Extraction of Torularhodin from *Rhodotorula rubra* Yeast Using Sunflower Oil

ALINA MIHALCEA[1, 2], ADRIAN ONU[1], CATALIN TUCUREANU[1], CAMELIA UNGUREANU[2]*, SILVIU RAILEANU[2], AURORA SALAGEANU[1], OVIDIU MUNTEAN[2]

[1]National Institute of Research "Cantacuzino", 103 Splaiul Independentei, 050096, Bucharest, Romania
[2]University "Politehnica" of Bucharest, 313 Splaiul Independentei Str., 060042, Bucharest, Romania

Yeasts are an important source of natural carotenoids (a group of oil soluble pigments). These solubilization characteristics of carotenoids have led to studies on recovery of these pigments from vegetable oils such as sunflower oil, soy oil, Palm oil, etc. Torularhodin is one of the carotenoid pigments produced by the yeast Rhodotorula sp., with a terminal carboxylic group considered now-a-days as a powerful antioxidant In this paper we extract the torularhodin from Rhodotorula rubra yeast using sunflower oil. Approximately 97% of the cellular mass is disintegrated over five cycles through the high pressure IKA WERKE homogenizer at a pressure from 1500 to 2000 bar. The HPLC analysis proves a good capacity for extracting the total carotenoid pigments in sunflower oil by obtaining a high value of 958.68 μg/mL medium from which a percentage of 70.29 % is represented by torularhodin.

Keywords: torularhodin, carotenoids, extraction, Rhodotorula rubra, sunflower oil

Most of the natural pigments are extracted from plants like paprika, grapes, etc. and microorganisms as *Monascus purpureus, Rhodotorula rubra, Phaffia rhodozyma, Sporobolomyces roseus* etc. [1-3]. Pigments like carotenoids, torularhodin and chlorophyll have been produced from microorganisms. Now-a-days there is growing interest in microbial pigments due to their natural character, nutritive and medicinal properties; production being independent of season, geographical conditions, and safety use [4-5]. Carotenoids are a group of fat-soluble pigments occurring widely in nature. These are highly unstable compounds and need to be protected by suitable storage conditions from exposure to light, excessive heat, and oxygen in order to prevent their breakdown [6]. An important stage in the extraction and separation of the carotenoid pigments is cell disruption (lysis). Cell lysis could be achieved through various approaches [7], such as optical [8], mechanical [9], chemical [10], and electric [11] methods. Sachindra and Mahendrakar [12] extracted carotenoid pigments from shrimp waste with vegetable oils and concluded that sunflower was the best oil for extraction. The advantage of oil extraction process is that the pigmented oil finds use as carotenoid source in many applications such as food, drugs and cosmetics industry.

Torularhodin is one of the carotenoid pigments produced by the yeast *Rhodotorula sp*., with a terminal carboxylic group considered now-a-days a powerful antioxidant to be included in food and drugs formulations. It is also useful as a stabilizing agent for foods, pharmaceuticals, cosmetics, etc., susceptible to oxidation and with antimicrobial activity [13-15]. It has been known that carotenoids undergo "bleaching" i. e., lose their colour, when exposed to radicals or to oxidizing species. Oxidation involves interruption of the conjugated double bond system either by cleavage or by addition to one of the double bonds. Oxygen may act either directly on the double bonds or through the hydroperoxides formed during lipid autoxidation [14].

Taking into account the above mentioned in this paper we extract the torularhodin from *Rhodotorula rubra* yeast with sunflower oil

Experimental part

Rhodotorula rubra cultivation was described in previous studies [1, 16].

Cell Treatment

The biological material subjected to cellular disruption consisted of 50 g of moist yeast *Rhodotorula rubra* type, which was separated from biomass through centrifugation in a stationary regime for 60 min. at 5000 rpm.

A good solution for the cellular disruption in order to obtain a therapeutic product is represented by the mechanical methods where other reactive are not involved [17]. These reactive could contaminate the product and additional purification stages might be added to the production process. Thus, cellular disruption through the mechanical by exposure to high pressure represents a rapid, safe and suitable technique in this situation. Cellular disintegration was done using a high pressure press Ika Werke (fig.) with the following characteristics: rotor operating speed: 137-344 rpm, maximum working pressure: 2000 bar, maximum working temperature: 60 °C, maximum working suprapressure in funnel: 6 bar, funnel volume: 0.4 L, working flow: 3 L/h, operating voltage: 200V and frequency: 50 - 60 Hz.

