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BIOENERGY/BIOFUELS/BIOCHEMICALS

Production of C4 and C5 branched-chain alcohols by engineered *Escherichia. coli*

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Abstract Higher alcohols, longer chain alcohols, contain more than 3 carbon atoms, showed close energy advantages as gasoline, and were considered as the next generation substitution for chemical fuels. Higher alcohol biosynthesis by native microorganisms mainly needs gene expression of heterologous keto acid decarboxylase and alcohol dehydrogenases. In the present study, branched-chain α-keto acid decarboxylase gene from *Lactococcus lactis subsp. lactis* CICC 6246 (Kivd) and alcohol dehydrogenases gene from *Zymomonas mobilis* CICC 41465 (AdhB) were transformed into *Escherichia coli* for higher alcohol production. SDS-PAGE results showed these two proteins were expressed in the recombinant strains. The resulting strain was incubated in LB medium at 37 °C in Erlenmeyer flasks and much more 3-methyl-1-butanol (104 mg/L) than isobutanol (24 mg/L) was produced. However, in 5 g/L glucose-containing medium, the production of two alcohols was similar, 156 and 161 mg/L for C4 (isobutanol) and C5 (3-methyl-1-butanol) alcohol, respectively. Effects of fermentation factors including temperature, glucose content, and α-keto acid on alcohol production were also investigated. The increase of glucose content and the adding of α-keto acids facilitated the production of C4 and C5 alcohols. The enzyme activities of pure Kivd on α-ketoisovalerate and α-ketoisocaproate were 26.77 and 21.24 μmol min^{-1} mg^{-1}, respectively. Due to its ability on decarboxylation of α-ketoisovalerate and α-ketoisocaproate, the recombinant *E. coli* strain showed potential application on isoamyl alcohol and isobutanol production.

Keywords 3-methyl-1-butanol · Isobutanol · α-keto acid decarboxylase · Alcohol dehydrogenase

Introduction

Due to the finite storage of fossil fuel and changeable oil price, developing alternative energies such as ethanol and other chemical compounds has become a potential approach. Branched-chain alcohols, including isobutanol and 3-methyl-1-butanol, can serve as biofuel and chemical feedstock and attracts a high interest from research and industrial areas. Compared to fuel ethanol, higher alcohols are perfect options for gasoline substitution due to their low vapor pressure, lower hygroscopicity, low water-solubility, and compatibility with current infrastructure, high energy density, and similar combustion properties to gasoline [1, 3, 14, 23]. In addition, isobutanol and 3-methyl-1-butanol are important chemical compounds widely used in chemical industry and food industry [9, 18].

The transformation of branched-chain amino acid to acids or alcohols was first described about a century ago [10]. The transaminations of these amino acids produce corresponding α-keto acids, which can be decarboxylated into aldehyde and subsequently reduced into fusel alcohol via the Ehrlich pathway by some microorganisms such as *Saccharomyces* and *Lactococcus* [10, 15]. The introduction of Ehrlich pathway into the model microorganism *E. coli* for higher alcohol biosynthesis from glucose was first initiated by Atsumi [1]. As depicted by Atsumi, *n*-butanol, isobutanol, 3-methyl-1-butanol, and 2-methyl-1-butanol can be

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produced through the biosynthesis pathway of norvaline, valine, leucine, and isoleucine, respectively. The intermediate of valine metabolism, α-ketoisovalerate (KIV), is the precursor of isobutanol and can be converted into alcohol by keto acid decarboxylase and alcohol dehydrogenases, the last two steps of Ehrlich pathway. Over-expression of genes of decarboxylase and dehydrogenase can enhance the production of isobutanol. This method had been applied to the synthesis of other higher alcohols successfully, though the alcohol yields were varied according to different alcohols. Numbers of organisms, including *E. coli*, *Bacillus subtilis*, and *Corynebacterium glutamicum,* were modified with this approach for C4 and C5 alcohols production [2, 5, 16, 23, 26].

