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Formation of Micella Containing Solubilized Sterols during Rehydration of Active Dry Yeasts Improves Their Fermenting Capacity

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During their rehydration in aqueous media, active dry yeasts (ADY) may be supplemented with inactive yeasts, yeast derivatives, or other optional complementary nutrients to improve their fermentation capacity. We found that yeast sterols solubilized in situ during ADY rehydration were particularly efficient for stimulating the fermenting capacity of ADY. Spontaneous solubilization of sterols during rehydration occurred by the formation of micelles by membrane phospholipids and specific cell wall polysaccharides and sterols, both compounds being provided by inactive dry yeasts (IDY). These micelles contained a specific distribution of the initial sterols from the inactive yeasts. Above a concentration of 100 mg L^{-1} in the rehydration medium, these micelles acted as emulsifiers. Their critical micellar concentration (cmc) was found to be about 4 g L^{-1}. During rehydration, purified micelles, at a concentration near the cmc, were able to interact quickly with yeast cell membranes by modifying the yeast plasma membrane order [monitored by steady-state fluorescence anisotropy of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluenesulfonate (TMA-DPH) probe] and by increasing the sterol contents of ADY. Such an enrichment of ADY by very low concentrations of solubilized sterols was very efficient for the completion of fermentations. This is useful when musts are limited in available phytosterols or when micro-oxygenation is not desirable during fermentation.

KEYWORDS: Alcoholic fermentation; sterols; yeast; rehydration

INTRODUCTION

Traditionally, indigenous yeast populations were used in winemaking. However, in the last 20 years winemakers have started to use pure *Saccharomyces cerevisiae* strains, in the form of active dry yeast (ADY) starters. This allows better control of fermentation and reduces the risk of negative organoleptic effects resulting from the growth and metabolism of other indigenous yeasts. However, even if ADY starters are used for must inoculation, nutrient deficiencies in the natural fermentation media, inhibitory substances, or certain technological practices may lead to stuck or sluggish fermentations (*1*, *2*). For sensory reasons, and to protect the final product from spoilage, the completion of fermentation is a prerequisite. Therefore, many studies have been conducted to determine the best nutrient supplementation of fermentation media. This is principally nitrogen (*3*−*5*), vitamins (*6*, *7*), and anaerobic growth factors or oxygen (*5*, *8*). Among the potential nutrient deficiencies of natural grape musts, sterol bioavailability is a very specific case: during winemaking, yeast cells, in the absence of oxygen, cannot achieve complete alcoholic fermentation by only incorporating grape phytosterols (*9*). In strongly anaerobic conditions, yeast cells easily incorporated grape phytosterols, promoting normal yeast growth and initiating fermentation. However, the grape phytosterols, being the predominant sterols, quickly perturbed the yeast membrane properties. Therefore, fermentation was only completed by the addition of oxygen. However, winemakers do not like adding oxygen to their musts, as it is difficult to precisely control the amount of oxygen transferred to the must or the wine (*10*, *11*). Moreover, oxygen dissolution was shown to occur easily during several of the preparation steps, such as tank filling and pumping over (*11*−*14*). As an alternative to oxygen addition, the addition to musts of suspended grape solids, which are well-known to passively adsorb fatty acids and sterols (*16*), has been suggested (*8*, *15*). It has been shown, in a wide variety of fermentation media, that rehydration of ADY in the presence of a high concentration (about 150 g L^{-1} (dw)) of inactive dry yeasts (IDY) increased the fermentation rate and thus diminished the duration of

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fermentation (*17*, *18*). However, this observation has never been attributed to a known physiological effect.

Here, we report the first analysis of the effect of inactive dry yeasts (IDY) during the rehydration of ADY, with particular emphasis on the bioavailability of sterols.

MATERIALS AND METHODS

Active Dry Yeast. *Saccharomyces cerevisiae* strain EC-1118 is a commercially available industrial dry yeast (Lallemand, Montreal, Canada).

Inactive Dry Yeast. Inactive dry yeasts (Go-Ferm) were obtained from Lallemand (Montreal, Canada).

Culture Media and Growth Conditions. The synthetic medium (SM) used in this study, strongly buffered to pH 3.3, simulated a standard grape juice (*19*). The medium contained (per liter) 200 g of glucose, 6 g of citric acid, 6 g of DL-malic acid, 750 mg of KH_2PO_4, 500 mg of K_2SO_4, 250 mg of $MgSO_4 \cdot 7H_2O$, 155 mg of $CaCl_2 \cdot 2H_2O$, 200 mg of NaCl, 4 mg of $MnSO_4 \cdot H_2O$, 4 mg of $ZnSO_4$, 1 mg of $CuSO_4 \cdot 5H_2O$, 1 mg of KI, 0.4 mg of $CoCl_2 \cdot 6H_2O$, 1 mg of H_3BO_3, 1 mg of $(NH_4)_6Mo_7O_{24} \cdot 2H_2O$, 20 mg of *myo*-inositol, 2 mg of nicotinic acid, 1.5 mg of calcium panthothenate, 0.25 mg of thiamin hydrochloride, 0.25 mg of pyridoxine hydrochloride, and 0.003 mg of biotin. It contained ammoniacal nitrogen and amino acids as nitrogen sources (w/w): 18.6% ammoniacal nitrogen (NH_4Cl), 20.5% L-proline, 16.9% L-glutamine, 1.25% l-arginine, 6.0% L-tryptophan, 4.9% L-alanine, 4.0% L-glutamic acid, 2.6% L-serine, 2.6% l-threonine, 1.6% L-leucine, 1.5% L-aspartic acid, 1.5% L-valine, 1.3% L-phenylalanine, 1.1% l-isoleucine, 1.1% L-histidine, 1.1% L-methionine, 0.6% L-tyrosine, 0.6% L-glycine, 0.6% L-lysine, and 0.4% L-cysteine. *S. cerevisiae* yeast strains under anaerobic growth conditions cannot incorporate proline (*17*, *18*), resulting in an available nitrogen concentration of SM medium (ammonium salts, α-amino acids) of 300 mg of N L^{-1}. The following anaerobic growth factors were added to the medium (per liter): 5 mg of oleic acid and 8 mg of stigmasterol (Sigma Chemicals, L'Isle-d'Abeau, France) dissolved in 1 mL of Tergitol NP40/absolute ethanol (50:50 v/v) (*6*). Strain EC-1118 was directly inoculated into SM after rehydration, as recommended by the manufacturer. Dry yeast (1 g) was suspended in 10 mL of water (37 °C) containing 0.5 g of glucose. Either 1.5 g of IDY or differing amounts of purified white colloid (WC) fractions were added. The suspension was kept at 37 °C for 30 min with vigorous stirring every 15 min. SM (1 L) was inoculated with 2 mL of the suspension, corresponding to a cell concentration of about 2.5×10^6 cells mL^{-1}. Filling conditions were controlled and fermentations were carried out under anaerobic and isothermal conditions (24 °C), and all fermentors (1 L) were fitted with fermentation locks (CO_2 bubbling outlets filled with water). All media were heat-sterilized (110 °C, 20 min). Fermentation media were deoxygenated by bubbling through with pure sterile argon for 30 min before inoculation.

