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Metabolic engineering of *Corynebacterium glutamicum* to enhance L-leucine production

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This work aimed to develop an efficient L-leucine industrial production strain of *Corynebacterium glutamicum* by using metabolic engineering. A recombinant *C. glutamicum* strain was constructed by expressing a feedback-resistant leuA-encoded 2-isopropylmalate synthase (IPMS) that carries three amino acid exchanges (R529H, G532D and L535V) from the mutant strain *C. glutamicum* ML1-9 which was obtained by screening for structural analogues. In order to improve the expression of IPMS, a strong promoter (*tac* promoter) was used to ensure efficient expression of the rate-limiting enzyme. In addition, reasonable metabolic modifications on the central carbon metabolic pathway and competitive metabolic pathways to optimize the L-leucine biosynthesis pathway by redistribution of various types of precursors and repression of negative regulation were used aimed for increased L-leucine production. The modifications involved (1) deletion of the gene encoding the repressor LtbR to increase expression of l*euBCD*, (2) deletion of the gene encoding the AlaT to decrease the concentration of extracellular L-alanine, and increased availability of pyruvate for L-leucine formation, (3) deletion of the gene encoding the threonine dehydratase to abolish L-isoleucine synthesis and to eliminate the intermediate precursor of L-isoleucine biosynthesis competing with L-leucine biosynthesis, (4) inactivation of the pantothenate synthetase to increase α- ketoisovalerate formation, and to enable its further conversion to L-leucine, and (5) inactivation of lactate dehydrogenase to decrease lactate production and its pyruvate consumption, concomitant to decreased glucose consumption rates and prevention of lactic acid to restrict cell growth. The production performance of the engineered strain MDLeu-19/pZ8-1*leuA*^{r} was characterized with cultivations in a bioreactor. Under fed-batch conditions in a 50-L automated fermentor, the best producer strain accumulated 38.1 g L^{-1} of L-leucine; the molar product yield being 0.42 mol L-leucine per mole of glucose (glucose conversion rate attained 26.4%). Moreover, during large-scale fermentation using a 150-m^{3} fermentor, this strain produced more than 37.5 g L^{-1} L-leucine and the glucose conversion rate was 25.8%, making this process potentially viable for industrial production.

Key words: *Corynebacterium glutamicum*, L-leucine, metabolic engineering, fermentation, industrial production.

INTRODUCTION

Corynebacterium glutamicum is a gram-positive, facultatively anaerobic, non-spore-forming, soil bacterium, which was originally discovered as a L-glutamic acid-secreting microorganism in the 1950s (Kinoshita et al.,

2004; Nakayama et al., 1961; Udaka, 1960) and initially known as *Micrococcus glutamicus*; later various isolates were identified (for example *Brevibacterium flavum*, *Brevibacterium lactofermentum*, *Brevibacterium divaricatum*, and *Corynebacterium lilium*) (Liebl et al., 1991). *C. glutamicum* is used in industrial biotechnology to produce several million tons of L-amino acids, such as the flavor enhancer L-glutamate (2,300,000 t/year) and the feed additive L-lysine (1,600,000 t/year) annually (Becker et al., 2011; Chen et al., 2014; Woo and Park, 2014). Thus, *C. glutamicum* has become a platform organism in industrial biotechnology (Becker et al., 2012). Based on the increasing knowledge about this organism, *C. glutamicum*, is a highly efficient host for the expression of heterologous proteins (Scheele et al., 2013), and gradually is being developed into an efficient industrial producer of branched chain amino acids (BCAAs) (Hasegawa et al., 2013; Vogt et al., 2014; Yin et al., 2014).

The BCAAs namely, L-valine, L-leucine, and L-isoleucine, have recently been attracting much attention due to their potential applications in various fields, including animal feed additives, cosmetics, pharmaceuticals, energy drinks and precursors in the chemical synthesis of herbicides (Becker and Wittmann, 2012; Kimball and Jefferson, 2006; Platell et al., 2000). The annual demand of BCAAs is more than 2500 tons, out of which demand of 1000 ton is for L-leucine alone and constantly increasing.

An important member of BCAAs, the role of L-leucine in stimulation of muscle protein synthesis and glucose homeostasis was described (Garlick, 2005; Kimball and Jefferson, 2006; Layman, 2003). L-leucine, in combination with the other BCAAs, has also been reported to be prescribed for patients with hepatic encephalopathy (Freund et al., 1982; Gluud et al., 2013, 2015). In addition, L-leucine is used in the condiment industry and as a lubricant in the pharmaceutical industry (Leuchtenberger, 2008; Mangal et al., 2015; Platell et al., 2000). Moreover, the pentanol isomers, such as 3-methyl-1-butanol, manufactured from the L-leucine precursor 2-ketoisocaproate have a potential application as biofuels (Cann and Liao, 2010; Li et al., 2010).

So far, most L-leucine producers, especially based on *C. glutamicum* production strains have been developed by random mutagenesis and screening of structural analogs and auxotrophic strains. One of the first L-leucine producers was a α-thiazolealanine-resistant, methionine-isoleucine-auxotrophic mutant derived from the glutamate-producing bacterium *B. lactofermentum* 2256, treated with nitrosoguanidine (Tsuchida et al., 1974). This strain was further optimized for higher yields by additional mutagenesis steps (Ambe-Ono et al., 1996; Tsuchida and Momose, 1986). Although this classical approach has been successful in terms of improving the yield of L-leucine by genetic manipulation of *C. glutamicum*; it has some limitations. The genetic alterations caused by random mutagenesis includes the region which is not directly related to amino acid biosynthesis, thereby causing some unwanted changes in cellular physiology such as, growth retardation and by-product formation (Woo and Park, 2014). In particular, the accumulation of large amounts of by-products is a problem in the production of the three BCAAs due to their overlapping biosynthetic pathways, which negatively affects the yield and downstream processing.

The market is growing substantially and requires efficient production processes, strategies for developing microbial strains efficiently producing L-leucine are now in transition from random mutagenesis towards systems metabolic engineering (Becker et al., 2016). The metabolism and regulatory circuits of L-leucine biosynthesis need to be thoroughly understood for designing system-wide metabolic engineering strategies (Park and Lee, 2010; Vogt et al., 2014).

In this study, we report the rational design of a *C. glutamicum* L-leucine producer ML1-9. The source strain used was a methionine prototrophic L-leucine-producing strain resistant to α-thiazolealanine (α-TA), α-amino butyric acid (α-AB) and γ-aminobutyric acid (γ-AB). In order to increase the L-leucine production, we developed genetically defined, highly efficient, and genetic stable producer strains. The main metabolic engineering strategies employed (Figure 1) are beneficial to the construction of engineering strains with the potential of industrial scale fermentation processes.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli JM109 was used for cloning. The *C. glutamicum* strains used are listed in Table 1 and plasmids and oligonucleotide primers are listed in Table 2.

