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5-Hydroxymethylfurfural induces *ADH7* and *ARI1* expression in tolerant industrial *Saccharomyces cerevisiae* strain P6H9 during bioethanol production

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HIGHLIGHTS

- Evolutionary engineering was used to select industrial *S. cerevisiae* tolerant to HMF.
- Expression levels of genes involved with HMF tolerance were studied.
- *ARI1* and *ADH7* genes were overexpressed in the presence of HMF.
- P6H9 showed higher ethanol productivities in HMF than sensitive strains.
- *S. cerevisiae* P6H9 has potential to be used for second-generation ethanol production.

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ABSTRACT

The aims of this work were to obtain, by evolutionary engineering, an industrial strain of *Saccharomyces cerevisiae* tolerant to high concentrations of HMF and to determine the expression levels of genes previously described as responsible for this tolerance. Cells were grown under anaerobic and oxygen limited conditions, in the presence of glucose or sucrose as carbon sources. P6H9 strain presented high expression levels for genes *ADH7* and *ARI1* in presence of HMF. This tolerant strain also showed higher ethanol productivity, biomass formation and alcohol dehydrogenase activity comparing to sensitive strains. Results suggest that *S. cerevisiae* P6H9 strain presents potential to be used for second-generation ethanol production.

1. Introduction

The development of second-generation bioethanol from lignocellulosic biomass has become widely important and is attracting global attention, since it is a renewable energy source and does not compete for areas designated for food production (Menon and Rao, 2012). The production of bioethanol from lignocellulosic feedstock requires pretreatments and hydrolyses steps to release sugars for yeast fermentation. Steam explosion and dilute-acid hydrolysis are already in use at pilot scale, but these technologies release, in addition to sugars, a wide range of compounds, which are inhibitory of yeasts metabolism. Therefore, the main challenge to turn this into an economically feasible process, is to minimize sugar degradation and the formation of furanic and phenolic compounds (Almeida et al., 2008; Margeot et al., 2009).

The main yeast metabolism inhibitors are the 2-furaldehyde (furfural), formed by pentoses dehydration, and 5-hydroxymethyl-2-furaldehyde (5-hydroxymethylfurfural–HMF), which is formed by hexoses dehydration (Liu et al., 2009). The generation of furfural during sugarcane bagasse acid hydrolysis can reach up to 5 g L^{-1}, (Aguilar et al., 2002), while HMF can accumulate to 6 g L^{-1} in hydrolysates from chipped pine wood (Larsson et al., 1999). Furans interfere with microbial growth and subsequent fermentation, interfering with glucolytic enzymes such as triose

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phosphate dehydrogenase, alcohol dehydrogenase, aldehyde dehydrogenase, and pyruvate dehydrogenase, as well as with protein and RNA synthesis (Modig et al., 2002; Sanchez and Bautista, 1988). In general, the effects of furans can be explained by a redirection of cell flux energy to damage repairing caused by these compounds and by reduced intracellular ATP and NAD(P)H levels, caused by enzymatic inhibition or consumption/regeneration of cofactors (Almeida et al., 2007).

On the other hand, HMF toxicity is associated with yeast cell damages, since the presence of its hydroxymethyl group leads to a reduced hydrophobicity and membrane permeability, which causes a low conversion rate of this compound (Taherzadeh et al., 2000). In *S. cerevisiae*, HMF toxicity was reported to be dose-dependent (Liu et al., 2004). The reduction of the aldehyde into a less toxic corresponding alcohol is a common detoxification strategy, which is mainly catalyzed by alcohol dehydrogenases. The main product described from HMF conversion in *S. cerevisiae* is 2,5-bis-hydroxymethylfuran (HMF alcohol) under aerobic and anaerobic conditions (Almeida et al., 2007; Liu et al., 2004). The compound 5-hydroxymethyl furan carboxylic acid (HMFCA) has also been identified (Taherzadeh et al., 2000).

