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Development of efficient D-lactic acid-producing *Saccharomyces cerevisiae* strains by evolutionary and rational metabolic engineering

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백 승 호

Development of efficient D-lactic acid-producing *Saccharomyces cerevisiae* strains by evolutionary and rational metabolic engineering

by

Seung-Ho Baek

Advisor : Professor Ji-Sook Hahn, Ph.D.

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School of Chemical and Biological Engineering

Graduate School

Seoul National University

ABSTRACT

Development of efficient D-lactic acid-producing *Saccharomyces cerevisiae* strains by evolutionary and rational metabolic engineering

Seung-Ho Baek
School of Chemical and Biological Engineering
The Graduate School
Seoul National University

There is an increasing demand for microbial production of optically pure lactic acid (LA) as a monomer for biodegradable poly lactic acid (PLA). *Saccharomyces cerevisiae*, having high acid tolerance, has emerged as a promising LA-producing host. In this dissertation, efficient D-LA-producing strains of *S. cerevisiae* were developed using rational metabolic engineering and adaptive evolution.

Firstly, to generate D-LA-producing strain, highly stereospecific D-lactate dehydrogenase gene (ldhA, designated *Lm.ldhA*) from *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293 was selected and expressed in *S. cerevisiae*

lacking natural LA production activity. To prevent D-LA utilization upon glucose depletion, *DLD1* encoding D-lactate dehydrogenase and *JEN1* encoding monocarboxylate transporter were disrupted in combination. Ethanol formation was reduced by deleting *PDC1* and *ADH1* genes encoding major pyruvate decarboxylase and alcohol dehydrogenase, respectively. In addition, glycerol production was eliminated by deleting *GPD1* and *GPD2* genes encoding glycerol-3-phosphate dehydrogenase, resulting in a gradual increase in D-LA production level up to 12.9 g/L with a yield of 0.65 g/g glucose.

Next, in order to overcome growth inhibition by LA in the engineered LA-producing strain adaptive evolution was carried out by gradual increase in LA concentrations in the culture medium. As a result, the evolved strain showed higher glucose consumption rate with an improved D-LA production level compared with the unevolved strain. By additional genome-integration of the *Lm.ldhA* gene into the evolved strain, a D-LA production level was further improved up to 38.3 g/L in acidic fermentation condition without pH control. In neutralization condition, this strain produced 118.6 g/L D-LA, showing an increased yield (0.79 g/g glucose). Through whole genome sequencing analysis, mutations of five genes, including two nonsense mutations (*GSF2* and *SYN8*) and three point mutations (*STM1*, *SIF2*, *and BUD27*) were detected in the evolved strain. Deletion of *GSF2* in the unevolved strain led to improved growth rate and glucose consumption ability, resulting in D-LA production level comparable to that of the evolved strain. It was also demonstrated that deletion of *STM1* or *SIF2* increased resistance to LA in *S.*

cerevisiae as well as deletion of *SYN8*, which is already known to increase LA tolerance.

Lastly, D-LA production level was further improved by integrating an additional copy of *HAA1* into the evolved strain. This Haa1-overexpressing strain consumed 62.2 g/L glucose and produced 48.9 g/L D-LA with a yield of 0.79 g/g glucose under acidic fermentation. In a flask fed-batch fermentation under neutralizing conditions, this final strain showed the highest productivity of 2.2 g/(L·h). In addition, it was demonstrated that Haa1 is phosphorylated by CK2 in *S. cerevisiae*. Although the function of CK2-dependent phosphorylation should be further elucidated, this result suggests that CK2 might be implicated in LA stress adaptation through regulating Haa1.

Keywords : D-Lactic acid, *Saccharomyces cerevisiae*, Metabolic engineering, Acid tolerance, Growth inhibition, Haa1, Adaptive evolution

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LIST OF ABBREVIATIONS

3AT	3-amino-1,2,4-triazole
ADH	alcohol dehydrogenase
ATP	adenosine triphosphate
$CaCO_3$	calcium carbonate
DHAP	dihydroxyacetone phosphate
EDTA	ehylenediaminetetraacetic acid
EMP	Embden-Meyerhoff-Parnas
G3P	glycerol-3-phosphate
GAP	glyceraldehyde-3-phosphate
GATK	genome analysis toolkit
GPD	glycerol-3-phosphate dehydrogenase
GST	glutathione S-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	histidine
HPLC	high performance liquid chromatography
HRE	Haa1 recognition element
LA	lactic acid
LAB	lactic acid bacteria
LB	Luria-Bertani
LDH	lactate dehydrogenase
Leu	leucine
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis

PCR	polymerase chain reaction
PDC	pyruvate decarboxylase
PLA	poly lactic acid
PMSF	phenylmethylsulfonyl fluoride
PP	pentose phosphate
PK	phosphoketolase
qRT-PCR	quantitative reverse transcription PCR
RI	refractive index
SC	synthetic complete
SDS	sodium dodecyl sulfate
SNV	single nucleotide variant
Trp	tryptophan
Ura	uracil
YPD	yeast extract-peptone-dextrose

Chapter 1.

Research background and objective

In recent decades, microbial production of lactic acid (LA) has emerged as an attractive alternative approach and LA has received many attentions as a noteworthy precursor due to its wide range of properties. Especially, production of poly lactic acid (PLA) has become a main purpose of LA production from microorganisms (Abbott et al. 2009; Martinez et al. 2013). Most LA fermentations have been carried out using lactic acid bacteria (LAB), but they require complex medium compositions and fastidious culture conditions. Above all, they mainly accumulate lactate rather than lactic acid preferred for industrial applications by pH control. These led to the research about *Saccharomyces cerevisiae* as a new host for microbial LA production (Abbott et al. 2009; Branduardi et al. 2008; Lee et al. 2015).

S. cerevisiae has been regarded as an important eukaryotic model organism and an interesting platform organism for industrial applications because its genetic information and tool is well established as well as several innate characteristics including strong fermentative ability and high stress tolerance (Branduardi et al. 2008; Sauer et al. 2010). Since *S. cerevisiae* does not make LA naturally, optically pure LA production is possible if a heterologous stereospecific lactate dehydrogenase (LDH) gene was introduced. Especially it has higher acid tolerance compared with other microorganisms, undissociated LA production with reducing use of neutralizing agent is possible. After generating the first LA-producing yeast strain (Dequin and Barre 1994), various engineered strains have been developed to improve LA production level. Until now, these studies have been focused on

enriching the carbon flux from pyruvate to LA, reducing ethanol and glycerol formation (Ishida et al. 2006a; Ishida et al. 2005; Ishida et al. 2006b; Saitoh et al. 2005; Tokuhiro et al. 2009).

So far, most engineered yeast strains produced optically pure L-LA because PLLA, polymerized using only L-LA, has been a major form of PLA production. However, there is an increasing demand for D-LA production since several properties of PLA are involved in combination ratio of PLLA and PDLA (Garlotta 2001; Tsuji 2002). Several studies have been reported that the metabolic-engineered strains, especially when eliminated *ADH1* gene, showed severe growth defects with reduction of glucose consumption rate because accumulated LA acts as a detrimental factor induced stress response (Ida et al. 2013; Ishida et al. 2006a; Lee et al. 2015; Sauer et al. 2010; Song et al. 2015). Although LA yield is increased, these problems lead to the overall decrease of final LA production level and productivity. Therefore, overcoming of growth retardation and insufficient glucose consumption is necessary for high-titer production. In addition, some researches have been focused on identifying the cellular response mechanism to LA stress. So far, it was confirmed that Haa1 and Aft1 participate in transcriptional responses to undissociated LA and dissociated lactate, respectively. Several genes related LA resistance have been also reported, but understanding of LA adaptation response in *S. cerevisiae* is still insufficient (Abbott et al. 2008; Fernandes et al. 2005; Hirasawa et al. 2013; Suzuki et al. 2013).

The ultimate aim of this study is to develop the efficient D-LA-producing

strain of *S. cerevisiae* by improving its acid tolerance. The first objective is the development of metabolic engineered strain producing pure D-LA. The second objective is the increase in growth and glucose consumption rates by adaptive evolution. The last objective is the demonstration of Haa1 overexpression effect and CK2-dependent Haa1 phosphorylation.

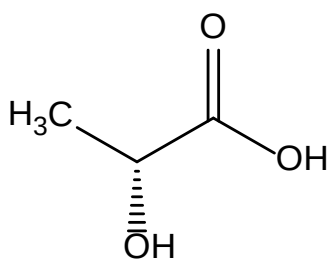
Chapter 2.

Literature review

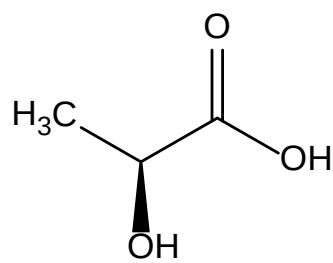
2.1. Microbial production of lactic acid

2.1.1. Lactic acid

Lactic acid (LA, 2-Hydroxypropanoic acid in IUPAC name) is the simplest three-carbon α-hydroxycarboxylic acid classified as a weak acid (pKa of 3.86) with the molecular formula $C_3H_6O_3$. In nature, there are two stereoisomers, D-LA and L-LA with a single chiral carbon (Fig. 2.1). Due to various properties, LA has a wide range of applications not only traditional food industry, but also agriculture, cosmetic, and pharmaceutical industries as an important compound (Eiteman and Ramalingam 2015; Juturu and Wu 2015; Martinez et al. 2013; Wang et al. 2015; Wee et al. 2006). Lactate, dissociated form of LA, is also an interesting chemical for hydrogenation to 1,2-propanediol or dehydration to acrylic acid (Gao et al. 2011). Currently, LA is principally utilized as a feedstock for poly lactic acid (PLA), an environmentally-friendly biodegradable polymer, which can be used for production from renewable packaging materials to biocompatible devices (Eiteman and Ramalingam 2015; Juturu and Wu 2015; Wang et al. 2015). LA has been generally manufactured by chemical synthesis based on lactonitrile (Martinez et al. 2013). In brief, lactonitrile was hydrolyzed by H_2SO_4, and then methyl lactate, esterified with methanol, was separated by distillation. Finally, racemic LA and methanol were produced by hydrolysis with H_2O. Other LA synthesis routes including sugar degradation, propylene glycol oxidation, choloropropionic acid hydrolysis, and nitric acid oxidation of propylene have been also developed



D-Lactic acid



L-Lactic acid

Figure 2.1 Structures of D- and L-isomers of lactic acid

(Martinez et al. 2013), but the resulting racemic D/L-LA mixture is not practical for food and medical applications because D-LA may be harmful to be assimilated by human and induced decalcification or acidosis. Optically pure LA production is also important to produce PLA because thermochemical and physical properties, and biodegradability can be improved by regulating of combination ratio of PDLA and PLLA, polymerized from D-LA and L-LA, respectively (Garlotta 2001). Since microbial LA production was firstly developed in 1881, fermentative LA production using microorganisms has received a great deal of interest because of an increasing need for PLA production. Indeed, it was reported that the worldwide demands for PLA were estimated to increase from 248.8 to 870.8 million kilograms per year to 2016 (Juturu and Wu 2015). Therefore, several commercial manufacturers such as Corbion Purac (Netherlands), Nature Works LLC (USA), Pyramid Bioplastics Guben GmBH (Germany), Galactic S.A. (Belgium), Archer Daniels Midland Company (USA) and Chinese companies have produced a large amount of LA using appropriate microorganisms, reaching approximately 90% of total LA production (Becker et al. 2015; Martinez et al. 2013). Although microbial LA production levels reached approximately 90% of total amount of LA production, but current LA production level is still insufficient to meet the LA consumption level. Therefore, numerous efforts have been focused on improving D-LA production yield and productivity by developing the efficient fermentation process and screening a new microorganism with lactate dehydrogenase gene. To reduce high production costs, there have been many attempts to use raw and

renewable materials from agricultural wastes to algal biomass (Juturu and Wu 2015). Furthermore, fermentation condition containing inexpensive nitrogen source, product recovery, and purification technologies in microbial LA production process have many challenges.

2.1.2. LA production by lactic acid bacteria

In nature, a variety of microorganisms including bacteria, fungi, and algae possess LA-producing properties by lactate dehydrogenase (LDH), which reduces pyruvate to D- or L-LA using NADH as a cofactor. Traditionally, lactic acid bacteria (LAB) have been predominantly used as a host strain for microbial LA production. LAB, which produce LA as a major fermentation product with generating ATP, are classified by two groups, homo-fermentative and hetero-fermentative strain based on glucose metabolism (Fig. 2.2). Homo-fermentative LAB produce two LA molecules converting one molecule of hexose by Embden-Meyerhoff-Parnas (EMP) pathway or one molecule of pentose including xylose and arabinose by pentose phosphate (PP) and glycolic pathways without byproducts formation. The theoretical yield of LA in this group is 1.0 g/g (2.0 mol/mol) from hexose and 1.0 g/g (1.67 mol/mol) from pentose, respectively. On the other hand, in hetero-fermentative LAB, both hexose and pentose are converted by phosphoketolase (PK) pathway, producing one molecule of LA with one molecule of ethanol or acetate depending on the redox potential. In terms of hexose utilization, hetero-fermentative LAB initially cleave glucose to ribulose-5-phosphate and CO_2 by

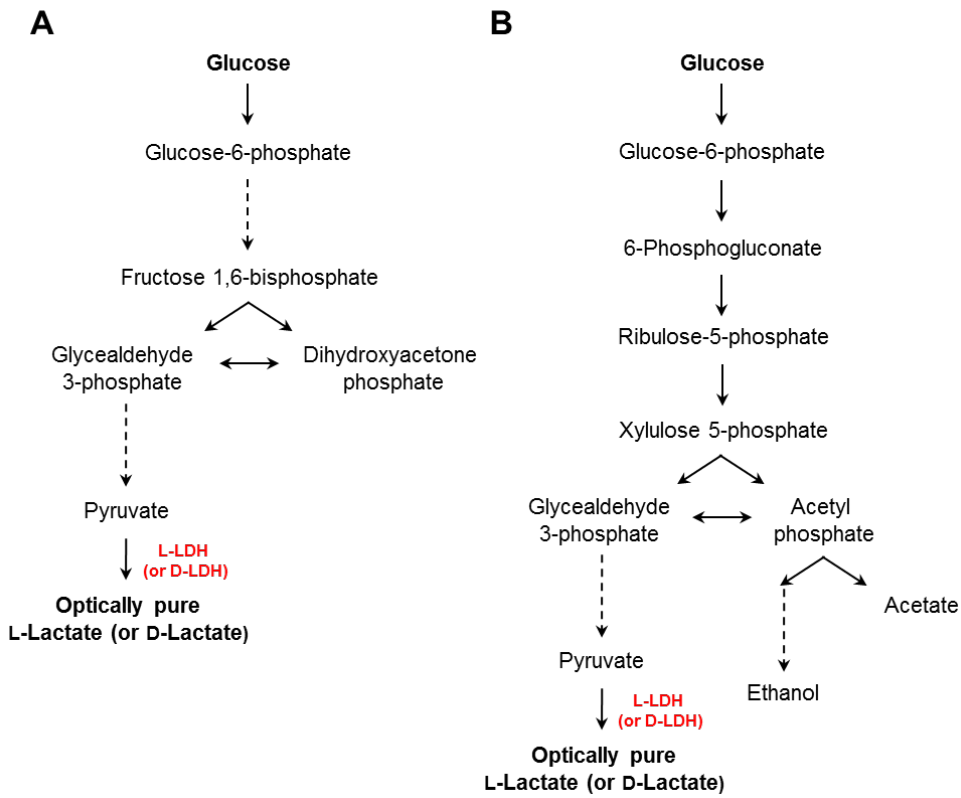


Figure 2.2 Metabolic pathways of LAB for LA fermentation

A. LA production with homo-fermentative LAB

B. LA production with hetero-fermentative LAB

several enzymes. As a result, maximum theoretical yields for LA reach 0.5 g/g (1.0 mol/mol) and 0.6 g/g (1.0 mol/mol) from hexose and pentose, respectively. Therefore, homo-fermentative LAB have been preferred as a host strains for commercial LA production than hetero-fermentative LAB (Juturu and Wu 2015; Martinez et al. 2013). So far, many researches have been investigated to reduce byproduct formation for improving the yield to the maximum theoretical value because LAB also express various enzymes, which compete with LDH for pyruvate utilization. However, genetic engineering approaches are difficult in LAB. Therefore, many studies have been attempted to search of the LDH gene having improved properties such as thermo-stability and substrate-specificity (Wee et al. 2006).

There are several factors to improve LA fermentation by LAB. Although glucose is the most attractive carbon source, it leads to glucose catabolite repression to restrict a simultaneous use of other carbon sources. To date, several genetic engineered LAB have been generated for efficient lactic acid fermentation from mixed sugars. Metabolically engineered *Lactobacillus plantarum* NCIMB 8826 strain showed the ability to metabolize glucose, xylose, and arabinose simultaneously for D-LA homo-fermentation (Yoshida et al. 2011). Other nutrients are an important factor for efficient LA fermentation in LAB. These microorganisms generally require complex nutritional compositions including nitrogen sources, vitamins, and minerals because they do not generate enough levels of these components needed for growth and maintenance. The complex

nitrogen sources such as yeast extract are used as the efficient nutrients for enhancing LA fermentation ability of LAB, but use of them may lead to increase of LA production cost in industrial scale (Tejayadi and Cheryan 1995; Wang et al. 2015). Therefore, several studies have been investigated to replace of complex nitrogen source with alternative nitrogen source. *Lactobacillus sp*. MKTLC878 showed higher productivity by using corn steep liquor as a nitrogen source (Hetényi et al. 2008). Fermentation condition is also one of the important factors to improve LA productivity of LAB. Fermentation below the pH 3.8 is more preferred for industrial LA production because high proportion of undissociated LA is more beneficial to simplify next step such as purification, but LAB generally grow well at pH level from 5 to 7. Therefore, medium pH control should be necessary to maintain LA productivity and yield in LAB cultivation, but use of neutralizing agent for pH control may cause additional problems such as gypsum formation, LA titer reduction, and extra process expense (Pal et al. 2009). Therefore, some studies have been investigated for development of acid-tolerant LAB strain by using genome shuffling or error-prone PCR of whole genome (Patnaik et al. 2002; Ye et al. 2013). In addition, inoculation volume and growth temperature should be considered for cell growth and LA productivity (Idris and Suzana 2006). Development of cost-effective fermentation process is also necessary for commercial LA production in industrial scale.

Table 2.1 LA production of LAB using various carbon sources

Microorganism	**Substrate**	**LA titer (g/L)**	**Reference**
Lactobacillus brevis	Corncob	39.1	(Guo et al. 2010)
Lactobacillus casei NCIMB 3254	Cassava bagasse	83.8	(John et al. 2006)
Lactobacillus delbrueckii mutant Uc-3	Molasses	166	(Dumbrepatil et al. 2008)
Lactobacillus lactis RM2-24	Cellobiose	80.0	(Singhvi et al. 2010)
Lactobacillus lactis IL 1403 *ptk::tkt*	Xylose	50.1	(Shinkawa et al. 2011)
Lactobacillus pentosus ATCC 8041	Corn stover	74.8	(Zhu et al. 2007b)
Lactobacillus plantarum (engineered)	Xylose	41.2	(Okano et al. 2009b)
Lactobacillus plantarum NCIMB 8826 *ΔldhL1-xpk1::tkt*	Arabinose	38.6	(Okano et al. 2009a)
Lactobacillus sp. RKY2	Rice and wheat bran	129	(Yun et al. 2004)

2.1.3. LA production by other bacteria

As mentioned above, LAB are conventional host strain for microbial LA production, but it is difficult to obtain high optical purity and yield of LA from LAB. To overcome these disadvantages, other bacteria such as *Corynebacterium* and *Bacillus* have been studied for applying LA production. In terms of cost-efficiency, *Bacillus* sp. are more suitable for LA fermentation compared with LAB because *Bacillus* sp. can grow in inexpensive medium with high temperature up to 50˚C (Wang et al. 2011b). *Corynebacterium glutamicum* has been also used as a host strain for LA fermentation. This gram positive microorganism naturally produces several organic acids including LA, succinic acid, and acetic acid. By reducing byproduct formation using metabolic engineering approaches, LA productivities and titers of *C. glutamicum* were improved. For example, it was reported that 120 g/L of optically pure D-LA was obtained by introducing heterologous LDH gene into *C. glutamicum* (Okino et al. 2008).

E. coli, natively producing D-LA, is another prominent platform for LA production. This can grow in simple medium containing pentose or hexose carbon source and its genetic manipulation tools are well known. It was also reported that LA has little inhibitory effect in proliferation of *E. coli*. Many metabolically-engineered strains have been generated and applied for improving LA production level and yield. In *E. coli*, deletion of *pflB* gene, encoding pyruvate formate lyase, is an effective manipulation to improve LA titer. A double knock out mutant strain lacking phosphoenolpyruvate carboxylase and D-LDH gene showed 45 g/L of L-

LA production by expressing L-LDH gene obtained from *Lactobacillus casei* (Chang et al. 1999). The engineered strain deleting *pflB*, *frdBC* (fumarate reductase), *adhE* (alcohol dehydrogenase), and *ackA* (acetate kinase) showed highest yield of LA close to theoretical value in defined medium (Zhu et al. 2007a). Additional knock-out *frdABCD* genes encoding fumatate reductase led to 138 g/L of LA production with a yield of 0.99 g/g (Zhu et al. 2007a). In recent decade, use of alternative carbon sources has been also concerned in LA production from *E. coli*. By metabolic engineering approaches, the homo-fermentative *E. coli* strain consuming glycerol as a carbon source was generated by overexpressing glycerol kinase and glycerol-3-phosphate dehydrogenase enzymes (Mazumdar et al. 2010). For sucrose utilization, an operon cluster containing invertase, fructokinase, anion symport, and repressor protein was overexpressed in *E. coli*, resulting 0.5M of D-LA production (Shukla et al. 2004). Although LA titer was improved by genetic manipulations, it still remains several problems such growth inhibitory effect and low LA productivity under acidic fermentation (Yu et al. 2011).

2.1.4. LA production by fungi

In order to use fungi as a LA-producing strain, *Rizhopus* sp., which naturally produce LA on different carbon sources under aerobic condition, has been investigated (Meussen et al. 2012). *R. oryzae* has two L-LDH genes and their activities are controlled by carbon sources. When fermentable sugar such as glucose or xylose is existed in culture medium, Ldh-A is activated to produce L-LA.

Table 2.2 LA production from bacteria and fungi

Microorganism	Substrate	LA titer (g/L)	Reference
Bacillus coagulans P4-102B QZ19	Glucose	90	(Wang et al. 2011a)
Corynebacterium glutamicum R	Glucose	120	(Zhou et al. 2003)
C. glutamicum R	Glucose	195	(Tsuge et al. 2015)
Escherichia coli ATCC11303 TG114	Glucose	118	(Grabar et al. 2006)
E. coli ATCC700926 LA02	Glycerol	32	(Mazumdar et al. 2010)
E. coli W3110 SZ40	Glucose	51	(Zhou et al. 2003)
Lactococcus lactis IL 1403	Xylose	50.1	(Shinkawa et al. 2011)
Rhizopus oryzae NRRL 395	Glucose	77.5	(Skory 2004)
R. oryzae NRRL 395	Glucose	140	(Liu et al. 2008)
R. oryzae NRRL 395	Glycerol	48	(Vodnar et al. 2013)
R. oryzae NBRC 5384	Glucose	231	(Yamane and Tanaka 2013)

Ldh-B is activated by non-fermentable carbon sources such as ethanol, glycerol, and lactate (Skory 2000). In addition, effective LA production is possible using simple culture medium without complex nitrogen source because *R. oryzae* has lower auxotrophy than LAB. (Liu et al. 2008). To improve L-LA formation, recombinant *R. oryzae* strains were developed for reducing ethanol and malate formation, resulting in improving LA production level up to 140 g/L (Liu et al. 2008; Vodnar et al. 2013).

2.1.5. LA production by engineered yeast

Although *S. cerevisiae* have no LA production ability, many researches have been investigated for LA production using *S. cerevisiae* because of its beneficial properties to be a prominent LA-producing microorganism. Especially, higher acid tolerance is the most attractive feature for the production of undissocated LA form under acidic condition. In addition, complex nutrients and culture condition are not required for this microorganism and optically pure LA production is possible by expressing a stereospecific LDH gene. The first study for LA production using *S. cerevisiae* was carried out expressing *Lactobacillus casei* L-Ldh (Dequin and Barre 1994), and many genetically engineered *S. cerevisiae* strains have been generated.

S. cerevisiae naturally spends a large part of pyruvate for ethanol production, there has been a lot of interests to reduce or eliminate ethanol formation for improving pyruvate pool. In *S. cerevisiae*, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) are involved in ethanol fermentation. It was

reported that deletion of only *PDC1* gene showed an impact for improvement of LA production level, but this manipulation was not sufficient for a significant reduction of ethanol formation (Ishida et al. 2005; Ishida et al. 2006b). Deletion of *ADH1*, a major alcohol dehydrogenase under fermentative growth, showed a significant reduction of ethnoal production, but this manipulation induced growth inhibition, following low LA productivity (Skory 2003). Combination of *PDC1* and *ADH1* deletions led to improving LA titer up to 74.1 g/L and a yield of 0.69 g/g glucose with a decrease in ethanol formation comapred with *PDC1* deletion strain (Tokuhiro et al. 2009). Although *PDC1* and *PDC5* double deletion and triple PDC knock-out strains showed higher yield and LA production level with no ethanol production than other engineered strains, but they commoly showed the severe growth retardation in glucose containing medium (Dato et al. 2014; Ishida et al. 2006a).

Although *S. cerevisiae* possesses high acid resistance, improving LA tolerance is still a main issue for LA production in *S. cerevisiae* because LA titer, productivity, and production rate are decreased in acidic fermentation condition. Thus, *S. cerevisiae* strain having higher acid tolerance is necessary for further improvement of production efficiency. By mutagenesis approaches, a mutant strain maintaining high intracellular pH was obtained. This strain produced 70 g/L LA with 0.9 g/g yield without pH control (Valli et al. 2006). Several LA stress-inducible genes and transcription factors have been identified and LA stress-response genes have been also detected by genome-wide analysis (Abbott et al.

Table 2.3 LA production from recombinant *S. cerevisiae* strains

Genotype	LDH	Titer (g/L)	Yield (g/g)	Productivity (g/(L·h))	Culture condition	Reference
pH < pKa (acidic fermentation)						
gal7Δ	*Lactobacillus plantarum* L-LDH	58.0	0.30		Batch (fermenter)	(Colombié et al. 2003)
pdc1Δ (diploid)	*B. taurus* L-LDH	50.6	0.65	0.70	Batch (shake flask)	(Ishida et al. 2005)
pdc1Δpdc5Δpdc6Δ (haploid)	*L. plantarum* L-LDH	70.0	0.93	0.97	Batch (baffled-shake flask)	(Valli et al. 2006)
pdc1Δpdc5Δpdc6Δ sam2Δ (haploid)	*L. plantarum* L-LDH	69.2	0.88	0.96	Batch (baffled-shake flask)	(Dato et al. 2014)
pdc1Δcyb2Δgpd1Δ nde1Δnde2Δ (haploid)	*Pelodiscus sinensis* L-LDH	117.0	0.58	2.4	Fed-batch (fermenter)	(Lee et al. 2015)
pdc1Δ (diploid)	*L. mesenteroides* D-LDH	53.2	0.53	0.74	Batch (shake flask)	(Ishida et al. 2006b)
dld1Δjen1Δadh1Δ gpd1Δgpd2Δpdc1Δ (haploid)	*L. mesenteroides* D-LDH	48.9	0.79	0.41	Batch (conical tube)	This study
pH > pKa (neutralizing fermentation)						
pdc1Δ (diploid)	*B. taurus* L-LDH	55.6	0.62	0.77	Batch (shake flask)	(Ishida et al. 2005)
pdc1Δ (diploid)	*B. taurus* L-LDH	122.0	0.61	2.5	Batch (fermenter)	(Saitoh et al. 2005)
pdc1Δpdc5Δ (diploid)	*B. taurus* L-LDH	82.3	0.82	0.43	Batch (shake flask)	(Ishida et al. 2006a)
pdc1Δadh1Δ (diploid)	*B. taurus* L-LDH	74.1	0.69	1.5	Batch (shake flask)	(Tokuhiro et al. 2009)
pdc1Δ (diploid)	*L. mesenteroides* D-LDH	61.5	0.61	0.85	Batch (shake flask)	(Ishida et al. 2006b)
dld1Δjen1Δadh1Δ gpd1Δgpd2Δpdc1Δ (haploid)	*L. mesenteroides* D-LDH	112.0	0.80	2.2	Fed-batch (shake flask)	This study

2008; Kawahata et al. 2006; Sugiyama et al. 2014). In addition, it was demonstrated that Jen1 and Ady2 might be involved in LA transort mechanism in *S. cerevisiae* (Lodi et al. 2002; Pacheco et al. 2012).

