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ORIGINAL RESEARCH PAPER

Activation of alternative metabolic pathways diverts carbon flux away from isobutanol formation in an engineered *Escherichia coli* strain

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Abstract
Objective Metabolic engineering efforts are guided by identifying gene targets for overexpression and/or deletion. Isobutanol, a biofuel candidate, is biosynthesized using the valine biosynthesis pathway and enzymes of the Ehrlich pathway. Most reported studies for isobutanol production in *Escherichia coli* employ multicopy plasmids, an approach that suffers from disadvantages such as plasmid instability, increased metabolic burden, and use of antibiotics to maintain selection pressure. Cofactor imbalance is another issue that may limit production of isobutanol, as two enzymes of the pathway utilize NADPH as a cofactor.
Results To address these issues, we constructed *E. coli* strains with chromosomally-integrated, codon-optimized isobutanol pathway genes (*ilvGM*, *ilvC*, *kivd*, *adh*) selected on the basis of their cofactor preferences. Genes involved in diverting pyruvate flux toward fermentation byproducts were deleted. Metabolite analyses of the constructed strains revealed extracellular accumulation of significant amounts of isobutyraldehyde, a pathway intermediate, and the overflow metabolites 2,3-butanediol and acetol.
Conclusions These results demonstrate that the genetic modifications carried out led to activation of alternative pathways that diverted carbon flux toward formation of unwanted metabolites. The present study highlights how precursor metabolites can be metabolized through enzymatic routes that have not been considered important in previous studies due to the different strategies employed therein. The insights gained from the present study will allow rational genetic modification of host cells for production of metabolites of interest.

Keywords *E. coli* · Isobutanol · Genome integration · Alternative metabolic pathways · Overflow metabolites

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Introduction

Isobutanol (2-methyl-1-propanol) is a four-carbon alcohol that is a potential biofuel candidate. It is a superior biofuel compared to ethanol because of its higher energy content, lower vapour pressure, lesser hygroscopicity and greater blend volume (Atsumi and Liao 2008; Tashiro et al. 2015; Yan and Liao 2009). Apart from its use in the transportation and biofuel sector, isobutanol also serves as a precursor for the production of a variety of polymers, paints, plastics and synthetic rubber (Kolodziej and Scheib 2012).

Isobutanol production using engineered *Escherichia coli* was first reported by Atsumi et al. (2008), who exploited the host's native branched-chain amino acid (BCAA) biosynthesis pathways for higher alcohol synthesis (Fig. 1). A number of studies have since reported using different approaches to produce isobutanol in *E. coli* at high yields and titres (Atsumi et al. 2010; Baez et al. 2011; Bastian et al. 2011; Huo et al. 2011; Liu et al. 2016; Shi et al. 2013; Trinh et al. 2011). Most approaches for isobutanol bioproduction utilize plasmids to overexpress genes of the isobutanol biosynthesis pathway. It is advantageous to express genes directly from the chromosome due to drawbacks such as segregational instability and copy-number effects associated with plasmid-based systems (Ajikumar et al. 2010; Lemuth et al. 2011; Sabido et al. 2013). Apart from a few reports (Akita et al. 2015; Trinh et al. 2011), most studies have employed plasmids to overexpress enzymes, which is not feasible for scale up due to the disadvantages associated with using plasmids (Atsumi et al. 2008; Baez et al. 2011; Savrasova et al. 2011).

The isobutanol pathway is cofactor imbalanced under anaerobic conditions as two enzymes of the pathway, acetohydroxy isomeroreductase (AHAIR) and some alcohol dehydrogenases (ADHs), utilize NADPH as cofactor (Fig. 1). A number of studies have focused on addressing this imbalance by increasing cofactor availability either by using enzymes engineered using directed evolution (Bastian et al. 2011) or by employing expression of NADPH-generating pathways (Liu et al. 2016; Shi et al. 2013; Trinh et al. 2011). However, overexpression of membrane-bound transhydrogenases such as PntAB is known to exert metabolic burden on the cell (Chin et al. 2009; Kabus et al. 2007).

The present study sought to address the above concerns by integrating genes of the isobutanol pathway in the *E. coli* genome and by expressing NADH-utilizing heterologous AHAIR and native ADH isozymes. The plasmid-free strains thus constructed were found to produce isobutyraldehyde, the immediate precursor of isobutanol, as well as metabolites that arise from pathways that branch off at various nodes of the glycolytic and BCAA pathways. Carbon

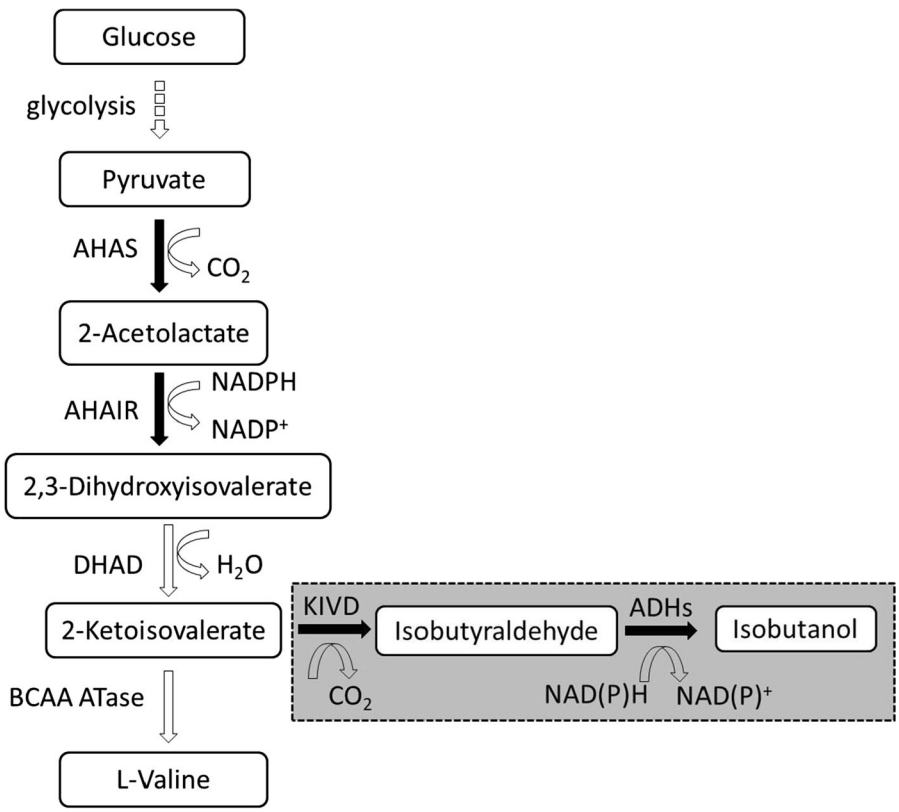

Fig. 1 Non-native biochemical pathway used for production of isobutanol in *E. coli*. Enzymatic reactions in the grey box divert flux from 2-ketoisovalerate toward isobutanol. BCAA ATase, branched-chain amino acid aminotransferase. Enzymes overexpressed in this study are indicated by solid arrows

flux through these pathways has not been demonstrated in earlier studies to divert flux from isobutanol formation, illustrating the role of alternative pathways in impacting production of metabolites of interest.

Materials and methods

Strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Primers used are listed in Table 2. All plasmids were maintained in *E. coli* DH5α. Genetic manipulations were carried out in *E. coli* BW25113. *E. coli* strains were routinely cultivated in LB medium at 37 °C. M9 minimal medium contained 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 1 ml of 1 M $MgSO_4$ and 0.1 ml of 1 M $CaCl_2$ (per litre). Antibiotics were used at the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 25 μg/ml. L-Rhamnose and L-arabinose were used at concentrations of 1 mM and 0.2%, respectively. For screening of integrants in the *melAB* and *mtlADR* loci, melibiose and D-mannitol were added to MacConkey agar base at a final concentration of 1%, respectively.

