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# Biological Active Compound Biosynthesis in a $Cu^{2+}$-Contaminated Culture Media

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## ABSTRACT

In the present study we monitor the effect of copper-contaminated culture media on development and active compound biosynthesis of two *Rhodotorula mucilaginosa* strains, assigned as R1 and R2. The media was prepared in aqueous extracts of *Asclepias syriaca* stems and different copper ions concentrations were added. The data reveals that the presence of 10 mg/ L $Cu^{2+}$ for the *Rhodotorula mucilaginosa* strain-1 (R1) and 50 mg/ L $Cu^{2+}$ for the *Rhodotorula mucilaginosa* strain-2 (R2) strain fermentation increases the biomass yield (24.7 g and 14.8 g wet biomass/ L culture medium, respectively) and biosynthesis of carotenoid pigments (approximately 1.4 µg/ g dry biomass) compared with references. The chelating properties of polyphenol compounds from aqueous extracts of *Asclepias syriaca* stems was confirmed by detection of 17.9 mg/ L $Cu^{2+}$ in extracts with 100 mg/ L $Cu^{2+}$ added, after 24 hours at 4°C. In the residual culture media, we have determines that the $Cu^{2+}$ concentrations decreased at 14.0 mg/L for *Rhodotorula mucilaginosa* strain-1 (R1) and 13.8 mg/ L for *Rhodotorula mucilaginosa* strain-2 (R2) strain fermentation, respectively, which means that the copper was trapped by the yeasts cells. Likewise, the polyphenols are digested and used as a carbon source. These results bring a significant contribution to the possibility of yeast fermentations in a low cost-vegetal polyphenol and copper ions system.


**Keywords:** *Asclepias syriaca*; Carotenoid pigments; Copper ions; Folin-ciocalteu method; Polyphenols; *Rhodotorula mucilaginosa*

## INTRODUCTION

Asclepias species has a high potential as an alternative crop. *Asclepias syriaca* can provide a proper development home for Monarch butterfly Danaus plexippus larval stage and the development of this species in the world [1] beside the economically aspects of providing latex for rubber production and biodiesel from the seed's oil. The silky seed floss is currently used to produce hypoallergenic pillows, comforters, and insulating fibre that can be used as a substitute for wood in pressed panels [2]. The de-flossed seeds have in turn been used to develop skin care products and nematocides/pesticides and contain potentially valuable health-benefitting lipids (e.g. specifically the sterols and fatty acids) [3].

Milkweed seeds contain 21% oil and 30% crude protein (dry basis). The oil is similar in quality to soybean oil. The dominant protein classes are water-soluble (22%) and salt-soluble (15%). The kernel contains 39% protein, which accounts for 45% of total protein, including 22% albumins which the water-soluble [4]. These results show that milkweed seed proteins have functional properties as thickeners, protein extender in adhesives or emulsifier in paints. Bleached milkweed seed oil consists in predominately unsaturated fatty acids (34% oleic, 50% linoleic, 1% linolenic) and has very low oxidative stability, but the oil could be used as an alternative triglyceride source for biodiesel [5]. Also, some compounds isolated from Asclepias subulata. Have anti-proliferative properties being highly selective to human cancer cells lines [6]. Despite numerous uses listed the entire plant is not completely exploited. In this perspective in previous studies performed in our research group, a crude aqueous extract from the plant's stems containing polyphenol compounds was used as a plants growth regulator and microorganism mediator. The aqueous extracts modulate some heavy metals bioaccumulation in plants and also the biosynthesis of photosynthesizing pigments, plant growth and development [7].

Copper is an essential micronutrient present in the active centre of some enzymes like Cu/ Zn superoxide dismutase, ascorbate oxidase, polyphenol oxidase and cytochrome C oxidase. Without this element, the plants growth becomes very difficult [8]. Also copper acts as cofactor in the catalytically biological processes [9]. Alternatively high concentrations of copper interfere with the development and metabolic systems of organisms [10]. On the opposite presence of free copper ions in high amounts in natural systems becomes one of the most severe environmental problems today.

