

Role of pH and Nitrogen Limitation in the Elaboration of the Extracellular Polysaccharide Pullulan by *Pullularia pullulans*

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During the growth of the yeastlike fungus *Pullularia pullulans* on glucose as sole carbon source, the cell-mass does not increase concomitantly with the elaboration of the extracellular polysaccharide pullulan, but precedes it. The conditions generated in the culture medium which activate the secretion of polysaccharide have been sought for, and, in particular, the roles of extracellular pH and nitrogen limitation are examined.

The structure of pullulan, an extracellular α -glucan elaborated by the yeastlike fungus *Pullularia pullulans*, has been shown to be predominantly a polymaltotriose linked through α -1,6-bonds on the terminal glucose residues of the trisaccharide (11). During an investigation into the utilization of carbon sources by *P. pullulans* and the ability of the organism to elaborate extracellular polysaccharides therefrom, it was observed that pullulan did not appear concomitantly with increase of cell mass but began at some point in the middle phase of growth. It is the purpose of this communication to describe two parameters of culture medium that might control pullulan elaboration.

MATERIALS AND METHODS

Quartermaster strain no. 3092 of *P. pullulans* was maintained at 4 C on agar slopes containing the nutrients described below and 2.5% sucrose as sole carbon source. The medium used is that of Ueda et al. (10), i.e., 2.5% glucose (140 mM), 0.5% K_2HPO_4 (29 mM), 0.1% NaCl (17 mM), 0.02% $MgSO_4 \cdot 7H_2O$ (0.8 mM), 0.06% $(NH_4)_2SO_4$ (4.5 mM), and 0.04% yeast extract (Difco).

For the study of glucose uptake, cells grown in a medium initially containing 2.5% glucose as sole carbon source were harvested and thoroughly washed with sterile water, and portions were resuspended in sterile medium containing 0.25% glucose (14 mM), 0.008 μ Ci of uniformly labeled ^{14}C -glucose (New England Nuclear Corp.) per ml, and salts at the concentration of the shake culture, but no yeast extract. HCl (1 N) was added to uptake media where a more acid pH was required, and an equivalent amount of 1 M KCl was added to the neutral media. Media were agitated on a gyratory shaker at 200 rev/min at 25 to 27 C. Samples were centrifuged, and portions of the

supernatant fluid were spotted on 1-cm squares of filter paper, extracted with 66% aqueous ethanol, dried, and counted by standard procedures of liquid scintillation spectrometry. This provided a measure of labeled extracellular polymer content (9). Cell weights were obtained by collecting and washing the pellet on tared 0.8- μ m membrane filters (Millipore Corp., Bedford, Mass.). Counting these papers by scintillation spectrometry indicated the extent of ^{14}C incorporation into the cell. The pullulan content of extracellular polymer was determined by the addition of unlabeled pullulan to supernatant fluid samples, followed by ethanol to a final concentration of 66%. The washed precipitate was digested with pullulanase, an enzyme specific for the hydrolysis of α -1,6-glucosidic bonds (3, 1), and the products were separated by descending paper chromatography irrigated with ethyl acetate-pyridine-water (10:4:3, v/v). The developed chromatogram was cut into strips which were immersed in scintillation fluid and counted. The action of pullulanase on pullulan is to effect complete depolymerization to maltotriose (3) and minor traces of tetrasaccharides (6). The ratio of counts migrating with tri- and tetrasaccharides to those at the origin provides a measure of pullulan content of polymer.

Glucose was measured by the glucose oxidase reagent (7), and extracellular polysaccharide estimations (Fig. 1) were determined by an alcohol precipitation technique described elsewhere (5).

RESULTS AND DISCUSSION

The appearance of extracellular pullulan is not concomitant with the increase in cell mass of growing *P. pullulans* cultures (Fig. 1), and for an understanding of the processes whereby this glucan is synthesized it is necessary to inquire into the environmental conditions controlling the rate of its elaboration.

