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RESEARCH ARTICLE

THE METABOLIC RESPONSE OF YEAST AS A MODEL ORGANISM TO *EPICOCCUM NIGRUM* PIGMENTS' ANTIOXIDANT ACTIVITY

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Abstract:

Two *Epicoccum nigrum* parental and mutant strains were used as good producers of carotenoid and flavonoid intracellular complex of pigments biosynthesized in solid state cultivation system. In the previous researches a high antioxidant activity of the fungal pigments was found. The aim of the work was to investigate the antioxidant action of carotenoids and flavonoids complex extracted from the *Epicoccum nigrum* mycelia upon the cells of the *Saccharomyces cerevisiae* strain as a model organism. Yeast was cultivated in submerged system on Malt Extract medium enriched with different concentrations of *Epicoccum nigrum* mycelial extract containing carotenoids and flavonoids in variable ratios. Yeast growth was monitored by measuring optical density and by direct counting with Thoma cytometer. Cell multiplication was monitored at different moments using kinetic parameters, like number of generations, rate of multiplication and generation time. Cell viability and stability in time were estimated through microscopic observation of cell suspended in methylene blue, used as redox indicator. Results showed that *Epicoccum nigrum* pigments increased the cell multiplication and metabolic activity and decreased the intracellular oxidation with positive effects on cell stability and viability.

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Keywords: carotenoids, flavonoids, *Epicoccum nigrum*, antioxidant activity, *Saccharomyces cerevisiae,* model organism, metabolic response

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Introduction

Antioxidants are of great interest because of their involvement in important biological and industrial processes. In general, compounds with antioxidant activity proved to possess anticancer, anti-cardiovascular, anti-inflammation and many other activities (Reis et al., 2007). It is thought that oxidative stress leads to an increased risk of developing several diseases in mammals, e.g. mutations, cancer, inflammation, cardiovascular disease. In particular, some of the strong oxidants that occur in the bodies of both mammals are reactive oxygen species such as superoxide radical, singlet oxygen and lipid peroxides (LPO). Aerobic organisms have both enzymatic and non-enzymatic defense systems against oxidative stress. In mammals insufficient ingestion of nutritional antioxidants has been linked to a decrease in the ability to defend against and increased susceptibility to oxidative stress and some diseases (Nakano et al., 1999). Both natural and synthetic antioxidants have been shown to enhance product stability, quality, and shelf life. Many research works have mentioned the disadvantage of synthetic antioxidants, and their possible injurious properties for human health in addition to their possible toxicity as well as general consumer rejection led to the decreased use of synthetic antioxidants such BHA or BHT which were reported to have several side effects. Consequently, the development of alternative antioxidants from natural origin has attracted considerable attention and many researchers have focused on the discovery of new natural antioxidants aimed at counteracting biologically harmful radicals (Es-Safi et al., 2007).

Of all known carotenoids - about 600 different types are found in nature – among which about 40 are regularly consumed by humans. Carotenoids are known to be biologically important micronutrients with a many functions. Around 50 carotenoids exhibit pro-vitamin A activity and may serve as precursors of retinoids. It has been demonstrated that β-carotene may provide protection against cancer development in human. Therefore, intake of β-carotene is recommended, especially in the form of high- dose supplements. Carotenoids supplementation has been further recommended for the prevention and treatment of degenerative diseases related to oxidative stress. Most of the evidence supports the hypothesis that β-carotene acts as *in vivo* antioxidant. The polyene chain is responsible for the chemical reactivity toward oxidizing agents and free radicals and for their role as antioxidant. Carotenoids are thought to increase the membrane's mechanical strength. Moreover, at low oxygen pressure in most tissues under physiological conditions, some investigators report that certain kinds of carotenoids exhibit potent radical trapping activity. Some types of carotenoids show positive biological activities in mammals without exhibiting any cytotoxicity. Therefore, the carotenoids appear to have special properties that no other antioxidants show (Nakano et al., 1999). β-carotene is a powerful scavenger of singlet oxygen and acts at low oxygen concentrations as chain-breaking antioxidant towards lipid peroxyl radicals. However, at higher oxygen concentrations carotenoid peroxyl radicals are generated, which could act as a prooxidant in an autoxidation process. Thus, β-carotene can act as a pro-oxidant as well as an anti-oxidant in the mitochondria (Siems et al., 2005). Flavonoids are a group of common phenolic plant pigments that are ubiquitous in all vascular plants. They occur naturally in a broad range of fruits and vegetables as well as beverages such as tea, red wine, coffee, and beer. People from European countries consume up to approximately 1 g of different flavonoids per day in the common diet, depending on the food source. Flavonoids have been reported to exert multiple biologic effects including anti-inflammatory, anti-allergic, antiviral, and anticancer activities. The property of many flavonoids to alter the expression and activities of numerous enzymes involved in the regulation of cell cycle and of apoptosis has been studied by several authors (Rusak, 2005).

