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Regulation of the ethanol stress response by transcription factor Znf1 in yeast *Saccharomyces cerevisiae*

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Abstract

High ethanol concentration is a critical problem in industrial fermentation since it leads to the inhibition of ethanol production by *Saccharomyces cerevisiae*. Ethanol stress affects *S. cerevisiae* leading to growth inhibition and low level of bioethanol production. To survive, *S. cerevisiae* induces ethanol stress responses including trehalose and glycogen pathway. Here, we focused on characterization of zinc cluster transcriptional regulator Znf1, a member of Zinc cluster DNA-binding proteins. Mostly, zinc cluster proteins are required to regulate transcription in *S. cerevisiae*. In this study, increasing ethanol concentrations impaired growth of the Δ*znf1* strain indicating that high ethanol concentration affects growth of the yeast. Similarly, impaired growth was observed in the strains lacking genes in trehalose and glycogen pathway when ethanol concentrations were increased, suggesting important role in modulating high ethanol stress response. This role was further examined via a gene expression analysis, using quantitative realtime-PCR during the exposure to 10% ethanol. In the Δ*znf1* strain, genes involved in ethanol stress response, including *ugp1* (encoding for UDP-glucose pyrophosphorylase) and *gph1* (encoding for Glycogen phosphorylase), showed down-regulated expression. Our results showed that Znf1 controls ethanol stress response by function as the activator of genes in glycogen pathway. Therefore, this study provides important knowledge for future improvement of ethanologenic yeast for better effective application in bioethanol fermentation.

Introduction

Yeast (*Saccharomyces cerevisiae*) is a major organism widely used in several industries. *S. cerevisiae* are important organism for fermentation and used in many applications including bioethanol and another biofuels production. These processes the ethanol is the target product of yeast fermentation; however, high ethanol concentration also causes a critical problem in industrial fermentation. High of ethanol concentrations within system fermentation have effects to yeasts such as low of growth and fermentation limited. However, yeast *Saccharomyces cerevisiae* have function to modulate cellular ethanol stress response involved in numerous pathways for this environmental stress response. Several genes are associated with ethanol tolerance involving a broad range of functional categories including glycerol metabolism, amino acid metabolism, glycolysis, and carbohydrate metabolisms.

Storage carbohydrate of yeast from trehalose and glycogen pathway, trehalose biosynthesis could be subtly affected by glycogen biosynthesis and catabolism. Glycogen involved in cell wall components such as glucan molecules and also reserves energy inside the cells [1]. Reported, genes expression of trehalose metabolism were up-regulated, including *TPS1* (Trehalose-6-Phosphate Synthase), *TPS2* (Trehalose-6-Phosphate synthase/phosphatase), *TSL1* (Trehalose synthase long chain), *PGM2* (Phosphoglucomutase),

NTH1 (Neutral trehalase), *ATH* (Acid trehalase), and *UGP1* (UDP-glucose pyrophosphorylase) results to trehalose accumulation under ethanol stress [2]. High of intracellular trehalose accumulation showed in overexpression of *TPS1* and deletion of *nth1* recombinant strain of *S. cerevisiae* leads to provide the ability protect against environmental stress [3]. Similarly, simultaneously induced expression of other genes involved in glycogen biosynthesis such as *GSY1* (Glycogen synthase) and *GSY2* (Glycogen synthase), and glycogen degradation such as *GPH1* (Glycogen phosphorylase) were also observed under ethanol stress [4]. Moreover, glycogen storage in yeast cells when grown in media with higher of stress concentration conditions. High level of glycogen and other carbohydrate content exhibited in presence of increasing ethanol concentrations [1]. These intracellular metabolizes can stabilize membranes, proteins and inhibit protein aggregation during refolding process, which responsible for effective protectant for yeast cells against ethanol stress [5].

The newly Zinc cluster protein Znf1 transcription factor belongs to one of the most important families of DNA-binding proteins [6, 7]. Reported that the Znf1 is transcriptional regulators regulated other genes obviously in alternative carbon source glucose-ethanol shift. It demonstrated that Znf1-binding enrichment at these promoters during the glucose–ethanol shift and also required for stress responses under pH and osmotic stresses in *S. cerevisiae* [6].

