World J Microbiol Biotechnol (2011) 27:2117–2124
DOI 10.1007/s11274-011-0675-9

ORIGINAL PAPER

D-lactic acid production by a genetically engineered strain *Corynebacterium glutamicum*

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Received: 17 September 2010 / Accepted: 24 January 2011 / Published online: 4 February 2011

Abstract Based on its ability to produce lactic acid from glucose in mineral salt medium under anaerobic conditions, genetic modifications on *Corynebacterium glutamicum* Res 167 were carried out with the aim of producing optical pure D-lactic acid, involving the knockout of L-lactate dehydrogenase gene from *C. glutamicum* and the heterologous expression of D-lactate dehydrogenase gene from *Lactobacillus bulgaricus* into *C. glutamicum*. D-lactic acid production of the genetically engineered strain *C. glutamicum* Res 167Δ*ldh*/*ldhA* was 17.92 g/l (optical purity higher than 99.9%) after 16 h fermentation, which was 32.25% higher than the lactic acid production of the parental strain.

Keywords D-lactic acid · *Corynebacterium glutamicum* · Genetic engineering · Gene knockout · Heterologous expression

Introduction

As an important synthetic precursor of many chiral compounds, lactic acid has been widely used in chemical, agriculture, food, medicine and environmental protection industries (John et al. 2007; Vallander and Erikson 1985; Wee et al. 2006). Moreover, lactic acid is a potential alternative of the non-biodegradable plastics from petrochemicals in poly lactic acid (PLA) synthesis (Akerberg and Zacchi 2000). Depending on its optical property, lactic acid can be divided into D-lactic, L-lactic and racemized DL-lactic acid. Recent research showed that the thermal stability of PLA was enhanced up to 200°C, which was 20°C above the melting temperature of the homo-polymer, when the poly D-lactic acid and poly L-lactic acid were mixed under a molar ratio of 1:1 (Sawai et al. 2007). Studies on D-lactic acid production become more and more popular since the above phenomenon was observed.

Currently, D-lactic acid production strains under research mainly include *Lactobacillus* species, *Escherichia coli* and *Saccharomyces cerevisiae*. Being the first industrialized strains such as *L. delbrueckii* (Hofvendahl and Hahn-Hägerdal 2000), *L. coryniformis* (Yáñez et al. 2003) and *L. lactis* (Joshi et al. 2010) etc. for D-lactic acid production, lactobacilli have drawn most researchers' focus (Lu et al. 2009). So far as we known, the highest yield (up to 1.0 g D-lactic acid/g glucose) and productivity (up to 18 g/l/h) were obtained by *L. delbrueckii* (Tashiro et al. 2010). However, the fermentation processes by these strains required complicated nutrients-contained media thus increased the cost of the substrate consumption and downstream separation. To solve this problem, researchers tried to produce D-lactic acid by engineered *E. coli* (Zhou et al. 2003), which could use minimal salt medium for cell growth. But the alternative approaches lacked promise not only for engineered *E. coli* which had a low productivity and a low tolerance to lactic acid (Portnoy et al. 2008); but also for engineered *S. cerevisiae* whose separation cost was low but yield and productivity was low, either (Tokuhiro et al. 2009). Thus the major problem now is to realize the

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high efficient D-lactic acid production based on cheap resources.

Corynebacterium glutamicum has been widely used for production of amino acid derivatives in industries (Kinoshita et al. 1957; Leuchtenberger et al. 2005). Under anaerobic conditions, growth of *C. glutamicum* was repressed but organic acids (mainly lactic acid, succinic acid and acetic acid) were produced with high conversion yield and productivity in mineral salt medium containing glucose as carbon source (Inui et al. 2004; Okino et al. 2005). If we can take advantage of these abilities of *C. glutamicum* and direct the carbon flux to D-lactic acid production as much as possible, by using molecular biology methods to block the byproducts production (such as L-lactic acid, succinic acid and acetic acid) during the organic acid metabolism, the maximum production of D-lactic acid may be achieved in a low-cost and high-efficient way. However, there are few studies and reports in this area to date (Okino et al. 2008).

In this paper, genetic modifications on *C. glutamicum* were carried out in the aim of obtaining an engineered strain to produce high-purity D-lactic acid, involving two steps: first, the knockout of L-lactate dehydrogenase gene from *C. glutamicum* and second, the heterologous expression of D-lactate dehydrogenase gene from *L. bulgaricus* into *C. glutamicum*. The present work was a primary but important step to construct a high-efficiently and low-costly D-lactic acid producing strain in a long term study.

