TRANSFORMATION OF POLYPHENOLS FROM BIOMASS BY SOME YEAST SPECIES

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Concerns on the complex processing of biomass have increased lately, being mainly aimed at fractional separation of all compounds, based on biorefinery principles. This technique allows the isolation and complete recovery of both primary and secondary components, using various chemical or biochemical agents. The application of an aqueous extraction procedure leads to the recovery of various types of extracts from the vegetable biomass, which could have either inhibitory or stimulating effects on the growth of microorganisms. From this point of view, studies were carried out on the influence of the aqueous extracts containing aromatic compounds with polyphenolic structure, separated from spruce bark, *Asclepias syriaca* plant and red grape seeds, on the development of yeast species which biosynthesize carotenoid pigments. Thus, two strains of yeast belonging to the *Rhodotorula* genus were cultivated on media containing the above-mentioned extracts. The obtained results have shown a different behavior of the two strains, which may be explained by their characteristic features, but also by the composition and concentration of polyphenols specific to the biomass source used for extraction. It was also found out that the yeasts used the polyphenolic compounds as a carbon and energy source, their concentration being reduced when increasing the duration of cultivation. The metabolism of polyphenols can be correlated with the carotenoid pigment content and composition.

Keywords: spruce bark, *Asclepias syriaca*, red grape seeds, aqueous extracts, polyphenolic compounds, *Rhodotorula sp.*

INTRODUCTION

The recovery of substances with antioxidant and nutritional potential from plant biomass is an economic issue with special relevance to pharmaceutical and food industries. At this moment, scarce information is available on the use of polyphenolic compounds in yeast fermentation. However, some research[1] reveals that yeasts have the ability to fragment and use polyphenolic compounds as a carbon source.

Polyphenols include several classes of compounds, such as phenols, phenolic acids, flavonoids, anthocyanins, and others, with more complex structures – tannins and lignins. Polyphenols are secondary metabolites produced by plants in response to stress conditions, such as infections, large amounts of UV rays or other factors.

So far, many research groups have attempted an efficient separation of polyphenol compounds from waste plant material, but in very few cases a qualitative and quantitative characterization of such compounds has been made.

Oxidized polyphenols also inhibit growth and development of certain microbial strains. The toxicity mechanism of polyphenols may be explained by the inhibition of hydrolytic enzymes, or by other interactions, such as blocking protein transport, non-specific interactions with carbohydrates, etc.[2]

Asclepias syriaca is a native plant of North America, with large, opposite elliptical leaves, containing a large amount of latex toxic to animals. In Romania, it is commonly known as "bee's flower", being cultivated as an ornamental and bee plant, but also

growing in the wild, in Ostrovul, Moldova Veche.[3] The plant, selected and cultivated for its high content of hydrocarbons, was used as a model for the complex processing of biomass. [4]

Dănăilă *et al*.[1] tested the influence of an ethanolic extract derived from grape seeds on the development of the yeast *Rhodotorula glutinis* 9.3. The authors used this extract in the culture medium in different concentrations, to study its influence on both biomass yield and carotenoid pigment biosynthesis. The researchers concluded that the extract could be used as an additional carbon source for the growth of these yeasts.

Another waste material was spruce bark, used for several considerations, related to economic and environmental protection. Each year, timber processing plants spread hundreds of kilograms of spruce bark on agricultural areas covering cultivated land. Dănăilă *et al*.[1] conducted some studies to optimize the extraction of this plant material with different solvents, including water, and tested these extracts by using them as a carbon source for the growth of yeast with technological importance. The results were favorable, these extracts being degraded to a simple carbon source by yeast enzyme systems.[5]

On the other hand, after the extraction with hot water, the inorganic salts, oligosaccharides, sugars and polyphenols[6] are removed, thus resulting extracts that can be successfully used as a carbon source in fermentation processes.

Another property of the *Rhodotorula* yeast species is to metabolize polysaccharides.[7]

To test the influence of polyphenolic compounds on the growth and biosynthesis of carotenoid pigments by *Rhodotorula* yeast, aqueous extracts obtained from the above-mentioned sources were used.

Thus, the consumption of polyphenolic compounds from the culture medium was observed, and their influence on the biosynthesis of carotenoid pigments was assessed.

