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COMPARATIVE STUDY OF AUREOBASIDIUM (PULLULARIA) PULLULANS AND RHODOTORULA GLUTINIS PIGMENTS

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ABSTRACT

A comparative study of Aureobasidium (Pullularia) pullulans and Rhodotorula glutinis pigments has been made. These pigments were revealed to be insoluble substances and generally chemically unreactive. Both pigments were closely associated with protein and contained small percentages of carbohydrate. Absorption peaks characteristic of carotenoid structures were absent. The infrared spectra of both pigments showed vibrational frequencies distinct from those reported for natural and synthetic melanins.

INTRODUCTION

Colonies of most species of yeast appear white, or white with a grayish or yellowish cast, as they age, certain species produce pigments. Comparatively few yeast produce appreciable amounts of pigment and those that do almost exclusively exhibit red to yellow pigments even though the various coloring matters may be chemically unrelated.

Luteraan et al. (1947) obtained evidence that yeast-like fungi synthesize pigments, carotenes, and red quinoid compounds which were involved in the mediation of oxygen through the respiratory process. As the quinoid compounds were eventually fully oxidized to melanic derivatives they become useless for respiratory purposes and were stored in the membrane or excreted.

Aureobasidium (Pullularia) pullulans, commonly called a "black yeast," is important industrially because it causes paint deterioration, discoloration of lumber, injury to plants and plant products. It has also been implicated in human infections (Wynne and Gott, 1956). Initially the appearance of a young colony is from white to tan to light pink; upon aging, colonies turn greenish and finally black. Information suggesting

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media for producing high concentrations of this pigment has been published (Lingappa et al., 1963; and Merdinger, 1964).

Rhodotorula glutinis has been obtained from human and animal sources. It has been found in the soil together with other yeast. Species of the genus Rhodotorula are by definition all pigmented, the color varying from a pale to deep salmon pink. The color has been reported to be due to the presence of carotenoid pigments (Bonner et al. 1946, Nakayama et al., 1954 and Phaff et al., 1952). In certain cases however, demonstration of the presence of carotenoids was made by visible observation of the color or non-specific tests.

This investigation was undertaken to isolate and characterize pigments from Aureobasidium (Pullularia) pullulans and Rhodotorula glutinis cultures. The comparative study of certain chemical properties of these pigments was made in order to determine whether there exists any similarities between these substances in the hope of ultimately gaining greater knowledge and improved understanding of the nature of these substances.

MATERIALS AND METHODS

Organism and growth conditions.

Aureobasidium (Pullularia) pullulans (strain NRRL YB-4515) was grown in a medium consisting of 0.3% malt and yeast extracts (Difco, Detroit, Mich. U.S.A.), 0.01% K_2HPO_4, 0.05% $MgSO_4$, and 0.01% KH_2PO_4.

Rhodotorula glutinis (strain NRRL YB-4027) was kindly furnished by Dr. C. P. Kurtzman, United States Department of Agriculture, Agriculture Research Service, Peoria, Illinois. The yeast was grown in a medium consisting of 0.3% malt and yeast extracts, 0.5% peptone (Difco, Detroit, Mich. U.S.A.), and 1% glucose.

Harvesting and extraction of pigment.

Details of growth of cells, isolation and preparation of Pullularia pigment have been described previously (Merdinger, 1969).

A 48 h Rhodotorula glutinis culture was checked for contamination microscopically and by plating on nutrient agar. If free of contamination, 100 ml of the culture were used as inoculum and added to one liter of broth medium. The cultures were shaken in a Lab-line Constant Temperature Shaker, with a rheostat setting of 3.0, at 25 C, for 7 days. The 7 day culture was checked for contamination as previously described and if free of contamination, 100 ml were aseptically transferred to a one liter of fresh broth medium. Both cultures were then shaken at 25 C for 7 days. The cultures were again checked for contamination and if free of contamination, the 14 day cultures were immediately stored at 7 C until harvesting. A 100 ml culture was taken aseptically from the 7 day culture and transferred to one liter broth medium. Both cultures were then shaken for 7 days and processed as previously described. This procedure was repeated until a sufficient quantity of 14 day cells were harvested.

