Bioprocess Biosyst Eng (2012) 35:1467–1475
DOI 10.1007/s00449-012-0736-y

ORIGINAL PAPER

Isobutanol production in engineered *Saccharomyces cerevisiae* by overexpression of 2-ketoisovalerate decarboxylase and valine biosynthetic enzymes

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Received: 20 February 2012 / Accepted: 8 April 2012 / Published online: 28 April 2012

Abstract Engineering of *Saccharomyces cerevisiae* to produce advanced biofuels such as isobutanol has received much attention because this yeast has a natural capacity to produce higher alcohols. In this study, construction of isobutanol production systems was attempted by overexpression of effective 2-keto acid decarboxylase (KDC) and combinatorial overexpression of valine biosynthetic enzymes in *S. cerevisiae* D452-2. Among the six putative KDC enzymes from various microorganisms, 2-ketoisovalerate decarboxylase (Kivd) from *L. lactis* subsp. *lactis* KACC 13877 was identified as the most suitable KDC for isobutanol production in the yeast. Isobutanol production by the engineered *S. cerevisiae* was assessed in micro-aerobic batch fermentations using glucose as a sole carbon source. 93 mg/L isobutanol was produced in the Kivd overexpressing strain, which corresponds to a fourfold improvement as compared with the control strain. Isobutanol production was further enhanced to 151 mg/L by additional overexpression of acetolactate synthase (Ilv2p), acetohydroxyacid reductoisomerase (Ilv5p), and dihydroxyacid dehydratase (Ilv3p) in the cytosol.

Keywords Isobutanol · Engineered *Saccharomyces cerevisiae* · 2-Ketoisovalerate decarboxylase · Valine biosynthetic enzymes

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Introduction

Bioethanol is considered the most common and effective biofuel that can be produced from renewable biomass via microbial production systems [1–3]. As a transportation fuel, however, bioethanol has limitations such as low energy density and high hygroscopicity. Several efforts have been made to produce advanced biofuels including *n*-butanol, isobutanol, and other higher alcohols [4–7]. Isobutanol has particularly received great attention as a potential biofuel because it has higher energy density, lower hygroscopicity, and higher octane value than ethanol. In addition, isobutanol can be readily used as a precursor for a number of valuable chemical syntheses [8, 9].

Saccharomyces cerevisiae can be a promising host for isobutanol production since it has already been used for ethanol production and is known to be tolerant to higher alcohols. Recently, *S. cerevisiae* has been demonstrated to have substantial tolerance to *n*-butanol, the straight chain isomer of isobutanol [10]. Since the difference between isobutanol and *n*-butanol is only in conformational structure, *S. cerevisiae* may be able to tolerate high concentrations of isobutanol by a similar mechanism [11]. In addition, *S. cerevisiae* is known to naturally produce various higher alcohols, which are involved in tastes and flavors of beer and wine [12–14]. Consequently, it may have more advantages in isobutanol production than other host microorganisms.

Isobutanol is synthesized through the Ehrlich degradation pathway [13, 15, 16], in which amino acids can be converted to their corresponding 2-keto acids by branched-chain amino-acid aminotransferase. 2-Keto acids can be further converted to aldehydes by 2-keto acid decarboxylase (KDC). Through the decarboxylation step of the Ehrlich degradation pathway, isobutyraldehyde can be synthesized from 2-ketoisovalerate, a precursor for valine biosynthesis. Then, isobutyraldehyde can be converted to isobutanol by alcohol dehydrogenase (ADH). The metabolic pathway for isobutanol biosynthesis in *S. cerevisiae* is illustrated in Fig. 1 [13, 17]. Pyruvate is synthesized from glucose through glycolysis. After transportation into the mitochondria, pyruvate is converted to 2-ketoisovalerate by three enzymes: acetolactate synthase (Ilv2p), acetohydroxyacid reductoisomerase (Ilv5p), and dihydroxyacid dehydratase (Ilv3p). Then, valine is synthesized from 2-ketoisovalerate by branched-chain amino-acid aminotransferase (Bat1p). Because valine synthesis usually occurs in the mitochondria [17], cytosolic expression of the *ILV* genes may be required for efficient isobutanol production even if 2-ketoisovalerate may be secreted from the mitochondria [22]. Cytosolic 2-ketoisovalerate can be converted to isobutyraldehyde by KDC-like enzymes such as pyruvate decarboxylase (Pdc1p, Pdc5p, Pdc6p), phenylpyruvate decarboxylase (Aro10p), and putative α-ketoisocaproate decarboxylase (Thi3p) [13, 18, 19]. Then, isobutyraldehyde can be converted to isobutanol by alcohol dehydrogenase (Adh2p) [8, 13, 20].