The conversion of α-keto acids to alcohols was catalyzed by α-keto acid decarboxylases (KDCs) and alcohol dehydrogenases (ADHs), among which KDC is the rate-limiting enzyme in Ehrlich pathway. Therefore, regulation of these enzymes offers good prospects for higher alcohol production. A number of KDCs have been identified in various organisms, including pyruvate decarboxylase (EC 4.1.1.1) [21], phenylpyruvate decarboxylase (EC 4.1.1.43) [11, 24], branched-chain 2-oxoacid decarboxylase (EC 4.1.1.72) [19, 25], 2-oxoglutarate decarboxylase (EC 4.1.1.71) [20], and indole-3-pyruvate decarboxylase (IPD) (EC 4.1.1.74) [12], which are classified by their substrate specificities. KDCs responsible for KIV conversion from *Lactococcus lactis*, *Psychrobacter cryohalolentis* and other organisms were identified, over-expressed, and characterized [8, 22, 25]. In the present study, KDC, named Kivd, from *Lactococcus lactis* and ADH from *Zymomonas mobilis* were transformed into *Escherichia coli* for higher alcohol production; the resulted strain can produce both isobutanol and 3-methyl-1-butanol. The fermentation factors were studied in detail, and the Kivd was also purified and partially characterized.

Materials and methods

Bacterial strains, media, and growth conditions

Lactococcus lactis subsp. lactis CICC 6246 and *Z. mobilis* CICC 41465 were purchased from China Center of Industrial Culture Collection. *E. coli* strains trans1-T1 and BL21(DE3) (TransGen, Beijing, China) were employed for gene cloning and protein expression, respectively.

Lactococcus lactis subsp. lactis CICC 6246 was cultured in MRS broth containing 1 % casamino acids, 1 % beef extract, 0.5 % yeast extract, 0.5 % sodium acetate, 0.2 % disodium citrate, 0.1 % Tween 80, 0.2 % K_2HPO_4, 0.02 % $MgSO_4{\cdot}7H_2O$, and 0.005 % $MnSO_4{\cdot}H_2O$. An overnight culture was inoculated by 1 % of inoculum into 15 mL of fresh medium in a 100-mL Erlenmeyer flask and grown at 37 °C in a rotary shaker overnight.

Zymomonas mobilis CICC 41465 was grown in CM medium containing 10 % glucose, 0.5 % Yeast Extract, 0.1 % $(NH_4)_2SO_4$, 0.1 % KH_2PO_4, 0.1 % $MgSO_4$. $7H_2O$. An overnight culture was inoculated into a 3-mL fresh medium in a 1-mL screw-cap flask, and still cultured at 30 °C for 48 h.

DNA manipulation

The cells of *L. lactis subsp. lactis* CICC 6246 and *Z. mobilis* CICC 41465 were pestled with liquid nitrogen, and the genomic DNA were extracted using TIANamp bacteria DNA kit (Tiangen, Beijing, China).

The DNA sequence of *kivd* gene was cloned from *L. lactis subsp. lactis* CICC 6246 genomic DNA with expression primer set of Kivd-F (CG*GGATCC*GATGTATACAGTAGGAGATTACC) and Kivd-R (GC*GTCGAC*TTATGATTTATTTTGTTCAGC), with the restriction sites of *Bam*HI and *Sal*I in italics. To clone *adhB* gene, *Z. mobilis* CICC 41465 genomic DNA was used as a PCR template with prime pair adhB-F (GC*GTCGAC*GGTTGTTTTCGGGTTGTTGC) and adhB-R (GG*GCGGCCGC*TTAGAAAGCGCTCAGGAAGAG), with the restriction sites of *Sal*I and *Not*I in italics. PCR products were ligated into pEASY-T3 vector for sequencing.

The correct *kivd* gene fragment was amplified from sequencing vector and digested with *Bam*HI and *Sal*I and cloned into vector pET-28a(+)(Novagen, Darmstadt, Germany) with the same enzyme digestion, creating pET-*kivd*. *AdhB* gene fragments were digested with *Sal*I and *Not*I and cloned into pET-*kivd* cut with the same enzymes, creating pET-*kivd-adhB*. The pET-*kivd-adhB* plasmid was verified by restriction enzyme digestion using *Bam*HI, *Sal*I, and *Not*I, and then sequencing performed. The recombinant plasmid pET-*kivd-adhB* was transformed into *E. coli* BL21(DE3) competent cells, creating recombinant strain *E. coli*-Kivd-AdhB.