Excepting *S. cerevisiae*, several yeast strains have been used as a host for LA production . It was reported that *Candida boidinii* showed higher L-LA production level (85.9 g/L) under neutral pH condition (Osawa et al. 2009). *Pichia stipitis* was also used for L-LA production, resulting in 58 g/L and 41 g/L of LA from xylose and glucose, respectively (Ilmen et al. 2007). Although some yeast such as *Kluyveromyces marxianus* possess higher tolerance than *S. cerevisiae*, but genetic tools should be established by further researches for applying the LA production strain.

2.2. LA stress adaptation in *S. cerevisiae*

2.2.1. Adaptive response to weak acid in *S. cerevisiae*

As mentioned above, *S. cerevisiae* has the remarkable resistance in low pH level compared with other microorganisms, which grows under acidic condition. Elucidating the adaptation and tolerance mechanisms to weak acid can provide useful information to enhance the robustness of acid-producing yeast and design the fermentation conditions, thus many studies have examined about the adaptation mechanism of weak acid stresses on *S. cerevisiae*. Toxicity of weak acids is involved in its chemical properties such as hydrophobicity. In particular, the pKa value is also an important determinant because undissociated form of the acid may

permeate the plasma membrane by facilitated diffusion and then lead to intracellular acidification by releasing protons. Furthermore, weak acids also affect function of membrane proteins and cellular permeability and they are also implicated in decrease of internal pH level (Fernandes et al. 2005; Stevens and Hofmeyr 1993), resulting in growth retardation and reduction of cell viability. In addition, lipid organization of cellular membrane is also influenced by weak acid stress. When an inhibitory concentration of the weak acid is existed in medium, *S. cerevisiae* tries to adapt itself to the change of growth condition by entering the lag phase, and then resumes normal growth. Along with this period, several cellular responses such as pH recovery, detoxification and energy level recovery are activated in *S. cerevisiae*.

Intracellular acidification is involved in releasing protons by the dissociation of weak acid and increasing the proton influx by perturbation of plasma membrane structures. This change leads to not only reduction of cellular activity and plasma membrane potential but also decrease of DNA and RNA synthesis rate. To maintain internal pH homeostasis within neutral level and membrane potential, the activities of plasma membrane H^+-ATPase and vacuolar membrane V-ATPase are increased in response to weak acid. Especially, Pma1 extrudes proton accumulating in cytosol with ATP hydrolysis, responding to lipophilic acids such as sorbic acid, octanoic acid, and decanoic acid (Alexandre et al. 1996; Holyoak et al. 1996), and V-ATPase activity is also an important factor to the recovery of cytosolic pH and respond to various weak acids by accumulating protons into the vacuole (Kawahata

et al. 2006; Mira et al. 2009).

Although decrease of intracellular pH level by ionize-proton is the major factor to induce weak acid toxicity, dissociated counter-ions inside of the cell is also led to various deleterious effects into the cellular functions such as change of membrane fluidity, aggregation of membrane protein, lipid peroxidation, oxidative stress, turgor pressure, perturbation of plasma and vacuolar membrane organization (Mira et al. 2010b; Piper et al. 2001). Therefore, *S. cerevisiae* uses several specific transporters implicated in multidrug resistance to reduce cytoplasmic concentrations of weak acid anion. For instance, Pdr12, a plasma membrane ATP-binding cassette transporter, is important for the active transport of counter-ions from sorbic acid, propionic acid, and levulinic acid (Holyoak et al. 1999; Piper et al. 1998). In addition, it was also reported that resistance of acetic acid and formic acid is improved by deleting of *PDR12* (Nygard et al. 2014). On the other hand, Pdr12 shows no activity to transport of more lipophilic weak acid including octanoic acid and decanoic acid (Hatzixanthis et al. 2003). Various major facilitator superfamily genes encoding drug-H^+ anti-porter are also related to weak acid tolerance. The expressions of *AQR1* and *AZR1* genes contribute to increase in tolerance of acetic acid and propionic acids in *S. cerevisiae* (Tenreiro et al. 2002; Tenreiro et al. 2000). *TPO2* and *TPO3* genes also require for improving acid resistance against acetic acid, propionic acid, and benzoic acid (Fernandes et al. 2005), whereas it remains to identify the specific transporter for lactate anion export.

In a recent decade, alteration of lipid and protein composition in plasma membrane has been demonstrated as an essential factor involved in weak acid tolerance. The efflux of dissociated weak acid from the cell interior is energy-dependent cellular mechanism and more energy expenditure is required if the released anion was reentered into the cell by passive diffusion with re-protonation form. Therefore, to limit the reentrance of the undissociated form is also important mechanism for weak acid adaptation response. The up-regulation of *SPI1*, encoding a glycosylphosphatidylinositol-anchored cell wall protein involved in remodeling of cell wall structure, was could reinforce the cell wall structure for reducing the diffusion rate and membrane damage by regulating membrane porosity (Simoes et al. 2006). Fps1, involving efflux of glycerol and xylitol, is a major plasma membrane channel to regulate diffusional entry of undissociated acetic acid (Kawahata et al. 2006; Mollapour and Piper 2007). Although plasma membrane components is a general response to overcome weak acid-induced stress (Mira et al. 2010a; Ro et al. 2008), the key factor may differ depending on the structure of weak acid (Kawahata et al. 2006; Suzuki et al. 2013). Lipid composition also contributes to improve weak acid tolerance by altering the plasma membrane structure.

Cellular energy level is also an important factor to improve acid tolerance in *S. cerevisiae*. Based on results from transcriptional regulation analysis, stresses induced by weak acids such as acetic acid, propionic acid, sorbic acid, benzoic acid, and LA could lead to up-regulation of genes involved in glycolysis, TCA cycle,

and ATP synthesis (Abbott et al. 2007; Abbott et al. 2008; Kawahata et al. 2006; Mira et al. 2010a). As mentioned above, a severe ATP depletion is induced in weak acid-stressed cells because ATPase membrane transporters and ATP-binding cassette pumps are involved in ATP consumption. Therefore, reduction of ATP consumption and increase of ATP level may be implicated in enhancing weak acid tolerance. Amino acid synthesis pathways were also activated in acid-stress conditions related acetic acid, propionic acid, and sorbic acid (Mira et al. 2010a; Mira et al. 2009). In addition, intracellular levels of metal cations are crucial for improving acid-tolerance because these ions are depleted by chelating with dissociated form (Abbott et al. 2008).

Based on genome-wide transcriptional regulation analyses with bioinformatics tools, novel transcription factors including War1, Msn2 and 4, Rim101, and Haa1 have been identified as a mediator for weak acid adaptation response (Kren et al. 2003; Mira et al. 2010a; Mira et al. 2009; Schuller et al. 2004). War1 mediates the adaptive responses from acid stresses induced lipophilic weak acids such as propionic acid, sorbic acid, and benzoic acid by regulating Pdr12 transcription (Gregori et al. 2008; Hazelwood et al. 2006; Kren et al. 2003). Msn2 and 4 are homologous transcription factors participating adaptive responses to various weak acids including acetic acid and propionic acid (Simoes et al. 2006). Haa1 is involved in responses to acetic acid and LA stresses (Mira et al. 2010a). This protein has a group of genes related to cell wall structure and multidrug pump. These genes may contribute to activate acid stress defense mechanism.

Transcription repressor Rim101 was implicated in propionic acid stress to regulate several genes including cell wall functions and internal pH homeostasis (Mira et al. 2009).

2.2.2. Adaptive response to LA stress in *S. cerevisiae*

As mentioned above, several studies have examined about adaptation mechanism involved in growth inhibition by sorbic acid or benzoic acid on *S. cerevisiae* and these researches indicated that Pdr12 transporter is important to respond acidic stress (Bauer et al. 2003; Holyoak et al. 1999; Nygard et al. 2014; Piper et al. 1998). Although acetic acid and LA are also classified as weak organic acids, but they have been thought to have different adaptation mechanism in *S. cerevisiae*. Therefore, several studies have been investigated to elucidate the stress response involved in short-chain organic acids.

In previous study, it was reported that growth inhibition mechanisms related acetic acid and LA are different in *S. cerevisiae* by comparison of Pma1 activity. Pma1 was activated by acetic acid stress, whereas a decrease of activity was observed from LA stress (Narendranath et al. 2001). LA stress leads to inequality of intracellular amino acid homeostasis with oxidative stress as well as growth retardation (Kawahata et al. 2006). Based on transcriptional regulation analysis, it was suggested that different transcriptional regulators were implicated in LA stress depending on pH level. When high concentrations of undissociated LA form were existed in the medium, Haa1 induced transcriptional activation of a group of genes,

designated Haa1-regulon. Instead, cells in weak acidic medium containing high portion of lactate, anion form of LA, showed up-regulation of several genes regulated by Aft1, which is the transcription factor involved in iron uptake and metabolism (Abbott et al. 2008). That is, adaptation response to LA stress in *S. cerevisiae* can be determined by pH level with ratio of undissociated and dissociated LA.

In aerobic condition, LA might lead to form hydroxyl-radicals from hydrogen peroxide. It was reported that cytosolic catalase expression level was increased in reduction of intracellular hydrogen peroxide level (Abbott et al. 2009). As mentioned above, LA stress is also involved in intracellular acidification by de-protonation and decrease of intracellular pH causes growth inhibition. Although higher level of cytosolic pH is an important factor to generate LA-producing strain (Valli et al. 2006), the mechanism and responsible factors to maintain higher pH level is currently unclear.

To elucidate adaptive response to LA in yeast, genome-wide identification approaches were also conducted (Hirasawa et al. 2013; Kawahata et al. 2006; Suzuki et al. 2013). These researches suggest the novel genes involved in LA tolerance and adaptive response.

2.2.3. The transciptional activator Haa1

Haa1 is a transcription factor involved in acid stress response induced by hydrophilic weak acids, and Haa1-dependent adaptive response is mostly dedicated

in acetic acid and lactic acid (Abbott et al. 2008; Fernandes et al. 2005; Keller et al. 2001). Haa1 has a high level of homology with Ace1, a transcription factor related to intracellular copper concentration, with the copper-activated DNA binding domain in N-terminal locus. However, activation mechanism of Haa1 is independent of the intracellular copper status (Keller et al. 2001). Although LA is also one of stress factor involved in Haa1 activation in *S. cerevisiae* (Mira et al. 2010a), acetic acid is thought as a major stress factor of Haa1. So far, various studies including Haa1 binding site, designated Haa1-responsive element (HRE), and Haa1-regulon identifications have been demonstrated in acetic acid stress condition (Keller et al. 2001; Mira et al. 2010a; Mira et al. 2011). Based on these results, practical applications of Haa1 have been carried out developing bioethanol production strain (Sakihama et al. 2015). To improve cellulosic-bioethanol production levels in *S. cerevisiae*, they constructed *HAA1*-overexpressing strain and confirmed that *HAA1* overexpression is an effective strategy for improving bioethanol production level in the presence of high acetic acid concentration. Against the up-regulation of Haa1 target genes, Haa1 expression level was not increased in acetic acid stress condition (Inaba et al. 2013; Mira et al. 2010a).

In recent decade, since the interest of *S. cerevisiae* as a LA-producing host strain has been increased, several studies for identifying Haa1 activation and response mechanisms depending on LA stress has been also investigated for

Table 2.4 List of previously identified genes designated Haa1-regulon

Gene or ORF	Biological function
TPO2	Polyamine transporter of the major facilitator superfamily
TPO3	Polyamine transporter of the major facilitator superfamily
YGP1	Cell wall-related secretory glycoprotein
YRO2	Protein with a putative role in response to acid stress
SPI1	GPI-anchored cell wall protein involved in weak acid resistance
GRE1	Hydrophilin essential in desiccation-rehydration process
PHM8	Lysophosphatidic acid phosphatase and nucleotidase
NRE1	Putative cytoplasmic short-chain dehydrogenase/reductase
COM2	Transcription factor that binds *IME1* upstream activation signal (UAS)
TDA6	Putative protein of unknown function
YLR297W	Protein of unknown function

development of LA-tolerant strain possessing higher LA production ability. As mentioned above, it was identified that transcriptional regulation by Haa1 was induced at LA stress condition existing undissociated LA as a predominant form. *HAA1* overexpression lead to improvement of LA resistance in *S. cerevisiae*, but, against the expectations, Haa1 expression was not increased at LA stress condition (Abbott et al. 2008). In recent study, it was reported that nuclear-exporter Msn5 is implicated in Haa1 localization from nucleus to cytoplasm and change of phosphorylation status of Haa1 might be involved in Haa1 translocation from cytoplasm to nucleus in LA stress (Sugiyama et al. 2014). However, the detailed activation mechanism of Haa1 to LA stress is still insufficient for logical approaches for improving LA tolerance (Abbott et al. 2008; Kawahata et al. 2006). Haa1 overexpression showed the positive effect for improving LA tolerance (Inaba et al. 2013; Sugiyama et al. 2014), but disruption of target genes, individually or in combination, did not show any effect to understand how Haa1 overexpression confers the LA resistance in *S. cerevisiae* (Abbott et al. 2008; Sugiyama et al. 2014). Unfortunately, kinase or phosphatase associated Haa1 modifications were also not yet identified.

2.2.4. Development of LA-tolerant *S. cerevisiae* strains

Likewise other organic acids, LA toxicity is also caused by decreasing pH level by LA accumulation. In brief, LA, produced from pyruvate, exists as the dissociated form inside of the cell because intracellular pH is higher compared with pKa value

of LA. Lactate is not enough to transport outside of cells because and *S. cerevisiae* has a limited active transport system, so lactate accumulated in cytoplasm. Since lactate has the negative charge after de-protonation, its transport involves ATP expenditure, cellular metabolism such as cell growth may be affected. If LA production level is enough to decrease the medium pH to below pKa value of LA, however, exported lactate is re-protonated and can diffuse from medium to cells. This re-protonated LA may be immediately deprotonated to lactate and then it is also exported by equal route using ATP consumption. If this cycle was repeated, both intracellular pH value and ATP level are significantly decreased and lactate accumulating cells showed growth retardation. Finally they should die out. For this reason, constructing a strain having higher tolerance against LA stress is beneficial to increase LA production level. Already mentioned above, unfortunately, the knowledge about adaptation and resistance responses to LA stress is still inadequate for logical approach to develop the engineered strain. Until now, some studies for acid-tolerant strain development have been investigated genome-wide identification using disruption strains library. For instance, a research group performed a genome-wide screening to find target genes conferring LA resistance, and they found that disruption of multiple genes including *DSE2*, *SCW11*, *EAF3*, and *SED1* showed LA tolerance under 6% LA stress condition and 27% increase of LA productivity (Suzuki et al. 2013). Genome-wide screening method is also used for searching target genes to improve L-LA production level (Hirasawa et al. 2013). Another group used cells sorting method for determining the strain having a high

intracellular pH level. This tolerant strain showed a significant improvement LA yield (Branduardi et al. 2006).

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* strain Rosetta-gami2 (DE3) pLysS [*Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL* (DE3) F′ [*lac+ lacIq pro*] *gor522::Tn10 trxB* pLysSRARE2 (CamR, StrR, TetR)] was used for expression of various proteins. *E. coli* strains were 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.

All yeast strains used in this study were derived from BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) or CEN.PK2-1C (*MATa ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8C SUC2*) strain obtained EUROSCARF. Various genetically manipulated strains were generated by PCR-mediated method using the Cre/loxP recombination system. To construct disruption strains, deletion cassettes were amplified from pUG27 or pUG72 plasmid using target-specific primer pairs (d_target gene ORF F and d_ target gene ORF R) and introduced into *S. cerevisiae* strains. After confirmation of the correct integration of the cassette at the target gene locus through PCR-based analysis using the primer pairs (c_ORF F and c_ORF R), the selection marker was removed by introducing pSH63 plasmid harboring Cre recombinase gene. The selection marker gene excision was also confirmed by PCR using confirmation primer pairs. To generate the *HAA1* mutant

strains possessing HAA1^{S291A}, HAA1^{S291D}, or HAA1$^{T159/S291/S378}$, an integration cassette containing *HAA1* promoter-controlled *HAA1* mutant gene and *HIS3* marker was amplified from each plasmid derived from pUG27MCS-HAA1 plasmid, and then integrated into the chromosome by substituting the endogenous *HAA1* ORF locus by homologous recombination. After confirmation of the correct integration of the cassette, the selection marker was removed by introducing pSH47 plasmid. To generate JHY5210 strain, an integration cassette containing *TEF1* promoter-controlled *Lm.ldhA* gene and URA3 marker was amplified from pUG72MCS-TEF-Lm.ldhA INT plasmid, and then integrated into the chromosome of *dld1Δjen1Δadh1Δgpd1Δgpd2Δ* strain (JHY5160), replacing the *PDC1* ORF. The selection marker *URA3* gene was excised subsequently by using Cre recombinase. To construct JHY5220 strain possessing an additional copy of *Lm.ldhA* gene, an integration cassette amplified by PCR from pUG72MCS-GPD-Lm.ldhA INT plasmid was integrated into the *JEN1* locus of JHY5210, where the *JEN1* ORF had been replaced with loxP. The selection marker *URA3* gene was also excised subsequently by using Cre recombinase. To develop strains having higher tolerance to D-LA, JHY5210 strain was subjected to adaptive evolution by growing cells in YPD medium with a gradual increase in LA concentrations from 1.3% to 3.95% during 11 subcultures. During the evolution, growth rate and the titers of metabolites were monitored to identify the properties of the evolved strains. From the final culture, LA-tolerant colonies were isolated on solid medium containing 3.8% LA and tested for D-LA production and glucose consumption. Among the

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*	
Rosetta-gami2 (DE3) pLysS	*Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL* (DE3) F′[*lac+ lacIq pro*] *gor522::Tn10 trxB* pLysSRARE2 (CamR, StrR, TetR)	
S. cerevisiae		
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
cka1Δ	BY4741 *cka1Δ::KanMX6*	EUROSCARF
cka2Δ	BY4741 *cka2Δ::KanMX6*	EUROSCARF
haa1Δ	BY4741 *haa1Δ::KanMX6*	EUROSCARF
$Haa1^{S291A}$	BY4741 $Haa1^{S291A}$*::loxP*	This study
$Haa1^{S291D}$	BY4741 $Haa1^{S291D}$*::loxP*	This study
$Haa1^{T159/S291/S378A}$	BY4741 $Haa1^{T159/S291/S378A}$*::loxP*	This study
L40	*MATa his3Δ200 trp1-901 leu2-3112 ade2 LYS::(4lexAop-HIS3) URA3::(8lexAop-LacZ)GAL4*	
CEN.PK2-1C	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8C SUC2*	EUROSCARF
JHY5100	CEN.PK2-1C *gsf2Δ::loxP-HIS3-loxP*	This study
JHY5110	CEN.PK2-1C *dld1Δ::loxP*	This study
JHY5120	CEN.PK2-1C *jen1Δ::loxP*	This study
JHY5130	CEN.PK2-1C *dld1Δ::loxP jen1Δ::loxP*	This study
JHY5140	CEN.PK2-1C *dld1Δ::loxP jen1Δ::loxP adh1Δ::loxP*	This study

Table 3.1 Strains used in this study (*Continued*)

Strain	Genotype	Reference
JHY5150	CEN.PK2-1C *dld1Δ::loxP jen1Δ::loxP adh1Δ::loxP gpd1Δ::loxP*	This study
JHY5160	CEN.PK2-1C *dld1Δ::loxP jen1Δ::loxP adh1Δ::loxP gpd1Δ::loxP gpd2Δ::loxP*	This study
JHY5161	CEN.PK2-1C *dld1Δ::loxP jen1Δ::loxP adh1Δ::loxP gpd1Δ::loxP gpd2Δ::loxP gsf2Δ::loxP-HIS3-loxP*	This study
JHY5210	JHY5160 *pdc1Δ::*P_{TEF1}-*Lm.ldhA*-T_{CYC1}	This study
JHY5211	JHY5210 *gsf2Δ::loxP-HIS3-loxP*	This study
JHY5212	JHY5210 *syn8Δ::loxP-HIS3-loxP*	This study
JHY5213	JHY5210 *stm1Δ::loxP-HIS3-loxP*	This study
JHY5214	JHY5210 *sif2Δ::loxP-HIS3-loxP*	This study
JHY5215	JHY5210 *bud27Δ::loxP-HIS3-loxP*	This study
JHY5220	JHY5210 *jen1Δ::*P_{TDH3}-*Lm.ldhA*-T_{CYC1}	This study
JHY5310	Evolved strain from JHY5210	This study
JHY5320	JHY5310 *jen1Δ::* P_{TDH3}-*Lm.ldhA*-T_{CYC1}	This study
JHY5330	JHY5320 *ura3::* P_{ADH1}-*HAA1*-T_{CYC1}	This study
JHY602	CEN.PK2-1C *adh1Δ::loxP adh2Δ::loxP adh3Δ::loxP adh4Δ::loxP adh5Δ::loxP*	(Kim and Hahn 2015)
JHY604	CEN.PK2-1C *adh1Δ::loxP gpd1Δ::loxP gpd2Δ::loxP*	(Kim and Hahn 2015)
JHY5401	JHY602 *gsf2Δ::loxP-URA3-loxP*	This study
JHY5402	JHY604 *gsf2Δ::loxP-URA3-loxP*	This study

evolved strains, the most efficient strain was selected and named as JHY5310. The construction of JHY5320 strain possessing an additional copy of *Lm.ldhA* gene was conducted using the same procedure of generating JHY5220 strain.

To generate JHY5330 strain having an additional copy of *HAA1* gene under the control of *ADH1* promoter, a linearized p306ADH-HAA1 plasmid digested with StuI was integrated into the *ura3-52* locus of JHY5320 strain. All genetic modifications were verified by PCR using specific primer pairs. Yeast cells were cultivated in YP medium (20 g/L peptone, 10 g/L yeast extract) supplemented with 20, 50, 70, or 100 g/L glucose or synthetic complete (SC) medium (6.7 g/L yeast nitrogen base without amino acids, 20 or 50 g/L glucose, 1.67 g/L amino acids dropout mixture lacking His, Trp, Leu, and Ura) supplemented with auxotrophic amino acids as required.

Table 3.2 Primers used for strain construction (gene manipulation)

Primers	Sequence (5'-3')
d_ADH1 F	*TTCAAGCTATACCAAGCATACAATCAACTATCTCATATACA* CAGCTGAAGCTTCGTACGC
d_ADH1 R	*CTTATTTAATAATAAAAATCATAAATCATAAGAAATTCGC* GCATAGGCCACTAGTGGAT
d_BUD27 F	*AGAATTTTATAGTAAACAGGTATCCTCAGACTGTAATAGCC* CAGCTGAAGCTTCGTACGC
d_BUD27 R	*TGTTAATATAGATTCTGATTTACTTTCTGTCTCCATATGGG* GCATAGGCCACTAGTGGAT
d_DLD1 F	*TGTACATCATTCCGATCCAGCTGGAAACAAAAGCAAGAACA* CAGCTGAAGCTTCGTACGC
d_DLD1 R	*TTTCAGGTTTACGTGAAGGGTGAAAAAGGAAAATCAGATAC* GCATAGGCCACTAGTGGAT
d_GPD1 F	*CACCCCCCCCCTCCACAAACACAAATATTGATAATATAAAG* CAGCTGAAGCTTCGTACGC
d_GPD1 R	*AAGTGGGGGAAAGTATGATATGTTATCTTTCTCCAATAAAT* GCATAGGCCACTAGTGGAT
d_GPD2 F	*TCTCTTTCCCTTTCCTTTTCCTTCGCTCCCCTTCCTTATCA* CAGCTGAAGCTTCGTACGC
d_GPD2 R	*GGCAACAGGAAAGATCAGAGGGGGAGGGGGGGGGAGAGTGT* GCATAGGCCACTAGTGGAT
d_GSF2 F	*GAGATCGGTGGACTTTGTTTTGATAGAGGGCGATTGCAAGC* CAGCTGAAGCTTCGTACGC
d_GSF2 R	*AATAAAAAAAAAGTCTGGATGGTAGTGTTTTGGTTTTACAA* GCATAGGCCACTAGTGGAT
d_JEN1 F	*AAAGTTTTTCCTCAAAGAGATTAAATACTGCTACTGAAAAT* CAGCTGAAGCTTCGTACGC
d_JEN1 R	*CATAGAGAAGCGAACACGCCCTAGAGAGCAATGAAAAGTGA* GCATAGGCCACTAGTGGAT
d_PDC1 F	*GTTTGTTCCCTTTATTTTCATATTTCTTGTCATATTCCTTTCT CA*CAGCTGAAGCTTCGTACGC
d_PDC1 R	*TCCATGGTAAGTGACAGTGCAGTAATAATATGAACCAATTTAT TT*GCATAGGCCACTAGTGGAT
d_SIF2 F	*CAGAAACAAAAAAAAGGTAGGGAAGGCCCATCACACGGAAA* CAGCTGAAGCTTCGTACGC

Table 3.2 Primers used for gene manipulation (*Continued*)

Primers	Sequence (5'-3')
d_SIF2 R	*AGAATGATAAAATTCATCTGTTTATGTACTGTACCTAGTTA* GCATAGGCCACTAGTGGAT
d_STM1 F	*AAGTAGAAATAAACCAAGAAAGCATACACATTTTATTCTCA* CAGCTGAAGCTTCGTACGC
d_STM1 R	*GTTATTGGATTCTTTCAGTTGGAATTATTCATATATAAGGC* GCATAGGCCACTAGTGGAT
d_SYN8 F	*CAGATCTCACGACAGCAAATAGATGCGTAAGCACACACGGT* CAGCTGAAGCTTCGTACGC
d_SYN8 R	*AACCAAACTTCGTATTCGAGCCTAAAAAACAGAATATAATG* GCATAGGCCACTAGTGGAT
i_JEN1-*Lm.ldhA* F	*AAAGTTTTTCCTCAAAGAGATTAAATACTGCTACTGAAAAT* GGGAACAAAAGCTGGAGCT
i_PDC1-*Lm.ldhA* F	*CTCATAACCTCACGCAAAATAACACAGTCAAATCAATCAAA* GGGAACAAAAGCTGGAGCT
i_PDC1-*Lm.ldhA* R	*AATGCTTATAAAACTTTAACTAATAATTAGAGATTAAATC* GCATAGGCCACTAGTGGAT
c_ADH1 F (-300)	ACAGCACCAACAGATGTCG
c_ADH1 R (+200)	TTGCTCGGCATGCCGGTA
c_BUD27 F (-276)	GTGGTCGGATCGTGCTTT
c_BUD27 R (+282)	AGAGTCCTTGGTTGGGGG
c_DLD1 F (-300)	TCTTGTCAACCCAGGTCCGT
c_DLD1 R (+300)	AGGAAGTGATGTAAGCTGCT
c_GPD1 F (-300)	CGCCTTGCTTCTCTCCCCTT
c_GPD1 R (+300)	CCGACAGCCTCTGAATGAGT
c_GPD2 F (-300)	TACGGACCTATTGCCATTGT
c_GPD2 R (+300)	TTAAGGGCTATAGATAACAG
c_GSF2 F (-259)	GGTCACTCCTTGTTCTTCT
c_GSF2 R (+212)	AGTAGATTCGTGAGGAATTG
c_HAA1 F (-235)	GGGATAACAGCACCAGCAC

