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Research Article

Genome-wide transcriptional regulation in *Saccharomyces cerevisiae* in response to carbon dioxide

Lin-Rui Tan[1,2], Jing-Jing Liu[2], Anshu Deewan[2,3], Jae Won Lee[2], Peng-Fei Xia[1], Christopher V. Rao[2,3], Yong-Su Jin[2,4,*], Shu-Guang Wang[1,5,*]

[1]School of Environmental Science and Engineering, Shandong University, Qingdao 266237, P. R. China
[2]Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, United States
[3]Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801, United States
[4]Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, IL 61801, United States
[5]Sino-French Research Institute for Ecology and Environment, Shandong University, Qingdao 266237, P. R. China
*Corresponding author: 1206 West Gregory Drive, Urbana, IL 61801, United States. Tel: 1-217-333-7981; E-mail: ysjin@illinois.edu; 72 Binhai Road, Qingdao 266237, P. R. China. Tel: 86-0532-58630921; E-mail: wsg@sdu.edu.cn
One sentence summary: Carbon dioxide could be an external stimulus for modulating metabolic activities in yeast and a transcriptional effector for diverse applications.
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Abstract

Sugar metabolism by *Saccharomyces cerevisiae* produces ample amounts of CO_2 under both aerobic and anaerobic conditions. High solubility of CO_2 in fermentation media, contributing to enjoyable sensory properties of sparkling wine and beers by *S. cerevisiae*, might affect yeast metabolism. To elucidate the overlooked effects of CO_2 on yeast metabolism, we examined glucose fermentation by *S. cerevisiae* under CO_2 as compared to N_2 and O_2 limited conditions. While both CO_2 and N_2 conditions are considered anaerobic, less glycerol and acetate but more ethanol were produced under CO_2 condition. Transcriptomic analysis revealed that significantly decreased mRNA levels of *GPP1* coding for glycerol-3-phosphate phosphatase in glycerol synthesis explained the reduced glycerol production under CO_2 condition. Besides, transcriptional regulations in signal transduction, carbohydrate synthesis, heme synthesis, membrane and cell wall metabolism, and respiration were detected in response to CO_2. Interestingly, signal transduction was uniquely regulated under CO_2 condition, where upregulated genes (*STE3*, *MSB2*, *WSC3*, *STE12*, and *TEC1*) in the signal sensors and transcriptional factors suggested that MAPK signaling pathway plays a critical role in CO_2 sensing and CO_2-induced metabolisms in yeast. Our study identifies CO_2 as an external stimulus for modulating metabolic activities in yeast and a transcriptional effector for diverse applications.

Introduction

Increasing evidences suggested that carbon dioxide (CO_2) might affect microbial metabolic activities due to its role as a biosynthetic substrate of carboxylation reactions and a metabolic product of decarboxylation reactions (Kluger 2015). Researchers have observed the effects of CO_2 on growth and metabolism of microorganisms, suggesting that CO_2 might affect gene expression to some degrees (Shen *et al.* 2004, Cheng *et al.* 2013). However, the effects of CO_2 on metabolism have not been elucidated comprehensively.

Yeast as a promising microbial cell factory releases CO_2 during sugar metabolism. Rapid CO_2 production, which is a desirable trait of yeast for beer and dough fermentation (Albertin *et al.* 2011, Bigey *et al.* 2021), may affect yeast metabolism. In particular, *Saccharomyces cerevisiae* grows well on glucose under both aerobic and anaerobic conditions, and has been widely used for the production of beer and dough which requires CO_2 (Gallone *et al.* 2018, Nielsen 2019, Opalek and Wloch-Salamon 2020). While transcriptional regulatory networks (TRNs) of *S. cerevisiae* in response to different glucose concentrations and oxygen levels have been studied extensively (Gomar-Alba *et al.* 2015, Shin *et al.* 2021, Zhang *et al.* 2021), the roles and TRNs associated with levels of CO_2 have not been studied. We hypothesized that CO_2 might play profound roles in regulating gene expression in *S. cerevisiae* as CO_2 is an inevitable metabolic product of sugar metabolism in yeast. Identification of the TRNs associated with the levels of CO_2 might allow to exploit CO_2 as an external stimulus for transcriptional control of target genes or pathways.

Most *S. cerevisiae* fermentations are conducted with sugars under aerobic conditions with oxygen (O_2) supply or anaerobic conditions with nitrogen (N_2) purging. Even though CO_2 is continuously generated and consumed by *S. cerevisiae* during the sugar metabolism, its effects might not be outstanding as other gases, such as O_2 and N_2 are major factions of the atmosphere with ventilation (Bracher *et al.* 2019). As such situations cause difficulties in distinguishing the role of CO_2 on metabolism, a fermentation experiment with only CO_2 in a headspace will be necessary. We attempted to capture the changes of mRNA levels in response to the alteration of the headspace gas from N_2 to CO_2 for a comprehensive understanding of cellular response to CO_2.

Specifically, glucose fermentation by *S. cerevisiae* was examined under CO_2 condition and transcriptomic analysis by RNA-seq was conducted. Identical fermentations under O_2 limited (abbreviated as O_2 unless otherwise specified) and N_2 conditions were performed as baselines. Concentrations of external metabolites (glycerol, acetate, and ethanol) and their yields from glucose under CO_2 as compared to O_2 and N_2 conditions were elucidated. Also, the expression levels of the genes involved in glycolysis were ana-

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lyzed, which has a direct impact on the production of the external metabolites. In order to reveal the global transcriptional changes in response to CO_2, differentially expressed genes (DEGs) based on KEGG and PFAM database in yeast were further identified. The aim of this study is to explore CO_2 effects on yeast fermentation with detailed information of TRNs, which are activated under CO_2 condition. We envision that the identified TRNs can be utilized to control gene expression in yeast using CO_2 as an external stimulus.

Materials and methods

Yeast strain and media

Saccharomyces cerevisiae BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) was used in this study. Yeast was cultured in yeast extract–peptone (YP) medium (10 g/l yeast extract and 20 g/l peptone) or synthetic complete (SC) medium (1.7 g/l yeast nitrogen base with 5 g/l ammonium sulfate and amino acids). Potassium phthalate buffer (50 mM) was added to SC medium to maintain a pH of 6.0. Certain concentration of glucose was supplied as carbon source.

