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Title: Improved robustness of an ethanologenic yeast strain through adaptive evolution in acetic acid is associated with its enzymatic antioxidant ability.

Short running head: Evolutionary engineering of yeast in acetic acid

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Abstract:

Aims: To investigate multiple tolerance of *Saccharomyces cerevisiae* obtained through a laboratory strategy of adaptive evolution in acetic acid, its relation with enzymatic ROS detoxification and bioethanol 2G production.

Methods and Results: After adaptive evolution in acetic acid, a clone (Y8A) was selected for its tolerance to high acetic acid concentrations (13 g l^{-1}) in batch cultures. Y8A was resistant to multiple stresses: osmotic, thermic, oxidative, saline, ethanol, organic acid, phenolic compounds and slow freeze-thawing cycles. Also, Y8A was able to maintain redox homeostasis under oxidative stress, whereas the isogenic parental strain (Y8) could not, indicating higher basal activity levels of antioxidative enzyme Catalase (CAT) and Gluthatione-S-Transferase (GST) in Y8A. Y8A reached higher bioethanol levels in a fermentation medium containing up to 8 g l^{-1} of acetic acid when compared to parental strain Y8.

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Conclusions: A multiple-stress-tolerant clone was obtained using adaptive evolution in acetic acid. Stress cross-tolerance could be explained by its enzymatic antioxidative capacity, namely CAT and GST.

Significance and Impact of the Study: We demonstrate that adaptive evolution used in *S. cerevisiae* was a useful strategy to obtain a yeast clone tolerant to multiple stresses. At the same time, our findings support the idea that tolerance to oxidative stress is the common basis for stress co-tolerance, which is related to an increase in the specific enzymes CAT and GST but not in Superoxide dismutase (SOD), emphasizing the fact that detoxification of H_2O_2 and not $O_2^{\bullet}$ is a key condition for multiple stress tolerance in *S. cerevisiae*.

Keywords*: Robustness, adaptive evolution, acetic acid, multiple tolerance, bioethanol 2G production,* yeast*, Saccharomyces cerevisiae, antioxidative enzymes.*

Introduction

Lignocellulosic biomass-based bioethanol production is constantly increasing, and *S. cerevisiae* is frequently used industrially for that purpose (Bathia *et al.* 2012). As a consequence of standard acid pre-treatment of the substrate, several toxic and growth-inhibiting compounds are produced. Consequently, the ability to tolerate these as well as a wide variety of stress conditions and maintain an adequate metabolic performance is essential for industrial applications of this yeast in order to reduce operational costs and enhance ethanol yields. A common inhibitor produced during pre-treatment is acetic acid (Trček *et al*. 2015).

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It is possible to increase the yeast performance in the presence of inhibitors or stressors by employing different methods such as genetic engineering (Ukibe *et al*. 2009; Jun and Jiavi 2012; Swidah *et al*. 2015; Guan *et al*. 2015; Chen *et al*. 2016), but the methodology of adaptive evolution/evolutionary engineering seems to offer multiple advantages as an efficient way to select yeast populations resistant to different stress conditions, and to expand their tolerance range (Çakar *et al*. 2005; Wallace-Salinas and Gorwa-Grauslund 2013; Jiang *et al*. 2016; Baek *et al*. 2016; Gonzalez-Ramos *et al*. 2016). The methodology is suitable for obtaining microorganisms with desired phenotypes not present in their genetic background, or with a complex base that is multi-gene-encoded (Perrone *et al*. 2005).

Reactive oxygen species (ROS) are generated during a fermentative aerobic process from molecular oxygen due to the different environmental stress conditions and can produce cell damage, with superoxide anion radical ($O_2•^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) being the most important ones that cause damage to proteins (like carbonylation), nucleic acids, lipids, and other cellular components, eventually leading to yeast cell death (Mendes-Ferreira *et al*. 2010).

The yeast *S. cerevisiae* has a network of defense mechanisms to protect against oxidative stress. The primary defense includes proteins that remove ROS or act by sequestering metal ions (Moradas-Ferreira *et al.* 1996). Thereby, primary defense mechanisms combine antioxidant enzymes such as catalase (CAT), glutathione S-transferase (GST), and superoxide dismutase (SOD) and ROS scavengers such as glutathione and thioredoxin (Moradas-Ferreira and Costa 2000).

Considering the evidence that the occurrence of an apoptotic phenotype in *S. cerevisiae* is inducible by oxidative stress, the same phenotype could be induced by acetic acid at high

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concentrations (Ludovico *et al.* 2001). This phenotype might be avoided by subjecting yeast cells to mineral acids that activate ROS detoxifying enzymes, like catalase, since the ability to resist multiple stresses is linked to yeast cells' capacity for H_2O_2 detoxification (Lewis *et al.* 1997; Giannattasio *et al.* 2005).

Thus, in view of the above-mentioned evidence, as well as the principle of stress co-tolerance in this yeast species (Attfield 1997), the objectives of this study were, firstly, to improve the fitness of an industrial osmotolerant *S. cerevisiae* strain selected for its tolerance to high acetic acid concentration through an adaptive evolution strategy to be used in bioethanol 2G production. Secondly, to investigate whether it is possible, using this method, to obtain a yeast cell population that is co-tolerant to different industrial stress conditions; and finally, to gain a first insight into the relation of oxidative stress resistance mechanisms to other stressors.

MATERIALS AND METHODS

Strain

The industrial osmotolerant *S. cerevisiae* strain Y8 (BAFC 3084) was kindly provided by CALSA (Compañía Argentina de Levaduras S.A.) and used throughout this study for the **adaptive evolution** experiments. The strain was maintained on Petri dishes containing YPD agar medium at 4°C. The selected clone obtained experimentally was stored in glycerol 25% v v^{-1} at -80°C.

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Media and culture conditions

Unless otherwise indicated, all experiments were carried out in submerged cultures in 100 ml Erlenmeyer flasks at a 1:5 medium-to-flask volume ratio containing YPD (1% yeast extract, 2% meat peptone, 2% glucose, adjusted to pH 5.5), and maintained under continuous agitation (250 rpm, in an orbital shaker), at 28°C. All tests were performed in triplicate, and repeated at least twice/once.

