QUANTITATIVE DETERMINATION OF THE CAROTENOIDS IN YEASTS OF THE GENUS *RHODOTORULA*[1]

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Though 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), no routine procedure seems to have been developed for the extraction, separation, and quantitative determination of the carotenoids in yeasts.

The carotenoid character of the pigments of the Rhodotorulae has been well established (Bonner *et al.*, 1946; Etchells *et al.*, 1953; Fink and Zenger, 1934; Fromageot and Tchang, 1938; Goodwin, 1952; Karrer and Rütschmann, 1943; Lederer, 1933, Peterson *et al.*, 1954). Bonner *et al.* (1946), in quantitative resolutions 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 (11 per cent); γ-carotene (9 per cent); and an unidentified carotenoid (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 $C_{37}H_{48}O_2$), from *Rhodotorula* species. Nakayama *et al.* (1954) determined the content of individual carotenoids present in several species of *Rhodotorula* and found that they contained torularhodin, torulene, and β- and γ-carotenes as the principal pigments.

In an earlier qualitative study from this laboratory (Peterson *et al.*, 1954) it was found possible to extract appreciable quantities of the carotenoids of yeasts directly with cold acetone, without hydrolysis, provided the yeasts were grown on a rotary shaker in the nitrogen base broth proposed by Wickerham (1951), plus 2 per cent glucose. In the present report, the above procedure, combined with several of the excellent analytical techniques of Nakayama *et al.* (1954) have been extended and improved to provide a routine procedure for the quantitative analysis of yeast carotenoids. The procedure developed has been used successfully with the seven species and one variety of yeasts in the genus *Rhodotorula* as defined by Lodder and Kreger-Van Rij (1952).

MATERIALS AND METHODS

The species and the one variety of *Rhodotorula* used for the various phases of this study are listed in table 1.

Since it was contemplated that the procedure developed would be used in future studies of the mechanisms involved in the biosynthesis of carotenoids by yeasts, it seemed desirable to use the simplest synthetic medium which would assure good growth. Exhaustive studies revealed that the rather simple basal medium presented in table 2 would provide excellent growth of *R. glutinis* and *R. glutinis* var. *rubescens* without added vitamins. With this basal medium *R. mucilaginosa*, *R. rubra*, and *R. flava* appeared to need only thiamin as an added vitamin, while *R. aurantiaca*, *R. pallida*, and *R. minuta*, the three species which grew rather poorly on all media, required *p*-aminobenzoic acid (23 μg per L) in addition to the thiamin. These three species, as well as *R. flava*, also produced lesser amounts of total pigments and best growth was obtained in a synthetic nitrogen base medium[3] proposed by Wickerham (1951) with 2 per cent glucose and 0.1 per cent yeast extract added.

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[3] Obtained from Difco Laboratories, Detroit, Michigan. Mention of trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture or the N. C. Agricultural Experiment Station over similar products not mentioned.

TABLE 1

Species of the genus Rhodotorula analyzed and the media in which they were grown

NRRL No.*	*Rhodotorula* spp.	Synonyms†	Medium Used
Y-1595	*R. mucilaginosa*	*R. sanguinea* *R. mucilaginosa* var. *sanguinea* *R. mucilaginosa* var. *carbonei* *R. sanniei* *R. mucilaginosa* var. *pararosea* *R. aclotiana*	Basal medium‡ with 400 μg of thiamin per L added
Y-1596	*R. glutinis*	*R. bronchialis* *R. suganii* *R. glutinis* var. *infirmo-miniata* *R. gracilis*	Basal medium‡
Y-1581	*R. aurantiaca*	*R. colostri* *R. longissima*	Wickerham's (1951) N-base medium with 2 per cent glucose and 0.1 per cent yeast extract added.
Y-1621	*R. glutinis* var. *rubescens*	*R. rufula* *R. glutinis* var. *rufula* *R. glutinis* var. *saitoi* *R. rubella* *R. glutinis* var. *lusitanica*	Basal medium‡
Y-1591	*R. rubra*	*R. mucilaginosa* var. *plicata* *R. rubra* var. *longa* *R. rubra* var. *curvata*	Basal medium‡ with 400 μg of thiamin per L added
Y-339	*R. pallida*	None (Note: Only one strain studied and it was obtained from Ciferri in 1926 labeled *Mycotorula muris*. Renamed by Lodder.	Same as for Y-1581 above
Y-1589	*R. minuta*	None (Note: Only one strain (*Torula minuta*) studied; isolated by Saito in 1922 (Tokyo); renamed *Torulopsis minuta* by Ciferri and in 1928 classified as *R. minuta* by Harrison.)	Same as for Y-1581 above
Y-1585	*R. flava*	None (Note: Only one strain (*Torula flava*) above studied; isolated by Saito in 1922. It was later classified by Harrison as *Chromotorula flava* and in 1934 classified by Lodder as *R. flava*.)	Same as for Y-1581

