

Comparison of *Yarrowia lipolytica* and *Pichia pastoris* Cellular Response to Different Agents of Oxidative Stress

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Abstract Yeast cells exposed to adverse conditions employ a number of defense mechanisms in order to respond effectively to the stress effects of reactive oxygen species. In this work, the cellular response of *Yarrowia lipolytica* and *Pichia pastoris* to the exposure to the ROS-inducing agents' paraquat, hydrogen peroxide, and increased air pressure was analyzed. Yeast cells at exponential phase were exposed for 3 h to 1 mM paraquat, to 50 mM H₂O₂, or to increased air pressure of 3 or 5 bar. For both strains, the cellular viability loss and lipid peroxidation was lower for the cells exposed to increased air pressure than for those exposed to chemical oxidants. The glutathione induction occurred only in *Y. lipolytica* strain and reached the highest level as a response to PQ exposure. In general, antioxidant enzymes were more expressed in *Y. lipolytica* than in *P. pastoris*. The enzyme superoxide dismutase was induced in both strains under all the oxidant conditions but was dependent on the cellular growth phase, being undetectable in non-growing cells, whereas glutathione reductase was more induced in those conditions. Hydrogen peroxide was the most efficient inducer of catalase. Both yeast cultures underwent no cellular growth inhibition with increased air pressure, indicating that these yeast species were able to adapt to the oxidative stressful environment.

Keywords Oxidative stress · Antioxidant enzymes · Increased air pressure · *Yarrowia lipolytica* · *Pichia pastoris*

Introduction

All aerobic organisms use molecular oxygen for respiration and oxidation of nutrients to obtain energy efficiently. During the reduction of molecular oxygen to water through

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acceptance of four electrons, aerobic microorganisms have to face the toxic effects of oxygen, once active oxygen species such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals are generated. These reactive oxygen species (ROS) are highly noxious to all biological molecules, including DNA, proteins, and lipids [1].

The rise of total air pressure leads to an increase of oxygen partial pressure, which generates ROS [2]. Pro-oxidant compounds in the culture medium, such as paraquat (PQ) and hydrogen peroxide also have the capacity to generate intracellular ROS.

Paraquat (1,1'-dimethyl-4,4'-bipyridylum dichloride) is a quaternary nitrogen herbicide and in the presence of a sufficient supply of reducing equivalents, repeated cycles of herbicide reduction and reoxidation can occur, producing large amounts of reactive oxygen species, oxidative stress, and lipid peroxidation [3].

The addition of exogenous hydrogen peroxide to microbial cultures may result in oxidative stress. The adaptation to this radical requires protein synthesis and the expression of at least 21 proteins increased following H_2O_2 adaptation [4].

The inadequate mixing and mass transfer that cells might face in an industrial submerged culture process may expose yeast cells to variations in dissolved oxygen, including transient exposure to regions of high dissolved oxygen, leading to oxidative stress. Despite the industrial significance, few studies were performed to simulate the impact of oxidative stress incurred by exposure to increased oxygen partial pressure and elevated dissolved oxygen on *Saccharomyces cerevisiae* [5], *Yarrowia lipolytica* [6], and *Kluyveromyces marxianus* [7] cultures.

Cells possess several defensive enzymatic (such as superoxide dismutase, catalase, and glutathione reductase) and nonenzymatic (such as glutathione) mechanisms to protect their cellular constituents and maintain cellular redox state.

Y. lipolytica, a non-conventional yeast, is a strictly aerobic microorganism with the ability to grow in a variety of hydrophobic compounds including alkanes, oils, and fatty acids, as well as in olive mill wastewater. It is most used as an adequate model for dimorphism studies in yeasts and is capable of producing important industrial metabolites, such as lipase, citric acid, biosurfactants, aroma, and microbial lipids [8]. The methylotrophic *Pichia pastoris* can grow to high-cell density and has the potential for high-level expression of recombinant proteins, such as β -glucosidase [9], β -galactosidase [10], and frutalin [11], among others. *P. pastoris* expression system offers the advantages of having a strong methanol-induced promoter (alcohol oxidase 1 promoter) and the capability of performing many eukaryotic posttranslational modifications, such as glycosylation, disulfide bond formation, and proteolytic processing [12].