Adaptive evolution experiments in acetic acid

For inoculum preparation, the Y8 strain was grown until late exponential phase was reached. This culture was used to inoculate flasks with YPD to an initial OD_{600nm} of 0.5. When the culture reached midlog phase, an aliquot was transferred to another flask with YPD fresh medium. Each batch was started with low initial biomass concentration (OD_{600nm}≈0.1) to select cells with better adaptability or detoxification capacity in acetic acid. The initial acetic acid concentration of the serial batch cultures was 3 g l^{-1}, and this concentration was subsequently increased up to 13 g l^{-1}. Growth was followed spectrophotometrically (OD_{600nm}) with a UV-Visible Spectrophotometer T60 PG Instrument. In the last batch culture grown, samples were withdrawn and stored at −80°C in 25% (v v^{-1}) glycerol solution.

Selection of acetic acid tolerant clones.

Glycerol stock populations obtained after the adaptation procedure were thawed and streaked on YPD agar plates containing 5 g l^{-1} acetic acid. The first colony developed (named Y8A) was selected for further characterization.

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Aerobic growth in acetic acid

Y8 and Y8A were cultured in YPD medium supplemented with 6, 8 or 10 g l^{-1} of acetic acid. Adequate samples were taken at different times (2, 4 and 6 h) for viability determination (CFU ml^{-1} after incubation for 48 h).

Resistance to stress conditions

Y8 and Y8A culture dilutions were made to obtain a final cell concentration with OD_{600nm} of 1.0. Then, cells were washed three times with distilled water, centrifuged at 5,000 x ***g*** for 10 min. and resuspended in 0.1 mol l^{-1} potassium phosphate buffer (pH 6.0) before being subjected to the following stress conditions: ethanol (10% v v^{-1}, 1 h), aliphatic acid (5 g l^{-1} acetic acid, 6 h, or 20 mmol l^{-1} formic acid, 30 min), temperature (42°C, 2 h), osmotic shock (3 mol l^{-1} sorbitol, 3 h), slow freeze-thawing - two cycles for 24 h (Kronberg *et al*. 2007), saline treatment (1.5 mol l^{-1} NaCl, 4 h), phenolic acid (15 mmol l^{-1} gallic acid, 4 h) and oxidative stress (5 mmol l^{-1} H_2O_2, 1 h). After treatments, stressors were removed by washing with 0.1 mol l^{-1} potassium phosphate buffer (pH 6.0) and/or by ending the experiment in a water bath at 25°C. Afterwards, serial dilutions were carried out (10^0-10^{-4}). Cell viability was determined in quadruplicate after each treatment by plating 100 µl of each dilution on YPD agar at 28°C for 48 h. The colonies formed were counted and viability was reported as the survival percentage (Adams and Moss 2002). All experiments, unless otherwise indicated, were carried out at 28°C.

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Oxidative stress parameters

Enzymatic assay

The parental strain Y8 and the adapted clone Y8A were used to determine ROS (Reactive Oxygen Species), as well as Catalase (CAT), Glutation-S-Transferase (GST) and Superoxide dismutase (SOD) enzyme activities. Subsequently, 5 ml aliquots of Y8 and Y8A cultures with an OD_{600nm} of 10 were centrifuged at 5,000 x ***g*** for 10 min, the supernatants were discarded and the cells resuspended in a plastic tube containing 5 ml of YP medium (1% yeast extract and 2% meat peptone). Thus, the stress conditions evaluated were: Control (without H_2O_2); low concentration of H_2O_2 (5 mmol l^{-1}) and high concentration of H_2O_2 (100 mmol l^{-1}). All these conditions were maintained for 2 h at 28°C and performed in triplicate.

Reactive oxygen species assay

Intracellular ROS production was measured using the oxidant-sensitive probe $2^{\prime},7^{\prime}$-dichlorofluorescein diacetate (H_2DCFDA). Fluorescence was measured using a spectrofluorophotometer at an excitation wavelength of 485 nm and emission wavelength of 520 nm using a FLUOstar OPTIMA fluorescence plate reader (BMG Labtech) (Zhang *et al*. 2003). A calibration curve was made with different concentrations of H_2O_2. The ROS were calculated as peroxide equivalents and the results expressed as mmol per mg $protein^{-1}$.

Antioxidant enzyme activities

Cells were harvested by centrifugation at 5,000 x ***g*** for 20 min, washed with 0.134 mol l^{-1} potassium phosphate buffer (pH 6.5) and resuspended in 0.5 ml of the same buffer

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containing protease inhibitors (0.2 mmol l^{-1} benzamidine and 0.5 mmol l^{-1} phenyl methyl sulfonyl fluoride). The cells were then disrupted by sonication using a Cole Parmer CP600 4710 Ultrasonic homogeniser. The homogenates were centrifuged at 10,000 x ***g*** for 30 min, and the supernatant was used as the enzyme sample. All procedures were performed at 4°C.

Catalase (CAT) activity was determined by following the dismutation of hydrogen peroxide spectrophotometrically at 240 nm, in a reaction mixture containing 50 mmol l^{-1} potassium phosphate buffer (pH 7.4) and 10 mmol l^{-1} hydrogen peroxide (Aebi, 1984). Results were expressed as units of CAT per mg proteins. One CAT unit was defined as the amount of enzyme required to catalyze the dismutation of 1 mmol of H_2O_2 per min.

Glutathione-S-Transferase (GST) activity was measured according to Habig *et al*. (1974), by detecting the conjugation product of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB). The reaction mixture contained enzyme sample, 100 mmol l^{-1} phosphate buffer (pH 6.5) and 10 mmol l^{-1} CDNB. The absorbance of GS-DNB complex was monitored at 340 nm (extinction coefficient GST-CDNB: 9.6 mmol l^{-1} cm^{-1}). Results were expressed as units of GST per mg proteins. One GST unit was defined as the amount of enzyme required to catalyze the formation of 1 μmol of GS-DNB per min.

Superoxide dismutase (SOD) activity was determined according to Beauchamp and Fridovich (1971). This method is based on the inhibition of the photochemical reduction of nitro blue tretrazolium (NBT). The reaction mixture contained enzyme sample, 13 mmol l^{-1} methionine, 0.1 mmol l^{-1} EDTA, 75 μmol l^{-1} NBT and 2 μmol l^{-1} riboflavin in 50 mmol l^{-1} potassium phosphate buffer (pH 7.8). The samples were exposed to intense cool-white light for 15 min and then the absorbance was measured at 560 nm. Results were expressed as units of SOD per mg proteins. One-unit of SOD was defined as the amount of enzyme

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necessary to inhibit the NBT reduction rate by 50%. Results were expressed as units of SOD per mg proteins.

