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EXPERIMENTAL ARTICLES

Pigmented Basidiomycetous Yeasts Are a Promising Source of Carotenoids and Ubiquinone Q_{10}

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Abstract—Strains of basidiomycetous yeasts isolated from different sources were studied in order to determine the content of carotenoid pigments and ubiquinone Q_{10} for subsequent selection work to obtain producers of these substances. The high specific productivity of carotenoids (600–700 mg/g) was revealed in the representatives of the following species: *Cystofilobasidium capitatum, Rhodosporidium diobovatum, R. sphaerocarpum, Rhodotorula glutinis, Rh. minuta*, and *Sporobolomyces roseus.* The ratio of the major pigments (torulene, torularhodine, and β-carotene) in the representatives of different species was studied. Certain specific features of pigment formation in relation to the taxonomic position of the yeasts were determined. Eurybiont species with substantial ecological lability are the most active producers of carotenoids and ubiquinone Q_{10} among the epiphytes. It is the first time a comparative analysis of the coenzyme Q_{10} content in different taxa has been performed using several strains of the same species. The maximal coenzyme Q_{10} production (1.84 mg/g of dry biomass) was found in the yeast species *R. sphaerocarpum.*

Key words: yeasts, carotenoid pigments, coenzyme Q_{10}, ecological lability.

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Carotenoid pigments are common to a broad range of organisms. They frequently occur in plants, animals, and microorganisms. The chemical structure of carotenoids (the presence of double bonds) determines specific properties of these compounds. Being strong antioxidants, carotenoids perform the function of quenching free radicals in many organisms; in some bacteria, carotenoids are also involved in photosynthesis. Carotenoids are actively used in agriculture (e. g., as pigments in an aquiculture and for staining egg yolks), as well as in medicine in the treatment of certain types of cancer, atherosclerosis, and coronary artery disease.

Pigmented yeasts are an interesting subject from the biotechnological point of view. As a rule, these yeasts have a broad spectrum of utilization of different carbon sources and grow well at high temperatures, forming a large biomass [1, 2].

Except for the anamorphous stage of the archiascomycete yeasts of the genera *Taphrina* and *Protomyces*, all known pigmented yeasts are related to three classes of basidiomycetes. Among pigmented yeasts, the representatives of only some small taxonomic groups have been investigated for the carotenoid pigment content. Along with the most known producer *Phaffia rhodozyma*, there is evidence of the capacity for carotene formation by other well-known pigmented yeasts of the genus *Rhodotorula* (order *Sporidiobolales*) [3, 4]. The composition and amount of the carotenoid pigments in numerous natural isolates of the genera *Rhodotorula/Rhodosporium* and *Sporobolomyces/Sporidiobolus* were studied in sufficient detail in the 1970s [1]. Unfortunately, accurate interpretation and application of these data in respect to the modern concept of the taxonomy of basidiomycetous yeasts is not possible. Moreover, some data exist of the amount and composition of carotenoid pigments in the representatives of the genera *Cryptococcus* (*Tremellales*) [5], *Cystofilobasidium* [6], and *Dioszegia* [7].

Apart from pigments, coenzyme Q_{10} is also an interesting product for biotechnology. This compound is widely used in medicine and cosmetology, and demand for it is constantly increasing. In yeasts, different kinds of ubiquinones occur: Q_5–Q_{10} [8]. Comparative analysis of the coenzyme Q_{10} content in basidiomycetous yeasts of different taxa involving several strains of different origin for each species has not been previously performed.

The aim of this study was to search for new producer strains of carotenoids and coenzyme Q_{10}, which are promising for further selection work.

