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Evolutionary Engineering of *Saccharomyces cerevisiae* for Enhanced Tolerance to Hydrolysates of Lignocellulosic Biomass

María P. Almario, Luis H. Reyes, Katy C. Kao

Department of Chemical Engineering, Texas A&M University, 3122 TAMU, College Station, Texas, 77843-3122; telephone: +979-845-5571; fax: +979-845-6446; e-mail: kao.katy@gmail.com

ABSTRACT: Lignocellulosic biomass has become an important feedstock to mitigate current ethical and economical concerns related to the bio-based production of fuels and chemicals. During the pre-treatment and hydrolysis of the lignocellulosic biomass, a complex mixture of sugars and inhibitors are formed. The inhibitors interfere with microbial growth and product yields. This study uses an adaptive laboratory evolution method called visualizing evolution in real-time (VERT) to uncover the molecular mechanisms associated with tolerance to hydrolysates of lignocellulosic biomass in *Saccharomyces cerevisiae*. VERT enables a more rational scheme for isolating adaptive mutants for characterization and molecular analyses. Subsequent growth kinetic analyses of the mutants in individual and combinations of common inhibitors present in hydrolysates (acetic acid, furfural, and hydroxymethylfurfural) showed differential levels of resistance to different inhibitors, with enhanced growth rates up to 57%, 12%, 22%, and 24% in hydrolysates, acetic acid, HMF and furfural, respectively. Interestingly, some of the adaptive mutants exhibited reduced fitness in the presence of individual inhibitors, but showed enhanced fitness in the presence of combinations of inhibitors compared to the parental strains. Transcriptomic analysis revealed different mechanisms for resistance to hydrolysates and a potential cross adaptation between oxidative stress and hydrolysates tolerance in several of the mutants.

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KEYWORDS: lignocellulosic biomass; HMF; furfural; acetic acid; evolutionary engineering; complex phenotypes; genomics

Introduction

There is an increased interest in the use of renewable feedstock for the production of second-generation biofuels and chemicals. Lignocellulosic biomass is an abundant renewable resource that is estimated to reach more than 1 billion dry tons annually in the US by year 2030 (Energy, 2011). In general, the lignocellulosic biomass needs to be pretreated and hydrolyzed to convert its cellulose and hemicellulose into simple sugars for fermentation. The breakdown of lignin produces phenolic compounds (Almeida et al., 2007). Hydrolysates of lignocellulosic biomass are complex mixtures of different pentose and hexose sugars, inhibitors (e.g., furfural, 5-hydroxymethyl furfural, phenolic compounds, etc.), and salts (e.g., $MgSO_4$, NaCl) (Mosier et al., 2005; Sun and Cheng, 2002). Dilute acid pretreatment is a commonly used method to facilitate enzymatic hydrolysis and improve sugar yield (Chen et al., 2012; Mosier et al., 2005). The pretreatment process generates numerous inhibitors that affect cellular growth and fermentation performance (Almeida et al., 2007; Liu et al., 2008; Ma and Liu, 2010). The byproducts generated after dilute acid pretreatment that have the most potent inhibitory effects include acetic acid (AA), formic acid, phenolic compounds and the furan aldehydes furfural and 5-hydroxymethylfurfural (HMF) (Larsson et al., 2001; Liu et al., 2004; Martin et al., 2007). AA is formed by de-acetylation of hemicelluloses (Almeida et al., 2007) and several mechanisms have been determined to be involved in acetic acid tolerance in yeast (Casal et al., 1996; Ludovico et al., 2002). The furans are derived from dehydration of hexoses and pentoses (Almeida et al., 2007; Liu et al., 2004). The inhibitors present in hydrolysates cause reduction in biological and enzymatic functions in yeast and *E. coli* (Liu et al., 2004; Modig et al., 2002; Sanchez and Bautista, 1988). In addition, synergistic effects between different inhibitors have been found to further enhance the negative impact on the growth rate of *Saccharomyces cerevisiae* (Liu et al., 2004, 2008). Several mechanisms to overcome inhibition have been identified, one of which is the *in situ* detoxification of HMF and furfural to less toxic compounds, furan di-methanol and

Data Availability: The microarray data has been deposited in the Gene Expression Omnibus with accession number GSE44085.
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furan methanol respectively, through NADPH-dependent reductions (Almeida et al., 2007; Liu et al., 2008; Ma and Liu, 2010). Mechanisms to overcome the toxic effects of weak acids, such as acetic acid, appear to be complex, involving the reduced uptake of extracellular acetate, increased activity of plasma membrane H^+-ATPase and efflux of the acetate through multidrug resistance transporters (MDR) (Mira et al., 2010; Zhang et al., 2011). However, the basic mechanisms to overcome inhibition in the presence of the complex mixtures of potential inhibitors present in hydrolysates of lignocellulosic biomass are not well known (Almeida et al., 2007; Liu et al., 2004, 2005).

