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ORIGINAL ARTICLE

L-Valine production with minimization of by-products' synthesis in *Corynebacterium glutamicum* and *Brevibacterium flavum*

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Abstract *Corynebacterium glutamicum* ATCC13032 and *Brevibacterium flavum* JV16 were engineered for L-valine production by over-expressing *ilvEBN*^{r}*C* genes at 31 °C in 72 h fermentation. Different strategies were carried out to reduce the by-products' accumulation in L-valine fermentation and also to increase the availability of precursor for L-valine biosynthesis. The native promoter of *ilvA* of *C. glutamicum* was replaced with a weak promoter MP*ilvA* (P-*ilvA*M1CG) to reduce the biosynthetic rate of L-isoleucine. Effect of different relative dissolved oxygen on L-valine production and by-products' formation was recorded, indicating that 15 % saturation may be the most appropriate relative dissolved oxygen for L-valine fermentation with almost no L-lactic acid and L-glutamate formed. To minimize L-alanine accumulation, *alaT* and/or *avtA* was inactivated in *C. glutamicum* and *B. flavum*, respectively. Compared to high concentration of L-alanine accumulated by *alaT* inactivated strains harboring *ilvEBN*^{r}*C* genes, L-alanine concentration was reduced to 0.18 g/L by *C. glutamicum* ATCC13032MP*ilvA*△*avtA* pDXW-8-*ilvEBN*^{r}*C*, and 0.22 g/L by *B. flavum* JV16*avtA*::*Cm* pDXW-8-*ilvEBN*^{r}*C*. Meanwhile, L-valine production and conversion efficiency were enhanced to 31.15 g/L and 0.173 g/g by *C. glutamicum* ATCC13032MP*ilvA*△*avtA* pDXW-8-*ilvEBN*^{r}*C*, 38.82 g/L and 0.252 g/g by *B. flavum* JV16*avtA*::*Cm* pDXW-8-*ilvEBN*^{r}*C*. This study provides combined strategies to improve L-valine yield by minimization of by-products' production.

Keywords L-Valine · *Corynebacterium glutamicum* · *Brevibacterium flavum* · *alaT* · *avtA*

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Introduction

L-Valine, an essential hydrophobic and branched-chain amino acid, is used as a component of cosmetics and pharmaceuticals as well as animal feed additives (Park et al. 2007; Leuchtenberger et al. 2005; Eggeling 2001; Patek 2007; Eggeling and Bott 2005). L-Valine has been produced by employing bacteria belonging to the genera *Brevibacterium* and *Corynebacterium* (Hermann 2003; Yamada et al. 1972). L-Valine is synthesized from pyruvate in a pathway comprising four reactions (Fig. 1), catalyzed by acetohydroxyacid synthase (AHAS, *ilvBN* gene product), acetohydroxyacid isomeroreductase (AHAIR, the *ilvC* gene product), dihydroxyacid dehydratase (DHAD, *ilvD* gene product), and transaminase B (TA, *ilvE* gene product) (Blombach et al. 2008; Patek 2007; Park and Lee 2010). Over-expression of *ilvBNC* operon (AHAS feedback-resistant by three branched-chain amino acids) and *ilvE* gene exhibited maximum L-valine production (Elisakova et al. 2005; Hou et al. 2012; Radmacher et al. 2002). However, the problem of by-products' formation emerged, especially L-isoleucine, L-leucine, and L-alanine, which share the almost same isoelectric point with L-valine, bring

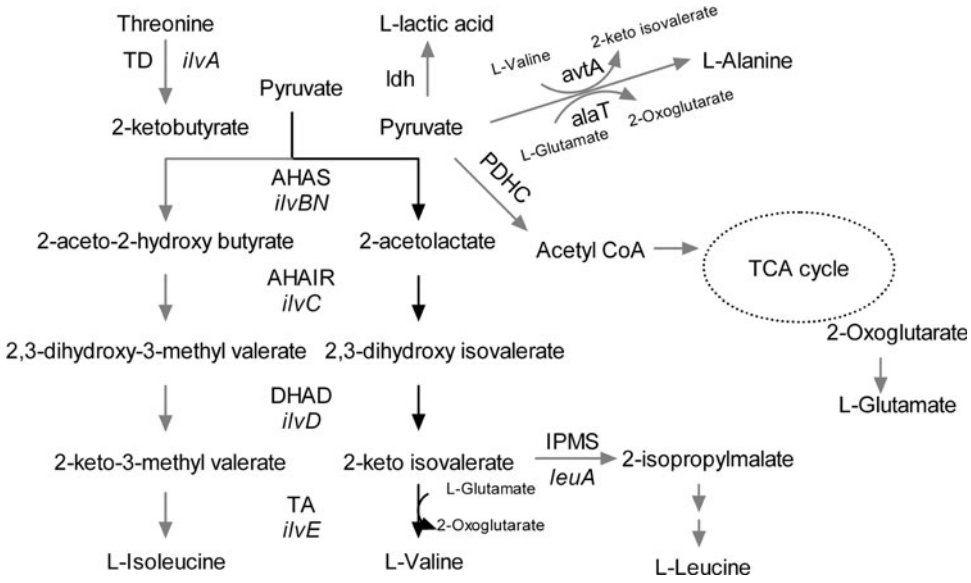

Fig. 1 Biosynthesis of L-valine and the main by-products in L-valine fermentation. *TD (ilvA)* threonine deaminase, *AHAS (ilvBN)* acetohydroxy acid synthase, *AHAIR (ilvC)* acetohydroxy acid isomeroreductase, *DHAD (ilvD)* dihydroxyacid dehydratase, *TA (ilvE)* transaminase B, *IPMS (leuA)* isopropymalate synthase, *ldh* lactate dehydrogenase, *PDHC* pyruvate dehydrogenase, *TCA* tricarboxylic acid, *avtA (avtA)* enzyme which can convert pyruvate to L-alanine with L-valine as amino donor, *alaT (alaT)* enzyme which can convert pyruvate to L-alanine with L-glutamate as amino donor

difficulty to L-valine industrial recovery processes. In addition, L-valine production will be affected by the formation of by-products to some extent.

