The Influence of Operating Conditions on the Growth of the Yeast *Rhodotorula Rubra* ICCF 209 and on Torularhodin Formation

ALINA MIHALCEA[1], CAMELIA UNGUREANU[2*], MARIANA FERDES[2], ANA AURELIA CHIRVASE[2], CRISTINA TANASE[2]

[1]National Institute of Research and Development for Microbiology and Immunology "Cantacuzino", 103 Splaiul Independentei, 050096, Bucharest, Romania

[2]University Politehnica of Bucharest,1-3 Polizu Str., 011061, Bucharest, Romania

The aim of the present work is to study the formation of the intracellular carotenoid pigment - torularhodin depending on culture medium composition and other cultivation factors. The paper presents the influence of shaker cultivation conditions on the growth and torularhodin formation of the yeast Rhodotorula rubra ICCF 209 and the procedure for the extraction, separation, and quantitative determination of the carotenoids, mainly beta-carotene and torularhodin, with antioxidant potential characteristics.

Keywords: carotenoids, torularhodin, Rhodotorula rubra, β-carotene

The ever-increasing demand for food containing only natural ingredients is responsible for the market trend towards the use of natural rather than synthetic pigments [1]. Amongst pigments of natural origin, carotenoids seem to play a fundamental role, their presence in the human diet being considered positively because of their action as pro-vitamin [2], antioxidant or possible tumors-inhibiting agents [3].

Carotenoid biosynthesis is a specific feature of the *Rhodotorula* species [4-6] and *Phaffia* genera [7-8].

The major carotenoid pigments obtained by biotechnological methods are torularhodin, β-carotene and torulene produced in various concentrations by *Rhodotorula* [9-10] and astaxanthin produced by *Phaffia rhodozyma* [11-12].

Culture media have a considerable influence on the yeast biomass accumulation and carotenoid pigments biosynthesis, particularly torularhodin (3', 4'-didehydro-β, γ-caroten-16'-oic acid), component with a high-level antioxidant potential.

The aim of the present work is to study the formation of the intracellular carotenoid pigment - torularhodin with the yeast *Rhodotorula rubra* ICCF 209 depending on culture medium composition and different cultivation factors.

Experimental part

The experiments were carried out in 1000 mL conical flasks, with 200 mL culture medium, on a rotary shaker (Gerhardt Laboshake) at 250 rpm, during 5-6 days of discontinuous aerobic bioprocess, at 28 °C in most cases, for the carotenoids mixture formation, mainly containing the torularhodin, in the stationary phase of the yeast *Rhodotorula rubra* ICCF 209 growth curve.

The medium composition, defined as MS3, was obtained by previous research work [13] with the formula: 40 g/L glucose, 1.5 g/L yeast extract, 5 g/L NH_4NO_3, 1 g/L KH_2PO_4, 0.4 g/L $MgSO_4$ x7H_2O and 0.4 g/L NaCl. Trace elements are assumed to be taken from the tap water.

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

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 or elongated budding yeast cells or blastoconidia, 2.5-6.5 x 6.5-14.0 μm in size.

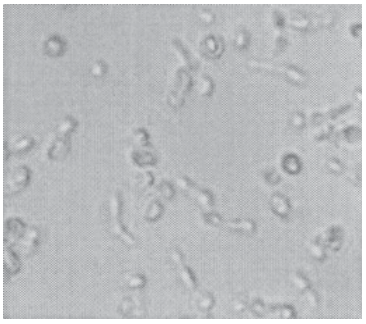

Fig. 1 Microscopic morphology of the Rhodotorula rubra ICCF 209 yeast cells

The cells growth was quantified by: Optical Density (O.D.) determination at Λ= 600 nm, evolution of *p*H and dried biomass concentration. Dry matter concentration determination was done after biomass separation from the culture medium by centrifugation. The biomass drying was achieved in the oven at 105°C until constant mass.

After cells separation by centrifugation three freeze-thaw cycles were performed. The pigments extraction procedure was done in accordance with the dedicated literature [14], comprising acetone extraction of the total pigments mixture including water soluble species, followed by n-hexane extraction to separate the total carotenoids content; another extraction with alkaline methanol allowing the torularhodin (the only pigment with acid structure) component isolation.

The total carotenoids concentration and the torularhodin concentration were determined based on the spectrometric recording of the extracts on the spectrophotometer UV-VIS (Jenway Spectrophotometer).

