Brazilian Journal of Microbiology 45, 4, 1477-1483 (2014)
ISSN 1678-4405

Research Paper

Reducing lactate secretion by *ldhA* Deletion in L-glutamate- producing strain *Corynebacterium glutamicum* GDK-9

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Submitted: March 5, 2013; Approved: March 14, 2014.

Abstract

L-lactate is one of main byproducts excreted in to the fermentation medium. To improve L-glutamate production and reduce L-lactate accumulation, L-lactate dehydrogenase-encoding gene *ldhA* was knocked out from L-glutamate producing strain *Corynebacterium glutamicum* GDK-9, designated GDK-9Δ*ldhA*. GDK-9Δ*ldhA* produced approximately 10.1% more L-glutamate than the GDK-9, and yielded lower levels of such by-products as α-ketoglutarate, L-lactate and L-alanine. Since dissolved oxygen (DO) is one of main factors affecting L-lactate formation during L-glutamate fermentation, we investigated the effect of *ldhA* deletion from GDK-9 under different DO conditions. Under both oxygen-deficient and high oxygen conditions, L-glutamate production by GDK-9Δ*ldhA* was not higher than that of the GDK-9. However, under micro-aerobic conditions, GDK-9Δ*ldhA* exhibited 11.61% higher L-glutamate and 58.50% lower L-alanine production than GDK-9. Taken together, it is demonstrated that deletion of *ldhA* can enhance L-glutamate production and lower the unwanted by-products concentration, especially under micro-aerobic conditions.

Key words: *Corynebacterium glutamicum*, L-glutamate, L-lactate dehydrogenase, gene knockout, fermentation.

Introduction

L-glutamate, a widely-used flavoring agent throughout the world and a starting material for the synthesis of various chemicals, is predominately produced by *Corynebacterium glutamicum* (Calik *et al.*, 2001). The current annual production of L-glutamate is approximately 2 500 000 tons with large increases each year. To date, several major strategies have been discovered that can induce L-glutamate overproduction, including biotin limitation, addition of antibiotics, surfactants, detergents, or cerulenin, and temperature triggering. The L-glutamate producers are characterized by a low pentose phosphate pathway flux and low conversion of α-ketoglutarate to oxaloacetate in the tricarboxylic acid (TCA) cycle, likely due to low activity of the α-ketoglutarate dehydrogenase complex (Stansen *et al.*, 2005).

In *C. glutamicum*, pyruvate is a branch point of glucose catabolism, since it is the portal to several metabolic pathways (Chen *et al.*, 2009). Significantly, pyruvate can enter the TCA cycle in two ways: by condensation with CO_2 to form a dicarboxylic acid, or by oxidative decarboxylation to acetyl CoA. In addition, pyruvate can be consumed in other reactions, including L-lactate and L-alanine synthesis by Lactate dehydrogenase (LDH) and transaminase (AlaT), respectively. Although the theoretical yield of L-glutamate from glucose is 81.74% (w/w), the actual yield is much lower, due to high biomass and synthesis of by-products (Takac *et al.*, 1998).

Oxygen supply is known to largely affect aerobic amino acid production by microorganisms, because aerobic

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microorganisms generally require sufficient oxygen concentrations to re-oxidize NAD (P)H_2 or $FADH_2$ and thereby generate adequate ATP for metabolism (Akashi *et al.*, 1979). The dissolved oxygen (DO) levels largely determine the interactions between metabolic reactions and genetic regulatory mechanisms, as well as product and by-products formation during L-glutamate fermentation. For example, in addition to growth rate and protein synthesis, nutrient consumption and waste production rate are sensitive to DO levels (Zupke *et al.*, 1995). The aerobic amino acid producing bacterium *C. glutamicum* accumulates lactate, succinate and acetate under oxygen-limited growth conditions (Dominguez *et al.*, 1993; Toyoda *et al.*, 2009).

In the L-glutamate fermentation process, due to the rapid growth and high cellular metabolism requirements of the bacteria, the oxygen supply is frequently insufficient and leads to L-lactate accumulation, which inhibits L-glutamate production. To meet the sufficient oxygen demand, the agitation rate in fermentation reactors is constantly increased during the glutamate production phase, adding cost to the already high-energy-consuming production process. To resolve this problem, we knocked out the L-lactic dehydrogenase encoding gene *ldhA* from the L-glutamate-producing strain *C. glutamicum* GDK-9 (GDK-9). Here we investigate the effect of *ldhA* gene deletion on the physiological characteristics and L-glutamate production of GDK-9.