These properties of flavonoids may be the reason for the observed cytostatic properties and the induction of apoptosis in many cell types, yet the molecular targets of flavonoids relevant for the control of these processes are not known. Microorganisms are useful models for studying different aspects of oxidative stress on biochemical, molecular-biological and cellular level.

Oxidative damages to proteins, lipids, nucleic acids and other cell components as well as defense systems against oxidative stress are basically almost similar to all levels of cell organization (Jamnik et al., 2007).

However, *in vivo* assays are also necessary to have a more accurate evaluation of the antioxidant agents' potential. *Saccharomyces cerevisiae,* the preferred model to study the response to stress in eukaryotic cells, is a useful organism for the identification of antioxidant agents. The use of lower organisms, like yeast cells, as model systems is particularly attractive because of the ease of genetic manipulation, the availability of the complete *Saccharomyces cerevisiae* genomic sequence and the apparent conservation of molecular mechanisms between yeast and human cells. About 30% of the human disease-associated genes significantly match yeast genes and, in contrast to humans, yeast genes can be easily manipulated through molecular biology techniques (Silva et al., 2005; Lopez et al., 2007). The yeast model system is ideal for these studies since (1) yeast are a simple eukaryotic organism with significant homology to mammalian systems; (2) the yeast genome is fully sequenced allowing facile manipulation of their genetics to predictably control their susceptibility/resistance to quinone toxicity; (3) yeast can survive under a variety of conditions including anaerobic conditions and varying *p*H, which allows an evaluation of growth conditions/cell environment on toxicity; and (4) yeast manipulation is simple and inexpensive (Rodriguez, 2004).

In this study, the possible antioxidant actions of *Epicoccum nigrum* pigments as carotenoids and flavonoids complex were examined *in vitro*, using the yeast *Saccharomyces cerevisiae* as a model organism. Further evaluation of the antioxidant activity of fungal pigments was determined using several *in vitro* assays focusing on the effects of pigments on yeast physiology.

Materials and Methods

Yeast strain The strain *Saccharomyces cerevisiae*, bread-making yeast, product made by SC ROMPAK SA Pascani Romania.

The inoculum was obtained after a 24hour aerobic cultivation on liquid malt and was $5x10^5$ cells/ml culture medium.

Pigments' biosynthesis and extraction Two strains of *Epicoccum nigrum*, from the microorganisms collection of the Microbiology Department of BIOALIMENT research center from "Dunarea de Jos" University of Galati (coded MIUG) produce complexes of pigments rich in carotenoids and flavonoids; their composition is variable depending on the strain and fermentation conditions (Bahrim and Soptica, 2004). The parental strain *Epicoccum nigrum* MIUG 2.15 produces a complex of carotenoid:flavonoid coloring agents in a 20:1 ratio (orange colorant) while the mutant strain *Epicoccum nigrum* MIUG 2.15m, derived from the parental strain by chemical mutagenesis, has a pigment ratio 1:1 (red colorant) (Barbu et al., 2006). Cultivation of moulds for pigment biosynthesis was performed in solid state fermentation system for 10 days, at 25°C and in darkness (Soptica and Bahrim, 2005). The mycelium thus obtained was dried at 40°C and milled; from the resulting powder pigments were extracted in the sterile distilled water to obtain concentrated extracts. The pigment concentrations in the coloring extracts were given in color units (CU) determined on the basis of the maximum wavelength measured by spectrum analysis as follows: orange extract (coded P), DO_{max} = 424 nm, CU/ml extract =8.875 and red extract (coded R), DO_{max} 399 nm, CU/ml extract =21.232. Thus, colour units (*CU*) were calculated using the following formula: $CU = OD \cdot d$, where: *OD* is the absorbance of the diluted extract at 424 nm or 399 nm; *d* is the dilution factor. Depending on the pigment concentration in the concentrated R and P extracts, several experimental variants were established by varying both concentration and type of majority pigments in the yeast growth medium (Table 1).

Yeast culture conditions The study focused on the yeast physiologic behavior and cell stability by cultivation in aerobiosis in a liquid medium based on malt extract (pH=5.0) to which pigment- enriched extracts were added according to the medium culture and pigment composition: P extract – 1.5%, 2.5%, 5.0% and 7.0%; R extract – 0.5%, 1.0%, 2.0% and 3.0%.

