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Vanadium pentoxide effects on stress responses in wine *Saccharomyces cerevisiae* strain UE-ME_3

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Abstract Vanadium pentoxide mainly used as catalyst in sulphuric acid, maleic anhydride and ceramics industry, is a pollutant watering redistributed around the environment. Research on biological influence of vanadium pentoxide has gained major importance because it exerts toxic effects on a wide variety of biological systems. In this work we intent to evaluate the effects of vanadium pentoxide ranging from 0 to 2 mM in culture media on a wine wild-type *Saccharomyces cerevisiae* from Alentejo region of Portugal. Our results show that 2.0 mM vanadium pentoxide in culture medium induced a significant increase of malonaldehyde level and Glutathione peroxidase activity, a slightly increase of Catalase A activity as well as a decrease of wet weight and mitochondrial NADH cit c reductase of *S. cerevisiae* UE-ME_3. Also our results show that cycloheximide prevent cell death when cells grows 30 min in presence of 1.5 mM of vanadium pentoxide.

Keywords Vanadium · Malonaldehyde · Catalase A · Mitochondrial NADH cit c reductase · Glutathione peroxidase · *Saccharomyces cerevisiae*

Abbreviations

V_2O_5	Vanadium pentoxide
MDA	Malonaldehyde
CAT A	Catalase peroxisomal
GPx	Glutathione peroxidase
ROS	Reactive oxygen species

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Introduction

Wine fermentation is a complex ecological and biochemical process involving the sequential development of different yeast species. The non-*Saccharomyces* yeasts grow well during early stages of fermentation, but are subsequently replaced during the following stages by *Saccharomyces* yeasts (Fleet and Heard 1992; Pretorius 2000). Traditionally the wine production by natural fermentation is greatly influenced by the yeast resistance to the stress conditions, including the osmotic stress imposed by the high sugar content of the must and the ethanol produced during fermentation, otherwise by pesticides and metals intakes by grapes from the soil or present in winery steel equipment (Bauer and Pretorius 2000; Querol et al. 2003). Vanadium is a rare, soft, ductile grey-white element (Nechay 1984) found combined in certain minerals and used mainly in steel or pesticides production. The level of vanadium occurrence in the earth's crust is around 150 ppm. Moreover, Human activities as fuel oil burning, steel empowerment, manufacturing of sulphuric acid and maleic anhydride and pesticide use, increase its environmental level in the air, soil, food and water, reaching in several cases 6,000 ppm (Penuelas and Filella 2002). This metal exhibits a wide range of stable oxidation states, two of which, vanadate (V^{5+}), and the less vanadyl (V^{4+}), are considered to be predominant in living systems (Bode et al. 1990). It is well known that vanadium exerts toxic, mutagenic, and genotoxic effects on a wide variety of biological systems, inducing alterations of many important metabolic functions

