A Thesis for the Degree of Master of Science

Enhancement of isobutanol production in engineered *Saccharomyces cerevisiae* by optimizing cytosolic valine biosynthesis

세포질 내 발린 생합성 경로 최적화를 통한 재조합 효모의 아이소부탄올 생산성 향상에 관한 연구

By

Kyung-Hye Park

Department of Agricultural Biotechnology
Seoul National University
February 2016

A Thesis for the Degree of Master of Science

Enhancement of isobutanol production in engineered *Saccharomyces cerevisiae* by optimizing cytosolic valine biosynthesis

Advisor : Professor Jin-Ho Seo

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

By

Kyung-Hye Park

Department of Agricultural Biotechnology
Seoul National University
February 2016

農 學 碩 士 學 位 論 文

Enhancement of isobutanol production

in engineered *Saccharomyces cerevisiae*

by optimizing cytosolic valine biosynthesis

세포질 내 발린 생합성 경로 최적화를 통한
재조합 효모의 아이소부탄올 생산 증가에 관한 연구

指導敎授 徐 鎭 浩

이 論文을 農學碩士學位論文으로 提出함
2016年 2月

서울大學校 大學院
農生命工學部 食品生命工學 專攻
朴 暻 蕙

朴 暻 蕙 의 農學碩士 學位論文을 認准함
2016年 2月

委 員 長 문 래 화 (인)
副委員長 서 진 호 (인)
委 員 최 영 진 (인)

ABSTRACT

Global environmental problems due to fossil fuel and its depletion have promoted the development of microorganisms for synthesizing alternative liquid biofuels. Compared to ethanol, a traditional biofuel, biobutanol has significant advantages such as higher energy density, lower hygroscopicity and compatibility with existing transportation infrastructures. Especially, isobutanol has a higher octane number, a standard measure of the performance of gasoline. Also, it has been applied in various industrial fields and used as an important platform chemical.

In this study, *Saccharomyces cerevisiae* which has been traditionally used for industrial ethanol production was engineered to produce isobutanol. Naturally *S. cerevisiae* produces isobutanol at low concentrations by the valine biosynthesis pathway and the Ehrlich pathway. However, there are some problems because of different

location of the valine biosynthesis pathway and the Ehrlich pathway. Therefore, this thesis was focused on optimizing the cytosolic valine biosynthesis pathway for improving isobutanol production.

First, investigations of various genes from bacteria involved in the valine biosynthesis pathway were conducted. The *alsS* gene from *Bacillus subtilis* which has high specificity to pyruvate was selected for expression of acetolactate synthase (ALS). The genes coding for ketolacid reductoisomerase (KARI) and dihydroxyacid dehydratase (DHAD) from *Escherichia coli* and *Corynebacterium glutamicum* were cloned and introduced to the D452-2 strain with keto acid decarboxylase (KDC) of *Lactococcus lactis*. All of genes which have the mitochondria targeting sequences were modified. The yeast strain containing the endogenous genes of ALS, KARI and DHAD was used as the control strain. Flask fermentations with 40 g/L glucose was carried out under micro-aerobic conditions. As a result, the isobutanol

titer and yield of the strain expressing *alsS* from *B. subtilis*, *ilvC* and *ilvD* from *C. glutamicum* and *kivD* from *L. lactis* were the highest among engineered strains used in this study.

Second, to improve the activity of DHAD, engineering of the Fe-S cluster was conducted. The Grx3 protein controls iron sensing. Cfd1 is an important factor of the cytosolic Fe-S cluster assembly. The CRISPR/Cas9 system was used to replace the *GRX3* gene in the chromosome of *S. cerevisiae* with the *CFD1* gene to construct the D_FeS strain. The D_FeS-SK_CCMDC strain was constructed by introducing *alsS* from *B. subtilis*, *ilvC* and *ilvD* from *C. glutamicum* and *kivD* from *L. lactis*. Compared to the D-SK_CCMDC, the strain D_FeS-SK_CCMDC produced isobutanol titer more than 60%.

Finally, the D_FeS-SK_CCMDC was cultivated in a reactor with gas trapping and the fermentation condition was controlled by altering agitation speed and aeration. The final concentration of isobutanol of

the D_FeS-SK_CCMDC was 246 mg/L with isobutanol yield (6.15 mg isobutanol/g glucose), corresponding to a 25% increase in titer and a 32% increase in yield than those obtained in flask fermentation.

These results suggested that modulation of the cytosolic valine biosynthetic pathway in combination with optimization of a fermentation process can be a successful strategy for producing isobutanol in *S. cerevisiae*.

Keywords : biofuels, isobutanol, *S. cerevisiae*, valine biosynthesis pathway, Fe-S cluster, gas trapping, *in situ* removal system

Student Number: 2014-20699

CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

1. Isobutanol

1.1. Advanced biofuel

Growing concerns about the acceleration of global warming and the depletion of fossil fuels have stimulated increased efforts towards production of biofuels from biomass, a renewable energy source (Baez, Cho et al., 2011).

Ethanol is a traditional biofuel, with existing commercial production. However, ethanol is not an ideal fuel because of several chemical properties, such as lower energy density compared to gasoline and a high tendency to absorb water. Especially, blending of ethanol cannot use an existing transportation infrastructure. Also, it cannot be used as a jet fuel because of high hygroscopicity (Table 1) (Atsumi, Hanai et al., 2008; Atsumi and Liao, 2008; Chen, Nielsen et al., 2011). Therefore, higher or branched alcohols have received much attention as alternative fuels and fuels additives (Kondo, Tezuka et al., 2012).

Among several alternative fuels, biobutanol has shown great promise because of its very similar properties to gasoline (Table 1) (Ranjan and Moholkar, 2012). Butanol possesses superior fuel

properties relative to ethanol, such as increased energy density and low hygroscopicity (Connor and Liao, 2008). In addition, it can easily mix with gasoline in any proportion and the air to fuel ratio and the energy content of butanol are close to gasoline (García, Päkkilä et al., 2011). These advantages allow safer handing and more efficient blending than ethanol (Blombach, Riester et al., 2011).

Isobutanol, one of the butanol isomers, is similar to 1-butanol but has lower toxicity and higher octane values because it is a branched-chain alcohol (Figure 1) (Table 1) (Matsuda, Ishii et al., 2013). A high-octane hydrocarbon can burn in a combustion engine like gasoline without adversely affecting the performance (Bastian, Liu et al., 2011).

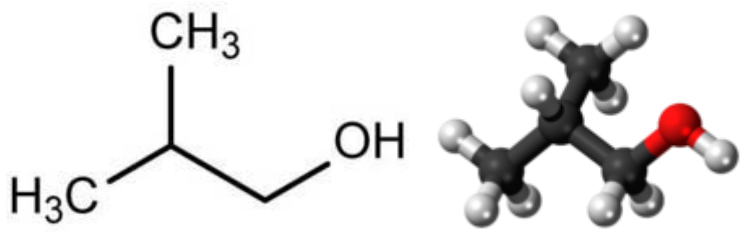


Figure 1. The structure of isobutanol

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
Higroscopicity	High	Low	Low
Fits current infrastructure?	No	Yes	Yes

1.2. Building block chemical

Isobutanol and its derivatives are used in a variety of industrial applications, including solvents, ink ingredients, paint, additives, and raw materials for organic compounds (Figure 2) (Akita, Nakashima et al., 2015). Furthermore, isobutanol can be readily used as a precursor for a number of valuable chemical syntheses (Lee, Seo et al., 2012). Isobutene, a dehydrated form of isobutanol, is exclusively produced in a large scale by petroleum refining and is used as a gasoline additive and for the production of butyl rubber and specialty chemicals. When isobutene is oligomerized to C8 alkene, it can be used for plastic polyethylene terephthalate (PET) by dehydrocylization and oxidation (Blombach, Riester et al., 2011; Lin, Mi et al., 2015). Isobutanol is used as feedstock of isobutyl acetate, which has used for producing lacquer and similar coatings. In the food industry, it is used as a flavouring agent. Also, isobutanol is a precursor of isobutyl esters – such as diisobutyl phthalate (DIBP), plasticizer agents in plastics, rubbers, and other dispersions.

Figure 2. Various usages of isobutanol

2. Microbial production of isobutanol

Isobutanol is a normal by-product of fermentations, but only in very small amounts (Brat, Weber et al., 2012). Therefore, there have been a lot of efforts to produce isobutanol efficiently by metabolic engineering. As a result, engineered *E. coli* produced ~22 g/L of isobutanol after 110 h at a yield (g isobutanol per g glucose) of 86% of the theoretical maximum (Atsumi, Hanai et al., 2008). Production of isobutanol from *C. glutamicum* and *B. subtilis* was up to 4.9 g/L and 2.62 g/L, respectively (Smith, Cho et al., 2010; Li, Wen et al., 2011) (Table 2).

Although the engineered bacterial strains produced high concentration of isobutanol, isobutanol toxicity is the major bottleneck for their industrial scale application (Park, Kim et al., 2014). Since *S. cerevisiae* is a workhorse in industrial manufacture of biofuels and chemicals and has the natural capacity to secrete fusel alcohols as by-products, this organism is supposed to be an attractive host for the production of higher alcohols (Bisaria and Kondo, 2014; Ida, Ishii et al., 2015). Also, it is tolerant to high concentrations of n-butanol, the straight chain isomer of isobutanol, by the same mechanisms it tolerates ethanol (Fischer, Klein-Marcuschamer et al.,

2008; Steen, Chan et al., 2008; Li, Wen et al., 2011).

The common approach to engineer *S. cerevisiae* for isobutanol production is to overexpress the native or truncated genes of the L-valine biosynthetic pathway. These strains were further improved by overexpressing the genes of the Ehrlich pathway as well as the *BAT2* gene, encoding yeast cytoplasmic branched-chain amino acid transferase (Chen, Nielsen et al., 2011; Brat, Weber et al., 2012; Lee, Seo et al., 2012; Matsuda, Ishii et al., 2012; Kondo, Tezuka et al., 2012). To improve pyruvate availability by blocking production of ethanol, a major by-product in production of isobutanol, the pyruvate dacarboxylase gene *PDC1* was deleted (Kondo, Tezuka et al., 2012). However the titer, yield and productivity of isobutanol should be developed because it is still low amount (Table 2).

Table 2. Microbial production of isobutanol

Strains	Substrates	Methods	Isobutanol Concentration	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
Corynebacterium glutamicum	Cellulose	Flask	660 mg/L	66 mg/g	Higashide et al., 2011
	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
	Glucose	Flask	224 mg/L	12 mg/g	Ida, Ishii et al., 2015

3. Isobutanol biosynthesis pathway

3.1. The valine biosynthesis pathway

The isobutanol biosynthesis pathway in *S. cerevisiae* consists of glycolysis, anabolic synthesis of 2-ketoisovalerate (an intermediate of valine biosynthesis) in the mitochondria and catabolism of this 2-ketoisovalerate into isobutanol in the cytosol (the Ehrlich pathway) (Figure 3) (Buijs, Siewers et al., 2013).

After conversion of glucose into pyruvate, pyruvate can be converted into 2-ketoisovalerate by the valine biosynthesis pathway. First, pyruvate is imported into the mitochondria and two molecules are condensed to 2-acetolactate by acetolactate synthase (ALS). It has a valine-responsive regulator, encoded by *ILV6*. In the subsequent step, 2-acetolactate is reduced to 2,3-dihydroxy isovalerate by ketoacid reductoisomerase (KARI). Finally, 2,3-dihydroxy isovalerate is converted to 2-ketoisovalerate by dihydroxyacid dehydratase (DHAD), an iron sulfur (FeS) cluster containing enzyme. For isobutanol synthesis, 2-ketoisovalerate has to be transported into the cytosol (Generoso, Schadeweg et al., 2015).

To enhance the valine biosynthesis pathway, the *alsS* gene from *B.*

subtilis coding for ALS, the *ilvC* and *ilvD* genes from *C. glutamicum* coding for KARI and DHAD were used in this study.

3.2. The Ehrlich pathway

Isobutanol is produced from 2-ketoisovalerate by two successive steps of the Ehrlich pathway, which involves decarboxylation by keto acid decarboxylase (KDC) and reduction of an aldehyde group by alcohol dehydrogenase (ADH) (Figure 3) (Park, Kim et al., 2014).

KDC is known as a critical enzyme for removing the carboxylic group of 2-keto acid to produce aldehyde (König, 1998). 2-ketoisovalerate, an important intermediate for the valine biosynthesis pathway, can be converted to isobutyraldehyde by KDC. And then isobutyraldehyde can be converted to isobutanol by ADH (Hazelwood, Daran et al., 2008).

Among various putative KDC enzymes, the *kivD* gene from *Lactococcus lactis subsp. lactis* KACC13877 was identified as the most suitable KDC for isobutanol production in *S. cerevisiae* (Lee, Seo et al., 2012). Wild *S. cerevisiae* has several ADHs, studies for determining the most suitable dehydrogenase to isobutyraldehyde, the precursor of isobutanol, were conducted. *ADH2* showed the highest activity with isobutyraldehyde (Brat, Weber et al., 2012).

