Rapid Diazonium Blue B Test to Detect Basidiomycetous Yeasts

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More than 100 known eucaryotic microorganisms were examined for capacity to form red to violet pigments with diazonium blue B after alkaline hydrolysis and an ethanol wash. All 50 basidiomycetes gave rapid and unequivocal positive reactions when 1- to 6-day-old cultures were tested, whereas all ascomycetes and algae gave negative reactions. Extracellular enzymes generally considered to be typical of basidiomycetous yeasts were detected in several ascomycetous taxa.

A variety of physiological and morphological characteristics distinguish the anamorphs of ascomycetous and basidiomycetous yeasts. Ascomycetous yeasts have two-layered cell walls that are attacked by (1,3)- β -glucanases to release protoplasts, have deoxyribonucleic acid guanine-plus-cytosine contents of about 30 to 60%, frequently are fermentative, usually lack extracellular urease and deoxyribonuclease (DNase), and have respiratory enzymes of coenzyme Q types 6 through 9 (2, 3, 11, 14, 20, 25). Basidiomycetous yeasts have laminar cell walls that resist (1,3)- β -glucanase activity, have deoxyribonucleic acid guanine-plus-cytosine contents of about 50 to 70%, with rare exception are strictly oxidative, generally have extracellular urease and DNase, have respiratory enzymes of coenzyme Q types 7 through 10, and give a red to violet color reaction with diazonium blue B (DBB) (2, 3, 6, 8, 9, 11, 14, 17, 20-22, 26; C. E. Sonck and E. Tunnela, *Antonie van Leeuwenhoek J. Microbiol. Serol.* **35**(Suppl.):E27, 1969). Except for the cell wall properties, these characteristics overlap among the species of these two groups.

The DBB reaction appears to be the most practical test for establishing basidiomycetous relationships in laboratories, but unfortunately the DBB test has not been standardized. A variety of different media and incubation times of several weeks have been employed, with some species giving questionable results. Therefore, the use of the DBB test is not widespread. This report describes an improved DBB test to detect basidiomycetous relationships of yeasts and yeastlike organisms and correlates the results of the DBB test with extracellular enzyme production.

MATERIALS AND METHODS

Organisms. All strains used in this study were authenticated cultures obtained from the freeze-dried culture collection at Georgia State University or from other laboratories specializing in the taxonomy of yeasts (Table 1). Routinely, cultures were maintained by monthly transfers on Sabouraud dextrose agar; the cultures were incubated for 3 days at 25°C and were then stored at 4°C.

DBB reagent. The DBB reagent was prepared by adding 15 ml of cold 0.25 M tris(hydroxymethyl)aminomethane buffer (pH 7.0) to 15 mg of DBB (*o*-dianisidine, tetrazotized; practical grade; 20% pure; Sigma Chemical Co.). DBB is unstable in warm and moist conditions, but 15-mg portions of the dye can be stored in well-sealed test tubes at 4°C for several weeks. The dissolved reagent was maintained in an ice bath and was used either before it turned dark yellow or within about 30 min.

Alkali-ethanol-DBB test. Cultures were grown in 5.0 ml of yeast nitrogen base (YNB) (Difco Laboratories) fortified with 0.5% glucose; the initial pH was adjusted to 7.0. Cultures were incubated on a roller drum (30 rpm) at 25°C for 3 days. Psychrophiles, which were grown at 15°C, and a few slow-growing mesophiles were incubated for 6 days to obtain sufficient growth. The cells were sedimented in a clinical centrifuge, and the spent medium was removed. The pellet was suspended in 0.5 ml of 0.05 M KOH, and this suspension was placed in a boiling water bath for 10 min, cooled to ambient temperature, and mixed well with 2.5 ml of 95% ethanol. Then this suspension was centrifuged briefly at low speed, the supernatant was discarded, and the pellet was suspended in 0.3 ml of DBB reagent. Usually a violet color was evident within a few seconds for positive strains. Immediately after the color appeared, 1.0 ml of 95% ethanol was added.

