J Ind Microbiol Biotechnol (2012) 39:63–72
DOI 10.1007/s10295-011-1000-1

ORIGINAL PAPER

Improvement of L-valine production at high temperature in *Brevibacterium flavum* by overexpressing *ilv*EBN^{r}C genes

Xiaohu Hou · Xiangyang Ge · Di Wu · He Qian · Weiguo Zhang

Received: 17 March 2011 / Accepted: 7 June 2011 / Published online: 25 June 2011

Abstract *Brevibacterium flavum* ATCC14067 was engineered for L-valine production by overexpression of different *ilv* genes; the *ilv*EBN^{r}C genes from *B. flavum* NV128 provided the best candidate for L-valine production. In traditional fermentation, L-valine production reached 30.08 ± 0.92 g/L at 31°C in 72 h with a low conversion efficiency of 0.129 g/g. To further improve the L-valine production and conversion efficiency based on the optimum temperatures of L-valine biosynthesis enzymes (above 35°C) and the thermotolerance of *B. flavum*, the fermentation temperature was increased to 34, 37, and 40°C. As a result, higher metabolic rate and L-valine biosynthesis enzymes activity were obtained at high temperature, and the maximum L-valine production, conversion efficiency, and specific L-valine production rate reached 38.08 ± 1.32 g/L, 0.241 g/g, and 0.133 g g^{-1} h^{-1}, respectively, at 37°C in 48 h fermentation. The strategy for enhancing L-valine production by overexpression of key enzymes in thermotolerant strains may provide an alternative approach to enhance branched-chain amino acids production with other strains.

Keywords *Brevibacterium flavum* · L-Valine · *ilv*EBN^{r}C genes · High-temperature fermentation

X. Hou (✉) · X. Ge · D. Wu · H. Qian · W. Zhang
The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, JiangNan University, 1800# Lihu Road, Wuxi 214122, JiangSu Province, People's Republic of China
e-mail: houxiaohu828@163.com

Introduction

Brevibacterium flavum, subspecies of *Corynebacterium glutmicum*, one of the most used corynebacteria in industrial fermentation, has been widely used to produce L-glutamate [27], L-lysine [24], L-threonine [10], and branched-chain amino acids [16]. L-Valine, an essential amino acid, is used for infusion solutions, for cosmetics, and as a precursor for the chemical synthesis of some herbicides [3, 13].

In corynebacterium, L-valine is synthesized from pyruvate in a pathway comprising four reactions catalyzed by acetohydroxyacid synthase (AHAS, *ilv*BN gene product), acetohydroxyacid isomeroreductase (AHAIR, *ilv*C gene product), dihydroxyacid dehydratase (DHAD, *ilv*D gene product), and transaminase B (TA, *ilv*E gene product) [3, 6, 18]. The *ilv*B gene encoding a catalytic subunit and the *ilv*N gene encoding a regulatory subunit form an operon with the *ilv*C gene [11], and the *ilv*BNC operon is a target for overexpression for L-valine accumulation [1, 2, 4, 21]. TA catalyzes the conversion of 2-oxo-isovalerate into L-valine with L-glutamate as the amino group donor [15]. Overexpression of the *ilv*BNC operon with the *ilv*E gene afforded higher L-valine production than overexpression of the *ilv*BNC operon the with *ilv*D gene [1, 3]. AHAS is feedback-inhibited by three branched-chain amino acids; the 50% inhibitory concentrations for AHAS with valine, isoleucine, and leucine have been determined (0.9, 3.1, and 6 mM, respectively) [14]. However, site-directed mutagenesis carried out within the regulatory subunit showed that substitution of Gly-Ile-Ile (positions 20–22) with Asp-Asp-Phe removed the AHAS feedback resistance to the aforementioned three branched-chain amino acids [7].

The optimum temperatures were found to be between 30 and 35°C for TA, 37°C for DHAD, 45°C for AHAIR, and 50°C for AHAS [14]. The optimal temperatures of the

enzymes involved in biosynthesis of L-valine are much higher than traditional fermentation temperature (30–33°C), because commercially potent L-valine producers were traditionally constructed by repeating random mutation and selection and could not grow rapidly above 35°C [5, 8]. Compared with industrial producers, *B. flavum* ATCC14067, one of the wild-type strains, exhibits high growth and sugar consumption and even grows rapidly at 40°C [9]. The wild-type strain *C. glutmicum* ATCC13032 has been used in L-lysine fermentation at 40°C [17].

Because high-temperature fermentation in L-valine production has not yet been established, different *ilv* genes were overexpressed in *B. flavum* ATCC14067 to determine the best candidate for L-valine production at high temperatures, and traditional fed-batch fermentations were carried out at 31, 34, 37, and 40°C. The results obtained here may be helpful for the industrial production of branched-chain amino acids.

Materials and methods

Construction of candidate strains

Strains, plasmids, and oligonucleotides used in this study are listed in Table 1. Cloning and site-directed mutagenesis were carried out based on the standard method [22]. Using *B. flavum* ATCC14067 genome and *B. flavum* NV128 genome as template, we amplified the *ilv*BNC operon (3.6 kb) with primers *ilv*BNC-F containing an SD sequence [23] and *ilv*BNC-R; *ilv*BN gene (2.4 kb) was amplified with primers *ilv*BNC-F and *ilv*BN-R; *ilv*E gene (1.14 kb)

