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Regulation of Melanin Production by *Cryptococcus neoformans*

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Species of *Filobasidiella*, the agents of cryptococcosis, produced melanin-like pigments within 4 to 48 h with diphenol, aminophenol, and diaminobenzene compounds as substrates. The rate of phenyloxidase activity was found to be regulated by glucose and nitrogen catabolite repression. Increased glucose concentration reduced pigmentation of all serotypes of *Filobasidiella*, whereas repression by nitrogen sources varied with the strain. Glutamine repressed the phenyloxidases of all isolates except those of serotype B, and $(NH_4)_2SO_4$ repressed the phenyloxidase of all isolates except that of serotype A. Tyrosine and glycine appeared to be near optimal for phenyloxidase activity but not necessarily for growth of all strains examined. Representatives of serotype C were unique in that their phenyloxidase system was adaptive in contrast to the constitutive system found in the other serotypes. No single medium was found to support pigmentation of all strains of *Cryptococcus neoformans* within a 72-h incubation period; false-negative reactions can occur.

Cryptococcus neoformans (Sanfelice) Vuillemin is the yeast stage of a basidiomycetous fungus pathogenic for humans and animals. Kwon-Chung et al. (10) recognized two imperfect species and two perfect stages within the complex previously considered a single species. *Filobasidiella neoformans* Kwon-Chung is the perfect stage of isolates of the A and D serotypes of *C. neoformans*, whereas *F. bacillispora* Kwon-Chung is the perfect stage of *C. bacillisporus* Kwon-Chung, comprised of isolates of the B and C serotypes.

The etiological agents of cryptococcosis are presumptively identified by their ability to produce dark, melanin-like pigments with various phenolic compounds as substrates. The phenyloxidase of the *C. neoformans* complex cannot utilize tyrosine as a substrate, but does use a wide variety of compounds, including ortho- and paradiphenols, aminophenols, and diaminobenzenes with structural similarity to 3,4-dihydroxyphenylalanine (DOPA) as substrates, (2, 4-6, 8, 9, 11, 12, 14-16). A generalized scheme adapted from pathways proposed for the formation of melanins by fungi by Raper (13) and Graham and Jeffs (7) is given in Fig. 1.

Chaskes and Tyndall (5) showed that glutamine-glycine-asparagine provided an optimal nitrogen source for pigmentation by nine isolates of *C. neoformans* with para- or orthodiphenols as substrates. Pigment formation from diphenols was found exclusively for *C. neoformans*, but other *Cryptococcus* species were able to use hydroquinone as a substrate. Of ten aminophenol and diaminobenzene compounds tested, 4-dihydroxymetanilamide and 3-aminotyrosine were found to be substrates for pigmentation by nine isolates of *C. neoformans*. Recently, Paliwal and Randhawa (11) showed that a simplified niger seed medium lacking glucose and creatinine was superior to the complete medium for the rapid development of pigment by *C. neoformans*. Later, these investigators (12) proposed the use of a rapid, 1-h test based on pigment formation in a DOPA-ferric citrate broth without added glucose or nitrogen source.

Several media that utilize pigment production for the presumptive identification of *C. neoformans*, such as "bird" seed, caffeic acid, and various DOPA agars, were compared by Bowman and Ahearn (3). A few strains failed to develop characteristic pigment within the recommended incubation periods on all media examined. To clarify the conditions which regulate the phenyloxidase of *C. neoformans*, we have examined the effects of glucose and various nitrogen sources on melanization.

