공학박사 학위논문

Improvement of glucose and fructose uptake and furan aldehydes tolerance in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae 의 포도당과 과당 수송 및 퓨란 알데히드 저항성 증대 연구

2016 년 8 월

서울대학교 대학원

화학생물공학부

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Improvement of glucose and fructose uptake and furan aldehydes tolerance in *Saccharomyces cerevisiae*

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Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

in Seoul National University

August, 2016

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ABSTRACT

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Saccharomyces cerevisiae is a multi-platform strain for the production of useful chemicals and fuels. In this thesis, *S. cerevisiae* was genetically engineered to improve utilization of various carbon sources including glucose and fructose. Also, yeast strain with enhanced tolerance to lignocellulosic furan aldehydes was developed.

Firstly, glucose uptake rate in *S. cerevisiae* was improved by overexpression of hexose transporters (HXTs) and a growth-promoting transcription factor Gcr1.

Among the five tested HXTs (Hxt1, Hxt2, Hxt3, Hxt4, and Hxt7), overexpression of a high-affinity transporter Hxt7 was the most effective in increasing the glucose uptake rate, followed by moderate-affinity transporters Hxt2 and Hxt4. Deletion of *std1* and *mth1*, encoding corepressors of *HXT* genes, exerted differential effects on the glucose uptake rate, depending on the culture conditions. In addition, improved cell growths and glucose uptake rates were achieved by overexpression of *GCR1*, which led to increased transcription levels of *HXT1* and ribosomal protein genes. All these genetic modifications that enhanced the glucose uptake rate also resulted in the increased the ethanol production rate, compared to wild-type *S. cerevisiae*. Furthermore, the growth-promoting effect of *GCR1* overexpression was successfully applied to lactic acid production in an engineered lactic acid-producing strain, resulting in a significant improvement of productivity and titers of lactic acid production under acidic fermentation conditions

Secondly, the fructose uptake rate was improved by overexpression of a heterologous high affinity fructose transporter and hexokinases (HXKs). To improve the fructose uptake, a high-affinity fructose transporter from fructophilic yeast was introduced into *S. cerevisiae*. *S. cerevisiae* strain CEN.PK2-1C expressing Fsy1 originated from *Candida magnoliae* showed increased fructose uptake in the medium containing either fructose as a sole carbon source or low concentrations of both fructose and glucose, but not in the medium containing high concentrations of both fructose and glucose. Furthermore, to improve intracellular fructose consumption to increase its diffusive intake, hexokinases were

overexpressed. Since overexpression of *HXK2* repressed yeast cell growth, other *HXK* genes, such as *HXK1*, *GLK1*, and a putative hexokinase *YLR446w* were overexpressed. Overexpression of these genes increased the glucose/fructose uptake rate. Especially, the overexpression of *HXK1* improved the uptake of glucose and fructose most, followed by the overexpression of the putative hexokinase Ylr446w. Although overexpression of each hexokinase gene was effective in glucose or fructose uptake, overexpression of CmFsy1 decreased the glucose uptake of all strains in the media containing high concentrations of both glucose and fructose.

Lastly, yeast strains tolerant to 5-hydroxymethylfurfural (HMF) and furfural, inhibitors generated during the pretreatment of lignocellulosic biomass, were developed. In this part, HMF and furfural were identified as thiol-reactive electrophiles, thus directly activating the Yap1 transcription factor via the H_2O_2-independent pathway, depleting cellular glutathione (GSH) levels, and accumulating reactive oxygen species in *S. cerevisiae*. However, furfural showed higher reactivity than HMF toward GSH *in vitro* and *in vivo*. In line with such toxic mechanisms, overexpression of $YAP1^{C620F}$, a constitutively active mutant of *YAP1*, and Yap1 target genes encoding catalases (*CTA1* and *CTT1*) increased tolerance to furfural and HMF. However, increasing GSH levels by the overexpression of genes for GSH biosynthesis (*GSH1* and *GLR1*) or by the exogenous addition of GSH to the culture medium enhanced tolerance to furfural but not to HMF.

Keywords : Metabolic engineering, lignocellulose, tolerance to furan aldehyde, glucose uptake, fructose uptake, lactic acid production, *Saccharomyces cerevisiae*

Student Number **:** 2010-23323

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LIST OF ABBREVIATIONS

ADH	alcohol dehydrogenase
ARS	autonomously replicating sequence
A-ALD	acetaldehyde dehydrogenase
CEN	centromere
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DTT	dithiothreitol
EDTA	ehylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorter
GAP	glyceraldehyde-3-phosphate
GC	gas chromatography
GPD	glycerol-3-phosphate dehydrogenase
GRX	glutaredoxin
GSH	glutathione
GSSG	glutathione disulfide
HA	hemagglutinin
His	histidine
HMF	hydroxymethylfurfural
HPLC	high performance liquid chromatography
HXT	hexose transporter
H_2DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
Leu	leucine
LDH	lactate dehydrogenase
NEM	*N*-ethylmaleimide
OD	optical density

ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDC	pyruvate decarboxylase
Phe	phenylalanine
PPP	pentose phosphate pathway
qRT-PCR	quantitative reverse transcription PCR
RBS	ribosome binding sites
RFU	relative fluorescence units
RI	refractive index
ROS	reactive oxygen species
RP	ribosomal protein
SC	synthetic complete
SD	synthetic defined
SD-CAA	synthetic dextrose casamino acids
SG-CAA	synthetic galactose casamino acids
Trp	tryptophan
TRX	thioredoxin
Tyr	tyrosine
UAS	upstream activation sequence
Ura	uracil
YP	yeast extract-peptone

Chapter 1.

Research background and objective

Metabolic engineering of microorganism has been a very attractive method for the production of various materials, such as chemicals, fuels, and pharmaceuticals. To establish these objectives, combinations of deletion or overexpression of innate genes or introduction of heterologous genes has been used as gene manipulation methods.

Sugar uptake is an obligatory and rate-limiting step for metabolic engineering. Since microbial nutrient supply system is tightly regulated in response to intracellular needs and rapidly changing extracellular environments to maintain homeostasis, the artificial enhancement of this carbon source uptake is a very difficult but important topic for increasing the efficiency of microbial fermentation.

Yeast *S. cerevisiae*, known as a platform strain for microbial fermentation, is an important microorganism with various advantages, such as abundant genetic and physiological information, easiness of genetic manipulation, and higher tolerance to industrial conditions and alcohols.

The first objective of this study is to increase the glucose uptake in yeast by overexpression of hexose transporters and a transcription factor *GCR1*. Yeast can utilize various types of carbon sources, among which glucose is the most preferred carbon source for its fermentation. Glucose uptakes in *S. cerevisiae* is complexly regulated to adapt cellular metabolisms in response to ever-changing environmental conditions. By construction of a yeast strain which exhibits more diverse passages and faster consumption rates for glucose than wild-type strain does, production of ethanol or lactic acid was improved.

The second objective of this study is to increase fructose uptakes from glucose-fructose mixed media. Much researches have been conducted to use less purified carbon sources, most of which are mixture of various kinds of monosaccharides. Among them, some materials such as raw sugar and sugarcane molasses have high contents of glucose and fructose. Since yeast favors glucose over fructose as a carbon source, the development of the efficient uptake system for both glucose and fructose was conducted by overexpression of heterologous fructose transporters from the fructophilic yeast and hexokinases.

The last objective of this research is the development of a tolerant yeast strain to furan aldehyde, such as furfural and hydroxymethylfurfural (HMF), which is classified as one of the byproducts generated in the pretreatment step of lignocellulosic biomass. Lignocellulosic biomass is composed of two polysaccharides, cellulose and hemicelluloses, and lignin. For the dissociation of these components, the pretreatment step is necessary, but generation of byproducts is inevitable. These byproducts decrease enzymatic and microbial activities in the following steps, which lead to decreases in process efficiency and increases in overall costs. To remove these negative effects, development of the tolerant *Saccharomyces cerevisiae* strains to those inhibitors is an important issue for lignocellulosic ethanol production. We uncovered yeast response mechanism to furan aldehyde and developed the tolerant strain to them.

The objectives of this study are summarized as follows.

- To increase glucose uptake in yeast *S. cerevisiae* by overexpression of hexose transporter and a transcription factor *GCR1*.
- To increase yeast fructose uptake in fructose-glucose mixed media by overexpression of a heterologous fructose transporter and a few hexokinases.
- To develop tolerant yeast strain to furan aldehyde for efficient usage of lignocellulosic hydrolysate as a carbon source.

Chapter 2.

Literature review

2.1. Glucose and fructose uptake in *S. cerevisiae*

2.1.1. Overview of glucose and fructose uptake in *S. cerevisiae*

Yeasts are able to use a variety of sugars for the production of cellular biomass and metabolic energy. For the utilization of these carbon compounds, their transport is an obligatory and rate-limiting step, which is processed directly or after degradation to monosaccharides by extracellular hydrolases into cells, specifically mediated by their own transporters. These transporters across the plasma membrane belong to the highly homologous sugar-transporter family of the major superfamily (30).

Among the fermentable sugars, a few hexoses, such as glucose and fructose, are preferentially utilized by the cell, as when they exist in the growth media, the mechanism for their transportation and utilization is activated, and those for other carbon sources are repressed. The activated genes are related to the expression of glycolytic genes, ribosomal proteins and hexose transporters, which are necessary for efficient glucose metabolism. And the repressed genes are related to the expression of the genes required for the transport and utilization of the other carbon sources, gluconeogenesis and respiration.

2.1.2. The hexose transporter family in *S. cerevisiae*

In its natural habitat, a wide range of sugar concentrations are encountered by yeast *S. cerevisiae*, changing from almost zero to more than 1 M in fruit molasses for

wine fermentation (31). To survive from these extremely fluctuating sugar levels, complex regulatory systems for expression of different hexose transporters specific for each environmental condition exists in *S. cerevisiae*. By this regulation, the yeast cells are allowed to adapt to each sugar condition for the optimization of cell growth and metabolism.

This sugar transporter family consists of Hxt1p to Hxt17p for glucose and fructose, Gal2p for galactose, and Snf3p and Rgt2p for glucose sensors (32-36). These transporters belong to the major facilitator superfamily (MFS) consisting of various cellular transporters for a variety of metabolites (30).

The hexose transporters contain 12 putative membrane-spanning domains transporting hexoses across the plasma membrane via facilitated diffusion. The Snf3p and Rgt2p lost their ability to transport hexoses, but has a role in the glucose sensing for the *HXT* gene expression. And overexpression of any one of the *HXT1-17* (except of *HXT12*) or *GAL2* restored growth of *hxt-null* yeast strain, which is unable to grow on the culture media containing any sugar components (38, 39).

By analysis of kinetics and genetics of glucose transport in yeast *S. cerevisiae*, each individual HXT possess different substrate affinities, and their expression patterns depend on various factors, especially the extracellular concentration of glucose. And all of these HXTs can also mediate transport of the other sugars, such as fructose and mannose, with low affinities (38). Hxt1 (K_m ~ 100 mM) and Hxt3 (K_m ~ 30-60 mM) are classified as low-affinity glucose transporters. *HXT1* is highly induced in the presence of high concentrations of glucose (>1%), whereas

HXT3 is induced by both low and high levels of glucose. When the glucose concentrations are lowered to around 0.1%, *HXT2* and *HXT4*, encoding moderate-affinity glucose transporters (K_m ~ 5-10 mM), are expressed (43). *HXT6* and *HXT7*, encoding highly homologous glucose transporters with very high glucose affinity (K_m ~ 1 mM), are expressed just before the depletion of glucose in the medium (44). In contrast to the upper mentioned genes, expression of *HXT5* encoding moderate-affinity transporter (K_m ~ 10 mM) is not regulated by extracellular glucose concentrations but by various environmental conditions affecting growth rate (45-49).

Although the expression of other HXTs, *HXT8* to *HXT17*, is still poorly demonstrated, it was revealed that deletion of each or all of the genes does not affect to the growth on the media with various carbon sources, and all of these transporters (except to Hxt12p) is able to mediate hexose transport only if overexpressed (38). The Hxt12p has a similar sequence with Hxt11p, but its inability to transport hexose is likely caused by a 2-bp insertion. These results show that Hxt8p–Hxt17p can operate as hexose transporters, but cannot contribute to actual glucose transport under the normal condition. In addition to this, the other role of these minor HXTs is known as the target of Pdr1p and Pdr3p, which are transcription factors for the expression of ATP-binding cassette (ABC) transporters (50, 51). Since overexpression of these transcription factors can improve the tolerance to antifungals or xenobiotics when overexpressed, *HXT9* and *HXT11* genes are also expected to play a role as a kind of efflux pumps against the

inhibitors.(52).

2.1.3. The glucose signal in *S. cerevisiae*

Despite its highly similar sequence to the hexose transporters, the capability of Snf3 and Rgt2 for sugar uptake seems to be lost (38, 53), but their function as receptors for sensing extracellular glucose was operated instead. Rgt2p functions as a low affinity sensor for increasing the induction level of *HXT1* (low affinity glucose transporter) in high glucose condition, while Snf3p does as a high affinity sensor for the expression of *HXT2* and *HXT4* (intermediate affinity glucose transporter) in low glucose condition (53, 54).

Both Snf3 and Rgt2 have an additional domain compared to HXTs, which is a C-terminal tail in cytosol, as about 200 amino acid lengths for signaling from extracellular glucose into the yeast cell (53, 55, 56). Each C-terminal extension has a similar motif, which appears twice in Snf3 and once in Rgt2, but their overall sequences are quite different. This common motif is a key domain for the signaling for HXT expression, since deletion of this motif let the sensor proteins lose the ability. This additional extension in C-terminal of the sensor proteins is necessary for the glucose signaling, and it is possible that attaching them to normal Hxt proteins or their individual expression confer on the ability of glucose sensing (53, 55-57). However, it is not strictly indispensible, because overexpression of Rgt2 without C-terminal tail domains was functional (57).

2.1.4. Regulation of hexose transporter expression

The glucose signal from the Snf3/Rgt2 sensor proteins is transferred into Rgt1, a bifunctional transcriptional factor which both activates or represses the expression of *HXT* genes, depending on extracellular sugar (Fig. 2.1).

Rgt1p is a constitutively expressed protein located only in the nucleus (54, 58), containing an N-terminal C_6Zn_2 zinc cluster DNA-binding domain (59). This domain allows to bind to a number of sites in the upstream of *HXT* genes for repressing their expressions via recruitment of the general repressor complex, consisting of Ssn6 and Tup1, in the glucose depletion media (54, 59-63). In addition to this, this repression activity of Rgt1 in HXT expression requires Mth1 and Std1 (62, 64-66).

The co-repressors, Mth1 and Std1 are able to bind to Rgt1p. Mth1 operates primarily as a repressor against the transcription of *HXT* in absence of glucose, and Std1 did the same functions in the media containing almost exhausted glucose (67-69). Although it was known that Mth1 and Std1 can bind to Rgt1 in the nucleus (66) and to sensor proteins at the cell surface (65, 70), it has not yet been revealed whether the regulation in subcellular localization exists or not (71).

At the glucose-containing media, glucose binds to the Snf3/Rgt2 sensor proteins on the plasma membranes, leading to the phosphorylation of the corepressors, Mth1 and Std1, which primes them to the ubiquitination mediated by SCF-Grr1 complex for subsequent degradation via 26S proteasome (57, 62, 67, 72, 73).

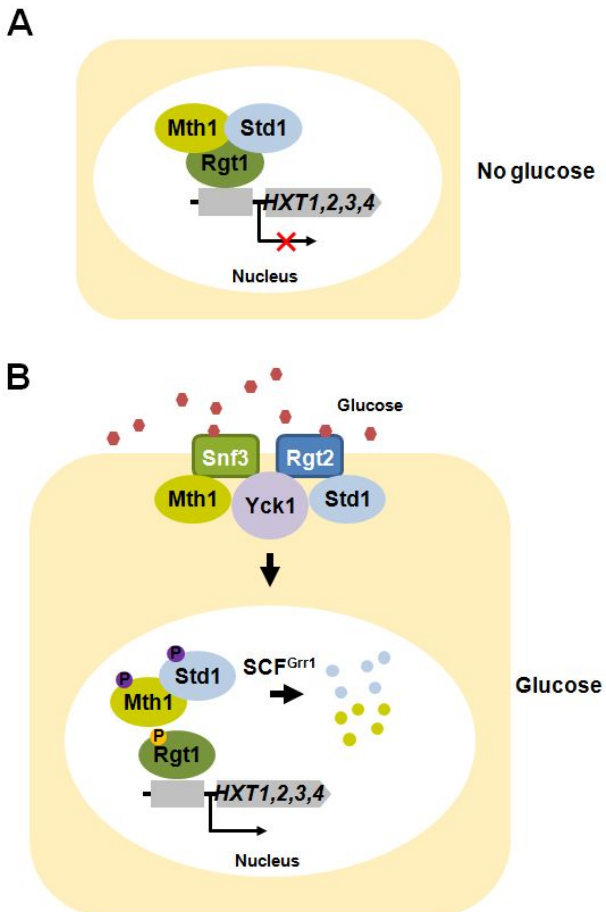

Figure 2.1 The Snf3p/Rgt2p–Rgt1p pathway in *S. cerevisiae*

A. In the absence of glucose, Rgt1p with corepressor Mth1p and Std1p repress the transcription of the *HXT* genes. B. When glucose binds to Snf3/Rgt2 sensor proteins, Yck1/2p are activated, phosphorytlating Mth1p and Std1p, which are bound to the Snf3p/Rgt2p C-terminal tails. This phosphorylation is led to the ubiquitination by SCF-Grr1 complex for degradation by the 26S proteasome. With the phosphorylation of Rgt1 by PKA, this releases Rgt1p from promoter sites of *HXTs* and results in derepression of *HXT* genes.

For phosphorylation of the corepressor proteins, Yck1/2p, a pair of type I casein kinases in membrane involved in many cellular processes, are required (57). The glucose signaling from the sensor protein to Yck1/2p is transferred from the plasma membrane into the nucleus (74). By the degradation of Mth1 and Std1 (62, 67), Rgt1 domain for phosphorylation by PKA is exposed, converting the activity of Rgt1 from repressor to activator for the expression of HXT (62, 67) (57). When Rgt1p is phosphorylated by PKA, phosphorylation in its four N-terminal putative PKA consensus sequences [RR/KSXS(T)] (S146, S202, S283 and S284) may be occurred, which is led to decreasing DNA-binding activity and repression power of Rgt1p (75).

In media containing non-fermentable carbon sources, PKA becomes an inactive heterotetrameric holoenzyme, which consists of two catalytic subunits encoded by one TPK (redundancy among *TPK1/2/3*) and two regulatory subunits, encoded by *BCY1*. In media containing glucose, PKA is activated via adenylate cyclase Cyr1p, the role of which is synthesizing cAMP. Increased intracellular cAMP level induces dissociation of PKA catalytic subunits from the inactive complex, resulting in the activation of PKA. Cyr1 is activated by two glucose-responsive systems; (i) extracellular glucose sensed by the plasma-membrane gPCR complex, a receptor Gpr1p with a G protein Gpa2; (ii) Ras1/2-mediated phosphorylation (76-81). Therefore, these two distinctive glucose-triggered events should be happened for the removal of the Rgt1 repressor from the *HXT* promoters. Elimination of Mth1 and Std1 must be preceded by degradation via the Snf3/Rgt2-

Rgt1 glucose sensing pathway, led to Rgt1p phosphorylation via the cAMP-PKA glucose sensing process.

Ultimately, this signaling process is transferred to the induction of *HXTs* depending on the different levels of glucose. In the media containing low concentration of glucose, Rgt1 is not completely phosphorylated because of not fully activated PKA, inducing *HXT* genes encoding high affinity glucose transporters, *HXT2* and *HXT4*. And in the media containing high concentration of glucose, fully activated PKA phosphorylates Rgt1 completely, inducing low affinity transporters, *HXT1* and *HXT3*, by intramolecular interaction of Rgt1 (67, 72).