Media and growth conditions

E. coli JM109 was cultivated in Luria-Bertani (LB) medium (Bertani, 1951), with kanamycin (25 mg mL^{-1} for *C. glutamicum* strains, 50 mg mL^{-1} for *E. coli* JM109) where appropriate, at 37°C. Bacterial growth was assessed by measuring optical density at 560 nm (OD_{560nm}). Slant cultures of *C. glutamicum* was maintained in LBG (LB plus w/v 0.5% glucose), with kanamycin if necessary at 30°C. In cases where threonine dehydratase (*ilvA*), ketopantoate hydroxymethyl transferase (*panB*), and pantothenate synthetase (*panC*) had to be inactivated, the growth medium was supplemented with L-isoleucine and D-pantothenate or vitamin B_5.

The seed medium for producing L-leucine contained the following

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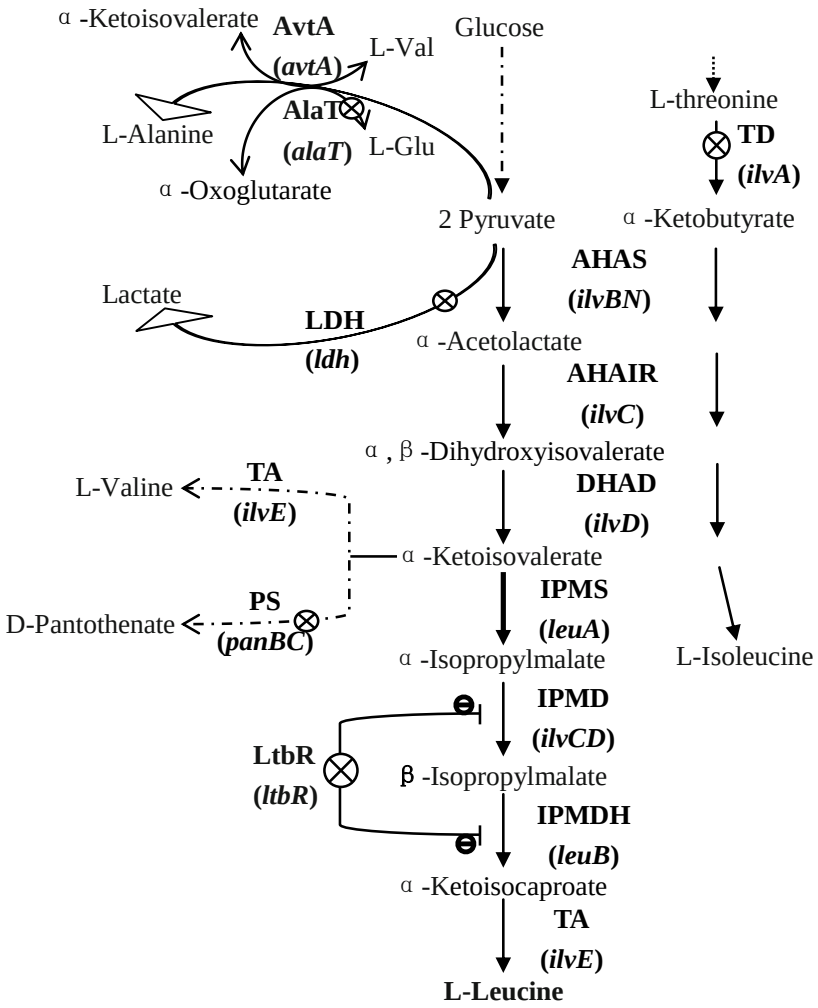

Figure 1. Biosynthetic pathways of L-leucine and their linkage to synthesis of other amino acids and organic acids in *C. glutamicum.* The metabolic engineering strategies for constructing the L-leucine producer (see text). AHAS, acetohydroxyacid synthase; AHAIR, acetohydroxyacid isomeroreductase; DHAD, dihydroxyacid dehydratase; IPMS, 2-isopropylmalate synthase; IPMD, 3-isopropylmalate dehydratase; IPMDH, 3-isopropylmalate dehydrogenase; LtbR, leucine and tryptophan biosynthesis regulator; AT, aminotransferase; TD, threonine dehydratase; AvtA and AlaT, Aminotransferases interacting while AlaT converts pyruvate to L-alanine in a glutamate-dependent reaction, AvtA is able to convert pyruvate to L-alanine in an L-valine dependent manner; LDH, lactate dehydrogenase; PS, pantothenate synthetase. Relevant gene names are given in parentheses. Thick arrows indicate increased gene expression. The "⊖" indicate repression of gene expression. The "⊗ indicate deletion of genes and the respective proteins.

components: 25 g L^{-1} glucose, 2.5 g L^{-1} yeast extract, 25g L^{-1} $(NH_4)_2SO_4$,1.5g L^{-1} Urea, 50 mL L^{-1} Soybean hydrolysate, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.7 g L^{-1} KH_2PO_4, 0.3 g L^{-1} K_2HPO_4 , 0.01 g L^{-1} $FeSO_4 \cdot 7H_2O$, 0.01 g L^{-1} $MnSO_4 \cdot H_2O$, 0.3 mg L^{-1} vitamin B_1, 0.5 mg L^{-1} vitamin B_5 (for Δ*panBC* strains), 0.3 mg L^{-1} vitamin C and 0.15 g L^{-1} L-isoleucine (for Δ*ilvA* strains).

The fermentation medium for producing L-leucine contained the following components: 80 g L^{-1} glucose, 1.5 g L^{-1} yeast extract, 10 g L^{-1} $(NH_4)_2SO_4$, 20 mL L^{-1} Soybean hydrolysate, 0.5 g L^{-1} $MgSO_4 \cdot 7H2O$, 0.7 g L^{-1} KH_2PO_4, 0.3 g L^{-1} K_2HPO_4 , 0.01 g L^{-1} $FeSO_4 \cdot 7H_2O$, 0.01 g L^{-1} $MnSO_4 \cdot H_2O$, 0.3 mg L^{-1} vitamin B_1, 0.5 mg L^{-1} vitamin B_5, 0.3 mg L^{-1} vitamin C and 0.15 g L^{-1} L-isoleucine.

Both seed and fermentation media were adjusted to pH 7.0 with 4 mol L^{-1} NaOH, and 25 μg mL^{-1} kanamycin (Km) was added, as required.

L-leucine batch fermentations were performed in a 50-L automatic fermentor (Zhenjiang East Biotech Equipment and Technology CO., Ltd, Jiangsu, China). 2 mL bacterial suspensions (1×10^8 bacteria per mL) of each test strain was inoculated into a 250-mL flask which contained 30 mL seed medium. The suspension was cultivated at 30°C with shaking at 220 rpm for 16 h.

Two flasks of 60 mL inoculum of this culture were added

Table 1. The main strains and plasmids involved in this study.