Table 1 Strains, plasmids, and oligonucleotides used in this study

	Relevant characteristic(s) or sequence	Reference or purpose
Strains		
E.coli JM109	recA1 end1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)/ F,(traD36 proAB$^+$ lac^q lacZ ΔM15)	Stratagene
B. flavum ATCC14067	Wild-type *B. flavum*	ATCC
B. flavum NV128	L-Valine producer (Leu^l α-AB^{hr} AHV^{hr} 2-TA^r)	Our lab
ATCC14067/pDXW-8-*ilv*BN^r	ATCC14067 harboring pDXW-8-*ilv*BN^r	This work
ATCC14067/pDXW-8-*ilv*BN^{r}C	ATCC14067 harboring pDXW-8-*ilv*BN^{r}C	This work
ATCC14067/pDXW-8-*ilv*EBN^{r}C	ATCC14067 harboring pDXW-8-*ilv*EBN^{r}C	This work
ATCC14067/pDXW-8-*ilv*EBNC	ATCC14067 harboring pDXW-8-*ilv*EBNC	This work
ATCC14067/pDXW-8-*ilv*E	ATCC14067 harboring pDXW-8-*ilv*E	This work
Plasmids		
pDXW-8	Km^r *E.coli-B. flavum* shuttle vector	Xu et al. [26]
pDXW-8-*ilv*BN^r	Plasmid carrying the *ilv*BN^r genes	This work
pDXW-8-*ilv*BN^{r}C	Plasmid carrying the *ilv*BN^{r}C genes	This work
pDXW-8-*ilv*EBN^{r}C	Plasmid carrying the *ilv*EBN^{r}C genes	This work
pDXW-8-*ilv*EBNC	Plasmid carrying the *ilv*EBNC genes	This work
pDXW-8-*ilv*E	Plasmid carrying the *ilv*E gene	This work
Oligonucleotides	5′ → 3′ sequence	
*ilv*BNC-F	CCGGAATTCGAAAGGAGATATACCGTGAATGTGGCAGCTTCTC	Amplifying *ilv*BNC and *ilv*BN
*ilv*BN-R	CCCAAGCTTTTAGATCTTGGCCGGAGC	Amplifying *ilv*BN
*ilv*BNC-R	CCCAAGCTTTTAAGCGGTTTCTGCG	Amplifying *ilv*BNC
MN-F	GTTCAGGACGTAGACG**ATGA**C**T**TTTCCCGCGTATCAGG	*ilv*N mutation
MN-R	CCTGATACGCGGGAAAA**GTCAT**CGTCTACGTCCTGAAC	*ilv*N mutation
*ilv*E-F	CCGGAATTCGAAAGGAGATATACCGTGTATCTGTCAGGTAGCAGGTGT	Amplifying *ilv*E
*ilv*E-R	CCGGAATTCTTAGCCAACCAGTGGGTAAAGCCAT	Amplifying *ilv*E
EM-F	AAACCCTCACCGG**C**ATTCAGCAAGGAAAC	Eliminating *EcoR*I site in *ilv*E
EM-R	GTTTCCTTGCTGAAT**G**CCGGTGAGGGTTT	Eliminating *EcoR*I site in *ilv*E

Mutated bases are in boldface; restriction sites (*EcoR*I, *Hind*III) are underlined

was amplified with primers *ilv*E-F containing the same SD sequence and *ilv*E-R. Site-directed mutagenesis in *ilv*N (MN-F and MN-R as primers) and *ilv*E (EM-F and EM-R as primers) was carried out with polymerase chain reaction (PCR) and *Dpn* I digestion and screening.

The *ilv*BN^r fragment, *ilv*BN^{r}C fragment, and *ilv*BNC fragment digested by *EcoR*Iand *Hind*III were ligated into pDXW-8 which was similarly digested. The resulting plasmids were 11,955, 13,152, and 13,152 bp in size, respectively, and were designated pDXW-8-*ilv*BN^r, pDXW-8-*ilv*BN^{r}C, and pDXW-8-*ilv*BNC. The *ilv*E fragment was ligated into pDXW-8, pDXW-8-*ilv*BN^{r}C, and pDXW-8-*ilv*BNC in the *EcoR*I site, the resulting plasmids were designated pDXW-8-*ilv*E, pDXW-8-*ilv*EBN^{r}C, and pDXW-8-*ilv*EBNC (*ilv*E before *ilv*BN^{r}C and *ilv*BNC), and were 9,703, 14,313, and 14,313 bp in size, respectively. Figure 1 shows a map of pDXW-8-*ilv*EBN^{r}C. The plasmids were transformed to *B. flavum* ATCC14067 based on the method of Xu et al. [26], and the strains were designated *B. flavum* ATCC14067/pDXW-8-*ilv*BN^r, *B. flavum* ATCC14067/pDXW-8-*ilv*BN^{r}C, *B. flavum* ATCC14067/pDXW-8-*ilv*EBNC, *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C, and *B. flavum* ATCC14067/pDXW-8-*ilv*E, respectively.

Culture conditions

Escherichia coli JM109 was used for the construction of pDXW-8-*ilv*BN^r, pDXW-8-*ilv*BN^{r}C, pDXW-8-*ilv*EBNC, pDXW-8-*ilv*EBN^{r}C, and pDXW-8-*ilv*E, grown in LB media at 37°C with 200 rpm. *B. flavum* was grown on LBG plate (LB supplemented with 5 g/L glucose) at 31°C. The concentration of kanamycin used was 50 μg/mL for *E. coli*, and 30 μg/mL for *B. flavum*.

The medium used for seed culture consisted of (per liter) 25 g glucose, 35 g corn steep liquor, 5 g $(NH_4)_2SO_4$, 2 g urea, 1 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, and 30 g $CaCO_3$. Fermentation medium contained (per liter) 125 g glucose, 8 g corn steep liquor, 12 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 50 μg D-biotin, and 100 μg thiamine-HCl. The initial pH of all media was adjusted to 7.3. $CaCO_3$ was sterilized by dry heat sterilization at 160°C for 90 min before being added to the medium.

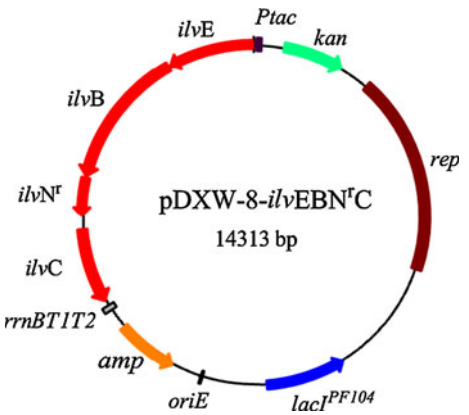

Fig. 1 Map of pDXW-8-*ilv*EBN^{r}C

Fed-batch fermentations were carried out in a 7-L jar fermentor (KF-7 l, Korea Fermentor Co., Inchon, Korea), containing 3.5 L medium with an inoculum size of 8% (v/v) from the seed culture grown to exponential phase (OD_{600} = 15). Ammonia water was used to balance the pH at 7.0 and to provide a nitrogen source. Glucose solution (800 g/L) was used to maintain the glucose concentration between 20 and 30 g/L in the late fermentation phase by adjusting the feeding speed according to glucose concentration checked every 4 h. Because high relative dissolved oxygen will result in high concentration of metabolites in the citric acid (TCA) cycle, especially L-glutamate [12, 25], relative dissolved oxygen was controlled between 10 and 20% by adjusting rotating speed and ventilation rate. Isopropyl-β-D-thio-galactoside (IPTG) inducer concentration was 1 mM at 12 h. Traditional 31°C fermentation and 34, 37, and 40°C fermentations, were carried out based on the growth of the strains at different temperatures.

