Contribution of *PRS3*, *RPB4* and *ZWF1* to the resistance of industrial *Saccharomyces cerevisiae* CCUG53310 and PE-2 strains to lignocellulosic hydrolysate-derived inhibitors

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HIGHLIGHTS

- Expression analyses revealed *PRS3*, *RPB4* and *ZWF1* roles in adaptation to inhibitors.
- *PRS3*, *RPB4* and *ZWF1* were overexpressed in two industrial *S. cerevisiae* strains.
- Fermentations were conducted in *Eucalyptus globulus* wood and corn cob hydrolysates.
- *PRS3* overexpression revealed to be advantageous for lignocellulosic fermentation.
- *PRS3*, *RPB4* and *ZWF1* overexpression results differed depending on strain/hydrolysate.

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ABSTRACT

PRS3, *RPB4* and *ZWF1* were previously identified as key genes for yeast tolerance to lignocellulose-derived inhibitors. To better understand their contribution to yeast resistance to the multiple stresses occurring during lignocellulosic hydrolysate fermentations, we overexpressed these genes in two industrial *Saccharomyces cerevisiae* strains, CCUG53310 and PE-2, and evaluated their impact on the fermentation of *Eucalyptus globulus* wood and corn cob hydrolysates. *PRS3* overexpression improved the fermentation rate (up to 32%) and productivity (up to 48%) in different hydrolysates. *ZWF1* and *RPB4* overexpression did not improve the fermentation performance, but their increased expression in the presence of acetic acid, furfural and hydroxymethylfurfural was found to contribute to yeast adaptation to these inhibitors. This study expands our understanding about the molecular mechanisms involved in industrial yeast tolerance to the stresses occurring during lignocellulosic bioethanol production and highlights the importance of selecting appropriate strain backgrounds/hydrolysates combinations when addressing further improvement of these processes.

1. Introduction

One of the major challenges faced in the production of bioethanol and other value-added products from lignocellulosic biomass is the generation of a wide range of compounds during the pre-treatment and hydrolysis process that affect the physiology and metabolism of microorganisms, decreasing their viability and productivity (Chandel et al., 2013; Parawira and Tekere, 2011). Therefore, the commercial success of lignocellulosic biomass conversion necessarily depends on the development of microorganisms able to cope with these inhibitors while simultaneously produce satisfactory amounts of the desired product (Chandel et al., 2013).

The inherent ability of yeast cells to withstand and detoxify the main inhibitory compounds present in lignocellulosic hydrolysates (acetic acid, furfural and hydroxymethylfurfural (HMF)) is variable among strains and determinant for efficient lignocellulosic bioethanol production (Modig et al., 2008; Pereira et al., 2014a). Depending on their genetic background, yeast cells activate distinct gene expression programs to help them counteract the negative impact of these inhibitors on their metabolism (Liu et al., 2009). Strains isolated from harsh industrial environments, such as *Saccharomyces cerevisiae* CCUG53310 (Purwadi et al., 2007) and PE-2 (Basso et al., 2008) (isolated from second and first generation bioethanol plants, respectively), have been shown to be more efficient in detoxifying and fermenting lignocellulosic hydrolysates

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than other industrial and laboratory background strains (Pereira et al., 2014a; Westman et al., 2012). However, the molecular characterization of these strains under relevant process conditions is limited, which hampers the understanding of the molecular mechanisms underlying their tolerance to inhibitory hydrolysates and the further improvement of their resistance to these compounds.

Results obtained from chemogenomic (Alriksson et al., 2010; Gorsich et al., 2006; Mira et al., 2010; Pereira et al., 2011, 2014b) and transcriptomic (Bajwa et al., 2013; Liu et al., 2009; Ma and Liu, 2010) analyses have been helpful in identifying the genetic determinants of yeast tolerance to lignocellulose-derived inhibitors. Overexpression of some target genes identified through these approaches in laboratory background strains has already been shown to be associated with improved growth, fermentation rate and/or ethanol production in the presence of these inhibitors (Alriksson et al., 2010; Gorsich et al., 2006; Park et al., 2011; Petersson et al., 2006). Based on chemogenomic analyses, we have previously identified *PRS3* and *RPB4* as key genes necessary for yeast growth and maximal fermentation rate in wheat straw hydrolysate (Pereira et al., 2011, 2014b), but the outcome of their overexpression in *S. cerevisiae* lignocellulosic-based fermentations has not yet been assessed. *ZWF1*, which had been previously shown to confer resistance to furfural and HMF (Gorsich et al., 2006; Park et al., 2011), was also found to be important for maximal yeast resistance to wheat straw hydrolysate (Pereira et al., 2014b).

Genetic strategies addressing strain tolerance improvement to lignocellulose-derived inhibitors have been developed mainly through gene overexpression in laboratory background strains and tested using synthetic hydrolysates (Gorsich et al., 2006; Park et al., 2011). Nevertheless, strains with robust genetic backgrounds have been shown to already display enhanced background expression of several genes involved in the detoxification of some of these inhibitors (Liu et al., 2009), and therefore their extra expression may result in different outcomes from those reported for laboratorial background strains. Moreover, the inhibitory load of lignocellulosic hydrolysates, which varies depending on the raw material and operational conditions of pretreatment (Chandel et al., 2013), has been shown to differentially influence the outcome of genetic manipulations (Alriksson et al., 2010; Park et al., 2011; Wallace-Salinas et al., 2014), highlighting the importance of evaluating their effect under process-like conditions.

In this work, we aimed at evaluating the contribution of *PRS3*, *RPB4* and *ZWF1* for the resistance of the industrial *S. cerevisiae* CCUG53310 and PE-2 strains to lignocellulosic hydrolysate-derived inhibitors, as a means to elucidate their role on yeast response to the multiple stresses occurring during lignocellulosic ethanol production and to better understand the molecular mechanisms underlying the robustness of these strains. For that, we analysed the expression profiles of these genes in fermentations with and without acetic acid, furfural and HMF, and evaluated the effect of their overexpression on the fermentation performance of both strains in corn cob and *Eucalyptus globulus* wood (EGW) hydrolysates, which contain different inhibitory loads.

2. Methods

2.1. Strains

Two industrial *S. cerevisiae* strains were used in this work: CCUG53310, flocculating strain isolated from a Swedish second generation bioethanol plant (Purwadi et al., 2007); and PE-2, isolated from a Brazilian first generation bioethanol plant (Basso et al., 2008). Overexpressing strains (Table 1) were generated using CCUG53310 and PE-2 as parental strains, as described below. *S. cerevisiae* CEN.PK113-7D was the source of the genomic DNA for

Table 1

Strains, plasmids and primers used in this study. Upper case sequences correspond to sequences complementary to the template. Lower case sequences correspond to additions for restriction sites (underlined).