Evolutionary engineering is an efficient strategy, which has been used to increase or modify characteristics of interest in bioprocesses, including resistance to furaldehydes by yeasts. Tolerance towards furfural, and to lignocellulosic hydrolysates in general, was described for *S. cerevisiae* strain TMB3400, which showed increased resistance to furfural after 300 generations of adaptation in the presence of this toxic (Heer and Sauer, 2008). Tolerance towards HMF and furfural was also improved for *Pichia. stipitis* strain NRRL Y-7124 and for *S. cerevisiae* strain NRRL Y-12632, using a directed adaptation method that consisted of increasing the toxics concentration and posterior selection after 100 re-inoculations to obtain a stable population. Adapted strains presented enhanced biotransformation ability to reduce HMF and furfural, and reach growth stationary phase earlier than the control (Liu et al., 2005).

It would be desirable that, in an industrial ethanol production, strains characterized as HMF resistant should have same ethanol productivities, either in the presence or absence of this aldehyde; so far, however, there has been no reports as such strains. Petersson et al. (2006) used genome-wide transcription analysis in the TMB3000 *S. cerevisiae* strain, which is known to tolerate inhibiting lignocellulose hydrolysates, and observed that *ADH2, ADH6* and *SFA1* genes presented increased expressions levels. *ADH6* gene was overexpressed in a laboratory strain, and results showed that HMF was reduced by Adh6p using NADPH as co-factor (Petersson et al., 2006). It has also been demonstrated that yeast clones overexpressing *ADH6* and *ADH7* genes were able to grow in the presence of HMF (Liu et al., 2008). Recently, the *ARI1* gene, coding for an NADPH-dependent aldehyde reductase from *S. cerevisiae*, was identified as presenting reduction activities towards at least 14 aldehyde substrates, including HMF (Liu and Moon, 2009). This dependence of NADPH induces a reprogramming of the yeast central metabolism by increasing the metabolic flux towards the pentose phosphate pathway in the presence HMF (Liu, 2011; Liu et al., 2009).

In the present study, four industrial strains of *S. cerevisiae* were evaluated regarding HMF tolerance. Evolutionary engineering was then applied to one strain aiming at increasing its tolerance towards HMF, Expression induction in the presence of HMF of four genes reported to be involved in its tolerance (*ADH6*, *SFA1*, *ADH7*, *ARI1*) were studied in anaerobic and oxygen limitation conditions, using either glucose or sucrose as carbon sources. Kinetics of yeasts cultures were conducted to compare the metabolisms of resistant and non-resistant strains in the presence of HMF.

2. Methods

2.1. Microorganisms and cell maintenance

The strains used in this study were *S. cerevisiae* P6, P18, and JP1 isolated from an ethanol production plant in Brazil (da Silva-Filho et al., 2005). HMF tolerant derivative strains P18R and JP1R were previously isolated by successive platting of their parental on YPD supplemented with 5 g L^{-1} HMF. The laboratory haploid strain BY4741 was used as reference. Yeasts were kept frozen at −20 °C in stock cultures of 20% glycerol and 80% of yeast culture (volume fraction).

2.2. Media and cultivation conditions

Oxygen limitation cultivations were performed in YPD medium (containing, in g L^{-1}: glucose, 20; yeast extract, 10; and peptone, 20) supplemented with HMF as indicated. Cultures were carried out in 250 mL flasks containing 60 mL of medium YPD (for item 2.3) or in 125 mL Erlenmeyer flasks containing 30 mL of medium (for item 2.4) at 30 °C and 150 rpm on a rotatory shaker. Initial inoculum of 0.1 OD (600 nm) was standardized. Fermentation assays used YPS (sucrose at 120 g L^{-1} instead of glucose) supplemented with HMF as indicated. Yeast cells were collected by centrifugation in graduated conical tubes until the equivalent of 5 mL corresponding to 10 % of inoculum (mass fraction, initial wet cell weight) and then the tubes were filled to 50 mL with YPS. Fermentations were carried out at 30 °C without agitation and samples were collected at defined time periods for determination of glucose, fructose, sucrose, ethanol, glycerol, acetate, and HMF concentration, and for biomass production.

2.3. Selection of strains and evolutionary engineering for HMF resistance

Yeast strains JP1, P6, and P18R were cultivated in YPD medium in the presence (4 g L^{-1}) or absence of HMF for 30 h and samples were taken at defined intervals for growth determination. Evolutionary engineering was used for further increment of the HMF resistance. Cells of JP1, P6, and P18R strains were submitted to an adaptation process by inoculating (OD of 0.1, 600 nm, 10% volume fraction) them in YPD medium with increments of HMF concentration of 0.5 g L^{-1} each batch, for 48 h at 30 °C. This procedure was repeated successive times until final cell biomass reached high and constant value. Samples of each culture cycle were stored on glycerol 20% at −18 °C.