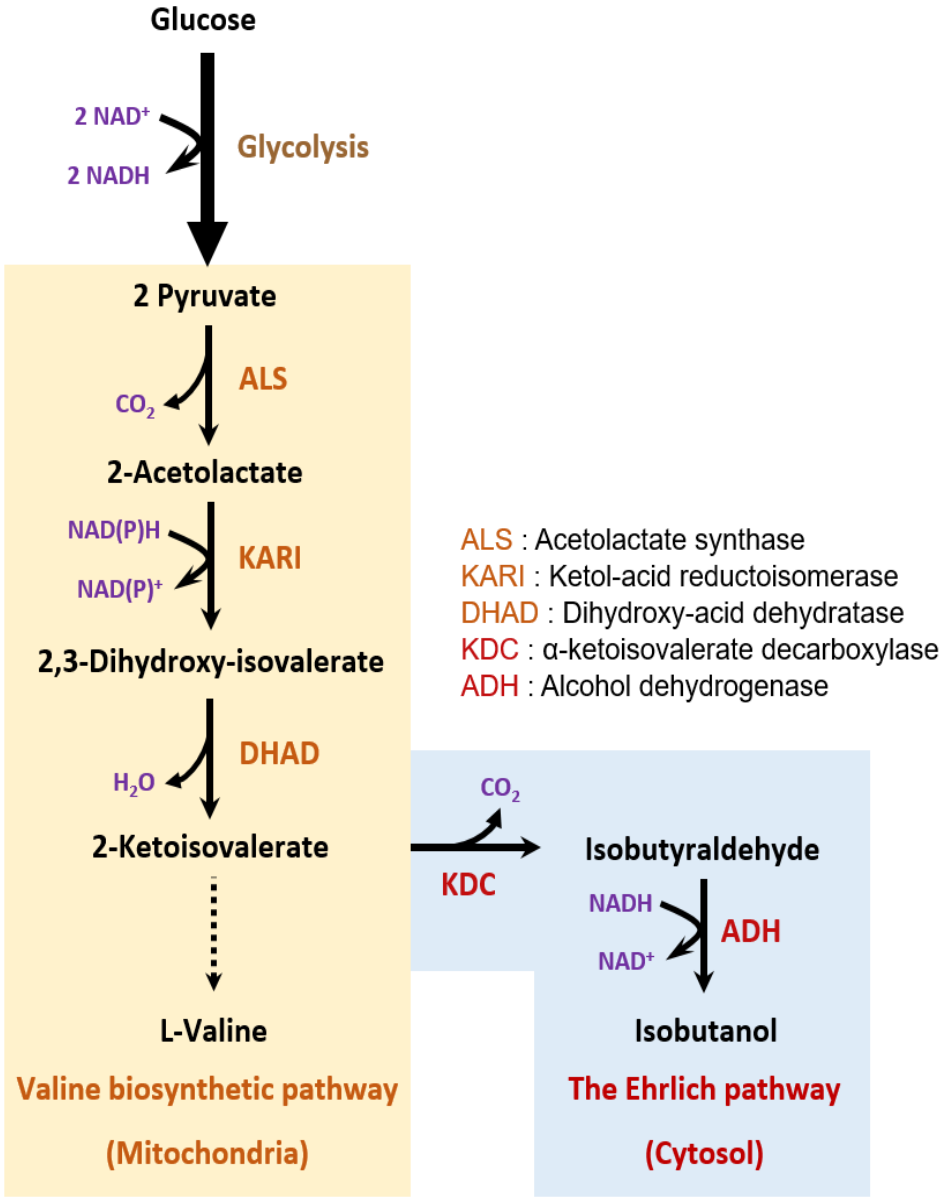


Figure 3. Isobutanol biosynthesis pathway

4. Cytosolic localization of the valine biosynthesis pathway in *S. cereivsiae*

The enzymes consisting of the valine biosynthesis pathway of *S. cerevisiae* are located in the mitochondria (Ryan and Kohlhaw, 1974). But the Ehrlich pathway is proceeded in the cytosol. In order to remove the need to transport intermediates between the mitochondria and cytosol and to increase availability of intermediates, there are many studies for compartmentalization of the isobutanol biosynthesis pathway into the mitochondria or cytosol.

Moving the complete pathway into mitochondria resulted in increased isobutanol production by 260%, compared with a strain overexpressing KDC and ADH in the cytosol. KDC and ADH were overexpressed in the mitochondria by fusing them with an N-terminal targeting sequence (Figure 4) (Avalos, Fink et al., 2013). On the other hand, a fully cytosolic pathway was also established. To re-localize ALS, KARI and DHAD into the cytosol, the mitochondrial targeting sequences of *ILV2*, *ILV5* and *ILV3* were deleted (Brat, Weber et al., 2012; Lee, Seo et al., 2012). The presence of all the enzymes in the same compartment can increase the production of isobutanol in *S. cerevisiae*.

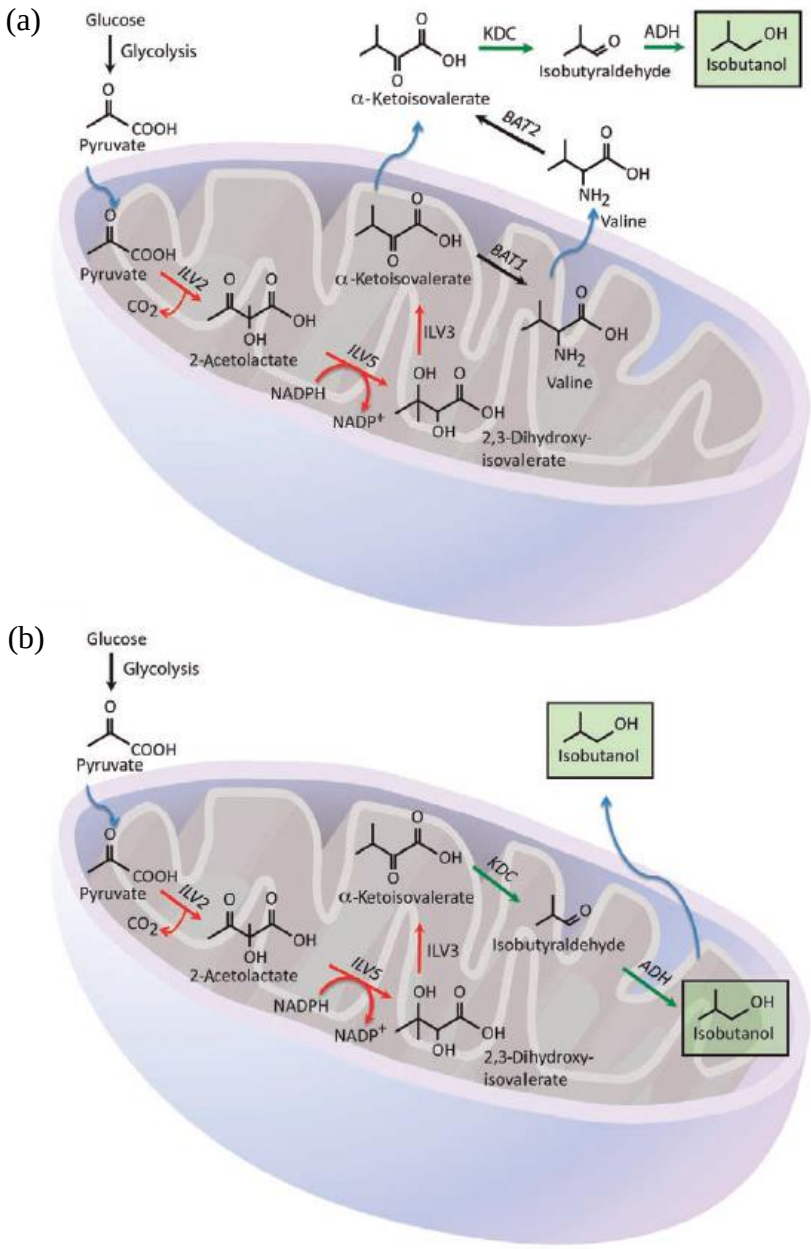


Figure 4. Compartmentalization of the whole isobutanol biosynthesis pathway into mitochondria of *S. cerevisiae* (Avalos, Fink et al., 2013)

In this study, the valine biosynthesis pathway was expressed in the cytosol by using the modified genes which were truncated the mitochondria targeting sequences (Figure 5). First, the *ILV* genes of *S. cerevisiae* were modified by Professor Yong-Su Jin's group at University of Illinois at Urbana-Champaign. They predicted the mitochondrial targeting sequences of the *ILV* genes by using MitoProt (http://ihg.gsf.de/ihg/mitoprot.html, Claros and Vincens, 1996). And then, the *ilvC* and *ilvD* genes from *E. coli* and the *ilvD* gene from *C. glutamicum* were modified in the same way. They encode KARI and DHAD and are known to be expressed in the cytosol of bacteria. When these genes were introduced in yeast, they have high possibility to locate in the mitochondrial matrix (Figure 6).

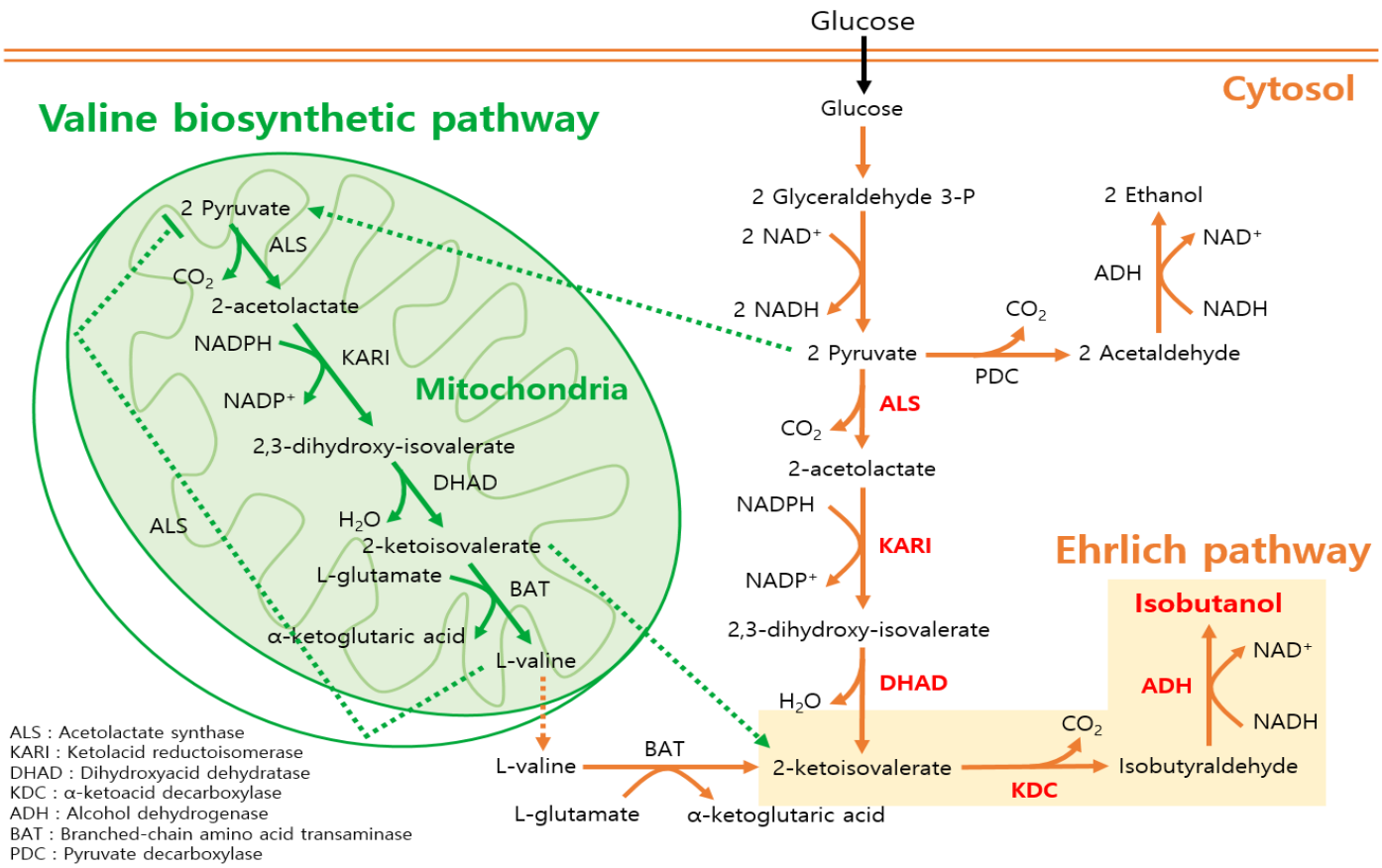


Figure 5. Strategy to produce isobutanol in cytosol of *S. cerevisiae*

(a) amino acid sequence encoded by *ILV2*, **0.9991**

MIRQSTLKNFAIKRCFQHIAYRNTPAMRSVALAQRFYSSSSRYYSASPLPASKRPEPAPSFNVDPLEQPAEPSKLAKKL
RAEPDMDTSFVGLTGGQIFNEMMSRQNVDTVFGYPGGAILPVYDAIHNSDKFNFVLPKHEQGAGHMAEGYARASG
KPGVVLVTSGPGATNVVTPMADAFADGIPMVVFTGQVPTSAIGTDAFQEADVVGISRSCTKWNVMVKSVEELPLRI
NEAFEIATSGRPGPVLVDLPKDVTAAILRNPIPTKTTLPSNALNQLTSRAQDEFVMQSINKAADLINLAKKPVLYVGA
GILNHADGPRLLKELSDRAQIPVTTTLQGLGSFDQEDPKSLDMLGMHGCATANLAVQNADLIIAVGARFDDRVTGNI
SKFAPEARRAAAEGRGGIIHFEVSPKNINKVVQTQIAVEGDATTNLGKMMSKIFPVKERSEWFAQINKWKKEYPYAY
MEETPGSKIKPQTVIKKLSKVANDTGRHVIVTTGVGQHQMWAAQHWTWRNPHTFITSGGLGTMGYGLPAAIGAQ
VAKPESLVIDIDGDASFNMTLTELSSAVQAGTPVKILILNNEEQGMVTQWQSLFYEHRYSHTHQLNPDFIKLAEAMG
LKGLRVKKQEELDAKLKEFVSTKGPVLLEVEVDKKVPVLPMVAGGSGLDEFINFDPEVERQQTELRHKRTGGKH*

(b) amino acid sequence encoded by *ILV5*, **0.9991**

MLRTQAARLICNSRVITAKRTFALATRAAAYSRPAARFVKPMITTRGLKQINFGGTVET
VYERADWPREKLLDYFKNDTFALIGYGSQGYGQGLNLRDNGLNVIIGVRKDGASWK
AAIEDGWVPGKNLFTVEDAIKRGSYVMNLLSDAAQSETWPAIKPLLTKGKTLYFSHG
FSPVFKDLTHVEPPKDLDVILVAPKGSGRTVRSLFKEGRGINSSYAVWNDVTGKAHEK
AQALAVAIGSGYVYQTTFEREVNSDLYGERGCLMGGIHGMFLAQYDVLRENGHSPSE
AFNETVEEATQSLYPLIGKYGMDYMYDACSTTARRGALDWYPIFKNALKPVFQDLYE
STKNGTETKRSLEFNSQPDYREKLEKELDTIRNMEIWKVGKEVRKLRPENQ*