	Relevant features	Source
S. cerevisiae strains		
CEN.PK113-7D	MATα, MAL2-8c, SUC2	INSA, France
CCUG53310	Flocculation	(Purwadi et al., 2007)
PE-2	Diploid	(Basso et al., 2008)
CC-Yep	CCUG53310, YEplac195KanMX	This work
CC-ZWF1	CCUG53310, YEpJCZ	This work
CC-PRS3	CCUG53310, YEpJCP	This work
CC-RPB4	CCUG53310, YEpJCR	This work
PE-Yep	PE-2, YEplac195KanMX	This work
PE-ZWF1	PE-2, YEpJCZ	This work
PE-PRS3	PE-2, YEpJCP	This work
PE-RPB4	PE-2, YEpJCR	This work
Plasmids		
pGEM-T Easy		Promega
YEplac195	*URA3* marker; 2-micron origin of replication	Gietz and Sugino (1988)
YEplac195KanMX	*URA3* and *KanMX* marker; 2-micron origin of replication	This work
YEpJCZ	YEplac195KanMX containing the *ZWF1* gene under the control of its native promoter	This work
YEpJCP	YEplac195KanMX containing the *PRS3* gene under the control of its native promoter	This work
YEpJCR	YEplac195KanMX containing the *RPB4* gene under the control of its native promoter	This work
Primers	Sequence (5′–3′)	
KanMX_FW	ggaattc<u>catatg</u>GAGATCTGTTTAGCTTGCCTC	
KanMX_RV	ggaattc<u>catatg</u>GCTCGTTTTCGACACTGG	
Z1	GTAAGGTGTAGTTTTGCACCC	
Z2	AAATTTTTGCAGACATTTTTGATATATAT	
P1	TTATCTTCATCACCGCCATAC	
P2	ACAAGAGAAACTTTTGGGTAAAATG	
R1	GATTGCTCAAATTAGCATGTGAA	
R2	AATCCTGTCCTTTTTCCTGTTAAATAG	
qPCR*ZWF1*_FW	CTGGTCTGTCAAATGCTACC	
qPCR*ZWF1*_RV	CCAGTAGGGCGTCTCTTAT	
qPCR*PRS3*_FW	GGCTAGGTCTACAGTTAACAAG	
qPCR*PRS3*_RV	GTCCCTAACAGATTCTCCAATAG	
qPCR*RPB4*_FW	ACGGGAGGAAATAATAAAGATTTG	
qPCR*RPB4*_RV	GACGGTTTCTTGGTCTCTAAAT	
qPCR*ACT1*_FW	GCCGAAAGAATGCAAAAGGA	
qPCR*ACT1*_RV	TAGAACCACCAATCCAGACG	

genes amplification. *Escherichia coli* NZY5α (Nzytech) was used as the recipient for all cloning steps.

2.2. Plasmids construction and yeast transformation

Expression plasmids containing the *S. cerevisiae* 2-micron replication origin were generated as follows. The *KanMX* cassette conferring resistance to geneticin/G418 was amplified from pUG6 (Guldener et al., 1996) with the KanMX primers pair (Table 1) and inserted into the *Nde*I site of YEplac195 (Gietz and Sugino, 1988), generating plasmid YEplac195KanMX. Based on the annotated sequence for the YNL241C (*ZWF1*), YHL011C (*PRS3*) and YJL140W (*RPB4*) open reading frames (NCBI BioProject Accession number PRJNA52955), their complete coding regions with corresponding native promoter and terminator sequences were amplified by PCR from *S. cerevisiae* CEN.PK113-7D genomic DNA using the primers pairs Z1/Z2, P1/P2 and R1/R2 (Table 1), respectively.

The PCR products were cloned into the pGEM-T Easy vector (PROMEGA) and the resulting constructs were digested with *Sac*I/*Sph*I, for isolation of the *ZWF1* and *RPB4* regions, or with *Eco*RI, for isolation of the *PRS3* region. The digested fragments were cloned into the YEplac195KanMX vector previously digested with the corresponding enzymes. The resulting *ZWF1*, *PRS3* and *RPB4* expression plasmids were named YEpJCZ, YEpJCP and YEpJCR, respectively. The orientation and sequence of the inserts in the plasmids were confirmed by restriction analyses and sequencing (Eurofins MWG Operon) with the primers pairs Z1/Z2, P1/P2 or R1/R2 (Table 1). Vectors were introduced into *S. cerevisiae* CCUG53310 and PE-2 using the lithium acetate method (Gietz et al., 1992). The empty vector (YEplac195KanMX) was also transformed into both yeast strains to serve as control in the fermentation assays. Transformants were selected in G418-containing media.

2.3. Preparation of the EGW, corn cob and synthetic hydrolysates

Lignocellulosic feedstocks (EGW and corn cob) were collected, milled and stored until its use. EGW and corn cob hydrolysates were prepared following optimized conditions previously described in Pereira et al. (2014a) and Romani et al. (2015), respectively. For this, water and lignocellulosic biomass were mixed at Liquid to Solid Ratio (LSR) equal to 8 kg/kg and submitted to hydrothermal treatment in a 3.7 L stainless steel reactor at 202 and 210 °C for corn cob and EGW, respectively. The hardness of treatment (expressed as "severity"; S_0) is defined as follow:

$$S_0 = \log R_0 = \log \int_0^t \exp \frac{T(t) - T_{REF}}{\omega} dt \tag{1}$$

where $T(t)$ stands for the time-temperature profile (including heating isothermal period and cooling). Calculations were made assuming the values usually employed in literature for T_{REF} and ω (100 °C and 14.75 °C, respectively) (Lavoie et al., 2010). The value of S_0 was 3.84 and 4.10 for corn cob and EGW treatments, respectively. The liquid fractions, hydrolysates containing hemicellulose and lignin-derived compounds, were recovered by filtration and analysed by HPLC (Section 2.7). The composition of these EGW and corn cob hydrolysates was according to that reported by Pereira et al. (2014a) and Romani et al. (2015), respectively. The pH of the hydrolysates was adjusted to 4.5 with 10 M sodium hydroxide. The hydrolysates were sterilized by filtration (0.2 μm pore size sterile filters) and supplemented with approximately 100 g/L glucose to improve the ethanol titer. Based on the inhibitors concentration of EGW hydrolysate, several synthetic media were prepared to test the susceptibility of the overexpressing strains to the main inhibitors present in real lignocellulosic hydrolysates, which consisted of a minimal medium containing, per liter, 1.7 g yeast nitrogen base without amino acids and ammonium sulfate, 5 g L-asparagine, ca. 100 g glucose, and: (1) acetic acid, furfural and HMF, or (2) only furfural. When indicated, real and synthetic hydrolysates were supplemented with 100 μg/mL G418 for plasmid maintenance.

2.4. Inoculum preparation

The strains used to inoculate the fermentation media (Table 1) were cultivated at 30 °C for 20-22 h, with orbital agitation (200 rpm), in YPD medium, containing, per liter, 10 g yeast extract, 20 g peptone and 20 g glucose. When indicated, 100 μg/mL of G418 were added to the media. Cells were recovered by centrifugation (15 min, 4000 g, 4 °C) and pellets were resuspended in ice-cold 0.9% (w/v) sodium chloride to obtain 200 mg of fresh yeast/mL. Before the resuspension, pellets from the CCUG53310 strain and from the CCUG53310-derived transformants were washed twice with 1.5% (w/v) sodium chloride, pH 3.0 to deflocculate. The concentrated cell suspensions were used to inoculate 30 mL of fermentation media, with a cellular concentration of 5 mg of fresh yeast/mL (mimicking the high initial cell densities used at the industrial scale).

