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Full Length Research Paper

Transcriptome analysis of *Saccharomyces cerevisiae* at the late stage of very high gravity (VHG) fermentation

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DNA microarray analysis was used to investigate the expression profile of *Saccharomyces cerevisiae* genes in glycolysis pathway, trehalose and steroid biosynthesis and heat shock proteins (HSP) in response to harsh environment under the late stage of very high gravity (VHG) fermentation. The data show that only a few genes (*GPM2*, *PGM1*, *GAL10* and *PGM1*) involved in glycolysis pathway and trehalose biosynthesis were up-regulated. Five genes that encode heat shock proteins (*HSP26*, *HSP10*, *HSP42*, *HSP78* and *HSP82*) were up-regulated. Among these five genes, there was a strong expression increase of about 84-fold for *HSP26.* The results of this study revealed adverse VHG fermentation conditions stress response pattern and suggested interesting information about the mechanisms involved in adaptation of cells to the complex VHG fermentation environment. This identification of genes provides information that will help to genetically modify yeast to further maintain the fermentation fitness and improve its fermentation capacity and process.

Key words: DNA microarray, gene expression, yeast, stress, very high gravity (VHG) fermentation.

INTRODUCTION

Fuel ethanol, the most common renewable liquid fuel from biomass resources conversion, is now a global energy commodity that is competitive with fossil fuels. High ethanol concentration and productivity have been continuously pursued goals in the fuel ethanol industry. However, most conventional ethanol fermentation processes are carried out with low carbohydrate concentration (usually less than 200 g L^{-1} of glucose) to prevent either glucose or ethanol stresses on yeast cells (Pham and Wright, 2007). As a result, final ethanol concentration is low at 6 to 10% (v/v), resulting in high energy inputs for the downstream distillation and waste distillage treatment (Pham and Wright, 2007). Among many ethanol fermentation technologies, one attractive approach that can significantly change industrial ethanol production is very high gravity (VHG) fermentation technology (Puligundla et al., 2011).

VHG fermentation technology is defined as using the medium containing sugar in excess of 250 g L^{-1} in order to achieve more than 15% (v/v) ethanol production (Thomas et al., 1996; Bai et al., 2008). Compared with conventional ethanol fermentation processes, the VHG fermentation has several distinct advantages such as the increase of ethanol concentration and process productivity, reduced capital costs, energy costs and the risk of bacterial contamination (Laopaiboon et al., 2009). Thus, VHG fermentation has been gradually applied by the ethanol industry to reduce costs (Theerarattananoon et al., 2008). However, it is frequently accompanied with stuck or sluggish characteristics near the end of fermentation process. Ethanol production is tightly coupled with yeast cell growth. In most cases, synergistic effects of environmental or physiological stress imposed upon the yeast cells are responsible for the stuck and sluggish fermentation. However, each type of problematic fermentation is associated with different types of stress for the yeast (Bisson and Butzke, 2000). The occurrence

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Abbreviations: VHG, Very high gravity; **HSP,** heat shock proteins.

of stuck and sluggish fermentations continues to be a challenge to the global ethanol industry.

Yeast strains of *Saccharomyces cerevisiae*, widely used in baking, brewing and wine making industries, are also used and extensively studied for fuel ethanol production. During VHG fermentation, yeasts are confronted with adverse environmental stresses such as high osmotic potential and high concentration of ethanol, which will impair yeast cells growth and viability and finally reduce its fermentation capacity (Puligundla et al., 2010). So, the capability of cells to tolerate multiple stresses is one of the important criteria for selecting industrial yeasts for efficient ethanol fermentation. Until now, many studies have been focused on yeast stress tolerance (Lindquist, 1992; Attfield, 1997; Estruch, 2000; Cakar et al., 2005; Gibson et al., 2007), but the molecular response mechanisms for stress and the correlation between ethanol production and stress response are very complicated and still remain elusive. After the completion of the genome sequence of *S. cerevisiae*, DNA microarrays were applied to investigate the intracellular state and characterize transcriptional responses to environmental stress during fermentation processes (Alexandre et al., 2001; Dinh et al., 2009; Hirasawa et al., 2007; Marks et al., 2008; James et al., 2003; Rossignol et al., 2003), but systematic stress response analysis for *S. cerevisiae* during VHG fermentation has been seldom studied (Devantier et al., 2005). Research on gene transcription of *S. cerevisiae* during VHG fermentation can not only improve our understanding for yeast in response to VHG fermentation, but also provide some clues for breeding of stress tolerant yeast strains.

Therefore, in this study, we employed yeast DNA microarray analysis to investigate and compare the transcriptional profile of *S. cerevisiae* near the end of fermentation under VHG and conventional ethanol fermentation. The functional analysis of specific genes identified in this study may help to provide important clues about the mechanisms involved in the adaption, survival, and maintenance of fermentation capacity under adverse VHG environmental conditions.

MATERIALS AND METHODS

Yeast strain and growth conditions

S. cerevisiae (CCTCC M206111) isolated from wine lees was used for this study. The strain was maintained on YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar). YPD medium (1% yeast extract, 2% peptone, 2% glucose) was used for pre-cultivation at 30°C with aeration and agitation (200 rpm). When it reached exponential growth phase (cell dry weight 3.0 to 3.5 g L^{-1}), the culture was used to inoculate fermentation medium with 10% (v/v).