Fermentations were carried out in fermentors (1 L) under isothermal conditions (24 °C) with gentle stirring.

Fermentation Kinetics. The amount of CO_2 released was calculated automatically every 20 min from the weight of the fermenter (*20*). Loss of ethanol and water due to CO_2 stripping accounted for less than 2% of the total weight loss. The CO_2 production rate was calculated by polynomial smoothing of the last 10 fermenter weight measurements. A high precision balance (±0.01 g) and frequent measurement of the fermenter weight allowed calculation of the CO_2 production rate with a high degree of precision and reproducibility [coefficient of variation for $d(CO_2/dt)_{max} = 0.8\%$; *20*]. The fermentation progress (FP) was calculated from the amount of CO_2 released from the culture medium according to the following equation:

$$FP = CO_{2,t}/CO_{2,max}$$

where $CO_{2,t}$ is the cumulative amount of CO_2 released at a time t and $CO_{2,max}$ is the total amount of CO_2 released after fermentation was complete. The use of fermentation progress instead of fermentation time allows the fermentation kinetics, which are closely linked to the disappearance of substrate from the medium, to be normalized. Fermentation was considered complete when the medium contained <2 g L^{-1} glucose. None of the fermentations were stopped if they were <99% complete.

Analytical Methods: *Cell Counting.* Culture growth and cell numbers were monitored on an electronic particle counter (ZBI model; Coulter-Counter Coultronics, Margency, France) linked to an analyzer (Channelyzer 254 model; Coulter-Counter Coultronics).

Fluorescence Depolarization Determination of Plasma Membrane Order. Plasma membrane lipid order was determined by measuring steady-state fluorescence anisotropy in yeast labeled with 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluenesulfonate (TMA-DPH; Molecular Probes, Eugene, OR). This cationic probe anchors primarily in the plasma membrane of intact cells (*23*). Active dry yeasts (200 mg) were added to 2 mL of 0.9% (w/v) NaCl solution containing 0.1 g of glucose and equilibrated with continuous stirring at 37 °C for 1.5 min in a 1-cm path length quartz cuvette. TMA-DPH (20 μL) from a 0.6 mM stock solution in methanol was then added to the suspension. After stabilization of the suspension, fluorescence measurements were made every 10 s om a Perkin-Elmer LS50-B fluorescence spectrometer with polarization turrets. The TMA-DPH probe was excited with vertically polarized light at 360 nm (3 nm bandwidth), and the vertical and horizontal components of the emitted light were measured at 450 nm (3 nm bandwidth). The fluorescence intensities were corrected for background fluorescence and light scattering from an unlabeled sample. Membrane order was expressed as the order parameter *S*, which gives a measure of the order of the membrane phospholipids: $S = (r/r_0)^{0.5}$ (*23*), 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.

Incorporation of Labeled Sterol. Dry yeast (1 g) was suspended in 10 mL of water (37 °C) containing 0.5 g of glucose and [4-^{14}C]-labeled cholesterol [2 μL, 55 mCi $mmol^{-1}$ (Amersham)], solubilized in 20 μL of tergitol NP40/ethanol (v/v). Aliquots of this medium (300 μL) were filtered under vacuum through glass fiber filters (Whatman GF/C), rinsed with 3 mL of distilled water, and kept at 4 °C. After addition of 20 mL of scintillation cocktail (Fluoransafe 2, BDH), the filters were counted for ^{14}C radioactivity with quenching correction on an LS 6500 counter (Beckman Instruments).

Fractionation of the White Colloid Fraction. The WC fraction was first precipitated with 80% (v/v) ethanol and then lyophilized. The dry residue was then hydrolyzed in the presence of NaOH (1 M) at 37 °C for 16 h. After precipitation with 80% (v/v) ethanol, the final residue was weighed and analyzed for polysaccharides. The supernatant was kept and extracted twice with dichloromethane for lipids analysis.

Total Yeast Sterols and Squalene Determination by GPC-MS. For each extraction, 50 μL of cholesterol (Sigma Chemical Co., L'Isle-d'Abeau, France) (0.5 mg mL^{-1} in pyridine) was used as an internal standard. All organic solvents were protected from oxidation by the addition of 0.01% (w/v) butylated hydroxytoluene (BHT). Total lipids from lyophilized yeast cells (1 g) were extracted overnight with methanol/chloroform (2:1 v/v), and the solid residue was extracted for 2 h with methanol/chloroform/water (2:1:0.8 v/v/v) (*24*). The organic extracts were combined and dried over Na_2SO_4, and the solvent was removed on a rotary evaporator. The total lipids were saponified and the sterols were extracted with hexane, washed three times with water/ethanol (1:1 v/v) (*25*), and dried over Na_2SO_4, and the solvent was removed on a rotary evaporator. Gravimetric analysis of the total recovered unsaponifiable material was then carried out. The total sterols were resuspended in 1 mL of hexane and were silylated by incubating 0.4 mL of the sterol solution and 50 μL of methyl silyl trifluoroacetamide (MSTFA, Pierce Chemicals, Perbio, Bezons, France) at 60 °C for 20 min. Sterol determination and quantification were carried out on a Hewlett-Packard 5989-II gas chromatograph equipped with a DB5 apolar column (J&W Scientific, Toulouse, France, 60 m × 0.32 mm × 1 μm) and coupled to a Hewlett-Packard 5890A mass spectrometer. Helium gas flow rate was set at 2.5 mL min^{-1}. Injector temperature rose from 30 to 320 °C at 180 °C min^{-1} and was kept at 320 °C for 50 min. The oven temperature rose from 250 to 320 °C at 6 °C min^{-1} and was kept at 320 °C for 40 min. A calibration curve was obtained by use of squalene, cholesterol, ergosterol, and lanosterol (Sigma Chemicals).

Quantification of Steryl Esters and Free Sterols. For quantification of steryl esters and free sterols, total cellular lipids, dissolved in hexane, were applied to silica gel 60 F254 TLC plates (Merck) and chromatograms were resolved by use of dichloromethane as the solvent. The spots were saponified and analyzed by gas chromatography as previously described (*26*).

Polysaccharide Composition as Neutral Monosaccharides. Neutral monosaccharides were released from the dry residue by hydrolysis with 2 M trifluoroacetic acid (75 min, 120 °C) (*27*). The monosaccharides were then converted to the corresponding alditol acetate derivatives (*28*) and quantified by GC analysis on a Hewlett-Packard Model 5890 gas chromatograph with a fused silica DB-225 (210 °C) capillary column (30 m × 0.32 mm i.d., 0.25 μm film, J & W Scientific) and H_2 as the carrier gas (*29*).

Polysaccharide Molecular Weight Distribution. The molecular weight distribution of the polysaccharides was determined on a high-performance size-exclusion chromatography (HPSEC) system composed of two serial Shodex OHpak KB-803 and KB-805 columns (0.8 × 30 cm; Showa Denkko, Japan) connected to an ERC-7512 refractometer (Erma, Japan) (*30*−*33*). The columns were equilibrated at 1 mL min^{-1} in 0.1 M $LiNO_3$. The average apparent molecular weight of each fraction was estimated from a universal calibration curve [ln (molecular mass) vs elution time] (*34*) established with a pullulan calibration kit (Showa Denko, Japan).

