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Oleic acid and ergosterol supplementation mitigates oxidative stress in wine strains of *Saccharomyces cerevisiae*

Sara Landolfo [a], Giacomo Zara [b], Severino Zara [b], Marilena Budroni [b], Maurizio Ciani [a], Ilaria Mannazzu [b,*]

[a] Dipartimento SAIFET, Sez. Microbiologia Alimentare, Industriale e Ambientale, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy
[b] Dipartimento di Scienze Ambientali, Agrarie e Biotecnologie Agroalimentari, Università degli Studi di Sassari, Viale Italia 39, 07100 Sassari, Italy

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ABSTRACT

During fermentation of high-sugar-containing medium lacking lipid nutrients, wine yeasts undergo oxidative stress and oxidative damage to cell membranes and proteins. Considering that cell membranes are important stress sensors, and that under hypoxic conditions wine yeasts modulate cell membranes composition by incorporating lipids available in the growth medium, in the present work, the effects of lipid nutrition on wine yeast oxidative stress response were evaluated on two strains of *Saccharomyces cerevisiae*. Biomarkers of oxidative stress, oxidative damage and antioxidant response were evaluated together with viability and acetic acid production during fermentation of a synthetic must lacking lipid nutrients as compared to added oleic acid and ergosterol. The results show that the availability of lipid nutrients causes a significant reduction in the intracellular content of reactive oxygen species and in the oxidative damage to membranes and proteins, as indicated by flow cytometry of cells stained with dihydroethidum (DHE) and propidium iodide (PI) and by Western blot of protein carbonyls. Accordingly, lipid nutrients feeding results in the increase in cell viability and superoxide activity, and the reduction in trehalose accumulation, proteinase A activity and production of acetic acid. In summary, these results are compatible with the hypothesis that the supplementation of lipid nutrients mitigates oxidative stress and oxidative damage in wine strains of *S. cerevisiae* during growth under unfavourable conditions.

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1. Introduction

The lipid composition of cell membranes strongly influences their physiological functions through effects on the activities of many membrane-associated enzymes and transporters (Avery et al., 1995; Vigh et al., 1998). Biosynthesis of sterols and fatty acids is involved in the maintenance of membrane efficiency when cells are subject to stress factors. In particular, yeast cells counteract ethanol stress by increasing the unsaturation index (Thomas et al., 1978, You et al., 2003) and ergosterol content (Shobayashi et al., 2005) of their cell membrane. The biosynthesis of unsaturated fatty acids and ergosterol is impaired during must fermentation, a process that generally occurs under hypoxic/anaerobic conditions. Indeed, oxygen deprivation negatively affects lipid biosynthesis, by decreasing the activity of oxygen-dependent enzymes (e.g. Δ9-desaturase, squalene epoxydase, the lanosterol demethylation complex), and by regulating the expression of acetyl-CoA carboxylase and hydroxymethylglutaryl-CoA reductase through the accumulation of saturated fatty acids and precursors of ergosterol, respectively (Bloomfield & Bloch, 1960; Hammond, 1993; Henry, 1982; Parks, 1978; Ratledge & Evans, 1989; Wakil et al., 1983).

Oxygen availability (Zara et al., 2009) and lipid nutrition (Redón et al., 2009) influence cell-lipid composition by acting on the biosynthesis and incorporation of lipids. In the absence of oxygen and exogenous lipids, *Saccharomyces cerevisiae* cells reduce the surface area of their organelle membranes and dilute their lipid content as necessary, to the limit of viability (Henry, 1982). Under these conditions, they undergo anaerobic stress, which results in the release of intermediates of lipid biosynthesis (Bardi et al., 1998) and oxidative damage to cell structures (Landolfo et al., 2008). In fact, in the absence of oxygen *S. cerevisiae* activates NAD(P)H-dependent pathways which produce significant levels of ROS (Rosenfeld and Beauvoit, 2003). Moreover, during the first stages of fermentation, in spite of the presence of repressive glucose concentrations, *S. cerevisiae* cells carry out a partial synthesis of mitochondrial cytochromes and consume oxygen through mitochondria-related activities (Salmon et al., 1998; Zara et al., 2009). These events, together with the variety of biotic and abiotic stresses to which wine yeasts are subject during must fermentation result in the production of ROS and the generation of a stress condition (Landolfo et al., 2008). Thus, *S. cerevisiae* viability is low during growth in the absence of oxygen and lipid nutrients (Fornairon-Bonnefond et al., 2002; Mannazzu et al., 2008, Landolfo et al., 2008). On the contrary, the achievement of a balanced lipid

