A PHYSIOLOGICAL COMPARISON OF THE DIMORPHIC FORMS OF *PULLULARIA PULLULANS*

by

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Short Title:

PHYSIOLOGY AND DIMORPHISM IN *PULLULARIA PULLULANS*

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INTRODUCTION

When medically important fungi were first studied, some pathogenic fungi which were unicellular *in vivo* were found to change to a mycelial form when cultured. This change in morphology was misleading as investigators believed they were not isolating the correct pathogenic organisms. The confusion continued until the pathogenic fungi were shown to change their morphology as a result of temperature changes. The term dimorphism was adopted to designate the phenomenon of a fungus existing in more than one morphological form. Technically, the word should only be used with medically important fungi, however a broader definition may be used. Cochrane (1958) defines "a dimorphic fungus as one in which a reversible transformation from a mycelial to a non-mycelial and unicellular growth type occurs." On this basis *Pullularia pullulans* is a dimorphic fungus.

The aim of this project is to compare the physiological activities of the dimorphic forms of *P. pullulans*. Chemical additives and growth conditions known to affect other dimorphic fungi were tested for their effect on the dimorphism of *P. pullulans*. From these tests, media were developed which supported predominantly mycelial or yeast-like growth. Carbon balances, Warburg respirometer experiments and experiments with labelled glucose were used to establish similarities and differences between the dimorphic forms.

LITERATURE REVIEW

Pullularia pullulans (de Bary) Berkhout

A. Classification

P. Pullulans is not easily classified and many different names have been given to this fungus. *Dematium pullulans* was included as a new species of *Dematium* when it was first described (de Bary, 1866). Bennett (1928) proposed the binomial *Anthostomella pullulans* after *Dematium pullulans* was shown to possess its own ascigerous stage, hence making it an ascomycete, however it was not like any other described species of the genus *Anthostomella*. No one has been able to repeat Bennett's work consequently the name has not been retained.

The name *Pullularia pullulans* was proposed when this organism was removed from the genus *Dematium* in an attempt to simplify nomenclature (Berkhout, 1923). This name was not accepted at first but since has been widely accepted especially when the organism is found as a saprobe (Miller *et al.*, 1957; Muskett and Calvert, 1940; Reynolds, 1950).

The name *Aureobasidium pullulans* (Arnaud, 1910) used by early phytopathologists in France and Italy antedates *Pullularia pullulans* and Cooke (1959) for this reason proposed it as a correct name for this fungus.

Only the most widely used names given to this fungus have been mentioned; there are many more. Since Pullularia pullulans is widely accepted as the name for this organism in its saprophytic form, it is used throughout this thesis.

Only two species are at present recognized in the genus Pullularia, P. pullulans and P. werneckii (Wynne and Gott, 1956); however, a proposed revision of the genus Pullularia created one more species. In addition to P. pullulans and P. werneckii, Wynne and Gott (1956) included a new species P. fermentans with seven varieties. Cultures that fermented one or more sugars were classified as P. fermentans and varietal differentiation was based on the combination of sugars fermented. Cultures that did not ferment any of the sugars tested were classified either as P. pullulans or P. werneckii. Species distinction was based on morphological characteristics such as relative sizes of blastospores, arthrospores and chlamydospores and pigment production. Thus, by using physiological characteristics an attempt was made to establish species in a genus exhibiting pronounced natural morphological variation.

B. Description

de Bary (1866) described P. pullulans as consisting of septate mycelium, from the segments of which, ellipsoidal cells budded freely at the ends or at the sides. The organism turned brown after the available food supply was exhausted.

Negroni and Fischer (1942) studied thirty strains of P. pullulans and described the colonies as black, folded, moist and glistening. The hyaline elliptical blastospores had an average measurement of 7 by 3.5 microns. They arose from the hyaline septate branched mycelium by budding and on small pedicels. The blastospores were disposed around the hyphae like a small cuff three or four cell layers in thickness. Other organs produced by the mycelium included arthrospores and hyaline chlamydospores.

A more recent description is based on one culture (Cooke, 1959). When grown on neopeptone-dextrose agar, colonies were white at first, then they usually became black throughout except at the margin, or occasionally blackened areas developed irregularly. Colonies were composed of a mat of mycelium which frequently appeared mucoid but sometimes dry and dull. The hyphae radiated from the center of the colony, becoming irregularly entangled. Young hyphae were thin-walled. Septa in young hyphae were simple; in older hyphae they became thickened and double-walled. Cells and hyphal segments in older hyphae broke apart at the thick, double-walled septa to form arthrospores or arthrospore-like hyphal segments. All cells were capable of producing conidia on papillae produced from the inner wall of the cell at irregular intervals. Occasionally, the papillae became short hyphal branches which resembled false basidia on which an irregular number of papillae produced conidiospores. The conidiospores were hyaline, ovate and drawn out at the base to a point

where it was attached to the papillae. The conidiospores gave rise to secondary conidia by yeast-like budding. These yeast-like spores were on the average slightly smaller than primary conidiospores. All hyphae were hyaline at first then the walls became pale green and finally they became black. The morphology of P. pullulans was found to be subject to considerable variation from strain to strain, from culture to culture, and from medium to medium. This variation indicates either an extremely unstable type of fungus or a highly heterocaryotic condition in which many possible strain types are represented in a single culture.

C. Habitat

P. pullulans has been isolated from many soil samples in low numbers (Jeffreys et al., 1953; Martin et al., 1956; Waksman, 1917). The organism is widely distributed as borne out by air sampling experiments in different parts of the world (Morrow and Lowe, 1943; Pady, 1951; Pady and Kapica, 1956). P. pullulans has been isolated from many different plants and plant products e.g. rye-grass seeds (Noble, 1939), pine needles (Langner, 1933), apples (Clark and Wallace, 1955), strawberries (Beneke et al., 1954), pears (English, 1940), cherries (English, 1945), tomatoes (Taylor and Shanor, 1945), and dew retting flax (Wieringa, 1956). Painted and varnished surfaces exposed to high humidites are subject to infestation by P. pullulans (Reynolds, 1950). The fungus grew on the oils in the paint, producing

black specks which gradually changed the general appearance of the paint; white paint changed to dull gray in about two years. P. pullulans has also been known to cause blue staining of wood (Jump, 1938). This organism also has been isolated from pulp used for paper manufacture (Pehrson, 1947). A number of plant diseases have been attributed to P. pullulans, however, it is better known as a mild plant pathogen or a secondary invader (Flor, 1936; Lewis, 1912; Muskett and Calvert, 1940). P. pullulans has been found as a symbiont with several insects such as scales and coccidae (Brues and Glaser, 1921; Steinhaus, 1955). Wynne and Gott (1956) isolated P. pullulans from lymph nodes in a case of Hodgkin's granuloma.

The above ecological resumé is by no means complete, but it serves to illustrate the ubiquitous and omniverous nature of this fungus. Thus, P. pullulans is of considerable importance economically as it causes discolouration of lumber products, paint deterioration and is the primary or secondary or co-operating organism causing injury to many plants and spoilage of numerous plant products.

D. Physiology

a). Physical Factors Affecting Growth

The effect of aeration on the growth of P. pullulans was studied by Luteraan (1954). An insufficient oxygen supply limited growth; whereas, in a well aerated medium growth was rapid and more

cellular material was obtained for the same amount of glucose consumed.

There are few references to temperature requirements of this fungus, however Wynne and Gott (1956) reported that nine out of twelve different strains tested grew at 37°C; whereas, Negroni and Fischer (1942) found the optimium temperature for growth was 23°C.

b). Chemical Factors Affecting Growth

A study of the abilities of some blue molds of wood to grow on special media revealed that P. pullulans grew slowly and produced only hyaline cells on 1.5% agar, 2% glucose and a salt mixture (Rennerfelt, 1940). Better growth was obtained on this medium when technical grade glucose was used, and best growth was obtained on 1.5% agar to which commercial malt extract was added.

The growth of P. pullulans was stimulated by a homoloque of thiamine in which the pyrimidine fraction contained an ethyl group in place of a methyl group (Schopfer, 1938). Recently, growth of P. pullulans in shake culture on a synthetic medium was shown to be dependent on a supply of thiamine (Ward, 1969). The thiamine could be replaced by a combination of equivalent amounts of thiazole and pyrimidine but not by either alone.

c). Metabolism

Negroni and Fischer (1942) from a study of thirty strains

of P. pullulans reported that they all utilized dextrose, maltose, lactose, raffinose and a small amount of galactose as sources of carbon. Nitrogen was supplied by peptone, asparagine, ammonium sulphate, potassium nitrate and to a slight extent, urea. The casein in milk was coagulated and peptonized and gelatin liquified.

Fermentation of sugars was used as a criterion for a proposed classification of the genus Pullularia (Wynne and Gott, 1956). Depending on their ability to ferment mannose, glucose, fructose, maltose, sucrose, raffinose, xylose, galactose and rhamnose, three species and seven varieties were established.

Strains of P. pullulans isolated from insects assimilated glucose, sucrose, maltose, raffinose and inulin (Steinhaus, 1955). They did not assimilate lactose and galactose although strains from other sources did assimilate these sugars. Strains isolated from insects fermented glucose to ethanol, with the production of acid and gas. Acid and gas were also formed from sucrose, maltose and raffinose.

A strain of P. pullulans studied by Clark and Wallace (1958a) possessed constitutive enzymes for the utilization of galactose, glucose, maltose, mannose, raffinose, sucrose and trehalose but not lactose.

In a study of pentose utilization a strain of P. pullulans utilized two pentoses, xylose and arabinose, to produce carbon dioxide, alcohol and cellular material (Rosa et al., 1929).

A medium of glucose, peptone and salts was used in a study of fermentation products of P. pullulans (Sumiki, 1929). From the fermented medium ethanol, acetic acid, succinic acid and D,L-lactic acid were isolated and identified. The quantities of the fermentation products were very small and the greater part of the glucose added was not consumed. Chrazaszaz and Schillak (1936) demonstrated that formic acid, acetic acid and propionic acid but no butyric acid were formed during calcium lactate utilization by P. pullulans.

Bernier (1958) studied P. pullulans in an investigation of polysaccharide production by litter and decay fungi grown on aqueous extracts of fresh leaves and on a glucose-salts-yeast extract media. Although polysaccharide was produced in the leaf extract media, only the polysaccharide produced in the glucose-salts-yeast extract medium was analyzed. Glucose, mannose, and traces of galactose, rhamnose and glucuronic acid were shown to constitute the polysaccharide. The polysaccharide which adhered closely to the mycelium contained only glucose and glucuronic acid. The polysaccharide contained nitrogen and phosphorus although a biuret test was negative. Electrophoresis revealed that two components were present in the polysaccharide.

Bender et al. (1959) described another polysaccharide of P. pullulans called pullulan, produced in Czapek-Dox medium with glucose, sucrose or fructose as a source of carbon. After hydrolysis, glucose was shown to be the only sugar present; whereas, after partial hydrolysis isomaltose and a small percentage of maltose were identified.

No amino acids were present after the polysaccharide was hydrolyzed. An infra-red spectrum analysis suggested a predominant percentage of α-1,4-linkages.

Studies with cell extracts of P. pullulans (Clark and Wallace, 1958a) indicated that this organism was capable of catabolizing glucose by reactions similar to those of the Embden-Meyerhof scheme as well as those of the pentose phosphate cycle. Evidence was presented that three enzymes of the glycolytic system; namely, phosphoglucomutase, fructose diphosphate aldolase and 3-phosphoglyceraldehyde dehydrogenase, were active in cell-free preparations. The reduction of TPN in the presence of glucose-6-phosphate and the utilization of ribose-5-phosphate by cell extracts indicated the presence of at least two enzymes of the pentose phosphate cycle.

