

# Transcriptional profiling of *Saccharomyces cerevisiae* T2 cells upon exposure to hardwood spent sulphite liquor: comparison to acetic acid, furfural and hydroxymethylfurfural

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Received: 15 November 2012 / Accepted: 20 March 2013 / Published online: 29 March 2013  
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**Abstract** Global gene expression was analyzed in *Saccharomyces cerevisiae* T2 cells grown in the presence of hardwood spent sulphite liquor (HW SSL) and each of the three main inhibitors in HW SSL, acetic acid, hydroxymethylfurfural (HMF) and furfural, using a *S. cerevisiae* DNA oligonucleotide microarray. The objective was to compare the gene expression profiles of T2 cells in response to the individual inhibitors against that elicited in response to HW SSL. Acetic acid mainly affected the expression of genes related to the uptake systems of the yeast as

well as energy generation and metabolism. Furfural and HMF affected the transcription of genes involved in the redox balance of the cell. On the other hand, the effect of HW SSL on *S. cerevisiae* T2 cells was distinct and considerably more diverse as compared to the effect of individual inhibitors found in lignocellulosic hydrolysates. This is not surprising as HW SSL contains a complex mixture of inhibitors which may act synergistically. HW SSL elicited significant changes in expression of genes involved in diverse and multiple effects on several aspects of the cellular structure and function. A notable response to HW SSL was decreased expression of the ribosomal protein genes in T2 cells. In addition, HW SSL decreased the expression of genes functioning in the synthesis and transport of proteins as well as metabolism of carbohydrates, lipids, vitamins and vacuolar proteins. Furthermore, the expression of genes involved in multidrug resistance, iron transport and pheromone response was increased, suggesting that T2 cells grown in the presence of HW SSL may have activated pheromone response and/or activated pleiotropic drug response. Some of the largest changes in gene expression were observed in the presence of HW SSL and the affected genes are involved in mating, iron transport, stress response and phospholipid metabolism. A total of 59 out of the 400 genes differentially expressed in the presence of HW SSL, acetic acid, HMF and furfural, belonged to the category of poorly characterized genes. The results indicate that transcriptional responses to individual

**Electronic supplementary material** The online version of this article (doi:[10.1007/s10482-013-9909-1](https://doi.org/10.1007/s10482-013-9909-1)) contains supplementary material, which is available to authorized users.

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lignocellulosic inhibitors gave a different picture and may not be representative of how the cells would respond to the presence of all the inhibitors in lignocellulosic hydrolysates such as HW SSL.

**Keywords** Biomass · Ethanol · Gene expression · Inhibitors · Lignocellulose · Microarray · Yeast

## Introduction

Overcoming the microbial stresses caused by pretreatment-derived inhibitors is one of the main challenges in achieving an efficient lignocellulose-to-ethanol process. Among the lignocellulosic hydrolysates, spent sulphite liquor (SSL) is produced by the acid bisulphite pulping of wood. The total sugar content of SSL ranges from 3 to 4 % (w/v), and varies depending on the source of the wood being pulped. Besides sugars, SSL also contains acetic acid, hydroxymethyl furfural (HMF) and furfural which inhibit yeast growth, viability and ethanol fermentation (Bajwa et al. 2009). SSL obtained from hardwoods (HW SSL) contains higher concentrations of inhibitors and is more toxic than SSL from softwoods.

Some researchers have devised physical or chemical methods to remove the inhibitors (Richardson et al. 2011). However, these approaches are not practical on a large scale. One strategy to overcome the effect of inhibitors is to develop fermenting yeasts with improved inhibitor tolerance. Some yeast strains have been designed, adapted, evolved or mutated to tolerate pretreatment-derived inhibitors (Amartey and Jeffries 1996; Nigam 2001; Bajwa et al. 2009, 2010; Pinel et al. 2011). However, the yeast strains obtained thus far are not robust enough for efficient ethanol production from lignocellulosic hydrolysates.

An understanding of how yeast metabolism, gene regulation, and stress responses are influenced by the inhibitors would be a good starting point for rational engineering and/or breeding of yeasts for efficient ethanol production in the presence of inhibitors in lignocellulosic hydrolysates. Some authors have examined the genetic mechanism(s) of tolerance to individual inhibitors. Investigation of genomic expression profiles of *Saccharomyces cerevisiae* demonstrated that several hundred genes were differentially

expressed in response to HMF (Liu and Slininger 2006). The comparative proteomic analysis in an industrial *S. cerevisiae* strain provided some understanding of the mechanism(s) involved in response to furfural (Lin et al. 2009). Mira et al. (2010) identified genes required for tolerance to acetic acid in *S. cerevisiae* by screening a set of mutants individually deleted for non-essential genes. Li and Yuan (2010) provided some insights into the inhibitory mechanisms of furfural and acetic acid at the transcriptional level in *S. cerevisiae*. In their study, furfural and acetic acid were added only after the yeast culture had reached mid-exponential growth. These conditions do not mimic realistic hydrolysate fermentation conditions. The study of Ma and Liu (2010) revealed a key role of transcriptional factor genes *YAP1*, *PDR1*, *PDR3*, *RPN4*, and *HSF1* in adaptation of *S. cerevisiae* to HMF. A recent proteomic study suggested the unfolded protein response may play a role in the detoxification of combined inhibitors (acetic acid, furfural and phenol) in an inhibitor-tolerant *S. cerevisiae* strain obtained by evolutionary adaptation (Ding et al. 2012). However, the proteomic expression profiles were obtained during growth of the yeast in a rich YPD medium which is unlikely to be encountered during fermentation of lignocellulosic hydrolysates. Despite these studies on the effect of inhibitors individually or in combination, the precise mechanism(s) by which pretreatment-derived inhibitors act during fermentation of sugars in lignocellulosic hydrolysates is not understood. In the present study, we analyzed global gene expression in an industrial *S. cerevisiae* strain exposed to HW SSL and to each of the three key inhibitors in HW SSL (acetic acid, HMF and furfural) using a yeast DNA oligonucleotide microarray to obtain information on HW SSL- and single inhibitor-associated gene expression.

## Materials and methods

### Yeast strain, culture conditions and chemicals

*Saccharomyces cerevisiae* cells, which were adapted to SSL and used in SSL fermentation at the Tembec Alcohol Plant, were provided as a cream by J. Strmen (formerly of Tembec; Témiscaming, Québec, Canada). A single strain (designated as T2) was isolated from the

cream and stored in glycerol for long-term storage. The yeast was maintained on potato dextrose agar (PDA) plates at 4 °C and subcultured every month. Hardwood spent sulphite liquor (HW SSL, pH 2.5) was provided by Tembec (Témiscaming, Québec, Canada) and stored at 4 °C until use. The HW SSL contained, in (w/v): 0.076 % arabinose, 2.2 % xylose, 0.25 % galactose, 0.33 % glucose, 0.55 % mannose, 1 % acetic acid, 0.18 % furfural and 0.11 % 5-hydroxymethyl furfural (HMF). The pH of HW SSL was raised to 5.5 with 10 M NaOH and the HW SSL was boiled for 5 min in a microwave oven and then cooled to room temperature before use (Bajwa et al. 2009). All other chemicals were purchased from Sigma-Aldrich.