In the present this report, we showed Znf1 regulated some genes in trehalose and glycogen metabolism, which candidate-ethanol stress response of yeast cells under high ethanol condition. Investigation of the cells lack genes in trehalose and glycogen pathways showed necessary requirement in presence of high ethanol. As controlling stress responsive genes, the genes important for adaptation to ethanol tolerance were identified. The aim of our study is to investigate the expression and effect on survival of genes in trehalose and glycogen pathways when yeast cells were challenged by high ethanol condition.

Methodology

Determination of ethanol stress sensitivity of the wild-type and deletion strains in the presence/ absence ethanol stress tolerance assay.

The *Saccharomyces cerevisiae* deletion strains (Δ*znf1*, Δ*pgm2*, Δ*gph1*, Δ*gsy1*, Δ*ath1*, and Δ*nth1*) were used in this experiment and result was analyzed by comparison with wild-type strains, FY73 (the generous gift from Dr. B. Turcotte, McGill University, Canada) and BY4742 (Open Biosystems, Dharmacon, Inc, Lafayette, CO, USA). After 18 h of culture in YPD medium at 30°C, yeast cells were harvested, and resuspended in distilled water to obtain OD_{600} of 0.1. Cell suspensions were serially diluted with 10-fold dilution (10^{-1}–10^{-4}) and kept at room temperature. 3 µl of each dilution was spotted onto YPD plate supplemented with 5%, 9%, and 12% (v/v) ethanol. Growth of yeast cells was assessed after incubation at 30 °C for 48 h.

Gene induction and quantitative realtime-polymerase chain reaction (qRT-PCR)

The FY73 (wild-type) and Δ*znf1* were grew in Yeast Extract-Peptone-Dextrose (YPD) at 30°C, overnight. Cells were adjusted to OD_{600} of 0.1 for use as starter culture and grew until OD_{600} reached 0.6. After that, cells culture was separated into two parts, untreated and 10% (v/v) ethanol. Cells were then incubated for 6 h. Cells were harvested and washed by DI water 2 times. Total RNAs were extracted with phenol as described [8] and purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis was carried out with the Super ScriptTM III first-strand synthesis kit (Life Technologies, USA). The qRT-PCR assay was performed using a CFX Connect™ Real-Time PCR Detection System and analyzed by CFX Manager™ software. The reaction mixtures contained Brilliant II SYBR Green QPCR Mix (Kapabiosystem). No reverse transcriptase and non-template controls were also included in the qRT-PCR analysis. Gene-specific oligonucleotides used in this study were shown in Table 1. The relative quantification of each transcript was calculated using the $2^{-\Delta\Delta Ct}$ method [9]. *ACT1* gene was an internal control.

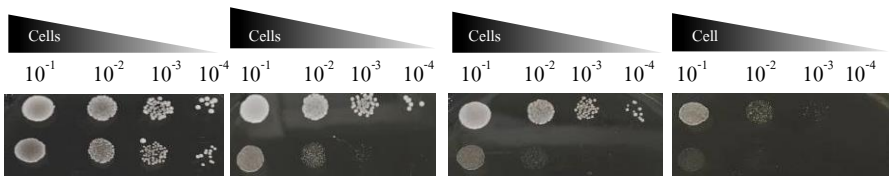
Table 1. Specific oligonucleotides used for quantitative real-time PCR.

Oligonucleotides DNA sequence 5' to 3'	
ACT1	ATTATATGTTTAGAGGTTGCTGCTTTGG
	and CAATTCGTTGTAGAAGGTATGATGCC
GSY1	CGAATGGAAGGCTGACCTA
	and ATGCCTCTCTTACCAGCTTC
UGP1	CAGTACGATAGCGACGTGC
	and GTGACCTGGTGGATACCAAG
NTH1	ACAGACTAGACGTGGTTCTG
	and CGGTATCCTCGATGGTCA
GPH1	GCTTATGAAGCTGCTTCG
	and CGGTTCTTGGTCCAAGACA
PGM2	AGATTGCCGCTATCGGTG
	and TTGTGACGGATTCAGGAGC

Results and Discussion

Firstly, this study investigated phenotypes of yeast cells when cultivated with media containing ethanol. The FY73 (wild-type) and lack *znf1* (Δ*znf1*) strains were induced to 5%, 9%, and 12% (v/v) ethanol containing YPD media. Cell survival was decreased, compared to untreated cells after 2 days of incubation (Figure 1). It was found that wild-type can survive better than Δ*znf1* strain in all conditions. The ability to survive of both strains was decreased by increasing ethanol concentration 5%, 9%, and 12% (v/v), respectively (Figure 1). Therefore, *ZNF1* encoding Znf1 transcription factor protein is necessary for yeast to survive under ethanol stress. It was suggested that the role of Znf1 transcription factor might regulate some genes involved in ethanol-tolerance mechanisms. Little is known for function of Znf1 in ethanol stress response; hence, target genes of Znf1 are required to be identified in the future. This knowledge will help to improve yeast strain to have more ethanol tolerance and ethanol productivity.