Recently, several achievements in isobutanol production have been reported using metabolically engineered microorganisms. Atsumi et al. [8] were able to produce 22 g/L isobutanol using engineered *Escherichia coli* with overexpression of AlsS (from *Bacillus subtilis*), IlvC-IlvD (from *E. coli*), Kivd (from *L. lactis*), and Adh2p (from *S. cerevisiae*) and deletion of several genes involved in by-product formation. The same group also reported ~5 g/L isobutanol was produced in metabolically engineered *Corynebacterium glutamicum* which is known to have a specialized capability for amino acid biosynthesis [21]. Meanwhile, it was reported that ~70 mg/L isobutanol was produced in recombinant *S. cerevisiae* by overexpressing the genes involved in valine metabolism [22].

In this study, construction of an isobutanol production system was attempted by overexpression of an effective KDC enzyme and additional overexpression of enzymes involved in valine biosynthesis in metabolically engineered *S. cerevisiae*. Investigations of the most specific KDC on 2-ketoisovalerate were performed by comparing intracellular activity levels of various KDCs from several microorganisms. Isobutanol production was assessed in batch fermentations of the recombinant *S. cerevisiae* overexpressing the most suitable KDC. In addition, combinatorial overexpression of Ilv2p, Ilv3p, and Ilv5p was performed to improve isobutanol biosynthesis.

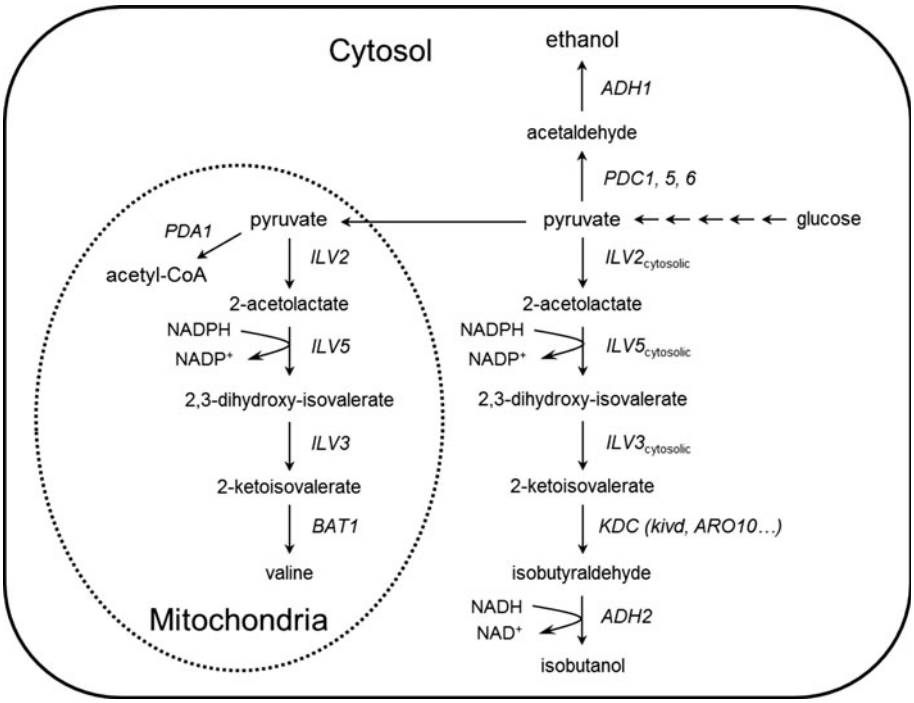

Fig. 1 Isobutanol biosynthetic pathway in recombinant *S. cerevisiae*

Materials and methods

Strains and plasmids

E. coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used for genetic manipulation. *S. cerevisiae* D452-2 (*Matα*, *leu2*, *his3*, *ura3*. and *can1*) [2] was used as the host strain for isobutanol production. Plasmids used for the expression of isobutanol biosynthetic genes were derived from pRS425GPD and pRS426GPD vectors. The KDC genes were obtained by the polymerase chain reaction (PCR) from the genomic DNA of six microorganisms. Primers and restriction enzymes used in this study are described in Table 1. After digestion with appropriate restriction enzymes, the *KDC* genes and pRS425GPD plasmid were ligated. The resulting plasmids are summarized in Table 2. The features of plasmids (promoter, terminator, origin, and markers) are also summarized in Table 2.

The *ILV2*, *ILV3*, and *ILV5* genes were obtained from the genomic DNA of *S. cerevisiae* D452-2. To obtain the *ILV* genes without the mitochondrial targeting sequence, PCR primers were designed to exclude the N-terminal amino acid residues, which are thought to be involved in mitochondrial targeting. The mitochondrial targeting sequences of the *ILV* genes were predicted by using MitoProt (http://ihg.gsf.de/ihg/mitoprot.html). The amplified *ILV2*, *ILV3,* and *ILV5* gene constructs lacked the coding sequences for the first 55, 20, and 34 amino acid residues. After digestion with *Bam*HI and *Xho*I, the *ILV* genes and pRS426GPD plasmid were ligated. The resulting plasmids were named as p426ILV2, p426ILV3, and p426ILV5, respectively.

For constructing a plasmid which can overexpress *ILV3* and *ILV5* genes, the DNA fragment containing the *GPD* promoter, *ILV3* gene and *CYC* terminator was released after digestion of p426ILV3 with *Eag*I and *Sac*I. The released fragment was blunt-ended and cloned into p426ILV5, which was digested with *Eag*I and blunt-ended. The resulting plasmid was named as p426ILV53.

