Improvement of high-temperature and inhibitor stress tolerance of xylose-fermenting yeast strains based on transcriptomic analysis

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July 2013

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CONTENTS

List of Abbreviations

ATP	adenosine triphosphate
CBP	consolidated bioprocessing
cDNA	complementary DNA
CSL	corn steep liquor
Cy3	cyanine3
DNA	deoxyribonucleic acid
E10	biofuel blend, 10% ethanol to 90% gasoline
GHG	greenhouse gases
GO	gene ontology
5-HMF	5-hydroxymethylfurfural
HPLC	high performance liquid chromatography
IME	inverse metabolic engineering
NADH	nicotinamide adenine dinucleotide
NAD(P)H	nicotinamide adenine dinucleotide phosphate
PCR	polymerase chain reaction
PPP	pentose phosphate pathway
qRT PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
SD	standard deviation
SEM	standard error of the mean
SSF	simultaneous saccharification and fermentation
XDH	xylitol dehydrogenase
XK	xylulokinase
XR	xylose reductase

List of Major Genes

ACT1	Actin, structural protein involved in cell polarization, endocytosis, and other cytoskeletal functions
ADH1	Alcohol dehydrogenase; fermentative isozyme active as homo- or heterotetramers; required for the reduction of acetaldehyde to ethanol, the last step in the glycolytic pathway
ADH7	Alcohol dehydrogenase; NADPH-dependent medium chain alcohol dehydrogenase with broad substrate specificity; member of the cinnamyl family of alcohol dehydrogenases; may be involved in fusel alcohol synthesis or in aldehyde tolerance
FIT2	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor, involved in the retention of siderophore-iron in the cell wall
FLO1	Flocculation; Lectin-like protein involved in flocculation; cell wall protein that binds mannose chains on the surface of other cells, confers floc-forming ability that is chymotrypsin sensitive and heat resistant
FLO5	Flocculation; Lectin-like cell wall protein (flocculin) involved in flocculation; binds mannose chains on the surface of other cells, confers floc-forming ability that is chymotrypsin resistant but heat labile
FLO10	Flocculation; Member of the FLO family of cell wall flocculation proteins; not expressed in most lab strains; overproduction induces flocculation that can be inhibited by mannose, sucrose, or glucose; overproduction also promotes haploid invasive growth and diploid filamentous growth
GND1	6-phosphogluconate dehydrogenase (decarboxylating); catalyzes an NADPH regenerating reaction in the pentose phosphate pathway; required for growth on D-glucono-delta-lactone and adaptation to oxidative stress
GRE2	3-methylbutanal reductase and NADPH-dependent methylglyoxal reductase
HSP78	Heat shock protein; Oligomeric mitochondrial matrix chaperone that cooperates with Ssc1p in mitochondrial thermotolerance after heat shock; able to prevent the aggregation of misfolded proteins as well as resolubilize protein aggregates
HSP104	Heat shock protein; Disaggregase; heat shock protein that cooperates with Ydj1p (Hsp40) and Ssa1p (Hsp70) to refold and reactivate

	previously denatured, aggregated proteins; responsive to stresses including: heat, ethanol, and sodium arsenite
HXT1	Low-affinity glucose transporter of the major facilitator superfamily, expression is induced by Hxk2p in the presence of glucose and repressed by Rgt1p when glucose is limiting
HXT4	High-affinity glucose transporter; member of the major facilitator superfamily, expression is induced by low levels of glucose and repressed by high levels of glucose
HXT5	Hexose transporter with moderate affinity for glucose; induced in the presence of non-fermentable carbon sources, induced by a decrease in growth rate
HXT9	Putative hexose transporter that is nearly identical to Hxt11p
INO1	Inositol-3-phosphate synthase, involved in synthesis of inositol phosphates and inositol-containing phospholipids; transcription is coregulated with other phospholipid biosynthetic genes
SSA4	Heat shock protein that is highly induced upon stress
TAL1	Transaldolase, enzyme in the non-oxidative pentose phosphate pathway; converts sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate to erythrose 4-phosphate and fructose 6-phosphate
TKL1	Transketolase; catalyzes conversion of xylulose-5-phosphate and ribose-5-phosphate to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate in the pentose phosphate pathway; needed for synthesis of aromatic amino acids
TPS1	Synthase subunit of trehalose-6-P synthase/phosphatase complex; synthesizes the storage carbohydrate trehalose; also found in a monomeric form; expression is induced by the stress response and repressed by the Ras-cAMP pathway
XYL1	Gene from *Scheffersomyces stipitis* encoding xylose reductase (XR)
XYL2	Gene from *Scheffersomyces stipitis* encoding xylitol dehydrogenase (XDH)
XKS1	Xylulokinase, converts D-xylulose and ATP to xylulose 5-phosphate and ADP; rate limiting step in fermentation of xylulose; required for xylose fermentation by recombinant *S. cerevisiae* strains
YGK3	Involved in control of Msn2p-dependent transcription of stress responsive genes and in protein degradation

CHAPTER 1

General Introduction

1.1 Overview of the strategic benefits of bioethanol production

In many applications, crude oil-derived fuel displaced coal and had long since dominated as a transport fuel. Recently, concerns have grown whether oil reserves have the capacity to service growing demand. According to Owen et al. (2010), supply and demand is likely to diverge between 2010 and 2015, while reserves that provide liquid fuels today will only have the capacity to cater half the demand by 2023. Not only that most of the reserves are produced in unstable regions of the world, we are also faced with greater environmental consequences if the energy usage pattern is not changed. There are mounting concerns on the buildup of carbon dioxide (CO_2) and other greenhouse gases (GHG) which could trap the heat that usually radiates from the earth and cause global climate change (Wyman, 1996).

As an alternative, biethanol, ethyl alcohol (C_2H_5OH) has been used as a modern biofuel. It is commonly blended with gasoline in concentrations of 10% ethanol to 90% gasoline, known as E10 and nicknamed 'gasohol'. It can also be used as a 5% blend with petrol which does not require engine modification (Balat, 2011). Thus, biofuels such as ethanol provide a more feasible technology than other renewable energy because it could serve immediately to substitute for petroleum products in transportation (Hira and de Oliveira, 2009). Also, the use of bioethanol-blended fuel for automobiles can significantly reduce petroleum use and reduce GHG emissions (Balat, 2011). Global biofuel production, including ethanol and biodiesel, has tripled from 4.8 billion gal in 2000 to 16 billion gal in 2007, but still accounts for less than 3% of global transportation fuel supply (Hira, 2011). According to Table 1.1, USA and Brazil are the current leaders in the world bioethanol production which utilizes starch from corn and sugarcane juice, respectively. Due to rapid growth in population and industrialization, worldwide ethanol demand is increasing continuously, despite some obstacles, for example debates on increasing food prices.

Table 1.1 2012 World Fuel Ethanol Production (Renewable Fuels Association, 2013).

Continent	Millions of Gallons
North & Central America	13,768
South America	5,800
Brazil	5,577
Europe	1,139
Asia	952
China	555
Canada	449
Australia	71
Africa	42

1.2 Lignocellulosic material as potential resources

The possible connection between ethanol production and food price inflation may happen in two ways, either by reallocating produced food crops to fuel production (eg. sugarcane being allocated to ethanol rather than to sugar) or by diverting agricultural land from food crops to energy crops (eg. wheat crops being substituted by corn or sugarcane) (Monteiro et al., 2012). But if the biofuel crops are cultivated only on unused or marginal land, the impact on food prices would be minimal. There are about 73.9 teragrams of crop residues in the world that could produce 49.1 gigaliters of ethanol per year (Kim and Dale, 2004). Lignocelluloses such as agricultural, industrial and forest residuals account for the majority of the total biomass present in the world, consists of lignin, cellulose, hemicelluloses, and various extractives (Jeffries and Jin, 2000). It is becoming one of the essential resources for bioethanol production since it is non-edible, thus does not compete with food supplies. Cellulose and hemicelluloses can be converted to ethanol by hydrolysis and fermentation. Besides glucose, the pentose sugar xylose is the major carbohydrate component of hemicellulose. The fermentation process would only be economically viable only if both hexose and pentose sugars present in the lignocellulosic hydrolysates are converted to ethanol (Kuhad et al., 2011). To achieve this, a microorganism capable of utilizing both glucose and xylose is very much anticipated and will be described in Section 1.4.

1.3 SSF and Consolidated bioprocessing

Simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP) simplifies the conventional process by involving less equipment, which would reduce the cost of investment. As shown in Figure 1.1, a conventional process would require two separate unit operation to cater for both saccharification and fermentation. SSF simplifies the process by combining saccharification and fermentation into a single unit operation. While SSF requires the addition of enzymes to hydrolyze the cellulose, CBP advances a step ahead by including the enzyme production into the same tank, instead of external enzyme supplement.

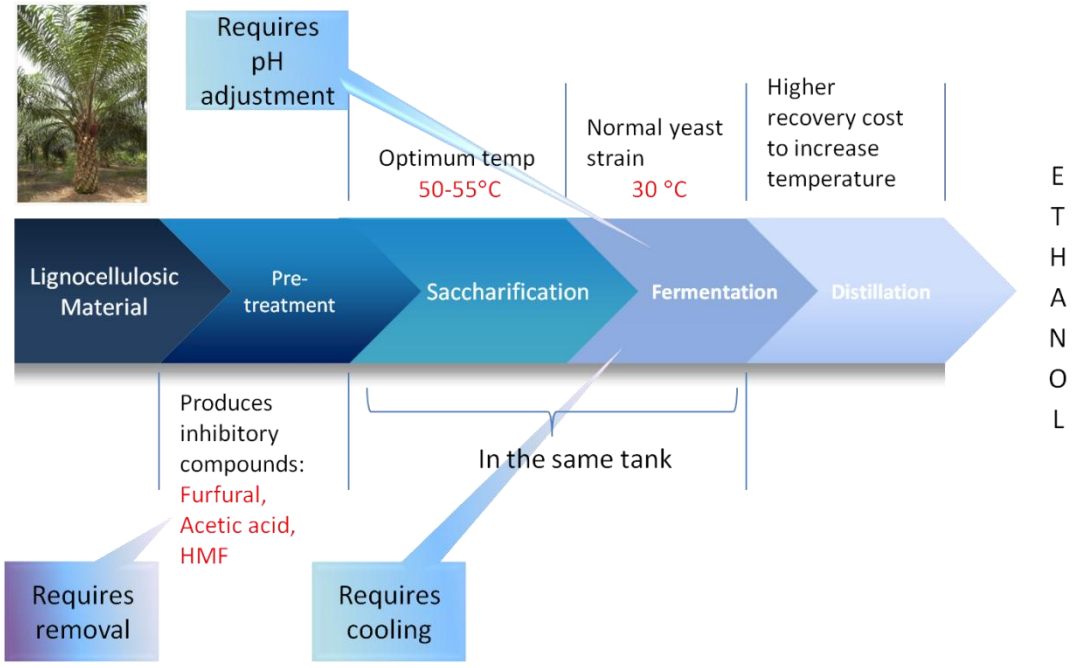

Figure 1.1 Scheme of SSF where saccharification and fermentation occurs in the same tank.

However, one of the major drawbacks in SSF and CBP is the optimum temperature required for the saccharification and fermentation stages. Saccharification is best done around 50°C, while most fermenting microbes have an optimum temperature between 28 and 37°C (Hasunuma and Kondo, 2012a). Therefore, thermotolerant microbial strains are essential to ensure the efficiency of SSF and CBP. Another drawback is the presence of inhibitors obtained from the lignocellulosic pretreatment stage. Depending

on the pretreatment process, hemicellulose sugars may be degraded to weak acids (acetic acid and formic acid) and furan derivatives (furfural and HMF) which can potentially inhibit the microorganisms during the ethanol fermentation step (Gírio et al., 2010). Here is where the inhibitor tolerant strains can help cut the cost of detoxification of the pretreated hydrolysates prior to fermentation.

1.4 Multi-stress tolerant xylose utilizing microorganism

As mentioned in Section 1.2, strains capable of utilizing not only glucose but xylose are very much needed in SSF and CBP. A few strains for xylose fermentation include *Pachysolen tannophilus, Candida shehatae, Scheffersomyces stipitis* and *Kluyveromyces marxianus*. However, they were observed to be less tolerant to pH, ethanol toxicity and hydrolysate inhibitors when compared to *Saccharomyces cerevisiae* (Kuhad et al., 2011). Naturally, *S. cerevisiae* can only consume glucose as the carbon source. Through genetic engineering, pentose sugar xylose utilization has been enabled in *S. cerevisiae* by expressing xylose reductase (XR) and xylitol dehydrogenase (XDH) from *S. stipitis* and xylulokinase (XK) from *S. cerevisiae* (Toivari et al., 2001). The simplified metabolic pathway for both glucose and xylose assimilation to ethanol is shown in Figure 1.2.

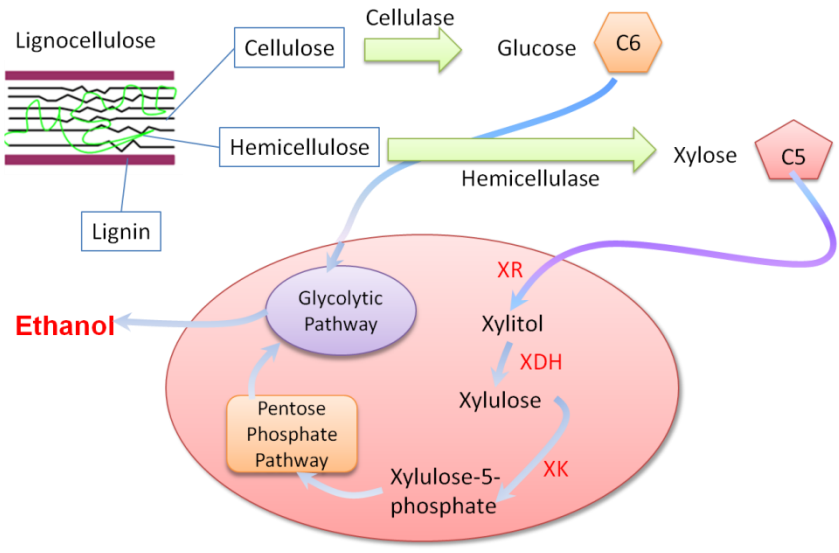

Figure 1.2 Simplified glucose and xylose assimilation to ethanol in genetically modified *S. cerevisiae.*

Apart from the challenge in developing a xylose utilizing strain, SSF and CBP also requires a robust microorganism with multiple stress resistance against high temperature, high osmotic stress and high ethanol concentration as well as fermentation inhibitors in lignocellulosic hydrolysates (Figure 1.3).

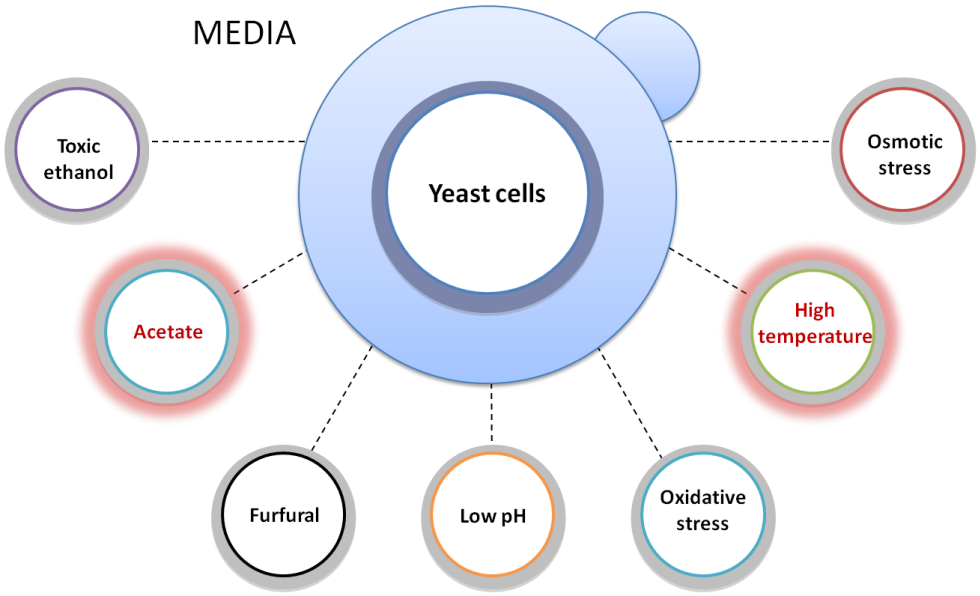

Figure 1.3 Yeast stresses during fermentation

Resistance to fermentation inhibitors such as weak acids and furfural would be significant since these compounds can interact with the yeast's cellular structures resulting in reduction of fermentation rate and reduction of ethanol productivity (Almeida et al., 2011). These stress tolerant phenotypes can be accomplished through mutagenesis, adaptation or genetic engineering. For the purpose of elucidating the underlying cause of the obtained phenotype, the study of gene expression would be necessary as demonstrated via inverse metabolic engineering (IME) strategy.

1.5 Inverse metabolic engineering by gene expression analysis

In the effort to develop a stress tolerant strain, IME is one of the techniques for evolving the desired phenotype. The strategy is done by first identifying the desired phenotype (eg. heat tolerant, inhibitor tolerant) followed by determination of the genetic

or environmental factors conferring that phenotype. The required phenotype was then achieved by directed genetic or environmental manipulation on the particular strain or microorganism.

The equipment used is a high throughput technology, DNA microarray which can provide a snapshot of the overall gene expression of the system under study (Karakach et al., 2010). The analysis might point to genes, whose changes in expression influences the phenotype positively or negatively (Bro and Nielsen, 2004). It would then lead to identification of one or a combination of target genes that may be causing the desired phenotype. The identified genes and factors can then be introduced into another strain or manipulated (deleted or overexpressed) to achieve a similar phenotype. Figure 1.4 shows the strategy of inverse metabolic engineering technique.

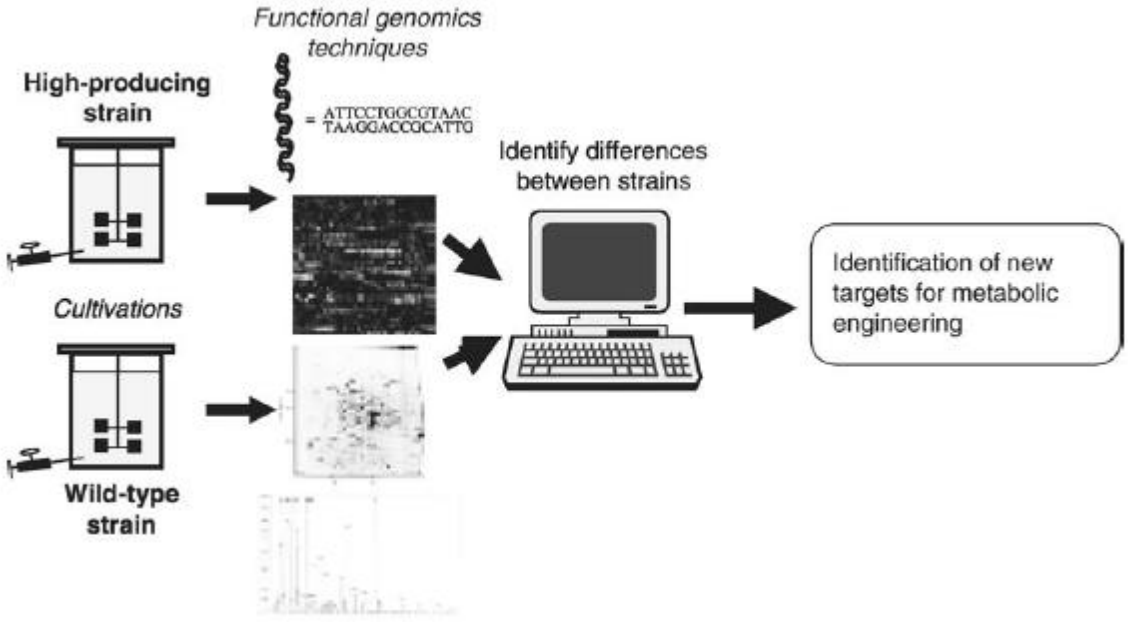

Figure 1.4 The strategy of inverse metabolic engineering technique (Bro and Nielsen, 2004).