Enzyme activity assay

The 4-mL samples were centrifuged at 12,000×*g* for 10 min, the supernatant was used to determine glucose and amino acid concentrations, the cell pellet was washed twice in cold 2% KCl, then, the cells were suspended in the disruption buffer (100 mM potassium phosphate buffer (pH 7.3) containing 0.5 mM dithiothreitol (DTT) and 20% glycerol (v/v) and stored at −20°C until analysis of enzyme activity [14]. Crude extracts were obtained by sonication of the cell suspension (20 kHz, 200 cycles spaced out by 2 and 3 s). Cellular debris was removed by centrifugation and the supernatant was kept on ice until the determination.

AHAS activity assay

The AHAS activity assay was a modification of the methods of Yang et al. [28] and Leyval et al. [14]. It is based on the conversion of pyruvate to acetoin through α-acetolactate. The reaction mixture contained 60 mM potassium phosphate buffer (pH 7.4), 50 mM sodium pyruvate, 10 mM $MgCl_2$, 100 μM thiamine pyrophosphate (TPP), and 100 μM flavin adenine dinucleotide (FAD). The reaction was initiated by the addition of 100 μL of crude extract to 2.5 mL of the mixture and was incubated at appropriate temperatures (31, 34, 37, 40°C) in accordance

with the fermentation temperatures. The reaction was terminated by acidification with 200 μL 3 M H_2SO_4. Then, the assay solution was incubated for 15 min at 60°C to allow the transformation of α-acetolactate into acetoin. The formed acetoin was quantified based on the Voges–Proskauer method; 1 mL 0.5% creatine and 2 mL 5% α-naphthol dissolved in 2.5 M NaOH were added and the mixture was incubated for 20 min at 60°C. The absorbance of the reddish reaction mixture (5.8 mL) was measured at 525 nm. One unit of AHAS activity was defined as the amount of enzyme required to form 1 μmol of α-acetolactate per minute.

AHAIR activity assay

The AHAIR assay was based on the method of Leyval et al. [14]. The reaction mixture (990 μL) contained 100 mM potassium phosphate buffer (pH 7.3), 10 mM α-acetolactate, 3 mM $MgCl_2$, and crude extract. The α-acetolactate was synthesized by mixing 0.1 M sodium hydroxide and 50 mM ethyl 2-acetoxy-2-acetoacetate for 30 min at room temperature under anoxic conditions. The AHAIR reaction was initiated by adding 10 μL of 10 mM NADPH in the assay solution and was performed at 31, 34, 37, and 40°C. NADPH consumption was followed at 340 nm. No decrease in the concentration of NADPH was observed when the assay was performed in the absence of α-acetolactate meaning that the endogenous consumption of NADPH was negligible.

One unit of AHAIR activity was defined as the amount of enzyme required to consume 1 μmol of NADPH per minute, using a molar extinction coefficient of 6,250 M^{-1} cm^{-1} for NADPH.

TA activity assay

The TA assay was based on the method of Leyval et al. [14]. The assay system contained 100 mM Tris–HCl (pH 9), 0.25 mM pyridoxal-5-phosphate, 5 mM α-ketoisovalerate, 10 mM potassium glutamate, and crude extract (in 1 mL). The reaction was performed at 31, 34, 37, and 40°C and 200-μL samples were collected at several time points over a period of 10 min. The reaction was terminated by mixing each sample with 60 μL of 21% perchloric acid. In order to remove protein, the sample was centrifuged (12,000×*g*, 5 min, 4°C). The supernatant was neutralized by addition of 5 M KOH and then centrifuged (12,000×*g*, 10 min, 4°C). L-Valine formation was then quantified by reversed-phase high-pressure liquid chromatography (HPLC). One unit of TA activity was defined as the amount of enzyme required to form 1 μmol of L-valine per minute.

Analytical methods

Five-milliliter samples were taken from the fermentor every 4 h. Then 1 mL was used to determine the biomass concentration by measuring the OD_{600} after an appropriate dilution or dry cell weight (DCW) per liter, where centrifuged at 12,000×*g* for 10 min then washed twice with distilled water, and dried at 105°C until achieving a constant weight. Under these experimental conditions, the correlation equation was DCW (g/L) = 0.42 × OD_{600} ($R^2 = 0.9881$).

Glucose concentration was determined by an SBA-40E immobilized enzyme biosensor. The amino acid concentrations were determined by reversed-phase HPLC.

The specific growth rate (μ) was determined by integrating over a time period of t_0 to t according to the equation for growth in a fed-batch system

$$\mathrm{d}(XV) = \mu XV\mathrm{d}t \quad (1)$$

producing an exponential equation of the type $y = ae^{\mu t}$. The specific glucose consumption rate (q_s) and the specific L-valine production rate (q_{val}) were calculated according to the equation in a fed-batch system

$$\mathrm{d}(SV) = -q_s XV\mathrm{d}t \quad (2)$$

$$\mathrm{d}(PV) = q_{val} XV\mathrm{d}t \quad (3)$$

where X and V are DCW and volume of the fermentor, S and P are the concentration of glucose and L-valine. The specific glucose consumption rate and the specific L-valine production rate also can be obtained from the slope of the semi-logarithmic plot of glucose concentration and L-valine production versus $(XV - X_0V_0)/\mu$ respectively.

Results and discussion

Effect of overexpression of different *ilv* genes on L-valine production at different temperatures

Thermotolerance of B. flavum

In order to verify the effect of temperature on the growth of *B. flavum*, we examined the thermotolerance of the classically derived industrial producer *B. flavum* NV128 and *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C at 31 and 40°C (Fig. 2a). Both strains were streaked on LBG plates, followed by incubation for 36 h at the temperatures indicated. The classically derived industrial producer *B. flavum* NV128 grew well at 31°C but showed no growth at 40°C; however, *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C grew well at both temperatures. This result indicated that the intrinsic thermotolerance has been lost in strain *B. flavum*