MATERIALS AND METHODS

Qualitative assay. Over 100 isolates of *C. neoformans*, including representatives of all known serotypes, were obtained from Bowman and Ahearn (3) and screened for their capacity to produce melanin pigments on agar medium containing extracts of niger seed, *Guizotia abyssinica* (16), and on the defined agar medium of Chaskes and Tyndall (5) containing

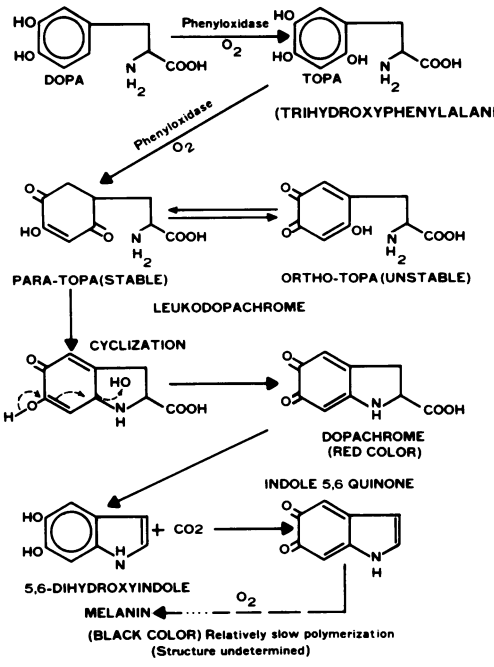

FIG. 1. *Proposed pathway of melanin synthesis by C. neoformans.*

DOPA and two sugar concentrations, either 0.5% or 0.05%. Twelve isolates representing all five serotypes and the extremes of pigment intensity for each serotype were selected for further studies (Table 1). The yeasts were maintained on Sabouraud dextrose agar slants with weekly transfers. The cultures were compared for their intensity of pigmentation with DOPA and three other substrates reported to allow pigmentation by *C. neoformans* (7). Isolates of *Filobasidium* known to lack phenyloxidase activity were used as controls.

The Chaskes and Tyndall (CT) medium was prepared in two separate solutions. The pigment substrate, either DOPA (0.2 g/liter), 5-aminosalicylic acid (0.5 g/liter), 3,4-diaminobenzoic acid (1.0 g/liter), or 4-hydroxymetanilamide (1.0 g/liter) (Aldrich Chemical Co., Milwaukee, Wis.), was combined in solution with asparagine (1.0 g), L-glutamine (1.0 g), and glycine (1.0 g) in 200 ml of deionized water. The pH was adjusted to 5.5 with 1 M K_2HPO_4, and the solution was sterilized by passage through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.).

The remaining ingredients, KH_2PO_4 (4.0 g), $MgSO_4 \cdot 7H_2O$ (2.5 g), thiamin-hydrochloride (10 mg), biotin (20 μg), glucose (0.5 to 5.0 g), and agar (25 g) were dissolved in 800 ml of deionized water (pH adjusted to 5.5), and the solution was autoclaved for 15 min at 121°C. The two solutions were brought to 55°C in a water bath and combined before plates were poured (the substrate solution was held at 55°C for the minimum of time necessary to prevent auto-oxidation of the pigment substrate.) The plates were stored under refrigeration and used within 1 week.

To prepare inocula, a loopful of cells was introduced into 5.0 ml of yeast carbon base (Difco Laboratories) without added nitrogen, and the culture was incubated on a roller drum (60 rpm) at 22 to 26°C for 24 h; 0.1 ml of the culture was used in a point inoculation. All tests were performed in duplicate with incubation in the dark, and results were recorded up to 72 h.

TABLE 1. *Isolates of C. neoformans selected for detailed study*[a]

Organism	Strain no.	Serotype	Mating type	Source
C. neoformans	1	A	α	CSF, Atlanta, Ga.
C. neoformans	14	A	α	Pulmonary, Oklahoma
C. neoformans	551	A	—	CDC
C. neoformans	444	B	α	NIH
C. neoformans	58	B	—	CSF, Brazil
C. neoformans	6289	B	α	CBS
C. neoformans	312	C	—	NIH
C. neoformans	191	C	a	NIH
C. neoformans	12	D	α	NIH
C. neoformans	430	D	a	NIH
C. neoformans	7221	AD	α	CSF, Venezuela
C. neoformans	295	AD	—	CDC
F. capsuligenum	325	NA	α	Sputum, Georgia
F. capsuligenum	370	NA	α	CDC, sputum

[a] —, No mating observed; CSF, cerebrospinal fluid; CBS, Centraalbureau voor Schimmelcultures; NIH, National Institutes of Health; CDC, Center for Disease Control; NA, not applicable.

