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

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ABSTRACT

Production of ethanol from xylose at high temperature would be an economical approach since it reduces risk of contamination and allows both the saccharification and fermentation steps in SSF to be running at elevated temperature. Eight recombinant xylose-utilizing *Saccharomyces cerevisiae* strains developed from industrial strains were constructed and subjected to high-temperature fermentation at 38 °C. The best performing strain was sun049T, which produced up to 15.2 g/L ethanol (63% of the theoretical production), followed by sun048T and sun588T, both with 14.1 g/L ethanol produced. Via transcriptomic analysis, expression profiling of the top three best ethanol producing strains compared to a negative control strain, sun473T, led to the discovery of genes in common that were regulated in the same direction. Identification of the 20 most highly up-regulated and the 20 most highly down-regulated genes indicated that the cells regulate their central metabolism and maintain the integrity of the cell walls in response to high temperature. We also speculate that cross-protection in the cells occurs, allowing them to maintain ethanol production at higher concentration under heat stress than the negative controls. This report provides further transcriptomics information in the interest of producing a robust microorganism for high-temperature ethanol production utilizing xylose.

1. Introduction

Ethanol derived from biomass sources is one of the options for renewable energy which is currently gaining prominence globally. Lignocellulosic biomass, which consists of lignin, cellulose, hemicelluloses, and various extractives (Jeffries and Jin, 2000) is becoming one of the essential resources for bioethanol production since it does not compete with food supplies. There are about 73.9 Tg of crop residues in the world that could produce 49.1 GL of ethanol per year (Kim and Dale, 2004). Besides glucose, the pentose sugar xylose is the major carbohydrate component of hemicellulose and is the carbon source of interest in this research.

To produce bioethanol at a reduced operating cost, the development of robust microorganisms for efficient fermentation must be directed toward 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.

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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; Kim et al., 2011). 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. Materials and methods

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 × *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. 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′-CGCGCGGCGCGCCGGTACCGTCGACGGCGCGCCGTTTAAACTCG-AGACTAGTTTAAACGC-3′ and 5′-CGCGCGGCCGCGTTTAAACTAGT-CTCGAGTTTAAACGGCGCGCCGTCGACGGTACCGGCGCGCCG-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.

2.3. 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 was carried out in a 100-mL bottle with a CO_2 outlet as previously described (Hasunuma et al., 2011a). 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 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. Cell growth was monitored by determination of optical density at 600 nm using a spectrophotometer UV-mini (Shimadzu).

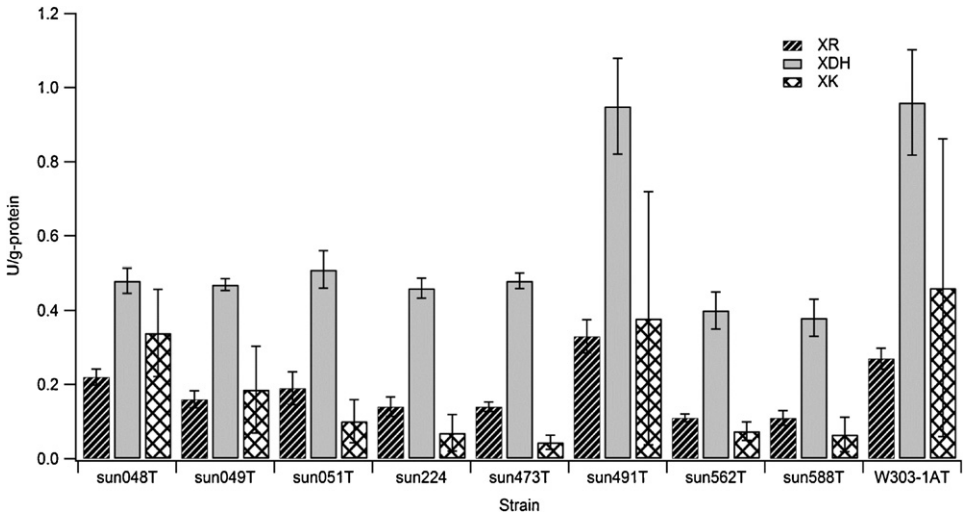

Fig. 1. Specific activities (U/g protein) of xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK) for 9 yeast strains.

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.

2.5. 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.

2.6. 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 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.