* NRRL: Northern Regional Research Laboratory (USDA), Peoria, Ill.
† From *The Yeasts* by J. Lodder and M. J. W. Kreger-Van Rij (1952).
‡ See table 2.

The yeasts were grown for 6 days at 24 to 26 C (room temperature) in 100 ml of synthetic broth (table 2) in 250-ml Erlenmeyer flasks on a rotary shaker[4] at 210 rpm.

Extraction of the pigments. Each flask was removed from the shaker and permitted to stand overnight in the refrigerator 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 to a 50 ml centrifuge tube and centrifuged for 20 min at 2500 rpm. After decanting the supernatant, 30 ml of distilled water were added and the packed cell mass broken up by vigorous stirring with a

[4] Model V, capacity 40 flasks, manufactured by the New Brunswick Scientific Company, New Brunswick, New Jersey.

TABLE 2

Composition of the basal synthetic medium employed

Ingredients	Quantity
	per L
Glucose	20 g
$(NH_4)_2SO_4$	3.5 g
Asparagine	1.0 g
KH_2PO_4	0.5 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NaCl	0.1 g
Zinc (as sulfate)	400 μg
Iron (as ferrous ammonium sulfate)	150 μg
Copper (as sulfate)	150 μg
Water to 1,000 ml	
pH after autoclaving generally in range	5.9–6.1

glass rod. The tube was centrifuged as before and the wash-water discarded. (Cells from a duplicate flask, similarly treated, were dried in centrifuge tubes overnight at 65 C in an air oven followed with drying under vacuum to constant weight for the determination of dry weight of cells.) For extraction of the pigments, 30 ml of acetone were added and the cells stirred vigorously. After centrifuging, the acetone was decanted into an Erlenmeyer flask and the procedure repeated with another 30 ml portion of acetone. To ensure complete extraction of the pigments, 20 ml of acetone were then added to the cells and the stoppered tube stored in the refrigerator overnight. The cells were then centrifuged and this acetone fraction combined with the two previous extracts.

This procedure provided complete extraction of the pigments from *R. glutinis* var. *rubescens* and *R. flava*, 80 to 90 per cent from *R. mucilaginosa* and *R. glutinis*, and only 50 to 60 per cent from *R. rubra*. The pigments of *R. aurantiaca* and *R. minuta* were poorly extracted. Acetone extracted no pigments at all from *R. pallida*.

In those cases in which complete extraction was not accomplished by acetone extraction alone, as indicated by a slight pinkish color of the cell residue, 20 ml of 0.5 N HCl were added to the centrifuge tube and the cell mass broken up with a stirring rod. Then the tube was placed into a vigorously boiling water bath for 15 min. Upon removal from the water bath, the tube was placed into ice water to cool for 10 min., after which the cells were separated by centrifugation. The pigments were then extracted from the cells with acetone as before and these extracts combined with those obtained from the direct extractions.

Separation of the pigments. The combined acetone extracts for a given sample were transferred to a 250 ml separatory funnel and 30 ml of *n*-hexane added with careful mixing. Water was added in 10 ml portions until a clear separation of the acetone-water phase and the *n*-hexane resulted with all the pigments in the upper hexane layer. The acetone-water layer was then drawn off and extracted separately with a few ml of hexane to make certain that no pigments still remained. If the hexane layer became pigmented, the acetone-water mixture was extracted again with *n*-hexane in another 250 ml separatory funnel, and the pigmented hexane combined with the previous hexane extract. The acetone-water layer was again checked for pigment. Usually one such extraction was sufficient.