The purpose of this study is to understand the mechanisms associated with tolerance of *S. cerevisiae* to hydrolysates of lignocellulosic biomass, using the adaptive laboratory evolution method called visualizing evolution in real time (VERT) (Reyes et al. 2012a, b). During adaptive evolution, mutants with enhanced fitness (adaptive mutants) arise and expand in the population. We refer to these expansions as adaptive events. VERT helps to identify adaptive events in an evolving population via tracking the changes in the relative frequencies of differentially labeled colored-subpopulations using flow cytometry. Thus, an observed expansion in a colored-subpopulation is indicative of the rise and expansion of an adaptive mutant within the expanding subpopulation. Adaptive mutants can then be isolated from the expanding colored-subpopulation for subsequent analyses. Using VERT, *S. cerevisiae* was evolved in the presence of hydrolysates and individual adaptive mutants were isolated throughout the course of the evolution. The relative resistances of the isolated adaptive mutants to the different inhibitory compounds found in the hydrolysates were assessed. In conjunction with the phenotypic analysis, transcriptome analysis was conducted for the different isolated mutants to elucidate the molecular mechanisms involved in hydrolysates tolerance.

Materials and Methods

Strains and Growth Conditions

Fluorescently marked *S. cerevisiae* strains were derived from FY2, a derivative of S288c, as previously described (Kao and Sherlock, 2008). Unless otherwise specified, yeast cells were cultured in YNB supplemented with no carbon source and mixed with different concentrations of hydrolysates (see Tables I and II) of lignocellulosic biomass at 30°C.

Preparation of Hydrolysates of Lignocellulosic Biomass

Hydrolysates of lignocellulosic biomass were obtained using dilute acid pretreated biomass slurry of corn stover (courtesy of Dr. Dan Schell and Dr. John Ashworth at the National Renewable Energy Laboratory). A determined amount (g) of this slurry (with solids loading of roughly 35%) was diluted in DI water for enzymatic hydrolysis. The detailed enzymatic hydrolysis procedure can be found in Supplementary

Table I. Concentration of each inhibitor and sugar present in various batches of hydrolysates.

Batch hydrolysates	Glucose % (w/v)	Xylose %(w/v)	Arabinose % (w/v)	Concentration of HMF (g/L)	Concentration of furfural (g/L)	Concentration of acetic acid (mM)
Batch 1	0.58	1.03	0.16	0.88	0.03	9.27
Batch 2	0.21	0.63	0.08	0.60	0.02	6.68
Batch 3	0.58	1.78	0.26	2.38	0.07	20.34
Batch 4	0.67	2.00	0.29	2.79	0.07	23.47
Batch 5	0.67	2.67	0.4	3.00	0.07	31
Batch 6	0.88	2.57	0.38	3.18	0.08	29.17

Table II. Composition of selective media used for evolution and phenotypic analyses.

Generation		Hydrolysates concentration								
From	To	Batch[b]	Dilution with YNB (%)[c]	Selective media	Glucose[a] % (w/v)	Xylose[a] % (w/v)	Arabinose[a] % (w/v)	Acetic acid[a] (mM)	HMF[a] (g/L)	Furfural[a] (g/L)
0	132	1	17	A	0.1	0.18	0.03	1.60	0.15	0.01
138	157	2	48	B	0.1	0.30	0.04	3.18	0.29	0.01
164	178	3	17	C	0.1	0.31	0.04	3.51	0.41	0.01
186	197	4	15	D	0.1	0.30	0.04	3.50	0.42	0.01
206	218	4	16	E	0.1	0.32	0.05	3.98	0.41	0.01
225	246	3	25	F	0.14	0.445	0.06	5.08	0.59	0.01
253	285	4	25	G	0.17	0.53	0.07	6.17	0.67	0.01
292	338	5	25	H	0.17	0.67	0.10	7.75	0.75	0.02
344	463	6	25	I	0.22	0.642	0.10	7.30	0.8	0.02

[a]Actual concentration in each batch of selective media.
[b]Batch number of hydrolysates from Table I used for preparation of selective media.
[c]Percent by volume of hydrolysate used in dilution with YNB (without glucose supplementation).