The nucleotide sequences were assembled and analyzed by Vector NTI advance 7.0 (Invitrogen). Alignment of nucleotide and deduced amino acid sequences was carried out with online Blastn and Blastp programs (http://www.ncbi.nlm.nih.gov/BLAST/), respectively.

Expression and purification of Kivd

The recombinant strains *E. coli*-Kivd-AdhB were inoculated in fresh LB and cultured for 2–3 h at 37 °C until the OD_{600} reached 0.6; final concentration of 0.6 mM of isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added for protein expression, and cells were then cultured at 30 °C for another 6 h. Cells were centrifuged at 12,000×g at 4 °C for 10 min.

The resulting pellet was resuspended in lysis buffer (20 mM Tris–HCl [pH 7.0]) and disrupted with an Ultrasonic processor VCX 750 (Sonics) on ice for 10 min with procedures of 10 s short burst followed by interval of 20 s for cooling. Cell debris was removed by centrifugation.

The supernatant containing Kivd was subjected to Ni^{2+}-nitrilotriacetic acid (NTA) chromatography and washed with a linear gradient of 2–300 mM imidazole in 50 mM Tris–HCl–0.5 M NaCl (pH 7.6) buffer. The elution fractions were pooled and assayed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [13]. The protein concentration was determined by the Bradford assay with bovine serine albumin as the standard [4].

α-Keto-acid decarboxylase activity assay

The reaction was performed in 50 mM sodium phosphate buffer (pH 6.0), containing 10 mM α-ketoisovalerate, 5 mM $MgCl_2$, 1.5 mM thiamin diphosphate (ThDP) and appropriately diluted enzyme solution. After incubation at 37 °C for 20 min, the reaction was stopped by lowering the pH to 2–3 with 6 N HCl. The production of isobutyraldehyde was quantified by high-performance liquid chromatography system (HPLC) (Waters2498, MA, US). A shodex sugar SH-1011 column (Waters2498, MA,US) equipped with UV-RI detector was used. The column was eluted with 0.005 M H_2SO_4 at a flow rate of 0.5 ml min^{-1} at 50 °C. Detector temperature was maintained at 50 °C.

The assay of enzyme activity towards α-ketoisocaproate was performed as that of α-ketoisovalerate, according to the production of isovaleraldehyde, which was measured to quantify the substrate specificity. One unit of decaroxylase activity (U) was defined as the amount of enzyme that produced 1 μmol aldehydes per min under standard conditions.

Production of alcohol compounds

To produce alcohol compounds, 1 % overnight cultures in LB broth of recombinant strain *E. coli*-Kivd-AdhB were inoculated into 100 mL fresh LB medium in 500 mL Erlenmeyer flask and grown at 37 °C in a rotary shaker for 3 h. The culture was then induced with 0.6 mM IPTG and continuously cultured for 12 h. Samples was taken for alcohol compound detection.

The glucose-containing medium, M9 medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 1 mM $MgSO_4{\cdot}7H_2O$, 1 mM $CaCl_2$, 5 g/L glucose), was used for further alcohol production fermentation. The influence of glucose concentration on alcohol production was also examined with 5 g/L and 10 g/L glucose, respectively.

To examine the effects of α-keto acid on alcohol production, 100 mg/L of α-ketoisovalerate (KIV) and α-ketoisocaproate (KIC) was separately added to the M9 medium with 10 g/L glucose.

Cell concentration measurement

The cell concentration was represented by OD_{600} and measured using a spectrophotometer (Libra S12, Biochrom, Cambridge, UK).

Detection of metabolites

The produced alcohol compounds were identified by gas chromatograph-mass spectrometry (GC–MS). The system consisted of model 6890A network GC system (Agilent Technologies, CA, US) and a model 5975C detector (Agilent Technologies, CA, US). A HP-INNOWAX column (30 m, 0.25 mm internal diameter, 0.25 mm film thickness; Agilent Technologies, CA, US) was used, with hydrogen as the carrier gas. Oven temperature was initially kept at 75 °C for 2 min, then raised to 240 °C at the rate of 10 °C min^{-1}, and maintained 2 min. The injector and detector were maintained at 250 °C. Chloroform solvent extraction was used to isolate alcohol compounds. 1 μL sample was injected in splitless injection mode.