Table 1. *Liquid medium variants for Saccharomyces cerevisiae submerged cultivation supplemented with pigments biosynthesized by Epicoccum nigrum*

Fungal pigments' extracts	Variants	Allowance of color extracts in fermentative medium	
		ml extract/ 100 ml fermentative medium	CU/ml fermentative medium
P Extract (carotenoid:flavonoid, 20:1)	P1	1.5	0.1331
	P2	2.5	0.2219
	P3	5.0	0.4438
	P4	7.0	0.6213
R Extract (carotenoid:flavonoid, 1:1)	R1	0.5	0.1062
	R2	1.0	0.2123
	R3	2.0	0.4246
	R4	3.0	0.6370

For evaluation purpose a sample was used with no pigments added. Cultivation was performed on a rotary shaker at 200 rpm, monitoring the yeast behavior during 48 hours cultivation.

Monitoring yeast multiplication and physiological state.

The effect of R and P extracts on yeast growth was monitored by measuring OD_{600} in the cell culture and using direct cytometry. After 6, 12, 24 and 48 hrs, biomass concentration, number of live cells, number of generations, rate of multiplication, generation time and mother cell were evaluated in the samples. Yeast viability assay was examined directly by microscopy in the presence of the blue metylene indicator, based on the live cells capacity of reducing the redox indicator from the blue oxidated form (blue) to the reduced form a leuco-derivative (colorless).

Results and discussions

Previous studies have shown the capacity of *Epicocum nigrum* selected strains (parental and mutant) of producing a pigment complex consisting of carotenoids and flavonoids, the biosynthesis potential being modified due to the chemically induced mutagenesis (Bahrim and Socaciu, 2006).

Evaluation of the anti-oxidative capacity of separate fractions, by chromatography and ion exchangers, have revealed the strong anti-oxidative activity featured by the pigments in the coloring complex (González-Sanjosé et al., 2006). *In vitro* evaluation the antioxidant activity of *Epicoccum nigrum* pigments on *Saccharomyces cerevisiae* cells show that in the presence of the extract containing flavonoid:carotenoid 1:1, for a pigment allowance equivalent to 0.1062 CU/mL culture medium (R1 variant), the yeasts culture features a similar evolution as that of the control (Figure 1). In variants with pigments allowance in concentration of 0.2123 – 0.4246 CU/mL, cultures are found to exhibit an almost ideal exponential multiplication within the interval 24-36 hrs cultivation (Figure 2), although the yeasts multiplication stagnation is extending up to 20-24 hrs, after which cell multiplication is visibly activated. The best yield of the viable cells is found in cultures having extract allowance in concentration of 0.4246 CU/mL. With pigment concentrations in medium of 0.6370 CU/mL, a decreased evolution of yeasts culture can be noted, probably induced by the yeast failure to adapt to a medium negatively affecting the multiplication speed. In the presence of the rich carotenoid extract (P extract), at concentrations of 0.1331 CU/mL culture medium (P1 variant) a stimulation of the yeasts physiologic activity becomes obvious, the viable cells concentration reached after 48 hrs cultivation being 1.22 times higher compared to the control (Figure 3). The extract rich in carotenoids also inhibits yeasts' multiplication in the first 12 hrs, but cells manage to adapt themselves within 15 hrs, the lag phase extends to 24 hrs, and after 30 hrs the live cell concentration is similar to that of the control (Figure 4). These measurements account for the need to optimize the antioxidant mixture in order to obtain the desired stimulation effect.

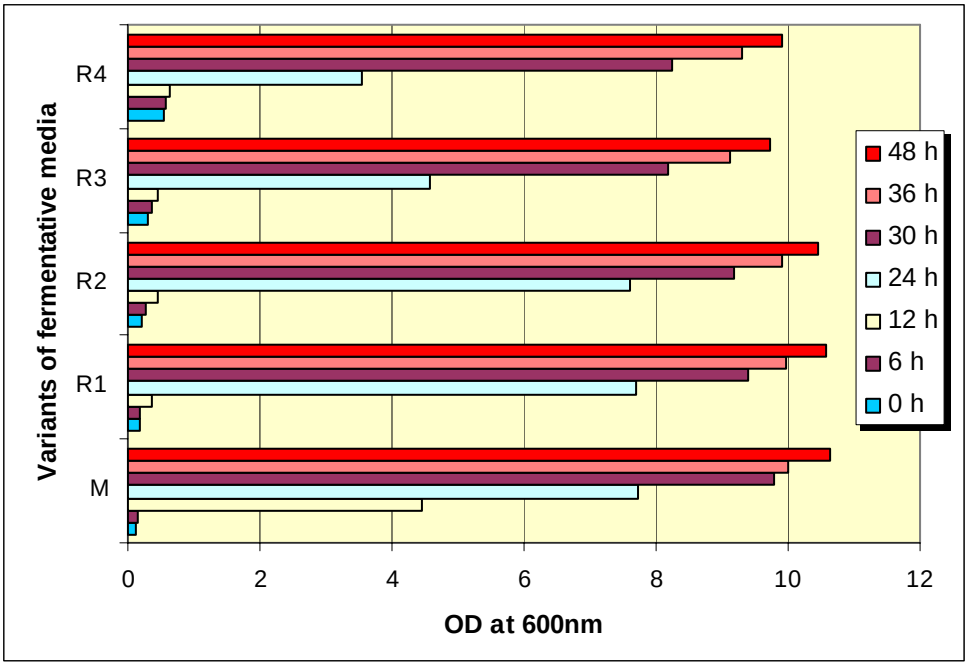

Figure 1. *Yeasts growth in aerobic condition in liquid medium supplemented with R extract*

