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Abstract

In red winemaking, polyphenols from grape berry pericarp and seed are extracted during fermentation and their interactions with yeast have been widely demonstrated. However, information concerning the impact of extracted polyphenols on yeast metabolism during fermentation is missing. The aim of this study was to further explore interactions between yeasts and polyphenols and to identify their effects on yeast metabolism and fermentation kinetics. This impact was studied in synthetic musts for four commercial *Saccharomyces cerevisiae* wine strains, using polyphenols purified from a thermovinification must, in both stressed (phytosterol deficient medium) and non-stressed conditions. Interactions between grape polyphenols and yeast cells were substantiated from the early stage of fermentation by means of epifluorescence and confocal microscopy. If these interactions were limited to yeast cell walls in non-stressed conditions, the passage of polyphenols through yeast envelope and their accumulation in the intracellular space of living cells was shown in phytosterol-deficient medium. Whatever the conditions used (stressed and non-stressed conditions) and for all strains, the presence of polyphenols led to a significant decrease of cell growth (50%), CO_2 production rate (60 to 80%) and nitrogen consumption (3 to 4 times less), resulting in increased fermentation lengths. The perturbation of yeast growth and metabolism due to polyphenol compounds was likely mostly linked to their interactions with the yeast plasma membrane. From the mid-stationary phase to the

end of the fermentation, an adaptive response was exhibited by yeast, resulting in lower mortality. This work evidenced a strong impact of polyphenols on yeast fermentative capacity and highlighted the importance of a better knowledge of the mechanisms involved to improve the management of fermentations in the context of red winemaking.

Keywords: grape polyphenols, yeasts, alcoholic fermentation, interactions, metabolism.

1. Introduction

Phenolic compounds are important to red wine quality because they impact colour, astringency, mouthfeel and flavour. They refer to a large group of chemical compounds including non-flavonoids (hydroxycinnamic acid esters, stilbenes) and flavonoids (anthocyanins, condensed tannins, flavanols monomers, flavonols, flavanonols). In red winemaking, polyphenols from the pericarp and seeds of grape berry, and especially anthocyanins and tannins, are extracted during the alcoholic fermentation. Several researches in enology have focused on the identification of yeast impact on wine phenolic composition and quality. Various yeast metabolites, such as pyruvic acid and acetaldehyde produced during alcoholic fermentation, were shown to react with different classes of phenolics (Fulcrand, Benabdeljalil, Rigaud, Cheynier, & Moutounet, 1998; Fulcrand, Cameira Dos Santos, Sarni-Manchado, Cheynier, & Fabre-bonvin, 1996; Morata et al., 2003; Morata et al., 2016; Morata, Gómez-Cordovés, Calderón, & Suárez, 2006). These reactions contribute to the stabilization of wine pigments. Furthermore, the adsorption of anthocyanins and pyranoanthocyanins by yeast cell walls through physico-chemical interactions during alcoholic fermentation may result in the modification of wine colour (Caridi, Sidari, Solieri, Cufari, & Giudici, 2007; Caridi, Cufari, Lovino, 2004; Monagas, Gómez-Cordovés, & Bartolomé, 2007; Morata et al., 2003). Physico-chemical interactions between yeast and polyphenols have also been studied in the context of wine aging on lees, a winemaking practice that consists in keeping the wine in contact with dead yeast cells (lees) after the completion of the fermentation (Mazauric & Salmon, 2005, 2006; Salmon, Vuchot, & Moutounet, 2003; Salmon, Fornairon-bonnefond, & Mazauric, 2002; Vasserot, Caillet, & Maujean, 1997). These physico-chemical interactions have long been thought to be limited to yeast cell wall. However, in our recent work, we showed that if polyphenols interact with cell walls, and especially mannoproteins, most of them pass through the

cell wall and plasma membrane of dead cells to interact with their intracellular components (Mekoue Nguela, Sieczkowski, Roi, & Vernhet, 2015; Mekoue Nguela, Vernhet, Sieczkowski, & Brillouet, 2015). The polyphenols involved were mainly high molecular weight tannins and derived pigments. This new information immediately raised the question of the ability of some polyphenols to enter in living yeast cells during fermentation and their potential impact on yeast metabolism.

Much effort has been devoted to the improvement of the fermentation step through the management of environmental conditions such as the control of oxygen concentration and temperature (Bisson, 1999; Sablayrolles, Dubois, Manginot, Roustan, & Barre, 1996), the use of selected yeasts and the correction of nutrient deficiencies such as nitrogen (Bely, Sablayrolles, & Barre, 1990; Bisson, 1999; Sablayrolles et al., 1996; Tesnière, Brice, & Blondin, 2015) and lipids (Bisson, 1999; Casalta, Vernhet, Sablayrolles, Tesnière, & Salmon, 2017; Ochando, Mouret, Humbert-Goffard, Sablayrolles, & Farines, 2017). By contrast, there is only sparse information concerning the impact of grape and wine polyphenols on yeast metabolism. A study has focussed on the effect of a crude tannin fraction on the metabolism of yeast during fermentation (Li, Du, Yang, & Huang, 2011). An inhibitory effect of tannins on the activity of the membrane enzyme H^+-ATPase was observed during the initial phase of the fermentation, along with a decrease in cell growth, CO_2 release, sugar consumption and ethanol production (Li et al., 2011). An adaptation step was observed after approximately three days of fermentation as a consequence of an enhancement of the activities of the enzymes involved in the glycolysis pathway (Li, Zhao, & Huang, 2014).

The aim of the present work was to further explore interactions between yeasts and grape polyphenols during alcoholic fermentation and to identify their effects on yeast metabolism and fermentation kinetics. This effect was studied using four commercial oenological *Saccharomyces cerevisiae* strains, in two different synthetic musts chosen to mimic non-stress (high phytosterol and usual sugar contents) and stress (low phytosterol and high sugar contents) fermentation conditions, supplemented or not (control) with polyphenols extracted from red musts obtained by thermovinification. The impact of polyphenols was evaluated through different phenotypic parameters: fermentation kinetics (conversion of sugar into CO_2 and ethanol), cell growth, cellular viability and nitrogen consumption. Light microscopy, epifluorescence and confocal

91 microscopy observations were achieved to identify the location of polyphenolic compounds in
92 yeast cells during fermentation.

93

94 ## 2. Materials and methods

95 ### 2.1. Yeast strains

96 This study was performed using four commercial *Saccharomyces cerevisiae* yeast strains from
97 the Lallemand yeast strain collection (Montreal, Canada), selected for winemaking and available
98 as active dry yeasts (Y1, Y2, Y3 and Y4). Fermentation flasks were inoculated with 10 g/hL
99 active dry yeast previously rehydrated for 30 min at 37 °C in a 50 g/L glucose solution (1 g of dry
100 yeast diluted in 10 mL of this solution).

101

102 ### 2.2. Polyphenol purification and analysis

103 #### 2.2.1. Polyphenol purification

104 Two polyphenol pools (PP1 and PP2) were purified from 18 L of a thermovinification must
105 (Merlot variety) produced in 2015 at the INRA Experimental Unit of Pech Rouge (Gruissan,
106 France) and stored at – 20 °C until use. Separation was achieved by adsorption/desorption using a
107 vinyl-divinylbenzen Diaion resin (RELITE SP411) and in batch mode. Considering (i) the
108 volumes of resin required to adsorb the phenolic compounds from a thermovinified must and (ii)
109 the volumes of solvent required for their elution, it was not possible to treat the whole must at the
110 same time. Therefore, two different purification steps were performed and two independent pools
111 PP1 and PP2, were obtained.
112 The first purification was performed on 10 L of the initial must. 2.5 L of must were mixed first
113 with 10 L resin until complete polyphenol adsorption (followed by UV-visible
114 spectrophotometry). The resin was then washed several times with acidified water (0.05%
115 trifluoroacetic acid, TFA) to remove non phenolic compounds (sugars, organic acids, salts,
116 polysaccharides, etc). The removal of non-phenolic compounds, followed by refractometry, was
117 considered completed when a value of 0° brix was obtained. Polyphenol desorption was achieved
118 by several washings with 96/4 v/v ethanol/water acidified with 0.05% TFA (total volume, 20 L).
119 The ethanolic extract was directly concentrated using a rotatory evaporator under vacuum at low
120 temperature (35 °C). The same protocol was applied four times to treat 10 L of must. The
121 polyphenol concentrated extracts obtained from the whole must volume (10 L) were then blended

to give the so-called PP1 polyphenol pool (2.4 L), aliquoted and stored at - 80 °C until use. PP1 was used to study the impact of polyphenols in stress conditions.

A second purification step was performed later on the remaining 8 L of must to study the impact of polyphenols in non-stress conditions. Changes were made in the extraction protocol to limit the volumes of organic solvent to be processed. The amount of must treated with 10 L resin was increased to 4 L. After adsorption, the resin was washed several times with acidified water (0.05% TFA), until a 0 ° brix was reached. Polyphenols were eluted with several washing with 96/4 v/v ethanol/water acidified with 0.05% TFA (total volume, 30 L). The same protocol was applied two times to treat 8 L of must. To decrease volumes to be treated by the rotatory evaporator, the two ethanolic extracts obtained after desorption of phenolic compounds (60 L) were stored at 4 °C and pooled before being concentrated first to 12 L by a vacuum distiller at 28 °C. After distillation, the extract was further concentrated by using rotatory evaporator under vacuum, at 35 °C to give the so-called PP2 polyphenol pool (2.3 L) that was stored at - 80 °C until use. PP2 was used to study the impact of polyphenols in non-stressed conditions.

Both PP1 and PP2 were used as such (liquid) to ensure a better solubilization in the synthetic must.

