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BIOSYNTHETIC PRODUCTION OF CAROTENOIDS USING YEAST STRAINS OF GENUS *RHODOTORULA* ON THE CHEAP BEER WORT SUBSTRATE

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ABSTRACT

Carotenoids having primary significance for animal husbandry, medicine and food industry are objects of studies to optimize their production conditions and to establish their complex role in living systems. Synthesis of carotenoids by microorganisms is preferential for industrial use due to their natural origin and possibility of combining production with waste disposal. Native microbial strains are the basis for selecting superproducers of organic pigments. In this study, the red yeasts of *Rhodotorula* genus were incubated in beer wort medium at initial cultural parameters (sugar concentration 6 degrees Balling, temperature 26°C, stirring rate 200 rpm, 4 days of fermentation). Analysis of biomass and carotenoid production by *Rhodotorula* yeast was performed under defined conditions. Two strains of *R. glutinis* BIM Y-253 and BIM Y-158 synthesizing over 100 µg carotenoids per 1 g biomass, the mark corresponding to the average level of production, were selected. The concentration of carotenoids generated by strain *R. glutinis* BIM Y-253 in the nutrient medium exceeded 1 mg / l. Screening of red yeast for the types of synthesized carotenoids showed predominance of torulene- and torularhodin-like compounds in isolated total pigment fractions. At the following experimental stage the effect of organic supplements on production of biomass and carotenoids by the selected strain *R. glutinis* BIM Y-253 was studied. Yeast incubation with 0.05% malic acid – the substrate for final reaction of TCA cycle, and fine grinding of biomass resulted in average carotenoid yield 3,5 mg/l. Our findings demonstrate the potential carotenoid productivity of red yeast at the initial values of incubation parameters, and illustrate the impact of organic compounds on biosynthetic efficiency of native yeast culture.

Keywords: Red yeast, *Rhodotorula*, carotenoids, natural substrate, microbial synthesis

INTRODUCTION

Carotenoids are lipophilic tetraterpenoid compounds showing red, orange or yellow pigmentation due to intense absorbance in the visible spectral area. To date isolated natural carotenoids embrace over 700 distinct molecules. Two major types of carotenoids are hydrocarbons $C_{40}H_{56}$ (carotenes) and oxygen-containing derivatives (xanthophyles). The lack of CH_3, CH_2 and CH groups in the terminal structure is denoted by prefix "nor", while contraction of carbon skeleton is designated as "apo". Carotenoids represented by geometric isomers are readily transformed (often from Z to E) under the impact of temperature, light and other factors, e.g. organic solvents (**Pechinsky and Kuregyan, 2013**). Despite the fact that large variety of carotenoids in the environment complicates their functional specification, carotenoids primarily perform the protective role in the cell. Owing to carotenoids microorganisms may colonize luminous habitats, the pigments are engaged in the mechanism governing resistance to ionizing radiation and promote adaptation to extreme conditions, like hydrogen sulphide atmosphere, geothermal springs, concentrated acid and salt solutions (**Ventosa *et al.*, 2010; Vsevolodov, 1988**). Currently vivid interest to carotenoids is determined by their broad application range as provitamin A and natural colorants in bakery, butter, refreshment drinks, cosmetics; feed premixes in fishery; antioxidants; organic reagents; drugs (**Frengova *et al.*, 2004; Jaswir *et al.*, 2011; Mata-Gómez *et al.*, 2014; Saddozai *et al.*, 2005**).