3. Results and discussion

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

Table 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

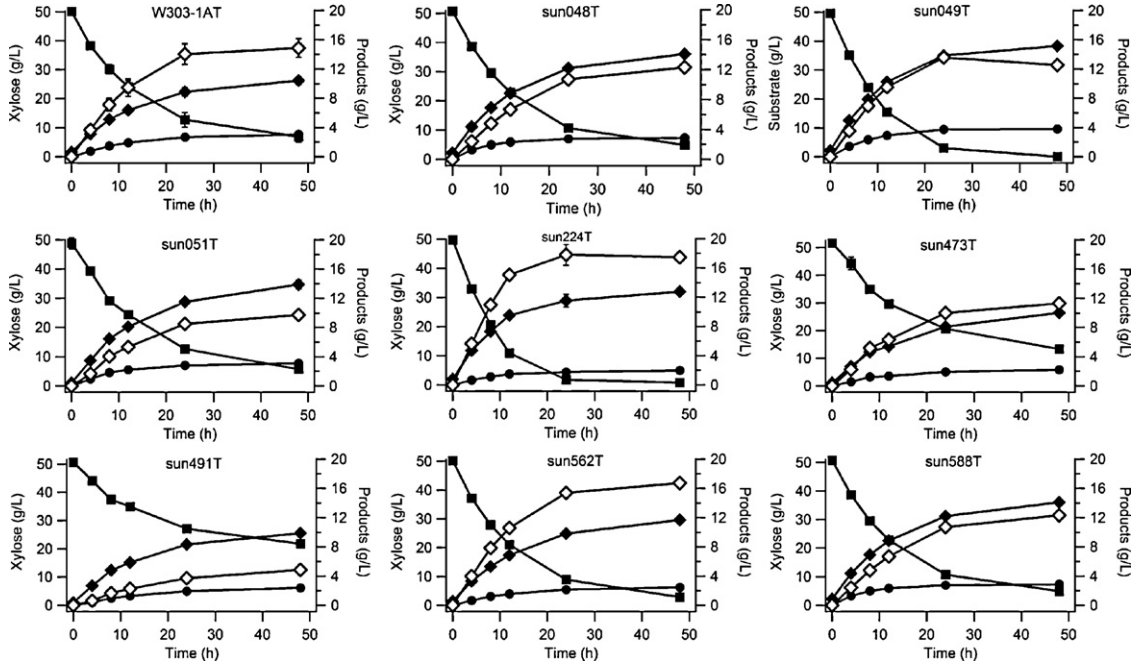

Fig. 2. Time course of fermentation for 9 strains cultivated at 38 °C for 48 h: (♦) ethanol, (■) xylose, (◊) xylitol and (●) glycerol. Values are averages of 3 independent experiments ± SD.

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. Fig. 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.

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 (Fig. 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. 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 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 Fig. 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

Table 2
Ethanol production, ethanol yield, ethanol production rate and xylose consumption at 30 °C and 38 °C for 9 yeast strains.

Strain	Ethanol production[a] (g/L)		Ethanol yield[b] (g/g)		Ethanol production rate[c] (g/L/h)		Xylose consumption[d] (g/L)	
	30 °C	38 °C	30 °C	38 °C	30 °C	38 °C	30 °C	38 °C
W303-1AT[e]	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.
[a] Maximum ethanol concentration after 48 h.
[b] Calculated based on g-ethanol produced/g-xylose consumed.
[c] Calculated based on change in ethanol production with respect to time from 0 h to 12 h of fermentation.
[d] Calculated based on xylose concentration difference between 0 h and 42 h of fermentation.
[e] Laboratory strain.

(between 0.38 and 0.48ýU/g protein), whereas sun473T's enzyme activity was comparable with those of the high ethanol producing strains.

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. Fig. 3 shows a Venn diagram highlighting the altered expression of genes by the top 3 thermo-tolerant ethanol producers. We used DNA microarrays with the capacity of identifying 6316 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.