The enzymes necessary for oxidizing most of the compounds of the Krebs cycle were shown to be present in P. pullulans (Clark and Wallace, 1958b). Intact cells oxidized 2-ketoglutarate, fumarate, malate and succinate; whereas, TPN stimulated the oxidation of malate in cell-free preparations. These results suggested that the citric acid cycle is active in P. pullulans.

Using glucose-1-C^{14} and glucose-6-C^{14}, Clark and Wallace (1958a) were able to demonstate that young cells of P. pullulans produced a greater amount of carbon dioxide from carbon "one" than from carbon "six" of glucose. As the cells became older this difference

was less pronounced. As the CO_2-$1^{14}/CO_2$-6^{14} ratio was not one, a way of glucose dissimilation other than the Embden-Meyerhof scheme must have occurred. In P. pullulans this could have been through the reactions of the pentose phosphate cycle. The existence of the glycolytic scheme cannot be eliminated on this basis and evidence indicates that both systems were probably active simultaneously.

E. Factors Affecting the Morphology of Dimorphic Fungi

a). Cysteine

The addition of cysteine to cultures of Candida albicans, C. tropicalis, Trichosporon capitatum and Hansenula anomala resulted in reduced mycelium formation (Nickerson and van Rij, 1949). The characteristic effect of cysteine on C. albicans was to produce short hyphal cells with very close spacing of blastospore clusters. This phenomenon could not be explained solely by redox potential differences since exposing these cultures to ascorbic acid had no effect on their morphology. It also appeared that some specificity was required of the thiol donor since sodium thioglycollate was not as effective as glutathione which in turn was less effective than cysteine. A supply of reduced organic sulphhydryl compounds is essential for rapid cell division and hence for maintenance of the unicellular condition (Nickerson and Mankowski, 1953). C. albicans grew entirely in the mycelial phase on a medium of starch which had been washed free of reducing sugars. Although the growth rate was slow on the poly-

saccharide medium, it was clear that the cellular division process was unable to keep pace with even the reduced growth rate. Upon addition of cysteine, yeast cells formed even on the polysaccharide medium; if some of the starch was replaced by glucose, then less cysteine was needed to achieve the same effect. It was proposed (Nickerson, 1948; Nickerson and Mankowski, 1953) that the action of cysteine was to establish an intracellular redox potential which maintains a critical concentration of thiol groups in the cell.

b). Selenium

Sodium selenite was found to promote cellular division of Candida albicans, Pullularia pullulans, Hormiscium dermatitidis and Geothrichum sp. (Nickerson et al., 1956). Preliminary experiments indicated that potassium tellurite also promoted cell division of C. albicans. With selenite ions present, the normal black pigmentation and filamentation of P. pullulans was completely suppressed and the growth remained in the budding yeast form. Mycelial growth from the periphery of colonies and streaks of Geotrichum sp. was prevented by selenite. Selenite ions also partially inhibited growth of H. dermatitidis but that which did occur was almost entirely in the yeast form. Thus, concentrations of selenite and tellurite ions which were somewhat inhibitory to the growth of yeast-like fungi caused a variety of filament producing organisms to grow in the yeast-phase condition. This effect was not a selective inhibition of the filamentous type of growth but a promotion of cellular division. This was believed to be

the first instance in which antimicrobial agents were shown to enhance division of normally filamentous cells.

c) Cobalt

Cobalt treated cells of C. albicans formed long mycelial fragments with no crosswalls and very wide spacing between blastospore clusters (Nickerson and van Rij, 1949). Cobalt has been reported to form complexes spontaneously with cysteine which are oxidized in air to stable compounds (Michaelis and Barron, 1929; Michaelis, 1929).

d) Oxygen

A taxonomic study of C. albicans (Wicherham and Rettger, 1939) disclosed than an abundance of oxygen resulted in a decrease in hyphal production; whereas, blastospore growth was enhanced. Bauer (1938) reported that the mycelial phase of P. pullulans was more pronounced under conditions of good aeration; whereas, conidial production was more pronounced under poor aeration.

MATERIALS AND METHODS

A. Cultures

Three cultures of *P. pullulans* were used in this study. Mac M 18 was previously in the Macdonald College culture collection. Mac M 21 was supplied by the Plant Research Institute, Dept. of Agriculture, Canada numbered, 46-666C. Mac M 23 was obtained from the Centraal Bureau voor Schimmelcultures, Baarn, Netherlands, labelled *Pullularia pullulans* (de Bary) Berkhout *var. fusca* (Browne) Berkhout.

Other cultures were collected, however they were not used in this investigation after preliminary screening trials. Mac M 15 was isolated from an apple and Mac M 16 and Mac M 17 were isolated from strawberries. Mac M 18, Mac M 20, Mac M 22 and Mac M 25 were supplied by the Plant Research Institute, Dept. of Agriculture, Canada numbered as 49-9, 45-189G, 47-375D and 41953 respectively. Mac M 24 was obtained from the Centraal Bureau voor Schimmelcultures, labelled *Anthostomella pullulans* (de Bary et Low) Bennett.

B. Maintenance of Cultures

Cultures for inoculum were grown as slants for four days at room temperature on malt extract agar (Difco). They were stored at 2°C and transfers to fresh medium were made every three weeks. During the three week period all necessary inocula were taken from these slants. Stock cultures were kept frozen at -10°C; these were used to start fresh slants whenever the morphology of the slants stored at 2°C deviated from the original description.

C. Medium

The basic growth medium contained: 0.1% ammonium nitrate, 0.05% magnesium sulphate, 0.1% potassium monohydrogen phosphate, 0.5%

yeast extract (Difco) and 2% glucose. The glucose, yeast extract and salts were autoclaved separately at 121°C for fifteen minutes.

D. Growth Conditions

All cultures were grown at 25°C on either a reciprocating shaker adjusted to 110 strokes per minute or a rotary shaker running at 250 revolutions per minute.

E. Separation of the Dimorphic Phases

Separation was accomplished by using a screen with openings equal to 0.074 mm. with 200 meshes to the inch (U.S. series equivalent No. 200). Directly under the screen a 600 ml. beaker was placed to catch the culture fluid and yeast-like cells as they passed through the screen. About one-fourth of the contents of each flask was poured onto the screen at one time. This material was mixed with a spatula to facilitate screening. When most of the culture liquor was through, the remaining material on the screen was washed with ca. 25 ml. distilled water dispensed under pressure from a wash bottle. The procedure was repeated using the rest of the contents of the flask in about three batches. The mycelium on the screen was checked microscopically to determine if the separation was satisfactory.

After separation of the two phases, the filtrate which contained the yeast-like cells, culture fluid and washings, was centrifuged at 6,000 revolutions per minute for fifteen minutes. The

supernatant was discarded and the yeast-like cells resuspended in 10 ml. distilled water to which 20 ml. acetone was added. The cellular material was collected on a tared filter with the aid of reduced pressure. The weight of the filter was taken as the average weight of five filters of the same size. The yeast cells on the filter were dried at 90°C and their weight determined by difference. The mycelium on the screen was placed on a tared filter with a spatula and the mycelium washed with 10 ml. acetone using reduced pressure to aid filtration. The mycelium was dried overnight at 90°C and its weight determined by the difference. In this way, the dry weight of the yeast and mycelial phase was determined. Only deviations from the control greater than 10% were considered significant.

F. Tests on Various Agents Affecting Dimorphism

a). Selenious acid, Cobalt sulphate and Cysteine

The effect of selenious acid, cobalt sulphate and cysteine on the dimorphism of Mac M 18, Mac M 21 and Mac M 23 was evaluated by adding each of these chemicals separately to the growth medium previously described; the basic growth medium with nothing added served as control. 1 ml. of inoculum was added to each 250 ml. Erlenmeyer flask containing 100 ml. medium. After five days growth on the reciprocating shaker the yeast-like cells and mycelium in each flask were separated and their dry weights determined.

b). Oxygen

The same procedure outlined above was repeated except 500 ml. Erlenmeyer flasks and the rotary shaker were used to increase aeration. Because of this increased aeration, the cultures were grown only for four days.

c). Thiamine

The cultures were grown in 500 ml. Erlenmeyer flasks on the rotary shaker using the following medium; 2 gm. glucose, 0.2 gm. L-asparagine, 10 µgm. thiamine, 0.05 gm. magnesium sulphate, 0.1 gm. potassium monohydrogen phosphate and 100 ml. distilled water. In order to grow Mac M 18 and Mac M 23, 2 mgm. yeast extract (Difco) were added to 100 ml. medium. Each constituent of the medium was autoclaved separately at 121°C for fifteen minutes. The dry weights of the yeast and mycelial phase were determined as already described.

G. Identification of End-Products of Glucose Metabolism

a). Gluconic Acid

Using 100 ml. of the appropriate medium (see results) in a 250 ml. Erlenmeyer flask, both phases of each culture were grown for four days on the reciprocating shaker. Daily samples of the culture liquors were analyzed using one dimensional ascending paper chromatography with butanol-pyridine-water (50:20:18) and isopropanol-pyridine-acetic acid-water (8:8:1:4) as the mobile phase. Alkaline silver

nitrate (Trevellyan et al., 1950) aniline phosphate (Bryson and Mitchell, 1951), and o-phenylene diamine (Block et al., 1950) were used as developing reagents.

b). Ethanol

After four days growth, the cellular material was centrifuged down and 5 ml. liquid was distilled from 200 ml. of the combined culture liquor of both growth phases. The three distillates containing ethanol produced by Mac M 18, Mac M 21 and Mac M 23 respectively, were used to synthesize ethyl-3,5-dinitrobenzoate by the method described for derivative formation of alcohols in an aqueous solution (Cheronis and Entrikin, 1958).

c). Pullulan

An extracellular polysaccharide, pullulan, was produced by each culture during glucose metabolism. It was precipitated by the addition of 1.2 volumes of acetone to the culture fluid (Bender et al., 1959). Biuret and xanthoproteic tests (Hawk et al., 1954) were applied to a portion of each polysaccharide. The remaining polysaccharide from each culture was separately hydrolyzed, neutralized and finally the hydrolyzate analyzed by paper chromatography for amino acids and sugars (Bender et al., 1959).

d). Organic acids

The yeast phase of Mac M 18 was grown for five days on a

reciprocating shaker (for medium, see results). The cells were removed by centrifugation at 6,000 revolutions per minute for fifteen minutes. The cell-free culture fluid (1000 ml.) was concentrated in a rotary evaporator to ca. 100 ml. This liquid was acidified, saturated with NaCl and extracted for 12 hours with ethyl ether (Neish, 1952). The ethyl ether was evaporated leaving a mixture of free organic acids. Using thionyl chloride and butanol, hydroxamates were formed from 0.5 ml. of the mixture of acids (Cheronis and Entrikin, 1958). Some of these hydroxamates were tentatively identified by paper chromatography using butanol-acetic acid-water (4:1:5) as the mobile phase and a ferric chloride spray for development (Block et al., 1958).

Using the method described by Neish (1952) without the boiling acid permanganate treatment, the silver salt of succinic acid was separated from 1 ml. of the organic acid mixture. The succinic acid was freed from its salt by acidification, then extracted with ethyl ether. The ethyl ether was evaporated and the succinic acid dissolved in 0.5 ml. distilled water. This solution was placed on a silicic acid column and eluted as described by Neish (1952). Whenever the silicic acid column was used the procedure described by Neish (1952) was followed exactly except that 0.1 N NaOH in a micrometer syringe burette (Neish, 1952) was used in place of the Machlett 5 ml. burette.

A few drops of the organic acid mixture were used to test for acetic acid using lanthanum nitrate (Feigel, 1956).

Lactic acid was identified by a colour reaction with p-hydroxydiphenyl (Neish, 1952). Using the silicic acid column, 0.5 ml. of the acid mixture was separated. The lactic acid fraction was evaporated down on the rotary evaporator to ca. 2 ml. and the p-hydroxydiphenyl colorimeter test applied.