2.4. Gene expression analysis

2.4.1. Cell treatments for gene expression analysis

The expression of genes previously described as responsible for HMF resistance were compared between treated and untreated cells grown under oxygen limitation condition and anaerobically to test the effects of physiology (see item 2.2). Cells were harvested at suitable intervals for analysis. They were centrifuged at 13 000*g* for 5 min, 4 °C and frozen at −18 °C for RNA extraction.

2.4.2. RNA extraction, cDNA synthesis and primer design

Yeast total RNA was isolated using NucleoSpin® RNA II kit following manufacturer's instructions (Macherey-Nagel, USA). RNA was quantified using spectrophotometric method (Nanovue, GE HealthCare). For cDNA synthesis, 1 μg of total RNA was used using ImProm-II™ Reverse Transcription System Promega II kit (Promega, USA). Different *S. cerevisiae* genes were used as reference according to validation experiments (Teste et al., 2009): for oxygen

Table 1
Primers used for gene expression analyses.

Primer	Sequence (5′–3′)
ADH6F	AGTGGGTTGAAAGTCGGTCAA
ADH6R	CAACGGTCACATTCCAAGCAT
ADH5F	GCTGCGGGACTGTAGGACTCT
ADH5R	TGTCAATGGCAATGATCTTGG
ADH7F	GCAAAGGATTGGAAGCATCC
ADH7R	CGCAGATACCACAGGCTTCA
ARI1F	GCCCATTTATTGACGTGCGT
ARI1R	TTGGCCGGTACATTCTGGTT

limitation cultivations (*TAF10* and *UBC6* for P6H9 strain; *TFC1* and *UBC6* for JP1 strain; *ALG9* and *TFC1* for BY4741 strain); and for anaerobic fermentation (*TAF10* and *UBC6* for all strains). Nucleotide sequences of *ADH6*, *ADH7*, *SFA1*, and *ARI1* genes are available at *Saccharomyces cerevisiae* Genome Database (http://www.yeastgenome.org/). Primers were designed using the software Primer Express (Applied Biosystems, Foster City, USA) and are described in Table 1.

2.4.3. Real time RT-PCR assays

Real-time PCR assays were performed using SYBR Green PCR Master Mix (Applied Biosystems, USA) and the parameters were previously reported (Elsztein et al., 2011). Temperature–time profile (95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min) was optimized for ABI Prism 7300 (Applied Biosystems). Amplification curves were analyzed with software SDS v.2.0 (Applied Biosystems). Negative PCR control (no template) and negative RT control (RNA not reverse-transcribed to cDNA) were run as internal controls. All samples were run in technical triplicates for each biological duplicate of cell cultivation. Normalization was performed for all genes at once using geNorm applet (Vandesompele et al., 2002), thus minimizing errors due to shifts in a single reference gene (Elsztein et al., 2011); the optimal number of reference genes to be used was also determined after submitting the raw data to the geNorm tool (http://medgen.ugent.be/genorm).

2.5. Analytical methods and kinetic parameters calculation

Growth rate of yeast cultures were measured as optical density (OD) at 600 nm. Cultivations were run for 48 h and samples were taken every 6 h. Cultures were centrifuged at 3500*g*, and cells were kept on −18 °C for further enzymatic activity assays. Yeast biomass was determined using a standard curve correlating the OD and cell dry weight (g L^{-1}). Glucose, glycerol, ethanol, and acetate concentrations were determined by HPLC (Shimadzu, Japan) equipped with a refractive index detector and Bio-Rad HPX-87H column (300 × 7.8 mm) using 5 mM sulfuric acid as eluent at 45 °C, flow rate of 0.6 mL min^{-1} and sample volumes of 20 μL. HMF concentration was determined by HPLC with a UV detector (at 276 nm) using a Nucleosil C18 column (250 × 4.6 mm) at room temperature, using acetonitrile–water (2:8) containing 10 g L^{-1} acetic acid as eluent, flow rate of 1.1 mL min^{-1} and sample volumes of 20 μL. The ethanol conversion yield ($Y_{P/S}$, g g^{-1}) was defined as the ratio of the concentration of ethanol produced and glucose consumed. The volumetric productivity (Q_p, g L^{-1} h^{-1}) was calculated using the ethanol production versus time.