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MATERIALS AND METHODS

Cultures. More than 90 yeast strains of basidiomycetous affinity from theAll-Russia Collection of Industrial Microorganisms (VKPM) and the Collection of the Soil Biology Department, Moscow State University (KBP) were studied for their carotenoid pigment and ubiquinone Q_{10} content. Both the strains stored in the collection for a long period of time and those isolated in the course of recent investigations were used in the work [9, 10]. The species which, according to the data in the literature, were expected to contain at least one of the target compounds were selected for screening. This category included the following yeasts: *Dioszegia hungarica* VKPM Y-3208, Y-3209; *Cryptococcus aerius* Y-2143, Y-2146, Y-2148; *Cr. laurentii* Y-229, Y-1974, Y-2150, Y-2151; *Cr. magnus* Y-2716, Y-2764, Y-2765, KBP 3836; *Cr. victoriae* Y-3203, KBP 3838, KBP 3839; *Cystofilobasidium capitatum* Y-3199, Y-3201, Y-3202; *Rhodosporidium babjevae* Y-996, Y-1565, Y-1566; *R. diobovatum* Y-305, Y-2235, Y-2450, Y-1001, Y-1561, Y-1562, Y-1564, Y-2419, Y-2420; *R. sphaerocarpum* Y-1559, Y-1560; *Rhodotorula aurantiaca* Y-985, Y-986, Y-2208; *Rh. glutinis* Y-30, Y-32, Y-77, Y-78, Y-80–Y-82, Y-358, Y-608, Y-689, Y-993, Y-994, Y-1051, Y-1724, Y-1760–Y-1762, Y-1930, Y-1944, Y-1949; *Rh. minuta* Y-983, Y-999, Y-2771–Y-2777, Y-2780–Y-2788; *Rh. mucilaginosa* Y-706; *Leucosporidiella muscorum* Y-2789–Y-2791; *Sporidiobolus salmonicolor* Y-2804, Y-2805; *Sporobolomyces pararoseus* Y-2799, Y-2801, Y-2802; and *Sp. roseus* Y-1599, Y-1804, Y-1805, Y-1810–Y-1812, Y-1814.

Cultivation. When carotenoids were studied, the yeasts were grown in a liquid medium of the following composition (g/l): glucose, 110; yeast extract, 2.0; peptone, 1.3; KH_2PO_4, 0.2; K_2HPO_4, 0.08; $MgSO_4$, 0.2; $(NH_4)_2SO_4$, 0.4; NaCl, 0.04; $CaCl_2$, 0.02. For the quantitative determination of ubiquinone, the yeasts were cultivated in a richer medium, with a composition promoting more active biomass growth (g/l): glucose, 110; peptone, 10; yeast extract, 5; and a mixture of trace elements. The flasks were inoculated with a 72-h yeast culture (20 ml of the medium in a 750-ml flask), grown for six days at 17°C on a rotary shaker at 260 rpm.

Isolation of the target compounds. Since some of the yeast strains studied contained a large amount of extracellular polysaccharides, the biomass was separated by centrifugation (1 min, 13400 *g*) and washed twice with distilled water (500 μl; 1.5 min, 12000 *g*). A portion of wet biomass (100 mg) was placed into a 2-ml test tube; 200 μl of glass beads (400–600 μm in diameter) and 200 μl of acetone were added. The test tubes were shaken at 2500 rpm in a vortex for 10–15 min; the extract was separated by centrifugation (1 min, 13400 *g*). The extraction procedure was repeated several times. The first extract was discarded, because it contained traces of water and exogenous polysaccharides and did not actually contain any carotenoids or coenzyme Q_{10}. The second, third, and fourth extracts were pooled and collected into 1.5-ml test tubes for subsequent analysis. In all the cases, sequential extraction of the carotenoid compounds was performed until the biomass became colorless. The extracts for ubiquinone determination were vacuum-concentrated to a volume of 50 μl at room temperature.

For quantitative calculations, the biomass moisture content was determined by lyophilic drying the weighed wet biomass portion obtained from 1 ml of a liquid culture.

Quantitative determination of the compounds. Total carotenoids was determined on a Thermo Spectronic (United States) spectrophotometer at two wave lengths corresponding to the β-carotene and torulene absorption maxima (452 and 460 nm, respectively). The measurements were carried out at the optical density values corresponding to the linear part of the calibration graph. For the calculations, the extinction coefficient values for these compounds (β-carotene, 25.6; torulene, 23.15) were used [11].

HPLC was used to determine the amount and spectrum of the main carotenoid pigments in the representatives of the taxonomic groups with the highest values of total carotenoids [6].

The quantitative determination of coenzyme Q_{10} was carried out using the method of thin-layer chromatography on glass plates (Merck, Switzerland). Vacuum-concentrated extracts were applied with a microsyringe (Gazokhrom 101, Russia) using the "dotting" technique. The standard ubiquinone concentrations (Coenzyme Q_{10}, reduced form, Sigma) were applied on the plate nearby. The separation was carried out in a glass chromatographic chamber using the following mixture as the mobile phase: hexane, 81; acetone, 6; methanol, 13 (parts by volume). The mobile phase used for highly pigmented extracts had a different composition: hexane, 95; acetone, 5 (parts by volume).

