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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY

Time-based comparative transcriptomics in engineered xylose-utilizing *Saccharomyces cerevisiae* identifies temperature-responsive genes during ethanol production

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Abstract Agricultural residues comprising lignocellulosic materials are excellent sources of pentose sugar, which can be converted to ethanol as fuel. Ethanol production via consolidated bioprocessing requires a suitable microorganism to withstand the harsh fermentation environment of high temperature, high ethanol concentration, and exposure to inhibitors. We genetically enhanced an industrial *Saccharomyces cerevisiae* strain, sun049, enabling it to uptake xylose as the sole carbon source at high fermentation temperature. This strain was able to produce 13.9 g/l ethanol from 50 g/l xylose at 38 °C. To better understand the xylose consumption ability during long-term, high-temperature conditions, we compared by transcriptomics two fermentation conditions: high temperature (38 °C) and control temperature (30 °C) during the first 12 h of fermentation. This is the first long-term, time-based transcriptomics approach, and it allowed us to discover the role of heat-responsive genes when xylose is the sole carbon source. The results suggest that genes related to amino acid, cell wall, and ribosomal protein synthesis are down-regulated under heat stress. To allow cell stability and continuous xylose uptake in order to produce ethanol, hexose transporter *HXT5*, heat shock proteins, ubiquitin proteins, and proteolysis were all induced at high temperature. We also speculate that the strong relationship between high temperature and increased xylitol accumulation represents the cell's mechanism to protect itself from heat degradation.

Keywords Bioethanol · Xylose · Thermotolerant · Transcriptomics · *Saccharomyces cerevisiae*

Introduction

Ethanol has a variety of favorable properties that are desirable for use as a neat or pure fuel for transportation. Enzymatic hydrolysis of plant carbohydrates has emerged as the most promising technology for the conversion of biomass into monomer sugars (glucose, xylose, arabinose, mannose, and galactose) for subsequent fermentation into bioethanol [29]. Lignocellulosic material is abundantly available, does not directly compete with food sources, and is considered a cheap raw material [25]. However, even if the raw material comes cheaply, the process economics will not be attractive if the cost of production remains high. Therefore, current bioethanol research is driven by the need to reduce the cost of production. One of the most potent emerging technologies is consolidated bioprocessing (CBP), where saccharification and fermentation of lignocellulosic biomass are featured in the same reactor [13]. However, the drawback associated with CBP is the different optimum temperature between saccharification and fermentation [9]. To address this problem, thermophilic ethanologens might be effective.

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Usage of thermophilic ethanologens in fermentation processes has advantages such as energy saving through reduced cooling costs, easier stripping of ethanol from broth and minimum risk of contamination [14]. Among the few thermotolerant strains known, *Clostridium* and *Thermoanaerobium* species have been investigated as ethanol producers. However, these strains have been consistently found to suffer from end-product inhibition and membrane damage [22]. *Saccharomyces cerevisiae* remains as the microorganism of choice for ethanol production due to its high tolerance to ethanol and ease in genetic modification. Hence, to engineer a robust microorganism for industrial use via inverse metabolic engineering, a better understanding of the genetic diversity of yeasts during high temperature pentose fermentation is deemed necessary. Examples of *S. cerevisiae* attaining thermotolerance have been compiled [6, 23, 26].

Despite being able to tolerate ethanol and high temperature, *S. cerevisiae* in its original metabolic pathway can only ferment glucose as the carbon source, which limits the maximum utilization of monomer sugars in lignocelluloses. This led to increased interest among researchers to reengineer the metabolic pathway, enabling *S. cerevisiae* to uptake xylose as a carbon source [8, 20]. To date, there have been several reports on transcriptomics analysis using recombinant *S. cerevisiae,* but no studies have yet reported the gene expression in *S. cerevisiae* utilizing xylose as the sole carbon source between two different temperatures. Bengtsson et al. [2] analyzed the gene expression between xylose-growing strains with their reference strains at 30 °C, while another group of researchers did gene expression analysis at high temperature, but with glucose as the carbon source of interest [18, 19, 24, 30]. In our previous work [12], we analyzed gene expression among the three best ethanol-producing strains modified from an industrial strain, compared to a negative control strain utilizing xylose at 38 °C only. The purpose then was to compare the transcriptomics between different strain backgrounds by cross-profiling to search for genes regulated in common among the best strains.

In the present report, we examined a strain of recombinant xylose-fermenting *S. cerevisiae* constructed from an industrial strain, sun049, which can grow naturally at high temperature. The performance of the strain in ethanol production was tested at two different temperatures, 30 and 38 °C. During ethanol production at high temperature, it is known that the yeast cells encounter several environmental stresses. Using xylose as the sole carbon source adds another stress to the yeast since *S. cerevisiae* were evolved to utilize hexoses. Therefore, genes responsible for enabling the cells to tolerate the elevated temperature while producing ethanol from xylose need to be revealed. It is worth mentioning that the heat stress in this study is focused on the long-term effects (up to 12 h after high temperature exposure) rather than short-term effects (a few minutes after high temperature exposure). The 12-h time-based transcriptomics would allow better analysis of gene-expression patterns during the course of fermentation compared to a single point transcriptomics analysis. No studies so far have investigated the temperature-responsive genes in xylose fermentation. Here, we highlighted genes that were up-regulated and down-regulated more than twofold at 38 °C compared to at 30 °C, and we present the transcriptomics analysis emphasizing the consistently up-regulated genes during the 12-h fermentation course.

Materials and methods

Strains and medium

The industrial strain (sun049) of *S. cerevisiae* was obtained from Suntory Limited (Tokyo, Japan). The strain was transformed with a YCp-type plasmid, pJHNX1X2XKN, harboring xylose-assimilating genes *XYL1* and *XYL2* from *Scheffersomyces stipitis* and *XKS1* from *S. cerevisiae* to yield sun049T [12]. It was grown in YPD medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose). Prior to use, the strains were pre-incubated in 5 ml of YPD medium to which was added 50 μg/ml clonNAT (Werner Bioagent, Jena, Germany) at 30 °C and 150 rpm. After 24 h of pre-incubation, the cells were transferred to a 1-l flask containing 500 ml of YPD medium with 50 μg/ml clonNAT. The cells were further incubated for 48 h at 30 °C and agitated at 150 rpm. The cells were then centrifuged at 3,000×*g* for 10 min and washed twice with sterile distilled water. After pelleting, the cells were adjusted to 50 g/l of wet cells with distilled water (10 g/l dry cell weight) and were ready to be inoculated to the fermentation.