2.5. Fermentation assays

Fermentations were carried out in 100 mL Erlenmeyer flasks fitted with perforated rubber stoppers enclosing glycerol-filled air-locks (to permit CO_2 exhaustion while avoiding the entrance of air). Prior to inoculation, the media were aerated by stirring with a magnetic bar (length of 3 cm) at >850 rpm for 20 min. Under these conditions, the oxygen concentration in the growth media was higher than 95% of air saturation. The fermentations were followed by measuring the reduction of mass loss resulting from CO_2 production.

CO_2 production was mathematically modeled following the equation described by Rodrigues et al. (2006):

$$P = \frac{P_0 P_{max} e^{P_r t}}{P_{max} - P_0 + P_0 e^{P_r t}}$$

where t is time (h), P is CO_2 concentration (g/L), P_{max} is maximum concentration of CO_2 (g/L), and P_r is the ratio between the initial volumetric rate of CO_2 formation (r_p) and the initial CO_2 concentration P_0 (g/L), which represents the maximum fermentation rate. The experimental data was fitted to the model and the parameters P_0, P_{max}, and P_r were calculated for each fermentation by nonlinear regression using the least-squares method using commercial software (Solver of Microsoft Excel 2010). The maximum CO_2 productivity (Qp_{max}; g/(L h)) was calculated at the time the CO_2 concentration reached its maximum. Samples were taken at the end of the fermentations and analysed by HPLC.

2.6. Gene expression analysis

For *PRS3*, *RPB4* and *ZWF1* expression analyses, YPD medium (control) and YPD medium supplemented with approximately 3.11 g/L acetic acid, 1.66 g/L furfural and 0.33 g/L HMF (concentrations similar to those present in the EGW hydrolysate) were used. Fermentations were carried out as indicated in Section 2.5. Samples containing approximately 2×10^7 cells were collected at different phases of the *S. cerevisiae* CCUG53310 and PE-2 fermentations: late lag-phase (ca. 1 g/L of CO_2 produced) and initial exponential phase (ca. 5 g/L of CO_2 produced). The cell pellet of each sample was immediately stored at −70 °C after washing with ice-cold 0.9% (w/v) sodium chloride, and the supernatant was stored at −20 °C for HPLC analysis (Section 2.7).

Total RNA was extracted from frozen cells with the RNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions and treated with DNase I (Fermentas). The concentration and purity of the total RNA was spectrometrically determined using a NanoDrop 1000TM (Thermo Scientific) and its integrity assessed on 1% agarose gel by visualization of the 25S/18S rRNA banding pattern after electrophoresis. Single-stranded cDNA was synthesized from 1 μg of total RNA with the SuperScript II Reverse Transcriptase (Invitrogen) and Oligo(dT) primers (NZYTech). Oligonucleotides for quantitative PCR (qPCR; primers with prefix qPCR inTable 1) were designed using the IDT PrimerQuest tool followed by a BLAST analysis against the *S. cerevisiae* genome sequence for specificity confidence. qPCR assays were performed in a CFX96 real-time PCR system (Bio-Rad). Each sample was tested in duplicate in 10 μL reaction mixes consisting of 5 μL of SsoFast Evagreen Supermix (Bio-Rad), 0.6 μL of each primer (600 nM final concentration), 2.8 μL of H_2O, and 1 μL of a 1:10 dilution of the

cDNA preparation. The absence of genomic DNA in RNA samples was checked by qPCR (minus RT control). A blank (no template control) was also incorporated in each assay. The thermocycling program consisted of an initial enzyme activation step at 95 °C during 30 s, followed by 40 cycles of 5 s denaturation at 95 °C and 5 s annealing/extension at 53.5 °C (*ZWF1*, *RPB4* and *ACT1*) or 55.7 °C (*PRS3* and *ACT1*). After completion of these cycles, data from the melting-curve were then collected to verify PCR specificity, lack of contamination and the absence of primer dimers. Relative expression levels were determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). For standardization, the results were expressed as target/reference ratio, being the reference gene the genome-encoded actin gene (*ACT1*).

2.7. Analytical methods

The concentrations of glucose, ethanol, acetic acid, furfural and HMF in the hydrolysates and in the samples from the fermentation runs were determined by HPLC using a Varian MetaCarb 87H column, eluted at 60 °C with 0.005 M sulfuric acid and at a flow rate of 0.7 mL/min. The peaks corresponding to glucose, ethanol and acetic acid were detected using a refractive index detector, whereas furfural and HMF were detected using an UV detector set at 210 nm.

2.8. Statistical analyses

GraphPad Prism for Windows version 6.01 was used to carry out the statistical analyses. Differences among gene expression for CCUG53310 and PE-2 strains were determined using multiple *t*-test. Differences between the fermentation profiles of each over-expressing strain and the control strain were tested by repeated measures two-way ANOVA, followed by Bonferroni post hoc test. Differences in kinetic parameters were determined using multiple *t*-test. Statistical significance was established at $P < 0.05$ for the comparisons.

3. Results and discussion

3.1. Differential expression of PRS3, RPB4 and ZWF1 during the initial phases of CCUG53310 and PE-2 fermentations in the presence and absence of lignocellulose-derived inhibitors

Strains adapted to tolerate superior concentrations of lignocellulose-derived inhibitors have been shown to display enhanced background expression of several genes involved in the detoxification of furfural and HMF (namely *ZWF1*), comparing to non-tolerant strains (Liu et al., 2009). Therefore, to better understand the role of *PRS3*, *RPB4* and *ZWF1* in the adaptation of the robust *S. cerevisiae* CCUG53310 and PE-2 strains to lignocellulose-derived inhibitors, we investigated the effect of the simultaneous presence of acetic acid, furfural and HMF (in concentrations mimicking the composition of EGW hydrolysates) on their expression at different fermentation phases (Fig. 1).

