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Modification of Potassium Nitrate Assimilation Test for Identification of Clinically Important Yeasts

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The modification of an auxanographic method used in yeast species identification to determine potassium nitrate (KNO_3) assimilation resulted in a simple and inexpensive KNO_3 assimilation test medium. The medium provided accurate and reliable results in less than 24 h.

Potassium nitrate (KNO_3) assimilation is a significant physiologic criterion for identifying yeasts (2, 4, 6), but conventional methods for determining KNO_3 assimilation (3, 5, 6) are often inconvenient to use and frequently give erroneous results. The Wickerham broth method (WBM; 7), which is the standard method, requires extensive preparation and a long incubation time (up to 31 days). Shorter incubations (≤7 days) are used with auxanographic procedures (AUXs), nitrate reductase determination methods, and the rapid nitrate swab test. However, these methods may give false-positive results owing to carry-over with the inoculum or to background color resulting from yeast cell pigments. False-negative results may be caused by the complete exhaustion of KNO_3 and its reduction to ammonium ions (3, 5, 7).

The KNO_3 assimilation test included in the Uni-Yeast-Tek (UYT; Flow Laboratories, Inc., McLean, Va.) yeast identification system provides reliable results, but frequently samples must be incubated for up to 7 days (1). Therefore, we modified the medium in the UYT system and devised a reliable KNO_3 test that is faster, less expensive, and easier to perform.

A total of 159 test organisms (Table 1), maintained as cell suspensions in sterile distilled water, were selected from the stock collection of the Analytab Products Mycology Laboratory. The purity and identity of each isolate were confirmed by standard morphologic and physiologic procedures (2).

For this investigation, a portion (0.2 ml) of each test isolate was removed aseptically from the stock tube with a sterile pipette, streaked over the surface of Sabouraud glucose agar (BBL Microbiology Systems, Cockeysville, Md.) in a 100-mm-diameter petri plate, and incubated for 48 to 72 h at 25 to 30°C. A portion of one of the colonies which developed was transferred aseptically and streaked over the surface of a second plate containing Sabouraud glucose agar and incubated as described above.

Colonies from the second Sabouraud glucose agar culture were used as inocula for four KNO_3 assimilation tests: the WBM, an AUX, the UYT system, and the modified KNO_3 agar (MKA) method. Growth in all four systems was assessed at least daily.

The WBM medium, containing 1.17% yeast carbon base (Difco Laboratories, Detroit, Mich.) and 0.078% KNO_3 (Fisher Scientific Co., Pittsburgh, Pa.) in aqueous solution, was used in initial comparative studies of our modified medium. These tests were conducted as outlined by van der Walt (6), with the tubes incubated at 25 to 30°C and examined daily for up to 31 days for growth (turbidity). A portion of the growth was removed aseptically from a tube with a turbidity equivalent to or greater than a Wickerham 2+ suspension. This portion was transferred to a second KNO_3 assimilation tube and incubated, and turbidity was measured as described above. Only isolates with growths ≥ a Wickerham 2+ suspension in two serial broth tests were considered positive for KNO_3 assimilation. The WBM medium without the nitrate source was used as a negative control to estimate background growth.

The AUX was used as described by van der Walt (6). Tubes containing 15.0 ml of sterile yeast carbon base (1.17%) in 1.2% Noble agar (Difco) were heated, and the molten medium was cooled to 50°C. A portion of the growth from a single colony of each test isolate was removed aseptically and suspended in the molten medium. The seeded agar was then poured into a 100-mm-diameter petri plate and allowed to solidify at 25°C. Sterile 3-mm-diameter paper disks were impregnated with KNO_3 by soaking overnight in a sterile 3.0% aqueous solution of KNO_3 and were dried for 2 h in a vacuum oven. Positive growth control disks were prepared similarly by soaking paper disks in a 3.0% solution of peptone (Difco). A control disk and an experimental disk were placed aseptically 4 cm apart on the surface of each seeded plate. The plates were incubated at 25 to 30°C and examined daily for growth (turbidity) around the KNO_3 and peptone disks.

The UYT plates were inoculated according to the instructions of the manufacturer. A portion of the growth of a single colony of each test isolate was transferred aseptically to sterile water to prepare an inoculum equivalent to a Wickerham 1+ suspension, and approximately 0.05 ml of the suspension was added to the KNO_3 well. The UYT test medium consisted of 0.125% KNO_3, 0.146% yeast carbon base, 0.008% bromthymol blue, and 1.5% agar (formulation provided by A. Zino, Flow Laboratories, Inc., through personal communication). During a 6-day incubation at 25 to 30°C, the plate was assessed at 2-h intervals on day 1 and then daily thereafter. Growth was detected by a color change of the pH indicator and by colony formation on the agar surface.

