USE OF A UREASE TEST FOR THE SCREENING AND IDENTIFICATION OF CRYPTOCOCCI

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The increase in mycological work by diagnostic laboratories has stimulated the development of simple and reliable procedures for routine screening and identification of fungus cultures.

The writer has investigated the possibility of applying procedures for the diagnosis of yeasts other than the widely used sugar fermentation and assimilation tests. During earlier investigations it was found that capsulated and red pigment-producing yeasts hydrolyzed urea in Christensen's medium (Christensen, 1946) while candida strains and saccharomycetes did not. Therefore studies were initiated to determine whether urea hydrolysis could be used with advantage in routine mycological work. For this study yeast cultures were procured from several recognized laboratories. The results of the examinations were finally compared with a collection of strains at the Communicable Disease Center.

Since the findings at both laboratories were in complete agreement and since the urease test offered improvement for the routine identification of yeasts and some yeast-like organisms, the results are given here. For the sake of completeness this report will also present the results with a number of yeasts from Dr. Wickerham's collection which were tested after the writer's return to Germany.

MATERIAL AND METHODS

Fungus strains. The number of strains of each species used is presented in table 1. All strains were checked for purity and for typical fermentation reaction in 1.0 per cent peptone-water containing 0.5 per cent sodium chloride, and one per cent of the Seitz-filtered sugar solution and bromthymol blue as indicator. Gas production was read after two and seven days in Durham tubes.

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The cultures were kept at room temperature on Sabouraud's agar and were transferred to fresh slants before the urease test was done. Most of the strains grew well at 37 C. A few strains which did not grow well at this temperature were incubated at 22 and 30 C.

Test procedure. After sufficient growth had developed on Sabouraud's agar (usually within 24 to 48 hours at the optimal temperature of growth) a heavy inoculum was transferred with a loop to the surface of the urea medium. Only the slanted part of the medium was inoculated.

Christensen's medium consists of the following base: peptone, 0.1 per cent; glucose, 0.1 per cent; NaCl, 0.5 per cent; KH_2PO_4, 0.2 per cent; agar, 1.5 per cent; and 0.012 g phenol red per 1000 ml. The ingredients were mixed and melted in a water bath. After adjusting the pH to 6.8 the medium was dispensed into test tubes in 4.5 ml amounts and autoclaved for 10 min at 121 C. To every tube of the autoclaved medium 0.5 ml of a 20 per cent Seitz-filtered solution of urea was added aseptically. After mixing with the base the contents of the tubes were allowed to solidify with a long slant and a deep butt.

After inoculation the tubes were incubated at the optimal temperature of the organism for 48 hr. Urea hydrolysis was indicated by a distinct color change of the indicator from orange-yellow to a deep pinkish red starting at the slanted part of the medium and progressing rapidly to the deep part. The color change could often be detected as early as 2 hr after inoculation.

RESULTS

The results of this study are summarized in table 1. From this table it can be seen that all strains of *Cryptococcus neoformans* including the variety *Cryptococcus uniguttulatus* (Zach) were able to hydrolyze urea rapidly regardless of whether the strains originated from human and animal sources or whether they were isolated from soil specimens from various states of the U.S.A. Likewise, all cultures belonging to the two nitrate-assimilating species: *C. albidus* and

C. diffluens (including Benham's *C. inocuus*) split urea within 18 to 14 hr. Some of the latter strains grew well only at temperatures below 30 C, and accordingly the urease test was positive with these strains only at 30 C and lower. Similarly a strain of *C. terreus* attacked urea at room temperature only. A positive reaction was also obtained with *C. laurentii* which was found (Seeliger, 1954) to be related serologically to *C. neoformans*.

Out of 49 cultures assigned to the genus *Cryptococcus*, only three (no. 14, 38, and 39) reacted consistently negative on the urea medium. Further tests showed that none of these three strains actually was a cryptococcus. No. 14, allegedly a transplant of the original culture of Busse and Buschke, fermented glucose rapidly with formation of gas; it lacked a capsule and was probably a mislabeled or contaminated culture. A second culture of the Busse strain (no. 7) reacted typically and differed markedly from no. 14. A saprophytic strain no. 38 did not ferment any sugars; it formed white colonies, grew without capsules, and belongs either to the torulopsis or to the debaryomyces group. The third urease-negative culture was *Cryptococcus glabratus*, a glucose-fermenting yeast that is now classified as *Torulopsis glabrata* (Lodder and Kreger-Van Rij, 1952). It therefore can be concluded that all 46 true cryptococcus strains were able to hydrolyze urea rapidly.

The same occurred with representative strains of 7 species of the genus *Rhodotorula*. The members of this genus had been included by earlier investigators in the genus *Cryptococcus*. The test strain of *R. flava*, however, lacked the ability to split urea rapidly.