To learn more about the response of these yeast strains to oxidative stress, the effect of different ROS-generating agents on cell viability and on the induction of antioxidant enzymes were studied. The content of malondialdehyde (MDA) and reduced glutathione (GSH) and the induction of antioxidant enzymes (superoxide dismutase (SOD), catalase, glutathione reductase (GR)) in response to H_2O_2 , PQ and increased air pressure were investigated. To our knowledge this is the first study concerning strains of *P. pastoris* and *Y. lipolytica* and their interaction with the pro-oxidant agents. Moreover, this is the first time that these yeast strains were exposed to oxidant agents in a non-respiratory medium (phosphate buffered saline (PBS) buffer) and a more detailed analyses of cellular antioxidant indicators was performed.

Material and Methods

Strains and Media

Y. lipolytica W29 (ATCC 20460) and *P. pastoris* CBS 2612 were grown in yeast peptone dextrose (YPD) (10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose) medium. The yeast strains were maintained in YPD agar plates and stored at 4 °C to a maximum of 1 month.

Oxidants Treatment

Yeasts cells were precultured during 9 h (exponential phase) in 250-mL Erlenmeyer flasks filled with 100 mL of YPD medium. Cells were then harvested by centrifugation, washed with PBS buffer, and resuspended in 400 mL of PBS buffer or YPD medium. In the experiments with chemical oxidants, PQ and H₂O₂ were added at nonlethal final concentration of 1 and 50 mM, respectively. The exposure to increased air pressure was performed in a 600-mL pressurized bioreactor (stainless steel stirred tank, PARR 4563, Parr Instruments, USA) under 3 or 5 bar of air pressure at 400 rpm and 1 vvm (volume of air/volume of liquid/minute) of aeration.

Batch Growth Under Increased Air Pressure

Yeasts cells were pre-grown overnight in 250-mL Erlenmeyer flasks with 100 mL of YPD at 140 rpm and at 27 °C (*Y. lipolytica*) or 30 °C (*P. pastoris*). Batch cultivations were carried out in the pressurized bioreactor, with 400 mL of each culture, at 27 or 30 °C and 400 rpm. Compressed air was continuously sparged into the culture at an aeration rate of 1 vvm. The values of air absolute pressure studied were 1, 3, and 5 bar. Details of the pressurized bioreactor are described elsewhere [6].

Analytical Methods

Yeasts samples were collected after 3 h of exposure to oxidants for analysis of cell viability, MDA, and GSH content and antioxidant enzymes activity. Cell viability was estimated by the methylene blue staining method [13]. Antioxidant enzymes were measured after cell disruption and dialysis of cell extracts, as described elsewhere [14]. Catalase was assayed using the method described by Beers and Sizer [15], SOD was quantified by the method of Marklund and Marklund [16] and glutathione reductase was analyzed according to the procedure described by Smith et al. [17]. MDA was measured by the method of thiobarbituric acid reactive species as described by Espindola et al. [18]. GSH was quantified in the neutralized extracts using DTNB reagent according to the procedure described by Jamnik and Raspor [19].

Samples from the batch cultures in the pressurized bioreactor were collected for analysis of cell concentration (optical density at 600 nm and converted to dry cell weight per liter) and glucose consumption. Glucose was quantified by HPLC with a Metacarb 67H column (Varian, Palo Alto, CA) and a RI detector. The eluent was H₂SO₄ 0.005 mol/L at 0.5 mL/min and the column temperature was 60 °C. Results are presented as the average ± standard deviation of three independent experiments.