To reduce the by-products' accumulation in L-valine fermentation, L-isoleucine and L-leucine should be the first step to be considered for engineering, because the enzymes involved in L-valine biosynthesis also catalyze the biosynthesis of L-isoleucine and L-leucine from L-threonine by threonine dehydratase (TD; *ilvA* gene product) and ketoisovalerate by 2-isopropylmalate synthase (IPMS; *leuA* gene product), respectively (Fig. 1). To solve this problem, two methods have been developed. The first one was deletion of the *ilvA* gene, resulting in L-isoleucine auxotrophy (Sahm and Eggeling 1999). The second one was down-modulation of the *ilvA* and *leuA* promoters, resulting in leak auxotroph of L-isoleucine and L-leucine (Holatko et al. 2009). The *C. glutamicum* strains with △*ilvA* produce L-valine when grown under conditions of L-isoleucine limitation. The minimal cultivation medium for these auxotrophic strains, however, must be supplemented with L-isoleucine. Adequate uptake of L-isoleucine by the auxotrophs could not therefore be ensured during cultivations for L-valine production (Radmacher et al. 2002). To avoid this effect, the native *ilvA* promoter was replaced by a mutant promoter Mp*ilvA* (P-*ilvA*M1CG). The activity of the mutant promoter was 13-fold lower than that of WT P-*ilvA*; the growth of *C. glutamicum* ATCC13032MP*ilvA* was similar to that of WT without L-isoleucine supplementation (Holatko et al. 2009).

Unwanted metabolites from pyruvate should be the second step to be considered for engineering. Pyruvate can act as precursors of numerous metabolites. Under oxygen deprivation, pyruvate can be catalyzed by lactate dehydrogenase to form L-lactic acid. If oxygen is supplied sufficiently, pyruvate can also be catalyzed by pyruvate dehydrogenase to form acetyl-CoA and then L-glutamate through TCA cycle. Oxygen supply has been considered to be one of the most important environmental factors affecting amino acid fermentation. Distribution of oxygen, in particular, could affect cell physiology to trigger undesirable stress responses such as switching biosynthesis from a desired amino acid to undesirable by-products like carbon dioxide, acids, and biomass (Ikeda 2003). Unlike sufficient oxygen supply for production of L-glutamate, L-arginine, and L-proline; L-valine formation is favorable under low dissolved oxygen tension.

L-Alanine should be the third step to be considered for engineering. L-Alanine was still the main by-product of constructed strains such as *C. glutamicum* 13032△*panBC*△*ilvA* pJC1*ilvBNCE*, *C. glutamicum* 13032△*aceE* pJC4*ilvBNCE*, and *B. flavum* ATCC14067 pDXW-8-*ilvEBN*^{r}*C* (Radmacher et al. 2002; Hou et al. 2012; Blombach et al. 2007). The mechanism of L-alanine synthesis has been demonstrated clearly (Fig. 1). AlaT (*alaT* gene product) converts pyruvate to L-alanine in an L-glutamate-dependent reaction, avtA (*avtA* gene product) is able to convert pyruvate to L-alanine in an L-valine-dependent manner (Marienhagen and Eggeling 2008; Marienhagen et al. 2005). However, with L-valine biosynthesis enhanced, effect of inactivation of *alaT* and/or *avtA* on L-valine production and L-alanine synthesis require further research in depth.

The purpose of this work was to minimize the by-procucts in L-valine fermentation and to further improve L-valine production in *C. glutamicum* and *B. flavum*. Down-modulation of *ilvA* promoter and inactivation of *alaT* and/or *avtA* combined with oxygen supply control were carried out for L-valine production with minimization of by-products' synthesis.

Materials and methods

Bacterial strains, plasmids, oligonucleotides, and culture conditions

B. flavum JV16 was engineered by repeating random mutagenesis and selection from wild type precursor strain *B. flavum* DSM 20411, which was deposited in Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). *B. flavum* JV16 was resistant to α-amino butyric acid and auxotrophic for L-leucine, L-isoleucine, and L-methionine, can produce 22 g/L L-valine, and was used as one of the working and parent strains.

The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. *E. coli* JM109 was grown in LB media at 37 °C with 200 rpm. *C. glutamicum* and *B. flavum* were grown on LBG plate (LB supplemented with 5 g/L glucose at 31 °C. The plasmids were transformed to *C. glutamicum* or *B. flavum* based on the method of Xu et al. (2010). The concentration of ampicillin used was 100 μg/mL for *E. coli*. The concentration of kanamycin used was 50 μg/mL for *E. coli* and 30 μg/mL for *C. glutamicum* and *B. flavum*. The concentration of chloramphenicol used was 20 μg/mL for *E. coli* and 5 μg/mL for *B. flavum*. DNA synthesis and sequencing were performed by Sangon (Shanghai, China).

Medium used for seed culture consisted of (per liter) 25 g of 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 containing (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 was used. Both media were adjusted to pH 7.3 with NaOH. $CaCO_3$ was sterilized by dry heat sterilization at 160 °C for 90 min before being added to the medium. For *B. flavum* JV16, 0.2 g/L of L-leucine, L-isoleucine, and L-methionine each was added to the fermentation medium.

Fed-batch fermentations were used to investigate the effect of different relative dissolved oxygen on L-valine production and by-products' formation, and to further investigate the mechanism of L-alanine synthesis with *ilvEBN*^{r}*C* genes over-expressed. 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 pH at 7.0 and to supply with nitrogen source. Glucose solution (800 g/L) was used to maintain 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. Relative dissolved oxygen was controlled by adjusting rotating speed and ventilation rate. IPTG was added to a final concentration of 1 mM at 12 h.

Construction of strains

Deletion of the chromosomal *alaT* and *avtA* genes in *C. glutamicum* were performed using crossover PCR and the suicide vector pK19mobsacB (Schafer et al. 1994). DNA fragments covering the 5′-end and the 3′-end of *alaT* and *avtA* were generated using the primer pairs *alaT*-L-F–*alaT*-L-R and *alaT*-R-F–*alaT*-R-R, *avtA*-L-F–*avtA*-L-R and *avtA*-R-F–*avtA*-R-R, respectively. The fragments were purified, mixed in equal amounts, and subjected to crossover PCR using primers *alaT*-L-F and *alaT*-R-R, *avtA*-L-F and *avtA*-R-R. The resulting fusion product △*alaT* (containing the *alaT* gene with an internal deletion of 938 bp) was ligated into *Xba*I and *Pst*I-restricted plasmid pK19mobsacB and transformed into *E. coli* and the resulting fusion product △*avtA* (containing the *avtA* gene with an internal deletion of 640 bp) was ligated into *Xba*I and *Hind*III-restricted plasmid pK19mobsacB and transformed into *E. coli*. The recombinant plasmids pK19mobsacB△*alaT* and pK19mobsacB△*avtA* were isolated from *E. coli* and electroporated into *C. glutamicum*. Clones were selected for kanamycin resistance to establish integration of the plasmid in the chromosome. In a second round of positive selection using sucrose resistance, clones were selected for deletion of the vector (Schafer et al. 1994). The deletions in the chromosomes were verified by PCR analysis.