EXPERIMENTAL

Two different yeast strains of *Rhodotorula sp*., denoted by R1 and R2, selected and purchased from Biotechnology Applied in Food Industry – Integrated Center for Research and Education – Bioaliment, "Dunarea de Jos" University of Galati, were cultivated. Prior to the experiment, yeast was cultivated in a medium with the following composition: 10 g/L glucose, 5 g/L peptone, 3 g/L malt extract, 3 g/L yeast extract. Fermentation was carried out on a thermostated stirring platform for 48 h, at 27 ºC and 120 rpm. The cells were recovered by centrifugation at 5000 rpm for 15 min, washed twice with distilled water and inoculated on a culture medium with the following composition: 15 g/L glucose, 2.5 g/L yeast extract, 3 g/L sodium acetate, 1 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.1 g/L $CaCl_2$, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.015 g/L $ZnSO_4$, 0.015 g/L $CuSO_4 \cdot 5H_2O$.

The components were dissolved in extracts with different contents of total polyphenols (Table 1), after which the culture medium was distributed in 100 mL volumes, in 250 mL Erlenmayer flasks, and inoculated with yeast. To determine the number of cells used for inoculation optical density was read at 620 nm. An absorbance of 0.5 is equivalent to 10^7 cells/1 mL inoculum.[8] Each sample was inoculated with $4x10^7$ CFU (CFU = colony forming unit). The experiment was carried out for each concentration of polyphenols extract from the plant material by a method described in a previous paper.[9]

The aqueous extraction for chemical characterization was realized with 20 g of dried material and 125 mL of distilled water, at 70 °C, for 45 min. The extraction was repeated until the water extract was colorless and the extracts were cumulated to a volume of 500 mL, with distilled water. The aqueous extracts were then subjected to analysis for determining the composition of total polyphenols, flavones, flavonoids, anthocyanins and tannins (Table 1). Also, the aqueous extract obtained was concentrated to 30 mL and fractionated by liquid-liquid extraction using ethyl acetate prior to HPLC analysis.

Estimation of total amount of phenolic compounds, tannins, flavonoids, flavonols and antocyanins

The total phenolic content of plant extracts was determined by the Folin-Ciocâlteu method. About 1 mL of extract was mixed with 500 µL of the Folin-Ciocâlteu reactive, 2 mL of 10% sodium carbonate and 5 mL of water. The mixture was shaken thoroughly and allowed to stay for 90 min. Then, the absorbance at 765 nm was determined against a blank, containing all reagents without samples or gallic acid, under the same conditions. The total phenolic content was expressed as the number of equivalents of gallic acid (GAE).

The total content of tannins was determined using the Folin-Ciocâlteu reactive. About 10 mL of diluted extracts (solution 1, S1) were mixed with 100 mg of casein by shaking for 2 h (adsorption of tannins) and then filtered (solution 2, S2). The total phenolic content for both solutions, S1 and S2, was determined with the Folin-Ciocâlteu method, as described before. The difference between the absorbancies of S1 and S2

corresponds to the concentration of casein-adsorbed tannins in the sample. The total casein-adsorbed tannins are expressed as the number of equivalents of gallic acid (GAE).[10]

The contents of flavonoids and flavonols were determined by the aluminium chloride method, using rutin as a reference compound.[10-11]

The antocyanins content was determined with the pH differential method described by Ribereau-Gayon.[12] The principle of this method involves decreasing of the extracts pH to values between 0.5 and 0.8, which causes transformation of all anthocyanins into a flavilium cation, which is red in colour. 1 mL of extract was pipetted into two tubes, and 1 mL of a 0.01% HCl solution in 95% ethanol was added to each tube. Further on, 10 mL of 2% aqueous HCl solution were added to the first tube (A1), and 10 mL of solution with pH 3.5 (prepared from 0.2M Na_2HPO_4 and 0.1M citric acid) to the other tube (A2). The absorbancies of both samples were measured at 520 nm against the blank sample (water instead of extract). The content of total anthocyanins mg/L:(A1-A2)xf; f = 396.598.

Extraction of carotenoid pigments

The amount of yeast wet biomass resulting from centrifugation was treated with 3 mL DMSO, and left at -20 °C for 24 h, after which it was subjected to sonication for 15 min and centrifuged, the supernatant being recovered in a centrifuge tube.

The procedure was performed 3 times, to destroy the whole yeast cell wall. After the DMSO treatment, the residual biomass was treated with acetone until it became colorless. The phases separated by acetone were mixed with those obtained with DMSO.

In the tube with both phases, 20% NaCl and 2 mL hexane were added, for achieving liquid-liquid extraction, until hexane became colorless. The hexane phases were collected and brought to the volumetric flask, to report the concentration of total carotenoid pigments. After extraction, the samples were stored at -20 °C until UV-VIS analysis.