For each extract corresponding to the indicated 3 stages extractions in specific solvents: acetone, n-hexane and basic methanol, adsorption spectra were drawn in 380-800 nm domain and the peaks were determined. To calculate the torularhodin concentration, the specific

* *email: ungureanucamelia@gmail.com; Tel.: +40723239120*

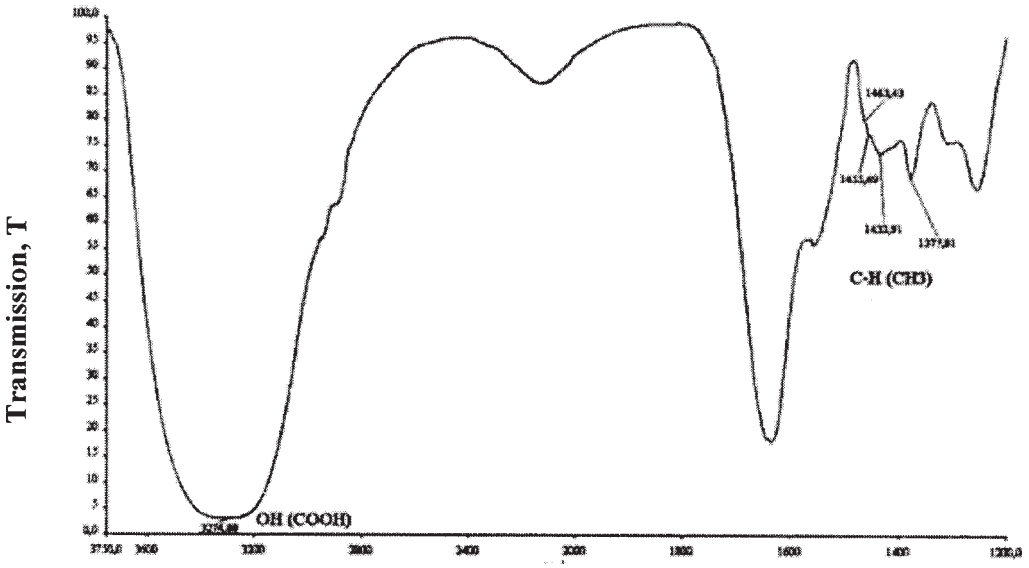

Fig. 2 Identification of the characteristic bands of torularhodin by FT-IR

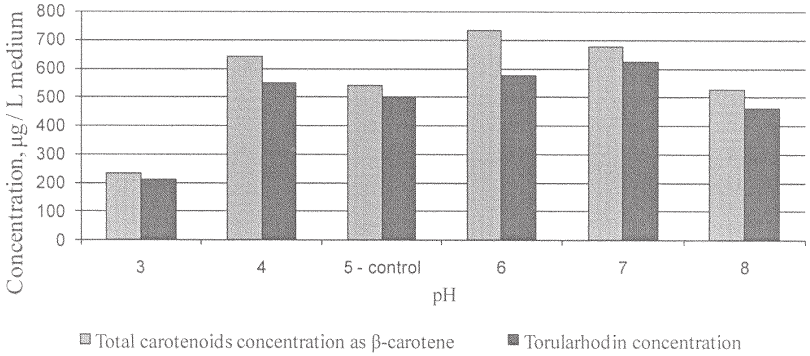

Fig. 3 Variation of total carotenoid and torularhodin concentration depending of initial pH values

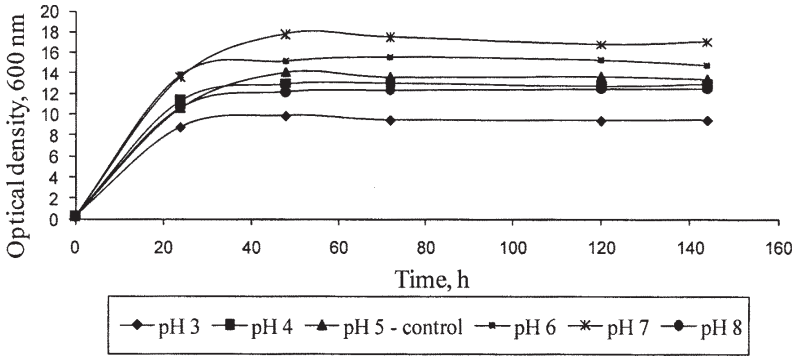

Fig. 4 Optical density variation depending of initial pH values of culture medium

absorption coefficient $E^{1\%}$ 1932 was applied to the difference between the absorbance of the hexane extract before and after methanol phase extraction, at 515 nm [15].