By analysing the CO_2 production profiles of these strains in inhibitory and non-inhibitory fermentations (Fig. 1A), longer lag phases were necessary for the adaptation of both strains to the presence of acetic acid, furfural and HMF, as expected (Pereira et al., 2014a). The CO_2 production profile of CCUG53310 was clearly more affected by inhibitory conditions than that of PE-2, reflecting the superior capacity demonstrated by the later to detoxify furfural and HMF (Fig. 1B). Indeed, PE-2 was able to completely degrade these compounds until the early exponential phase (EP), while CCUG53310 did not (Fig. 1B). When characterizing the fermentation performance of these strains in real EGW hydrolysate,
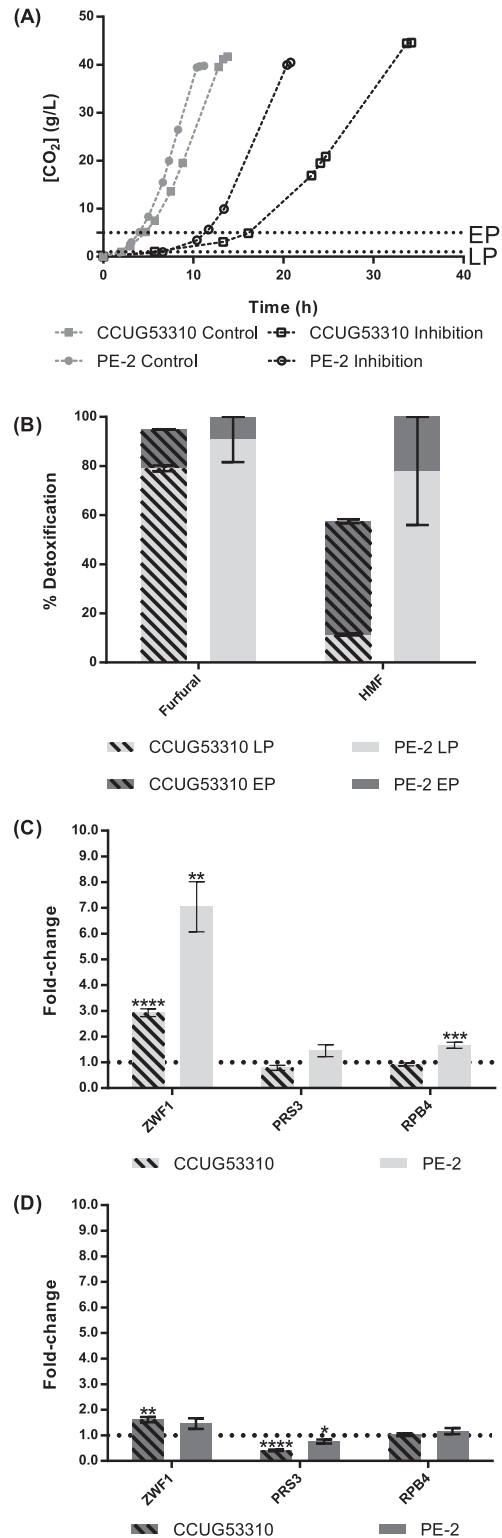

Fig. 1. Gene expression analyses of *S. cerevisiae* CCUG53310 and PE-2 during fermentations in the presence and absence of lignocellulose-derived inhibitors. (A) Profiles of CO_2 production from fermentations in YPD (Control) and in YPD supplemented with acetic acid (ca. 3.1 g/L), HMF (ca. 0.33 g/L) and furfural (ca.1.7 g/L) (Inhibition). Samples were collected at the late lag phase (LP) and early exponential phase (EP), corresponding to ca. 1 and 5 g/L of CO_2 produced, respectively (indicated by the dotted lines). (B) Furfural and HMF detoxification at LP and EP of fermentation in inhibitory media. (C, D) Differential expression of *PRS3*, *RPB4* and *ZWF1* at the LP (C) and EP (D) in inhibitory fermentations relative to the control (represented by the dotted line). Data represents the average ± SEM from two biological replicates. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.
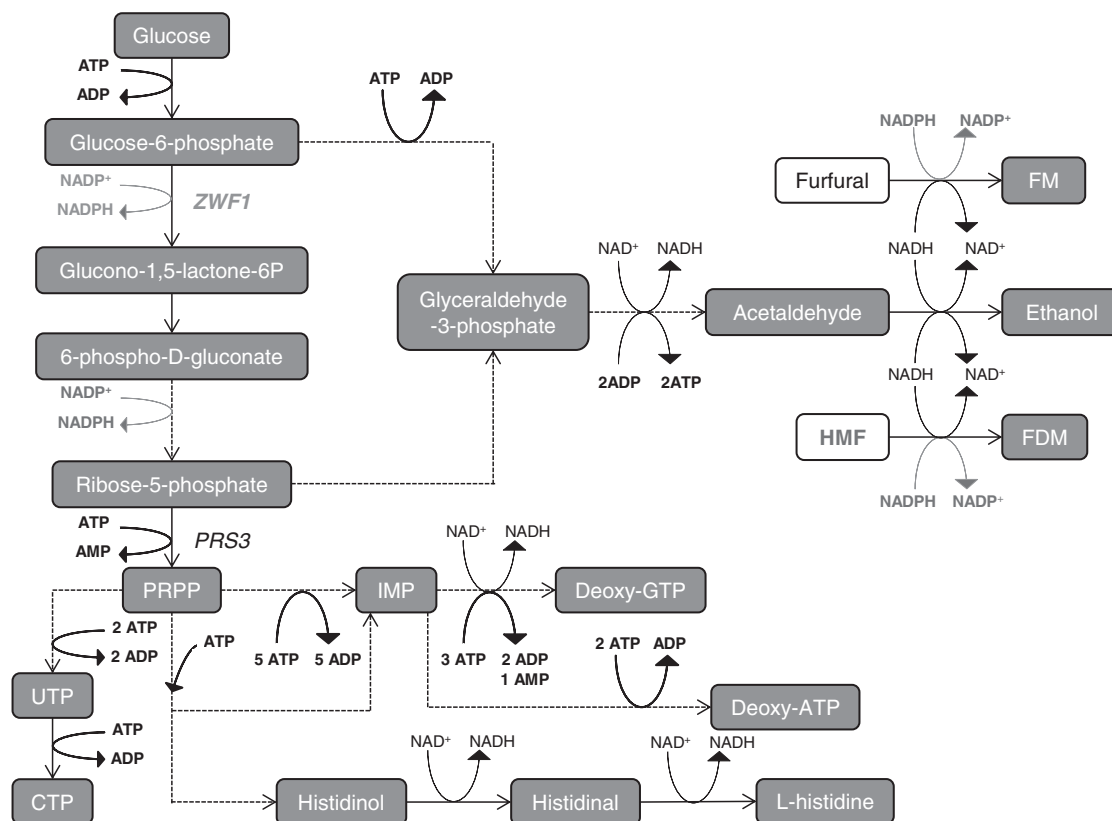
Fig. 2. Schematic representation of the glucose metabolic pathways and conversion of furfural and HMF by *S. cerevisiae*. Genes/cofactors involved in furfural and HMF detoxification are highlighted in black and grey, respectively. Bolded arrows and letters indicate ATP consuming/generating steps. PRPP: 5-phospho-ribosyl-1(alpha)-pyrophosphate. FM: 2-furanmethanol. FDM: 2,5-bis-hydroxymethylfuran[furan-2,5-dimethanol]. IMP: inosine monophosphate. UTP: uridine triphosphate. Deoxy-GTP: Deoxy-guanosine triphosphate.

Pereira et al. (2014a) previously reported a faster furfural detoxification rate of *S. cerevisiae* PE-2 comparatively to CCUG53310, which is in accordance with our observations.

