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Protein expression analysis revealed a fine-tuned mechanism of in situ detoxification pathway for the tolerant industrial yeast *Saccharomyces cerevisiae*

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Abstract

Inhibitory compounds liberated from lignocellulose pretreatment are representative toxic chemicals that repress microbial growth and metabolism. A tolerant strain of the industrial yeast *Saccharomyces cerevisiae* is able to detoxify a major class of toxic compounds while producing ethanol. Knowledge on the yeast tolerance was mostly obtained by gene expression analysis and limited protein expression evidence is yet available underlying the yeast adaptation. Here we report a comparative protein expression profiling study on Y-50049, a tolerant strain compared with its parental industrial type strain Y-12632. We found a distinctive protein expression of glucose-6-phosphate dehydrogenase (Zwf1) in Y-50049 but not in Y-12632, in the relatively conserved glycolysis and pentose phosphate pathway (PPP) in response to a combinational challenge of 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF). A group of proteins with aldehyde reduction activity was uniquely induced expressed in Y-50049 but not in Y-12632. Such evidence allowed fine-tuning a mechanism of the renovated in situ detoxification by Y-50049. As the key protein, Zwf1 drove the glucose metabolism in favor of the oxidative branch of the PPP facilitating in situ detoxification of the toxic chemicals by Y-50049. The activated expression of Zwf1 generated the essential cofactor nicotinamide adenine dinucleotide phosphate (NADPH) enabling reduction of furfural and HMF through a group of aldehyde reduction enzymes. In return, the activate aldehyde reductions released desirable feedbacks of $NADP^+$ stimulating continued oxidative activity of Zwf1. Thus, a well-maintained cofactor regeneration cycle was established to restore the cofactor imbalance caused by furfural-HMF. Challenges and perspectives on adaptation of significantly differential expressions of ribosomal proteins and other unique proteins are also discussed.

Keywords Adaptation · Industrial yeast · In situ detoxification · Protein expression · Stress tolerance

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Introduction

Developing the next-generation biocatalyst is vital for fermentation-based industrial applications and a sustainable bio-based economy. A pretreatment process is currently required to release fermentable sugars embedded in lignocellulosic materials for microbial utilization in production of biofuels and chemicals. Decomposition of lignocellulosic biomass by a pretreatment procedure commonly generates toxic compounds as byproducts that inhibit cell growth and subsequent fermentation (Palmqvist and Hahn-Hägerdal 2000; Luo et al. 2002). Currently, identified toxic chemicals associated with biomass pretreatment were classified into aldehydes, phenols, ketones, and weak organic acid based on their functional group of chemical compositions (Klinke et al. 2004; Liu and Blaschek 2010; Liu 2018). Among which, 2-furaldehyde (furfural) and 5-

hydroxymethyl-2-furaldehyde (HMF) were known as representative inhibitive aldehyde compounds commonly observed in lignocellulosic hydrolysates (Liu and Blaschek 2010). Furfural and HMF induce reactive oxygen species, cause cofactor imbalance, and damage DNA and RNA replication that significantly inhibit cell growth and metabolism (Allen et al. 2010; Liu 2006; 2018). In the presence of multiple inhibitory compounds, cells can be killed even at modest concentrations due to a combinational toxic damage (Liu et al. 2004; 2008; Gorsich et al. 2006). Overcoming the major classes of toxic compounds associated with lignocellulose-to-biofuels conversion possesses a significant challenge for new strain development.

A tolerant strain of *Saccharomyces cerevisiae* NRRL Y-50049 was previously developed by selection under pressure using an environmental engineering scheme of adaptive laboratory evolution method (Liu et al. 2005). Its parental strain NRRL Y-12632 is a diploid industrial yeast type strain, also known as CBS1171, ATCC18824, AWRI74, CCRC21447, DBVPG6173, DSM70449, IFO10217, IGC4455, JCM7255, and NCYC505 by worldwide collections (Bradbury et al. 2006). The evolved new strain NRRL Y-50049 is able to detoxify a broad range of toxic aldehyde compounds in situ while producing ethanol (Liu et al. 2005, 2008; Moon and Liu 2012; 2015).

A reprogrammed pathway was proposed for the in situ detoxification based on comparative gene expression analysis (Liu et al. 2009). Key gene regulatory elements and major MAPK pathways were identified to support the yeast tolerance by transcriptome analysis (Ma and Liu 2010; Lin et al. 2009a; Zhou et al. 2014; Liu et al. 2018). A previous protein expression study showed that industrial yeast displayed an immediate sensitive response in cell wall, plasma membrane, and nucleus compared with other subcellular locations when treated with the toxic chemicals (Lin et al. 2009b). However, limited evidence of protein expression is available on the mechanism of the yeast tolerance and adaptation to furfural-HMF.

Here we report a time-course study of comparative protein profile analysis between the evolved tolerant strain Y-50049 and its parental wild type strain Y-12632 for their expression under conditions with or without a combinational challenge of furfural and HMF. The distinctive protein profiles in center metabolic pathways between the two strains provided new evidence fine-tuning the in situ detoxification pathway by the tolerant yeast. The significantly differential expression of conserved ribosomal proteins observed in this study suggested their potential critical roles in the yeast adaptation. Results of this study provide a fined-tuned mechanism of in situ detoxification and new insight into underlying tolerant adaptation for the industrial yeast. Such knowledge aids the development of the next-generation biocatalyst for production of fuels and chemicals from lignocellulosic materials.

Materials and methods

Yeast strains and culture conditions

The diploid *S. cerevisiae* industrial yeast type strain NRRL Y-12632 and its evolved tolerant strain NRRL Y-50049 were obtained from USDA Agricultural Research Service Culture Collection, Peoria, IL USA. The lyophilized culture stock was recovered and maintained on an yeast mold (YM) medium containing 3 g yeast extract, 3 g malt extract, and 5 g peptone per liter. Cells were grown in a 2-L flask containing 1 L of YM broth at 30 °C with agitation at 275 rpm. Cell growth was monitored by absorbance at OD_{600} with periodic samples until 72 h. The inhibitor containing medium was added with a concentrated stock solution of furfural and HMF each adjusted at a final concentration of 15 mM in a medium. A medium without addition of furfural and HMF served as a control. Each strain was grown on these two different media for comparative studies. Cells grown on the non-toxin treated medium were sampled at 0, 24, and 48 h for protein preparations. Cell samples grown on the toxin treated medium were collected for protein preparation at 0, 24, 48, and 56 h after incubation. Cells were harvested by centrifugation at 8000×*g* at 4 °C for 5 min. The tube containing a cell pellet was immediately frozen on dry ice and store at − 80 °C until use.

Sample preparation and protein detection

Yeast cells were lysed using Y-PER plus dialyzable yeast protein extraction reagent (Thermo Scientific, Rockford, IL, USA) followed by dialysis. Protein concentrations were measured by Bradford assay (Bradford 1976) using bovine serum albumin as a standard. Protein samples were prepared following procedures in a previously described protocol (Batth et al. 2012). Briefly, a reducing buffer tris(2-carboxyethyl)phosphine was added in the sample to reduce the disulfide bonds in the protein at a final concentration of 5 mM. The mixture was incubated at room temperature for 30 min. Then, a blocking solution of indole-3-acetic acid was added to the mix at the final concentration at 1 m mol/mg protein and incubated at room temperature in dark for 30 min. Finally, trypsin was added at a ratio of 1:50 (trypsin:protein) for digestion at 37 °C for 16 h. Then the treated protein samples were stored at − 20 °C. Samples of proteins ranged from 25 to 28 μg were picked separately and shipped overnight with a dry ice package to Joint BioEnergy Institute (JBEI), US Department of Energy (Emeryville, CA), for protein detection. The protein detection assay was a very kind gift generally provided by Christopher Petzold from Joint BioEnergy Institute using their in-house developed protocol for tandem mass spectrometry (MS/MS)-based multiple reaction monitoring analysis coupled with liquid chromatographic separation (Batth et al. 2012).

