J Ind Microbiol Biotechnol (2012) 39:1549–1556
DOI 10.1007/s10295-012-1155-4

Effect of transport proteins on L-isoleucine production with the L-isoleucine-producing strain *Corynebacterium glutamicum* YILW

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Received: 19 December 2011 / Accepted: 31 May 2012 / Published online: 26 June 2012

Abstract Previous studies have shown that the deletion of *brnQ* from the *Corynebacterium glutamicum* chromosome results in a significant reduction in L-isoleucine uptake rates, while overexpression of *brnFE* leads to enhanced L-isoleucine export rates. Given that net excretion rates would be an important factor for high titers of L-isoleucine accumulation, we have tested the notion that decreased L-isoleucine uptake combined with increased L-isoleucine excretion will further improve high-yield strains that are currently used for the industrial-scale production of L-isoleucine. To examine the effect of the two carriers on L-isoleucine accumulation in L-isoleucine producer *C. glutamicum* YILW, we constructed a *brnQ* deletion mutant (*C. glutamicum* YILWΔ*brnQ*) and two *brnFE* overexpressors (*C. glutamicum* YILWpXMJ19*brnFE* and *C. glutamicum* YILWΔ*brnQ*pXMJ19*brnFE*). Compared to the original strain, the efflux rate of the *brnQ* mutant increased from 19.0 to 23.6 nmol min^{-1} mg (dry wt)$^{-1}$ and its L-isoleucine titer increased from 154.3 mM (20.2 g l^{-1}) to 170.3 mM (22.3 g l^{-1}). The efflux rates of *C. glutamicum* YILWpXMJ19*brnFE* and *C. glutamicum* YILWΔ*brnQ*pXMJ19*brnFE* were 33.5 and 39.1 nmol min^{-1} mg (dry wt)$^{-1}$, and their L-isoleucine production titers were 197.2 mM (25.9 g l^{-1}) and 221.0 mM (29.0 g l^{-1}), respectively. Our results suggest that modifications of the transport system could provide a promising avenue for further increasing L-isoleucine yield in the L-isoleucine producer.

Keywords *Corynebacterium glutamicum* · L-isoleucine · Excretion · Uptake carrier · Export carrier

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Introduction

As an essential branched-chain amino acid, L-isoleucine holds an important part of the amino acid market share. Given that it is not synthesized in mammals, its main application is in the field of synthetic nutrition for infusions or special diets. Microorganisms synthesize L-isoleucine via a multistep biosynthetic pathway, starting from the central precursor metabolite L-aspartate, with branches to L-lysine and L-methionine, as well as an additional one L-threonine as intermediates. Key enzymes involved in the biosynthesis of L-isoleucine include aspartate kinase, homoserine dehydrogenase, homoserine kinase, threonine dehydratase, and acetohydroxy acid synthase, with the latter serving as the first enzyme of a series of enzymes that catalyze parallel reactions to L-isoleucine and L-valine and both threonine dehydratase and acetohydroxy acid synthase being feedback-inhibited by L-isoleucine [19]. Due to this tight and complicated regulation, fermentative processes for L-isoleucine are in general not as well developed as those for L-lysine [3, 5, 24, 29].

Although earlier work through the direct manipulation of genes encoding key enzymes in the L-aspartate biosynthetic pathway has proven very useful for improving the biosynthesis of this amino acid, such as through

overexpression of the key enzymes for diverting carbon flow from these pathways to other products [2, 7, 22], in studies with *Corynebacterium glutamicum*, the internal isoleucine concentrations measured were always higher than the extracellular ones at all fermentation stages [20]. It is known that transaminase catalyzes the last step of the L-isoleucine biosynthetic pathway. However, increasing transaminase activity in strains using accumulative precursors did not affect the secretion of L-isoleucine from the strains tested [30], indicating that the strategy to adopt for improving a strain's biosynthetic capacity probably does not rely on the biosynthetic reaction, but rather on the secretion of the synthesized L-isoleucine from the cell. When cytoplasmic L-isoleucine levels exceed a certain threshold, the export of amino acid to the medium becomes rate limiting [14]. High-producing strains are subject to complex regulations at several steps in the L-isoleucine biosynthetic pathway. As it is difficult to remove all of the feedback controls in the L-isoleucine biosynthetic pathway, excreting L-isoleucine into the surrounding medium over time can decrease the intracellular L-isoleucine concentration, thus becoming an important target for further increasing L-isoleucine production.

