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ORIGINAL ARTICLE

Engineering *Corynebacterium glutamicum* Mutants for 3-Methyl-1-butanol Production

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Abstract

3-Methyl-1-butanol (3MB) is a promising biofuel that can be produced from 2-ketoisocaproate via the common ʟ-leucine biosynthesis pathway. *Corynebacterium glutamicum* was chosen as a host bacterium because of its strong resistance to isobutanol. In the current study, several strategies were designed to overproduce 3MB in *C. glutamicum* through a non-fermentation pathway. The engineered *C. glutamicum* mutant was obtained by silencing the pyruvate dehydrogenase gene complex (*aceE*) and deleting the lactic dehydrogenase gene (*ldh*), followed by mutagenesis with diethyl sulfate (DES) and selection with Fmoc-3-4-thiazolyl-ʟ-alanine (FTA). The mutant could produce 659 mg/L of 3MB after 12 h of incubation. To facilitate carbon flux to 3MB biosynthesis, the engineered recombinant was also constructed without branched-chain acid aminotransferase (*ilvE*) activity by deleting the *ilvE* gene. This recombinant could produce 697 mg/L of 3MB after 12 h of incubation.

Keywords 3-Methyl-1-butanol · ʟ-Leucine · *Corynebacterium glutanicum* · Mutation breeding · Non-fermentation pathway

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Introduction

Due to the exhaustion of petroleum resources and global warming, renewable and clean biofuels have become areas of intense research in recent years (Li 2011). Alcohol-based fuels are an environmentally friendly energy source with high octane value, which can completely or partly replace gasoline and ease the shortage of fossil fuels. Isoamyl alcohol, a higher chain alcohol, can not only be widely used in many industries, but can also prolong the life of automotive parts by effectively reducing carbon deposits in spark plugs, valves, and pistons (Li 2011; Liu et al. 2008). In addition, 3-methyl-1-butanol (3MB) is a very versatile solvent and chemical reagent that can also be used to synthesize many important chemical products (Wang et al. 2012). Presently, 3MB receives much interest as an ideal supplement or a sustainable replacement for gasoline due to its distinguished advantages, such as its ability to be blended with gasoline at any ratio; compatibility with current infrastructures; and higher energy density and lower hygroscopicity when compared with bioethanol (Jojima et al. 2013). With its wide application range and potential as a substitute fuel, the development of 3MB production processes is desirable.

In 2008, the non-fermentation-based pathways for synthesis of branched-chain higher alcohols were described (Atsumi et al. 2008). This new way of producing higher alcohols combined the amino acid synthesis pathways with decarboxylation and reduction pathways of α-ketoacid, which is a common precursor of amino acid synthesis (Zhang et al. 2008). On the other side, *Corynebacterium glutamicum*, isolated from soil, was described as a short, aerobic, gram-positive rod capable of converting glucose into high yields of L-glutamic acid within a few days under optimal conditions (Kalinowski et al. 2003). In previous studies, *Escherichia coli* and *Saccharomyces cerevisiae* were chosen as host bacteria because of the clear genetic background (Connor et al. 2010; Hohmann 2002); however, *C. glutamicum* has stronger resistance to isobutanol than *E. coli* (Smit et al. 2005). Nevertheless, there are some reports on the construction of high-yielding isoamyl alcohol strains. For instance, Xiao et al. (2016) reported that overexpression of *kivd* and *adh3*, and inactivation of *aceE* and *ldh*, led to the production of 41.42 mg/L/h of 3MB in *C. glutamicum*. By mutating the start codon of the gene *ilvE* to reduce its activity in a high-production L-leucine *C. glutamicum* strain, inhibition of *ldhA*, and overexpression of *aro10* and *yqhD* was achieved. This engineered strain could produce 2.76 g/L 3MB after 48 h (Vogt et al. 2014, 2015, 2016). Additionally, mutagenesis is a good way to improve the capacity of the expression host, which is important to further increase the production of higher alcohols by optimized metabolic flux. Undirected whole-cell mutagenesis (UWCM) and gene manipulation were employed to increase 3MB production in *C. crenatium* (Su et al. 2016). The biosynthesis pathway of 3MB is shown in Fig. 1 (Bueckle-Vallant et al. 2014; Vogt et al. 2016). Additionally, a knockout of *aceE* in the pyruvate dehydrogenase complex (PDHC) and *ldh* blocked the pyruvate metabolic bypass to lactate and acetyl-CoA while increasing the metabolic flux from pyruvate to α-ketoisovalerate, thereby improving 3MB production

