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Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways

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ABSTRACT

Production of higher alcohols via the keto-acid intermediates found in microorganism's native amino-acid pathways has recently shown promising results. In this work, an *Escherichia coli* strain that produces 1-butanol and 1-propanol from glucose was constructed. The strain first converts glucose to 2-ketobutyrate, a common keto-acid intermediate for isoleucine biosynthesis. Then, 2-ketobutyrate is converted to 1-propanol through reactions catalyzed by the heterologous decarboxylase and dehydrogenase, or to 1-butanol via the chemistry involved in the synthesis of the unnatural amino acid norvaline. We systematically improved the synthesis of 1-propanol and 1-butanol through deregulation of amino-acid biosynthesis and elimination of competing pathways. The final strain demonstrated a production titer of 2 g/L with nearly 1:1 ratio of butanol and propanol.

1. Introduction

The shortage of petroleum and the environmental perturbation due to its consumption have become a crucial problem facing the world in this century. In an attempt to lower the petroleum demand and to utilize renewable resources, bio-ethanol production has been intensively studied over the past 50 years. Despite the current use of ethanol as a transportation fuel, interest in butanol as the next-generation gasoline substitute has grown because of its higher energy density and lower vapor pressure compared to ethanol. In addition, its lower hygroscopicity allows it to be readily stored and distributed using existing infrastructure.

1-Butanol production from carbohydrates has been carried out using *Clostridium* through acetone–butanol–ethanol (ABE) fermentation (Lin and Blaschek, 1983; Nair and Papoutsakis, 1994; Formanek et al., 1997). However, *Clostridium*'s complex physiology and difficulty for genetic manipulation present challenges for further improvement in this organism. It is thus of interest to transfer the butanol production pathway from *Clostridium* to an easily manipulated organism, such as *Escherichia coli*. The initial success of this task has recently been demonstrated (Atsumi et al., 2007).

1-Propanol is another alcohol that can potentially be used as a gasoline substitute. It is currently used as a multi-purpose solvent in a variety of industrial products such as paint, cleaner and cosmetics. Microbial production of 1-propanol has been detected from certain species of *Clostridium* (Janssen, 2004) via threonine catabolism and from yeast (Eden et al., 2001) in beer fermentation. However, both resulted in only small quantities of 1-propanol (<70 mg/L). No existing microorganism has been reported to produce 1-propanol from sugars in significant amounts.

Instead of using the pathways naturally evolved for alcohol production in microorganisms, our group has devised a systematic approach (Atsumi et al., 2008) for the synthesis of higher alcohols utilizing the amino-acid biosynthetic pathways that are present in all organisms. Not only is this system readily transferable into other hosts but utilization of native amino-acid intermediates as alcohol production precursors also minimizes metabolic perturbation caused by toxic intermediates. With this strategy, Atsumi et al. (2008) have demonstrated a high level of isobutanol production in *E. coli*. Here, the same strategy is applied to produce 1-butanol and 1-propanol in *E. coli*.

As reported earlier (Atsumi et al., 2008), upon introduction of the promiscuous 2-ketoacid decarboxylase (Kivd) from *Lactococcus lactis* (Smit et al., 2005) and alcohol dehydrogenase 2 (ADH2) from *Saccharomyces cerevisiae* into *E. coli*, 2-ketobutyrate can be converted into 1-propanol (Fig. 1a). While 2-ketobutyrate is a common intermediate derived from threonine and a precursor for isoleucine biosynthesis, the 1-butanol precursor 2-ketovalerate is a rare metabolite in the cell leading to the synthesis of the unnatural amino acid, norvaline. Similar to the formation of 2-ketoisocaproate (McCourt and Duggleby, 2006), the precursor for leucine biosynthesis, production of 2-ketovalerate was catalyzed by the enzymes LeuABCD using 2-ketobutyrate as an alternative starting substrate (Fig. 1b) via the keto-acid chain elongation process (Bogosian et al., 1989; Kisumi et al., 1976).

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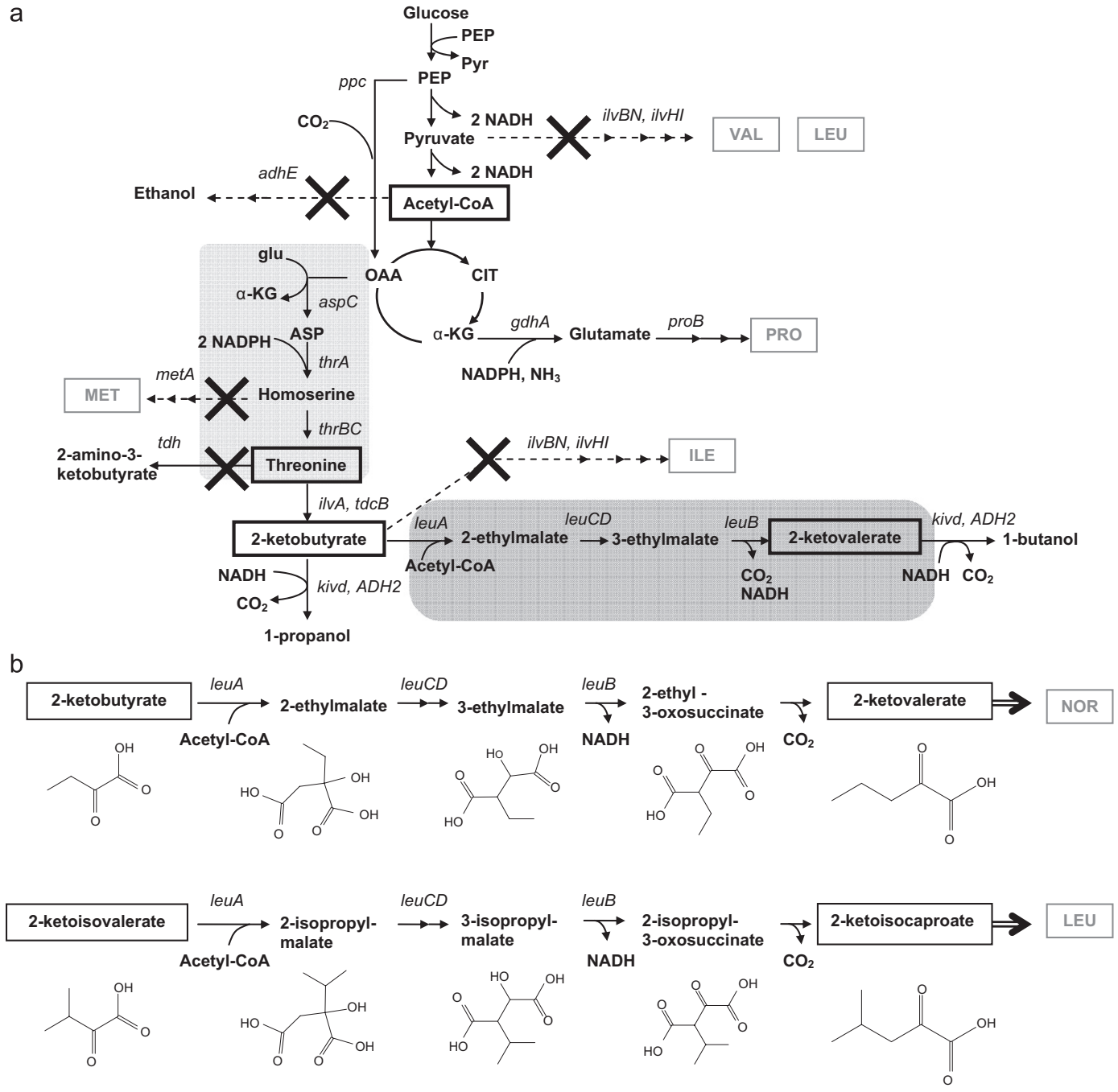