Under anaerobic conditions, *S. cerevisiae* can convert furfural and HMF into less toxic corresponding alcohols (Fig. 2). Reduction of furfural is preferentially NADH-dependent, while reduction of HMF has been mainly associated with the consumption of NADPH (Wahlbom and Hahn-Hagerdal, 2002). These reductions provide sufficient NAD(P)$^+$ for NAD(P)H regeneration, maintaining a redox balance during the detoxification of these compounds (Liu et al., 2009). Overexpression of *ZWF1* has been shown to be important for the detoxification of furfural and HMF during the lag phase (Gorsich et al., 2006; Liu et al., 2009), probably by increasing the glucose metabolism flux in favour of the pentose phosphate pathway, and thus contributing to accelerate the NAD(P)H regeneration required to supply the cofactors needed for reduction of these inhibitors (Liu et al., 2009) (Fig. 2). Accordingly, at the late lag phase (LP), *ZWF1* expression was highly enhanced under inhibitory conditions in both CCUG53310 and PE-2 (Fig. 1C). At the early exponential phase (EP) (Fig. 1D), when HMF and furfural had been completely degraded by PE-2 (Fig. 1B), the expression of *ZWF1* in this strain reversed to levels similar to those of the control. In contrast, at this phase, *ZWF1* remained slightly up-regulated in the CCUG53310 strain (Fig. 1D), probably reflecting the still incomplete furfural and HMF detoxification (Fig. 1B).

PRS3 expression at the LP (Fig. 1C) was unaffected by the simultaneous presence of acetic acid, furfural and HMF, whereas at the EP, when mainly acetic acid (PE-2) or HMF and acetic acid (CCUG53310) were present (Fig. 1B), this gene was down-regulated (Fig. 1D). In fact, the presence of acetic acid, and corresponding inhibitory effect, was permanent during the inhibitory fermentations, with the acetic acid concentration varying in the range of 1.9-3.1 g/L (average content of 2.5 g/L). Two mechanisms have been proposed to explain the inhibitory effect of weak acids (Russell, 1992): intracellular anion accumulation and depletion of ATP (uncoupling theory). The first proposes that the accumulation of the dissociated form of the acid inside the cell (due to the low extracellular pH) leads to an intracellular acidification and consequent toxic effects at various levels of the cellular metabolism (Russell, 1992). The uncoupling theory states that the cytoplasmic pH decrease resultant from the inflow of weak acids activates the ATP-dependent proton pumps to neutralize the pH, leading to a depletion of ATP (Russell, 1992). Thus, when acetic acid was the main inhibitor present (EP), the *PRS3* expression was possibly down-regulated to save ATP, as Prs3p synthesizes 5-phospho-ribosyl-1(alpha)-pyrophosphate (PRPP) in an ATP-dependent reaction and PRPP is in turn a precursor of nucleotide and histidine biosynthesis, pathways with high ATP consumption (Fig. 2). At the LP this possible down-regulating effect of acetic acid was likely counteracted by the necessity of Prs3p to maintain a redox balance for furfural and HMF detoxification, as purine nucleotide and histidine biosynthesis are also important pathways for NADH regeneration (Fig. 2). Interestingly, as demonstrated by comparative transcription dynamic analyses, most of the differentially expressed genes under inhibitor challenging conditions show repressed response (Liu et al., 2009; Ma and Liu, 2010). In fact, down-regulated expression under these conditions has been suggested to provide efficient means of energy utilization for economic pathway development (Ma and Liu, 2010).
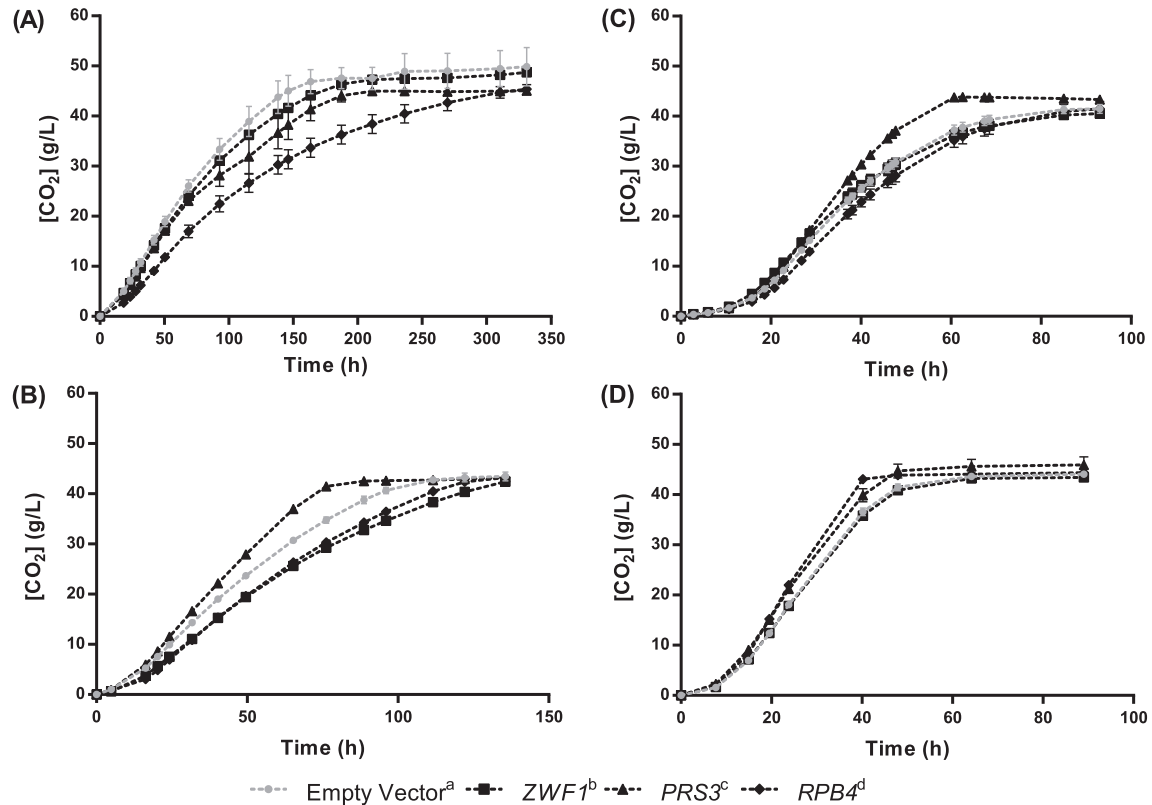
Fig. 3. CO_2 production profiles of *S. cerevisiae* CCUG53310 transformants (Empty Vector, and overexpressing *PRS3*, *RPB4* and *ZWF1*) in synthetic and real lignocellulosic hydrolysates with different inhibitors composition. Data represents the average ± range from two biological replicates. (A) EGW hydrolysate. a,d* (from 68.7 to 211.2 h). (B) Corn cob hydrolysate. a,b* (from 45.1 to 87.8 h). (C) Synthetic hydrolysate with acetic acid, HMF and furfural. a,c*. (D) Synthetic hydrolysate with furfural. a,d*. *$P < 0.05$.

Regarding the expression of *RPB4*, only at the LP of PE-2 fermentations was it altered by the simultaneous presence of acetic acid, furfural and HMF, where the synergistic effect of these inhibitors induced its expression by 1.7-fold (Fig. 1C). On the other hand, at the EP of the same fermentation, when only acetic acid was present, the expression of *RPB4* was unaltered (Fig. 1D). In fact, furfural and HMF were found to repress translation initiation in *S. cerevisiae* (Iwaki et al., 2013), a process that has been shown to be stimulated by Rpb4p/Rpb7p (Harel-Sharvit et al., 2010). Rpb4p also plays an important role in the transcription of genes involved in some stress responses (Sampath and Sadhale, 2005). Together, this may support the necessity for an up-regulated expression of *RPB4* at an early fermentation stage, when furfural and HMF were present. However, in the CCUG53310 strain the *RPB4* expression was unaffected by the presence of these stressors, which shows that different strains respond differently to the stress imposed by the toxic compounds present in lignocellulosic hydrolysates.