### Cell growth and RNA isolation

A loopful of T2 cells from an isolated colony on YPD agar plate was inoculated into 25 mL of 0.67 % (w/v) yeast nitrogen base without amino acids (YNB) and 2 % (w/v) glucose in a 125-mL Erlenmeyer flask. The inoculum culture was grown with shaking at 180 rpm and 28 °C for 48 h. One milliliter of this culture was transferred into 250-mL Erlenmeyer flasks each containing 50 mL of 0.67 % (w/v) YNB, 2 % (w/v) glucose and one of the following: 0.55 % (w/v) acetic acid, 0.1 % (w/v) furfural, 0.3 % (w/v) HMF or HW SSL diluted to 50 % (v/v) with sterile water. The concentrations of the inhibitors and HW SSL were chosen such that they were slightly lower than their respective concentrations that completely inhibit growth, thereby allowing yeast cells to grow. Also, the concentrations of acetic acid and furfural selected were similar to those present in 50 % (v/v) HW SSL. Since in previous studies, the toxicity of HMF was observed to be lower than furfural (Bajwa et al. 2010), the concentration for HMF used in the present study was slightly higher than that found in 50 % (v/v) HW SSL. Acetic acid and furfural were directly used to amend the medium while HMF was prepared as 8.8 M stock and was filter-sterilized before use as described (Bajwa et al. 2009). Cells in each culture were grown to late exponential phase with an optical density at 600 nm ( $OD_{600}$ ) of about 1.0 (about  $7 \times 10^6$  cells). The dark color of HW SSL precluded  $OD_{600}$  measurements; therefore, growth was measured by enumerating colony forming units (CFUs/mL) on PDA plates. Cells were harvested by centrifugation at  $6,000 \times g$  for 10 min. Total RNA was isolated and

purified using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. As control, total RNA was isolated from T2 cells grown in 0.67 % (w/v) YNB and 2 % (w/v) glucose without inhibitors.

### Microarray hybridization

For each aforementioned growth condition, four biological replicates each starting with an independently grown inoculum culture were used. Total RNA from each culture was isolated and subjected to microarray analysis independently. Indirect amino allyl dUTP labeling using oligonucleotide d(T) primers on total RNA was carried out in a reverse transcription reaction with Affinity Script HC reverse transcriptase, and subsequently coupled to fluorescent NHS-ester Cy3 (as per manufacturer, PA23001, GE HealthCare; FairPlay III Microarray Labeling Kit, Cat# 252012, Agilent Technologies). Labeled targets were hybridized to *S. cerevisiae* whole genome DNA long 70mer oligonucleotide microarray (*EBI-accession*: A-MEXP-327 or *GEO-accession*: GPL3549) at 45 °C for over 14 h in hybridization buffer (4× SSC or saline–sodium citrate buffer, 0.5 % SDS, 25 % formamide and 2× Denhardt's Solution), washed once in 2× SSC buffer with 0.2 % SDS (40 °C), and 2 times 0.2× SSC buffers (24 °C), for 10 min in each washing step; where a 20× stock solution of SSC consisted of 3 M sodium chloride and 300 mM trisodium citrate (adjusted to pH 7.0 with HCl).

### Data acquisition and analysis

Microarrays were scanned by using Axon GenePix 4000B. Quantification of spots was done using the software GenePix Pro version 6. Quantified fluorescence intensities for all spots were exported to Microsoft Office Excel and normalized by standardization in the following manner. The raw fluorescent intensities were corrected with local background (around each spot) subtracted, and then further adjusted for each array by taking the relative intensities against the minimum of the background-subtracted spots. Log-transformed values of these adjusted intensities (defined as  $X$ ) were obtained according to the formula:  $X = \log [Intensity - Intensity_{min} + 1]$  (where *Intensity* is the local background-subtracted intensity of the spot and *Intensity\_min* is the minimum of the background-subtracted intensity of the array), so

that the log value of the minimal intensity was set to zero. The  $Z$  scores were calculated as followed;  $Z$  score =  $(X - avg)/SD$  (where  $avg$  is the average values of  $X$  and  $SD$  is the standard deviation). In turn,  $Z$  test value for each experimental condition as compared to the control condition was calculated according to Cheadle et al. (2003). In particular, the formula for  $Z$  test statistics is:  $Z$  test = [(average of  $Z$  score for gene  $G$  in the experimental) – (average  $Z$  score for gene  $G$  in the control)]/( $\sqrt{[SD_{exp}^2/N_{exp} + SD_{con}^2/N_{con}]}$ ); where  $SD_{exp}$  and  $N_{exp}$  are, respectively, standard deviation and number of replicates of the experimental, while  $SD_{con}$  and  $N_{con}$  are the corresponding values of the control. In addition to  $Z$  test, the fold-changes were estimated; firstly the grand average values of the means and SD ( $Mean_{grand\_avg}$ ,  $SD_{grand\_avg}$ ) of adjusted log-transformed signal intensities for all microarray experiments were calculated, and secondly the normalized signal intensities (symbolized as  $X_{norm}$ ) were calculated according to the formula:  $X_{norm} = Z$  score  $\times$   $SD_{grand\_avg} + Mean_{grand\_avg}$ ; and finally log\_ratios were determined for each comparison by the difference between the averages of normalized log\_signal in the two treatments. Differentially expressed genes were defined as  $Z$  test at  $P < 0.06$  and fold-change  $> 2$ .

Annotations for the yeast genes were downloaded from the SGD database (2009). Enrichment of functional categories among differentially expressed genes was examined using DAVID, the database for annotation, visualization and integrated discovery (<http://david.abcc.ncifcrf.gov/>), and according to annotation from COGs, clusters of orthologous groups of proteins (<http://www.ncbi.nlm.nih.gov/COG/>). Analysis of transcription regulatory associations was performed using the YEASTRACT database (Teixeira et al. 2006b). Gene network analysis was done based on GENEMANIA (Warde-Farley et al. 2010). To systematically review, mine and integrate a large volume of information from pathways, gene ontology, and gene networks, the following workflow was implemented as a guideline to the present pathway and/gene function enrichment analysis. Step 1: the union set encompassed hits of active genes for all four treatments [furfural, HMF, acetic acid, HW SSL], was first compiled. Based on the overlaps of responses in different treatments, active genes were assigned to 15 sectors of a Venn diagram of 4 sets (Supplemental file S2). Step 2: for each combination of the two treatments, their

corresponding sectors in the Venn diagram were pooled together to yield three groups that represented the unique members and common members for the two treatments. Analyses for enrichment of KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways as well as “function annotation clustering” were then performed by uploading these three groups of genes separately onto the DAVID online analysis web-interface. Individual genes with exceptionally large fold-change or  $Z$  test scores were also subjected to an in-depth annotation analysis using DAVID. Step 3: the broader categorization of gene functions according to COG was performed for each member of the aforementioned three groups. Specific attention was given to the COG function categories that had multiple members in each Venn diagram sectors. Step 4: detailed literature search for interesting genes and members of enriched pathways was carried out initially on the SGD yeast knowledge base. Preliminary genetic and functional interactions were curated manually from the SGD database as well. Step 5: highly interactive genes were subjected to bioinformatics analysis using tools provided by YEASTRACT or GENEMANIA.