Carotenoid pigments have anti-oxidant properties, protecting cells from free radicals, preventing lipid peroxidation and promoting the metabolism homeostasis [11]. The carotenoids are abundantly found in fruits and vegetables such as pomegranate [12], carrots, tomatoes [13], apricots, jackfruits [14], but are also biosynthesised by algae [11] and by microorganisms like Rhodopseudomonas palustris [15], Sporobolomyces ruberrimus [16], Rhodobacter sphaeroides, Dunaliella, Rhodospirillum rubrum, Rhodotorula, Phaffia rhodozyma, Sporidiobolus pararoseus [17] etc. Since the main amounts of carotenoids are provided by chemical synthesis which does not meet population demands for natural foods, cosmetics and vitamin supplements, the biosynthesis of pigments from microbial sources has received greater interest lately.

Therefore the aim of this paper was to assess the synergic influence of an A. syriaca polyphenol aqueous extract and copper ions ($Cu^{2+}$) on two *Rhodotorula mucilaginosa* strains (assigned as R1 and R2) growth and development in a stressful media. Also, the carotenoids biosynthesis capacity of the yeasts was monitored in order to increase this biological active compound production.

## MATERIALS AND METHODS

### Microorganisms

Two *Rhodotorula mucilaginosa* yeast strains, assigned R1 and R2 were isolated and selected by Integrated Centre for Research Bioaliment, Biotechnology Applied in Food Industry, "Dunarea de Jos" University of Galati. Molecular identification was achieved in Department of Applied Biology, Section of Microbiology & Industrial Yeasts Collection DBVPG and University of Perugia [18].

### Chemicals

All the chemicals used were provided by Sigma-Aldrich.

### Aqueous extraction and experimental assay

The *Asclepias syriaca* biomass was obtained from an energetically crop (2008) in a research program of "Gheorghe Asachi" Technical University of Iasi [7]. The plant stems were grinded to 1 - 2 mm and 15 g of grounded material was extracted with 100 ml distilled water in a simple bath system, at 80°-C for 45 min. The aqueous extraction was repeated until the extract was colourless. All the extraction fractions were collected and cumulated to a final volume of 1000 ml using distilled water.

In the final extract, four different copper sulphate concentrations (10, 25, 50 and 100 mg $Cu^{2+}$/ L) were added. The solutions were kept at 4°C for 24h and then the $Cu^{2+}$ and total polyphenol concentrations

were evaluated. In order to assess the effect of the polyphenol and $Cu^{2+}$ on the yeasts development and biosynthesis of carotenoid pigments, the basic and minimal components of yeast culture medium were dissolved in this $Cu^{2+}$-polyphenol system and the fermentations of the *Rhodotorula mucilaginosa* yeast strains were performed.

### Culture medium preparation and fermentation conditions

Pre-culture medium was obtained following a previously published protocol [19]. The experimental culture medium was prepared in the *Asclepias syriaca* aqueous extracts containing also (g/ L): glucose - 1; $KH_2SO_4$ - 1; $(NH_3)_2SO_4$ - 1; $CaCl_2$ - 0.05; then it was sterilized for 20 minutes at 110°-C distributed 100 ml each in Erlenmeyer flasks and inoculated. The fermentation process was monitored for four days. Every 24 hours, the wet biomass amount, pH value, the total polyphenol concentration, free copper ions in the culture medium and the concentration of carotenoid pigments extracted was determined.

### Aqueous extracts characterization

The aqueous extract was characterized by the total polyphenol content using the Folin Ciocalteu method.

### Biomass determination and pH measurements

The wet biomass was separated by centrifugations at 5000 rpm for 20 min and the dry biomass was determined using a protocol adapted from Tinoi et al. [20], by drying at 105°-C until there were no variations in weight. At every 24 hours, the culture media pH was measured.

### $Cu^{2+}$ determination

The $Cu^{2+}$ concentrations were monitored using a GBS Avanta atomic absorption spectrophotometer, following a protocol reported by Stingu et al. [8]. The wavelength used for the measurement was 324.70 nm and the calibration curves were obtained in a range of 0.02- 5.00 µg/ ml $Cu^{2+}$. The equation used was $y = 0.059x + 0.0016$, $R^2 = 0.9996$ and the concentrations were expressed in µg $Cu^{2+}$/ ml residual culture medium after biomass separation by centrifugation.