1.6 Specific research objectives

Considering all the above information together, the main objective of this research is to develop a stress-tolerant xylose-utilizing microorganism that can fulfill the requirements to be used in SSF or consolidated bioprocessing for ethanol production. It should also be mentioned that the sole carbon source used throughout the research is xylose due to lack of information in the use of this sugar in ethanol production as compared to glucose at the gene level. It aims to focus on the high temperature tolerance as well as inhibitor (furfural and acetic acid) tolerance which are specifically described in the following chapters:

1.6.1 Objective of Chapter 2

To identify the genes regulated in common during high temperature fermentation (38°C) among the best xylose-utilizing *S. cerevisiae* strains by cross-profiling of the transcriptomics data.

1.6.2 Objective of Chapter 3

To elucidate heat responsive genes in a genetically-modified *S. cerevisiae* strain during high temperature ethanol production from xylose via time-based transcriptomics.

1.6.3 Objective of Chapter 4

To develop a furfural-tolerant yeast strain by co-expressing *TAL1* and *ADH1* genes for ethanol production from lignocellulosic hydrolysates.

1.6.4 Objective of Chapter 5

To enhance the ethanol production under acetic acid stress by metal ions supplementation.

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CHAPTER 2

Gene expression cross-profiling in genetically modified industrial S*accharomyces cerevisiae* strains during high-temperature ethanol production from xylose

2.1 Introduction

Ethanol derived from biomass sources is one of the options for renewable energy which is currently gaining prominence globally. To produce bioethanol at a reduced operating cost, the development of robust microorganisms for efficient fermentation must be directed towards critical performance targets since such microorganisms are the heart of the whole process. One of the critical characteristics a microorganism must have is the ability to survive high-temperature stress during fermentation. If the process is to undergo simultaneous saccharification and fermentation (SSF), it is often run at 37°C to compromise the optimal temperature for the yeast and that for cellulolytic enzymes, between 30 and 55°C (Olofsson et al., 2008). High-temperature fermentation offers advantages such as reduction of contamination risk, reduction of cooling cost due to unneeded chiller unit and suitability for use in tropical countries (Hasunuma and Kondo, 2012a).

For a process such as SSF to advance, the microorganism having a combination of both phenotypes; xylose utilizing and ability to grow at high temperature is considered advantageous. For xylose consuming yeast, the prominent microorganism is *Scheffersomyces stipitis* (Bajwa et al., 2011), but *S. stipitis* strains stifle their natural xylose-fermentation abilities at elevated temperature because of their low thermotolerance (Ryabova et al., 2003). As for the high-temperature growth microorganism, one of the ideal strain is *Kluyveromyces marxianus*, which can produce ethanol from glucose at 48°C (Wahlbom et al., 2003). However, *Saccharomyces cerevisiae* remains as the most suitable microorganism because it can tolerate inhibitors and is more robust for industrial application (Hasunuma and Kondo, 2012b). Also, tools for genetic recombination simplify the engineering of *S. cerevisiae* strains.

Significant advances in improving strain performance for bioethanol production have been developed in the past few decades since wild-type *S. cerevisiae* is not capable of utilizing xylose as a carbon source. This has been practiced by overexpressing the genes from *S. stipitis* encoding xylose reductase (XR) and xylitol dehydrogenase (XDH).

Ethanol production was enhanced by overexpressing the endogenous XK gene encoding xylulokinase (Chu and Lee, 2007; Jeffries and Jin, 2000). On the other hand, genetic engineering strategies that have addressed yeast tolerance to elevated temperature are rare (Benjaphokee et al., 2012; Prasetyo et al., 2011). To our knowledge, there have been no reports on the improvement of thermotolerance of xylose-fermenting *S. cerevisiae* strains. Thus, focused basic research is expected to identify genes responsible for high-temperature fermentation.

To elucidate the mechanism underlying the orchestration of gene expression under certain stress conditions, we used a high-throughput measurement technology, performing transcriptomics extensively with a DNA microarray system. A number of researchers have investigated the primary gene networks that control the following; adaptation to high sugar stress (Erasmus et al., 2003), the mechanism of alcohol tolerance (Hirasawa et al., 2007; Hong et al., 2010; Li et al., 2010a), the adaptation to cultivation in molasses medium in fed-batch culture (Shima et al., 2005), genes response during glucose and xylose co-fermentation (Sedlak et al., 2003), the heat shock response (Boy-Marcotte et al., 1999) and adaptation to high pressure environment (Fernandes et al., 2004). Most of the previous gene profiling work on thermotolerant *S. cerevisiae* has focused on glucose as the carbon source (Boy-Marcotte et al., 1999; Shahsavarani et al., 2011; Ye et al., 2009). However, the transcriptome of recombinant *S. cerevisiae* cultivated on xylose is different (Van Vleet and Jeffries, 2009). Thus, the understanding of how the cells produce ethanol from xylose under high-temperature stress is still lacking.

In this work, we isolated industrial *S. cerevisiae* strains that are able to tolerate temperatures up to 38°C. In addition to the competency of withstanding high temperature, the ability to ferment xylose was conferred to the thermotolerant strains through genetic engineering. We highlighted the performance of 8 genetically modified industrial strains and 1 genetically modified lab strain fermenting xylose at 38°C and characterized the expression of genes differentially regulated at high temperature that might lead to the thermotolerance phenotype. This was done by comparing the levels of gene expression between the best and poor strains by cross-profiling. An intense search for specific genes resulted in a list of genes commonly expressed by the top 3 ethanol producers. Therefore, the differentially expressed genes were identified, and we then hypothesized whether they are involved in tolerance to high temperature. This paper is

the first to elucidate the gene profile of *S. cerevisiae* genetically engineered from industrial strains to produce ethanol from xylose as the sole carbon source at 38°C.

2.2 Materials and methods

2.2.1 Strains and medium

Industrial strains (sun048, sun049, sun051, sun224, sun473, sun491, sun562 and sun588) of *S. cerevisiae* were obtained from Suntory Limited (Tokyo, Japan). Each strain has different backgrounds since they were cultivated under different industrial conditions. These strains were transformed with a YCp-type plasmid, pJHNX1X2XKN, harboring xylose assimilating genes *XYL1* and *XYL2* from *S. stipitis* and *XKS1* from *S. cerevisiae* to yield sun048T, sun049T, sun051T, sun224T, sun473T, sun491T, sun562T and sun588T, respectively. Laboratory strain W303-1A (Petrezselyova et al., 2010) was also transformed with pJHNX1X2XKN to yield W303-1AT. W303-1AT was used as the first control while sun473T and sun491T were used as negative controls (lowest ethanol producers at 38°C). The strains were transferred from -80°C and 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 YPD medium to which was added 5 μL clonNAT (Werner Bioagent, Jena, Germany) at 30°C and 150 rpm. After 24 h, the cells were grown aerobically in 500 mL YPD medium with 500 μL clonNAT for 48 h at 30°C and 150 rpm. The cells were then centrifuged at 3000 x 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 and were ready to be inoculated for fermentation.

2.2.2 Plasmid construction

Plasmid pJHNX1X2XKN for xylose assimilation was constructed as follows. Plasmid pIUMC was created by replacing the *Bss*HII fragment of pIUX1X2XK (Katahira et al., 2006) with the multilinkers 5'-CGCGCGGCGCGCCGGTACCGTCGACGGCGCGCCGTTTAAACTCGAGACTAGTTTAAACGC-3' and 5'-CGCGCGGCCGCGTTTAAACTAGTCTCGAGTTTAAACGGCGCGCCGTCGACG

GTACCGGCGCGCCG-3'. A gene cassette for clonNAT resistance was obtained by PCR using pAG36 (Goldstein and McCusker, 1999) as a template and 5'-GGCGCGCCCGATCTGTTTAGCTTGCCTCG-3' and 5'-GGCGCGCCCCGTTTTCGACACTGGATGGCG-3' as primers. The cassette was cloned into TOPOBluntII (Invitrogen, Tokyo, Japan), and its sequence was confirmed. In order to construct pJHNAT, the *URA3* fragment of pJHXSB (Hatanaka et al., 2009) was replaced with the clonNAT resistance cassette using the *Asc*I site, and then the *Not*I fragment was replaced with a linker oligonucleotide, 5'-GGCCGCACTCGAGTGTTTAAACACTCGAGTGC-3'. *XYL2* was removed from pIUX1X2XK by digestion with *Sac*I and *Bam*HI, and was inserted into the same sites of pYCGPY (Kodama et al., 2001), yielding pYCGPYX2. Plasmid pJHXSBP was created by inserting a *Pme*I site between the *Sac*I and *Bam*HI sites of pJHXSB. *XKS1* was removed from pIUX1X2XK by digestion with *Sma*I and *Sal*I, then blunt-ended and ligated into the *Pme*I sites of pJHXSBP to create pJHXK. The expression cassettes of *XYL1*, *XYL2*, and *XKS1* were removed from pIUX1X2XK, pYCGPYX2, and pJHXK by digestion with *Kpn*I and *Xho*I, *Sal*I and *Spe*I, and *Not*I, respectively, and inserted into the *Kpn*I and *Sal*I sites, the *Xho*I and *Spe*I sites, and the *Not*I site of pIUMC, respectively, yielding pIUX1X2XKN. The 3 tandem expression cassettes were removed from pIUX1X2XKN by digestion with *Pvu*II and inserted into the *Pme*I site of pJHNAT, resulting in pJHNX1X2XKN.

2.2.3 Fermentation conditions

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. Batch fermentation of 50 ml working volume was carried out in a 100-mL bottle with a CO_2 outlet. Temperature was controlled by placing the bottles in a water bath equipped with a magnetic stirrer. Fermentation temperature was set to 38°C with stirring at 500 rpm. Samples were taken at 0, 4, 8, 12, 24 and 48 h of fermentation. The xylose, ethanol, xylitol and glycerol concentrations were determined by high-performance liquid chromatography (Shimadzu, Kyoto, Japan) with a refractive index detector. 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. Cell growth was monitored by determination of optical density at 600 nm using a spectrophotometer UV-mini (Shimadzu).

2.2.4 Enzyme assays

The activities of XR and XDH were assayed by following the methods described by Katahira et al. (2006). XK activity assay was referred to previous work (Matsushika et al., 2008). One unit of enzyme activity was defined as the amount of enzyme that reduced or oxidized 1 μmol of NAD^+ or NAD(P)H per minute.

2.2.5 DNA microarray analysis

Samples obtained at 12 h of fermentation were used for RNA preparation to observe the gene expression during exponential phase. 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, Delaware, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. cDNA was generated by reverse transcription, labeled with Cy3 and hybridized to *S. cerevisiae* 4X44 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 microarray sample was analyzed in duplicate. Gene expression was calculated using normalized data and only 2-fold and above induction or reduction were reported.

2.2.6 Quantification of transcript levels using real-time PCR assay

The RNA samples used for microarray experiments were 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 2.1. The fold change of transcript levels was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). *ACT1* was used as the internal standard.

Table 2.1 Primers used in this study.

Genes	Product size	Primer sequences
FLO10	172 bp	Forward: TGTAAGCGAGCATACCAACG Reverse: TGGTGTGGAGACAACAGAGC
PHO12	179 bp	Forward: TGGTTGGTAGACACGGTGAA Reverse: GGCAAGTGTGGTTTCCATTT
REE1	177 bp	Forward: GTCCGTGTTCAGGGAAGCTA Reverse: AGCATTTCCGCCATAAACAG
ENA1	155 bp	Forward: GTCTGGTGAAGGTCGGTGAT Reverse: ACCCACGGAGGTTTCTTCTT
ARO3	189 bp	Forward: ACGCTTGGAAGACTGGAGAA Reverse: GGGTCATGTAGGGAACATGG
HXT9	180 bp	Forward: ACTACGGCACCACCATCTTC Reverse: CAAACACAGCAAAGCAGCAT
ACT1 (Internal standard)	72 bp	Forward: TGGATTCCGGTGATGGTGTT Reverse: TCAAAATGGCGTGAGGTAGAGA

2.3 Results and Discussion

2.3.1 Ethanol fermentation performance of the xylose utilizing yeast strains

The rationale for exploiting industrial strains for bioethanol production is that they had adapted to wide variations of environmental stress conditions during the industrial processes such as high sugar and ethanol concentration, nitrogen starvation, varying pH, osmotic pressure, anaerobiosis and temperature fluctuation (Drapcho et al., 2008). Even though high fermentation temperatures often inhibit *S. cerevisiae* growth

and its ability to produce ethanol, our results suggests that all 9 strains were naturally capable of growing and producing ethanol at 38°C at various efficiencies. Also worth mentioning is that the strains used in this study had not undergone any laboratory adaptation to high-temperature stress beforehand. Their genetic background may be advantageous for study of genes expressed in high-temperature bioethanol production utilizing xylose. The strain W303-1AT, obtained by the transformation of laboratory strain W303-1A with pJHNX1X2XKN, was used as one of the control strain.

The incorporation and functional expression of the XR and XDH genes was confirmed by enzyme assays. During fermentation, the sole carbon source, xylose, is reduced to xylitol by XR, and further oxidized to xylulose by XDH. Then, xylulose is phosphorylated to xylulose-5-phosphate by XK. Figure 2.1 shows that all 9 strains had measurable activities for XR, XDH and XK. Every strain showed the same relationship, where XDH activity was higher than XR activity, even though the levels varied. Strains sun048Tand sun049T showed higher XK than XR activity, while sun051T, sun224T, sun473T, sun562T and sun588T showed the opposite. Interestingly, the enzyme activities for the negative controls; W303-1AT and for sun491T were very high compared to the rest of the genetically modified industrial strains.

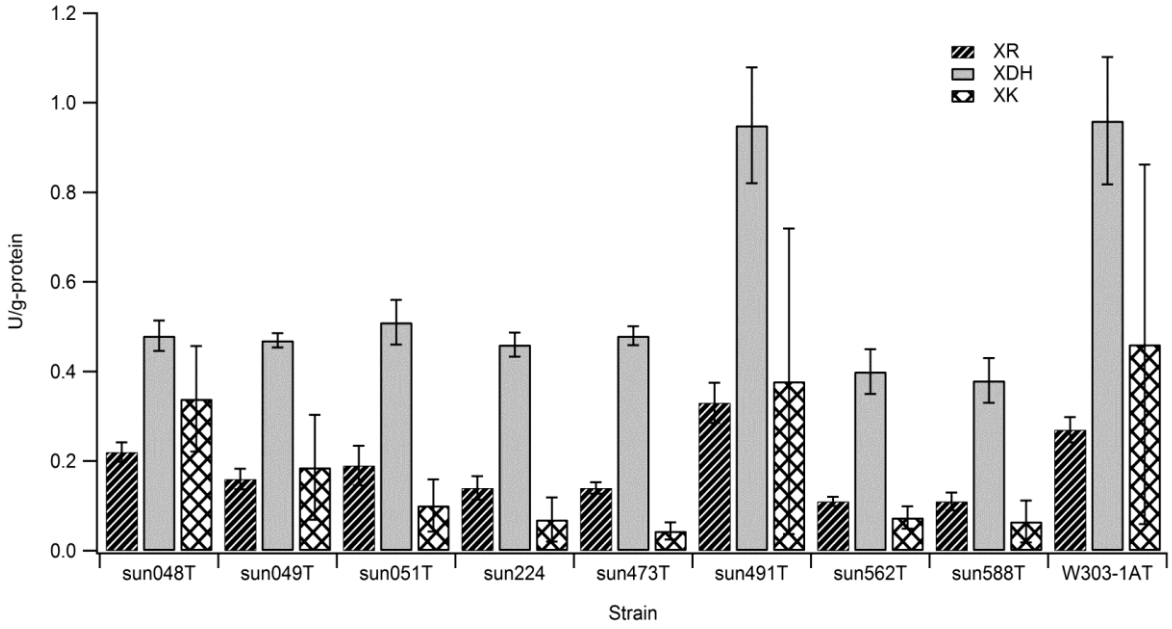

Figure 2.1 Specific activities (U/g protein) of xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK) for 9 yeast strains.

After inferring that the enzymes were functional, we characterized the strains in terms of fermentation performance. Fermentation by the 9 strains was successful at 38°C using xylose as the sole carbon source in the fermentation medium (Figure 2.2). The initial amount of xylose was 50 g/L, and all 9 strains were able to consume xylose efficiently except for strains sun473T and sun491T, for which xylose was still in excess after 48 h of fermentation. Strain sun224T demonstrated the fastest xylose consumption; xylose was almost fully consumed within 24 h. Among the genetically modified industrial strains, sun049T produced the highest amount of ethanol, 15.2 g/L, followed by sun048T and sun588T, both at 14.1 g/L ethanol. sun051T was also able to produce ethanol at an adequate amount of 13.9 g/L. On the other hand, the weakest ethanol producers were the negative control strains; sun491T at 9.9 g/L and sun473T at 10 g/L, followed by sun562T at 11.7 g/L. The performance of the lab strain W303-1AT was comparable to that of sun473T, generating only 10.4 g/L ethanol. Apart from ethanol, the major by-products produced during the fermentation were glycerol and xylitol. Glycerol excretion was considered minimal in this fermentation; however, for the high xylitol production, it remains another problem to be solved in future work.

