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Food Chemistry 71 (2000) 519–528

Food Chemistry

www.elsevier.com/locate/foodchem

Oxygen consumption by wine lees: impact on lees integrity during wine ageing

Jean-Michel Salmon *, Caroline Fornairon-Bonnefond, Jean-Paul Mazauric, Michel Moutounet

Unité Mixte de Recherches «Sciences pour l'oenologie», INRA-IPV, 2, place Viala, F-34060 Montpellier Cedex 1, France

Received 15 March 2000; received in revised form 24 May 2000; accepted 24 May 2000

Abstract

During wine ageing on lees, periodic stirring of the lees and repetitive additions of small amounts of oxygen to the wine is generally performed traditionally and empirically. Although this technological step is thought to have a significant impact on the organoleptic composition of the final product, little is known about the interaction between oxygen and lees during such a process. During simulation of wine ageing on lees, we now show that some membrane lipids of yeast lees, in contact with dissolved oxygen, even at very low concentration, undergo a mild oxidation. These oxidation reactions lead to the production of lipid peroxides and then to the production of unknown end-products. They are primarily due to the production of reactive oxygen species (ROS), and totally explain the capacity of yeast lees to consume oxygen during wine ageing. The lipid oxidations within the plasma membrane are responsible for strong modifications of plasma membrane order. Some of the final products of oxidation reactions may play a favourable role in the organoleptic equilibrium of wines aged on lees.

Keywords: Wine ageing; Yeast; Lees; Oxygen; Lipid peroxidation

1. Introduction

Wine lees are mainly constituted of yeast cells produced during alcoholic fermentation, deposited at the bottom of fermentation tanks and mixed with tartaric salts, bacteria and some plant cell debris. Wine ageing on their fine lees (essentially dead yeast cells) is a traditional enological practice used during the manufacture of wines in different countries (Boulton, Singleton, Bisson & Kunkee, 1996). This ageing option ranges from placing wines in barrels to placing wines in large cooperage systems and stainless steel tanks with their yeasts and some grape solids. Ageing provides the conditions, primarily several months of yeast contact, for many components within the yeast to leak into the wine. Although the organoleptic composition of wine changes during the ageing of wines in the presence of lees, recent studies indicate that neither cell wall nor cell membranes lose their integrity, as detected by microscopic techniques (Charpentier & Feuillat, 1993; Charpentier, Nguyen Van Long, Bonaly & Feuillat, 1986; Hernawan & Fleet 1995; Moreno-Arribas, Pueyo, Polo & Martin-Alvarez, 1998). During wine ageing on lees, the release of wall polysaccharides, nitrogen, fatty and nucleic acids has been demonstrated and clearly attributed to an autolysis phenomenon (Ferrari & Feuillat, 1988; Leroy, Charpentier, Duteurtre, Feuillat & Charpentier, 1990; Llauberes, 1997; Pueyo, Martinez-Rodriguez, Polo, Santa-Maria & Bartolome, 2000). Moreover, it has been shown that yeast lees during autolysis were able to interact with wine volatile thiols by making disulfuride bonds with wall mannoproteins (Lavigne & Dubourdieu, 1996).

There are many examples of periodic stirring of the lees. Such practice includes repetitive additions of small amounts of oxygen to the wines and is generally associated with a limited homogenisation of wine and lees on a traditional and empirical basis. However, there

* Corresponding author. Tel.: +33-499-61-25-05; fax: +33-499-61-28-57.

E-mail address: jmsalmon@ensam.inra.fr (J.-M. Salmon).

0308-8146/00/$ - see front matter
PII: S0308-8146(00)00204-1

appear to be only few reports indicating significant effects due to this treatment. From an organoleptic point of view, several studies indicate that wine ageing on lees led to well-balanced and smooth wines and, more generally, to the modification of the aromatic intensity of the wines (Feuillat, 1992; Lubbers, Voilley, Feuillat & Charpentier, 1994). Moreover it has been speculated that the presence of lees may confer, to the wine, a high cellaring capability (Feuillat, 1987).

However, most of the studies about ageing on lees have only been focused on compounds released into the wine by yeast autolysis. A recent study has revealed that yeast lees were able to exhibit specific oxygen utilization rates ranging from 1 to 4 μmol O_2 h^{-1} 10^{-10} cells from the second to the sixth month of wine ageing (Fornairon, Mazauric, Salmon & Moutounet, 1999). Such an oxygen consumption by *S. cerevisiae* lees was proved to be completely independent of any cellular viability. Although these oxygen consumption rates are submitted to a time-decay during wine ageing on lees, such oxygen utilization by yeast lees could be responsible for the total dissolved oxygen depletion of homogenised wines in less than one day at 14°C. It is important to notice that all the previous works done on lees autolysis have always been carried out without taking into account the potential interaction of lees with oxygen. This strong interaction of yeast lees with oxygen during wine ageing has been investigated neither in terms of impact on lees integrity, nor of final reaction products, which may exert strong organoleptic effects on the ultimate quality of the wine. In the present study, we present preliminary results obtained on the impact of oxygen on the membrane lipids and integrity of yeast lees during simulation of wine ageing on lees.

2. Materials and methods

2.1. Strains and culture conditions

Saccharomyces cerevisiae, strain K1, was an industrial diploid homothallic wine yeast commercialized as a dry yeast (K1 ICV-INRA, Montpellier, France).