Mini-scale fed-batch VHG fermentation

Ethanol fermentation medium is prepared as follows (all compounds are expressed in g L^{-1}): yeast extract, 5.0; peptone 5.0; $(NH_4)_2SO_4$, 1.5; KH_2PO_4 1.5; $MgSO_4.7H_2O$, 0.65; $CaCl_2$ 2.8; inositol 0.85; thiamin 0.35; pyridoxine 0.004; nicotinic acid 0.004; para-aminobenzoic acid 0.007; biotin 0.000024; and pantothenic acid 3.5 ml. Glucose was added at a desired level. Mini-scale fed-batch VHG fermentation was conducted in a 1L jar fermentor containing 240 ml starting medium with 150 g L^{-1} glucose. After inoculation with seed culture, cultivation was kept at 30°C and the stirring rate was set at 150 rpm. The fermentor was connected to a constant flow pump. Between 8 and 24 h in cultivation, 160 ml medium of the fed-batch phase containing 525 g L^{-1} glucose at a dilution rate of 0.01 L h^{-1} was fed in the jar fermentor. The overall glucose concentration of starting medium and fed-batch phase medium was 300 g L^{-1}. Meanwhile, the low gravity (160 g L^{-1} glucose) batch fermentation was carried out at 30°C and 150 rpm as control. Experiment was done in triplicates.

Fermentation monitoring

The growth of *S. cerevisiae* M 206111 was monitored by measuring the optical density at 620 nm (OD_{620}) by using spectrophotometer. Cell dry weight determination was performed by filtering a known volume of culture samples on 0.45 µm pore size polyamide membranes and drying at 105°C until constant weight. Cell viability was determined by the methylene blue staining method (Lee et al., 1981).

Fermentation processes were monitored by measuring the concentrations of residual sugar and ethanol in samples taken from fermentation broth at different time points. Ethanol concentrations were determined using a gas chromatograph (FULI 9770, China) equipped with a GDX103 packed column (FULI Corp, China), a flame ionization detector and a computing integrator system at 95°C column temperature, 150°C injection temperature and 150°C detector temperature. Before injection, the culture samples were centrifuged at 4000 g for 5 min to remove solid particles, then, propanol (2% v/v, final concentration) was added as an internal standard for determination (Antony, 1984). Residual sugar concentration was determined by 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959).

RNA isolation

For microarray analysis, yeast cells were harvested from fermentors at the late stage (48 h) of the VHG fermentation process by centrifugation, immediately frozen in liquid nitrogen, and stored at -80°C until the preparation of total RNA samples. Total RNA was extracted from yeast cells by using the hot-phenol method (Köhrer and Domdey, 1991). The quantity and quality of the extracted RNA were determined by measuring absorbance at 260 nm (A_{260}) and calculating the ratio of A_{260} to A_{280}. Then, total RNA was used to produce cy5/cy3-labeled cDNA as described in the Affymetrix GeneChip Expression Analysis technical manual (www.affymetrix.com) and prepared for microarray construction and analysis. Meanwhile, total RNA which was obtained from yeast cells at the end of (15 h) low gravity batch fermentation was taken as the control sample.

Microarray hybridization and scanning

RNA labeling and microarray hybridization, washing, staining and scanning were performed at CapitalBio Corp (Beijing, China). Biotin-labeled cDNA was hybridized to the Affymetrix GeneChip® Yeast Genome 2.0 Array after dilution and fragmentation according to the Affymetrix protocol. Expressed genes were scanned using GeneChip® Scanner 3000 (both from CapitalBio Corp, China).