Quantitative assay. Cells (1 loopful) from 96-h slants were introduced into 100 ml of various broths based on the formula of Chaskes and Tyndall (5). The media varied in their concentrations of glucose and in their nitrogen components and lacked a substrate for pigmentation. The nitrogen sources tested singly and in combination were $(NH_4)_2SO_4$, 0.5%; L-tyrosine (Tyr), 0.008%; creatinine, 0.1%; glycine (Gly), 0.1%; L-glutamine (Glu), 0.1%; and asparagine (Asp), 0.1%. The media were sterilized by filtration, kept under refrigeration, and used within 1 week. The cultures were incubated on a shaker (200 rpm) at 22 to 26°C. After 48 h, 20 ml of the culture was added to 100 ml of the same type of fresh broth and incubated for an additional 48 h. The cells were harvested from 20 ml of the culture broth by centrifugation and suspended in 0.03 M citrate buffer adjusted to pH 6.0 (or alkaline pH's with 5 M NaOH). To obtain acid pH's, 4 N HCl was employed.

The cell suspension (0.5 ml adjusted to an appropriate density) was added to a reaction mixture composed of 2.7 ml of a stock solution of DOPA (0.2 g of DL-DOPA in 100 ml of deionized water, kept under refrigeration in the dark) and 0.8 ml of 0.5 M sodium

citrate (pH 6.0) and adjusted with 0.03 M sodium citrate to a final volume of 4 ml. The optical density of the cell suspensions was 0.3 for glucose and 0.5 for nitrogen studies at 475 nm with a Bausch & Lomb Spectronic 20. Controls were cells suspended in buffer and substrate and in buffer only. The reaction systems were incubated in a shaking water bath at temperatures of 22, 30, and 37°C, and the optical density was determined at 475 nm at 10-min intervals up to 90 min. A near-linear reaction occurred during the first 30 to 40 min before the reaction rate decreased. Enzyme activity for nitrogen studies was recorded as specific activity (rate of reaction per dry weight of cell).

Cells of certain strains (including cells from 24-, 48-, 72-, and 96-h cultures) failed to show pigment formation in the assay described above. Therefore, to determine whether induction by the substrate occurred, 20 ml of a DOPA solution to give a final concentration of 0.02% was added to the 48-h cultures (restoring the volume to 100 ml), and incubation was extended for an additional 72 h. Pigmentation was observed visually.

RESULTS

The pigmentation by the representative isolates of *C. neoformans* on agar media was reduced or latent with all substrates at the higher glucose concentration (0.5%), particularly for the B and C serotypes. The control isolates of *Filobasidium* gave no evidence of pigmentation. The most rapid and intense pigmentation (orange to reddish) was produced with 4-hydroxy-metanilamide, but DOPA was chosen as the substrate for further studies because it gave a distinctive black color. DOPA is also an established intermediate in the synthesis of melanin (7). Random microscopic examination of cells grown in the presence of DOPA, or reacted with DOPA, demonstrated that the cell walls contained the obvious pigmentation. This association of pigment with the cell wall was found also for the other substrates.

With the quantitative assay system, optimal pigmentation of representative strains of *C. neoformans* was obtained with cells grown in CT broth with low glucose (0.05%) at pH 6.0 within 30 min. Of the temperatures tested (22, 30, and 37°C), maximal pigmentation within 60 min was obtained when the reaction mixture was incubated at 30°C. These conditions of pH and temperature were used subsequently. The synthesis of melanins by representative isolates was repressed with increasing concentrations of glucose (Fig. 2). Some strains with weak phenyloxidase activity and representatives of serotype C gave no evidence of melanization with this assay system.