(Willsky 1990). Moreover, vanadium can be a pollutant in urban, industrial and rural areas, as a consequence of industrial processes or chemical products application, fact which requires the elucidation of the mechanisms by which living organisms, like this wine yeast, answer to the presence of this metal ion in its growth medium during wine fermentation (Mannazzu 2001). Since metals environmental level is an issue of great environmental apprehension, we used *Saccharomyces cerevisiae* strain UE-ME_3, a vanadium resistant strain (Ferreira et al. 2006), for evaluate the vanadium stress responses in a wine wild-type yeast of Alentejo, a wine region of Portugal, having in account the environmental importance of characterization and preservation of wild-type strains of a winery region. Considering that metals, disturbs energetic metabolism of the cell, which is the main source of endogenous ROS production, mainly at NADH reoxidation mechanisms and fatty acids oxidation, we also intent evaluate how vanadium pentoxide affect several antioxidant responses of yeast cell, since reactive oxygen species has also been identified as essential in yeast cell death (Madeo et al. 1999) and NADH dehydrogenases display the role of regulates apoptosis in yeast (Wu et al. 2002; Wissing et al. 2004; Maris et al. 2001; Li et al. 2006). At least five mechanisms of NADH reoxidation exist in *S. cerevisiae*. These are: (a) alcoholic fermentation; (b) glycerol production; (c) respiration of cytosolic NADH via external mitochondrial NADH dehydrogenases (EC 1.6.5.3, NDE1 and NDE2); (d) respiration of cytosolic NADH via the glycerol-3-phosphate shuttle; and (e) oxidation of intramitochondrial NADH via a mitochondrial 'internal' NADH dehydrogenase (EC 1.6.5.3, NDI). (Luttik et al. 1998; Bakker et al. 2001; Jault et al. 1994; Moore et al. 1992; Crichton et al. 2007; Small and McAlister-Henn 1998; Davidson and Schiestl 2001). Although it has been recognized that peroxisomal structures and a number of their constituent proteins are inducible by lipids or fatty acids, very little is known about the mechanisms involved in induction of peroxisomes in any eucaryotic organism (Lazarow and Fujiki 1985; Lock et al. 1989; Tolbert 1981). Catalase A (EC 1.11.1.6, CAT A) has been demonstrated to be induced by fatty acids together with peroxisomal structures and a number of other peroxisomal proteins (Skoneczny et al. 1988; Veenhuis et al. 1987; Cohen et al. 1985; Cohen et al. 1988). Therefore it should be possible to use the expression of the Cta1 gene as an indicator for the induction or repression of peroxisome formation (Simon et al. 1991). Though it was determined CAT A, a peroxisomal enzyme involved in scavenging of H_2O_2 resulting from an eventual increase of mitochondrial free-radical flux induced by vanadium to evaluate the antioxidant response of peroxisomal fraction from lag yeast cells. In other hand, glutathione peroxidase (GPx) an enzyme of redox cycle of glutathione is expressed in yeast cells and plays a crucial role in the defence line against ROS (Izawa et al. 1995) under *S. cerevisiae* reducing H_2O_2 endogenously formed, as well as, LOOH to H_2O and corresponding alcohol (Inoue et al. 1999). Given that mitochondrial electron transport is a large contributor to oxidative stress, we have investigated how vanadium pentoxide influence yeast mitochondrial NADH dehydrogenases, using NADH cytochrome c reductase activity as marker, CAT A, and GPx as peroxisomal and cytoplasmactic enzymes markers of antioxidant response, as well as cytoplasmatic malonaldehyde generation during metal exposition assays to determine yeast cell damages caused by vanadium pentoxide. In addition, we attempt to determine if cycloheximide, a protein synthesis inhibitor, revert an eventual yeast growth inhibition caused by 1.5 and/or 2.0 mM V_2O_5 and infer an eventual apoptosis process (Ludovico et al. 2003), which can be correlate with events involving mitochondria and peroxisomes.

Materials and methods

Microorganisms and growth conditions

The eukaryotic model used was the wine wild-type *Saccharomyces cerevisiae* UE-ME_3, a strain isolated from regional wine (Alentejo-Portugal), belonging to the Enology laboratory collection of University of Évora, a greatly resistant strain to metal stress (Ferreira et al. 2006). The isolated colonies of this strain were stored in glycerol (30%, w/v) at −80°C. The cells were grown to mid-exponential phase in a water bath, with orbital stirring, at 28°C, in 250-ml flasks containing 100 ml of YEPD medium with 2% (w/v) of glucose (Atlas 2006). The cells (10^6 cells ml^{-1}) at mid-exponential phase were inoculated in the same condition and incubated during 200 min at 28°C in the absence or presence of 1.5 and 2.0 mM V_2O_5.

At the end of the experiment, samples from each treatment were used for biomass determination by wet weight. The cultures were used to obtain the peroxisomes enriched fraction, and mitochondria enriched fraction, as well as, post-peroxisomal supernatant which were used for determination of CAT A; mitochondrial NADH cit C reductase; GPx and Malondialdehyde (MDA) level, respectively.

Inhibition of protein synthesis

The effect of protein synthesis inhibition was also evaluated determining the dose-response curves in liquid medium and yeast growth in solid medium: Exponential-phase cells were harvest, suspended and grown at 28°C in liquid 2% glucose YEPD medium, containing V_2O_5 at 0, 1.5 and 2.0 mM and cycloheximide 50 mg/ml for 30 min. At the same time and conditions was performed a control assay

without cycloheximide. Samples from each treatment were diluted and plated in triplicate on YEPD medium, in order to obtain viable counts (cfu) and to observe the effect of protein synthesis inhibition (Ludovico et al. 2003; Matsuyama et al. 1998).

