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Use of the valine biosynthetic pathway to convert glucose into isobutanol

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Abstract Microbiological synthesis of higher alcohols (1-butanol, isobutanol, 2-methyl-1-butanol, etc.) from plant biomass is critically important due to their advantages over ethanol as a motor fuel. In recent years, the use of branched-chain amino acid (BCAA) biosynthesis pathways together with heterologous Ehrlich pathway enzyme system (Hazelwood et al. in Appl Environ Microbiol 74:2259–2266, 2008) has been proposed by the Liao group as an alternative approach to aerobic production of higher alcohols as new-generation biofuels (Atsumi et al. in Nature 451:86–90, 2008; Atsumi et al. in Appl Microbiol Biotechnol 85:651–657, 2010; Cann and Liao in Appl Microbiol Biotechnol 81:89–98, 2008; Connor and Liao in Appl Environ Microbiol 74:5769–5775, 2008; Shen and Liao in Metab Eng 10:312–320, 2008; Yan and Liao in J Ind Microbiol Biotechnol 36:471–479, 2009). On the basis of these remarkable investigations, we re-engineered *Escherichia coli* valine-producing strain H-81, which possess overexpressed *ilvGMED* operon, for the aerobic conversion of sugar into isobutanol. To redirect valine biosynthesis to the production of alcohol, we also—as has been demonstrated previously (Atsumi et al. in Nature 451:86–90, 2008; Atsumi et al. in Appl Microbiol Biotechnol 85:651–657, 2010; Cann and Liao in Appl Microbiol Biotechnol 81:89–98, 2008; Connor and Liao in Appl Environ Microbiol 74:5769–5775, 2008; Shen and Liao in Metab Eng 10:312–320, 2008; Yan and Liao in J Ind Microbiol Biotechnol 36:471–479, 2009)—used enzymes of Ehrlich pathway. In particular, in our study, the following heterologous proteins were exploited: branched-chain 2-keto acid decarboxylase (BCKAD) encoded by the *kdcA* gene from *Lactococcus lactis* with rare codons substituted, and alcohol dehydrogenase (ADH) encoded by the *ADH2* gene from *Saccharomyces cerevisiae*. We show that expression of both of these genes in the valine-producing strain H-81 results in accumulation of isobutanol instead of valine. Expression of BCKAD alone also resulted in isobutanol accumulation in the culture broth, supporting earlier obtained data (Atsumi et al. in Appl Microbiol Biotechnol 85:651–657, 2010) that native ADHs of *E. coli* are also capable of isobutanol production. Thus, in this work, isobutanol synthesis by *E. coli* was achieved using enzymes similar to but somewhat different from those previously used.

Keywords Biofuel · Isobutanol · Branched-chain amino acids · Branched-chain 2-keto acid decarboxylase · Alcohol dehydrogenase

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Introduction

Production of biofuel from renewable sources is of particular interest due to the limited reserves of traditional energy sources (such as fossil fuels), unstable oil prices, and the need to limit greenhouse gas emissions and air and water pollution. Plant biomass is one of the most important renewable energy resources. Significant steps have been taken to increase biomass use to produce hexoses and pentoses and to adapt microorganisms for the simultaneous

consumption of a variety of carbon sources. Conversion of C5 and C6 sugars into liquid energy carriers occurs in two major anaerobic biological processes: ethanol fermentation and mixed acetone, ethanol and butanol fermentation. Currently, biosynthesis of higher alcohols (1-butanol, isobutanol, 2-methyl-1-butanol, etc.) from plant biomass has gained significant attention due to advantages over ethanol as a motor fuel. In particular, isobutanol has higher energy density than ethanol, and it is less hygroscopic and less volatile. Up until now, among higher alcohols, only 1-butanol has been manufactured by microbiological synthesis, and this was achieved by using *Clostridium acetobutylicum* in a strictly anaerobic process [16, 18]. Microorganisms naturally capable of industrially relevant conversion of sugars into C4 and C5 alcohols have not yet been identified. The yeast *Saccharomyces cerevisiae* has been shown to synthesize small amounts of isobutanol and other higher alcohols by the Ehrlich pathway as result of catabolism of corresponding branched-chain amino acids (BCAA) valine, leucine, and isoleucine [10–12, 22].