Peroxide determination

H_2O_2 concentrations of the solutions used were determined in adequate aliquots with a UV-Visible Spectrophotometer T60 PG Instrument at 240 nm using the peroxide molar extinction coefficient (43.6 mol l^{-1} cm^{-1}).

Protein content

Total soluble protein content was determined in the supernatant according to Bradford (1976), using bovine serum albumin as a standard (Sigma Aldrich, St. Louis, Missouri, USA).

Ethanologenic fermentation in presence of acetic acid

All fermentations were carried out in microaerobic conditions (Nikel *et al*, 2006), in 100 ml Erlenmeyer flask using YPD in the absence or presence of acetic acid (6, 8 and 10 g l^{-1}). The initial cell concentration was ajusted to OD_{600nm} 0.5, and the cells were kept in suspension with a magnetic stirrer (50 rpm). Ethanol was determined in supernatant aliquots of the cultures using an enzymatic kit (R-Biopharm AG, Darmstadt, Germany, Cat. No. 10 176 290 035).

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Bioethanol 2G production

To evaluate the ethanologenic performance of the adapted strain in the presence of inhibitors commonly found in acid hydrolyzates of lignocellulosic biomass, we used a medium produced in our laboratory. Briefly, residual seed cake of jojoba (*Simmondsia chinensis,* Gayol *et al*. 2007) - alpha cellulose 40%, hemicellulose 23% and lignin 18%, was ground to less than 1 mm particles, hydrolyzed in diluted acid (1:10 w v^{-1} of 0.5 N H_2SO_4, 3 h and 100°C), pH was brought to 5.5 with NaOH, and then filtered. The hydrolyzate was supplemented with glucose, up to a final concentration of 120 g l^{-1}, named as $JH_{12\%}$. Fermentation performance was compared to that of the parental strain, and controls were carried out in YNBD (6.7 g l^{-1} YNB "BD™ Difco™ Yeast Nitrogen Base" and 120 g l^{-1} glucose), named as $YNBD_{12\%}$. Ethanol and glucose were determined in adequated supernatant aliquots after centrifugation of the fermentation samples at 7.500 **x *g*** for 10 min at 24, 48 and 72 h using two enzymatic kits (R-Biopharm AG, Darmstadt, Germany, Cat. No. 10 176 290 035 for ethanol and Wiener lab, Rosario, Argentina, Cat. No. 1400101 for glucose). Unless indicated, all the drugs used were of analytical grade or of the best commercial grade available.

Statistical analysis

Results from different treatments were compared statistically by two-way analysis of variance (ANOVA) followed by a Tukey *post hoc* test. The suppositions of normality and homogeneity of variances were tested with Lillieford and Bartlett tests, respectively (Sokal and Rohlf, 1999). All data were analyzed statistically using GraphPad Prism 5 software. The suppositions test was carried out with Statistica v.8 software.

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Results

Strain adaptation in acetic acid

The adaptation and selection procedures in the successive batches with acetic acid were performed over a period of 33 days at 28°C. In the last batch with a concentration of 13 g l^{-1} no growth was observed. Before the nineteenth day, the batch incubation periods were short (one or two days), from then on, the periods were longer in duration (3, 4 or 5 days). The Y8 strain was able to grow up to a concentration of 12 g l^{-1} of acetic acid. This growth protocol allowed the adaptation of resistant populations through metabolism adaptation or spontaneous mutations.

Evaluation of clone tolerance to acetic acid

After growing in YPD medium containing acetic acid, a pronounced decrease in CFU ml^{-1} was observed for the parental strain in 8 and 10 g l^{-1} of acetic acid, in contrast to the adapted clone, as indicated by viability and percentage of survival (Fig. 1 and Table 1).

In all conditions tested, the survival rate was greater in the adapted clone than in the parental strain. This result indicated that the tolerance to acetic acid in the adapted clone Y8A was certainly improved during the evolutionary engineering method in this acid.

Aerobic growth in different acetic acid concentration levels.

The batch cultures performed in the presence of 6, 8 and 10 g l^{-1} of acetic acid were carried out to study the behavior of the parental (Y8) and the adapted strain (Y8A) over a 96 h growth period in aerobic conditions. As shown in Fig. 2, there were no differences between clones in control conditions (0 g l^{-1} acetic acid), 6 and 10 g l^{-1} of acetic acid. The latter

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acetic acid concentration was found to be inhibitory to both strains in this period of time. On the other hand, clone Y8A reached significantly higher biomass levels after 48 h in YPD medium containing 8 g l^{-1} of acid acetic, a longer lag phase was observed during the first 48 h in both cell types. Thereafter, clone Y8A was able to grow up to OD_{600nm}≈10 at 96 h while the parental strain could not grow.

Multiple stress tolerance

In order to analyze the sturdiness of the adapted clone strain in relation to the parental strain, tolerance to a set of stress conditions that might be encountered during bioethanol industrial production was evaluated. The first set of stresses studied was the following: heat, ethanol, oxidative stress, osmotic shock and freezing-thawing. The second set of stresses studied consisted of inhibitors that might be present in the lignocellulosic hydrolyzate used for fermentation: aliphatic acids (acetic and formic), phenolic compounds (gallic acid) and saline solution (NaCl) (Fig. 3). The clone Y8A showed a significantly higher tolerance to all stress conditions tested.

Enzymatic assays in oxidative conditions

To evaluate the response to oxidative stress, we studied in cell free extract the activity of the enzymes Catalase (CAT), Glutathione S Transferase (GST) and Superoxide dismutase (SOD) and its relationship to intracellular ROS levels. In addition, these assays were used as another way to compare the capacity of yeast to tolerate various stress conditions through antioxidant response.

Results indicated that ROS levels of the parental strain showed significant differences between control and H_2O_2 treated cultures. While the adapted strain showed no significant

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differences between the control, and cells treated with 5 mmol l^{-1} and 100 mmol l^{-1} H_2O_2, in contrast, significant differences in ROS content were found between the parental and the adapted strain (Fig. 4).

