A Thesis for the Degree of Master of Science

Production of isobutanol by pyruvate decarboxylase-deficient *Saccharomyces cerevisiae*

pyruvate decarboxylase 결여 효모로부터

아이소부탄올 생산에 관한 연구

By

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Seoul National University

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아이소부탄올 생합성 경로를 포함한 pyruvate decarboxylase 결여 효모로부터 아이소부탄올 생산에 관한 연구

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ABSTRACT

Global environmental problems and high oil prices are driving the development of technologies for synthesizing alternative liquid biofuels from renewable resources as transportation energy. Compared to ethanol traditionally used as a gasoline additive, branched-chain higher alcohols exhibit significant advantages such as higher energy density, lower hygroscopicity, lower vapor pressure and compatibility with existing transportation infrastructures. Isobutanol is regarded as a next generation transportation fuel of good quality and so microbial production of isobutanol from cellulosic biomass has been done extensively.

In this study, *Saccharomyces cerevisiae* was metabolically engineered to produce isobutanol. This strain has been traditionally used for industrial production of ethanol because of high tolerance against alcohols and many genetic tools. Naturally *S. cerevisiae* produces a little isobutanol by the valine biosynthesis pathway and the Erlich pathway.

To strengthen the isobutanol biosynthetic pathway, the modified endogenous ILV2 gene from *S. cerevisiae* without the mitochondria targeting sequence, the *ilvC* and *ilvD* genes from *Escherichia coli* and

the *kivD* gene from *Lactobacillus lactis* were overexpressed in *S. cerevisiae*. *ILV2* is coding the acetolactate synthase (ALS), *ilvC* and *ilvD* are ketoacid reductoisomerase (KARI) and dihydroxyacid dehydratase (DADH) and *kivD* is ketoacid decarboxylase (ADH). ALS, KARI, DADH and ADH are the enzymes necessary fo isobutanol biosynthesis. The constructed strain produced 120 mg/L isobutanol from glucose, along with production of ethanol as a major metabolite.

To improve isobutanol production through eliminating ethanol production, a pyruvate decarboxylase (Pdc)-deficient mutant (SOS4) was used as a host for isobutanol production, which is a non-ethanol producing and pyruvate accumulating strain. Pyruvate is a key intermediate for isobutanol production. When the modified endogenous *ILV2* gene, *ilvC* and *ilvD* genes from *E. coli* and *kivD* gene from *L. lactis* were overexpressed in the SOS4, the resulting strain was able to produce 283 mg/L isobutanol from glucose in 144 h.

Acetohydroxyacid reductoisomerase and dihyroxyacid dehydratase encoded by *ilvC* and *ilvD* genes act in mitochondria of *S. cerevisiae* naturally. Also these enzymes are presumed to be expressed in mitochondria in the yeast because the *ilvC* and *ilvD* genes have the specific sequences for mitochondria targeting. So the modified *ilvC* and

ilvD genes without the specific sequences were used and the resulting strain produced 326mg/L isobutanol from glucose in 144 h.

Additionally, to increase an expression level of all four genes involved in the isobutanol biosynthetic pathway, an existing *GPD* promoter was replaced with the truncated *HXT7* promoter known as a strong promoter. The resulting strain produced 446 mg/L isobutanol from glucose in 144 h, which was about 15-fold higher than the wild type strain.

Isobutanol production from xylose that is abundant in lignocellulosic hydrolyzate would make the production of isobutanol more sustainable and economical. However *S. cerevisiae* cannot utilize xylose as a carbon source, the *XYL1*, *XYL2* and *XYL3* genes coding for xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK) derivied from *Schefferosomyces stipitis* were introduced into the SOS4 for xylose fermentation. The resulting strain (SOS4X) accumulated pyruvate by utilizing xylose without ethanol production. By introducing the isobutanol biosynthetic system into the SOS4X, the resulting strain produced 121 mg/L isobutanol from xylose in 144h. These results suggest that *S. cerevisiae* might be a promising host for producing isobutanol from lignocellulosic biomass for industrial applications.

Keywords : biofuel, isobutanol, xylose, lignocellulosic biomass, pyruvate decarboxylase (Pdc)-deficient *S. cerivisiae*

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

1. Advanced biofuel – Isobutanol

Largely in response to uncertain fuel supply, global environmental problems and efforts to reduce carbon dioxide emissions, bioethanol has become one of the most promising biofuels today (Atsumi, Cann et al., 2008).

Recently, production of "second generation" biofuels by motivated engineering microbes has been noted for its better properties than ethanol. Compared to the traditional ethanol, higher alcohols should offer advantages such as higher energy density, lower hygroscopicity, lower vapor pressure, and compatibility with existing transportation infrastructure. (Atsumi and Liao, 2008; Atsumi, Wu et al., 2010; Steen, Chan et al., 2008).

Thus branched-chain higher alcohols, such as isopropanol, isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol represent possible alternatives. Isobutanol, compared with n-butanol, has advantages of having a higher octane number and the possibility of usage outside the fuel industry as well (Chen, Nielsen et al., 2011; Kondo, Tezuka et al., 2012). (Figure 1)

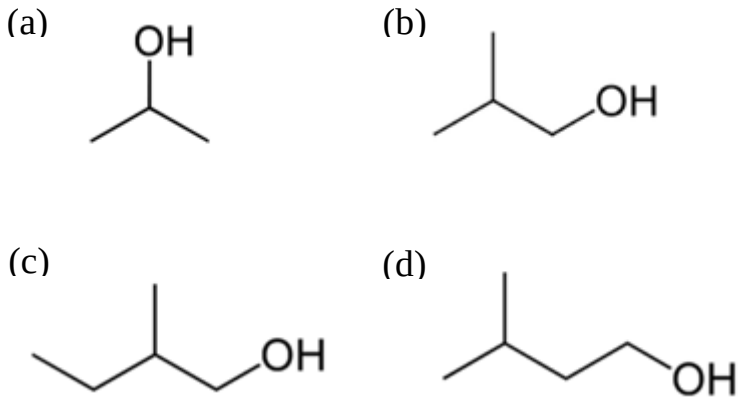


Figure 1. The structure of branched-chain higher alcohols

(a) isopropanol (b) isobutanol

(c) 2-methyl-1-butanol (d) 3-methyl-1-butanol

Isobutanol is an important platform chemical with broad applications in large chemicals and fuels markets and a “drop-in” product that should allow customers to replace petroleum-derived raw materials with isobutanol-derived raw materials without modification to their equipment or production processes. It is used as feedstock in the manufacture of isobutyl acetate, which is used for the production of lacquer and similar coatings, and for the food industry as a flavouring agent. Also, isobutanol is a precursor of derivative esters; isobutyl esters such as diisobutyl phthalate are used as plasticizer agents in plastics, rubbers, and other dispersions.

2. Isobutanol production in microorganisms

Production of isobutanol in microorganisms has been achieved by harnessing the highly active 2-keto acid pathways. Engineered *Clostridium glutamicum* using the 2-keto acid pathway produced 4.9 g/L isobutanol (Smith, Cho et al., 2010) and the engineered *Bacillus subtilis* produced up to 2.62 g/L isobutanol by the heterologous Ehrlich pathway (Li, Wen et al., 2011). Especially, production of isobutanol has been investigated in engineered *E. coli* to reach a concentration of 22 g/L isobutanol (Atsumi, Hanai et al., 2008). (Table 1)

S. cerevisiae was chosen as a host for isobutanol production because it is a genetically well-characterized organism and a current industrial strain as ethanol producer (Ro, Paradise et al., 2006; Steen, Chan et al., 2008). *S. cerevisiae* is able to tolerate 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).

Because its natural productivity of isobutanol is not significant, a strategy of genetic engineering to increase isobutanol production,

which is involved in valine biosynthesis was considered. Initially, the Ehrlich pathway and valine synthetic pathway was overexpressed and then, the *PDC1* gene encoding a major pyruvate decarboxylase with the intent of altering the abundant ethanol flux via pyruvate was deleted. Through these engineering steps, along with modification of culture conditions, the isobutanol titer of *S. cerevisiae* was elevated by 13-fold, from 11 mg/l to 143 mg/l, and the yield was 6.6 mg/g glucose, which is higher than any previously reported value for *S. cerevisiae* (Kondo, Tezuka et al., 2012).

Table 1. Isobutanol production from various microorganisms.

Microorganism	Isobutanol titer	Reference
Clostridium glutamicum	4.9 g/L	Smith, Cho et al., 2010
Corneybacterium glutamicum	660 mg/L	Higashide et al., 2011
Bacillus subtilis	2.62 g/L	Li, Wen et al., 2011
Escherichia coli	22 g/L	Atsumi, Hanai et al., 2008
	50 g/L	Baez et al., 2011
Saccharomyces cerevisiae	151 mg/L	WH Lee et al., 2012
	143 mg/L	Kondo, Tezuka et al., 2012
	630 mg/L	D Brat et al., 2012

3. Isotubanol biosynthetic pathway

3.1. The valine biosynthesis pathway

Isobutanol is produced by wild *S. cerevisiae* as a degradation product of valine metabolism (Hazelwood, Daran et al., 2008). Isobutanol can be produced by the valine biosynthesis pathway and ehrlich degradation pathway. (Figure 2)

Pyruvate to 2-ketoisovalerate involved in the valin biosynthesis pathway is catalyzed by acetolactate synthase (ALS), ketoacid reductoisomerase (KARI) and dihydroxyacid dehydratase (DADH).

ALS catalyses the first common step converting pyruvate to 2-acetolactate in isoleucine and valine biosynthesis and localizes in the mitochondria. 2-Acetolactate can be converted to 2,3-dihydroxy isovalerate and then converted to 2-ketoisovalerate by DADH (Lee et al., 2012).

To enhance the valine biosynthesis pathway, the endogenous *ILV2* gene coding for ALS, *ilvC* and *ilvD* genes from *E. coli* coding for KARI and DADH were used in this study.

In *E. coli*, valine, which is a branched-chain amino acid, is synthesized by the *ilvIHCDE* pathway (Atsumi, Hanai et al., 2008).

3.2. The Ehrlich pathway

Isobutanol can be biosynthesized via 2-ketoisovalerate catalyzed by ketoacid decarboxylase (KDC) and alcohol dehydrogenase (ADH) (Jia, Li et al., 2011). Previous studies utilized inherent KDC and ADH activities for isobutanol production (Hazelwood, Daran et al., 2008; Chen, Nielsen et al., 2011).

KDC is known to be a critical enzyme for removing the carboxylic group from 2-ketoacid to produce aldehyde (König 1998). An intermediate metabolite, 2-ketoisovalerate from the valine biosynthesis pathway, can be converted to isobutyraldehyde by KDC and then converted to isobutanol by ADH (Hazelwood, Daran et al., 2008). The *kivD* gene from *L. Lactis* subsp. *lactis* KACC13877 was determined as the most suitable KDC for isobutanol production in *S. cerevisiae* (Lee et al., 2012) and the effect of *kivD* gene from *L. lactis* was proved by determinate of KDC activity.

Wild *S. cerevisiae* has *ADH2* that showed the highest activity in transforming isobutyraldehyde to the corresponding alcohol (Atsumi, Hanai et al., 2008; Jia, Li et al., 2011).

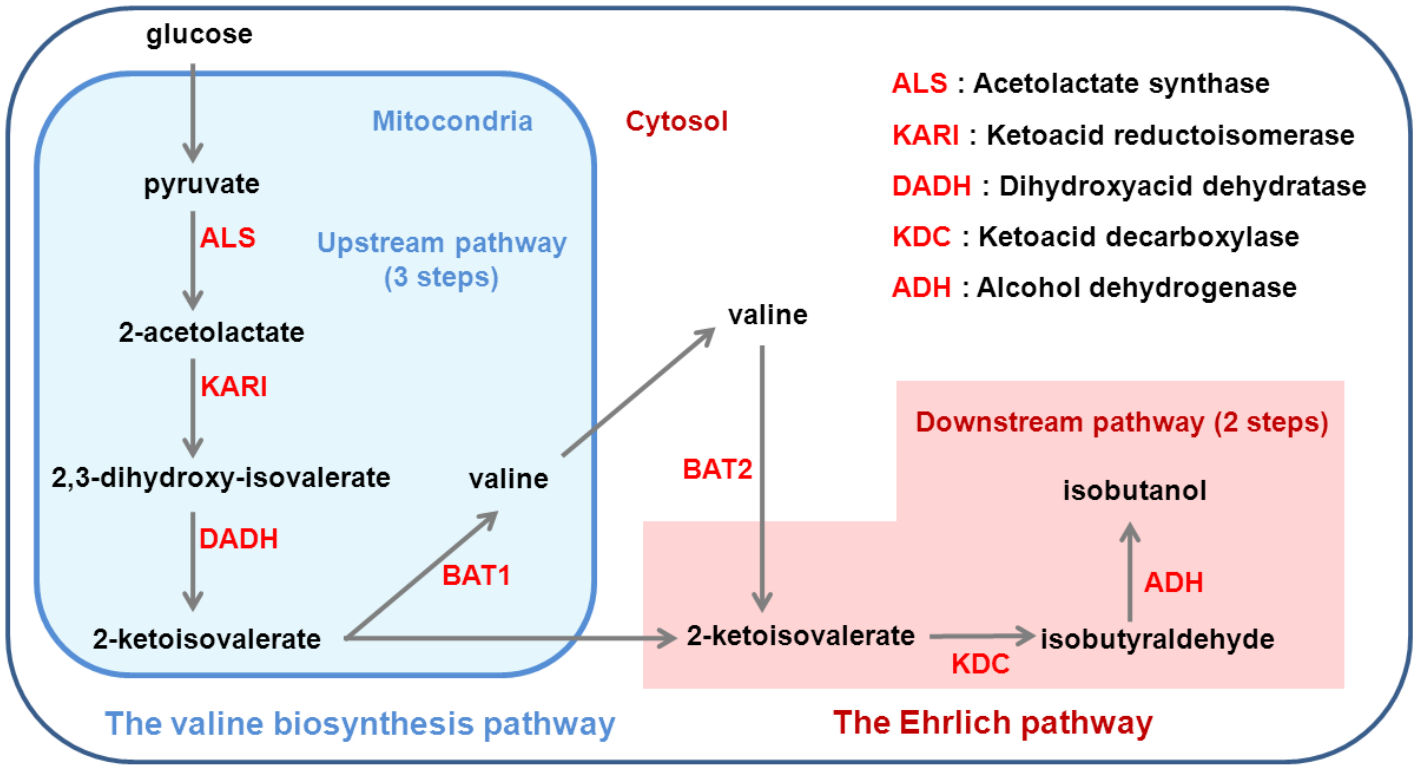


Figure 2. Isobutanol biosynthetic pathway

4. Compartmentalization of the whole isobutanol biosynthetic pathway into cytosol of *S. cerevisiae*

The enzymes involved in the valine synthesis pathway of *S. cerevisiae* are located in the mitochondrial matrix (Ryan and Kohlhaw, 1974). But the Ehrlich pathway proceeds in the cytosol.

Compartmentalization of the Ehrlich pathway into mitochondria increased isobutanol production by 260%, compared with a strain overproducing enzymes involved in only the first three steps of the biosynthetic pathway. (Figure 3) Compartmentalization may include increased availability of intermediates, removing the need to transport intermediates out of the mitochondrion and reducing the loss of intermediates to competing pathways. (Avalos J.L. et al., 2013)

In this study, compartmentalization of the valine biosynthesis pathway into cytosol was tried by overexpressing modified *ILV2* gene which was truncated mitochondria targeting sequence. (Figure 4) This modified *ILV2* gene was constructed by professor Jin Yong Su's group at University of Illinois at Urbana-Champaign. And then, for the next 2 steps involved in valine synthesis pathway, *ilvC* and *ilvD* genes from *E. coli* were used instead of endogenous *ILV5*

and *ILV3* genes. They were overexpressed to convert the 2-acetolactate produced from pyruvate by modified *ILV2* gene efficiently. It is also known to be expressed in the mitochondrial matrix in *E. coli*. When these genes were introduced in yeast, it has possibility to locate in mitochondrial matrix. Therefore, modified *ilvC* and *ilvD* genes truncated putative mitochondria targeting sequence were used in this study for constructing cytosolically re-localized isobutanol biosynthetic pathway in *S. cerevisiae.*

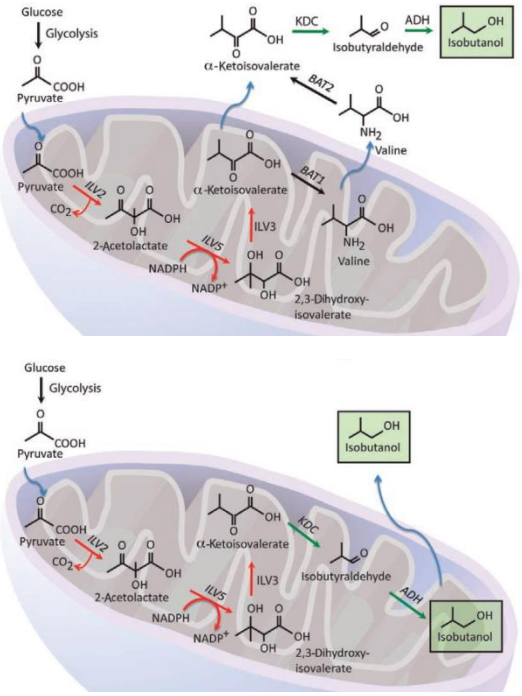


Figure 3. Compartmentalization of the Ehrlich pathway into mitochondria of *S. cerevisiae* (Avalos J.L. et al., 2013)

(a) amino acid sequence encoded by *ILV2*, **0.9993**

MIRQSTLKNFAIKRCFQHIAYRNTPAMRSVALAQRFYSSSSRYYSASPLPASKRPEPAPS
FNVDPLEQPAEPSKLAKKLRAEPDMDTSFVGLTGGQIFNEMMSRQNVDTVFGYPGGAILP
VYDAIHNSDKFNFVLPKHEQGAGHMAEGYARASGKPGVVLVTSGPGATNVVTPMADAFAD
GIPMVVFTGQVPTSAIGTDAFQEADVVGISRSCTKWNVMVKSVEELPLRINEAFEIATSG
RPGPVLVDLPKDVTAAILRNPIPTKTTLPSNALNQLTSRAQDEFVMQSINKAADLINLAK
KPVLYVGAGILNHADGPRLLKELSDRAQIPVTTTLQGLGSFDQEDPKSLDMLGMHGCATA
NLAVQNADLIIAVGARFDDRVTGNISKFAPEARRAAAEGRGGIIHFEVSPKNINKVVQTQ
IAVEGDATTNLGKMMSKIFPVKERSEWFAQINKWKKEYPYAYMEETPGSKIKPQTVIKKL
SKVANDTGRHVIVTTGVGQHQMWAAQHWTWRNPHTFITSGGLGTMGYGLPAAIGAQVAKP
ESLVIDIDGDASFNMTLTELSSAVQAGTPVKILILNNEEQGMVTQWQSLFYEHRYSHTHQ
LNPDFIKLAEAMGLKGLRVKKQEELDAKLKEFVSTKGPVLLEVEVDKKVPVLPMVAGGSG
LDEFINFDPEVERQQTELRHKRTGGKH

(b) amino acid sequence encoded by *ilvC*, **0.8706**

MANYFNTLNLRQQLAQLGKCRFMGRDEFADGASYLQGKKVVIVGCGAQGLNQGLNMRDSG
LDISYALRKEAIAEKRASWRKATENGFKVGTYEELIPQADLVINLTPDKQHSDVVRTVQP
LMKDGAALGYSHGFNIVEVGEQIRKDITVVMVAPKCPGTEVREEYKRGFGVPTLIAVHPE
NDPKGEGMAIAKAWAAATGGHRAGVLESSFVAEVKSDLMGEQTILCGMLQAGSLLCFDKL
VEEGTDPAYAEKLIQFGWETITEALKQGGITLMMDRLSNPAKLRAYALSEQLKEIMAPLF
QKHMDDIISGEFSSGMMADWANDDKKLLTWREETGKTAFETAPQYEGKIGEQEYFDKGVL
MIAMVKAGVELAFETMVDSGIIEESAYYESLHELPLIANTIARKRLYEMNVVISDTAEYG
NYLFSYACVPLLKPFMAELQPGDLGKAIPEGAVDNGQLRDVNEAIRSHAIEQVGKKLRGY
MTDMKRIAVAG

(c) amino acid sequence encoded by *ilvD*, **0.5454**

MPKYRSATTTHGRNMAGARALWRATGMTDADFGKPIIAVVNSFTQFVPGHVHLRDLGKLV
AEQIEAAGGVAKEFNTIAVDDGIAMGHGGMLYSLPSRELIADSVEYMVNAHCADAMVCIS
NCDKITPGMLMASLRLNIPVIFVSGGPMEAGKTKLSDQIIKLDLVDAMIQGADPKVSDSQ
SDQVERSACPTCGSCSGMFTANSMNCLTEALGLSQPGNGSLLATHADRKQLFLNAGKRIV
ELTKRYYEQNDESALPRNIASKAAFENAMTLDIAMGGSTNTVLHLLAAAQEAEIDFTMSD
IDKLSRKVPQLCKVAPSTQKYHMEDVHRAGGVIGILGELDRAGLLNRDVKNVLGLTLPQT
LEQYDVMLTQDDAVKNMFRAGPAGIRTTQAFSQDCRWDTLDDDRANGCIRSLEHAYSKDG
GLAVLYGNFAENGCIVKTAGVDDSILKFTGPAKVYESQDDAVEAILGGKVVAGDVVVIRY
EGPKGGPGMQEMLYPTSFLKSMGLGKACALITDGRFSGGTSGLSIGHVSPEAASGGSIGL
IEDGDLIAIDIPNRGIQLQVSDAELAARREAQDARGDKAWTPKNRERQVSFALRAYASLA
TSADKGAVRDKSKLGG

Figure 4. Putative mitochondria targeting sequence (highlighted in blue) of *ILV2*, *ilvC*, and *ilvD* genes and probability of export to mitochondria in yeast (Claros M.G., Vincens P., 1996)

5. Promoter for efficient gene expression in yeast

The efficient expression of heterologous proteins in yeast considerably relies on yeast promoters. Therefore, different promoters have been used to successfully direct expression of heterologous genes in yeast and well-characterized promoters are essential for pathway engineering and synthetic biology efforts in *S. cerevisiae* (Blazeck et al., 2012).

Constitutive promoters offer fairly constant gene expression levels at the single-cell level without the need for specific inducers or media formulations (Da Silva et al., 2012). The most widely used constitutive promoters have often been from the yeast glycolytic pathway. They are highly-expressed and used successfully to express a number of proteins. These glucose-dependent promoters include those for glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter (Holland et al., 1980), phosphoglycerate kinase (*PGK1*) promoter (Ogden et al., 1986), pyruvate decarboxylase (*PDC1*) promoter (Kellermann et al., 1986), triosephosphate isomerase (*TPI1*) promoter (Russell, 1985), alcohol dehydrogenase I (*ADH1*) promoter (Hitzeman et al., 1981) and pyruvate kinase (*PYK1*) promoter (Nishizawa et al., 1989). Other commonly used

native promoters include iso-1-cytochrome c (*CYC1*) promoter (Gallwitz et al., 1980), actin 1 (*ACT1*) promoter (Gallwitz et al., 1980), mating factor alpha-1 (*MFα1*) promoter (Brake et al., 1984) and those for hexose transport, for example *HXT7* promoter (Reifenberger et al., 1995). Under de-repressed conditions *HXT7* is by far the most strongly expressed *HXT* gene, but it is strongly repressed at high glucose concentrations (Reifenberger et al., 1997). However, 5' deletion of the *HXT7* promoter region, leaving only the 390 bp upstream of the ATG start codon leads to strong constitutive transcription of the gene on glucose media (Hauf et al., 2000). Therefore, a truncated *HXT7* promoter is used widely in glucose-based studies.