### Carotenoid pigments extraction and quantification

The carotenoid pigments extraction and quantification were carried out using a previously published protocol [19].

## RESULTS AND DISCUSSION

### Determination of polyphenol concentration in A. syriaca herbal aqueous extract. The polyphenol chelating properties

The total polyphenol concentration in the aqueous extract obtained from 15g of *Asclepias syriaca* plant stems was evaluated as being 213.58 mg GAE/ L of extract. The polyphenol extract has been supplemented with $Cu^{2+}$ (10, 25, 50 and 100 mg $Cu^{2+}$/ L) and kept at 4°-C for 24h. After 24h the polyphenol and $Cu^{2+}$ concentrations were determined. The confirmation of the polyphenol compounds chelating properties was obtained by measuring the polyphenol and $Cu^{2+}$ concentrations, both having decreased. The sample with the highest copper ions concentration, 100 mg/ L $Cu^{2+}$, had only 17.892 mg/ L free form $Cu^{2+}$ after 24 hours (Figure 1a). The same situation was observed for the polyphenol concentrations (Figure 1b).

In agreement with previously published studies, we found that the metal chelation leads to polyphenol redox cycle interruptions by engagement of all the coordination active sites and the formation of insoluble complexes [21]. The complexion efficiencies of metal ions is due to the polyphenol structures and the degree of polymerisation [22] and are also related to the hydroxyl group locations and number on the aromatic ring [23].

### Wet biomass measurements

In the synergic fermentation/ bioaccumulation process proposed in this study, wet biomass was evaluated. The wet biomass weigh variations during the bioprocess are presented in (Figure 2a and 2b). The polyphenol presence in the culture medium has a positive influence on the biomass development for the blank fermentation (19.61 g/ L wet biomass). A concentration of 10 mg $Cu^{2+}$/ L added in the culture medium leads to an increase of biomass for *Rhodotorula mucilaginosa*- 1 strain (24.7 g/ L wet biomass after 72h fermentation)