Determination of HPLC polypenols

A reversed-phase high-performance liquid chromatographic technique was developed to identify and quantify the major phenolic compounds contained in the aqueous extracts obtained from bark of *Picea abies*, red grape seeds of *Vitis vinifera* (Merlot) and *Asclepias syriaca* plant. To this end, a standard mixture solution of phenolic compounds was used.

Sample concentrations were calculated based on peak areas, and compared to those of each of the external standards. The HPLC chromatograph was a Dionex UltiMate 3000. The column was a Dionex Acclaim 120, C18 RP (4.6x150 mm, particle size 5 μm), and temperature was maintained at 30 °C. The flow rate was of 0.5 mL/min. The mobile phase used was 1% acetic acid in water (A) *versus* 1% acetic acid in methanol (B), for a total running time of 30 min, and the gradient was modified as follows: solvent B started at 10%, and increased to 40% within 30 min.

Table 1
Total amount of phenols, tannins, flavonoids, flavonols and antocyanins for concentrated extracts

Aqueous extracts from	Total polyphenols (mgGAE/100 g)	Tannins (mgGAE/100 g)	Flavonoids (mgRE/100 g)	Flavonols (mgRE/100 g)	Antocyanins (mg/L)
Bark of *Picea abies*	517.95	164.40	22.63	8.13	-
Red grape seeds of *Vitis vinifera* (Merlot)	506.25	198.38	27.73	7.11	18.52
Asclepias syriaca	287.85	159.27	8.04	9.02	-

UV-VIS measurements

Carotenoid pigment concentration, determined by reading sample absorbance at 450 nm, on a UV-VIS spectrometer, was calculated with a standard curve of β-carotene in hexane and expressed in mg pigment/g dry biomass.

RESULTS AND DISCUSSION

Estimation of total amount of phenolic compounds, tannins, flavonoids, flavonols and antocyanins

The concentrations of different classes of polyphenols were determined by the Folin-Ciocâlteu method for total phenols and tannins, by the aluminium chloride method, for the estimation of flavonoids and flavonols, and by a pH differential method, respectively, for the determination of antocyanins.

The concentration values for all extracts are presented in Table 1. The higher amount of total polyphenols (518 mg/100 g) was obtained for the *Picea abies* bark extract GAE, followed by *Vitis vinifera* red grape seeds (Merlot) (506 mg/100 g GAE), and

Asclepias syriaca aqueous extract (287 mg/100 g). The *Vitis vinifera* red grape seed extract shows a higher content of flavonoids and flavonols, compared to the *Picea abies* bark and *Asclepias syriaca* extracts. The antocyanins content for the red grape seeds of *Vitis vinifera* was of about 19 mg/100 g, while in the other two extracts, this class of compounds was not present.

HPLC determination

For the examination of extracts, 8 representative polyphenols (Fig. 1) were selected, and their contents were determined by reversed-phase HPLC, coupled to diode-array detection. The phenolic profiles of the aqueous extracts recorded at 280 nm are presented in Figures 2 to 4.

The major compounds of grape seed extract were gallic acid and catechine. Other representative compounds, with high intensity, may be observed, assumed to correspond to epichatechine, epicatechin gallate or other oligomers encountered in grape seeds.[13]

Vanillic, syringic and p-cumaric acids were the main compounds present in *Asclepias syriaca* extracts, but their concentrations were quite low, ranging from 0.11 to 0.98 mg/100 g GAE.

The phenolic compounds identified in the *Picea abies* bark extracts were gallic acid, catechine and vanillic acid. Several minor peaks, which could indicate the presence of different procyanidins, 518 mg/100 g, were also detected.

The aqueous extracts used to obtain the culture medium were diluted and brought to a 1 L volume. The amount of total polyphenols in these extracts is given in Table 3. For each plant material, extracts with two different concentrations of total polyphenols, equivalent to the quantity resulted from 0.5 and 5 g of extracted plant material, were made.

Behavior of polyphenolic compounds in culture medium

The data obtained[9] have shown that the polyphenolic compounds influence the development of two strains of yeast and, as shown in Figures 5A and B, the concentrations of these polyphenolic compounds decreasing continuously until the end of the experiment, whichever their concentration in the culture medium.

The different composition in polyphenolic compounds of the aqueous extracts, presented in Table 2, influences differently the evolution of the two strains of *Rhodotorula* yeasts, which is also the case of the yeast cultivated on a medium obtained with aqueous extracts from red grape seeds (Figs. 6A and B).

