

Metabolic activity stimulation of the wine yeasts by polyphenols extracted from red grapes

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Abstract

Two aqueous of 1% tartaric and citric acid solutions were used for polyphenols extraction from red grapes. The effect of polyphenols of the *Saccharomyces cerevisiae* var. *ellipsoideus* strain as a model organism was investigated. Yeast was cultivated in aerobic and facultative anaerobic conditions on grape must medium enriched with different concentrations of polyphenol extracts in variable ratios. Yeasts growth kinetics was evaluated by direct counting of the cells. Cells 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 polyphenols from red grapes stimulated the cell multiplication and metabolic activity and decreased the intracellular oxidation with positive effects on cell stability and viability. The maximum of fermentation intensity was reached for concentrations of polyphenols of 0,77 mg/mL extract in tartaric acid and 0,62 mg/mL extract in citric acid, at levels of 98.42% and 92.37% respectively.

Keywords: red grapes polyphenols, *Saccharomyces cerevisiae* var. *ellipsoideus*, cells multiplication, fermentation ability

Introduction

The polyphenolic compounds with antioxidant activity in red wines have been proposed as an explanation for the lower death rate from coronary heart disease in France referred to as “The French Paradox” [1]. In wine, the polyphenolic compounds play an antioxidant role in both biological and food systems with favourable effects on human health, such as inhibition of oxidation of low-density lipoprotein cholesterol, and inhibition of platelet aggregation [2], thereby decreasing heart disease risks.

The content of total phenolics and anthocyanins in red grapes can be strongly influenced by many variables, depending on variety, cultural factors, such as fertilization, ripening [3,4]. The antioxidant capacity of red wines have been demonstrated in biological systems “in vitro” and “in vivo” [5,6], being usually attribute to some bioactives compounds present in wines, such as polyphenols, and specially to the flavonoid compounds [4,7] such as anthocyanins.

Microorganisms are used to study different aspects of oxidative stress on biochemical, molecular-biological and cellular level useful models. 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 [8]. However, *in vivo* assays are also necessary to have a more accurate evaluation of the effect of red grape polyphenols on metabolic activity stimulation potential. Preferred model to study the response to stress in eukaryotic cells is *Saccharomyces cerevisiae*.

The actual trend in winemaking is the selection of starter cultures able to complement and optimize grape quality in order to obtain a wine, which could be the result of the optimal interaction yeast/grapes. The selection of starter cultures is mainly addressed to the principal yeast in wine fermentation, *Saccharomyces cerevisiae* var. *ellipsoideus*, characterized by high ethanol and sulphur dioxide tolerance, which allows dominating and completing grape must fermentation.

A naturally occurring polyphenols (i.e. tannic acid) from grapes may precipitate or complexes with different macromolecules, such as polysaccharides and proteins with H bond acceptors. It is well known that polyphenols primarily interact as colloids with proteins by Van der Waals bonds [9]. However, there are few studies on the adsorption of polyphenols on yeasts during alcoholic fermentations and little information regarding effect of polyphenols biotechnological properties of wine yeast.

The colour of coloured wines can be modified by yeasts through establishing weak and reversible interactions between anthocyanins and yeast walls or through periplasmic beta-glucosidase activity targeted to the one part of anthocyanins [10,11].

The interaction of yeast lees with wine polyphenols maintains the spherical morphology of yeast cells, although degradation of the yeast cell wall occurs during autolysis. The yeast cell wall gives mechanical stability and dictates shape and external morphology of yeasts. As polyphenols do not affect the release of cell wall mannoproteins in the medium, yeast cells remaining spherical after contact with polyphenols indicates that wine polyphenols interact with the cell wall of yeast [12]. This observation revealed that some domains of the yeast cell wall are protected from extracellular hydrolytic enzyme activity by wine polyphenols.

From technological point of view is interesting to know how metabolic activity stimulation of wine yeast by the red grape polyphenols addition will improve yeasts characteristics implied in wine biotechnology.

The aims of this study are to obtain polyphenol extracts from red grapes by using different conditions of extraction and to evaluate their effects on biotechnological properties of wine yeast *Saccharomyces cerevisiae* var. *ellipsoideus* as yeast kinetics multiplication and alcoholic fermentation ability.

