Bioresource Technology 172 (2014) 269–275

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Production of optically pure D-lactate from glycerol by engineered *Klebsiella pneumoniae* strain

Xinjun Feng [a,b], Yamei Ding [c], Mo Xian [a], Xin Xu [a], Rubing Zhang [a], Guang Zhao [a,*]

[a] CAS Key Laboratory of Biobased Materials, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 266101, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China
[c] Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China

## HIGHLIGHTS

- *K. pneumoniae* was engineered to produce optically pure D-lactate from glycerol.
- The aeration rate played a critical role in D-lactate production.
- The strain produced 142 g/L D-lactate under microaerobic conditions.
- The highest D-lactate production and yield from glycerol were achieved.

## ARTICLE INFO

*Article history:*
Received 14 August 2014
Received in revised form 14 September 2014
Accepted 15 September 2014
Available online 22 September 2014

*Keywords:*
D-Lactate
Glycerol
Engineered *Klebsiella pneumoniae* strain
Bioconversion

## ABSTRACT

In this study, glycerol was used to produce optically pure D-lactate by engineered *Klebsiella pneumoniae* strain. In the recombinant strain, D-lactate dehydrogenase LdhA was overexpressed, and two genes, *dhaT* and *yqhD* for biosynthesis of main byproduct 1,3-propanediol, were knocked out. To further improve D-lactate production, the culture condition was optimized and the results demonstrated that aeration rate played an important role in D-lactate production. In microaerobic fed-batch fermentation, the engineered strain accumulated 142.1 g/L optically pure D-lactate with a yield of 0.82 g/g glycerol, which represented the highest D-lactate production from glycerol so far. This study showed that *K. pneumoniae* strain has high efficiency to convert glycerol into D-lactate and high potentiality in utilization of crude glycerol from biodiesel industry.



## 1. Introduction

As one of the best choices among alternative fuels, biodiesel has attracted considerable attention. The tremendous growth of biodiesel industry has generated a surplus of glycerol, which led to a dramatic decrease in glycerol price (Posada et al., 2012). Therefore, it is an urgent need to convert glycerol into higher-value products. In the past few years, microbial conversions of glycerol into value-added chemicals such as 1,3-propanediol (1,3-PDO) (Menzel et al., 1997; Durgapal et al., 2014), 3-hydroxypropionate (Luo et al., 2012; Kwak et al., 2013; Jung et al., 2014), poly(3-hydroxypropionate) (Andreeßen et al., 2010; Wang et al., 2013) and succinate (Blankschien et al., 2010) were reported.

*Klebsiella pneumoniae* can use glycerol as sole carbon and energy source. Besides that, it is also a kind of well-studied microorganism exhibiting high growth rate without special nutritional requirements. Taken together, *K. pneumoniae* strains were widely used in the bioconversion of glycerol into 3-hydroxypropionate (Kumar et al., 2013) and 1,3-PDO (Seo et al., 2010). Recently, several studies to synthesize D-lactate from glycerol using *K. pneumoniae* strains arose (Wang and Meng, 2012; Rossi et al., 2013; Song et al., 2013).

D-Lactate is an important chiral intermediates and organic synthetic raw material, widely used in the fields of pharmaceutical, herbicides and cosmetics. D-Lactate is also a monomer for the production of polylactate (PLA), a biodegradable plastic which was usually produced from optically pure L-lactate. The incorporation of D-lactate into PLA was able to improve the mechanical performance, thermal resistance, and hydrolysis resistance of PLA-based materials (Fukushima et al., 2007). With the increase in the global market of PLA, there is a great demand for polymer-grade D-lactate.

In D-lactate biosynthesis, the fermentative D-lactate dehydrogenase encoded by *ldhA* gene contributes much to the lactate production. In previous studies synthesizing D-lactate from glycerol by

* Corresponding author. Tel.: +86 532 80662767; fax: +86 532 80662765.
*E-mail address:* zhaoguang@qibebt.ac.cn (G. Zhao).

http://dx.doi.org/10.1016/j.biortech.2014.09.074
0960-8524/

*K. pneumoniae* strain, D-lactate and 1,3-PDO were produced simultaneously, and the production of 1,3-PDO at high levels decreases the carbon flow towards D-lactate (Rossi et al., 2013; Song et al., 2013). In this study, overexpression of *ldhA* gene and knockout of *dhaT* and *yqhD* genes involved in 1,3-PDO synthesis were carried out in *K. pneumoniae* ATCC25955. The resultant strain showed much higher D-lactate production when compared with the wild-type strain. Under microaerobic conditions, the engineered strain produced 142.1 g/L D-lactate with a yield of 0.82 g/g glycerol, representing the highest D-lactate production from glycerol so far.