Native P-*ilvA* was amplified with primers P*ilvA*-F and P*ilvA*-R, site-directed mutagenesis in P-*ilvA* (MP*ilvA*-F and MP*ilvA*-R as primers) was carried out with PCR and *Dpn*I digestion and screening based on the standard site-directed mutagenesis method (Sambrook and Russel 2001). The resulting product MP*ilvA* (P-*ilvA*M1CG) was ligated into *EcoR*I and *Xba*I-restricted plasmid pK19mobsacB. The next steps were the same as procedure of deletion of the chromosomal gene in *C. glutamicum*. Substitution of native promoter P-*ilvA* with mutant promoter MP*ilvA* was verified by sequencing. The strategy used for allelic exchange in *C. glutamicum* was shown in Fig. 2a.

However, the procedure of deleting the chromosomal gene in *C. glutamicum* was not suitable for *B. flavum* due to lack of sucrose sensitivity. Inactivation of the chromosomal *alaT* and *avtA* genes in *B. flavum* were performed using integration vector (Ausubel et al. 2005), Pucm-T-*alaT*m-P*tac-cat* and Pucm-T-*avtA*m-P*tac-cat* (Fig. 2b). DNA fragment *cat* gene (ORF) was generated using primers *cat*-F and *cat*-R with plasmid PCP20 (Datsenko and Wanner 2000) as a template. The fragment was purified and digested by *Nhe*I and *Hind*III, and then was ligated into pDXW-8 which was similarly digested. The resulting plasmid was

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

Strain, plasmid, or oligonucleotide	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
*C. glutamicum*ATCC13032	Wild type *C. glutamicum*	ATCC
ATCC13032MP*ilvA*	*C. glutamicum* ATCC13032MP*ilvA*	This work
JV16	L-Valine producer; resistant to α-amino butyric acid; auxotrophic for L-leucine, L-isoleucine, L-methionine; can produce 22 g/L L-valine	Our lab
ATCC13032MP*ilvA* pDXW-8-*ilvEBN^{r}C*	*C. glutamicum* ATCC13032MP*ilvA* harboring pDXW-8-*ilvEBN^{r}C*	This work
JV16 pDXW-8-*ilvEBN^{r}C*	*B. flavum* JV16 harboring pDXW-8-*ilvEBN^{r}C*	This work
13032MP*ilvA*△*alaT*	*C. glutamicum* ATCC13032MP*ilvA*△*alaT*	This work
13032MP*ilvA*△*avtA*	*C. glutamicum* ATCC13032MP*ilvA*△*avtA*	This work
13032MP*ilvA*△*alaT*△*avtA*	*C. glutamicum* ATCC13032MP*ilvA*△*alaT*△*avtA*	This work
13032MP*ilvA*△*alaT* pDXW-8-*ilvEBN^{r}C*	*C. glutamicum* ATCC13032MP*ilvA*△*alaT* harboring pDXW-8-*ilvEBN^{r}C*	This work
13032MP*ilvA*△*avtA* pDXW-8-*ilvEBN^{r}C*	*C. glutamicum* ATCC13032MP*ilvA*△*avtA* harboring pDXW-8-*ilvEBN^{r}C*	This work
13032MP*ilvA*△*alaT*△*avtA* pDXW-8-*ilvEBN^{r}C*	*C. glutamicum* ATCC13032MP*ilvA*△*alaT*△*avtA* harboring pDXW-8-*ilvEBN^{r}C*	This work
JV16*alaT*::*Cm*	*B. flavum* JV16*alaT*::*Cm*	This work
JV16*avtA*::*Cm*	*B. flavum* JV16*avtA*::*Cm*	This work
JV16*alaT*::*Cm* pDXW-8-*ilvEBN^{r}C*	*B. flavum* JV16*alaT*::*Cm* harboring pDXW-8-*ilvEBN^{r}C*	This work
JV16*avtA*::*Cm* pDXW-8-*ilvEBN^{r}C*	*B. flavum* JV16*avtA*::*Cm* harboring pDXW-8-*ilvEBN^{r}C*	This work
Plasmids		
pDXW-8	*E. coli–B. flavum* shuttle vector; Km^R P*tac*	(Xu et al. 2010)
pDXW-8-*ilvEBN^{r}C*	Plasmid carrying the *ilvEBN^{r}C* genes	(Hou et al. 2012)
pK19mobsacB	Integration vector; Km^r *oriV$_{Ec}$ oriT sacB*	(Schafer et al. 1994)
pK19mobsacBMP*ilvA*	Plasmid to replace the *ilvA* native promoter with P-*ilvA*M1CG	(Holatko et al. 2009)
pK19mobsacB△*alaT*	Plasmid to delete a 938-nt fragment of *alaT* gene in the *C. glutamicum* chromosome	This work
pK19mobsacB△*avtA*	Plasmid to delete a 640-nt fragment of *avtA* gene in the *C. glutamicum* chromosome	This work
PCP20	Ap^R Cm^R repA(Ts) pSC101 based vector expressing the yeast Flp recombinase	(Datsenko and Wanner 2000)
Pucm-T	Ap^R pBR322 ori 2.7 kb	Sangon, Shanghai
pDXW-8-*cat*	Plasmid carrying the *cat* gene	This work
Pucm-T-P*tac-cat*	Pucm-T carrying P*tac-cat*-T*rrnBT1T2*	This work
Pucm-T-*alaT*m-P*tac-cat*	Plasmid to inactivate the *alaT* gene in the *B. flavum* JV16 chromosome	This work
Pucm-T-*avtA*m-P*tac-cat*	Plasmid to inactivate the *avtA* gene in the *B. flavum* JV16 chromosome	This work
Oligonucleotides	5′ → 3′ sequence	
P*ilvA*-F	CCGGAATTCACGCCTGGTTGCTGATCGTATC	*EcoR*I
P*ilvA*-R	AAGCTCTAGACGTTCCAGCAAAGAATCCAATCC	*Xba*I
MP*ilvA*-F	GTGCAATTCTAGGAGAAGAT***C***ACA***G***TAGTCAACCATGAGTGAAACA	
MP*ilvA*-R	TGTTTCACTCATGGTTGACTA***C***TGT***G***ATCTTCTCCTAGAATTGCAC	
alaT-L-F	AAGCTCTAGACACCAATCAAAGGACTTCTTCTTGTAGCGC	*Xba*I