2.1.1. Culture media

The synthetic medium MS300 used in this study was a simulated standard grape juice and was strongly buffered to pH 3.3 (Bely, Sablayrolles & Barre, 1990). This medium contained, in excess, all the vitamins essential for growth of the yeast strains. It contained the following (per litre): glucose 200 g; citric acid 6 g; DL-malic acid 6 g; mineral salts (mg): KH_2PO_4 750, KH_2SO_4 500, $MgSO_4{\cdot}7H_2O$ 250, $CaCl_2{\cdot}2H_2O$ 155, NaCl 200, $MnSO_4{\cdot}H_2O$ 4, $ZnSO_4$ 4, $CuSO_4{\cdot}5H_2O$ 1, KI 1, $CoCl_2{\cdot}6H_2O$ 0.4, H_3BO_3 1, $NaMoO_4{\cdot}2H_2O$ 1; vitamins (mg): myo-inositol 20, nicotinic acid 2, calcium panthothenate 1.5, thiamin hydrochloride 0.25, pyridoxine hydrochloride 0.25, biotin 0.003; anaerobic growth factors: ergosterol 15 mg, sodium oleate 5 mg, Tween 80 0.5 ml; nitrogen source: 385 mgN l^{-1} as (% w/w) ammoniacal nitrogen (NH_4Cl) 18.6 and amino acids: L-proline 20.5, L-glutamine 16.9, L-arginine 1.25, L-tryptophan 6, L-alanine 4.9, L-glutamic acid 4, L-serine 2.6, L-threonine 2.6, L-leucine 1.6, L-aspartic acid 1.5, L-valine 1.5, L-phenylalanine 1.3, L-isoleucine 1.1, L-histidine 1.1, L-methionine 1.1, L-tyrosine 0.6, L-glycine 0.6, L-lysine 0.6, and L-cysteine 0.4. Since the amino acid proline is not assimilable by the yeast strains used in this study under anaerobic growth conditions, the ammonium salts and α-amino acids (all amino acids except proline) in the medium were considered as assimilable nitrogen. As a consequence, the assimilable nitrogen concentration of MS300 medium is 300 mgN l^{-1}.

2.1.2. Growth conditions

K1 strain, which is available as a commercial dry yeast, was not precultured and was thus directly inoculated into fermentation medium after the rehydration procedure recommended by the manufacturer. Briefly, 1 g dry yeast was suspended in 10 ml warm water (37°C) containing 0.5 g glucose. This suspension was kept without stirring for 30 min at 37°C, except for one short stirring after 15 min incubation. Inoculation of fermentation media was realized by adding 0.5 ml of this suspension per litre of culture medium. Such yeast inoculum corresponded to the enological practice on the industrial scale (5 g hl^{-1}). Fermentation media were normally de-aerated by bubbling argon prior to inoculation (initial oxygen concentration below 1 mg l^{-1}). Filling conditions were controlled, and fermentations were carried out in fermenters (1.2 l) fitted with fermentation locks with permanent stirring under isothermal conditions (28°C) and anaerobiosis.

2.1.3. Cell harvesting and simulation of wine ageing on lees

Unless specified in the text, yeast cells were harvested by centrifugation exactly 100 h after the end of alcoholic fermentation, as determined by the absence of residual sugar in the culture medium (concentration < 2 g l^{-1}). This 100 h-lag time after the end of fermentation was previously found to be necessary in order to get a null cell viability ($< 10^{-3}$) within yeast cells (Fornairon et al., 1999). Such yeast cells were then further considered as yeast lees. Yeast lees were washed twice in a synthetic medium simulating a standard wine and suspended at the desired cell concentration in the same medium. This medium was buffered to pH 3.3 and contained the following (per litre): citric acid 6 g; DL-malic acid 6 g; mineral salts (mg): KH_2PO_4 750, KH_2SO_4 500, $MgSO_4.7H_2O$ 250, $CaCl_2.2H_2O$ 155, NaCl 200; ethanol 120 ml.

2.2. Analytical methods

2.2.1. Culture growth

Culture growth and cell number were monitored by using an electronic particle counter (Coulter-Counter Coultronics, ZBI model) linked to a channelyzer (Coulter-Counter Coultronics, Channelyzer 254 model).

2.2.2. Residual glucose

Glucose consumption during growth was measured with dinitrosalicylic acid reagent (Miller,1959).

2.2.3. Protein determination

Protein concentrations were determined by the BCA protein reagent method (Pierce chemicals), after precipitation with TCA. Bovine serum albumin was used as standard.

2.2.4. QO_2 measurements

After centrifugation (5 min, 500 *g*), cells were washed twice and suspended at a 10^9 cells ml^{-1} cell density in 100 mM phthalic acid buffer (pH 4.5). After a 15 min temperature equilibration, QO_2 measurements were performed according to the methodology previously described (Salmon, Fornairon & Barre, 1998) in a 1.8 ml thermostatted reaction vessel (30°C) fitted with a Clark oxygen probe (typeYSI 5331, Gilson, Middleton, USA).

2.2.5. Cellular viability

Cellular viability was obtained by plating about 1000 cells, as determined with the electronic particle counter, on YPD agar medium contained in Petri dishes (Agar 20 g l^{-1}, yeast extract (Difco, USA) 10 g l^{-1}, bactopeptone (Difco) 20 g l^{-1}, and glucose 20 g l^{-1}). Petri dishes were then incubated for 48 h at 28°C and examined for the presence of colonies.