Materials and Methods

Strains, plasmids, media and cultivation

All strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in LB medium at 37 °C, *C. glutamicum* was grown in BHI medium (Keihauer *et al.*, 1993) at 32 °C. The seed medium comprised glucose (25 g L^{-1}), KH_2PO_4 (2.2 g L^{-1}), $MgSO_4$ (0.9 g L^{-1}), corn steep liquor (30 mL L^{-1}), soybean hydrolysate (20 mL L^{-1}) and urea (3 g L^{-1}). The production medium comprised glucose (80 g L^{-1}), KH_2PO_4 (2.0 g L^{-1}), $MgSO_47H_2O$ (1.8 g L^{-1}), $MnSO_4H_2O$ (2.33 mg L^{-1}), $FeSO_47H_2O$ (2.33 mg L^{-1}), VB_1 (0.233 mg L^{-1}), corn steep liquor (5 mL L^{-1}) and soybean hydrolysate (10 mL L^{-1}).

Construction of recombinant plasmids

Plasmid for gene disruption in *C. glutamicum* was derived from pK18mobsacB. Upstream (*ldhA*I) and downstream (*ldhA*II) sequences of *ldhA* were PCR-amplified from the genomic DNA of the L-glutamate-producing strain using the primer sets ldhA717-1 (5'-ATCGGAATTCAGGTGCCGACA CTAATGC-3') plus ldhA717-2 (5'-ACCGACGGTTTCTTTCATTTTCGATCCCACTTC CTGATTTC CC-3'), and ldhA600-1 (5'-ATCGAAAATGAAAGAAACCGTCGGTAACTTT TTGGTTTACGGGC -3') plus ldhA600-2 (5'-TCGAAGCTTGCTTCCAGACGGTTTCATC-3'), respectively. Splicing by overlap extension was used to fuse upstream *ldhA*I and downstream *ldhA*II using the primer pair ldhA717-1 and ldhA600-2. The resulting fragment was digested by *Hind* III and *EcoR* I, cloned into vector pK18mobsacB and transformed into *E.coli* DH5αMCR. Transformants were selected on LB agar containing 50 μg mL^{-1} kanamycin. The resulting recombinant vector pK18mobsacBΔ*ldhA* was verified by DNA sequencing. The construction process of recombinant plasmid was showed in Figure 1.

Construction of the recombinant strains

The resulting plasmid pK18mobsacBΔ*ldhA* was isolated from *E. coli* DH5αMCR and electroporated into GDK-9 (Van der Rest *et al.*, 1999; Schäfer *et al.*, 1994). The intact chromosomal *ldhA* was replaced by the truncated Δ*ldhA* gene via homologous recombination (double crossover). Because the plasmid pK18mobsacB cannot duplicate in *C. glutamicum*, the recombinant strain alone could survive on the kanamycin-containing medium. The recombinants were picked up from the Petri dish and cul-

Table 1 - Strains, plasmids and primers used in this work.

Stains/plasmids	Characters	Source
Strains		
E. coli		
DH5αMCR	F^-φ80dlacZΔ(lacZYA-argF)U169 recA1 endA1 hsdR17(rk^- mk^+) supE44 λ^- thi-1 gyrA96 relA1	[Grant *et al*., 1990]
C. glutamicum		
GDK-9	The L-glutamate producing strain	Stored in our lab
GDK-9Δ*ldhA*	*C. glutamicum* GDK-9 with *ldhA* deletion	This work
Plasmids		
pK18mobsacB	Integration vector, Km^r oriV_{Ec}oriT sacB	[Jäger *et al*., 1992]
pK18mobsacBΔ*ldhA*	pK18mobsacB carrying the Δ*ldhA*	This work