Results and Discussion

Cell Viability

The effect of three different oxidative stress inductors, PQ, H₂O₂, and hyperbaric air, on yeast cell viability was tested. The experiments were conducted with cells at the exponential phase since these cells are metabolically more active than stationary phase cells and were likely to be more susceptible to the possible lethality of aerobic stressors [20]. For exposure assays the yeast cells were resuspended in two different culture media, in order to analyze the antioxidant response of respiring and non-respiring cells.

The presence of a carbon source allowed the cells to grow during the oxidant exposure, and led to more active and viable cells. In the experiments with YPD medium, there was no significant difference on the cellular viability between strains independent of the stressors (Table 1). However, the exposure to oxidants agents in PBS buffer demonstrated that the *P. pastoris* cells were more susceptible than the *Y. lipolytica*. Hassan and Fridovich [20] also observed that the *Escherichia coli* cells were strikingly less sensitive to PQ in complex medium than in minimal medium.

When cells of *Y. lipolytica* were exposed to H₂O₂ and PQ in PBS buffer, respectively, 87 and 90 % of the cells survived after 3 h of exposure, which shows that the strain is quite resistant to these oxidant agents. The exposure to hyperbaric air leads to a minor decrease in cell viability, indicating that the oxidative stress imposed by the partial oxygen pressure is less deleterious than the other stressors. The *P. pastoris* cells were more susceptible to the oxidative stress than the other strain, mainly in PBS buffer. When *Pichia* cells were subjected to the PQ and H₂O₂ treatment about 36 and 42 % were killed, respectively. In turn, there was 78 and 77 % of viable cells in treatments under 3 and 5 bar of air, respectively. Pinheiro et al. [21] reported that *K. marxianus* cells respond better to the PQ than to the H₂O₂ exposure during 24 h of growth. Also, cell viability was higher for the cells growing under increase air pressure up to 6 bar than for those cells exposed to chemical oxidants. Data suggest that different organisms respond differently to ROS and that they use different defense mechanisms against those substances, namely the nonprotein thiol GSH and a variety of antioxidant enzymes.

Antioxidant Markers

Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as MDA, a natural byproduct of lipid peroxidation. Regardless of the yeast strain, the exposure to oxidant agents leads to MDA production. This production was more pronounced with the PQ treatment (Fig. 1). It has been proposed that the cyclic reduction–oxidation of PQ initiates lipid peroxidation through

Table 1 Cell viability (%), defined as the ratio of final and initial viability, of two yeasts strains in exponential phase of growth, resuspended in PBS buffer and YPD medium, exposed to different oxidants for 3 h. Values are average ± standard deviation of three independent experiments

	PBS buffer				YPD medium			
	H ₂ O ₂	PQ	3 bar	5 bar	H ₂ O ₂	PQ	3 bar	5 bar
<i>Y. lipolytica</i> W29	87±11	90±10	95±12	95±13	92±15	94±13	97±14	96±15
<i>P. pastoris</i> CBS 2612	58±8	64±10	78±12	77±12	90±14	93±15	97±14	96±14

subsequent superoxide radical and singlet oxygen intermediates. The chemical cascades leading to the reduction of PQ and the generation of free radicals and lipid peroxidation are the main factors that lead to mitochondrial damage [22].

The MDA content in cells resuspended in PBS buffer and exposed to PQ was three- and tenfold higher than in the experiments under 5 bar for *Y. lipolytica* and *P. pastoris* cells, respectively. The exposure of cells in YPD medium to H₂O₂ led to a six and twofold enhancement in MDA production compared to 5 bar of air pressure for *Y. lipolytica* and *P. pastoris* cells, respectively. Interestingly, it seems that lipid peroxidation with hydrogen peroxide is minimized, being *P. pastoris* less sensitive to the stress caused by H₂O₂. Thus, it is reasonable to conclude that the stress caused by an increase of partial oxygen pressure did not lead to membrane destructive processes.