Protein Content. Protein content was measured according to Lowry et al. (*35*).

Detection of Glycolipids after Separation by Thin-Layer Chromatography. Analytical TLC was performed on silica gel 60 TLC plates (Merck, Darmstadt, Germany) with chloroform/methanol/water [60:40:9 v/v/v, containing 0.002% (w/v) $CaCl_2$] as mobile phase. WC fraction samples were dissolved in chloroform/methanol (2:1 v/v) and applied by streaking 5 μL on the plate. The plates were developed with Bial's orcinol reagent [orcinol 0.55% (w/v) and H_2SO_4 5.5% (v/v) in ethanol/water 9:1 (v/v)] and heated briefly to ~200−250 °C.

GPC-MS Fatty Acids Analysis. All samples were silylated before injection on GPC-MS as follows: 0.4 mL of sample, 50 μL of methylsilyl-trifluoroacetamide (MSTFA, Pierce Chemical Co.), and standard [50 μL of C11:0 (undecanoic acid) (20 mg mL^{-1} in pyridine)] were incubated at 50 °C for 20 min. The silylated compounds were then separated on a Hewlett-Packard 5989-II gas chromatograph fitted with a DB5 apolar column (J&W Scientific, 60 m × 0.32 mm × 1 μm) and coupled to a Hewlett-Packard 5890A mass spectrometer.

Dynamic Light Scattering Experiments. DLS measurements were carried out on a Malvern Zetasizer 3000 HS (Malvern Instruments, Malvern, U.K.), equipped with a 10 mW He−Ne laser, and at a wavelength of 633 nm. The sample cell was thermostated at 25 ± 0.1 °C. Measurements were carried out at an angle of 90° to the incident beam. The time dependence of the scattered light was monitored, and the autocorrelation function $G(t)$ of the particles was measured. The diffusion coefficient of the particle (D) was derived from this function, and hydrodynamic radii, R_H, of the particles were calculated from the Stokes−Einstein equation. It was assumed that all particles were spherical, and, thus, the equation can be written as

$$D = \frac{kT}{6\pi\eta R_H}$$

where k is the Boltzmann constant, T is the temperature, and η is the solvent viscosity.

The cumulant method was used to fit the autocorrelation curves, and the average particle size, Z_{av}, was determined. Time zero was considered to be the mixing of the stock solution with the buffer, and each measurement was the average of 10 subruns.

Incidence of WC Fraction on the Surface Tension of Water. The surface tension of water, γ_L, at different concentrations of the WC fraction was measured at 25 °C by the Wilhelmy plate method on a K12 tensiometer (Krüss).

RESULTS

Active Dry Yeasts Exhibit a High Capacity to Incorporate Lipidic Structures during Rehydration. We assessed the capacity of active dry yeasts (ADY) to incorporate hydrophobic structures from the rehydration medium during a rehydration time of 30 min. We monitored the lipid bilayer accessibility of the lipophilic fluorescent probe, TMA-DPH. An increase in probe accessibility to yeast plasma membrane was observed in less than 30 min of rehydration (**Figure 1**). This increase was not due to fluorescent stabilization, as probe equilibration into the reaction medium was observed in less than 12 s under the same experimental conditions (data not shown). We also looked at whether sterols could be incorporated in a similar way during ADY rehydration by following the incorporation of [4-^{14}C]-cholesterol during ADY rehydration (**Figure 2**). Under these conditions, about 14% of the labeled sterol was incorporated into ADY, showing that, during rehydration, ADY had the capacity to incorporate exogenous solubilized sterols.

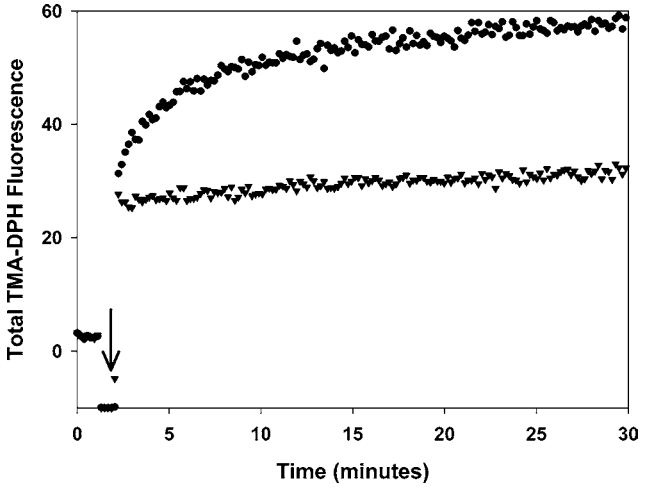

Figure 1. Total fluorescence kinetics of TMA-DPH probe during rehydration of EC 1118 ADY (1 g) in 10 mL of water containing 0.5 g of glucose in the presence (●) or the absence (▼) of 25 mg of purified white colloid (WC) fraction at 37 °C. The black arrow indicates the addition of 20 μL of TMA-DPH from a 0.6 mM stock solution in methanol.

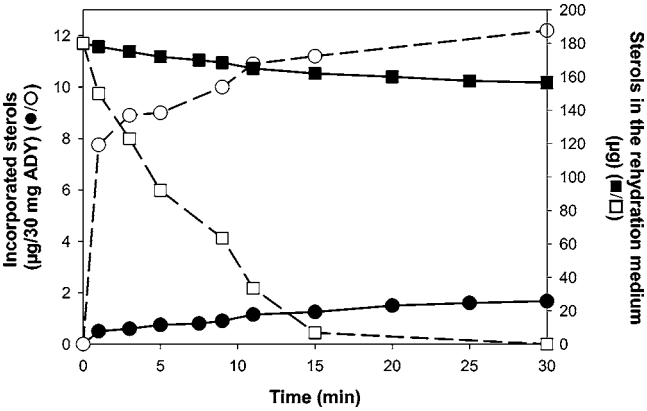

Figure 2. Incorporation of [4-^{14}C]cholesterol (180 μg, 55 mCi $mmol^{-1}$) during rehydration of EC 1118 ADY (1 g) in 10 mL of water containing 0.5 g of glucose in the presence (solid symbols) or the absence (open symbols) of 25 mg of purified white colloid (WC) fraction at 37 °C.