2.1.5. Glucose repression by Hxk2 in *S. cerevisiae*

It has been shown that in yeast cells, sensing the availability of intracellular glucose is mediated by the hexokinase isoenzyme 2 (Hxk2) (82, 83) (Fig. 2.2). It has been known that the signal originated from intracellular glucose represses several genes involved in gluconeogenesis, respiration, and usage of alternative carbon sources. However, not until later, it was revealed that Hxk2 is required for this process (84). In the cytosol, Hxk2 is known to phosphorylase 6^{th} carbon of glucose as an initial step of glycolysis. But about a decade ago, it was demonstrated that Hxk2 is also able to be localized to the nucleus (85, 86). In the nucleus, with the association with the transcription factor Mig1, it has been revealed that Hxk2 inhibits expression of several genes such as SUC2 invertase (87-89). In addition to

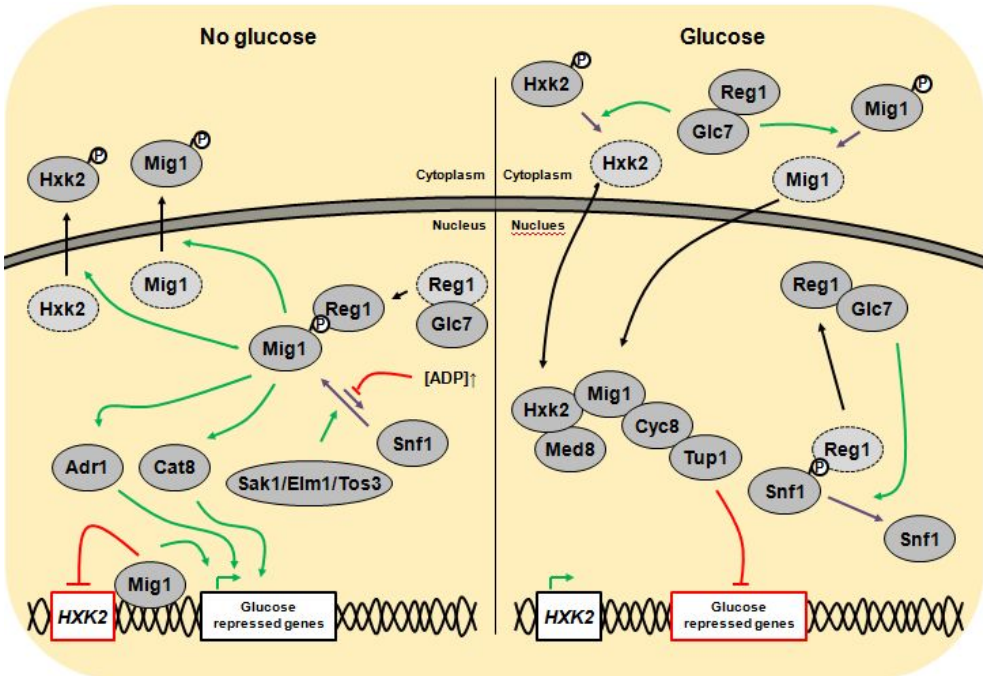

Figure 2.2 The glucose repression pathway of Mig1-Hxk2 and regulation of Snf1 in *S. cerevisiae.*

In glucose condition, Mig1 and Hxk2 are dephosphorylated by Reg1-Glc7 and moved into the nucleus. After that, Mig1 and Hxk2 represses multiple genes by interacting with Med8 and Cyc8-Tup1 via glucoes repression in nucleus. Moreover, the activity of Snf1 is decreased by an uncovered pathway, in which Reg1-Glc7 is likely to be relative. When glucose is depleted, both of Mig1 and Hxk2 are phosphorylated by Snf1 and move out of the nucleus. At last, Med8 derepresses transcription of glucose-repressed genes and blocks Hxk2 expression. In addition to that, Snf1 is activated under low glucose conditions and also participates in derepression of glucose-repressed genes. Activation of glucose-repressed genes partly occurred with the help of Adr1 and Cat8. The stimulation of Snf1 is mediated by ADP, which prevents Snf1 from dephosphorylation by Reg1-Glc7. A green arrow means activation, a red line or box means inhibition, a purple arrow points to a metabolic reaction, and a black arrow presents a relocation.

that, Hxk2 has been observed to negatively regulate Med8, a subunit of the RNA polymerase II mediator complex (90). It is interesting that the dual effects of Med8, a repressing effect on *HXK2* gene expression or a stimulating effect on *SUC2* (Fig. 2.2) (91). Besides, derepression of many other glucose-repressed genes, including those related to gluconeogenesis and respiration, needs the Snf1-mediated activation of transcriptional regulators, including Cat8 and Adr1 (92-94) (Fig. 2.2).

In the condition of high concentration of glucose, localization of Mig1 is in the nucleus (Fig. 2.2) (88). It has been known that Mig1 is able to directly interact with Hxk2, and the distribution of Hxk2 between the nucleus and the cytoplasm may depend on Mig1, but the mechanism has not been uncovered. (87, 89). And reversely, localization of Mig1 is also depends on Hxk2 by the coverage of Ser311 on Mig1. Blocking Ser311 inhibits nuclear export by phosphorylation on this specific residue (88). In addition to its interaction with Med8, Hxk2-Mig1 complex also binds to the repressors, Cyc8 and Tup1, thus forming the glucose-repressor complex (Fig. 2.2)(95). Especially, phosphorylation of Ser311 on Mig1 by Snf1 causes the dissociate of Mig1 from Cyc8-Tup1, and this specific residue on Mig1 is shown to be necessary for derepression of the SUC2 gene (88).

In addition to the phosphorylation of Mig1, it was known that Snf1 also phosphorylates Ser14 on Hxk2 in low glucose conditions (96, 97). Phosphorylation on Hxk2, like Mig1, induces its export from the nucleus (Fig. 2.2) (98). Moreover, it has been demonstrated that dephosphorylation of both Hxk2 and Mig1 is required to relocalize to the nucleus (88). Both Hxk2 and Mig1 is

dephosphorylated with the mediation of Glc7, the catalytic subunit of protein phosphatase 1 (PP1) (Fig. 2.2) (85, 86, 97, 99). There are many different targets of Glc7, but this dephosphorylation is specifically related to glucose repression by its regulatory subunit, Reg1 (99, 100). And the Snf1 protein kinase is another important target of this Reg1-Glc7 phosphatase complex. Snf1, the yeast homolog of AMPK, is also involved in the regulation of already demonstrated glucose metabolism, such as regulation of the *HXT*- and glucose-repressed genes, as well as many other intracellular processes, including lipid metabolism, aging, growth, and response to various kinds of stresses (101-103). The activation of Snf1 is mediated by phosphorylation of Thr210 performed by three redundant upstream kinases, Sak1, Elm1, and Tos3. On the contrary to this, inactivation of Snf1 depends on the dephosphorylation mediated by Reg1-Glc7 (Fig. 2.2)(99, 104-107). Although phosphorylation of Snf1 is increased under low glucose condition (104), but activity of Reg1-Glc7 complex or the three upstream kinases is shown to be independent of glucose concentrations (99). Instead, the accessibility of Reg1-Glc7 to Thr210 on Snf1 has been suggested as the mode of controlling Snf1 activity (99). Remarkably, it has previously appeared that Reg1 directly binds to Snf1 (108, 109). Whether Reg1 interacts with Glc7 or Snf1 has been suggesed as the important step in the regulation of Snf1 activity. Consequently, it has been recommended that the interaction between Reg1 and Snf1 blocks Thr210 on Snf1 and thereby inhibits Glc7-mediated dephosphorylation (Fig. 2.2) (99, 110). Also, it was recently shown that ADP, but not AMP, prevents dephosphorylation of Thr210 on Snf1, hence

activating the kinase (103). Likewise, it has also appeared that ADP guards against dephosphorylation in mammalian cells (111), proposing an evolutionarily conserved regulation of AMPK/Snf1.

2.2. Tolerance to Furan aldehydes in *S. cerevisiae*

2.2.1. Overview of lignocellosic biomass

Lignocellulosic biomass is one of the cheapest and most abundant material for the production of the feedstock of fuel and chemicals. This lignocelluloses are composed of three polymers, cellulose, hemicelluloses and lignin. The composition of these components can vary wildly (Table 2.2) (3-6, 112). Lignin and hemicellulose have crosslinked structures, surrounding cellulose. Cellulose is a kind of polysaccharides composed of from several hundreds to thousands of glucose with beta-1,4 glycosidic chains (113).

Cellulose has a crystalloid property, which hinders enzymes or chemicals access to itself. Hemicellulose consists of various kinds of monomer sugars, such as glucose, xylose, mannose, galactose and arabinose. This hemicellulose has branched and amorphous properties, by which its ease of hydrolysis is induced. Lignin is a cross-linked polymer, composed of ether and other carbon-bound prophanoid. The main components of this lignin are phenolic compounds, including *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol.

Table 2.1 The composition of common lignocellulosic-biomass materials

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40–55	24–40	18–25
Hardwoods barks	22-40	20-38	30-55
Softwood stems	45–50	25–35	25–35
Softwood barks	18-38	15-33	30-60
Nut shells	25–30	25–30	30–40
Corn cobs	45	35	15
Cornstalks	39-47	26-31	3-5
Grasses	25–40	35–50	10–30
Paper	85–99	0	0–15
Wheat straw	37-41	27-32	13-15
Sorted refuse	60	20	20
Leaves	15–20	80–85	0
Cotton seed hairs	80–95	5–20	0
Newspaper	40–55	25–40	18–30
Chemical pulps	60–80	20–30	2–10
Primary wastewater solids	8–15	*NA	24–29
Swine waste	6.0	28	*NA
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

*NA : not available

(2-7)

The overall process of lignocellulosic biomass is divided into four serial events; pretreatment, hydrolysis, fermentation and distillation (114). Pretreatment is necessary for the accessibility for the microbial process, and hydrolysis is a prerequisite of the saccharification for fermentation. Fermentation is the actual production step for target materials, and distillation is the purification of target.

2.2.2. Lignocellosic inhibitors

Lignocellulosic materials are recalcitrant to hydrolysis, so a pretreatment processing step is required, under severe conditions for the removal of the lignin and depolymerization of cellulose and hemicelluloses, making them more amenable biomass to hydrolytic enzymes (114).

The actual pretreatment steps are broadly divided into some areas; physical (milling and grinding) / physicochemical (steam pretreatment / autohydrolysis, hydrothermolysis, and wet oxidation) / chemical (alkali, dilute acid, oxidizing agents, and organic solvents) / biological / electrical / the mix of these methods (115). To apply these to the industrial process, economical efficiency is one of the most important thing, in which criterion the thermo-chemical treatment, such as dilute-acid or steam-explosion, is the most preferential treatment method. This treatment induces formation of byproducts that decrease the activities of next steps. These byproduct are categorized into 3 groups; furan aldehydes, phenolics, and weak acids (116-118).

Furan aldehydes, which are found in almost every hydrolysates, are 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF), formed by the dehydration of 5 or 6 carbon sugars (119, 120) (Table 2.1). Weak acids are including acetic acid, formic acid, and levulinic acid, formed by deacetylation of hemicellulose or HMF breakdown. And phenolics, such as vanillin, syringaldehyde, and conferyl aldehyde, are produced by the degradation of lignin (40, 121).

These inhibitors negatively affect the enzymatic degradation and fermentation process, and the level of the negative effects depends on property and concentration of each inhibitor. Among them, furfural and HMF decrease enzymatic activities, break down DNA, and inhibit protein and RNA synthesis (122, 123). Weak acid induces low pH stress, and formic acid is the most toxic because of the lowest pKa and small sizes, which is thought to enhance its transport via cell wall to lower cytosolic pH and inhibits the microbial growth. The phenolic compounds are also toxic, although their inhibitory mechanism is not fully known.

2.2.3. Response to furan aldehyde in *S. cerevisiae*

Furan aldehydes, representative of furfural and HMF, are produced in most of the pretreatment methods, regardless of the kind of lignocellulosic materials (Table 2.1). Furfural and HMF are respectively produced from the dehydration of 5-carbon and 6-carbon sugar (124).

In *S. cerevisiae*, Both Furfural and HMF reduce enzymatic and biological

Table 2.1 The contents of lignocellulosic inhibitors according to the pretreatment methods

Feedstock	Pretreatment	Acetate (g/L)	Formate (g/L)	Furfural (g/g)	5-Hydroxymethylfurfural (g/L)	Phenol (g/L)	Reference
Softwood							
Pine	Acid STEX	2	NA	0.7	1.7	NA	(11)
Fir	SO_2 STEX	NA	NA	2.3	1.8	NA	(15)
Spruce	SO_2 STEAM	4.2	NA	1.3	2.0	NA	(18)
Spruce	Acid STEAM	2.8	0.7	1.4	2.3	2.9	(22)
Spruce	Acid STEAM	2.4	1.6	1.0	5.9	0.64	(23)
Spruce	Acid STEX	2.4	NA	0.6	1.5	NA	(11)
Spruce bark	Acid STEX	0.0	NA	0.7	0.4	NA	(11)
Hardwood							
Alder	Acid STEX	10.7	NA	3.2	2.6	NA	(11)
Aspen	Acid STEX	9.1	NA	3.5	2.2	NA	(11)
Birch	Acid STEX	2	NA	0.5	0.2	NA	(11)
Willow	Acid STEX	NA	NA	0.3	0.7	NA	(11)
Willow	SO_2 STEAM	5	NA	NA	NA	NA	(37)
Willow	SO_2 STEAM	NA	NA	NA	NA	2.5	(40)
Poplar	STEX	NA	NA	NA	NA	1.8	(41)
Hardwood							
Alder	Alkaline WO	5.8	6.5	0.0	0.0	2.7	(42)

*NA : not available

activities, break down DNA, and inhibit protein and RNA synthesis. And furfural inhibits glycolytic enzymes and aldehyde dehydrogenase activity, resulting in accumulation of acetaldehyde that would be responsible for the lag phase during growth of yeast in the presence of furans (125).

With the help of microarray or omics analysis, genetic response mechanisms to those inhibitors have been investigated (126-128). Based on this precedent information, there are many trials to construct a tolerant strain to these furan aldehydes. Originally, it was shown that the detoxification by reduction of furfural or HMF is performed by both NADPH- and NADH-coupled reductase or dehydrogenase (Fig. 2.2) (129). And the pentose phosphate pathway genes, such as *ZWF1*, *GND1*, *RPE1* and *TKL1* in *S. cerevisiae* were found to be associated with tolerance to furfural-induced stress (130). Also, overexpression of innate or heterologous genes increased tolerance to furfural or HMF, which are shown in Table 2.3.

2.2.4. Response to oxidative stress in *S. cerevisiae*

Among researches that analyze the global data about yeast genetic response to furan aldehyde stress, there is a microarray analysis in HMF stress condition (126). It was shown that this HMF stress is categorized into 5 types in yeast; oxidative stress, protein degradation, protein protection, DNA repair and multidrug resistance. Notably, a transcription factor Yap1 belongs to all of these categories, which is

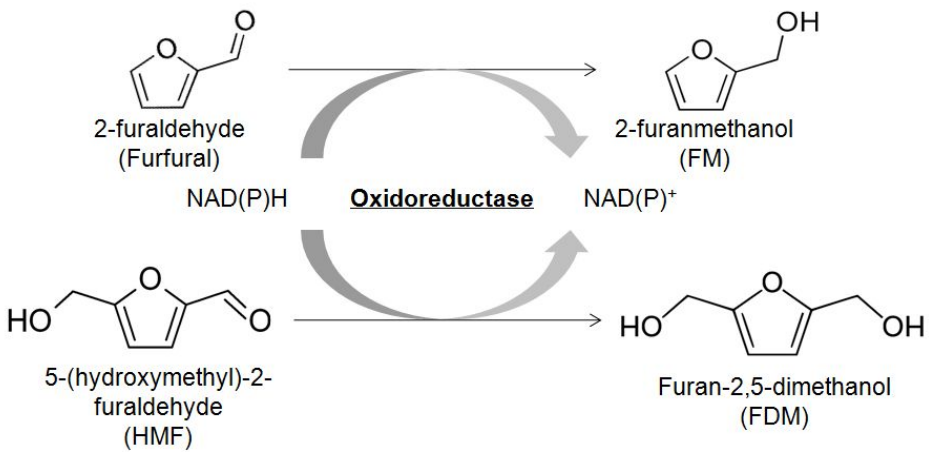

Fig 2.2 The Detoxification process of furan aldehyde by oxidoreductase with the help of NAD(P)H in *S. cerevisiae* (8)

Table 2.3 Strain improvement by targeted genetic engineering

Gene	Strain	Identification	Experimental conditions	Strain improvement	Reference
ADH6	*Saccharomyces cerevisiae*	Microarray analysis	Pulse addition of 1.5 g/L HMF in (an)aerobic batch cultivation in mineral medium	Four times higher specific uptake rate of HMF	(1)
			Batch fermentation of spruce hydrolysate	Four times higher specific uptake rate of HMF and 20% higher specific ethanol productivity	(13)
MUT-*ADH1*	*Saccharomyces cerevisiae*	Protein purification, MS, and gene isolation	Semi-aerobic growth in mineral medium supplemented with 20 mM HMF	Reduction of lag phase and two times faster HMF conversion	(17)
	TMB3000 strain		Batch fermentation of spruce hydrolysate	Four times higher specific uptake rate of HMF and 18% higher specific ethanol productivity	(13)
ADH7	*Saccharomyces cerevisiae*	Microarray analysis	Growth in minimal medium supplemented with 40 mM HMF	Exit lag phase after 94 h While the control failed to grow even after 156 h	(1, 20, 21)
XYL1	*Pichia stipitis*	Protein purification	Growth in minimal medium	Increased *in vivo* HMF	(25)
ZWF1	*Saccharomyces cerevisiae*	Screening of a *Saccharomyces cerevisiae* gene disruption library	Growth in SD-complete supplemented with 50 mM furfural	Allowed growth at this Furfural concentration, which was lethal for control strain	(26)
FFR	*Escherichia coli LYO1*	Protein purification	Data not available		(28)

* ADH6, alcohol dehydrogenase 6; ADH7, alcohol dehydrogenase 7; HMF, 5-hydroxymethyl-2-furaldehyde; FFR, furfural reductase; MS, mass spectrometry; MUT-ADH1, mutated alcohol dehydrogenase 1; SD, synthetic defined *medium*; XYL1, xylose reductase; ZWF1, glucose-6-phosphate dehydrogenase

known as the most important transcription factor for oxidative stress in yeast *S. cerevisiae* (131).

The activation process of Yap1p is divided into 2 types (132, 133). The first activation mechanism is originated from H_2O_2 stress, mediated with Gpx3p. Yap1p is normally bound with an nuclear exportin protein, Crm1p, which induces free transport between cytosol and nucleus. In H_2O_2 stress, cysteine on Gpx3p oxidized. This oxidized Gpx3p transfer this oxidizing power into Yap1p, which form disulfide bond between the cysteine-rich domains of both of its middle domain and C-terminal domain. Crm1p is dissociated by this Intramolercular disulfide bond, resting Yap1p in the nucleus to induce target genes for the response to the stress condition.

The second activation mechanism is mediated from the chemicals which have reactivity with thiols, such as electrophiles and divalent heavy metals cations, directly binding into cysteine-rich domain in C-terminal of Yap1p to dissociate Crm1p, independent of Gpx3p. And the target genes of Yap1 in response to each stress are a little different(133) .

2.2.5. Roles of Glutathione in *S. cerevisiae*

Glutathione(GSH) is a tri-amino acid peptide, composed of cysteine, glycine, and glutamate. For the biosynthesis of GSH in *S. cerevisiae*, *GSH1* firstly forms L-glutamyl-cysteine from glutamate and cysteine, and *GSH2* connects that with glycine (134). In addition to this endogenous biosynthesis, GSH is taken up from

the extracellular environment via Hgt1 (135).

GSH is used as a bullet for the activity of glutaredoxin(GRX), glutathione S-transferase(GST), glutathione transferase(GTT), and glutathione peroxidase(GPX). These enzymes are small proteins which acts as a thiol oxidoreductases responsible for reducing protein disulphides or producing glutathione–conjugated disulphides (136). The conjugated form(GS-X) is transported via the yeast vacuolar pump Ycf1p, conferring resistance to various inhibitors, such as some kinds of electrophiles and metals (137-139).

And with the help of GSH as hydrogen donor, the cysteine residues from the enzyme active site are involved in the reaction. After the oxidation of GSH, the dimer disulphide form(GSSG) are produced, which are regenerated by glutathione reductase(*GLR1*) with NADPH as the electron donor (134, 140).

Chapter 3.

Materials and methods

3.1. Strains and media

All strains used in this study are listed in Table 3.1. *E. coli* strain DH5α [F^- Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^-, m_K^+) *phoA supE44* λ^- *th*i-*1 gyrA96 relA1*] was used for genetic manipulations. *E. coli* was cultured in Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with 50 μg/mL ampicillin.

