EFFECT OF LIGHT AND MEDIA UPON GROWTH AND MELANIN FORMATION IN AUREOBASIDIUM PULLULANS (DE BY.) ARN. (=PULLULARIA PULLULANS)

by

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(23.II.1963)

(with 8 figs.)

Aureobasidium pullulans, commonly called a "black yeast", has been isolated from air, soil, poultry, fruits and other natural substrates. It is a common laboratory contaminent, a common soil-borne saprophyte (GILMAN 1957), a plant pathogen , and has been implicated in human infections as well (WYNNE & GOTT, 1956). BARNETT (1960) points out that the light-colored variants of this fungus resemble *Candida* spp. and also have been confused with other human pathogens such as *Cladosporium* and *Hormodendron*. This becomes evident by the listing of nearly eighty synonyms for this fungus by COOKE (1959) who prefers to call this fungus *Aureobasidium pullulans* because of nomenclatorial priority.

In addition to being ubiquitous and omnivorous, *Aureobasidium* also is polymorphic. Initially, colonies of this fungus are yeastlike añd colorless (white) and gradually turn black as the culture ages and develops mycelia. A culture of this fungus shows varying proportions of cells, conidia, pseudomycelia and irregular hyphae depending on the strain, media, cultural conditions, and age of the culture. In view of the variability of *Aureobasidium*, it is understandable how the nomenclatorial difficulties alluded to above have arisen.

An association between pigmentation and pleomorphism in this fungus has been assumed (COOKE, 1959) but very little work has been done on the subject. WYNNE & GOTT (1956) reported extracellular pigment production by several strains of this fungus under anaerobic conditions. CIFERRI *et al.* (1956) reported that pigment production varied with the strain of the fungus used and with the oxygen tension, and NICKERSON *et al.* (1956) showed that selenite (10^{-3}M) suppressed pigment production and promoted formation of

the yeast phase. The present work was undertaken to investigate further the nature of pigment production in *Aureobasidium,* and the effect of environmental variables upon its growth.

MATERIALS AND METHODS

A culture of a black yeast was isolated as a laboratory contaminant, and was identified as *Pullularia pullulans* by Dr. L. WICKER-HAM, Northern Regional Research Laboratory, Peoria, Illinois. The following media were used and any divergences are mentioned in the text.

I. Defined medium #1:

Asparagine	0.2 g
KH_2PO_4	0.15 g
Mg SO_4. 7 H_2O	0.05 g
$FeCl_3$	0.2 mg
$ZnCl_2$	0.1 mg
$MnCl_2$	0.02 mg
Biotin	0.1 μg
Carbohydrates	20.0 g
Water	1000 ml

II. Defined medium #2:

Casein hydrolysate	80.0 ml
KH_2PO_4	1.5 g
$MgSO_4$. $7H_2O$	0.5 g
Mineral stock soln.	0.4 ml
Carbohydrate	20.0 g
Water	1000 ml
Mineral stock solution:	
H_3BO_4	0.2 mg
$ZnSO_4.7H_2O$	0.4 mg
$NaMoO_4.2H_2O$	0.1 mg
$CaCl_2.6H_2O$	0.1 mg
$CuSO_4.5H_2O$	0.2 mg
$MnSO_4$. H_2O	0.1 mg
$CaCl_2$	0.2 mg
$FeSO_4.7H_2O$	0.25 mg
Water	

The tables show the different carbohydrates or other amendments used in the media. Media were adjusted to pH 6.7—6.8 using KOH. Erlenmeyer flasks, of 125 ml capacity, contained 45 ml of medium and were plugged and autoclaved at 15 lb pressure for 20 minutes. The flasks were inoculated with known amounts of washed cells taken from one-week-old cultures and were incubated at 21°C

on a rotary shaker in light or in total darkness. Unless otherwise noted, all experiments were run in duplicate. Organic addenda, such as amino acids, casein hydrolysate and beef or yeast extracts were added to the growing cultures as sterile solutions. Growth measurements were made by drying at 95°C overnight. Undefined media such as potato dextrose agar, prune agar and corn meal agar were prepared from commercial sources. Dextrine was purified by shaking with absolute acetone, for three days at 21°C, with daily changes of acetone after which it was air dried and shaken with several changes of distilled water.

Separation of melanin granules from fungal cells:

In the usual experiment, the contents of three 125 ml flasks were pooled and centrifuged at 2,000 × g for three minutes and the supernatant which contained most of the granules was decanted and saved. The sediment was resuspended in 0.01 Na_2CO_3 and centrifuged again as before. This procedure was repeated several times until the sediment consisted only of fungal cells. The combined supernatant suspensions were centrifuged for five minutes at 12,000 × g and the precipitate collected. Such precipitates contained mostly granules which appeared black in the aggregate, and very few fungal cells.

Extraction of melanin:

Granules, prepared as described above, were mixed with six times their volume of acid-washed glass beads (Minnesota Mining and Manufacturing Co. Super-Brite pavement markers) and disrupted in a Nossal disintegrator (NOSSAL, 1953) for 30 seconds at 1°C. Melanin was extracted in 15 volumes of 0.5N NaOH (based on the volume of the granule-bead mixture) with occasional shaking at room temperature for two hours (WHITE, 1958). This material was decanted and centrifuged at 2,000 × g for two minutes and the colored supernatant solution collected. This procedure was repeated until no more color could be extracted from the precipitate. The pH was adjusted to neutrality with HCl and the material was incubated at 4°C for one hour, upon which a precipitate of melanin was obtained. Dry weights of melanin were obtained after drying the precipitate at 96°C for 4—6 hours.

