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ORIGINAL PAPER

Genome-wide transcriptional analysis of *Saccharomyces cerevisiae* during industrial bioethanol fermentation

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Abstract *Saccharomyces cerevisiae* is widely applied in large-scale industrial bioethanol fermentation; however, little is known about the molecular responses of industrial yeast during large-scale fermentation processes. We investigated the global transcriptional responses of an industrial strain of *S. cerevisiae* during industrial continuous and fed-batch fermentation by oligonucleotide-based microarrays. About 28 and 62% of all genes detected showed differential gene expression during continuous and fed-batch fermentation, respectively. The overrepresented functional categories of differentially expressed genes in continuous fermentation overlapped with those in fed-batch fermentation. Downregulation of glycosylation as well as upregulation of the unfolded protein stress response was observed in both fermentation processes, suggesting dramatic changes of environment in endoplasmic reticulum during industrial fermentation. Genes related to ergosterol synthesis and genes involved in glycogen and trehalose metabolism were downregulated in both fermentation processes. Additionally, changes in the transcription of genes involved in carbohydrate metabolism coincided with the responses to glucose limitation during the early main fermentation stage in both processes. We also found that during the late main fermentation stage, yeast cells exhibited similar but stronger transcriptional changes during the fed-batch process than during the continuous process. Furthermore, repression of glycosylation has been suggested to be a secondary stress in the model proposed to explain the transcriptional responses of yeast during industrial fermentation. Together, these findings provide insights into yeast performance during industrial fermentation processes for bioethanol production.

Keywords Bioethanol · Transcriptome · Continuous fermentation · Fed-batch fermentation · *Saccharomyces cerevisiae*

Introduction

Saccharomyces cerevisiae is widely applied in industrial fermentation for bioethanol production [3]. Yeast cells used for industrial fermentation are subject to wide variations in diverse environmental factors, such as sugar concentration, ethanol concentration, nitrogen resource, pH, and osmotic pressure [12]. Systems for biology technologies have been developed to enable global characterization of molecular responses to such complex environmental variations at the level of the proteome [5, 18], the metabolome [10], and the lipidome [13, 41]. These technologies also can be used to elucidate yeast responses to environmental variations that occur during industrial fermentation for ethanol production. Proteomic studies revealed that multiple proteins involved in glycolysis and gluconeogenesis, stress response, and amino acid metabolism were more or less abundant during industrial ethanol fermentation [4, 14]. Metabolomic research has revealed intracellular changes in metabolites during continuous and fed-batch industrial fermentation processes [8]. However, limited information about the transcriptomic response of yeast during industrial bioethanol fermentation is available. Characterizing transcriptional responses of

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yeast during the industrial process might provide information useful for improving industrial fermentation.

Genome-wide transcriptional analysis has been employed to investigate yeast responses to a variety of stresses that arise during fermentation, such as glucose and ammonia limitations [17], salt stress [21], nitrogen concentration [22], unfolded protein stress [28], alterations in growth temperature [29], ethanol exposure, and hypoxia [36]. Most of these studies have focused on the response of yeast to one or several specific stresses; however, the impact of the combination of these stresses, such as occurs during industrial fermentation, is likely to be far more complex [12]. Microarray analysis of brewer's yeast subjected to batch fermentation in a 3-l fermentor has been carried out [16], and Varela et al. quantified gene expression profiles of industrial yeast under winemaking conditions in a 50-l fermentor [39]. Marks et al. used genome-wide expression analysis to study responses of yeast to stresses that occur during wine fermentation with a 1-l working volume [19]. However, these studies were conducted on a small scale and/or in well-controlled fermentors. Studies performed under standard laboratory conditions are inadequate to reveal the mechanisms of metabolic and regulatory changes that occur during industrial fermentation processes [19]. In industrial bioethanol fermentation, large-volume fermentors are used to achieve more economic benefit, and the complexity of full-scale fermentation cannot be replicated in small-scale wort fermentations [11]. The genome-wide transcriptional response of lager yeast during full-scale batch brewery fermentation has been reported [11], but no transcriptional data derived during continuous industrial fermentation have been reported.

The aim of this study was to investigate the global transcriptional changes of yeast during industrial bioethanol fermentation. The transcriptome of active dry yeast was monitored during industrial continuous and fed-batch fermentation processes for bioethanol production using oligonucleotide-based microarrays. The results indicate that the transcription of genes involved in a variety of functions was reprogrammed during industrial bioethanol fermentation. Based on the data collected herein, we propose a model for the transcriptional changes that take place during industrial fermentation. These results expand our understanding of industrial-strain response to the industrial bioethanol fermentation process.

Materials and methods

Strains

Angel® thermal-tolerant alcohol active dry yeast (product no. 80000012; Angel Yeast Co., Ltd.; Hubei, China), *S. cerevisiae*, was used for all experiments.

Fermentation conditions

Bioethanol fermentation was carried out on an industrial scale using continuous and fed-batch processes (Fig. 1). Conditions of fermentation processes were as described previously [4]. For the continuous fermentation system, 11 steel-tank fermentors in series were employed, each with a working volume of 320,000 l. The first fermentor was designed for cell growth with aeration (10 m^3/min), whereas the others were maintained under anaerobic conditions for ethanol production (Fig. 1a). Each fermentor was fitted with a circulating pump (180 m^3/h) that pumped the broth from the bottom to the top of the fermentor, making conditions throughout the fermentor less heterogeneous. Fed-batch fermentation was carried out in a 480,000-l steel-tank fermentor, and saccharified liquid feedstock was added during the first 18 h (Fig. 1b). Several parameters were the same for both processes: feedstock was composed of 20% grain and 80% maize; commercial active dry yeast was used directly as the inoculum; temperature was maintained between 30 and 35°C; pH and dissolved oxygen were not controlled during the fermentation processes.

Fermentation monitoring

Fermentation processes were monitored by measuring the concentrations of reducing sugar and ethanol in samples taken from different fermentors for continuous fermentation and at different time points for fed-batch fermentation. Ethanol was analyzed using a DB-5MS column (Agilent, Germany) on an SP 3420A gas chromatography instrument (Beifen Ruili, China) equipped with a flame ionization detector; reducing sugar concentration was determined by the dinitrosalicylic acid method [24].