Strains	Relevant characteristics	Source or reference
***C. glutamicum* strains**		
ATCC13032	Wild type	ATCC
[a] ML1-9	L-leucine producer *C. glutamicum* strain created by random mutagenesis	Fujian Maidan Biology Group Co., Ltd
ML1-9-*leuA*^{r}	ML1-9 containing shuttle expression vector pZ8-1*leuA*^{r}	This work
MDLeu-19	ML1-9 derivative with inactivated *ilvA*, *alaT*, *ldh*, *panBC* and *ltbR* gene	This work
[b]MDLeu-19/ pZ8-1*leuA*^{r}	Derived from MDLeu-19 which overexpression of *leuA*^{r} by vector pZ8-1	This work
Plasmids		
pZ8-1	Shuttle expression vector (oriV *E. coli*, oriV *C. glutamicum*), containing *tac* promoter, Km^r	(Kassing, et al., 1994)
pZ8-1*leuA*^{r}	pZ8-1 derivative containing *leuA*^{r} gene which increase resistance to feedback inhibition comprise to wide *leuA* gene	This work
pK18*mobsacB*	Shuttle vector for allelic exchange in *C. glutamicum*	(Schäfer, et al., 1994)
pK18*mobsacB*Δ*ilvA*	pK18*mobsacB* containing truncated *ilvA* gene	This work
pK18*mobsacB*Δ*alaT*	pK18*mobsacB* containing truncated *alaT* gene	This work
pK18*mobsacB*Δ*ldh*	pK18*mobsacB* containing truncated *ldh* gene	This work
pK18*mobsacB*Δ*panBC*	pK18*mobsacB* containing truncated *panBC* gene	This work
pK18*mobsacB*Δ*ltbR*	pK18*mobsacB* containing truncated *ltbR* gene	This work

[a]: The ML1-9 strain was stored at Industrial Microbiology, Ministry of Education Engineering Research Center (Fujian Normal University), Fuzhou, China. [b]: The MDLeu-19/ pZ8-1*leuA*^{r} strain was deposited at the China Center for Type Culture Collection under the accession number CCTCC NO: M 2014620.

Table 2. Primers and sequences.

Primers	Sequences
leuA-F	5'-*GAATTC*[b]ATGCCAGTTAACCGCTACATGCCT-3'
leuA-R	5'-*GTCGAC*[c]TTAAACGCCGCCAGCCAGGAC-3'
leuA^r-F	5'-ACGTCACCGTCGATGGCC*G*CGGCAACG*G*CCCAC*T*G-3'
leuA^r-R	5'-GGCCATCGACGGTGACGTCCTTGCCGTTGT-3'
ilvA-P1	5'-*GAATTC*AGGAGAAGATTACACTAGTCAACC-3'
ilvA-P2	5'-AACTACAGACCTAGAACCTATGCAGCCGATGCTTCGTCGAAG-3'
ilvA-P3	5'-TAGGTTCTAGGTCTGTAGTTATGATGAGCGCGACCGAGGGCGC-3'
ilvA-P4	5'-*GTCGAC*TTAGGTCAAGTATTCGTACTCAG-3'
alaT-P1	5'-*GAATTC*GTGACTACAGACAAGCGCAAAACCTC-3'
alaT-P2	5'-AACTACAGACCTAGAACCTATTGAGGAGTGCTTGGGTGGTCATG-3'
alaT-P3	5'-TAGGTTCTAGGTCTGTAGTTACTGGACCAAAGCAATACGCACGTGG-3'
alaT-P4	5'-*GTCGAC*CTACTGCTTGTAAGTGGACAGGAAG-3'
ldh-P1	5'-*GAATTC*CTGCAGGGCATAGATTGGTTTTG -3'
ldh-P2	5'-AACTACAGACCTAGAACCTAATGACATCGCCAACGATGGACTTC-3'
ldh-P3	5'-TAGGTTCTAGGTCTGTAGTT ATCGGCATGGGTCTTGCTCGCATC-3'
ldh-P4	5'-*GTCGAC*TTGGTGCGAAGATGCGCGTAATG-3'
panBC-P1	5'-*GAATTC*CATGTCAGGCATTGATGCAAAG-3'
panBC-P2	5'-AACTACAGACCTAGAACCTAAGCATCAACAATGCGTCGAATC-3'
panBC-P3	5'-TAGGTTCTAGGTCTGTAGTTGCTTATCGACGCCCTCCTCC-3'
panBC-P4	5'-*GTCGAC*CGATCAGGGCGCACCAAATTGAAC-3'
ltbR-P1	5'-*GAATTC*ATGACCTTGAAATACACGGTGAAG-3
ltbR-P2	5'-AACTACAGACCTAGAACCTA ATGCAGGGTCAGCAGCGCGC-3'
ltbR-P3	5'-TAGGTTCTAGGTCTGTAGTTAGCGCCGCGTGCACCCAATG-3'
ltbR-P4	5'-*GTCGAC*ATATCGTTTCATGGGACAGTATAGC-3'

[c]The underlined nucleotides, GAATTC, indicate the restriction enzyme cutting site of *EcoR* I, and the underlined nucleotides, GTCGAC, indicate the restriction enzyme cutting site of *Sal* I.

aseptically to a 30 L automatic fermentor (Zhenjiang East Biotech Equipment and Technology CO., Ltd, Jiangsu, China) containing 12 L seed medium and cultivated at 30°C for 16 h. The seed culture medium was inoculated into (20% inoculum) 30 L of production medium in a 50-L fermentor. The temperature and level of dissolved oxygen were maintained at 30°C and 25-35%, respectively, and the pH was maintained at about 7.0 with ammonium hydroxide (25 % NH_4OH) during the course of the cultivation period. When the initial glucose was consumed at 10 g L^{-1}, sterilization glucose solution (70 %w/v) was fed to the fermentor in order to maintain the glucose concentration in the fermentation broth at 10-15 g L^{-1} according to the batch-feeding strategy of pseudo-exponential growth, and at the end of the fermentation, controlled glucose concentration was 1.0 g L^{-1} or less.

Construction of the modified strains

Conventional techniques of molecular biology like PCR, restriction digestion and ligation were carried out according to the standard protocols (Sambrook, 2001). All enzymes for recombinant DNA work were obtained from TAKARA (Takara, Japan). All primers and DNA extraction and purification kits were obtained from Sangon Biotech (Sangon Biotech, Shanghai, China). Plasmid-based gene expressions were achieved using vector pZ8-1 carrying a *tac* promoter (Frunzke et al., 2008).

