A PROCEDURE FOR DEMONSTRATING THE PRESENCE OF CAROTENOID PIGMENTS IN YEASTS[1]

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The presence of carotenoid pigments is used in yeast taxonomy to differentiate the genus *Rhodotorula* from the genera *Cryptococcus* and *Torulopsis* (Lodder and Kreger-Van Rij, 1952). Certain difficulties are encountered, however, in attempts to demonstrate satisfactorily the presence of carotenoid pigments in yeasts. Procedures which have proved successful in the extraction of carotenoids from plants and other biological materials do not appear to be generally applicable. Several workers (Lodder and Kreger-Van Rij, 1952; Mrak *et al.*, 1949; Mackinney, 1940) have reported difficulties in extraction of pigments from certain species of *Rhodotorula* with methods employing alcoholic-potassium hydroxide. Mrak *et al.* (1949) found, however, that the pigments could be extracted with acetone if the cell mass was first hydrolyzed carefully with strong (1:1) hydrochloric acid. In our hands this method was not sufficiently definitive since as Mrak *et al.* (1949) have suggested, there is considerable variation between species with respect to the degree of heating necessary for proper breakdown of the cells. Further, as these authors point out, with this strength of acid overheating destroys the pigment, whereas underheating fails to give complete extraction.

It is of interest that the Dutch investigators, Lodder and Kreger-Van Rij (1952), in summarizing their results on extensive studies of existing methods say, "We found no practicable way to prove the carotenoid nature of the pigments by a simple test and we have been obliged to rely merely on the result of a visual observation."

Our own interest in this problem developed in the course of a study to determine the species of yeasts associated with different parts of the cucumber plant (*Cucumis sativus*). A total of 966 yeast isolates was obtained from 37 sets of staminate and pistillate flowers, and five samples of small, immature fruit. More than half of the yeast cultures obtained were asporogenic, nonfermentative, carotenoid pigment producing types placed in the genus *Rhodotorula*. With such a large collection of isolates it became necessary to develop improved cultural and chemical techniques adaptable to the routine screening of potentially pigmented species.

EXPERIMENTAL METHODS

The yeasts used for the various phases of this study and their sources are listed in table 1.

Preliminary experiments substantiated the findings of others that in most cases hydrolysis of pigmented yeasts with various concentrations of alcoholic or aqueous potash failed to liberate the carotenoid pigments. This was especially true if the yeasts were grown on solid or liquid media which were highly buffered or contained such materials as peptone or casein hydrolyzate. In these cases the pigments could be released only for subsequent extraction with acetone by the use of rather strong concentrations of acid. If, on the other hand, the yeasts were grown at 24 to 26 C (room temperature) in a synthetic broth proposed by Wickerham (1951) (100 ml of this nitrogen base medium[2] plus two per cent glucose in 250 ml Erlenmeyer flasks) for 72 hours on a rotary shaker[3] at 210 oscillations per minute,

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[2] Obtained from Difco Laboratories, Detroit, Mich.

[3] Model V, capacity 40 flasks, manufactured by the New Brunswick Scientific Company, New Brunswick, N. J.

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TABLE 1

Absorption maxima for total carotenoid pigments from species and strains of pigmented yeasts. Cultures grown in synthetic broth 72 hours on a rotary shaker

