Rapid Method for Determining Nitrate Utilization by Yeasts

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Received for publication 4 January 1977

A test for the nitrate-reductase activity in yeasts has been developed, in which the reaction may be read after only 10 min of incubation. The rapidity of the test is due to the optimization of pH, substrate concentration, and temperature for the reaction.

Nitrate utilization is an important criterion for identifying species of many medically important yeasts, particularly in the genera *Cryptococcus*, *Trichosporon*, and *Rhodotorula* (3, 6). Test methods used in the past have been unsatisfactory for reasons of lengthy incubation time and, in many cases, ambiguous results (5, 7, 8). The special nitrate broth of Rhodes and Roberts (5) has been to date the best way to determine nitrate assimilation. This test detects the presence of nitrite, the end product of nitrate reductase, in a semisolid medium via reaction with equal volumes of 0.5% α -naphthylamine and 0.8% sulfanilic acid each in 5 N acetic acid. These latter reagents are indicators of the nitrite anion and cause formation of a red azo dye in its presence (1, 4). The advantages of this test include that the results were obtained much faster than was possible with the previous methodologies and that the red azo dye complex provided a bright, easy-to-read test. The special nitrate broth method required only 40 to 72 h of incubation, and the test correlated at the 99% level with standard nitrate utilization tests (5). Assuming that the α -naphthylamine-sulfanilic acid reaction does accurately measure nitrate reductase, then parameters that affect the reaction rates of other enzymes should also influence chromophore production in the positive test. In reactions of purified enzymes, there exists some combination of pH, ionic strength, substrate concentration, and temperature for which the reaction proceeds at its maximum. The reaction rate of an enzyme within the intact cell should, in addition, be affected by other factors such as membrane permeability and substrate transport across cell membrane. In this study, we sought to optimize the effect of pH, substrate concentration, and temperature on the reaction rate of nitrate reductase in the intact yeast.

With the Rhodes-Roberts medium serving as a point of departure, the effect of a large inocu-

lum, hence large enzyme concentration, on the reaction rate was first determined. By introducing a heavy inoculum (approximately 10^8 cells) into 0.5 ml of special nitrate medium, nitrate utilization was detectable after 2 h of incubation at 37°C. With the same size inoculum and reaction volume as above, positive results were also obtainable within 2 h by using 0.1% aqueous KNO_3 alone as a substrate. The effect of pH on the reaction rate was then evaluated in phosphate buffers of varying pH containing 0.1% KNO_3 . The optimal pH range for the reaction was found to occur between 5.8 and 6.5, by visual grading of color change with α -naphthylamine and sulfanilic acid (Sigma Chemical Co.): i.e., 0 = no color change with respect to a negative control, 1+ = faint pink, 2+ = pink, 3+ = dark pink to light red, and 4+ = dark red. From this point on, all experiments were carried out with 0.1% KNO_3 in 0.1 M phosphate buffer, pH 5.8.

Time to positivity was determined at varying temperatures. Five minutes of incubation in a water bath at 50°C yielded 1+ to 2+ results with clinical isolates of *Cryptococcus albidus* var. *diffluens*; longer incubations at 50°C had no further effect. Increasing the nitrate concentration to 0.2% KNO_3 , then incubating 10 min at 45°C, yielded 2+ to 3+ results. These latter modifications were adopted as standard conditions for further testing.

Since nitrate reductase in the cell rather than cell growth was important to this study, the amount of substrate (nitrate) available to the enzyme would also control the reaction rate. To rule out any effect the cell membrane or transport mechanisms of the cell might have on substrate availability within the cell, substances affecting membrane permeability were evaluated for effect on the reaction rate. Amphotericin B (Fungizone; Squibb) was incorporated in varying concentrations in the medium, with the optimum concentration occurring be-

tween 30 to 60 $\mu\text{g/ml}$. All nitrate-positive species tested yielded 4+ reactions under standard incubation conditions, except *Rhodotorula glutinis* which remained 2+. Other substances tested such as Zephiran chloride (Winthrop Laboratories, 200 $\mu\text{g/ml}$), Tween 80 (BioQuest, 10,000 $\mu\text{g/ml}$), and saponin (Nutritional Biochemicals Co., 10,000 $\mu\text{g/ml}$) yielded 4+, 3+, and 3+ reactions, respectively, with all yeasts examined, including the rhodotorulas. In concentrations ranging up to 20%, neither sodium lauryl sulfate (Sigma) nor dimethylsulfoxide (J. T. Baker) enhanced the positive tests. Also, changes in membrane permeability did not change the reaction of nitrate-negative yeasts. Because of its effectiveness at low concentration, Zephiran chloride (200 $\mu\text{g/ml}$) was chosen as the agent to increase cell permeability to the reaction mixture.