In marked contrast to the predominantly positive results with cryptococci and *Rhodotorula* spp., it was found that all representative strains of the genera: *Kloeckera*, *Endomyces*, *Lipomyces*, *Saccharomyces*, *Saccharomycodes*, *Hansenula*, *Nematospora*, *Debaryomyces*, *Nadsonia*, *Schwanniomyces*, and *Pichia* failed to attack urea within 4 days on Christensen's urea medium. Out of 14 species of candida tested, only a culture of *Candida humicola* produced urease.

Of the other yeast-like fungi, all seven strains of *Geotrichum candidum* and related species did not split urea. The same applies to several species of the genus *Trichosporon* except *T. cutaneum* which gave a delayed positive reaction. Likewise a strain designated as *Endomyces vernalis* gave a

TABLE 1

Hydrolysis of urea by yeasts and yeast-like organisms on Christensen's urea agar

Genera and Species	Alkalinization of the Medium Within 4 Days
Cryptococcus neoformans (36)*	+
Cryptococcus neoformans var. uniguttulatus	
Cryptococcus diffluens (*C. inocuus*) (6)	
Cryptococcus albidus (4)	
Cryptococcus laurentii (1)	
Cryptococcus terreus (1)	
Rhodotorula rubra (2)	+
Rhodotorula glutinis (3)	
Rhodotorula glutinis var. rubescens (1)	
Rhodotorula mucilaginosa (4)	
Rhodotorula aurantiaca (1)	
Rhodotorula minuta (1)	
Rhodotorula pallida (1)	
Rhodotorula flava (1)	Weakly +
Torulopsis glabrata	−
Debaryomyces kloeckeri and related species (10)	
Lipomyces lipoferus (1)	
Saccharomyces delbrueckii (1)	
Saccharomyces cerevisiae (18)	
Saccharomyces carlsbergensis (3)	
Saccharomyces pastorianus (1)	
Saccharomyces logos (1)	
Saccharomyces willianus (1)	
Saccharomycodes ludwigii (1)	
Pichia farinosa (1)	
Hansenula anomala (3)	
Nematospora phaseoli (1)	
Nadsonia elongata (1)	
Schwanniomyces occidentalis (1)	
Kloeckera apiculata (1)	
Endomyces magnusii (1)	
Candida albicans (66)	−
Candida pseudotropicalis (4)	
Candida parapsilosis (6)	
Candida guilliermondii (3)	
Candida zeylanoides (1)	
Candida mycoderma (2)	
Candida solani (1)	
Candida stellatoidea (3)	
Candida tropicalis (6)	
Candida krusei (4)	
Candida utilis (3)	
Candida macedoniensis (1)	
Candida mesenterica (1)	
Candida humicola (1)	+

* Numbers in parentheses represent strains studied.

TABLE 1 (*Continued*)

Genera and Species	Alkalinization of the Medium Within 4 Days
Trichosporon capitatum (1) *Trichosporon sericeum* (1) *Trichosporon fermentans* (1) *Trichosporon cutaneum* (1)	−
Trichosporon pullulans (*Endomyces vernalis*) (1)	+
Geotrichum candidum and related species (7)	−
Pullularia pullulans (4)	+
Sporobolomyces holsaticus and related species (3)	−

strongly positive reaction in the urease test. According to Lodder and Kreger-Van Rij (1952) this nonfermenting yeast is a synonym of *Trichosporon pullulans*.

Four isolates of the black yeast *Pullularia pullulans* and three strains of the red pigment producing basidiomycete *Sporobolomyces holsaticus*, and related species hydrolyzed the compound as rapidly as the cryptococci and most rhodotorula strains.

DISCUSSION

The consistent results of the above experiments justify the following conclusions:

The urea hydrolysis test with Christensen's urea-agar medium permits a clear-cut differentiation among several genera of yeasts and yeast-like organisms. So far all urease-positive cultures tested belong to few nonfermenting genera, i. e. *Cryptococcus*, *Rhodotorula*, *Sporobolomyces*, *Pullularia*, *Trichosporon*, and *Candida*.

The various species and their varieties of the genus *Cryptococcus* cannot be distinguished from each other on the basis of the urease test. This test offers, however, great help in the rapid separation of cryptococci from other nonfermenting yeasts, for instance *Candida zeylanoides*, *C. curvata*, *Debaryomyces kloeckeri*, and *Lipomyces lipoferus*. There are many other procedures for identifying *C. neoformans*, which usually produces large capsules. But not all strains form capsules visible in India ink preparations. In addition, the demonstration of starch production requires several days. Although weakly encapsulated strains, particularly when they do not grow well at 37 C and are nonpathogenic to mice, are usually regarded as saprophytes, the writer has cultured such strains repeatedly from the spinal fluid of a patient with chronic meningoencephalitis and of two other patients with symptoms of meningitis. This might indicate that these strains of *C. diffluens* (*C. inocuus*) are not entirely devoid of pathogenic properties.