Recently, a strategy for aerobic production of the higher alcohols has been proposed [2, 3, 5, 7, 23, 27]. This approach includes using the highly active BCAA biosynthetic pathways as a source of keto acids (2-ketoisovalerate, 2-keto-3-methylvalerate, 2-ketoisocaproate, 2-ketovalerate, etc.) and subsequent conversion of these metabolites into the corresponding alcohols (isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-butanol, etc.) [2, 3, 5, 7, 23, 27]. In the cited works [2, 3, 27], to produce isobutanol, the conversion of carbon source into 2-ketoisovalerate in *Escherichia coli* was achieved, through an overexpression of *ilvCD* (*E. coli*) genes together with enhanced expression of genes encoding different types of acetolactate synthase, *ilvIH* (*E. coli*) or *alsS* (*Bacillus suibtilis*).

BCAAs are usually manufactured by fermentation by employing mutant strains of various bacteria, including *E. coli*. As the immediate precursors for BCAAs can also be used for the biosynthesis of higher alcohols, an opportunity to adapt the existing BCAA-hyperproducing strains for biofuel production is of particular interest. Overall in this work, redesign of valine producer H-81 for isobutanol synthesis was performed. In this producer, 2-ketoisovalerate supply for isobutanol production was ensured by overexpression of *ilvD* and *ilvGM* genes encoding feedback-resistant acetolactate synthase II of *E. coli*. Critical to isobutanol production strategy are the following two enzymes from the Ehrlich pathway: 2-keto acid decarboxylase (EC 4.1.1.72) and alcohol dehydrogenase (EC 1.1.1.1), with broad substrate specificity [2, 3, 5, 7, 23, 27]. Particularly, 2-keto acid decarboxylase Kivd from *Lactococcus lactis* [8], together with alcohol dehydrogenase Adh2 from *S. cerevisiae*, has been used for isobutanol synthesis in *E. coli* [2, 3, 27]. Considering substrate specificity, another well-characterized

2-keto acid decarboxylase from *L. lactis*, KdcA, seems to be also applicable for the same purpose [24, 28]. The substrate spectrum of KdcA is similar to Kivd, with which it shares approximately 90% sequence identity [8, 24, 28].

In this study, by introducing the *kdcA* gene from *L. lactis*, with rare codons substituted, and the *ADH2* gene from *S. cerevisiae* under the control of a regulated promoter into an *E. coli* valine-producing strain, we evaluated the possibility of producer re-engineering for aerobic conversion of sugar to higher alcohols, particularly isobutanol.

Materials and methods

Strains and media

All strains used in this study are listed in Table 1. XL1-Blue was the *E. coli* strain used for cloning. Strain H-81 was derived from *E. coli* MG1655 (*ilvG*^{ValR} *ileS17*) and contains four copies of the valine operon under the control of the P$_R$-promoter of phage λ, P$_R$-*ilvG*^{ValR}*MED*-2Tfd, integrated into the chromosome via mini-Mu vector [21, 26]. *E. coli* strains were grown aerobically at 37°C in either lysogeny broth (LB) medium [20] or M9 medium [20] containing 100 µg/ml of ampicillin for selection, if appropriate. Cultivation of valine-producing strains was carried out in fermentation medium (FM) at pH 7.0 containing the following (g/l): $(NH_4)_2SO_4$ 15; KH_2PO_4 1.5; $MgSO_4$ 1; $CaCO_3$ 20; B1 0.01; glucose 60. *S. cerevisiae* S288C strain was grown aerobically at 37°C in yeast peptone dextrose (YPD) medium [1].

Cultivation conditions

Preseeding cultures in test tubes containing 1 ml of LB medium were incubated at 32°C for 18 h on a rotary shaker (250 rpm). Overnight cultures were diluted 1:20 into 2 ml of FM medium in 20×200-mm test tubes by adding 0.5 mM isopropyl-thio-α-galactoside (IPTG) for Lac promoter induction. Cultivation of strain H-81 and its plasmid derivatives was performed at 32°C for 68 h on a rotary shaker (250 rpm).