CAT activity was significantly different in the two strains, with the activity of the adapted strain being greater than that of the parental strain under the 3 conditions tested (Fig. 5). On the other hand, treatments with H_2O_2 did not affect CAT activity in either of the two strains. SOD activity did not show significant differences between strains and between treatments (Fig. 6).

GST activity of the parental strain showed no significant differences under the different treatment conditions, while the adapted strain showed a decrease in GST activity that was statistically significant between the control and 100 mmol l^{-1} H_2O_2. The comparison of the GST activity between Y8 and Y8A showed a significantly higher value in the adapted Y8A compared to the parental Y8 under control conditions and with 5 mmol l^{-1} H_2O_2. In the treatment with 100 mmol l^{-1} H_2O_2, the adapted Y8A showed activity similar to the parental strain (Fig. 7).

Oxidative and non-oxidative stress biomarkers

To analyze the cellular features elicited by other stresses than oxidative ones in the strain used, and in order to assess the hypothesis that stress co-tolerance is based on oxidative stress tolerance, we investigated whether a non-oxidative stress like saline stress (See Materials and Methods) could produce an oxidative response such as those we found for hydrogen peroxide stress. The results obtained in this work showed that after saline stress, oxidative biomarkers levels were higher in Y8A respect to Y8 (ROS levels were 0.007 and 0.005 mmol mg protein^{-1} for Y8A and Y8 respectively, and CAT levels were 0.65 and 0.4

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Units mg protein^{-1} for Y8A and Y8 respectively). These results are in agreement with those found for oxidative stress

Growth and fermentation performance in the presence of acetic acid

The adapted clone Y8A and the parental strain Y8 were compared for ethanol production in YPD fermentation mediums with different acetic acid concentrations containing 20 g l^{-1} glucose at 28°C. As shown in Fig. 8, in control conditions, the ethanol concentration of both strains increased rapidly during the first 24 h of fermentation until it reached 9 g l^{-1} and then it remained stable after 48 hours. Notably, the yeast Y8A was most efficient in producing ethanol in the medium containing 6 g l^{-1} of acetic acid, reaching a maximum ethanol production of 4.15 g l^{-1} at 48 h, which represents a yield of 0.21 g g^{-1}, corresponding to 41.2% of the theoretical yield. Y8 produced about 2.71 g l^{-1} of ethanol, with a yield of 0.135 g g^{-1}, which corresponded to 26.5% of the theoretical yield. The acetic acid selected strain showed significantly higher ethanol yield than the parental strain. Also, in comparison with the parental Y8, the clone Y8A presented significant differences in ethanol production when we evaluated it at 8 g l^{-1} acetic acid after 48 h. In this case, Y8A was able to produce 2.02 g l^{-1} (0.10 g g^{-1} – 19.6% theoretical yield) versus 1.6 g l^{-1} (0.08 g g^{-1} – 15.7% theoretical yield) for Y8.

Bioethanol 2G production

To test the ability of Y8A to produce 2G bioethanol on a commercial scale, we formulated a fermentation medium consisting of lignocellulosic raw material in the form of acid-

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hydrolyzed Jojoba cake (See Materials and Methods) supplemented with glucose up to 120 g l^{-1} in order to reach a bioethanol concentration of about 60 g l^{-1}.

As shown in Figure 9a, the stress tolerant strain was able to ferment an agroindustrial residue like Jojoba cake ($JH_{12\%}$), reaching an ethanol volumetric production of 56.3 g l^{-1} after 72 hours. The bioethanol level reached by the non-adapted strain was 1.3-fold lower in these conditions. On the other hand, bioethanol production in $YNBD_{12\%}$ was higher for the non-adapted strain compared to the adapted one. Furthemore, glucose uptake accompanied ethanol production by each strain using the different substrates (Figure 9b).

As a whole, the results indicated that the adapted strain attained higher ethanol levels than the non-adapted one because it is tolerant to the toxic compounds present in the lignocellulosic raw material. Also, the non-adapted clone showed a prolonged lag phase, which again could be explained by the presence of inhibitors of yeast growth and metabolism present in the lignocellulosic material.

Discussion

In this work, by using the evolutionary adaptation strategy of metabolic engineering (Çakar *et al*. 2012) a clone of *S. cerevisiae* tolerant to acetic acid was obtained. When compared to the isogenic osmotolerant parental strain, the adapted strain was able to resist a higher acetic acid concentration (12 g l^{-1}) and also to withstand a wider range of stresses affecting yeast performance during the bioethanol 2G production process. These results imply that a common damage tolerance and repair mechanism must exist in order to tolerate these stresses. The improved performance of the tolerant strain could be attributed to physiological or genotype variations provoked during adaptation in acetic acid either by

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mutations or metabolic adaptations. Our results are consistent with the principle of stress co-tolerance found in *S. cerevisiae* (Attfield 1997, Lewis *et al*. 1997).

Gonzalez-Ramos *et al*. (2013) used a similar strategy to the one we applied in this work but based on an alternating cultivation cycle in the presence and absence of acetic acid and they obtained a constitutively resistant *S. cerevisiae* variant tolerant to acetic acid as the one we obtained. Likewise, they found that mutations in four genes were identified as causative for acetic acid tolerance.

Acetic acid triggers hydrogen peroxide production, resulting in apoptotic cell death of yeast. This means that a high expression of catalase is necessary to resist acetic acid stress by eliminating hydrogen peroxide (Giannattasio *et al*. 2005). Nevertheless, in the acetic acid-adapted clone, we found higher levels of ROS compared to the non-adapted strain, in spite of the fact that the adapted strain presented higher levels of CAT. Also, acetic acid causes an oxidative imbalance, with the consequent increase in antioxidant defenses (increase in CAT, among others) (Giannasttasio *et al*. 2015). Although it does not allow the redox state to return to the initial values (evidenced by the higher level of ROS than was present), it makes it possible to withstand the highest levels of ROS (Martani *et al.* 2013), found in the adapted strains.