L-isoleucine is more hydrophobic than L-lysine. The transport of L-isoleucine can be differentiated into diffusion, carrier-mediated uptake, and carrier-mediated export [31]. Diffusion depends on the concentration gradient across the plasma membrane and the diffusion constant K_d [4], while the transport activity of branched-chain amino acids in *C. glutamicum* depends on the presence of Na^+ ions and on the membrane potential [4]. In *C. glutamicum*, L-isoleucine uptake is modulated by the uptake carrier BrnQ, and the deletion of the gene *brnQ* from the chromosome was observed to result in a much lower L-isoleucine uptake rate compared to that of the wild-type strain [26]. BrnQ has been found to be inactive at a low intracellular L-isoleucine concentration but to be activated when intracellular L-isoleucine concentration exceeded 1 mM [1]. The L-isoleucine export carrier is a two-component permease, a LIV-E family transporter, encoded by *brnF* and *brnE*, which also exports L-methionine, L-leucine, and L-valine. Overexpression of *brnFE* has been found to result in a significantly increased export rate of these amino acids [15, 28]. These findings make it possible to compensate for the weakness of limited capacity of L-isoleucine efflux with the use of recombinant DNA technology.

Since both the *brnQ* deletion mutant and *brnFE* overexpressors result in significantly elevated L-isoleucine transport rates [15, 26, 28], it seems reasonable to suggest that an efficient biosynthetic pathway, together with a high transport activity would provide the conditions necessary for amino acids overproduction [10, 11, 17, 21]. We therefore decided to investigate the effects of transport systems on L-isoleucine production with the industrial production strain *C. glutamicum*. In the work presented here, we constructed a *brnQ* deletion mutant *C. glutamicum* YILWΔ*brnQ*, then cloned and overexpressed *brnFE* in both *C. glutamicum* YILW and *C. glutamicum* YILWΔ*brnQ*. Our results clearly demonstrate that manipulating the L-isoleucine transport systems BrnQ and BrnFE could lead to significantly improved yields of L-isoleucine in the microorganism strains currently used in industrial-scale production of L-isoleucine.

Materials and methods

Bacterial strains, plasmids, medium, and growth conditions

The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* was grown in LB medium at 37 °C, *C. glutamicum* was grown in BHI medium or CGXII minimal medium [13] at 32 °C. The seed medium contained glucose (30 g l^{-1}), yeast extract (5 g l^{-1}), $(NH_4)_2SO_4$ (3 g l^{-1}), $KH_2PO_4{\cdot}3H_2O$ (1.5 g l^{-1}), $MgSO_4{\cdot}7H_2O$ (0.6 g l^{-1}), $FeSO_4{\cdot}7H_2O$ (0.01 g l^{-1}), $MnSO_4{\cdot}H_2O$ (0.01 g l^{-1}), corn steep liquor (30 ml l^{-1}), and soybean hydrolysate (30 ml l^{-1}). The production medium contained glucose (80 g l^{-1}), $(NH_4)_2SO_4$ (4 g l^{-1}), $FeSO_4{\cdot}7H_2O$ (0.015 g l^{-1}), $MgSO_4{\cdot}7H_2O$ (0.5 g l^{-1}), $MnSO_4{\cdot}H_2O$ (0.015 g l^{-1}), $KH_2PO_4{\cdot}3H_2O$ (1.5 g l^{-1}), $K_2HPO_4{\cdot}3H_2O$ (3 g l^{-1}), biotin (100 μg l^{-1}), vitamin B_1 (5 mg l^{-1}), soybean hydrolysate (20 ml l^{-1}), and corn steep liquor (15 ml l^{-1}).

Construction of plasmids

Construction of the pK18mobsacBΔbrnQ

Both the upstream and downstream regions of *brnQ* were PCR-amplified from the genomic DNA of the L-isoleucine-producing strain using primer sets BrnQ743-1 (5′-CAGGGC-ATGCGATATTGCGGCGAAGTACG-3′) and BrnQ743-2 (5′-CACTCGTTGCTAGAAATCCTAAG A-TAGCTGGTAGAT-3′), and BrnQ761-1 (5′-TTAGGA TTTCTAGCAACGAGTGCTATCCCTGCGCT-GAAT-3′) and BrnQ761-2 (5′-GCTATCTAGAGCATACCCAACTG TGCTGCCAAT-3′), respectively. Splicing by overlap extension was used to fuse *brnQ* upstream and *brnQ* downstream with the primer pair BrnQ743-1 and BrnQ761-2. The resulting fragment was digested by *Sph*I and *Xba*I, cloned into vector pK18*mobsacB*, and transformed into *E. coli* DH5αMCR. Transformants were selected on LB agar containing 50 μg kanamycin ml^{-1}. The resulting recombinant vector was verified by DNA sequencing.