In contrast to the uniform repression of phenyloxidase activity by glucose, repression by nitrogen source varied with the compound and the strain. Glutamine as a sole nitrogen source repressed phenyloxidase activity of all but isolates of the B serotype, whereas $(NH_4)_2SO_4$ repressed phenyloxidase activity of isolates of all but the A serotype. Both glutamine and $(NH_4)_2SO_4$ supported good growth of all strains. Tyrosine, which was not necessarily a good growth substrate, promoted enzyme activity of most isolates, but strain variation was noted for the B serotype (Table 2 and Fig. 3).

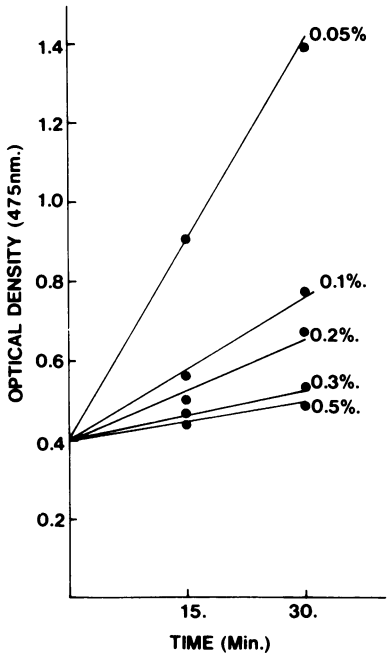

FIG. 2. *Effect of glucose concentration on phenyloxidase activity of C. neoformans (1A) with DOPA as the pigment substrate at 30°C.*

Combinations of repressive or promotive nitrogen sources did not always produce an additive effect on enzyme activity. For example, glutamine, glycine, and asparagine were individually promotive of enzyme activity by 58-B, but a combination of these nitrogen sources was repressive. On addition of tyrosine, which was repressive, enzyme activity for 58-B was enhanced (Table 2 and Fig. 3). Similar phenomena were noted for strains of serotype D. A combination of a repressive and promotive nitrogen source (glutamine and tyrosine for 14-A) masked the repression for some strains but not for others (295-AD, Table 2).

The effects of different nitrogen sources on the phenyloxidase activity of cells grown in the presence of DOPA are given in Table 3. All strains were not tested with all nitrogen sources; however, preliminary studies demonstrated that, when a medium yielded cells with a specific

TABLE 2. *Phenyloxidase activity of C. neoformans grown on various nitrogen sources*[a]

Nitrogen source	Sp act of the following cultures:[b]							
	551A	1A	14A	444B	58B	430D	12D	295AD
Tyr	ND	4.2	3.4	2.0	0	0.08	0	4.0
Gly	ND	ND	3.45	ND	1.92	ND	0	1.2
Glu	ND	ND	0.45	0.81	3.13	ND	0	0.1
Asp	ND	ND	0.91	ND	3.0	ND	0	0.12
Cr	0	0.51	2.0	0.92	3.1	0	0	6.1
$(NH_4)_2SO_4$	0	0.92	1.87	0	0.15	ND	0	0.3
Gly + Tyr	0.16	3.5	3.7	0.85	2.3	0.26	0.15	2.2
Glu + Tyr	ND	2.2	3.3	0.35	1.3	0.35	0.08	0.3
Asp + Tyr	ND	3.0	3.3	0.56	1.92	0.30	0.05	0.87
Cr + Glu	ND	2.5	0	0.74	3.0	0	0	1.2
Glu + Gly + Asp	ND	2.0	2.8	0.15	0.15	0.12	0	3.3
Tyr + Glu + Gly + Asp	ND	2.0	2.2	0.15	3.0	0.45	0.18	ND

[a] Cells grown for 48 h in DOPA-free modified basal medium of Chaskes and Tyndall with indicated nitrogen course before addition of DOPA.

[b] Specific activity determined from rate of reaction per dry weight of cells. ND, Not done.