It seems that there was no direct relation between the medium composition and the induction of lipid peroxidation. Although the *Y. lipolytica* strain exposed to PQ showed a 2.4-fold increase in MDA content when the cells were in PBS buffer compared to YPD medium, the addition of the H₂O₂ led to a threefold enhancement of MDA in PBS buffer.

The involvement of oxidative mechanisms to mediate the damaging effects of oxidant agents tested has been first shown by the analysis of GSH. According to our results, only the *Y. lipolytica* strain has shown the capacity to induce GSH production in the experimental conditions (Fig. 2). It is likely that *P. pastoris* possesses other defense mechanisms more important than GSH in antioxidant response to stressors, such as antioxidant enzymes SOD or catalase. Also, this low GSH production in *Pichia* might be responsible for the lower-cell viability in PBS medium observed for this yeast as compared to *Y. lipolytica*.

The exposure of *Y. lipolytica* cells to PQ led to a 30-fold enhancement on GSH induction comparatively to the experiments conducted with hydrogen peroxide. Also, GSH was more induced with air pressure than with H₂O₂. The cells exposed to hydrogen peroxide showed a minor GSH induction. The GSH content in experiments with H₂O₂ was 30- and 9-fold smaller than the value obtained with PQ and 5 bar of air pressure. Among the oxidative stress agents, PQ is a thiol-oxidizing agent resulting in fast oxidation of GSH to oxidized glutathione (GSSG). The comparison of GSH in cells treated with PQ and increased air pressure indicates that the thiol oxidation should be small up to 5 bar of air pressure.

Glutathione is an essential reductant during normal metabolic processes in yeasts. Grant et al. [23] demonstrated that a *S. cerevisiae* strain which lacks a functional copy of the *GSH1* gene

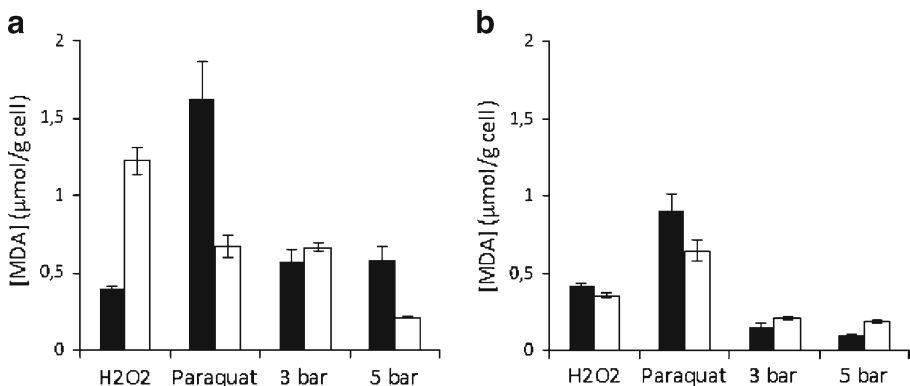


Fig. 1 MDA content of **a** *Y. lipolytica* W29 and **b** *P. pastoris* CBS 2612 cells in exponential phase of growth, resuspended in PBS buffer (black bars) and YPD medium (white bars) and exposed to different oxidants. Values are average \pm standard deviation of three independent experiments

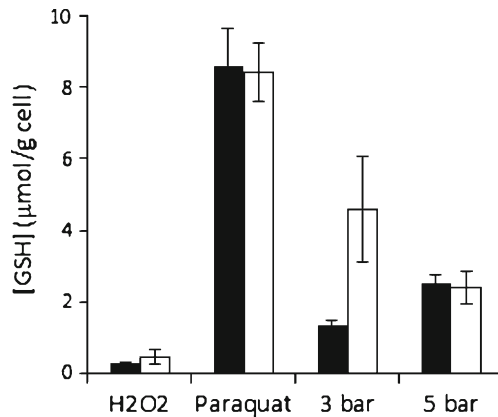


Fig. 2 GSH content of *Y. lipolytica* W29 cells in exponential phase of growth resuspended in PBS buffer (black bars) and YPD medium (white bars) and exposed to different oxidants. Values are average \pm standard deviation of three independent experiments

is hypersensitive to the peroxides and the oxidative stress conditions induced by the H₂O₂. Izawa et al. [24] demonstrated that intracellular GSH played an important role in the stress response to H₂O₂ in *S. cerevisiae* using glutathione-depleting agents and a glutathione-deficient mutant. This could explain the major decrease of yeast cells viability exposed to H₂O₂ comparatively to the others stressors agents, as reported above.