Materials and Methods. The composition of each batch of hydrolysates after enzyme hydrolysis was quantified (details below) and listed in Table I. The resulting hydrolysates were filter sterilized using 0.22 μm filters and subsequently diluted in YNB with no carbon source (5.95 g YNB without amino acids or ammonium sulfate and 17.5 g ammonium sulfate). The composition of each batch of hydrolysates/YNB mixture is listed in Table II. Hydrolysates composition was analyzed using high-performance liquid chromatography (HPLC; AgilentTechnologies, 1260 Infinity, Santa Clara, CA) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA). Glucose, xylose, arabinose, acetic acid and ethanol were quantified using the RI detector at 52°C, using 0.6 mL/min of 5 mM H_2SO_4 as mobile phase. Furfural and HMF were detected using the UV/vis detector at 254 nm.

Adaptive Evolution Experiment

The adaptive evolution experiments were performed in 125 mL flasks at 30°C in 10 mL of hydrolysates/YNB with three parallel populations. Roughly equal numbers of the three fluorescently marked (GFP, YFP, and DsRed) strains were used to seed each population. A serial transfer of 1% of the total volume into fresh medium was performed every 24–48 h. Samples were taken prior to each serial transfer to monitor the relative proportions of the three colored-subpopulations using a flow cytometer (FACS) (BD FACScan^{TM}) and to generate frozen stocks to be stored at −80°C in 17.5% glycerol for further analysis. The concentration of hydrolysates was increased in a step-wise fashion (see Table II). Table II lists the concentrations of sugars and inhibitors in each batch of selective media (selective media *A*–*I*) used for the evolution experiments. The concentration of hydrolysates was increased when an adaptive event (increase in more than two consecutive measurements in the relative proportion of a colored-subpopulation) was observed. This experiment was conducted for approximately 463 generations.

Isolation of Adaptive Mutants

For the isolation of adaptive mutants responsible for each of the observed adaptive events, five clones from each expanding colored-subpopulation were isolated and their growth kinetics measured in selective media *G* (hydrolysates/YNB medium) (see Table II) in 96-well plates using a microplate reader (TECAN^{TM} Infinite M200) at 30°C. The parameter "Relative increase in maximum specific growth rate" (Reyes et al., 2012a) for each clone was calculated using Equation (1), , where μ_i is the maximum specific growth rate for strain *i*.

Relative increase in μ_i

$$\frac{(\mu_{i@\text{Hydro}}/\mu_{i@\text{No Hydro}}) - (\mu_{\text{wildtype@Hydro}}/\mu_{\text{wildtype@No Hydro}})}{\mu_{\text{wildtype@Hydro}}/\mu_{\text{wildtype@No Hydro}}} \quad (1)$$

The clone with the highest "relative increase in μ_i" was chosen as the adaptive clone from the expanding subpopulation. Student's *t*-test using four biological replicates was used to assess significance.

The relative fitness coefficient, *s*, for each mutant was determined using Equation (2).

$$s = \left(\frac{\mu_{\text{adaptive}_{\text{mutant}}}}{\mu_{\text{wildtype}}}\right) - 1 \quad (2)$$

Thus a mutant with a relative fitness coefficient greater than "0" has higher fitness compared with the parental strain at that condition. Statistical significance for *s* was assessed by testing the null hypothesis that $\mu_{\text{adaptive\_mutant}}$ was not higher (or lower, depending on the sign of *s*) than $\mu_{\text{wild-type}}$ using a one-tailed student *t*-test (with *P*-value threshold of 0.05).

Growth Kinetics

Three biological replicates of the selected isolated mutants were grown in 125 mL flasks in batch cultures to determine the relative fitness coefficient, *s*, of each mutant against its parental strain in different conditions. The growth kinetics for each culture was determined by monitoring the OD_{600} every 2 h from an initial OD_{600} of ~0.05. The relative fitness coefficient *s* was determined using Equation (2). The analyzed conditions were: (a) hydrolysates of biomass with YNB, (b) YNB supplemented with 1% (w/v) glucose, (c) YNB supplemented with 1% (w/v) glucose and 0.9 g/L HMF, (d) YNB supplemented with 1% (w/v) glucose and 50 mM acetic acid, (e) YNB supplemented with 1% (w/v) glucose and 0.9 g/L HMF and 0.4 g/L furfural, and (f) YNB supplemented with 1% (w/v) glucose and 0.4 g/L furfural. Cultures grown in condition (b) were also analyzed for their rates of glucose consumption using the HPLC.