2.2.2. Polyphenol analysis

The total polyphenol indexes (TPI) of the two purified pools were determined by absorbency measurements with 1 cm optical path after an adequate dilution in water (TPI = A_{280nm}*dilution factor). This dilution factor was chosen to get absorbency values below 0.8. TPI values of 209 and 289 were obtained for the PP1 and PP2 extracts, respectively; this indicated a higher polyphenol concentration in PP2 than in PP1. The polyphenol content in PP1 and PP2 was determined by weighing freeze-dried aliquots. Before freeze-drying, these aliquots (5 mL) were further purified on C18 SPE SDB-L cartridges (Styrene Divinyl Benzene; strata phenomenex; 500 mg) to remove any traces of sugars, organic acids or salts. PP1 and PP2 extract aliquots were first diluted 3.5 times with water and the whole volume treated on 10*500 mg cartridges. Sugars and organic acids were eluted with 5 ml of water/acetic acid (98/2 v/v) and phenolic compounds recovered with 8 ml of methanol. The latter was evaporated to dryness under vacuum,

redissolved in water and freeze-dried. Phenolic compounds were found to be 10.3 and 17.7 g/L in PP1 and PP2, respectively.

The PP1 and PP2 extracts were diluted in an acidified methanolic solution (water/methanol/formic acid: 49/50/1) for polyphenol analysis. The analyses were performed by the polyphenol platform facility of the joint research unit SPO. Monomers were analysed by Ultra High Performance Liquid Chromatography, coupled to triple-quadrupole Mass Spectrometry (UHPLC-QqQ-MS) associated for detection in Multiple Reaction Monitoring (MRM) mode, according to the methods described by Lambert et al. (Lambert, et al., 2015) and Pinasseau et al. (Pinasseau, et al., 2016). Proanthocyanidins, ie oligomeric and polymeric flavan-3-ols, were analyzed by UHPLC-QqQ-MS after acid-catalyzed depolymerization in the presence of phloroglucinol coupled to DAD and MS detection (Pinasseau, et al., 2016).

The polyphenol compositions of PP1 and PP2 on a dry weight basis are summarized in table 1. The low analysis yields are related to the fact that some polyphenols in the concentrated fractions consisted of derived pigments and tannins, formed by chemical reactions during thermovinification, storage and/or purification steps and resistant to the usual depolymerization methods (Kennedy, Matthews, & Waterhouse, 2000; McRae, Falconer, & Kennedy, 2010; Poncet-Legrand et al., 2010; Vernhet et al., 2011). The two extracts differed in their composition, which is probably due to a longer storage time for PP2 and to the changes adopted in the extraction procedure.

2.3. Fermentations in stressed conditions

Stressed and non-stressed fermentation conditions were simulated by varying phytosterol and sugar levels in a synthetic must, supplemented (test) or not (control) with polyphenols. For fermentation in stressed conditions, sugars in the synthetic must (SM) were set at 250 mg/L (125 mg/L glucose, 125 mg/L fructose) and a phytosterol content of 2 mg/L. The synthetic must composition in terms organic acids, salts, and vitamins consisted of (Bely et al., 1990): 6 g/L malic acid; 6 g/L of citric acid; 750 mg/L KH_2PO_4; 500 mg/L K_2SO_4; 250 mg/L $MgSO_4.7H_2O$; 155 mg/L $CaCl_2.2H_2O$; 200 mg/L NaCl; vitamins (mg/L): myo-inositol (20), calcium pantothenate (1.5), thiamin hydrochloride (0.223), nicotinic acid (2), pyridoxine (0.25), and biotin (0.003); oligoelements (mg/L): $MnSO_4.H_2O$ (4), $ZnSO_4.7H_2O$ (4), $CuSO_4.5H_2O$ (1), $CoCl_2.6H_2O$ (0.4), H_3BO_3 (1), $(NH_4)_6Mo_7O_{24}$ (1) and $FeCl_3$ (14.5). The nitrogen source was composed of ammonium chloride and amino acids. The stock solution of amino acids was as

follows (in g/L): tyrosine (1.4), tryptophan (13.7), isoleucine (2.5), aspartate (3.4), glutamate (9.2), arginine (28.6), leucine (3.7), threonine (5.8), glycine (1.4), glutamine (38.6), alanine (11.1), valine (3.4), methionine (2.4), phenylalanine (2.9), serine (6.0), histidine (2.5), lysine (1.3), cysteine (1.0) and proline (46.8). A volume of 11.7 mL of this solution and 413 mg of NH_4Cl were added to 1 L of the medium to obtain 384 mg/L of assimilable nitrogen in the SM. This high assimilable nitrogen content was chosen to enhance the stress related to phytosterol deficiency (Ochando, Mouret, Humbert-Goffard, Sablayrolles and Farines, 2017). The pH of the synthetic must was adjusted to 3.3 with NaOH 10 M. All chemicals (analytical grade) were purchased from Sigma Aldrich. The SM including all components but phytosterols and polyphenols was autoclaved at 100 °C for 15 min. After the heat treatment, the SM was supplemented with the phytosterol solution for lipid requirements of yeast cells during anaerobic growth. Phytosterol content was adjusted to 2 mg/L using 0.133 mL/L of a stock solution composed of 15 g/L of phytosterols (85451, Sigma Aldrich) in Tween 80 and ethanol (1:1, v/v). Half of the SM volume was finally supplemented with the polyphenol pool PP1 to obtain a final polyphenol concentration of about 2 g/L (assay), the other half being intended as a control.

Fermentations were performed in 330-mL flasks, containing 250 mL of SM, at 24 °C and with continuous magnetic stirring (150 rpm). They were performed in strict anaerobiosis, obtained by bubbling argon in the medium for 10 min before yeast inocculation. Flasks were equipped with fermentation locks to maintain anaerobiosis. All fermentations were performed in duplicate with the four yeast strains in the presence (assay) and in the absence (control) of polyphenols. The CO_2 release was automatically and continually followed by measuring the weight loss of the fermenters hourly.

2.4. Fermentation in non-stressed conditions

A second series of experiments was performed in non-stressed conditions. To this end, sugar concentration in the synthetic must was reduced to 220 g/L and phytosterol concentration was increased to 11 mg/L. The composition of the synthetic must in organic acids, salts, vitamins, oligo-elements and nitrogen was not modified and the same phytosterol source was used, at a concentration of 0.7315 mL/L. Sterilization and phytosterol supplementation were performed as described above. Half of the SM volume was finally supplemented with the PP2 polyphenol pool at a concentration of 2 g/L (assays). In these experiments, fermentations (assays and controls)

were performed in 1-L flasks, containing 900 mL of SM, equipped with fermentation locks to maintain anaerobiosis at 24 °C, with continuous magnetic stirring (150 rpm). The use of a different volume was imposed by the availability of online fermentation monitoring tools. Anaerobiosis was obtained by bubbling argon for 10 min for the 330 mL flasks and for 20 min for the 1 L flasks. All fermentations were performed in duplicate with the four yeast strains. The CO_2 release was automatically and continually followed by measuring the weight loss of the fermenters after each 20 min.

2.5.Cell population

During the growth phase, 5 mL of fermentation medium were withdrawn each 2 h to determine cell population using a Coulter counter (Model Z2, Beckman-Coulter, Margency, France) fitted with a 100 µm aperture probe (Sablayrolles & Barre, 1992). The specific growth rate (µ) was evaluated by plotting ln (X) as a function of time, with X being cell number at a given time. The slope of the linear part of the curve represented µ.

2.6. Cell mortality

Cell mortality was determined by flow cytometry using a BD Accuri™ C6 cytometer (BD Biosciences, Le Pont de Claix, France). Fermentation medium was centrifuged for 5 min at 14000 × g. The supernatant was removed and the pellet diluted with Phosphate-Buffered Saline (1×) to reach 5 to 10 × 10^6 cells/mL. For staining, propidium iodide (PI) was added to the cell suspensions (5 µL of PI at 100 mg/mL in 500 mL of centrifuged and diluted sample), mixed by gentle shaking and incubated for 10 min at room temperature in the dark, then placed on ice before the fluorescence measurement by flow cytometry. PI is a fluorescent nucleic acid stain that cannot penetrate intact cell membranes. PI fluorescence (with excitation using a 488 nm laser and emission at 575 nm) was collected via a 670 nm large-pass filter (Fluorescence 3). Mortality was determined as the percentage of dead cells among all the cells (Delobel, Pradal, Blondin, & Tesnière, 2012).

2.7. Measurement of assimilable nitrogen

To avoid polyphenol interference with enzymatic tests, assimilable nitrogen was calculated from total nitrogen, determined by the Kjeldahl method (Scheiner, 1976), assuming that it accounts for 80% of it. Samples were withdrawn during the stationary phase of the fermentation.

2.8. Determination of metabolite concentrations

Ethanol and sugar concentrations were determined by HPLC (HPLC 1290 Infinity, Agilent™ Technologies, Santa Clara, California, USA) on a Phenomenex Rezex ROA column (Phenomenex™, Le Pecq, France) at 60 °C. The column was eluted with 0.005 N H_2SO_4 at a flow rate of 0.6 mL/min. Concentrations were determined using a refractive index detector. The analysis was performed with an Agilent™ EZChrom software package. The yield of ethanol production was calculated from the amount of sugar (g) required to produce 7,89 g of ethanol (1% ethanol, v/v) in 1 L of fermented medium. Yield ethanol = 7,89/consumed sugar (g).