Table 1 List of bacterial strains and plasmids used

Strains and plasmids	Genotype/relevant characteristics	Reference
Bacterial strains		
DH5α	F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ80*dlacZ*ΔM15 Δ*(lacZYA-arg F)U169, hsdR17*(r_K^- m_K^+), λ^-	Hanahan (1983)
BW25113	F^- λ^- *lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Datsenko and Wanner (2000)
B wild type	F^-	Coli Genetic Stock Center, #5365
E. coli Δ*frsA*	BW25113 Δ*frsA*	This study
E. coli Δ5	BW25113 Δ*frsA* Δ*ldhA* Δ*ackA* Δ*frdBC* Δ*adhE*	This study
E. coli Δ51	*E. coli* Δ5 *melAB::rhaP*$_{BAD}$*-ilvGM*	This study
E. coli Δ52	*E. coli* Δ51 *ilvC::T. phaeum ilvC*	This study
E. coli Δ54	*E. coli* Δ51 *mltADR::rhaP*$_{BAD}$*-kivd*	This study
E. coli Δ55	*E. coli* Δ52 *mltADR::rhaP*$_{BAD}$*-kivd*	This study
E. coli Δ54.P	*E. coli* Δ54 P_{adhP}*::rhaP*$_{BAD}$	This study
E. coli Δ54.K	*E. coli* Δ54 P_{yahK}*::rhaP*$_{BAD}$	This study
E. coli Δ54.A	*E. coli* Δ54 P_{dkgA}*::rhaP*$_{BAD}$	This study
E. coli Δ54.O	*E. coli* Δ54 P_{fucO}*::rhaP*$_{BAD}$	This study
E. coli Δ54.D	*E. coli* Δ54 P_{yqhD}*::rhaP*$_{BAD}$	This study
E. coli Δ55.P	*E. coli* Δ55 P_{adhP}*::rhaP*$_{BAD}$	This study
E. coli Δ55.K	*E. coli* Δ55 P_{yahK}*::rhaP*$_{BAD}$	This study
E. coli Δ55.A	*E. coli* Δ55 P_{dkgA}*::rhaP*$_{BAD}$	This study
E. coli Δ55.O	*E. coli* Δ55 P_{fucO}*::rhaP*$_{BAD}$	This study
E. coli Δ55.D	*E. coli* Δ55 P_{yqhD}*::rhaP*$_{BAD}$	This study
Plasmids		
pKD46	Amp^R; temperature-sensitive replication, λ Red recombinase expression	Datsenko and Wanner (2000)
pCP20	Amp^R and Cm^R; temperature-sensitive replication, thermal induction of FLP recombinase synthesis	Cherepanov and Wackernagel (1995)
pSD111	Kan^R; genomic integration plasmid, T7 promoter	Deb et al. (2016)
pSD112	Kan^R; genomic integration plasmid, *rhaP*$_{BAD}$ promoter	Deb et al. (2016)
pSD212	Kan^R and Cm^R; promoter replacement plasmid, *rhaP*$_{BAD}$ promoter	Deb et al. (2016)

Table 2 List of primers used

Primer	Sequence
Primers for gene deletion	
frsA-del-N[a]	*AGTTTCCAGTAAGTATTCTGGAGGCTGCATCCATGACACAGGCAAACCTG*GTGTAGGCTGGAGCTGCTTC
frsA-del-C	*AATTTAGCAAATTTTTAACACAAGCGTTTTTCGATCCAGTCGGTGATTTC*CATATGAATATCCTCCTTA
ackA-del-N	*TGGCTCCCTGACGTTTTTTTAGCCACGTATCAATTATAGGTACTTCCATG*ATTCCGGGGATCCGTCGACC
pta-del-C	*GCAGCGCAAAGCTGCGGATGATGACGAGATTACTGCTGCTGTGCAGACTG*TGTAGGCTGGAGCTGCTTCG
ldhA-del-N	*TATTTTTAGTAGCTTAAATGTGATTCAACATCACTGGAGAAAGTCTTATG*ATTCCGGGGATCCGTCGACC
ldhA-del-C	*CTCCCCTGGAATGCAGGGGAGCGGCAAGATTAAACCAGTTCGTTCGGGCA*TGTAGGCTGGAGCTGCTTCG
frdB-del-N	*GGATGCAGCCGATAAGGCGGAAGCAGCCAATAAGAAGGAGAAGGCGAATG*ATTCCGGGGATCCGTCGACC
frdC-del-C	*TTGGATTTGGATTAATCATCTCAGGCTCCTTACCAGTACAGGGCAACAAA*TGTAGGCTGGAGCTGCTTCG
adhE-del-N	*CGAGCAGATGATTTACTAAAAAAGTTTAACATTATCAGGAGAGCATTATG*ATTCCGGGGATCCGTCGACC
adhE-del-C	*CCGTTTATGTTGCCAGACAGCGCTACTGATTAAGCGGATTTTTTCGCTTT*TGTAGGCTGGAGCTGCTTCG
Primers for confirmation of gene deletion	
conf-frsA-N	CGCACTTTGTCACCATCTTC
conf-frsA-C	GTGCCGGTTTCACGTTGAC
conf-ackA-N	CAGGTATCCTTTAGCAGCCTG
conf-pta-C	GATTCAGTGATTGCGGACATAG
conf-ldhA-N	GCATTCAATACGGGTATTGTG
conf-ldhA-C	GGCGTAACAGCAATTTTGTC
conf-frdB-N	GACGTCAACTTCCTCAAAC
conf-frdC-C	GCATACGGTGTAAACCACAC
conf-adhE-N	GGATCACGTAATCAGTACCC
conf-adhE-C	GTGTTCTGCAAATAGTTGTGC
Primers for ilvGM SOE PCR and cloning in template plasmid pSD112	
fwd_GM (NdeI)[b]	GGAATTCCATATGAATGGCGCACAGTGGGT
rev_GM1_SOE	ACTGGCGTGTGGTGCGAAG
fwd_GM2_SOE	CTGAACACCTTCGCACCACACGCCAGTGTTATCCACATGGATATCGAC
rev_GM (SacI)	CCGAGCTCTCAGGCGCGGATTTGTTGT
Primers for genome integration in melAB locus	
fwd_mel_int	*CGGCGCATATTGCCCTGATGGACATTGACCCCACCCGCCTGGAAGAGTCGCATATTGT*GAACATAATGGTGCAGGGCGC
rev_mel_int	*AGCGCAACGATGGCTTTAAGTGTCAGATGGCTTCCTTCAGCAGACGGTTGATTGTCTG*ATCCGGATATAGTTCCTCCTTTC
Primers for replacement of native ilvC	
fwd_CTP_rep	*CTCGCAAACGCGAACCGAACAATAAGAAGCACAACATCACGAGGAATCACC*ATGAAAATCTATTATGACCAAG
rev_CTP_rep	*GTTTTCTCCCTCTCCCTGTGGGAGAGGGTCGGGGTGAGGGCATCAGCGCGCAC*ATCCGGATATAGTTCCTCCTTTC
fwd_CIA_rep	*CTCGCAAACGCGAACCGAACAATAAGAAGCACAACATCACGAGGAATCACC*ATGGCGAAAATCTACAAAGATG
rev_CIA_rep	*GTTTTCTCCCTCTCCCTGTGGGAGAGGGTCGGGGTGAGGGCATCAGCGCGCAC*ATCCGGATATAGTTCCTCCTTTC