## 2. Methods

### 2.1. Strains and growth conditions

Bacterial strains, plasmids, and primers are listed in Table 1. *Escherichia coli* DH5α was used as the host to construct and store recombinant plasmids, and *E. coli* χ7213 was used for preparation of suicide vectors. *K. pneumoniae* ATCC25955 was used for D-lactate production. Bacteria were grown at 37 °C in Luria-Bertani (LB) broth unless specified. Pure glycerol was used for D-lactate production unless specified. Diaminopimelic acid (DAP) (50 mg/ml) was used for the growth of χ7213 strain. When necessary, antibiotics were added at final concentration of 50 mg/ml for kanamycin and 34 mg/ml for chloramphenicol. LB agar containing 10% sucrose was used for selection in allelic exchange experiments.

### 2.2. Development of K. pneumoniae mutant strains

The mutations were constructed using suicide vector pRE112 as previously described (Edwards et al., 1998). For the *dhaT* deletion, PCR amplification of approximately 500-bp fragments upstream and downstream of this gene was carried out using the appropriate primers, respectively (Table 1). These two fragments were ligated using overlapping PCR to generate an engineered deletion segment, which was cloned into the pRE112 vector to generate plasmid pRE112-Δ*dhaT*. The Δ*dhaT* mutation introduced into *K. pneumoniae* by allelic exchange using suicide vector pRE112-Δ*dhaT*, and the resultant strain named as Q1189. A similar strategy was used to introduce *yqhD* mutation using suicide vector pRE112-Δ*yqhD* into Q1189 strain, and the double mutant strain was named as Q1533. Those strains were confirmed by PCR and DNA sequencing.

### 2.3. ldhA Cloning

The plasmid pBAD18-*ldhA* was constructed using PCR fragments containing the *ldhA* gene generated with primers 738 and 739 and *K. pneumoniae* chromosomal DNA as a template, which were digested with *Sac*I and *Xba*I and then ligated with pBAD18-kan digested by the same enzymes.

### 2.4. Shake flask cultivation

For D-lactate production in shake flask cultivation and fed-batch fermentation, a modified minimal medium was used, which contained the following components (per liter): glycerol, 20 g; citric acid, 0.42 g; $KH_2PO_4$, 2 g; $KH_2PO_4$, 1.6 g; $NH_4Cl$, 5.4 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; and 1 mL of trace elements solution ($FeCl_3{\cdot}6H_2O$, 5 g; $MnCl_2{\cdot}4H_2O$, 2 g; $ZnCl_2$, 0.684 g; $CoCl_2{\cdot}6H2O$, 0.476 g; $CuCl_2{\cdot}2H_2O$, 0.17 g; $H_3BO_3$, 0.062 g; $Na_2MoO_4{\cdot}2H_2O$, 0.005 g; and 10 mL of concentrated HCl per liter).

**Table 1**
Bacterial strains, plasmids, and primers used in this study.

| Strain, plasmid and primers | Description | Source |
|---|---|---|
| *Strains* | | |
| *E. coli* DH5α | Cloning host | Lab collection |
| *E. coli* χ7213 | Host strain for pRE112, DAP auxotrophic strain | Roland et al. (1999) |
| Q1188 | *K. pneumoniae* ATCC25955 | ATCC |
| Q1189 | *K. pneumoniae* ATCC25955 Δ*dhaT* | This study |
| Q1533 | *K. pneumoniae* ATCC25955 Δ*dhaT* Δ*yqhD* | This study |
| Q1904 | Q1188 pBAD18-*ldhA* | This study |
| Q1905 | Q1189 pBAD18-*ldhA* | This study |
| Q1906 | Q1533 pBAD18-*ldhA* | This study |
| *Plasmids* | | |
| pRE112 | Suicide vector, R6K origin, chloramphenicol resistant | Edwards et al. (1998) |
| pRE112-Δ*dhaT* | Suicide vector for construction of Δ*dhaT* mutant | This study |
| pRE112-Δ*yqhD* | Suicide vector for construction of Δ*yqhD* mutant | This study |
| pBAD18-kan | Cloning vector, pBR322 origin, *araBAD* promotor, kanamycin resistant | NBRP-*E. coli* at NIG |
| pBAD18-*ldhA* | pBAD18-kan carrying *ldhA* gene, kanamycin resistant | This study |
| **Primers** | **Sequence (5′–3′)** | **Restriction enzymes** |
| *ΔdhaT construction* | | |
| 400 | CGGGGTACCATGAGCTATCGTATGTTTG | KpnI |
| 401 | GATGAGGCGGATCGCCTGCGATCACAAACTTCACTTTG | |
| 402 | CAAAGTGAAGTTTGTGATCGCAGGCGATCCGCCTCATC | |
| 403 | GTCCGAGCTCTCAGAATGCCTGGCGGAAAATCG | SacI |
| 405 | GCATTATAACCTGAAGCGAG | |
| 406 | TACGCCTGGCGGTGAAAGCGAC | |
| *ΔyqhD construction* | | |
| 377 | ATGAGCTCTACAGCAGGCGGACGTCAGGC | SacI |
| 378 | GGCTCGATGCCGCCAAATTC | |
| 379 | GAATTTGGCGGCATCGAGCCGTATCGATGCCGCTATTGCC | |
| 380 | ATTCTAGATGGTACGCGGCGGCGGTGTC | XbaI |
| 381 | GGTGATGAACAGCTCATCGC | |
| 437 | GTCATCTGGCAGCGGTATCGT | |
| *ldhA cloning* | | |
| 738 | CGAGCTCTATAATCACTGGAGAAAAGTC | SacI |
| 739 | GCTCTAGAGAATCAGACGATGGCGTTCG | XbaI |

