The Influence of the Carbon Source on Torularhodin Pigment Biosynthesis

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The aim of the present work is to study the formation of the intracellular carotenoid pigment - torularhodin with the yeast Rhodotorula rubra ICCF 209 in a culture medium depending on the type and concentration of carbon source.

Keywords: torularhodin, Rhodotorula rubra, carotenoid pigment

Facing the growing economic significance of carotenoids, due to their use as food colorants, nutritional supplements, in cosmetics or in human therapy as antioxidants, much interest has been dedicated to new supplies of this class of pigments [1,2]. In particular, the development of carotenoid-producing bioprocesses is regarded as a competitive solution, as it can provide important quantities of pigments such as torularhodin and β-carotene produced by *Rhodotorula rubra* [3,4] or astaxanthin from *Phaffia rhodozyma* [5,6] without facing the typical problems generated by the weather dependency of the agriculture production.

Recently, raw materials and by-products of agro industrial origin have been proposed as low cost alternative carbohydrate sources for microbial metabolite production. Bacteria, yeasts and fungi are able to synthesize carotenoids, but the pigment of interest torularhodin is only produced by some yeasts cultivated in a rich medium with peptone and yeast extract as nitrogen source and specific carbon sources to be replaced by agricultural by-products [7,8].

The aim of the present work is to study the formation of the intracellular carotenoid pigment - torularhodin with the yeast *Rhodotorula rubra* ICCF 209 in a culture medium depending on the type and concentration of carbon source.

Experimental part

The experiment was carried out in 500 mL flasks, each containing 150 mL culture medium, on a rotary shaker (Gerhardt Laboshake) at 150 rpm, for 7 days, at 30° C, for the pigment formation in the stationary phase of growth curve. The pH was not adjusted during the process.

A general medium composition, finally defined as MS3, was determined by previous research work [9] with the formula: carbon sources to be tested, 1.5 g/L yeast extract, 5 g/L NH_4NO_3, 1 g/L KH_2PO_4, 0.4 g/L $MgSO_4x7H_2O$ and 0.4 g/L NaCl. Trace elements are assumed to be taken from tap water.

The other tested culture medium, M1, has the next composition: 67 g/L glycerol (carbon source), 20 g/L $(NH_4)_2SO_4$, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L Na_2HPO_4, 4 g/L KH_2PO_4 and 0.5 g/L peptone.

A suspension of yeast cells in sterile water is used for the inoculum preparation. Inoculum is analyzed in terms of number of cells / mL.

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On MS3 agar-medium the cells are coral pink, usually smooth, sometimes reticulate and rugose. Microscopic morphology on Olympus U-CMAD 3, 500X shows spherical to elongate budding yeast-like cells or blastoconidia, 2.5-6.5 x 6.5-14.0 μm in size.

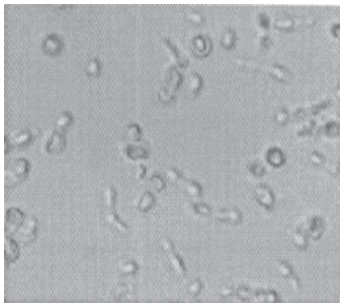


Fig. 1. Microscopic morphology of the *Rhodotorula rubra* ICCF 209 yeast cells

The cells growth is quantified by: OD determination at Λ=600 nm, *p*H, medium dry weight % and cells' nr./ mL measured with a microscopic grid. Dry matter determination was done after biomass separation from the culture medium by centrifugation or by filtration on a filter with 0.45 micrometers pores. Determination of dry biomass was achieved in the oven at 105°C.

After cells' separation by centrifugation three freeze-thaw cycles were performed. Disintegration of cells was enhanced by mechanical treatment with IKA WERKE press. The pigments extraction procedure was done in accordance with the dedicated literature [10,11], comprising acetone extraction of the total pigments content including water soluble types, followed by n-hexane extraction to separate the carotenoid pigments; another extraction with alkaline methanol allowed the torularhodin (the only pigment with acid structure) isolation from the carotenoid pigments mixture. The torularhodin concentration was calculated using the specific absorption coefficient, $E^{1\%}1932$ [12] considering the absorbance values and the dry biomass.

Results and discussions

Cultivation of Rhodotorula rubra in media with glucose and glycerol

This first experiment aimed to study the growth of *Rhodotorula rubra* ICCF 209 cells in MS3 medium, with 4%

glucose as carbon source and NH_4NO_3 as nitrogen source and in the M1 medium with 67 g/L glycerol as carbon source and $(NH_4)_2SO_4$ as nitrogen source.