Cultures were harvested by centrifugation at 755 x g at 4 C and were washed first with 0.9% NaCl and then with distilled water until free from chloride. Test for the presence of chloride in water washings was made using 1% aqueous silver nitrate solution. Washed cells were broken by adding to each gram of cells 3.0 g of 0.1 mm glass beads and 3.0 ml 0.2 M phosphate buffer at pH 7.0 in a Sorvall Omni-mix. Rupturing of cells was performed at 5 C at a rheostat setting of 100 for 1 h, at which point microscopic examination indicated 80-85% breakage. The homogenate was centrifuged 10 min at 600 x g and 4 C. Supernatant fluid was decanted and sequentially centrifuged at 8,000 and 25,000 x g for 10 min, followed by centrifugation at 105,000 x g for 1 h. The pigment residue isolated at 105,000 x g was washed with 0.1 N NaOH followed by 0.2 M phosphate buffer, pH 7.0 and then several times with deionized water. The sediment was dialyzed overnight against deionized water, and lyophilized.

The dried yeast pigments as well as synthetic melanin (K-K Laboratories, Plainview, N. Y.) were further purified before analysis by washing. Melanin and the Pullularia pigment were washed in saline with mixing on a Vortex Mixer for 5 min; followed by centrifugation at 480 x g for 30 min and removal of the saline wash. They were then washed three times with deionized water with mixing; followed by centrifugation at 480 x g for 30 min in each case. The Rhodotorula pigment was washed in a similar manner except for centrifugation at 105,000 x g for 1 h after each wash. The pigments were then dried by lyophilization. These procedures have been previously shown to yield pure pigment preparations free of cellular debris (Merdinger, 1964).

Mild Mineral Acid Hydrolysis.

To a 25 ml round bottom flask was added for every 2 mg of pigment, 1 ml of 2 N HCl. The flask was attached to a reflux condenser, placed in a heating manifold, and refluxed for 1 h. After cooling, the mixture was removed from the flask and placed in a conical test tube and centrifuged for 10 min at 280 x g. The hydrolysate was removed from the tube. To approximately every 2 mg of residue was added 1 ml 2 N HCl. This mixture was again placed in a 25 ml round bottom flask, heated under reflux for 1 h and the acid hydrolysate was removed in the same manner as previously described. The remaining residue was washed several times with deionized water and freeze dried. Residues were analyzed by IR Spectroscopy. Hydrolysates were dried under vacuo in a rotary evaporator and redissolved in a minimum volume of distilled water for further analysis.

Analytical Methods.

Solubility and qualitative analysis of the elements were determined according to Shriner et al.(1967).

Protein was estimated by a microadaptation of the method of Lowry et al. (1951). Spectrophotometer readings were made at 250 nm in a Beckman DU spectrophotometer and converted to micrograms of protein per milliliter by a serum albumin standard curve.

Hexose content was determined quantitatively by the anthrone reaction (Scott and Melvin, 1953) with glucose solutions used as standards.

Paper and Thin Layer Chromatography.

Hydrolysates were chromatographed on Whatman No. 1 filter paper and Eastman silica and cellulose thin layer sheets (without fluorescent indicator): and compared with authentic carbohydrate and amino acid standards. The developing systems were n-butyl alcohol/ethyl alcohol/water (57:34.5:8.5) and ethyl acetate/pyridine/water (10:4:3). Carbohydrate and amino acid spots were detected by aniline oxalate and ninhydrin spray reagents.

Amino Acid Analysis.