Materials and Methods

Polyphenolic extracts. Red Grapes (*Chile, South America*) were purchased directly from a local supermarket. A quantity of 150 g of fresh skin were soaked in 350 ml of a 1% aqueous tartaric (T) and citric (C) acid solution and stirring at 25°C for 60 minutes. Immediately the extracts were separated from solids and concentrated in a Rotavapor R-120 Büchi, at the temperature of 35°C, in the vacuum conditions at p = 0,9 bar, from 4.5% soluble solids to 23.5% soluble solids for tartaric acid extract and from 6.5% soluble solids to 22% soluble solids for citric acid extract.

The obtained extracts were analyzed for total polyphenols content by reaction with Folin-Ciocalteu reagent, and were expressed as mg/L of gallic acid [13]. Total polyphenols were evaluated on two extracts.

Yeast strain. Lyophilised wine yeast, strain *Saccharomyces cerevisiae* var. *ellipsoideus* - SIHA Levactive B - Bourgogne, Germany, was reactivated, by stationary cultivation in sterile grape must, during 24 h at room temperature. A concentration of $5 \cdot 10^7$ CFU/mL fermentative medium, of activate culture was used as the vegetative inoculum both in multiplication and alcoholic fermentation samples.

Yeast culture conditions. The study focused on the yeast multiplication was realized by cells cultivation in submerged conditions in a liquid medium based on 100 mL of sterile grape must (20.2 soluble solids), enriched with 0.5% ammonium sulphate g, to which polyphenolic extracts were added in different concentrations according to extract composition, as follows: T1(C1) = 0.31mg/mL, T2(C2) = 0.46mg/ mL, T3(C3) = 0.62mg/mL and T4(C4) = 0.77mg/mL total polyphenols. A volume of 1.1 mL of vegetative inoculum was then added for each sample. For evaluation purpose a sample (M) was used with no extract added. Cultivation was performed on a rotary shaker at 200 rpm, observing the yeast behaviour during 48 hours cultivation.

Similar, to evaluate wine yeast fermentative capacity stimulation on 200 mL of sterile grape must (20.2 soluble solids), enriched with 0.5% ammonium sulphate were added the equivalent quantities of polyphenolic extracts and inoculum. For evaluation purpose a sample was used with no extract added. Fermentation was realized in stationary cultivation conditions, during 48 hours at room temperature, by using the same concentrations of vegetative inoculum.

Yeast metabolic activity stimulation. The effect of two extracts T and C on yeast growth was monitored by using direct cytometry. Colony forming units per ml (CFU/mL) were calculated, and microbial population vs. time was modelled according to the reparametized Gompertz equation proposed by ref. [14] by using model:

$$y = a * \exp \{ -\exp [((\mu_{\max} * e) / A) * (\lambda + t)] + 1 \}$$

where: $y = \ln(N / N_0)$, N_0 is the initial microbial population; N the microbial population at time t , $A = \ln(N_{\infty} / N_0)$ is the maximum value reached with N_{∞} as the asymptotic maximum population, $\mu_{\max}$ is the maximum specific growth rate, and λ the lag phase period.

After 12, 24, 36 and 48 h, biomass concentration, number of viable cells, number of generations, rate of multiplication, generation time and mother cells were evaluated. Yeast viability assay was examined by microscopy in the presence of the blue metylene indicator, based on the viable capacity of reducing the redox indicator from the blue oxidated form (blue) to the reduced form a leuco-derivative (colorless).

To evaluate the effect of polyphenols on wine yeast fermentative capacity after 6, 12, 24 and 48 h, the fermentation rate and fermentation intensity (% fermented sugar/initial sugar) were calculated.

Results and discussions

Variability of polyphenolic extracts with extraction conditions

Citric acid was more efficient than tartaric acid for polyphenols extraction from fresh red grape skins. The content in total polyphenols evaluated by Folin Ciocalteau index was 607.5 mg/L gallic acid for tartaric acid extract and 309.9 mg/L gallic acid for citric acid extract, respectively. The nature of acid affected the extraction yield. This difference could be due to the larger molecular size of citric with respect of tartaric acid. The extraction process is based on the stability of polyphenols in polar solvents.