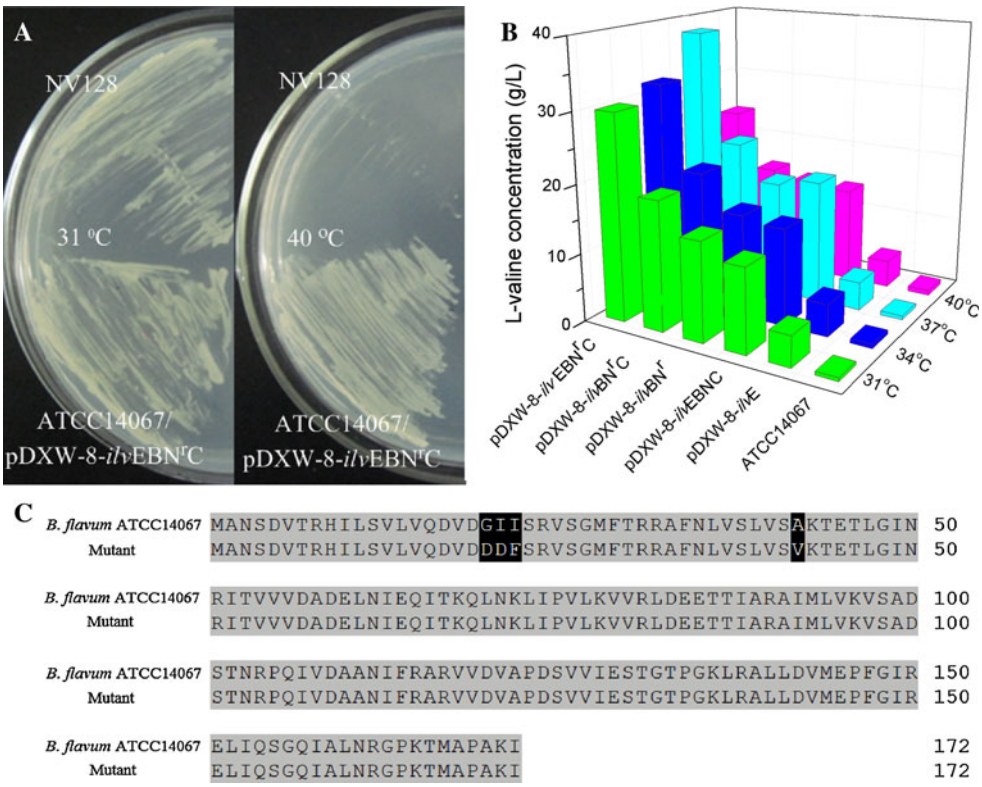

Fig. 2 Effect of overexpression of different *ilv* genes on L-valine production at different temperatures. **a** Growth of *B. flavum* at 31 and 40°C, **b** L-valine production by overexpressing different *ilv* genes at different temperatures, **c** comparison of wild-type and mutant sequences of regulatory subunit of AHAS

NV128 during the process of repeated random mutation and selection. On the other hand, *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C exhibited growth at 40°C, indicating the potential application of this strain to high-temperature fermentation of L-valine.

Effect of overexpression of different ilv genes on L-valine production at different temperatures

Bartek et al. [1] and Blombach et al. [3] demonstrated that overexpression of the *ilv*BNC operon with the *ilv*E gene afforded higher L-valine production than overexpression of the *ilv*BNC operon with the *ilv*D gene. To study the separate contributions of *ilv*B, *ilv*N^r, *ilv*C, and *liv*E genes to the production of L-valine, the stains of *B. flavum* ATCC14067/pDXW-8-*ilv*BN^r, *B. flavum* ATCC14067/pDXW-8-*ilv*BN^{r}C, *B. flavum* ATCC14067/pDXW-8-*ilv*EBNC, *B. flavum* ATCC 14067/pDXW-8-*ilv*EBN^{r}C, and *B. flavum* ATCC14067/pDXW-8-*ilv*E were constructed, fermentations with the resulting strains were carried out at 31, 34, 37, and 40°C with the fermentation with *B. flavum* ATCC14067 as control experiment. As shown in Fig. 2b, compared with L-valine production by *B. flavum* ATCC14067 (less than 1 g/L at each temperature), the maximum L-valine production by *B. flavum* ATCC14067/pDXW-8-*ilv*EBNC and *B. flavum* ATCC14067/pDXW-8-*ilv*E reached 18 ± 0.67 g/L at 37°C and 4.8 ± 0.41 g/L at 34°C, indicating that L-valine production can be enhanced to some extent by overexpression of wild-type *ilv* genes.

With *ilv*N^r gene overexpressed, the maximum L-valine production by *B. flavum* ATCC14067/pDXW-8-*ilv*BN^r, *B. flavum* ATCC14067/pDXW-8-*ilv*BN^{r}C, and *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C was increased to 16.82 ± 0.41, 22.23 ± 0.67, and 38.08 ± 1.32 g/L at 37°C, respectively. The results suggested that overexpression of *ilv*N^r and *ilv*E genes played an important role in the high production of L-valine, and *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C may be a good candidate for L-valine production at high temperatures. Compare to wild-type enzymes (*ilv*EBNC products), there were no mutants in the catalytic subunit of AHAS, AHAIR, and TA, but 4 mutants (G20D, I21D, I22F, A42V) in the regulatory subunit of AHAS (*ilv*N^r product). The *ilv*N^r gene was amplified from *B. flavum* NV128 and then site-directed mutagenesis was performed; a comparison of wild-type and mutant sequences of the regulatory subunit of AHAS is shown in Fig. 2c.

Kinetic analysis of L-valine production by *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C in different fermentations

In order to determine the reasons for the enhanced L-valine production at 37°C, *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C was cultured at different temperatures and the