The only culture of the genus *Lipomyces* tested, a typical strain of *L. lipoferus*, reacted consistently negative on the urea agar. *L.lipoferus* has been shown to produce a starch-like substance like the cryptococci. Recently it was suggested by Connell, Skinner and Hurd (1954) to reduce its name to synonymy with *Lipomyces starkeyi*. All of their strains, one of which was isolated from the human skin, produced the starch-like material mentioned above, and a phylogenetic relationship between the genera *Lipomyces* and *Cryptococcus* was discussed by these authors. The resemblance of *C. neoformans* with *L. starkeyi* was also emphasized by Benham (1955) who found similar ascus-like structures in both species. In the light of the negative urease test with one typical lipomyces strain and the consistently positive reactions by all true cryptococci, it seems that the ability to attack urea may serve as a basis for considering these two genera as distinct. This concept would also be supported by the fact that the writer's strain of *L. lipoferus* was antigenically not related to representative serotypes of the cryptococcus group (Seeliger 1954). However, more lipomyces strains must be examined before any definite conclusions can be drawn.

Starch production and hydrolysis of urea are constant properties of all true cryptococci. These two characteristics are apparently not linked to each other in the lipomyces group. But they do occur together in the starch-producing and urea-hydrolyzing *Candida humicola*, which by these two properties can easily be distinguished from other pellicle producing nonfermenting candidas. Likewise certain strongly red pigmented strains of the rhodotorula group do produce starch and thereby show the same combination of biochemical characteristics as the cryptococci. According to Wickerham (1955, *personal communication*) the genus *Trichosporon* consists of two distinct types of species: one group assimilates many carbon sources, as do cryptococci, and some of them likewise synthesize starch; the others do not produce starch,

assimilate only a few carbon sources and are related to *Geotrichum candidum*. A positive urease test among members of the first group would certainly be in accordance with Wickerham's suspicion of a strong taxonomic relationship between the starch producing strains of trichosporon and cryptococci. Among the trichosporon strains tested, *T. pullulans* and two similar isolates proved to be rapid urea hydrolyzers and *T. cutaneum* gave a delayed positive test. On the other hand *T. fermentans*, *T. capitatum*, and *T. sericeum* failed to break down urea under the conditions of test.

Candida humicola may be suspected of taking an intermediary position between the urease positive and starch producing strains of trichosporon and the cryptococci.

The urease test may be useful in further investigations concerning possible phylogenetic relationships among the black dematiaceous fungi, for instance between the urease-positive *Pullularia pullulans* and other species such as *Cladosporium werneckeii* and *Torula bergeri*, both resembling Pullularia and sharing common antigens with *P. pullulans* (Trejos and Seeliger, 1955, *unpublished data*). An identical biochemical pattern would substantiate the assumption of a possible close relationship.

Such a relationship has also been postulated for strains of rhodotorula and sporobolomyces (Janke and Hansa, 1954). The view that certain rhodotorula may be degraded variants of the basidiomycete *Sporobolomyces* is compatible with their identical biochemical pattern and with the results of the urea hydrolysis test. However, serological investigations of 10 strains of both genera failed so far to show any serological relationship between them (Seeliger, 1954).

The urease test has been used for more than three years, and was found to be of definite value. Within 18–48 hours it answers the question whether a noncapsulated or poorly capsulated nonfermenting yeast might belong to the genus *Cryptococcus*. In diagnostic work the test can be performed simultaneously with sugar fermentation tests. Among the fermenting cultures which are urease negative, may be *C. albicans*, which usually can be readily distinguished by its ability to produce chlamydospores and by its fermentation reactions. The urease-positive cultures must be identified further. If no pigment is formed on isolation media, such strains most likely belong to the genus *Cryptococcus*. They have to be further classified by other procedures. The urease test therefore helps in eliminating saprophytes and in the rapid recognition of possible pathogens.

Hydrolysis of urea has not been used as a means of differentiation between genera or species of yeasts by Lodder and Kreger-Van Rij (1952). Urea has, however, been utilized by earlier workers in assimilation studies. Wickerham (1946) has shown that urea assimilation will occur with all yeast strains previously designated as unable to utilize this compound, when an adequate vitamin supply is provided. He also has drawn attention to the toxicity of urea in simple synthetic media. In Christensen's medium, urea does not have a toxic effect upon yeasts even in a concentration of 2.0 per cent. The urease reaction described in this paper, is different from the assimilation tests performed by Wickerham (1946) and others. It may serve as an additional tool for taxonomic studies.

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SUMMARY

A urease test is described for the differentiation of yeasts and yeast-like organisms. It was found that among the nonfermenting yeasts tested, all members of the genera *Cryptococcus*, *Rhodotorula*, *Pullularia*, and *Sporobolomyces*, *Candida humicola*, *Trichosporon cutaneum*, and *T. pullulans* were able to split urea when inoculated on the surface of Christensen's urea agar. Nonfermenting yeasts belonging to *Debaryomyces kloeckeri* and related species, *Candida zeylanoides*, *Lipomyces lipoferus*, and several species of the genus *Trichosporon*, did not hydrolyze urea. Neither did any of the fermenting yeasts hydrolyze the compound. The practical applications of the test and its use in laboratory and taxonomic work is discussed.

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