All of them, *GPD* promoter, *PGK1* promoter and truncated *HXT7* promoter are known as powerful promoters in expression of heterologous proteins relatively.

In this study, to increase expression level of 4 genes involved in isobutanol biosynthetic pathway, *GPD* promoter and truncated *HXT7* promoter were used and compared the effect in isobutanol production.

6. Pyruvate decarboxylase-deficient *S. cerevisiae*

S. cerevisiae, which is generally used to ferment sugar to ethanol, has been utilized as a host for the production of therapeutic proteins or chemicals with commercial value by metabolic engineering. Because the yield of the desired product should be maximized in respect of the economy, it is necessary to redirect carbon fluxs away from ethanol production towards the desired products, pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae* has been employed for the production for lactic acid, glycerol, and malic acid (Geertman et al., 2006; Ishida et al., 2006; Zelle et al., 2008).

Pyruvate decarboxylase which is located at the branch point between fermentative and respiratory metabolism can convert pyruvate to acetaldehyde, which is further reduced into ethanol by alcohol dehydrogenase. In *S. cerevisiae*, there are three structural genes (*PDC1*, *PDC5* and *PDC6*) involved as active pyruvate decarboxylase isoenzymes. Among these genes, disruption of *PDC1* and *PDC5* or all *PDC* genes led to elimination of pyruvate decarboxylase activity completely (Flikweert et al., 1996).

The Pdc-deficient strains have potential defects for industrial fermentations. Firstly, the Pdc-deficient mutant needs external

supplement of C_2 compound such as acetate or ethanol for synthesis of cytosolic acetyl-CoA which is required to synthesize lysine and fatty acid synthesis (Flikweert et al., 1996; Pronk et al., 1996). Cytosolic acetyl-CoA is synthesized from acetaldehyde via acetate in the cells. Synthesis of acetaldehyde, however, is blocked due to the elimination of Pdc activity, which leads to the shortage of cytosolic acetyl-CoA. Secondly, the Pde-deficient mutant showed lower growth rate a glucose-containing media than wild *S. cerevisiae*. While respiration is necessary for re-oxidation of cytosolic NADH in the Pdc-deficient strains in the absent of ethanol fermentation, glucose represses respiration, which makes Pdc-deficient strains suffer from redox imbalance. A C_2-independent and glucose-tolerant Pdc-deficient strain (SOS4) was constructed by evolutionary engineering. Genome sequencing of the SOS4 revealed a point mutation (A81P) in the *MTH1* gene leading to an amino acid change from alanine to proline (Ala81Pro). Point mutation of the *MTH1* gene in the SOS4 respectively is likely to reduce glucose uptake, which alleviated the pyruvate accumulation and redox imbalance from eliminating Pdc activity in the evolved Pdc-deficient strain (Kim et al., 2013).

7. Production of isobutanol from xylose

Lignocellulosic biomass is an attractive feedstock as a second generation source for biofuel production since it is readily available, e.g., as a waste from the pulp and paper or agricultural industries, and also due to the fact that it is renewable with cycles many orders of magnitude shorter compared with those of fossil fuels. Lignocellulose, which is composed of cellulose, hemicellulose, and lignin, is often hydrolyzed by acid treatment and then the hydrolysate obtained is used for ethanol fermentation by microorganisms such as yeast and bacteria. *S. cerevisiae* that offers numerous benefits in terms of readily availability and large-scale fermentation can be considered as a host for producing ethanol.

However *S. cerevisiae* is unable to metabolize xylose, which is the dominant pentose sugar in hydrolysates of lignocellulosic biomass (Matsushika et al., 2009). A number of different strategies have been applied to engineer yeasts capable of efficiently producing ethanol from xylose, including the introduction of initial xylose metabolism and xylose transport, changing the intracellular redox balance, and overexpression of xylulokinase and pentose phosphate pathways (Chu and Lee; Ha et al., 2011; Hahn-Hagerdal et al.,

2007; Jeffries and Jin, 2004; Jin et al., 2005). Among these attempts, *S. cerevisiae* can efficiently produce ethanol from xylose as a carbon source by introducing xylose utilization pathway, the *XYL1*, *XYL2* and *XYL3* genes coding for xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK) derivied from *S. stipitis*.

Isobutanol production from xylose that is abundant in lignocellulosic hydrolyzate would make the production of isobutanol more sustainable and economical.

Simultaneous overexpression of an optimized, cytosolically localized valine biosynthesis pathway together with overexpression of xylose isomerase *XylA* from *Clostridium phytofermentans*, transaldolase *Tal1* and xylulokinase *Xks1* enabled recombinant *S. cerevisiae* cells to complement the valine auxotrophy of *ilv2,3,5* triple deletion mutants for growth on D-xylose as the sole carbon source. Moreover, after additional overexpression of ketoacid decarboxylase *Aro10* and alcohol dehydrogenase *Adh2*, the cells were able to ferment D-xylose directly to isobutanol. The strain consumed about 12 g D-xylose and produced up to 1.36 mg/L of isobutanol (D Brat et al., 2013).

Although *S. cerevisiae* is able to metabolize xylose, they could be produce low amounts of isobutanol and mainly produce ethanol as a by-pruduct. Therefore it is necessary to redirect the carbon flux from ethanol to isobutanol production.

In this study, *S. cerevisiae* capable of utilizing xylose and accumulating pyruvate by introducing xylose assimilation pathway into a Pdc-deficient *S. cereviesiae* (SOS4) was used as host for isobutanol production from xylose. By introducing efficient isobutanol biosynthetic system into the host, it could be possible to produce isobutanol more successfully from xylose than the previous results. (Figure 5 a)

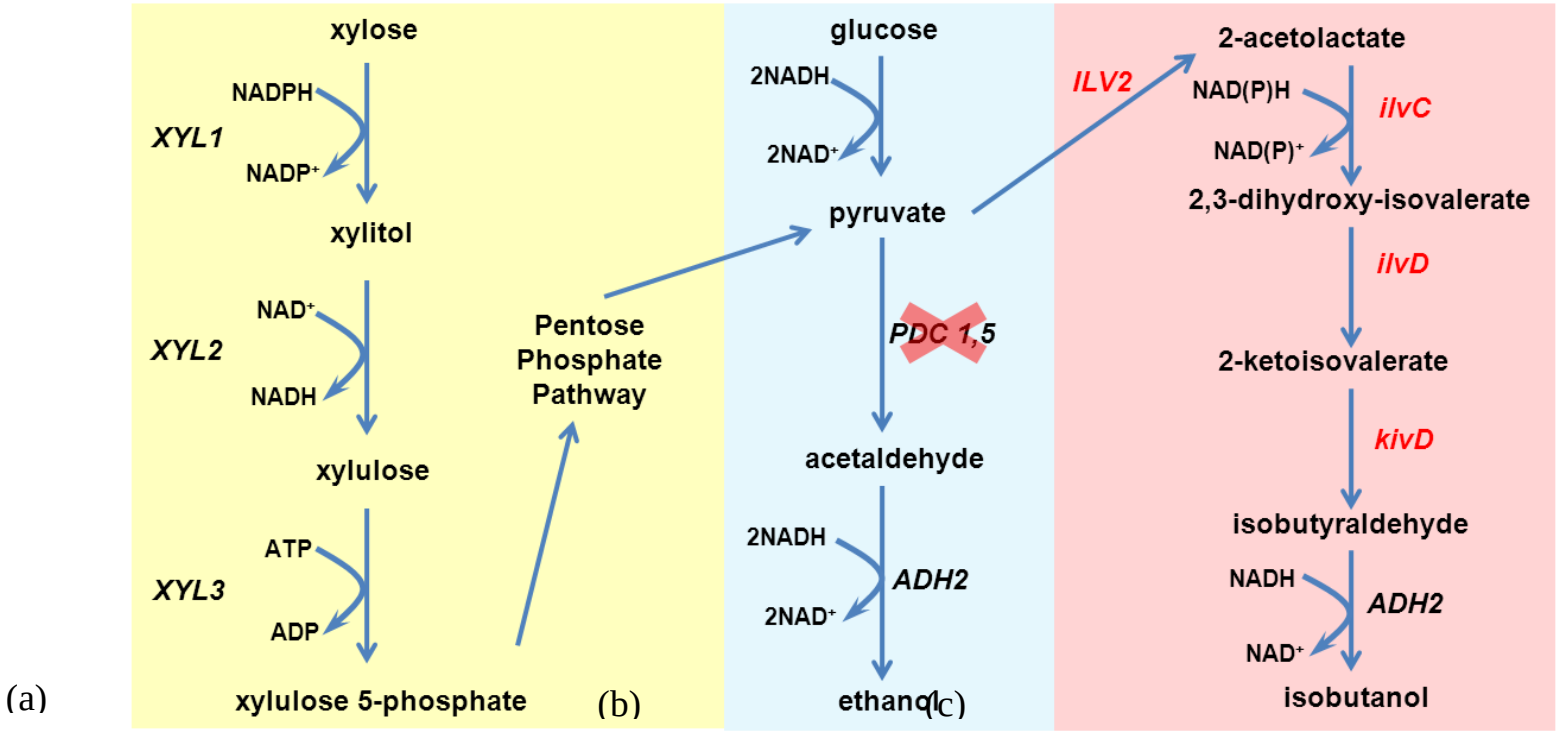


Figure 5. Metabolic pathway in engineered *S. cerevisiae* (a) xylose metabolizing pathway, (b) blocked ethanol producing pathway, (c) isobutanol biosynthetic pathway (red box : in cytosol) in this study

8. Research objectives

For production of isobutanol in recombinant *S. cerevisiae*, several factors should be considered including elimination competing pathway and enhancement isobutanol producing flux from carbon source.

This study was focused on pyruvate accumulating strain as a host and then, enhancing isobutanol biosynthesis pathway.

The specific objectives of this study are as follows.

(1) To lead carbon flux from ethanol to isobutanol production by employing pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae*,

(2) To improve isobutanol biosynthetic pathway by introducing heterologous genes and overexpressing endogenous gene in cytosol under control of strong promoter,

(3) To develop Pdc-deficient *S. cerevisiae* capable of fermenting xylose as a carbon source, accumulating pyruvate and producing isobutanol.

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), protease inhibitor cocktail and 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); 2N NaOH and 2N 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*] and the pyruvatedecarboxylase (Pdc)-deficient *S. cerevisiae* D452-2 strain (SOS4) was used as host strains for the expression of isobutanol

biosynthetic pathway. (Table 2, 3)

Also SOS4X which is introduced xylose assimilation pathway into the SOS4 was used as host strain in fermentation of xylose. (Table 3)

S. cerevisiae D452-2 was donated (Hosaka, Nikawa et al., 1992). And the SOS4 and SOS4X were constructed by S. J. Kim at Seoul National University in Korea (Kim et al., 2013). Other strains in Table 2, 3 were constructed in this study.

The constructed strains were stored on YEPD and YNB selective medium respectively in a deep freezer at - 80℃ suspended in 15% glycerol.

Table 2. List of the *S. cerevisiae* D452-2 strains used in this study

Name	Description
D452-2	*Saccharomyces cerevisiae* (*Matα, leu2 his3 ura3 can1*)
D-CON-$_{GPD}$	D452-2 (p423GPD, p425GPD, p426GPD)
D-2K-$_{GPD}$	D452-2 (p423GPD, p425GPD-MI2-K, p426GPD)
D-2KCD-$_{GPD}$	D452-2 (p423GPD-C, p425GPD-MI2-K, p426GPD-D)

Table 3. List of the SOS4 and SOS4X strains used in this study

Name	Description
SOS4	D452-2 Δ*PDC1* Δ*PDC5*
SOS4-CON-$_{GPD}$	SOS4 (p423GPD, p425GPD, p426GPD)
SOS4-2K-$_{GPD}$	SOS4 (p423GPD, p425GPD-MI2-K, p426GPD)
SOS4-2KCD-$_{GPD}$	SOS4 (p423GPD-C, p425GPD-MI2-K, p426GPD-D)
SOS4-BKCD-$_{GPD}$	SOS4 (p423GPD-C, p425GPD-SB-K, p426GPD-D)
SOS4-KKCD-$_{GPD}$	SOS4 (p423GPD-C, p425GPD-SK-K, p426GPD-D)
SOS4-PKCD-$_{GPD}$	SOS4 (p423GPD-C, p425GPD-SP-K, p426GPD-D)
SOS4-2KCD-$_{CYTO\text{-}GPD}$	SOS4 (p423GPD-MC, p425GPD-MI2-K, p426GPD-MD)
SOS4-2KCD-$_{CYTO\text{-}HXT}$	SOS4 (p423HXT-MC, p425HXT-MI2-K, p426HXT-MD)
SOS4X	SOS4 (p306-XYL123)
SOS4X-CON-$_{HXT}$	SOS4X (p423HXT, p425HXT)
SOS4X-2KCD-$_{CYTO\text{-}HXT}$	SOS4X (p423HXT-MC-MD, p425HXT-MI2-K)

2.2. Plasmids

6 plasmids were used as mother vectors which has the *GPD* promoter and truncated *HXT7* promoter respectively and *CYC1* terminator from *S. cerevisiae.*(Figure 6) These are cloning vectors for episomal expression system of isobutanol biosynthetic pathway, endogenous *ILV2* gene, *alsS* genes from *Bacillus subtilis, Klebsiella pneumoniae* and *Penibacillus polymyxa, ilvC* and *ilvD* genes from *E. coli* and *kivD* gene from *L. lactis.* (Table 4, 5) There are oligonucleotide sequence of primers used cloning. (Table 6)

Abbreviations and the meaning used in this study are as follows.

MI2 is modified *ILV2* gene which means truncated *ILV2* gene by removing mitochondria. SB means *alsS* gene from *B. subtilis.* SK means *alsS* gene from *K. pneumonia.* SP means *alsS* gene from *P. polymyxa.* C means *ilvC* gene from *E. coli*. D means *ilvC* gene from *E. coli*. MC is modified *ilvC* gene which means truncated *ilvC* gene for expression in cytosol. MD is modified *ilvD* gene which means truncated *ilvD* gene for expression in cytosol. K means *kivD* gene from *L. lactis.* XYL123 means *XYL1, XYL2* and *XYL3* genes from *S. stipitis.*

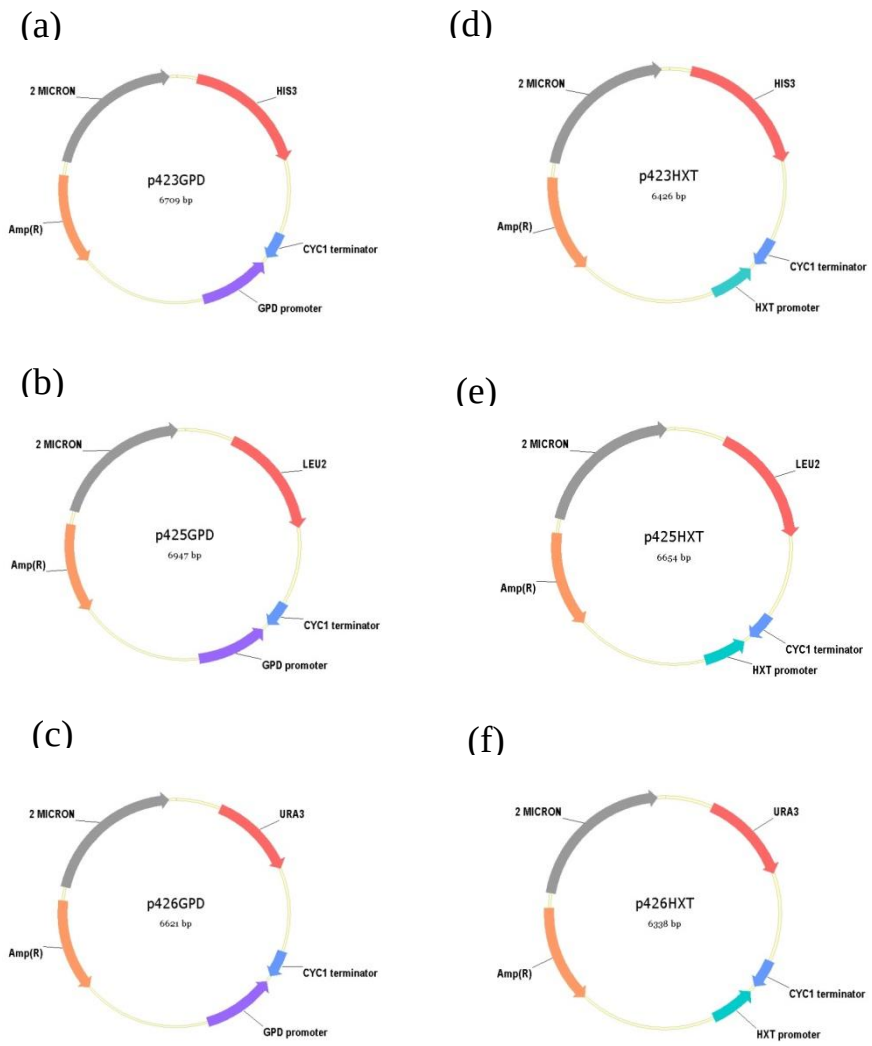


Figure 6. Mother vectors used in this study

(a) p423GPD, (b) p425GPD, (c) p426GPD,

(a) p423HXT, (b) p425HXT, (c) p426HXT

Table 4. List of the plasmids with *GPD* promoter used in this study

Name	Description
p423GPD	HIS3, *GPD* promoter, *CYC1* terminator, 2 μ origin, Amp^r
p425GPD	LEU2, *GPD* promoter, *CYC1* terminator, 2 μ origin, Amp^r
p426GPD	URA3, *GPD* promoter, *CYC1* terminator, 2 μ origin, Amp^r
p425GPD-MI2-K	pRS425GPD harboring modified *ILV2* from *S. cerevisiae* and *kivD* from *L. lactis*
p425GPD-SB-K	pRS425GPD harboring *alsS* from *B. subtilis* and *kivD* from *L. lactis*
p425GPD-SK-K	pRS425GPD harboring *alsS* from *K. pneumoniae* and *kivD* from *L. lactis*
p425GPD-SP-K	pRS425GPD harboring *alsS* from *P. polymyxa* and *kivD* from *L. lactis*
p423GPD-C	pRS423GPD harboring *ilvC* from *E. coli*
p426GPD-D	pRS423GPD harboring *ilvD* from *E. coli*
p423GPD-MC	pRS423GPD harboring modified *ilvC* from *E. coli*
p426GPD-MD	pRS423GPD harboring modified *ilvD* from *E. coli*
p306-XYL123	pRS306 harboring *XYL1*, *XYL2* and *XYL3* from *S. stipitis*

Table 5. List of the plasmids with truncated *HXT7* promoter used in this study

Name	Description
p423HXT	HIS3, truncated *HXT7* promoter, *CYC1* terminator, 2 μ origin, Amp^r
p425HXT	LEU2, truncated *HXT7* promoter, *CYC1* terminator, 2 μ origin, Amp^r
p426HXT	URA3, truncated *HXT7* promoter, *CYC1* terminator, 2 μ origin, Amp^r
p425HXT-MI2-K	pRS425HXT harboring modified *ILV2* from *S. cerevisiae* and *kivD* from *L. lactis*
p423HXT-MC	pRS423HXT harboring modified *ilvC* from *E. coli*
p426HXT-MD	pRS426 HXT harboring modified *ilvD* from *E. coli*
p423HXT-MC-MD	pRS423HXT harboring modified *ilvC* and modified *ilvD* from *E. coli*

pRS423GPD, pRS425GPD and pRS426GPD were donated (Christianson et al., 1992).

p306_XYL123 was constructed by professor Jin Yong Su's group at University of Illinois at Urbana-Champaign.

Other plasmids in Table 4, 5 were constructed in this study.

Table 6. List of oligonucleotide used in this study

Primer name	Oligonucleotide sequence (5’ → 3’)
F-MI2-BamHI	CGGGATCCAAAATGGAGCCTGCTCCAAGTTT
R-MI2-XhoI	CCGCTCGAGTTAGTGCTTACCGCCTGTAC
F-K-BamHI	CGGGATCCATGTATACAGTAGGAGATTAC
R-K-XhoI	CCGCTCGAGTTATGATTTATTTTGTTCAGCAA
F-SB-BamHI	CGGGATCCATGTTGACAAAAGCAACAAAAGA
R-SB-XhoI	CCGCTCGAGCTAGAGAGCTTTCGTTTTCA
F-SK-BamHI	CGGGATCCATGGACAAACAGTATCCGGTA
R-SK-XhoI	CCGCTCGAGTTACAGAATCTGACTCAGATGCA
F-SP-BamHI	CGGGATCCTTGAGTACAAAAGTGCAAGCTGT
R-SP-XhoI	CCGCTCGAGTTAGTTTAATTGGTTAGGCAGCA
F-C-BamHI	CGGGATCCAAAATGGCTAACTACTTCAATACACT
R-C-XhoI	CCGCTCGAGTTAGTGGTGATGGTGATGATGACCCGCAACAGCAATACG
F-MC-BamHI	CGGGATCCAAAATGATGGGCCGCGATGAATTC
R-MC-XhoI	CCGCTCGAGTTAGTGGTGATGGTGATGATGACCCGCAACAGCAATACG
F-D-BamHI	CGGGATCCAAAATGCCTAAGTACCGTTCCG
R-D-EcoRI	CGGAATTCTTAGTGGTGATGGTGATGATGACCCCCCAGTTTCGATTTATC
F-MD-BamHI	CGGGATCCAAAATGACCGGAATGACCGACGC
R-MD-EcoRI	CGGAATTCTTAGTGGTGATGGTGATGATGACCCCCCAGTTTCGATTTATC
SacI-GPDp	CGAGCTCAGTTTATCATTATCAATACTCGCCA
SacI-HXTp	CGAGCTCTCGGGCCCCTGCTTCTG
SacI-CYCt	CGAGCTCGGCCGCAAATTAAAGCCTTC

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. Transformation of *E. coli*

Transformation of *E. coli* was carried out as described by Sambrook et al., (1989). *E. coli* Top10 was cultured in 5 mL LB medium for 12hr. 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.

3.3. Preparation of plasmid DNA and yeast 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 microorganism genomic DNA to obtain a template for the gene was carried out using using DNeasy Blood & Tissue Kit from QIAGEN (Düsseldorf, 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 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).

3.5. 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 45 sec, 55 ℃ for 30 sec, 72 ℃ for 1 min, 1 cycle of 72 ℃ for 10 min. The amplified gene was confirmed by gel electrophoresis.

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 (1 % tryptone, 0.5 % yeast extract, 1 % NaCl) with 50 μg/mL ampicillin was used for recombinant *E. coli* cultivation.

YEPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose) and YNB 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. Inoculum

Recombinant *S. cerevisiae* stock was transferred to a test tube containing YNBD selection medium and incubated overnight at 30 ℃, 250 rpm in shaking incubator (Vision, Korea). Pre-culture was performed in a 250 mL flask with 100 mL working volume at 30 ℃, 250 rpm for appropriate time. The inocula were prepared by growing cells overnight to an OD_{600} of 5~10. The cells were harvested by centrifugation at 3,000 rpm for 10 min and washed in 5 mL of sterilized DDW. The washed cells were transferred to the 250 mL glass flask containing 50 mL YEPD medium. The initial OD_{600} of main flask culture was 1.0. in glucose medium and 10.0 in xylose medium. In bioreactor cultivation initial OD_{600} was approximately 5.0.

4.3. Cultivations

Batch fermentation in flask was carried out 50 mL working volume at 30 ℃ in shaking incubator (Vision Korea), and shaking rate was maintained at 100 rpm for creating micro-aerobic conditions.

Large-scale batch fermentation was performed using a bench-top fermentor (KoBioTech, Korea). Cultivations were performed in 500 mL YEPD medium at 30 ℃ and pH 5.5 (adjustment by 5N HCl and 2N NaOH). The fermentation experiments were performed under separate cultivation conditions. (Table 7)

Table 7. Summary of cultivation conditions in bioreactor

Agitation (rpm)	Aeration (vvm)
300	0.25
300	0.5
300	1.0

5. Analysis

5.1. Dry cell mass

Dry cell mass concentration was estimated by measuring absorbance at 600 nm by a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). Optical density was converted into dry cell mass by using the following conversion equation.

$$\text{Dry cell mass (g/L)} = 0.345 \times OD_{600}$$

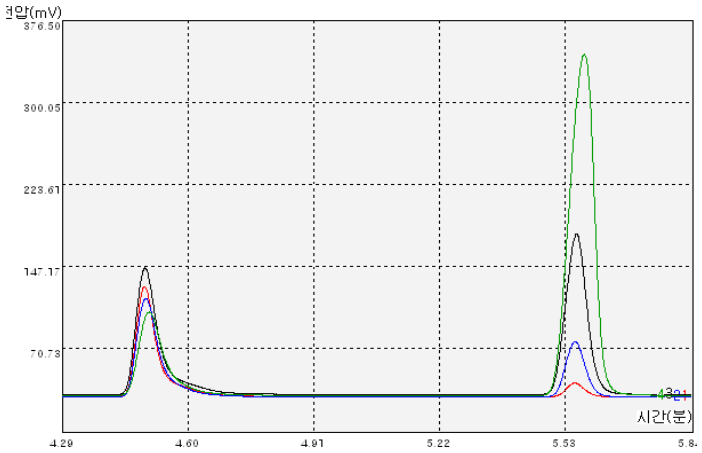
5.2. Metabolite detection

Concentrations of glucose and ethanol were measured by a high performance liquid chromatography (Agilent 1100LC, U.S.A) equipped with the Carbohydrate Analysis column (Phenomenex, USA). The carbohydrate analysis ion exclusion column heated at 60°C was applied to analyze the 20 uL of diluted culture broth. Detection was made with a reflective index detector at 35 °C. HPLC operation conditions were set according to the instruction manual of the column supplier. H_2SO_4 (5 mM) solution was used as mobile phase at a 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 A 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℃. 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 content was determined by extrapolation from standard curves using the internal standard to normalize the values. (Figure 7)

(a)



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

(b)

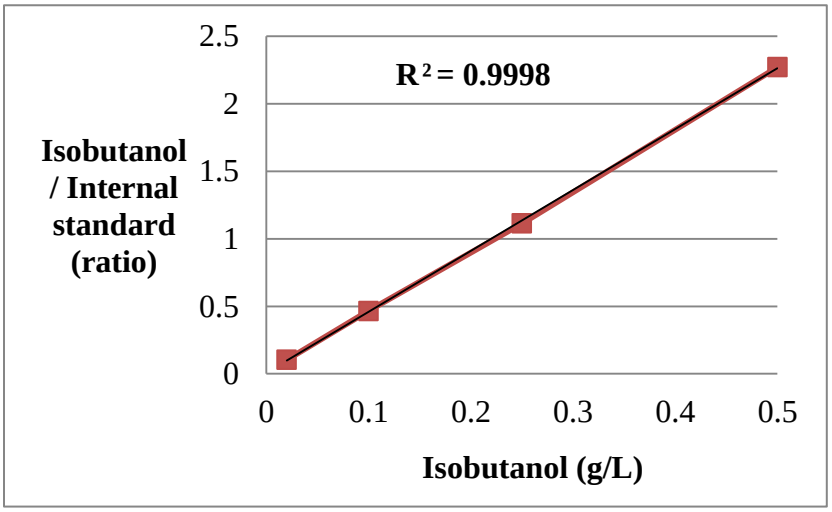


Figure 7. (a) Standard peak of GC analysis : n-propanol, isobutanol

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

III. RESULTS AND DISCUSSIONS

1. Production of isobutanol by pyruvate decarboxylase-deficient *S. cerevisiae* (SOS4) with the isobutanol biosynthetic pathway

1.1. Production of isobutanol in *S. cerevisiae*

In previous studies by S.J. Baek, modified *ILV2* gene and *kivD* gene were tested by combination of integration and episomal expression systems. So in this study, additional *ilvC* and *ilvD* genes from *E. coli* were overexpressed to construct the whole valine biosynthetic pathway in *S. cerevisiae*. Therefore, when modified *ILV2*, *ilvC*, *ilvD* and *kivD* genes were introduced, the isobutanol biosynthetic pathway from pyruvate to isobutylaldehyde was constructed. And the final step from isobutylaldehyde to isobutanol was catalyzed by endogenous ADH which is abundant in *S. cerevisiae*.

Genetic map of plasmids constructed in this part is in Figure 8 and D-CON-$_{GPD}$, D-2K-$_{GPD}$ and D-2KCD-$_{GPD}$ were constructed using these plasmids. The flask fermentation profiles of constructed strains are in Figure 9.

While D-CON$_{-GPD}$ produced 30 mg/L of isobutanol and D-2K$_{-GPD}$

produced 72 mg/L of isobutanol, D-2KCD$_{-GPD}$ produced 120 mg/L of isobutanol from 20 g/L of glucose within 144 h in micro-aerobic conditions. The strain overexpressed modified *ILV2*, *ilvC*, *ilvD* and *kivD* genes produced isotuanol 4-fold more than wild type strain with empty plasmids. And additional introduction of *ilvC* and *ilvD* genes is slightly effective, but the amount of isobutanol concentration was an immaterial increase. That is because about 7 g/L of ethanol still produced as by-product competing with isobutanol. To eliminate ethanol production pathway and improve isobutanol production, pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae* was used in next part.

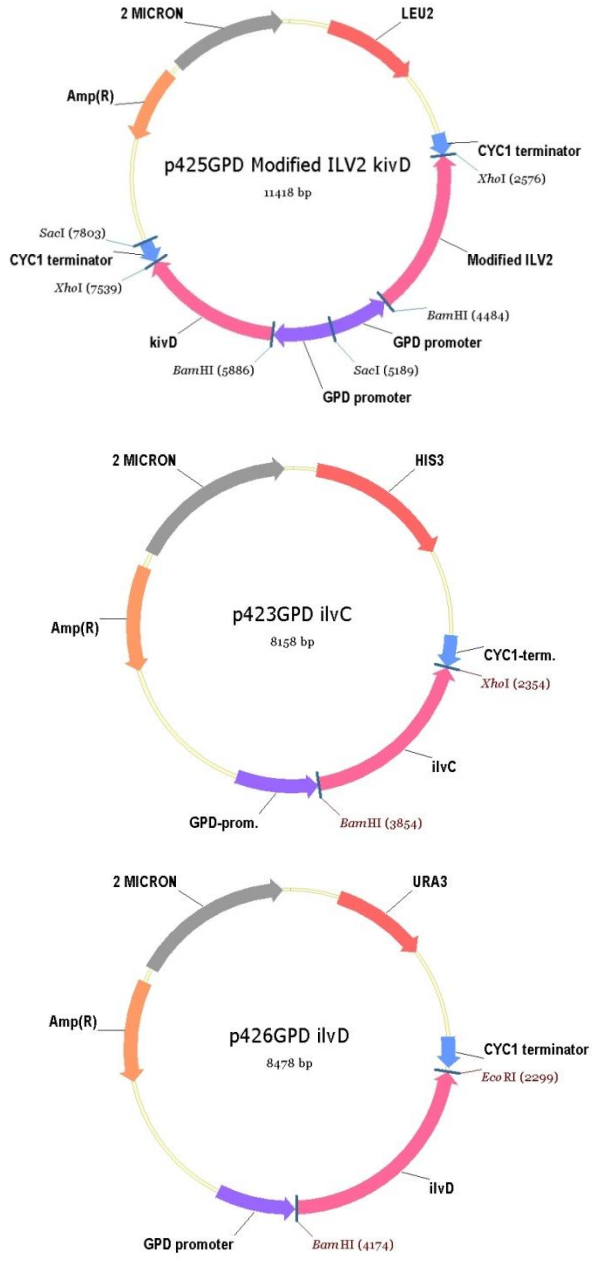


Figure 8. Genetic map of plasmids

(a) p425GPD-MI2-K, (b) 423GPD-C, (c) p426GPD-D

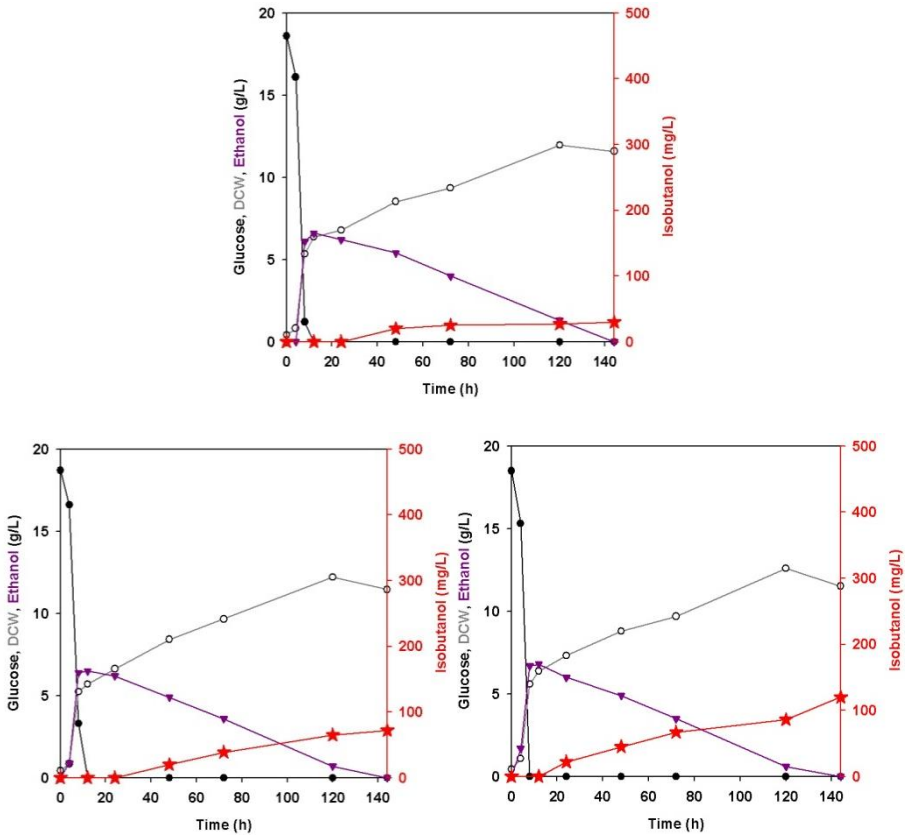


Figure 9. Flask fermentation profiles of

(a) D-CON-$_{GPD}$, (b) D-2K-$_{GPD}$, (c) D-2KCD-$_{GPD}$

Symbols : Glucose ( ), DCW ( ), Pyruvate ( ),

Glycerol ( ), Acetate ( ), Ethanol ( ), Isobutanol ( )

1.2. Production of isobutanol in the SOS4

SOS4-CON$_{-GPD}$, SOS4-2K$_{-GPD}$, SOS4-2KCD$_{-GPD}$ were constructed using those plasmids constructed in previous part and the flask fermentation profiles of constructed strains are in Figure 10.

When SOS4 with empty plasmids consumed only 15.8 g/L glucose from initial 20 g/L glucose within 144 h under micro-aerobic conditions and accumulated 4.2 g/L of pyruvate without production of ethanol. The SOS4-2K$_{-GPD}$ produced 152 mg/L of isobutanol and accumulated 3.65 g/L of pyruvate and SOS4-2KCD$_{-GPD}$ produced 283 mg/L of isobutanol and accumulated 3.06 g/L of pyruvate with slightly rapid glucose consumption. These results suggest that accumulated pyruvate can be converted into isobutanol by isobutanol biosynthetic pathway. While *S. cerevisiae* showed an immaterial increase resulted from additional overexpressen of *ilvC* and *ilvD* genes in previous part, the improved amount of isobutanol in SOS4 was worthy increment. However, a difference between reduced amount of accumulated pyruvate and increased amount of isobutanol was unbalanced because reduced pyruvate was used in respiration mainly. To convert pyruvate to isobutanol effectively, it is necessary to enhance the isobutanol biosynthetic pathway.

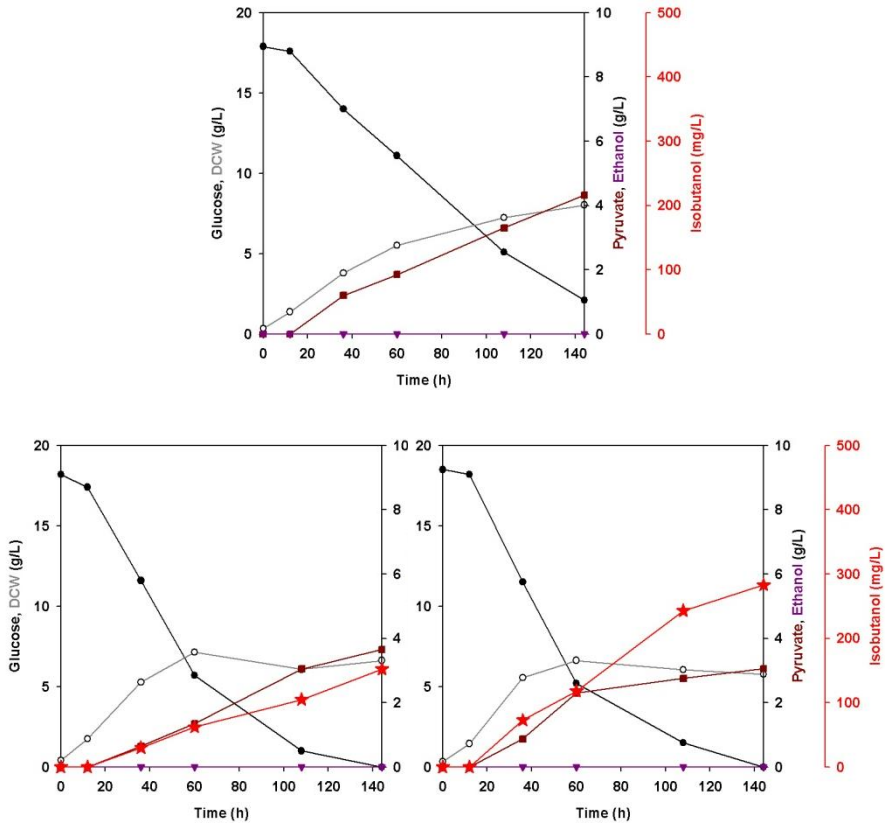


Figure 10. Flask fermentation profiles of

(a) SOS4-CON$_{-GPD}$, (b) SOS4-2K$_{-GPD}$, (c) SOS4-2KCD$_{-GPD}$

Symbols : Glucose ( ), DCW ( ), Pyruvate ( ),

Glycerol ( ), Acetate ( ), Ethanol ( ), Isobutanol ( )

Table 8. Summary of flask fermentation in part 1.1. and 1.2.

Strain	Maximum dry cell weight (g/L)	Consumed glucose (g/L)	Pyruvate (g/L)	Concentration of isobutanol (mg/L)	Yield of isobutanol ($mg_{isobutaol}/g_{glucose}$)
D-CON_{-GPD}	12.0	18.6	0	30	1.61
D-$2K_{-GPD}$	12.2	18.7	0	72	3.85
D-$2KCD_{-GPD}$	12.6	18.5	0	120	6.49
SOS4-CON_{-GPD}	8.0	15.8	4.3	0	-
SOS4-$2K_{-GPD}$	7.1	18.2	3.7	152	8.35
SOS4-$2KCD_{-GPD}$	6.6	18.5	3.1	283	15.30

2. Construction of the efficient isobutanol biosynthetic system

2.1. Evaluation of the acetolactate synthases from various microorganisms

ALS is a key enzyme for isobutanol production because it catalyze the first step for conversion of pyruvate to isobutanol. *alsS* genes from other bacteria strains were known to have strong activity and readily used in other studies. Especially, *alsS* gene from *B. subtilis* is used in 2,3-butanediol production in *S. cerevisiae* (Kim et al., 2013).

In this study, three different *alsS* genes from *Bacillus subtilis*, *Klebsiella pneumoniae* and *Penibacillus polymyxa* were tested for isobutanol production.

Genetic map of plasmids constructed in this part is in Figure 11 and SOS4-BKCD$_{-GPD}$, SOS4-KKCD$_{-GPD}$ and SOS4-PKCD$_{-GPD}$ were constructed using these plasmids. The flask fermentation profiles of constructed strains are in Figure 11.

Each strain containing various *alsS* genes, p425GPD-SB-K, p425GPD-SK-K and p425GPD-SP-K, produced 269 mg/L, 237 mg/L, 223 mg/L of isobutanol, and the results maens that modified *ILV2* gene was slightly more effective than *alsS* gene from bacteria.

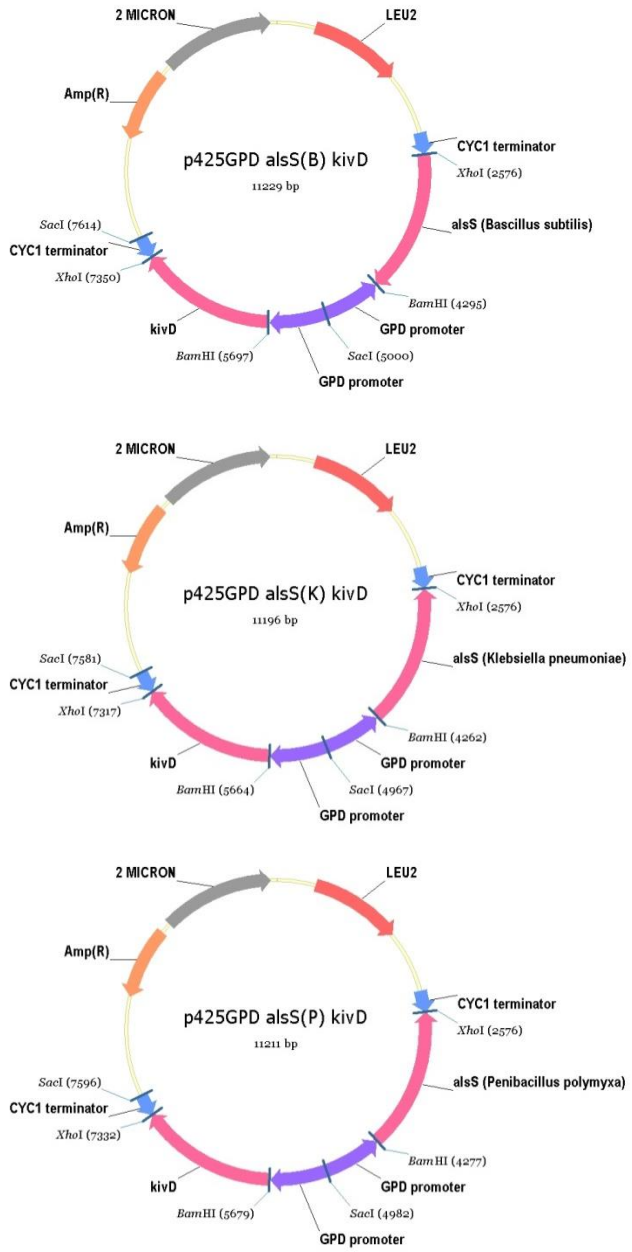


Figure 11. Genetic map of plasmids

(a) p425GPD-SB-K, (b) p425GPD-SK-K, (c) p425GPD-SP-K

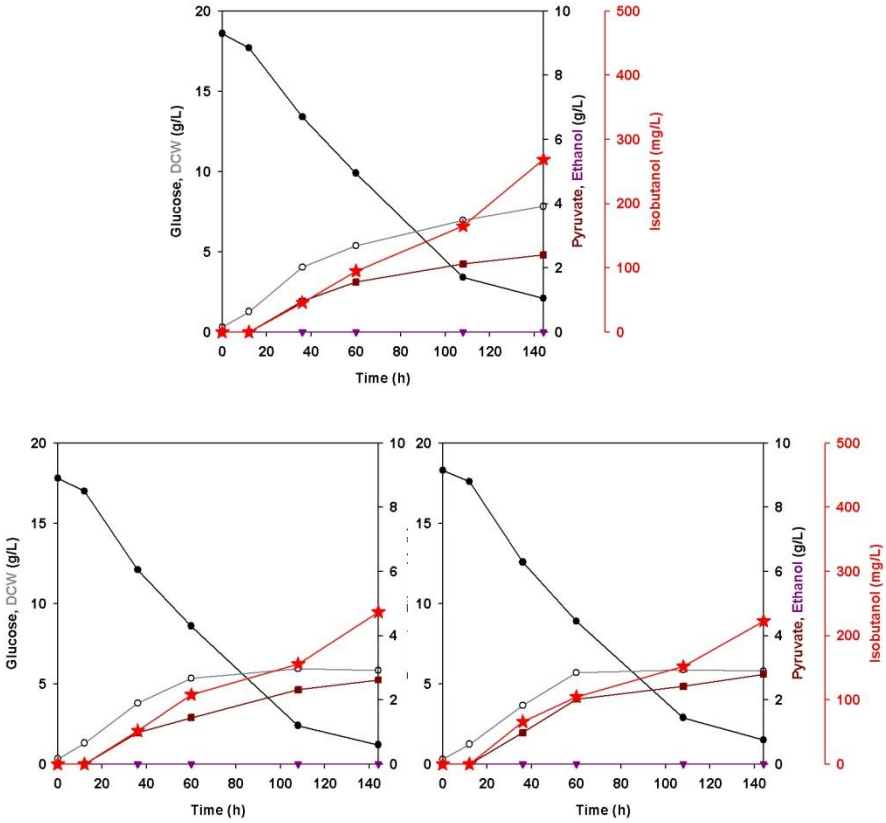


Figure 12. Flask fermentation profiles of

(a) SOS4-BKCD$_{-GPD}$, (b) SOS4-KKCD$_{-GPD}$, (c) SOS4-PKCD$_{-GPD}$

Symbols : Glucose ( ), DCW ( ), Pyruvate ( ), Glycerol ( ), Acetate ( ), Ethanol ( ), Isobutanol ( )

Table 9. Summary of flask fermentation in part 2.1.

