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Further Modifications of the Auxanographic Method for Identification of Yeasts

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A modified auxanographic carbohydrate assimilation procedure for the identification of medically important yeasts is described. This method employs a heavy inoculum of unstarved yeasts, autoclaved yeast assimilation medium, pour plates of shallow depth, and commercially available carbohydrate-impregnated disks. The accuracy of this procedure was established in a comparison with the Wickerham broth method.

The role of yeasts as agents of opportunistic infection has steadily gained in prominence during the last decades (7, 10, 11). Because yeasts often colonize man, determining the significance of isolates from clinical specimens is usually difficult. Proper identification of yeasts allows an appreciation of the pathogenic potential and epidemiological character of the isolate. This information may assist the physician in evaluating the medical significance of the yeast, as well as in determining the proper course of therapy and the patient's prognosis.

Carbohydrate assimilation reactions are among the primary tests used to differentiate genera and species of yeasts (9). In many laboratories, the identification to the species level of yeasts other than *Candida albicans* is avoided because methods avaliable for assimilation testing are cumbersome, time consuming, and often difficult to interpret. Recently, new procedures for determining the carbohydrate assimilation reactions of yeasts have been described (1, 5, 8). These methods represent modifications of the auxanographic technique of Beijerinck (3) and/or the standard broth method of Wickerham (14). Although these modifications appear to be satisfactory, they possess several disadvantages. They use pH indicators to detect assimilation of carbohydrates. This requires buffering, manipulating, and monitoring the pH of the medium. Misinterpretation of test results may occur with reversion of the pH indicator or from the diffusion of acid in the medium (5). In addition, some methods require preparation of sterile carbohydrate or yeast nitrogen base (YNB) carbohydrate-impregnated disks (5, 8), which is time consuming.

We have developed a modification of the auxanographic method for carbohydrate assimilation testing that requires minimal preparation time and is easily interpreted. The accuracy of our modified method and its value in identifying yeasts isolated from clinical materials was evaluated against the standard Wickerham broth technique.

MATERIALS AND METHODS

Development of the modified auxanographic method. Several representative yeast species were used to examine selected variables in the auxanographic carbohydrate assimilation technique. Variations in the yeast inoculum and the basal media were tested in the preliminary studies. These included: inoculum consisting of nonstarved yeasts and of yeast starved for 24 and 48 h in distilled water, 1× YNB, 0.1 and 0.5% dextrose in distilled water or 1× yeast nitrogen base; an inoculum size ranging from 0.1 ml of a MacFarland no. 1 nephlometry standard to 1.0 ml of a MacFarland no. 5; the use of pour plates and plates inoculated by swabbing the surface with the yeast suspension; the alteration of agar depth, i.e., 25, 35, 50, or 70 ml of medium per 150-mm sterile petri dish; variation in the medium's agar concentration between 1.5 and 2.0%; and preparation of the medium with filter-sterilized or autoclaved YNB in concentrations of 1× (6.7 g of YNB/1,000 ml of medium) or 0.1×.

Species examined in these trials were laboratory stock cultures that included one isolate each of *Candida albicans, C. guilliermondii, C. tropicalis, Cryptococcus albidus* var. *albidus, C. albidus* var. *diffluens, C. neoformans, C. uniguttulatus, Rhodotorula rubra, Saccharomyces cerevisiae, Torulopsis glabrata,* and *Trichosporon cutaneum*. The modified auxanographic method described below was derived from the information obtained from these studies.

Modified auxanographic method. Medium for the modified auxanographic procedure was prepared by dissolving 6.7 g of YNB (Difco) in 100 ml of distilled water and then adding this to 1 liter of a 2.0% aqueous solution of Noble agar (Difco) that had been boiled for 1 to 2 min. Aliquots (25 ml) of this medium were dispensed into tubes (25 by 150 mm), which

were then autoclaved at 15 lb/in² at 121°C for 15 min. Medium either was used after cooling to 45 to 50°C or stored in a refrigerator at 5°C for a maximum of 6 weeks. Before use, two tubes of medium for each isolate to be identified were melted in a boiling water bath and then cooled to 45 to 50°C.

Growth from a 48-h culture of the yeast on Sabouraud dextrose agar was suspended in 5.0 ml of sterile distilled water and homogenized with a Vortex mixer. The turbidity was adjusted to that of a MacFarland no. 5 nephlometry standard. Each tube of cooled medium was inoculated with 1.0 ml of the standardized yeast suspension, mixed, and then poured into a sterile petri plate (150 by 15 mm). After the medium was allowed to harden at room temperature, six Minitek (BBL) carbohydrate-impregnated disks were placed on each of the plates. Carbohydrates tested include cellobiose, dextrose, dulcitol (galactitol), galactose, inositol, lactose, maltose, melibiose, raffinose, sucrose, trehalose, and xylose. Inoculated plates were incubated at 25°C and examined by indirect light every other day for 14 days. Assimilation of a carbohydrate was indicated by a zone of growth around the carbohydrate disk.