Shake flask cultivation was carried out in a 250-mL flask containing 100 mL medium at 37 °C in an orbital incubator shaker at 100 rpm. All the cultures were induced at 0.6 ~ 0.8 $OD_{650}$ with 0.1% (w/v) L-arabinose. The temperature was set at 30 °C after induction. The pH was maintained at 7.0 with ammonia every 12 h along with antibiotic supplemented. 20 g/L glycerol was added into the medium after 24 h. The samples were withdrawn to determine the D-lactate, by-products and glycerol. All shake experiments were carried out in triplicates. To evaluate the effect of organic nitrogen sources on D-lactate production, beef extract, peptone and yeast extract were added into the minimal medium at final concentration of 3 g/L, respectively.

### 2.5. Fed-batch fermentation

Fed-batch fermentation was carried out in a Biostat B plus MO5L fermentor (Sartorius Stedim Biotech GmbH, Germany) containing 3 L of medium as described above. During the fermentation process, pH was controlled automatically at 7.0 with ammonia. The expression of plasmid-borne gene was initiated at an $OD_{650}$ of 3 by adding 0.1% L-arabinose. After the initial carbon sources were nearly exhausted, fed-batch mode was commenced by feeding a solution containing 10 M glycerol at 15 mL/h. For microaerobic condition, the sterile air was sparged at 0.5, 1.0, 1.5, 2.0 and 2.5 vvm with an agitation speed of 400 rpm. For fully aerobic condition, the dissolved oxygen (DO) concentration was maintained at 20% saturation. Anaerobic condition was performed as previously described (Huang et al., 2012). For two-step process (fully aerobic-microaerobic), fully aerobic condition as described above was used until the cell concentration reached an $OD_{650}$ of 30, followed by a microaerobic phase (2.5 vvm). Samples were withdrawn every 4 h to determine cell mass and metabolites concentration.

### 2.6. Analytical methods

Biomass was determined by measuring the optical density of samples at 650 nm with a UV visible spectroscopy system (Varian Cary 50 Bio, US). The value of the density was converted to cell dry weight (CDW) based on that one unit of $OD_{650}$ was equivalent to 0.284 g CDW/L. The substrate and metabolites including glycerol, D-lactate, 1,3-PDO, ethanol, acetate, succinate and 3-hydroxypropionate, were determined by HPLC (Huang et al., 2012). The optical purity of D-lactate was measured with a chiral column and a UV detector (Zou et al., 2013). Carbon distribution was calculated based on the carbon of total glycerol consumption and that contained in the end products. The fraction for cell growth was determined assuming the elemental composition of $C_4H_7O_2N$ with an ash content of 3.6% (Huang et al., 2013).

## 3. Results and discussion

### 3.1. Strain construction and characterization

The strain *K. pneumoniae* ATCC 25955 is able to produce optically pure D-lactate, and no L-lactate was detected in culture of this strain, indicating high enzyme specificity of D-lactate dehydrogenase of *K. pneumoniae* strain. To improve the D-lactate production, D-lactate dehydrogenase gene *ldhA* was amplified by PCR and cloned into vector pBAD18-kan to generate plasmid pBAD18-*ldhA*, which was tested in *K. pneumoniae* strain along with empty vector. With L-arabinose induction, LdhA was observed as a distinct band with the expected molecular weight on SDS–PAGE, demonstrating that this protein was properly expressed in recombinant *K. pneumoniae* strain.

The D-lactate production of *K. pneumoniae* strains carrying empty vector or pBAD18-*ldhA* was tested using a modified minimal medium under shake flask condition. Only 0.41 ± 0.01 g/L D-lactate was produced by the control strain, and LdhA overexpression increased the D-lactate production significantly to 2.16 ± 0.04 g/L. However, K. *pneumoniae* strains accumulated 1,3-PDO as a principle product when using glycerol as carbon source, and the strains carrying empty vector and pBAD18-*lahA* produced 9.58 ± 0.01 and 7.96 ± 0.19 g/L 1,3-PDO, respectively.