Data analysis

Raw data were received from JBEI on a Scaffold template. Data was analyzed using Scafford 4 purchased from Proteome Software, Inc. (Portland, OR, USA). Frequency of protein detection was recorded based on multiple amino acid alignment. A protein with a detection frequency of 100% with all amino acid fragments was identified as a positive response. A protein was also considered as significant if it was consistently expressed over time with high levels of detection frequency. Functional analysis was based on Saccharomyces Genome Database (https://www.yeast genome.org/).

Genome sequence analysis

Genome sequencing was performed by SeqWright DNA Technology Service (Houston, TX), an Illumina Certified Service Provider working under Good Laboratory Practice conditions. Illumina pair-end reads of 151 bp at 42-49X genome coverage were generated for each of the industrial yeast *S. cerevisiae* strains NRRL Y-12632 and NRRL Y-50049. These whole-genome sequence data are available at the NCBI Sequence Read Archive under accession number PRJNA484962. Additional confirmation genome sequences for the two strains were also obtained from Qingdao Institute of BioEnergy and Bioprocess Technology by Solexa GA-IIx sequencing platform with a pair-ended strategy. This produced paired reads of 57 bp with an average insert size of 300 bp.

Detection of single nucleotide variations

A genome assembly for strain Y-12632 was produced with the PCAP. Solexa program (Huang 2017) on the pair-end reads from the strain. The genome assembly was used as a reference for mapping the paired-end reads from strain Y-50049 onto the reference with Bowtie2 (Langmead and Salzberg 2012). Genes encoding differentially expressed proteins in the genome assembly were located by computing best alignments between the genome assembly and the coding sequences of these genes with the DDS2 program (Huang et al. 2004). Single nucleotide variations between strains Y-12632 and Y-50049 in these genes were detected by examining the coverage of reads from strain Y-50049 in each gene locations of the Y-12632 genome assembly. The examination was carried out in the IGV viewer (Robinson et al. 2011). A sequence mutation was acceptable if its minimum depth of read coverage was 20 and its minimum frequency for the minor allele in the reads was equal to or greater than 20%. Non-synonymous mutations were identified by aligning the genomic region of the gene to the protein sequence of the gene at the codon level with the AAT package (Huang et al. 1997).

Results

Phenotype of cell growth

Cell growth of the tolerant strain NRRL Y-50049 and its parental wild type strain NRRL Y-12632 was compared in response to the presence or absence of the combinational challenge of furfural-HMF. On a standard YM medium without addition of furfural-HMF, both strains showed an almost identical cell growth and reached a stationary phase no later than 24 h after incubation (Fig. 1a). The cell density slightly increased to near an OD_{600} reading of 1.5 at 72 h after incubation for both strains. On a YM medium amended with furfural and HMF at 15 mM each, the wild type strain Y-12632 failed to grow and was unable to establish a culture after 72 h (Fig. 1b). In contrast, the tolerant Y-50049 displayed a growth trend similar to the untreated cultures, reaching a stationary phase no later than 24 h. The cell density was typically less than that of the non-furfural-HMF treated cells. Mechanisms behind such a difference are currently unclear. Its highest cell density

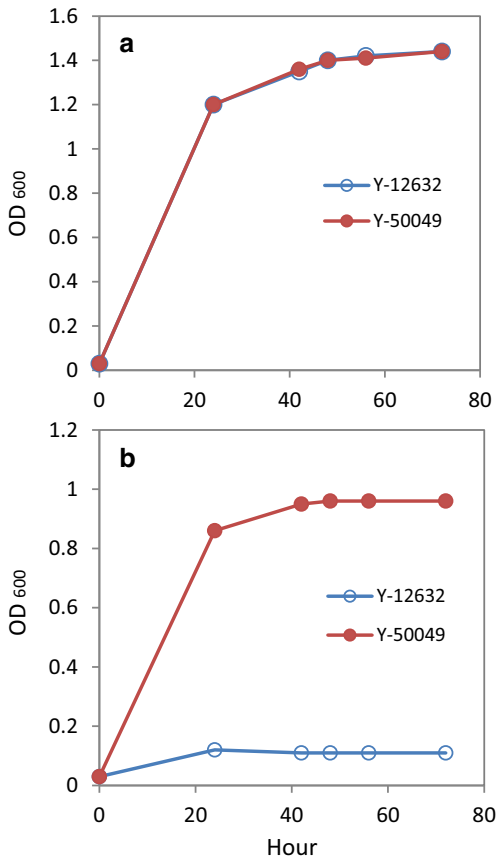

Fig. 1 Comparative cell growth response to a combinational challenge of furfural-HMF. **a** Cell growth of *Saccharomyces cerevisiae* NRRL Y-50049 and its parental strain NRRL Y-12632 under normal conditions on an YM medium without treatment of furfural and HMF. **b** Cell growth in response to the combinational challenges of 15 mM each of furfural and HMF on an YM medium showing a nearly normal growth of the tolerant strain NRRL Y-50049

was around an OD_{600} reading of 1 at 48 h after incubation. As demonstrated in previous studies, furfural and HMF were completely depleted from the medium prior to entering a stationary phase of cell growth by the tolerant strain Y-50049, such as 24 h treated with 12 mM or 48 h treated with 20 mM (Liu et al. 2008, 2009). In this study, when treated with 15 mM each of furfural and HMF, strain Y-50049 quickly reached a stationary phase well before 48 h and the toxic chemicals should be completely depleted prior to the station phase.

Overview of protein expression

Expressed proteins for cells treated or untreated with the toxins were identified by Joint BioEnergy Institute, US Department of Energy (Emeryville, CA), using tandem mass spectrometry (MS/MS)-based multiple reaction monitoring analysis coupled with liquid chromatographic separation method. Using Scafford 4 proteome software analysis, we identified a total of 298 proteins from cell samples without treatment of furfural-HMF for strain Y-50049 and Y-12632. The protein expression profiles for the untreated samples observed over time between the two strains are essentially the same (Supplementary Table S1). For cells treated with furfural-HMF, we identified 297 proteins for these strains (Supplementary Table S2). Proteins identified from the treated and the untreated experimental sets were highly consistent with over 90% of proteins in common. This indicated a consistency and reproducibility of the protein identification method applied in this study. Unlike observed from non-treated cells, we found about 30% of the proteins displayed significantly differential expressions between the two strains for cells treated with furfural-HMF. We present those uniquely expressed proteins in Y-50049 but not expressed in Y-12632 overtime in response to the combinational challenge of furfural-HMF. Specifically, here we focus on the signature expressions in the altered center metabolic pathways. An outstanding group of ribosomal proteins and other distinctive proteins expressed in Y-50049 that potentially impact adaptation of the yeast tolerance are also reported.

Signature expression of Zwf1 in glycolysis-PPP

In this study, it was interesting to find that major proteins in glycolysis and pentose phosphate pathway (PPP) were persistently expressed in both strains treated with furfural-HMF. It appeared that unlike the more sensitive gene expression response, the eventual protein expression in the center metabolic pathway was robust against the furfural-HMF challenge. However, there was a distinct exception of Zwf1 which was only expressed in the adapted strain Y-50049 but not in its parental strain after the toxic treatment throughout the entire course of the study (Table 1). The lack of expression of Zwf1in Y-12632 was closely associated with its cell growth response. Cell growth of strain Y-12632 was ceased by furfural-HMF and no additional increased cell mass was observed, in contrast to the health growth of Y-50049 after 24 h (Fig. 1b). The unique expression of Zwf1 in Y-50049 in such a conserved center metabolic pathway was highly significant for the adapted strain Y-50049.