Determination of amino acid concentration

Accumulated L-valine was measured by thin-layer chromatography (TLC). TLC plates (10×15) cm were coated with 0.11-mm layers of Sorbfil silica gel containing no fluorescent indicator (Stock Company Sorbpolymer, Krasnodar, Russia). The Sorbfil plates were developed with a mobile phase consisting of isopropanol-ethylacetate-25% aqueous ammonia-water (16:16:5:10 v/v). A solution of

Table 1 Strains and plasmid used in this study

Strain or plasmid	Relevant genotype	Source or reference
Strains		
XL1-Blue	*E. coli* (*recA1 endA1* gyrA96 (Nal^R) *thi-1 hsdR17* (r$_k^-$ m$_k^-$) *glnV44 relA1 lac* [*F* '::Tn*10*(Tet^R)'*proAB lacI^q* Δ(*lacZ*)*M15*])	Stratagene
H-81	*E. coli*—MG1655 (*ileS17,* 4 copies of mini-Mu::P$_R$- *ilvG*^{ValR}*MED*-2T$_{fd}$) (alternative names VKPM B-8066, V-2)	[21], [26] VKPM
S288C	*S. cerevisiae* (MATα mal gal2)	NBRC
Plasmids		
pPCRScript-kdcA-L.la	pMB1 ori; Amp^R; *kdcA*	This work
pMW118	pSC101 ori; Amp^R; MCS	GenBank accession number AB005475
pMW118-kdcA	pSC101 ori; Amp^R; P$_{lac}$-*kdcA*	This work
pMW118-kdcA-adh2	pSC101 ori; Amp^R; P$_{lac}$-*kdcA-ADH2*	This work

ninhydrin (2% w/v) in acetone was used as the visualizing reagent.

Reagents

Restriction enzymes, T4 DNA ligase, and high-fidelity polymerase chain reaction (PCR) enzyme mix were obtained from Fermentas (Vilnius, Lithuania). QIAquick Gel Extraction Kits and QIAGEN Plasmid Mini Kits were obtained from QIAGEN GmbH (Germany). Oligonucleotides were obtained from Sintol (Moscow, Russia). Schiff's reagent for aldehyde determination was from Fluka. Isobutanal, 2-methylbutanal, isobutanol and 2-methylbutanol were from Sigma.

DNA handling procedures

Protocols for genetic manipulation of *S. cerevisiae* [1] and *E. coli* [20], as well as techniques for isolating and manipulating nucleic acids were as described. Plasmid DNA was isolated with a QIAGEN Plasmid Mini Kit and genomic DNA was isolated with a Bacterial Genomic DNA Kit (Sigma). DNA fragments were isolated from agarose gels using a QIAquick Gel Extraction Kit (QIAGEN). Synthesis of the *kdcA* gene was performed by Sloning BioTechnology GmbH (Germany).
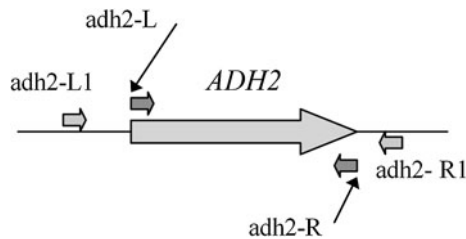
Plasmid construction

All plasmids used or constructed in this study are listed in Table 1.

1. Construction of pPCRScript-kdcA-L.la: To clone *kdcA*, synthesis of a *kdcA* gene (GenBank accession number AY548760) from *L. lactis* with a modified nucleotide sequence codon-optimized for *E. coli* and with EcoRI and BamHI restriction sites (5′-GAATTCGAAGGAGGGAAT