Table 3.2 Primers used for gene manipulation (*Continued*)

Primers	Sequence (5'-3')
c_HAA1 R (+241)	GAGATGGGGAGGCCATTC
c_JEN1 F (-300)	AACGGTCTTTTGCCCCCCCT
c_JEN1 R (+216)	CTTGCTAGTGTTAACGGC
c_JEN1-Lm.ldhA R (+473)	AAGCATACATCCCCCTTC
c_PDC1 F (-340)	TTGAGTACTTTTCTTCATAATTGCATAATA
c_PDC1 R (-300)	AAAAAAAGAGGTATCCTTGATTAAGGAACA
c_PDC1-Lm.ldhA F (-484)	GACTTTTCGTGTGATGAGGC
c_PDC1-Lm.ldhA R (+478)	TTTACATGGACCGCACCAAG
c_SIF2 F (-246)	CATGGAGCGGAACTTAGC
c_SIF2 R (+192)	GCTCATTGTCTTCTCTCATCG
c_STM1 F (-272)	GATATCATCGTTGCGTAGAG
c_STM1 R (+282)	CAAACAAACTACACGCTTGC
c_SYN8 F (-260)	GCTGACTCATCTGCCACG
c_SYN8 R (+254)	GGTCCTTTTCTCCGGTAGG

3.2. Plasmids

All Plasmids used in this study are listed in Table 3.3. Plasmids for expressing heterologous D-LDH genes were constructed by general molecular cloning. The DNA fragments of *D-ldh1*, *D-ldh2*, and *D-ldh3* genes from *Lactobacillus jensenii* strains (indicated as *Lj.ldh1*, *Lj.ldh2*, and *Lj.ldh3* in this study) and *ldhA* gene from *Lactobacillus delbrueckii subsp. bulgaricus* ATCC 11842 (indicated as *Ld.ldhA*) were prepared by PCR from plasmids provided by Dr. Y. H. Kim (Kwangwoon University, Korea). pETldhD plasmid containing *ldhA* (LEUM_1756) gene from *L. mesenteroides subsp. mesenteroides* ATCC 8293 (indicated as *Lm.ldhA*) was kindly provided by Dr. N. S. Han (Chungbuk National University, Korea). *Lj.ldh1*, *Lj.ldh3*, *Ld.ldhA*, and *Lm.ldhA* genes were cloned between BamHI and PstI sites of p425ADH plasmid. The amplified *Lj.ldh2* gene was cloned between BamHI and SalI sites of p425ADH plasmid. *Lm.ldhA* gene was also cloned between BamHI and SalI sites of p415ADH, p415TEF, p415GPD, p425TEF, and p425GPD plasmids by sub-cloning. To construct the integration plasmid containing *Lm.ldhA* gene, a DNA fragment spanning from *TEF1* promoter to *CYC1* terminator (P_{TEF1}-*Lm.ldhA*-T_{CYC1}) was amplified by PCR from p425TEF-Lm.ldhA and cloned between ApaI and NheI sites of pUG72MCS, resulting in pUG72MCS-TEF-Lm.ldhA INT. pUG72MCS is generated by inserting 56-bp DNA fragment containing additional cloning sites (PacI, NheI, BamHI, SmaI, EcoRI, ApaI, MluI, and AscI) between HindIII and PstI sites of pUG72. pUG72MCS-GPD-Lm.ldhA

Table 3.3 Plasmids used in this study

Plasmid	Description	Reference
pUG27	Plasmid containing *loxP-Sp.his5*$^{+}$*-loxP* deletion cassette	EUROSCARF
pUG72	Plasmid containing *loxP-Kl.URA3-loxP* deletion cassette	EUROSCARF
pSH47	CEN/ARS, *URA3*, P_{GAL1}-cre-T_{CYC1}	EUROSCARF
pSH63	CEN/ARS, *TRP1*, P_{GAL1}-cre-T_{CYC1}	EUROSCARF
p306	*URA3*	
p415ADH	CEN/ARS, *LEU2*, P_{ADH1}, T_{CYC1}	
p415GPD	CEN/ARS, *LEU2*, P_{TDH3}, T_{CYC1}	
p415TEF	CEN/ARS, *LEU2*, P_{TEF1}, T_{CYC1}	
p423GPD	2μ, *LEU2*, P_{TDH3}, T_{CYC1}	
p425ADH	2μ, *LEU2*, P_{ADH1}, T_{CYC1}	
p425GPD	2μ, *LEU2*, P_{TDH3}, T_{CYC1}	
p425TEF	2μ, *LEU2*, P_{TEF1}, T_{CYC1}	
p306ADH-HAA1	plasmid containing P_{ADH1}-*HAA1*-T_{CYC1}	This study
p414GPD-HXT1	CEN/ARS, *TRP1*, P_{TDH3}-*HXT1*-T_{CYC1}	(Kim et al. 2015)
p415ADH-Lm.ldhA	CEN/ARS, *LEU2*, P_{ADH1}-*Lm.ldhA*-T_{CYC1}	This study
p415GPD-Lm.ldhA	CEN/ARS, *LEU2*, P_{TDH3}-*Lm.ldhA*-T_{CYC1}	This study
p415TEF-Lm.ldhA	CEN/ARS, *LEU2*, P_{TEF1}-*Lm.ldhA*-T_{CYC1}	This study
p423GPD-CKA1	2μ, *LEU2*, P_{TDH3}-*CKA1*-T_{CYC1}	This study
p425ADH-Lj.ldh1	2μ, *LEU2*, P_{ADH1}-*Lj.ldh1*-T_{CYC1}	This study
p425ADH-Lj.ldh2	2μ, *LEU2*, P_{ADH1}-*Lj.ldh2*-T_{CYC1}	This study
p425ADH-Lj.ldh3	2μ, *LEU2*, P_{ADH1}-*Lj.ldh3*-T_{CYC1}	This study
p425ADH-Ld.ldhA	2μ, *LEU2*, P_{ADH1}-*Ld.ldhA*-T_{CYC1}	This study
p425ADH-Lm.ldhA	2μ, *LEU2*, P_{ADH1}-*Lm.ldhA*-T_{CYC1}	This study
p425GPD-Lm.ldhA	2μ, *LEU2*, P_{TDH3}-*Lm.ldhA*-T_{CYC1}	This study

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

Plasmid	Description	Reference
p425GPD-CKA2	2μ, *LEU2*, P_{TDH3}- *CKA2*-T_{CYC1}	This study
p425TEF-Lm.ldhA	2μ, *LEU2*, P_{TEF1}-*Lm.ldhA*-T_{CYC1}	This study
pUG27MCS	pUG27 plasmid containing additional restriction enzyme sites	This study
pUG72MCS	pUG72 plasmid containing additional restriction enzyme sites	This study
pUG27MCS-HAA1	plasmid carrying P_{HAA1}-*HAA1* -T_{HAA1}	This study
pUG27MCS-HAA1^{S291A}	plasmid carrying P_{HAA1}-*HAA1*^{S291A} -T_{HAA1}	This study
pUG27MCS-HAA1^{S291D}	plasmid carrying P_{HAA1}-*HAA1*^{S291D}-T_{HAA1}	This study
pUG27MCS-HAA1$^{T159/S291/S378A}$	plasmid carrying P_{HAA1}-*HAA1*$^{T159/S291/S378A}$-T_{HAA1}	This study
pUG72MCS-TEF-Lm.ldhA INT	plasmid carrying P_{TEF1}-*Lm.ldhA*-T_{CYC1}	This study
pUG72MCS-GPD-Lm.ldhA INT	plasmid carrying P_{TDH3}-*Lm.ldhA*-T_{CYC1}	This study
pGEX-4T-1	GST fusion plasmid	GE Healthcare
pGEX-4T-1-HAA1 1-694	plasmid containing *HAA1* 1-694	This study
pGEX-4T-1-HAA1 1-150	plasmid containing *HAA1* 1-150	This study
pGEX-4T-1-HAA1 151-400	plasmid containing *HAA1* 151-400	This study
pGEX-4T-1-HAA1 1-400	plasmid containing *HAA1* 1-400	This study
pGEX-4T-1-HAA1 401-694	plasmid containing *HAA1* 401-694	This study
pGEX-4T-1-HAA1 151-400^{T159A}	plasmid containing *HAA1* 151-400^{T159A}	This study
pGEX-4T-1-HAA1 151-400^{S291A}	plasmid containing *HAA1* 151-400^{S291A}	This study

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

Plasmid	Description	Reference
pGEX-4T-1-HAA1 151-400^{S378A}	plasmid containing *HAA1* 151-400^{S378A}	This study
pGEX-4T-1-HAA1 151-400$^{T159/S291A}$	plasmid containing *HAA1* 151-400$^{T159/S291A}$	This study
pGEX-4T-1-HAA1 151-400$^{S291/S378A}$	plasmid containing *HAA1* 151-400$^{S291/S378A}$	This study
pGEX-4T-1-HAA1 1-400^{S291A}	plasmid containing *HAA1* 1-400^{S291A}	This study
pGEX-4T-1-HAA1 1-400^{S330A}	plasmid containing *HAA1* 1-400^{S330A}	This study
pGEX-4T-1-HAA1 1-400$^{S291/S330A}$	plasmid containing *HAA1* 1-400$^{S291/S330A}$	This study
pBTM116	2μ, *TRP1*, P_{ADH1}, T_{ADH1}	
JHP021	2μ, *TRP1*, P_{ADH1}- LexA-*CKA1*- T_{ADH1}	(Cho et al. 2014)
JHP022	2μ, *TRP1*, P_{ADH1}- LexA-*CKA2*- T_{ADH1}	(Cho et al. 2014)
JHP023	2μ, *TRP1*, P_{ADH1}- LexA-*CKB1*- T_{ADH1}	(Cho et al. 2014)
JHP024	2μ, *TRP1*, P_{ADH1}- LexA-*CKB2*- T_{ADH1}	(Cho et al. 2014)
pVP16	2μ, *LEU2*, P_{ADH1}, T_{ADH1}	
pVP16-HAA1	2μ, *LEU2*, P_{ADH1}-*HAA1*- T_{ADH1}	This study

INT plasmid was also generated by cloning a P_{TDH3}-*Lm.ldhA*-T_{CYC1} DNA fragment amplified from p425GPD-Lm.ldhA into pUG72MCS. To construct the plasmid for *HAA1* integration, *HAA1* gene from *S. cerevisiae* BY4741 was cloned between BamHI and SalI sites of p416ADH, and then an amplified DNA fragment containing P_{ADH1}-*HAA1*-T_{CYC1} was also cloned between SacI and KpnI sites of p306 plasmid, resulting in p306ADH-HAA1.

pGEX-4T-1-HAA1 plasmid for expressing GST-fused Haa1 protein was constructed as follows. The DNA fragment of *HAA1* gene was amplified by PCR from *S. cerevisiae* BY4741 genomic DNA, and then cloned between BamHI and SalI sites of pGEX-4T-1 plasmid. The expression vectors for truncated derivatives of GST-Haa1 were constructed by cloning of the PCR fragments of Haa1 1-150, Haa1 151-400, Haa1 1-400, and Haa1 401-694 into pGEX-4T-1 plasmid, respectively. DpnI-mediated site-directed mutagenesis was conducted to generate various expression vectors for GST-Haa1. pGEX-4T-1-HAA1$^{T159/S291/S378A}$ plasmid was constructed by repeated DpnI-mediated mutagenesis. To construct the integration plasmid for generating strains containing *HAA1* mutant genes instead of endogenous *HAA1* gene, the DNA fragment from HAA1 promoter (-612) to HAA1 terminator (+518) was amplified by PCR, and cloned between BamHI and KpnI sites of pUG27MCS plasmid, resulting in pUG27MCS-HAA1 and its derivatives.

Table 3.4 Primers used for gene cloning

Primers	Sequence (5'-3')
Gsf2 F	GGCGGGATCCATGGAGATTTACATTAGACTTAACG
Gsf2 R	GGCGGTCGACTTAATTAGATTTTTTCAAATCATTCTTTTTT
Haa1 F	GGCGGGATCCATGGTCTTGATAAATGGCAT
Haa1 R	GGCGGTCGACTCATAACGAAGACATGAAATT
Haa1 1-150 R	GGCGGTCGACTCATGATAGTGCTCCGTGGC
Haa1 1-400 R	GGCGGTCGACTCAAAGCGTTGAAATCGACGA
Haa1 151-400 F	GGCGGGATCCATGGATACCTCTAGCATACTGACG
Haa1 401-694 F	GGCGGGATCCATGTCCCGTGCAAACTTATTAT
Haa1^{T159A} F	TCTAGCATACTGACGAGCGCATTTTTAGACAGTGAGCCGG
Haa1^{T159A} R	CCGGCTCACTGTCTAAAAATGCGCTCGTCAGTATGCTAGA
Haa1^{S291A} F	CTAGAAGAATATATTCCTGCTGACATTGATGGGGTTGGAA
Haa1^{S291A} R	TTCCAACCCCATCAATGTCAGCAGGAATATATTCTTCTAG
Haa1^{S330A} F	ACCGCTGCAACTGGTGAAGCTAAGTTCGACATTAACGACA
Haa1^{S330A} R	TGTCGTTAATGTCGAACTTAGCTTCACCAGTTGCAGCGGT
Haa1^{S378A} F	CAAGAACAATAACAACTCTGCTAGACAAGAACATCAAGGA
Haa1^{S378A} R	TCCTTGATGTTCTTGTCTAGCAGAGTTGTTATTGTTCTTG
HIS-tag ldh R	GGCGCTGCAGTCAGTGGTGGTGGTGGTG
Ld.ldhA F	GGCGGGATCCATGACTAAGATTTTTGCT
Ld.ldhA R	GGCGGTCGACTTAGCCAACTTTAACCGGTGT
Lj.ldh1 F	GGCGGGATCCATGCTGCACAATAAATCC

Restriction enzyme sites are underlined

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

Primers	Sequence (5'-3')
Lj.ldh1 R	GGCGGTCGACTCAACCCAGTTTGACCGGC
Lj.ldh2 F	GGCGGGATCCATGACCAAAATTTTCGCC
Lj.ldh2 R	GGCGGTCGACTCAACCCAGTTTAACCGGCG
Lj.ldh3 F	GGCGGGATCCATGAAAATCGCTGTCTTC
Lj.ldh3 R	GGCGGTCGACTCAGCGGGACACAACAAAATG
Lm.ldhA F	GGCGGGATCCATGAAGATTTTTGCTTACGG
Lm.ldhA R	GGCGCTGCAGTTAATATTCAACAGCAATAG
Lm.ldhA_INT F	GGCGGCTAGCGGGAACAAAAGCTGGAGCTC
Lm.ldhA_INT R	GGCGGGGCCCGGGGGATGTGCTGCAAGG
Sif2 F	GGCGGGATCCATGAGTATAACAAGTGAAGAACTAAAC
Sif2 R	GGCGGTCGACTTATATGGCTACAACTGAACCT
Stm1 F	GGCGGGATCCATGTCCAACCCATTTGATTTG
Stm1 R	GGCGGTCGACTTAAGCCAAAGATGGCAAGT
Syn8 F	GGCGGGATCCATGGATGTGTTGAAGCTGG
Syn8 R	GGCGGTCGACTTATAATACTAATAGAAGCAACAGGAGCA

Restriction enzyme sites are underlined

3.3. Culture conditions

CEN.PK2-1C strains harboring plasmids were pre-cultured in SC-Leu medium containing 20 g/L glucose, and then inoculated to OD_{600} of 0.5 into SC-Leu medium containing 20 g/L glucose or to OD_{600} of 1 into SC-Leu medium containing 50 g/L glucose. Strains without plasmids were pre-cultured in YPD medium containing 20 g/L glucose, and then inoculated to OD_{600} of 1 into YPD medium containing 50 or 70 g/L glucose. Cells were cultivated in 5 mL medium in a 50 mL screw cap conical tube at 30ºC with shaking at 170 rpm. For shake flask fed-batch fermentation, JHY5330 strain was pre-cultured in YPD medium containing 20 g/L glucose, and then cultured in 10 mL YPD medium containing 100 g/L glucose in a 100 mL flask at 30ºC with shaking at 170 rpm. Approximately 50 g/L of sterilized calcium carbonate ($CaCO_3$) was used as a neutralizing agent in fed-batch fermentation. A feed solution, containing 800 g/L glucose, was added to the culture medium when glucose was exhausted.

To investigate the induction of Haa1 target genes by lactic acid, BY4741 strains were pre-cultured in YPD medium containing 20 g/L glucose, and then inoculated to OD_{600} of 0.1 into 20 mL of YPD medium containing 20 g/L glucose in a 100 mL shake flask. When cell density was reached approximately OD_{600} of 0.8 ~ 1, 10ml of culture medium was transferred and cultured with 20 g/L Lactic acid solution (Duksan, Korea) in a 50ml shake flask for 1 h.

3.4. Whole genome sequencing analysis

The genomic DNA of evolved strain was isolated using HiGene^{TM} Genomic DNA Prep Kit For Yeast (BIOFACT, Korea) according to the manufacturer`s recommendation. DNA library for genomic sequencing was prepared using the TruSeq DNA sample preparation kits (Illumina, USA) and sequenced using Illumina Hiseq-2000 (Illumina, USA) at 2 x 75 bp read pairs. The raw reads were processed with Trimmomatic (version 0.3) to remove adapters and poor quality reads (quality score < 20). Reads shorter than 36 bp after processing were discarded. The quality of the processed data was evaluated using FastQC. Reads were mapped to the reference genome (CEN.PK 113-7D) using Burrows-Wheeler Aligner (BWA) software (ver 0.7.1). Duplicates were removed with the MarkDuplicates program of the Picard package (http://picard.sourceforge.net). Indels were located and realigned with Realigner Target Creator/Indel Realigner of Genome Analysis Toolkit (GATK). Single nucleotide variants (SNVs) were detected using MuTect (ver 1.1.7). Indels were detected using VarScan software (ver. 2.3.7).

3.5. Proteins preparation

In order to purify GST-fused Haa1 proteins, *E. coli* Rosetta-gami2 (DE3) pLysS strain was transformed with various HAA1 expression plasmids, and the proteins were induced with 1 mM IPTG for 4 h at 30ºC. GST-Haa1 derivative proteins were purified using glutathione-agarose resin, and dialyzed against dialysis buffer

containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 15% glycerol. All purified proteins were stored at - 75ºC.

3.6. *In vitro* kinase assay

In vitro kinase assays using GST-Ck2 kinase protein were performed with GST-Haa1 proteins purified from *E. coli*. 1 μg of GST-Ck2 with 5 μg of GST-Haa1 and GST-Haa1 derivatives (GST-Haa1 1-150, GST-Haa1 151-400, GST-Haa1 151-400^{T159A}, GST-Haa1 151-400^{S291A}, GST-Haa1 151-400^{S378A}, GST-Haa1 151-400$^{T159/S291A}$, GST-Haa1 151-400$^{S291/S378A}$, GST-Haa1 1-400, GST-Haa1 1-400^{S291A}, GST-Haa1 1-400^{S330A}, GST-Haa1 1-400$^{S291/S330A}$, and GST-Haa1 401-694) were incubated in 20 μl of reaction buffer containing 25 mM HEPES (pH 7.5), 10mM $MgCl_2$, 50 μM ATP, and 2 μCi of [γ-^{32}P]-ATP at room temperature for 1 h. Kinase-substrate protein reaction was terminated by boiling in SDS-PAGE sample buffer and the reaction products were separated by SDS-PAGE. The phosphorylated proteins were detected using phosphorimage analyzer (BAS-2500, Fujifilm).

3.7. Yeast two hybrid assay

To discover the interactions between Haa1 and CK2 subunits including catalytic and regulatory subunits, yeast two hybrid assays were carried out using *S. cerevisiae* L40 strain with plasmids expressing VP16 AD and LexA DBD-fusion proteins. Cells harboring each plasmid were cultured in SC-Trp-Leu medium containing 20 g/L glucose until cell growth entered exponential phase. After

dilution of the harvested cells to OD_{600} of 1, its 10 and 100-fold dilution with water, each cell was spotted on a control solid medium (SC-Trp-Leu) and a selective solid medium (SC-His-Trp-Leu) containing 0.3 mM 3-Amino-1,2,4-triazole (3-AT) for selecting false-positive colonies, respectively.

3.8. RNA preparation and quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from yeast cells using the hot acidic phenol RNA isolation method. The 5ml of cells was harvested and frozen at -75ºC in 350 μL of lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.5% SDS. 350 μL of acidic phenol was added directly on top of frozen cells and incubated at 65ºC for 20 min with occasional vortexing. After each sample was chilled in ice for 10 min, supernatant solution was extracted using chloroform. After centrifugation and ethanol precipitation, the isolated RNA pellets were dissolved in RNase-free water. The relative amount of mRNA was determined by qRT-PCR as previously described (Cho et al. 2014). Briefly, 800 ng of total RNA was subjected to reverse transcription with 0.1 μg of oligo-(dT) for 1 h at 42ºC using 200 unit of M-MLV reverse transcriptase (M-biotech, Inc., Korea), and then followed by heat inactivation for 10 min at 75ºC. For qRT-PCR analysis, 0.5 μL of RT reaction solution was subsequently used for the PCR mixture preparation with 1X SYBR master mix (Roche Diagnostics), and gene-specific primers. PCR amplification was carried out with 45 cycles of 95ºC for 20 s, 55ºC for 30 s, and 72ºC for 30 s

using Roche LightCycler 480 real-time PCR system (Roche Diagnostics). The *ACT1* transcripts were used as a control for quantification of transcripts of indicated genes. Primer sequences used for qRT-PCR are as follows:

q_ACT1 F, 5'-GCCGAAAGAATGCAAAAGGA-3';

q_ACT1 R, 5'-TAGAACCACCAATCCAGACGG-3';

q_HAA1 F, 5'-CGTGTGGGCGAAGTTAGCG-3';

q_HAA1 R, 5'-CACCAGTTGCAGCGGTTGC-3'

q_TDA6 F, 5'-GTCCCCTGTGGGATCCATCTTTG-3';

q_TDA6 R, 5'-CGCACCATTTCTGTCTTGGATCTC-3'

q_TPO2 F, 5'-CCTTGTTTACCATTCAAATGTACCACAAATTGAATT-3';

q_TPO2 R, 5'-ATTTTTACCGGAAACTTGCACCAG-3'

3.9. Spotting assay

Yeast cells were pre-cultured in YPD medium containing 20 g/L glucose, and then inoculated to OD_{600} of 0.2 into YPD medium containing 20 g/L glucose. Cells were cultivated in 4 mL medium in a 50 mL screw cap conical tube at 30ºC until growth phase entered early exponential phase (OD_{600} of 0.8 ~ 1.0). After dilution of the harvested cells to OD_{600} of 1, each cell was tested for growth by spotting serially diluted cultures onto YPD medium with or without lactic acid.

3.10. Analytical methods

Cell growth was monitored by measuring optical density at 600 nm using spectrophotometer (Varian Cary50 UV/Vis spectrophotometer, Agilent). Samples

collected from culture supernatant were filtered through a 0.22 μm syringe filter and the concentrations of acetate, ethanol, glucose, glycerol, and lactate were determined by high performance liquid chromatography (HPLC). HPLC was performed in UltiMate 3000 HPLC system (Thermo Fishers Scientific) equipped with Bio-Rad Aminex HPX-87H column (300 mm x 7.8 mm, 5 μm) and 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.

Chapter 4.

Construction of the D-lactic acid producing *Saccharomyces cerevisiae* strain by inhibiting D-lactic acid consumption and byproduct formation

4.1. Introduction

In recent years, there has been a growing interest in poly lactic acid (PLA) as a biodegradable polymer produced from renewable resources. Lactic acid (LA), a monomer for PLA production, has been mainly produced from the fermentation of lactic acid bacteria, the natural producers of LA (Abdel-Rahman et al. 2013; Hofvendahl and Hahn–Hägerdal 2000). However, because of the acid sensitivity of lactic acid bacteria, industrial-scale production of LA requires neutralization during fermentation and recovery of LA from the resulting lactate salt (Vaidya et al. 2005). Therefore, *Saccharomyces cerevisiae*, having high acid tolerance, has emerged as a promising alternative to produce LA. Since *S. cerevisiae* does not synthesize LA naturally, optically pure L-LA or D-LA can be produced by heterologous expression of stereospecific lactate dehydrogenase (LDH) genes in *S. cerevisiae* (Abdel-Rahman et al. 2013; Porro et al. 1995). Polymerization of L-LA and D-LA leads to PLLA and PDLA, respectively, and PLLA has been the major form of PLA produced so far. However, thermochemical and physical properties, and biodegradation rate of PLA can be improved by making stereo-complex PLA with different combination ratios of PLLA and PDLA (Garlotta 2001; Ikada et al. 1987). Therefore, there is an increasing demand for D-LA production as well as L-LA.

To date, metabolic engineering of *S. cerevisiae* for LA production has been focused on the reduction of a large carbon flux from pyruvate to ethanol, facilitating LA production from pyruvate (Fig. 1). Ethanol production from

pyruvate involves two enzymatic steps; conversion of pyruvate to acetaldehyde by pyruvate decarboxylase (PDC) followed by the conversion of acetaldehyde to ethanol by alcohol dehydrogenase (ADH). Therefore, genes encoding PDC (*PDC1*, *PDC5*, and *PDC6*) and ADH (*ADH1*, *ADH2*, *ADH3*, *ADH4*, *ADH5*, and *SFA1*) have been deleted in various combinations to increase LA production (Ida et al. 2013; Ishida et al. 2006a; Ishida et al. 2005; Ishida et al. 2006b; Lee et al. 2015; Porro et al. 1995; Tokuhiro et al. 2009; Valli et al. 2006). Although blocking PDC by deleting all three or two major genes (*PDC1* and *PDC5*) is the most effective way of increasing pyruvate availability for LA production, the use of PDC-negative strains is limited due to their severe growth defects on glucose (Ishida et al. 2006a; van Maris et al. 2004a). Inhibition of ethanol production reduces ADH-dependent NAD^+ regeneration, resulting in an increase in glycerol production via the action of glycerol-3-phosphate dehydrogenase (GPD) as a compensation mechanism to maintain redox balance (Fig. 1) (Skory 2003). Therefore, deletion of *GPD1* and *GPD2* genes encoding GPD also has been shown to reduce byproduct formation (Ida et al. 2013; Kim and Hahn 2015).

To develop an efficient D-LA-producing *S. cerevisiae* strain, a heterologous D-LDH gene (ldhA, LEUM_ 1756) from *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293, showing the highest activity when expressed in *S. cerevisiae*, was selected among five D-LDH genes from different lactic acid bacteria. Thereafter, metabolic pathway engineering was used to inhibit D-LA consumption upon glucose depletion and minimize byproduct formation such as

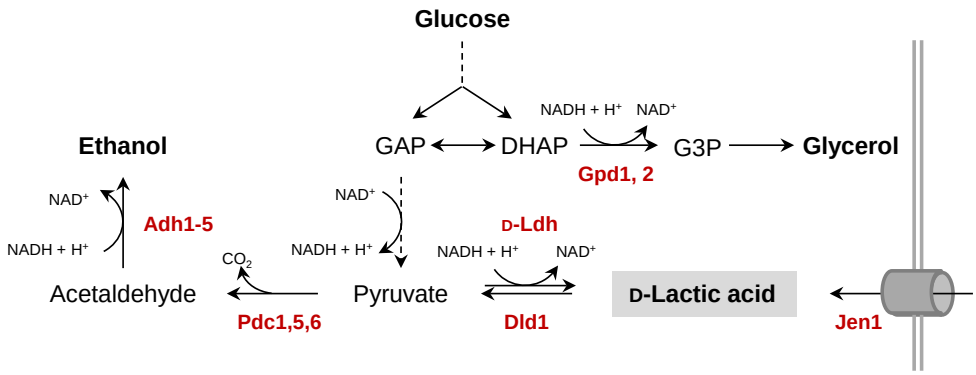


Figure 4.1 Metabolic pathways for the production of D-LA in *S. cerevisiae*

Pyruvate generated from glycolysis is converted to D-LA by heterologous D-Ldh or ethanol by native PDC and ADH. Under respiratory growth conditions, -LA can be imported by Jen1 and oxidized to pyruvate by Dld1 to be consumed as an alternative carbon source. Dashed arrows indicate multiple enzymatic steps. DHAP, dihydroxyacetone phosphate; GAP, glycealdehyde-3-phosphate; G3P, glycerol-3-phosphate.

ethanol and glycerol. D-LA production was also increased by the appropriate combination of promoter and plasmid type.