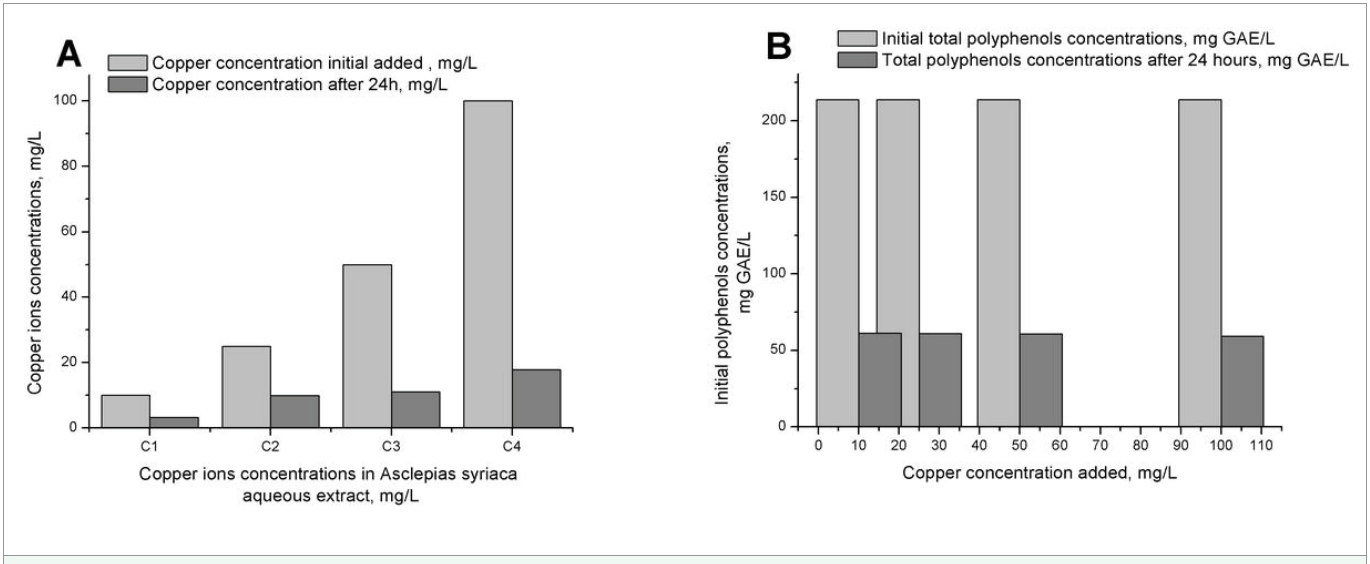


**Figure 1:** $Cu^{2+}$ concentrations in *A. syriaca* aqueous extract after 24 hours. Total polyphenol concentrations in *A. syriaca* extract after 24 hours in contact with $Cu^{2+}$.

while high $Cu^{2+}$ concentrations added (100 mg/ L) inhibit the strain growth. The behaviour of *Rhodotorula mucilaginosa*- 2 strain in the same conditions is different. The higher amount of wet biomass (14.8 g/ L) was obtained for the fermentation process in which 50 mg $Cu^{2+}$/ L were added in the medium, but the biomass yield is lower compared with *Rhodotorula mucilaginosa*- 1 (R1) strain in the same conditions.

### pH determination

The pH variation in media during the fermentation process was evaluated (Figure 3a and 3b). The pH value significantly decreases from 6 to 3 in the first 24 hours. This change is correlated with the yeast metabolism (formation of acidic metabolites during microbial growth) creating appropriate condition for $Cu^{2+}$ bioaccumulation.

In the *Rhodotorula mucilaginosa*- 1 (R1) yeast strain fermentation, the pH increases to around 6 after 24 hours. The increased pH value corresponds to the maximum concentration of biomass. The same observations are consistent for the *Rhodotorula mucilaginosa*- 2 (R2) strain. High amounts of wet biomass (R2AS15Cu50 and R2AS15Cu100) are obtained when the pH value is around 6.