Fermentation experiment

Precultures were kept in YP medium with 20 g/l glucose until stationary phase before the final cells were inoculated. Batch cultures were in SC medium with 40 g/l glucose in 100 ml serum bottles. Air was kept in the bottle before sealing with butyl rubber stoppers to regard as O_2 condition. To prepare N_2 and CO_2 conditions, ergosterol and Tween 80 were added initially to provide nutrients for long-term anaerobiosis before sealing, and N_2 or CO_2 of ultrahigh purity was used as purging gas to remove air and fill the bottle (Verduyn *et al.* 1990). After purging for 20 min, oxyrase was injected into N_2 and CO_2 filled bottles, respectively, to further remove O_2 effect (Knabel and Thielen 1995). All culture volume was adjusted to 2 ml with initial OD_{600}~1.0 to maintain the dominant role of headspace gas under each condition during fermentation. Temperature and agitation were maintained at 30°C and 100 rpm, and experiment was conducted for 48 h until nearly all glucose was consumed up. The growth profiles were recorded at the wavelength of 600 nm using a spectrophotometer, and fermentation product concentrations were measured by high-performance liquid chromatography (HPLC, Agilent Technologies 1200 Series) equipped with a refractive index detector and a Rezex ROA-Organic Acid H^+ (8%) column (Phenomenex Inc., USA). The column was eluted with 0.005 N of H_2SO_4 at a flow rate of 0.6 ml/min at 50°C.

RNA sequencing

S. cerevisiae with three independent replicates under each fermentation condition was employed for determination of mRNA level. Cells were collected at an exponential phase with 15.5 ± 0.5 g/l glucose left in the medium for RNA extraction, when transcriptional perturbations in yeast were dominated by gas effect rather than other interference from cellular environment, such as glucose or other nutrient concentration. Specifically, cells were cultivated for 20 h under O_2 condition and 16 h under CO_2 and N_2 conditions before collection. The subsequent RNA isolation used glass-bead method as described and was treated with DNase according to the manufacturer's protocol (Qiagen). The quality of total RNA was confirmed by bioanalyzer (Agilent) and the concentration was measured by fluorometry (Qubit). Library construction and RNA sequencing using Illumina NovaSeq 6000 machine were performed at the Keck Center at the University of Illinois at Urbana-Champaign. Each sample yielded over 50 million reads and totally about 550 million single reads were produced for nine samples. Average quality scores per base were higher than 30. Reads were trimmed based on quality score and adapters (Liu *et al.* 2021). The trimmed reads were then mapped to S288C reference sequence from the Genebank. Gene expression levels were denoted by read counts in each experimental condition. Normalization procedure was performed with TMM method (Robinson and Oshlack 2010). Genes were identified as differentially expressed with Log2 of fold change (FC) ≥ 1 or ≤ -1 and *P*-value < 0.05.

Quantitative real-time PCR (qRT-PCR)

At least three independent RNA extractions were assayed for each condition. Complementary DNAs were obtained using the Invitrogen SuperScript™ III First-Strand Synthesis SuperMix for RT-PCR kit (Invitrogen, USA). qRT-PCR was performed in 96-well plates on a Lightcycler 480 instrument (Roche Applied Science, USA) using SYBR Green I Master (Roche) according to the manufacturer's protocol. Primers were listed in Table S1 (Supporting Information). mRNA levels of specific genes were normalized by geometric mean of mRNA level of *ACT1* based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001, Halbeisen and Gerber 2009).

Statistical analysis

Fermentation experiments were conducted in triplicate. Error bars denote the standard deviation from the means of independent experiments. The differences between data were evaluated using Student's *t*-test with $P < 0.05$ (*) and $P < 0.01$ (**).

Results and discussion

Effect of headspace CO_2 on glucose fermentation and byproduct formation

Glucose fermentation of *S. cerevisiae* was conducted in tightly sealed vials with a headspace gas of CO_2. To identify any changes in the production of extracellular metabolites in response to CO_2, identical fermentation tests were performed in yeast but under the headspace gas condition of either N_2 or air (regarded as O_2 condition). *S. cerevisiae* had similar growth profiles under CO_2 and N_2 conditions, which are considered as anaerobic conditions (Fig. 1A). Moreover, the glycerol and ethanol productions increased and the acetate production decreased under CO_2 or N_2 conditions as compared under O_2 condition, which is considered as aerobic condition (Fig. 1). The results are consistent with the previous reports that anaerobiosis increases glycerol and ethanol production but decreases acetate production in yeast (Hagman and Piškur 2015, Henningsen *et al.* 2015). Interestingly, even though both CO_2 and N_2 conditions are regarded as anaerobic conditions, the production of glycerol and acetate decreased, while ethanol production increased in *S. cerevisiae* in response to CO_2 as compared to N_2 (Fig. 1). Specifically, 6.15 ± 0.15 g/l glycerol with a yield of 0.14 ± 0.00 g/g glucose was produced under N_2 condition, while only 4.24 ± 0.08 g/l of glycerol was produced with a yield of 0.10 ± 0.00 g/g glucose under CO_2 condition at the end of the fermentation (Fig. 1B and 1C). Less acetate was also observed (0.80 ± 0.01 g/l vs. 1.14 ± 0.02 g/l) under CO_2 than under N_2 conditions (Fig. 1D and 1E). Moreover, final ethanol concentrations were different (*P*-value < 0.05) with productions of 17.86 ± 0.23 g/l of under CO_2 condition and 16.65 ± 0.54 g/l under N_2 condition (Fig. 1F and 1G).

To further confirm the differences in the formation of byproducts under different headspace gases, the produced amounts of