As torularhodin is the unique carotenoid with a carboxylic group, it can be easily identified by a characteristic OH band, included in the carboxylic acid function, which approximately absorbs between 2500 and 3700 cm^{-1} by FT-IR (Fourier Transformed Infra Red) method, with Perkin Elmer Spectrum 100 series (Universal ATR Sampling Accessory) [16]. This was verified by FT-IR spectrum showing the presence of a hydroxyl function (in a carboxylic group) but also of CH_3 groups, with absorption band between 1315 and 1475 cm^{-1}.

Results and discussion

Identification of the structure of tourlarhodin by FT-IR determination

The FT-IR spectrum (fig. 2) shows the presence of a hydroxyl function (in a carboxylic group) in the region already mentioned, but also CH_3 groups can be identified, with an absorption band between 1315 and 1475 cm^{-1}.

Influence of the initial pH

Several experiments were realized to determine the influence of the initial cultivation *p*H, in the range 3 – 8, on the yeast growth and both – total carotenoids and torularhodin formation, considering the control medium MS3 with initial *p*H 5.

The growth curves and the pH evolution for the mentioned experimental variants are represented in the figures 4 and 5.

The studied characteristics – yeast growth, *p*H evolution, the total carotenoids formation and the torularhodin formation recommend the initial *p*H range of 6-7 as being favorable, the torularhodin fraction from the total carotenoids' content being more than 90% for the *p*H of 7. The *p*H 3 is not favorable for the pigments formation and the *p*H 8 is limiting the yeast growth.

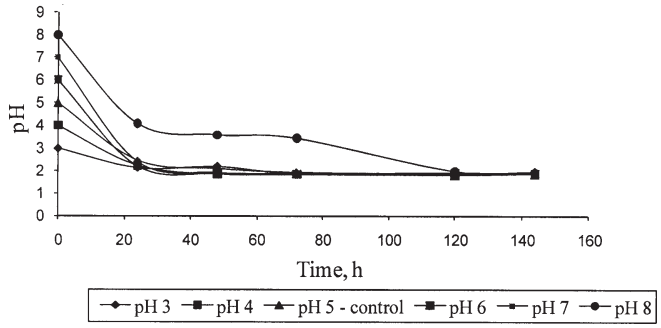

Fig. 5. Variation of pH depending of growth time

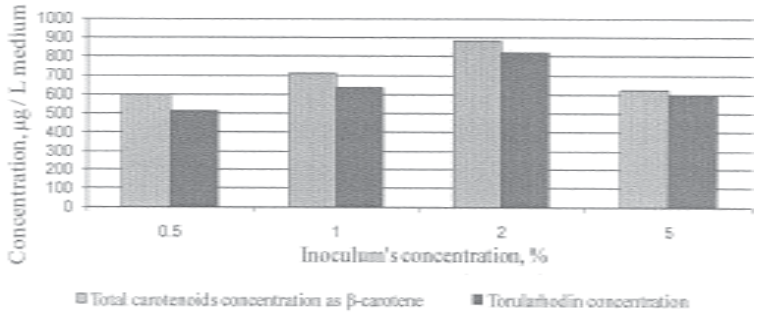

Fig.6. Variation of total carotenoid and torularhodin concentration depending on the inoculum size

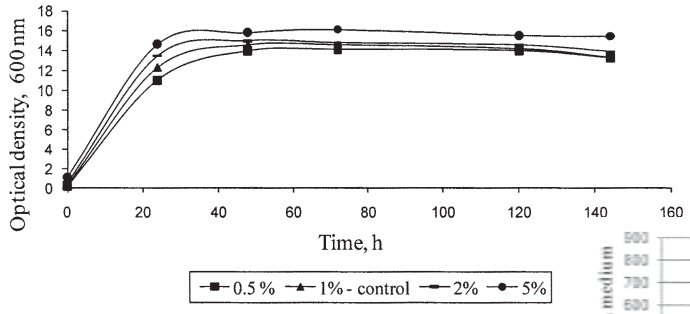

Fig. 7. Optical density variation depending of the inoculum size

Table 1
EXPERIMENTAL VARIANTS NOTATION FUNCTION OF THE USE OF DIFFERENT NITROGEN SOURCES

Experimental variants	Nitrogen source, 5 g/L
1	NH_4NO_3 - control
2	$NH_4H_2PO_4$
3	$(NH_4)_2SO_4$
4	NH_4Cl
5	NH_4NO_3 + 0.1% tryptophan
6	NH_4NO_3 + 0.1% threonine
7	NH_4NO_3 + 0.1% glutamic acid
8	NH_4NO_3 + 0.1% cysteine
9	NH_4NO_3 + 0.1% alanine
10	NH_4NO_3 + 0.1% tyrosine
11	NH_4NO_3 + 0.1% proline
12	NH_4NO_3 + 0.1% leucine
13	NH_4NO_3 + 0.1% valine

Influence of the initial inoculum concentration

Also the growth and the carotenoids production, mainly torularhodin with the same yeast *Rhodotorula rubra* ICCF 209 were investigated for different initial inoculum cellular density on the control medium MS3. The growth evolution is done in the figure 7 and the pigments formation results are presented in the figure 6.