Strain	Maximum dry cell weight (g/L)	Consumed glucose (g/L)	Pyruvate (g/L)	Concentration of isobutanol (mg/L)	Yield of isobutanol ($mg_{isobutanol}/g_{glucose}$)
SOS4-2KCD$_{-GPD}$	6.6	18.5	3.1	283	15.30
SOS4-BKCD$_{-GPD}$	7.8	16.5	2.4	269	16.30
SOS4-KKCD$_{-GPD}$	5.9	16.6	2.6	237	14.27
SOS4-PKCD$_{-GPD}$	5.9	16.8	2.8	223	13.27

2.2. Cytosolic re-localization of the whole valine biosynthesis pathway of *S. cereviae*

ilvC and *ilvD* genes from *E. coli* were overexpressed to convert the 2-acetolactate produced from pyruvate by modified *ILV2* gene more efficiently. But, it is also known to be expressed in the mitochondrial matrix in *E. coli*. It has possibility to locate enzymes encoded by these genes into mitochondrial matrix in yeast. Therefore, modified *ilvC* and *ilvD* genes truncated putative mitochondria targeting sequences were used in this study for constructing cytosolically re-localized isobutanol biosynthetic pathway in *S. cerevisiae*.

Genetic map of plasmids constructed using modified *ilvC* and *ilvD* genes in this part is in Figure 13 and SOS4-2KCD$_{\text{-CYTO-GPD}}$ was constructed using these plasmids. The flask fermentation profiles of constructed strains are in Figure 14.

The results was small range of increase in isobutanol titer, which is increased up to 326 mg/L of isobutanol from 283 mg/L of isobutanol consuming 20 g/L of glucose within 144 h under micro-aerobic conditions, and equally confirmed in repeated experiments. Cytosolic re-localized expression of these enzymes encoded modified genes was proved in previous research by indirect

immunofluorescence microscopy of wild-type and N-terminally truncated proteins carrying a C-terminal 6His-tag. α-His antibodies were applied for the visualisation of Ilv enzymes and α-Hsp70 antibodies were applied for cytosolic staining (D Brat et al., 2012).

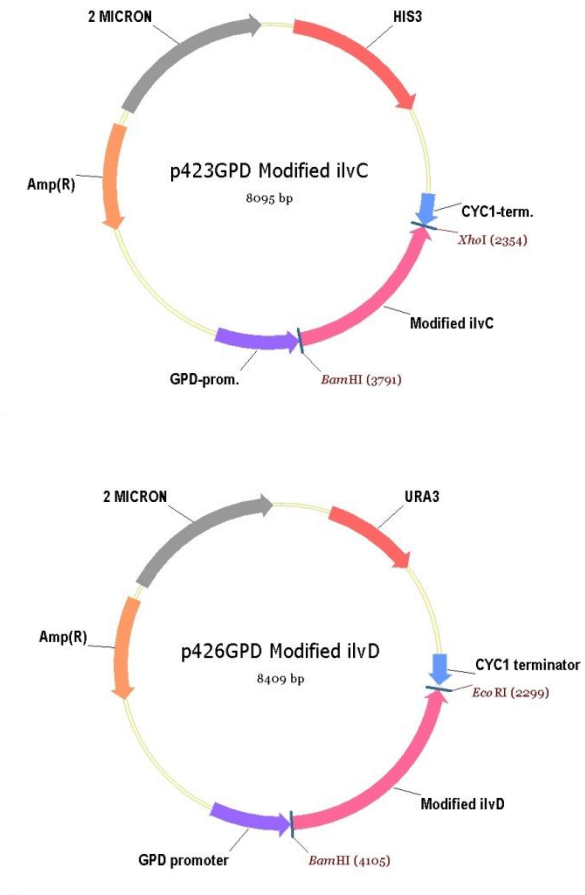


Figure 13. Genetic map of plasmids

(a) p423GPD-MC, (b) p426GPD-MD

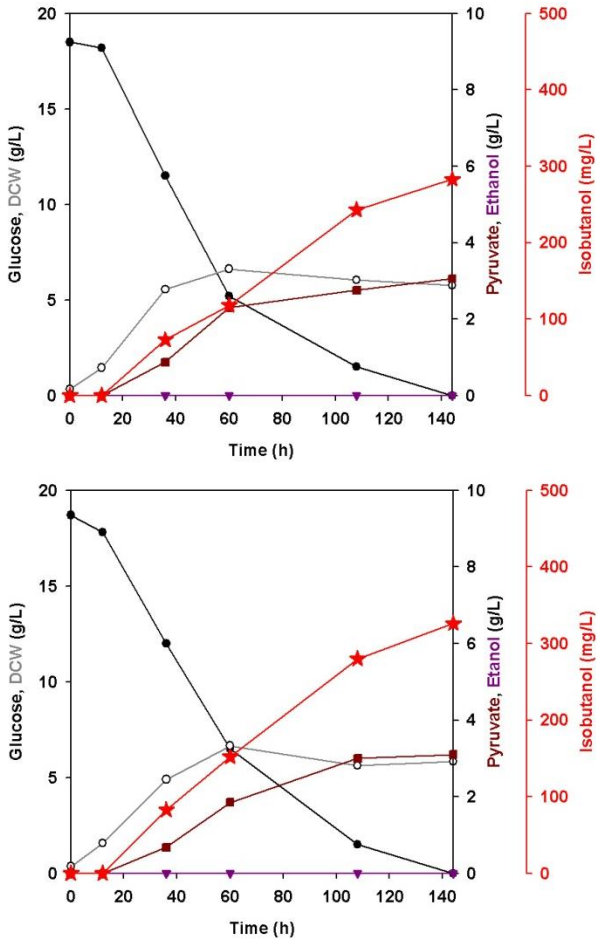


Figure 14. Flask fermentation profiles of

(a) SOS4-2KCD$_{-GPD}$, (b) SOS4-2KCD$_{-CYTO-GPD}$

Symbols : Glucose ( ), DCW ( ), Pyruvate ( ), Glycerol ( ), Acetate ( ), Ethanol ( ), Isobutanol ( )

2.3 Promoter replacement to increase isobutanol biosynthetic genes expression level

In previous parts of this study, all genes were controlled under *GPD* promoter. To enhance the isobutanol biosynthetic pathway, *GPD* promoter was replaced with truncated *HXT7* promoter. These constitutive promoters are normally used for overexpression of heterologous gene in yeast. Particularly, truncated *HXT7* promoter is known as 30 ~ 40 % stronger than *GPD* promoter in yeast.

Genetic map of plasmids constructed using mother vectors containing truncated *HXT7* promoter in this part is in Figure 15. And SOS4-2KCD$_{\text{-CYTO-HXT}}$ was constructed using these plasmids. The flask fermentation profiles of constructed strains are in Figure 16.

The results was 1.37-fold increase in isobutanol titer between SOS4-2KCD$_{\text{-CYTO-GPD}}$ and SOS4-2KCD$_{\text{-CYTO-HXT}}$, which is increased up to 446 mg/L of isobutanol from 326 mg/L of isobutanol from 20 g/L of glucose within 144 h under micro-aerobic conditions, and equally confirmed in repeated experiments.

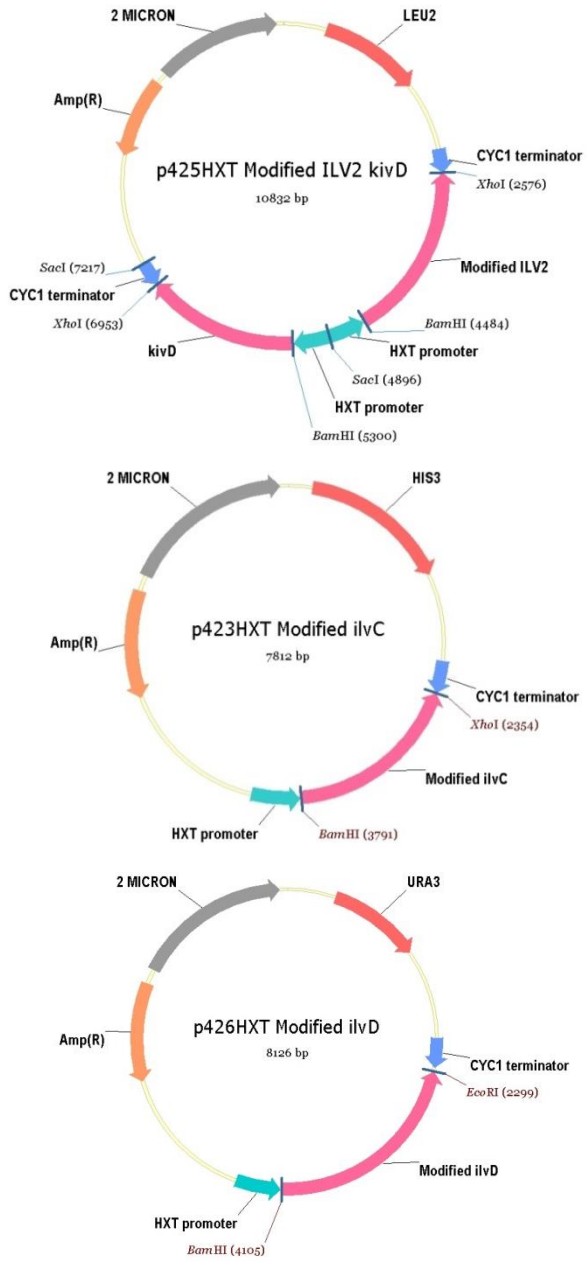


Figure 15. Genetic map of plasmids

(a) p425HXT-MI2-K, (b) p423HXT-MC, (c) p426HXT-MD

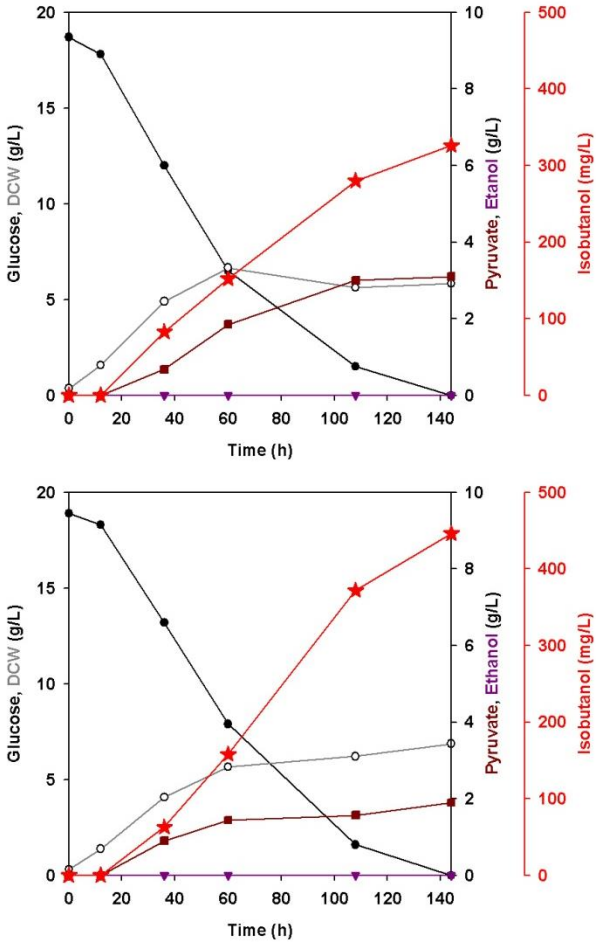


Figure 16. Flask fermentation profile of

(a) SOS4-2KCD$_{\text{-CYTO-GPD}}$, (b) SOS4-2KCD$_{\text{-CYTO-HXT}}$

Symbols : Glucose ( ), DCW ( ), Pyruvate ( ), Glycerol ( ), Acetate ( ), Ethanol ( ), Isobutanol ( )

Table 10. Summary of flask fermentation in part 2.2. and 2.3.

Strain	Maximum dry cell weight (g/L)	Consumed glucose (g/L)	Pyruvate (g/L)	Concentration of isobutanol (mg/L)	Yield of isobutanol ($mg_{isobutanol}/g_{glucose}$)
SOS4-2KCD$_{-GPD}$	6.6	18.5	3.1	283	15.30
SOS4-2KCD$_{-CYTO-GPD}$	6.7	18.7	3.1	326	17.43
SOS4-2KCD$_{-CYTO-HXT}$	6.9	18.9	1.9	446	23.60

3. Production of isobutanol from xylose by pyruvate decarboxylase-deficient *S. cerevisiae* (SOS4) with xylose fermenting pathway and the isobutanol biosynthetic system

In order to obtain xylose-fermenting ability, SOS4X that p306-XYL123 harboring *XYL1*, *XYL2* and *XYL3* genes under the control of constitutive promoters was integrated into the URA3 locus of the SOS4 genome was constructed in previous study of 2,3-butanediol production (Kim et al., 2013a).

When SOS4X with empty plasmids cultured in complex medium with 20 g/L of xylose within 144 h under micro-aerobic conditions, SOS4X-CON$_{-HXT}$ consumed only 17.5 g/L xylose from initial 20 g/L xylose and accumulated 3.9 g/L of pyruvate without production of ethanol after 144 h. The accumulation of pyruvate by the elimination of ethanol production in the SOS4X suggest that the SOS4X might be a good strain to produce isobutanol from xylose.

As the SOS4 was able to consume xylose and accumulate pyruvate instead of ethanol, the isobutanol biosynthetic system constructed in previous parts introduced into the SOS4X to convert pyruvate accumulated from xylose into isotubanol.

Genetic map of plasmid constructed in this part is in Figure 17. SOS4X-CON$_{\text{-HXT}}$ and SOS4-2KCD$_{\text{-CYTO-HXT}}$ was constructed and the flask fermentation profiles of constructed strains are in Figure 18.

Specially, SOS4-2KCD$_{\text{-CYTO-HXT}}$ produced 120 mg/L of isobutanol from 20 g/L of xylose within 144 h under micro-aerobic conditions without ethanol production. This results show that Pdc-deficient *S. cerevisiae* containing a cytosolically located isobutanol biosynthetic pathway under strong promoter and a xylose assimilating pathway are able to convert xylose into isobutanol.

Simultaneous overexpression of an optimized, cytosolically localized valine biosynthesis pathway together with overexpression of xylose isomerase *XylA* from *Clostridium phytofermentans*, transaldolase *Tal1* and xylulokinase *Xks1* enabled recombinant *S. cerevisiae* cells to complement the valine auxotrophy of *ilv2,3,5* triple deletion mutants for growth on D-xylose as the sole carbon source. Moreover, after additional overexpression of ketoacid decarboxylase *Aro10* and alcohol dehydrogenase *Adh2*, the cells were able to ferment D-xylose directly to isobutanol. The strain consumed about 12 g D-xylose and produced up to 1.36 mg/L of

isobutanol (D Brat et al., 2013).

In this study, increased isobutanol production from xylose was achived by eliminating of competing ethanol production pathway. However, the isobutanol production rates and titers from xylose are still low. The SOS4-2KCD-$_{CYTO-HXT}$ strain have not yet been optimized and need to be improved by further genetic or evolutionary engineering. Further optimization of the strain should greatly enhance isobutanol production from xylose.

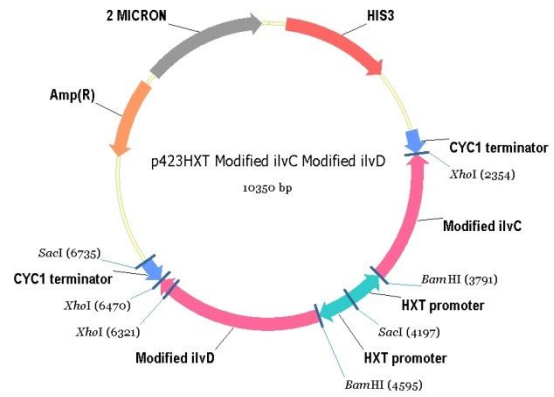


Figure 17. Genetic map of plasmid p423HXT-MC-MD

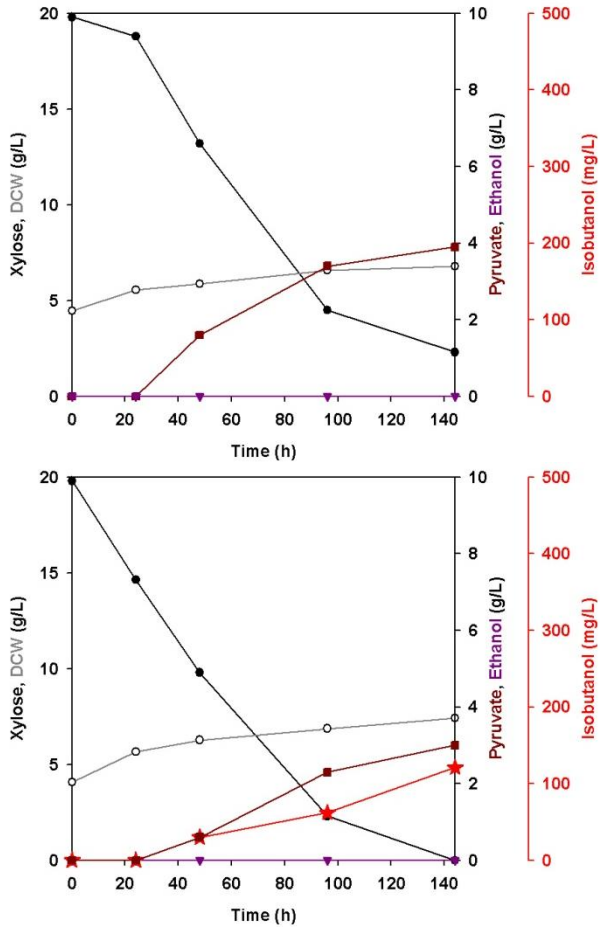


Figure 18. Flask fermentation profiles of

(a) SOS4X-CON$_{\text{-HXT}}$, (b) SOS4-2KCD$_{\text{-CYTO-HXT}}$

Symbols : Xylose ( ), DCW ( ), Pyruvate ( ), Glycerol ( ), Acetate ( ), Ethanol ( ), Isobutanol ( )

Table 11. Summary of flask fermentation in part 3.

Strain	Maximum dry cell weight (g/L)	Consumed xylose (g/L)	Pyruvate (g/L)	Concentration of isobutanol (mg/L)	Yield of isobutanol ($mg_{isobutanol}/g_{xylose}$)
SOS4X-CON_{-HXT}	6.8	17.5	3.9	0	-
SOS4X-$2KCD_{-CYTO-HXT}$	7.4	19.8	3.0	120	6.06

IV. CONCLUSIONS

This thesis can draw the following conclusions :

(1) In micro-aerobic batch fermentation, *S. cerevisiae* harboring the modified *ILV2*, *ilvC*, *ilvD* and *kivD* genes produced 120 mg/L isobutanol along with 7 g/L ethanol as a major metabolite. However, the Pdc-deficient *S. cerevisiae* strain harboring the modified *ILV2*, *ilvC*, *ilvD* and *kivD* genes produced 283 mg/L isobutanol without ethanol production.

(2) To enhance the isobutanol biosynthetic pathway, all enzymes involved in isobutanol production were cytosolically re-localized by modification of the *ilvC* and *ilvD* genes and the *GPD* promoter was replaced with the truncated *HXT7* promoter. The Pdc-deficient *S. cerevisiae* harboring three modified *ILV2*, *ilvC* and *ilvD* genes and *kivD* under the control of the truncated *HXT7* promoter produced 446mg/L isobutanol in micro-aerobic batch fermentation.

(3) To construct *S. cerevisiae* able to metabolize xylose without ethanol production, *XYL1*, *XYL2* and *XYL3* were introduced into the Pdc-deficient *S. cerevisiae*. When the isobutanol biosynthetic system was overexpressed in this strain, 120 mg/L of isobutanol was produced from xylose in micro-aerobic batch fermentation.

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국 문 초 록

화석연료의 사용으로 인한 에너지자원 고갈과 지구온난화와 같은 환경문제가 야기되면서 미생물을 이용하여 재생 가능한 생물자원으로부터 바이오 에너지를 생산하고자 하는 연구가 활발히 진행되고 있다. 현재 상용화된 대표적인 바이오 연료는 에탄올지만, 최근에는 탄소사슬이 많은 알코올들이 주목을 받고 있다. 그 중 부탄올은 에탄올에 비해 에너지 밀도가 높고, 흡습성 및 증기압이 낮아 운반에 용이해서 다양하게 적용이 가능한 연료이다. 특히 이소부탄올은 n-부탄올에 비해 더 많은 옥탄가를 가지고 있고 가솔린과 어떤 비율로도 혼합이 가능하여 기존의 가솔린 엔진과 수송 파이프라인에 그대로 적용 가능하다는 장점을 갖고 있다. 또한 수송연료뿐만 아니라 플라스틱, 연료 첨가제 등을 합성하는 전구체로서 사용되는 고부가가치 물질로 주목 받고 있다.

본 연구에서는 재조합 균주 개발에 용이하고, 부탄올과 당화액에 대한 내성이 높은 효모 *Saccharomyces cerevisiae*를 사용하여 대사공학적인 접근을 통해 이소부탄올을 생산하고자 하였다.

와일드 타입의 *S. cerevisiae*는 발린 생합성 경로와 에이리치 경로를 통하여 미토콘드리아에서 이소부탄올을 소량 생산할 수 있다. 이소부탄올 생합성에 관여하는 *S.*

cerevisiae 유래의 *ILV2, E. coli* 유래의 *ilvC*와 *ilvD, L. lactis* 유래의 *kivD* 유전자를 도입하여 120 mg/L의 이소부탄올을 생산하였다. 그러나 여전히 에탄올이 주된 산물로 생성되는 문제점이 있었다.

따라서 주요 부산물인 에탄올 생성을 억제하고자 피루브산탈카르복시효소 활성이 완전히 저해된 균주를 모균주로 하여 이소부탄올 생합성 경로를 도입하였고 결과적으로 에탄올의 생성 없이, 축적된 피루브산으로부터 283 mg/L의 이소부탄올을 생산하였다.

하지만 여전히 미량의 이소부탄올이 생성됨에 따라 이소부탄올의 생합성 경로를 강화하고자 이소부탄올 합성과 관련된 모든 효소를 미토콘드리아가 아닌 세포질에서 발현되도록 조작하였으며, 효모에서 강력하다고 알려진 프로모터를 이용하였다. 그 결과, 아이소부탄올의 생산량을 446 mg/L까지 증가시켰다.

이와 더불어, 경제적 측면에서 볼 때, 목질계 바이오매스로부터 이소부탄올을 생산하는 것이 이소부탄올의 지속 가능한 상업화를 위해 해결해야 할 주요 연구 과제이다. 하지만 *S. cerevisiae*는 목질계 바이오매스에 다량으로 존재하는 자일로스를 대사하지 못한다는 단점이 있다. 이를 개선하고자 *S. stipitis* 유래의 자일로스 대사 관련 유전자인 *XYL1, XYL2, XYL3*를 피루브산탈카르복시효소 활성이 완전히 저해된 균주에 도입하였다. 포도당에서와

마찬가지로 에탄올의 생성 없이 자일로스를 대사하여 피루브산이 축적됨을 확인하였다. 위에서 구축한 이소부탄올 생합성 경로를 도입하여 자일로스로부터 120 mg/L의 이소부탄올이 생성됨을 확인하였다. 이는 재조합 S. cerevisiae를 이용하여 목질계 바이오매스로부터 이소부탄올의 생산의 가능성을 제시한 연구 결과이다.

주요어 : 바이오에너지, 바이오부탄올, 이소부탄올,
피루브산, 피루브산탈카르복시효소 결여 효모,
자일로스, 목질계 바이오매스,

학 번 : 2012-21150

감사의 글

대학을 졸업하고 어리기만 했던 제가 대학원이라는 작은 사회를 경험하며 많은 것을 보고 느꼈습니다.

