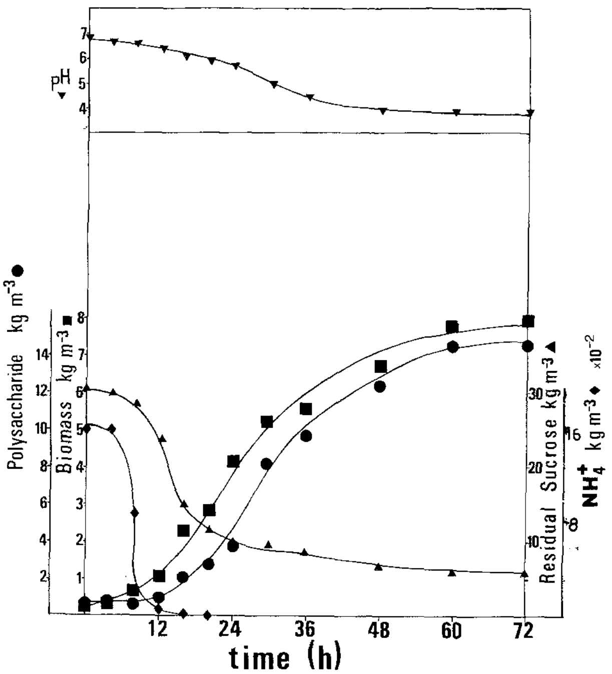

Fig. 1. Batch fermentation for polysaccharide production by *A. pullulans* with an initial concentration of 0.6 kg · m^{-3} $(NH_4)_2SO_4$ in the medium. Culture conditions and analytical methods as described in text

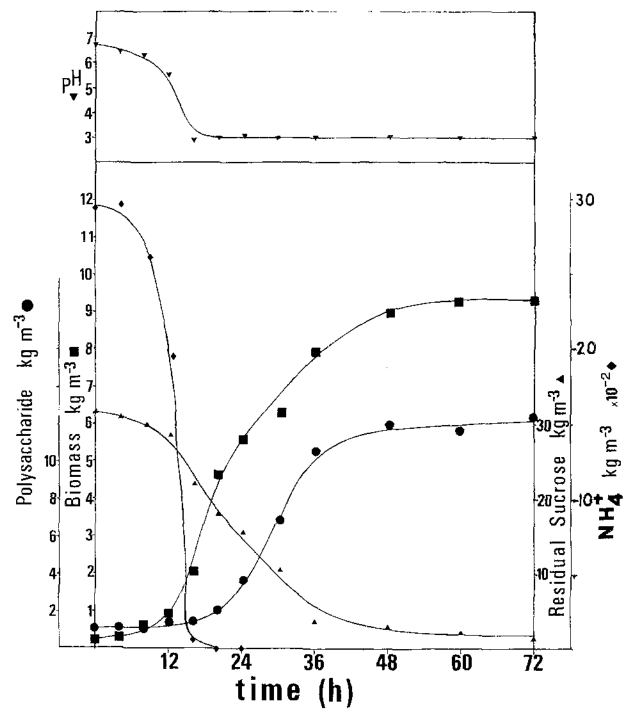

Fig. 2. Polysaccharide production by *A. pullulans* with an initial $(NH_4)_2SO_4$ concentration of 1.2 kg · m^{-3}

fore, the presence of free NH_4^+ in the medium appeared to suppress polysaccharide elaboration. After this lag period the polysaccharide concentration increased in parallel with biomass until 72 h when no further increases were observed. This cessation was not caused by sucrose limitation, which only slowly disappeared from the medium after 24 h and about 6.25 kg · m^{-3} sucrose was left at the end of the fermentation, but possibly due to nitrogen limitation or unfavourable pH (Catley 1973).

Effect of Varying Ammonium Sulphate Concentration

Increasing the initial level of $(NH_4)_2SO_4$ in the medium had a marked effect on polysaccharide production by *A. pullulans*. For example, a twofold increase to 1.2 kg · m^{-3} gave the results shown in Fig. 2. Again, no increase in polysaccharide formation was detectable until NH_4^+ had almost disappeared from the medium. The lag period now lasted 16–20 h. However, although sucrose utilisation increased under these conditions, more carbon was diverted into biomass, and polysaccharide production dropped. Little further production occurred after 36 h although biomass continued to increase up to 60 h after inoculation. Overall, this represented a decrease of ca. 13% and an increase of ca. 18% in polysaccharide and biomass production respectively. The rapid fall in pH to a steady value of 2.9 after 16 h mirrored the NH_4^+ uptake.