Zwf1 is a glucose-6-phosphate dehydrogenase enzyme that catalyzes the first step of the oxidative branch of the pentose phosphate pathway (PPP) generating nicotinamide adenine dinucleotide phosphate (NADPH). Zwf1 activity was found to be inhibited by accumulated NADPH and stimulated with decreased cytosolic levels of NADPH (Llobell et al. 1988). The challenge of furfural-HMF reduced cytosolic levels of cofactors in yeast and such a cofactor imbalance caused severe cell inhibition (Liu et al. 2009). The activated Zwf1 expression resulted in production of NADPH, a necessary cofactor needed to the first step of the irreversible oxidative branch of PPP. In the presence of furfural-HMF, the dominant expression of Zwf1 in the tolerant strain Y-50049 appeared to drive the glucose metabolism away from the conventional glycolysis in favor to the oxidative branch of PPP (Fig. 2). Such a critical step provided necessary NADPH needed for furfural-HMF reduction by numerous aldehyde reductases. Although furfural-HMF reduction using cofactor NADH was also observed, however, in fact, the NADPH-dependent aldehyde reductase activity was the only reliant function for remediation of furfural-HMF at higher concentrations (Heer et al. 2009; Liu and Moon 2009; Liu 2018).

Induced expression of critical aldehyde reductase

In *S. cerevisiae*, nearly 100 proteins were identified to possess coordinating sites with an NADP-binding domain of Zwf1. Many of these were commonly known involved in the center metabolic pathways as observed in this study (Table 2). Most of these protein encoding genes were reported to have enhanced expressions in response to the inhibitor challenges (Liu et al. 2008; Liu and Moon 2009; Ma and Liu 2010; Moon and Liu 2015). In this study, we found all these proteins such as Ari1, Adh6, Adh7, Gre2, Ydr541c, Ygl039w, and Ymr315w were constantly expressed in Y-50049 in response to the toxic challenge compared with its parental strain Y-12632 (Table 3). These proteins were documented showing higher levels of aldehyde reduction activity by direct enzyme assays (Liu et al. 2008, 2009; Hector et al. 2009; Moon and Liu 2015). And evidently, most of these proteins were NADPH-dependent as the most efficient aldehyde reductase. Expression of numerous proteins was clearly induced by furfural-HMF, such as a newly characterized aldehyde reductase Ari1 (Liu and Moon 2009; Bowman et al. 2010; Jordan et al. 2011) and a putative aldehyde reductase Ypr1 (Ford and Ellis 2002). These induced expression was highly consistent with previous observed by gene expression analysis for the

Table 1 Major proteins expressed in frequency (%) in the reprogrammed glycolysis and pentose phosphate pathway (PPP) for the tolerant industrial yeast *Saccharomyces cerevisiae* strain NRRL Y-50049 compared with its parental wild type NRRL Y-12632 over time in response to synergistic challenges of furfural and HMF

Pathway	Gene/ORF	Protein	MW (kD)	Treated with furfural-HMF							
				0 h		24 h		48 h		56 h	
				Y-12632	Y-50049	Y-12632	Y-50049	Y-12632	Y-50049	Y-12632	Y-50049
Glycolysis	*HXK1 / YFR053C*	Hxk1	54	98	0	100	100	100	100	100	100
	HXK2 / YGL253W	Hxk2	54	100	100	100	100	100	100	98	100
	PGI1 / YBR196C	Pgi1	61	100	100	100	100	100	100	100	100
	PFK2 / YMR205C	Pfk2	105	92	99	100	100	100	100	96	100
	FBA1 / YKL060C	Fba1	40	100	100	100	100	100	100	100	100
	TPI1 / YDR050C	Tpi1	27	0	0	100	100	100	100	100	100
	TDH1 / YJR052W	Tdh1	36	100	100	100	100	100	100	100	100
	TDH2 / YJR009C	Tdh2	36	92	88	89	100	80	100	92	100
	TDH3 / YGR192C	Tdh3	36	100	100	100	100	100	100	100	100
	PGK1 / YCR012W	Pgk1	45	100	100	100	100	100	100	100	100
	GPM1 / YKL152C	Gpm1	28	100	100	100	100	100	100	100	100
	ENO1 / YGR254W	Eno1	47	100	100	100	100	100	100	100	100
	ENO2 / YHR174W	Eno2	47	100	100	100	100	100	100	100	100
	PYK1 / YAL038W	Pyk1	55	100	100	100	100	100	100	100	100
	PDC1 / YLR044C	Pdc1	61	100	100	100	100	100	100	100	100
	ALD6 / YPL061W	Ald6	54	9	66	100	100	100	100	100	100
PPP-oxidative branch	***ZWF1 / YNL241C***	**Zwf1**	**58**	**0**	**0**	**0**	**100**	**0**	**100**	**0**	**100**
	SOL3 / YHR163W	Sol3	28	0	95	0	100	85	100	100	100
	GND1 / YHR183W	Gnd1	54	0	86	100	100	100	100	100	100
PPP-nonoxidative phase	*TKL1 / YPR074C*	Tkl1	74	100	0	98	100	45	100	96	100
	TAL1 / YLR354C	Tal1	37	0	75	100	100	100	100	100	100

industrial yeast (Liu and Moon 2009; Sehnem et al. 2013). With typical aldehyde reduction activity, activated expression of these enzymes was able to convert furan aldehydes of furfural and HMF into less toxic compounds of furan methanol (FM) and furan dimethanol (FDM), respectively. These enzymes are critical biocatalysts consisting of a new component in the reprogrammed detoxification pathway for the adapted Y-50049.

An important transcription factor Hyr1, a regulator of transcription factor Yap1, was also differentially expressed in Y-50049 during the entire course of the study (Table 3). The signature gene expression of a master regulator gene *YAP1* was found to lead key gene regulatory elements and most oxidative reductase activities through varied cross interactions (Ma and Liu 2010; Liu 2011; Zhou et al. 2014; Zhang et al. 2015). In this work, we did not observe protein expression of Yap1 likely due to the limited detection coverage. However, the identification of the persistent expression of Hyr1 in Y-50049 is interesting and convincing. Hyr1 is a transcription factor and a thiol peroxidase not only protects cells directly from oxidative stress, but also acts as a hydroperoxide sensor (Delaunay et al. 2002; Avery et al. 2004; Ma et al. 2007). This redox signaling agent activated transcription factor Yap1 and subsequent Yap1p-dependent transcriptions (Delaunay et al. 2002). It appeared the expression of Hyr1 in Y-50049 may act with a twofold merit in the detoxification reactions (Fig. 2). On the other hand, the repressed Y-12632 eventually lost Hyr1 expression after 24 h in response to the toxic challenge (Table 3).