One of the principal antioxidant enzymes is superoxide dismutase that is involved in the dismutation of superoxide anions to dioxygen and hydrogen peroxide. In the experiments with yeast cells resuspended in PBS buffer, both strains were unable to induce the SOD enzyme. This result may suggest that only in the presence of a respiratory carbon source, the yeast strains used on this work have the capacity to induce this enzyme. The general non-induction of SOD observed after oxidant agents treatment in PBS buffer could also be the result of oxidative damage of this enzyme, because it is known to be repressed by various peroxides. This probably reflects the decreased ability of the cells to adapt efficiently to the oxidative stress. Whereas SOD is considered to be an essential antioxidant enzyme, at the same time it can have pro-oxidant effects in vivo [25], and thus, SOD inactivation can also be the result of a cellular defense mechanism. Hassan and Fridovich [20] found that *E. coli* cells responded to PQ by increasing their content of SOD in the presence of yeast extract, but not in its absence, leading to the assumption that yeast extract might have exerted its effect by eliciting the SOD biosynthesis.

Although there were differences in the other defensive mechanisms between the two yeasts tested (e.g., GSH and catalase), SOD induction was quite similar for both strains, with each oxidant treatment (Fig. 3). This may be due to the fact that this enzyme is one of the primarily induced antioxidant mechanisms involved in stress defense.

The experiments under air pressure up to 5 bar produced a clear increase in SOD activity compared to the exposure to H₂O₂ (69 % high for *Y. lipolytica* and 65 % high for *P. pastoris*) and PQ (57 % high for *Y. lipolytica* and 65 % high for *P. pastoris*). This high SOD activity in the experiments under 3 and 5 bar of air pressure may explain the higher cell viability of the both strains. Previous work demonstrated that the increase of total air pressure up to 6 bar leads to an enhancement of intracellular SOD of *Y. lipolytica* W29 [6]. The importance of yeasts superoxide dismutase enzyme on triggering ROS generation by oxygen was also proved by Lushchak et al. [26], who observed that *S. cerevisiae* strains carrying mutations in *SOD 1* and *SOD 2* genes were hypersensitive to oxygen, causing slow aerobic growth.

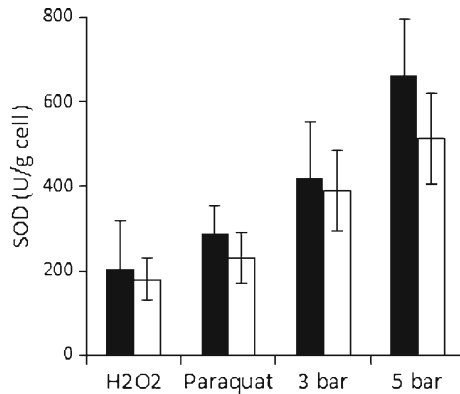


Fig. 3 Superoxide dismutase specific activity of *Y. lipolytica* W29 (black bars) and *P. pastoris* CBS 2612 (white bars) cells in exponential phase of growth resuspended in YPD medium and exposed to different oxidants. Values are average \pm standard deviation of three independent experiments

The low SOD activity in yeast cells incubated with 50 mM H₂O₂ suggests that this enzyme does not participate in the cellular acclimatization to drastic oxidative H₂O₂-induced challenges. Abbeg et al. [27] observed only a slight induction of SOD with the addition of H₂O₂ to the various *Candida* strains medium and Biryukova et al. [28] found a slight induction of SOD with the pretreatment of *Y. lipolytica* cells with 0.3 mM H₂O₂.