The results of OD measurements, *p*H, medium d. w. %, and the cell number are presented in figures 2-5.

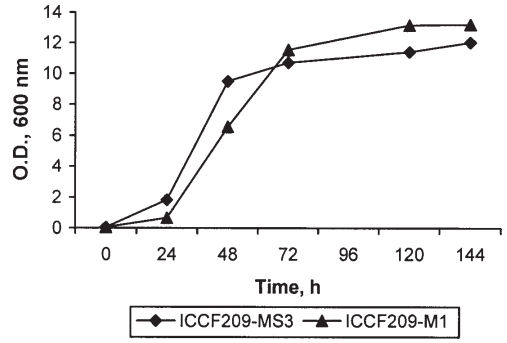


Fig. 2. OD values for the cultures of *Rhodotorula rubra* ICCF 209 in MS3 and M1 media

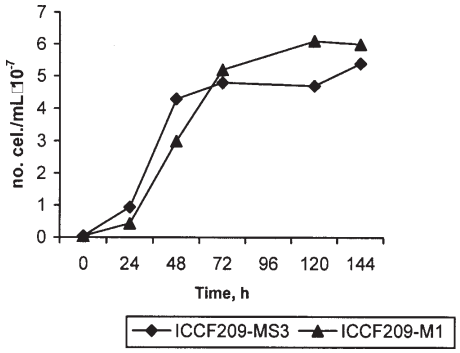


Fig. 3. Cell number/mL values for the cultures of *Rhodotorula rubra* ICCF 209 in MS3 and M1 media

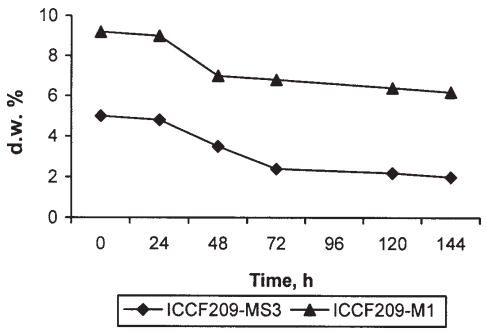


Fig. 4. Consumption of nutrients measured as dry weight medium for the cultures of *Rhodotorula rubra* ICCF 209 in MS3 and M1 media

The variation of measured data during experiments considerably depends on the type of culture medium, MS3 or M1. In the case of the containing glucose medium, OD values are higher by about 14% by comparison with the values reached in the M1 medium with glycerin. It was observed a strong decrease in pH medium, until *p*H=2 for MS3, in which yeast growth is best.

Cultivation of Rhodotorula rubra ICCF 209 yeast in MS3 medium with different carbohydrates as carbon sources

The experiment was achieved to study the influence of different carbohydrates as carbon sources on yeast growth and on carotenoid pigment formation, mainly torularhodin, in the MS3 medium. Moreover the influence of different concentrations of glucose in the same medium was also determined.

There was an important growth of yeast cells in all cases during the first 48 h (exponential phase), excepting the lactose containing medium.

It is possible that the strain *R. rubra* ICCF 209 could synthesize some glycozidases involved in maltose and sucrose hydrolysis, but it can not assimilate lactose. Accumulation of pink-orange pigmented cells began to be observed after the first two days.

Main variables evolutions are presented in the next figures.

The different carbon substrates have a considerable influence on the cell growth and on the carotenoid pigments biosynthesis, particularly torularhodin. The yeast strain growth has been stimulated by these carbon sources: glucose, fructose, sucrose and maltose, but inhibited by lactose. The stationary phase characterized by an important production of carotenoid pigments starts after 48 h and shows a deep decrease of *p*H to acidic values. The concentrations of glucose in the range of 1-6 % did not influence largely the accumulation of cells and the pigment formation.