The organic acids produced by Mac M 23 were identified solely on the basis of elution from the silicic acid column.

e). Partial Characterization of Unidentified Organic Acids

Organic acids produced by the yeast phase of Mac M 23 were extracted with ethyl ether and the ether evaporated. During evaporation a light brown solid separated out; it was centrifuged and washed with ca. 0.5 ml. ethyl ether. The decomposition point of the solid was determined and its ultra violet absorption spectrum plotted. It was analyzed by paper chromatography using the butanol layer of a butanol-formic acid-water (10:2:15) mixture as the mobile phase and p-dimethyl-amino benzaldehyde as the developing agent (Block et al., 1950). Citric acid, isocitric acid, cis-aconitic acid and itaconic acid were used as standards during paper chromatography analysis.

After ether extraction the organic acids from the mycelial phase of Mac M 18 and Mac M 23 were also analyzed by paper chromatography using the same solvent and developing agent.

H. Quantitative Determination of the End-Products of Glucose Metabolism

As P. pullulans is an aerobic organism, a continuous supply of air had to be provided during growth in such a way that respiratory carbon dioxide could be measured. Therefore, it was necessary to construct a fermentation train (Figure 1) which would sterilize the air entering the train, remove carbon dioxide from it, and finally collect the carbon dioxide produced during glucose metabolism. Since the fermentations were aerobic it was impossible to calculate an oxidation-reduction ratio. The method used for these aerobic fermentations was as follows. Both phases of each culture were grown in a 500 ml. Erlenmeyer flask constructed so that air could be forced through the flask (Neish, 1952). The air sterilizer was made from glass tubing 2.4 cm. x 18 cm. packed firmly with absorbent cotton. Care was taken that the cotton was not compressed too tightly thus stopping the air flow. The ends of the glass tube were fitted with rubber stoppers, each containing one glass tube 8 cm. x 0.7 cm. These glass tubes protruded into the cotton ca. 4 cm. when the rubber stoppers were fitted tightly so that the air could not escape. Rubber tubing was attached to the 8 cm. x 0.7 cm. glass tubes and an adapter to hold a B-D Yale hypodermic needle, size 32, length 3 inches, was placed in the longer end of the rubber tubing. The hypodermic needle was attached and this end of the tubing placed in a 20 cm. x 2.5 cm. test tube. Cotton was packed between the top of the test tube and the

Figure 1 Fermentation train used for carbon balances.

rubber tubing; then the air sterilizer was wrapped in paper and autoclaved at 121°C for 15 minutes.

The medium was placed in the fermentation flask in the following manner; ca. 2 gm. glucose were weighed out and placed in a 100 ml. volumetric flask. The flask was made to volume with distilled water and shaken until all of the glucose was dissolved. From this glucose solution, 2 ml. were removed, placed in a 10 ml. volumetric flask and diluted to volume. With this solution the amount of glucose added to the fermentation flask was determined by the method of Somogyi and Nelson (Neish, 1952). Triplicate determinations were made for all samples and preparation of the standard curve. The remaining glucose solution in the 100 ml. volumetric flask was poured through the side arm into the fermentation flask using a glass funnel. The 100 ml. volumetric flask and funnel were rinsed twice with ca. 5 ml. distilled water; the washings were added to the fermentation flask. The side arm and the top of the fermentation flask were fitted with cotton plugs and the flask autoclaved for fifteen minutes at 121°C. The yeast extract, salts and, where applicable (see results), cysteine, thiamine and asparagine were sterilized separately in test tubes and added aseptically through the side arm. After addition of these substances the fermentation flask was then inoculated and the side arm fitted with a sterile rubber serum bottle cap. The sterile needle from the air sterilizer was pushed through the rubber cap and the fermentation flask placed on a reciprocating shaker. The top of the

fermentation flask was then connected with rubber tubing to a glass bead tower gas washer containing 20 ml. carbonate-free 6 N NaOH. Rubber tubing from the air sterilizer was attached to a Fisher-Milligan gas washer filled with 200 ml. 20% NaOH to complete the fermentation train. The Fisher-Milligan gas washer in turn was attached by rubber tubing to an air supply under a pressure of ten pounds per square inch. The flow of air was adjusted so that 600 ml. flowed through the fermentation train in one hour. The fermentation flask was shaken on the reciprocating shaker for five days.

a). Carbon Dioxide

At the end of this period the hypodermic needle was pulled out of the rubber cap in the fermentation flask, then the air was turned off. The rubber tubing was disconnected from the top of the fermentation flask and the 20 ml. 6 N NaOH in the bead tower gas washer transferred to a 200 ml. volumetric flask. The gas washer was rinsed three times with CO_2-free water and the washings added to the 200 ml. volumetric flask. Using CO_2-free water the volumetric flask was made to volume and shaken. The amount of CO_2 absorbed by the NaOH was determined by titration after $BaCl_2$ was added (Neish, 1952). Three 20 ml. aliquots of the NaOH solution and blank were titrated.

b). Carbon Assimilated

The culture fluid in the fermentation flask was placed in a centrifuge bottle, then the fermentation flask was washed twice with 10 ml. distilled water and the washings added to the culture fluid. After the culture fluid was centrifuged at 6,000 revolutions per minute for fifteen minutes, the supernatant was poured into a 200 ml. volumetric flask. The cellular material was washed twice with 10 ml. distilled water; the washings were added to the supernatant. The volumetric flask was then made to volume with distilled water and shaken. The cellular material was suspended in 10 ml. distilled water and ca. 20 ml. acetone were added. The cells were collected on a tared filter with the aid of reduced pressure and washed with pure acetone. The acetone-prepared cells were dried overnight at 90°C and weighed. Using 10 ml. Van Slyke-Folch combustion fluid (Neish, 1952), the amount of carbon in 10 mgm. dried cells was determined by the combustion and direct titration method of Baker et al. (1954). All combustion flasks were autoclaved for one hour at 121°C. Blanks and cell carbon content were determined in triplicate.

c). Residual Glucose

Residual glucose was determined by the same method used for initial glucose determination (Neish, 1952) after appropriate dilution of 10 ml. of the culture liquor in the 200 ml. volumetric flask.

d). Ethanol

Ethanol was distilled from 20 ml. of alkaline fermentation liquor and collected in a 10 ml. volumetric flask. The distillation was continued until ca. 9 ml. distillate was collected. The flask was made to volume, shaken and after appropriate dilution, the ethanol was determined in duplicate by the acid dichromate oxidation method (Neish, 1952).

e). Pullulan

Pullulan was precipitated out of 20 ml. culture liquor by the addition of 24 ml. acetone. The precipitated pullulan was collected on a tared filter using reduced pressure, washed with pure acetone, dried overnight at 90°C and weighed.

f). Organic Acids

The organic acids were prepared for separation and quantitative determination by the silicic acid column method (Neish, 1952) as follows; 40 ml. of the culture fluid were concentrated to about 10 ml. on a rotary evaporator. The solution was saturated with NaCl and extracted with ethyl ether for 12 hours. The ethyl ether was evaporated leaving the free organic acids for column chromatography. Because there were unidentified organic acids present which were not eluted by the solvents used, five times the recommended number of milliequivalents of organic acids were placed on the column.

This has no effect on the operation of the column except possibly in the separation of lactic acid and succinic acid; however, lactic acid was produced only by Mac M 18 during yeast-like growth. The quantity of lactic acid in this case was checked by a colorimetric determination (Neish, 1952).

The calcium salt of gluconic acid was precipitated between pH 9.4 and pH 10.2 after addition of calcium nitrate and 120 ml. 95% ethanol to 40 ml. culture fluid (Herrick and May, 1928). The precipitate was collected in a tared crooch crucible, dried at 90°C and weighed.

I. Oxidation of Various Substrates by the Dimorphic Forms

Standard Warburg respirometer experiments were carried out using the "direct method" described by Umbreit et al., (1959). Each Warburg flask contained; 0.5 ml. 0.02 M substrate, 1 ml. cell suspension, 0.2 ml. 20% NaOH, and 1.5 ml. 0.02 M phosphate buffer, pH 6.5. The flasks were shaken at 90 strokes per minute at 25°C. The following substrates were tested for oxidation by both growth phases of Mac M 18, Mac M 21 and Mac M 23; glucose, gluconic acid, 2-ketogluconic acid, glycolic acid, pyruvic acid, cis-aconitic acid, fumaric acid and succinic acid; in addition, a preparation of crude ribulose which contained at least one other pentose was used.

The yeast and mycelial phases of each culture were grown in the appropriate medium for 48 hours on a rotary shaker. The growth material was separated by centrifugation and, using the screening technique previously described, one growth phase was washed free of the other. The cellular material was examined microscopically to verify that only one dimorphic form was present. The cells or mycelium were suspended in 50 ml. 0.02 M phosphate buffer,

and aerated on a rotary shaker for 2 hours to decrease endogenous respiration. Any large pellets of mycelium still remaining after aeration were removed. The cellular material was centrifuged at 6,000 revolutions for 15 minutes, the supernatant discarded and the centrifuge bottle inverted for ten minutes to drain excess water. The cellular material was placed in a tared 50 ml. Erlenmeyer flask and weighed. A calculated volume of 0.02 M phosphate buffer, pH 6.5, was added so that 1 ml. of the suspension contained 75 mgm. (wet weight) cellular material. A substrate was considered utilized if the oxygen uptake was 10% higher than endogenous.

J. Fermentation of Glucose-1-C^{14} and Glucose-6-C^{14}

One hundred microcuries of D-glucose-1-C^{14} and D-glucose-6-C^{14} were obtained from Atomic Energy of Canada, Ltd. Glucose was added to each radioactive sample to give a total weight of 1 gm., then the glucose was recrystallized as the monohydrate. A 0.02 M glucose solution was made with each type of radioactive glucose. The quantity and radioactivity of the glucose in each solution was checked by plating three 0.1 ml. quantities of each 0.02 M glucose solution on glass planchets. After drying under an infra-red heat lamp, the radioactivity was determined by a Berkley 1000-B decimal scaler. Following duplicate counting, the quantity of glucose on each glass planchet was determined by the method of Somogyi and Nelson (Neish, 1952).

The Warburg respirometer was used for the radioactive

experiments, the pH, temperature, agitation speed, and flask contents being the same as outlined previously. The yeast and mycelial phase of cultures Mac M 18 and Mac M 21 were grown, prepared and suspended in the same manner as previously described. Six Warburg flasks were used for each growth phase of each culture; two were used to measure endogenous respiration, two contained glucose-1-C^{14} and two contained glucose-6-C^{14} as substrates. When about 15% of the substrates were utilized, 1 ml. of 30% formaldehyde was added to each flask to stop respiration. The NaOH in the center well and in the filter paper was removed with adequate rinsing, then 1 ml. 1 M $BaCl_2$ was added to precipitate the radioactive carbon dioxide. By centrifuging, the precipitate was washed once with 5 ml. distilled water, once with 5 ml. 95% ethanol and finally suspended in a few drops of 95% ethanol. Infinitely thin glass planchets were made from this suspension by evenly plating ca. 1.5 mgm. of the radioactive barium carbonate (Calvin et al., 1949). Each planchet was counted twice and the barium carbonate on the planchets was determined by direct titration (Baker et al., 1954).

RESULTS

A. Morphology of the Dimorphic Forms

Figures 2 to 7 are representative photographs illustrating the morphology of the dimorphic forms of each culture. The photographs were taken of cultures grown predominantly in one dimorphic form which was separated from the other phase by the washing technique previously described. Figures 2, 4 and 6 present yeast-like cells of Mac M 18, Mac M 21 and Mac M 23, respectively. These cells multiplied by budding and as shown in Figure 4 by bipolar budding. Figures 3, 5 and 7 present mycelium of Mac M 18, Mac M 21 and Mac M 23, respectively. Mac M 18 (Figure 3) had markedly septate mycelium; whereas, the septation in mycelium of Mac M 21 and Mac M 23 was not as pronounced (Figures 5 and 7).