The melanin of the fungal cells was extracted by crushing cells from which the granules had been removed, as described above, in a Nossal disintegrator for two minutes (SCHAEFFER, 1953). Thereafter, the melanin was extracted in 0.5N NaOH as described previously.

Tests for the presence of melanin: (WHITE, 1958)

"Positive" tests—
1. solubility in hot 0.5N NaOH— Melanin precipitates dissolve completely in hot 0.5N NaOH.

2. presence of colorless or grayish particles after extraction with hot 0.5N NaOH— Animal melanins are known to form such granules and it is believed that this residuum is a protein which accompanies the melanin (PEARSE, 1961).
3. solubility in cold 1N Na_2CO_3— Melanin is dissolved in cold Na_2CO_3 but colorless particles remain in the residuum, as in the case of treatment with NaOH.
4. reaction with $FeCl_3$— In the presence of a pinch of Fe_2CO_3 melanin solutions show a thick brown precipitate which disappears upon the addition of more of the salt.
5. absorption spectrum— Melanin in solution gives a characteristic absorption curve between 400—600mμ.

"Negative" tests—
1. solubility of melanin in water— Neither hot nor cold distilled water extracts any color from melanin.
2. solubility of melanin in organic solvents— No color is extracted from melanin in ethanol, chloroform, diethylether, acetone, or mixtures of these solvents.

According to PEARSE (1961, p. 633), "The most striking characteristics of melanins . . . is their complete insolubility in organic solvents or in anything that is not markedly destructive to the tissues which contain them". Moreover, melanins are soluble in strong NaOH and KOH but lipofuchsin is not. Another characteristic of melanins is the readiness with which they may be bleached. Thus, strong oxidizing agents such as H_2O_2, $HClO_4$, $KMnO_4$, and $FeCl_3$ accomplish this but the process requires about 24 hours. Because of the difficulty in analyzing melanin pigments their chemistry is not well known so that there is a degree of non-specificity in their identification. Nevertheless, melanins are usually defined as being pigments that are insoluble in substances that leave tissues intact, are soluble in NaOH, are bleached by strong oxidizing agents, and are reduced ammoniacal silver nitrate.

Total nitrogen determination:

As a part of the measurement of growth, total nitrogen determinations of the oven-dried cells and granules were performed according to a modification of the method of KOCH & McMEEKIN (1924). Ten mg aliquots of cells and granules were digested with sulphuric acid and H_2O_2. Appropriate dilutions of the digest were then made with water and Nessler's reagent was added. The resulting color was determined in a colorimeter and the total nitrogen of the samples was calculated with reference to a standard curve.

RESULTS

Growth and melanin production in Aureobasidium pullulans:

Growth of *Aureobasidium pullulans* in the dark and light was

studied using defined medium $\neq 1$ containing 0.02% asparagine and 2% of a number of carbohydrates. The data in table I reveal that among the monosaccharides, glucose is a better source of energy than is fructose or galactose. Among the disaccharides, sucrose and maltose are better carbon sources than trehalose, while lactose is the poorest of this class of compounds. As for the polysaccharides, the order of effectiveness is dextrine, starch, pectin, inulin. Except

TABLE I.

Growth and melanin formation of Aureobasidium pullulans in defined medium $\neq 1$ with asparagine (0.2 g/liter). Cells grown for 14 days at 21°C.

Carbohydrates	Culture color	Cell dry wt. g	Characteristics of cells	Melanin pigment test
Glucose	dark green to black	light: .1668 Dark: .1580	Large, thick-walled and with brown granular contents	Black precipitate from cell extract
Fructose	dark green to black	Light: .1089 Dark: .1072	Same as above	Brown precipitate from cell extract
Galactose	greenish black	Light: .0960 Dark: .0852	Mostly thin-walled and a few giant thick walled	No precipitate
Sucrose	dark green to black	Light: .1849 Dark: .1780	Same as in glucose	Same as in fructose
Lactose	Colorless	Light: .0119 Dark: .0039	Thin-walled colorless cells	No precipitate
Trehalose	pale brownish green in dark and colorless in light	Light: .1405 Dark: .1281	Thin-walled small cells	No precipitate
Maltose	dark green to black	Light: .1726	Same as in glucose	Brown precipitate from cell extract
Dextrine	,, ,,	Light: .2443 Dark: .1265	,, ,, ,, Abundant extracellular dark granules	Brown pigment from cell extract and from granules
Starch	,, ,,	Light: .2077 Dark: .1361	Same as in glucose	Dark brown precipitate from cells
Inulin	Colorless	Light: .0333 Dark: .0262	Small thin-walled cells.	No precipitate
Pectin	dark green to black	Light: .1227 Dark: .1127	Same as in glucose	Precipitate from culture fluid but not from cells.

Differences in color of the cultures in light and darkness are not mentioned when not significant.

for lactose, upon which *A. pullulans* grows poorly, the disaccharides are a better source of carbohydrate than are the monosaccharides.

