Utilization of Carbon Sources by *Pullularia pullulans* for the Elaboration of Extracellular Polysaccharides

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The yeastlike fungus *Pullularia pullulans* utilizes simple mono- and disaccharides both in the production of cell mass and the elaboration of extracellular polysaccharide. The utilization pattern of these sugars and the effect obtained by varying the *p*H of the medium are studied, and the ability of the organism to utilize and elaborate extracellular polysaccharides from noncarbohydrate sources is explored.

The production of extracellular polysaccharides by the yeastlike fungus *Pullularia pullulans* from a variety of glycosidic carbon sources has been widely reported (6–9, 32, 34), and in some instances (6, 9, 34) the elaboration of pullulan has been specifically described. Pullulan is an extracellular neutral glucan, the structure of which has been determined by Bender and Wallenfels (6) and by Wallenfels et al. (34) as consisting of α-maltotriose units polymerized in linear fashion through $1 \rightarrow 6$ linkages on the terminal glucose residues of the trisaccharide. Minor tetrasaccharide components occupying both internal and external positions have been reported (13, 14, 34), and in one case evidence for a small proportion of $1 \rightarrow 3$ linkages has been established (28).

This report describes the utilization by *P. pullulans* of sucrose, maltose, glucose, fructose, and the nonglycosidic substrates, glycerol and acetate, for cell growth and production of pullulan. The effect of extracellular *p*H on polysaccharide synthesis is discussed, and the circumstances leading to the production of pullulan are considered.

MATERIALS AND METHODS

Quartermaster strain no. 3092 of *P. pullulans* was kindly supplied by Elwyn T. Reese of the U.S. Army Natick Laboratories, Natick, Mass. The medium used is that of Ueda et al. (32), i.e., 0.5% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.06% $(NH_4)_2SO_4$, and 0.04% yeast extract (Difco), together with the appropriate carbon source. Where the starting *p*H of the medium was required to be other than normal, i.e., *p*H 7.5, an equivalent amount of phosphoric acid replaced the potassium phosphate and was titrated to the desired *p*H before sterilization. Cultures were maintained at 4 C on an agar slope containing the above nutrients together with 2.5% sucrose. Suspensions of cells from these slopes were transferred to a medium containing 2.5% glucose as carbon source

and grown for 3 to 4 days at room temperature (25 to 27 C). Transfers of 0.5 ml of this culture into 50 ml of starter culture containing the appropriate carbon source were then made, and these were grown for 3 to 4 days. These cultures (0.5 ml) were now used as innocula for 50-ml cultures to be subjected to analysis. In the appropriate experiments (for details see figures), these cultures were supplemented with approximately 2 μCi of ^{14}C of the carbon source under consideration, i.e., uniformly labeled sucrose, glucose, fructose, glycerol, or acetate obtained from New England Nuclear Corp. or maltose obtained from Calatomic. Cultures were grown on a gyratory shaker at 200 rev/min in flasks fitted with septa-covered side arms, and samples were withdrawn by needle and syringe.

Cell weights were obtained by centrifugation of culture samples at 17,000 × *g* for 10 min, followed by three washes with distilled water and collection of the cells on 0.8-μm membrane filters (Millipore Corp., Bedford, Mass,) which were then dried for 18 hr at 50 C over phosphorus pentoxide in vacuo. Counting this filter paper by standard procedures of liquid scintillation spectrometry provided a measure of ^{14}C incorporation into the cells. The scintillation medium for all ^{14}C assays was 0.5% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(5,phenyloxazolyl)-benzene in toluene.

Elaboration of polysaccharide during fungal growth was monitored by two methods. A relatively unspecific technique utilized the method of Thomas et al. (30). A 100-μliter sample of culture supernatant fluid was spotted on a 1-cm² piece of filter paper (Whatman E. T.), extracted with 66% ethanol, and dried. The paper was then counted by liquid scintillation spectrometry and provided a measure of labeled macromolecule in the supernatant fluid. For a more thorough analysis of extracellular polysaccharide and to demonstrate specifically the presence of pullulan, the culture supernatant fluid was made 66% with respect to ethanol, and the precipitated material was washed three times with 66% ethanol. The washed precipitate was dissolved in water at 100 C, and samples of the solution were assayed as follows. The total carbohydrate was determined either by the phenol-

sulfuric acid procedure (18) with glucose as standard or by acid hydrolysis (25) and estimation of the liberated sugars by Nelson's method (23) or estimation of glucose by the glucose oxidase reagent (21). Pullulanolysis was carried out at pH 5.0 and 37 C as by Abdullah et al. (1) until no further increase in reducing power (23) was observed. The products of pullulanolysis were fractionated by descending paper chromatography (Whatman no. 1), irrigating with ethyl acetate-pyridine-water (10:4:3, v/v), and the sugar zones were located by the silver nitrate-sodium hydroxide dip (31). For the assay of ¹⁴C-labeled products, chromatography in the same system was performed by using Whatman 3MM paper. Material was applied to a 10-cm base line, and the developed chromatogram was cut into strips (1 by 10 cm) which were immersed in scintillation fluid and counted by standard procedures of scintillation spectrometry.