2.9. Epifluorescence and confocal microscopy

Fermentation media were withdrawn (1 mL) at the end of the growth phase (24 h) and yeast cells recovered by centrifugation (5 min at 1500 ×g), washed four times with 1 mL of Phosphate-Buffered Saline to remove non-sorbed polyphenols and re-suspended in 1 mL of the same buffer. Samples and controls were treated in the same conditions and submitted to microscopy observations. They were first observed under normal light and epifluorescence. Imaging was performed with a Zeiss Axiophot microscope using a Rhodamine filter (λexc = 500−600 nm, λem =540−650 nm). Confocal imaging was performed with a LEICA SP8 microscope fitted with a Plan-Apochromat ×63/1.4 NA oil DIC objective. Excitations were obtained for polyphenols with a 405 nm blue diode, and emissions were collected with a 530−600 nm bandpass filter. For the images acquired in lambda scanning mode, emission spectra were obtained on sample regions of interest (ROIs) by spectral acquisition (lambda stack, excitation at 405 nm). The detection bandwidth was set to collect emissions from 420 to 750 nm, using an array of 32 photomultiplier tube (PMT) detectors, each with a 10 nm bandwidth. The method of linear unmixing was applied with advanced iterative and one residual channel.

2.10. Statistical analysis

Data are expressed as the average of three measurements. ANalysis Of VAriance (ANOVA) and Principal Component Analysis (PCA) were carried out with the software XLSTAT (Addinsoft, Paris, France). Two-factors ANOVA was performed to evaluated effects of polyphenols and

yeast strains on each parameter measured in stressed and non-stressed conditions separately. Difference among mean values was determined using Tukey's test, significant results were considered at $p<0.05$.

3. Results

In the present work, we have evaluated in two synthetic musts the impact of polyphenols on the fermentative kinetics of different wine yeast strains. Population levels, growth rate, cell viability and nitrogen consumption were also determined. The composition of the two synthetic musts was chosen to mimic non-stressed and stressed conditions. Polyphenol location in yeast cells during fermentation was investigated through epifluorescence and confocal microscopy.

3.1. Fermentation kinetics

Fermentation kinetics observed in both stressed and non-stressed conditions and in the absence (control) or in the presence of polyphenols are shown Figure 1.

3.1.1. CO_2 production rate

Results obtained with the controls (Figure 1) are in accordance with previous works concerning the influence of sterol concentrations on fermentation kinetics (Casalta et al., 2017; Ochando et al., 2017). It is well known that phytosterols lead to better viability of yeasts at the end of fermentation enabling thus to avoid stuck fermentations. As expected, the maximum rates of CO_2 production (Vmax) were lower in stressed conditions than in non-stressed conditions (Figure 1). In both conditions, the addition of red must polyphenols led to a significant decrease of the V_{max} (60 to 80%) and to an increase of the fermentation duration (Figure 1). The latter was mostly evidenced in non-stressed conditions, where we observed an increase of the fermentation durations from 150 to at least 300 h. In stressed conditions, this increase was limited to around 50 h.

3.1.2. Cell growth

As shown in Table 2, the presence of polyphenols led for all strains and in both stressed and non-stressed conditions to a significant decrease ($p<0.0001$) in the specific growth rate. Its value was about half as low in the presence of polyphenols compared to the corresponding control. Likewise, cell populations during the stationary phase were significantly ($p<0.0001$) lower in the presence of polyphenols (Table 2). The population decrease was not strain-dependent; however, in stressed conditions, specific growth rate was impacted by yeast strains (Table 2).

3.1.3. Specific CO_2 production rate and sugar consumption

The specific CO_2 production rate ($1/X.dCO_2/dt$, X being cell number in 1 L of fermentation medium) was calculated and plotted according to sugar consumption (%) (Figure 2). The latter was strongly impacted by polyphenols. Depending on yeast strain, different behaviors were observed. For Y1 and Y2, the specific rate of CO_2 production was lower in the assays than in the controls from the beginning of the fermentation to the mid-stationary phase, corresponding to about 40% of sugar consumption. This demonstrated a lower sugar consumption rate by cells during this period in the presence of polyphenols. Then, close specific rates of CO_2 production were measured in the assays and the corresponding controls until the end of fermentation. This could reflect an adaptive response by yeast against polyphenol stress. Y3 presented a behavior similar to that of Y1 and Y2 when fermentation was performed in stressed conditions. However, in non-stressed conditions, specific CO_2 production after mid-stationary phase and until the end of fermentation appeared to be higher in the presence of polyphenols. In that case and as the fermentation progressed, the metabolic activity of the cells decreased more rapidly in the absence of polyphenols. This suggests a stronger resistance to ethanol in the presence of polyphenols. Similar results were observed with Y4 in stressed conditions: sugar consumption was low before mid-stationary phase in the presence of polyphenols, but an adaptive response was observed with fermentation progress. Despite the stress induced by polyphenols, sugar was widely consumed at the end of the fermentation (Figure 2) in non-stressed conditions with all strains but Y4, which exhibited a high residual sugar level (> 20 g/L) (Table 2). In stressed conditions, residual sugar was high with and without polyphenol for all strains, probably due to the low phytosterol concentration.

3.2. Ethanol production

Ethanol was quantified by HPLC at the end of fermentations. Very close ethanol contents and production yields were found between the controls and the treatments in stressed and non-stressed conditions. No significant impact of polyphenols was observed in stressed conditions, but the impact of yeast strain was significant ($p<0.05$), especially Y4 (Table 2). In non-stressed conditions, no significant impact of yeast was observed, ethanol content and production yields were only impacted by polyphenols (Table 2).

3.3. Nitrogen consumption

In this study, assimilable nitrogen was measured at the beginning of the stationary phase (Table 2), when nitrogen consumption has ceased (Crepin, Nidelet, Sanchez, Dequin, & Camarasa, 2014) and before the occurrence of nitrogen release as a result of cell mortality. Assimilable nitrogen consumption was significantly impacted ($p<0.0001$) by polyphenols in both stressed and non-stressed conditions (Table 2). Total residual assimilable nitrogen in the controls ranged from 32 to 93 mg/L, depending on the strain and fermentation conditions (Table 2). This indicated a non-consumption of about 11 to 24% of the initial assimilable nitrogen. In the presence of polyphenols, residual assimilable nitrogen ranged from 164 to 275 mg/L. These values were 3 to 4 times higher than in the controls and indicated a non-consumption of about 43 to 72% of the initial assimilable nitrogen.

3.4. Cell mortality

Cell mortality was followed during the stationary phase, from 30 to 99% of sugar consumption (Figure 3). In most cases and before 80% consumed sugar, mortality was higher with polyphenols than without, showing an increase in mortality during the stationary phase. This effect was more pronounced with Y4, Y3 and Y1 in non-stressed conditions. An opposite behavior was observed at the end of fermentation, from 80% and at 99% of sugar consumption in stressed and non-stressed conditions, respectively Once again, this suggested an adaptation of the cells to the stress caused by polyphenols, resulting in increased resistance to ethanol.

3.5. Direct interactions between polyphenols and yeasts evidenced by microscopy

Yeast cells were sampled at the end of the growth phase and submitted to microscopy observations. They were observed in epifluorescence microscopy (Figure 4) as regular spheres (average diameter = 3 μm). Yeast cells morphology was not affected by the presence of polyphenols but stacks were observed in the treatments (Figure 4). Under rhodamine excitation (λexc = 500−600 nm, λem =540−650 nm), control cells exhibited an extremely faint fluorescence, whereas assay cells fluoresced strongly in red in both stressed and non-stressed conditions. This fluorescence was within the range of that of polyphenols in the applied experimental conditions. Additional confocal microscopy observations (λexc = 405 nm, λem = 530−600 nm) were performed to get further information on polyphenol location. In stressed conditions, control cells showed an almost invisible green fluorescence, whereas an intense green fluorescence was observed in the inner core of cells grown in the presence of polyphenols (Figure 5). This intense green fluorescence was not observed for cells grown in non-stressed conditions (Figure 6). Yeast cells were also observed by confocal spectral analysis. Observations performed with Y1 are presented in Figure 7. Control cells in stressed and non-stressed conditions (Figure 7C, G) exhibited an autofluorescence emission spectrum in the 460−480 nm range (Figure 7D,H) with a very low mean intensity (20). The assay cells in stressed conditions exhibited a different autofluorescence emission spectrum, with several maxima in the 500−600 nm range (Figure 7A B) and a very high intensity. Spectral analysis of this fluorescence in comparison with that of purified grape polyphenols indicated this was due to these compounds (Brillouet et al., 2013; Mekoue Nguela et al., 2015). This revealed the presence of polyphenols inside the cells in stressed conditions at an early stage of fermentation. In non-stressed conditions, cells grown in the presence of polyphenols exhibited only a faint fluorescence (Figure 7 E, F). Considering the observations made by epifluorescence microscopy, this showed that in non-stressed conditions, polyphenols at the end of the growth phase were only present on the yeast surface.