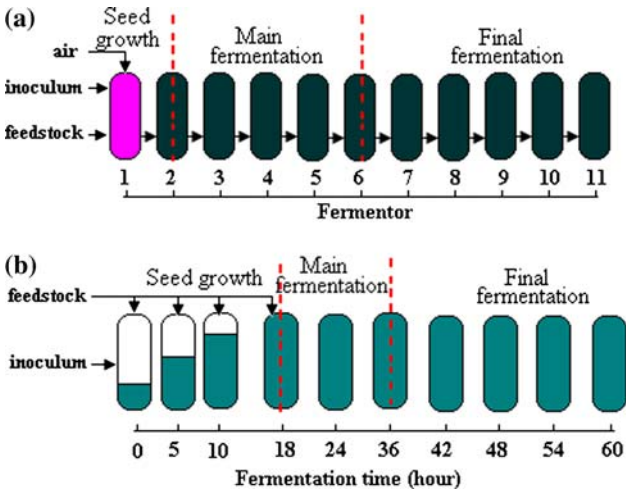

Fig. 1 Continuous (**a**) and fed-batch (**b**) fermentation processes

Experimental design

Based on reducing sugar consumption and ethanol production, we identified seed growth stage, early main fermentation stage, and late main fermentation stage for each fermentation process. To characterize transcriptional changes that occurred during fermentation processes, we chose three sampling points for each fermentation process that would represent the three fermentation stages, respectively. For continuous fermentation, samples taken from the first, third, and fifth fermentors were used for microarray analysis. The first fermentor was used as the control. For fed-batch fermentation, samples taken at 5, 24, and 36 h after inoculation were used for microarray analysis; the 5-h sample served as the control. For continuous fermentation, two individual samples taken from the same system served as replicates; for fed-batch fermentation, samples taken from two different batch fermentations served as the duplicates. Twelve microarrays were used.

Sampling for microarray analysis

For both fermentation processes, samples were taken from the bottom of the circulating pumps in the fermentors. In order to remove the solid feedstock in the broth, 50-ml samples were centrifuged at 800 rpm for 2 min, and then the supernatant was collected and centrifuged at 1,200 rpm for 2 min. Cell pellets were resuspended in 25 ml Milli-Q water, and 1 ml of the cell mixture was transferred to a centrifuge tube and centrifuged at 4,000 rpm for 5 min. The tube with cell pellets was frozen in liquid nitrogen for RNA extraction. All centrifugations were performed at room temperature.

RNA preparation and labeling

Total RNA was isolated using the hot phenol method [34]. The concentration of total RNA was determined by measuring absorbance at 260 nm (A_{260}), and the quality of RNA was determined by calculating the ratio of A_{260} to A_{280} and by agarose gel electrophoresis. Total RNA (tRNA) was labeled as described in the Affymetrix GeneChip Expression Analysis technical manual (www.affymetrix.com). Briefly, tRNA was used to synthesize double-stranded complementary DNA (dscDNA), which was purified and used as the template for synthesis of biotin-labeled complementary (cRNA). The cRNA was purified by QIAGEN RNeasy Total RNA Isolation kit (QIAGEN, USA), and the quality of cRNA was determined by calculating A_{260}:A_{280}.

Hybridization and scanning of microarrays

RNA labeling and microarray hybridization and scanning were performed at Shanghai Biochip Co., Ltd. Biotin-labeled cRNA was hybridized to the GeneChip® Yeast Genome 2.0 Array from Affymetrix (Santa Clara, CA, USA) after dilution and fragmentation according to the Affymetrix protocol (www.affymetrix.com). Microarrays were scanned with the Genechip Scanner 3000 controlled by the GeneChip Operating Software (GCOS) system.

Data analysis

Microarray data were processed in the EXpression ANalyzer and DisplayER (EXPANDER) 4.1 [35]. Data from different microarrays were normalized by quintile method in EXPANDER 4.1, and differentially expressed genes for continuous or fed-batch fermentation process were selected using two criteria: (1) those for which $P < 0.05$ in t tests performed on samples taken at different time points; (2) those that showed more than a two-fold change based on minimal gene expression for at least one time point. All differentially expressed genes in each fermentation process were sorted into four clusters using the k-means method according to gene expression patterns. Functional enrichment of genes in each cluster was explored using EXPANDER 4.1 and MIPS Function Catalogue (http://mips.gsf.de). Information about specific gene functions and biological pathways was obtained from KEGG (http://www.genome.jp/kegg) and the *Saccharomyces* Genome Database (http://www.yeastgenome.org).

Results

Changes in reducing sugar and ethanol concentrations during fermentation processes

Saccharified liquid from starch was used as the feedstock for both continuous and fed-batch fermentation processes. Reducing sugar and ethanol concentrations in the broth were measured to monitor the fermentation process. In continuous fermentation, the third fermentor produced ethanol at the fastest rate. Very little ethanol was produced after the fifth fermentor, indicating the beginning of the final fermentation stage (Fig. 2). In fed-batch fermentation, the fastest stage for ethanol production was at 24 h postinoculation, and the final fermentation stage began after 36 h. Changes in ethanol and reducing sugar concentrations indicated that the third and fifth fermentors of the continuous process represented the same fermentation stage as at 24 h and 36 h postinoculation in the fed-batch process, respectively. At the seed-growth stage in fed-batch fermentation, the ethanol concentration increased slowly (Fig. 2). This could be explained by the addition of feedstock during the first 18 h (Fig. 1), which diluted the concentration of the final fermentation product. Bioethanol

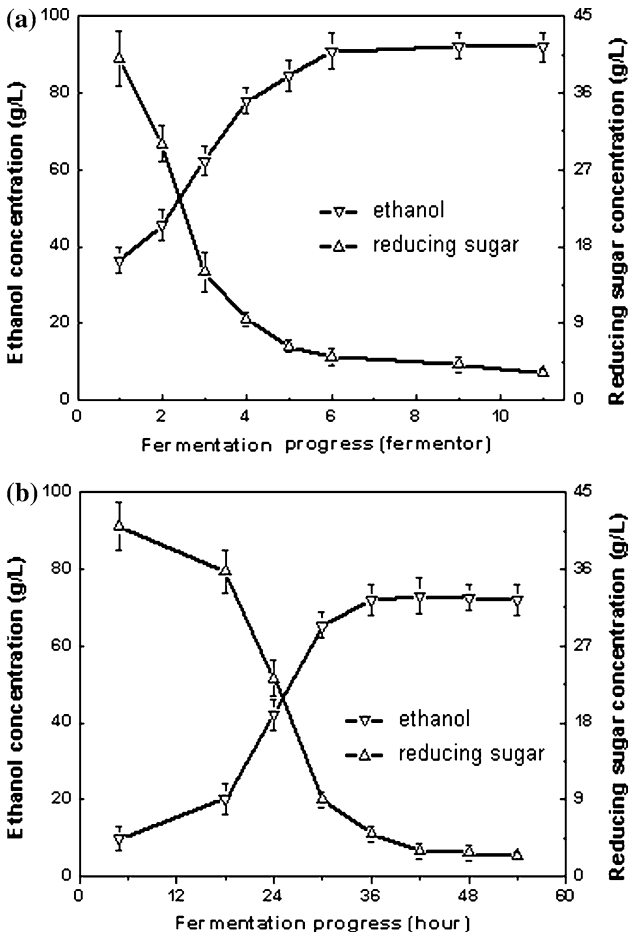

Fig. 2 Changes in ethanol and reducing sugar concentrations during continuous (**a**) and fed-batch (**b**) fermentation. Reducing sugars include mainly glucose, fructose, and maltose