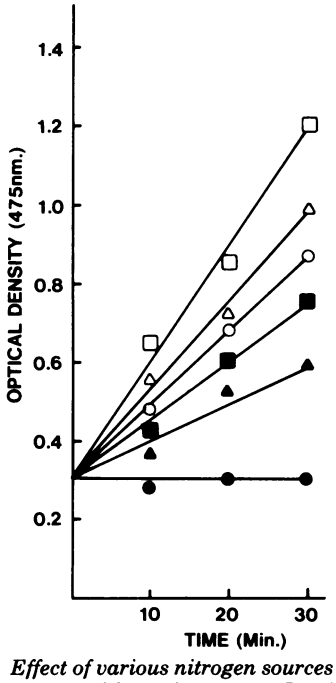

FIG. 3. *Effect of various nitrogen sources on phenyloxidase activity of C. neoformans (58B) with DOPA as the pigment substrate at 30°C. Symbols: □, Glu-Gly-Asp-Tyr; △, Gly; ○, Asp; ■, Glu-Gly-Asp; ▲, Glu; ●, Tyr.*

enzyme activity near or above 1.0, maximal pigmentation was obtained in the same medium with added DOPA by 24 h. The adaptive nature and sensitivity to repression of the phenyloxidase of the C serotype was obvious, but repression in instances of strains with profuse pigmentation [$(NH_4)_2SO_4$ for 295-AD] was not visually observable (Table 3).

The intensity of pigmentation with a particular nitrogen source did not necessarily correlate with the capability of that nitrogen source to support growth. For example, creatinine, which has been reported to be preferentially utilized as a nitrogen source by the B and C as contrasted to A and D serotypes (10), promoted phenyloxidase activity of 14-A but supported only weak-to-latent growth of the culture. Tyrosine and glycine gave good pigmentation for the majority of the strains examined.

Cultures of all serotypes grown in CT broth (0.05% glucose with DOPA present as substrate) continued to synthesize melanin upon transfer to CT agar supplemented with tyrosine but lacking DOPA. In these experiments, a light inoculum without visually detectable pigmentation was employed. Cells grown on CT broth without DOPA failed to produce melanin on the CT-tyrosine agar.

DISCUSSION

Paliwal and Randhawa (11) reported that glucose appeared to be the main factor responsible for delayed pigmentation of *C. neoformans* on niger seed agar. They further recommended that creatinine be omitted from *G. abyssinica*-based selective medium. The underlying mechanisms for the delayed pigmentation were not determined. Interpretation of results obtained with niger seed extract is difficult, since it contains various substrates for pigmentation, e.g., 3,4-dihydroxycinnamic acid (caffeic acid), and orthodiphenol (9, 18). This mixed and variable substrate pool in the extract probably accounts for the lack of specificity sometimes noted for niger seed agar for distinguishing *C. neoformans* from other yeasts. The regulation of melanization in other species of *Cryptococcus* has not been examined.

This preliminary screening of over 100 isolates representing *F. neoformans*, *F. bacillispora*, *C.*

TABLE 3. *Conversion of DOPA to melanin by C. neoformans grown on various nitrogen sources*[a]

Nitrogen source (culture no.)	Time (h) of black pigmentation[b]										
	Cre	Glu	Tyr	$(NH_4)_2SO_4$	Cre + Glu	Tyr + Asp	Tyr + Glu	Glu + Gly + Asp	Cre + Glu + Gly + Asp	Tyr + Glu + Gly + Asp	Tyr + Gly
551A	>72[b]	ND	ND	>72	ND	ND	ND	>72	ND	ND	72
1A	72	>72	4	4	4	48	>72	4	4	4	4
14A	24	>72	4	4	72	48	4	72	ND	48	4
444B	4	4	4	>72	48	>72	>72	4	ND	4	4
58B	24	ND	48	ND	4	4	48	4	4	4	4
6289B	24	4	ND	>72	4	ND	ND	ND	ND	ND	ND
312C	24	>72	4	ND	72	>72	>72	>72	72	24	48
191C	24	>72	4	>72	72	>72	>72	24	ND	48	24
430D	>72	>72	72	ND	48	24	24	4	>72	4	4
12D	ND	>72	72	>72	>72	48	>72	48	ND	24	24
295AD	4	ND	ND	4	24	ND	ND	4	24	ND	ND
7221AD	4	>72	ND	4	>72	ND	ND	ND	ND	ND	ND