For constructing a plasmid which can overexpress *ILV2*, *ILV3*, and *ILV5* genes, the DNA fragment containing the GPD promoter, *ILV2* gene and CYC terminator was amplified by PCR from p426ILV2. The fragment was ligated with p426ILV53 after digestion with *Sac*I. The resulting plasmid was named as p426ILV253.

Table 1 List of primers used in this study

Target DNA	Primer name	Sequence	Restriction enzyme	Source
kdc	*F-Spe*I_*kdc_mtu*	GGACTAGTATGACACCCCAGAAGAGCGA	*Spe*I	*Mycobacterium tuberculosis* H37Rv
	*R-Hin*dIII_*kdc_mtu*	CCCAAGCTTTCACTGCGGCGCCATGGATC	*Hin*dIII	
ARO10	*F-Bam*HI_*ARO10_sce*	CGGGATCCATGGCACCTGTTACAATTG	*Bam*HI	*S. cerevisiae* D452-2
	*R-Xho*I_*ARO10_sce*	CCGCTCGAGCTATTTTTTATTTCTTTTAAGTGC	*Xho*I	
ipdc	*F-Spe*I_*ipdc_bce*	GGACTAGTATGAAAAAACAATATACTGTAAG	*Spe*I	*Bacillus cereus* ATCC 14579
	*R-Hin*dIII_*ipdc_bce*	CCCAAGCTTTTAAGAATTTTGCTGACCAGA	*Hin*dIII	
kivd	*F-Bam*HI_*kivd*	CGGGATCCAAAATGTATACAGTAGGAGATTAC	*Bam*HI	*Lactobacillus lactis* subsp. *lactis* KCTC 3926
	*R-Xho*I_*kivd*	CCGCTCGAGTTATGATTTATTTTGTTCAGCAA	*Xho*I	*L. lactis* subsp. *hordniae* KACC 13442
				L. lactis subsp. *lactis* KACC 13877
ILV2	#075	AATGCCGGATCCAAAATGGAGCCTGCTCCAAGTTT	*Bam*HI	*S. cerevisiae* D452-2
	#076	AATGCCCTCGAGTTAGTGCTTACCGCCTGTACG	*Xho*I	
ILV3	#077	AATGCCGGATCCAAAATGAAGAAGCTCAACAAGTACTC	*Bam*HI	*S. cerevisiae* D452-2
	#078	AATGCCCTCGAGTTAAGCATCTAAAACACAACCGTTGG	*Xho*I	
ILV5	#079	AATGCCGGATCCAAAATGGCTGCCCGTTTCGTTAA	*Bam*HI	*S. cerevisiae* D452-2
	#080	AATGCCCTCGAGTTATTGGTTTTCTGGTCTCAACTTTCT	*Xho*I	
P_{GPD}-*ILV2*-T_{CYC}	#132	GGCGAGCTCGTAATACGACTCACTATAGGGCGA	*Sac*I	p426ILV2
	#133	GGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTC	*Sac*I	

Table 2 List of plasmids used in this study

Plasmids	Relative feature	Gene source
pRS425GPD	*LEU2*, GPD promoter-MCS-CYC terminator, 2 μ origin, Amp^r	
pRS426GPD	*URA3*, GPD promoter-MCS-CYC terminator, 2 μ origin, Amp^r	
p425KDCmtu	*LEU2*, P_{GPD}-*KDC*-T_{CYC}, 2 μ origin, Amp^r	*M. tuberculosis* H37Rv
p425ARO10sce	*LEU2*, P_{GPD}-*ARO10*-T_{CYC}, 2 μ origin, Amp^r	*S. cerevisiae* D452-2
p425IPDCbce	*LEU2*, P_{GPD}-*IPDC*-T_{CYC}, 2 μ origin, Amp^r	*B. cereus* ATCC 14579
p425kivd3926	*LEU2*, P_{GPD}-*kivd*-T_{CYC}, 2 μ origin, Amp^r	*L. lactis* subsp. *lactis* KCTC 3926
p425kivd13442	*LEU2*, P_{GPD}-*kivd*-T_{CYC}, 2 μ origin, Amp^r	*L. lactis* subsp. *hordniae* KACC 13442
p425kivd13877	*LEU2*, P_{GPD}-*kivd*-T_{CYC}, 2 μ origin, Amp^r	*L. lactis* subsp. *lactis* KACC 13877
p426ILV2	*URA3*, P_{GPD}-*ILV2*-T_{CYC}, 2 μ origin, Amp^r	*S. cerevisiae* D452-2
p426ILV3	*URA3*, P_{GPD}-*ILV3*-T_{CYC}, 2 μ origin, Amp^r	*S. cerevisiae* D452-2
p426ILV5	*URA3*, P_{GPD}-*ILV5*-T_{CYC}, 2 μ origin, Amp^r	*S. cerevisiae* D452-2
p426ILV53	*URA3*, P_{GPD}-*ILV5*-T_{CYC}-P_{GPD}-*ILV3*-T_{CYC}, 2 μ origin, Amp^r	*S. cerevisiae* D452-2
p426ILV253	*URA3*, P_{GPD}-*ILV2*-T_{CYC}-P_{GPD}-*ILV5*-T_{CYC}-P_{GPD}-*ILV3*-T_{CYC}, 2 μ origin, Amp^r	*S. cerevisiae* D452-2