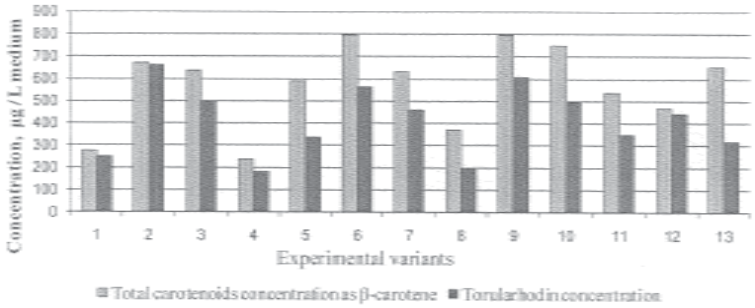

Fig. 8 Variation of the torularhodin concentration, and the total carotenoids concentration (as β-carotene) depending of the nitrogen source from the broth

An inoculum concentration of 1-2 v/v is favourable for both yeast growth and carotenoids formation including the torularhodin accumulation.

Study of the nitrogen source influence

In a first step, growth and carotenoids synthesis, including the torularhodin formation with the *Rhodotorula rubra* yeast were investigated based on the use of different salts as mineral nitrogen sources such as NH_4NO_3, $NH_4H_2PO_4$, $(NH_4)_2SO_4$, NH_4Cl and NH_4NO_3 (as the control salt initially present in MS3 medium) with the concentration of 5 g/L for all the variants. At the same time for the control medium composition variant the organic nitrogen source, the yeast extract, was supplemented with a concentration of 0.1% from the following amino acids: tryptophan, threonine, glutamic acid, cysteine, alanine, tyrosine, proline, leucine and valine.

The experiment lasted for 144 h.

The pigments formation results are presented in the fig. 8 and the growth curves are indicated in the figure 9 a and b).

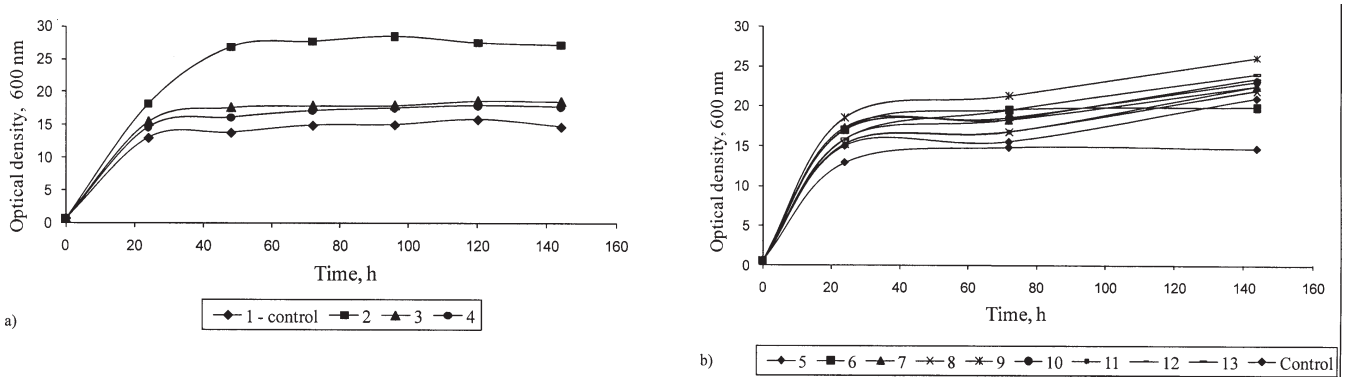

Fig.. 9. The growth of *Rhodotorula rubra* ICCF 209 yeast for medium composition variants with different nitrogen sources; a) medium variants 1-4, b) medium variants 5-13