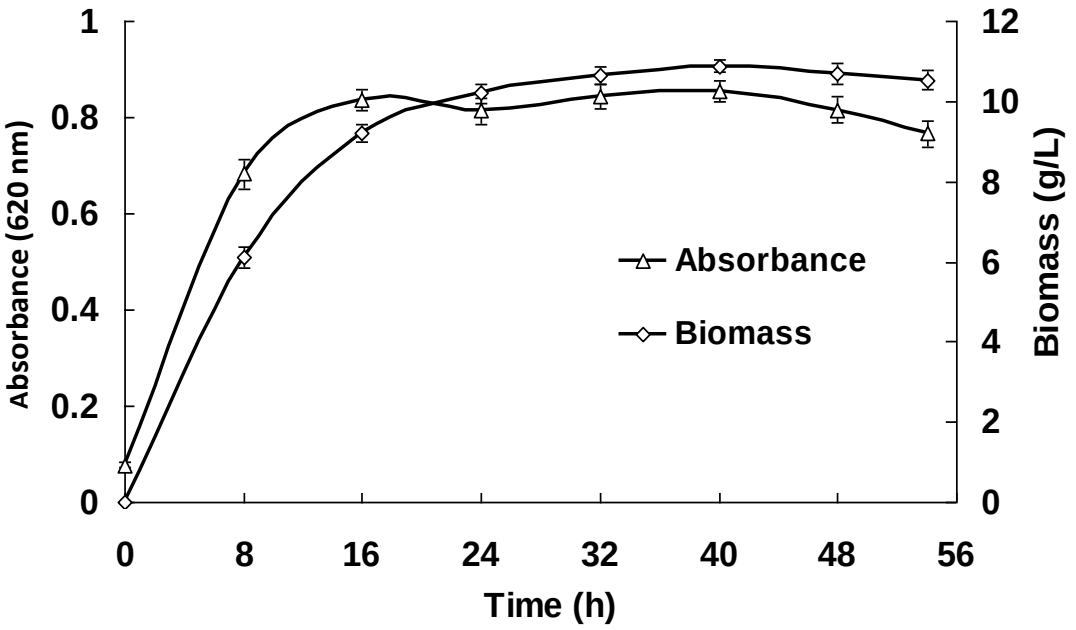

Figure 1. Growth curves of *S. cerevisiae* during fed-batch very high gravity fermentation. Data represent the average of duplicate fermentations.

Microarray data analysis

Microarray data were processed and extracted with Affymetrix GeneChip Operating Software Version 1.4 (GCOS) (CapitalBio Corp, China). To identify genes that are differentially expressed in VHG fermentation samples compared to the control samples, fold change analysis was performed in which the ratios of geometric means of the expression intensities of the corresponding genes in VHG fermentation samples relative to control samples were calculated. Genes whose ratio changed > 2-fold with $q < 0.05$ were determined to have significantly different gene expression. The ratios were reported as the up- or down-fold change. Gene descriptions and annotations were found in the *Saccharomyces* Genome Database (SGD; www.yeastgenome.org).

To eliminate the biological variance, three biologically independent experiments of each of the samples were analyzed. Only genes having consistently altered expression in two independent experiments were selected for further analysis.

RESULTS AND DISCUSSION

VHG fermentation profiles

In order to avoid the damage of high sugar osmotic stress exerted on *S. cerevisiae* cells at the beginning of VHG fermentation, fed-batch experiment was adopted in VHG fermentation process as described earlier.

To clarify the growth characteristic of *S. cerevisiae* during VHG fermentation, the growth was monitored by measuring OD_{620} and cell dry weight (Figure 1). Effect of yeast fermentation ability was determined by the comparison of changes in glucose and ethanol concentration in the broth (Figure 2). Maximum glucose concentration was observed at fermentation, initially of about 120 g L^{-1}, and from 8 to 24 h; glucose concentrations did not exceed 110 g L^{-1} which was well below the level of substrate inhibition as described above 150 g L^{-1} (Erdei et al., 2010).

In the first 16 h (logarithmic phase), a great increase in ethanol formation in the broth was detected, accompanied by the *S. cerevisiae* cells growing quickly, and during which more than half of the ethanol was produced. Then, it showed a significant productivity decline to the fermentation end. This phenomenon could be caused by the growth, and fermentation progression was hampered by a decline in yeast viability and fermentation capacity of *S. cerevisiae* which appeared to be a consequence of osmotic pressure, ethanol stress and other metabolic inhibitors accumulation in broth. At 48 h, the maximum ethanol concentration reached 137 g L^{-1}, after which fermentation ended with the residual glucose at approximately 4.71 g L^{-1} and the volumetric productivity at approximately 2.54 g L^{-1} h^{-1}. After 48 h cultivation, yeast cells were at a decline phase and had suffered a series of multiple stresses. Also at 48 h, the cell viability was determined as 32 ± 0.3%. So, at this point, in order to better understand the metabolic response of *S. cerevisiae*, gene transcriptional profiles using microarray were carried out.

The low gravity (160 g L^{-1}) batch fermentation was also analyzed. After 15 h fermentation, the ethanol concentration reached 70 g L^{-1} and did not increase, while the residual glucose concentration was 15 g L^{-1}. Meanwhile, cell viability was determined as 88 ± 0.2%. So, at the end of fermentation (15 h), total RNA of the yeast cells was extracted as control sample for microarray analysis.