All single-and multi-gene in-frame deletions of DNA sequences in *C. glutamicum* strains were done via two-step homologous recombination using the suicide vector pK18mobsacB (Schäfer et al., 1994). The *ilvA* deletion was achieved by constructing pK18*mobsacB*Δ*ilvA* plasmid. The Δ*ilvA* gene fragment (in-frame deletion) was obtained by polymerase chain reaction (PCR) and extension PCR using primers *ilvA*-1, *ilvA*-2 and *ilvA*-3, *ilvA*-4.The fragment was first subcloned into simple pMD18-T (pMD™18-T Vector Cloning Kit, TAKARA) and ligated into pK18*mobsacB,* digested by *EcoRI* and *SalI*. The final plasmid pK18*mobsacB*Δ*ilvA* of 6.56 kb was characterized by restriction analysis. The plasmid was transformed into *C. glutamicum* cells by electroporation. Clones were selected for kanamycin resistance to establish integration of the plasmid in the chromosome through homologous recombination. In a second round of positive selection using sucrose resistance, clones were selected for deletion of the vector and *ilvA*. Disruption of *panBC, alaT* and *ltbR* in the parental strain were performed using the same method as described for disrupting *ilvA* using the primers *panBC*-P1, *panBC*-P2, *panBC*-P3 and *panBC*-P4 for the *panBC gene*; *ldh*-P1, *ldh*-P2, *ldh*-P3 and *ldh*-P4 for the *ldh* gene; primers *alaT*-P1, *alaT*-P2, *alaT*-P3 and *alaT*-P4 for the *alaT* gene; and primers *ltbR*-P1, *ltbR*-P2, *ltbR*-P3 and *ltbR*-P4 for the *ltbR* gene. For strain construction, the techniques specific for *C. glutamicum*, for example transformation of strains via electroporation, were carried out according to the published method (Eggeling and Bott, 2005; van der Rest et al., 1999). All constructed plasmids as well as chromosomal deletions and integrations in the engineered strains were verified by PCR analysis and the nucleotide deletions in the chromosomes were verified by sequencing (Sangon Biotech).

Construction of plasmid pZ8-1*leuA*^{r}

Analysis of *leuA* gene sequences, show that *leuA* sequence of *C. glutamicum* ML1-9 has six sites mutated compared to the wildtype strain. Three of the six mutations were silent and the others involved three amino acid substitutions (R529H, G532D and L535V). Studies have shown that not only these three amino acids are located in binding region of IPMS, but their substitutions in the protein can significantly enhance its activity and relieve the feedback-inhibition by L-leucine (Pátek et al., 1994), consistent with our results. Moreover, L535V substitution is located in the enzyme binding pocket of IPMS, which may increase its affinities towards its substrates (Nayden et al., 2004). Overall, improved resistance to the feedback inhibition effect of IPMS activity by L-leucine and increased expression of *leuA*^{r} are necessary to obtain high L-leucine concentrations.

The *leuA*^{r} was PCR-amplified with the primers *leuA*^{r}-P1and *leuA*^{r}-P2, and ML1-9 chromosomal DNA as the template. The PCR product was purified using a purification kit (Sangon Biotech). Plasmid DNA was also extracted by an isolation kit (Sangon Biotech). The purified leuA^r fragment was first subcloned into simple pMD18-T vector, then digested with *EcoR* I and *Sal* I, and finally inserted into pZ8-1 vector to construct pZ8-1*leuA*^{r}.

Analysis of fermentation products

Bacterial growth was determined by measuring optical density at 560 nm (OD_{560nm}) against distilled water with an UNICO 2802PCS UV/VIS spectrophotometer (Unico Instrument Co., Ltd. Shanghai, China). The cell density was characterized by OD_{560nm}. The concentrations of glucose and lactate were monitored using an SBA-40C biosensor analyzer (Biology Institute of Shandong Academy of Sciences, Jinan, China). Concentrations of amino acids and other organic acids in the culture supernatants were determined by Automatic Amino Acid Analyzer L-8900 (Hitachi, Japan) and Bioprofile 300A biochemical analyzer (Nova Biomedical, USA), respectively.

Enzyme assays

Crude cell extracts were prepared for the determination of IPMS and 3-isopropylmalate dehydratase (IPMD) activity. After 48 h of cultivation, cells were harvested by centrifugation (10 min, 4°C, 10000 rpm), washed twice with 20 mL of the enzyme assay buffer (200 mM Tris-HCl, pH 7.0, 20 mM KCl, 5 mM $MnSO_4$, 0.1 mM ethylenediamine tetraacetic acid, and 2 mM dithiothreitol), subsequently resuspended in 1 mL 200 mM potassium phosphate buffer (pH 7.0) with 1% lywallzyme (w/v) and incubated at 30°C for 3 h. Cells were disrupted by sonication for 5 min on ice bath using an ultrasonic processor Scientz-IID (Ningbo Scientz Biotechnology Co., Ningbo, China). The supernatants of the crude extracts, separated from the cellular debris by centrifugation (15 min, 4°C, 13000 rpm), were used for enzymatic assays. Protein concentration was determined by the Bradford assay using bovine serum albumin as the standard. Each assay was replicated thrice. IPMS catalyzes the following reaction:

2-Ketoisovalerate + acetyl-CoA → 2-isopropylmalate + CoA

The activity of IPMS can be determined by using a continuous spectrophotometric assay measuring coenzyme A (CoA) formation with 5,5′-dithiobis-(2-nitrobenzoic acid)(DTNB) (Kohlhaw, 1988; Kohlhaw and Robert, 1988; Ulm et al., 1972).

The total volume of the reaction solution was 1 mL, containing 500 µL 50 mM Tris–HCl buffer (pH 7.5) containing 20 mM KCl, 100 µL 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) solution (1 mM in 50 mM Tris–HCl, pH7.5), 50 µL acetyl-CoA solution (3 mM in 50 mM Tris–HCl, pH7.5), 295 µL ddH_2O and 50 µL crude extract. The reaction was started by adding 5 µL 2-ketoisovalerate solution (40 mM in 50 mM Tris–HCl, pH7.5) and terminated by adding a final concentration of 75% ethanol. The liberated CoA was measured at 412 nm with an UNICO 2802PCS UV/VIS spectrophotometer. Enzyme activities were calculated using an extinction coefficient of 13,600 $M^{-1}cm^{-1}$ for the yellow-colored 5-thio-2-nitrobenzoate dianion. The specific activity was defined as the micromoles of CoA released by per milligram of protein per minute.

Table 3. Comparison of the maximum biomass (OD_{560nm}), specific IPMS activities and L-leucine production of different strains*.

Strains	OD_{560nm}×100	L-leucine(g L^{-1})	Specific IPMS activity (μmol min^{-1} mg^{-1})
ML1-9	0.985±0.005	18.5±0.5	0.33±0.01
ML1-9/pZ8-1	0.969±0.006	17.1±0.3	0.32±0.01
ML1-9/pZ8-1*leuA*^{r}	0.976±0.004	23.6±0.7	0.63±0.02

*Values for the calculation of standard deviations were derived from three independent measurements.

The activity of IPMD was determined according to the published method (Kohlhaw and Robert, 1988; Vogt et al., 2014). It was also used A continuous spectrophotometric assay method was used and IPMD activity was determined by measuring the optical absorption of the reaction intermediate 2-isopropylmaleate at 235 nm.

The 1 mL reaction mixture, contained 400 μL potassium phosphate buffer (200 mM, pH 7.0), 40 μL 3-isopropylmalate solution (40 mM), 510 μL ddH_2O and 50 μL crude cell extract was incubated at 30°C for 10 min. The optical density of the increase of absorbance at 235 nm was measured by UNICO 2802PCS UV/VIS spectrophotometer. Enzyme activities were calculated using an extinction coefficient of 4530 $M^{-1}cm^{-1}$ for 2-isopropylmaleate. The specific activity was defined as the micromoles of 2-isopropylmalate formation by per milligram of protein per minute.