CULTURE NUMBER AND SOURCE	YEAST	ABSORPTION MAXIMA IN PETROLEUM ETHER	CULTURE NUMBER AND SOURCE	YEAST	ABSORPTION MAXIMA IN PETROLEUM ETHER
		mμ			mμ
NRRL no.*	*Rhodotorula* spp		FFL no.†	*Rhodotorula* spp	
Y-1581	*R. aurantiaca*	455	SY-85	*R. glutinis*	480
Y-1583	*R. bronchialis*	450, 480	SY-875	*R.* species A (red-orange)	450
Y-1585	*R. flava*‡	450	SY-810	*R. flava*	450
Y-1586	*R. glutinis*	neg.§	SY-836	*R.* species B (yellow)	450
Y-1621	*R. glutinis* var. *rufula*	450, 480	SY-629	*R.* species C (yellow)	445
Y-1588	*R. longissima*	450	SY-1054	*R.* species D (yellow-black)	440
Y-1589	*R. minuta*	450, 480	SY-413	*R.* species E (yellow)	450
Y-1590	*R. mucilaginosa* var. *pararosea*	480	NRRL no.*	*Torulopsis* spp	
			Y-1401	*T. flavescens*	neg.§
Y-1591	*R. mucilaginosa* var. *plicata*	480	Y-346	*T. neoformans*	neg.§
Y-1592	*R. rubra*	480	Y-1501	*T. albida* var. *japonica*	450
			FFL no.†	(Controls)	
Y-1593	*R. rubra* var. *curvata*	480	SY-161	*Candida pulcherrima*	neg.§
Y-1594	*R. rubra* var. *longa*	480	SY-188	*Candida* species A	neg.§
Y-1595	*R. sanniei*	450	SY-91	*Candida* species B	neg.§

* NRRL: Northern Regional Research Laboratory (USDA), Peoria, Ill.
† FFL: Food Fermentation Investigations Laboratory (USDA), Raleigh, N. C.
‡ Nonpigmented culture when received.
§ Culture negative for carotenoids.

the pigments could be extracted readily by the following procedure:

Method A—cells from synthetic broth, direct extraction with acetone, no acid, no heat. The flask was removed from the shaker and permitted to stand overnight at room temperature for settling of the cells. Then as much supernatant liquid was poured off and discarded as could be conveniently accomplished without loss of cells. The remaining solution and cells were transferred then to a 50 ml centrifuge tube and centrifuged for 5 minutes at 2,000 rpm. After decanting the supernatant, 20 ml of acetone were added and the packed cell mass broken up by vigorous stirring with a glass rod. Upon standing for 10 to 15 minutes, the tube was centrifuged as before. The acetone was decanted then into a small separatory funnel and the cells extracted as before with 20 ml of acetone. To the combined acetone extracts 20 to 25 ml of petroleum ether (or "skellysolve B") were added, and the mixture shaken gently. Upon addition of 15 to 20 ml of water, a clear-cut separation of the two phases occurred, and if carotenoid pigments were present, the upper (epiphasic) petroleum ether layer was colored (yellow, orange, pink, or intense red, depending on the yeast being extracted). After an additional washing with water, the petroleum layer was filtered into a test tube through a small quantity (about 5 g) of anhydrous sodium sulfate and brought to the nearest convenient volume. Absorption spectra were obtained for all extracts for the wavelength region 400 to 520 mμ, using a Beckman B spectrophotometer.

Although the above procedure worked very well for all carotenoid containing yeasts grown in the synthetic broth, it seemed desirable to devise an alternative method which would prove satisfactory for the same yeasts when grown in

less clearly defined media, whether liquid or solid. Repeated experiments with the method of Mrak *et al.* (1949), using 6 N HCl (1:1), gave positive results in that the epiphase was usually colored, but there was evidence of considerable pigment destruction and radical modification of absorption spectra. Therefore, it was decided to modify this method by determining the weakest concentration of HCl as well as the shortest and least rigorous heat treatment which would accomplish a satisfactory extraction of carotenoid pigments. Replicated samples of centrifuged yeast cells obtained as under Method A were heated in a boiling water bath with 20 ml of various concentrations of HCl (0.01 N to 2 N) for various lengths of time, ranging from one minute to 25 minutes. Maximum extraction with least destruction of pigments was obtained by the following method.