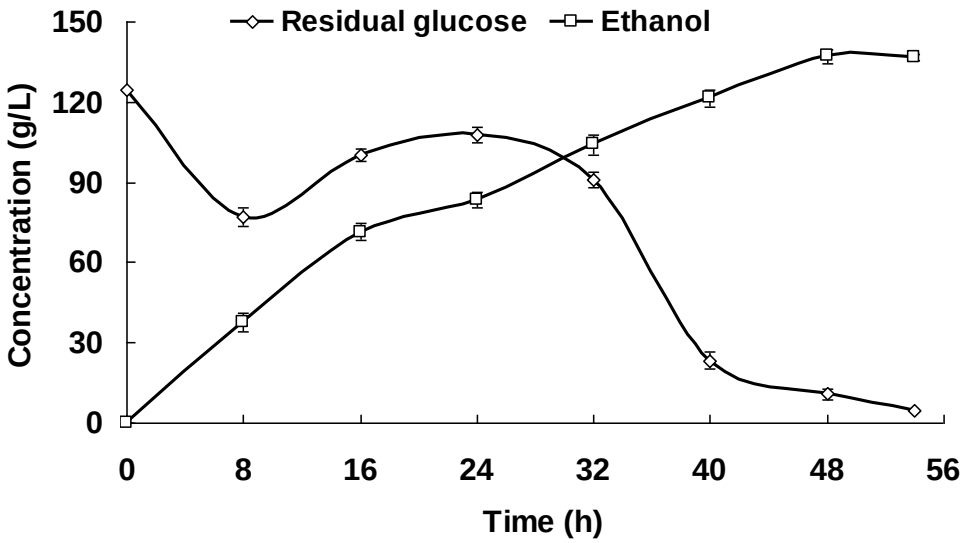

Figure 2. The concentration of ethanol and residual glucose in the broths during fed-batch very high gravity fermentation. Data represent the average of duplicate fermentations.

Microarray analysis

The gene transcriptional profiles of *S. cerevisiae* cells at a single time point of the late stage of VHG fermentation was analyzed by whole genome microarray analysis. Among the 5841 known or predicted genes identified at the time of our analysis, approximately 2265 genes were affected, of these genes, 816 genes were ≥2 fold up-regulated and 1449 were ≥2 fold down-regulated. Genes were distributed in 94 pathways (metabolism; genetic information processing; environmental information processing; cellular processes) according to the KEGG pathway database (http://www.genome.jp/kegg/pathway.html). In the large amount of data generated by microarray analysis, special attention was focused on the genes involved in ethanol generation and stress tolerance to ethanol which was dominant at the end of VHG fermentation.

Glycolysis/gluconeogenesis

The glycolysis pathway is critical for ethanol production. The transcriptome analysis results revealed that in general, expression levels for 16 glycolysis/ gluconeogenesis genes were down-regulated and 3 genes (*GPM2*, *PGM1* and *GAL10*) were up-regulated more than 2-fold compared to the control.

The gene *GPM2* related to gluconeogenesis was up-regulated at the late stage of VHG fermentation. Similar results have been observed for high hydrostatic pressure and ethanol stress, but the function of this gene is largely unknown (Fernandes et al., 2004; Ma and Liu, 2010). The up-regulation of *PGM1* and *GAL10* were the first observed during VHG fermentation. The gene *PGM1* encodes phosphoglucomutase and its function is involved in the metabolism of glycogen and trehalose. It has been reported that *PGM1* and *PGM2* double null mutants accumulated lower levels of trehalose in *S. cerevisiae* than wild type (Boles et al., 1994). Trehalose is known for its important role in protecting cell membrane against harsh environment stresses (Sharma, 1997; Bandara et al., 2009), therefore, the up-regulation of *PGM1* suggests that it may be considered as multiple stress-tolerance related gene. *GAL10* encodes a bifunctional enzyme with mutarotase and UDP galactose 4-epimerase activities (Majumdar et al., 2004). Until now, there was no additional information in literature on the high expression of *GAL10* correlated with the yeast adaptation of adverse environment. This is the first report that the *GAL10* correlated with the VHG fermentation and it indicated that the high expression of *GAL10* may be involved in response to adverse environment. Therefore, in further studies, it would be interesting to examine whether *GAL10* expression correlates to the adverse environment response.

Pyruvate decarboxylases and alcohol dehydrogenases play important roles in the conversion of pyruvate to ethanol as a terminal product of glycolysis. Genes *PDC5*, *PDC6*, *ADH2* and *ADH4*, encoding pyruvate decarboxylases and alcohol dehydrogenases respectively exhibited significant down-regulation at the late stages of VHG fermentation. On the contrary, at early time of VHG fermentation, as observed by Rautio et al. (2007), the *ADH4* expression levels increased and led to a decrease in residual glucose concentration and an increase in ethanol concentration. Similarly, the expression of two genes specifying glucose kinase (*HXK1* and *HXK2*) and *PYK2* encoding for pyruvate kinase were also found expressed at lower levels (Table 1). Meanwhile, acetyl-

Table 1. Genes of glycolysis/gluconeogenesis induced at the late of very high gravity (VHG) fermentation.

Open reading frame (ORF) code	Gene name	Gene description	Induction fold
Up-regulated			
YBR019c	*GAL10*	UDP-glucose 4-epimerase	6.25
YDL021w	*GPM2*	Non-functional derivative of phosphoglycerate mutase	2.45
YKL127w	*PGM1*	Phosphoglucomutase, minor isoform	3.59
Down-regulated			
YMR303c	*ADH2*	Alcohol dehydrogenase II	−6.25
YGL256w	*ADH4*	Alcohol dehydrogenase IV	−5.26
YMR169c	*ALD3*	Stress inducible aldehyde dehydrogenase	−2.00
YOR374w	*ALD4*	Aldehyde dehydrogenase, mitochondrial	−3.45
YER073w	*ALD5*	Aldehyde dehydrogenase (NAD+), mitochondrial	−2.50
YPL061w	*ALD6*	Aldehyde dehydrogenase, cytosolic	−7.14
YLR377c	*FBP1*	Fructose-1,6-bisphosphatase	−10.00
YOR347c	*PYK2*	Pyruvate kinase, glucose-repressed isoform	−5.88
YER178w	*PDA1*	Pyruvate dehydrogenase alpha chain precursor	−2.44
YBR221c	*PDB1*	Pyruvate dehydrogenase beta chain precursor	−2.33
YLR134w	*PDC5*	Pyruvate decarboxylase, isozyme 2	−2.94
YGR087c	*PDC6*	Pyruvate decarboxylase 3	−4.34
YNL071w	*LAT1*	Dihydrolipoamide S-acetyltransferase	−2.33
YFR053c	*HXK1*	Hexokinase I	−2.04
YGL253w	*HXK2*	Hexokinase II	−2.00
YLR153c	*ACS2*	Acetyl-coenzyme A synthetase	−11.11