In the engineered clone, CAT and GST activity was higher in both control and oxidative conditions. Indeed, CAT seems to play a relevant role as an antioxidant defense in this yeast. The increase observed in the Y8A strain, when exposed to the highest H_2O_2 concentration applied, could be indicative of an antioxidant response. High CAT activity probably provides a yeast with higher tolerance to the other stress conditions used in this work (Lewis *et al*. 1997).

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While the adapted strain had higher levels of GST activity, treatment with the highest hydrogen peroxide concentration used diminished the activity of the enzyme. This could be due to either a direct effect on the structure, or on the active site of the enzyme, as well as to the inhibition of certain synthesis steps, caused by the oxidative imbalance induced by H_2O_2 (Bray *et al.* 1974).

Assuming a common basis for the stress co-tolerance of *S. cerevisiae*, these types of antioxidant enzyme activity would be able to neutralize or decrease the effects of the stresses on the yeast cell.

SOD enzyme activity of the parental and adapted strains was found to be at comparable levels under the all the conditions tested. This study is further experimental evidence for the hypothesis first postulated by Giannattasio *et al*. 2005 that in *S. cerevisiae,* H_2O_2 detoxification rather than superoxide detoxification, plays a major role in preventing yeast cell damage or death in response to acetic acid and probably to other stresses

The link found between saline and oxidative biomarker production could apply to other stresses such as those used in this work. This must be verified with other non-oxidative stresses, but the results presented here support the hypothesis that tolerance to oxidative stress is a good indicator of multiple stress tolerance in this yeast species.

Taken as a whole, our results suggest that the ability of *S. cerevisiae* to co-tolerate stresses, indicative of its general robustness, could be linked to its capacity for H_2O_2 detoxification, and also point to the fact that the maintenance of intracellular redox balance is pivotal for *S. cerevisiae* to resist multiple stressors.

The adapted strain not only produced bioethanol in a synthetic medium ($YNBD_{12\%}$) but also in a lignocellulosic based medium ($JH_{12\%}$). The results found are indicative that the adapted strain was specifically tolerant to inhibitors present in the acid hydrolysate and

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could be used to produce bioethanol in this non-detoxified agroindustrial residue. Moreover, if the cellulosic raw material contains appropriate levels of fermentable sugars, bioethanol production could reach adequate commercial levels.

Finally, we obtained a robust clone with better ethanol fermentation performance in the presence of acetic acid when compared with the isogenic parental strain.

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CONFLICTS OF INTEREST

The author(s) declare that they have no competing interests.

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Table 1 Survival rate percentages in different acetic acid concentration and times of exposure.

Strain	6 g l^{-1} acetic acid			8 g l^{-1} acetic acid			10 g l^{-1} acetic acid		
	2h	**4h**	**6h**	**2h**	**4h**	**6h**	**2h**	**4h**	**6h**
Y8	83.6±8.9	67.05±11.3	19.1±1.3	61.6±9.3	13.7±3.9	1.03±0.03	21.2±6.8	0.4±0.04	0.09±0.009
Y8A	113.1±15.7	101.5±13.6	75.3±13.3	108.6±12.8	66.6±7.5	16.3±0.1	96.6±5.8	54.9±5.5	13.1±1.9

Experiments have been performed in duplicate. The results represent the mean values ± SD of two independent experiments.

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1 **Figure legends:**

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3 **Figure 1.** Growth of yeast cells under different acetic acid concentration (0, 6, 8 and 10 g l^{-1}) and time of exposure (2, 4 and 6 h). After treatments in acetic acid, parental strain Y8 and the adapted clone Y8A were serially diluted, and 10 µl of each dilution 10^0–10^{-4} (from left to right) was spotted onto YPD plates at 28°C for 48 h.

7 **Figure 2.** Cell growth of Y8 and Y8A in YPD medium with increasing concentrations of acetic acid: (a) Control or without acetic acid. (b) 6 g l^{-1}. (c) 8 g l^{-1}. (d) 10 g l^{-1}. Open triangles and open circles indicate Y8 and Y8A respectively. The results represent the mean values ± SD of three independent experiments.

11 **Figure 3.** Survival percentages of the Y8 and Y8A after being subjected to different stress conditions: Heat, ethanol, osmotic, freezing-thawing, oxidative, acetic acid, formic acid, saline and gallic acid. See Materials and Methods section for details. Empty bars and black filled bars represent Y8 and Y8A respectively. The results indicate the mean values ± SD of three independent experiments.

16 **Figure 4.** Effect of H_2O_2 on ROS levels of the Y8 and Y8A strains. Empty bars and black filled bars represent Y8 and Y8A respectively. The results represent the mean values ± SD of three independent experiments. Significant differences ($p < 0.05$) are indicated by different letters.

20 **Figure 5.** Effect of H_2O_2 on Catalase (CAT) activity of the Y8 and Y8A strains. Empty bars and black filled bars represent Y8 and Y8A respectively. The results represent the mean values ± SD of three independent experiments. Significant differences ($p < 0.05$) are indicated by different letters.

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24 **Figure 6.** Effect of H_2O_2 on Superoxide dismutase (SOD) activity of the Y8 and Y8A
25 strains. Empty bars and black filled bars represent Y8 and Y8A respectively. The results
26 represent the mean values ± SD of three independent experiments.

27 **Figure 7.** Effect of H_2O_2 on Glutathione-S-Transferase (GST) activity of the Y8 and Y8A
28 strains. Empty bars and black filled bars represent Y8 and Y8A respectively. The results
29 represent the mean values ± SD of three independent experiments. Significant differences
30 ($p < 0.05$) are indicated by different letters.

31 **Figure 8.** Bioethanol production profile for Y8 and Y8A in different acetic acid
32 concentrations. Empty bars and black bars represent Y8 and Y8A respectively (after 24 h).
33 Vertical lines bars and horizontal lines bar represent Y8 and Y8A respectively (after 48 h).
34 The values are the average ± SD of at least three independent experiments.

35 **Figure 9.** Bioethanol (a) and glucose (b) concentration for Y8 and Y8A. Close circles and
36 triangles represent Y8 in $YNBD_{12\%}$ and $JH_{12\%}$ respectively. Open triangles and circles
37 indicate Y8A in $YNBD_{12\%}$ and $JH_{12\%}$ respectively. The results represent the mean values ±
38 SD of two independent experiments.