Table 2 continued

Primer	Sequence
Primers for confirmation of ilvC replacement	
conf_C_rep_f	CGGCTTTCCGCCAGATGC
conf_C_rep_r	CAGATGGTTCCGGCGTTCG
Primers for cloning kivd in template plasmid pSD112	
fwd_KLL (NdeI)	GGAATTCCATATGTATACGGTGGGTGACTACCTGCT
rev_KLL (SacI)	CCGAGCTCTTAGCTTTTGTTTTGTTCCGCAAACAG
Primers for genome integration in mtlADR locus	
fwd_mtl_int	*CCGCGTTATTTATTCCAACAGGGTGGTTACCGAACGAGACGCTGGCGAAGCTGGTC*GAACATAATGGTGCAGGGCGC
rev_mtl_int	*GCTGAATTTGCATTGCATACAAACTGGAGTCTGCTGGTTCAAACTGTGGCGGCGG*ATCCGGATATAGTTCCTCCTTTC
Primers for promoter replacement of native alcohol dehydrogenases	
fwd_adhP_PR	*CCTCCATCTAAGCCTGCGCCCGGTAGTGAAGGCTACCGGGCTATTTCCCTCTCCCTTTT*CATTCTGCTAACCAGTAAGG
rev_adhP_PR	*GCGCAGTGTTTTATACGTAACGTCAACATGATGATCCTTCGTAACAACTGCAGCCTT*CATATGTATATCTCCTTCTTATA
fwd_yahK_PR	*CGCATTTGAAACGCCTGCAACGGTGAATAGTAAGAGATTTAAGCCCCAGGGAGTGGGGCA*CATTCTGCTAACCAGTAAGG
rev_yahK_PR	*GTGATATCCATCGGTTCAAGTGGTTGTTTAGCGGAATATGCACCAACAGCTTTGATCTT*CATATGTATATCTCCTTCTTATA
fwd_dkgA_PR	*GTTGGATGTCAGCCGCCGTATATACGAAGCCGCCCGCTAAGCTTTTTACGCCTCAAACTTT*CATTCTGCTAACCAGTAAGG
rev_dkgA_PR	*CAGTCCCAGCTGGGGCATGACATTGCCATCCTGTAGCTTAATAACGGTTGGATTAGC*CATATGTATATCTCCTTCTTATA
fwd_fucO_PR	*CTTAACCGATCGATCCCCGCTATTCACTACATGATTGCGGCGGCTGGCGGTAATTCTATTC*CATTCTGCTAACCAGTAAGG
rev_fucO_PR	*GTTAAAGCCCCAACAGCACCCCGACCAAACCATGCCGTTTCGTTCAGAATCATTCTGTTAGC*CATATGTATATCTCCTTCTTATA
fwd_yqhD_PR	*GATTTTATGCCCGGAAAAGAGAATTATGATGCCAGGCTCGTACATCACCGGTGTACGTGCC*ATTCTGCTAACCAGTAAGG
rev_yqhD_PR	*CAGCGATTGCGCCTTTACCAAACAGAATGCGGGTTGGGGTGTGCAGATTAAAGTTGTT*CATATGTATATCTCCTTCTTATA
Primers for confirmation of promoter replacement of native alcohol dehydrogenases	
conf_P_rep_f	CATTTTGCCAACCGCAAACGC
conf_P_rep_r	GCGAAGCCCTGCTGAAAATGG
conf_K_rep_f	GACGCCAGCTTTGTGCTGG
conf_K_rep_r	CTACCTGATCACCAACGGCTACC
conf_A_rep_f	GCTCCATCCCGGCTTTGC
conf_A_rep_r	CGCGGGGGCGCTTGTGGTCG
conf_O_rep_f	GCGTTGGCATCCGGTCTG
conf_O_rep_r	GTGACCGATAAGATGGATGCTGCAG
conf_D_rep_f	GACATTGAGACGCAACCCTG
conf_D_rep_r	CTCAATACCGCCAAATTCCAG

[a]Homology extensions are in italics

[b]Restriction sites are underlined

Gene deletion

The *frsA*, *ackA-pta*, *ldhA*, *frdBC* and *adhE* genes were sequentially deleted from the *E. coli* BW25113 genome using the one-step gene inactivation protocol (Datsenko and Wanner 2000) to construct *E. coli* Δ5. Briefly, the disruption cassette from template plasmid pKD13 (Datsenko and Wanner 2000), comprising of an FRT-flanked kanamycin resistance gene, was amplified using primers that included 50 nt homology extensions upstream and downstream of the genes targeted for deletion. The purified PCR products were electroporated in *E. coli* BW25113 carrying the pKD46 Red helper plasmid (Datsenko and Wanner 2000). Kanamycin-resistant transformants were tested using PCR to confirm gene deletion, and the FRT-flanked disruption cassette was eliminated by transforming mutants with plasmid pCP20 (Cherepanov and Wackernagel 1995) and incubating at 42 °C for Flp-mediated elimination of the kanamycin resistance gene.

Cloning and genome integration of *ilvGM*

The gene coding for acetohydroxy acid synthase (AHAS) II, *ilvGM*, was amplified from the *E. coli* B genome (GenBank accession CP000819.1) using Pfu DNA polymerase (Thermo Scientific, USA). Splice overlap extension (SOE) PCR was carried out using primers that carried a silent mutation to remove an internal NdeI site from the *E. coli* B *ilvGM* gene. The amplified SOE PCR product was cloned in template plasmid pSD112 (Deb et al. 2016) to construct plasmid pSD112-ilvGM. The genome integration cassette from pSD112-ilvGM was amplified using primers for integration within the *melAB* locus, and the purified PCR product was electroporated in *E. coli* Δ5 transformed with plasmid pKD46 to construct *E. coli* Δ51. Transformants were selected on MacConkey agar plates containing kanamycin and melibiose (Albermann et al. 2010), and the FRT-flanked kanamycin resistance gene was eliminated as described above.

To confirm expression of AHAS, *E. coli* Δ5 (control) and Δ51 were cultivated in LB medium at 37 °C in an orbital shaker (180 rpm). When cultures reached $OD_{600} \sim 0.6$, L-rhamnose was added at a final concentration of 0.2%, and incubation was allowed to continue overnight. The next day, cell-free supernatant was analyzed by the Voges-Proskauer method (Westerfield 1945) to detect formation of acetoin due to overexpression of AHAS.

Replacement of native *ilvC* with heterologous *ilvC* homolog

The acetohydroxy acid isoreductomerase (AHAIR) gene from *Thermacetogenium phaeum* (Brinkmann-Chen et al. 2014) (GenBank accession NC_018870.1) was codon optimized and synthesized by GenScript (Piscataway, NJ, USA) and cloned in template plasmid pSD111 (Deb et al. 2016) to construct plasmid pSD111-CTP. The genome integration cassette (downstream from the T7 promoter) from plasmid pSD111-CTP was amplified using primers for replacement of the native *E. coli ilvC* gene. The purified PCR product was electroporated in *E. coli* Δ51 transformed with plasmid pKD46 (Datsenko and Wanner 2000) to construct *E. coli* Δ52. Transformants were selected on LB agar plates containing kanamycin, and the FRT-flanked kanamycin resistance gene was eliminated.

Replacement of native *ilvC* with heterologous *ilvC* was confirmed using PCR. Functional complementation of native *ilvC* by the heterologous homolog was confirmed by patching *E. coli* Δ52 on M9 agar plates supplemented with 1% glucose (with no added valine) and observing growth after overnight incubation at 37 °C. *E. coli* Δ51 was used as control and patched on the same plate.

Genome integration of *kivd*

The gene coding for ketoisovalerate decarboxylase (KIVD) from *Lactococcus lactis* (de la Plaza et al. 2004) (GenBank accession NC_013656.1) was codon optimized and synthesized by GenScript (Piscataway, NJ, USA) and cloned in template plasmid pSD112 (Deb et al. 2016) to construct plasmid pSD112-KLL. The genome integration cassette from plasmid pSD112-KLL was amplified using primers for integration within the *mltADR* locus. The purified PCR product was electroporated in *E. coli* Δ51 and Δ52 transformed with plasmid pKD46 to construct *E. coli* Δ54 and Δ55, respectively. Transformants were selected on MacConkey agar plates containing kanamycin and mannitol, and the FRT-flanked kanamycin resistance gene was eliminated.