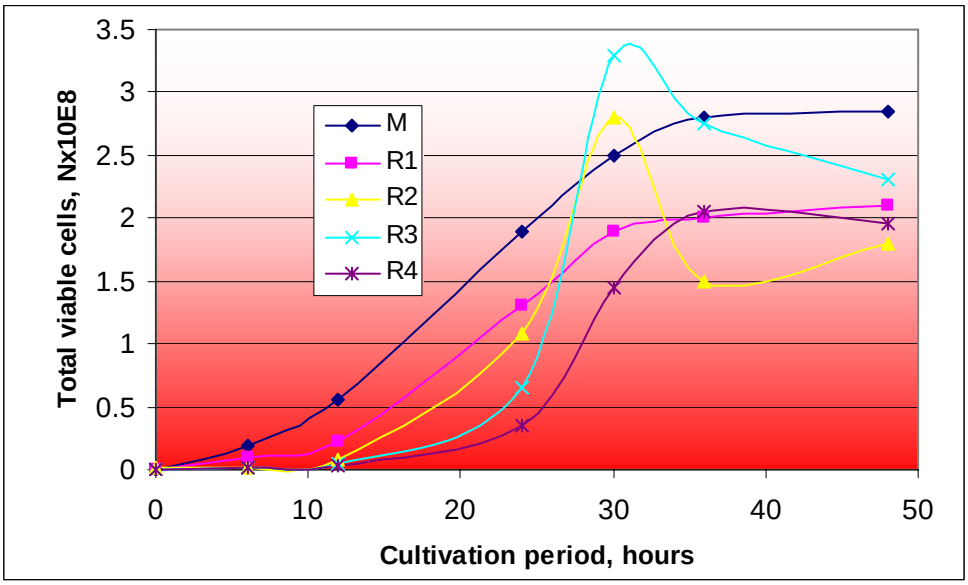

Figure 2. *Dynamics of the yeast multiplication by submerged cultivation in liquid medium supplemented with R extract*

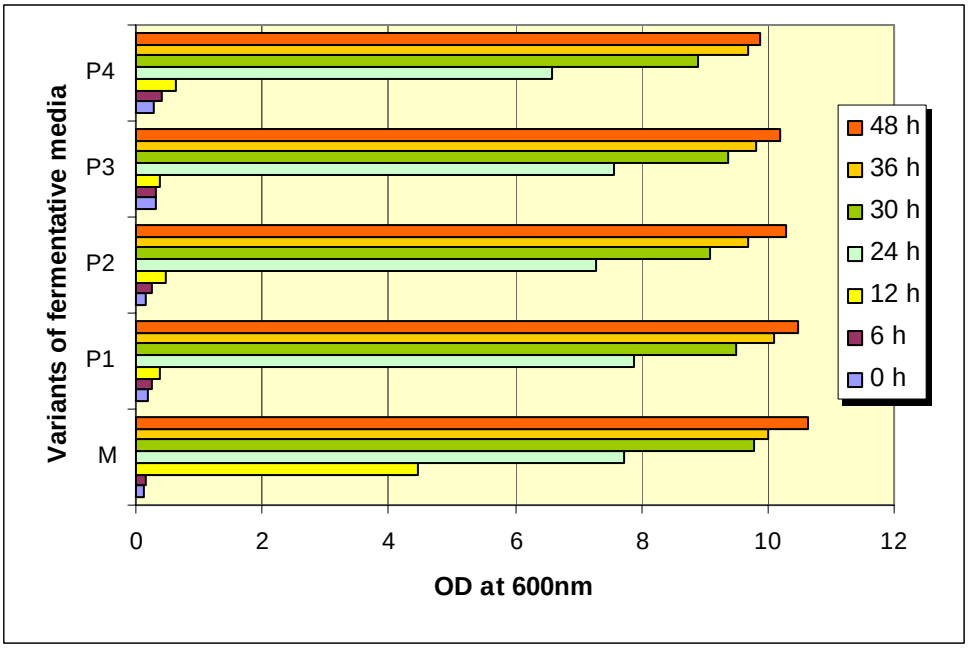

Figure 3. *Yeasts growth in aerobic condition in liquid medium supplemented with P extract*