Rehydration of Active Dry Yeasts in the Presence of High Concentrations of Inactive Dry Yeasts Is Similar to Sterol Complementation of the Culture Medium. Rehydration of ADY in the presence of high concentrations (about 150 g L^{-1} (dw)) of inactive dry yeasts (IDY) is known to increase the fermentation rate and, consequently, to diminish the duration of fermentation in a wide variety of fermentation media (*17*, *18*). In a rich, deaerated synthetic fermentation medium, with

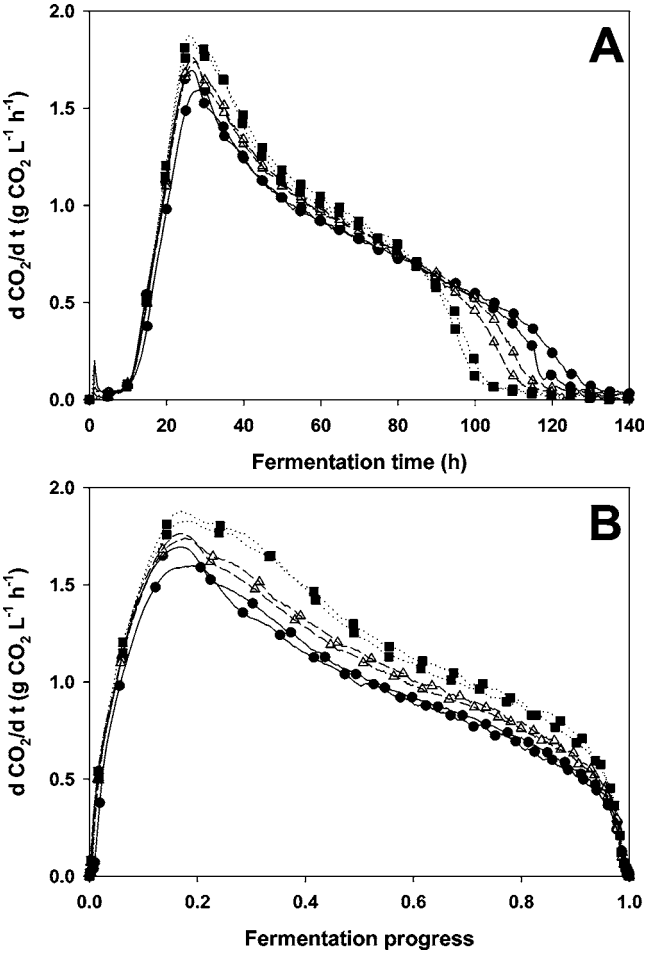

Figure 3. Variation in the CO_2 production rate by *S. cerevisiae* strain EC-1118 in SM at 24 °C (●,△). Ergosterol dissolved in 1 mL of Tergitol NP40/absolute ethanol (50:50 v/v) was added to the medium at a concentration of 0.4 mg L^{-1} (■). EC-1118 ADY strain was rehydrated in the presence (△) or the absence (●,■) of 150 g L^{-1} (dw) IDY. The CO_2 production rate is represented as a function of (A) fermentation time and (B) fermentation progress. Only 90 data points from about 900 are represented for clarity of the graph. Results of two different fermentations are presented for both fermentation time and fermentation progress.

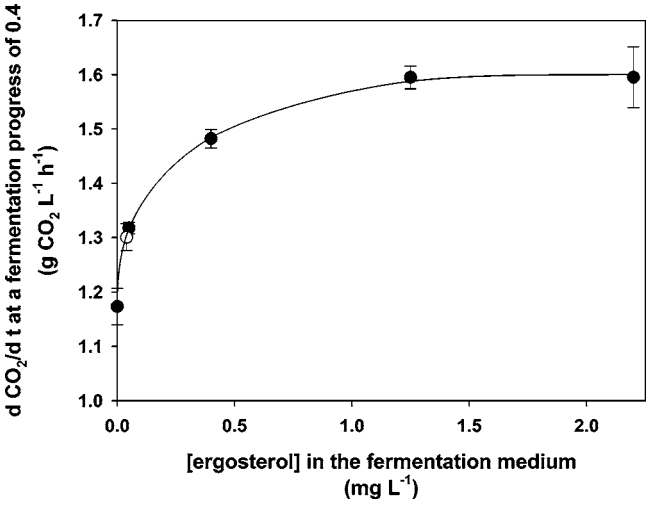

Figure 4. Effect of adding ergosterol to SM on the CO_2 production rate by *S. cerevisiae* strain EC-1118 at 24 °C, measured at a fermentation progress of 0.4 (●). Means and range of two different experiments are shown. The open circle represents the effect of rehydratation in the presence of 150 g L^{-1} (dw) IDY on the CO_2 production rate by *S. cerevisiae* strain EC-1118, measured at a fermentation progress of 0.4 in the absence of added ergosterol (equivalent to the addition of 40 μg L^{-1} ergosterol to SM).

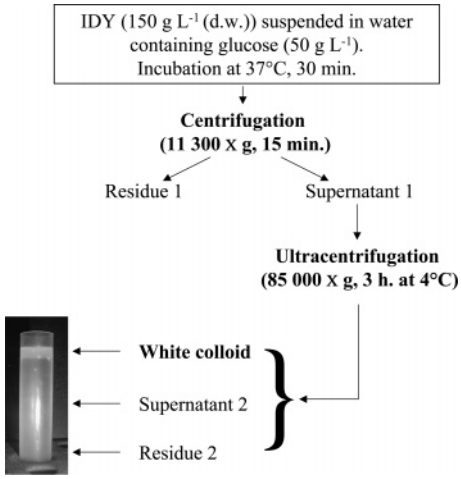

Figure 5. Extraction scheme used for the purification of the white colloid (WC) fraction from inactivated dry yeasts [150 g L^{-1} (dw)] incubated in the presence of glucose (50 g L^{-1}) for 30 min at 37 °C.

stigmasterol added as a unique source of phytosterol (*9*), ADY was efficiently rehydrated in the presence of a high concentration of IDY (**Figure 3**). A similar efficiency was obtained by adding very small amounts of ergosterol to the fermentation medium. This suggests that IDY aids ADY rehydration due to the transfer of sterols from IDY to ADY, leading to better fermentations when there is a sterol deficiency. At a fermentation progress of 0.4, the fermentation rates suggested that the positive effect of 150 g L^{-1} (dw) IDY during rehydration corresponded to the equivalent addition of 40 μg L^{-1} ergosterol to the fermentation medium (**Figure 4**).

Formation of Micelles by Membrane Phospholipids, Specific Cell Wall Polysaccharides, and Sterols during Rehydration of Inactive Dry Yeasts. Sterols are strongly hydrophobic compounds. Therefore, the transfer of sterols from IDY to ADY should involve a complex macromolecular structure that allows the solubilization of yeast sterols in the aqueous phase. A particular fraction was easily purified from rehydrated IDY by successive centrifugation steps (**Figure 5**). This fraction was purified as a white colloid (WC), floating on the aqueous phase after ultracentrifugation at 4 °C. It represented from 1.1% to 1.6% of the initial weight of IDY.