An examination of parameters presented in

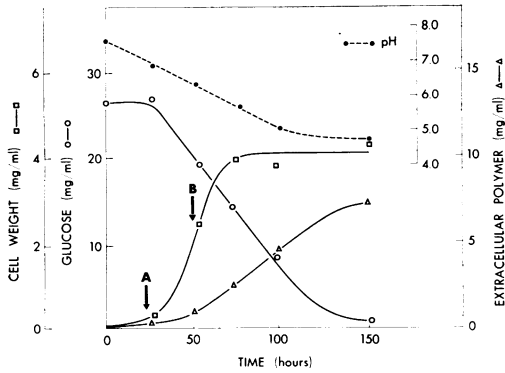


FIG. 1. Growth curve of *P. pullulans* in a medium containing 2.5% glucose (140 mM) as sole carbon source. Cultures were shaken on a gyratory shaker at 200 rev/min at 25 to 27 C. Cells were harvested in the early (A) and middle (B) phases of growth. Symbols: □, dry cell weight (milligrams/milliliter); ○, extracellular glucose (milligrams/milliliter); △, extracellular polysaccharide (milligrams/milliliter); ●, extracellular pH.

Fig. 1 shows a progression to an acidic environment during the course of culture and a cessation of cell growth before complete utilization of glucose. It might be presumed, then, that utilization of the ammonium ion as nitrogen source produced an expulsion of protons from the cell, causing the medium to become acid, and that cell growth ceased because of nitrogen limitation. This latter supposition was later confirmed (Fig. 4). It is the effect of extracellular pH and nitrogen limitation on pullulan production which is examined in this communication.

Medium, inoculated with 1% of its volume by a 48-hr-old culture, was harvested in the middle phase of its growth (Fig. 1, point B), and the ability of the cells to consume glucose and elaborate extracellular polymer was examined in two regions of pH (Fig. 2), corresponding to acidity conditions prior to and during the production of pullulan (Fig. 1). Analyses of these experiments together with the pullulan content of extracellular polysaccharide are described in Table 1. The consumption rate of glucose for cultures at this phase of growth (calculated from the data in Fig. 1) is 140 μ g per hr per mg (dry weight) of cell and is seen to be in good agreement with data in this table. It is apparent that the increased uptake of glucose at the more acid pH is diverted to the synthesis of extracellular polysaccharide, the composition of which is predominantly pullulan. This observation has been paralleled elsewhere when it was shown that extracellular polysaccharide elaborated from a culture growing on 2.5% fructose, as sole carbon source, is 29% pullulan, but that elaborated from fructose in cultures growing on 2.5% glucose and 2.5% fructose as sole carbon sources is 80%. In the latter system, glucose is preferentially metabolized with the consequence that fructose is utilized in a later stage of culture which has become acidic. The elaboration rate of other extracellular polysaccharide material appears to be independent of pH in the ranges examined. This other product may be separated from pullulan by depolymerization of the latter to tri- and tetrasaccharides with pullulanase (6) and separation of the undegraded polymer from these oligosaccharides by gel frac-

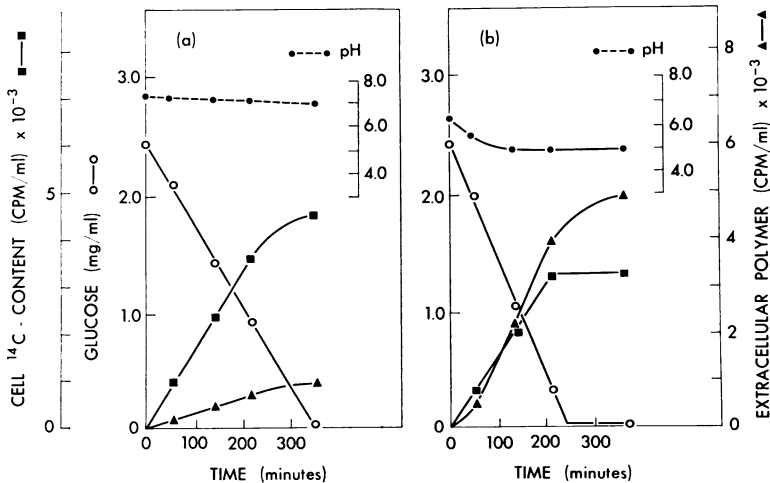


FIG. 2. Utilization of glucose by cells harvested in the middle phase of growth (48 hr, Fig. 1, point B). Extracellular pH ranges: (a) 7.2 to 7.0; (b) 6.1 to 5.0. Initial dry cell weights were 3.9 mg/ml. Symbols: ■, cell ¹⁴C content (counts per minute of cells/ml of culture); ○, extracellular glucose (milligrams per milliliter); ▲, extracellular polymer (counts per minute per milliliter); ●, extracellular pH.