To observe the effect of temperature stress on the 9 strains, a comparison between the strains' ethanol production, ethanol yield, ethanol production rate and xylose consumption at 30°C and 38°C are summarized in Table 2.2. It can be seen that by increasing the temperature to 38°C, all 9 strains resulted in reduced ethanol production. However, the best strain that can maintain its performance and seems unaffected by the temperature stress was sun588T (only 1.5% reduction in ethanol production), As for the negative control strains, sun491T suffered the most by showing a 40% reduction in ethanol production, followed by sun473T (23% reduction). In terms of ethanol yield, the lab strain experienced the highest drop (11%) due to high temperature, while sun588T showed higher ethanol yield at 38°C (0.31 g/g) compared to the yield during 30°C (0.29 g/g). Evaluating the strains' performance at 38°C, even though the highest ethanol yield, 0.34 g ethanol/g xylose consumed, was achieved by sun491T, its xylose consumption was the poorest of all strains at only 28.9 g/L. The second highest ethanol yield was obtained from strains sun051T at 0.32 g ethanol/g xylose consumed, corresponding to 63% of the theoretical yield, and from sun048T, sun049T and sun588T, each at 0.31 g ethanol/g xylose consumed (61% of theoretical yield). The remaining strains yielded between 0.24 and 0.26 g ethanol/g xylose

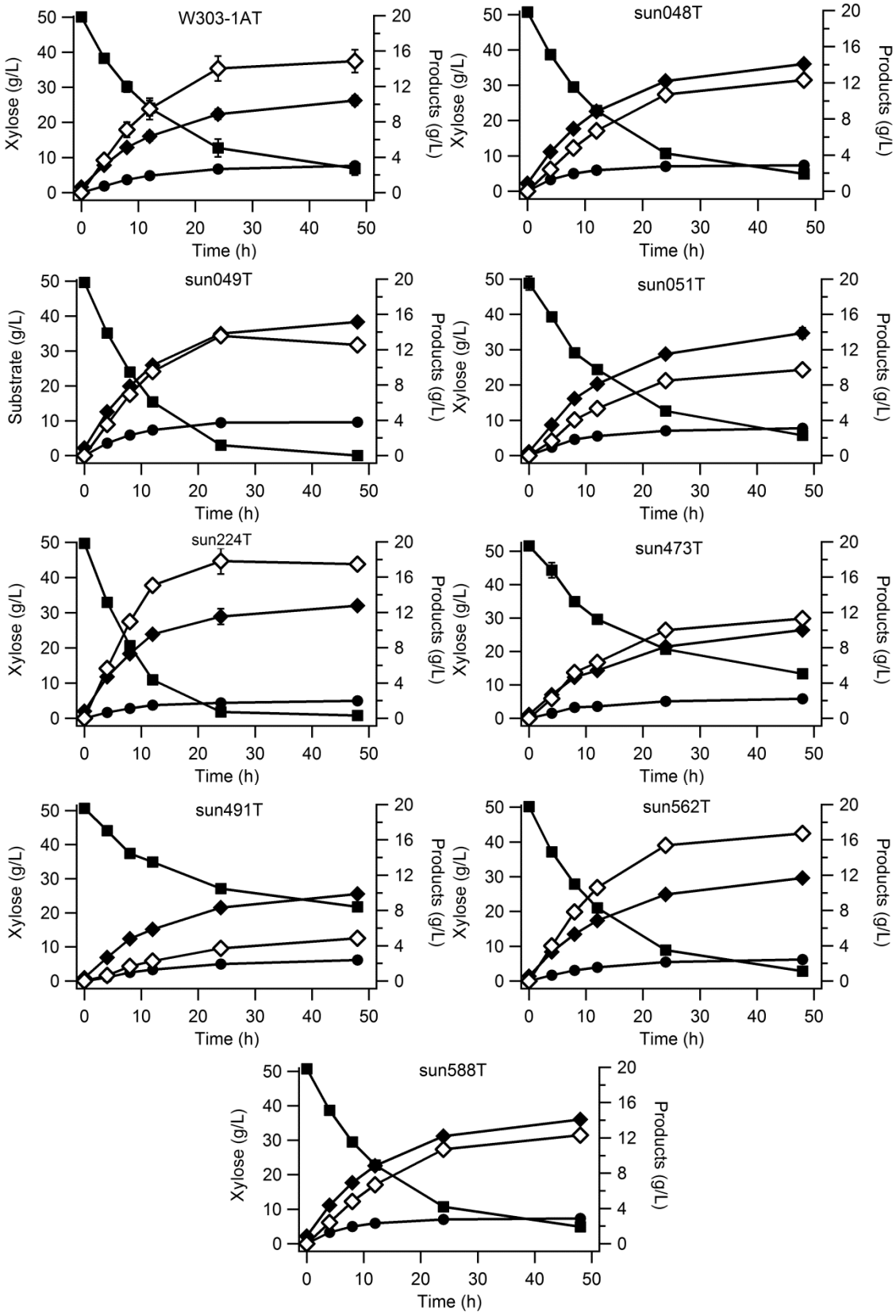

Figure 2.2 Time course of fermentation for 9 strains cultivated at 38°C for 48 h. (◆) ethanol, (■) xylose, (◇) xylitol and (●) glycerol. Values are averages of 3 independent experiments ± SD.

consumed (between 47% and 51% of theoretical). sun049T showed the highest ethanol production rate at 0.87 g ethanol/L/h, followed by sun224T at 0.82 g ethanol/L/h. The lab strain, W303-1AT showed a similar ethanol production rate (0.56 g/L/h) to sun491T.

Based on the results of fermentation over time at 38°C, the best ethanol producer is suggested to be sun049T due to its high ethanol production, highest production rate and 100% carbon source utilization. Its performance was also evaluated by comparing its ethanol yield at high temperature with the results from the 30°C fermentation, which were almost the same, indicating that an increase in temperature does not give major effect on its production. To investigate what underlies the ability of the genetically modified industrial strains to produce ethanol at high temperature, a negative control was chosen as a benchmark. Though we used 3 negative control strains; W303-1AT, sun473T and sun491T, to make a fair comparison in gene expression analysis, sun473T was chosen as the most suitable negative control since it is also a genetically modified industrial strain and its enzyme activity level were most similar to the high ethanol producers, sun049T, sun048T and sun588T. This was based on Figure 2.1, where the XDH activity level of sun491T was found to be too high (0.95 U/g protein) in comparison with the three best high temperature ethanol producer strains (between 0.38 – 0.48 U/g protein), whereas sun473T's enzyme activity was comparable with those of the high ethanol producing strains.

Table 2.2 Ethanol production, ethanol yield, ethanol production rate and xylose consumption at 30°C and 38°C for 9 yeast strains.

Strain	Ethanol Production[1], g/L		Ethanol Yield[2], g/g		Ethanol Production rate[3], g/L/h		Xylose Consumption[4], g/L	
	30°C	38°C	30°C	38°C	30°C	38°C	30°C	38°C
W303-1AT[5]	12.2 ± 1.70	10.4 ± 0.54	0.27 ± 0.02	0.24 ± 0.01	0.48 ± 0.13	0.56 ± 0.11	45.3 ± 3.20	43.2 ± 0.20
sun048T	16.6 ± 0.66	14.1 ± 0.29	0.34 ± 0.01	0.31 ± 0.02	0.71 ± 0.10	0.66 ± 0.10	49.2 ± 0.85	45.8 ± 0.32
sun049T	16.1 ± 0.23	15.2 ± 0.38	0.32 ± 0.01	0.31 ± 0.03	0.80 ± 0.10	0.87 ± 0.10	49.8 ± 0.24	49.6 ± 0.10
sun051T	16.5 ± 0.42	13.9 ± 0.61	0.33 ± 0.01	0.32 ± 0.01	0.66 ± 0.10	0.76 ± 0.10	49.8 ± 0.47	43.2 ± 0.31
sun224T	14.0 ± 0.33	12.8 ± 0.02	0.28 ± 0.00	0.26 ± 0.00	0.76 ± 0.10	0.82 ± 0.01	50.6 ± 1.08	49.0 ± 0.90
sun473T	13.0 ± 0.91	10.0 ± 0.15	0.29 ± 0.00	0.26 ± 0.00	0.51 ± 0.08	0.53 ± 0.02	44.4 ± 2.46	38.3 ± 1.10
sun491T	16.4 ± 0.24	9.9 ± 0.15	0.35 ± 0.00	0.34 ± 0.00	0.58 ± 0.01	0.55 ± 0.01	46.8 ± 1.07	28.9 ± 0.90
sun562T	12.8 ± 0.31	11.7 ± 0.05	0.26 ± 0.00	0.25 ± 0.00	0.53 ± 0.01	0.59 ± 0.01	49.5 ± 1.14	47.3 ± 0.50
sun588T	14.3 ± 0.11	14.1 ± 0.29	0.29 ± 0.00	0.31 ± 0.00	0.64 ± 0.01	0.76 ± 0.01	49.5 ± 0.07	45.8 ± 0.10

Values are averages of three independent experiments ± SD

[1]Maximum ethanol concentration after 48h.

[2]Calculated based on g-ethanol produced/g-xylose consumed

[3]Calculated based on change in ethanol production with respect to time from 0 h to 12 h of fermentation

[4]Calculated based on xylose concentration difference between 0 h to 42 h of fermentation

[5]Laboratory strain

2.3.2 Elucidation of novel genes enhancing ethanol production at elevated temperature by cross-profiling

The expression profiles between strains enable us to screen genes which might be important for growth at high temperature. Recent findings in search of thermotolerance-conferring genes in *S. cerevisiae* suggested that *CDC19*, encoding pyruvate kinase (Benjaphokee et al., 2012), and *RSP5*, encoding ubiquitin ligase (Shahsavarani et al., 2011), were essential for achieving a high temperature growth strain. A transcriptomic analysis by Ye et al. (2009) utilizing glucose as the carbon source at 42°C suggested that extensive oxidative stress was involved in heat shock. Genes involved in trehalose and glycogen metabolisms were up-regulated. However, work on establishing gene expression profiles for genetically modified industrial thermotolerant strains has not yet been reported. A similar study was done by Runquist et al. (2009), but at 30°C.

The 3 best ethanol producers at 38°C; sun049T, sun048T and sun588T, were chosen for transcriptomics analysis and compared to the negative control, sun473T. Genes with expression up-regulated or down-regulated after 12 h of fermentation at 38°C were identified using DNA microarrays. The most productive phase for these strains was the exponential phase and during this phase, ethanol was produced under the exposure to heat stress. Therefore, it was the best time to investigate the gene regulation after the cells adapted to heat stress. According to Dinh et al. (2009), a certain fraction of genes regained their original expression after the cells adapted to the stress. Thus, in strategizing to find which genes were responsible for ethanol production under heat stress conditions, we screened genes which were induced or reduced after 12 h of heat exposure. Choosing target genes by relying on the fold change in expression only within the same strain might be tedious because of the long list of genes expressed, and sometimes ends up being a blind guess. Therefore, we implemented cross-profiling, in which the target genes were selected based on fold change integrated across strains having the desired phenotype. Figure 2.3 shows a Venn diagram highlighting the altered expression of genes by the top 3 thermo-tolerant ethanol producers.

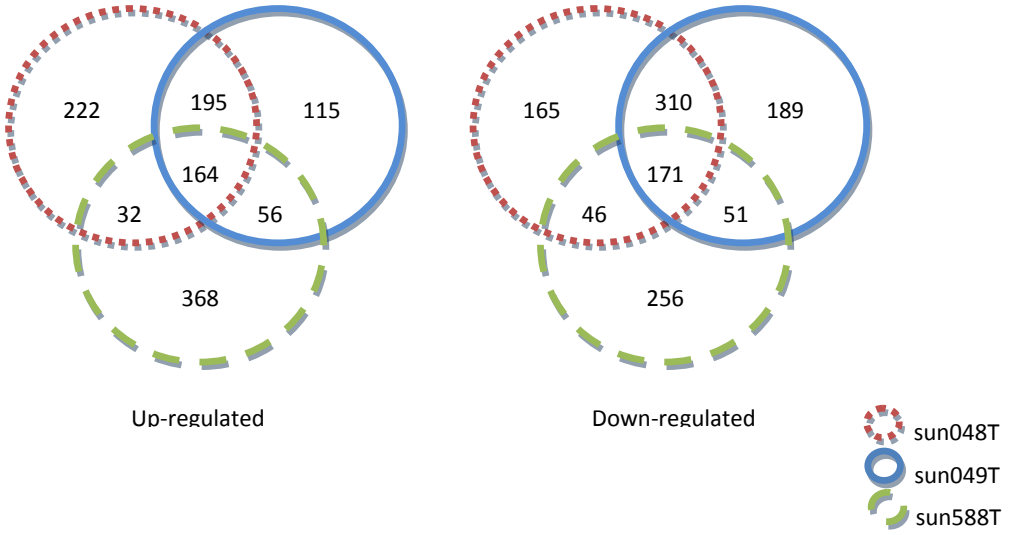

Figure 2.3 Venn diagram showing the number of genes differentially regulated among the best ethanol producers, sun048T, sun049T and sun588T.

We used DNA microarrays with the capacity of identifying 6,316 genes at a time. Only expression changes greater than 2-fold were taken into consideration. When comparing the genes among sun049T, sun048T and sun588T, we found that 164 genes were commonly induced which suggests that target genes involved in heat tolerance might be among this group, while 171 genes were suppressed, correspondingly.

2.3.2.1 Genes regulated in common among the best ethanol-producing strains

The 164 genes up-regulated in common need to be validated to determine whether they confer thermotolerance in xylose-utilizing yeast. To begin our quest for candidate genes, we focused on the most highly regulated genes. The 20 most highly induced and the 20 most highly suppressed genes in sun049T, sun048T and sun588T when grown at high temperature with xylose as the carbon source are documented in Tables 2.3, 2.4 and 2.5, respectively. Summarizing the 3 tables, the genes induced in common were mainly from the carbohydrate metabolism and cell wall maintenance functional groups. This is an important finding because a critical component during cell survival is the management of the energy source. In this case, the energy source was xylose. It is not only important for the cells to survive growing under heat stress, but also to continuously produce ethanol as in the absence of heat stress.

The uptake of xylose in *S. cerevisiae* has been proposed to be mediated by its hexose-transport system, encoded by the *HXT* genes (Hamacher et al., 2002). In our study, all three high ethanol-producing strains showed very high up-regulation of *HXT9*. Up-regulation of genes encoding a high-affinity sugar transporter and other sugar-repressible genes seems to be a response to sugar limitation (Li et al., 2010b), but does not seem to apply in our conditions since the up-regulation occurred at 12 h of fermentation, when xylose is still available. The functions of genes *HXT8* to *HXT17* are not well understood and remain a challenge for further investigation (Ozcan and Johnston, 1999). Thus, this induction of *HXT9* might be related to the cell response to high-temperature fermentation. Apart from *HXT9*, *HXT1* and *HXT4* were up-regulated in strains sun048T and sun049T, likely for the purpose of ATP production. *HXT4* was recently identified as up-regulated in a *TPS1*-overexpressing trehalase deletion strain under heat stress conditions (Mahmud et al., 2012). This raises the possibility that the higher ethanol producers would have higher xylose uptake compared to the control strains, which was reflected by their xylose consumption (Table 2.2).

Table 2.3 Ranking of 20 most highly up-regulated and down-regulated genes in sun049T compared to sun473T after 12 h of fermentation at 38°C. Fold change is the ratio of expression in sun049T relative to sun473T.

Up-regulated (530 genes)				Down-regulated (721 genes)			
Genes	**Category**	**Description**	**Fold**	**Genes**	**Category**	**Description**	**Fold**
PCS60	RNA processing	Peroxisomal AMP-binding protein	268	*ARO3*	Carbohydrate metabolism	3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase	2544
HXT9	Carbohydrate metabolism	hexose transporter	267	*AIF1*	Cell stress	Mitochondrial cell death effector	1318
REE1	Carbohydrate metabolism	regulation of enolase (ENO1)	167	*ENA5*	ATPase	Protein with similarity to P-type ATPase	930
AIM24	Genes of unknown function	Protein of unknown function	122	*RDS1*	Transcription factor	Zinc cluster transcription factor	681
RAS1	Signal transduction	GTPase involved in G-protein signaling in the adenylate cyclase activating pathway	108	*MAL13*	Carbohydrate metabolism	MAL-activator protein	553
ENB1	Small molecule transporter	Endosomal ferric enterobactin transporter	100	*PHO12*	Phosphate metabolism	One of three repressible acid phosphatases,	544
FLO10	Cell wall maintainance	Lectin-like protein with similarity to Flo1p	88	*RNQ1*	Unknown	Prion, an infectious protein	486

YKR104W	Cell stress	Putative transporter of the multidrug resistance-associated protein (MRP) subfamily	82	*COS10*	Genes of unknown function	Protein of unknown function	472
YLR460C	Other metabolism	Member of the quinone oxidoreductase family	82	*YCR102C*	Genes of unknown function	Putative protein of unknown	379
YIL014C-A	Genes of unknown function	Putative protein of unknown function [YIL014C-A]	82	*AVL9*	Unknown	Conserved protein involved in exocytic transport from the Golgi	278
YIL082W-A	Unknown	Retrotransposon TYA Gag and TYB Pol genes	42	*ENA1*	ATPase	ATPase sodium pump	269
GIP1	Meiosis	Meiosis-specific regulatory subunit of the Glc7p protein phosphatase	36	*FLO5*	Cell wall maintainance	Lectin-like cell wall protein (flocculin) involved in flocculation	251
AAD16	Carbohydrate metabolism	Putative aryl-alcohol dehydrogenase	31	*YAR064W*	Genes of unknown function	Putative protein of unknown function [YAR064W]	209
COS1	Genes of unknown function	Protein of unknown function	31	*MPH2*	Carbohydrate metabolism	Alpha-glucoside permease	158
YOL159C	Genes of unknown function	Protein of unknown function	30	*MPH3*	Carbohydrate metabolism	Alpha-glucoside permease, transports maltose	134

ASI1	Unknown	Putative integral membrane E3 ubiquitin ligase	25	*COS8*	Unknown	Nuclear membrane protein	128
VID24	Carbohydrate metabolism	Peripheral membrane protein located at Vid (vacuole import and degradation) vesicles	25	*GIT1*	Small molecular transporter	Plasma membrane permease, mediates uptake of glycerophosphoinositol	126
PUF3	Unknown	Protein of the mitochondrial outer surface	25	*YFR057W*	Genes of unknown function	Putative protein of unknown function [YFR057W]	104
PHO89	Phosphate metabolism	Na+/Pi cotransporter, active in early growth phase	22	*SFG1*	Transcription factor	Putative transcription factor	85
SUI1	Translation factor	Component of a complex involved in recognition of the initiator codon	22	*YHR214C-D*	Genes of unknown function	Putative protein of unknown	78

Table 2.4 Ranking of 20 most highly up-regulated and down-regulated genes in sun048T compared to sun473T after 12 h of fermentation at 38°C. Fold change is the ratio of expression in sun048T relative to sun473T.

Up-regulated (613 genes)				Down-regulated (692 genes)			
Genes	**Category**	**Description**	**Fold**	**Genes**	**Category**	**Description**	**Fold**
ATP6	ATP synthesis	Mitochondrially encoded subunit a of the F0 sector of mitochondrial F1F0 ATP synthase	1496	*ARO3*	Carbohydrate metabolism	3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase	965
PCS60	Unknown	Peroxisomal AMP-binding protein	355	*ENA5*	ATPase	P-type ATPase sodium pumps	592
HXT9	Carbohydrate metabolism	Putative hexose transporter that is nearly identical to Hxt11p	253	*RDS1*	Transcription factor	Zinc cluster transcription factor	470
YIL014C-A	Genes of unknown function	Putative protein of unknown function	250	*RNQ1*	Unknown	Prion, an infectious protein	394
RAS1	Signal transduction	GTPase involved in G-protein signaling in the adenylate cyclase	173	*PHO12*	Phosphate metabolism	One of three repressible acid phosphatases	326
REE1	Carbohydrate metabolism	Cytoplasmic protein involved in the regulation of enolase (ENO1)	169	*COS12*	Genes of unknown function	Protein of unknown function,	284
FLO1	Cell wall maintainance	involved in flocculation, cell wall protein	116	*YRF1-4*	Unknown	Helicase encoded by the Y' element of subtelomeric regions	282

AIM24	Genes of unknown function	Protein of unknown function	108	*YGL262W*	Genes of unknown function	Putative protein of unknown function	281
YLR460C	Other metabolism	Member of the quinone oxidoreductase	102	*YHR219W*	Genes of unknown function	Putative protein of unknown function	279
YKR104W	Cell stress	Putative transporter of the multidrug resistance-associated protein	90	*YRF1-5*	Unknown	Helicase encoded by the Y' element of subtelomeric regions	270
YLR162W	Genes of unknown function	Putative protein of unknown function	78	*YLL066C*	Genes of unknown function	Putative protein of unknown function	265
MUC1	Cell wall maintainance	GPI-anchored cell surface glycoprotein (flocculin)	59	*YRF1-1*	Unknown	Helicase encoded by the Y' element of subtelomeric regions	260
FLO10	Cell wall maintainance	Lectin-like protein with similarity to Flo1p	50	*YRF1-8*	Unknown	One of several telomeric Y' element-encoded DNA helicases	247
AAD16	Carbohydrate metabolism	Putative aryl-alcohol dehydrogenase	49	*YCR102C*	Genes of unknown function	Putative protein of unknown function	220
COS6	Genes of unknown function	Protein of unknown function	49	*AVL9*	Transporter	Conserved protein involved in exocytic transport	188
SEO1	Small molecule transporter	Putative permease, member of the allantoate transporter	41	*YHL042W*	Genes of unknown function	Putative protein of unknown function	133

GIP1	Meiosis	Meiosis-specific regulatory subunit of the Glc7p protein phosphatase	33	*ENA1*	ATPase	P-type ATPase sodium pump	104
VID24	Carbohydrate metabolism	Peripheral membrane protein	30	*MPH2*	Carbohydrate metabolism	Alpha-glucoside permease	100
VAC14	Small molecule transporter	Maintenance of vacuole size and acidity	30	*MPH3*	Carbohydrate metabolism	Alpha-glucoside permease	97
AAD6	Other metabolism	Involved in the oxidative stress response	29	*GIT1*	Membrane transporter	Plasma membrane permease	83

Table 2.5 Ranking of 20 most highly up-regulated and down-regulated genes in sun588T compared to sun473T after 12 h of fermentation at 38°C. Fold change is the ratio of expression in sun588T relative to sun473T.