High ethanol concentration is an inhibitor of yeast cells that can slow growth, disrupt cellular proteins and enzymes, and leads to the membrane damage by change composition of cell wall and cell membrane [10, 11]. Generally, yeast resists to environmental stress, including high ethanol concentration, by modulation of cellular stress response pathways such as trehalose and glycogen metabolisms [3]. In this study, function of genes involved in trehalose and glycogen pathway were examined under ethanol stress. Ethanol stress sensitivity of the wild-type and deletion strains, including *pgm2*, *gph1*, *gsy1*, *ath1*, and *nth1* were performed by spotted assay. Cells were induced to 5%, and 12% (v/v) ethanol containing YPD media (Figure 2). Deletion of *pgm2*, *gph1*, *gsy1*, *ath1*, and *nth1* which involved in trehalose and glycogen pathways showed defective growth more than wild-type under ethanol stress. While, survival of wild-type (BY4742) and deletion strains induced by 5% and 12% (v/v) ethanol was impaired as clearly observed at spot of 10^{-4} -fold (Figure 2). The ability to survive of all strains was decreased by increasing ethanol concentration at 5% and 12%, respectively. Overall, deletion strains of *ZNF1* gene and genes in trehalose and glycogen pathway showed significantly decrease of survival under high ethanol concentrations interpreted that Znf1 and these genes were important to ethanol tolerance. Furthermore, these genes have been up-regulate in ethanol tolerate strain, they are necessary to cellular ethanol stress response [4].



YPD YPD+5%Ethanol YPD+9%Ethanol YPD+12%Ethanol

Figure 1. Phenotypes of yeast strains in present of ethanol stress. FY73 (wild-type: WT) and Δ*znf1* strains were spotted on YPD solid media supplemented 5%, 9%, and 12% (v/v) ethanol. Cells were serially diluted with 10-fold dilution from 10^{-1} to 10^{-4} with an initially OD_{600} of 0.1. Cells were incubated at 30°C, 48 h.

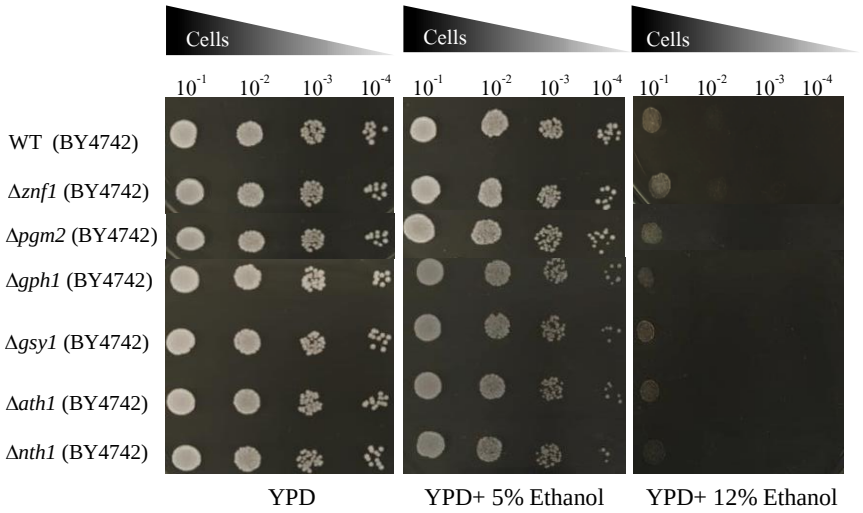


Figure 2. Phenotypes of BY4742 (wild-type: WT) and trehalose as well as glycogen deleted strains under ethanol stress. These strains were spotted on YPD solid media supplemented 5% and 12% (v/v) ethanol. Cells were serially diluted with 10-fold dilution from 10^{-1} to 10^{-4} with an initially OD_{600} of 0.1. Cells were incubated at 30°C, 48 h.