Promoter replacement of native alcohol dehydrogenase genes

The promoter replacement cassette from template plasmid pSD222 (Deb et al. 2016), comprising of the FRT-flanked chloramphenicol resistance gene and the downstream $rhaP_{BAD}$ promoter, was amplified using primers that included 50 nt homology extensions upstream and downstream of the promoter regions of *adhP*, *yahK*, *dkgA*, *fucO* and *yqhD* alcohol dehydrogenase genes. Purified PCR products were individually electroporated in *E. coli* Δ54 and Δ55 transformed with plasmid pKD46 (Datsenko and Wanner 2000). Transformants were selected on LB agar plates containing chloramphenicol, and promoter replacement was confirmed using PCR.

GC–MS analysis for metabolite detection

For detection of metabolites, *E. coli* strains were cultivated in 10 ml LB broth supplemented with 0.5% glucose and 0.5% L-rhamnose at 30 °C in an orbital shaker (180 rpm). After 24 h of cultivation (OD_{600} = 1.8), cells were collected by centrifugation and resuspended in 1 ml fresh LB broth supplemented with 1% glucose in a screw capped 15 ml tube and incubated at 30 °C in an orbital shaker (180 rpm). After 4 h, when glucose in the medium was exhausted, cells were separated from the medium by centrifugation at 9000×*g* for 10 min at 4 °C, and the supernatant extracted with 1 ml ethyl acetate. 5 µl of the ethyl acetate layer was injected in an Agilent Technologies (USA) 7890A gas chromatograph (GC) system used with an Agilent 5975C mass selective detector equipped with an Agilent DB-Wax column (30 m × 320 µm × 0.50 µm). Helium was used as carrier gas (1 ml/min flow rate). The GC oven temperature was held at 40 °C for 2 min, initially ramped to 110 °C at 8 °C/min and held for 2 min, and then increased to 220 °C at 20 °C/min with a run time of 18.25 min. For detection of isobutyraldehyde, an Agilent 19091 J-413 HP-5 column (30 m × 320 µm × 0.25 µm) was used. The GC oven temperature was held at 40 °C for 2 min, ramped to 230 °C at 10 °C/min and held for 2 min with a run time of 23 min.

Sample peaks were identified by comparing with authentic standards, and by using the NIST Mass Spectral Search Program (version 2.0 f)

Results and discussion

Deletion of non-essential fermentative pathways in *Escherichia coli* BW25113

To conserve pyruvate and reduce carbon flux through fermentative pathways, the gene coding for FrsA was deleted from the *E. coli* BW25113 genome. FrsA binds to the unphosphorylated state of IIA^{Glc} and acts as a fermentation-respiration switch that regulates the switch between fermentation and respiration pathways (Koo et al. 2004). Deletion of this gene leads to reduced formation of fermentation by-products, whereas overexpression leads to decreased rate of cellular respiration (Koo et al. 2004).

To further increase availability of pyruvate for diversion toward isobutanol synthesis, genes coding for production of acetate, lactate, succinate and ethanol (*ackA-pta, ldhA, frdBC* and *adhE*, respectively) were sequentially deleted to construct *E. coli* Δ5. Formation of lactate, formate and succinate was eliminated in *E. coli* Δ5, while acetate production was reduced (Fig. 2).

Expression of enzymes of the valine biosynthesis pathway

Acetohydroxy acid synthase (AHAS; EC 2.2.1.6) catalyzes the first step in the valine biosynthesis pathway and is the rate limiting step (Park et al. 2007). Of the three AHAS isozymes present in *E. coli*, AHAS II (coded by *ilvGM*) is resistant to feedback inhibition by L-valine (Gedi and Yoon 2012; Hill et al. 1997). As AHAS II is inactive in *E. coli* K-12 strains due to a frameshift mutation (Lawther et al. 1981), AHAS II from *E. coli* B was chosen for overexpression. *ilvGM* from *E. coli* strain B was integrated in *E. coli* Δ5 under control of the L-rhamnose inducible $rhaP_{BAD}$ promoter, the resultant integrant being designated *E. coli* Δ51. Functionality of the integrated gene was confirmed by performing enzyme assay for AHAS, which is based on the formation of acetoin through 2-acetolactate and further detection of acetoin using the Voges-Proskauer method. The formation of a cherry red color confirmed the functionality of AHAS (Fig. 3).

The $rhaP_{BAD}$ promoter is an inducible, strong and tunable promoter with low basal transcriptional activity that provides a varied range of expression levels

depending on the concentration of inducer used (Brautaset et al. 2009; Egan and Schleif 1993; Giacalone et al. 2006; Haldimann et al. 1998). The advantage of using the $rhaP_{BAD}$ promoter for the isobutanol pathway is that it provides a possibility to tune gene expression levels by varying the inducer concentrations. This is an important parameter to consider since isobutanol is toxic to *E. coli* at concentrations > 8 g/l (Brynildsen and Liao 2009). Moreover, as L-rhamnose is not metabolized by the host strain *E. coli* BW25113 due to deletion of the chromosomal *araBAD* genes, the inducer concentration remains constant in the medium. Therefore, this promoter was chosen for overexpression of *ilvGM* and other downstream genes of the pathway.

The second step of the valine biosynthesis pathway is catalyzed by the enzyme acetohydroxy acid isomeroreductase (AHAIR; EC 1.1.1.86), encoded by the *ilvC* gene. Native AHAIR from *E. coli* is NADPH-dependant (Chunduru et al. 1989). Based on its low K_m values and cofactor preference toward NADH (Brinkmann-Chen et al. 2014), we chose to express AHAIR from *Thermacetogenium phaem*.

2-Acetolactate, the product of AHAS II, is a substrate for AHAIR. A 100-fold increase in binding of RNA polymerase to the *ilvC* promoter is mediated by the positive activator protein IlvY in the presence of 2-acetolactate (Opel and Hatfield 2001; Rhee et al. 1998, 1999; Wek and Hatfield 1988). Therefore, expression of heterologous NADH-dependant *ilvC* gene from the native *ilvC* promoter may be sufficient to drive flux through the rest of the pathway. Based on this reasoning, *ilvC* from *T. phaem* was integrated in strain *E. coli* Δ51 under control of the native promoter of *ilvC* with the simultaneous replacement of the native *ilvC* gene to construct *E. coli* Δ52.

Functionality of heterologous *ilvC* was confirmed by growing the transformants containing the heterologous *ilvC* gene in minimal medium lacking L-valine. *E. coli* Δ52 showed similar growth as compared to the control strain *E. coli* Δ51, suggesting that the heterologous *ilvC* was able to functionally complement the replaced native *E. coli ilvC* gene (Fig. 4).