Up-regulated (613 genes)				Down-regulated (692 genes)			
Genes	**Category**	**Description**	**Fold**	**Genes**	**Category**	**Description**	**Fold**
REE1	Carbohydrate metabolism	Cytoplasmic protein involved in the regulation of enolase (ENO1)	1500	*YFL054C*	Small molecule transporter	Putative channel-like protein	4302
COS6	Unknown function	Protein of unknown function	1170	*AIF1*	Cell stress	Mitochondrial cell death effector that translocates	1498
HXT9	Carbohydrate metabolism	Putative hexose transporter	777	*ALR2*	Small molecule transporter	Probable Mg(2+)transporter	1139
YKR104W	Cell stress	Putative transporter of the multidrug resistance-associated protein (MRP)	427	*YFL052W*	Transcription factor	Putative zinc cluster protein	1078
FLO10	Cell wall maintainance	Lectin-like protein with similarity to Flo1p	360	*ARO3*	Carbohydrate metabolism	3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase	948
ENB1	Small molecule transporter	Endosomal ferric enterobactin transporter	355	*UIP3*	Genes of unknown function	Protein of unknown function	853
TFA1	Pol II transcription	TFIIE large subunit, involved in recruitment of RNA polymerase II to the promoter	306	*ENA5*	ATPase	Protein with similarity to P-type ATPase sodium pumps	634
PCS60	Unknown	Peroxisomal AMP-binding protein	202	*YNR071C*	Genes of unknown function	Putative protein of unknown function	542
SEO1	Small molecule transport	Putative permease, member of the allantoate transporter	171	*RNQ1*	Unknown	Prion, an infectious protein	360

YIL014C-A	Genes of unknown function	Putative protein of unknown function	157	*DAK2*	Carbohydrate metabolism	Dihydroxyacetone kinase,	299
AIM24	Genes of unknown function	Protein of unknown function	157	*DOG2*	Carbohydrate metabolism	2-deoxyglucose-6-phosphate phosphatase	181
YLR460C	Other metabolism	Member of the quinone oxidoreductase family	138	*ENA1*	ATPase	P-type ATPase sodium pump	178
YOL159C-A	Genes of unknown function	Putative protein of unknown function	90	*YFR057W*	Genes of unknown function	Putative protein of unknown function	98
COS1	Genes of unknown function	Protein of unknown function	90	*AGP3*	Amino acid metabolism	Low-affinity amino acid permease	91
YOL159C	Genes of unknown function	Soluble protein of unknown function	70	*PUT4*	Amino acid metabolism	Proline permease, required for high-affinity transport of proline	55
AI2	Reverse transcriptase	Splicing of the COX1 pre-mRNA	65	*AVL9*	Molecule transportation	Conserved protein involved in exocytic transport from the Golgi	51
VAC14	Other metabolism	maintenance of vacuole size and acidity	59	*EHD3*	Other metabolism	3-hydroxyisobutyryl-CoA hydrolase	50
FLO1	Cell wall maintainance	Lectin-like protein involved in flocculation	53	*YFL051C*	Genes of unknown function	Putative protein of unknown function	46
RAS1	Signal transduction	GTPase involved in G-protein signaling in the adenylate cyclase	35	*RPC82*	Pol III transcription	RNA polymerase III	43
DAN1	Cell wall maintenance	Cell wall mannoprotein	32	*PHO12*	Phosphate metabolism	Acid phosphatases	40

Cell wall maintenance has also been reported to be a major reaction of cells towards heat. Heat and ethanol stress adversely affect membrane integrity. The composition of a cell's membrane lipids shifts with temperature, ethanol concentration and stage of cultivation (Eliasson et al., 2001). This was supported by our findings where two *FLO* genes, *FLO1* and *FLO10,* were up-regulated in the higher ethanol producers. These genes are thought to be involved in cell flocculation (Soares, 2011). According to Tofalo et al. (2010), flocculation is one of the most important characteristics of *S. cerevisiae* strains used for industrial purposes, and ours were originated from industrial strains. Among the *FLO* genes, *FLO1* can induce the strongest flocculating ability when introduced into a non-flocculating yeast strain (Zhao et al., 2011). Our microarray data show high induction of *FLO1* in sun048T and sun588T (Tables 2.4 and 2.5), while *FLO10* was induced in all 3 top ethanol producers. In sun049T, even though *FLO10* was up-regulated, *FLO5* was down-regulated. The reason for this contradictory regulation of *FLO* genes remains unclear. Based on our observations after 48 h of fermentation, the yeast cells flocculated faster at the bottom of the bottle after fermentation at 38°C than at 30°C. High temperature might cause individual cells to change from free-floating to being attached to substrates or other cells (Bester et al., 2012). Flocculation ability at higher temperature is useful for bioethanol production because cells can be separated from the medium without centrifugation or filtration, both of which are expensive procedures (Watanabe et al., 2009).

Another candidate gene that showed high regulation among the top 3 strains was *REE1*. *REE1,* a cytoplasmic protein involved in the regulation of enolase encoded by *ENO1,* was highly up-regulated in the top strains. Enolase is a phosphopyruvate hydratase that catalyzes the conversion of 2-phosphoglycerate to phospho*enol*pyruvate during glycolysis and the reverse reaction during gluconeogenesis. *ENO1* was up-regulated during co-fermentation of glucose and xylose (Sedlak et al., 2003) and in response to galactose induction (Choi et al., 2008). Thus, *REE1* has been thought to be involved in carbon metabolism. Enolase is also the enzyme most severely affected by acidic stress (Pampulha and Loureiro-Dias, 1990). Therefore, *REE1* might be induced for boosting sugar phosphate utilization when it is inhibited by high temperature. Since *ENO1* was not detected in our microarray analysis, we ran a validation check using qRT-PCR (data not shown). *ENO1* was up-regulated in sun048T and sun049T, but

down-regulated in sun588T. The reason for this different expression between the strains was unknown. According to Edwards et al. (1999), *ENO1* may have alternative functions on cell wall and it may be related to the effect of heat. Thus we speculated that the strains' cell walls reacted differently by changing the expression of *ENO1*. In the case of *ARO3* gene, encoding 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, it was highly down-regulated in the best ethanol-producing strains, which might suggest that its function in xylose assimilation was halted. ARO3p catalyzes the first step in aromatic amino acid synthesis from erythrose-4-phosphate produced in the pentose phosphate pathway. From previous work by Li et al. (2010a), *ARO3* was found to be significantly repressed in the presence of ethanol. Higher levels of expression of *ARO* genes involved in tryptophan synthesis were earlier identified as increasing ethanol tolerance in diploid and haploid yeasts (Li et al., 2010a). However, no literature has reported any impact of high temperature on ARO3p function. *PHO12* and *ENA1* were also down-regulated greatly in all 3 strains. *PHO12* is involved in phosphate metabolism, while *ENA1* is thought to encode the primary plasma membrane Na^+-ATPase exporter in *S. cerevisiae*. Another study by Alepuz et al. (1997) found that elevated *ENA1* expression was a result of glucose repression. In our case, the down-regulation of *ENA1* is more likely to be related to the ATP generation rather than glucose repression. It is known that heat stress causes a high demand for ATP generation by cells (Li et al., 2009). Therefore, the evidence shows that the negative control strain required more ATP compared to the high ethanol producing strains.

To validate the microarray data, a few genes thought to have an effect on tolerating high temperature during ethanol production were chosen for qRT-PCR analysis. Figure 2.4 shows the qRT-PCR output for 6 selected genes; both analyses were in agreement even though there were differences in quantification due to different detection sensitivity. All gene expression values were normalized to those of the negative control strain, sun473T.

2.3.2.2 High-temperature condition up-regulates other stress genes

Regarding other regulated genes, the *HSP12* gene, which is induced by heat shock, was up-regulated as expected, but was not included in the top 20 highly induced genes. The induction of *HSP12* was suggested to increase membrane stability (Welker

et al., 2010). A group of genes known to be induced under other stresses was up-regulated in sun048T and sun049T; for example, *TRP1*, encoding phosphoribosylanthranilate isomerase, which catalyzes the third step in tryptophan biosynthesis, was highly induced in the best ethanol producers, sun048T (+14.9), sun049T (+10.9) and sun588T (+3.5). Even though *TRP1* has been speculated to confer ethanol tolerance (Hirasawa et al., 2007), its induction shows that it might also confer heat tolerance.

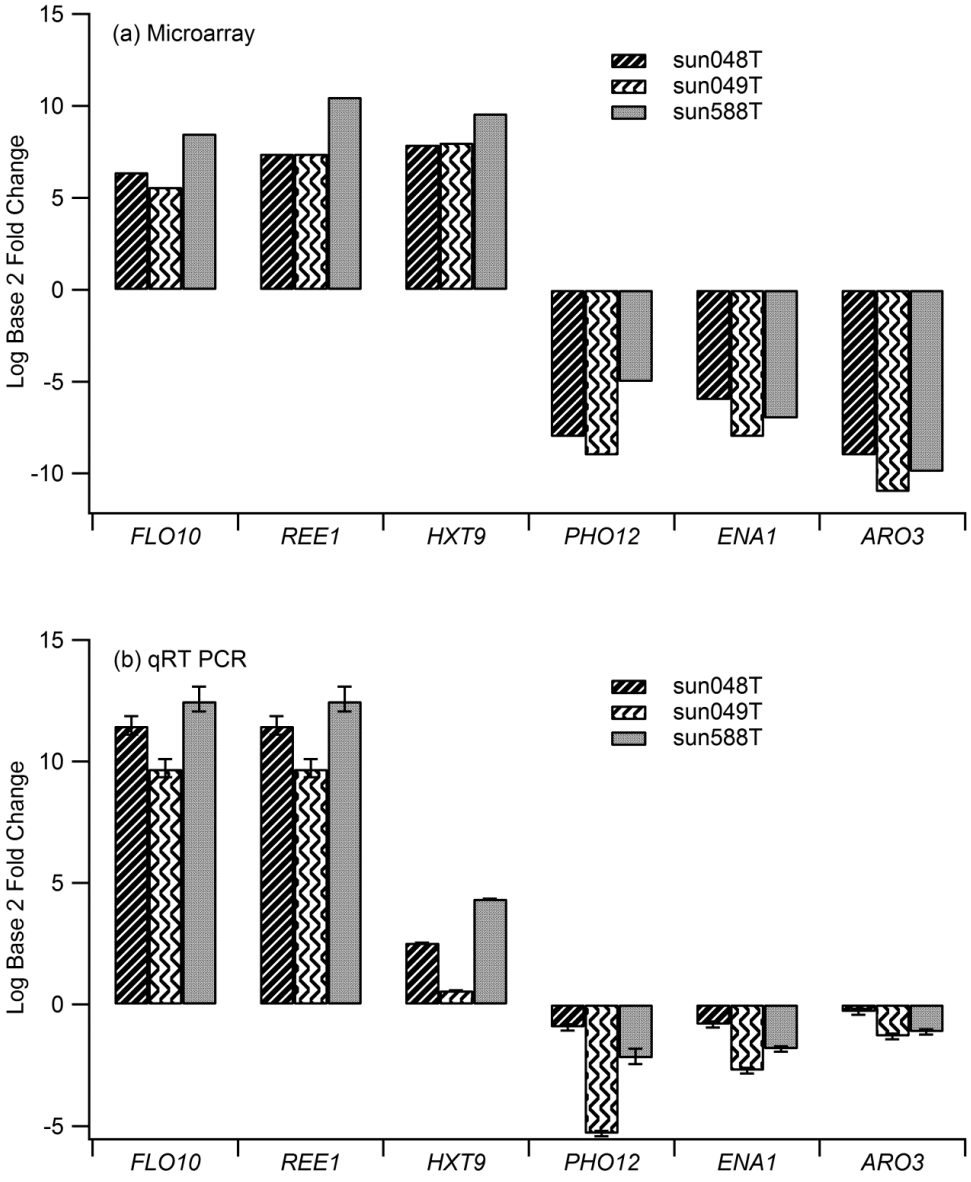

Figure 2.4 Validation of microarray data using qRT-PCR for sun048T, sun049T and sun588T normalized to sun473T. *ACT1* was used as an internal standard.

GPD1, responsible for glycerol synthesis, was also turned on during the fermentation course by sun048T (+2.9) and sun049T (+3.3). Glycerol is needed by the yeast in order to protect and repair cellular structures from damage caused by high temperature. Interestingly, *FDH1*, suggested to protect cells against formic acid (Hasunuma et al., 2011b), was also up-regulated in 3 strains, sun049T (+5.6), sun 048T (+2.2) and sun588T (+6.7). *INO1*, which has relevance for ethanol stress, was also up-regulated by all 3 strains, but especially strong in sun049T (+3.2) and sun 588T (+2.2). INO1p has been implied to contribute to the production of phospholipid barriers against ethanol (Hong et al., 2010), which we suspect would have the same mechanism as that for protecting cells from heat stress.

Considering the above information together, even though the 3 best strains have different genetic backgrounds, they all reacted to high temperature by enhancing the xylose metabolism pathway and proteins involved in maintaining cell integrity. At 38°C, other genes believed to confer tolerance to other stress conditions were also up-regulated, indicating the occurrence of cross-protection. Therefore, we agree with the suggestions made by Zheng et al. (2011) and Hohmann and Mager (2003) that cross-protection occurs under stress conditions and allows the cells to survive. Our findings also support the notion that temperature has a clear impact on the product formation pathway (Olofsson et al., 2008).

2.4 Conclusion

The performance of 8 genetically modified industrial strains with enhanced ability to consume xylose was tested in fermentation at elevated temperature. Using a cross-profiling strategy, the 3 best ethanol-producing strains at 38°C were found to have the same reaction towards high temperature by strengthening their cell walls and manipulating expression of metabolic pathway genes. FLO genes were highly induced under high temperature conditions with the yeast strains, probably as a defense mechanism to protect the cells from high temperature. Most genes which showed substantial up-regulation during the heat stress condition were the same genes that enhance ethanol tolerance. So far, it seems that the genetically modified industrial xylose-utilizing yeasts have a similar mechanism for tolerating heat stress as other microorganisms. However, many details such as the interactions between the genes and

the roles of certain regulated genes remain to be discovered. This report serves to provide additional details on global gene profiling in the interest of producing a robust microorganism for high-temperature ethanol production. The direction of future hemicellulose conversion research would be much simplified by the development of such a robust biocatalyst.

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CHAPTER 3

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

3. 1 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 (Van Dyk and Pletschke, 2012). Lignocellulosic material is abundantly available, does not directly compete with food sources and is considered a cheap raw material (Sarkar et al., 2012). 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 (Kim et al., 2011). However, the drawback associated with CBP is the different optimum temperature between saccharification and fermentation (Hasunuma and Kondo, 2012a). To address this problem, thermophilic ethanologens might be effective.

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 (Kumar et al., 2009). 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 (Mielenz, 2001). *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 (Edgardo et al., 2008; Prasetyo et al., 2011; Shahsavarani et al., 2011).

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 had led to increased interest among researchers to reengineer the metabolic pathway, enabling *S. cerevisiae* to uptake xylose as a carbon source (Hahn-Hagerdal et al., 2007; Matsushika et al., 2009a). 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. (2008) 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 (Lu et al., 2009; Sakaki et al., 2003; Ye et al., 2009). In our previous work (Ismail et al., 2013), we have 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°C 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 two-fold 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.

3.2 Materials and Methods

3.2.1 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 (Ismail et al., 2013). 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 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 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 3000 × *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.

3.2.2 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°C 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 3000 *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 (Hasunuma et al., 2011a). 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.

3.2.3 DNA microarray analysis

1 ml of cell samples obtained at 3, 6, 9 and 12 h (log phase) of fermentation were quenched by 1.4 ml cold-methanol at -40°C. Cells harvested by centrifugation at 6000 × *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, Delaware, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. cDNA was generated by reverse transcription, labeled with Cy3 and hybridized to *S. cerevisiae* 4x44 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 microarray sample was analyzed in duplicate. Gene expression was calculated using normalized data and only 2-fold and above induction or reduction were reported. Differentially-expressed genes more than 2-fold were sorted into 4 clusters using the K-means method (Euclidean as distance metric) according to their time-based expression patterns. For every cluster, the GO was obtained using the GeneSpring GX software.

3.2.4 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 3.1. Thermocycling conditions were as follows: 95°C for 10 min (1 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 $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). *ACT1* was used as the internal standard.

Table 3.1 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
ACT1 (Internal standard)	72	Forward: TGGATTCCGGTGATGGTGTT Reverse: TCAAAATGGCGTGAGGTAGAGA

3.3 Results

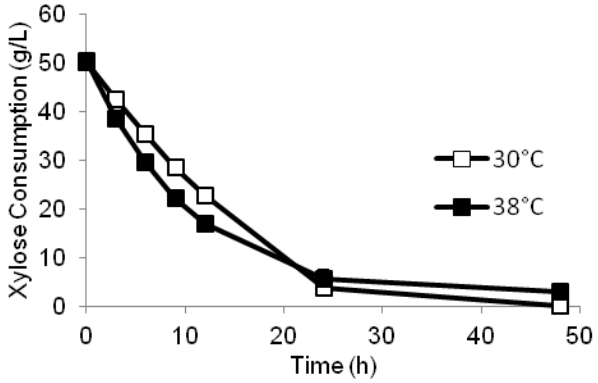
3.3.1 Xylose fermentation at 30℃ and 38℃

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 then be phosphorylated by XK to xylulose-5-phosphate, which would then be metabolized through the non-

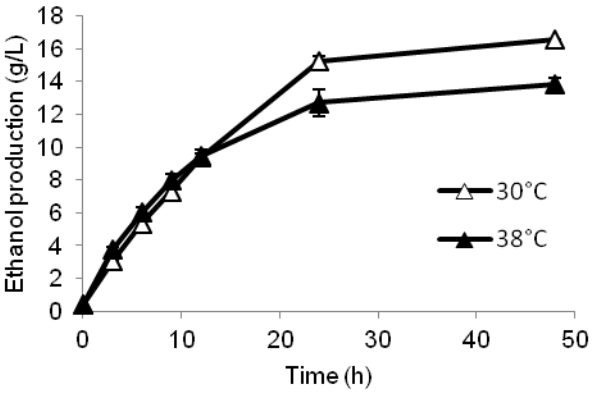
oxidative pentose phosphate pathway (PPP) and glycolysis pathway (Hasunuma et al., 2011a). The ability of the sun049T strain to produce ethanol from xylose was tested at 30°C and 38°C. Figure 3.1 presents the result of ethanolic fermentation utilizing xylose as the only carbon source. It shows that sun049T 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 (Ismail et al., 2013). 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.

3.3.2 Comparative transcriptomics analysis for xylose fermentation at 30℃ and 38℃ based on different gene clusters

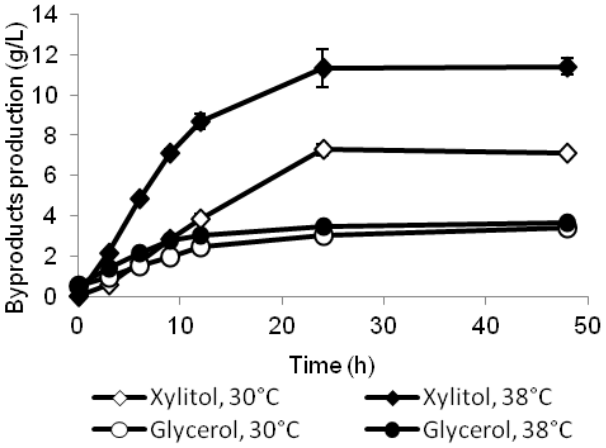
The simplest approach to identifying differentially-regulated genes is to consider the fold change between control and experimental, i.e., 30°C and 38°C, conditions, respectively. Figure 3.2 shows a 4-way Venn diagram representing the number of genes induced and reduced during the four sampling time (3 h, 6 h, 9 h and 12 h) under heat stress. It shows that more genes remained down-regulated (1373 genes) than up-regulated (868 genes) during the 12 h high temperature fermentation, including genes with unknown functions. After the fold change analysis was done, differentially-expressed genes were grouped into four clusters according to their expression patterns. As shown in Figure 3.3, four clusters were obtained using the K-means algorithm.