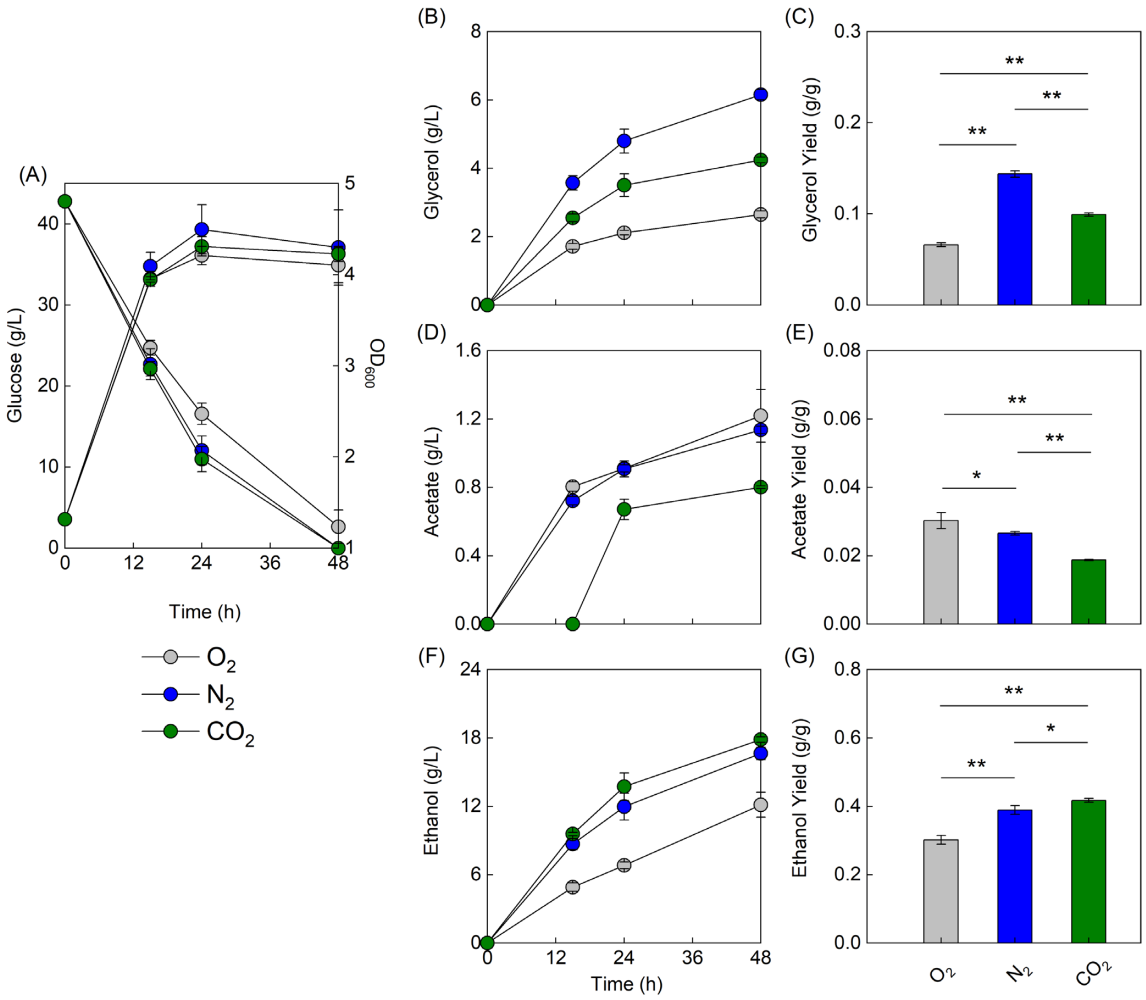

Figure 1. Time-course glucose fermentation profiles of *S. cerevisiae* under different headspace gases. Fermentation was conducted at 30°C and 100 rpm in SC media with 40 g/l glucose under O_2, N_2, and CO_2 conditions. Yields were calculated based on consumed glucose. **(A)** Glucose consumption and growth profiles, **(B)** glycerol production profiles, **(C)** glycerol yields, **(D)** acetate production profiles, **(E)** acetate yields, **(F)** ethanol production profiles, and **(G)** ethanol yields. Experiments were conducted in triplicate and error bars denoted the standard deviation from the means of independent experiments. * represents significant difference with *P*-value < 0.05, ** represents significant difference with *P*-value < 0.01.

glycerol, acetate and ethanol were normalized by the cell densities (Table 1). While the normalized amounts of consumed glucose (~0.33 mol/l/OD) were similar under all the conditions, the calculated carbon fluxes into fermentation products were higher (~66%) under CO_2 and N_2 conditions than under O_2 condition (~48%), which agrees with a previous result that glycolytic flux increased during anaerobiosis (van den Brink *et al.* 2008). Carbon flux calculations were also conducted in yeast cells at the exponential and early stationary phases and similar results were obtained, suggesting CO_2 effect on glucose fermentation in yeast was stable and growth-independent (Tables S2 and S3, Supporting Information). Even though total carbon fluxes into fermentation products (glycerol, acetate, and ethanol) were similar under anaerobic conditions, the distribution patterns of carbon fluxes were significantly changed in response to CO_2.

Global transcriptional changes of *S. cerevisiae* in response to CO_2

We hypothesized the differences in the production of external metabolites might be caused by transcriptional regulation in yeast in response to CO_2. To dissect the role of CO_2 in controlling carbon flux distribution on glucose fermentation and to understand how *S. cerevisiae* responds transcriptionally to CO_2, transcriptomic analysis was performed. Particularly, as glucose concentration could affect the transcriptomes of *S. cerevisiae* (Gancedo 2008, Shashkova *et al.* 2017, Kim and Rodriguez 2021), cells under CO_2, N_2, or O_2 conditions for transcriptomic analysis were collected when similar amounts of glucose (15.5 ± 0.5 g/l) were left in the medium to minimize the effect of glucose (Table S4, Supporting Information). Besides, fermentation results of the collected cells were similar as those detected in Fig. 1 (Table S4 and Figure S1, Supporting Information), and carbon flux calculation was also consistent with Table 1 (Table S5, Supporting Information), suggesting that transcriptomes of the collected cells could explain for the different phenotypes detected under each gas condition with minimized glucose effect.

Through principal component analysis (PCA) of the transcriptomes, distinct clusters of genes expressed under CO_2 condition as compared to N_2 and O_2 conditions indicated that gene expression levels were significantly changed in *S. cerevisiae* in response to CO_2 (Figure S2, Supporting Information). The identified DEGs with Log2 of fold change (FC) ≥ 1 or ≤ −1 and *P*-value < 0.05 under each condition were then examined. First, we explored the genes which are perturbed significantly under CO_2 as compared to O_2 condi-

Table 1. Carbon flux calculation under different fermentation conditions.

Conditions	$C_{Consumed\ glucose}$ (mol/l/OD)	$C_{Ethanol}$ (mol/l/OD)	$C_{Glycerol}$ (mol/l/OD)	$C_{Acetate}$ (mol/l/OD)	C_{E+G+A} (mol/l/OD)	% ($C_{E+G+A}/C_{Consumed\ glucose}$)
O_2	0.3259 ± 0.0031	0.1282 ± 0.0055	0.0211 ± 0.0005	0.0099 ± 0.0008	0.1591 ± 0.0062	48.84 ± 1.92
N_2	0.3337 ± 0.0328	0.1695 ± 0.0196	0.0470 ± 0.0057	0.0090 ± 0.0008	0.2254 ± 0.0259	67.47 ± 1.70
CO_2	0.3372 ± 0.0068	0.1834 ± 0.0043	0.0327 ± 0.0005	0.0063 ± 0.0001	0.2225 ± 0.0048	65.97 ± 0.88

tions. Among the 500 DEGs which showed up- or down-regulation when CO_2 is filled instead of air (Figure S3A, Supporting Information), many of them were involved in the seripauperin (PAU) and TIP1 family, mitogen-activated protein kinases (MAPK) signaling pathway, glycolysis, starch and sucrose metabolism, and steroid biosynthesis based on KEGG and PFAM database (Figure S3B, Supporting Information). Similar results were obtained from the analysis of DEGs when N_2 is filled in the headspace of cultures as compared to O_2 condition since the N_2 condition is also considered as anaerobic like the CO_2 condition (Figure S3C and S3D, Supporting Information). However, the expression levels of genes involved in the MAPK signaling pathway did not change under N_2 condition unlike under CO_2 condition. When we examined the genes which are differentially expressed only under CO_2 compared to N_2 conditions, 249 DEGs were identified (Fig. 2A) including the genes involved in the MAPK signaling pathway (Fig. 2B).