2.6. Enzyme activity assay

Crude protein extracts were prepared using the glass beads method. Cells were resuspended in 400 μL potassium phosphate buffer 100 mM, pH 7.0, and 2 μL of a solution of 100 mM PMSF with an equal volume of glass beads (450 × 500 μm). Cells were disrupted by vortexing six times for 60 s, and the samples were cooled on ice for 60 s in between the vortex steps. Protein extracts were collected by centrifugation at 13000*g* for 5 min at 4 °C and the concentration was determined using Lowry assay method. Alcohol dehydrogenase activity was monitored by recording decreased absorbance at 340 nm using NADPH as cofactor. The reaction mixture consisted of a final concentration of 10 mM HMF substrate and 100 μM of NADPH in 100 mM potassium phosphate buffer, pH 7.0. All reagents were maintained at 25 °C prior to use. Assays were carried out in a volume of 1 ml at 25 °C for 1 min. The protein samples were kept on ice. To start the reaction, 25 μl of crude extract protein was added to the reaction mix. The NADPH molar absorption coefficient was 6.22 mM^{-1} cm^{-1}.

3. Results and discussion

3.1. Selection of industrial strains with HMF tolerance

Four *S. cerevisiae* strains were tested for their tolerance to HMF under oxygen limitation metabolism. Fig. 1 A shows the extended lag growth phase for all strains when in the presence of HMF, with

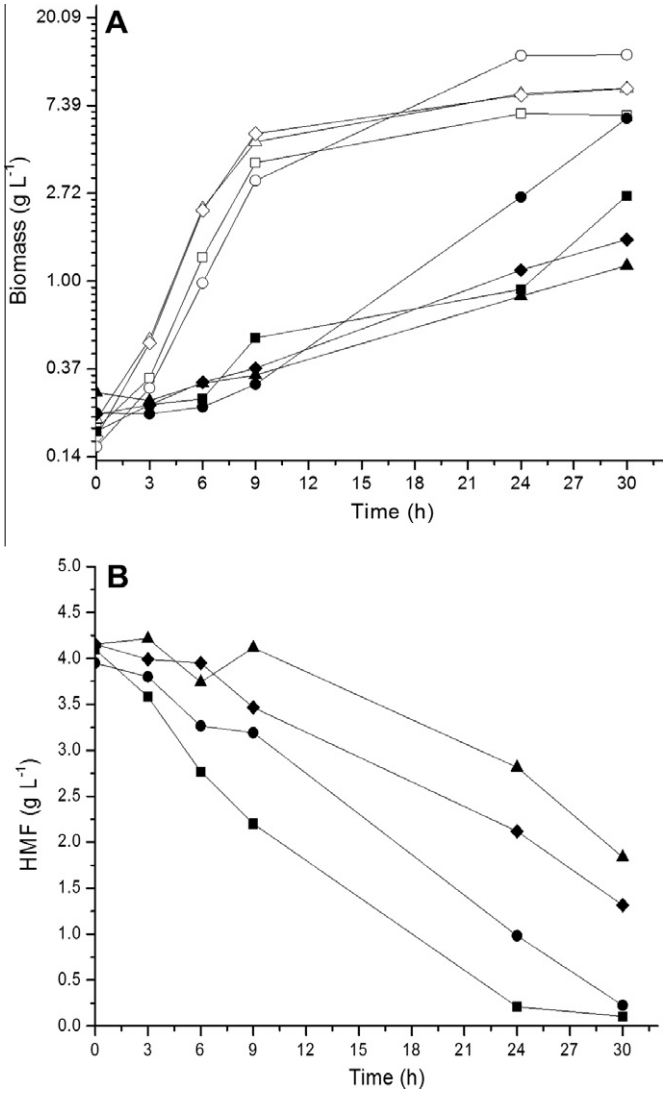

Fig. 1. Kinetics of biomass production (A) and HMF assimilation (B) of *S. cerevisiae* strains JP1 (▲), JP1R (♦), P6 (■), and P18R (●). Open symbols, condition control; filled symbols, presence of 5 g L^{-1} HMF. Results represent the mean of triplicate.