Table 1 Strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Reference or source
Corynebacterium glutamicum		
YILW	The L-isoleucine-producing strain (Leu^L + AHV^r + SG^r + Leu-ME^r)	[25][a]
YILWΔ*brnQ*	*C. glutamicum* YILW with *brnQ* deletion	This work
YILWpXMJ19	*C. glutamicum* YILW harboring pXMJ19	This work
YILWpXMJ19*brnFE*	*C. glutamicum* YILW harboring pXMJ19*brnFE*	This work
YILWΔ*brnQ*pXMJ19*brnFE*	*C. glutamicum* YILWΔ*brnQ* harboring pXMJ19*brnFE*	This work
Escherichia coli		
DH5αMCR	F^- φ80d*lacZ*Δ(lac*ZYA-argF*)U169 *recA1 endA1 hsdR17*(rk^- mk^+) *supE44* λ^- thi-1 *gyrA*96 *relA1*	[8]
Plasmids		
pK18*mobsacB*	Integration vector, Km^r $oriV_{Ec}$*oriT sacB*	[23]
pK18*mobsacB*Δ*brnQ*	Plasmid carrying the Δ*brnQ*	This work
pXMJ19	Expression vector, p*tac*, $lacI^q$, Cm^r	[12]
pXMJ19*brnFE*	Plasmid carrying the *brnFE* genes encoding L-isoleucine export carrier protein	This work

[a] The original strain, *C. glutamicum* HL41, was stepwise mutated by UV and diethyl sulfate (DES); the L-isoleucine-producing strain, *C. glutamicum* YILW contained a genetic marker (Leu^L + AHV^r + SG^r + Leu-ME^r) and was selected by genetic marker testing and single clone isolation

Construction of the pXMJ19brnFE

The genes *brnFE* were PCR-amplified from the genomic DNA of *C. glutamicum* YILW using primers BrnFE-1 (5′-ACAAGGATCCACTGGCAACAAAACTACCC-3′) and BrnFE-2 (5′-GGT- AGGTACCAAAATCCGCATCCC CTTC-3′). The resulting fragment was digested by *Kpn*I and *Bam*HI, inserted into the expression vector pXMJ19, and transformed into *E. coli* DH5αMCR. Transformants were selected on LB agar containing 25 μg chloramphenicol ml^{-1}. The resulting recombinant vector was verified by DNA sequencing.

Construction of strains

The plasmid pK18*mobsacB*Δ*brnQ* was extracted and electroporated into *C. glutamicum* YILW as described by Schäfer et al. [23]. The intact chromosomal *brnQ* was replaced by the truncated Δ*brnQ* gene via homologous recombination (double crossover). The constructed plasmid pXMJ19*brnFE* isolated from *E. coli* was electroporated into *C. glutamicum* YILW and *C. glutamicum* YILWΔ*brnQ* [27].