These trends became much more pronounced as the initial $(NH_4)_2SO_4$ level was further increased to 2.4 kg · m^{-3}. Very little polysaccharide was detectable at any time during the fermentation (Fig. 3), and final yield fell by ca. 95% while biomass increased by ca. 70% over values achieved with 0.6 kg · m^{-3} $(NH_4)_2SO_4$. Although sucrose was still available, no polysaccharide production phase followed exhaustion of NH_4^+ from the medium, unlike the previous runs. There are several possible explanations for this. The cell may become irreversibly committed, in response to high initial NH_4^+ concentrations to a continued carbon flow into biomass at the expense of polysaccharide synthesis, or the low pH of 2.1 under these conditions may inhibit its production.

The second possibility was investigated by repeating the fermentation at a constant pH of 4.5, chosen since it is the optimal pH for polysaccharide production (Harvey and Kristiansen, unpublished results). Results obtained (Fig. 4) clearly demonstrate the supression of polysaccharide elaboration was due largely to increased NH_4^+ concentration and not low pH. Although a short production phase followed complete NH_4^+ assimilation at this constant pH (Fig. 4) the culture did not achieve yields comparable to the earlier batch runs with low NH_4^+

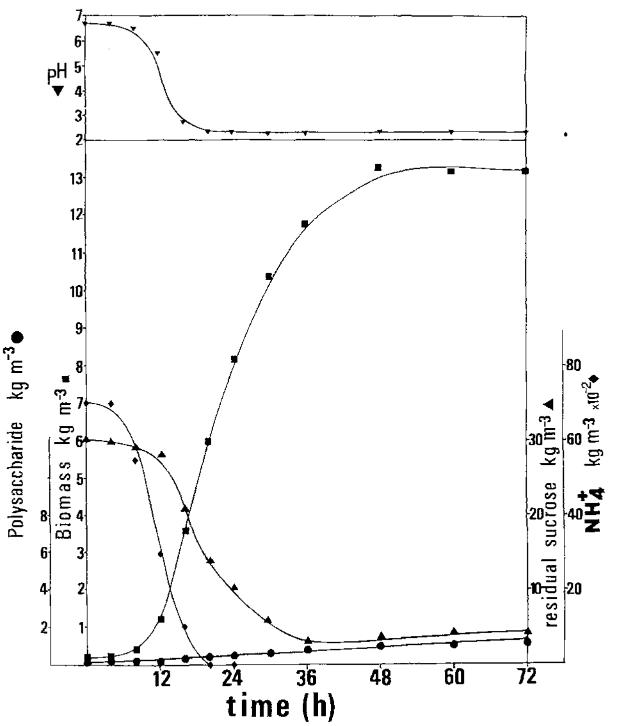

Fig. 3. Polysaccharide production by *A. pullulans* with an initial $(NH_4)_2SO_4$ concentration of 2.4 kg · m^{-3}

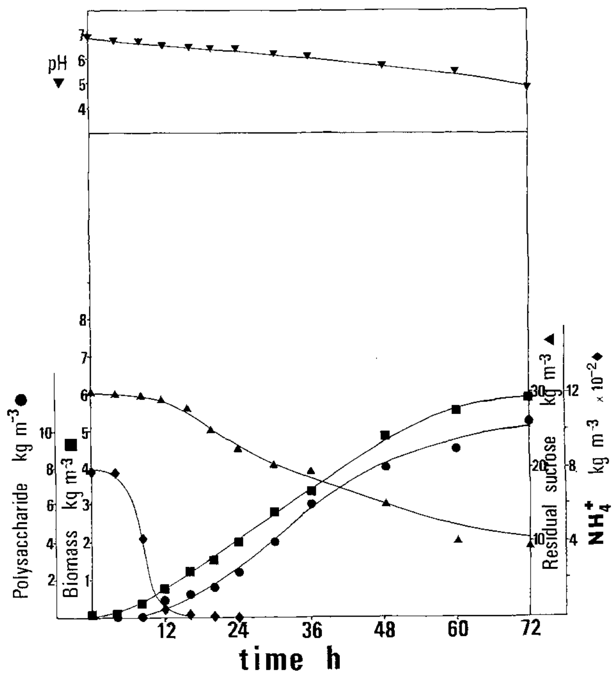

Fig. 5. Polysaccharide production by *A. pullulans* with an initial $(NH_4)_2SO_4$ concentration of 0.3 kg · m^{-3}