The produced alcohol compounds were quantified by a gas chromatograph equipped with flame ionization detector (GC-FID). The GC-2014 gas chromatograph was purchased from Shimadzu (Kyoto, Japan). The separation of alcohol compounds was carried out by A DB-FFAP capillary column (30 m, 0.32 mm internal diameter, 0.25 mm film thickness; Agilent Technologies (CA, US)). GC oven temperature was initially held at 60 °C for 1 min, raised with a gradient of 20 °C min^{-1} until 200 °C, and held for 3 min. Argon was used as the carrier gas. The injection and detector temperatures were maintained at 180 °C and 250 °C, respectively. A 10-μL sample was injected with 30:1 split ratio injection mode.

Nucleotide sequence accession number

The nucleotide sequence of the α-keto acid decarboxylase gene (*kivd*) from *L. lactis subsp. lactis* CICC 6246 was deposited in GenBank database under accession no. KR347479.

Results

Gene cloning and sequence analysis

Herein we cloned a α-keto acid decarboxylase encoding gene of 1647 bp, named *kivd,* from *L. lactis subsp. lactis* CICC 6246, and *adhB* gene of 1152 bp length from *Z.*

mobilis CICC 41465. Sequence analysis showed that kivd shared the highest identification with an indole-3-pyruvate decarboxylase from *Lactococcus lactis* (100 %, WP_012897921) and pyruvate decarboxylase from *Lactococcus lactis* (99 %, WP_023189172), followed by alpha-ketoisovalerate decarboxylase from *Lactococcus lactis subsp. Lactis* IFPL730 (98 %, CAG34226) [7] and a branched-chain keto acid decarboxylase (Kdca) from *Lactococcus Lactis* NIZO B1157 (88 %, AY548760) [22].

Gene expression of *kivd* and *adhB* in *E. coli*

The *kivd* and *adhB* gene fragments were cloned and linked to vector pET-28a(+), creating pET-*kivd-adhB* (Fig. 1) and then transformed into *E. coli* BL21(DE3) competent cells under the manufacturer's instruction. SDS-PAGE results showed that two thick protein bands with molecular weight of 61 and 40 kDa were found in the recombinant strains (Fig. 2), which indicated gene expression of *kivd* and *adhB* in *E. coli* cells.

Production of alcohols on LB medium

The cells harboring pET-*kivd-adhB* were inoculated into fresh LB medium and induced by IPTG for alcohol production. The alcohol mixtures identified by GC–MS were 2-methyl-1-propanol (isobutanol) and 3-methyl-1-butanol (isoamyl alcohol). The fermentation broth was sampled at interval of 2 h to quantify the yield of alcohols, as shown in Fig. 3. After 12 h of fermentation, the concentrations of 3-methyl-1-butanol and isobutanol were 104 mg/L and 24 mg/L, respectively.

Effects of temperature on cell growth and 3-methyl-1-butanol production

To determine the effects of temperature on cell biomass growth and alcohol production, the fermentation broth was cultured at 30 and 37 °C separately. Cell growth curve showed that recombinant *E. coli* reached saturated period within 8 h at 37 °C, which is faster than that at 30 °C (12 h) (Fig. 4).

The production rate of 3-methyl-1-butanol was sharply influenced by fermentation temperature. It took about 8 and 12 h to achieve the highest alcohol titer of 3-methyl-1-butanol at 37 °C and 30 °C, respectively. The faster accumulation of alcohol at 37 °C may be due to the higher cell biomass and metabolic rate. Because of the faster alcohol producing rate at 37 °C, this temperature was applied for further studies.