2.2.6. Quantification of hydroperoxides in yeast lees by ferrous ion oxidation in the presence of xylenol orange (FOX method)

As this method is described as more sensitive than the widely employed thiobarbituric acid assay (TBA) for the direct measurement of lipid peroxides (Jiang, Woollard & Wolff, 1991), we used this method when yeast lees were used at high concentrations (beyond 10^8 cells ml^{-1}). One hundred microlitres yeast lees suspensions were added to 900 µl of pure methanol containing the lipid-soluble antioxidant butylated hydroxytoluene (BHT) (4 mM). One hundred µl of methanol-solubilized sample was added to 900 µl of the following reaction mixture and incubated at 28°C for 30 min prior to measurement at 560 nm : 100 µM xylenol orange, 250 µM Fe^{2+} ($Fe(NH_4)_2(SO_4)_2$), 25 mM H_2SO_4, and 4 mM BHT in 90% (v/v) methanol. (The reaction is complete after 15 min and stable overnight at room temperature). According to previous calibrations performed with standard peroxides (hydrogen peroxide, linoleic hydroperoxide, *t*-butyl hydroperoxide and cumene hydroperoxide), a mean apparent extinction coefficient of 4.5 10^4 l mol^{-1} cm^{-1} was used for peroxide concentration calculations (Jiang, Hunt & Wolff, 1992).

2.2.7. Quantification of lipid hydroperoxides in yeast lees by detection of diphenyl-1-pyrenylphosphine (DPPP) fluorescence

The production of lipid hydroperoxides within yeast lees was determined by measuring the oxidation of the nonfluorescent probe diphenyl-1-pyrenylphosphine (DPPP, Molecular Probes, Eugene, OR) into the corresponding strongly fluorescent phosphine oxide. The very high solubility of DPPP in lipids allowed the detection of hydroperoxides in the membranes of cells and lipid vesicles (Noguchi, Numano, Kaneda & Niki, 1998). Two hundred microlitres of yeast lees suspension were added to 1800 µl of 0.9% (w/v) NaCl and equilibrated with continuous stirring at 30°C for 30 min in a 1 cm-pathlength quartz cuvette. Twenty microlitres of DPP from a 1 mM stock solution in pure acetonitrile were then added to the lees suspension. When measurements were stabilized, fluorescence determinations were made every 12 s by using a Perkin-Elmer LS50-B fluorescence spectrometer with the following settings : 358 nm excitation (10 nm bandwidth), 379 nm emission (2.5 nm bandwidth). Calibration of the probe was achieved by adding known amounts of hydrogen peroxide during the assay.

2.2.8. Detection of reactive oxygen species (ROS) in yeast lees by using the H_2O_2-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA)

The H_2O_2-sensitive dye DCFH-DA (Molecular Probes, Eugene, OR) was used to measure hydrogen peroxide production in yeast lees. DCFH-DA staining was conducted by including the dye (10 µM) from a 100 mM stock solution in dimethylsulfoxide in the culture medium throughout the entire culture period. Utilization of this probe to measure H_2O_2 in hypoxic cultures of *Saccharomyces cerevisiae* has been previously described (Yurkow & Mckenzie, 1993):DCFH-DA, a non-fluorescent analogue of fluorescein, entered the cells by passive diffusion and was concentrated within cells by an esterase-dependent process (Bass, Parce, Dechatelet, Seeds & Thomas, 1983). Once inside the cell, the functional groups of DCFH-DA were subject to modification by endocellular peroxides, resulting in a molecule with intense green fluorescence, when excited with 488 nm light. One hundred microlitres of DCFH-DA-loaded cells were suspended in 900 µl NaCl 0.9% (w/v) under a thin layer of mineral oil (1 ml) in a 1 cm-pathlength quartz cuvette with permanent stirring. Fluorescence determinations were made every 12 s by using a Perkin-Elmer LS50-B fluorescence spectrometer with the following settings:488 nm excitation (2.5 nm bandwidth),

525 nm emission (20 nm bandwidth). Since the fluorescence of DCFH-DA-labelled cells is dependent on the amount of peroxide produced within the cells (Bass et al., 1983), maximal dye loading was difficult to determine directly. As a consequence, prior to the assays, each batch of DCFH-DA-labelled cells was submitted to a calibration of DCFH-DA response by addition of known amounts of hydrogen peroxide to the cells.

2.2.9. Fluorescence depolarization determination of plasma membrane order

Plasma membrane lipid order was determined by measuring steady-state fluorescence anisotropy in yeast lees labelled with 1-(4 trimethylammoniumphenyl)-6 phenyl-1,3,5-hexatriene p-toluene sulfonate (TMA-DPH, Molecular Probes, Eugene, OR). This cationic probe anchors primarily in plasma membrane of intact cells (Gille, Sigler & Hofer, 1993). One hundred microlitres of yeast lees suspension were added to 1800 μl of 0.9% (w/v) NaCl and equilibrated with continuous stirring at 30°C for 30 min in a 1 cm-pathlength quartz cuvette. Twenty microlitres of TMA-DPH from a 0.6 mM stock solution in pure methanol were then added to the lees suspension. When measurements were stabilized, fluorescence determinations were made every 12 s by using a Perkin-Elmer LS50-B fluorescence spectrometer with polarization turrets. TMA-DPH probe was excited with vertically polarized light at 360 nm (3 nm bandwidth), and the vertical and horizontal vectors of emitted light were measured at 450 nm (3 nm bandwidth). The measured fluorescence intensities were corrected for background fluorescence and light scattering from an unlabelled sample. Membrane order was expressed as the order parameter S, which reflects the orderliness of membrane phospholipids: $S = (r/r_0)^{0.5}$ (Gille et al., 1993), where r_0 is the theoretical limiting anisotropy (0.395 for TMA-DPH) in the absence of rotational motion and r is the steady-state anisotropy measured in the membrane.