Fermentation conditions and HPLC analysis

Batch fermentation was carried out in a 100-ml bottle with a CO_2 outlet. Fermentation medium consisted of: 0.5 % (v/v) corn steep liquor (CSL) (Sigma-Aldrich, Tokyo, Japan), 5 g/l urea, 50 g/l xylose, 1 μg/ml pyridoxin-HCl, 1 μg/ml thiamine-HCl, 1 μg/l $MgSO_4$, 2 μg/l $ZnSO_4$, 10 μg/ml pantothenate and 0.1 μg/l biotin. The total working volume was 50 ml. Temperature was controlled by placing the bottles in a water bath equipped with a magnetic stirrer. Fermentation temperatures were set to 30 and 38 °C with stirring at 500 rpm. Samples for high-performance liquid chromatography (HPLC) analysis were taken at 0, 3, 6, 9, 12, 24, and 48 h of fermentation. Samples were centrifuged at 3,000×*g* for 5 min at 4 °C. The supernatant was checked for xylose, ethanol, xylitol, and glycerol concentration by

HPLC (Shimadzu, Kyoto, Japan) with a refractive index detector as described previously [10]. The eluent used was MilliQ water with a flow rate of 0.6 ml/min. The column used was a Shim-Pack SPR-Pb (Shimadzu) with the oven temperature set to 80 °C. Experiments were performed in triplicate.

DNA microarray analysis

One milliliter of cell samples obtained at 3, 6, 9, and 12 h (log phase) of fermentation was quenched by 1.4 ml of cold methanol at −40 °C. Cells harvested by centrifugation at 6,000 × *g* for 5 min at −20 °C were then freeze-dried at −20 °C. The dried cells were used for RNA preparation. Total RNA was obtained by following the protocol provided for the Total RNA Isolation Mini Kit (Agilent Technologies, Palo Alto, CA, USA). RNA concentration and quality were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. cDNA was generated by reverse transcription, labeled with Cy3 and hybridized to *S. cerevisiae* 4 × 44 microarrays. Prior to scanning, hybridization was performed at 65 °C for 17 h. The arrays were scanned by an Agilent Single Color DNA Microarray Scanner (Agilent Technologies); GeneSpring GX ver. 11.5.1 software (Agilent Technologies) was used to analyze data such as fold change in expression. Every biological sample for microarray analysis was analyzed in duplicate. Gene expression was calculated using normalized data and only twofold and above induction or reduction were reported. Differentially expressed genes more than twofold were sorted into four clusters using the *K* means method (Euclidean as distance metric) according to their time-based expression patterns. For every cluster, the gene ontology (GO) was obtained using the GeneSpring GX software.

Real-time PCR assay

The RNA samples used for microarray experiments were also used for real-time PCR validation. cDNA was generated by reverse transcription with a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Quantitative PCR experiments were done in triplicate with a Thunderbird SYBR qPCR Mix (Toyobo) using a Stratagene Mx3005P Real-Time PCR system (Agilent Technologies). The sequences of the forward and reverse primers for the specific genes are listed in Table 1. Thermocycling conditions were as follows: 95 °C for 10 min (one cycle), followed by 40 cycles of the following conditions: 95 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min. The fold change of transcript levels was calculated by the $2^{-\Delta\Delta C_t}$ method [17]. *ACT1* was used as the internal standard.

Results

Xylose fermentation at 30 and 38 °C

The xylose assimilation ability by *S. cerevisiae* was conferred by co-expressing the genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH), which originated from *S. stipitis,* and xylulokinase (XK) from *S. cerevisiae*. By reconstruction of this new pathway, consumed xylose could be reduced to xylitol by XR, and then XDH would oxidize xylitol into xylulose. Xylulose would

Table 1 Primers used in this study

Genes	Product size (bp)	Primer sequences
HSP104	154	Forward: AAGGACGACGCTGCTAACAT
		Reverse: CACTTGGTTCAGCGACTTCA
INO1	164	Forward: AGAGATTGCTCCTTCCACGA
		Reverse: ACTTGGTTTGTCCCGACTTG
ADH2	170	Forward: GCTGCTGGTGGTCTAGGTTC
		Reverse: GCCTTAACGACTGCGCTAAC
GRE2	150	Forward: GCCTTCCAAAAGAGGGAAAC
		Reverse: ATGGGTAGCACCAGAACCTG
XYL1	191	Forward: AGTTAGTTGGTGCCGGTGTC
		Reverse: GAAGGTGACTGGGAAGTGGA
XYL2	248	Forward: GGTGGTCGTTTCGTTCAAGT
		Reverse: GCTCTGACCAAGTCGTAGGC
XKS1	173	Forward: GATTCAAACGCAAGCTCACA
		Reverse: GCACCAATGACTTGAGCAAA
ACT1 (Internal standard)	72	Forward: TGGATTCCGGTGATGGTGTT
		Reverse: TCAAAATGGCGTGAGGTAGAGA

then be phosphorylated by XK to xylulose-5-phosphate, which would then be metabolized through the non-oxidative pentose phosphate pathway (PPP) and glycolysis pathway [10]. The ability of the sun049T strain to produce ethanol from xylose was tested at 30 and 38 °C. Figure 1 presents the result of ethanolic fermentation utilizing xylose as the only carbon source. It shows that sun049T was able to consume xylose within 48 h even under heat stress. In terms of ethanol production, 16.5 g/l was obtained at 30 °C with a yield of 0.33 g ethanol per gram of xylose consumed. This corresponds to 65 % of the theoretical yield. Under heat stress, ethanol production suffered a 15 % reduction after 48 h of fermentation. However, despite a decrease in performance under heat stress, this strain appeared to be the best at producing ethanol at 38 °C based on our previous round of analysis [12]. The cell concentration remained constant throughout the fermentation course and was unaffected by high temperature (data not shown). What makes the strain interesting for investigation is that during the first 12 h of fermentation, its performance is similar to that at the control temperature. Another important observation made was regarding xylitol production, where the rate of xylitol production increased dramatically with temperature. Glycerol production, however, seemed to be unaffected by high temperature.