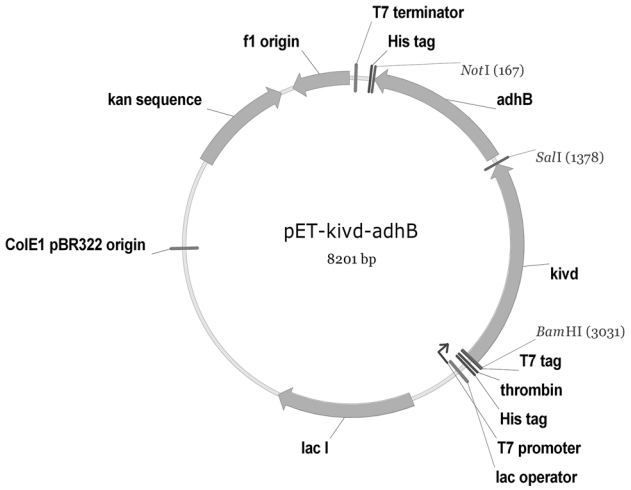

Fig. 1 Plasmid map of the recombinant pET-*kivd-adhB*

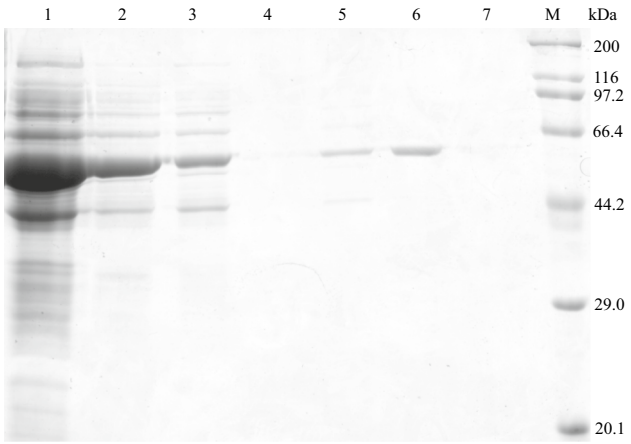

Fig. 2 SDS-PAGE analyses of expression and purification of the recombinant Kivd. Lanes: *M* protein standard; *1*, total intracellular protein of recombinant *E. coli* containing pET-kivd-adhB; *2–7*, the elution fractions

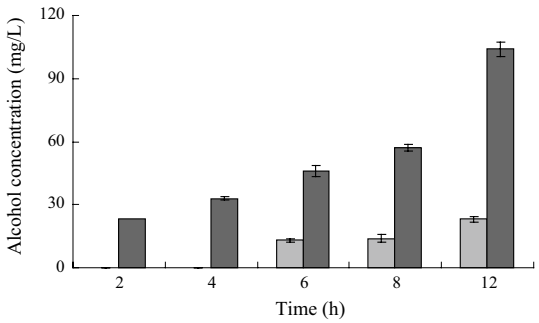

Fig. 3 Alcohol production in LB medium at 37 °C by recombinant *E. coli* harboring pET-kivd-adhB. *Black square*, Isobutanol; *deep black square* 3-methyl-1-butanol

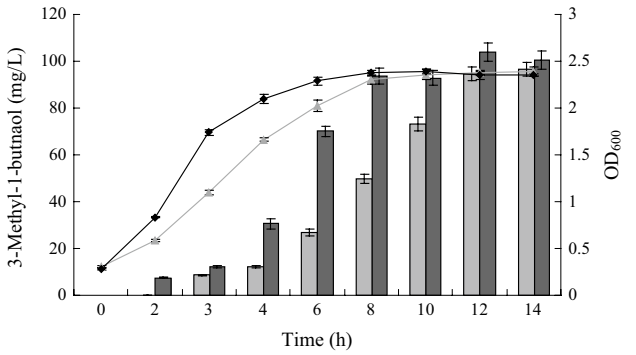

Fig. 4 Effects of temperature on cell growth rate and 3-methyl-1-butanol production of recombinant *E. coli* strain in LB medium. *Triangle* OD600 value at 30 °C; *Diamond* OD600 value at 37 °C; *black square* alcohol content at 30 °C; *deep Black square*, alcohol content at 37 °C