Such microorganisms as algae, fungi, bacteria, yeast may serve as sources of carotenoids on a par with plants. Some carotenoids of microbial and alternative origin were licensed for legal use in many countries as food and feed additives. For instance, β-carotene of carrot, zigomycete fungi *Blakeslea trispora* and unicellular algae *Dunaliella salina* is applied as alimentary dye (**Nelis and Leenheer, 1990**). Production of carotenoids by microbial synthesis is distinguished by the following advantages: presence in the cell of the sole pigment group (not accompanied by chlorophylles), possibility of incubation in the fermenters, fast and controllable growth rates of microbial culture, supply of inexpensive nutrient substrates (**Frengova and Beshkova, 2009; Latha *et al.*, 2005**). Carotenoid supplements derived from yeast may be admixed as freeze-dried biomass, yet hardly digestible rigid cell walls of yeast species (e.g. *Phaffia rhodozyma*) urge technologists to resort to preliminary cell disintegration (**An and Choi, 2003**). The promising agents for biotechnological synthesis of carotenoids are red yeast of *Rhodotorula* genus. Investigations are focused on this microbial group to evaluate economic prospects of pigment production biotechnology. The major types of *Rhodotorula* yeast carotenoids are α-, β-, γ-carotenes, torulene, torularhodin, lycopene (**Bogdanovskaya *et al.*, 1978**), with some substances possessing remarkable practical and commercial value. Noteworthy, that carotene and lycopene have been introduced on a large scale in food industry (bioadditive E160 was approved in European community, Russia, Eastern Europe (**Matthäus *et al.*, 2014**). Aim of this study was to investigate capacity to synthesize carotenoids on beer wort nutrient medium in 22 strains of red yeast deposited at Belarusian collection of non-pathogenic microorganisms. Brewery wort is alcohol fermentation intermediate of relatively low cost. Research objectives included assessment of biomass and carotenoid production by tested yeast strains and their differentiation in the spectrum of generated pigments, as well as analysis of effect of organic compound supply into the medium on biomass and carotenoid productivity.

MATERIAL AND METHODS

Yeast strains

Twenty-two yeast strains of genus *Rhodotorula*, belonging to the species: *R. glutinis*, *R. lactosa*, *R. minuta*, *R. aurantiaca*, *R. mucilaginosa* and *R. rubra* provided from the stock of the Belarusian collection of non-pathogenic microorganisms were used in this study. A list of tested strains is presented in Table 1. The "red" yeast usually colonize the terrestrial, aqueous and air habitats. The collection strains were isolated from samples of soil, high moss peat (*R. glutinis*), water (*R. minuta*), air (*R. mucilaginosa, R. lactosa*).

Table 1 List of yeast strains of genus *Rhodotorula* deposited at Belarusian collection of non-pathogenic microorganisms

Species and the BIM number		
R. glutinis BIM Y -10	*R. lactosa* BIM Y -118	*R. mucilaginosa* BIM Y -161
R. glutinis BIM Y-33	*R. glutinis* BIM Y -138	*R. mucilaginosa* BIM Y -162
R. mucilaginosa BIM Y-47	*R. aurantiaca* BIM Y -156	*R. glutinis* BIM Y -167
R. mucilaginosa BIM Y-50	*R. glutinis* BIM Y -157	*R. glutinis* BIM Y- 243
R. lactosa BIM Y -113	*R. glutinis* BIM Y -158	*R. glutinis* BIM Y- 253
R. minuta BIM Y-114	*R. glutinis* BIM Y -159	*R. mucilaginosa* BIM Y- 254
R. lactose BIM Y -116	*R. glutinis* BIM Y -160	*R. rubra* BIM Y- 268
R. minuta BIM Y -117		

Cultivation of yeast strains of Rhodotorula genus

The yeast cells were incubated in 500 ml flasks containing 200 ml of beer wort (6 ° B) with constant stirring (200 rpm) at 26 ° C for four days, and in 250 ml flasks containing 100 ml of beer wort (6 ° B) with constant stirring (180 rpm) for the experiment with organic additives. The accumulated biomass was freeze-dried.

Recovery of carotenoids and evaluation of their production level

Yeast cell walls were disrupted in a glass mortar, carotenoids were extracted using acetone and petroleum ether (**Rodriguez-Amaya, 2001**).

Analysis of the carotenoid synthetic ability was carried out by express method based on measuring absorbance in the visible wavelength area using spectrophotometer "Solar" UV (PB2201). Pigment production level was calculated as β-carotene via optical absorbance at 450 nm according to the formula (**Sahabi, 2012; Sindhua and Sherief, 2011**):

$$C = \frac{A \times D \times 10^6}{100 \times G \times d \times E}$$

where:
C - Carotenoid concentration, μg/g;
A - Optical density of the solution at 450 nm;
D - Eluate volume, ml;
E - β-carotene extinction coefficient (2590 at 450 nm);
d – Cuvette width (1 cm);
G - Portion of dry biomass collected for carotenoid extraction, g.