Fig. 1. (a) Schematic illustration of 1-propanol and 1-butanol production via the threonine and norvaline biosynthetic pathways in the genetically engineered *E. coli*. Disruptions of the specific pathways are indicated by the crosses. Rectangular boxes with thick lines are placed around the essential precursors for the alcohol production. The threonine pathway is shaded with light gray while the norvaline pathway is shaded with dark gray. Pathways leading to the biosynthesis of valine, leucine, isoleucine, proline and methionine are indicated by their corresponding abbreviations. (b) Reactions of 2-ketoacids catalyzed by the leucine biosynthetic enzymes LeuABCD. Top panel shows the synthesis of 1-butanol precursor 2-ketovalerate utilizing the unnatural norvaline pathway. Bottom panel shows the synthesis of leucine precursor 2-ketoisocaproate. 2-ketoisovalerate is the natural substrate for LeuA. The 2-ketoacids are enclosed by rectangular boxes.

In the proposed pathway, 2-isopropylmalate synthase (LeuA) is responsible for the Aldol addition of acetyl CoA to 2-ketobutyrate, which differs from its natural substrate 2-ketoisovalerate by a methyl group at the beta position (Fig. 1b). Then, Isopropylmalate isomerase, consisting of two subunits LeuC and LeuD, catalyzes the transfer of the hydroxyl group between adjacent carbons, converting 2-ethylmalate into 3-ethylmalate. Finally, oxidation and decarboxylation of 3-ethylmalate are performed by the metal-dependent 3-isopropylmalate dehydrogenase (LeuB) using NAD+ as the electron acceptor to yield 2-ketovalerate, NADH and CO_2. Instead of being transaminated into norvaline, the resulting 2-ketovalerate is subsequently turned into 1-butanol by Kivd and ADH2 (Fig. 1a).

In this work, we achieved co-production of 1-butanol and 1-propanol by metabolically engineering *E. coli*. This work demonstrates that the vast amount of knowledge accumulated for amino-acid hyper-productions can be readily transferred and applied to the production of higher alcohols such as 1-propanol

and 1-butanol using the 2-ketoacid pathways (Atsumi et al., 2008).

2. Materials and methods

2.1. Plasmid construction

pSA40, pSA55 and pSA62 were designed and constructed by S. Atsumi as described in Atsumi et al. (2008). The *lacI* gene was amplified with primers *lacI* SacI f and *lacI* SacI r from *E. coli* MG 1655 genomic DNA. The PCR product was then digested with SacI and ligated into the pSA55 open vector cut with the same enzyme behind the promoter of the ampicillin resistance gene, creating pSA55I.

WT *thrABC* was amplified by PCR using primers *thrA* f Acc65 and *thrC* r HindIII from *E. coli* BW25113 WT genomic DNA. The resulting product was digested with Acc65 and HindIII and cloned into pSA40 cut with the same pair of enzymes, creating pCS41.

To replace the replication origin of pCS41 from colE1 to pSC101, pZS24-MCS1 (Lutz and Bujard, 1997, NAR) was digested with *Sac*I and AvrII. The shorter fragment was gel purified and cloned into plasmid pCS41 cut with the same enzymes, creating pCS59.

The feedback resistant mutant (denoted by fbr) $thrA^{fbr}$ was amplified by PCR along with *thrB* and *thrC* from the genomic DNA isolated from the threonine over-producer ATCC 21277 using primers *thrA* f Acc65 and *thrC* r HindIII. The resulting product was digested with Acc65 and HindIII and cloned into pSA40 cut with the same pair of enzymes, creating pCS43.

To replace the replication origin of pCS43 from colE1 to pSC101, pZS24-MCS1 (Lutz and Bujard, 1997, NAR) was digested with SacI and AvrII. The shorter fragment was gel purified and cloned into plasmid pCS43 cut with the same enzymes, creating pCS49.

The gene *tdcB* was amplified with PCR using primers *tdcB* f Acc65 and *tdcB* r SalI from the genomic DNA of *E. coli* BW25113 WT. The resulting PCR product was gel purified and digested with Acc65 and SalI. The digested fragment was then ligated into the pSA40 open vector cut with the same pair of enzymes, creating pCS14.

To replace the replication origin of pCS14 from colE1 to p15A, pZA31-luc (Lutz and Bujard, 1997, NAR) was digested with SacI and AvrII. The shorter fragment was gel purified and cloned into plasmid pCS14 cut with the same enzymes, creating pCS16.

