A Thesis for the Degree of Master of Science

Enhanced production of isobutanol in engineered *Saccharomyces cerevisiae* by blocking competitive pathways

경쟁 경로의 차단을 통한 재조합 효모의 아이소부탄올 생산성 향상에 관한 연구

By

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Department of Agricultural Biotechnology
Seoul National University
February 2017

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Advisor : Professor Jin-Ho Seo

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

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農 學 碩 士 學 位 論 文

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ABSTRACT

As the global warming and climate change caused by petroleum-based fuels are intensifying, the research has actively conducted to develop bio-based fuels. Among the various candidates for ideal biofuels, isobutanol has valuable properties as a fuel such as high energy density, high octane number, low hygroscopicity and low vapor pressure. Also, isobutanol is an important platform chemical used as precursors of various chemicals.

In this study, *Saccharomyces cerevisiae*, which has various advantages for industrial production, was used as a host strain to produce isobutanol. Isobutanol is synthesized via the L-valine biosynthesis pathway and Ehrlich pathway in *S. cerevisiae*. In previous studies, engineered *S. cerevisiae*, which expresses the enzymes involved in isobutanol biosynthesis in the cytosol, was constructed. But an isobutanol yield of the engineered *S. cerevisiae* was still low. Also,

there are several competitive pathways for isobutanol production in *S. cerevisiae*; biosynthetic pathways for L-valine, L-isoleucine, L-leucine, isobutyrate and ethanol biosynthesis. In this study, the competitive pathways were eliminated by the CRISPR-Cas9 gene editing method and by overexpressing acetolactate synthase (ALS) from *Bacillus subtilis*, ketolacid reductoisomerase (KARI) from *Escherichia coli*, dihydroxyacid dehydratase (DHAD) and ketoacid decarboxylase (KDC) from *Lacococcus lactis*, an isobutanol yield based on glucose was improved a 8.6-fold higher than the corresponding value of the control strain. In batch cultivation in a bioreactor with gas trapping, this strain produced 662 mg/L isobutanol with 6.7 mg/g of yield.

This study indicates that competitive pathways are one of the causes for the low production of isobutanol in *S. cerevisiae*. By totally blocking the ethanol synthesis and screening another KDC with higher enzyme activity, it would be possible to improve the production of

isobutanol in *S. cerevisiae*.

Keywords : Biofuels, Isobutanol, *Saccharomyces cerevisiae*, Branched-chain amino acid biosynthesis, Alcohol dehydrogenase, CRISPR-Cas9, Gas trapping

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I. INTRODUCTION

1. Isobutanol – promising biofuel & biochemical

Production of eco-friendly biofuels from biomass is getting interests as concerns about global warming and climate change are growing. (Chen, Nielsen et al., 2011)

Typically, ethanol has been produced from biomass commercially and used as a mixture with gasoline. However, ethanol has several defects to replace the gasoline such as high water content, high hygroscopicity and low energy density relative to gasoline. (Atsumi, Cann et al., 2008; Atsumi, Hanai etal., 2008)

On the other hand, because the carbon-hydrogen bonds can release energy through combustion, higher alcohols which have more carbons and hydrogens than ethanol have benefits as gasoline substitutes (Savage, 2011; Kondo, Tezuka et al., 2012).

In particular, biobutanol has outstanding fuel characteristics compared to ethanol such as high energy density, low hygoscopicity, low reid vapor pressure and similar energy contents to gasoline. (Ranjan and Moholkar, 2012; Connor and Liao, 2008; García, Päkkilä et al., 2011).

Among the isomers of butanol, isobutanol, which is a branched-chain alcohol, has the same fuel properties as other butanol isomers, but lower toxicity and higher octane values caused by its branched chain. (Figure 1) (Table1) (Blombach, Eikmanns et al., 2011). A high-octane hydrocarbon can be applied to conventional engines like gasoline without adversely affecting the performance (Bastian, Liu et al., 2011). Therefore, isobutanol is more affordable to be applied as an alternative for gasoline, compared to other butanol isomers such as n-butanol (Table 2).

In addition, isobutanol is an important platform chemical. It can be used for various industrial applications including solvents, ink ingredients, paint, additives, and raw materials for organic compounds (Akita, Nakashima et al., 2015). Also, isobutanol can be readily used as a precursor for a number of valuable chemical syntheses such as isobutyl acetate (lacquer and coatings), diisobutyl phthalate (DIBP, plasticizer) and isobutylene, a monomer of butyl rubber (Lee, Seo et al., 2012).

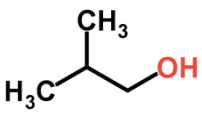


Figure 1. Structure of isobutanol.

Table 1. Chemical properties of liquid fuels (Bisaria, Kondo, 2014)

Fuel	**Ethanol**	**Isobutanol**	**Gasoline**
Volumetric energy density (MJ/L)	20.8	29.0	32.0
Vapor pressure (psi)	1.1	0.17	0.1-30
Average octane number	116	110	90
Hygroscopicity	High	Low	Low
Fits current infrastructure	No	Yes	Yes

Table 2. Physiochemical properties of n-butanol and isobutanol (Michael, Martin et al., 2016)

	n-butanol	isobutanol
Research octane number (RON)	94-96	113
Density [kg·m-3]	810	806
Lower heating value [MJ·kg-1]	33.3	33.3
Volumetric lower heating value [MJ·dm-3]	27.0	26.8
Latent heat of vaporization [kJ·kg-1]	716	579
Chemical formula	C_4H_9OH	C_4H_9OH
Mass share of C [%]	65	65
Mass share of H [%]	13.5	13.5
Mass share of O [%]	21.5	21.5
Viscosity [mPa·s]	2.57	3.33
Boiling point [°C]	118	108
Stoichimetric air/fuel ratio	11.2	11.2

2. Production of isobutanol in microorganisms

As shown in Table 3, several bacterial strains including *Escherichia coli*, *Corynebacterium glutamicum* and *Bacillus subtilis* were reported to produce isobutanol. By introducing the ketoacid decarboxylase (KDC) and alcohol dehydrogenase (ADH) of the Ehrlich pathway and by overexpressing acetolactate synthase (ALS), ketolacid reductoisomerase (KARI) and dihydroxyacid dehydratase (DHAD) of the L-valine biosynthesis pathway, those microorganisms produced high concentrations of isobutanol successfully (Atsumi, Hanai et al., 2008; Blombach, Riester et al., 2011; Qi, Li et al., 2014). However, these bacterial strains are not proper to produce isobutanol in an industrial scale because of the toxicity of isobutanol. (Weber, Christian et al., 2010)

On the other hand, *S. cerevisiae* is tolerant to high concentrations of alcohol such as ethanol and n-butanol (Fischer, Klein-Marcuschamer et al., 2008). Also, *S. cerevisiae* is genetically well-characterized and has been proven robust in industrial ethanol fermentation. Therefore, *S. cerevisiae* was chosen as a host strain in this study.

In previous studies, *S. cerevisiae* was engineered to overexpress the

enzymes of the L-valine biosynthesis pathway and Ehrlich pathway in the cytosol (Lee, Seo et al., 2012). In order to eliminate competitive pathways, some enzymes were deleted, but isobutanol production was still low (Park, Kim et al., 2014; Kondo, Tezuka et al., 2012; Ida, Ishii et al., 2015; Brat, Weber et al., 2012). In several studies, the enzymes of the L-valine pathway and Ehrlich pathway (ALS, KARI, DHAD, KDC and ADH) were overexpressed in the mitochondria for sufficient supply of the [Fe-S] cluster used as a cofactor by DHAD (Avalos, Fink et al., 2013; Park, Kim et al., 2016).

Also, KH Park engineered the [Fe-S] cluster synthesis and assembly system in *S. cerevisiae* in a previous study. First, *GRX3* which controls *AFT1*, a transcription factor concerned in iron uptake, was deleted for constitutive uptake of the iron. Second, *CFD1,* an enzyme which transfers the [Fe-S] cluster into cytosolic [Fe-S] proteins, was overexpressed. Consequently, 246 mg/L isobutanol was produced in bioreactor by engineered *S. cerevisiae* (Park, 2016). However, since isobutanol production of *S. cerevisiae* is still low compared to other bacterial strains, additional studies are necessary.

Table 3. Production of isobutanol in microorganism

Strains	Substrates	Methods	Concentration of isobutanol	Yield	References
Bacillus subtilis	Glucose	Flask	2.62 g/L	0.07 g/g	Li, Wen et al., 2011
	Glucose, Acetate	Fed-batch	5.5 g/L	0.19 g/g	Li, Huang et al., 2012
	Glucose	Fed-batch	6.1 g/L	0.23 g/g	Qi, Li et al., 2014
Corynebacterium glutamicum	Cellulose	Flask	660 mg/L	66 mg/g	Higashide et al., 2011
	Glucose, Acetate	Flask	4.9 g/L	97 mg/g	Smith, Cho et al., 2010
	Glucose, Acetate	Fed-batch	13 g/L	0.2 g/g	Blombach, Riester et al., 2011
Escherichia coli	Glucose	Flask	22 g/L	0.35 g/g	Atsumi, Hanai et al., 2008
	Glucose	Fed-batch	50.8 g/L	0.28 g/g	Baez, Cho et al., 2011
Saccharomyces cerevisiae	Glucose	Flask	151 mg/L	3.8 mg/g	Lee, Seo et al., 2012
	Glucose	Flask	377 mg/L	3.8 mg/g	Park, Kim et al., 2014

(be continued)

Strains	Substrates	Methods	Concentration of isobutanol	Yield	References
	Glucose	Flask	143 mg/L	6.6 mg/g	Kondo, Tezuka et al., 2012
	Glucose	Flask	224 mg/L	12 mg/g	Ida, Ishii et al., 2015
	Glucose	Flask	331 mg/L	17 mg/g	Park, Kim et al., 2016
	Glucose	Flask	630 mg/L	15 mg/g	Brat, Weber et al., 2012
	Glucose	Flask	635 mg/L	6.4 mg/g	Avalos, Fink et al., 2013
	Glucose	Bioreactor	246 mg/L	6.2 mg/g	Park, 2016, thesis

3. Pathway for isobutanol biosynthesis in *S. cerevisiae*

Isobutanol is produced via the L-valine biosynthesis pathway and Ehrlich pathway in engineered *S. cerevisiae* (Buijs, Siewers et al., 2013) (Figure 2). The L-valine pathway exists in the mitochondria. Glucose is converted to pyruvate by glycolysis in the cytosol and the pyruvate is transferred to the mitochondria through the mitochondrial pyruvate carrier (Bender, Pena et al., 2015). In the mitochondria, acetolactate synthase (ALS), ketolacid reductoisomerase (KARI), dihydroxyacid dehydratase (DHAD) and branched-chain amino acid aminotransferase (BAT) synthesize L-valine from pyruvate (Ryan, Kohlhaw et al., 1974).