Table 3 List of strains used in this study

Strains	Genotype	Gene source
S. cerevisiae D452-2	*Mat*α, *leu2*, *his3*, *ura3* and *can1*	
D-56	D452-2/pRS425GPD/pRS426GPD	
D-K	D452-2/p425kivd13877/pRS426GPD	*kivd* from *L. lactis* subsp. *lactis* KACC 13877
D-I3K	D452-2/p425kivd13877/p426ILV3	*kivd* from *L. lactis* subsp. *lactis* KACC 13877, *ILV3* from *S. cerevisiae* D452-2
D-I53K	D452-2/p425kivd13877/p426ILV53	*kivd* from *L. lactis* subsp. *lactis* KACC 13877, *ILV5*, *ILV3* from *S. cerevisiae* D452-2
D-I253K	D452-2/p425kivd13877/p426ILV253	*kivd* from *L. lactis* subsp. *lactis* KACC 13877, *ILV2*, *ILV5* and *ILV3* from *S. cerevisiae* D452-2
D-I253	D452-2/pRS425GPD/p426ILV253	*ILV2*, *ILV5* and *ILV3* from *S. cerevisiae* D452-2

All plasmids used in this study are listed in Table 2. All constructed plasmids were subjected to DNA sequencing. PCR, general DNA manipulation, bacterial transformation, and yeast transformation were done according to the methods described previously [23]. All strains used in this study are listed in Table 3.

Culture condition

Luria-Bertain (LB) medium (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl) with 100 μg/mL ampicillin was used for plasmid preparation from *E. coli*.

Synthetic complete (SC) medium (6.7 g/L yeast nitrogen base without amino acid, 20 g/L glucose, and 0.625 mg/L CSM without Leu, Trp, and Ura) with appropriate nucleotides and amino acids was used for selection of the transformants, seed cultivation, and pre-cultivation.

Main cultivation was performed in 250 ml flasks containing 50 ml of YPD medium (10 g/L yeast extract, 20 g/L Bacto peptone, and 40 g/L glucose, pH 6.0). Cells at the middle of exponential growth (12–16 h of cultivation) in the pre-cultivation were harvested and used in the main cultivation. Fermentations were begun with the inoculation of cells at an initial concentration of 0.35 g/L. Temperature was maintained at 30 °C. For microaerobic cultivation, agitation speed was maintained at 100 rpm during the fermentation.

Determination of KDC activity

Cells from the cultivation were harvested and adjusted around 7 g/L. They were resuspended in reaction buffer (50 mM potassium phosphate buffer containing 2.5 mM $MgSO_4$ and 0.1 mM ThDP, pH 6.8) and disrupted by vortexing (Voltex genie 2, Scientific industries, Bohemia,

USA) with acid-washed glass beads (0.45–0.52 mm, Sigma, St Louis, USA). To prevent degradation of enzymes, Protease Inhibitor Cocktail (Roche, Basel, Switzerland) was added. After centrifugation at 12,000 rpm for 10 min, the supernatant was separated and used as crude enzyme solution for KDC activity assay. Measurements of KDC activity were done following the method described previously [24]. Since the decarboxylation of 2-ketoisovalerate cannot be measured directly, the reduction of isobutyraldehyde to isobutanol by aldehyde dehydrogenase (ADH), which requires NADH as a cofactor, was assumed to correspond to the KDC activity. The crude cell extract and 2-ketoisovalerate (120 mM) were added into a reaction mixture containing NADH and ADH from the baker's yeast (Sigma-Aldrich). Oxidation of NADH at 340 nm was monitored with a 96-well microplate reader (Molecular Devices Co., Sunnyvale, CA, USA) at 30 °C. One unit of KDC activity was defined as the amount of an enzyme that can oxidize one micromole of NADH per minute. Specific KDC activity (U/mg cellular protein) was estimated by dividing enzyme activity by the total intracellular protein concentration.

Analytical methods

Cell concentration was measured using a spectrophotometer (Biomate 5, Thermo, NY, USA). Glucose and ethanol concentrations were determined by a high-performance liquid chromatography (HPLC) system (Agilent Technologies 1200 Series) equipped with a Rezex ROA Organic Acid H^+ column (Phenomenex, Torrance, CA, USA) and a refractive index (RI) detector (Agilent, Palo Alto, CA, USA). The column was eluted with 0.01 M H_2SO_4 at a flow rate of 0.6 ml/min at 50 °C. Isobutanol concentration was determined by a gas chromatography (GC) system (Agilent Technologies 7890 Series) equipped with a HP-INNOWAX column (Agilent). Column temperature was controlled by the following gradient program: 60 °C for 1 min; increase at a rate of 15 °C/min; 225 °C for 5 min.