RESULTS AND DISCUSSION

Recombinant strains and plasmids

The deletions of *ilvA*, *ldh*, *alaT*, *panBC* and *ltbR* in *C. glutamicum* ML1-9 were confirmed by colony PCR using relevant primers P1 and P4. The sequence analysis results revealed that the obtained PCR product did not contain unwanted mutations.

The expression vector pZ8-1*leuA*^{r} was transformed into the ML1-9 strain and the IPMS activity of crude extracts was determined. The expression of pZ8-1*leuA*^{r} was assessed by analysis of fed-batch cultivation in a 30-L automatic fermentor. As shown in Table 3, the *C. glutamicum* ML1-9-*leuA*^{r} strain have an improved yield of L-leucine, indicating that *leuA*^{r} gene was successfully expressed by pZ8-1*leuA*^{r}, with increase of the activity of IPMS (In the ML1-9/pZ8-1*leuA*^{r} strain, there is about a two-fold higher specific IPMS activity than the ML1-9 and ML1-9-pZ8-1 levels, respectively). The phenomenon results in enhanced carbon flux for L-leucine biosynthesis. These data suggest that the feedback-resistant IPMS is crucial to the accumulation of L-leucine.

Effect of gene deletions on cell growth and L-leucine production

The ML1-9 strain that overexpressed *leuA*^{r} accumulated more L-leucine than the other ML1-9 strains. Therefore, the known feedback inhibition, especially of the *leuA*-encoded IPMS is not a major problem, probably due to the partial feedback inhibition and repression of the other enzymes like *ilvBN*-encoded AHAS.

Fed-batch fermentations of ML1-9Δ*ilvA*, ML1-9Δ*ldh*, ML1-9Δ*alaT*, ML1-9Δ*panBC*, and ML1-9Δ*ltbR* strains (without overexpression of *leuA*^{r}) were performed, and their fermentation growth curve and L-leucine production were measured (Figure 2). All modified strains produced more L-leucine than the control strain ML1-9. It is of note that although all the modified strains had quite similar growth patterns, significant differences were observed in their maximum cell densities (OD_{560nm}) and time to reach the stationary phase (Figure 2a). ML1-9ΔltbR strain that reached the maximum cell density (OD_{560nm}=115) after 18 h of fermentation had the highest cell density among these strains, including the control strain, which reached the maximum cell density after 22 h. Additionally, the highest concentration of L-leucine (20.5 g L^{-1}) was also produced by ML1-9Δ*ltbR*. It was reported that *ltbR* encoding a transcriptional regulator of IclR protein family, which was involved in transcriptional regulation of the L-leucine biosynthesis pathways of *C. glutamicum*, repressed expression of *leuCD* and *leuB* in the presence of high concentration of L-leucine in the medium (Iris et al., 2007). We therefore deleted *ltbR* to increase the IPMD and IPMDH activity significantly by increasing the expression of *leuCD* and *leuB,* which released the negative transcriptional regulation of the *leu* genes of ML1-9Δ*ltbR* (Table 5). Moreover, *ltbR* deletion strain did not need extra supplemental ingredients in the fermentation medium. In comparison to other auxotrophic strains, ML1-9ΔltbR improved the fermentation, by increasing the fermentation index and had a positive effect on the cell growth (Figure 2a).

The ML1-9Δ*ldh* strain was constructed by deletion of *ldh* which encodes the key enzyme for lactate synthesis (Garvie, 1980). Consistent with previous reports, synthesis of lactate was greatly reduced from 0.85 to 0.05% by the deletion of *ldh* (Wieschalka et al., 2012). Moreover, the deletion of *ldh* was not the limiting step in the metabolic flow, since the growth rate and the L-leucine concentration of the *ldh* deletion strains were higher than the control. This indicates that the inactivation of LDH prevented lactic acid inhibition of growth. Not only that, due to inactivation of LDH, increased precursor metabolite concentrations are available with low L-lactic acid production resulting in an increase in the yield of L-leucine (Figure 2b).

Unlike deleted *ltbR* and *ldh* gene, *ilvA*, *alaT* and *panBC*

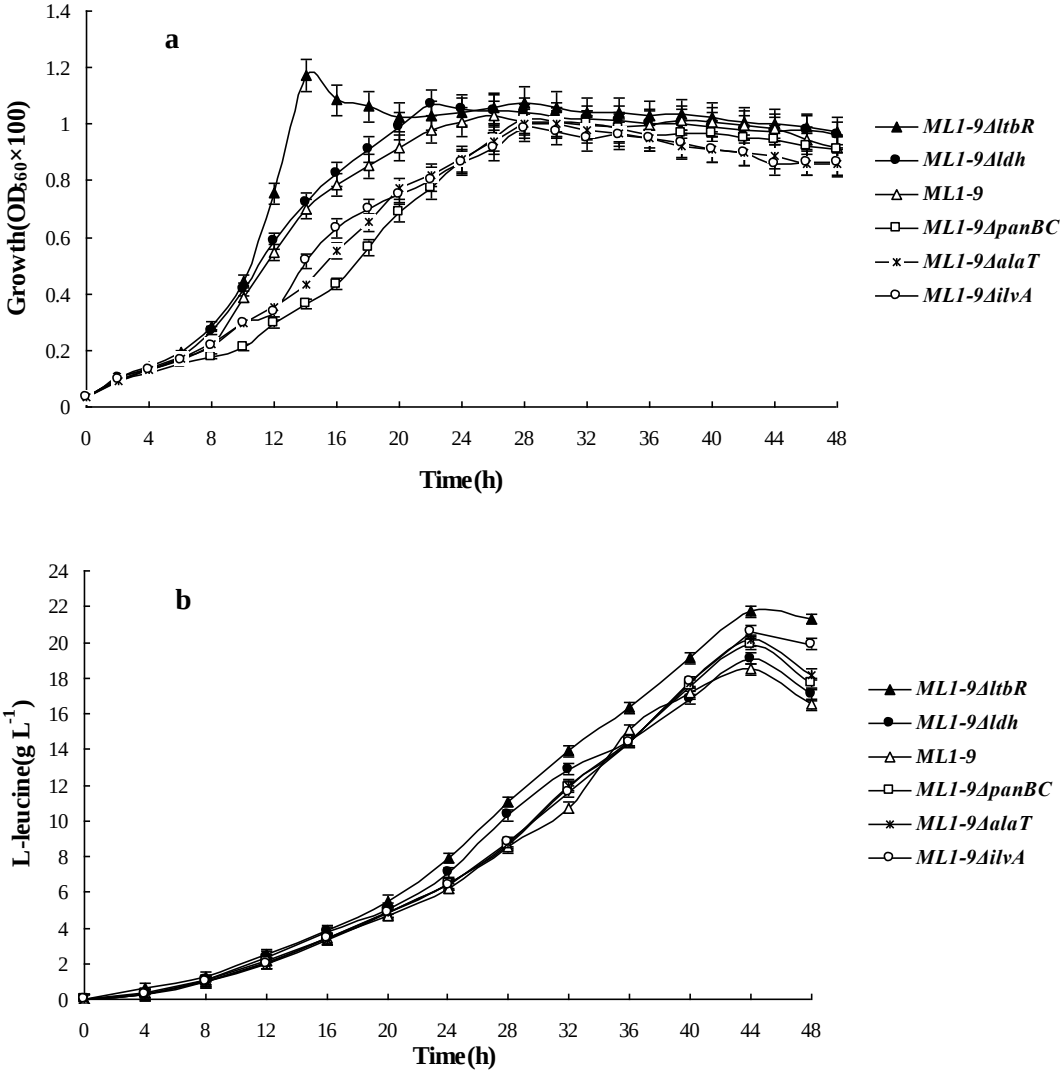

Figure 2. Fed-batch fermentations of different strains derived from the ML1-9. (a) Fermentation growth curves. (b) L-Leucine concentration in the fermentation process.