Glucose transport and degradation in *S. cerevisiae* under CO_2 condition

As glucose is mainly metabolized *via* glycolysis to generate biomass precursors and external metabolites (Dashko *et al.* 2014), we examined the expression levels of the genes coding for glucose transporters and glycolytic enzymes. Hxt1-7 are considered as the major glucose transporters in *S. cerevisiae* (Reifenberger *et al.* 1995). Among these transporters, *HXT6* and *HXT7* coding for high-affinity glucose transporters were upregulated under CO_2 as compared to O_2 conditions (Table S6, Supporting Information). The expression levels of *HXT6* and *HXT7* were still higher than those under N_2 condition (Fig. 3). Upregulated hexose transport contributes to the biofilm formation of yeast (Yeater et al. 2007). Previous research has confirmed that biofilm formation was enhanced in yeast in high CO_2 (Pentland *et al.* 2021), which was partially due to the upregulated *HXT6* and *HXT7* in response to CO_2. In addition, *S. cerevisiae* might be able to sense CO_2 and control the expression levels of the high-affinity glucose transporters even though glucose was not depleted in the culture medium (Reifenberger *et al.* 1995). Among the glycolytic enzymes, many genes were highly expressed under CO_2 and N_2 conditions as compared to O_2 condition (Table S6, Supporting Information), which can be classified as anaerobic-specific genes (Rodriguez *et al.* 2001, Choo *et al.* 2018). As a result, the higher glycolytic carbon flux can be maintained under CO_2 and N_2 conditions (Table 1). However, the expression level of *TDH3* coding for glyceraldehyde-3-phosphate dehydrogenase was downregulated under CO_2 compared to N_2 conditions (Fig. 3), which might be responsible for the changes in external metabolite production in response to the headspace CO_2 gas. Moreover, the expression levels of *PDC1* coding for pyruvate decarboxylase and *PCK1* coding for phosphoenolpyruvate carboxykinase were decreased under CO_2 compared to N_2 conditions (Fig. 3). As CO_2 is released during the decarboxylation reaction catalyzed by Pck1 and Pdc1 (Meyer *et al.* 2011, Tang *et al.* 2018), sufficient CO_2 may send a signal to repress the expression of *PCK1* and *PDC1* under CO_2 condition.

To further investigate how the altered metabolite production under CO_2 condition is connected with gene expression levels, the enzymes which are directly involved in the production of glycerol, ethanol, and acetate were studied. In glycerol biosynthesis, *GPD1* coding for glycerol-3-phosphate dehydrogenase was significantly upregulated (P-value < 0.05) with Log2 (FC) of 1.58 under CO_2 and Log2 (FC) of 1.95 under N_2 conditions compared to O_2 condition (Table S6, Supporting Information). This is consistent with the increased glycerol production under anaerobic conditions (Fig. 1B and 1C; Semkiv *et al.* 2017). Upregulation of *GPD1* was further confirmed by qRT-PCR (Fig. S4, Supporting Information). Among glycerol-3-phosphate phosphatases (Gpp1 and Gpp2) which are involved in glycerol biosynthesis (Pahlman *et al.* 2001), *GPP1* was downregulated under CO_2 with FC of 1.59 and P-value < 0.05 as compared to N_2 conditions (Table S6, Supporting Information). However, no significant change was detected in the expression level of *GPP2* (Table S6, Supporting Information). The decreased expression level of *GPP1* might cause less production of glycerol under CO_2 than N_2 conditions (Pahlman *et al.* 2001, Lindner *et al.* 2012). Glycerol formation is essential to reoxidize the NADH produced in synthesis of biomass and secondary fermentation products (Guadalupe Medina et al. 2010). As less glycerol was produced under CO_2 condition, *S. cerevisiae* may need to reoxidize NADH by the reduction of acetate to ethanol *via* NADH-dependent reactions, thus decreasing the acetate yield and increasing the ethanol yield (Guadalupe Medina *et al.* 2010). However, when referring to the transcript abundance of the genes involved in the acetate and ethanol biosynthesis, *ALD4* and *ALD6* coding for aldehyde dehydrogenase in the acetate synthesis were upregulated (Fig. 3). Ald4 and Ald6 preferred $NADP^+$ as a cofactor instead of NAD^+ (Miyagi *et al.* 2009, Papapetridis *et al.* 2016). Therefore, the upregulation of *ALD4* and *ALD6* might not play a dominant role for the production of acetate due to the reduced glycerol production under CO_2 condition.

Transcriptional changes in the MAPK signaling pathway in response to CO_2

CO_2 as an environmental signal may be sensed *via* signaling transduction pathways, thus affecting yeast metabolism. Transcriptomic analysis showed that genes in the MAPK signaling pathway were significantly upregulated under CO_2 condition, while they were not regulated under both O_2 and N_2 conditions (Table S7, Supporting Information). The MAPK signaling pathway consists of a signal receptor in cell membrane and signaling proteins, which relay phosphorylation for the control of gene expression in nucleus (Seger and Krebs 1995). Upon initiation by a signal receptor on the cell surface, the MAPK cascade is activated by phosphorylation of several kinases and then induces the expression levels of target proteins in the nucleus of the cell (Fig. 4A; Saito 2010). Interestingly, under CO_2 condition, most of the genes involved in the MAPK signal receptors were affected with higher expression levels (Table 2). Specifically, *STE3* coding for a pheromone receptor in α cell was highly expressed (P-value < 0.05) under CO_2 condi-