4.2. Expression of heterologous D-lactate dehydrogenase (D-LDH) genes in *S. cerevisiae*

To select more appropriate *S. cerevisiae* strain as the host for D-LA production, the D-LDH gene (LEUM_1756) from *L. mesenteroides* subsp. *mesenteroides* (*Lm.ldhA*) was expressed in BY4741 and CEN.PK2-1C strain. D-LA was detected in the both strains harboring p425ADH-*Lm.ldhA* plasmid, and no D-LA production was detected in the control strains harboring p425ADH plasmid (Fig. 4.2C). The D-LA-producing cells showed slower glucose uptake and growth rates than control strains (Fig. 4.2A and B). Compared to BY4741 strain, CEN.PK2-1C strain showed higher D-LA, accompanying lower ethanol production level. From these results, CEN.PK2-1C strain was selected as the host strain for generating an efficient D-LA-producing *S. cerevisiae* strain.

To evaluate the activities of different D-LDH genes in *S. cerevisiae*, five D-LDH genes including *D-ldh1*, *D-ldh2*, and *D-ldh3* from *L. jensenii* (*Lj.ldh1*, *Lj.ldh2*, and *Lj.ldh3*), *ldhA* from *L. delbrueckii* subsp. *bulgaricus* (*Ld.ldhA*), and *Lm.ldhA* were expressed in CEN.PK2-1C strain under the control of *ADH1* promoter from high copy number plasmid. All these D-LDH genes have been shown to be highly stereospecific for D-LA production (Jun et al. 2013; Li et al. 2012; Okino et al.

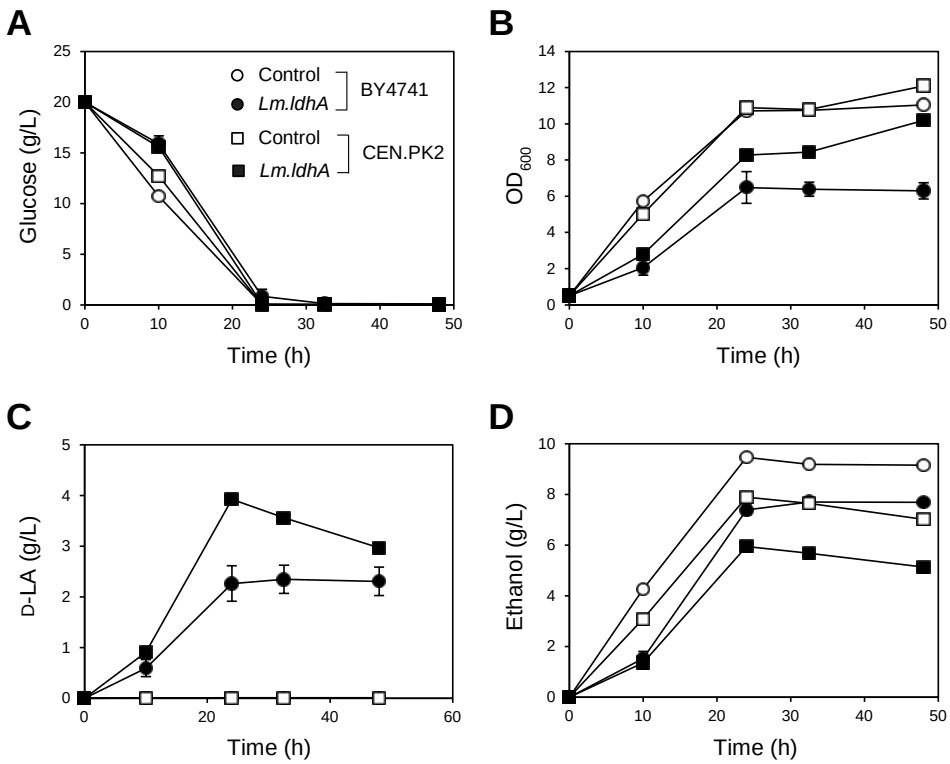


Figure 4.2 D-LA production in defferent *S. cerevisiae* strains

S. cerevisiae BY4741 and CEN.PK2-1C cells expressing the *Lm.ldhA* gene from p425ADH plasmid vector were cultivated in SC-Leu medium containing 20 g/L of glucose. Each strain harboring empty plasmid was used as a control, respectively. The amounts of glucose (A), D-LA (C), and ethanol (D) were measured by HPLC. Error bars indicate standard deviations of three independent experiments.

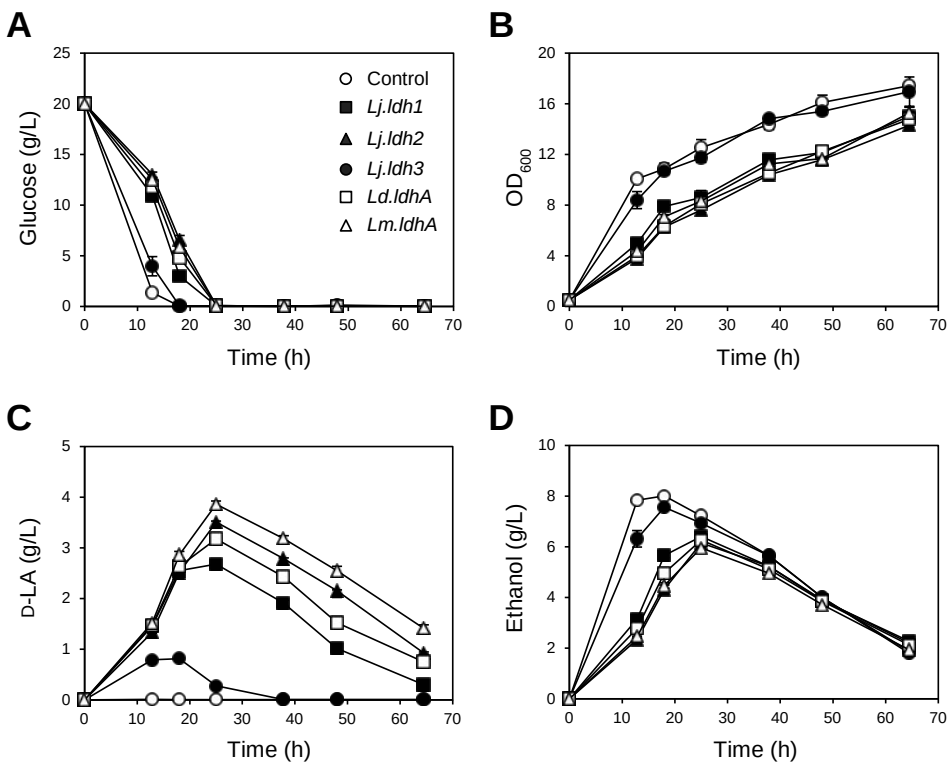


Figure 4.3 D-LA production activities of different D-LDH genes

S. cerevisiae CEN.PK2-1C cells expressing the indicated D-LDH genes from p425ADH plasmid vector were cultivated in SC-Leu medium containing 20 g/L of glucose. Cells harboring empty plasmid were used as a control. The amounts of glucose (A), D-LA (C), and ethanol (D) were measured by HPLC. Error bars indicate standard deviations of three independent experiments.

2008). *S. cerevisiae* expressing *Lm.ldhA* showed the highest level of D-LA production (3.9 g/L), followed by cells expressing *Lj.ldh2* (3.5 g/L), *Ld.ldhA* (3.2 g/L), and *Lj.ldh1* (2.7 g/L) after 25 h (Fig. 4.3C). Expression of *Lj.ldh3* resulted in the smallest amount of D-LA production (0.8 g/L) (Fig. 4.3C). The D-LA-producing cells, especially the high-level producers, showed a significant reduction in glucose uptake and growth rates, and ethanol production level (Fig. 4.3A, B, and D), suggesting that the newly introduced D-LA production pathway can compete with the ethanol production pathway, while exerting growth inhibitory effects. Upon glucose depletion, a gradual decrease in D-LA and ethanol levels was observed, indicating the utilization of these metabolites under our culture conditions (Fig. 4.3C and D). Based on the D-LA production activity in *S. cerevisiae*, we selected *Lm.ldhA* for further experiments.

4.3. Increase in D-LA production by disrupting genes *DLD1* and *JEN1*

As shown in Fig. 4.3C, D-LA can be catabolized upon glucose depletion in *S. cerevisiae*. Monocarboxylate transporters, Jen1 and Ady2, have been shown to play a role in LA uptake upon glucose depletion (Pacheco et al. 2012). Next, the conversion of D-LA to pyruvate is catalyzed by D-lactate dehydrogenase encoded by *DLD1* gene.

Therefore, to minimize D-LA consumption, we deleted *DLD1* and *JEN1*,

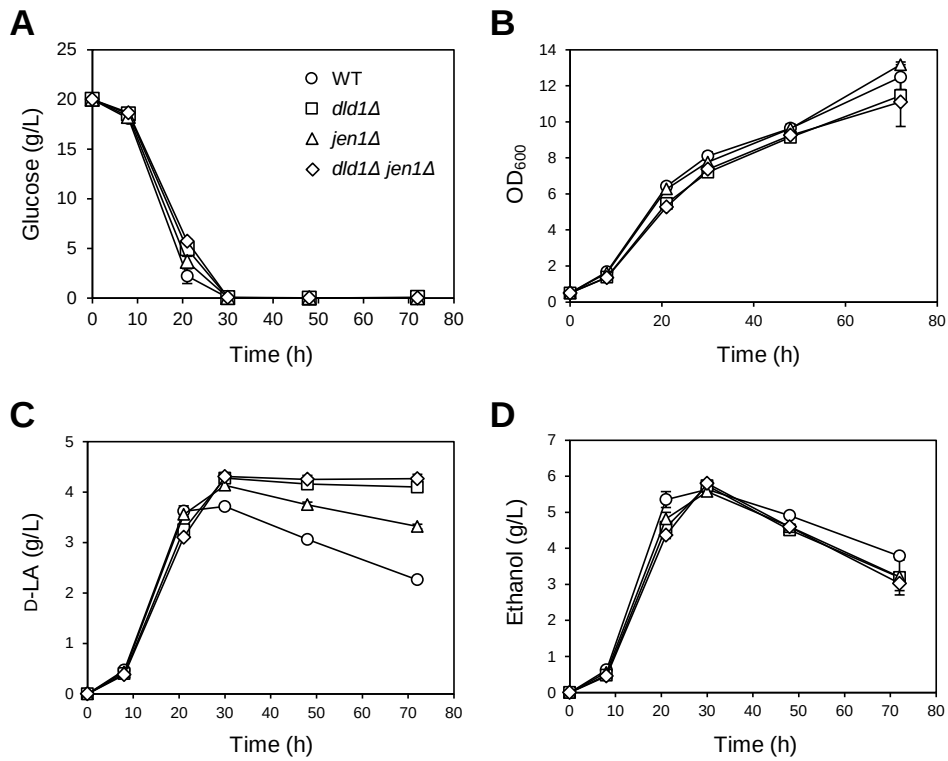


Figure 4.4 D-LA production in deletion strains of *DLD1* and/or *JEN1*

CEN.PK2-1C wild type (WT), *dld1Δ*, *jen1Δ*, and *dld1Δjen1Δ* strains harboring p425ADH-Lm.ldhA were cultivated in SC-Leu medium containing 20 g/L glucose (A), and D-LA (C) and ethanol (D) levels were detected. Error bars indicate standard deviations of three independent experiments.

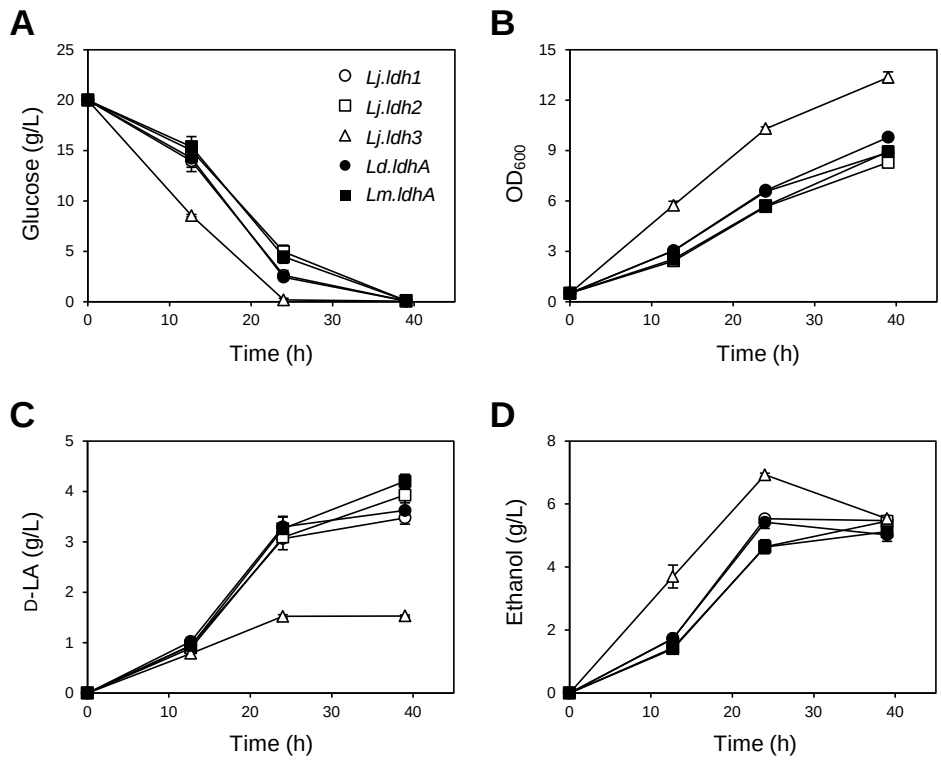


Figure 4.5 D-LA production activities of different D-LDH genes in *dld1Δjen1Δ* strain

dld1Δjen1Δ strains expressing the indicated D-LDH genes from p425ADH plasmid vector were cultivated in SC-Leu medium containing 20 g/L of glucose. The amounts of glucose (A), D-LA (C), and ethanol (D) were measured by HPLC. Error bars indicate standard deviations of three independent experiments.

individually or in combination, and D-LA production was monitored in each strain harboring p425ADH-*Lm.ldhA* plasmid. In agreement with the previous report, D-LA consumption after glucose depletion was reduced, although not completely inhibited, in *jen1Δ* strain compared with wild type (Fig. 4.4C) (Pacheco et al. 2012). On the other hand, D-LA consumption was almost completely inhibited in *dld1Δ* and *dld1Δjen1Δ* strains, suggesting that Dld1 plays an essential role in D-LA utilization. However, prevention of D-LA utilization in *dld1Δ* and *dld1Δjen1Δ* strains exerted a marginal effect on ethanol consumption after glucose depletion (Fig. 4.4D). Deletion of *DLD1* or *JEN1* gene had no effect aspect of the reduction of growth rate (Fig. 4.4B). To confirm the tendency of the activities of D-LDH genes, five genes were expressed in *dld1Δjen1Δ* strain. In agreement with the previous result, expression of *Lm.ldhA* resulted in the largest amount of D-LA production (4.2 g/L), increasing 0.3 g/L compared with that of CEN.PK2-1C (Fig. 4.5C).

4.4. Expression of *Lm.ldhA* gene by using different promoters and plasmid types

To further optimize D-LA production in *dld1Δjen1Δ* strain, *Lm.ldhA* was expressed under the control of different promoters, *ADH1*, *TEF1*, and *TDH3* (also known as GPD) from either CEN/ARS-based low copy number plasmid (p415) or 2μ-based high copy number plasmid (p425). In the case of p415-based expression, D-LA

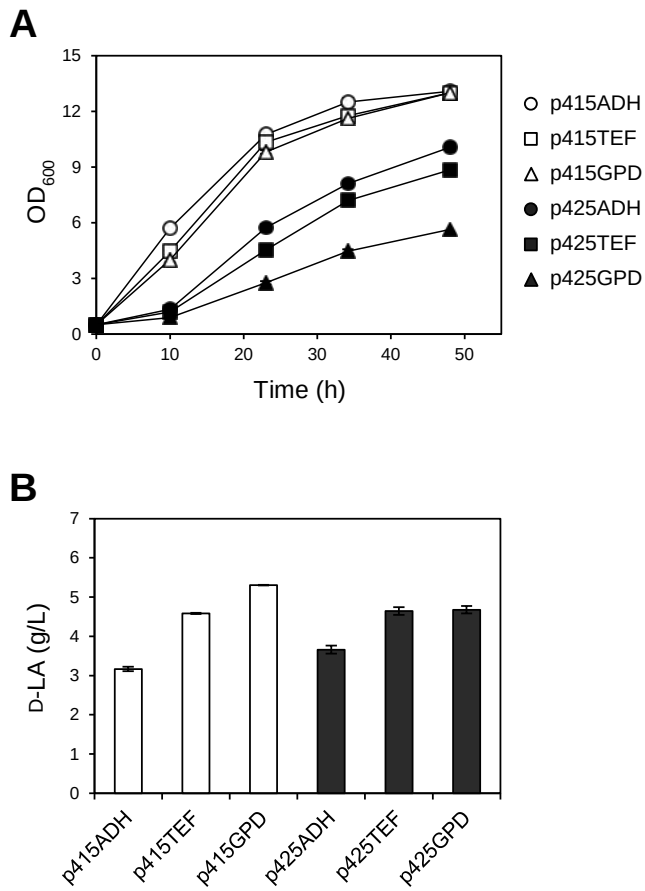


Figure 4.6 Investigation of the effects of promoters and plasmid types on D-LA production.

dld1Δjen1Δ strain was transformed with plasmids expressing *Lm.ldhA* gene from the indicated plasmids. Cell growth (A) was monitored during the cultivation in SC-Leu medium containing 20 g/L glucose and D-LA production level (B) was determined after 48 h. Error bars indicate standard deviations of three independent experiments.

production levels showed a correlation with the promoter strengths (Fig. 4.6B), exhibiting the highest D-LA level when *Lm.ldhA* was expressed from the *TDH3* promoter (p415GPD), followed by the expression from the *TEF1* promoter (p415TEF) and *ADH1* promoter (p415ADH). Higher D-LA production levels were accompanied by lower ethanol production levels (data not shown) and slower growth rates (Fig. 4.6A). p425-based expression of *Lm.ldhA* also led to a promoter strength-dependent D-LA production, but exerted more severe growth inhibitory effects than did the expression from p415, without significant improvement of D-LA production levels. These results suggest that maintaining high copy number plasmid might cause metabolic burden in D-LA-producing cells consuming large amounts of ATP to export lactate anions and protons (van Maris et al. 2004b). As a result, we selected p415GPD-*Lm.ldhA* plasmid for further experiments.

4.5. Improvement of D-LA production by deleting ethanol and glycerol formation pathways

Ethanol production from pyruvate is the major competing pathway in D-LA production (Fig. 4.1). Therefore, to further enhance D-LA production in *dld1Δjen1Δ* strain, we first deleted *ADH1* gene encoding the major alcohol dehydrogenase because a strain deleting additional ADH genes might shows more severe growth defect than a strain lacking *ADH1*. The resulting strain *dld1Δjen1Δadh1Δ* harboring p415GPD-*Lm.ldhA* (JHY5141) had growth defects but showed a 40% increase in

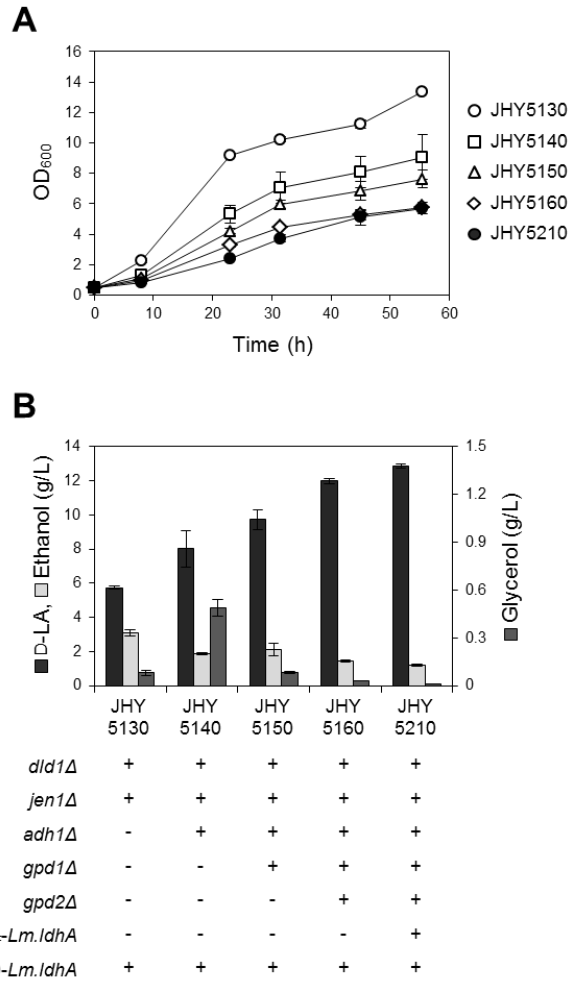


Figure 4.7 Improvement of D-LA production by deleting genes involved in byproduct formation

OD_{600} of 0.5 cells were cultivated in SC-Leu medium containing 20 g/L glucose. Error bars indicate standard deviations of three independent experiments.

A. Growth curves of the indicated D-LA-production strains.

B. D-LA, ethanol, and glycerol production levels in the indicated strains after 55.5 h.

D-LA production (8.0 g/L) and a 39% decrease in ethanol production (1.9 g/L) compared with *dld1Δjen1Δ* strain harboring p415GPD-*Lm.ldhA* (JHY5133) (Fig. 4.8B). However, deletion of *ADH1* led to an increase in glycerol production from 0.1 g/L to 0.5 g/L (Fig. 4.7B) as a compensation mechanism to maintain redox balance by regenerating NAD^+ (Fig. 4.1) (Skory 2003). Additional sequential deletion of *GPD1* and *GPD2* genes encoding glycerol-3-phosphate dehydrogenase eliminated glycerol accumulation, resulting in enhanced D-LA production up to 12.0 g/L (JHY5161) (Fig. 4.7B).

Next, we deleted *PDC1* encoding the major pyruvate decarboxylase by integrating *TEF1* promoter-controlled *Lm.ldhA* into the *PDC1* locus of the *dld1Δjen1Δadh1Δgpd1Δgpd2Δ* strain (JHY5160). The resulting strain *dld1Δjen1Δadh1Δgpd1Δgpd2Δpdc1Δ::Lm.ldhA* (JHY5210) harboring p415GPD-*Lm.ldhA* plasmid led to the highest level of D-LA production (12.9 g/L) among the engineered strains in SC-Leu medium containing 20 g/L glucose (Fig. 4.7B).

4.6. Conclusions

In this chapter, five heterologous D-LDH genes from *Lactobacillus* and *Leuconostoc* species, which have been demonstrated to be highly stereospecific for D-LA production, was expressed to produce optically pure D-LA in *S. cerevisiae*. Among the genes tested, *Lm.ldhA* was selected as the most active D-LDH gene to develop D-LA-producing strains. For the purpose of preventing D-LA consumption

after glucose depletion, genes involved in LA uptake (*JEN1*) and D-LA degradation (*DLD1*) were deleted. To further improve D-LA production in *dld1Δjen1Δ*, we reduced the formation of byproducts including ethanol and glycerol by deleting *ADH1, GPD1,* and *GPD2* genes. Thereafter, we additionally deleted *PDC1* in *dld1Δjen1Δadh1Δgpd1Δgpd2Δ* strain by integrating *Lm.ldhA* with *TEF1* promoter to reduce ethanol production. The resulting strain JHY5210 showed increased in D-LA production (12.9 g/L) and decrease in ethanol and glycerol production compared with *dld1Δjen1Δadh1Δgpd1Δgpd2Δ* strain from 20 g/L glucose, accompanying an increased yield of 0.65 g/g glucose.

Chapter 5.

Improvements of production level and yield of D-lactic acid using the adaptive-evolved strain and whole genome sequencing analysis

5.1. Introduction

In recent decades, a variety of metabolically engineered *S. cerevisiae* have received tremendous interests and attentions as an attractive alternative for the production of high value-added products such as pharmaceuticals, organic acid, and fuels (Sauer et al. 2010). Especially, LA production requires an acid-tolerant host strain because their undissociated form needed for PLA polymerization exists predominantly at low pH values (Martinez et al. 2013). Thus the construction of an efficient LA-producing *S. cerevisiae* strain have been studied to replace the LA-producing prokaryotic strain such as lactic acid bacteria (LAB).

Although *S. cerevisiae* has high acid tolerance, overcoming the growth inhibitory effect of LA is necessary for high-titer production. Inside of the cytosol with neutral pH, LA molecules dissociate into the acid anions and protons. Accumulation of both species induces multiple stress conditions via cytosolic acidification and modifications of membrane lipids and proteins (Abbott et al. 2008; Valli et al. 2006). Furthermore, exporting the protons and acid anions through H^+-ATPase and efflux pumps requires high cellular energy consumption (van Maris et al. 2004b). Some biological functions such as cell wall structure and membrane efflux pumps are commonly implicated in stress tolerance to various weak acids such as acetic acid, lactic acid, benzoic acid, and sorbic acid. However, cellular adaptive responses and tolerance mechanisms are quite variable depending on the chemical properties of weak acids (Mira et al. 2010a).

In order to investigate yeast genes involved in tolerance to LA, to date, several strategies such as genome-wide screening using deletion strain libraries and transcriptome analysis of the LA-treated yeast cells have been conducted for generating LA-tolerant *S. cerevisiae* strains (Hirasawa et al. 2013; Suzuki et al. 2013). One of the conventional strategies to improve tolerance is the adaptive laboratory evolution approach by inducing unpredictable genetic modifications using specific stress. This can be a powerful tool with whole genome sequencing and reverse metabolic engineering to reveal the association between mutated gene and phenotypic change (Kildegaard et al. 2014).

In this chapter, LA-tolerant strain generated by adaptive laboratory evolution was used for improving LA production level and yield in both acidic and neutralizing fermentation conditions. Through whole genome sequencing analysis, the five genes, having point mutation by single-base substitution, were identified from the evolved strain and then functional analysis was performed for deciphering what mutated gene confers the improved properties in the evolved strain.

5.2. Improvement of LA tolerance of JHY5210 strain by adaptive laboratory evolution

As shown in Fig. 4.7A, D-LA production levels were improved by deleting competing pathways, but growth defects were observed from strains eliminating ethanol and glycerol synthesis pathways. The growth of JHY5210 strain containing

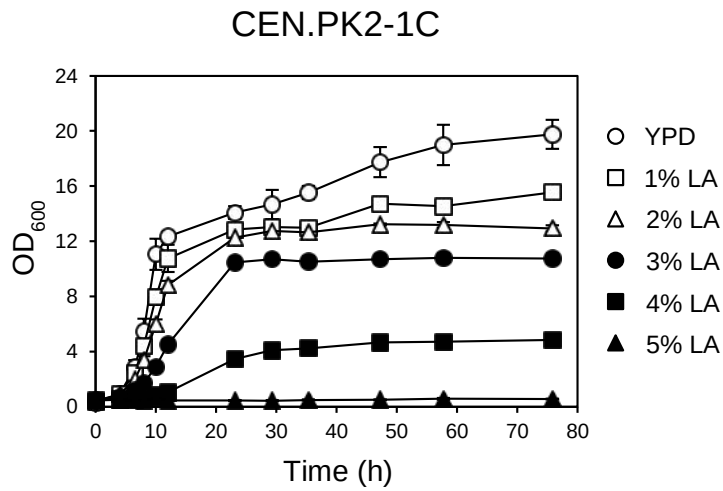


Figure 5.1 Growth defects of CEN.PK2-1C strain in YPD medium containing LA

OD_{600} of 0.2 cells were cultivated in YPD medium containing 20 g/L glucose with LA from 1% to 5% (w/v) except control medium (YPD). Error bars indicate standard deviations of two independent experiments.