Results

Investigation of inherent tolerance of *S. cerevisiae* to isobutanol

In order to check the tolerance of *S. cerevisiae* to isobutanol, the growth of *S. cerevisiae* D452-2 under different isobutanol concentrations was compared. As more isobutanol was added to the YPD medium, a lower growth rate was observed as expected. When isobutanol concentrations in the medium were 0.5 % (w/v) and 1 % (w/v), *S. cerevisiae* was able to grow with decent growth rates (80 or 50 %) as compared with the growth rate without isobutanol addition. However, *S. cerevisiae* could not grow at all with 20 g/L isobutanol, suggesting that isobutanol is more toxic than ethanol to *S. cerevisiae*. While current titers of isobutanol produced by the engineered yeast are low enough to prevent any inhibition on cell growth and metabolism, cellular and metabolic engineering approaches for improving tolerance of *S. cerevisiae* to isobutanol need to be considered as the isobutanol concentrations produced exceed 10 g/L.

Identification of a specific KDC on 2-ketoisovalerate

Keto-acid decarboxylase (KDC) is known to be a crucial enzyme for removing the carboxylic group from 2-keto acid to produce aldehyde. As shown in Fig. 1, 2-ketoisovalerate, an intermediate metabolite of valine biosynthesis, can be converted to isobutyraldehyde by KDC and then converted to isobutanol by alcohol dehydrogenase (ADH) [13]. As decarboxylation from 2-ketoisovalerate is known to be catalyzed by several types of KDC [13, 24, 25], identification of KDC showing the highest specific activity may be required for effective production of isobutanol in yeast. Genes coding for KDC from four microorganisms, *Mycobacterium tuberculosis*, *Bacillus cereus*, *L. lactis,* and *S. cerevisiae*, were cloned. Specifically, first, three 2-ketoisovalerate decarboxylase genes (*kivd*) from *L. lactis* subsp. *lactis* KCTC3926, KACC13877, and *L. lactis* subsp. *hordniae* KACC13442 [24, 25] were obtained using degenerate primers (Table 1). Second, the gene encoding phenylpyruvate decarboxylase, *ARO10* from *S. cerevisiae* [19], was also cloned as Aro10p has been demonstrated to show a higher affinity to 2-ketoisovalerate over other 2-keto acids [8]. Third, two genes (*ipdc* and *kdc*) encoding putative KDC enzymes (indole-pyruvate decarboxylase and KDC) were cloned from *B. cereus* and *M. tuberculosis*, based on high similarities to the KDC from *L. lactis* subsp. *lactis*.

After constructing expression cassettes for overexpressing these genes under the control of a strong promoter (P_{GPD}) using multi-copy plasmid (pRS425) (Table 2), the transformants of the expression cassettes were obtained using *S. cerevisiae* D452-2 as a host strain. In order to confirm the functional expression of the *KDC* genes, in vitro KDC activities of the transformants were measured (Fig. 2). Unexpectedly, the control strain containing an empty plasmid showed a considerable level of KDC activity (0.46 U/mg). It is presumed that such a basal level KDC activity is due to non-specific KDC activities by other decarboxylases. There are five KDC-like enzymes in *S. cerevisiae* (Aro10p, Thi3p, Pdc1p, Pdc5p, and Pdc6p), all of

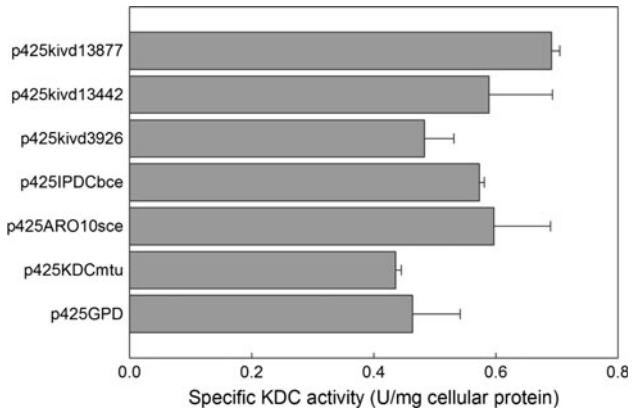

Fig. 2 Comparison of specific KDC activity in recombinant *S. cerevisiae* D452-2 strains overexpressing putative *KDC* genes from six microorganisms