The operon *leuABCD* was amplified using primers A106 and A109 (Atsumi et al., 2008) and *E. coli* BW25113 WT genomic DNA as the template. The PCR product was cut with SalI and BglII and ligated into pCS16 digested with SalI and BamHI, creating pCS20.

To create an expression plasmid identical to pSA40 but with p15A origin, the p15A fragment obtained from digesting pZA31-luc with SacI and AvrII was cloned into pSA40 open vector cut with the same restriction enzymes, creating pCS27.

The $leuA^{fbr}$ G462D mutant (feedback resistant) (Gusyatiner et al., 2002) was constructed using Splice Overlap Extension (SOE) (Horton et al., 1989) with primers G462Df and G462Dr and the *E. coli* BW25113 WT genomic DNA as a template to obtain the $leuA^{fbr}BCD$ operon. Then the SOE product was digested and cloned into the restriction sites Acc65 and *Xba*I to create PZE_leuABCD (constructed by K. Zhang, unpublished data). The resulting plasmid was next used as a template to PCR out the $leuA^{fbr}BCD$ with primers A106 and A109 (Atsumi et al., 2008). The product was cut with SalI and BglII and ligated into pCS27 digested with SalI and BamHI, creating pCS48.

The gene *tdcB* from the genomic DNA of *E. coli* BW25113 WT was amplified with PCR using primers *tdcB* f Acc65 and *tdcB* r SalI. The resulting PCR product was gel purified, digested with Acc65 and SalI and then ligated into the pCS48 open vector cut with the same pair of enzymes, creating pCS50.

The gene *ilvA* was amplified from *E. coli* BW25113 WT genomic DNA with primers A110 and A112 (Atsumi et al., 2008). Next, it was cut with Acc65 and XhoI and ligated into the pCS48 open vector digested with Acc65 and SalI, creating pCS51.

DNA polymerase KOD for PCR reactions was purchased from EMD Chemicals (San Diego, CA). All restriction enzymes and Antarctic phosphatase were from New England Biolabs (Ipswich, MA). Rapid DNA ligation kit was obtained from Roche (Manheim, Germany). Oligonucleotides were ordered from Operon (Huntsville, AL). A list of oligonucleotides used is given in Table 1.

2.2. Media and cultivation

For all of the alcohol production experiments, single colonies were picked from LB plates and inoculated into 3 ml of LB media contained in test tubes with the appropriate antibiotics (ampicillin 100 μg/ml, kanamycin 50 μg/ml and spectinomycin 50 μg/ml). The overnight culture grown in LB at 37 °C in a rotary shaker (250 rpm) was then inoculated (1% vol/vol) into 20 ml of M9 medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 1 mM $MgSO_4$, 10 mg vitamin B_1 and 0.1 mM $CaCl_2$ per liter of water) containing 30 g/L glucose, 5 g/L yeast extract, appropriate antibiotics, and 1000 × Trace Metal Mix A5 (2.86 g H_3BO_3, 1.81 g $MnCl_2 \cdot 4H_2O$, 0.222 g $ZnSO_4 \cdot 7H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.079 g $CuSO_4 \cdot 5H_2O$, 49.4 mg $Co(NO_3)_2 \cdot 6H_2O$ per liter water) in 250 ml conical flask. The culture was allowed to grow at 37 °C in a rotary shaker (250 rpm) to an OD_{600} of 0.4–0.6, then 12 ml of the culture was transferred to a 250 ml screw capped conical flask and induced with 1 mM IPTG. The induced cultures were grown at 30 °C in a rotary shaker (240 rpm). Samples were taken throughout the next 3 to 4 days by opening the screwed caps of the flasks, and culture broths were either centrifuged or filtered to retrieve the supernatant. In some experiments as indicated, 8 g/L of threonine was added directly into the cell culture at the same time of induction. All antibiotics and reagents in media were purchased from either Sigma Aldrich (St. Louis, MO) or Fisher Scientifics (Houston, TX).

2.3. Bacterial strains

E. coli BW25113 ($rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$) was designated as the wild type (WT) (Datsenko and Wanner, 2000) for comparison. XL-1 Blue (Stratagene, La Jolla, CA) was used to propagate all plasmids. Construction of BW25113 F' (or JCL 16) was described in Atsumi et al. (2007) to supply the $lacI^q$. Host gene deletions of *metA*, *tdh*, *ilvB*, *ilvI* and *adhE* were achieved with P1 transduction using the Keio collection strains (Baba et al., 2006) as donor. The kan^R inserted into the target gene region was removed with pCP20 (Datsenko and Wanner, 2000) in between each consecutive knock out. Then, removal of the gene segment was verified by colony PCR using the appropriate primers.

2.4. Metabolite detections

The produced alcohol compounds were quantified by a gas chromatograph (GC) equipped with flame ionization detector. The system consisted of model 5890A GC (Hewlett-Packard, Avondale, PA) and a model 7673A automatic injector, sampler and controller (Hewlett-Packard). Supernatant of culture broth was injected in split injection mode (1:15 split ratio) using methanol as the internal standard. Detailed procedures are described in Atsumi et al. (2007).

For other secreted metabolites, filtered supernatant was applied (0.02 ml) to an Agilent 1100 HPLC equipped with an