Materials and methods

Strains, plasmids, media and cultivation

All strains and plasmids used in this study were listed in Table 1. *E. coli* DH5α was used for plasmids propagation, which was cultured in Luria–Bertani (LB) medium containing (per liter): 10 g peptone, 5 g yeast extract and 10 g NaCl (pH 7.2–7.5) at 37°C. *L. bulgaricus* was used as the *ldhA* source, which was cultured in de Man-Rogosa-Sharp (MRS) medium containing (per liter): 20 g glucose, 10 g peptone, 10 g meat extract, 5 g yeast extract, 2 g K_2HPO_4, 5 g NaAc, 2 g triammonium citrate, 0.58 g $MgSO_4{\cdot}7H_2O$, 0.25 g $MnSO_4{\cdot}H_2O$ and 1 ml Tween 80 (pH 6.2–6.4) at 40°C. All corynetacteria strains used were derived from *C. glutamicum* Res 167 and cultured in nutrient-rich medium (A-medium) containing (per liter): 40 g glucose, 2 g urea, 2 g yeast extract, 7 g casamino acid, 7 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 6 mg $FeSO_4{\cdot}7H_2O$, 4.2 mg $MnSO_4{\cdot}H_2O$, 0.2 mg biotin and 0.2 mg thiamine (pH 7.0) at 30°C. Steam sterilization of the above media was performed under 0.05 MPa, 115°C for 30 min. Appropriate concentrations of antibiotics were supplemented in corresponding media when genetic manipulations were carried out: for *E. coli*, 50 μg/ml of kanamycin (final concentration, similarly hereinafter) and 20 μg/ml of chloramphenicol; for *C. glutamicum*, 25 μg/ml of kanamycin and 10 μg/ml of chloramphenicol. For transformation,

Table 1 Strains and plasmids used in this work

Stains/plasmids	Characters	Source
Strains		
E. coli DH5α	*F*$^-$, *φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk*$^-$*, mk*$^+$*), phoA, supE44,* λ^-*, thi-1, gyrA96, relA1*	Bethesda Research Laboratories (1986)
L. bulgaricus ATCC 11842	Type strain; source of *ldhA* gene	ATCC 11842
C. glutamicum Res 167	Restriction-deficient mutant of *C. glutamicum* ATCC 13032; Δ*cglIM* Δ*cglIR* Δ*cglIIR*	University of Bielefeld
C. glutamicum Res 167Δ*ldh*	*C. glutamicum* Res 167 without *ldh* gene	This work
C. glutamicum Res 167Δ*ldh/ldhA*	*C. glutamicum* Res 167Δ*ldh* harboring pXMJ19-*ldhA*	This work
Plasmids		
pEASY-T1	Amp^r; *lacZ* TA cloning vector	Takara Biotechnology Co., Dalian, China
pEASY-T1-*ldh*	pEASY-T1 containing *ldh1-ldh2* insert	This work
pEASY-T1-*ldhA*	pEASY-T1 containing *ldhA* insert	This work
pK18*mobsacB*	Mobilizable vector, allows for selection of double crossover in *C. glutamicum*	Schäfer et al. (1994)
pK18*mobsacB-ldh*	Km^r; pK18*mobsacB* containing *ldh1-ldh2* insert	This work
pXMJ19	Cm^r P*tac lacI*^{q} pBL1 *oriV*$_{C.\ glutamicum}$ pK18 *oriV*$_{E.\ coli}$; shuttle vector	Jakoby et al. (1999)
pXMJ19-*ldhA*	pXMJ19 containing *ldhA* insert	This work

C. glutamicum Res 167 was cultured in Brain Heart Infusion-Sorbitol (BHIS) medium containing: 7.4 g brain heart infusion, 18.2 g sorbitol in 200 ml distilled water (pH 7.2–7.5) at 30°C, sterilized by filtration (filer pore size of 0.22 μm).