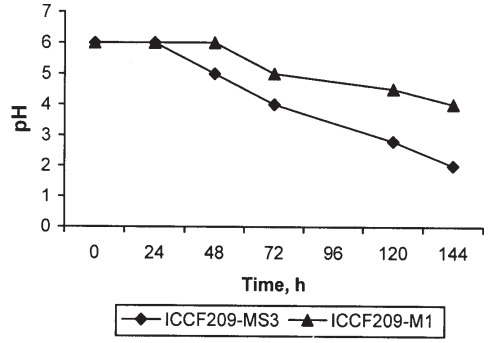


Fig. 5. *p*H values for the cultures of *Rhodotorula rubra* ICCF 209 in MS3 and M1 media

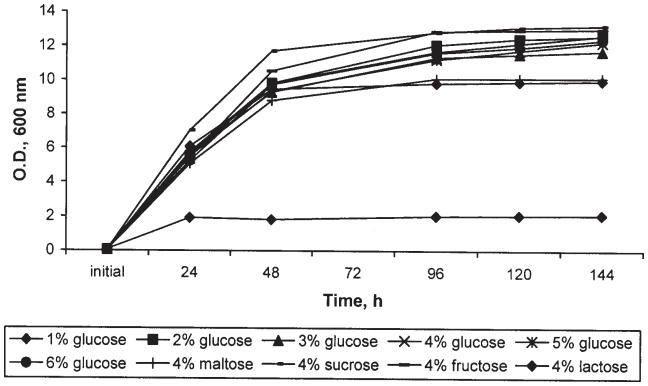


Fig. 6. OD evolution depending on the type of carbohydrates and glucose concentration, for the strain *Rhodotorula rubra* ICCF 209, in MS3 medium

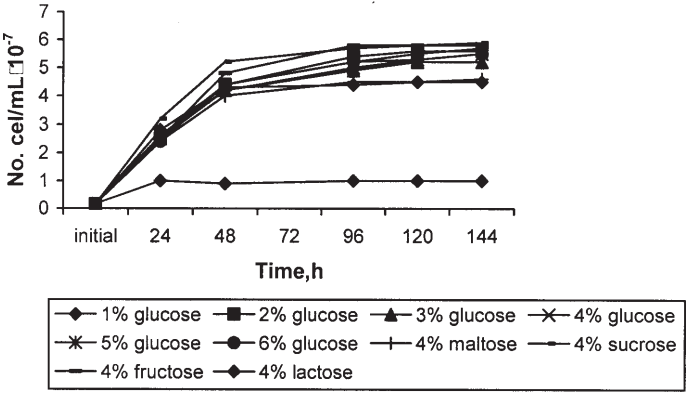


Fig. 7. Cell number evolution depending on the type of carbohydrates and glucose concentration, for the strain *Rhodotorula rubra* ICCF 209, in MS3 medium

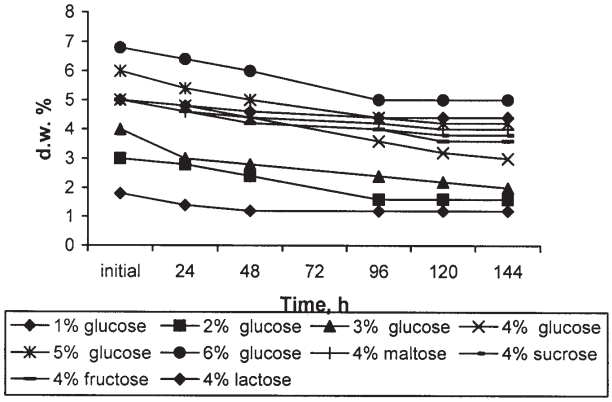


Fig. 8. Dry weight medium evolution depending on the type of carbohydrates and glucose concentration, for the strain *Rhodotorula rubra* ICCF 209, in MS3 medium

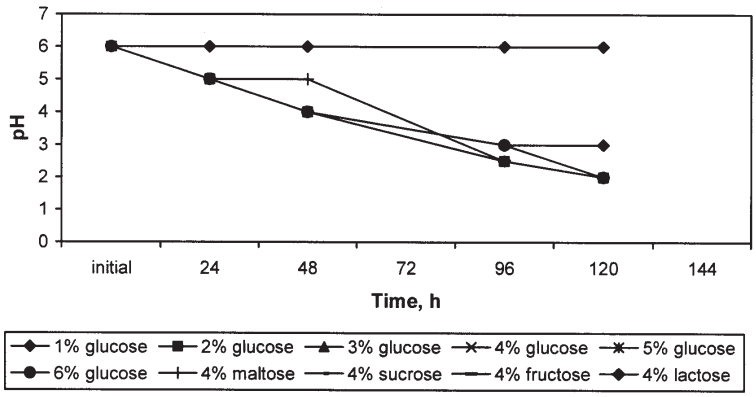


Fig. 9. pH evolution depending on the type on carbohydrates and glucose concentration, for the strain *Rhodotorula rubra* ICCF 209, in MS3 medium