For most strains tested, the results of the optimized test for the reductase were as clear as the Rhodes-Roberts procedure. However, when determining nitrate-reductase activity of red yeasts with a small reaction volume and large inoculum, a color control was often required to separate the red of the positive test from the red yeast inoculum. The color control consisted of a duplicate yeast suspension minus the nitrite-developing reagents. To eliminate the color control, the nitrate assimilation test was varied as follows: the optimal medium as described above was prepared in a 5 $\times$ concentration: KNO_3 , 2 g; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 11.7 g; Na_2HPO_4 , 1.14 g; Zephiran chloride, 1.2 ml of a 17% solution; and 200 ml of water. Standard cotton swabs (Johnson and Johnson, 6-inch [ca. 15.24 cm] Cotton Buds) were saturated in the 5 $\times$ concentrate, absorbing 0.1 ml of the medium. Thus, the swab contained the equivalent solids of the 0.5-ml reaction mixture. The saturated swabs were then dried in vacuo at room temperature for 24 h and autoclaved.

The tip of a swab prepared in the preceding manner was coated with yeasts by sweeping it across several colonies on a plate. Whereupon, the inoculated swab was swirled against the bottom of an empty test tube (13 by 150 mm) to assure adequate contact between organism and substrate within the swab. The tube containing the swab was incubated for 10 min at 45°C; the swab was removed from the tube and inserted into a second tube containing two drops each of the α -naphthylamine and sulfanilic acid reagents. The test remained quite easy to read, and the cotton tip of the swab containing the organisms turned bright cherry red. The swab technique had several advantages over liquid methods. Since each swab was a self-contained test unit, by eliminating the need for solid me-

dium or pipetting, required for other nitrate tests, many could be prepared beforehand. Also, since they were dehydrated, swabs were less prone to contamination than liquid medium and could be stored in a sealed container for a longer time. Furthermore, when red yeasts were tested for nitrate assimilation, a separate color control was not necessary. If the organisms were confined solely to the swab tip, the reagents soaked vertically through the swab into the white area above the inoculum, separating the bright red color of the positive test from the red yeasts.

The influence of prior growth conditions on nitrate utilization was determined with the swab test on several positive and negative isolates grown at room temperature for 48 h on media commonly used for the primary isolation of microorganisms (Table 1). For the most part, the nitrate assimilation of these organisms was unaffected by prior growth conditions. *Candida utilis* (ATCC 22023) nitrate reductase seemed affected by previous growth conditions, including temperature of growth, in that media containing blood products and incubation at room temperature seemed to inhibit nitrite production. In a survey of 287 isolates of 25 different yeast species, the rapid nitrate test correlated 100% with the Rhodes-Roberts test (Table 2). Several isolates, which were adjudged nitrate positive by the rapid test, had previously been considered nitrate negative by both the Delft and Wickerham nitrate tests. Upon retesting these isolates via the Rhodes-Roberts method, they were reclassified as nitrate positive.

In summary, a rapid technique for detecting nitrate utilization by yeasts has proved advantageous. The swab nitrate method yields results within 15 min as opposed to 72 h for the special nitrate broth of Rhodes and Roberts, and 7 to 14 days for either the Wickerham or Delft method. The rapidity of swab technique is due to: (i) direct detection of the enzyme conversion of nitrate to nitrite within the inoculum, rather than inferring nitrate assimilation from growth on a special medium, resulting in an increased time to positivity; (ii) optimization of parameters known to affect any enzyme reaction; and (iii) elimination of measuring or pipetting of liquids during the test. Another advantage is that addition of zinc dust is unnecessary to eliminate false-negative reactions, as has been the case in earlier tests with the α -naphthylamine and sulfanilic acid reagents (4). The nitrate concentration within the swab has been increased so that it no longer limits the reaction, thereby eliminating the possibility of a complete reduction of nitrate to ammonium ion, which would not be detectable by the devel-

TABLE 1. Effect of different primary plating media on yeast nitrate reductase as determined by the rapid nitrate assimilation test

Media	Reaction of organism in media ^a					
	<i>Cryptococcus</i> <i>albidus</i> var. <i>diffluens</i>	<i>Cryptococcus</i> <i>albidus</i> var. <i>albidus</i>	<i>Rhodotorula</i> <i>glutinis</i>	<i>Candida utilis</i>		<i>Candida</i> <i>albicans</i>
				Growth at 25°C	Growth at 37°C	
SDA ^b	4+	4+	4+	1+	2+-3+	0
GSDA ^c	4+	4+	3+	0-1+	3+	0
SABHI ^b	4+	4+	3+	1	1+-2+	0
CMA ^d	3+	4+	3+	3+	4+	0
TOC ^e	3+	3+	2+	3+	3+	0
BLOOD ^f	3+	3+	3+	0	0-1+	0
CHOC ^f	3+	3+	3+	0	0-1+	0

^a 0, No color change; 1+, faint pink; 2+, pink; 3+, dark pink to light red; 4+, dark red.

^b Sabouraud dextrose agar (Difco).