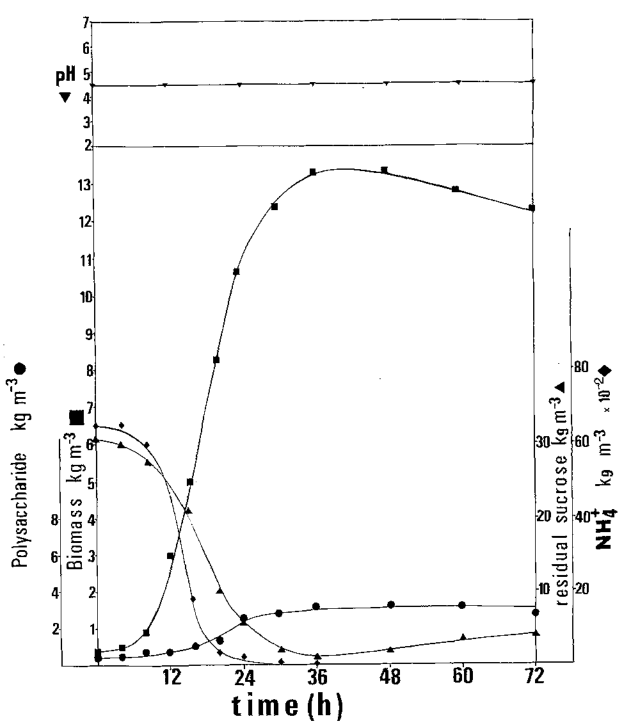

Fig. 4. Polysaccharide production by *A. pullulans* with an initial $(NH_4)_2SO_4$ concentration of 2.4 kg · m^{-3}, and pH controlled at 4.5

concentration. Most of the sucrose was again incorporated into biomass which was formed in very similar quantities to the previous experiment (Fig. 3). There was also some evidence of cell lysis occurring at the later stages as evidenced by gradual drop in biomass and a slight but consistent increase in sucrose levels in the medium.

On the other hand, no corresponding increase in polysaccharide formation occurred if the initial $(NH_4)_2SO_4$ concentration was decreased from 0.6 to 0.3 kg · m^{-3}. Instead, although biomass and polysaccharide production showed their usual relationship to each other (Fig. 5), final yields in both cases were depressed. This may have been because less sucrose was assimilated under these conditions, caused possibly by severe nitrogen limitation or high pH (Catley 1973), but was not investigated further.

Discussion

The initial NH_4^+ concentration appears to affect both the production of biomass and the production of pullulan. The final biomass concentration increases with inlet NH_4^+. The substantial increase in dry weight after NH_4^+ exhaustion is considered to be a result of carbon accumulation by the cell, a phenomenon well documented for filamentous fungi.

No attempt was made in this study to routinely chemically characterise the polysaccharide material produced by *Aureobasidium pullulans*. However, the physiology of its production in a 6L fermenter agreed in some respects to the formation of pullulan in shake flask cultures (Catley 1971, 1973). Namely, an increase in NH_4^+ concentrations enhances carbon flow into biomass formation with a corresponding decrease in polysaccharide levels so that at 2.5 $kg \cdot m^{-3}$ $(NH_4)_2SO_4$ polymer synthesis had almost ceased. Although low pH inhibits pullulan synthesis (Ono et al. 1977) its effect in this study was less substantial than NH_4^+ concentrations (Figs. 3, 4).

Results presented here further demonstrate that NH_4^+ must be exhausted from the medium before exopolysaccharide production can commence, but in contrast to Catley's (1981) study, the duration of this lag in polymer production, not noticed with biomass, appeared to depend on the initial NH_4^+ level in the medium.

The repressive effect of excess ammonium ions on metabolite production including antibiotics in microbes is well documented (Aharonowitz 1980), and clearly the regulatory role of these ions in *A. pullulans* deserves closer examination. Whether they exert their effect directly or indirectly through inhibition of enzyme synthesis as shown in several other fungi (Marzluf 1981) or as alosteric effectors of one or several enzymes in carbon metabolism is not clear. The immediate appearance of polysaccharide in the medium after NH_4^+ exhaustion is possibly circumstantial evidence in favour of the second hypothesis. Indeed, the physiology of accumulation of this polymer may resemble citric acid production in *Aspergillus niger* (Kubicek et al. 1979) where both phosphofructokinase and pyruvate kinase are activated by NH_4^+, whose presence would therefore favour biomass formation.

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