Comparative transcriptomics analysis for xylose fermentation at 30 and 38 °C based on different gene clusters

The simplest approach to identifying differentially regulated genes is to consider the fold change between control and experimental, i.e., 30 and 38 °C, conditions, respectively. Figure 2 shows a four-way Venn diagram representing the number of genes induced and reduced during the four sampling time (3, 6, 9, and 12 h) under heat stress. It shows that more genes remained down-regulated (1,373 genes) than up-regulated (868 genes) during the 12-h high-temperature fermentation, including genes with unknown

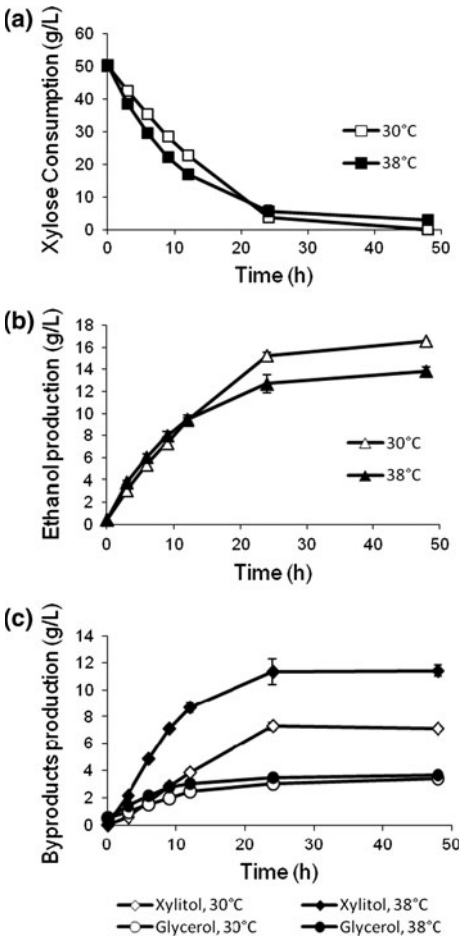

Fig. 1 Time course of xylose fermentation by sun049T at 30 and 38 °C. **a** Xylose consumption (g/l). **b** Ethanol production (g/l). **c** By-products, xylitol and glycerol production (g/l). Values are the average of three independent experiments ±SD

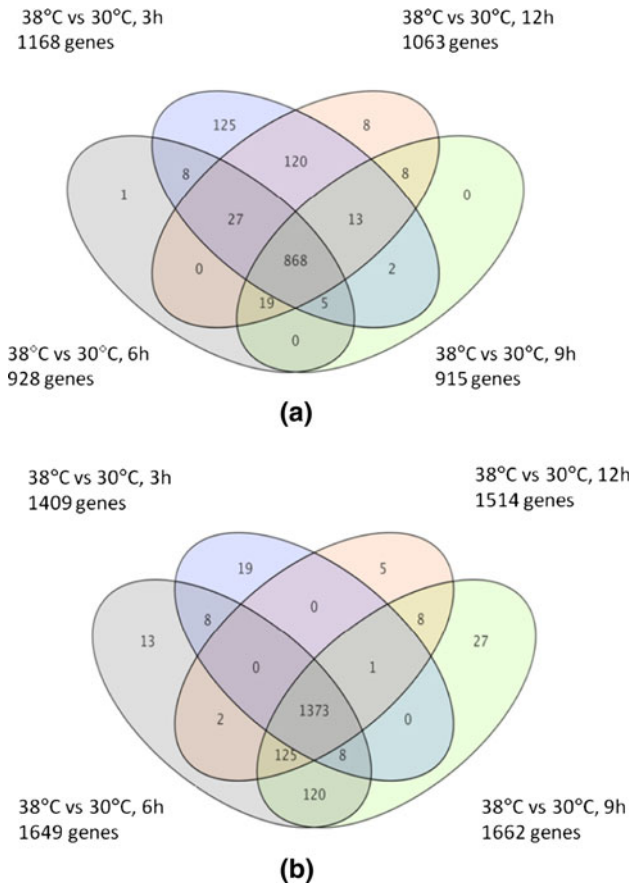

Fig. 2 Four-way Venn diagram showing the relationship among genes regulated at 3, 6, 9, and 12 h of xylose fermentation. **a** Up-regulated genes at 38 °C relative to 30 °C. **b** Down-regulated genes at 38 °C relative to 30 °C

functions. After the fold change analysis was done, differentially expressed genes were grouped into four clusters according to their expression patterns. As shown in Fig. 3, four clusters were obtained using the K-means algorithm.

Cluster 1 (Fig. 3a) represents genes that are up-regulated throughout the fermentation course under heat stress compared to the control. We speculate that the genes in this cluster are the genes needed for a cell protection mechanism. The enriched GO annotations for the genes in cluster 1 are listed in Table 2. The consistently up-regulated gene ontologies with statistically significance were the genes related to protein catabolic processes, proteolysis, protein folding, ubiquitin-dependent protein catabolic processes, and response to stress and response to stimuli. There are overlapping genes in different gene ontologies, meaning that the same gene might have more than a single function activated during heat stress. Among the genes up-regulated were those involved in protein folding and refolding (*HSP10, HSP60, HSP78, HSP82, HSP104, SSE1, SSE2*, and *SSA1-4*). In conjunction with the up-regulation of protein folding genes, genes involved in ubiquitin-dependent protein catabolic processes also tended towards up-regulation (*HUL5, UBC4, UBC6, UBP3, UBP6*, and *UBP9*). Stress regulated genes such as *GRE2* and *GRE3* are also included in this cluster.

Cluster 2 (Fig. 3b) consists of genes that have a mixture of increasing and decreasing expression levels throughout the 12-h fermentation at 30 °C, but were down-regulated at 38 °C with expression levels remaining almost unchanged. The significant GO annotations in this cluster (Table 3) are related to ribosomal protein- and cytoplasmic-associated genes. The strongest GO enrichment under "cellular component" is "cytosolic ribosome" ($p = 2.87\text{E}{-16}$).