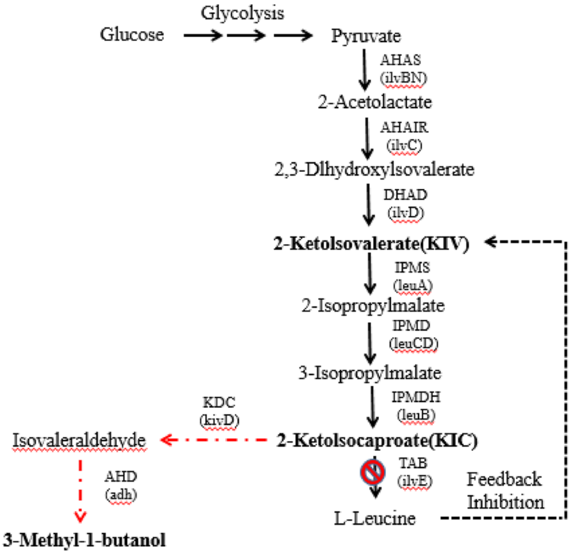


Fig. 1 The pathway from pyruvate towards 3MB in *C. glutamicum.* Enzymes and their corresponding genes are shown in parentheses. *AHAS* acetohydroxyacid synthase, *AHAIR* acetohydroxyacid isomeroreductase, *DHAD* dihydroxyacid dehydratase, *IPMS* isopropylmalate synthase, *IMPD* isopropylmalate dehydratase, *IPMDH* isopropylmalate dehydrogenase, *TAB* transaminase B, *DH* alcohol dehydrogenase, *KDC* α-Ketoisovalerate decarboxylase. The red dash lines indicate the synthesis pathway of 3-methyl-1-butanol after pEC-kivd-rbs-adh3 vectors are transformed into the strains

(Xiao et al. 2016). Gene ilvE could catalyze the conversion of 2-ketoisoacetic acid to L-leucine, which were knocked out could block the L-leucine assimilation pathway and improve the 3MB production. Thanks to the availability of the completed *C. glutamicum* genome and amino acid synthesis technology, we propose a novel idea for 3MB biosynthesis through a non-fermentation pathway using *C. glutamate*'s α-ketoacid metabolism. To achieve 3MB overproduction in *C. glutamate*, two main strategies are adopted in the current study. Firstly, an Fmoc-3-4-thiazolyl-L-alanine (FTA; a structural analog to L-leucine)-resistant mutant of *C. glutamicum* ATCC13032Δ*aceE*Δ*ldh* was developed for 3MB over production via diethyl sulfate (DES)-induced mutagenesis, and then expression of *kivD* and *adh3* to increase 3MB yield. Secondly, a genetically engineered mutant was constructed with an inactivated aminotransferase gene *ilvE* (Fig. 1), which encodes transaminase B.

Materials and Methods

Microorganisms, Plasmids, Reagents, and Growth Conditions

All microorganisms and plasmids used in this study, along with their related properties, are listed in Table 1. *E.coli* Trans1-T1, used to construct plasmids, was grown in Luria–Bertani (LB) complete medium at 37 °C. A wild-type *C. glutamicum* ATCC13032 strain was used as the parent strain in this study. It and its recombinant derivatives, which were used to express key genes, were aerobically cultivated in LBG (LB with 5 g/L glucose) at 30 °C. When appropriate, 50 µg/mL kanamycin and/or 1 mmol/L isopropyl-β-D-thiogalactoside (IPTG) was added to the medium.