We carried out further analysis of the WC fraction. Preliminary investigations showed that it could be precipitated by 80% (v/v) ethanol, indicating that it contained macromolecular material (data not shown). This fraction reacted positively to Bial's reagent, indicating that it also contained glycolipids (data not shown). Protein analysis revealed that the WC fraction contained less than 1% (dw) protein. Precipitation by 80% (v/v) ethanol and further alkaline hydrolysis [NaOH (1 M), 37 °C, 16 h] resulted in about 70% of the initial dry mass being recovered as a solid residue, indicating that the WC fraction contained about 30% nonpolysaccharide material. After hydrolysis, the recovered solid residue was no longer amphiphilic and became completely soluble in water. The polysaccharide composition, as neutral monosaccharides, was determined on the lyophilized WC fraction before and after alkaline hydrolysis (**Table 2**). About 62% of the detected monosaccharides were glucose and about 33% were mannose, confirming that the WC fraction came from the yeast cell walls. We also detected inositol

Table 1. Quantification and Characterization of Squalene and Sterols in the Original Inactive Dry Yeast and in the Lyophilized White Colloid Fraction[a]

	original IDY			WC fraction	
compounds detected	contents [mg g^{-1} (dw)]	free sterols (% total)	contents [% total (dw)]	contents [mg g^{-1} (dw)]	contents [% total (dw)]
squalene	1.15	-	15.7	0.52	7.6
lanosterol	0.1	77.6	1.4	0.44	6.4
zymosterol	2.58	10.2	35.1	1.77	25.9
ergosterol	2.99	96.9	40.7	1.55	22.6
fecosterol	0.37	64.3	5.0	0.72	10.5
ergosta-5,7-dienols[b]	0.15	72.5	2.0	2.97	27.0

[a] Obtained after rehydration of IDY for 30 min at 37 °C. [b] All ergosta-5,7-dienols (dihydroergosterol, methylzymosterol, desmethyllanosterol, and ergosta-7,22-dien-3-ol) contained two conjugated double bonds in the B ring, exhibiting a strong absorption peak at 284 nm.

Table 2. Oligosaccharide Analysis (as Alditol Acetates) of the Lyophilized WC Fraction before and after Alkaline Hydrolysis[a]

	mass % (anhydromoles)	
compounds detected	before alkaline hydrolysis	after alkaline hydrolysis
mannose	62.9	61.4
glucose	33.4	33.0
galactose	1.6	2.1
rhamnose	0.8	1.5
arabinose	0.8	1.0
inositol	0.5	0.9

[a] NaOH (1 M), 37 °C, 16 h.

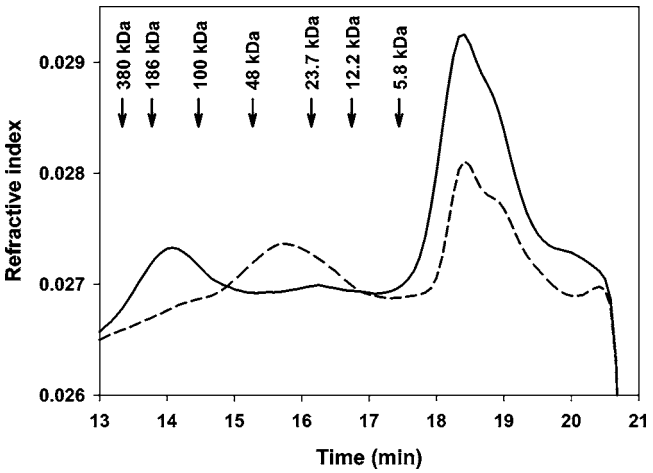

Figure 6. HPSEC profiles of WC fraction before (solid line) and after (dashed line) alkaline hydrolysis. Elution times and molecular masses (in kilodaltons) of the pullulan standards are shown.

in low amounts, suggesting that a glycosylphosphatidylinositol (GPI) anchor was also potentially present in the WC fraction. High-performance size-exclusion chromatography (HPSEC) of the polysaccharides in the WC fraction before alkaline hydrolysis showed one large peak corresponding to a molecular mass of about 167 kDa and a much smaller peak corresponding to about 20 kDa (**Figure 6**). After alkaline hydrolysis, the large peak disappeared from the chromatogram, with a new peak appearing, corresponding to a molecular mass about 31.7 kDa. Analysis of the 30% nonpolysaccharidic material obtained after alkaline hydrolysis revealed that it primarily consisted of C17:1, C16:0, and C18:0 fatty acids (about 95%) and sterols (about 5%). These were likely to be the lipid counterparts of the detected glycolipids (data not shown).

We used dynamic light scattering (DLS) to analyze the lyophilized WC fraction (**Figure 7**). Aggregation was seen above a concentration of 5 mg L^{-1} WC fraction. The aggregates had an average size of 380 nm and were polydisperse (polydispersity index ranging from 0.5 to 0.8). The formation of such aggregates by suspension of the WC fraction in water was mostly irreversible (data not shown). We tested the ability of such aggregates to modify the surface tension of water by use of the Wilhelmy plate method and a tensiometer. The total superficial tension of water strongly decreased as the WC fraction concentration increased to a concentration of 100 mg L^{-1}, showing that aggregates formed micellelike particles at high concentrations. Its critical micellar concentration (cmc) was about 4 g L^{-1} in water (**Figure 8**).

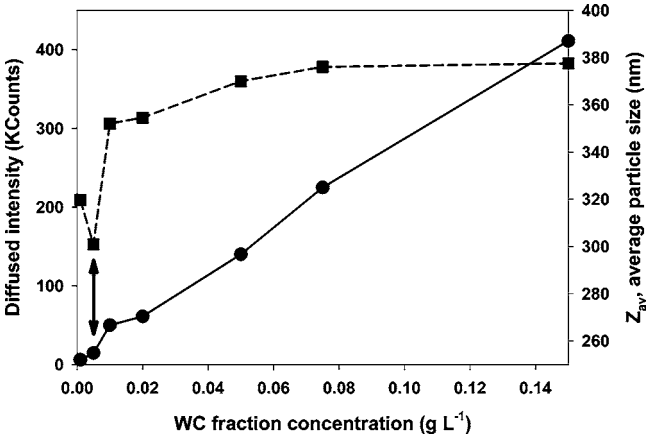

Figure 7. Dynamic light scattering (DLS) analysis of the WC fraction solubilized in pure water: diffused intensity of scattered light (●) and average particle size Z_{av} (■). The double black arrow indicates the beginning of aggregation.

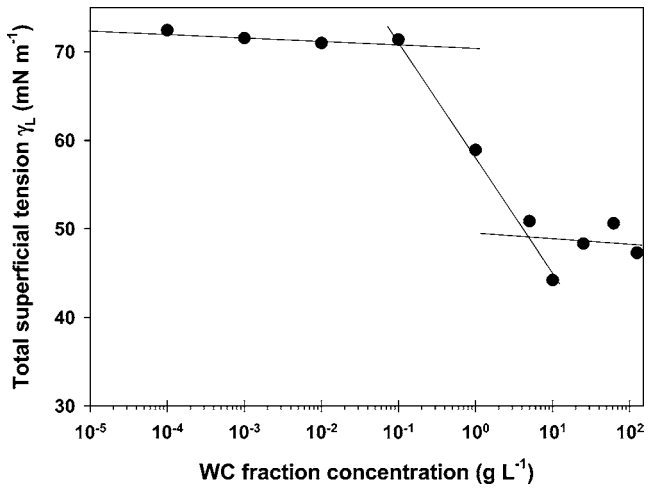

Figure 8. Evolution of the total superficial tension of water (γ_L, mN m^{-1}) in the presence of different concentrations of the WC fraction at 25 °C.