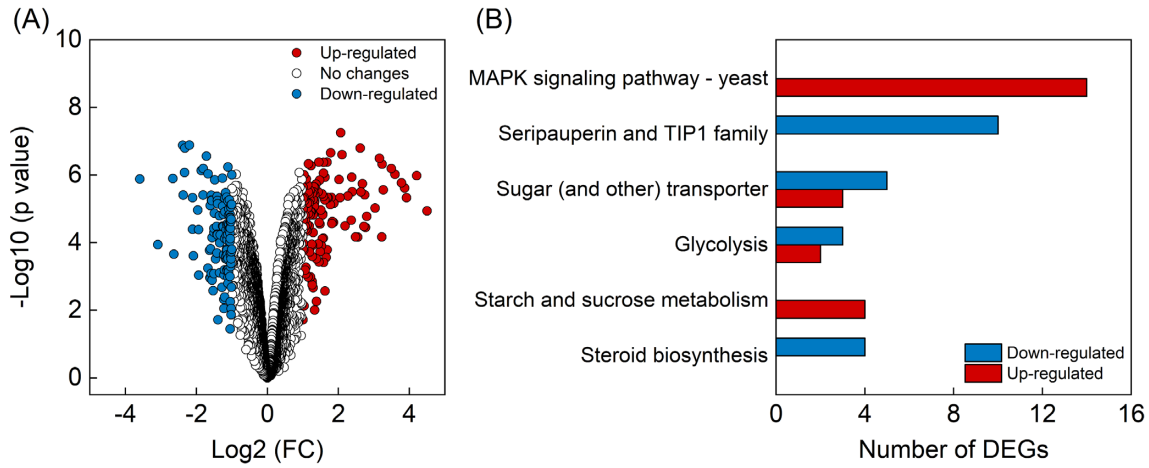

Figure 2. Volcano plot of DEGs under CO_2 compared to N_2 conditions **(A)** and quantitative distribution of DEGs based on KEGG and PFAM database **(B)**. Red represents upregulated genes; white represents genes with no significant changes; and blue represents downregulated genes. FC, fold change; DEGs, differentially expressed genes.

Table 2. Expression level of membrane sensor coding genes in MAPK signaling pathway.

		Log2 (FC)					
SGD	**Gene name**	**CO_2 vs. O_2**	***P*-value**	**CO_2 vs. N_2**	***P*-value**	**N_2 vs. O_2**	***P*-value**
S000001661	*STE3*	2.11	2.39E-06	1.77	7.47E-06	0.34	8.91E-02
S000003246	*MSB2*	1.82	7.40E-07	0.93	5.62E-05	0.89	7.64E-05
S000005465	*WSC3*	1.63	2.25E-06	1.32	8.54E-06	0.31	5.07E-02

tion with Log2 (FC) of 2.11 compared to O_2 and Log2 (FC) of 1.77 compared to N_2 conditions (Hagen *et al.* 1986). *MSB2* coding for an osmosensor and signal receptor under starvation was also upregulated with *P*-value < 0.05 under CO_2 compared to O_2 conditions with Log2 (FC) of 1.82 or N_2 conditions with Log2 (FC) of 0.93 (Table 2; Tanaka *et al.* 2014). *WSC3* coding for a sensor-transducer of heat shock or other stressor signals (Sekiya-Kawasaki *et al.* 2002) was 2.50-fold and 3.09-fold upregulated with *P*-value < 0.05 under CO_2 condition compared to that under N_2 and O_2 conditions. These highly expressed genes in the MAPK signal receptors suggest that *S. cerevisiae* under CO_2 might be more sensitive to environmental stimuli (Adhikari *et al.* 2015). Further, the genes involved in mating were upregulated under CO_2 condition (Table S7, Supporting Information). A protein–protein interaction (PPI) network revealed that Ste12, a transcription factor that positively activates mating related genes, is a hub of the associated gene regulation under CO_2 condition (Fig. 4B; Dolan and Fields 1990, Stone *et al.* 2000). The highly expressed *STE12* explains for the increased expression levels of mating genes (*FUS1*, *FAR1*, *STE4*, *MF(ALPHA)1*, *STE3*, *GPA1*, and *FUS3*) under CO_2 condition. We then confirmed the high expression level of *STE12* under CO_2 condition using qRT-PCR (Figure S5, Supporting Information). In addition, Ste12 and Tec1 have been reported to interact and coregulate genes. Indeed, the expression level of *TEC1*, which is known to regulate gene for the filamentous growth in *S. cerevisiae* were 4.36-fold and 4.30-fold higher under CO_2 condition than O_2 and N_2 conditions (Table S7, Supporting Information; Madhani and Fink 1997, Chou, Huang and Liu 2004). A previous study has demonstrated that CO_2 can play a critical role for the efficient sporulation during the filamentous growth in *S. cerevisiae* (Jungbluth *et al.* 2012), which might be caused by the upregulated *STE12* and *TEC1* under CO_2 condition. Even though the MAPK signaling pathway is responsible for the transient induction of target genes, it is remarkable that the genes coding for multiple signal receptors and transcription factors in the MAPK cascade were upregulated under CO_2 condition. CO_2 sensing in yeast has been extensively studied (Klengel *et al.* 2005, Hall *et al.* 2010, Pohlers *et al.* 2017), while CO_2-responsive MAPK signaling pathway was rarely reported. The regulated MAPK signaling pathway under CO_2 condition suggested that external CO_2 may be sensed by an integration of the MAPK signaling pathway with other well-studied signaling pathways.

Improved carbohydrate synthesis in response to CO_2

Once receiving signal from various stresses, yeast might respond by synthesizing carbohydrates (Gomar-Alba *et al.* 2015, Pereira et al. 2018). Glycogen and trehalose are two reserve carbohydrates in yeast (François and Parrou 2001). When analyzing the transcriptomes of the cells grown under CO_2, N_2, and O_2 conditions, the genes (*GSY1*, *GSY2*, *GPH1*, *UGP1*, *GLC3*, *GDB1*, and *PGM2*) involved in the glycogen synthesis, *TSL1* and *TPS2* involved in the trehalose synthesis showed higher expression levels under N_2 and CO_2 than O_2 conditions (Table S8, Supporting Information), which was consistent with a previous report (Li *et al.* 2010). Among these genes, the expression levels of *GSY1*, *GSY2*, *UGP1*, and *GPH1* increased more in response to CO_2 (Fig. 5). Several researches have reported that carbohydrate metabolic pathways play critical roles in biofilm formation (Yeater *et al.* 2007). Based on our result, the promoted biofilm formation of yeast in high CO_2 reported previously may be due to the upregulated carbohydrate synthesis under CO_2 condition (Pentland *et al.* 2021). Moreover, glycogen serves as a carbon and energy reserve in yeast and its presence confers survival advantages to the cell (François and Parrou 2001). The in-

