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ORIGINAL PAPER

Respiratory capacity of the *Kluyveromyces marxianus* yeast isolated from the mezcal process during oxidative stress

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Abstract During the mezcal fermentation process, yeasts are affected by several stresses that can affect their fermentation capability. These stresses, such as thermal shock, ethanol, osmotic and growth inhibitors are common during fermentation. Cells have improved metabolic systems and they express stress response genes in order to decrease the damage caused during the stress, but to the best of our knowledge, there are no published works exploring the effect of oxidants and prooxidants, such as H_2O_2 and menadione, during growth. In this article, we describe the behavior of *Kluyveromyces marxianus* isolated from spontaneous mezcal fermentation during oxidative stress, and compared it with that of *Saccharomyces cerevisiae* strains that were also obtained from mezcal, using the W303-1A strain as a reference. *S. cerevisiae* strains showed greater viability after oxidative stress compared with *K. marxianus* strains. However, when the yeast strains were grown in the presence of oxidants in the media, *K. marxianus* exhibited a greater ability to grow in menadione than it did in H_2O_2. Moreover, when *K. marxianus* SLP1 was grown in a minibioreactor, its behavior when exposed to menadione was different from its behavior with H_2O_2. The yeast maintained the ability to consume dissolved oxygen during the 4 h subsequent to the addition of menadione, and then stopped respiration. When exposed to H_2O_2, the yeast stopped consuming oxygen for the following 8 h, but began to consume oxygen when stressors were no longer applied. In conclusion, yeast isolated from spontaneous mezcal fermentation was able to resist oxidative stress for a long period of time.

Keywords Yeast · Oxidative stress · Mezcal · Spontaneous fermentation · Respiration · México

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Introduction

Mezcal is a distilled alcoholic beverage derived from the fermented and cooked core of different agave species (*Agave salmiana*, *Agave angustifolia*, *Agave cupreata*, *Agave duranguensis*, and *Agave tequilana*) that is produced within a limited region of México (NOM-070-SCFI 1997). Mezcal is produced by means of an unaltered handcrafted process that is several centuries old. The mezcal process can be divided into six principal stages: agave harvesting; cooking; milling; fermentation; distilling, and bottling (Gschaedler et al. 2004). Agave species have oligofructans that are hydrolyzed during the cooking stage using stone ovens (a hole in the ground, filled with stones, heated with wood, and covered with soil) (Lappe-Oliveras et al. 2008). The temperature is not well controlled and the other variables of this stage are dependent on the particular methods of each production house. Higher levels of mallard and caramel compounds are frequently found in cooked agave. In the next stage, the cooked agaves are manually shredded to obtain the fiber, which is placed in fermentation tanks with no water. For 2–10 days, the fiber is maintained in the

tank as a spontaneous, semisolid fermentation and when the temperature increases or the fiber emits the smell of ethanol, water is added to the tank (Arellano et al. 2012). The fermentation stage takes 1–3 weeks, after which the fermented juice is distilled twice in a copper alembic still. Finally, the alcoholic proof is adjusted to 35°–50° Gay-Lussac, and finally the mezcal is bottled (Gschaedler et al. 2004).

During the fermentation stage, no commercial yeasts are used and the yeast present in the agave wort carries out natural fermentation. These yeasts are exposed to extreme conditions because the agave must possess between 200 and 350 g of reducing sugars/kg of agave fiber (Arellano et al. 2012), which causes osmotic stress, and the yeast must produce ethanol in order to decrease the osmotic stress. The agave must also contain growth inhibitors such as furfurals (Palmqvist et al. 1999), vanillin (Fitzgerald et al. 2004), and saponins (De León-Rodríguez et al. 2006; Eskander et al. 2010; Pereira da Silva et al. 2006), all of which cause stress in the yeast during fermentation. The majority of the resistant yeasts survives and grows in the fermentation tank (Pérez-Torrado et al. 2009).

Different types of environmental and physiological stress conditions constantly challenge yeasts during fermentation, such as heat shock, ethanol stress, osmotic stress, oxidative stress, and others (Cardona et al. 2007; Carrasco et al. 2001; Gille and Sigler 1995). To cope with the deleterious effects of stress, the yeast is required to develop rapid molecular responses to repair the damage and protect against further exposure to the same and other forms of stress (Carrasco et al. 2001). It is frequently assumed that synthesis of stress proteins is intended for yeast survival and its adaptation to adverse conditions; for example, the Hsp104p protein has been found to be activated during heat shock (Sánchez et al. 1992); but this protein has also been detected during ethanol stress and oxidative stress, meaning that stress proteins are not specific for just one particular stress (Kim et al. 2006). Transcription factors, such as Msn2p and Msn4p, are also involved in the recognition of activated genes during different stresses (Martínez-Pastor et al. 1996; Cohen et al. 2003; Cardona et al. 2007).