Spontaneous Solubilization of Sterols during Rehydration of Inactive Dry Yeasts at High Concentration. Spectropho-

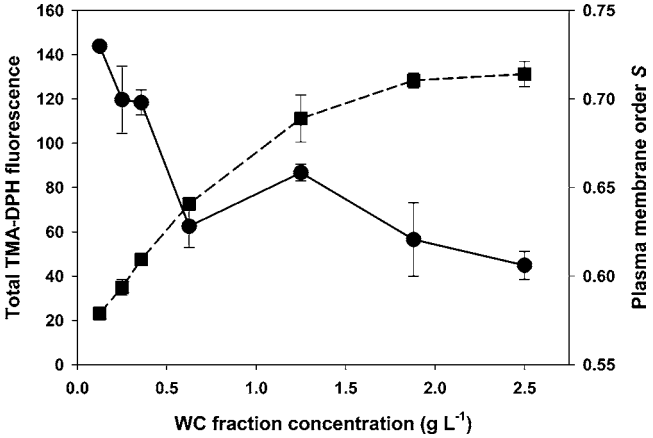

Figure 9. Evolution of the total fluorescence of TMA-DPH probe (■) and of the plasma membrane order (*S*,●) during rehydration of EC-1118 ADY (1 g) in 10 mL of water containing 0.5 g of glucose in the presence of various concentrations of purified white colloid (WC) fraction. Measurements were performed after 30 min of rehydration at 37 °C. Mean and range of two different experiments are presented.

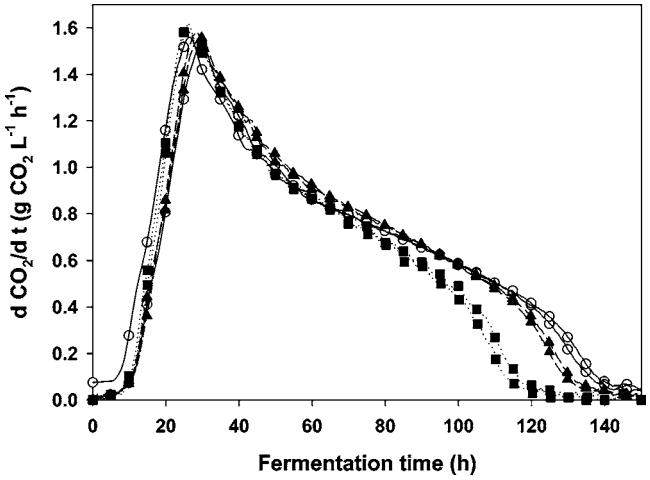

Figure 10. Variations in the CO_2 production rate by *S. cerevisiae* strain EC-1118 in SM at 24 °C. EC-1118 ADY strain was rehydrated in the absence (○) or in the presence of 1.2 g L^{-1} (▲) or 2.4 g L^{-1} (■) of lyophilized WC fraction. The CO_2 production rate is represented as a function of fermentation time. Only 90 data points from about 900 are represented for clarity of the graph. Results of two different fermentations are presented.

tometric analysis (200−300 nm) of different amounts of the lyophilized WC fraction in chloroform showed an absorption peak at 284 nm (data not shown), which is characteristic of sterol compounds containing two conjugated double bonds in their B ring (ergosterol and ergosta-5,7-dienols). We estimated, from comparison with ergosterol solutions in chloroform, that such sterols represented 4−5 μg/g of lyophilized WC fraction (data not shown). As sterols are initially present in IDY, we compared the sterols from IDY and lyophilized WC fractions. The sterol content of the WC fraction was complex and different from the original IDY. This suggests that specific IDY sterols are solubilized during the formation of the WC fraction (**Table 1**). The WC fraction contained about 15 mg g^{-1} (dw) total sterols, and about 240 μg of sterols was extracted from 1 g of IDY during rehydration at 37 °C.

Micelles Formed during Rehydration Act as Bioemulsifiers of Sterols during Rehydration of Active Dry Yeasts in the Presence of Inactive Dry Yeasts. We studied the effect of the WC fraction on the capacity of the ADY cell membranes to incorporate TMA-DPH to determine whether the WC fraction micelles could act as sterol bioemulsifiers during ADY rehydration. We saw a large increase in the accessibility of TMA-DPH to the cellular lipid bilayers when the WC fraction is added to the rehydration medium (**Figure 1**). This could be due to the effective incorporation of lipids from the WC fraction (sterols and other lipids) within the membranes of ADY, thus changing the overall properties of cell membranes. Therefore, we examined the plasma membrane order (*S*) of ADY by TMA-DPH fluorescence depolarization during ADY rehydration. The presence of between 0.1 and 1.0 g L^{-1} of the WC fraction in the rehydration medium led to a strong and continuous decrease in the plasma membrane order and to a corresponding increase of TMA-DPH probe accessibility in the membranes (**Figure 9**). This is consistent with an increase in total lipid bilayer surface area due to the incorporation of WC fraction lipids by ADY. We also followed the levels of [4-^{14}C]cholesterol present in ADY during rehydration in the presence of the WC fraction (2.5 g L^{-1}) to determine its effect on the incorporation of sterols into ADY (**Figure 2**). The labeled sterol was completely incorporated into ADY. This suggested that the WC fraction acted as a sterol bioemulsifier, allowing ADY to incorporate exogenous solubilized sterols. We also studied the ADY fermentation kinetics in the presence of the WC fraction (**Figure 10**). A rich, deaerated synthetic fermentation medium, with stigmasterol added as a unique source of phytosterol, was used to simulate ergosterol deficiency (*9*). As expected, ADY was efficiently rehydrated in the presence of a high concentration of the WC fraction, particularly with respect to duration of fermentation.

DISCUSSION

The rehydration of ADY is essential for highly viable yeast inoculum (*36*). Metal ions (magnesium and zinc) were known to affect the initiation of fermentation by ADY (*37*−*39*). However, the composition of the rehydration medium was shown to have very little effect on the efficiency of rehydration (*40*).

This study showed that, during the rehydration of ADY, yeast cells could incorporate a limited amount of lipophilic probes or solubilized sterols in their cellular membranes. Beker and Rapoport (*41*) demonstrated previously that dehydrated yeasts had damaged cytoplasm and plasma membranes when in anabiosis (*42*). During rehydration, such membrane damage led to an increase in cellular membrane permeability (*36*, *43*). The cellular membranes rapidly repaired themselves during rehydration, and a fast mobilization of lipid storage within the cells was previously observed by electron microscopy (*44*). Therefore, the incorporation of extracellular solubilized sterols by ADY as demonstrated in our experiments during rehydration was consistent with the known physiology of yeast rehydration.