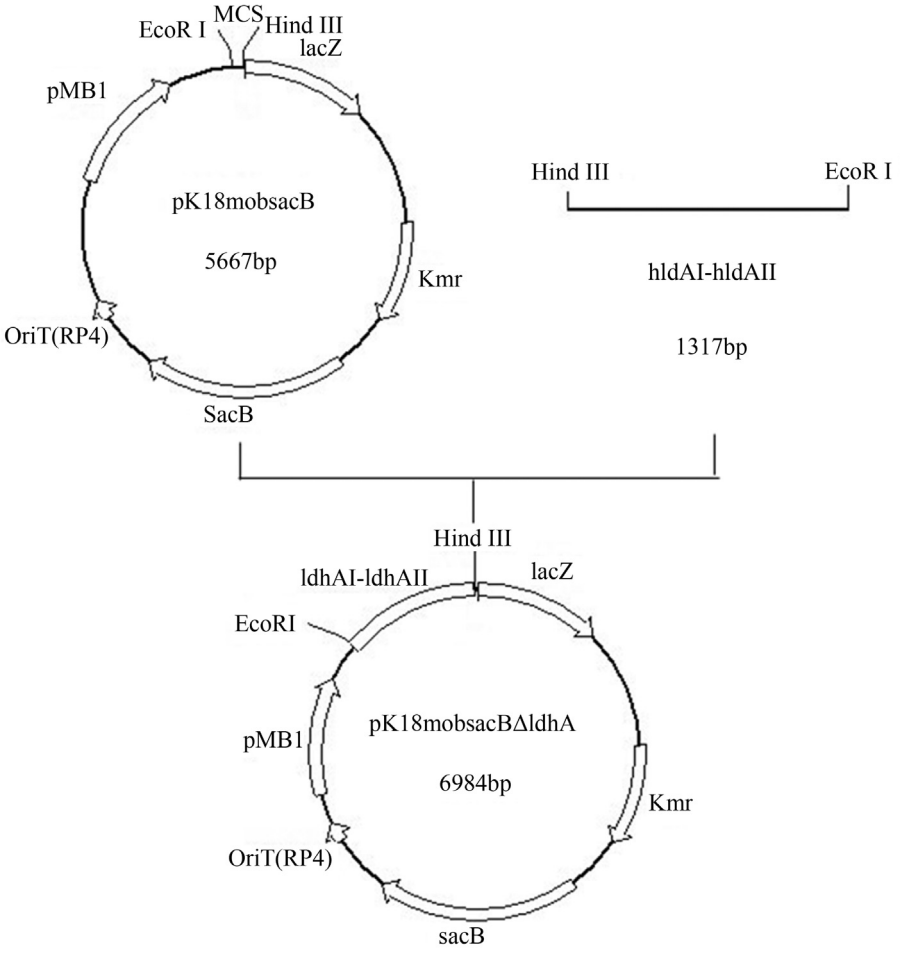

Figure 1 - The construction process of recombinant plasmid.

tured in BHIS liquid nutrient medium for 14 h. Following incubation, the strain was spread onto BHIS solid nutrient supplemented with 10% sucrose. *SacB* gene from *Bacillus subtilis* encodes the enzyme levansucrase, which catalyzes sucrose hydrolysis and levan synthesis. *C. glutamicum* strains possessing a *sacB* gene is lethal in the presence of sucrose probably due to too much levan accumulating in periplasm of cells. (Jäger *et al.*, 1992). And therefore only the double crossover homologous recombinants could survive on the sucrose-contaning medium. From these survivors, the *ldhA* gene knockout recombinant GDK-9Δ*ldhA* was isolated using primer pairs ldhA713-1 plus ldhA600-2 and upldh (5'-CAGGAGATGTTGGAGTTG-3') plus dpldh (5'-TTGAAGCGTTCCATCTCG-3').

Fermentations

For shake flask experiments, 30 mL culture broth in a 500 mL baffled flask was cultivated at 200 rpm for 30 h. When the initial glucose was depleted, the 500 g L^{-1} glucose solution was fed into the shake flask to keep the glucose concentration approximately at 20 g L^{-1}. The pH was maintained at 7.0 and controlled by the addition of 25.0% NH_4OH.

