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# RADIORESISTANCE AND OTHER ASPECTS OF *AUREOBASIDIUM PULLULANS* (DE BARY) GROWN AEROBICALLY AND UNDER NITROGEN.

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## ABSTRACT

The radioresistance of the blastospores and chlamydospores of *Aureobasidium pullulans* (de Bary) grown in shake culture was increased by cultivation at constant pH and nitrogen tension and further enhanced by following this with aerobic growth conditions. The ultrastructure of the blastspore and chlamydospore grown under nitrogen and oxygen were compared. Changes of cell wall and mitochondria were evident. Studies of the composition of the aerobically produced pigment were made.

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## INTRODUCTION

One of the most important representatives within a group of radio-resistant microorganisms is the yeastlike fungus Pullularia pullulans (de By.). Due to its polymorphic form, dependent upon growth conditions, it was isolated and described under some synonyms like Aureobasidium pullulans, Dematium pullulans, and Pullularia pullulans(3).

The nonspecific nutritional requirements of P. pullulans makes it a commonly and frequently encountered organism. Bauer (1) reported about its osmophilic character, being able to divide in saturated sugar solutions. Bender (2) and Cooke (3) described growth conditions and the formation of an extracellular polysaccharide which was extensively produced in media with glucose or sucrose, the amounts of which depend on the sugar concentration. Merdinger (10) studied their growth in high glucose concentrations and showed that trehalase was its main carbohydrase and that it has ability to grow over a broad pH range.

The present work investigated the survival of blastospores and chlamydospores after irradiation with $^{60}Co$ and describes the cytology of anaerobically and aerobically grown cells by electronmicroscopic techniques. Finally, some attempts were made to determine what relation pigment formation plays in the above.

## MATERIALS AND METHODS

Pullularia pullulans, (NrrL YB-4515) a Danish strain, was cultivated on potato-dextrose agar pH 4.6 at 23°C. For investigative work the organism was grown in shaken cultures or in a pH static system in potato-dextrose broth (Difco). Shaken cultures were incubated for three weeks in a Brunswick Gyrotory shaker at 30°C (1000 ml Erlenmeyer flasks with 400 ml medium). The blastospores were harvested by centrifugation at 3000 rev/min and washed three times with 0.15 M phosphate buffer pH 7.0. The coated chlamydospores could be separated from the blastospores by decantation after centrifugation because they did not sediment at 3000 rev/min as the uncoated blastospores. Separated chlamydospores were then partially sedimented at 4000 rev/min.

Static Growth Conditions: A 14 liter Brunswick microfermentor, adapted with a Metrohm pH meter for automatic control of the pH to 4.6 and containing 12 liters of potato-dextrose media, was inoculated with 400 ml of a 12-hr-old suspension of P. pullulans. The system was held anaerobically by a flow rate of 2 liters per min of sterile nitrogen. After four days the unpigmented blastospores were harvested from 6 liters of medium by centrifugation. Aeration of the remaining 6 liters of medium was continued with a flow rate of 2 liters per min. After 1.5 hr of aeration cell material was collected from 3 liters of medium for study of ratio-resistance, while aeration of the remaining medium was continued for electronmicroscopic studies.

Irradiation: Irradiation experiments were carried out in a $^{60}Co$ source with a dose rate of 540,000 rad per hr at 20°C. Cells were suspended in 0.15 M phosphate buffer pH 7.0. After irradiation the suspension were diluted and plated on potato-dextrose agar. For every irradiation dose three plates with three dilutions were used. Every experiment was repeated three times. The sterile plastic Petri dishes were incubated at 30°C and after three days the colonies counted.

Electronmicroscopy: The ultrastructure of blastospores and chlamydospores was investigated, in the sections after embedding in epon and vestopal resin (1, 13), and with freeze etching method (12). Thin sections were produced with an automatic Reichert-Ultramicrotome. Glass knives were obtained with a LKB knife maker. Freeze-etched preparations were produced with Balzers instruments. After etching, the replicas were treated with sulfuric acid and Dakins solution to remove the remaining organic particles. After final washing with distilled water the copies of the broken cell surfaces were picked up on formvar coated grids. Electronmicroscopy was performed with a Siemens UM 60. Samples were taken at different times during both anaerobic and aerobic growth for the cytological investigations.

Pigment: Pigmented Cells were broken by homogenization with a Sorvall Omin-mixer by adding to each gram of cells 3 g of 0.1 mm glass beads and 3 ml of 0.1 M acetate buffer (pH 5.5). The pigment was recovered by centrifugation at 40,000 rev/min. The pigment was dialyzed exhaustively versus water and subjected to Molisch and Benedicts tests before and after 1 hr with hydrolysis 1 N HCl. Paper chromatograms were run ascendingly with Whatman #1 employing t-amyl alcohol:i-propanol:water:4:1:1.5; v:v:v, to identify hydrolysis products. Spots were detected using an ammoniacal silver nitrate spray.