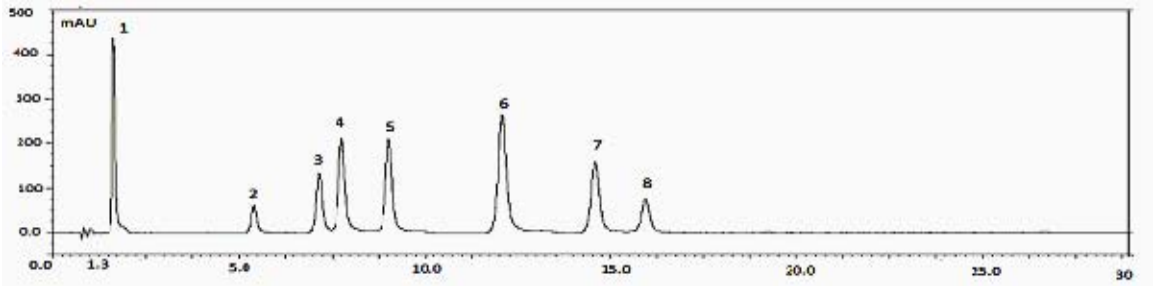


Figure 1: Typical chromatogram at 280 nm obtained for polyphenol standards. Identified compounds of peaks 1-8 are gallic acid, catechine, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid and sinapic acid, respectively

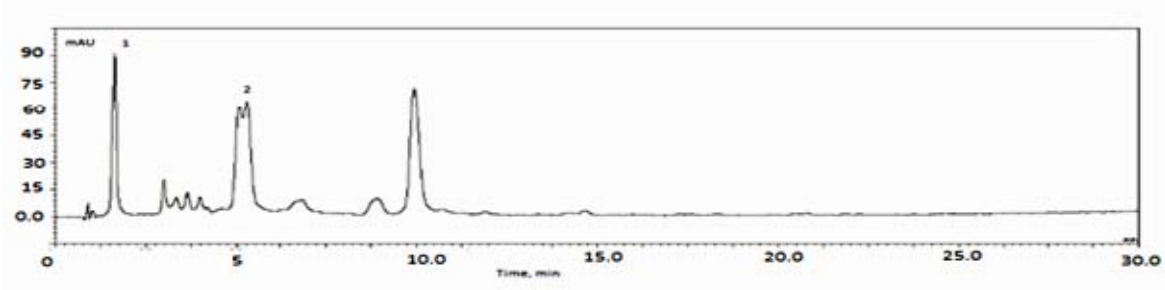


Figure 2: HPLC profile of grape seed aqueous extract; identified compounds: 1 – gallic acid; 2 – catechine

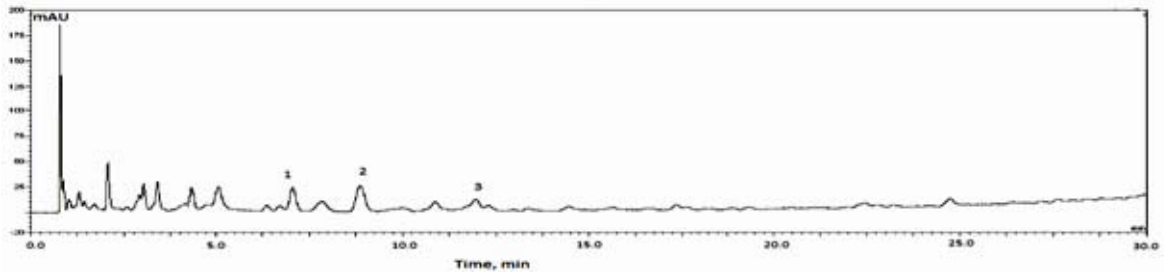


Figure 3: HPLC profile of *Asclepias syriaca* aqueous extract; identified compounds: 1 – vanillic acid; 2 – syringic acid; 3 – p-coumaric acid