(c) amino acid sequence encoded by *ILV3*, **0.9462**

MGLLTKVATSRQFSTTRCVAKKLNKYSYIITEPKGQGASQAMLYATGFKKEDFKKPQVGVGSCWWSGNPCNM
HLLDLNNRCSQSIEKAGLKAMQFNTIGVSDGISMGTKGMRYSLQSREIIADSFETIMMAQHYDANIAIPSCDKN
MPGVMMAMGRHNRPSIMVYGGTILPGHPTCGSSKISKNIDIVSAFQSYGEYISKQFTEEEREDVVEHACPGPG
SCGGMYTANTMASAAEVLGLTIPNSSSFPAVSKEKLAECDNIGEYIKKTMELGILPRDILTKEAFENAITYVVAT
GGSTNAVLHLVAVAHSAGVKLSPDDFQRISDTTPLIGDFKPSGKYVMADLINVGGTQSVIKYLYENNMLHGNT
MTVTGDTLAERAKKAPSLPEGQEIIKPLSHPIKANGHLQILYGSLAPGGAVGKITGKEGTYFKGRARVFEEEGA
FIEALERGEIKKGEKTVVVIRYEGPRGAPGMPEMLKPSSALMGYGLGKDVALLTDGRFSGGSHGFLIGHIVPEA
AEGGPIGLVRDGDEIIIDADNNKIDLLVSDKEMAQRKQSWVAPPPRYTRGTLSKYAKLVSNASNGCVLDA*

(d) amino acid sequence encoded by *ilvC* of *E. coli*, **0.8172**

MANYFNTLNLRQQLAQLGKCRFMGRDEFADGASYLQGKKVVIVGCGAQGLNQGLNMRDSGLDISYALR
KEAIAEKRASWRKATENGFKVGTYEELIPQADLVINLTPDKQHSDVVRTVQPLMKDGAALGYSHGFNIVE
VGEQIRKDITVVMVAPKCPGTEVREEYKRGFGVPTLIAVHPENDPKGEGMAIAKAWAAATGGHRAGVLES
SFVAEVKSDLMGEQTILCGMLQAGSLLCFDKLVEEGTDPAYAEKLIQFGWETITEALKQGGITLMMDRLSN
PAKLRAYALSEQLKEIMAPLFQKHMDDIISGEFSSGMMADWANDDKKLLTWREETGKTAFETAPQYEGKIG
EQEYFDKGVLMIAMVKAGVELAFETMVDSGIIEESAYYESLHELPLIANTIARKRLYEMNVVISDTAEYGN
YLFSYACVPLLKPFMAELQPGDLGKAIPEGAVDNGQLRDVNEAIRSHAIEQVGKKLRGYMTDMKRIAVAG*

(e) amino acid sequence encoded by *ilvD* of *E. coli*, **0.5040**

MPKYRSATTTHGRNMAGARALWRATGMTDADFGKPIIAVVNSFTQFVPGHVHLRDLGKLVAEQIEAAG
GVAKEFNTIAVDDGIAMGHGGMLYSLPSRELIADSVEYMVNAHCADAMVCISNCDKITPGMLMASLRL
NIPVIFVSGGPMEAGKTKLSDQIIKLDLVDAMIQGADPKVSDSQSDQVERSACPTCGSCSGMFTANSMN
CLTEALGLSQPGNGSLLATHADRKQLFLNAGKRIVELTKRYYEQNDESALPRNIASKAAFENAMTLDIA
MGGSTNTVLHLLAAAQEAEIDFTMSDIDKLSRKVPQLCKVAPSTQKYHMEDVHRAGGVIGILGELDRA
GLLNRDVKNVLGLTLPQTLEQYDVMLTQDDAVKNMFRAGPAGIRTTQAFSQDCRWDTLDDDRANGCI
RSLEHAYSKDGGLAVLYGNFAENGCIVKTAGVDDSILKFTGPAKVYESQDDAVEAILGGKVVAGDVVVI
RYEGPKGGPGMQEMLYPTSFLKSMGLGKACALITDGRFSGGTSGLSIGHVSPEAASGGSIGLIEDGDLIA
IDIPNRGIQLQVSDAELAARREAQDARGDKAWTPKNRERQVSFALRAYASLATSADKGAVRDKSKLGG*

(e) amino acid sequence encoded by *ilvD* of *C. glutamicum*, **0.9282**

MIPLRSKVTTVGRNAAGARALWRATGTKENEFGKPIVAIVNSYTQFVPGHVHLKNVGDIVADAVRKAG
GVPKEFNTIAVDDGIAMGHGGMLYSLPSREIIADSVEYMVNAHTADAMVCISNCDKITPGMLNAAMRL
NIPVVFVSGGPMEAGKAVVVDGVAHAPTDLITAISASASDAVDDAGLAAVEASACPTCGSCSGMFTANS
MNCLTEALGLSLPGNGSTLATHAARRALFEKAGETVVELCRRYYGEEDESVLPRGIATKKAFENAMAL
DMAMGGSTNTILHILAAAQEGEVDFDLADIDELSKNVPCLSKVAPNSDYHMEDVHRAGGIPALLGELN
RGGLLNKDVHSVHSNDLEGWLDDWDIRSGKTTEVATELFHAAPGGIRTTEAFSTENRWDELDTDAAK
GCIRDVEHAYTADGGLVVLRGNISPDGAVIKSAGIEEELWNFTGPARVVESQEEAVSVILTKTIQAGEVLV
VRYEGPSGGPGMQEMLHPTAFLKGSGLGKKCALITDGRFSGGSSGLSIGHVSPEAAHGGVIGLIENGDIV
SIDVHNRKLEVQVSDEELQRRRDAMNASEKPWQPVNRNRVVTKALRAYAKMATSADKGAVRQVD*

Figure 6. Putative mitochondria targeting sequences (highlighted in red) of various genes and probability of export to mitochondria in yeast (Claros and Vincens, 1996)

5. Cytosolic Fe-S cluster related to activity of dihydroxyacid dehydratase

DHAD, catalyzing conversion of 2,3-dihydroxy-isovalerate to 2-ketoisovalerate, possesses Fe-S clusters. In eukaryotes, Fe-S proteins are present in mitochondria, cytosol, and nucleus, although the initial assembly of this co-factor takes place inside the mitochondria. Therefore, simple overexpression of a Fe-S protein in the cytosol could be meaningless and it is needed to modulate expression of components related in the Fe-S assembly machinery (Generoso, Schadeweg et al., 2015). To increase activated form of DHAD, cells should take more irons and make Fe-S proteins available in the cytosol.

The regulation of iron metabolism is a critical function for cells and is achieved at the transcriptional level. The transcription of the genes for iron uptake and storage in *S. cerevisiae* is primarily regulated by Aft1. Aft1 is the most important transcription factor in regulating the expression of the genes involved in iron homeostasis. Nuclear localization of Aft1 induces the expression of the iron regulon genes in iron-deficient cells but are inactivated in iron-replete cells by moving to the cytosol. Iron inhibition of Aft1 is controlled by monothiol

glutaredoxins Grx3 and Grx4. They physically bind with Aft1 and lead to deactivation of the Aft1-regulated genes. As a result, iron uptake is inhibited. Indeed, cells lacking Grx3 and Grx4 show constitutive expression of the iron regulon genes to increase iron uptake (Figure 7) (Ojeda, Keller et al., 2006; Lill and Mühlenhoff, 2008; Li, Mapolelo et al., 2009; Outten and Albetel, 2013).

Meanwhile, synthesis of the Fe-S protein is controlled by iron content in the cell. Biogenesis of the Fe-S cluster is accomplished by three distinct machineries in eukaryotes; the iron-sulfur cluster (ISC) assembly machinery of mitochondria, ISC export machinery and cytosolic iron-sulfur protein assembly (CIA) machinery. Among these machineries, the CIA machinery is not linked to iron sensing by Aft1. Therefore, in order to not only synthesize Fe-S proteins but also mature cytosolic Fe-S proteins, further engineering of the CIA machinery should be conducted. The first step of the CIA machinery is Cfd1. It forms a complex with Nbp35 and binds up to three [4Fe-4S] clusters. These labile Fe-S clusters can be rapidly transferred and incorporated into target Fe-S apoproteins (Figure 8) (Rutherford, Ojeda et al., 2005; Lill, Dutkiewicz et al., 2006; Netz, Pierik et al., 2007; Sharma, Pallesen et al., 2010).

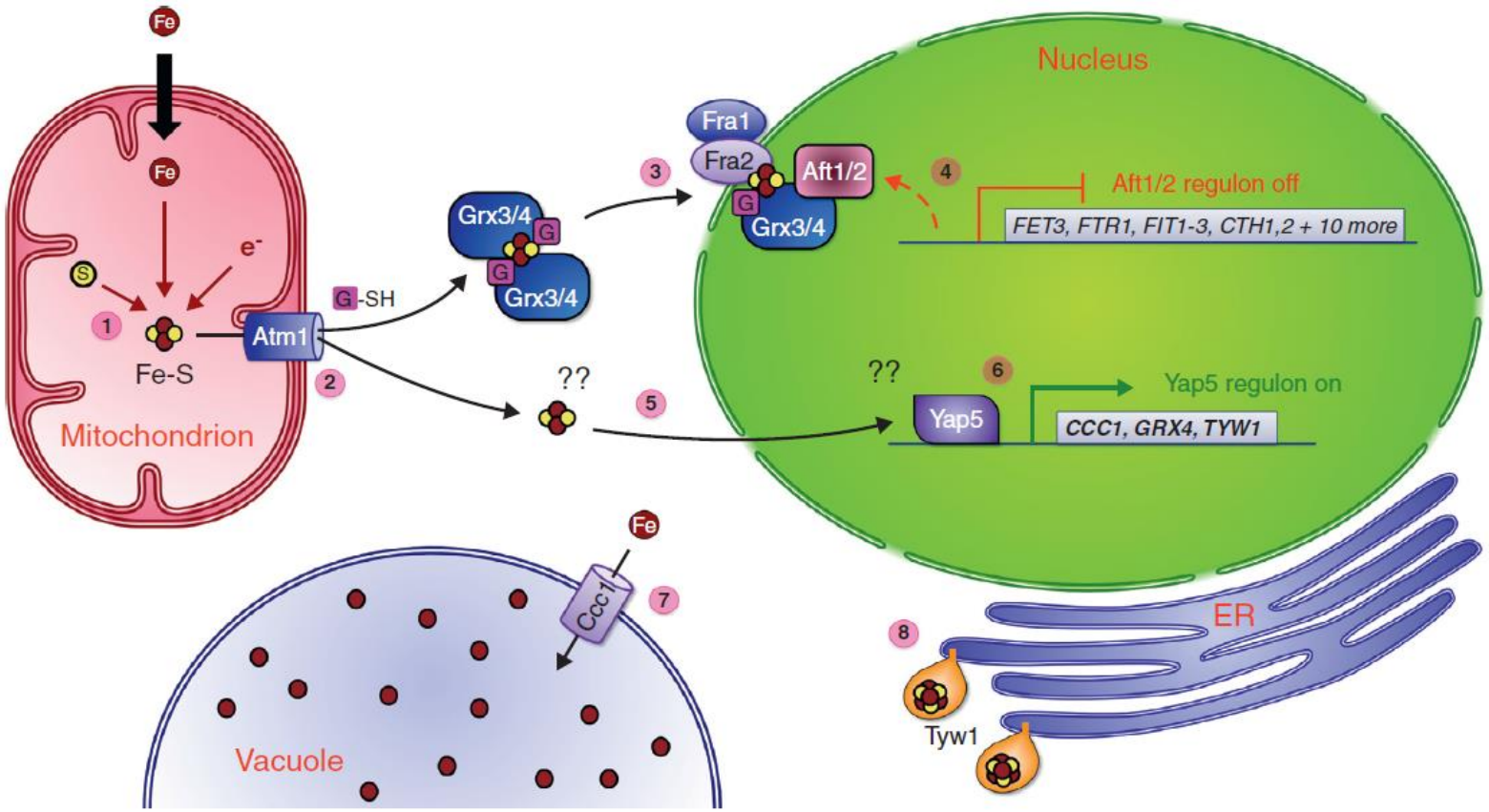


Figure 7. A working model for the iron homeostasis (Outten and Albetel, 2013)

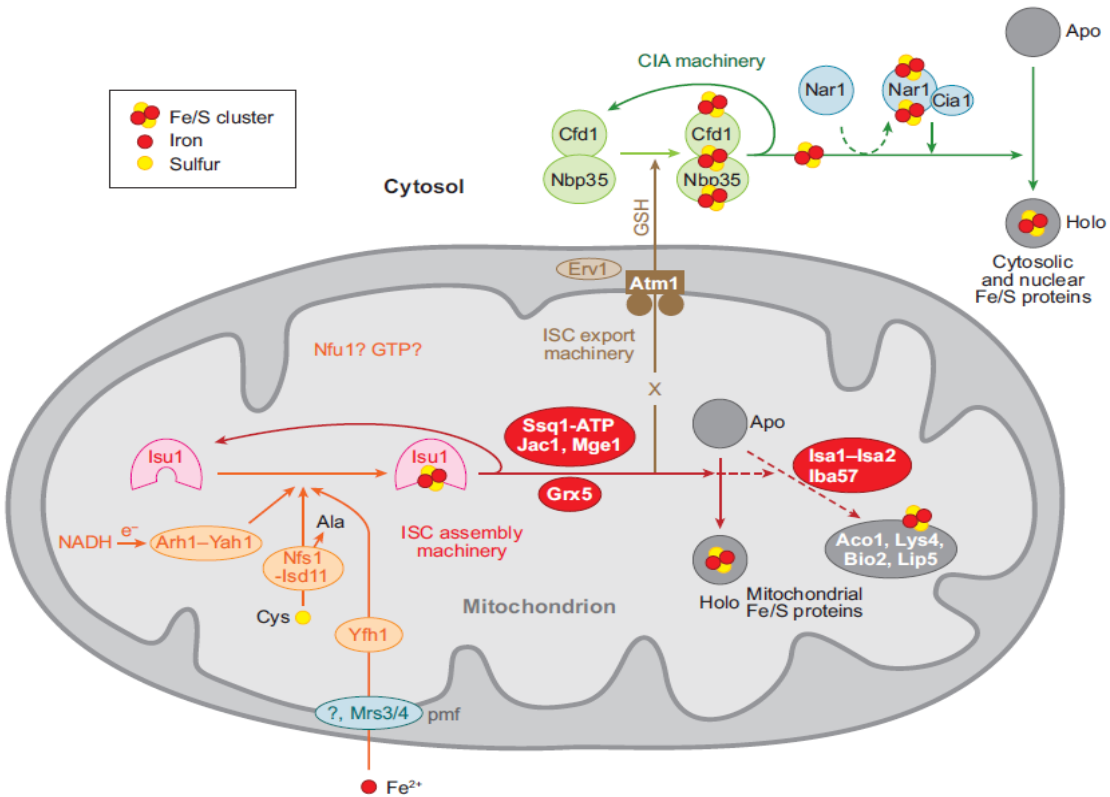
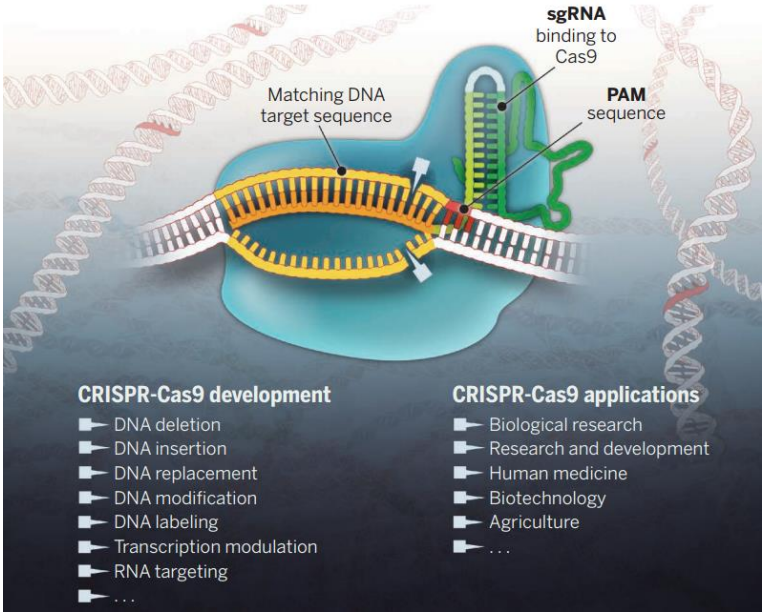