In isobutanol production, pyruvate is converted into 2-ketoisovalerate (2-KIV) via the L-valine biosynthesis pathway and 2-KIV should be transported into the cytosol for the Ehrlich pathway. A different compartment of the L-valine biosynthesis pathway and Ehrlich pathway is inefficient to produce isobutanol. Therefore, in a previous study, the L-valine biosynthesis pathway was re-localized into the cytosol by deleting the mitochondria targeting sequence (MTS) of ALS, KARI and DHAD (Lee, Seo et al., 2012).

In the Ehrlich pathway, 2-KIV is converted to isobutanol by ketoacid decarboxylase (KDC) and alcohol dehydrogenase (ADH) (Hazelwood, Daran et al., 2008). Especially, KDC is a key enzyme to remove the carboxylic group of 2-ketoisovalerate and produce isobutyraldehyde (König, 1998). Among the various putative KDCs, *kivD* from *Lactococcus lactis* subsp. lactis KACC13877 was selected in the previous study (Lee, Seo et al., 2012). Also, endogenous *ADH2* of *S. cerevisiae* was identified to have the highest activity for isobutyraldehyde (Atsumi, Hanai et al., 2008; Brat, Weber et al., 2012)

In this study, to enhance the pathway for isobutanol synthesis, the enzymes involved in isobutanol synthesis were overexpressed in the cytosol; *alsS* from *Bacillus subtilis* as ALS, *ilvC* from *Escherichia coli* as KARI, *ilvD* from *Lactococcus lactis* as DHAD and *kivd* from *L. lactis* as KDC. Mitochondria targeting sequences (MTS) of used enzymes were predicted by MitoProt (http://ihg.gsf.de/ihg/mitoprot.html, Claros, Vincens, 1996). And *ilvC* from *E. coli* was modified to truncate MTS by KH Park (Park, 2016).

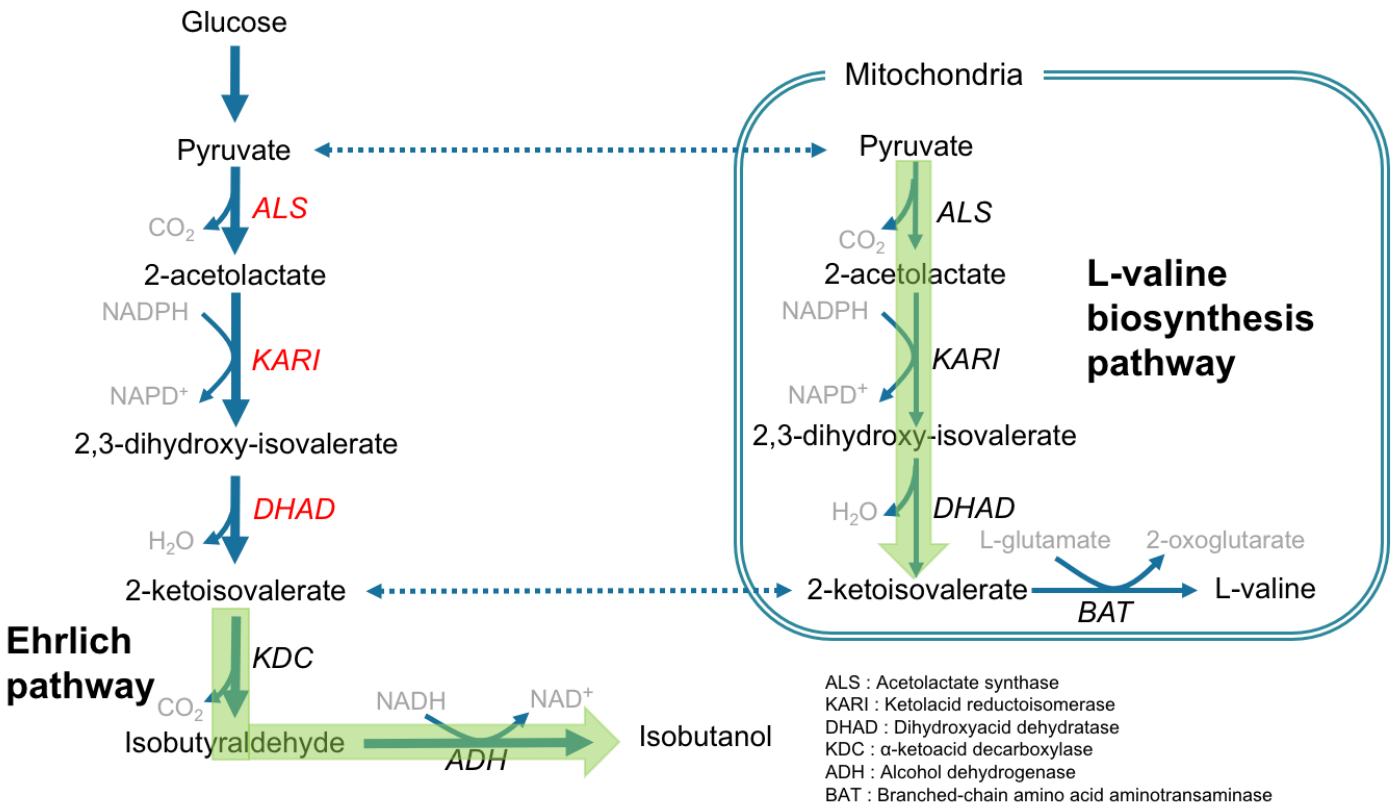


Figure 2. Pathway for isobutanol biosynthesis in *S. cerevisiae*.

4. Competitive pathways for isobutanol production

Isobutanol production has several competitive pathways both in the mitochondria and cytosol. L-valine is one of the branched-chain amino acids (BCAA) and the L-valine biosynthesis pathway shares the intermediates with the other BCAA biosynthesis pathways. L-valine and L-isoleucine are synthesized in the mitochondria. Although isobutanol production uses the L-valine biosynthesis pathway, the key compound is 2-KIV, not L-valine. Also, as L-isoleucine is synthesized from the mitochondrial pyruvate, L-isoleucine is one of the by-products for production of isobutanol. The other BCAA is L-leucine. 2-KIV produced in the mitochondria is converted to L-leucine in the cytosol via the L-leucine biosynthesis pathway (Dickinson, 1999; Holmberg, Petersen, 1988). To increase the amounts of 2-KIV for isobutanol production, eliminating the BCAA biosynthesis pathway is essential.

2-KIV is converted to isobutyraldehyde in the cytosol by ketoacid decarboxylase (KDC). As isobutyraldehyde is one of the toxic aldehyde in a cell, it is converted to isobutyrate by aldehyde dehydrogenase (ALD). This reaction interrupts the hydrogenation of isobutyraldehyde to isobutanol. In addition, ethanol is a major metabolite in *S. cerevisiae*. Pyruvate from glucose is converted to ethanol by pyruvate

decarboxylase (PDC) and alcohol dehydrogenase (ADH) (Flikweert, van et al., 1996). As most of the pyruvates are used to produce ethanol naturally in *S. cerevisiae*, it is necessary to reduce ethanol production for other compounds such as 2,3-butanediol and lactic acid. There were several efforts to eliminate the ethanol biosynthesis pathway by deletion of PDC or ADH (Kim, Seo et al., 2015; Ida, Furusawa et al., 2012). The competitive pathways in the mitochondria and cytosol are displayed in Figure 3.

In this study, BCAA, isobutyrate and ethanol synthesis were blocked. To improve isobutanol production, the enzymes involved in the competitive pathways were disrupted by the CRISPR-Cas9 system.

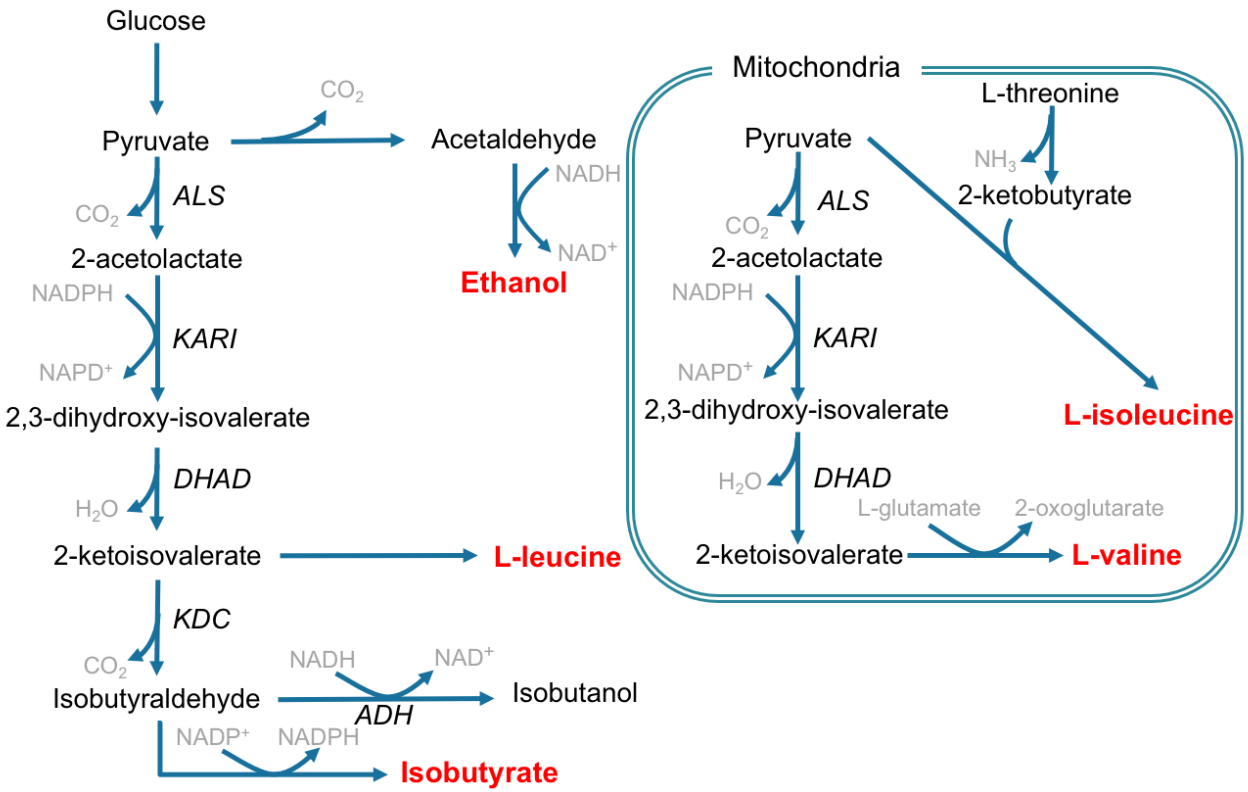


Figure 3. Competitive pathways for isobutanol synthesis.