DBB test on solid media. The solid media used for DBB tests were skim milk agar (1), Sabouraud dextrose agar containing 0.5% yeast extract, chalk agar (12), Christensen urea agar (12) containing 20 mg of acid fuchsine per liter rather than phenol red, and YNB agar containing 0.5% glucose in various combinations with tryptophane (50 mg/100 ml), casein (0.5%), and CaCO₃ (0.5%). Cultures were incubated for 5 to 7 days (rarely longer) at 25°C (or 15°C for psy-

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TABLE 1. List of strains studied, DBB reaction, and extracellular enzyme activities of yeasts and yeastlike organisms^a

Strain no.	Species	Designation	Source ^b	Division ^c	DBB reaction ^d	Urease test ^e	DNase test ^e	Proteinase test ^e
1	<i>Aessosporon salmonicolor</i>	CBS 5937	UM	B (23)	1	+	+	+
2	<i>Bullera globospora</i>	CBS 6981	UM	B (23)	2	+	+	w
3	<i>Bullera aurantiaca</i>	CBS 6714	UM	B (23)	2	+	+	+
4	<i>Candida amytolenta</i>	CBS 6273	GSU	B	2	+	+	+
5	<i>Candida humicola</i>	UM 610	GSU	B (14)	2	+	+	+
6	<i>Candida philya</i>	CBS 6272	GSU	B (22)	3	+	+	w
7	<i>Cryptococcus ater</i>	Y 6661	NRRL	B	2	+	+	+
8	<i>Cryptococcus diffluens</i>	32-71	GSU	B (14)	3	+	+	+
9	<i>Cryptococcus laurentii</i>	87-2	GSU	B (14)	3	+	w	+
10	<i>Cryptococcus neoformans</i>	372 A	GSU	B (23)	3	+	+	w
11	<i>C. neoformans</i>	76 B	GSU	B (23)	3	+	+	+
12	<i>C. neoformans</i>	836 C	GSU	B (23)	2	+	+	w
13	<i>C. neoformans</i>	433 D	GSU	B (23)	3	+	+	w
14	<i>C. neoformans</i>	295 AD	GSU	B (23)	3	+	+	+
15	<i>Fissuricella filamenta</i>	B 1273 a	CDC	B (18)	2	+	+	+
16	<i>Filobasidiella neoformans</i>	F 3502 a	GSU	B (23)	3	+	+	w
17	<i>Filobasidium capsuligenum</i>	CDC 325	GSU	B (23)	2	+	w	+
18	<i>Leucosporidium gelidum</i>	AH 177	GSU	B (23)	2	+	w	+
19	<i>Leucosporidium nivalis</i>	AH 176	GSU	B (23)	2	+	+	+
20	<i>Malassezia pachydermatis</i>	506594	CDC	B (10)	3	+	+	+
21	<i>Phaffia</i> sp.	Ph 4	GSU	B (16)	3	+	+	+
22	<i>Rhodospiridium infirmo-miniatum</i>	IE 293	GSU	B (23)	3	+	+	+
23	<i>Rhodospiridium sphaerocarpum</i>	CBS 5941	GSU	B (23)	3	+	w	+
24	<i>Rhodotorula acheniosum</i>	CBS 6324	GSU	B	3	+	+	+
25	<i>Rhodotorula rubra</i>	RJ 1	UFRJ	B (14)	3	+	+	+
26	<i>Sporidiobolus johnsonii</i>	CBS 5470	GSU	B (23)	2	+	+	+