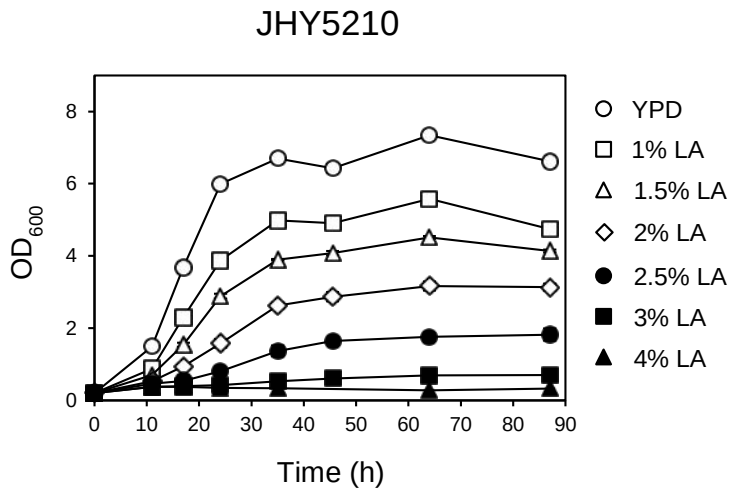


Figure 5.2 Growth defects of JHY5210 strain in YPD medium containing LA

OD_{600} of 0.2 cells were cultivated in YPD medium containing 20 g/L glucose with LA from 1% to 4% (w/v) except control medium (YPD). Error bars indicate standard deviations of two independent experiments.

a genome integrated *Lm.ldhA* gene was halved compared with JHY5130 strain. The growth retardation was also observed when JHY5210 strain was cultured in YPD medium containing only 1% LA and this strain showed reduced acid tolerance compared with CEN.PK2-1C (Fig. 5.1 and 5.2). That is, D-LA production was limited by the growth inhibitory effect of D-LA. To further improve D-LA production level by enhancing lactic acid tolerance of JHY5210 strain, I used the evolved strain JHY5310, a selected strain based on glucose consumption ability and D-LA production level from adaptive laboratory evolution approach with a gradual increase in LA concentrations.

To examine the advancement of acid tolerance in evolved strain, JHY5210 and JHY5310 strains were tested for growth by spotting cultures onto YPD solid media containing 1.5% and 2%LA, respectively. The unevolved strain JHY5210 showed growth defect in the presence of LA, but the evolved strain JHY5310 grew well up to 2% LA concentration (Fig. 5.3). The different growth rates between unevolved and evolved strains were also observed in the control medium without LA. From these results, it was confirmed that the evolved strain JHY5310 possessed an increased growth rate and LA tolerance by adaptive evolution.

5.3. D-LA production using the evolved strains

To determine the improvement of glucose consumption and D-LA production abilities of the evolved strain JHY5310, JHY5210 and JHY5310 strains harboring

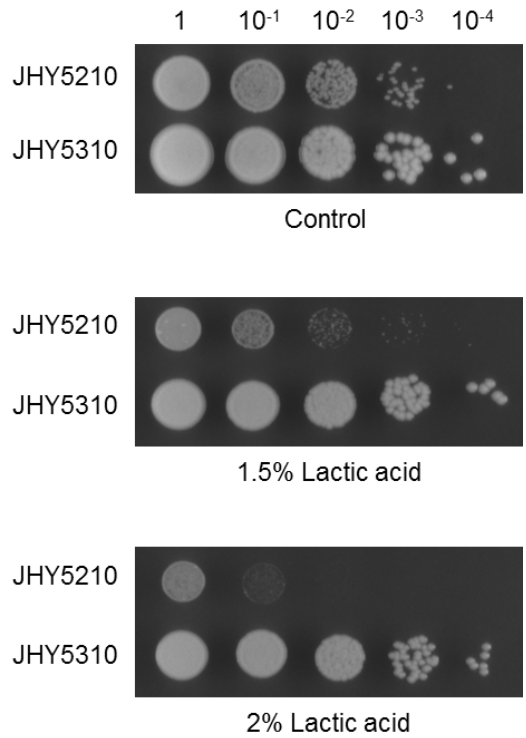


Figure 5.3 LA tolerance of JHY5310 strain compared with JHY5210 in YPD solid medium containing LA

OD_{600} of 1 cells cultivated in YPD medium containing 20 g/L glucose were tested by spotting serially diluted cultures onto YPD media with 1.5% or 2% LA, respectively.

p415GPD-*Lm.ldhA* plasmid were cultured in SC-Leu medium containing 50g/L glucose. The unevolved strain JHY5210 consumed only 32.7 g/L of glucose for 120 h, producing 21.3 g/L D-LA. On the other hand, the evolved JHY5310 strain showed 41% higher glucose consumption (47.1 g/L) and increased D-LA production up to 31.7 g/L (Fig. 5.4).

Next, I constructed the evolved strain JHY5320 containing an additional genome-integrated *Lm. ldhA* gene controlled by *TDH3* promoter into *JEN1* locus of JHY5310 strain. Additional *Lm. ldhA* gene expression led to an increase in D-LA production level up to 38.3 g/L in YPD medium containing 50 g/L glucose. In addition, this strain showed reduced ethanol formation from 2.8 g/L to 1.9 g/L compared with JHY5310 (Fig. 5.5). This result suggests that the expression level of *Lm. ldhA* gene in only one-copy of *Lm.ldhA* gene integrating strains might be not sufficient for improving metabolic flux from pyruvate to D-LA with decreasing byproduct formations.

Although D-LA production levels were improved by deleting competing pathways with adaptive laboratory evolution and additional integration of *Lm.ldhA* gene, JHY5320 strain was not completely consumed glucose in acidic fermentation. It was observed that acidity of YPD medium including 20 g/L LA was drastically decreased from pH 6.5 to pH 2 (data not shown) and the acidic condition of culture medium might be a major factor to trigger the glucose consumption limitation in D-LA producing strains such as JHY5210. Therefore, D-LA production level was monitored under neutralizing fermentation condition. The evolved strain JHY5320

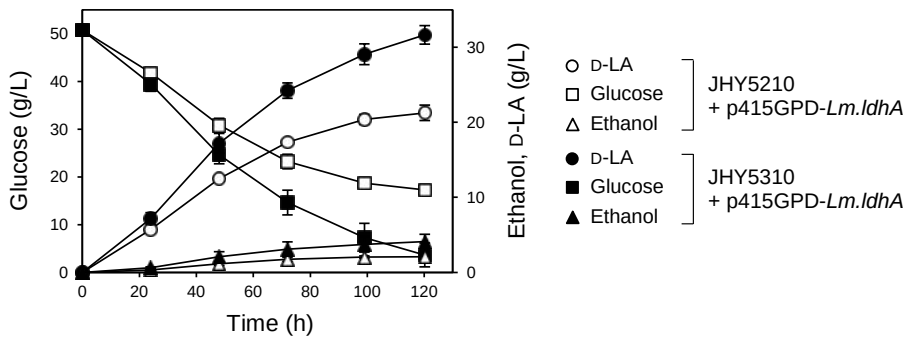


Figure 5.4 D-LA production in the evolved LA-tolerant strain (JHY5310) compared with the unevolved strain JHY5210

OD_{600} of 1 cells of JHY5210 and JHY5310 strains harboring p415GPD-*Lm.ldhA* plasmid were cultivated in SC-Leu medium containing 50 g/L of glucose and D-LA, glucose, and ethanol levels were detected by HPLC. Error bars indicate standard deviations of three independent experiments.

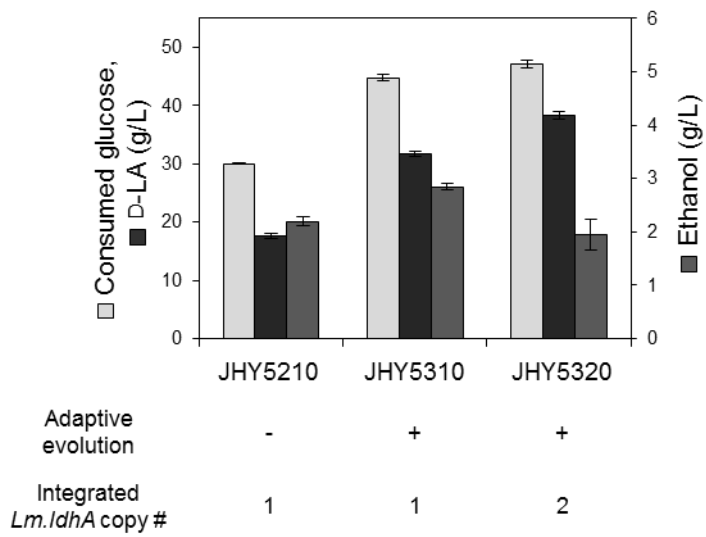


Figure 5.5 Investigation of the effect from additional integration of *Lm.ldhA* in the evolved JHY5310 strain.

OD_{600} of 1 cells each strain were cultivated in YPD medium containing 50 g/L of glucose. D-LA, glucose, and ethanol levels were detected by HPLC. Error bars indicate standard deviations of three independent experiments.

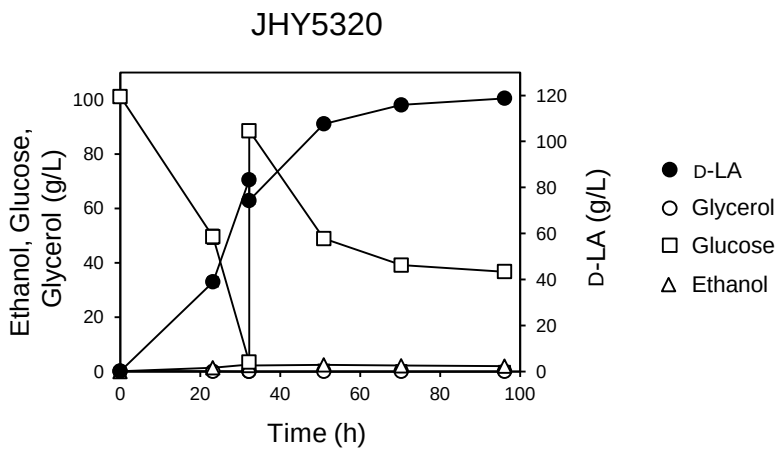


Figure 5.6 D-LA production profiles in fed-batch fermentation under neutralizing condition

Strain JHY5330 was inoculated in the YPD medium containing 100 g/L glucose with 50 g/L of $CaCO_3$ and additional feeding of glucose was conducted when glucose in the medium was depleted. D-LA, ethanol, and glycerol production levels and residual glucose concentrations in the medium were detected for 96.1 h. Error bars indicate standard deviations of three independent experiments.

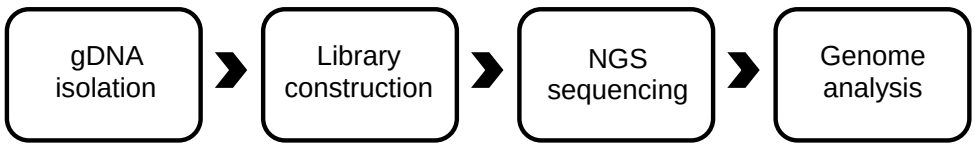
was cultured in flask fed-batch fermentation in YPD medium containing 100g/L glucose with 50 g/L of $CaCO_3$. After 32 h cultivation, as expected, this strain consumed almost 100 g/L glucose, producing 83.3 g/L D-LA (Fig. 5.6). Further cultivation up to 96.1 h with additional glucose supplement, D-LA titer was increased up to 118.6 g/L from 149.6 g/L glucose accompanying low levels of ethanol and glycerol (1.8 g/L and < 0.01 g/L, respectively). In addition, JHY5320 strain showed comparable yield (0.79 g/g glucose) compared with previously reported LA-producing strains.

5.4. Whole genome sequencing analysis of the evolved strain

As shown in the previous results, the evolved strain JHY5310 possessed a higher glucose consumption ability compared with unevolved strains, but it was not identified what factors confer this distinction with growth recovery to the evolved strains. To identify the main factor involved in increase of acid tolerance, whole genome sequencing analysis of JHY5310 was performed with JHY5210, fourth, and eighth sub-cultured strains. Genomic DNA of each strain was prepared using genomic DNA isolation kit, and then isolated genomic DNA samples were used for construction of library and analysis of genome sequencing. The genomes map were generated and analyzed against the genome sequences of the CEN.PK113-7D as the reference (Fig. 5.7).

Compared to genome sequences of the unevolved strain JHY5210,

A


gDNA isolation
Library construction
NGS sequencing
Genome analysis

B

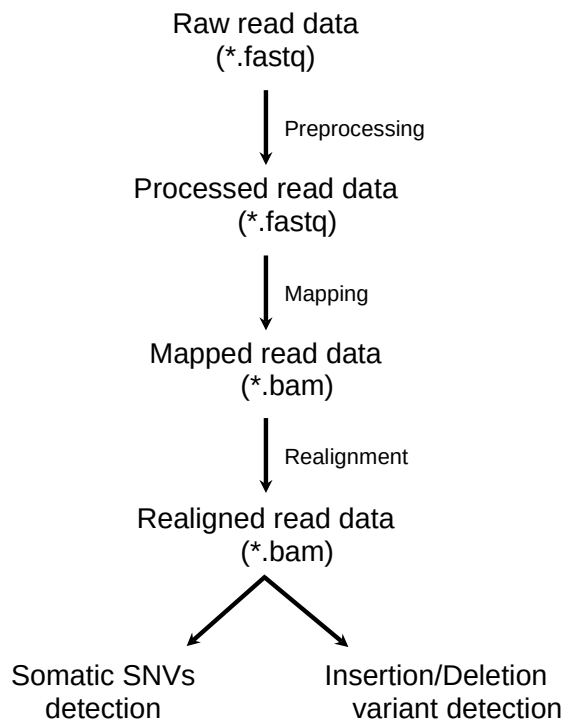

Raw read data (*.fastq)
Preprocessing
Processed read data (*.fastq)
Mapping
Mapped read data (*.bam)
Realignment
Realigned read data (*.bam)
Somatic SNVs detection
Insertion/Deletion variant detection

Figure 5.7 Overall steps for whole genome sequencing analysis

In brief, each DNA sample was isolated by using genomic DNA prep kit for yeast and then sequenced using Illumina Hiseq-2000 (A). Raw read data from genome analysis was preprocessed using Trimmomatic trimming tool for removing low quality base and adapter. Next, processed read data was mapped by BWA software and then mapped sequence was religned for eliminating gene insertion and deletion using Realigner Target Creator/Indel Realigner of Genome Analysis Tool Kit (GATK). Based on realigned data, single nucleotide variants (SNVs) and insertions/deletions were detected by MuTect and VarScan software, respectively (B).

Table 5.1 Results of whole genome sequencing analysis of the evolved strain JHY5310

Gene	Type of mutation	Position
GSF2	Nonsense Mutation	E2**Stop** (GAG→TAG)
SYN8	Nonsense Mutation	E121**Stop** (GAG→TAG)
SIF2	Point Mutation	M66**I** (ATG→ATT)
STM1	Point Mutation	P144**L** (CCA→CTG)
BUD27	Point Mutation	P549**H** (CCT→CAT)

the five genes having a base-substitution were observed in the evolved strain JHY5310. Fourth and eighth sub-cultured strains also had four and five mutated genes, respectively. Interestingly, all three evolved strains in these analyses had a same point-mutated *SIF2* gene encoding WD40 repeat-containing subunit of Set3C histone deacetylase complex, where nucleotide at position 198 was changed from G to T. This missense mutation led to a change of amino acid sequence from methionine to isoleucine. The eighth and JHY5310 strain had *GSF2* gene, inserting the novel stop codon, encoding glucose signaling factor, but substitution position was different. The nucleotide position 886 of *GSF2* was changed from glycine to TGA stop codon in eighth sub-cultured strain, whereas TAG stop codon was observed at the second amino acid position of G*SF2* in the JHY5310 strain. Excepting *GSF2* gene, the other nonsense-mutated gene was detected from *SYN8* gene encoding endosomal SNARE related to mammalian syntaxin 8. In addition, missense mutation in *STM1* gene encoding a protein required for optimal translation under nutrient stress and *BUD27* gene, translation initiation in yeast, were also detected in the evolved strain JHY5310. In contrast, fourth sub-cultured strain had a point-mutated *HXK2* gene and silent-mutated *AGA1* and *RPN5* genes. In eighth sub-cultured strain, point-mutated *SUR1* and *MET4* genes with silent-mutated *HSP82* were observed. Gene duplication was not detected in any evolved strains.

5.5. Funtional analysis of the five mutated genes

I supposed that five genes found in the evolved strain JHY5310 might lead to the particular properties such as improving glucose consumption ability. To investigate the functions of these genes, each gene was overexpressed using p416GPD plasmid individually in the unevolved strain JHY5210. All overexpressing cells showed no significant effects to glucose consumption level (Fig. 5.8). Next, each deletion strain was also constructed in the unevolved strain JHY5210 by integrating deletion cassette including *HIS3* gene as a selection marker and cultured in YPD medium containing 50 g/L glucose to measure glucose consumption levels. Among them, only JHY5211 strain deleting *GSF2* gene showed significant improvements of glucose consumption level and yield compared with the unevolved strain (Fig. 5.9). On the contrary, deletion of *BUD27* gene led to decrease of growth rate and glucose consumption ability. JHY5212, JHY5213, and JHY5214 strains deleting *SYN8*, *STM1*, and *SIF2*, respectively, showed no significant changes in glucose consumption rate compared with JHY5210 strain (Fig. 5.9).

When the unevolved strain JHY5210 and these deletion strains were tested for growth by spotting cultures onto YPD solid medium containing 1.5% LA, the enhanced LA tolerance was observed from JHY5212, JHY5213, and JHY5214 compared with unevolved strain (Fig. 5.10). The effect of *SYN8* gene deletion for LA tolerance was already reported from previous researches using genome-wide screening (Kawahata et al. 2006; Suzuki et al. 2013), however, the positive effect

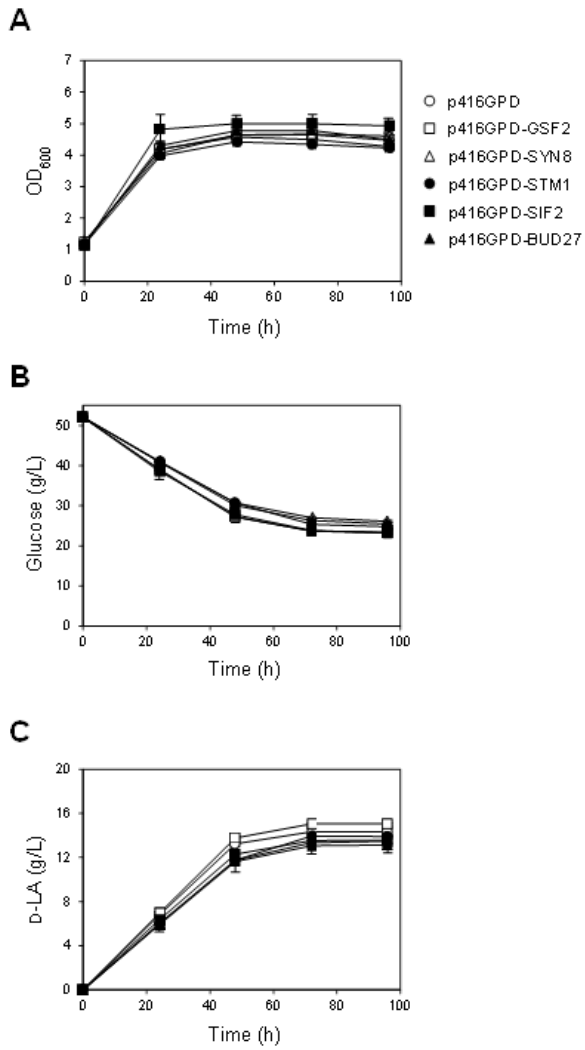


Figure 5.8 Overexpression effects of the five genes detected from whole genome sequencing anaylses in JHY5210 strain

Each gene found from sequencing analysis of JHY5310 strain was overexpressed using p416GPD based plamids. OD_{600} of 1 cells each transforment were cultivated in SC-Ura medium containing 50 g/L glucose. D-LA, glucose, and ethanol levels were detected by HPLC. Error bars indicate standard deviations of three independent experiments.

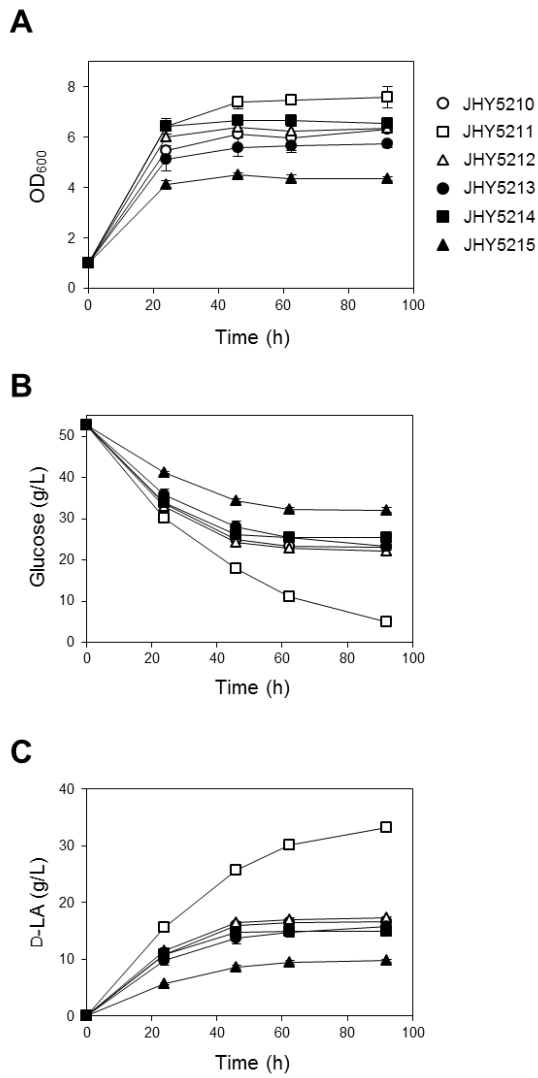


Figure 5.9 Investigation of deletion effects of the five genes detected from whole genome sequencing anaylses

Deletion stains derived from were cultivated in YPD medium containing 50 g/L glucose. D-LA, glucose, and ethanol levels were detected until 96 h. Error bars indicate standard deviations of three independent experiments.

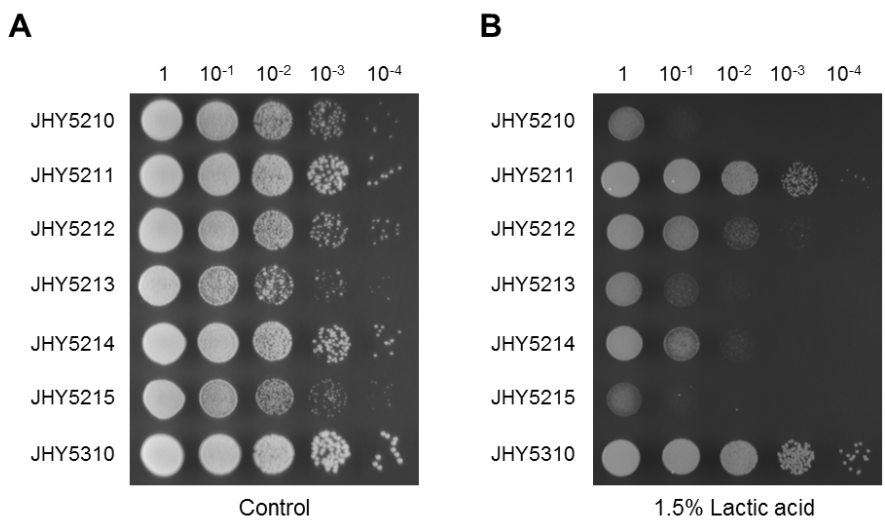


Figure 5.10 LA tolerance test in deletion strains and the evolved strain

Deletion stains and the evolved strain were cultivated in YPD medium and then each strain was tested for growth rate analysis by spotting onto YPD solid medium containing 1.5% LA with JHY5210 strain as a control.

of *STM1* or *SIF2* deletion for improving LA tolerance is not reported yet. The *GSF2* deletion strain JHY5211 showed higher growth rate compared with the unevolved JHY5210 and other strain not only YPD medium, but also LA-containing YPD medium.

5.6. *GSF2* deletion effects in other engineered strains

By observing overexpression or deletion effect of five mutated genes, it was investigated that deletion of *GSF2, SYN8, STM1,* or *SIF2* gene conferred LA tolerance to the evolved strain, but improvement of D-LA production level was observed in only *GSF2* deletion strain. In previous study, it was reported that Gsf2, an integral membrane protein located in endoplasmic reticulum (ER), might be involved in relieving glucose repression of several genes regulated by glucose (Sherwood and Carlson 1997; Sherwood and Carlson 1999). When GSF2 was deleted, expression levels of *SUC2* and *GAL10*, encoding invertase and galactose transporter, respectively, was significantly improved in the presence of glucose (Sherwood and Carlson 1997). It was also reported that *GSF2* deletion led to hexose transporter 1 (Hxt1) accumulation in ER and *GSF2* deletion effects were suppressed by Hxt1 overexpression. Therefore, it was suggested that relief of glucose repression by deleting *GSF2* might be associated with abnormal localization of Hxt1 (Sherwood and Carlson 1999).

As shown in the Fig. 5.9, glucose consumption of unevolved strain was

considerably recovered by *GSF2* deletion. In order to investigate glucose consumption and D-LA production levels of *GSF2* deletion strain, the unevolved strain JHY5211 and the evolved strain JHY5310 were cultured in YPD medium containing 50 g/L glucose with the unevolved strain JHY5210 as a reference. In agreement with previous result, JHY5210 strain still showed limited glucose consumption, remaining 19.1 g/L glucose, whereas JHY5211 strain consumed 50.4 g/L glucose, producing 35.6 g/L LA until 97 h cultivation (Fig. 5.11). In the case of the evolved strain JHY5310, glucose in the medium was completely consumed, producing D-LA up to 37.6 g/L. Compared to JHY5310 strain, JHY5211 strain showed slightly lower glucose consumption and D-LA production levels, but showed reduced ethanol formation from 3.7 g/L to 2.5 g/L. In addition, D-LA production yield of JHY5211 strain was improved from 0.58 g/g glucose to 0.71 g/g glucose compared with the unevolved strain JHY5210. I supposed that the mutation into *GSF2* gene might be applied to the engineered *S. cerevisiae* strains, deleting ethanol and glycerol synthesis pathways, for improving glucose consumption ability and growth rate.