Table 1 continued

Strain, plasmid, or oligonucleotide	Relevant characteristic(s) or sequence	Reference or purpose
alaT-L-R	**AGGTCGTAGATGGACTGTGTCGGTGGTCTTAGA**GGTTTTGCGCTTGTCT	
alaT-R-F	**TCTAAGACCACCGACACAGTCCATCTACGACCT**CACTGGCGAACACG	
alaT-R-R	GACACTGCAGCTTGTTTGCGCCATAGGGATATATTGG	*Pst*I
avtA-L-F	CTAGTCTAGACTCCAAGGCTTTCGCTAAGGATG	*Xba*I
avtA-L-R	**GGGTAGGCATTGCCACATAAT**CCCCGTGATCCAAGGTGGCGATAA	
avtA-R-F	**ATTATGTGGCAATGCCTACCC**CGGGAGGTGTTCGTCGATAAGCTC	
avtA-R-R	CACAAGCTTTGGCCGGAAAGATCAGCAACCAT	*Hind*III
cat-F	GTAGCTAGCGAAAGGACATCAACGATGGAGAAAAAAATCACTGG	*Nhe*I
cat-R	CGCAAGCTTTTACGCCCCGCCCTGCCAC	*Hind*III
P*tac-cat*-F	ACCGATATCCCGTTCTGGATAATGTTTTTTGC	*Eco*RV
P*tac-cat*-R	GATGATATCGGGTTATTGTCTCATGAGCG	*Eco*RV
*alaT*m-F	CGCGGATCCTCCACCTCCAAAGGCATTATTCC	*BamH*I
*alaT*m-R	CACGGATCCGCCCTCAATAAATCCACGTGC	*BamH*I
*avtA*m-F	CTAGTCTAGAGGTCCTTTGGGATACACCGAGGT	*Xba*I
*avtA*m-R	CTAGTCTAGAGGAGCACACAAGGAAAGAGAAGC	*Xba*I

Mutated bases are in italic boldface, crossover PCR primers are in boldface, restriction sites are underlined

Ap ampicillin, *Cm* chloramphenicol, *Km* kanamycin, *ATCC* American type culture collection

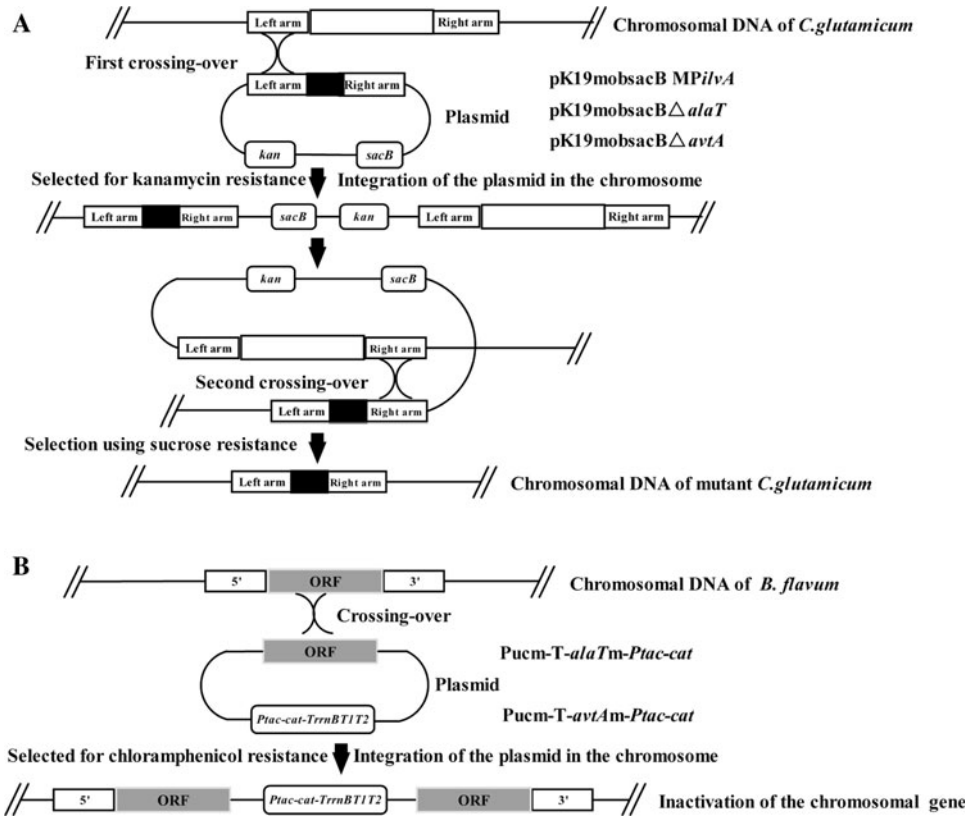

Fig. 2 Strategy used for genetic manipulations in *C. glutamicum* and *B. flavum*. **a** Allelic exchange in *C. glutamicum*, **b** inactivation of gene in *B. flavum*

designated as pDXW-8-*cat*. DNA fragment P*tac-cat*-T*rrnBT1T2* generated from pDXW-8-*cat* using primers P*tac-cat*-F and P*tac-cat*-R ligated into *EcoR*V-restricted plasmid Pucm-T. DNA fragments *alaT*m (600 bp, target to insert the integration vector into the ORF of *alaT* gene by single homologous recombination) and *avtA*m (600 bp, target to insert the integration vector into the ORF of *avtA* gene by single homologous recombination) were generated using primer pairs *alaT*m-F–*alaT*m-R and *avtA*m-F–*avtA*m-R, and then were ligated into *BamH*I-restricted or *Xba*I-restricted plasmid Pucm-T-P*tac-cat*. The recombinant plasmids Pucm-T-*alaT*m-P*tac-cat* and Pucm-T-*avtA*m-P*tac-cat* were isolated from *E. coli* and electroporated into *B. flavum*. Clones were selected for chloramphenicol resistance to guarantee integration of the plasmid in the chromosome. Inactivation of the specific chromosomal gene in *B. flavum* was verified by PCR analysis.

Analytical methods

Two milliliter of samples was taken from the fermentor every 4 h. One milliliter 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 correlated equation was DCW (g/L) = 0.36 × OD_{600} (R^2 = 0.9901) for *C. glutamicum*, and DCW (g/L) = 0.38 × OD_{600} (R^2 = 0.9891) for *B. flavum*. Another 1 mL of the culture was harvested by centrifugation (12,000×*g* for 10 min), and the supernatant was used for determination of glucose, amino acid, and/or organic acid concentrations in the culture fluid. Glucose concentration was determined by SBA-40E immobilized enzyme biosensor. The amino acids' concentrations were determined by reversed-phase high-pressure liquid chromatography (Agilent 1200, USA) with DAD detection (338 nm) after automatic precolumn derivatization with *ortho*-phthaldialdehyde (Lindroth and Mopper 1979). Separation was carried out at 40 °C on a column of Thermo dC_{18} (particle size 5 μm, 4.6 mm × 250 mm). The elution buffer consisted of a polar phase (0.1 M sodium acetate, pH 7.2) and a nonpolar phase (methanol and acetonitrile). Quantification was done by calculation of the concentration using an internal standard. The organic acids' concentrations were determined by reversed-phase high-pressure liquid chromatography (Agilent 1200, USA) with UV detection (215 nm). Separation was carried out at 25 °C on a column of waters dC_{18} (particle size 5 μm, 4.6 mm × 250 mm). The elution buffer consisted of a polar phase (0.01 moL/L KH_2PO_4) and a nonpolar phase (5 % acetonitrile). Quantification was done by calculation of the peak area by the external standard method.