Evaluation of the modified auxanographic method. Yeast isolates used in this study were obtained from clinical material submitted to the bacteriology and mycology laboratories of the North Carolina Memorial Hospital, Chapel Hill. Two hundred and thirteen yeasts were examined (Table 1), which represented 7 genera and 15 species of medically important yeasts. After confirmation of its purity, each yeast was tested by the modified auxanographic method described above and by the Wickerham broth method without agitation (13). To prepare the Wickerham assimilation medium, 4.0 ml of distilled water was dispensed into test tubes and sterilized. To this was added 0.5 ml of a solution (6.7 g/100 ml) of YNB (Difco) which had been filter sterilized. A 6% solution of each carbohydrate to be tested (raffinose and dulcitol, 12%) was filter sterilized, and 0.5 ml was added to each tube. A control tube containing YNB but no carbohydrate was used for each isolate. Each tube was inoculated with 0.1 ml of a MacFarland no. 1 suspension of yeast that had been starved in distilled water at 25°C for 48 h. Tests were incubated at 25°C and were observed for 21 days. Assimilation tests were considered positive when more growth was observed in the tubes containing carbohydrate than in the control tube.

Isolates that gave conflicting reactions for a particular carbohydrate were retested by both assimilation methods to determine which procedure resulted in the initial incorrect reaction. Other parameters used to identify yeasts included fermentation of dextrose, galactose, lactose, maltose, sucrose, and trehalose (13); nitrate assimilation; urea hydrolysis; pellicle formation on Sabouraud dextrose broth; production of germ tubes in human serum; chlamydospore production on corn meal agar; and characteristic microscopic morphology (12). Identification was based on the reactions for yeast species described in reference 9.

RESULTS

Method development. During preliminary studies, variations in the auxanographic procedure that affected the accuracy or interpretation of results were noted. Medium containing autoclaved 1× YNB gave readings comparable to those of media containing filter-sterilized 1× and 0.1× YNB. Although medium containing autoclaved 0.1× YNB was satisfactory for testing yeasts, it failed to support abundant growth of *Cryptococcus uniguttulatus*.

Variation in inoculum density did not significantly reduce the completion time of assimilation tests. However, the use of 2% agar and a heavy inoculum, 1.0 ml of a MacFarland no. 5 suspension, resulted in the most compact, well-defined zones around carbohydrate disks.

Nonstarved yeasts and yeasts starved in various media for 24 and 48 h gave the same readings for assimilation tests. Background growth, which made the interpretation of some tests difficult, occurred with certain genera of yeasts, notably *Cryptococcus* and *Trichosporon*. This could not be eliminated by altering inoculum size or by prolonged starvation of yeasts. Commercially prepared YNB contains a small amount of inositol, which can be assimilated by members of these genera. The use of YNB prepared without inositol in our laboratory failed to reduce background growth. Decreasing the volume of agar in pour plates to 25 ml minimized background growth, thus permitting unambiguous and accurate readings. Because assimilations were generally complete within a few days, desiccation of the pour plates was not a problem. Inoculation of plates by swabbing the surface, despite the aforementioned variations, invariably resulted in troublesome background growth, particularly with *Cryptococcus* and *Trichosporon*.

Evaluation of the modified auxanographic method. Two hundred and thirteen yeasts were tested for their ability to assimilate 12 carbohydrates by the modified auxanographic and Wickerham broth methods. A total of 2,556 paired assimilations were performed (Table 1). Agreement between the two methods on initial testing was 98.2%. Of the 45 (1.8%) reactions that gave conflicting results initially, 44 showed agreement by both methods when the discrepant carbohydrate assimilations were repeated. In one instance, the difference in assimilation reactions could not be resolved by retesting. Final agreement between the two methods was 99.9%. The Wickerham procedure was responsible for 37 (82.2%) of the 45 initial incorrect results, whereas the auxanographic

method was in error in 7 (15.6%) instances. The auxanographic method averaged 3.3 days for completion of assimilation reactions, whereas the Wickerham broth procedure averaged 5.0 days. No changes in the assimilation patterns of the isolates tested were observed after 10 days with the auxanographic method or after 14 days with the Wickerham method.