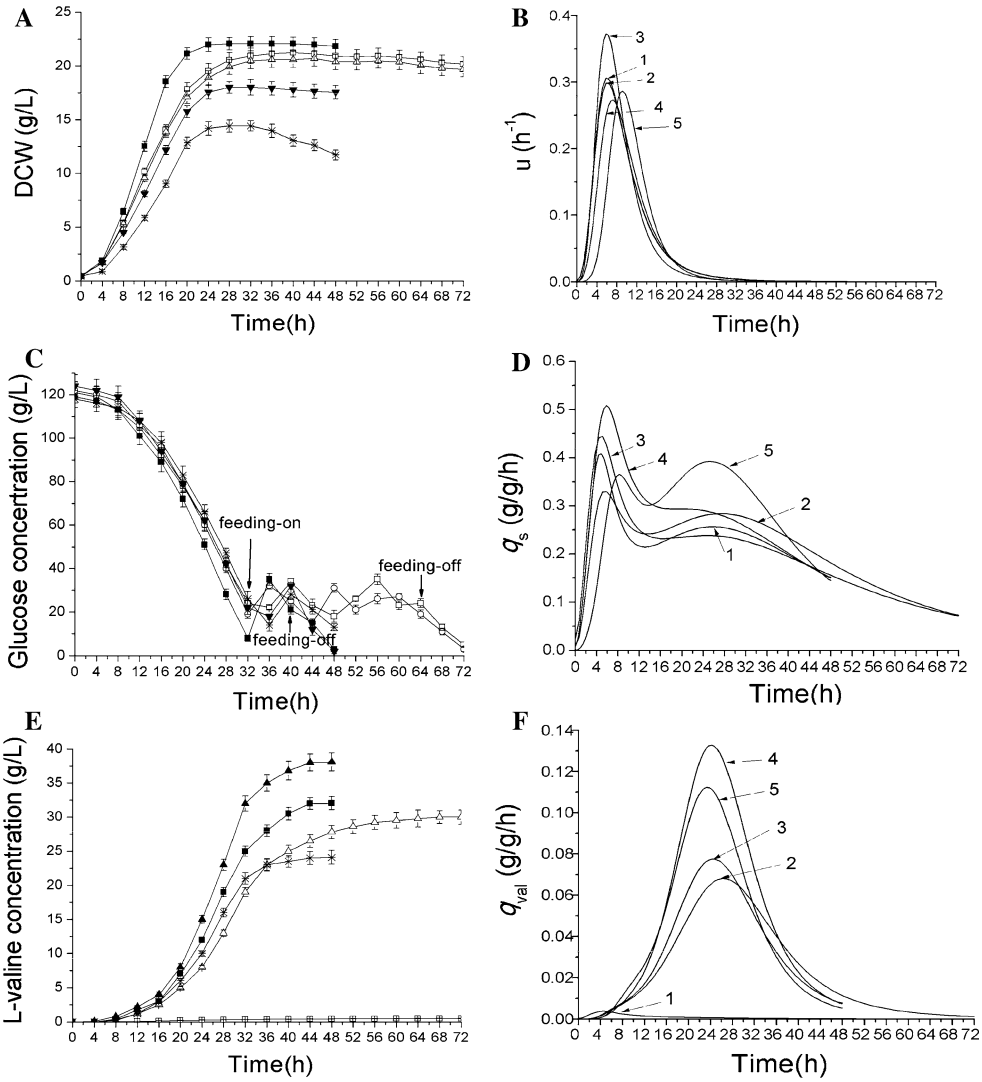

Fig. 3 Parameters of L-valine production by *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C in different fermentations. **a** DCW, **b** specific growth rate, **c** glucose concentration, **d** specific glucose consumption rate, **e** L-valine production, **f** specific L-valine production rate. *B. flavum* ATCC14067 at 31°C in 72 h fermentation (*open squares* and *curve* 1); *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C at 31°C in 72 h fermentation (*open triangles* and *curve* 2), 34°C in 48 h fermentation (*filled squares* and *curve* 3), 37°C in 48 h fermentation (*filled inverted triangles* and *curve* 4), and 40°C in 48 h fermentation (*asterisks* and *curve* 5)

fermentation kinetics was further analyzed. As shown in Fig. 3, in traditional 31°C fermentation (32 h feeding-on, 64 h feeding-off), the maximal DCW was 21.24 ± 0.74 and 20.70 ± 0.72 g/L, the maximal specific growth rate reached 0.306 h^{-1} at 6 h and 0.298 h^{-1} at 6.5 h, and the L-valine concentration reached 0.51 ± 0.03 and 30.08 ± 0.92 g/L for *B. flavum* ATCC14067 and *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C respectively, The results indicated that overexpression of the *ilv*EBN^{r}C genes had little influence on the growth of *B. flavum* ATCC14067, however, it dramatically contributed to L-valine production.

At newly elevated temperatures (32 h feeding-on, 40 h feeding-off), for *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C only, the maximal DCW was 22.05 ± 0.66 g/L for 34°C, 18.02 ± 0.73 g/L for 37°C, and 14.42 ± 0.58 g/L for 40°C; the maximal specific growth rate at different temperatures was 0.298 h^{-1} for 31°C, 0.372 h^{-1} for 34°C, 0.273 h^{-1} for 37°C, and 0.287 h^{-1} for 40°C. These results demonstrated the optimum growth temperature was near to 34°C. Both OD_{600} and DCW decreased after 32 h at 40°C, suggesting some negative thermal influences on *B. flavum*. However, as temperature increased, specific glucose consumption rate increased, as shown in Fig. 3d. Compared with 0.322 g/g/L at 31°C, the maximal specific glucose consumption rate was increased to 0.449 g/g/L at 34°C, 0.512 g/g/L at 37°C, 0.449 g/g/L at 34°C, and 0.485 g/g/L at 40°C. This suggested that high metabolic rate can be obtained at high temperature, which also can be used to the shorten fermentation time.

Maximum L-valine production of 38.08 ± 1.32 g/L and the maximal specific L-valine production rate of 0.133 g g^{-1} h^{-1} were obtained at 37°C in 48 h fermentation. An L-valine production of 32.05 ± 1.05 and 24.12 ± 1.05 g/L and maximal specific L-valine production rate of 0.078 and 0.112 g g^{-1} h^{-1} were obtained at 34 and 40°C in 48 h fermentations respectively. This finding

suggested that it was necessary to maintain an appropriate temperature (near 37°C) to reach high L-valine production and specific L-valine production rate.

As shown in Fig. 3, L-valine production was coupled with cell growth. Unlike in the uncoupled, dual growth and production process, in which cell growth and production can be optimized separately, it may be difficult to reach a balance between cell growth and L-valine production for maximizing product formation. Maximum L-valine production and maximum specific L-valine production rate were obtained at 37°C, whereas maximum DCW and maximum specific growth rate were obtained at 34°C. Therefore, if the fermentation temperature were maintained at 37°C, the process would be easily controlled, but high biomass would not be reached. A two-stage temperature control strategy may provide an alternative approach to maintain high cell growth rate to prolong L-valine production for higher titers under high-temperature conditions.

Metabolic flux analysis of L-valine production by *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C in different fermentations

To further investigate the reasons for high production of L-valine at 37°C, AHAS, AHAIR, and TA activity and by-product concentration were checked and used for metabolic flux analysis. As shown in Fig. 4, AHAS, AHAIR, and TA activity were dramatically improved after IPTG induction at 12 h. Maximum AHAS activity of 180.23 ± 7.08 U/mL and maximum AHAIR activity of 68.75 ± 2.48 U/mL were reached at 40°C, whereas maximum TA activity of 46.23 ± 1.38 U/mL was obtained at 34°C; the results obtained here were also in accordance with the reported optimum temperatures of AHAS, AHAIR, and TA [14].