Table 1
Strains, plasmids and oligonucleotides used

Strain	Genotype	Reference
BW25113	*rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR*514 Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Datsenko and Wanner (2000)
XL-1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}*ZΔM15 Tn10* (Tet^R)]	Stratagene
JCL 16	BW25113/F' [*traD36, proAB+, lacI*^{q} *ZΔM15* (Tet^R)]	Atsumi et al. (2007)
CRS 21	JCL16 Δ*metA*	This study
CRS 22	JCL16 Δ*metA*, Δ*tdh*	This study
CRS 23	JCL16 Δ*metA*, Δ*tdh*, Δ*ilvB*	This study
CRS 24	JCL16 Δ*metA*, Δ*tdh*, Δ*ilvB*, Δ*ilvI*	This study
CRS 31	JCL16 Δ*metA*, Δ*tdh*, Δ*ilvB*, Δ*ilvI*, Δ*adhE*	This study
CRS-BuOH 2	JCL16 Δ*metA*, Δ*tdh*+pCS49/pSA62/pSA55I	This study
CRS-BuOH 11	JCL16 Δ*metA*, Δ*tdh*, Δ*ilvB*, Δ*ilvI*+pCS49/pSA62/pSA55I	This study
CRS-BuOH 12	JCL16+pCS49/pSA62/pSA55I	This study
CRS-BuOH 18	JCL16 Δ*metA*, Δ*tdh*, Δ*ilvB*, Δ*ilvI*+pCS49/pCS51/pSA55I	This study
CRS-BuOH 19	JCL16 Δ*metA*, Δ*tdh*, Δ*ilvB*, Δ*ilvI*+pCS49/pCS20/pSA55I	This study
CRS-BuOH 20	JCL16 Δ*metA*, Δ*tdh*, Δ*ilvB*, Δ*ilvI*+pCS49/pCS50/pSA55I	This study
CRS-BuOH 23	JCL16 Δ*metA*, Δ*tdh*, Δ*ilvB*, Δ*ilvI*, Δ*adhE*+pCS49/pSA62/pSA55I	This study
CRS-BuOH 31	JCL16+pSA62/pSA55I	This study
CRS-BuOH 32	JCL16 Δ*metA*+pCS49/pSA62/pSA55I	This study
Plasmid	Genotype	Reference
pZA31-luc	$P_L tetO_1$:: *luc* (VF); p15A ori; Cm^R	Lutz and Bujard (1997)
pZS24-MCS1	$P_L lac/ara_1$:: MCS1; pSC101 ori; Kan^R	Lutz and Bujard (1997)
pCS20	$P_L lacO_1$:: *tdcB*(EC)-*leuABCD*(EC); p15A ori; Kan^R	This study
pCS27	$P_L lacO_1$:: MCS1; p15A ori; Kan^R	This study
pCS49	$P_L lacO_1$:: *thrA*^{fbr}*BC* (EC ATCC 21277); pSC101 ori; Spec^R	This study
pCS50	$P_L lacO_1$:: *tdcB* (EC)-*leuA*^{fbr}*BCD*(EC G462D mut); p15A ori; Kan^R	This study
pCS51	$P_L lacO_1$:: *ilvA* (EC)-*leuA*^{fbr}*BCD*(EC G462D mut); p15A ori; Kan^R	This study
pCS59	$P_L lacO_1$:: *thrABC* (EC); pSC101 ori; Spec^R	This study
pSA40	$P_L lacO_1$:: MCS1; ColE1 ori; Kan^R	Atsumi et al. (2008)
pSA55	$P_L lacO_1$:: *kivd* (LL)-*ADH2* (SC); ColE1 ori; Amp^R	Atsumi et al. (2008)
pSA55I	$P_L lacO_1$:: *kivd* (LL)-*ADH2* (SC), *lacI*; ColE1 ori; Amp^R	This study
pSA62	$P_L lacO_1$:: *ilvA* (EC)-*leuABCD*(EC); p15A ori; Kan^R	Atsumi et al. (2008)
Primer name	Sequence 5′→3′	Reference
lacI SacI f	CTAGAGCTCGAAGGAGATATACCATGAAACCAGTAACGTTATACGATG	This study
lacI SacI r	CTAGAGCTCTCACTGCCCGCTTTCCAGTC	This study
tdcB f Acc65	CGAGCGGTACCATGCATATTACATACGATCTGCCGG	This study
tdcB r SalI	ACGCAGTCGACTTAAGCGTCAACGAAACCGGTGATT	This study
thrA f Acc65	TCAGGTACCATGCGAGTGTTGAAGTTCGGCGGTACAT	This study
thrC r HindIII	TCAAAGCTTTTACTGATGATTCATCATCAATTTACGCAA	This study

auto-sampler (Agilent Technologies) and a BioRad (Biorad Laboratories, Hercules, CA) Aminex HPX87 column (0.5 mM H_2SO_4, 0.6 mL/min, column temperature at 65 °C). Glucose was detected with a refractive index detector while organic acids were detected using a photodiode array detector at 210 nm. Concentrations were determined by extrapolation from standard curves.

3. Results

3.1. Deregulation of threonine biosynthesis

As shown in Fig. 1a, 2-ketobutyrate and 2-ketovalerate are the precursor for 1-propanol and1-butanol production, respectively. In order to increase the pool of the rare metabolite 2-ketovalerate for 1-butanol synthesis, the gene *ilvA* and *leuABCD* from *E. coli* were over-expressed using plasmid pSA62 to (i) direct higher metabolic flux towards 2-ketobutyrate and (ii) utilize the norvaline synthetic chemistry as the major 2-ketovalerate production route (Fig. 1a). Kivd and ADH2 were also over-expressed from pSA55I to convert the two keto-acids into their corresponding alcohols. Over-expression of *ilvA* and *leuABCD* in addition to *kivd* and *ADH2* raised both the 1-propanol and 1-butanol levels by nearly five-fold in JCL 16, from a virtually non-detectable amount (data not shown) to about 60 and 30 mg/L respectively (Fig. 2a). The concentration of 1-butanol and 1-propanol plateaued after 24 h while isobutanol and ethanol continued to increase steadily.

To test whether threonine production was the bottleneck, 8 g/L threonine was added to the *E. coli* culture at induction. The result verified our hypothesis: the accumulated amount of propanol and butanol in the culture broth was raised to 2 g/L, which was about a 20-fold increase for both alcohols (Fig. 2b). Since transcription attenuation and allosteric feedback inhibition of ThrA by threonine are the major regulatory mechanisms (Szczesiul and Wampler, 1976; Debabov, 2003), expressing a feedback-resistant mutant of ThrA behind a non-native promoter might help deregulate threonine synthesis and therefore improve production of the downstream alcohols. A feedback resistant ThrA (designated as ThrA^{fbr}) was reported previously in the threonine hyperproducer ATCC 21277 (Shiio et al., 1971). Sequencing results indicated that the mutation on ThrA responsible for the feedback resistance occurs at the 1297th base pair, changing a glycine into an arginine. The *thrA*^{fbr}*BC* operon of this strain was then cloned and expressed from plasmid pCS49 under the control of $P_L lacO_1$ promoter. As a comparison, the WT *thrABC* operon was also cloned and expressed from pCS59 under $P_L lacO_1$. With the addition of ThrA^{fbr}BC over-expression, the production levels of both 1-propanol and 1-butanol were increased three- to four-fold higher relative to the case without ThrA^{fbr}BC (Fig. 2a). Strains with WT ThrABC over-expressed showed a 10–20% decrease in the two target alcohols (data not shown) compared to the levels produced by the same strains over-expressing ThrA^{fbr}BC (Figs. 2a and 5a), both in the JCL 16 background and in CRS 31 (JCL16 Δ*metA*, Δ*tdh*, Δ*ilvB*, Δ*ilvI*, Δ*adhE*). This result demonstrated that