Moreover, the yeast *Saccharomyces cerevisiae* has been utilized as a model to study how eukaryotic cells respond to stress, to understand the function of stress-induced proteins, and to explain why, in view of the high degree of evolutionary conservation of stress pathways between yeast and eukaryotic cells, this yeast can be used as a suitable model system for characterizing the stress response in more complex organisms (Estruch 2000). During stress, the yeast induces not only the proteins used to repair damaged components or to afford protection, but also metabolic enzymes, indicating that a reorganization of metabolic fluxes is required or that it is appropriate for ensuring survival and adaptation to the stress condition (Blomberg 2000).

Yeast requires oxygen during growth, similar to other aerobic cells, including higher organisms (Ojovan et al. 2011); thus, cells can produce toxic compounds derived from molecular oxygen, which are known as reactive oxygen species (ROS). High levels of these compounds result in oxidative stress. Among ROS compounds we find the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\cdot}$), which cause damage to different cell macromolecules (lipids, proteins, and DNA) that have been associated with a number of human diseases (Halliwell and Gutteridge 2007). The cells have developed a highly regulated complex of enzymes and compounds as antioxidant defenses; mainly superoxide dismutase (SOD), catalase (Cat), glutathione reductase (Grx), thioredoxins (Trx), glutathione, and carotenoids. These enzymes and compounds reduce the ROS produced by the cell to non-lethal levels. Antioxidant capacity, gene activation, and cascade signaling during oxidative stress have been developed in different cells, such as in yeast (Cyrne et al. 2010; Jamieson 1992; Westwater et al. 2005). Experiments have been developed to study antioxidant capacity in vitro, employing compounds such as H_2O_2, menadione, paraquat, cumen hydroperoxide, *t*-butylhydroperoxide, Fe^{2+} ($FeSO_4$), etc.

Indigenous mezcal yeasts are under constant stress during fermentation (Arellano et al. 2012) and they have developed an adaptation system to survive that condition. The aim of this work was to establish the mezcal yeast capacity for undergoing oxidative stress at different stages of aerobic growth (exponential, stationary, and late-stationary) in comparison with the *S. cerevisiae* W303-1A reference strain, to determine the yeast's growth capacity after an oxidative stress event and its physiological behavior during oxidative stress in the presence of the oxidants menadione and H_2O_2. Our results indicate that to apply oxidative stress, it is also necessary to know the amount of time required for the desired effect—not just the time needed for viability or cell growth—and these must be the parameters used for evaluating the oxidative stress that is produced.

Materials and methods

Yeast strains and media

Yeast strains were obtained from the culture collection of the CIATEJ (Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, México) (Gschaedler et al. 2004) and from the ATCC (American Type Culture Collection, Rockville, MD, USA). CIATEJ

Table 1 Yeasts isolated in spontaneous mezcal fermentations

Yeast strain	Specie	Mexican state
MC4	*S. cerevisiae*	Oaxaca
OFF1	*K. marxianus*	Guerrero
SLP1	*K. marxianus*	San Luis Potosí
W303-1A	*S. cerevisiae*	Reference strain (ATCC 208352)

yeast strains were isolated from spontaneous mezcal fermentation in different Mexican states (Table 1).

All of the yeast strains were grown for 12 h at 30 °C and 250 rpm in a YPD medium (yeast extract 1 %, bacteriological peptone 2 %, and glucose 2 %; DIFCO); the yeasts were then collected, washed twice with salt solution (0.75 %), and finally added to a glycerol-water solution (50/50) and maintained at −70 °C until use.

Effect of oxidative stress on yeast viability during different growth phases

Oxidative stress was applied as described (Thorpe et al. 2004), with some modifications. Briefly, yeast strains were grown in Erlenmeyer flasks in a YPD medium for 24 h at 30 °C and 250 rpm; the yeasts were then collected and inoculated 1×10^7 cell/mL in a fresh YPD medium. The culture was incubated for 72 h under the same conditions, and samples were taken at 12, 24, 48, and 72 h. The cells for each sample were quantified by the Neubauer chamber; then, 1×10^7 cells/mL were inoculated into fresh media and either menadione sodium bisulphate (Sigma) or H_2O_2 (Sigma) was added, as previously noted for each experiment. After 3 h, the yeasts were counted and the colony forming units (CFU) were determined (Demasi et al. 2006).

Effect of oxidative stress on growth rate

The yeast strains were grown as previously mentioned, but in this case, the yeasts were inoculated into microplates with fresh YPD, to which we added 0, 10, 50, 100, and 200 mM of menadione sodium bisulphate (Menadione) or the same concentration of H_2O_2. Work volume was 200 µL and optic density (OD) was evaluated at 600 nm every hour for 8 h in Lexmark microplate readings. From these results, the rate growth was calculated according to the logistic method. Each experiment was done in triplicate for the statistical analysis.