B. Effect of Various Agents on Dimorphism

a). Selenious acid, Cobalt sulphate and Cysteine

Dry weight determinations of the yeast and mycelial phases of *P. pullulans* (Table 1) shows that cysteine favoured yeast-like growth of all cultures tested. Additional yeast extract enhanced this effect for Mac M 18 and Mac M 21, but had no effect on Mac M 23. Too much cysteine had an antagonistic effect on yeast-like growth of Mac M 21, however this effect was counteracted by additional yeast extract. Selenious acid had no effect on Mac M 18, but the more dilute concentration

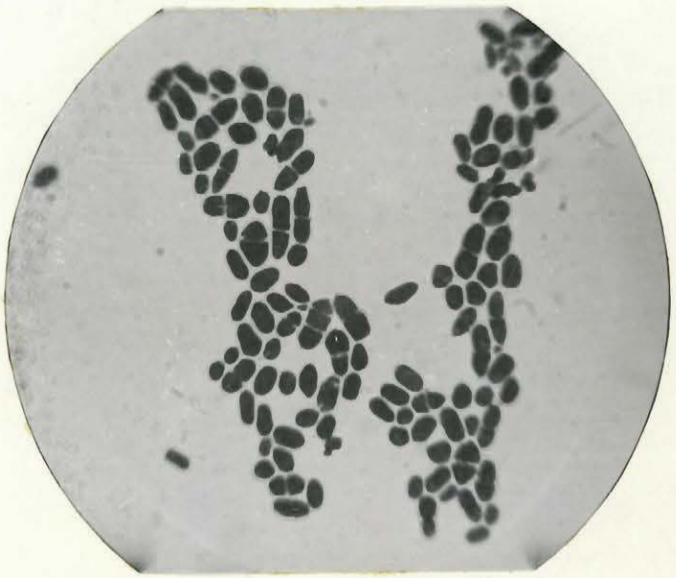

Figure 2 Yeast-like cells of Mac M 18. (700 X)

Figure 3 Mycelium of Mac M 18. (700 X)

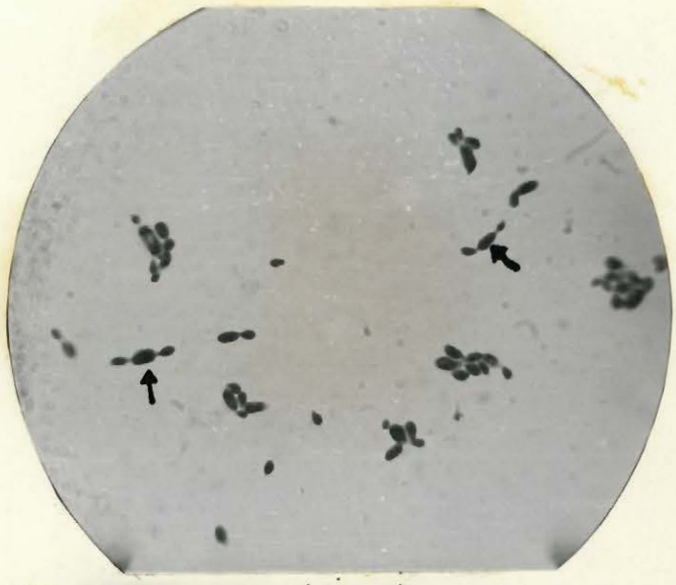

Figure 4 Yeast-like cells of Mac M 21. (700 X)
← - bipolar budding.

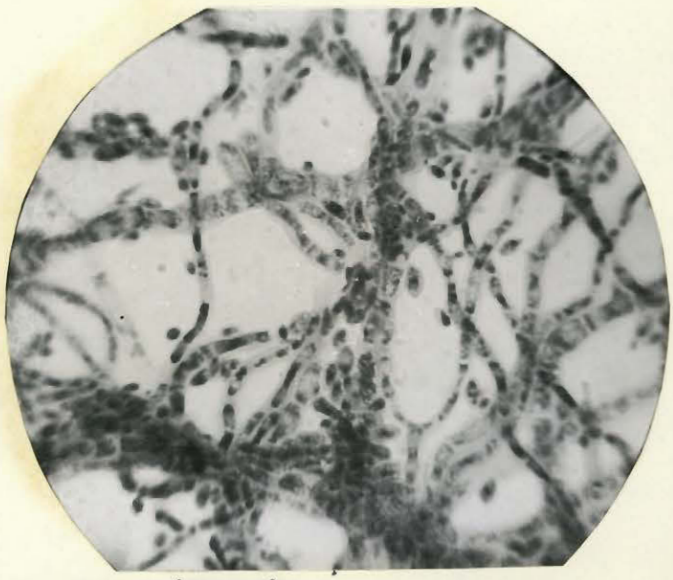

Figure 5 Mycelium of Mac M 21. (700 X)

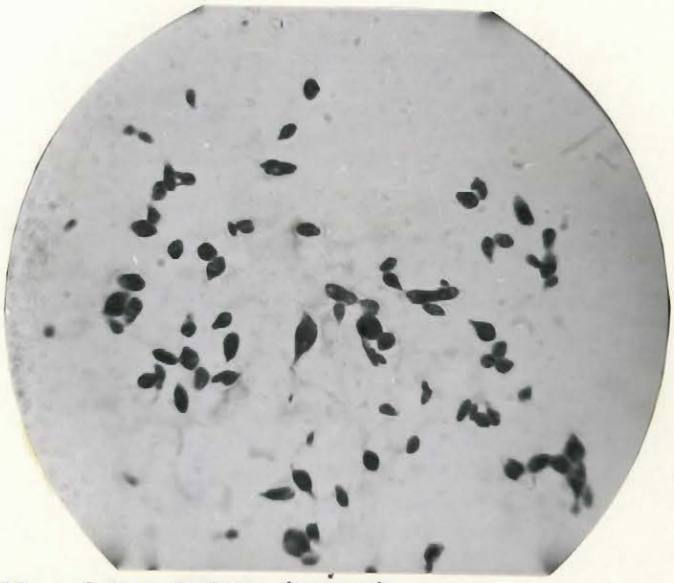

Figure 6 Yeast-like cells of Mac M 23. (700 X)

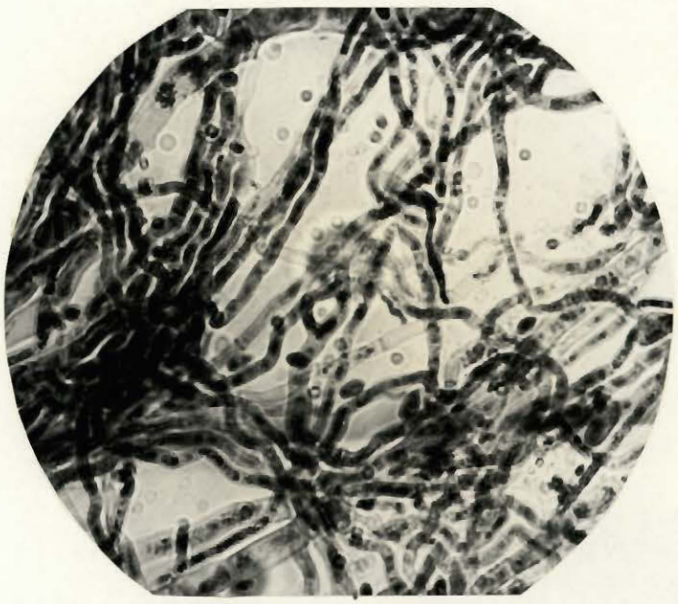

Figure 7 Mycelium of Mac M 23. (700 X)

TABLE I

Effect of Various Agents on the Dimorphism of *P. pullulans*.

Agent	Concentration (mgm./100 ml.medium)	Ratio of dry weight of yeast growth to mycelial growth as per cent.					
		Mac M 18		Mac M 21		Mac M 23	
		Yeast	Mycelium	Yeast	Mycelium	Yeast	Mycelium
Control	-	4	96	40	60	3	97
Cysteine	50	18	82	91	9	17	83
Cysteine	100	55	45	76	24	30	70
Cysteine and extra yeast extract	100 50	78	22	97	3	32	68
Selenious acid	0.003	1	99	36	64	5	95
Selenious acid	0.0003	4	96	52	48	96	4
Cobalt sulphate	0.03	6	94	27	73	4	96

of selenious acid favoured yeast-like growth of Mac M 21 and Mac M 23. Cobalt sulphate favoured mycelial growth of Mac M 21 but had no effect on Mac M 18 or Mac M 23.

b). Oxygen

Increased aeration had no effect on Mac M 21 or Mac M 23, but favoured yeast-like growth of Mac M 18 (Table 11). This was also true when cysteine and extra yeast extract were added to the medium. Selenious acid at the concentration tested favoured yeast-like growth of Mac M 21, but it did not affect Mac M 18 or Mac M 23. Cobalt sulphate appeared to have no effect on any culture.

c). Thiamine

Thiamine increased yeast-like growth of all cultures. The percent dry weight of the yeast phase of Mac M 18, Mac M 21 and Mac M 23 was 70%, 78% and 83% respectively, when these cultures were grown in the thiamine-asparagine medium. When cobalt sulphate was added to this medium the percent dry weight of the yeast phase of Mac M 21 was 7%.

C. Media Used for Predominantly Yeast-like or Mycelial Growth

From the above results media (Table III) of different composition were devised to support yeast-like growth or mycelial growth of the three cultures.

TABLE II

Effect of Increased Aeration* on the Dimorphism of *P. pullulans*.

Agent	Concentration (mgm./100 ml. medium)	Ratio of dry weight of yeast growth to mycelial growth as per cent					
		Mac M 18		Mac M 21		Mac M 23	
		Yeast	Mycelium	Yeast	Mycelium	Yeast	Mycelium
Control (Table I)		4	96	40	60	3	97
Control	-	36	64	38	62	7	93
Cysteine and extra yeast extract	100 50	100	0	100	0	36	64
Selenious acid	0.003	43	57	60	40	10	90
Cobalt sulphate	0.03	28	72	43	57	15	85

* Aeration was increased by using a larger surface to volume ratio and a rotary shaker.

TABLE III

Media Used for Predominately Yeast-like or Mycelial Growth.

Culture	Yeast Phase		Mycelial Phase	
Mac M 18	Glucose	2.0 gm.	Glucose	2.0 gm.
	Yeast extract	0.1 gm.	Yeast extract	0.05 gm.
	Cysteine	0.1 gm.	Potassium monohydrogen phosphate	0.1 gm.
	Potassium monohydrogen phosphate	0.1 gm.	Magnesium sulphate	0.05 gm.
	Magnesium sulphate	0.05 gm.	Ammonium nitrate	0.05 gm.
	Distilled Water	100 ml.	Cobalt sulphate	0.05 mgm.
			Distilled Water	100 ml.
Mac M 21	Glucose	2.0 gm.	Glucose	2.0 gm.
	Yeast extract	0.1 gm.	L-Asparagine	0.1 gm.
	Cysteine	0.05 gm.	Potassium monohydrogen phosphate	0.1 gm.
	Potassium monohydrogen phosphate	0.1 gm.	Magnesium sulphate	0.05 gm.
	Magnesium sulphate	0.05 gm.	Ammonium nitrate	0.05 gm.
	Distilled Water	100 ml.	Cobalt sulphate	0.05 mgm.
			Thiamine	10 ugm.
			Yeast extract	1 mgm.
			Distilled Water	100 ml.
Mac M 23	Glucose	2.0 gm.	Glucose	2.0 gm.
	Yeast extract	0.05 gm.	Yeast extract	0.05 gm.
	Cysteine	0.05 gm.	Potassium monohydrogen phosphate	0.1 gm.
	L-Asparagine	0.05 gm.	Magnesium sulphate	0.05 gm.
	Potassium monohydrogen phosphate	0.1 gm.	Ammonium nitrate	0.05 gm.
	Magnesium sulphate	0.05 gm.	Distilled Water	100 ml.
	Thiamine	10 ugm.		
	Distilled Water	100 ml.		