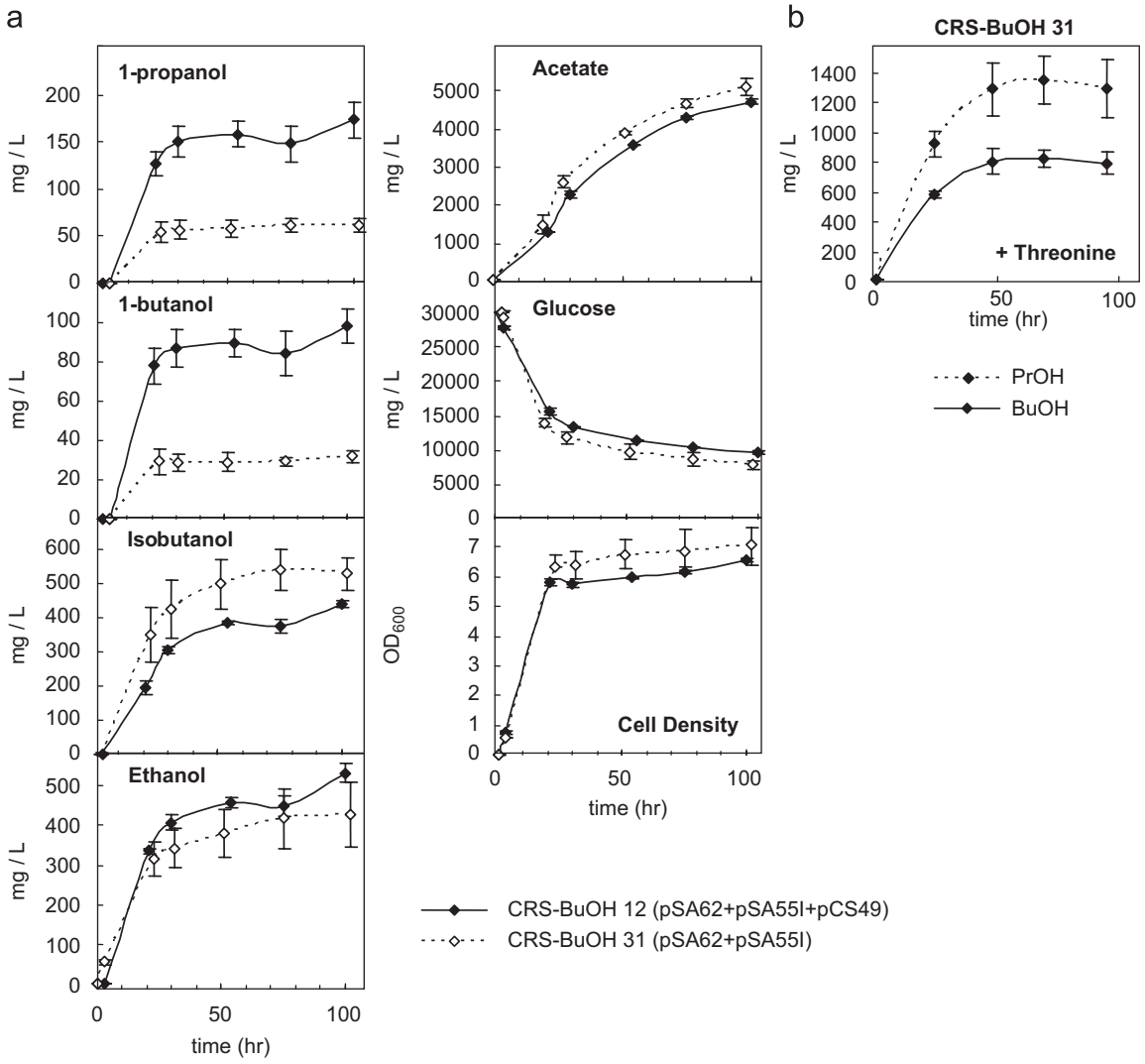

Fig. 2. Effects of ThrA^{fbr}BC over-expression and threonine addition on the production of target alcohols and major metabolites. Cells were cultured as described in Materials and methods. *X*-axis represents the time since inoculation of the overnight culture into 20 ml of fresh media. (a) Time courses of 1-propanol, 1-butanol and major by-products produced by CRS-BuOH 12 (filled diamond) and CRS-BuOH 31 (open diamond). Cell growth and concentrations of glucose present in the media are also shown. Both CRS-BuOH 12 and 31 are based on JCL 16. CRS-BuOH 31 contained pSA62 and pSA55I while CRS-BuOH 12 contained an additional plasmid pCS49 which carried the *thrA^{fbr}BC* behind $P_L lacO_1$. (b) 8 g/L of threonine was added to the culture of CRS-BuOH 31 at the time of induction. Solid line indicates butanol while dashed line represents propanol.

the intracellular threonine accumulation was minor but still affected the WT ThrA activity. As indicated by the decrease in isobutanol production (Fig. 2a), the presence of ThrA^{fbr}BC helped direct the metabolic flux more towards the threonine pathway, thus improved the overall 1-propanol and 1-butanol productivity.

3.2. Elimination of competing pathways

To further increase the propanol and butanol production titer, genes involved in the competing side reactions were removed to avoid the consumption or degradation of the desired intermediates. Since threonine production is the major limitation in the synthesis of 2-ketobutyrate, homoserine *O*-succinyltransferase *metA* and threonine dehydrogenase *tdh* were first deleted to minimize the loss of desired precursors to methionine biosynthesis and to block the catabolism of threonine into 2-amino-3-ketobutyrate. This approach has been employed in the threonine hyper-production (Debabov, 2003). With *metA* and *tdh* disrupted, the combined production of 1-propanol and 1-butanol increased to about 1.2 g/L as seen from Fig. 3a, with the major contribution primarily coming from 1-propanol. Less significant effect of these two gene deletions was seen on 1-butanol production.