Expression of enzymes for isobutanol production

2-Ketoisovalerate is the penultimate metabolite in valine biosynthesis. This intermediate can be diverted toward isobutanol production by overexpression of genes of the Ehrlich pathway (Atsumi et al. 2008). The first step of the non-native isobutanol pathway is catalyzed by a 2-keto acid decarboxylase (KIVD; EC 4.1.1.72) encoded by the gene *kivd* from *L. lactis*, which leads to the formation of isobutyraldehyde by decarboxylation of 2- ketoisovalerate (de la Plaza et al. 2004).

kivd from *L. lactis* was integrated at the *mtlADR* locus in the *E. coli* Δ51 and *E. coli* Δ52 strains, the resultant integrants being designated *E. coli* Δ54 and *E. coli* Δ55, respectively. GC–MS analysis was carried

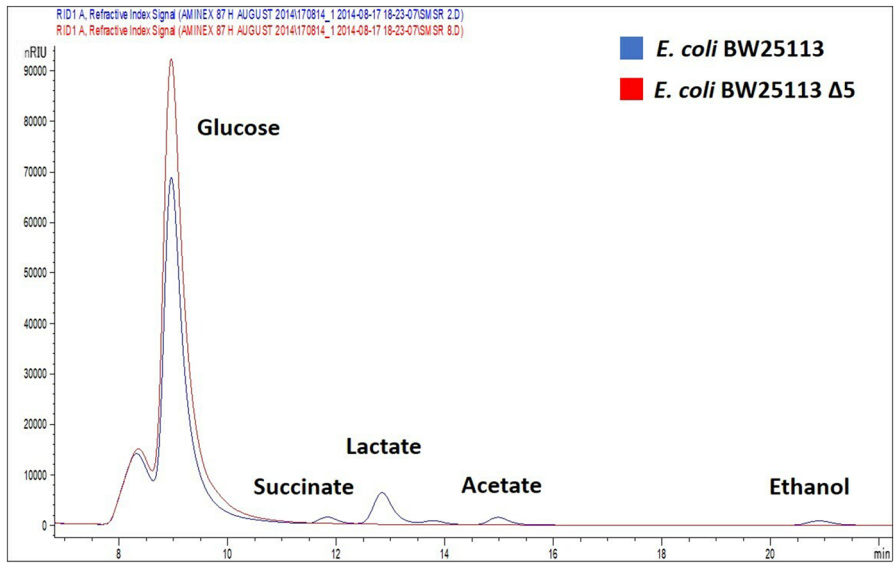

Fig. 2 HPLC chromatogram of culture supernatants of *E. coli* BW25113 and Δ5 showing elimination of fermentation by-products in *E. coli* Δ5

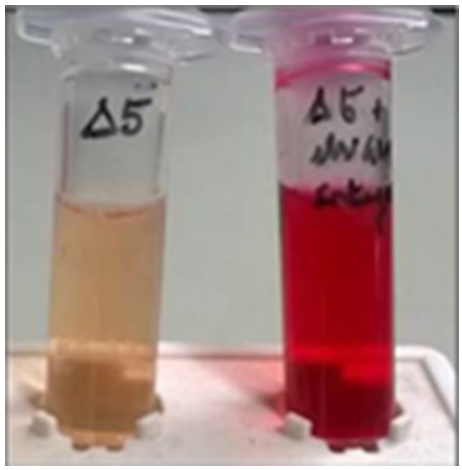

Fig. 3 Confirmation of functionality of *ilvGM* integrated in *E. coli* Δ51 using enzyme assay. A positive Voges-Proskauer test for *E. coli* Δ51 (right) confirmed AHAS activity. *E. coli* Δ5 used as control (left) tested negative for the Voges-Proskauer test

out to detect isobutyraldehyde formation in *E. coli* Δ54 and *E. coli* Δ55 (data not shown).

The last step of the isobutanol pathway involves the conversion of isobutyraldehyde to isobutanol by alcohol dehydrogenases. Both native and non-native alcohol dehydrogenases have been assessed for isobutanol production (Atsumi et al. 2010). Overexpression of heterologous enzymes may sometimes lead to protein misfolding and formation of inclusion bodies (Atsumi et al. 2010). Overexpression of native enzymes may avoid issues of incompatible codon usage, misfolding and protein aggregation.

The isobutanol pathway is a NADPH- or a NADH-dependant pathway depending on the cofactor preferences of the AHAIR and ADH isozymes expressed. NADH-dependant enzymes are preferred since the intracellular levels of NADH are higher than those of NADPH (Bastian et al. 2011; Chin et al. 2009). Both NADPH- and NADH-dependant alcohol dehydrogenases have been used for isobutanol production (Atsumi et al. 2008, 2010; Bastian et al. 2011; Shi et al. 2013; Savrasova et al. 2011).

To assess the effect of different alcohol dehydrogenases on isobutanol production, five native *E. coli* alcohol dehydrogenases, based on their cofactor dependence, kinetic parameters and substrate preference, were chosen as candidates for overexpression.

YqhD is a NADPH-dependant aldehyde reductase that is active toward a variety of substrates, including

Fig. 4 Confirmation of functionality of *ilvC* from *T. phaem* integrated in *E. coli* Δ51. *E. coli* Δ52 grown in minimal media lacking L-valine (lower panel of the plate) displayed similar growth as *E. coli* Δ51, used as control (upper panel)

isobutyraldehyde, butyraldehyde, methylglyoxal, furfural and acetol (Jarboe 2011). YqhD has been shown to be responsible for isobutanol production in the absence of overexpression of heterologous ADH, indicating its ability to convert isobutyraldehyde to isobutanol (Atsumi et al. 2010). AdhP is a NADH-dependant medium chain dehydrogenase/reductase that is active toward isobutyraldehyde (Rodriguez and Atsumi 2012; Shafqat et al. 1999). YahK is a NADPH-dependant medium chain dehydrogenase active toward a multitude of substrates including isobutyraldehyde, butyraldehyde and other medium chain aldehydes, with highest activity for isobutyraldehyde (Pick et al. 2013; Rodriguez and Atsumi 2014). DkgA (previously known as YqhE) is a NADPH-dependant oxidoreductase that is involved in ketogluconate metabolism (Habrych et al. 2002; Yum et al. 1999). DkgA is known to be active toward medium chain substrates including isobutyraldehyde and has no activity toward acetaldehyde (Rodriguez and Atsumi 2014). FucO is a well-characterized NADH-dependant lactaldehyde:propanediol oxidoreductase with activity toward isobutyraldehyde (Boronat and Aguilar 1979; Rodriguez and Atsumi 2014).

YqhD, AdhP, YahK, DkgA and FucO were screened for isobutanol production by individually expressing each of these genes by replacing their native promoters with the strong $rhaP_{BAD}$ promoter in *E. coli* Δ54 and *E. coli* Δ55.

Analysis of metabolite production in constructed strains

E. coli Δ54 and *E. coli* Δ55 individually overexpressing YqhD, AdhP, YahK, DkgA and FucO were grown

Table 3 Isobutyraldehyde production by *E. coli* Δ54[a] and *E. coli* Δ55[b] overexpressing YqhD, AdhP, YahK, DkgA or FucO

ADH overexpressed	Isobutyraldehyde produced (mg)/g glc consumed	
	E. coli Δ54	E. coli Δ55
YqhD	17.26 ± 0.7	7.24 ± 3.76
AdhP	23.93 ± 1.34	8.32 ± 4.96
YahK	15.54 ± 0.14	10.21 ± 7.39
DkgA	17.58 ± 6.75	14.14 ± 7.29
FucO	18.95 ± 13.79	19.63 ± 4.55

[a]*E. coli* Δ54 overexpresses AHAS, KIVD and the indicated ADH genes

[b]*E. coli* Δ55 overexpresses AHAS, *T. phaeum* AHAIR, KIVD and the indicated ADH genes

in LB broth supplemented with glucose, and extracellular metabolites produced were analyzed using GC–MS. Isobutyraldehyde, the penultimate metabolite in the isobutanol biosynthesis pathway, was detected (Table 3), along with isovaleraldehyde, which is the penultimate metabolite in the pathway leading to 3-methyl-1-butanol (Fig. 5). However, isobutanol was not detected. *E. coli* Δ54.P, expressing NADPH-dependent native AHAIR and NADH-dependent AdhP, produced the highest amount of isobutyraldehyde, which represents ~ 6% of theoretical maximum (0.4 g/g glucose). This indicates a low flux through the isobutanol pathway; isobutanol formed from such low amounts of isobutyraldehyde may be difficult to detect, and would require special equipment (Rodriguez and Atsumi 2012) to trap and measure (for e.g., a gas stripping apparatus).