General DNA manipulation

To clone corresponding DNA, *C. glutamicum* and *L. bulgaricus* were treated with 4 mg/ml lysozyme at 37°C for 30 min. PCR reaction system contained 50 ng DNA, 0.2 mM deoxynucleoside triphosphates, 2% dimethylsulfoxide in LA Taq polymerase buffer with $MgCl_2$ and 4 U of LA Taq polymerase (Takara Biotechnology Co., Dalian, China) for 30 cycles at temperatures of 94°C for denaturation (1 min), 52°C for annealing (30 s), and 72°C for extension (1.5 min). Genetic manipulations such as restriction and ligation were carried out as described by Sambrook and Russell (2001). *C. glutamicum* were transformed by electroporation as described by van der Rest et al. (1999). Transformation of *E. coli* was performed by heat shock as described by Sambrook and Russell (2001).

Construction of recombinant plasmids

Plasmids for gene disruption and expression in *C. glutamicum* were derived from pK18*mobsacB* and pXMJ19, respectively. Primers used for DNA amplification were listed in Table 2. To clone *ldh1* and *ldh2* (cloning conditions described in above section), *C. glutamicum* Res 167 genomic DNA was used as PCR template with two pairs of primers uldh1, dldh1 and uldh2, dldh2 (After obtaining *ldh* (945 bp) sequence of *C. glutamicum* from NCBI, two homology arms *ldh1* (723 bp) and *ldh2* (784 bp) were designed by Primer Premier 5.0. The pair of primers uldh1 and dldh1 was used to generate *ldh1* and the pair of primers uldh2 and dldh2 was used to generate *ldh2*). The overlapped PCR products were amplified with a pair of primers uldh1 and dldh2 using *ldh1* and *ldh2* mixture as PCR template. The PCR-amplified fragment of *ldh1-ldh2* whose 3′-A overhang was added before ligation was sub-cloned into pEASY-T1 (procedures performed according to the manufacture's instructions), obtaining the recombinant plasmid pEASY-T1-*ldh*. The *Hind*III-*EcoR*I target fragment was cloned into the suicide vector pK18*mobsacB* cut with the same enzymes, creating *ldh* gene knockout vector pK18*mobsacB-ldh* (Fig. 1). *ldhA* was amplified using *L. bulgaricus* ATCC 11842 genomic DNA as PCR template with a pair of primers uldhA and dldhA. The PCR-amplified fragment of *ldhA* whose 3′-A overhang was added before ligation was sub-cloned into pEASY-T1 (procedures performed according to the manufacture's instructions), obtaining the recombinant plasmid pEASY-T1-*ldhA*. The *Xba*I-*Sac*I target fragment was cloned into the shuttle vector pXMJ19 cut with the same enzymes, creating *ldhA* gene expression vector pXMJ19-*ldhA* (Fig. 2). All cloned DNA fragments were confirmed to be correct by sequencing.

Construction of the recombinant strains

The gene knockout plasmid pK18*mobsacB-ldh* was transformed by electroporation into *C. glutamicum* Res 167 and the positive strains were selected by kanamycin resistance. Because pK18*mobsacB* could not duplicate in *C. glutamicum*, only the recombinant strain could survive on the petri dish with kanamycin (Schäfer et al. 1994). Positive strains were picked up and cultured in LB liquid nutrient medium

Table 2 Primers used in this work

Name	Sequence[a]	Notes
uldh1	5′-CCAAGGTGCCGACACTAAT-3′	To generate *ldh1* from *C. glutamicum* Res 167
dldh1	5′-CGGTGATTTCGCAACTCCAACATCTCCTG-3′	
uldh2	5′-TTGGAGTTGCGAAATCACCGACCACGAGA-3′	To generate *ldh2* from *C. glutamicum* Res 167
dldh2	5′-GCTTCCAGACGGTTTCATC-3′	
uldhA	5′-GGCTAGGAGCTCTGTAAGAAAATCTGTAGGT-3′	To generate *ldhA* from *L. bulgaricus* ATCC 11842; to amplify *ldhA* in *C. glutamicum* Res 167Δ*ldh/ldhA*
dldhA	5′-TGAGCTCTAGAAAAGGAGGAGGGACAATTAATGACT-3′	
u1	5′-AGAAACCGTCGGTAACAAG-3′	To amplify *ldh* in *C. glutamicum* Res 167Δ*ldh*
d1	5′-TTGAAGCGTTCCATCTCGT-3′	
up1	5′-GCTTGTAAAACAGCCAGG-3′	To amplify internal fragment of *ldh1-ldh2* in *C. glutamicum* Res 167Δ*ldh*
down1	5′-GTGGTAGTCAAGCGGGTAG-3′	
upXMJ	5′-TGCGCCGACATCATAACG-3′	To amplify *ldhA* and part of the plasmid in *C. glutamicum* Res 167Δ*ldh/ldhA*
dpXMJ	5′-GGGAGACCCCACACTACC-3′	