Assay of amino acid excretion

Four different *C. glutamicum* strains cultivated in BHI medium were harvested by centrifugation, washed twice with 0.9 % NaCl at 4 °C, and suspended in 30 ml of pre-warmed CGXII minimal medium (32 °C) at about 3.0 mg (dry wt) ml^{-1} medium. L-isoleucine excretion rates were determined by first adding 100 mM L-isoleucine and 10 mM L-isoleucyl-L-isoleucine dipeptide to the medium to simulate the actual L-isoleucine production process conditions, then incubating the cells at 32 °C for 20 min to allow the uptake of the dipeptide and subsequent internal hydrolysis. Samples of 1 ml were withdrawn every 15 min for 2 h to determine external and internal L-isoleucine concentrations. Cells were separated from the medium by silicone oil centrifugation, and the internal L-isoleucine was extracted using the established method [14]. The efflux rate was calculated by determining the intracellular and extracellular L-isoleucine concentrations at short time intervals, which were calculated based on a previous report that the average cytoplasmic volume of *C. glutamicum* was 1.8 μl mg^{-1} (dry wt) [14]. A parallel sample was taken and pretreated at 55 °C for 8 min to inactivate the carriers and then cultivated at 32 °C for 7 min to determine the diffusion rate [31]. The diffusion rates were calculated according to $K_d \times ([aa]_{out} - [aa]_{in})$ (the known diffusion constant K_d was 0.13 for L-isoleucine) [16, 18]. Given that the L-isoleucine uptake rate for strains with a functional *brnQ* was invariable under all conditions assayed [4, 9, 26, 31], we used the reported maximum of 1.1 ± 0.3 nmol min^{-1} mg (dry wt)$^{-1}$ [4, 15] as the L-isoleucine uptake rates of *C. glutamicum* YILW and *C. glutamicum* YILWpXMJ19*brnFE* and data on the L-isoleucine uptake rates for our *brnQ* mutants were from Touch et al. [26]. As the extracellular L-isoleucine concentrations determined were higher than the intracellular concentrations, diffusion and carrier-mediated uptake all

Table 2 L-isoleucine transport rates with different strains ($p < 0.05$)

Strains	Diffusion [nmol min^{-1} mg (dry wt)$^{-1}$]	Uptake rate [nmol min^{-1} mg (dry wt)$^{-1}$][a]	Export rate [nmol min^{-1} mg (dry wt)$^{-1}$]	Efflux rate [nmol min^{-1} mg (dry wt)$^{-1}$]
YILW	5.3 ± 0.2	1.1 ± 0.3	25.4 ± 0.9	19.0 ± 0.7
YILWΔ*brnQ*	5.0 ± 0.1	0.0 ± 0.1	28.6 ± 1.3	23.6 ± 1.1
YILWpXMJ19*brnFE*	5.5 ± 0.1	1.1 ± 0.3	40.1 ± 2.0	33.5 ± 1.4
YILWΔ*brnQ*pXMJ19*brnFE*	5.4 ± 0.2	0.0 ± 0.1	44.5 ± 2.2	39.1 ± 1.9

Values are presented as the mean ± standard deviation (SD)

[a] The uptake rates are based on the data of Ebbighausen et al. [4] and Touch et al. [26]

contributed to the L-isoleucine influx. The efflux rate plus the influx rate is the export rate.

Fermentations

Fermentations were carried out in 5-l bioreactors (Shanghai Baoxing Bio-engineering Equipment Co., Shanghai, China). The *C. glutamicum* seed culture was grown in shake-flasks (100 ml seed medium in a 1-l shake flask, 200 rpm, 32 °C) to an OD_{600} about 12.0–16.0 within 12 h and was transferred to the 5-l production medium for L-isoleucine production with 10 % (v/v) inoculum size. Glucose concentration was kept close to 2.0 % (20.0 g l^{-1}). The pH was controlled by the addition of 25.0 % NH_4OH and maintained at approximately 7.0. The ammonium concentration was kept above 0.8 mol l^{-1}. The concentration of dissolved oxygen was 20.0–30.0 % during the whole process.

Analytics

During the fermentation process, 1-ml samples were taken from the cultures and centrifuged at 4 °C and 13,000 g for 5 min. For biomass determination, cell dry weight was determined gravimetrically. Glucose was determined with a biosensor (Institute of Biology, Shandong Academy of Science, Shanghai, China). Amino acids were analyzed as 2, 4-fluoro-dinitrobenzene derivatives by using high-performance liquid chromatography. For the detection of metabolites, analytical methods were performed as described by Kelle et al. [14].

Statistical analysis

All experiments were conducted in triplicate, and data were averaged and presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test were used to determine significant differences. Statistical significance was defined as $p < 0.05$.