Results

Effect of different relative dissolved oxygen on L-valine production and by-products' formation

To investigate the effect of different relative dissolved oxygen on L-valine production and by-products' formation, *C. glutamicum* ATCC13032MP*ilvA*, *C. glutamicum* ATCC13032MP*ilvA* pDXW-8-*ilvEBN*^{r}*C*, and *B. flavum* JV16, *B. flavum* JV16 pDXW-8-*ilvEBN*^{r}*C* were cultured under relative dissolved oxygen 5–30 % in fed-batch fermentations, respectively, to determine the concentrations of L-valine and by-products. The concentrations of L-isoleucine and L-leucine did not exceed 0.5 g/L. There were no significant changes in biomass for *C. glutamicum* and *B. flavum* under different relative dissolved oxygen, but the L-valine production and by-products' distribution differed. As shown in Fig. 3, when relative dissolved oxygen was adjusted to 5 % saturation, L-lactic acid became the main by-product. L-Glutamate also can become the main by-product when relative dissolved oxygen was adjusted to high saturation (25–30 %). However, the concentrations of L-lactic acid and L-glutamate dramatically decreased under 15–20 % saturation. The results suggested that L-lactic acid and L-glutamate can be removed through oxygen supply control strategy. The 15 % saturation may be the most appropriate relative dissolved oxygen for L-valine fermentation. L-Valine production was 22.52 g/L (L-lactic acid 0.09 g/L, L-glutamate 0.31 g/L) by *C. glutamicum* ATCC13032MP*ilvA* pDXW-8-*ilvEBN*^{r}*C*, and 30.21 g/L (L-lactic acid 0.12 g/L, L-glutamate 0.28 g/L) by *B. flavum* JV16 pDXW-8-*ilvEBN*^{r}*C*.

However, L-alanine was always the main by-product whatever the saturation of the relative dissolved oxygen was; further, gene manipulation in the pathway of L-alanine synthesis should be carried out.

Effect of *alaT* inactivation on L-valine production and L-alanine synthesis

To reduce the accumulation of L-alanine and also to increase the availability of pyrurate for L-valine biosynthesis, inactivation of *alaT* gene was carried out in *C. glutamicum* ATCC13032MP*ilvA* and *B. flavum* JV16, respectively. As shown in Fig. 4 and Table 2, in fed-batch fermentations, L-valine production and conversion efficiency reached 28.15 g/L and 0.158 g/g by *C. glutamicum* ATCC13032MP*ilvA*△*alaT* pDXW-8-*ilvEBN*^{r}*C*, 34.42 g/L and 0.218 g/g by *B. flavum* JV16*alaT*::*Cm* pDXW-8-*ilvEBN*^{r}*C*. The concentration of L-alanine by *C. glutamicum* ATCC13032MP*ilvA*△*alaT* was reduced by 78.06 % (from 7.52 to 1.65 g/L), indicating alaT is the principal L-alanine-supplying enzyme, in conformity with the result of Marienhagen and Eggeling (2008). However, it cannot

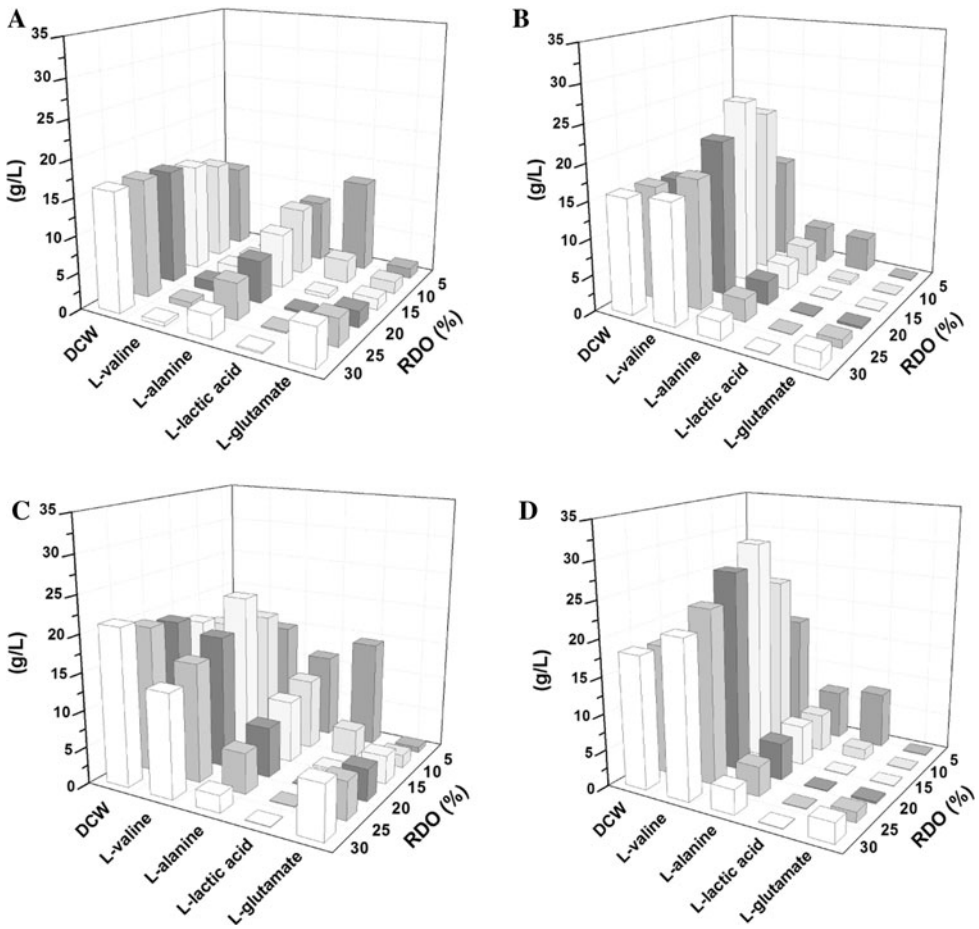

Fig. 3 Effect of different relative dissolved oxygen on L-valine production and by-products' formation. **a** *C. glutamicum* ATCC13032MP*ilvA*, **b** *C. glutamicum* ATCC13032MP*ilvA* pDXW-8-*ilvEBN*^{r}*C*, **c** *B. flavum* JV16, and **d** *B. flavum* JV16 pDXW-8-*ilvEBN*^{r}*C*. *RDO* relative dissolved oxygen

be ignored that the concentration of L-alanine was 3.05 g/L by *C. glutamicum* ATCC13032MP*ilvA*△*alaT* pDXW-8-ilv*EBN*^{r}*C*. The same phenomenon was found in *B. flavum* JV16, the concentration of L-alanine was 4.38 g/L by *B. flavum* JV16*alaT*::*Cm* compared to 8.55 g/L by *B. flavum* JV16, 6.81 g/L by *B. flavum* JV16*alaT*::*Cm* pDXW-8-*ilvEBN*^{r}*C*. The concentration of L-alanine increased while L-valine production was enhanced by over-expressing *ilvEBN*^{r}*C* genes, requiring further research in depth.