GTG-3′, 5′-GGATCCTTA-3′) was carried out by Sloning BioTechnology. In the modified *kdcA* sequence, the following 52 codons were optimized: 16 Thr codons—positions 2, 70, 113, 130, 137, 142, 185, 186, 188, 231, 237, 287, 291, 371, 385, and 459 (codon ACA was replaced with ACG); 8 Arg codons—positions 10, 133, 147, 156, 320, 421, 443, and 462 (codons CGA, CGG and AGA were replaced with CGC); 14 Pro codons—positions 23, 91, 99, 128, 158, 164, 175, 207, 250, 348, 352, 398, 408, and 448 (codons CCA, CCT, and CCC were replaced with CCG); 9 Leu—114, 153, 233, 238, 276, 302, 358, 440, and 523 (codon CTA was replaced with CTG); and 5 Ile—255, 304, 308, 332, and 453 (codon ATA was replaced with ATT). The resulting DNA fragment containing the *kdcA* gene was digested with EcoRI and BamHI and cloned into pPCR-Script (GenBank accession number U46017.1) cut with the same enzymes, creating pPCRScript-kdcA-Lla. The *kdcA* gene was cloned in the opposite orientation relative to the Lac promoter to reduce potential toxicity from gene expression.

2. Construction of pMW118-kdcA: To clone *kdcA*, pPCRScript-kdcA-Lla was digested with EcoRI and BamHI. The DNA fragment containing the *kdcA* gene was cloned into the pMW118 plasmid cut with the same enzymes, creating pMW118-kdcA.

3. Construction of pMW118-kdcA-adh2: The structural parts of the *ADH2* and *ADH1* genes from *S. cerevisiae* S288C have nearly 100% homology; therefore, cloning the *ADH2* gene was done by two-step PCR amplification using two pairs of primers (Fig. 1). Initially, to amplify the 1,520-bp DNA fragment containing the *ADH2* gene, *S. cerevisiae* S288C genomic DNA was used as a PCR template with primers adh2-L1 (5′-GACAAAAAAAATGATGGAAGACAC-3′) and adh2-R1 (5′-CAGGAAAGAGTTACTCAAGAAC-3′). PCR products were purified and used as a PCR template to amplify the *ADH2* gene with primers adh2-L (5′-CTGACTGGATCCAAGAAGGAGATATATATG
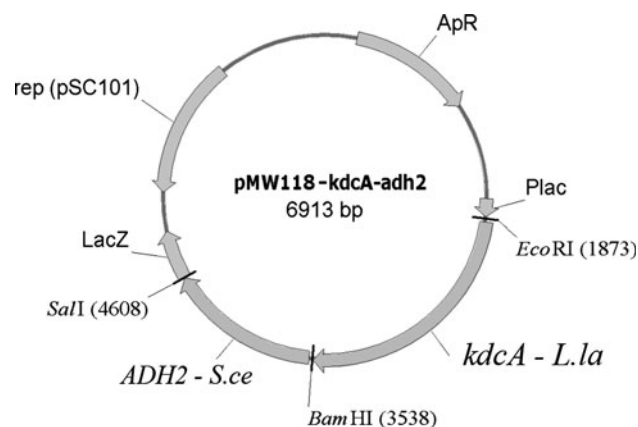
Fig. 1 The two-step polymerase chain reaction (PCR) amplification scheme for the *ADH2* gene from *Saccharomyces cerevisiae* S288C genomic DNA

TCTATCCCAGAAACTCAAAAAG -3′) and adh2-R (5′-GCCATAGTCGACTTATTTAGAAGTGTCAACAA CGTAT-3′). PCR products were digested with BamHI and SalI and cloned into pMW118-kdcA cut with the same enzymes, creating pMW118-kdcA-adh2 (Fig. 2).

Preparation of cell extracts

A culture of strain H-81 harboring either pMW118-kdcA or pMW118-kdcA-adh2 was grown overnight, and 0.1 ml was used to inoculate fresh medium (10 ml of M9 medium supplemented with 1/10 v/v LB). The cells were induced with 0.5 mM IPTG after inoculation. Inoculated cultures were grown for 4.5 h until an optical density at 540 nm of 0.8 was reached. Cells were harvested by centrifugation, washed twice in 1M sodium chloride and then in 0.1 M potassium–phosphate buffer (pH 6.3). Cells were suspended in 0.1 M potassium–phosphate buffer (pH 6.3) and disrupted by sonication. The supernatant after centrifugation at $12,000\times$ for 30 min was used as the cell extract. All steps were performed at temperatures <4°C. Protein concentrations were determined by the Bradford assay (Bio-Rad protein assay, GmbH).