Results

Manipulation of transport proteins affects L-isoleucine excretion

To investigate the effect of BrnQ and BrnFE on L-isoleucine transport in the L-isoleucine-producing *C. glutamicum* YILW, we calculated the L-isoleucine transport rate in different strains under standard conditions (Table 2). The deletion of *brnQ* resulted in a 24.2 % increase in the efflux rate [from 19.0 to 23.6 nmol min^{-1} mg (dry wt)$^{-1}$], while overexpression of *brnFE* in *C. glutamicum* YILWpXMJ19*brnFE* led to a notable increase in the L-isoleucine efflux rate from 19.0 to 33.5 nmol min^{-1} mg (dry wt)$^{-1}$. Notably, the efflux rate of *C. glutamicum* YILWpXMJ19*brnFE* [33.5 nmol min^{-1} mg (dry wt)$^{-1}$] was much higher than that of the *brnQ* gene-disrupted mutant, indicating that export carrier *brnFE* plays the more significant role in L-isoleucine transport from the cell to the medium. Also, interestingly, the L-isoleucine efflux rate of *C. glutamicum* YILWΔ*brnQ*pXMJ19*brnFE* with *brnFE* overexpression was higher (39.1 nmol min^{-1} mg (dry wt)$^{-1}$) than that of either *C. glutamicum* YILWΔ*brnQ* or *C. glutamicum* YILWpXMJ19*brnFE*.

Relevance of L-isoleucine uptake carrier *brnQ* for L-isoleucine production

To investigate the relevance of the uptake carrier *brnQ* for L-isoleucine production in L-isoleucine-producing strains, we carried out 5-l fed-batch fermentations with *C. glutamicum* YILW and *C. glutamicum* YILWΔ*brnQ*. Off-line analysis results of biomass, glucose concentrations, and L-isoleucine production are presented in Fig. 1. The biomass of *C. glutamicum* YILWΔ*brnQ* was slightly higher than that of the original strain in the first exponential growth phase, i.e., within 20 h of fermentation initiation, and for the following period, namely 20–35 h after fermentation initiation. *C. glutamicum* YILWΔ*brnQ* exhibited no further growth after 20 h, while the original strain continued to grow slowly,

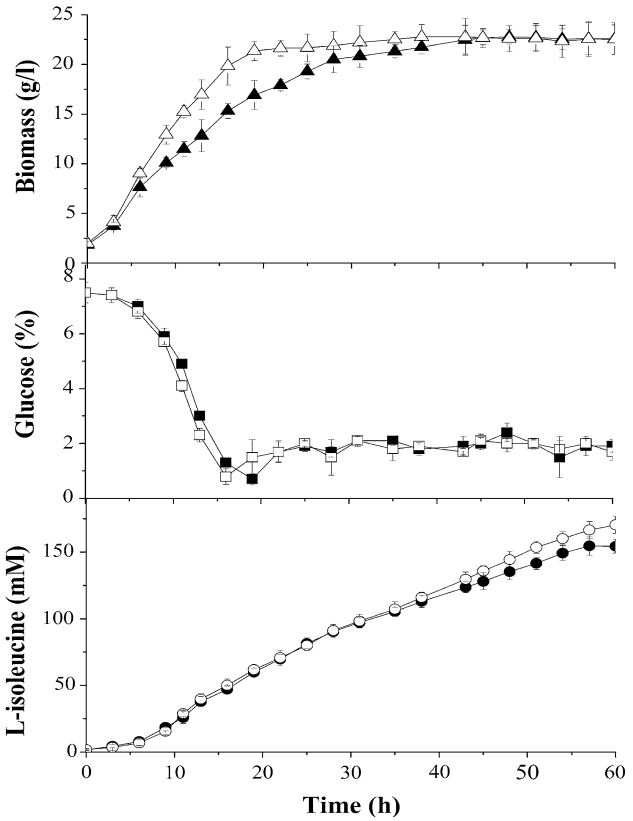

Fig. 1 Glucose-controlled L-isoleucine fed-batch fermentations of *Corynebacterium glutamicum* YILW (*solid symbols*) and *C. glutamicum* YILWΔ*brnQ* (*open symbols*) ($p < 0.05$)