[a] Restriction enzyme sites underlined

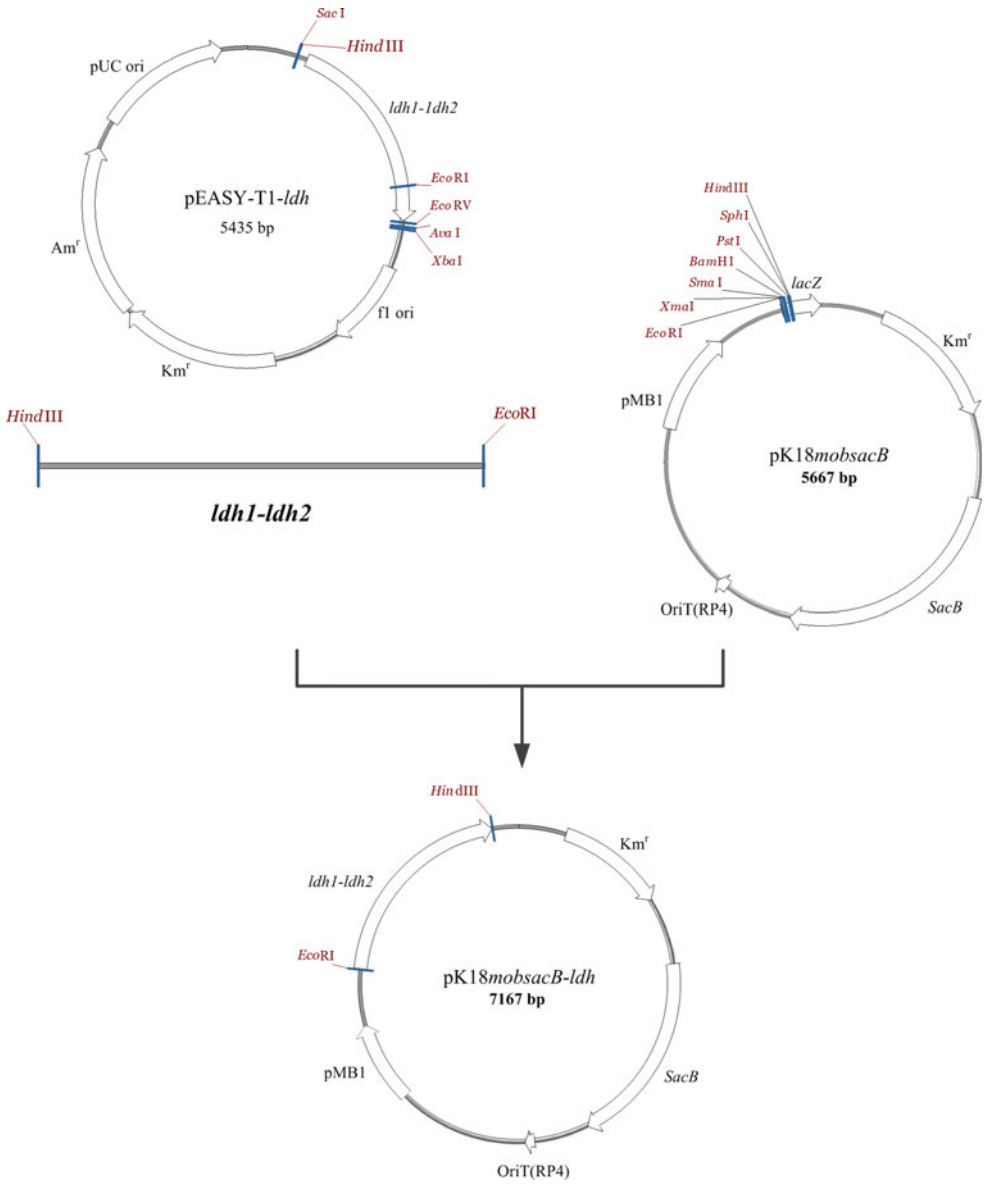

Fig. 1 Construction of the *ldh* gene knockout plasmid pK18*mobsacB-ldh*

for 16 h, then the strain suspensions was spread onto solid medium with 20% sucrose. Only wild-type *C. glutamicum* and recombinants with double crossover homologous recombination could survive on this solid medium because the *C. glutamicum* strains with *sacB* gene were not able to growing. Pairs of primers up1, down1, uldh1, dldh2 and u1, d1 were used to do bacterial colony PCR to select the *ldh* gene knockout recombinant *C. glutamicum* Res 167Δ*ldh* among the ones with sucrose resistance.