Cluster 3 (Fig. 3c) groups the genes that were significantly down-regulated throughout the high-temperature fermentation period as depicted in Table 4. The most down-regulated gene annotation under "biological process" is "small molecule biosynthesis process" ($p = 3.15\text{E}{-08}$). A number of *ERG* genes encoding proteins involved in ergosterol biosynthesis are included in this group. Genes involved in amino acid, amine, carboxylic acid, and organic acid metabolic processes and cell wall structure were also affected.

Lastly, cluster 4 represents the unique pattern of gene expression at 38 °C compared to 30 °C. It shows that these genes increased their expression levels at 30 °C throughout the 12-h fermentation. However, these same genes that were drastically down-regulated after 3 h of heat stress recovered their expression levels after the sixth hour. This particular cluster, comprising a unique gene expression

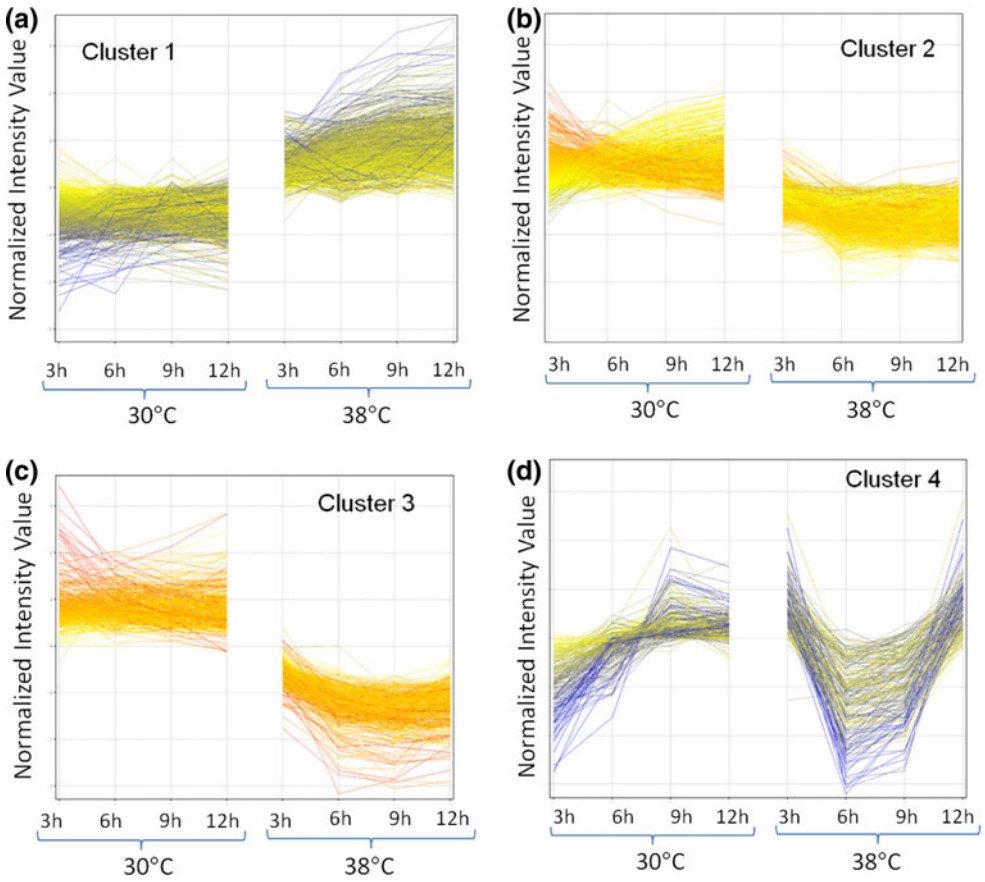

Fig. 3 Clustered genes based on time-based expression pattern at 30 and 38 °C after normalization. **a** Cluster 1 consists of consistently up-regulated genes at 38 °C relative to 30 °C. **b** Cluster 2 consists of down-regulated genes with constant expression levels at 38 °C relative to 30 °C. **c** Cluster 3 consists of down-regulated genes at 38 °C relative to 30 °C. **d** Cluster 4 consists of uniquely regulated genes at 38 °C relative to 30 °C. The clustering algorithm used was a *K*-means (Euclidean) using GeneSpring. The *horizontal axis* indicates the time of gene expression (3, 6, 9, and 12 h) for fermentation temperatures of 30 and 38 °C, and the *vertical axis* indicates the normalized gene expression ratio in log terms (base 2). Genes with unknown functions are included

Table 2 Summary of GO annotated in cluster 1 (up-regulated genes throughout high-temperature fermentation with xylose as the carbon source)