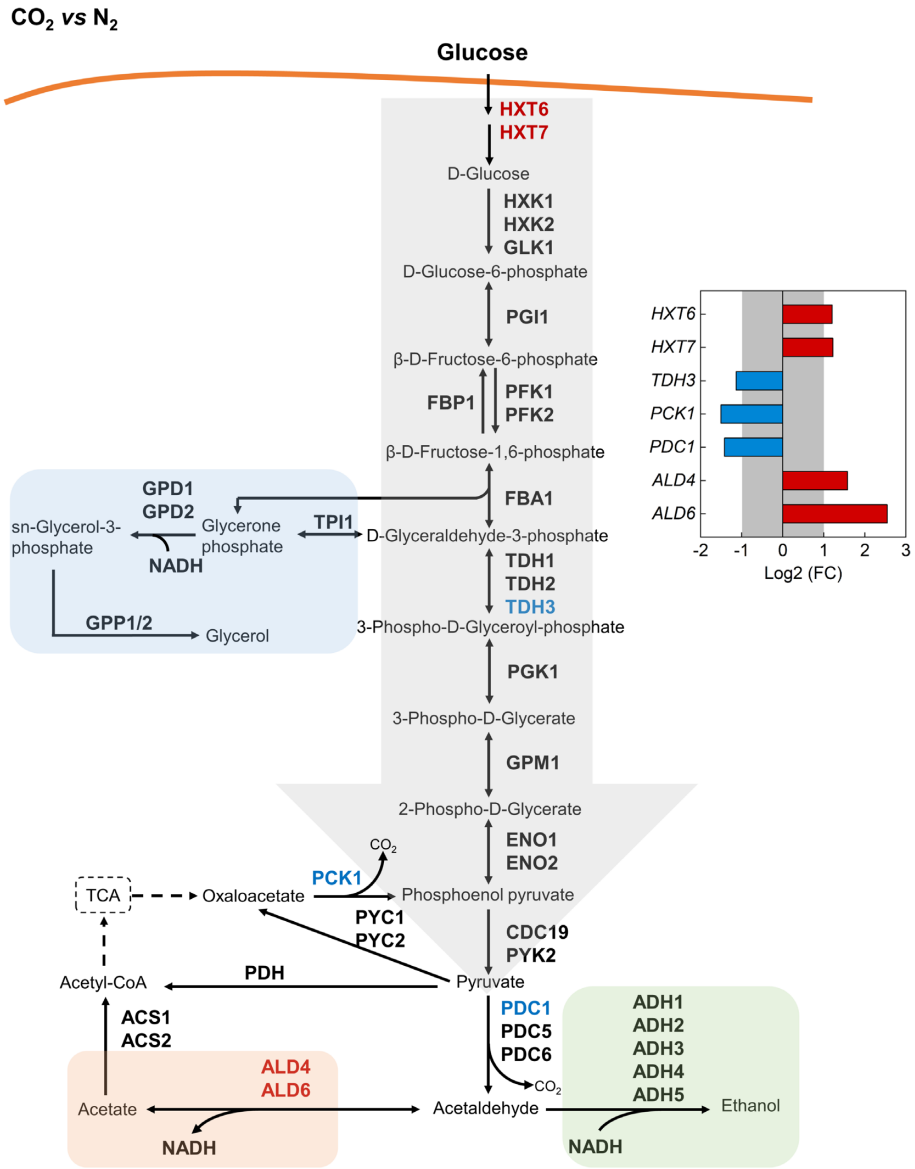

Figure 3. DEGs in glucose transport and degradation pathway under CO_2 compared to N_2 conditions. Red represents upregulated coding genes with Log2 (FC) ≥ 1 and *P*-value < 0.05 and blue represents downregulated coding genes with Log2 (FC) ≤ −1 and *P*-value < 0.05. HXT6/7, hexose transporter; HXK1/2, hexokinase; GLK1, glucokinase; PGI1, phosphoglucoisomerase; FBP1, fructose-1,6-bisphosphatase; PFK1/2, phosphofructokinase; FBA1, fructose 1,6-bisphosphate aldolase; TPI1, triose-phosphate isomerase; GPD1/2, glycerol-3-phosphate dehydrogenase; GPP1/2, glycerol-3-phosphate phosphatase; TDH1/2/3, triose-phosphate dehydrogenase; PGK1, 3-phosphoglycerate kinase; GPM1, glycerate phosphomutase; ENO1/2, enolase; PCK1, phosphoenolpyruvate carboxykinase; CDC19, pyruvate kinase; PYK2, paralog of CDC19; PYC1/2, pyruvate carboxylase; PDH, pyruvate dehydrogenase multienzyme complex; PDC1/5/6, pyruvate decarboxylase; ALD4/6, aldehyde dehydrogenase; ACS1/2, acetyl CoA synthetase; and ADH1/2/3/4/5, alcohol dehydrogenase.

creased abundance of mRNA levels of the enzymes in the glycogen synthesis pathway may lead to more glycogen accumulation in cells under CO_2 condition, thus conferring a better survival of *S. cerevisiae* under stress conditions.

Other anaerobically induced genes involved in heme synthesis, cell wall and membrane metabolism, and respiration in *S. cerevisiae* in response to CO_2

As CO_2 is also considered as an anaerobic gas, many anaerobically induced genes were detected under CO_2 condition as that under N_2 condition. For instance, heme is depleted and the heme biosynthetic genes, such as *HEM13*, are upregulated under anaerobic conditions (Keng 1992). This is consistent with our results that *HEM13* had higher expression level in *S. cerevisiae* under both CO_2 and N_2 conditions during anaerobiosis, while decreased mRNA level was detected under CO_2 compared to N_2 conditions with Log2 (FC) of 1.04 and *P*-value < 0.05. In addition, biosynthetic ergosterol (ERG) genes (*e.g. ERG3* and *ERG6*) coding for proteins in the plasma membrane and the *PAU* gene family (*e.g. PAU10* and *PAU11*) including the *TIP* subfamily (e.g. *TIP1*) related with cell wall physiology, have been reported to be upregulated under anaerobic condition (Rachidi *et al.* 2000, Abramova *et al.* 2001). Specifically, expression levels of *ERG* genes determine the fluidity and permeability of the plasma membrane (Jordá and Puig 2020), and changes of *PAU* gene family are expected to affect the cell wall porosity (Kwast *et al.* 2002). Upregulations of these genes maintain or activate metabolic fluxes in order to meet nutritional and en-

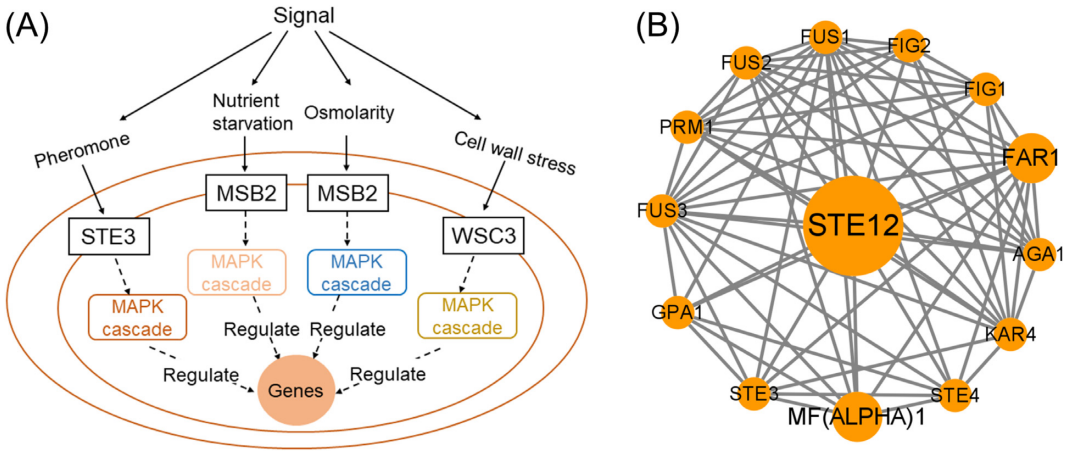

Figure 4. Membrane sensor for MAPK signaling pathway (A) and the PPI network of upregulated encoding genes for mating under CO_2 condition by Cytoscape (B). Node and font size indicated the correlations with other genes and bigger size suggests higher scores by Bottleneck ranking.