production lasted about 60 h for both processes, but ethanol concentrations at the final stage differed: 91.9 g/l for continuous fermentation and 72.0 g/l for fed-batch fermentation (Fig. 2).

Overview of transcriptional profiles of yeast during continuous and fed-batch industrial fermentation

The industrial ethanol fermentation process can be divided into different fermentation stages according to operation conditions and chemical concentrations (Fig. 1). For microarray experiments, yeast cells were taken from the first, third, and fifth fermentors in continuous fermentation to represent seed fermentation, early main fermentation, and late main fermentation stages, respectively, whereas the corresponding samples in fed-batch fermentation were taken at 5, 24, and 36 h postinoculation. The correlation coefficient of the two replicates for each experimental point exceeded 0.97, except for the 5-h point in fed-batch fermentation (0.92). Additionally, we assessed the microarray platform used here, and microarray data obtained from this platform were consistent with real-time polymerase chain reaction (PCR) results (Fig. S1).

Transcript levels of 1,626 genes [28% of the detected open reading frames (ORFs)] in the yeast subjected to continuous fermentation and 3,621 genes (62% of the detected ORFs) in the yeast subjected to fed-batch fermentation changed significantly. The differentially expressed genes for each fermentation process were sorted into different clusters according to their expression pattern during the fermentation process (Fig. 3). Because establishing a greater number of clusters would favor defining more specific expression patterns and having more genes in one cluster (fewer clusters) would favor extracting specific and significant functional categories, we established four clusters. The functionally enriched categories of genes in each cluster are presented in Tables 1 and 2. Many over-represented functional categories overlapped between the two processes. The repressed functional categories in continuous and fed-batch fermentation processes included ribosomal biogenesis, aminoacyl tRNA synthesis, and ergosterol biosynthesis. Likewise, similar categories of functions were induced in each process, including stress response and metabolism of energy reserves.

Transcriptional changes of genes involved in ergosterol synthesis

Genes involved in ergosterol metabolism were repressed significantly in both fermentation processes (Tables 1, 2). Ergosterol, as the product of the sterol biosynthetic pathway, is the main sterol in *S. cerevisiae*. Fifteen genes encoding enzymes involved in ergosterol synthesis were downregulated in both fermentation processes (Fig. 4). The transcription of most of these genes, such as *ERG10*, *HMG1*, *ERG12*, *ERG8*, *IDI1*, *ERG9*, *ERG1*, *ERG24*, *ERG26*, *ERG6*, *ERG2*, and *ERG4*, was repressed more significantly during the early main fermentation stage in continuous fermentation than in fed-batch fermentation.

Transcriptional changes of genes related to energy reserve metabolism

Accumulation and mobilization of energy reserves (trehalose and glycogen) represent general stress responses. Genes involved in trehalose and glycogen metabolism were upregulated simultaneously during both fermentation processes (Fig. 5). Four genes, *TPS1*, *TPS2*, *TSL1,* and *PGM2*—which encode enzymes involved in trehalose biosynthesis from glucose-1-phosphate through glucose-6-phosphate and alpha, alpha-trehalose-6-phosphate—were upregulated during both fermentation processes (Fig. 5a). In *S. cerevisiae*, glycogen biosynthesis involves three

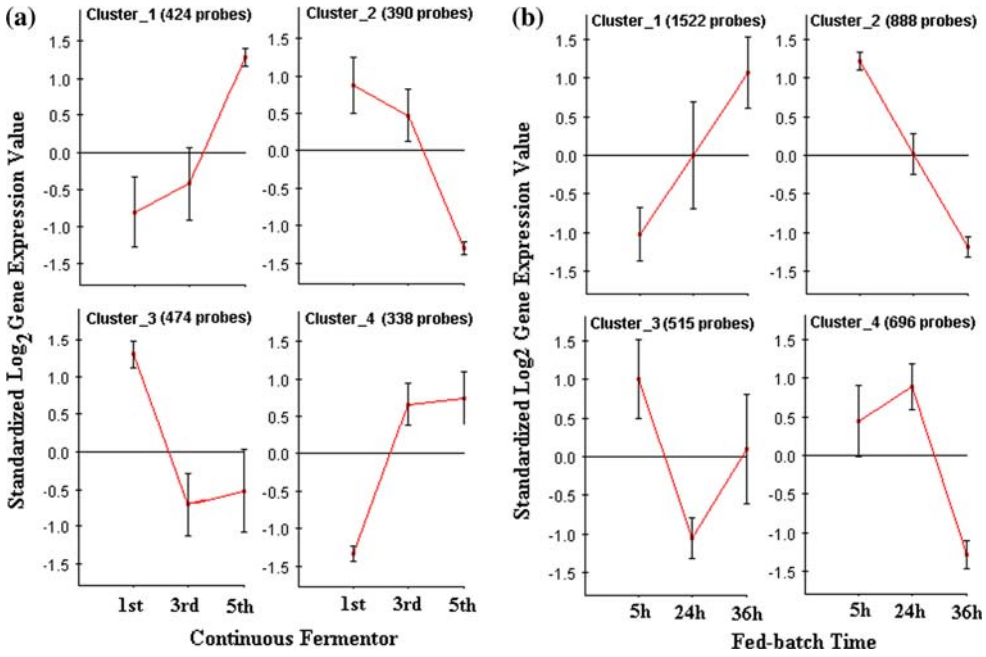

Fig. 3 Cluster analysis of differentially expressed genes in industrial *Saccharomyces cerevisiae* during continuous (**a**) and fed-batch (**b**) fermentation

processes: nucleation, elongation, and ramification or branching [9]. Genes involved in each of the three steps, including *GLG1*, *GSY1*, *GSY2*, *GLC3*, and *GLP2*, were upregulated during both fermentation processes (Fig. 5b). Interestingly, genes involved in degradation of trehalose and glycogen were also upregulated during both fermentation processes. These genes included *NTH1* and *NTH2*, which encode neutral trehalases, and *GDB1*, which encodes a glycogen debranching enzyme (Fig. 5). The simultaneous upregulation of genes involved in trehalose and glycogen mobilization and accumulation indicates that that reserve metabolism was being fine-tuned during fermentation.