Fig. 2 Map of the plasmid pMW118-kdcA-adh2

BCKAD assay

For branched-chain 2-keto acid decarboxylase (BCKAD) assays, cell extracts were incubated for 3 h at 30°C in a closed 2-ml vial at pH 6.3 (0.1 M potassium–phosphate buffer) with 0.1 M 2-ketoisovalerate (2-keto-3-methylvalerate) as the substrate and with 1 mM $MgCl_2$ and 0.1 mM thiamine pyrophosphate (TPP) as cofactors in a total volume of 1 ml. Then, 0.2 ml of Schiff's reagent was added into the reaction mixture to determine aldehyde concentration. After color development, samples were placed on ice. The concentrations of isobutanal and 2-methyl-butanal were determined spectrophotometrically at 553 nm using a spectrophotometer (Spectronic GENESYS 2, Thermo Electron Corporation). Relative 2-keto acid decarboxylase activity was calculated by dividing the amount of product formed (in nanomoles) by the reaction time (in minutes) and the protein content in the sample (in milligrams).

Branched-chain amino acid transaminase assay

Cell extracts were incubated for 15 min at 37°C in 2-ml vial at pH 7.5 [0.5 M Tris hydrochloride (HCl) buffer + 1 mM dithiothreitol (DTT)] with 50 mM Phe and 50 mM 2-ketoisovalerate (2-keto-3-methylvalerate) as the substrates and with 0.5 mM pyridoxal phosphate (PLP) as cofactor in a total volume of 200 μl. Then, the samples were placed on ice and 0.8 ml of 1.25 N NaOH was added to stop the reaction. The formation of phenylpyruvate was analyzed at 320 nm against a control to which NaOH had been added at 0 min. The OD was read immediately after addition of NaOH. A molar extinction coefficient of $17,500 \text{ M}^{-1} \text{ cm}^{-1}$ was used [6]. Specific activity is defined as nanomoles of phenylpyruvate formed per minute per milligram of protein.

Determination of isobutanol concentration in FM medium

The concentration of isobutanol in FM medium was determined by gas chromatography (GC) analysis. GC flame ionization detector (GC-FID) analysis was accomplished on a Varian 3600 gas chromatograph with 3 m × 3-mm (i.d.) stainless-steel column packed with 15% CARBOWAX 1500 on CHROMATON N-AW 0.15–0.2 mm. The injector temperature was 180°C, and injection volume was 1 μl. Nitrogen was used as the carrier gas with a flow rate of 30 ml/min. The column temperature was initially kept at 50°C for 3 min and then increased to 120°C at 10°C/min and held for 3 min. The detector temperature was 250°C. Methanol, ethanol, 1-propanol, isobutanol, and 1-butanol were separated under these conditions.

Table 2 Accumulation of valine and isobutanol by strain H-81 with the plasmid pMW118-kdcA

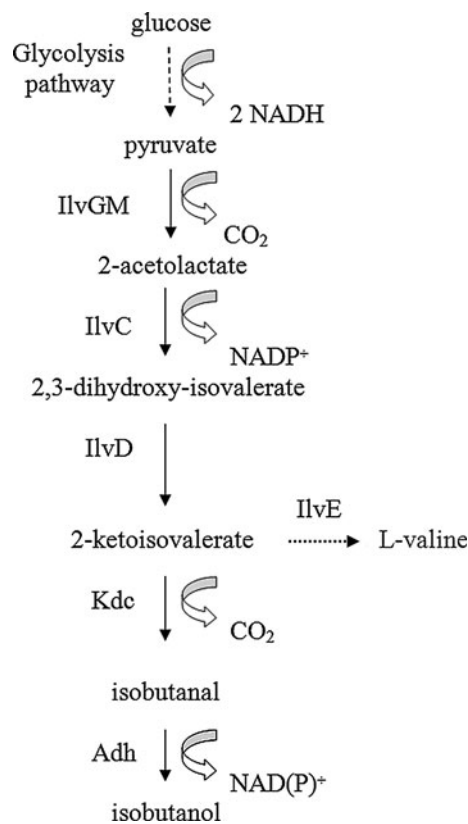| Strain	Inducer	Val (g/l)	OD$_{540}$	Isobutanol (g/l)
H-81/pMW118-kdcA	−IPTG[a]	8.5	31	0.29
	+IPTG	3.5	29	0.49
H-81/pMW118-kdcA[b]	+IPTG	3	16	6.35
H-81		12.5	33	<0.05
H-81[b]		8	18	<0.05

