USE OF SHAKEN CULTURES IN THE ASSIMILATION TEST FOR YEAST IDENTIFICATION[1]

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The assimilation test, using liquid media in standing culture, is a fundamental diagnostic test in the taxonomy of yeasts (Wickerham and Burton, 1948; Wickerham, 1951). The incubation period for this method has been standardized at 20 to 24 days. This long incubation is necessitated by the occurrence of slow or adaptive metabolic reactions of certain species, especially those which are oxidative rather than fermentative. Often some of these nonfermentative isolates show a variable reaction at the conclusion of the incubation period, thus making an interpretation of the assimilatory response of the organism quite difficult.

In the course of extensive investigations of the marine-occurring yeast flora of Biscayne Bay Florida, studies were begun on the effect of shaking on the assimilation test. Many of our marine isolates, especially members of Rhodotorula, exhibited latent, weak, or variable assimilatory patterns, thus making it desirable to establish whether more definitive reactions could be developed by using agitation during the growth of the yeast.

MATERIALS AND METHODS

Cultures. The following species of yeasts used in this study were collected from various sediments in Biscayne Bay, Florida. These isolates included: *Hansenula anomala* (MY-38), *Debaryomyces kloeckeri* (MY-19), *Cryptococcus laurentii* (MY-76), *Candida tropicalis* (MY-139), and the following species of *Rhodotorula*: *R. graminis* (MY-105), *R. texensis* (MY-44), *R. mucilaginosa* (MY-1, 130, 180), *R. glutinis* (MY-21, 95), and *R. minuta* (MY-166, 177). One isolate, *R. marina* (MY-50) was taken from a deep sea core at a depth of 455 fathoms off Bimini, The Bahamas.

Stock cultures of the marine-occurring isolates were maintained on a sea water medium (M-6) of 2 per cent glucose, 1 per cent yeast extract (Difco), and 2.3 per cent nutrient agar (Difco). An additional 0.2 per cent agar was used in the preparation of this medium.

The morphological and physiological characteristics used in the identification of the isolates are those described by Wickerham (1951) and by Lodder and Kreger-Van Rij (1952).

Media and growth conditions. Assimilation tests were run in yeast carbon and nitrogen base media (Difco) in accordance with Wickerham's formula. In the nitrate assimilation test, the stored nitrogen of the inoculum was reduced by prior incubation of the organism in 2.0 per cent glucose for 72 hr. Unless noted otherwise, all media were prepared with distilled water.

Inocula consisted of 0.1 ml of cell suspensions prepared in sterile distilled water adjusted to a standard turbidity, about 70 per cent transmittancy (T) at 365 mμ. Cultures were incubated at laboratory room temperature, 27 to 31 C.

A Gyrotory shaker (model G 10, New Brunswick Scientific Company), 55 rpm, fitted with special test tube racks, was used for the shake cultures. Cultures were grown in 18 by 150 mm tubes, provided with stainless steel closures (Bellco Glass, Inc.). Growth was observed at 24-hr intervals over 7 days, and for 24 days in standing culture.

Measurement of growth. Growth response was measured with a Bausch and Lomb Spectronic 20 colorimeter set at 365 mμ. Readings are expressed as percentage transmittancy (T), using the sterile medium as the reagent blank.

The degree of assimilation was determined turbidimetrically by using the colorimeter as well as the visual card method described by Wickerham (1951). A comparative study of these two methods was made to standardize the values

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of the turbidimetric readings with the arbitrary ranges established by Wickerham. The relationship between per cent transmittancy and the degree of assimilation, as determined by the Wickerham visual turbidimetric method, is indicated in table 1.

RESULTS AND DISCUSSION

Agitation of the culture during growth permits a more constant and uniform supply of oxygen as well as greater uniformity throughout the medium (Gavin, 1957). In a standing culture, the gaseous concentration varies from the air-liquid interface to the bottom of the tube. Furthermore, the medium is modified in the immediate area of cell growth as depletion of food substances and accumulation of metabolic products occurs. Though normal diffusion occurs within the stationary medium, the rates may not be sufficient to establish optimal conditions for reproduction.

In tables 2 and 3, terminal degrees of assimilation, or maximal growth, are given for the various species of yeasts in both standing and shake cultures. The 4-day incubation period required for maximal development of the rhodotorulas tested, using agitation, is necessitated by the rather slow growth of most of the isolates of this group in galactose, and by some in maltose and lactose. However, growth of these species in glucose and in sucrose generally was comparable to the rate of growth of species of the other genera tested.