Table 1 Microorganisms and plasmids used in this study

Name	Related properties	Sources
Strains		
E.coli		
Trans1-T1	*F-φ80 (lac Z)ΔM15 ΔlacX74 hsdR17(rk-,mk+) ΔrecA1398 endA1 tonA*	TRANS
C. glutamicum		
CG0	*C. glutamicum* ATCC13032	Xiao et al. (2016)
CG1	CG0 Δ*aceE*Δ*ldh*	Xiao et al. (2016)
CG2	CG0/ pEC-kivd-rbs-adh3	Xiao et al. (2016)
CG3	CG1/ pEC-kivd-rbs-adh3	Xiao et al. (2016)
CG4	DES-created mutant from CG0	This work
CG5	DES-created mutant from CG1	This work
CG6	CG4/pEC-kivd-rbs-adh3	This work
CG7	CG5/ pEC-kivd-rbs-adh3	This work
CG8	CG1/pk18-ΔilvE	This work
CG9	CG4/pk18-ΔilvE	This work
CG10	CG5/pk18-ΔilvE	This work
CG11	CG8/pEC-kivd-rbs-adh3	This work
CG12	CG9/pEC-kivd-rbs-adh3	This work
CG13	CG10/pEC-kivd-rbs-adh3	This work
Plasmids		
pEC-kivd-rbs-adh3	aph(3′)-IIa oriV lacIq Ptrc *kivd-rbs adh3*	Xiao et al. (2016)
pk18mobsacB	Kan+ LacZ sacB oriV/T	Xiao et al. (2016)
pk18-ΔilvE	Kan+ LacZ sacB oriV/T Δ*ilvE*	This work

DES and FTA were purchased from Aladdin (Shanghai, China). Oligonucleotides were obtained from Invitrogen (Shanghai, China). All restriction enzymes and rapid DNA ligation kits were purchased from Takara (Dalian, China). TransStart Taq DNA polymerases were purchased from TRANS (Beijing, China). All other chemicals used in this study were of analytical grade.

Screening of Overproduction of L-Leucine Mutants

Mutations were induced by treatment with DES. From 5 mL LBG overnight cultures of the parental strains (CG0, CG1), 2% of cultures were inoculated into 20 mL of fresh LBG medium and cultivated at 30 °C with shaking at 180 rpm until bacterial culture reached a stationary-phase stable stage. Cultures (1.8 mL) were centrifuged at 7378g (30 °C). Cell pellets were washed twice with 1 mL phosphate buffer (pH 7.0) and then treated with an equal volume of phosphate buffer containing 0.5% or 0.375% DES. The bacterial resuspensions were incubated at approximately 30 °C for 30 min. Cells were then pelleted by centrifugation and the supernatant was discarded. The remaining cell pellets were washed twice with 1 mL phosphate buffer to remove the mutagen. Control cells were subjected to the same protocol but were only treated with phosphate buffer. Cells were diluted to 108 CFU/mL and then spread on LBG plates to calculate the lethal rate.

Preliminary screening was performed by culturing the induced bacteria in screening medium (10 g/L glucose, 5 g/L urea, 5 g/L $(NH4)_2SO_4$, 1 g/L $KH_2PO_4 \cdot 3H_2O$, 1 g/L K_2HPO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.01 g/L $MnSO_4 \cdot H_2O$, 50 µg/L biotin, 200 µg/L vitamin B1, 20 g/L agar powder) containing 0.3% L-leucine analogs FTA (Tsuchida et al. 1975). After incubation at 30 °C for 4–6 days, the colonies were collected.

Secondary screening was performed by inoculating the L-leucine-analog-resistant mutants into the fermentation medium LBG and incubating at 30 °C for 24 h to test L-leucine production using paper chromatography–mottle elution chronometry.

