ENHANCED RHODOTORULA SP. CAROTENOIDS PRODUCTION BY NATURAL ANTIOXIDANTS

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Carotenoids are substances with very special and remarkable properties that no other groups of substances possess and that form the basis of their many, varied functions and actions in all kinds of living organisms. Often traditionally thought of as plant pigments, the carotenoids have a much wider distribution and occur extensively also in animals and microorganisms. Carotenoid biosynthesis is a specific feature of the Rhodotorula species, Rhodosporidium and Phaffia genera.

The recovery of high added-value products from waste plant material has been an important issue with economic relevance for the pharmaceutical and food industries. Based on utilizations of natural antioxidants like polyphenolic compounds, it should be noted that there is very little information on the interaction between yeasts and polyphenols, although some researchers accept the fact that yeasts plays a direct part in the breakdown of these substances and recent studies sustain the existence of an inhibitory effect on carotenoids microbial biosynthesis.

Response of both strains to natural antioxidants was different and the level of the polyphenolic compounds show that it is possible to use them partially as a carbon energy source by the yeasts, as shown by the evolution of both mass quantity and yeast dry substance. Glucose was utilized at a higher rate in the case of antioxidants added and the total carotenoids produced by yeasts in the case of incubation with different initial concentrations of vegetal extract were higher with 66%.

***Keywords**: Rhodotorula, polyphenols, carotenoids, grape seed extract.*

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. 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 antioxidant or possible tumour-inhibiting agents. Carotenoids are important antioxidants, photoprotective agents and source of provitamin-A [1]. The antigenotoxic and anticarcinogenic properties of carotenoids have been reviewed [2]. Studies [3] have demonstrated that *Rhodotorula glutinis* is seif and non-toxic when used in feed. There are relationships between structure, the chemical and physical properties and the varied biological functions of carotenoids [4].

Carotenoid-synthesizing yeasts are aerobes and the culture conditions, such such as cultivation temperature [5], lightening [6], induced substances [7], and inhibitors [8] play important roles in the carotenoid-forming activity of yeasts. Optimization of the medium and environmental conditions is necessary in microbial fermentations to fully exploit the potential of selected microbial strains [9]. The conventional medium optimization is usually time-consuming and often fails to assess the additive response of the variables [10].

Given these factors, the aim of the present study is to verify whether any interaction occurs between yeasts and polyphenols, and if so, to test the *Rhodotorula* carotenoids production under the influence of yeast genera, culture medium and the natural antioxidants concentrations.

MATERIALS AND METHODS

Microorganisms *Rhodotorula glutinis 9.3.* and *Rhodotorula glutinis 9.1.* were maintained on malt agar slants at 4^0C and transferred every month.

Grape seeds from the *Chambourcin* type were selected, dried and sorted at dimension between 1-2 mm. The vegetal material before extraction was grounded, resulting a brown-red powder. Because of the various specific solvents reactions are different with each compound fractions, for grape seeds it was used a successive extraction of the vegetal material, first with ethyl-ether at a ratio solvent-to-sample 1:3.5, to degrease the vegetal material and to obtain the vegetal oils. The obtained residue was treated with ethanol for the extraction of polyphenolic compounds, at ratio 1:3.5, in order to obtain the alcoholic extract. All extracts were concentrated on a rotavapory to dryness and alcoholic extracts was later utilized in the study.

Determination of extractive yield was done in accordance with the organic substance quantity obtained from the extractive process, compared with the initial quantity of vegetal material. Total Polyphenols Content of the extracts was determined by the Folin-Ciocalteu method and expressed as mg eq. GAE /L. Determination of total extractive yield (T.E.Y) was done after Soxhlet extraction, when dry extracts were obtained by evaporating the solvent at room temperature and folowed by dryness at 150 º C, until it reached constant mass. Results are expressed as mg total extract/mL. For total anthocyanins a classic spectrophotometric method was used, based on the a pH- differential structure [13]. Concomitant to the determination of the total polyphenols, the non-tannin polyphenols were quantified, largely using the same procedure. The only difference was the use of a probe reaction using a solution of methyl – cellulose 0.4%, followed by the Folin-Ciocalteu reactive reaction [14]. Total flavonoids was measured by aluminum chloride colorimetric assay. The HPLC analyses were performed at room temperature using a 4.6 -250 mm Beckmann column (Ultrasphere) C18. The mobile phase was a mixture of two solvents A (water: formic acid 2.5: 97.5 ratio) and B (A : acetonitrile 20:80 ratio).