## RESULTS AND DISCUSSION

The survival curves of three-week-old blastospores and chlamydospores harvested from shaken cultures are different in their radioresistance at low doses (Fig. 1). Growth inhibition of chlamydospores (Curve A) appeared at 135 krad whereas the blastospores (Curve B) began to be affected at 220 krad. The $LD_{99}$ was the same (650 krad) for both cell forms. The separated blastospores (Curve C) cultivated anaerobically at constant pH, were more resistant ($LD_{99}$ 860 krad) than the three-week-old cells from shaken cultures. The resistance was enhanced (Curve D) when the anaerobically grown cells were further cultivated aerobically for 1.5 hr. The $LD_{99}$ of these cells increased to 1.4 Mrad. Growth of cells was slow and pigment formation was suppressed for the 48 hr duration of anoxic conditions. But after four hours under oxygen the cells grew rapidly and

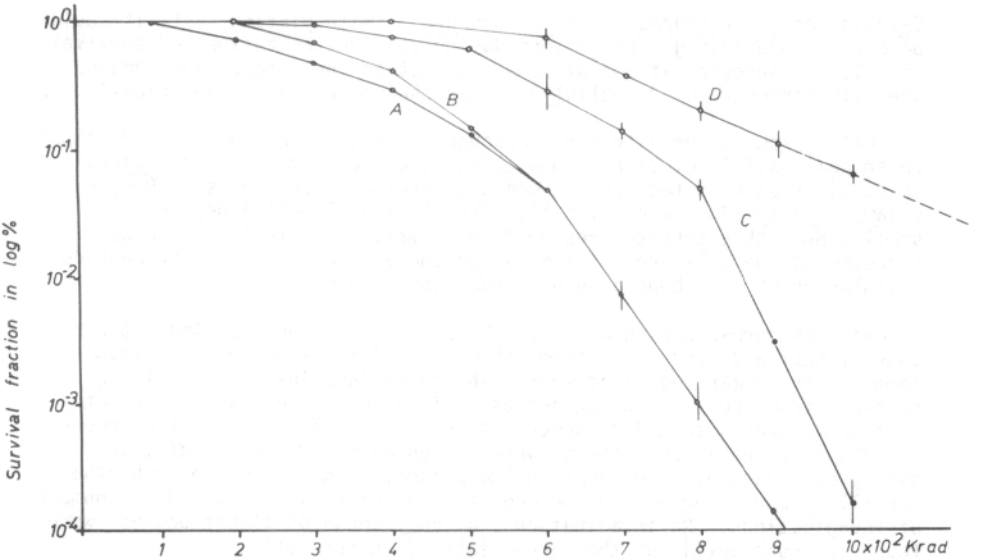


Fig. 1. The radioresistance of blastospores and chlamydospores of A. pullulans at low doses is illustrated in their survival curves. Curve A shows that inhibition of chlamydospores appeared at 135 Krad, while inhibition of the blastospores (Curve B) began at 220 Krad. Curve C shows blastospores grown under nitrogen and constant pH. Curve D shows blastospores grown as in Curve C but having 1.5 hr of aerobic growth prior to irradiation.

formed pigment suddenly. Preliminary attempts at characterizing the pigment of the aerobic cells has yielded the following findings. The pigment is most likely a macromolecule since it was sedimented at 40,000 rev/min and was not dialyzable after eight days. The isolated pigment gave a negative Benedict's but postive Molisch test. After acid hydrolysis the Benedict's test became positive for the neutralized hydrolysate. The color and amount of the pigment remained unchanged after the hydrolysis. Chromatograms of the concentrated hydrolysate showed that glucose was the only detectable monosaccharide in the system employed.

Neither the black pigment nor the coating of chlamydospores protected the cells from the effects of ionizing radiation since the nitrogen-grown cells lack these factors but are nevertheless more radioresistant than the coated and black-pigmented cells of a shake culture. Merdinger (10) previously reported the conditions for formation of pigment by A. pullulans, which seemed not to be identical with melanin, as suggested by Bender

et al. (2) and Durrell (6). Whether the carbohydrate, which is an integral part of the pigment is mono-, di-, or polysaccharide was still to be established.

Cytology of blastospores and chlamydospores: With plating tests it could be established that gas conditions during cultivation influenced survival of cells. Therefore it was of some interest to investigate and compare the ultrastructure of *P. pullulans* grown anaerobically and aerobically.