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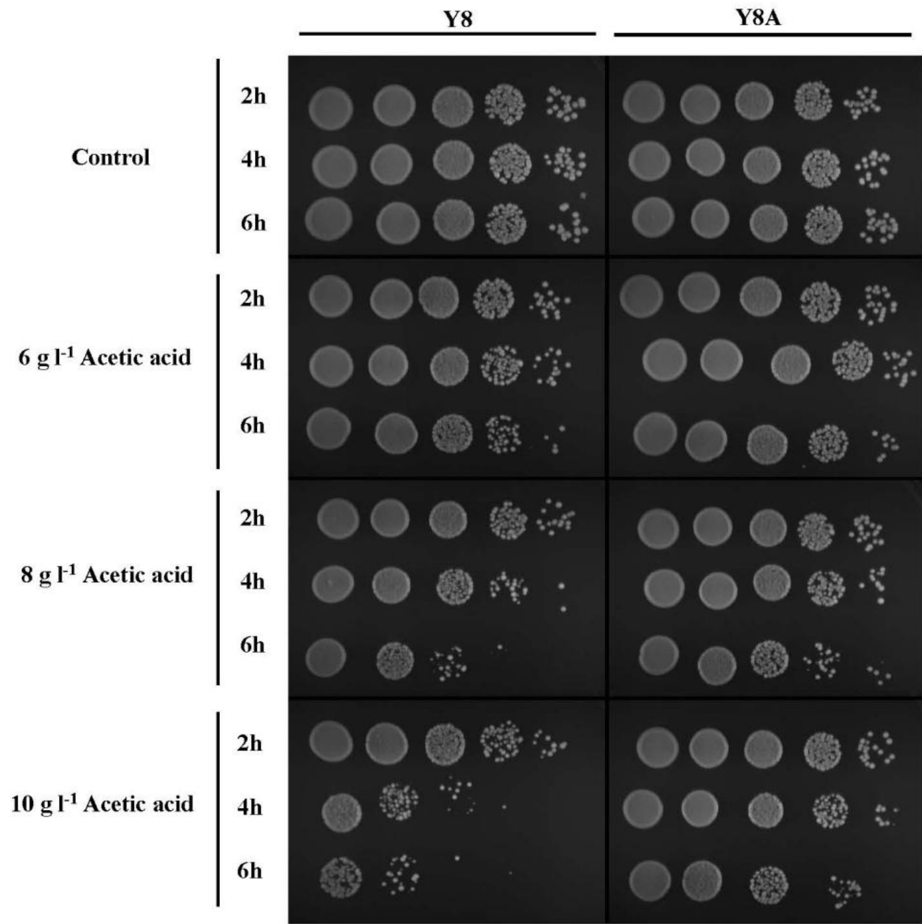

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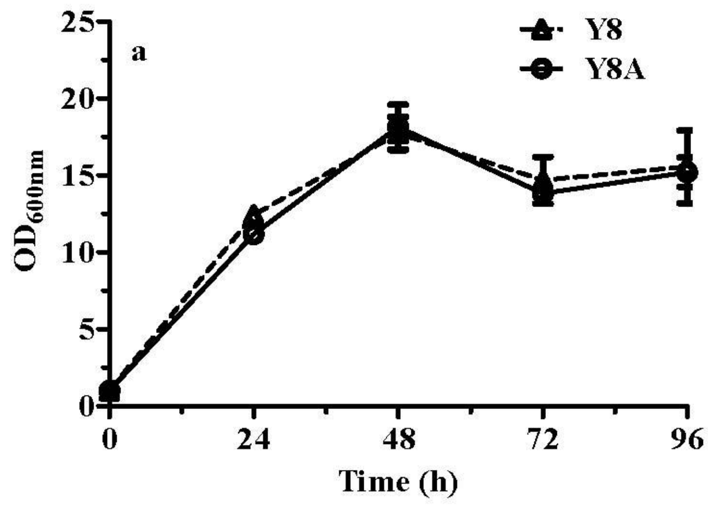

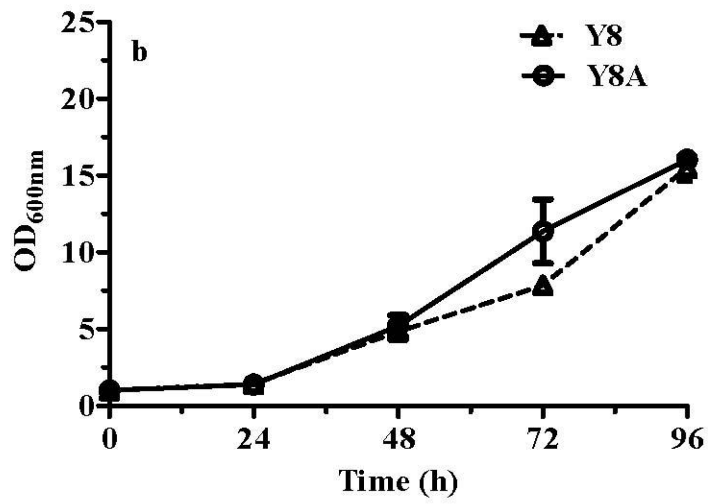

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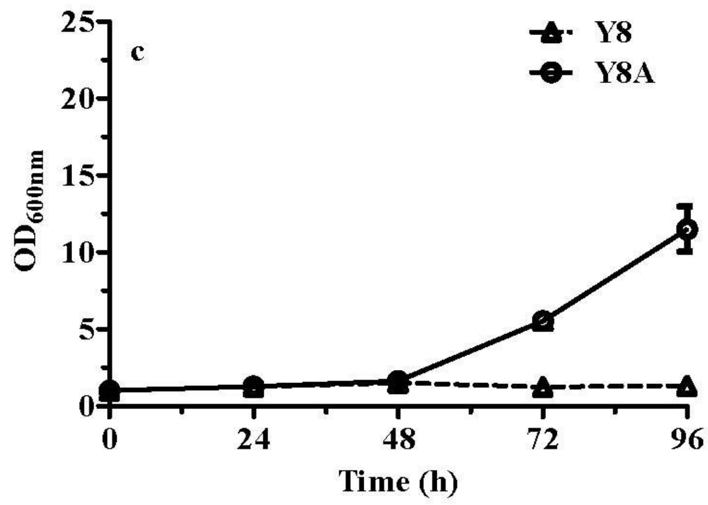