Transcriptional changes of genes related to protein folding and glycosylation

During continuous fermentation, protein folding was significantly overrepresented in cluster 4 (Table 1), and 17 differentially expressed genes related to protein folding were upregulated beginning at the early main fermentation stage (Table 3). In fed-batch fermentation, 33 differentially expressed genes in cluster 1 were involved in protein folding and stability (Table 3), and these genes were upregulated gradually as fermentation progressed. Most proteins related to protein folding are located in endoplasmic reticulum (ER). Glycosylation, an important modification for protein folding, is also carried out in ER. Glycoprotein metabolic processes were overrepresented in cluster 2 for fed-batch fermentation (Table 2). Most genes encoding enzymes involved in glycosylation, such as *ALG1*, *ALG2*, *ALG5*, *ALG6*, *ALG7*, *ALG8*, *ALG9*, *ALG12*, and *DIE2*, were significantly downregulated during the late main fermentation stage in fed-batch fermentation (Fig. 6). Several genes involved in glycosylation were also repressed in continuous fermentation, including *ALG1*, *ALG6*, *CAX4*, and *DPM1* (Fig. 6).

Transcriptional changes of genes involved in carbohydrate metabolism

Carbohydrate metabolism provides energy for physiological activities and precursors for metabolites important for maintaining cellular viability. Many genes involved in carbohydrate metabolism were upregulated during both fermentation processes (Tables 1, 2). Figure 7 shows the transcriptional changes of genes involved in glycolysis and gluconeogenesis during both fermentation processes. *HXK2*, which encodes hexokinase isoenzyme 2, was downregulated during both fermentation processes, and the decrease in the transcript level of *HXK2* occurred much more quickly and more significantly in fed-batch fermentation than in continuous fermentation. Transcription of *GLK1*, which encodes glucokinase, was upregulated during late main fermentation in both processes. *HXK1*, which encodes hexokinase isoenzyme 1, which catalyzes the same reaction with Hxk2p and Glk1p, was induced significantly beginning at the early main fermentation stage in fed-batch fermentation but was induced only marginally until the late main fermentation stage in continuous fermentation (Fig. 7).

Differentiation between glycolysis and gluconeogenesis pathways occurs at two different irreversible steps. *PFK1/2* and *CDC19* encode the enzymes that catalyze the irreversible steps in glycolysis; the transcription of these genes

Table 1 Overrepresented functions of differentially expressed genes in each cluster during continuous fermentation

Cluster	Enriched function[a]	No. of genes[b]	*P* value[c]
1	Carbohydrate metabolism	49	2.25E-18
1	Energy derivation by oxidation of organic compounds	33	2.03E-12
1	Energy reserve metabolism	16	1.83E-10
1	Acetyl-coenzyme A metabolism	10	5.25E-08
1	Uridine diphosphate−glucosyltransferase activity	7	3.11E-06
1	Coenzyme metabolism	22	4.95E-06
1	Response to chemical stimulus	41	1.45E-05
1	Monocarboxylic acid metabolism	20	2.11E-05
2	Ribosomal subunit	103	0
2	Structural constituents of ribosomes	101	0
2	Translation	117	0
2	Ribosome biogenesis and assembly	69	1.30E-20
2	Macromolecule metabolism	226	1.17E-15
2	Ribosome assembly	23	4.68E-12
2	Cellular metabolism	248	2.24E-11
2	Organelle organization and biogenesis	130	4.60E-09
2	Regulation of translational fidelity	8	5.00E-08
2	Ribosomal small subunit assembly and maintenance	8	7.40E-07
2	RNA binding	36	9.87E-07
3	Ergosterol metabolism	15	2.55E-11
3	Oxidoreductase activity	39	2.04E-06
3	Cellular lipid metabolism	34	1.92E-05
4	Protein folding	17	2.45E-07
4	Spore wall assembly (sensu Fungi)	12	3.02E-07
4	Unfolded protein binding	12	1.15E-05

[a] Enriched function includes MIPS functional category description

[b] Number of genes in our gene list involved in corresponding functional categories

[c] *P* value calculated based on hypergeometric distribution

changed very little in both fermentation processes (Fig. 7). In contrast, *FBP1* and *PCK1*, which encode the corresponding enzymes for the irreversible steps in gluconeogenesis, were upregulated gradually beginning in the early stage of main fermentation in both processes. Transcription of *MDH2*, which encodes a malate dehydrogenase isoenzyme, increased similarly as *PCK1* during both fermentation processes. *CAT8*, which encodes a transcriptional factor necessary for derepression of a variety of genes under nonfermentative growth conditions [37], was induced beginning at the early stage of main fermentation in both processes. Furthermore, the transcription of 16 glucose-repressible genes, including *HXT7*, *HXT10*, *HXT13*, *HXT17,* and *HAP4*, was upregulated in both fermentation processes (Fig. 8; Table S1). Figure 8 also shows that upregulation of these genes was more significant and occurred earlier in fed-batch fermentation than in continuous fermentation.