[a] Cells were grown in DOPA-free modified CT medium of Chaskes and Tyndall with indicated nitrogen source before addition of DOPA; pigmentation was recorded visually from this point over an additional 72 h.
[b] Visual observation of maximal black pigmentation of the strain with recordings at 4, 24, 48, and 72 h. ND, Not done.

neoformans, and *C. bacillisporus* indicates that the phenyloxidases of these species are characteristically subject to carbon and nitrogen catabolite repression. The repressive effect of glucose was found to be similar for all strains studied, but generally strains of the B and C serotypes seemed more sensitive to regulation. The repressive effect of glucose applied to other substrates of phenyloxidase of *C. neoformans*, and the repression was reinforced or altered, depending on the strain, by the nitrogen source. The phenyloxidase of the C serotype was distinct in requiring induction by DOPA for activity to be expressed. Tyrosine, the biological precursor of DOPA in melanin synthesis by *Mucor* (7), enhanced pigmentation (also found for tryptophan; unpublished data) by most isolates of *C. neoformans* with DOPA as the substrate. Tyrosine is not used as a substrate for pigmentation by *C. neoformans* (14, 18). With induction by DOPA, however, representatives of all serotypes of *C. neoformans* produced melanin-like pigments from tyrosine.

The repression or promotion of the pigmentation by a particular nitrogen source was strain related. The promoting effect of a nitrogen source on pigmentation for certain strains was masked by the presence of a repressor. This was most noticeable for the AD, B, and C serotypes grown in the presence of creatinine (promotive) and creatinine plus glutamine (repressive). This type of phenomenon may be attributed to preferential transport of the repressor (or in some instances the promoter) across the cell membrane. Paliwal and Randhawa (12) suggested creatinine as a possible repressor of pigmentation of *C. neoformans*. Subsequently, they recommended a medium without glucose and creatinine for rapid pigmentation by *C. neoformans*. Most probably, their investigation, like most others, examined mainly (or only) the common A or D serotypes and not the rare B and C serotypes whose melanization appears enhanced by creatinine. Creatinine does not appear to be a repressor of phenyloxidase by the A and D serotypes; however, as a sole nitrogen source it supported only slow growth and concomitantly relatively weak phenyloxidase activity by some isolates. Kwon-Chung et al. (10) showed that B and C serotypes readily utilized creatinine for growth as contrasted with isolates of serotypes A and D.

In this study, a combination of tyrosine plus glycine appeared to be optimal for pigmentation; however, considerable strain variation in intensity of pigmentation occurred within a serotype. In particular, the various agar media used for the detection of *C. neoformans* by pigmentation which contain from 0.5 to 1.0% glucose (5, 6, 9, 15–17) would not distinguish the adaptative nature of the enzyme of the C serotype from that of the constitutive nature of lesser-pigmented strains of the other serotypes.

The differences between the serotypes in the regulation of phenyloxidase activity may explain the discrepancies in studies which report on the validity of pigment formation for the presumptive identification of *C. neoformans*. Since most isolates of A and D, the more common serotypes in the United States (1; D. G. Ahearn and R. A. Schlitzer, *in* A. Balows (ed.), Procedures in bacterial, mycotic, and parasitic infections, 6th ed.,

in press), give rapid development of melanin on appropriate media, the validity of using melanin production for presumptive identifications is supported. With DOPA as a substrate and with defined growth conditions for the inoculum, false-positive reactions do not appear to be a difficulty. However, as stated previously, no single medium was found to support pigmentation by all strains of *C. neoformans* within a 72-h incubation period; false-negative reactions can occur.