* Corresponding author. Tel.: +39 79 229385; fax: +39 79 229370.
E-mail address: i.mannazzu@uniss.it (I. Mannazzu).

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composition under oxygen availability is accompanied by an increase in viability and biomass production (Zara et al., 2009).

During winemaking, wine yeasts can assimilate exogenous lipids (Luparia et al., 2004; Redón et al., 2009) and unsaturated fatty acids (Chen, 1980), and incorporate them into their cell membranes to promote cell growth and fermentative activity (Thurston et al., 1981; Herraiz et al., 1990; Belviso et al., 2004). However, not all lipids have the same effects on cell membranes. According to Redón et al. (2009), C16:1 supplementation has a positive effect on wine-yeast fitness, as it reduces the fermentation length and increases yeast viability. On the contrary, the outcome of ergosterol feeding is rather controversial, as it can result in a dramatic reduction in cell viability and an increase in fermentation rate (Redón et al., 2009).

As the plasma membrane is an important sensor of stress conditions (Vigh et al., 1998; Redón et al., 2009) and a target for intracellular ROS (Landolfo et al., 2008), the aim of this study was to determine whether lipid nutrition can serve as a means to mitigate oxidative stress during fermentation of a high-sugar-containing must. To do this, oleic acid and ergosterol were added to high-sugar-containing medium, and the oxidative damage to the yeast cell structures and the antioxidant response of two wine-yeast strains were analysed and compared to the absence of these lipid nutrients.

2. Materials and methods

2.1. Strains, media and culture conditions

The study was conducted on EC1118, a commercial enological strain of *S. cerevisiae* (Lallemand, Montreal, Canada), and M25, a wine-yeast strain deposited with the Culture Collection of DiSAABA (University of Sassari, Italy). The media used were SJ (0.2% YNB w/o amino acids, 0.7% ammonium sulphate, 12% glucose, 12% fructose, 30 mg/L leucin, 20 mg/L histidine, 20 mg/L uracil, and 2% agar when required) and ergosterol/oleic acid supplemented SJ (FSJ: SJ with 15 mg/L ergosterol and 6 mg/L oleic acid dissolved in tergitol/ethanol 50/50). Bench-scale fermentations were carried out in triplicate in SJ and FSJ medium. Briefly, the yeast strains were pre-cultured aerobically in YEPD (2% glucose, 1% yeast extract, 2% peptone) (liquid:air ratio, 1:10), and 5×10^5 cells/mL were inoculated into 100 mL flasks containing 75 mL SJ/FSJ medium (liquid:air ratio, 7.5:10) equipped with glass capillary stoppers. These inoculations were incubated statically at 20 °C for 20 days to promote the occurrence of hypoxic conditions. A flask for each sampling time was inoculated, and both the culture broths and the cells were analysed as described below. Yeast growth was determined by viable plate counting and total cell counting in a hemocytometer. All of the experiments were carried out in triplicate from independent pre-cultures.

2.1. Analytical determination of acetic acid

The acetic acid content was evaluated at day 20 by steam distillation, according to official analytical methods (EC, 2000).