The recovered cellular mass was suspended in 100 mL of distilled water and mixed until a homogenous suspension was obtained. The obtained suspension is passed through the high pressure homogenizer five times at pressures ranging from 1500 to 2000 bar.

From the initial suspension and after each breaking cycle, samples were collected to be analyzed and processed at microscope in order to sustain the efficiency of the cellular lysis. Data had been processed using both the image

* *email: ungureanucamelia@gmail.com; Tel.: 0040723239120*

Fig. 1 Experimental setup High pressure homogeniser - Ika Werke

processing software ImageJ 1.41 and the statistical processing software R version 3.0.3 (R is a free software environment for statistical computing and graphics). Cellular perimeter and circularity had been analyzed.

3 images per sample were acquired at random positions on a Nikon TE2000U microscope using a 5MP cooled CCD camera and a 20x objective. The images were automatically analyzed using an in-house algorithm developed in ImageJ in order to identify and count intact cells (based on size and circularity).

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Taking into account the properties of the fat-soluble carotenoid pigments, which are located in the double fat layer of the cellular membrane, it is considered that a similar environment to the natural condition of biosynthesis assures stability and preserves the antioxidant properties of these compounds. Thus, to the suspension of disintegrated cells is added 50 mL of food oil made from sunflower, the mixture being afterwards emulsified in the high pressure homogenizer at 1500 bar. It is thus obtained a pink stable emulsion consisting of an oily part (food oil) and of an aqueous part. During the emulsion the major part of torularhodin and the carotenoid pigments are extracted in the oil phase of the suspension. The separation of the oil extract containing the total carotenoid pigments from the aqueous part and the cellular remains requires a previous breaking of this emulsion. The emulsion break is realized through freezing at -20°C and defrosting at ambient temperature followed by 60 min of *centrifuge at* 5000 rpm. The oil phase was recovered and the extraction, separation and analysis of the carotenoid pigment were realized by *Reverse Phase* - HPLC (*Reverse Phase* - High-performance liquid chromatography). The carotenoid pigments were extracted in acetone from the oil extract and analyzed through *Reverse Phase* - HPLC. After the extraction from acetone, the pigment separation was realized utilizing a water/acetone gradient as eluting system (method adapted from Weber et al.) [18]. The extract in acetone was frozen at -80 p C over night for the deposing of the lipids. After freezing the extract had been centrifuged for 10 min at 10000, the clear organic phase is collected using a syringe and is filtered through a Millex GV (4 mm) membrane of 0.22 μm. For the HPLC analysis in the inverse phase dilutions are realized in acetone and 20μL volumes are used for injection. In a similar manner the control sample from sunflower food oil is prepared.

For the separation and analysis of the extract it was used a system composed of: chromatography column Agilent Eclipse Plus C18 (4.6 x 250 mm; 5 μm) for the reverse phase, pumps HPLC Waters 501, control module for Waters pumps, *in-line* vacuum *degasser* and a *photodiode array detector.* Data processing was realized using the software Empower II Build 2154 Waters. As mobile phase a gradient with 70 to 100 % acetone for 20 min with a debit of 1 mL/min at 40 p C was used. The detection was realized on a wide spectrum ranging from 280 to 780 nm. For data processing the chromatogram was extended to 450 nm (specific to beta-carotene) and the spectrums of separate peaks were analyzed.

The computation of the torularhodin and of the total carotenoid pigments expressed as beta-carotene was done according to the standard beta-carotene curve as shown in figure 2.

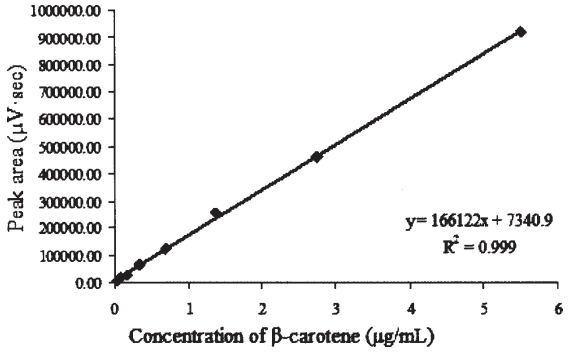

Fig. 2 Calibration curve of β-carotene

For the determination of the standard curve it was used a type II crystalline beta-carotene ≥ 95% (HPLC) from Sigma. Binary dilutions in acetone were realized which were subjected to the same HPLC separation methods as were the samples.