Paper Chromatography–Mottle Elution Chronometry

Paper chromatography–mottle elution chronometry (Yu and Chen2005) was used to quantitatively determine the concentration of L-leucine. The paper chromatography filter was prepared by marking sample points with a pencil every 2 cm along the long edge of the filter. L-leucine standard solution and culture supernatant were applied on the corresponding filter paper points and then dried. The filter was placed in a chromatography cylinder containing an expansion agent [butyl alcohol:acetic acid:water (4:1:2)]. The filter paper was removed and placed in a fume hood to dry when the expansion agent reached the top of filter paper (2 cm). The chromatographic agent (0.5% ninhydrin solution) was sprayed on the chromogenic zone. After the filter paper was completely colored, the L-leucine-colored spots were cut to the same size and then into thin strips and placed in test tubes. Eluent [0.1% CuSO4:75% ethanol = 2.38 (V/V)] was added to the tube and oscillational elution was performed for 30 min. The elution's OD value was measured with a UV spectrophotometer (Biochrom, Libras 12) at a wavelength of 520 nm. A standard curve was drawn with the OD values of different concentrations of L-leucine standard liquid as the vertical axis and L-leucine content as the horizontal axis.

Deletion of *C. glutamicum* Chromosomal *ilvE* Gene

Inactivation of the aminotransferase gene *ilvE* in CG4 and CG5 was executed using crossover PCR and the suicide vector pk18mobsacB (Schafer et al. 1994). The upstream fragment *ilvE1* and the downstream segment *ilvE2* were cloned with the primer pairs F1/F2 and F3/F4 (Table 2), respectively. The two fragments were recycled and saved as crossover PCR templates to augment Δ*ilvE* with primer pairs F1/F2. The resulting fusion product Δ*ilvE* was ligated into the SalI-BamHI-restricted plasmid pK18mobsacB and transformed into *E. coli* Trans1-T1. The recombinant plasmid was isolated from *E. coli* and then electroporated into *C. glutamicum* CG4 and CG5, which were first inoculated into LBHIS medium (0.5% tryptone, 0.25%

Table 2 List of oligonucleotides used in this work

Name	Sequence (5′ → 3′)
F1	ACGCGTCGACATTCCACTACGGACAGGCAA
F2	GGATAGGTGATTGGAAGTTGGATGACCAGGAACTTGTAGGC
F3	CCAACTTCCAATCACCTATCCCATCACCCGCAAGTCA
F4	CGCGGATCCTAGCCAACCAGTGGGTAA

yeast extract, 0.5% NaCl, 1.85% brain heart infusion, 9.1% sorbitol, and 2% agar) with 50 µg/mL kanamycin, and secondly incubated a small colony into LBHIS medium with 10% sucrose. Through homologous recombination, the intact chromosomal *ilvE* genes in *C. glutamicum* CG4 and CG5 were replaced by Δ*ilvE*. Successful recombination was verified by PCR.

Production of 3-Methyl-1-butanol

The recombinant plasmid pEC-kivd-rbs-adh3 was transformed into the *C. glutamicum* mutant with high production of L-leucine to produce 3MB (van der Rest et al. 1999). *C. glutamicum* recombinants were streaked onto LBG plates containing 50 µg/mL kanamycin. A single colony was pre-cultured in a gas-tight test tube containing 3 mL of LBG medium at 30 °C overnight in a rotary shaker (200 rpm). The overnight culture was diluted to a 1:100 ratio with 10 mL of fresh LBG medium in a 50-mL plastic test tube. Cells were then incubated at 30 °C for 3–4 h (OD_{600} up to 0.6 ~ 0.8), followed by addition of 1 mM IPTG. After 12 h, the culture supernatant was analyzed for metabolites by gas chromatography (GC-2014, Shimadzu). The GC oven temperature was initially held at 60 °C for 1 min, then raised to 260 °C at 10 °C/min, and held for 2 min. The detector was maintained at 250 °C. A 1:50 split ratio was performed. A 1-µL sample was injected after passing through a 0.22-µm filter membrane. The hydrogen pressure was 0.4 Mpa, the air pressure was 0.4 Mpa, and argon pressure was 0.6 Mpa.