Effect of *avtA* inactivation on L-valine production and L-alanine synthesis

To further investigate the mechanism of L-alanine synthesis with *ilvEBN*^{r}*C* genes over-expressed, inactivation of *avtA* gene was carried out in *C. glutamicum* ATCC13032MP*ilvA*, *C. glutamicum* ATCC13032MP*ilvA*△*alaT*, and *B. flavum* JV16, respectively. As shown in Fig. 5 and Table 3, in fed-batch fermentations, the concentration of L-alanine was 5.18 g/L by *C. glutamicum* ATCC13032MP*ilvA*△*avtA*. Noticeably, the maximal concentration of L-alanine by *C. glutamicum* ATCC13032MP*ilvA*△*avtA* pDXW-8-*ilvEBN*^{r}*C* was 1.28 g/L at 12 h, after IPTG induction, the concentration of L-alanine was dramatically reduced and was 0.31 g/L finally. *C. glutamicum* ATCC13032MP*ilvA*△*alaT*△*avtA* exhibited a low DCW of 12.41 g/L, L-valine production was 25.55 g/L with L-alanine concentration of 0.18 g/L by *C. glutamicum* ATCC13032 MP*ilvA*△*alaT*△*avtA* pDXW-8-*ilvEBN*^{r}*C*. These results demonstrated that the biosynthesis of L-alanine in *C. glutamicum* ATCC13032MP*ilvA*△*avtA* was catalyzed by alaT, and L-alanine concentration was reduced owing to the over-expression of *ilvEBN*^{r}*C* genes.

L-Valine production was enhanced to 27.25 g/L by *B. flavum* JV16*avtA*::*Cm* compared to 24.42 g/L by *B. flavum* JV16*alaT*::*Cm*, the concentration of L-alanine was 1.48 g/L (4.38 g/L by *B. flavum* JV16*alaT*::*Cm*). With *ilvEBN*^{r}*C* genes over-expressed, L-valine production and conversion efficiency were enhanced to 38.82 g/L and 0.252 g/g by *B. flavum* JV16*avtA*::*Cm* pDXW-8-*ilvEBN*^{r}*C*, and the concentration of L-alanine was reduced to 0.22 g/L. Taken together, in *alaT* inactivation strains, L-alanine was accumulated through the reaction catalyzed by avtA when L-valine biosynthesis was enhanced. On the other hand, L-alanine concentration can be dramatically reduced by *avtA* inactivation when L-valine biosynthesis was enhanced.

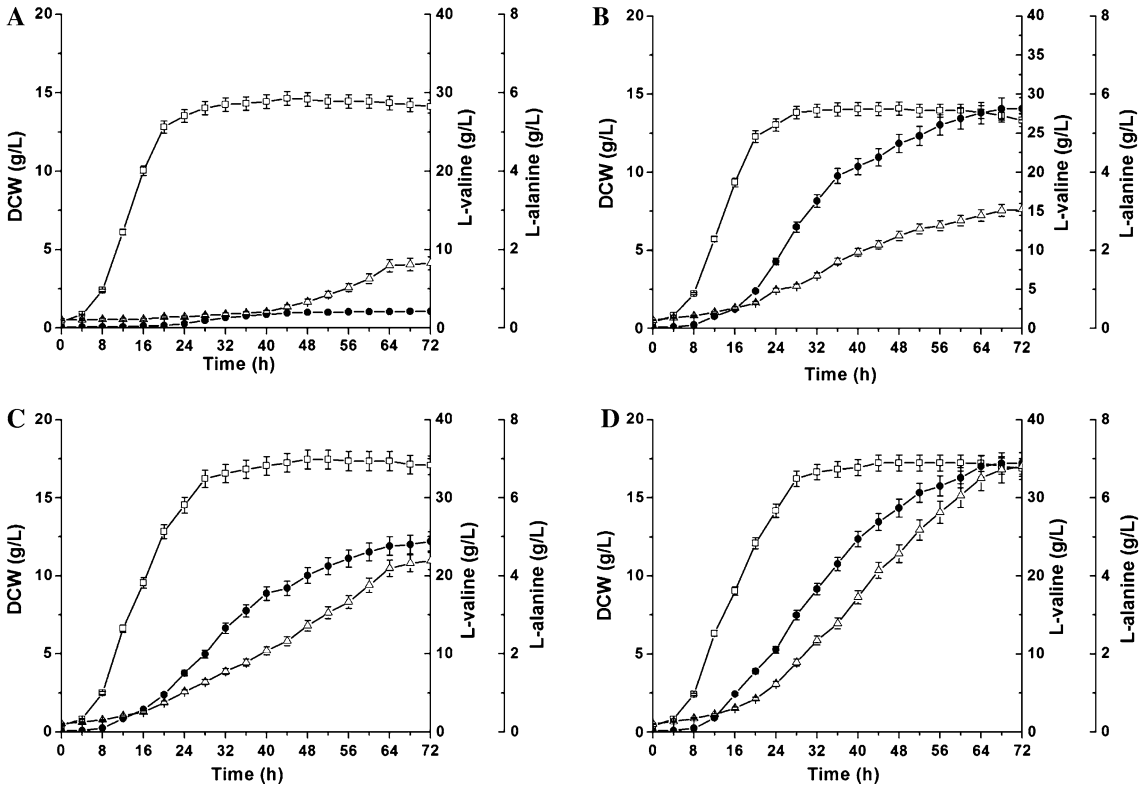

Fig. 4 Effect of *alaT* inactivation on L-valine production and L-alanine synthesis. **a** *C. glutamicum* ATCC13032MP*ilvA*△*alaT*, **b** *C. glutamicum* ATCC13032MP*ilvA*△*alaT* pDXW-8-*ilvEBN*^{r}*C*, **c** *B. flavum* JV16*alaT*::*Cm*, and **d** *B. flavum* JV16*alaT*::*Cm* pDXW-8-*ilvEBN*^{r}*C*. *Open squares* DCW, *filled circles* L-valine, *open triangles* L-alanine

Table 2 Fed-batch culture parameters of L-valine fermentation by *alaT* inactivation strains