The plate was placed into the saturated chromatographic chamber. After 8–9 min, when the length of solvent front run was 75.0 ± 5.0 mm, the chromatogram was taken out and dried at room temperature for 5 min. The spots of coenzyme Q_{10} were found to be stable in the chromatogram for 75 min. Visualization was carried out with the gel documentation system (UVP, United States) with external UV illumination at 254 nm. The image was photographed. The amount of ubiquinone was determined by the intensity of the spot coloration using the Photoshop 6.0 (Adobe Systems, Inc., United States) program in the grayscale (8 bit) color scheme. The calibration graph was constructed using the standard concentrations, and the coenzyme Q_{10} content was determined in the experimental extracts. In order to calculate the average values for each taxon, the threshold value detectable by this method (0.01 mg/g, considering the weight and dilution of the samples), rather than zero, was assigned to the samples in which ubiquinone concentration could not be determined.

Table 1. Carotenoid content and biomass yield in the yeast species studied

Taxonomic group	Species	Number of strains studied	Dry biomass, g/l (minimal/maximal)	Carotenoids, mg/g (minimal/average/maximal)
Filobasidiales	*Cr. aerius*	3	15.0–16.5	0.00
	Cr. magnus	3	15.2–20.6	0.00/0.04/0.14
Tremellales	*Cr. laurentii*	4	15.0–17.4	0.00
	Cr. victoriae	3	24.3–27.3	0.09/0.10/0.10
	D. hungarica	2	35.3–36.5	0.20/0.21/0.22
Cystofilobasidiales	*Cys. capitatum*	3	30.3–34.2	0.15/0.44/0.70
Leucosporidiales	*Leu. muscorum*	3	25.5–26.0	0.00
Sporidiobolales	*Rh. aurantiaca*	3	16.7–26.0	0.10/0.18/0.31
	Rh. mucilaginosa	1	26.4	0.23
	Rh. glutinis	21	20.7–38.2	0.05/0.20/0.56
	R. babjevae	3	26.3–33.1	0.25/0.28/0.32
	R. diobovatum	11	33.8–38.6	0.09/0.33/0.63
	R. sphaerocarpum	2	27.0–27.2	0.31/0.49/0.76
	Sp. pararoseus	3	26.3–27.3	0.09/0.10/0.11
	Sp. roseus	7	29.6–37.5	0.09/0.23/0.43
	S. salmonicolor	2	20.1–20.5	0.05/0.06/0.08
Naohidea-Minuta	*Rh. minuta*	18	18.7–36.3	0.04/0.15/0.53

RESULTS AND DISCUSSION

The value of the total carotenoids varied significantly in all the taxa studied (Table 1). The maximal values of over 0.6 mg/g of dry biomass (calculated by β-carotene) and were detected in the representatives of the species *Cys. capitatum, R. sphaerocarpum, R. diobovatum,* and *Rh. glutinis*. These species and those closely related to them are the typical epiphytes and are frequently isolated from different plant material [1, 12–15]. A slightly lower total carotenoid value (about 0.5 mg/g of dry biomass) was typical of the strains of *Sp. roseus*, the other well-known species of epiphytic ballistospore-producing yeasts.

It is noteworthy that among the red basidiomycetes of the genus *Rhodotorula*, the total carotenoid pigment value was reliably higher in the representatives of *Sporidiobolales* than in the *Naohidea–Rhodotorula minuta* clade (0.25 and 0.15 mg/g, respectively; $F = 196.4$; $p < 0.001$). Moreover, the latter accumulated a noticeably smaller biomass. The dimorphous yeasts assigned to this clade are related to the phytopathogenic fungi of the genera *Occultifur* and *Naohidea*; they possibly require specific growth factors. In contrast, the eurybiontic yeasts of the order *Sporidiobolales* are more labile and able to develop effectively under different ecological conditions.

The teleomorphous genus *Cystofilobasidium* has long been known among pigmented yeast fungi. However, the content of carotenoid pigments in these yeasts was analyzed only once, practically simultaneously with our investigation [6]. We observed high values of the total carotenoids in these yeasts; the highest value was 0.7 mg/g of dry biomass. This is the highest value for the yeast strains used in this study.

In the anamorphous yeasts of the genus *Cryptococcus*, carotenoid pigments are present in significantly lower amounts. *Tremellales* cryptococci (*Cr. laurentii, Cr. victoriae*) were, on average, characterized by greater productivity than *Filobasidiales* ones (*Cr. magnus, Cr. aerius*). The highest values were found in the widespread species *Cr. victoriae* (0.14 mg/g). In the species *Cr. laurentii*, which is not closely related to it, the level was significantly lower.