2.3. Resistance to plumbagine

About 5×10^6 cells were seeded for inclusion in SJ and FSJ plates and 6 mm blank disks (Par Test, Oxoid) that had been soaked with 10 μL 125 mM plumbagine (Sigma P7262) were put on the agar surface. Plumbagine is an oxidative stress inducer that generates superoxide anions (Rodrigues-Pousada et al., 2005). The effect of plumbagine was measured as the diameter of the inhibition haloes around the disks after six days of incubation at 20 °C and was defined as the mean of three independent replicates.

2.4. Flow cytometry

A Coulter Epics XL (Beckman Coulter, Inc. Fullerton, CA, USA) equipped with a 15 mW, air-cooled, argon-ion laser (emission, 488 nm) with five sensors for the detection of forward and side light scatter of green (525 nm, channel 1), yellow (575 nm, channel 2), and orange-red (620 nm, channel 3) fluorescence was used. Calibration of yeast cell size was performed using the flow cytometry size-calibration kit (Molecular Probes, Inc. Eugene, OR, USA). Yeast cells were harvested at the indicated times, and ethanol-treated cells (70%, 2 h) were included as a positive control for oxidation. The samples were then washed and re-suspended in phosphate-buffered saline (PBS; 8.0 g/L NaCl, 0.20 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, pH 7.4) to a final concentration of $1–2\times10^6$ cells/mL (OD_{600}, 0.08–0.20). The cell suspensions were stained with propidium iodide (PI) and dihydroethidium (DHE), according to the procedures described by Landolfo et al. (2008). At least 1.5×10^4 cells were analysed for each experiment. The data were visualized using the WinMDI flow cytometry software (Joseph Trotter, Salk Institute for Biological Studies, La Jolla, CA, USA).

2.5. Preparation of crude cell extracts

Crude yeast-cell extracts for the antioxidant enzyme assays were prepared in 0.1 M Tris (pH 7.6) following a mechanical lysis protocol (Mannazzu et al., 1998). Crude extracts for the proteinase A activity assays were prepared in 0.1 M Tris/HCl (pH 7.6) by vigorous shaking of the cell suspensions in the presence of glass beads, for 3 min. Total protein contents were assayed according to Bradford (1976).

2.6. Measurement of antioxidant enzyme activities

Superoxide dismutase (SOD) activities were measured as described by Oyanagui (1984), and are expressed as U/mg protein. Proteinase A (Pep4) activities were evaluated as described by Jones (1990), and are expressed as μg Tyr/min/mg protein.

2.7. Western blotting of protein carbonyls

Protein carbonyls were detected using a 2,4-dinitrophenylhydrazine (DNPH)-binding method, as described by Levine et al. (1994). Aliquots of crude extracts containing 15 μg protein were derivatized with 20 mM DNPH in 10% trifluoroacetic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and loaded onto polyacrylamide gels (10%) Laemmli et al. (1970). After electrophoresis, the proteins were transferred to Polyvinylidene fluoride (PVDF) membranes and probed with anti-DNP IgG (Sigma-Aldrich Inc., St Louis, MO, USA) at a 1:5000 dilution as the first antibody, and goat anti-rabbit IgG linked to alkaline phosphatase (Sigma-Aldrich Inc., St Louis, MO, USA) at a 1:5000 dilution as the second antibody. Immunodetection was performed by the BCIP/NBT staining method (Sigma-Aldrich Inc., St Louis, MO, USA). Densitometric analysis of Western blot was carried out by using Quantity one 1D analysis Software (vers. 4.6.7) (BioRad Laboratories, Hercules, CA, USA).

2.8. Trehalose evaluation

Trehalose was evaluated as described by Parrou et al. (1997). The glucose produced by trehalase hydrolytic activity was measured with the Glucose Oxidase (GO) Assay kit (Sigma-Aldrich, Inc., St Louis, MO, USA).

2.9. Data analysis

All the experiments were carried out in triplicate from independent pre-cultures. Statistical analyses of the data were performed using

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