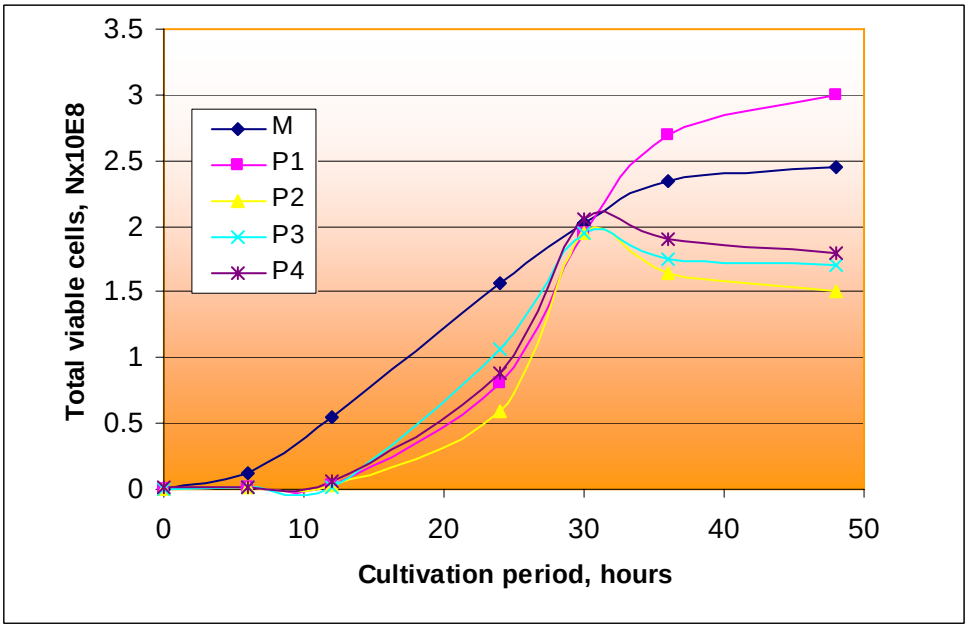

Figure 4. *Dynamics of the yeast multiplication by submerged cultivation in liquid medium supplemented with R extract*

The best yeast multiplication has been reached after 30 hrs cultivation in the variants with 0.2123-0.4246 CU/mL culture medium, the extract having a balanced composition of flavonoids and carotenoids (R2 variant) (Figure 5). When orange colored extract is added in concentration of 0.62125UC/mL culture medium (P4 variant) a stimulation of the cells multiplication, after 24 hrs of submerged cultivation, as compared with the control was observed (Figure 6). At this concentration, the pigments' presence in the fermentative medium results in slower yeast multiplication process in the first 12 hrs, as compared with the antioxidant- free variant