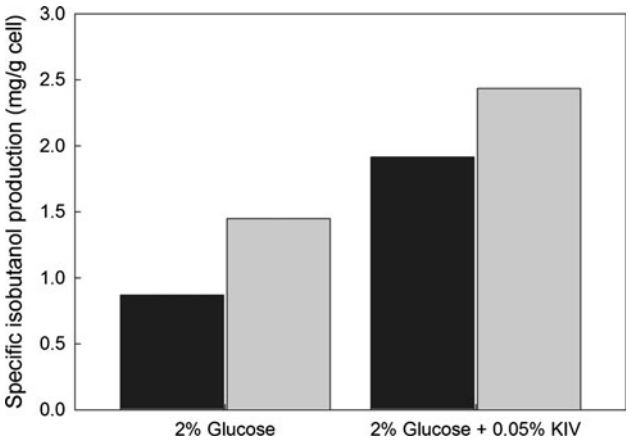

Fig. 3 Confirmation of isobutanol production in 5 ml test tube fermentation of recombinant *S. cerevisiae* D-56 (*black bar*) and D-K (*white bar*) with 2 % (w/v) glucose or 2 % (w/v) glucose with 0.05 % (w/v) 2-ketoisovalerate (KIV)

which might catalyze the conversion of 2-ketoisovalerate to isobutyraldehyde [13, 18, 19]. While the control strain showed the basal level of KDC activity, all transformants with the introduced Kivd and putative KDC showed significantly higher KDC activity. Among the enzymes tested in this study, Kivd from *L. lactis* subsp. *lactis* KACC13877 had the highest decarboxylase activity on 2-ketoisovalerate (0.69 U/mg), nearly 1.5-times higher than the control. Kivd from *L. lactis* subsp. *hordniae* KACC13442, Ipdc from *B. cereus* and Aro10p from *S. cerevisiae* showed similar decarboxylase activity. Thus, Kivd from *L. lactis* subsp. *lactis* KACC13877 was determined as the most suitable KDC for isobutanol production in *S. cerevisiae*.

Isobutanol production by engineered *S. cerevisiae*

It was tested whether the engineered yeast overexpressing the selected Kivd could produce isobutanol from glucose. The Kivd overexpressing strain was able to produce 66 % more isobutanol than its parental strain containing an empty plasmid. Inefficient production of isobutanol from glucose in this engineered yeast seems to be limited by supply of 2-ketoisovalerate, a precursor for the valine biosynthesis pathway. Therefore, 0.5 g/L 2-ketoisovalerate was added into the medium to determine if exogenous supply of the precursor could enhance isobutanol production. As expected, the addition of 2-ketoisovalerate improved production of isobutanol, suggesting the endogenous pathway for producing 2-ketoisovalerate in yeast is limiting for efficient isobutanol production (Fig. 3). Therefore, we constructed expression cassettes for overexpressing *ILV2*, *ILV3*, and *ILV5* genes with various combinations (Table 2) to improve the supply of 2-ketoisovalerate.

Effect of overexpression of endogenous valine biosynthetic enzymes on isobutanol production

In order to overexpress Ilv2p, Ilv3p, and Ilv5p in the cytosol rather than the mitochondria where those enzymes are localized for valine biosynthesis [17], we designed PCR primers to exclude the N-terminal amino acid residues, which were predicted to be involved in mitochondrial targeting. As a result, the truncated *ILV* genes without the targeting sequences were overexpressed in engineered *S. cerevisiae* expressing Kivd. Three engineered strains expressing Ilv3p, Ilv3p-Ilv5p, or Ilv2p-Ilv3p-Ilv5p in the background of Kivd overexpression were constructed to confirm that the supply of 2-ketoisovalerate is a limiting step of isobutanol production. The three engineered strains (D-I3K, D-I53K, and D-I253K) expressing the *kivd* and *ILV* genes with various combinations (Table 3) as well as the engineered strain expressing the *kivd* gene only (D-K) were subjected to fermentation experiments using 40 g/L glucose as the sole carbon source (Fig. 4). In order to allow sufficient metabolic fluxes towards the isobutanol pathway rather than ethanol production at the branch point of pyruvate, the fermentation experiments were performed under micro-aerobic conditions.

The control strains (D-56, the parental strain and D-I253, engineered strain expressing only Ilv2p-Ilv3p-Ilv5p) produced only small amounts (22 and 17 mg/L, respectively) of isobutanol (Fig. 4a, f) but overexpression of Kivd led to increased isobutanol production up to 93 mg/L (Fig. 4b). This result suggests that Kivd is the essential enzyme for isobutanol production in *S. cerevisiae* because of its higher specificity to 2-ketoisovalerate. Even if *S. cerevisiae* has various kinds of KDC-like enzymes, significant amounts of isobutanol cannot be synthesized without overexpression of the specific 2-ketoisovalerate