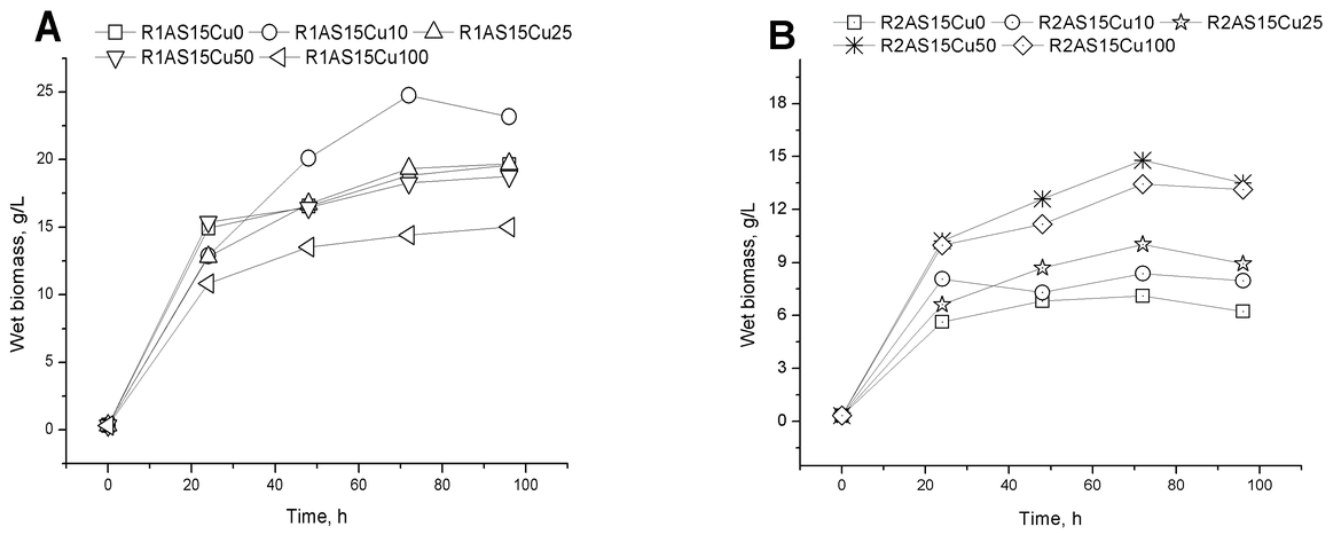


**Figure 2:** Variation of wet biomass yield during the experiment; a) - R1AS15 Cu0 - R1, strain grown in the extract from 15 g/ L *A. syriaca* plant stems containing 0 mg $Cu^{2+}$/ L, R1AS15Cu10 - R1 strain grown in the extract from 15 g/ L *A. syriaca* plant stems containing 10 mg $Cu^{2+}$/ L, R1AS15Cu25 - R1 strain grown in the extract from 15 g/ L *A. syriaca* plant stems containing 25 mg $Cu^{2+}$/ L, R1AS15Cu50 - R1 strain grown in the extract from 15 g/ L *A. syriaca* plant stems containing 50 mg $Cu^{2+}$/ L, R1AS15Cu100 - R1 strain grown in the extract from 15 g/ L *A. syriaca* plant stems containing 100 mg $Cu^{2+}$/ L, b) – the R2 strain grown in the same aqueous extract with the same copper ions concentrations as R1 strain.

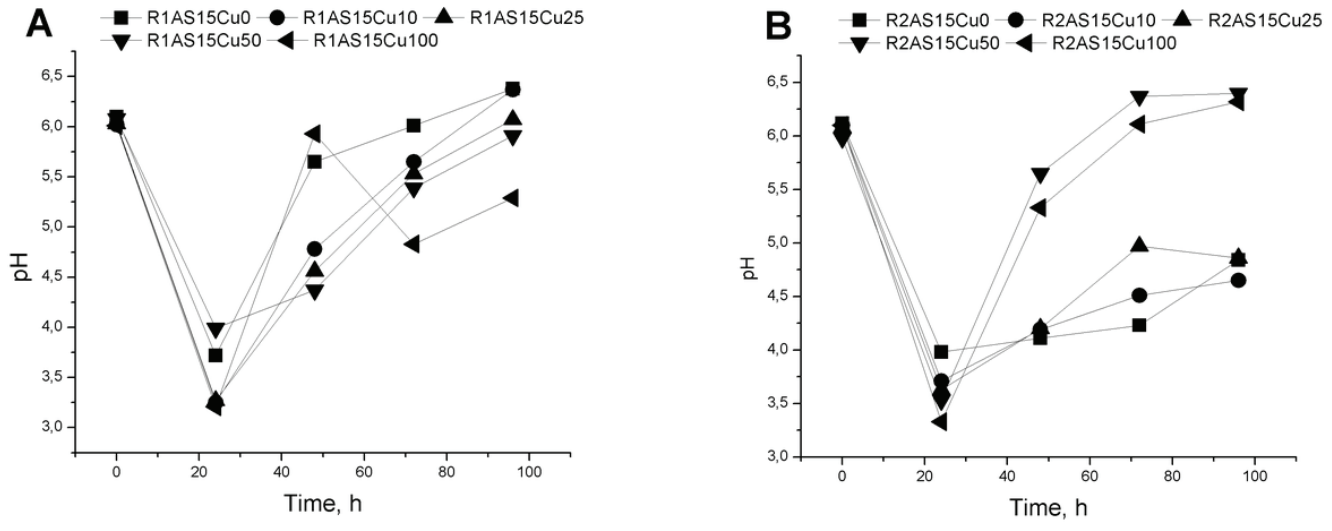


**Figure 3:** pH variation in media during the fermentation process.

### Variation of total polyphenol concentration from residual fermentative culture medium

The natural polyphenols from the culture medium have a positive influence on yeast development. High concentrations of polyphenols (approximately 10 mg GAE/ L total polyphenol for R1AS15Cu10 experiment) are used in the *Rhodotorula mucilaginosa*- 1 (R1) strain fermentation process (Figure 4a). For the *Rhodotorula mucilaginosa*-2 (R2) fermentation, (R2AS15Cu50 and R2AS15Cu100), high yields of wet biomass and more evident polyphenol intake from culture medium was observed (Figure 4b).