Results and discussions

Cell disruption

As shown in figure 3, the appearance is typical for healthy cell culture yeast from the *Rhodotorula* type: spherical, ellipsoid, ovoid or elongate, well defined, in accordance with the literature [19]. All the characteristics mentioned before being well outlined using the image analysis software ImageJ.

After the first cycle to a pressure of 1500-2000 bar, a large part of the cell are disintegrated; these cells gain a diffuse aspect with a weak and irregular shape which indicates the presence of cellular debris. At the end of the disintegration process the images acquired with the

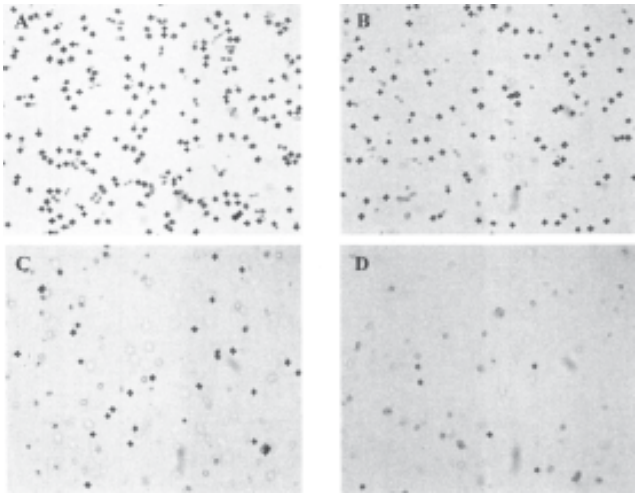

Fig. 3 Automated image analysis using an "in-house" algorithm written for ImageJ, A – initial cell suspension and the suspension after, B – 1st breaking cycle; C - the 3rd breaking cycle and D - the 5th breaking cycle

Table 1
THE PARAMETERS VALUES PROCESSED USING THE IMAGE ANALYSIS SOFTWARE Image AND THE STATISTICAL ANALYSIS SOFTWARE R

Stage	Number of cells±S.D.	Cellular perimeter±S.D.	Circularity±S.D.
Initial	190 ±6	89±0.91	0.35±0.2
1st Cycle	124±5	86±0.41	0.31±0.04
2nd Cycle	98±6	85±1.96	0.30±0.4
3rd Cycle	33±4	83±2.23	0.26±0.31
4th Cycle	28±1	80±1.02	0.25±0.08
5th Cycle	6±3	78±0.38	0.23±0.01

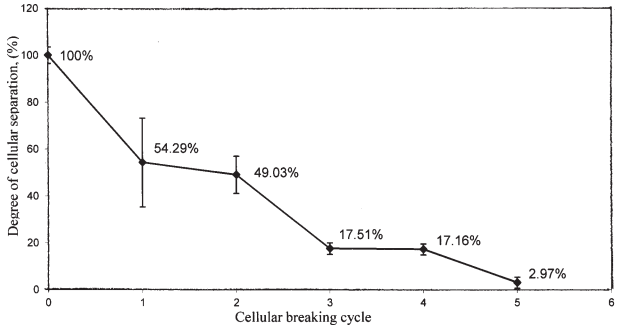

Fig. 4 The evolution of the cellular breaking efficiency as a function of the number of breaking cycles

microscope and the analysis revealed a very small number of unbroken cells which are resistant to the high pressure they were subjected. Shape factors like cellular perimeter and the circularity (a function of perimeter and area) are dimensionless quantities used in image analysis and microscopy that numerically describe the shape of a particle, independent of its size [20]. Table 1 presents the parameters values processed using the image analysis software ImageJ and the statistical analysis software R.

After the first pass through the high pressure homogenizer almost 50 % of the cells are broken, a value which is maintained with a small decrease for the second pass through the pump. A sudden drop in the percentage of broken cells occurs after the third breaking cycle when only 17.5% cells are identified. This value is maintained until the end of the breaking procedure; after the fifth passage through the pump statistics show that 97 % of the cellular mass is disintegrated. Approximately 97% of the cellular mass is disintegrated after five passages through the high pressure Ika Werke homogenizer at a pressure of 1500-2000 bar. Cellular disintegration by subjecting the cellular mass to high pressure is an efficient and rapid method which is adequate to the inclusion in the technologies for obtaining pharmaceutical bioproducts based on torularhodin and associated pigments.

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The efficiency of the cell breaking for yeast must also be evaluated from the extraction capacity of the torularhodin and of the total carotenoid pigments.

Concentration of torularhodin (table 2) in the acetone extract was calculated using the calibration curve of β-carotene (fig. 2).