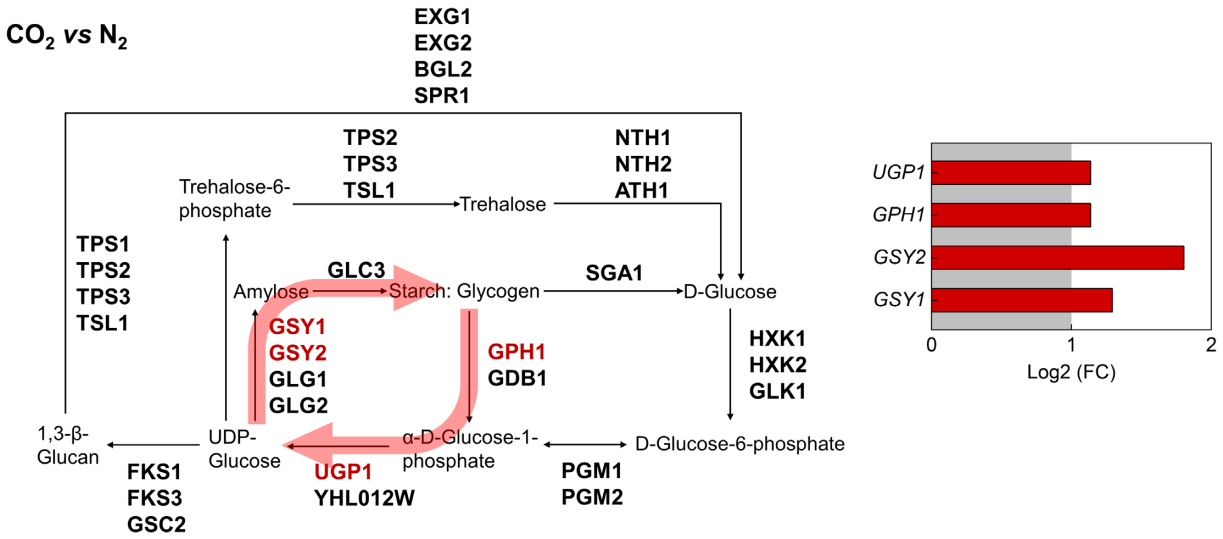

Figure 5. Schematic carbohydrate metabolism pathway with upregulated coding genes under CO_2 compared to N_2 conditions. Red represents upregulated coding genes with Log2 (FC) ≥ 1 and *P*-value < 0.05. PGM1/2, phosphoglucomutase; UGP1, UDP-glucose pyrophosphorylase; YHL012W, paralog of UGP1; GSY1/2, glycogen synthase; GLG1/2, glycogenin glucosyltransferase; GLC3, glycogen branching enzyme; SGA1, sporulation-specific glucoamylase; GPH1, glycogen phosphorylase; GDB1, glycogen debranching enzyme; TPS1, trehalose-6-phosphate synthase; TPS2, trehalose-6-phophate synthase/phosphatase; TPS3, trehaslosephosphate synthase; TSL1, paralog of TPS3; NTH1/2, neutral trehalase; ATH1, acid trehalase; FKS1, catalytic subunit of 1,3-beta-D-glucan synthase; FKS3, protein involved in spore wall assembly; GSC2, paralog of FKS1; EXG1/2, exo-1,3-beta-glucanase; BGL2, beta-glucanase; and SPR1, sporulation-specific exo-1,3-beta-glucanase.

ergetic requirements under anaerobic condition (Andreasen and Stier 1953, 1954). We observed that most of the genes (*e.g. ERG3*, *ERG6*, *ERG5*, and *ERG25*) in the ergosterol biosynthesis and *PAU* genes (*e.g. PAU24*, *PAU13*, *DAN1*, and *TIR4*) showed higher expression levels under both CO_2 and N_2 conditions (Fig. 6A). However, expressions of these genes were significantly repressed under CO_2 condition ($P < 0.05$) compared to N_2 condition (Tables S9 and S10, Supporting Information).

Upc2 as a transcription factor was reported to be an activator of many anaerobically induced genes, including *ERG* and *PAU* gene families (Joshua and Höfken 2017), and a PPI network of Upc2 with the proteins of the ERG and PAU families was shown in Fig. 6B, indicating that Upc2 could be responsible for the transcriptional regulation of the *ERG* and *PAU* genes under CO_2 condition. When comparing the expression levels of *UPC2* under different conditions, mRNA level of *UPC2* under CO_2 was higher than that under O_2 conditions, while lower than N_2 conditions (Fig. 6C). The expression level of *UPC2* was further verified by qRT-PCR and consistent results were obtained (Figure S6, Supporting Information). This relatively lower expression level of *UPC2* under CO_2 condition caused less transcript abundance of the *ERG* and *PAU* genes than N_2 condition during anaerobiosis.

Gene *CYC7* coding for cytochrome c isoform in respiration was also anaerobically induced, while showed higher expression level with Log2 (FC) of 1.62 under CO_2 than N_2 conditions, which was different from most anaerobically induced genes with relatively lower expression levels detected under CO_2 condition. The expression level of *CYC7* was further verified by qRT-PCR and highest expression level was still obtained under CO_2 condition (Figure S7, Supporting Information). Cyc7 plays a protective role on re-oxygenation in yeast (Kwast *et al.* 2002). As such, *S. cerevisiae* with higher expression level of *CYC7* under CO_2 condition may be more