27	<i>Sporidiobolus ruinenii</i>	CBS 5001	GSU	B (23)	3	+	+	+
28	<i>Sporobolomyces albo-rubescens</i>	CBS 842	GSU	B (23)	3	+	+	+
29	<i>Sporobolomyces antarcticus</i>	Y 8295	NRRL	B (23)	3	+	+	+
30	<i>Sporobolomyces holsaticus</i>	CBS 1522	GSU	B (23)	2	+	+	+
31	<i>Sporobolomyces pararoseus</i>	CBS 484	GSU	B (23)	3	+	+	+
32	<i>Sporobolomyces roseus</i>	CBS 486	GSU	B (23)	2	+	+	+
33	<i>Sporobolomyces salmonicolor</i>	CBS 490	GSU	B (23)	3	+	+	+
34	<i>Sporobolomyces singularis</i>	CBS 5109	GSU	B (23)	3	+	+	+
35	<i>Sporobolomyces</i> sp.	S	GSU	B (23)	3	+	+	w
36	<i>Sporobolomyces</i> sp.	ML 2277	GSU	B (23)	3	+	+	+
37	<i>Sterigmatomyces aphidus</i>	Y 7954	NRRL	B	3	+	+	+
38	<i>Sterigmatomyces elviae</i>	Y 7504	NRRL	B (20)	2	+	w	+
39	<i>Sterigmatomyces indicus</i>	Y 6837	NRRL	B	3	+	+	+
40	<i>Sterigmatomyces halophilus</i>	YB 4619	NRRL	B	3	+	+	+
41	<i>Sterigmatomyces nectairii</i>	Y 8296	NRRL	B	2	+	w	+
42	<i>Sterigmatomyces penicillatus</i>	Y 8284	NRRL	B	3	+	w	+
43	<i>Sterigmatomyces polyborus</i>	Y 7955	NRRL	B (20)	2	+	w	+
44	<i>Sterigmatomyces</i> sp.	CDC 188	GSU	B	3	+	w	+
45	<i>Sterigmatomyces</i> sp.	Y 7956	NRRL	B	3	+	+	+
46	<i>Tremella foliaceae</i>	CBS 6969	CBS	B	2	+	+	+
47	<i>Trichosporon cutaneum</i>	RJ 77	UFRJ	B (6)	2	+	w	+
48	<i>T. cutaneum</i>	4-14	GSU	B (6)	2	+	+	+
49	<i>Trichosporon oryzae</i>	Y 7808	NRRL	B	3	+	+	+
50	<i>Ustilago maydis</i>	B 13166	CDC	B	2	+	+	+
51	<i>Ustilago zeae</i>	B 10086	CDC	B	2	+	+	+
52	<i>Aureobasidium pullulans</i>	C 83	GSU	A (7)	—	+	w	+
53	<i>A. pullulans</i>	PL	GSU	A (7)	—	+	+	+
54	<i>Botryascus synnaedendrus</i>	CBS 6393	UM	A (23)	—	—	—	—
55	<i>Brettanomyces anomalus</i>	Y 1415	NRRL	A (12)	—	—	—	—
56	<i>Brettanomyces bruxellensis</i>	Y 1411	NRRL	A (12)	—	—	—	—
57	<i>Brettanomyces clausenii</i>	Y 1414	NRRL	A (12)	—	—	—	—
58	<i>Brettanomyces lambicus</i>	Y 1413	NRRL	A (12)	—	—	—	—
59	<i>Brettanomyces</i> sp.	177 And	GSU	A (12)	—	—	—	—
60	<i>Candida albicans</i>	595	GSU	A (14)	—	—	—	—
61	<i>Candida glabrata</i>	RJ 39	UFRJ	A (14)	—	—	—	—
62	<i>Candida krusei</i>	RJ 66	UFRJ	A (14)	—	—	—	—
63	<i>Candida maltosa</i>	R 42	GSU	A (13)	—	—	—	—
64	<i>Candida parapsilosis</i>	22019	GSU	A (14)	—	—	—	—
65	<i>Candida tropicalis</i>	231	GSU	A (13)	—	—	—	—