Results and Discussion

Mutagenesis with Different Concentrations of DES

The DES mutagen doses used in this study ensured a lethal rate of approximately 70–80%, which is more likely to produce mutants (Zhu 1994). In the present work, *C. glutamicum* ATCC13032 CG0 and CG1 with deletion of chromosomal *aceE* and *ldh* genes were selected as the mutation parental strains. Parental strains were treated with different concentrations of DES for 30 min (Table 3). Under the effect of DEC, the growth of engineered strain became slowly apparently. The higher the used DEC concentration, the less the growth rate and the more the

Table 3 Cell death rates with DES

Concentration of DES (%)	CG0 fatality rate (%)	CG1 fatality rate (%)
0.375	19.7	69.8
0.5	79.6	100

lethal rate. For CG0, 0.5% DES was suitable, which resulted in a lethal rate up to 79.6%, while for CG1 0.375% DES was preferable. The deletion of gene caused some effects on growth features of engineered strains probably due to the difference of DES does.

Screening of *C. glutamicum* Mutants with High-accumulation of L-Leucine

2-isopropylmalate synthase, encoded by gene *leuA*, catalyzes the conversion of 2-ketoisovalerate to 2-isopropylmalate during L-leucine biosynthesis, which is regulated by L-leucine feedback inhibition (Park et al. 2014; Patek et al. 1994; Smith et al. 2010). FTA, a structural analog to L-leucine, was selected for screening of resistant mutant strains, because it could reduce the feedback inhibition to repress 2-isopropylmalate synthase from L-leucine. Eight strains mentioned above (CG4 and CG5) that were grown on LBG medium with 0.3% FTA were subjected to L-leucine production testing. The OD_{520} value of L-leucine is shown in Fig. 2. By comparing with the original strains, the mutants increased L-leucine productivity to varying extents, with the OD_{520} of CG4-4 strain reaching 0.593 and CG5-4 reaching 0.598. These results show the significance of feedback-resistant *leuA* for L-leucine overproduction and illustrate the preliminary success of high-yield 3MB strain construction.

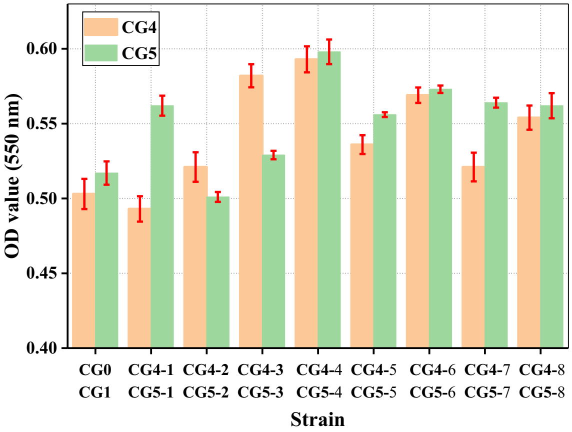


Fig. 2 L-Leucine OD_{520} value of CG4 and CG5 strains

Production of 3MB with Expression of *kivD* and *adh* Genes

High l-leucine-yielding mutants CG4 and CG5 were transformed with pEC-*kivd-rbs-adh3* vectors to form CG6 and CG7, respectively. The 3MB production of the culture supernatant for CG6 and CG7 was then investigated. The 3MB production from mutants CG6 and CG7 significantly increased in yield when compared to parental strains CG2 and CG3 (Fig. 3b). The cell growth trend was very similar for all strains, however, the occurrence time of the lag, exponential, and stable phases presented a little difference among each strain (Fig. 3a). Moreover, 3MB production increased 300 mg/L (from 182 to 482 mg/L) above CG2 parental strain levels after deletion of *aceE* and *ldh*, while 3MB production increased 293 mg/L (from 182 to 475 mg/L) above CG2 parental strain levels after mutagenesis with DES. This indicates that gene knockout had a better effect than mutagenesis to some extent because gene knockouts are targeted while mutagenesis is random. However, mutagenesis reduced the yield differences caused by *aceE* and *ldh* gene knockout. The increase of *aceE* and *ldh* gene knockout was 300 mg/L 3MB (from 182 to 482 mg/L) before mutagenesis, and 184 mg/L 3MB (from 475 to 659 mg/L) after mutagenesis, which shows that mutagenesis is irreversible and probably caused the increase of metabolic flux from α-ketoisovalerate to α-ketoisocaproate. Moreover genetic modification was the main reason for the increase in *leuA* expression, which is consistent with previous reports (Park et al. 2014; Zhang et al. 2008).