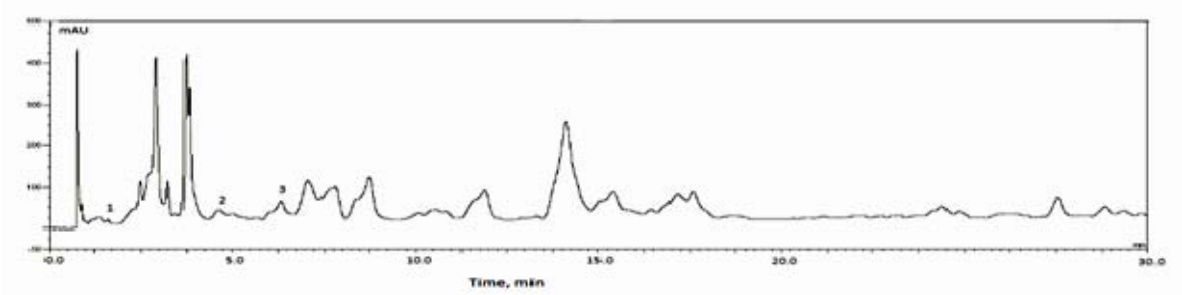


Figure 4: HPLC profile of *Picea abies* bark aqueous extract; identified compounds: 1 – gallic acid; 2 – catechin; 3 – vanillic acid

The reduction in the concentration of polyphenols during yeast cultivation takes place at different rates, depending on its initial value, which was influenced by the quantity of material used for extraction.

When using an aqueous extract with a concentration in total polyphenols equivalent to that obtained from 0.5 g grape seeds, the decrease in concentration until the end of the process for the two strains is of about 20 mg/L, while when the yeasts were grown on a medium prepared using extract with a concentration of total polyphenols equivalent to that obtained from 5 g grape seeds, the concentration of total polyphenols was reduced by 55 (Fig. 6A) and 100 mg/L (Fig. 6B). Therefore, the polyphenols present in this extract are consumed by yeast, being used as a carbon source.

Figure 7 shows that the extract with a concentration in total polyphenols equivalent to that obtained from 0.5 g bark of *Picea abies* is used preferentially by the R2 strain (Fig. 7B), prompting the use of this extract as a carbon source in yeast cultivation.

In the case of strain R1, the consumption of polyphenols with a rate approximately equal for both concentrations of the extracts used may be noticed (Fig. 7A). If one chooses to use polyphenols as a carbon source, the choice of concentration for the extract used to prepare the culture medium should be correlated with the biomass yield as final results.

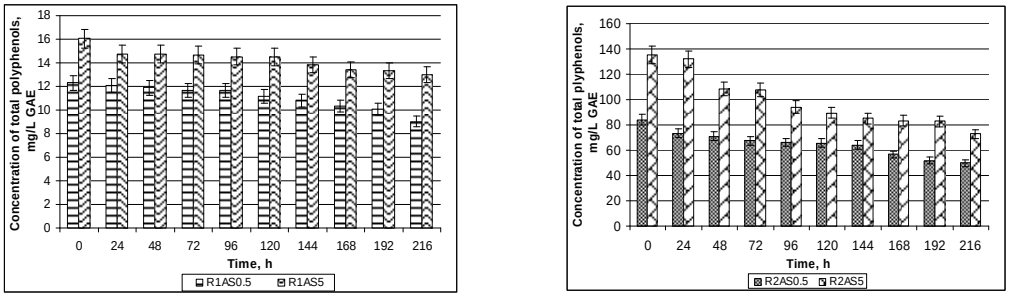
The metabolism of polyphenolic compounds may be a characteristic of the *Rhodotorula* yeast, as confirmed by literature data referring to the degradation of simple polyphenolic compounds. Thus, Gupta *et al.*[14] studied sinapic acid degradation by the *Rhodotorula glutinis* yeast.

Table 2
Concentrations of individual phenolic compounds in *Picea abies* bark, *Asclepias syriaca* and grape seed extracts (mg/100 g dry plant)

Phenolic compounds	*Picea abies* bark extracts	*Vitis vinifera* red grape seed extract (Merlot)	*Asclepias syriaca* extract
Gallic acid	3.19	6.12	-
Catechine	31	44.36	-
Vanillic acid	39.4	-	0.87
Syringic acid	-	-	0.11
p-Coumaric acid	-	-	0.11

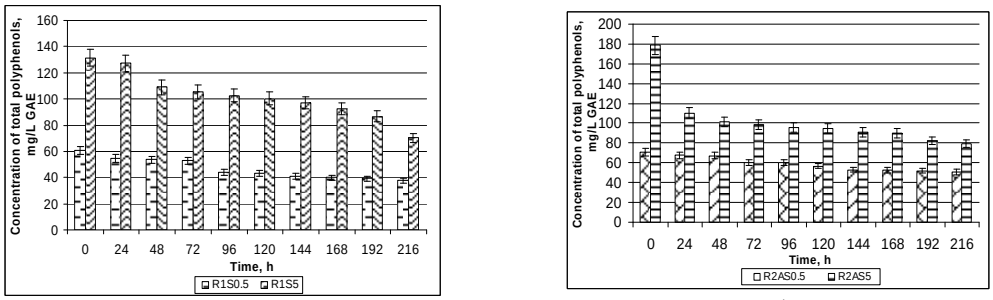
Table 3
Total polyphenols content in the extracts used for yeast cultivation