Fructose was measured by the method of Dische (17). Sucrose was determined by the incubation of samples with yeast invertase at pH 5.0 and 37 C with measurement of released glucose by the glucose oxidase reagent (21). Maltose was determined by the incubation of samples with amyloglucosidase from *Aspergillus niger* (2) in 60 mM citrate phosphate buffer (pH 5.0) at 37 C with measurement of released glucose by the glucose oxidase method (21). The amyloglucosidase was adjusted to a final concentration of 0.02 international units per ml based upon the hydrolysis of maltose. These conditions were sufficient to cleave maltose but were shown to have a negligible effect on an equal amount of pullulan. Uronic acids were measured by the carbazole method of Dische (16) and phosphate, by the method of Sumner (29).

Alkali-soluble material was extracted from washed lyophilized cells with 30% potassium hydroxide at 100 C for 30 min. The digest was diluted with 2 volumes water and centrifuged; 6 volumes of ethanol were added to the supernatant fluid to precipitate extracted material. For alcohol extraction, three extractions of washed cells were made with 95% ethanol for 20 min at 60 C. The combined extracts were evaporated to dryness at 40 C and dissolved in water.

RESULTS AND DISCUSSION

An examination of the literature describing pullulan (6, 9, 28, 32, 34) suggests that different strains of *P. pullulans* do not elaborate polysaccharides of a common structure. The investigations reported here define pullulan as a glucan insoluble in 33% aqueous ethanol, which is depolymerized by the α-1→6-glucosidic bond endohydrolase, pullulanase (1), to yield a single major product of maltotriose.

Carbon source utilization The growth of *P. pullulans*, production of extracellular polymeric material, culture pH, and utilization of carbon substrate of a shake culture grown on a medium containing 2.5% glucose as sole carbon source are described in Fig. 1a. After about 100 hr, the culture gradually darkens and eventually becomes black through the elaboration of melanin pig-

ments (19). This pigmentation also appeared in cultures grown on sucrose, maltose, and fructose, but was absent in those grown on glycerol or acetate.

Polymeric material isolated at 100 hr of growth from the supernatant fluid of glucose-grown cultures was dried to constant weight and subjected to acid hydrolysis and depolymerization by pullulanase. The results, together with similar analyses of polysaccharides isolated at 100 hr from cultures grown on maltose, sucrose, and fructose, are listed in Table 1. Correspondence between glucose assayed by glucose oxidase and glucose equivalents estimated by Nelson's method indicated the polymer to be essentially polyglucose in constitution, a fact confirmed by paper chromatography of these hydrolysates which indicates glucose as the major monosaccharide together with a trace of galactose. Depolymerization with pullulanase followed by
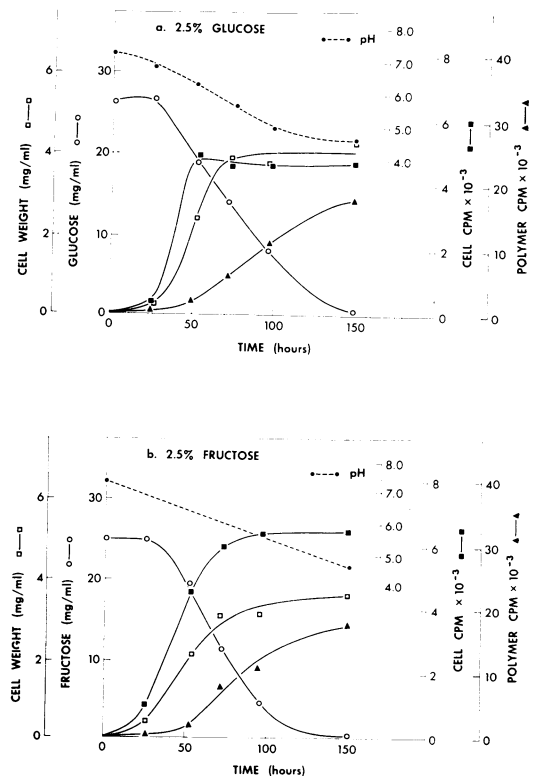

