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The effect of ammonium ions and pH on the elaboration of the fungal extracellular polysaccharide, pullulan, by *Aureobasidium pullulans*

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Summary. Effects of the absence and presence of NH_4^+ (50 mM) and proton concentration (pH range 4—8) on pullulan elaboration by *Aureobasidium pullulans* have been examined. The action of NH_4^+ is thought to be not as an effector of metabolic pathways, but rather as an influence on protein synthesis. The short-term effect of changes in environmental pH can be dramatic but pullulan elaboration rates, after a period of adjustment, typically about 90 min, appear to be largely the same within the pH range studied.

Introduction

Studies of environmental factors that effect production of microbial extracellular products are necessary in that they contribute to understanding control of cellular metabolism and the optimizing of yields of commercially interesting products. There is, therefore, an interest in examining the environmental parameters controlling the production of pullulan, an extracellular α-glucan elaborated by the polymorphic deuteromycete *Aureobasidium pullulans* that has been reported to possess properties suitable for food packaging material (Yuen 1974).

Parameters effecting polysaccharide production have been reviewed (Catley 1979) and both the presence of NH_4^+ and extracellular pH are discussed as important influences. More recently Seviour and Kristiansen (1983) have examined pullulan production in fermentations of 6 l and come to the same general conclusion, namely that depletion of NH_4^+ is the signal for commencement of pullulan elaboration. There are, however, no explanations as to the rationale of suppression or activation of polysaccharide synthesis. This communication presents results which contribute to understanding the mechanisms that control the appearance of extracellular pullulan in submerged cultures.

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Materials and methods

Aureobasidium pullulans strain ATCC9348 was grown in shake culture medium of the following composition: glucose, 250 mM; KCl, 5.0 mM; $MgCl_2$, 4.0 mM; Na_2SO_4, 4.0 mM; Na_2HPO_4, and NaH_2PO_4, 20 mM; NH_4Cl, 20 mM; $FeCl_3$, 2.0 μM; $MnCl_2$, 2.0 μM; $ZnCl_2$, 2.0 μM; $CaCl_2$, 50 μM; $CuSO_4$, 0.2 μM; and yeast nitrogen base (Difco) 0.65% (w/v). Continuous culture was in a 3 l fermentor at a working volume of 1.5 l. Growth temperature was at 27° C and the pH was held at 5.5 by automatic titration with 1 M NaOH. Air flow was at 0.5 l min^{-1}. The medium was the same as in shake culture but with no yeast nitrogen base and NH_4Cl as a growth limiting component at 4 mM. Foaming was controlled by polypropylene glycol 2000 at a final dilution of 1 to 10^4.

Trehalose was measured using trehalase (Catley and Kelly 1976) and glycogen by extraction with trichloroacetic acid followed by digestion with amyloglucosidase. In both cases glucose was estimated using glucose oxidase (Lloyd and Whelan 1969). Pullulan was estimated using pullulanase as described by Catley (1971). NH_4^+ was assayed with an ammonium ion probe. Cell dry-weights were obtained by filtration through cellulose acetate filters (0.8 μm pore size) and dried over P_2O_5 at 45° C for 18 h. Yeast cell counts were measured using a haemocytometer.

For studying the effect of NH_4^+ and proton concentration, cells from shake cultures were harvested after 36 h of growth (mid-exponential growth phase), washed, and the hyphal form removed by filtration through nylon mesh of 45 μm square porosity. Cellular uptake of glucose or pullulan elaboration were both estimated by ^{14}C-isotope counting as described previously (Catley 1980). The shake medium used for environmental studies conducted at 27° C, was that described above except that glucose was at an initial concentration of 50 mM, yeast extract and trace metals were omitted and

NH_4^+ was present, when required, at 50 mM concentration. MES, (2-[N-morpholino] ethanesulphonic acid) (50 mM) was used to maintain the pH of cultures in the pH 7—8 region. It was established that this buffer did not alter glucose assimilation or pullulan elaboration by the cells. Alteration of pH was by addition of 2 M NaOH or HCl. The absence of NH_4^+ in the studies of proton concentration effects meant that there was little or no acidification of the medium by the cells.