4. Discussion

In the conditions used in the present study, polyphenols induced significant changes in yeast metabolism and fermentation kinetics. Whether in a stressed environment (low phytosterol and high sugar content) or not, we observed a decrease of the specific growth rates and final populations, leading to a high content of residual assimilable nitrogen. In the presence of

polyphenols, cell mortality appeared in the early stages of the fermentations and yeasts presented a lower capacity to convert sugar into ethanol (illustrated by the low specific rate of CO_2 production on Figure 2). These combined factors led to a substantial increase of the fermentation durations compared to controls. After a long stress period, starting from the beginning of the fermentation to the mid-stationary phase, yeasts ended up adapting to environmental conditions and exhibited a fermentation activity close to that of the controls. Despite the stress suffered by yeast in the presence of polyphenols, about 98% of the initial sugar was consumed at the end of the fermentation when the phytosterol content was high and the sugar content equal to 220 g/L. Surprisingly, during the last part of the alcoholic fermentation, cells exhibited a higher viability at the same fermentation stage, suggesting a better resistance to ethanol stress at the end of the process. This is confirmed by the principal component analysis (PCA) (Figure 8), performed to evaluate the correlation between the variables analysed during the fermentations (variable factor map) and the proximity between samples (individual factor map). Polyphenols were the first parameter that differentiated samples into two main groups: fermentations performed without polyphenol (first group, in blue), and with polyphenols (second group, in red). The first group is characterized by a high fermentation activity (V_{max}, specific CO_2 rate), high cell growth (specific growth and final population) and late mortality. These factors were strongly positively correlated between them. The second group regroups samples with polyphenols, characterized by high residual nitrogen and fermentation duration, the latter being strongly correlated. Each group was divided into two subgroups according to environmental conditions. Fermentations performed in stressed conditions were characterized by higher residual sugar content. All strains presented similar behaviours in the presence of polyphenols, except Y3 in non-stressed conditions. This strain differed from the other ones by the high concentration and production yield of ethanol. In general, present results are in accordance with those of Li et al. (2011), who showed an inhibition effect of grape proanthocyanidins on cell growth, CO_2 release, sugar consumption and ethanol production during the initial phase of the fermentation. An improvement of yeast metabolism was observed at the mid-exponential phase, suggesting an adaptive response mechanism.

Regarding present results, different mechanisms can be hypothesized to explain the impact of polyphenols on yeast metabolism: physico-chemical interactions between polyphenols and

nutrients such as amino acids and sterols in musts, decreasing their availability for yeast cells, or direct interactions of some polyphenols with living cells, modifying their metabolic activity.

- **Interactions between polyphenols and nutrients in musts**

Interactions between polyphenols and nitrogen compounds have mainly been investigated in the context of interactions between proanthocyanidins and proteins (Bate-Smith, 1954; Maury, Sarni-Manchado, Lefebvre, Cheynier, & Moutounet, 2001). Non-covalent physico-chemical interactions between proanthocyanidins and proteins are attributed to hydrophobic attraction between the aromatic rings of polyphenols and aromatic amino-acids (proline, phenylalanine, tyrosine, tryptophan, histidine), strengthened by hydrogen bonds between polyphenol hydroxyl groups (H-donors) and another adjacent atom bearing a lone pair of electrons such as oxygen of carboxylic groups or nitrogen of aromatic amino acids of proteins, leading to the formation of mostly irreversible soluble or insoluble complexes (Charlton et al., 2002; Siebert, Troukhanova, & Lynn, 1996). When dealing with tannins and proteins, the irreversible character of non covalent interactions is related to the formation of multiple bonds. In this work, interactions between amino acids of the must and polyphenols could be involved in the formation of soluble and reversible complexes making amino acids less available or less recognizable by yeast nutrient sensors. On the other hand, interactions between sterols and polyphenols in solution have been observed (Marquez, Millan, Souquet, & Salmon, 2009). The effect of ergosterol in a solubilized form in a model solution of (+)-catechin/acetaldehyde was checked in the context of the study of the adsorption of phenolic compounds by yeast and more precisely of the adsorption of phenolic compounds involved in the browning of oxidized white wines. The analysis of the supernatant after interactions and centrifugation indicated direct interactions between ergosterols and polyphenolic compounds (mainly the colorless intermediate compounds of the browning reactions). In our case, interactions in solution between phytosterols and polyphenols can also occur. Such interactions, by decreasing phytosterol availability, could also explain the results obtained. Indeed, phytosterols are essential nutrients for the building of yeast plasma membranes in anaerobic conditions, as cells are unable to synthesize their own sterols.

- **Direct interactions between polyphenols and living cells**

The present study demonstrated interactions between grape polyphenols and yeast cells from the early stage of the fermentation (growth phase). If these interactions were limited at the outer part of the yeast in non-stressed conditions, the passage of some polyphenols through the yeast envelope and their accumulation in the intracellular space of almost all living cells was evidenced with the sterol deficient medium. This could be related to the different compositions of the polyphenol pools used in stressed and non-stressed conditions (Table 1). However, the same polyphenol families were found in PP1 and PP2. Another possibility is that a different membrane permeability is caused by the lack of sterols. In any case, the fact that some polyphenols enter cells under stressed conditions does not increase cell mortality compared to what is observed under non-stressed conditions. This would indicate the involvement of a transport system that protects yeast internal environment (trapping inside vesicles for example) against interactions that would be harmful such as interactions between polyphenols and internal functional proteins.

Polyphenol adsorption on yeast cell outer surface, demonstrated in both tested conditions, may have detrimental consequences on the cell metabolic activity by impairing cell signalling functions and nutrient transport.

Indeed, different studies have demonstrated the disorganization of plasma membrane as the result of polyphenol binding. Such binding could affect its integrity and fluidity, leading to the modification of its function. Thus, direct interactions between flavanol monomer (catechin) or colourless intermediates resulting from (+)-catechin/glyoxylic acid and (+)-catechin-/acetaldehyde reactions with the sterols of yeast plasma membrane have been evidenced. These interactions strongly altered the physical and chemical properties of functional reconstituted plasma membrane vesicles, especially for vesicles enriched with grape phytosterols (Marquez, Millan, & Salmon, 2009). Another study dealing with flavanol-lipid interactions in the context of astringency perception also showed interactions between membrane lipids and catechin (C), epicatechin (EC) and epigallocatechin-gallate (EGCG) (Furlan, Castets, et al., 2014; Furlan, Jobin, et al., 2014). In that work, the authors mimicked the membranes of the oral cavity by micrometric size liposomes and lipid droplets in food by nanometric isotropic bicelles. Deuterium and phosphorus solid-state NMR demonstrated the membrane hydrophobic core disordering promoted by flavanol monomers. C and EGCG destabilized isotropic bicelles and convert them

493 into an inverted hexagonal phase. Flavanols were shown to be located at the membrane interface
494 and to stabilize the lamellar phases.
495 Other studies (Khan et al., 2013; Brüning, 2013; Wu & Liu, 2013) have also shown that some
496 flavonoids can directly interact with cell plasma membrane and modulate various signalling
497 pathways affecting cell proliferation, viability and metabolism. These properties are used in
498 cancer treatment and prevention. Several flavonoids affect the proliferation of cancer cells by
499 inhibiting the TORC2 signalling complex (protein kinase targeted by rapamycin), which is
500 located in the cell membrane and plays a major role in controlling cell division (actin
501 cytoskeleton synthesis) and endocytosis (Khan et al., 2013). This property has been shown for
502 small polyphenols present in grapes and wines such as quercetin (Brüning, 2013), resveratrol
503 (Wu & Liu, 2013) and epigallocatechine gallate (Wang, Sun, & Yakisich, 2013). The binding of
504 these polyphenols on TORC2 led to the suppression of the signalling function, associated with a
505 strong decrease of cell growth (Khan et al., 2013). As resveratrol (stilbene) and small flavonoids
506 are present in the polyphenol pools used in this work, their binding on the TORC2 signalling
507 complex could explain the results obtained: a lower nitrogen consumption resulting in lower cell
508 growth, a higher mortality in the early stage of the fermentation and thus an increased
509 fermentation duration.
510
511
512 **Conclusion**
513 This present work showed the impact of polyphenols purified from a thermovinification must on
514 yeast cell growth and fermentation capacity in the frame of the chosen conditions: a synthetic
515 must, the early addition of a high concentration (2 g/L) of polyphenols and a complete anaerobic
516 environment. It will be of interest to evaluate the genericity of these results in real enological
517 conditions, especially in red wine traditional winemaking process where grape polyphenols are
518 released progressively and where the fermentation is generally performed in a partially aerobic
519 environment. The results obtained highlighted an impact of polyphenols on yeast metabolism
520 during fermentation that deserves to be studied in depth. It would be necessary to study separately
521 the different families of grape polyphenols on yeast metabolism and fermentation kinetics and to
522 further explore their impact on yeast signaling and plasma membrane integrity/functionality.
523 Interactions in solution between grape polyphenols and must nutrients such as amino acids,

phytosterols and vitamins also deserve to be better understood for a better control of red wine fermentations.

Abbreviation used

H^+-ATPase	Proton adenosintriphosphatase
INRA	Institut National de la Recherche Agronomique
DAD	Diode Array Detector
MS	Mass spectrometry
KH_2PO_4	Potassium dihydrogen phosphate
K_2SO_4	Potassium sulfate
$MgSO_4.7H_2O$	Magnesium Sulfate Heptahydrate
$CaCl_2.2H_2O$	Calcium chloride dihydrate
oNaCl	Sodium hypochlorite
$MnSO_4.H_2O$	Manganese sulfate monohydrate
$ZnSO_4.7H_2O$	Zinc sulfate heptahydrate
$CuSO_4.5H_2O$	Cupric sulfate pentahydrate
$CoCl_2.6H_2O$	Cobalt(II) chloride hexahydrate
H_3BO_3	Boric acid
$(NH_4)6M_{O7}O_{24}$	Ammonium heptamolybdate
FeCl3	Iron trichloride
NaOH	Sodium hydroxide
NH_4Cl	Ammonium chloride
H_2SO_4	Sulfuric acid

549	$TORC_2$	Target of rapamycin complex 2
550	NMR	Nuclear magnetic resonance
551	λexc	Exitation wavelength
552	λem	Emission wavelength
553		

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562

Tables

Table 1: Composition of the two polyphenolic extracts, expressed in mg/g of dried weight.

	PP1	PP2
Total anthocyanins	376	159
Hydroxycinnamic acids	38	15
Flavonols	29	39
Pyranoanthocyanins	2	1.4
Stilbenes	60	36
Proanthocyanidins	140	163
*aDP**	*5.9*	*6.0*
*% EgC**	*29*	*29*
*% Ec-G**	*3.7*	*3.4*
Yield (% mass conversion)	65 %	44 %

*Proanthocyanidin characteristics: aDP, average degree of polymerization; %EgC, percentage in epigallocatechin units; % Ec-G, percentage in epicatechin-gallate units.