^c Gentamicin (Schering), Sabouraud dextrose agar (Difco).

^d Cornmeal agar (Difco).

^e Experimental medium for *C. albicans* morphology and *C. neoformans* pigmentation (2).

^f BBL prepared media.

TABLE 2. Nitrate-reductase activity in yeasts as determined by both the rapid nitrate assimilation test (swab test) and the special nitrate broth method

Organism	No. of iso- lates tested	Swab test		Special nitrate broth	
		No. posi- tive	No. nega- tive	No. posi- tive	No. nega- tive
Nitrate-positive organisms ^a					
<i>Cryptococcus terreus</i>	10	10	0	10	0
<i>C. albidus</i> var. <i>albidus</i>	24	24	0	24	0
<i>C. albidus</i> var. <i>diffluens</i>	39	39	0	39	0
<i>Candida utilis</i>	3	3	0	3	0
<i>Rhodotorula glutinis</i>	14	14	0	14	0
Total	90	90	0	90	0
Nitrate-negative organism ^a					
<i>Cryptococcus neoformans</i>	32	0	32	0	32
<i>C. laurentii</i> var. <i>laurentii</i>	9	0	9	0	9
<i>C. uniguttulatus</i>	2	0	2	0	2
<i>Rhodotorula rubra</i>	1	0	1	0	1
<i>Trichosporon cutaneum</i>	4	0	4	0	4
<i>T. capitatum</i>	1	0	1	0	1
<i>Candida albicans</i>	48	0	48	0	48
<i>C. guilliermondii</i>	6	0	6	0	6
<i>C. tropicalis</i>	26	0	26	0	26
<i>C. zeylanoides</i>	2	0	2	0	2
<i>C. parapsilosis</i>	7	0	7	0	7
<i>C. rugosa</i>	2	0	2	0	2
<i>C. stelladoidea</i>	5	0	5	0	5
<i>C. aaseri</i>	3	0	3	0	3
<i>C. pseudotropicalis</i>	2	0	2	0	2
<i>C. krusei</i>	5	0	5	0	5
<i>Torulopsis glabrata</i>	18	0	18	0	18
<i>Saccharomyces cerevisiae</i>	4	0	4	0	4
<i>S. chevalieri</i>	8	0	8	0	8
<i>S. champagnii</i>	12	0	12	0	12
Total	197	0	197	0	197

^a As previously determined by the Delft or Wickerham nitrate assimilation tests.

oping reagents. Since substrate is no longer a rate-limiting step of the reaction, swabs can be incubated at 45°C from 10 min to at least 18 h without affecting test results. While the swab technique for nitrate assimilation yields rapid results, it sacrifices neither accuracy nor clarity. Accumulated results of this technique have, in every case, correlated with the special nitrate-broth test, which had been shown to be a superior method of determining nitrate utilization in yeasts (4).

This research was supported by funds from the Estate of Leroy R. Kinney Charitable Trust, New York, New York.

We also wish to express gratitude to G. D. Roberts, J. W. Murphy, and B. H. Cooper for their generous gift of cultures.

ADDENDUM

Although α -naphthylamine may be obtained from Sigma Chemical Co., St. Louis, Mo., some difficulty may arise in the future purchase of this chemical since it has been labeled a controlled substance. We have evaluated several alternative reagents on a limited scale and have found the pair, 0.5% *p*-arsanilic acid in 5 N acetic acid and 0.8% *N*-naphthylethylenediamine dihydrochloride in 5 N acetic acid, to be satisfactory. These reagents can be used in place of

the α -naphthylamine and sulfanilic acid reagents if so desired.

LITERATURE CITED

1. Blazevic, D. J., and G. M. Ederer. 1975. Principles of biochemical tests in diagnostic microbiology, p. 79-82. John Wiley & Sons, New York.
2. Fleming, W. H., J. M. Hopkins, and G. A. Land. 1977. New culture medium for the presumptive identification of *Candida albicans* and *Cryptococcus neoformans*. J. Clin. Microbiol. 5:236-243.
3. Lodder, J. 1971. Utilization of nitrate, p. 84. In J. Lodder (ed.), The yeasts. North-Holland Publishing Co., Amsterdam.
4. Morrison, R. T., and R. N. Boyd. 1959. Diazonium salts, p. 570-584. Organic chemistry. Allyn & Bacon Inc., Boston, Mass.
5. Rhodes, J. G., and G. D. Roberts. 1974. Comparison of four methods for determining nitrate utilization by cryptococci. J. Clin. Microbiol. 1:9-10.
6. Silva-Hutner, M., and B. H. Cooper. 1974. Medically important yeasts, p. 491-507. In E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D. C.
7. Webb, D. C., C. Papageorge, and C. T. Hall. 1971. Identification of yeasts. Center for Disease Control, Atlanta, Ga.
8. Wickerham, L. J. 1951. Taxonomy of yeasts. Dep. Agric. Tech. Bull. 1029:1-56.