D. Identification of End-Products of Glucose Metabolism

a). Gluconic Acid

Gluconic acid was produced during mycelial growth of Mac M 18 and Mac M 21 after 48 hours; it was not produced by Mac M 23 in either growth phase. Gluconic acid was identified by paper chromatography, having an R_F of 0.05 in the butanol-pyridine-water solvent and 0.42 in the isopropanol-pyridine-acetic-acid-water solvent. Both glucose and gluconic acid were detected with alkaline silver nitrate and glucose was identified with aniline phosphate. The absence of α-keto acids was demonstrated with o-phenylenediamine.

b). Ethanol

The ethyl-3,5-dinitrobenzoate was made from ethanol produced by Mac M 18, Mac M 21 and Mac M 23; the three samples had melting points of 89-91°C, 89-91°C and 89.5-91°C respectively. Authentic ethyl-3,5-dinitrobenzoate melts at 93°C. The distillation characteristics and the melting points of the derivatives indicated that all cultures produced ethanol.

c). Pullulan

Pullulan was recovered from the fermentation liquor and

biuret and xanthroproteic tests demonstrated that the polysaccharide was essentially protein free. After hydrolysis of pullulan from Mac M 18, Mac M 21 and Mac M 23, glucose was the only sugar found and no amino acids were present. These cultures then produce a similar polysaccharide to that described by Bender et al. (1958).

d). Organic Acids

Silicic acid column chromatography indicated that acetic acid, formic acid, succinic acid and lactic acid were produced during yeast-like growth of Mac M 18. A positive colour test with p-hydroxydiphenyl was obtained before column chromatography and with the solvent containing lactic acid after column chromatography. This confirmed the presence of lactic acid.

The formation of silver succinate which was eluted from the silicic acid column by the correct solvent established the presence of succinic acid.

Paper chromatography of hydroxamates and a positive lanthanum nitrate test indicated that acetic acid was present.

Formic acid was identified by paper chromatography of its hydroxamate derivative.

e). Partial Characeterization of Unidentified Organic Acids

An organic acid produced by the yeast phase of Mac M 23

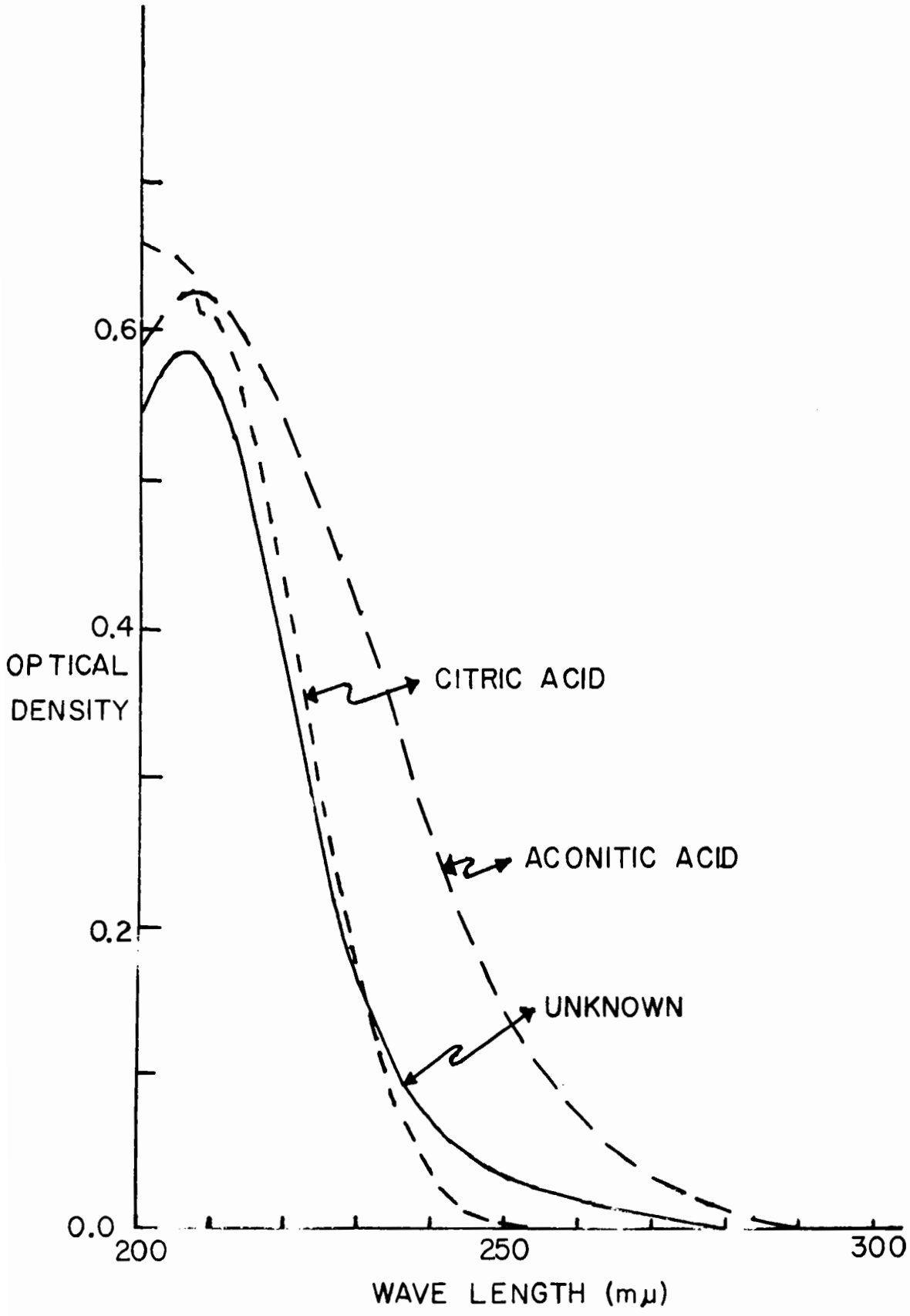

Figure 8 Absorption spectrum of unidentified compound.

decomposed between 210-220°C. It had the same ultra-violet absorption pattern as cis-aconitic acid (Figure 8) and anlysis with paper chromatography indicated only one compound. This compound reacted with Erhlich's reagent to produce a yellow spot (R_F 0.89) which strongly absorbed ultra-violet light (citric acid, isocitric acid, and cis-aconitic acid gave blue spots and they had lower R_F values). Itaconic acid gave the same yellow colour with Erhlich's reagent and its R_F value was similar but the melting point of itaconic acid is 162-164°C. Since the unidentified acid gave the same colour with Erhlich's reagent as itaconic acid and itaconic acid was oxidized by glucose grown resting cells of Mac M 23 (Figure 12) then perhaps the unidentified acid was formed from itaconic acid oxidation.

Analysis of the organic acids produced by mycelial growth of Mac M 23 revealed a spot which corresponded to the unknown acid described in the preceding paragraph (i.e. it showed the same R_F value, yellow colour, and strong absorption of ultra-violet light). There was another acidic compound present which had an R_F of 0.52 and gave a yellow spot with Erhlich's reagent.

Mycelial growth of Mac M 18 produced an acid which had the same R_F value and yellow colour reaction as the latter compound.

E. Quantitative Determination of End-Products of Glucose Metabolism

The two end-products of glucose fermentation which

TABLE IV

Carbon Balances for P. pullulans.

Product	Mac M 18		Mac M 21		Mac M 23	
	Yeast	Mycelium	Yeast	Mycelium	Yeast	Mycelium
	(mM product/100 mM glucose utilized)					
Cells *	170.6	116.0	247.4	147.7	211.4	161.2
Carbon dioxide	174.4	159.7	302.8	145.5	116.7	165.6
Ethanol	68.6	18.4	0.0	74.5	2.2	13.5
Pullulan **	2.6	3.7	4.1	2.0	9.0	9.5
Acetate	2.0	0.0	3.1	0.0	0.0	0.0
Formate	2.2	0.0	4.9	1.0	2.0	0.0
Succinate	3.3	0.0	0.9	1.4	0.2	0.0
Lactate	2.7	0.0	0.0	0.0	0.0	0.0
Gluconate	0.0	6.6	0.0	16.2	0.0	0.0
Percent carbon recovered	88	62	98	93	65	68
Percent glucose utilized	80	45	98	30	99	80
Amount glucose utilized (gm.)	2.58	1.09	2.06	0.81	2.52	2.18

* Measured as m moles carbon

** Measured as m moles glucose

accounted for most of the carbon recovered were cells and carbon dioxide (Table IV). Ethanol accounted for a large portion of carbon during yeast-like growth of Mac M 18 and mycelial growth of Mac M 21 and Mac M 23. Pullulan was produced in the largest quantities by Mac M 23; Mac M 21 and Mac M 18 produced about the same quantity of the dextran. There was no difference in the quantity of pullulan produced by either dimorphic form of the three cultures. Lactic acid was produced only by Mac M 18 during yeast-like growth. Mac M 23 produced no acetic acid in either growth form; whereas, Mac M 18 and Mac M 21 produced acetic acid only during yeast-like growth. All three cultures produced succinic acid and formic acid during growth of one or both growth phases. Gluconic acid was produced by Mac M 18 and Mac M 21 only during mycelial growth.

F. Oxidation of Various Substrates by the Dimorphic Forms

The results of resting cell oxidation of various substrates are presented in Figures 9 to 14. Endogenous respiration was four to nine times higher in yeast-like cells of all cultures. Gluconic acid and 2-ketogluconic acid were not oxidized by any of the cellular preparations. In general, there was very little difference in substrate oxidation by the two dimorphic forms.

Yeast-like cells of Mac M 18 oxidized cis-aconitic acid after a short lag and pyruvic acid after a long lag period. Mycelium of Mac M 18 did not utilize cis-aconitic acid but did oxidize pyruvic