To further improve the D-lactate biosynthesis, genes associated with 1,3-PDO synthesis, *dhaT* and *yqhD*, were knocked out using suicide vector pRE112. After 48-h cultivation, the *dhaT* mutant strain Q1905 produced 4.11 ± 0.08 g/L D-lactate and 5.07 ± 0.15 g/L 1,3-PDO, and the *dhaT yqhD* double mutant strain Q1906 accumulated 4.72 ± 0.03 g/L D-lactate and 4.05 ± 0.27 g/L 1,3-PDO. Deletion of *dhaT* and *yqhD* genes did enhance the D-lactate biosynthesis, however there was still a considerable amount of 1,3-PDO produced by strain with deletion of *dhaT* and *yqhD* genes. This result was consistent with previous research (Wang et al., 2003; Ashok et al., 2013), indicating that unknown 1,3-PDO oxidoreductase(s) may be exist in *K. pneumoniae*. The *dhaT yqhD* double mutant strain carrying pBAD18-*ldhA* was used for D-lactate production in the following study.

### 3.2. Effect of organic nitrogen on the production of D-lactate

The composition of culture media plays an important role in the microbial production of desired products. To improve the D-lactate yield, organic nitrogen source was optimized in this study. Beef extract, peptone, and yeast extract were added into the minimal medium at a final concentration of 3 g/L, respectively, and the D-lactate productions were compared. The D-lactate concentration increased dramatically when organic nitrogen source was used. Addition of beef extract increased D-lactate production to 5.47 ± 0.03 g/L, and peptone supplement resulted in production of 7.44 ± 0.07 g/L D-lactate. Especially, 8.33 ± 0.09 g/L D-lactate was produced with addition of yeast extract, about 1.8 times higher than that in the reference experiment with $NH_4Cl$ as the sole nitrogen source (4.72 ± 0.03 g/L).

### 3.3. Fed-batch fermentations at different aeration rates

To investigate the effect of aeration on the production of D-lactate, fed-batch fermentations were conducted at different conditions including anaerobic, microaerobic (with various aeration rates of 0.5, 1.0, 1.5, 2.0 and 2.5 vvm), and fully aerobic conditions. As shown in Fig. 1, there was no significant difference of cell mass observed when under anaerobic and microaerobic conditions, and the peak biomass were less than 5 g/L. Further enhancement of aeration improved the growth rate and cell mass dramatically. The maximum cell mass was 10.08 g/L under fully aerobic conditions (DO $\geqslant$ 20%). In all experiments, the cell mass decreased slightly at late stationary phase.

The aeration condition played an important role in the D-lactate production. Under anaerobic conditions, D-lactate production was 47.33 g/L with a yield of 0.25 g/g glycerol. With increasing aeration rates, the D-lactate production was remarkably improved and reached the maximum of 142.11 g/L with a yield of 0.82 g/g glycerol at 2.5 vvm (Table 2 and Fig. 1). However under well-oxygenated conditions, the glycerol consumption was low and only 2.20 g/L D-lactate was formed after 48 h fermentation (Table 2 and Fig. 1), probably correlated with the low D-lactate dehydrogenase activity under fully aerobic condition (Tarmy and Kaplan, 1968).

Similarly to the microbial conversion of glycerol to 1,3-PDO, a two-step fermentation, comprising an initial aerobic biomass

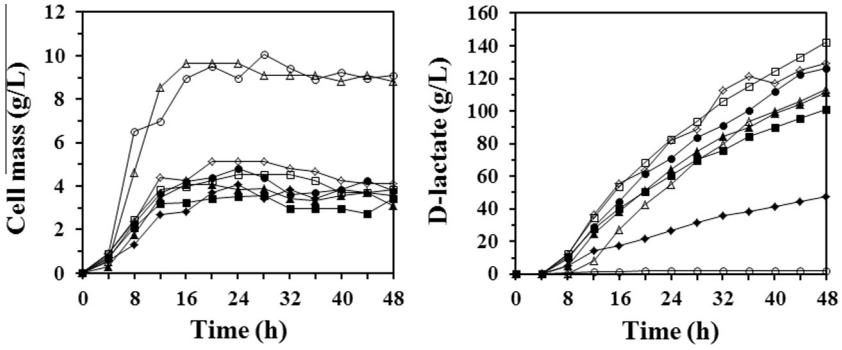


**Fig. 1.** Cell mass and D-lactate production in fed-batch fermentation at different aeration rates in a 5-L bioreactor. Symbols: (♦) anaerobic; (■) 0.5 vvm; (▲) 1.0 vvm; (●) 1.5 vvm; (◇) 2.0 vvm; (□) 2.5 vvm; (△) two-step; (○) aerobic.