Cells grown under nitrogen atmosphere showed the absence of a distinct outer cell wall (Fig. 2) as compared to aerobically grown *P. pullulans* (Fig. 3) or as reported for the conidiospores and ascospores of *Byssochlamys fulva* (13). Where visible, the outer cell wall appeared as a small line with a density greater than normal. The fluffy particles surrounding the cell are not similar to the extracellular polysaccharide pullulan which is produced under oxygen conditions.

Nitrogen grown blastospores exhibited invaginations varying between short linear and large bent forms (Fig. 4). After aeration the plasmalemma of these aerated blastospores showed no invaginations or similar surface structures. This conversion of invaginated membranes to smooth-surface membranes seemed to occur within 4 h. Differences in the number and shape of the invaginations, which depended on the gas conditions during cultivation, were found in the present study. Moor and Muhlethaler (11) found a correlation between the age of yeast cells and the number of invaginations. No invaginations, or only those of slight degree, were found in young cells but they accumulated in older cells.

In some mitochondria (Fig. 5) the cristate are only small lines, and in some there is only a diffused zone, which may indicate the residue of the mitochondrial cristae. Comparing Figure 5, showing an early stage of growth under oxygen, to Figure 3 showing cells cultivated under oxygen for a longer period, we find that the number of hypertrophic mitochondria is approximately equal to the number of lipid bodies in the boated cells. The presence of lipid and its enhanced synthesis in aerated cultures may be an additional factor in the radioresistance of *A. pullulans*. With the progress of the culture all mitochondria may have changed to lipid bodies. That great changes in cell metabolism occur is also indicated by a fast increase in KOH consumption. Similarily with the activation of the cell metabolism the formation of pigment and pullulan start within a short time.

Acid production, as evaluated by consumption of KOH, occurred in a linear fashion under nitrogen growth conditions for 4 days but increased rapidly upon aeration. Bauer (1) found a relative independence of pH on the growth of *A. pullulans*. Greater radiosensitivity may be related to unfavorable growth conditions, to the decrease of the viability with age, and to deficiency of media and simultaneous accumulation of extracellular products especially organic acids which may act on cell growth and enzyme systems. It is assumed that the repair-enzyme systems may also be affected by long cultivation in shake culture. Serianni (14) found that the cells of *Micrococcus radiodurans* were more sensitive to x-rays in the logarithmic growth phase than in the stationary one. Contrariwise, the

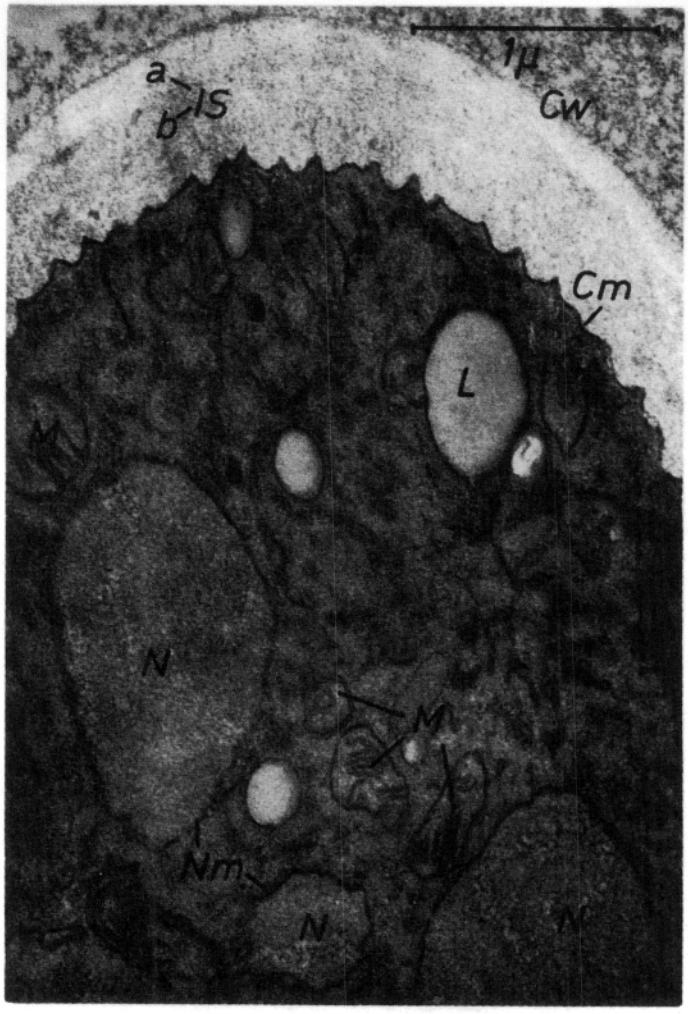


Fig. 2.* A blastospore of Aureobasidium pullulans showing three nuclei and a great number of mitochondria, perhaps in a promitochondrial stage. Mitochondria with different forms of cristae may be noted. The outer cell wall appears as a small line with a density greater than normal. The fluffy particles coating the cell are pigmentation granules.

