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Rapid Detection of Yeast Enzymes by Using 4-Methylumbelliferyl Substrates

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In a preliminary study with a limited number of isolates, the usefulness of 17 4-methylumbelliferyl substrates for identifying yeast isolates was investigated. Substrates to detect acid phosphatase, glucosidase, and pyrophosphate diesterase showed promise.

In 1975, Maddocks and Greenan (5) reported the use of 4-methylumbelliferyl conjugated substrates for the rapid detection of bacterial enzymes. The hydrolysis product of enzymatic action on these conjugates, 4-methylumbelliferone, produces a light blue fluorescence when viewed with the long wavelength of a Wood's lamp. The 4-methylumbelliferyl substrates also have been used to study the enzyme activities of mycobacteria (4) and mycoplasmas (1), as well as in the study of a wide range of metabolic problems in humans (2, 7-9). Considering these reports, it seemed important to investigate the usefulness of fluorogenic substrates for aiding in the rapid identification of medically significant yeast isolates.

The organisms used in this study were obtained from the Northwestern Hospital (Minneapolis, Minn.) and the Cleveland Clinic Foundation (Cleveland, Ohio) mycology stock culture collections and from fresh clinical isolates recovered by the University of Minnesota Hospitals Mycology Laboratory. Each isolate was subcultured onto Emmon-modified Sabouraud dextrose agar for 48 h before testing. The distribution of species of the 65 organisms evaluated included the following: 10 of *Candida albicans*, 5 of *Candida guilliermondii*, 4 of *Candida krusei*, 10 of *Candida parapsilosis*, 4 of *Candida pseudotropicalis*, 3 of *Candida stellatoidea*, 9 of *Candida tropicalis*, 10 of *Candida* (*Torulopsis*) *glabrata*, 3 of *Cryptococcus laurentii*, and 7 of *Cryptococcus neoformans*.

The 4-methylumbelliferyl substrates (Koch-Light, Colnbrook, England) and the enzymes assayed for are listed in Table 1. A 15-μmol sample of each substrate was dissolved in 0.2 ml of dimethyl sulfoxide (J. T. Baker, Phillipsburg, N.J.). *N',N*-Dimethylformamide (J. T. Baker) was used to dissolve 4-methylumbelliferyl butyrate, elaidate, nonanoate, and palmitate, since these substrates precipitated in dimethyl sulfoxide. Each substrate was diluted to 10 ml with selected buffers or dilute acids, which included 0.1 M sodium acetate-hydrochloride buffer (pH 5.1), 5 μM sodium citrate-citric acid buffer (pH 4.5), and 0.01 M acetic acid (pH 3.4). The diluted substrates were sterilized by filtration (Millipore Corp., Bedford, Mass.), dispensed in 0.3-ml portions into test tubes (13 by 100 mm), and used for testing immediately or frozen at −20°C for use during a 1-week period. A heavy, milky suspension of each yeast isolate was made in the specific substrates. The suspensions were incubated in a 37°C heating block for 30 min, 2 h, and 4 h. After incubation, the tubes were removed and inspected for the presence or absence of fluorescence, using the long wavelength of a Wood's lamp in a partially darkened room. In positive tests, the substrate-organism mixtures produced a light blue fluorescence, indicating the release of 4-methylumbelliferone from the hydrolyzed substrates; no fluorescence was observed with negative tests. An uninoculated tube of substrate and an inoculated tube of solvent without conjugate were used as controls to make certain that the conjugate had not deteriorated or that the solvent had not caused the organisms to fluoresce.