Method B—cells from glucose broth (Difco), heated with 0.5 N HCl. The pigmented yeast to be studied was grown in glucose broth[4] (100 ml supplemented with an additional 1.5 per cent glucose in a 250 ml Erlenmeyer flask) for 72 hours on the rotary shaker and separated in a 50 ml centrifuge tube by centrifugation as in Method A. After removing the supernatant broth, 20 ml of 0.5 N HCl were added and the cell mass broken up by stirring with a glass rod. Then the tube was placed in a vigorously boiling water bath and heated for 15 minutes. Upon removal from the water bath the tube was placed immediately into ice water to cool for 10 minutes, after which the cells were separated by centrifugation. Pigments were extracted then from the cells with acetone and petroleum ether as before (Method A).

Method C—cells from glucose broth (Difco), heated with 6 N HCl. The yeasts were grown and separated from the broth as under Method B. Next the cells were suspended in 8 to 10 ml of 6 N HCl (1:1) and the mixture carefully heated to a boil and promptly cooled. Acetone (10 to 15 ml) and 5 to 10 ml of petroleum ether were added and the mixture shaken gently. After transferring to a small separatory funnel, the upper (epiphasic) layer was separated and treated as in Method A.

[4] A similar medium containing casein hydrolyzate also was used throughout the study for growing the test yeasts. Results for pigment extraction were essentially the same as those reported for cells grown in glucose broth (Difco); for that reason the results are not reported.

RESULTS

The influence of the three extraction methods (A, B, and C) used, on the absorption spectra of total petroleum ether extracts of three representative yeasts, is shown in figure 1. The species selected—*Rhodotorula rubra*, *R. bronchialis*, and *R. flava*—were typical of the red, red-orange, and yellow pigmented types in the large number of yeast cultures studied. Since all pigmented yeasts grown in synthetic broth on a rotary shaker for 72 hours yielded their pigments readily by the mild treatment involving extraction with cold acetone only (Method A), it was assumed that the absorption spectra of these petroleum ether extracts might be used as standards in testing the efficacy of two other methods.

Absorption spectra of the carotenoid pigment extracts of *R. rubra* and *R. bronchialis* were essentially the same whether obtained by direct extraction with acetone when grown in synthetic broth (figure 1, A) or after preliminary treatment at 100 C for 15 minutes in 0.5 N HCl when grown in glucose broth (figure 1, B).

Although the weak acid treatment (0.5 N HCl) was very effective with the red or red-orange yeasts studied, it evidently modified the pigment composition in the case of the yellow yeast, *R. flava*, in that the pronounced absorption maximum at 450 mμ disappeared almost entirely (figure 1, B). Similar results were obtained also with a yellow yeast, SY-836. As mentioned earlier, the present paper was prompted by the need for proof of the carotenoid nature of pigmented yeasts isolated from the cucumber plant. Over one-half of the 500 yeasts obtained were of the yellow pigmented type, and these were confirmed as belonging to the *Rhodotorula* genus only by Method A in that typical carotenoid absorption spectra were obtained.

The method of Mrak *et al.* (1949), involving preliminary treatment of the yeast cells with strong HCl (1:1), caused drastic changes in the absorption spectra of the total pigment extracts. This is illustrated in figure 1, C. In the cases of *R. bronchialis* and *R. flava*, the absorption maxima in the visible region disappeared entirely.