Results

Pullulan production in continuous culture

Changes in dry-cell weight, NH_4^+ and pullulan concentrations produced by variation in dilution rate are seen in Fig. 1. Preliminary experiments had established that feed-medium containing glucose (250 mM) and NH_4^+ (4 mM) produced growth conditions limiting in nitrogen. Glucose concentration never fell below 190 mM. Both yeast and hyphal forms were observed, the yeast-cell number varying between 1 and 4.5×10^7 ml^{-1}. Trehalose, as a percentage of dry-cell weight, rose to 20% at a dilution rate of 0.15 h^{-1} but fell to less than 5% below dilution rates of 0.1 or greater than 0.25 h^{-1}. Small amounts of glycogen were observed with a maximum of 2% of dry-cell weight at a dilution rate of 0.1 h^{-1}. The pH was held at 5.5 throughout. As can be seen in Fig. 1 the production of pullulan commences with the exhaustion of NH_4^+.

Pullulan production in shake culture — Effect of NH_4^+

The effect on glucose assimilation and pullulan accumulation by yeast and hyphal forms in the presence and absence of 50 mM NH_4^+ is shown in Figs. 2 and 3, respectively. Cellular growth is monitored in both cases by the uptake of ^{14}C-isotope into the cell-mass. In the case of yeasts, this was augmented by cell number counts showing a rise from 4.5×10^7 to 8.0×10^7, and 6×10^7 cells ml^{-1} over 6 h in the presence and absence of NH_4^+ respectively. In the absence of NH_4^+ the pH remained constant at 5.0, whereas in the presence of NH_4^+ and consequent production of acidity the medium required re-adjusting to pH 5.0 every 30 min. No morphological changes were seen and there was no difference in the accumulation rate of pullulan in the presence or absence of NH_4^+ over the 6 h period, but differences in glucose assimilation by the cells are obvious.

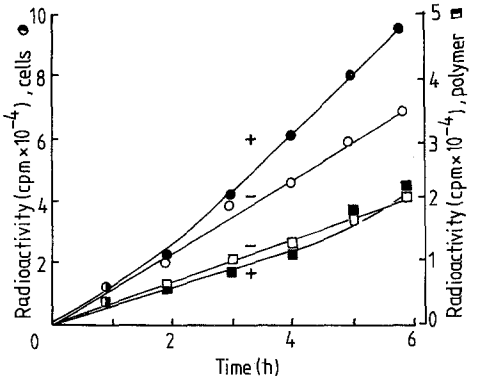

Fig. 2. Shake culture of *A. pullulans* (yeast form) utilizing ^{14}C-glucose (50 mM; 3.7 KBq ml^{-1}) in the absence (−), or presence (+) of NH_4Cl (50 mM). Cellular ^{14}C-isotope incorporation (●, ○); pullulan accumulation (■, □)

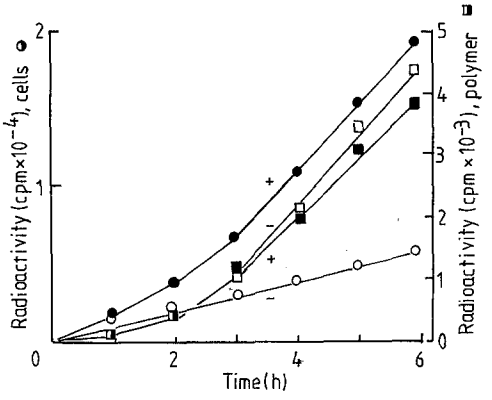

Fig. 3. Shake culture of *A. pullulans* (mycelial form) utilizing ^{14}C-glucose (50 mM; 3.7 KBq ml^{-1}) in the absence (−), or presence (+) of NH_4Cl (50 mM). For symbols, see Fig. 2