## Results and discussion

### Growth of *S. cerevisiae* T2 cells in the presence of individual inhibitors and HW SSL

When *S. cerevisiae* T2 cells were grown in diluted HW SSL or in the presence of acetic acid, furfural and HMF, cell growth was inhibited and a prolonged lag phase was observed as compared to the control culture. In the absence of any inhibitors, the yeast growth reached  $OD_{600}$  of 1.0 within 13 h. In the presence of 0.55 % (w/v) acetic acid, 0.1 % (w/v) furfural and 0.3 % (w/v) HMF; it took 30, 70 and 79 h, respectively, to reach  $OD_{600}$  of 1.0. In 50 % (v/v) diluted HW SSL, the cell number increased by about 1 log unit in 24 h.

### General trends in the transcriptional responses of *S. cerevisiae* T2 cells growing in the presence of inhibitors

A distinct whole genome transcriptional response was detected when T2 cells were grown in the presence of individual inhibitors or HW SSL as compared to control. Based on  $Z$  test scores ( $P < 0.06$ ) and

estimated fold-changes ( $>2$ ), a total of 400 genes (out of a total of 6,307 genes in *S. cerevisiae*) showed significant expression changes (Supplemental file S1). This accounts for about 6.3 % of the *S. cerevisiae* genome. Of these 400 differentially expressed genes, around 55 % belong to the yeast eukaryotic core (YEC) genes which constitute the minimal gene set in yeast required to sustain a functioning cellular life form. The number of genes differentially expressed in the presence of different individual inhibitors and HW SSL is shown in a Venn diagram (Supplemental file S2). The alphabets correspond to the Venn diagram sectors mentioned in Supplemental file S1. The numeric values represent the numbers of differentially expressed genes in the sectors. In the presence of HW SSL, 242 genes showed significant expression changes. Out of these 242 genes, 206 were responses seen only in the presence of HW SSL; 27 genes were responses common to both HW SSL and acetic acid; 6 genes were responses common to both HW SSL and furfural while 10 genes were responses common to both HW SSL and HMF. In the presence of acetic acid, 153 genes showed significant expression changes. Of these, 120 genes were responses specific to acetic acid. In the presence of furfural and HMF, 27 and 31 genes, respectively, showed significant expression changes. Of these, 16 were differentially expressed only in response to furfural while 12 genes showed significant expression changes only in HMF-grown cells.

Cells grown in the presence of HW SSL noticeably showed the highest number of genes with significant changes and a trend towards decreasing gene expression (94 up vs. 148 down) as compared to those seen with individual inhibitors (acetic acid: 94 genes up, 59 down; furfural: 19 up and 8 down; HMF: 12 up and 19 down, respectively). The volcano plot of Z test scores against the log of fold-changes also shows these trends (Supplemental file S3).

The microarray results also showed that the effect of individual inhibitors (acetic acid, HMF or furfural) on gene expression in T2 cells was very different from the effect of HW SSL. Only 14.9 % of the 242 responsive genes in HW SSL were shared with those seen in any of the other individual inhibitors (Supplemental file S2). A selection of notable genes that were differentially expressed in T2 cells exposed to HW SSL and individual inhibitors was obtained by functional annotation clustering and pathways enrichment analysis (see workflow in “Materials and methods”

section). They were grouped by functional categories and listed in Tables 1, 2, 3, 4. Growth inhibition by acetic acid seems to affect genes associated with biosynthesis of amino acids, particularly methionine and other sulfur containing amino acids; uptake of inorganic nutrients, sugars, vitamins and amino acids; and regulation of transcriptional machinery (Supplemental file S1). In the presence of HMF or furfural, genes related to redox balance, glyoxylate cycle, and gluconeogenesis were differentially expressed (Supplemental file S1). Furfural also induced the expression of genes related to vacuole functions. On the other hand, HW SSL exerted effects on genes involved in multiple aspects of cellular metabolism at the transcriptional level. Most of these responses were observed only in HW SSL-grown cells. For example, HW SSL decreased the expression of a large number of ribosomal protein (RP) genes (Fig. 1) and genes related to initiation factors, elongation factors, tRNA synthetases and ribosomal biogenesis and assembly. HW SSL also affected transcription of genes related to mating, stress response, cell wall organization, actin cytoskeleton, cell division, carbohydrate metabolism, fatty acid and nitrogen compound biosynthesis and nucleo-cytoplasmic transport (Table 1).

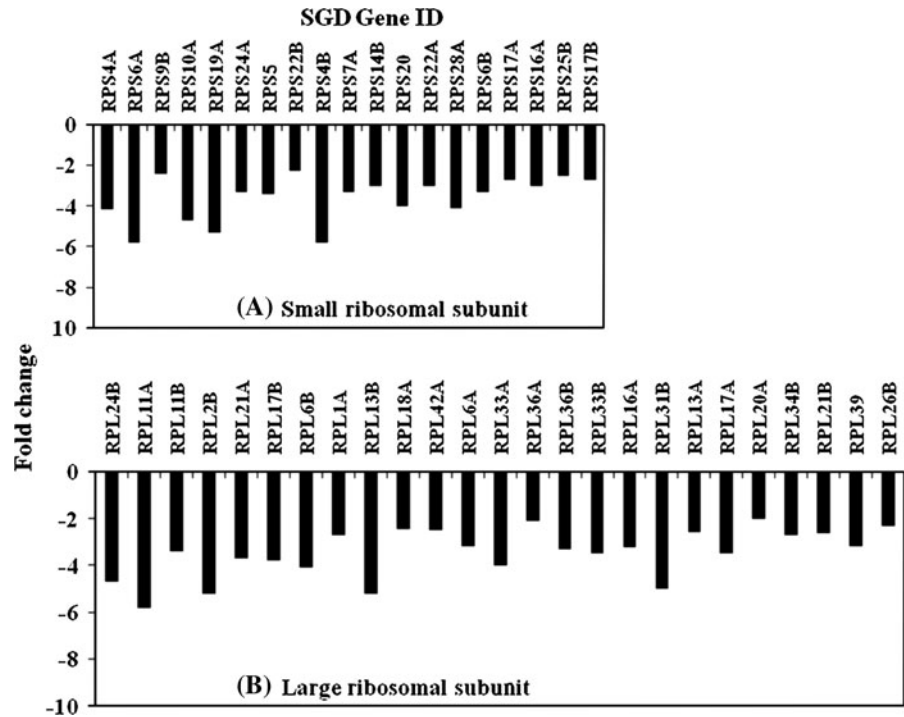
Discussed below are the selected notable genes found to be differentially expressed in T2 cells exposed to HW SSL and individual inhibitors.