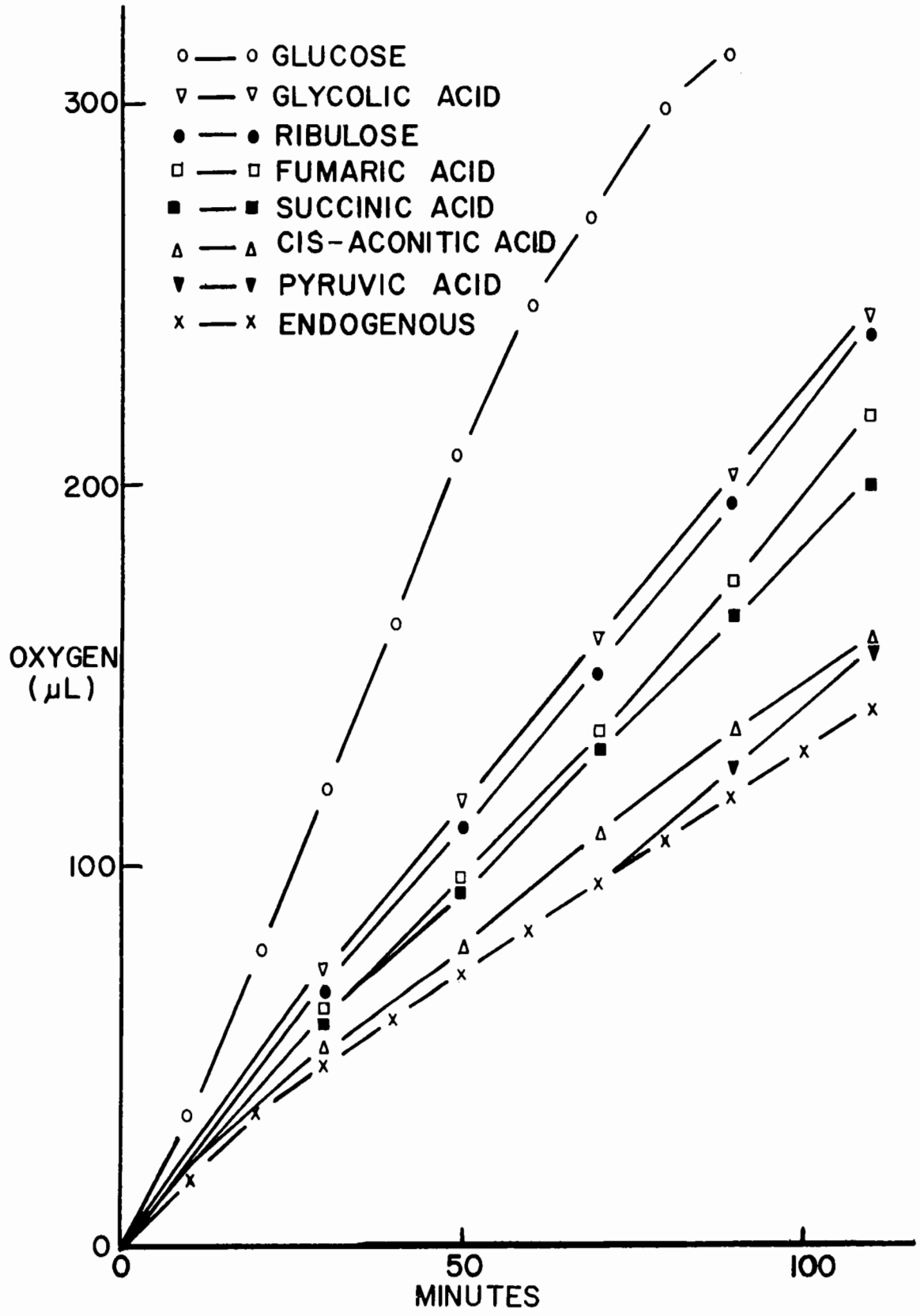

Figure 9 Oxidation of substrates by yeast-like cells of Mac M 18.

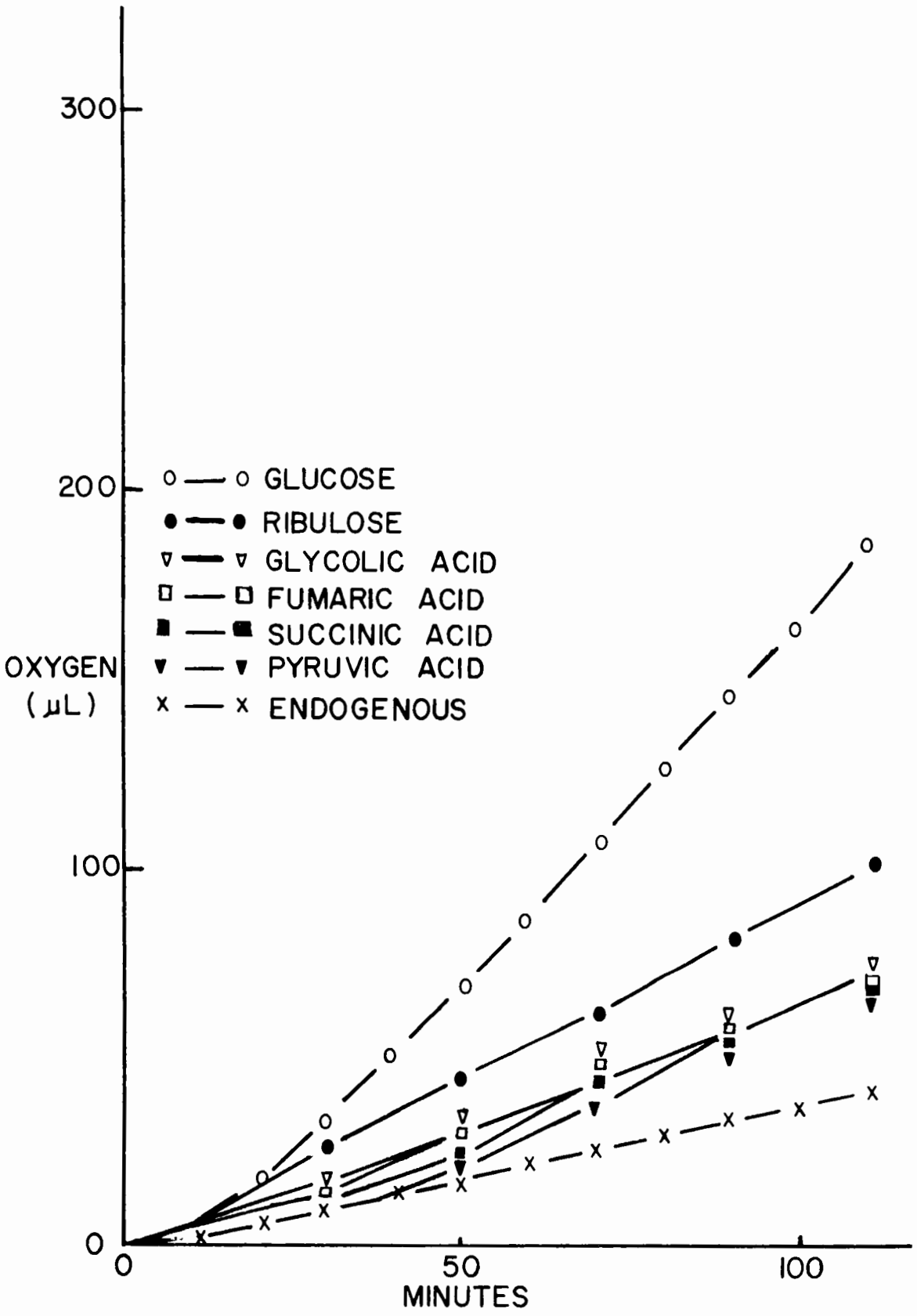

Figure 10 Oxidation of substrates by mycelium of Mac M 18.

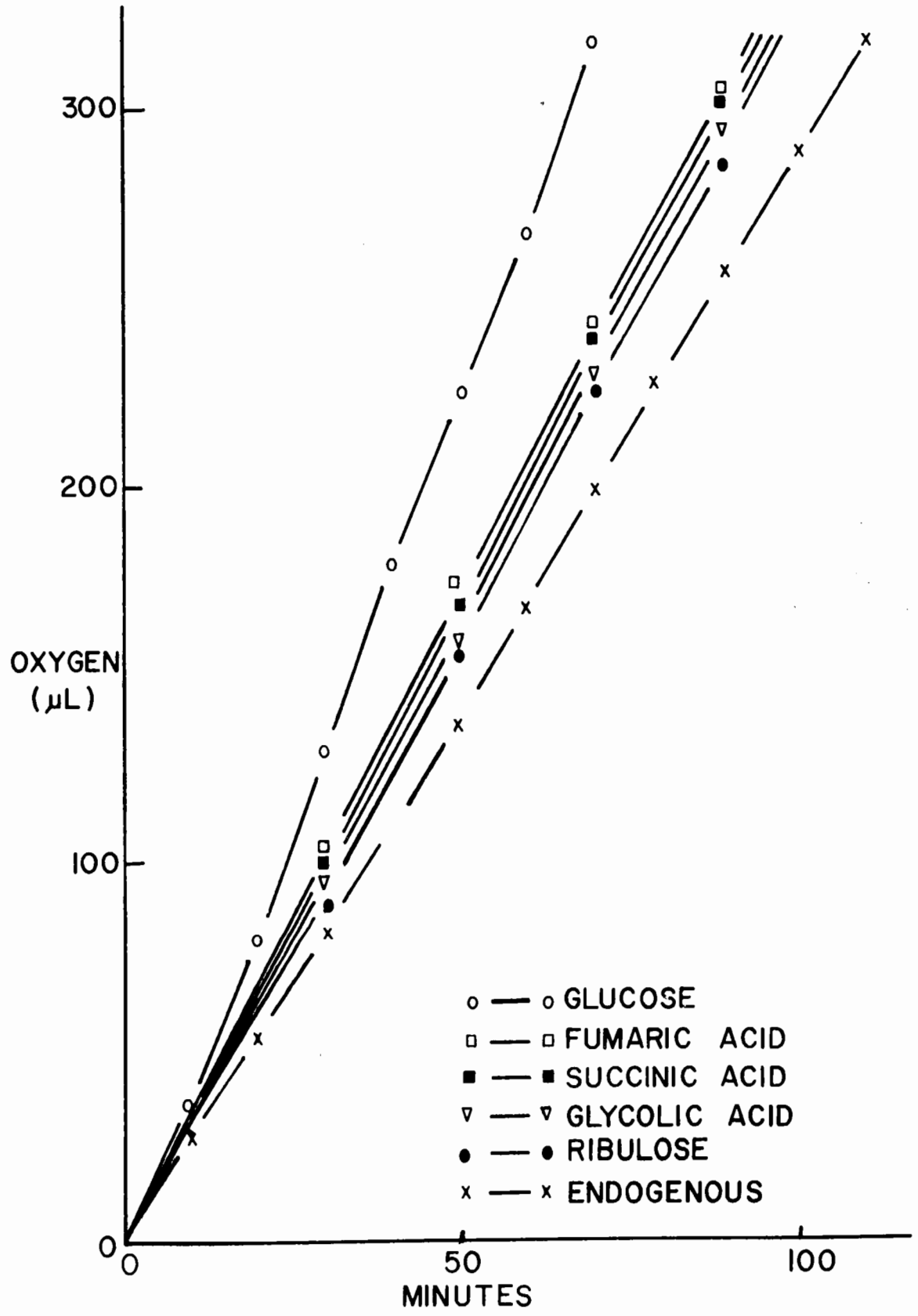

Figure 11 Oxidation of substrates by yeast-like cells of Mac M 21.