Extraction variant	Dry weight (g%)	Wet biomass in 100 mL medium (g)	Dry biomass in 1000 mL medium (g)
A: 2% glucose	15.65	2.260	3.53
B: 4% glucose	15.41	2.3255	3.57
C: 4% maltose	14.40	2.1956	3.15
D: 4% sucrose	13.88	3.9033	5.41
E: 4% fructose	18.57	2.6705	4.95
F: 4% lactose	-	0.3132	-

Table 1
DETERMINATION OF THE CELLULAR DRY WEIGHT VALUES FOR A-F VARIANTS OF CARBON SOURCES, RHODOTORULA RUBRA ICCF 209 STRAIN

Table 2
DETERMINATION OF THE TORULARHODIN, µg/L AND TORULARHODIN % FROM TOTAL CAROTENOID PIGMENTS AND FOR Á-E VARIANTS OF CARBON SOURCES, *RHODOTORULA RUBRA* ICCF 209 STRAIN

Carbon source	Dry biomass %, w/w	Dry biomass, g/L medium	Abs hexane 1 (452 nm)	Carote-noid pigments as beta caroten, µg/L	Abs hexane 1 (515 nm)	Abs hexane 2 (515 nm)	Torular -hodin, µg/L	Torularhodin, % from total carotenoid pigments
Glucose 2%	15.65	3.53	0.641	288	0.661	0.211	262	90
Glucose 4%	15.41	3.57	0.783	362	0.891	0.342	328	90
Maltose 4%	14.40	3.15	0.307	134	0.334	0.094	53	40
Sucrose 4%	13.88	5.41	0.686	534	0.775	0.275	277	52
Fructose 4%	18.57	4.95	1.296	690	1.511	0.591	407	59

Extraction of total carotenoid pigments and torularhodin separation

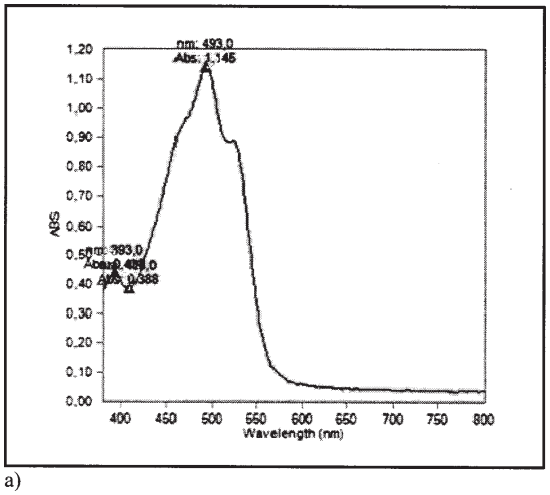
In order to quantify the carotenoid pigments concentration, the cell dry biomass was determined for the final samples of the above mentioned experimental variants and the dry biomass/culture liter was calculated.

The results are presented in the table 1 for 144 h growth in discontinuous bioprocess, MS3 medium composition.

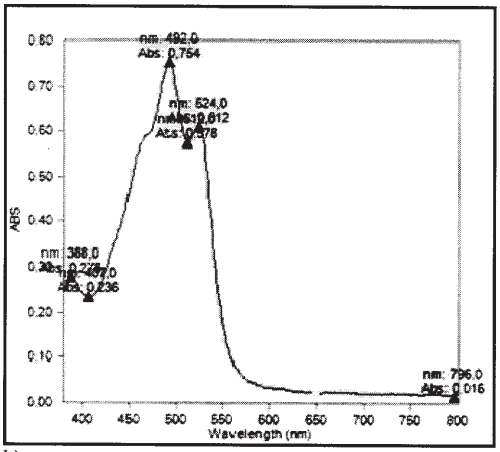
Because the yeast can not assimilate the lactose, the amount of biomass is very low for the corresponding variant.

The pigment extraction was carried out in 3 stages in specific solvents: acetone, n-hexane and basic methanol. For each extract absorption spectra were drawn in 380-800 nm domain and the peaks were determined. To calculate the torularhodin concentration, the specific absorption coefficient $E^{1\%}$ was used for the difference between the absorbances of hexane extract before and after methanol extraction, at 515 nm [13].

The torularhodin concentration function of the extraction models realised for the studied medium composition MS3 variants is also represented in the figure 13.