Light increases growth markedly when this organism is grown upon polysaccharides like dextrine and starch, but not when the other sugars are used. Although there is an effect of light when lactose and inulin are the carbon sources, growth is so slight that the effect is doubtful.

Fig. 1. Cells grown in the light on defined medium #1 containing 2.0 gm asparagine per liter and 2% lactose, trehalose or inulin as the carbon source. Magnified about 500 ×.

Several classes of cells are found in these cultures, including colorless ones with thin walls and yeast-like morphology, and dark, relatively large, thick-walled ones with brown granular contents. These types are illustrated in figs. 1—4. The colorless cells are produced upon lactose, inulin, galactose and trehalose whereas the dark ones are found upon the other sugars. With the exception of trehalose, those sugars which induce the formation of the colorless

cells are the ones upon which *Aureobasidium* grows least well. Growth upon trehalose differs from that upon the other sugars which support growth in that the medium remains colorless in the light in contrast to the dark color obtained in the media when the other sugars are used.

Dark-colored extracellular granules, like those illustrated in fig. 5 are found when dextrine is the carbon source. These particles
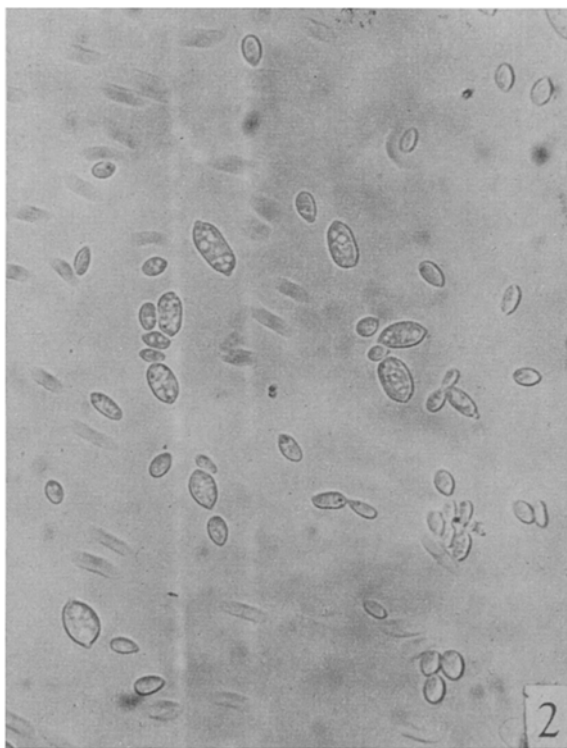

Fig. 2. Cells grown in the light in defined medium ≠1 containing 0.2 gm asparagine per liter and 2% glucose. Culture medium appears pink. Magnified about 500 ×.

appear after about 4 days of growth. They are not found in any of the other media.

The presence of melanin was investigated by grinding and extracting the cells as described in Methods. One test for melanin, the results of which are included in table I, depends upon the formation of a precipitate upon neutralization of the alkaline extract. Precipitates, suggesting the presence of melanin, were obtained in

extracts from thick-walled brown cells found in all carbohydrates. Conversely, no precipitate could be found in extracts from thin-walled cells.

Another test for melanin depends upon the absorption spectrum between 400 and 600 mμ of suspensions of this material. A straight line usually is obtained over this range whose slope is characteristic of the melanin being examined (SCHAEFFER, 1953). As the data in
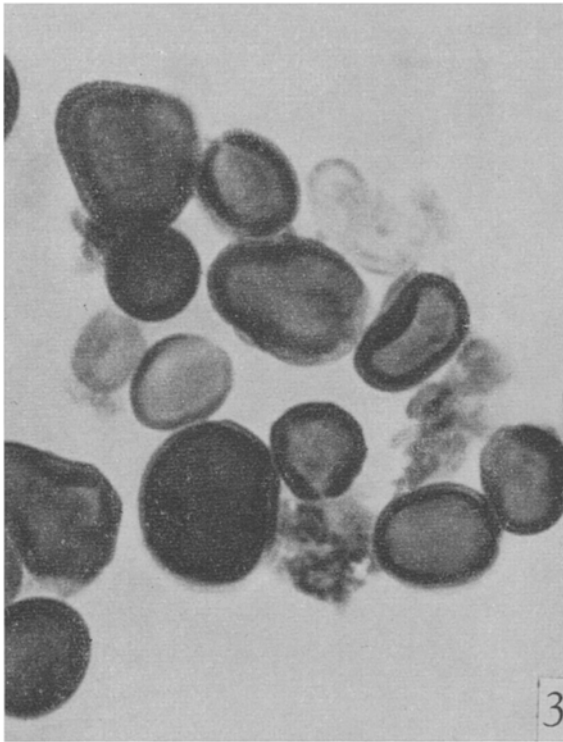

Fig. 3. Cells grown for 6 days in the light in defined medium $\neq$1 containing 0.2 gm asparagine per liter and 2% dextrine. Greenish-black, thick-walled cells with large globules are formed, as well as some thin-walled yeast-like cells and extra-cellular granules. Magnified about 500 ×.

fig. 6 disclose, the extracts from the dark, thick-walled cells form a straight line, as would be expected of melanins. The slopes of these lines were calculated and recorded in table II.