Consistent with the fact that *PRS3*, *RPB4* and *ZWF1* play important, but different, roles in yeast tolerance to the multiple stresses present in lignocellulosic biomass fermentation (Pereira et al., 2011, 2014b), their expression was here shown to be differentially susceptible to the presence of acetic acid, furfural and HMF (Fig. 1C and D). The different inhibitor concentrations at different fermentation stages were also shown to elicit distinct gene expression responses (Fig. 1B–D), which also varied between the two strains studied. *S. cerevisiae* PE-2, which displayed a faster adaptation to the inhibitory conditions here tested (Fig. 1A), as well as a more efficient furfural and HMF detoxification capacity than *S. cerevisiae* CCUG53310 (Fig. 1B), also presented a more highly induced expression of *ZWF1* and *RPB4* in the presence of acetic acid, furfural and HMF than the later (Fig. 1C), highlighting the importance of these genes for maximal yeast tolerance to these inhibitors.

3.2. Effect of PRS3, RPB4 and ZWF1 overexpression on the fermentation performance of S. cerevisiae CCUG53310 in lignocellulosic hydrolysates

Since the strain background and the hydrolysate inhibitory load have been shown to differentially influence the outcome of genetic manipulations targeting the improvement of strain resistance to lignocellulose-derived inhibitors (Alriksson et al., 2010; Park et al., 2011; Wallace-Salinas et al., 2014), we here assessed the effect of *PRS3*, *RPB4* and *ZWF1* overexpression on the fermentation performance of two already robust background strains under process-like conditions.

The CO_2 production profiles of *S. cerevisiae* CCUG53310 independently overexpressing *PRS3*, *RPB4* or *ZWF1* were compared with the control fermentation profiles in different real and synthetic hydrolysates (Fig. 3). For each hydrolysate, the lag phase, the maximum fermentation rate, the maximum CO_2 productivity and the final ethanol concentration of each transformant are indicated in Table 2.

PRS3 overexpression improved the *S. cerevisiae* CCUG53310 fermentation performance in all the hydrolysates tested, with the exception of EGW hydrolysate (Fig. 3, Table 2), where no significant differences where observed between the fermentative profiles of the *PRS3*-overexpressing strain and the control strain (Fig. 3A, Table 2). However, in the synthetic hydrolysate containing acetic acid, furfural and HMF in concentrations similar to those of the EGW hydrolysate (Fig. 3C), the overexpression of this gene led to a 20% faster fermentation rate and to a 48% higher CO_2 productivity than the control (Table 2). Similarly, *PRS3* overexpression also led to improved fermentation rate (32%) and CO_2 productivity (42%) in corn cob hydrolysate (Fig. 3B, Table 2). The total inhibitory load of the corn cob hydrolysate is lower than that of the EGW hydrolysate, particularly in what concerns acetic acid concentration

Table 2
Lag phase, maximum fermentation rate (Pr), maximum CO_2 productivity (Qp_{max}) and final ethanol concentration of each *S. cerevisiae* CCUG53310 transformant strains' fermentation in synthetic and real hydrolysates. CCUG53310 transformed with the empty vector (control; CC-Yep), and overexpressing *ZWF1* (CC-ZWF1), *PRS3* (CC-PRS3) and *RPB4* (CC-RPB4). $^*P < 0.05$; $^{**}P < 0.01$.