Medium composition variants	Fatty acids addition	Total carotenoids concentration as β-carotene, μg / L medium	Torularhodin concentration, μg /L medium
C	Control (MS3)	239.79	185.11
L	Glucose + 0.1% linoleic acid	169.8	161.26
O	Glucose + 0.1% oleic acid	356.43	315.33
S	Glucose + 0.1% stearic acid	351.79	307.81
P	Glucose + 0.1% palmitic acid	324.18	258.03

Table 2
INFLUENCE OF THE FATTY ACIDS ADDITION ON THE CAROTENOIDS FORMATION MAINLY TORULARHODIN

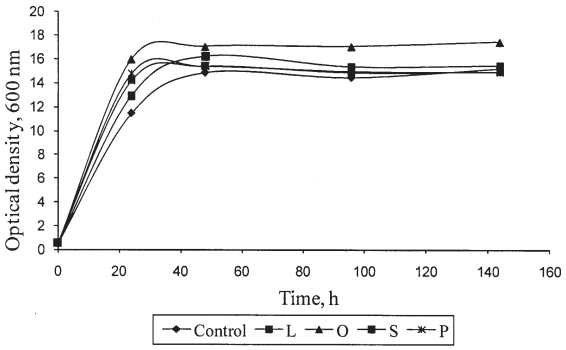

Fig. 10. Influence of fatty acids addition on the growth of *Rhodotorula rubra* ICCF 209 yeast

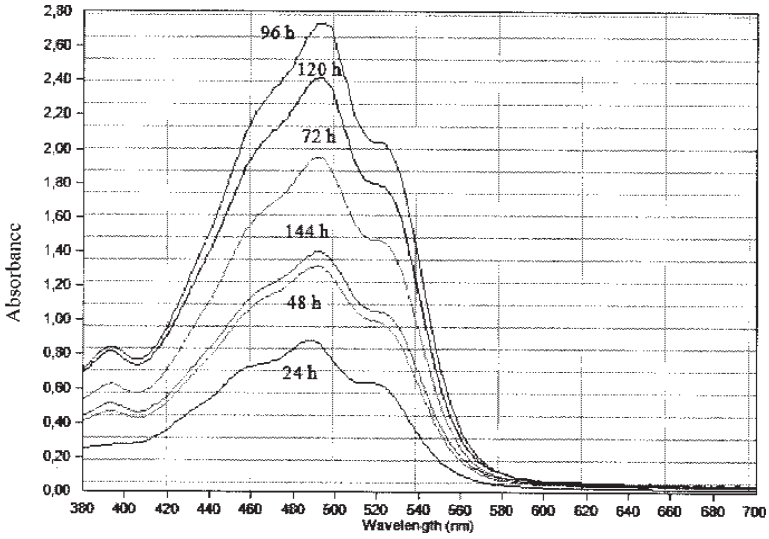

Fig. 11. Absorption spectra of hexane extract for the Rhodotorula rubra ICCF 209 culture in MS3

In figure 9 a the growth was higher when acid ammoniu-monophosphate was used and as it is indicated in the previous figure, the highest carotenoids concentration was measured in the same case with a fraction of about 95 % torularhodin in the pigments mixture.

In figure 9 b the growth was higher when 0.1 % alanine was added to MS3 medium followed by the variant with 0.1% threonine; conforming to the previous figure, the highest total carotenoids concentration as well as the torularhodin concentration were determined for the same variants (70-75% from the total carotenoids content represents the torularhodin).

Compared to control medium (MS3) all the variants characterized by small additions of amino acids have better growths. The introduction of alanine and threonine seems to influence more the pigments formation than the use of $NH_4H_2PO_4$ instead of the control mineral nitrogen source.

Influence of fatty acids additions

In order to study the possible influence of the introduction of small concentrations of fatty acids on growth and pigments formation considering the *Rhodotorula* yeasts capacity to assimilate these acids as valuable carbon sources [17] the control medium formula was supplemented with 0.1% of fatty acids: linoleic/oleic/stearic/palmitic. Again the growth and the total carotenoids concentration compared with the torularhodin concentration were determined. The carotenoids formation, including torularhodin is presented in the table 2, and the growth curves for the same experimental variants are represented in the figure 10.

The growth was better when oleic acid was used and the carotenoids and torularhodin concentrations are the highest for this variant.