Respiratory capacity during oxidative stress with menadione or H_2O_2

The yeasts were grown for 24 h in a YPD medium at 30 °C and 250 rpm. The yeast was then collected and inoculated 1×10^7 cells/mL in a 150-mL minibioreactor with a working volume of 100 mL of YPD medium at 30 °C; a stirrer speed of 500 rpm and an airflow rate of 0.10 l/min were added. The minibioreactors were equipped with pH and dissolved oxygen sensors (Applikon), which were connected to an Applikon AD1030 biocontroller on a computer with bioexpert software to obtain online data. The exhaust gases were monitored with bluesens O_2 and CO_2 sensors, and the data were obtained with Bacvis software. After the yeast was inoculated, the process continued for 3 h to allow the yeast to adapt; then, either 50 mM of H_2O_2 or 100 mM of menadione sodium bisulphate was added. Samples were taken for 24 h and analyzed for OD at 600 nm using the cell count obtained with a Neubauer chamber and the reducing sugars obtained through a modified Miller method.

Results

Effect of oxidative stress on yeast viability during different growth phases

In order to determine the effect of oxidative stress at different concentrations of H_2O_2 and menadione in *Kluyveromyces marxianus* and *S. cerevisiae* yeast isolated from the mezcal process at different growth phases (12, 24, 48 and 72 h), viability was determined after the oxidants were added. All the yeast grew up with almost 100 % of viability when no oxidant was present, but when 10 mM H_2O_2 was added to the samples, the viability decreased more than 70 % at 24 and 48 h. Additionally, at 50 mM and 100 mM the viability decreased more than 99 %.

The effect of the oxidants was dose-dependent, because viability decreased as the concentration increased (Figs. 1, 2). All of the yeast species tested were more resistant to menadione than to H_2O_2, but *S. cerevisiae* was more resistant than *K. marxianus*; the MC4 strain isolated from the mezcal process was the most resistant. Its viability decreased only 70 % at 100 mM of H_2O_2 during the exponential phase (12 h of growth), and the oxidant effect was reduced during the stationary phase (48 and 72 h of growth). *K. marxianus* showed little difference between strains because the OFF1 strain was more resistant at 10 mM of H_2O_2 in all of the growth stages (Fig. 1), while the viability of the SLP1 strain increased during the stationary phase at 72 h.

As previously mentioned, the effect produced with menadione was dosage-dependent and it did not produce high oxidative stress, as did H_2O_2 (Fig. 2). Viability was >10 % at 100 mM of menadione in all of the stages of growth. The oxidative stress produced by menadione demonstrated that the late-stationary phase (72 h) was not any more resistant than the earlier stages.