FIG. 1. *Growth of P. pullulans on carbon sources of 2.5% glucose (a), or 2.5% fructose (b). Each contains 0.04 or 0.05 μCi, respectively, of ¹⁴C-labeled substrate per ml. Symbols:* □, *dry cell weight (mg/ml);* ■, *cell ¹⁴C content (counts per minute of cells/milliliter of culture);* ○, *extracellular glucose or fructose (milligrams/milliliter);* ▲, *extracellular polymer (counts per minute per milliliter);* ●, *extracellular pH.*

TABLE 1. *Analysis of polymer precipitated from the supernatant fluid of P. pullulans culture*[a]

Carbon source (5%)	Extra-cellular polymer (mg/ml)	Per cent polysaccharide content of polymer[b]			Per cent pullulan content of polysac-charide[c]	Per cent malto-triose content of poly-mer[d]
		I	II	III		
Sucrose....	14.8	72	71	76	89	76
Maltose...	4.9	53	53	61	46	52
Glucose...	8.8	65	69	66	65	61
Fructose..	6.8	56	57	57	60	29

[a] Grown at 25 to 27 C for 100 hr.

[b] Polysaccharide content estimated by acid hydrolysis and measurement of released sugars by Nelson's method, I, glucose oxidase, II, and phenol-sulfuric acid procedure, III.

[c] Per cent pullulan is based on the glucose content of the polymer and is described in text.

[d] ^{14}C-maltotriose isolated from chromatograms separating the pullulanolysis products of labeled polymers as described in text.

TABLE 2. *Analysis of polymer precipitated from supernatant fluid of P. pullulans cultures*[a]

Time (hr)	Extracellular polymer (mg/ml)	Per cent polysaccharide content[b]	Per cent pullulan content[c]
63	3.2	61	107
88	8.7	70	95
111	11.6	60	97
135	17.4	64	88
160	20.7	65	107

[a] Grown at 25 to 27 C on a 5% sucrose carbon source.

[b] Polysaccharide estimated as glucose content of polymer; see Table 1, footnote b.

[c] See footnote c, Table 1.

paper chromatography of the products revealed maltotriose as the major oligosaccharide product together with traces of tetrasaccharide (13). Similar chromatographic analyses of ^{14}C-labeled polymer that had been subjected to pullulanolysis indicated that as well as tri- and tetrasaccharide products a proportion of the label remained at the origin (Table 1). Uronic acids or phosphate, if present in these polymers, constitute less than 5% of their dry weight.

A quantitative analysis, by pullulanolysis of polysaccharides isolated at 63, 88, 111, 135, and 160 hr from cultures utilizing sucrose, indicated that 88 to 107% of the polyglucose content could be accounted for as polymaltotriose (Table 2).

The growth of *P. pullulans* utilizing 2.5% concentration of fructose containing 0.04 μCi of ^{14}C-fructose per ml is illustrated in Fig. 1b. The

parameters of cell weight, extracellular pH, utilization of carbon substrate, and extracellular polymer production appear to be comparable to the glucose-grown culture of Fig. 1a. In particular, the rate of fructose uptake is essentially the same as glucose. However, the analyses provided in Table 1 show fructose to be less efficient as a source of pullulan.

The characteristics of growth on a 5% sucrose medium are shown in Fig. 2a, and demonstrate the preference of the cell for the utilization of glucose over fructose, the rate of uptake of each sugar in the absence of the other being approximately the same (Fig. 1). The almost complete hydrolysis of sucrose before commencement of the log phase of growth is catalyzed by the elaboration of an induced invertase. This enzyme cannot be detected in cultures grown on 2.5% glucose or mixtures of 2.5% glucose and 2.5%
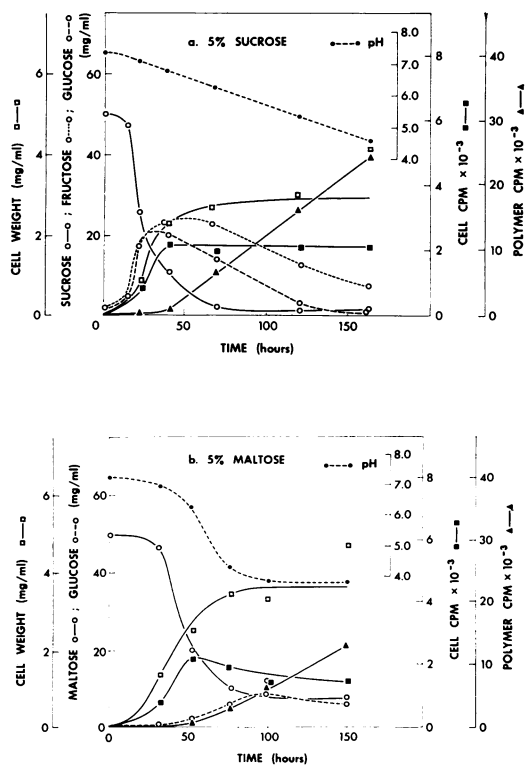