deletion strains had an impact on metabolic flux distribution and cell growth, thereby affecting the production of L-leucine. ML1-9Δ*ilvA*, an isoleucine auxotroph strain, was constructed by inactivating the threonine dehydratase (deleted *ilvA*) in order to block the biosynthesis of L-isoleucine. It is to be noted that a certain amount of L-isoleucine (0.15 g L^{-1} for this work) was supplemented into the medium in order to maintain cell growth. The result shows that the cell growth of ML1-9Δ*ilvA* was limited, prolonging the logarithmic growth phase relatively compared to the prototrophic strain, ML1-9. Moreover, the cell densities of the strain were also the lowest among all the other strains including ML1-9 (Figure 2a). It is noteworthy that despite L-isoleucine supplementation, the effects on growth were long lasting, both the cell growth rate and the highest cell density were decreased, and the adaptation period was prolonged compared with the original strain ML1-9. On the contrary, the prevention of L-isoleucine synthesis not only relieved the feedback inhibition of acetohydroxy acid synthase, but also increased the carbon metabolism, with increased precursor metabolite concentrations needed for the L-leucine biosynthesis pathway (Eggeling et al., 1997; Sahm and Eggeling, 1999). Therefore, we were able to obtain more L-leucine production through ML1-9Δ*ilvA* strain, which resulted in the second highest concentration of L-leucine compared to other strains (Figure 2b).

Similarly, AlaT (encoded by *alaT* gene) is the main enzyme for L-alanine synthesis. While AlaT converts pyruvate (amino acceptor) to L-alanine in a glutamate-dependent reaction (glutamate as amino donor), AvtA does the same in L-valine dependent manner (Jan et al., 2005). When compared to the control strain (ML1-9), the concentration of L-alanine in ML1-9Δ*alaT* strain was

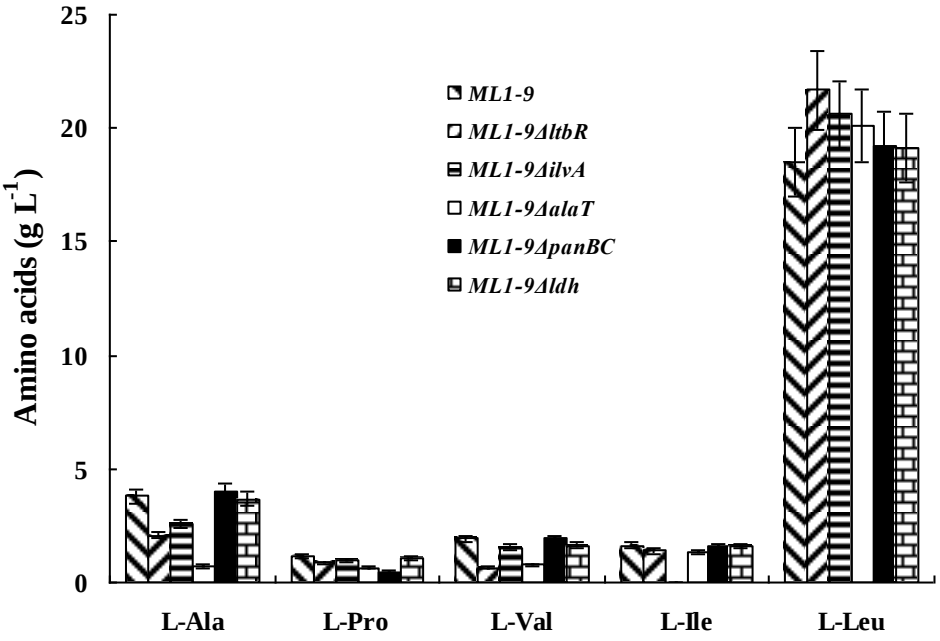

Figure 3. Comparison of L-leucine and related amino acids among the different strains.

greatly reduced because of *alaT* deletion (Figure 3). In this study, we investigated cell growth and accumulation of L- leucine by AvtA and/or AlaT inactivation. A double deletion mutant, auxotrophic for L-alanine, was generated by deletion of *avtA* and *alaT* in the same strain. Cell growth of these strains is severely limited, which could not be revived by L-alanine supplementation. These results showed that ATs (AvtA and AlaT) are not only responsible for the synthesis of L-alanine, but also play a crucial role in balancing the intracellular amino acid pools essential for normal cell growth. These results are consistent with the previous studies (Marienhagen and Eggeling, 2008). Concentration of L-alanine was reduced from 3.81 to 3.09 g L^{-1} by deletion of *avtA*, whereas L-valine concentration increased and L-leucine concentration decreased (data not shown). Unlike the strain with AvtA inactivation, deletion of *alaT* alone blocked L-alanine synthesis, impaired cell growth and extended the logarithmic growth phase (Figure 3). However, *alaT* deletion resulted in more precursor flow to L-leucine pathway, and accumulation of more L-leucine. Additionally, the L-valine concentration also decreased, which is an added advantage for L-leucine downstream processing.

The ML1-9Δ*panBC* strain was constructed by deleting *panBC*, which must be grown in a medium supplemented with 0.45 mg L^{-1} D-pantothenate or 0.5 mg L^{-1} vitamin B5. Compared to the control strain ML1-9, the adaptation period of ML1-9Δ*panBC* strain was extended, and also the growth rate became lower. It took 28 h (approximately 22 h in the control strain) for the ML1-9Δ*panBC* strain to reach the highest cell density, which was reduced compared to the control strain (Figure 2a). However, this result indicates that it can accumulate more L-leucine (Figure 2b). It has been reported that blocking D-pantothenate biosynthesis affects the TCA cycle by reducing CoA formation which ultimately affects central energy metabolism or even cellular flux properties (Lothar and Hermann, 2001; Riedel et al., 2001; Sahm and Eggeling, 1999; Shimizu et al., 1985). In addition, a reduced flux through the pyruvate dehydrogenase probably results due to the D-pantothenate limitation, leading to an increase of pyruvate availability (L-alanine synthesized directly from pyruvate). Furthermore, blocking of D-pantothenate synthesis results in increased amounts of precursors (more ketoisovalerate availability) for BCAAs synthesis pathways (including L-valine and L-leucine).Of note, more L-alanine is available than L-valine and L-leucine.