In the absence of oxygen, yeast growth and fermentation occurred in a wide variety of natural fermentation media. During wine fermentation, the yeast incorporated grape phytosterols, which are principally located in the cuticular wax and berry skins of grapes. However, as seen under laboratory conditions (*45*), the phytosterols promoted normal yeast growth and inhibited fermentation. Being the predominant sterols, they also rapidly perturbed the yeast membrane properties, leading to sluggish fermentations (*9*). Therefore, under anaerobic conditions, yeast cells incorporating only grape phytosterols could not achieve complete fermentation. We have previously shown that supplementing the culture medium with small amounts of solubilized ergosterol compensates for the deleterious effect of

phytosterols (*9*). We have shown, in this present study, that rehydration of ADY in the presence of IDY had a similar effect, suggesting the transfer of sterols between IDY and ADY during rehydration. We identified an active component of IDY after purification of a white colloid (WC) fraction found floating over the aqueous phase after ultracentrifugation at 4 °C. This fraction represented about 1.5% of the initial weight of IDY, and contained, as dry weight, 70% polysaccharides, 15% fatty acids, and 15% sterols. We found glucose and mannose were the major monosaccharides present in the polysaccharidic fraction, confirming the yeast cell wall as the origin of this WC fraction. The WC fraction had an initial molecular mass of about 167 kDa and a molecular mass of about 31.7 kDa after lipid loss by alkaline hydrolysis. Inositol detected in the oligosaccharides after acid hydrolysis suggested the existence of a glycosylphosphatidylinositol (GPI) anchor (*46*), which may act as a functional link between fatty acids and polysaccharides in this fraction. It has been proposed that each outer-layer mannoprotein of the yeast cell wall is posttranslationally modified by addition of a GPI anchor, which allows a specific targeting of the GPI-anchored mannoprotein to the outer leaflet of the plasma membrane (*47*). Once on the outer leaflet of the plasma membrane, the anchor is cleaved within the C-terminal glycan and the GPI is transferred to form a glycosidic linkage with the branched β-1,6-glucan (*47*). Therefore, the WC fraction may be a specific fraction of the cell wall attached to fragments of the cell plasma membrane. These are released during the damage of the surface of the cell wall during IDY dehydration (*48*). However, further analysis of the WC fraction needs to be done. The purified WC fraction showed self-aggregation in an aqueous phase above a concentration of 5 mg L^{-1}. The aggregates had an average size of 380 nm. We demonstrated that these aggregates modified the surface tension of water above a concentration of 100 mg L^{-1} and formed micellelike particles above a concentration of 4 g L^{-1} in water. A recent study identified two complexes of mannoproteins from the cell wall of *S. cerevisiae* as potential components of a bioemulsifier fraction extracted from yeast biomass by strong heat treatment (autoclaving for 3 h; *49*). These components had a very high protein content (15−46% (dw)) and had poorly characterized molecular masses (from 25 to 1000 kDa). In this present study, the almost complete absence of proteins in the recovered WC fraction was remarkable. Therefore, the WC fraction components are clearly different, and further analysis needs to be done to determine their nature.

The effect of IDYs on the rehydration of ADY and on fermentation relies on their ability to release fragments of yeast cell wall in the rehydration medium. Used at high concentrations, IDY releases fragments that can form micellelike particles. These can act as bioemulsifiers by decreasing the total superficial tension of water and by solubilizing a specific fraction of IDY sterols in the rehydration medium. During rehydration, cellular membranes damaged during dehydration are rapidly repaired. The incorporation of the formed micelles in ADY, and consequently of IDY yeast sterols, then leads to a better membrane lipid composition of the yeasts used as inoculum.

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LITERATURE CITED

(1) Alexandre, H.; Charpentier, C. Biochemical aspects of stuck and sluggish fermentation in grape must. *J. Ind. Microbiol. Biotechnol*. **1998**, *20*, 20−27.

(2) Bisson, L. F. Stuck and sluggish fermentations. *Am. J. Enol. Vitic*. **1999**, *50*, 107−119.

(3) Jiranek, V.; Langridge, P.; Henschke, P. A. Amino acid and ammonium utilization by *Saccharomyces cerevisiae* wine yeasts from a chemically defined medium. *Am. J. Enol. Vitic*. **1995**, *46*, 75−83.

(4) Sablayrolles, J. M.; Dubois, C.; Manginot, C.; Roustan, J. L.; Barre, P. Effectiveness of combined ammoniacal nitrogen and oxygen additions for completion of sluggish and stuck wine fermentations. *J. Ferment. Bioeng.* **1996**, *82*, 377−381.

(5) Julien, A.; Roustan J. L.; Dulau, L.; Sablayrolles, J. M. Comparison of nitrogen and oxygen demands of enological yeasts: technological consequences. *Am. J. Enol. Vitic*. **2000**, *51*, 215−222.

(6) Eglinton, J.; Henschke, P. Can the addition of vitamins during fermentation be justified? *Aust. Grapegrow. Winemak.* **1993**, *352*, 47−52.

(7) Bataillon, M.; Rico, A.; Sablayrolles, J. M.; Salmon, J. M.; Barre, P. Early thiamin assimilation by yeasts in enological conditions: impact on alcoholic fermentation kinetics. *J. Ferment. Bioeng*. **1996**, *82*, 145−150.

(8) Delfini, C.; Costa, A. Effects of the grape must lees and insoluble materials on the alcoholic fermentation rate and the production of acetic acid, pyruvic acid and acetaldehyde. *Am. J. Enol. Vitic*. **1993**, *44*, 86−92.

(9) Luparia, V.; Soubeyrand, V.; Berges, T.; Julien A.; Salmon J. M. Assimilation of grape phytosterols by *Saccharomyces cerevisiae* and their impact on enological fermentations. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 25−32.

(10) Blateyron, L.; Aguera, E.; Dubois, C.; Gerland, C.; Sablayrolles, J. M. Control of oxygen additions during alcoholic fermentations. *Vitic. Enol. Sci*. **1998**, *53*, 131−135.

(11) Ribereau-Gayon, P.; Glories, Y.; Maujean, A.; Dubourdieu, D. L'influence des différentes opérations du travail du vin. In *2-Chimie du vin. Stabilisation et traitements*; Ribereau-Gayon, P., Glories, Y., Maujean, A., Dubourdieu, D., Eds.; Dunod: Paris, 1998; pp 459−462.

(12) Vidal, J. C.; Dufoucq, T.; Boulet, J. C.; Moutounet, M. Les apports d'oxygène au tours des traitements des vins. Bilan des observations sur site, I^{ère} partie. *Rev. Fr. Oenol*. **2001**, *190*, 24−31.

(13) Vidal, J. C.; Boulet, J. C.; Moutounet, M. Les apports d'oxygène au cours des traitements des vins. Bilan des observations sur site. 2ème partie. *Rev. Fr. Oenol*. **2003**, *201*, 32−38.

(14) Vidal, J. C.; Boulet, J. C.; Moutounet, M. Les apports d'oxygène au cours des traitements des vins. Bilan des observations sur site. 3ème partie. *Rev. Fr. Oenol*. **2004**, *205*, 25−33.

(15) Delfini, C.; Cocito, C.; Ravaglia, S.; Conterno, L. Influence of clarification and suspended grape solid materials on sterol content of free run and pressed grape musts in the presence of growing yeast cells. *Am. J. Enol. Vitic*. **1993**, *44*, 452−458.