Quantitative total amino acid analyses were performed on a Spinco Model 120C Amino Acid Analyzer by the method of Spackman et al. (1958). Pigment preparations of approximately 1-3 mg were hydrolyzed for 22 h in 1.0 ml 6 N HCl in evacuated and sealed Pyrex tubes at 110 C. Times of hydrolysis ranged between 22 and 120 h. After hydrolysis acid was removed at room temperature in vacuo over NaOH.

Spectroscopy.

Infrared spectra on pigments incorporated into KBr disks were obtained with a Perkin-Elmer model 700 spectrophotometer; and ultraviolet and visible spectra were obtained with a Beckman DU spectrophotometer.

RESULTS

Optimal growth and pigment formation occurred between five to twelve days in Aureobasidium (Pullularia) pullulans cultures and between twelve to fourteen days in Rhodotorula glutinis cultures. The isolated pigments were non-granular.

The elementary composition of the pigments consisted of carbon, hydrogen, nitrogen, and sulfur. The presence of nitrogen and sulfur were not detected by chromatographic analysis.

The most striking characteristic of the pigments seemed to be their complete insolubility in water and their almost complete insolubility in most of the common organic solvents as shown in Table 1. Both pigments were slightly soluble in acetone with no color being extracted. They were slightly soluble in alkali and acid with color extracted and (or) formation, in most cases. This solubility behavior is similar to melanin (Wolman, 1969).

Pigment hydrolysates obtained from mild HCl hydrolysis produced positive color development with the Lowry assay indicating the presence of protein nitrogen.

TABLE 1. Comparison of the solubility of pigments from Pullularia pullulans and Rhodotorula glutinis

Solubility	Pigments	
	Pullularia pullulans	Rhodotorula glutinis
Cold distilled water	-	-
Hot distilled water	-	-
Acetone	Slt. S	Slt. S
Benzene	-	-
Butanol	-	-
Carbon Disulfide	-	Slt. S
Chloroform	-	Slt. S (pale pink)
Cyclohexanone	-	"
Dioxane	-	"
Ethanol	-	"
Hexane	-	"
Methanol	-	"
Petroleum Ether	-	"
Propanol	-	"
Sodium Hydroxide, 5%	Slt. S (green)	Slt. S
Sodium Carbonate, 5%	-	-
Glacial Acetic Acid, 97%	Slt. S (brown)	Slt. S
Hydrochlorid Acid, 37%	Slt. S (brown-red)	Slt. S
Nitric Acid, 71%	Slt. S (brown)	Slt. S (green)
Sulfuric Acid, 95%	"	S (amber)
6 N HCl, followed by 6 N NaOH	Slt. S (red change to green)	Slt. S. (no color change)

(-) Indicates Insolubility
Slt. S Indicates Slightly Soluble
S Indicates Soluble

Carbohydrate analysis of concentrated pigment hydrolysates indicated that Pullularia pigment had a carbohydrate content of 0.5 per cent, while Rhodotorula pigment contained a carbohydrate content of approximately 3.0 per cent.

Glucose was the only monosaccharide detected by paper and thin layer chromatography in Pullularia pigment hydrolysates. Galactose, glucose, mannose, and a fast moving component with the same mobility ratio as ribose were detected in Rhodotorula pigment hydrolysates.

The color of the Pullularia pigment remained unchanged after hydrolysis in strong mineral acid; 16 per cent of the pigment starting material was recovered after 120 h hydrolysis. Rhodotorula glutinis (105,000 x g) was easily hydrolyzable with strong acid. Approximately 100 per cent of this material was hydrolyzed with 6 N HCl in 24 h as shown in Table 2.

TABLE 2. Mineral acid hydrolysis of pigments with 6 N HCl

Pigment	Time hydrolyzed* (hr)	Quantity of pigment hydrolyzed (mg)	Quantity of pigment after hydrolysis (mg)†	% Pigment Recovered after hydrolysis
Pullularia	24	3.0	1.30	43.3
	48	3.0	0.95	31.6
	72	3.0	0.65	21.6
	96	3.0	0.40	13.6
	120	2.5	0.40	16.0
Rhodotorula	24	3.0	$\leq$0.005	$\leq$0.1
	72	3.0	$\leq$0.005	$\leq$0.1
	120	3.0	$\leq$0.005	$\leq$0.1

*Conditions as for amino acid analysis - see Methods.
†Residues were recovered, washed with distilled water, dried and weighed.