The 48-h fermentation at 40°C afforded maximum AHAS and AHAIR activity but did not have ideal TA activity, biomass concentration (Fig. 3a), and specific glucose consumption rate (Fig. 3d). The 48-h fermentation at 34°C afforded maximum TA activity and biomass concentration but did not have ideal AHAS and AHAIR activity or specific glucose consumption rate. Therefore, both processes failed to reach high L-valine production with maximal specific L-valine production rates of 0.112 and 0.078 g g^{-1} h^{-1} respectively (Table 2). In contrast, the balance of biomass concentration, specific glucose consumption rate, and enzymes activity was better controlled at 37°C in 48 h fermentation than other fermentations processes, so the maximum L-valine production and maximum specific L-valine production rate were obtained at 37°C.

Figure 4d shows the by-products distribution, which are also listed in Table 2. L-Alanine, L-glutamate, L-leucine, and L-isoleucine were the main by-products; other by-products were not listed in the table, because their

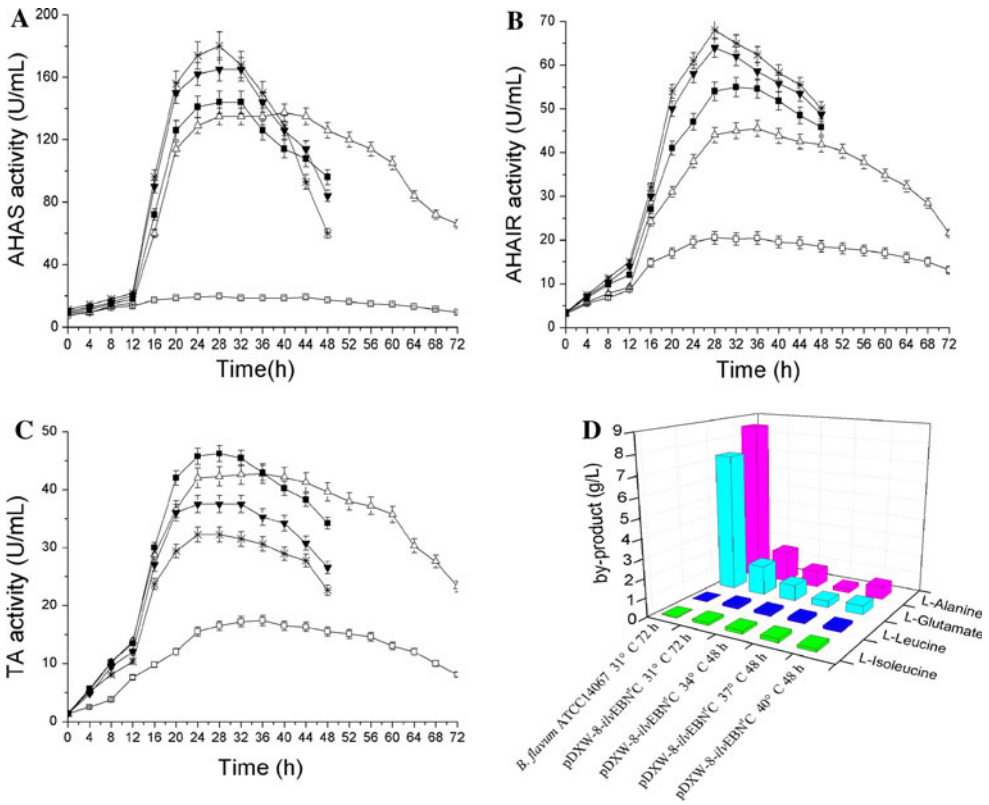

Fig. 4 Enzymes activity and by-products distribution of L-valine production by *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C in different fermentations. **a** AHAS activity, **b** AHAIR activity, **c** TA activity, **d** by-products distribution. *B. flavum* ATCC14067 at 31°C in 72 h fermentation (*open squares*); *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C at 31°C in 72 h fermentation (*open triangles*), 34°C in 48 h fermentation (*filled squares*), 37°C in 48 h fermentation (*filled inverted triangles*), and 40°C in 48 h fermentation (*asterisks*)

Table 2 Comparison of fed-batch culture parameters of L-valine production in different fermentations

Parameters	*B. flavum* ATCC14067 at 31°C in 72 h	ATCC14067 pDXW-8-*ilv*EBN^{r}C at 31°C in 72 h	ATCC14067 pDXW-8-*ilv*EBN^{r}C at 34°C in 48 h	ATCC14067 pDXW-8-*ilv*EBN^{r}C at 37°C in 48 h	ATCC14067 pDXW-8-*ilv*EBN^{r}C at 40°C in 48 h
L-Isoleucine (g/L)	0.034 ± 0.002	0.153 ± 0.003	0.165 ± 0.003	0.195 ± 0.004	0.164 ± 0.004
L-Leucine (g/L)	0.024 ± 0.001	0.165 ± 0.002	0.168 ± 0.003	0.185 ± 0.003	0.155 ± 0.003
L-Glutamate (g/L)	7.25 ± 0.101	1.56 ± 0.082	0.83 ± 0.055	0.35 ± 0.021	0.48 ± 0.028
L-Alanine (g/L)	8.52 ± 0.120	1.68 ± 0.071	0.88 ± 0.037	0.23 ± 0.012	0.68 ± 0.034
L-Valine production (g/L)	0.51 ± 0.03	30.08 ± 0.92	32.05 ± 1.05	38.08 ± 1.32	24.12 ± 1.05
Glucose consumption (g/L)	212 ± 7	232 ± 8	180 ± 5	158 ± 3	141 ± 3
Conversion efficiency (g/g)	0.002	0.129	0.178	0.241	0.171
Maximal specific L-valine production rate ($g\ g^{-1}\ h^{-1}$)	0.037	0.068	0.075	0.133	0.112

concentration did not exceed 0.1 g/L. The concentration of L-isoleucine and L-leucine was little improved at elevated fermentation temperatures because the enzymes in the L-valine biosynthesis pathway were also the enzymes for L-Isoleucine and L-leucine biosynthesis. For *B. flavum* ATCC14067, L-glutamate concentration was 7.25 ± 0.101 g/L and L-alaine concentration was 8.52 ± 0.120 g/L after traditional fermentation at 31°C for 72 h. Over-expressing *ilv*EBN^{r}C genes in *B. flavum* ATCC14067 remarkably decreased by-products formation (1.56 ± 0.082 g/L for L-glutamate and 1.68 ± 0.071 g/L for L-alanine). Compared to traditional fermentation at 31°C for 72 h with *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C, L-glutamate and L-alanine were further decreased at elevated fermentation temperatures (Table 2; Fig. 4d); L-glutamate was decreased by 46.79% at 34°C, 77.61% at 37°C, and 69.34% at 40°C; and L-alanine was decreased by 47.78% at 34°C, 86.36% at 37°C, and 59.63% at 40°C. Minimum concentrations of L-alanine and L-glutamate were obtained at 37°C in 48 h fermentation, 0.23 ± 0.012 and 0.35 ± 0.021 g/L respectively.