Discussion

Continuous and fed-batch fermentation are the most common industrial fermentation technologies. In this study, both processes were divided into different fermentation stages, including seed-growth stage, main fermentation stage, and final fermentation stage. Continuous fermentation goes through the different fermentation stages in the spatial dimension (i.e., in different fermentors), whereas fed-batch fermentation does so in the temporal dimension (over time; Fig. 1). Thus, we could compare transcriptional changes at the same fermentation stage between the two processes. In the scatter plots showing transcriptional changes at the late main fermentation stage for both processes (Fig. S1), we found that yeast cells exhibited similar but stronger transcriptional changes during the fed-batch process than during the continuous process. We also compared transcriptional responses of yeast at the early main fermentation stage (not shown), but linearity of transcriptional responses of yeast during the two fermentation processes was very poor. Therefore, we hypothesized that changes in gene expression in fed-batch fermentation became more significant than those in continuous fermentation after the early main fermentation stage. Evidence for a wider dynamic range of transcription in fed-batch fermentation compared with continuous fermentation was also found by comparing gene transcript levels for the overrepresented functions, including genes related to

Table 2 Overrepresented functions of differentially expressed genes in each cluster during fed-batch fermentation

Cluster	Enriched function[a]	No. of genes[b]	*P*-value[c]
1	Spore wall assembly (sensu Fungi)	29	1.16E-10
1	Carbohydrate metabolism	80	1.22E-08
1	External encapsulating structure organization and biogenesis	75	2.20E-07
1	Sporulation	51	4.84E-07
1	Monosaccharide transport	17	5.10E-07
1	Sugar transmembrane transporter activity	15	1.13E-05
1	Energy reserve metabolism	21	1.42E-05
2	Translation	138	1.28E-27
2	Structural constituents of ribosomes	98	2.14E-27
2	Macromolecule biosynthesis	180	3.56E-26
2	Biosynthetic processes	247	5.40E-24
2	Cellular protein metabolism	253	2.99E-11
2	Amine metabolism	71	1.33E-10
2	Ligase activity, forming carbon–oxygen bonds	17	7.26E-07
2	Amino acid activation	16	7.89E-07
2	Golgi vesicle transport	47	6.89E-06
2	Glycoprotein metabolism	27	1.52E-05
3	Amino acid metabolism	38	6.17E-05
4	Ribonucleoprotein complex biogenesis and assembly	103	8.02E-17
4	Ribosome biogenesis and assembly	90	2.46E-16
4	RNA processing	90	1.35E-09
4	Maturation of 5.8S ribosomal RNA (rRNA) from tricistronic rRNA transcript	17	2.37E-08
4	Biopolymer metabolism	283	2.94E-08
4	Ergosterol metabolism	15	3.71E-08
4	Organelle organization and biogenesis	209	7.93E-08
4	Lipid biosynthesis	37	1.97E-07
4	Small nucleolar RNA (snoRNA) binding	14	1.08E-06
4	Nucleobase\side\tide and nucleic acid metabolism	221	2.44E-06
4	Total RNA (tRNA) processing	25	5.05E-06
4	RNA metabolism	157	5.46E-06
4	Maturation of small subunit rRNA (SSU-rRNA)	16	9.84E-06
4	Cellular component organization and biogenesis	307	9.92E-06
4	RNA modification	22	1.03E-05
4	RNA methylation	10	1.17E-05

[a] Enriched function includes MIPS functional category description

[b] Number of genes in our gene list involved in corresponding functional categories

[c] *P* value calculated based on hypergeometric distribution

reserve metabolism (Fig. 5) and glycosylation (Fig. 6), and genes involved in glycolysis and gluconeogenesis (*HXK1*, *HXK2*, *FBP1*, and *PCK1*; Fig. 7). Many aspects of the industrial fermentation process could have contributed to gene transcription modulation, such as ethanol and sugar concentrations, pH and temperature changes, and even the process technology (continuous or fed-batch fermentation). The reason for the more significant transcription changes during fed-batch fermentation is unclear, and further investigation in this regard is warranted.

Ergosterol is a membrane component and is important for cell viability [27] and resistance to ethanol [2]. Transcriptional profiles showed that most genes encoding proteins of ergosterol biosynthesis were repressed in both processes but more significantly in continuous fermentation. Lack of oxygen can repress ergosterol synthesis [1], and ethanol was found to have negative effects on total and free ergosterol content in yeast [36]. A synergistic effect of ethanol and hypoxia on ergosterol content has also been shown [36]. In this study, the extended time used to add the saccharified liquid feedstock might have provided extra oxygen during fed-batch fermentation, which could have partly alleviated ergosterol synthesis repression. However, in continuous fermentation, the oxygen present in the

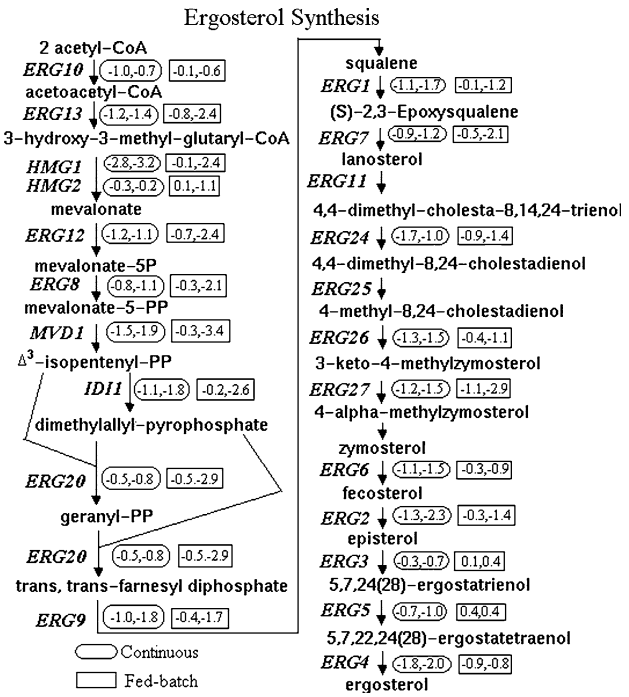

Fig. 4 Differential expression of genes involved in ergosterol synthesis in continuous and fed-batch fermentation. *Numbers in rounded rectangles* represent $\log_2$ changes in the third and fifth fermentors relative to the first fermentor (control) under continuous fermentation. *Numbers in rectangles* represent $\log_2$ changes at 24 and 36 h relative to 5 h (control) under fed-batch fermentation