3. Results

Since wine ageing on lees lasts from six up to 18 months, we designed an experimental device to simulate interactions between yeast lees and oxygen. In this device, depicted in Fig. 1, lees at high concentration (up to 10^9 cells ml^{-1}) were simply left in contact with air by the surface of the medium under low-speed stirring. This stirring was set up in order to obtain a low oxygen transfer from the air to the medium. Oxygen consumption by lees was, therefore, easily calculated from instantaneous oxygen concentration measurements in the medium and from previous calibration of global volumetric oxygen transfer to the medium ($K_La = 0.15$ h^{-1}). As shown in Fig. 2, oxygen consumption by yeast

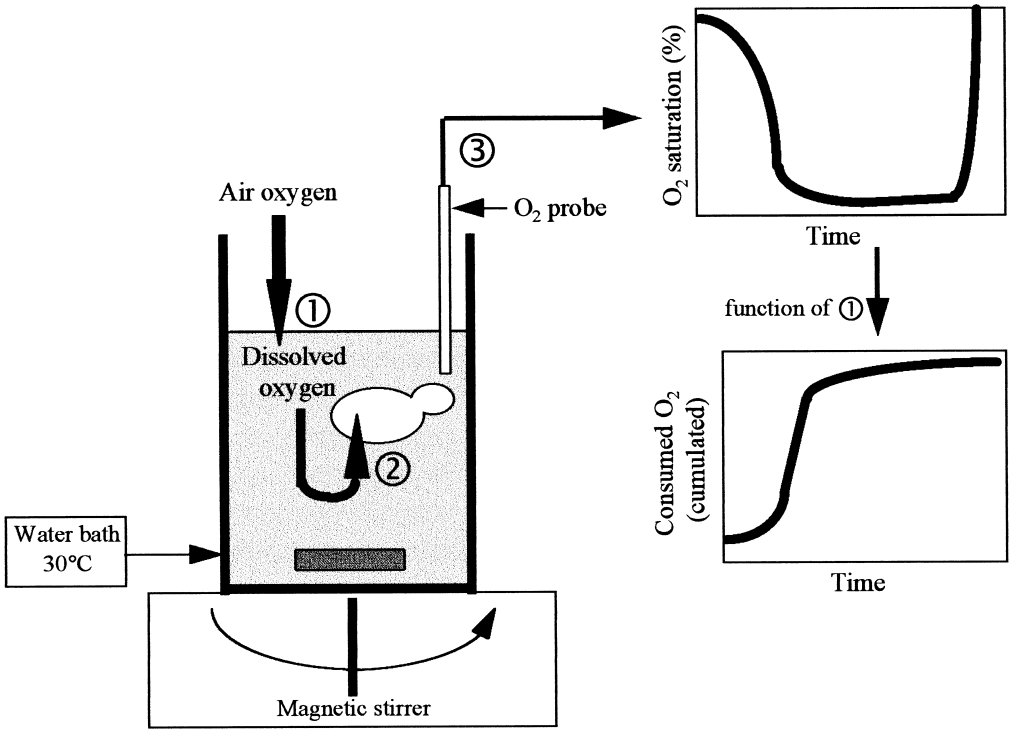

Fig. 1. Experimental device (volume:250 ml) used for the study of oxygen consumption by *S. cerevisiae* lees at high concentration (5.10^8 to 10^9 cells ml^{-1}). The instantaneous dissolved oxygen concentration in the medium (③) is measured by an oxygen probe (Clark electrode). This instantaneous oxygen concentration is the resultant of both saturation of the medium by atmospheric oxygen (①) and oxygen consumption by lees (②). The oxygen transfer capacity (K_La) of the device was calculated to be 0.15 h^{-1}.

lees first increased exponentially, rapidly reached a steady-state and finally completely stopped when about 20 μmol O_2 were consumed by 10^{10} cells. Simultaneous detection of peroxides within biomass by ferrous ion oxidation in the presence of xylenol orange (FOX method) revealed that the exponential rise of oxygen consumption was associated with a transient accumulation of peroxides in the lees. However, oxygen consumption by lees continued after the total disappearance of peroxides from the biomass. Further experiments, performed on the same experimental basis (Fig. 3) revealed that generation of biomass peroxides is indeed directly and linearly correlated with the initiation of oxygen consumption by lees. Addition of redox-active metals, such as Cu^{++} ions, which are well known to be capable of inducing free-radical production and therefore to act as autooxidation catalysts (Schutz, Day & Sinnhuber, 1962), markedly increased this phenomenon. In order to study interactions between oxygen and lees at lees concentrations equivalent to those encountered at the end of alcoholic fermentation (about 10^8 cells ml^{-1}), we used a second experimental device (Fig. 4), where medium was submitted to a continuous air flushing. This device exhibited a better oxygen transfer to the medium than the previous one ($K_La = 2\ h^{-1}$) and allowed comparison with lees conserved in a similar device protected from air by flushing the head-space with pure argon. These devices were severely protected against any microbiological spoilage consecutive to the experiment duration (up to 600 h). Ethanol and water losses by air-stripping were daily checked and corrected by addition of water and ethanol when necessary. At the end of alcoholic fermentation (time 0 on Fig. 5), cellular viability decreased sharply from about 50% to a null value in less than 100 h. In lees protected from oxygen, this loss of viability was less marked and null viability was reached later. Accumulation of biomass peroxides was noticeable when oxygen was fed to the medium (Fig. 5A), and was well correlated to the oxygen consumption by lees (Fig. 5B). However, detection of peroxides by the FOX method appeared insufficiently sensitive to correctly detect peroxides at the early stage of the experiment when lees were used at low concentrations (about 10^8 cells ml^{-1}) (Fig. 5A). As detection of peroxides by the FOX method requires a biomass solubilization with pure methanol, we supposed that peroxides were mainly produced at the cell membrane level. As a consequence, in a similar experiment, we attempted to directly detect peroxide production within the membrane compartment of lees, by using the lipid soluble probe diphenyl-1-pyrenylphosphine (DPPP). This nonfluorescent probe is quickly oxidised by hydroperoxides present in the membranes into the corresponding strongly fluorescent phosphine oxide (Noguchi et al., 1998). As shown in Fig. 6, production of hydroperoxides in lees was strictly dependent on the presence of oxygen. The initial peak of membrane hydroperoxides production is well correlated with the cell viability remaining (Fig. 5A). However, after the complete loss of cell viability, the production of membrane hydroperoxides continued to rise steadily for a long period of time, while oxygen consumption by lees remained noticeable. Since incubation of yeast suspension after the end of alcoholic fermentation in hydro-alcoholic medium at 30°C is known to lead to yeast autolysis (Charpentier & Feuillat, 1993), we tried to evaluate the effect of oxygen on the autolysis phenomenon by measuring protein release from lees in the medium. Surprisingly, as shown in Fig. 7, the interaction between oxygen and lees had no significant effect on yeast autolysis, as revealed by protein degradation. Further experiments revealed that the amino acid composition of proteins was also not affected by the presence of oxygen (data not shown). Since membrane hydroperoxides were