Commercial melanin was found to be resistant to hydrolysis since 35 per cent of the pigment could still be recovered after 168 h of hydrolysis. The color of melanin remain unchanged even after this extensive period of hydrolysis.

Results of quantitative total amino acid analysis of pigments are recorded in Tables 3 and 4. The mean value of amino acids released during 24, 72 and 120 h hydrolysis was taken as representing the tru content of the amino acids in the Pullularia pigment hydrolysate, while the average of 24 and 62 h hydrolysis was taken as representing the true amino acid content in the Rhodotorula pigment hydrolysate. Quantitative total amino acid analysis of melanin is recorded in Table 5. Tyrosine accounted for 63.5 per cent of the amino acid composition of the hydrolyzable protein in the melanin sample.

Visible absorption spectra of the yeast pigments in hexane and carbon disulfide revealed very little about their structure. Over the visible wavelengths, pigment solutions had little absorbance with no apparent absorption bands. Plain curves without specific absorption maxima were produced (Fig. 1), similar to those of melanin.

The infrared spectra of the Pullularia pigment; raw, washed, and acid treated, were all similar to each other. This was also true for the Rhodotorula pigment (105,000 x g). The Pullularia and Rhodotorula pigments have spectra that are similar to each other (Fig. 2). Both had characteristic absorption which could be attributed to the amine or aromatic hydroxy bond (at about 3300 cm^{-1}), C-H stretching which could be attributed to an alkane (at about 3300 cm^{-1}), carbonyl vibration

TABLE 3. Results obtained in Amino Acid Analysis of *Pullularia pullulans* Pigment in µg%

Amino Acid	Acid Hydrolysis (hours) 24	72	120	Amino Acid Composition Average
Lysine	4.2	5.3	6.1	5.20
Histidine	1.7	2.2	3.2	2.37
Arginine	3.9	3.9	5.0	4.27
Aspartic	11.0	11.3	11.4	11.23
Threonine	9.1	9.2	8.6	8.97
Serine	8.5	7.7	6.5	7.57
Glutamic	9.1	10.1	10.7	9.97
Proline	5.1	Tr*	Tr	1.70
1/2 Cyst.	Tr			Tr
Glycine	5.5	7.1	7.0	6.53
Alanine	7.8	9.7	9.2	8.90
Valine	7.1	7.3	8.5	7.63
Methionine	Tr	Tr	Tr	Tr
Isoleucine	5.6	6.6	7.1	6.43
Leucine	6.7	7.7	7.8	7.40
Tyrosine	3.5	1.8	2.8	2.70
Phenylalanine	5.0	6.7	6.0	5.90
Glucosamine	6.3	3.3		3.20
Cysteic Acid	Tr	Tr	Tr	Tr
	100.1	99.9	99.9	100.1
Total Sample, mg	3.00	3.00	2.50	2.83
Total µg recovered	62.85	49.36	38.54	50.25
Per cent total sample recovered	2.09	1.64	1.28	1.64

*Tr: Trace or too little to quantitate (less than 5 nM).

(at about 1700 cm^{-1}), C-O, CN, or O-H vibration (at about 1700 cm^{-1}), and C, C-N, or O-H vibration (at about 1080 cm^{-1}). These spectra showed vibrational frequencies distinct from the infrared spectra of natural and synthetic melanins which have been previously reported (Van Woert *et al.* 1967), and the commercial melanin used in this study.