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A Thesis for the Degree of Master of Science

Production of isobutanol by pyruvate decarboxylase-deficient *Saccharomyces cerevisiae*

pyruvate decarboxylase 결여 효모로부터

아이소부탄올 생산에 관한 연구

By

Min-Ji Kim

Department of Agricultural Biotechnology

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農 學 碩 士 學 位 論 文

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아이소부탄올 생합성 경로를 포함한 pyruvate decarboxylase 결여 효모로부터 아이소부탄올 생산에 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
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金 玟 志 의 農學碩士 學位論文을 認准함
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ABSTRACT

Global environmental problems and high oil prices are driving the development of technologies for synthesizing alternative liquid biofuels from renewable resources as transportation energy. Compared to ethanol traditionally used as a gasoline additive, branched-chain higher alcohols exhibit significant advantages such as higher energy density, lower hygroscopicity, lower vapor pressure and compatibility with existing transportation infrastructures. Isobutanol is regarded as a next generation transportation fuel of good quality and so microbial production of isobutanol from cellulosic biomass has been done extensively.

In this study, *Saccharomyces cerevisiae* was metabolically engineered to produce isobutanol. This strain has been traditionally used for industrial production of ethanol because of high tolerance against alcohols and many genetic tools. Naturally *S. cerevisiae* produces a little isobutanol by the valine biosynthesis pathway and the Erlich pathway.

To strengthen the isobutanol biosynthetic pathway, the modified endogenous ILV2 gene from *S. cerevisiae* without the mitochondria targeting sequence, the *ilvC* and *ilvD* genes from *Escherichia coli* and

the *kivD* gene from *Lactobacillus lactis* were overexpressed in *S. cerevisiae*. *ILV2* is coding the acetolactate synthase (ALS), *ilvC* and *ilvD* are ketoacid reductoisomerase (KARI) and dihydroxyacid dehydratase (DADH) and *kivD* is ketoacid decarboxylase (ADH). ALS, KARI, DADH and ADH are the enzymes necessary fo isobutanol biosynthesis. The constructed strain produced 120 mg/L isobutanol from glucose, along with production of ethanol as a major metabolite.

To improve isobutanol production through eliminating ethanol production, a pyruvate decarboxylase (Pdc)-deficient mutant (SOS4) was used as a host for isobutanol production, which is a non-ethanol producing and pyruvate accumulating strain. Pyruvate is a key intermediate for isobutanol production. When the modified endogenous *ILV2* gene, *ilvC* and *ilvD* genes from *E. coli* and *kivD* gene from *L. lactis* were overexpressed in the SOS4, the resulting strain was able to produce 283 mg/L isobutanol from glucose in 144 h.

Acetohydroxyacid reductoisomerase and dihyroxyacid dehydratase encoded by *ilvC* and *ilvD* genes act in mitochondria of *S. cerevisiae* naturally. Also these enzymes are presumed to be expressed in mitochondria in the yeast because the *ilvC* and *ilvD* genes have the specific sequences for mitochondria targeting. So the modified *ilvC* and

ilvD genes without the specific sequences were used and the resulting strain produced 326mg/L isobutanol from glucose in 144 h.

Additionally, to increase an expression level of all four genes involved in the isobutanol biosynthetic pathway, an existing *GPD* promoter was replaced with the truncated *HXT7* promoter known as a strong promoter. The resulting strain produced 446 mg/L isobutanol from glucose in 144 h, which was about 15-fold higher than the wild type strain.

Isobutanol production from xylose that is abundant in lignocellulosic hydrolyzate would make the production of isobutanol more sustainable and economical. However *S. cerevisiae* cannot utilize xylose as a carbon source, the *XYL1*, *XYL2* and *XYL3* genes coding for xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK) derivied from *Schefferosomyces stipitis* were introduced into the SOS4 for xylose fermentation. The resulting strain (SOS4X) accumulated pyruvate by utilizing xylose without ethanol production. By introducing the isobutanol biosynthetic system into the SOS4X, the resulting strain produced 121 mg/L isobutanol from xylose in 144h. These results suggest that *S. cerevisiae* might be a promising host for producing isobutanol from lignocellulosic biomass for industrial applications.

Keywords : biofuel, isobutanol, xylose, lignocellulosic biomass, pyruvate decarboxylase (Pdc)-deficient *S. cerivisiae*

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

1. Advanced biofuel – Isobutanol

Largely in response to uncertain fuel supply, global environmental problems and efforts to reduce carbon dioxide emissions, bioethanol has become one of the most promising biofuels today (Atsumi, Cann et al., 2008).

Recently, production of "second generation" biofuels by motivated engineering microbes has been noted for its better properties than ethanol. Compared to the traditional ethanol, higher alcohols should offer advantages such as higher energy density, lower hygroscopicity, lower vapor pressure, and compatibility with existing transportation infrastructure. (Atsumi and Liao, 2008; Atsumi, Wu et al., 2010; Steen, Chan et al., 2008).

Thus branched-chain higher alcohols, such as isopropanol, isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol represent possible alternatives. Isobutanol, compared with n-butanol, has advantages of having a higher octane number and the possibility of usage outside the fuel industry as well (Chen, Nielsen et al., 2011; Kondo, Tezuka et al., 2012). (Figure 1)

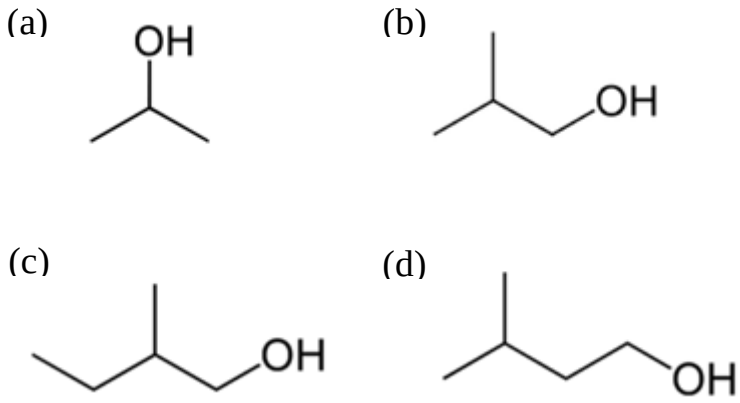


Figure 1. The structure of branched-chain higher alcohols

(a) isopropanol (b) isobutanol

(c) 2-methyl-1-butanol (d) 3-methyl-1-butanol

Isobutanol is an important platform chemical with broad applications in large chemicals and fuels markets and a “drop-in” product that should allow customers to replace petroleum-derived raw materials with isobutanol-derived raw materials without modification to their equipment or production processes. It is used as feedstock in the manufacture of isobutyl acetate, which is used for the production of lacquer and similar coatings, and for the food industry as a flavouring agent. Also, isobutanol is a precursor of derivative esters; isobutyl esters such as diisobutyl phthalate are used as plasticizer agents in plastics, rubbers, and other dispersions.

2. Isobutanol production in microorganisms

Production of isobutanol in microorganisms has been achieved by harnessing the highly active 2-keto acid pathways. Engineered *Clostridium glutamicum* using the 2-keto acid pathway produced 4.9 g/L isobutanol (Smith, Cho et al., 2010) and the engineered *Bacillus subtilis* produced up to 2.62 g/L isobutanol by the heterologous Ehrlich pathway (Li, Wen et al., 2011). Especially, production of isobutanol has been investigated in engineered *E. coli* to reach a concentration of 22 g/L isobutanol (Atsumi, Hanai et al., 2008). (Table 1)

S. cerevisiae was chosen as a host for isobutanol production because it is a genetically well-characterized organism and a current industrial strain as ethanol producer (Ro, Paradise et al., 2006; Steen, Chan et al., 2008). *S. cerevisiae* is able to tolerate 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).

Because its natural productivity of isobutanol is not significant, a strategy of genetic engineering to increase isobutanol production,

which is involved in valine biosynthesis was considered. Initially, the Ehrlich pathway and valine synthetic pathway was overexpressed and then, the *PDC1* gene encoding a major pyruvate decarboxylase with the intent of altering the abundant ethanol flux via pyruvate was deleted. Through these engineering steps, along with modification of culture conditions, the isobutanol titer of *S. cerevisiae* was elevated by 13-fold, from 11 mg/l to 143 mg/l, and the yield was 6.6 mg/g glucose, which is higher than any previously reported value for *S. cerevisiae* (Kondo, Tezuka et al., 2012).

Table 1. Isobutanol production from various microorganisms.

Microorganism	Isobutanol titer	Reference
Clostridium glutamicum	4.9 g/L	Smith, Cho et al., 2010
Corneybacterium glutamicum	660 mg/L	Higashide et al., 2011
Bacillus subtilis	2.62 g/L	Li, Wen et al., 2011
Escherichia coli	22 g/L	Atsumi, Hanai et al., 2008
	50 g/L	Baez et al., 2011
Saccharomyces cerevisiae	151 mg/L	WH Lee et al., 2012
	143 mg/L	Kondo, Tezuka et al., 2012
	630 mg/L	D Brat et al., 2012

3. Isotubanol biosynthetic pathway

3.1. The valine biosynthesis pathway

Isobutanol is produced by wild *S. cerevisiae* as a degradation product of valine metabolism (Hazelwood, Daran et al., 2008). Isobutanol can be produced by the valine biosynthesis pathway and ehrlich degradation pathway. (Figure 2)

Pyruvate to 2-ketoisovalerate involved in the valin biosynthesis pathway is catalyzed by acetolactate synthase (ALS), ketoacid reductoisomerase (KARI) and dihydroxyacid dehydratase (DADH).

ALS catalyses the first common step converting pyruvate to 2-acetolactate in isoleucine and valine biosynthesis and localizes in the mitochondria. 2-Acetolactate can be converted to 2,3-dihydroxy isovalerate and then converted to 2-ketoisovalerate by DADH (Lee et al., 2012).

To enhance the valine biosynthesis pathway, the endogenous *ILV2* gene coding for ALS, *ilvC* and *ilvD* genes from *E. coli* coding for KARI and DADH were used in this study.

In *E. coli*, valine, which is a branched-chain amino acid, is synthesized by the *ilvIHCDE* pathway (Atsumi, Hanai et al., 2008).

3.2. The Ehrlich pathway

Isobutanol can be biosynthesized via 2-ketoisovalerate catalyzed by ketoacid decarboxylase (KDC) and alcohol dehydrogenase (ADH) (Jia, Li et al., 2011). Previous studies utilized inherent KDC and ADH activities for isobutanol production (Hazelwood, Daran et al., 2008; Chen, Nielsen et al., 2011).

KDC is known to be a critical enzyme for removing the carboxylic group from 2-ketoacid to produce aldehyde (König 1998). An intermediate metabolite, 2-ketoisovalerate from the valine biosynthesis pathway, can be converted to isobutyraldehyde by KDC and then converted to isobutanol by ADH (Hazelwood, Daran et al., 2008). The *kivD* gene from *L. Lactis* subsp. *lactis* KACC13877 was determined as the most suitable KDC for isobutanol production in *S. cerevisiae* (Lee et al., 2012) and the effect of *kivD* gene from *L. lactis* was proved by determinate of KDC activity.

Wild *S. cerevisiae* has *ADH2* that showed the highest activity in transforming isobutyraldehyde to the corresponding alcohol (Atsumi, Hanai et al., 2008; Jia, Li et al., 2011).

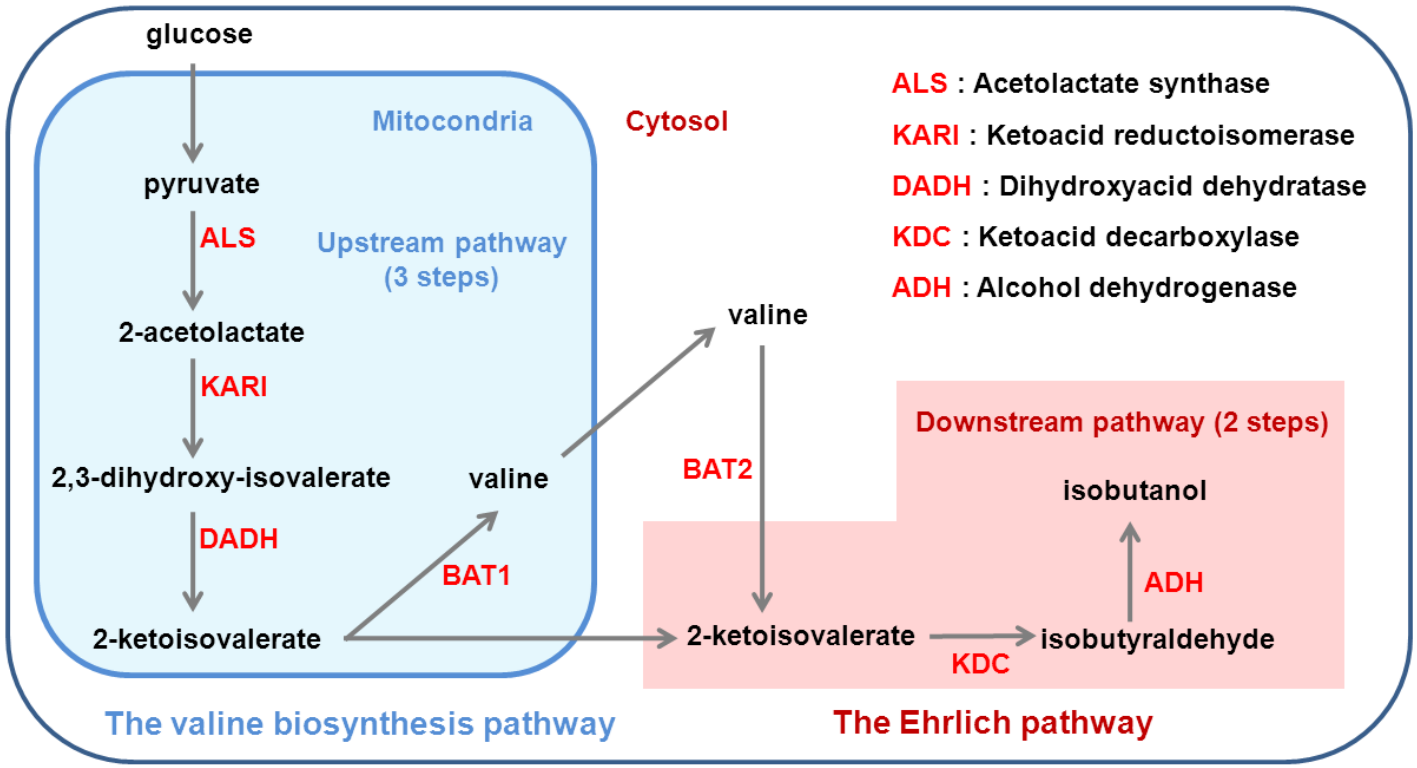


Figure 2. Isobutanol biosynthetic pathway

4. Compartmentalization of the whole isobutanol biosynthetic pathway into cytosol of *S. cerevisiae*

The enzymes involved in the valine synthesis pathway of *S. cerevisiae* are located in the mitochondrial matrix (Ryan and Kohlhaw, 1974). But the Ehrlich pathway proceeds in the cytosol.

Compartmentalization of the Ehrlich pathway into mitochondria increased isobutanol production by 260%, compared with a strain overproducing enzymes involved in only the first three steps of the biosynthetic pathway. (Figure 3) Compartmentalization may include increased availability of intermediates, removing the need to transport intermediates out of the mitochondrion and reducing the loss of intermediates to competing pathways. (Avalos J.L. et al., 2013)

In this study, compartmentalization of the valine biosynthesis pathway into cytosol was tried by overexpressing modified *ILV2* gene which was truncated mitochondria targeting sequence. (Figure 4) This modified *ILV2* gene was constructed by professor Jin Yong Su's group at University of Illinois at Urbana-Champaign. And then, for the next 2 steps involved in valine synthesis pathway, *ilvC* and *ilvD* genes from *E. coli* were used instead of endogenous *ILV5*

and *ILV3* genes. They were overexpressed to convert the 2-acetolactate produced from pyruvate by modified *ILV2* gene efficiently. It is also known to be expressed in the mitochondrial matrix in *E. coli*. When these genes were introduced in yeast, it has possibility to locate in mitochondrial matrix. Therefore, modified *ilvC* and *ilvD* genes truncated putative mitochondria targeting sequence were used in this study for constructing cytosolically re-localized isobutanol biosynthetic pathway in *S. cerevisiae.*

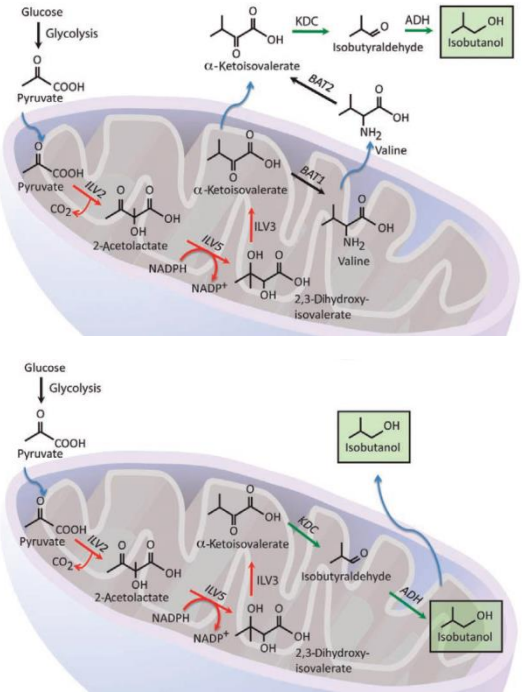


Figure 3. Compartmentalization of the Ehrlich pathway into mitochondria of *S. cerevisiae* (Avalos J.L. et al., 2013)

(a) amino acid sequence encoded by *ILV2*, **0.9993**

MIRQSTLKNFAIKRCFQHIAYRNTPAMRSVALAQRFYSSSSRYYSASPLPASKRPEPAPS
FNVDPLEQPAEPSKLAKKLRAEPDMDTSFVGLTGGQIFNEMMSRQNVDTVFGYPGGAILP
VYDAIHNSDKFNFVLPKHEQGAGHMAEGYARASGKPGVVLVTSGPGATNVVTPMADAFAD
GIPMVVFTGQVPTSAIGTDAFQEADVVGISRSCTKWNVMVKSVEELPLRINEAFEIATSG
RPGPVLVDLPKDVTAAILRNPIPTKTTLPSNALNQLTSRAQDEFVMQSINKAADLINLAK
KPVLYVGAGILNHADGPRLLKELSDRAQIPVTTTLQGLGSFDQEDPKSLDMLGMHGCATA
NLAVQNADLIIAVGARFDDRVTGNISKFAPEARRAAAEGRGGIIHFEVSPKNINKVVQTQ
IAVEGDATTNLGKMMSKIFPVKERSEWFAQINKWKKEYPYAYMEETPGSKIKPQTVIKKL
SKVANDTGRHVIVTTGVGQHQMWAAQHWTWRNPHTFITSGGLGTMGYGLPAAIGAQVAKP
ESLVIDIDGDASFNMTLTELSSAVQAGTPVKILILNNEEQGMVTQWQSLFYEHRYSHTHQ
LNPDFIKLAEAMGLKGLRVKKQEELDAKLKEFVSTKGPVLLEVEVDKKVPVLPMVAGGSG
LDEFINFDPEVERQQTELRHKRTGGKH

(b) amino acid sequence encoded by *ilvC*, **0.8706**

MANYFNTLNLRQQLAQLGKCRFMGRDEFADGASYLQGKKVVIVGCGAQGLNQGLNMRDSG
LDISYALRKEAIAEKRASWRKATENGFKVGTYEELIPQADLVINLTPDKQHSDVVRTVQP
LMKDGAALGYSHGFNIVEVGEQIRKDITVVMVAPKCPGTEVREEYKRGFGVPTLIAVHPE
NDPKGEGMAIAKAWAAATGGHRAGVLESSFVAEVKSDLMGEQTILCGMLQAGSLLCFDKL
VEEGTDPAYAEKLIQFGWETITEALKQGGITLMMDRLSNPAKLRAYALSEQLKEIMAPLF
QKHMDDIISGEFSSGMMADWANDDKKLLTWREETGKTAFETAPQYEGKIGEQEYFDKGVL
MIAMVKAGVELAFETMVDSGIIEESAYYESLHELPLIANTIARKRLYEMNVVISDTAEYG
NYLFSYACVPLLKPFMAELQPGDLGKAIPEGAVDNGQLRDVNEAIRSHAIEQVGKKLRGY
MTDMKRIAVAG

(c) amino acid sequence encoded by *ilvD*, **0.5454**

MPKYRSATTTHGRNMAGARALWRATGMTDADFGKPIIAVVNSFTQFVPGHVHLRDLGKLV
AEQIEAAGGVAKEFNTIAVDDGIAMGHGGMLYSLPSRELIADSVEYMVNAHCADAMVCIS
NCDKITPGMLMASLRLNIPVIFVSGGPMEAGKTKLSDQIIKLDLVDAMIQGADPKVSDSQ
SDQVERSACPTCGSCSGMFTANSMNCLTEALGLSQPGNGSLLATHADRKQLFLNAGKRIV
ELTKRYYEQNDESALPRNIASKAAFENAMTLDIAMGGSTNTVLHLLAAAQEAEIDFTMSD
IDKLSRKVPQLCKVAPSTQKYHMEDVHRAGGVIGILGELDRAGLLNRDVKNVLGLTLPQT
LEQYDVMLTQDDAVKNMFRAGPAGIRTTQAFSQDCRWDTLDDDRANGCIRSLEHAYSKDG
GLAVLYGNFAENGCIVKTAGVDDSILKFTGPAKVYESQDDAVEAILGGKVVAGDVVVIRY
EGPKGGPGMQEMLYPTSFLKSMGLGKACALITDGRFSGGTSGLSIGHVSPEAASGGSIGL
IEDGDLIAIDIPNRGIQLQVSDAELAARREAQDARGDKAWTPKNRERQVSFALRAYASLA
TSADKGAVRDKSKLGG

Figure 4. Putative mitochondria targeting sequence (highlighted in blue) of *ILV2*, *ilvC*, and *ilvD* genes and probability of export to mitochondria in yeast (Claros M.G., Vincens P., 1996)

5. Promoter for efficient gene expression in yeast

The efficient expression of heterologous proteins in yeast considerably relies on yeast promoters. Therefore, different promoters have been used to successfully direct expression of heterologous genes in yeast and well-characterized promoters are essential for pathway engineering and synthetic biology efforts in *S. cerevisiae* (Blazeck et al., 2012).

Constitutive promoters offer fairly constant gene expression levels at the single-cell level without the need for specific inducers or media formulations (Da Silva et al., 2012). The most widely used constitutive promoters have often been from the yeast glycolytic pathway. They are highly-expressed and used successfully to express a number of proteins. These glucose-dependent promoters include those for glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter (Holland et al., 1980), phosphoglycerate kinase (*PGK1*) promoter (Ogden et al., 1986), pyruvate decarboxylase (*PDC1*) promoter (Kellermann et al., 1986), triosephosphate isomerase (*TPI1*) promoter (Russell, 1985), alcohol dehydrogenase I (*ADH1*) promoter (Hitzeman et al., 1981) and pyruvate kinase (*PYK1*) promoter (Nishizawa et al., 1989). Other commonly used

native promoters include iso-1-cytochrome c (*CYC1*) promoter (Gallwitz et al., 1980), actin 1 (*ACT1*) promoter (Gallwitz et al., 1980), mating factor alpha-1 (*MFα1*) promoter (Brake et al., 1984) and those for hexose transport, for example *HXT7* promoter (Reifenberger et al., 1995). Under de-repressed conditions *HXT7* is by far the most strongly expressed *HXT* gene, but it is strongly repressed at high glucose concentrations (Reifenberger et al., 1997). However, 5' deletion of the *HXT7* promoter region, leaving only the 390 bp upstream of the ATG start codon leads to strong constitutive transcription of the gene on glucose media (Hauf et al., 2000). Therefore, a truncated *HXT7* promoter is used widely in glucose-based studies.