coenzyme gene *ACS2*, an essential gene for yeast growth (Van Den Berg and Steensma, 1995) also revealed a down-regulation of 11-fold. These down regulated genes in glycolysis indicated that yeast respond to adverse environment of VGH fermentation by adjusting their metabolic activities to avoid viability loss and becoming adapted to stressful conditions.

Genes involved in heat shock protein (HSP)

The environment of VHG fermentation is not constant but continuously changing. To cope with adverse effects of stress, *S. cerevisiae* developed rapid molecular response to repair the damage and protect against further detriment by stress. Among them, heat shock proteins (HSPs) mainly acting as molecular chaperones or facilitating the degradation of proteins denatured by stress protect proteins from the deleterious stresses (Piper, 1997).

The expression patterns of some HSPs at the late stages of VHG fermentation are specified in Table 2. Five genes that encode for HSPs, *HSP26*, *HSP10*, *HSP42*, *HSP78* and *HSP82*, were up-regulated. *HSP26*, *HSP10* and *HSP42* are small HSPs which have important functions in thermostability, disaggregation, and proteolysis inhibition (Han et al., 2008). Similarly, Devantier et al. (2005) showed that *HSP26* and *HSP42* were also up-regulated in a laboratory strain and an industrial strain of *S. cerevisiae* under high substrate concentration (280 g L^{-1} maltodextrin initially) fermentation. For the HSP70 family, *FES1*, *SSA2*, *SSA3*, *SSA4* and *SSE1* were more highly expressed. *SSA2*, *SSA3* and *SSA4* encode the cytosolic *HSP70*, form an essential group for cells normal growth and are directly related to thermotolerance (Craig et al., 1994). Their increased expression levels in adverse conditions suggest that they play important roles in combined stress tolerance in VHG fermentation.

HSP26, the most strongly induced gene, is the principal small HSP of *S. cerevisiae*, which encodes a cytoplasmic protein involved in response to several forms of stress. Its high induction level was also observed in the following stress conditions: ethanol stress, high hydrostatic pressure, osmotic stress, low pH medium, high-nitrogen culture and in sake brewing and wine fermentation (Alexandre et al., 2001; Fernandes et al., 2004; Erasmus et al., 2003; Kawahata et al., 2006; Backhus et al., 2001; Wu et al., 2006; Marks et al., 2008). Its expression is regulated under stress conditions by the Msn2/4p, Hsf1p and Gis1p transcription factors, with particular traits depending on the kind of stress affecting yeast cells (Amorós and Estruch, 2001). In *S. cerevisiae*, the deletion of *HSP26* causes an abnormal cell shape and accumulation of protein aggregates under stress

Table 2. Genes involved in heat shock protein of *S. cerevisiae* at the late of VHG fermentation.

ORF code	Gene name	Gene description	Induction fold
Up-regulated			
YBR072w	*HSP26*	Heat shock protein	84.38
YBR101c	*FES1*	Hsp70 nucleotide exchange factor	4.74
YLL024c	*SSA2*	Heat shock protein of HSP70 family, cytosolic	3.81
YER103w	*SSA4*	Heat shock protein of HSP70 family, cytosolic	4.61
YDR258c	*HSP78*	Heat shock protein of clpb family of ATP-dependent proteases, mitochondrial	3.19
YBL075c	*SSA3*	Heat shock protein of HSP70 family, cytosolic	3.44
YNL281w	*HCH1*	ZHeat shock protein regulator that binds to Hsp90p and may stimulate ATPase activity	3.57
YDR214w	*AHA1*	Stress-regulated cochaperone	3.04
YMR186w	*HSC82*	Heat shock protein	2.18
YPL240c	*HSP82*	Heat shock protein	2.52
YNL007c	*SIS1*	Heat shock protein	2.16
YOR020c	*HSP10*	Chaperonin, mitochondrial	2.01
YPL106c	*SSE1*	Heat shock protein of HSP70 family	2.25
YOR027w	*STI1*	Stress-induced protein	2.13
YDR171w	*HSP42*	Heat shock protein	2.42
YNL064c	*YDJ1*	Mitochondrial and ER import protein	2.12
Down-regulated			
YNL310c	*ZIM17*	ZInc finger Motif protein, localized to the mitochondria	−3.13
YHR064c	*SSZ1*	Regulator protein	−2.56
YDL229w	*SSB1*	Heat shock protein of HSP70 family	−2.70
YNL209w	*SSB2*	Heat shock protein of HSP70 family, cytosolic	−2.50