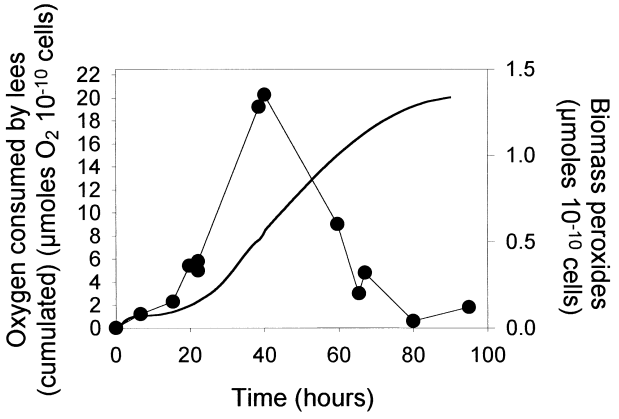

Fig. 2. Kinetics of peroxide production in lees at 30°C during an accelerated oxygen consumption by *S. cerevisiae* lees at a concentration of 5.10^8 cells ml^{-1} using the experimental device described in Fig. 1. (●): Biomass peroxides as determined by the FOX method, (–): total oxygen consumed by lees (cumulated).

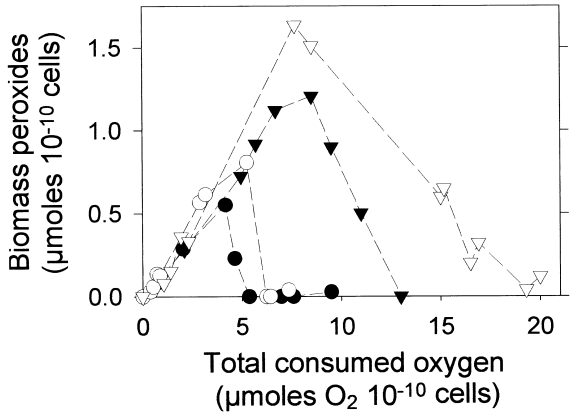

Fig. 3. Relationship between peroxide production in lees (as determined by the FOX method) and the total oxygen consumed by lees at 30°C during accelerated oxygen consumption by *S. cerevisiae* lees at different concentrations using the experimental device described in Fig. 1. (●): $2.5.10^8$ cells ml^{-1}, (○): $3.5.10^8$ cells ml^{-1}, (▼): $5.1.10^8$ cells $ml^{-1} + Cu^{++}$ 5 μM, (▽): 5.10^8 cells $ml^{-1} + Cu^{++}$ 20 μM.