Figure 5 shows the metabolic pathway of *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C in high-temperature fermentations; L-valine is synthesized from two molecules of pyruvate, and L-isoleucine is synthesized from one molecule of pyruvate and one molecule of 2-ketobutyrate catalyzed by AHAS, AHAIR, DHAD, and TA. L-Leucine is synthesized from 2-keto-isovalerate. Meanwhile, pyruvate can also undergo aminotransferase (*ala*T)-catalyzed transformation to L-alanine, and also dehydrogenase (*ace*E)-catalyzed transformation to acetyl-CoA and then L-glutamate through TCA cycle. Owing to overexpression of *ilv*E before the *ilv*BN^{r}C operon, TA with high activity catalyzed the conversion of 2-keto-isovalerate to L-valine rather than forming L-leucine via reactions catalyzed by the enzymes encoded by *leu*ABCD. In high-temperature fermentations, AHAS, AHAIR, DHAD, and TA activity were enhanced, which improves the metabolic flux to the L-valine biosynthesis pathway from pyruvate instead of L-glutamate and L-alanine biosynthesis pathways. L-Valine was synthesized preferentially instead of L-isoleucine because L-threonine was limited. L-Glutamate is also used as an amino group donor in L-valine formation [15], so L-glutamate concentration decreased as L-valine formed.

Park et al. used *Escherichia coli* W3110 at 31°C in 40–80 h fed-batch fermentations for the production of L-valine based on transcriptome analysis, in silico gene knockout simulation [20], and in silico flux response analysis [19]; L-valine production and conversion efficiency were finally increased to 32.3 g/L and 0.38 g/g respectively. *C. glutamicum*Δ*aceE*Δ*pqo*Δ*pgi* (pJC4*ilv*BNCE) constructed by Blombach et al. [3] was used in 90 h fed-batch fermentation at 30°C in which L-valine production reached 48 g/L, which also was the maximum L-valine

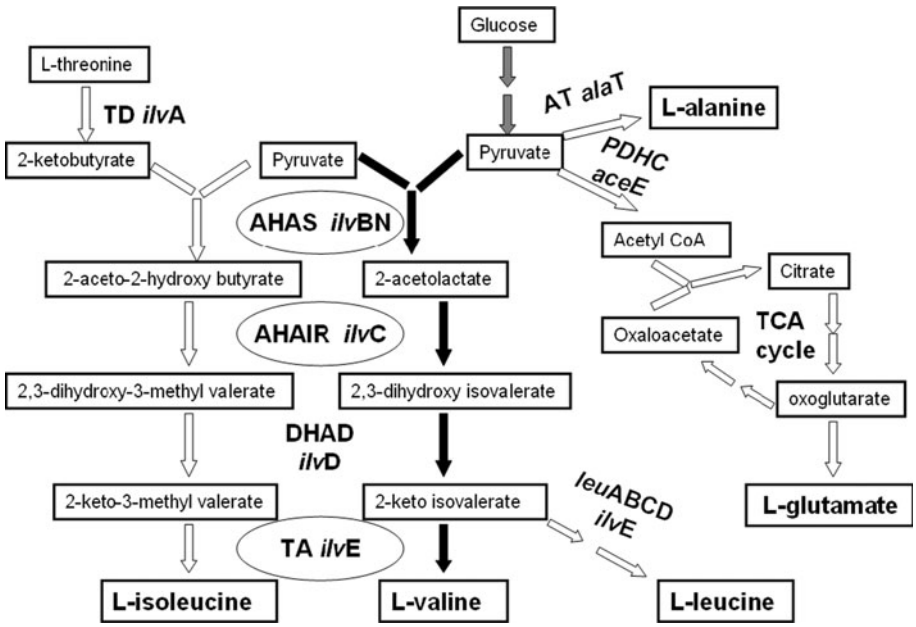

Fig. 5 Metabolic flux distribution maps for *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C in high-temperature fermentations

production with *C. glutamicum*. In contrast, 48 h fermentation with *B. flavum* ATCC14067/pDXW-8-*ilv*EBN^{r}C at 37°C afforded a high L-valine production of 38.08 ± 1.32 g/L, a low glucose consumption of 158 ± 3 g/L, and a high conversion efficiency of 0.241 g/g (Table 2). Low glucose consumption and high conversion efficiency would significantly decrease the overall product cost. High-temperature and short-time fermentation also saves a large quantity of cooling water which is necessary in classical fermentation to maintain the correct fermentation temperature, especially in tropical regions and in summer.

The mechanism of improving L-valine productivity at high temperatures cannot be explained well at present. However, the discovery of the balance between biomass concentration, specific glucose consumption rate, and enzymes activity and its influence on L-valine productivity opens up a new avenue for further studies.

Conclusions

Brevibacterium flavum ATCC14067/pDXW-8-*ilv*EBN^{r}C is a good candidate for high-temperature fermentation. As this study demonstrates, to achieve high L-valine production and specific L-valine production rate, it is necessary to reach a balance between biomass concentration, specific glucose consumption rate, and the activity of key enzymes. A maximum L-valine production of 38.08 ± 1.32 g/L and maximal specific L-valine production rate of 0.133 g g^{-1} h^{-1} were obtained at 37°C in 48 h fermentation with a maximum conversion efficiency of 0.241 g/g. High-temperature fermentation may be helpful for the industrial production of L-valine.

Acknowledgments This project was financially supported by the Program of Chinese 863 National High-Tech Research and Development Plan Project (No. 2008AA02Z212).