	p value	Major genes
Biological process		
Proteolysis involved in cellular protein catabolic process	3.08E−07	*CDC48, CSR1, DAS1, DOC1, DSK2, HLJ1, HUL5, KAR2, MET30, PUP2, RPN1/2/3/5/7/8/9/10/11/13, STS1, UBC4/6, UBP3/6/9, UBX4, UFD1, UMP1, VID24, VPS24, YDJ1, YGK3*
Modification-dependent protein catabolic process	4.75E−07	
Ubiquitin-dependent protein catabolic process	4.75E−07	
Proteolysis	7.32E−07	
Modification-dependent macromolecule catabolic process	7.65E−06	
Protein catabolic process	1.47E−05	
Cellular protein catabolic process	2.31E−05	
Protein refolding	2.07E−05	*HSC82, HSP10/60/78/82/104, MDJ1, SSA1, SSC1, SSE1/2, YDJ1*
Protein folding	4.3E−05	*AHA1, BUD27, CCT2/3/5, ERO1, FES1, GSF2, HCH1, HLJ1, HSC82, HSP10/26/42/60/78/82/104, KAR2, MDJ1, PDI1, SBA1, SIS1, SSA1-4, SSC1, SSE1/2, STI1, TAH1, TCP1, YDJ1*
Cellular catabolic process	0.003	*ADH2, CDC48, CSR1, CUL3, CUP2, DOC1, ENA1, GLO1/4, GRE3, HSP60/78/82/104, HUL5, INO4, KAR2, MDH2, MET30, MND2, NQM1, PRE4/6/7/8/10, PYC1, RAS1, RPN1/2/3/4/5/7/8/10/11/13, RPT1-6, SEM1, SSA1-4, STS1, UBC4/6, UBP3/6/9, UMP1, VID24, YDJ1, YPI1*
Cellular macromolecule catabolic process	0.005	
Macromolecule catabolic process	0.005	
Response to stress	0.009	*AHA1, ALD6, ARO3, CDC7/48, CUP1-1, CUP1-2, ENA1, GRE3, HSC82, HSP26,30,42,60,78,82,104, INO2, KAR2, MET22, MSN1, PHO4, RAD4/5/6/10/14/16/23/33/51/52/54, SKN7, SSA1-4, SSE1/2, SSK2, UBA4, UBC4, UMP1, YAR1, YDJ1, YGK3, YPI1*
Response to stimulus	0.01	
Cellular component		
Proteasome complex	2.07E−10	*ECM29, HUL5, PRE4/6/7/8/10, PUP2, RAD6/23, RPN2/3/5/7/8/9/10/11/13, RPT1-6, SCL1, SEM1, UBC4/6, UMP1*
Proteasome storage granule	1.56E−08	*PRE4/6/7/8/10, PUP2, RPN1/2/3/5/7/8/9/10/11/13, RPT1/4/5/6, SCL1, SEM1*
Cytosolic proteasome complex	1.57E−08	
Proteasome regulatory particle	1.00E−07	*RPN1/2/3/5/7/8/9/10/11/13, RPT1-6, SEM1, UBP6*
Proteasome accessory complex	1.00E−07	
Proteasome regulatory particle, lid subcomplex	4.06E−05	
Proteasome regulatory particle, base subcomplex	0.001	
Nucleus	0.005	*AAH1, ACS1, ARO3, CDC7/28/40/48, COS8, CUP2, ECM11/29, FLO8, GCR2, GLO1, GRE2/3, HSP26/104, HUL5, INO2/4, KAR3, MET4/30, MSN1, NQM1, NUS1, PHO4/81, PRE4/6/7/8/10, RAD4/5/6/10/14/16/23/33/51/52/54, RPN1/2/4/7/11/13, RPT1-6, SEM1, SGD1, SKN7, SOL1, SSA1/4, SUT1, UBX4, UMP1, YPI1*
Molecular function		
Unfolded protein binding	1.59E−04	*HSC82, HSP10/26/42/60/82/104, KAR2, MDJ1, SSA1-4, SSC1, SSE1/2, YDJ1*
ATPase regulatory activity	0.001	*AHA1, MDJ1, SSE1/2, YDJ1*

pattern, did not have any overexpressed gene ontology due to many of the genes having unknown functions.

Transcriptional changes of genes with increase in fold change over time

Table 5 indicates some of the consistently induced genes during the 12-h fermentation course. Highlighted in bold are the genes with an increase in fold change at every sampling point. We speculate that the genes whose expression levels were not only up-regulated, but increased over time are the genes that enable the cells to sustain the ability to withstand heat stress. The stress inducible gene, *STI1*; the glycolysis gene, *GRE2*; and the stationary phase gene, *SPG4* were constantly up-regulated during the high temperature period. *HSP78* and *HSP104* showed increasing expression levels during the 12-h fermentation. *INO1,* which has previously been linked to ethanol stress [11],

Table 3 Summary of GO annotated in cluster 2 (down-regulated genes with constant expression level throughout high-temperature fermentation with xylose as the carbon source)

	p value	Major genes
Biological process		
Translation	7.56E−06	*ARG1, BIO2, CDC60, COQ2, DSE1, FIT2, HMS2, MET2/6/13/14/32, RPL3/5/30, RPS2/13/20, RSM25, SED1*
Regulation of translation	2.49E−05	*CDC19/55/60, PFK1, PGI1, SSB1, VAS1*
Posttranscriptional regulation of gene expression	3.94E−04	
Regulation of cellular protein metabolic process	1.81E−04	*BMH2, CDC19/55/60, PFK1, PGI1, RPL3/5/30, SSB1, VAS1*
Cellular component		
Cytosolic ribosome	2.87E−16	*ARD1, GCN2, RPL2A/2B/3/11A/11B/12A/12B/13A/13B/15A/16A/16B/17A/17B/18A/18B/20B/22A/22B/23B/26A/26B/27B/29/30/31A/31B/33A/34A/34B/39, RPS2/5, STM1*
Ribosomal subunit	6.84E−11	*MRP4/13/51, MRPL1/6/11/17/25/32/38/44, RPL3/5/30/39, RPS2/5/13, RSM25*
Cytosolic part	3.75E−10	*ASC1, ENO1, PFK1, RPL3/5/29/30/39, RPS2/5/20, YKE2*
Cytoplasmic part	4.24E−08	*ADH1, ALD5, ALG2/6/9, ALO1, ALT1, ANB1, ANP1, ARD1, ARG1/2, ARV1, ATP14/19, CAT5, CCW14, CDC10/14/15/19/25/50, CHS3/6, COQ2/6/9, CYR1, DDR2, DSS1, EMC1/2/4, ENO1, FRT2, GRX7, HMF1, HXK1, LEU4, PFY1, PGK1, PST2, SOL4, TDH1, TPS3*
Ribosome	1.04E−07	*ARD1, MRP4, RPS2, SED1, STM1*
Cytosolic small ribosomal subunit	1.08E−07	*ASC1, RPS2/5/13/15/20*
Cytosolic large ribosomal subunit	1.92E−07	*RPL3/5/29/30/39*
Cytosol	1.13E−06	*ADH1, CDC19/25, ENO1, HMF1, HXK1, RPL3/5/30, RPS2/5/13/20, TSA1*
Large ribosomal subunit	7.92E−05	*MRPL1/6/11/17.25/32, RPL3/5*
Small ribosomal subunit	1.32E−04	*MRP4/13, RPS2/13/15/20, RSM25*
Ribonucleoprotein complex	0.001	*DBP6, MRP4/13, RPS2/13/15/20, RSM25, SED1, SSB1, TSA1*
Molecular function		
Structural constituent of ribosome	4.56E−11	*MRP4/13, RPL3/5/30, RPS2/13/15/20, RSM25*
Structural molecule activity	3.75E−10	*CCW14, CDC10, MRP4/13, PIR3, RPS2/5/13/15/20, RSM25, SED1, YLR194C*

was also induced in our analysis. The induction rate rose to +7.65 at 12 h of heat stress. Another gene that showed a high fold change is *ADH2,* which at 3 h of heat stress was induced almost threefold, and then increased to eight times higher than the control at 12 h. Another gene, *YGK3,* a protein kinase involved in the control of Msn2p-dependent transcription of stress responsive genes and in protein degradation, was also induced during the 12-h fermentation. One gene of unknown function (*YNL019C*) also showed an increase in fold change with time. The importance of this gene in relation to heat stress management has not been tested previously.