Preparation of peroxisomes enriched fraction

Peroxisomes enriched fraction was obtained by a modification of the procedure of Petrova et al. (2004). Cells growing in YEPD medium with 2% (w/v) of glucose and, incubated during 200 min at 28°C, in the absence or presence of 1.5 and 2.0 mM V_2O_5 were harvested and ultra-sonic disrupt in chilled lysis buffer (0.2 M sorbitol, 20 mM HEPES-KOH pH 7.0, 50 mM potassium acetate, 2 mM EDTA). The resulting extracts were differentially centrifuged at 3,000*g*, 4°C, 10 min, and at 12,000*g*, 4°C, 30 min. The crude organelle fraction obtained from this centrifugation was suspended in 50 mM phosphate buffer pH 7.5 at a protein concentration of 150 μg per ml.

Preparation of mitochondria enriched fraction

Mitochondria enriched fraction was obtained by a modification of the procedure of Tzagoloff (1971). Cells growing in YEPD medium with 2% (w/v) of glucose and incubated during 200 min at 28°C in the absence or presence of 1.5 and 2.0 mM V_2O_5 were harvested and suspended in a medium consisting of 0.25 M mannitol, 0.05 M Tris–acetate, pH 7.5, and 1 mM EDTA (MTE) at a concentration of 1–5 g, wet weight, per 30 ml of medium. The suspension (2.5 ml) was mechanical homogenized with 1 ml of glass beads (0.45–0.50 mm diameter) for 45 s. The homogenate was centrifuged at 800*g* for 20 min. The supernatant was centrifuged at 12,000*g* for 30 min. The mitochondria enriched fraction obtained was suspended in a solution containing 0.25 M sucrose and 0.01 M Tris–acetate, pH 7.5, concentration of 200 μg per ml. All steps were carried out at 0–4°C.

Malonaldehyde and protein determination

The obtained post-peroxisomal supernatant was used for malonaldehyde determination (MDA, nmol/mg wet weight) as an index of lipid peroxidation according to the spectrofluorometric method of Uchiyama and Mihara (1978) and Kitamura et al. (2006). In brief, 0.05 ml of post-peroxissomal supernatant, 0.2 ml of 8.1% SDS and 3.0 ml of 0.4% thiobarbiturate in 10% acetic acid solution (pH 3.5) were added and heated in a water bath at 95°C for 60 min. After cooling, 5.0 ml of *n*-butanol and 1.0 ml distilled water were added to the sample, which was centrifuged at 3,000 rpm for 10 min. The fluorescence of TBARS was determined in the butanol phase at 553 nm using a single beam Shimadzu RF-5001 PC spectrofluorophotometer with the excitation at 515 nm. MDA was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane (10 mmol; Aldrich) by HCl (10 ml, 100 mM) in a total volume of 50 ml at 60°C for 1 h. A series of MDA standards ranging from 2.5 to 100 μM were treated like de samples and used to prepare a standard calibration curve. The solutions were stored at 4°C and assayed spectrofluorophotometrically immediately prior to use.

Protein concentration was determined according to Lowry et al. (1951) using BSA as standard.

Enzymatic assays

CAT A activity was determined by measuring the decrease in absorbance at 240 nm due to H_2O_2 consumption according to Beers and Sizer (1952). The reaction mixture consisted of: 30 mM H_2O_2 and adequate concentration of peroxisomal pellet ($\sim$5 μg per ml) in 50 mM phosphate buffer pH 7.5.

NADH-cytochrome *c* reductase activity was determined in mitochondria enriched fraction ($\sim$15 μg per ml) at 30°C in 10 mM K_2HPO_4, pH 7.5, containing 100 μM KCN by following the reduction of Cyt C (22.5 μM) at 550 nm in the presence of 0.5 mM NADH, according to Tzagoloff et al. (1975) and Ludovico et al. (2002).

GPx activity in post-peroxissomal supernatant were assayed in reaction buffer comprised of 100 mM phosphate pH 7.0, 5 mM EDTA, 1.60 mM, NADPH, 5.0 mM GSH and 0.24U glutathione reductase. Post-peroxissomal supernatant in adequate dilution (25 μg per ml) was added to 1 ml of total volume. The reaction mixture was pre-incubated for 10 min to 37°C with continuous stirring. After the addition of NADPH, the decay of the absorbance at 340 nm was followed for 3 min to obtain a line of control test. The overall reaction was then initiated by addition of 1.0 mM of t-BHP, preheated to 37°C and the decrease in absorption at 340 nm was monitored for 5 min according to standard protocols (Chaudiere et al. 1984; Flohé and Gunzler 1984). The rate of reaction depends on GPx was obtained when the effects of independent and non-enzymatic hydroperoxide were subtracted from the total rate of reaction, according to descriptions in literature (Flohé and Gunzler 1984). As GSH was continuously regenerated by glutathione reductase, the concentration of GSH in the trial was held in the initial level. Consequently, the GPx reaction proceeds in accordance with an order kinetics of pseudo-zero. To calculate enzymatic activity, it was used the coefficient of molar extinction 6.22 mM^{-1} cm^{-1} of NADPH + H^+ (Holme and Peck 1993).