The gene expression plasmid pXMJ19-*ldhA* was transformed by electroporation into *C. glutamicum* Res 167Δ*ldh* and the positive strains were selected by chloramphenicol resistance. As pXMJ19 was an *E. coli-C. glutamicum* shuttle vector, recombinants harboring pXMJ19-*ldhA* could survive on the petri dish with chloramphenicol. Positive strains were picked up and cultured in LB liquid nutrient medium with 10 μg/l chloramphenicol for 16 h. Pairs of primers uldhA, dldhA and upXMJ, dpXMJ were used to do bacterial colony PCR to select the *ldhA* gene expression recombinant *C. glutamicum* Res 167Δ*ldh/ldhA*.

Lactic acid production

For lactic acid/D-lactic acid production, *C. glutamicum* Res 167/Res 167Δ*ldh/ldhA* were cultured in A-medium at 30°C for 8 h with a constant rotation speed of 200 rpm. 500 ml of the culture were harvested (OD_{600} = 0.8–1.0) by centrifugation (5,000 × *g*, 4°C, 10 min). The cell pellet was subsequently washed once with mineral salt medium (BT-medium) containing (per liter): 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 6 mg $FeSO_4{\cdot}7H_2O$, 4.2 mg $MnSO_4{\cdot}H_2O$, 0.2 mg biotin and 0.2 mg thiamine (pH 7.5