FIG. 2. *Growth of P. pullulans on carbon sources of 5% sucrose (a) or 5% maltose (b). Each contains 0.05 or 0.04 μCi, respectively, of ^{14}C-labeled substrate per ml. Symbols:* □, *dry cell weight (milligrams/milliliter);* ■, *cell ^{14}C content (counts per minute of cells/milliliter of culture);* O—O, *extracellular sucrose or maltose (milligram/milliliter);* O—O, *extracellular glucose (milligrams/milliliter), and* O-----O, *fructose (milligrams/milliliter);* ▲, *extracellular polymer (counts per minute per milliliter);* ●, *extracellular pH.*
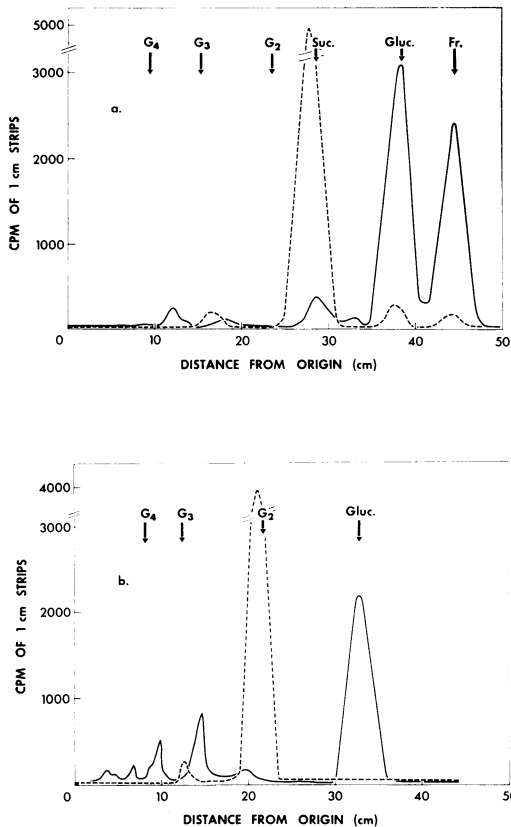
FIG. 3. *Extracellular hydrolase and transferase activities of sucrose-grown cultures acting upon sucrose (a) and maltose-grown cultures acting upon maltose (b). Each figure is a combination of two plots representing the distribution of ^{14}C-substrate [at 0 hr (-----)] and ^{14}C-products [after 24 hr of incubation at 37 C (—)] on a paper chromatogram developed and monitored as described in the text. Positions of fructose, Fr; glucose, Gluc; sucrose, Suc; and the maltodextrins, G_2 (maltose), G_3 etc., are indicated at the top of the figure.*

fructose, a fact that probably reflects catabolite repression that has been demonstrated for invertase in other systems (20). It hydrolyzes raffinose to fructose and galactosylglucose, but has a negligible effect on maltose. A sample of supernatant fluid, isolated from a sucrose-grown culture at 100 hr and adjusted to a sucrose concentration of 1% containing ^{14}C-sucrose, was incubated for 24 hr at 37 C. Portions of this digest sampled at 0 and 24 hr were fractionated on paper (Whatman 3MM). The ^{14}C profile (Fig. 3a) revealed a major pattern of hydrolysis to glucose and fructose with a minor portion of the sucrose (5%) involved in transfer reaction to yield oligosaccharides. The complete absence of

any label at the origin indicated that no polysaccharide synthesis had taken place. Primer, should it have been needed, was present as unlabeled pullulan.

Samples of whole supernatant fluid isolated at 40, 63, 88, 111, 135, and 160 hr from sucrose-grown cultures were fractionated on Sephadex G-200 in 10 mM phosphate buffer (pH 7.0) containing 100 mM sodium chloride. Column effluent, monitored for carbohydrate by the phenol-sulfuric acid assay, revealed that polysaccharide, when present, was completely excluded by the gel. This evidence, together with that cited above, indicates that pullulan cannot be synthesized in a cell-free system but is produced by the organism as a completed macromolecule which may later undergo modification (13).