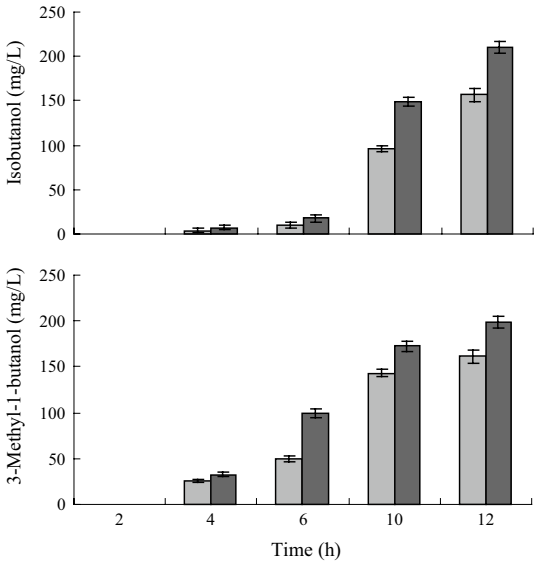

Fig. 5 Effects of glucose content on the production of isobutanol and 3-methyl-1-butanol by recombinant *E. coli* in M9 medium at 37 °C, *black square* 5 g/L glucose; *deep black square* 10 g/L glucose

Production of branched-chain alcohols on M9 medium

To attain higher concentration of 3-methyl-1-butanol, M9 medium containing glucose was used for alcohol production. It was found that isobutanol and 3-methyl-1-butanol with close concentration were produced in M9 medium with recombinant *E. coli* containing pET-*kivd-adhB*. The influence of initial glucose concentration on alcohol production was also studied. 5 g/L and 10 g/L initial glucose content resulted in maximum isobutanol and 3-methyl-1-butanol of 157 and 162 mg/L, 211 and 199 mg/L, respectively (Fig. 5).

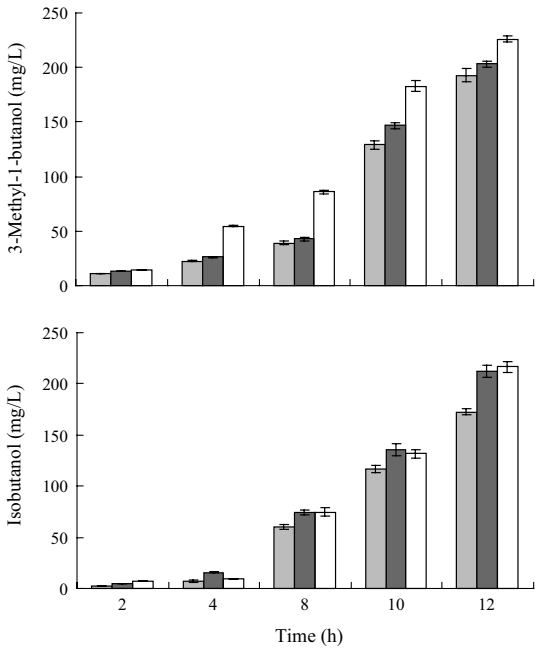

Fig. 6 Effects of α-keto acid on the production of alcohols by recombinant *E. coli* in M9 medium at 37 °C, *black square* fermentation broth without α-keto acid; *deep black square* fermentation broth with KIV; *white square* fermentation broth with KIC

It has been reported that the production of isobutanol is about 3 times of 3-methyl-1-butanol after introduction of *kivd* gene into *E. coli* strain [1, 6]. Our results showed that the concentration of these two alcohols were almost similar, which is different from previous reports.

Effects of α-keto acid precursor on production of 3-methyl-1-butanol

Due to the close productivities of isobutanol and 3-methyl-1-butanol by recombinant *E. coli* containing Kivd, 100 mg/L KIV and KIC was added to the alcohol fermenting medium with IPTG. The addition of KIV and KIC resulted in increase of isobutanol production by 23 and 25 %, respectively. 3-Methyl-1-butanol production increased about 17 % by adding KIC and only slightly increased (5 %) with the presence of KIV (Fig. 6).