The solubility of melanins is used frequently to characterize these materials. Therefore, extracts of cells of *Aureobasidium* grown in dextrine were prepared as described above and their solubility in various solvents studied. Table III records the results obtained in
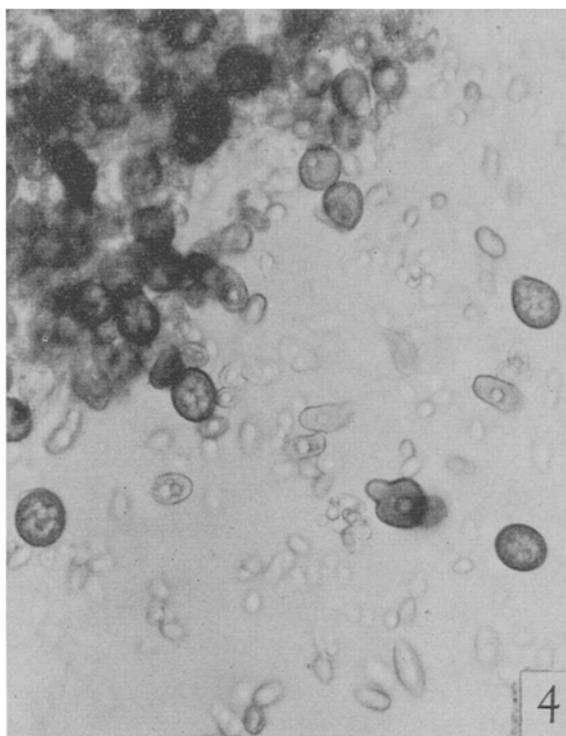
Fig. 4. Cells grown as in figure 3 except for 14 days. Note dark thick-walled cells
as in figure 3, but absence of granules inside the cells or in the medium.
Magnified about 1,000 ×.

TABLE II.

*Slopes of absorption spectra obtained from extracts of cells of Aureobasidium pullulans
and from extracellular granules prepared as described in the text. Data taken from
figure 1 and from similar graphs plotted for extracellular granules.*

Material		Slope
extracts of cells grown on:		
maltose		-0.0015
galactose		-0.0011
fructose		-0.0012
glucose		-0.0017
sucrose		-0.0018
starch		-0.0014
pectin		-0.0021
dextrine		-0.0016
extracellular granules	-0.0047,	-0.0030
(prepared during six	-0.0035,	-0.0040
different experiments)	-0.0044,	-0.0046

TABLE III.

Comparison of the properties of melanin from cells of A. pullulans, extracellular granules and dextrine. Cells were grown in basal medium containing 0.2 g asparagine per liter and dextrine, and granules were harvested after six days in this medium.

Test	Cellular extract	Extracellular granules	Dextrine
Solubility in:			
Cold H_2O	—	—	Slt. Sol.
Hot H_2O	—	—	—
Ethanol	—	—	—
Acetone	—	—	—
Chloroform	—	—	Slt. Sol.
Cold 0.5N NaOH	+	?	+
Cold 0.5N Na_2CO_3	+ [a]	+ [a]	+
Hot 0.5N Na_2CO_3	—	?	+
Hot 0.5N NaOH (FeCl₃)	— [b]	— [b]	— [c]
Cold 0.5N NaOH, followed by			
HCl to pH 7	—	—	+.
Anthrone-positive materials calculated as glucose (g/mg dry wt.)	not tested	260	1340
Nitrogen content (g/mg dry wt.)	not tested	2.5	4.5
Ratio of glucose/nitrogen	not tested	104	298

[a] incompletely soluble
[b] precipitate redissolves in excess $FeCl_3$
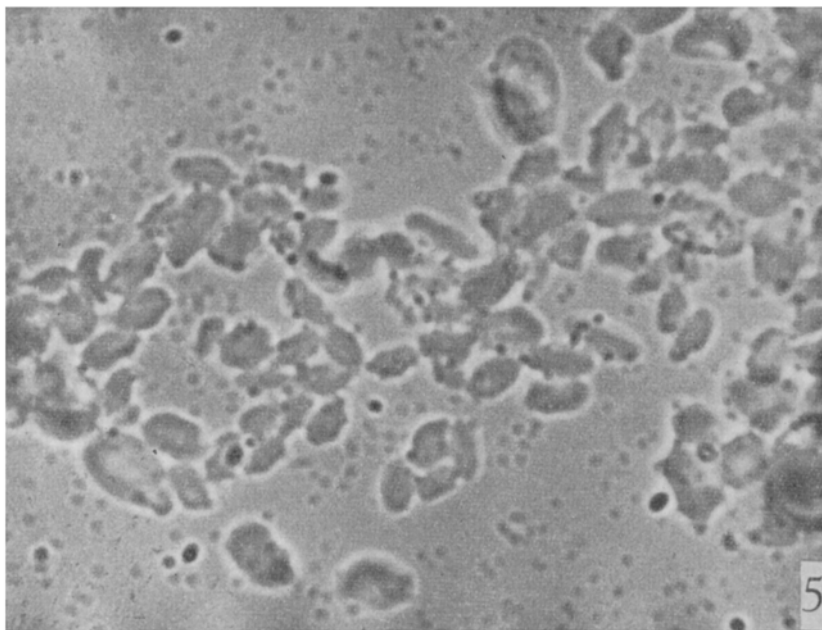[c] precipitate does not redissolve in excess $FeCl_3$