(a)

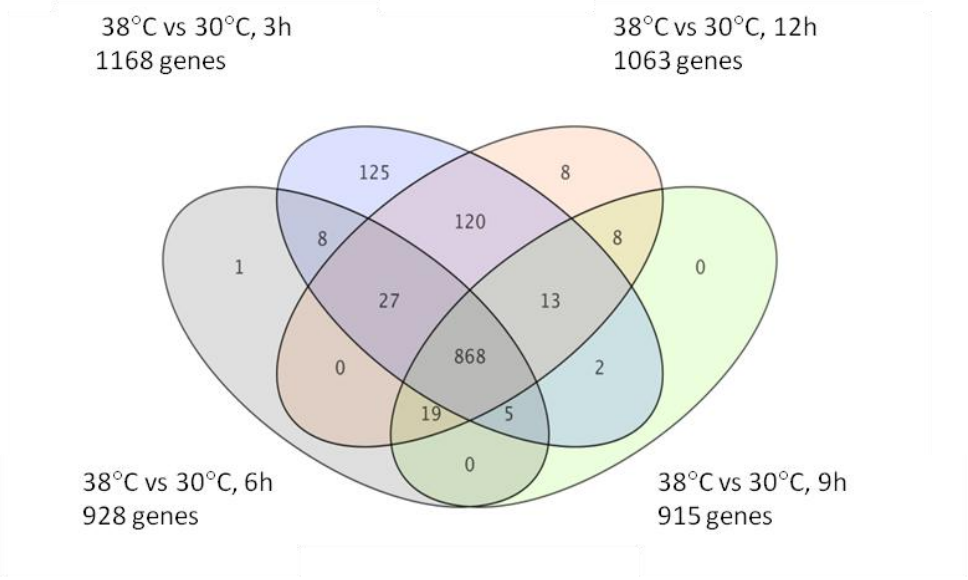


(b)

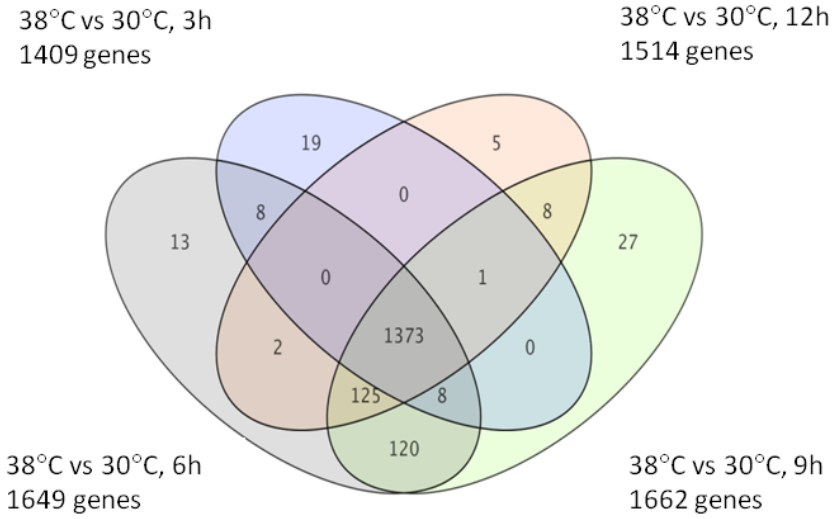


(c)

Figure 3.1 Time course of xylose fermentation by sun049T at 30°C 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.



(a)



(b)

Figure 3.2 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.

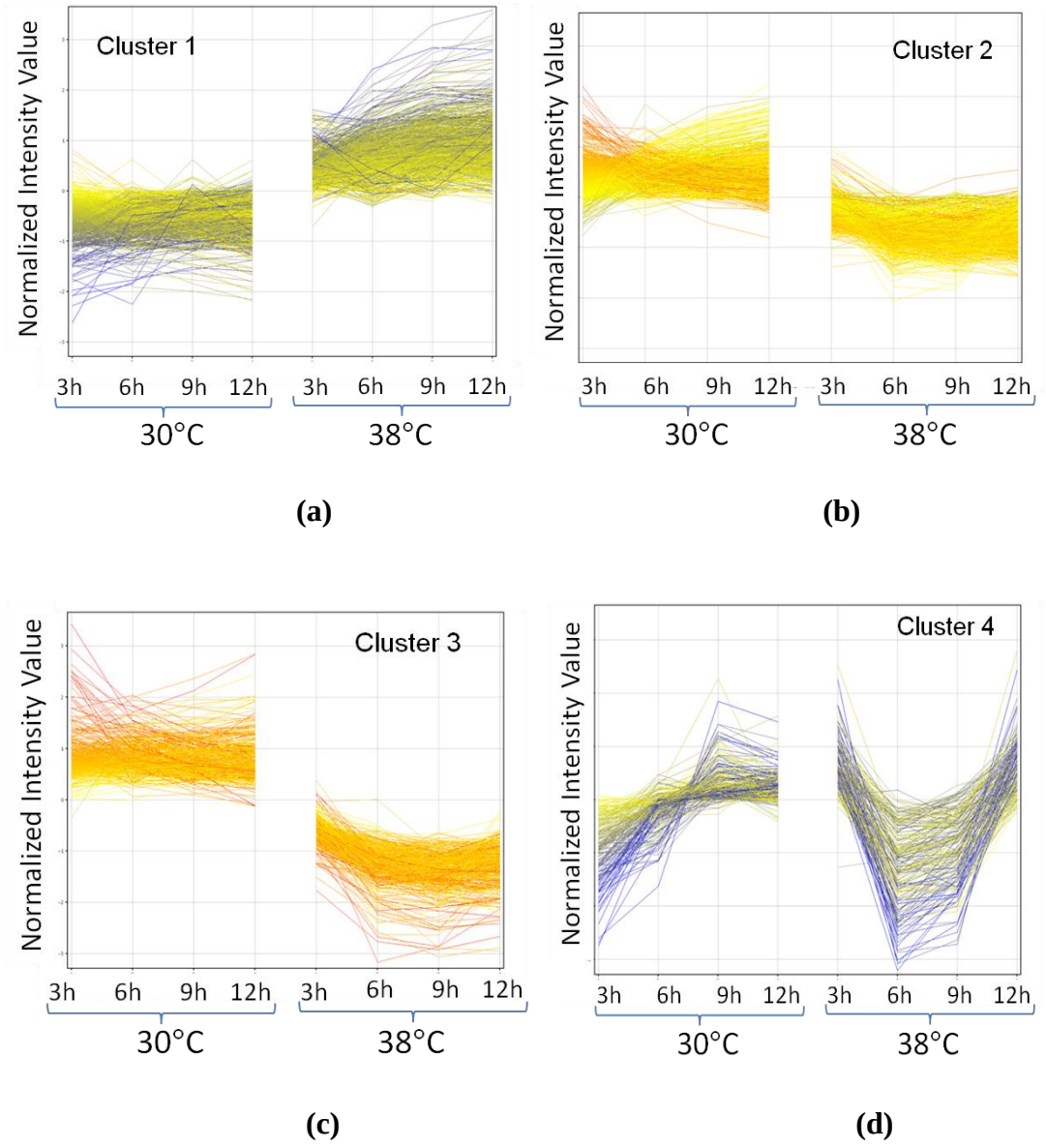


Figure 3.3 Clustered genes based on time-based expression pattern at 30°C and 38°C after normalization. (a) Cluster 1 consists of consistently up-regulated genes at 38°C relative to 30°C. (b) Cluster 2 consists of down-regulated genes with constant expression levels at 38°C relative to 30°C. (c) Cluster 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°C and 38°C, and the vertical axis indicates the normalized gene expression ratio in log terms (base 2). Genes with unknown functions are included.

Cluster 1 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 3.2. 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 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 3.3) are related to ribosomal protein- and cytoplasmic-associated genes. The strongest GO enrichment under 'cellular component' is 'cytosolic ribosome' ($p = 2.87E-16$).

Cluster 3 groups the genes that were significantly down-regulated throughout the high temperature fermentation period as depicted in Table 3.4. The most down-regulated gene annotation under 'biological process' is 'small molecule biosynthesis process' ($p = 3.15E-08$). 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 pattern, did not have any overexpressed gene ontology due to many of the genes having unknown functions.

Table 3.2 Summary of GO annotated in Cluster 1 (Up-regulated genes throughout high temperature fermentation with xylose as the carbon source)

Biological process	**P value**	**Major genes**
Proteolysis involed 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	**P value**	**Major genes**
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	**P value**	**Major genes**
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*

Table 3.3 Summary of GO annotated in Cluster 2 (Down-regulated genes with constant expression level throughout high temperature fermentation with xylose as the carbon source)

Biological process	**P value**	**Major genes**
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	**P value**	**Major genes**
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	**P value**	**Major genes**
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*

Table 3.4 Summary of GO annotated in Cluster 3 (Down-regulated genes throughout high temperature fermentation with xylose as the carbon source)

Biological process	P value	Major genes
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 Thiamin and derivative metabolic process Thiamin biosynthetic process	5.09E-04 8.10E-04 0.002	*RPI1, SNO2/3, THI4/12/20*
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	**P value**	**Major genes**
Fungal type cell wall Cell wall External encapsulating structure	5.16E-07 5.16E-07 5.16E-07	*AGA2, CIS3, CRH1, CWP2, DAN4, DSE2/4, EGT2, EXG1, FIT1, FLO10, MKC7, MUC1, PST1, RRT12, SCW4/11, SRL1, SUN4, TIR2, TOS6*
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	P value	Major genes
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	

3.3.3 Transcriptional changes of genes with increase in fold change over time

Table 3.5 indicates some of the consistently induced genes during the 12-h fermentation course.

Table 3.5 Selected genes constantly found to be induced during the 12h heat stress

Genes	3h	6h	9h	12h
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 increase with time

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 (Hong et al., 2010), 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 3-fold, 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.

3.3.4 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. The genes involved in PPP and glycolysis are depicted in Table 3.6.

Table 3.6 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**	**3h**	**6h**	**9h**	**12h**
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

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*.

3.3.5 Microarray data validation

We chose 4 genes (*HSP104*, *INO1*, *ADH2* and *GRE2*) that showed a clear response to heat stress for validation using qRT PCR. Figure 3.4 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 microarray data and the qRT PCR data was observed with differences in magnitude attributable to differences in detection sensitivity between each type of equipment.

3.4 Discussion

With the aim of developing a robust biocatalyst for cost-effective ethanol production, we successfully constructed *S. cerevisiae* strains that can grow on and consume xylose as the sole carbon source (Ismail et al., 2013). 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. (2012), which produced only 7 g/L ethanol when xylose was the only carbon source. However, the MA-R4 strain used by Matsushika et al. (2009b) 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 (Hahn-Hagerdal et al., 2007). 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 (Chu and Lee, 2007), *HXT5* and *HXT7*

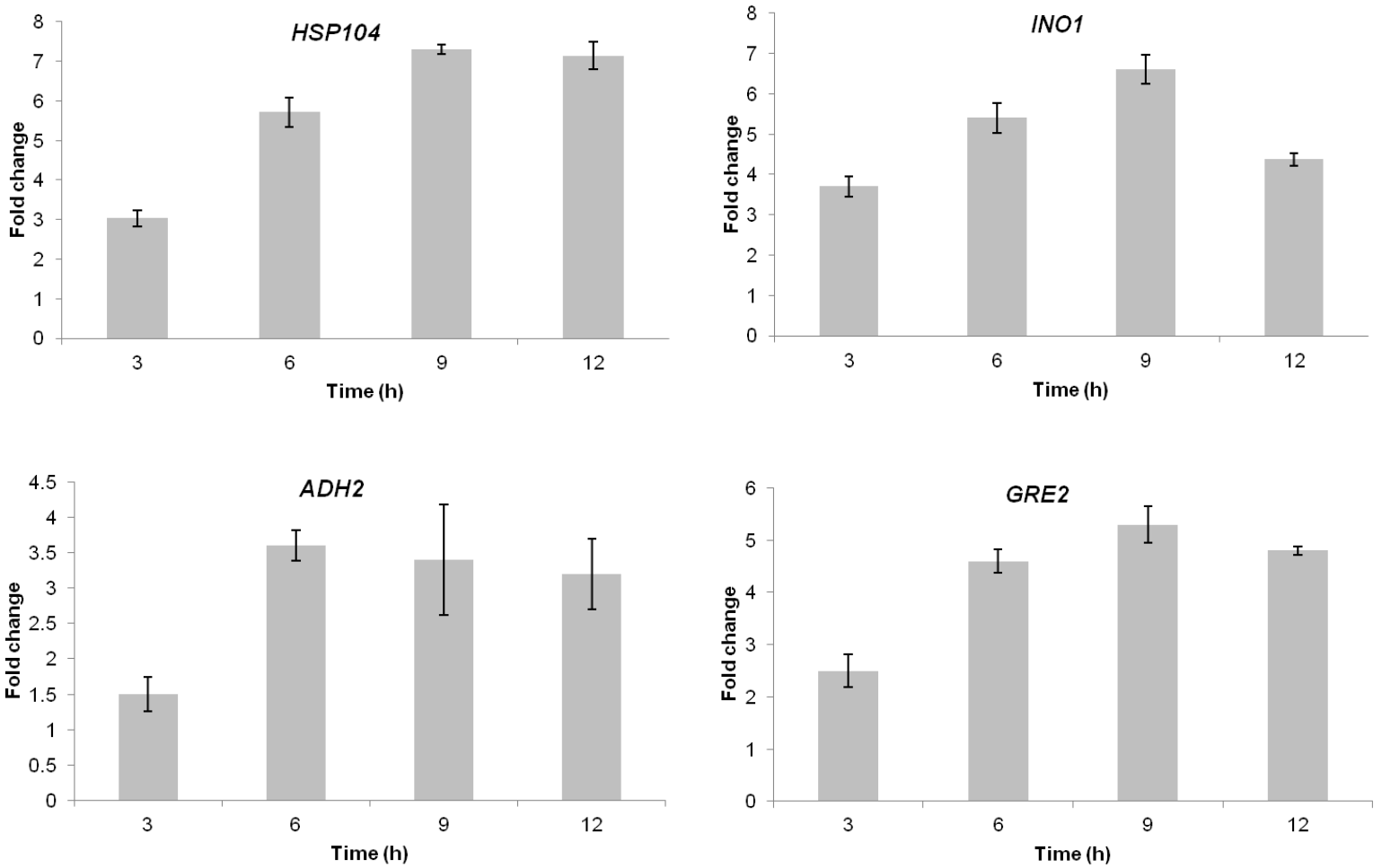


Figure 3.4 Validation of time-based microarray data by qRT PCR for *HSP104*, *INO1*, *ADH2* and *GRE2*. Fold change is calculated based on gene expression at 38°C divided by the values at 30°C. *ACT1* was used as an internal standard. Values are the average of three independent experiments ± SD.

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. PPP could be one of the limiting steps during high temperature ethanol production.

An interesting observation was the strong correlation between temperature and the amount of xylitol produced. According to the transcriptomics data, severely down-regulated PPP genes might indicate the accumulation of certain intermediates. For instance, when the *TKL1* gene is down-regulated there would be an accumulation of sedoheptulose 7-phosphate. This intermediate accumulation would later affect the subsequent genes 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 were to have a significant control on the flux (Toivari et al., 2001). Another possibility, based on a previous xylose fermentation study utilizing *S. stipitis,* is that xylitol production could be temperature dependent to some degree (du Preez et al., 1986). 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. In our work, *GRE2* (3-methylbutanal reductase and NADPH-dependent methylglyoxal reductase) and *GRE3* were both induced during heat stress. This conforms with the study of Traff et al. (2001) 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 for the decrease in ethanol production at high temperature might be the high induction of *ADH2*. High *ADH2* activity can presumably lead to rapid ethanol re-assimilation and may explain the low observed accumulated ethanol (Toivari et al., 2001). 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 (Boy-Marcotte et al., 1999). In our results, the co-induction of heat shock proteins with ubiquitination genes is in agreement with the findings of Gasch (2003). 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 (Gasch, 2003). Apart from the induction of HSP genes, other genes induced under other type of stresses were also detected. *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 (Ye et al., 2009). 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 (Sakaki et al., 2003). 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 (Li et al., 2010b). It was found that lack of oxygen can repress ergosterol synthesis (Li et al., 2010b). This might be true since oxygen is necessary for xylose uptake (Toivari et al., 2001). The need for oxygen would be relatively higher at high temperature since the cells need more respiration for ATP generation (Ye et al., 2009). 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. (2012), 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.

3.5 Conclusion

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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CHAPTER 4

Co-expression of *TAL1* and *ADH1* in recombinant xylose-fermenting *Saccharomyces cerevisiae* improves ethanol production from lignocellulosic hydrolysates in the presence of furfural

4.1 Introduction

Industrial bioethanol production from lignocellulosic materials has received much attention recently. Lignocellulosic materials and agricultural residues are low-cost sources for ethanol production. However, hurdles must be overcome before these residues can be utilized efficiently by microorganisms. A major problem in biomass conversion to ethanol is the inhibitory compounds generated during the pretreatment of biomass. Depending on the pretreatment process, the inhibitory compounds can be classified into three major groups: (a) furan derivatives; (b) weak carboxylic acids, mainly acetic, formic and levulinic acid; and (c) phenolic compounds such as vanillin and syringaldehyde from the degradation of lignin (Nicolaou et al., 2010). The furan derivatives 5-hydroxymethylfurfural (5-HMF) and furfural are formed at high temperatures and pressures as degradation products of hexoses and pentoses, respectively (Liu et al., 2009). Furfural and 5-HMF can interfere with microbial growth and are considered the most potent inhibitors in bioethanol production (Liu, 2006). One strategy to minimize the harmful effects of these inhibitors is by physical, chemical or biological detoxification of the biomass prior to fermentation (Heer and Sauer, 2008). However, incorporation of detoxification procedures into an overall process increases its complexity, increases the amount of sugar lost, and in practice raises cost (Tian et al., 2011) and fouls process equipment (Hahn-Hagerdal et al., 2007). On top of that, the hemicellulosic fractions of lignocellulosic materials often contain monomeric xylose, which cannot be assimilated by *Saccharomyces cerevisiae*. Hence, yeast with the ability to assimilate xylose and tolerate inhibitors such as furfural is very much needed in industrial bioethanol production since complete substrate utilization is one of the prerequisites for economic competitiveness of the process (Hahn-Hagerdal et al., 2007). Improvement of *S. cerevisiae* to allow consumption of xylose was achieved by reconstruction of the xylose assimilating pathway. This was done by heterologous expression of genes for xylose reductase (XR) and xylitol dehydrogenase (XDH)

derived from *Scheffersomyces stipitis* along with overexpression of xylulokinase (XK) from *S. cerevisiae* (Hasunuma et al., 2011a). On the other hand, *S. cerevisiae* is capable of detoxifying low concentrations of furfural under anaerobic conditions by reduction to furfuryl alcohol (Gorsich et al., 2006). Thus, to enhance tolerance to inhibitors, many researchers have deleted or overexpressed target genes (Hasunuma and Kondo, 2012b). In terms of tolerance limited to furfural, deletion of *PHO13* (Fujitomi et al., 2012) and overexpression of *MSN2* (Sasano et al., 2012) and *ZWF1* (Gorsich et al., 2006) proved to be essential. Two genes manipulated in this study were *ADH1* (alcohol dehydrogenase 1) and *TAL1* (transaldolase 1). *ADH1* has been described as the main fermentative enzyme responsible for ethanol formation in *S. cerevisiae* (Laadan et al., 2008). Based on previous studies, overexpression of *ADH1* variant (m6Adh1) has been identified in industrial yeast strain TMB3000 as an NADH-dependent enzyme capable of reducing both furfural and 5-HMF (Ishii et al., 2013). While *TAL1* (transaldolase 1) is one of the genes in the non-oxidative pentose phosphate pathway (PPP), chosen to be overexpressed to improve xylose assimilation during fermentation in the presence of inhibitor. To date, the overexpression of alcohol dehydrogenase (ADH) gene as enzyme for reducing levels of furfural and 5-HMF was only tested on its single capacity and its synergistic effects with other genes is still unknown. Here, we report the use of a recombinant xylose-utilizing *S. cerevisiae* strain co-expressing the variant *TAL1* and *ADH1* genes that shows improved ethanol production from lignocellulosic hydrolysate in the presence of furfural. Fermentation using the genetically modified strain was followed by transcriptomics analysis to reveal cellular responses during exposure to furfural. Such an evaluation has not been reported previously, and the approach offers promise to allow construction of a cost-effective biocatalyst for ethanol production from lignocellulosic materials.