Effect of *ilv*E Deletion on 3MB Biosynthesis

To improve the production of 3MB, a truncated Δ*ilvE* fragment was constructed to replace the *C. glutamicum* chromosomal gene *ilvE* to avoid l-leucine production (Connor et al. 2010; Nozzi et al. 2014). BACT, a branched-chain acid aminotransferase (Ichihara and Koyama 1966; Taylor and Jenkins 1966), encoded by gene *ilvE*, specifically catalyzes amino group transferring with 2-ketoacid to form l-leucine, l-isoleucine, and l-valine using pyridoxal phosphate as a coenzyme. With the

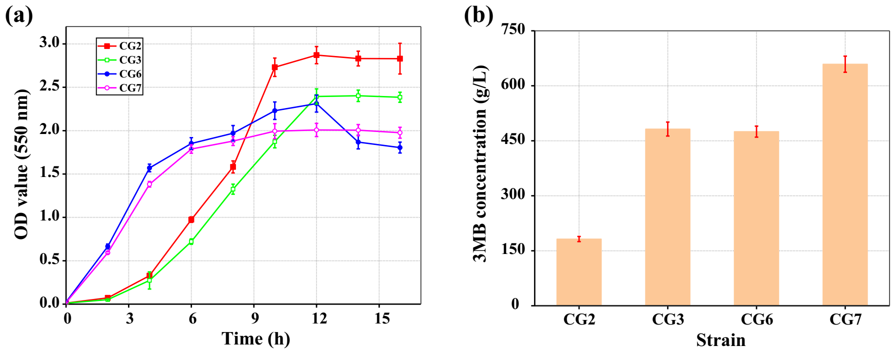


Fig. 3 Comparison of engineered strains on cell growth (**a**) and 3-methyl-1-butanol production (**b**)

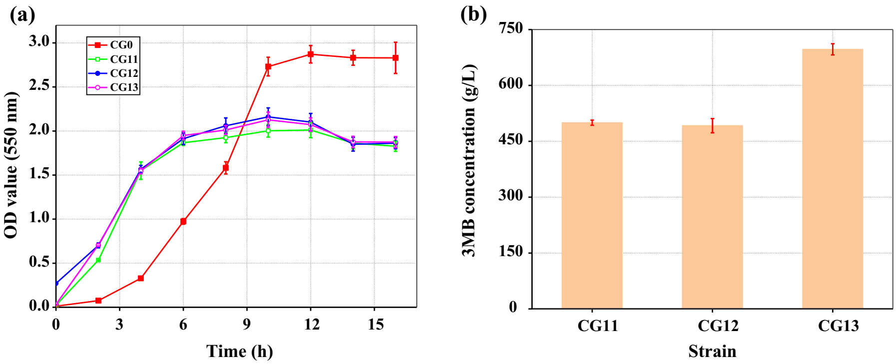


Fig. 4 Growth curve of strains (**a**) and concentration graph of 3-methyl-1-butanol (**b**)

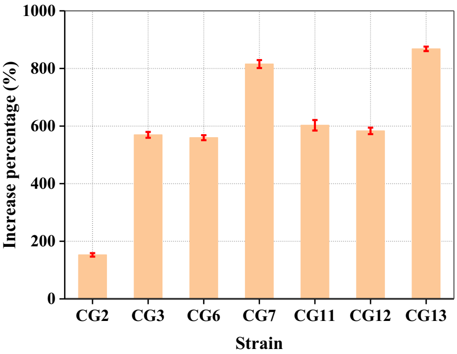
Table 4 Results of metabolic engineering studies on 3MB productivity by different microorganisms