Another species of *Tremellales* yeasts investigated was *Dioszegia hungarica* (syn. *Bullera armeniaca, Cryptococcus hungaricus*). These pigmented ballistospore yeasts have been isolated from the phylloplane samples in various regions of the world. The closely related yeast identified as *D. takashimae* has recently been studied for pigment composition [7]. Its total carotenoid pigment content was comparable to the maximal values revealed for *Tremellales* yeasts: 0.2 mg/g of dry biomass.

Pigmented yeasts occur in a variety of natural substrates: plant, soil, and aquatic. The presence of pigments in these yeasts is conventionally explained as a protective reaction against the effect of solar radiation [8]. However, no data exist concerning the relation between the level of the pigments accumulated within cells of yeast species and their habitat and the degree of insolation. Among the natural isolates selected by us were those isolated from the most contrast substrates:

Table 2. Ratio of the main carotenoid pigments in some yeast species

Taxon	Average share of pigments in extract, %		
	Torulene	Torularhodine	β-carotene
Sp. roseus	55	38	7
R. sphaerocarpum	18	4	78
R. diobovatum	84	11	5
Rh. minuta	81	10	9
Rh. glutinis	63	4	33
Cys. capitatum	17	20	63

soil and the phillosphere. To evaluate the influence of the initial substrate on the total content of carotenoid pigments, we performed a factor analysis ANOVA, with the total content of carotenoid pigments calculated from the maxima of torulene (460 nm) and β-carotene (452 nm) absorption as the dependent variable, and substrate (soil or the phyllosphere) as the grouping variable. The strains isolated from soil and plant substrates differed significantly in their carotenoid level ($F = 6.03$; $p < 0.0001$). The selective conditions of the phyllosphere environment probably favor the selection of strains with a more pronounced capacity for the synthesis of photoprotectors, such as carotenoids. The role of the level of pigmentation as an adaptive reaction to environmental conditions requires more detailed consideration and additional experiments.

The distribution of the value of total carotenoid pigments was different for the representatives of different taxonomic groups. Among the representatives of *Sporidiobolales* and *Cystofilobasidiales*, the most frequent occurrence was noted for the average total carotenoid values (normal distribution). Tremelloid yeasts and the species of the *Naohidea–Rhodotorula minuta* clade were characterized by a lognormal distribution with the maximum probability at low values of total carotenoid pigments. To put it differently, the strains with high carotenoid production more likely to occur among the representatives of *Sporidiobolales* and *Cystofilobasidiales*.

It was shown that β-carotene, torulene, and torularhodine are the main carotenoid pigments contained in yeasts [1, 4, 12]. However, the ratio of these pigments has not yet been studied in detail for the representatives of most taxa.

In order to determine the carotenoid pigment ratio in the extract, strains from different taxonomic groups were chosen which had relatively high levels of total carotenoids (for their taxa). Apart from these criteria, special attention was given to little-studied species which may appear to be potential producers of pigmented compounds. A total of 11 yeast strains were analyzed: *Rh. glutinis* VKPM Y-358; *Rh. minuta* VKPM Y-983; *R. diobovatum* VKPM Y-305; *R. sphaerocarpum* VKPM Y-1559, Y-1560; *Sp. roseus* VKPM Y-1811, Y-1812; *D. hungarica* VKPM Y-3208, Y-3209; *Cr. victoriae* VKPM Y-3203; and *Cys. capitatum* VKPM Y-3202.

The main pigments in most of the yeast species studied are carotene, torulene, and torularhodine. They account for a total of 90% of all the compounds revealed in the extracts.

Although the ratio of these pigments varied in the strains of the same species, certain patterns can be established (Table 2). The yeasts identified as the representatives of the *Rh. glutinis* sensu lato group [12] tend to accumulate predominantly torulene (60–80%), while the β-carotene content varied between 5 and 35%. This group is the best studied one in the search of microorganisms which are the potential pigment producers: a similar carotenoid ratio was described at a qualitative and semiquantitative level [4, 16–18]. In the *Rh. minuta* species, torulene was the predominant pigment; these yeasts are very similar to *R. diobovatum* in the pigment ratio. Among the previously unstudied *Sporidiobolales* species, *Sp. roseus* produce similar amounts of torulene and torularhodine. The yeasts of the teleomorph species *R. sphaerocarpum* synthesize predominantly β-carotene (up to 78%). Moreover, the strains of these species accumulate considerable biomass, which allows them to be considered as potential producers, especially *R. sphaerocarpum.*