References

1. Bartek T, Zonnchen E, Klein B, Gerstmeir R, Makus P, Lang S, Oldiges M (2010) Analysing overexpression of L-valine biosynthesis genes in pyruvate-dehydrogenase-deficient *Corynebacterium glutamicum*. J Ind Microbiol Biotechnol 37(3):263–270. doi:10.1007/s10295-009-0669-x
2. Blombach B, Arndt A, Auchter M, Eikmanns BJ (2009) L-Valine production during growth of pyruvate dehydrogenase complex-deficient *Corynebacterium glutamicum* in the presence of ethanol or by inactivation of the transcriptional regulator SugR. Appl Environ Microbiol 75(4):1197–1200. doi:10.1128/AEM.02351-08
3. Blombach B, Schreiner ME, Bartek T, Oldiges M, Eikmanns BJ (2008) *Corynebacterium glutamicum* tailored for high-yield L-valine production. Appl Microbiol Biotechnol 79(3):471–479. doi:10.1007/s00253-008-1444-z
4. Blombach B, Schreiner ME, Holatko J, Bartek T, Oldiges M, Eikmanns BJ (2007) L-Valine production with pyruvate dehydrogenase complex-deficient *Corynebacterium glutamicum*. Appl Environ Microbiol 73(7):2079–2084. doi:10.1128/AEM.02826-06
5. Demain AL (2000) Microbial biotechnology. Trends Biotechnol 18(1):26–31. doi:S0167-7799(99)01400-6
6. Eggeling L, Bott M (2005) Handbook of *Corynebacterium glutamicum*. CRC, Boca Raton
7. Elisakova V, Patek M, Holatko J, Nesvera J, Leyval D, Goergen JL, Delaunay S (2005) Feedback-resistant acetohydroxy acid synthase increases valine production in *Corynebacterium glutamicum*. Appl Environ Microbiol 71(1):207–213. doi:10.1128/AEM.71.1.207-213.2005
8. Ikeda M (2003) Amino acid production processes. Adv Biochem Eng Biotechnol 79:1–35
9. Ikeda M, Ohnishi J, Hayashi M, Mitsuhashi S (2006) A genome-based approach to create a minimally mutated *Corynebacterium glutamicm* strain for efficient L-lysine production. J Ind Microbiol Biotechnol 33(7):610–615. doi:10.1007/s10295-006-0104-5

10. Ishida M, Sato K, Hashiguchi K, Ito H, Enei H, Nakamori S (1993) High fermentative production of L-threonine from acetate by a *Brevibacterium flavum* stabilized strain transformed with a recombinant plasmid carrying the *Escherichia coli* thr operon. Biosci Biotechnol Biochem 57:1755–1756
11. Keilhauer C, Eggeling L, Sahm H (1993) Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. J Bacteriol 175(17):5595–5603
12. Kimura E (2003) Metabolic engineering of glutamate production. Adv Biochem Eng Biotechnol 79:37–57
13. Leuchtenberger W, Huthmacher K, Drauz K (2005) Biotechnological production of amino acids and derivatives: current status and prospects. Appl Microbiol Biotechnol 69(1):1–8. doi:10.1007/s00253-005-0155-y
14. Leyval D, Uy D, Delaunay S, Goergen JL, Engasser JM (2003) Characterisation of the enzyme activities involved in the valine biosynthetic pathway in a valine-producing strain of *Corynebacterium glutamicum*. J Biotechnol 104(1–3):241–252. doi:10.1016/S0168-1656(03)00162-7
15. Marienhagen J, Kennerknecht N, Sahm H, Eggeling L (2005) Functional analysis of all aminotransferase proteins inferred from the genome sequence of *Corynebacterium glutamicum*. J Bacteriol 187(22):7639–7646. doi:10.1128/Jb.187.22.7639-7646.2005
16. Miyajima R, Shiio I (1972) Regulation of aspartate family amino acid biosynthesis in *Brevibacterium flavum* VI. Effects of isoleucine and valine on threonine dehydratase activity and its formation. J Biochem 71:951–960
17. Ohnishi J, Hayashi M, Mitsuhashi S, Ikeda M (2003) Efficient 40°C fermentation of L-lysine by a new *Corynebacterium glutamicum* mutant developed by genome breeding. Appl Microbiol Biotechnol 62(1):69–75. doi:10.1007/s00253-003-1254-2
18. Park J, Lee S (2010) Fermentative production of branched chain amino acids: a focus on metabolic engineering. Appl Microbiol Biotechnol 85(3):491–506. doi:10.1007/s00253-009-2307-y
19. Park JH, Kim TY, Lee KH, Lee SY (2011) Fed-batch culture of *Escherichia coli* for L-valine production based on in silico flux response analysis. Biotechnol Bioeng 108(4):934–946. doi:10.1002/bit.22995
20. Park JH, Lee KH, Kim TY, Lee SY (2007) Metabolic engineering of *Escherichia coli* for the production of L-valine based on transcriptome analysis and in silico gene knockout simulation. Proc Natl Acad Sci USA 104(19):7797–7802. doi:10.1073/pnas.0702609104
21. Radmacher E, Vaitsikova A, Burger U, Krumbach K, Sahm H, Eggeling L (2002) Linking central metabolism with increased pathway flux: L-valine accumulation by *Corynebacterium glutamicum*. Appl Environ Microbiol 68(5):2246–2250. doi:10.1128/AEM.68.5.2246-2250.2002
22. Sambrook J, Russel DV (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
23. Srivastava P, Deb JK (2005) Gene expression systems in corynebacteria. Protein Express Purif 40(2):221–229. doi:10.1016/j.pep.2004.06.017
24. Tada K, Kishimoto M, Omasa T, Katakura Y, Suga K (2000) L-Lysine production by exponential feeding of L-threonine. J Biosci Bioeng 90:669–674
25. Wittmann C (2010) Analysis and engineering of metabolic pathway fluxes in *Corynebacterium glutamicum*. Adv Biochem Eng Biotechnol 120:21–49. doi:10.1007/10_2009_58
26. Xu D, Tan Y, Huan X, Hu X, Wang X (2010) Construction of a novel shuttle vector for use in *Brevibacterium flavum*, an industrial amino acid producer. J Microbiol Methods 80(1):86–92. doi:10.1016/j.mimet.2009.11.003
27. Yamada K, Kinoshita S, Tsunoda T, Aida K (1972) The microbial production of amino acids. Halsted, New York
28. Yang YT, Peredelchuk M, Bennett GN, San KY (2000) Effect of variation of Klebsiella pneumoniae acetolactate synthase expression on metabolic flux redistribution in *Escherichia coli*. Biotechnol Bioeng 69(2):150–159. doi:10.1002/(SICI)1097-0290(20000720)69:2<150::AID-BIT4>3.0.CO;2-N