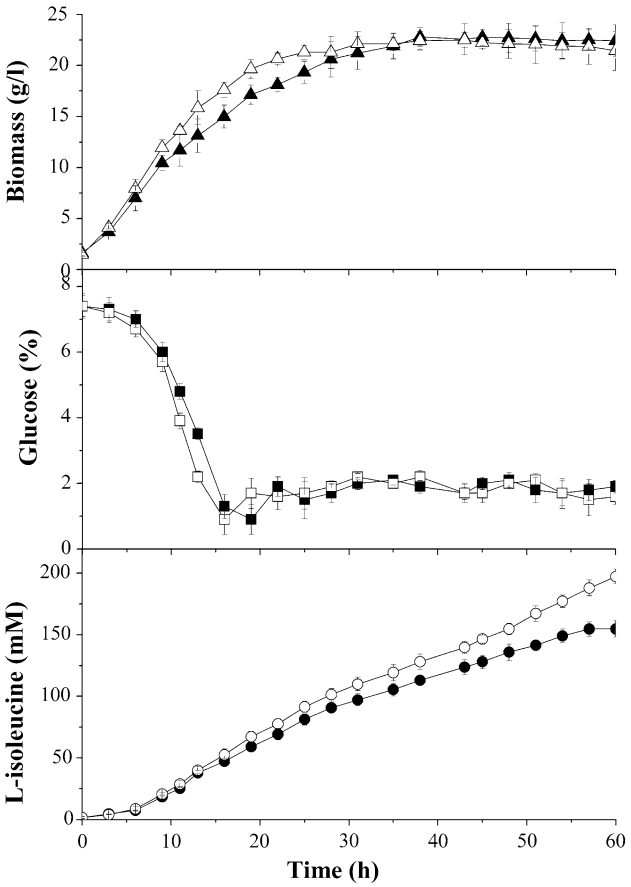

Fig. 2 Glucose-controlled L-isoleucine fed-batch fermentations of *C. glutamicum* YILWpXMJ19 (*solid symbols*) and *C. glutamicum* YILWpXMJ19*brnFE* (*open symbols*) ($p < 0.05$)

with both strains attaining a final biomass up to 23.0 g l^{-1} at 45 h, indicating that deletion of *brnQ* had nearly no effect on the optimal growth of *C. glutamicum* YILWΔ*brnQ*. The L-isoleucine concentrations accumulated by *C. glutamicum* YILW and *C. glutamicum* YILWΔ*brnQ* were approximately identical from the beginning of fermentation to 35 h post-fermentation initiation ($p > 0.05$); thereafter, the *C. glutamicum* YILWΔ*brnQ* mutant accumulated slightly higher levels of L-isoleucine than *C. glutamicum* YILW. At the end of fermentation, L-isoleucine production by *C. glutamicum* YILWΔ*brnQ* was 10.4 % higher than that of *C. glutamicum* YILW [170.3 mM (22.3 g l^{-1}) vs. 154.3 mM (20.2 g l^{-1}), respectively]. Moreover, L-isoleucine by *C. glutamicum* YILWΔ*brnQ* was approximately 16.6 % higher than that by *C. glutamicum* YILW (0.14 vs. 0.12 g isoleucine/g glucose, respectively).

Relevance of L-isoleucine export carrier *brnFE* for L-isoleucine production with *C. glutamicum* YILW

The fermentation results revealed that the deletion of *brnQ* led to an enhanced L-isoleucine accumulation in the producer; however, the effect of the export carrier BrnFE on L-isoleucine production remained unclear. To clarify this point, we performed 5-l fed-batch L-isoleucine fermentations with *C. glutamicum* YILWpXMJ19 and *C. glutamicum* YILWpXMJ19*brnFE*, respectively. Characteristic profiles for fermentations were as shown in Fig. 2. Although both strains grew to almost the same final biomass (about 22.5 g l^{-1}), *C. glutamicum* YILWpXMJ19*brnFE* constantly exhibited a higher production of L-isoleucine during the whole process, while *C. glutamicum* YILWpXMJ19*brnFE* produced 27.8 % more L-isoleucine than *C. glutamicum* YILWpXMJ19 at the end of fermentation period [197.2 mM (25.9 g l^{-1}) vs. 154.3 mM (20.2 g l^{-1}), respectivley]. The yield of the *C. glutamicum* YILWpXMJ19*brnFE* was determined to be 0.18 g/g of glucose.