4.2 Materials and Method

4.2.1 Microbial strains and media

Escherichia coli NovaBlue (Novagen, Inc., Madison, WI) was used as the host strain for recombinant DNA manipulation. *E. coli* was grown in Luria-Bertani medium (10 g/L peptone, 5 g/L yeast extract, and 5 g/L sodium chloride) containing 100 mg/L

ampicillin. *S. cerevisiae* strains were routinely cultivated at 30ºC in synthetic medium [SD medium; 6.7 g/L yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI), 20 g/L glucose] supplemented with appropriate amino acids and nucleotides, and in YPD medium (20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose).

4.2.2 Construction of plasmids and yeast transformation

Standard techniques for nucleic acid manipulation described by Sambrook et al. (Sambrook et al., 1989) were followed. The *TAL1* gene, encoding transaldolase (TAL), was amplified using genomic DNA of *S. cerevisiae* strain MT8-1 (*MATa ade his3 leu2 trp1 ura3*) (Tajima et al., 1985) as a template and the primer set P1 (5'-ATCAGGACTAGTATGTCTGAACCAGCTCAAAAG-3')/P2 (5'-AATCGCGGATCCTTAAGCGGTAACTTTCTTTTCAATC-3'). The PCR-amplified *TAL1* gene was digested with *Spe*I and *Bam*HI, then ligated into the *Spe*I-*Bam*HI site of pGK404 (Ishii et al., 2009) to yield plasmid pGK404ScTAL1. The *m6ADH1* gene, encoding a variant *ADH1* protein, was obtained by digestion of pEWm6ADH1 (Ishii et al., 2013) with *Xho*I and *Not*I, followed by ligation into the *Xho*I-*Not*I site of pRS405+2 μm (Ishii et al., 2009) to yield plasmid pRS405m6ADH1. Yeast was transformed by the lithium-acetate method (Hasunuma et al., 2011a). The plasmids pGK404ScTAL1 and pGK404 were digested with *Eco*RV and the linear products were transformed into *S. cerevisiae* strain MT8-1X (Katahira et al., 2008) to yield MT8-1X/pGK404ScTAL1 and MT8-1X/pGK404, respectively. The plasmid pRS405+2 μm was transformed into *S. cerevisiae* strains MT8-1X/pGK404ScTAL1 and MT8-1X/pGK404 to yield MT8-1X/TAL-405 and MT8-1X/404-405, respectively. The plasmid pRS405m6ADH1 was transformed into *S. cerevisiae* strain MT8-1X/pGK404ScTAL1 to yield MT8-1X/TAL-ADH. Transformants were selected on SD medium supplemented with appropriate amino acids and nucleotides.

4.2.3 Ethanol fermentation

The yeast transformants were aerobically cultivated in YPD medium for 48 h at 30ºC. Cells were collected by centrifugation at 1,000 × g for 5 min at 4ºC and washed twice with distilled water. The cells were inoculated into 50 mL of fermentation

medium (YPX medium: 10 g/L yeast extract, 20 g/L peptone, and 50 g/L xylose) containing 0 or 70 mM furfural. All fermentations were performed at 30ºC with mild agitation in 100 mL closed bottles equipped with a bubbling CO_2 outlet as described previously (Hasunuma et al., 2011a). The initial cell concentration was adjusted to 50 g of wet cells/L. Wet cell weight was determined by weighing a cell pellet that was harvested by centrifugation at 1,000 × *g* for 5 min. For determination of the concentrations of ethanol, glucose, glycerol, xylitol and xylose in the fermentation medium, the supernatant obtained by centrifugation at 14,000 × *g* at 4ºC for 5 min was separated by high performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) equipped with a Shim-pack SPR-Pb column (7.8 mm × 250 mm; Shimadzu) and an RID-10A refractive index detector (Shimadzu) (Hasunuma et al., 2011a). The HPLC system was operated at 80ºC, with water at a flow rate of 0.6 ml/min as the mobile phase. Cell growth was monitored via optical density at 600 nm using a UVmini-1240 spectrophotometer (Shimadzu). Furfural and furfuryl alcohol were determined as described previously (Ishii et al., 2013).

4.2.4 Fermentation of lignocellulosic hydrolysate

Lignocellulosic hydrolysates were obtained by hot water pretreatment and subsequent enzyme treatment as described previously (Sanda et al., 2011). The hydrolysate was composed of sugars (12.0 g/L glucose, 9.7 g/L xylose, 2.6 g/L arabinose, 0.9 g/L galactose and 4.7 g/L fructose), 27.1 mM acetic acid, 20.1 mM formic acid, 7.8 mM furfural, 0.5 mM 5-HMF, 0.6 mM vanillin, and 0.4 mM syringaldehyde, and was used as a fermentation medium after supplementation with 10 g/L yeast extract and 20 g/L peptone. The fermentation was performed as described above.

4.2.5 Gene expression profiling and analysis

Total RNA in the yeast cells was obtained after 6 h of fermentation using the Total RNA Isolation Mini Kit (Agilent Technologies, Palo Alto, CA, USA) following the manufacturer's protocol. cDNA was generated by reverse transcription, labeled with Cy3 dye, and hybridized to *S. cerevisiae* 4×44k microarrays. Array scans and data

analyses were performed with the Agilent DNA Microarray Scanner and Genespring GX software, respectively (Agilent Technologies). Gene expression levels were normalized per chip. Microarray data from the present analysis has been deposited in the Gene Expression Omnibus repository at the U.S. National Center for Biotechnology Information (NCBI) under accession no. GSE45273.

4.3 Results

4.3.1 Fermentation performance of TAL-ADH co-expressing S. cerevisiae strains in the presence and absence of furfural

We determined the effects of furfural on ethanol fermentation with 50 g/L xylose as the sole carbon source in defined medium. Figure 4.1 compares the fermentation performance of three strains: *S. cerevisiae* MT8-1X/404-405 (control strain), MT8-1X/TAL-405 (TAL-expressing strain) and MT8-1X/TAL-ADH (TAL/ADH-co-expressing strain) in the absence and presence of furfural. In the absence of furfural, 50 g/L xylose was fully consumed by MT8-1X/404-405 (Figure 4.1A) and MT8-1X/TAL-405 within 48 h (Figure 4.1B), while it was almost fully consumed by MT8-1X/TAL-ADH within 24 h (Figure 4.1C). MT8-1X/TAL-ADH also produced the highest amount of ethanol (Figure 4.1C), 14.6 g/L, of the three strains. Then, 70 mM furfural was added to the same medium to observe the ability of the three strains to produce ethanol in the presence of furfural. The xylose consumption of the control strain was severely inhibited (only 50% of the xylose consumed) by 70 mM furfural (Figure 4.1D). However, xylose consumption was accelerated by the expression of the *TAL* gene in MT8-1X/TAL-405 (77% xylose consumed), and further improved by the co-expression of the *TAL* and *ADH* genes in MT8-1X/TAL-ADH (94% xylose consumed) (Figures 4.1E-F). The same trend was observed in ethanol production, with the TAL-expressing strain and TAL/ADH-co-expressing strain respectively producing 1.7-fold and 2.3-fold more ethanol than the control strain in the presence of furfural. This shows that the overexpression of a single gene, *TAL1*, is a relevant strategy for improving ethanol production in the presence of furfural, and that co-expression of *TAL1* and *ADH1* is an even better strategy. As shown in Figure 4.2A, the control strain

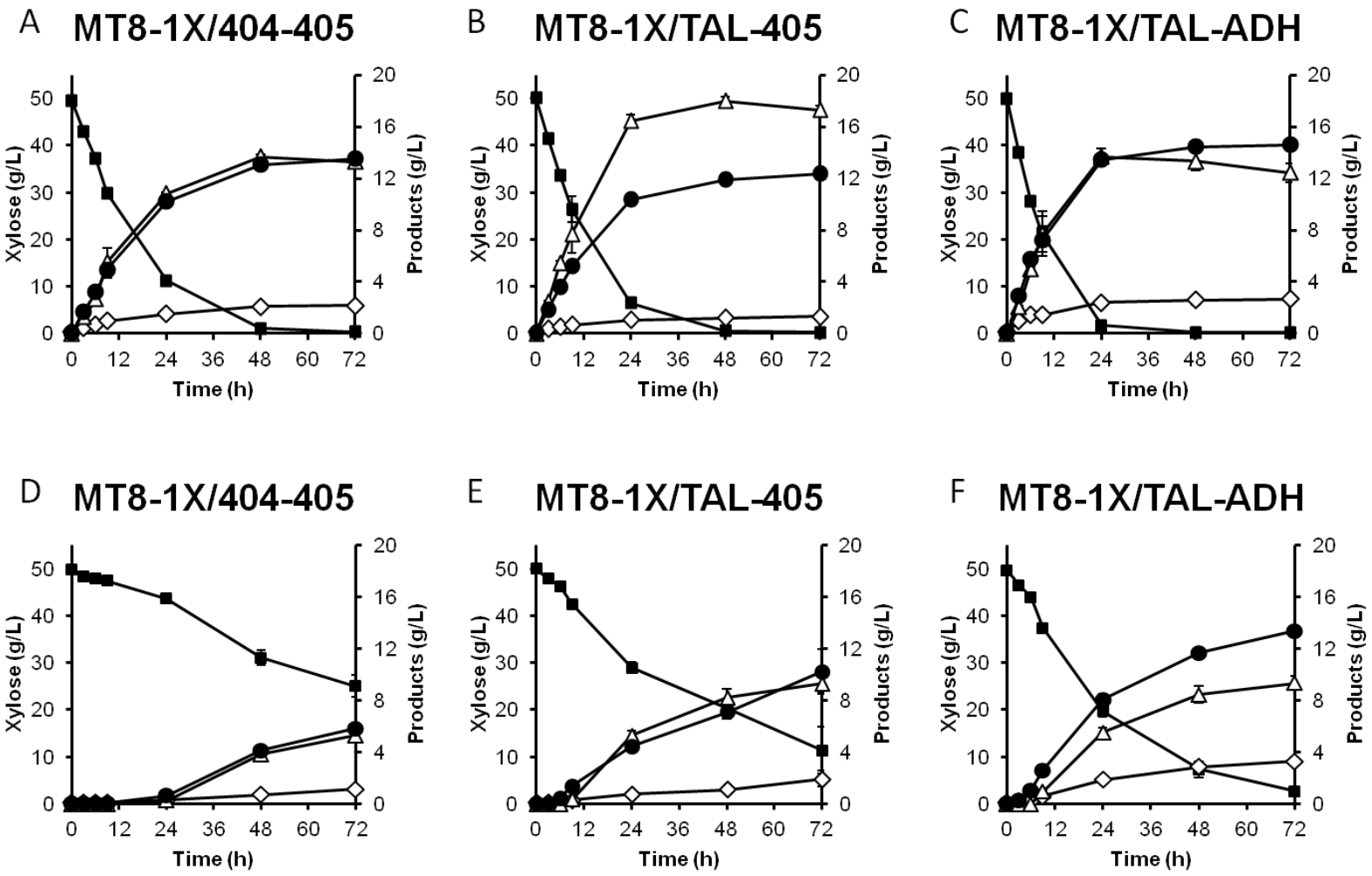

Figure 4.1 Effect of furfural on ethanol fermentation from xylose in defined media at 30°C using *S. cerevisiae* MT8-1X/404-405 (A,D), MT8-1X/TAL-405 (B,F) and MT8-1X/TAL-ADH (C,F). (A-C) 0 mM furfural and (D-F) 70 mM furfural. Ethanol (filled circle), xylose (filled square), glycerol (open diamond) and xylitol (open triangle). Values are averages of 3 independent experiments ± SEM.

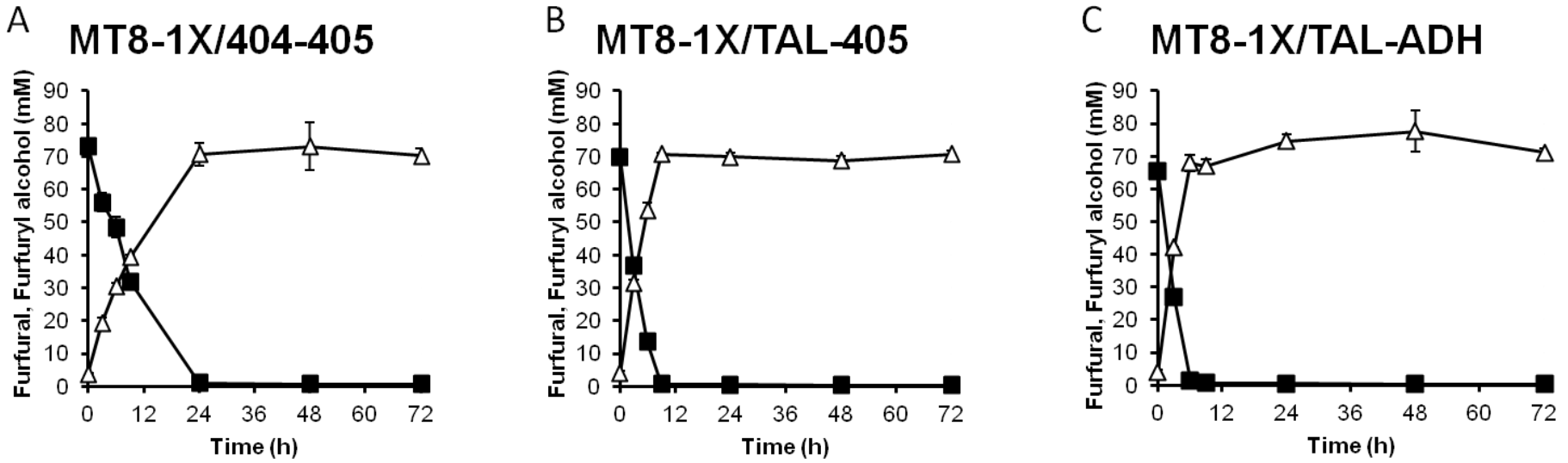

Figure 4.2 Conversion of 70 mM furfural to furfuryl alcohol by *S. cerevisiae* (A) MT8-1X/404-405, (B) MT8-1X/TAL-405 and (C) MT8-1X/TAL-ADH in defined media. Furfural (filled square) and furfuryl alcohol (open triangle). Values are averages of 3 independent experiments ± SEM.

completely converted 70 mM furfural to furfuryl alcohol only after 24 h, compared to MT8-1X/TAL-405 (Figure 4.2B) and MT8-1X/TAL-ADH (Figure 4.2C), which converted it by 9 h and 6 h, respectively. In all three strains, xylose consumption and ethanol production accelerated after the furfural concentration decreased. Table 4.1 compares the ethanol, xylose and glycerol yields for the three strains based on the xylose consumed. The TAL/ADH-co-expressing strain not only yielded the most ethanol in the presence of furfural, but also accumulated the least xylitol, 0.20 g/g, compared to the TAL-expressing and control strains. The addition of 70 mM furfural seemed to negatively affect the three strains' cell growth slightly compared to the control condition (0 mM furfural) (Figure 4.3). Also, there was almost no difference between the growth profiles of the three recombinant strains in the presence of 70 mM furfural. This shows that the improved ethanol production by MT8-1X/TAL-405 and MT8-1X/TAL-ADH was not related to cell growth, but rather to a shift in metabolic response due to the presence of furfural.

4.3.2 Fermentation performance of the co-expressed TAL/ADH genes in undetoxified hemicellulosic hydrolysate

We then tested the performance of the three strains in undetoxified hemicellulosic hydrolysates having xylose, glucose and fructose as the major sugars. Since the hydrolysates were obtained via hot water treatment, the concentrations of the inhibitors were determined to be as follows: 7.8 mM furfural, 0.5 mM 5-HMF, 27.1 mM acetic acid, 10.2 mM formic acid, 0.2 mM vanillin and 0.4 mM syringaldehyde. As depicted in Figure 4.4, the three strains fully metabolized the fructose and glucose in the hydrolysate, but not the xylose. The TAL/ADH-co-expressing strain (Figure 4.4C) and the TAL-expressing strain (Figure 4.4B) consumed 82% and 75% of the xylose, respectively, compared to the control strain (Figure 4.4A), 58%. The ethanol production followed the same trend, where the TAL/ADH-co-expressing and TAL-expressing strains produced 13.4 g/L and 12.6 g/L ethanol, respectively, compared to the control strain, 11.5 g/L. This indicates that the TAL/ADH-co-expressing strategy improved the ethanol titer by 16% compared to the control strain during hemicellulosic fermentation containing furfural.

Table 4.1 Ethanol concentration, ethanol yield, xylitol yield and glycerol yield after 72 h of fermentation of 50 g/L xylose in the presence or absence of furfural by recombinant *S. cerevisiae* strains

Strain	Furfural	Ethanol (g/L)	[a]Yield (g/g)		
			Ethanol	Xylitol	Glycerol
MT8-1X/404-405 (control)	0	13.5 ± 0.2	0.27 ± 0.01	0.27 ± 0.01	0.04 ± 0.00
	70	5.9 ± 0.4	0.24 ± 0.01	0.21 ± 0.01	0.05 ± 0.01
MT8-1X/TAL-405	0	12.3 ± 0.4	0.25 ± 0.01	0.35 ± 0.01	0.03 ± 0.01
	70	10.2 ± 1.7	0.26 ± 0.02	0.24 ± 0.04	0.05 ± 0.02
MT8-1X/TAL-ADH	0	14.6 ± 0.1	0.29 ± 0.00	0.25 ± 0.03	0.05 ± 0.01
	70	13.4 ± 0.2	0.29 ± 0.02	0.20 ± 0.02	0.07 ± 0.01

Values are mean of three independent experiments ± SD.
[a] Yields are based on consumed xylose

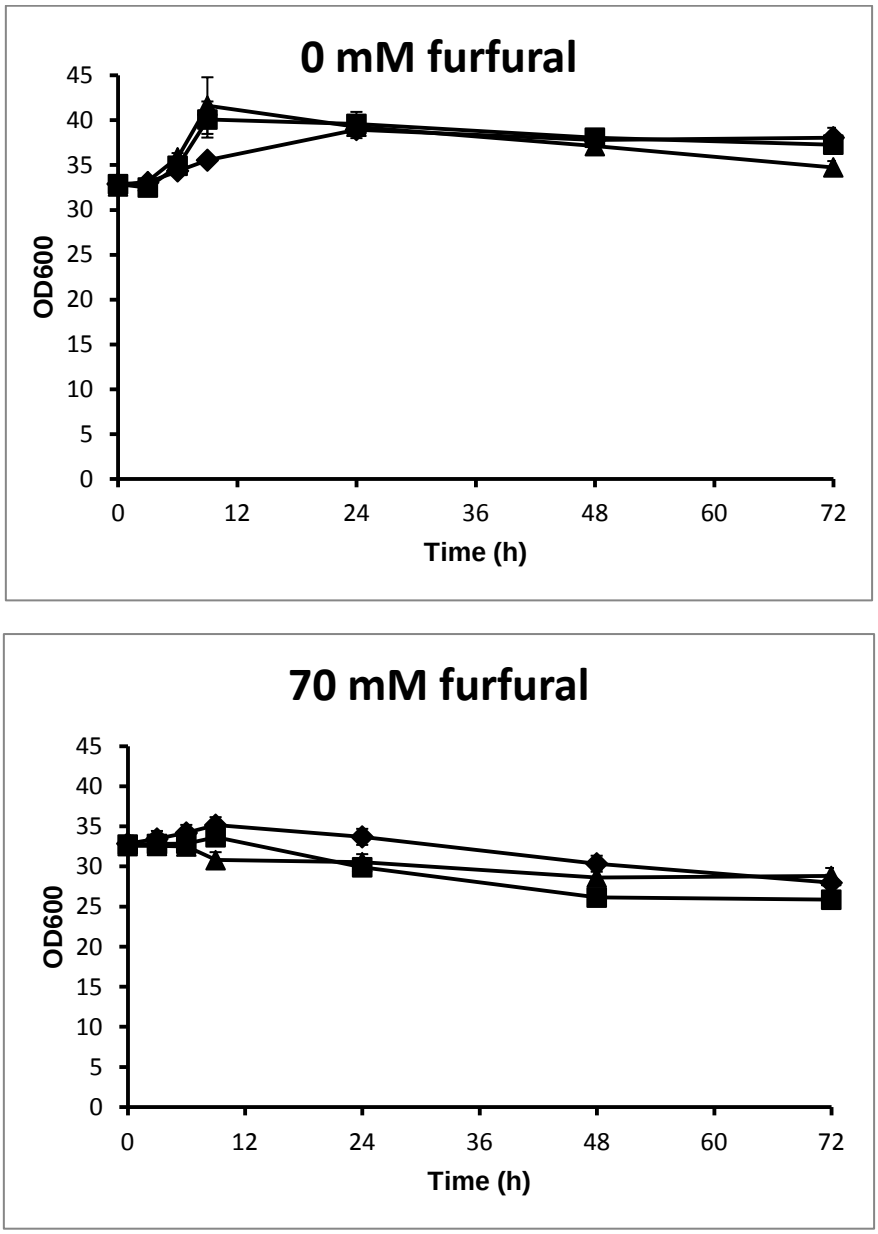

Figure 4.3 Cell growth for *S. cerevisiae* MT8-1X/404-405 (closed diamond), MT8-1X/TAL-405 (closed square) and MT8-1X/TAL-ADH (closed triangle) during the absence and presence of furfural.