For L-glutamate fermentation, a 5-l bioreactor (Shanghai Baoxing Bio-engineering Equipment co, Ltd) with an operating volume of 3l was used as fermenter. The seed cultures were grown for 8 h in shake-flasks (100 mL seed medium inoculated into a 1-l shake-flask, 200 rpm, 32 °C) to reach an OD_{600} about 12.0-16.0. Mature cultures were transferred to 3-l production medium for L-glutamate production (inoculum size 10% v/v), and cultivated for 24 h. During the whole process, glucose concentration was maintained at approximately 20 g L^{-1} by adding 900 g L^{-1} glucose solution and the glucose feeding rate was adjusted according to glucose consumption rate of each hour. The pH was maintained at 7.0 and controlled by the addition of 25.0% NH_4OH. DO concentration was maintained at 20% during the growth phase and was turned to 10% during glutamate-producing period. For assay of effect of DO on L-glutamate production by GDK-9Δ*ldh* , DO was adjusted as required by setting the agitation speed to a specified value (within the range 300 to 900 rpm) during glutamate-producing period.

Analysis methods

During the fermentation process, 1 mL samples were taken from the cultures and centrifuged at 4 °C and 13,000 rpm for 5 min. For biomass assay, dry cell weight was determined gravimetrically. Glucose concentration was determined with a biosensor (Institute of Biology, Shandong Academy of Science). Amino acids were analyzed as 2, 4-fluoro-dinitrobenzene derivatives using high-performance liquid chromatography. Metabolites were detected by analytical methods as described previously (Kelle *et al.*, 1996). Organic acids were screened by HPLC and an UV detector at 215 nm (HPLC, Series 1100, Agilent Technologies, USA, XDB-C8 column). HPLC was undertaken at 25 °C and pH 3.0, with 100 mM L^{-1} KH_2PO_4 solution as mobile phase.

Enzymatic assay for L-lactate dehydrogenase

Bacterial cells were harvested and re-suspended in 50 mM sodium phosphate buffer (pH 7.5) supplemented with 10 mM $MgCl_2$, and grown to 20 OD_{600} units. Cells were disrupted by sonication at 400 W for 10 min with 3 s pulse and 2 s pulse-off cycling (JY92-2D, SEIENTZ, Ningbo, China), followed by centrifugation at 8,000 rpm for 15 min. The resulting supernatant was used as the cyto-soluble fraction. Total protein concentration in cell extracts was measured by Lowry's method (Sigma Kit) using bovine serum albumin as standard. Lactate dehydrogenase (LDH) assay was performed as previously described (Inui *et al.*, 2004).

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

Effect of *ldhA* gene deletion on L-glutamate production by GDK-9 in shake flask cultures

To investigate the effect of *ldhA* deletion on L-glutamate production by the L-glutamate- producing GDK-9, the gene was knocked out from the bacterium. Successful ldhA deletion was validated by PCR and sequencing (data not shown). Moreover, measured enzymatic activity of LDH was 1.5 U mg $protein^{-1}$ in cell extract of the GDK-9, and negligible in GDK-9Δ*ldh* (Table 2). These results further verified that the *ldhA* gene had been successfully knocked out in GDK-9Δ*ldhA*.

Next, levels of L-glutamate, L-lactate and L-alanine produced by the *ldhA*-knockout strain GDK-9Δ*ldhA* in shake flask (Table 2) were measured. Both strains yielded approximately the same biomass; however, L-glutamate production reached 70.4 ± 1.33 g L^{-1} by GDK-9Δ*ldhA*, 9.15% more than that attained by the GDK-9 (64.5 ± 1.91 g L^{-1}) at 2630 h. Moreover, the deletion of *ldhA* resulted in a 97.6% and 44.6% decrease in L-lactate and L-alanine relative to the GDK-9, respectively. These data show that *ldhA* gene deletion markedly improved L-glutamate production in GDK-9 and dramatically lower by-products formation.

Effect of *ldhA* gene deletion on L-glutamate production by GDK-9 in fermenters

To further examine the impact of the *ldhA* deletion on L-glutamate production, we carried out 5-l feed-batch fermentations using GDK-9 and GDK-9Δ*ldh*. Biomass, glucose concentration, and L-glutamate productions as functions of incubation time are presented in Figure 2. In early-stage cultures, the biomass of GDK-9Δ*ldh* was slightly lower than that of the GDK-9. However, as the incubation time increased, both strains yielded similar biomass, indicating that *ldhA* deletion did not adversely affect the growth of the bacteria. In addition, the glucose consumption rate of GDK-9Δ*ldh* was slower than that of the GDK-9 during the growth phase, although this situation reversed when entering the stabilization growth phase. Despite these differences, the final L-glutamate titer reached 120 g L^{-1} by GDK-9Δ*ldh*, 10.1% more than that of by GDK-9 (109 g L^{-1}) after 24 h of culture.