Results and Discussion

Evolutionary Dynamics of *S. cerevisiae* During the Adaptive Evolution in Hydrolysates of Lignocellulosic Biomass

Approximate equal numbers of fluorescently (GFP, YFP and DsRed) marked strains were used to seed three hydrolysates-challenged populations (P1, P2, and P3) in batch cultures in selective media *A* (Table II). The concentration of the hydrolysates was increased in a step-wise manner over the course of 463 generations (see Table II and Fig. 1). The relative proportions of the three different colored-subpopulations were monitored using FACS. As described in (Reyes et al., 2012a), the expansions and contractions of different colored-subpopulations observed using VERT are the results of rise and expansions of adaptive mutants (fitter mutants) within the different colored-subpopulations. Thus, an expansion in a colored-subpopulation is indicative of the occurrence and expansion of an adaptive mutant in that colored-subpopulation, and fitter mutants can then be isolated from the expanding colored-subpopulation for subsequent analyses.

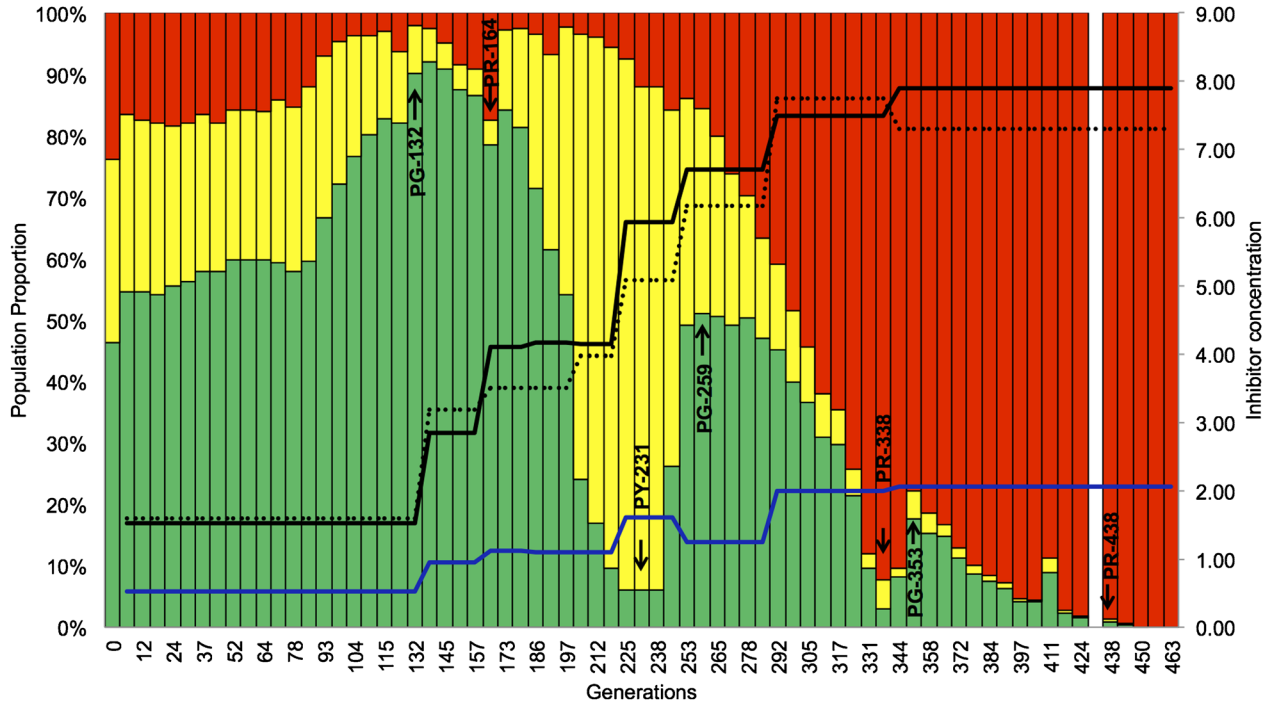

Figure 1. The evolutionary dynamics of population P3 during *in vitro* evolution in hydrolysates-challenged condition. The yellow bars indicate the relative proportion of the yellow subpopulation, green bars indicate the proportion of the green subpopulation, and red bars indicate the proportion of the red subpopulation. The black line indicates the approximately concentration of HMF(×10, g/L), black dashed line corresponds to acetic acid (mM), and blue line is furfural (×100, g/L). The arrows indicate where the adaptive mutants were isolated.

Upon conclusion of the adaptive evolution experiments, population P3, which reached the highest concentration of hydrolysates, was chosen for further analysis. Seven adaptive events were identified in P3, where the expanding subpopulation reached their maximum proportion at generations 132, 164, 231, 259, 338, 353, and 438 (Fig. 1). To isolate the adaptive mutants responsible for each expansion, five clones were randomly picked from each expanding subpopulation at the above-mentioned generations. The clone with the highest specific growth rate in hydrolysates/YNB was chosen as the adaptive mutant from the expanding subpopulation. These adaptive mutants were named according to their color and generation from which they were isolated: PG-132, PR-164, PG-259, PY-231, PR-338, PG-353, and PR-438.