#### Genes differentially expressed in the presence of HW SSL

HW SSL had effects on multiple aspects of cellular metabolism at the transcriptional level (Supplemental file S1). The expression of 94 genes was increased while that of 148 genes was decreased when T2 cells were grown in the presence of HW SSL. Most of these responses (85 %) were observed only in HW SSL-grown cells (Supplemental file S1).

A notable response to the presence of HW SSL was decreased expression of the RP genes in T2 cells. These included 19 and 25 genes encoding proteins of the small (Fig. 1a) and large (Fig. 1b) ribosomal subunits, respectively. Furthermore, expression of several genes (*SES1*, *UTP7*, *HYP2*, *ANB1*, *TEF4* and *NOP58*) encoding tRNA synthetases, initiation and elongation factors, and ribosome biogenesis-related proteins, was also reduced (Table 1). All these belong to the category of YEC genes. These responses are characteristic of the inhibition

**Fig. 1** Fold-change in the expression of genes encoding RPs of the **a** small and **b** large ribosomal subunit



of the TOR signaling pathway which modulates the balance between protein synthesis and degradation depending on nutrient availability, and is recognized as an important mechanism that controls cell growth in eukaryotes (Rohde and Cardenas 2003). The production of ribosomes is an energetically costly process that requires the action of all three RNA polymerases and involves more than 100 gene products. In *S. cerevisiae*, TOR signalling regulates ribosome biogenesis at both the transcriptional and translational levels and plays an important role in coupling nutrient availability to the transcription of genes involved in the formation of ribosomes. Inhibition of the TOR pathway leads to reduced transcription of RP genes (Warner 1999).

The transcript levels of *SSE2*, *HSP104*, *TSA1* and *EGD1* were reduced in T2 cells exposed to HW SSL. These genes are related to protein folding. Misfolded or damaged proteins, especially aggregated proteins are highly toxic to cells. Degradation of misfolded and damaged proteins by the ubiquitin-mediated proteasome pathway plays an important role in maintaining normal cell function and viability. In the present study, the expression of five genes related to ubiquitination (*BRO1*, *BSD2*, *RAD6*, *UBC8* and *PAI3*) was increased in T2 cells exposed to HW SSL.

Among the genes whose expression increased in the presence of HW SSL, the most striking were the 78- and 98-fold increases in the transcription of *PRM1* and *YLR168C*. *PRM1* codes for a pheromone-regulated protein involved in mating while Ylr168cp is a mitochondrial protein involved in phospholipid metabolism. It is notable that *PRM4*, encoding another pheromone-regulated protein proposed to be involved in mating, was also up-responsive in T2 cells grown in the presence of HW SSL. Prm1p facilitates plasma membrane fusion during yeast mating. During pheromone-induced cell cycle arrest in the G1 phase, the mating pathways ensure tight controls for small cell size (Goranov et al. 2009). In the present study, transcription of several genes related to the progression of cell size and cell cycle were differentially expressed in the presence of HW SSL. Expression of *CLN3*, coding for one of the yeast cyclins was decreased in response to HW SSL-induced stress. Cln3p activates Cdc28p kinase to promote the G1 to S phase transition and plays a role in regulating transcription of the other G1 cyclins genes, *CLN1* and *CLN2*. Transcription of a number of other genes (*BFR1*, *CDC39*, *CLN3* and *TSA1*) functioning in the regulation of cell cycle was decreased in the presence

**Table 1** Selected genes differentially expressed in response to HW SSL

Functional categories and gene names	Functions	Fold-change	Z score <sup>a</sup>
<b>Pleiotropic drug response</b>			
<i>PDR5</i>	Pleiotropic ABC efflux transporter of multiple drugs	4.7	1.96
<i>PDR16</i>	Protein involved in multidrug transport	3.0	2.8
<i>SNQ2</i>	Protein SNQ2	3.4	3.25
<i>QDR2</i>	Multidrug transporter	4.3	2.4
<b>Iron and copper uptake</b>			
<i>FIT2</i>	Facilitator of iron transport	38.7	7.6
<i>FIT3</i>	Facilitator of iron transport	120	8.59
<i>FET3</i>	High-affinity iron uptake and multicopper oxidase	2.3	2.2
<i>FET4</i>	Low-affinity Fe <sup>(2+)</sup> transport protein	6.3	2.56
<i>TIS11</i>	mRNA-binding protein	3.0	2.4
<b>Protein synthesis</b>			
<i>SES1</i>	Seryl-tRNA synthetase, cytoplasmic	-2.7	-2.3
<i>UTP7</i>	U3 small nucleolar RNA-associated protein 7	-6.25	-6.0
<i>HYP2</i>	Eukaryotic translation initiation factor 5A-2	-4.5	-4.3
<i>ANB1</i>	Eukaryotic translation initiation factor 5A-1	-4.0	-3.76
<i>TEF4</i>	Elongation factor 1-gamma 2	-3.8	-3.58
<i>NOP58</i>	Nucleolar protein 58	-2.7	-2.00
<b>Nucleoporins</b>			
<i>NUP192</i>	Nucleoporin NUP192	-2.2	-2.53
<b>Actin and myosin</b>			
<i>TPM1</i>	Tropomyosin-1	-4.0	-3.18
<i>MYO2</i>	Myosin-2	-2.1	-2.08
<i>ARP2</i>	Essential component of the actin nucleation center	-2.6	-3.0
<i>ARP5</i>	Nuclear actin-related protein involved in chromatin remodeling	-2.5	-2.57
<i>COF1</i>	Cofilin	-2.5	-3.0
<i>ABP140</i>	Non-essential protein that binds actin filaments	-4.76	-3.26
<b>Cell wall organization</b>			
<i>PIR1</i>	O-glycosylated protein required for cell wall stability	-5.26	-2.64
<i>FLO1</i>	Cell wall protein	-5.26	-2.54
<i>FLO5</i>	Cell wall protein	-3.8	-2.18
<i>ECM33</i>	GPI-anchored protein	-4.0	-2.38
<b>Cell division</b>			
<i>NKP1</i>	Kinetochore protein	-7.7	-3.85
<i>CLN3</i>	G1 cyclin involved in cell cycle progression	-2.5	-3.3
<i>CBF5</i>	Pseudouridine synthase	-5.26	-4.05
<b>Glycolysis and pentose phosphate pathway</b>			
<i>PFK2</i>	Phosphofructokinase	-6.6	-3.0
<i>ADH1</i>	Alcohol dehydrogenase	-7.7	-2.05
<i>ADH2</i>	Alcohol dehydrogenase	-5.88	-2.46
<i>ADH5</i>	Alcohol dehydrogenase	-3.12	-2.0
<i>ALD3</i>	Aldehyde dehydrogenase	-3.03	-3.1
<i>FBA1</i>	Fructose 1,6-bisphosphate aldolase	-4.5	-2.05
<i>PGI1</i>	Phosphoglucose isomerase	-5.5	-2.72
<i>PDC1</i>	Pyruvate decarboxylase isozymes	-5.26	-3.05
<i>CDC19</i>	Pyruvate kinase	-12.6	-4.5
<i>TP11</i>	Triose phosphate isomerase	-5.26	-3.0
<i>GND1</i>	6-Phosphogluconate dehydrogenase	-3.3	-2.15
<i>SOL3</i>	6-Phosphogluconolactonase	-3.0	-3.17