Polyphenols were added aseptically to the culture flask Erlenmeyer of 500 mL containing 100 mL of the medium, in concentrations of 8, 16, 32 and 64 g/L. After inoculation with the pre-cultivated cell suspensions of *Rhodotorula glutinis*, flask cultures were performed at 20^oC on a rotary shaker at 120 rpm. The culture broth was harvested by centrifugation at 10.000 g for 15 minutes and determining the biomass accumulations monitored the cell growth. The cells were harvested and then washed twice with distilled water. Acetone was added and then the mixture was vortexed for 10 sec. The acetone fractions were collected in a separatory funnel and hexane, saturated

NaCl, and water were added successively to extract the carotenoids. After separation, the hexane phase was withdrawn, and absorbance detection was conducted by using the spectrophotometer at 501 nm. The concentration of total carotenoids was calculated with an extinction coefficient of 2500. For HPLC analysis was used to determine the beta-carotene concentrations. For elution, a mixture of hexane and acetone (84:16, v/v) was used. Total proteins were determined thru Biuret method and the results were quantified thru a BSA standard cure and express as mg/mL [15].

RESULTS AND DISCUSSION

The physical and chemical characterization of the grape seeds extract (tab. 1) shows an extremely rich phenolic composition, which promotes the used raw material on a higher rank in the hierarchy of the available vegetable resources that contain polyphenolic compounds.

Tabel 1

Physical and chemical characteristics of the ethanolic grape seeds extract

Characteristics	Value
Extractive yield	5,2
Shape and colour	Clear liquid with a red-brown color
T.E.Y. (mg total extract/mL)	53,2
Flavones (mg/L)	791,03
Anthocyanis (mg/L)	240,36
T.P.C. (mg eq. GAE/L)	3975,11
Tannin polyphenols(mg eq. GAE/L)	1303,99

The results of HPLC analyses are shown as a typical chromatogram in fig. 1. Based on these experimental data, it is possible to draw the conclusion that the phenolic compounds in grape seed extract are represented primarily by gallic acid.

Growth and carotenoids biosynthesis of the yeasts *Rhodotorula glutinis 9.1* and *Rhodotorula glutinis 9.3* was studied as a function of medium supplementation with natural antioxidants from grape seeds.

The results, given as the units of total carotenoids concentration at the end of growth, showed that the presence of polyphenols in culture media influenced the biosynthesis and accumulation of these compounds depending on the used dose and yeast strain. Polyphenols inhibited the cell growth to some extent and the total concentration of carotenoids was smaller in this cases. As can be seen, when compare to control sample, the *Rhodotorula gracilis 9.3*. strain is much more sensitive to the presence of natural antioxidants in culture media. The level of total carotenoids reached a maximum at 8 g/L polyphenolic extract, but beyond this value the inhibition process is stronger as the maximum dose is reached. On the other hand, in the case of *Rhodotorula gracilis 9.1.* strain although the level of total carotenoids is smaller, she is much less sensitive to polyphenolic compounds.

As shown in fig. 3, the ratio of β-carotene, compared with control samples, increased with increasing vegetal extract concentration. The β-carotene content increased up to 85% when polyphenols were added to culture media at 16 g/L concentration for *Rhodotorula gracilis 9.3.* and with 81% for *Rhodotorula gracilis*

9.1 at 16 g/L. It was clearly observed that polyphenols added to a YPG medium inoculated with *Rhodotorula glutinis* 9.1 and 9.3. had an stimulatory effect on the β-carotene biosynthesis.