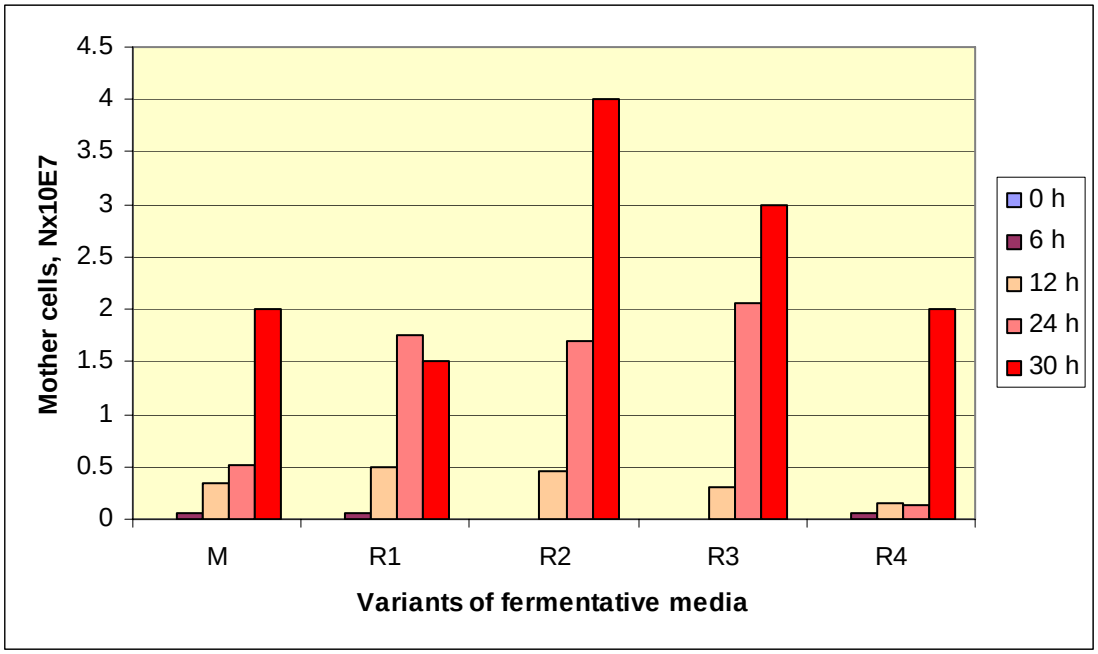

Figure 5. *Yeasts budding in the presence of R extract*

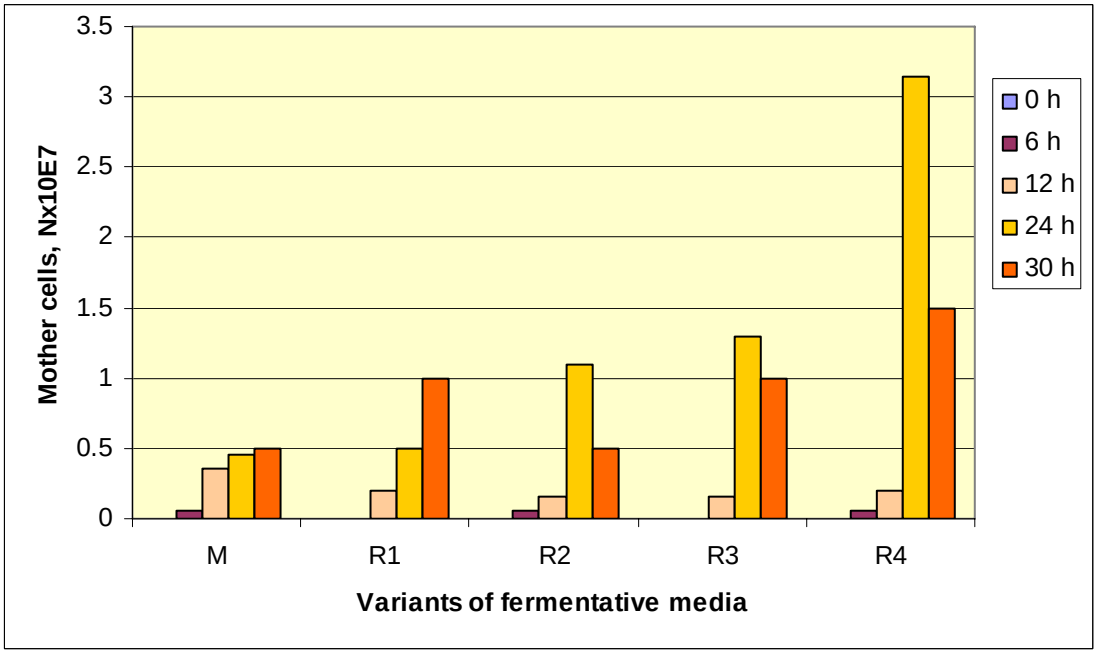

Figure 6. *Yeasts budding in the presence of P extract*

The number of cell divisions is 3.456 times higher than the control in the presence of the red extract, at a concentration of red extract equivalent at 0. 424UC/mL culture medium (R3 variant) (Figure 7). In contrast, in the presence of extract P the highest number of cell divisions is reached with pigments of concentration 0.621UC/mL culture medium (P4 variant).

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A similar variation is also obtained from the rate of multiplication (Figure 8). The highest rate of multiplication, compared with the control, was found for the yeasts grown in the presence of red color of concentration 0.424UC/mL culture medium. In the case of P extract, the highest rate of multiplication is reported for a concentration 0.6370 C/mL culture medium