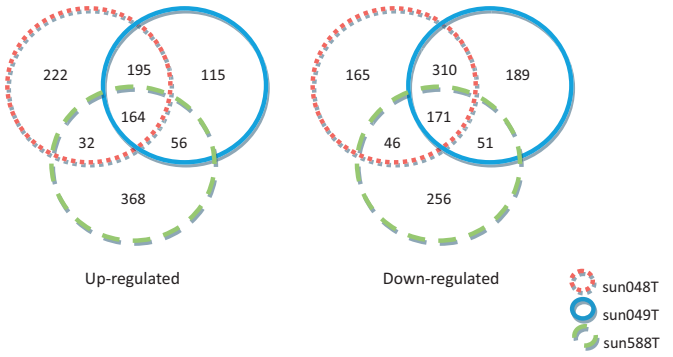

Fig. 3. Venn diagram showing the number of genes differentially regulated among the best ethanol producers, sun048T, sun049T and sun588T. 164 and 171 genes were commonly up-regulated and commonly down-regulated, respectively.

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 3–5, 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

Table 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	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 maintenance	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	82	*AVL9*	Molecule transportation	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 maintenance	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	25	*GIT1*	Membrane transporter	Plasma membrane permease	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 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	RNA processing	Peroxisomal AMP-binding protein	355	*ENA5*	ATPase	Protein with similarity to P-type ATPase	592
HXT9	Carbohydrate metabolism	Hexose transporter	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	Regulation of enolase (ENO1)	169	*COS12*	Genes of unknown function	Protein of unknown function	284
FLO1	Cell wall maintenance	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 (MRP) subfamily	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 maintenance	GPI-anchored cell surface glycoprotein (flocculin)	59	*YRF1-1*	Unknown	Helicase encoded by the Y′ element of subtelomeric regions	260
FLO10	Cell wall maintenance	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*	Molecule Transportation	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	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 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	Regulation of enolase (ENO1)	1500	*YFL054C*	Small molecule transporter	Putative channel-like protein	4302
COS6	Genes of unknown function	Protein of unknown function	1170	*AIF1*	Cell stress	Mitochondrial cell death effector	1498
HXT9	Carbohydrate metabolism	Hexose transporter	777	*ALR2*	Small molecule transporter	Probable Mg(2+) transporter	1139
YKR104W	Cell stress	'Putative transporter of the multidrug resistance-associated protein (MRP) subfamily	427	*YFL052W*	Transcription factor	Putative zinc cluster protein	1078
FLO10	Cell wall maintenance	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	634
PCS60	RNA processing	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	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	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 maintenance	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	One of three repressible acid phosphatases	40

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).

Cell wall maintenance has also been reported to be a major reaction of cells toward 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 4 and 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

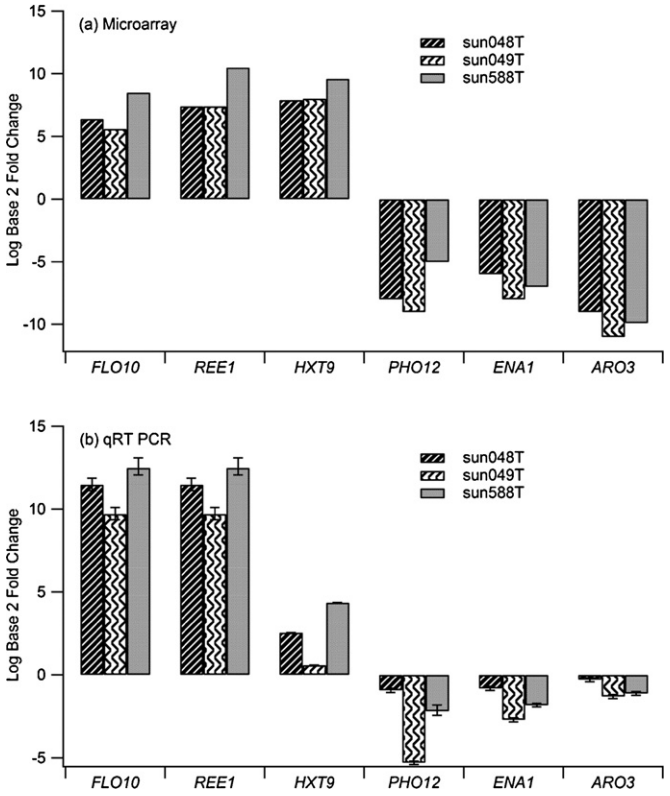

Fig. 4. Validation of microarray data using qRT-PCR for sun048T, sun049T and sun588T normalized to sun473T. *ACT1* was used as an internal standard.

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. Fig. 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.

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 (Supplementary data). 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. *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), sun048T (+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 sun588T (+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.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2012.10.017.

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).

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 toward 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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