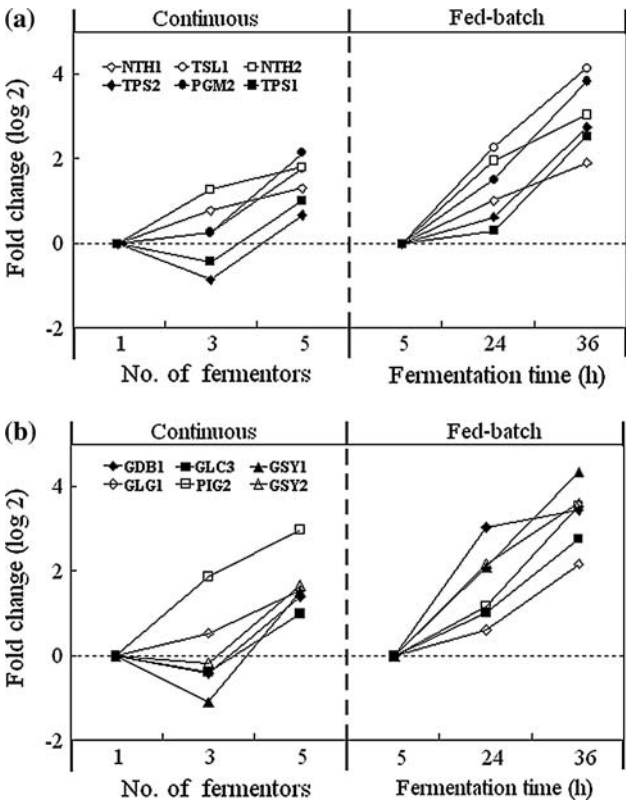

Fig. 5 Changes in expression of genes related to reserve carbohydrate metabolism in continuous and fed-batch fermentation. Genes related to trehalose metabolism (**a**). Genes related to glycogen metabolism (**b**). *First, third,* and *fifth* refer to expression in samples taken from the first, third, and fifth fermentors, respectively, in continuous fermentation; *5, 24,* and *36 h* refer to expression in samples taken at 5, 24, and 36 h, respectively, in fed-batch fermentation

saccharified liquid would have been exhausted in the second fermentor, and no extra oxygen would have been transferred into the following fermentors. At the same fermentation stage, the ethanol concentration in continuous fermentation samples was higher than that in fed-batch fermentation samples (Fig. 2). These two factors could partly explain the more significant repression of ergosterol synthesis in continuous fermentation than in fed-batch fermentation. There are few side pathways leading from the intermediate metabolites in the ergosterol synthesis pathway, and the accumulation of the intermediate metabolites is usually harmful to yeast. Therefore, as observed in this study, transcription of most genes involved in ergosterol synthesis is coordinated (Fig. 4). In previous studies, downregulation of genes related to ergosterol synthesis was observed during sake brewing and fermentation of industrial yeast in synthetic media [31]. In fact, to maintain yeast viability during long-term wine making, aeration is often employed [31]. One group reconstructed the ergosterol synthesis pathway from *Escherichia coli* in *S. cerevisiae* and was able to sustain yeast growth under conditions that would usually inhibit its constitutive ergosterol biosynthetic pathway [20].

Trehalose and glycogen are the major reserve carbohydrates in *S. cerevisiae*. We found that not only the expression of genes related to reserve synthesis but also the expression of genes involved in reserve degradation was induced during industrial fermentation processes. Trehalose has been shown to be involved in surviving osmotic, ethanol, and oxidative stresses [15] and is very important for maintaining cell viability under stress conditions [33]. Glycogen is also involved in responses to a wide variety of environmental stresses, including heat stress; nutrient starvation; or exposure to sodium chloride, hydrogen peroxide, copper sulfate, high levels of ethanol, and weak organic acids [9]. Similarly increased transcription of these genes has been observed in many fermentation processes, including sake brewing [40], full-scale lager yeast fermentation [11], and mimicking alcohol fermentation using commercial industrial yeast [31]. Significant accumulation of glycogen and trehalose was also observed during experiments mimicking alcohol fermentation, although genes encoding enzymes for reserve degradation, including *GBD1*, *NTH1,* and *NTH2*, were also upregulated during the fermentation process [31]. These observations indicate that

Table 3 Genes involved in protein folding that were upregulated in continuous and fed-batch fermentation processes

Processes	Continuous fermentation	Fed-batch fermentation
Genes	*CPR6, ERO1, HCH1, HSC82, YDJ1, HSP26, HSP60, HSP78, HSP82, PAC2, SIS1, SLU7, SSA1, SSA2, STI1, HSP104*	*AHA1, APJ1, CDC37, CIN1, COX17, CPR6, ECM10, ERO1, HCH1, HLJ1, HSC82, HSP10, HSP104, HSP26, HSP42, HSP60, HSP78, HSP82, JJJ3, KAR2, MDJ1, MPD1, PAC2, SGT1, SSA1, SSA2, SSA3, SSA4, SSC1, SSE1, SSE2, STI1, YDJ1*

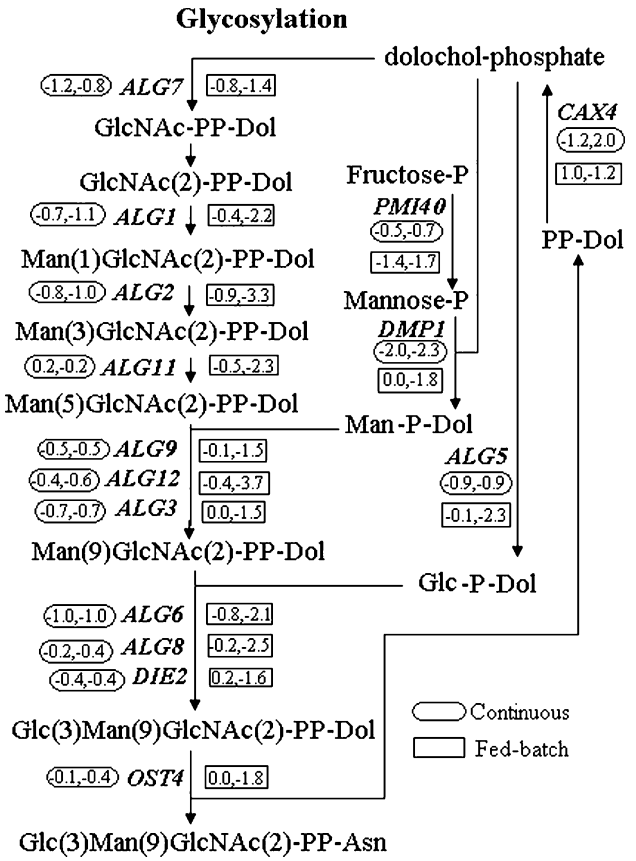

Fig. 6 Transcriptional changes of genes involved in glycosylation in continuous and fed-batch fermentation. *Numbers in rounded rectangles* represent $\log_2$ changes in the third and fifth fermentor relative to the first fermentor (control) under continuous fermentation. *Numbers in rectangles* represent $\log_2$ changes at 24 and 36 h relative to 5 h (control) under fed-batch fermentation

yeast responded to the stress of the fermentation process not only by accumulating reserves but also by increasing metabolic fluxes related to reserves.