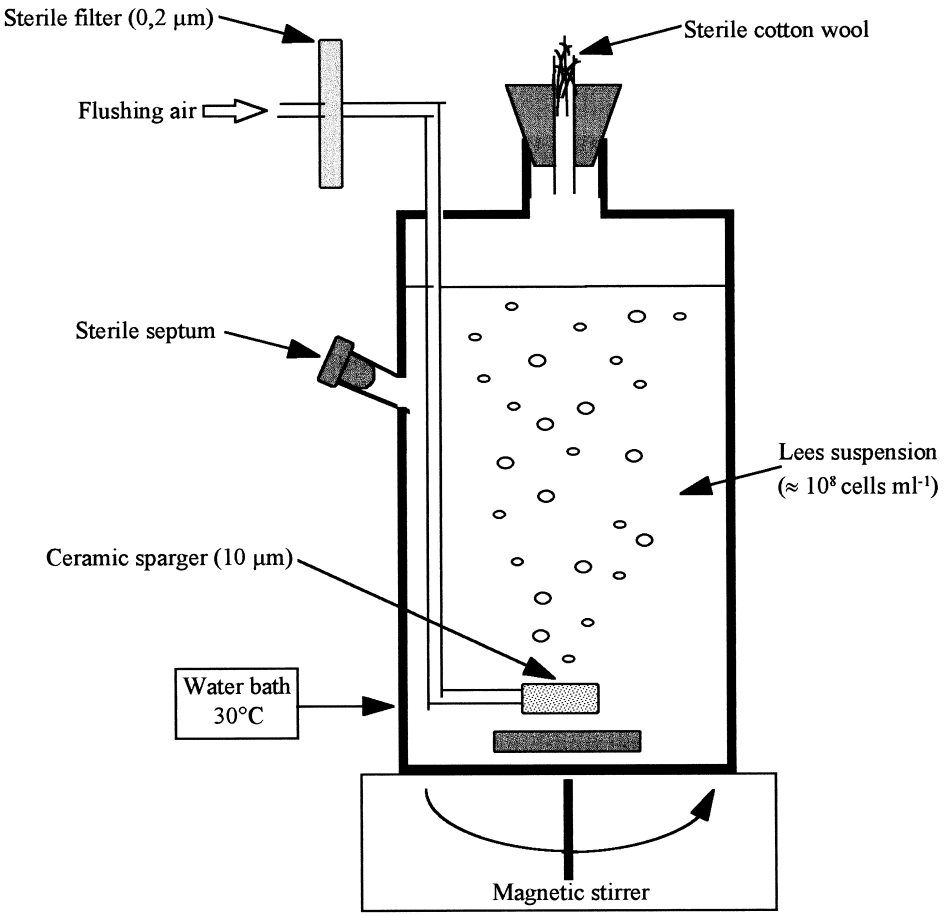

Fig. 4. Experimental device (volume: 1 l) used for the study of oxygen consumption by *S. cerevisiae* lees at low concentration (10^8 cells ml^{-1}). The oxygen transfer capacity (K_La) of the device was calculated to be 2.0 h^{-1}.

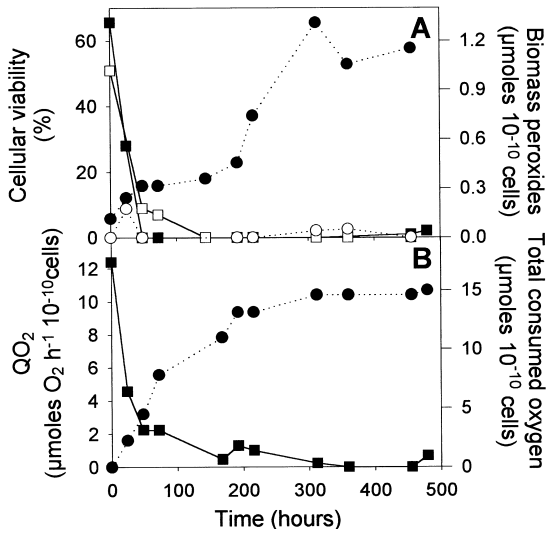

Fig. 5. (A) Evolution of biomass viability (–) and biomass peroxides contents as determined by the FOX method (· · · ·), (B) specific oxygen consumption rate (–) and total oxygen consumed by lees (· · · ·) during conservation at 30°C of *S. cerevisiae* lees (lees concentration: $2.5.10^8$ cells ml^{-1}) using the experimental device described in Fig. 4. (●/■): stirred lees + oxygen, (○/□): stirred lees + argon.

produced during such ageing on lees in the presence of oxygen without affecting the overall autolysis phenomenon, we further focused experiments on the estimation of consecutive alterations at the membrane level. We used the property of the cationic fluorescent TMA-DPH probe to intercalate within lipid bilayers to monitor both lipid bilayer accessibility (total fluorescence, Fig. 8A) and plasma membrane order (steady-state fluorescence anisotropy, Fig. 8B). Oxygen interaction with lees (reinforced or not by Cu^{++} ion addition) initially led to a dramatic decrease of probe accessibility to plasma membrane when compared to argon-protected lees, as measured by total fluorescence of the probe (Fig. 8A). After about 400 h of ageing, when consumption of oxygen by lees reached a null value (Fig. 5B), both argon- and oxygen-treated lees exhibited a similar value of total TMA-DPH fluorescence. This result indicated that, in both cases, plasma membranes had reached a similar final physical state. When lees are aged in the presence of oxygen, the observed initial decrease of fluorescence is associated with a strong change in the orderliness of membrane phospholipids, as measured by fluorescence anisotropy of the probe (Fig. 8B). Such a

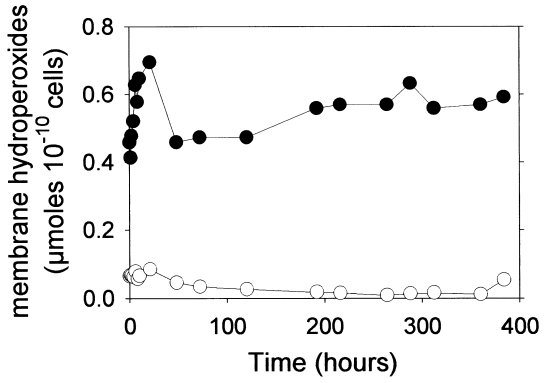

Fig. 6. Kinetics of lipid hydroperoxide production in lees (detection by DPPP probe fluorescence) during conservation at 30°C of *S. cerevisiae* lees (lees concentration: 10^8 cells ml^{-1}) using the experimental device described in Fig. 4. (●): stirred lees + oxygen, (○): stirred lees + argon.

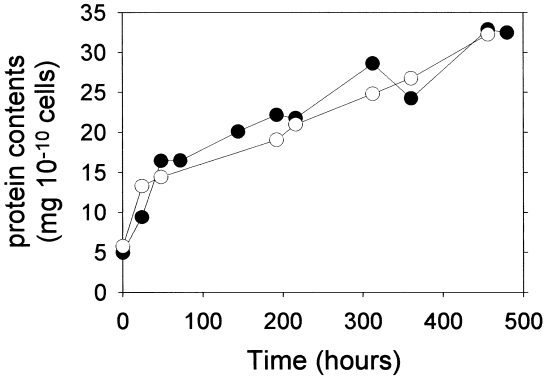

Fig. 7. Protein release kinetics by lees in the medium during conservation at 30°C of *S. cerevisiae* lees (lees concentration: 10^8 cells ml^{-1}) using the experimental device described in Fig. 4. (●): stirred lees + oxygen, (○): stirred lees + argon.