All of them, *GPD* promoter, *PGK1* promoter and truncated *HXT7* promoter are known as powerful promoters in expression of heterologous proteins relatively.

In this study, to increase expression level of 4 genes involved in isobutanol biosynthetic pathway, *GPD* promoter and truncated *HXT7* promoter were used and compared the effect in isobutanol production.

6. Pyruvate decarboxylase-deficient *S. cerevisiae*

S. cerevisiae, which is generally used to ferment sugar to ethanol, has been utilized as a host for the production of therapeutic proteins or chemicals with commercial value by metabolic engineering. Because the yield of the desired product should be maximized in respect of the economy, it is necessary to redirect carbon fluxs away from ethanol production towards the desired products, pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae* has been employed for the production for lactic acid, glycerol, and malic acid (Geertman et al., 2006; Ishida et al., 2006; Zelle et al., 2008).

Pyruvate decarboxylase which is located at the branch point between fermentative and respiratory metabolism can convert pyruvate to acetaldehyde, which is further reduced into ethanol by alcohol dehydrogenase. In *S. cerevisiae*, there are three structural genes (*PDC1*, *PDC5* and *PDC6*) involved as active pyruvate decarboxylase isoenzymes. Among these genes, disruption of *PDC1* and *PDC5* or all *PDC* genes led to elimination of pyruvate decarboxylase activity completely (Flikweert et al., 1996).

The Pdc-deficient strains have potential defects for industrial fermentations. Firstly, the Pdc-deficient mutant needs external

supplement of C_2 compound such as acetate or ethanol for synthesis of cytosolic acetyl-CoA which is required to synthesize lysine and fatty acid synthesis (Flikweert et al., 1996; Pronk et al., 1996). Cytosolic acetyl-CoA is synthesized from acetaldehyde via acetate in the cells. Synthesis of acetaldehyde, however, is blocked due to the elimination of Pdc activity, which leads to the shortage of cytosolic acetyl-CoA. Secondly, the Pde-deficient mutant showed lower growth rate a glucose-containing media than wild *S. cerevisiae*. While respiration is necessary for re-oxidation of cytosolic NADH in the Pdc-deficient strains in the absent of ethanol fermentation, glucose represses respiration, which makes Pdc-deficient strains suffer from redox imbalance. A C_2-independent and glucose-tolerant Pdc-deficient strain (SOS4) was constructed by evolutionary engineering. Genome sequencing of the SOS4 revealed a point mutation (A81P) in the *MTH1* gene leading to an amino acid change from alanine to proline (Ala81Pro). Point mutation of the *MTH1* gene in the SOS4 respectively is likely to reduce glucose uptake, which alleviated the pyruvate accumulation and redox imbalance from eliminating Pdc activity in the evolved Pdc-deficient strain (Kim et al., 2013).

7. Production of isobutanol from xylose

Lignocellulosic biomass is an attractive feedstock as a second generation source for biofuel production since it is readily available, e.g., as a waste from the pulp and paper or agricultural industries, and also due to the fact that it is renewable with cycles many orders of magnitude shorter compared with those of fossil fuels. Lignocellulose, which is composed of cellulose, hemicellulose, and lignin, is often hydrolyzed by acid treatment and then the hydrolysate obtained is used for ethanol fermentation by microorganisms such as yeast and bacteria. *S. cerevisiae* that offers numerous benefits in terms of readily availability and large-scale fermentation can be considered as a host for producing ethanol.

However *S. cerevisiae* is unable to metabolize xylose, which is the dominant pentose sugar in hydrolysates of lignocellulosic biomass (Matsushika et al., 2009). A number of different strategies have been applied to engineer yeasts capable of efficiently producing ethanol from xylose, including the introduction of initial xylose metabolism and xylose transport, changing the intracellular redox balance, and overexpression of xylulokinase and pentose phosphate pathways (Chu and Lee; Ha et al., 2011; Hahn-Hagerdal et al.,

2007; Jeffries and Jin, 2004; Jin et al., 2005). Among these attempts, *S. cerevisiae* can efficiently produce ethanol from xylose as a carbon source by introducing xylose utilization pathway, the *XYL1*, *XYL2* and *XYL3* genes coding for xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK) derivied from *S. stipitis*.

Isobutanol production from xylose that is abundant in lignocellulosic hydrolyzate would make the production of isobutanol more sustainable and economical.

Simultaneous overexpression of an optimized, cytosolically localized valine biosynthesis pathway together with overexpression of xylose isomerase *XylA* from *Clostridium phytofermentans*, transaldolase *Tal1* and xylulokinase *Xks1* enabled recombinant *S. cerevisiae* cells to complement the valine auxotrophy of *ilv2,3,5* triple deletion mutants for growth on D-xylose as the sole carbon source. Moreover, after additional overexpression of ketoacid decarboxylase *Aro10* and alcohol dehydrogenase *Adh2*, the cells were able to ferment D-xylose directly to isobutanol. The strain consumed about 12 g D-xylose and produced up to 1.36 mg/L of isobutanol (D Brat et al., 2013).

Although *S. cerevisiae* is able to metabolize xylose, they could be produce low amounts of isobutanol and mainly produce ethanol as a by-pruduct. Therefore it is necessary to redirect the carbon flux from ethanol to isobutanol production.

In this study, *S. cerevisiae* capable of utilizing xylose and accumulating pyruvate by introducing xylose assimilation pathway into a Pdc-deficient *S. cereviesiae* (SOS4) was used as host for isobutanol production from xylose. By introducing efficient isobutanol biosynthetic system into the host, it could be possible to produce isobutanol more successfully from xylose than the previous results. (Figure 5 a)

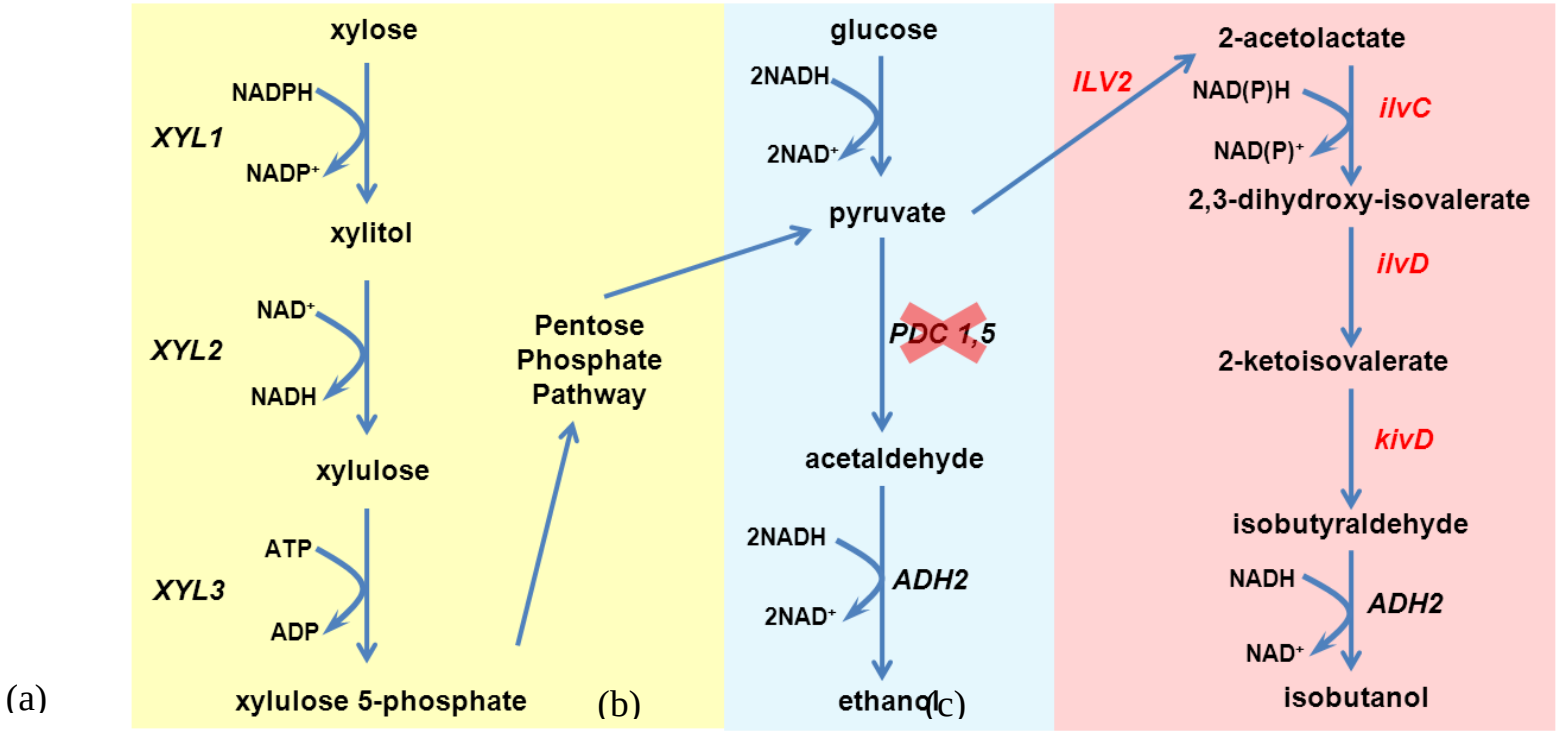


Figure 5. Metabolic pathway in engineered *S. cerevisiae* (a) xylose metabolizing pathway, (b) blocked ethanol producing pathway, (c) isobutanol biosynthetic pathway (red box : in cytosol) in this study

8. Research objectives

For production of isobutanol in recombinant *S. cerevisiae*, several factors should be considered including elimination competing pathway and enhancement isobutanol producing flux from carbon source.

This study was focused on pyruvate accumulating strain as a host and then, enhancing isobutanol biosynthesis pathway.

The specific objectives of this study are as follows.

(1) To lead carbon flux from ethanol to isobutanol production by employing pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae*,

(2) To improve isobutanol biosynthetic pathway by introducing heterologous genes and overexpressing endogenous gene in cytosol under control of strong promoter,

(3) To develop Pdc-deficient *S. cerevisiae* capable of fermenting xylose as a carbon source, accumulating pyruvate and producing isobutanol.

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), protease inhibitor cocktail and 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); 2N NaOH and 2N 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*] and the pyruvatedecarboxylase (Pdc)-deficient *S. cerevisiae* D452-2 strain (SOS4) was used as host strains for the expression of isobutanol

biosynthetic pathway. (Table 2, 3)

Also SOS4X which is introduced xylose assimilation pathway into the SOS4 was used as host strain in fermentation of xylose. (Table 3)

S. cerevisiae D452-2 was donated (Hosaka, Nikawa et al., 1992). And the SOS4 and SOS4X were constructed by S. J. Kim at Seoul National University in Korea (Kim et al., 2013). Other strains in Table 2, 3 were constructed in this study.

The constructed strains were stored on YEPD and YNB selective medium respectively in a deep freezer at - 80℃ suspended in 15% glycerol.

Table 2. List of the *S. cerevisiae* D452-2 strains used in this study

Name	Description
D452-2	*Saccharomyces cerevisiae* (*Matα, leu2 his3 ura3 can1*)
D-CON-$_{GPD}$	D452-2 (p423GPD, p425GPD, p426GPD)
D-2K-$_{GPD}$	D452-2 (p423GPD, p425GPD-MI2-K, p426GPD)
D-2KCD-$_{GPD}$	D452-2 (p423GPD-C, p425GPD-MI2-K, p426GPD-D)

Table 3. List of the SOS4 and SOS4X strains used in this study

Name	Description
SOS4	D452-2 Δ*PDC1* Δ*PDC5*
SOS4-CON-$_{GPD}$	SOS4 (p423GPD, p425GPD, p426GPD)
SOS4-2K-$_{GPD}$	SOS4 (p423GPD, p425GPD-MI2-K, p426GPD)
SOS4-2KCD-$_{GPD}$	SOS4 (p423GPD-C, p425GPD-MI2-K, p426GPD-D)
SOS4-BKCD-$_{GPD}$	SOS4 (p423GPD-C, p425GPD-SB-K, p426GPD-D)
SOS4-KKCD-$_{GPD}$	SOS4 (p423GPD-C, p425GPD-SK-K, p426GPD-D)
SOS4-PKCD-$_{GPD}$	SOS4 (p423GPD-C, p425GPD-SP-K, p426GPD-D)
SOS4-2KCD-$_{CYTO\text{-}GPD}$	SOS4 (p423GPD-MC, p425GPD-MI2-K, p426GPD-MD)
SOS4-2KCD-$_{CYTO\text{-}HXT}$	SOS4 (p423HXT-MC, p425HXT-MI2-K, p426HXT-MD)
SOS4X	SOS4 (p306-XYL123)
SOS4X-CON-$_{HXT}$	SOS4X (p423HXT, p425HXT)
SOS4X-2KCD-$_{CYTO\text{-}HXT}$	SOS4X (p423HXT-MC-MD, p425HXT-MI2-K)

2.2. Plasmids

6 plasmids were used as mother vectors which has the *GPD* promoter and truncated *HXT7* promoter respectively and *CYC1* terminator from *S. cerevisiae.*(Figure 6) These are cloning vectors for episomal expression system of isobutanol biosynthetic pathway, endogenous *ILV2* gene, *alsS* genes from *Bacillus subtilis, Klebsiella pneumoniae* and *Penibacillus polymyxa, ilvC* and *ilvD* genes from *E. coli* and *kivD* gene from *L. lactis.* (Table 4, 5) There are oligonucleotide sequence of primers used cloning. (Table 6)

Abbreviations and the meaning used in this study are as follows.

MI2 is modified *ILV2* gene which means truncated *ILV2* gene by removing mitochondria. SB means *alsS* gene from *B. subtilis.* SK means *alsS* gene from *K. pneumonia.* SP means *alsS* gene from *P. polymyxa.* C means *ilvC* gene from *E. coli*. D means *ilvC* gene from *E. coli*. MC is modified *ilvC* gene which means truncated *ilvC* gene for expression in cytosol. MD is modified *ilvD* gene which means truncated *ilvD* gene for expression in cytosol. K means *kivD* gene from *L. lactis.* XYL123 means *XYL1, XYL2* and *XYL3* genes from *S. stipitis.*

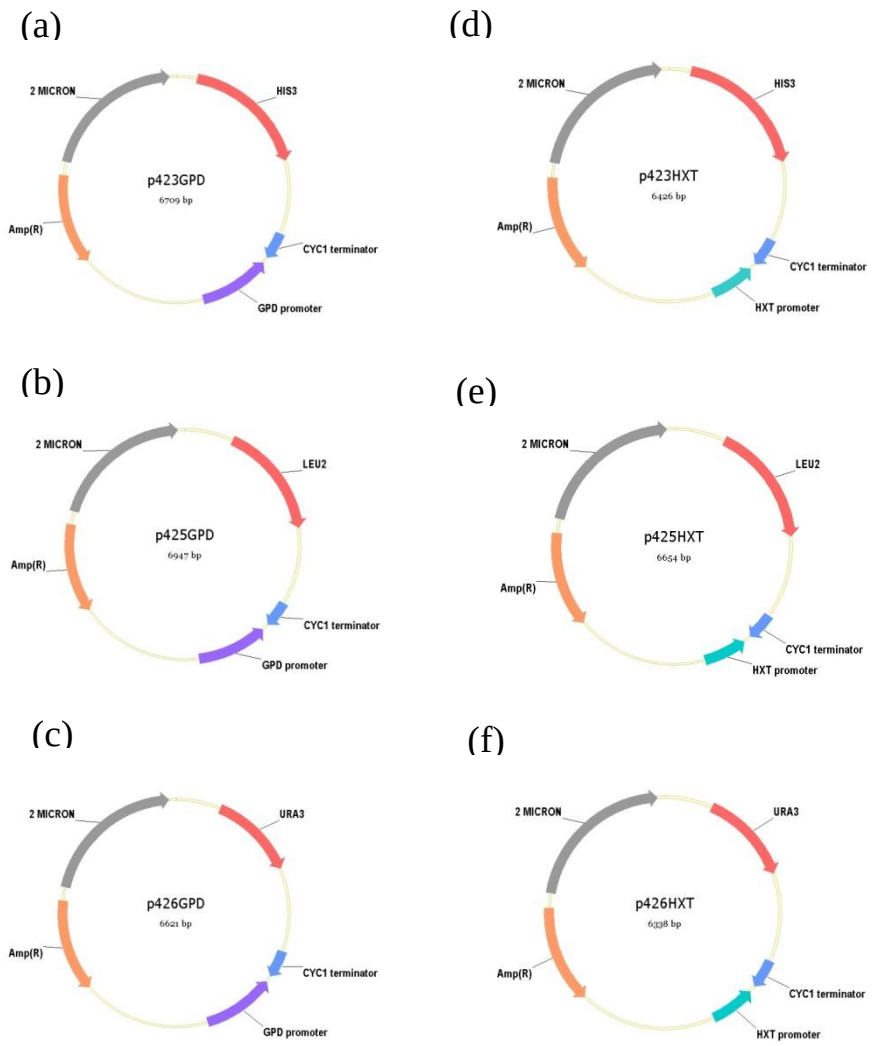


Figure 6. Mother vectors used in this study

(a) p423GPD, (b) p425GPD, (c) p426GPD,

(a) p423HXT, (b) p425HXT, (c) p426HXT

Table 4. List of the plasmids with *GPD* promoter used in this study

Name	Description
p423GPD	HIS3, *GPD* promoter, *CYC1* terminator, 2 μ origin, Amp^r
p425GPD	LEU2, *GPD* promoter, *CYC1* terminator, 2 μ origin, Amp^r
p426GPD	URA3, *GPD* promoter, *CYC1* terminator, 2 μ origin, Amp^r
p425GPD-MI2-K	pRS425GPD harboring modified *ILV2* from *S. cerevisiae* and *kivD* from *L. lactis*
p425GPD-SB-K	pRS425GPD harboring *alsS* from *B. subtilis* and *kivD* from *L. lactis*
p425GPD-SK-K	pRS425GPD harboring *alsS* from *K. pneumoniae* and *kivD* from *L. lactis*
p425GPD-SP-K	pRS425GPD harboring *alsS* from *P. polymyxa* and *kivD* from *L. lactis*
p423GPD-C	pRS423GPD harboring *ilvC* from *E. coli*
p426GPD-D	pRS423GPD harboring *ilvD* from *E. coli*
p423GPD-MC	pRS423GPD harboring modified *ilvC* from *E. coli*
p426GPD-MD	pRS423GPD harboring modified *ilvD* from *E. coli*
p306-XYL123	pRS306 harboring *XYL1*, *XYL2* and *XYL3* from *S. stipitis*

Table 5. List of the plasmids with truncated *HXT7* promoter used in this study

Name	Description
p423HXT	HIS3, truncated *HXT7* promoter, *CYC1* terminator, 2 μ origin, Amp^r
p425HXT	LEU2, truncated *HXT7* promoter, *CYC1* terminator, 2 μ origin, Amp^r
p426HXT	URA3, truncated *HXT7* promoter, *CYC1* terminator, 2 μ origin, Amp^r
p425HXT-MI2-K	pRS425HXT harboring modified *ILV2* from *S. cerevisiae* and *kivD* from *L. lactis*
p423HXT-MC	pRS423HXT harboring modified *ilvC* from *E. coli*
p426HXT-MD	pRS426 HXT harboring modified *ilvD* from *E. coli*
p423HXT-MC-MD	pRS423HXT harboring modified *ilvC* and modified *ilvD* from *E. coli*

pRS423GPD, pRS425GPD and pRS426GPD were donated (Christianson et al., 1992).

p306_XYL123 was constructed by professor Jin Yong Su's group at University of Illinois at Urbana-Champaign.

Other plasmids in Table 4, 5 were constructed in this study.

Table 6. List of oligonucleotide used in this study

Primer name	Oligonucleotide sequence (5’ → 3’)
F-MI2-BamHI	CGGGATCCAAAATGGAGCCTGCTCCAAGTTT
R-MI2-XhoI	CCGCTCGAGTTAGTGCTTACCGCCTGTAC
F-K-BamHI	CGGGATCCATGTATACAGTAGGAGATTAC
R-K-XhoI	CCGCTCGAGTTATGATTTATTTTGTTCAGCAA
F-SB-BamHI	CGGGATCCATGTTGACAAAAGCAACAAAAGA
R-SB-XhoI	CCGCTCGAGCTAGAGAGCTTTCGTTTTCA
F-SK-BamHI	CGGGATCCATGGACAAACAGTATCCGGTA
R-SK-XhoI	CCGCTCGAGTTACAGAATCTGACTCAGATGCA
F-SP-BamHI	CGGGATCCTTGAGTACAAAAGTGCAAGCTGT
R-SP-XhoI	CCGCTCGAGTTAGTTTAATTGGTTAGGCAGCA
F-C-BamHI	CGGGATCCAAAATGGCTAACTACTTCAATACACT
R-C-XhoI	CCGCTCGAGTTAGTGGTGATGGTGATGATGACCCGCAACAGCAATACG
F-MC-BamHI	CGGGATCCAAAATGATGGGCCGCGATGAATTC
R-MC-XhoI	CCGCTCGAGTTAGTGGTGATGGTGATGATGACCCGCAACAGCAATACG
F-D-BamHI	CGGGATCCAAAATGCCTAAGTACCGTTCCG
R-D-EcoRI	CGGAATTCTTAGTGGTGATGGTGATGATGACCCCCCAGTTTCGATTTATC
F-MD-BamHI	CGGGATCCAAAATGACCGGAATGACCGACGC
R-MD-EcoRI	CGGAATTCTTAGTGGTGATGGTGATGATGACCCCCCAGTTTCGATTTATC
SacI-GPDp	CGAGCTCAGTTTATCATTATCAATACTCGCCA
SacI-HXTp	CGAGCTCTCGGGCCCCTGCTTCTG
SacI-CYCt	CGAGCTCGGCCGCAAATTAAAGCCTTC

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. Transformation of *E. coli*

Transformation of *E. coli* was carried out as described by Sambrook et al., (1989). *E. coli* Top10 was cultured in 5 mL LB medium for 12hr. 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.

3.3. Preparation of plasmid DNA and yeast 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 microorganism genomic DNA to obtain a template for the gene was carried out using using DNeasy Blood & Tissue Kit from QIAGEN (Düsseldorf, 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 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).

3.5. 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 45 sec, 55 ℃ for 30 sec, 72 ℃ for 1 min, 1 cycle of 72 ℃ for 10 min. The amplified gene was confirmed by gel electrophoresis.

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 (1 % tryptone, 0.5 % yeast extract, 1 % NaCl) with 50 μg/mL ampicillin was used for recombinant *E. coli* cultivation.

YEPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose) and YNB 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. Inoculum

Recombinant *S. cerevisiae* stock was transferred to a test tube containing YNBD selection medium and incubated overnight at 30 ℃, 250 rpm in shaking incubator (Vision, Korea). Pre-culture was performed in a 250 mL flask with 100 mL working volume at 30 ℃, 250 rpm for appropriate time. The inocula were prepared by growing cells overnight to an OD_{600} of 5~10. The cells were harvested by centrifugation at 3,000 rpm for 10 min and washed in 5 mL of sterilized DDW. The washed cells were transferred to the 250 mL glass flask containing 50 mL YEPD medium. The initial OD_{600} of main flask culture was 1.0. in glucose medium and 10.0 in xylose medium. In bioreactor cultivation initial OD_{600} was approximately 5.0.

4.3. Cultivations

Batch fermentation in flask was carried out 50 mL working volume at 30 ℃ in shaking incubator (Vision Korea), and shaking rate was maintained at 100 rpm for creating micro-aerobic conditions.

Large-scale batch fermentation was performed using a bench-top fermentor (KoBioTech, Korea). Cultivations were performed in 500 mL YEPD medium at 30 ℃ and pH 5.5 (adjustment by 5N HCl and 2N NaOH). The fermentation experiments were performed under separate cultivation conditions. (Table 7)

Table 7. Summary of cultivation conditions in bioreactor

Agitation (rpm)	Aeration (vvm)
300	0.25
300	0.5
300	1.0

5. Analysis

5.1. Dry cell mass

Dry cell mass concentration was estimated by measuring absorbance at 600 nm by a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). Optical density was converted into dry cell mass by using the following conversion equation.

$$\text{Dry cell mass (g/L)} = 0.345 \times OD_{600}$$

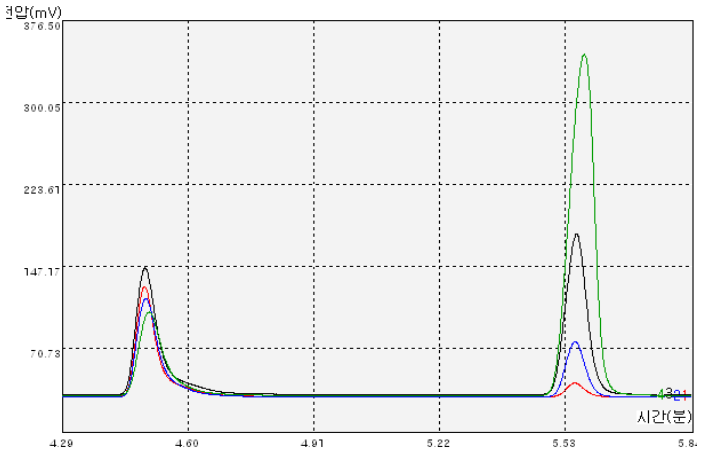
5.2. Metabolite detection

Concentrations of glucose and ethanol were measured by a high performance liquid chromatography (Agilent 1100LC, U.S.A) equipped with the Carbohydrate Analysis column (Phenomenex, USA). The carbohydrate analysis ion exclusion column heated at 60°C was applied to analyze the 20 uL of diluted culture broth. Detection was made with a reflective index detector at 35 °C. HPLC operation conditions were set according to the instruction manual of the column supplier. H_2SO_4 (5 mM) solution was used as mobile phase at a 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 A 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℃. 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 content was determined by extrapolation from standard curves using the internal standard to normalize the values. (Figure 7)

(a)



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

(b)

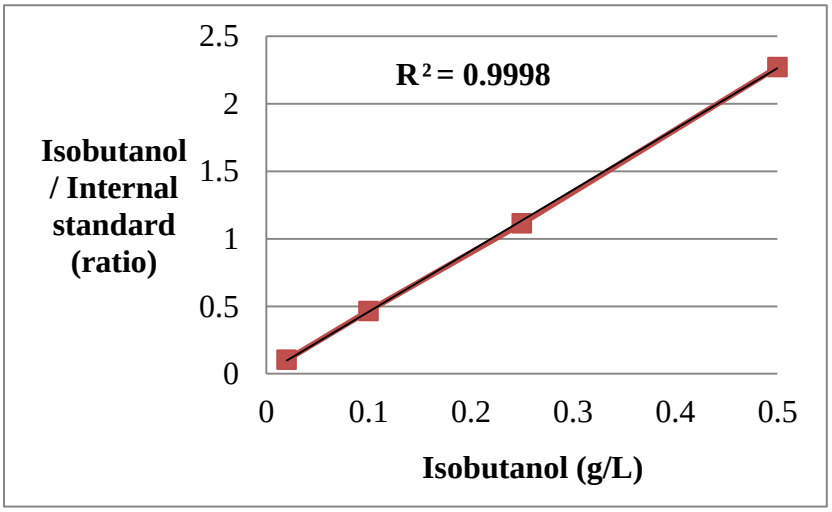


Figure 7. (a) Standard peak of GC analysis : n-propanol, isobutanol

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

III. RESULTS AND DISCUSSIONS

1. Production of isobutanol by pyruvate decarboxylase-deficient *S. cerevisiae* (SOS4) with the isobutanol biosynthetic pathway

1.1. Production of isobutanol in *S. cerevisiae*

In previous studies by S.J. Baek, modified *ILV2* gene and *kivD* gene were tested by combination of integration and episomal expression systems. So in this study, additional *ilvC* and *ilvD* genes from *E. coli* were overexpressed to construct the whole valine biosynthetic pathway in *S. cerevisiae*. Therefore, when modified *ILV2*, *ilvC*, *ilvD* and *kivD* genes were introduced, the isobutanol biosynthetic pathway from pyruvate to isobutylaldehyde was constructed. And the final step from isobutylaldehyde to isobutanol was catalyzed by endogenous ADH which is abundant in *S. cerevisiae*.

Genetic map of plasmids constructed in this part is in Figure 8 and D-CON-$_{GPD}$, D-2K-$_{GPD}$ and D-2KCD-$_{GPD}$ were constructed using these plasmids. The flask fermentation profiles of constructed strains are in Figure 9.

While D-CON$_{-GPD}$ produced 30 mg/L of isobutanol and D-2K$_{-GPD}$

produced 72 mg/L of isobutanol, D-2KCD$_{-GPD}$ produced 120 mg/L of isobutanol from 20 g/L of glucose within 144 h in micro-aerobic conditions. The strain overexpressed modified *ILV2*, *ilvC*, *ilvD* and *kivD* genes produced isotuanol 4-fold more than wild type strain with empty plasmids. And additional introduction of *ilvC* and *ilvD* genes is slightly effective, but the amount of isobutanol concentration was an immaterial increase. That is because about 7 g/L of ethanol still produced as by-product competing with isobutanol. To eliminate ethanol production pathway and improve isobutanol production, pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae* was used in next part.

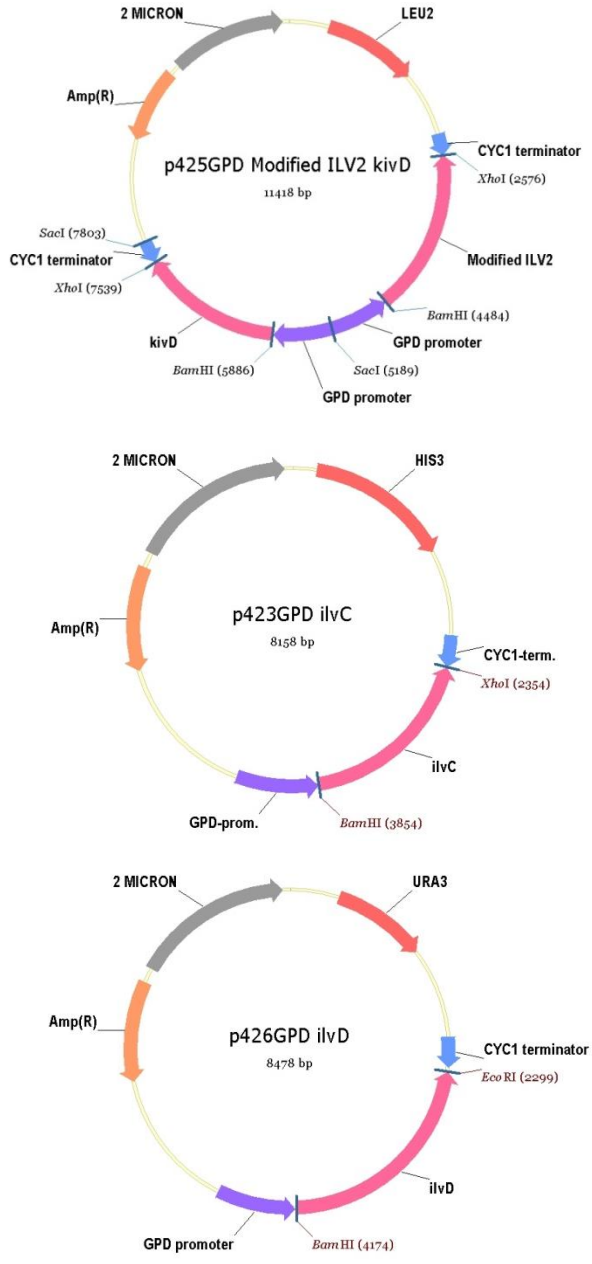


Figure 8. Genetic map of plasmids

(a) p425GPD-MI2-K, (b) 423GPD-C, (c) p426GPD-D

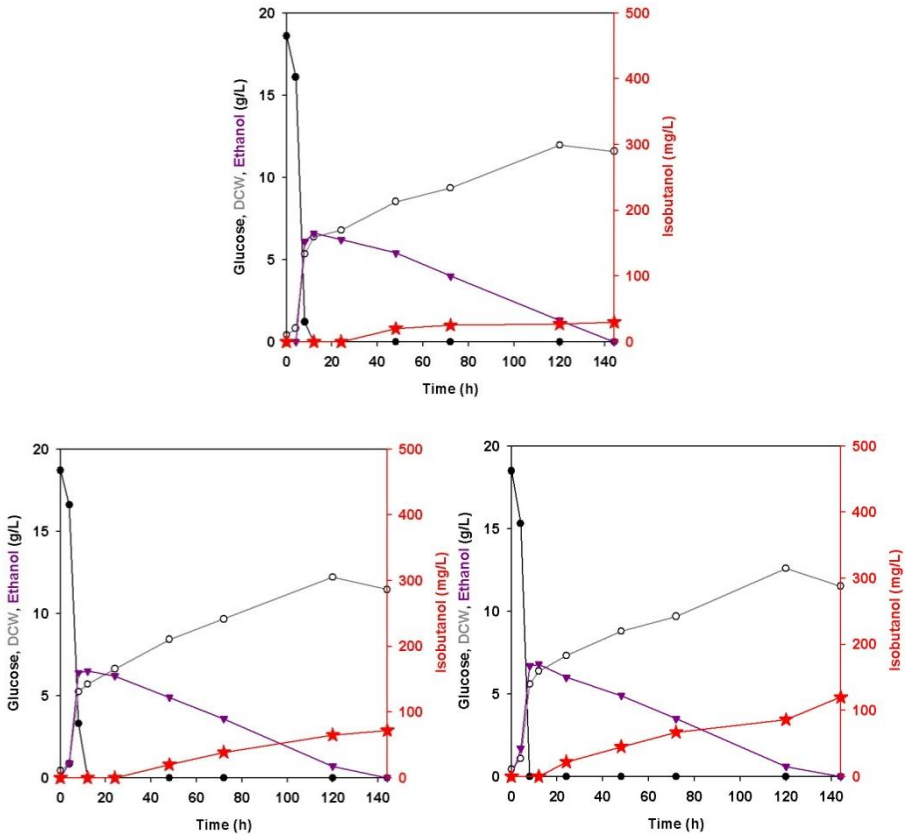


Figure 9. Flask fermentation profiles of

(a) D-CON-$_{GPD}$, (b) D-2K-$_{GPD}$, (c) D-2KCD-$_{GPD}$

Symbols : Glucose ( ), DCW ( ), Pyruvate ( ),

Glycerol ( ), Acetate ( ), Ethanol ( ), Isobutanol ( )

1.2. Production of isobutanol in the SOS4

SOS4-CON$_{-GPD}$, SOS4-2K$_{-GPD}$, SOS4-2KCD$_{-GPD}$ were constructed using those plasmids constructed in previous part and the flask fermentation profiles of constructed strains are in Figure 10.

When SOS4 with empty plasmids consumed only 15.8 g/L glucose from initial 20 g/L glucose within 144 h under micro-aerobic conditions and accumulated 4.2 g/L of pyruvate without production of ethanol. The SOS4-2K$_{-GPD}$ produced 152 mg/L of isobutanol and accumulated 3.65 g/L of pyruvate and SOS4-2KCD$_{-GPD}$ produced 283 mg/L of isobutanol and accumulated 3.06 g/L of pyruvate with slightly rapid glucose consumption. These results suggest that accumulated pyruvate can be converted into isobutanol by isobutanol biosynthetic pathway. While *S. cerevisiae* showed an immaterial increase resulted from additional overexpressen of *ilvC* and *ilvD* genes in previous part, the improved amount of isobutanol in SOS4 was worthy increment. However, a difference between reduced amount of accumulated pyruvate and increased amount of isobutanol was unbalanced because reduced pyruvate was used in respiration mainly. To convert pyruvate to isobutanol effectively, it is necessary to enhance the isobutanol biosynthetic pathway.

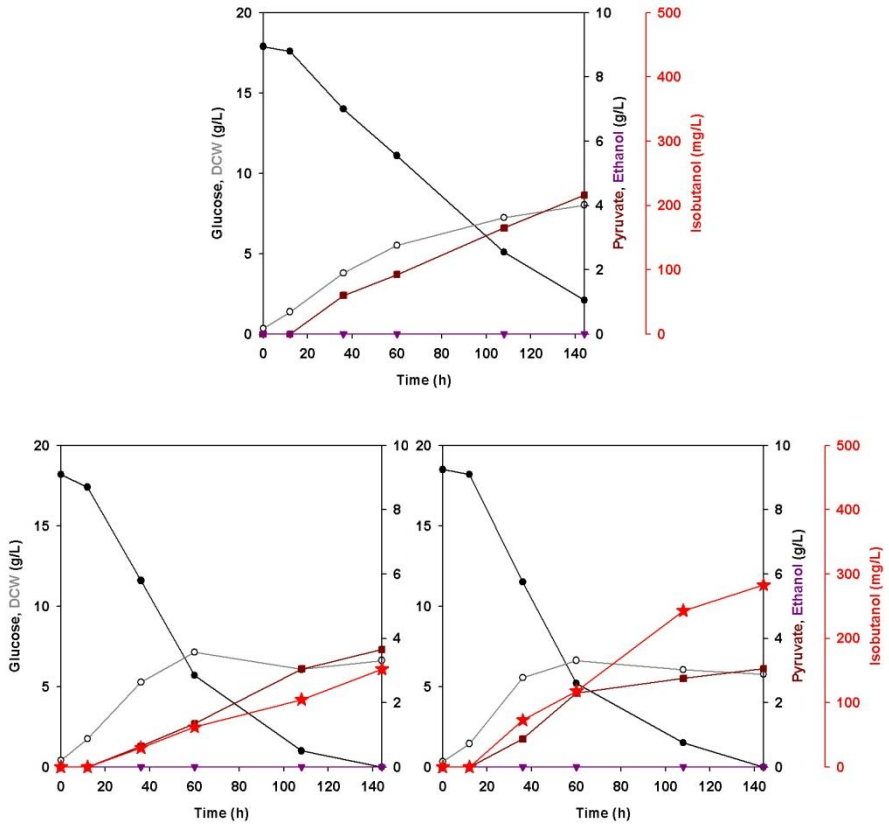


Figure 10. Flask fermentation profiles of

(a) SOS4-CON$_{-GPD}$, (b) SOS4-2K$_{-GPD}$, (c) SOS4-2KCD$_{-GPD}$

Symbols : Glucose ( ), DCW ( ), Pyruvate ( ),

Glycerol ( ), Acetate ( ), Ethanol ( ), Isobutanol ( )

Table 8. Summary of flask fermentation in part 1.1. and 1.2.

Strain	Maximum dry cell weight (g/L)	Consumed glucose (g/L)	Pyruvate (g/L)	Concentration of isobutanol (mg/L)	Yield of isobutanol ($mg_{isobutaol}/g_{glucose}$)
D-CON_{-GPD}	12.0	18.6	0	30	1.61
D-$2K_{-GPD}$	12.2	18.7	0	72	3.85
D-$2KCD_{-GPD}$	12.6	18.5	0	120	6.49
SOS4-CON_{-GPD}	8.0	15.8	4.3	0	-
SOS4-$2K_{-GPD}$	7.1	18.2	3.7	152	8.35
SOS4-$2KCD_{-GPD}$	6.6	18.5	3.1	283	15.30

2. Construction of the efficient isobutanol biosynthetic system

2.1. Evaluation of the acetolactate synthases from various microorganisms

ALS is a key enzyme for isobutanol production because it catalyze the first step for conversion of pyruvate to isobutanol. *alsS* genes from other bacteria strains were known to have strong activity and readily used in other studies. Especially, *alsS* gene from *B. subtilis* is used in 2,3-butanediol production in *S. cerevisiae* (Kim et al., 2013).

In this study, three different *alsS* genes from *Bacillus subtilis*, *Klebsiella pneumoniae* and *Penibacillus polymyxa* were tested for isobutanol production.

Genetic map of plasmids constructed in this part is in Figure 11 and SOS4-BKCD$_{-GPD}$, SOS4-KKCD$_{-GPD}$ and SOS4-PKCD$_{-GPD}$ were constructed using these plasmids. The flask fermentation profiles of constructed strains are in Figure 11.

Each strain containing various *alsS* genes, p425GPD-SB-K, p425GPD-SK-K and p425GPD-SP-K, produced 269 mg/L, 237 mg/L, 223 mg/L of isobutanol, and the results maens that modified *ILV2* gene was slightly more effective than *alsS* gene from bacteria.

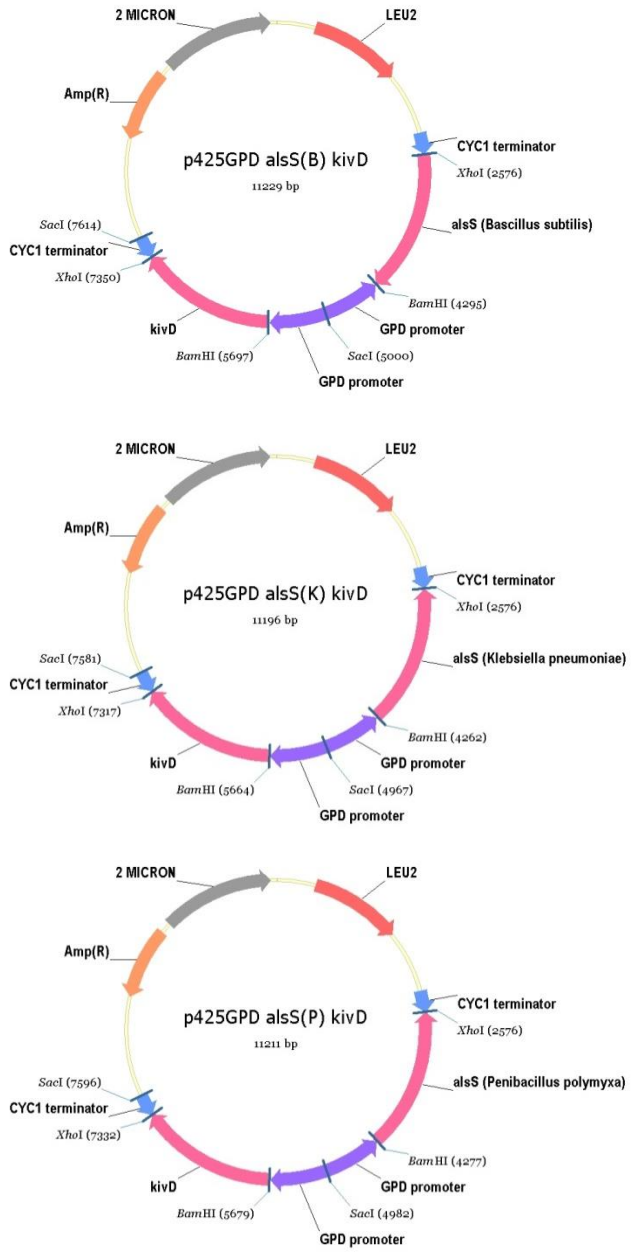


Figure 11. Genetic map of plasmids

(a) p425GPD-SB-K, (b) p425GPD-SK-K, (c) p425GPD-SP-K

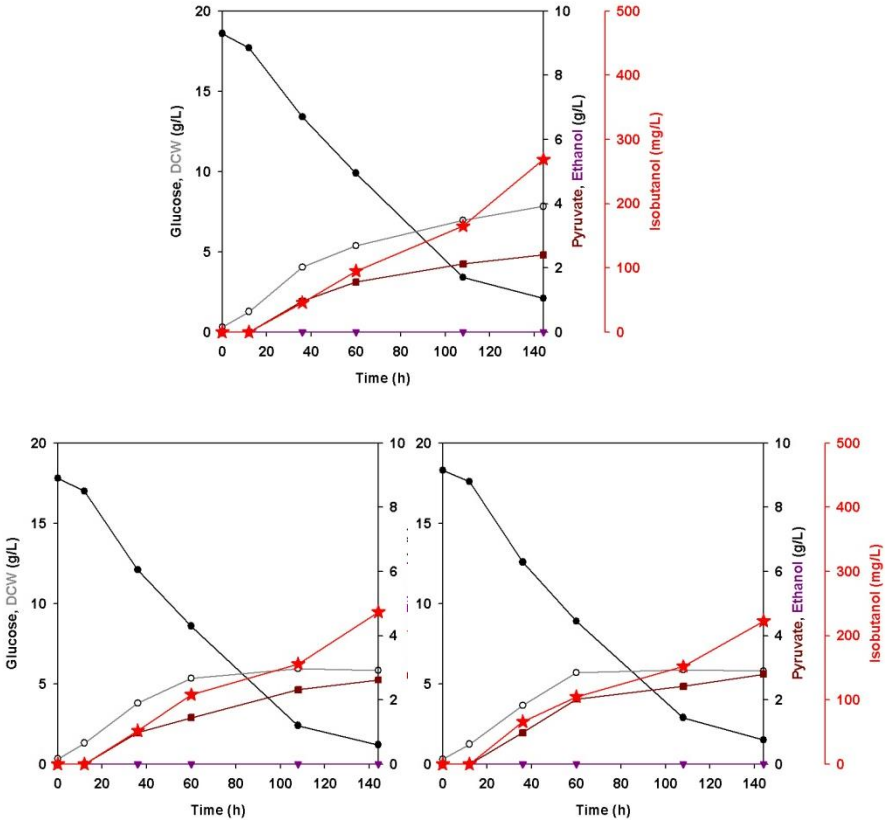


Figure 12. Flask fermentation profiles of

(a) SOS4-BKCD$_{-GPD}$, (b) SOS4-KKCD$_{-GPD}$, (c) SOS4-PKCD$_{-GPD}$

Symbols : Glucose ( ), DCW ( ), Pyruvate ( ), Glycerol ( ), Acetate ( ), Ethanol ( ), Isobutanol ( )

Table 9. Summary of flask fermentation in part 2.1.