**Table 2**
Comparison of glycerol consumption, biomass formation and metabolites production at different aeration rates.

| | Anaerobic | Microaerobic | | | | | Aerobic DO ⩾ 20% | Two-step |
|---|---|---|---|---|---|---|---|---|
| | | 0.5 vvm | 1.0 vvm | 1.5 vvm | 2.0 vvm | 2.5 vvm | | |
| Glycerol consumed (g/L) | 189.46 | 144.44 | 168.90 | 156.58 | 152.30 | 173.91 | 77.00 | 149.58 |
| Biomass (g/L) | 3.58 | 3.41 | 3.12 | 3.78 | 4.12 | 3.83 | 10.08 | 9.66 |
| D-Lactate (g/L) | 47.33 | 101.25 | 111.62 | 126.30 | 129.31 | 142.11 | 2.20 | 113.23 |
| Yield of lactate (g/g) | 0.25 | 0.70 | 0.66 | 0.81 | 0.85 | 0.82 | 0.03 | 0.76 |
| Lactate volumetric productivity (g/L/h) | 0.99 | 2.11 | 2.33 | 2.63 | 2.69 | 2.96 | 0.05 | 2.36 |
| 1,3-PDO (g/L) | 31.49 | 16.8 | 13.83 | 12.33 | 11.97 | 13.19 | ND[a] | 1.99 |
| Acetate (g/L) | 0.27 | 0.30 | 0.24 | 1.3 | 0.5 | 2.5 | 5.82 | 1.73 |
| Ethanol (g/L) | 12.6 | 4.15 | 3.40 | 3.42 | 3.46 | 2.66 | 2.65 | 2.15 |
| Succinate (g/L) | 2.89 | 2.39 | 1.81 | 1.55 | 1.93 | 1.90 | 0.41 | 1.31 |
| 3-HP (g/L) | 1.00 | 0.75 | 1.80 | 1.09 | ND | ND | ND | ND |

[a] ND, not detected.

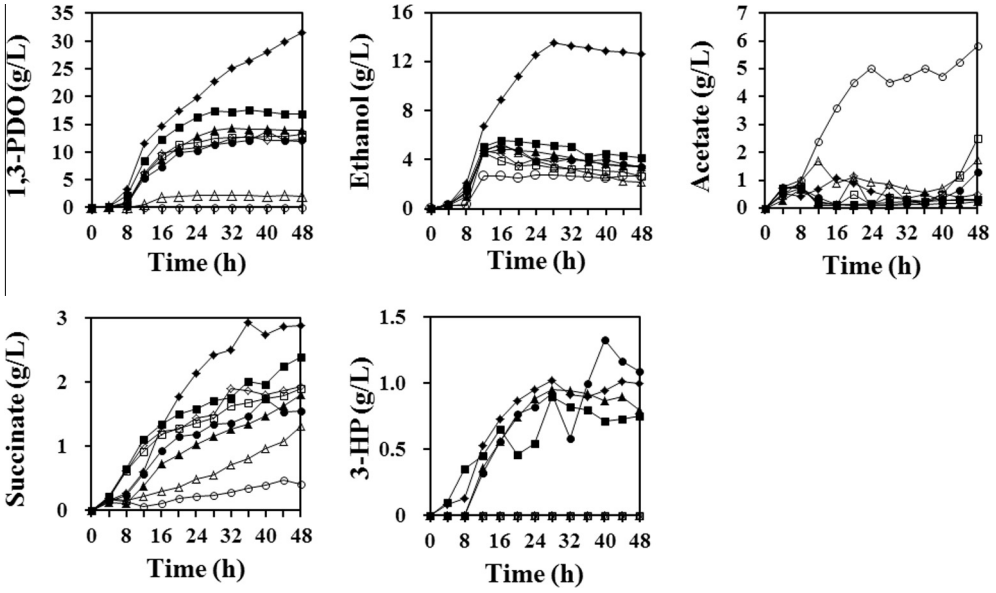


**Fig. 2.** By-products produced in fed-batch fermentation at different aeration rates in a 5-L bioreactor. Symbols: (♦) anaerobic; (■) 0.5 vvm; (▲) 1.0 vvm; (●) 1.5 vvm; (◇) 2.0 vvm; (□) 2.5 vvm; (△) two-step; (○) aerobic.

formation step followed by a microaerobic D-lactate synthesis step at 2.5 vvm, was also carried out. Compared with the cultures under anaerobic and microaerobic conditions, the cells grew much better and the peak biomass reached 9.66 g/L. After 48-h fermentation, 113.23 g/L of D-lactate was produced with a yield of 0.76 g/g glycerol (Table 2 and Fig. 1). Although much higher than those under aerobic conditions, the D-lactate production and yield were lower than those under microaerobic conditions at 2.5 vvm.

As shown in Table 2, the D-lactate yield and productivity varied with the degree of oxygen availability. The yield exhibited a similar trend with the production of D-lactate, and the maximum yield 0.85 g/g glycerol was reached at the aeration of 2.0 vvm. The

highest overall volumetric productivity was 2.96 g/L/h, achieved at the aeration rate of 2.5 vvm and higher than previous reports.