Table 2
Comparison between yield and productivity of ethanol of four industrial strains of *S. cerevisiae* growing in presence or absence of 5 g L^{-1} HMF.

Strain	JP1		JP1R		P6		P18R	
HMF	−	+	−	+	−	+	−	+
$Y_{P/S}$ (g g^{-1})	0.45	0.04	0.46	0.06	0.37	0.06	0.13	0.06
Q_p (g L^{-1} h^{-1})	0.64	0.08	0.55	0.05	0.40	0.05	0.10	0.04

P18R strain showing the highest biomass production at the end of cultivation. Although all strains showed the ability to reduce HMF concentration of media, P18R and P6 were able to completely deplete the toxic at the end of run (Fig. 1B). Glucose consumption was not affected, independent of strains (data not shown). In the presence of HMF, three out of four strains presented only about 10 % of the ethanol yield ($Y_{P/S}$) and volumetric productivity (Q_p) compared to controls without HMF (Table 2). P18R strain, already presenting lower fermentative capacity in the reference medium, showed 50 % yield and productivity reductions. The dose-dependent toxicity of HMF has been reported for *S. cerevisiae* ATCC 211239 and NRRL Y-12632 strains and even at the lowest concentration evaluated (1.2 g L^{-1}) the yeast final biomass did not reach that in the reference condition without HMF (Liu et al., 2004). Ethanol production was also affected in *S. cerevisiae* T1 and Y-1528 strains upon exposure to 3 and 4 g L^{-1} HMF, respectively (Keating et al., 2006). Finally, when furfural and HMF were added to cultures of *Spathaspora arborariae* strain UFMG-HM19.1A, their presence caused significant reductions on biomass formation and ethanol productivity (da Cunha-Pereira et al., 2011). The biochemical explanation seems to be the inhibition of essential enzymes, such as triose phosphate dehydrogenase and alcohol dehydrogenase (Sanchez and Bautista, 1988). HMF and furans were reported to affect CO_2 evolution by yeast cells (Banerjee et al., 1981). Due to their ability to form biomass in the presence of HMF, and to show HMF assimilation, P18R and P6 strains were selected for new rounds of evolutionary engineering for HMF resistance. JP1 was also selected, but in this case for its ability to produce high concentrations of ethanol in absence of HMF.

3.2. Evolutionary engineering for HMF resistance

Evolutionary engineering was performed aiming at selecting strains hyper-resistant to HMF. After 24 days of successive cultivations with increasing concentrations of HMF, it was possible to increase the tolerance of JP1 strain to 3.5 g L^{-1} HMF, while P18R strain was improved to 6 g L^{-1} HMF (results not shown). Moreover, a tolerant mutant of P6 strain was able to grow up to 9 g L^{-1} HMF after 36 days of cultivations. This mutant was named P6H9 and it was selected for further experiments. In the present work it was, therefore, possible to further increase HMF tolerance of JP1R strain by 20 % and to select a hyper-tolerant strain by increasing the HMF tolerance of P6 by 360%. Evolutionary engineering has been efficiently applied to obtain several interesting mutants of *S. cerevisiae* showing improved xylose-glucose co-assimilation by recombinant xylose-fermenting *S. cerevisiae* strain (Kuyper et al., 2005), and for the selection of sulfuric acid-tolerant JP1 strain (de Melo et al., 2010), which was also used in this work.

3.3. Gene expression analyses

Gene expression analyses were performed comparing the three *S. cerevisiae* strains BY4741, JP1, and P6H9. In the reference medium without HMF, these strains showed similar growth rates, reaching 0.5 DO in 3 h of oxygen limitation cultivation. However, in the presence of HMF there was a marked difference among strains to reach that cell concentration: 12.5 h for BY4741; 5 h for JP1; and 3 h for the mutant P6H9. In JP1, high expression level was observed for *ADH6* gene (>11-fold), while a moderate induction (>2-fold) was observed for *ADH7*, *ARI1*, and *SFA1* genes. On the other hand, there were surprisingly high inductions of *ADH7* (>550-fold) and *ARI1* (>20-fold) genes detected for the P6H9 mutant. Comparatively, the sensitive BY4741 strain showed discreet (>2-fold) inductions of *ADH7* and *ARI1* genes.