5. CRISPR-Cas9 system for genome editing

CRISPR (clusted regularly interspaced short palindromic repeats)-Cas9 (CRISPR associated protein 9) is adaptive immune defenses against invading foreign nucleic acids in bacteria and archaea (Marraffini, Luciano et al., 2010). In the biotechnology, CRISPR-Cas9 is used as the effective genome editing tool because of its site-specificity and simplicity. The Cas9, an RNA-guided endonuclease, generates double-strand break (DSB) in the genomic DNA. The broken nucleic acid is repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR). Therefore, indel mutation or precise genome editing is possible using the CRISPR-Cas9 system (DiCarlo, Noville et al., 2013).

In previous studies, a genome editing method for *S. cerevisiae* using CRISPR-Cas9 was constructed (DiCarlo, Noville et al., 2013; Zhang, Kong et al., 2014). In this study, gene disruption to block the production of by-products was performed using the genome editing method based on the CRISPR-Cas9 system.

6. Gas trapping system

Isobutanol is a volatile compound, so it is evaporated during the cultivation. Therefore, it is important to trap the evaporated isobutanol, especially in industrial production. In previous studies, gas trapping was developed and recognized as a relatively easy, efficient and low-cost method for collecting volatile compounds, including isobutanol. (Inokuma, Liao et al., 2010)

In this study, batch cultivation in a bioreactor was performed with a gas trapping system equipped by KH Park to trap the isobutanol produced by engineered *S. cerevisiae* (Park, 2016).

7. Research objectives

This study was focused on the production of isobutanol by engineered *S. cerevisiae*. The specific objectives of this study are listed:

1) To eliminate the competitive pathways in the mitochondria such as the L-valine and L-isoleucine biosynthesis pathway and to improve the supply of 2-ketoisovalerate for isobutanol production.

2) To block the competitive pathways in the cytosol such as the isobutyrate, L-leucine and ethanol biosynthesis pathway and to enhance isobutanol production.

3) To produce isobutanol with improved yield in a bioreactor using a gas trapping system.

Ⅱ. Materials and Methods

1. Reagents

All used chemicals were of reagent grade. Yeast nitrogen base (YNB, w/o amino acid), yeast synthetic drop-out supplement, glucose, antifoam and isobutanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bacto-peptone, bacto-trypton, yeast extract and bacto-agar were obtained from Difco (Detroit, MI., USA); ethanol and n-propanol from Merck (Darmstadt, Germany); ampicillin from Fisher Scientific (Hampton, NH, USA); hygromycin B from Duchefa (Haarlem, Netherlands); aureobasidin A from Clontech Laboratories (Mountain view, CA, USA); ethidium bromide from Bioneer (Daejeon, Korea); agarose from Dongin Genomic (Seoul, Korea); NaOH, HCl, NaCl and H_2SO_4 from Duksan (Ansan, Korea).

2. Strains and plasmids

2.1. Strains

E. coli TOP10 (Invitrogen, Carlsbad, CA, U.S.A) was used for the propagation and preparation of plasmid DNA for gene cloning. *S. cerevisiae* D452-2 [*Matα, leu2 his3 ura3 can1*], [Fe-S] cluster engineered *S. cerevisiae* D452-2 strain (WΔG) and pyruvate decarboxylase (PDC)-deficient *S. cerevisiae* D452-2 strain (SOS5) were used as host strains for the genome editing and expression of the isobutanol biosynthesis pathway. Strains used in this study are described in Table 4.

The constructed strains were stored on YPD or YNBD medium in a deep freezer at -80℃ suspended in 15% glycerol.

Table 4. List of the strains used in this study

Strains	Description	Reference
E. coli TOP10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen, Carlsbad, CA, USA
S. cerevisiae D452-2	*Matα, leu2 his3 ura3 can1*	Hosaka, *et al*., 1992
WΔG	D452-2 *ΔGRX3*::*CFD1*	Constructed by KH Park
WΔGB	D452-2*ΔGRX3*::*CFD1ΔBAT1*	In this study
WΔGBI	D452-2*ΔGRX3*::*CFD1ΔBAT1ΔILV1*	In this study
WΔGBIA	D452-2*ΔGRX3*::*CFD1ΔBAT1ΔILV1ΔALD6*	In this study
WΔGBIAL	D452-2*ΔGRX3*::*CFD1ΔBAT1ΔILV1ΔALD6ΔLEU1*	In this study
WΔGBIALA1	D452-2*ΔGRX3*::*CFD1ΔBAT1ΔILV1ΔALD6ΔLEU1ΔADH1*	In this study
WΔG_3vec	WΔG, p423TDH3_M.*ilvC*, p425TDH3_*alsSkivd*, p426TDH3_*ilvD*	In this study
WΔGB_3vec	WΔGB, p423TDH3_M.*ilvC*, p425TDH3_*alsSkivd*, p426TDH3_*ilvD*	In this study
WΔGB_2vec	WΔGB, p423TDH3_M.*ilvCilvD*, p426TDH3_*alsSkivd*	In this study

(be continued)

Strains	Description	Reference
WΔGBI_2vec	WΔGBI, p423TDH3_M.*ilvCilvD*, p426TDH3_*alsSkivd*	In this study
WΔGBIA_2vec	WΔGBIA, p423TDH3_M.*ilvCilvD*, p426TDH3_*alsSkivd*	In this study
WΔGBIAL_2vec	WΔGBIAL, p423TDH3_M.*ilvCilvD*, p426TDH3_*alsSkivd*	In this study
WΔGBIALA1_2vec	WΔGBIALA1, p423TDH3_M.*ilvCilvD*, p426TDH3_*alsSkivd*	In this study
SOS5	D452-2 *ΔPDC1 ΔPDC5 ΔPDC6*	Kim, Seo et al., 2015
SOS5ΔG	SOS5*ΔGRX3*::*CFD1*	Constructed by KH Park
SOS5ΔGB	SOS5*ΔGRX3*::*CFD1 ΔBAT1*	In this study
SOS5ΔGB_3vec	SOS5ΔGB, p423TDH3_M.*ilvC*, p425TDH3_*alsSkivd*, p426TDH3_*ilvD*	In this study
SOS5ΔGB_PDC1	SOS5ΔGB _p426TDH3_*PDC1*	In this study
SOS5ΔGB_PDC5	SOS5ΔGB _p426TDH3_*PDC5*	In this study
SOS5ΔGB_PDC6	SOS5ΔGB _p426TDH3_*PDC6*	In this study

2.2. Plasmids

Yeast episomal plasmids (p423TDH3, p425TDH3, p426TDH3) harboring the 2 μ origin, constitutive *TDH3* promoter and *CYC1* terminator from *S. cerevisiae* were used as mother vectors. To express the isobutanol biosynthesis pathway, *alsS* from *Bacillus subtilis*, M.*ilvC* from *Escherichia coli*, *ilvD* from *Lactococcus lactis* and *kivd* from *L. lactis* are introduced to the mother vectors, respectively.

To disrupt the genes involved in the competitive pathways using CRISPR-Cas9 system, a yeast centromere plasmid harboring the Cas9 expression cassette under the constitutive *TEF1* promoter and *CYC1* terminator from *S. cerevisiae* and yeast episomal plasmids harboring the guide-RNA expression cassette under the *SNR52* promoter and *SUP4* terminator are used. The plasmids and oligonucleotide sequences as the polymerase chain reaction (PCR) primer used in this study are described in Table 5 and 6, respectively.

Abbreviations and significations used in this study are as follows. ‘M’ means the modification to express the genes in the cytosol by removing mitochondria targeting sequence (MTS). ‘3vec’ or ‘2 vec’ means the number of plasmids used to express the isobutanol

biosynthesis pathway. In this study, histidine, leucine and uracil auxotroph marker set or histidine and uracil auxotroph marker set was used.

Table 5. List of the plasmids used in this study

Strains	Description	Reference
p423TDH3	*LEU2*, *TDH3* promoter, *CYC1* terminator, 2 μ origin, Amp^R	Mumberg *et al*., 1995
p425TDH3	*URA3*, *TDH3* promoter, *CYC1* terminator, 2 μ origin, Amp^R	Mumberg *et al*., 1995
p426TDH3	*LEU2*, *TDH3* promoter, *CYC1* terminator, 2 μ origin, Amp^R	Mumberg *et al*., 1995
p423TDH3_M.ilvC	p423TDH3 harboring modified *ilvC* from *E. coli*	Constructed by KH Park
p425TDH3_alsSkivd	p425TDH3 harboring *alsS* from *B. subtilis* and *kivd* from *L. lactis*	Constructed by KH Park
p426TDH3_ilvD	p426TDH3 harboring *ilvD* from *L. lactis*	In this study
p423TDH3_M.ilvCilvD	p423TDH3 harboring modified *ilvC* from *E. coli* and *ilvD* from *L. lactis*	In this study
p426TDH3_alsSkivd	p426TDH3 harboring *alsS* from *B. subtilis* and *kivd* from *L. lactis*	In this study
pAUR_Cas9	*AUR1-C*, *CEN6*, *ARS4*, Amp^R, P_{TEF1}_*Cas9*_T_{CYC1}	Constructed by JW Kim
p42H_g*BAT1*	Hyg^R, 2 μ origin, Amp^R, P_{SNR52}_*gBAT1*_T_{SUP4}	Constructed by KH Park
p42H_g*ILV1*	Hyg^R, 2 μ origin, Amp^R, P_{SNR52}_*gILV1*_T_{SUP4}	In this study
p42H_g*ALD6*	Hyg^R, 2 μ origin, Amp^R, P_{SNR52}_*gALD6*_T_{SUP4}	In this study