S. cerevisiae strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), *bdh1Δ* (BY4741, *bdh1Δ::KanMX6*), and CEN.PK2-1C (*MATa ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8C SUC2*) were obtained from EUROSCARF. The gene disruption mutants were constructed by using the Cre/*loxP* recombination system (141). The gene deletion cassette was obtained by PCR amplification from pUG27, pUG72 or pUG73 as template, using a gene-specific primer pair of d_ORF F and d_ORF R. After confirmation of the correct integration of the cassette at the target gene locus through PCR analysis using the confirmation primers (c_ORF F and c_ORF R), the marker gene was removed by transformation of Cre recombinase-expression vector, pSH63 or p414G-Cre. Additional gene deletion was sequentially conducted using the same procedure. Lactic acid-producing strains, SP2006 and SP1130, possessing lactate dehydrogenase (*LDH*) genes originating from *Pelodiscus sinensis* subspecies *japonicus* (*PsjLDH*) or *Bos taurus* (*BtLDH*) were described previously (142). In CEN.PK2-1C, open reading frames (ORFs) of *ADH1*, *PDC1*, *GPD1*, and *CYB2* genes were replaced with either *BtLDH* or

Table 3.1 Strains used in this study

Strain	Genotype	Reference
E. coli		
DH5α	F$^-$ Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^-, m_K^+) *phoA supE44* λ$^-$ *th*i-*1 gyrA96 relA1*	
S. cerevisiae		
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
CEN.PK2-1C	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8C SUC2*	EUROSCARF
gpx3Δ	BY4741, *gpx3Δ::KanMX6*	EUROSCARF
hgt1Δ	BY4741, *hgt1Δ::KanMX6*	This study
JHY701	CEN.PK2-1C *mth1Δ::loxP*	This study
JHY702	CEN.PK2-1C *std1Δ::loxP*	This study
JHY703	CEN.PK2-1C *mth1Δ::loxP std1Δ::loxP*	This study
SP2006	CEN.PK2-1C *pdc1Δ::*P_{ccw12}*-BtLDH cyb2Δ::*P_{ccw12}*-BtLDH gpd1Δ::*P_{ccw12}*-PsjLDH adh1Δ::*P_{ccw12}*-PsjLDH*	This study
SP2018	SP2006 *his3Δ::*P_{TDH3}*-GCR1*	This study
SP1130	SP2006 *ald6Δ::loxP leu2Δ*::P_{TDH3}*-mhpF pdc6Δ*::P_{TDH3}*-eutE*	This study
SP1141	SP1130 *his3Δ*::P_{TDH3}*-GCR1*	This study

PsjLDH gene controlled by the *CCW12* promoter, resulting in strain SP2006 (142). To generate strain SP1130, P_{TDH3}-controlled *E. coli mhpF* and *eutE* genes, encoding acetylating acetaldehyde dehydrogenase, were introduced into the chromosome of SP2006, and *ALD6* was additionally deleted (142). To generate strains SP2018 and SP1141, overexpressing *GCR1*, the P_{TDH3}-*GCR1*/*HA*-*URA3*-*HA* cassette was PCR amplified using primers i_*GCR1* F and i_*GCR1* R from the plasmid pUC57-URA3-*GCR1*, and integrated into the chromosome of SP2006 and SP1130, respectively, by replacing *his3Δ1*. After confirmation of the correct integration of the cassette by PCR analysis using a primer pair of ch_*HIS3* F and ch_*GCR1* R, the *URA3* marker flanked with HA tags was popped-out (143).

Yeast cells were cultured in YP medium (10 g/L yeast extract and 20 g/L bacto-peptone) supplemented with 20, 50, or 80 g/L glucose (YPD10), in synthetic complete (SC) medium (6.7 g/L yeast nitrogen base without amino acids, 1.4 g/L amino acids dropout mixture lacking His, Trp, Leu, and Ura) supplemented with auxotrophic amino acids as required and 20, 50, or 80 g/L glucose, or in synthetic defined (144) medium (20 or 50 g/L glucose, 6.7 g/L yeast nitrogen base without amino acids) supplemented with auxotrophic requirements (120 μg/mL Leu and 50 μg/mL each of His, Met, and Ura).

Table 3.2 Primers used for strain construction (gene manipulation)

Primers	Sequence (5'-3')
d_MTH1 F	GAATTTTATTCGAACGCATAGAGTACACACACTCAAAGGACAG CTGAAGCTTCGTACGC
d_MTH1 R	TCCAAAAAAACCATCGGGAAGGTTTCTTTTTAGTATCTGCATA GGCCACTAGTGGATCTG
d_STD1 F	GTAGGAGGTTTTGCACTACTTAACAGACAAATAAAACGAGCAG CTGAAGCTTCGTACGC
d_STD1 R	AGGACATTCCATCAGGCTTCCAATCTAACCCCAACCTTGCATA GGCCACTAGTGGATCTG
i_GCR1 F	TATACTAAAAAATGAGCAGGCAAGATAAACGAAGGCAAAGGGT TTCCCGACTGGAAAGC
i_GCR1 R	ATATATCGATTGCTGCAGCTTTAAATAATCGGTGTCACTACCA GTCACGACGTTGTAAAA
ch_MTH1 F	TTCCTTTCTTCTCAAACTTT
ch_MTH1 R	TCTTTACTGCGATAACGGT
ch_STD1 F	GTTCCATAACGGAAAAATT
ch_STD1 R	ACTTAATCTTAAAGGTCGT
ch_HIS3 F	ATGCCTCGGTAATGATTTTC
ch_GCR1 R	CCGCTAGTAGCGTAATCTG
Ura-B	CAGACCGATCTTCTACCC
Ura-C	TTGGCTAATCATGACCCC

3.2. Plasmids

Plasmids used in this study are listed in Table 3.3. *YAP1* and *ZWF1* ORFs were amplified by PCR and cloned into the XbaI and XhoI sites of a pRS415ADH vector. $YAP1^{C620F}$ mutant was generated by overlapping PCR, and cloned into the XbaI and XhoI sites of pRS415ADH. PCR-amplified *CTA1* and *CTT1* ORFs were cloned into the HindIII and XhoI sites of pRS416ADH, and *GSH1*, *GSH2*, and *GLR1* ORFs were cloned into the XbaI and XhoI sites of pRS416ADH.

HXT1, *HXT2*, *HXT3*, and *HXT4* ORFs were amplified by PCR using the primers shown in Table 2, and inserted between the BamHI and ClaI sites of the pRS414GPD plasmid vector (10). *HXT7* ORF was inserted between the BamHI and XhoI sites of the pRS414GPD, and *GCR1* and *GCR2* ORFs were inserted between the XbaI and XhoI sites of the pRS416GPD and pRS415GPD, respectively. The P_{TDH3}-*GCR1*-T_{CYC1} fragment was amplified by PCR using primers c_*GCR1* F and c_*GCR1* R from the pRS416GPD-*GCR1* and cloned into the KpnI site of the pUC57-*URA3* (142), generating pUC57-*URA3*-*GCR1*.

Table 3.3 Plasmids used in this study

Plasmid	Description	Reference
p414GPD	CEN/ARS, *TRP1*, P_{TDH3}, T_{CYC1}	(10)
P415GPD	CEN/ARS, *LEU2*, P_{TDH3}, T_{CYC1}	(10)
p416GPD	CEN/ARS, *URA3*, P_{TDH3}, T_{CYC1}	(10)
p415ADH	CEN/ARS, *LEU2,* P_{ADH1}, T_{CYC1}	(10)
p416ADH	CEN/ARS, *URA3,* P_{ADH1}, T_{CYC1}	(10)
pUG72	Plasmid containing *loxP-Kl.URA3-loxP* deletion cassette	EUROSCARF
pUG73	Plasmid containing *loxP-Kl.LEU2-loxP* deletion cassette	EUROSCARF
pSH63	CEN/ARS, *TRP1*, P_{GAL1}-*cre*-T_{CYC1}	EUROSCARF
p415ADH-YAP1	CEN/ARS, *TRP1*, P_{ADH1}-*YAP1*-T_{CYC1}	This study
p415ADH-ZWF1	CEN/ARS, *TRP1*, P_{ADH1}-*ZWF1*-T_{CYC1}	This study
p415ADH-YAP1^{C620F}	CEN/ARS, *TRP1*, P_{ADH1}-*YAP1*^{C620F}-T_{CYC1}	This study
p416ADH-CTA1	CEN/ARS, *TRP1*, P_{ADH1}- *CTA1*-T_{CYC1}	This study
p415ADH-CTT1	CEN/ARS, *TRP1*, P_{ADH1}- *CTT1*-T_{CYC1}	This study
p415ADH-GSH1	CEN/ARS, *TRP1*, P_{ADH1}- *GSH1*-T_{CYC1}	This study
p415ADH-GSH2	CEN/ARS, *TRP1*, P_{ADH1}- *GSH2*-T_{CYC1}	This study
p415ADH-GLR1	CEN/ARS, *TRP1*, P_{ADH1}- *GLR1*-T_{CYC1}	This study
p415ADH-ZWF1	CEN/ARS, *TRP1*, P_{ADH1}-*ZWF1*-T_{CYC1}	This study
p414GPD-HXT1	CEN/ARS, *TRP1*, P_{ADH1}- *HXT1*-T_{CYC1}	This study
p414GPD-HXT2	CEN/ARS, *TRP1*, P_{ADH1}- *HXT2*-T_{CYC1}	This study
p414GPD-HXT3	CEN/ARS, *TRP1*, P_{ADH1}- *HXT3*-T_{CYC1}	This study
p414GPD-HXT4	CEN/ARS, *TRP1*, P_{ADH1}- *HXT4*-T_{CYC1}	This study
p414GPD-HXT7	CEN/ARS, *TRP1*, P_{ADH1}- *HXT7*-T_{CYC1}	This study
p416GPD-GCR1	CEN/ARS, *TRP1*, P_{ADH1}- *GCR1*-T_{CYC1}	This study

Table 3.3 Plasmids used in this study (*Continued*)

Plasmid	Description	Reference
p416GPD-GCR1	CEN/ARS, *TRP1*, P_{ADH1}- *GCR1*-T_{CYC1}	This study
p416GPD-GCR2	CEN/ARS, *TRP1*, P_{ADH1}- *GCR2*-T_{CYC1}	This study
pUC57-URA3	Plasmid containing *HA*-*URA3*-*HA* deletion cassette	This study
pUC57-URA3–*GCR1*	P*TDH3*-*GCR1*-T*CYC1* cloned into the KpnI site of pUC57-URA3	This study

Table 3.4 Primers used for gene cloning

Primers	Sequence (5'-3')
YAP1F	GCGTCTAGAATGAGTGTGTCTACCGCC
YAP1R	GCGCTCGAGTTAGTTCATATGCTTATTCAA
ZWF1F	GCGTCTAGAATGAGTGAAGGCCCCGTC
ZWF1R	GCGCTCGAGCTAATTATCCTTCGTATCTTC
CTA1F	GCGAAGCTTATGTCGAAATTGGGACAA
CTA1R	GCGCTCGAGTCAAAATTTGGAGTTACTCGA
CTT1F	GCGAAGCTTATGAACGTGTTCGGTAAA
CTT1R	GCGCTCGAGTTAATTGGCACTTGCAATGGA
GSH1F	GCGTCTAGAATGGGACTCTTAGCTTTG
GSH1R	GCGCTCGAGTTAACATTTGCTTTCTATTGA
GSH2F	GCGTCTAGAATGGCACACTATCCACCT
GSH2R	GCGCTCGAGCTAGTAAAGAATAATACTGTC
GLR1F	GCGTCTAGAATGCTTTCTGCAACCAAA
GLR1R	GCGCTCGAGTCATCTCATAGTAACCAATTC
Forward fragment of YAP^{C620F} R	GCCATTAGCTCGGAA*A*ATAAACCATCGACATC
Backward fragment of YAP1^{C620F} F	GATGTCGATGGTTTAT*T*TTCCGAGCTAATGGC
HXT1 F	CGCGGATCCATGAATTCAACTCCCG
HXT1 R	CGCATCGATTTATTTCCTGCTAAACAAAC
HXT2 F	CGCGGATCCATGTCTGAATTCGCTACTAG
HXT2 R	CGCATCGATTTATTCCTCGGAAACTCT
HXT3 F	CGCGGATCCATGAATTCAACTCCAGATTT
HXT3 R	CGCATCGATTTATTTCTTGCCGAACAT

Restriction enzyme sites are underlined

Table 3.4 Primers used for gene cloning (*Continued*)

Primers	Sequence (5'-3')
HXT4 F	CGCGGATCCATGTCTGAAGAAGCTGC
HXT4 R	CGCATCGATCTACTTTTTTCCGAACATC
HXT7 F	CGCGGATCCATGTCACAAGACGCTG
HXT7 R	CGCCTCGAGTTATTTGGTGCTGAACA
GCR1 F	CGCTCTAGAATGGTATGGTATGATCATAACA
GCR1 R	CGCCTCGAGTTAAGATGGTGTATTATGTCG
GCR2 F	CGCTCTAGAATGCATCACCAAACTAAGT
GCR2 R	CGCCTCGAGTCATCTTTGTAAATCCCTTAA
c_GCR1 F	CGCGAGCTCTCATTATCAATACTCGCCATTTCA
c_GCR1 R	CCCGAGCTCCAAATTAAAGCCTTCGAGCG

Restriction enzyme sites are underlined

Table 3.5 Primers used for RT-PCR

Primers	Sequence (5'-3')
YAP1 F	ATGATGTCGTTCCATCTAAGGAAGG
YAP1 R	CAACCCCTCTTTCTGAACATTTTGC
TRX2 F	AAAGTTTGCAGAACAATATTCTGACG
TRX2 R	TTGGCACCGACGACTCTGGTAACC
MET16 F	AACAGTATATAGATGCAAACAATGTAC
MET16 R	CACACTCGGTCTTGGCCTTGCCC
ACT1 F	GCCGAAAGAATGCAAAAGGA
ACT1 R	TAGAACCACCAATCCAGACGG
HXT1 F	CCATTAAGAGTCAAATCC
HXT1 R	GTAAGCGAAAACCATACA
HXT2 F	TCTTCTTCGCTATTAGTT
HXT2 R	TTGTAATGAAGGGAGTGA
HXT3 F	TTGTGGGGTTTCTTGATT
HXT3 R	TTCTTCCAAAGTCAAACC
HXT4 F	ATGGCTATTGCTCAAGCT
HXT4 R	TGGAACGAAGAAGAAGAC
ADH1 F	TTATTCAGATCCATCGGT
ADH1 R	GTATCTGGTAGAAGCTTC
ENO1 F	CAACCCAAAGAGAATTGC
ENO1 R	AGATCTGTGGGAAACCAT
GPM1 F	GAATCTTTGGCTTTGGTC
GPM1 R	AGCATCAGAGATACCTTC
PGK1 F	CAATTGATTGACAACTTG
PGK1 R	TTCCATCAACTTTGGAAC

Table 3.5 Primers used for RT-PCR (*Continued*)

Primers	Sequence (5'-3')
RPL11A F	CGGTATTGACGAACACATTGACTTGG
RPL11A R	TACCCTTACATCTCTTTCTTCTAGTG
RPS18B F	TCAAAACGACATTACTGATGGTAAGG
RPS18B R	CCAGAAGTGTCTAATACCACGGTGG
RPL30 F	CATCATTGCCGCTAACACTCCAGTTTTG
RPL30 R	CCAGCTTCCAAAATAGAGACAACACCG

Restriction enzyme sites are underlined

3.3. Culture conditions

3.3.1. Improving glucose uptake in *S. cerevisiae*

CEN.PK2-1C yeast cells were precultured in YPD (1% yeast extract and 2% bacto peptone) medium or SC (0.67% yeast nitrogen base without amino acids and 0.2% amino acid dropout mixture lacking appropriate components for selection) medium containing 4% glucose, and inoculated to an A_{600} of 5 in YPD or SC medium containing 8% glucose. Cells were cultivated in 5 ml volume in 50 ml screw-capped conical tubes at 30°C with shaking at 170 rpm. For lactic acid fermentation, lactic acid-producing strains were inoculated from an overnight seed culture to an A_{600} of 0.5 into 50 ml YPD medium containing 4% glucose with or without 5 g/L $CaCO_3$ in 250 ml flat-capped flasks, and grown at 30°C under micro-aerobic conditions with shaking at 90 rpm.

3.3.2. Improving fructose uptake in *S. cerevisiae*

CEN.PK2-1C yeast cells were precultured in YPD (1% yeast extract and 2% bacto peptone) medium or SC (0.67% yeast nitrogen base without amino acids and 0.2% amino acid dropout mixture lacking appropriate components for selection) medium containing 2% glucose, and inoculated to an A_{600} of 0.3 in YPD or SC medium containing 2% or 5% glucose or fructose. Cells were cultivated in 5 ml volume in 50 ml screw-capped conical tubes at 30°C with shaking at 170 rpm.

3.3.3. Increasing tolerance to furan aldehydes in yeast

Cells were grown overnight at 30°C in a 96 deep-well plate and reinoculated to A_{600} of 0.01 in medium containing 20 to 40 mM furfural (Sigma-Aldrich, USA) or 30 to 40 mM HMF (Sigma-Aldrich, USA). Cell growth was monitored by using a 96-well plate spectrophotometer (Multiskan GO, Thermo Scientific, USA).

3.4. Fluorescence-activated cell sorter (FACS) analysis

Levels of intracellular ROS were measured using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Sigma-Aldrich, USA) (145). Cells exponentially grown in YPD medium were treated with 20 mM furfural, 15, 30, or 45 mM HMF, or 5 mM H_2O_2 for 8 h, and then H_2DCF-DA was added to a final concentration of 10 μg/L and incubated for 60 min at 30°C. The cells were washed twice in PBS and were analyzed using flow cytometry. The fluorescence of cells from each sample was determined by using a FACSCanto flow cytometer (Becton Dickinson, USA) equipped with a 488-nm blue laser. Among the 10,000 cells analyzed for each sample, those cells with fluorescence intensities ranging from 10^2 to 10^4 were counted.

3.5. *In vitro* Glutathione assays

The concentration of sulfhydryl groups of GSH was measured using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (Sigma-Aldrich, USA) (146). 5 mM GSH

(Calbiochem, USA) were incubated with 30 or 60 mM furfural and 30 or 60 mM HMF in PBS buffer at 30°C. During the incubation, aliquots were taken and mixed with 150 µM DTNB in a buffer containing 30 mM Tris-HCl (pH 8.0) and 3 mM EDTA to 200 µl of total assay volume. Each sample was incubated for 5 min, and the color development was determined by using a 96-well plate spectrophotometer (Multiskan GO, Thermo Scientific, USA) at 412 nm.

3.6. *In vivo* Glutathione assays

Cells were grown in YPD medium to early exponential phase and 30 mM of HMF or furfural was treated for 5 and 10 h. Harvested cells were washed and resuspended in cold 5% 5-sulfosalicylic acid in a 50 mM potassium phosphate buffer (pH 7.8) containing 3 mM EDTA, and disrupted with glass beads. The lysates were centrifuged (4°C, 13,200 x g) for 15 min and the supernatant was obtained for the measurement of total and oxidized glutathione. Glutathione quantification was performed as described previously (147). For the estimation of total glutathione, 10 µl of 4 M triethanolamine was added to a 100 µl cell extract, and aliquots were taken and mixed with 150 µM DTNB in a buffer containing 50 mM potassium phosphate buffer (pH 7.8), 3 mM EDTA, 0.2 mM NADPH, and 0.25 U glutathione reductase (Sigma-Aldrich, USA) in 200 µl of a total assay volume. For the determination of oxidized glutathione disulfide (GSSG) levels, glutathione quantification assays were performed after eliminating GSH by

incubating 2 μl of 1 M 2-vinylpyridine in 100 μl cell lysates for 1 h.

3.7. RNA preparation and quantitative reverse transcription PCR

The 1 mL of cells was harvested and frozen at -80ºC in 300 μL of lysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.5% SDS]. 300 μL of acidic phenol was added to each sample and incubated at 65ºC for 20 min with occasional vortexing. Prior to chloroform extraction, the solution was chilled on ice for 10 min. After centrifugation and ethanol precipitation, the resulting RNA pellets were dissolved in RNase-free water. The relative amount of mRNA was determined by quantitative reverse transcription PCR (qRT-PCR) as previously described (148). Reverse transcription (RT) of 2 μg of total RNA was carried out with 0.1 μg of oligo-(dT) for 1 h at 42ºC using M-MLV reverse transcriptase (M-biotech, Inc., Korea), followed by heat inactivation for 10 min at 75ºC. PCR mixture containing 1 μL of 20 μL RT reaction solution, 1xSYBR master mix (Roche Diagnostics), and gene-specific primers, was subjected to qPCR reaction with 45 cycles of 95ºC for 10 s, 55ºC for 20 s, and 72ºC for 20 s using Roche LightCycler 480 real-time PCR system (Roche Diagnostics). Primer sequences used for qRT-PCR are at Table 3.5

3.8. Analytic methods

Cell growth was monitored by optical density at 600 nm (OD_{600}) using a

spectrophotometer (Cary 50 Conc, Varian). To quantify the concentration of metabolites, culture supernatants were collected and filtered through a 0.22 μm syringe filter. The concentrations of glucose, lactate, glycerol, acetate and ethanol were determined by using high performance liquid chromatography (HPLC).. HPLC analysis was performed in UltiMate 3000 HPLC system (Thermo Scientific, Dionex) equipped with an Aminex HPX-87H column (300 mm x 7.8 mm, 5μm, Bio-Rad) and a refractive index (RI) detector. The column was eluted with 5 mM H_2SO_4 as a flow rate of 0.6 mL/min at 60ºC and RI detector was kept at 35ºC. Chemicals for standard solution were purchased from Sigma-Aldrich.