(16) Alexandre, H.; Nguyen Van Long, T.; Feuillat, M.; Charpentier, C. Contribution à l'étude des bourbes: Influence sur la fermentation des moûts. *Rev. Fr. Oenol*. **1994**, *146*, 11−20.

(17) Trioli, G. Effect of Fermaid addition to white grape juice on the behavior of several commercial yeast strains. *Wein−Wiss.* **1996**, *51*, 204−209.

(18) Dulau, L.; Ortiz-Julien, A.; Trioli, G. Method for active dry yeast rehydration, and rehydration medium. International Patent PCT/FR02/01949, 2002.

(19) Bely, M.; Sablayrolles, J. M.; Barre, P. Description of alcoholic fermentation kinetics: its variability and significance. *Am. J. Enol. Vitic*. **1990**, *40*, 319−324.

(20) Ingledew, W. M.; Magnus, C. A.; Sosulski, F. W. Influence of oxygen on proline utilization during the wine fermentation. *Am. J. Enol. Vitic*. **1987**, *38*, 246−248.

(21) Salmon, J. M.; Vincent, O.; Mauricio, J. C.; Bely, M.; Barre, P. Sugar transport inhibition and apparent loss of activity in *Saccharomyces cerevisiae* as a major limiting factor of enological fermentations. *Am. J. Enol. Vitic.* **1993**, *44*, 56−64.

(22) Sablayrolles, J. M.; Barre, P.; Grenier, P. Design of a laboratory automatic system for studying alcoholic fermentations in anisothermal enological conditions. *Biotechnol. Tech.* **1987**, *1*, 181−184.

(23) Gille, G.; Sigler, K.; Hofer, M. Response of catalase activity and membrane fluidity of aerobically grown *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* to aeration and the presence of substrates. *J. Gen. Microbiol.* **1993**, *139*, 1627−1634.

(24) Folch, J.; Lees, M.; Stanley, G. H. S. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497−509.

(25) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911−917.

(26) Ness, F.; Bourot, S.; Regnacq, M.; Spagnoli, R.; Berges, T.; Karst, F. SUT1 is a putative Zn[II]2Cys6-transcription factor whose upregulation enhances both sterol uptake and synthesis in aerobically growing *Saccharomyces cerevisiae* cells. *Eur. J. Biochem.* **2001**, *268*, 1585−1595.

(27) Albersheim, P.; Nevins, D. J.; English, P. D.; Karr, A. A method for the analysis of sugars in plant cell wall polysaccharides by gas−liquid chromatography. *Carbohydr. Res.* **1967**, *5*, 340−345.

(28) Harris, P. J.; Henry, R. J.; Blakeney, A. B.; Stone, B. A. An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydr. Res.* **1984**, *127*, 59−73.

(29) Saulnier, L.; Brillouet, J. M.; Joseleau, J. P. Structural studies of pectic substances from the pulp of grape berries. *Carbohydr. Res.* **1988**, *182*, 63−78.

(30) Doco, T.; Brillouet, J. M. Isolation and characterization of a rhamnogalacturonan II from red wine. *Carbohydr. Res.* **1993**, *243*, 333−343.

(31) Pellerin, P.; Vidal, S.; Williams, P.; Brillouet, J. M. Characterization of five type II arabinogalactan−protein complexes from red wine with increasing uronic acid content. *Carbohydr. Res.* **1995**, *277*, 135−143.

(32) Pellerin, P.; Doco, T.; Vidal, S.; Williams, P.; Brillouet, J. M.; O'Neill, M. A. Structural characterization of red wine rhamnogalacturonan II. *Carbohydr. Res.* **1996**, *190*, 183−197.

(33) Vidal, S.; Williams, P.; O'Neill, M. A.; Pellerin, P. Polysaccharides from grape berry cell walls. Part I: tissue distribution and structural characterization of the pectic polysaccharides. *Carbohydr. Polym.* **2001**, *45*, 315−323.

(34) Grubisic, Z.; Rempp, P.; Benoit, H. A universal calibration for gel permeation chromatography. *J. Polym. Sci., Part B: Polym. Phys.* **1967**, *5*, 753−759.

(35) Lowry, O. H.; Rosebrough, A. L.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

(36) Beker, M. J.; Blumbergs, J. E.; Ventina, E. J.; Rapoport, A. I. Characteristics of cellular membranes at rehydration of dehydrated yeast *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **1984**, *19*, 347−352.

(37) Walker, G. M. Magnesium as a stress-protectant for industrial strains of *Saccharomyces cerevisiae*. *J. Am. Soc. Brew. Chem.* **1998**, *56*, 109−113.

(38) Walker, G. M. The role of metal ions in optimising yeast fermentation performance. In *Nutritional aspects II. Synergy between yeasts and bacteria*; Lallemand, Ed.; Lallemand: Toulouse, France, 2002; pp 17−22.

(39) Walker, G. M.; Smith, G. D. Metal ion preconditioning of baker's yeast. In *Proceedings of the fifth Aviemore conference of malting, brewing and distilling*; Campbell, I., Ed.; Institute of Brewing: London, 1999; pp 311−315.

(40) Radler, F.; Dietrich, K.; Schoenig, I. Microbiological testing of active dried yeast preparations for winemaking. *Dtsch. Lebensm.*−Rundsch. **1985**, *81*, 73−77.

(41) Beker, M. J.; Rapoport, A. I. Conservation of yeasts by rehydration. *Adv. Biochem. Eng. Biotechnol.* **1987**, *35*, 127−171.

(42) Rapoport, A. I. Rejection of areas of damaged cytoplasm by microorganisms in a state of anabiosis. *Microbiology* **1973**, *42*, 317−318.

(43) Rapoport, A. I.; Khroustalyova, G. M.; Camanis, G. J.; Beker, M. J. Yeast anhydrobiosis: permeability of the plasma membrane. *Microbiology* **1995**, *64*, 229−232.

(44) Saulite, V. A.; Rapoport, A. I.; Beker, M. E. Lipid inclusions in cells and changes in them during dehydration and reactivation of yeasts. *Microbiology* **1986**, *55*, 99−104.

(45) Hossak J. A.; Rose, A. H. Fragility of plasma membranes in *Saccharomyces cerevisiae* enriched with different sterols. *J. Bacteriol.* **1976**, *127*, 67−75.

(46) McConville, M. J.; Ferguson, M. A. J. The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochem. J.* **1993**, *294*, 305−324.

(47) Lipke, P. L.; Ovalle, R. Cell wall architecture in yeast: new structure and new challenges. *J. Bacteriol.* **1998**, *180*, 3735−3740.

(48) Rapoport, A. I.; Biryuzova, V. I.; Beker, M. E. Effect of dehydration on the surface structure of the yeast cell wall. *Microbiology* **1983**, *52*, 204−207.

(49) Barriga, J. A. T.; Cooper, D. G.; Idziak, E. S.; Cameron D. R. Components of the bioemulsifier from *S. cerevisiae*. *Enzyme Microb. Technol.* **1999**, *25*, 96−102.

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