The pigmented hexane layer was then washed with three 50-ml portions of distilled water. These washings may be accomplished with vigorous shaking with no danger of emulsions being formed. After the final water wash was discarded, the hexane layer was filtered into 100-ml volumetric flasks through a small amount of anhydrous sodium sulfate over a small filter of glass wool. The separatory funnels and filters were rinsed with hexane. The clear pigmented hexane filtrate was then brought to volume with hexane and its optical density determined on a Beckman spectrophotometer model DU at 500 mμ against a hexane blank. The pigmented hexane was then transferred from the 100-ml flasks to 250-ml separatory funnels. The pigment, torularhodin, was separated from the hexane by extracting with 50-ml portions of 90 per cent methanolic potash (0.1 N) until no color appeared in the methanolic layer. One to three alkaline extractions were required for complete removal of the torularhodin, depending upon the species of *Rhodotorula* being analyzed. The hexane layer was then washed alkali-free with distilled water (attested by a water-white wash solution upon the addition of 1 or 2 drops of phenolphthalein), and filtered into 100-ml volumetric flasks through anhydrous sodium sulfate, as previously described. The optical density was again determined at 500 and 484 mμ after the contents of the flasks were brought to volume with hexane.

The optical density value at 500 mμ obtained before the alkaline extraction, minus the optical density at 500 mμ after extraction, was used to determine the torularhodin concentration, using the specific absorption coefficient[5] 258 (Karrer and Rutschmann, 1945; Nakayama *et al.*, 1954). (In separate experiments with each of the species tested, the alkaline-methanol extracts were acidified, the torularhodin taken up in hexane and identified by its absorption spectrum. Concentrations determined on these extracts were identical with those obtained by the "difference" method above.)

A 50-ml aliquot of each hexane extract was then pipetted onto an adsorption column (2 cm in diameter) containing a 10 to 15 cm layer of a uniformly packed MgO-Supercel[6] mixture (2:1) which had previously been washed with *n*-hexane. When the pigmented hexane was drawn through the column under vacuum, all of the pigments were adsorbed near the top. The β- and γ-carotenes were then readily eluted from the column with absolute ethanol, while the torulene fraction, pink in color, remained firmly adsorbed near the top of the column.

The eluted pigmented solution containing the β- and γ-carotenes was then transferred to 125-ml separatory funnels; 25 ml of distilled water and 25 ml of *n*-hexane were added, forcing the pigments into the hexane layer. The hexane was washed free of ethanol and dried with anhydrous sodium sulfate as before, being filtered into 50-ml volumetric flasks. These were then made up to volume with hexane and the optical densities determined at the wave lengths 461.5, 478, and 484 mμ.

The concentration of torulene was determined by subtracting the optical density value obtained at 484 mμ after chromatography from the optical density obtained at 484 mμ before chromatography (assuming the same volume relationships), and using the specific absorption coefficient 268 (Lederer, 1933; Nakayama *et al.*, 1954). The adequacy of this method of determining the concentration of torulene was established by separate studies with each species in which the torulene was carefully eluted from the column, and its absorption spectrum shown to be that of torulene. Concentrations determined on these extracts were, within experimental errors, identical with those obtained by the "difference" method.

The concentrations of the β- and γ-carotenes were found by using the following equations:

$$\beta\text{-carotene, conc in g/L} = \frac{\text{O.D.}^{478} - 0.7\ \text{O.D.}^{461.5}}{75}$$

$$\gamma\text{ carotene, conc in g/L} = \frac{\text{O.D.}^{461.5} - 220C_1}{272}$$

Where C_1 = Conc of β-carotene in g/L.

Starting with the specific absorption coefficients presented by Zechmeister *et al.* (1943) and Zechmeister and Polgár (1943), the above equations were developed using the principles and formulae derived by Knudson *et al.* (1940) for colorimetric analysis of two component color systems.

Preliminary experiments with each of the species tested revealed that torularhodin, torulene, β- and γ-carotenes were the only pigments present which absorbed in the visible region. β-and γ-Carotenes were identified chromatographically and spectrophotometrically in separate discrete studies. No lycopene was identified.