Chapter 4.

Improvement of glucose uptake and fermentation by overexpressing hexose transporters and a transcriptional activator Gcr1 in *Saccharomyces cerevisiae*

4.1. Introduction

Saccharomyces cerevisiae has been used as an important cell factory for production of fuels and chemicals (149, 150). Although yeast can utilize a wide range of carbon sources, glucose is the most preferred carbon source for yeast fermentation (81). Glucose uptake in *S. cerevisiae* is elaborately regulated to adapt cellular metabolism in response to ever changing environmental conditions (81, 151). However, to achieve high titer and productivity of target chemicals using metabolically engineered yeast cells, it may be beneficial to inactivate, circumvent, or modify some of such natural regulatory mechanisms.

The first regulatory step of glucose uptake is the facilitated diffusion of glucose through hexose transporters (*HXTs*) in the plasma membrane (151, 152). *S. cerevisiae* contains 18 *HXT* family members of hexose transporters, Hxt1 to Hxt17 and Gal2, among which Hxt1 to Hxt7 function as major glucose transporters (153). Expression of each HXT, having different glucose affinity, is differentially regulated depending on extracellular glucose concentrations (39, 154, 155). Hxt1 (K_m ~ 100 mM) and Hxt3 (K_m ~ 30-60 mM) are classified as low-affinity glucose transporters. *HXT1* is highly induced in the presence of high concentrations of glucose (>1%), whereas *HXT3* is induced by both low and high levels of glucose. When the glucose concentrations are lowered to around 0.1%, *HXT2* and *HXT4*, encoding moderate-affinity glucose transporters (K_m ~ 5-10 mM), are expressed (43). *HXT6* and *HXT7*, encoding highly homologous glucose transporters with very

high glucose affinity (K_m ~ 1 mM), are expressed just before the depletion of glucose in the medium (44). Expression of *HXT5* encoding moderate-affinity transporter (K_m ~ 10 mM) is not regulated by extracellular glucose concentrations but by various environmental conditions affecting growth rate (45, 46).

The glucose concentration-dependent expression of *HXT* genes is achieved by complex interconnection of both glucose induction and glucose repression mechanisms (77, 156). Induction of *HXT1/2/3/4* genes in the presence of glucose is mainly regulated by plasma membrane glucose sensors Snf3 and Rgt2, and a transcriptional repressor Rgt1 (62, 64). In the absence of glucose, *HXT* genes are repressed by Rgt1 in association with corepressors Mth1 and Std1 (65, 66). In the presence of glucose, binding of glucose to Snf3 and Rgt2 triggers a signal to the degradation of Mth1 and Std1, resulting in the release of Rgt1 from the promoters and subsequent derepression of *HXT* genes (61). On the other hand, *HXT2*/*3*/*4* and *HXT6*/*7* are repressed by Mig1 in the presence of glucose (43, 71). Upon glucose starvation, activated Snf1 kinase phosphorylates Mig1, leading to its nuclear export and derepression of its target genes.

Previously, it has been shown that overexpression of *HXT1* or *HXT7* in *S. cerevisiae* increases the rates of glucose uptake and ethanol production (157). Moreover, overexpression of *HXT1* or *HXT7* led to increased productivity and titer of lactic acid in *S. cerevisiae* cells engineered for lactic acid production, suggesting the possibility of manipulating glucose uptake rate to improve fermentation performance for the production of various target chemicals if optimal metabolic

pathways toward the target product are provided (157). In addition to an increase in HXT protein levels in the plasma membrane, an increase in glycolytic flux might serve as a driving force to enhance glucose uptake rate. However, so far, overexpression of glycolytic enzymes individually or in combination was not successful to significantly improve glycolytic flux (158-162). In general, the highest yield of a desired chemical can be reached by maximizing the metabolic flux to the desired product while minimizing biomass synthesis. However, the drain of metabolites from essential pathways and/or product toxicity could inhibit cell growth, exerting a negative effect on the target chemical production by decreasing volumetric productivity. Therefore, a proper balance between growth rate and production flux is important to achieve both high yield and productivity (163).

In this study, we improved glucose uptake rate in *S. cerevisiae* by overexpressing *HXTs* with various glucose affinities. In addition, improved cell growth and glucose uptake rates could be achieved by overexpressing a transcriptional activator Gcr1, which led to increased expression levels of *HXT1* and genes encoding ribosomal proteins (RPs). Overexpression of *GCR1* was successfully applied to lactic acid production in an engineered strain, resulting in a significant improvement of lactic acid production under acidic fermentation conditions, where overcoming the growth inhibitory effect is critical to improve lactic acid production.

4.2. Effects of overexpressing *HXTs* with different glucose affinities on glucose uptake rate

In a previous study, it has been shown that overexpression of *HXT1* and *HXT7*, encoding low- and high-affinity transporters, increases glucose uptake rate with similar efficiencies (157). We reinvestigated the effects of *HXT* overexpression on glucose uptake rate by individually overexpressing 5 *HXT* genes, *HXT1*/2/3/4 and *HXT7*, encoding glucose transporters with different affinities. The *HXT* genes were overexpressed under the control of a strong constitutive *TDH3* promoter from a CEN/ARS-based low copy number plasmid vector. In SC-Trp minimal medium containing 80 g/L glucose, CEN.PK2-1C cells expressing each *HXT* gene showed faster glucose uptake and ethanol production rates than did a control strain harboring an empty plasmid vector (Fig. 4.1). The amounts of consumed glucose in each strain after 10- and 24-h cultivation were correlated with the amounts of produced ethanol, reflecting the fact that ethanol production is the major carbon flux during the yeast fermentation. The overexpression effect of high-affinity glucose transporter Hxt7 was most effective, followed by moderate affinity glucose transporters Hxt2 and Hxt4. Overexpression of low-affinity transporters Hxt1 and Hxt3 showed marginal effects on glucose uptake and ethanol production (Fig. 4.1).

4.3. Effects of deleting Mth1 and Std1 corepressors of *HXT* genes on glucose uptake rate

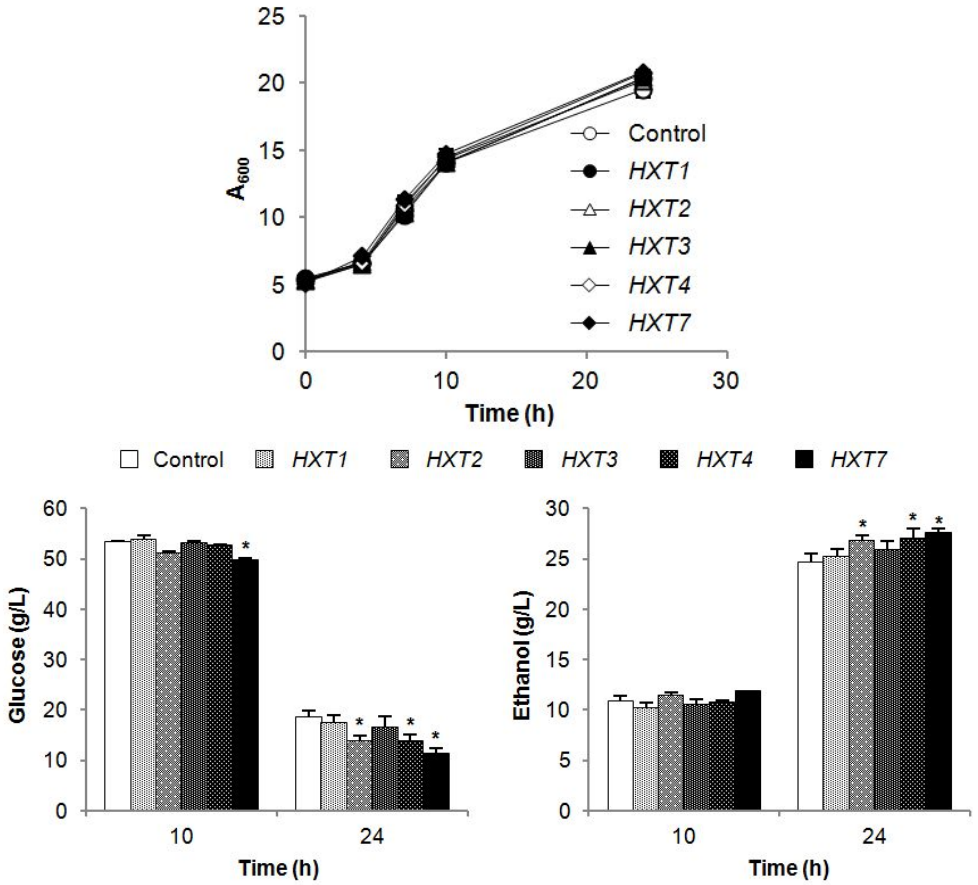

Figure 4.1 Increased glucose uptake and ethanol production rates by overexpression of *HXT* genes.

CEN.PK2-1C cells overexpressing each *HXT* gene (*HXT1* to *HXT4* and *HXT7*) were grown in SC-Trp medium with 80 g/liter glucose, and glucose and ethanol levels in the medium were detected after 10 and 24 h of cultivation. Cells harboring empty pRS414GPD vector were used as a control. Mean values of the results of triplicate experiments are shown with error bars indicating the relative standard deviations. *, $P<0.05$ (compared to the wild type).

Mth1 and Std1 act as co-repressors of the repressor Rgt1 whose target genes include *HXT1* to *HXT4* and *HXT7* (64, 66). Although Std1 and Mth1 are mainly functional in the absence of glucose, deletion of *STD1* and/or *MTH1* has been shown to derepress *HXT* genes simultaneously to different extents depending on glucose concentrations (65). Therefore, we deleted *STD1* and *MTH1* individually or in combination to examine the effects of multiple up-regulation of *HXT* genes on glucose uptake.

In SC medium, glucose uptake and ethanol production rates were slightly higher in *mth1Δ* and *std1Δ* strains than in the wild type (Fig. 4.2A). The effect of double deletion was very subtle compared with those of single deletions (Fig.4. 2A). However, in rich YPD medium, *std1Δ* and *mth1Δstd1Δ* strains showed a significant increase in glucose uptake and ethanol production rates compared with wild type (Fig. 4.2B). The *mth1Δstd1Δ* strain, showing the fastest glucose uptake rate, also showed faster growth rate than those of other strains. To elucidate *HXTs* responsible for the differences in glucose uptake rates, we measured mRNA levels of *HXT* genes in each strain after 6-h cultivation. *HXT1* mRNA levels showed the most noticeable derepression in *std1Δ* and *mth1Δstd1Δ*, suggesting that the increased expression of *HXT1* might be mainly responsible for the enhanced glucose uptake in these strains in YPD medium (Fig. 4.2C). *HXT3* expression also slightly increased in *std1Δ*, but not in *mth1Δstd1Δ* (Fig. 4.2C). On the contrary, expression levels of *HXT2* and *HXT4* rather decreased in *std1Δ* and *mth1Δstd1Δ* (Fig. 4.2C).

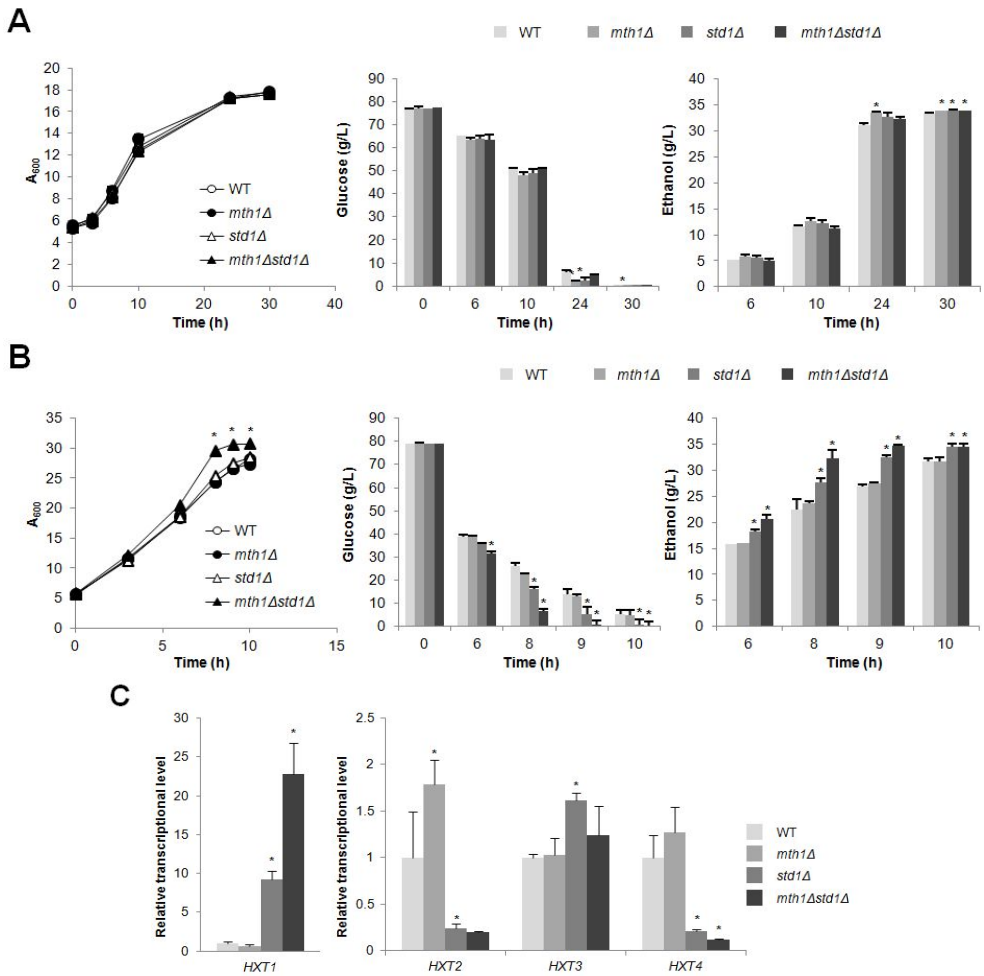

Figure 4.2 Effects of deleting corepressor genes *MTH1* and *STD1* on glucose uptake and ethanol production rates.

Wild-type (WT; CEN.PK2-1C) and *mth1Δ*, *std1Δ*, and *mth1Δ std1Δ S. cerevisiae* cells were grown in SC (A) or YPD (B) medium with 80 g/liter glucose, and glucose and ethanol levels were detected during growth. (C) Expression levels of *HXT* genes. The indicated strains were grown in YPD (80 g/liter) medium for 6 h, and the mRNA expression levels of each *HXT* gene were measured by qRT-PCR and normalized to that of the *ACT1* gene. The mRNA levels relative to that of the wild-type control are indicated with standard deviations.of the results of three independent experiments. *, P _ 0.05 (compared to the wild type).

Taken together, the effects of deleting *MTH1* and/or *STD1* on the expression of *HXT* genes and glucose uptake appear to be variable depending on culture conditions, which might be due to condition-dependent expression levels of Mth1 and Std1 as well as complex regulatory networks of Mth1/Std1-Rgt1 transcriptional regulators, which regulate not only *HXT* genes but also other target genes.

4.4. Improvement of glucose uptake rate by overexpressing *GCR1*

In previous studies, overexpression of genes encoding glycolytic enzymes was not effective in significantly improving glycolytic flux (158-162). Therefore, we tested whether overexpression of transcription factor Gcr1, which activates not only glycolytic genes, but also other glucose-responsive target genes such as ribosomal protein (RP) genes, could improve glucose uptake rate through facilitating glucose utilization and cell growth (164). Gcr1 binds to CT (CTTCC) box in the promoters of glycolytic genes and Gcr2 interacts with Gcr1 for the transcriptional activation. However, Gcr2 is dispensable for the activation of RP gene transcription by Gcr1 (164). CEN.PK2-1C cells overexpressing *GCR1* showed increased glucose uptake and ethanol production rates (Fig. 4.3A). On the contrary, overexpression of *GCR2* rather decreased glucose uptake rate, and overexpression of both *GCR1* and *GCR2* exerted significant defects in growth and glucose uptake (Fig. 4.3A).

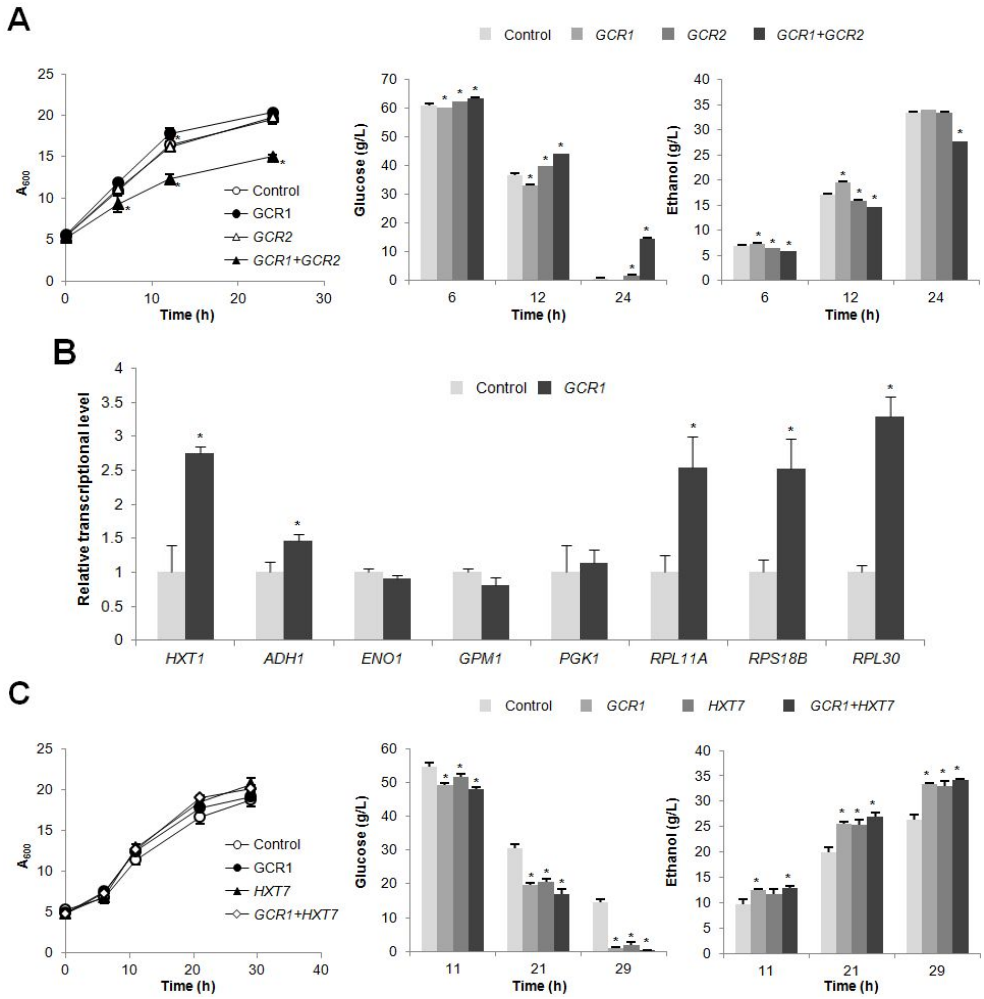

Figure 4.3 Improvement of glucose uptake rate by overexpressing *GCR1*.

Effects of overexpressing *GCR1* and/or *GCR2* on the glucose uptake rate. CEN.PK2-1C cells overexpressing *GCR1* and *GCR2* alone or in combination were grown in SC-Leu-Ura medium with 80 g/liter glucose and monitored for glucose uptake and ethanol production. Cells expressing empty vectors pRS415GPD and pRS416GPD were used as a control. (B) Effects of overexpression of the *GCR1* gene on the transcription levels of its target genes. CEN.PK2-1C cells overexpressing *GCR1* or harboring pRS416GPD (control) were grown in SC-Ura medium with 80 g/liter glucose for 12 h, and mRNA expression levels of Gcr1 target genes were measured by qRT-PCR and normalized to the *ACT1* level. The mRNA levels relative to those of the control are indicated with

Figure 4.3 Improvement of glucose uptake rate by overexpressing *GCR1*.(continued)

standard deviations of the results of three independent experiments. *, *P* _ 0.05 (compared to control). (C) Effects of overexpressing *GCR1* alone or in combination with *HXT7*. Cells overexpressing the indicated genes were grown in SC-Trp-Ura medium with 80 g/liter glucose and monitored for glucose uptake and ethanol production. Cells expressing empty vectors (pRS414GPD and pRS416GPD) were used as a control. Mean values of triplicate experiments are shown with error bars indicating standard deviations. *, *P* _ 0.05 (compared to control).