TABLE 1—Continued

Strain no.	Species	Designation	Source ^b	Division ^c	DBB reaction ^d	Urease test ^e	DNase test ^e	Proteinase test ^e
66	<i>Chlorella</i> sp.	CDC 79-078	GSU	Al (4)	—	—	—	—
67	<i>Chyrosporium xerophilum</i>	CDC 80-0615	CDC	A	—	—	—	+
68	<i>Cryptococcus melibiosum</i>	CBS 2102	GSU	A (19)	—	—	—	—
69	<i>Debaryomyces hansenii</i>	RJ 52	UFRJ	A (23)	—	—	—	—
70	<i>Geotrichum candidum</i>	14PS	GSU	A (23)	—	—	—	—
71	<i>Geotrichum penicillatum</i>	RJ 260	UFRJ	A (23)	—	—	—	—
72	<i>Hanseniaspora occidentalis</i>	CBS 2592	GSU	A (15)	—	—	—	—
73	<i>Hanseniaspora osmophila</i>	ATCC 24231	GSU	A (15)	—	—	—	—
74	<i>Hanseniaspora vineae</i>	CBS 6555	GSU	A (15)	—	—	—	—
75	<i>Hansenula anomala</i>	RJ 510	UFRJ	A (23)	—	—	w	—
76	<i>Hansenula polymorpha</i>	AH 277	GSU	A (23)	—	—	—	—
77	<i>Hansenula saturnus</i>	UM 305	GSU	A (23)	—	—	—	—
78	<i>Kloeckera africana</i>	CBS 6706	GSU	A (15)	—	—	—	—
79	<i>Kloeckera javanica</i>	ATCC 10637	GSU	A (15)	—	—	—	—
80	<i>Kluyveromyces fragilis</i>	333	AC	A (12)	—	—	—	—
81	<i>Metschnikowia bicuspidata</i>	RJ 763	UFRJ	A (12)	—	—	—	—
82	<i>Nadsonia fulvescens</i>	UM 815	GSU	A (12)	—	—	—	—
83	<i>Nematospora coryli</i>	Y 984	GSU	A (12)	—	—	+	+
84	<i>Pichia membranaefaciens</i>	DF 5586	GSU	A (12)	—	—	—	—
85	<i>Pichia ohmeri</i>	RJ 420	UFRJ	A (12)	—	—	—	—
86	<i>Pichia spartinae</i>	p 14	GSU	A (12)	—	—	—	+
87	<i>Prototheca zopfii</i>	RJ 328	UFRJ	Al (4)	—	—	—	—
88	<i>Saccharomyces cerevisiae</i>	RJ 200	UFRJ	A (12)	—	—	—	—
89	<i>Saccharomyces exiguus</i>	RJ 30	UFRJ	A (12)	—	—	—	—
90	<i>Saccharomycopsis fibuligera</i>	CDC 419-72	GSU	A (23)	—	—	+	+
91	<i>Saccharomycopsis lipolytica</i>	RJ 71	UFRJ	A (23)	—	+	+	+
92	<i>S. lipolytica</i>	RJ 257	UFRJ	A (23)	—	+	+	+
93	<i>S. lipolytica</i>	SOLS	GSU	A (23)	—	+	+	+
94	<i>S. lipolytica</i>	SOLR	GSU	A (23)	—	+	+	+
95	<i>S. lipolytica</i>	1A	GSU	A (23)	—	+	+	+
96	<i>S. lipolytica</i>	37-1	GSU	A (23)	—	+	+	+
97	<i>Schizosaccharomyces octosporus</i>	UM 300	GSU	A (12)	—	+	—	—
98	<i>Sporopachydermia lactativora</i>	Cr 234	GSU	A (19)	—	—	—	—
99	<i>Sporothrix schenckii</i>	B 2560	CDC	A	—	+	+	+
100	<i>Taphrina potentillar</i>	T 422	GSU	A	—	+	+	w
101	<i>Trichosporon ericense</i>	CBS 5974	GSU	A (24)	—	—	—	—
102	<i>Trichosporon fennicum</i>	CBS 5928	GSU	A	—	—	—	+
103	<i>Trichosporon terrestre</i>	CBS 6697	GSU	A (24)	—	—	w	—

^a For the DBB test, cells were grown in YNB containing 0.5% glucose (pH 7.0).

^b UM, University of Miami Marine Laboratory; GSU, Georgia State University Department of Biology; UFRJ, Universidade Federal do Rio de Janeiro, Instituto de Microbiologia; NRRL, Northern Regional Research Laboratory, United States Department of Agriculture, Peoria, Ill.; CDC, Centers for Disease Control, Atlanta, Ga.; AC, Animal Diseases Research Institute, Nepean, Ontario, Canada; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands.

^c B, Basidiomycete; A, ascomycete; Al, alga. The numbers in parentheses are reference numbers.

^d 1, Pink violet; 2, violet; 3, dark violet to purple; —, yellow or brown.

^e +, Positive; w, weak; —, negative.

chrophiles). DBB reagent (1 or 2 drops) was placed directly onto the surface of each culture. In some tests the plates were incubated at 55 to 60°C for 16 h before application of DBB.

Extracellular enzyme tests. Production of urease was determined with Christensen urea agar (Difco), and results were recorded after 2, 3, and 5 days. After 6 days of incubation, DNase production was detected by using DNase test agar (Difco) supplemented with 1.0% glucose and 1.0% yeast extract. Proteinase activity was detected on YNB agar containing 0.5% glucose and 0.5% casein (pH 7.0) after 7 days of incubation. Proteinase and DNase plates were washed of growth with either 95% ethanol or distilled water, and the plates were flooded with 1 N HCl for 1 hr; this was followed by observation for clear zones against a dark

background. Proteinase production was also determined by clearing of skim milk agar (1).

RESULTS

Cells of most of the known basidiomycetous yeasts produced pink to red or violet color reactions with DBB on at least several of the solid growth media used; however, there was considerable variation between isolates as to the intensity of the color and the age of the culture necessary for obtaining a positive reaction. For example, the isolates of *Cryptococcus laurentii* and *Candida amylo lenta* required about 2 weeks of incubation on the optimal solid medium

before they gave positive DBB tests. The incubation times required for these and other yeasts to produce positive DBB reactions were reduced to 5 days or less by incubating the cultures for about 16 h at 55 to 60°C just before DBB was added. Generally, most known ascomycetes gave negative reactions, except when grown on chalk agar. Also, some ascomycetes turned pink after the addition of DBB when they were grown on skim milk agar. During growth on tryptophane-supplemented YNB agar containing glucose, various microorganisms produced pink to dark brown pigments which interfered with subsequent DBB tests. The most intense DBB color reactions for basidiomycetes grown on solid media were obtained with urea agar and YNB agar containing glucose and CaCO_3 , but growth on these media was usually weak.