The L-leucine biosynthesis pathway produces 2-ketoisocaproate, which is the precursor for L-leucine. 2-ketoisocaproate is a substrate for KIVD and can be further converted to 3-methyl-1-butanol by the action of various alcohol dehydrogenases (Atsumi et al. 2008). Detection of isovaleraldehyde in cell culture supernatants indicates diversion of carbon from 2-ketoisovalerate, which is the precursor for isobutanol synthesis, toward L-leucine biosynthesis (Fig. 5). Deletion of genes of the L-leucine pathway may prevent diversion of 2-ketoisovalerate into the L-leucine pathway. However, this was not attempted as deletion of the *leuABCD* operon (encoding the first three steps of the L-leucine pathway) would lead to L-leucine auxtrophy and therefore necessitate supplementation of growth media with L-leucine.

Overflow metabolites originating at two branch points, dihydroxyacetone phosphate (DHAP) and 2-acetolactate, were also observed in the culture supernatants of *E. coli* Δ54 and *E. coli* Δ55 expressing YqhD, AdhP, YahK, DkgA and FucO (Fig. 5). Detection of acetol and 1,2-propanediol (originating from DHAP) and diacetyl, acetoin and 2,3-butanediol (originating from 2-acetolactate) suggest that alternative pathways were activated due to the genetic modifications made in the constructed strains. The observation of overflow metabolites originating at the DHAP and 2-acetolactate nodes is in agreement with the results reported by Milne et al. (2016) in *Saccharomyces cerevisiae* engineered to produce isobutanol.

Overflow metabolites observed at dihydroxyacetone phosphate branch point

There are two routes for production of 1,2-propanediol (1,2-PDO): one route occurs via the glycolytic intermediate DHAP, and the other route involves the 6-deoxysugars fucose and rhamnose (Bennett and San 2001).

The 1,2-PDO pathway via DHAP involves the latter's conversion to methylglyoxal by methylglyoxal synthase encoded by *mgsA* (Cooper 1984). An increased rate of carbon influx leads to elevated amounts of methylglyoxal (Tötemeyer et al. 1998). Methylgloxal is converted to 1,2-PDO (Fig. 5) via a L-lactaldehyde or an acetol intermediate (Cameron et al. 1998; Ko et al. 2005).

The alcohol dehydrogenase enzymes (YqhD, AdhP, YahK, DkgA and FucO) overexpressed in this study exhibit broad substrate specificities and can act on a range of substrates, including methylglyoxal, acetol and L-lactaldehyde (Jarboe 2011; Ko et al. 2005; Altaras and Cameron 1999, 2000; Lee et al. 2010). For instance, YqhD is a NADPH-dependant *E. coli* aldehyde reductase that has been used for production of

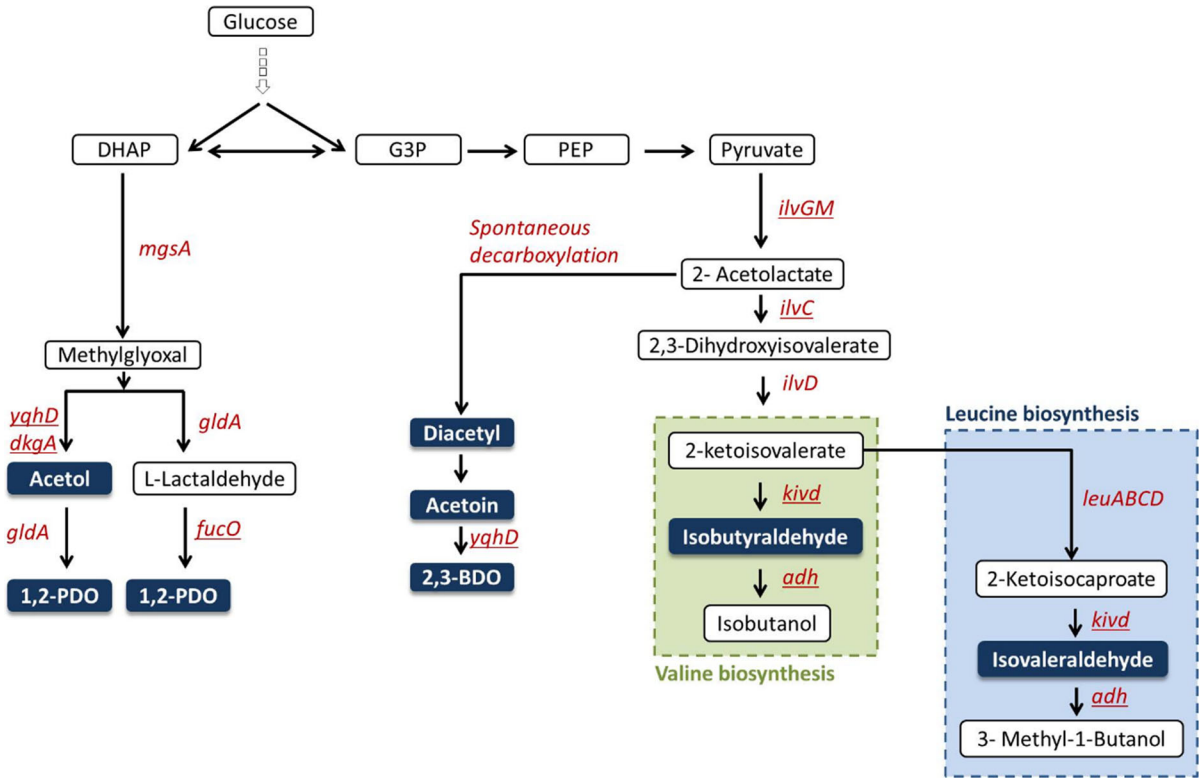

Fig. 5 Pathway intermediates (in red boxes) and overflow metabolites (in blue boxes) observed in culture supernatants of *E. coli* Δ54 and *E. coli* Δ55 individually overexpressing YqhD, AdhP, YahK, DkgA and FucO. Enzyme activities reported in literature are indicated. Genes overexpressed in this study are underlined

1,2-PDO, and is active toward both isobutyraldehyde and methylglyoxal (Atsumi et al. 2010; Jarboe 2011; Lee et al. 2010). The $K_m^{\text{isobutyraldehyde}}$ and $K_m^{\text{methylglyoxal}}$ values of YqhD are 2 mM and 2.6 mM, respectively, indicating that it can use either of the two substrates to form either isobutanol or acetol. However, YqhD has a higher k_{cat} value for methylglyoxal (4.7 s^{-1}) than isobutyraldehyde (1 s^{-1}), implying a higher catalytic efficiency with the former. In addition, the specificity constant is k_{cat}/k_m of YqhD toward methylglyoxal is 3.6-fold higher than that toward isobutyraldehyde (Atsumi et al. 2010; Jarboe 2011; Lee et al. 2010).

Similarly, YahK, DkgA and FucO are broad range aldehyde reductases that are active toward a broad range of substrates such as L-lactaldehdye, methylglyoxal and acetol, which are precursors of 1,2-PDO (Pick et al. 2013; Rodriguez and Atsumi 2014; Boronat and Aguilar 1979; Chen and Lin 1984; Niu and Guo 2015; Zhu and Lin 1989). L-Rhamnose has been reported to induce synthesis of FucO (Boronat and Aguilar 1979; Chen and Lin 1984); as L-rhamnose has been used to induce transcription of the integrated genes, FucO expression may be an artefact of the strategy employed in this study. Interestingly, the *mgsA* locus, coding for methylglyoxal synthase, has been used to integrate and overexpress *ilvC* for isobutanol production, which would have prevented formation of acetol and 1,2-PDO (Shi et al. 2013).

Therefore, the detection of acetol and 1,2-PDO in cell culture supernatants can be attributed to the broad substrate range of the alcohol dehydrogenases that have been employed in this study for the conversion of isobutyraldehyde to isobutanol in the engineered *E. coli* strain.

Overflow metabolites observed at 2-acetolactate branch point

GC–MS analysis of cell culture supernatants revealed presence of metabolites of the 2,3-butanediol pathway

(diacetyl and acetoin) and 2,3-butanediol (2,3-BDO). This indicated diversion of carbon flux at the 2-acetolactate branch point toward 2,3-BDO synthesis, which may have occurred due to overexpression of native alcohol dehydrogenases exhibiting broad substrate ranges.