TABLE 4. Results obtained in Amino Acid Analysis of *Rhodotorula glutinis* Pigment in µg%

Amino Acid	Acid Hydrolysis (hours) 24	72	Amino Acid Composition Average
Lysine	6.71	7.09	6.90
Histidine	1.56	2.41	1.99
Arginine	5.87	6.14	6.00
Aspartic	10.31	9.87	10.09
Threonine	5.30	4.98	5.14
Serine	3.18	4.99	4.09
Glutamic	13.93	11.83	12.88
Proline	5.99	5.52	5.76
1/2 Cyst	--	--	--
Glycine	5.49	5.40	5.44
Alanine	9.07	8.20	8.63
Valine	6.68	7.60	7.14
Methionine	--	--	--
Isoleucine	4.78	5.77	5.28
Leucine	9.12	9.71	9.41
Tyrosine	3.25	2.32	2.79
Phenylalanine	4.77	5.36	5.07
Glucosamine	2.12	.59	1.35
Custeic Acid	.88	1.12	1.00
Methionine Sulfoxide	1.07	1.09	1.08
	100.08	99.99	100.04
Total Sample, mg	3.00	3.00	3.00
Total µg recovered	278.6	212.6	245.6
Per cent total sample	9.29	7.08	8.19

TABLE 5. Results obtained in Amino Acid Analysis of Melanin in µg%

Amino Acid	Acid Hydrolysis (hours) 24	72	Amino Acid Composition Average
Lysine	2.6	3.0	2.80
Histidine	--	--	--
Arginine	--	--	--
Aspartic	5.4	4.6	5.00
Threonine	2.4	1.2	1.80
Serine	3.7	1.8	2.74
Glutamic	3.9	4.6	4.25
Proline	Tr	Tr	Tr
1/2 Cyst	--	--	--
Glycine	3.3	3.1	3.2
Alanine	2.6	1.5	2.05
Valine	1.4	2.4	1.90
Methionine	3.5	3.6	3.55
Isoleucine	2.3	1.4	1.85
Leucine	2.7	2.3	2.50
Tyrosine	62.4	64.6	63.50
Phenylalanine	3.9	5.7	4.80
	100.1	99.9	99.95
Total sample, mg	2.2	2.0	2.1
Total µg recovered	34.2	28.8	31.5
Percent total sample	1.55	1.44	1.50

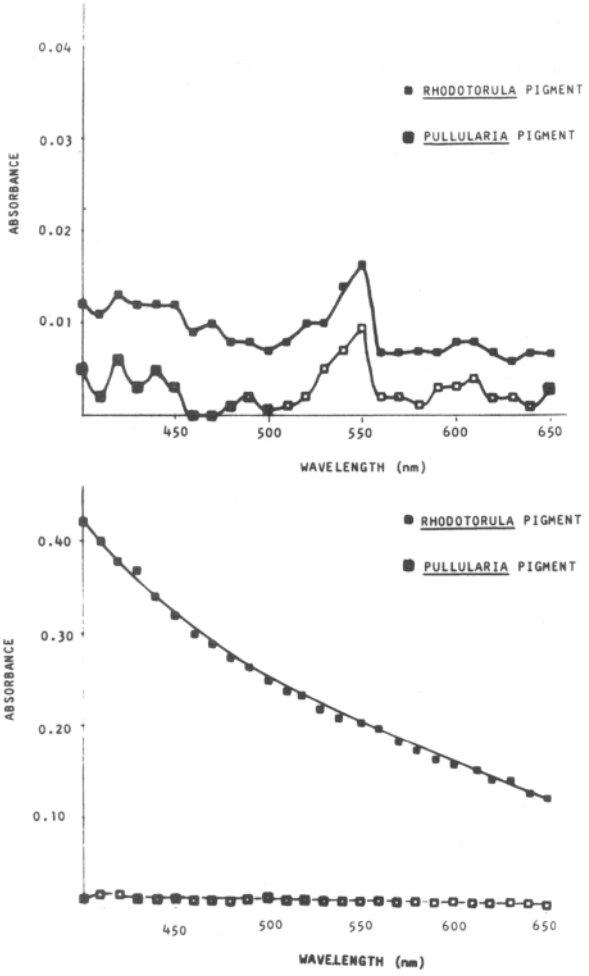

Fig. 1. Visible adsorption spectra of the yeast pigments in hexane (upper curves) and in carbon disulfide, lower curves. Concentrations were 1.0 mg Rhodotorula pigment in either 3.0 ml carbon disulfide or 5.0 ml of hexane. The concentration of the Pullularia pigment was 1.5 mg in 3.0 ml carbon disulfide and 1.4 mg in 5.0 ml hexane.