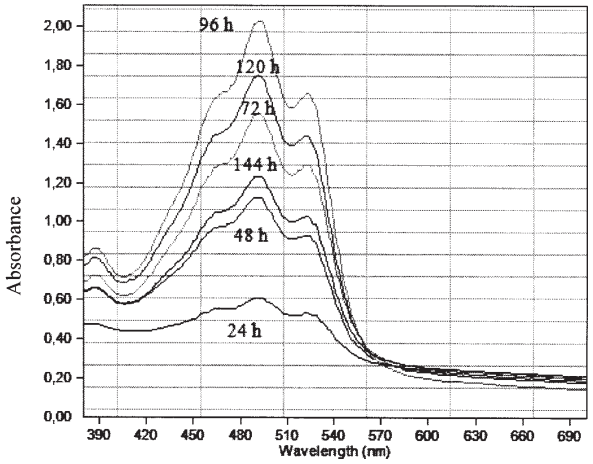

Fig. 12 Absorption spectra of methanol extracts for the Rhodotorula rubra ICCF 209 culture in MS3

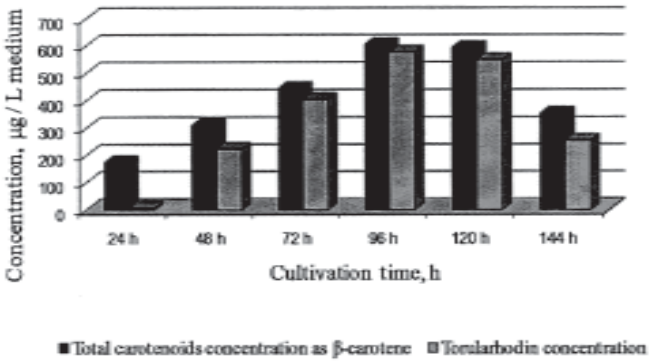

Fig. 13 Variation of the torularhodin concentration, and the total carotenoids concentration (as β-carotene) depending on the cultivation time

It is to mention that the influence of the addition of small concentrations of amino acids alanine or threonine was more important.

Influence of cultivation time

Several experiments were realized to determine the influence of the cultivation time, in the range 1 – 6 days, on the yeast growth and both – total carotenoids and torularhodin formation were measured. The stationary phase characterized by an important production of carotenoid pigments starts after 48 h in all cases.

The total carotenoids concentration and the torularhodin concentration were determined based on the spectrometric recording of the extracts on the UV-VIS spectrophotometer (Jenway Spectrophotometer).

The total carotenoids concentration was determined as β-carotene content by using the extract absorbance values (fig. 11), similar the torularhodin concentration was calculated using methanol extracts absorbance values (fig. 12) with the specific absorption coefficient, $E^{1\%}1932$ [18].

Until 48 h the ratio between the torularhodin concentration and the total carotenoids concentration is small, so the yeast biosynthesizes more the other pigments; then during the growth stationary phase the concentration of torularhodin can represent so far 90% from total carotenoids content. The carotenoids concentration increases until 96-120 h, but after this duration the trend is towards the diminution of any carotenoids content.

The typical growth curve for the MS3 medium composition is represented in figure 9 a.

Study of the general medium formula optimization

In a first stage it was necessary to get the most favourable concentrations of the previous determined amino acids (threonine and alanine) and fatty acid (oleic acid), as the addition substrates with important influence on the yeast growth and carotenoids, mainly torularhodin formation.

Table 3
SUPPLEMENTATION VARIANTS OF MS3 MEDIUM

Supplementation variant of MS3 medium	Notation
alanine 0.05%	1
alanine 0.1%	2
alanine 0.2%	3
threonine 0.05%	4
threonine 0.1%	5
threonine 0.2%	6
oleic acid, 0.1 %	7
oleic acid, 0.2 %	8
oleic acid, 0.5 %	9

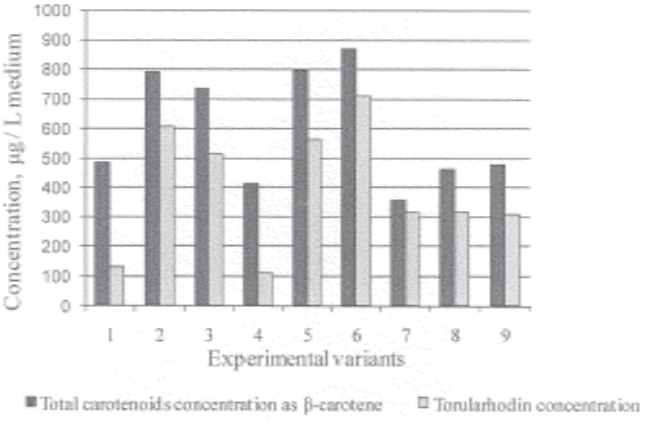

Fig.14 Influence of different concentration of alanine, threonine and oleic acid on the total carotenoids and torularhodin formation

The results demonstrate that the concentrations of 0.1-0.2 % of threonine or alanine are indicated to stimulate beside the yeasts growth both the total carotenoids formation and the torularhodin formation. The introduction of concentrations of 0.2-0.5 % oleic acid influences more than the amino acids the yeast growth, but less the carotenoids formation, so finally the threonine or alanine are necessary to get higher concentrations in carotenoids, mainly torularhodin in concentrations of about 75-80% from the total pigments mixture. It was to study the combined effect of the three substrates (alanine, threonine, and oleic acid) especially on the carotenoids formation.