| Strains	Lag phase (h)	Pr (h^{-1})	Qp_{max} (g/(L h))	Ethanol (g/L)
EGW hydrolysate: 2.33 g/L acetic acid, 1.77 g/L furfural, 0.26 g/L HMF and 113 g/L glucose				
CC-Yep	18.3 ± 0.0	0.0352 ± 0.0002	0.254 ± 0.016	52.0 ± 5.6
CC-ZWF1	18.3 ± 0.0	$0.0319 \pm 0.0002^{**}$	0.224 ± 0.004	50.7 ± 0.7
CC-PRS3	18.3 ± 0.0	0.0298 ± 0.0066	0.213 ± 0.003	46.9 ± 0.6
CC-RPB4	18.3 ± 0.0	$0.0238 \pm 0.0018^*$	$0.144 \pm 0.005^*$	47.2 ± 1.3
Corn cob hydrolysate: 1.57 g/L acetic acid, 1.54 g/L furfural, 0.12 g/L HMF and 112 g/L glucose				
CC-YEp	16.3 ± 0.0	0.0570 ± 0.0048	0.384 ± 0.006	45.2 ± 1.3
CC-ZWF1	16.3 ± 0.0	0.0484 ± 0.0005	$0.313 \pm 0.005^{**}$	44.1 ± 0.7
CC-PRS3	16.3 ± 0.0	$0.0750 \pm 0.0010^*$	$0.544 \pm 0.007^{**}$	45.0 ± 0.4
CC-RPB4	16.3 ± 0.0	0.0505 ± 0.0014	$0.347 \pm 0.005^*$	45.0 ± 0.8
Syntethic hydrolysate 1: 2.71 g/L acetic acid, 1.28 g/L furfural, 0.34 g/L HMF and 120 g/L glucose				
CC-YEp	15.8 ± 0.0	0.102 ± 0.000	0.486 ± 0.013	43.1 ± 1.3
CC-ZWF1	15.8 ± 0.0	0.0995 ± 0.0023	0.474 ± 0.009	42.1 ± 0.8
CC-PRS3	15.8 ± 0.0	$0.122 \pm 0.004^*$	$0.721 \pm 0.010^{**}$	45.0 ± 0.9
CC-RPB4	15.8 ± 0.0	$0.0973 \pm 0.0005^{**}$	0.481 ± 0.024	43.1 ± 2.2
Syntethic hydrolysate 2: 1.95 g/L furfural and 112 g/L glucose				
CC-YEp	7.7 ± 0.0	0.137 ± 0.007	0.680 ± 0.001	45.8 ± 0.3
CC-ZWF1	7.7 ± 0.0	0.133 ± 0.002	$0.673 \pm 0.001^*$	45.2 ± 0.0
CC-PRS3	7.7 ± 0.0	0.144 ± 0.001	$0.934 \pm 0.041^*$	47.8 ± 2.3
CC-RPB4	7.7 ± 0.0	$0.179 \pm 0.004^*$	$1.07 \pm 0.02^{**}$	46.1 ± 0.0
Syntethic hydrolysate base: minimal medium with 120 g/L glucose				
CC-Yep	3.6 ± 0.0	0.297 ± 0.013	1.95 ± 0.01	46.2 ± 0.2
CC-ZWF1	3.6 ± 0.0	0.286 ± 0.005	1.91 ± 0.07	45.3 ± 1.8
CC-PRS3	3.6 ± 0.0	0.283 ± 0.009	1.95 ± 0.01	46.1 ± 0.3
CC-RPB4	3.6 ± 0.0	0.284 ± 0.001	1.90 ± 0.02	45.4 ± 0.6
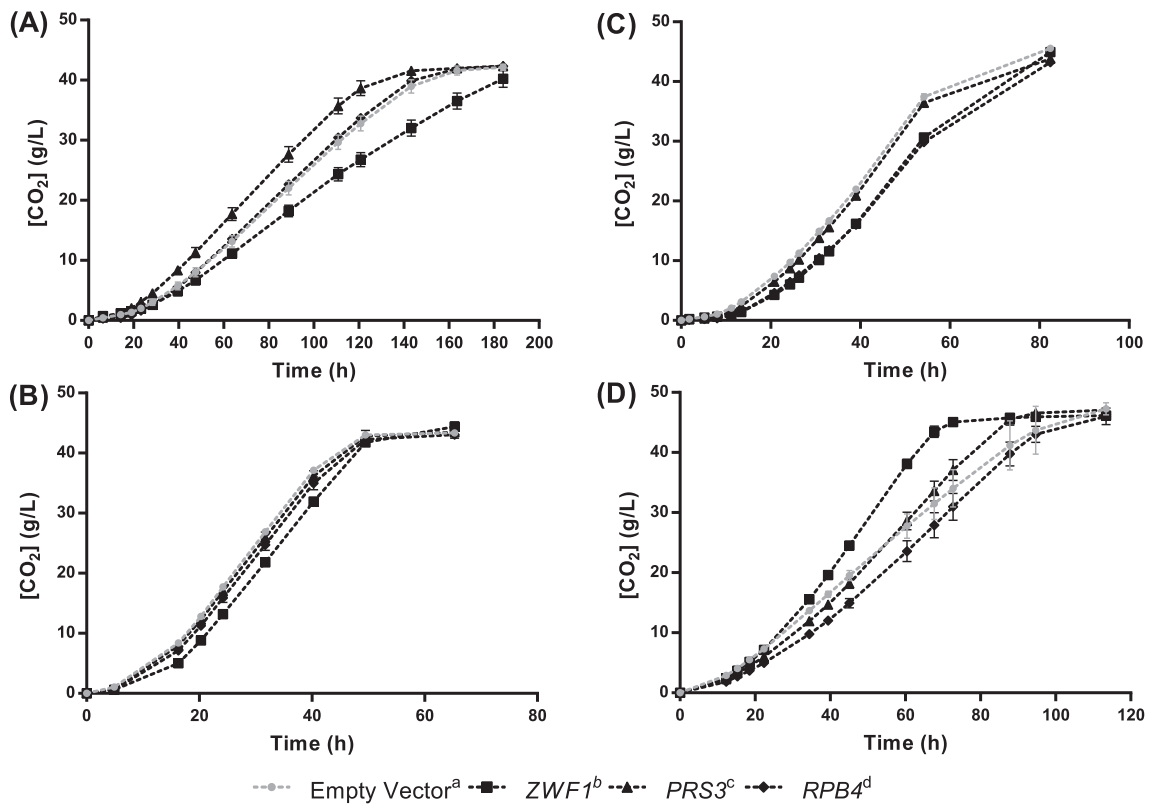
Fig. 4. CO_2 production profiles of *S. cerevisiae* PE-2 transformants (Empty Vector, and overexpressing *PRS3*, *RPB4* and *ZWF1*) in synthetic and real lignocellulosic hydrolysates with different inhibitors composition. Data represents the average ± range from two biological replicates. (A) EGW hydrolysate. a,b*** (from 88.8 to 163.6 h); a,c* (from 63.7 to 120.8 h). (B) Corn cob hydrolysate. a,b***. (C) Synthetic hydrolysate with acetic acid, HMF and furfural. a,b**; a,d**. (D) Synthetic hydrolysate with furfural. a,b* (from 45.1 to 87.8 h). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 3
Lag phase, maximum fermentation rate (Pr), maximum CO_2 productivity (Qp_{max}) and final ethanol concentration of each *S. cerevisiae* PE-2 transformant strains' fermentation in synthetic and real hydrolysates. PE-2 transformed with the empty vector (control; PE-Yep), and overexpressing *ZWF1* (PE-ZWF1), *PRS3* (PE-PRS3) and *RPB4* (PE-RPB4). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Strain	Lag phase (h)	Pr (h^{-1})	Qp_{max} (g/(L h))	Ethanol (g/L)
EGW hydrolysate: 1.80 g/L acetic acid, 1.11 g/L furfural, 0.24 g/L HMF and 111 g/L glucose				
PE-Yep	28.4 ± 0.0	0.0400 ± 0.0000	0.254 ± 0.007	43.8 ± 0.7
PE-ZWF1	28.4 ± 0.0	0.0338 ± 0.0017*	0.219 ± 0.011	41.8 ± 2.0
PE-PRS3	28.4 ± 0.0	0.0471 ± 0.0021*	0.290 ± 0.004*	44.1 ± 0.1
PE-RPB4	28.4 ± 0.0	0.0422 ± 0.0001***	0.256 ± 0.000	44.0 ± 0.3
Corn cob hydrolysate: 1.57 g/L acetic acid, 1.54 g/L furfural, 0.12 g/L HMF and 112 g/L glucose				
PE-YEp	16.3 ± 0.0	0.132 ± 0.002	0.869 ± 0.007	45.1 ± 0.4
PE-ZWF1	16.3 ± 0.0	0.123 ± 0.005	0.844 ± 0.003*	46.2 ± 1.1
PE-PRS3	16.3 ± 0.0	0.129 ± 0.003	0.865 ± 0.027	45.2 ± 1.5
PE-RPB4	16.3 ± 0.0	0.128 ± 0.001	0.854 ± 0.018	44.8 ± 1.0
Syntethic hydrolysate 1: 3.00 g/L acetic acid, 1.46 g/L furfural, 0.37 g/L HMF and 120 g/L glucose				
PE-YEp	13.4 ± 0.0	0.0977 ± 0.0017	0.553 ± 0.001	47.4 ± 0.1
PE-ZWF1	20.8 ± 0.0	0.0900 ± 0.0007*	0.546 ± 0.006	43.7 ± 0.5
PE-PRS3	13.4 ± 0.0	0.103 ± 0.001	0.532 ± 0.005*	45.6 ± 0.5*
PE-RPB4	20.8 ± 0.0	0.0893 ± 0.0001*	0.524 ± 0.001**	44.9 ± 0.1**
Syntethic hydrolysate 2: 2.52 g/L furfural and 106 g/L glucose				
PE-YEp	12.3 ± 0.0	0.0535 ± 0.0050	0.417 ± 0.009	49.2 ± 1.0
PE-ZWF1	12.3 ± 0.0	0.0895 ± 0.0010**	0.620 ± 0.005**	48.0 ± 0.3
PE-PRS3	12.3 ± 0.0	0.0632 ± 0.0034	0.518 ± 0.011**	48.9 ± 0.3
PE-RPB4	15.3 ± 0.0	0.0555 ± 0.0057	0.406 ± 0.012	47.9 ± 1.4
Syntethic hydrolysate base: minimal medium with 120 g/L glucose				
PE-YEp	6.5 ± 0.0	0.168 ± 0.003	1.12 ± 0.01	45.4 ± 0.4
PE-ZWF1	6.5 ± 0.0	0.176 ± 0.002	1.15 ± 0.01	47.1 ± 0.4*
PE-PRS3	6.5 ± 0.0	0.169 ± 0.008	1.13 ± 0.05	45.8 ± 2.5
PE-RPB4	6.5 ± 0.0	0.170 ± 0.002	1.13 ± 0.01	45.8 ± 0.4

(Table 2). As mentioned above, *PRS3* overexpression may contribute to increase the carbon flux in favour of metabolic pathways important for the regeneration of NADH, a cofactor required for the detoxification of furfural and HMF, and for ethanol production (Fig. 2). However, some of these pathways compete for ATP with cellular mechanisms that are activated to counteract the cytoplasmic acidification promoted by the inflow of acetic acid (Russell, 1992). Therefore, in the presence of relatively high concentrations of acetic acid, *PRS3* overexpression may not be particularly advantageous.