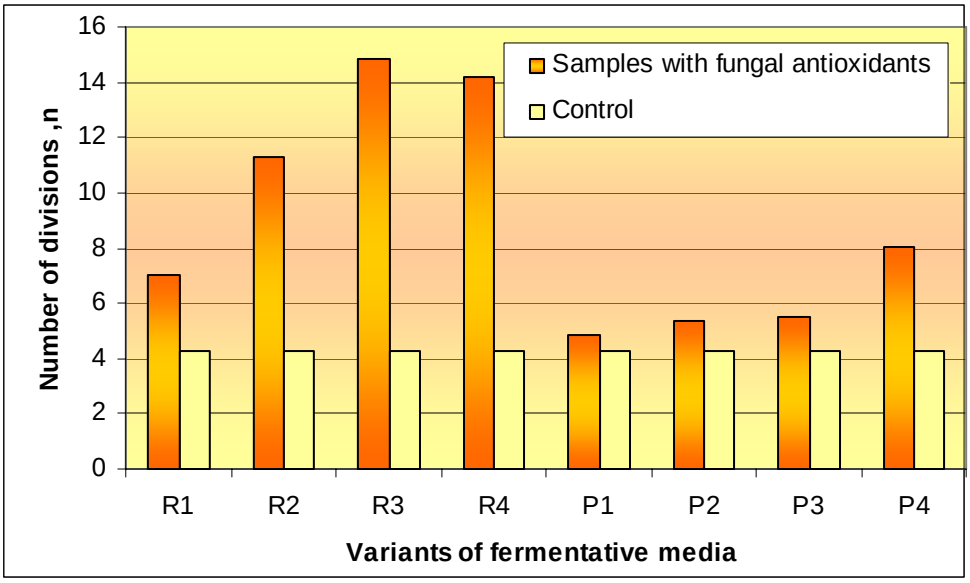

Figure 7. *Number of cell division for yeasts cultivated in the presence of fungal antioxidants*

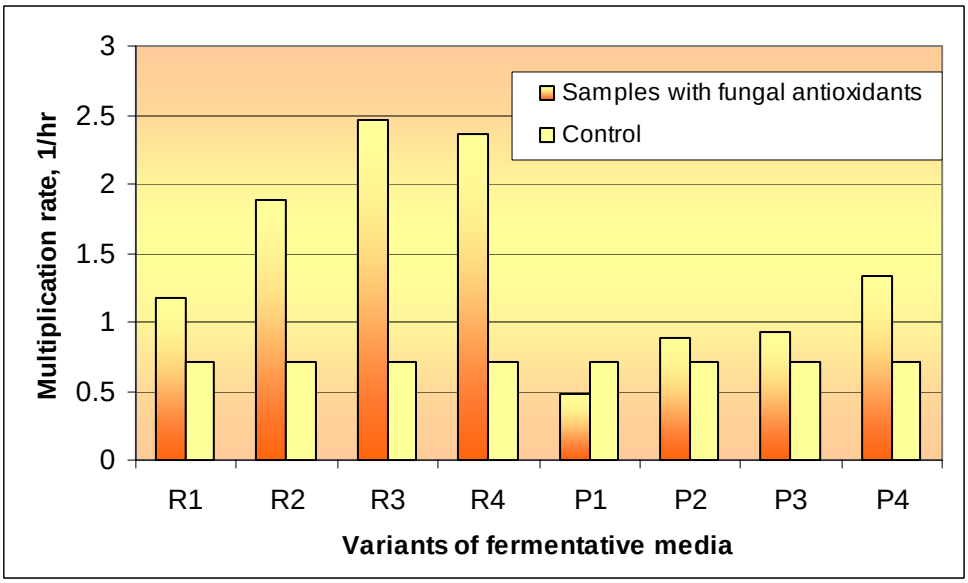

Figure 8. *Rate of multiplication of yeasts cultivated in the presence of fungal antioxidants*

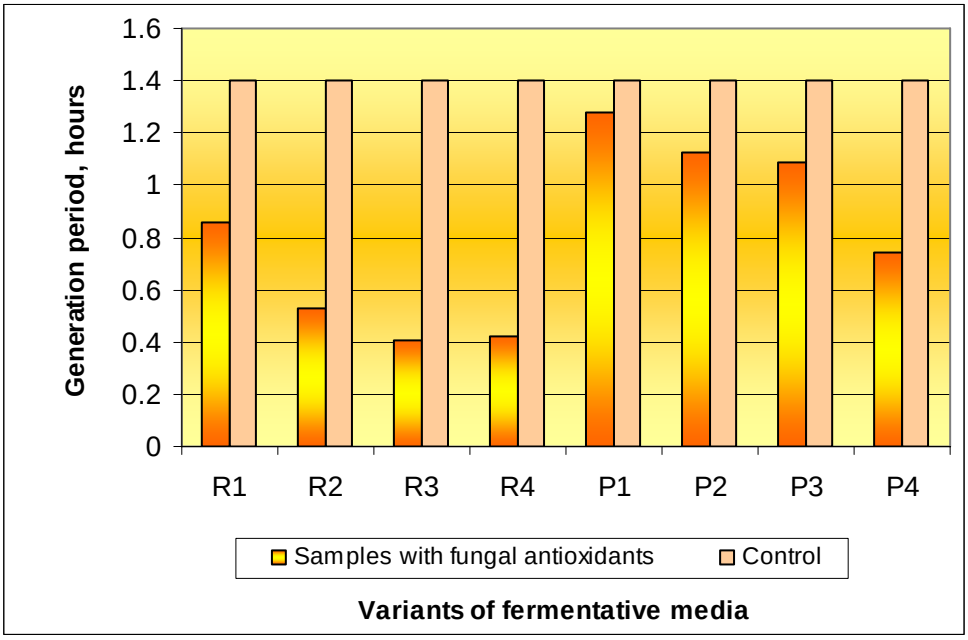

Figure 9. *Generation time for yeasts cultivated in the presence of fungal antioxidants*