Figure 8. Three distinct systems participate in the generation of Fe-S proteins in eukaryotes (Lill and Mühlenhoff, 2008)

In this study, to enhance DHAD activity by increasing synthesis of the Fe-S cluster, the *GRX3* in chromosome of *S. cerevisiae* was replaced with the *CFD1* cassette which from *GPD* promoter to *CYC1* terminator by the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR associated protein 9) system. Bacteria and archaea have evolved adaptive immune defenses, termed CRISPR/Cas systems, which use short RNA to direct degradation of foreign nucleic acids. Because of its site-specificity and simplicity, it has emerged as a powerful genome-editing tool. A Double-Strand Break (DSB) is generated precisely by Cas9, an RNA-guided endonuclease, and repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR). As a result, various genome engineering such as site-specific mutation, gene knockout and DNA replacement is able to be conducted. (Figure 9) (DiCarlo, Norville et al., 2013; Mali, Esvelt et al., 2013; Mali, Yang et al., 2013; Hsu, Lander et al., 2014; Zhang, Kong et al., 2014)

(a) Overview of CRISPR/Cas9 system as genome editing tool (Doudna and Charpentier, 2014)



(b) Cellular DNA repair mechanisms (Hsu, Lander et al., 2014)

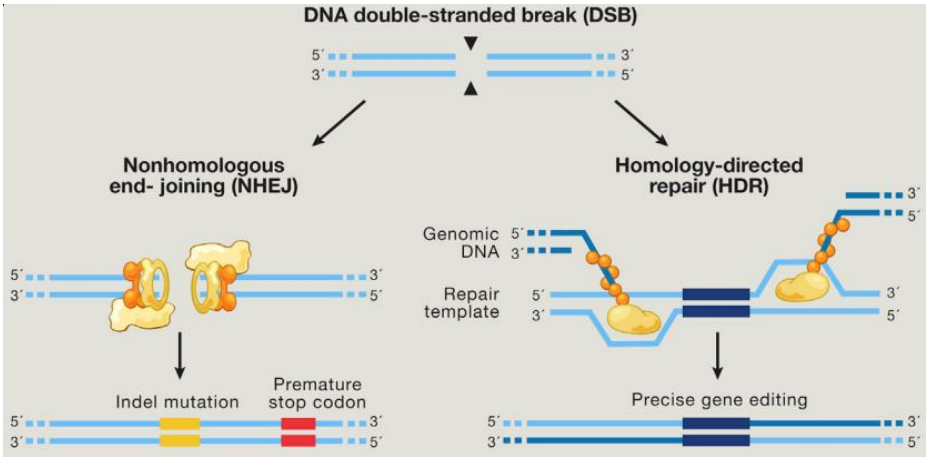


Figure 9. The CRISPR/Cas9 system for targeted genome editing

6. Gas trapping based *in situ* removal system

The accumulation of butanol caused drastically reducing production yields. Pervaporation is one of the most promising methods for continuous separation and concentration of volatile fermentation products. However, pervaporation is a complex process that is not easily applied to commercial scale-up. Several *in situ* solvent recovery strategies have been developed and a much simpler technique, termed gas trapping, has been recognized as a relatively easy, efficient and low-cost method for removing volatile organic compounds, including isobutanol, from aqueous solutions. (Matsumura, Takehara et al., 1992; Inokuma, Liao et al., 2010)

In this study, to improve isobutanol production by metabolically engineered *S. cerevisiae*, the optimization of fermentation conditions and isobutanol removal by gas trapping were performed. (Baez, Cho et al., 2011)

7. Research objectives

For production of isobutanol in metabolically engineered *S. cerevisiae*, several factors should be considered including searching for better enzymes of the valine biosynthetic pathway and enhancement of DHAD activity by engineering the Fe-S cluster synthesis.

The specific objectives of this research are listed:

1) To search for heterologous α-acetolactate synthase, ketolacid reductoisomerase and dihydroxyacid dehydratase for isobutanol production in *S. cerevisiae*,

2) To enhance cytosolic DHAD expression by engineering the Fe-S cluster synthesis in *S. cerevisiae*,

3) To improve titer and yield of isobutanol by optimizing fermentation conditions in a bioreactor.

Ⅱ. Materials and Methods

1. Reagents

All chemicals used were of reagent grade. Glucose, agarose, ampicillin, ethidium bromide, yeast synthetic drop-out supplement, yeast nitrogen base (YNB, w/o amino acid), isobutanol were purchased from Sigma-Aldrich Chemical Company (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); HPLC-grade water from J.T. Baker (Phillipsburg, NJ, USA); NaOH and HCl, NaCl, H_2SO_4, and potassium phosphate 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. *S. cerevisiae* D452-2 [*Matα, leu2 his3 ura3 can1*] was used as host strains for the expression of the genes involved in isobutanol biosynthesis pathway and genome editing.

The detailed procedure of construction of D_FeS is described below, and the schematic map is shown in Figure 10. In step 1, pAur_cas9 was transformed into the D452-2. After that, cells were plated on selective medium (YPDA plate) and allowed to grow until colonies were ready to pick. The resulting strain was named D_cas9. In step 2, a double-stranded oligonucleotide repair DNA for *CFD1* cassette insertion was PCR amplified with primers F3_repair_dGRX3_GPDp and R3_repair_dGRX3_CYCt (Table 6) and pCFD1 as template. The pdGRX3 (~500 ng) was then transformed together with the donor DNA (~2 μg) into D_cas9. Cells were plated on an YPDAH plate and allowed to grow until transformants were ready to pick. In step 3, all of the colonies were

PCR amplified with primers F_GRX3_up_seq and R_GRX3_down_seq (Table 6) to screen insertion of *CFD1* cassette and sequenced. The confirmed *GRX3* deletion and *CFD1* cassette insertion mutant was named D_FeS-cas9 (pdGRX3). In step 4, in order to drop out the pAur_cas9 and pdGRX3, D_FeS-cas9 (pdGRX3) was cultured in liquid YPD medium and then streaked onto a YPD plate for single colonies, which were then PCR amplified with primers F_cas9_seq and R_cas9_seq for pAur_cas9 and F1_SacI_gBlock and R2_KpnI_gBlock for pdGRX3 (Table 6) to confirm the release of the plasmids (Zhang, Kong et al., 2014).

Strains used in this work are described in Table 3. The constructed strains were stored on YPD or YNBD medium respectively in a deep freezer at -80℃ suspended in 15% glycerol.

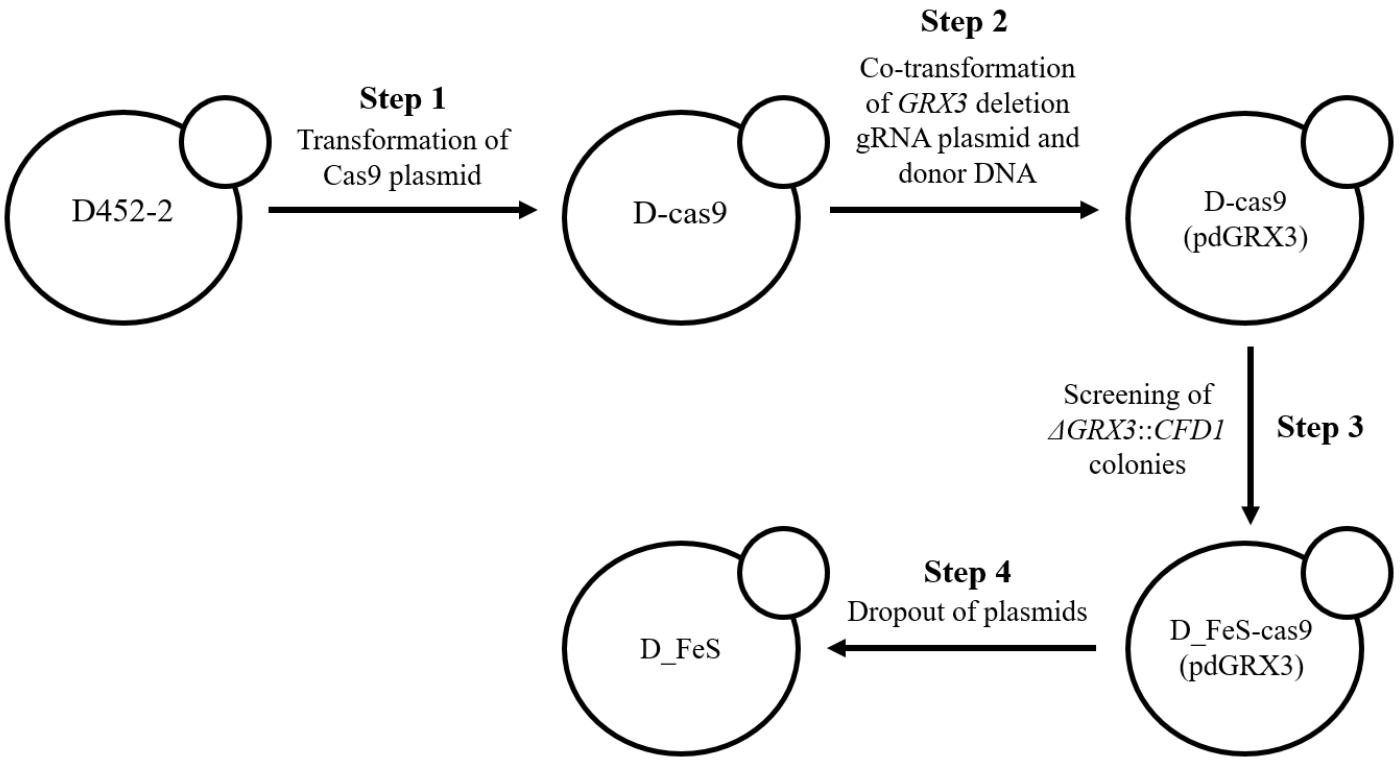


Figure 10. Flow chart for replacement of *GRX3* to *CFD1* cassette in *S. cerevisiae* D452-2

Table 3. List of the strains used in this study

Name	Description
D452-2	*Saccharomyces cerevisiae* (*Matα, leu2 his3 ura3 can1*)
D-M2K_M5M3	D452-2 (pM2_K, pM5_M3)
D-SK_M5M3	D452-2 (pS_K, pM5_M3)
D-SK_MCEMDE	D452-2 (pS_K, pMCE_MDE)
D-SK_CCMDC	D452-2 (pS_K, pCC_MDC)
D-cas9	D452-2 (p414TEF1_aur_cas9)
D_FeS	D452-2 *ΔGRX3*::*CFD1*
D_FeS-SK-CCMDC	D452-2 *ΔGRX3*::*CFD1* (pS_K, pCC_MDC)

2.2. Plasmids

Three plasmids were used as mother vectors which have the *GPD* promoter or truncated *HXT7* promoter and *CYC1* terminator from *S. cerevisiae* (Figure 11). These are cloning vectors for an episomal expression system of the isobutanol biosynthesis pathway, endogenous *ILV2* gene, *alsS* gene from *Bacillus subtilis*, *ilvC* and *ilvD* genes from *E. coli* and *C. glutamicum* and *kivD* gene from *L. lactis*. p425GPD is used for construction of *CFD1* cassette (Table 4). There are oligonucleotide sequence of primers used cloning (Table 5, 6).