(be continued)

Strains	**Description**	**Reference**
p42H_g*LEU1*	Hyg^R, 2 μ origin, Amp^R, P_{SNR52}_*gLEU1*_T_{SUP4}	In this study
p42H_g*ADH1*	Hyg^R, 2 μ origin, Amp^R, P_{SNR52}_*gADH1*_T_{SUP4}	Constructed by JW Kim
p426TDH3_PDC1	p426TDH3 harboring *PDC1* from *S. cerevisiae*	Constructed by JW Kim
p426TDH3_PDC5	p426TDH3 harboring *PDC5* from *S. cerevisiae*	Constructed by JW Kim
p426TDH3_PDC6	p426TDH3 harboring *PDC6* from *S. cerevisiae*	Constructed by JW Kim

Table 6. List of oligonucleotides used in this study

Name	Oligonucleotide sequence (5' → 3')
F_BamHI_alsS	CGGGATCCATGTTGACAAAAGCAACAAAAGA
R_XhoI_alsS	CCGCTCGAGCTAGAGAGCTTTCGTTTTCA
F_BamHI_MilvCe	CGGGATCCAAAATGATGGGCCGCGATGAATTC
R_XhoI_ilvCe	CCGCTCGAGTTAACCCGCAACAGCAATACGTTTC
F_BamHI_ilvDl	CGGGATCCATGGAATTCAAATATAACGGAAAAGTTGAATC
R_XhoI_ilvDl	CCGCTCGAGCTATAAATCTGTAACGCAACCTTCAC
F_ SacI_TDH3	CGAGCTCAGTTTATCATTATCAATACTCGCCATTTC
R_ SacI _CYC	CGAGCTCGGCCGCAAATTAAAGCCTTCG
F_inf_p426_TDH3	GGAACAAAAGCTGGAGCTCAGTTTATCATTATCA
R_inf_CYC_TDH3	GATAATGATAAACTGAGCTCGGCCGCAAATTAAA
F1_SacI_gRNA	GGGGTACCTCTTTGAAAAGATAATGTATG
R2_KpnI_gRNA	ATAGCGAGCTCAGACATAAAAAACAAAAAAAGCACCACC
R1_gRNA_ILV1	ATTTCAATTCAGAGTGTAGTGATCATTTATCTTTCACTGCG
F2_gRNA_ILV1	ACTACACTCTGAATTGAAATGTTTTAGAGCTAGAAATAGCAAGT
R1_gRNA_ALD6	CGGGCTAAGGCCAAAGTTTTGATCATTTATCTTTCACTGCG
F2_gRNA_ALD6	AAAACTTTGGCCTTAGCCCGGTTTTAGAGCTAGAAATAGCAAGT
R1_gRNA_LEU1	AGTGTCTGTCGATATACAACGATCATTTATCTTTCACTGCG
F2_gRNA_LEU1	GTTGTATATCGACAGACACTGTTTTAGAGCTAGAAATAGCAAGT
F_repair_BAT1	GGTCAGCCAAAGAAGGGTGGGGCACTCCACACATCAAGCCTTAC GGTTAACTTTCTCTTG
R_repair_BAT1	ATGCATAATGGAATACACAAGCAGATGGGTCAAGAGAAAGTTAA CCGTAAGGCTTGATGT
F_repair_ILV1	ACACCTGTCACCTTCCTTGATAAAACTACACTCTGAATTGAAATA GGATGAGCTGCAAAC
R_repair_ILV1	ACTAAACGGACGTAATCAGGGGTGTTATCAGTTTGCAGCTCATCC TATTTCAATTCAGAG
F_repair_ALD6	CCATTGAAGCTTTGGACAATGGTAAAACTTTGGCCTTAGCCCGTT GAGATGTTACCATTG
R_repair_ALD6	TAGGCAGCAGCATCTCTTAGACAGTTGATTGCAATGGTAACATCT CAACGGGCTAAGGCC

(be continued)

Name	Oligonucleotide sequence (5’ → 3’)
F_repair_LEU1	CAAGATGAAAATGGTTCCTTTTTGTTGTATATCGACAGACACTA GGTTCATGAAGTCACC
R_repair_LEU1	ATTTTCTAGGCCTTCGAAAGCTTGTGGAGAGGTGACTTCATGA ACCTAGTGTCTGTCG
F_repair_ADH1	CCCAGAAACTCAAAAAGGTGTTATCTTCTACGAATCCCACGGT AAGTAGGAATACAAAGA
R_repair_ADH1	CAATTCGTTGGCCTTTGGCTTTGGAACTGGAATATCTTTGTATTC CTACTTACCGTGGGA
F_check_BAT1	CGCATGTTGCAGAGACATTCCTTGAAGTT
R_check_BAT1	CCGTTAGTTCAAGTCGGCAACAGTTTTTG
F_check_ILV1	ATGTCAGCTACTCTACTAAAGCAACC
R_check_ILV1	GCACCAGATGGTTCTACAATACTTC
F_check_ALD6	ATGACTAAGCTACACTTTGACACTG
R_check_ALD6	GTTAAGATCTTGGCGCCTTCTTTC
F_check_LEU1	ATGGTTTACACTCCATCCAAGG
R_check_LEU1	GATGCTCTTTAGCGGTGTGTTAGG
F_check_ADH1	ATGTCTATCCCAGAAACTCAAAAAGG
R_check_ADH1	CTTTTCGTAAATTTCTGGCAAGGTAG
F_cas9_seq	ATGGACAAGAAGTACTCCATTG
R_cas9_seq	TCACACCTTCCTCTTCTTCTT

3. DNA manipulation and transformation

3.1. Enzymes

Restriction enzymes and calf intestinal alkaline phosphatase (CIP) were purchased from New England Biolabs (Beverly, MA, USA). T4 DNA ligation mix (Mighty Mix) was obtained from Takara (Shiga, Japan).

3.2. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) to amplify the gene for cloning was performed with the PrimeSTAR® HS PCR PreMix (Takara, Shiga, Japan) in GeneAmp PCR System 2720 (Applied Biosystems, CA, USA). PCR solution was composed of 10 pmol of forward and reverse primers, and 10 ng of plasmid DNA as a template. PCR amplification was performed as follows; 1 cycle of 95℃ for 5 min; 30 cycles of 94℃ for 30 sec, 55℃ for 30 sec, 72℃ for 1 kb/min, 1 cycle of 72℃ for 10 min. The amplified gene was confirmed by gel electrophoresis.

Colony PCR to confirm the yeast transformation of plasmids was performed with the TOPsimple™ DyeMIX-Tenuto PCR premix

(enzynomics, Daejeon, Korea) in the same PCR machine. PCR solution was composed of 10 pmol of forward and reverse primers, and picked yeast colony as a template. PCR amplification was performed as follows; 1 cycle of 98℃ for 10 min; 35 cycles of 94℃ for 30 sec, 50℃ for 30 sec, 72℃ for 1 kb/min, 1 cycle of 72℃ for 10 min. The amplified gene was confirmed by gel electrophoresis.

3.3. Preparation of bacterial genomic DNA

Preparation of the genomic DNA to obtain a template for the gene was carried out using DNeasy Blood & Tissue Kit from QIAGEN (Hilden, Germany) according to the manufacturer's instruction.

3.4. Isolation of DNA fragments and DNA sequencing

DNA was digested with restriction enzymes and separated on a 1% (w/v) agarose gel. After full separation of the desired DNA band, the gel containing the DNA fragment was solubilized and further purified by using Qiaquick® gel extraction kit from QIAGEN (Hilden, Germany). DNA sequencing was performed by SolGent (Daejeon, Korea).

3.5. Transformation of *E. coli*

Transformation of *E. coli* was carried out as described by Sambrook (Sambrook and Russell, 1989). *E. coli* Top10 was cultured in 5 mL LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) for 12 hr. 0.5 mL of the culture was transferred to fresh 50 mL LB medium and cultured until $O.D._{600}$ reached 0.5. Cells harvested by centrifugation at 6000 rpm for 5 min at 4℃ were resuspended in 5 mL of cold 100 mM $CaCl_2$ solution containing 15% (v/v) glycerol. Resuspended cells were aliquoted to 100 μL, mixed with DNA, and kept on ice for 30 min. They were subjected to heat-shock at 42℃ for 45 sec and 1 mL of LB medium was added to the tubes and incubated at 37℃ for 40 minutes to allow the bacteria to express the antibiotic resistance. Transformed cells was spread on LB agar plates with an ampicillin selection marker.

3.6. Preparation of plasmid DNA

Mini-scale preparation of plasmid DNA was carried out using DNA-spin™ Plasmid DNA purification Kit from iNtRON Biotechnology (Seongnam, Korea) according to the manufacturer's instruction.

3.7. Yeast transformation

Transformation of expression vectors was performed using the yeast EZ-Transformation kit (BIO 101, Vista, Calif.). Transformants were selected on YNB medium containing 20 g/L glucose. Amino acids, nucleotides and antibiotics were added as necessary.

4. Genome editing by CRISPR-Cas9 system

Genome editing was performed by CRISPR-Cas9. First, a plasmid (pAUR_Cas9) harboring the Cas9 protein from *Streptococcus pyogenes* was transformed to *S. cerevisiae*. Protospacers of target genes were selected using CRISPRdirect (http://crispr.dbcls.jp, Naito, Hino et al., 2015). For construction of a gRNA expressing plasmid, overlap-PCR was used. PCR primers including protospacer sequences (R1 and F2) were synthesized. The first fragment was amplified with F1 and R1 primer, and the other fragment was amplified with F2 and R2 primer. The full amplified fragment was obtained by the overlap-PCR using F1 and R2 primer. This fragment was cut by *Sac* Ⅰ and *Kpn* Ⅰ, and then ligated with the p42H plasmid.

Gene disruption was carried out by inserting a stop codon in the open reading frame. Repair DNA was designed to contain about 50 bp homology with the target gene and stop codon. By introducing the gRNA expressing plasmid (p42H_gRNA) and a repair DNA, gene disruption was performed. The inserted stop codon was confirmed by DNA sequencing (Zhang, Kong et al., 2014).

5. Media and culture conditions

5.1. Media

LB medium with 75 μg/mL of ampicillin was used for recombinant *E. coli* cultivation.

YPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose) with 0.1 μg/mL of aureobasidin A and 375 μg/mL of hygromycin B and YNBD $his^{-}leu^{-}ura^{-}$ (6.7 g/L yeast nitrogen base without amino acid, 2.0 g/L amino acids mixture without histidine, tryptophan, leucine and uracil, 20 mg/L tryptophan and 2% glucose) or YNBD $his^{-}ura^{-}$ medium (6.7 g/L yeast nitrogen base without amino acid, 2.0 g/L amino acids mixture without histidine, tryptophan, leucine and uracil, 20 mg/L tryptophan, 76 mg/L leucine and 2% glucose) were used for selection of yeast strains.

For flask cultivaion, YPD medium containing 40 g/L glucose or YNBD medium containing 40 g/L glucose and for bioreactor cultivation, YPD medium containing 100 g/L glucose was used.

5.2. Cultivations in flask

To prepare the inoculums, engineered *S. cerevisiae* stock was cultivated in a 5 mL test tube with YNBD medium containing 20 g/L glucose at 30 ℃, 250 rpm for 48 h. The grown cells were transferred to 500 mL baffled-flask with 100 mL YNBD medium containing 20 g/L glucose at 30 ℃, 250 rpm until the mid-exponential phase ($O.D._{600}$ 5-6). The cells were harvested by centrifugation at 7,000 rpm for 1 min and washed twice with double-distilled water (DDW). The harvested cells were inoculated into the main culture at the initial $O.D._{600}$ 1.0. In the case of Pdc-deficient *S. cerevisiae*, initial $O.D._{600}$ was 10.0.

Main culture was carried out in 250 mL flask with 50 mL working volume of YNBD medium (All of the tested strains except for Pdc-deficient strains) or YPD medium (Pdc-deficient strains) containing 40 g/L glucose in 30℃ shaking incubator (Vision, Daejeon, Korea). The aeration condition was micro-aerobic (100 rpm) which was determined as the best condition to produce isobutanol in previous study by SJ Baek.

5.3. Cultivations in bioreactor with gas trapping

Large-scale batch cultivation was carried out using a bench-top fermentor (KF-1L, KoBioTech, Incheon, Korea). Cultivations were performed in 500 mL YPD medium containing 100 g/L glucose at 30℃ and pH 6.0 (adjusted by 2 N HCL and 2 N NaOH). For micro-aerobic conditions, an agitation speed of 300 rpm and aeration of 1 vvm were maintained.

The air out of the bioreactor was connected to the gas trapping system. The air passed the bottle (500 mL of water) and condenser sequentially. As the bottles and condensers were cooled with ice and 4℃ water, the evaporated isobutanol from bioreactor was trapped in the bottles. (Figure 4) (Baez et al., 2011).

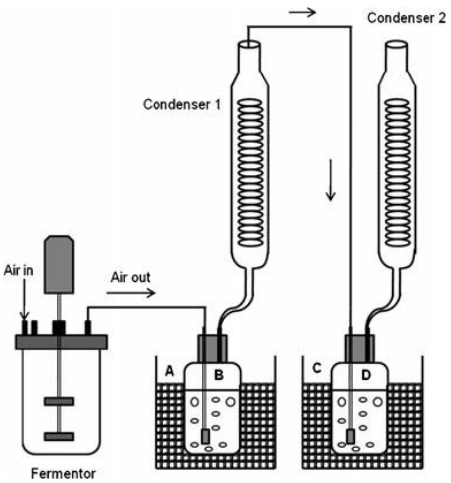


Figure 4. Schematic diagram of gas trapping (Baez et al., 2011).

6. Analysis

6.1. Dry cell weight

Cell growth was estimated by measuring optical density (O.D.) at 600 nm using a spectrophotometer (OPTIZEN POP, Mecasys, Daejeon, Korea). Dry cell weight (DCW) was calculated by pre-estimated conversion equation.

$$\text{Dry cell weight (g/L)} = 0.3 \times \text{O.D.}_{600}$$

6.2. Metabolite detection

Concentrations of glucose, glycerol, acetate and ethanol were measured by a high performance liquid chromatography (Agilent Technologies 1260 Series, Santa Clara, CA, USA) with a Rezex ROA-organic acid column (Phenomenex, CA, USA). The column maintained at 60℃ was eluted with 5 mM sulfuric acid at flow rate of 0.6 ml/min. Detection was made with a reflective index (RI) detector at 35℃.

6.3. Isobutanol detection

Concentration of isobutanol was analyzed by a gas chromatograph (YL6100, YoungLin, Incheon, Korea) equipped with flame ionization detector (FID). The separation of alcohol compounds was carried out by HP-FFAP capillary column (30 m, 0.25 mmID., 0.25 μm film thickness) purchased from Agilent Technologies (Santa Clara, CA, USA). Oven temperature was initially held at 60℃ for 4 min and raised with a gradient of 6℃/min until 200℃ and held for 2 min. Helium was used as the carrier gas at a 40 cm/sec constant flow. The FID was fed by a mixture of high purity air, hydrogen and helium. The injector and detector were maintained at 250℃ and 300℃, respectively. The column was injected with 1 μL of the supernatant of culture broth in a splitless injection mode. n-propanol was used as the internal standard, and alcohol content was determined by extrapolation from standard curves using the internal standard to normalize the values.

III. RESULTS AND DISCUSSIONS

1. Blocking the competitive pathways in mitochondria

1.1. Elimination of L-valine biosynthesis pathway

2-Ketoisovalerate (2-KIV) is the key intermediate of isobutanol synthesis since it links the L-valine biosynthesis pathway with the Ehrlich pathway. Pyruvate is synthesized through glycolysis in the cytosol and transferred to the mitochondria. In the mitochondria, pyruvate is converted to 2-KIV by acetolactate synthase (ALS, *ILV2*), ketolacid reductoisomerase (KARI, *ILV5*) and dihydroxyacid dehydratase (DHAD, *ILV3*). Then, branched-chain amino acid transferase (BAT, *BAT1*) synthesizes L-valine from 2-KIV (Lee, Seo et al., 2012; Schoondermark-Stolk and Tabernero et al., 2005). In previous study, isobutanol production was improved by deletion of *BAT1* in *S. cerevisiae* CEN.PK2-1C (Park, Kim et al., 2014). Because the L-valine biosynthesis pathway competes with isobutanol production, *BAT1* was disrupted by the CRISPR_Cas9 system to enhance 2-KIV availability used to synthesize isobutanol in this study. The control strain was WΔG_3vec and the *BAT1* disrupted strain was WΔGB_3vec. Both strains overexpressed the

enzymes involved in the isobutanol biosynthesis pathway using three vectors which have different amino acid auxotroph markers (*alsS* from *B. subtilis*, M.*ilvC* from *E. coli*, *ilvD* from *L. lactis* and *kivd* from *L. lactis*). Profiles are displayed for the batch culture of the constructed strains in Figure 5.

While WΔG_3vec produced 20 mg/L of isobutanol, WΔGB_3vec produced 70 mg/L of isobutanol from 40 g/L glucose. The isobutanol yield of the *BAT1* disrupted strain was 1.65 mg/g which was a 3.4-fold higher than the corresponding value of the control strain (0.49 mg/g) (Table 7). This result suggests the increased availability of mitochondrial 2-KIV exerted a positive effect on isobutanol production.

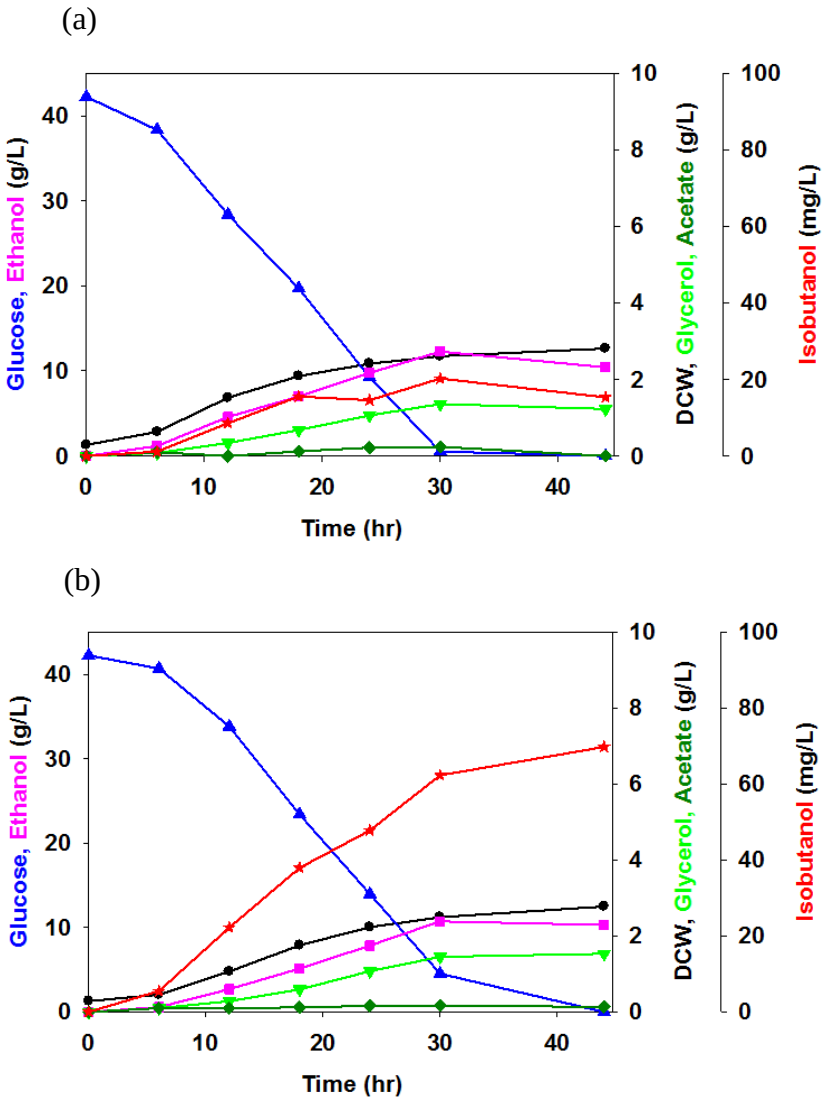


Figure 5. Profiles of cell mass and metabolites in batch culture of (a) WΔG_3vec, (b) WΔGB_3vec.