MKA was prepared by separately dissolving 1.4 g of

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TABLE 1. Distribution of 159 test organisms in KNO_3 assimilation studies

Organism	No. of isolates
Aureobasidium pullulans	6
Candida albicans	15
C. ciferrii	1
C. guilliermondii	3
C. krusei	3
C. lusitaniae	2
C. norvegensis	1
C. parapsilosis	1
C. pseudotropicalis	1
C. steatolytica	1
C. tropicalis	6
C. utilis	4
Cryptococcus albidus	13
C. laurentii	11
C. neoformans	7
C. terreus	5
C. uniguttulatus	3
Hansenula anomala	9
H. fabianii	3
H. polymorpha	5
Hyphopichia burtonii	1
Kloeckera apiculata	2
Kluyveromyces fragilis	1
K. vanudenii	1
K. veronae	1
Malassezia pachydermatis	2
Pichia farinosa	1
Rhodotorula glutinis	7
R. minuta	4
R. pilimanae	5
R. rubra	5
Rhodotorula sp.	1
Saccharomyces cerevisiae	2
S. microellipsodes	1
Sporobolomyces salmonicolor	5
Torulopsis glabrata	9
T. haemulonii	2
T. magnoliae	4
Trichosporon beigelii	4
T. pullulans	1

KNO_3, 1.6 g of yeast carbon base, 0.12 g of bromthymol blue, and 16.0 g of Noble agar in 1.0 liter of distilled water. The final pH was adjusted to 5.9 to 6.0. Compared with the most recent formulation of the KNO_3 medium in the UYT kit, MKA contained slightly higher concentrations of KNO_3, yeast carbon base, pH indicator, and agar. Portions (6 ml) of MKA were dispensed into screw-cap tubes (16 by 125 mm), autoclaved for 15 min, and allowed to solidify at a slant. A portion of a single colony of each test isolate was removed aseptically with a sterile applicator stick, streaked over the slant agar surface, incubated at 25 to 30°C, and examined daily. Positive results were indicated by a change of the medium from greenish yellow to blue-green or blue.

There was complete agreement of results with MKA, the AUX, and the WBM for 30 test isolates. Definitive results were obtained with MKA and the AUX within 7 days after inoculation. For the WBM, with daily growth readings, an average incubation of 14 days (range, 6 to 31 days) was required.

Identical results were also obtained with MKA and the AUX for an additional 61 isolates when the media were assessed after 7 days of incubation at 25 to 30°C. We found MKA easier to prepare, inoculate, and read.

TABLE 2. Comparison of two agar-based methods for rapidity

Organism	No. of strains	Incubation time (days)[a]	
		MKA	AUX
Nitrate-positive yeasts			
Aureobasidium pullulans	4	1	3
Candida utilis	4	1	1.3
Cryptococcus albidus	5	1	1.6
C. terreus	3	1	1.7
Hansenula anomala	4	1	1
H. polymorpha	2	1	2
Rhodotorula glutinis	5	1	1.6
Sporobolomyces salmonicolor	3	1	1
Torulopsis magnoliae	3	1	2
Trichosporon pullulans	1	6	8[b]
Nitrate-negative yeasts			
Candida albicans	3	1	3
C. guilliermondii	1	1	3
C. tropicalis	3	1	3
Cryptococcus laurentii	5	1	3
C. neoformans	4	1	3
Rhodotorula minuta	4	1	3
R. pilimanae	4	1	3
R. rubra	4	1	3
Torulopsis glabrata	3	1	3
Trichosporon beigelii	3	1	3

[a] Time required to obtain definitive results with the isolates tested. For the AUX, negative results were recorded on day 3, since the maximum incubation period needed to obtain a positive reaction was 3 days, except for *T. pullulans*. For both systems, samples were incubated at 25 to 30°C.

[b] Negative results were obtained after 8 days of incubation.

In a study to compare the rapidities of the MKA method and the AUX, definitive results with the 68 remaining test isolates were obtained with MKA within 1 day of inoculation (Table 2), except with *Trichosporon pullulans*. The AUX required an average of 2 days to produce similar results.

In similar rapidity studies with the UYT system, positive results were obtained, on the average, in 1 day or less with MKA, compared with 2 days with the UYT system (Table 3). Weak positive responses were much easier to detect with MKA. Carry-over with the inoculum occasionally biased the AUX and UYT results, but no such interference was found with MKA. Because one isolate of *T. pullulans* failed to assimilate KNO_3 with the AUX, the overall correlation between results from MKA and the AUX was 99.4%.

TABLE 3. Comparison of the MKA and UYT methods for rapidity

Organism	No. of isolates	Incubation time (h)[a]	
		MKA	UYT
Aureobasidium pullulans	3	12	48
Candida utilis	4	6	24
Cryptococcus albidus	3	18	48
C. terreus	1	24	48
Hansenula anomala	4	10	24
H. polymorpha	2	15	36
Rhodotorula glutinis	5	10	48
Sporobolomyces salmonicolor	3	24	48
Torulopsis magnoliae	3	18	48

[a] Average time required to obtain definitive results with the isolates tested. Growth was assessed at 2-h intervals on day 1 and then daily thereafter.

These studies have shown that the MKA procedure is rapid, reliable, relatively simple, and inexpensive. Definitive results were obtained, on the average, in 1 day or less with MKA, compared with 2 days with the UYT system and the AUX and 14 days with the WBM. We found nearly complete agreement of results from the four tests with 159 isolates. MKA was simpler and easier to prepare than other media. The final cost per test ($0.20 to $0.25) was approximately one-half that of the AUX or UYT KNO_3 test and one-fourth that of the WBM test. In addition, MKA was easier to inoculate, and results were easier to interpret. Weak reactions could be more readily detected, and there was no carry-over interference.

The advantage of our MKA procedure can best be ascribed to (i) the use of tubes or plates with larger surface areas, resulting in faster detection of KNO_3 utilization, and (ii) the inoculation of the medium with heavier suspensions of yeast cells, resulting in more rapid assimilation of the nitrogen source.

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