The alkali-ethanol-DBB test was the one procedure that provided a complete correlation between positive DBB reactions with basidiomycetes and negative reactions with other organisms (Table 1). An initial pH of 7.0 was necessary for a complete correlation because the basidiomycetous yeasts *Aessosporon salmonicolor* and *Sporidiobolus johnsonii* failed to give positive DBB reactions with an initial pH of 5.5 and because several other basidiomycetes gave weak results when grown in YNB broth containing glucose at an initial pH of 5.5. On complex media, such as Sabouraud broth, the number of basidiomycetes failing to react with DBB increased. With the alkali-ethanol-DBB method, basidiomycetes typically gave violet pigmentation with cells 72 h old. Weaker but still obviously positive reactions were obtained with 24-h-old growth, even with *C. laurentii* and *C. amylolepta*. Weak reactions sometimes appeared pink when the cells were in suspension, but the violet color was evident in the pellet after centrifugation. The concentration of the dye was not critical, since preliminary testing demonstrated that concentrations of 0.2 and 1.0 mg/ml gave the same results. Washing the cells with 95% ethanol markedly reduced the production of brown pigments, which otherwise occurred after DBB was added. The ethanol wash also extracted some of the carotenoids of the red yeasts, facilitating subsequent detection of violet pigments after DBB was added. The violet color was retained by the cells for several hours at room temperature and up to several days under refrigeration. Without the ethanol treatment, the violet color usually faded and was masked by brown pigments within a few minutes. No violet, pink, or red pigment was observed with the alkali-ethanol-DBB method for any of the ascomycetes.

All basidiomycetous strains produced extracellular urease, DNase, and proteinase. Of the 52 ascomycetes, only 12 produced extracellular urease, 14 were positive for DNase, and 15 were positive for proteinase. Proteinase activity of most red-pigmented yeasts was detected only with the synthetic casein medium.

DISCUSSION

All basidiomycetous yeasts (established as such on the basis of life cycle, laminar cell wall, and high nuclear deoxyribonucleic acid guanine-plus-cytosine contents) gave rapid and unequivocal positive reactions with the alkali-ethanol-DBB procedure, whereas all ascomycetes were negative. The mechanism of the DBB reaction is unknown, but van der Walt and Hopsu-Havu have speculated that the color formation involves tryptophane degradation intermediates and ketones associated with the cell wall (21). Tryptophane-supplemented media shortened the incubation time necessary for achieving a positive test with some basidiomycetes, but many strains also produced pigments before DBB was added. Production of pigments by yeasts grown on medium containing tryptophane has been reported previously (5). An initial pH of the medium near neutrality was more important for a positive DBB reaction than the use of media containing tryptophane, beef extract, or peptone. The alkaline hydrolysis step seemed to facilitate exposure and formation of substances which reacted with DBB and resulted in more intense colors than the color obtained after the natural growth aging autolysis process. Usually, a 72-h growth period was employed, but this was for convenience. Shorter or longer incubation periods could be employed; sufficient growth was the principal criterion. An important aspect of the alkali-ethanol-DBB method was the stabilization of the color reaction by ethanol. This permitted time for comparative readings of numerous strains.

Previous studies have shown that extracellular enzyme production is common in basidiomycetous yeasts (8, 20). Our results support the observation by van der Walt et al. (22) that some ascomycetous yeasts, such as *Saccharomycopsis* spp., also produce extracellular enzymes. The yeastlike ascomycetous fungi *Aureobasidium pullulans*, *Sporothrix schenckii*, and *Taphrina potentillar* are of particular note because they produced all of the enzymes tested.

Currently, the only definitive way to assess the basidiomycetous nature of the anamorph state of a yeast is to elucidate the life cycle. Considerable evidence has now accumulated that the ultrastructural nature and chemical na-

ture of the cell wall are also determinative (11, 21). However, procedures used to study these characteristics require technology not practical in routine identifications. The alkali-ethanol-DBB procedure is a simple and apparently reliable test for determining the basidiomycetous affinity of a yeast.

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REPRINT REQUESTS

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