decrease of fluorescence anisotropy of TMA-DPH probe within lipid bilayers was consistent with the angular reorientation of the lipid acyl chains (Kleinfeld, Dragsten, Klausner, Pjura & Matayoshi, 1981), and thus indicated that changes in the composition of membrane lipids occurred. Finally, we tried to directly monitor the production of reactive oxygen species (ROS) in yeast lees by using the H_2O_2-sensitive fluorescent probe DCFH-DA. DCFH-DA loaded cells were aged for 100 h after the end of alcoholic fermentation in order to reach no detectable viability. These cells were then submitted to known H_2O_2 additions in order to calibrate the probe response to peroxide detection (Fig. 9). For the final experiment, DCFH-DA-loaded yeast lees were simply diluted in air-saturated hydroalcoholic medium and left to consume dissolved oxygen. Evolution of oxygen concentration and DCFH-DA fluorescence were recorded through three cycles of oxygen consumption (Fig. 10). Oxygen consumption by lees is clearly accompanied by the production of reactive oxygen species (ROS). This ROS generation rose as long as

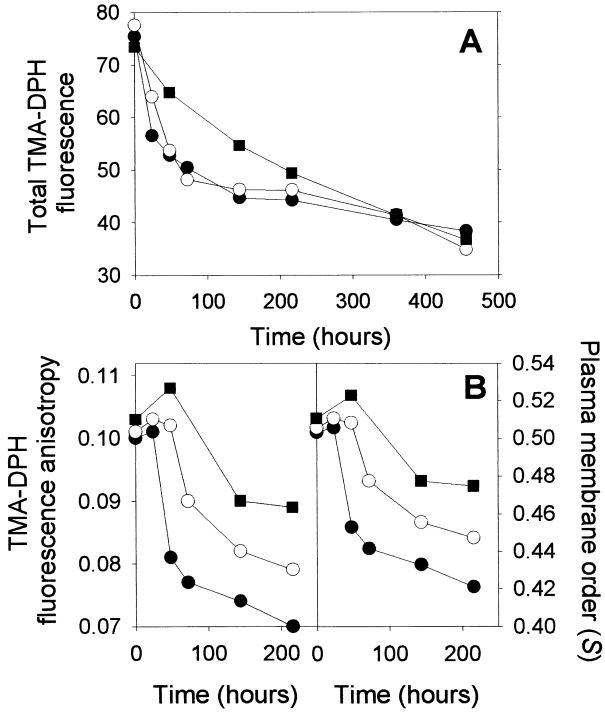

Fig. 8. Total fluorescence (A) and fluorescence anisotropy (B) kinetics of TMA-DPH probe, and plasma membrane order (*S*) evolution (B) during conservation at 30°C of *S. cerevisiae* lees (lees concentration: 10^8 cells ml^{-1}) using the experimental device described in Fig. 4. (●) : stirred lees + oxygen, (○): stirred lees + oxygen + Cu^{++} 20 µM; (■): stirred lees + argon.

oxygen was consumed by lees. However, although oxygen consumption tended to decrease as a function of time, the ROS generation appeared quasi exponential.

4. Discussion

Wine ageing on their yeast lees is a traditional enological practice used during the manufacture of wines in different countries (Boulton et al., 1996). During this ageing, repetitive additions of small amounts of oxygen to the wines is generally associated with a limited homogenisation of wine and lees on a traditional and empirical basis. Since oxygen consumption by *S. cerevisiae* lees was proved to occur independently of any cellular viability (Fornairon et al., 1999), we tried to educe any repercussion of this oxygen consumption on lees integrity.

Peroxides were the first easily detectable chemical species during oxygen consumption by lees. They appeared to be mainly localised at the plasma membrane level. Unsaturated fatty acids from membrane lipids, and especially their peroxidation products, can play an essential role in the initiation and propagation of radical reactions in the core of yeast membranes (Noguchi et al., 1998). In fact, oxidation of lipids and lipoproteins may arise, in any living organism, either by the action of free radicals or by the action of lipoxygenase

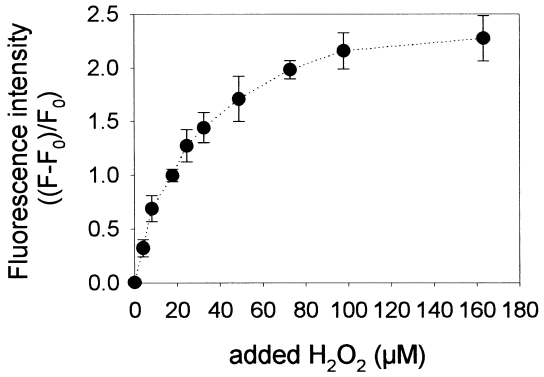

Fig. 9. Typical calibration curve of the response of DCFH-DA-loaded *S. cerevisiae* cells (lees concentration: 2.10^8 cells ml^{-1}) to the addition of known amounts of hydrogen peroxide (mean value and standard errors of triplicate are shown).