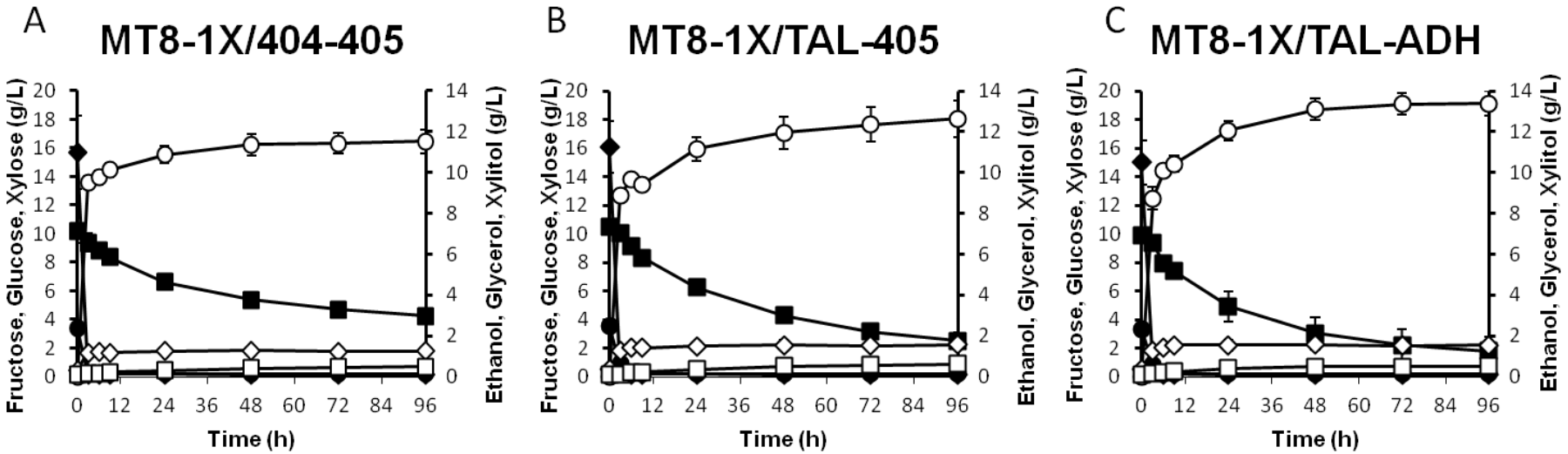

Figure 4.4 Ethanol fermentation from hemicellulosic hydrolysate containing furfural at 30°C using *S. cerevisiae* (A) MT8-1X/404-405, (B) MT8-1X/TAL-405 and (C) MT8-1X/TAL-ADH. Ethanol (open circle), xylose (closed square), glucose (closed diamond), fructose (closed circle), xylitol (open square) and glycerol (open diamond). Values are averages of 3 independent experiments ± SEM.

4.3.3 Transcriptomic analysis of gene regulation in response to furfural

Apart from co-expressing TAL-ADH in yeast to improve ethanol production from xylose, it is also important to observe the other genes' regulation under furfural stress. The induction or reduction of certain genes with certain functions might also lead to improved furfural reduction between the modified strains. Thus, global transcriptomic analysis was carried out for MT8-1X/TAL-ADH, MT8-1X/TAL-405 and control strain after 6 h (the mid-phase during furfural conversion) of xylose fermentation in the presence of 70 mM furfural. Our approach in finding the differentially regulated genes is by calculating the fold change between the experiment and control, where the gene expression from the experiment is divided with the gene expression of the control. Upregulation and downregulation in gene expression are indicated by a plus and minus sign, respectively. We compared the genes that were upregulated in our strains to those upregulated in Heer et al.'s (2009) analysis. The furfural concentration used by Heer et al. (Heer et al., 2009) was a maximum of 36 mM, with glucose as the carbon source. In our control strain, genes with the highest induction due to the presence of 70 mM furfural were *YKL071W* (+84), *GRE2* (+79), *AAD4* (+40), *YML131W* (+13), *OYE2* (+5.3), *OYE3* (+13) and *YDL124W* (+6). These same genes were also upregulated by the TAL-expressing strain at 70 mM furfural. *ALD4*, a major mitochondrial aldehyde dehydrogenase that is required for growth on ethanol and the conversion of acetaldehyde to acetate (Liu et al., 2008) was induced 8-fold in the control strain but was not detected in the other two strains. The expression of *ADH7* was induced 4.9-fold in the TAL-expressing strain relative to the control strain under the same conditions. The pentose phosphate pathway (PPP) genes *TAL1* and *TKL1* were induced 4.3- and 2.1-fold, respectively, in the TAL-expressing strain compared to the control strain in the presence of furfural. *SOL3*, encoding 6-phosphogluconolactonase, was induced 2.8- and 4.1-fold by TAL-expressing and TAL/ADH-co-expressing strains, respectively, compared to the control strain. When analyzing the transcriptomes of the co-expressing *TAL*/*ADH* strain and the control strain, the same genes were induced even more: *ADH7* (+6.0), *TAL1* (+8.0) and *TKL1* (+3.3) under the same fermentation conditions. In the absence of furfural, the gene upregulation of the TAL- expressing strain compared to the control strain was as follows: *TAL1* (+2.7) and *TKL1* (+1.1). Heat shock proteins (HSP) are also induced by a wide variety of stressants including solvents

and toxic chemicals (Nicolaou et al., 2010). Surprisingly, heat shock protein genes such as *HSP78, HSP26, HSP30* and *HSP12* were only induced in the control strain when stressed with furfural, and not in the other strains.

4.4 Discussion

Most biorefineries have been developed for the production of bioethanol from biomass, which requires utilization of xylose, but its hydrolysates also negatively affect microbial fermentation due to the presence of highly toxic compounds (Hasunuma et al., 2011b). We constructed a recombinant *S. cerevisiae* strain not only capable of utilizing xylose but also of producing higher amounts of ethanol in the presence of as high as 70 mM furfural. Previously, overexpression of an ADH-related gene reduced levels of toxic compounds such as furfural and 5-HMF in hydrolysates of pretreated lignocelluloses (Ishii et al., 2013), while the overexpression of *TAL1* reduced the inhibitory effect of weak acids such as acetic and formic acids (Hasunuma et al., 2011a). To provide additional robustness of yeast to fermentation inhibitors, we hypothesized that the combination of both genes would improve *S. cerevisiae*'s ability to consume xylose and produce ethanol under conditions of furfural-induced stress. Previously, co-expression of two genes, *TAL1* and *FDH1*, proved successful to improve ethanol production from xylose in the presence of acetate and formate (Sanda et al., 2011). In media containing 40 mM furfural, overexpression of *m6ADH1* and wild type *ADH1* in xylose utilizing strain YPH499XU by Ishii et al. (Ishii et al., 2013) completely converted furfural within 12 h, and ethanol production was improved compared to their control strain. This shows that *ADH1* on its own capacity have the catalytic potential to reduce furfural. Thus, the aim of this study was to investigate the synergistic effect of *ADH1* and *TAL1* when co-expressed in *S. cerevisiae* during ethanol production in the presence of furfural. We tested three strains' performance in both defined medium (containing 70 mM furfural) and undetoxified lignocellulosic hydrolysates (containing 7.8 mM furfural). In the defined medium, 70 mM furfural was high enough to inhibit the control strain, and a 12 h lag phase in furfural conversion was observed relative to the TAL-expressing and TAL/ADH co-expressing strains. Toxicity of the furan was also exhibited by the cells' slower xylose consumption and ethanol production during the initial exposure to the high furfural concentration. In the control strain, full

reduction of furfural, achieved after 24 h, might be associated with the induction of the *YKL071W* gene. Heer et al. (2009) overexpressed the uncharacterized *YKL071W* and oxidoreductase *ADH7*, and found them to be the key molecular components of their furfural-resistant strain. We agree with Heer et al. (2009) that *ADH7*, which was greatly induced in our TAL-expressing strain, is relevant in responding to furfural stress during ethanol production. Since TAL alone does not directly convert furfural to furfuryl alcohol, the improved furfural conversion in the TAL-expressing strain might be linked to the function of *ADH7*, which encodes an NADPH-dependent, medium-chain-length alcohol dehydrogenase with a broad substrate specificity that includes aromatic compounds (Heer et al., 2009). Apart from that, based on our results, the depletion time for furfural was the fastest with the TAL-ADH expressing strain resulting in notable improvement in ethanol production and xylose consumption which suggests that the strategy was successful. This could be explained by the function of *ADH1*, an NADH-dependent alcohol dehydrogenase, which according to Gorsich et al. (2006), provides free NADH, needed to detoxify furfural. The same conclusion was also reached by Lin et al. (2009), who noted that *ADH1, ADH5* and *ADH6* may catalyze the reduction of furfural to furfural alcohol even under aerobic conditions. It is believed that the reduction of furfural to furfuryl alcohol depends on NADH, although there are strong indications that NADPH-dependent processes are also involved (Heer et al., 2009; Jarboe et al., 2011; Liu et al., 2008).

Since the carbon source was xylose, we focused on the transcriptomics of the PPP genes. Transcription analysis performed on the TAL-expressing strain indicated that the upregulation of central metabolism was necessary for growth in the presence of furfural. In both TAL-expressing and TAL-ADH-co-expressing strains, induction of *TAL1, TKL1* and *SOL3* in the presence of furfural might assist xylose consumption in the presence of furfural, leading to higher ethanol production. Acceleration of xylose assimilation may be a response of the strains recovering from inhibitor stress. Thus, based on the transcriptomics, we suggest that PPP may have a direct correlation with furfural detoxification when xylose is the carbon source, as proposed by Gorsich et al. (2006). When glucose is the carbon source, PPP may not be directly correlated with furfural conversion, due to the absence of changes in gene expression observed by Lin et al. (2009).

We assume that heat shock gene expression is a general stress response triggered by exposure to furfural in the control strain. As the expression of heat shock protein genes was unchanged in the TAL-ADH co-expressing strain, the strains may have responded differently to the same furfural concentration. MT8-1X/TAL-ADH might not detect furfural as a stress factor due to its robust nature or it may effectively utilize its high activity of TAL and ADH to detoxify furfural and similar inhibitory compounds.

To conclude, our findings demonstrated that by co-expressing the *TAL1* and *ADH1* genes, ethanol production could be improved by 127% in the presence of 70 mM furfural. The results of fermentation using undetoxified hydrolysates indicate that the synergistic effects of *TAL1* and *ADH1* were capable of improving ethanol production in the presence of 7.8 mM furfural, which mimics real industrial applications. Expressing only the *TAL* gene can confer higher ethanol titer in the presence of furfural, but not as well as the combination of expressing both the *TAL1* and *ADH1* genes. Transcriptomics analysis showed that the improved conversion of furfural was also attributed by strong expression of *ADH7*, where higher expression of this gene resulted in faster furfural conversion. Ethanol production was also improved by the upregulation of xylose metabolism genes which might in turn accelerated the xylose uptake in the presence of furfural. It is hoped that the outcome of this investigation will assist in filling the knowledge gap in developing a multi-tolerant yeast strain for a sustainable and cost-competitive biomass-to-ethanol industry.

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CHAPTER 5

Zinc, magnesium and calcium ions supplementation confers tolerance to acetate stress in industrial *Saccharomyces cerevisiae* utilizing xylose

5.1 Introduction

Global concerns on the sustainability of the crude oil supply and environmental issues associated with the over-consumption of petroleum-based products have revitalized biofuels production. Due to these circumstances, biofuels from lignocellulosic materials is becoming an increasingly important alternative to fossil fuel. However, in ensuring that the process economics for the industrial production to be feasible, the biocatalyst involved in the fermentation process must be capable of fermenting all the sugars present from the hydrolyzates with high ethanol yields and productivities (Sanda et al., 2011). This included pentose sugar, xylose which is not naturally utilized by *Saccharomyces cerevisiae*. To add to the challenge in fermenting xylose to ethanol, inhibitors contained in the undetoxified hydrolysates must be tolerated. Acetyl groups cleaved off from the hemicelluloses will be found in the hydrolysates as acetate/acetic acid. The acetyl content is normally higher in pentose-rich materials than in materials with low pentose content (Almeida et al., 2011). Together with high ethanol concentration and other toxic compounds, acetic acid may contribute to fermentation arrest and reduced ethanol productivity (Mira et al., 2010). Thus, stress caused by acetic acid is more important in xylose-rich hydrolysates. Efforts to induce the ethanol production from lignocellulosic hydrolysates in the presence of acetic acid has been successfully done by previous researchers via genetic modification (Fujitomi et al., 2012; Hasunuma et al., 2011a; Lu et al., 2011; Sanda et al., 2011). Apart from genetic modification, media composition is also important. In dealing with the stress related challenge, we used a xylose-utilizing recombinant strain (Ismail et al., 2013) with the aid of metal ions supplementation into the fermentation media. Previously, magnesium, calcium and zinc ions had shown to help the cells in tolerating ethanol toxicity (Zhao and Bai, 2012) which was caused by increasing levels of ethanol during yeast fermentation, and also heat shock (Birch and Walker, 2000). The combinations of the three metal ions have even been tested in an optimized media to confer ethanol tolerance (Xue et al., 2008). According to Birch and Walker (2000), the protective

responses induced by heat shock and ethanol showed a high degree of similarity, for example induction of two major integral plasma membrane protein, ATPase and heat shock proteins. Since these three metals showed protective effects in several stresses, we hypothesize that they would show the same effects towards acetic acid. In terms of improving acetic acid tolerance, only zinc supplementation was tested to be successful but with glucose as the carbon source (Xu et al., 2012). To date, this is the first report on the effect of zinc, magnesium and calcium ions supplementation on ethanol production from xylose under acidified media. In this work, media containing 30 mM acetic acid were supplemented with different concentrations of the three metal ions, and their effect on ethanol fermentation was observed. We then analyzed the transcriptional responses between zinc, magnesium and calcium addition during the presence of acetic acid to find the underlying changes in gene regulation that might lead to improved yeast performance.

5.2 Materials and Methods

5.2.1 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 (Ismail et al., 2013). YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) was used as the growth medium. Prior to use, the strains were pre-incubated in 5 mL YPD medium added with 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 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 3000 × *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.

5.2.2 Fermentation conditions and HPLC analysis

Batch fermentation was carried out in a 100-mL bottle with a CO_2 outlet. Fermentation medium with a total working volume of 50 mL consists of (g/L): xylose 50, yeast extract 5, peptone 4 and acetic acid 1.8 (30 mM). Cell concentration was inoculated at 25 g/L (wet weight). Metal supplementation concentrations were added as follows: 100, 180 and 300 μM of $ZnSO_4$, 1.5, 3.5 and 7 mM of $MgSO_4$ and 1, 2 and 5 mM of $CaCl_2$. Two control medium was used: medium added with 30 mM acetic acid without metal, and medium without both acetic acid and metals. Temperature was controlled at 30°C by placing the bottles in a water bath equipped with a magnetic stirrer (500 rpm). Samples for high-performance liquid chromatography (HPLC) analysis were taken at 0, 3, 6, 9, 12, 24, 48, 72 and 96 h of fermentation. Samples were centrifuged at 3000 *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 (Hasunuma et al., 2011a). 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.

5.2.3 Metal ions analysis

1 mL sample of yeast cells was taken every 24 h of fermentation. The cells were washed two times with distilled water and freeze-dried overnight. The cell dry weight was measured. Then, 100 μL of 1M nitric acid was added to the freeze-dried cells and mixed for 1 min. After centrifugation (12 000 rpm, 5 min), the supernatant was diluted with 1M nitric acid solution up to the Atomic Absorption Spectroscopy (Hitachi, Japan) detection limit and tested for zinc, magnesium and calcium contents in yeast cells. The standard curve was obtained using the standard solution for zinc, magnesium and calcium. Metal ion content in yeast cells was calculated as micromole per cell dry weight.

5.2.4 Cell viability

Cell viability was measured using a disposable hemocytometer chamber (C-Chip, Korea) using a microscope (Zeiss, Germany). Trypan blue was used in the vital staining.

5.2.5 DNA microarray analysis

1 ml of cell samples obtained at 6 h (log phase) of fermentation were harvested and washed with sterile distilled water by centrifugation at 6000 × *g* for 5 min at 4°C. 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, Delaware, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. cDNA was generated by reverse transcription, labeled with Cy3 and hybridized to *S. cerevisiae* 4x44 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 microarray sample was analyzed in duplicate. Gene expression was calculated using normalized data.