As shown in Figure 2, L-leucine showed the highest yield after 44h fermentation, However, the results presented in the 50-L fermentation did not exhibit a significant improvement in line with expectations in the production of L-leucine with single modified strains, and the increase in L-leucine yield of single modified strains were also lower than the proper integrattion strain (Table 4). Possible reasons for this failure could be the genetic background ML1-9 strain and composition of the medium that may affect the capacity of L-leucine accumulation. Another possibility could be that a single gene modification often influences the metabolic networks rather than merely affecting a single metabolic reaction, thereby limiting the target metabolic flows. A third possibility would be a general growth limitation introduced by some auxotrophs or metabolic defects.

In addition, the L-leucine accumulation, and biomass of

Table 4. Fed-batch culture parameters of L-leucine production by MDLeu-19.

Strain	Amino acid concentration (g L^{-1})				
	L-Ala	L-Pro	L-Val	L-Ile	L-Leu
MDLeu-19	0.51	0.58	0.65	0	28.5±0.5

Table 5. Enzymes activities and by-product concentrations with L-leucine production in different strains.

Strain	Specific activity(μmol min^{-1} mg^{-1} protein)			Lactic acid (g L^{-1})	D-pantothenate (g L^{-1})
	IPMS	IPMD	IPMDH		
ML1-9	0.343±0.02	0.211±0.02	0.190±0.03	10.2±0.5	0.76±0.03
ML1-9/pZ8-1	0.341±0.02	0.208±0.02	0.185±0.03	10.7±0.5	0.79±0.03
ML1-9/pZ8-1 *leuA*^{r}	0.645±0.05	0.213±0.03	0.188±0.03	10.5±0.7	0.64±0.05
ML1-9Δ*ltbR* /pZ8-1 *leuA*^{r}	0.86±0.05	0.45±0.05	0.380±0.05	9.2±0.5	0.47±0.05
ML1-9Δ*ilvA*/pZ8-1 *leuA*^{r}	0.644±0.05	0.211±0.03	0.199±0.03	10.6±0.5	0.66±0.05
ML1-9Δ*alaT*/pZ8-1 *leuA*^{r}	0.643±0.05	0.216±0.02	0.201±0.04	11.8±0.7	0.83±0.05
ML1-9Δ*panBC*/pZ8-1 *leuA*^{r}	0.645±0.05	0.209±0.02	0.191±0.03	10.9±0.6	0
ML1-9Δ*ldh*/pZ8-1 *leuA*^{r}	0.651±0.05	0.234±0.02	0.216±0.04	0	0.66±0.05
MDLeu-19/pZ8-1 *leuA*^{r}	0.91±0.05	0.53±0.03	0.421±0.06	0.16±0.03	0

strains (OD_{560nm}) decreased between 44 and 48 h, respectively. Therefore, the fermentation was terminated at 48 h. Both the L-leucine titer and production rate of the mutant strains were higher than those of the control strain (Figures 2 and 3). Based on this, we have integrated a number of favorable modifications and deleted *ltbR*, *ilvA*, *alaT*, *panBC,* and *ldh* in a ML1-9 strain in order to obtain a modified strain named MDLeu-19, based on metabolic engineering. The fermentation characteristics of this strain were investigated by the same methods.

The L-leucine concentration of MDLeu-19 (Table 4) was the highest among the strains tested in the current study, which was 54.1% higher than the control strain ML1-9 (18.5±0.5 g L^{-1}). On the the other hand, the concentrations of the other amino acids, especially L-isoleucine, L-valine, and L-alanine, were low. This was beneficial to the post-fermentation process and gives an improved glucose conversion. The results also demonstrat that some of these combinations could lead to significantly improved L-leucine production.

Activity analysis of some key enzymes in different modified strains

Our observation suggests that cloning and overexpression of *leuA*^{r} gene can significantly improve the IPMS activity by nearly two-fold (Table 3) leading to increase in the carbon flux to the L-leucine pathway. With *leuA*^{r} gene overexpressed, the maximum L-leucine productions by ML1-9Δ*ltbR*, ML1-9Δ*ilvA*, ML1-9Δ*alaT*, ML1-9Δ*panBC*, ML1-9Δ*ldh,* and MDLeu-19 strain were also increased up to 26.7±0.3, 24.7±0.5, 23.8±0.7, 22.1±0.6, 21.8±0.4 and 38.1±0.6 g L^{-1}, respectively. Moreover, the acticity of IPMS, IPMD, and IPMDH, as well as the concentrations of extracellular lactic acid and D-pantothenate were also determined (Table 5).

The above results indicate that L-leucine producing strains show elevated activities of enzymes required for L-leucine synthesis. Expression of the *leuA*^{r} gene by pZ8-1 enhanced the IPMS activity nearly two-fold in *C. glutamicum*, whereas no significant increase was observed by other metabolic modifications except deletion of *ltbR*.

L-leucine overproduction by significant increase in the enzyme activity was achieved by deletion of the leucine transcriptional repressor LtbR. Compared to the strains with overexpressed *leuA*^{r}, IPMS activity was enhanced 32.3% by deletion of *ltbR*. Besides this, the IPMD and IPMDH activity was doubled in some of the strains, which is consistent with previous studies (Iris et al., 2007; Vogt et al., 2014). Furthermore, the MDLeu-19/pZ8-1*leuA*^{r} strain has the highest L-leucine synthesis enzyme activities among these strains. The inactivation of the transcriptional regulator has directly enhanced transcription of *leuABCD* operon. However, the decisive factor is the proper integration of activities in the central metabolism and the L-leucine biosynthesis pathway. On the one hand, the use of metabolic modifications to improve precursor supply by reducing the consumption of pyruvate based on inactivation of AlaT and LDH. On the other hand, increased L-leucine synthesis enzyme activity (expression of *leuA*^{r} and deletion of the leucine transcriptional repressor LtbR) and weaken the competitive metabolism to redirect metabolic flux towards L-leucine instead L-isoleucine or D-pantothenate. Both forces provided potential for a strong increase in L-leucine production.

L-leucine originates directly from two pyruvate molecules, whereas D-pantothenate is a precursor in the biosynthesis of CoA. Deletion of *panBC* blocked D-pantothenate synthesis leading to a reduction in metabolic flux from pyruvate to acetyl-CoA. This eventually limits the cell growth, which probably requires D-pantothenate supplementation (Table 5). This also indicates that more L-leucine was accumulated just by directly increasing the availability of precursors rather than promoting the L-leucine synthesis activity e.g., deletion of *ltbR* (IPMD and IPMDH activity were not significantly increased by deletion of *panBC*).