Strain	Maximum dry cell weight (g/L)	Consumed glucose (g/L)	Pyruvate (g/L)	Concentration of isobutanol (mg/L)	Yield of isobutanol ($mg_{isobutanol}/g_{glucose}$)
SOS4-2KCD$_{-GPD}$	6.6	18.5	3.1	283	15.30
SOS4-BKCD$_{-GPD}$	7.8	16.5	2.4	269	16.30
SOS4-KKCD$_{-GPD}$	5.9	16.6	2.6	237	14.27
SOS4-PKCD$_{-GPD}$	5.9	16.8	2.8	223	13.27

2.2. Cytosolic re-localization of the whole valine biosynthesis pathway of *S. cereviae*

ilvC and *ilvD* genes from *E. coli* were overexpressed to convert the 2-acetolactate produced from pyruvate by modified *ILV2* gene more efficiently. But, it is also known to be expressed in the mitochondrial matrix in *E. coli*. It has possibility to locate enzymes encoded by these genes into mitochondrial matrix in yeast. Therefore, modified *ilvC* and *ilvD* genes truncated putative mitochondria targeting sequences were used in this study for constructing cytosolically re-localized isobutanol biosynthetic pathway in *S. cerevisiae*.

Genetic map of plasmids constructed using modified *ilvC* and *ilvD* genes in this part is in Figure 13 and SOS4-2KCD$_{\text{-CYTO-GPD}}$ was constructed using these plasmids. The flask fermentation profiles of constructed strains are in Figure 14.

The results was small range of increase in isobutanol titer, which is increased up to 326 mg/L of isobutanol from 283 mg/L of isobutanol consuming 20 g/L of glucose within 144 h under micro-aerobic conditions, and equally confirmed in repeated experiments. Cytosolic re-localized expression of these enzymes encoded modified genes was proved in previous research by indirect

immunofluorescence microscopy of wild-type and N-terminally truncated proteins carrying a C-terminal 6His-tag. α-His antibodies were applied for the visualisation of Ilv enzymes and α-Hsp70 antibodies were applied for cytosolic staining (D Brat et al., 2012).

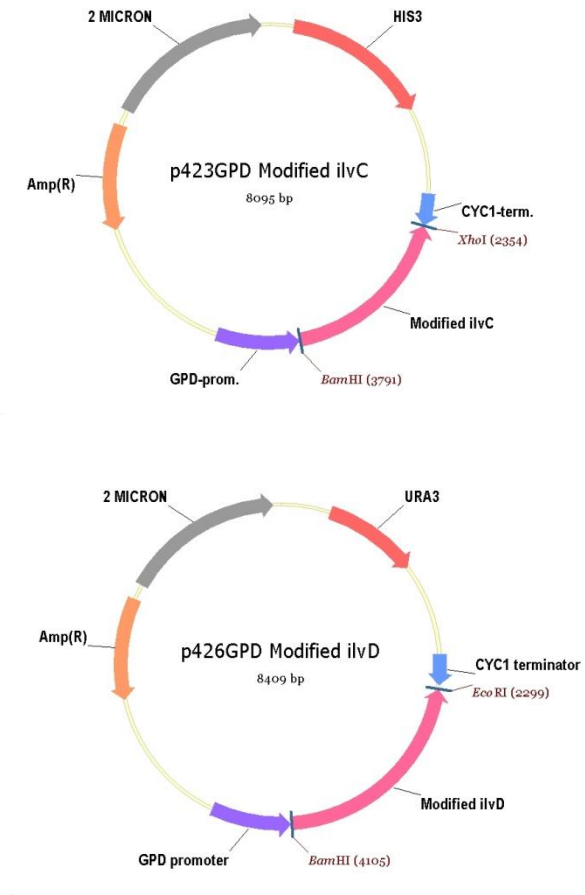


Figure 13. Genetic map of plasmids

(a) p423GPD-MC, (b) p426GPD-MD

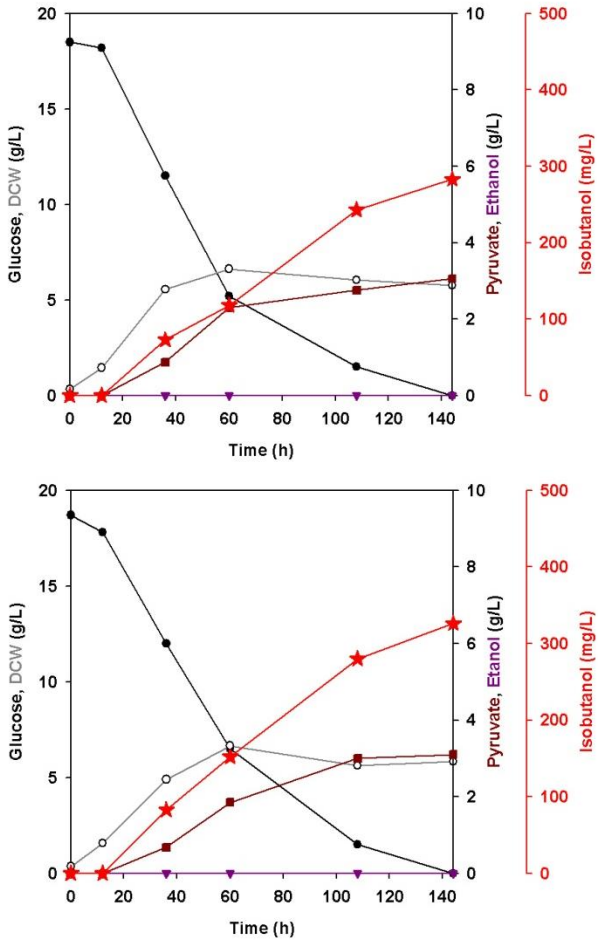


Figure 14. Flask fermentation profiles of

(a) SOS4-2KCD$_{-GPD}$, (b) SOS4-2KCD$_{-CYTO-GPD}$

Symbols : Glucose ( ), DCW ( ), Pyruvate ( ), Glycerol ( ), Acetate ( ), Ethanol ( ), Isobutanol ( )

2.3 Promoter replacement to increase isobutanol biosynthetic genes expression level

In previous parts of this study, all genes were controlled under *GPD* promoter. To enhance the isobutanol biosynthetic pathway, *GPD* promoter was replaced with truncated *HXT7* promoter. These constitutive promoters are normally used for overexpression of heterologous gene in yeast. Particularly, truncated *HXT7* promoter is known as 30 ~ 40 % stronger than *GPD* promoter in yeast.

Genetic map of plasmids constructed using mother vectors containing truncated *HXT7* promoter in this part is in Figure 15. And SOS4-2KCD$_{\text{-CYTO-HXT}}$ was constructed using these plasmids. The flask fermentation profiles of constructed strains are in Figure 16.

The results was 1.37-fold increase in isobutanol titer between SOS4-2KCD$_{\text{-CYTO-GPD}}$ and SOS4-2KCD$_{\text{-CYTO-HXT}}$, which is increased up to 446 mg/L of isobutanol from 326 mg/L of isobutanol from 20 g/L of glucose within 144 h under micro-aerobic conditions, and equally confirmed in repeated experiments.

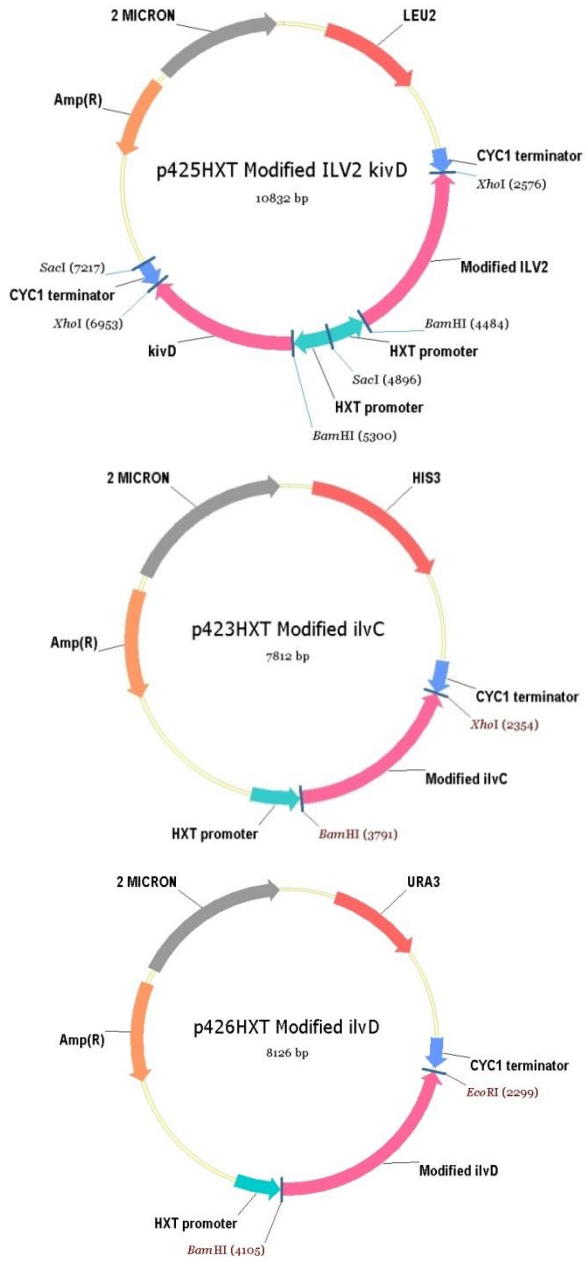


Figure 15. Genetic map of plasmids

(a) p425HXT-MI2-K, (b) p423HXT-MC, (c) p426HXT-MD

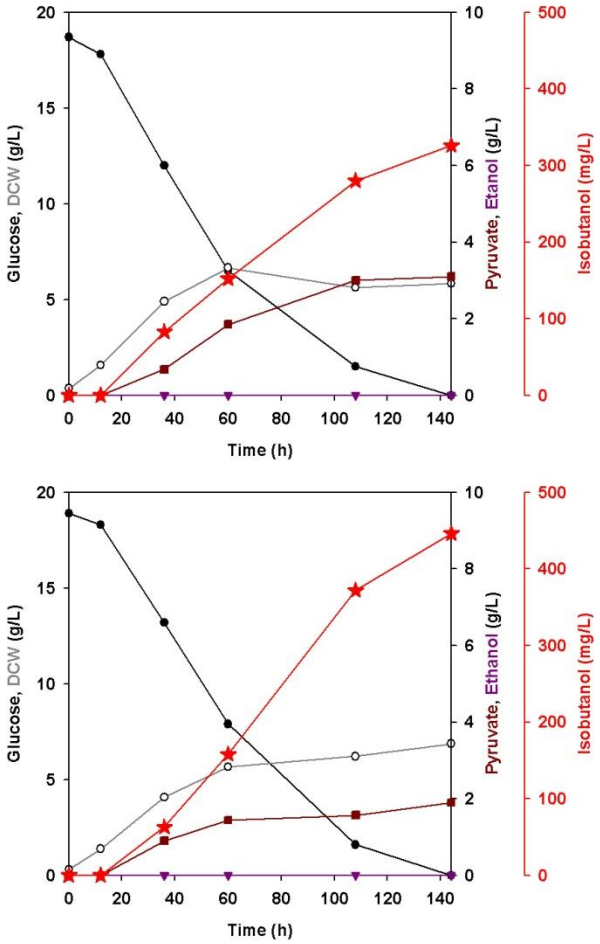


Figure 16. Flask fermentation profile of

(a) SOS4-2KCD$_{\text{-CYTO-GPD}}$, (b) SOS4-2KCD$_{\text{-CYTO-HXT}}$

Symbols : Glucose ( ), DCW ( ), Pyruvate ( ), Glycerol ( ), Acetate ( ), Ethanol ( ), Isobutanol ( )

Table 10. Summary of flask fermentation in part 2.2. and 2.3.

Strain	Maximum dry cell weight (g/L)	Consumed glucose (g/L)	Pyruvate (g/L)	Concentration of isobutanol (mg/L)	Yield of isobutanol ($mg_{isobutanol}/g_{glucose}$)
SOS4-2KCD$_{-GPD}$	6.6	18.5	3.1	283	15.30
SOS4-2KCD$_{-CYTO-GPD}$	6.7	18.7	3.1	326	17.43
SOS4-2KCD$_{-CYTO-HXT}$	6.9	18.9	1.9	446	23.60

3. Production of isobutanol from xylose by pyruvate decarboxylase-deficient *S. cerevisiae* (SOS4) with xylose fermenting pathway and the isobutanol biosynthetic system

In order to obtain xylose-fermenting ability, SOS4X that p306-XYL123 harboring *XYL1*, *XYL2* and *XYL3* genes under the control of constitutive promoters was integrated into the URA3 locus of the SOS4 genome was constructed in previous study of 2,3-butanediol production (Kim et al., 2013a).

When SOS4X with empty plasmids cultured in complex medium with 20 g/L of xylose within 144 h under micro-aerobic conditions, SOS4X-CON$_{-HXT}$ consumed only 17.5 g/L xylose from initial 20 g/L xylose and accumulated 3.9 g/L of pyruvate without production of ethanol after 144 h. The accumulation of pyruvate by the elimination of ethanol production in the SOS4X suggest that the SOS4X might be a good strain to produce isobutanol from xylose.

As the SOS4 was able to consume xylose and accumulate pyruvate instead of ethanol, the isobutanol biosynthetic system constructed in previous parts introduced into the SOS4X to convert pyruvate accumulated from xylose into isotubanol.

Genetic map of plasmid constructed in this part is in Figure 17. SOS4X-CON$_{\text{-HXT}}$ and SOS4-2KCD$_{\text{-CYTO-HXT}}$ was constructed and the flask fermentation profiles of constructed strains are in Figure 18.

Specially, SOS4-2KCD$_{\text{-CYTO-HXT}}$ produced 120 mg/L of isobutanol from 20 g/L of xylose within 144 h under micro-aerobic conditions without ethanol production. This results show that Pdc-deficient *S. cerevisiae* containing a cytosolically located isobutanol biosynthetic pathway under strong promoter and a xylose assimilating pathway are able to convert xylose into isobutanol.

Simultaneous overexpression of an optimized, cytosolically localized valine biosynthesis pathway together with overexpression of xylose isomerase *XylA* from *Clostridium phytofermentans*, transaldolase *Tal1* and xylulokinase *Xks1* enabled recombinant *S. cerevisiae* cells to complement the valine auxotrophy of *ilv2,3,5* triple deletion mutants for growth on D-xylose as the sole carbon source. Moreover, after additional overexpression of ketoacid decarboxylase *Aro10* and alcohol dehydrogenase *Adh2*, the cells were able to ferment D-xylose directly to isobutanol. The strain consumed about 12 g D-xylose and produced up to 1.36 mg/L of

isobutanol (D Brat et al., 2013).

In this study, increased isobutanol production from xylose was achived by eliminating of competing ethanol production pathway. However, the isobutanol production rates and titers from xylose are still low. The SOS4-2KCD-$_{CYTO-HXT}$ strain have not yet been optimized and need to be improved by further genetic or evolutionary engineering. Further optimization of the strain should greatly enhance isobutanol production from xylose.

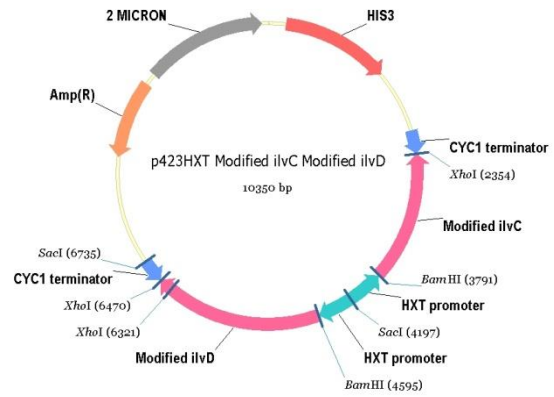


Figure 17. Genetic map of plasmid p423HXT-MC-MD

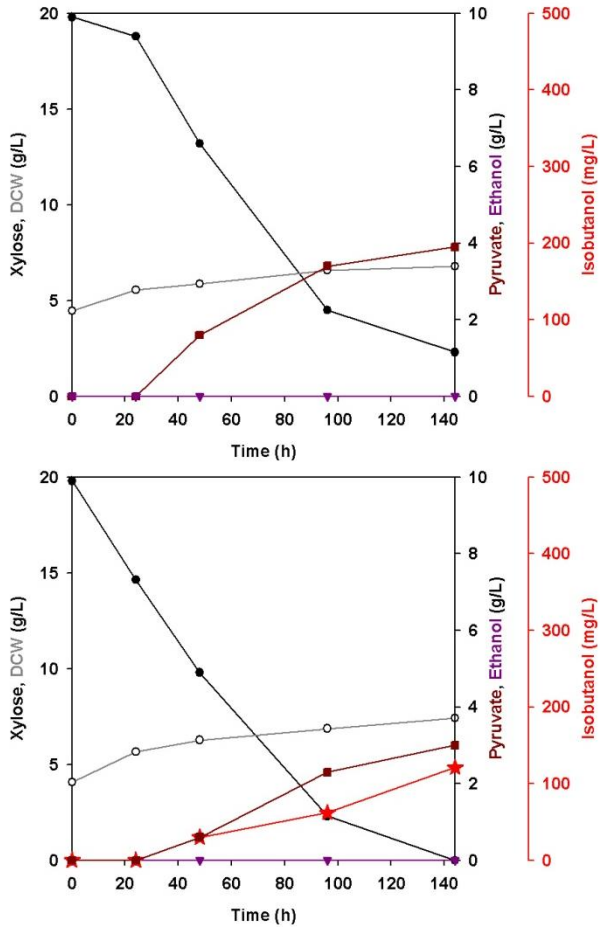


Figure 18. Flask fermentation profiles of

(a) SOS4X-CON$_{\text{-HXT}}$, (b) SOS4-2KCD$_{\text{-CYTO-HXT}}$

Symbols : Xylose ( ), DCW ( ), Pyruvate ( ), Glycerol ( ), Acetate ( ), Ethanol ( ), Isobutanol ( )

Table 11. Summary of flask fermentation in part 3.

Strain	Maximum dry cell weight (g/L)	Consumed xylose (g/L)	Pyruvate (g/L)	Concentration of isobutanol (mg/L)	Yield of isobutanol ($mg_{isobutanol}/g_{xylose}$)
SOS4X-CON_{-HXT}	6.8	17.5	3.9	0	-
SOS4X-$2KCD_{-CYTO-HXT}$	7.4	19.8	3.0	120	6.06

IV. CONCLUSIONS

This thesis can draw the following conclusions :

(1) In micro-aerobic batch fermentation, *S. cerevisiae* harboring the modified *ILV2*, *ilvC*, *ilvD* and *kivD* genes produced 120 mg/L isobutanol along with 7 g/L ethanol as a major metabolite. However, the Pdc-deficient *S. cerevisiae* strain harboring the modified *ILV2*, *ilvC*, *ilvD* and *kivD* genes produced 283 mg/L isobutanol without ethanol production.

(2) To enhance the isobutanol biosynthetic pathway, all enzymes involved in isobutanol production were cytosolically re-localized by modification of the *ilvC* and *ilvD* genes and the *GPD* promoter was replaced with the truncated *HXT7* promoter. The Pdc-deficient *S. cerevisiae* harboring three modified *ILV2*, *ilvC* and *ilvD* genes and *kivD* under the control of the truncated *HXT7* promoter produced 446mg/L isobutanol in micro-aerobic batch fermentation.

(3) To construct *S. cerevisiae* able to metabolize xylose without ethanol production, *XYL1*, *XYL2* and *XYL3* were introduced into the Pdc-deficient *S. cerevisiae*. When the isobutanol biosynthetic system was overexpressed in this strain, 120 mg/L of isobutanol was produced from xylose in micro-aerobic batch fermentation.

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국 문 초 록

화석연료의 사용으로 인한 에너지자원 고갈과 지구온난화와 같은 환경문제가 야기되면서 미생물을 이용하여 재생 가능한 생물자원으로부터 바이오 에너지를 생산하고자 하는 연구가 활발히 진행되고 있다. 현재 상용화된 대표적인 바이오 연료는 에탄올지만, 최근에는 탄소사슬이 많은 알코올들이 주목을 받고 있다. 그 중 부탄올은 에탄올에 비해 에너지 밀도가 높고, 흡습성 및 증기압이 낮아 운반에 용이해서 다양하게 적용이 가능한 연료이다. 특히 이소부탄올은 n-부탄올에 비해 더 많은 옥탄가를 가지고 있고 가솔린과 어떤 비율로도 혼합이 가능하여 기존의 가솔린 엔진과 수송 파이프라인에 그대로 적용 가능하다는 장점을 갖고 있다. 또한 수송연료뿐만 아니라 플라스틱, 연료 첨가제 등을 합성하는 전구체로서 사용되는 고부가가치 물질로 주목 받고 있다.

본 연구에서는 재조합 균주 개발에 용이하고, 부탄올과 당화액에 대한 내성이 높은 효모 *Saccharomyces cerevisiae*를 사용하여 대사공학적인 접근을 통해 이소부탄올을 생산하고자 하였다.

와일드 타입의 *S. cerevisiae*는 발린 생합성 경로와 에이리치 경로를 통하여 미토콘드리아에서 이소부탄올을 소량 생산할 수 있다. 이소부탄올 생합성에 관여하는 *S.*

cerevisiae 유래의 *ILV2, E. coli* 유래의 *ilvC*와 *ilvD, L. lactis* 유래의 *kivD* 유전자를 도입하여 120 mg/L의 이소부탄올을 생산하였다. 그러나 여전히 에탄올이 주된 산물로 생성되는 문제점이 있었다.

따라서 주요 부산물인 에탄올 생성을 억제하고자 피루브산탈카르복시효소 활성이 완전히 저해된 균주를 모균주로 하여 이소부탄올 생합성 경로를 도입하였고 결과적으로 에탄올의 생성 없이, 축적된 피루브산으로부터 283 mg/L의 이소부탄올을 생산하였다.

하지만 여전히 미량의 이소부탄올이 생성됨에 따라 이소부탄올의 생합성 경로를 강화하고자 이소부탄올 합성과 관련된 모든 효소를 미토콘드리아가 아닌 세포질에서 발현되도록 조작하였으며, 효모에서 강력하다고 알려진 프로모터를 이용하였다. 그 결과, 아이소부탄올의 생산량을 446 mg/L까지 증가시켰다.

이와 더불어, 경제적 측면에서 볼 때, 목질계 바이오매스로부터 이소부탄올을 생산하는 것이 이소부탄올의 지속 가능한 상업화를 위해 해결해야 할 주요 연구 과제이다. 하지만 *S. cerevisiae*는 목질계 바이오매스에 다량으로 존재하는 자일로스를 대사하지 못한다는 단점이 있다. 이를 개선하고자 *S. stipitis* 유래의 자일로스 대사 관련 유전자인 *XYL1, XYL2, XYL3*를 피루브산탈카르복시효소 활성이 완전히 저해된 균주에 도입하였다. 포도당에서와

마찬가지로 에탄올의 생성 없이 자일로스를 대사하여 피루브산이 축적됨을 확인하였다. 위에서 구축한 이소부탄올 생합성 경로를 도입하여 자일로스로부터 120 mg/L의 이소부탄올이 생성됨을 확인하였다. 이는 재조합 S. cerevisiae를 이용하여 목질계 바이오매스로부터 이소부탄올의 생산의 가능성을 제시한 연구 결과이다.

주요어 : 바이오에너지, 바이오부탄올, 이소부탄올,
피루브산, 피루브산탈카르복시효소 결여 효모,
자일로스, 목질계 바이오매스,

학 번 : 2012-21150

감사의 글

대학을 졸업하고 어리기만 했던 제가 대학원이라는 작은 사회를 경험하며 많은 것을 보고 느꼈습니다.

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