To investigate whether *GSF2* gene deletion can confer the increase in growth rate and glucose consumption ability to other engineered strains, several *GSF2* deletion strains were constructed and cultured in YPD medium containing 20g/L glucose. Although *gsf2Δ* strain derived from CEN.PK2-1C showed higher cell density after 14.5 h, growth rate was not improved. When JHY5160 and JHY5161 strains deleting *ADH1*, *GPD1*, and *GPD2* genes with *DLD1* and *JEN1* were

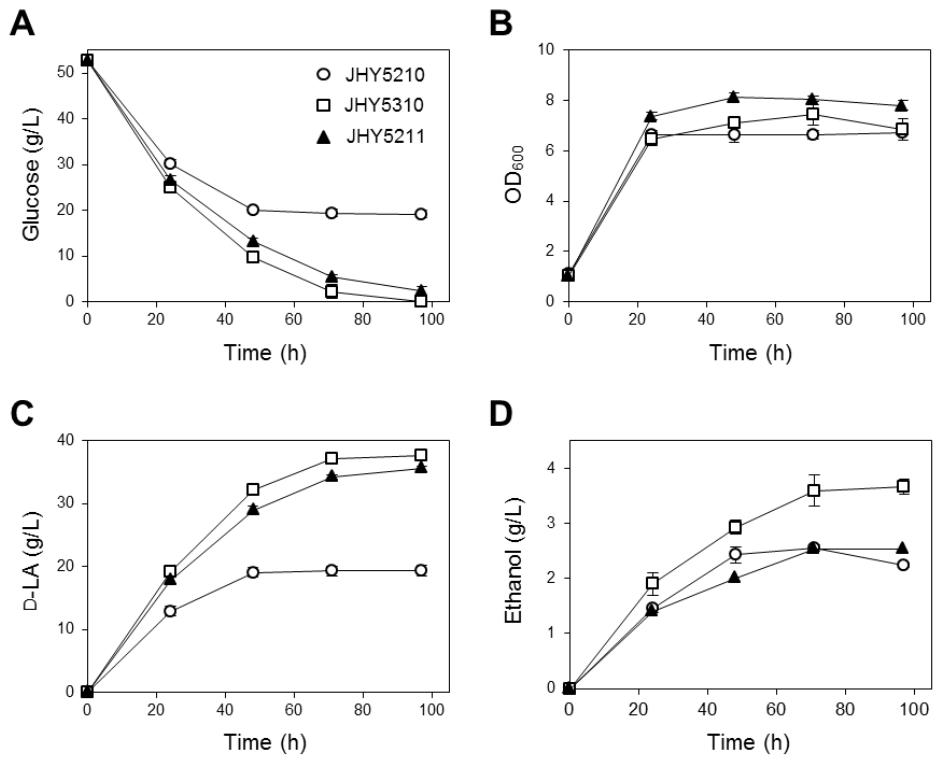


Figure 5.11 Analysis of D-LA production and glucose consumption levels in JHY5211 strain compared with the evolved strain (JHY5310) and the unevolved strain (JHY5210)

OD_{600} of 1 cells of each strain were cultivated in YPD medium containing 50 g/L glucose. Residual glucose concentrations (A)and D-LA (C) and ethanol (D) production levels in the medium were determined for 97h by HPLC. Error bars indicate standard deviations of three independent experiments.

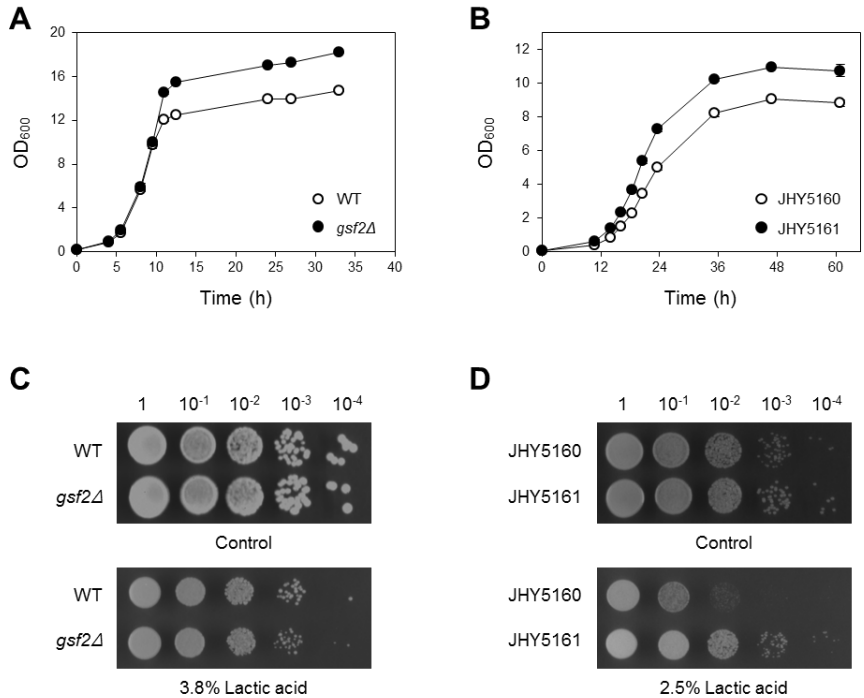


Figure 5.12 Investigation of *GSF2* deletion effect in strains lacking LA production ability

A. OD_{600} of 0.2 cells of CEN.PK2-1C and *gsf2Δ* strains were cultivated in YPD medium containing 20 g/L glucose to measure cell densities for 33 h. Error bars indicate standard deviations of three independent experiments.

B. OD_{600} of 0.05 cells of JHY5160 strain and JHY5161 strain deleting *GSF2* gene were cultivated in YPD medium containing 20 g/L glucose until 60.9 h. Error bars indicate standard deviations of three independent experiments.

C. CEN.PK2-1C and *gsf2Δ* strains were tested for LA tolerance by spotting cultures onto YPD solid medium with 3.8% LA.

D. JHY5160 and JHY5161 strains were tested for LA tolerance by spotting cultures onto YPD solid medium with 2.5% LA.

cultured in YPD medium, in contrast, *GSF2* deletion strain showed improved growth rate accompanying higher cell density compared with JHY5160 strain. *GSF2* deletion effect was also tested by culture onto YPD solid medium containing LA. As shown in Fig. 5.12D, JHY5160 strain showed severe growth inhibition in YPD solid medium containing 2.5% LA, but JHY5161 strain showed the overcoming growth defect. In addition, improvement growth rate was also observed from JHY602 strain deleting five ADH genes from *ADH1* to *ADH5* and JHY604 strain deleting *ADH1* with *GPD1* and *GPD2* genes, respectively (Fig. 5.13). These results suggest that growth defects caused by eliminating ethanol or ethanol and glycerol synthesis pathways can be overcome by deletion of *GSF2* gene.

5.7. Conclusions

Although D-LA production levels were improved by deleting competing pathways, D-LA production was limited by the growth inhibitory effect of D-LA. Deletion of *PDC1* gene may affect the cytosolic level of acetyl-CoA, but inability to synthesize acetyl-CoA is not a main reason because other PDC genes including *PDC5* and *PDC6* are also involved in acetaldehyde formation from pyruvate. Therefore, the evolved strain developed from JHY5210 strain by adaptive evolution was used for improving D-LA production levels. The evolved strain JHY5310 showed higher acid tolerance and glucose consumption ability compared with the unevolved strain

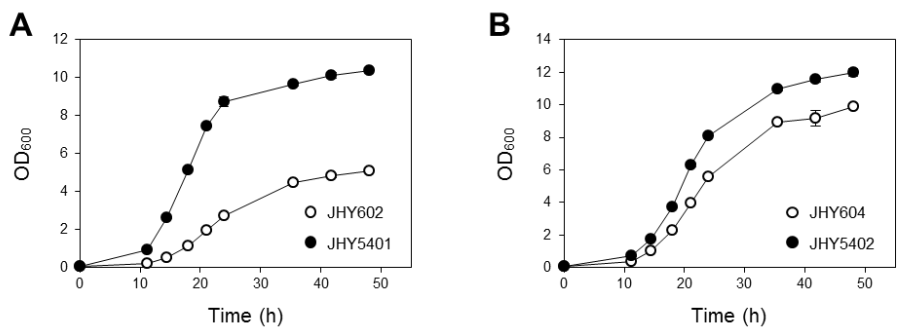


Figure 5.13 Investigation of *GSF2* deletion effect in other engineered strains

A. OD_{600} of 0.05 cells of JHY602 and JHY5401 deleting *GSF2* in JHY602 were cultivated in YPD medium containing 20 g/L glucose to measure cell densities for 48 h. Error bars indicate standard deviations of three independent experiments.

B. OD_{600} of 0.05 cells of JHY604 strain and JHY5402 strain deleting *ADH1*, *GPD1*, and *GPD2* with *GSF2* were cultivated in YPD medium containing 20 g/L glucose until 48 h. Error bars indicate standard deviations of three independent experiments.

JHY5210. Although it is difficult to reveal what is the major factor, LA and proton might be suggested as factors to confer the advanced properties into the evolved strain because growth retardation can be involved in both proton and LA levels in culture medium. JHY5320 strain containing two copies of genome-integrated *Lm. ldhA* genes produced up to 38.3 g/L D-LA with a yield of 0.81 g/g glucose in acidic fermentation condition without pH control, exhibiting an increase in D-LA titer by 21% compared with JHY5310 strain. Under neutralizing fed-batch fermentation condition, 118.7 g/L of D-LA was produced with a yield of 0.79 g/g glucose for 96.1 h. Through whole genome sequencing analysis of the evolved strain JHY5310, the five mutated genes were found. By deletion effect test, it was confirmed that single deletion of *SYN8*, *STM1*, or *SIF2* gene could contribute to improve LA tolerance in the evolved strain. Especially, deletion of *GSF2* gene restored growth retardation and glucose consumption ability in the unevolved strain. Compared to the evolved strain, *gsf2Δ* strain showed similar levels of growth rate and glucose consumption, accompanying D-LA production up to 35.6 g/L. The *GSF2* deletion effect was also demonstrated in JHY5161 strain without genome-integration of *Lm.ldhA* as well as JHY5401 and JHY5402 strains. Based on these results, I suppose that the positive effect may be associated with the insufficient glucose catabolite repression in *GSF2* deletion strains. In previous research, it was reported that Hxt1 in *gsf2Δ* strain was partially accumulated in the ER, not plasma membrane. If abnormal localization of Hxt1 was involved in decrease of glucose consumption, sufficient glucose repression may be not triggered in a cell and then

relieving glucose repression can support the overcoming growth defect in recombinant *S. cerevisiae*. To demonstrate the effect of *GSF2* deletion into the engineered *S. cerevisiae* strains having severe growth defects, further study should be focused on identifying the Gsf2 and Hxt1 mechanism of growth rate recovery and increase of glucose consumption ability.

Chapter 6.

Development of Haa1-overexpressing strain for improving lactic acid tolerance and D-lactic acid production level

6.1. Introduction

In *Saccharomyces cerevisiae*, various weak acids act as stress factors causing decrease of intracellular pH level and growth retardation (Sauer et al. 2010; Suzuki et al. 2012b; Ullah et al. 2012). To overcome these challenges, *S. cerevisiae* possesses the adaptive response and tolerance mechanisms by regulating specific proton/anion efflux pump expressions or altering of plasma membrane compositions (Abbott et al. 2008; Gregori et al. 2008; Kawahata et al. 2006; Nygard et al. 2014). To further understand of adaptation mechanism about each weak acid, many studies have been investigated focusing on transcription factors and its specific target genes.

LA also leads to weak acid stress such as ROS stress. Although *S. cerevisiae* has high acid tolerance, elucidation of adaptive response mechanism to LA stress is crucial for development of the efficient LA-producing *S. cerevisiae* strain. However, the current knowledge of LA stress mechanism and adaptation mechanism is not enough to rational approaches for strain development. So far, it has been reported that several LA stress-inducible genes are regulated by transcription factors Aft1 and Haa1, which are involved in iron metabolism and adaptation response to acid stress, respectively (Abbott et al. 2008; Kawahata et al. 2006; Sugiyama et al. 2014).

Especially, Haa1 is a transcriptional activator involved in LA stress condition when undissociated form predominantly exists. Haa1 was implicated in regulation

of 160 target genes, including *TPO2* encoding polyamine transporter and *YRO2* that are known to be involved in acetic acid tolerance, directly or indirectly (Mira et al. 2010a; Mira et al. 2011; Takabatake et al. 2015). In recent decade, overexpression of Haa1 has been shown to increase LA tolerance under acidic condition, whereas the overexpression effect of target genes for improving LA tolerance and LA production level was not identified yet. (Inaba et al. 2013; Sakihama et al. 2015). It was also reported that localization of Haa1 from nucleus to cytosol regulated by Msn5 and change of phosphorylation status were related to function of Haa1 in the cell under low pH condition, but specific kinase or phosphatase involved in Haa1 was not reported (Sugiyama et al. 2014).

Casein kinase 2 (CK2), a highly conserved essential protein kinase in all eukaryotes, is composed by two catalytic subunits (*CKA1* and *CKA2*) and two regulatory subunits (*CKB1* and *CKB2*) in *S. cerevisiae* (Litchfield 2003). The deletion of two regulatory subunits does not affect cell viability, whereas all catalytic subunits deletion strain does not survive in *S. cerevisiae*. General phosphorylation site of CK2 is known for serine or threonine residues containing several acidic amino acids such as aspartic acid or glutamic acid between -2 and +5 positions. (Berkey and Carlson 2006; Glover 1998; Meggio and Pinna 2003). This kinase has various substrates involved in cellular processes including transcription regulation, cell survival, cell cycle regulation, and RNA polymerases (Glover 1998; Litchfield 2003; Pinna 2002). Among them, several substrates have been identified as a genuine protein phosphorylated by CK2 *in vivo*. This kinase also plays an

important role for stress response such as ethanol stress, osmotic stress, and nutrient starvation stress in *S. cerevisiae* (Berkey and Carlson 2006; Burns and Wente 2014; Cho et al. 2014).

In this chapter, D-LA production in the evolved strain JHY5320 was further improved by integrating *HAA1* gene for enhancing LA tolerance. Through *in vitro* kinase assays and qRT-PCR analyses, Haa1 was identified as a new substrate of CK2 and Cka2 subunit particularly involved in Haa1 activity under LA stress. I focused on investigating CK2-dependent phosphorylation site in Haa1 and demonstrating the function of phosphorylated residue against LA stress.

6.2. Construction of *HAA1*-integrated strain for improving D-LA production ability

As an effort to further increase LA tolerance of JHY5320, *HAA1*, a transcription activator involved in LA stress response, was integrated into the genome of JHY5320 under the control of *ADH1* promoter, generating JHY5330. In YPD medium with 70 g/L glucose, JHY5330 consumed 62.2 g/L glucose and produced 48.9 g/L, indicating 11% and 10% increases, respectively, compared with JHY5320, reaching a yield of 0.79 g/g glucose and a productivity of 0.41 g/(L·h) (Fig. 6.1). These results demonstrate that strain JHY5330 is highly efficient in D-LA production even under acidic fermentation conditions without pH control. The positive effect of *HAA1* overexpression was more prominent at the later stage of

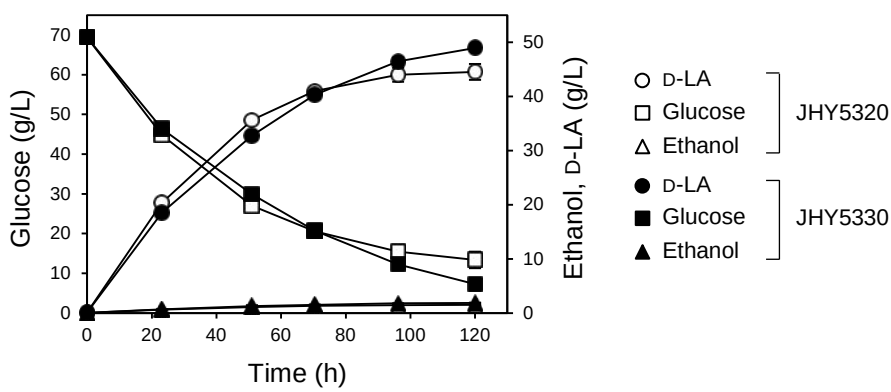


Figure 6.1 Improvement of D-LA production by integrating *HAA1* gene into the evolved strain JHY5320

OD_{600} of 1 cells of JHY5320 and *HAA1*-integrated strain JHY5330 were cultivated in YPD medium containing 70 g/L glucose. D-LA and ethanol production levels and residual glucose concentrations in the medium were detected for 120 h. Error bars indicate standard deviations of three independent experiments.

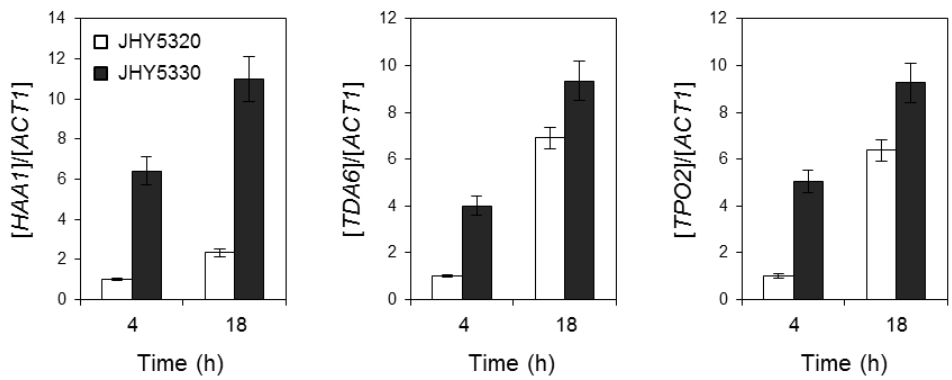


Figure 6.2 Determinations of transcription levels of *HAA1* and its target genes by qRT-PCR analysis

OD_{600} of 1 cells of JHY5320 and JHY5330 were cultivated in YPD medium containing 70g/L glucose, and mRNA levels of the indicated genes were determined by qRT-PCR using *ACT1* mRNA level as a control. Error bars indicate standard deviations of three independent experiments.

fermentation, which might reflect the protective role of Haa1 under acidic conditions.

I confirmed the effect of *HAA1* overexpression on the transcriptional activation of its target genes by qRT-PCR analysis. As expected, JHY5330 showed higher *HAA1* mRNA levels than those shown in JHY5320 (Fig. 6.2). The mRNA levels of Haa1 target genes *TDA6* and *TPO2* were also higher in JHY5330 than in JHY5320, suggesting that the increased expression levels of Haa1 target genes might contribute to enhancing acid tolerance and D-LA production ability in strain JHY5330. Expression levels of *TDA6* and *TPO2* increased during the cultivation from 4 to 18 h in both JHY5320 and JHY5330 strains, which might reflect the activation of Haa1 in response to D-LA accumulation as well as the increase in *HAA1* transcription itself by yet unidentified regulatory mechanisms (Fig. 6.2).

To compare D-LA production ability of *HAA1* integrated strain with previously developed strains that were evaluated under pH-controlled conditions, I also investigated D-LA production levels of JHY5330 in the presence of neutralizing agent during the fermentation. I carried out flask fed-batch fermentation in YPD medium containing 100 g/L glucose with 50 g/L of $CaCO_3$. After 50.9 h of cultivation, strain JHY5330 produced 112.0 g/L D-LA from 140.2 g/L glucose with a yield of 0.80 g/g glucose and high productivity of 2.2 g/(L·h), while showing limited byproduct formation (2.6 g/L ethanol and < 0.01 g/L glycerol) (Fig. 6.3). Further cultivation up to 96.1 h led to an increase in D-LA production up to 124.4

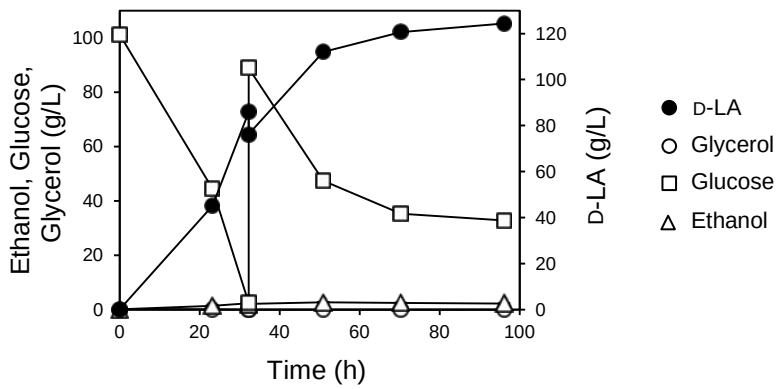


Figure 6.3 D-LA production profiles in fed-batch fermentation under neutralizing condition

JHY5330 strain was inoculated in the YPD medium containing 100 g/L glucose with 50 g/L of $CaCO_3$ and additional feeding of glucose was conducted when glucose in the medium was depleted. D-LA, ethanol, and glycerol production levels and residual glucose concentrations in the medium were detected for 96.1 h by HPLC. Error bars indicate standard deviations of three independent experiments.

g/L from 154.7 g/L glucose, but productivity was reduced to 1.3 g/(L·h) due to a decrease in glucose uptake rate after 50.9 h.

6.3. CK2-dependent phosphorylation of Haa1

The possibility of phosphorylation of Haa1 has been reported based on mass spectrometric results in previous studies and it was recently reported that the change of phosphorylation level of Haa1 was observed under the LA-induced stress conditions. However, it is still not examined the kinase mediated phosphorylation of Haa1 and its activation mechanism to LA stress. Because CK2 is known for the essential kinase implicated cell viability as well as various stress tolerance in yeast, I confirmed whether CK2 is involved in the phosphorylation of Haa1. By *in vitro* kinase assay using GST-Haa1 with GST-CK2, CK2-dependent phosphorylation of Haa1 was observed. When 4,5,6,7-tetrabromobenzotriazole (TBB) was treated as a CK2-specific inhibitor, Haa1 phosphorylation by CK2 was clearly abolished (Fig. 6.4). I examined the interaction of Haa1 and CK2 *in vivo*. In yeast two hybrid assay result, CK2 regulatory subunits, Ckb1 and Ckb2, showed the interaction with Haa1, but not any catalytic subunit (Fig. 6.5).

Next, to investigate growth retardation of BY4741 WT, *cka1Δ*, *cka2Δ*, and *haa1Δ* strains in the presence of LA, all strains were cultured in YPD medium containing 3.5% LA. Compared to growth of BY4741 WT, deletion of *CKA2* or *HAA1* exerted growth defect in LA stress condition, whereas *cka1Δ* strain showed a

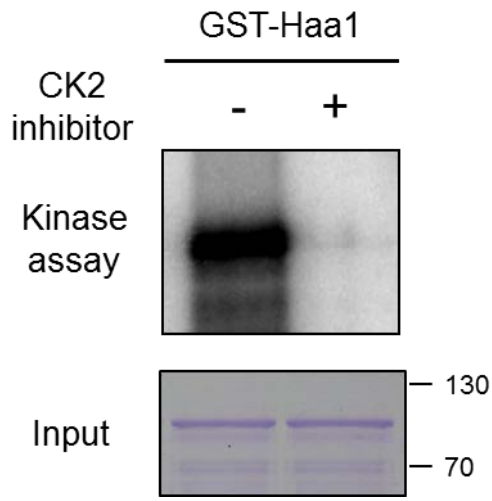


Figure 6.4 Haa1 phosphorylation by CK2 *in vitro*

In vitro kinase assay was performed using GST-CK2 and GST-Haa1 purified *S. cerevisiae* and *E.coli*, respectively. 20 μM of TBB was used for inhibition CK2 activity. The input proteins were detected by Coomasie-Blue staining.

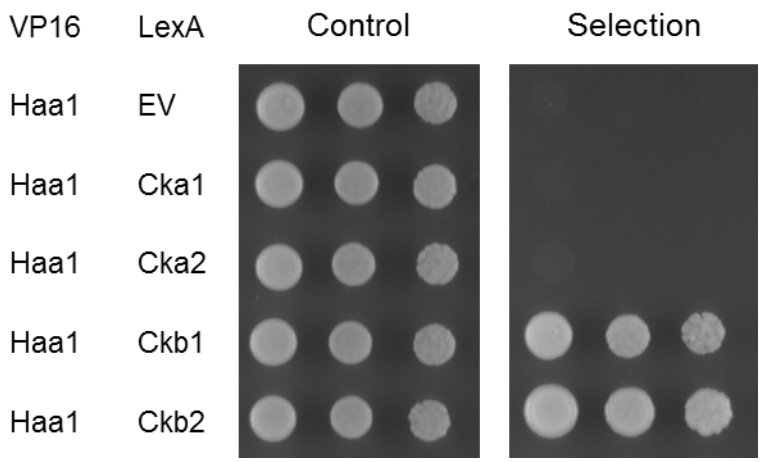


Figure 6.5 Yeast two hybrid assay by CK2 and Haa1

S. cerevisiae strain L40 cells expressing LexA-fused CK2 subunits with VP16-fused Haa1 were spotted on a control solid medium containing histidine and a selection solid medium containing 0.3 mM 3-AT but lacking histidine to detect protein-protein interactions *in vivo*.

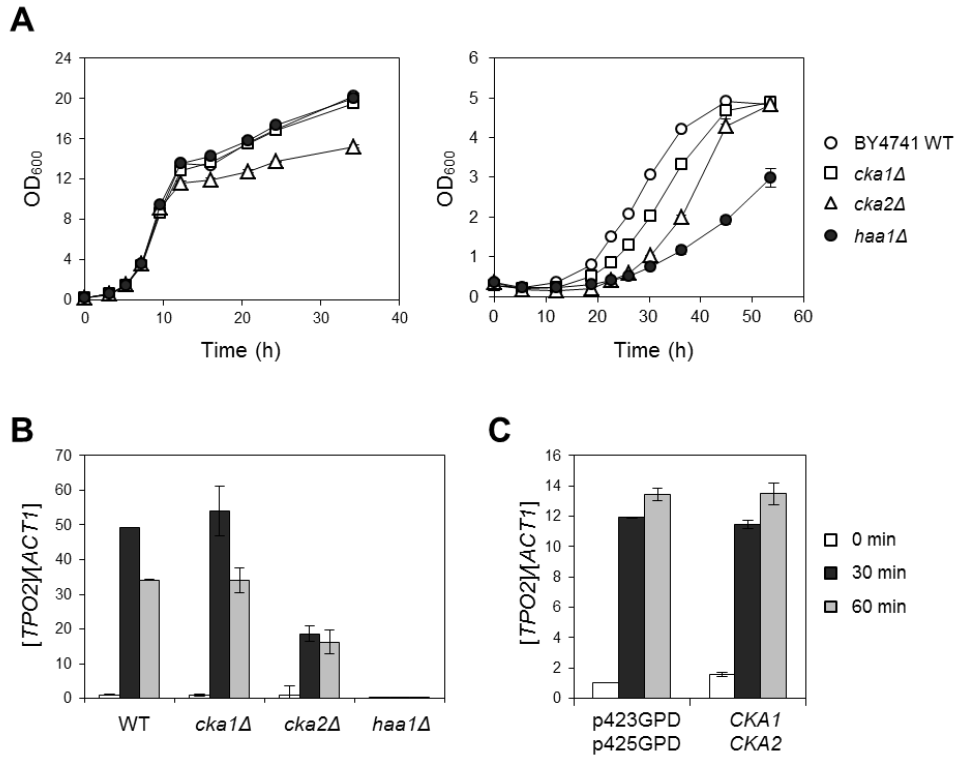


Figure 6.6 Investigation of the*CKA2* dependence of *HAA1* in LA stress conditions

A. OD_{600} of 0.2 cells were inoculated were cultured in YPD medium containing 20 g/L glucose with (right) or without 3.5% LA (left). Error bars indicate standard deviations of three independent experiments.

B. All strains were treated 2% LA for 1 h, and transcription levels of *TPO2* were determined by qRT-PCR analysis with *ACT1* as the reference gene. Error bars indicate standard deviations of three independent experiments.

C. WT cells harboring the empty vectors or plasmids overexpressing *CKA1* and *CKA2* were treated 2% LA for 1 h, and transcription levels of *TPO2* were determined by qRT-PCR normalized with *ACT1*.

slightly decrease of growth rate than BY4741 WT (Fig. 6.6A). Deletion effects of *CKA2* and *HAA1* were also investigated by quantification of transcription levels of Haa1-specific target gene, *TPO2*, from each strain. Transcription levels of *TPO2* from WT and *cka1Δ* strains showed no difference in both 30 min and 60 min upon LA stress, but *CKA2* deletion strain exhibited lower transcription level compared with WT and *cka1Δ* strains (Fig. 6.6B). Against my expectations, overexpression of catalytic subunits using high copy plasmids in BY4741 WT showed no effect to change the *TPO2* transcription levels compared with cells harboring the empty vectors in LA stress condition (Fig. 6.6C). Taken together, it was confirmed that CK2 is one of the kinase of Haa1 and especially Cka2 may be involved in Haa1 under LA stress condition.