Protein and all enzymatic measurements were carried out with a double beam spectrophotometer, Hitachi-U2001 with temperature control.

Statistical analysis

All the data presented are mean values achieved in five independent experiments ± SD. The normality and homogeneity of variance were assessed by the P-Plot and Levene's test, respectively. The statistical analysis of results were performed by ANOVA I and Dunett's test to determine significant differences ($P < 0.01$) between treatments, using SPSS for windows, version 16, licensed to University of Évora (Sokal and Rohlf 1997).

Results

In this work we have been studied the stress response to vanadium pentoxide a pollutant resulting from chemical industry like, sulphuric acid and pesticides production or ceramic making processes, using a wine wild-type yeast of Alentejo region of Portugal. Figure 1 shows a significantly decrease of biomass production for 200 min by wine *Saccharomyces cerevisiae* UE-ME$_3$ cultures exposed to V_2O_5 ($P < 0.01$), occurring a decline of 19 and 53% of wet weight for cells growing in culture medium with 1.5 and 2.0 mM V_2O_5, respectively. This fact appoints us vanadium pentoxide, in this range concentration, as a potent growth inhibitor of this wild-type yeast *S. cerevisiae*.

In order to evaluate the effects of vanadium pentoxide on de novo protein synthesis, the dose-response curves were determined, growing yeast cells in liquid medium containing V_2O_5 at 0, 1.5 and 2.0 mM with or without cycloheximide 50 mg/ml for 30 min, plating samples from each treatment on YEPD medium and counting colony forming units (cfu) in solid medium. Figure 2 shows that these concentration of cycloheximide is not cytotoxic after 30 min incubation and increased cell viability in cultures treated with 1.5 mM vanadium pentoxide, whereas for 2.0 mM V_2O_5 occur a reverse response.

Having in account that oxidative damages resulting from ROS formation in metal stress conditions and a subsequent fail of antioxidant and energetic mechanisms of the cell will be implicated in cell death, it was also select as a significant goal of this study to prospect the possibility of MDA level of the cell, antioxidant peroxisomal catalase, mitochondrial NADH cytochrome c reductase and glutathione peroxidase activities of wine wild-type yeast *Saccharomyces cerevisiae* UE-ME$_3$ will be used as biomarkers of vanadium toxicity.

Figure 3 shows that yeast cells growing in presence of vanadium pentoxide have got an high significantly increase of MDA level ($P < 0.01$) with values 19- and 52-fold higher than control, for 1.5 and 2.0 mM V_2O_5, respectively.

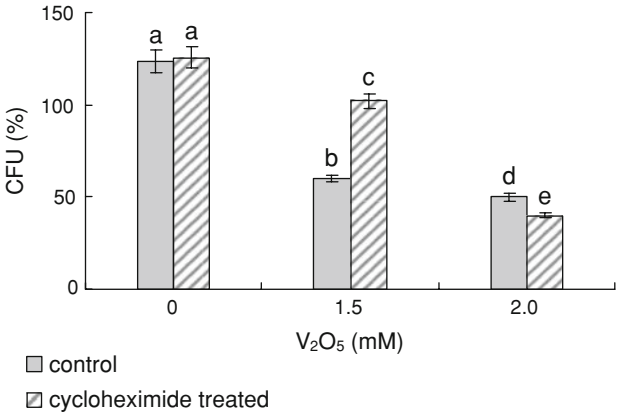

Fig. 2 Relative survival (% cfu) of wine wild-type *Saccharomyces cerevisiae* UE-ME$_3$, for 30 min, V_2O_5 0, 1.5 and 2.0 mM in the absence or presence of cycloheximide. *Each bar* represents the mean of five independent experiments (*bars* with *no common letter* are significantly different, $P < 0.01$)