Strain	Maximal DCW (g/L)	L-Alanine (g/L)	L-Valine (g/L)	Glucose consumption (g/L)	Conversion efficiency (g/g)
13032MP*ilvA*△*alaT*	14.64 ± 0.82	1.65 ± 0.28	2.12 ± 0.65	155 ± 3	0.014
13032MP*ilvA*△*alaT* pDXW-8-*ilvEBN*^{r}*C*	14.08 ± 0.94	3.05 ± 0.75	28.15 ± 1.08	178 ± 5	0.158
JV16*alaT*::*Cm*	17.45 ± 0.77	4.38 ± 0.93	24.42 ± 0.96	150 ± 4	0.162
JV16*alaT*::*Cm* pDXW-8-*ilvEBN*^{r}*C*	17.24 ± 0.87	6.81 ± 1.22	34.42 ± 1.52	158 ± 3	0.218

Discussion

The first main strategy used in the construction of the L-valine-producing *C. glutamicum* and *B. flavum* strains was cloning the genes *ilvBNC*, *ilvD*, and *ilvE* in multi-copy plasmids (Hou et al. 2012; Blombach et al. 2007, 2008; Radmacher et al. 2002), but the by-products like L-alanine, L-isoleucine, L-leucine, and L-glutamate existed in these strains to some extent. The second main strategy was constructing gene deletions, which can increase the availability of pyruvate for L-valine biosynthesis (Sahm and Eggeling 1999; Blombach et al. 2007, 2008, onRef>), requiring specific supplementation such as L-isoleucine for △*ilvA* and acetate for △*aceE*. Another strategy for improving amino acid production is the modulation of gene expression using promoter tuning (Hammer et al. 2006). This strategy avoids two extremes, strong over-expression of the gene for the enzyme that is supposed to be rate limiting and elimination of branching off or competing pathways by gene deletions (Holatko et al. 2009). Using constitutive promoters of various strengths, all of the genes involved in a pathway may be expressed to the level which ensures an optimum flux through the pathway (Solem et al. 2007). Recently, promoter activity modulation has been used in L-valine-producing *C. glutamicum* (Holatko et al. 2009) and the activity of the promoters of *ilvA* (encoding

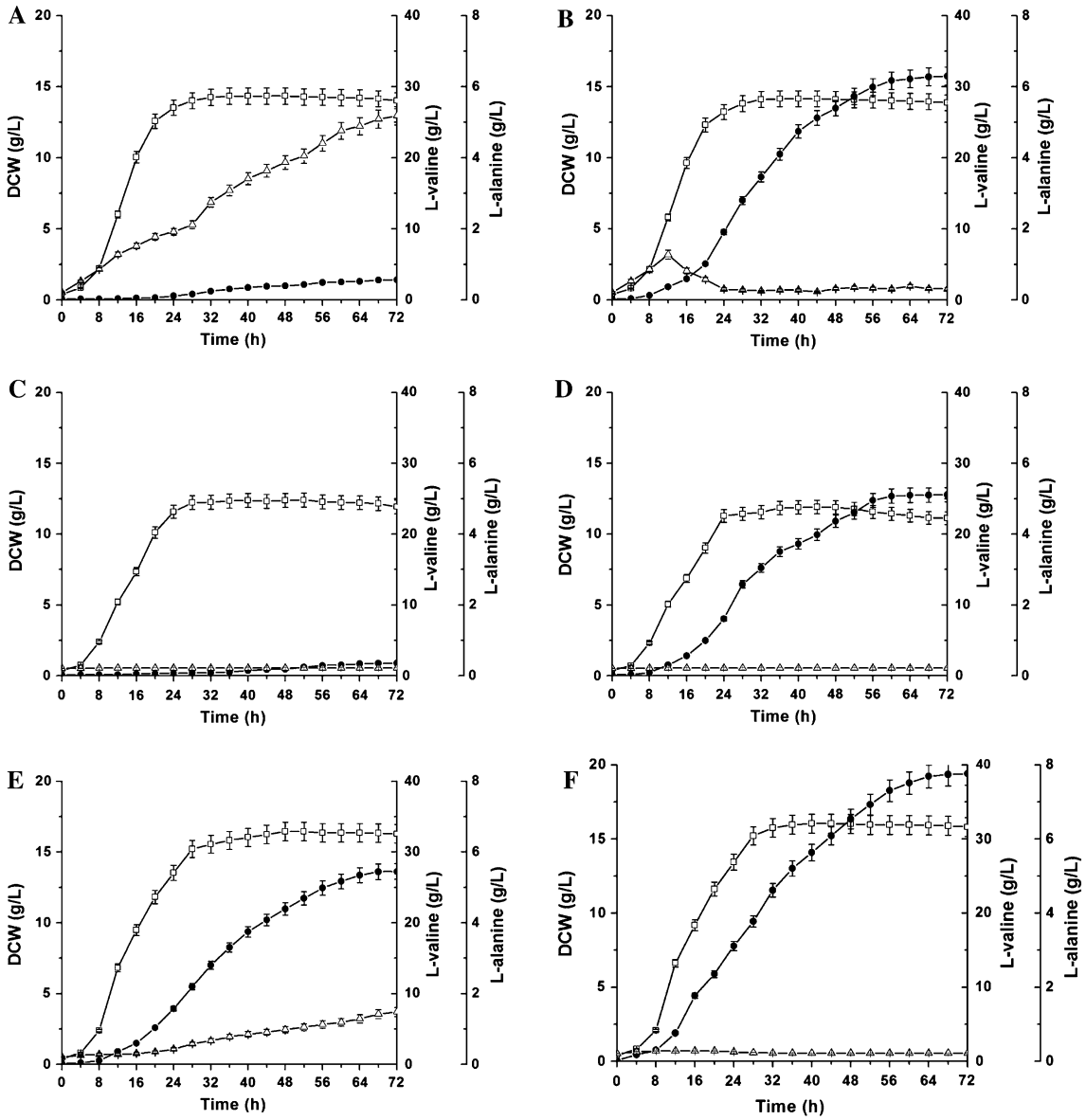

Fig. 5 Effect of *avtA* inactivation on L-valine production and L-alanine synthesis. **a** *C. glutamicum* ATCC13032MP*ilvA*△*avtA*, **b** *C. glutamicum* ATCC13032MP*ilvA*△*avtA* pDXW-8-*ilvEBN*^{r}*C*, **c** *C. glutamicum* ATCC13032MP*ilvA*△*alaT*△*avtA*, **d** *C. glutamicum* ATCC13032MP*ilvA*△*alaT*△*avtA* pDXW-8-*ilvEBN*^{r}*C*, **e** *B. flavum* JV16*avtA*::*Cm*, and **f** *B. flavum* JV16*avtA*::*Cm* pDXW-8-*ilvEBN*^{r}*C*. *Open squares* DCW, *filled circles* L-valine, *open triangles* L-alanine

threonine dehydratase) gene was down-modulated by site-directed mutagenesis. The introduced weak promoter of *ilvA* gene increased L-valine production by reducing the biosynthetic rate of L-isoleucine. The weak promoter of P-*ilvA*M1CG was adopted in this work, and L-isoleucine concentration was successfully reduced when *ilvEBN*^{r}*C* genes were over-expressed in *C. glutamicum*. It is worth mentioning that the L-valine producer *B. flavum* JV16 (Leu^{-} Ile^{-} Met^{-}) obtained by repeated mutagenesis and selection is a good candidate for further metabolic engineering for high L-valine production.