The profiles of by-products are shown in Fig. 2. Under anaerobic conditions, the major by-products are 1,3-PDO and ethanol, with the concentrations of 31.49 and 12.60 g/L, respectively. With increasing oxygen availability, production of all these by-products decreased dramatically. The reduced 1,3-PDO production was assumed to be related with the decreased activity of glycerol dehydratase, which converts glycerol into 3-hydroxypropionaldehyde as precursor of 1,3-PDO and is sensitive to oxygen.

Increased oxygen availability also led to enhanced acetate accumulation. The acetate concentration reached 2.50 and 5.82 g/L at 2.5 vvm and fully aerobic condition, respectively. Along with acetate production, the reaction catalyzed by acetate kinase also provides ATP, which will benefit biomass synthesis and could contribute to better cell growth. Succinate and 3HP were also detected under anaerobic conditions with the concentration of 2.89 and 1.00 g/L respectively, and showed similar changing tendency with 1,3-PDO and ethanol under various degrees of oxygen accessibility. The productions of all those by-products were also presented in Table 2.

### 3.4. Redox balance and carbon distribution under microaerobic conditions

In bacteria cells, glycerol was converted into phosphoenolpyruvate (PEP), which enters glycolysis and TCA cycle, providing energy and precursors for biosynthesis. The transformation of glycerol to each mol of PEP generates 2 mol of NADH, which has to be re-oxidized for this process to continue. Under microaerobic conditions, NADH is partially oxidized via the respiratory chain, dependent on oxygen availability, and the rest is consumed by formation of some metabolites such as lactate, ethanol and succinate. Each mol of succinate and ethanol formed from PEP consumes 2 mol of NADH, while one mol of NADH is oxidized with lactate formation. Besides that, one mol of NADH is produced during the conversion of glycerol into 3-HP, and one mol of NADH is consumed for 1,3-PDO production. In this study, the redox state (Table 3) was calculated based on the pathways and stoichiometric relationships for each metabolite produced from glycerol, and the unbalanced NADH was assumed to be oxidized by respiration and hydrogen formation.

Under anaerobic conditions, about 90% of produced NADH was used for the production of lactate, 1,3-PDO, ethanol and succinate. However at 2.5 vvm, about half of produced NADH was oxidized for the production of those products. With elevated aeration rate, the production of reduced products 1,3-PDO, ethanol and succinate decreased significantly probably due to the inactivation of related enzymes by oxygen. However, the production of D-lactate increased, consistent with previous report (Huang et al., 2013). This result indicates that the lactate dehydrogenase has higher oxygen tolerance and the formation of D-lactate is a suitable pathway to re-oxidize NADH under microaerobic conditions.

With increased oxygen accessibility, more NADH entered oxidative phosphorylation pathway, resulting in increased ATP formation, which could contribute to better cell growth. Given that *K. pneumoniae* achieved the maximum hydrogen yield under microaerobic conditions (Chen et al., 2003), formation of hydrogen could be additional way to balance the produced NADH.

To further understand the metabolism in the engineered strain, the carbon distribution (Table 4) was determined under various aeration conditions. As shown in Table 2, the biomass formation increased with aeration, consistent with previous report (Huang et al., 2013), in which the *K. pneumoniae* biomass was 2.2-times higher at 1.5 vvm than that under anaerobic conditions. In this study, the total carbon recovery was only 59.0% under anaerobic

**Table 3**
Balance analysis of NADH in fed-batch fermentations under different conditions.

| Metabolites | Anaerobic | | Microaerobic | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0.5 vvm | | 1.0 vvm | | 1.5 vvm | | 2.0 vvm | | 2.5 vvm | |
| | Produced (mmol) | Consumed (mmol) | Produced (mmol) | Consumed (mmol) | Produced (mmol) | Consumed (mmol) | Produced (mmol) | Consumed (mmol) | Produced (mmol) | Consumed (mmol) | Produced (mmol) | Consumed (mmol) |
| Biomass | 102.4 | 0 | 97.5 | 0 | 89.4 | 0 | 108.0 | 0 | 117.8 | 0 | 109.7 | 0 |
| D-Lactate | 3152.5 | 1576.3 | 6744.0 | 3372.0 | 7434.7 | 3717.4 | 8412.2 | 4206.1 | 8613.0 | 4306.5 | 9465.6 | 4732.8 |
| 1,3-PDO | 0 | 1241.4 | 0 | 662.3 | 0 | 545.2 | 0 | 486.1 | 0 | 471.9 | 0 | 520.0 |
| Ethanol | 1641.0 | 1641.0 | 540.5 | 540.5 | 442.8 | 442.8 | 445.4 | 445.4 | 450.6 | 450.6 | 346.4 | 346.4 |
| Acetate | 27.0 | 0 | 30.0 | 0 | 24.0 | 0 | 129.9 | 0 | 50.0 | 0 | 249.8 | 0 |
| Succinate | 146.8 | 146.8 | 121.4 | 121.4 | 92.0 | 92.0 | 78.8 | 78.8 | 98.1 | 98.1 | 96.5 | 96.5 |
| 3-HP | 33.3 | 0 | 25.0 | 0 | 26.6 | 0 | 36.3 | 0 | 0 | 0 | 0 | 0 |
| Total | 5103.0 | 4605.5 | 7558.4 | 4696.2 | 8109.5 | 4797.3 | 9210.6 | 5216.3 | 9329.4 | 5327.1 | 10268.0 | 5695.7 |