In yeast, carbohydrate metabolism is the central and essential metabolic process used for ethanol-producing fermentation. Reprogramming of carbohydrate metabolism would be an essential response to stress-inducing environmental variations. Transcription of many genes related to glycolysis and gluconeogenesis changed during industrial fermentation. Hxk1p, Hxk2p, and Glk1p catalyze the first irreversible step in glucose metabolism and control sugar flux into glycolysis. *HXK2* is highly expressed and is the predominant isoenzyme expressed during growth in glucose. *HXK1* and *GLK1* transcription is repressed by Hxk2p in the presence of glucose [30]. *HXK2* transcription would be repressed and *GLK1* and *HXK1* transcription would derepress from Hxk2p at low glucose concentration [30]. As shown in Fig. 7, increases in transcript levels of *GLK1* and *HXK1* were more profound in fed-batch than in continuous fermentation, which is consistent with *HXK2* repression.

Upregulation of key genes in gluconeogenesis (*FBP1* and *PCK1*) and *MDH2* were observed in both fermentation processes. Both *FBP1* and *PCK1* can be repressed by glucose through multiple signaling pathways [42]. *MDH2* was shown to be a glucose-repressible gene and to be derepressed through Cat8p or Sip4p [32]. Upregulation of *MDH2* would increase the generation of oxaloacetate from malate and increase the metabolic flux into the gluconeogenesis pathway (Fig. 7). These results indicate that glucose uptake from the fermentation broth during fermentation was not sufficient. Upregulation of genes encoding high-affinity glucose transporters (*HXT7*, *HXT10*, *HXT13*, and *HXT17*) and other glucose-repressible genes seemed to be the response to glucose limitation. However, upregulation of these genes occurred at the early main fermentation stage, when the concentration of reducing sugar was still high (15 g/l in continuous fermentation and 23 g/l in fed-batch fermentation). During fermentation of grape juice by an industrial yeast strain, 23 glucose-repressible genes were upregulated [19]. However, no similar variations in the expression of these genes were observed during alcohol fermentation by an industrial strain in synthetic media [31]. Thus, transcriptional regulation of these glucose-repressible genes might be related to the composition of media. Several hypotheses explaining the derepression of these genes have been proposed, including genetic differences between industrial and laboratory strains, alterations in the structure of proteins involved in signaling due to disruption of membrane structure by increasing concentrations of ethanol, activation of the retrograde response pathway, unknown specific ethanol-sensing mechanism in the presence of excess glucose, and the regulatory function of the depressed *HXK2* [19]. Further studies are needed to determine the regulatory

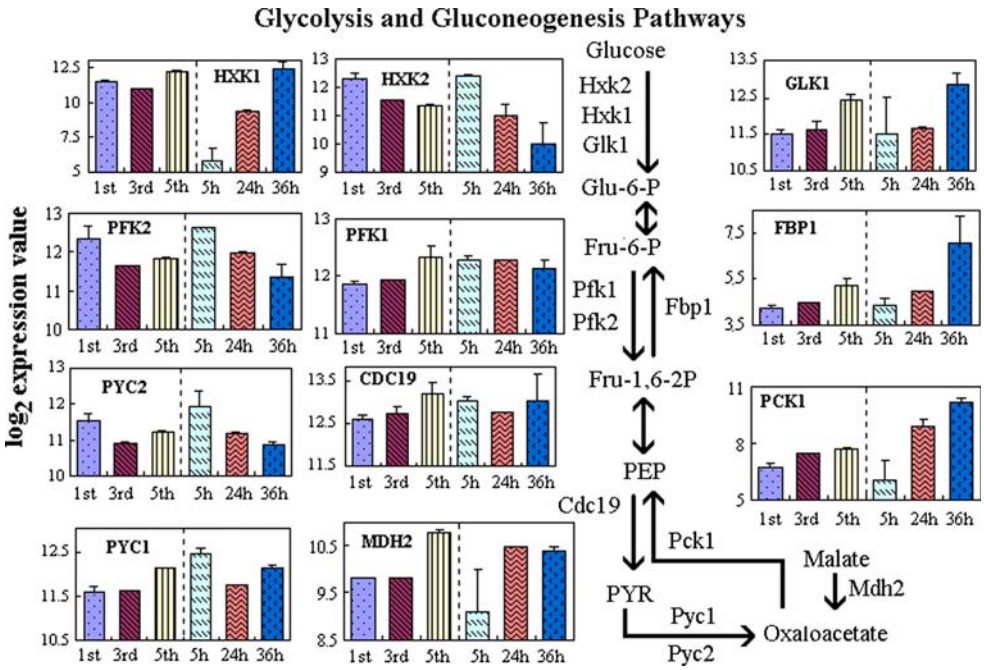

Fig. 7 Changes in expression of genes involved in glycolysis and gluconeogenesis pathways. *First, third,* and *fifth* refer to expression in samples taken from the first, third, and fifth fermentors, respectively, in continuous fermentation; *5, 24* and *36 h* refer to expression in samples taken at 5, 24, and 36 h, respectively, in fed-batch fermentation

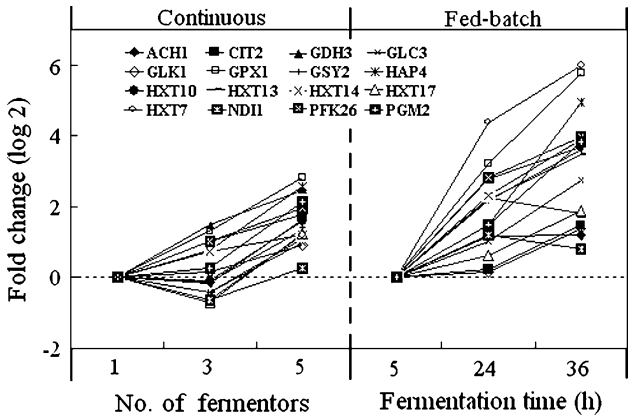

Fig. 8 Changes in expression of glucose-repressible genes. *First, third,* and *fifth* refer to expression in samples taken from the first, third, and fifth fermentors, respectively, in continuous fermentation; *5, 24,* and *36 h* refer to expression in samples taken at 5, 24, and 36 h, respectively, in fed-batch fermentation

mechanisms involved in the altered transcription of these glucose-repressible genes during industrial fermentation.