conditions, but does not affect thermotolerance (Franzmann et al., 2008). Also, the overexpression of *HSP26* showed that the viability under particular stress conditions was not improved (Jiménez-Martí et al., 2009). Moreover, some HSP proteins work together as partners with co-chaperons or co- factors to resist adverse conditions, thus, the manipulation of one special gene was not effective for improving the stress resistance phenotype of yeast cells. It has been demonstrated that *HSP26* functionally interacts with the molecular chaperone *HSP104* and deletion of both *HSP26* and *HSP104* from yeast cells reduces the survival rate after a severe heat shock 5-fold (Cashikar et al., 2005). Similarly, the molecular chaperon *HSP78*, a member of the Clp/Hsp100 family localized in the mitochondria of *S. cerevisiae* can cooperate with *HSP70* in refolding protein and in maintaining mitochondrial functions under heat stress (Krzewska et al., 2001). The synergistic effect was also found in *HSP10* whose function is strictly related to another mitochondrial chaperone *HSP60*. Consequently, it is likely that these up-regulated proteins may play a role in protecting yeast cells, either independently or in conjunction with other protective agents when facing adverse conditions. However, the connections between HSPs and their function with stress tolerance remain elusive.

Genes involved in trehalose and steroid

Table 3 shows changed genes (≥2 fold) involved in biosynthesis of trehalose and steroids. Trehalose levels have been correlated with cell survival under adverse conditions. The protective role of trehalose is an obvious interpretation of its production in response to stress. It is well known that trehalose accumulates in yeast cells under nutrient starvation, heat stress and ethanol stress (Parrou et al., 1999; Sharma, 1997; Bandara et al., 2009). However, trehalose metabolism genes, except *PGM1*, were mostly neither induced nor repressed at the end of VHG fermentation.

It was demonstrated that sterols were important for cell membrane protection (Bloch, 1983). Ergosterol, the main sterol in yeast, is an essential component of yeast plasma membranes which affects membrane fluidity, permeability and activity of membrane-bound enzymes (Parks and Casey, 1995). Previous studies have shown that sterol biosynthesis is an important process to ensure cell viability during brewing fermentation and ergosterol plays an important role in ethanol tolerance and thermotolerance (James et al., 2003; Fernandes et al., 2004). Most notably, ten genes in steroid biosynthesis were down-regulated under VHG fermentation (Table 3).

Table 3. Gene folder change in the biosynthesis of steroid and trehalose.

ORF code	Gene name	Gene description	Induction fold
Steroid biosynthesis			
Down-regulated			
YPL117c	*IDI1*	Isopentenyl-diphosphate delta-isomerase	−3.13
YMR220w	*ERG8*	Phosphomevalonate kinase	−3.57
YGR175c	*ERG1*	Squalene monooxygenase	−4.55
YHR190w	*ERG9*	Farnesyl-diphosphate farnesyltransferase	−2.04
YHR072w	*ERG7*	Lanosterol synthase	−11.11
YNR043w	*MVD1*	Mevalonate pyrophosphate decarboxylase	−5.00
YML075c	*HMG1*	3-hydroxy-3-methylglutaryl-coenzyme A reductase 1	−2.27
YLR450w	*HMG2*	3-hydroxy-3-methylglutaryl-coenzyme A reductase 2	−3.45
YJL167w	*ERG20*	Farnesyl-pyrophosphate synthetase	−5.00
YMR208w	*ERG12*	Mevalonate kinase	−2.63
Trehalose biosynthesis			
Up-regulated			
YKL127W	*PGM1*	Phosphoglucomutase, minor isoform	3.59

Taken together, the up-regulated or down-regulated genes under the given fermentation conditions are recognized as the responsible genes adaptation to the adverse environmental stress. The microarray analysis suggested that the highly repressed genes (*ERG7*, *ERG20*, *ERG1* and *ERG8*) which were corresponded to adverse conditions for yeast survival and may lead the viability of yeast cells, sharply reduced at the end of VHG fermentation. With fermentation processing, yeasts, at high cell densities are faced with high concentrations of ethanol and nutrients starvation, where cell populations survive in mostly metabolic non proliferation state. As shown in Figure 1, the yeast cells concentration change was minimal during late fermentation process (Figure 1). In adverse environment, cells are aging or declining and probably changing their metabolic state which may result in the down-regulation of most trehalose and steroid biosythesis genes. For most genes down-regulation, these results may impair yeast cells activities and substrate assimilation and result in problematic fermentation and yeast could not be used for recycling into subsequent fermentations.

Conclusions

This study investigates the gene expression changes in *S. cerevisiae* at the late stage of VHG fermentation using DNA microarray. The data show genes whose expression was significantly up-regulated or down-regulated (≥2-fold) in glycolysis pathway, trehalose, steroid and heat shock proteins synthesis. For example, we have shown that only a few genes involved in glycolysis pathway and trehalose synthesis are up-regulated and most genes involved in steroid biosynthesis are down-regulated. Similarly, we demonstrated that a whole set of HSP genes expression are induced.