Expression of genes related to xylose metabolism

As xylose is the only carbon source available for the cells, verification of expression of the xylose metabolizing genes is necessary. Xylose is transported in the cells by facilitated diffusion through the hexose transporter, a member of the *HXT* gene family. Based on the transcriptomics data, only *HXT5* was induced during 9 h (+2.2) and 12 h (+2.8) of fermentation (Supplementary data). Then, by using qRT PCR, expression of *XYL1* and *XYL2* from *S. stipitis* and *XKS1* in the recombinant *S. cerevisiae* strain was measured from the samples at 38 °C fermentation relative to those of the 30 °C fermentation. The fold change was found to be less than twofold, thus considered not significantly affected by high temperature (Figure S1). The genes involved in PPP and glycolysis are depicted in Table 6. According to the transcriptomics data, *TKL1, TKL2, TAL1*, and *GND1* are down-regulated due to heat stress. *TKL1, TAL1*, and *GND1* showed a greater decrease in expression over the 12 h of heat stress. Our results also show that most glycolytic genes are down-regulated at high temperature except for *GLO1* and *GCR2*.

Table 4 Summary of GO annotated in cluster 3 (down-regulated genes throughout high-temperature fermentation with xylose as the carbon source)

	p value	Major genes
Biological process		
Small molecule biosynthetic process	3.15E−08	*ARG4/7, ARO1/4/8, CBS2, DED81, DUG1, GDH1, GLT1, GTO3, GTT2, HIS1/2/4/7, HOM2/3, LEU2, LYS2/9/12/21, MET3/5/10/17/28, PAN5, PDC6, SER33, SNO2/3, THI3/4/12/20/80, THR1, THS1, TRP2, TYS1*
Cellular amino acid and derivative metabolic process	4.55E−07	
Cellular amino acid metabolic process	1.35E−06	
Amine metabolic process	1.93E−06	
Cellular amine metabolic process	1.38E−05	
Cellular amino acid biosynthetic process	1.52E−05	
Cellular nitrogen compound biosynthetic process	2.06E−05	
Amine biosynthetic process	3.83E−05	
Carboxylic acid biosynthetic process	2.06E−05	*ACP1, ARG4/7, ARO1/4, AYR1, ERG3, FEN1, GDH1, GLT1, HIS1/2/4/7, HOM2/3, LEU2, LYS2/9/12/21, MET3/5/10/17/28, SER33, THR1, TRP2*
Organic acid biosynthetic process	2.06E−05	
Sulfur compound metabolic process	3.06E−05	*ACP1, DUG1, GDH1, GTO3, GTT2, HOM2/3, MET3/5/10/17/28, RPI1, SNO2/3, THI4/12/20/80, THR1, TRX1*
Organic acid metabolic process	4.49E−05	*ACP1, ARG4/7, ARO1/4/8, DED81, ERG3, FDH1, FEN1, FRS1, GDH1, GLT1, GRS1, GUS1, HIS1/2/4/7, HOM2/3, LEU2, LYS2/9/12/21, MET3/5/10/17/28, PDC6, PYC2, SER33, SNO2/3, THI3, THR1, TRP2, TYS1*
Oxoacid metabolic process	1.38E−04	
Carboxylic acid metabolic process	1.38E−04	
Cellular ketone metabolic process	1.54E−04	
Thiamin and derivative biosynthetic process	3.08E−04	*RPI1, SNO2/3, THI4/12/20/80*
Sulfur compound biosynthetic process	4.14E−04	*ACP1, HOM2, MET3/5/10/17/28, RPI1, SNO2/3, THI4/12/20/80*
Thiamin metabolic process	5.09E−04	*RPI1, SNO2/3, THI4/12/20*
Thiamin and derivative metabolic process	8.10E−04	
Thiamin biosynthetic process	0.002	
Aromatic compound biosynthetic process	8.56E−04	*ARO1/4, RPI1, SNO2/3, THI4/12/20/80, TRP2*
Cellular aromatic compound metabolic process	0.002	*ACO2, ARG4/7, ARO1/4/8, HIS1/4/7, HOM2/3, LYS21, PDC6, RPI1, SNO2/3, THI4/12/20/80, TRP2, YMC1*
Aspartate family amino acid biosynthetic process	0.004	*HOM2/3, LYS2/9/12/21, MET3/5/10/17/28, THR1*
Cytokinetic process	0.006	*BUD8, CHS2, DSE2/4, ERV15, MYO1/2/5, RAX1/2, SUN4, TWF1*
Cell wall organization or biogenesis	0.008	*ARG7, CHS2, CIS3, CRH1, CWH41, CWP2, DSE2/4, EXG1, KRE11, MET5, MKC7, PSA1, PST1, ROT2, RRT12, SCW4/11, SPO21/73, SRL1, SUN4, SWI4, TPM1, YEA4*
Cellular component		
Fungal type cell wall	5.16E−07	*AGA2, CIS3, CRH1, CWP2, DAN4, DSE2/4, EGT2, EXG1, FIT1, FLO10, MKC7, MUC1, PST1, RRT12, SCW4/11, SRL1, SUN4, TIR2, TOS6*
Cell wall	5.16E−07	
External encapsulating structure	5.16E−07	
Extracellular region	1.87E−06	*CIS3, CRH1, CWP2, DAN4, DSE2/4, EGT2, EXG1, FIT1, FLO10, MUC1, PST1, SCW4/11, SRL1, SUC2, SUN4, TIR2, TOS6*
Anchored to membrane	6.94E−04	*CRH1, CWP2, DAN4, DSE2, EGT2, FIT1, FLO10, MKC7, MUC1, PST1, TIR2, TOS6*
Molecular function		
Hydrolase activity, hydrolyzing *O*-glycosyl compounds	2.91E−06	*CRH1, CWH41, DSE2, EGT2, EXG1, MAL12/32, ROT2, SCW4/11, SUC2, SUN4*
Hydrolase activity, acting on glycosyl bonds	3.05E−05	
Glucosidase activity	1.51E−05	