Cultures provided with equivalent amounts of glucose and fructose exhibit growth characteristics similar to sucrose-grown cultures. With inclusion of either ^{14}C-glucose or fructose, a preferential use of glucose is seen both in the uptake of label by the cell and the production of extracellular polymer (Fig. 4). The appearance of oligosaccharide in the sucrose-grown cultures (Fig. 3a), presumably arising through disproportionation of the substrate, was not observed in cultures grown on a combination of glucose and fructose. Examination of ^{14}C-polymer produced from ^{14}C-fructose in this combination of glucose and fructose indicated that 80% of the label was found in maltotriose after depolymerization with pullulanase. This is to be contrasted with the 29% found from fructose alone (Table 1).
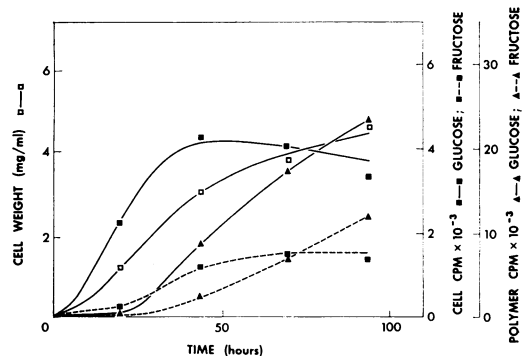
The utilization of a 5% maltose carbon source

FIG. 4. *Combination of data from two cultures, each utilizing both 2.5% glucose and 2.5% fructose, but labeled in only one source with 0.04 µCi of ^{14}C-glucose or fructose per ml. Symbols: □, dry cell weight (milligrams/milliliter); cell ^{14}C content (counts per minute of cells per milliliter of culture) ■—■ from ^{14}C-glucose or ■----■ fructose; extracellular polymer (counts per minute per milliliter) ▲—▲ derived from ^{14}C-glucose or ▲-----▲ fructose.*

is described in Fig. 2b. The characteristics of cell growth are comparable with sucrose, but it is apparent that the rate of glucose utilization by the cells is at least equal to its production from maltose. Glucose is only detected in the culture medium towards the end of the log phase. It is not known from these studies whether maltose itself is transported into the cell prior to hydrolysis. Fuente and Sols (15) have provided evidence that this is so with the metabolism of lactose and maltose by *Saccharomyces cerevisiae*. Measurements of maltase activity in the supernatant fluid of maltose-grown *Pullularia* cells indicates the release of 0.11 mg of glucose per hour per ml of culture at 40 hr. The utilization of substrate by cells growing on glucose is 0.14 mg of glucose per hour per ml of culture at a comparable period of cell growth, and although this is a comparison of data from two separate cultures it would appear that the maltose is capable of providing enough glucose by way of extracellular hydrolysis for the cells' requirements. Though not looked for, there may exist a bound maltase located at the cell surface, a possible precursor of the extracellular enzyme (20), which could augment the hydrolysis of extracellular disaccharide. However, the transport of maltose into the cell prior to metabolism cannot be ruled out.

Examination of the supernatant carbohydrase activity indicates an ability to hydrolyze sucrose and raffinose as well as maltose. No maltase activity is present in cultures grown solely on glucose. The hydrolysis of maltose is accompanied by considerable transferase activity as judged by comparison of the release of glucose assayed by glucose oxidase with the increase in reducing power measured by the Nelson reagent. Figure 3b illustrates the disproportionation of a 1% maltose digest containing ^{14}C-maltose and catalyzed by supernatant fluid enzymes isolated after 100 hr of culture on maltose. In 24 hr, 40% of the original maltose has been transformed to products of higher molecular weight with a chromatographic mobility suggesting the formation of glucosidic bonds other than α-1→4. Correspondence between extracellular enzymes and those associated with the cell is well documented (20). Ueda et al. (33) have reported the property of acetone-dried cells of *P. pullulans* to produce panose and higher molecular weight oligosaccharides from maltose, and it is probable that this cell-bound transferase is related to that reported in this communication. Reference to Fig. 3b shows that no polysaccharide is produced by this cell-free system as evidenced by the absence of ^{14}C label at the chromatogram origin. However, this disproportionation was observed

in a cell-free system. The released glucose in maltose-grown cultures is immediately metabolized, but the extracellular transfer products, detectable at all stages of culture growth by paper chromatography, apparently are not. Thus, in the complete system some extracellular polysaccharide may be synthesized through a shift in equilibrium of the transfer system brought about by the continuous removal of glucose. The analysis of polysaccharide material from maltose-grown cultures (Table 1) indicated that the polymaltotriose content of the polymer is but slightly lower than that obtained from glucose.