The induced HSP genes and the role of their products in protecting cells against the damage caused by multiple stressful conditions are interesting aspects in the study. However, the relationship between the induction HSPs and the acquisition of stress tolerance is still a controversial subject (Estruch, 2000). To further research such problems in VHG fermentation, our future study will focus on the synergistic effect of induction of HSPs with stress tolerance.

Therefore, the results of DNA microarray analysis provide a deeper understanding of stress defense molecular mechanisms of *S. cerevisiae* to withstand multiple stressful conditions of VHG, and can be applied to improve the resistance of industrial yeast strains.

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REFERENCES

Alexandre H, Ansanay-Galeote V, Dequin S, Blondin B (2001). Global gene expression during short-term ethanol stress in *Saccharomyces cerevisiae*. FEBS Lett. 498: 98-103.

Amorós M, Estruch F (2001). Hsf1p and Msn2/4p cooperate in the expression of *Saccharomyces cerevisiae* genes HSP26 and HSP104 in a gene- and stress type-dependent manner. Mol. Microbiol. 39: 1523-1532.

Antony JC (1984). Malt beverages and malt brewing materials: Gas chromatographic determination of Ethanol in beer. J. Assoc. Annal. Chem. 67: 192-193.
Attfield PV (1997). Stress tolerance: the key to effective strains of industrial baker's yeast. Nat. Biotechnol. 15: 1351-1357.
Backhus LE, DeRisi J, Brown PO, Bisson LF (2001). Functional genomic analysis of a commercial wine strain of *Saccharomyces cerevisiae* under differing nitrogen conditions. FEMS Yeast Res. 1: 111-125.
Bai FW, Anderson WA, Moo-Young M (2008). Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnol. Adv. 26: 89-105.
Bandara A, Fraser S, Chambers PJ, Stanley GA (2009). Trehalose promotes the survival of *Saccharomyces cerevisiae* during lethal ethanol stress, but does not influence growth under sublethal ethanol stress. FEMS Yeast Res. 9: 1208-1216.
Bisson LF, Butzke CE (2000). Diagnosis and Rectification of Stuck and Sluggish Fermentations. Am. J. Enol. Viticult. 51: 168-177.
Bloch KE (1983). Sterol structure and membrane function. CRC Crit. Rev. Biochem. 14: 47-92.
Boles E, Liebetrau W, Hofmann M, Zimmermann FK (1994). A family of hexosephosphate mutases in *Saccharomyces cerevisiae*. Eur. J. Biochem. 220: 83-96.
Cakar ZP, Seker UO, Tamerler C, Sonderegger M, Sauer U (2005). Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae*. FEMS Yeast Res. 5: 569-578.
Cashikar AG, Duennwald M, Lindquist SL (2005). A chaperone pathway in protein disaggregation. Hsp26 alters the nature of protein aggregates to facilitate reactivation by Hsp104. J. Biol. Chem. 280: 23869-23875.
Craig EA, Baxter BK, Becker J, Halladay J, Zigelhoffer T (1994). Cytosolic hsp70 of *Saccharomyces cerevisiae*: roles in protein synthesis, protein translocation, proteolysis, and regulation. In: Morimoto RI et al., (Eds) The Biology of Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 31-52.
Devantier R, Pedersen S, Olsson L (2005). Transcription analysis of *S. cerevisiae* in VHG fermentation: The genome-wide transcriptional response of *Saccharomyces cerevisiae* during very high gravity ethanol fermentations is highly affected by the stationary phase. Ind. Biotechnol. 1: 51-63.
Dinh TN, Nagahisa K, Yoshikawa K, Hirasawa T, Furusawa C, Shimizu H (2009). Analysis of adaptation to high ethanol concentration in *Saccharomyces cerevisiae* using DNA microarray. Bioproc. Biosyst. Eng. 32: 681-688.
Erasmus DJ, van der Merwe GK, van Vuuren HJ (2003). Genome-wide expression analyses: Metabolic adaptation of *Saccharomyces cerevisiae* to high sugar stress. FEMS Yeast Res. 3: 375-399.
Erdei B, Barta Z, Sipos B, Réczey K, Galbe M, Zacchi1 G (2010). Ethanol production from mixtures of wheat straw and wheat meal. Biotechnol. Biofuels, 3: 16-25.
Estruch F (2000). Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol. Rev. 24: 469-486.
Fernandes PM, Domitrovic T, Kao CM, Kurtenbach E (2004). Genomic expression pattern in *Saccharomyces cerevisiae* cells in response to high hydrostatic pressure. FEBS Lett. 556: 153-160.
Franzmann TM**,** Menhorn P**,** Walter S, Buchner J (2008). Activation of the chaperone Hsp26 is controlled by the rearrangement of its thermosensor domain. Mol. Cell. 29: 207-216.
Gibson BR, Lawrence SJ, Leclaire JP, Powell CD, Smart KA (2007). Yeast responses to stresses associated with industrial brewery handling. FEMS Microbiol. Rev. 31: 535-569.