Relative Fitness in the Presence of Hydrolysates and Individual Inhibitors

The growth kinetics for each adaptive mutant was determined in batch cultures to compare their growth rates against their respective parental strain (of the same color) in several different conditions and their relative fitness coefficients were calculated. First, the specific growth rates of the mutants and parental strains were measured in selective media *H* (Table II). Six out of seven of the isolated adaptive mutants showed a statistically significant positive relative fitness coefficient compared with the parental strain (see Fig. 2). The largest improvement was observed in mutant PR-164 with a 57% improvement in specific growth rate over the parental strain in hydrolysates. Since hydrolysates of biomass contain a complex mixture of inhibitors, adaptation of the evolved mutants may be a result of enhanced resistance to one or more of the inhibitors present. To determine which of the most commonly studied inhibitors (furfural, HMF, and AA) the evolved mutants have enhanced resistance to, the relative fitness coefficients of each strain in the presence of each of the inhibitors were determined in YNB supplemented with 1% (w/v) glucose (see Fig. 2). All but one mutant (PY-231) showed enhanced fitness in at least one of the conditions tested. The concentration of HMF used in the test was 0.9 g/L (the highest concentration of HMF in the hydrolysate was 0.8 g/L). Since the highest concentration of furfural present in the hydrolysates (0.02 g/L) was not inhibitory by itself, the relative fitness coefficient measurements were carried out in a concentration of 0.4 g/L of furfural. Adaptive mutants PG-132 and PR-164 showed a statistically significant increase in relative fitness in the presence of furfural, and mutants PR-164 and PR-438 exhibited higher tolerance to HMF compared to their parental strains. All adaptive mutants from the red subpopulation (PR-164, PR-338, and PR-438)

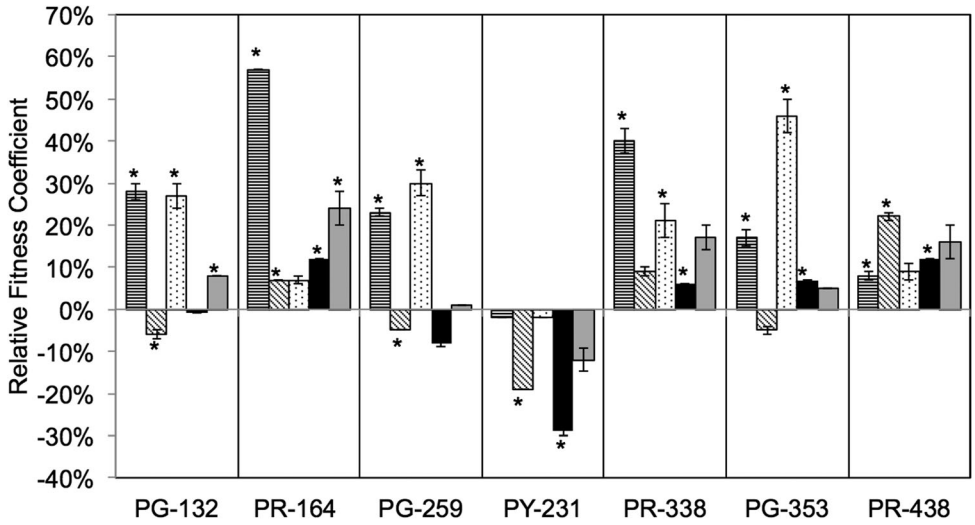

Figure 2. The relative fitness coefficients of isolated mutants from population P3 in the presence of each inhibitor. Horizontal lined bars: Selective media *H* (hydrolysates in YNB), diagonal lined bars: HMF (0.9 g/L), dotted bars: HMF (0.9 g/L) and furfural (0.4 g/L), solid black bars: AA (50 mM), and solid gray bars: furfural (0.4 g/L). YNB supplemented with 1% glucose is the base media used for conditions containing individual inhibitors. Asterisks indicate statistical significance between the specific growth rates of isolated mutant and parental strains with a *P*-value threshold of 0.05 (using a one-tailed student *t*-test with a minimum of three biological replicates).

and the green mutant PG-353 showed higher tolerance to AA (50 mM). Adaptive mutants PR-164 and PR-438 were the most resistant to AA with relative fitness coefficients of 12%. In addition to individual inhibitors, we also tested the resistances of each mutant in the presence of both HMF (0.9 g/L) and furfural (0.4 g/L). Four out of seven adaptive mutants showed statistically significantly improved fitness in the presence of both HMF and furfural, with PG-353 exhibiting the highest tolerance with a fitness improvement of 46% over the parental strain.