Microorganisms	Genetic modifications	Substrate	Productivity (mg/L/h)	Reference
C. glutamicum	Deleted *ldh* and *aceE* + mutagenesis + deleted *ilvE*	Glucose	58.08	Present study
C. glutamicum	Expressed *aro10* and *yqhD*	Glucose	57.5	Vogt et al. (2016)
C. glutamicum	Expressed *kivd* and *adh3* + deleted *aceE* and *ldh*	Glucose	41.42	Xiao et al. (2016)
C. crenatium	UWCM + expressed *LEU1*, *LEU2*, *kivd*, and *ADH6*	Glucose	29.72	Su et al. (2016)
C. crenatium		Duckweed	25.86	
C. glutamicum	Expressed *alsS*, *ilvC*, *ilvD*, *kivd,* and *adhA*	Glucose	8.3	Smith et al. (2010)

inactivation of *ilvE,* L-leucine was not detected in the culture supernatant of strains CG8, CG9, and CG10. Consequently, plasmid pEC-*kivd*-*rbs*-*adh3* was transformed into CG8, CG9, and CG10; however, the introduction of the plasmid did not affect the growth curve (Fig. 4a) when compared to the previous strain. By overexpressing *kivD* and *adh3*, 3MB accumulation increased to 500 mg/L, 492 mg/L, 697 mg/L in CG11, CG12, and CG13, respectively (Fig. 4b). 3MB productivity of different engineered microorganisms is shown in Table 4. Compared with CG6 and CG7, 3MB yield slightly increased, which shows that *ilvE* has less influence on the production of 3MB. In addition, isobutanol was detected up to 1.06 g/L (data not shown), which shows that the high L-leucine-yielding strains obtained were vital to increasing the production of 3MB.

Many studies demonstrate that high concentrations of higher alcohols inhibit bacterial growth (Isken and de Bont 1998; Sikkema et al. 1995). However, it is obvious that a high concentration of higher alcohols is not the reason that deletion of *ilvE* has a little effect on the production of 3MB. Compared with CG3, CG6, and CG7, 3MB accumulation increased from 482 to 506 mg/L in CG11, 475 to 492 mg/L in CG12, and 659 to

Fig. 5 The 3MB production increase between various mutants and raw *C. glutamicum*



697 mg/L CG13. Compared to raw CG0, the increase was significant, and the maximum increase percentage from CG13 reached above 850% (Fig. 5). The yield of all strains slightly increased whether or not the concentration of 3MB was high. Another possibility is that 2-ketoisovalerate decarboxylase (Kivd) has a stronger binding capacity for 2-ketoisocaproate than branched-chain acid aminotransferase (encoded by *ilvE*). In other words, the introduction of pEC-*kivd-rbs-adh3* vectors inhibits the activity of branched-chain acid aminotransferase. This work used mutagenesis to screen a high-yield L-leucine *C. glutamicum* strain, thereby partially relieving L-leucine feedback inhibition. However, the *ilvE* knockout did not significantly increase 3MB production, which indicates that mutagenesis altered isopropylmalate synthase *leuA* and transcriptional regulator *LtbR* expression. Thus, *ilvE* gene knockout did not significantly affect 3MB yield. The high-yield L-leucine strain is a significant factor for high 3MB production.

Conclusion

Based on the common metabolic pathways of L-leucine and 3MB biosynthesis, high L-leucine-yielding strains were obtained through mutation breeding and gene deletion. After 12 h of induction with IPTG in LBG medium, the mutant accumulated 659 mg/L of 3MB. Furthermore, with the inactivation of the transaminase gene *ilvE* in the mutant, the final titer of 3MB reached 697 mg/L. To our knowledge, this is the highest reported productivity of 3MB using engineered *C. glutamicum*, which also demonstrates the utility and potential in using *C. glutamicum* as a host for high 3MB production.

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