Improved Auxanographic Method for Yeast Assimilations: a Comparison with Other Approaches

G. A. LAND,* E. C. VINTON, G. B. ADCOCK, AND J. M. HOPKINS

Departments of Clinical Microbiology and Medical Mycology, Wadley Institutes of Molecular Medicine, Dallas, Texas 75235

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An improved pour-plate auxanographic method has been developed for determining the assimilation of 14 different carbohydrates by medically important yeasts. Reduction of a dye incorporated into the agar has been correlated with the growth and carbohydrate assimilation of the yeasts, allowing the speciation of many yeasts within 24 to 48 h. This technique has been found to compare more than favorably with existing yeast assimilation techniques, in terms of rapid identification, total cost, and technician time in preparing and inoculating the plates. The dye pour-plate auxanographic technique provides an easier-to-interpret, rapid, and reproducible method of mycological identification for small clinical laboratories.

In debilitated hosts, such as cancer patients, time spent in the identification of microorganisms is of the utmost importance (1, 22, 30). Often the decision between which organism is a probable pathogen and which is a probable contaminant becomes critical when deciding upon addition of antimicrobials to the chemotherapy of these patients. For example, amphotericin B, the antimicrobial of choice for most systemic fungal infections, is not a drug to be promiscuously given to seriously ill patients, due to possible toxic side effects, especially nephrotoxicity (27). Thus, to prescribe this treatment on the basis of isolating an unspecified yeast from a patient could further compromise an already debilitated host (29). On the other hand, identification of fungal isolates is notoriously slow, and for this reason, antimicrobial therapy is often delayed beyond the point where it would be beneficial to the patient in case of a very real infection.

Most techniques for fungal identification involve laboratory demonstration of their morphology under various environmental conditions, as well as testing for certain biochemical traits they might have (2, 15, 21). The latter is particularly important in speciating medically important yeasts (1, 30). Identifying organisms in this manner, however, is tedious and usurps a great deal of technician time. Consequently, most small clinics or hospital laboratories do not take the time to do more than a cursory identification, i.e., *Candida albicans* versus *Candida* species not *albicans* (10). There has been, however, an increase in the number of hospitalized patients with disseminated oppor-

tunistic fungal infections (31) due to altered host defense mechanisms (8, 9, 20), surgical procedures (26, 29), and manipulation of indwelling catheters (35). With this higher incidence of opportunistic infections, then, it has become imperative to identify organisms quickly (13, 25) and to quantitate them (22) so that the time needed for confirmatory cultures without endangering the patient is available (G. L. Dorn, G. Burson, and J. Haynes, manuscript in preparation).

The ability of a yeast cell to assimilate various carbohydrates has long been used as the standard method to speciate medically important yeasts (7). Since the auxanographic technique of carbohydrate assimilation first appeared (7), several methods have been developed to improve the technique and obtain more definitive results. Recent advances in yeast assimilative techniques have included a modified auxanogram using sugar-impregnated disks (11) and a turbidimetric system in a broth, which has become the standard by which all other techniques are measured (33). Newer modifications of the Wickerham and Burton technique (33) include aeration of the broth (3), solidifying the broth as an agar slant (23), and modification of the agar slant by the addition of a pH indicator dye (1).

This latter technique (agar slant and indicator dye hereafter referred to as the Adams-Cooper technique) would be attractive to a small laboratory for several reasons: (i) a good correlation of indicator change with growth, making assimilations easier to determine; (ii) ease of preparation (the carbohydrate-contain-

ing medium may be autoclaved together in batch quantities and stored for up to 6 months); and (iii) cost in technician time compared favorably with existing methods.

In employing the Adams-Cooper technique (1) of yeast assimilation at Wadley Institutes and Granville C. Morton Hospital, we experienced a continual problem of contamination (approximately 10 to 15%). Contamination might be encouraged in the Adams-Cooper procedure, since the carbohydrate concentration of the medium is high and the sterilization procedure has been drastically reduced to avoid hydrolysis of some of the more labile carbohydrates.

Even though the dye assimilation slant has achieved a considerable timesaving advantage over other assimilation methods, identification of medically important yeasts still requires 1 to 2 weeks. The first several days of any procedure used for the identification of yeasts is usually concerned with starving the yeasts (4, 33), the rationale for this procedure being to deplete the large metabolite pools that are known to occur in fungi (19). Thus, upon subculture to the complete assimilation medium, the starved cells would be forced to feed off of the carbohydrates provided. The Adams-Cooper technique avoids some time loss as a result of starving the yeasts, by using a very dilute cell suspension as the inoculum. It takes, however, 2 to 3 days for this dilute inoculum to grow sufficiently to be noticeable on the slant, which does increase identification time. This present study was initiated to develop a method of yeast assimilation, based on current technology, but with faster identification capabilities. We also felt that any system of yeast assimilation should be applicable to small clinical laboratories in terms of costs, space, technologist time, and ease in identification of the specimen.

MATERIALS AND METHODS

Microorganisms. All yeast isolates came from the following sources: The Tulane University Mycology Collection; The Mycology Laboratories, Texas State Department of Health; Department of Microbiology, Temple University School of Medicine; Department of Microbiology and Immunology, Duke University Medical Center; Clinical Microbiology Laboratory, Granville C. Morton Cancer and Research Hospital.

Dye pour-plate auxanographic (DPPA) technique. A medium containing a pH indicator dye, which provided the optimum conditions for rapid carbohydrate assimilation, was determined by varying the pH and concentrations of either the nitrogen source or the carbon source. The composition of the medium supporting the most rapid assimilation of glucose by both *C. albicans* and *Cryptococcus neoformans* was as follows (per liter): 20 g of agar (Davis Gelatin, Christ Church, New Zealand); 20 mg of

bromocresol purple (Sigma Chemical Company, St. Louis, Mo.); 0.67 g of yeast nitrogen base (YNB; Difco Laboratories, Detroit, Mich.). The above dye agar medium was brought to a pH of 7 while molten, then dispensed into 4-ounce (0.121 liter) prescription bottles (60 ml per bottle) and autoclaved at 15 pounds pressure for 15 min. The sterile agar in bottles was used either immediately or after solidification and storage at 4 C.

The melted medium was allowed to cool to 45 to 50 C, seeded with a heavy suspension made from two to three colonies from a pure culture of the yeast (approximately 10^7 to 10^8 yeasts/ml, Table 1), poured into plates, and allowed to solidify. After the inoculated agar had solidified, carbohydrate-impregnated disks were placed on the surface of the plates, six along the outer perimeter of the plate, approximately 1 inch (2.54 cm) from the rim, and one disk in the center. The disks were arranged so that the first seven carbohydrates (see Table 3) were on one plate and the last seven on a second plate.

Carbohydrate disks. All sugars (Table 2) were obtained from the Sigma Chemical Company (St. Louis, Mo.) and were made up into 100-mg/ml stock solutions and filter-sterilized (0.20 μ m Nalge Lab Ware, Division Sybron Corporation, Rochester, N. Y.). Various concentrations (3 μ g to 30 mg) of these filter-sterilized stock solutions were dispensed on sterile concentration blanks (1/4-inch [0.635 cm] diameter, Difco Laboratories), placed in sterile plastic plates (60 by 15 mm, Kimble Plastics), and allowed to dry.

Assimilation slants. Assimilation slants were made and inoculated according to the procedure of Adams and Cooper (1). Briefly, the medium contained (per 100 ml): 2 g of agar (Noble agar, Difco Laboratories); 0.2 ml of a 1.6% solution of bromocresol purple; 1.0 ml of 0.1 N NaOH, 0.67 g of YNB (Difco Laboratories); 1 g of a specific carbohydrate (Table 2); and 100 ml of deionized water. The medium had a final pH of 7.0. The molten medium was

TABLE 1. Evaluation of the influence of size of the inoculum in the rapid identification of *C. albicans* by yeast assimilations

Dilution of original inoculum	No. of organisms per 0.1 ml ^a	Absorbance at 600 nm of the yeast suspension	Identification: time elapsed for complete yeast assimilations (h)
0	TN ^b	1.135	17
1:10	TN	0.185	17
1:10 ²	TN	0.025	24
1:10 ³	970		72
1:10 ⁴	88		>96

^a Done by averaging replicate spread plate cultures of 0.1 ml of each dilution (Sabouraud dextrose agar, incubated at 37 C).

^b Too numerous to count on spread plates.

^c The original suspension contained approximately 10^7 organisms/ml.

TABLE 2. Comparison of various carbohydrate assimilation techniques for rapidity in identifying known yeast isolates

Organism ^a	Medium ^b	Sugars ^c														Total elapsed time for identification ^d
		1 glu	2 gal	3 sorb	4 suc	5 malt	6 cello	7 tre	8 lac	9 mel	10 raff	11 mlz	12 xyl	13 dul/glc	14 inos	
<i>Candida albicans</i>	DPPA	0.25	0.25	0.58	0.25	0.25	0.5	0.5				0.25	0.5			0.5
	AC	2	3	3	3	3	14	5				8	5			8
	SA	2	6	5	3	7	13	7				10	13			13
	WICK	5	6	15	8	8		12				21	18			21
<i>C. pseudotropicalis</i>	DPPA	0.25	0.25		0.25		0.5		0.25		0.25	0.25				0.5
	AC	2	2		2		14		2	3	3	5				14
	SA	2	2		5		13		6	12	12	14				14
	WICK	5	5		8		18			18	14	14				18
<i>C. guilliermondii</i>	DPPA	1	1	2	1	1	0.5	2	2.5	2.5	3	2	3			3
	AC	2	5	10	5	5	12	5	14	5	5	5	14			14
	SA	2	10	5	5	5	14	12	14	5	5	10	12			14
	WICK	5	5	14	5	5		14	18	18	10	14	18			18
<i>Cryptococcus neoformans</i>	DPPA	0.25	1	1	1	1		1		0.25	0.25	1	1		0.5	1
	AC	3	3	5	3	3		5		3	3	5	3		3	5
	SA	5	5	5	5	5		5		5	5	5	7		5	7
	WICK	5	5	5	5	5		12		10	10	5	14		14	14
<i>C. albidos</i> var. <i>diffluens</i>	DPPA	0.5	3		0.25	0.5	0.5	0.3	3	1	1	0.5	0.25	0.5	2	3
	AC	3			3	3		5	7	14	5	3	14		3	>21
	SA	3	14		3	5		5	14	18	5	7	7		7	>21
	WICK	7	14		14	14		14	14	7	7	7	7		7	>21
<i>C. albidos</i> var. <i>albidus</i>	DPPA	0.25	3		1	1	1	1	3	1	1	1	1		1	3
	AC	3	14		3	3	14	14	14	3	3	3	14		3	14
	SA	3			3	3	18	18	14	14	7	7	7		7	>21
	WICK	7	7		7	7	20	16	14	18	7	7	7		7	>21
<i>Rhodotorula graminis</i>	DPPA	0.25	0.25	0.5	0.25		0.5	0.25		0.5		0.5	1		1	1
	AC	3	8	8	3		3	3		14	5	5	5		5	14
	SA	3	3	7	3		3	3		10	5	14	14		14	14
	WICK	7	7	5	5		7	7		14	14	7	14		14	14
<i>R. rubra</i>	DPPA	0.25	0.5	1	0.25	1	1	0.5		0.5	1	0.25			1	1
	AC	3	3	3	3	3	7	3		7	3	3	3		3	7
	SA	3	5	3	3	3	7	5		5	3	3	3		3	5
	WICK	7	7	7	7	7	7	7		7	7	7	7		7	7

<i>Torulopsis glabrata</i>	DPPA	0.25																0.5
	AC	3																3
	SA	3																3
	WICK	7																7
<i>Trichosporon cutaneum</i>	DPPA	0.25	0.25	1	0.25	0.5	1	0.5	0.25	0.5	0.25	1	0.5	0.25	0.5			1
	AC	5	5	14	3	3	14	5	5	5	14	5	5	5	5			14
	SA	5	5	7	7	7	14	5	7	7	7	7	7	7	7			>21
	WICK	7	7	14	14	14	21	14	7	7	14	7	7	7	7			>21

^a Results obtained by averaging the assimilation data from 10 strains of each organism. In cases where assimilation can be positive or negative, only the positive results are averaged.

^b AC, Assimilation slant; SA, swab auxanogram; WICK, Wickerham broth.

^c Data expressed as time in days for a positive reading of the sugar. A positive sugar assimilation for DPPA and AC media was dye change, whereas a positive for SA and WICK media was as defined by Lodder (21), i.e., noticeable growth on the substrate. Abbreviations: glu, glucose; gal, galactose; sorb, sorbose; suc, sucrose; malt, maltose; cello, cellobiose; tre, trehalose; lac, lactose; mel, melibiose; raff, raffinose; mlz, melizitose; xyl, xylose; dul, dulcitol; glc, galactol; inos, inositol.

^d Data expressed as the day the last sugar assimilation became positive.

dispensed (4.5 ml) into screw-capped tubes (16 by 125 mm; Owens-Illinois, Toledo, Ohio), sterilized by autoclaving at 10 pounds pressure for 10 min, and slanted. Slants were inoculated by pipetting 0.1 ml of a dilute yeast suspension onto the surface of the medium as described by Adams and Cooper (1).

Swab auxanographic technique. YNB was prepared in a 10× concentration in 100 ml of deionized water according to the manufacturer's directions (Difco Laboratories). The 10× solution was then diluted with 900 ml of deionized water and 20 g of agar (Davis Gelatin) was added. The medium was autoclaved at 15 pounds pressure for 15 min and allowed to cool to 50 C. Plates (155 by 15 mm, Kimble Plastics, Division Owens-Illinois) were poured to a 1/4-inch depth (approximately 60 ml). The plates were allowed to solidify and were incubated (37 C) overnight to remove moisture. The YNB agar plates were inoculated from a dilute yeast suspension by a modified swab technique (6). The swab was immersed in this yeast suspension and then streaked over the entire surface of the agar several times. Commercially prepared carbohydrate-impregnated disks (Difco Laboratories; BBL, Cockeysville, Md.) were arranged in the same pattern used for the DPPA technique on two plates. All inoculated plates and slants were incubated at either 37 C or room temperature and read at appropriate intervals.

Wickerham broth technique. YNB (10×) and carbohydrate stock (10%) solutions were prepared as described by Wickerham and Burton (33). The YNB solution was appropriately diluted, dispensed in 4.5-ml aliquots to screw-capped test tubes, and sterilized by autoclaving. The carbohydrate stock solutions were filter sterilized, and 0.5 ml of each carbohydrate was added aseptically to the cooled broth in tubes, making the final carbohydrate concentration in the medium 1%. The carbohydrate-broth tubes were inoculated as described by Wickerham and Burton, and assimilation results were also judged by his criteria (33).

Evaluation of techniques. The four yeast assimilation techniques were compared with each other for their ability to provide rapid assimilation results for known yeasts. The data collected were the elapsed time for each carbohydrate used in a particular assimilation technique to become positive and the total time required for the complete assimilation profile of the organism.

A more detailed comparison of the DPPA and swab auxanographic techniques was made in the following double-blind study. Specimens sent to the clinical laboratory of Granville C. Morton Cancer and Research Hospital were streaked for isolation on Sabouraud agar plates and incubated at 37 C and at room temperature. Single isolated yeast colonies were used to inoculate two Sabouraud slants. The inoculated slants were equally divided between two groups of technologists for identification. In addition, both groups were provided with known organisms to serve as internal controls for the experiment. Both groups followed the same schema for identification (1), differing only in their method of yeast assimilation (one group used the DPPA technique and the other group used the swab auxano-

gram). Data were expressed as the total time taken by each group for the identification of known or unknown yeasts.

Growth studies. Five-milliliter suspensions of *C. albicans* were made as described for the DPPA technique and the absorbance of each suspension was determined at 650 nm. Tenfold dilutions of these suspensions were made and their absorbance at 650 nm was also determined. One-tenth milliliter of each dilution was spread on the surface of a Sabouraud dextrose agar plate; the process was repeated in triplicate for each dilution. The remaining 4.7 ml of each yeast dilution was mixed with molten DPPA agar and allowed to solidify. Carbohydrate-impregnated disks were placed on the solidified plates for assimilation auxanography. The numbers of yeasts in each dilution were determined by the aforementioned Sabouraud spread plates and numbers were correlated with a specific absorbance value at 650 nm, as well as time required for each yeast dilution to give the complete carbohydrate assimilation pattern for *C. albicans*.

A second study involved correlating changes in absorbance at 600 nm with color change in the bromocresol purple dye and with yeast growth. Sealed quartz cuvettes (Gilford Instruments, Oberlin, Ohio) were sterilized and aseptically filled with one of the following: (i) a molten dye-agar combination, also containing 1.5 mg of glucose and 10^7 organisms per ml; (ii) the same dye-agar-glucose combination containing 10^4 organisms per ml; (iii) YNB glucose agar without dye inoculated with 10^7 organisms per ml; (iv) YNB glucose agar without dye inoculated with 10^4 organisms per ml; (v) a YNB-agar reference

standard containing no dye, glucose, or yeast. The agar in the cuvettes was allowed to solidify as quickly as possible, without temperature shocking the yeasts in the suspension. The changes in absorbance at 600 nm for each cuvette were monitored and recorded automatically at 2-min intervals for 24 h. The temperature of the spectrophotometric chamber during the experiment was 36 ± 1 C.

RESULTS

A medium was formulated which would permit the rapid assimilation of carbohydrates by *C. albicans* as evidence by a pH change from purple to yellow in an agar pour plate containing bromocresol purple (Table 1). Furthermore, it was our experience that a visible (purple to yellow) color change was apparent 2 to 4 h after inoculation with not only glucose but with several of the other carbohydrate substrates as well (Fig. 1B and C). By varying concentrations of each of the carbohydrates (Table 2), we determined the optimal concentrations for their rapid assimilation via the DPPA technique (expressed as milligrams/disk): (i) glucose, galactose, sucrose, and lactose, 3 to 6 mg; (ii) sorbose, maltose, and dulcitol, 5 to 7 mg; (iii) cellobiose, 10 to 14 mg; (iv) trehalose, raffinose, melizitose, and xylose, 10 to 12 mg; (v) melibiose, 15 to 20 mg; (vi) inositol, 2 to 4 mg.

The rapid assimilation of carbohydrates by *C. albicans* also appeared to be in direct propor-

FIG. 1. The assimilation of various carbohydrates by *C. albicans* using an improved DPPA technique: (A) 1 h, (B) 3 h, (C) 5 h, (D) 10 h, (E) 18 h, (F) 24 h. Numbers correspond to the carbohydrates listed in Table 2.

tion to the size of the inoculum (Table 1). Results obtained from plating 10-fold dilutions of a 5-ml yeast suspension for enumeration as well as carbohydrate assimilation indicated that an absorbance of 0.2 to 1.1 at 600 nm, which correlated with 10^6 to 10^7 yeasts per ml in the suspension, gave a complete carbohydrate assimilation profile within 17 h (Fig. 1). If the concentration of yeasts in the suspension was increased 10-fold, i.e., from 10^7 to 10^8 organisms per ml, the time for a complete assimilation pattern was reduced to around 12 to 14 h.

There also appeared to be a correlation between size of the inoculum, growth, and a change in color of the dyed agar from purple to yellow (Fig. 2). When the DPPA agar in a cuvette was inoculated with 10^5 organisms per ml, a steady decrease in absorbance at 600 nm was noted through 18 h, whereas during this same time a parallel cuvette containing yeast agar suspension without dye increased in absorbance at 600 nm. Upon direct observation, the dye agar yeast suspension was completely yellow by h 14. Continuous spectrophotometric monitoring showed that the rates of change of absorbance at 600 nm for the growth cuvette (YNB plus yeasts) and dye reduction cuvette (dye plus YNB plus yeasts) were approximately the same through 12 h (Fig. 2); i.e., time of

FIG. 2. Correlation of dye reduction with growth during pour-plate auxanography. Orgs/ml, Number of organisms in 1 ml of inoculum; BCP, bromocresol purple.

maximum growth correlated with the time of maximum dye reduction. Changes in growth and dye reduction continued until h 20, whereupon a stationary and parallel phase for both was noted. Dye agar inoculated with 10^7 organisms per ml showed an immediate change from purple to yellow (approximately 1 h) and a companion culture without dye showed a slight increase in turbidity at 600 nm during h 1. By direct observation at h 4, dye reduction was apparently completed. The cuvette containing yeasts plus YNB showed a modest increase in turbidity by this time; and, by h 4, the absorbance of both cuvettes at 600 nm was approximately the same. By h 8, both cuvettes showed a very limited rate of change and the rates of absorbance of both cuvettes paralleled each other.

In determining the rate of carbohydrate assimilations by known yeasts with either the DPPA technique or with the assimilation slant, swab auxanogram, or Wickerham broth, the DPPA technique consistently gave more rapid and easier-to-read results (Fig. 3, Table 3). The average time for the complete assimilation profiles of all yeasts in this study was as follows: DPPA, 1.4 days; assimilation slant, 10.9 days; swab auxanogram, 13.4 days; Wickerham broth, 16.7 days. The data broken down into generic groups show the same trends in rapidity of carbohydrate assimilation techniques; i.e., DPPA results were faster than assimilation slant and swab auxanogram, with Wickerham broth the slowest of the four. The genera of *Candida* and *Rhodotorula* as groups were all speciated within 1 day by the DPPA technique, as was *Torulopsis glabrata* and *Trichosporon cutaneum*. Final results with the assimilation slants for these fungi took 7 to 10 days, the swab plates 11 to 14 days, and the Wickerham broth greater than 14. Species of *Cryptococcus*, although following the same declension in time

FIG. 3. The auxanographic assimilation of various carbohydrates on DPPA by *Cryptococcus neoformans*. Numbers correspond to the carbohydrates listed in Table 2.

for complete yeast assimilation on the various agars as the other fungi had, were slower as a group in giving complete assimilation patterns. For example, the *Cryptococcus* group averaged 2 days for complete assimilation patterns on the DPPA. The other carbohydrate assimilation

techniques also required correspondingly longer time to give complete assimilation results.

The comparison of the DPPA with the swab technique in the identification of yeasts isolated from clinical material also showed that

TABLE 3. Comparison of the DPPA technique of yeast assimilation with a swab auxanographic (SA) technique in identifying either known yeasts or unknown yeasts isolated from patient material

Organism	Medium	No. of isolates	Days required for complete assimilation			
			1	2	3	>3
<i>Candida albicans</i>	DPPA	56	88 ^a	100		
	SA	56			86	100
<i>C. aaseri</i>	DPPA	1	100			
	SA	1			100	
<i>C. utilis</i>	DPPA	1 ^b			100	
	SA	1 ^b				100
<i>C. tropicalis</i>	DPPA	11	73	100		
	SA	11			55	100
<i>C. stellatoidea</i>	DPPA	22	92	100		
	SA	22			77	100
<i>C. parasilosis</i>	DPPA	12 ^b	55	100		
	SA	12 ^b			50	100
<i>C. krusei</i>	DPPA	2	100			
	SA	2			50	100
<i>C. guilliermondii</i>	DPPA	10	60	80	100	
	SA	10			60	100
<i>C. pseudotropicalis</i>	DPPA	14	71	94	100	
	SA	14			57	100
<i>Cryptococcus neoformans</i>	DPPA	22	59	77	100	
	SA	22			68	100
<i>C. albidus</i> var. <i>albidus</i>	DPPA	5		20	100	
	SA	5				100
<i>C. albidus</i> var. <i>laurentii</i>	DPPA	4			75	100
	SA	4				100
<i>C. uniguttulatus</i>	DPPA	1	100			
	SA	1			100	
<i>C. terreus</i>	DPPA	3 ^b		33		100
	SA	3 ^b			33	100
<i>C. luteolus</i>	DPPA	1			100	
	SA	1				100
<i>Rhodotorula glutinis</i>	DPPA	1	100			
	SA	1			100	
<i>R. graminis</i>	DPPA	2	50	100		
	SA	2			100	

TABLE 3—Continued

Organism	Medium	No. of isolates	Days required for complete assimilation			
			1	2	3	>3
<i>R. rubra</i>	DPPA	1		100		
	SA	1			100	
<i>Sporobolomyces salmonicolor</i>	DPPA	4		25	75	100
	SA	4			25	100
<i>S. roseus</i>	DPPA	1			100	
	SA	1				100
<i>Saccharomyces cerevisiae</i>	DPPA	19	42	79	100	
	SA	19		21	52	100
<i>S. champagnii</i>	DPPA	3		33		100
	SA	3				100
<i>S. rouxii</i>	DPPA	2			100	
	SA	2				100
<i>Torulopsis glabrata</i>	DPPA	26	50	66	100	
	SA	26		38	69	100
<i>Trichosporon cutaneum</i>	DPPA	7	100			
	SA	7			57	100
<i>T. pullulan</i>	DPPA	1		100		
	SA	1				100
<i>Aureobasidium pullulan</i>	DPPA	2		100		
	SA	2				100
<i>A. prunorum</i>	DPPA	1 ^b		100		
	SA	1 ^b			100	
<i>Geotrichum candidum</i>	DPPA	2	50	100		
	SA	2			100	
<i>Ustilago zeae</i>	DPPA	2 ^b			50	100
	SA	2 ^b				100

^a Data expressed as percentage identified within a specified time period.

^b Cultures provided from either the Tulane University Mycology Collection or the mycology laboratories, Texas State Department of Health.

the DPPA approach gave a faster assimilation profile (Table 3). This was a double-blind study, with two separate groups of technologists isolating and identifying yeasts from either sputum, biopsies, autopsy tissue, or blood, in addition to some known fungal cultures for reference strains. The DPPA group averaged 95% identification by 3 days and 100% identification by 4 days. The swab plate group averaged 45% identification of all fungi by 3 days, 65% by day 7, and 100% by day 13. The complete data from both groups showed a 90% correlation on identification (only 243 of the 300 strains or isolates

identified are reported in this paper). Results from this study also demonstrated that pure yeast colonies obtained from various primary isolation media (i.e., Sabouraud dextrose agar, eosin methylene blue agar, blood agar plates, corn meal plus Tween 80, rice extract plus Tween 80, and *Salmonella-Shigella* agar) could be made into suspensions and be used for the DPPA technique without a prior period of growth and starvation before their assimilation pattern was determined. However, the swab plates tended to show some background growth when prepared directly from an isolation plate.

DISCUSSION

The increased incidence of opportunistic fungal infections in patients undergoing treatments with corticosteroids (24, 25), surgical manipulations (26, 29), catheterization (35), immunosuppression (17, 22), or in the advanced state of malignancy (8, 9, 14, 18, 31) has made it necessary for clinics and hospitals to improve their fungal identification techniques. Most small clinics and laboratories, however, have not taken the time in the past for proper fungal identification, for reasons of work overload and lack of technical expertise (1, 10). Although many techniques and media have been developed to streamline the identification of bacteria from clinical samples, particularly the *Enterobacteriaceae* (16), few techniques have been developed to provide the same service in identifying fungi.

Traditionally, yeasts have been identified by characteristic fermentation (gas production) in a carbohydrate-containing broth and by growth without gas production in a carbohydrate-containing broth, i.e., assimilation (1, 4, 21, 33). Yeast carbohydrate assimilations done in the broth system, as well as in a solidified version (23), require some type of starvation procedure (4, 33) prior to inoculation into a medium containing the specific carbohydrate. The starvation procedure was deemed necessary to decrease background growth, made possible by carbohydrate pools within the yeast, which could give false positive growth on latent or weakly assimilated substrates. The swab plate technique provided an example of background growth during the present study if the plates were seeded directly with cells grown on a rich medium, but not if the inoculum had been previously starved. However, the starvation procedure increases the time required for yeast identification.

A recent modification of the standard methodology for assimilations involved the addition of a dye (bromocresol purple) to a solidified Wickerham medium (1). Results obtained with this technique suggested that a change in color from purple to yellow could be correlated with the growth of the yeast on the substrate. Although the dye-slant technique for yeast assimilation provides faster, easier-to-interpret, and more reproducible results, there are some inherent problems with the procedure. One disadvantage is that several days are lost as the dilute inoculum on the slant grows enough to be noticed by the technologist. Another problem is that with a multitude of tubes (13 to 15 depending upon the number of sugars used) for each test, and with several yeasts per day to iden-

tify, the technologist could easily lose track of which tube has or has not been inoculated. A final disadvantage, at least in our experience, is a consistent 10 to 15% contamination rate in the uninoculated assimilation slants.

The evolution of the DPPA technique was, as stated in the introduction, to provide a rapid, more reproducible, and less sophisticated method of yeast assimilation (1, 10). The use of a dye-agar auxanogram was also explored by Adams and Cooper (1); and the auxanograms compared favorably with their slants. However, they used a swab technique to inoculate the plate which might tend to give ambiguous results for weak or latent assimilations of carbohydrates. The original auxanographic technique was a pour plate (7); we felt that the pour plate would also provide a tightly defined ring of growth around the substrate-impregnated disk, since the size of the ring would be controlled by the diffusion of substrate from the disk into the agar.

Dye was added to the agar based on the suggestion of Adams and Cooper (personal communication) that growth seemed to be correlated with a pH change in the medium (1), with the bromocresol purple indicator changing from purple to yellow. Studies presented in this paper confirm that observation, for both a high inoculum and a low inoculum showed dye reduction and a concomitant increase in turbidity; the latter observation has classically been interpreted as growth. Admittedly, the changes in turbidity of either inoculum was not striking, but then cellular division would not be expected to occur at a rapid rate with a large inoculum in the microaerophilic environment of a pour plate. However, in comparing results from the turbidity experiments and the dilution plates with regard to time needed for the assimilation pattern of a yeast to be completed, our results suggest that dye reduction occurs rapidly in the presence of 10^6 to 10^8 cells per ml and has unfailingly correlated with the assimilation of a particular carbohydrate.