Under aerobic conditions, 2-acetolactate spontaneously decarboxylates to form diacetyl, which is further converted to acetoin by the action of acetolactate decarboxylase (Nielsen et al. 2010). In the last step, butanediol dehydrogenase catalyzes the conversion of acetoin to 2,3-BDO. The presence of 2,3-BDO pathway intermediates suggests that native *E. coli* enzymes may be responsible for catalyzing these reactions. Interestingly, Silber et al. (1974) reported purification of a NADPH-dependant reductase capable of reducing diacetyl to acetoin, the gene for which has not been identified.

Native and heterologous AHAIR, which catalyzes the second step in the valine biosynthesis pathway, were expressed from the native *ilvC* promoter. It is possible that expression of *ilvC* from its native promoter was not sufficient to pull carbon flux toward the isobutanol pathway and resulted in 2-acetolactate being diverted toward 2,3-BDO synthesis. Increased expression of *ilvC* from a stronger promoter may prevent carbon overflow from the valine biosynthesis pathway. Additionally, overexpression of *ilvD* encoding for DHAD may further drive flux through the isobutanol pathway and prevent formation of overflow metabolites at the 2-acetolactate branch point.

Conclusion

In the present study, *E. coli* BW25113 was rationally engineered to construct plasmid-free isobutanol producing strains. Three major strategies were adopted: conserving pyruvate, channelling flux toward valine production, and balancing cofactor usage. Analysis of performance of the constructed strains revealed diversion of carbon to alternative pathways, leading to formation of unwanted metabolites. This may be attributed to a low carbon flux through the valine biosynthesis pathway due to the use of the native promoter to express AHAIR, and the broad substrate ranges of the ADH isozymes overexpressed.

The low concentration of isobutyraldehyde observed in this study warrants using strong promoters to overexpress the valine biosynthesis pathway. Savrasova et al. (2011) reported using a valine-producing *E. coli* strain expressing four copies of genome-integrated *ilvGMED* operon from the strong λ phage P_R promoter to produce isobutanol, while Akita et al. (2015) expressed genome-integrated valine and isobutanol biosynthesis genes from the strong T7 promoter. This allows the isobutanol-producing pathway to divert the high levels of 2-ketoisovalerate toward isobutanol formation.

The choice of ADH expressed to catalyze the last step of the isobutanol pathway influences the metabolites formed. Most studies have utilized the native YqhD alcohol dehydrogenase, which has been reported to have a high affinity for isobutyraldehyde (Atsumi et al. 2010). However, YqhD utilizes NADPH as a cofactor; in our attempt to use NADH-utilizing ADHs, we overexpressed the native *E. coli* enzymes AdhP and FucO. The results obtained suggest that in conjunction with our approach to increase flux through the valine pathway, this strategy led to the activation of alternative pathways, an issue not encountered by other researchers in earlier studies. This observation underscores the importance of alternative pathways in diverting carbon flux from molecules sought to be overproduced.

The insights gained from the present study will allow rational genetic modification of *E. coli* for production of metabolites of interest.

References

Ajikumar PK, Xiao WH, Tyo KEJ, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G (2010) Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. Science 330:70–74

Akita H, Nakashima N, Hoshino T (2015) Bacterial production of isobutanol without expensive reagents. Appl Microbiol Biotechnol 99:991–999

Albermann C, Trachtmann N, Sprenger GA (2010) A simple and reliable method to conduct and monitor expression cassette integration into the *Escherichia coli* chromosome. Biotechnol J 5:32–38

Altaras NE, Cameron DC (1999) Metabolic engineering of a 1,2-propanediol pathway in *Escherichia coli*. Appl Environ Microbiol 65:1180–1185

Altaras NE, Cameron DC (2000) Enhanced production of (R)-1,2-propanediol by metabolically engineered *Escherichia coli*. Biotechnol Prog 16:940–946

Atsumi S, Liao JC (2008) Metabolic engineering for advanced biofuels production from *Escherichia coli*. Curr Opin Biotechnol 19:414–419

Atsumi S, Hanai T, Liao JC (2008) Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature 451:86–89

Atsumi S, Wu TY, Eckl EM, Hawkins SD, Buelter T, Liao JC (2010) Engineering the isobutanol biosynthetic pathway in *Escherichia coli* by comparison of three aldehyde reductase/alcohol dehydrogenase genes. Appl Microbiol Biotechnol 85:651–657

Baez A, Cho KM, Liao JC (2011) High-flux isobutanol production using engineered *Escherichia coli*: a bioreactor study with in situ product removal. Appl Microbiol Biotechnol 90:1681–1690

Bastian S, Liu X, Meyerowitz JT, Snow CD, Chen MM, Arnold FH (2011) Engineered ketol-acid reductoisomerase and alcohol dehydrogenase enable anaerobic 2-methylpropan-1-ol production at theoretical yield in *Escherichia coli*. Metab Eng 13:345–352

Bennett GN, San KY (2001) Microbial formation, biotechnological production and applications of 1,2-propanediol. Appl Microbiol Biotechnol 55:1–9

Boronat A, Aguilar J (1979) Rhamnose-induced propanediol oxidoreductase in *Escherichia coli*: purification, properties, and comparison with the fucose-induced enzyme. J Bacteriol 140:320–326

Brautaset T, Lale R, Valla S (2009) Positively regulated bacterial expression systems. Microb Biotechnol 2:15–30

Brinkmann-Chen S, Cahn JK, Arnold FH (2014) Uncovering rare NADH-preferring ketol-acid reductoisomerases. Metab Eng 26:17–22

Brynildsen MP, Liao JC (2009) An integrated network approach identifies the isobutanol response network of *Escherichia coli*. Mol Syst Biol 5:277

Cameron DC, Altaras NE, Hoffman ML, Shaw AJ (1998) Metabolic engineering of propanediol pathways. Biotechnol Prog 14:116–125

Chen YM, Lin EC (1984) Dual control of a common L-1,2-propanediol oxidoreductase by L-fucose and L-rhamnose in *Escherichia coli*. J Bacteriol 157:828–832

Cherepanov PP, Wackernagel W (1995) Gene disruption in *Escherichia coli*: tcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene 158:9–14

Chin JW, Khankal R, Monroe CA, Maranas CD, Cirino PC (2009) Analysis of NADPH supply during xylitol production by engineered *Escherichia coli*. Biotechnol Bioeng 102:209–220

Chunduru SK, Mrachko GT, Calvo KC (1989) Mechanism of ketol acid reductoisomerase–steady-state analysis and metal ion requirement. Biochemistry 28:486–493

Cooper RA (1984) Metabolism of methylglyoxal in microorganisms. Annu Rev Microbiol 38:49–68

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97:6640–6645

de la Plaza M, Fernández de Palencia P, Peláez C, Requena T (2004) Biochemical and molecular characterization of alpha-ketoisovalerate decarboxylase, an enzyme involved in the formation of aldehydes from amino acids by *Lactococcus lactis*. FEMS Microbiol Lett 238:367–374

Deb SS, Reshamwala SMS, Lali AM (2016) A series of template plasmids for *Escherichia coli* genome engineering. J Microbiol Methods 125:49–57

Egan SM, Schleif RF (1993) A regulatory cascade in the induction of *rhaBAD*. J Mol Biol 234:87–98

Gedi V, Yoon MY (2012) Bacterial acetohydroxyacid synthase and its inhibitors–a summary of their structure, biological activity and current status. FEBS J 279:946–963

Giacalone MJ, Gentile AM, Lovitt BT, Berkley NL, Gunderson CW, Surber MW (2006) Toxic protein expression in *Escherichia coli* using a rhamnose-based tightly regulated and tunable promoter system. Biotechniques 40:355–364