Statistics

The presented data sum up results of three experiments. For statistical interpretation of experimental findings confidence range of arithmetic means at 95% significance level was computed. Data processing and analysis was performed using software package Microsoft Excel.

RESULTS AND DISCUSSION

Production of biomass by yeast strains of genus Rhodotorula

Biomass production is illustrated in **Figure 1**. After 3 experimental series the highest level of biomass production (more than 10 g/l) was shown by the yeast strains *R. glutinis* BIM Y-10, *R. mucilaginosa* BIM Y-50, *R. lactosa* BIM Y-118, *R. aurantiaca* BIM Y-156, *R. glutinis* BIM Y-157, *R. mucilaginosa* BIM Y-162, *R. glutinis* BIM Y-167, *R. rubra* BIM Y-268.

Production of carotenoids by yeast strains of genus Rhodotorula

Since yeast strains of genus *Rhodotorula* differ in carotenoid extractivity, it is expedient to consider yields of carotenoids recovered by the afore-mentioned method as a consequence of pigment production and subsequent release from cells. In this study the highest yields of carotenoids (over 80 μg/g) were recorded for the strains *R. glutinis* BIM Y-158 and BIM Y-253, with the peak value achieved in *R. glutinis* BIM Y-253 culture (150.9 ± 78.4 μg/g). Carotenoid production data are presented in **Figure 1**.

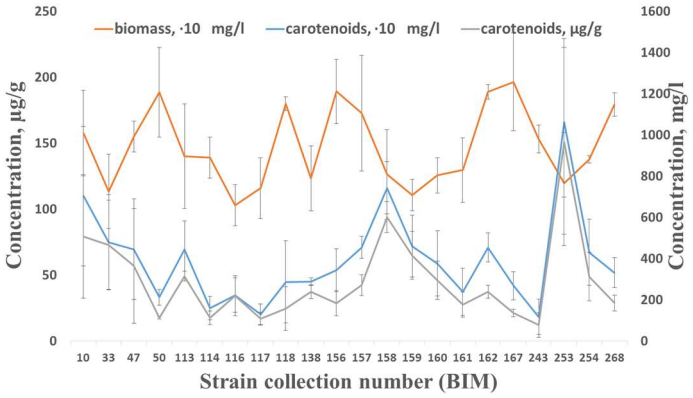

Figure 1 Production of biomass and carotenoids by yeast strains of genus

Rhodotorula

Yeast strains *R. glutinis* BIM Y-158 and BIM Y-253 also showed enhanced biomass and carotenoid production capacity: concentration of the pigments exceeded 0.7 and 1 mg/l, respectively.

The absorbance spectra of total carotenoid fractions

The spectra of yeast pigment fractions demonstrate the presence of typical for carotenoids three peaks of absorbance, with the exception of five strains lacking clear peaks (*R. glutinis* BIM Y-33 and BIM Y-157, *R. lactosa* BIM Y-113, BIM Y-116 and BIM Y-118). Carotenoid spectra of the studied microbial group are heterogeneous in distribution of absorbance maxima, indicating the synthesis of diverse prevalent pigments by yeast strains belonging to genus *Rhodotorula*. According to the revealed absorbance maxima, the examined strains can be divided into several groups presented in **Table 2**. The absorbance spectra of group representatives in the visible region are shown in **Figure 2**.