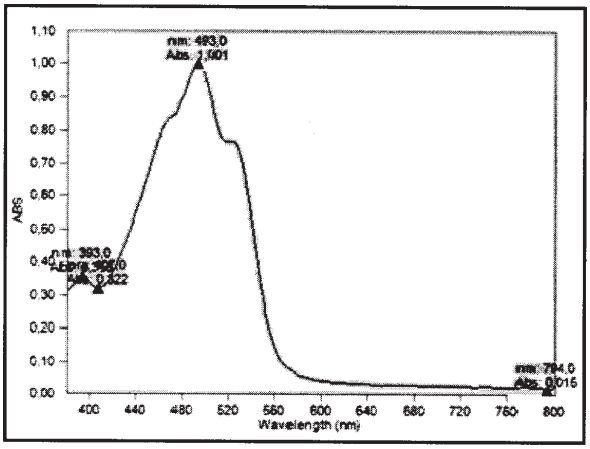


a)

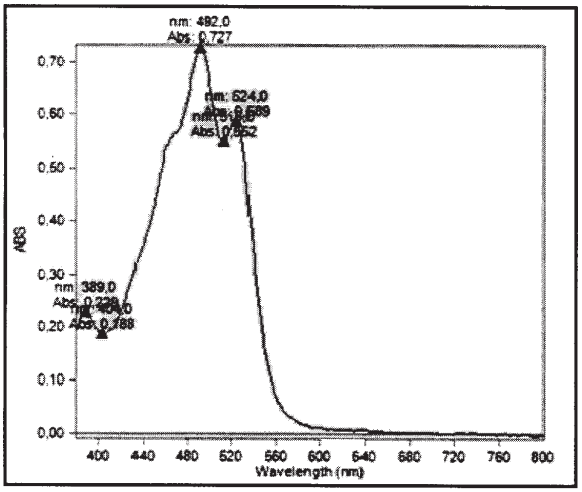


b)

Fig. 10. Absorption spectra of hexane (a) and methanol (b) extracts for the *Rhodotorula rubra* ICCF 209 culture in 4% glucose medium

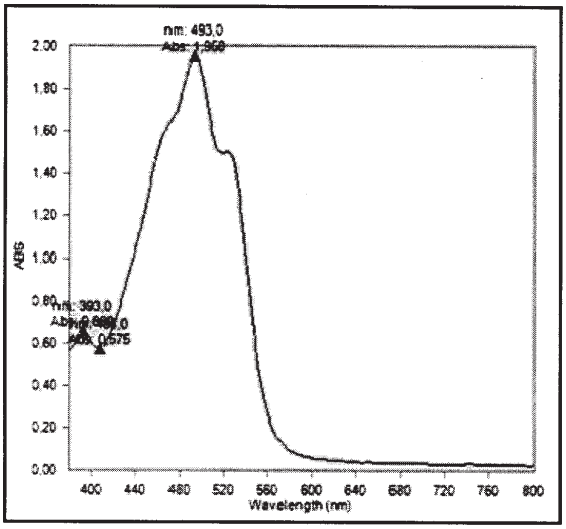


a)

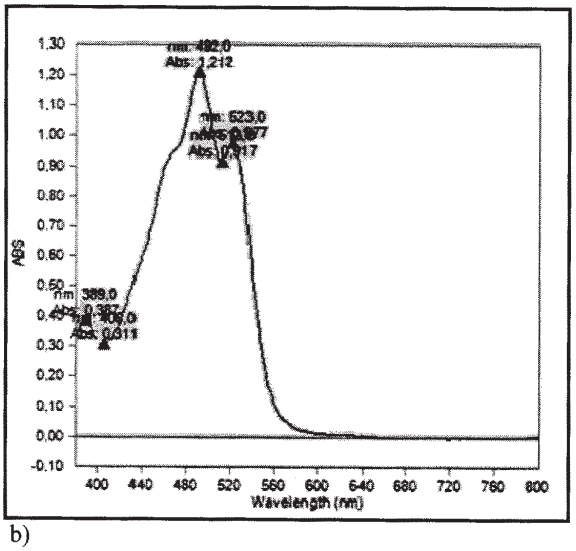


b)

Fig. 11. Absorption spectra of hexane (a) and methanol (b) extracts for the *Rhodotorula rubra* ICCF 209 culture in 4% sucrose medium



a)



b)