LITERATURE CITED

1. **Bennett, J. E., K. J. Kwon-Chung, and T. S. Theodore.** 1978. Biochemical differences between serotypes of *Cryptococcus neoformans*. Sabouraudia **16**:167-174.
2. **Botard, R. W., and D. C. Kelley.** 1968. Modified Littman oxgall agar to isolate *Cryptococcus neoformans*. Appl. Microbiol. **16**:689-690.
3. **Bowman, P. I., and D. G. Ahearn.** 1977. Ecology of *Cryptococcus neoformans* in Georgia, p. 258-268. Proceedings of the Fourth International Conference on the Mycoses, PAHO, scientific publication no. 356.
4. **Breyer, D., F. Staib, M. Senska, and A. Blisse.** 1972. Suitability of O-diphenoloxidase demonstration by means of catechol for the differentiation of *Cryptococcus neoformans*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I. Orig. **222**:540-543.
5. **Chaskes, S., and R. L. Tyndall.** 1975. Pigment production by *Cryptococcus neoformans* from para- and orthodiphenols: effect of the nitrogen source. J. Clin. Microbiol. **1**:509-514.
6. **Chaskes, S., and R. L. Tyndall.** 1978. Pigment production by *Cryptococcus neoformans* and other *Cryptococcus* species from aminophenols and diaminobenzenes. J. Clin. Microbiol. **7**:146-152.
7. **Graham, J. G., and P. W. Jeffs.** 1977. The role of 2,4,5-trihydroxyphenylalanine in melanin biosynthesis. J. Biol. Chem. **252**:5729-5734.
8. **Hopfer, R. L., and D. Groschel.** 1975. Six-hour pigmentation test for the identification of *Cryptococcus neoformans*. J. Clin. Microbiol. **2**:96-98.
9. **Korth, H., and G. Pulverer.** 1971. Pigment formation for differentiating *Cryptococcus neoformans* from *C. albicans*. Appl. Microbiol. **21**:541-542.
10. **Kwon-Chung, K. J., J. E. Bennett, and T. S. Theodore.** 1978. *Cryptococcus bacillisporus* sp. nov.: serotype B-C of *Cryptococcus neoformans*. Int. J. Syst. Bacteriol. **28**:616-620.
11. **Paliwal, D. K., and J. S. Randhawa.** 1978. Evaluation of a simplified *Guizotia abyssinica* seed medium for differentiation of *Cryptococcus neoformans*. J. Clin. Microbiol. **7**:346-348.
12. **Paliwal, D. K., and H. S. Randhawa.** 1978. A rapid pigmentation test for identification of *Cryptococcus neoformans*. Antonie van Leeuwenhoek J. Microbiol. Serol. **44**:243-246.
13. **Raper, H. W.** 1927. The tyrosinase-tyrosine reaction of 5,6-dihydroxy-indole and 5,6-dihydroxyindole-2-carboxylic acid—the precursor of melinin. Biochemistry **21**: 89-96.
14. **Shaw, C. E., and L. Kapica.** 1972. Production of diagnostic pigment by phenyloxidase activity of *Cryptococcus neoformans*. Appl. Microbiol. **24**:824-830.
15. **Shields, A. B., and L. Ajello.** 1966. Medium for selective isolation of *Cryptococcus neoformans*. Science **151**: 208-209.
16. **Staib, F.** 1962. *Cryptococcus neoformans* and *Guizotia abyssinica* (syn. *G. oleifera* D. C.) (Farbreaktion fur *C. neoformans*) Z. Hyg. **148**:466-475.
17. **Staib, F., and M. Senska.** 1973. Brown pigmentation (brown color effect, BCE) of *Cryptococcus neoformans* on *Guizotia abyssinica*-creatinine-agar as dependent on the initial pH value. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **225**:113-124.
18. **Strachan, A. A., R. J. Yu, and F. Blank.** 1971. Pigment production of *Cryptococcus neoformans* grown with extracts of *Guizotia abyssinica*. Appl. Microbiol. **22**: 478-479.