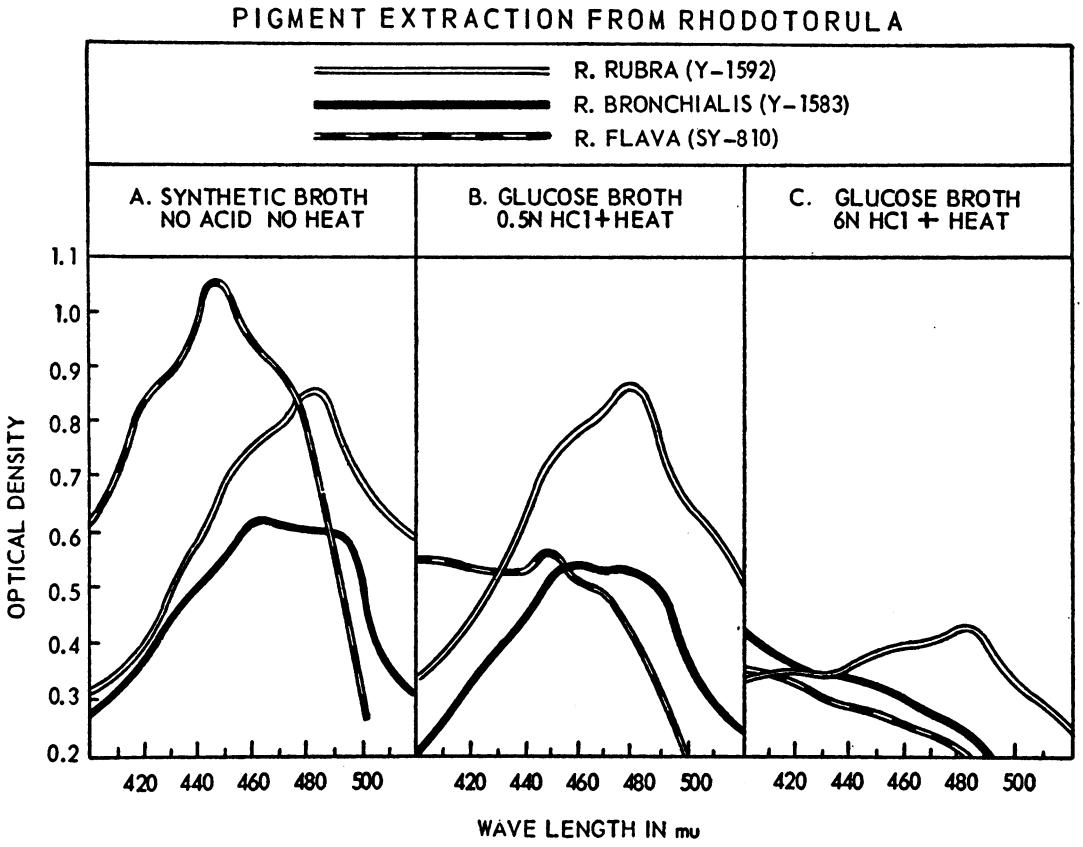

Figure 1. Influence of method of extraction on absorption spectra of *Rhodotorula* pigments. Left: Method A, cells grown in synthetic broth and extracted with acetone only. Center: Method B, cells grown in glucose broth and treated with 0.5 N HCl for 15 minutes at 100 C before acetone extraction. Right: Method C, cells grown in glucose broth and brought to a boil in 6 N HCl (1:1) before acetone extraction.

The method of choice is clearly that of growing potentially pigmented species in synthetic broth for 72 hours on a rotary shaker and extracting the centrifuged cells with acetone (Method A). Absorption maxima of the petroleum ether extracts of species and strains of *Rhodotorula* extracted in this manner are given in table 1. Although it may be suggested that absorption spectra determined on total carotenoid extracts of yeasts provide only limited information since they represent composite spectra of several different pigments, they are none the less adequate for screening pigmented species. The results presented in table 1 demonstrate that total extracts of most red to orange colored types have a dominant maximum at 480 mμ, whereas most yellow types usually have a maximum at about 450 mμ.