Microarray data validation

We chose four genes (*HSP104, INO1, ADH2*, and *GRE2*) that showed a clear response to heat stress for validation using qRT PCR. Figure 4 shows the fold change of the selected genes at 38 °C relative to 30 °C at 3-, 6-, 9-, and 12-h time points during the course of fermentation. Agreement of fold change, and direction, between the

Table 5 Selected genes constantly found to be induced during the 12-h heat stress

Genes	3 h	6 h	9 h	12 h
HSP30	+8.17	+6.08	+5.90	+4.80
HSP26	+2.86	+2.17	+2.35	+2.08
HSP104	**+2.28**	**+2.53**	**+2.89**	**+3.21**
HSP60	+3.82	+3.95	+3.38	+3.15
HSP78	**+2.22**	**+2.61**	**+3.20**	**+4.08**
HSP82	+4.44	+4.06	+4.02	+3.89
INO1	**+3.56**	**+6.69**	**+6.58**	**+7.65**
YGK3	**+3.95**	**+6.61**	**+7.08**	**+8.31**
SSA1	+3.70	+3.38	+3.32	+3.42
STI1	+3.97	+3.89	+3.06	+3.89
ADH2	**+2.78**	**+4.22**	**+6.48**	**+8.11**
GRE2	+2.27	+3.52	+4.26	+4.24
SPG4	+2.10	+3.48	+6.54	+6.49
CUP1	+3.20	+2.55	+3.08	+3.54
YNL019C (unknown function)	+3.13	+4.62	+5.92	+5.94

Genes in *bold*—their fold change increases with time

Table 6 Fold change of genes involved in the pentose phosphate pathway (PPP) and glycolysis at high temperature (38 °C) relative to control temperature (30 °C)

Pathway	Genes	3 h	6 h	9 h	12 h
PPP	*TKL1*	−2.0	−4.1	−5.3	−6.3
	TKL2	−2.6	−5.4	−4.8	−4.2
	TAL1	ND	−2.7	−3.5	−3.9
	RPE1	ND	ND	ND	ND
	RKI1	ND	ND	ND	ND
	GND1	ND	−3.9	−7.2	−7.2
Glycolysis	*TDH1*	ND	ND	ND	−2.11
	GLO1	ND	ND	+2.4	+2.2
	GCR1	−2.1	−3.9	−3.8	−3.5
	GCR2	ND	ND	+2.0	+2.2
	PGK1	ND	ND	ND	−2.4
	PFK27	ND	+3.6	+3.0	+3.6
	ENO1	ND	ND	ND	−2.5
	FBA1	ND	ND	ND	ND

ND not detected

microarray data and the qRT PCR data was observed with differences in magnitude attributable to differences in detection sensitivity between each type of equipment.

Discussion

With the aim of developing a robust biocatalyst for cost-effective ethanol production, we successfully constructed *S. cerevisiae* strain sun049T, which can grow on and consume xylose as the sole carbon source. Comparing the results for the 30 °C fermentation with data from other similar reports, strain sun049T obtained higher ethanol production, 16.5 g/l, than the mutant strain, $SX3^{MUT}$ used by Lee et al. [15], which produced only 7 g/l ethanol when xylose was the only carbon source. However, the MA-R4 strain used by Matsushika et al. [21] achieved a slightly higher ethanol yield (0.35 g ethanol per gram of xylose consumed) compared to sun049T. To our knowledge, fermentation by *S. cerevisiae* using xylose as the sole carbon source at high temperature has not been tested. Thus, the strain's performance at 38 °C cannot be compared with other studies. Industrial strains can tolerate many hydrolysates better than laboratory strains, indicating that generating a pentose-fermenting *S. cerevisiae* strain with an industrial background would be preferable [8]. Sun049T is considered to be thermotolerant based on its ability to fully consume xylose at high temperature. There are a large number of target genes that could be responsible for the desired traits and it is not an easy task to pinpoint relevant genes at a molecular level. In this study, we identified gene responses that might be of importance to heat tolerance for xylose fermentation.

An important question is how the strain managed to continue producing ethanol under high temperature. Clearly, the uptake of xylose by the strain was not affected by temperature increase. According to Chu and Lee [4], *HXT5* and *HXT7* are particularly important to *S. cerevisiae* when xylose is the only available carbon source. Based on this data, the induction of *HXT5* during the late log phase was used to indicate an increased diffusion of xylose into the cells as a response to high temperature. On the other hand, the overall ethanol production was negatively affected under heat stress even though it was well tolerated during the first 12 h of fermentation. Based on the results, the ethanol production was not cell concentration-dependant, but rather metabolically dependant. The contradiction between PPP gene expressions and the ethanol production during the first 12 h indicated that the genes' regulation actually occurs faster than the cell's change in phenotype, which was shown later at 24 h by lower ethanol productivity. Also, PPP could be one of the limiting steps during high temperature ethanol production where severely down-regulated PPP genes might indicate the accumulation of certain intermediates. For instance, when the *TAL1* gene is down-regulated, there would be an accumulation of sedoheptulose 7-phosphate. This intermediate accumulation would later affect the metabolic flux in PPP. As a result, aromatic amino acid synthesis was also affected by being down-regulated. This reflects the fall in the biosynthetic machinery for proteins in the cells during high-temperature stress. Accumulation of pentose phosphate intermediates as well as low ATP levels can be detrimental, but could be attenuated if the xylose uptake rate