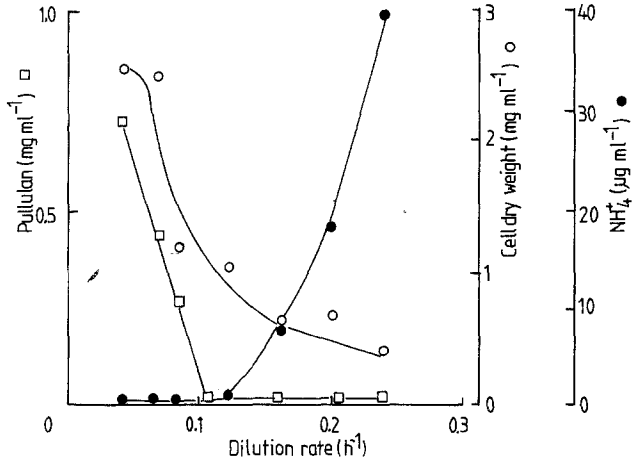

Fig. 1. Continuous culture of *A. pullulans*. See text for conditions

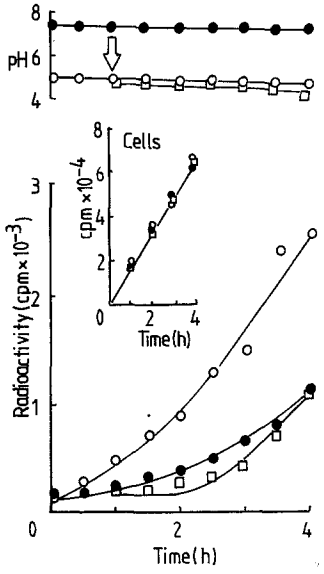

Fig. 4. Accumulation of pullulan and cellular ^{14}C-isotope (*inset*) at pH 7.5 (●); 5.0 (○); and when shifted from pH 7.5 to 5.0 after 1 h (□)

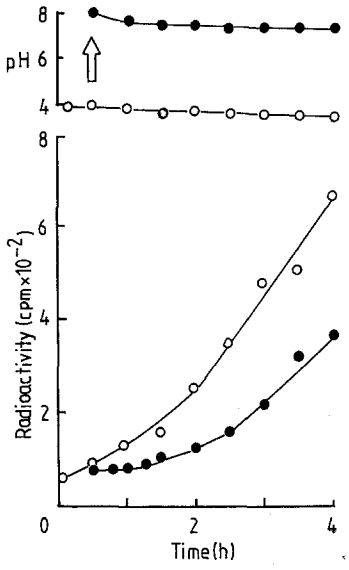

Fig. 5. Accumulation of pullulan at pH 4.0 (○) and when shifted from pH 4.0 to 8.0 after 1 h (●)

Pullulan production in shake culture — Effect of pH

The effect of pH on pullulan accumulation is shown in Figs. 4 and 5 and is derived from yeast cells shaken in the absence of NH_4^+ but similar results were obtained when NH_4^+ (50 mM) was present. The selected pH was maintained throughout the 4 h period and there appeared to be no change in the growth rates of the cells (Fig. 4) as measured by cellular ^{14}C-isotope accumulation. The previously reported (Catley 1971) effect of pH on pullulan elaboration was observed, namely that at alkaline pH the elaboration rate was less than at the more acid pH. What is seen in the present work is that in adjusting the medium from an acid to an alkaline pH there is an immediate cessation of polysaccharide elaboration followed by a recovery of production (Fig. 5). The change from alkaline to acid medium (Fig. 4) does not produce an immediate elaboration of polysaccharide, but a recovery period, again after approximately 1 h, is seen.