The activation of catalase observed following each oxidant stressors treatment, is one of the most common cellular responses to redox alterations, because this enzyme is easily induced by a wide range of stimuli often related to the energy status of the cell [29].

In general, the oxidant treatments with cells resuspended in PBS buffer showed a higher catalase activity compared to those with YPD medium (Fig. 4). A 1.7-, 2.9-, 5-, and 3.8-fold enhancement in enzyme activity was obtained when *Y. lipolytica* cells resuspended in PBS buffer were exposed to H₂O₂, PQ, 3 and 5 bar of air pressure, respectively, compared to the activities with YPD medium. The glucose concentration can exert a negative pressure on the catalase activity of *Y. lipolytica* W29. In fact, Braconi et al. [29] reported the same effect in the early phase of *S. cerevisiae* growing in high glucose concentration medium. The

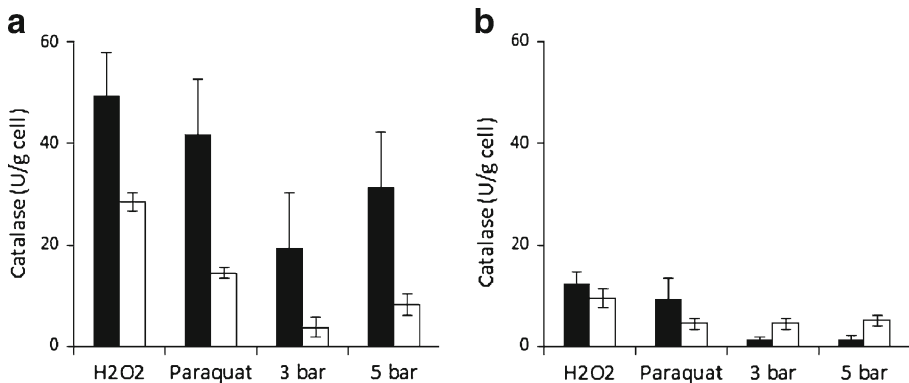


Fig. 4 Catalase specific activity of **a** *Y. lipolytica* W29 and **b** *P. pastoris* CBS 2612 cells in exponential phase of growth, resuspended in PBS buffer (black bars) and YPD medium (white bars) and exposed to different oxidants. Values are average \pm standard deviation of three independent experiments

differences found in the experiments with *P. pastoris* were not so significant. On the other hand, as both yeast cells resuspended in PBS buffer were not capable to induce SOD, it can be concluded that both SOD and catalase operate in concert to protect cells from oxidative stress. Such relationship can be connected with the compensatory response of the yeast cells to maintain the steady state level of ROS inside the cells [30]. Semchyshyn and Lozinska [31] observed that hydrogen peroxide increased catalase activity in SOD-deficient mutant.

When comparing the two yeasts, it was observed that *Yarrowia* showed a higher capacity to induce catalase after exposure to oxidant agents. A 4-, 16-, and 23-fold enhancement was obtained in *Y. lipolytica* with H₂O₂ and PQ, 3 and 5 bar of air pressure, respectively, compared to the activities obtained with *P. pastoris*.

Particular attention should be paid to H₂O₂, the stress treatment that leads to a higher catalase synthesis in both yeast strains, regardless of the medium where yeast cells were resuspended. The exposure of *Y. lipolytica* cells to H₂O₂, resuspended in YPD medium, led to a 1.9-, 7.5-, and 3.5-fold increase in catalase activity as compared to PQ, 3 and 5 bar of air pressure treatments. The enzyme activity of *P. pastoris* cells resuspended in PBS medium and treated with H₂O₂ was 1.3, 9.9, and 8.9 times higher than that obtained in exposures to PQ, 3 and 5 bar of air pressure, respectively. Other authors have demonstrated that the treatment with H₂O₂ induced increased levels of catalase activity in *P. pastoris* [17], *Candida albicans* [32], various *Candida* strains [27], *Aspergillus niger* [33], *Y. lipolytica* [28], and *S. cerevisiae* [34].