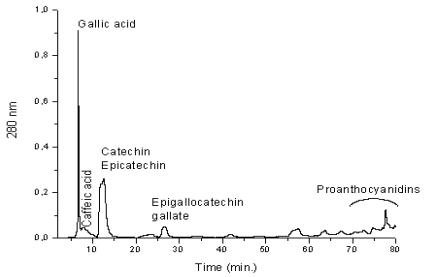

Figure 1**. Chromatogram of grape seed ethanolic extract**

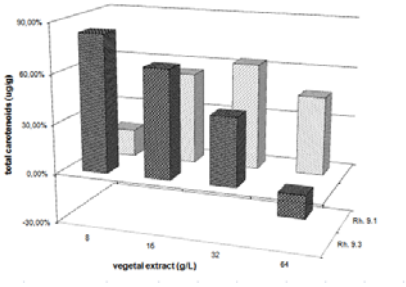

Figure 2. **The total carotenoids**

Figure 4 shows the growth behaviours of *Rhodotorula gracilis 9.1* and *Rhodotorula gracilis 9.3* in the media containing different concentration of grape seed extract. As can be seen in all cases the yeast biomass is grater than the controls' one, but we must keep in mind that polyphenols are substances that can form complexes with different molecules, including yeast cells walls. Among the possible mechanisms, there can be mentioned the physical ones (that involve the establishment of weak and reversible interactions between the polyphenolic compounds and the yeast wall) and the chemical ones (by co-pigmentation and condensation reactions between the yeast metabolic products and different polyphenols classes) (16).

As evident from figure 5, the level of the polyphenolic compounds had an ascendant evolution, but the evolution is still strongly influenced by the strain used. In this study, the *Rhodotorula gracilis* 9.1. strain produced more biomass and the level of polyphenolic compounds is smaller, than in the case of *Rhodotorula gracilis* 9.3.

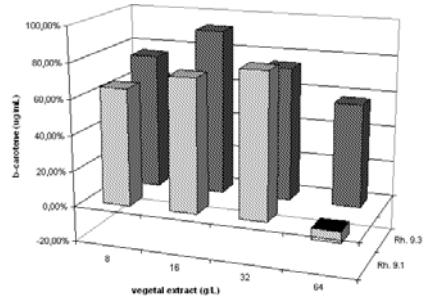

Figure 3. **The ratio of β-carotene**

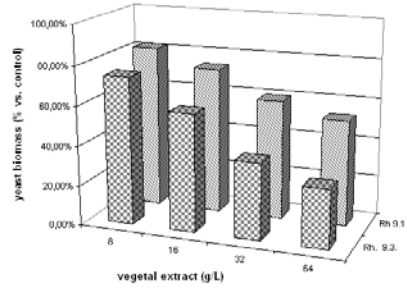

Figure 4. **Yeast biomass accumulation**

The pH-value of growth medium influences not only biosynthesis activity of culture, but also culture growth rate. The final pH significantly affected the growth and total carotenoids production properties of *Rhodotorula gracilis 9.1 and 9.3.* as shown in figure 6. As the pH decreased, specific growth and carotenoid production

rates increased more evident a polyphenol -dose- response being in the *Rhodotorula gracilis 9.1.*

However, a relationship between the ratio of carotenoids, the initial concentrations of polyphenols and the total proteins in the medium at the end of the process was observed (figure 7). This fact may lead to the conclusion that polyphenols determine, probably through pH reduction, the intensification of the yeast autolysis processes.

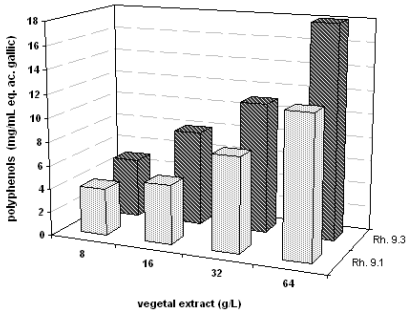

Figure 5. **Total polyphenolic compounds in YPG medium**

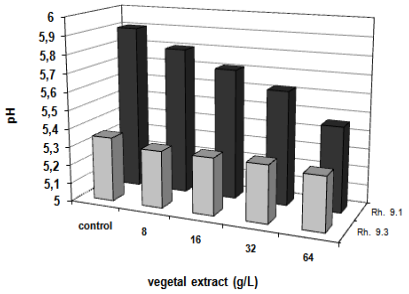

Figure 6. **pH level in YPG medium at the end of the process**

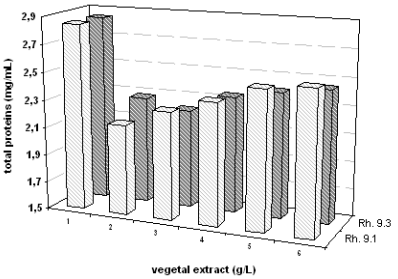

Figure 7. **Total proteins in YPG medium with polyphenols**

CONCLUSIONS

The results showed that the presence of polyphenols in culture media influenced the biosynthesis and accumulation of carotenoids depending on the used dose and yeast strain. It was clearly observed that polyphenols added to a YPG medium inoculated with *Rhodotorula glutinis* 9.1 and 9.3. had an stimulatory effect on the β-carotene biosynthesis.

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