Table 2: Effect of polyphenols on cell growth (cell population and specific growth rate), metabolite consumption (nitrogen compounds, sugar) and ethanol production.

	Stressed conditions			Non-stressed conditions		
Parameters	Yeast strains	Polyphenols		Yeast strains	Polyphenols	
		-Polyphenol	+Polyphenol		-Polyphenol	+Polyphenol
Cell population (x 10^7/mL)	Y1 a	15.0 ± 0.2 a	4.0 ± 0.5 b	Y1 a	9.5 ± 0.0 a	4.5 ± 0.5 b
	Y2 a	15.1 ± 0.7 a	6.2 ± 0.7 b	Y2 a	9.0 ± 0.0 a	4.8 ± 0.2 b
	Y3 a	18.1 ± 0.2 a	3.1 ± 0.1 b	Y3 a	7.6 ± 0.1 a	6.3 ± 1.3 b
	Y4 a	13.2 ± 1 a	4.1 ± 0.3 b	Y4 a	12.3 ± 3.8 a	6.3 ± 1.2 b
*p- Value**	*P>0.05*	*p <0.0001*		*P>0.05*	*p <0.0001*	
Specific growth rate	Y1 ab	0.13 ± 0.01 a	0.06 ± 0.00 b	Y1 a	0.16 ± 0.00 a	0.09 ± 0.01 b
	Y2 b	0.11 ± 0.01 a	0.05 ± 0.00 b	Y2 a	0.17 ± 0.00 a	0.11 ± 0.01 b
	Y3 a	0.14 ± 0.00 a	0.11 ± 0.00 b	Y3 a	0.19 ± 0.01 a	0.07 ± 0.02 b
	Y4 b	0.10 ± 0.01 a	0.07 ± 0.00 b	Y4 a	0.11 ± 0.01 a	0.04 ± 0.02 b
*p- Value**	*p <0.0001*	*p <0.0001*		*p >0.05*	*p <0.0001*	
Assimilable residual nitrogen (mg/L)	Initial must	382 ± 11	382 ± 13	Initial must	382 ± 11	382 ± 13
	Y1 a	83 ± 2 a	208 ± 5 b	Y1 a	74 ± 5 a	266 ± 6 b
	Y2 a	93 ± 4 a	214 ± 4 b	Y2 a	61 ± 9 a	164 ± 16 b
	Y3 a	32 ± 3 a	186 ± 6 b	Y3 a	51 ± 4 a	244 ± 3 b
	Y4 a	40 ± 5 a	275 ± 3 b	Y4 a	66 ± 10 a	273 ± 17 b
*p- Value**	*p >0.05*	*p <0.0001*		*p >0.05*	*p <0.0001*	
Residual sugar (g/L)	Initial must	220 ± 0	220 ± 0	Initial must	250 ± 0	250 ± 0
	Y1 a	22 ± 2 a	24 ± 1 a	Y1 a	0.5 ± 0.1 a	2.7 ± 1.6 b
	Y2 ab	20 ± 2 a	23 ± 2 a	Y2 a	0.5 ± 0.1 a	5.4 ± 1.8 b
	Y3 b	20 ± 2 a	17 ± 1 a	Y3 a	0.4 ± 0.0 a	2.7 ± 0.7 b
	Y4 c	0.7 ± 0 a	8 ± 0 a	Y4 a	0.5 ± 0.0 a	26.6 ± 7.5 b
*p- Value**	*p <0.0001*	*p >0.05*		*p >0.05*	*P<0.01*	
Ethanol production (g/L)	Y1 a	105 ± 1 a	108 ± 0 a	Y1 a	100 ± 13 a	114 ± 1 b
	Y2 a	103 ± 0 a	105 ± 1 a	Y2 a	101 ± 15 a	113 ± 0 b
	Y3 a	102 ± 1 a	108 ± 2 a	Y3 a	107 ± 5 a	139 ± 7 b
	Y4 b	112 ± 1 a	112 ± 1 a	Y4 a	111 ± 2 a	105 ± 1 b
*p- Value**	*p <0.05*	*p >0.05*		*p >0.05*	*p <0.05*	
Yield of ethanol production (g/L)	Y1 a	0.42 ± 0.00 a	0.43 ± 0.00 a	Y1 a	0.45 ± 0.05 a	0.52 ± 0.00 b
	Y2 b	0.41 ± 0.00 a	0.42 ± 0.00 a	Y2 a	0.46 ± 0.07 a	0.51 ± 0.00 b
	Y3 ab	0.41 ± 0.00 a	0.43 ± 0.01 a	Y3 a	0.49 ± 0.02 a	0.63 ± 0.03 b
	Y4 c	0.45 ± 0.00 a	0.45 ± 0.00 a	Y4 a	0.50 ± 0.01 a	0.48 ± 0.00 b
*p- Value**	*p <0.0001*	*p >0.05*		*p >0.05*	*p <0.05*	

Determinations have been performed in triplicate. *P value**: significance test of polyphenols and yeast trains effects on cell growth and metabolite consumption. Different letters indicate significant difference ($p<0.05$) among quantitative or qualitative variables.

Figure caption

Figure 1. Fermentation kinetics in stressed and non-stressed conditions. Two replicates without polyphenol (blue and green curves), two replicates with polyphenols (red and yellow curves).

Figure 2. Change in the specific fermentation rate as a function of consumed sugar in stressed and non-stressed conditions. Two replicates without polyphenol (blue and green curves), two replicates with polyphenols (red and yellow curves).

Figure 3. Evolution of cell mortality during fermentations in stressed and non-stressed conditions performed without polyphenols (controls, white bars) and with polyphenols (grey bars) .

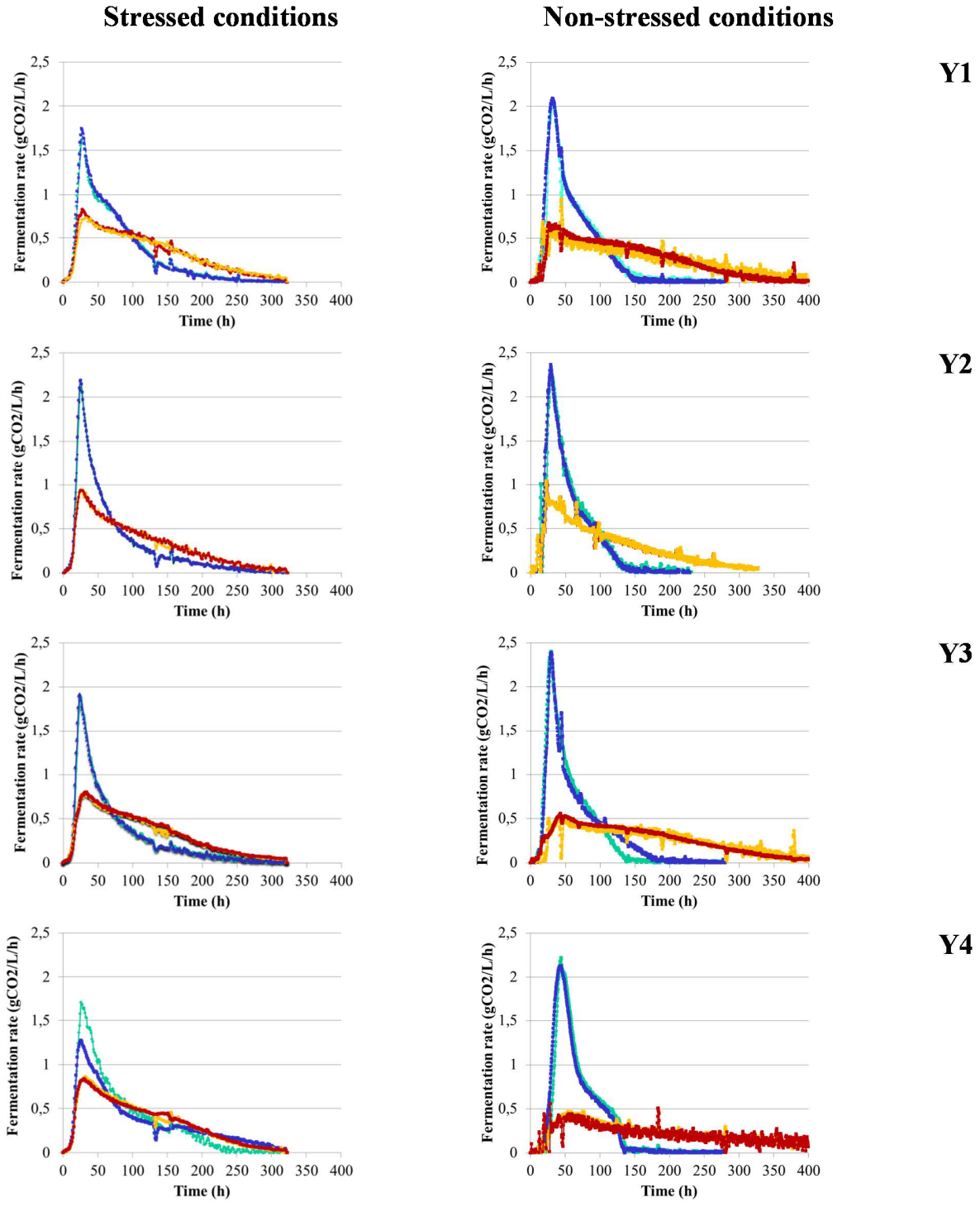
Figure 4. Epifluorescence images of yeast cells at the end of growth phase (λexc = 500−600 nm, λem =540−650 nm).