Fig. 5. Appearance of 4-day-old culture of *Aureobasidium pullulans* in defined medium ≠1. Note colorless thin-walled cells and small granules. Approx. × 1,200.
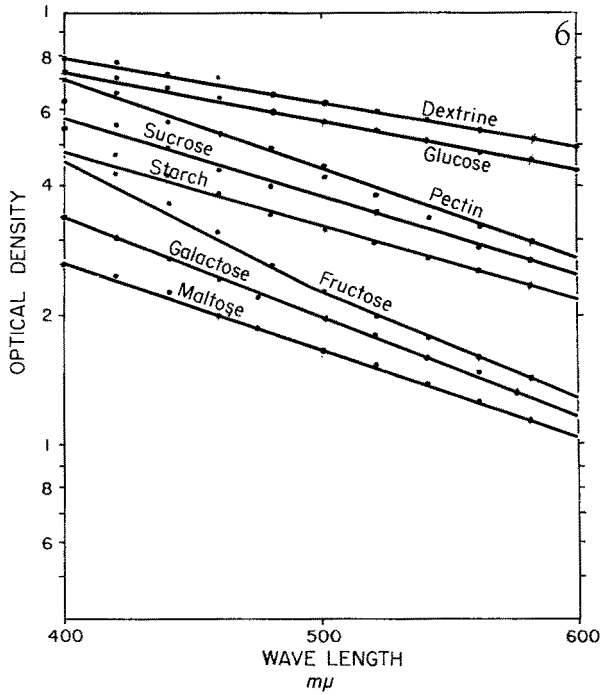
Fig. 6. Absorption spectra of alkaline extracts of cells of *Aureobasidium pullulans* grown on the basal medium containing 0.2 g asparagine per liter and 2% of various carbohydrates. Cells were harvested after 14 days and extracts were prepared as described in the section on Methods. Suspensions in 0.5 N NaOH were examined in a Beckman DU-spectrophotometer, in 1.0 cm cells.
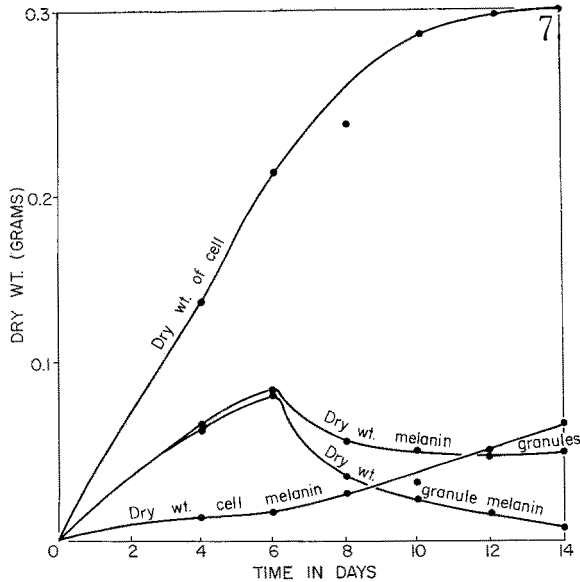

Fig. 7. Kinetics of melanin production in cells of *Aureobasidium pullulans*, and of the formation of extracellular granules by this organism. The basal medium was used with 2% dextrine and cultures were grown in the light at 21°C, on a rotary shaking machine.

these experiments and suggests again that the pigment in *Aureobasidium* is melanin.

Properties of extracellular granules and their formation:

As was noted previously, extracellular granules are seen conspicuously only when the basal medium contains dextrine. Consequently the kinetics of their formation were studied in this medium through the analysis of cultures harvested at various times after inoculation. Extracellular granules were separated from cells and the dry weights recorded. Then, the melanin was extracted from the cells

TABLE IV.

Time-course of the Development of Cells and Extracellular Granules in Aureobasidium pullulans.
Basal medium contained 0.2 g asparagine and 0.05 g MgSO₄ per liter.

Age of culture	Cell dry wt.[a]	Granule dry wt.[a] (1)	Cell melanin[b]	Granule melanin[b] (2)	(2) — (1)	Remarks
4 days	.2662 g	.1302 g	.0246 g	.1314 g	1.0	Cells small, colorless, thin-walled. Abundant extra-cellular granules.
6 days	.4350	.1682	.0318	.1674	0.99	Same as above, more granules and a few cells turned brown.
8 days	.4624	.1124	.0574	.0654	0.58	Granules appeared less dense, more cells were brown but without granular deposit on the cell walls.
10 days	.5674	.1060	.0652	.0492	0.46	Granules, less conspicuous walls and contents of cells brown and conspicuous.
12 days	.585	.1058	.097	.0228	0.21	Same as above, the granules much smaller, and aggregating, dark granular deposits on cell walls.
14 days	.5938	.101	.137	.0198	0.19	Dark brown cell walls with dark granular deposits

[a] Dry weight of cells obtained from 136 ml culture, in g.
[b] Dry weight of melanin precipitate obtained from crushed cells or from granules, in g, obtained from 136 ml culture.

and weighed in the form of a precipitate from the neutralized suspension. The extracellular granules were extracted in alkali, as in the case of the cellular melanin, neutralized and the precipitate weighed. These results are presented in table IV and fig. 7.