Preliminary experiments have shown that petroleum ether extracts of *Rhodotorula* obtained by Method A can be used successfully for the chromatographic separation and identification of yeast carotenoids. A portion of this work has been given in a recent report by Etchells *et al.* (1953). The latter publication also demonstrates pictorially, in natural color, the striking influence of the cultural medium on pigment production by certain species of *Rhodotorula.*

DISCUSSION

In providing the yeast taxonomist with a simple, rapid procedure for determining the presence of carotenoid pigments in yeasts, it is doubtful if any more rigorous criteria of identity are needed than demonstration of their solubility in petroleum ether (colored either yellow, orange,

pink, or red); their absorption in the region 400 mμ to 520 mμ; and, generally, their epiphasic behavior when partitioned between 90 per cent methanol and petroleum ether (this does not imply exclusion of the possibility that hypophasic carotenoids will be detected in similar organisms). Many confirmatory spectroscopic and chromatographic analyses of pigments of certain red types of *Rhodotorula* (notably *R. rubra*) have been made and their carotenoid character established (Bonner *et al.*, 1946; Etchells *et al.*, 1953; Fink and Zenger, 1934; Fromageot and Tchang, 1938; Goodwin, 1952; Karrer and Rutschmann, 1943; Lederer, 1933).

Bonner *et al.* (1946), in a recent study in which they made a quantitative chromatographic resolution of pigments from *R. rubra* and several of its mutant strains, found four major hydrocarbon carotenoids. These were identified as torulene (about 76 per cent of the total carotenoids); β-carotene, $C_{40}H_{56}$ (11 per cent); γ-carotene, $C_{40}H_{56}$ (9 per cent); and an unidentified carotenoid, "pigment A" (4 per cent). Other workers (Fink and Zenger, 1934; Fromageot and Tchang, 1938; Karrer and Rutschmann, 1943; Lederer, 1933) have isolated, in addition, an acid pigment, torularhodin (probably, $C_{37}H_{46}O_2$), from *Rhodotorula*. It appears, however, that yeasts having principally yellow pigments, such as *R. flava*, have not been studied. Extraction Method A, as outlined in this paper, should offer workers a satisfactory procedure for studying the nature of the pigments of the yellow species as well as yeasts with questionable pigmentation now classified in other genera.

In this connection, it is of interest that Phaff *et al.* (1952) recently have reported the isolation of a culture of *Torulopsis albida* from shrimp, and petroleum ether extracts of the cells showed a yellowish-green color, indicating the presence of carotenoid pigments. Our culture of *T. albida* var. *japonica* (see table 1) not only gave a yellow color in the petroleum ether extract but also gave an absorption maximum at 450 mμ. The two yeasts are considered identical in the new classification by Dutch workers (Lodder and Kreger-Van Rij, 1952) and are listed as *Cryptococcus albida*. In view of the presence of the carotenoid pigments demonstrated for this species, it should be placed in the genus *Rhodotorula*.

It has become evident from this work that carotenoid production covers a wider range of yeast types than has been previously suspected. Indeed it appears that Wickerham (1952) has summarized the situation adequately in a recent review on yeast taxonomy when he stated, "there is a possibility that the taxonomic value of the ability to produce carotenoid pigment has been exaggerated".

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SUMMARY

When pigmented yeasts of the *Rhodotorula* genus were grown in nonsynthetic media which were highly buffered or contained complex organic materials, such as peptone or casein hydrolyzate, the pigments could only be released for subsequent extraction with acetone by the use of rather strong concentrations of acid and heat. Saponification procedures were completely ineffective.

However, when various species of *Rhodotorula* (including both the red and the yellow pigmented types) were grown in Wickerham's synthetic broth (containing two per cent glucose) for 72 hours on a rotary shaker, the pigments could be extracted readily and directly from the cells with cold acetone and transferred to petroleum ether for characterization by chromatographic and spectrophotometric methods.

Carotenoid pigments could be demonstrated in all red to orange species of *Rhodotorula* tested and grown in nonsynthetic media (i.e., glucose broth) by a preliminary treatment of the cells with 0.5 N HCl heated for 15 minutes at 100 C followed by extraction with acetone. However, this acid treatment (as well as the one using 6 N HCl) destroyed or measurably altered the pigments obtained from certain yeast species of the yellow pigmented type (i.e., *Rhodotorula flava* group).

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