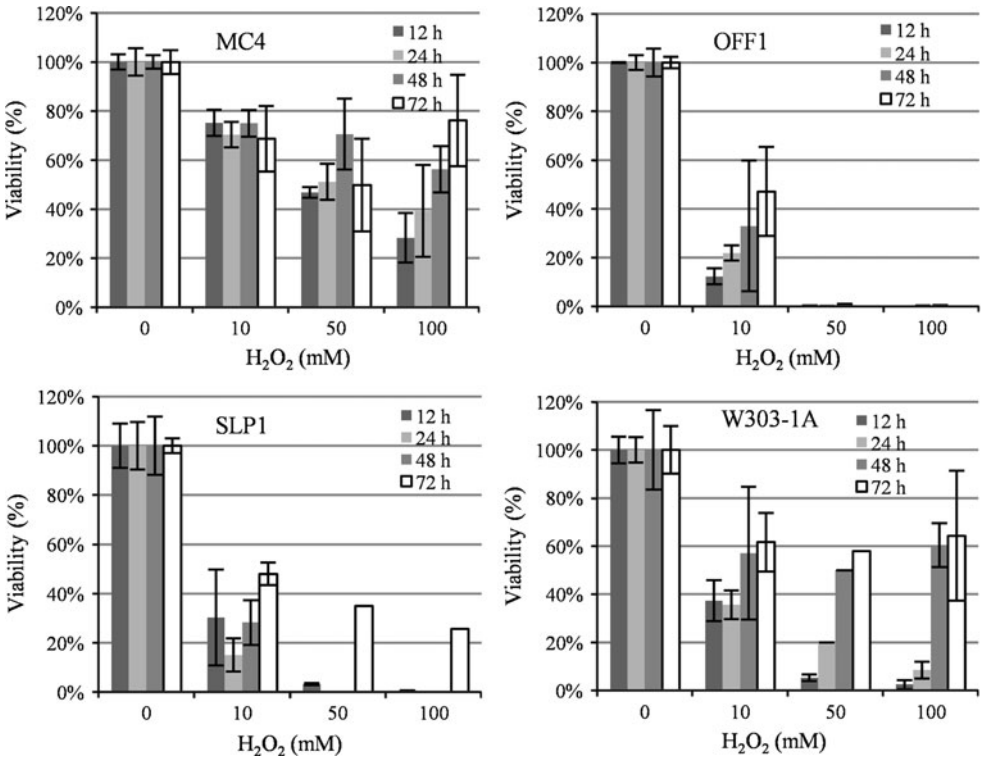

Fig. 1 Cell viability after an oxidative stress event using H_2O_2 at different concentrations (0, 10, 50, and 100 mM) in autochthonous yeast *S. cerevisiae* (MC4), *K. marxianus* (SLP1 and OFF1), and the *S. cerevisiae* (W303-1A) reference strain in different stages of growth

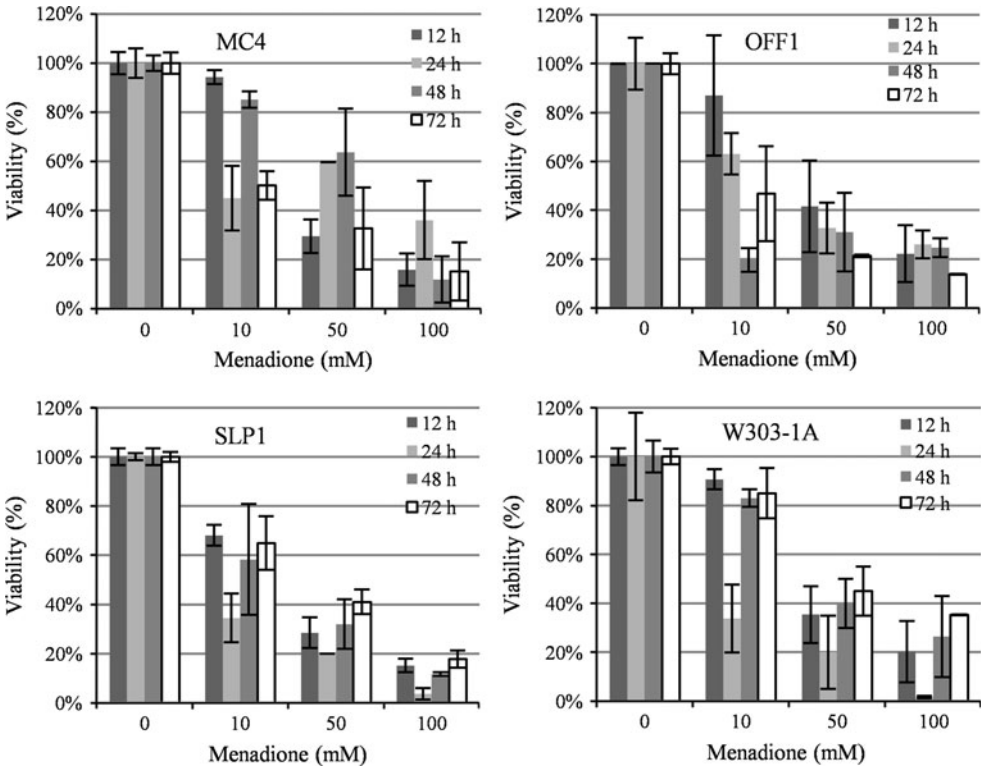

Fig. 2 Cell viability after an oxidative stress event using H_2O_2 at different concentrations (0, 10, 50, and 100 mM) in autochthonous yeast *S. cerevisiae* (MC4) and *K. marxianus* (SLP1 and OFF1) in different stages of growth

Effect of oxidative stress on the growth rate

Because the oxidative stress produced by H_2O_2 and menadione did not decrease viability by >99 %, we needed to determine whether the oxidants had an effect on the growth rate. The yeasts were inoculated into an YPD medium and different concentrations of menadione or H_2O_2 were added (0, 10, 50, 100, and 200 mM). Then, OD was evaluated at