Differential expressions of ribosomal proteins

S. cerevisiae has a total of 79 ribosomal proteins and many are essential for cell growth (Steffen et al. 2012). In this study, we identified 65 ribosomal proteins, including 32 ribosomal proteins for the 40S subunit and 33 ribosomal proteins for the 60S subunit from both strains. There was no significant difference for ribosomal protein expression between the two strains without the treatment of furfural-HMF. However, we observed at least 40 ribosomal proteins, including 21 of 40S and 19 of 60S ribosomal proteins were persistently expressed over time in

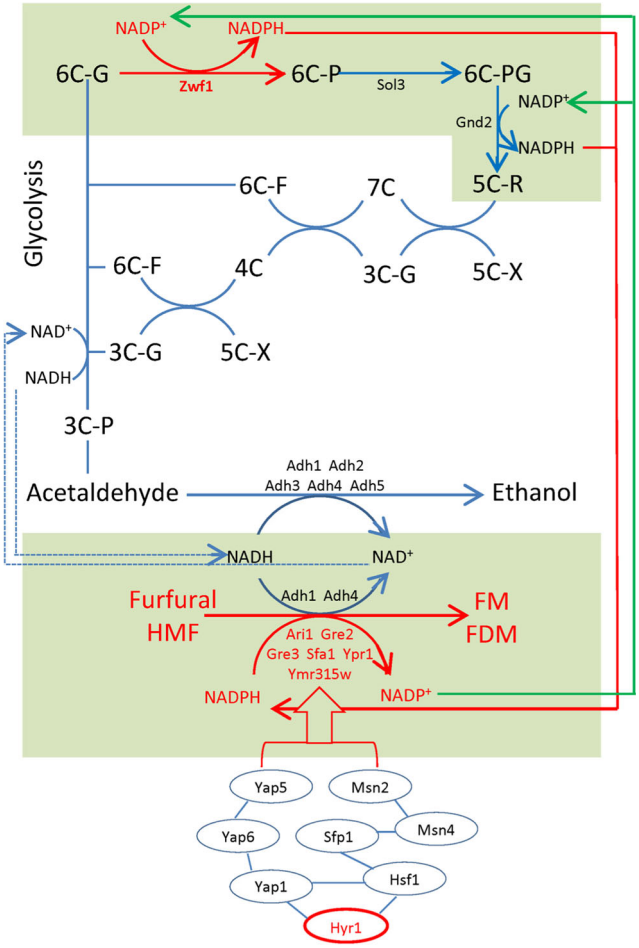

Fig. 2 Fine-tuned in situ detoxification pathway of adapted industrial yeast. The tolerant industrial yeast strain NRRL Y-50049 displayed a unique expression of Zwf1 in response to a combinational challenge of furfural-HMF throughout the entire course of this study. Such an expression drove the sugar metabolism in favor to the irreversible oxidative branch of pentose phosphate pathway (PPP). Y-50049 also gained enhanced expression of a group of aldehyde reductases that enabled reduction of furfural and HMF into less toxic furan methanol (FM) and furan dimethanol (FDM), respectively. Such a biotransformation by these enzymes consumed NADPH and released $NADP^+$. The adapted Zwf1 reaction provided the essential cofactor NADPH for reduction of furfural and HMF (red line). In return, the biotransformation pathway released $NADP^+$ supplying a smooth feedback to the oxidative branch of PPP (green line) that restored the cofactor imbalance caused by the toxic chemicals. The distinctly activated Hyr1, a redox signaling agent activating key transcription factor Yap1, is also presented in red in relationships with representative transcription factors at the bottom. In the illustrative conserved pathways, 3C-G stands for glyceraldehydes 3-phosphate; 3C-P, pyruvate; 4C, erythrose 4-phosphate; 5C-R, ribose 5-phosphate; 5C-X, xylulose 5-phosphate; 6C-F, fructose 6-phosphate; 6C-G, glucose 6-phosphate; and 7C, sedoheptulose 7-phosphate

Y-50049 but not in Y-12632 in response to the combinational challenge of furfural-HMF (Table 4).

The 40 differentially expressed ribosomal proteins involved in six functional categories under GO terms of biological process, five under cellular component, and four under molecular functions (Fig. 3). Yet, many functions are not clearly defined. Many of these ribosomal proteins were found to have high levels of statistical significance in the interactive cellular process and molecular process, nucleic acid transport, and ribosome assembly and biogenesis (Supplementary Fig. S1a; Supplementary Fig. S2a); under categories of cytoplasm, intracellular organelle, nucleus, organelle part, and ribosome dominated the entire section of cellular component (Supplementary Fig. S1b; Supplementary Fig. S2b); and under the section of molecular functions such as structural molecular activity, molecular functions, and binding (Supplementary Fig. S1c; Supplementary Fig. S2c). Furthermore, among 40 significantly differentially expressed ribosomal proteins, 33 were identified as essential ribosomal proteins (Table 4).

Other uniquely expressed proteins

In addition to a large number of ribosomal proteins and signature expressions of proteins involved in the detoxification pathways, we identified 40 other significantly expressed proteins in Y-50049 but not in Y-12632 in response to the furfural-HMF challenge (Table 5). These proteins represented functions in a wide range of categories covering membrane, energy, mitochondria, vacuole, fatty acids, and amino acid biosynthesis. About a half of these proteins were stress related and at least 15 of them were activated against DNA replication stress. In addition, at least 10 proteins including Ade1, Ade17, Aco1, Afr1, Car1, Gcv3, Gph1, Pnc1, Rdl1, and Rtn2 were also induced by furfural-HMF. These proteins cover functions of purine nucleotide biosynthesis and mitochondrial maintenance as exampled by Ade1, Ade17, Aco1, and Rdl1.

Sequence variations

In order to seek potential structure evidence supporting the tolerant functions of Y-50049, we compared genomic sequence variations between the evolved Y-50049 and its parental strain Y-12632. Out of selected 92 genes encoding the uniquely expressed proteins in Y-50049, we found a total of 15 single nucleotide polymorphism (SNP) variations. Among them, six were detected as non-synonymous mutations that changed corresponding amino acids of the encoding proteins (Table 6). Since Y-50049 is a derivative of a diploid industrial yeast, the detected frequency of the nucleotide variation was presented in both alleles of the genes against its wild type. Ribosomal protein Rps9B and Rpl23A encoding genes *RPS9B* and *RPL23A* showed relatively higher levels of variation as measured by SNP at 78% and 39%, respectively, compared with its parental strain Y-12632. It was unexpected to observe such higher levels of sequence variations in the conserved ribosomal protein (essential) genes. We also found a relatively lower level of sequence variation frequency at 22% in an uncharacterized ORF *YMR208W*. The primary functions

Table 2 Representative proteins of *Saccharomyces cerevisiae* showing coordinating sites with NAD(P)-binding domain of Zwf1

ORF	Gene	Protein	Function	Sequence location
YOL086C	*ADH1*	Adh1	Alcohol dehydrogenase	145-316
YMR303C	*ADH2*	Adh2	Alcohol dehydrogenase	145-316
YMR083W	*ADH3*	Adh3	Alcohol dehydrogenase	171-342
YBR145W	*ADH5*	Adh5	Alcohol dehydrogenase	147-320
YMR318C	*ADH6*	Adh6	Alcohol dehydrogenase	154-320
YCR105W	*ADH7*	Adh7	Alcohol dehydrogenase	155-321
YGL157W	*ARI1*	Ari1	NADPH-dependent aldehyde reductase	3-341
YHR183W	*GND1*	Gnd1	6-Phosphogluconate dehydrogenase	3-175
YGR256W	*GND2*	Gnd2	6-Phosphogluconate dehydrogenase	7-178
YOL151W	*GRE2*	Gre2	Methylglyoxal reductase, DNA replication stress	1-335
YKL085W	*MDH1*	Mdh1	Mitochondrial malate dehydrogenase	8-164
YOL126C	*MDH2*	Mdh2	Cytoplasmic malate dehydrogenase	13-158
YDL078C	*MDH3*	Mdh3	Peroxisomal malate dehydrogenase	3-144
YDL168W	*SFA1*	Sfa1	Formaldehyde dehydrogenase, DNA replication stress	170-346
YJL052W	*TDH1*	Tdh1	Glyceraldehyde-3-phosphate dehydrogenase isozyme 2	1-168
YJR009C	*TDH2*	Tdh2	Glyceraldehyde-3-phosphate dehydrogenase isozyme 2	1-168
YGR192C	*TDH3*	Tdh3	Glyceraldehyde-3-phosphate dehydrogenase isozyme 3	1-168
YDR541C	*YDR541C*	Ydr541cp	Aldehyde reductase	2-337
YGL039W	*YGL039W*	Ygl039wp	Aldehyde reductase	3-341
YMR315W	*YMR315W*	Ymr315wp	Oxidoreductase, DNA replication stress	3-152
YNL241C	*ZWF1*	Zwf1p	Glucose-6-phosphate dehydrogenase	9-199

of *YMR208W* are related to mitochondria and ribosome interactions (Fleischer et al. 2006; Reinders et al. 2006).