During the course of the growth of these cultures, the yeast-like cells, with thin walls, appear first. These are accompanied, as soon as three days after inoculation, by extracellular granules. Some brown cells appeared at six days, coinciding with the peak in the

number of extracellular granules that are formed. After eight days there is an obvious decrease in the number of granules. This precedes the time when the peak of growth is reached. At this time, the extracellular granules are much less conspicuous than they have been before, and many cells with thick walls and brown contents are present. Dark granular deposits appear on the cells after 12 days and the walls become noticeably brown. Melanin is accumulated even after the peak in growth is reached so it is possible that cells formed previously synthesize the pigment at this time.
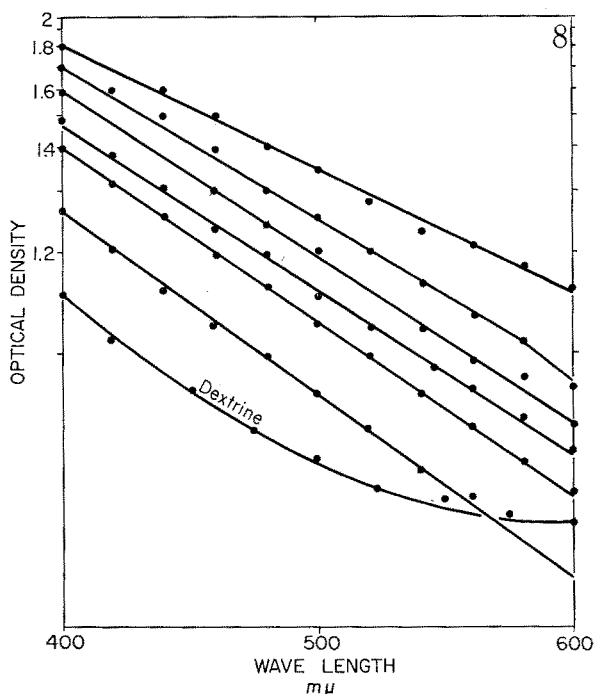

Fig. 8. Absorption spectra of extracellular granules obtained upon growth of *Aureobasidium pullulans* for 6 days at 21°C, on the basal medium with 2% dextrine. Conditions of observation as described for results in Figure 5.

When the extracellular granules first appear, on the third and fourth day, they are light-green and average about 0.7μ in size (200 granules measured), but many exceed 1.0μ. However, after the sixth or seventh day, they become stickier, tend to aggregate, and have an average size of 0.3μ, with none in excess of 0.8μ.

The dark color of the extracellular granules found in media containing dextrine suggested that they might contain melanin. Therefore, the granules were extracted as described in Methods, and the weight of the precipitate measured after neutralization. As the data in table IV disclose, the entire content of the granule isolated

through the sixth day is recoverable by the means used to extract melanin. On the other hand, the proportion of these granules that contains extractable melanin decreases markedly after the sixth day until it levels off at 14 days. It is possible that the pigment disappears from the granules at this time, or that it is altered to a form which is less soluble in the basic solution used for the extraction. Therefore, the extracellular granules change in their color, stickiness, size and content of extractable melanin during the 14 days they were observed.

Absorption spectra of extracellular particles were obtained between 400 and 600 mμ and plotted in figure 8. As in the case of the melanin extracted from the cells, straight lines were obtained whose slopes are provided in table II.

Inasmuch as the extracellular granules are found only in media containing dextrine, the granules were compared with dextrine, *per se*, in various ways. The results are provided in table III and reveal that there are several differences between the dextrine and extracellular granules. Thus, the solubility of the two are different in water, chloroform, and neutral solutions after alkaline extraction. Furthermore, the extracellular granules contain less carbohydrate and more nitrogen per glucose equivalent than does dextrine. Finally, as the data in figure 8 reveal, the shape of the absorption spectrum of dextrine granules in 0.5N NaOH differs markedly from that of the extracellular granules and melanin from cells in not being a straight line between 400 to 600 mμ.

Growth and Melanin production by *Aureobasidium pullulans* under varying conditions:

The *Neurospora* "minimal" medium (RYAN, 1950) was used for the growth of *Aureobasidium pullulans* in light and dark. Growth was permitted for 14 days in a rotary shaker at 21°C and the weight of cells and of the melanin extracted from them was recorded in table V. Light enhances the growth of the cells when dextrine is the carbon source but not very greatly when glucose is used. Moreover, the color of the medium and the cells is markedly darker when the culture is in the light as compared with when it is kept in the dark. Thus, in the light, both carbohydrates support the development of dark, greenish-brown media in which large thick-walled brown cells are found. In the dark, however, with both carbohydrates, the media were light buff in color and thin-walled, colorless small cells are formed. These data are supported by the finding that melanin was extractable only from cells grown in the light, as can be seen in Table V. In no case were extracellular granules formed in this medium.

When defined medium #2 was used, there was no significant difference found between cultures grown in the light and dark. The results given in Table V are for light-grown cultures in which it is shown that the amount of NH$_4$NO$_3$ used when dextrine is the carbon

TABLE V.