When simulating industrial conditions (high-sugar, high-biomass inoculum, anaerobic fermentation), *ADH6* gene was repressed and *ARI1* gene was moderately induced in JP1 strain (>2-fold). Similar to observations for the oxygen limitation condition, under anaerobiosis, *ADH7* was also over-expressed in P6H9 strain, followed by *ARI1* gene. *ADH6* was the first gene reported to be involved with HMF tolerance in yeast (Petersson et al., 2006). Genome-wide DNA Microarray showed the over-expression of *ADH6* gene in *S. cerevisiae* TMB3000 strain exposed to HMF and its importance to cell tolerance was demonstrated by overexpressing *ADH6* in the recombinant strain CEN.PK 113-5D, also resulting in an increase of NAPDH-dependent alcohol dehydrogenase activity by the cells (Petersson et al., 2006). Adh6p enzyme is active towards a wide spectrum of linear, branched-chain and aromatic primary alcohols and aldehydes such as cinnamaldehyde, benzaldehyde, veratraldehyde, and panisaldehyde using NADPH as the coenzyme (Larroy et al., 2002a). *SFA1* gene overexpression from *S. cerevisiae* TMB3000 was also evaluated, and the NADH-dependent HMF-reducing activity was observed. However, this activity did not result in increased HMF uptake rate (Petersson et al., 2006). Sfa1p presents molecular structure and biological function similar to alcohol dehydrogenases (Wehner et al., 1993), and has been proposed to be a bifunctional enzyme with glutathione-dependent formaldehyde dehydrogenase and long-chain alcohol dehydrogenase activities (Dickinson et al., 2003).

The product of *ARI1* gene was first described as a NADPH-dependent reductase accepting ethyl acetoacetate as substrate (Katz et al., 2003). This gene was also used to produce the 2-phenylethanol from phenylpyruvate, with carbonyl reductase function (Hwang et al., 2009). Recently, Liu and Moon (2009) showed that the partially purified Ari1p displayed NADPH-dependent reduction activities toward at least 14 aldehyde substrates, including furfural, HMF, acetaldehyde, butyraldehyde, cinnamic aldehyde, and phenylacetaldehyde. The expression of *ARI1* gene showed a 10-fold increase in *S. cerevisiae* Y-12632 strain upon exposure to HMF (Liu and Moon, 2009), similar to the results for P6H9 strain in this work.

The product of *ADH7* gene, a reductase from the NADPH-dependent cinnamyl alcohol dehydrogenase family, has been pointed as an important enzyme for detoxification of cinnamaldehyde, furfural, phenylacetaldehyde, vanillin, and 3-methylbutanal (Larroy et al., 2002b), and the *ADH7* overexpression confers yeast tolerance to HMF (Liu et al., 2008). The HMF-tolerant *S. cerevisiae* Y-50049 strain showed a 35-fold increased *ADH7* transcription when compared to sensitive strain Y-12632 (Liu et al., 2009).

3.4. Culture kinetics in presence and absence of HMF

Physiological analysis under oxygen limitation cultivation was performed for P6H9 strain and compared with the industrial JP1 and laboratory BY4741 strains (Fig. 2). The three strains showed similar growth rate (Fig. 2A), ethanol (Fig. 2B) and glycerol (Fig. 2C) production in the reference medium, showing that the genetic mutation that conferred HMF hyper-tolerance did not affect yeast fermentative capacity. Yet, the production of acetate in the reference medium, which was already low for industrial strain JP1, was practically abolished for P6H9 tolerant strain compared to BY4741 (Fig. 2D). In the presence of HMF, the yeast cells