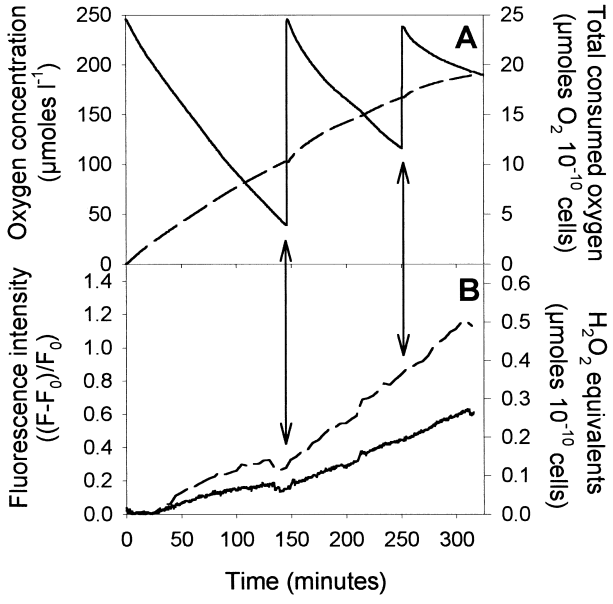

Fig. 10. Evolution of: (A) oxygen concentration (–) and cumulated oxygen consumed by lees (····), (B) DCFH-DA normalized fluorescence (–) and correspondent reactive oxygen species equivalents (····) during a classical QO_2 measurement at 30°C on *S. cerevisiae* lees (lees concentration: 2 10^8 cells ml^{-1}) issued from DCFH-DA-loaded cells (black arrows indicate saturation of medium with air to 100% saturation).

activities (Halliwell & Chirico, 1993; Yamashita, Nakamura, Nogushi, Niki & Kuhn, 1999). Moreover, we have shown that addition of redox-active metals, such as Cu^{++} ions, which are well known to be capable of inducing free-radical production and, therefore, acting as auto-oxidation catalysts (Schutz et al., 1962), markedly increased the generation of membrane peroxides in lees.

It is well known that, in the presence of dissolved oxygen (even at very low concentration) in a culture medium, free radical formation occurs (Aoshima, Kadoya, Tanigushi, Satoh & Hatanaka, 1999). Such free radical generation is normally scavenged in viable *S. cerevisiae* cells by enzymatic reactions, such as Cu-, Zn- and Mn-superoxide dismutase activities, even in anaerobiosis (Galiazzo & Labbe-Bois, 1993 ; Ohmori et al., 1999). These enzymatic activities lead to the production of peroxides, that are normally detoxified by means of catalase and glutathione peroxidase activities (Grant, Perrone & Dawes, 1998). However, in yeast lees, characterised by a null viability, one can expect such enzymatic reactions to be not completely functional. Generation of reactive oxygen species (ROS) was indeed detectable in lees in contact with oxygen, even at very low oxygen concentration. This ROS generation paralleled the ability of yeast to consume oxygen and easily explained the generation of peroxides in biomass (Kanner, German & Kinsella, 1987). Nevertheless, in addition to these types of enzymatic reactions, the abundance of tannins, phenols and pigments, already found in wine, would create a favourable environment for the development of such oxidation reactions (Okuda, Kimura, Yoshida, Hatano, Okuda & Azrichi, 1983).

Observation of ROS generation may also be associated with lipoxygenase activities. However, the existence of a specific lipoxygenase activity in anaerobic *Saccharomyces cerevisiae* cells remains questionable: only a few studies have examined the existence of such an enzymatic activity in *S. cerevisiae* (Bisakowski, Perraud & Kermasha, 1997; Schechter & Grossman, 1983).

It is important to note that the considerable amount of oxygen uptake observed in yeast lees can be roughly accounted for by total peroxide formation and, once peroxide degradation occurs, the oxygen consumption by lees gradually stops. The similarities between the short initiation scales and the relative extents, of oxygen consumption by lees and lipid peroxidation observed, were consistent with the latter process being associated with a deterioration of membrane integrity (van Ginkel & Sevanian, 1994). In fact, plasma membrane order (*S*) values decreased roughly in the same order as that predicted from lipid peroxidation experiments. The discrepancy observed for the experiment performed in the presence of Cu^{++} ions is not surprising, since Howlett and Avery (1997) proved that values of the plasma membrane order parameter do not provide a linear index of membrane integrity or membrane contents. However, it is important to note that, once lees interact with oxygen, modification of plasma membrane integrity is not related to any changes in the overall extent of autolysis. Such results may indicate that the expected products of membrane lipid oxidation were retained within the plasma membrane and not released in the medium. Such an hypothesis is in accordance with the recent work of a spanish group (Pueyo et al., 2000), which clearly did not detect any phospholipid release during yeast autolysis in a model wine system. Amongst *S. cerevisiae* membrane lipids, polyunsaturated fatty

acids [such as linoleate (18:2) and linolenate (18:3)], which may be present in yeast cells as free fatty acids or as constituents of acylglycerol derivatives of phospholipids, were the best targets for lipid peroxidation. Oxidation of such lipids, either by the action of free radicals or by the action of lipoxygenase activity, led to the formation of volatile compounds as final products, which might exert a strong organoleptic effect (Gardner, 1985). However, decomposition of unsaturated fatty acid hydroperoxides required the action of several enzymatic activities (mainly hydroperoxide lyase and alcohol dehydrogenase activities) in order to produce the corresponding volatile aldehydes (Hatanaka, Kajiwara & Sekiya, 1986). Such enzymatic reactions seemed, consequently, not functional in yeast lees. Experimental work is already in progress in order to determine the exact chemical composition of the final products of lipid oxidation in the membrane lipids of yeast lees exposed to oxygen. One can expect that some of these final products may play a favourable role in the organoleptic equilibrium of wines aged on lees, once these end-products are liberated in the medium, either by the action of the classical yeast autolysis on lipids (Pueyo et al., 2000) or by the action of a presently unknown enzymatic step. Future work has to be conducted to test such a hypothesis. Finally, this membrane lipid oxidation phenomenon offers an explanation of the occurrence of cyanide resistance and SHAM-sensitivity in oxygen utilization previously observed in viable anaerobic yeast cells during alcoholic fermentation (Salmon et al., 1998).

Acknowledgement

C.F. was a recipient of a doctoral fellowship from the Boccard company (Lyon, France).

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