Growth and melanin production of Aureobasidium pullulans when grown on Neurospora "minimal" medium containing 2% carbohydrate, and in defined medium #2. Cultures were grown for 14 days at 21°C. Those in defined medium #2 were grown only in the light.

Carbohydrate	Color of medium	Cell dry wt. in g, 45 ml medium	Wet wt. of melanin ppt. 45 ml culture	Remarks
Neurospora "minimal":				
Dextrine				
light	dark, greenish brown	.1541	.090	Dark, thick-walled cells with brown contents.
dark	light bluff	.1035	nil	Thin-walled, colorless small cells.
Glucose				
light	dark greenish brown	.1452	.060	Dark, thick-walled cells with brown contents.
dark	light buff	.1225	nil	Thin-walled, colorless small cells.
Defined medium #2:				
Dextrine				
0.2 NH_4NO_3.	dark brown	.2060	positive	Thin-walled cells.
2.0 g NH_4NO_3	light brown	.2689	negative	Same as above.
Glucose				
0.2 g NH_4NO_3	light brown	.1882	negative	Colorless thin-walled cells
2.0 g NH_4NO_3	Colorless	.2003	negative	Same as above.

TABLE VI.

Effect of temperature upon growth of Aureobasidium pullulans grown in basal medium containing 0.2 g asparagine per liter and 2% glucose of dextrine as carbon source. Cultures were harvested after 24 hours in the dark.

Carbohydrate	Incubation temperature	Growth, dry wt. g	Remarks
Glucose	27°C	0.1087	Cells light-tan in color, no extracellular granules.
Glucose	30°C	0.0361	Thin-walled, yeast-like, colorless cells; no extracellular granules.
Dextrine	27°C	0.1753	Thin-walled, dark cells; extracellular granules present.
Dextrine	30°C	0.061	Cells light-tan in color; no extracellular granules

TABLE VII.

Effect of temperature and light on A. pullulans grown on different undefined media. Observations were made on agar slant cultures, containing 1% glucose, grown in the dark unless otherwise noted.

Media	Temperature	Remarks
Corn meal agar	6°C	Very little growth, white.
	8°C	Moderate growth, culture white; cells yeast-like.
	21°C; light	Good growth, culture black with mycelia along the margins.
	21°C; darkness	Good growth, white cultures without mycelia in the margin.
	27°C	Good growth; cultures white with mycelia along the margins.
	32°C	Poor growth, light brown.
	37°C	No growth.
Potato-dextrose agar	6°C	Moderate growth, culture gray.
	8°C	Good growth, culture black and no mycelia.
	21°C; light	Very good growth; cultures black with mycelia along the margins, soon after inoculation.
	21°C; darkness	Very good growth; cultures gray and turning black after 3 weeks, with mycelia along the periphery.
	27°C	Good growth; culture buff-colored and long mycelia along the margin.
	32°C	Poor growth; cultures white to light brown.
	37°C	No growth.
Prune agar	6°C	No growth.
	8°C	Poor growth, cultures yeast-like and white.
	21°C; light	Good growth, cultures yeast-like and white.
	21°C; darkness	Same as above.
	32°C	Same as above.
	37°C	No growth.
Nutrient agar	6°C	Poor growth, culture yeast-like and white.
	8°C	Same as above, but with mycelial filaments along the margins.
	21°C; light	Good growth, yeast-like and white even after three weeks.
	21°C; darkness	Same as above.
	32°C	Poor growth, white, yeast-like cultures.
	37°C	No growth.
1% yeast extract	6°C	Moderate growth of spreading yeast-like white colonies.
	8°C	Same as above; better growth.
	21°C; light	Same as above; good growth.
	21°C; darkness	Same as above.
	32°C	Same as above; poor growth.
	37°C	No growth.

source, determines whether melanin is produced. In no case were extracellular granules produced in this type of defined medium.

The effects of temperature upon the growth of *A. pullulans* and upon the production of extracellular granules were studied upon the defined medium #1 containing 0.2 g asparagine per liter and 2%

of either glucose or dextrine as the carbon source. Table VI discloses that growth is greatly restricted at 30°C, when compared to that at 27°C. Furthermore, extracellular granules are not produced at 30°C nor do cells become as dark in dextrine at this temperature.

These experiments pointed to the marked influence of the medium and the physical environment upon the growth and melanization of *Aureobasidium* so a survey of several undefined media was carried out. Cultures were prepared in duplicate test tubes of slanted agar and observations of the color and morphology of the colonies and cells were recorded in Table VII. These data corroborate the previous data which suggested that the medium determines whether light influences the development and color of *Aureobasidium*. Thus, light induces rapid blackening and mycelial formation in the organism when it is grown on corn meal agar. A similar effect is found in cultures on potato dextrose agar but blackening and mycelium formation occur eventually even in the dark on this medium. Other experiments have revealed considerable variations in the appearance of cultures grown upon potato-dextrose agar at different times. In contrast to the cultures grown on corn meal and potato dextrose agars, those grown on prune, nutrient and yeast-extract agars were unaffected by light. These latter media permitted the formation of mycelial, instead of yeast-like colonies in only one case, that of cultures grown at 8°C in nutrient agar. Even the temperature maxima and minima for growth appear to be affected by the medium, although the results are only qualitative. Still, considerable growth was visible at 6°C in potato-dextrose and yeast extract agars but in no others of the media used. At the other end of the temperature range, prune agar is the only medium that supports growth at 32°C. However, in no case was growth visible at 37°C.