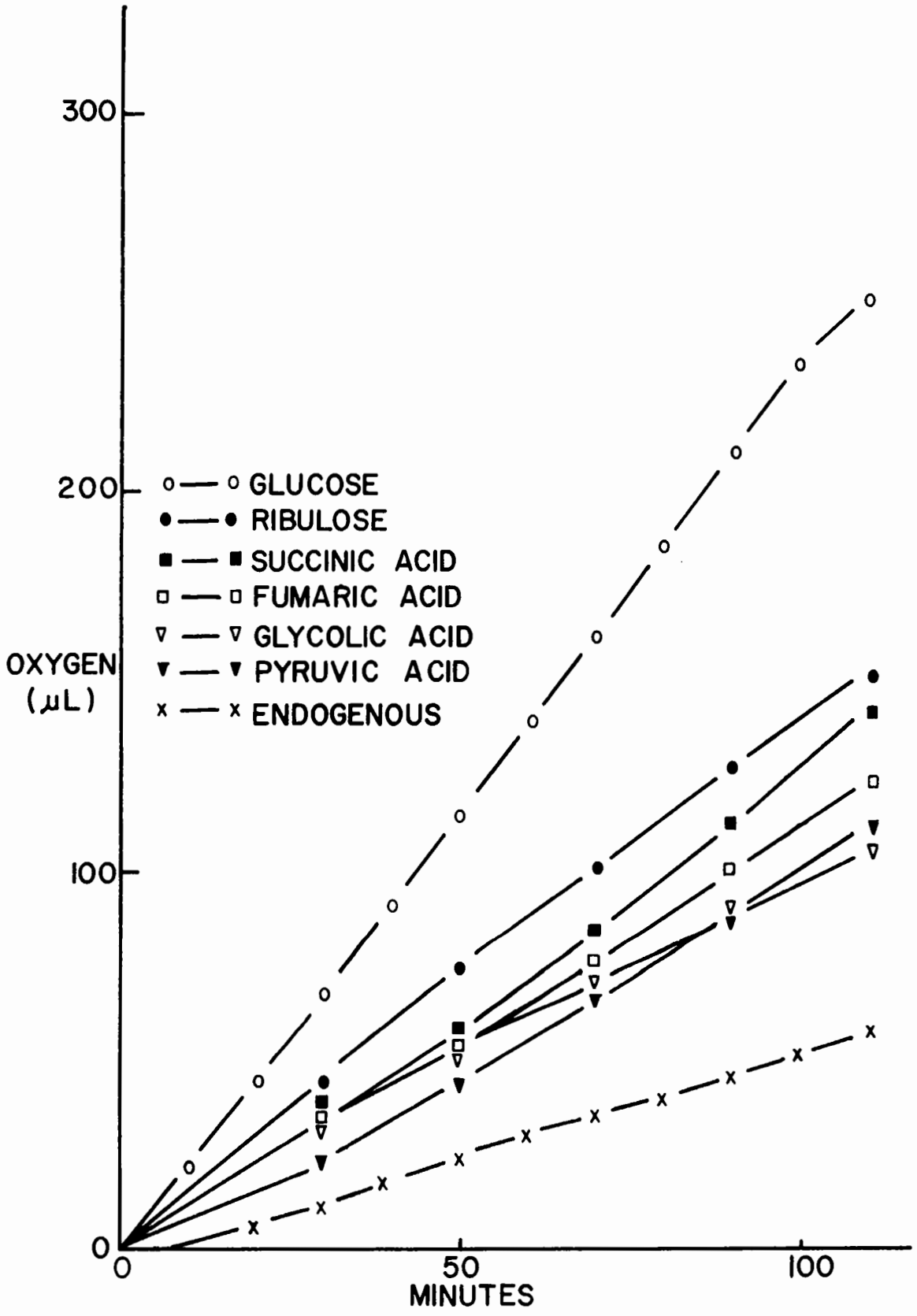

Figure 12 Oxidation of substrates by mycelium of Mac M 21.

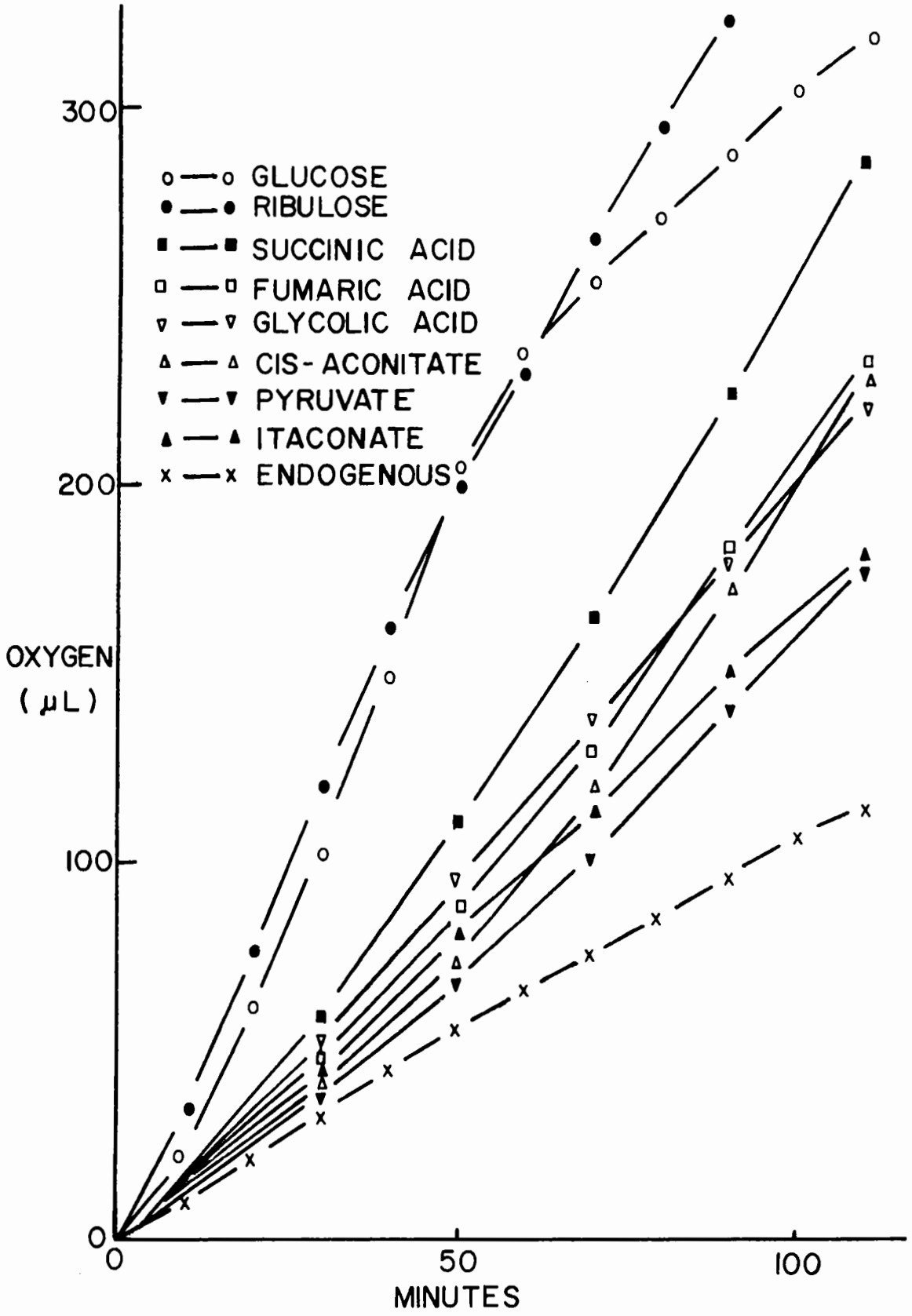

Figure 13 Oxidation of substrates by mycelium of Mac M 23.

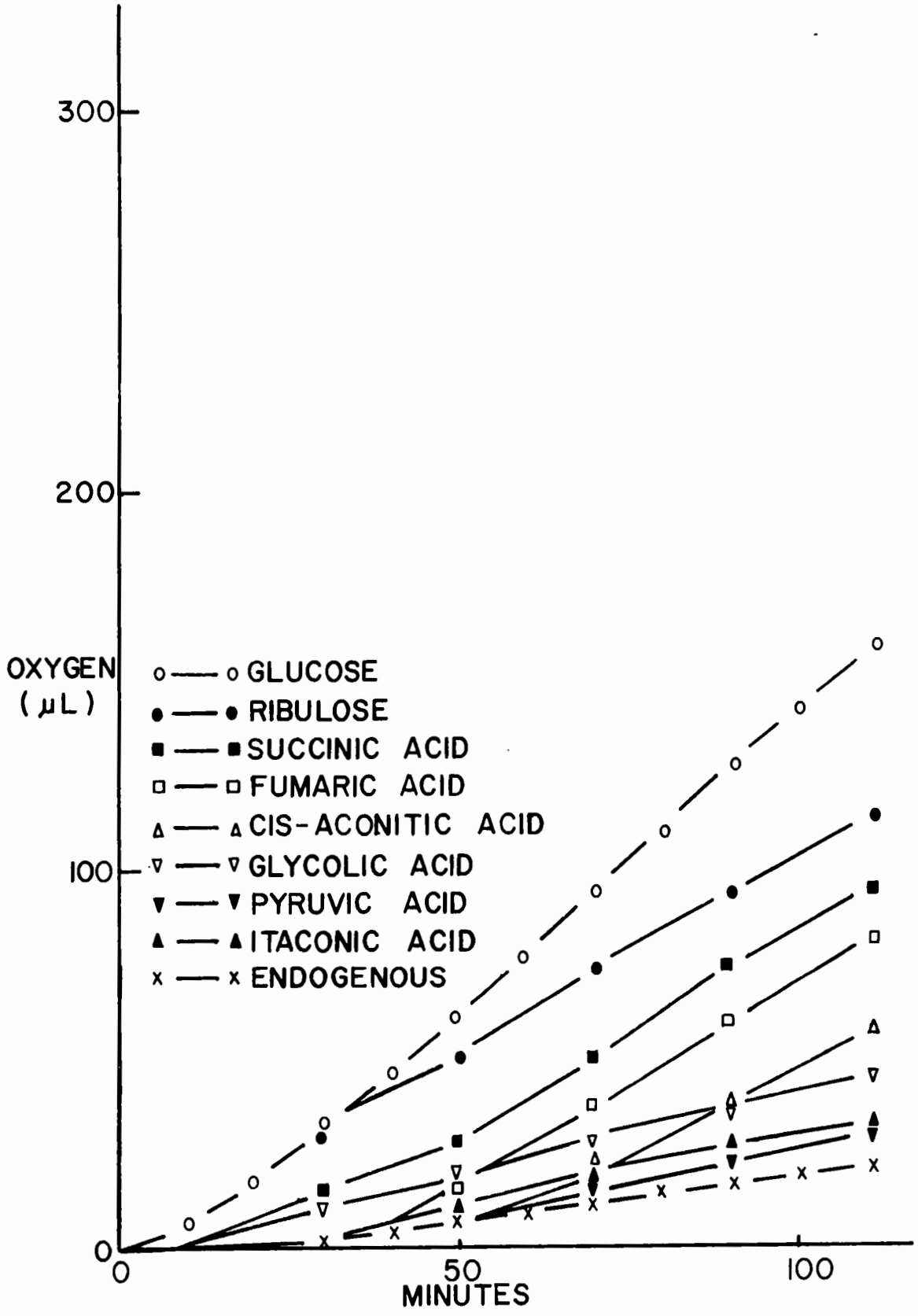

Figure 14 Oxidation of substrates by mycelium of Mac M 23.

acid after a short lag period.

cis-Aconitic acid was not utilized by either phase of Mac M 21; whereas, pyruvic acid was utilized by the mycelium but not by the yeast-like cells.

Pyruvic acid and cis-aconitic acid were utilized more rapidly by the yeast phase of Mac M 23 than by the mycelial phase. Both phases of Mac M 23 could oxidize itaconic acid but the culture could not grow on itaconic acid as the sole carbon source.

G. Fermentation of Glucose-1-C^{14} and Glucose-6-C^{14}

The amount of radioactivity added to each Warburg flask either as glucose-1-C^{14} or as glucose-6-C^{14} was the same because measurements of the radioactivity of equal portions of the sugar stock solutions showed that the C-6^{14} / C-1^{14} ratio was 1.026 (Table V).

The results (Table VI) show that the amount of carbon dioxide coming from the "one" position of glucose is higher than the amount coming from the "six" position on all trials. This occurrence is more pronounced in the mycelium than the yeast cells of Mac M 18 and Mac M 21.

TABLE V

Ratio of counts from Glucose-1-C^{14} and Glucose-6-C^{14}.

Sample	Weight(ugm.)	Counts per minute	c.p.m./(ugm.)glucose	Ratio $\frac{6\text{-}C^{14}}{1\text{-}C^{14}}$
Glucose-6-C^{14}	305	1,836	6.020	
	302	1,799	5.956	
	305	1,807	5.926	
				1.026
Glucose-1-C^{14}	316	1,863	5.897	
	302	1,766	5.780	
	297	1,715	5.774	

TABLE VI

Oxygen Consumed and Radioactive Carbon Dioxide Produced by the Dimorphic Forms of Mac M 18 and Mac M 21.