Excretion of byproducts during fermentations

During L-glutamate fermentation process, L-lactic acid, L-alanine and α-ketoglutarate are the most common byproducts excreted into the medium (Jojima *et al.*, 2010). To investigate the effect of *ldhA* deletion on by-product production, concentrations of these three by-products were measured at the end of the fermentation process. As shown in Table 3, levels of L-lactic acid, L-alanine and α-keto-

Table 2 - Concentration of L-glutamate, byproducts, and LDH specific activity, in shake flask studies of GDK-9 and GDK-9Δ*ldhA*.

Strain	DCW (g L^{-1})	L-glutamate (g L^{-1})	L-lactate (g L^{-1})	L-alanine (g L^{-1})	LDH-specific activity (U mg $protein^{-1}$)
GDK-9	9.97 ± 0.56	64.5 ± 1.33	8.5 ± 0.22	1.95 ± 0.05	1.50.06
GDK-9Δ*ldhA*	9.88 ± 0.47	70.4 ± 1.91	0.2 ± 0.02	1.08 ± 0.04	0.05 ± 0.01

DCW: dry cell weight. Each datum represents the average value of at least three independent measurements.

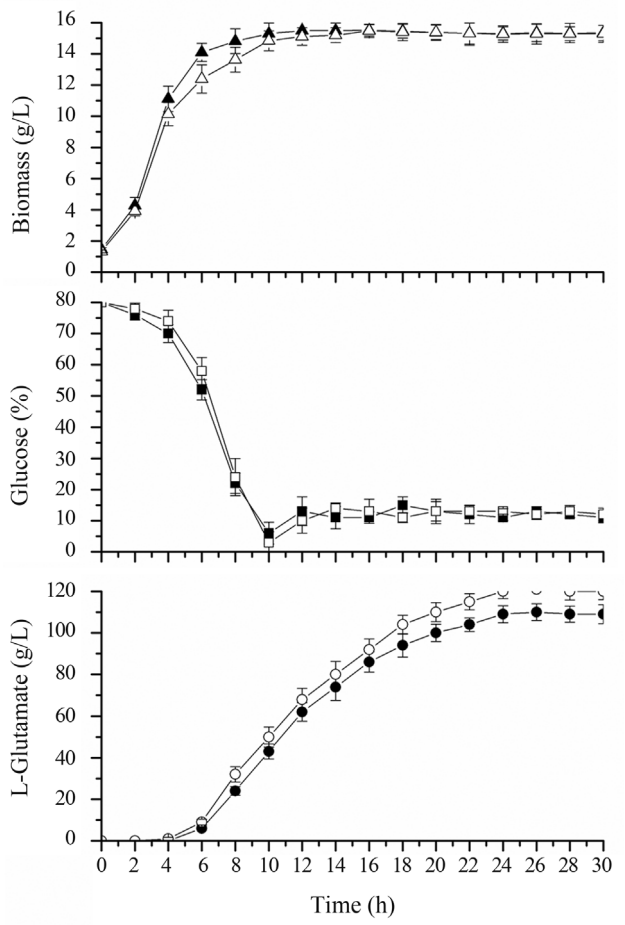

Figure 2 - Biomass, glucose and L-glutamate concentrations in glucose-controlled L-glutamate fed-batch fermentations of GDK-9 (solid symbols) and GDK-9Δ*ldh* (open symbols).

glutarate secreted by GDK-9Δ*ldh* were dramatically reduced relative to the wild-type (97.1%, 50.91% and 20.45% reduction, respectively).

Effect of the *ldhA* gene deletion on L-glutamate production by the GDK-9 under different DO conditions

DO play an important role in L-glutamate production as well as by-products accumulation (Calik *et al.*, 2001). To study the differences in performance between the *ldhA* knockout strain and GDK-9 exposed to different DO levels, 5-l fed-batch L-glutamate fermentations was performed under oxygen-deficient conditions (DO = 0%), micro-aerobic conditions (DO = 10%) and high oxygen conditions (DO = 30%) imposed at the L-glutamate production phase.