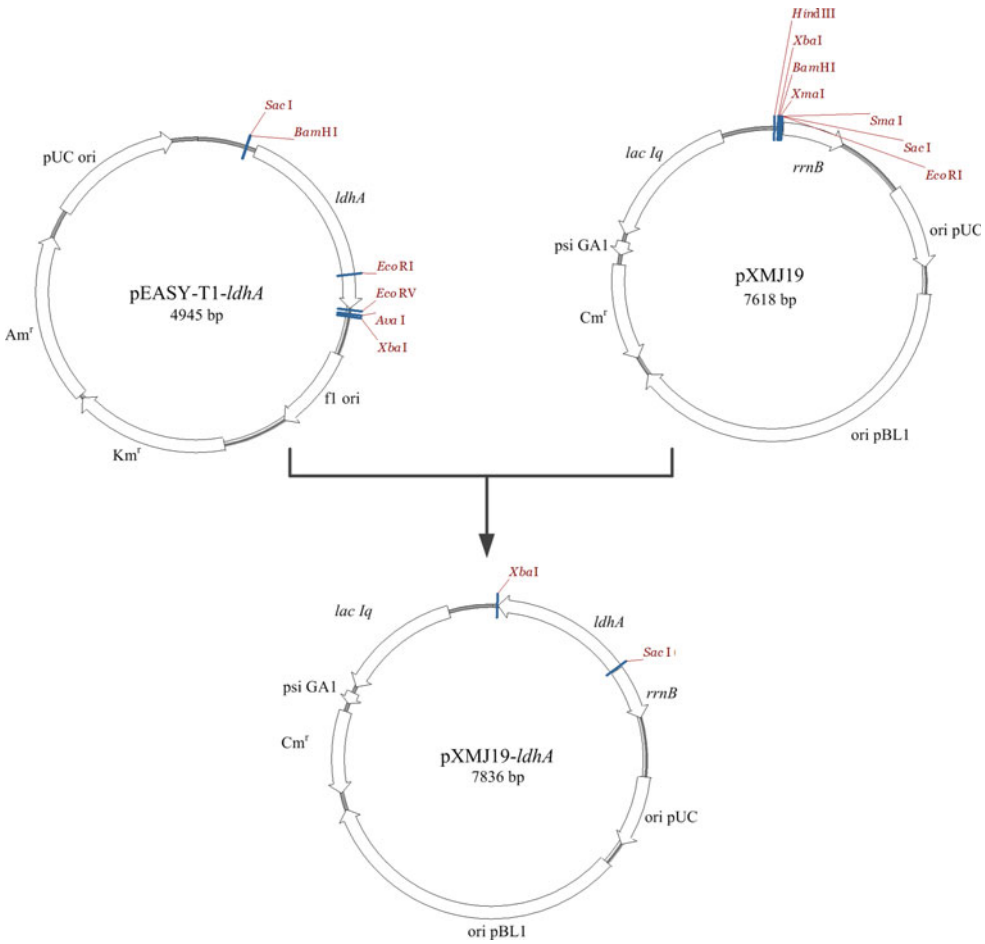

Fig. 2 Construction of the *ldhA* gene expression plasmid pXMJ19-*ldhA*

before sterilization), which was steam sterilized under 0.05 MPa, 115°C for 30 min. All of the washed cells were re-suspended in 50 ml of BT-medium with 4% (w/v) glucose in a lidded 100 ml bottle and incubated in a rotary shaker (THZ-C, Taicang Experiment Equipment, China) at 30°C for 16 h with a constant rotation speed of 150 rpm. The pH was monitored using a pH controller (PH-101, Hotec Instruments Co., Taiwan) and maintained at pH 7.0 by automatic supplementing of NH_4OH. The fermentation experiments were all done in three repeats under the same operating conditions.

Analysis of glucose, lactic acid and cell concentrations

Samples were centrifuged (10,000 × *g*, 4°C, 10 min) and the supernatants were analyzed for sugars and organic acids. Glucose concentration was measured by a biosensor analyzer (SBA-40B, Institute of Biology, Shandong Province Academy of Sciences, China). Lactic acid concentration (D-lactic acid concentration + L-lactic acid concentration) was determined by high performance liquid chromatography (HPLC, Series 1200, Agilent Technologies, USA), equipped with a ZORBSX SB-C18 column (250 mm × 4.6 mm, Agilent Technologies, USA) and a UV detector (210 nm). The mobile phase was 5 mM H_2SO_4 at a flow rate of 1 ml/min. The oven temperature was 65°C. D-lactic acid concentration was measured by a lactate assay kit (Sigma Diagnostics Lactate Procedure No. 735, Sigma Co., St. Louis, MO, USA). Then L-lactic acid concentration was the difference between the measured lactic acid concentration and D-lactic acid concentration. The optical purity of the produced lactic acid was calculated by: (D-lactic acid concentration − L-lactic acid concentration)/lactic acid concentration ×100% (Okano et al. 2010). Cell concentration was measured through optical density at 600 nm (OD_{600}) by an ultraviolet spectrophotometer (ZF1-II, Shanghai Jiapeng Tech. Co., China).

Results and discussion

Confirmation of the recombinant strains

Confirmation of *ldh* deletion in the mutant *C. glutamicum* Res 167Δ*ldh* by PCR and electrophoresis was shown

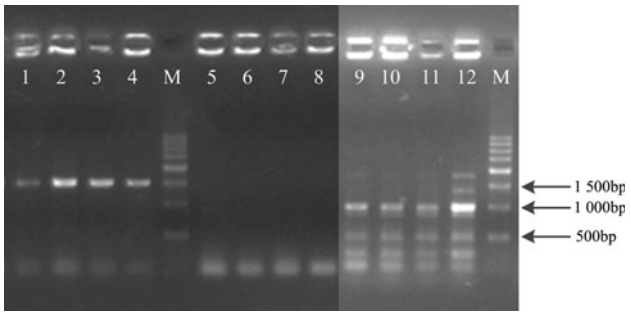

Fig. 3 Confirmation of *ldh* gene knockout in the mutant *C. glutamicum* Res 167Δ*ldh* by PCR and electrophoresis (*lanes* 1–4: amplifying *ldh1-ldh2* with pair of primers uldh1 and dldh2; *lanes* 5–8: amplifying *ldh* with pair of primers u1 and d1; *lanes* 9–12: amplifying internal fragment of *ldh1-ldh2* with pair of primers up1 and down1; *lane* M: 500 bp plus ladder)

