A SIMPLE TEST FOR CAROTENOID PIGMENTS IN YEASTS

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The imperfect yeasts are divided by Lodder (1934) into two families, *Rhodotorulaceae* and *Torulopsidaceae*, distinguished by the occurrence of carotenoid pigments in the former. Lodder employed a form of the Molisch test to demonstrate the carotenoid nature of the pigments in *Rhodotorulaceae*. This involved covering a large mass of cells with an alcoholic potassium hydroxide solution and storing in the dark for several days or even weeks. After such treatment, Lodder found well-defined carotenoid crystals under the microscope, which turned blue when treated with concentrated sulfuric acid. Over a number of years, working with numerous cultures of several species of *Rhodotorula*, the writers have had uniformly negative results with the foregoing procedure. Mackinney (1940) observed that several species of *Rhodotorula* and *Sporobolomyces* resist prolonged boiling in alcoholic potassium hydroxide, without liberation of pigment and without apparent change in the cells under the microscope. The pigments may be extracted, however, after careful hydrolysis of the cell mass with hydrochloric acid, with acetone, and are then readily transferred to petroleum ether.

Bonner, Sandoval, Tang, and Zechmeister (1946) extracted the pigment from *Rhodotorula* mutants with benzene by shaking the cell suspension in a benzene-alcoholic potassium hydroxide mixture for 1 to 1.5 hours. This procedure is readily applicable in most cases, but with some species producing a heavy mucilaginous slime there is scarcely perceptible color in the benzene layer, and the treatment (involving vigorous mechanical shaking) has had no visible effect on the microscopic appearance of the cells.

The purpose of this note is to provide the yeast taxonomist with a simple, quick procedure for determining the presence of carotenoid pigments in yeasts. When the pigments have been brought into solution, there are obviously many tests required for rigorous proof of identity. However, in the case of all *Rhodotorula* and *Sporobolomyces* species examined, solubility of the pigments in petroleum ether is virtually an adequate criterion by itself, since a large number of confirmatory spectroscopic and chromatographic analyses have been made. The conclusion can be reinforced, if the petroleum ether solution is not too dilute, by the test with concentrated sulfuric acid, and, of course, by such other methods and equipment as may be available.

The details of the simple test, which we found very useful for laboratory classes, are as follows: Yeast growth on a heavily inoculated plate is removed with the end of a glass slide. Care should be exercised to avoid removing any of the agar substrate. The yeast on the slide is transferred with the aid of a spatula to a small beaker, or test tube, and suspended evenly in 8 to 10 ml of 1:1 hydrochloric

acid. The mixture is heated to a boil and promptly cooled. The purpose of the acid treatment is to break down the mucilaginous material usually surrounding the cells of the red yeasts and to make possible the subsequent extraction of carotenoid pigments from the cell material. The hydrochloric acid treatment is the most critical part of the procedure. Overheating destroys the pigment, whereas underheating fails to give complete extraction. There is some variation between species with respect to the degree of heating necessary for proper breakdown of the cells. In a few cases it may be necessary to prolong the heating somewhat, and in other cases to use temperatures of 80 or 90 C rather than boiling.

When the contents of the tube have been cooled to room temperature, 10 to 15 ml of acetone and 3 to 5 ml of petroleum ether or benzene are added, and the mixture is shaken gently. A 50-ml glass-stoppered Erlenmeyer flask or a small separatory funnel is most suitable, though a stoppered test tube may be used. Water (15 to 20 ml) is then added to effect clear-cut separation of two phases. If carotenoids are present, the upper (epiphasic) petroleum ether or benzene layer is colored. Benzene has certain disadvantages as compared to petroleum ether, particularly if the sulfuric acid test is to be applied, as it is appreciably miscible with water, which must of course be removed, and there is also a tendency to form emulsions. Whereas the few ml of petroleum ether can readily be evaporated under the hot water faucet, the benzene solution cannot be so easily handled. It has, however, the advantage of being much less inflammable, and the test is in some respects more sensitive, since carotenoid solutions in benzene possess greater tinctorial power than corresponding solutions in petroleum ether. The difference between orange-red in petroleum ether and pink, the color in benzene, is quite pronounced.

Confirmation of the presence of carotenoids in the surface layer may be obtained as follows: The contents of the test tube or flask are poured into a small separatory funnel, and the lower aqueous layer is discarded. The petroleum ether or benzene layer is transferred to a dry test tube and sufficient anhydrous sodium sulfate is added (2 to 3 grams are usually adequate) to take up all dissolved water. The mixture is shaken gently and allowed to stand 1 to 2 minutes. If necessary, the clear solution can be decanted into another test tube, and the petroleum ether layer concentrated under the hot water tap, away from flames. The solution is carefully poured on the surface of 1 to 2 ml concentrated sulfuric acid in a third tube and a blue coloration will be observed. This confirmatory step may offer some difficulty, especially in cases in which there is very little pigment. Interfering solutes may color the sulfuric acid dark brown, and atypical colors, reddish or purple, may also be obtained. These usually develop more slowly. If the ether layer is gently poured over the sulfuric acid, a definite bluish tint can be observed shortly after addition, at least near the interface. Larger quantities of yeast may be worked up when pigmentation is weak.

The writers have tried this extraction procedure on a large number of species and varieties of *Rhodotorula*, *Sporobolomyces*, *Torulopsis*, *Pichia*, and other yeasts producing pink, red, orange, or yellow pigments. The test has always given

positive results for cultures of *Rhodotorula* and *Sporobolomyces* and negative for pigmented strains of *Taphrina deformans*, *Torulopsis pulcherrima*, *Torulopsis lipofera*, *Torulopsis luteola*, *Pichia*, *Zygosaccharomyces*, and *Pullularia*. The pigments of the latter group are noncarotenoid and frequently diffuse into the medium.

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