A higher level of variation of 48% was also observed in the sequence of an ATP synthase encoding gene *ATP16*. Compared with a conserved homologous allele of *YMR315W* for the parental Y-12632, the frequency of the single nucleotide mutations increased from 0 to 27%, resulting in amino acid alteration from glutamine to arginine (Table 6). Ymr315w was found to have NADPH-specific reductase activity (Hector et al. 2009). It has a NADP-binding domain of Zwf1 and was involved in the newly defined in situ detoxification branch of center metabolic pathways by this study. A similar level of sequence mutation was observed in *FAS2*, a fatty acid synthetase gene for long-chain saturated fatty acids, in Y-50049 up to 28% against Y-12632. As discussed above, increased species content of long-chain fatty acid was considered as a self-defending mechanism against the toxic chemicals (Yang et al. 2012).

Discussion

Using a time-course comparative protein profiling analysis in this study, we identified distinctive expression of Zwf1 and a group of proteins with aldehyde reduction activity against the combinational challenge of furfural-HMF for an adapted industrial yeast strain. New evidence of the protein expression revealed a fine-tuned mechanism of an in situ detoxification pathway for the tolerant industrial yeast. We also identified differential expressions of a large group of ribosomal proteins, including many essential ribosomal proteins, and other uniquely expressed proteins in the tolerant Y-50049 but not in its parental strain Y-12632. Perspectives and new challenges on continued in-depth investigation underlying yeast adaptation are also discussed. New knowledge obtained from this research and insight into the tolerance of the industrial yeast aid the development of the next-generation biocatalyst for production of fuels and chemicals from lignocellulosic materials.

Yeast tolerance is commonly measured using a complete synthetic medium, since an enriched medium often lessen cell susceptibility to the chemical treatment (Liu et al. 2004). Since the wild type is unable to grow on the complete synthetic medium under the combinational toxicity of furfural plus HMF, we applied a standard YM medium, an enriched medium, to allow Y-12632 growing and producing a necessary amount of cell mass for protein sampling in this study. Under the same YM medium conditions, these two strains displayed significantly different phenotypes of cell growth

Table 3 Protein expression of representative transcription factor Hyr1 and reductase enzymes involving in the reprogrammed detoxification pathway for the tolerant industrial yeast *Saccharomyces cerevisiae* strain NRRL Y-50049 compared with its parental wild type NRRL Y-12632 over time in response to synergistic challenges of furfural and HMF

Gene / ORF	Protein	MW (kD)	Treated with furfural-HMF							
			0 h		24 h		48 h		56 h	
			Y-12632	Y-50049	Y-12632	Y-50049	Y-12632	Y-50049	Y-12632	Y-50049
ADH1 / YOL086C	Adh1	37	100	100	100	100	100	100	100	100
ADH4 / YGL256W	Adh4	41	95	100	0	92	0	100	0	100
ADH6 / YMR318C	Adh6	40	18	96	100	100	97	100	100	100
ARI1 / YGL157W	Ari1	38	0	0	100	100	100	100	100	99
GRE2 / YOL151W	Gre2	38	0	0	0	72	100	100	14	99
GRE3 / YHR104W	Gre3	37	0	0	100	93	8	100	0	100
SFA1 / YDL168W	Sfa1	41	0	0	0	18	94	100	0	100
YPR1 / YDR368W	Ypr1	35	0	0	0	100	0	100	0	92
YMR315W / YMR315W	Ymr315w	38	0	96	0	100	0	100	0	100
HYR1 / YIR037W	Hyr1	19	100	100	100	100	0	100	0	100

response, which were similar as observed on a synthetic medium at relatively lower concentrations of a single furan aldehyde compound (Liu et al. 2004, 2005). Strain Y-50049 displayed a clear tolerant response against the challenge of furfural-HMF. This type of resistance was demonstrated to be stable and inheritable, and the tolerant industrial yeast was suggested as a potential delivery vehicle for the next-generation biocatalyst development (Liu 2018).

In a previous gene expression study, we observed a significant differential gene expression response, including repressed expressions, for the industrial yeast treated with furfural-HMF (Liu et al. 2009). It was interesting to find that most proteins involved in glycolysis and PPP were more robust than the sensitive gene expression response to furfural-HMF. *ZWF1* was previously identified as a tolerance candidate gene for yeast by previous studies. A *ZWF1* single gene deletion mutation of *S. cerevisiae* was highly sensitive to furfural and HMF (Gorsich et al. 2006). Overexpression of *ZWF1* allowed cells to grow in the presence of furfural and HMF that are otherwise lethal to the yeast. Expression of *ZWF1* was induced by furfural and HMF in the tolerant yeast; and the activated *ZWF1* expression was suggested among critical cofactor regeneration steps in the activation of the reprogrammed in situ detoxification pathway (Liu et al. 2009). In this study, we observed new evidence of Zwf1 as a key protein with constant differential expressions over time against the toxic chemicals in the conserved glycolysis and PPP compared with its parental strain. A few other proteins were not initially observed but eventually expressed at 24 h, such as Sol3 and Tal1. The outstanding expression of Zwf1 allowed fine-tuning the previously proposed in situ detoxification pathway by gene expression analysis.

During this biotransformation process, the activated aldehyde reductase activity in Y-50049 consumed NADPH stimulating Zwf1 activity. The enhanced Zwf1 reaction activity at the PPP-oxidative branch provided the essential cofactor NADPH for the reduction of furfural and HMF (Fig. 2). In return, the biotransformation reaction by these reductases released $NADP^+$ which served desirable feedbacks that are needed for the oxidative reaction by Zwf1. Consequently, the activated Zwf1 reactions released NADPH to continuously support the aldehyde reductions. Thus, a well-maintained cofactor recycling balance was established and the damage of cofactor imbalance caused by the toxic chemicals was restored. These unique reactions in the adapted Y-50049 were complementary to each other that constituted a renovated path for in situ detoxification of furfural and HMF (Fig. 2). In contrast, the lack of Zwf1 expression and aldehyde reductase activity in Y-12632 appeared to cease the viable activities of the parental wild type strain although many other proteins in the center metabolic pathways remained intact.