The 45 assimilation reactions yielding initial disagreement between the two methods are shown in Table 2. Incorrect assimilation results for certain carbohydrates were frequently related to an assimilation method. In addition, similar errors occurred with several species of yeasts. Cellobiose most frequently resulted in initial errors with both methods (40.0%). Sixteen of the 18 initial incorrect cellobiose assimilation reactions were negative, with 14 occur-

TABLE 1. *Assimilation results obtained by the Wickerham broth and modified auxanographic methods*

Organism	No. of strains tested	No. of paired assimilation tests	No. of tests in agreement on initial test	No. of conflicting tests	No. of incorrect results with:		
					Wickerham method	Modified auxanographic method	No. unresolved by retest
Candida albicans	45	540	538	2	0	2	0
C. guilliermondii	6	72	71	1	1	0	0
C. krusei	11	132	132	0	0	0	0
C. parapsilosis	18	216	214	2	1	1	0
C. pseudotropicalis	7	84	81	3	1	2	0
C. tropicalis	54	648	634	14	14	0	0
Cryptococcus neoformans	8	96	93	3	3	0	0
C. albidus var. *albidus*	2	24	23	1	1	0	0
C. laurentii	2	24	24	0	0	0	0
C. terreus	1	12	12	0	0	0	0
Geotrichum candidum	1	12	12	0	0	0	0
Rhodotorula rubra	5	60	55	5	2	2	1
Saccharomyces cerevisiae	11	132	131	1	1	0	0
Torulopsis glabrata	35	420	420	0	0	0	0
Trichosporon cutaneum	7	84	71	13	13	0	0
Total	213	2,556	2,511 (98.2)[a]	45 (1.8)[a]	37 (82.2)[b]	7 (15.6)[b]	1 (2.2)[b]

[a] Percentage of total number of assimilation tests performed.
[b] Percentage of 45 conflicting tests.

TABLE 2. *Assimilation reactions for yeast species that were incorrect on initial testing*

Organism	Assimilation of:[a]								No. of unresolved reactions	Total
	Cellobiose	Dulcitol	Inositol	Lactose	Melibiose	Raffinose	Sucrose	Trehalose		
Candida albicans	1*/0			1*/0						2
C. guilliermondii					0/1					1
C. parapsilosis						1*/1*				2
C. pseudotropicalis	1/1							0/1*		3
C. tropicalis	0/12					0/1*	0/1			14
Cryptococcus neoformans				0/1*	0/1*			0/1		3
C. albidus var. *albidus*				0/1						1
Rhodotorula rubra	1/1 + 1**				0/1*	1/0			1	5
Saccharomyces cerevisiae								0/1		1
Trichosporon cutaneum		0/4*	0/1	0/1	0/3*	0/4*				13
Subtotal										
False-positive reactions	1*/0*	0*/4*	0*/0*	1*/1*	0*/5*	1*/6*	0*/0*	0*/1*		
False-negative reactions	2/14	0/0	0/1	0/2	0/1	1/0	0/1	0/2		
Unresolved	1	0	0	0	0	0	0	0		
Total	18	4	1	4	6	8	1	3		45

[a] Data expressed as incorrect modified auxanographic result/incorrect Wickerham result. Symbols: *, False-positive reactions; **, unresolved.

ring in the Wickerham method. *Candida tropicalis* accounted for 12 of these 14 negative reactions. Additional discrepancies with cellobiose occurred with *C. albicans*, *C. pseudotropicalis* and *Rhodotorula rubra*. The unresolved discrepancy occurring between both methods was observed with cellobiose.

A total of 18 incorrect assimilation tests were attributed to dulcitol, melibiose, and raffinose. Sixteen of the 18 reactions were false positive, occurring almost exclusively by the Wickerham method. Eleven of the 16 incorrect positive assimilation reactions for these carbohydrates were observed with *Trichosporon cutaneum*. The remaining nine initial discrepancies between the two methods were noted with inositol, lactose, sucrose, and trehalose. Other discrepancies occurred randomly with various organisms.

In this study, assimilation reactions for each isolate were considered correct (i) only after they were obtained by both methods after initial testing or retesting and (ii) when these results were consistent with published reactions (9). Correct assimilation patterns were obtained on initial testing for 209 (98.1%) of the 214 isolates by the auxanographic procedures and for 185 (86.4%) with the Wickerham method (Table 3). Initial incorrect results were observed most frequently with carbohydrates that may or may not be assimilated by the particular species of yeast tested. Assimilation of cellobiose by *C. tropicalis* and *R. rubra* and of melibiose, raffinose, and dulcitol by *T. cutaneum* is variable within these species. Therefore, in most instances an incorrect assimilation reaction obtained by either method would not have resulted in the incorrect identification of an isolate.