5.3 Results and Discussion

5.3.1 Optimum metal ions supplementation improves ethanol production and cell growth

Ethanol fermentation using xylose as the sole carbon source was tested in the presence of acetic acid. The strain used was *S. cerevisiae* genetically-modified to overexpress the genes from *S. stipitis* encoding xylose reductase (XR) and xylitol dehydrogenase (XDH), enabling it to consume xylose. Ethanol production was enhanced by overexpressing the endogenous XK gene encoding xylulokinase. As

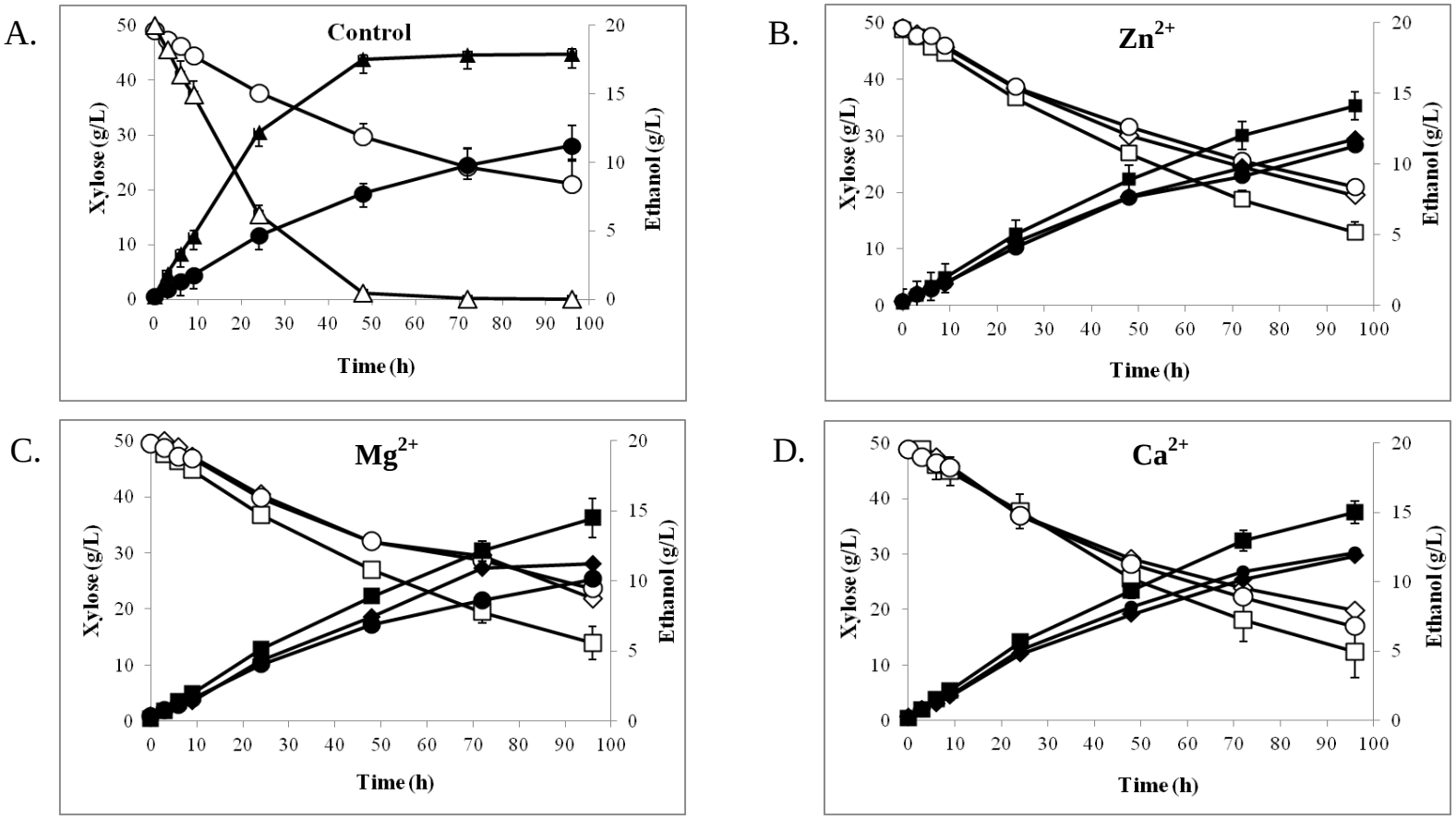

Figure 5.1 Effect of metal ion concentrations on ethanol production and xylose production under 30 mM acetate stress. (A) Control condition: 30 mM acetate, without metal supplementation (circle) Without acetate, without metal supplementation (triangle). (B) Zn^{2+} concentration: 100 μM (diamond), 180 μM (square), 300 μM (circle). (C) Mg^{2+} concentration: 1.5 mM (diamond), 3.5 mM (square), 7 mM (circle). (D) Ca^{2+} concentration: 1 mM (diamond), 2 mM (square), 5 mM (circle). Closed symbol - ethanol production, open symbol – xylose consumption. Values are average of three runs ± SD.

shown in Figure 5.1A, 30 mM acetic acid arrested the ethanol production by 37%, and the xylose consumption by 44% compared to the condition absence of acetic acid. Three types of metal ions, zinc, magnesium and calcium were tested on their ability to confer acetic acid tolerance during ethanol production with xylose as the carbon source. Based on Figure 5.1B, of the three zinc concentrations tested, 180 μM zinc increased the ethanol production by 26 % and xylose consumption by 28%. Zinc concentrations lower (100 μM) and higher (300 μM) than 180 μM showed no improvement compared to the negative control. This result is in agreement with the work done by Xu et al. (2012) where they discovered that 0.03 g/L zinc sulfate showed the best protective effect in a batch bioreactor. The only difference was the carbon source used, which was glucose. Figure 5.1C shows that 3.5 mM magnesium provided the highest ethanol production (14.5 g/L) compared to 1 mM and 7 mM magnesium. Xylose consumption improved by 29% compared to the negative control. Of all the metals tested, 2 mM calcium illustrated the best supplementation performance under acetic acid stress where 34% and 38% increment in ethanol production and xylose consumption was achieved, respectively (Figure 5.1D). Evaluating the three metals together, the best metal concentrations were 180 μM, 3.5 mM and 2 mM of zinc, magnesium and calcium, respectively. Concentrations lower or higher than the latter did not show significant improvement when compared to the control condition without metal supplementation. No change in pH was observed during the 96 h fermentation period in the media supplemented with the three metals.

The increment in ethanol production was believed to be linked to the increment in cell viability which seemed applicable to the three metals tested. According to Figure 5.2, media supplemented with 180 μM zinc, 3.5 mM magnesium and 2 mM of calcium gained the highest cell viability under 30 mM acetic acid stress after 96 h of fermentation course. The cell viability improved for all the concentrations tested when compared to the negative control (without metal), especially in 3.5 mM magnesium and 2 mM calcium, 54% and 51% increment, respectively. This indicates that the metal supplementation stimulated cell growth but its concentration must be optimized. Improved cell viability by zinc supplementation was also achieved by Wu et al. (2013) and Zhao et al. (2009) using *Clostridium acetobutylicum* and self-flocculating yeast, respectively.

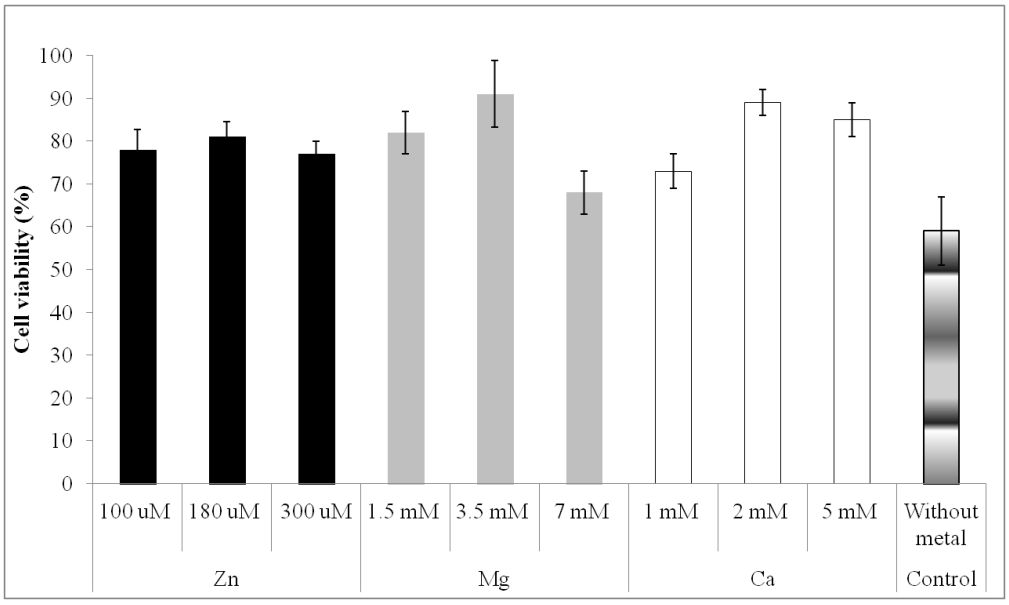

Figure 5.2 Effect of metal concentrations on cell viability in the presence of 30 mM acetic acid after 96 h of fermentation. Results are average of 3 runs ± SD.

Very high concentration of metal supplementation might not be a good strategy since it may lead to toxicated cells, as demonstrated by cells added with 7 mM magnesium. As stated by Zhao et al. (2009), zinc has a protective effect against ethanol stress, and this result shows that it might be used to tolerate acetic acid as well. Not limited to zinc ions, magnesium and calcium ions showed better protective effects under acidic stress. Thus, to better understand the functions of the metals towards cell protection, transcriptomic analysis was done to observe the gene regulations after 6 h of supplementation.

5.3.2 Comparative transcriptomic analysis in response to metal ions addition

The transcriptomics was compared between the cells supplemented by the three optimum metal ions concentration (180 μM zinc, 3.5 mM magnesium and 2 mM of calcium) relative to the control condition (without metal addition). Figure 5.3 is showing the Venn diagram for the up-regulated and down-regulated genes among the three metal ions. Only genes regulated more than 1.5-folds are taken into consideration. One gene, *FIT2* was found to be up-regulated in common between zinc, magnesium and calcium ions, while 11 genes were commonly down-regulated. It also indicated that calcium and zinc ions resulted in higher number of similar genes compared to

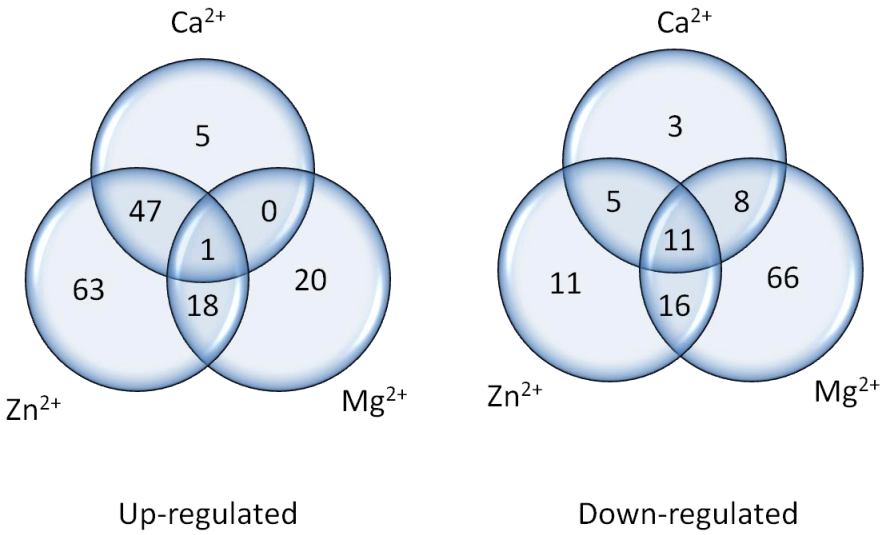

Figure 5.3 Venn diagram indicating the number of genes commonly up-regulated and down-regulated (more than 1.5-fold change) between Ca^{2+}, Zn^{2+} and Mg^{2+}.

magnesium. Table 5.1 shows the overrepresented functional categories in response to metal ions supplementation in media containing 30 mM acetic acid. We found that the addition of magnesium ions resulted in up-regulation of 'intrinsic to membrane' category. This includes *COX2, COX3, HXT8, HXT10* and *PHO84*. We performed analysis on the down-regulated genes under magnesium addition but no particular functional categories were found. In terms of zinc addition, the most significant functional category is 'sulfur compound metabolic process' which includes *MET6* (involved in methionine biosynthesis). Genes related to cell wall and encapsulating structure was also induced. However, genes related to 'cellular zinc ion homeostasis' and 'mitochondrial part' were down-regulated as a result of zinc addition. Despite showing good cell viability and ethanol production, the transcriptomics for the calcium supplemented yeast showed no genes being induced more than 2-fold compared to the control. However, one of the functional categories for the down-regulated genes was 'energy derivation by oxidation of organic compound' which consists of *ADH2*. The down-regulation of *ADH2* (-2.0), encoding alcohol dehydrogenase which catalyzes the conversion of ethanol to acetaldehyde might be assisting the ethanol production under calcium supplementation.

Some genes related to cell wall and hexose transporter are shortlisted in Table 5.2, showing the fold change obtained between the three metal ions relative to the control.

Table 5.1 Overrepresented functional categories in response to metal ions supplementation in media containing 30 mM acetic acid

	Functional category	P-value
Up-regulated in response to Mg supplementation	Intrinsic to membrane	0.045
Up-regulated in response to Zn supplementation	Fungal type cell wall	0.022
	External encapsulating structure	0.039
	Cell wall	0.039
	Sulfur compound metabolic process	3.349E-06
	Thiamin biosynthetic process	4.367E-06
	Aromatic compound biosynthetic process	6.466E-06
	Vitamin metabolic process	0.001
	Cellular nitrogen compound biosynthetic process	7.227E-04
	Cellular amino acid metabolic process	0.004
	Vitamin B6 biosynthetic process	0.02
	Pyridoxine metabolic process	0.02
	Amine metabolic process	0.039
	Cellular aromatic compound metabolic process	1.753E-05
	Vitamin biosynthesis process	3.414E-04
Down-regulated in response to Ca supplementation	Energy derivation by oxidation of organic compound	0.01
	Glyoxylate cycle	0.01
	Aerobic respiration	0.019
Down-regulated in response to Zn supplementation	Cellular zinc ion homeostasis	1.313E-06
	Mitochondrial part	0.005

Table 5.2 Transcriptional changes of some genes in response to the metal ions supplementation under acetic acid stress. Values are fold-change relative to the control condition (without metal ion supplementation)

Gene	Zn^{2+}	Mg^{2+}	Ca^{2+}
FIT2	+2.4	+1.7	+1.5
HXT1	+2.2	+1.8	-1.0
CRR1	+2.5	+1.8	+1.4
GAS2	+2.6	-1.5	+1.7
TKL1	+2.0	-1.0	+1.4
MKC7	+2.4	+1.2	+1.9
FRE7	+2.6	+2.8	+1.4
RCK1	+4.4	+2.6	-1.3
ADH2	+1.3	-1.5	-2.0

The only similar significant response between the three metal ions was the up-regulation of *FIT2*, encoding facilitator of iron transport. Genes related to iron uptake are required for yeast tolerance to acetic acid which is in agreement with the findings of Mira et al. (2010). Zinc might also improve the cell wall structure by up-regulating cell wall genes, *GAS2*, *CRR1* and *SUN4*. The remodeling of the yeast cell wall structure in response to acetic acid or other weak acids is known to be essential to reduce the diffusion rate of the undissociated weak acid forms into the cell interior (Mira et al., 2010). According to Li and Yuan (2010), acetic acid specifically repressed the genes related to mitochondria ribosomal protein, where it is the center for many redox reactions including detoxification. Also, it interrupts with the carbohydrate metabolism and regulation. Thus, it might indicate that the improvement of xylose consumption was not caused by acetic acid detoxification by zinc addition, but might be due to the increased expression of a hexose transporter *HXT1* (+2.2), while ethanol production might be improved by the induction of transketolase *TKL1* (+2.0), which is one of the genes in the pentose phosphate pathway. Magnesium supplementation also might improve membrane structure which correlates well with the highest cell viability. Calcium's role in improving the ethanol production under acetic acid stress is probably related to cell wall maintenance also. This is based on *MKC7*, the highest up-regulated gene after calcium addition, which encodes GPI-anchored aspartyl protease; member of the yapsin family of proteases involved in cell wall growth and maintenance, which shares functions with Yap3p.

Taken together, tolerance to acetic acid can be alleviated by the supplementation of zinc, magnesium and calcium ions where each of these metal ions has a specific role in regulating different genes.

5.4 Conclusion

Fermentation of lignocellulosic hydrolysates to ethanol is very challenging to yeast because of the inhibitory compounds derived from cellulose, hemicellulose and lignin degradation during pretreatment of the biomass are present in the broth. Acetic acid is among these compounds and it can be released from hemicellulose to such an extent that yeast fermentation is completely compromised. This study shows that protective measures against acetic acid stress can be achieved by metal ions supplementation at optimum concentrations. Ethanol production was most improved by 34% when supplemented with 2 mM calcium, followed by 180 μM zinc (26% improvement) and 3.5 mM magnesium (29% improvement). The higher ethanol production obtained was linked to the high cell viability percentage after the metal ion addition. Comparative transcriptomics between the supplemented cultures and the control suggest that the cell viability was improved by upregulation of cell wall and membrane genes. Upregulation of hexose transporter genes and genes in metabolic pathways might also enhanced ethanol production in the presence of acetic acid.

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CHAPTER 6

General Conclusion

With the increasing demands for energy and the shrinking energy resources, the utilization of lignocellulosic biomass for the production of biofuel offers a renewable alternative. It is anticipated that the transition from first generation (starch-based) to second generation (lignocellulosic-based) biofuel processes must involve a move from conventional wild-type production strains towards genetically engineered variants with superior phenotypes to increase fermentation efficiency. Crucially, transformation, gene transfer and genomic integration systems are now emerging that will allow the potential of thermotolerant and inhibitor tolerant microorganisms to be developed and used in simultaneous saccharification and fermentation or consolidated bioprocessing system. Different from previous investigations in developing thermotolerant and inhibitor tolerant strains, this work focused more on xylose utilization, rather than the typical fermentable sugar, glucose. For the lignocellulosic bioethanol process to be economically feasible, both glucose and xylose must be fully converted to ethanol. Successful xylose fermentation would increase the ethanol production. Also, xylose fermentation is affected to a greater degree by hydrolysates inhibitors as compared to glucose fermentation. Thus, lack of information on the effects of heat and inhibitors at the gene level when xylose is the carbon source encouraged the kick-start of this project. Equipped with high-throughput technology, novel findings were obtained and the expression of the key genes related to heat response and inhibitor stress response was studied.

Chapters 2 and 3 were dedicated to the study of heat responsive genes in the interest to develop a thermotolerant yeast strain. Eight industrial strains from different industrial backgrounds were engineered to be capable of consuming xylose. The first transcriptome analysis strategy was done across three best performing strains (sun048T, sun049T and sun588T) with the aim to search for genes regulated in common. The common genes induced proved that cross-protection occurred during high temperature stress condition. Then, one superior strain, sun049T was chosen to be further investigated based on its natural capacity in tolerating heat stress. The second

transcriptome analysis strategy was by observing the gene expression pattern during high temperature fermentation and control temperature in sun049T along the first 12 h. The result showed several genes responsive to heat with their expression increased by time. Xylitol production was also showed to be linked with heat stress and speculated to be the cells protective mechanism. Combining both transcriptome strategy results, the list of genes for the subsequent step was narrowed down. However, more investigation needs to be done to ensure whether a single gene manipulation is enough, or the genes function in network in order to achieve thermotolerance. The report on the global gene expressions under high temperature in xylose-utilizing industrial strains was the first of its kind and hopefully would provide useful information on the gene functions under heat stress conditions.

Chapters 4 and 5 were devoted to the development of strains tolerant to inhibitors, namely furfural and acetic acid. The strategy implemented was to co-express *TAL1* and *ADH1* genes in the xylose utilizing strain, MT8-1X/TAL-ADH, which proved to be successful in tolerating furfural. The strain was also tested successful in producing ethanol from real undetoxified hydrolysates, bringing the strain close to commercial value. Besides applying genetic engineering to manipulate the strains phenotype, media supplementation strategy showed promising. Zinc, magnesium and calcium supplementation demonstrated to act as a protective shield to the yeast sun049T under acetic acid stress, which resulted in higher cell viability. Transcriptomic analysis identifies the genes related to cell walls and membrane being altered by the metal ions addition which might lead to improved ethanol production under acetic acid stress.

Based on the evidence from the above investigations, it is hoped that the information provided would somehow contribute to the successive work in developing the perfect strain for lignocellulosic biofuel production. Further work should clarify the possibility of combining the thermotolerant and inhibitor tolerant phenotype into a single strain with the interest to develop a multi-stress tolerant strain.

PUBLICATION LIST

Ismail, K.S.K., Sakamoto, T., Hasunuma, T., Hatanaka, H. and Kondo, A. (2013) Gene expression cross-profiling in genetically modified industrial S*accharomyces cerevisiae* strains during high-temperature ethanol production from xylose. Journal of Biotechnology. 163(1): 50-60.

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Ismail, K.S.K., Hasunuma, T., X.Q. Zhao and Kondo, A. Zinc, magnesium and calcium ions supplementation confers tolerance to acetate stress in industrial *Saccharomyces cerevisiae* utilizing xylose. – Soon to be submitted.

Other publication:

Napier grass extrusion coupled with steamed aerosolized acid hydrolysis improved total sugar released with low inhibitor content for high-solid ethanol production. – To be submitted.