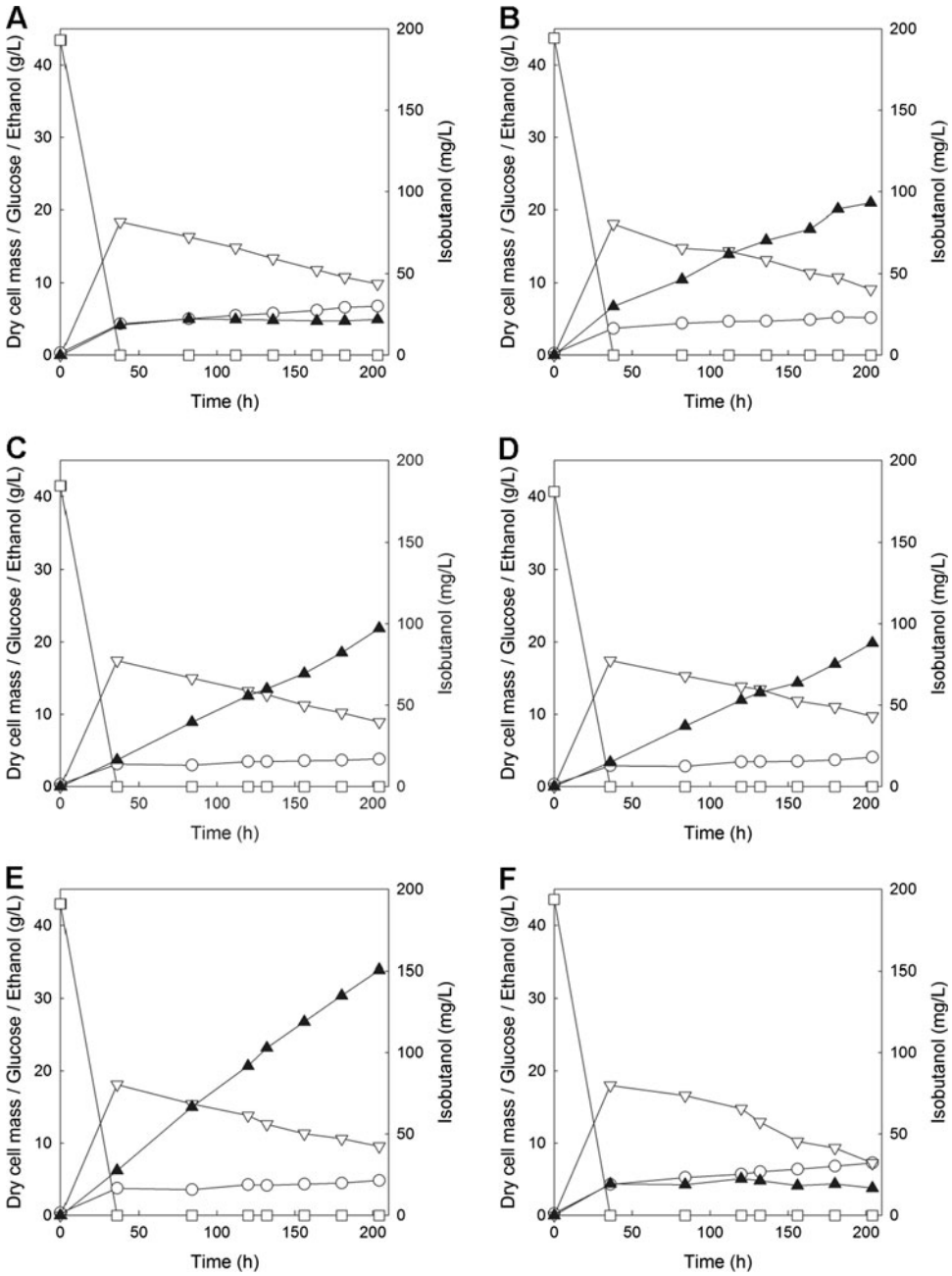

Fig. 4 Profiles of micro-aerobic batch fermentations of recombinant *S. cerevisiae* D-56 (**a**), D-K (**b**), D-I3K (**c**), D-I53K (**d**), D-I253K (**e**) and D-I253 (**f**). Dry cell mass (*open circle*), glucose (*open square*), ethanol (*inverted triangle*) and isobutanol (*closed triangle*). Measurements of cell, glucose, ethanol, and isobutanol concentrations were done by three independent experiments of the six strains. *Symbols* in the figures show the representative values of batch fermentations

decarboxylase. Additional overexpression of Ilv2p-Ilv3p-Ilv5p resulted in even higher isobutanol production up to 151 mg/L (Fig. 4e), but partial overexpression of Ilv3p or Ilv3p-Ilv5p failed to improve isobutanol production further (Fig. 4c, d).

Discussion

Wild-type *S. cerevisiae* can produce small amounts of higher (C3–C6) alcohols, which are also known as fusel oil, during ethanol fermentation. However, these higher alcohols are not direct fermentative products of sugars but degraded products of amino acids (valine, leucine, isoleucine, methionine, and phenylalanine) via the Ehrlich pathway [12–14]. While isobutanol can be produced through deamination, decarboxylation, and reduction steps from valine, only small amounts of isobutanol can be produced through the Ehrlich pathway because of the limited supply of valine and lack of efficient KDC in *S. cerevisiae*. Indeed, about 20 mg/L isobutanol production was observed by a parental yeast strain at the early stage of fermentation (Fig. 4a), but isobutanol titer did not increase further. It was assumed the 20 mg/L isobutanol was

produced from valine present in YPD medium via the Ehrlich pathway. The overexpression of the selected Kivd from *L. lactis* increased isobutanol production up to 93 mg/L, suggesting that endogenous KDC activity for decarboxylating 2-ketoisovalerate is critical in *S. cerevisiae*. Therefore, we can postulate that metabolic engineering of *S. cerevisiae* for producing isobutanol requires introduction of efficient Kivd and sufficient supply of its precursor, 2-ketoisovalerate, in addition to selective amplification for isobutanol biosynthesis and deletion of the pathways for by-product formation.