TABLE 1. Utilization of glucose by *P. pullulans* isolated in the early and middle phases of growth

pH range during uptake	Consumption of extracellular glucose (μg per mg of cell ^a per hr)	Glucose carbon incorporated into cell ^b (μg per mg of cell ^a per hr)	Glucose incorporated into extracellular polymer ^b (μg per mg of cell ^a per hr)	Per cent pullulan in extracellular polymer
Early phase				
7.3-7.0	330	188	9	
5.8-3.5	375	202	4	
Middle phase				
7.2-7.0	120	47	10	36
6.1-5.0	151	44	53	89

^a Based on initial dry weight of cells.

^b Based on specific activity of glucose.

tiation on Sephadex G-200. Acid hydrolysis of the polymer followed by paper chromatography indicates half of the hydrolytic products travelling with the mobility of uronic acids and the remainder with that of glucose. This suggests that it is the acidic fraction of extracellular polysaccharide described by Bouveng et al. (4).

The consumption of glucose and elaboration of polymer by cells are identical at concentrations 10 times that depicted in Fig. 2, and it is presumed that translation of glucose into the cell is mediated by a transport mechanism. This rate, at least over the period of the uptake experiments, is unaffected by a fourfold increase in the concentration of ammonium sulfate. Addition of 2-deoxyglucose to a concentration of 40 mM in

uptake experiments containing 140 mM glucose reduces the rate of glucose assimilation by 40%, but completely abolishes extracellular polymer elaboration. The rationale for these observations and the increased uptake of glucose at acid pH is being examined in terms of transport phenomena.

In an attempt to delay the increase in acidity of a growing culture, the initial phosphate concentration was increased from 29 to 116 mM. An adequate rate of growth with a final density of 2.4 mg (dry cell weight)/ml was observed. However, the extracellular pH did not drop below 7.4, and only 18% of the extracellular polymer observed in Fig. 1 was elaborated. Analysis of cells grown under these conditions and harvested in the middle phase of growth revealed a full capacity for the production of pullulan at an acid pH. Thus, although the potential for pullulan production is generated during growth, a high extracellular pH prevents its expression.

Cells growing in the initial stages of culture (Fig. 1, point A) were harvested and their ability to assimilate glucose was examined. The results (Fig. 3 and Table 1) indicate a different utilization pattern of the carbon source from that observed in the middle phase of culture. Little or no polysaccharide was elaborated at either pH 7.3 to 7.0 or 5.8 to 3.5, and the growth rate, judged by the rate of ¹⁴C incorporation into the cells, was some 4.5 times faster. It is apparent, then, that although extracellular pH plays a role in the expression of pullulan elaboration when the culture is in the later stages of growth, it has no effect when it is in the earlier.

A culture parameter that may stimulate or retard pullulan elaboration is the availability of a

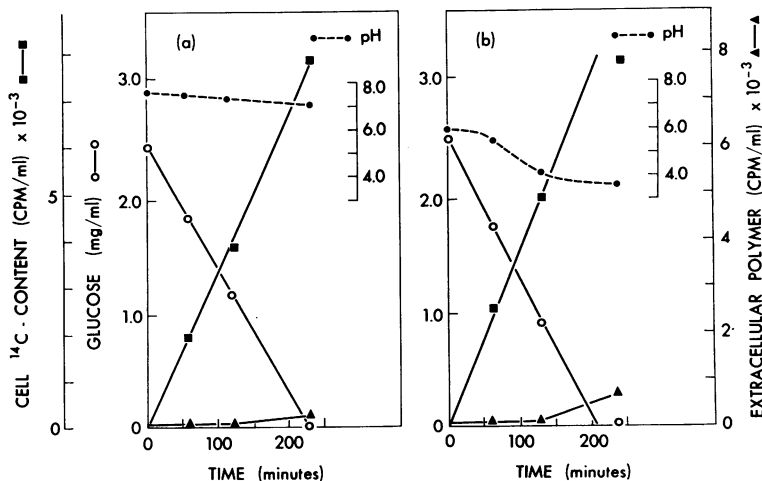


FIG. 3. Utilization of glucose by cells harvested in the early phase of growth (24 hr, Fig. 1, point A). Extracellular pH ranges: (a) 7.3 to 7.0; (b) 5.8 to 3.5. Initial dry cell weights were 2.1 mg/ml. For key, see Fig. 2.