**Table 1** continued

Functional categories and gene names	Functions	Fold-change	Z score <sup>a</sup>
<b>Oxidative phosphorylation</b>			
<i>QCR7</i>	Subunit VII of the ubiquinol cytochrome c reductase complex	5.72	3.32
<i>QCR8</i>	Subunit VIII of the ubiquinol cytochrome c reductase complex	7.25	1.9
<i>COX 5B</i>	Subunit Vb of cytochrome c oxidase	2.15	1.64
<i>COX7</i>	Subunit VII of cytochrome c oxidase	10.5	6.14
<i>COX10</i>	Protein required for cytochrome c oxidase	2.5	2.3
<i>COX13</i>	Subunit VIa of cytochrome c oxidase	5.7	2.3
<i>COX15</i>	Essential prosthetic group for cytochrome c oxidase	2.6	1.65
<i>NDE1</i>	NADH dehydrogenase	3.47	2.2
<b>Ubiquitination</b>			
<i>BRO1</i>	Vacuolar protein sorting (VPS) factor	2.8	2.2
<i>BSD2</i>	Controls metal transport to vacuole	2.06	2.0
<i>RAD6</i>	Ubiquitin-conjugating enzyme	2.1	3.0
<i>UBC8</i>	Ubiquitin-conjugating enzyme	2.24	3.5
<i>PA13</i>	Cytoplasmic proteinase A inhibitor	17.5	3.6
<b>Protein folding</b>			
<i>SSE2</i>	Heat shock protein	-2.0	-2.1
<i>HSP104</i>	Heat shock protein	-5.0	-2.5
<i>TSA1</i>	Thioredoxin peroxidase	-3.5	-3.0
<i>EGD1</i>	Subunit beta1 of the nascent polypeptide-associated complex	-4.5	-2.8
<b>Mating</b>			
<i>PRM1</i>	Protein involved in membrane fusion during mating	77.3	7.9
<i>PRM4</i>	Pheromone-regulated protein	2.6	1.7
<b>Cell cycle</b>			
<i>BFR1</i>	Component of mRNP complexes	-4.7	-2.6
<i>CDC39</i>	Component of the CCR4-NOT complex	-2.7	-2.1
<i>CLN3</i>	G1 cyclin involved in cell cycle progression	-2.5	-3.3
<i>TSA1</i>	Thioredoxin peroxidase	-3.5	-3.0
<b>Phosphate metabolism</b>			
<i>YLR168C</i>	Protein required for phospholipid metabolism	94.4	8.64
<b>Stress response</b>			
<i>DDR2</i>	Multistress response protein	17.0	2.7
<b>Biosynthesis of nitrogen compounds</b>			
<i>ARG1</i>	Arginosuccinate synthetase	-5.26	-2.25
<i>BAT1</i>	Branched-chain amino acid aminotransferase	-2.0	-2.0
<i>ILV5</i>	Acetohydroxyacid reductoisomerase	-4.34	-2.23
<i>URA5</i>	Major orotate phosphoribosyltransferase	-3.1	-2.3
<i>URA10</i>	Minor orotate phosphoribosyltransferase	-3.0	-2.6
<i>ARO4</i>	3-Deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase	-2.85	-2.21
<i>SNZ2</i>	Member of a stationary phase-induced gene family	-5.5	-2.1
<i>RNR2</i>	Ribonucleotide-diphosphate reductase	-3.0	-2.1
<b>Lipid biosynthesis</b>			
<i>ACC1</i>	Acetyl-CoA carboxylase	-5.9	-2.4
<i>FAS1</i>	Beta subunit of fatty acid synthetase	-5.9	-2.7
<i>ERG11</i>	Lanosterol 14-alpha-demethylase	-2.6	-3.7
<b>Regulation of transcription</b>			
<i>CLN3</i>	G1 cyclin involved in cell cycle progression	-2.6	-3.4
<i>CDC39</i>	Component of the CCR4-NOT complex	-2.7	-2.0
<i>EGD1</i>	Subunit beta1 of the nascent polypeptide-associated complex	-4.8	-2.8
<i>SUB1</i>	Transcriptional coactivator	-2.94	-3.1



**Table 1** continued

Functional categories and gene names	Functions	Fold-change	Z score <sup>a</sup>
<i>MPT5</i>	Member of the Puf family of RNA-binding proteins	−2.4	−2.5

<sup>a</sup> Z score is based on standardized Z test

**Table 2** Selected genes differentially expressed in response to furfural

Functional categories and gene names	Functions	Fold-change	Z score <sup>a</sup>
Oxidoreductases			
<i>AAD4</i>	Probable aryl-alcohol dehydrogenase	3.4	3.5
<i>AAD14</i>	Probable aryl-alcohol dehydrogenase	2.7	2.1
<i>AAD16</i>	Probable aryl-alcohol dehydrogenase	2.3	2.4
<i>GRE2</i>	NADPH-dependent methylglyoxal reductase	2.3	2.2
<i>OYE3</i>	NADPH oxidoreductase	3.7	3.4
<i>PRX1</i>	Thioredoxin peroxidase	2.1	2.3
<i>GCY1</i>	Glycerol dehydrogenase	2.3	2.5
Vacuole functioning			
<i>PRC1</i>	Vacuolar carboxypeptidase	2.5	2.3
<i>VMA2</i>	Vacuolar H <sup>+</sup> -ATPase	2.3	2.0
<i>FIG4</i>	Phosphatase	6.9	2.1
Glyoxylate cycle			
<i>ICL1</i>	Isocitrate lyase	−3.2	−4.2
<i>MLS1</i>	Malate synthase 1, glyoxysomal	−2.0	−2.7
<i>CIT2</i>	Citrate synthase, peroxisomal	−2.0	−1.9
Mating, cell fusion and cell wall organization			
<i>KEL1</i>	Protein required for proper cell fusion and cell morphology	2.5	2.0
<i>CWP2</i>	Cell wall mannoprotein	2.4	2.4
<i>FIG4</i>	Phosphatase required for mating	6.5	2.1
Glucose sensing			
<i>SNF3</i>	Plasma membrane glucose sensor	2.2	3.1

<sup>a</sup> Z score is based on standardized Z test

of HW SSL. Another gene, *LGE1* coding for an unknown protein was up-responsive in response to HW SSL. Lge1p plays role in regulating the cell size as null mutant forms abnormally large cells. Cell cycle is tightly regulated by cell size. For the cell to divide, it must grow to a critical size before cell cycle progression occurs (Goranov et al. 2009). Although highly speculative, it seems that HW SSL activates the pheromone signalling in T2 cells exposed to HW SSL. Activation of the pheromone response attenuates protein synthesis in *S. cerevisiae* (Goranov et al. 2009). This may partly explain the decreased expression of several genes related to ribosome biogenesis in T2 cells.