Fig. 12. Absorption spectra of hexane (a) and methanol (b) extracts for the the *Rhodotorula rubra* ICCF 209 culture in 4% fructose

The final results indicate that the highest torularhodin yield was obtained for the experimental variant cultivation medium MS3 with 4% fructose as C source (407 μg / L medium). Due to the fact that this carbohydrate is expensive enough, it is of economic importance to consider the cultivation medium variants with 4% glucose or sucrose. The determined torularhodin concentrations are in agreement with the values presented in the specific literature [14-16], demonstrating that both the yeast *Rhodotorula rubra* ICCF 209 and the cultivation medium with the composition MS3 can be considered as valuable solutions for the torularhodin preparation.

Regarding the medium M1 with the glycerin as the carbon source the carotenoid pigments formation was very

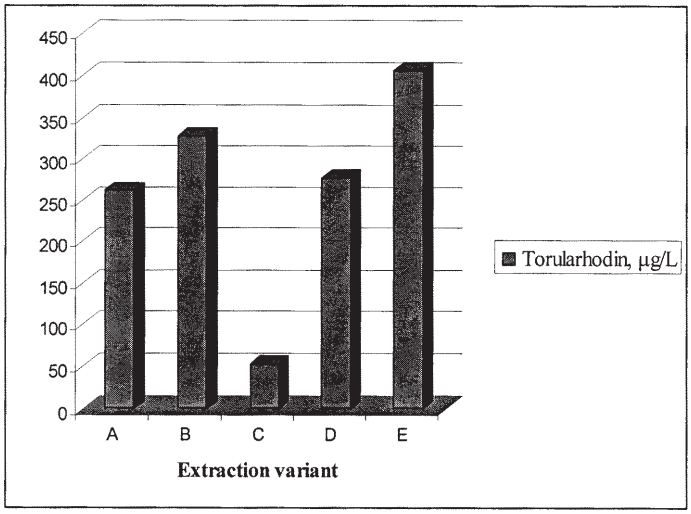


Fig. 13 The torularhodin concentration function of the extraction variants

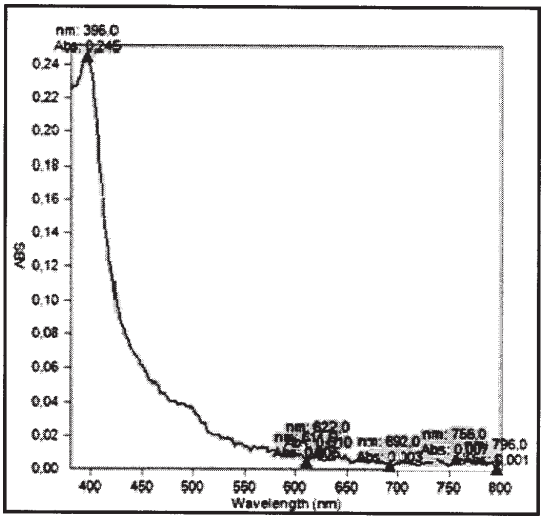


Fig. 14. The absorption spectrum of the acetone extract (total pigments content) obtained from the *Rhodotorula rubra* ICCF 209 cultivated in M1 medium

low conforming to the absorption spectrum presented in the figure 14.

Conclusions

Culture media have a considerable influence on the yeast biomass accumulation and carotenoid pigments biosynthesis, particularly torularhodin, component with a high-level antioxidant potential.

It was found that the carbon source represented by carbohydrates or glycerin resulted in different behaviours of yeast population.

For all carbon sources tested the *Rhodotorula rubra* ICCF 209 strain presented a good growth as can be seen from the OD 600 nm values. The yeast strain growth is stimulated by glucose, fructose, sucrose and maltose, but inhibited by lactose. The stationary phase, when the carotenoid pigments formation is the most important process, starts after 48 h and it is characterized by deep decrease of pH to acidic values. The variation of initial glucose concentration in the range of 1-6 % did not show a major influence on the pigment formation.

The pigments extraction was achieved in n-hexane for total carotenoid pigments and in basic methanol for torularhodin, the unique acid component.

For the tested *Rhodotorula* strain it has been determined the highest pigments yield in the case of fructose medium; the total carotenoid pigments was 690 µg/L and the torularhodin concentration was 407µg/L, followed by the concentrations accumulated when glucose and sucrose were used, the results being comparable with the literature results for other *Rhodotorula* yeasts.

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