In the presence of a modest concentration of a toxic compound, yeast strains often show a lag phase in growth response. Once passing through this delay, the cell growth would increase significantly with an accelerated rate than it would occur in the absence of the toxic compounds (Liu et al. 2004). The rebalanced cofactor regeneration cycle by Y-50049 presented in this study illustrated the key mechanism of the yeast resistance behind this phenomenon. Glycolysis and PPP were found among the most significantly affected pathways for Y-50049 in comparative transcriptome analysis (Zhang et al. 2015). Building upon these previous observations and proposed reprogrammed pathway based on gene expression analysis (Gorsich et al. 2006; Liu and Moon

Table 4 Differentially expressed ribosomal proteins in tolerant strain NRRL Y-50049 of *Saccharomyces cerevisiae* compared with its parental strain Y-12632 in response to furfural-HMF challenges

New name*	Protein	ORF	MW	Description	Furfural-HMF treated							
					0 h		24 h		48 h		56 h	
					Y-12632	Y-50049	Y-12632	Y-50049	Y-12632	Y-50049	Y-12632	Y-50049
eS1A	RPS1A**	YLR441C	29 kDa	40S rp S1-A	100	100	91	100	53	99	65	96
eS1B	RPS1B**	YML063W	29 kDa	40S rp S1-B	100	100	99	100	48	100	53	97
uS5	RPS2**	YGL123W	27 kDa	40S rp S2	100	100	0	0	0	100	0	100
eS6A	RPS6A**	YPL090C	27 kDa	40S rp S6A	100	100	0	100	0	100	32	100
eS7B	RPS7B	YNL096C	22 kDa	40S rp S7-B	100	100	0	100	0	100	0	100
eS8A	RPS8A**	YBL072C	22 kDa	40S rp S8	100	100	0	76	0	100	0	100
uS4B	RPS9B**	YBR189W	22 kDa	40S rp S9-B	100	91	0	95	0	100	0	84
eS10A	RPS10A**	YOR293W	13 kDa	40S rp S10-A	100	100	75	100	0	100	67	100
uS15	RPS13**	YDR064W	17 kDa	40S rp S13	100	100	100	100	0	100	0	100
uS11B	RPS14B**	YJL191W	15 kDa	40S rp S14-B	100	100	0	100	0	100	0	100
uS9A	RPS16A**	YMR143W	16 kDa	40S rp S16A	100	100	0	100	97	100	0	100
eS17A	RPS17A**	YML024W	16 kDa	40S rp S17-A	100	100	96	100	0	100	0	100
uS13A	RPS18A**	YDR450W	17 kDa	40S rp S18A	100	100	29	100	0	100	96	96
eS19A	RPS19A**	YOL121C	16 kDa	40S rp S19-A	100	100	84	100	18	99	9	95
eS21A	RPS21A**	YKR057W	10 kDa	40S rp S21-A	100	100	71	100	8	93	6	92
eS21B	RPS21B**	YJL136C	10 kDa	40S rp S21-B	100	100	71	100	8	93	6	92
uS8A	RPS22A**	YJL190C	15 kDa	40S rp S22-A	100	100	94	100	0	100	0	100
eS25A	RPS25A	YGR027C	12 kDa	40S rp S25-A	100	100	0	0	0	93	0	100
eS28A	RPS28A**	YOR167C	8 kDa	40S rp S28-A	100	100	79	100	8	99	7	100
eS28B	RPS28B**	YLR264W	8 kDa	40S rp S28-B	100	100	79	99	8	87	7	87
uS14A	RPS29A**	YLR388W	7 kDa	40S rp S29-A	100	100	0	93	0	95	0	96
P2A	RPP2A	YOL039W	11 kDa	60S acidic rp P2-alpha	100	67	88	100	97	100	0	100
uL2A	RPL2A	YFR031C-A	27 kDa	60S rp L2A	100	100	0	95	0	100	0	100
uL18	RPL5**	YPL131W	34 kDa	60S rp L5	85	0	100	100	63	100	0	74
eL6B	RPL6B**	YLR448W	20 kDa	60S rp L6-B	99	96	0	100	0	95	6	76
eL8B	RPL8B**	YLL045C	28 kDa	60S rp L8-B	100	91	79	96	0	93	0	72
uL6A	RPL9A**	YGL147C	22 kDa	60S rp L9-A	100	100	0	45	0	100	0	100
uL16	RPL10**	YLR075W	25 kDa	60S rp L10	100	100	91	100	0	100	0	96
uL5A	RPL11A**	YPR102C	20 kDa	60S rp L11-A	100	100	100	100	0	100	0	100
eL14A	RPL14A**	YKL006W	15 kDa	60S rp L14-A	100	100	100	100	97	100	12	100
uL22A	RPL17A**	YKL180W	21 kDa	60S rp L17-A	100	83	0	99	0	100	0	100
eL19A	RPL19A**	YBR084C-A	22 kDa	60S rp L19A	100	100	84	100	0	100	0	100

Table 4 (continued)

New name*	Protein	ORF	MW	Description	Furfural-HMF treated					
					0 h		24 h		48 h		56 h	
					Y-12632	Y-50049	Y-12632	Y-50049	Y-12632	Y-50049	Y-12632	Y-50049
eL20A	RPL20A**	YMR242C	20 kDa	60S rp L20A	100	100	93	100	0	100	0	100
eL21A	RPL21A**	YBR191W	18 kDa	60S rp L21-A	100	100	0	100	0	100	0	75
uL14A	RPL23A**	YBL087C	14 kDa	60S rp L23A	100	100	76	100	0	100	0	100
eL24A	RPL24A	YGL031C	18 kDa	60S rp L24-A	100	81	0	92	0	76	0	74
eL24B	RPL24B**	YGR148C	18 kDa	60S rp L24-B	100	92	0	0	0	91	0	68
eL31A	RPL31A	YDL075W	13 kDa	60S rp L31-A	100	100	96	100	89	100	0	100
uL29A	RPL35A**	YDL191W	14 kDa	60S rp L35A	100	100	85	100	0	100	75	100
eL38	RPL38	YLR325C	9 kDa	60S rp L38	100	100	0	100	0	8	0	100

* New name system following the proposal by Ban et al. (2014)

** Essential ribosomal proteins

2009; Liu et al. 2009; Zhang et al. 2015), the distinctive evidence of comparative protein expression presented in this study further suggests a fine-tuned mechanism of in situ detoxification of furfural and HMF through the reprogrammed pathway by the tolerant Y-50049.

Another significant finding was that a large number of conserved ribosomal proteins showed differential expression against furfural-HMF compared with its parental strain Y-12632. Most of these are essential ribosomal proteins. Cells are unable to grow without an essential ribosomal protein (Steffen et al. 2012). All ribosomal proteins interact with rRNA in the mature ribosome directly. They also serve as direct binding sites for translation factors. The absence of any one ribosomal protein leads to defect in a distinct pre-rRNA processing step (Woodford Jr and Baserga 2013). Ribosomal proteins are essential for ribosome biogenesis. Ribosomes as conserved nanomachines translate information in the genome to create the proteome in yeast cells (Woodford Jr and Baserga 2013; De la Cruz et al. 2015). Lost functions of ribosomal proteins in ribosomal assembly are lethal in yeast (Lecompte et al. 2002; Woodford Jr and Baserga 2013). In the presence of furfural-HMF, the wild type Y-12632 lost expression of many essential ribosomal proteins during this time-course study and appeared dead. It was interesting to observe the adapted Y-50049 showing active expressions of all of these ribosomal proteins against furfural-HMF.