Figure 5. Confocal images of yeast cells at the end of growth phase in stressed conditions. White arrows: destroyed cells. λexc = 405 nm, λem = 530−600 nm.

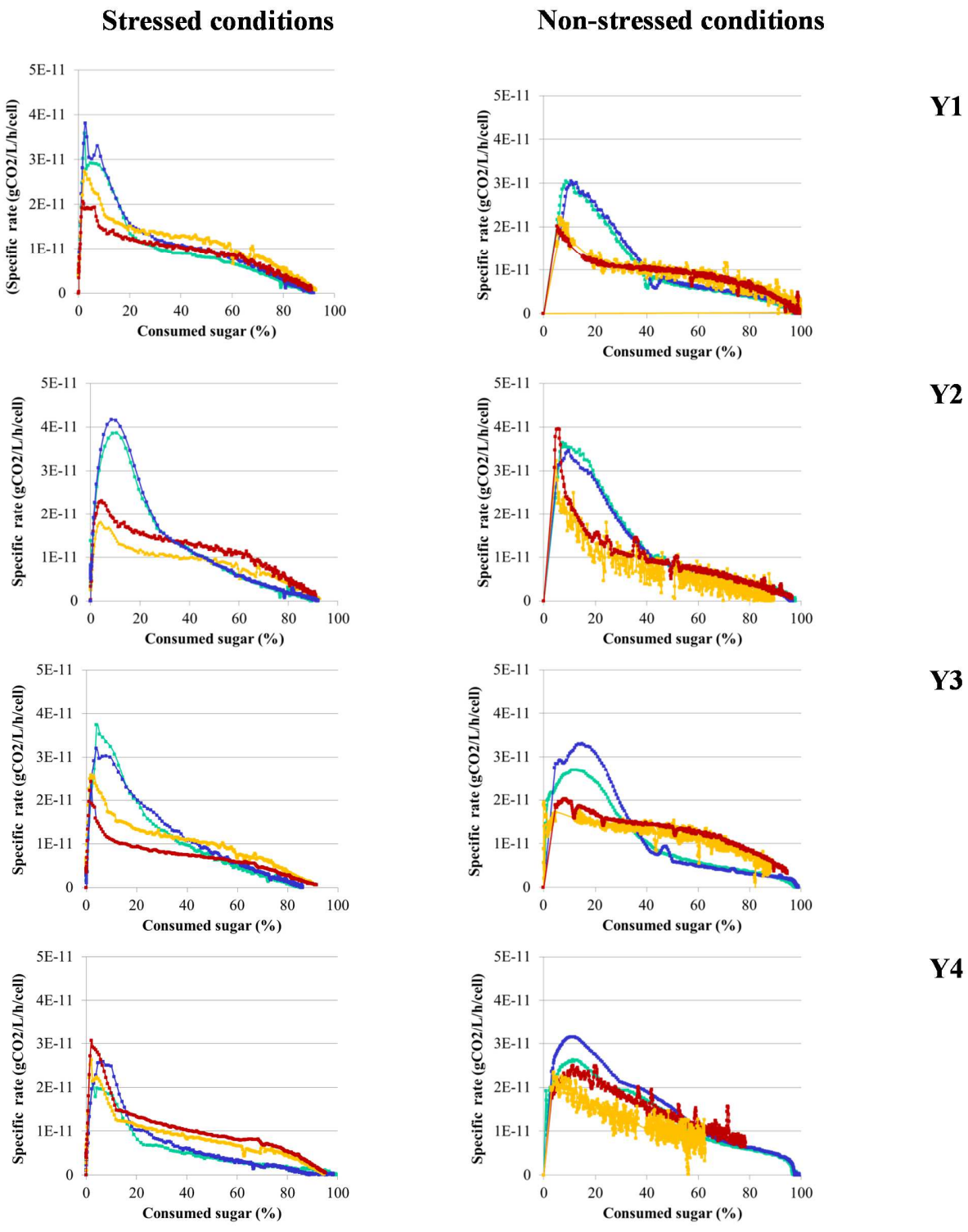
Figure 6. Confocal images of yeast cells at the end of growth phase in non-stressed conditions. λexc = 405 nm, λem = 530−600 nm.

Figure 7. Confocal images of Y1 (first column) and spectral analysis (second column) showing their autofluorescence emission spectrum (λem = 420−750 nm range). Y1 in stressed conditions with polyphenols (A and B) and without polyphenol (C and D); Y1 in non-stressed conditions with polyphenols (E and F) and without polyphenol (G and H).

Figure 8. Principal component analysis (PCA) of kinetic parameters (duration, specific growth rate, specific rate of CO_2 production, Vmax), total cell number (cell population), mortality at the early stage of fermentation (early mortality), mortality at the end of fermentation (late mortality), residual nitrogen and ethanol production. On the individual factor map, yeasts without polyphenol in stressed and non-stressed conditions (a and c respectively), yeasts with polyphenols in stressed and non-stressed conditions (b and d respectively).



Mekoue et al. figure 1



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643 *Mekoue et al. figure 2*
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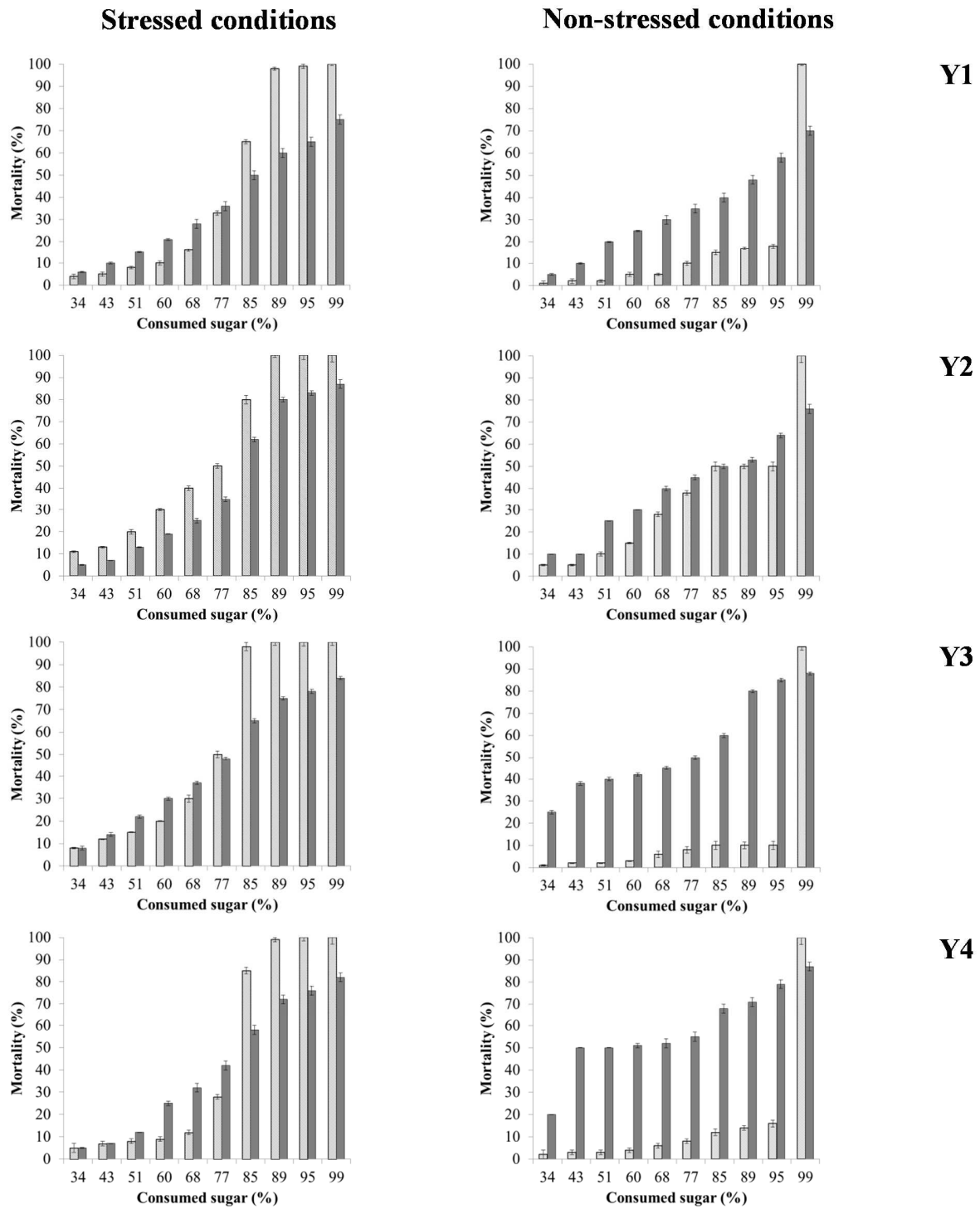