Table 2 Grouping of *Rhodotorula* yeast strains in accordance with absorbance maxima of intracellular pigments

The absorbance peak values	**458-465/ 485-490/ 516-521**	**424-428/ 456-466/ 487-490**	**437-439/ 463-467/ 485-489**	**460-465/ 487-490**	**459-468/ 477-490**
BIM collection strain number	Y-10, Y-47, Y-50, Y-114, Y-117, Y-138, Y-156, Y-158, Y-159, Y-160, Y-161, Y-162, Y-167, Y-243, Y-253, Y-254, Y-268	Y-33, Y-116	Y-113	Y-157	Y-118

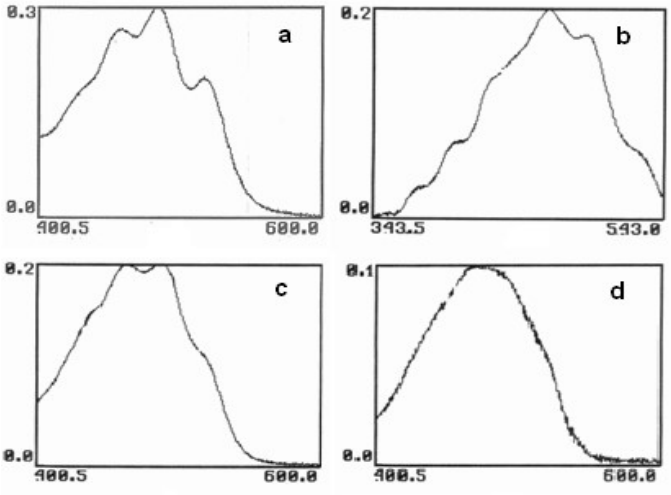

Figure 2 The spectrum of total carotenoids extracted from the cultures: a) *R. glutinis* BIM Y-158 with absorbance maxima at wavelengths 460, 486 and 518 nm; b) *R. glutinis* BIM Y-33 with absorbance maxima at wavelengths 424, 459 and 490 nm; c) *R. lactosa* BIM Y-113 with absorbance maxima at wavelengths 438, 463 and 488 nm; d) *R. lactosa* BIM Y-118 with absorbance maximum at wavelength 468 nm, "shoulder" in the area 477-490 nm.

Effect of organic additives on production of carotenoid-containing biomass by yeast strain R. glutinis BIM Y-253

Biotechnological production of carotenoids envisages supply of various substances to optimize the process. As a rule, the nutrient medium is supplemented with foam-quenching agents (surfactants), antioxidants (citric acid), vitamins (thiamin) and promoters of biosynthesis (β-ionone, 2,6,6-trimethyl-1-acetyl hexane) (**Tikhonov *et al.*, 2008**). Our study examined effect of organic compounds on biomass and carotenoid synthesis by yeast strain *R. glutinis* BIM Y-253 selected as the best producer on beer wort medium. To stimulate carotenogenesis TCA cycle intermediates (citric, fumaric and malic acids in 0.05% concentrations) or seed oil in concentration 500 µl/l were fed into the same medium (6 ° B). Extraction of carotenoids followed vigorous milling of biomass. The results are presented in **Figure 3**.

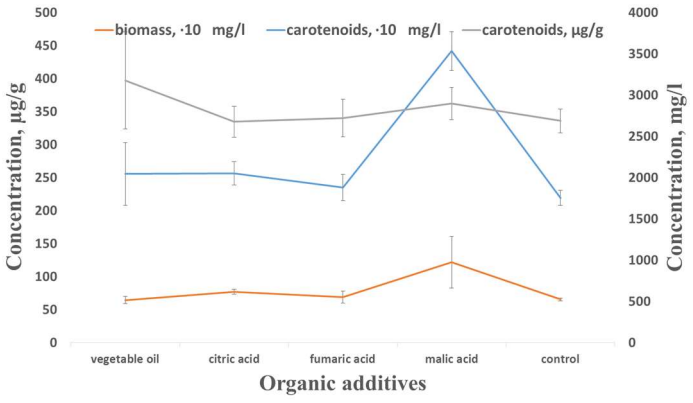

Figure 3 Production of biomass and carotenoids by yeast strain *R. glutinis* BIM Y-253 on beer wort medium enriched with organic acids and vegetable oil

Carotenoid and biomass biosynthetic capacity of strain BIM Y-253 on beer wort medium supplemented with sunflower seed oil, fumaric, citric and malic acids constituted 2.3 mg/l, 1.7 mg/l, 2.1 mg/l, 3.5 mg/l, respectively. Thus, supply of 0.05% malic acid into the medium promotes 2.2 times carotenoid yield as compared to the control variants. Enhanced carotenoid ratio in yeast biomass was recorded after addition of sunflower seed oil (397.22±73.72 µg/g) and malic acid (362.01±24.3 µg/g).