Aqueous extracts	Total polyphenols content in the extract from 0.5 g plant material (mg/L GAE)	Total polyphenols content in the extract from 5 g plant material (mg/L GAE)
Picea abies bark extract	89.98	148.78
Vitis vinifera red grape seed extract (Merlot)	69.19	143.39
Asclepias syriaca extract	53.47	112.93



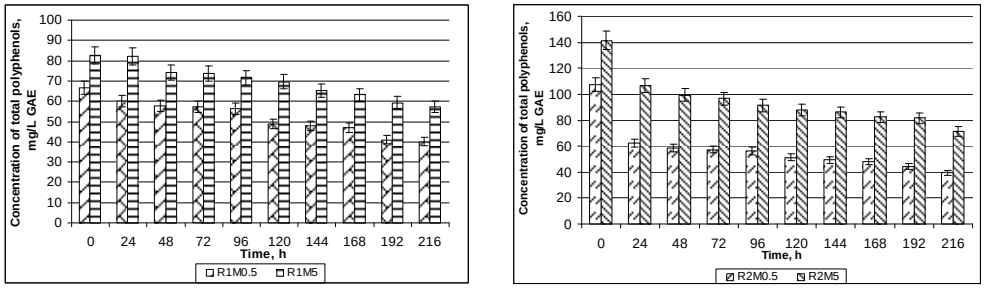
A) B)

Figure 5: Variation of total polyphenols concentrations during yeast cultivation on medium containing aqueous extract from *Asclepias syriaca* plant; A) R1 strain, B) R2 strain



A) B)

Figure 6: Variation of total polyphenols concentrations during yeast cultivation using medium containing aqueous extract from red grape seeds of *Vitis vinifera* (Merlot); A) strain R1, B) R2 strain



A) B)

Figure 7: Variation of total polyphenols concentrations during yeast cultivation using medium containing aqueous extract from bark of *Picea abies*; A) strain R1, B) R2 strain

It was found out that the sinapic acid was completely consumed only in the presence of glucose in the culture medium, because yeast requires an additional carbon source to activate the enzyme complex. The above-mentioned authors proposed a reaction mechanism for the formation of intermediates such as syringic acid, 3-O-methyl gallic acid, gallic acid and 2,6-dimethoxy-1,4-benzoquinone (Fig. 8).

At the same time,[15] after numerous experiments carried out with bacteria and

fungi, a mechanism of vanillin degradation from vanillic acid under the action of vanillin dehydrogenase was proposed. Vanillic acid was then converted into protocatechuic acid by demethylation, and metabolized by the enzymatic attack of the aromatic ring (Fig. 9).

Such literature data advocate for the degradation of polyphenols from the plant extracts studied by similar mechanisms – information that will be ascertained in further studies. Also, they highlight the role that glucose may play as a primary carbon source for the necessary degradation enzymes and for the metabolism of polyphenolic compounds.

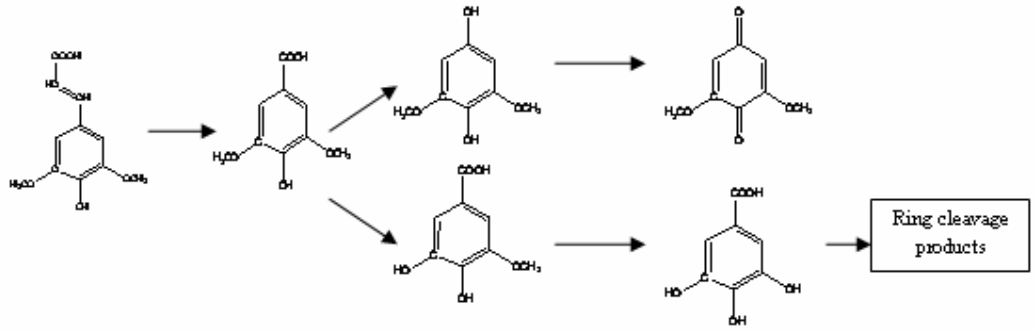


Figure 8: Proposed sequence of reactions for the degradation of sinapic acid[14]