To elucidate the effects of *GCR1* overexpression, transcription levels of its target genes were investigated after 12-h cultivation. Unlike our initial expectation, transcription levels of glycolytic genes (*ENO1*, *GPM1*, and *PGK1*) were not elevated in cells overexpressing *GCR1* (Fig. 4.3B). On the other hand, overexpression of *GCR1* led to an increase in expression levels of RP genes (*RPL11A*, *RPS18B*, and *RPL30*), *HXT1* and *ADH1* encoding alcohol dehydrogenase (Fig. 4.3B). Expression levels of *HXT2/3/4* were not significantly changed by *GCR1* overexpression (data not shown). Therefore, *GCR1* overexpression might increase glucose uptake rate mainly by enhancing ribosome biogenesis, which is tightly linked to cell growth, rather than by elevating the expression levels of glycolytic genes.

Overexpression of *GCR1* was more efficient in facilitating glucose uptake rate than overexpression of *HXT7* (Fig. 4.3C). Slight additive effects were observed when *HXT7* gene was overexpressed together with *GCR1* (Fig. 4.3C). Although the control cells harboring empty vector(s) showed slower glucose uptake rates in SC-Trp (Fig. 4.1A) and SC-Trp-Ura medium (Fig. 4.3C) compare to that in SC-Leu-Ura medium (Fig. 4.3A), the overexpression effects of *HXT7* and *GCR1* on glucose uptake rate were reproducibly observed independent of the types of medium.

4.5. Improvement of lactic acid production by overexpressing *GCR1*

Since overexpression of *GCR1* was proven to be effective in increasing glucose uptake and ethanol production rates in wild type *S. cerevisiae*, we next applied *GCR1* overexpression to lactic acid production in engineered strains expressing lactate dehydrogenase. We used two lactic acid-producing strains, SP2006 and SP1130 (142). For high-yield lactic acid production, the pathways for glycerol and ethanol formation had been attenuated in SP2006 by deleting *GPD1* encoding glycerol-3-phosphate dehydrogenase, *PDC1* encoding pyruvate decarboxylase, and *ADH1*. SP1130, an improved derivative of the construction of SP2006, was generated by increasing cellular supply of acetyl-CoA via introducing bacterial genes for acetylating acetaldehyde dehydrogenase (A-ALD) enzymes, MphF and EutE, while deleting *PDC6* encoding aldehyde dehydrogenase (142). In the present work, *GCR1* was overexpressed in SP2006 and SP1130 by integrating the gene into the genome under the control of the *TDH3* promoter, generating SP2018 and SP1141, respectively.

Interestingly, overexpression of *GCR1* exhibited different effects depending on lactic acid-production ability of each strain. Note that the amounts of glucose uptake in the lactic acid-producing strains are limited, consuming only 27.4 g/L and 30.2 g/L glucose, respectively, when SP2006 and SP1130 strains were grown in YPD medium containing 40 g/L glucose (Fig. 4.4A and B). The volumetric productivity of lactic acid decreased over time both in SP2006 and SP1130, but

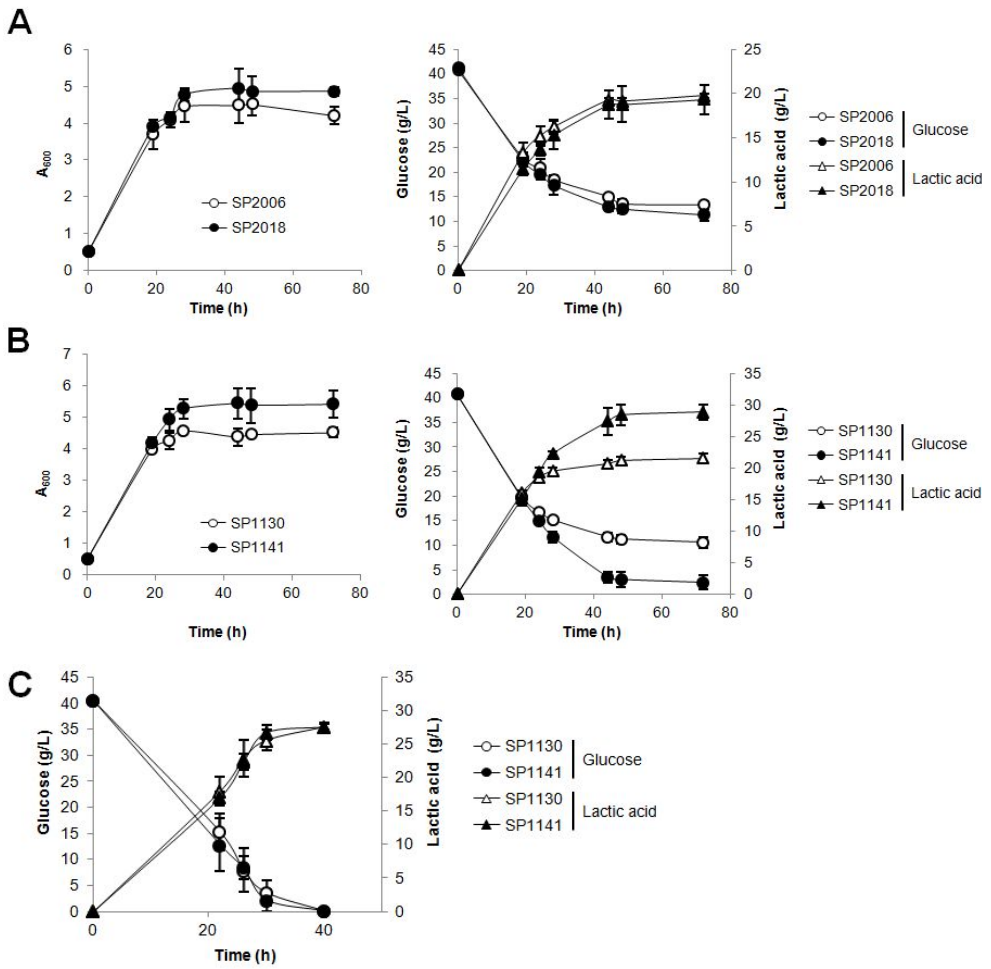

Figure 4.4 Improvement of lactic acid production by overexpressing *GCR1*.

Effects of *GCR1* overexpression in strain SP2006 background. SP2006 and SP2018 (SP2006 overexpressing *GCR1*) strains were grown in YPD medium containing 40 g/liter glucose under microaerobic conditions. (B and C) Effects of *GCR1* overexpression in strain SP1130 background. Glucose uptake and lactic acid production were measured in SP1130 and SP1141 (SP1130 overexpressing *GCR1*) strains during growth in YPD medium containing 40 g/liter glucose without CaCO3 (B) or with 5 g/liter CaCO3 (C) under microaerobic conditions. Mean values of the results of triplicate experiments are shown with error bars indicating standard deviations.

SP1130 showed about 20% higher productivitiy than that of SP2006 up to 28-h cultivation (Fig. 4.4A and B). Overexpression of *GCR1* in SP2006 (strain SP2018) led to a slight increase in glucose uptake level and final cell density (Fig. 4.4A). However, the increased amount of glucose transported into the SP2018 cells was not efficiently converted to lactic acid, showing rather slightly decreased lactic acid production levels. On the other hand, *GCR1* overexpression in SP1130 background (strain SP1141) resulted in a significant increase in glucose consumption and lactic acid production levels (Fig. 4.4B). By overexpressing *GCR1*, glucose consumption increased from 30.2 g/L to 38.4 g/L, and lactic acid production increased from 21.5 g/L to 28.9 g/L compared to strain SP1130. The yield also increased from 0.71 g/g glucose to 0.75 g/g glucose.

Next, we investigated whether the growth-promoting effect of *GCR1* overexpression could also improve lactic acid production when the growth inhibitory acid stress is relieved by neutralization. When SP1130 cells were grown in YPD medium containing 40 g/L glucose and 5 g/L CaCO3 as a neutralizing reagent, cells consumed all glucose in the medium after 40 h, producing 27.6 g/L lactic acid. Under these fermentation conditions, SP1141 showed glucose uptake rate and lactic acid production levels similar to those of SP1130, suggesting that *GCR1* overexpression might provide a selective advantage when cell growth is impaired.

Taken together, *GCR1* overexpression might contribute to improving cell growth by facilitating glucose uptake and protein synthesis. However, the positive

correlation between the growth-promoting effect and the production of a target chemical might be prominent only in the presence of efficient and strong metabolic flux toward the target chemical, and when growth inhibition is one of the limiting factors for the production of the target chemical.

4.6. Conclusions

Glucose uptake is the first rate limiting step to control cellular metabolism depending on extracellular glucose availability and intracellular metabolic capacity. Very sophisticated regulatory mechanisms for glucose uptake have been evolved to ensure cell survival in natural environments with high fluctuation of glucose availability (153). However, engineering the natural regulatory networks to allow higher glucose uptake rate might be beneficial for the production of various fuels and chemicals using yeast as a cell factory if the engineered strain has an efficient metabolic flux toward a target chemical.

As an effort to increase glucose uptake rate, we tested various genetic modifications related to glucose uptake and cell growth. The most direct strategy was overexpressing *HXTs* with various affinities to glucose. Among the *HXTs* tested (Hxt1/2/3/4 and Hxt7), high-affinity transporter Hxt7 was most effective in increasing glucose uptake rate, followed by moderate-affinity transporters Hxt2 and Hxt4. The effects of eliminating Mth1 and Std1 corepressors of *HXT* genes were more complicated due to the complex nature of the regulatory networks and

multiple targets regulated by Rgt1 (66). Mth1 and Std1 are homologous proteins acting as corepressors of Rgt1, but their cellular functions are distinguished mainly based on their differential expression levels depending on glucose concentrations (69). Although expression levels of Std1 are not much affected by glucose concentrations, Mth1, whose expression is repressed by Snf1-Mig1 glucose repression pathway, is abundant only under glucose starvation conditions ((62, 69, 70). The deletion effects of *MTH1* and/or *STD1* on glucose uptake were more prominent in YPD medium than in SC medium. In SC medium, deletion of *MTH1* was most effective in increasing glucose uptake rate, but in YPD medium, *std1Δ* and *std1Δmth1Δ* strains, but not *mth1Δ* strain, showed increased glucose uptake rate. Such culture condition-dependent effects of *MTH1* and/or *STD1* deletions on glucose uptake might reflect different expression levels of Mth1 and Std1 as well as their slightly different regulatory mechanisms, which lead to different contribution of each corepressor to the regulation of Rgt1 target genes in a given condition. Different nutritional compositions and pH between YPD and SC media and faster pH drop in SC medium than in YPD medium might be responsible for the differential roles of Mth1 and Std1. In YPD medium, derepression of *HXT1* seems to be mainly responsible for the increase in glucose uptake rate in *std1Δ* and *std1Δmth1Δ* under our experimental conditions, although we cannot rule out the contributions of other Rgt1 target genes. Unexpectedly, *std1Δ* and *std1Δmth1Δ* exhibited reduced expression levels of *HXT2* and *HXT4*, which might be due to

indirect effects of derepressing other Rgt1 target genes such as *MIG2*, which encodes a repressor for *HXT2* and *HXT4* ((43, 69).

As another strategy to increase glucose uptake rate, we overexpressed *GCR1*. Gcr1 plays an important role in glucose metabolism by activating glycolytic genes, *HXT* genes, and *ADH1* (165). Furthermore, Gcr1 also promotes cell growth by activating the expression of translational components such as RP genes (164). Gcr2, a Gcr1-binding coactivator, is essential only for the expression of CT box-containing glycolytic genes, but not for RP genes, which do not have CT box (164). Although overexpression of *GCR1* was effective in increasing glucose uptake and ethanol production rates, overexpression of *GCR2* alone or in combination with *GCR1* showed a negative effect on glucose uptake. Overexpression of *GCR1* led to increased expression levels of *HXT1*, *ADH1*, and RP genes, but not glycolytic genes. These results might be in part related to the fact that transcriptional activation of glycolytic genes requires both Gcr1 and Gcr2, but Gcr1 alone can activate RP gene transcription. Therefore, the increase in glucose uptake rate by *GCR* overexpression might be caused by improving cell growth through promoting translational ability and by increased expression of *HXT1* rather than by increasing glycolytic flux through activating the expression of glycolytic gene.

Overexpression of *GCR1* was successfully applied to increase lactic acid production in an engineered yeast strain SP1141. Inside of lactic acid-producing cells, lactic acid molecules dissociate into the acid anions and protons, inducing multiple stress conditions via cytosolic acidification and modifications of cellular

components ((166, 167). Furthermore, exporting the protons and acid anions through H^+-ATPase and efflux pumps is highly energy-consuming process (168). Because of such toxic effects of lactic acid, cell growth and glucose uptake halt when lactic acid production reaches a certain level. SP1130, the parental strain of SP1141, consumed only 30.2 g/L glucose even in the presence of 40.0 g/L glucose in the medium, producing 21.5 g/L lactic acid. On the other hand, strain SP1141, overexpressing *GCR1,* showed increased glucose uptake up to 38.4 g/L, and the imported glucose was successfully converted to lactic acid, reaching 28.9 g/L. Therefore, the growth-promoting effect of *GCR1* overexpression might increase the threshold level of the growth inhibitory effects of lactic acid, leading to higher titer, yield, and productivity of lactic acid production. However, *GCR1* overexpression was not effective when applied to another lactic acid-producing strain SP2006, which has lower acetyl-CoA level and weaker lactic acid producing capability than those of SP1130. Therefore, in SP2018 having inefficient metabolic flux to lactic acid, the increased amount of imported glucose might be mainly used to biomass synthesis rather than for lactic acid production.

Although *GCR1* overexpression was effective in improving cell growth, it failed to exert a positive role for lactic acid production when the growth inhibitory effect of the acid stress was alleviated by neutralizing the medium during the fermentation. Therefore, Gcr1-overexpression could be applied to improve lactic acid production during acidic fermentation. Acidic fermentation is preferred for

lactic acid production to reduce the cost of neutralization during fermentation and recovery of lactic acid from the resulting lactate salt (169) .

Taken together, increase in glucose uptake and cell growth rates might be a very useful strategy to improve the production of target metabolites if efficient metabolic pathways were provided to synthesize the desired molecules. Especially, *GCR1* overexpression, which enhances cell growth, might be a promising strategy for many other metabolic engineering applications where growth inhibition is one of the major bottlenecks to improve production.

Chapter 5.

Increasing fructose uptake by overexpression of hexokinases and *Candida magnoliae* Fsy1 in *Saccharomyces cerevisiae*

5.1. Introduction

S. cerevisiae is one of the most important cell factory for microbial fuel production, which has high tolerance to industrial conditions originated from high gravity fermentation, such as high acid, alcohol, osmotic stresses (32, 33). The most preferred carbon source in *S. cerevisiae* has been known as glucose, uptake of which is mediated by the expression of various hexose transporters(*HXTs*) depending on the extracellular glucose concentration .

Fructose is another highly fermentable sugar that widely appears in nature and its production occurs from various sources (170). Enhancing the ability of fructose uptake of yeast is a potentially important factor for efficient fermentation of fructose-rich culture or co-fermentation of glucose and fructose. From now on, most of the proteins that have been experimentally investigated for transporting fructose in yeasts and fungi have been known to prefer glucose to fructose and only a few genes have shown for encoding fructose-specific transporters in yeasts (36).

The first identified fructophlilic transporters were in several *Saccharomyces* strains, and other high-affinity fructose symporters were identified in the aerobic milk yeast *Kluyveromyces lactis* and fungi *Botrytis cinerea* (Table 5.1) (13, 20, 22, 200). In wine yeast, mutant *HXT3* in Fermichamp strain and a high affinity fructose symporter in *S. cerevisiae* EC 1118 was reported (15). Except the mutant *HXT3*,

Table 5.1 Kinetic parameters of fructose-specific transporters characterized in yeasts and fungi

Yeasts and fungi	Gene	Function	Km (mM)	Vmax (mmol h^{-1} $[gdw]^{-1}$)	Reference
Saccharomyces pastorianus	*FSY1*	H^+ symporter	0.16±0.02	3.8±0.2	(9)
Saccharomyces cerevisiae EC1118	*FSY1*	H^+ symporter	0.24±0.04	0.93±0.08	(12)
Kluyveromyces lactis	*FRT1*	H^+ symporter	0.16±0.02	0.10±0.02	(14)
Botrytis cinerea	*FRT1*	H^+ symporter	1.1	0.66	(16)
ZygoSaccharomyces rouxii	*FSY1*	H^+ symporter	0.45±0.07	0.57±0.02	(19)
Candida magnoliae JH110	*FSY1*	H^+ symporter	0.13±0.01	2.1±0.3	(24)
ZygoSaccharomyces bailii	*FFZ1*	Facilitator	80.4	3.3	(27)
ZygoSaccharomyces rouxii	*FFZ1*	Facilitator	424.2±163.1	12.7±3.3	(19)
Candida magnoliae JH110	*FFZ1*	Facilitator	105±12	8.6±0.4	(24)
Fermichamp	*HXT3 mutant*	Facilitator	123± 22	0.01452	(29)

these transporters actively transport fructose via a proton symport mechanism but not glucose. Also, some fructose-specific transporters were isolated and characterized from various fructophilic yeasts (27, 34, 36). These yeast strains prefer fructose to glucose, unlike the other yeast strains. And in *Candida magnoliae* and *ZygoSaccharomyces rouxii*, several transporters were also identified characterized. Among them, it was shown that Fsy1 and Ffz1 of *C. magnoliae* can make *hxt*-null *S. cerevisiae* strain survive in glucose and fructose mixed media, which showed the maintanence of stablility of those heterologous transporter proteins in *S. cerevisiae*.

And after uptake of the sugars into the cell, their phosphorylations by sugar kinases, such as Hxk1, Hxk2 and Glk1 (171) are occurred. The actual sugar phosphorylation is very complex, according to the changes of the relative phosphorylation rate of glucose and fructose by these hexokinases with different substrate affinity. Glk1, the glucokinase, mainly phosphorylates glucose. And Hxk1 has three times higher Vmax with fructose than with glucose. Inversely, Hxk2 has a little higher Vmax with glucose than with fructose (172, 173). In case of the affinity, the affinity of Hxk1 for glucose (Km= 0.12 mM) is higher than that for fructose (Km=1.5 mM). And in case of Hxk2, this hexokinase showed higher affinity for glucose (Km=0.25 mM) than for fructose (Km= 1.5 mM).

In the glucose condition, glucose repression by Hxk2 inhibits the expression of other hexokinases, which decreases affinity to fructose due to low fructose affinity of Hxk2 than that of the other hexokinases (82). In this case, additional expresssion

of other hexokinase with high affinity to fructose might increase the fructose uptake and consumption. Actually, overexpression of *HXK1* improved the glucose and fructose uptake in the media containing both glucose and fructose, but that of *HXK2* did not (174).

The objective of this part is to improve fructose uptake of yeast in the media containing both glucose and fructose. For this purpose, heterologous fructophilic transporters and innate hexokinases were overexpressed. And finally, the synergy effect of overexpression of both hexokinase and fructophilic transporter was also investigated.

5.2. Glucose and fructose uptake in WT strain

It was already known that yeast prefer glucose to fructose. At first, to confirm the difference in the uptake rate of each hexose in yeast, we investigated growth of CEN.PK2 WT strain in the media containing glucose or fructose as carbon sources. (Fig. 5.1)

These results showed that glucose uptake rate was faster than that of fructose. And in the media with glucose or fructose as a sole carbon source, uptake rate of each hexose was faster than that in the media containing both glucose and fructose. This confirmed that in yeast, uptake process of each hexose is competitive, and affinity to glucose is higher than that of fructose.