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* Abbreviations used for figures. Cw Cell wall, IS intermediate space, C Cytoplasm, N Nucleus, Nm Nuclear membrane, M Mitochondria, ER Endoplasmic reticulum, L Lipid, I invagination of the cytoplasm membrane, P Pores of the Nucleus, Cm Cytoplasm membrane.

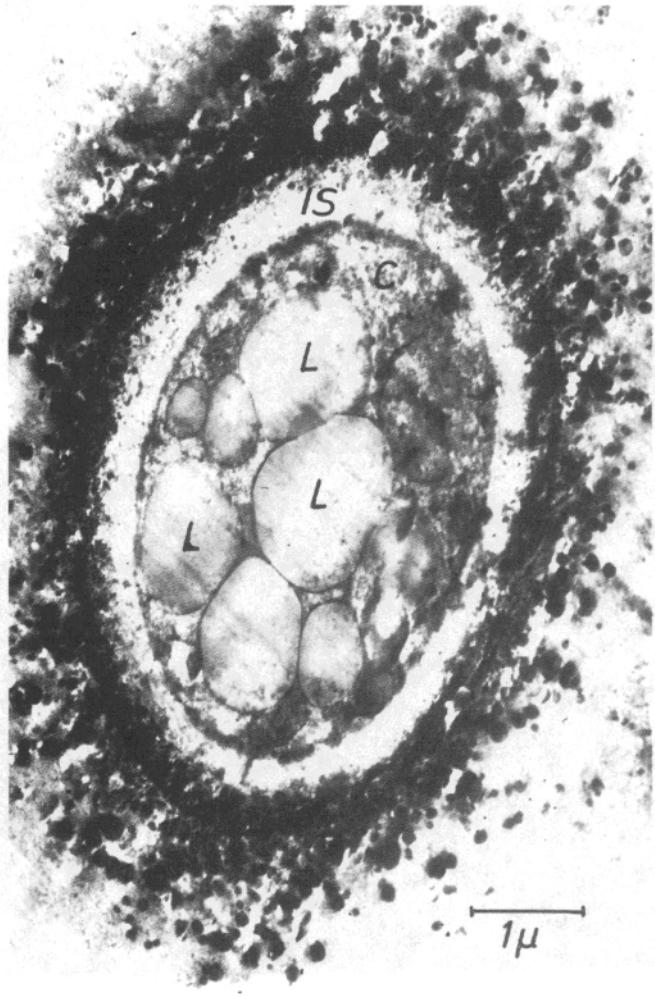


Fig. 3. Aureobasidium pullulans grown under aeration. The cell, surrounded by pullulan shows the cytoplasm filled with lipid bodies. The portion of the cell where the formation of pullulan was observed to originate was the IS as reported by Durrell (6). In the thin sections, black points of pullulan were found only in the outer portion, whereas the inner portion of the intermediate space seemed unchanged. Pigment initiation also seemed to occur in the IS.

Fig. 4. The surface of the cytoplasm membrane. In the freeze-etched cell peculiar formed invaginations are visible which are not present within aerobically grown cells.

Fig. 5. A double cell in the early stage of aeration following anoxic cultivation. Lipid droplets are accumulating and the blastospore contains a great number of hypertrophic mitochondria. The inner mitochondrial space of the cristae contains only small lines and in some units are only found diffused zones, the inner zone seems lysed. This is observed to be the first stage of conversion to lipid bodies. The final stage-lipid bodies are also present.

present investigations revealed an increase in the radioresistance of A. pullulans in the logarithmic growth phase at constant pH and high aeration.

Swelling effects of mitochondria were described as occurring in many tissues, as heart, liver and others (7). David (4) gave a very complete picture of this effect. Hirano and Lindgren (8) noted the disappearance of mitochondria at the transition of aerobic to an anaerobic way of life, whereby the endoplasmatic structures increased. With longer aeration of A. pullulans mitochondria decreased in number proportionately with the increase of lipid in the cytoplasm. Though Duncan and Hild (5) discussed the question of whether mitochondria are able to change directly into lipid bodies, our experiments seem to present this as a definite possibility. That is, with the progress of the culture all mitochondria seemed to have changed to lipid bodies.

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