The data show that the yeast's enzymatic equipment was adjusted promptly and the metabolic pathways were directed toward polyphenol consumption as carbon source for growth and development in the presence of oxygen.

### Variation of copper ions concentrations from residual culture medium

The $Cu^{2+}$ concentrations were evaluated by analysing the residual culture medium every 24 hours for four days. The obtained data confirm a continuous decrease of $Cu^{2+}$ concentrations during the fermentation (Figure 5a and 5b).

Some yeasts are very resistant to stressful environment [24]. The $Cu^{2+}$ from culture medium could be trapped by yeasts through different mechanisms. It can be used as a catalyst in the metabolic processes or adsorbed on the cellular membrane. In the first case, the fate of copper ions depends on the metabolic pathways of the strain. One of the copper uptake mechanisms in yeast is the sequestration of the ions by metathionin due to the high cysteine content, followed by the absorption of cationic ions in the cells [25]. Rhodotorula species adsorbs the heavy metal on the wall cell surface by an independent mechanism [26]. At the same time, the ions can

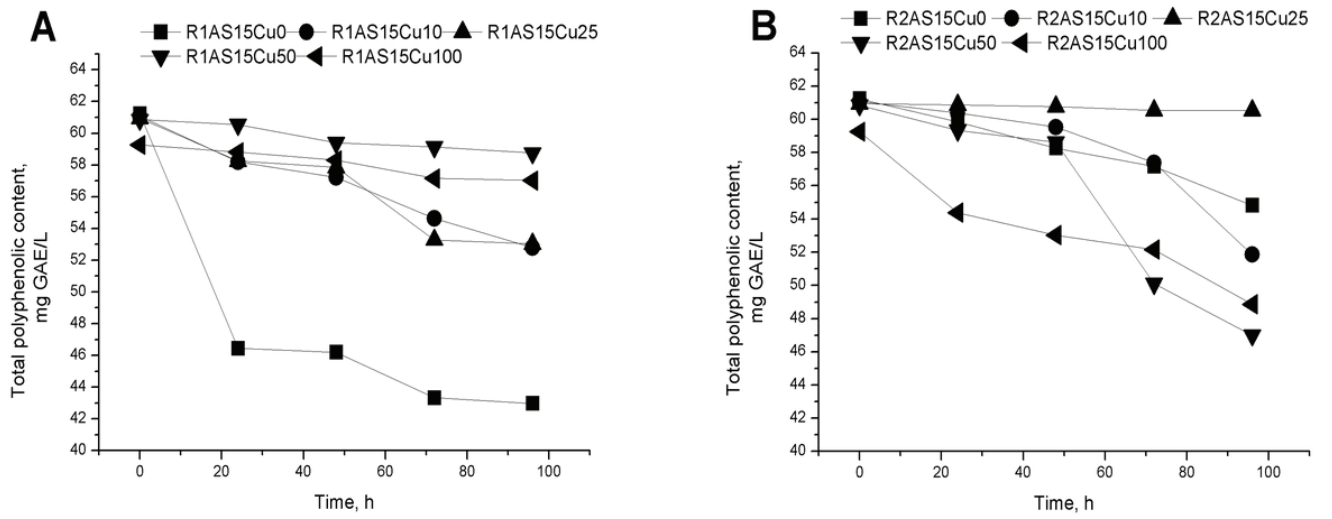


**Figure 4:** Variation of the total polyphenol concentration from residual culture medium.

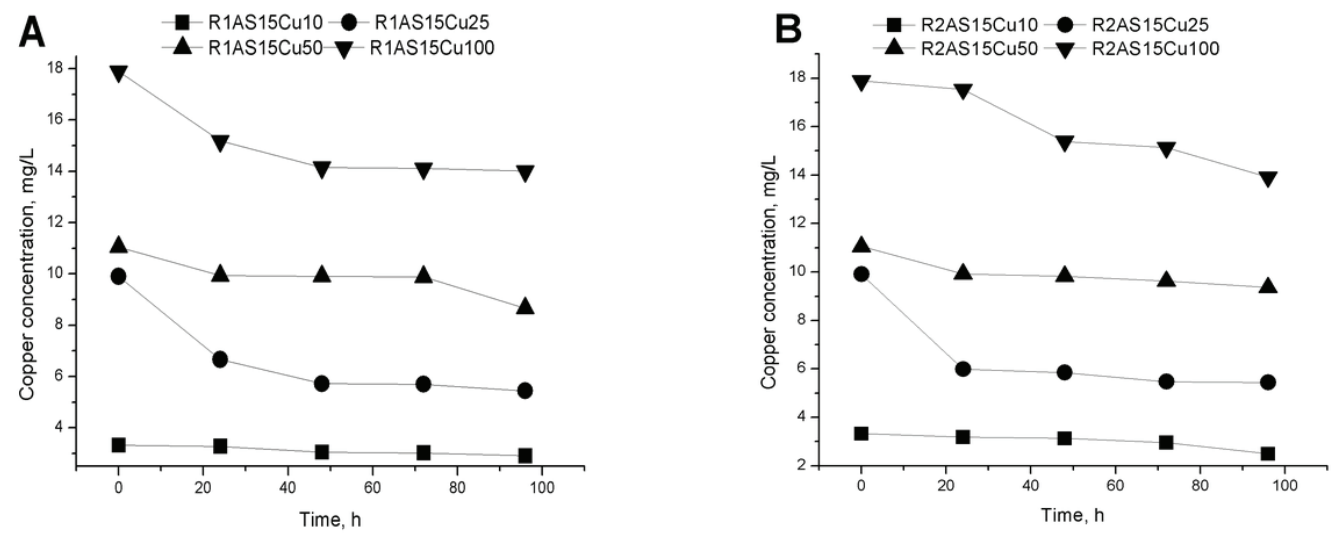


**Figure 5:** Variation of $Cu^{2+}$ concentrations from the residual culture medium at different time intervals.