0.05% level of malic acid in the medium proved optimal. Dose-dependent effect of malic acid supply into medium on biomass generation of strain BIM Y-253 was observed, with cell-productivity tending to decline at lower pH values - ≤ 3.0 (**Figure 4**).

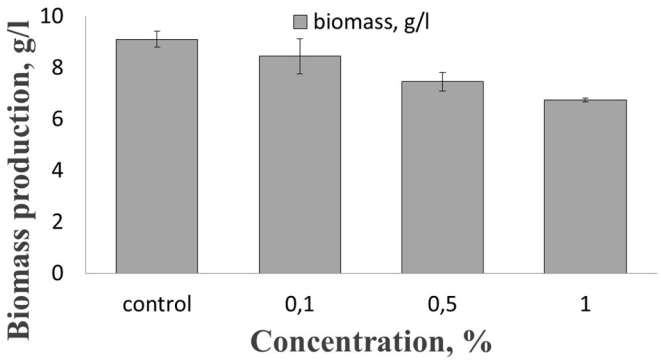

Figure 4 Dose-dependent biomass productivity of yeast strain BIM Y-253 upon supply of malic acid into beer wort nutrient medium

It should be noted that the tested organic compounds did not affect carotenoid spectra.

DISCUSSION

The highest carotenoid yields were attained by the following strains: *R. glutinis* BIM Y-253 (the ultimate productivity was 150.9 ± 78.4 µg/g), and *R. glutinis* BIM Y-158 (94.1 ± 11.7 µg/g), respectively. The results of 4 day yeast incubation in beer wort medium (6 ° B) correspond to the average carotenoid productivity (at least 100 µg/g) of *Rhodotorula* strains. It should be noted that initial values of fermentation parameters, like concentration of the nutrient medium, agitation rate, temperature, incubation time provide for minimal levels of carotenoid productivity. For comparison, the optimum cultivation time for strain *Rhodotorula gracilis* equalled 9 days in preference to tested 3 and 6 days (**Govindaswamy *et al.*, 1999**). It was shown for strain *R. glutinis* that the ratio of cell carotenoids plateaued on day 4 of the culture (**Tinoi *et al.*, 2005**). The maximum growth rate and metabolic activity of *R. glutinis* strains was observed at 29 and 30 ° C (**Mata-Gómez *et al.*, 2014**). Due to the fact that carotenogenesis is an aerobic process, stirring rates can also be increased. In experiments optimizing conditions of carotenoid synthesis by mutant strain *R. glutinis* the effect of medium composition was evaluated at elevated agitation rate - 250 rpm (**Bhosale and Gadre, 2001**). Previous studies on synthesis of carotenoids were conducted on natural substrates as carbon sources, such as grape juice, grape must, peat extract and hydrolyzate, sugar cane juice and molasses, milk whey, corn hydrolyzate (**Mata-Gómez *et al.*, 2014**). For example, when incubated in molasses medium for 3 days at 30 ° C and stirring rate 200 rpm, the strain *R. rubra* generated 2.74 mg/L carotenoids (**Banzatto *et al.*, 2013**). Production of carotenoids by *R. glutinis* on brewery wastes constituted 0.6 mg/L, 1.2 mg/L and 1.0 mg/L (crude wastewater, glucose enriched wastewater and synthetic sugar medium, respectively). In the raw and glucose-upgraded wastewater major carotenoids were represented by torularhodin and β-carotene in approximately equal proportions, whereas torulene, torularhodin and β-carotene prevailed in a synthetic sugar medium (**Schneider *et al.*, 2013**). *R. glutinis* cells displayed different color at temperature 5 ° C and 25 ° C (yellow vs red or pink, respectively). The established distinctions were attributed to high β-carotene and γ-carotene levels in cells at 5 ° C, whereas rising temperatures increased the ratio of torulene and torularhodin (**Bogdanovskaya *et al.*, 1978**).