We hypothesized that the low improvement in 1-butanol production was attributable to the divergence of 2-ketobutyrate into the isoleucine pathway and/or the availability of acetyl-CoA. To further conserve acetyl-CoA and 2-ketobutyrate, the two key precursors for 1-butanol formation, the first enzymatic reaction in the biosynthesis of valine, leucine and isoleucine was disrupted. Elimination of the larger catalytic subunit (coded by *ilvB*) of the acetohydroxy acid synthase isozyme (AHAS I) and the catalytic subunit (coded by *ilvI*) of the AHAS III led to auxotroph of the above amino acids (Pátek, 2007). These two additional deletions resulted in a two-fold increase in the 1-butanol production (Fig. 3a) while 1-propanol level remained unchanged. It also nearly abolished the production of isobutanol, 3-methyl-butanol and 2-methyl-butanol by removal of their precursors. The minute accumulation of isobutanol might have been resulted from the reverse reaction in the last step of valine synthesis catalyzed by

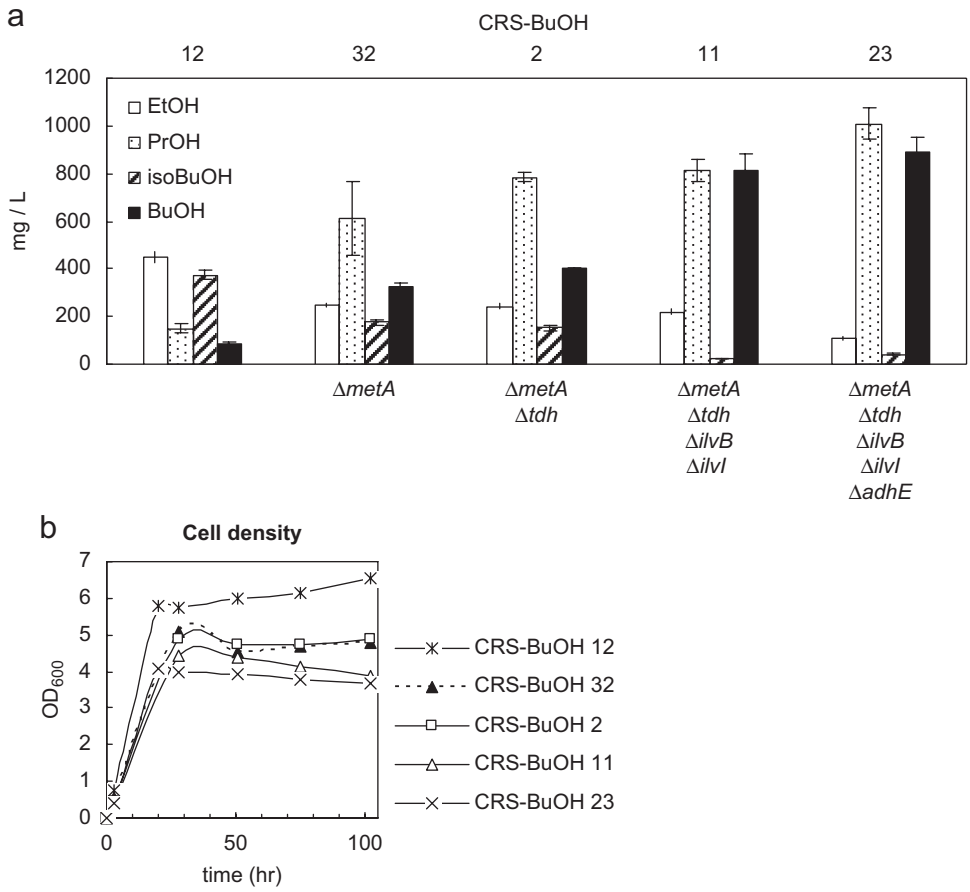

Fig. 3. Comparison of alcohol productions in various knock-out strains. Cells were cultured for 72 h as described in Materials and methods. (a) Δ indicates gene deletions. All strains contained the same set of plasmids pCS49, pSA62 and pSA55I. (b) Growths for each of the production strains shown in (a).

IlvE, which took valine present in the media (supplemented with yeast extract) and converted it back into 2-ketoisovalerate (Kuramitsu et al., 1985).

To reduce ethanol production, we deleted the *E. coli adhE* gene. Although disruption of *adhE* did not improve the overall C3 and C4 alcohol production much, it did increase the specificity by lowering ethanol formation from 0.25 g/L down to approximately 0.1 g/L. With these genes eliminated from the genome, the final strain (JCL 16 Δ*metA*, Δ*tdh*, Δ*ilvB*, Δ*ilvI*, Δ*adhE*) showed a near 1:1 co-production of 1-propanol and 1-butanol with minor accumulation of ethanol and insignificant levels of isobutanol and the methyl-butanols (< 50 mg/L).

3.3. Assessment of alternative feedback resistant enzymes

Transcriptional attenuation, one of the major mechanisms in amino-acid regulation, had minimal effect on the key enzymes here since all essential genes were cloned and expressed behind a non-native promoter without the leader sequence. On the other hand, allosteric feedback inhibition of the enzymes by their own amino-acid products could not be neglected, specifically IlvA (Eisenstein, 1991) and LeuA (Pátek, 2007). TdcB, *E. coli*'s catabolic threonine dehydratase, provided an alternative to IlvA for catalyzing the deamination of threonine into 2-ketobutyrate while being naturally insensitive to isoleucine feedback inhibition (Umbarger and Brown, 1957). In addition, it has recently been shown to be an excellent candidate for isoleucine hyper-production (Guillouet et al., 1999). To assess the benefit of this alternative enzyme towards our production, *tdcB* was over-expressed behind $P_L lacO_1$ with *leuABCD* on pCS20. Results showed