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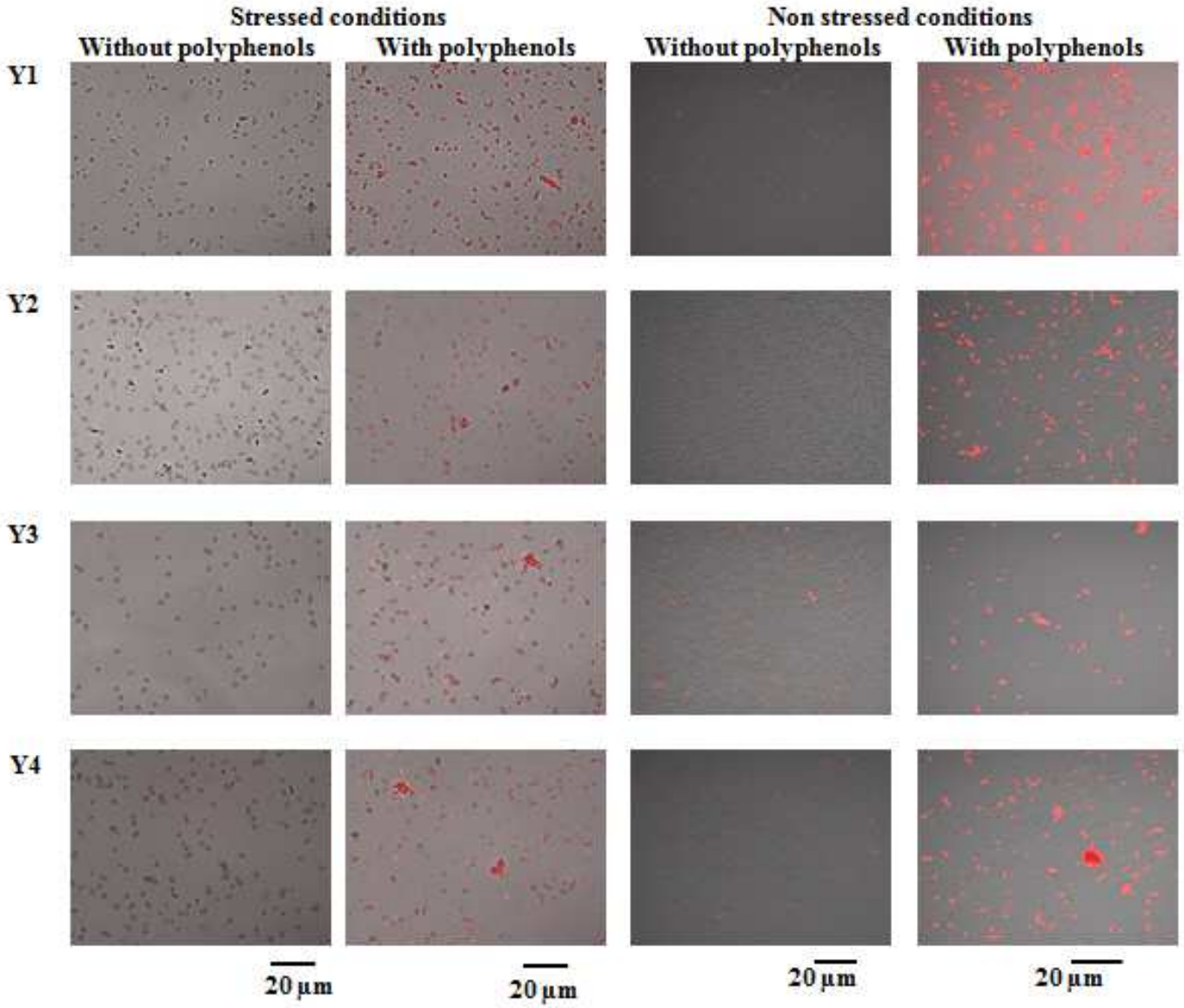

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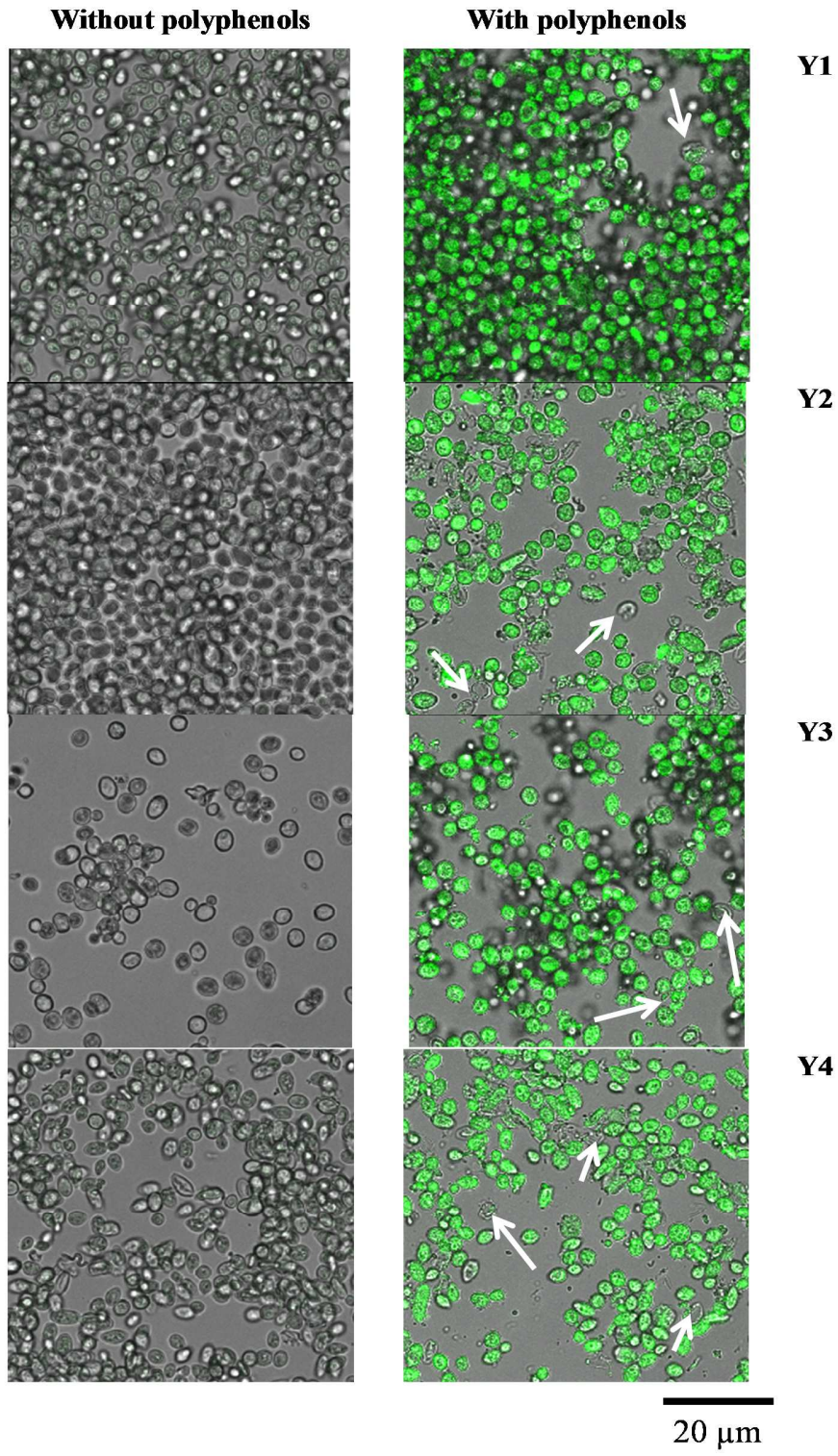

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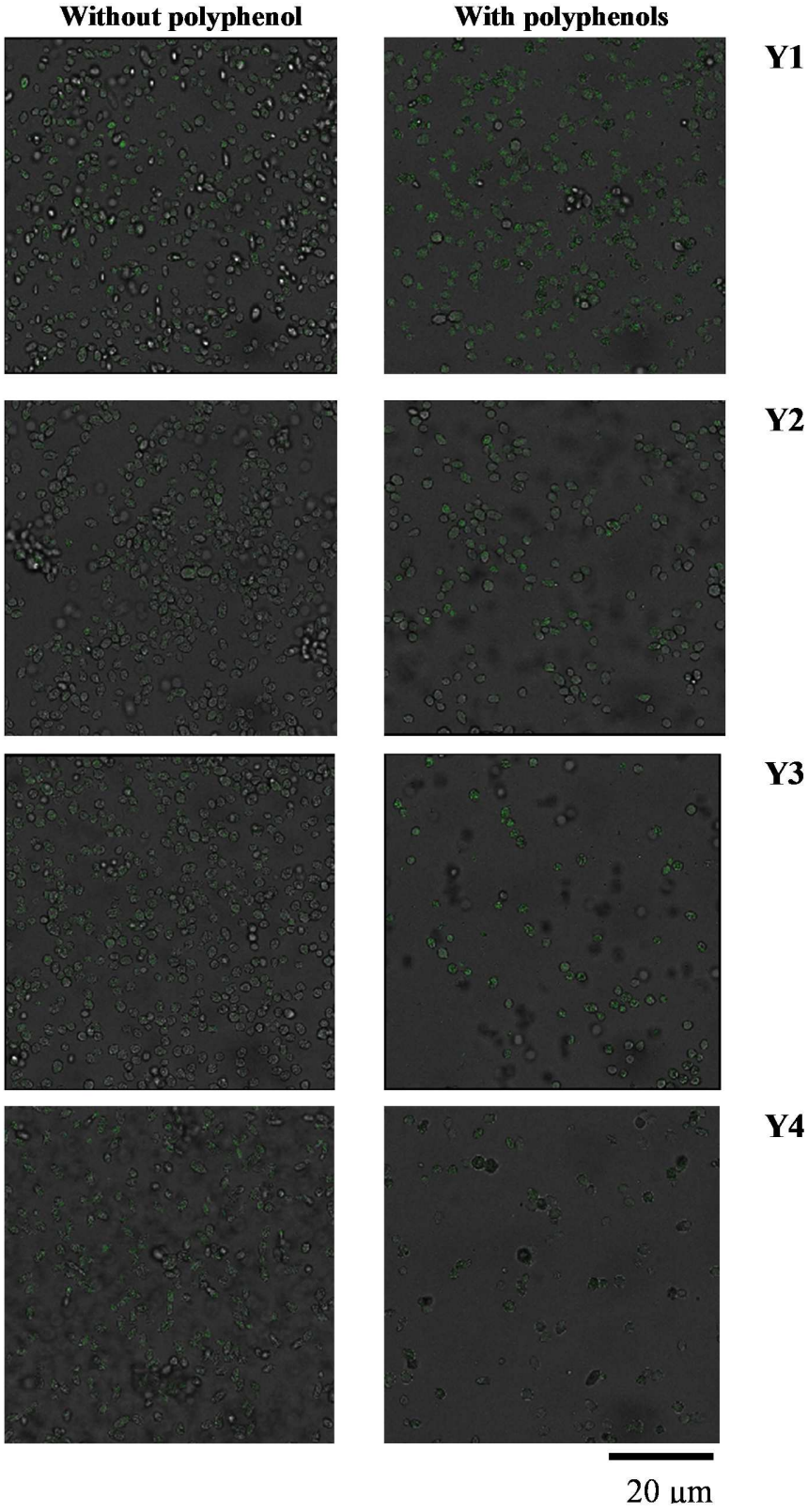