Transcriptome Analysis

Transcriptome analysis using DNA microarrays was used to identify any differences in relative transcript abundances between the isolated mutants and the parental strains in the presence of hydrolysates. The results showed significant perturbations in different metabolic pathways in the mutants, and there exists significant differences in transcriptional regulation between linages. The transcriptome data was analyzed based on the individual performance of each adaptive mutant to the major inhibitors present in hydrolysates. Some of the key perturbations are described below. Transcription factors activities were assessed using Network Component Analysis (NCA) (Liao et al., 2003; Tran et al., 2005) (see Supplemental Materials and Methods for more informationon microarray data analysis).

Acetic Acid

The adaptive mutants with a statistically significant positive relative fitness coefficient against their parental strains in the presence of AA were PR-164, PR-338, PG-353 and PR-438. Commonly up-regulated genes between these mutants were *ATP5*, *VMA3*, *VPH1*, and *SPI1*. *ATP5* is a required gene for ATP synthesis-coupled proton transport. *VPH1*and *VMA3* are genes involved with vacuolar ATPase acidification. *SPI1* is a gene involved in weak acid resistance and its expression is controlled by the transcription factors Msn2p/Msn4p (Teixeira et al., 2006). Depletion of ATP (uncoupling theory) and intracellular anion accumulation have been suggested to be responsible for the toxicity effects of weak acids (Russell, 1992). The uncoupling theory states that the decrease in cytoplasmic pH generated by the influx of AA results in the activation of ATP-dependent proton pumps to neutralize the pH, leading to a depletion of ATP (Russell, 1992). The intracellular anion accumulation theory, on the other hand, states that accumulation of dissociated AA inside the cell (since only protonated or associated AA can pass across the cytoplasmic membrane) due to the lower extracellular pH causes a toxicity effect (Palmqvist and Hahn-Hägerdal, 2000). Thus, the overexpression of ATP synthase and H^+-ATPase observed in the adaptive mutants are potential mechanisms for their enhanced tolerance to AA. Indeed, the H^+-ATPase present in the vacuolar membrane has been shown to be important for weak acid tolerance (Mira et al., 2010). Table III shows selected up-regulated or down-regulated genes for the isolated mutants related with acetic acid tolerance. Detoxification through multidrug resistance transporters (MDR) has also been found to play a role in yeast tolerance to weak acids (Mira et al., 2010). Using NCA, we identified several transcription factors whose activities were significantly perturbed. The activities of MSN4, RIM101, PDR1, and PDR3 were increased and the

Table III. Selected up-regulated (bold) or down-regulated (non-bolded) genes in the isolated mutants related with acetic acid tolerance.

Strain	Cell wall and protein function	Lipid metabolism	Plasma membrane and vacuolar H+ATpase
PR-164	**HSP82**		**ATP15, ATP16, ATP17, ATP5**
PR-338			**VMA3, ATP14, ATP5, VPH1**
PG-353	SPI1	**YPC1, YDC1, PDR16**	**VPH1, PMP1, ATP5, ATP4**
PR-438	**SPI1, HSP78, HSP104, SSE2**	**YPC1**	**SIA1, ATP14, ATP17, ATP4, ATP5**

p-value < 0.05.

Table IV. Transcription factors whose activities were increased (bold) or decreased (non-bold) in adaptive mutants associated with resistance to acetic acid.

Strain	Transcription factors
PR-164	**MSN4,** WAR1, **RIM101, PDR1**
PR-338	WAR1, **PDR1**
PG-353	**MSN4,** WAR1,**PDR1, PDR3**
PR-438	**MSN4,** WAR1,**PDR3**

activity of WAR1 was decreased (Table IV). All aforementioned transcription factors have been found to be related with adaptive response of yeast to drug and weak acids (Kolaczkowska and Goffeau, 1999; Mira et al., 2010).

HMF and Furfural

HMF and furfural affect cell growth by similar mechanisms, including inhibition of alcohol dehydrogenase (*ADH*), pyruvate dehydrogenase (*PDH*), and aldehyde dehydrogenase (*ALDH*) and damages to cell membrane (Almeida et al., 2007). Adaptive mutants PG-132 and PR-164 were statistically significantly better than the wild type in 0.4 g/L furfural. Additionally, adaptive mutants PR-164 and PR-438 (from the red subpopulation) have positive relative fitness coefficients in presence of 0.9 g/L HMF. Interestingly, adaptive mutants PG-259, PR-338, and PG-353 have statistically significant positive fitness coefficients only in the combination of HMF and furfural. Table V shows a summary of the differentially regulated genes in the mutants that are potentially associated with HMF and/or furfural tolerance.