Fermentation with the *brnFE*-overexpression in *C. glutamicum* YILWΔ*brnQ*pXMJ19*brnFE*

To determine whether deletion of *brnQ* and overexpression of *brnFE* had any synergistic effects on L-isoleucine production, we carried out 5-l fed-batch fermentations with *C. glutamicum* YILWΔ*brnQ*pXMJ19*brnFE*. The results are shown in Fig. 3. *C. glutamicum* YILWΔ*brnQ* harboring

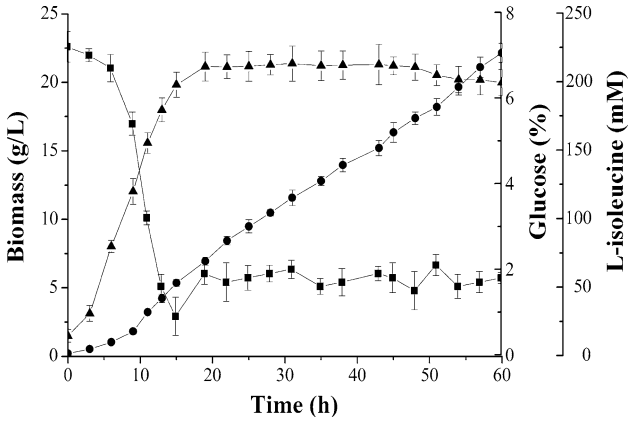

Fig. 3 Growth, residual glucose, and L-isoleucine production during the fed-batch cultivation of *C. glutamicum* YILWΔ*brnQ*pXMJ19*brnFE*. *Filled triangle* Biomass, *filled square* concentration of glucose, *filled circle* L-isoleucine ($p < 0.05$)

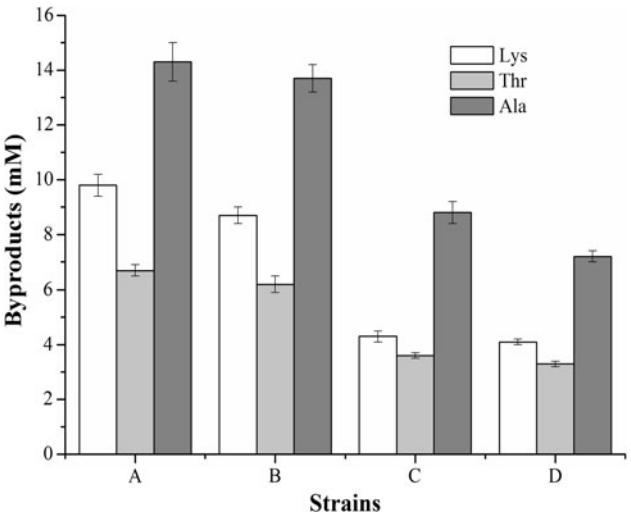

Fig. 4 Concentration of byproducts in the medium at the end of the fermentation period. *C. glutamicum* YILW (*A*), *C. glutamicum* YILWΔ*brnQ* (*B*), *C. glutamicum* YILWpXMJ19*brnFE* (*C*), *C. glutamicum* YILWΔ*brnQ*pXMJ19*brnFE* ($p < 0.05$) (*D*)

pXMJ19*brnFE* grew to a biomass of 21.2 g l^{-1} within 20 h (Fig. 3). Overexpression of *brnFE* in *C. glutamicum* YILWΔ*brnQ*pXMJ19*brnFE* resulted in a 29.8 % increase in L-isoleucine concentration [from 170.3 mM (22.3 g l^{-1}) to 221.0 mM (29.0 g l^{-1})] and in a higher L-isoleucine yield compared with *C. glutamicum* YILWΔ*brnQ* and *C. glutamicum* YILWpXMJ19*brnFE* (0.24 vs. 0.14 and 0.18 g/g of glucose, respectively). Taken together, these results show that a decreased L-isoleucine uptake on the one hand, an increased L-isoleucine export on the other hand and, finally, an increase in L-isoleucine efflux are highly beneficial for L-isoleucine overproduction with *C. glutamicum* YILW.

Excretion of byproducts during fermentations

During the fermentations, the main amino acid byproducts excreted into the medium were L-alanine, L-lysine, and L-threonine. The results of the 5-l fed-batch fermentations are shown in Fig. 4. The strain *C. glutamicum* YILW produced mainly L-alanine, L-lysine, and L-threonine, with respective final concentrations of 14.3, 9.8, and 6.7 mM. Interestingly, the *brnFE* overexpressors excreted relatively lower amounts of L-alanine, L-lysine, and L-threonine, while the deletion of *brnQ* seemed make no significant contribution changes in the excretion of byproducts. The final L-isoleucine producer *C. glutamicum* YILWΔ*brnQ*pXMJ19*brnFE* accumulated 7.2, 4.1, and 3.3 mM L-alanine, L-lysine, and L-threonine, which was a decrease of 49.6, 56.1, and 50.7 % compared with the original strain, respectively.