As shown in Figure 3, the biomass of both strains under oxygen-deficient conditions was slightly lower than that obtained under micro-aerobic and high oxygen conditions. Oxygen-deprived GDK-9Δ*ldh* produced 4.04% more L-glutamate but 53.26% more L-alanine than GDK-9. While under micro-aerobic conditions, GDK-9Δ*ldh* produced 11.6% more L-glutamate and 58.5% less L-alanine than the GDK-9. However, under the high-oxygen condition, GDK-9Δ*ldh* produced approximately 43.23% less L-alanine than the GDK-9, and produced similar amount of L-glutamate which is much lower than that produced under oxygen-deficient and micro-aerobic conditions. Collectively, these results show that deleting the *ldhA* gene has little effect on L-glutamate production under oxygen-deficient and high oxygen conditions, but exerts a pronounced effect under micro-aerobic conditions.

Discussion

From detailed studies of branched pyruvate metabolism, by-products have been pinpointed as the bottleneck for L-glutamate production in a high-yield strain (Uy *et al.*, 2003). And it is suggested that molecular genetic manipula-

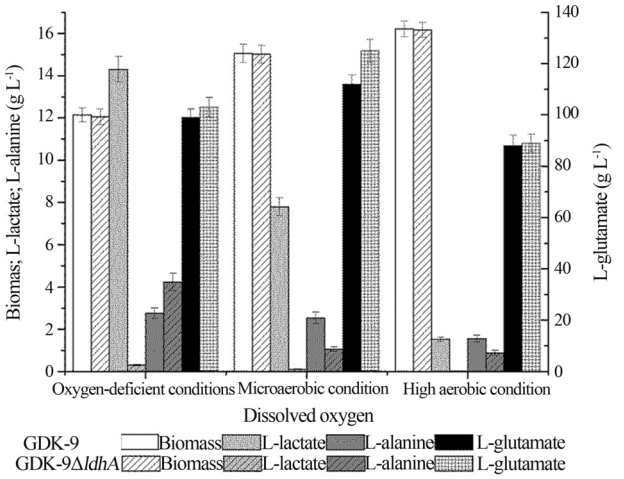

Figure 3 - Biomass gain, by-product and L-glutamate production under different dissolved oxygen conditions.

Table 3 - Concentration of byproducts in the medium at the end of the fermentation process.

Strains	L-lactate (g L^{-1})	L-alanine(g L^{-1})	α-ketoglutarate(g L^{-1})
GDK-9	7.87 ± 0.31	2.12 ± 0.18	0.44 ± 0.05
GDK-9Δ*ldhA*	0.22 ± 0.04	1.04 ± 0.15	0.35 ± 0.02

Each datum represents the average value of at least three independent measurements.

tion and fermentation optimization are viable approaches to increasing the L-glutamate yield (Ikeda, 2003; Kirchnera *et al.*, 2003). L-lactate and L-alanine are common by-products of glutamate production (Marienhagen *et al.*, 2005). Minimizing the formation of such by-products is desirable in industrial-scale production of L-glutamate (Amin *et al.*, 1993).

To enhance L-glutamate production, we here proposed to eliminate concurrent L-lactate formation by deleting the *ldhA* gene encoding the NAD-dependent LDH enzyme, which is the sole L-lactate generating enzyme in *C. glutamicum* (Stansen *et al.*, 2005). In this study, the *ldhA* gene was deleted from the L-glutamate-producing strain GDK-9. The GDK-9Δ*ldh* produced more L-glutamate than the GDK-9 in shake-flask cultures, probably because this strain secreted less L-lactate (Table 2) and more metabolic flux was drawn to L-glutamate synthesis. As shown in Figure 2, the GDK-9Δ*ldh* produced 10.1% more L-glutamate than the GDK-9 in 5-l fed-batch fermentation studies, although the final biomass of the two strains was similar. Generally, a metabolically engineered microbe has a reduced growth rate compared to its parental strain (Jung *et al.*, 2012). Studies report that *ldhA* deletion in severals microbes reduced cell growth (Yang *et al.*, 1999; Chen *et al.*, 2009; Fiedler *et al.*, 2011). Although the biomass of GDK-9Δ*ldh* was slightly lower than that of the GDK-9 in early-stage cultures, both strains yielded similar biomass as the incubation time increased, indicating that *ldhA* deletion did not adversely affect the bacterial growth. However, the glucose consumption rate of the GDK-9Δ*ldhA* was slightly slower than GDK-9 during the period of 2-6 h, which may be probably due to two reasons: on one hand, the biomass of GDK-9Δ*ldhA* was smaller but both strains accumulated similar amount of glutamate in that period, thus less glucose was consumed; and on the other hand, metabolic flux from pyruvate to lactate was cut and therefore glucose wasted on lactate synthesis was prevented.