in Fig. 3. It was expected that with pair of primers uldh1 and dldh2, there should be an about 1,500 bp product (*ldh1-ldh2*) which was amplified from *C. glutamicum* Res 167Δ*ldh*. If reverse mutation occurred in the strain, there would be an about 2,500 bp product (*ldh1-ldh-ldh2*). With pair of primers up1 and down1, there should be an about 1,000 bp product (internal fragment of *ldh1-ldh2*) which was amplified from *C. glutamicum* Res 167Δ*ldh*. If reverse mutation occurred, there would be an about 2,000 bp product (internal fragment of *ldh1-ldh-ldh2*). With pair of primers u1 and d1, there should be no product amplified from *C. glutamicum* Res 167Δ*ldh*. If reverse mutation occurred, there would be an about 1,000 bp product (*ldh*). From Fig. 3, it can be seen that there were the 1,500 bp bands with pair of primers uldh1 and dldh2 in lanes 1–4 and the 1,000 bp bands with pair of primers up1 and down1 in lanes 9–12; while no bands with pair of primers u1 and d1 were obtained in lanes 5–8. These PCR and electrophoresis results were in good consistent with the expected results, which meant that the L-lactate dehydrogenase gene of *C. glutamicum* Res 167 had been successfully knocked out in *C. glutamicum* Res 167Δ*ldh*.

Confirmation of *ldhA* expression in the recombinant *C. glutamicum* Res 167Δ*ldh/ldhA* by PCR and electrophoresis was shown in Fig. 4a (amplifying *ldhA* with pair of primers uldhA and dldhA) and Fig. 4b (amplifying *ldhA* and part of the plasmid with pair of primers upXMJ and dpXMJ, selecting bacterial colonies 1–4, 6–7, 9 and 11 of Fig. 4a with obvious PCR products). It was expected that if the shuttle vector pXMJ19-*ldhA* was successfully transformed into *C. glutamicum* Res 167Δ*ldh*, there should be an about 1,000 bp product (*ldhA*) with pair of primers uldhA and dldhA and an about 1,500 bp product (*ldhA* and part of the plasmid) with pair of primers upXMJ and dpXMJ; otherwise, there would be no products for both cases. It can be seen from Fig. 4a that there were the 1,000 bp bands with pair of primers uldhA and dldhA in eight lanes (1–4, 6–7, 9 and 11; the 1,000 bp band in lane X was the PCR product of *ldhA* amplified from *E. coli* harboring pXMJ19-*ldhA* as positive control); and it was also confirmed in Fig. 4b that there were the 1,500 bp bands with pair of primers upXMJ and dpXMJ in all eight lanes. These PCR and electrophoresis results were in good consistent with the expected results, which meant that the plasmid pXMJ19-*ldhA* had been successfully transformed into *C. glutamicum* Res 167Δ*ldh*, obtaining the final engineered strain *C. glutamicum* Res 167Δ*ldh/ldhA* in this work. Strain of colony 3 of Fig. 4b was picked up for further D-lactic acid fermentation experiments.

Lactic acid production

Fermentation experiments of the parental strain *C. glutamicum* Res 167 and genetically engineered strain *C. glutamicum* Res 167Δ*ldh/ldhA* were carried out under the ways as described above, and the results were shown in Fig. 5, including the glucose consumption of both strains, and the lactic acid production of the parental strain as well as D-lactic acid production of the engineered strain. Data shown in this figure were the averaged results of the repeated experiments and the relative standard deviations were all with ±3%, which ensured the reliability of the strains. It can be seen from the figure that the lactic acid production of the parental strain *C. glutamicum* Res 167