Abbreviations and significations used in this study are as follows. M is modified gene which means truncated gene by removing mitochondria targeting sequence for expression in the cytosol. 2 is *ILV2* gene from S. cerevisiae. S means *alsS* gene from *B. subtilis.* 5 is *ILV5* gene from *S. cerevisiae*. 3 is *ILV3* gene from *S. cerevisiae*. CE means *ilvC* gene from *E. coli*. CC means *ilvC* gene from *C. glutamicum*. DE is *ilvD* from *E. coli*. DC is *ilvD* from *C. glutamicum*. K means *kivD* gene from *L. lactis.*

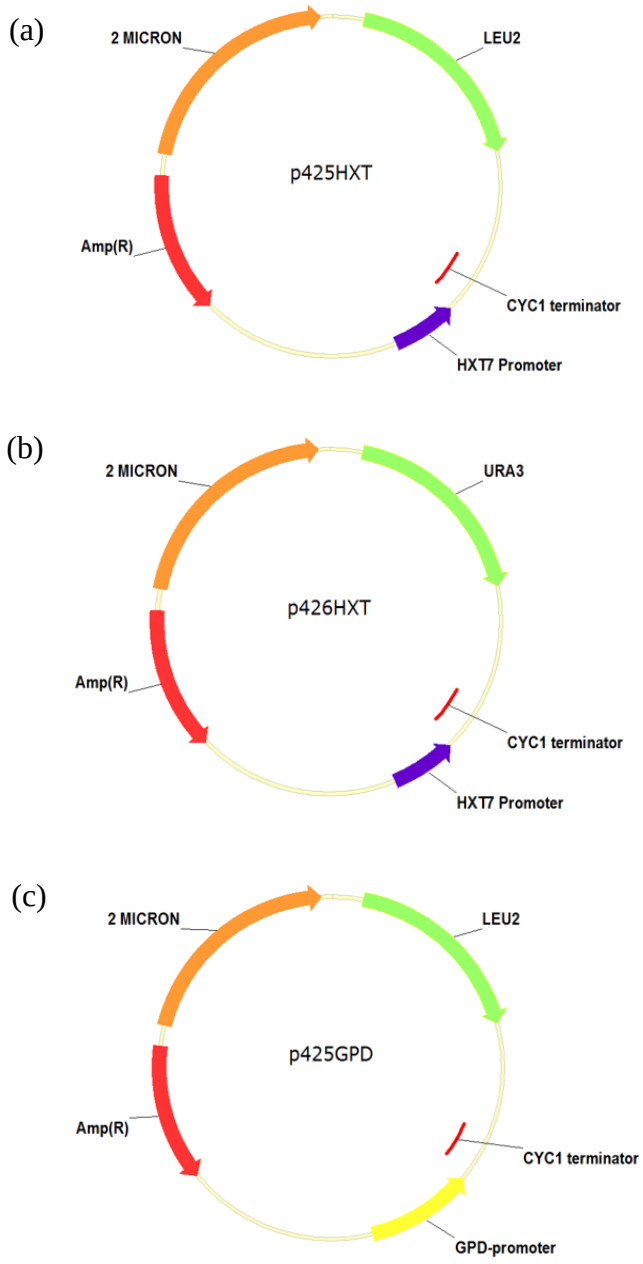


Figure 11. Mother vectors used in this study.

(A) p425HXT, (B) p426HXT, (C) p425GPD

Table 4. List of the plasmids used in this study

Name	Description
p425HXT	LEU2, truncated *HXT7* promoter, *CYC1* terminator, 2 μ origin, Amp^r
p426HXT	URA3, truncated *HXT7* promoter, *CYC1* terminator, 2 μ origin, Amp^r
p425GPD	LEU2, *GPD* promoter, *CYC1* terminator, 2 μ origin, Amp^r
pM2_K	p425HXT harboring modified *ILV2* from *S. cerevisiae* and *kivD* from *L. lactis*
pS_K	p425HXT harboring *alsS* from *B. subtilis* and *kivD* from *L. lactis*
pM5_M3	p426HXT harboring modified *ILV5* and modified *ILV3* from *S. cerevisiae*
pMCE_MDE	p426HXT harboring modified *ilvC* and modified *ilvD* from *E. coli*
pCC_MDC	p426HXT harboring *ilvC* and modified *ilvD* from *C. glutamicum*
pAur_cas9	p414TEF1 harboring Cas9 from Streptococcus pyogenes, *AUR1-C*
pdTRP	p42H harboring TRP1 disruption gRNA cassette, hphNT1
pdGRX3	p42H harboring GRX3 disruption gRNA cassette, hphNT1

pM2_K was constructed by M.J. Kim at Seoul National University in Korea.

pAur_cas9 was constructed by J.W. Kim at Seoul National University in Korea.

pdTrp was constructed by professor Yong-Su Jin's group at University of Illinois at Urbana-Champaign.

Other plasmids in Table 4 were constructed in this study.

Table 5. List of oligonucleotides used in this study

Primer name	Oligonucleotide sequence (5' → 3')
F_BamHI_alsS	CGGGATCCATGTTGACAAAAGCAACAAAAGA
R_XhoI_alsS	CCGCTCGAGCTAGAGAGCTTTCGTTTTCA
F_BamHI_M5	CGCGGATCCAAAATGGCTGCCCGTTTCGTTAA
R_XhoI_M5	CCGCTCGAGTTATTGGTTTTCTGGTCTCAACTTTCT
F_BamHI_M3	CGCGGATCCAAAATGAAGAAGCTCAACAAGTACT
R_XhoI_M3	CCGCTCGAGTTAAGCATCTAAAACACAACCGTTG
F_BamHI_MCE	CGGGATCCAAAATGATGGGCCGCGATGAATTC
R_XhoI_CE	CCGCTCGAGTTAACCCGCAACAGCAATACGTTTC
F_BamHI_MDE	CGGGATCCAAAATGACCGGAATGACCGACGC
R_EcoRI_DE	CCGGAATTCTTAACCCCCCAGTTTCGATTTATCG
F_BamHI_CC	CGCGGATCCATGGCTATTGAACTGCTTTATGATGCT
R_XhoI_CC	CCGCTCGAGTTAAGCGGTTTCTGCGCGAG
F_ClaI_MDC	CGCATCGATATGACCGGCACCAAGGAAAATG
R_XhoI_DC	CCGCTCGAGTTAGTCGACCTGACGGACTG
F_BamHI_CFD1	CGCGGATCCATGGAGGAACAGGAGATAGG
R_XhoI_CFD1	CCGCTCGAGTTATTTTGGAGATTCTATCTGGGG
SacI-HXTp	CGAGCTCTCGGGCCCCTGCTTCTG
SacI-CYCt	CGAGCTCGGCCGCAAATTAAAGCCTTC

Table 6. List of oligonucleotides used for CRISPR/Cas9 system

Primer name	Oligonucleotide sequence (5' → 3')
F1_SacI_gBlock	TCTACAGCGGCCGCGAGCTCTCTTTGAAAAGATAATGTAT
R1_dGRX3_gBlock	TAGTATGAGTTTGTGATGATGATCATTTATCTTTCACTGCG
F2_dGRX3_gBlock	ATCATCACAAACTCATACTAGTTTTAGAGCTAGAAATAGCAAGT
R2_KpnI_gBlock	TATAGAGCGGCCGCGGTACCAGACATAAAAAACAAAAAAAG
F3_repair_dGRX3_GPDp	ATAAACGCCACCAAGCAAGATATTACACAGCAGCAAAACTTTTTC AGGAAAGTTTATCATTATCAATACTCGCCA
R3_repair_dGRX3_CYCt	TGGAAATGGAGACTTTTCTGTTTCAATTTACACTAAATACATCTATT TATGGCCGCAAATTAAAGCCTTC
F_cas9_seq	ATGGACAAGAAGTACTCCATTG
R_cas9_seq	TCACACCTTCCTCTTCTTCTT
F_GRX3_up_seq	GAGATGCCAAACTAAGAGGTCAT
R_GRX3_down_seq	TACCATTAGTTGGAAATGGAGACTTT

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 was obtained from Takara (Tokyo, Japan).

3.2. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was performed with the Accupower™ PCR PreMix (Bioneer Co., Daejon, Korea) in GeneAmp PCR System 2400 (*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.

3.3. Isolation of DNA fragments and DNA sequencing

DNA was digested with restriction enzymes and separated on a 0.1% (w/v) agarose gel. After full separation of the desired DNA band from

the gel, the gel containing the DNA fragment was solubilized and further purified by using Gel Extraction Kit from Takara (Tokyo, Japan). DNA sequencing was performed by SolGent (Daejon, Korea).

3.4. 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 OD_{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 test tubes and incubated at 37℃ for 1 hour to allow the bacteria to express the antibiotic resistance. Transformed cells was spread on LB agar plates with an ampicillin selection marker.

3.5. Preparation of plasmid DNA and bacteria genomic DNA

Mini-scale preparation of plasmid DNA was carried out using *Dyne* ™ Plasmid Miniprep Kit from Dyne Bio Co. (Seongnam, Korea) according to the manufacturer's instruction.

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

3.6. 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 and nucleotides were added as necessary.

4. Media and culture conditions

4.1. Media

LB medium with 50 μg/mL ampicillin was used for recombinant *E. coli* cultivation.

YPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose) and YNBD medium which lacked appropriate amino acid were used for selection of yeast strains. YNB Synthetic Complete medium (6.7 g/L yeast nitrogen base without amino acid, 2.0 g/L amino acids mixture without histidine, tryptophan, leucine or uracil) was used for cultivation of yeast strain.

4.2. Cultivations in flask

Batch fermentation was performed in 250 mL flask with 50 mL working volume of YP medium containing 40 g/L glucose at 30℃ in shaking incubator (Vision Korea), and shaking rate was maintained at 100 rpm for creating micro-aerobic conditions.

Seed cultures were prepared by culturing 1% recombinant *S. cerevisia*e stock in 5 mL test tube of YNB medium containing 20 g/L glucose and incubated 48 h at 30℃, 250 rpm in shaking incubator

(Vision, Korea). Yeast cells were harvested at a mid-exponential phase and inoculated into main cultures with initial OD_{600} of ~1.0.

4.3. Cultivations in bioreactor with gas trapping

To optimize fermentation conditions and obtain isobutanol removed from medium by gas trapping, batch fermentation was carried out using a bench-top fermentor (KoBioTech, Korea). Cultivations were performed in 500 mL YP medium with 40 g/L glucose at 30℃ and pH 6.0 (adjustment by 5 N HCL and 2 N NaOH). For aerobic conditions, an agitation speed of 500 rpm and aeration of 1 vvm were maintained until all of glucose was consumed. After that, aeration of 0.2 vvm was maintained for oxygen-limited condition and agitation speed was controlled from 250 rpm to 350 rpm.

The evaporated isobutanol from fermentor was condensed using two condensers connected in series. The exhaust gases from the fermentor were bubbled in a trap (picker B containing 500 mL of water) cooled with ice and then circulated through condenser 1 maintained at 4℃. After that, gas continued circulating through a second equal loop (D receiver and condenser 2) (Figure 12) (Baez,

Cho et al., 2011).

Seed cultures were prepared by culturing 1% recombinant *S. cerevisiae* stock in a 5 mL test tube of YNB medium containing 20 g/L glucose in a shaking incubator at 30℃ and 250 rpm for 48 h. Pre-cultures were prepared by inoculating the seed cultures in a 500 mL flask with 100 mL working volume of YNB medium containing 20 g/L glucose and grown in a shaking incubator at 30℃ and 250 rpm for 72 h. Yeast cells were prepared by growing cells to an OD_{600} of 5~10. The cells were harvested by centrifugation at 3000 rpm for 10 min and washed in 5 mL of sterilized DDW and inoculated into the bioreactor with initial OD_{600} of ~10.

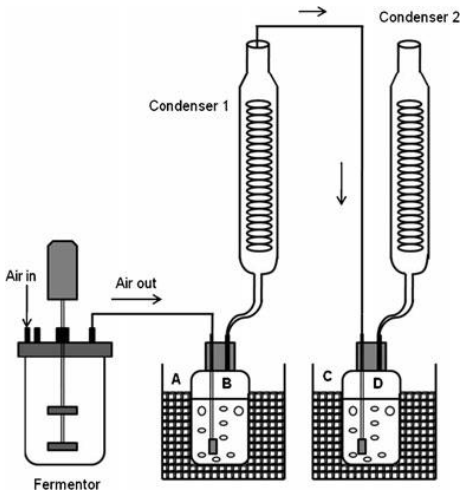


Figure 12. Schematic diagram of fermentation/gas trapping

5. Analysis

5.1. Dry cell weight

Cell growth was monitored by optical density (OD) at 600 nm using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). Dry cell weight (DCW) was estimated by using a conversion factor.

$$\text{Dry cell weight (g/L)} = 0.3 \times OD_{600}$$

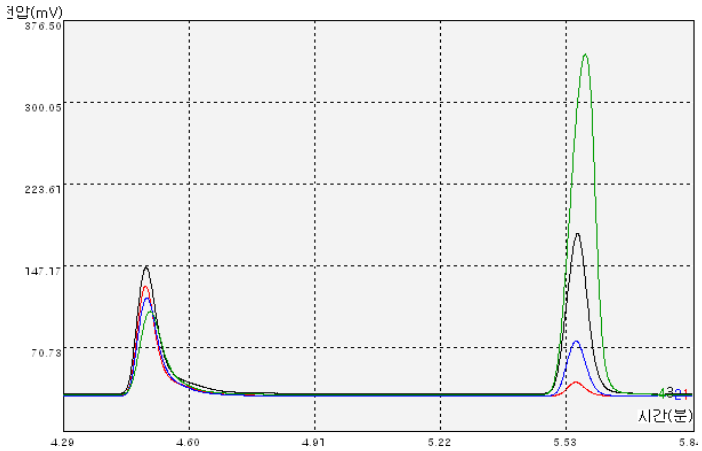
5.2. Metabolite detection

Concentrations of glucose, glycerol, acetate and ethanol were measured by a high performance liquid chromatography (Agilent Technologies 1200 Series, U.S.A) with a Bio-Rad Aminex HPX-87H column. The carbohydrate analysis ion exclusion column heated at 60℃ was applied to analyze the 20 μL of diluted culture broth. Detection was made with a reflective index detector at 35℃. HPLC operation conditions were set according to the instruction manual of the column supplier. The column was eluted with 5 mM sulfuric acid at flow rate of 0.6 ml/min.

5.3. Isobutanol detection

The produced isobutanol were quantified by a gas chromatograph (GC) equipped with flame ionization detector (FID). The model is YL6100 GC (YoungLin Inc, Incheon, Korea) and 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). GC 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. The internal standard used was 1-propanol, and alcohol content was determined by extrapolation from standard curves using the internal standard to normalize the values (Figure 13).