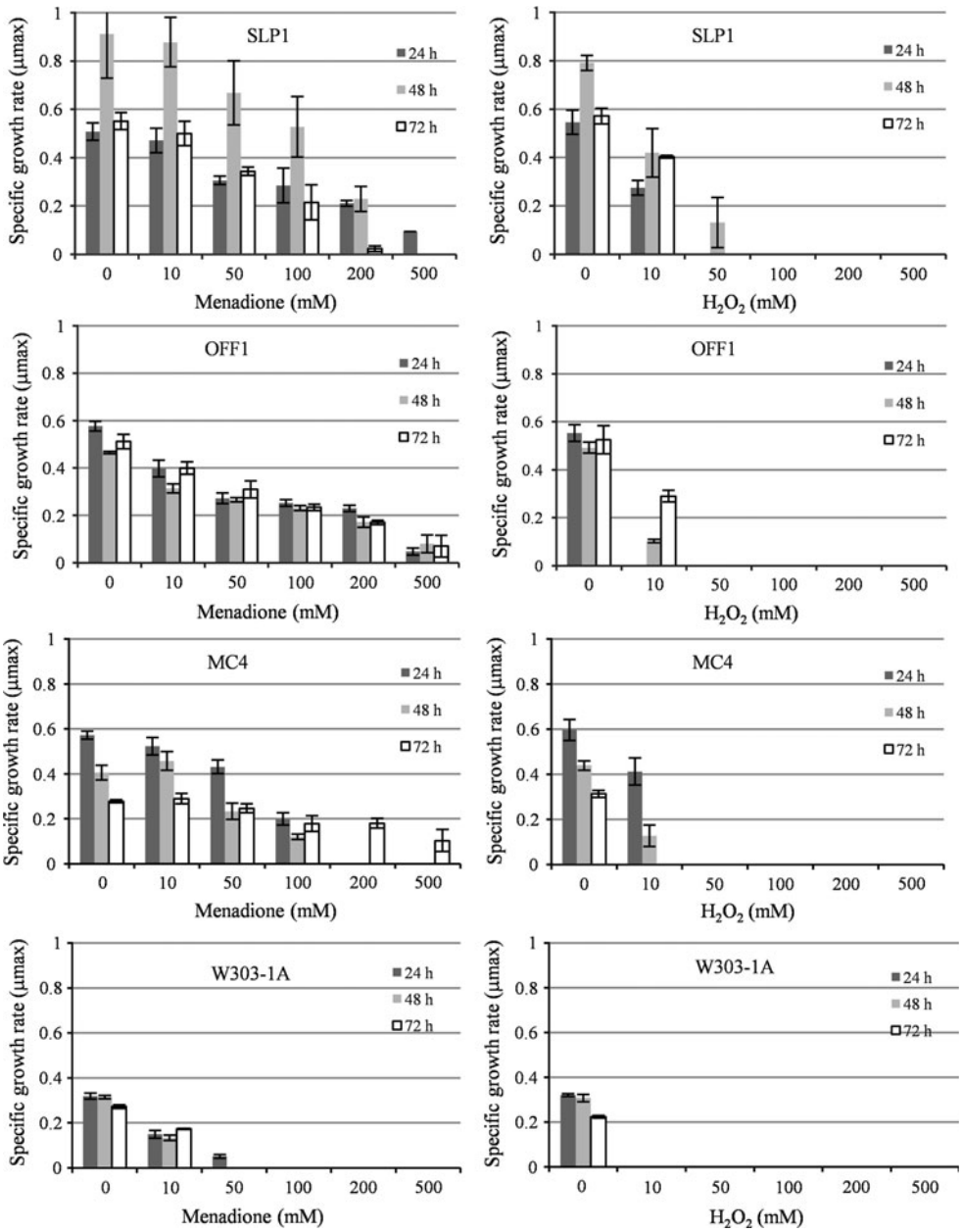

Fig. 3 Specific growth rate of *K. marxianus* (SLP1 and OFF1) and *S. cerevisiae* (MC4 and W303-1A) during oxidative stress at different concentrations of menadione and H_2O_2. Oxidative stress was produced in yeast collected at different stages of growth

600 nm every hour for the next 8 h. The growth rate was calculated and the results are shown in Fig. 3. The oxidative stress was dosage-dependent and, surprisingly, the yeast had the capacity to grow with the oxidants. The most resistant yeast was *K. marxianus* SLP1, which was able to increase OD at 200 mM of menadione and 50 mM of H_2O_2. Moreover, all of the autochthonous mezcal yeasts possessed the capacity to grow at higher concentrations of menadione.