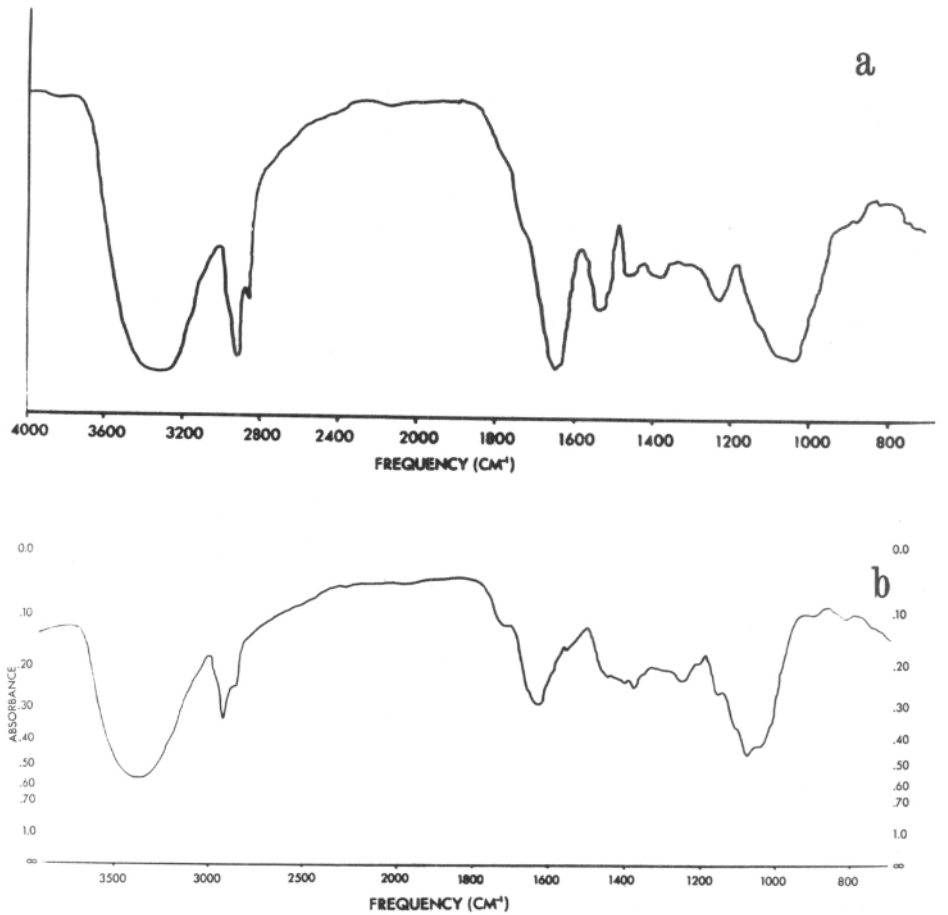

Fig. 2 Infrared spectra of Pullularia and Rhodotorula pigments.
Fig. a is 1.5 mg Rhodotorula pigment in 200 mg KBr.
Fig. b is 1.0 mg Pullularia pigment in 200 mg KBr.

DISCUSSION

Solubility behavior of the pigments indicated that these materials were not readily soluble, were chemically unreactive, and perhaps high molecular weight or polymeric materials containing acidic and basic functional groups.