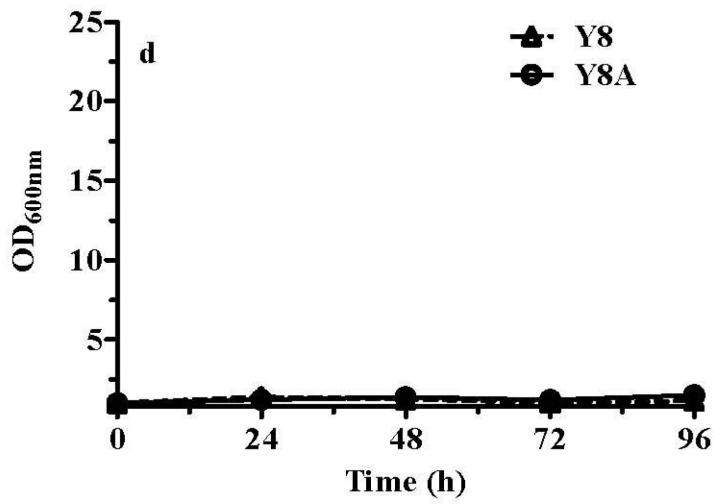

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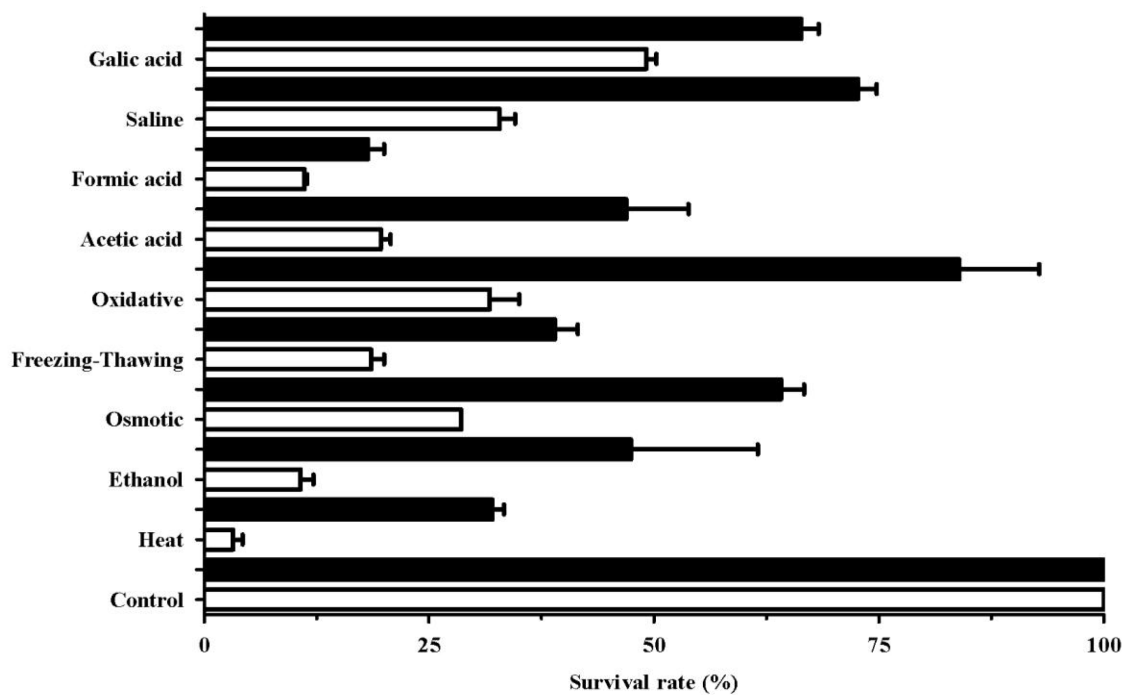

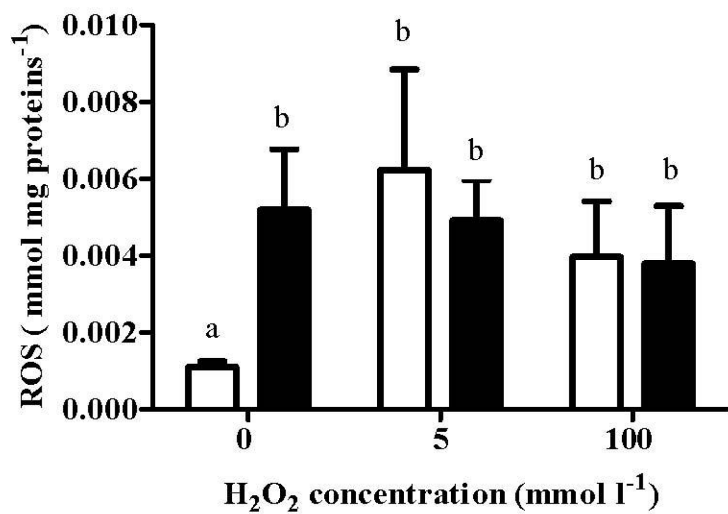

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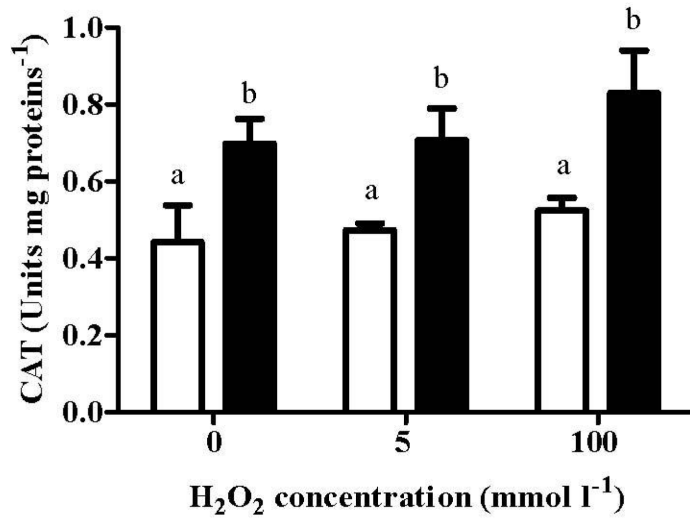