Symbols : glucose (▲), DCW (●), glycerol (▼), ethanol (■), isobutanol (★)

Table 7. Summary of flask cultivation in part 1.1

Strain	Maximum dry cell weight (g/L)	Concentration of Ethanol (g/L)	Concentration of isobutanol (mg/L)	Yield of isobutanol (mg $_{isobutanol}$/g $_{glucose}$)
WΔG_3vec	2.8	12.3	20	0.49
WΔGB_3vec	2.8	10.3	70	1.65

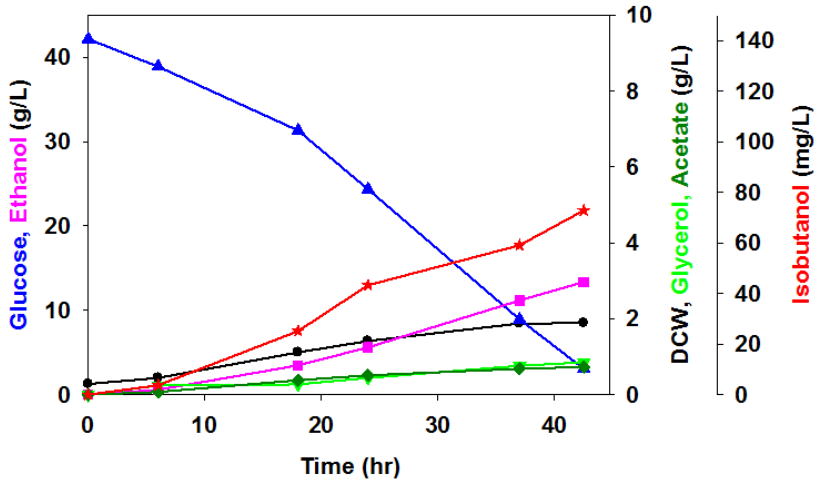
1.2. Elimination of L-isoleucine biosynthesis pathway

Like L-valine, L-isoleucine is also synthesized from pyruvate in the mitochondria. L-Threonine is converted to 2-ketobutyrate by threonine deaminase (*ILV1*). 2-Ketobutyrate and pyruvate are precursors for L-isoleucine biosynthesis. Also, the L-isoleucine biosynthesis pathway shares the key enzymes for isobutanol production such as ALS, KARI and DHAD. Therefore, it would be possible to consolidate the isobutanol biosynthesis pathway by eliminating L-isoleucine production. In the previous study, *ILV1* was deleted in *S. cerevisiae* BY4741 and YPH499 and isobutanol production was improved (Ida, Ishii et al., 2015). In addition to block the L-valine biosynthesis pathway by disruption of *BAT1*, *ILV1* was disrupted by the CRISPR-Cas9 system in this study. The control strain was WΔGB_2vec and the *ILV1* disrupted strain was WΔGBI_2vec. Both strains overexpressed the enzymes involved in the isobutanol biosynthesis using two vectors which have different amino acid auxotroph markers (*alsS* from *B. subtilis*, M.*ilvC* from *E. coli*, *ilvD* from *L. lactis* and *kivd* from *L. lactis*). Profiles are

displayed for the batch culture of the constructed strains in Figure 6.

The control strain (WΔGB_2vec) produced 73 mg/L of isobutanol with 1.86 mg/g of isobutanol yield. The *ILV1* disrupted strain (WΔGB_2vec) produced 139 mg/L with 3.29 mg/g of yield, which are 1.9-fold and 1.8-fold higher than the corresponding values of the control strain, respectively (Table 8). Prevention of the competitive carbon flux in the mitochondria is an effective strategy to enhance isobutanol production in *S. cerevisiae*.

(a)



(b)

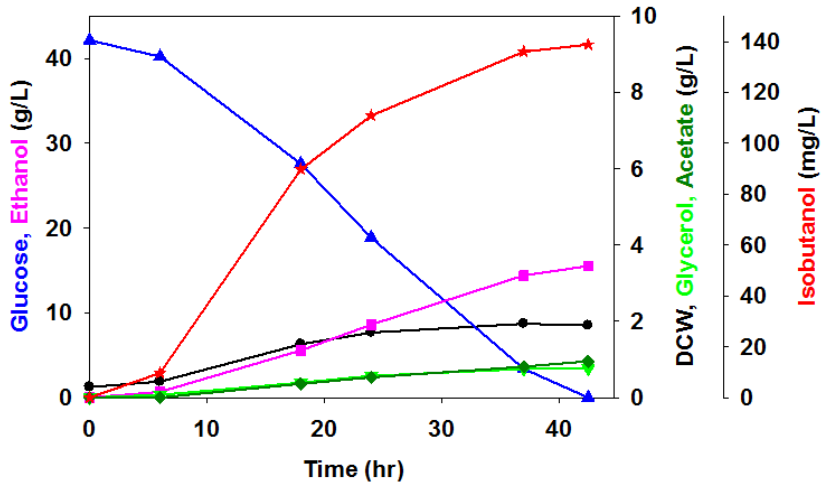


Figure 6. Profiles of cell mass and metabolites in batch culture of (a) WΔGB_2vec, (b) WΔGBI_2vec.

Symbols : glucose (▲), DCW (●), glycerol (▼), ethanol (■), isobutanol (★)

Table 8. Summary of flask cultivation in part 1.2

Strain	**Maximum dry cell weight (g/L)**	**Concentration of Ethanol (g/L)**	**Concentration of isobutanol (mg/L)**	**Yield of isobutanol ($mg_{isobutanol}/g_{glucose}$)**
WΔGB_2vec	1.9	13.4	73	1.86
WΔGBI_2vec	1.9	15.5	139	3.29

2. Blocking competitive pathways in cytosol

2.1. Elimination of isobutyrate and L-leucine biosynthesis pathway

Although the competitive carbon flux in the mitochondria is eliminated, there are several by-products for isobutanol production in the cytosol. Isobutyrate is one of the by-products in the cytosol which is converted from isobutyraldehyde, a precursor of isobutanol, by aldehyde dehydrogenase (ALD, *ALD6*) (Hazelwood, Daran et al., 2008). In previous studies, isobutanol production was improved by *ALD6* deletion in *S. cerevisiae* CEN.PK2-1C and BY4741 (Park, Kim et al., 2014; Ida, Ishii et al., 2015).

L-leucine is one of the branched-chain amino acids. In contrast to L-valine and L-isoleucine, L-leucine is synthesized in the cytosol, not the mitochondria. For L-leucine biosynthesis in the cytosol, 2-KIV is utilized as a key precursor by *LEU4*. Therefore, the L-leucine biosynthesis pathway competes with the isobutanol biosynthesis pathway. There are four enzymes in the cytosol for L-leucine biosynthesis (*LEU4*, *LEU1*, *LEU2* and *BAT2*) (Baichwal, Cunningham et al., 1983). In a previous study, *LEU4*, the first

enzyme of the L-leucine biosynthesis pathway, was deleted in *S. cerevisiae* CEN.PK2-1C, but the result was not effective (Park, Kim et al., 2016).

On the other hand, *LEU1* is a typical cytosolic enzyme using the [Fe-S] cluster as a cofactor. Because DHAD in isobutanol biosynthesis uses the [Fe-S] cluster of which production is highly regulated in *S. cerevisiae*, *LEU1* might be an appropriate deletion target. Deletion of *LEU1* in *S. cerevisiae* and the consequential effect on activity of DHAD was mentioned in the patent before (US 8241878 B2). In this study, *LEU1* was disrupted by the CRISPR-Cas9 system for two reasons. One is elimination of the L-leucine biosynthesis pathway, and the other is an enhancement of DHAD activity by suppling the additional cytosolic [Fe-S] clusters.

The *BAT1* and *ILV1* disrupted strain (WΔGBI_2vec) was modified to eliminate production of isobutyrate and L-leucine production (WΔGBIA_2vec and WΔGBIAL_2vec) in this study. Both strains overexpressed the enzymes involved in the isobutanol biosynthesis using two vectors which have different amino acid auxotroph markers (*alsS* from *B. subtilis*, M.*ilvC* from *E. coli*, *ilvD* from *L. lactis* and *kivd* from *L. lactis*). Profiles are displayed for the batch

culture of the constructed strains in Figure 7.

While the control strain (WΔGBI_2vec) produced 139 mg/L of isobutanol with 3.29 mg/g of isobutanol yield, WΔGBIA_2vec and WΔGBIAL_2vec produced 147 mg/L and 155 mg/L of isobutanol with 3.51 mg/g and 3.71 mg/g of yield, respectively (Table 9). Titer and yield of isobutanol were 11% and 13% higher than the corresponding values of the control strain. Elimination of competitive pathways in the cytosol was not that effective compared to mitochondria. Because most of pyruvate in the cytosol is converted to ethanol in *S. cerevisiae*, it is very hard to increase the production of isobutanol without deleting the ethanol biosynthesis pathway.