DISCUSSION

Our method for carbohydrate assimilation testing uses modifications of the basic auxanographic technique described by Silva-Hutner and Cooper (12), which are similar to those used by other investigators. Adams and Cooper (1) obtained satisfactory results when YNB was sterilized by autoclaving. Although Land and co-workers (8) have recommended the use of assimilation agar prepared with autoclaved 0.1× YNB, we observed reduced growth of *Cryptococcus uniguttulatus* on this medium and would recommend the use of 1× YNB. Our method uses a heavy inoculum of unstarved yeasts in pour plates of shallow agar depth. Huppert et al. (5) also recommend the use of plates with shallow agar depth in order to obtain more clearly defined end points. Huppert et al. (5) and Land et al. (8) have also used a heavy inoculum of unstarved yeasts without encountering difficulties due to carry-over of stored carbon sources. Thus, our modifications and those of other investigators represent procedural alterations that do not comprise, and apparently enhance, the accuracy of assimilation determinations while allowing these test

TABLE 3. *Comparison of correct assimilation patterns for yeast obtained on initial test with the modified auxanographic and Wickerham broth methods*

Organism	No. of strains tested	No. of correct assimilation patterns on initial test with:			No. unresolved by retest
		Both methods	Modified auxanographic method	Wickerham method	
Candida albicans	45	44	44	45	
C. guilliermondii	6	5	6	5	
C. krusei	11	11	11	11	
C. parapsilosis	18	16	17	17	
C. pseudotropicalis	7	4	6	5	
C. tropicalis	54	40	54	40	
Cryptococcus neoformans	8	6	8	6	
C. albidus var. *albidus*	2	1	2	1	
C. laurentii	2	2	2	2	
C. terreus	1	1	1	1	
Geotrichum candidium	1	1	1	1	
Rhodotorula rubra	5	2	3	3	1
Saccharomyces cerevisiae	11	10	11	10	
Torulopsis glabrata	35	35	35	35	
Trichosporon cutaneum	7	0	7	0	
Total	213	178 (83.5)[a]	209 (98.1)	185 (86.9)	1 (0.5)

[a] Numbers in parentheses are percentages.

procedures to be more convenient and practical.

In our evaluation of the Wickerham broth and modified auxanographic methods, agreement between the two methods was greater than 98% on initial testing. However, when inconsistent results occurred, they were more commonly observed with certain organisms and substrates. These results were obtained most frequently with the Wickerham assimilation method. The majority of false-positive errors obtained on initial testing of *T. cutaneum* probably resulted from the poorly defined end points observed with nonassimilated carbohydrates when the broth method was used. Despite the use of a carbohydrate-free control tube, discrimination between weak-positive results and background growth of *T. cutaneum* was often difficult. The use of plates with a shallow agar depth with the auxanographic method minimized the problem of background growth so that positive and negative results with this and other organisms were easily differentiated.

With both methods, initial negative cellobiose results were observed more frequently than any other incorrect reaction. Initial negative Wickerham assimilation tests for cellobiose were most often obtained with *Candida tropicalis*. We did not observe this problem with *C. tropicalis* when we used our modified auxanographic procedure. The dependence of cellobiose assimilation upon the presence of an inducible enzyme system (4, 6) may account for the frequency of false-negative reactions. The discrepancy with cellobiose between the two assimilation methods also may reside in the degree of aeration of cultures. Agitation of Wickerham broth tubes, which was not done in this study, has been shown to enhance the assimilation of carbohydrates by yeasts (2). The more successful detection of positive cellobiose assimilation results for *C. tropicalis* by the auxanographic method may be due to growth of the yeast on the pour plate surface. These inconsistencies, which were encountered with the Wickerham assimilation method, may be of interest to those using the procedure for yeast identification.

The agreement between the modified auxanographic and Wickerham broth methods was found to be 98.2% on initial testing and 99.9% when discrepancies were reevaluated. The auxanographic procedure, in our hands, was more reproducible than the reference Wickerham method, yielding correct assimilation results and accurate identification of isolates more frequently on initial testing.

The high degree of reproducibility evidenced by the modified auxanographic procedure may be attributed to its simplicity and the ease of interpreting results. Other advantages of the auxanographic method include: the elimination of inoculum starvation; use of easily prepared reagents that can be sterilized by autoclaving and stored before use; and minimal time and skill requirements for the performance of the test. The shorter time required for completion of assimilation reactions represents an additional advantage of our method over the reference method. The modified auxanographic procedure is an accurate and practical means of assimilation testing that may be useful for the identification of medically important yeasts in diagnostic laboratories.

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