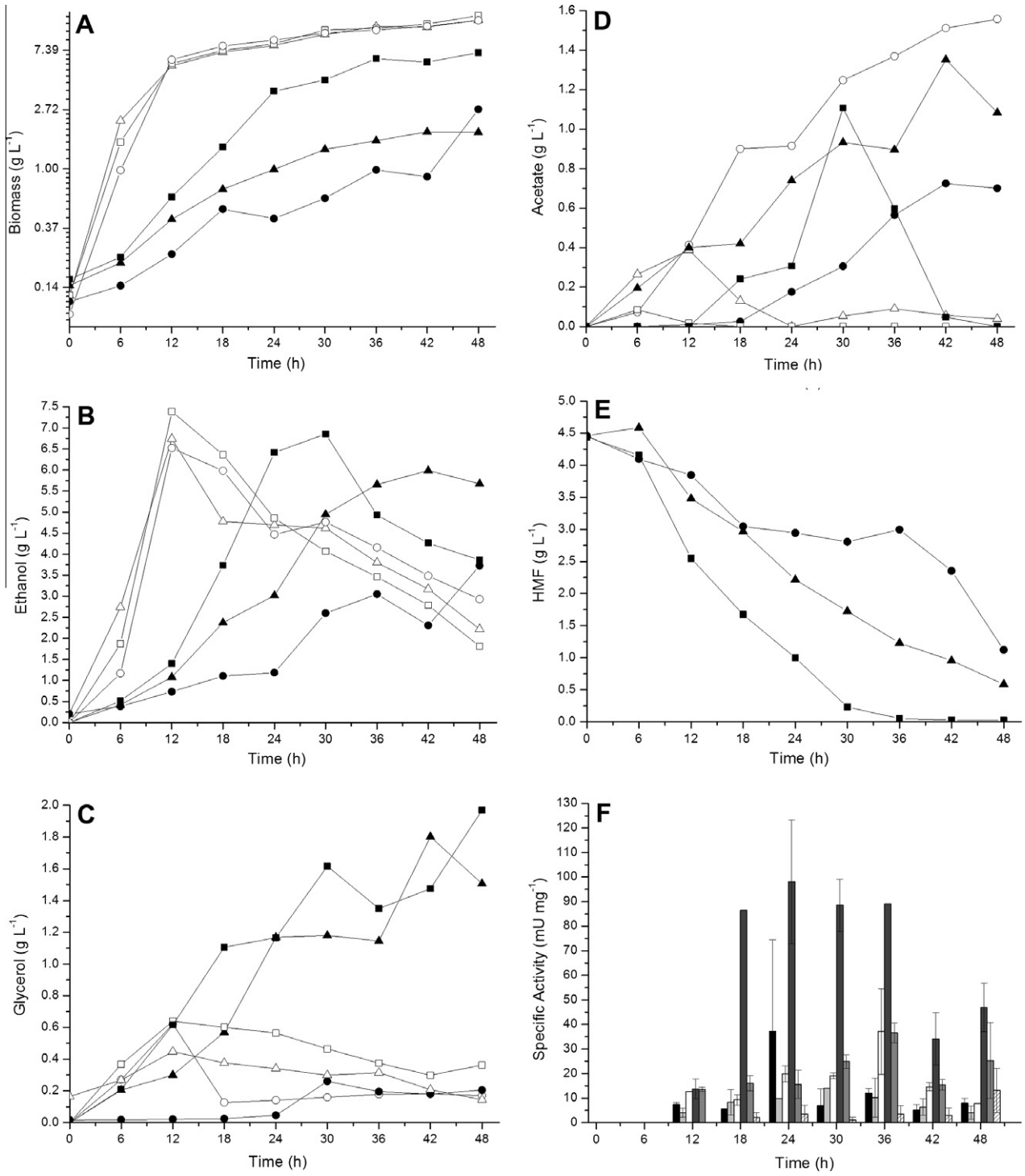

Fig. 2. Physiological analysis of of *S. cerevisiae* strains JP1 (▲), P6H9 (■), and BY4741 (●) cultivated in YPD (open symbols) or YPD containing HMF at 5 g L^{-1} (filled symbols). Production of biomass (A), ethanol (B), glycerol (C), acetate (D), HMF assimilation (E), and NADPH-dependent HMF reduction activity (F) are presented. Results represent the mean of duplicate.

experienced a change from reductive to oxidative metabolism. The highest growth rate in HMF medium observed for P6H9 strain (Fig. 2A) was also correlated with the highest kinetics of HMF assimilation by this strain (Fig. 2E) and the highest enzymatic activity of its cell-free extract to reduce this compound using NADPH as cofactor (Fig. 2F). This intense metabolic capacity can be explained by the greatly enhanced up-regulation of *ADH7* gene in P6H9 strain. The industrial strain JP1 showed a moderate tolerance towards HMF (Fig. 2A), also showing some limited assimilation of this toxic compound (Fig. 2E), possibly caused by the up-regulation of *ADH6* gene. However, no increased NADPH-dependent enzymatic activity was observed for this strain (Fig. 2F).