Enzyme activity specificity

To verify the enzyme specificity for the substrate, the Kivd protein was purified by Ni^{2+}-NTA agarose chromatography system, and one pure band about 61 KDa was confirmed according to SDS-PAGE analysis (Fig. 2). The pure recombinant Kivd was applied for enzyme assay with the substrates of KIV and KIC. The decarboxylase activity of Kivd on KIV and KIC was 26.77 and 21.24 μmol min^{-1} mg^{-1},

respectively, which showed very close decarboxylation ability on KIV and KIC.

Discussion

Kivd gene encoding α-keto acid decarboxylanse from *L. lactis subsp. lactis* CICC 6246 and *adhB* gene encoding alcohol dehydrogenase from *Z. mobilis* CICC 41465 were cloned into *E. coli* BL21(DE3). The resulting recombinant strain can produce isobutanol and 3-methyl-1-butanol as expected. Previous reports about *E. coli* or other microorganisms with heterologous decarboxylanse can produce much more isobutanol than isoamyl alcohol [1, 17, 23], which is very different from our study reports. The *E. coli* JCL16 with the entire metabolic pathway from pyruvate to 3-methyl-1-butanol can produce about 2 g/L isobutanol and only 56 mg/L 3-methyl-1-butanol after 18 h of induction with IPTG [6]. The same phenomenon was also observed in recombinant *C. glutamicum* strain harboring *alsS-ilvCD* and *kivd*, which can produce 2.2 g/L isobutanol and 0.4 g/L 3-methyl-1-butanol [23]. However, it was very interesting for us to find that the amount of 3-methyl-1-butanol (104 mg/L) was much higher than isobutanol (24 mg/L) when the recombinant harboring pET-*kivd-adhB* was incubated on LB medium. Even in glucose containing medium fermentation, the titers of these two alcohols were almost similar.

The KIVD-LL protein was further purified and characterized with substrate specificity. Enzyme assay results showed that the activity of α-keto acid decarboxylase on KIC (21.24 μmol min^{-1} mg^{-1}) was very close to that on KIV (26.77 μmol min^{-1} mg^{-1}). The known reports about the activity of branched-chain α-keto acid decarboxylase all showed that the enzyme exhibited highest activity towards KIV. de la Plaza [7] identified and characterized an α-ketoisovalerate decarboxylase from *L. lactis* IFPL730 with the highest specific activity (80.7 U mg^{-1}) for KIV, which is about fourfold and sixfold less active with KIC (18.3 U mg^{-1}) and a-ketomethylvalerate (13.5 U mg^{-1}), respectively. Branched-chain α-keto acid decarboxylase (KdcA) from *L. lactis* B1157 [22] also exhibited the highest activity for KIV, the decarboxylation for KIC is about 30 % of the highest. Although the Kivd exhibited highest specific activity for KIV in present studies, the decarboxylase activity for KIC was about 80 % of the highest.

Due to the high specificities for KIV and KIC, the decarboxylase has great potential application on 3-methyl-1-butanol production, which was well verified by the recombinant *E. coli* strain containing pET-*kivd-adhB*. The low concentration of alcohol compounds may be due to the lack of α-keto acid precursors. The artificial addition of KIV and KIC to the fermentation broth obviously improved both alcohol formations. It was reported that the addition of α-keto acid can efficiently increase the corresponding alcohol flux by 2– to 23-fold and decrease other metabolites markedly [1]. However, the addition of KIV and KIC can improve the production of both C4 and C5 alcohols by about 5–25 % in present studies. The low increase level may be due to the low adding amount of keto acid (100 mg L^{-1}). The effects of KIC addition on isobutanol production (25 %) may because of the accumulation of KIC, which had feedback inhibition on the LeuABCD metabolic pathway, the leucine biosynthesis pathway, which further facilitated KIV conversion into isobutanol. To attain more C5 alcohols, the production enhancement of KIV with acetyl-coenzyme-A is needed. Reports showed that low expression level of *leuA*, the enzyme catalyzes reaction mentioned above, was the bottleneck for KIC production [6]. Further modification including elimination of end inhibition of *ilvE* (encoding branched-chain-amino-acid transferase) may contribute to carbon transfer to leucine biosynthesis and further transfer to 3-methyl-1-butanol.

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