Reverse-Phase HPLC is based on analytes' partition coefficients between a polar mobile phase (a flowing liquid: acetone-water mixture) and a hydrophobic (nonpolar) stationary phase (sorbents packed inside a column whit octadecyl (C18) bonded group), where polar analytes elute first while nonpolar analytes interact more strongly with the hydrophobic C18 groups.

In the chromatogram the first compound eluted by mobile phase is torularhodin, which is pigment with the highest polarity of carotenoid pigments from acetone extract. This is due to the chemical structure which has a -COOH group (fig. 5-6).

Concentration of torularhodin and total carotenoid pigments relative to the moist and dried cellular mass (table 3) was calculated by the method outlined in the previous article [16].

The HPLC analysis proves a good capacity for extracting the total carotenoid pigments in sunflower oil by obtaining a high value of 958.68 μg/mL medium from which a percentage of 70.29 % is represented by torularhodin.

Acetone extraction and analysis by Reverse-Phase HPLC method represents both a qualitative and quantitative study of carotenoid pigments, but can be used as a method for monitoring and controlling the bioprocess for obtaining the torularhodin and associated carotenoids pigments, the results obtained in a relative short time can be utilized for taking quick process decisions and the process performance and product quality can be achieved.

Table 2
CONCENTRATION OF TORULARHODIN IN THE ACETONE EXTRACT

Peak surface (μV·sec)	Torularhodin percentage from total carotenoid pigments (%)	Surface of total peaks (μV·sec)	Torularhodin concentration (μg / mL extract)
42377	70.29	60289	21.1

Table 3
CONCENTRATION OF TORULARHODIN AND TOTAL CAROTENOID PIGMENTS RELATIVE TO THE MOIST AND DRIED CELLULAR MASS

Final volume of cellular suspension, mL	Total moisted biomass, g	Dried biomass, %	Dried biomass, g/L	Dried biomass, mg/g moisted substance	Torular-hodin, μg /L of medium	Total pigments expressed as β-carotene, μg / L of medium	Total pigments expressed as β-carotene, μg / g dried substance
6000	191.73	18.84	6.02	9420	673.86	958.68	159.26

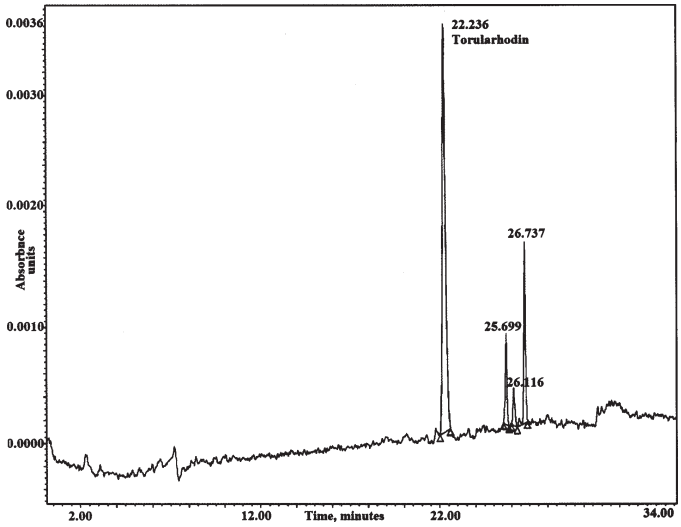

Fig. 5 Chromatogram at 450 nm for the extract of total carotenoid pigments in sunflower oil

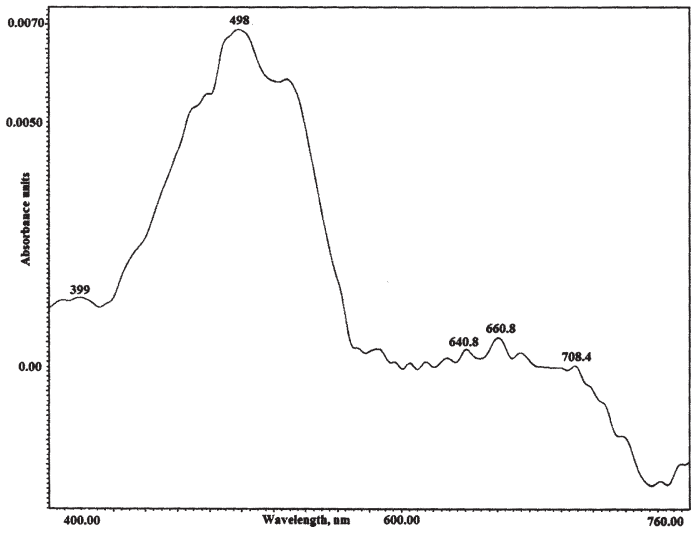

Fig. 6 Chromatogram of torularhodin for the extract of sunflower oil

Conclusions

In all methods applied for the identification or for the dosage of carotenoid pigments it is necessary the reference to the specific values of a standardized product. In its absence the results are reported in total carotenoids expressed as beta-carotene.