6.4. Investigation of phosphorylation residues in Haa1 by CK2

So far, several serine residues such as Ser271 or Ser274 in Haa1 were predicted as the phosphorylation sites based on phosphor-proteomic analyses using mass spectrometry. However, the phosphorylation residue confirmed by experimental result has not been reported yet. Therefore, identification of CK2-dependent phosphorylation residues of Haa1 was conducted by using *in vitro* kinase assay. First of all, phosphorylation site was predicted from PhosphoGrid online database analysis based on the CK2 consensus sequence (SXXE/D) and total 12 candidate

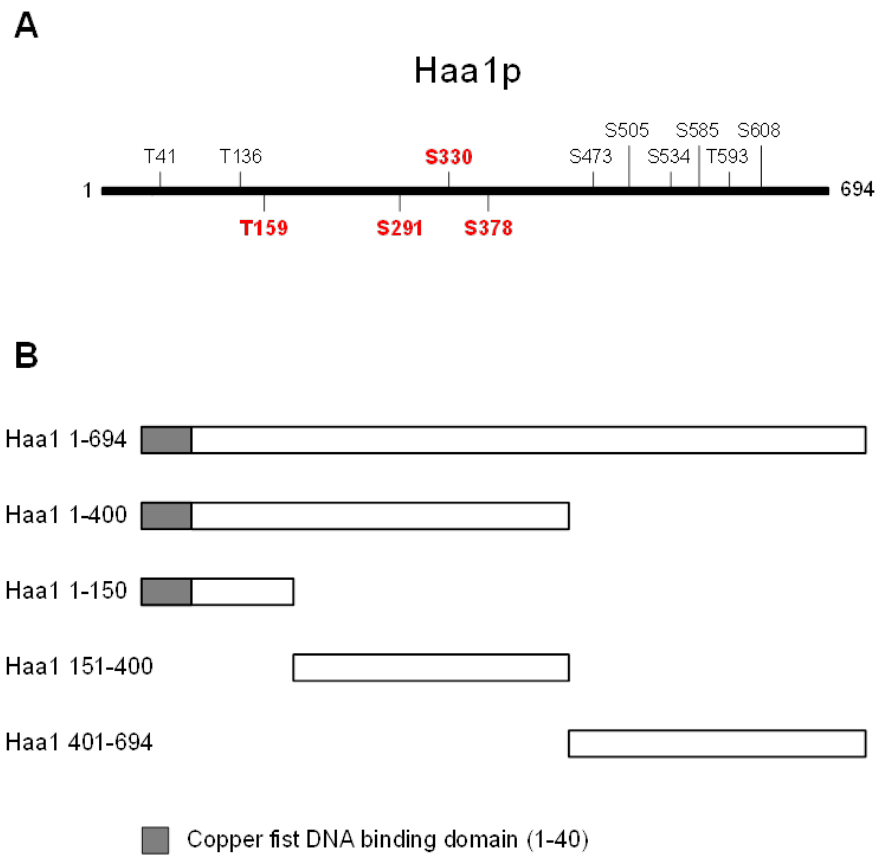


Figure 6.7 CK2-dependent phosphorylation sites in Haa1 and its derivatives used in this study

A. Mapping of CK2-dependent phosphorylation residues in Haa1 based on the prediction results. Bold letters indicate the determined residues to test phosphorylation status in this study.

B. Schematic diagram of Haa1 structure and trunacted Haa1 derivatives.

serine and threonine residues were determined (Fig. 6.7A). Next, Haa1 1-400 and Haa1 401-694 fragments were generated by truncation of Haa1 based on mapping the prediction sites in Haa1 and used as substrates for kinase assay for excluding the insignificant candidates. Since Haa1 1-400 fragment was strongly phosphorylated by CK2 compared with Haa1 401-694, Haa1^{S291A}, Haa1^{S330A}, and Haa1$^{S291/S330A}$ mutants were generated by amino acid substitution because S291 and S330 were expected as reliable phosphorylation candidates in 1-400 residues (Fig. 6.7A). GST-Haa1^{S291A} showed a significant decrease of phosphorylation level by CK2, implying that S291 might be a major CK2-dependent phosphorylation residue. However, phosphorylation levels of GST-Haa1^{S330A} and GST-Haa1$^{S291/S330A}$ were similar with Haa1 and GST-Haa1^{S291A}, respectively (Fig. 6.7B). To identify the function of S291 residue to LA stress, a strain expressing Haa1^{S291A} instead of Haa1 was constructed by substituting endogenous *HAA1* gene to *HAA1^{S291A}* gene bearing an additional copy of own terminator and qRT-PCR analysis was performed. Unfortunately, *TPO2* transcription levels of cells expressing Haa1^{S291A} exhibited no difference with Haa1^{WT}, generating as a control strain, in LA stress condition. Haa1^{S291D} strain, mimicking phosphorylation status in S291 residue, did not also showed significant change of *TPO2* transcription level (Fig. 6.9).

Although S291 was expected as a major CK2-dependent phosphorylation site, the change of transcription levels of *TPO2* followed S291 phosphorylation status was not identified. To investigate the possibility of existing another residues

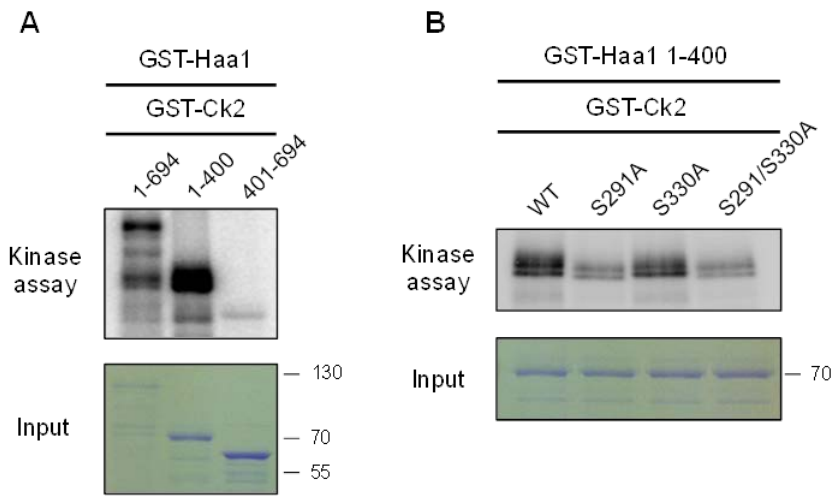


Figure 6.8 CK2-dependent phosphorylation of Haa1 mutants *in vitro*

A. *In vitro* kinase assay was performed using GST-Haa1, GST-Haa1 1-400, and GST-Haa1 401-694 with GST-CK2. The input proteins were detected by Coomasie-Blue staining.

B. *In vitro* kinase assay was performed using same method described panel A. Haa1^{S291A}, Haa1^{S330A}, and Haa1$^{S291/S330A}$ fused GST-tag were subjected to test as substrate proteins. GST-Haa1 1-400 was also used in this test as a control. The input proteins were detected by Coomasie-Blue staining.

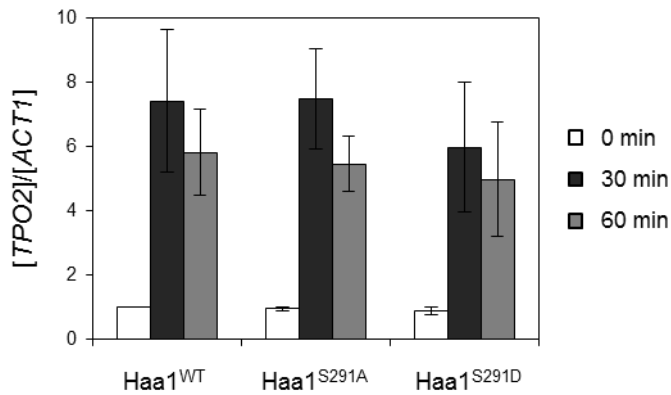


Figure 6.9 *TPO2* transcription levels of Haa1^{S291A} and Haa1^{S291D} strains under LA stress condition

Cells expressing Haa1^{WT}, Haa1^{S291A}, and Haa1^{S291D}, respectively, were cultured in YPD medium until early exponential phase, and then incubated in the presence of 2% LA contained YPD medium for 1 h. Transcription levels of *TPO2* were determined by qRT-PCR analysis with *ACT1* as the reference gene. Error bars indicate standard deviations of three independent experiments.

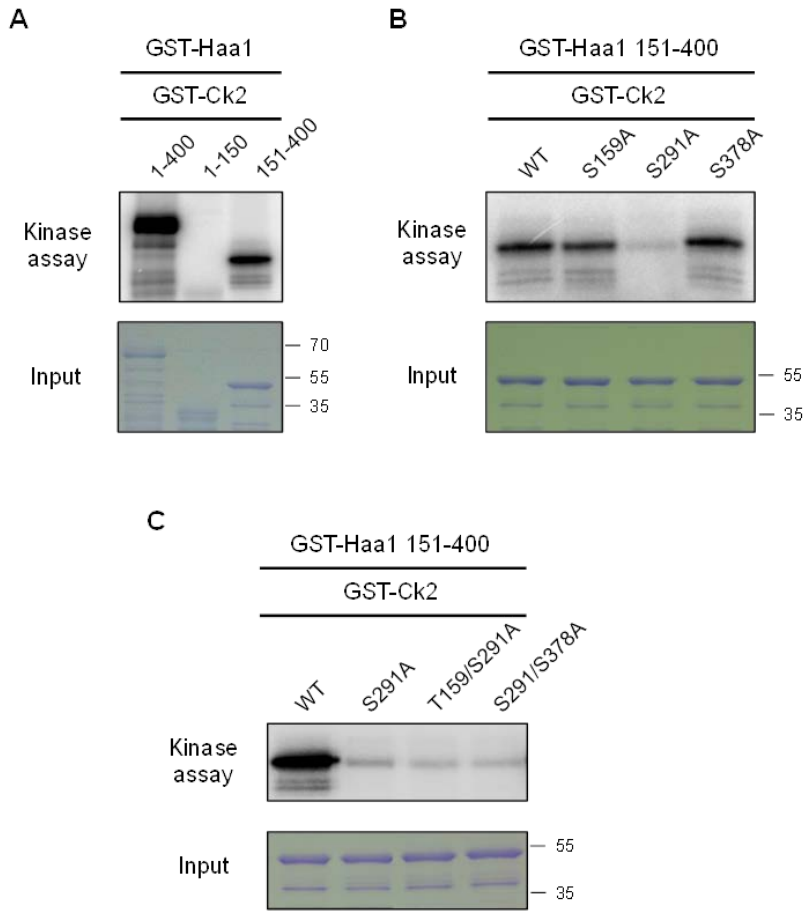


Figure 6.10 Determination of phosphorylation levels of T159A and S378A in combination with S291A of Haa1 151-400 derivative

A. *In vitro* kinase assay was performed using GST-Haa1 1-150 and GST-Haa1 151-400 proteins purified *E. coli* with GST-CK2. Input proteins were detected by Coomasie-Blue staining.

B. Single amino acid substituted mutants (Haa1^{T159A}, Haa1^{S291A}, and Haa1^{S378A}) from Haa1 151-400 were used as substrates for CK2-dependent phosphorylation sites by *in vitro* kinase assay.

C. GST-Haa1$^{T159/S291A}$ and GST-Haa1$^{S291/S378A}$ were purified and used to determine the difference of phosphorylation levels by *in vitro* kinase assay.

phosphorylated by CK2 except S291, *in vitro* kinase assay performed with Haa1 1-150 and Haa1 151-400 as the substrates (Fig. 6.10A). Thereafter, *in vitro* kinase assays were also carried out using GST fused Haa1^{T159A} and Haa1^{S378A} or GST-Haa1 $^{T159/S291A}$ and GST-Haa1 $^{S291/S378A}$ generated by Haa1 151-400, respectively. Unfortunately, all mutants showed ambiguous changes of phosphorylation level compared with GST-Haa1^{S291A} 151-400 because the extent of phosphorylation was substantially decreased only alanine substitution of S291 (Fig. 6.10B and C). Therefore, a mutant strain possessing *HAA1*$^{T159/S291/S378A}$ gene instead of *HAA1* gene was constructed and used for the test identifying the difference of *TPO2* transcription levels compared with *HAA1*^{WT} strain under 2% LA stress condition. As shown in Fig. 6.11, *TPO2* transcription level of mutant strain showed no significant difference compared to that of *HAA1*^{WT} strain. Taken together, the function of CK2-dependent phosphorylation under LA stress condition is still incompletely understood.

6.5. Conclusions

In this chapter, it was demonstrated that overexpression of *HAA1* could further improve LA production when introduced into the evolved strain JHY5320. Although initial glucose consumption rate was lower than that of JHY5320, the *HAA1*-overexpressing strain JHY5330 showed improved glucose consumption at the later stage of fermentation, resulting in higher D-LA production levels under

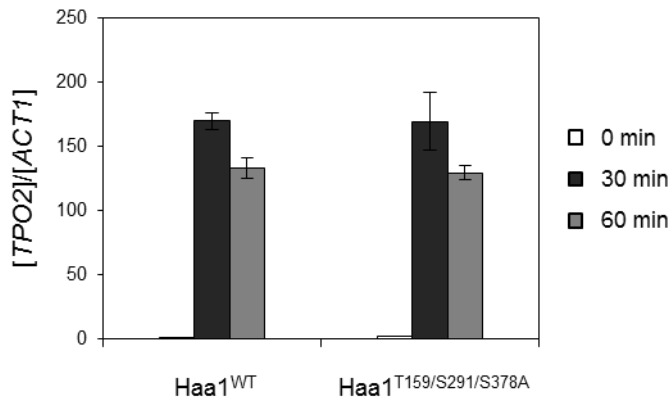


Figure 6.11 Investigation of difference of *TPO2* transcription levels between Haa1^{WT} and Haa1$^{T159/S291/S378A}$ strains

Cells expressing Haa1^{WT} or Haa1$^{T159/S291/S378A}$ were treated 2% LA for 1 h. Transcription levels of *TPO2* were determined by qRT-PCR with *ACT1* as the reference gene. Error bars indicate standard deviations of three independent experiments.

acidic fermentation condition without pH control. As mentioned above, it is reported that Haa1 can be activated by undissociated LA, not the dissociated proton from LA. Therefore, these advanced properties might be also involved in undissociated LA in culture medium. On the other hand, under neutralizing fed-batch fermentation conditions, 112.0 g/L of D-LA was produced with a yield of 0.80 g/g glucose and a productivity of 2.2 g/(L·h). Compared with a previous study producing D-LA in *S. cerevisiae*, this strain showed superior performance especially in terms of yield. This also showed D-LA titer, yield, and productivity comparable to those of previously developed *S. cerevisiae* strains producing L-LA. These results suggest that overexpression of *HAA1* might provide a selective advantage under the conditions of medium acidification caused by LA accumulation.

To further understand Haa1 response to LA stress, I searched the kinase involved in Haa1 phosphorylation and it was confirmed that CK2 phosphorylated Haa1 *in vitro* and interacted with regulatory subunits *in vivo*. Especially, Haa1 activity was dependent on Cka2 catalytic subunit when LA stress was induced. Based on the results from phosphorylation sites prediction, I constructed truncated Haa1 fragments and amino acid substituted Haa1 derivatives and used to investigate CK2-dependent phosphorylation site. From kinase assay results, S291 was considered as a major phosphorylation residue by CK2, unfortunately, functions of Haa1 phosphorylation by CK2 under LA stress condition was not demonstrated yet. Therefore, further investigation should be required to find the

CK2-dependent phosphorylation residue and elucidate the function of CK2 kinase into activation of Haa1.

Chapter 7.

Overall discussion and recommendations

In this dissertation, an efficient D-LA-producing *S. cerevisiae* strain was developed by using metabolic engineering strategies, including rational pathway manipulations for reduction of byproducts formation and adaptive laboratory evoluation with Haa1 overexpression for improving LA production level and acid tolerance. To enhance the understanding of Haa1 response mechanism under LA stress condition, CK2-dependent phosphorylation of Haa1 was investigated.

In the first part, *L. mesenteroides* subsp. *mesenteroides* ATCC 8293 (*Lm.ldhA*) *ldhA* gene was selected and genes implicated D-LA consumption (*JEN1* and *DLD1*) and byproducts production including ethanol (*ADH1*) and glycerol (*GPD1* and *GPD2*) were sequencially disrupted for the purpose of improving optically pure D-LA production level in *S. cerevisiae*. The resulting strain (JHY5210) showed a significant improvement of the D-LA production yield accompanying no consumption with no glycerol accumulation. Glycerol is one of the impurities to purify LA from fermenation broth, therefore, elimination of glycerol formation can be an attrative feature for commercial LA production.

In the second part, the LA-tolerant evolved strain was developed by adaptive laboratory evolution with additional integration of *Lm.ldhA* gene. The resulting strain JHY5320 showed increase in D-LA production level and yield with alleviating growth retardation. Although *S. cerevisiae* inherently has high stress tolerance, it has been reported that elimination of glycerol formation leads to a reduction in growth rate and stress tolerance towards heat, osmotic, and oxidative stresses. Therefore, LA resistance is still a limiting factor to further improve D-LA

production. Through whole genome sequencing analysis, it was confirmed that deletion of several genes is involved in improving LA tolerance in the evovled strain. In particular, deletion of *GSF2* conferred an increase of growth rate and glucose consumption ability in not only unevolved LA-producing strain, but also other engineered strains possessing growth defects by reducing ethanol formation. These results suggest that *GSF2* gene manipulation can be a promising strategy for various pyruvate-derived chemical production by overcoming growth defect of engineered yeast strain. Although the inspiring results were obtained, future works should be focused on elucidating the function and mechanism of *GSF2* deletion for constructing more robust and efficient LA-producing strain of *S. cerevisiae*.

In the third part, overexpression of Haa1 was introduced into the evolved strain JHY5320 to further improve D-LA production. Advancing LA tolerance has been already reported, but its effect on LA production has not yet been demonstrated. This manipulation led to upregulation of Haa1 target genes expressions and improving LA production level under acidic fermentation condition. Therefore, *HAA1* overexpression might be also an attractive approach for improving LA fermentation ability in *S. cerevisiae*. To elucidate Haa1 response mechanism to LA stress, I confirmed the CK2-dependent phosphorylation of Haa1 with physical interactionHaa1 and CK2. As a result, I suppose that CK2, especially Cka2 subunit, might be involved in Haa1 response mechansim to LA stress, but the function of CK2-dependent phosphorylation should be demonstrated by further studies.

By introducing of rational metabolic engineering with adaptive evolution strategy, an efficient LA-producing *S. cerevisiae* strain was developed. This resulting strain showed the advanced D-LA titer, yield, and productivity with acid tolerance. Optimization of various fermentation conditions such as fermentor operation controlling pH level under pKa of LA, inoculating cell density, and aeration rate may be useful approaches to further improve the fermentation performance to produce D-LA. Further elucidating of LA tolerance and Haa1 response mechanism in *S. cerevisiae* should be also necessary for practical application in industrial process.

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Appendix

Cellulosic ethanol production by combination of cellulase-displaying yeast cells

A.1 Introduction

Cellulosic biomass is considered as one of the most promising alternatives to fossil fuels for the production of biofuels and other useful chemicals (Fortman et al. 2008). Hydrolysis of crystalline cellulose to glucose requires the sequential reactions of three groups of cellulases, endoglucanase (EG), exoglucanase (mostly cellobiohydrolase, CBH), and β-glucosidase (BGL), which are produced in cellulolytic fungi and bacteria (Demain et al. 2005; Foreman et al. 2003). EG randomly cleaves internal β-1,4-glycosidic bonds in the amorphous regions of crystalline cellulose. From the reaction products of EG, CBH produces cellodextrins such as cellobiose and cellotriose, which are finally hydrolyzed to glucose by BGL (Kumar et al. 2008).

The high cost of cellulase is the main obstacle for the utilization of cellulosic biomass. Therefore, a great deal of effort has been made to screen and develop cellulases with high enzymatic activity and stability (Li et al. 2009; Percival Zhang et al. 2006). On the other hand, consolidated bioprocessing (CBP), combining cellulase production, cellulose degradation, and fermentation in a single step, is considered as the most cost-effective way to utilize cellulosic biomass (Lynd et al. 2005; Wilson 2009). One of the key requirements for cellulosic ethanol production by CBP is to develop microbial strains equipped with the properties of efficient cellulose degradation as well as ethanol production. *Saccharomyces cerevisiae*, having a high capacity of ethanol production, is one of the strong candidates for

CBP. So far, to develop cellulolytic yeast strains, various fungal or bacterial cellulases have been expressed in yeast as secreted forms (Jeon et al. 2009; van Wyk et al. 2010)or displayed on the yeast surface (Fujita et al. 2004; Ito et al. 2009; Murai et al. 1998; Yamada et al. 2010; Yanase et al. 2010). Especially, the surface display of cellulases enables to mimic cellulosome structure found on the surface of anaerobic cellulolytic bacteria such as *Clostridium thermocellum*, *Clostridium cellulovorans*, and *Ruminococcus flavefaciens* (Hyeon et al. 2010; Tsai et al. 2010). Cellulosome is generated by assembly of multiple dockerin-containing cellulases and xylanases into a scaffoldin, an integrating protein containing cohesion domains, through interaction between dockerin and cohesion domains (Fontes and Gilbert 2010). Such a multifunctional cellulosome structure allows highly efficient cellulose degradation through synergistic effects of various enzymes. To date, several fungal cellulases such as *Trichoderma reesei* EGII and CBHII, and *Aspergillus aculeatus* EGI and BGLI were expressed on the yeast surface (Fujita et al. 2004; Murai et al. 1998; Wen et al. 2010). In addition, some bacterial cellulases such as *C. thermocellum* CelA and BglA, and *Clostridium cellulolyticum* CelE and CelG were purified from *Escherichia coli* or secreted from yeast cells, and then attached to a chimeric scaffoldin expressed on the yeast surface (Tsai et al. 2010; Tsai et al. 2009).

To generate efficient cellulolytic yeast strains, it is important to select cellulases based on their enzymatic properties when displayed on the yeast surface. However, comparative analysis of various cellulases expressed on the yeast surface

has not yet been conducted. Among the three types of cellulases, EG shows the highest diversity, which might be necessary to degrade various types of cellulases in nature. Therefore, as an effort to search for enzymes suitable to develop cellulolytic yeast strains, we compared the activities of fungal and bacterial EGs expressed on the yeast surface. For fungal EGs, we selected *T. reesei* EGII, which has been widely studied on account of its high enzymatic activity, and EGI from a thermophilic fungus, *Thermoascus aurantiacus*. For bacterial EGs, we selected CelA and CelD from *C. thermocellum*, anaerobic and thermophilic bacteria. Except for EGII, other EGs have not yet been directly expressed on the yeast surface. Based on the characterized enzymatic properties, we chose *T. aurantiacus* EGI for ethanol production from PASC in combination with *T. reesei* CBHII and *A. aculeatus* BGLI. Previously, multiple cellulases were co-displayed in a single cell for ethanol production (Fujita et al. 2004; Yamada et al. 2010). However, in this study, we adapted a new strategy of combining three types of yeast cells each displaying different cellulase, which allows convenient optimization of ethanol production by adjusting the combination ratio of each cell type.

A.2 Materials and methods

A.2.1 Strains and culture conditions

Saccharomyces cerevisiae EBY100 strain (*MATa GAL1-AGA1::URA3 ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS2 prb1Δ1.6R can1* GAL) containing pCTCON

plasmid were grown in a selective SD-CAA medium (20 g/L dextrose, 6.7g/L yeast nitrogen base, 5 g/L casamino acids, 5.4 g/L Na_2HPO_4 and 8.56 g/L $NaH_2PO_4 \cdot H_2O$). For the induction of cellulase gene cloned in pCTCON vector, OD_{600} of 1 cells grown in SD-CAA medium were harvested and resuspended in SG-CAA medium containing galactose instead of glucose, and further incubated for 18 h at 30°C.

A.2.2 Plasmids

The yeast surface display vector pCTCON was kindly provided by Dr. Y. -S. Kim (Ajou University, Korea) and *T. reesei* EGII, CBHII and *A. aculeatus* BGLI cDNAs were provided by Dr. H. Zhao (University of Illinois at Urbana-Champaign, USA). For the surface display, *T. reesei* EGII and CBHII genes lacking a signal sequence were amplified by PCR, and then cloned into the NheI and BamHI sites of pCTCON vector. The surface display vectors for CelA, CelD, EGI, and BGLI were generated by homologous recombination in yeast cells. *C. thermocellum* CelA and CelD genes lacking signal sequences were amplified from genomic DNA (ATCC 27405) and EGI and BGLI genes were amplified from each cDNA by using PCR primers, each containing 35 nt or 37 nt region homologous to the pCTCON vector. Each PCR product was transformed into *S. cerevisiae* EBY100 together with the pCTCON vector digested with NheI and BamHI, leading to an insertion of the PCR product into a pCTCON vector by homologous recombination. The plasmid was isolated from the transformant and confirmed by DNA sequencing. The primer sequences used for each EG are shown in Table A.1.

Table A.1 Primer sequences of cellulases used in this study

Primers	Sequence (5'-3')
CelA: F	*GTGGTGGTGGTTCTGGTGGTGGTGGTTCT***GCTAGC**GCAGGTG TGCCTTTTAACACA
CelA: R	*AAGTCCTCTTCAGAAATAAGCTTTTGTTC***GGATCC**ATAAGGT AGGTGGGGTATGCT
CelD: F	*GTGGTGGTGGTTCTGGTGGTGGTGGTTCT***GCTAGC**GCAAAAA TAACGGAGAATTAT
CelD: R	*AAGTCCTCTTCAGAAATAAGCTTTTGTTC***GGATCC**TATTGGT AATTTCTCGATTAC
EGI: F	*TGGTGGTGGTGGTTCTGGTGGTGGTGGTTCT***GCTAGC**ATGAA GCTCGGCTCTCTCGT
EGI: R	*ACAAGTCCTCTTCAGAAATAAGCTTTTGTTC***GGATCC**AAGAT ACGGAGTCAAAATAG
EGII: F	CCG**GCTAGC**CAGCAGACTGTCTGGGGC
EGII: R	AAT**GGATCC**CTTTCTTGCGAGACACGA
CBHII: F	CCG**GCTAGC**CAAGCTTGCTCAAGCGTC
CBHII: R	AAT**GGATCC**CAGGAACGATGGGTTTGC
BGLI: F	*GTGGTGGTGGTTCTGGTGGTGGTGGTTCT***GCTAGC**GATGAAC TGGCGTTCTCTCCT
BGLI: R	*AAGTCCTCTTCAGAAATAAGCTTTTGTTC***GGATCC**TTGCACC TTCGGGAGCGCCGC

Restriction enzyme sites are shown in bold and the sequences homologous to pCTCON vector are shown in italic. (F, forward primer; R, reverse primer).

A.2.3 Immunofluorescence microscopy

Yeast cells expressing cellulases were washed with wash buffer (PBS and 0.1% BSA) and incubated with anti-HA mouse monoclonal antibody (Roche) in PBSF buffer at room temperature. The cells were washed twice, and then incubated with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC, Sigma-Aldrich) for 10 minutes at 4°C. After washing the cells, cellular localizations of the cellulases were observed by fluorescent microscope (Eclipse 80i, Nikon Instruments). The whole cell fluorescence intensity was measured by fluorescence microplate reader (AT/Genios Pro, TEKAN) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

A.2.4 Endoglucanase activity assay

OD_{600} of 10 cells harboring the surface-display plasmid for EG were grown in SG-CAA medium, harvested, and washed with distilled water and then with a reaction buffer with various pH values. 50 mM citrate/sodium citrate buffer was used for the pH range of 3 to 6, and 50 mM Tris-HCl buffer was used for pH 7 to 9. Cells were resuspended in a reaction buffer containing 10 g/L carboxymethyl cellulose (CMC, Sigma-Aldrich), or 5 g/L phosphoric acid swollen cellulose (PASC) generated from Avicel PH-101 (Fluka), and then incubated for 6 h at various temperatures, from 30 to 80°C. The amount of reducing sugar in the reaction supernatant was determined by dinitrosalicylic acid (DNS) method as described

elsewhere (Nevoigt and Stahl 1997). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar from substrate per min.