In a second stage the same growth and products formation characteristics were studied by comparing the evolutions at 30 and at 28 °C and considering the improved medium formula in accordance with the above presented findings. The growth evolution is done in the figure 16 and the pigments formation results are presented in the figure 17.

The medium composition variants are indicated in the table 4.

The results indicate some important trends:

- the medium composition variant M2, where there were both - the replacement of NH_4NO_3 as anorganic nitrogen source with the same concentration of $NH_4H_2PO_4$ and the supplementation with alanine (0.1%), threonine (0.2%), and oleic acid (0.1%) - influence more the yeast growth,

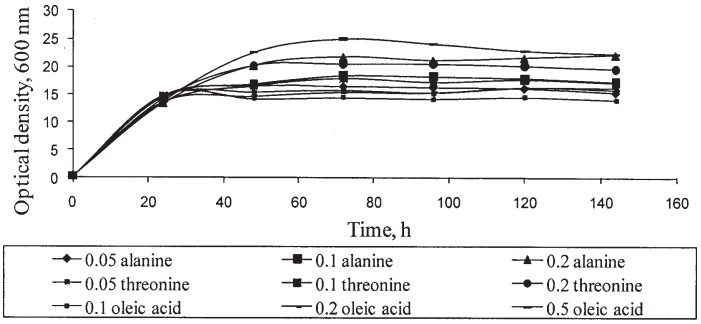

Fig.15. Influence of different concentrations of alanine, threonine and oleic acid on *Rhodotorula rubra* ICCF 209 yeast growth

Table 4

COMPOSITION MODIFICATIONS OF THE CONTROL MEDIUM MS3

Medium variant	Cultivation medium characteristics
Control	MS3
M1	(a) Replacement of NH_4NO_3 0.5% with same concentration of $(NH4)_2SO_4$ (b) Supplementation with: - alanine 0.1% - threonine 0.2% - oleic acid 0.1%
M2	(a) Replacement of NH_4NO_3 0.5% with same concentration of $NH_4H_2PO_4$ 0.5% (b) Supplementation with: - alanine 0.1% - threonine 0.2% - oleic acid 0.1%
M3	Supplementation of the yeast extract concentration from 0.15 to 0.5%

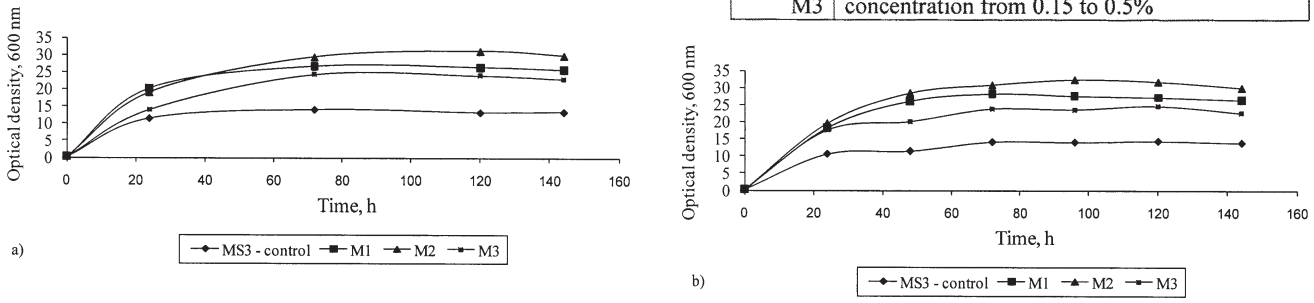

Fig.16. Growth of *Rhodotorula rubra* ICCF 209 yeast versus of the cultivation temperature and the composition of the supplemented media (a) 30 °C; (b) 28 °C