Additionally, deletion of the *ldh*, encoding lactate dehydrogenase (LDH) not only minimized lactate production and increased the availability of precursor, but also moderately enhanced the transcription and expression of *leuB* and *leuCD* (Table 5). As expected, inactivation of LDH effectively blocked the synthesis of lactic acid and reduced the adverse effects of this organic acid on cell growth, especially in the area where there is lack of dissolved oxygen at high-density fermentation environment. Notably, an *ldh*-deficient mutant was not able to produce lactate, and synthesis of acetic acid compared to the parent strain did not increase. When acetic acid synthesis pathway was further modified by knock out the *pta* gene, the growth of the strain was severely suppressed (date not shown). This observation could perhaps be ascribed to ML1-9 strain, which has undergone multiple generations of mutation breeding. This suggests that the activity of the protein encoded by *pta* genes are not limiting L-leucine production in the ML1-9 strain background. This set of observations corroborates the view that the ability of acetic acid biosynthesis may already have been greatly reduced in ML1-9 strain. Further modifications of the acetic acid pathway may affect the TCA cycle of central metabolism. On the one hand, acetic acid may be activated by a series of enzymes to produce acetyl-CoA, further into the TCA cycle, and then promote cell pyruvate metabolism. On the other hand, acetic acid may also help to improve the critical enzyme activity of the TCA cycle, promote the efficiency of TCA cycling, increase the TCA cycle flux, and supply the carbon skeleton of a variety of compounds for cell biosynthesis (Gerstmeir et al., 2003; Sorger-Herrmann et al., 2015; Wolfe, 2005).

In conclusion, the modifications of transcriptional regulator and bypassing metabolic pathways could further improve the yield of L-leucine in such a way that the direct negative regulation of L-leucine synthesis is reduced and the metabolic flux of L-leucine biosynthesis pathway is probably redistributed.

L-leucine production and glucose conversion rate of the MDLeu-19/pZ8-1*leuA*^{r} strain

MDLeu-19/pZ8-1*leuA*^{r} strain was characterized in 50-L fed-batch cultivations in order to determine the influence of different process parameters on the productivity and to analyze the behavior of the strain under such controlled conditions. Because of a relatively higher yield and glucose conversion rate in 50-L automatic fermentor, MDLeu-19/pZ8-1*leuA*^{r} strain was carried out in 150-m^3 fermentor to explore the industrial production of L-leucine.

Cells show similar growth curves in 50-L automatic fermentor and 150-m^3 industrial fermentor. Glucose consumption and cell growth were substantially synchronous. After 8-18 h of fermentation, the cells reach the logarithmic phase with a sharp increase in glucose consumption. After 18 h the cells reached the highest cell biomass (OD_{560nm}=110.6), it entered the stationary phase. Here, the concentration of L-leucine in the fermentation broth continued to gradually increase as shown in Figure 4. After 48-h fermentation, maximum L-leucine concentration by the MDLeu-19/pZ8-1*leuA*^{r} strain was 37.5 g L^{-1}, with a glucose conversion rate of 25.8%. Both the L-leucine yield and glucose conversion rate were lower than that in 50-L automatic fermentor. First, industrial fermentor microenvironments are unfavorable and gradients are formed not found in the 50-L fermentor. Lack of oxygen in parts in these fermentors lead to unsynchronized cell growth and metabolism. There is also a possibility that the results can be interpreted as loss in plasmid stability in the industrial fermentor which might be relatively poor.

Conclusions

It has been demonstrated that modification methods to engineer the parental strain of *C. glutamicum* described here are useful for constructing novel strains that produce and export high levels of BCAAs (Blombach et al., 2008; Hasegawa et al., 2013; Wang et al., 2013; Kennerknecht et al., 2002; Radmacher et al., 2002; Satoshi et al., 2012; Vogt et al., 2014; Yin et al., 2014).

In order to achieve high L-leucine production and specific L-leucine production rate, it is necessary to reach a balance between biomass concentration, specific glucose consumption rate, and the activity of key enzymes.

The present study demonstrates the capability for greater production of L-leucine by a strain named MDLeu-19/pZ8-1*leuA*^{r}, based on the mutation breeding of L-leucine producing strain ML1-9. This was achieved by overexpression of *leuA*^{r} and reasonable modifications of L-leucine biosynthesic pathways by redistribution of various types of precursors and repression of negative regulation. Using the optimized conditions described above, the final maximum output in the 50-L automatic fermentor was 38.1 g L^{-1}. The maximal specific L-leucine production rate of 0.794 g L^{-1} h^{-1} was obtained for a 48 h fermentation with a maximum conversion efficiency of

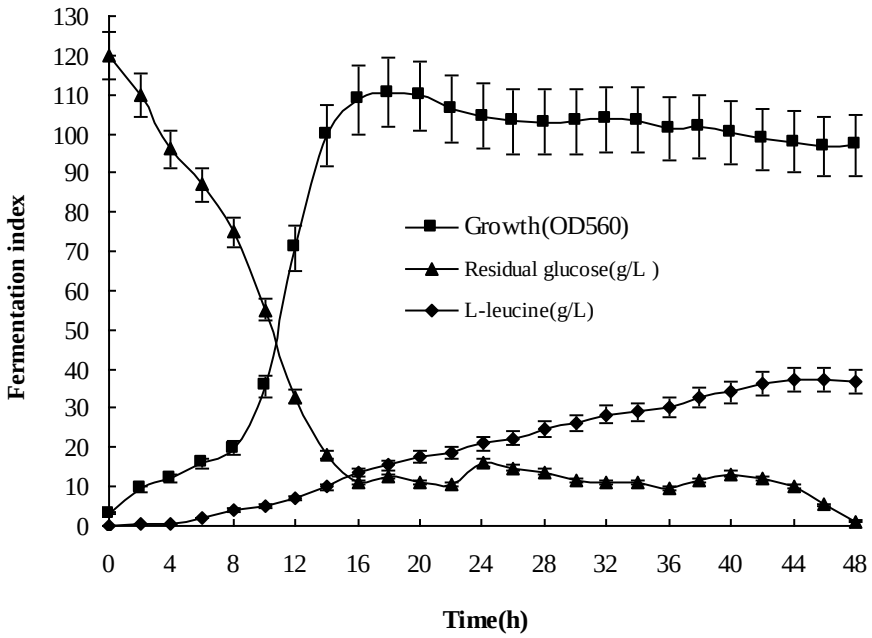

Figure 4. Strain MDLeu-19/pZ8-1*leuA*^{r} fermentation in a 150-m^3 fermentor.

0.306 g g^{-1}, with an increase compared to the original strain by 105.9 and 50.9%, respectively. Moreover, we have successfully achieved industrial–scale production of L-leucine in 150-m^3 fermentor with a yield of 37.5 g L^{-1} and a glucose conversion rate of 25.8%. Furthermore, the total content of 'contaminating' amino acids was less than 12.5% which influences the costs for downstream processing and product purification, making this strain potentially ideal for industrial application.

To the best of our knowledge, this is the first report of MDLue-19/pZ8-1*leuA*^{r} strain producing the highest yield and conversion efficiency for a bacterial L-leucine in industrial-scale production. Furthermore, in order to increase the yield of L-leucine, improve industrial fermentation efficiency and reduce cost, the use of comparative transcriptomics and proteomics analyses will allow for confirmation that the increased expression of these genes are correlated to L-leucine biosynthesis and also provide additional targets for strain improvement.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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