In contrast to the rest of the yeasts, the dimorphic basidiomycetes of the species *Dioszegia hungarica* were found to synthesize only one specific pigment. Earlier, selective synthesis of the rare pigment plectaniaxanthin (1',2'-dihydroxylated monocyclic carotenoid) was described for a representative of this genus, *D. takashimae* [7]. The authors indicate that the detection of this compound is of interest, because it confirms the taxonomic isolation of the *Dioszegia* clade. The data on the carotenoid composition in other tremelloid yeasts have been extremely scanty up to now. There is only evidence of the capacity of *Cr. flavescens* (syn. *Cr. laurentii* var. *flavescens*) to synthesize a spectrum of various pigmented compounds [5]. The analyzed *Tremellales* cryptococcus *Cryptococcus victoriae* (not related to *Cr. flavescens*) synthesized, apart from torulene (14%), torularhodine (12.5%), and β-carotene (6%), a large amount of other carotenoids (a total of over 60%), which we did not succeed in identifying due to the absence of standards. However, plectaniaxanthin was not detected among these substances. Thus, our investigation provides additional confirmation of the aforementioned specific characteristics of the yeasts of the *Dioszegia* subclade, namely, their ability to produce one predominant pigment plectaniaxanthin (>95%), whereas other *Tremellales* yeasts are capable of synthesizing a spectrum of various carotenoid compounds.

No comparative studies of the ubiquinone Q_{10} content in different species of pigmented yeasts have previously been conducted [19]. Most of the strains studied had a low coenzyme Q_{10} content in the biomass,

Table 3. Coenzyme Q_{10} content in the yeasts studied

Taxon	Number of strains*	Coenzyme Q_{10} content, mg/g	
		Average**	Maximum
R. sphaerocarpum	2/2	0.92	1.84
Cr. magnus	2/4	0.59	1.18
Sp. roseus	3/7	0.25	0.72
Leu. muscorum	1/3	0.44	0.44
Rh. minuta	5/18	0.20	0.40
R. diobovatum	2/11	0.18	0.29
Rh. glutinis	7/21	0.09	0.22

Notes: * The numerator denotes the number of strains in which we succeeded in determining the coenzyme Q_{10} content; the denominator denotes the total number of strains.

** The average value is calculated for the strains in which we succeeded in determining the coenzymeQ_{10} content.

which was lower than the detectable level of 0.1 μg/g of dry biomass. The highest value was recorded for *R. sphaerocarpum* VKPM Y-1559 (1.85 μ/g), which also exhibited high values of total carotenoid pigments. The average and maximal ubiquinone content values in different yeast species are presented in Table 3.

As in the case with the carotenoid pigments, the most active coenzyme Q_{10} producers are the eurybiontic species with high ecological lability. It is noteworthy that the nonpigmented species *Leucosporidiella muscorum* (syn. *Rh. muscorum*), which is the anamorph of the teleomorphic genus *Leucosporidium* widespread in the boreal zone, belongs to this group.

Among the representatives of widespread pigmented species, we found the strains actively synthesizing carotenoid pigments and (or) ubiquinone. The only exception was the eurybiont species *Cr. magnus*, which was characterized by very weak pigment production and high coenzyme Q_{10} content (1.2 mg/g).

The results obtained for the species represented by a large number of strains demonstrate that the coenzyme Q_{10} content values are distributed in a similar way: about 50% of the strains have a level exceeding the minimally determined content (0.01 mg/g), of which about 50% exhibit the maximal values for a given species.

Application of the results of our screening enabled us to reveal new species which may be regarded as potential β-carotene producers: *Cys. capitatum* and *R. sphaerocarpum*. Strains which have a high content of both carotenoid pigment and coenzyme Q_{10} were found: the representatives of the species *R. sphaerocarpum* and *Sp. roseus*. The yeast species *R. diobovatum* and *Rh. glutinis* are often regarded as carotenoid producers; they had a low ubiquinone Q_{10} content. It was noted that the level of carotenoid pigments production was not a species-specific feature [6, 20] and varied significantly in different strains. At the same time, using the large data experimental array of, we revealed the correlation between the total carotenoid content values and the taxonomic position of the isolates at a level exceeding the species level.

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