TABLE 1

Degree of assimilation in relation to per cent light transmission

Degree of Assimilation	Transmittancy (*T*)
	%
3+	0–11
2+	12–24
1+	25–33
−	34–100

TABLE 2

Degree of assimilation in shake culture for 2 days and in standing culture for 14 days

Organism	Glucose		Galactose		Sucrose		Lactose		Maltose		KNO_3	
	2	14	2	14	2	14	2	14	2	14	2	14
	Per cent transmittancy											
Hansenula anomala (MY-38)	4	4	8	2	3	3	95	83	3	4	2	6
Debaryomyces kloeckeri (MY-39)	5	10	6	11	6	7	96	90	9	13	91	53
Cryptococcus laurentii (MY-76)	3	3	3	3	4	0	2	2	3	2	78	62
Candida tropicalis (MY-139)	3	4	3	8	4	4	97	90	2	5	90	77

TABLE 3

Degree of assimilation of 8 isolates of Rhodotorula in shake culture for 4 days and standing culture for 14 days

Organism	Glucose		Galactose		Sucrose		Lactose		Maltose		KNO_3	
	4	14	4	14	4	14	4	14	4	14	4	14
	Per cent transmittancy											
R. mucilaginosa (MY-130)	8	12	19	20	12	14	81	82	20	25	91	90
R. mucilaginosa (MY-180)	2	0	24	9	0	3	90	69	9	4	81	70
R. glutinis (MY-21)	8	2	20	17	5	4	90	91	22	12	7	12
R. graminis (MY-105)	4	3	5	20	4	6	84	64	95	90	4	14
R. texensis (MY-44)	3	7	10	19	4	7	20	10	79	66	87	88
R. minuta (MY-166)	7	19	19	21	17	16	91	80	71	78	90	91
R. minuta (MY-177)	5	4	21	16	8	4	94	80	80	78	87	91
Rhodotorula sp. (MY-95)	8	10	20	28	21	35	78	80	5	29	4	27

Though the use of agitation often obscures the occurrence of weak, latent, or variable growth responses, it is considered that the elimination of such ill-defined reactions facilitates the correct identification of the organism. If desired, weak or latent reactions are still determinable in shake vessels by observation of growth during the incubation period at 12- to 24-hr intervals.

Under standardized conditions, a latent reaction will demonstrate a lengthened lag phase prior to the exponential growth phase, whereas a weak reaction exhibits a protracted exponential growth phase after a normal lag phase. Because of the variability of conditions that may affect a weak or latent response, such reactions, unless definitely characteristic of a particular species, are not sufficient of themselves to permit definitive differentiation of species. The use of such reactions in dichotomous taxonomic keys generally should be avoided.

In reference to the above, a strain (MY-1) of *R. mucilaginosa*, showed a latent reaction in maltose in both standing and in shake cultures, with approximately equal amounts of growth for 24 days and 4 days, respectively. However, a vigorous increase in growth, noted in the shaker series after an incubation of 3 to 4 days, did not occur in the standing cultures until 21 to 24 days.

One obvious advantage of using agitation in the assimilation tests is the shortening of the period necessary for maximal growth of the organism. The time periods designated in this study are not suggested as standards, as other genera and species of yeasts may require a longer incubation to achieve maximal development. A 7-day period was selected arbitrarily as the maximal incubation time, since this period was approximately 24 hr longer than the time required for the slowest growing marine forms (MY-50, 95) examined to attain maximal development. The isolate MY-95, while resembling *R. glutinis*, could not be classified as one of the recognized species of *Rhodotorula* because of the weak assimilation reactions of the organism in standing culture. This was particularly marked in KNO_3 broth, which is a critical characteristic in species differentiation of the organism. However, with agitation, the organism exhibited more clearly definitive reactions, permitting identification of the species as *R. glutinis*. The isolate, MY-50, collected from a deep sea core, showed a variable reaction in lactose at the conclusion of the normal incubation period of 20 to 24 days. The assimilation pattern thus expressed was atypical of any described species of *Rhodotorula* showing comparable morphological characteristics. In shake culture, MY-50 exhibited a positive lactose assimilation reaction between 6 and 7 days. This reaction was not observed in standing cultures until after a growth period of 30 days. The isolate then was identified as a strain of *R. marina*.

A preliminary investigation of comparable yeast growth rates of our marine isolates with terrestrial counterparts indicated that, in general, slow growing species from the marine habitat required a longer growth period than did their corresponding slow growing terrestrial species. This was true particularly of certain strains of Rhodotorula.

The method discussed here is not intended as a substitute for the standard assimilation test currently used. Rather, it offers a rapid means of obtaining the assimilatory spectrum of yeasts as well as a more critical evaluation of the growth of slow and latent forms.

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SUMMARY

The growth of yeasts in various assimilation media was examined in both standing and shake cultures. The use of agitation was found to reduce the incubation time from 3 weeks to approximately 1 week or less. Nonfermentative yeasts, particularly those of the genus *Rhodotorula*, which commonly assimilate carbohydrates by adaptive or slow growth, and often give indefinite results in standing culture, were found to increase their assimilation under agitation.

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