	Mac M 18				Mac M 21			
	Yeast		Mycelium		Yeast		Mycelium	
Flask *	c.p.m. 10uM CO_2	uL O_2	c.p.m. 10uM CO_2	uL O_2	c.p.m. 10uM CO_2	uL O_2	c.p.m. 10uM CO_2	uL O_2
Experiment I								
Endogenous		84		34		206		20
Glucose-1-C^{14}	740	203	1,097	197	844	289	681	138
Glucose-6-C^{14}	378	199	446	201	430	297	339	150
Ratio $\frac{CO_2\text{-}1^{14}}{CO_2\text{-}6\ 14}$	1.96		2.46		1.96		2.01	
Time (Minutes)	60		110		80		100	
Experiment II								
Endogenous		89		38		209		38
Glucose-1-C^{14}	705	205	1,060	204	944	291	1,021	169
Glucose-6-C^{14}	389	207	443	205	435	298	376	155
Ratio $\frac{CO_2\text{-}1^{14}}{CO_2\text{-}6^{14}}$	1.81		2.39		2.17		2.72	
Time (Minutes)	60		110		80		100	

* Each Warburg flask contained; 1.5 ml. 0.02M phosphate buffer pH 6.5, 0.5 ml. 0.02 M substrate, 1 ml. cellular preparation, and 0.2 ml. 20% NaOH.

DISCUSSION

Microscopical examination revealed that the method of separating the dimorphic forms of P. pullulans was satisfactory. The method was time consuming, however reduced pressure to aid filtering and washing would greatly speed up the procedure. The separation method, coupled with the dispersion caused during aeration to reduce endogenous respiration, broke the mycelium into fragments which could easily be suspended. This permitted the mycelium to be dispensed in the same way as yeast-like cells during Warburg experiments.

Cysteine is known to favour yeast-like growth of several dimorphic fungi (Nickerson and van Rij, 1949) and during this investigation cysteine was shown to facilitate yeast-like growth of P. pullulans. At least two enzymatic reduction mechanisms linking glucose metabolism with disulphide reduction are known. The disulphide of oxidized glutathione is reduced by the enzymatic transfer of hydrogen from TPNH (Conn and Vennesland, 1951 a,b); whereas, cysteine is enzymatically reduced by the transfer of hydrogen from DPNH (Nicherson and Romano, 1952). Nickerson and Mankowski (1953) have shown that Candida albicans will grow in the mycelial form on starch agar but yeast-like growth could be induced by adding either glucose or cysteine. The action of glucose

and cysteine was believed to provide DPNH and TPNH in quantities adequate enough to maintain the thiol groupings associated with cellular division (Nickerson, 1948; Nickerson, 1954). Since yeast-like growth of P. pullulans, like C. albicans, responds to cysteine it appears that P. pullulans requires similar thiol groupings for cell division to occur.

Selenious acid favoured yeast-like growth of two cultures of P. pullulans. These results agree with those reported for P. pullulans and C. albicans by Nickerson et al., (1956) who demonstrated the effect was not a selective inhibition of filamentous growth but a promotion of cellular division. Sulphur contained in protein occurs mostly in an oxidized state of disulphide (-S-S-) linkages; the sulphhydryl group (-SH) which is essential for the activity of many enzymes and hence possibly cell division, is readily subject to oxidation both in the cell and in isolated proteins. In contrast to the lability of -SH, protein bound selenium has been reported to occur in the reduced state (-SeH) and to be oxidized with difficulty. This explanation seems doubtful when applied to the results of this investigation because of the low concentrations of selenious acid found to affect dimorphism.

Cobalt sulphate supported mycelial growth of one of the cultures tested. Cobalt treated cells of C. albicans have been shown to form long mycelial fragments with no crosswalls and very

wide spacing between blastospore clusters (Nickerson and van Rij, 1949). A possible explanation is that cobalt forms complexes spontaneously with cystine and cysteine thus making them unavailable to the organism (Michaelis, 1929; Michaelis and Barron, 1929).

Aeration was found to favour yeast-like growth of one of the three cultures tested. This result disagrees with what Bauer (1938) reported for P. pullulans but it does agree with Wickerham and Rettger's (1939) results for C. albicans.

Thiamine promoted yeast-like growth of all cultures. Since P. pullulans has a requirement for thiamine (Ward, 1960) then a limited supply of this vitamin would slow down glucose metabolism with a corresponding decrease in reduced TPN and DPN. This could cause mycelial growth. A sufficient supply of thiamine would increase glucose metabolism thus supplying enough reduced TPN and DPN to induce cell division. If the concentration of thiol groupings associated with cellular division was critical then the difference in concentration of thiamine needed for growth and cell division and that needed for growth alone, need not be great.

Cells and carbon dioxide are the main products of glucose metabolism by P. pullulans but ethanol is sometimes a major product. Since itaconic acid can be oxidized by Mac M 23 and itaconic acid as well as the unidentified organic acids give the same colour

reaction with Erhlich's reagent, it appears that the unidentified organic acids are similar to itaconic acid. If one assumes that the unidentified organic acids are three carbon fragments or larger, it is evident that yeast-like cells can oxidize glucose further than can mycelium in the same growth period. The only other major difference between the two phases is the production of gluconate by the mycelial phase of P. pullulans. Nickerson (1954) found that a filamentous mutant of C. albicans could accumulate and reduce a tetrazolium dye; whereas, cells of the parent strain growing as a normally budding yeast accumulated the dye but did not reduce it. Nickerson concluded that the morphological mutant had an impairment of a cellular oxidation mechanism at a flavoprotein locus. This locus was the site at which a reaction essential for cellular division was coupled via an oxidation-reduction system to cellular metabolism. The production of gluconate by the mycelial phase but not by the yeast phase of P. pullulans seems very similar to the reduction of tetrazolium dyes by mycelium of C. albicans. The fact that gluconate and 2-ketogluconate were not oxidized by resting cells of P. pullulans and 2-ketogluconate was never detected in the culture fluid nullifies the existence of a catabolic scheme involving gluconate. The radioactive glucose experiments indicated that the pentose phosphate cycle operates well in both phases of P. pullulans and thus a partial blockage of the shunt

mechanism in the mycelial phase is unlikely. Also, the fact that gluconate is produced only by the mycelium of P. pullulans eliminates the possibility of a partial blockage. Therefore, there is a possible similarity between the production of gluconate by P. pullulans and tetrazolium dye reduction by C. albicans.

The pullulan produced by the cultures studied in this investigation was more like the dextran described by Bender et al., (1959) than the dextran obtained by Bernier (1958) in that glucose was the only sugar found to constitute the polysaccharide. The polysaccharide was precipitated free of protein by the method used.

Endogenous respiration is from four to nine times higher in the yeast phase than in the mycelial phase of P. pullulans yet the rate of glucose utilization is only one and one-half times higher in the yeast phase. In one instance, glucose utilization was actually faster in the mycelial phase. On this basis it appears that most of the reserve material is stored by the yeast-like cells. Possibly yeast-like cells would be more likely to survive unfavourable conditions.

Both phases of P. pullulans metabolized glucose, ribulose, fumaric acid, succinic acid and glycolic acid in a similar manner indicating a basic similarity between the dimorphic forms. Pyruvic acid and cis-aconitic acid were metabolized differently by each phase. It is difficult to explain anything from these differences although

the most obvious explanation is a difference in permeability of the cell membrane of the dimorphic forms.

The significance of the rapid utilization of glycolic acid is interesting because it is related to the carbon balances. All of the cultures produced formic acid during glucose metabolism. However, there are a number of possible routes of formate formation. Formic acid may be formed from pyruvic acid, oxalic acid, glycolic acid, glyoxalic acid, formylglutamic acid, formylaspartic acid, imidazole acetic acid and via the glycine-succinate cycle (Umbreit, 1960). Although none of these pathways may be ruled out, the glycolic acid-glyoxalic acid route appears to be the most probable since glycolic acid was utilized by glucose-grown cells of P. pullulans without a lag period. If P. pullulans possesses formic dehydrogenase, there is a possibility of glucose being oxidized to carbon dioxide by this route.

By measuring the radioactivity of carbon dioxide produced from specifically labelled glucose, the E.M.P. scheme and the pentose phosphate shunt were shown to be active in both dimorphic forms of P. pullulans. Katz and Wood (1960) have presented some of the difficulties encountered in evaluating the relative participation of different pathways during glucose utilization. Bloom et al. (1953) and Bloom and Stetten (1955) were the first to recognize and attempt to correct for the most important

source of error. By using $C^{14}O_2$ yields from lactate-3-C^{14} and lactate-1-C^{14}, they hoped to correct for the incomplete oxidation of the acetyl-CoA formed from pyruvate during glucose catabolism. To apply the correction they assumed that all the triose phosphate formed was oxidized to pyruvate and that it in turn was all decarboxylated to carbon dioxide and acetyl-CoA. Since a considerable amount of C^{14} of the triose phosphate may be incorporated into compounds without decarboxylation of pyruvate, the correction was without doubt too low. Another source of error is recycling of fructose-6-PO_4 through glucose-6-PO_4 in the pentose phosphate cycle but, by far the most important error is incomplete oxidation of acetyl-CoA. This source of error has lead Katz and Wood (1960) to conclude that by itself the determination of the $C^{14}O_2$ yields from glucose-1-C^{14} and glucose-6-C^{14} provides no useful quantitative and only very limited qualitative information on the patterns of glucose metabolism. The amount of carbon dioxide coming from carbon "one" of glucose is slightly higher when glucose is metabolized by the mycelial form of P. pullulans. The carbon balances indicate that the mycelial phase cannot oxidize glucose as completely as the yeast phase during the same time period. This, according to Katz and Wood, would result in a slightly higher CO_2-$1^{14}/CO_2$-6^{14} ratio for the mycelial phase, which agrees with the results. Otherwise, the ratios of CO_2-$1^{14}/CO_2$-6^{14} are very close indicating that glucose was metabolized via the same pathway by the dimorphic forms. No attempt was made during this investigation to evaluate the relative

participation of the E.M.P. scheme and the pentose phosphate scheme. The investigation merely demonstrated that the participation of each scheme was the same in both dimorphic forms.

No relationship between morphological form and glucose metabolism could be established except possibly the formation of gluconate. Thus it seems that metabolism and cell division are completely divorced from one another. These results support the basic contention that "the manifold processes of growth and cell division that comprise cellular multiplication are not inextricably linked." (Falcone and Nickerson, 1958).

SUMMARY

A method of spearating the dimorphic forms of Pullularia pullulans was developed and used to study the effect which cysteine, selenious acid, cobalt sulphate and increased aeration have on the dimorphism of the fungus. Cysteine favoured yeast-like growth of all cultures tested; whereas, selenious acid promoted yeast-like growth in two of three cultures. Cobalt sulphate fostered mycelial growth of one culture and increased aeration favoured yeast-like growth of one culture. From these data media were developed which supported growth of predominantly one or the other dimorphic form of each culture.

Products of glucose catabolism by both dimorphic forms of three cultures were identified and measured quantitatively. Most of the glucose was either assimilated or converted to carbon dioxide, however in some instances ethanol was produced in substantial quantities. The three cultures studied also produced from the fermentation of glucose, formic acid, succinic acid and the extracellular polysaccharide, pullulan. In addition, two cultures produced acetic acid and one culture produced lactic acid. The only significant difference in the end-products of glucose metabolism by the dimorphic forms of P. pullulans was the production of gluconic acid by the mycelial phase of two of the cultures.

Warburg respiration experiments revealed that glucose, ribulose, succinic acid, fumaric acid and glycolic acid were utilized equally well by both dimorphic forms. Endogenous respiration was considerably higher in the yeast-like cells. Gluconic acid and α-ketogluconic acid were not utilized by either phase of any culture. The utilization of pyruvic acid and cis-aconitic acid varied considerably from culture to culture and from one dimorphic form to another.

The measurement of radioactive carbon dioxide produced from glucose-1-C^{14} and glucose-6-C^{14} revealed that the pentose phosphate shunt and the Embden-Meyerhof scheme operate similarly in both dimorphic forms of two of the cultures of P. pullulans.

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