Transcription of *DDR2* was increased 17-fold in T2 cells grown in HW SSL. This gene codes for a

multistress response protein and its expression is activated by various xenobiotic agents and environmental or physiological stresses (Kobayashi and McEntee 1990; Kobayashi et al. 1996). Transcript levels of several genes encoding components of oxidative phosphorylation (*QCR7*, *QCR8*, *COX 5B*, *COX7*, *COX10*, *COX13*, *COX15* and *NDE1*) were increased when T2 cells were exposed to HW SSL. Interestingly, exposure to HW SSL increased the expression of a number of genes encoding multidrug resistance (MDR) transporters belonging to the ATP-binding cassette (ABC) transporters and major facilitator superfamily. These included *PDR5*, *PDR16*, *QDR2* and *SNQ2* (Table 1). Increased expression of several genes encoding MDR transporters has also been observed in *S. cerevisiae* cells exposed to the

**Table 3** Selected genes differentially expressed in response to HMF

Functional categories and gene names	Functions	Fold-change	Z score <sup>a</sup>
Oxidoreductases			
<i>AAD4</i>	Probable aryl-alcohol dehydrogenase	2.8	3.0
<i>AAD14</i>	Probable aryl-alcohol dehydrogenase	2.1	2.3
<i>ADH7</i>	Alcohol dehydrogenase	5.8	2.6
Ribosomal proteins			
<i>RPS8B</i>	Protein component of the small (40S) ribosomal subunit	2.1	1.9
<i>RPL10</i>	Protein component of the large (60S) ribosomal subunit	2.07	2.3
Glyoxylate cycle			
<i>ICL1</i>	Isocitrate lyase	−3.4	−4.2
<i>MLS1</i>	Malate synthase 1, glyoxysomal	−2.3	−3.4
Amino acid metabolism			
<i>LYS9</i>	Saccharopine dehydrogenase	2.3	2.05
<i>LEU9</i>	Alpha-isopropylmalate synthase II	3.0	2.4
<i>AGP1</i>	Low-affinity amino acid permease	3.0	3.6

<sup>a</sup> Z score is based on standardized Z test

herbicide 2,4-D (Teixeira et al. 2006a). Pdr5p efflux pump activity mediates resistance to many xenobiotic compounds including mutagens, fungicides, steroids, and anticancer drugs. Increased expression of MDR genes observed in our study suggests a possible function of MDR in pumping out toxic metabolites from T2 cells exposed to HW SSL.

Another notable response of T2 cells to HW SSL was the increased expression of genes involved in iron uptake. These included *FIT2*, *FIT3*, *FET3* and *FET4*. It is speculated that T2 cells grown in HW SSL may have increased requirement for iron. Increased transcription of *FIT2* and *FIT3* has been reported in a furfural-adapted *S. cerevisiae* strain and its parent strain exposed to furfural (Heer et al. 2009).

It is notable that some of the genes (*SCS7*, *LPX1* and unidentified ORFs *YGR035C* and *YLL056C*) up-responsive in the presence of HW SSL are regulated by *YRM1*, the transcription factor that activates genes involved in MDR. A gene network analysis based on GENEMANIA revealed that these up-responsive genes, along with the drug transporter genes, interact with oxidative stress response genes or genes encoding oxido-reductases/proteins involved in active transmembrane transporter activity.

Transcription of several genes related to cell's biosynthetic activity, such as biosynthesis of nitrogen compounds, lipids, actin cytoskeleton and cell wall organization was decreased in the presence of HW

SSL. Also, genes related to glycolysis and pentose phosphate pathway, cell cycle and regulation of transcription were down-responsive in T2 cells exposed to HW SSL (Table 1).

Taking together the broadly differential expression observed for several genes that are crucial to regulation of cell cycle, cell size, mating, cell polarization, as well as organization of actin cytoskeleton, we hypothesize that exposure to HW SSL activates the pheromone signaling pathway in T2 cell, and causes repression of protein synthesis. The shutdown of growth and biosynthetic activities in turn conserves resources in the cells to energize multidrug transporters, and allows cells to better adapt to the toxic environment.

#### Genes differentially expressed in the presence of HMF or furfural

In T2 cells grown in furfural, 19 genes were up-responsive and 8 were down-responsive. In cells grown in HMF, 12 genes were up-responsive and 19 were down-responsive. The up-responsive genes mainly included those encoding dehydrogenases and oxido-reductases. A complete list of genes differentially expressed in cells grown in the presence of HMF or furfural is given in Supplemental file S1. Of these, a few selected differentially expressed genes are shown in Tables 2 and 3.

**Table 4** Selected genes differentially expressed in response to acetic acid

Functional categories and gene names	Functions	Fold-change	Z score <sup>a</sup>
Biosynthesis of amino acids			
<i>MET2</i>	Homoserine <i>O</i> -acetyltransferase	3.8	2.11
<i>MET17</i>	Methionine and cysteine synthase	3.5	2.6
<i>MET3</i>	ATP sulfurylase	3.4	5.3
<i>MET22</i>	Bisphosphate-3'-nucleotidase	2.6	2.3
<i>ARG8</i>	Acetylornithine aminotransferase	3.1	2.31
<i>CYS4</i>	Cystathionine beta-synthase	2.9	2.3
<i>MET16</i>	3'-Phosphoadenylsulfate reductase	3.5	3.36
<i>ASN2</i>	Asparagine synthetase	2.13	3.0
<i>GCN4</i>	Transcriptional activator of amino acid biosynthetic genes	2.8	2.2
<i>GLY1</i>	Threonine aldolase	2.13	2.3
<i>LPD1</i>	Dihydroliipoamide dehydrogenase	2.14	2.6
Transporters			
<i>MEP3</i>	Ammonium transporter	3.6	3.2
<i>MCH5</i>	Riboflavin transporter	2.05	2.7
<i>TPO2</i>	Polyamine transporter	2.8	2.1
<i>PHO84</i>	High-affinity inorganic phosphate transporter	7.74	3.83
<i>HXT1</i>	Low-affinity glucose transporter	4.8	2.4
Phosphate metabolism			
<i>PHO5</i>	Repressible acid phosphatase	3.0	3.1
<i>PHO11</i>	Acid phosphatase	2.75	2.41
<i>PHO12</i>	Acid phosphatase	2.3	3.1
<i>PHM8</i>	Lysophosphatidic acid phosphatase	2.3	3.8
Respiratory chain			
<i>ATP4</i>	Subunit b of F1F0 ATP synthase	2.4	2.2
<i>ATP19</i>	Subunit k of F1F0 ATP synthase	2.8	3.4
<i>COX9</i>	Subunit VIIa of cytochrome c oxidase	2.3	2.1
<i>COX20</i>	Protein required for assembly of cytochrome c oxidase	2.3	4.6
<i>AAC1</i>	ADP/ATP translocator	2.9	2.1
<i>RIP1</i>	Ubiquinol cytochrome c reductase	2.7	2.6
<i>PET10</i>	Protein required for ATP/ADP exchange	3.3	2.1
<i>SDH2</i>	Succinate dehydrogenase	2.2	3.1
Regulation of gene expression			
<i>SMI1</i>	Protein involved in the regulation of cell wall synthesis	2.1	2.9
<i>YAP1</i>	AP-1-like transcription factor YAP1	2.14	2.9
<i>GCN4</i>	General control protein	2.8	2.2
<i>HST2</i>	NAD-dependent deacetylase	2.3	2.05
<i>MIG2</i>	Regulatory protein	4.1	5.3
<i>ASH1</i>	Transcriptional regulatory protein	2.9	3.82