The effect of polyphenols from red grapes on wine yeasts growth ability and cells metabolic stability in aerobic conditions

In submerged cultivation in aerobic conditions, dynamics of wine yeasts multiplication in the presence of red grapes polyphenols is depicted in Figure 1(a) for tartaric acid extract (T) and Figure 1 (b) for citric acid extract (C).

In all variants after 24 hours the exponential phase is finished. A multiplication cells stimulation was observed for a concentration of 0.31- 0.62 mg/mL polyphenols in T extract and 0.46-0.77 mg/mL polyphenols in C extract. With a concentration of 0.46 mg/mL polyphenols in medium (T 2 variant), a initial decreased evolution of yeasts culture can be noted, probably induced by the yeast failure to adapt to a medium negatively affecting the multiplication rate.

Cells multiplication is higher with 7.3% for T1 variant and 2.12% for C4 variant when compared with control sample M.

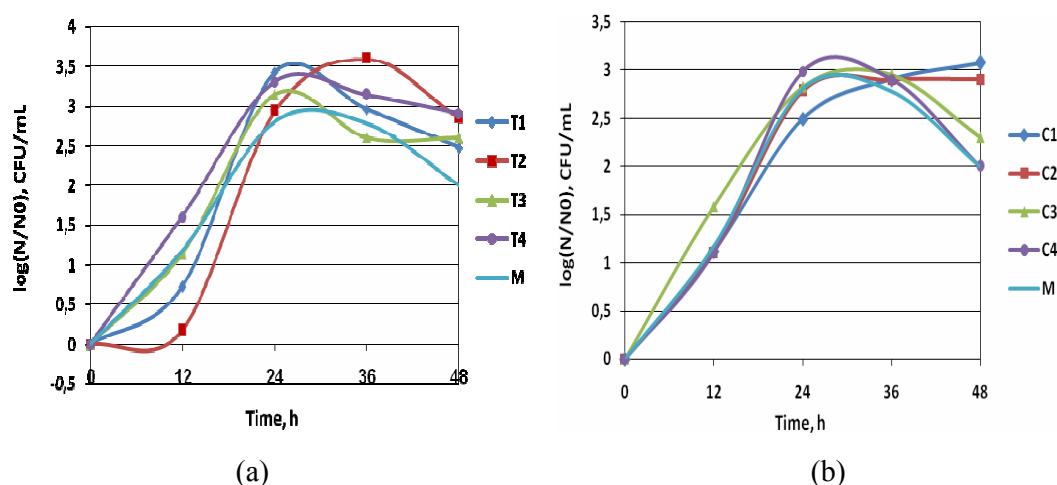


Figure 1. Dynamics of the yeasts multiplication by aerobic cultivation in liquid medium supplemented with polyphenolic extract: (a) tartaric extract (T) and (b) citric extract (C)

Dry biomass yield (g dry biomass/100 mL medium) after 60 hours of cultivation was also evaluated (Figure 2). These results confirm the previous one when the best multiplication rate were obtained for samples T1 (0.31 mg/mL polyphenols extract in 1% tartaric acid) and C4 (0.77 mg/mL polyphenols extract in 1% citric acid).

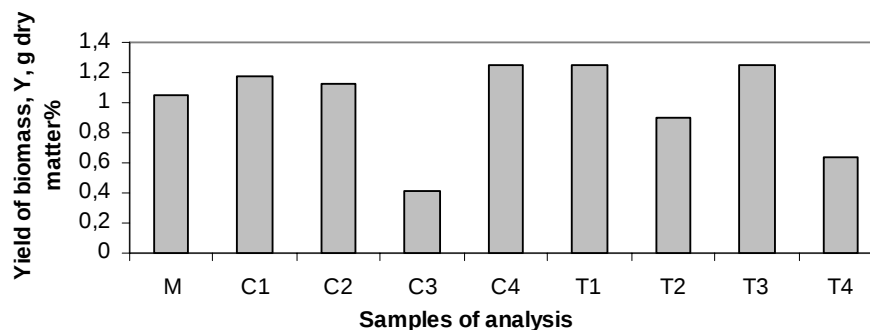


Figure 2. Yield of dry biomass by submerged aerobic cultivation of yeasts in media with red grape polyphenols

Kinetic parameters of yeasts multiplication were calculated and are **illustrated** in Table 1.