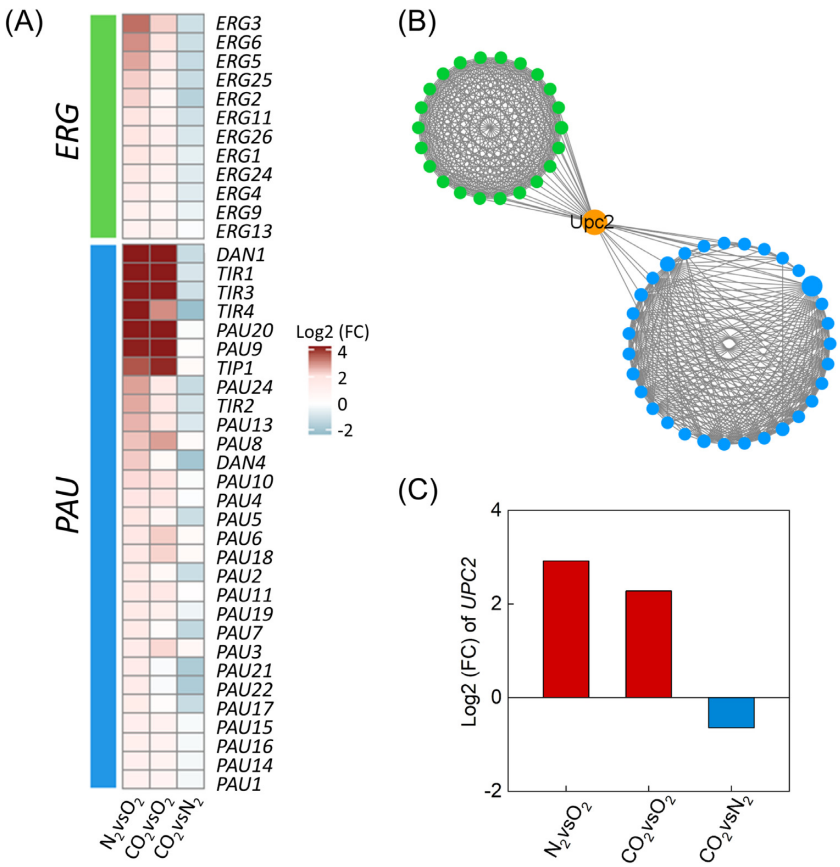

Figure 6. Comparison of mRNA levels of *ERG* and *PAU* gene family in *S. cerevisiae* **(A)**. PPI network of ERG (green) and PAU (blue) family with Upc2 (orange) using Cytoscape software **(B)**. Node size indicated the correlations with other genes and bigger size suggests higher scores by Bottleneck ranking. Expression level of *UPC2* with *P*-value < 0.05 (C). CO_2 vs. N_2 indicates comparisons between CO_2 and N_2 conditions; CO_2 vs. O_2 indicates comparisons between CO_2 and O_2 conditions; N_2 vs. O_2 indicates comparisons between N_2 and O_2 conditions.

resistant against the stressful condition of O_2 reintroduction (Verbelen *et al.* 2009), which makes the cells easily adapt to the transition from anaerobic to aerobic conditions.

Perspective for yeast in response to CO_2

CO_2 is generated during yeast fermentation, which acts as an environmental cue and plays critical roles in cellular metabolism and stress response. Extensive studies have investigated the CO_2 sensing in nonpathogenic *S. cerevisiae* and pathogenic *Candida albicans*, including the cAMP/PKA (protein kinase A)-induced filamentation and lipid/Pkh1/2-regulated CO_2 adaption (Klengel *et al.* 2005, Pohlers *et al.* 2017). Particularly, a CO_2-promoted transition from yeast to filamentous growth is essential for the pathology of *C. albicans* (Pentland et al. 2021). Specifically, CO_2 activates the adenylate cyclase, resulting in the increased levels of cAMP and the PKA-dependent activation of hyphal-specific genes (Pentland *et al.* 2021). The MAPK signaling pathway is another crucial pathway that relays signals to evoke cellular metabolism in yeast, including filamentation (Chen and Thorner 2007). Our results showed that mRNA levels of transcriptional factors involved in filamentation in the MAPK signaling pathway were significantly increased in response to CO_2, suggesting the filamentous growth of yeast under CO_2 condition may be due to the crosstalk between cAMP/PKA and MAPK. In addition, genes encoding multiple signal receptors in MAPK, and genes involved in carbohydrate synthesis and reoxygenation were upregulated, which explained for the increased stress response of yeast in high CO_2 (Pentland *et al.* 2021).

Other pathways in response to CO_2, such as hexose transport, which were confirmed to be upregulated in *C. albicans* (Pentland et al. 2021), were detected with upregulation in *S. cerevisiae* as well. However, even though genes involved in the cell wall and membrane metabolism were upregulated under CO_2 condition as previously reported (Hakkaart *et al.* 2020), higher expression levels of specific genes were detected under N_2 condition, suggesting these metabolisms may be dominated by anaerobiosis rather than particular CO_2 regulation. Combined with previous results, the information obtained in this study will be essential for a better understanding of CO_2 as a signal to regulate the intertwined pathways in yeast.

Conclusion

In this study, we observed phenotypic differences in the production of glycerol, acetate, and ethanol from glucose fermentation by *S. cerevisiae* under different headspace gases (CO_2, N_2, and O_2). While CO_2 and N_2 conditions are considered as anaerobic conditions, different amounts of glycerol, acetate, and ethanol were produced. To explain the phenotypic differences, we conducted a genome-wide expression analysis in *S. cerevisiae* under CO_2, N_2, and O_2 conditions. Under CO_2 condition, mRNA levels of the enzymes in the glycerol synthesis pathway decreased, which is consistent with the reduced production of glycerol under CO_2 condition.

Other TRNs in the signal transduction pathway, carbohydrate synthesis, heme synthesis, membrane and cell wall metabolism, and respiration were regulated in response to CO_2, indicating that CO_2 is an environmental stimulus/signal which orchestrates the TRNs. Intriguingly, the MAPK signaling pathway was distinc-

tively regulated under CO_2 condition. The significantly upregulated genes in signal receptors and transcription factors indicated that MAPK signaling pathway plays a critical role in CO_2 sensing in yeast.

Our study characterized not only external metabolites of glucose fermentation and TRNs under different headspace gases (CO_2, N_2, and O_2), but also provided insights for the practical applications of the identified TRNs. For instance, yeast has great potentials to efficiently increase the production of biofuels and decrease the effect of byproducts under CO_2 condition. The robustness of *S. cerevisiae* might be enhanced through both upregulation of glycogen synthesis and increased resistance against reoxygenation by exploiting the TRNs in response to CO_2 for industrial biomanufacturing. However, most anaerobically induced genes showed lower expression level under CO_2 condition during anaerobiosis, which may inhibit the anaerobic reactions for desired chemicals. This detailed information of TRNs in *S. cerevisiae* in response to CO_2 allows researchers to evaluate the cellular environment during fermentation process, thus modifying cells and facilities due to diverse goals.

Supplementary data

Supplementary data are available at FEMSYR online.

Authors' contributions

Y.S.J., S.G.W., and L.R.T. conceived the investigation. J.J.L. and L.R.T. performed the experiments. A.D., C.V.R., and L.R.T. analyzed the data. J.W.L., P.F.X., and L.R.T. discussed the results. Y.S.J. and L.R.T. wrote the manuscript.

Data availability statement

RNA-seq data are available at the NCBI Sequence Read Archive (SRA) under accession number PRJNA774407.

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Conflict of interest statement. None declared.

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