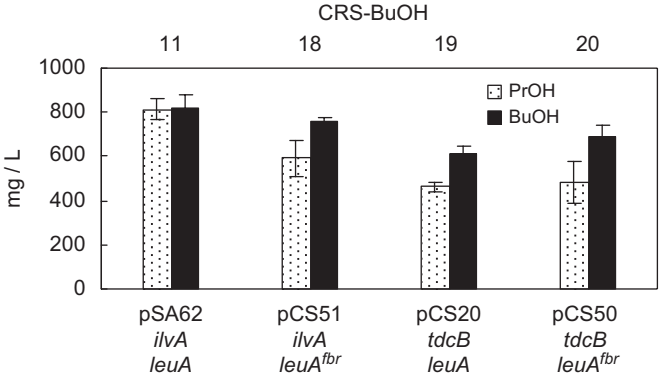

Fig. 4. Comparison of 1-propanol and 1-butanol production using alternative feedback resistant threonine dehydratase and 2-isopropylmalate synthase Cells were cultured for 72 h as described in Materials and methods. CRS 24 (JCL16 Δ*metA*, Δ*tdh*, Δ*ilvB*, Δ*ilvI*) was used as the root strain for the comparison. All strains contained pSA62 and pSA55I in addition to the plasmids indicated below the figure. Gene names of the specific threonine dehydratase and 2-isopropylmalate synthase expressed on the plasmids are listed below the plasmid number.

that TdcB led to a 30% lower production in each of the two target alcohols compared to IlvA (Fig. 4). It is possible that the minute amount of isoleucine brought about by the addition of yeast extract was insignificant to inhibit IlvA enzymatic activity. As a result, the insensitivity towards feedback inhibition of TdcB became less important than the activity of the enzyme itself under the given experimental condition.

Similarly, suspicions of feedback inhibition on LeuA by leucine present in the yeast extract led to the construction and testing of the *leuA* feedback insensitive mutant G462D (Gusyatiner et al., 2002), designated as *leuA*^{fbr}. The point mutation on *leuA*^{fbr} was introduced by site-directed mutagenesis using SOE and the resulting operon *leuA*^{fbr}*BCD* was over-expressed on plasmid pCS51. As shown in Fig. 4, the feedback insensitive LeuA^{fbr} failed to increase the production of 1-propanol and 1-butanol. Again, this demonstrated that the amount of leucine present in the cells was probably below the inhibitory level to cause an adverse effect on the LeuA enzymatic activity.

3.4. Time course of 1-propanol and 1-butanol production

Time courses of the alcohol and metabolite productions in the final strain CRS-BuOH 23 (JCL 16 Δ*metA*, Δ*tdh*, Δ*ilvB*, Δ*ilvI*, Δ*adhE*/pCS49, pSA62 and pSA55I) are shown in Fig. 5. Both propanol and butanol production steadily increased in almost a linear fashion throughout the 72 h period and appeared to plateau by the end of the third day. The same behavior was also observed in the ethanol production. Ethanol formation in the Δ*adhE* background might be due to the slight affinity of Kivd towards pyruvate. On the other hand, extracellular levels of the major metabolites acetate and lactate continued to increase significantly after the alcohol production period, which might be a result of excess acetyl-CoA and NADH, respectively. As seen in Fig. 5d, major consumption of glucose occurred in the alcohol production period and appeared to be independent of growth alone. After the first 24 h, cells stopped growing and remained somewhat stationary during the next few days of alcohol production.

4. Discussion

Biofuel production utilizing the organism's native amino-acid pathways offers several advantages over the existing methods.