Table 1. Kinetic parameters of yeasts multiplication

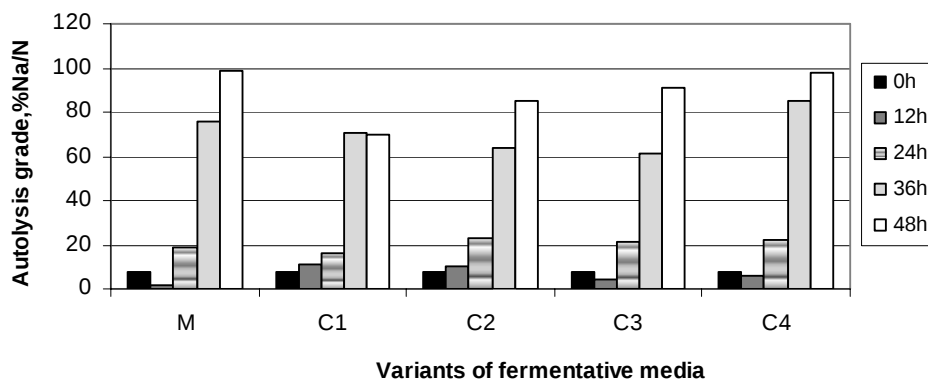
Samples	Generation number (n)	Multiplication rate (v), 1/h	Generation time, h
M	6.03	0.5023	1.99
C1	4.56	0.380	2.63
C2	5.57	0.464	2.15
C3	4.12	0.344	2.91
C4	6.24	0.520	1.92
T1	8.98	0.749	1.34
T2	9.17	0.765	1.31
T3	6.69	0.557	1.80
T4	5.68	0.474	2.11

Polyphenolic extracts in tartaric acid have a positive effect upon yeasts multiplication kinetic when compared with polyphenolic extracts in citric acid at the similar polyphenols concentrations.

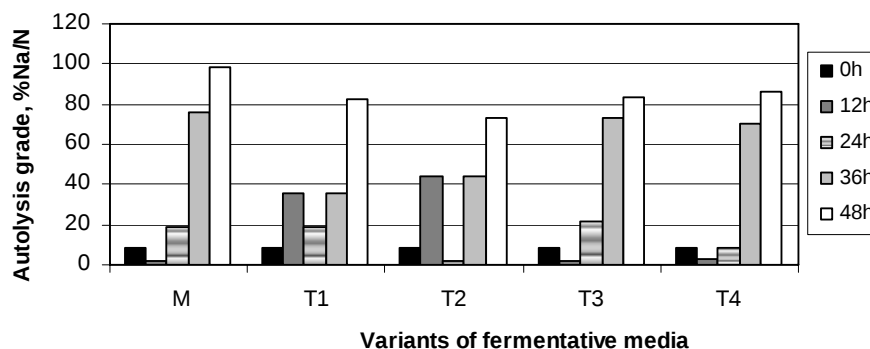
However, when the content of polyphenols in fermentative media is 0.31 mg/mL (T1) the number of generation was increased 1.48-fold than control sample and 1.43-fold than the most efficient concentration of polyphenols in citric acid extract (C4).

Metabolic stability of wine yeast in presence of the red grape polyphenols

Studies on cell stability have shown that in the absence of polyphenolic extracts the yeasts cells undergo to fast autolysis after 24 h of submerged cultivation in aerobic conditions.



(a)



(b)

Figure 3. Autolysis rate of yeasts in the presence of polyphenolic extract: (a) tartaric extract (T) and (b) citric extract (C)

Examining the autolysis, the percentage of autolysed cells with respect to the total number of cells in the culture is visibly reduced after 36 h by cultivation in the samples with 0.31mg/mL tartaric extract (variant T1) and 0.62 mg/mL citric extract (variant C3) (Figures 3a, and 3b). These data are well correlated with the previous results and imply a higher protection effect of the yeasts cell with polyphenolic extract in tartaric acid solution than in citric acid solution at the same concentration of polyphenols.