The selective absorbance of waves in the visible range is a vital diagnostic key used for identification of carotenoids by spectrophotometry in the UV and visible wavelength bands. The absorbance maxima of main carotenoids synthesized by *Rhodotorula* yeast are provided in **Table 3**. Spectral characteristics of carotenoid fractions from 22 yeast strains (presented in **Table 2**) evidence the presence of ranges in the values of absorbance maxima of total carotenoids. This observation may be explained by high lability of terpenoid compounds affecting their absorbing properties. Absorbance maxima of torulene and torularhodin correspond to wavelength peak ranges of the first group of yeast (**Table 2**), the absorbance maximum of β-carotene – to the second group, γ-carotene – to the third group. The inference may be deduced from these findings on the predominant synthesis of specific pigments in different carotenogenic yeasts.

Table 3 Carotenoid absorbance maxima in petroleum ether[a]

Pigment	Absorbance maxima in petroleum ether		
α-carotene	421	445	474
	422	444	473
β-carotene	425	453	484
	425	451	482
	421	451	478
	425	448	475
γ-carotene	437	461	494
	437	462	494
Lycopene	446	472	505
Torulene	460	484	518
Torularhodin	465	492	523

[a]The data are cited from **Kudrytskaya, 1990; Maldonado *et al.*, 2008.**

The following experimental series has revealed promoters of growth and carotenoid synthesis for yeast strain *Rhodotorula glutinis* BIM Y-253. Diverse reports stated stimulating effect of sunflower seed oil and TCA cycle intermediates – citric and malic acids (**Kiritsa, 2005**). In our study the highest stimulating activity was shown by 0.05% malic acid. Noteworthy that malate may serve as direct precursor of pyruvate in process mediated by malic enzyme (**Garay *et al.*, 2014; Zelle *et al.*, 2010**). In turn, pyruvate is located at the junction of major assimilation and dissimilation metabolic pathways (**Zhu *et al.*, 2012**).

CONCLUSION

Screening of 22 yeast strains of genus *Rhodotorula* for carotenoid production capacity allowed to sort out 2 variants with maximal biosynthetic activity referred to species *R. glutinis*. The results shown by these 2 strains match the medium level of carotenoid productivity (above 100 μg/g) among *Rhodotorula* yeast under the set conditions and therefore appear attractive for further upgrading of strain characteristics to raise the level of carotenoid production and to optimize pigment generation parameters. Native microbial cultures limited in carotenoid productivity serve as the basis for selection of pigment hyperproducers (**Emmerstorfer-Augustin *et al.*, 2016; Guo *et al.*, 2015**). For instance, mutagenic treatment of parent strains by nitrosoguanidine, ethyl methanesulfonate, UV irradiation resulted in 2.8-fold rise of synthesized amounts of β-carotene and other carotenoids by modified *R. glutinis* strains (**Cong *et al.*, 2007; Cutzu *et al.*, 2013**). In our study the effect of added organic compounds on biomass and carotenoid productivity of yeast strain *R. glutinis* BIM Y-253 was estimated. 4-day strain incubation in beer wort medium supplemented with 0.05% malic acid at agitation rate 180 rpm in artificial light culture resulted in 3.5 mg/l carotenoid yield, i.e. 2.2 times up the control values. Under experimental conditions the studied 22 yeast strains displayed prevailing synthesis of torulene and torularhodin. So far the latter pigments are inferior to β-carotene in volume of industrial output (**Braunwald, 2013**), yet they possess certain preferential properties, e.g. superior antioxidant activity of torulene due to enhanced number of double bonds in the molecular structure (**Mata-Gómez *et al.*, 2014**). Elucidation of torularhodin role in acquired tolerance of *R. mucilaginosa* to UV-B-exposure led the researchers to assume significant contribution of other molecular mechanism, apart from antioxidant action, in promoting cell survival during UV-B ray treatment (**Moliné *et al.*, 2010**). Discovery of new vital functions of modified carotenoid compounds might spur up motivation in biological production of diverse pigments and enlarge the range of microbial cultures involved in manufacturing technologies.

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