enter into the cells by membrane delivery, internal distribution or extracellular precipitations by excretion of some extracellular metabolites [27,28]. The yeast is a very good absorption system due to high growth rate and unicellular nature [29]. A second advantage of yeasts that the efficiency of copper retention is more pronounced with the expanse of the wet biomass [30]. In this context, the highest $Cu^{2+}$ amount consumed by *Rhodotorula mucilaginosa*- 1 (R1) strain was in the R1AS15Cu10 experiment, (22.11% from total $Cu^{2+}$ added) and for *Rhodotorula mucilaginosa*- 2 (R2) strain in the R2AS15Cu50 experiment (3.4% from total $Cu^{2+}$ added) (Figure 5a and 5b).

Regarding the dependence of the $Cu^{2+}$ amount trapped by the yeast cells, the decrease in culture medium pH value to between 4 and 5 is correlated with the increased amount of copper retained by the yeast [26,29].

### The carotenoid pigments biosynthesis

The amount of carotenoid pigments biosynthesised was evaluated in order to highlight the polyphenol and $Cu^{2+}$ influence at the end of every fermentation process.

The biomass was subjected to carotenoid pigments extraction and the pigments were quantified by UV measurements (Figure 6a and 6b). The presence in the culture medium of only polyphenols (213.50 mg GAE/ L) is not well tolerated (R1As15Cu0 and R2As15Cu0). When 10 mg $Cu^{2+}$/ L are added, a higher amount of pigment (1.3 µg pigment/ g dry biomass) was biosynthesised by *Rhodotorula mucilaginosa*-1 (R1). This is explained by the fact that polyphenols bind the copper in the medium and the polyphenol concentrations and available copper ions are important for yeast development and carotenoid pigments biosynthesis. For R1AS15Cu10 fermentation, a higher amount of wet biomass was obtained, while a higher amount of polyphenol was degraded.