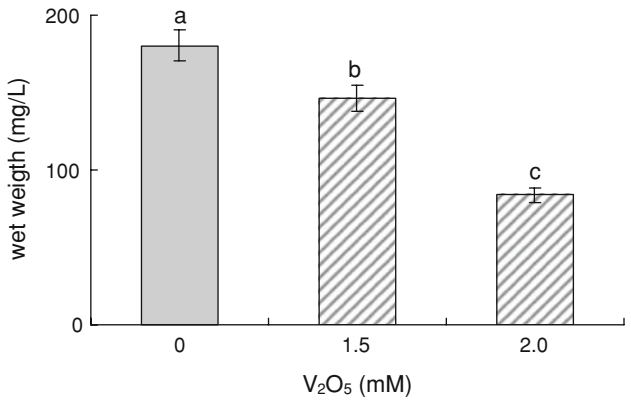

Fig. 1 Effect of V_2O_5 on biomass production for 200 min by wine wild-type *Saccharomyces cerevisiae* UE-ME$_3$ cell. *Each bar* represents the mean ± SD of five independents experiments (*bars* with *no common letter* are significantly different, $P < 0.01$)

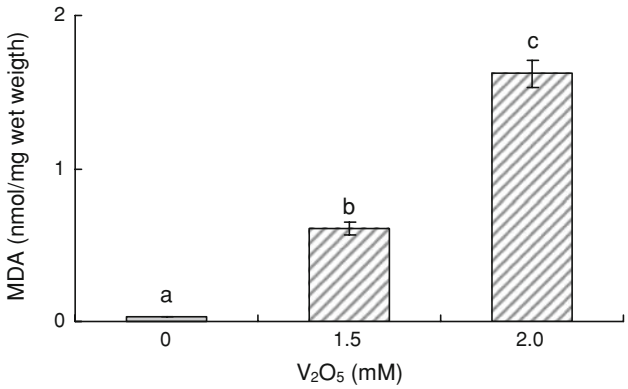

Fig. 3 Effect of V_2O_5 on MDA level of wine wild-type *Saccharomyces cerevisiae* UE-ME$_3$. *Each bar* represents the mean ± SD of five independents experiments (*bars* with *no common letter* are significantly different, $P < 0.01$)

Figure 4 only shows an increase of antioxidant CAT A activity for the treatment with 2.0 mM V_2O_5, but without statiscal significance.

Figure 5 shows a significantly decrease of mitochondrial NADH cyt C reductase in both vanadium pentoxide treatments, occurring a decline of 30 and 33% for 1.5 and 2.0 mM V_2O_5 in culture medium.

Figure 6 shows a significantly and reverse increase of glutathione peroxidase in *S. cerevisae* UE-ME_3 grown in presence of vanadium pentoxide, occurring a raise of 3, 5× and 2× for 1.5 and 2.0 mM V_2O_5 in culture medium, respectively.

Discussion

The aim of this study was to evaluate the effect of 1.5 and 2.0 mM vanadium pentoxide on cell growth, de novo protein synthesis, lipid peroxidation, catalase A, mitochondrial NADH cytochrome C reductase and cytosolic glutathione peroxidase activities of wild-type wine yeast *Saccharomyces cerevisiae* UE-ME_3.The results show that vanadium pentoxide is a potent growth inhibitor of wine wild-type yeast *Saccharomyces cerevisiae* UE-ME_3, in both concentration assayed (Fig. 1). In order to address the question if the cell death process, induced in *S. cerevisiae* UE-ME_3 by vanadium pentoxide, is an active process, which is characteristic of apoptosis (Ludovico et al. 2002), we analysed the dependence of the vanadium pentoxide induced yeast killing on de novo protein synthesis. The presence of cycloheximide attenuated the toxic effect of 1.5 mM vanadium pentoxide, increasing cell viability of yeast exposed 30 min to 1.5 mM vanadium pentoxide, an indication that such cell death is an active process (Fig. 2). During oxidative stress, a proportion of the ROS evades the host defences and can cause oxidative damage to nucleic acids, lipids, and proteins (Halliwell 1991; Kappus 1987; Sies and de Groot 1992). As a result of the oxidative damage to lipids, a variety of lipid hydroperoxides are formed, whose subsequent breakdown products may well be just as toxic as the ROS themselves (Esterbauer 1993). MDA is a commonly detected aldehyde (Esterbauer et al. 1991; Steels et al. 1994) which has been used as a measure of lipid peroxidation (Turton et al. 1997). Consequently, the highly significant increase of MDA level detected in yeast cells grown in presence of vanadium pentoxide (Fig. 3) advise that oxidative damages caused by ROS (Cabiscol et al. 2000), linked to an approximately null response of CAT A (Fig. 4) and a reverse increase of GPx (Fig. 6) to lipid peroxides stress, are also implicated in *S. cerevisiae* UE-ME_3 growth inhibition. This response is correlate with loss of vital functions, like cytotoxic inhibition of mitochondrial dehydrogenases (Fig. 5), which block cytosolic