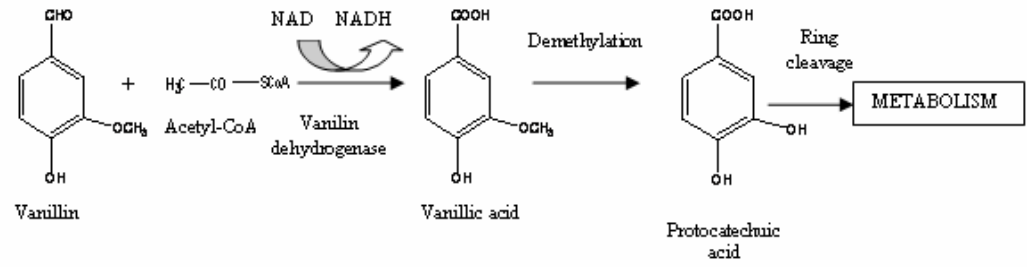


Figure 9: Proposed pathway of vanillin metabolism[15]

Variation in concentration of carotenoid pigments biosynthesized by yeast

The two species of *Rhodotorula* yeast (R1 and R2) were cultivated in media containing aqueous extracts of *Asclepias syriaca*, *Vitis vinifera* red grape seeds (Merlot) and *Picea abies* bark, with different contents of total polyphenols. A variation of polyphenols concentration has been carried out with different amounts of plant material for extraction, 0.5 and, respectively, 5.0 g (denoted by AS05, AS5, S5 S05, M05 and M5).

The results on the content of carotenoid pigment synthesized by these two yeast species during cultivation are shown in Figures 10A-F. When using extracts of *Asclepias syriaca* (Figs. 10A and B), it was observed that the R1 species produced lower amounts of carotenoid pigments, compared to the control sample, in which microorganisms were grown on a standard medium.

At the same time, the R2 species was more productive than R1 in this extract, the content of carotenoid pigment coming closer to that of the control, or overreaching it after 192 h. After this period, a different behavior was observed, probably caused by the changing composition of the culture medium as a result of the carbon source metabolism. Meanwhile, a continuous increase of the cultivation time, up to 120 h, manifested by an increasing concentration of the polyphenol extract, could be noticed.

The utilization of an aqueous extract of *Vitis vinifera* seeds determined a different behavior of the two yeast species (Figs. 10 C and D). Thus, the R1 species produced large quantities of carotenoid pigments in all situations, in comparison with the R2 strain. Lower concentrations of polyphenols were favorable to the R1 species in the

biosynthesis process. Therefore, the R2 species biosynthesized a maximum content of carotenes, at high concentrations of the extract, after 120 h.

When yeast was grown on a medium containing extracts of spruce bark, inhibitory effects were observed in terms of carotenoid pigment biosynthesis (Figs. 10E and F), especially in the case of yeast R1. This phenomenon can be correlated with the particular species of yeast, and also with the composition of polyphenols in the extract. The R2 species behaves differently, and produces a greater quantity of pigment, compared with the control, after 48 h, when grown in a culture medium with a lower content of polyphenols (variant M 0.5). Subsequently, the pigment content is reduced with increasing the duration of cultivation, at higher values of extract concentration.