Figure 5a describes the growth of *P. pullulans* on 2.5% glycerol as carbon source. Cell growth is adequate but slower, and there is no production of extracellular polysaccharide. Addition of glucose to a concentration of 2.5% restored the
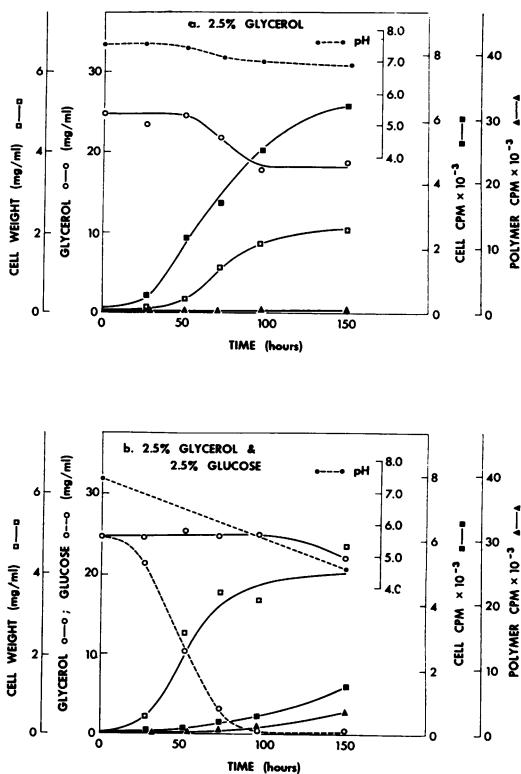

FIG. 5. *Growth of P. pullulans on carbon sources of 2.5% glycerol (a) or 2.5% glycerol and 2.5% glucose (b). Each contains 0.05 μCi of ^{14}C-glycerol per ml. Symbols:* □, *dry cell weight (milligrams/milliliter);* ■, *cell ^{14}C content (counts per minute of cells/milliliter of culture);* ○——○, *extracellular glycerol, estimated from aqueous ethanol-extractable ^{14}C in the supernatant fluid (milligrams/milliliter) and* ○---○, *glucose (milligrams/milliliter);* ▲, *extracellular polymer (counts per minute per milliliter);* ●, *extracellular pH.*
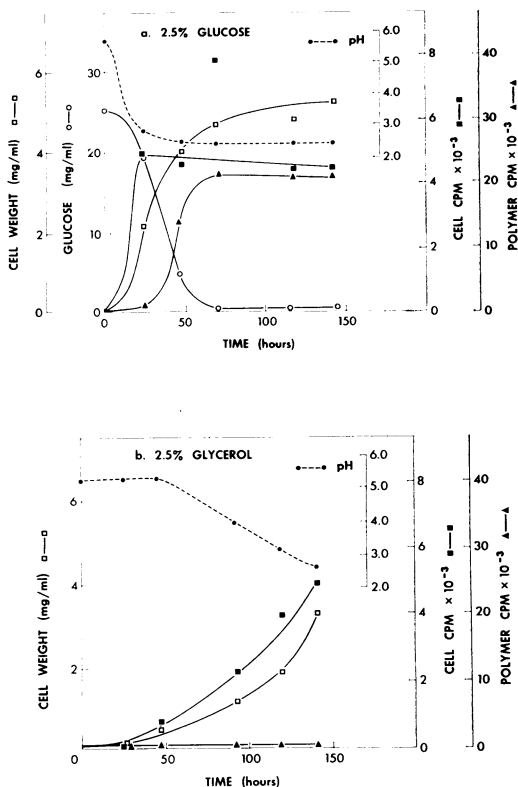
FIG. 6. *Growth of P. pullulans on carbon sources of 2.5% glucose (a) or 2.5% glycerol (b) in acidic media. Each contains 0.04 μCi of ¹⁴C-substrate per ml. Symbols: □, dry cell weight (milligrams/milliliter); ○, extracellular glucose (milligrams/milliliter); ■, cell ¹⁴C content (counts per minute of cells/milliliter of culture); ▲, extracellular polymer content (counts per minute per milliliter); ●, extracellular pH.*