(a)



0.02 g/L **0.1 g/L** **0.25 g/L** **1.0 g/L**

(b)

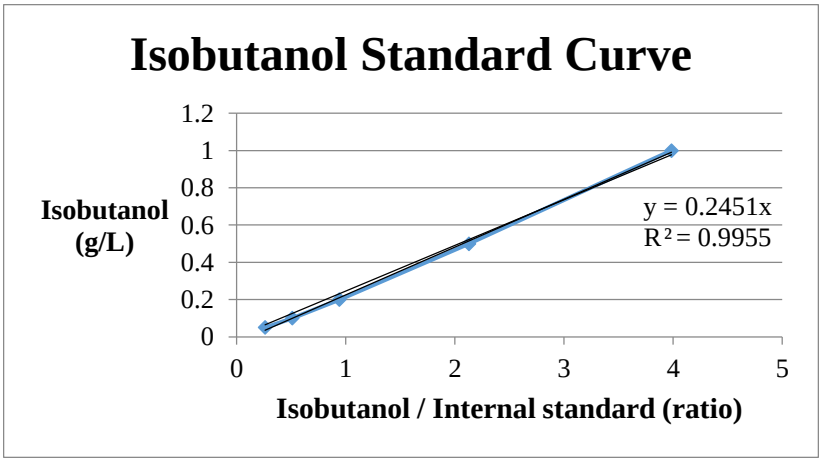


Figure 13. (a) Standard peak of GC analysis : 1-propanol, isobutanol

(b) Standard curve of isobutanol ratio with constant 1-propanol

III. RESULTS AND DISCUSSIONS

1. Construction of the efficient isobutanol biosynthesis system

1.1. Comparison of acetolactate synthases from *S. cerevisiae* and *B. subtilis*

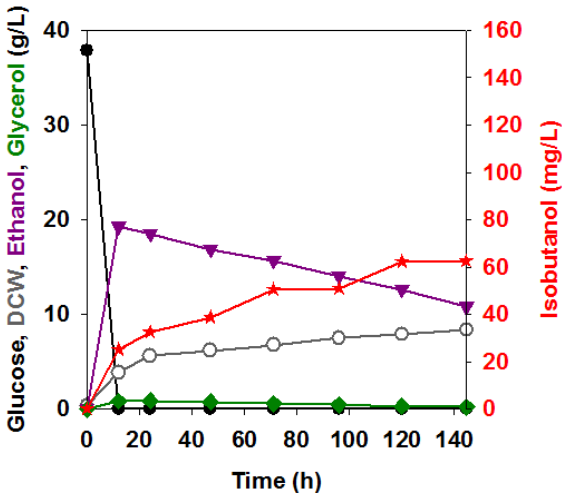
In the previous study by M.J. Kim, the endogenous *ILV2* was overexpressed in *S. cerevisiae*. However, *S. cerevisiae* native ALS encoded by the *ILV2* gene has higher affinity for 2-ketobutyrate over pyruvate. To produce 2-acetolacte, an intermediate of isobutanol, two moles of pyruvate should be reacted by ALS. If one pyruvate and one 2-ketobutyrate are reacted, they form 2-aceto-2-hydroxy-butyrate, a precursor of isoleucine (Gollop, Damri et al., 1990). Also, ALS of *S. cerevisiae* has a large subunit containing the catalytic machinery and a small subunit with a regulatory role encoded by *ILV6*. This small subunit regulates the activity of ALS by end-product (valine) feedback inhibition (Pang and Duggleby, 1999).

ALS of *B. subtilis* encoded by the *alsS* gene has stronger preference to pyruvate than 2-ketobutyrate and it has no regulatory subunit (Gollop, Damri et al., 1990). Therefore, *alsS* from *B. subtilis* was

investigated for improving isobutanol production. The D-SK_M5M3 strain was constructed by introducing ALS from *B. subtilis* and overexpressing the endogenous *ILV5* and *ILV3* and *kivD* of *L. lactis* in D452-2 used as a host. The control strain was D-M2K_M5M3. Batch fermentation profiles of the constructed strains are displayed in Figure 14.

While D-M2K_M5M3 produced 62.5 mg/L of isobutanol, D-SK_M5M3 produced 112 mg/L of isobutanol from 40 g/L of glucose within 144 h under micro-aerobic conditions. The D-SK_M5M3 strain produced isobutanol almost double than the control strain, D-M2K_M5M3. Also, the D-SK_M5M3 strain uses glucose for isobutanol and cell mass rather than production of ethanol, the major by-product of isobutanol fermentation (Table 7). Production of ethanol of D-M2K_M5M3 strain and D-SK_M5M3 strain is 19.3 g/L and 15.8 g/L, respectively. To improve isobutanol production and to accumulate less ethanol, ALS of *B. subtilis* was used for subsequent experiments.

(a)



(b)

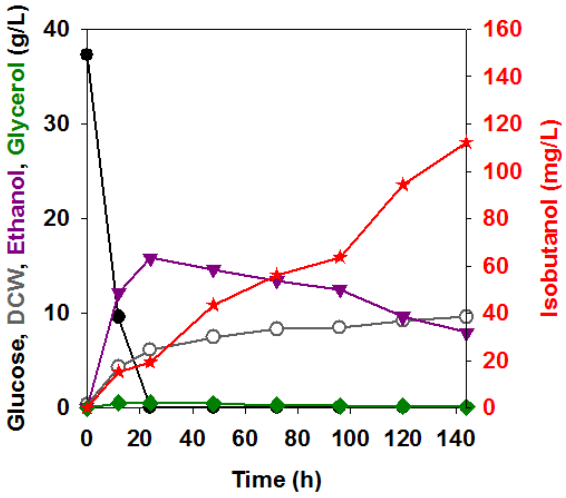


Figure 14. Flask fermentation profiles of
(a) D-M2K_M5M3, (b) D-SK_M5M3
Symbols : Glucose ( ), DCW ( ), Glycerol ( ),
Ethanol ( ), Isobutanol ( )

Table 7. Summary of flask fermentation in part 1.1

Strain	**Maximum dry cell weight (g/L)**	**Consumed glucose (g/L)**	**Maximum Ethanol (g/L)**	**Concentration of isobutanol (mg/L)**	**Yield of isobutanol (mg $_{isobutanol}$/g $_{glucose}$)**
D-M2K_M5M3	8.34	37.9	19.3	62.5	1.65
D-SK_M5M3	9.60	37.3	15.8	112	3.01

1.2. Evaluation of ketolacid reductoisomerase and dihydroxyacid dehydratase from various microorganisms

The KARI and DHAD genes from *E. coli* and *C. glutamicum* were selected for increasing isobutanol production in yeast. *E. coli* is the most widely used strain for isobutanol production and recombinant *E. coli* produced successfully isobutanol by using their KARI and DHAD encoded by *ilvC* and *ilvD*, respectively (Atsumi and Liao, 2008). *C. glutamicum* is known as a workhorse for production of the valine (Blombach, Riester et al., 2011; Hasegawa, Uematsu et al., 2012). Because the isobutanol biosynthetic pathway consists of valine biosynthetic pathway and Ehrlich pathway, KARI and DHAD of *C. glutamicum* are supposed to improve production of isobutanol.

According to the previous results, D_SK_MCEMDE and D_SK_CCMDC strains were constructed by combination of ALS from *B. subtilis* and KARIs and DHADs from *E. coli* and *C. glutamicum* in D452-2. Additionally, *kivD* of *L. lactis* was overexpressed to enforce the Ehrlich pathway. The control strain was D_SK_M5M3, which includes the endogenous KARI and DHAD.

Batch fermentation profiles of the constructed strains are displayed in Figure 15. Experiments were carried out in triplicate.

The D_SK_MCEMDE strain produced 104 ± 9.8 mg/L isobutanol from 40 g/L glucose, and it is the lowest isobutanol titer among three strains. On the contrary, D_SK_M5M3 and D_SK_CCMDC produced 116 ± 22.9 mg/L and 128 ± 19.3 mg/L isobutanol, respectively (Table 8). These results might be due to the expression of KARI and DHAD in active forms in *S. cerevisiae*. Because *ilvC* and *ilvD* of *E. coli* are original from prokaryote they may be not expressed in yeast properly. Also, *ilvC* of *C. glutamicum* does not have the mitochondria targeting sequence. So it does not need to modify itself for expressing KARI in the cytosol of *S. cerevisiae*. Therefore, *ilvC* and *ilvD* of *C. glutamicum* are good sources for isobutanol production of yeast.

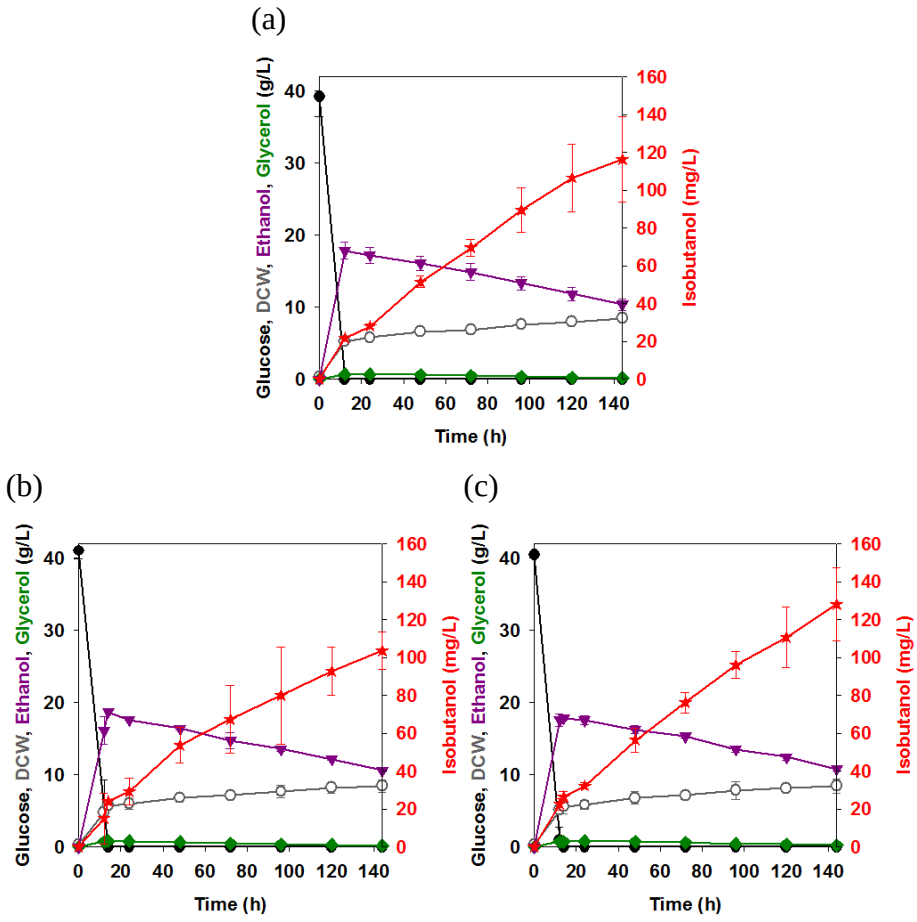


Figure 15. Flask fermentation profiles of

(a) D-SK_M5M3, (b) D-SK_MCEMDE, (c) D_SK_CCMDC

Symbols : Glucose ( ), DCW ( ), Glycerol ( ),

Ethanol ( ), Isobutanol ( )

Table 8. Summary of flask fermentation in part 1.2

Strain	**Maximum dry cell weight (g/L)**	**Consumed glucose (g/L)**	**Maximum Ethanol (g/L)**	**Concentration of isobutanol (mg/L)**	**Yield of isobutanol ($mg_{isobutanol}/g_{glucose}$)**
D-SK_M5M3	8.43 ± 0.53	39.2 ± 2.84	17.9 ± 1.14	116 ± 22.9	2.96 ± 0.45
D-SK_MCEMDE	8.43 ± 0.87	41.1 ± 1.07	18.3 ± 0.64	104 ± 9.8	2.53 ± 0.30
D-SK_CCMDC	8.45 ± 0.96	40.4 ± 0.29	18.1 ± 0.15	128 ± 19.3	3.17 ± 0.51

2. Engineering Fe homeostasis and Fe-S cluster synthesis

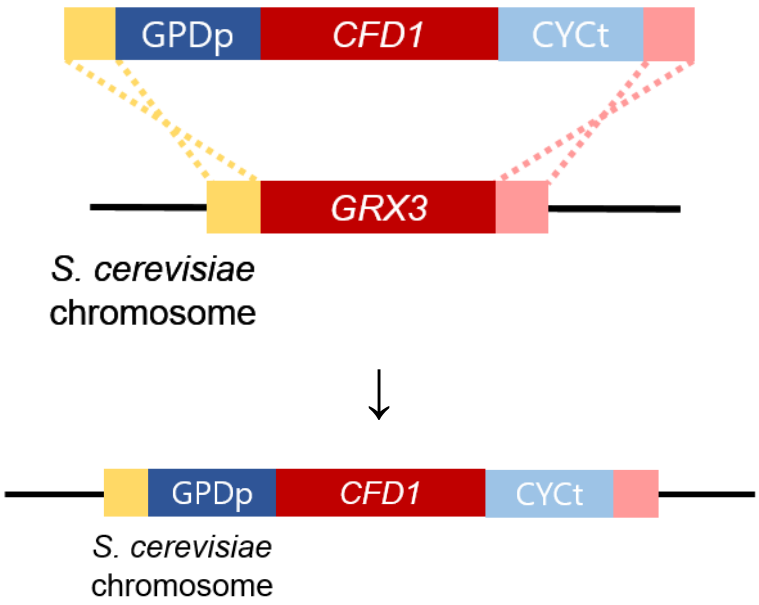
2.1. Construction of engineered strain by using CRISPR/Cas9 system

Although KARI and DHAD of *C. glutamcium* could be expressed successfully in yeast, engineering of the Fe-S cluster, an important factor of activity of DHAD, is necessary to increase cytosolic DHAD activity. Fe-S cluster biogenesis is tied to Fe homeostasis in yeast and iron sensing is dependent on the presence of the Grx3 and Grx4 proteins (Ojeda, Keller et al., 2006; Sharma, Pallesen et al., 2010). And it was found that the ratio of active DHAD increased when *GRX3* was deleted (19~31%). In addition, specific activity of DHAD increased more than 3 fold when *GRX3* was deleted (Anthony, Flint et al., 2012; Flint, Paul et al., 2012). Cfd1 plays the critical role of promoting iron entry into the CIA pathway (Sharma, Pallesen et al., 2010).

To delete *GRX3* and introduce *CFD1* in the chromosome of *S. cerevisiae* at the same time, the CRISPR/Cas9 system is used. Replacement of *GRX3* to *CFD1* cassette can be conducted by

homologous recombination. The schematic diagram of this procedure is displayed in Figure 16 (a). DNA replacement is confirmed by colony PCR. When wild *S. cereivisae* was PCR amplified with primers F_GRX3_up_seq and R_GRX3_down_seq (Table 6), the PCR product is about 1000 bp. If the *CFD1* cassette is inserted into the location of *GRX3*, the PCR product will be about 2000 bp because the *CFD1* cassette is about 1800 bp (Figure 16 (b)). After pAur_cas9 and pdGRX were drop out from D452-2, D_FeS was constructed.