**Table 4**
Carbon balance in fed-batch fermentations under different conditions.

| Substrate or metabolites | Anaerobic (mmol) | Microaerobic | | | | |
|---|---|---|---|---|---|---|
| | | 0.5 vvm (mmol) | 1.0 vvm (mmol) | 1.5 vvm (mmol) | 2.0 vvm (mmol) | 2.5 vvm (mmol) |
| Glycerol | 18516.0 | 14116.2 | 16506.7 | 15302.6 | 14884.4 | 16996.6 |
| Biomass | 409.45 | 389.95 | 357.45 | 432.19 | 471.19 | 438.69 |
| D-Lactate | 4728.8 | 10116.0 | 11152.1 | 12618.3 | 12919.5 | 14198.4 |
| 1,3-PDO | 3724.2 | 1986.9 | 1635.6 | 1458.2 | 1415.6 | 1559.9 |
| Ethanol | 1641.0 | 540.5 | 442.8 | 445.4 | 450.6 | 346.4 |
| Acetate | 27.0 | 30.0 | 24.0 | 129.9 | 50.0 | 249.8 |
| Succinate | 293.7 | 242.9 | 183.9 | 157.5 | 196.1 | 193.1 |
| 3-HP | 99.9 | 74.9 | 79.9 | 108.9 | 0 | 0 |
| Carbon recovery (%) | 59.0 | 94.8 | 84.1 | 100.3 | 104.2 | 99.9 |

**Table 5**
Comparison of D-lactate production by different strains.

| Carbon source | Strains | Relevant genotype | D-Lactate production (g/L) | Yield (g/g glycerol) | Volumetric productivity (g/L/h) | Optical purity (%) | References |
|---|---|---|---|---|---|---|---|
| Glycerol | *K. pneumoniae* | Δ*dhaT* Δ*yqhD* pBAD18-*ldhA* | 142.1 | 0.82 | 2.96 | ~100 | This study |
| Glycerol | *E. coli* | Δ*ack-pta* Δ*pps* Δ*pflB* Δ*dld* Δ*poxB* Δ*adhE* Δ*frdA* pUC-*ldhA* | 100.3 | 0.75 | 2.78 | 99.97 | Chen et al. (2014) |
| Glycerol | *K. pneumoniae* | – | 33.4 | 0.19 | 1.04 | NA[a] | Song et al. (2013) |
| Glycerol | *K. pneumoniae* | – | 59.0 | 0.47 | 1.13 | NA | Rossi et al. (2013) |
| Glycerol | *K. pneumoniae* | Δ*budC* pKP-*ldhA* | 68.4 | 0.78 | 1.22 | NA | Wang et al. (2013) |
| glycerol | *E. coli* | Δ*pta* Δ*adhE* Δ*frdA* Δ*dld* pZS*glpKglpD* | 32.0 | 0.80 | 0.54 | 99.9 | Mazumdar et al. (2010) |
| Glycerol | *E. coli* | Δ*pflA* | NA | 0.61 | NA | NA | Zhu and Shimizu (2004) |
| Fresh sweet potato | *Lactobacillus coryniformis* | – | 186.4 | 0.85 | 3.11 | NA | Nguyen et al. (2013) |
| Raw corn starch | *Lactobacillus plantarum* | Δ*LdhL1*::*amyA* | 73.2 | 0.73 | 1.53 | 99.6 | Okano et al. (2009) |
| Glucose | *Corynebacterium glutamicum* | Δ*ldhL*, *ldhA* | 120 | 0.86 | 4.0 | 99.9 | Okino et al. (2008) |
| Glucose | *Saccharomyces cerevisiae* | Δ*pdC1*, *ldhA* | 61.5 | 0.61 | 0.85 | 99.9 | Ishida et al. (2006) |
| Glucose | *E. coli* | Δ*ack*, Δ*pps*, Δ*pflB*, Δ*dld*, Δ*poxB*, Δ*adhE*, Δ*frdA* | 125 | 0.19 | 0.61 | NA | Zhou et al. (2011) |

[a] Not available.

conditions, probably due to the highest production of 1,3-PDO and ethanol. Part of 1,3-PDO and ethanol is synthesized using NADPH-dependent enzymes, and NADPH is mainly produced through oxidative pentose phosphate (PP) pathway along with $CO_2$ emission, resulting in the low carbon recovery. With the increase in aeration, more D-lactate and NADH were formed, and less 1,3-PDO and ethanol were produced. Taken together that NADPH can be synthesized from $NADP^+$ and NADH by transhydrogenase PntAB (Clarke and Bragg, 1985), the flow through PP pathway and $CO_2$ emission were reduced, and then the carbon recovery was enhanced accordingly. At 2.0 vvm, the maximum carbon recovery of 104.2% was achieved. Because of use of yeast extract, the glycerol requirement for growth was reduced, and the real carbon recovery should be lower than the calculated value.