In this study, we screened and identified a KDC exhibiting a relatively higher activity on 2-ketoisovalerate through measuring in vitro activity of KDC using crude extracts of the transformants overexpressing KDCs from various microorganisms. While the highest KDC activity with 2-ketoisovalerate was observed from the transformant expressing Kivd from *L. lactis* subsp. *lactis* KACC13877, the parental strain (*S. cerevisiae* D452-2) with an empty plasmid also showed a considerable level of KDC activity unexpectedly. This result indicates that endogenous enzymes exhibiting KDC activity exist in *S. cerevisiae*. Five isozymes of pyruvate decarboxylase (Aro10p, Thi3p, Pdc1p, Pdc5p, and Pdc6p) might be responsible for the relatively high KDC activity [13, 18, 19]. Because of this endogenous KDC activity in *S. cerevisiae*, the control strain (D-56) was able to produce about 20 mg/L isobutanol even without overexpression of Kivd. However, the Kivd overexpressing strain (D-K) produced 4-times more isobutanol than the control strain, suggesting that Kivd is required for enhanced production of isobutanol in yeast.

Unfortunately, simultaneous production of isobutanol and ethanol was not observed in our fermentation experiments by the engineered strains. During the glucose consumption period, there was a slight difference in isobutanol production among the control strain (D-56), Kivd overexpressing strain (D-K), and Kivd/Ilv2p-Ilv3p-Ilv5p overexpressing strain (D-I253K). While these three strains produced 20–30 mg/L isobutanol during the glucose consumption period, more accumulation of isobutanol by both D-K and D-I253K strains was observed after the yeast strains ceased ethanol production. These results indicate that the isobutanol produced after glucose depletion might be produced from ethanol rather than glucose. In order to facilitate isobutanol production from glucose, a forced change of the carbon flux from ethanol to valine biosynthesis by deletion of the *PDC* genes involved in ethanol production might be required.

2-Ketoisovalerate is used as a precursor of valine biosynthesis. Amplification of the valine biosynthetic pathway (from pyruvate to 2-ketoisovalerate) might further improve isobutanol production. It was hypothesized that an increase in the expression level of *ILV3*, *ILV5*, and *ILV2* genes might improve isobutanol production by enhancing the carbon flux from pyruvate to 2-ketoisovalerate. Previously, efficient supply of 2-ketoisovalerate was attempted by increasing carbon flux from pyruvate to 2-ketoisovalerate through a mitochondrial overexpression of valine biosynthetic enzymes, Ilv2p, Ilv3p, and Ilv5p [22]. However, conversion of 2-ketoisovalerate to isobutyraldehyde occurs in the cytosol, which means additional manipulation may be required for efficient supply of cytosolic 2-ketoisovalerate. Moreover, valine is known to be a feedback inhibitor on Ilv2p, which indicates in situ removal of mitochondrial valine may be required for isobutanol production. Consequently, they also overexpressed a cytosolic valine degrading enzyme, Bat2p, which converts valine to 2-ketoisovalerate. We speculated that the cytosolic overexpression of valine biosynthetic enzymes may be more efficient than a mitochondrial overexpression for isobutanol production as transport of pyruvate and 2-ketoisovalerate through the mitochondrial membrane could be limited. Hence, truncated *ILV* genes were overexpressed in this study for efficient supply of cytosolic 2-ketoisovalerate. Even though the overexpression of Ilv3p (D-I3K) or Ilv3-Ilv5p (D-I53K) did not improve isobutanol production significantly, simultaneous overexpression of three genes (*ILV2*, *ILV3*, and *ILV5*) led to more than a 60 % improvement in isobutanol production. It is notable that isobutanol production by the D-I253 strain showed even lower isobutanol production than the control strain. This result strongly suggests that isobutanol cannot be produced efficiently without overexpression of Kivd in spite of the amplified flux from pyruvate to 2-ketoisovalerate. Again, these results indicate that Kivd is the most essential enzyme for isobutanol production in *S. cerevisiae*.

This study was undertaken to construct an isobutanol production system by overexpression of the most effective KDC enzyme and combinatorial overexpression of valine biosynthetic enzymes in engineered *S. cerevisiae*. More research is in progress to modulate the carbon flux from ethanol production to isobutanol production.

Conclusion

In this study, construction of an isobutanol production system was attempted through the metabolic engineering of *S. cerevisiae*. Overexpression of the most effective KDC enzyme and combinatorial overexpression of valine biosynthetic enzymes were performed to activate isobutanol biosynthesis in *S. cerevisiae*. In order to facilitate isobutanol production in yeast, more research such as modulation of carbon flux from ethanol to isobutanol should be conducted.

Acknowledgments This research was supported by the Advanced Biomass R&D Center (ABC) of Korea Grant funded by the Ministry of Education, Science and Technology (2010-0029799) and the Marine Biotechnology Program funded by the Ministry of Land, Transport and Maritime Affairs of Korean Government.

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