In addition to the important functions of ribosomal protein in ribosome assembly, extra-ribosomal functions of ribosomal proteins are widely expected in all life forms (Wool 1966; Warner and Mclntosh 2009; Zhou et al. 2015). In *S. cerevisiae*, many additional functions beyond the ribosome assembly were summarized including functions at least in categories of translation, replicative life span, DNA repair, and growth and morphological transformation (Lu et al. 2015). All of these functions may impact the adaptation of the tolerant Y-50049. Furthermore, since the tedious and complicated interactions of ribosomal proteins involved in high speed and super accuracy, it is very difficult to define the complete functions of ribosomal proteins (Nikolay et al. 2015). It is expected that many unknown functions and interactions remain to be clarified. The challenge was why these conserved ribosomal proteins were missing in Y-12632 but persistently expressed in the adapted Y-50049 against furfural-HMF? What kind of roles have the ribosomal proteins involved in the interactions of the yeast adaptation to the chemical stress? This is indeed a new perspective in understanding the yeast adaptation.

Cells are adapted when they have evolved molecular mechanisms that allow them to grow optimally under varied challenging environmental conditions (Brooks et al. 2011). In this study, we pinpointed evidence of the unique Zwf1 expression in Y-50049 against an almost intact

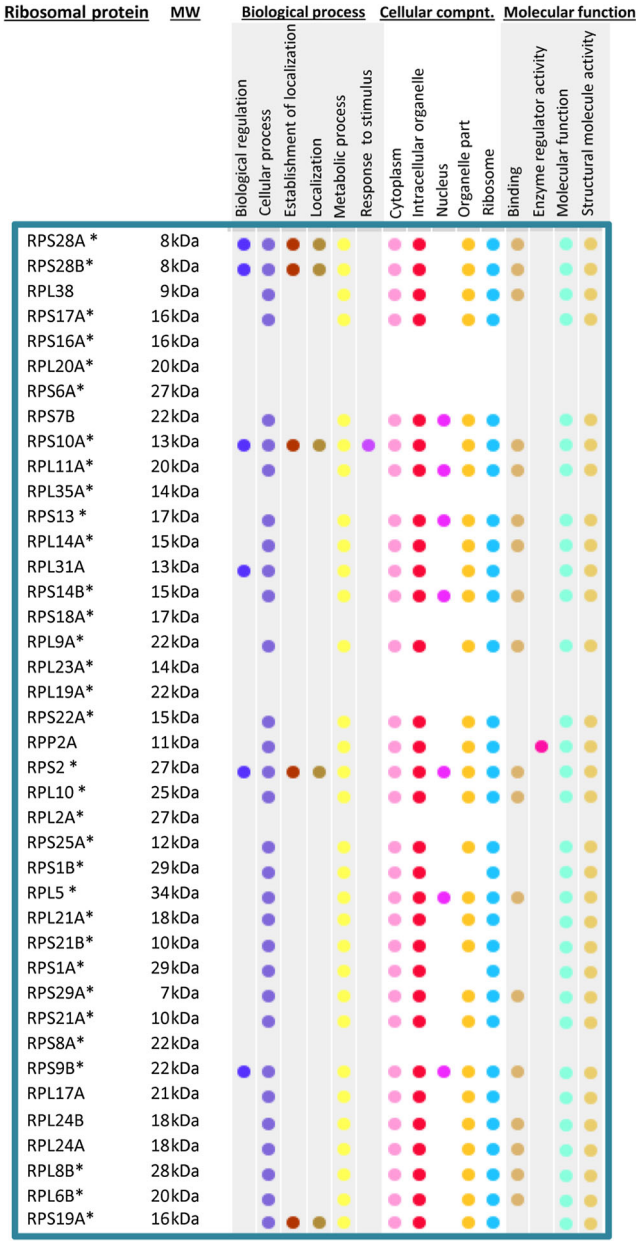

Fig. 3 Functional categories of differentially expressed ribosomal proteins in Y-50049. GO functional categories for ribosomal proteins identified in this work that potentially impact adaptation and evolution of the tolerance of *S. cerevisiae* strain NRRL Y-50049 against a combinational challenge of furfural-HMF. Essential ribosomal proteins are marked with asterisks (*)

background of most robust proteins in glycolysis and PPP. This is a distinctive partial change of the center metabolic pathways of Y-50049. We view this outstanding change as a significant local adaptation in the conserved center metabolic pathways. On the other hand, the altered yeast response against the toxic chemicals in the adaptation was indeed documented at the global level, including rewired networks (Ma and Liu 2010; Lin et al. 2009a; Zhou et al. 2014; Zhang et al. 2015; Jung et al. 2017; Liu 2018). The finding of the large number of differentially expressed ribosomal proteins between the two strains was astonishing. Since these ribosomal proteins are highly conserved, the altered expression of these ancient proteins, as a significant signature expression of Y-50049, might indicate more deeply ingrained changes for the yeast adaptation against the toxic chemicals. Currently, such knowledge is unknown. Findings of this study are expected to trigger interests in continued in-depth investigations on adaptation of the tolerance for the industrial yeast.

New evidence of other unique protein expression obtained in this study was consistent and supportive to previous observations by gene expression analysis. For example, in this study, we found that numerous proteins in various amino acid metabolisms were actively engaged in Y-50049 but not in Y-12632, such as Thr4 for biosynthesis of threonine. Previous genome expression analysis studies suggested that amino acid metabolism and biosynthesis were among the significantly impacted downstream pathways by the toxic chemicals in Y-50049 (Ma and Liu 2010; Zhou et al. 2014; Zhang et al. 2015). In this study, we found that Fas2, a fatty acid synthetase for long-chain saturated fatty acids, was constantly expressed in Y-50049 but diminished in Y-12632 over time after the furfural-HMF challenges (Table 5). Increased species content with a longer fatty acyl chain for industrial yeast was observed by a phospholipidomic study when treated with the toxic chemicals (Yang et al. 2012). It was suggested as a self-defensive response to facilitate cell survival with adjusted permeability and fluidity by the yeast. The active expression of Fas2 and possibly its sequence mutations can be a supporting evidence for the enhanced long-chain fatty acid biosynthesis. However, the functions and direct impact of these sequence mutations need to be further defined. Cell stress genes encoding molecular chaperones that facilitate protein folding and confirmation were identified as common environmental response genes by comprehensive genome expression analysis (Causton et al. 2001; Ma and Liu 2010). In this study, we also identified induced expressions of this group of proteins such as Hsp12 and Hsp90 cochaperone Sti1.

Sequence variations found in Y-50049 indicated that an active mutation process in these genes occurred under the selection pressure, although the entire cell population of the adapted yeast was not completely homogenized yet. Again, whether these mutations directly changed the gene functions is currently unclear. In addition, variations beyond gene coding region such as promoter regions and intron regions are also worth to be investigated in the future. Overall, we found relatively lower levels of non-synonymous mutations in Y-50049 against Y-12632. Such lower levels of mutations suggested that variation of the sequence structure in *S. cerevisiae* is not the only revenue leading to altered resistant phenotypes for Y-50049.