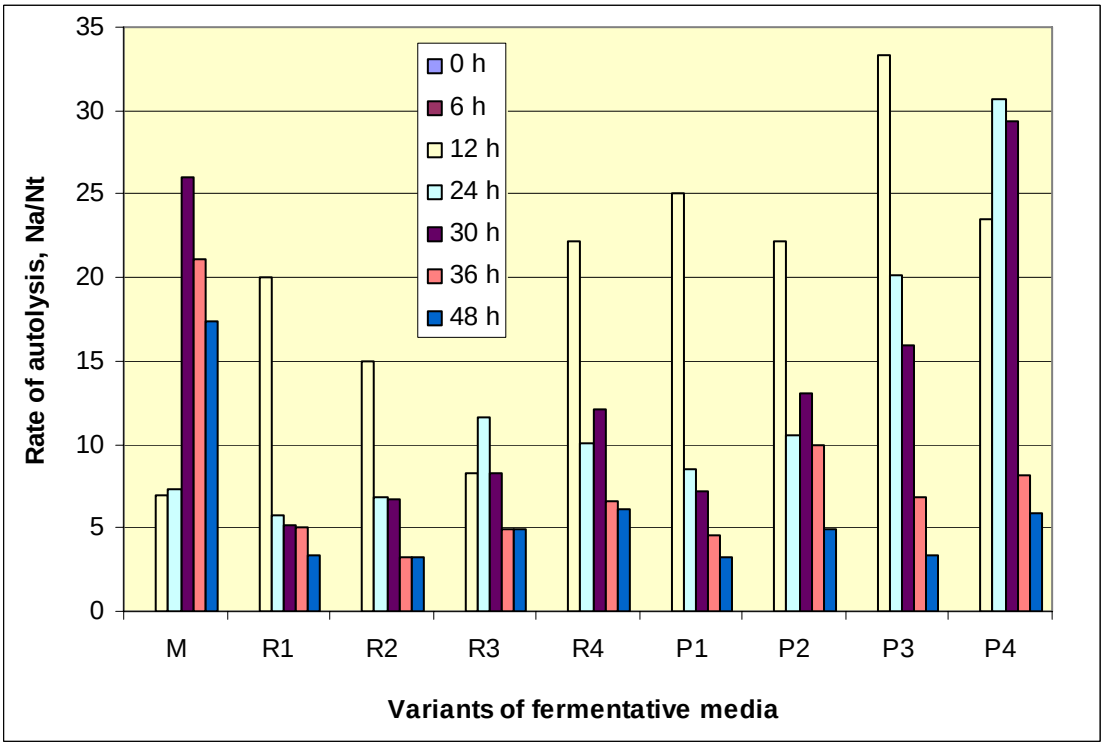

Figure 10. *Autolysis rate of yeasts cultivated in the presence of fungal antioxidants*

The generation time evolution is perfectly consistent with the previous results, which accounts for their accuracy (Figure 9).

Studies on cell stability have shown that in the absence of antioxidants the yeasts cells undergo fast autolysis after 24 hrs cultivation. Examining the autolysis, the percentage of autolyzed cells with respect to the total number of cells in the culture is visibly reduced after 24-30 hrs cultivation in the samples with 0.2123 CU/ml, R extract and 0.4438 CU/ml, P extract (Figure 10). These data are well correlated with the previous results and imply a higher protection effect of the yeasts cell with R extract containing higher flavonoid concentrations.

Conclusions

S. cerevisiae could be proposed as a cell model to provide an initial and rapid screening of the antioxidant potential of fungal pigments. A stimulation of the cell multiplication and a better vitality of yeast cells in presence of the fugal extracts, rich in carotenoids and flavonoids were demonstrated. According to results, the yeast *S. cerevisiae* was confirmed as being useful in evaluating the antioxidant activity of fungal metabolites with antioxidant activity. Furthermore, this *in vitro* methodology could be complemented *in vivo* by assays, producing a more complete analysis of the antioxidant capacity of carotenoids and flavonoids produced by *Epicoccum nigrum* strains. There may, of course, be limitations in extrapolating results obtained with yeast cells, due to differences in the molecular environment and more complex genetic interactions. There are several other *in vitro* methodologies that measure different kinds of free radical species such as lipid peroxidation, deoxyribose damage assay, superoxide scavenger capacity, among others. Nevertheless, it is important to use an *in vivo* methodology to assess the real capacity of an antioxidant to protect cells. In this way, the present work should be supplemented with further studies in order to understand the functionality *in vivo* of pigments produced by *Epicoccum nigrum*.

Acknowledgements

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