Discussion

The effect of NH_4^+

The continuous fermentation data presented in Fig. 1 support the notion that pullulan is a secondary metabolite produced at a point where NH_4^+ is of negligible concentration and glucose is in excess. Reports on the effect of NH_4^+ on catabolic pathways in yeast agree on the influence but differ in the proposed mechanism. Rothman and Cabib (1969) suggested that in *Saccharomyces cerevisiae* NH_4^+ stimulates glycolysis through its activating effect on phosphofructokinase and that loss of stimulation upon NH_4^+ removal allows glucose to be diverted to glycogen. On the other hand Saita and Slaughter (1984) propose that NH_4^+ stimulation of fermentation is by virtue of its acting as a substrate for protein synthesis. A combination of both events is proposed by Lloyd et al. (1983). A third effect, distinct from direct action as an effector or general source of nitrogen, has been proposed by Dickinson and Dawes (1983).

That pullulan elaboration in liquid culture is influenced by NH_4^+ has been clearly demonstrated (Catley 1971; Seviour and Kristiansen 1983). In order to gain some insight into the rationale of NH_4^+ suppression it was decided to look at the cells response over 6 h. The results are presented in Fig. 2 for yeast cells and in Fig. 3 for hyphal cells. In both cases there is no difference in the accumulation rate but since both cellular assimilation of glucose and cell number increase more in the presence of NH_4^+ the elaboration rate, defined as polymer produced per unit cell mass, is clearly reduced under these conditions.

In separate experiments on yeast cells it was shown that after 24 h the accumulation rate of pullulan was maintained at the same initial rate in the absence, but had ceased in the presence, of NH_4^+. From this it may be concluded that for the first 6 h the production of pullulan by cells already producing polysaccharide is not inhibited but that new cells produced during that period do not have the ability to elaborate polysaccharide. Thus the role of NH_4^+ acting as an effector on a metabolic pathway can be discounted. Cycloheximide at concentrations of 3.6 μM had no effect on elaboration rate over the first 2 h but synthesis ceased by 3 h as did the ability of the cell to utilise glucose.

The effect of pH

Influence of environmental pH on the accumulation of pullulan was first reported by Catley (1971) and then by Ono et al. (1977). It was subsequently shown (Catley 1980) that the yeast, and not the mycelial, form is the predominant producer of pullulan. Thus a distinction must be made between the effect, if any, of environmental pH on the morphological balance and the ability of the yeast cell to produce pullulan. That increasing acidity influences morphology has been shown by Bermejo et al. (1981) and by Heald and Kristiansen (1985) who showed that the more acidic the environment the less the proportion of yeast cells were produced with a concomittant drop in the accumulation of pullulan.

However, it remained unclear as to whether the previously observed effect of reduced acidity (Catley 1971) was only of short term influence or lasted longer over the typical fermentation periods of 2—3 days. Yeast cells were now maintained at a fixed pH, or switched to another, in the presence of excess glucose and absence of NH_4^+. The ability of cells to produce pullulan at pH 7.5 or 5.0 is seen in Fig. 4. For cells maintained at these pH values the initial elaboration rate is quite different, but after 1 h the accumulation of pullulan in the more alkaline milieu begins to approach that of the acidic culture. Cells rapidly adjusted after the first hour from an environmental pH of 7.5 to 5.0 do not show an immediate ability to elaborate pullulan but again exhibit a lag-period. In contrast cells elaborating pullulan at pH 4.0 that are shifted to pH 8.0 show an immediate cessation of polysaccharide synthesis (Fig. 5) followed once more by a period of about 1 h before the accumulation approaches the previous rate. The accumulation rate of ^{14}C-radioisotope by the cells is not affected by pH (Fig. 4) from which it is presumed that acidity in the range of pH 7.5 to 5.0 does not effect the cells ability to assimilate glucose.

Thus, although there may be a short term (1—2 h) effect of pH there is probably little or no effect of milieu acidity in the longer times of fermentation runs (48—72 h). This is of significance in determining conditions for promoting the yield of pullulan for it now seems likely that the effect of acidity is in altering the morphological balance of *A. pullulans* (Heald and Kristiansen 1985) rather than control of elaboration of those cells producing it.

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