elaboration of polysaccharide, and, by the use of ¹⁴C-glycerol in this two-substrate culture, it was established that 4.3% of the polysaccharide originated from the triol (Fig. 5b). This is to be compared with 25% of glucose that is diverted to extracellular polymer in a culture utilizing 2.5% of that substrate as carbon source. Depolymerization followed by paper chromatography of the products established that 58% of this labeled material was pullulan. The pH profile of the glucose-glycerol culture, unlike growth on glycerol alone which did not fall below 6.9, indicated increasing acidity during the course of cell growth and was comparable to glucose-grown cultures (Fig. 1a). It could be argued that a drop in pH of the growth medium might be the initiator of pullulan elaboration and that a consequence of including glucose in a growth medium containing glycerol is the provision of a

favorable acidic environment for the conversion of glycerol into pullulan. The relationship between extracellular pH and metabolic activity has been reported for polysaccharide synthesis in *S. cerevisiae* (5) and the elaboration of extracellular polysaccharide from *Cryptococcus laurentii* (3). The growth and polysaccharide production of glucose- or glycerol-grown cultures adjusted to an acid pH of 5.7 or 5.4, respectively, are shown in Fig. 6. It is apparent that pH does indeed have an effect on glucose utilization with an increase in rate of 2.5 times in the log phase of growth. This is reflected in an increase in the rate of cell growth and polysaccharide production. However, comparison with Fig. 1a indicates only a 15% stimulation of extracellular material after 150 hr, a quantitative analysis of which shows 90% to be pullulan. In contrast, the increased acidity of the glycerol medium does not induce pullulan elaboration. Maintenance of glucose-grown cultures above pH 8 produces adequate cell growth with a final density of 3 mg/ml, but no extracellular material. Reinoculation of this growth into a glucose medium that is allowed to progress to acidity during growth will again produce pullulan.

The utilization of acetate, provided as 5% sodium acetate trihydrate (i.e. 2.2% acetate), as carbon source appears to be effective for cell growth but not in production of extracellular polysaccharide (Fig. 7). Cultures grown on 2.2% acetate and supplemented with a 2.5% glucose concentration do not elaborate polysaccharide from either source although, as seen in the labeling profiles of Fig. 8, both substrates are
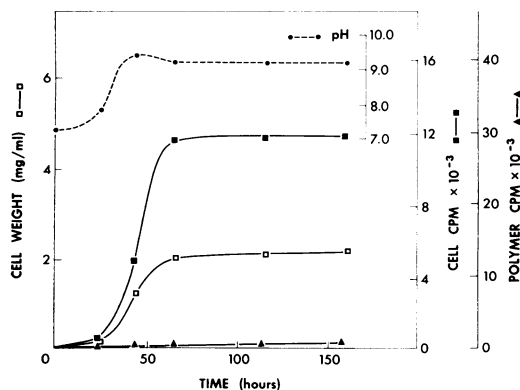

FIG. 7. *Growth of P. pullulans on 5% sodium acetate trihydrate (2.2% acetate) containing 0.04 μCi of ¹⁴C-substrate per ml. Symbols: □, dry cell weight (milligrams/milliliter); ■, cell ¹⁴C content (counts per minute of cells/milliliter of culture); ▲, extracellular polymer content (counts per minute per milliliter); ●, extracellular pH.*
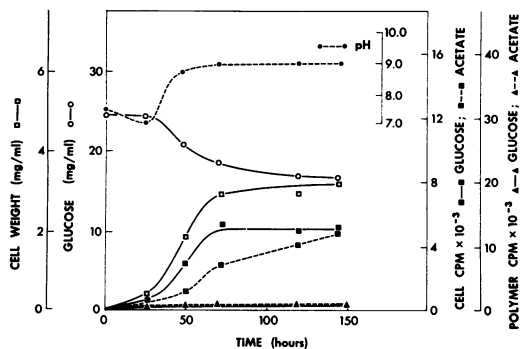
FIG. 8. *Combination of data from two cultures, each utilizing both 2.5% glucose and 5% sodium acetate trihydrate but labeled in only one source with 0.04 μCi of ^{14}C-glucose or acetate per ml. Symbols: □, dry cell weight (milligrams per milliliter); ○, glucose content (milligrams per milliliter); cell ^{14}C content (counts per minute of cells/milliliter of culture)* ■—■ *from ^{14}C-glucose or* ■----■ *acetate; extracellular polymer (counts per minute per milliliter)* ▲—▲ *derived from ^{14}C-glucose or* ▲—▲*-acetate;* ●*, extracellular pH.*

used for cell growth. The pH profile of the acetate-grown culture (Fig. 7) indicates a shift to alkalinity upon commencement of the log phase of culture, a process which is but slightly delayed by the presence of 2.5% glucose (Fig. 8) and which probably accounts for the absence of extracellular polysaccharide elaboration.