Although both pigments were completely insoluble in water, they were slightly soluble in aqueous solutions of acid and base. The Pullulariapigment behaved as an indicator. Addition of base to an aqueous solution of the pigment produced a greenish coloration, and on acidification the solution changed to red. The fact that this pigment behaved as an indicator is an interesting phenomenon that has also been observed by earlier workers (Merdinger, 1964 and Ciegler and Wickerham, personal communication). In marked contrast, the Rhodotorula pigment did not behave as an indicator, remaining red throughout the testing. Neither pigment gave blue-green colors in concentrated sulfuric acid as do certain carotenoids (Florkin and Stotz, 1962).

The Rhodotorula pigment appeared to be significantly more soluble in the common organic solvents tested than the Pullularia pigment which was only slightly soluble in acetone with no color being extracted. Color was extracted from the Rhodotorula pigment in all the organic solvents tested except for acetone. In benzene and butanol the pigment appeared to be insoluble.

Carbohydrate appeared to be an integral part of the pigments. These findings agree with those of Ciegler and Wickerham (personal communication) who also found sugar moieties separable from pigment extracts.

Carbohydrate analysis of concentrated pigment hydrolysates indicated that the Rhodotorula pigment had approximately 3.0 per cent carbohydrate as galactose, glucose, mannose, and possibly ribose. While the Pullularia pigment had approximately 0.5 per cent carbohydrate as glucose. Whether this carbohydrate occurs as mono, di, or polysaccharide has still to be established for each pigment.

Hydrolysis data suggest that protein may be closely associated with the pigments. The protein moieties associated with the chromophores of melanin and the Pullularia pigment appear to be more protected from acid reagent and may consist of more stable bonds than that of the Rhodotorula pigment, which was easily hydrolyzable. Approximately 100 per cent of the Rhodotorula pigment was hydrolyzed in 24 h whereas 65 per cent of melanin was hydrolyzed in 168 h and 84 per cent of the Pullularia pigment was hydrolyzed in 120 h.

The yeast pigments produced plain absorption curves without specific absorption maxima or peak intensities in hexane and carbon disulfide similar to melanin. Comparison of these spectra with the absorption peaks of carotenoids (Florkin and Stotz, 1962) indicated that the pigments did not have any of the maxima characteristic of carotenoids.

The infrared spectra of the Pullularia and Rhodotorula pigments were similar to each other. There was no change in these even after the pigments were extensively washed or acid treated. The infrared spectra of the pigments showed some characteristic bands. A characteristic absorption which could be attributed to the amine or aromatic hydroxy bond (at about 3300 cm^{-1}), alkane C-H stretching (at about 2920-2840 cm^{-1}), a carbonyl group (at about 1625 cm^{-1}), O-H bending or C-N vibration or C-O stretching vibration (at about 1080 cm^{-1}), and indication of aromaticity between 1500-1400 cm^{-1}).

The pigments were in the solid phase, and there was a considerable amount of line brading and overlapping in the spectra which precluded a detailed interpretation. Comparison of these spectra with those of natural and synthetic melanins showed the pigment spectra to be distinct from those of melanins showed the pigment spectra to be distinct from those of melanins.

Although additional data are needed (especially data of carbohydrate and protein analyses), the spectral results however suggest that the Pullularia and Rhodotorula pigments are closely related structures. Carbohydrate and protein appear to be an integral part of both pigment molecules, however, each are distinct in having amino acid compositions of any typical protein whereas melanin reflects its probable biosynthetic origin with its 63% tyrosine content. In this respect both pigments are unique from melanin.

The implications of this study seem in accord with Merdinger (1964) who reported the conditions for formation of pigment by Pullularia pullulans. He indicated that the pigment did not appear to be identical with melanin. This is in accord with the findings of Bender et al. (1959) and Durell (1968), but in clear distinction to the report of Lingappa et al. (1963) who claimed the black pigment was melanin. The data presented here clearly rule out melanin as the pigment in Aureobasidium pullulans. Also it is clear that the Rhodotorula pigment is not a carotene.

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