Respiratory capacity during oxidative stress

The yeasts were able to grow at different oxidant concentrations, as previously mentioned, leading us to pose the following question: If the yeast can grow, is it possible to change the respiration in a culture medium containing oxidants? To answer this question, microbioreactors were designed to evaluate oxygen consumption, pH, glucose, and OD during growth. Figure 4 depicts the results obtained without the addition of oxidants. When the process began, the sensors were maintained constant and the yeast was then inoculated. It is clear that the dissolved oxygen was consumed during the following 8 h and that the pH decreased to 3.9; subsequently the dissolved oxygen increased, probably because the yeast stopped the respiration process. However, the pH increased to nearly 7.0; this effect was not detected using *S. cerevisiae* (data not shown). The pH in this case only increased to 5.0. Other

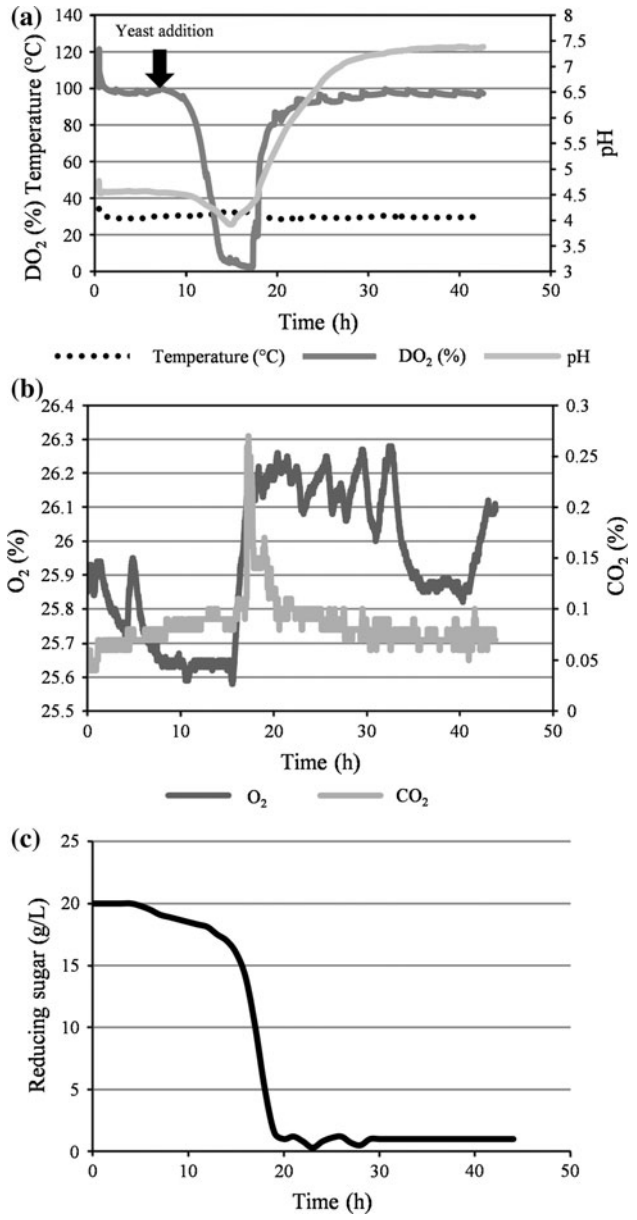

Fig. 4 Respiratory capacity and sugars consumed using a *K. marxianus* strain SLP1. **a** Temperature, dissolved oxygen, and pH behavior during growth in a minibioreactor (the *arrow* indicates the time at which the yeast was inoculated). **b** Behavior of CO_2 and O_2 in exhaust gases during growth in a minibioreactor. **c** Reducing sugars during growth

variables were measured; such as O_2 percentage and CO_2 in exhaust gases. CO_2 increased and sugars were consumed at high rates only when the dissolved oxygen decreased 8 h after the experiment was started. At that point, CO_2 was not detected at high concentrations and there were no sugars.

Figure 5 illustrates the results obtained when the oxidants menadione and H_2O_2 were added. Menadione was observed to exert a different effect than H_2O_2. When menadione was added, the yeast continued to consume oxygen, but after some time (5 h for the 24-h inocula, 10 h for the 48-h inocula, and 4 h for the 72-h inocula), respiration stopped. The pH decreased during the first hours after menadione was added, and when the dissolved oxygen increased to nearly 100 %, the pH was stable at an acidic pH.

When hydrogen peroxide was added to the culture, dissolved oxygen increased by nearly 400 %, a few minutes after the oxidant was added. The dissolved oxygen decreased slowly over the next 8 h, but then it was consumed, similarly to when oxidants were not added to the culture. In this case, the pH decreased during the first half of the experiment and then increased. pH behavior was similar to the conduct when no oxidants were added, but it did not increase to pH 7.

Discussion

During spontaneous fermentation, yeast cells are affected by different stress conditions. Improvement under these conditions can be a useful way to obtain better fermentative behavior, and according to the literature, these conditions can affect growth capacity and consequently, fermentation. In order to determine the effect of oxidative stress on *K. marxianus* and *S. cerevisiae* strains isolated from mezcal production facilities and to compare it with that of *S. cerevisiae* W303-1A, we developed a strategy to ascertain the yeast's behavior during oxidative stress. Viability has been used to evaluate resistance to oxidative stress (Gershon and Gershon 2000). In some works, oxidants were added to the solid media and inoculated with the yeast (Thorpe et al. 2004). In others, minidiscs with solutions containing the oxidants have been employed (Carrasco et al. 2001). In this study, we attempted to use these strategies to analyze an oxidative stress event (data not shown), but the effect with H_2O_2 was not reproducible and sometimes we were unable to reduce the viability. To cause a desired effect, we used YPD liquid media and prior to the yeast inoculations, we added the oxidants before the yeast inocula. This methodology provided us with reproducible results, showing the need to establish the correct method for evaluating oxidative stress, principally with H_2O_2, because it is volatile and has a short lifetime.