Ethanol production was delayed in the presence of HMF for all tested strains, but P6H9 strain was less affected and the final maximal concentration of ethanol achieved was almost the same in the presence or absence of HMF. As expected, ethanol volumetric productivity fell from 0.62 g L^{-1} h^{-1} to 0.23 g L^{-1} h^{-1} in the presence of HMF (Table 3).

Table 3
Comparison between yield and productivity of ethanol for *S. cerevisiae* industrial strains P6H9, JP1, and BY4741, in presence or absence of HMF.

Strain	BY4741		JP1		P6H9	
HMF	–	+	–	+	–	+
$Y_{P/S}$ (g g^{-1})	0.32	0.26	0.32	0.32	0.35	0.33
Q_p (g L^{-1} h^{-1})	0.54	0.07	0.54	0.16	0.62	0.27

Another important observation is the fact that glycerol production was greatly induced by HMF in the industrial strain JP1 and in the HMF-tolerant P6H9 strain (Fig. 2C), despite the reduced growth rate (Fig. 2A), linking its formation to a stress-induced phenomenon caused directly by the assimilation of HMF or indirectly, by some sort of metabolic imbalance. Studies on glycerol production reported that lowered activity of different ADH isoenzymes, especially Adh1p, results in an increase of glycerol production in *S. cerevisiae* (Johansson and Sjostrom, 1984). The protein coded by *ADH1* is an enzyme responsible for the anaerobic cytosolic reduction of acetaldehyde to ethanol (Taherzadeh et al., 2002). Analysis of the glucose metabolism of adh^0 cells shows that the lack of all known ADH isozymes results in the formation of glycerol as a major fermentation product, followed by a significant production of acetaldehyde and acetate (DREWKE et al., 1990). In an earlier study, *ADH1*, *ADH2*, *ADH3*, and *ADH4* genes expression did not change in tolerant aldehyde *S. cerevisiae* strain. However, glycerol production was not reported for the same strain in that study (Liu et al., 2009).

Except for strain BY4741, which showed a poor growth in the presence of HMF, all other tested strains showed an increased production of acetate in the presence of this toxic (Fig. 2D). Increased acetate production was reported for *ADH6*-overexpressing strain in batch fermentation (Almeida et al., 2008). In *S. cerevisiae*, there are two main metabolic routes responsible for NADPH formation, namely the pentose-phosphate pathway, and the acetaldehyde dehydrogenase reaction. The first route drains carbon from glycolysis and together with the stress-associated glycerol-3P dehydrogenase reduces the supply of NADH; the second pathway drains acetaldehyde for the production of acetate. Thus, the metabolic requirement for NADPH could lead to increased production of acetate, and decreased production of ethanol as consequence.

In this study, the sensitive strain BY4741 presented low expression levels for all four genes previously described as being involved with HMF tolerance; JP1 strain, however, showed increased expression levels of *ADH6* gene, but this was not reflected in HMF reduction during cultivation, or ethanol and biomass production. These results contrast with results of a previous study, in which tolerance towards HMF in yeast strains overexpressing *ADH6* and *ADH7* genes led to improved growth rate in the presence of this toxic (Liu et al., 2008). However, in the present study, the mutant strain P6H9 showed the highest HMF tolerance so far described for a yeast strain, at the same time capable of producing ethanol. In the presence of this toxic compound, high expression levels for *ADH7* and *ARI1* genes were observed in the two different metabolic conditions tested. In particular, *ADH7* gene showed a significant increase in its expression levels. This was reflected in the biomass and ethanol production and in the enzymatic activity.

4. Conclusion

A high-tolerant *S. cerevisiae* mutant strain P6H9 was obtained by evolutionary engineering from the industrial strain P6, which was isolated from molasses-using ethanol producing plant, and this phenotype was correlated to the high expression levels of *ADH7* and *ARI1* genes and increased enzymatic activity for NADPH-dependent HMF reduction. In the presence of HMF, this strain showed a better physiological performance than JP1 industrial strain and BY4741. Overall, the results obtained in this work shows the importance of several genes on the phenotype for HMF tolerance and strain P6H9 can be further exploited in research for second generation ethanol production.

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