An in-depth understanding of molecular biology and physiology of relevant organisms is essential for the development of genetically engineered production strains because the metabolic network of an organism is an extremely complex system. In reality, however, most of the metabolic reactions are rather inactive and only selected fluxes dominate the metabolism, whereas the regulation of these fluxes depends strongly on cultivation conditions (Almaas et al. 2004). In particular, it is generally known that the central metabolism reveals significant differences under fully aerobic and oxygen-limited conditions (Zimmermann

Table 3 Fed-batch culture parameters of L-valine fermentation by *avtA* inactivation strains

Strain	Maximal DCW (g/L)	L-Alanine (g/L)	L-Valine (g/L)	Glucose consumption (g/L)	Conversion efficiency (g/g)
13032MP*ilvA*△*avtA*	14.35 ± 0.89	5.18 ± 0.78	2.82 ± 0.45	148 ± 2	0.019
13032MP*ilvA*△*avtA* pDXW-8-*ilvEBN*^{r}*C*	14.16 ± 0.83	0.31 ± 0.02	31.51 ± 1.03	182 ± 4	0.173
13032MP*ilvA*△*alaT*△*avtA*	12.41 ± 0.77	0.22 ± 0.02	1.78 ± 0.22	132 ± 2	0.013
13032MP*ilvA*△*alaT*△*avtA* pDXW-8-*ilvEBN*^{r}*C*	11.91 ± 0.93	0.18 ± 0.02	25.55 ± 0.93	145 ± 3	0.176
JV16*avtA*::*Cm*	16.45 ± 0.67	1.48 ± 0.25	27.25 ± 1.05	144 ± 4	0.189
JV16*avtA*::*Cm* pDXW-8-*ilvEBN*^{r}*C*	16.04 ± 0.72	0.22 ± 0.02	38.82 ± 1.27	154 ± 3	0.252

et al. 2006). L-Glutamate and L-lactic acid are the main by-products with *C. glutamicum* and *B. flavum* under aerobic and oxygen-limited conditions in L-valine fermentation. Optimization of oxygen supply, which can easily remove unnecessary by-products such as L-glutamate and L-lactic acid in this work, also reduces the burden of metabolic engineering of L-valine production.

The ATs alaT and avtA of *C. glutamicum* have substrate specificity for L-alanine in common. Both the △avtA and △alaT mutants of *C. glutamicum* exhibit reduced and slightly variable growth on minimal medium-containing plates, which can be fully restored by the addition of 1 mM L-alanine (data not shown). However, a double mutant was auxotrophic for L-alanine, showing that both ATs can provide L-alanine and that they are the only ATs involved (Marienhagen and Eggeling 2008). In this work, with *alaT* inactivated, the concentration of L-alanine was reduced by 78.06 % (from 7.52 to 1.65 g/L) by *C. glutamicum* ATCC13032M*PilvA*△*alaT* and by 78.06 % (from 7.52 to 1.65 g/L) by *B. flavum* JV16*alaT*::*Cm*. With *avtA* inactivated, the concentration of L-alanine was 5.18 g/L by *C. glutamicum* ATCC13032M*PilvA*△*avtA* and 1.48 g/L by *B. flavum* JV16*avtA*::*Cm*. The results indicate that alaT is the principal L-alanine-supplying enzyme. However, while L-valine production was enhanced by over-expressing *ilvEBN*^{r}*C* genes, it cannot be ignored that the concentration of L-alanine increased by *alaT* inactivation strains (3.05 g/L by *C. glutamicum* ATCC13032M*PilvA*△*alaT* pDXW-8-*ilvEBN*^{r}*C*; 6.81 g/L by *B. flavum* JV16*alaT*::*Cm* pDXW-8-*ilvEBN*^{r}*C*). Meanwhile, in *avtA* inactivation strains, L-alanine was successfully reduced to 0.2–0.3 g/L, suggesting that over-expression of *ilvEBN*^{r}*C* can effectively reduce the biosynthesis of L-alanine catalyzed by alaT. Under normal flux conditions, alaT have higher flux efficiency than avtA toward L-alanine synthesis (Marienhagen and Eggeling 2008); however, the concentration of L-glutamate as amino donor for alaT was reduced in this work and avtA will play the major role in L-alanine synthesis when L-valine biosynthesis is enhanced. Nevertheless, avtA is the only AT with exceptionally high activity toward L-alanine as an amino donor not preferably using L-glutamate but L-valine (Marienhagen et al. 2005). Therefore, to construct effective L-valine-producing strain without L-alanine accumulated, *avtA* gene must be disrupted. On one hand, it can increase the availability of pyruvate for L-valine biosynthesis; on the other hand, it can effectively prevent the decomposition of L-valine accumulated.

Under our experimental conditions, all strains entered the stationary phase after approximately 24 h (shown in Figs.4, 5). The same phenomenon was found in the work of Blombach et al. (2008) and Marienhagen and Eggeling (2008). The results suggested that over-expression of the *ilvEBN*^{r}*C* genes and *alaT* or *avtA* inactivation had little influence on the growth of the strains. The plasmid pDXW-8-*ilvEBN*^{r}*C* (*ilvE* before *ilvBN*^{r}*C*; four mutations in the regulatory subunit of AHAS; *tac* promoter) was constructed by Hou et al. (2012), and the strain *B. flavum* ATCC14067 pDXW-8-*ilvEBN*^{r}*C* can produce 30.08 g/L L-valine at 31 °C in 72 h fermentation. The plasmid pDXW-8-*ilvEBN*^{r}*C* was also adopted in this work, L-valine production was enhanced to 31.15 g/L by *C. glutamicum* ATCC13032MP*ilvA*△*avtA* pDXW-8-*ilvEBN*^{r}*C* and 38.82 g/L by *B. flavum* JV16*avtA*::*Cm* pDXW-8-*ilvEBN*^{r}*C*. The combined results indicate that the effective L-valine-producing strains of *C. glutamicum* and *B. flavum* can be attained by inactivation of *avtA* gene and increasing the intracellular content of L-valine biosynthetic enzymes AHAS, AHAIR, and TA. Further, work will focus on engineering of central metabolic pathways and L-valine export (Park and Lee 2010), and systems metabolic engineering which has been successfully implemented for L-threonine (Lee et al. 2007) and L-lysine (Becker et al. 2011) may be the best choice.

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