Of the 17 fluorogenic substrates used to detect enzymatic activity, the substrates for the detection of acid phosphatase, β-D-glucosidase, and pyrophosphate diesterase produced results which would be helpful in the identification of yeasts. The results, including pH of substrates and incubation times, are shown in Table 2. With a pH of 3.4 and an incubation time of 4 h, acid phosphatase activity was detected in all of the yeast isolates with the exception of *C.* (*Torulopsis*) *glabrata*. Only the 10 strains of the two species of *Cryptococcus* were positive for β-D-glucosidase activity after 30 min at pH 5.1, thus separating this genus of yeast from *Candida* species. When pyrophosphate diesterase

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TABLE 1. *The 4-methylumbelliferyl substrates and corresponding enzymes assayed*

4-Methylumbelliferyl substrate	Enzyme assayed
Phosphate	Acid phosphatase
α-L-Arabinopyranoside	α-L-Arabinopyranosidase
Sulfate K salt	Aryl sulfatases
Butyrate	Butyrate esterase
β-D-Cellobiopyranoside monohydrate	β-Cellobiosidase
Elaidate	Elaidate esterase lipase
β-L-Fucopyranoside	β-L-Fucosidase
2-Acetamido-2-deoxy-β-D-galactopyranoside	*N*-Acetyl-β-D-galactosaminidase
β-D-Galactopyranoside (anhydrous)	β-D-Galactosidase
2-Acetamido-2-deoxy-β-D-glucopyranoside	*N*-Acetyl-β-D-glucosaminidase
β-D-Glucopyranoside	β-D-Glucosidase
β-D-Glucuronide (trihydrate)	β-D-Glucuronidase
α-D-Mannopyranoside	α-D-Mannosidase
Nonanoate	Nonanoate esterase lipase
Palmitate	Palmitate esterase lipase
Pyrophosphate diester disodium salt	Pyrophosphate diesterase
β-D-Xylopyranoside	β-D-Xylosidase

TABLE 2. *Enzyme activity in 65 yeast isolates*[a]

Strain	No. of isolates examined	No. of isolates positive for the following enzyme:		
		Acid phosphatase[b]	β-D-Glucosidase[c]	Pyrophosphate diesterase[d]
Candida albicans	10	10	0	0
C. guilliermondii	5	5	0	0
C. krusei	4	4	0	0
C. parapsilosis	10	10	0	0
C. pseudotropicalis	4	4	0	0
C. stellatoidea	3	3	0	0
C. tropicalis	9	9	0	9
C. (Torulopsis) glabrata	10	0	0	0
Cryptococcus laurentii	3	3	3	0
C. neoformans	7	7	7	0

[a] Tests used 4-methylumbelliferyl substrates under specific conditions.
[b] Assayed at pH 3.4, 4 h.
[c] Assayed at pH 5.1, 30 min.
[d] Assayed at pH 4.5, 2 h.

activity was determined at pH 4.5 after 2 h, only the nine strains of *C. tropicalis* tested were positive.

None of the 65 yeast isolates showed detectable β-D-galactosidase, β-L-fucosidase, or α-L-arabinopyranosidase activity at the three pH values and incubation times described. The results of the assays for the remaining enzymes, using these fluorogenic substrates, were variable, showing strain variation within the species of a genus.

It is important to be aware of the fact that the intensity of the fluorescence of the hydrolysis product, 4-methylumbelliferone, has been reported to be increased at an alkaline pH (3). Although the addition of alkali to the organism–4-methylumbelliferyl substrate complexes after incubation worked well in several quantitative and qualitative investigations (1, 2, 4, 5, 7–9), in our study the addition of alkali produced fluorescence in all of the tests including the negative controls. We also found that the 4-methylumbelliferyl substrates hydrolyzed spontaneously in the pH range of 6.0 to 7.0, resulting in nonspecific fluorescence. These were the reasons why we performed the tests using substrates with pH values of 3.4, 4.5, and 5.1.

The techniques used in this study were easy to perform and consistent with the principles of rapid testing (6). The results of this limited preliminary investigation suggest that umbelliferyl substrates may have usefulness for detecting specific enzyme activity in clinically significant yeast isolates, thus aiding in the rapid identification of these organisms. Further investigation of diluent content and pH with many more isolates may confirm the usefulness of these substrates.

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