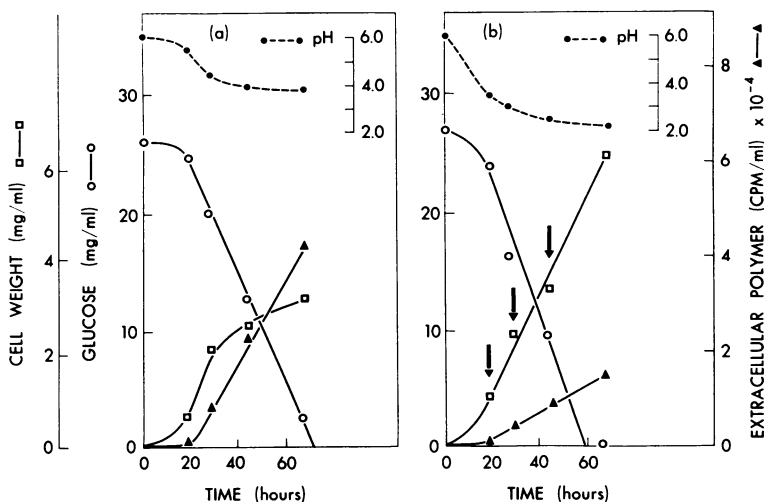


FIG. 4. Growth of *P. pullulans* in normal medium but containing (a) 2.2 mM ammonium sulfate; (b) initially 4.5 mM ammonium sulfate, followed by additions of this nitrogen source ↓ at 18, 27, and 43 hr increasing the molarity to equivalent initial concentrations of 9.0, 13.5, and 18.0 mM, respectively. The medium contained 0.04 μCi of ^{14}C -glucose per ml. Symbols: □, dry cell weight (milligrams/milliliter); ○, extracellular glucose (milligrams/milliliter); ▲, extracellular polymer (counts per minute per milliliter); ●, extracellular pH.

TABLE 2. Effect of nitrogen levels on glucose utilization and pullulan production by *P. pullulans*^a

Amt (NH ₄) ₂ SO ₄ added (mmoles/50 ml of culture)	Per cent initial glucose diverted to extracellular polymer	Per cent pullulan in extracellular polymer	Specific activity of cells (counts per min per mg)
0.11	54	89	1,730
0.9	17	87	1,620

^a Analysis after 67 hr of growth.

nitrogen source, and so the effect of ammonium ion concentration on carbon utilization patterns was studied. Shaken cultures (50 ml) containing one half or the normal level of ammonium sulfate were adjusted to pH 5.9 to 6.0 so that pullulan elaboration could be immediately expressed, inoculated with 0.5 ml of a 3-day-old shake culture, and analyzed over a period of 67 hr. A 0.5-ml amount of 0.45 M sterile ammonium sulfate solution was added aseptically at 18, 27, and 43 hr to the culture containing the normal level of ammonium ion (4.5 M), thus raising the total amount of nitrogen source added to four times the initial level. The analyses (Fig. 4 and Table 2) indicate that for similar rates of carbon utilization the diversion of glucose from incorporation into cellular material to the elaboration of polysaccharide is dependent on ammonium ion concentration. The analysis also indicates that the initial growth of cells at a pH which allows the

full expression of pullulan production does not immediately produce organisms elaborating polysaccharide.

Pullulan elaboration then, providing the extracellular pH is sufficiently acidic, appears to be dictated by the availability of nitrogen source, but there may be other culture parameters not examined as yet, e.g., oxygen tension, which contribute to the change in glucose utilization patterns of the cell. It is seen that cells actively producing pullulan in the middle phase of a culture depleted in nitrogen source continue to do so when resuspended in a medium containing excess nitrogen but then readjust their metabolism during growth in that medium with a cessation of polysaccharide elaboration. It is seen then that the rate of pullulan elaboration is under at least two forms of control. The response to extracellular pH is a rapid one and has been shown in unreported experiments to occur within at least 15 min of lowering the pH. It is most probably related to glucose transport phenomena. A much slower response is effected by the level of ammonium ion in the culture medium and demonstrates a nutritional control of polysaccharide elaboration. Moreover, it is seen (Fig. 4) that, although the rate of pullulan production is markedly different at different nitrogen levels, the apparent time of commencement of elaboration is the same.

Microscopic observation of *P. pullulans* cells at different phases of culture show a change from the hyphal form in the early stages of

growth to a unicellular yeast form in later culture. Though difficult to quantitate, the change appears to coincide with the onset of pullulan production and can be similarly inhibited or stimulated by levels of ammonium ion. It would thus appear that *P. pullulans* exhibits a form of nutritional dimorphism (8). Microscopic examination of both of these cell forms in India ink reveals that a minor portion of both forms is capsulated, an observation made by Bender et al. (2), but whether this capsule is related to pullulan is not known.

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