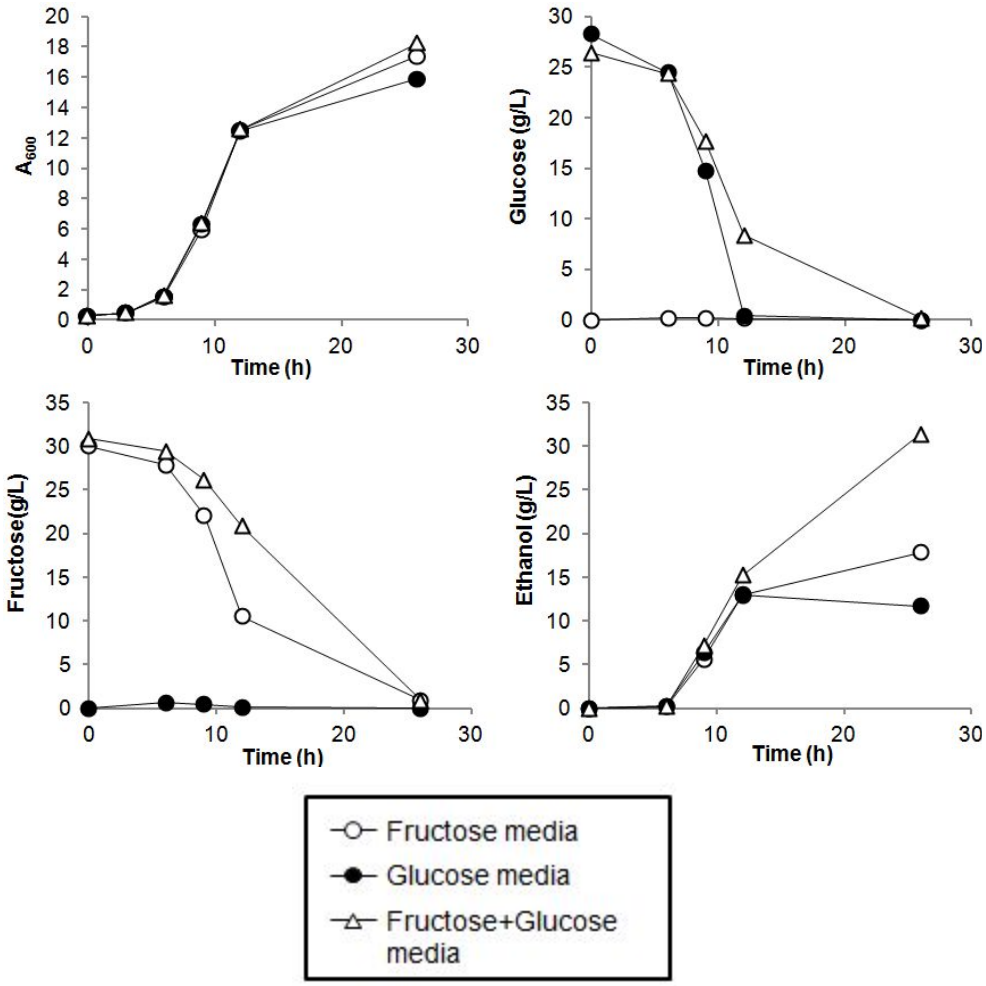

Figure 5.1 Glucose and fructose consumption of WT CEN.PK2 strain in glucose or fructose containing media.

CEN.PK2-1C cells were grown in YP medium with 30 g/liter glucose or fructose. Glucose, fructose and ethanol levels in the medium were detected at each estimated time of cultivation. Mean values of the results of triplicate experiments are shown without error bars.

5.3. Effects of overexpressing CmFsy1 on glucose and fructose uptake rate

In a previous study, it has been shown that expression of fructophilic transporters could make *hxt*-null *S. cerevisiae* survive in the condition of fructose as the sole carbon source. In our experiments, however, overexpression of Ffz1 from *Z. rouxii* as well as *C. magnoliae* was not effective (data not shown). And we also investigated the overexpression effect of Fsy1 originated from both strains. Overexpression of the gene was done under the control of a strong constitutive *TDH3* promoter from a *CEN*/*ARS*-based low copy number plasmid vector. In SC-Ura minimal medium containing 18g/L of fructose or 18g/L of both glucose and fructose, CEN.PK2-1C cells expressing *FSY1* of *C. magnoliae* showed increased fructose uptake and ethanol production rates (Fig. 5. 2). Although glucose uptake rate was similar or a little lowered at each time point, uptake rate of total sugar was increased, which was led to increased ethanol production. And overexpression of *FSY1* of *Z. rouxii* showed marginal effects in glucose and fructose mixed media.

5.4. Effects of overexpressing hexokinases on glucose and fructose uptake rate

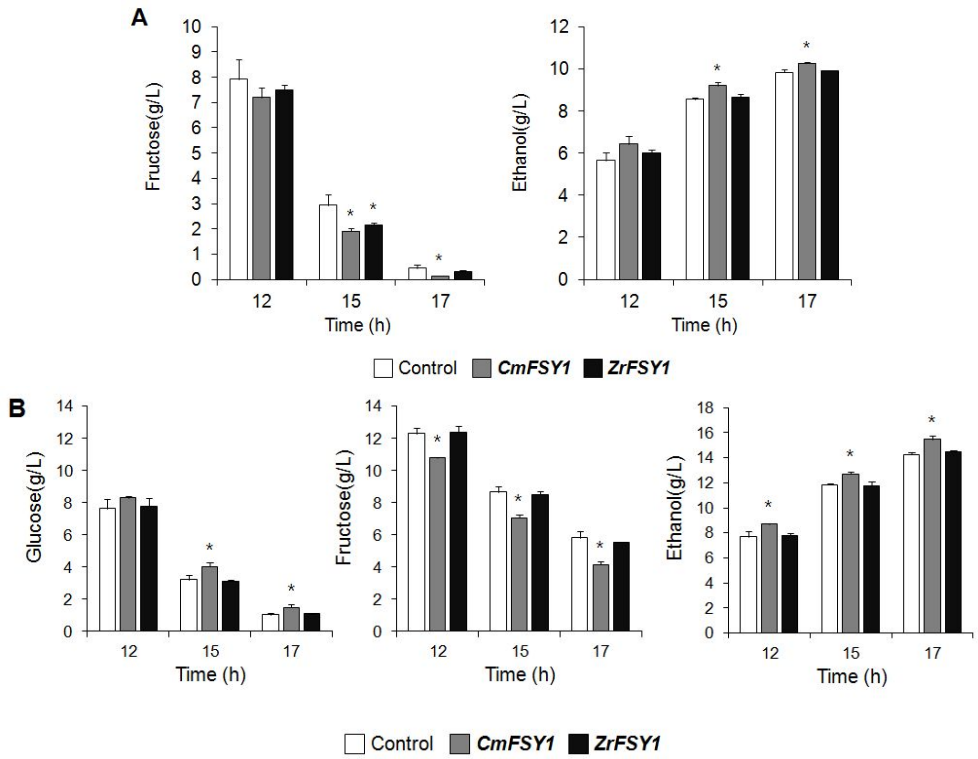

Figure 5.2 Glucose and fructose consumption of heterologous fructose transporter overexpressed strain in fructose sole source or glucose-fructose mixed media.

Effects of overexpressing *CmFsy1* or *ZrFsy1* on fructose sole carbon source (A) or glucose and fructose mixed carbon source (B). CEN.PK2-1C cells overexpressing *CmFsy1* or *ZrFsy1* were grown in SC-Ura medium with about 20/L fructose with or without 30g/L glucose, and monitored for glucose and fructose uptake and ethanol production. Cells expressing empty vector pRS416GPD were used as a control. Mean values of biological triplicates are shown with error bars indicating standard deviations. *P < 0.05 compared to control.

Glucose phosphorylation, which is the first step in glycolysis, is mediated by Hxk2 in glucose and fructose containing media. The roles of *HXK2* are phosphorylation of glucose or fructose and glucose repression, which is the transcriptional repression of utilization of other carbon sources except glucose when the culture media contains glucose. By glucose repression, Hxk2 also inhibit the expression of other hexokinases, such as Hxk1, Glk1. These hexokinase are expressed in glucose-depletion media, and Hxk1 can replace the role of Hxk2 in *hxk2* deletion strain (175). So we identified the effect of overexpression of these hexokinases for inducing rapid influx of each hexose by facilitated diffusion.

Among them, we already saw that Hxk2 overexpression decreased yeast growth and metabolism, so Hxk2 was excluded from the target. Other hexokinases, Hxk1, Glk1, and a putitive hexokinase Ylr446 were overexpressed. They were overexpressed under the control of a strong constitutive *TDH3* promoter from a *CEN*/*ARS*-based low copy number plasmid vector. In SC-Leu minimal medium containing about 15 g/L glucose and fructose, CEN.PK2-1C cells expressing each hexokinase gene showed faster glucose or fructose uptake and ethanol production rates than did control strain harboring an empty plasmid vector (Fig. 5.3). These might be due to increased diffusion by rapid consumption of intracellular hexose. Especially, *HXK1* overexpression showed the most rapid hexose uptake and ethanol production. And overexpression of the putative hexokinase, Ylr446w, also increased glucose and fructose uptake. The amounts of total consumed hexose

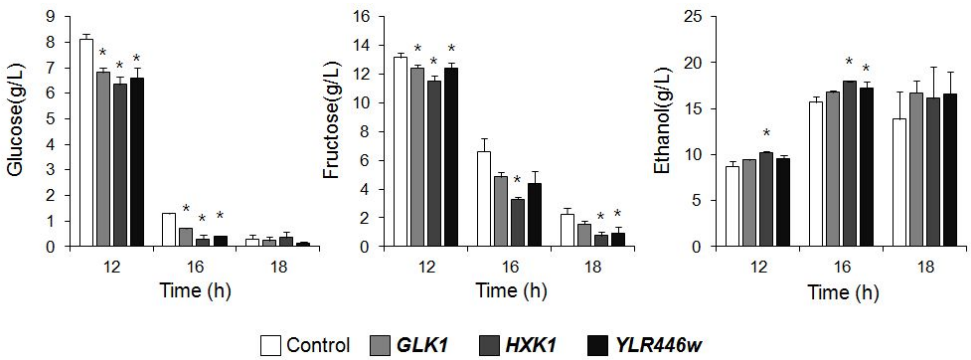

Figure 5.3 Glucose and fructose consumption of hexokinases overexpressed strain in glucose-fructose mixed media.

Effects of overexpressing *GLK1*, *HXK1, YLR446w* on glucose and fructose mixed carbon source. CEN.PK2-1C cells overexpressing *GLK1*, *HXK1, YLR446w* were grown in SC-Leu medium with about 15g/L glucose and fructose, and monitored for glucose and fructose uptake and ethanol production. Cells expressing empty vector pRS415GPD were used as a control. Mean values of biological triplicates are shown with error bars indicating standard deviations. *P < 0.05 compared to control.

of each strain at all estimated times were correlated with the amounts of produced ethanol.

5.5. Synergy effect of overexpression of hexokinase with CmFsy1

To maximize fructose or glucose uptake rate in yeast, each hexokinase and CmFsy1 were overexpressed under the control of a strong constitutive *TDH3* promoter from a *CEN*/*ARS*-based low copy number plasmid vector (Fig 5.4). And to set the concentration of each hexose with the actual sugar cane molasses (70-80g/L of glucose and fructose), concentration of each hexose was increased to 50g/L (176).

Like the results in low concentration of glucose and fructose mixed media (Fig. 5.2), CmFsy1 overexpression showed increased fructose uptake at 24h early time point. But at 40h, it showed decreased glucose uptake a little, which resulted in the similar effect with that of WT strain. And in case of hexokinase, only overexpression of Hxk1 increased uptake of both of glucose and fructose. Glk1 overexpression increased only glucose uptake, and Ylr446w overexpression increased only fructose uptake. And co-overexpression of hexokinase with CmFsy1 reduced fructose uptake ability of hexokinase overexpression strains, which appeared as their lower fructose uptake at 40h than that at 24h. *HXK1* overexpression showed the most rapid sugar uptake rate, and this tendency

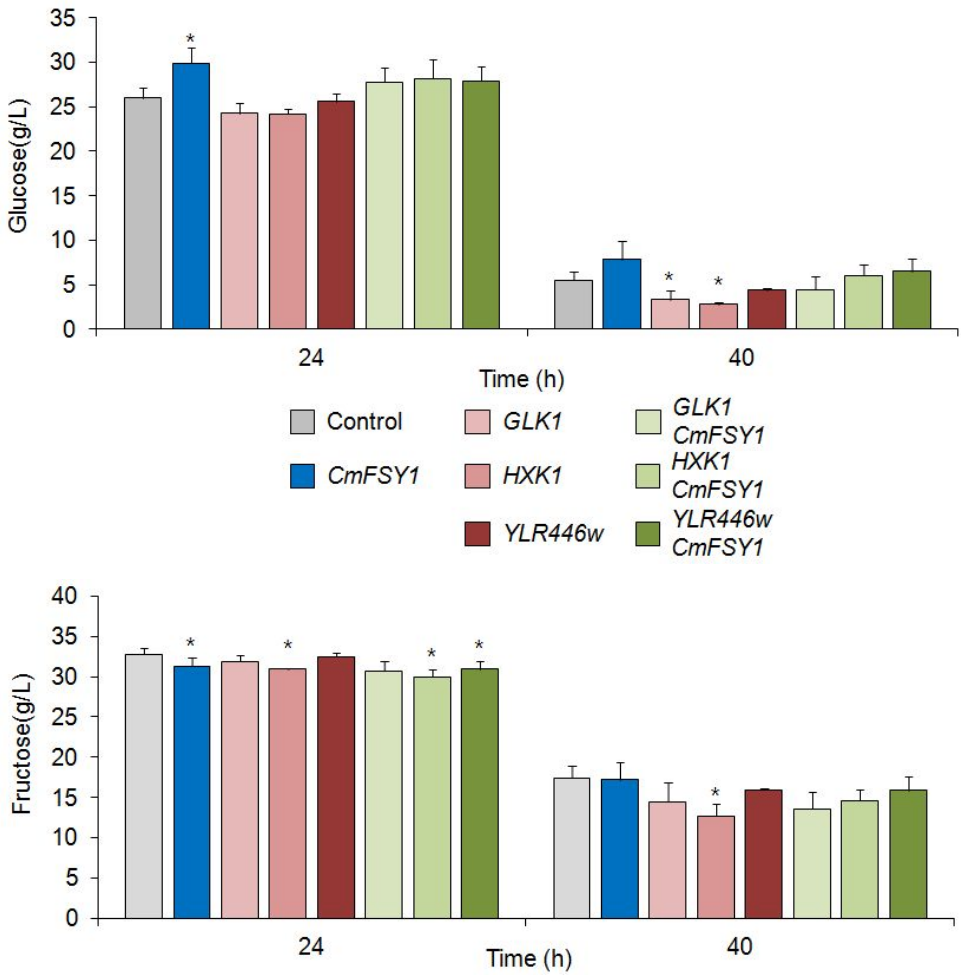

Figure 5.4 Glucose and fructose consumption of hexokinases with *CmFSY1* overexpressed strain in glucose-fructose mixed media.

Effects of overexpressing Hexokinase alone or in combination with *CmFSY1*. CEN.PK2-1C cells overexpressing *GLK1*, *HXK1, YLR446w* with or without *CmFSY1* were grown in SC-Leu-Ura medium with 50g/L glucose and fructose, and monitored for glucose and fructose uptake and ethanol production. Cells expressing empty vector pRS415GPD and pRS416GPD were used as a control. Mean values of biological triplicates are shown with error bars indicating standard deviations. *$P < 0.05$ compared to control.

was the same in the late time point.

5.6. Conclusions

Glucose and fructose are the most preferable carbon sources in yeast. But in the media containing both sugars, their uptakes are competitive and fructose uptake is a little more restricted than that of glucose. To solve this problem, we introduced fructophlic symporter Fsy1 from *C. magnoliae* and *Z. rouxii* at first. Although overexpression of CmFsy1 increased fructose uptake in the culture media containing low concentration of both glucose and fructose or fructose as the sole carbon source, it didn't show any effects in the media containing high concentration of glucose and fructose as carbon sources, which showed that reduced glucose uptake by overexpression of fructophilic transporter could be led to offset the effect of enhanced fructose uptake. This might be originated from decreased space capacity in yeast surface, which reduced space for glucose transporter by overexpression of fructose transporter might be led to defects in glucose uptake. And we overexpressed hexokinases Hxk1 and Glk1, which are not related to the glucose repression, and a putative hexokinase Ylr446w. Their overexpression resulted in the improvement of uptake of glucose or fructose, according to each target hexokinase. It was already revealed that overexpression of Hxk1 can increase uptake of both glucose and fructose, and we saw that Ylr446w, a putative hexokinase, could operate as a hexokinase in yeast. Although its

transcriptional or translational regulation and specific activity in general metabolism has not been revealed, we found that its overexpression could enhance the hexose uptake and metabolism. These results might be applied to other engineered yeast strain, leading to improvement of carbon source uptake and productivity.

Chapter 6.

Increasing tolerance to furan aldehyde by Yap1 as thiol-reactive electrophiles generating oxidative stress

6.1. Introduction

Bioethanol production from lignocellulosic biomass is currently one of the key subjects of the biofuel industry. However, because of the extremely rigid and complex nature of lignocellulose, pretreatment is required to make this material available for subsequent enzymatic digestion and microbial fermentation (177). The commonly used dilute acid pretreatment generates numerous chemical byproducts such as furan aldehydes, weak acids, and phenol derivatives, which inhibit microbial cell growth and ethanol fermentation (178). To overcome the inhibitory effects of these compounds, various physical and chemical methods of medium detoxification have been developed (177). However, because of the high cost of medium detoxification methods, biodetoxification of inhibitors by using tolerant microorganisms is considered a more practical alternative (178).

Furfural and 5-hydroxymethylfurfural (HMF) are derived from the dehydration of pentoses and hexoses, respectively (179). The amounts of furfural and HMF generated after pretreatment are variable depending on the types of raw materials and pretreatment methods, and up to 26 mM furfural and 47 mM HMF were detected in the dilute acid hydrolysates (129). These furan aldehydes are known as the most potent inhibitors of microbial cell growth (129, 180). The toxic aldehyde groups of furfural and HMF can be reduced to hydroxyl groups by several oxido-reductases including alcohol dehydrogenases (Adh1, Adh6, and Adh7) (1, 129, 181), aldehyde reductase (Ari1) (182), and methylglyoxal

reductases (Gre2 and Gre3) in yeast cells (181, 183-186), and aldehyde dehydrogenase (YqhD) and methylglyoxal reductase (DkgA) in *Escherichia coli* (187). These enzymes consume NADH and NADPH as cofactors during the reduction process. On the other hand, furfural and HMF can also be detoxified by oxidation by Ald6 aldehyde dehydrogenase (186). In line with these detoxification mechanisms, furfural and/or HMF tolerant yeast strains could be generated by the overexpression of *ADH6*, *ADH7*, and *ALD6* (1, 13, 186). In addition, overexpression of *ZWF1*, involved in NADPH regeneration through a pentose phosphate pathway, also enhanced furfural tolerance (130).

In order to develop strains tolerant to furan aldehydes, it is important to understand the cellular toxic mechanisms of furan aldehydes. Genome-wide transcriptome analyses have revealed a wide range of cellular functions regulated by furfural and HMF in yeast (126, 188). Because different yeast strains and conditions were used for these previous experiments, little overlap exists among the genes identified to be up-regulated by furfural and HMF. However, induction of stress-responsive genes was commonly observed when cells were treated with either furfural or HMF. Especially, Yap1, a major oxidative stress regulator, was identified as one of the key regulators involved in genomic adaptation to HMF in *Saccharomyces cerevisiae*, suggesting that HMF might elicit oxidative stress (126, 189). In agreement with the role for Yap1 in adaptation to HMF, the overexpression of YAP1 has been shown to increase tolerance to HMF and dilute acid spruce hydrolysates (189). Furthermore, furfural has been shown to induce the

accumulation of reactive oxygen species (ROS), leading to damages in cell components including mitochondrial and vacuolar membranes, and chromatin in yeast (190). However, how furfural and HMF induce oxidative stress has not yet been elucidated. In addition, the inhibition of biosynthetic pathways by depletion of NADPH during the reduction of furfural and HMF might also be responsible for their toxicity. In *E. coli*, the NADPH-dependent sulfur assimilation pathway was shown to be inhibited in furfural-treated cells, and supplementation of sulfur-containing amino acids, cysteine and methionine, could increase furfural tolerance (191). Although furfural and HMF share the same toxic aldehyde group, furfural has higher toxicity than HMF (123, 192). However, detailed mechanisms for the differences in toxicity between furfural and HMF have not yet been clarified.

In this study, as an effort to develop tolerant strains to furfural and HMF, we investigated the mechanisms by which furfural and HMF induce oxidative stress. We have demonstrated that thiol-reactivity of furfural and HMF contribute to inducing oxidative stress, but furfural has higher thiol-reactivity than HMF. Based on the identified mechanisms we generated yeast strains tolerant to HMF and/or furfural.

6.2. Induction of intracellular ROS by furfural and HMF in *S. cerevisiae*

In previous studies, it was shown that furfural induces ROS accumulation, resulting in damages of various cellular components in *S. cerevisiae* (190). Therefore, we

investigated whether HMF can also exert the same ROS accumulation effect as furfural. The cellular ROS levels were detected by incubating cells with 2'7'- DCF diacetate which is converted to a fluorescent compound upon ROS-dependent oxidation. 2'7'- DCF diacetate is known to detect H_2O_2, peroxynitrite ($ONOO^-$), and hydroxyl radical (•OH) (145). As a positive control, the cells were treated with 5 mM H_2O_2, and the fluorescent cells were counted by FACS analysis. In agreement with the previous report, ROS accumulation was detected after incubation of yeast cells with 30 mM furfural for 8 h (Fig. 6.1). Furthermore, treatment of HMF also induced ROS accumulation in a dose-dependent manner, indicating that furfural and HMF share a common effect of inducing intracellular ROS accumulation (Fig. 6.1).