<sup>a</sup> Z score is based on standardized Z test

Furfural is known to induce oxidative stress in yeast cells and the primary mechanism of yeast resistance is converting it to less toxic furfuryl alcohol and furoic acid (Heer and Sauer 2008; Taherzadeh et al. 2000) by oxido-reductases. Not surprisingly, growth on furfural led to increased expression of genes coding for oxido-reductases in the present study. These included *GRE2*, *OYE3*, *PRX1*, *GCY1*, *AAD4*, *AAD14* and *AAD16*. Heer et al. (2009) also reported induction of these genes under furfural-induced stress. The expression of *AAD4* and *AAD14*, encoding aryl-alcohol dehydrogenases, was also increased in HMF-grown cells. Aryl-alcohol dehydrogenases convert aromatic aldehydes, such as veratraldehyde and anisaldehyde, into their corresponding alcohols. These aromatic compounds may be released during lignin oxidation by fungal lignin peroxidases (Haemmerli et al. 1987). Induction of *AAD4* by furfural in *S. cerevisiae* was also reported by Heer et al. (2009).

Furfural also increased expression of genes (*PRC1*, *VMA2* and *FIG4*) involved in proper functioning of the vacuole. Furfural-induced stress is known to cause damage to vacuolar membrane (Allen et al. 2010). A few genes involved in efficient mating, cell fusion and cell wall organization (*KEL2*, *CWP2* and *FIG4*) were also up-responsive in T2 cells grown in the presence of furfural. *SNF3*, coding for plasma membrane low glucose sensor that regulates glucose transport, was also up-responsive under furfural-induced stress. Using the YEASTRACT database (Teixeira et al. 2006b), it was found that most of the up-responsive genes under furfural stress in T2 cells are known targets of *Yap1*, a major oxidative stress regulator in *S. cerevisiae*.

Gorsich et al. (2006) demonstrated that a knockout of *ZWF1* and *GND1*, genes related to pentose phosphate pathway, in *S. cerevisiae* leads to increased sensitivity to high concentrations (50 mM) of furfural. These genes were not differentially expressed in *S. cerevisiae* T2 cells under the conditions used (defined medium supplemented with 10 mM furfural) in our study.

In the presence of HMF, transcription of *ADH7* in T2 cells was increased. Transcriptomic analysis by Ma and Liu (2010) also reported induction of *ADH7* in *S. cerevisiae* cells exposed to HMF. Yeast clones over-expressing *ADH7* show high reduction capability towards HMF (Pettersson et al. 2006). *ADH7* encodes the NADPH-dependent medium-chain alcohol dehydrogenase with broad substrate specificity. It is a

member of the cinnamyl family of alcohol dehydrogenases and is thought to be involved in the buildup of fusel alcohols (Larroy et al. 2002). Kitagawa et al. (2003) demonstrated that dithiocarbamate fungicides, thiuram, zineb, and maneb, and other fungicides such as tetrachloroisophthalonitrile and pentachlorophenol, also induced aryl-alcohol dehydrogenase genes. Thus, increased expression of these genes may not be directly related to induction of aromatic aldehyde metabolism.

The expression of several genes involved in amino acid metabolism was increased in T2 cells grown in the presence of HMF. These included: *LYS9* encoding an enzyme in lysine biosynthesis, *LEU9* encoding an enzyme in leucine biosynthesis and *AGP1* encoding low-affinity amino acid permease. Other significantly induced genes in T2 cells grown in the presence of HMF included *RPS8B* and *RPL10* that code for RPs.

In T2 cells, growth in furfural or HMF led to decreased expression of *PCK1*, encoding phosphoenolpyruvate carboxykinase, a key enzyme in gluconeogenesis and two genes (*ICL1* and *MLS1*) encoding enzymes of the glyoxylate cycle.

#### Genes differentially expressed in the presence of acetic acid

Out of the 72 genes up-responsive only in acetic acid-grown T2 cells, 11 (*ARG8*, *ASN2*, *CYS4*, *GCH4*, *GLY1*, *LPD1*, *MET2*, *MET3*, *MET16*, *MET17* and *MET22*) are involved in amino acid biosynthesis (Table 4). The genes involved in the biosynthesis of cysteine, glutamate, methionine and glycine have been identified as determinants of acetic acid tolerance in *S. cerevisiae* (Mira et al. 2010). Increased expression of these genes is also consistent with a cellular response to decreased concentration of these amino acids inside acetic acid-grown cells (Almeida et al. 2009). Acetic acid has been shown to inhibit methionine biosynthesis in *E. coli* (Roe et al. 2002). Interestingly, in *E. coli* cells, growth inhibition in the presence of acetic acid could be reversed by the addition of methionine (Han et al. 1993; Roe et al. 2002). Ding et al. (2012) have reported an increased abundance of proteins involved in methionine metabolism in response to the combined presence of acetic acid, furfural and phenol in *S. cerevisiae*.

The expression of genes encoding components of the respiratory chain (*ATP4*, *ATP19*, *COX9*, *COX20*,

*AAC1*, *RIP1*, *PET10* and *SDH2*) was also increased in T2 cells in the presence of acetic acid. These genes have been implicated in providing protection against acetic acid (Mira et al. 2010). A fourfold increase in the expression of *TPO2* that codes for plasma membrane MDR transporter of the major facilitator superfamily was observed in T2 cells exposed to acetic acid. Expression of Tpo2p in *S. cerevisiae* confers resistance to acetic acid; this is thought to be mediated by the active expulsion of acetate from yeast cells (Mira et al. 2010). In another study, *TPO2* was reported to be transcriptionally activated in response to acetic acid in *S. cerevisiae* (Fernandes et al. 2005).