Table 5 Other unique protein expressions over time in Y-50049 compared with its parental strain Y-12632 against synergistic challenges of furfural and HMF

Gene / ORF	Protein	MW (kDa)	Description	0 h		24 h		48 h		56 h	
				Y-12632	Y-50049	Y-12632	Y-50049	Y-12632	Y-50049	Y-12632	Y-50049
ACB1 / YGR037C	Acb1	10	Acyl-CoA-binding protein, DNA replication stress	100	100	1	100	0	100	0	100
OLA1 / YBR025C	Ola1	44	P-loop ATPase, hydrogen peroxide response, DNA replication stress	97	100	0	100	0	100	0	100
PBI2 / YNL015W	Pbi2	9	Vacuole inheritance, DNA replication stress	0	97	89	100	0	100	0	100
PFY1 / YOR122C	Pfy1	14	Conserved protein, cytoskeleton organization, DNA replication stress	0	100	95	53	0	100	0	100
PRB1 / YEL060C	Prb1	70	Vacuolar proteinase B involved in protein degradation, DNA replication stress	0	0	33	0	2	100	0	91
RNR4 / YGR180C	Rnr4	40	dNTP synthesis, DNA replication stress	98	0	0	100	0	100	0	100
VMA4 / YOR332W	Vma4	26	Vacuolar H+-ATPase, DNA replication stress	100	100	96	100	0	100	0	100
YPL225W / YPL225W	Ypl225w	17	Interact with ribosomes, DNA replication stress	94	96	0	37	0	100	0	76
HSP12 / YFL014W	Hsp12	12	Membrane protein, heat shock, oxidative stress, osmostress, DNA replication stress HOG and Ras-Pka pathways	100	100	100	0	0	100	0	100
SGT2 / YOR007C	Sgt2	37	Glutamine-rich cytoplasmic cochaperone, DNA replication stress	56	0	100	100	0	100	0	100
RBL2 / YOR265W	Rbl2	12	Protection from excess free beta-tubulin, DNA replication stress	100	100	24	99	0	100	0	100
ADK1 / YDR226W	Adk1	24	Purine metabolism, DNA replication stress	100	100	100	100	0	100	84	100
GLO1 / YML004C	Glo1	37	Catalyzes detoxification of methylglyoxal, regulated by methylglyoxal levels and osmotic stress	17	0	95	0	0	100	0	100
STI1 / YOR027W	Sti1	66	Stress inducible Hsp90 cochaperone	82	100	97	100	0	100	0	100
RTN1 / YDR233C	Rtn1	33	Nuclear pore assembly and maintenance of tubular ER morphology; promotes membrane curvature, ER stress	100	100	100	100	0	100	0	100
RTN2 / YDL204W	Rtn2	44	Nuclear pore assembly and maintenance of tubular ER morphology; promotes membrane curvature, ER stress	0	0	0	0	0	100	0	100
RDL1 / YOR285W	Rdl1	15	Mitochondrial outer membrane, DNA replication stress	0	0	0	69	0	100	0	96
ADE1 / YAR015W	Ade1	35	Ribotide synthetase required for de novo purine nucleotide biosynthesis, DNA replication stress	0	0	0	0	0	100	0	76
ADE17 / YMR120C	Ade17	65	Enzyme of de novo purine nucleotide biosynthesis	0	0	0	0	0	100	0	100
PNC1 / YGL037C	Pnc1	25	NAD+ salvage pathway, stimulus response, DNA replication stress	0	0	0	0	0	100	0	100
GPH1 / YPR160W	Gph1	104	Mobilization of glycogen, regulated by stress-response elements and HOG MAO kinase pathway	0	0	0	0	0	100	0	99
	Car1	36		0	0	0	0	0	100	0	99

Table 5 (continued)

Gene / ORF	Protein	MW (kDa)	Description	0 h		24 h		48 h		56 h	
				Y-12632	Y-50049	Y-12632	Y-50049	Y-12632	Y-50049	Y-12632	Y-50049
CAR1 / YPL111W			Catabolizes arginine to ornithine and urea, arginine and nitrogen catabolite repression, freeze tolerance								
ACO1 / YLR304C	Aco1	85	Aconitase, TCA cycle, mitochondrial genome maintenance	0	0	0	0	0	100	59	98
GCV3 / YAL044C	Gcv3	19	Catabolism of glycine, protein lipoylation	0	0	0	95	0	100	0	99
ARF1 / YDL192W	Afr1	21	ADP-ribosylation factor, regulation of coated vesicle formation	0	7	0	0	0	100	0	100
THR4 / YCR053W	Thr4	57	Conserved threonine synthase, amino acid control pathway	100	91	97	100	0	100	0	100
FSH1 / YHR049W	Fsh1	27	Putative serine hydrolase, localizes to both the nucleus and cytoplasm	100	100	0	6	0	100	0	96
ADO1 / YJR105W	Ado1	36	Adenosine kinase, utilization of S-adenosylmethionine Adenosine kinase, utilization of S-adenosylmethionine	100	100	100	100	0	100	0	100
EGD2 / YHR193C	Egd2	19	Involved in protein sorting and translocation; associated with cytoplasmic ribosomes	100	100	96	100	0	100	0	100
YNL208W / YNL208W	Ynl208w	20	Interact with ribosomes	100	100	14	95	0	95	0	91
SFM1 / YOR021C	Sfm1	25	rRNA processing and ribosome biogenesis and in biopolymer catabolism	100	100	0	82	0	100	0	83
RIB3 / YDR487C	Rib3	23	Riboflavin biosynthesis, mitochondrial respiration	99	96	100	100	0	100	0	96
RIB4 / YOL143C	Rib4	19	Catalyzes synthesis of immediate precursor to riboflavin	0	70	0	94	0	100	0	100
ATP16 / YDL004W	Atp16	17	Conserved ATP synthase	95	96	37	95	0	100	0	96
FAS2 / YPL231W	Fas2	207	Fatty acid synthetase for long-chain saturated fatty acids	100	98	6	82	0	100	0	100
DUG1 / YFR044C	Dug1	53	Glutathione degradation	61	84	0	0	0	100	0	89
CSS1 / YIL169C	Css1	100	Induced by both high and low pH environment	100	100	0	0	0	95	16	82
MCR1 / YKL150W	Mcr1	34	NADH-cytochrome b5 reductase, ergosterol biosynthesis	100	76	0	100	12	100	45	100
TPM1 / YNL079C	Tpm1	24	Binds to and stabilizes actin cables and filaments for polarized cell growth and organelle distributions		77	88	99	0	100	0	100
ZEO1 / YOL109W	Zeo1	13	Regulates the cell wall integrity pathway	58	100	0	9	0	100	0	

Table 6 Sequence variations of the adapted tolerant industrial yeast *Saccharomyces cerevisiae* NRRL Y-50049 showing non-synonymous mutations compared with its parental wild type strain NRRL Y-12632

ORF	Gene	Function	Chromosome	Position	SNP	Frequency Y-12632	Frequency Y-50049	Amino acid	Codon	Strand
YBL087C	*RPL23A*	60S ribosomal protein	II	60178	G/A	93/7	58/39	G/S	GGT/AGT	Reverse
YBR189W	*RPS9B*	40S ribosomal protein	II	605183	A/T	59/41	19/78	E/V	GAA/GTA	Forward
YNL208W	*YNL208W*	Interact with ribosome	XIV	254808	G/A	89/11	78/22	G/S	GGC/AGC	Forward
YDL004W	*ATP16*	ATP synthase	IV	443281	A/T	79/17	52/48	T/S	ACT/TCT	Forward
YMR315W	*YMR315W*	Reductase	XIII	903653	A/G	100/0	71/27	Q/R	CAA/CGA	Forward
YPL231W	*FAS2*	Long-chain fatty acids	XVI	109841	A/T	97/3	72/28	K/I	AAA/ATA	Forward

Since the chemical-resistance of Y-50049 is a stably heritable phenotype, sequence-independent functions may exist for the yeast. Recently, a growing area of research on epigenetics has been clearly defined (Berger et al. 2009; Fedoroff 2012). *S. cerevisiae* has been demonstrated to have epigenetic-regulated functions like most other complex eukaryotes although it is single celled (Grunstein and Gasser 2013). Today, the epigenetic state of the yeast tolerance is unknown. As another new perspective, it would be very attractive and certainly interested to explore the epigenetic-regulated yeast tolerance in fully understanding mechanisms of adaptation for the industrial yeast.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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