6.3. Activation of Yap1 as thiol-reactive electrophiles by furfural and HMF

Previous DNA microarray experiments have revealed that Yap1 is one of the key regulators involved in genomic adaptation to HMF in *S. cerevisiae* (126). Yap1 is activated by H_2O_2 and thiol-reactive electrophiles through different mechanisms (193). The H_2O_2-dependent activation of Yap1 is mediated by a glutathione peroxidase Gpx3 which acts as a H_2O_2 sensor and induces intramolecular disulfide bond formation between N- and C-terminal Cys residues in Yap1. On the other hand, thiol-reactive electrophiles such as N-ethylmaleimide (NEM), acrolein, and

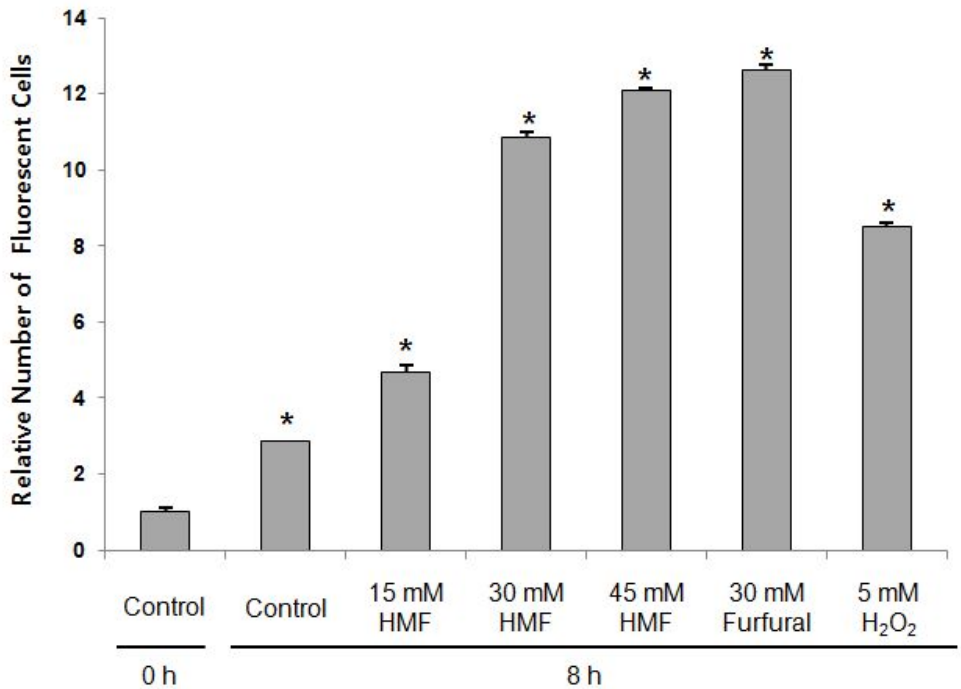

Figure 6.1 Both furfural and HMF induce ROS accumulation.

Exponentially growing yeast cells were treated with the indicated amounts of HMF, furfural, or H_2O_2 for 8 h and treated with 2',7'-DCF diacetate, a ROS indicator. The number of fluorescent cells was analyzed by FACS. Data represent an average of three experiments, and bars indicate plus standard deviations.

4-hydroxynonenal directly activate Yap1 by covalent modification of Cys residues residues in the C-terminal domain (132). Furthermore, H_2O_2 and thiol-reactive reagents have been shown to activate different subsets of Yap1 targets as well as common targets, eliciting distinct adaptive responses (133). Therefore, we examined which pathway is responsible for the HMF-dependent activation of Yap1. In addition, we investigated whether furfural could also activate Yap1.

In order to understand the Yap1 activation pathway, we compared the expression levels of Yap1 targets, *TRX2*, *YAP1*, and *MET16*, in wild type and *gpx3Δ* upon treatment of HMF or furfural (Fig. 6.2). The expression of *TRX2* and *YAP1* was induced when cells were treated with 0.4 mM H_2O_2, but their induction folds were dramatically reduced in *gpx3Δ*, confirming the role for Gpx3 in the H_2O_2-dependent activation of Yap1 (Figs. 6.2A and B). The expression of *TRX2* and *YAP1* was also induced by 30 mM HMF and 20 mM furfural, although the induction folds were lower than those of H_2O_2-dependent induction. However, HMF- and furfural-dependent inductions of *TRX2* and *YAP1* were largely unaffected by the lack of *GPX3*, suggesting that H_2O_2 might play a minimal role in the HMF- and furfural-dependent Yap1 activation pathways. Therefore, HMF and furfural might directly activate Yap1 as thiol-reactive electrophiles.

Unlike *TRX2* and *YAP1*, *MET16* is known to be responsive to thiol-reactive reagents rather than H_2O_2 (133). Accordingly, *MET16* was less sensitive to H_2O_2-dependent induction compared with *TRX2* and *YAP1* (Fig. 6.2C). Moreover, HMF and furfural were more effective than H_2O_2 for the induction of *MET16*, further

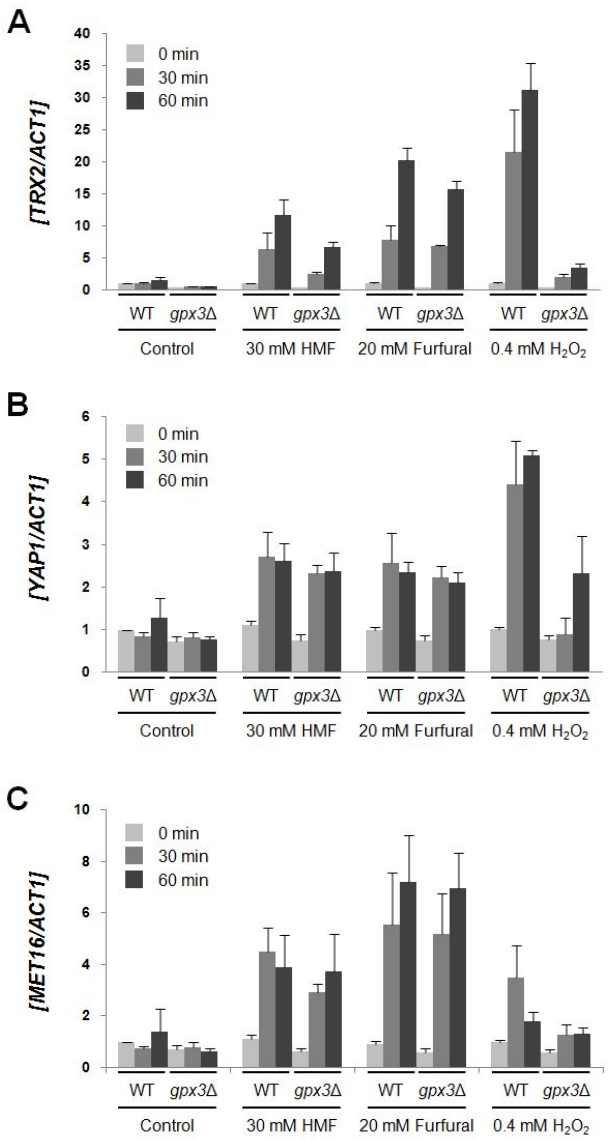

Figure 6.2 Furfural and HMF activate Yap1 as thiol-reactive electrophiles.

BY4741 (WT) and *gpx3Δ* cells were grown in YPD medium until early exponential phase and then treated with the indicated amounts of HMF, furfural, and H_2O_2 for 30 or 60 min. The mRNA expression levels of (A) *TRX2*, (B) *YAP1*, (C) *MET18* were detected by qRT-PCR and normalized to *ACT1*. The relative mRNA levels to those of untreated wild type control were indicated.

supporting the notion that HMF and furfural directly activate Yap1 as thiol-reactive reagents (Fig. 6.2C). Although HMF and furfural induce the accumulation of ROS, which include H_2O_2, Yap1 seems to be directly activated by HMF and furfural before the accumulation of H_2O_2.

6.4. Furfural has higher reactivity than HMF towards GSH *in vitro* and *in vivo*.

If HMF and furfural act as thiol-reactive electrophiles, they might react not only with Yap1 but also with other cellular sulfhydryl groups present in proteins and small molecular thiols such as GSH. GSH, the most abundant thiols in cells, serves as a redox buffer and plays a central role in cellular protection against oxidative stress (194). To investigate the effects of furfural and HMF on GSH, we incubated HMF or furfural with 5 mM GSH and detected the reactivity by measuring the decrease in thiol concentration of GSH. After 15 min of incubation, GSH concentrations were reduced to 4.93 mM and 4.82 mM in the presence of 30 mM and 60 mM furfural, respectively (Fig. 6.3A). However, no significant reduction of GSH concentrations was detected in the presence of HMF for up to 1 h, suggesting higher reactivity of furfural than HMF towards GSH (Fig. 6.3A). Upon longer incubation of up to 96 h, HMF-dependent reduction of GSH concentration was also observed (Fig. 6.3B). The higher thiol-reactivity of furfural than HMF agrees with the predicted electrophilicity of these compounds. HMF has an additional

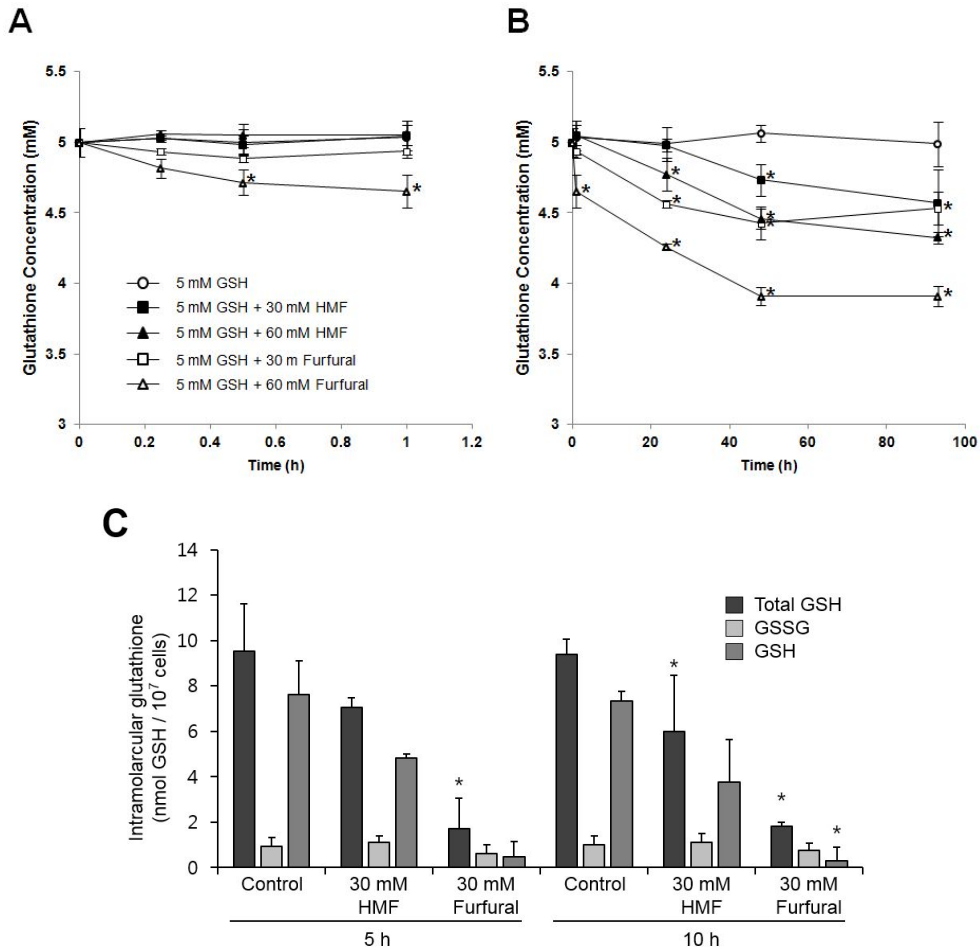

Figure 6.3 Furfural shows higher reactivity than HMF towards GSH *in vitro* and *in vivo*.

The indicated concentrations of furfural or HMF were incubated with 5 mM GSH at 30°C, and the concentrations GSH sulfhydryl groups were measured up to 1 h (A) and up to 96 h (B). (C) Early exponential phase cells grown in YPD medium were treated with 30 mM HMF or furfural for 5 h and 10 h. Deproteinated cell lysates were assayed for the measurement of total GSH and GSSG levels, and GSH levels were calculated by abstracting the amounts of GSSG from those of total GSH. *, significantly lower than the control.

hydroxymethyl group, which acts as an electron donating group reducing electrophilicity.We also investigated the effects of furfural and HMF on GSH and GSSG levels *in vivo* (Fig. 6.3C). In agreement with the fact that most of the cellular glutathione is present in a reduced form, the untreated control cells showed a GSH:GSSG ratio of 8:1. Treatment of 30 mM HMF reduced the GSH levels by 37%-49% without affecting the GSSG levels, resulting in a GSH:GSSG ratio of 4.3:1 and 3.4:1 after 5 h and 10 h, respectively. 30 mM furfural exerted more dramatic depletion of GSH to levels even lower than those of GSSG, resulting in a GSH:GSSG ratio of 0.74:1 and 0.41:1 after 5 and 10 h, respectively. These results suggest that although both furfural and HMF reduce cellular GSH levels possibly by direct interaction, furfural is much more effective in GSH depletion.

6.5. Overexpression of an active YAP1 mutant increases tolerance to furfural and HMF.

Since both furfural and HMF act as thiol-reactive electrophiles which can induce oxidative stress, the overexpression of *YAP1* might confer resistance to furfural and HMF by activation of genes involved in resistance to oxidative stress and reduction of furan aldehydes (126, 195). Indeed, overexpression of *YAP1* has been shown to convey resistance to HMF, although the detailed mechanisms have not been investigated (189).

Based on our observation that furfural can also activate Yap1, we examined whether overexpression of *YAP1* can also increase tolerance to furfural. In addition to wild type *YAP1*, we overexpressed $YAP1^{C620F}$ mutant under the *ADH1* promoter, and examined cell growth in the presence of furfural or HMF. $YAP1^{C620F}$ is a gain-of-function allele of *YAP1*, originally isolated as a mutant conferring resistance to diazaborine (196). *ZWF1*, involved in NADPH production through the pentose phosphate pathway, was also overexpressed as a positive control (130). As shown in Fig. 4, cells overexpressing *ZWF1* showed slightly better growth than the control in the presence of 30 mM or 40 mM HMF. However, overexpression of *YAP1* was more efficient than *ZWF1* overexpression for enhancing the HMF resistance. Furthermore, $YAP1^{C620F}$-overexpressed cells showed higher HMF tolerance than *YAP1*-overexpressed cells, reflecting the higher activity of the $Yap1^{C620F}$ mutant.

Although overexpression of *YAP1*, $YAP1^{C620F}$, and *ZWF1* also increased tolerance to furfural, the effect of each gene slightly differed depending on the furfural concentration (Fig. 6.4). In the presence of 20 mM furfural, cells overexpressing *ZWF1* showed better growth than cells overexpressing $YAP1^{C620F}$ or *YAP1*. However, at a higher concentration of 30 mM, the overexpression of $YAP1^{C620F}$ or *YAP1* was more efficient than the overexpression of *ZWF1* in increasing furfural tolerance.

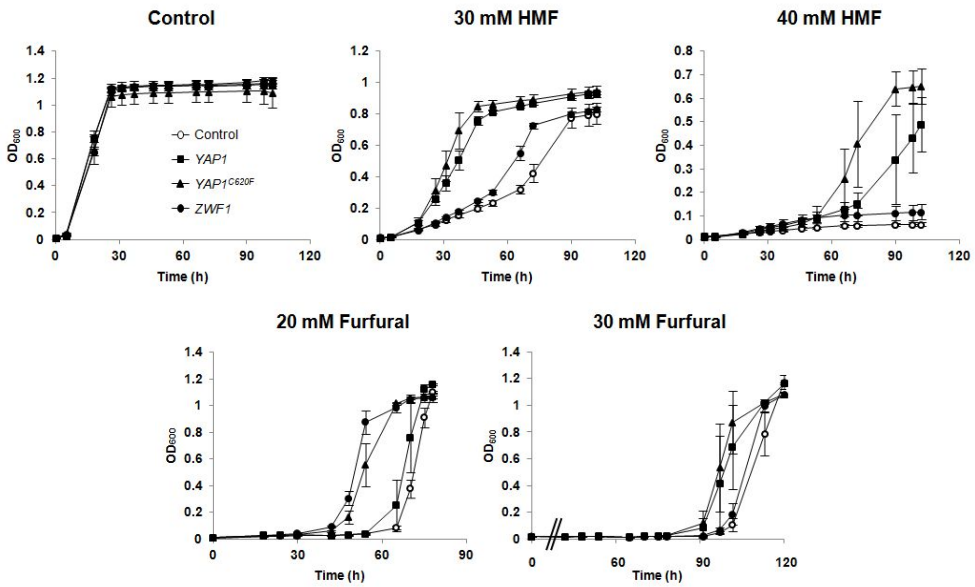

Figure 6.4 Overexpression of *YAP1* or *YAP1^{C620F}* increases tolerance to HMF and furfural.

Cells overexpressing *YAP1*, *YAP1^{C620F}*, or *ZWF1* were grown in SC-Leu medium containing 30 mM or 40 mM HMF, or 20 mM or 30 mM furfural. Cells containing pRS415ADH empty vector were used as a control. All the experiments were performed in triplicate.

6.6. Overexpression of catalase increases furfural and HMF tolerance.

Although H_2O_2 does not seem to play a major role in the activation of Yap1 in the presence of furfural or HMF (Fig. 6.2), the accumulated H_2O_2 could be one of the mediators exerting the toxicity of furfural and HMF. To test this possibility, we overexpressed catalases that decompose H_2O_2, and examined the effects on furfural and HMF tolerance. *CTA1* encoding mitochondrial catalase and *CTT1* encoding cytosolic catalase are known targets of Yap1 (133). Overexpression of *CTA1* and *CTT1* commonly led to furfural and HMF tolerance, indicating that H_2O_2 indeed plays a part in the toxicity of furfural and HMF (Fig. 6.5A). For HMF tolerance, *CTA1* was more effective than *CTT1*.

6.7. Increase in cellular GSH levels enhances tolerance to furfural, but not to HMF.

Since furfural and HMF react with cellular GSH (Fig. 6.3), GSH might play a protective role against furfural and HMF. We investigated this possibility by overexpressing *GSH1* and *GSH2* involved in glutathione biosynthesis, and *GLR1* encoding glutathione reductase that recycles the oxidized GSSG back to GSH (197). The first and rate-limiting step of GSH biosynthesis is the production of γ-glutamyl cysteine (γ-GC) by Gsh1, and Gsh2 catalyzes the production of GSH from γ-GC and glycine (197). The expression of *GSH1*, *GSH2*, and *GLR1* genes

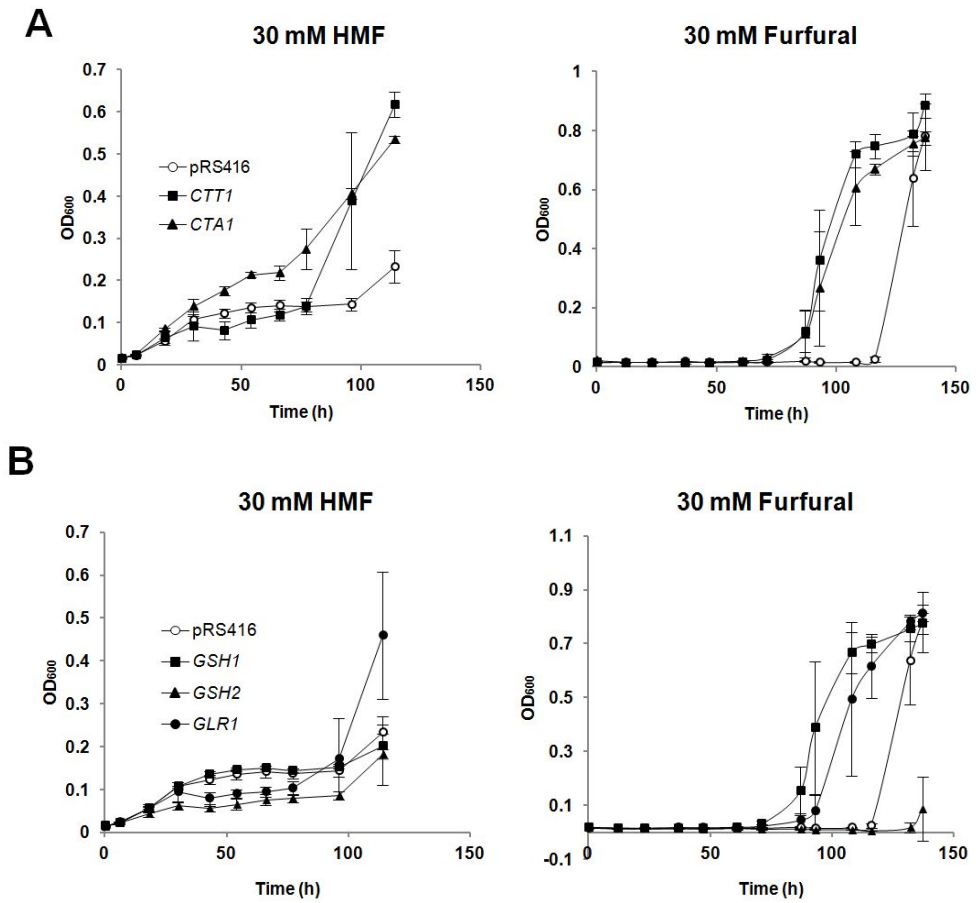

Figure 6.5 Effects of overexpression of antioxidant genes on tolerance to furfural and HMF.