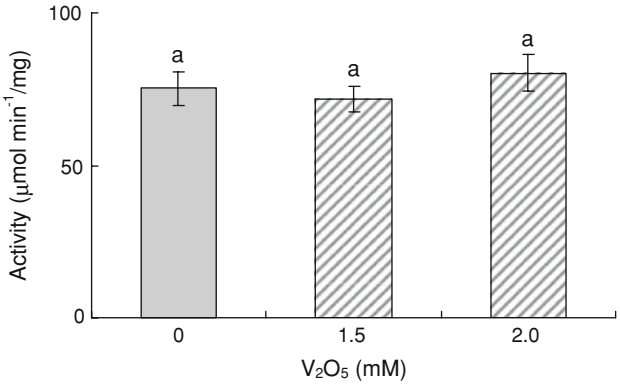

Fig. 4 Effect of V_2O_5 on CAT A activity of wine wild-type *Saccharomyces cerevisiae* UE-ME_3. *Each bar* represents the mean ± SD of five independents experiments (*bars* with *no common letter* are significantly different, $P < 0.01$)

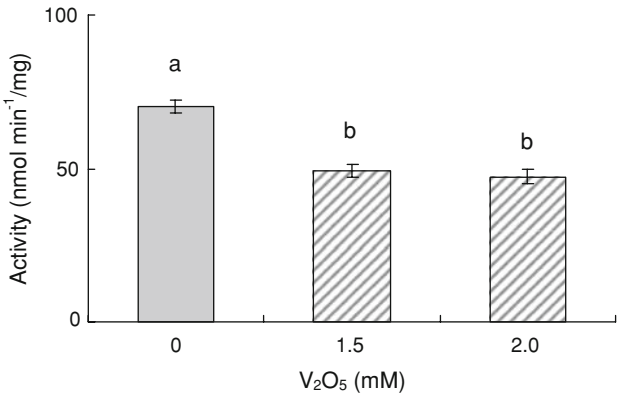

Fig. 5 Effect of V_2O_5 on mitochondrial NADH cyt C reductase activity of wine wild-type *Saccharomyces cerevisiae* UE-ME_3. *Each bar* represents the mean ± SD of five replicates (*bars* with *no common letter* are significantly different, $P < 0.01$)

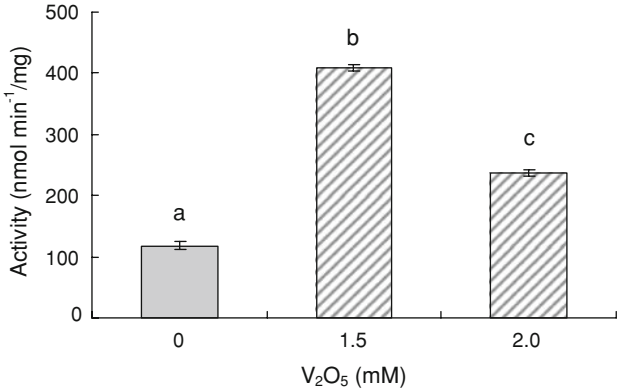

Fig. 6 Effect of V_2O_5 on GPx activity of wine wild-type *Saccharomyces cerevisiae* UE-ME_3. *Each bar* represents the mean ± SD of five replicates (*bars* with *no common letter* are significantly different, $P < 0.01$)

NADH reoxidation with disturb of energetic metabolism of the *S. cerevisiae* UE-ME_3 cells, generating ROS, like is described by several authors (Herrero et al. 2008; Sedensky and Morgan 2006; Wang et al. 2004; Davidson and Schiestl 2001; Overkamp et al. 2000). Bearing in mind that cytosolic ROS, namely hydrogen peroxide and/or lipid peroxides can be implicated in the yeast cell death (Madeo et al. 2002), we presume that oxidative stress generate by 1.5 mM vanadium pentoxide can cause apoptosis in *S. cerevisiae* UE-ME_3. Additionally we also suppose that yeast MDA levels and mitochondrial dehydrogenases activity can be sensitive markers of vanadium toxicity.

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