Of the carbon sources investigated, whether disaccharide, monosaccharide, glycerol, or acetate, P. pullulans appears capable of their utilization for cell growth. In this investigation, it was decided to maintain the supply of carbon at approximately the same level, e.g., 2.5% glucose (40% C); 2.5% glycerol (39% C); 5% sodium acetate trihydrate (18% C), rather than maintain these substrates at equimolarity. Ethanolic extraction of cells that have been grown on glucose, glycerol, or acetate, followed by paper chromatographic separation of the extracted sugars revealed trehalose to be present in all three cells. It is apparent, then, that the reserve disaccharide of P. pullulans (22) is readily synthesized from 6-3- and 2-carbon substrates. However, the production of extracellular pullulan is not so facile, and it is of interest to attempt a definition of the conditions under which it is elaborated.

Conditions for polysaccharide elaboration. Reference to growth characteristics of P. pullulans on glucose (Fig. 1a) and fructose (Fig. 1b) indicates that pullulan elaboration appears to commence only when the cells are in the late log phase and does not stop when cell growth has reached the stationary phase. ^{14}C-glucose added

to cells in the late log phase is immediately observed in elaborated polysaccharide. It is supposed, then, that glucose transported into the cell is not diverted to an extracellular destination until the basic growth requirements of the cell are met. Pullulan is not produced by cells growing in media containing a 0.5% glucose carbon source, and, since the maximum cell density under these conditions is 47% that of growth on 2.5% glucose, it is presumed that the lower glucose concentration presents conditions of carbon limitation to the growth of the organism. The conditions for the production of pullulan then are reminiscent of the accumulation of polysaccharide in bacteria which occurs as a result of limiting growth conditions in the presence of excess carbon source (27). However, by using procedures of alkaline extraction in 30% potassium hydroxide at 100 C, pullulan has not been detected within the cell and does not appear to be metabolized upon elaboration (13). By contrast, growth upon excess glycerol does not lead to pullulan production. It is apparent from Fig. 5a that glycerol uptake is mediated by a different form of control than that of glucose utilization since, upon growth limitation by an as yet unspecified nutritional component, glycerol, unlike glucose or fructose, ceases to be metabolized. The production of extracellular polysaccharide by cells growing on maltose occurs only when the requirements of the cell for glucose are exceeded by the production of that substrate from maltose.

Pullulan, then, appears to be elaboratred under conditions favorable for the accumulation of glucose or fructose metabolites within the cells. Regulation mechanisms have been proposed for *Saccharomyces cerevisiae* (4) which control the initial interaction of the cell with monosaccharide, i.e., membrane transport of glucose is under control from an immediate metabolite, glucose-6-phosphate. Romano and Kornberg (26), studying A. nidulans, have postulated the mediation of a glucose-transport mechanism by acetyl coenzyme A, an observation which provides another possible explanation for the lack of glucose utilization for extracellular polysaccharide production in the presence of acetate, since the uptake of hexoses by P. pullulans may now be under a more strict control. It is perhaps this type of transport control function which is missing in P. pullulans. The conversion of glycerol into pullulan in the presence of glucose undergoing a similar transformation reflects that proportion of glycerol metabolites that are channeled into polymer production under these conditions. The effect of pH on the ability of the cell to utilize glucose for extracellular polysaccharide production may reflect transport

dependence on *p*H and provide another reason for the apparent inability of the cell to produce pullulan from glucose in the presence of acetate.

Cellular pullulan. The occurrence of pullulan was sought within cells that were actively elaborating pullulan. Washed and lypholized preparations were extracted with hot 30% potassium hydroxide. Acid hydrolysis of the isolated polysaccharides indicated that 5.7% of the dry weight of glucose-grown cells could be accounted for as polysaccharide of which 50% was comprised of glucose. Depolymerization of this material with pullulanase indicated that pullulan, if present, comprised less than 2% of the isolated polysaccharide. Pullulan itself was shown to tolerate these extraction conditions. Similarly, examination of polysaccharides extracted from glycerol or acetate-grown cells revealed no detectable pullulan. Correspondence between extracellular polysaccharides and the structure of the cell walls of the elaborating organism has been suggested. Such a relationship has been reported for five strains of *Candida* (12), and in some yeasts there appears to be a correspondence between elaborated mannans and the cell wall architecture of these organisms (24). However, Brown et al. (10, 11), in the examination of cell wall constituents of *P. pullulans*, have not reported the presence of pullulan, and the similar alkaline extraction procedures described above have failed to demonstrate the presence of the polysaccharide.

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