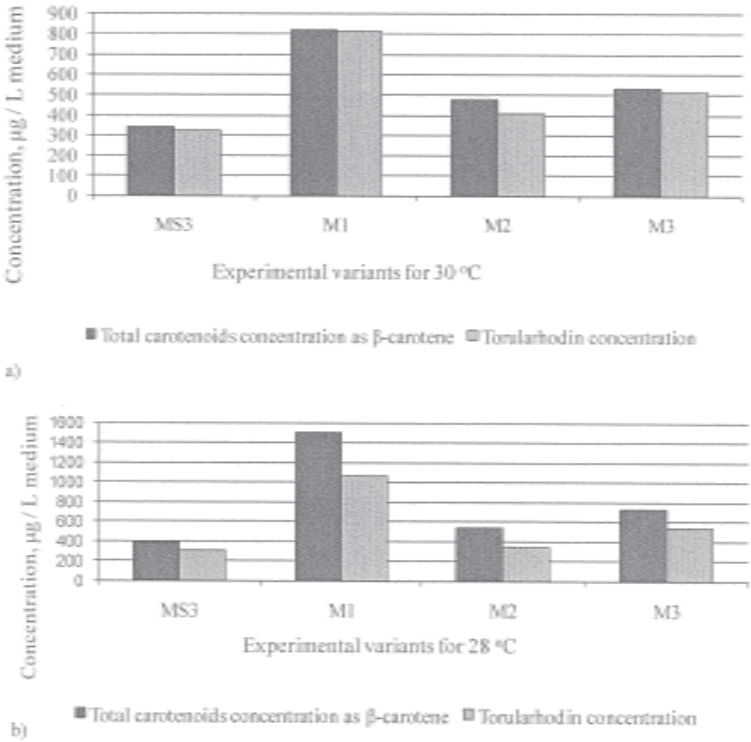

Fig. 17. Influence of cultivation temperature and the composition of the supplemented media on the total carotenoids and torularhodin formation (a) 30 °C; (b) 28 °C

but also the total carotenoids and torularhodin formation, higher concentration of about 1 mg / L torularhodin is formed on this variant;

- though the growth is not influenced by the temperature change from 28 to 30°C, the carotenoids formation is influenced for all the medium variants, this indicating a general metabolic trend. Both the total carotenoids, but also the torularhodin concentrations are higher for the diminished temperature, with the limit that the ratio between the torularhodin concentration and the total carotenoids concentration is bigger for the temperature of 30°C;

- the increase in yeast extract concentration until 0.5% determine a rise in both the total carotenoids concentration and the torularhodin content, but the influence is less important than that measured for the medium variant M2.

Conclusions

Cultivation media have a considerable influence on the yeast growth and carotenoid pigments biosynthesis,

particularly torularhodin, component with a high-level antioxidant potential.

The studied characteristics – yeast growth, the total carotenoids formation, and the torularhodin formation recommend the initial pH range of 6-7 as being favorable, the torularhodin ratio from the total carotenoids content being greater for the *p*H of 7.

At the same time an inoculum concentration of 1-2 % is favorable for both yeast growth and carotenoids formation.

When NH_4NO_3 is replaced in the control medium with other mineral nitrogen sources ($NH_4H_2PO_4$, $(NH_4)_2SO_4$, or NH_4Cl) both the growth and the carotenoids formation are higher when acid ammonium phosphate was used; in this case a fraction of about 95% torularhodin is formed in the pigments mixture.

The results demonstrate the concentrations of 0.1-0.2 % of threonine or alanine are indicated to stimulate beside the yeasts growth both the total carotenoids formation and the torularhodin formation. The introduction of concentrations of 0.2-0.5% oleic acid influences more the amino acids the yeast growth, but less the carotenoids formation.

The bioprocess duration is important: until 48 h the ratio between the torularhodin concentration and the total carotenoids concentration is small, so the yeast biosynthesizes more the other pigments; then during the growth stationary phase the concentration of torularhodin can represent so far 90% from total carotenoids content and the carotenoids concentration increases until 96-120 h, but after this duration the trend is towards the diminution of any carotenoids content.

The medium composition variant, where there were both - the replacement of NH_4NO_3 as anorganic nitrogen source with the same concentration of - $NH_4H_2PO_4$ and the supplementation with alanine (0.1%), threonine (0.2%), and oleic acid (0.1%) – represents the optimum composition studied so far for torularhodin formation.

Though the growth is not influenced by the temperature change from 28 to 30 °C, the carotenoids formation is influenced for all the medium variants, this indicating a general metabolic trend. Both the total carotenoids, but also the torularhodin concentrations are higher for the diminished temperature, with the limit that the ratio between the torularhodin concentration and the total carotenoids concentration is bigger for the temperature of 30°C.

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