A.2.5 Ethanol Fermentation from PASC

Yeast cells, each harboring the surface-display plasmid for EGI, CBHII, or BGLI, were grown in SD-CAA medium and then transferred to SG-CAA medium for 48 h to express cellulase. OD_{600} of 50 of cells were washed with distilled water and cells expressing different cellulases were mixed in various ratios. A mixture of cells was incubated in 10 ml of YP medium (20 g/L peptone and 10 g/L yeast extract) for 1 h to remove residual carbon source, and then resuspended in YP-PASC medium (YP medium containing 10 g/L PASC as the sole carbon source). Ethanol fermentation was carried out at 30°C in anaerobic condition. Cell mixture was incubated in a 30 mL serum bottle with 10 mL reaction volume. Ethanol concentration was measured by high performance liquid chromatography (HPLC) (Finnigan Surveyor Plus, thermo scientific, USA). HPLC was operated using a BioRad Aminex HPX-87H column (300 mm x 7.8 mm, 5 μm) at 60°C with 5 mM H_2SO_4 as eluant at a flow rate of 0.8 mL/min. Refractive index (RI) detector (Finnigan Surveyor RI Plus detector, thermo scientific, USA) was used to determine the ethanol quantification.

A.3 Results

A.3.1 Expression of cellulases on the yeast surface

Two bacterial endoglucanases (EGs), *C. thermocellum* CelA and CelD, and two fungal EGs, *T. aurantiacus* EGI and *T. reesei* EGII, were expressed under the control of Gal1 promoter as Aga2-fusion proteins (Fig. A.1). Aga2-fused EGs were displayed on the surface of *S. cerevisiae* EBY100 through a pair of disulfide bond between Aga2 and Aga1 (Ueda and Tanaka 2000), a yeast cell wall protein. CelA and CelD contain dockerin domains involved in the generation of cellulosome complex. The catalytic domains of CelA and CelD are classified as GH8 and GH9 glycoside hydrolase (GH) family, respectively. Both EGI and EGII have GH5 modules, and EGII has an additional carbohydrate binding module (CBM), promoting substrate binding. We also expressed *T. reesei* CBHII (exoglucanase) and *A. aculeatus* BGLI (β-glucosidase) on the yeast surface to be used for ethanol production.

After induction in a galactose medium for 18 h, the expression of cellulases was evaluated by western blotting (data not shown). Because of the fusion partners, each cellulase exhibited a molecular weight of about 30 kDa higher than the predicted size. We also confirmed the expression of cellulases on the yeast surface by immunofluorescent microscopy. As shown in Fig. A.2, all cellulases were successfully displayed on the yeast surface.

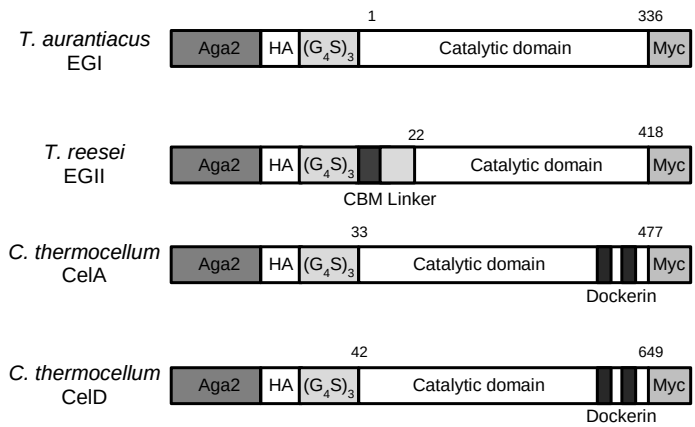


Figure A.1. Schematic diagram of endoglucanase structures for the yeast surface display

Each gene was cloned into pCTCON vector after deletion of a signal sequence except for *T. aurantiacus* EGI. The numbers indicate amino acid residues of each protein. Each EG is fused with Aga2 for linking to the yeast surface by a pair of disulfide bond, and contains an HA (N-terminal) tag, a Myc (C-terminal) tag, and a $(G4S)_3$ linker domain. *C. thermocellum* CelA and CelD have a pair of dockerin (CelA, 417-440, 449-472; CelD, 585-608, 621~644), and *T. reesei* EGII contains a carbohydrate binding module (CBM) and a linker (CBM, 22-57; linker, 58-91).

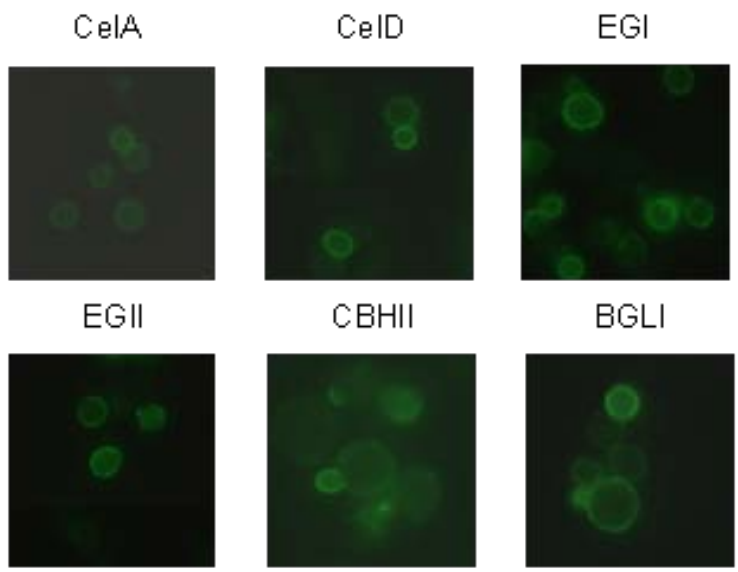


Figure A.2. Immunofluorescence microscopy of cellulases displayed on the yeast surface

Expression of cellulases in yeast EBY100 strain was induced in galactose media for 18 h. Cellulases were labeled with mouse anti-HA antibody and goat anti-mouse IgG conjugated with FITC, and their cellular localizations were detected by fluorescence microscope. Each image was rescaled the brightness and contrast. The brightness and contrast of all images except BGLI were increased 10% and 20%, respectively.

A.3.2 Characterization of the enzyme activities EGs displayed on the yeast surface

A3.2.1 Effect of pH on the activities of the surface-displayed EGs

In order to measure the EG activity displayed on the yeast surface, the same number of cells (OD_{600}=10) were harvested after galactose induction, and then enzyme assay was carried out by using carboxymethyl cellulose (CMC) as a substrate. To evaluate the effect of pH on each EG, activity assay was performed at 37°C and over a broad range of pH (Fig. A.3A). Among the four EGs, *T. reesei* EGII showed the highest activity in overall pH range except for pH 9. *T. aurantiacus* EGI showed the second highest activity up to pH 6. In general, the two fungal EGs showed highest activities at pH 3 and 4, but their activities decreased rapidly at higher pH.

On the other hand, *C. thermocellum* CelA and CelD were almost inactive at pH3, but showed the highest activities at pH 4. In addition, although CelA and CelD also showed pH-dependent inactivation over a pH range from 4 to 9, their inactivation was less sensitive to the increase in pH compared with EGI and EGII.

A3.2.2 Effect of temperature on the activities of the surface-displayed EGs

We also examined the optimal temperature of each EG displayed on the yeast surface. All the EGs showed increase in activity upon increase in temperature from

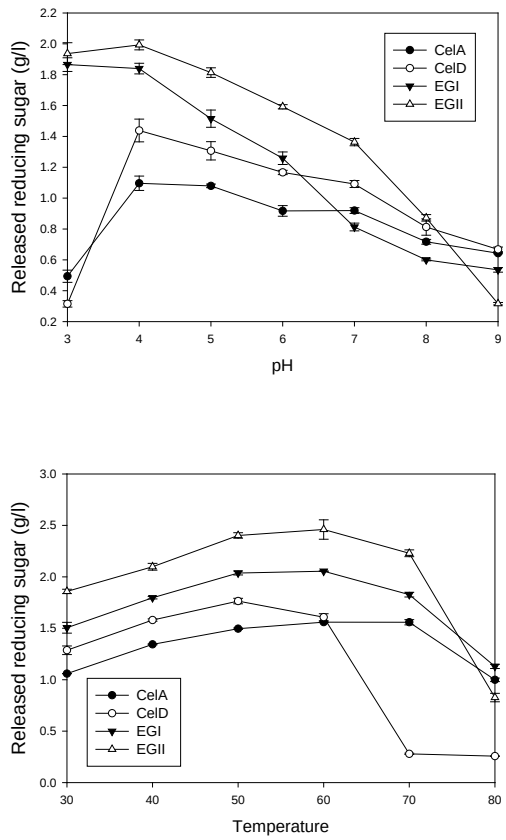


Figure A.3. Effect of pH and temperature on the activities of the surface-displayed EGs

A. Activity of the surface-displayed EG was determined by using CMC as a substrate. After 6 h incubation of the EG-displaying yeast cells at 37 °C in a buffer with the indicated pH, the released reducing sugars were measured by DNS method.

B. Activity of the surface-displayed EG was determined at pH 5.2 and the indicated temperatures using CMC as a substrate. The experiments were performed independently at three times.

30 to 50°C (Fig. A.3B). CelA showed the highest temperature optimum of 70°C, while CelD activity was maximized at 50°C and dropped rapidly after reaching 60°C. The temperature optimums for EGI and EGII were about 60°C, similar to the previously reported temperature optimums measured using purified enzymes (Adachi et al. 1998; Dato et al. 2014), and their activities were still maintained up until 70°C.

A3.2.3 Specific activities of EGs displayed on the yeast surface

Since the surface expression level of each enzyme can be different, the activity we measured by using the same amount of cells does not necessarily reflect the specific activity of each enzyme. Therefore, to normalize the enzyme activity to its protein level, protein expression levels on the surface were measured by detecting whole cell fluorescent intensities after treating the cells with mouse anti-HA antibody and FITC-conjugated anti-mouse IgG antibody. The fluorescence intensity was highest in cells expressing EGI (100%), followed by the cells expressing EGII (96%), CelA (90%), and CelD (71%). When the EG activities were normalized to their expression levels, CelD exhibited a comparable activity to EGII in overall pH range (Fig. A.4A), and in the temperature range from 30 to 50°C (Fig. A.4B). Therefore, CelD could be as active as EGII if expressed at equal levels.

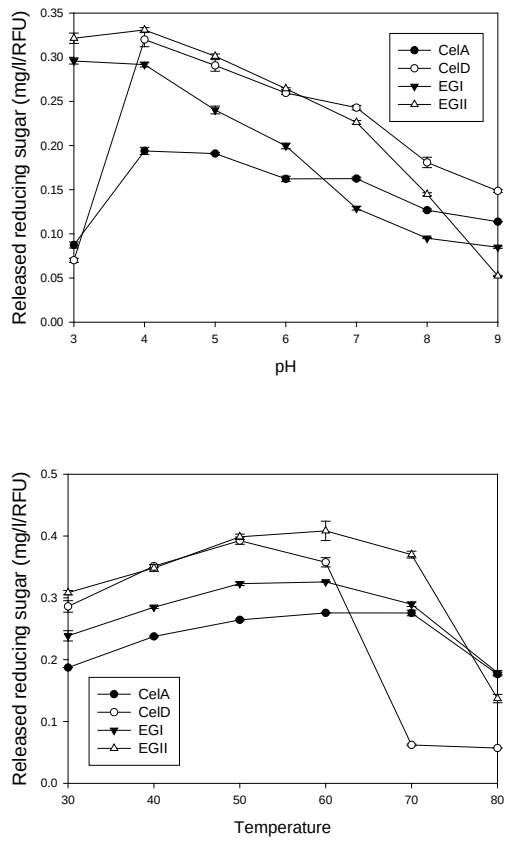


Figure A.4. Specific activity of EGs normalized to the protein expression levels on the yeast surface

A. The enzyme activities shown in Figure 3A were normalized to the whole cell fluorescent intensities of the corresponding EGs, which were measured by fluorescence microplate reader after labeling the cells with mouse anti-HA antibody and FITC-conjugated anti-mouse IgG antibody.

B. The enzyme activities shown in Fig. A.3B were normalized to the whole cell fluorescent intensities of the corresponding EGs.

A.3.3 Substrate specificity of the surface-displayed EGs

Next, we examined the substrate specificity of the four EGs displayed on the yeast surface by using two types of cellulosic materials, CMC and PASC. Activity assays were performed under two different conditions, the optimal CMC hydrolysis condition (pH 4, 50°C) (Fig. A.5A) as determined above and general yeast culture condition (pH 6, 30°C) (Fig. A.5B). EGI and EGII could degrade both CMC and PASC under both conditions. Although EGII showed higher CMC hydrolysis activity than EGI, EGI showed better PASC hydrolysis activity than EGII.

On the other hand, PASC served as a poor substrate for bacterial EGs. Although CelA and CelD showed efficient CMC hydrolysis activity, CelD did not show any PASC hydrolysis activity, and CelA hydrolyzed PASC only under the condition of pH 4 and 50°C (Fig. A.5A and B). These results indicate that each EG has a unique substrate specificity, which might be affected by reaction conditions.

A.3.4 Cellulosic ethanol production by combination of cells displaying different types of cellulases

Since *T. aurantiacus* EGI seems to have enzyme activity comparable to the widely used *T. reesei* EGII, and showed better activity toward PASC, we explored the possibility of ethanol production from PASC by using EGI. For this purpose, we also expressed *T. reesei* CBHII (exoglucanase) and *A. aculeatus* BGLI (β-

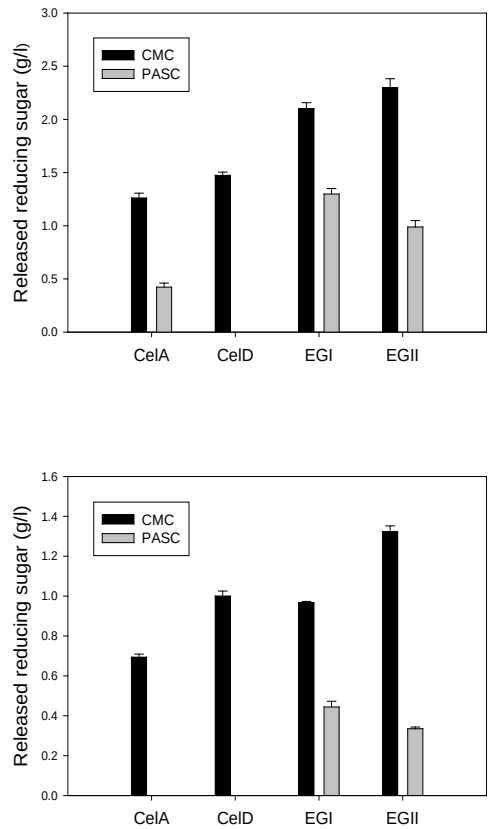


Figure A.5. Substrate specificity of the surface-displayed EGs

Enzyme activity was determined by using 10g/L CMC and 5 g/L PASC at pH 4 and 50°C (**A)**, and at pH 6 and 30°C (**B**).

glucosidase) on the yeast surface (Fig. A.2B), and investigated the interplay of EGI with CBHII and BGL in the hydrolysis of PASC.

In previous studies, multiple cellulases were co-displayed in a single cell for ethanol production (Eberhardt et al. 1999; Tokuhiro et al. 2008; Yamada et al. 2010). In that case, protein expression levels can be limited because of the metabolic burden. Furthermore, it is difficult to control the expression levels of each cellulase for optimal ethanol production. Therefore, instead of co-displaying the cellulases, we displayed only one type of cellulase in one cell, and then mixed the cells displaying different cellulases to induce a synergy in cellulose degradation. To confirm the cooperative actions of the three types of cellulases, we mixed an equal amount of cells, each displaying a different cellulase, and measured the released reducing sugars from PASC. Although CBHII itself showed little PASC hydrolysis activity, CBHII greatly enhanced reducing sugar production in combination with EGI (EGI + CBHII), indicating that CBHII can hydrolyze the degradation products generated by EGI (Fig. A.6). However, the reducing sugars generated by EGI and CBHII were almost completely abolished by the addition of cells expressing BGLI (EGI + CBHII + BGLI) (Fig. A.6). This result suggests that BGL1 converts the reducing sugar to glucose which can be immediately consumed by yeast cells. Taken together, PASC can be successfully degraded into glucose by combining cells, each displaying EGI, CBHII, and BGLI.

Next, we produced ethanol from PASC by using this cell combination system. First, we optimized the EGI:CBHII ratio for efficient generation of reducing sugars.

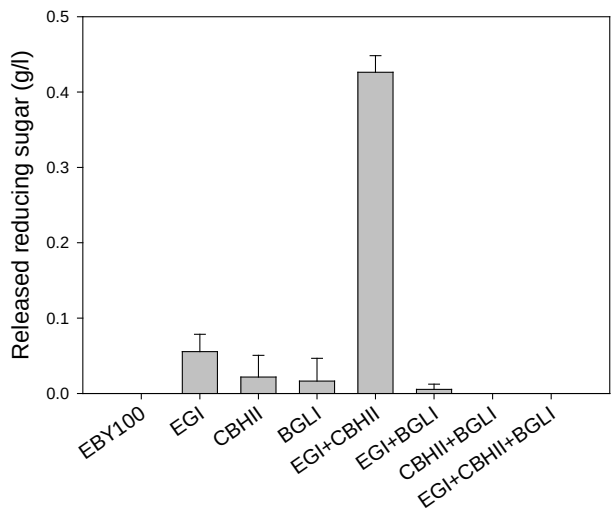
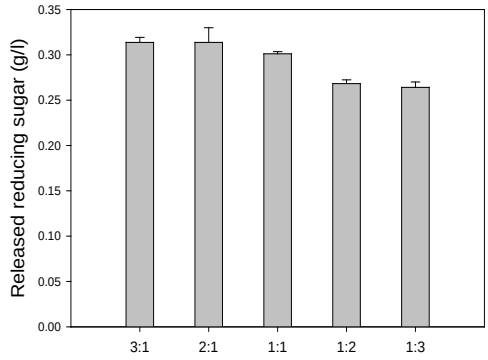
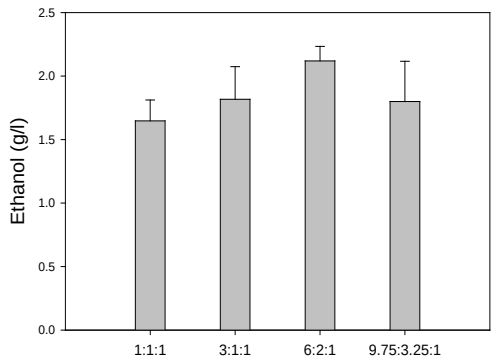


Figure A.6. Synergistic hydrolysis of PASC by combination of the surface-displayed cellulases

Equal number of cells, each displaying EGI, CBHII, and BGLI were mixed to OD_{600} of 5 as the indicated combinations, and EBY100 host cells were added to adjust the cell density. Reducing sugars generated from the hydrolysis of 5 g/L PASC was measured at pH 4.7 and 30 °C.


Released reducing sugar (g/l)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
3:1
2:1
1:1
1:2
1:3


Ethanol (g/l)
2.5
2.0
1.5
1.0
0.5
0.0
1:1:1
3:1:1
6:2:1
9.75:3.25:1

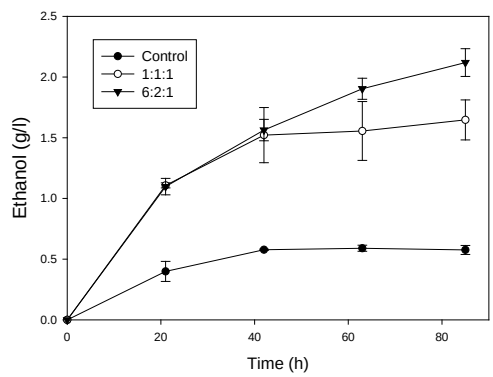

Ethanol (g/l)
Control
1:1:1
6:2:1
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
Time (h)

Figure A.7. Optimization of the cellulase combination ratio and ethanol production from PASC

A. Optimization of EGI:CBHII ratio for PASC hydrolysis.

B. Optimization of EGI:CBHII:BGLI ratio for ethanol production from PASC. Cell mixtures composed of the indicated ratios of EGI, CBHII and BGLI were incubated with 10g/L PASC and ethanol production was detected after 85 h.

C. Time course of ethanol production from negative control (closed circle), EGI:CBHII:BGLI ratio of 1:1:1 (open circle), and the optimized 6:2:1 ratio (closed triangle).

Cells displaying EGI and CBHII were mixed in various ratios and the released reducing sugars from PASC were measured. As shown in Fig. A.7A, EGI:CBHII in a ratio of 3:1 or 2:1 produced the highest amounts of reducing sugars compared with other mixing ratios. Therefore, we fixed the EGI:CBHII ratio to 3:1, and then added cells displaying BGLI with various ratios to find an optimal condition for ethanol production. A mixture of cells with EGI:CBHII:BGLI ratio of 6:2:1 produced the highest amount of ethanol (2.12 g/L) after 85 h incubation with 10 g/L PASC (Fig. A.7B and C). A mixture of cells composed of an equal amount of each cell type produced 1.65 g/L ethanol after 85 h, indicating about 1.3-fold improvement of ethanol production by optimizing the cell ratio (Fig. A.7B and C).

A.4 Discussion

In this study, we developed an ethanol production system in which yeast cells displaying different types of cellulases are combined in an optimized ratio. We first selected an EG suitable for the yeast surface display based on the characterization of the surface-immobilized enzyme activities, rather than those of free enzymes. We analyzed enzymatic properties of four fungal and bacterial EGs expressed on the yeast surface. The pH optimums of all the tested EGs attached on the yeast surface were around pH 4. For the surface-displayed EGII, its pH-dependent changes in the enzyme activity are very similar to those reported for native EGII or recombinant EGII purified after secretion from *S. cerevisiae* (Adachi et al. 1998). Therefore, attachment of this enzyme on the yeast surface might not induce large

changes in its overall pH-dependent activities. The previously reported pH optimums for CelA and CelD purified from *E. coli* are in the range of pH 5.5 to 6.5 (Schwarz et al. 1986; Sikorski and Hieter 1989), which are higher than the pH optimums observed for the surface-displayed enzymes in our study. The substrate specificities of CelA and CelD are also different compared previous researches. CelA and CelD purified from *E. coli* showed the enzymatic function about CMC and PASC in pH 4 (Lee et al. 2011; Schwarz et al. 1986). And CelA expressed in yeast was confirmed to be functional about two different substrates (Eberhardt et al. 1999). However, there was no PASC degradation activity at pH 6 both CelA and CelD. It is not clear yet whether these differences are caused by enzyme immobilization or by using a different host for expression. It might be possible that the glycosylation mechanism influences the enzyme activity. In a recent research, CelA expressed by yeast showed lower activity than purified CelA from *E. coli* because of the glycosylation mechanism of yeast (Suzuki et al. 2012a). To inhibit bacterial contamination, low initial pH was used for ethanol fermentation, and most yeast ethanol fermentation is operated in the pH range of 4.0 to 5.0 (Kadar et al. 2007). Therefore, the pH optimums of these EGs might be suitable for their future usage in CBP.

All the EGs tested showed high temperature optimums ranging from 50 to 70°C. Such high temperature optimums of these enzymes could be a disadvantage for the operation of CBP using yeast cells whose usual cultivation temperature is 30°C. Therefore, to ensure higher enzyme activities of many cellulases with high

temperature optimums, fermentation at higher temperature using heat-resistant yeast strains could be considered as one of the solutions.

Higher expression levels of fungal EGs than those of bacterial enzymes suggest that fungal EGs could have advantages in expression in yeast cells although the expression levels can also be affected by other gene-specific factors. Therefore, considering both expression level and activity, *T. aurantiacus* EGI might be as useful as *T. reesei* EGII which have been widely studied and used for ethanol production.

For cellulosic ethanol production, instead of expressing multiple cellulases in one cell, we adopted a new strategy of combining three types of cells, each displaying a different cellulase. Such a strategy allows optimization of the ethanol production by simply changing the ratio of cells expressing each type of cellulase. Furthermore, the number of different cellulases expressed in a single cell can be limited because of the difficulties in molecular cloning as well as poor expression levels due to the metabolic burden (van Rensburg et al. 2012). However, unlimited number of cellulases can be included for ethanol production by using this cell combination system. Further characterization of a wide range of cellulases on the yeast surface and improvement the surface expression efficiency would facilitate the development of CBP using cellulolytic yeast strains.

Abstract in Korean

국 문 초 록

생분해성 폴리젖산 중합에 단량체로 사용되는 고순도의 입체특이적 젖산을 얻기 위한 미생물 발효에 대한 관심이 높아짐에 따라 본래 높은 내산성을 가지는 *Saccharomyces cerevisiae* 가 젖산 발효를 위한 미생물로 각광받고 있다. 본 연구에서는, 대사공학 및 적응진화 전략을 통한 효모의 내산성 향상을 통해 고순도의 D 형 젖산을 효율적으로 생산할 수 있는 효모 균주를 개발하였다.

첫 번째로, 입체특이성이 매우 높은 *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293 유래의 D 형 젖산 탈수소효소를 도입하고, 효모의 카복실산 운반단백질 유전자인 *JEN1* 과 D 형 젖산 탈수소효소 유전자인 *DLD1* 을 함께 제거함으로써 포도당 고갈 시 발생되는 젖산 소비능이 억제된 D 형 젖산 생산용 균주를 개발하였다. 이와 함께 피루브산 탈탄산효소 유전자인 *PDC1* 과 알코올 탈수소효소 유전자인 *ADH1* 을 함께 제거하여 젖산 발효시 경쟁적 대사산물로 작용하는 에탄올의 생성량을 줄이고, 글리세롤 합성을 억제하기 위해서 효모 내 글리세롤-3-탈수소효소 유전자인 *GPD1* 과 *GPD2* 를 모두 제거하였다. 이를 통하여 0.65 g/g 포도당의 높은 수율로 12.9 g/L 의 D 형 젖산을 생산하는 균주를 개발하였다.

다음으로, 적응진화 전략을 통해 젖산 내산성이 증가되고 제한된 포도당 소비능과 생장 저해 현상이 극복된 효모 균주를 개발하였다. 최종선별된 진화 균주는 진화 전 균주에 비해 높은 포도당 소비 속도와 D 형 젖산 생산량을 보였다. 추가적인 D 형 젖산 탈수소효소의 유전체

내 삽입을 통해서 배지 내 pH 조절이 없는 산성 발효 조건에서는 38.3 g/L, 중화제를 처리한 발효 조건에서는 118.6 g/L 까지 D 형 젖산 생산량이 증대됨과 동시에 높은 수율(0.79 g/g 포도당)로의 생산이 가능한 균주가 개발되었다. 그리고 선별된 진화 균주의 전체 유전서열에 대한 해독을 통해 종결코돈이 삽입된 두 유전자 (*GSF2*, *SYN8*)과 아미노산 치환이 삽입된 세 종류의 유전자 (*STM1*, *SIF2*, *BUD27*)을 발견하였다. *GSF2* 유전자의 제거는 진화 전 균주의 생장 속도 향상과 포도당 소비능 증대 효과를 나타냈으며, 진화 균주에 상응하는 D 형 젖산 생산량 증대를 보였다. *STM1* 과 *SIF2* 유전자의 제거 또한 이미 알려진 *SYN8* 유전자 제거 효과와 동일하게 효모 내 젖산 내성의 증대에 효과적인 것으로 확인되었다.

마지막으로, 추가적인 *HAA1* 유전자의 유전체 내 삽입을 통해 개발된 균주는 산성 발효 조건에서 62.2 g/L 의 포도당으로부터 48.9 g/L 의 D 형 젖산을 생산하였다. 이 *HAA1* 과발현 균주는 중화제를 처리한 유가 배양 조건에서는 가장 높은 2.2 g/L·h 의 생산성을 나타내었다. 이와 더불어 Haa1 이 효모 내 CK2 인산화효소에 의해서 인산화됨이 확인되었다. 비록 CK2 에 의한 Haa1 인산화의 기능은 추후 연구를 통해 더 밝혀져야 하지만, 본 결과를 통해 젖산 스트레스에 대한 효모의 적응 기작에 CK2 인산화효소가 Haa1 을 조절함으로써 연관될 수 있는 가능성이 확인되었다.

주요어 : D 형 젖산, *Saccharomyces cerevisiae*, 대사공학, 내산성, 생장저해, Haa1, 적응진화

학 번 : 2009-20995