The higher carotenoid concentrations obtained for the *Rhodotorula mucilaginosa*-2 (R2) strain (R2As15Cu50) are correlated with the highest amount of wet biomass obtained and also the highest amount of polyphenol degraded. The carotenoid pigments concentrations are close with those obtained for the *Rhodotorula mucilaginosa*- R1 strain fermentation, 1.4µg pigments/ g dry biomass.

## CONCLUSIONS

The culture media with *Asclepias syriaca* stems aqueous extract and 10 mg $Cu^{2+}$ / L have a positive influence on the wet biomass yield (24.7 g/ L at 72h), the polyphenol compounds degradation and the carotenoid biosynthesis (1.3 µg pigment/ g dry biomass) by a *Rhodotorula mucilaginosa*- 1 (R1) strain. The higher amount of wet biomass for *Rhodotorula mucilaginosa*- 2 (R2) strain is obtained for 50 mg $Cu^{2+}$/ L added in the media (14.8 g/ L) and 1.4 µg pigment/ g dry biomass are biosynthesised. The $Cu^{2+}$ concentrations retained increase until the end of the experiments, and it is a process that depends on pH variation and biomass yield during fermentations. The polyphenol compounds were digested by the yeast's enzymatic systems and used in the energetic metabolism. Also the water used in the vegetable extract and culture media preparations may be copper contaminated.

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## AUTHOR CONTRIBUTIONS STATEMENT

A. R. Petrovici - conceptualization, methodology, validation, investigation, resources, data duration, writing-original draft preparation, writing-review and editing, supervision, project administration I. Stoica - formal analysis, Irina Volf - visualization, writing - review and editing, Valentin I. Popa - writing-review and editing, supervision, project administration.

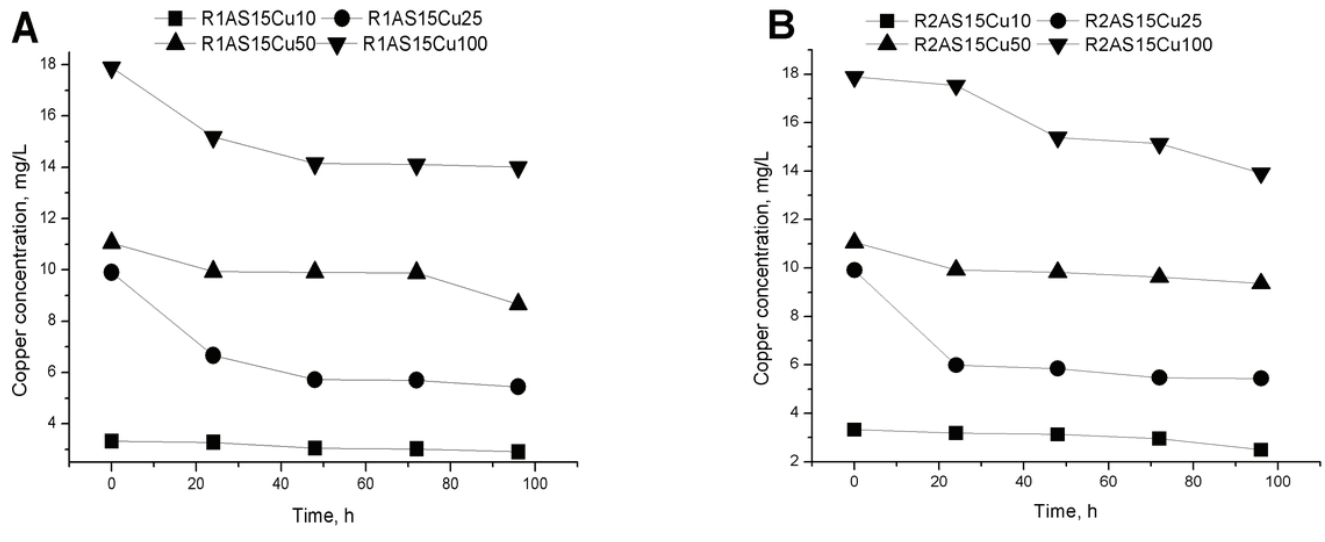


**Figure 6:** Variation of carotenoid pigment biosynthesis during the fermentative process.

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