In T2 cells, genes encoding transporters of glucose (*HXT1*), ammonium (*MEP3*), riboflavin (*MCH5*), inorganic phosphate (*PHO84*) and iron–sulfur cluster (*ATM1*) were up-responsive in the presence of acetic acid. Yeast cells seem to have a higher demand for phosphate under acetic acid-induced stress as the expression of several genes (*PHO5*, *PHO11*, *PHO12* and *PHM8*) involved in phosphate metabolism increased in the presence of acetic acid. Expression of *SFA1*, encoding S-hydroxymethylglutathione dehydrogenase, was also increased in T2 cells exposed to acetic acid. Over-expression of *SFA1* is particularly interesting because Sfa1p can act as a HMF reductase in HMF detoxification. Surprisingly, the expression of this gene was not increased significantly in HMF-grown cells.

The expression of a number of genes related to cell wall organization (*EXG1*, *KRE2*, *ROT1*, *SMI1*), oxidative stress response (*GLR1*, *HMX1*, *YAP1*, *ZWF1*) and genes coding for transcriptional activators (*YAP1*, *GCN4*, *SMI1*, *HST2*, *MIG2* and *ASH1*) was increased in T2 cells grown in the presence of acetic acid. Another notable feature of acetic acid-induced stress was the increased expression of *YAP1*, the major oxidative stress regulator in yeast. Using the YEASTRACT database it was found that 53 % of the genes that encode proteins up-responsive under acetic acid stress were known targets of *Yap1*.

Interestingly, most of the genes down-responsive in the presence of acetic acid were related to the retrotransposons. Out of the 50 retrotransposon genes known in *S. cerevisiae*, the expression of 39 genes was reduced in T2 cells in the presence of acetic acid (Supplemental file S1).

Li and Yuan (2010) examined the transcriptomic shifts in response to furfural and acetic acid in

*S. cerevisiae*. In the presence of acetic acid, they observed reduced expression of genes related to mitochondrial proteins, respiration and carbohydrate metabolism and increased expression of genes involved in the biosynthesis of arginine, histidine, glycine and serine. In the same study, furfural-induced stress caused a down-regulation of the genes related to the transcriptional and translational control and upregulation of genes related to stress response. These gene expression responses were not observed in our study. The discrepancy likely resulted from different experimental conditions used. Li and Yuan (2010) used a rich (YPD) high sugar [10 % (w/v) glucose] medium in contrast to the minimal defined medium used in our study. Also, Li and Yuan (2010) added the inhibitors when the growing culture reached mid-exponential phase and cells were collected after 20 min of exposure to the inhibitors. Furfural and acetic acid were added at much higher concentrations (1.4 and 1.8 %, w/v, respectively) compared to our study (0.1 and 0.55 %, w/v).

#### Gene expression responses common to individual inhibitors and HW SSL

Results from the microarray experiment identified many common responses in T2 cells elicited by the individual inhibitors and HW SSL. Around 27 genes differentially expressed in acetic acid-grown cells were also differentially expressed in HW SSL while 6 genes differentially expressed in cells grown in furfural were also differentially expressed in HW SSL-grown cells. Also, 10 genes differentially expressed in cells grown in HMF overlapped with those in the HW SSL-grown cells (Supplemental file S1).

Transcription of *OYE3*, coding for a conserved NADPH oxido-reductase was up-responsive in T2 cells in the presence of furfural, acetic acid and HW SSL. This enzyme has been shown to be involved in furfural reduction by Heer et al. (2009). Another notable common response was the decreased expression of *CTT1*, coding for a cytosolic catalase, in T2 cells exposed to HMF, acetic acid or HW SSL. Ctt1p activity is important during oxidative stress in protecting proteins against oxidative inactivation. Catalase activity is also increased in *S. cerevisiae* during oxidative stress caused by aging and acid stress adaptation (Agarwal et al. 2005; Giannattasio et al.

2005). Transcription of *FMP43*, coding for a mitochondrial pyruvate carrier, was decreased in T2 cells exposed to each of the three inhibitors. *PUT1*, coding for proline oxidase, was down-responsive in the presence of furfural, HMF and HW SSL. *STF2* and *GPH1* were down-responsive in the presence of HMF as well as HW SSL. *STF2* codes for a protein involved in regulation of the mitochondrial F1F0-ATP synthase while *GPH1* codes for non-essential glycogen phosphorylase.

T2 cells responded to acetic acid or HW SSL by increasing the expression of genes related to citric acid cycle (*IDH1*, *IDH2* and *CIT1*), oxidative phosphorylation and electron transport chain (*ATP18*, *QCR2*, *QCR9* and *CYC*) and iron transport (*FIT2* and *FET4*). Transcription of *TIS11*, encoding one of the mRNA-binding proteins expressed during iron starvation and involved in iron homeostasis, was also increased in T2 cells in the presence of HW SSL as well as acetic acid.

Among the 400 genes which were differentially expressed in T2 cells in the presence of 1 of the 3 inhibitors or HW SSL, 59 belong to the category of poorly characterized genes or genes with unknown function as well as genes related to unnamed proteins (Supplemental file S1). Of these 59 genes, 18 belong to the category of YEC genes. One of the uncharacterized ORFs, *YLL056C*, has been reported to be induced in *S. cerevisiae* cells in response to furfural (Heer et al. 2009). In the present study, this gene was induced in cells exposed to HW SSL, but not furfural. Transcription of this gene is activated by genes involved in pleiotropic drug resistance. The deduced protein sequence of *YLL056C* contains an NAD(P)H binding site, suggesting that *YLL056C* encodes a protein involved in the oxido-reduction reactions. Identification and characterization of these genes encoding proteins of unknown function may provide information on their relationship(s) and/or mechanism(s) of action. Additional experiments focused on comparison of gene expression analysis during different stages of growth may reveal a better picture of the overall yeast response to stress induced by a mixture of pretreatment-derived inhibitors in lignocellulosic hydrolysates.

In summary, HW SSL elicited significant changes in the expression of genes involved in diverse and multiple effects on several aspects of the cellular structure and function in *S. cerevisiae* T2 cells. The distinct HW SSL-induced transcriptional responses

differed substantially from those elicited by each of the three main inhibitors commonly found in lignocellulosic hydrolysates. Our study is a first step towards defining the overall transcriptional response of *S. cerevisiae* in the presence of HW SSL and should contribute to a better understanding of the mechanism(s) of inhibition of yeast growth and fermentation in the presence of a host of inhibitors in lignocellulosic hydrolysates.

**Acknowledgments** This research was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada. We thank J. Strmen (formerly of Tembec) for providing the *S. cerevisiae* T2 strain and HW SSL.

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