DISCUSSION

The plastic morphology of *Aureobasidium* has been shown to respond to environmental variables like aeration and pH (BAUER, 1938), nitrogen source (LUTERAAN, 1954b), and sulfhydryl compounds (NICKERSON *et al.*, 1956). In addition, it depends upon the type of inoculum (LUTERAAN, 1954a) and upon the age of the culture (BAUER, 1938; STEINHAUS, 1955). Sugars influence the morphology of *Aureobasidium* in shaken cultures, as can be seen in table I. Thus, trehalose induces the formation of colorless yeast-like cells whereas dark, thick-walled cells are formed in the other good sources of carbon. Light also influences the morphology of this organism as was shown in table V. In *Neurospora* "minimal" medium, containing either dextrine or glucose, cells of *Aureobasidium* are dark and thick-walled in the light, but yeast-like and uncolored in the dark.

These data underscore the importance of the medium in determining whether light affects this fungus. Thus, growth or melanin

formation are profoundly affected in *Aureobasidium* when poly-saccharides and trehalose are used with defined medium #1 (table I), *Neurospora* "minimal" medium, corn meal and potato dextrose agars. To our knowledge, this is the first report of light effects upon growth in *Aureobasidium*.

According to the several criteria outlined in the section on Results, *Aureobasidium* produces melanin pigments in certain cells. This conclusion is in accord with the finding of ROUKHELMAN (1937) that phenolic pigments could be extracted from *Torula schoenii (Pullularia)*. She claimed that the pigment was formed first in the vacuole and was deposited later in the cell walls. These observations also agree with our own. The slope of absorption spectra of basic solutions of the melanin from *Neurospora* mycelium averages about –0.0027 according to SCHAEFFER (1953) and that of the ascospore pigment –0.0020, values that are in good agreement with those obtained in the present experiments with *Aureobasidium* (table II). On the other hand, the slope of the pigment from the extracellular granules produced by *Aureobasidium* averages about –0.0040 (table II) so that it differs from the intracellular pigments in this way at least. It should be noted that the melanins are an heterogenous group of pigments having in common quinonoid polymers in a protein matrix. Therefore the chemical identity of the melanins with which we have worked has not been established and the significance of a difference in absorption spectrum is uncertain. However as was pointed out above, the melanin(s) of *Aureobasidium* appear to belong to the same general class of pigments as those described in other organisms on the basis of several types of test.

Extracellular granules are formed by *Aureobasidium* only when dextrine is the carbon source and when asparagine is the nitrogen source. Because of the absolute requirement for dextrine the properties of this polysaccharide and the granules were compared (table III). It is concluded as a result of these experiments that the properties of the extracellular granules resemble those of melanin and differ from those of dextrine as far as solubility, absorption spectrum etc. are concerned.

Aureobasidium is shown to be able to use lactose slowly as a carbon source for growth. This conclusion is supported by the work of FRASER (1934) who found that a strain of *A. pullulans* utilized lactose slowly. In addition ROUKHELMAN (1937) and NEGRONI & FISCHER (1942), the latter working with 30 strains, report that lactose is used as a carbon source by this organism. In direct contrast, STEINHAUS (1955) reported that strains of this organism that he isolated from insects failed to utilize lactose or galactose. Similarly, CLARK & WALLACE (1958) claim that this organism cannot oxidize lactose, nor can it "ferment" (aerobically) this sugar, according to WYNNE & GOTT (1956). No explanation of these discrepancies is available as yet but it is possible that strain differences, or selection of variants during long term growth experiments,

account for the data.

According to COOKE (1961), the organism with which *Aureo-basidium* is most often confused is *Cladosporium*. However, many other organisms form stages that are similar to those of *Aureo-basidium*, leading COOKE to suggest that *Pullularia* (*Aureobasidium*) *pullulans* "may in reality be the imperfect state of a number of different species of Ascomycetes." Further confusion is caused by the pleomorphic nature of *Aureobasidium*, and its great adaptability in response to chemical and physical factors in the environment. That these may be diverse genetic types of this organism, as well as heterocaryotic mixtures, leads to the conclusion that much remains to be understood about the genetics and physiology of this fungus and related creatures.

Summary

Polysaccharides like dextrine and starch are shown to be the best carbon sources for the growth of *Aureobasidium pullulans* although growth is good upon a variety of other carbon sources. Light increases growth markedly when polysaccharides are the carbon source but not when other sugars are used. Variation in cell morphology is described in response to sugars and light. Extracellular granules, whose properties resemble those of melanin, are produced when dextrine is the carbon source in a defined medium containing asparagine as the source of nitrogen. The dark pigment was extracted from the walls of thick-walled brown cells of *A. pullulans* and characterized as a melanin on the basis of several tests, including solubility and absorption spectrum. *A. pullulans* was grown on several defined and undefined media and the response of the fungus to light is shown to be determined by the medium, and the temperature at which the cultures are grown.

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