The investigation gene expressions in trehalose and glycogen mechanisms adapted to the high ethanol concentration. *Saccharomyces cerevisiae* responds to ethanol stress condition by reprogramming the expression of genes in various pathways [11]. Several hundred genes are identified associated with ethanol tolerance involving a broad range of functional categories including trehalose and glycogen biosynthesis, membrane and cell wall organization [2]. Here, the relative of mRNA expression level of ethanol/glucose in wild-type (FY73) and Δ*znf1* were investigated by qRT-PCR analysis. These included some genes encoding key enzymes in trehalose and glycogen pathways as well as related pathways such as cell wall and membrane stabilization. Expression of *GSY1*, *UGP1*, *NTH1*, *GPH1*, and *PGM2* genes in wild-type (FY73) were non-significantly different between ethanol stress and glucose condition. In the Δ*znf1*, however, expression of *UGP1* and *GPH1* genes under ethanol stress were significantly down-regulated for 2.04-fold and 5.00-fold, respectively, when compare with the glucose condition obvious in Figure 3. Therefore, the Znf1 might be an activator of genes in trehalose and glycogen synthesis that lead to accumulation of trehalose and glycogen under ethanol stress. Amount of accumulate trehalose and glycogen under ethanol stress in *S. cerevisiae* will be investigated to confirmed function of Znf1. In next, we will add investigate the role of functional Znf1 regulate in these pathways. Our results agreed with previous reports showing elevated mRNA level of induced expression of *UGP1* and *GPH1* as in glycogen biosynthesis [2]. Increasing level of carbohydrate and trehalose in cells, it is possible that yeast cells have to adapt the newly evolved intracellular environment by actively balancing glycogen concentrations to state in proper cellular functions and enhance glucose metabolism. In addition, storage carbohydrates under ethanol stress condition have been reported to function by reducing membrane permeability, which supported strongly at cell wall compositions for survival [4].

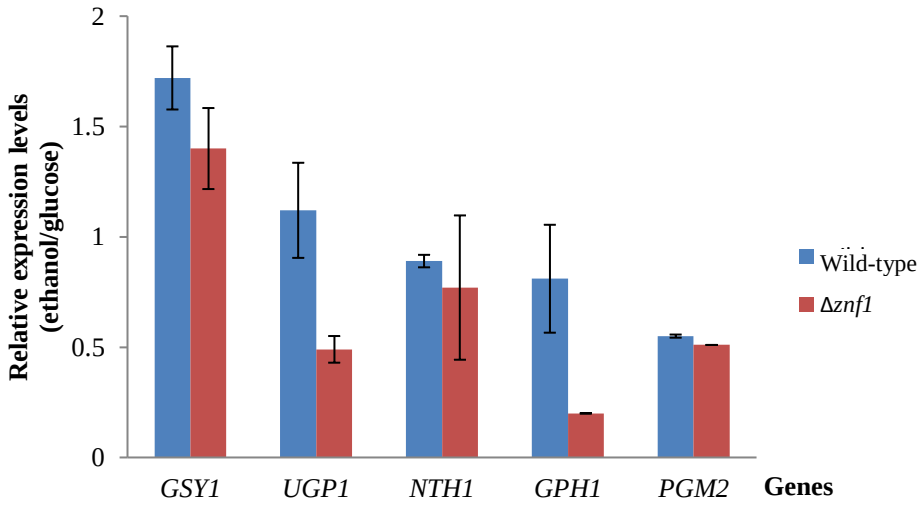


Figure 3. Relative expression levels of genes involved in trehalose and glycogen metabolism adapted to the high ethanol concentration. Wild-type and Δ*znf1* strain were grew in media containing 10% (v/v) ethanol. The relative expression levels were obtained via the comparative C_t method for quantification of the $2^{-\Delta\Delta Ct}$. Error bars indicate standard deviations calculated from at least two independent experiments.

Conclusion

In conclusion, ethanol stress is critical to cell survival and effect depends on increasing ethanol concentrations. The *UGP1* and *GPH1* genes, which encode key enzymes in trehalose and glycogen metabolism and also involved in ethanol stress response, are necessary to enhance the survival of cell in ethanol stress. Expression of these genes under ethanol stress might be regulated by Znf1. Therefore, knowledge provided by this study can be applied to improve cell ability in tolerance to ethanol stress which is one of the most important problem in industrial ethanol production. Future construction of ethanologenic yeast strains can lead to increase ethanol production.

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