The effect of polyphenols from red grapes on wine yeast fermentative ability

During the alcoholic fermentation the total CO₂ loss and fermentation rate was evaluated. The higher total loss of CO₂ was observed at variants T4 and C3 when compared with control sample M (Figure 4). The highest total loss of CO₂ at variant T4 (0.77 mg/mL polyphenols) was achieved.

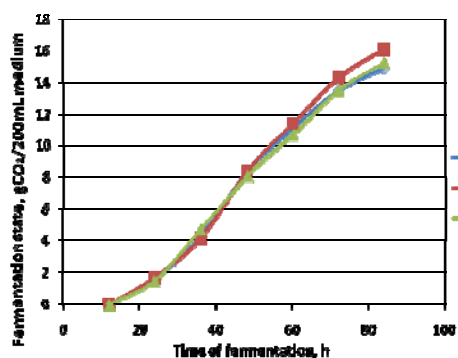


Figure 4. Dynamic of the alcoholic fermentations of grape must in presence of polyphenols extracts

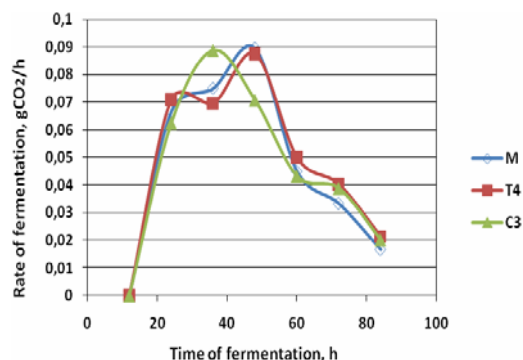


Figure 5. Rate of the alcoholic fermentations of grape must in presence of polyphenols extracts

The rate of fermentation based on CO₂ loss at different fermentation time was also evaluated and it was observed to be maximum after 48 h for all variants with optimum for variants T4 and C3 (Figure 5).

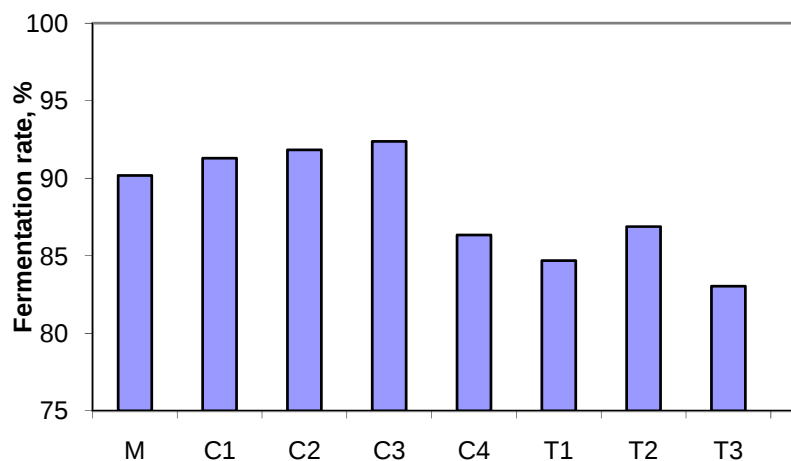


Figure 6. Intensity of grape must fermentation in the presence of polyphenols extracts

The intensity of fermentation was also calculated and the maximum was reached for variants T4 and C3 where 98.42% and 92.37% respectively of initial sugar was fermented, according with initial sugar content in the fermentative media (Figure 6).

Conclusions

- Presence of polyphenols in fermentative media stimulate metabolic activity and increase the stability of wine yeasts.
- Effect of polyphenols extracted from red grapes upon yeasts metabolism may vary depending on type of extractant used, its concentration and also is direct correlated with concentration of polyphenols that fermentative media is supplemented.
- Yeasts behaviour in fermentative media supplemented with polyphenols, and the optimal concentration with beneficial effects on physiological activity is depending on fermentation conditions, in aerobic conditions for cells multiplications and facultative anaerobic conditions for alcoholic fermentation respectively.
- According to this, the results are significant only for the alcoholic fermentation conducted by selected dry yeast. Moreover, as long as the studied strain has been selected already from an oenological environment, it can be already an adapted strain to the polyphenols content.

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