Data represent the mean of three separate experiments

[a] Isopropyl-thio-α-galactoside

[b] Rubber stopper instead of routinely used cotton stopper was applied; in this case glucose was not utilized completely and the level of residual glucose was 1% (in other cases the sugar was consumed completely)

Results and discussion

The valine-producing strain H-81 [21, 26] was used as a model host for isobutanol production from glucose. This strain converts glucose into valine mainly due to the expression of *ilvGMED* genes under control of the "strong" lambda phage P$_R$ promoter [19]. Additionally, this artificial operon (P$_R$-*ilvGMED*) contains the full-length gene *ilvG*. Due to this mutation, strain H-81 (in contrast to *IlvG*-deficient wild-type *E. coli* K-12) possesses a type II acetolactate synthase that is resistant to feedback inhibition by valine. This strain accumulated 12–13 g/l of valine from 60 g/l of glucose in test-tube cultivation (Table 2), thereby indicating the potential for equimolar synthesis of 2-ketoisovalerate. The latter compound can act not only as a keto precursor of valine but also as an isobutanol precursor (Fig. 3).

E. coli does not possess an enzyme capable of decarboxylating branched-chain keto acids (BCKAs). The possibility to use for this purpose heterologous *kivd* gene (GenBank Accession Number AJ746364) from *L. lactis* [8] was shown by Liao group [2, 3, 27]. In our work, we used the *kdcA* gene (GenBank accession number AY548760) from *L. lactis* [24], which shares almost 90% sequence identity with the *kivd* gene *L. lactis* [28]. The *kdcA* gene from *L. lactis* encodes a BCKA decarboxylase (EC 4.1.1.72) with broad substrate specificity [24, 28]. This enzyme enables conversion of BCKAs (2-ketoisovalerate, 2-keto-3-methylvalerate and 2-ketoisocaproate), which are the immediate keto precursors of valine, isoleucine, and leucine into the corresponding aldehydes (isobutanal, 2-methylbutanal and 3-methylbutanal, respectively) [24, 28]. The native nucleotide sequence of *kdcA* contains a number of codons that are rare in *E. coli*. These codons were optimized for expression in *E. coli* and are listed in "Materials and methods." Synthesis of the modified *kdcA* sequence was performed by Sloning Biotechnology GmbH. The

Fig. 3 Pathway of isobutanol biosynthesis. *IlvGM* acetolactate synthase II, *IlvC* acetohydroxy acid isomeroreductase, *IlvD* dihydroxy-acid dehydratase, *IlvE* branched-chain amino acid aminotransferase, *Kdc* 2-keto acid decarboxylase, *Adh* alcohol dehydrogenase

kdcA-containing EcoRI-BamHI DNA fragment was cloned into a high-copy-number pPCR-Script vector. The resulting plasmid (pPCRScript-kdcA-Lla) contains the *kdcA* coding region oriented away from the lactose promoter to avoid possible toxicity from *kdcA* gene expression.

The *kdcA* gene with rare codons substituted was subcloned into the low-copy-number vector pMW118. The resulting plasmid (pMW118-kdcA) contains the *kdcA* gene under the control of the P$_{lac}$ promoter. Introduction of this plasmid into the valine-producing H-81 strain and subsequent induction with IPTG resulted in a drop in valine production accompanied by low-level isobutanol accumulation during cultivation in test tubes under aerobic conditions (Table 2). Using a rubber stopper for cultivation of isobutanol-accumulating strains prevented alcohol evaporation and thereby increased isobutanol accumulation in culture broth (Table 2). No detectable isobutanol synthesis was observed in the absence of pMW118-kdcA (Table 2). Induction of *kdcA* expression initiated 2-ketoisovalerate decarboxylation, thereby decreasing valine production and yielding isobutanal. It is worth noting that the further conversion of isobutanal into the corresponding alcohol, isobutanol, is apparently due to the native *E. coli* alcohol dehydrogenases'