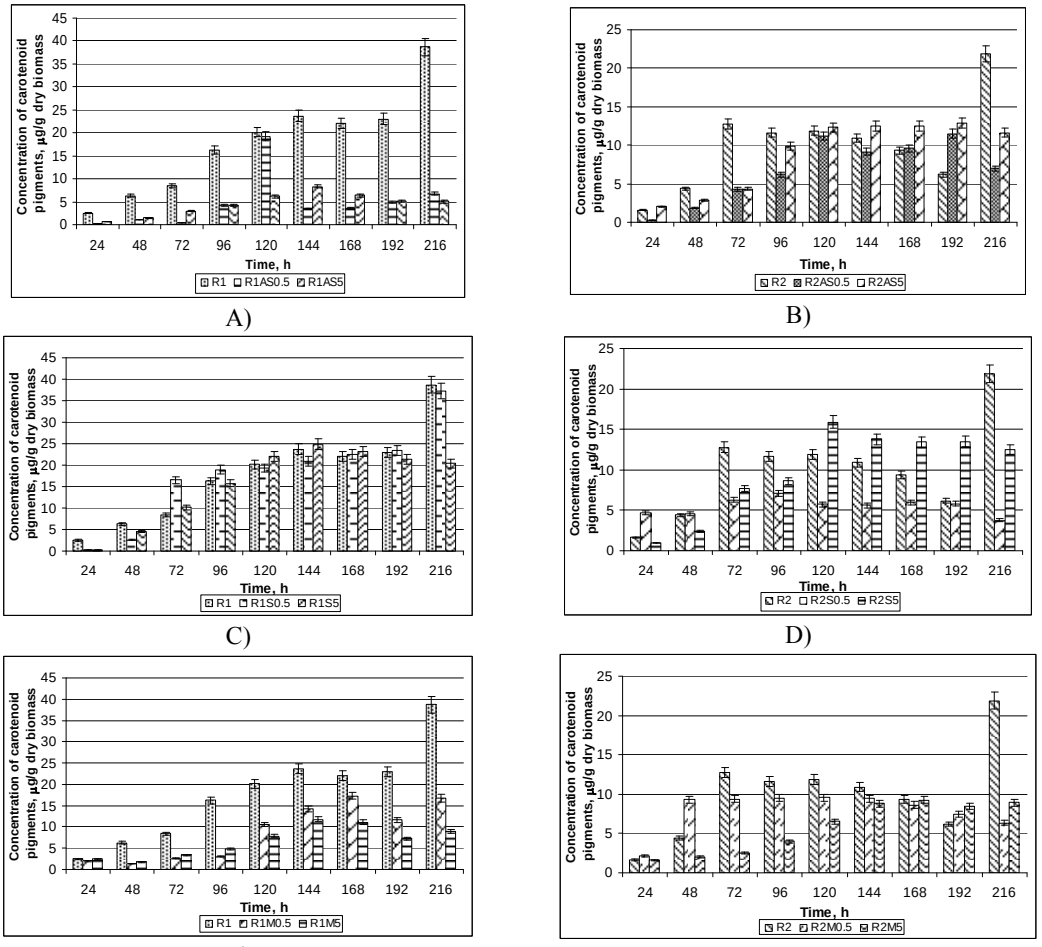


Figure 10: Variation of carotenoid pigment content during the process; A) R1 in aqueous extract of *Asclepias syriaca*, B) R2 in aqueous extract of *Asclepias syriaca*, C) R1 in aqueous extract of *Vitis vinifera* red grape seeds (Merlot), D) R2 in aqueous extract of *Vitis vinifera* red grape seeds (Merlot), E) R1 in aqueous extract of *Picea abies* bark, F) R2 in aqueous extract of *Picea abies* bark

One can therefore estimate that the use of plant extracts in culture media highlights the particular behaviors of yeast, as depending on the nature of species, composition and concentration of polyphenols. Thus, for attaining maximum efficiency in carotenoid pigments, the R2 species is recommended, with an aqueous extract of red grape seeds with a total polyphenol concentration of 143.39 mg/L GAE. Only in this situation, significant amounts of carotenoid pigments can be obtained.

CONCLUSIONS

Aqueous extracts were obtained and characterized using spruce bark, red grape seeds and *Asclepias syriaca* plant as raw materials. The highest concentrations in the total polyphenolic compounds were found characteristic of spruce bark extracts,

followed by those of red grape seeds of *Vitis vinifera* (Merlot) and *Asclepias syriaca*. Also, differences in the compositional spectrum of polyphenols were evidenced.

Polyphenol extracts with different concentrations were used to obtain the culture medium for two species of *Rhodotorula sp*. yeast. The results recorded showed that yeast consumes the polyphenolic compounds. The decrease in their concentration during the process is specific to yeast species, and can be correlated with the specific concentration and composition of polyphenols in the used extracts.

From this perspective, red grape seed extracts are efficiently consumed by yeasts. Polyphenolic compounds are successfully degraded by the enzymatic equipment of the *Rhodotorula* yeasts. According to literature, simple polyphenolic compounds are metabolized in the presence of glucose by *Rhodotorula glutinis*, through a mechanism to be discussed in further studies, on tested plant extracts.

With respect to the effect of the compounds from aqueous extracts of *Asclepias syriaca*, they affect adversely the biosynthesis of carotenoid pigments, compared to the control.

Aqueous grape seed extracts have a positive effect on the biosynthesis of carotenoid pigments for the two strains.

Aqueous extracts of spruce bark can be used only for the development of strain R2, when positive results are obtained after 48 h of culture, compared to the control, as for strain R1, these extracts have a negative effect on the biosynthesis of carotenoid pigments.

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