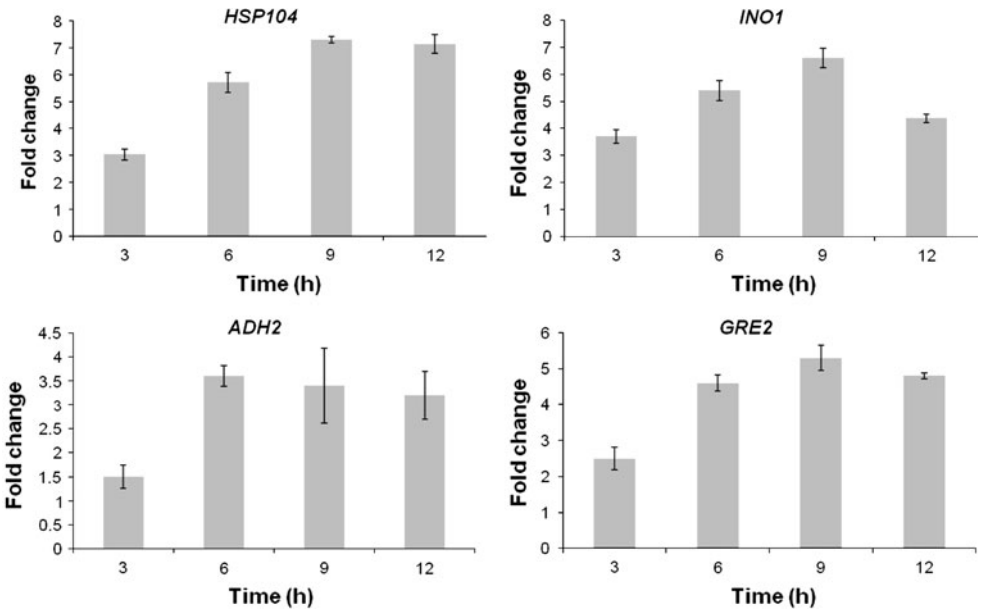

Fig. 4 Validation of time-based microarray data by qRT PCR for *HSP104, INO1, ADH2*, and *GRE2*. Fold change is calculated based on gene expression at 38 °C divided by the values at 30 °C. *ACT1* was used as an internal standard. Values are the average of three independent experiments ±SD

were to have a significant control on the flux [27]. Based on a previous xylose fermentation study utilizing *S. stipitis,* xylitol production could be temperature-dependent to some degree [5]. This xylitol production might be one of the contributing factors leading to the decrease in ethanol production at high temperature, and the phenomenon might also be related to the induction of *GRE* genes. *GRE3* is an aldose reductase involved in xylose metabolism that is also stress-induced. The upregulation of *GRE2* and *GRE3* conforms to the study of Traff et al. [28] in which deleted *GRE3* resulted in decreased xylitol formation. Therefore, future study on fine tuning *GRE3* expression in high-temperature fermentation needs to be carried out to further investigate this hypothesis. Another possibility is that high *ADH2* activity can presumably lead to rapid ethanol re-assimilation and may explain the low observed accumulated ethanol [27]. Taken together, we speculate that the strain increased its xylitol production as a mechanism to cope with heat stress. As for glycerol production, it appears that the glycerol pathway was not activated under heat stress.

A recognized cellular response at high temperature is to increase heat shock protein (HSP) expression. It was also anticipated that some genes regulating Msn2p would be induced during high-temperature fermentation. *YGK3* is one of the stress genes regulating *MSN2*. Msn2p is a transcription factor that binds to stress-response elements (STRE). As thoroughly investigated by previous researchers, Msn2p is activated by heat shock [3]. In our results, the co-induction of heat shock proteins with ubiquitination genes is in agreement with the findings of Gasch [7]. Cells respond to the heat-denatured protein by inducing genes encoding protein-folding chaperones such as *HSP26, HSP78, HSP104*, and *SSA4*. Denatured proteins that cannot be properly refolded are targeted for degradation by ubiquitination [7]. *INO1* was induced at high temperature, maybe as a response mechanism via production of phospholipid barriers. This induction shows that the same gene can be regulated by various types of stresses. Our strategy in obtaining transcriptomics data within a 12-h period was to observe the expression level for an extended period. Evidently, based on gene behavior, *HSP104, HSP78, INO1*, and *YGK3* are the genes partly responsible for long-term thermotolerance. We speculate that *HSP104* and *HSP78* worked together with *YGK3* to become induced over time to cope with the increasing volume of heat-denatured proteins. Since the cell wall is also affected by heat, *INO1* was up-regulated as a mechanism to protect cell structure.

On the other hand, cytoplasmic and ribosomal protein genes significantly repressed due to heat stress are believed to comprise the cell's mechanism to save energy [30]. This is thought to be a response to maintain the fidelity of protein translation and folding at the expense of the rate of protein synthesis [24]. Ergosterol is one of the products from the sterol biosynthetic pathway, also down-regulated. It is a membrane component and important for cell viability and resistance to ethanol [16]. It was found that a lack of oxygen can repress ergosterol synthesis [16]. This might be true since oxygen is necessary for xylose uptake [27]. The need for oxygen would be relatively higher at high temperature since the cells need more respiration for ATP generation [30]. Thus, reduced amounts of oxygen in the fermentation bottle may halt ergosterol synthesis.

Comparing our work with that of others, the up-regulation of *HSP26, SSA4, HSP82*, and *HSP104* is in agreement with the results by Auesukaree et al. [1], even though the carbon source differs. Immediate transfer of the yeast cells from room-temperature conditions to a 38 °C fermentation broth is experienced as stress. However, after hours of heat exposure, the cells managed to adapt to the stressful conditions. This might explain why some genes highlighted by other researchers were not found to be regulated in our transcriptomics analysis. However, this does not mean that they were not functional. Since our earliest transcriptomics analysis was done at 3 h after exposure to heat stress, the undetected genes might have regulated their expression levels soon, perhaps a few minutes, after stress adaptation. It is therefore necessary to investigate the gene behavior for a longer duration to study how the genes change their expression levels over certain periods and conditions.

Conclusions

We used a time-based transcriptomics approach to identify a large number of long-term temperature responsive genes. We found that a strain consuming xylose exhibited partially similar gene regulation to those strains inoculated in glucose media at high temperature. The down-regulation of amino acid synthesis and cell wall genes under heat stress indicates a negative effect on the cells. To cope with the stress, the cells induce the hexose transporter and heat shock proteins together with the ubiquitin-dependent protein genes to protect proteins and cellular functions from the harmful effects of heat. Ribosomal proteins were down-regulated for energy saving purposes, and xylitol accumulation at high temperature might function as a protectant, helping the external cell to withstand heat stress. It is hoped that the temperature-responsive gene behavior identified in this study will accelerate future research to uncover the thermotolerance limitations on ethanol production from xylose at high temperature, with a view to making consolidated bioprocessing more realistic and economically feasible.

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