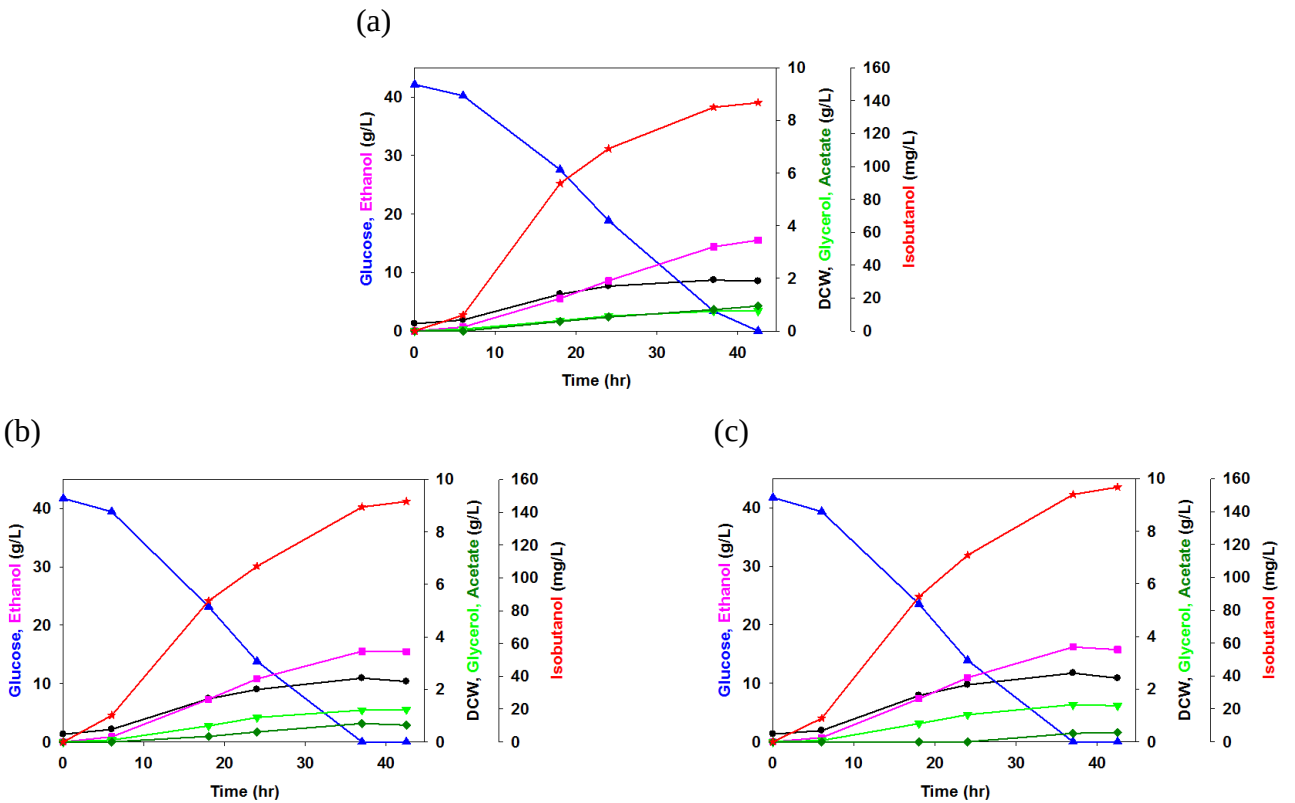


Figure 7. Profiles of cell mass and metabolites in batch culture of (a) WΔGBI_2vec, (b) WΔGBIA_2vec, (c) WΔGBIAL_2vec.

Symbols : glucose (▲), DCW (●), glycerol (▼), ethanol (■), isobutanol (★)

Table 9. Summary of flask cultivation in part 2.1

Strain	**Maximum dry cell weight (g/L)**	**Concentration of Ethanol (g/L)**	**Concentration of isobutanol (mg/L)**	**Yield of isobutanol ($mg_{isobutanol}/g_{glucose}$)**
WΔGBI_2vec	1.9	15.5	139	3.29
WΔGBIA_2vec	2.3	15.5	147	3.51
WΔGBIAL_2vec	2.4	15.8	155	3.71

2.2. Elimination of ethanol biosynthesis pathway by using pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae*

Pyruvate decarboxylase and alcohol dehydrogenase are the two key enzymes for ethanol production in *S. cerevisiae*. To block ethanol production, *PDC1,5,6*-deficient *S. cerevisiae* D452-2 (SOS5) was constructed in a previous study (Kim, Seo et al., 2015). Also, KH Park engineered the SOS5 strain to synthesize the [Fe-S] clusters and assemble it with cytosolic enzymes more efficiently (SOS5⊿G). In this study, *BAT1* of SOS5⊿G was disrupted (SOS5⊿GB) and the strain expressing the enzymes for isobutanol biosynthesis (SOS5⊿GB_3vec) was constructed. This strain was tested in YPD medium with 40 g/L glucose and supplementation of 0.2 % ethanol. However, SOS5⊿GB_3vec cannot produce isobutanol at all. In order to explore a plausible reason for not producing isobutanol, the additional experiment was performed. Although 4 g/L 2-KIV was added as a starting compound to the medium, no isobutanol production was detected. (data not shown)

Because isobutanol was not produced from 2-KIV, it’s possible to

hypothesize pyruvate decarboxylase is the actual enzyme to convert 2-KIV into isobutyraldehyde. In previous studies, ketoacid decarboxylase (KDC) activity of pyruvate decarboxylase was reported (Atsumi, Hanai et al., 2008; Dickinson, Harrison et al., 1998).

To confirm the ketoacid decarboxylase (KDC) activity of *PDC1,5,6*, plasmids harboring *PDC1,5,6*, respectively, were introduced to SOS5ΔGB (SOS5ΔGB_PDC1, SOS5ΔGB_PDC5 and SOS5ΔGB_PDC6). The constructed strains were tested in YPD medium with 40 g/L glucose and 4 g/L KIV. Profiles are displayed for the batch culture of the constructed strains in Figure 8.

In batch cultivation, SOS5ΔGB_PDC1, SOS5ΔGB_PDC5 and SOS5ΔGB_PDC6 produced 1.0 g/L, 1.3 g/L and 1.2 g/L of isobutanol, respectively (Table 10). These results indicate pyruvate decarboxylase (PDC*1,5,6*) could catalyze the conversion of 2-KIV into isobutyraldehyde. Therefore, deletion of *PDC1,5,6* is not an affordable strategy to block ethanol biosynthesis for isobutanol production. Instead of pyruvate decarboxylase, alcohol dehydrogenase is a more reasonable target for deletion to block ethanol biosynthesis.

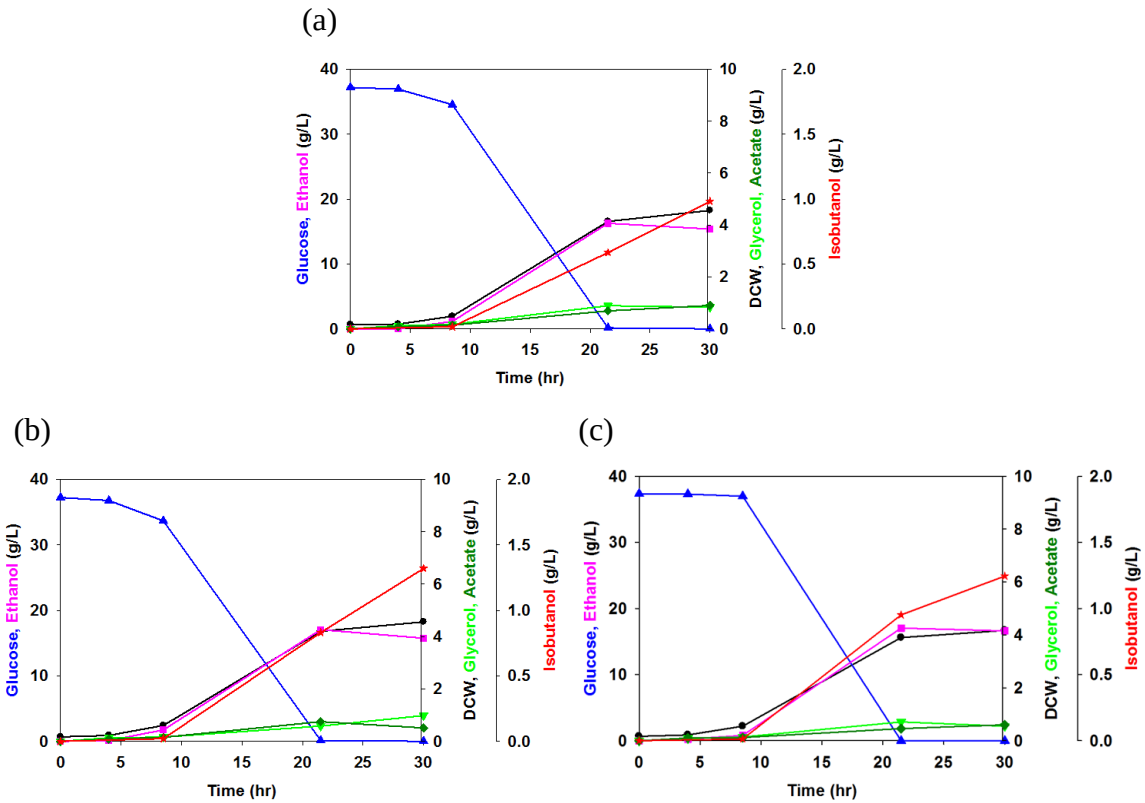


Figure 8. Profiles of cell mass and metabolites in batch culture of (a) SOS5ΔGB_PDC1, (b) SOS5ΔGB_PDC5, (c) SOS5ΔGB_PDC6.

Symbols : glucose (▲), DCW (●), glycerol (▼), ethanol (■), isobutanol (★)

Table 10. Summary of flask cultivation in part 2.2

Strain	**Maximum dry cell weight (g/L)**	**Concentration of Ethanol (g/L)**	**Concentration of isobutanol (g/L)**
SOS5ΔGB_PDC1	4.6	15.4	1.0
SOS5ΔGB_PDC5	4.6	15.7	1.3
SOS5ΔGB_PDC6	4.2	16.6	1.2

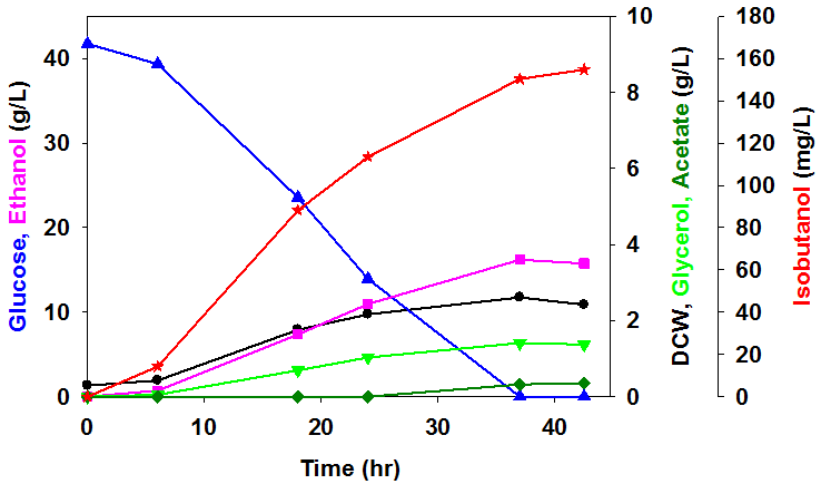
2.3. Elimination of ethanol biosynthesis pathway by disrupting alcohol dehydrogenase gene

There are seven alcohol dehydrogenase isozymes (*ADH1-7*) in *S. cerevisiae*. Among the alcohol dehydrogenase isozymes, *ADH1* is the major isozyme for ethanol production (Dorsey, Peterson et al., 1992). Although deletion of *ADH1* is not sufficient for totally blocking ethanol production (Ida, Furusawa et al., 2012), *ADH1* was disrupted by the CRISPR-Cas9 to reduce the production of ethanol. The control strain was WΔGBIAL_2vec and the *ADH1* disrupted strain was WΔGBIALA1_2vec. Both strains overexpressed the enzymes involved in the isobutanol biosynthesis using two vectors which have different amino acid auxotroph markers (*alsS* from *B. subtilis*, M.*ilvC* from *E. coli*, *ilvD* from *L. lactis* and *kivd* from *L. lactis*). Profiles are displayed for the batch culture of the constructed strains in Figure 9.