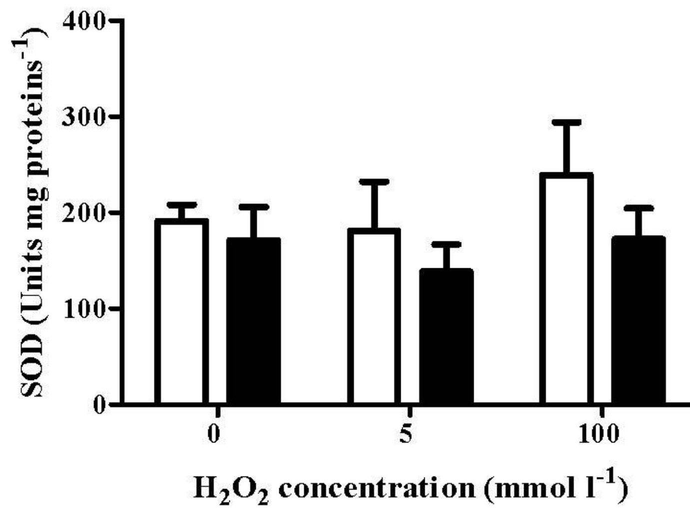

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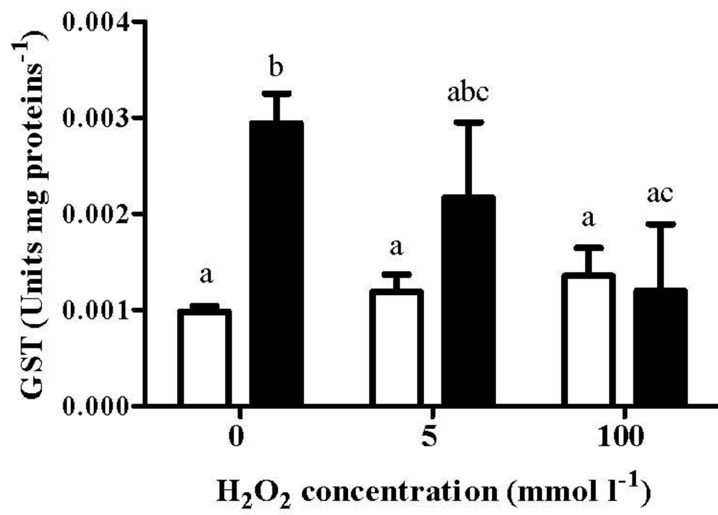

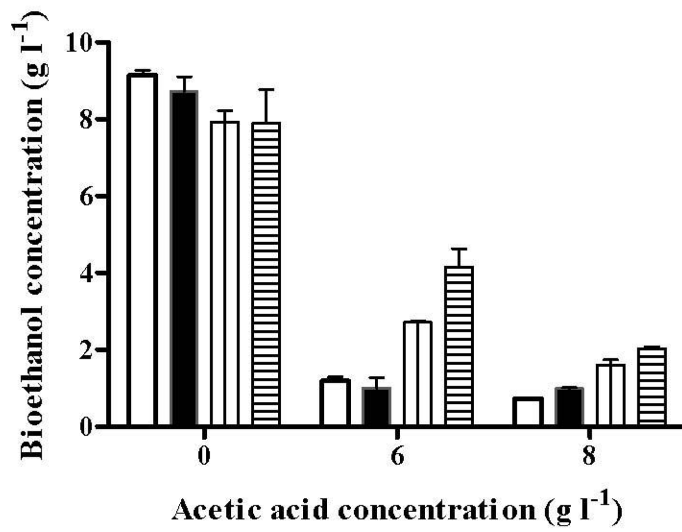

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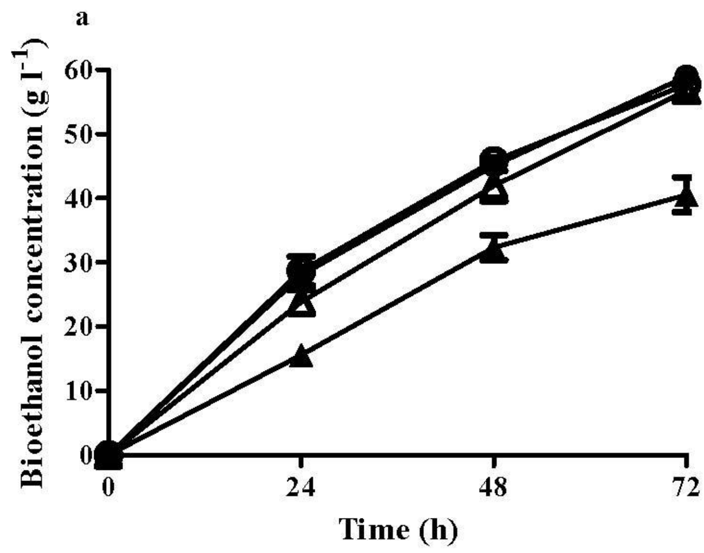

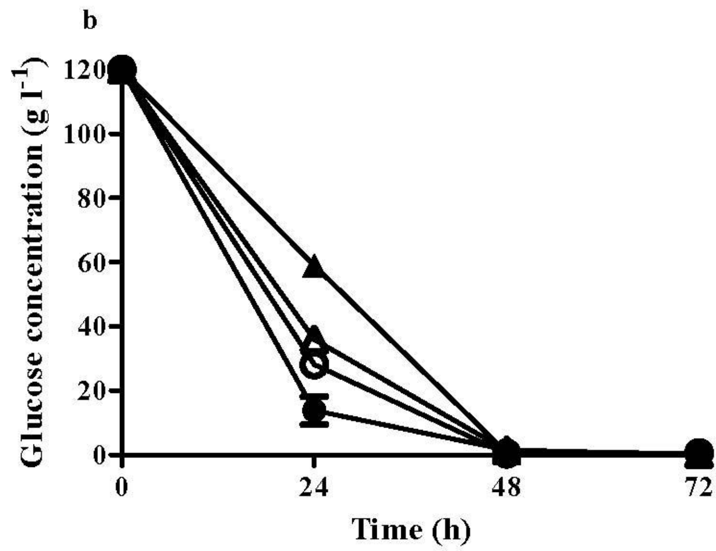

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