S. cerevisiae has the ability to convert furfural to furan methanol and HMF to furan di-methanol using *in situ* detoxification as a response mechanism in the presence of these inhibitors (Judy et al., 2008; Liu et al., 2004, 2008; Ma and Liu, 2010). It has previously been proposed that the detoxification is through multiple NADPH-dependent aldehyde reduction, such as the reductases ALD4 and GRE3 (Liu et al., 2008), which was up-regulated in mutant PR-164, potentially causing NAD(P)H depletion. Serine, lysine, and arginine biosynthesis were down-regulated in all adaptive mutants with enhanced tolerance to HMF (PR-164 and PR-438). The repression of these amino acid biosynthesis pathways have been found to increase regeneration of ATP and NAD(P)H in the TCA cycle (Ma and Liu, 2010), possibly as compensatory mechanism for NAD(P)H depletion.

Transcription factors involved in pleiotropic drug resistance (PDR) and oxidative stress tolerance were significantly activated in almost all of the adaptive mutants with enhanced tolerance to furfural and/or HMF (see Table VI), with the latter suggesting the presence of oxidative stress during growth on hydrolysate. To determine which of the isolated adaptive mutants are also more tolerant to oxidative stress, the growth kinetics of each of the adaptive mutants were determined in the presence of 2 mM of H_2O_2. All the adaptive mutants from the red subpopulation (PR-164, PR-338, and PR-438) and one mutant from the green subpopulation (PG-353) showed significantly enhanced tolerance to hydrogen peroxide compared to their parental strain (see Table VII). In addition, all these adaptive mutants have also enhanced tolerance to AA. All the other adaptive mutants did not show a significant improvement in the presence of hydrogen peroxide. The heat map of expressions from select genes for the adaptive mutants from the red lineage (Fig. 3) shows that the higher up-regulated genes correspond to drug resistance (*IMD2*), cytochrome *c* oxidase (*COX13, COX9*), DNA replication stress (*SAP155*), phosphate metabolism (*PHO11*), and mitochondrial inner membrane protein (*FCJ1*). The most down-regulated genes are related with cell wall mannoprotein of the Srp1p/Tip1p family of serine-alanine-rich proteins

Table V. Selected up-regulated (bold) or down-regulated (non-bolded) genes in the isolated mutants associated with resistance to HMF and/or furfural.

Strain	Aldehyde reductase	Amino acid biosynthesis
PG-132	GRE3, **ADH6**	**ARG7, ARG8, ARG4**
PR-164	**ALD4**	ARG3
PG-259	GRE3	**ARG7, ARG2, ARG8, ARG4**
PR-338	ADH7	ARG1
PG-353	GRE3, **ADH6**	**CPA1, ARG8, ARG4**
PR-438	**GRE3**, ADH6, ADH7	CPA2, CPA1, ARG3, ARG1, **ARG4**

Table VI. Transcription factors whose activities were increased (bold) or decreased (non-bold) in adaptive mutants associated with resistance to HMF and/orfurfural.

Strain	Transcription factors pleiotropic drug resistance	Transcription factors oxidative stress
PG-132	—	—
PR-164	**PDR1, YRR1, YAP6**	**YAP1**
PG-259	**PDR1, PDR3, YRR1**	**—**
PR-338	**PDR1, YRR1,**	**YAP1**
PG-353	**PDR1, PDR3, YRR1,**	**YAP1**
PR-438	**PDR3, YRR1, YAP6**	**YAP1**

Table VII. Percent improvement in relative fitness in the presence of 2 mM H_2O_2.

Adaptive Mutants	% Improvement in H_2O_2 (2 mM)
PR-164	33%
PR-338	40%
PG-353	70%
PR-438	39%

P-value < 0.05.

(*TIR3*), pyruvate decarboxylase (*PDC5*), and alpha-agglutinin (*SAG1*).

The adaptive mutants PG-132 and PG-259 exhibited negative relative fitness coefficients in the presence of the individual inhibitor HMF, but positive relative fitness coefficients in the presence of multiple inhibitors (HMF/furfural and hydrolysates). These adaptive mutants were isolated early on during the evolution. It is possible that the mutations acquired for these strains only allow them to grow in presence of more than one inhibitor at a time.