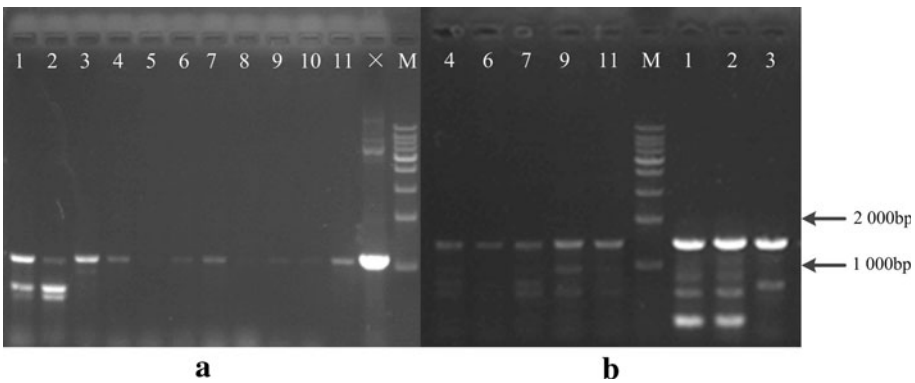

Fig. 4 Confirmation of plasmid pXMJ19-*ldhA* transformation in the recombinant *C. glutamicum* Res 167Δ*ldh/ldhA* by PCR and electrophoresis (**a** amplifying *ldhA* with pair of primers uldhA and dldhA; *lane* X: *E. coli* harboring pXMJ19-*ldhA* as positive control; *lane* M: 1,000 bp plus ladder. **b** amplifying *ldhA* and part of the plasmid with pair of primers upXMJ and dpXMJ; *lane* M: 1,000 bp plus ladder)

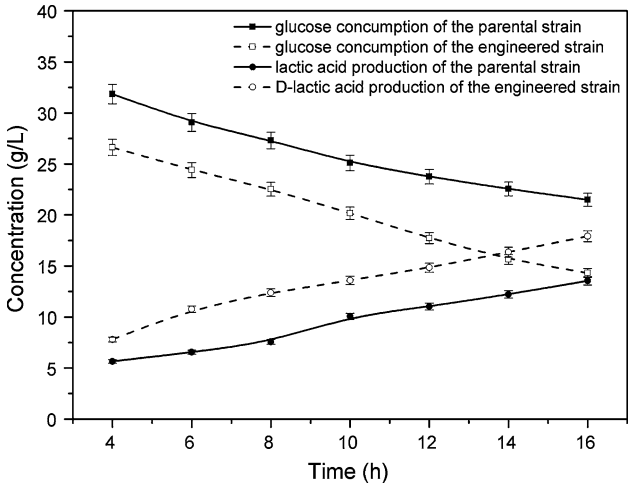

Fig. 5 Fermentation profiles of the parental strain *C. glutamicum* Res 167 and engineered strain *C. glutamicum* Res 167Δ*ldh/ldhA*

was 13.55 g/l after 16 h fermentation, while the D-lactic acid production of the engineered strain *C. glutamicum* Res 167Δ*ldh/ldhA* was 17.92 g/l after 16 h fermentation, which was about 32.25% higher than the lactic acid production of the parental strain. It should be pointed out that the engineered strain did not produce L-lactic acid and the optical purity of the final product was higher than 99.9%.

In this study, a genetically engineered strain *C. glutamicum* Res 167Δ*ldh/ldhA* was successfully constructed which had the ability to produce high optical pure D-lactic acid with a higher production compared with the lactic acid production of the parental strain, by knockout of the L-lactate dehydrogenase gene and heterologous expression of D-lactate dehydrogenase gene from *L. bulgaricus*, as validated by the bacterial colony PCR and electrophoresis results. However, this was only the very first step to obtain the high-efficiently and low-costly D-lactic acid producing strain. Current engineered strain was still not comparable in D-lactic acid producing ability with the already industrialized strains whose production was up to 120 g/l (Calabia and Tokiwa 2007), not to mention the production cost since the substrate was glucose and the fermentation process had not been optimized yet in this work. Moreover, compared with other genetically engineered strains reported in literature whose D-lactic acid productions were around 1.27–138 g/l and yields were around 0.53–0.99 (Zhou et al. 2010), the recombinant obtained in this work (D-lactic acid production: 17.92 g/l, yield: 0.82 g/g) also needed further improvement for future utilization.

It was noticed that there were many other byproducts such as succinic acid and acetic acid apart from D-lactic acid in the products. Genetic engineering approach could be adopted to disrupt corresponding genes to reduce or even eliminate the production of these byproducts, increasing the carbon availability toward D-lactic acid. It is also promising to further enhance the production ability of the strain through metabolic engineering application and fermentation technique optimization.

Acknowledgments The authors would like to acknowledge the generous donation of the strain *C. glutamicum* Res 167 and plasmids pK18*mobsacB* and pXMJ19 by Prof. Shuangjiang Liu of the Institute of Microbiology, Chinese Academy of Sciences. This work was financially supported by the National High Technology Research Development Program of China (No. 2006AA020102), the National Natural Science Foundation of China (No. 20906070 and No. 20976124), the Innovation Foundation of Tianjin University, and Program of Introducing Talents of Discipline to Universities (No. B06006).

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