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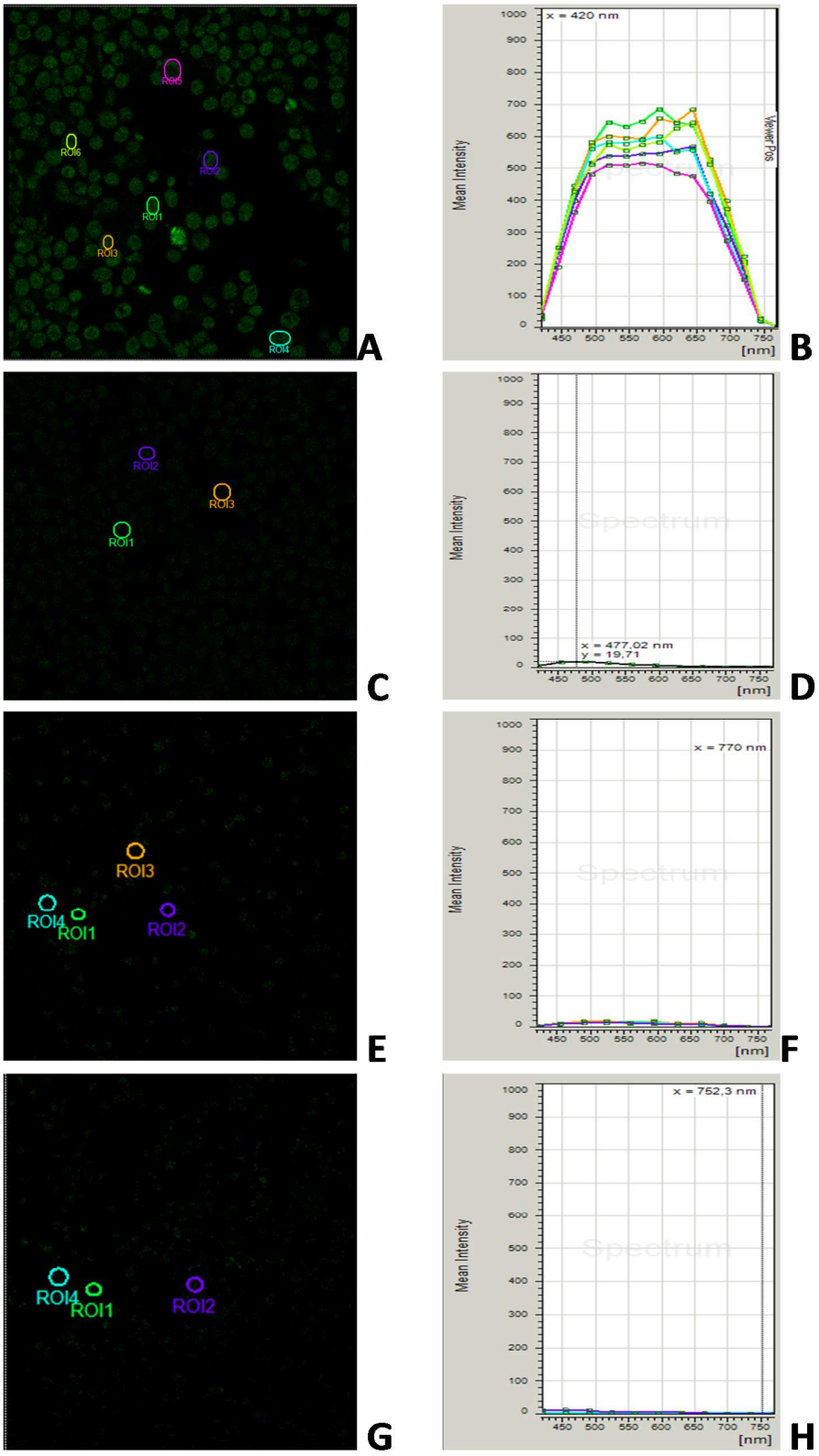

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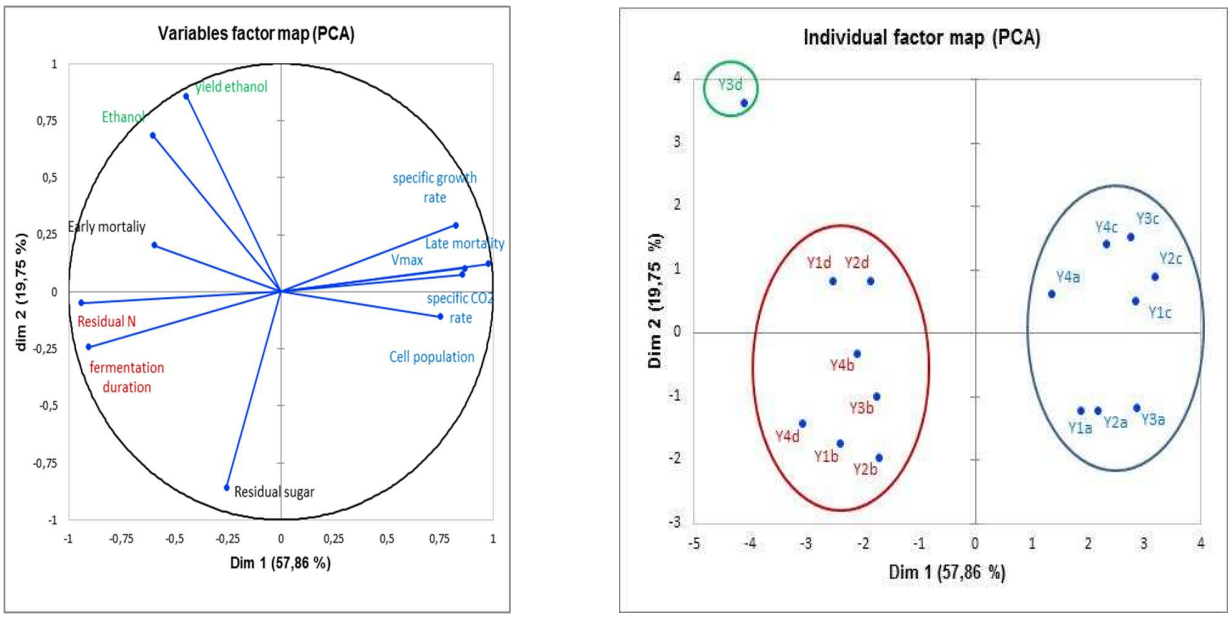
658 *Mekoue et al. figure 7*



Mekoue et al. figure 8

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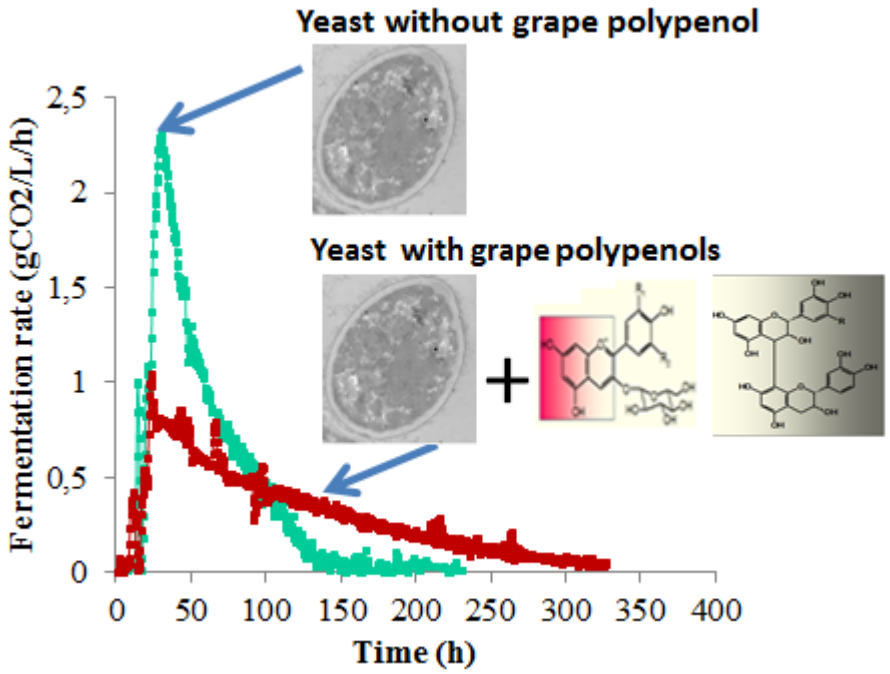
Yeast without grape polypenol
Yeast with grape polypenols
Fermentation rate (gCO2/L/h)
2,5
2
1,5
1
0,5
0
0
50
100
150
200
250
300
350
400
Time (h)