Discussion

Detailed studies of metabolic flux and intracellular amino acid concentrations have revealed that excretion may be the bottleneck for increasing L-isoleucine production in a high-yield strain [20]. In this case, the problem is likely to be overcome by increasing the excretion of the cytoplasmic L-isoleucine to the level at which feedback control does not operate or decrease feedback controls. In our study presented here, we examined the effect of manipulating transport systems in *C. glutamicum* YILW on L-isoleucine production.

Ikeda and Katsumata [10] showed that the defect in the uptake of aromatic amino acids contributed to an increasing production of these aromatic amino acids, demonstrating that prevention of amino acid uptake as well as deregulation of the biosynthetic pathway is one of the strategies that can be adopted to increase the yield and productivity of amino acid high-producing strains. These authors also showed that reduced L-tryptophan uptake activity resulted in an 10–20 % L-tryptophan yield increase, while increased uptake activity led to a drastic decrease in yield in an L-tryptophan high-producer of *C. glutamicum* [11]. In our study, we also proved that the *brnQ* mutant increased both the L-isoleucine efflux rate and L-isoleucine yield, which may be due to the prevention of L-isoleucine (that has been excreted to the medium) re-uptake by the *brnQ* deletion. Our investigation of biomass production with the *brnQ* mutant revealed that there was no obvious negative effect associated with the L-isoleucine uptake deficiency, possibly because the internal synthesized L-isoleucine is present in sufficient quantities to maintain the growth of strains.

Kruse et al. [17] increased L-threonine production by overexpression of the L-threonine excretion carrier, the *E. coli rhtB*, *rhtC* genes, and *thrE* (heterologous gene in *C. glutamicum*) in the *E. coli* L-threonine producer.

Hermann and Krämer [9] showed that the outward-directed flux was due to the activity of the excretion carrier, which was characterized with respect to its energy dependence and its regulation at the level of expression. In our study, overexpression of *brnFE* in *C. glutamicum* YILWpXMJ19*brnFE* resulted in an increase of L-isoleucine production, and the increase was even higher in *C. glutamicum* YILWΔ*brnQ*pXMJ19*brnFE*. A possible explanation is that increasing L-isoleucine excretion via *brnFE* overexpression can decrease the feedback control from the high-level intracellular L-isoleucine. These results indicate that, compared with the influence of *brnQ*, the export carrier *brnFE* had a more significant influence on transporting L-isoleucine from cell to the medium.

During the fermentations, the main byproducts excreted in the medium were L-alanine, L-lysine, and L-threonine, and improvement of the transport system resulted in a concentration decrease of byproducts. The L-isoleucine export carrier *brnFE* in *C. glutamicum* also exports L-leucine and L-methionine with comparable activity [6, 28]. The determinations of fermentative byproducts showed that traces of L-leucine were found with the original strain, but that the concentrations of L-leucine with the *brnQ* mutant and the *brnFE* overexpressor were below the lower limit of detection. In addition, the concentrations of L-alanine, L-threonine, and L-lysine decreased. One possible explanations is that L-isoleucine excretion increase could direct the carbon flux more efficiently from glucose to L-isoleucine, thereby reducing the accumulation of byproducts. Also, it is known that ketol-acid reductoisomerase (EC:1.1.1.86) catalyzes the two reversible reactions of the downstream branched-chain pathway (catalyzing parallel reactions to L-isoleucine and L-valine) and that excretion of synthesized L-isoleucine increases the catalytic efficiency of those reactions and avoids further degradation of intermediates and the end product. Taking all our results together, we conclude that a decreased L-isoleucine uptake or (and) an increased L-isoleucine export could increase L-isoleucine accumulation.

It has been reported that moderate expression of a gene proved to be more useful for amino acid production than overexpression of the same gene carried by a multicopy plasmid [22]. Consequently, to further improve the already relatively efficient production of L-isoleucine in *C. glutamicum*, one approach may be to focus on obtaining recombinants with a stable phenotype and moderate expression of *brnFE*. This could be achieved by obtaining a stable and limited number of gene copies using chromosomal integration and different copy-number shuttle vectors.

Acknowledgments We gratefully acknowledge the excellent support of Wenting Huo and Qian Liu in their operation of the fermentation process control. This work was supported by Tianjin Research Program of Application Foundation and Advanced Technology (Grant No. 08JCZDJC15400).

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