(a)



(b)

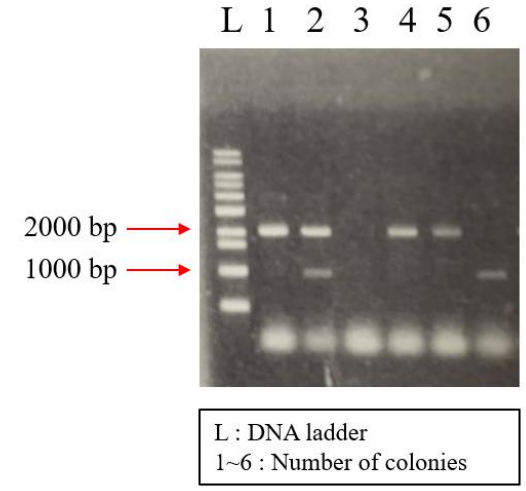


Figure 16. (a) Schematic diagram of procedure for D_FeS strain

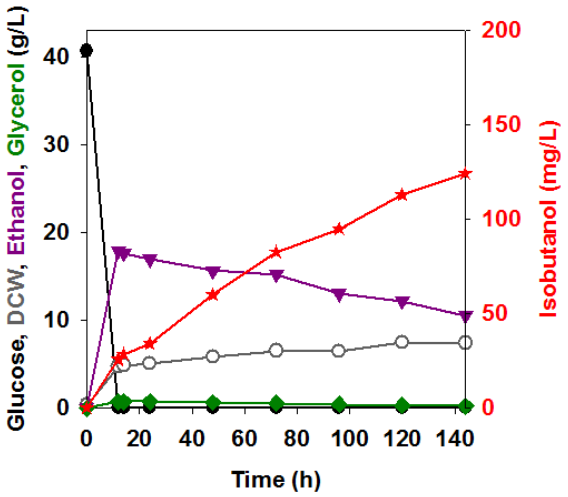
(b) Gel electrophoresis for confirmation of DNA replacement

2.2. Production of isobutanol from the strain engineered for Fe-S cluster

D_FeS-SK_CCMDC was constructed using plasmids containing *alsS* of *B. subtilis*, *ilvC* and *ilvD* of *C. glutamicum* and *kivD* of *L. lactis*. The control strain was D-SK_CCMDC and these strains cultured in YP medium with 40 g/L glucose under micro-aerobic conditions. Batch fermentation profiles of the constructed strains are displayed in Figure 17.

While the control strain produced 124 mg/L isobutanol, D_FeS-SK_CCMDC produced 197 mg/L isobutanol. The strain manipulated for increasing activity of DHAD showed concentration of isobutanol more than 60% than the control strain (Table 9). Deletion of *GRX3* may have an effect of increasing the amount of Fe-S cluster because of continuously uptake Fe into the cell. In addition, overexpression of *CFD1* could help DHAD bind to the Fe-S cluster in the cytosol. Therefore, the active forms of cytosolic DHAD were increased and production of isobutanol was improved.

(a)



(b)

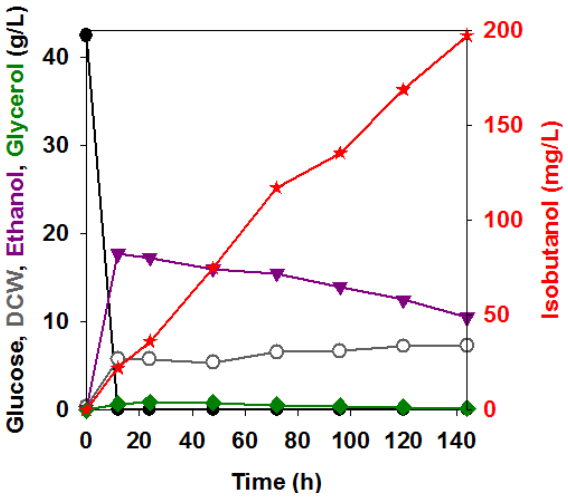


Figure 17. Flask fermentation profiles of

(a) D-SK_CCMDC, (b) D_FeS-SK_CCMDC

Symbols : Glucose ( ), DCW ( ), Glycerol ( ),

Ethanol ( ), Isobutanol ( )

Table 9. Summary of flask fermentation in part 2.2

Strain	**Maximum dry cell weight (g/L)**	**Consumed glucose (g/L)**	**Maximum Ethanol (g/L)**	**Concentration of isobutanol (mg/L)**	**Yield of isobutanol ($mg_{isobutanol}/g_{glucose}$)**
D-SK_CCMDC	7.38	40.6	17.9	124	3.05
D_FeS-SK_CCMDC	7.26	42.4	17.7	197	4.65

3. Isobutanol production in bioreactor with gas trapping

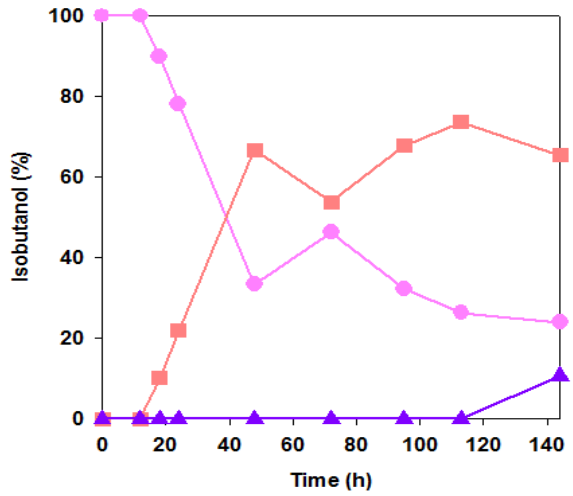
3.1. Confirmation of effect of gas trapping in different conditions

To confirm how much isobutanol produced in fermentor could be trapped by gas trapping, batch fermentation was performed under different conditions. And the ratios of concentration of isobutanol in fermentor or picker to total concentration of isobutanol were established. Profiles of isobtuanol ratio under different fermentation conditions are displayed in Figure 18.

In anaerobic batch fermentation (200 rpm, 0.2 vvm), the amount of isobutanol in first picker is more than in fermentor after 40 h. On the other hand, in fermentation under more aerobic condition (300 rpm, 2.0 vvm), the concentration of isobutanol in pickers is higher than in fermentor after 20 h. In addition, there is little isobutanol in second picker of fermentation in anaerobic condition. However, the ratio of isobutanol in second picker of aerobic condition increased and it was almost 40% at the end of the fermentation. These results indicate that isobutanol, a volatile compound, can be collected by using *in situ*

removal system with gas trapping. Also, this system has been used to produce fermentation products over its inhibitory threshold. So continuous product removal and recovery from the culture is needed (Inokuma, Liao et al., 2010).

(a)



(b)

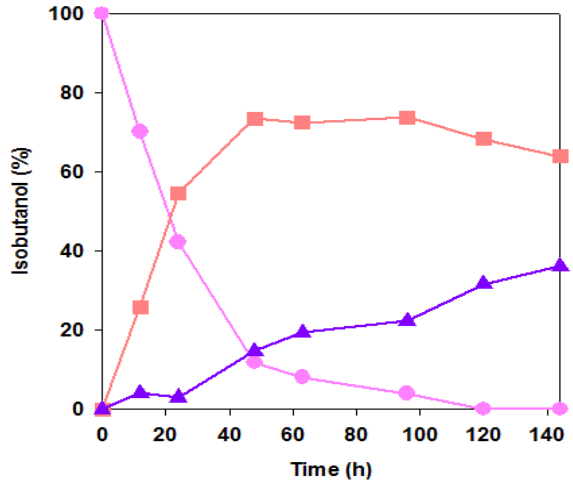


Figure 18. Profiles of isobutanol ratio in

(a) 200 rpm, 0.2 vvm, (b) 300 rpm, 2.0 vvm

Symbols : Fermentor ( ), 1st picker ( ), 2nd picker ( )

3.2. Optimization of fermentation condition

Batch fermentation in a bioreactor with gas trapping was carried out by using the strain showing the highest yield of isobutanol, D_FeS-SK_CCMDC. The fermentation condition is optimized during fermentation for improving production of isobutanol. Before all of glucose was consumed, the fermentation condition was maintained aerobically in order to reduce production of ethanol. After 6 h, the time when the concentration of glucose was detected 0, aeration was reduced from 1.0 vvm to 0.2 vvm because DHAD is oxygen sensitive (McCourt and Duggleby, 2006). The batch fermentation profile of the D_FeS-SK_CCMDC strains are displayed in Figure 19.

The final concentration of isobutanol of the D_FeS-SK_CCMDC strain was 246 mg/L after 144 h cultivation, with yield of isobutanol (6.15 mg isobutanol/g glucose) (Table 10). The titer and yield of isobutanol were higher than results of flask fermentation, 197 mg/L and 4.65 mg/g, respectively. And the reasons might be lower ethanol yield and proper fermentation condition. The more anaerobic condition was, the more ethanol was produced in *S. cerevisiae*. Indeed, while the flask fermentation maintained to micro-aerobic condition of D_FeS-SK_CCMDC showed 17.7 g/L ethanol, the

aerobic bioreactor fermentation showed 15.5 g/L ethanol (Table 10). Since ethanol biosynthetic pathway is the competitive pathway of isobutanol, it was assumed that more pyruvate used for production of isobutanol in aerobic fermentation. Optimizing fermentation condition is also important to improve production of isobutanol. Oxygen destroys the [4Fe-4S] cluster in DHAD and inactivates the enzyme. As a result, inactivated DHAD limits the biosynthesis of branched-chain amino acids (Flint, Tuminello et al., 1993; Baez and Shiloach; 2013). If the aeration 1.0 vvm maintained during fermentation, isobutanol could be produced well in latter part of fermentation.

In this study, increased isobutanol production was achieved by construction of the efficient isobutanol biosynthesis pathway and manipulation of mechanism of Fe-S cluster. However, the isobutanol production titer and rates were still low. Therefore, further metabolic engineering including elimination of by-product, such as ethanol and branched-chain amino acids, and resolving cofactor imbalance should be conducted.

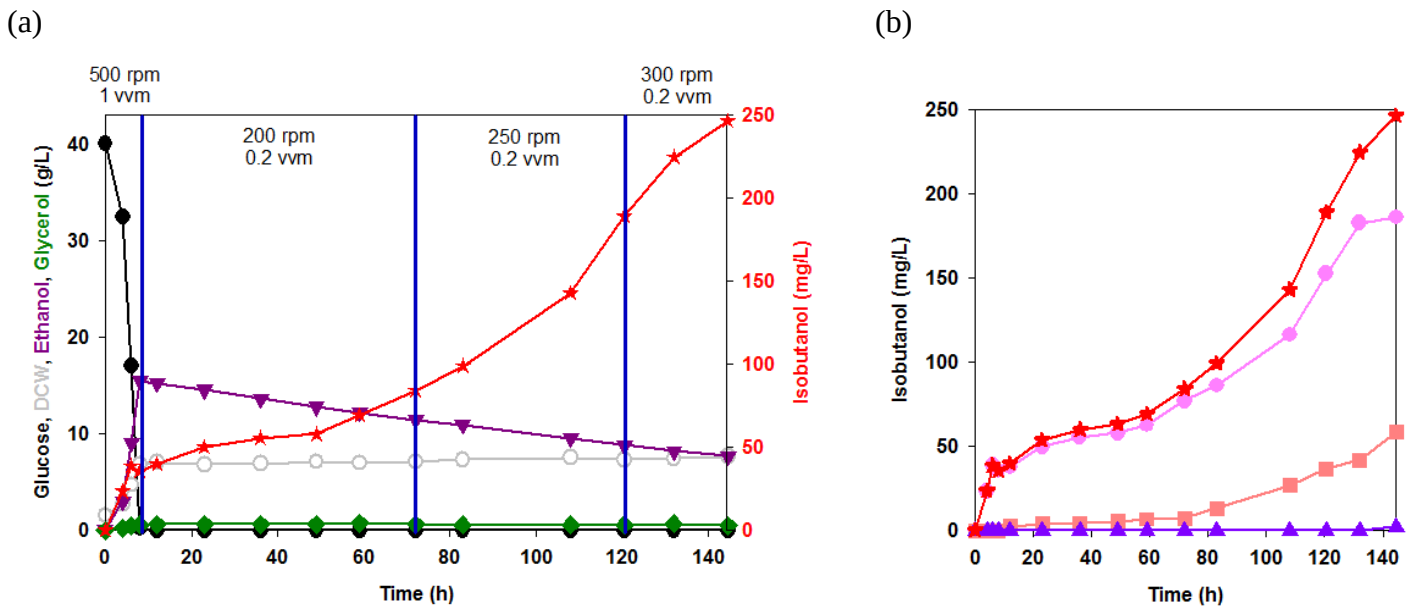


Figure 19. (a) Batch fermentation profile of D_FeS-SK_CCMDC,

(b) Isobutanol profile of D_FeS-SK_CCMDC

Symbols : Glucose ( ), DCW ( ), Glycerol ( ), Ethanol ( ), Total isobutanol ( )

Fermentor ( ), 1st picker ( ), 2nd picker ( )

Table 10. Summary of flask fermentation and bioreactor fermentation of D_FeS-SK_CCMDC

Strain	**Maximum dry cell weight (g/L)**	**Consumed glucose (g/L)**	**Maximum Ethanol (g/L)**	**Concentration of isobutanol (mg/L)**	**Yield of isobutanol (mg $_{isobutanol}$/g $_{glucose}$)**
Flask fermentation	7.26	42.4	17.7	197	4.65
Bioreactor fermentation	7.65	40.0	15.5	246	6.15

IV. CONCLUSIONS

This thesis can draw the following conclusions :

(1) For isobutanol production in *S. cerevisiae*, combination of the *alsS* gene from *B. subtilis*, the *ilvC and ilvD* genes from *C. glutamicum* and the *kivD* gene from *L. lactis* was the best in terms of titer and yield of isobutanol.

(2) The D_FeS-SK_CCMDC strain, replacing the *GRX3* gene to the *CFD1* gene for continuously uptake of iron and synthesis of cytosolic Fe-S cluster, produced 197 mg/L of isobutanol and it is more than 60% than the control strain, D-SK_CCMDC, in flask fermentation. It was found that improvement of Fe-S cluster synthesis for increasing activity of DHAD was effective to production of isobutanol in *S. cerevisiae*.