One interesting finding was the downregulation of genes related to glycosylation, which could explain, at least in part, the upregulation of proteins related to the stress response to unfolded proteins. Downregulation of glycosylation and upregulation of unfolded protein stress response proteins suggest dramatic changes in the ER environment during both fermentation processes. In *S. cerevisiae*, 70% of proteins processed through ER and Golgi are glycosylated [26]. Previous research has suggested that glycosylation was related to protein folding and that covalently linked glycan could facilitate oligomerization by mediating intersubunit interactions in the protein or stabilizing the oligomer in other ways [25]. Thus, glycosylation repression could lead to the accumulation of unfolded and misfolded proteins, which would trigger the unfolded protein response, as observed in this study. The increased expression of genes related to protein folding and stability signals the accumulation of unfolded or misfolded proteins. The unfolded protein response is also required for the coordinated disposal of misfolded proteins [38], and the unfolded protein response decreases the ER output of unfolded protein stress to close a negative feedback loop [23]. In our study, downregulation of glycosylation might have been triggered through an unknown mechanism during fermentation processes, which resulted in unfolded protein stress.

Another interesting finding was that transcriptional changes that occurred during industrial fermentation were similar in many aspects to changes that occurred in response to protein glycosylation deficiency in yeast [6]. Changes consistent with the response to protein glycosylation deficiency included upregulation of genes involved in the unfolded protein response and reserve metabolism and glucose-repressible genes [6]. Therefore, we proposed a model to explain transcriptional changes observed in this study (Fig. 9). In this model, glycosylation repression is one of the secondary stresses in yeast cells and affects upregulation of glucose-repressible genes, genes involved in reserve metabolism and the unfolded protein response, and *HXK2* downregulation. However, downregulation of ergosterol synthesis is not the downstream response of glycosylation repression. According to this model, some

unknown mechanisms during fed-batch fermentation repress glycosylation more significantly than during continuous fermentation, thereby explaining the more significant transcriptional changes observed in fed-batch fermentation than in continuous fermentation. Further investigation of the trigger responsible for signaling repression of glycosylation might help uncover some underlying regulatory mechanisms of gene transcription during industrial fermentation.

Proteomic analysis using the same fermentation systems used in this study showed significant changes in most enzymes involved in glycolysis [4]. However, the transcriptional level of genes encoding these enzymes did not change significantly. Such inconsistencies between relative changes in protein versus messenger RNA (mRNA) expression could be due to posttranscriptional mechanisms, i.e., the regulation of protein synthesis and degradation, which are the predominant mechanisms of glycolytic enzyme regulation [7]. Another cluster of differentially expressed proteins were stress-related proteins, and Hsp26p was included in this cluster [4]. The high expression level of Hsp26p was consistent with *HSP26* upregulation beginning at the early main fermentation stage in both continuous and fed-batch processes.

We compared our results with previous studies on transcriptional profiles of yeast during brewery fermentation. Gibson et al. investigated the lager yeast transcriptome during full-scale brewery fermentation using microarrays [11]. Because both studies were based on full-scale industrial fermentation, many similarities were observed, such as the upregulation of genes related to reserve carbohydrate metabolism, upregulation of genes involved in gluconeogenesis (*FBP1* and *PCK1*), and induction of genes involved in sugar transport. However, a significant increase in the transcriptional level of genes involved in glycolysis, such as *TPI1*, *TDH1*, *TDH3*, *PGK1*, *ENO1*, was observed during lager yeast fermentation [11], whereas these genes were not significantly upregulated in this study. These different observations might be related to the different strains (active dry yeast and lager yeast) and/or different media. In addition, in the lager yeast fermentation study, only genes related to sugar metabolism were reported. However, in our study, transcriptional changes of genes related to other many processes, such as glycosylation and ergosterol synthesis, were also observed. We also compared our results with those from a study that mimicked industrial fermentation using synthetic media and active dry yeast [31]. This mimicking fermentation was representative of industrial fermentation in most aspects, and most transcriptional changes observed in the mimicking process were similar to those observed in this study, such as downregulation of ergosterol synthesis, weak regulation of genes related to carbohydrate metabolism,

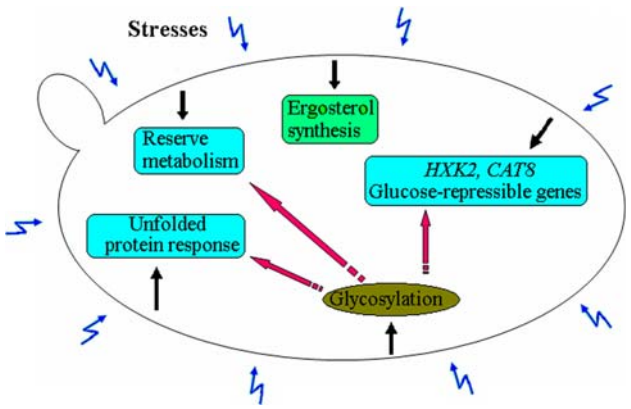

Fig. 9 Model depicting transcriptional changes of yeast during industrial fermentation. Expression of genes involved in ergosterol synthesis, the unfolded protein response, reserve metabolism, and glycosylation, as well as glucose-repressible genes, responded to industrial fermentation stresses. Glycosylation downregulation might be one of the secondary stresses that modulate expression of glucose-repressible genes and genes involved in the unfolded protein response and reserve metabolism

upregulation of trehalose and glycogen metabolism, and upregulation of genes related to the unfolded protein response [31]. Strains used in both studies were active dry yeast, which might partly explain the similarity in the observations. However, we observed glycosylation downregulation and significant glucose-repressible gene upregulation, which were not detected in the mimicking fermentation process. Thus, the small-scale mimicking fermentation process does not fully represent industrial fermentation in every aspect.

Yeast cells suffered from a variety of stresses during the fermentation process [12]. Therefore, transcriptional changes of genes involved in many metabolic pathways were global responses to all stresses during industrial fermentation. However, the exact sequence of these responses was not obtained, and it was essential to characterize the response regulation during industrial fermentation. Further study is needed to determine response regulation characteristics, such as further research on time-course transcriptome with shorter interval and research on transcriptomic research on response-specific stresses during industrial fermentation.

Conclusion

Genome-wide transcriptional responses of an industrial strain of *S. cerevisiae* during industrial fermentation processes were evaluated by DNA microarray analysis. Data showed that multiple metabolic processes were reprogrammed at the transcriptional level during both continuous and fed-batch fermentation processes, including

glycosylation, ergosterol synthesis, reserves metabolism, and glucose metabolism. Based on these data, we proposed a model for transcriptional changes of yeast during industrial fermentation. The results expand our understanding of yeast responses during industrial bioethanol fermentation and may be helpful for further molecular-level studies aimed at improving the efficiency of bioethanol production.

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