ability to carry out this reaction [3]. The *E. coli* genome includes at least the following six genes annotated as alcohol dehydrogenases (ADHs) or putative ADHs: *adhE, adhP, eutG, yiaY, yqhD* and *yjgB* [3, 14] (www.ecocyc.org). Higher alcohols containing more than three carbon atoms in the chain have been identified as preferred substrates for *yqhD*-encoded ADH [25]. Our data concerning isobutanol production by the valine-producing strain under conditions of *kdcA* expression are also in good agreement with the recent observation that the YqhD protein can act as an isobutanol dehydrogenase [3].

In addition to the native *E. coli* putative isobutanol dehydrogenases, a range of heterologous enzymes can be used for more effective conversion of isobutanal into isobutanol. A wide range of microorganisms possess this kind of activity. In particular, the yeast *S. cerevisiae* is responsible for degradation of BCAAs into fusel alcohols via the Ehrlich pathway [12, 13]. The *S. cerevisiae* genome harbors genes for a range of ADHs, which are carefully reviewed in [9, 13], some of which can participate in fusel alcohols biosynthesis. Five ethanol dehydrogenases, Adh1, Adh2, Adh3, Adh4, Adh5, and two nicotinamide adenine dinucleotide phosphate, reduced (NADPH)-dependent medium-chain alcohol dehydrogenases, Adh6 and Adh7, with broad substrate specificity are among them [9, 13]. From among these enzymes, we chose to use Adh2 for heterologous expression in *E. coli*. It is necessary to note that quite recently, due to careful investigation by Atsumi et al. [3], AdhA *L. lactis* and YqhD *E. coli* were established as preferable enzymes for isobutanol synthesis [3]. The *S. cerevisiae* isozymes Adh1 and Adh2 are practically identical except for one amino acid residue; Adh1 contains Met at position 294, whereas Adh2 contains Leu at this position. Nucleotide sequence analysis revealed the presence of several codons that are rare in *E. coli* in the structural part of *ADH2* (8 Arg codons encoded by AGA and 3 Leu codons encoded by CTA), but none were located in the beginning of the gene; therefore *ADH2* was cloned in its native form. The DNA fragment containing *ADH2* was obtained by two-step PCR amplification (as shown in Fig. 1) by using the genomic DNA of *S. cerevisiae* S288C as a template for the initial step. This fragment was subcloned into the plasmid pMW118-kdcA just downstream of *kdcA*. The resulting plasmid, pMW118-kdcA-adh2, contains an artificial inducible operon, P_{lac}-*kdcA-ADH2*. The level of both valine and isobutanol accumulation during test tube cultivation of the recombinant strain H-81/pMW118-kdcA-adh2 under IPTG induction was tested (Table 3). Data show that induction of the operon decreased valine synthesis to a level sixfold less than that of the control parent strain, H-81. The drop in valine synthesis was accompanied by isobutanol accumulation. Despite the variation in valine accumulation with and without induction, isobutanol production did not differ

Table 3 Accumulation of valine and isobutanol by strain H-81 with the plasmid pMW118-kdcA-adh2

Strain	Inducer	Val (g/l)	OD$_{540}$	Isobutanol (g/l)
H-81/pMW118-kdcA-adh2	−IPTG[a]	11	34	0.56
	+ IPTG	2	30	0.91
H-81		13	32	<0.05

Data represent the mean of three separate experiments

[a] Isopropyl-thio-α-galactoside

Table 4 Relative activity of branched-chain 2-keto acid decarboxylase (BCKAD) in strain H-81 with the plasmids pMW118-kdcA and pMW118-kdcA-adh2

Strain	Relative activity of BCKAD, nmol/min·mg			
	2-ketoisovalerate		2-keto-3-methylvalerate	
	−IPTG[a]	+IPTG	−IPTG	+IPTG
H-81/pMW118-kdcA	30	150	40	205
H-81/pMW118-kdcA-adh2	30	185	30	280