RESULTS AND DISCUSSION

The total content of carotenoid pigments (in μg per g of dry cells) present in each of the seven species and one variety of yeasts in the genus *Rhodotorula*, and the percentage distributions of the contributing pigments in each case, are presented in table 3. Analytical techniques and procedures were developed primarily with *R. glutinis* and *R. glutinis* var. *rubescens* as the test species. The values shown are the means for 10 samples of *R. glutinis* and 13 samples of its variety *rubescens*. Values for other species are the means of 3 determinations. A statistical analysis of the data for which means are reported in table 3 revealed that the standard error (s.d.) of a single determination within a batch (samples of the same species grown under identical condi-

[5] Specific absorption coefficient as used in this publication refers to α in the following equation:

$$C = \frac{\log \frac{Io}{10I}}{\alpha x}$$

Where C = concentration in g per L, $\log \frac{Io}{10I} = -\log T$ or optical density, and x = depth of solvent in cm.

[6] Westvaco magnesia no. 2642.

TABLE 3

Distribution of carotenoid pigments in species of yeasts in the genus Rhodotorula

Species	Wt of Dry Cells per 100 ml of Medium	Torularhodin	Torulene	β-Carotene	γ-Carotene	Total Carotenoids per g of Dry Cells
	g	%	%	%	%	μg
R. mucilaginosa	0.5943	28.6	59.3	9.0	3.1	357.7
R. glutinis	0.4481	66.8	27.2	3.5	2.3	332.0
R. aurantiaca	0.3909	0	84.8*	6.2	9.0	112.8
R. glutinis var. *rubescens*	0.2346	29.8	37.8	23.9	8.5	494.0
R. rubra	0.3806	49.3	27.5	14.6	8.5	186.7
R. pallida	0.6755	0	89.5	4.7	5.8	51.0
R. minuta	0.7181	37.8	41.2	13.9	7.1	93.4
R. flava	0.4919	0	0	61.5	38.5	19.1

* Probably not torulene: no maximum at 500 mμ; more soluble in methanol than torulene.

tions) was as follows: torularhodin, 3.5; torulene, 4.4; β-carotene, 1.64; and γ-carotene, 2.6. The differences observed between species with respect to the pigment distribution are for the most part, clear-cut and not attributable to determination and sampling variability. The largest concentrations of carotenoids (per g of dry cells) were found in *R. glutinis* var. *rubescens* *R. mucilaginosa*, and *R. glutinis*. Total pigment concentrations in *R. pallida* and *R. flava* were very small in comparison.

It is of interest that each of the species had its own characteristic pigment make-up. *R. glutinis* and *R. rubra* were characterized by their proportionately high percentages of the acid pigment, torularhodin, while *R. aurantiaca*, *R. pallida*, and *R. flava* did not appear to develop this pigment under our conditions. Bonner *et al.* (1946) could not demonstrate this pigment in any marked quantity in their strain of *R. rubra*. Lodder and Kreger-Van Rij (1952) found the acidic pigment in several but not all red strains of *Rhodotorula*. Nakayama *et al.* (1954) found that 32.3 per cent of the total pigment of *R. rubra* and 42.5 per cent of the total pigment of *R. glutinis* consisted of torularhodin. In the present study, *R. flava* was the only species tested which did not contain torulene. The highest concentration of β-carotene was found in *R. glutinis* var. *rubescens*. *R. flava*, however, had the highest relative amount of this pigment.

The results reported herein are generally in good agreement with those of Nakayama *et al.* (1954), who reported that the combined torularhodin-torulene fraction accounted for 57 to 85 per cent of the total pigment. The comparable range in this study was 67 to 94 per cent.

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SUMMARY

A procedure has been presented for the extraction, separation, and quantitative determination of the carotenoids in species of the genus *Rhodotorula*. The method has been applied successfully to the determination of the concentrations of the individual carotenoids produced by representatives of the seven species and one variety of yeasts in the genus *Rhodotorula* as defined in 1952 by Lodder and Kreger-Van Rij.

β-Carotene and γ-carotene were found in all of the eight yeasts studied; torulene was observed in seven (absent in *R. flava*) and torularhodin in five (absent in *R. aurantiaca*, *R. pallida* and *R. flava*). The largest concentrations of carotenoids were found in *R. glutinis*, its variety *rubescens* and in *R. mucilaginosa*.

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