Throughout the fermentations, the main by-products excreted into the medium were L-lactic, L-alanine and α-ketoglutarate. Compared with the GDK-9, the GDK-9Δ*ldhA* yielded lower levels of by-products (Tables 2 and 3). One possible explanation is that, in the GDK-9Δ*ldhA*, more pyruvate was fluxed to the TCA cycle for L-glutamate biosynthesis, leaving less carbon source for byproduct conversion.

The DO concentration has been shown to affect final L-glutamate production and the composition of extracellular amino acids produced (Zupke *et al.*, 1995). In this study, the *ldhA* deletion strain accumulated more L-alanine instead of L-lactate than the GDK-9 under oxygen-deficient conditions. A possible explanation is that oxygen deprivation inhibits the TCA pathway (Cocaign-Bousquet *et al.*, 1995). In this situation, pyruvate would accumulate, along with its derivatives L-alanine, L-lactate and ethanol. And the deletion of the *ldhA* gene from GDK-9 favors L-alanine accumulation. Here, we observed that deleting the *ldhA* gene produced no obvious positive effect on L-glutamate production, possibly because the accumulated L-alanine inhibits L-glutamate synthesis under oxygen-deficient conditions (Dominguez *et al.*, 1993). We further demonstrated that L-alanine accumulation is reduced in GDK-9Δ*ldh* relative to GDK-9 under micro-aerobic and high dissolved oxygen conditions.

It is reported that higher DO can strengthen TCA cycles but reduces activities of phosphoenolpyruvate carboxylase and glutamate dehydrogenase, and thus carbon-fixation (supplying oxaloacetate to the TCA cycle) and glutamate synthesis were weaken (Gao *et al.*, 2005; Yamamoto *et al.*, 2011). And therefore L-glutamate concentration was higher under micro-aerobic conditions than under high aerobic conditions. Moreover, pyruvate carboxylase (PC) as another enzyme involved in oxaloacetate synthesis need biotin as its coenzyme. Hasegawa (2008) reported that PC activity under a biotin-limited condition was ~20% of that under the condition with sufficient biotin. GDK-9 is a biotin-auxotroph strain and strategy used for this strain to trigger L-glutamate production is biotin limitation. As Figure 3 showed biomass of GDK-9 strains under high aerobic condition is higher and so more biotin was consumed than that under microaerobic condition and thus less PC activity was left. This is maybe another reason for the higher L-glutamate production under micro-aerobic conditions. These results indicate that the *ldhA* mutant enhances L-glutamate production most effectively under micro-aerobic conditions.

It has been reported that increasing the activity of pyruvate dehydrogenase by over-expressing the respective genes or by introducing alleles for deregulated pyruvate dehydrogenase might improve L-glutamate production, and the reduced pyruvate dehydrogenase activity observed during L-glutamate production is purportedly responsible for by-product formation (Uy *et al.*, 2003). Consequently, to further improve the already relatively efficient L-glutamate production in GDK-9, enhancing the activity of pyruvate dehydrogenase presents as a viable approach. Other promising approaches are optimization of fermentation technique and metabolic engineering. We have consolidated the validity of the latter approach in the results presented here.

Acknowledgments

We gratefully acknowledge the support from Jingbo Liang and Chunqian Guo. This work was supported by National Key Technology Research and Development Program of the Ministry of Science and Technology (2011BAC11B03), National High Technology Research and Development Program (2012AA02A703, 2013AA102106), Tianjin Municipal Science and Technol-

ogy Commission (Grant No. 12ZCZDSY01900), and Program for Chang jiang Scholars and Innovative Research Team in University (IRT1166).

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