Data represent the mean of three separate experiments

[a] Isopropyl-thio-α-galactoside

greatly between these two cases. Evidently, this fact indicated that a considerable part of the newly synthesized isobutanol evaporated during aerobic cultivation. The absence of a marked drop in biomass due to toxic levels of isobutanol after IPTG induction supports this idea. Aerobic incubation of test tubes with cell-free medium initially containing 10 g/l of isobutanol resulted in the evaporation of >80% of the initial alcohol (the final concentration of isobutanol after incubation was <2 g/l). Thus, our data indicate that expression of two heterologous genes encoding BCKAD and ADH in *E. coli* redirected the carbon flux from the synthesis of valine to that of isobutanol; but for all tested aerobic conditions, the yield of isobutanol was 1.5% (g isobutanol/g glucose). To accumulate higher levels of isobutanol in the culture broth, it is necessary to perform cultivation under special conditions that prevent evaporation. In this case, when microaerobic conditions arise, it is necessary to disrupt a number of genes coding for pyruvate-degrading enzymes that are activated anaerobically, as was suggested by Liao and coworkers [2].

Measurement of BCKAD activity in recombinant strains containing the *kdcA* gene from *L. lactis* under control of the P_{lac} promoter confirmed the presence of IPTG-inducible decarboxylase activity (Table 4). Also, it is important to note that the enzymatic activity toward 2-keto-3-methylvalerate, the keto precursor of isoleucine, was 1.5 times higher than the activity toward 2-ketoisovalerate, the keto precursor of valine (Table 4). These data agree with the results of

detail analysis of substrate specificity of KdcA *L. lactis* performed by Yep and coworkers [28].

Expression of the P_{lac}-*kdcA-ADH2* operon did not completely prevent valine oversynthesis (Table 3). Apparently there exists competition between the IlvE-mediated amination of 2-ketoisovalerate and utilization of this keto acid by BCKAD and further conversion by ADH. The level of IlvE activity for H-81 was 340 nmol/min/mg. It is necessary to take into account that the host strain H-81 contains several chromosomal copies of the *ilvGMED* operon under control of the "strong" lambda phage P_R promoter, whereas the *kdcA* and *ADH2* genes are in an artificial operon under control of the "moderate" P_{lac} promoter. The moderate promoter was chosen as a first approach to prevent possible accumulation of toxic aldehyde in case of imbalance between its synthesis and further consumption. Therefore, imbalance between effective 2-ketoisovalerate synthesis and its further insufficient utilization in the Ehrlich pathway seems quite possible, thereby indicating the need to optimize expression of the *kdcA-ADH2* artificial operon.

Conclusions

This work highlights the possibility of using BCAA biosynthetic pathways for biofuel production from renewable resources. Using as a model the *E. coli* valine-producing strain with the overexpressed genes encoding acetolactate synthase II, the aerobic synthesis of isobutanol from glucose was achieved by coupling BCAA biosynthetic pathways in the host with heterologous Ehrlich pathway, which completely support the data first obtained by Liao et al. [2, 3, 27]. It was shown that the valine-producing *E. coli* strain H-81 can be re-engineered for isobutanol production by introducing an artificial operon consisting of two heterologous genes: *kdcA* from *L. lactis* and *ADH2* from *S. cerevisiae*. Also, the ability of *E. coli* to convert isobutanal into isobutanol by a native ADH was confirmed, and YqhD is considered to be one of the promising candidates. To further improve isobutanol production, the balance between 2-keto acid synthesis and utilization should be maintained by optimizing *kdcA* and *ilvE* gene expression. A high level of *ilvE* expression is not needed. Toxicity of higher alcohols remains a difficult problem. Isobutanol tolerance of *E. coli* was studied in detail [4]. Only few reports have addressed the potential of alternative hosts for alcohol production and focused on the selection of microorganisms tolerant to these compounds [15, 17]. Considering the significant recent progress in the development of membrane technologies for higher alcohol separation and the promising results of screening for and characterizing alcohol-tolerant species, further engineering of microorganisms for effective conversion of biomass-derived sugars into fusel alcohols should create a basis for implementing this approach to the production of biotechnological fuels.

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