Han MJ, Yun H, Lee SY (2008). Microbial small heat shock proteins and their use in biotechnology. Biotechnol. Adv. 26: 591-609.
Hirasawa T, Yoshikawa K, Nakakura Y, Nagahisa K, Furusawa C, Katakura Y, Shimizu H, Shioya S (2007). Identification of target genes conferring ethanol stress tolerance to *Saccharomyces cerevisiae* based on DNA microarray data analysis. J. Biotechnol. 131: 34-44.
James TC, Campbell S, Donnelly D, Bond U (2003). Transcription profile of brewery yeast under fermentation conditions. J. Appl. Microbiol. 94: 432-448.
Jiménez-Martí E, Zuzuarregui A, Ridaura I, Lozano N, del Olmo M (2009). Genetic manipulation of HSP26 and YHR087W stress genes may improve fermentative behaviour in wine yeasts under vinification conditions. Int. J. Food Microbiol. 130: 122-130.
Kawahata M, Masaki K, Fujii T, Lefuji H (2006). Yeast genes involved in response to lactic acid and acetic acid: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p. FEMS Yest Res. 6: 924-936.
Köhrer K, Domdey H (1991). Preparation of high molecular weight RNA. Method. Enzymol. 194: 398-405.
Krzewska J, Langer T, Liberek K (2001). Mitochondrial Hsp78, a member of the Clp/Hsp100 family in *Saccharomyces cerevisiae*, cooperates with Hsp70 in protein refolding. FEBS Lett. 489: 92-96.
Laopaiboon L, Nuanpeng S, Srinophakun P, Klanrit P, Laopaiboon P (2009). Ethanol production from sweet sorghum juice using very high gravity technology: Effects of carbon and nitrogen supplementations. Bioresour. Technol. 100: 4176-4182.
Lee SS, Robinson FM, Wang HY (1981) Rapid-determination of yeast viability. Biotechnol. Bioeng. Symp. 11: 641-649.
Lindquist S (1992). Heat-shock proteins and stress tolerance in microorganism. Curr. Opin. Genet. Dev. 2: 748-755.
Majumdar S, Ghatak J, Mukherji S, Bhattacharjee H, Bhaduri A (2004). UDPgalactose 4-epimerase from *Saccharomyces cerevisiae*. A bifunctional enzyme with aldose 1-epimerase activity. Eur. J. Biochem. 271: 753-759.
Ma M, Liu LZ (2010). Quantitative transcription dynamic analysis reveals candidate genes and key regulators for ethanol tolerance in *Saccharomyces cerevisiae*. BMC Microbiol. 10: 169-188.
Marks VD, Ho Sui SJ, Erasmus D, van der Merwe GK, Brumm J, Wasserman WW, Bryan J, van Vuuren HJ (2008). Dynamics of the yeast transcriptome during wine fermentation reveals a novel fermentation stress response. FEMS Yeast Res. 8: 35-52.
Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426-428.
Parks LW, Casey WM (1995). Physiological implications of sterol biosynthesis in yeast. Annu. Rev. Microbiol. 49: 95-116.
Parrou JL, Enjalbert B, Plourde L, Bauche A, Gonzalez B, Francois J (1999). Dynamic responses of reserve carbohydrate metabolism under carbon and nitrogen limitations in *Saccharomyces cerevisiae*. Yeast, 15: 191-203.
Pham TK, Wright PC (2007). Proteomic analysis of calcium alginate-immobilized *Saccharomyces cerevisiae* under high-gravity fermentation conditions. J. Proteome Res. 7: 515-525.
Piper P (1997). The yeast heat shock response. In: Hohmann S, Mager WH (ed) Yeast stress responses. Chapman & Hall, New York, pp 75-99.
Puligundla P, Smogrovicova D, Obulam VS, Ko S (2011). Very high gravity (VHG) ethanolic brewing and fermentation: a research update. J. Ind. Microbiol. Biotechnol. 38: 1133-1144.
Rautio JJ, Huuskonen A, Vuokko H, Vidgren V, Londesborough J (2007). Monitoring yeast physiology during very high gravity wort fermentations by frequent analysis of gene expression. Yeast, 24: 741-760.
Rossignol T, Dulau L, Julien A, Blondin B (2003). Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast, 20: 1369-1385.
Sharma SC (1997). A possible role of trehalose in osmotolerance and ethanol tolerance in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. 152: 11-15.
Theerarattananoon K, Lin YH, Peng DY (2008). Metabolic heat evolution of *Saccharomyces cerevisiae* grown under very-high-gravity conditions. Process Biochem. 43: 1253-1258.
Thomas KC, Hynes SH, Ingledew WM (1996). Practical and theoretical considerations in the production of high concentration of alcohol by fermentation. Process Biochem. 31: 321-331.
Van Den Berg MA, Steensma HY (1995). ACS2, a *Saccharomyces cerevisiae* gene encoding acetyl-coenzyme A synthetase, essential for growth on glucose. Eur. J. Biochem. 231: 704-713.
Wu H, Zheng XH, Araki Y, Sahara H, Takagi H, Shimoi H (2006). Global gene expression analysis of yeast cells during sake brewing. Appl. Environ. Microb. 72: 7353-7358.