Viability decreases linearly when oxidant concentration is increased, but resistance to oxidative stress increases during the stationary phase (48 and 72 h). Under these conditions, it was necessary to add 100 mM of menadione and 50 mM of H_2O_2 to decrease viability to 20 %. These results are contrary to those obtained by others who have performed similar studies (Rodríguez-Manzaneque et al. 1999; Kim et al. 2006); those authors used a lower concentration of menadione and H_2O_2 to decrease viability. However, the results concur with those of Dellomonaco

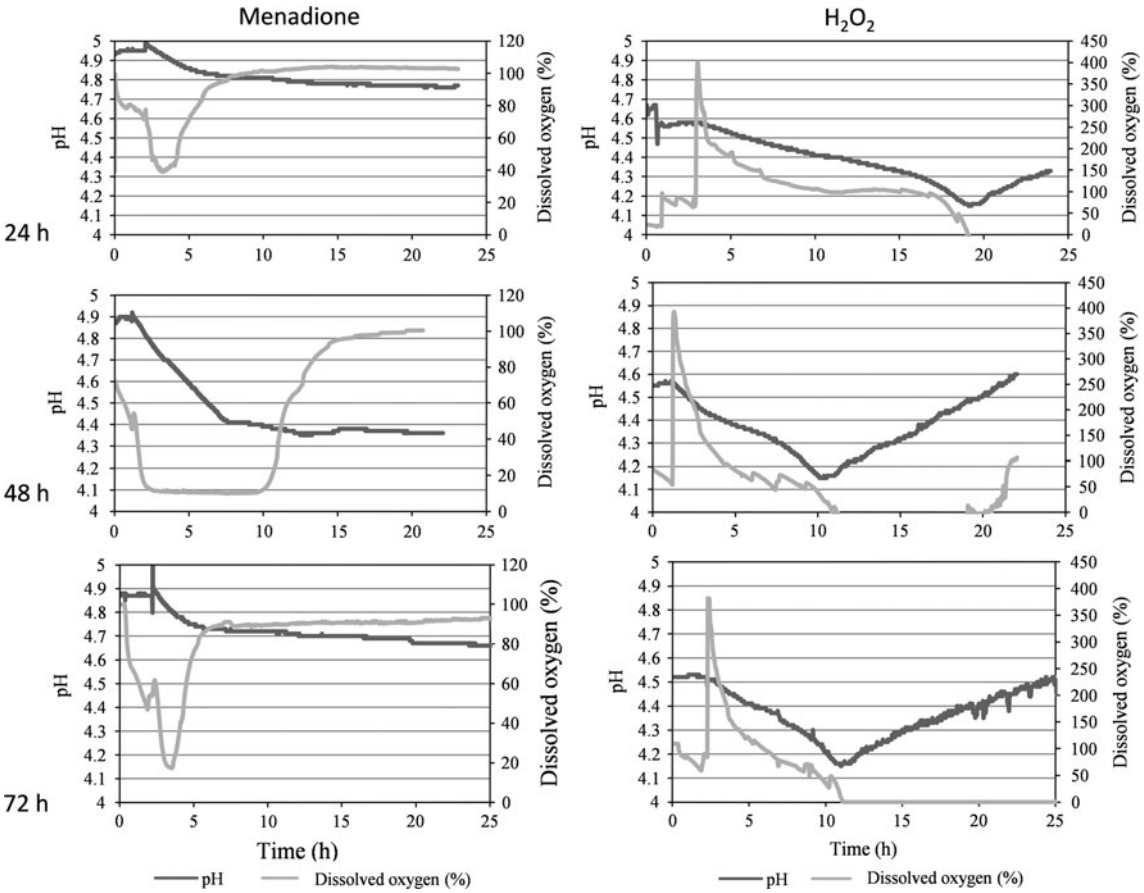

Fig. 5 Respiratory capacity for *K. marxianus* SLP1 yeast during oxidative stress with H_2O_2 (50 mM) and menadione (100 mM). The yeasts were collected at different stages of growth