Habrych M, Rodriguez S, Stewart JD (2002) Purification and identification of an *Escherichia coli* beta-keto ester reductase as 2,5-diketo-D-gluconate reductase YqhE. Biotechnol Prog 18:257–261

Haldimann A, Daniels LL, Wanner BL (1998) Use of new methods for construction of tightly regulated arabinose and rhamnose promoter fusions in studies of the *Escherichia coli* phosphate regulon. J Bacteriol 180:1277–1286

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557–580

Hill CM, Pang SS, Duggleby RG (1997) Purification of *Escherichia coli* acetohydroxyacid synthase isoenzyme II and reconstitution of active enzyme from its individual pure subunits. Biochem J 327:891–898

Huo YX, Cho KM, Rivera JG, Monte E, Shen CR, Yan Y, Liao JC (2011) Conversion of proteins into biofuels by engineering nitrogen flux. Nat Biotechnol 29:346–351

Jarboe LR (2011) YqhD: a broad-substrate range aldehyde reductase with various applications in production of biorenewable fuels and chemicals. Appl Microbiol Biotechnol 89:249–257

Kabus A, Georgi T, Wendisch VF, Bott M (2007) Expression of the *Escherichia coli pntAB* genes encoding a membrane-bound transhydrogenase in *Corynebacterium glutamicum* improves L-lysine formation. Appl Microbiol Biotechnol 75:47–53

Ko J, Kim I, Yoo S, Min B, Kim K, Park C (2005) Conversion of methylglyoxal to acetol by *Escherichia coli* aldo-keto reductases. J Bacteriol 187:5782–5789

Kolodziej R, Scheib J (2012) Bio-isobutanol: the next-generation biofuel. hydrocarbon processing. http://www.hydrocarbonprocessing.com/magazine/2012/september-2012/special-report-refining-developments/bio-isobutanol-the-next-generation-biofuel. Accessed on 9 Dec 2018

Koo BM, Yoon MJ, Lee CR, Nam TW, Choe YJ, Jaffe H, Peterkofsky A, Seok YJ (2004) A novel fermentation/respiration switch protein regulated by enzyme IIA^{Glc} in *Escherichia coli*. J Biol Chem 279:31613–31621

Lawther RP, Calhoun DH, Adams CW, Hauser CA, Gray J, Hatfield GW (1981) Molecular basis of valine resistance in *Escherichia coli* K-12. Proc Natl Acad Sci USA 78:922–925

Lee C, Kim I, Lee J, Lee KL, Min B, Park C (2010) Transcriptional activation of the aldehyde reductase YqhD by YqhC and its implication in glyoxal metabolism of *Escherichia coli* K-12. J Bacteriol 192:4205–4214

Lemuth K, Steuer K, Albermann C (2011) Engineering of a plasmid-free *Escherichia coli* strain for improved in vivo biosynthesis of astaxanthin. Microb Cell Fact 10:29

Liu Z, Liu P, Xiao D, Zhang X (2016) Improving isobutanol production in metabolically engineered *Escherichia coli* by co-producing ethanol and modulation of pentose phosphate pathway. J Ind Microbiol Biotechnol 43:851–860

Milne N, Wahl SA, van Maris AJA, Pronk JT, Daran JM (2016) Excessive by-product formation: a key contributor to low isobutanol yields of engineered *Saccharomyces cerevisiae* strains. Metab Eng Commun 3:39–51

Nielsen DR, Yoon SH, Yuan CJ, Prather KL (2010) Metabolic engineering of acetoin and meso-2, 3-butanediol biosynthesis in *E. coli*. Biotechnol J 5:274–284

Niu W, Guo J (2015) Stereospecific microbial conversion of lactic acid into 1,2-propanediol. ACS Synth Biol 4:378–382

Opel ML, Hatfield GW (2001) DNA supercoiling-dependent transcriptional coupling between the divergently transcribed promoters of the *ilvYC* operon of *Escherichia coli* is proportional to promoter strengths and transcript lengths. Mol Microbiol 39:191–198

Park JH, Lee KH, Kim TY, Lee SY (2007) Metabolic engineering of *Escherichia coli* for the production of L-valine based on transcriptome analysis and in silico gene knockout simulation. Proc Natl Acad Sci USA 104:7797–7802

Pick A, Rühmann B, Schmid J, Sieber V (2013) Novel CAD-like enzymes from *Escherichia coli* K-12 as additional tools in chemical production. Appl Microbiol Biotechnol 97:5815–5824

Rhee KY, Senear DF, Hatfield GW (1998) Activation of gene expression by a ligand-induced conformational change of a protein-DNA complex. J Biol Chem 273:11257–11266

Rhee KY, Opel M, Ito E, Hung SP, Arfin SM, Hatfield GW (1999) Transcriptional coupling between the divergent promoters of a prototypic LysR-type regulatory system, the *ilvYC* operon of *Escherichia coli*. Proc Natl Acad Sci USA 96:14294–14299

Rodriguez GM, Atsumi S (2012) Isobutyraldehyde production from *Escherichia coli* by removing aldehyde reductase activity. Microb Cell Fact 11:90

Rodriguez GM, Atsumi S (2014) Toward aldehyde and alkane production by removing aldehyde reductase activity in *Escherichia coli*. Metab Eng 25:227–237

Sabido A, Martínez LM, de Anda R, Martínez A, Bolívar F, Gosset G (2013) A novel plasmid vector designed for chromosomal gene integration and expression: use for developing a genetically stable *Escherichia coli* melanin production strain. Plasmid 69:16–23

Savrasova EA, Kivero AD, Shakulov RS, Stoynova NV (2011) Use of the valine biosynthetic pathway to convert glucose into isobutanol. J Ind Microbiol Biotechnol 38:1287–1294

Shafqat J, Höög JO, Hjelmqvist L, Oppermann UC, Ibáñez C, Jörnvall H (1999) An ethanol-inducible MDR ethanol dehydrogenase/acetaldehyde reductase in *Escherichia coli*: structural and enzymatic relationships to the eukaryotic protein forms. Eur J Biochem 263:305–311

Shi A, Zhu X, Lu J, Zhang X, Ma Y (2013) Activating transhydrogenase and NAD kinase in combination for improving isobutanol production. Metab Eng 16:1–10

Silber P, Chung H, Gargiulo P, Schulz H (1974) Purification and properties of a diacetyl reductase from *Escherichia coli*. J Bacteriol 118:919–927

Tashiro Y, Rodriguez GM, Atsumi S (2015) 2-Keto acids based biosynthesis pathways for renewable fuels and chemicals. J Ind Microbiol Biotechnol 42:361–373

Tötemeyer S, Booth NA, Nichols WW, Dunbar B, Booth IR (1998) From famine to feast: the role of methylglyoxal production in *Escherichia coli*. Mol Microbiol 27:553–562

Trinh CT, Li J, Blanch HW, Clark DS (2011) Redesigning *Escherichia coli* metabolism for anaerobic production of isobutanol. Appl Environ Microbiol 77:4894–4904

Wek RC, Hatfield GW (1988) Transcriptional activation at adjacent operators in the divergent-overlapping *ilvY* and *ilvC* promoters of *Escherichia coli*. J Mol Biol 203:643–663

Westerfield WW (1945) A colorimetric determination of blood acetoin. J Biol Chem 161:495–502

Yan Y, Liao JC (2009) Engineering metabolic systems for production of advanced fuels. J Ind Microbiol Biotechnol 36:471–479

Yum DY, Lee BY, Pan JG (1999) Identification of the *yqhE* and *yafB* genes encoding two 2,5-diketo-D-gluconate reductases in *Escherichia coli*. Appl Environ Microbiol 65:3341–3346

Zhu Y, Lin EC (1989) L-1,2-propanediol exits more rapidly than L-lactaldehyde from *Escherichia coli*. J Bacteriol 171:862–867