(3) In batch fermentation with a gas trapping based *in situ* removal system, the maximum isobutanol concentration of the D_FeS-SK_CCMDC strain was obtained at 246 mg/L, corresponding to 25% enhancement with flask fermentation.

V. REFERENCES

Akita, H., *et al.* (2015). "Bacterial production of isobutanol without expensive reagents." Applied Microbiology and Biotechnology 99(2): 991-999.

Anthony, L. C., *et al.* (2012). Recombinant yeast host cell with Fe-S cluster proteins and methods of using thereof, Google Patents.

Atsumi, S., *et al.* (2008). "Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels." Nature 451(7174): 86-89.

Atsumi, S. and J. C. Liao (2008). "Metabolic engineering for advanced biofuels production from *Escherichia coli*." Current Opinion in Biotechnology 19(5): 414-419.

Avalos, J. L., *et al.* (2013). "Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols." Nature Biotechnology 31(4): 335-34.

Baez, A., *et al.* (2011). "High-flux isobutanol production using engineered *Escherichia coli*: a bioreactor study with *in situ* product removal." Applied Microbiology and Biotechnology 90(5): 1681-1690.

Baez, A. and J. Shiloach (2013). "*Escherichia coli* avoids high dissolved oxygen stress by activation of SoxRS and manganese-superoxide dismutase." Microbial Cell Factories 12: 23.

Bastian, S., *et al.* (2011). "Engineered ketol-acid reductoisomerase and alcohol dehydrogenase enable anaerobic 2-methylpropan-1-ol production at theoretical yield in *Escherichia coli*." Metabolic Engineering 13(3): 345-352.

Bisaria, V., *et al.* (2014). “Bioprocessing of renewable resources to commodity bioproducts.” Willy.

Blombach, B., *et al.* (2011). "*Corynebacterium glutamicum* tailored for efficient isobutanol production." Applied and Environmental Microbiology 77(10): 3300-3310.

Brat, D., *et al.* (2012). "Cytosolic re-localization and optimization of valine synthesis and catabolism enables inseased isobutanol production with the yeast *Saccharomyces cerevisiae*." Biotechnology for Biofuels 5(1): 65.

Buijs, N. A., *et al.* (2013). "Advanced biofuel production by the yeast *Saccharomyces cerevisiae*." Current Opinion in Chemical Biology 17(3): 480-488.

Chen, X., *et al.* (2011). "Increased isobutanol production in *Saccharomyces cerevisiae* by overexpression of genes in valine metabolism." Biotechnology for Biofuels 4(21): 2089-2090.

Claros, M. G. and P. Vincens (1996). "Computational method to predict mitochondrially imported proteins and their targeting sequences." European Journal of Biochemistry 241(3): 779-786.

Connor, M. R. and J. C. Liao (2008). "Engineering of an *Escherichia coli* strain for the production of 3-methyl-1-butanol." Applied and Environmental Microbiology 74(18): 5769-5775.

DiCarlo, J. E., *et al.* (2013). "Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems." Nucleic Acids Research: 135.

Fischer, C. R., *et al.* (2008). "Selection and optimization of microbial hosts for biofuels production." Metabolic Engineering 10(6): 295-304.

Flint, D. H., *et al.* (1993). "The inactivation of Fe-S cluster containing hydro-lyases by superoxide." Journal of Biological Chemistry 268(30): 22369-22376.

Flint, D., *et al.* (2012). “Activity of Fe–S cluster requiring proteins.” United States Patent Application US 2012/0064561 A1.

García, V., *et al.* (2011). "Challenges in biobutanol production: How to improve the efficiency?" Renewable and Sustainable Energy Reviews 15(2): 964-980.

Generoso, W. C., *et al.* (2015). "Metabolic engineering of

Saccharomyces cerevisiae for production of butanol isomers." Current Opinion in Biotechnology 33: 1-7.

Gollop, N., *et al.* (1990). "Physiological implications of the substrate specificities of acetohydroxy acid synthases from varied organisms." Journal of Bacteriology 172(6): 3444-3449.

Hasegawa, S., *et al.* (2012). "Improvement of the redox balance increases L-valine production by *Corynebacterium glutamicum* under oxygen deprivation conditions." Applied and Environmental Microbiology 78(3): 865-875.

Hazelwood, L. A., *et al.* (2008). "The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism." Applied and Environmental Microbiology 74(8): 2259-2266.

Higashide, W., *et al.* (2011). "Metabolic engineering of Clostridium cellulolyticum for production of isobutanol from cellulose." Applied and Environmental Microbiology 77(8): 2727-2733.

Hsu, P. D., *et al.* (2014). "Development and applications of CRISPR-Cas9 for genome engineering." Cell 157(6): 1262-1278.

Ida, K., *et al.* (2015). "Eliminating the isoleucine biosynthetic pathway to reduce competitive carbon outflow during isobutanol production by *Saccharomyces cerevisiae*." Microbial Cell Factories 14(1): 62.

Inokuma, K., *et al.* (2010). "Improvement of isopropanol production by metabolically engineered *Escherichia coli* using gas stripping." Journal of Bioscience and Bioengineering 110(6): 696-701.

Kondo, T., *et al.* (2012). "Genetic engineering to enhance the Ehrlich pathway and alter carbon flux for increased isobutanol production from glucose by *Saccharomyces cerevisiae*." Journal of Biotechnology 159(1): 32-37.

König, S. (1998). "Subunit structure, function and organisation of pyruvate decarboxylases from various organisms." Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular

Enzymology 1385(2): 271-286.

Lee, W.-H., *et al.* (2012). "Isobutanol production in engineered *Saccharomyces cerevisiae* by overexpression of 2-ketoisovalerate decarboxylase and valine biosynthetic enzymes." Bioprocess and Biosystems Engineering 35(9): 1467-1475.

Li, H., *et al.* (2009). "The yeast iron regulatory proteins Grx3/4 and Fra2 form heterodimeric complexes containing a [2Fe-2S] cluster with cysteinyl and histidyl ligation." Biochemistry 48(40): 9569-9581.

Li, S., *et al.* (2011). "Engineering *Bacillus subtilis* for isobutanol production by heterologous Ehrlich pathway construction and the biosynthetic 2-ketoisovalerate precursor pathway overexpression." Applied Microbiology and Biotechnology 91(3): 577-589.

Li, S., *et al.* (2012). "Rational improvement of the engineered isobutanol-producing Bacillus subtilis by elementary mode analysis." Microbial Cell Factories 11(1): 101.

Lill, R., *et al.* (2006). "Mechanisms of iron–sulfur protein maturation in mitochondria, cytosol and nucleus of eukaryotes." Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1763(7): 652-667.

Lill, R. and U. Mühlenhoff (2008). "Maturation of iron-sulfur proteins in eukaryotes: mechanisms, connected processes, and diseases." Annual Review of Biochemistry. 77: 669-700.

Lin, P. P., *et al.* (2015). "Consolidated bioprocessing of cellulose to isobutanol using *Clostridium thermocellum*." Metabolic Engineering 31: 44-52.

Mali, P., *et al.* (2013). "Cas9 as a versatile tool for engineering biology." Nature Methods 10(10): 957-963.

Mali, P., *et al.* (2013). "RNA-guided human genome engineering via Cas9." Science 339(6121): 823-826.

Matsuda, F., *et al.* (2013). "Increased isobutanol production in *Saccharomyces cerevisiae* by eliminating competing pathways and

resolving cofactor imbalance." Microbial Cell Factories 12: 119.

Matsumura, M., *et al.* (1992). "Continuous butanol/isopropanol fermentation in down-flow column reactor coupled with pervaporation using supported liquid membrane." Biotechnology and Bioengineering 39(2): 148-156.

McCourt, J. and R. Duggleby (2006). "Acetohydroxyacid synthase and its role in the biosynthetic pathway for branched-chain amino acids." Amino Acids 31(2): 173-210.

Netz, D. J., *et al.* (2007). "The Cfd1–Nbp35 complex acts as a scaffold for iron-sulfur protein assembly in the yeast cytosol." Nature Chemical Biology 3(5): 278-286.

Ojeda, L., *et al.* (2006). "Role of glutaredoxin-3 and glutaredoxin-4 in the iron regulation of the Aft1 transcriptional activator in *Saccharomyces cerevisiae*." Journal of Biological Chemistry 281(26): 17661-17669.

Outten, C. E. and A.-N. Albetel (2013). "Iron sensing and regulation in *Saccharomyces cerevisiae*: ironing out the mechanistic details." Current Opinion in Microbiology 16(6): 662-668.

Pang, S. S. and R. G. Duggleby (1999). "Expression, purification, characterization, and reconstitution of the large and small subunits of yeast acetohydroxyacid synthase." Biochemistry 38(16): 5222-5231.

Park, S.-H., *et al.* (2014). "Metabolic engineering of *Saccharomyces cerevisiae* for the production of isobutanol and 3-methyl-1-butanol." Applied Microbiology and Biotechnology 98(21): 9139-9147.

Ranjan, A. and V. S. Moholkar (2012). "Biobutanol: science, engineering, and economics." International Journal of Energy Research 36(3): 277-323.

Rutherford, J. C., *et al.* (2005). "Activation of the iron regulon by the yeast Aft1/Aft2 transcription factors depends on mitochondrial but not cytosolic iron-sulfur protein biogenesis." Journal of Biological Chemistry 280(11): 10135-10140.

Ryan, E. and G. Kohlhaw (1974). "Subcellular localization of isoleucine-valine biosynthetic enzymes in yeast." Journal of Bacteriology 120(2): 631-637.

Sharma, A. K., *et al.* (2010). "Cytosolic iron-sulfur cluster assembly (CIA) system: factors, mechanism, and relevance to cellular iron regulation." Journal of Biological Chemistry 285(35): 26745-26751.

Smith, K. M., *et al.* (2010). "Engineering *Corynebacterium glutamicum* for isobutanol production." Applied Microbiology and Biotechnology 87(3): 1045-1055.

Steen, E. J., *et al.* (2008). "Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol." Microbial Cell Factories 7(1): 36.

Zhang, G.-C., *et al.* (2014). "Construction of a Quadruple Auxotrophic Mutant of an Industrial Polyploid *Saccharomyces cerevisiae* Strain by Using RNA-Guided Cas9 Nuclease." Applied and Environmental Microbiology 80(24): 7694-7701.

국 문 초 록

화석 연료로 인한 환경문제와 자원의 고갈을 해결하기 위해 미생물로부터 대체 연료를 생산하는 연구가 활발히 진행되고 있다. 바이오부탄올은 대표적인 바이오 연료인 에탄올보다 에너지 밀도가 높고, 흡습성이 낮으며 운반이 쉬워 연료로 사용되기에 더 적합하다. 특히, 아이소부탄올은 연료의 성능을 결정하는 기준인 옥탄가가 높다. 또한, 다양한 산업 분야에서 이용되어 왔기 때문에 아이소부탄올은 고부가가치 물질로 주목받고 있다.

본 연구에서는 알코올에 대한 내성이 높고 재조합 균주 개발이 용이하여 산업용 에탄올을 생산하는 데 이용되어 온 효모 *Saccharomyces cerevisiae*를 이용하여 아이소부탄올을 생산하였다. 야생 *S. cerevisiae*는 발린 생합성 경로와 에를리히 경로를 통하여 아이소부탄올을 소량 생산할 수 있다. 그러나 발린 생합성 경로와 에를리히 경로의 위치가 달라 중간체들의 전환이 효율적이지 않다는 문제점이 있었다. 따라서

본 연구에서는 발린 생합성 경로를 세포질에서 발현되도록 조작하여 아이소부탄올의 생산성을 증진시키고자 하였다.

먼저 발린 생합성 경로를 강화하기 위하여 여러 박테리아 유래의 유전자들을 도입해 가장 높은 아이소부탄올 생산 수율을 보이는 유전자 조합을 선정하였다. 이 때, 발린 생합성 경로를 세포질에서 발현시키고자 각 유전자들의 미토콘드리아 표적 서열을 알아본 뒤 유전자들을 조작하였다. 그 결과, acetolactate synthase (ALS)를 암호화하는 *Bacillus subtilis* 유래의 *alsS*, ketolacid reductoisomerase (KARI)와 dihydroxyacid dehydratase (DHAD)를 암호화하는 *Corynebacterium glutamicum* 유래의 *ilvC*, *ilvD*, ketolacid decarboxylase (KDC)를 암호화하는 *Lactococcus lactis* 유래의 *kivd*를 발현한 균주에서 가장 높은 농도의 아이소부탄올을 생산하였다.

다음으로 DHAD의 활성에 관여하는 Fe-S cluster와 관련된 균주 개량을 진행하였다. 세포의 Fe 조절 기작의 중요 조절인자인 *GRX3*의 발현을 억제하여 세포 내 Fe 흡수를 증진시

키고자 하였고 세포질 내 Fe-S cluster 조합 기작의 첫 번째 단계인 *CFD1*을 과발현하여 세포질 내에 있는 DHAD를 더 많은 Fe-S cluster와 결합시키고자 하였다. 이를 위해 CRISPR/Cas9 시스템을 이용하여 효모의 염색체 상의 *GRX3*를 *CFD1*과 교체하였다. 이렇게 조작한 균주에 앞서 선별한 발린 생합성 경로 유전자들을 과발현한 뒤 플라스크 수준에서 발효를 진행한 결과, 대조군 대비 60% 가량 아이소부탄올 생산성이 향상되었다.

이 균주를 이용해 가스 포집 장치를 이용해 발효기 수준에서 회분식 배양을 진행하였다. 발효를 수행하기 전, 서로 다른 발효 조건에서 가스 포집 장치를 이용한 in situ removal system의 효과가 어떻게 바뀌는 지에 대한 실험도 진행되었다. 그 뒤, 아이소부탄올 발효의 주요 부산물이 에탄올의 생산을 줄이고 더 높은 아이소부탄올 농도를 얻기 위하여 발효 조건을 조절하며 회분식 배양을 진행하여 최종적으로 246 mg/L의 아이소부탄올을 생산할 수 있었다. 이는 플라스크 수준에서의 발효보다 32% 증가한 수치이다.

본 연구에서 수행한 일련의 실험 결과를 통해 세포질 내 발린 생합성 경로를 강화하고 발효 과정을 최적화하는 것은 효모로부터 아이소부탄올을 생산성을 증진할 수 있음을 확인하였다.

주요어 : 바이오 연료, Isobutanol, 효모, 발린 생합성 경로, Fe-S cluster, gas trapping, *in situ* removal system

학 번 : 2014-20699