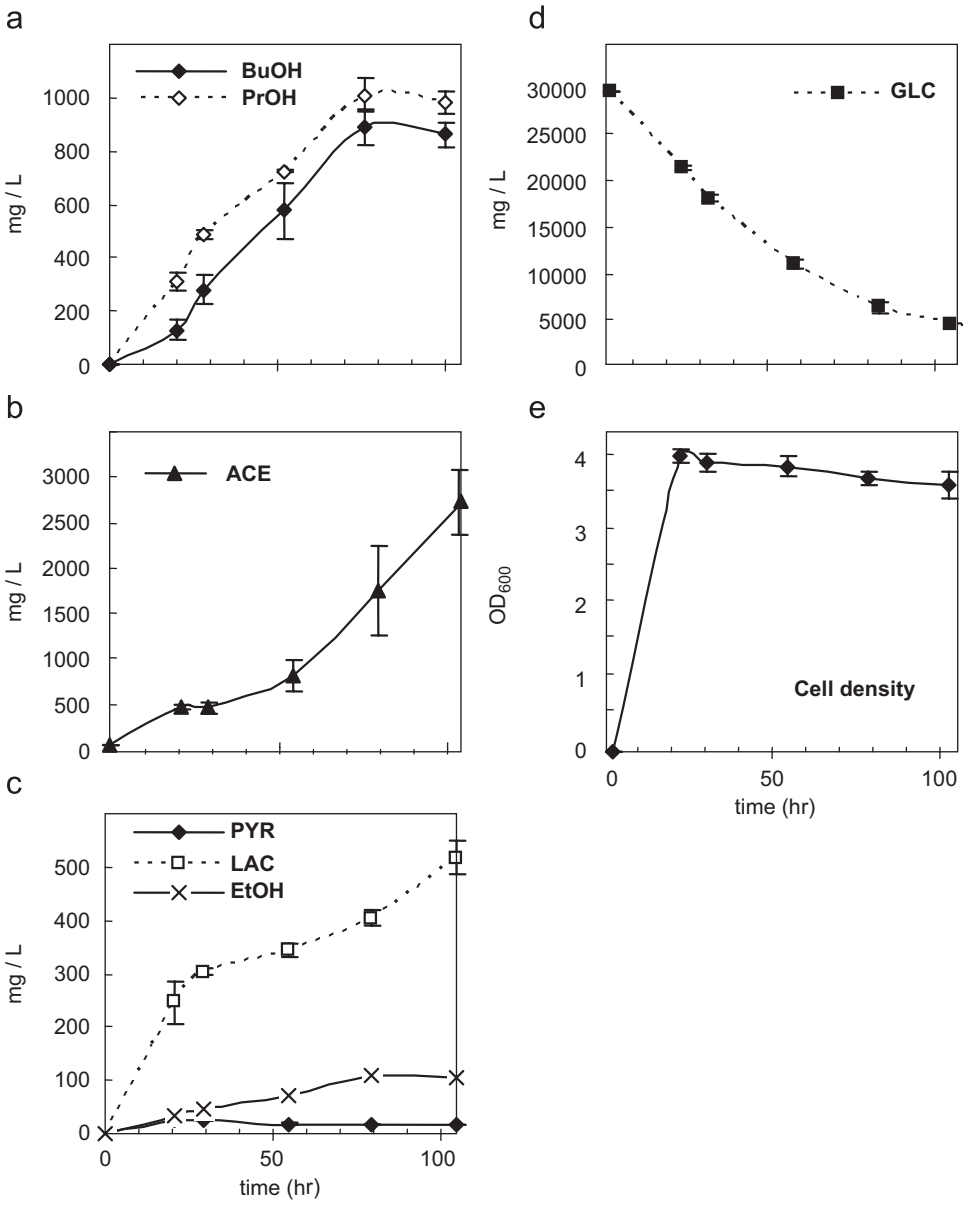

Fig. 5. Production of 1-propanol, 1-butanol and the metabolic by-products in CRS-BuOH 23. Cells were cultured as described in Materials and methods. *X*-axis represents the time since inoculation of the overnight culture into 20 ml of fresh media. (a) Production of 1-propanol and 1-butanol. Filled diamonds represent butanol and opened diamonds indicate propanol. (b) Production of the by-product acetate. (c) Production of by-products pyruvate, lactate and ethanol. Filled diamond, opened square and cross represent pyruvate, lactate and ethanol respectively. (d) Glucose concentration in the media. (e) Growth of CRS-BuOH 23.

In contrast to the butanol production pathway found in many species of *Clostridium*, our engineered amino-acid biosynthetic routes for biofuel production circumvent the need to involve CoA-dependent intermediates. Intracellular accumulation of poorly metabolized xenobiotic acyl-CoA is known to cause metabolic toxicity in the host by sequestering the supply of CoA that is essential for protein synthesis and growth (Brass, 1994). Therefore, a more host-friendly biofuel production system utilizing the organism's native metabolites in the amino-acid biosynthetic pathway is desirable.

Upon over-expression of *kivd* (*L. lactis*), *ADH2* (*S. cerevisiae*), and the *E. coli ilvA*, *leuABCD*, *thrA*^{fbr}*BC*, the engineered *E. coli* strain produces 1-butanol and 1-propanol. The production of 1-butanol requires 2-ketovalerate, whose formation involves 2-ketobutyrate and the unnatural norvaline biosynthetic pathway. Dependence of 2-ketovalerate synthesis on the leucine biosynthetic enzymes LeuABCD was confirmed in this work by the lack of 1-butanol production in the Δ*leuABCD* strain (data not shown). Competition of the essential intermediate 2-ketobutyrate exists between Kivd and LeuA, which respectively leads to the formation of 1-propanol and the 1-butanol precursor 2-ketovalerate (Fig. 1a). Since Kivd has similar affinity towards both 2-ketoacids (Atsumi et al., 2008), and 2-ketobutyrate is an unnatural substrate for LeuA (Bogosian et al., 1989), 1-propanol was co-produced with 1-butanol in similar amounts. Deregulation of the threonine biosynthesis and removal of the diverging pathways catalyzed by *metA* and *tdh* successfully improved both 1-propanol and 1-butanol production titer. Lysine biosynthesis, on the other hand, although also branches off from the threonine pathway, was not eliminated in this study. Main progress in the 1-butanol productivity was made upon interruption of the valine, leucine and isoleucine biosynthesis pathway by inactivating AHAS I and III, which led to a two-fold increase in 1-butanol but insignificant effect on 1-propanol. This selective improvement was attributed to (i) the increase in the availability of 2-ketobutyrate and acetyl-CoA, (ii) the release of the essential enzymes LeuABCD from their natural substrates in the leucine pathway, and (iii) relief of IlvA and LeuA from possible feedback inhibition by elimination of the leucine and isoleucine biosynthesis.

The lack of improvement using the alternative enzymes TdcB and LeuA^{fbr} raised the possibility that the intracellular levels of leucine and isoleucine were low in CRS24. TdcB, the biodegradative threonine dehydratase, is activated by its allosteric effector AMP by decreasing its K_m for threonine (Hirata et al., 1965; Simanshu et al., 2007). Since high concentrations of pyruvate and some 2-ketoacids (including 2-ketobutyrate) were shown to inactivate TdcB enzymatically (Feldman and Datta, 1975), build-up of these intermediates might have had detrimental impact on its enzymatic activity when there was not enough AMP in the cells to counteract the negative effect. Also, the higher K_m value for threonine associated with TdcB than IlvA (Hirata et al., 1965; Eisenstein, 1991; Simanshu et al., 2007) in the absence of significant intracellular AMP level could result in a slower deamination rate, thus contribute to the poorer performance on the overall alcohol production as seen in Fig. 4 ($K_m = 8$ mM for purified *E. coli* IlvA in the absence of isoleucine and 20 mM for *E. coli* TdcB in the absence of AMP). The lack of effect observed in the leucine-feedback insensitive mutant LeuA^{fbr} (G462D) may be attributed to a possible decrease in its affinity towards 2-ketobutyrate due to the mutation. However, the detailed cause remains to be elucidated.

In this work, we have successfully demonstrated the potential of C3 and C4 alcohol production using the microorganism's native amino-acid biosynthetic pathway. Through successive streamlining of the metabolic network, improvement of 1-butanol and 1-propanol production was obtained. Increasing strain tolerance to 1-propanol and 1-butanol using novel and existing methods (Borden and Papoutsakis, 2007; Alper et al., 2006; Yomano et al., 1998) may be essential towards higher productivity. Elimination of the fermentative by-products and further optimization of culture conditions may also be important. Although the combined butanol and propanol production is still sub-optimal, it is believed that through further strain improvement, bioreaction optimization, and toxicity tolerance, high productivity and selectivity of each biofuel can be achieved using the amino-acid biosynthetic pathway.

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