Cells overexpressing *CTA1* or *CTT1* (A) or *GSH1*, *GSH2*, or *GLR1* (B) were grown in SC-Ura media containing 30 mM HMF or furfural. Cells harboring pRS416ADH vector was used as a control. All the experiments were performed in triplicate.

are all regulated by Yap1 (140, 198). As shown in Fig. 6.5B, the overexpression of *GSH1* and *GLR1*, but not *GSH2*, increased cellular tolerance to furfural, suggesting the protective effect of GSH against furfural toxicity. However, neither *GSH1* nor *GSH2* overexpression increased tolerance to HMF, while *GLR1* overexpression slightly increased the tolerance (Fig. 6.5B). Therefore, the protective role of GSH seems to be specific to furfural which is more effective than HMF in GSH depletion.

6.8. Addition of GSH and dithiothreitol (DTT) to the medium reduces toxicity of furfural, but not HMF.

Next, we examined whether GSH added to the medium could also elicit protective effects against furfural and HMF. In accordance with the effect of GSH overexpression *in vivo*, the addition of GSH in rich YPD medium exerted a protective effect on cell growth only in the presence of furfural, but not HMF. Although furfural tolerance was increased by the addition of GSH in a dose-dependent manner, the cells became more sensitive to HMF in the presence of GSH (Fig. 6.6A).

We also tested the effects of DTT on furfural and HMF tolerance. DTT is a cell-permeable reducing agent that can prevent disulfide bond formation (199). Treatment of DTT has been shown to induce reductive stress (200). Accordingly, cell growth was slightly reduced in the presence of DTT in a dose-dependent

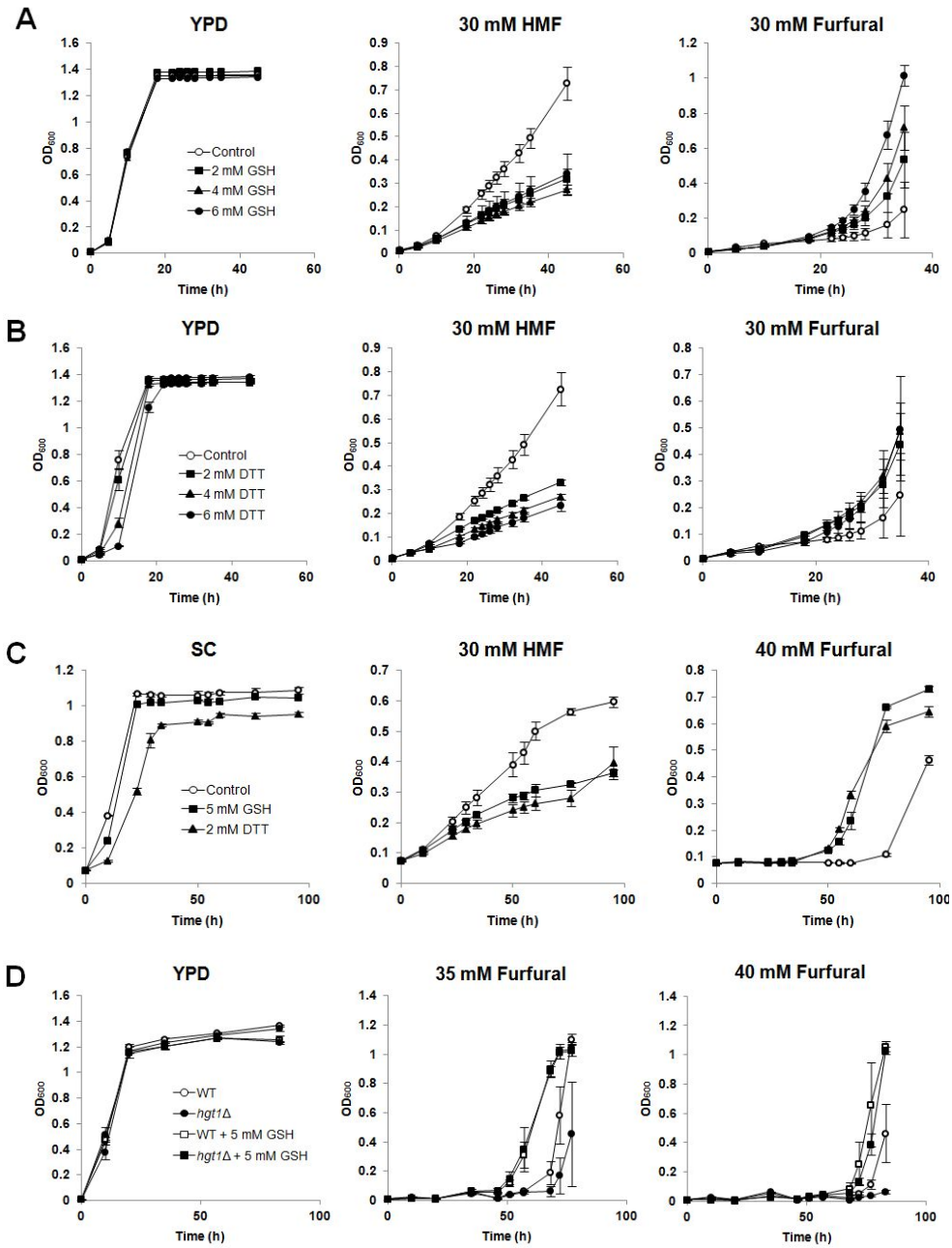

Figure 6.6 Addition of GSH or DTT into medium increases tolerance to furfural.

Cells were grown in YPD medium containing 30 mM HMF or furfural in the presence of the indicated concentrations of GSH (A) or DTT (B). (C) Cells were grown in SC medium

Figure 6.6 Addition of GSH or DTT into medium increases tolerance to furfural.(Continued)

(D) containing 30 mM HMF or 40 mM furfural in the presence of 5 mM GSH or 2 mM DTT. Wild type and *hgt1Δ* cells were grown in YPD medium containing 35 mM or 40 mM furfural in the absence or presence of 5 mM GSH. All the experiments were performed in triplicate.

manner (Fig. 6.6B). Even with such a growth inhibitory effect, DTT facilitated cell growth in the presence of furfural, suggesting that thiol oxidation is one of the toxic effects of furfural. On the contrary, DTT exerted a negative effect on cell growth in the presence of HMF (Fig. 6.6B).

To examine whether the effects of GSH and DTT on furfural and HMF tolerance could be dependent on culture medium, we also tested the effects of GSH and DTT in a synthetic complete (SC) medium, where furfural and HMF show higher toxicity than YPD medium. As shown in the YPD medium, both GSH and DTT showed protective effects against furfural, while inhibiting cell growth in the presence of HMF in SC medium (Fig. 6.6C).

Next, we investigated whether the protective role of GSH against furfural requires transport of GSH inside the cell. Hgt1 is a high-affinity GSH transporter, and *hgt1Δ* deletion mutant showed no detectable plasma membrane GSH transport (135). Therefore, we compared the protective effect of GSH in wild type and *hgt1Δ*. In the presence of furfural, *HGT1* deletion mutant showed longer lag phase than wild type, implying that GSH uptake from the medium has an advantage in furfural tolerance (Fig. 6.6D). However, when 5 mM GSH was provided in the medium, *hgt1Δ* as well as wild type showed increased furfural tolerance, indicating that GSH can play a protective role against furfural even outside of the cell. The protective role of extracellular GSH against furfural might be the result of the GSH-dependent protection of surface-exposed thiols. In addition, GSH could directly react with furfural, reducing the effective concentration of furfural.

6.9. Conclusions

Furfural and HMF are known to induce oxidative stress, but the underlying mechanisms have not yet been clarified. In this study, we demonstrated that furfural and HMF act as thiol-reactive electrophiles, thus activating the Yap1 transcription factor and reducing cellular GSH levels. In addition to the consumption of NADPH during the reductive detoxification of furfural and HMF, such thiol-reactivity might be responsible for the induction of oxidative stress and ROS accumulation by these compounds.

Covalent modification or oxidation of sulfhydryl groups can affect the structure and function of enzymes and other proteins with various biological functions. The redox status of protein sulfhydryl groups are mainly regulated by glutaredoxins (GRXs) and thioredoxins (TRXs) which are reduced by GSH and NADPH-dependent thioredoxin reductase, respectively (197). The oxidized GSSG is reduced by glutathione reductase, using NADPH as a reducing power (140). GSH also acts as a reductant for glutathione peroxidase involved in the reduction of H_2O_2 and organic hydroperoxides (136). Therefore, maintenance of GSH and NADPH levels is important for cellular protection against oxidative stress.

We have shown that furfural has higher thiol-reactivity than HMF, thus more effectively depleting GSH levels *in vitro* and *in vivo*. Cellular GSH levels were reduced by 16-fold with a 5 h treatment of 30 mM furfural, but only by 1.6-fold with 30 mM HMF. Such higher thiol-reactivity might be partly responsible for the

higher toxicity of furfural than HMF. Considering the central role of GSH in cellular protection against oxidative stress (194), the depletion of GSH might be one of the major toxic mechanisms of furfural. In support of this notion, the overexpression of genes for GSH synthesis (*GSH1*) and GSSG reduction (*GLR1*), or addition of GSH in the medium, conferred resistance to furfural. The *hgt1Δ* strain, which cannot uptake GSH, was more sensitive to furfural than wild type, but still showed increased furfural resistance in the presence of extracellular GSH. Therefore, GSH seems to play a protective role against furfural both inside and outside the cell. In addition to its role against oxidative stress, GSH is also involved in the detoxification of electrophilic xenobiotics through the formation of GSH S-conjugates, which are subsequently transported into the vacuole or out of the cell (138). Therefore, we cannot rule out the possibility that GSH might also be involved in the detoxification of furfural by this pathway.

Although HMF could also reduce cellular GSH levels, HMF tolerance was largely unaffected by increasing expression of genes for GSH biosynthesis. Therefore, the relatively mild reduction in GSH levels seems not to be critical for the toxicity of HMF. Considering the fact that HMF has weaker thiol-reactivity than furfural, other effects such as NADPH depletion, ROS accumulation, and modifying other cellular targets might play larger roles for HMF toxicity. Unexpectedly, the addition of GSH or DTT in the medium further inhibited cell growth in the presence of HMF. Further studies are needed to elucidate the mechanisms for the negative effect of GSH and DTT on HMF tolerance.

Although furfural and HMF commonly act as thiol-reactive reagents inducing oxidative stress, their differences in reactivity might affect a different range of cellular targets, thus exhibiting differential toxic mechanisms. Therefore, pleiotropic gene expression by overexpression of $YAP1^{C620F}$ might be an effective strategy to generate furfural- and HMF- tolerant yeast strain. Overexpression of *YAP1* might confer resistance to furfural and/or HMF by activation of genes for antioxidant enzymes including *CTA1*, *CTT1*, *GSH1*, and *GLR1* as demonstrated in this study. Furthermore, Yap1 targets also include *ADH7* and *GRE2* which are involved in the reduction of furan aldehydes (126, 195). Yap1 also regulates the expression of efflux pumps including *FLR1*, *YCF1*, and *SNQ2*, which might be implicated in the detoxification of furfural and/or HMF (196) (126). The furfural- and HMF- tolerance could be further increased by optimizing $YAP1^{C620F}$ expression levels and by screening more *YAP1* mutants with enhanced activity.

Taken together, for the first time we elucidated the thiol-reactivity of furfural and HMF as the underlying mechanisms by which oxidative stress is induced by these compounds. Furthermore, we provided new evidence that furfural and HMF might cause differential cytotoxic effects due of the differences in thiol-reactivity. The similarities and differences in toxicity between furfural and HMF might provide useful information for the future development of tolerant strains for cellulosic ethanol production.

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Chapter 7.

Overall discussion and recommendations

In this thesis, yeast strains which have the ability of an improved uptake of glucose and fructose or higher tolerance to lignocellulosic furan aldehydes were developed. These strategies are expected to be able to apply to various yeast strains for metabolic engineering.

In the first part, glucose uptake was enhanced by overexpressing the hexose transporter and a transcription factor *GCR1* for the ethanol and lactic acid production in *S. cerevisiae*. Among several *HXTs*, *HXT7* overexpression was the most effective in glucose uptake and ethanol production, and *GCR1* overexpression was also effective. And the simultaneous overexpression of these two genes increased the effect of overexpression of each gene. Finally, application to lactic acid production strain showed that *GCR1* overexpression increased the uptake rate and capacity of glucose and lactic acid productivity.

In the second part, for the development of the yeast strain that have improved fructose uptake in the media containing both glucose and fructose as the carbon source, heterologous fructose transporters and hexokinases was overexpressed in *S. cerevisiae*. Although Fsy1 originated from *C. magnoliae* increased fructose uptake in both low (20g/L) and high (50g/L) concentration of fructose and glucose containing media, it decreased glucose uptake, especially reducing the overall growth and productivity in the condition of high concentration of the sugars. Overexpression of the hexokinase, Hxk1, Glk1, and a putative hexokinae Ylr446w, which are not related to glucose repression, increased uptake of glucose or fructose and production of ethanol in low and high glucose and fructose containing media.

At last, to construct tolerant yeast strain to furan aldehydes, the response mechanism to HMF and furfural was investigated and several resistant strains to them were developed. It was confirmed that both furfural and HMF induce the generation of ROS, and activate Yap1 as a thiol-reactive electrophile. And overexpression of constitutively active $Yap1^{C620F}$ mutant increased tolerance to furan aldehydes, and among the target of Yap1, catalase or glutathione production enzymes also decreased the lag phase induced by those inhibitors, which showed the importance of Yap1-branched pathway. In case of glutathione, it is specifically effective in tolerance to furfural, not HMF, and a strong reductant, dithiothreitol also showed similar effects. By these results, it was shown that furfural and HMF induce similar but different effects in yeast, and we constructed tolerant strain to them.

Conclusively, we established the strategy for the improvement of uptake of conventional carbon sources, glucose and fructose, and showed the effectiveness of each strategy in WT or lactic acid production strain, which is able to be applied to other various strains for metabolic engineering. And by the development of tolerant yeast strain to lignocellulosic furan aldehydes, it is expected to reduce the overall cost of fuel production from lignocellulose and contribute to the practical application of the microbial fermentation.

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Abstract in Korean

국 문 초 록

Saccharomyces cerevisiae 는 차세대 연료 생산을 위한 미생물로, 대장균과 함께 미생물을 이용한 연구에 있어서 대표적인 모델로 이용되어 왔다. 본 연구에서는 대사공학적 전략을 이용하여 포도당, 과당 및 목질계 바이오매스 전처리 물질에서 에탄올 및 젖산을 효율적으로 생산할 수 있는 연구를 진행하였다.

첫 번째로, 효모 균주의 에탄올 및 젖산 생산을 증가시키기 위해서 포도당 흡수가 증진된 균주를 제작했다. 효모에서 포도당은 포도당 수송체 (*HXT*)를 통한 확산을 통해 흡수되며, 이러한 *HXT* 의 발현은 외부 포도당 농도를 인지한 포도당 센서 단백질에서 전달된 신호에 의해 조절된다. 이러한 포도당 수송체의 직접적인 과발현을 통해 포도당 흡수가 증진됨을 관찰했다. 테스트한 5 개의 *HXT* (Hxt1, Hxt2, Hxt3, Hxt4, and Hxt7) 중 포도당에 가장 높은 친화도를 갖는 Hxt7 의 과발현이 가장 효과적인 것으로 관찰되었고, 보통의 친화도를 갖는 Hxt2 와 Hxt4 의 과발현이 또한 효과를 보였다. *HXT* 발현의 억제 인자로 알려진 *mth1* 과 *std1* 의 결손은 배지 조건에 따라서 포도당 흡수 정도에 다른 영향을 미쳤다. 또, *HXT1* 과 리보솜의 전사를 증가시키는 전사 조절 인자인 Gcr1 의 과발현은 증가된 세포 성장과 포도당 흡수 증진을 유도했다. 이러한 포도당 흡수 속도를 강화하는 모든 전략은 에탄올 생산 증가로 이어졌으며, *GCR1* 의 경우 젖산 생산을 위해 변이된 균주에 적용했을 때도 산성 발효 조건에서 포도당 흡수 속도 및 젖산의 생산성과 생산량에 있어서 상당한 증가를 보였다.

두 번째로, 포도당과 과당이 함께 섞인 배지에서 과당 흡수를 증진시키기 위해서 당 인산화 효소와 이형의 과당 수송체를 과발현한 효모 균주를 제작했다. 효모는 포도당이 있는 배지에서 다른 탄소원을 대사 및 흡수하는 과정이 억제되는 단점이 있다. 이는 효모 내 특이적인 과당 수송체가 없기 때문이며, 이를 보완하기 위해 포도당에 친화적인 균주에 과당에 특이적인 수송체를 도입하여 과당을 탄소원으로 이용하는 배지에서 흡수가 증진되는 것을 확인했으며, 과당과 포도당이 섞인 배지에서는 농도에 따라 다른 결과를 보였다. 또한 세포 내부에서 과당 소모를 촉진하도록 6 탄당 인산화 효소를 과발현하여 포도당 및 과당이 섞인 배지에서 둘 모두가 빠르게 소모되는 것을 관찰했다. 포도당이 있는 배지에서 가장 주요하게 작용하는 *HXK2* 의 경우 glucose repression 을 일으키고 과발현 시 성장 및 활성 억제를 초래하기 때문에, *HXK2* 에 의해 평소에 억제되는 *HXK1*, *GLK1* 과 아직 그 기능이 제대로 밝혀지지 않은 인산화 효소인 *YLR446w* 를 타겟으로 하였다. 여기서 기존에 알려진 바가 있는 *HXK1* 이 가장 효과적으로 당 소모를 증진시키는 것을 확인했으며, *YLR446w* 과발현 역시 과당의 소모를 증진시켰으며, 이를 통해 이것이 당 인산화 효소로 작용할 수 있음을 간접적으로 확인할 수 있었다. 앞선 포도당 흡수 증진 실험에서와 동일하게, 여기서도 당 소모의 증진은 에탄올 생산으로 모두 이어짐이 확인되었다.

마지막으로, 목질계 바이오매스 기반 에탄올 생산을 위해 푸르푸랄(furfural)과 5-히드록시메틸푸르푸랄(5-hydroxymethylfurfural, HMF)에 대한 저항성 균주를 제작하였다. HMF 와 furfural 은 산화적인 스트레스를 유발한다고 알려져 있으나, 효모 내에서 어떤 유전적 응답 기작으로 탈 독성화가 이루어지는지 밝혀지지 않았다. 효모 내에서 산화적인 스트레스에 대한 응답 반응은 두 가지 종류로 나뉘는데,

thiol 에 대한 높은 반응성을 지닌 물질을 통한 방식이나 과산화수소에 의한 방식으로 나뉘게 된다. 각각의 스트레스에서 알려진 타겟의 전사량 변화를 통해서, HMF 와 furfural 은 thiol 과 반응성이 높은 친 전자성 물질로 작용하며, 직접적으로 산화적인 스트레스에 대한 중추로 작용하는 Yap1 을 활성화시킨다. 이는 과산화물과는 독립적인 방식으로 작용하며, 세포 내 글루타치온(GSH)를 고갈시키면서 ROS 를 축적시키게 되는데, 특히 furfural 은 GSH 와 *in vivo* 와 *in vitro* 모두에서 매우 활발하게 반응한다는 것이 확인되었다. 그리고 이러한 알아낸 독성 기작을 통해서, 항상적으로 활성화되어 있는 $YAP1^{C620F}$ 의 돌연변이, 그리고 Yap1 의 타겟인 카탈라아제(*CTA1* 과 *CTT1*)의 과발현을 통해 furfural 과 HMF 에 대한 저항성이 증진됨을 확인했다. 하지만, 글루타치온 합성이나 재생에 관련된 유전자(*GSH1* 과 *GLR1*)의 과발현에 의하거나, 세포에 직접적으로 이를 첨가함으로써 세포 내 글루타치온을 높이는 것은 furfural 에 대한 저항성만을 증진시켰으며, HMF 에 대해서는 효과를 보이지 못하는 것이 확인되었다.

주요어 : 대사공학, 포도당 흡수, 과당 흡수, 사탕수수 당액, 목질계바이오매스, 퓨란 알데히드 저항성, 젖산, *Saccharomyces cerevisiae*

학 번 : 2010-23323