### 3.5. D-Lactate production from crude glycerol

To evaluate the feasibility of the engineered strain to synthesize D-lactate from crude glycerol from biodiesel production, the D-lactate production experiment in shake flasks was performed using crude glycerol, which contains 73% glycerol, 2% fatty acids and 25% water, as sole carbon source. When pure glycerol was used, the recombinant strain consumed 10.51 ± 0.40 g/L glycerol, and produced 0.99 ± 0.10 g/L biomass and 8.15 ± 0.25 g/L D-lactate after 48-h cultivation. The glycerol consumption, biomass, and D-lactate production were 9.52, 0.97, and 7.41 g/L, respectively, when crude glycerol was used. The D-lactate produced from crude glycerol was optically pure, and no L-lactate was detected in culture. Those results suggest that crude glycerol could be directly converted to D-lactate without any purification and the constructed strain has high potentiality in utilization of crude glycerol from biodiesel industry.

Some studies of D-lactate biosynthesis were summarized in Table 5. In early stage, readily usable carbohydrates, such as glucose and sucrose, were used as carbon source. To achieve lower production cost of D-lactate, inexpensive carbon sources have attracted growing interest. As a by-product of biodiesel production, glycerol has become an inexpensive and abundant carbon source for microbial fermentation. Several attempts to synthesize D-lactate from glycerol were reported. Compared with previous reports, the strain constructed in this study showed higher enzyme substrate specificity and no L-lactate was detected in the culture. Furthermore, this engineered *K. pneumoniae* strain also represented the highest D-lactate production, yield and volumetric productivity from glycerol so far. In respect of utilization of inexpensive carbon sources, the most inspiring result was reported recently (Nguyen et al., 2013). *Lactobacillus coryniformis* strain produced 186.4 g/L D-lactate using fresh sweet potato through simultaneous saccharification and fermentation.

## 4. Conclusions

In this study, *K. pneumoniae* strain was metabolically engineered and successfully applied in D-lactate production from glycerol. Overexpression of lactate dehydrogenase LdhA and knockout of genes associated with 1,3-PDO synthesis significantly improved the D-lactate production. The fermentation condition was optimized and aeration rate played an essential role in D-lactate accumulation. Under microaerobic conditions with 2.5 vvm, the recombinant strain produced 142.1 g/L D-lactate with a yield of 0.82 g/g glycerol, representing the highest D-lactate production and yield from glycerol so far. This study also proved the feasibility to produce D-lactate from crude glycerol from biodiesel industry.

## Acknowledgements

This research was financially supported by the 100-Talent Project of CAS (for GZ) and Natural Science Foundation of Shandong Province (ZR2013EMZ002). We acknowledge Dr. Roy Curtiss III (Arizona State University) for strain χ7213 and pRE112, Dr. Xuebing Li (QIBEBT, CAS) for crude glycerol, and NBRP-*E. coli* at NIG for the clone vector pBAD18-kan.

## References

Andreeßen, B., Lange, A.B., Robenek, H., Steinbüchel, A., 2010. Conversion of glycerol to poly(3-hydroxypropionate) in recombinant *Escherichia coli*. Appl. Environ. Microbiol. 76, 622–626.

Ashok, S., Sankaranarayanan, M., Ko, Y., Jae, K.E., Ainala, S.K., Kumar, V., Park, S., 2013. Production of 3-hydroxypropionic acid from glycerol by recombinant

*Klebsiella pneumoniae* Δ*dhaT*Δ*yqhD* which can produce vitamin B12 naturally. Biotechnol. Bioeng. 110, 511–524.
Blankschien, M.D., Clomburg, J.M., Gonzalez, R., 2010. Metabolic engineering of *Escherichia coli* for the production of succinate from glycerol. Metab. Eng. 12, 409–419.
Chen, X., Xiu, Z.L., Wang, J.F., Zhang, D.J., Xu, P., 2003. Stoichiometric analysis and experimental investigation of glycerol bioconversion to 1,3-propanediol by *Klebsiella pneumonia*e under microaerobic conditions. Enzyme Microb. Technol. 33, 386–394.
Chen, X., Tian, K., Niu, D., Shen, W., Algasan, G., Singh, S., Wang, Z., 2014. Efficient bioconversion of crude glycerol from biodiesel to optically pure D-lactate by metabolically engineered *Escherichia coli*. Green Chem. 16, 342–350.
Clarke, D.N., Bragg, P.D., 1985. Cloning and expression of the transhydrogenase gene of *Escherichia coli*. J. Bacteriol. 162, 367–373.
Durgapal, M., Kumar, V., Yang, T.H., