Rapid Urea Broth Test for Yeasts

GLENN D. ROBERTS,¹ CARLYLE D. HORSTMEIER,¹ GEOFFREY A. LAND,^{2*} AND
JANIE H. FOXWORTH²

Section of Clinical Microbiology, Department of Laboratory Medicine, Mayo Clinic, Rochester, Minnesota 55901,¹ and Department of Microbiology, Wadley Institutes of Molecular Medicine, Dallas, Texas 75235²

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A rapid, miniaturized, urea broth test useful for detecting urease activity of yeasts was compared to Christensen urea agar. All urease-producing yeasts tested were positive on both media; however, 60% were reactive in the urea R broth within 30 min, and the remainder were reactive within 4 h. This urea multiwell test may be useful as a rapid screening method for detecting urease-producing yeasts recovered from clinical specimens and as an adjunct test with other rapid methods of yeast identification.

Urea hydrolysis has been considered a prominent point at which dichotomy occurs in the classification of yeasts (1, 12). In conjunction with morphology on vegetable-polysaccharide agars, urea hydrolysis has been used to separate several medically important genera from other yeasts, namely, *Cryptococcus* species, *Trichosporon* species, and to a lesser extent *Rhodotorula* species (1). The conventional method, Christensen urea agar (CUA), was originally formulated to separate various bacterial species (2-4, 11, 13) and was adopted shortly thereafter for yeast identification (12). The mechanism of the test is simple, relying upon the release of ammonia during urea hydrolysis to change the pH of the medium to alkaline, converting the light-orange phenol red indicator to pink. The reaction is much slower in yeasts than in bacteria, usually requiring 18 to 48 h for the positive test. We felt that the time to positivity for CUA might possibly be shortened to a 1-workday test to serve as a screening technique (9) and to augment several rapid tests recently developed for yeast identification (1, 5, 7).

Since many hospitals have experienced an influx of patients susceptible to yeast colonization (6), there has been a concomitant increase in the varieties as well as numbers of yeasts isolated by clinical laboratories. This plethora of yeasts has necessitated that laboratorians become more proficient in yeast identification and more efficient in their ability to rapidly select for isolates that are of medical importance. The following report demonstrates how the rapid test for urea hydrolysis might be used to fulfill these requirements.

MATERIALS AND METHODS

Isolates: The organisms used in this study were stock cultures, isolates from the Center for Disease

Control and New York City Department of Health Proficiency Testing Programs, or clinical isolates from the mycology laboratories of the Mayo Clinic, the Wadley Institutes of Molecular Medicine, the Baylor University Medical Center at Dallas, and the University of Oklahoma at Norman. Additionally, several American Type Culture Collection (ATCC) isolates were used: ATCC 26310, *Candida albicans*; ATCC 24064, *Cryptococcus neoformans*; ATCC 16725, *Rhodotorula glutinis*; ATCC 10663, *Trichosporon capitatum*; and ATCC 9331, *Trichosporon pullulans*.

Inoculum. An inoculum was prepared by covering the tip of a wooden applicator stick or the wooden end of a Johnson & Johnson 6-inch (15.24-cm) cotton bud with cells from one large yeast colony (approximately 10 mm in diameter) or three or four small colonies (1 to 4 mm in diameter).

Urea broth. Urea broth was made according to Christensen's original formula (2). The basal medium contained, per liter of water: peptone (Difco Laboratories, Detroit, Mich.), 1 g; sodium chloride, 5 g; monopotassium phosphate, 2 g; phenol red, 0.012 g; glucose, 1 g; and urea, 20 g. The latter chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. In some experiments, the concentration of either phosphate (from 10^{-1} to 10^{-4} M) or urea (from 2 to 0.002%) was varied to ascertain concentrations of each necessary for rapid urea hydrolysis. All broths were sterilized by filtration (0.22- μ m; Falcon, Oxnard, Calif.), decanted into 1-dram (ca. 1.2-g) vials (3 ml/vial), and stored at -70°C until needed. The broth was stable under these conditions for at least 2 months. Once thawed, the urea broth was used immediately or refrozen at -20°C if any remained. After 5 days, all used and refrozen broth was discarded.

URB. Dehydrated urea R broth (URB; Difco) was reconstituted with 3 ml of sterile distilled water per the manufacturer's directions. Once reconstituted, the broth was used within 1 workday.

Rapid urea hydrolysis. Two-tenths milliliter of test medium was pipetted into each well of a clean, nonsterile 96-well microtiter test plate (Falcon, Microtiter II). Test plates prepared with an automatic micropipetter (Sigma, 100 ml) equipped with nonsterile

tips were found to be satisfactory. Inocula from unknown and known organisms were prepared as above, transferred to the microtiter wells, and mixed vigorously by agitation. Wells were sealed with Scotch tape and incubated for a maximum of 4 h at 37°C, with readings taken hourly. Any change in the indicator to pink indicated the presence of urease (Fig. 1). Additionally, slants of CUA (Difco) were made as directed, and each slant was inoculated in parallel with the rapid multiwell test.

RESULTS

To determine optimum conditions for rapid urea hydrolysis by yeasts, the buffering capacity, substrate concentration, and reaction temperature were varied. In preliminary studies, the time for urea hydrolysis was indirectly proportional to the buffering capacity of the medium (Fig. 2). When the phosphate concentration of the medium approached that of CUA (0.38 M phosphate), the reaction took 24 h to occur. If the $\log_{10}$ of phosphate concentration was plotted against time to positivity, a typical buffer titration curve was observed. The fastest specific reaction took place within a range of 3×10^{-3} to 10^{-3} M phosphate, whereas below 10^{-3} M many false positives were observed. Raising the phosphate concentration to 10^{-2} M removed the false positive reactions and only slightly decreased the reaction rate to an average of 90 to 120 min. In extending the study at 10^{-2} M phosphate to

include several replications of over 600 isolates, no false positive reactions were observed. Changes in urea concentration within a range of 0.02 to 2% did not influence the positive reaction; however, at concentrations of $\leq 0.002\%$, false negative reactions occurred and became more frequent with further substrate limitation. The optimal temperature for rapid urea hydrolysis

FIG. 2. Relationship of phosphate concentration of medium to time positive in the yeast urease reaction. Average of three replications of (—) four different *Cryptococcus* species (*C. neoformans*, *C. laurentii*, *C. albidus*, and *C. diffluens*) and (-----) *Candida* species (*C. albicans*, *C. stellatoidea*, *C. tropicalis*, and *C. parapsilosis*).

FIG. 1. Urea hydrolysis by unknown yeasts using URB in a microtiter test plate incubated at 37°C for 4 h. Plates should be read at hourly intervals up to the 4-h limit. In the figure, a positive test is read as any change in contrast from light gray (wells B-11, F-7) to dark gray (wells C-3, D-3) or black (wells D-4, E-4). In the actual test a positive is indicated by any change of the straw-colored medium to purple-pink.

was 37°C, whereas higher temperatures led to both false negative and positive reactions and lower temperatures slowed down the reaction.

URB has a formula consistent with the component concentrations promoting rapid urease reactions found in the above studies. Therefore, additional studies included parallel experiments with the commercial and synthetic broth. Under test conditions, the time to positivity with URB proved to be faster than that predicted by the concentration studies. Sixty minutes were required for a positive test instead of the 120 min expected from the phosphate titration curve (Fig. 2, Table 1). Sixty-nine percent of all isolates positive by CUA were positive on URB within 30 min, with an additional 16% positive at 60 min and the majority of the remaining cultures (10%) positive by 120 min (Table 1). No false positive reactions were observed in these experiments, and 4 h was adopted as the maximum incubation time for the test. Further testing of 2,900 unknown clinical isolates and known stock cultures from other sources confirmed the 4-h incubation period (Table 2).

In determining urease activity in the above 2,900 strains, a few false positive and negative reactions were seen (Table 2). Three percent of all stock cultures previously found to be positive by growth on CUA were negative by the multiwell test (Table 2). However, when subcultured onto Sabouraud agar plates, incubated overnight, and retested, the organisms proved to be positive on URB within 4 h. When cultures were permitted to age for 5 days or more on Sabouraud dextrose agar, false positive reactions would occur, i.e., some 2% of 2,709 urea-negative isolates (Table 2). Finally, pregrowth of these unknown and known yeasts on commonly used isolation media such as blood, chocolate, brain heart infusion, Trypticase soy, Sabouraud, and

corn meal agars had no effect upon urea multiwell test results.

DISCUSSION

Urea decomposition was previously deemed a critical characteristic for separating species within the genus *Proteus* (11, 13). The medium of Rustigian and Stuart (11), devised for *Proteus* identification, permitted differentiation between fast and slow urease producers. By lowering the concentration of phosphate and optimizing the inoculum size and temperature, Christensen demonstrated that most urease-producing bacteria could hydrolyze urea within 2 to 4 h (2). However, the subtle differentiation between fast and slow urease producers, a characteristic felt to be of importance to earlier investigators, was sacrificed for the rapid test. The role of urea hydrolysis in yeast taxonomy was emphasized by Seeliger's use of the test as a screen for the identification of cryptococci (12). Even though the test was essentially the same as Christensen's rapid method, yeasts did not hydrolyze urea as quickly as bacteria, resulting in a 24- to 48-h test. For this reason, the urea slant test for yeasts is held for 4 to 5 days (8, 12) before it is considered negative. Although urea hydrolysis has proven an excellent characteristic for yeast classification, it has never been fully adapted to the physiological requirements of the yeasts.

By following the same logic Christensen used for bacteria, we determined the optimum conditions for the yeast urease reaction. First, the buffering capacity of the medium was found to play an important role in reaction rate. If the phosphate concentration was limited, the reaction rate increased until a concentration of 10^{-3} M was reached. Below this concentration, reactions went too quickly, and false positives were noted. This was especially true of older cultures

TABLE 1. Time for urea hydrolysis in an optimized liquid medium^a

Organism	No. of strains	Time positive (min)							
		30	60	90	120	150	180	210	240
<i>Cryptococcus neoformans</i>	96	91 ^b	5						
<i>C. albidus</i> var. <i>albidus</i>	49	42	6		1				
<i>C. albidus</i> var. <i>diffluens</i>	20	14	3	2	1				
<i>C. terreus</i>	9		3	1	3	1	1		
<i>C. luteolus</i>	4				2		2		
<i>C. uniguttulatus</i>	3	2	1						
<i>C. skinneri</i>	2		1		1				
<i>Trichosporon cutaneum</i>	27	7	13		3		2	1	1
<i>T. pullulans</i>	10	2	3	1	2	1	1		
<i>T. capitatum</i>	8	1	2	1				2	2

^a Average of three replications for each isolate in URB and in a synthetic medium (see Materials and Methods). Results were the same with either medium.

^b Number of isolates positive at unit time.

TABLE 2. Urea hydrolysis by yeasts in stock culture as determined by the urea multiwell test and CUA slants

Organisms	No. of strains	No. of isolates positive or negative by:			
		CUA		URB	
		+	−	+	−
Urease-positive organisms					
<i>Cryptococcus neoformans</i>	121	119	2	119	2 ^a
<i>C. albidus</i> var. <i>albidus</i>	40	39	1	39	1
<i>C. albidus</i> var. <i>diffluens</i>	42	40	2	40	2
<i>C. laurentii</i>	27	27		27	
<i>C. terreus</i>	8	8		7	1
<i>C. uniguttulatus</i>	3	2	1	3	
<i>C. skinneri</i>	2	2		1	1
<i>Rhodotorula</i> species	19	13	6	19	
<i>Trichosporon cutaneum</i>	6	2	4	5	1
<i>T. capitatum</i>	4	2	2	4	
<i>Prototheca wickerhamii</i>	1	1		1	
Total	273	255	18	265	8
Urease-negative organisms	2,709 ^a		2,709	48 ^b	2,661

^a Includes isolates of *Candida albicans*, *C. tropicalis*, *C. krusei*, *C. stellatoidea*, *C. parapsilosis*, *C. pseudotropicalis*, *Torulopsis glabrata*, *Saccharomyces cerevisiae*, *S. rouxii*, *S. chevalieri*.

^b All false negative or false positive organisms were found to be from 7-day or older cultures. When these were subcultured onto Sabouraud dextrose agar, incubated overnight, and then retested, all subcultures reverted to their expected urease activity.

pregrown on a medium rich in peptone and may have been the result of nonspecific indicator changes due to the natural alkalinity of an older colony. The alkalinity of an older colony merely reflects a buildup of released ammonia during extended growth on peptone. Urea concentration and reaction temperature for the yeast enzyme generally agreed with values obtained with the bacterial system. Similarly to the bacterial enzyme, the substrate concentration range was fairly broad, with 0.02 to 2% urea sufficient substrate to carry the reaction. However, excess urea (2%) was provided to drive the reaction toward ammonia release and alkalinity rather than permitting reversal to an acid pH upon substrate exhaustion. The reaction rate was found to increase linearly from 25 to 37°C, plateau at 40°C, and then begin to decline at 45°C. Presumably, these latter results were due to the higher temperature either driving off ammonia before indicator change or inactivating the intracellular enzyme itself. At 37°C, enzyme activity appeared independent of growth since some organisms incapable of growing at 37°C showed competent urease production. Specific enzyme activity in the absence of yeast growth has been reported elsewhere (5) and is suggestive of a temperature-insensitive constitutive enzyme whose activity is independent of replication.

The optimum combination of buffer and substrate in the synthetic medium, 10⁻² M phosphate and 2% urea, was found to be closely

approximated by URB. In comparing the performance of URB with that of synthetic medium, the commercial broth also worked within the 4-h incubation period. Most positive isolates could be determined within 30 to 120 min on URB, with a few recalcitrant isolates taking a full 4 h. There were a few false negatives with both CUA (6.6%) and URB (3%), as well as several false positives (2%) with the latter test. The aberrant results were always found in testing older cultures and could be abrogated by subculturing and retesting the fresh isolates. For this reason we suggest that isolates no older than 3 days be used for this test.

A recent paper has suggested the use of the Bertholet reaction to determine urease production (10). Although offering clear-cut results in 50 min, there are several disadvantages to the system. (i) Preparation of the reagents requires stringent chemical measurements and techniques, which take unnecessary time. (ii) Reagents must be freshly prepared for each day of testing. (iii) A large inoculum is used for the Bertholet test, requiring several days of pre-growth, offsetting the advantage of a faster test. The urea multiwell system offers the advantages of a prepared and lyophilized medium, or, if formulated in house, a medium quickly made up from easily obtained and stable ingredients. When made in large batches, the medium may be divided into smaller aliquots and then frozen at -70°C until needed. Moreover, the inoculum

may be derived from single colonies of pure isolates, doing away with pregrowing the inoculum several days before testing.

The urea multiwell test provides results on an important characteristic in yeast identification within 1 workday. In conjunction with other rapid tests (1, 5, 7), an unknown yeast in pure culture may be identified within 24 h. Finally, since the test has been reduced to a small volume and hourly readings may be taken with ease, the rapid urease test may be used as a fast and efficient screen for some medically important yeasts (9).

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