et al. (2007) and Pinheiro et al. (2002), who utilized 50 mM of hydrogen peroxide to produce a small amount of oxidative stress in *K. marxianus*, and viability was <70 %. However, cell viability only shows the cells that survive after treatment (in those studies, 3 h after the addition of menadione or H_2O_2). To determine whether the yeast has the ability to grow in the presence of the oxidant in the medium, we developed an experiment in which oxidants were added and growth was determined. The results showed that the yeast strain was able to grow in nearly all of the menadione treatments, but it could only grow at 10 mM in the H_2O_2 treatments. The *K. marxianus* strain was more resistant than the *S. cerevisiae* strains, and the weakest was the W303-1A reference strain, signifying that the yeast isolated from the mezcal possesses an antioxidant system that can be activated to decrease cell damage and to grow. The growth phase played a different role in the yeasts (Romano et al. 2006). For example, the OFF1 and W303-1A strains were not significant in this regard; but the growth phase was significant for the SLP1 and MC4 strains. Menadione and H_2O_2 have been used to produce oxidative stress, and it is known that they induce different cell responses (Flattery-O'Brien et al. 1993). At 72 h the yeast shown a different behavior, because has the ability to grow after an oxidative stress in the presence of 50 and 100 mM H_2O_2, thus it is possible that the yeast induced the antioxidant system by decreasing the effect of the oxidant. As is mentioned by Zakrajsek et al. (2011) during the stationary phase the yeast show high stability in the oxidation levels and consequently in the cell energy metabolic activity. Moreover, the yeast capacity to obtain resistance is the capacity of maintains the oxidation in levels to let the yeast live for more time. Finally, a study by Joon et al. (1995) in *Schizosaccharomyces pombe*, found a correlation between the increase in resistance to oxidative stress at later growth phase with an increase in the antioxidant enzymes activities. The addition of non-lethal concentrations of H_2O_2 or thermal shock prior to lethal levels of oxidants such as menadione or H_2O_2 supplies the yeast with resistance (Stephen and Jamieson 1997). In its natural environment during the mezcal process, the yeast frequently undergoes high temperatures and has elevated sugar concentrations and antimicrobial compounds from the agave juice (such as furfural and saponins, etc.)

(Palmqvist et al. 1999; De León-Rodríguez et al. 2006). It is probable that the stress produced during fermentation provides the yeasts with resistance to the higher concentrations of menadione and H_2O_2 (Moradas-Ferreira et al. 1996), such as those obtained in this study.

The results obtained show a different behavior of the yeast strain when the oxidative stress is produced in short period (Figs. 1, 2), compared to a long period (Fig. 3). The survival yeasts after 3 h of oxidative stress has the capacity to grow, but when the oxidant is maintained in the media the yeasts could not grow. The capacity of growth during the oxidative stress was different in each yeast strain, because the ability to activate cell protection mechanisms to adapt to environmental changes and to repair the cellular components damaged. Kim et al. (2006) and others has reported that trehalose is produced in high concentrations during the oxidative stress and this component could be guarding cell membranes by lipid peroxidation (Herdeiro et al. 2006). Simultaneously, the antioxidant enzymes and all the defense systems are activated to maintain not only the viability and inclusive to grow during the oxidative stress.

The respiration patterns in *K. marxianus* demonstrated that menadione produces a slow oxidative stress because the yeast retained the dissolved oxygen consumed during the first 4 h after the addition of the oxidants; the dissolved oxygen even increased slowly until 100 % recovery. During this time, the yeast grew and its population increased, but it finally stopped consuming oxygen and the pH remained constant, probably indicating that it was unable to survive after that. In contrast, the H_2O_2 produced an effect only during the first 12 h; afterward, the yeast was able to consume oxygen and to produce the same change in pH as when oxidants were not added to the media, indicating that the yeast could grow. It has been demonstrated that H_2O_2 decreased the ATP levels, but that menadione did not (Osorio et al. 2003). Hydrogen peroxide affects the oxygen consumed; the ATP levels then decreased because the electron transport chain had no oxygen to produce ATP. Moreover, the addition of non-lethal levels of H_2O_2 prior to an oxidative stress event increased the yeast's resistance, but this did not occur with menadione (Flattery-O'Brien et al. 1993). This treatment could not be effective because the exposure time to menadione was 1 h, and in our case, the test time was increased to 4 h in order for the yeast to gain resistance.

These results indicate that to apply oxidative stress, it is necessary to know the amount of time required for the desired effect, and not only that which is necessary for viability or cell growth; these must be the parameters for evaluating the oxidative stress produced. The behavior of the oxygen consumed and the pH could be used to describe the status of oxidative stress in the yeast. And finally, we demonstrated that the yeast isolated from spontaneous mezcal fermentation was able to resist oxidative stress for a long period of time.

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Conflict of interest The authors declare that they have no conflict of interest.

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