The enzyme glutathione reductase is involved in the glutathione recycling system. This enzyme enables the cell to sustain adequate levels of cellular GSH, once it is primarily responsible for the reduction of GSSG to reduced glutathione at the expense of nicotinamide adenine dinucleotide phosphate (NADPH).

Both yeast strains were able to induce glutathione reductase (Fig. 5). However, the GR was considerably smaller than the SOD and catalase activities, suggesting that glutathione reductase plays a minor role on antioxidant defense against the agents tested. On the other hand, as the process of redox-cycling depletes intracellular NADPH (cofactor for glutathione reductase), the enzyme activity was likely to be affected by oxidative stress. It seems that there is a relation between the medium composition and the glutathione reductase induction once the cells resuspended in PBS buffer showed higher activity, whatever the oxidant agent tested and yeast strain may be.

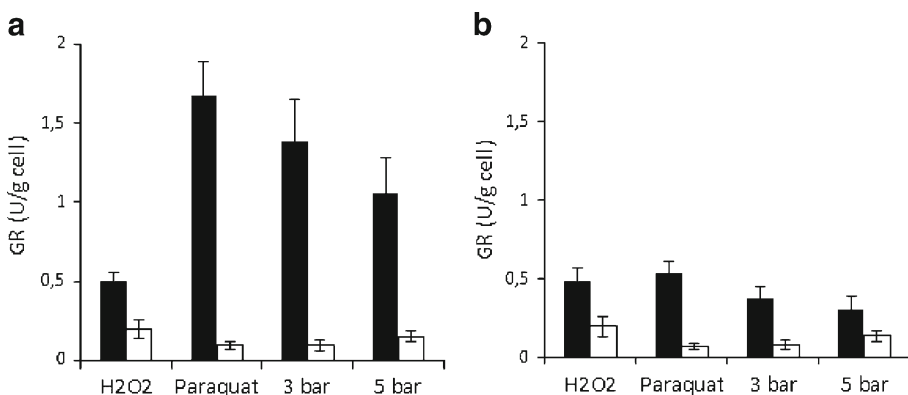


Fig. 5 Glutathione reductase specific activity of **a** *Y. lipolytica* W29 and **b** *P. pastoris* CBS 2612 cells in exponential phase of growth, resuspended in PBS buffer (black bars) and YPD medium (white bars) and exposed to different oxidants. Values are average \pm standard deviation of three independent experiments

In all the experiments with *P. pastoris* cells there was no significant difference in GR enzyme activity with any of the pro-oxidant agents. However, with the *Y. lipolytica*, cells treated with H_2O_2 showed less glutathione reductase activity. A 3.3-fold enhancement in enzyme activity was observed in the treatment with PQ compared to H_2O_2 exposure. It was not surprising that the higher activity of this enzyme was found for the agents that also lead to a more pronounced induction of GSH (PQ and increased air pressure) once this enzyme participates in the reduction of GSSG to GSH in the presence of NADPH.

Growth Under Increased Air Pressure

Since *Y. lipolytica* and *P. pastoris* showed to be able to adapt to pressures of 3 and 5 bar and it was showed that antioxidant defense mechanisms are induced under these conditions, batch cultures of the yeast strains under increased air pressure were performed to validate the resistance of the yeasts under this stress condition. Typical batch growth and glucose consumption profiles are shown in Fig. 6. Both strains were able to grow for 24 h under air pressure values 5-fold higher than the atmospheric pressure.