Glucose Consumption

To determine if the adaptive mutants evolved not only to be more fit in the presence of hydrolysates but also able to more efficiently utilize and metabolize nutrients in the media, the rate of glucose consumption was measured using HPLC for cultures grown in YNB supplemented with 1% glucose in batch cultures. All the adaptive mutants from the red subpopulation showed significantly higher rate of glucose consumption compared to the ancestral strain. All the other mutants exhibited similar rate of glucose consumption compared to the parental strains, suggesting that the non-red mutants are not more adaptive as a result of enhanced glucose utilization.

Conclusion

In conclusion, we isolated adaptive mutants with enhanced fitness in hydrolysates using a previously published adaptive evolution method, VERT. All but one of the isolated mutants showed increased relative fitness compared with the parental strains in the presence of hydrolysates, with the best mutant exhibiting a relative fitness coefficient of 57%. The mutants showed differential resistances to the inhibitors present in the hydrolysates and some had increased glucose uptake rates, indicative of the complex nature of adaptation to this feedstock. Using transcriptome analysis, we elucidated some potential mechanisms for enhanced tolerance in the isolated adaptive mutants.

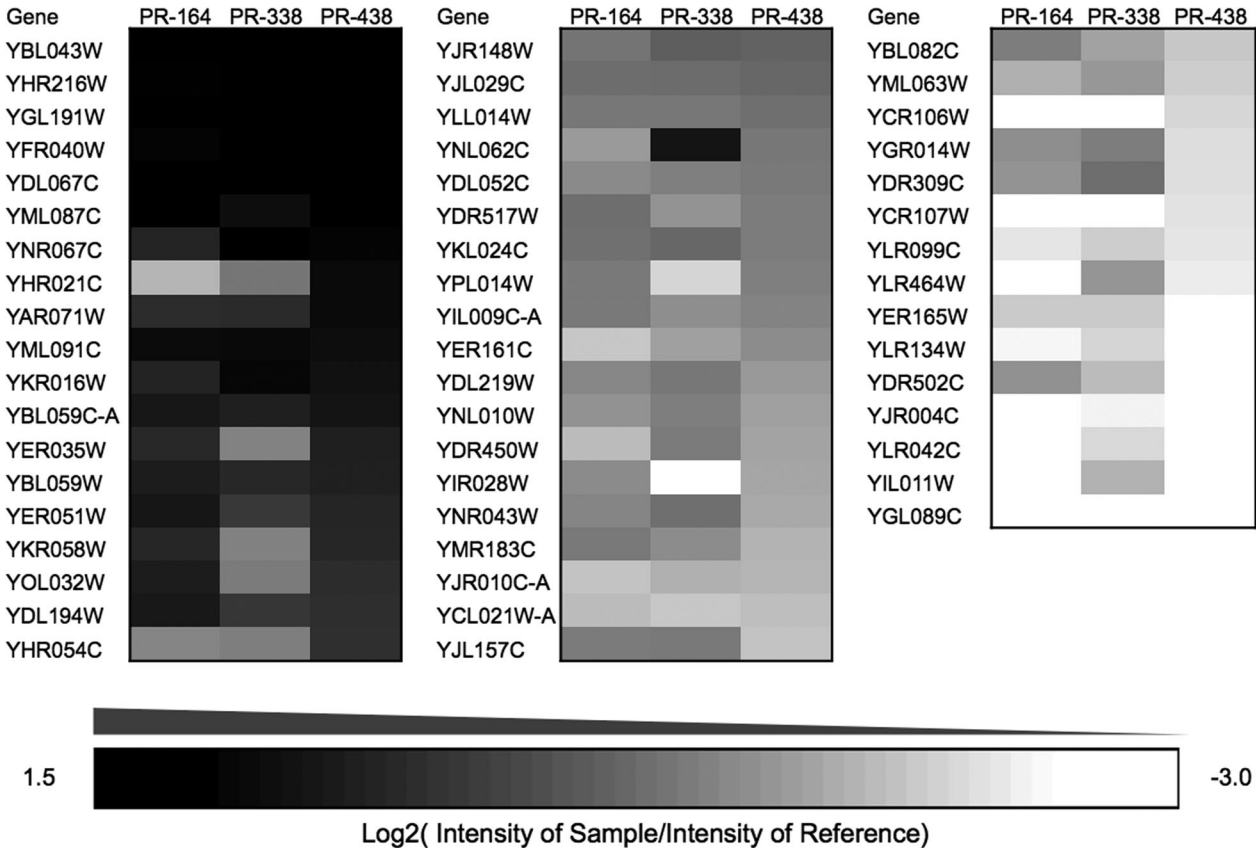

Figure 3. Heat map of gene expression changes of isolated mutants from the red subpopulation.

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