The nitrogen source also affected the rate of carbohydrate assimilation. For example, concentrations of YNB suggested by the manufacturer to be optimal for growth appeared to cause a considerable delay in the production of acid from carbohydrate metabolism. Decreasing the concentration of YNB to 0.1 or 0.01 times the suggested concentration permitted a more rapid appearance of a change in pH indicator to yellow. There are several good reasons why the limitation of nitrogen could affect carbohydrate assimilation. For instance, the nitrogen source could affect the metabolism of the

cell: first of all, limited nitrogen would create starvation conditions within the cells after several metabolic turnovers, especially if the yeasts are inoculated in high numbers. In addition, a decrease in YNB would mean a concomitant decrease in the amount of NH_4^+ ion in the medium, as ammonium sulfate is one of the medium's constituents (0.5%). A decrease in the NH_4^+ ion has been shown to affect carbohydrate transport in yeast and other fungi as well as influencing their carbohydrate metabolism by affecting some key enzymes for control of glycolysis and energy production (reviewed in 5, 12, 32, 38).

There are some disadvantages to the DPPA technique. The pour plate itself is felt by some to be an extremely messy technique and more open to contamination. Although the latter is true, the large inoculum used in this method would in most cases favor overgrowth of the plate by the inoculated organisms rather than by chance airborne or shed contaminants, nor have we been bothered with undue contamination since employing the DPPA technique in our laboratory. The heavy yeast suspension used as an inoculum requires several colonies, which may be a disadvantage if the sample is limited. However, most primary plates have enough growth for the test to be done adequately and current research indicates that the DPPA technique may be adapted to several micro-techniques using one colony as an inoculum (Land, Hopkins, and W. H. Fleming III, manuscript in preparation). Temperature control of the molten agar is also critical, and to avoid mistakes requires a water bath specifically set for that one temperature. If the agar

is too hot (above 50 C), labile organisms such as some of the *Cryptococcus* species will be heat-killed to a level that would make rapid and correct interpretations of the plate difficult. If the agar is too cool (below 45 C), the agar will begin to gel, giving a very heterogenous suspension and plate, again making interpretation of results difficult. The use of two plates may also be a disadvantage, but it has been our experience that two plates and seven sugars per plate produce the easiest results to read and the most complete information. On occasion, we have used one plate and 14 sugars, and it proved a bit crowded and required some experience in reading results, especially if sugars were weakly or latently assimilated.

We do feel that the advantages of this technique outweigh the disadvantages. For example, the use of the DPPA technique with 14 sugars can rapidly speciate 90% or better of the common yeasts a clinical laboratory will receive, without having to do the time-consuming fermentations. The shelf life in prescription bottles is at least 6 months. The DPPA may be inoculated from a primary plate without starvation, thus decreasing total identification time. The composition of the medium as well as the inoculum have also been formulated to give rapid assimilation results, and in the case of most medically important yeasts, identification within 24 h is not uncommon. Organisms which grow at 37 C may be tested at that temperature, in most cases achieving more rapid yeast assimilation profiles than at room temperature. A cost comparison of the DPPA and the assimilation slant (Table 4) shows that they both are comparable. Admittedly, cost comparisons are

TABLE 4. Comparison of cost and time per test in preparing the dye auxanogram plates and the dye assimilation slants

Auxanogram		Assimilation slants	
Item	Cost	Item	Cost
Petri plates	0.46	Tubes ^a	3.45
YNB	0.01	YNB	0.04
Davis agar	0.03	Noble agar	0.09
4-oz prescription bottles ^a	0.21	Bromocresol purple	0.005
Bromocresol purple	0.01	Technician time ^b	
Technician time ^b		Preparation and autoclaving of medium	18.00
Preparation and autoclaving of medium	10.50	Labeling and preparation of suspensions	5.00
Labeling and preparation of suspensions	2.00	Inoculation of medium for 20 tests	5.20
Inoculation of medium for 20 tests	3.20	Cost/test	1.59
Cost/test	0.82		

^a Items autoclavable and reusable.
^b Medium for 20 tests and evaluated at \$4.00 per h.

somewhat artificial and the disparity of cost per test between the two techniques probably is not that striking.

However, the ease of preparation, ease of inoculation, the saving of space, and the quick, reproducible, and easy-to-interpret results of the DPPA suggest that this technique might be considered by a small laboratory for yeast assimilations. It certainly fulfills the requirements (1, 10) for a less sophisticated and more timesaving method of mycological identification for use in clinical laboratories. Thus, a laboratory using an easy-to-follow schema of generic identification, such as proposed by Silva-Hutner and Cooper (30), and the DPPA technique to speciate the organisms could provide an adequate mycological identification service with a minimum expenditure of time and money.

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