Regardless of the yeast strain, the rise of total air pressure from 1 to 3 and 5 bar led to an increase in the final cell dry weight. 3.3-fold and 1.9-fold improvement in biomass production was obtained with the increase of air pressure up to 5 bar compared to 1 bar, for *Y. lipolytica* and *P. pastoris*, respectively. Among the yeast strains studied, the highest biomass yield was obtained with 5 bar of air pressure. An improvement in biomass yield of *Y. lipolytica* and *P. pastoris* cultures from 0.3 to 1.1 mass of cells per mass of glucose and from 0.3 to 0.6 mass of cells per mass of glucose, respectively, was achieved with increase air pressure up to 5 bar. The values of specific growth rate obtained at 1 and 5 bar were 0.18 and 0.28 h^{-1} , respectively. The rise of air pressure from 1 to 5 bar led to a 1.6-fold improvement in specific growth rate. However, no significant differences were obtained on growth rates of *P. pastoris* when the pressure varies from 1 to 5 bar (0.27–0.29 h^{-1}). Nevertheless, no inhibitory effects were observed in the cellular activity under high air pressures of 3 and 5 bar. These results confirmed the ability of the yeast to cope with oxidative stress conditions that can arise from the air pressure increase, since air pressure at 5 bar is less harmful than other oxidant agents and cells were able to induce their antioxidant defenses.

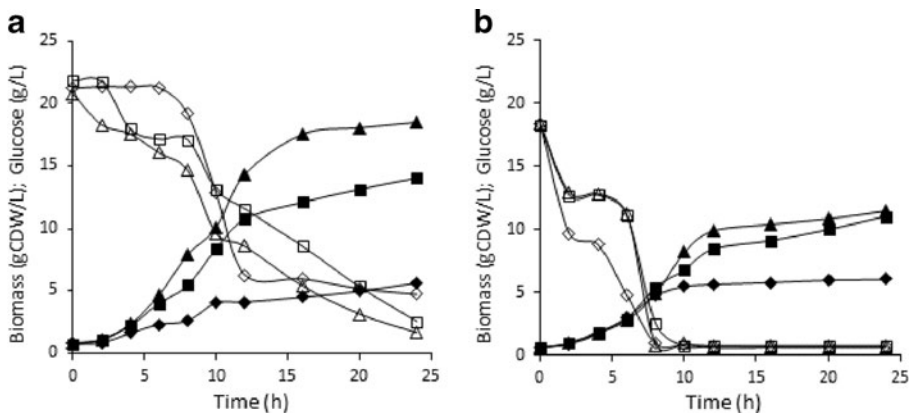


Fig. 6 Batch growth (*close symbols*) and glucose consumption (*open symbols*) of *Y. lipolytica* (**a**) and *P. pastoris* (**b**) under different air pressures: 1 bar (\blacklozenge), 3 bar (\blacksquare), and 5 bar (\blacktriangle)

Conclusions

Under normal physiological conditions, the toxic effects of ROS are minimized by enzymatic and nonenzymatic antioxidants. However, under stressful conditions, oxidant levels may increase to overwhelm the antioxidants, resulting in cell damage.

Our results suggest that *Y. lipolytica* have a more potent antioxidant system than *P. pastoris*, which was proved by the higher cell viability and enzymatic mechanisms induction. Moreover, the results herein reported demonstrated that both strains were more susceptible to oxidant agents resuspended in PBS buffer than in YPD medium.

Cell responses against both superoxide and peroxide stresses include enhanced expression of SOD and catalase, which are key enzymes for direct ROS scavenging. Under superoxide stress (PQ and air pressure), the SOD induction was the main observed mechanism. In contrast, and as expected, the effect of H₂O₂ treatment on antioxidant enzyme synthesis was much more pronounced for catalase than for SOD.

The results reported herein proved that an air pressure raise up to 5 bar can be successfully applied to the batch cultivation of both *Y. lipolytica* and *P. pastoris* strains with increased biomass yields. Moreover, the induction of antioxidant defenses against the superoxide anion, demonstrated that yeast strains can cope with possible oxidative stress caused by increased air pressure. These two combined results, offer an opportunity to perform industrial bioprocesses based in these yeast strains, under increased air pressure.

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