The overexpression of *RPB4* had contrasting effects over the fermentation performance of *S. cerevisiae* CCUG53310 in different inhibitory media (Fig. 3). Its overexpression was advantageous for the fermentation in synthetic hydrolysate containing only furfural (Fig. 3D), allowing a 57% higher CO_2 productivity and a 31% faster fermentation rate than the control. However, *RPB4* overexpression negatively affected the fermentation performance of *S. cerevisiae* CCUG53310 in more complex hydrolysates (Table 2). In the synthetic hydrolysate containing acetic acid, furfural and HMF (Fig. 3C), the overexpression of *RPB4* slowed down the CCUG53310 fermentation rate (Table 2). *RPB4* overexpression also reduced the CCUG53310 fermentation rate in EGW hydrolysate by 48%. Furthermore, the CO_2 productivity in corn cob and EGW hydrolysate fermentations was also negatively affected by the overexpression of *RPB4* (Table 2). Therefore, although *RPB4* overexpression was advantageous for the fermentation performance of *S. cerevisiae* CCUG53310 at relatively high initial furfural concentrations, under more complex inhibitory conditions it had a deleterious effect over this strain physiology.

ZWF1 overexpression in laboratorial *S. cerevisiae* strains was previously described to allow growth in the presence of otherwise lethal concentrations of furfural (Gorsich et al., 2006) and to improve furfural and HMF conversion rates, with consequent increase in ethanol production and cell growth (Park et al., 2011). Nevertheless, *ZWF1* overexpression was here shown to negatively affect the fermentation performance of the *S. cerevisiae*

CCUG53310 strain in real hydrolysates (Fig. 3A and B, Table 2). It is worth noting that this gene is already naturally overexpressed by CCUG53310 upon exposure to lignocellulosic inhibitors (Figs. 1C and D), and our results show that its additional overexpression brings no extra advantage.

3.3. Effect of PRS3, RPB4 and ZWF1 overexpression on the fermentation performance of S. cerevisiae PE-2 in lignocellulosic hydrolysates

The CO_2 production profiles of *S. cerevisiae* PE-2 independently overexpressing *PRS3*, *RPB4* or *ZWF1* were also compared with control fermentation profiles in different real and synthetic hydrolysates (Fig. 4). For each hydrolysate, the lag phase, the maximum fermentation rate, the maximum CO_2 productivity and the final ethanol concentration of each transformant are indicated in Table 3.

As seen in Fig. 4A and Table 3, the overexpression of *PRS3* improved the *S. cerevisiae* PE-2 fermentation rate (18%) and CO_2 productivity (14%) in EGW hydrolysate. A 24% increase on the CO_2 productivity of the PE-2 *PRS3*-overexpressing strain was also observed in synthetic hydrolysate containing only furfural at a relatively high concentration (Fig. 4D, Table 3). Similarly, the PE-2 CO_2 productivity in this hydrolysate was also significantly improved (49%) by the overexpression of *ZWF1*, as was its fermentation rate (Fig. 4D, Table 3). However, *ZWF1* overexpression had a negative effect over the *S. cerevisiae* PE-2 fermentation performance in all the other hydrolysates tested (Fig. 4, Table 3). Depending on the inhibitory composition of the hydrolysate, the overexpression of *RPB4* also had contrasting outcomes in the fermentation performance of PE-2. While in synthetic hydrolysates its overexpression increased the lag-phase, in EGW hydrolysate it slightly improved the PE-2 fermentation rate (Table 3).

Similarly to what was observed in the *S. cerevisiae* CCUG53310 strain, *ZWF1* and *RPB4* overexpression in PE-2 also resulted mainly in negative or no effects over its fermentation performance. On the other hand, *PRS3* overexpression produced more consistent and

advantageous effects over the fermentation capacities of these strains.

The overexpression of *PRS3*, *RPB4* and *ZWF1* was here shown to present different outcomes that were clearly dependent on the inhibitory composition of the fermentation media, but also on the background strain. For instance, in the hydrolysate containing only furfural, the overexpression of *RPB4* had a positive effect in CCUG53310 (Fig. 3D), but a negative effect in the PE-2 strain (Fig. 4D). Since Rpb4p has been suggested to be involved in the regulation of the cellular response to certain stress conditions (Sampath and Sadhale, 2005), our results hint at the existence of regulatory differences between these strains. In fact, an alignment of the promoter region of *PRS3*, *RPB4* and *ZWF1* from several laboratory and industrial background *S. cerevisiae* strains (S288, SIGMA1278b, CEN.PK113-7D, AWRI1631, EC1118, CBS7960 and JAY291, haploid derivative of PE-2) revealed relevant differences between strains, namely in terms of TATA box location and transcription factors' binding sites. Moreover, slight differences in the amino acid sequence of Zwf1p were also found between these strains and Moon and Liu (2012) showed that even slight alterations in the coding sequence of another gene involved in furfural and HMF detoxification, *GRE2*, resulted in increased growth rates in the presence of HMF. Although the CCUG53310 genome sequence is not publically available, similar differences may also exist between this strain and PE-2, which may help explain the different responses of CCUG53310 and PE-2 to similar fermentation conditions and to the overexpression of *PRS3*, *RPB4* and *ZWF1*.

Considering the results presented here, it is clear that the engineering of yeast strains for increased tolerance to inhibitory lignocellulosic biomass must be carefully addressed, considering the different background of each *S. cerevisiae* strain and the specific composition of the raw material and hydrolysate to use.

4. Conclusions

S. cerevisiae CCUG53310 and PE-2 were here shown to differentially express *PRS3*, *RPB4* and *ZWF1* in response to different lignocellulosic inhibitor loads. Increased expression of *ZWF1* and *RPB4* in the presence of acetic acid, furfural and HMF was found to contribute to yeast adaptation to these inhibitors, but their overexpression did not improve the fermentation performance in real lignocellulosic hydrolysates. In contrast, *PRS3* overexpression improved the fermentation rate and productivity of both strains. The heterogeneous outcomes of these genes overexpression in different hydrolysates show that tolerance engineering must be customized to the strain background and hydrolysate used in the process.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.05.006.

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