The *ADH1* disrupted strain (WΔGBIALA1_2vec) produced 177 mg/L of isobutanol with 4.20 mg/g of yield, which are 14 % and 13 % higher than the corresponding values of the control strain, respectively (Table 11). Even though ethanol titer was similar to the

control strain, isobutanol titer was improved. This result suggests that totally elimination of ethanol production by deletion of other alcohol dehydrogenase isozymes would lead a considerable improvement of isobutanol production (Ida, Furusawa et al., 2012).

(a)



(b)

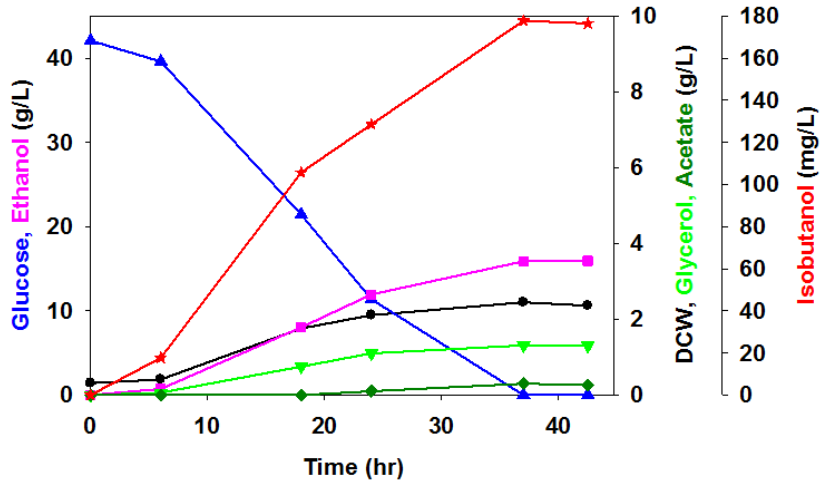


Figure 9. Profiles of cell mass and metabolites in batch culture of (a) WΔGBIAL_2vec, (b) WΔGBIALA1_2vec.
Symbols : glucose (▲), DCW (●), glycerol (▼), ethanol (■), isobutanol (★)

Table 11. Summary of flask cultivation in part 2.3

Strain	**Maximum dry cell weight (g/L)**	**Concentration of Ethanol (g/L)**	**Concentration of isobutanol (mg/L)**	**Yield of isobutanol ($mg_{isobutanol}/g_{glucose}$)**
WΔGBIAL_2vec	2.4	15.8	155	3.71
WΔGBIALA1_2vec	2.4	15.9	177	4.20

3. Isobutanol production in bioreactor with gas trapping

To improve the isobutanol production, batch cultivation was performed in a bioreactor with a gas trapping system equipped by KH Park (Baez, Cho et al., 2011; Park, 2016). In micro-aerobic (300 rpm, 1 vvm) cultivation with YPD medium, WΔGBIALA1_2vec produced 662 mg/L of isobutanol with 6.71 mg/g of yield, which were 3.8-fold and 1.6-fold higher than the corresponding values in flask cultivation. (Figure 10; Table 12). This improvement of isobutanol production indicates that isobutanol, a volatile compound, can be collected by the gas trapping system. However, 39.3 g/L of ethanol was produced with 0.40 g/g of yield, which means most of consumed glucose was converted to ethanol. In further studies, it is necessary to block the ethanol production by eliminating other genes encoding alcohol dehydrogenase isozymes. Furthermore, another ketoacid decarboxylase (KDC) which has enhanced activity to 2-KIV should be screened to consolidate the isobutanol biosynthesis.

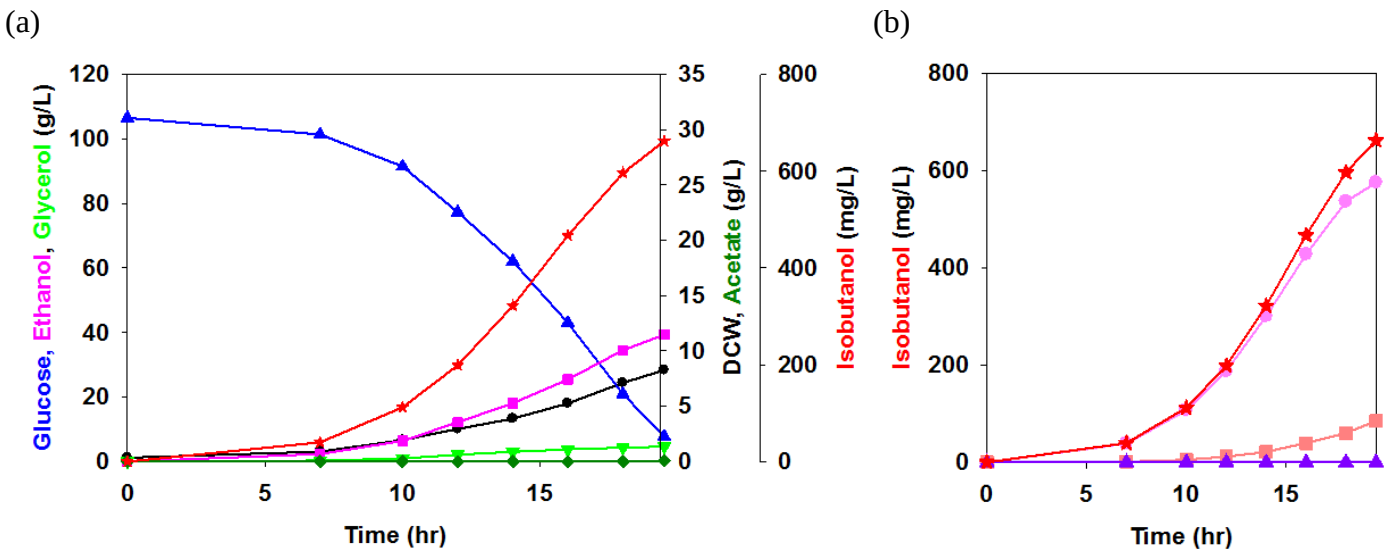


Figure 10. (a) Profile of cell mass and metabolites in batch culture of WΔGBIALA1_2vec, (b) Profile of trapped isobutanol in batch culture of WΔGBIALA1_2vec.

Symbols : glucose (▲), DCW (●), glycerol (▼), ethanol (■), total isobutanol (★), bioreactor (●), 1st picker (■), 2nd picker (▲)

Table 12. Summary of bioreactor cultivation of WΔGBIALA1_2vec with gas trapping

Strain	**Maximum dry cell weight (g/L)**	**Consumed glucose (g/L)**	**Concentration of Ethanol (g/L)**	**Concentration of isobutanol (mg/L)**	**Yield of isobutanol ($mg_{isobutanol}/g_{glucose}$)**
WΔGBIALA1_2vec	8.3	98.7	39.3	662	6.71

IV. CONCLUSIONS

This thesis can draw the following conclusions :

(1) To eliminate L-valine, L-isoleucine, L-leucine, isobutyrate and ethanol synthesis, *BAT1*, *ILV1*, *LEU1*, *ALD6* and *ADH1* were disrupted by CRISPR-Cas9. In flask culture, the constructed *S. cerevisiae* WΔGBIALA1_2vec produced 177 mg/L of isobutanol with 4.20 mg/g of yield.

(2) In batch culture with gas trapping, 662 mg/L of isobutanol was produced with 6.7 mg/g of yield by WΔGBIALA1_2vec.

(3) As WΔGBIALA1_2vec still produced high concentration of the ethanol, additional disruption of the genes encoding alcohol dehydrogenase isozymes would be necessary.

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국 문 초 록

석유 기반의 연료에 의한 지구 온난화와 기상변화가 심화됨에 따라 생물자원 기반의 연료를 생산하는 연구가 활발히 진행되고 있다. Isobutanol은 에너지 밀도 및 옥탄가가 높고, 흡습성 및 증기압이 낮아 연료로서의 가치가 높다. 또한 다양한 화학물질의 전구체로 사용될 수 있어 화학적 가치 또한 높다.

본 연구에서는 산업적 생산에 유리한 미생물인 *Saccharomyces cerevisiae*를 이용하여 isobutanol을 생산하고자 하였다. Isobutanol은 L-valine 생합성 경로와 에를리히 경로를 통해 생산된다. 기존 연구를 통해 세포질에서 해당 경로의 효소를 과발현하는 *S. cerevisiae*가 구축되었지만, 여전히 isobutanol의 생산 수율이 낮다는 문제점이 있었다. 따라서 isobutanol 생산 경로의 경쟁 경로로 작용하는 L-valine, L-isoleucine, L-leucine, isobutyrate 그리고 ethanol 생합성 경로를 CRISPR-Cas9 시스템을 통해

차단하고자 하였다. 이 균주에 isobutanol 생합성 경로인 *Bacillus subtilis* 유래의 acetolactats synthase (ALS), *Escherichia coli* 유래의 ketolacid reductoisomerase (KARI) 그리고 *Lactococcus lactis* 유래의 dihydroxy-acid dehydratase (DHAD)와 ketoacid decarboxylase (KDC)를 도입하였다. 그 결과, 경쟁 경로를 차단하지 않은 대조군 대비 8.6배 향상된 생산 수율로 isobutanol을 생산할 수 있었다. 또한 이 균주로 발효기 수준에서 가스 포집 장치를 이용한 회분식 배양을 수행한 결과, 662 mg/L의 isobutanol을 6.7 mg/g 의 수율로 생산할 수 있었다.

본 연구를 통해 경쟁 경로가 *S. cerevisiae*의 낮은 isobutanol 생산성의 원인 중 하나임을 알 수 있었다. 이후에는 에탄올 생산의 완전한 차단과 높은 활성의 KDC에 대한 탐색을 통해 isobutanol의 생산성을 향상시킬 수 있을 것이다.

주요어 : 바이오 연료, Isobutanol, *Saccharomyces cerevisiae*, Branched-chain amino acid biosynthesis,

Alcohol dehydrogenase, CRISPR-Cas9, Gas trapping

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