Approximately 97 % of the cellular mass is disintegrated over five passages through the high pressure Ika Werke homogenizer at a pressure from 1500 to 2000 bar.

Cellular disintegration by subjecting the cellular mass to high pressure is an efficient and rapid method which is adequate to the inclusion in the technologies for obtaining pharmaceutical bioproducts based on torularhodin and associated pigments.

The fat-soluble proprieties of the carotenoid pigments permit their extraction in sunflower food oil. These conditions assure a stability of the antioxidant proprieties of its compounds and the efficiency and the safety from a pharmaceutical and alimentary point of view.

The HPLC analysis proves a good capacity for extracting the total carotenoid pigments in sunflower oil by obtaining a high value of 958.68μg/mL medium from which a percentage of 70.29 % is represented by torularhodin.

The oil control sample analyzed proves that this does not contain traces of carotenoid pigments which can influence the concentration or the evaluation of the extract.

References

1.UNGUREANU C., FERDES M., CHIRVASE A. A., Rev. Chim. (Bucharest), **63**, no. 3, 2012, p. 316.
2.FERDEŞ M., UNGUREANU C., RADU N., CHIRVASE A. A., Chem. Eng. Transactions, **17**, 2009, p. 1089.
3.ANANDA N., VADLANI P.V., J. Ind. Microbiol Biot., **37**, 2010, p. 1183.
4.BERMAN J., ZORRILLA-LÓPEZ U., FARRÉ G., ZHU C., SANDMANN G., TWYMAN R. M., CHRISTOU P., Phytochem Rev, 2014, in press
5.BARBINTA-PATRASCU M. E., UNGUREANU C., IORDACHE S. M., BUNGHEZ I. R., BADEA N., RAU I., J. Mater. Chem. B., **2**(21), 2014, p. 3221.
6.DIAS M. G., CAM ES M. F. G. F. C., OLIVEIRA L., Food Chem., **156**, 2014, p.37.
7.BROWN R.B., AUDET J., J. R. Soc. Interface, **5**, 2008, p. 131.
8.RAU K.R., GUERRA A., VOGEL, A., VENUGOPALAN V., Appl. Phys. Lett., **84**, 2004, p. 2940.
9.DI CARLO D., JEONG K.H., LEE L.P., Lab. Chip, **3**, 2003, p. 287.
10.IRIMIA D., TOMPKINS R.G., TONER M., Anal. Chem., **76**, 2004, p. 6137.
11.MC CLAIN M.A., CULBERTSON C.T., JACOBSON S.C., ALLBRITTON N.L., SIMS C.E., RAMSEY J.M., Anal. Chem., **75**, 2003, p. 5646.
12.SACHINDRA N.M., BHASKAR N., MAHENDRAKAR NS., J. Sci. Food Agric., **85**, 2005, p. 167.
13.UNGUREANU C., POPESCU S., PURCEL, G., TOFAN V., POPESCU M., SÎLÍGEANU A., PÎRVU, C., Mat. Sci. Eng. C., **42**, 2014, p. 726.
14.UNGUREANU C., FERDES M., Adv. Sci. Lett., **18**(1), 2012, p. 50.
15.SAKAKI H., NAKANISHI T., SATONAKA K.Y., MIKI W., FUJITA T., KOMEMUSHI S., J. Biosci. Bioeng., **89**, 2000, p. 203.
16.UNGUREANU C., FERDES, M., CHIRVASE, A. A., MOCANU, E., Chem. Eng. Transactions, **24**, 2011, p. 943.
17.BROOKMAN J., Biotechnol. Bioeng., **16**, 1974, p. 371.
18.WEBER R.W.S., ANKE H., DAVOLI P., J. Chromatogr. A, **1145**, 2007, p. 118.
19.COMAN I., MARES M., Micologie medicală aplicată, Ed. Junimea; Iasi, 2000, pp. 236-237.
20.WOJNAR L., KURZYDŁOWSKI K.J., Practical Guide to Image Analysis, ASM International, 2000, pp. 157-160.

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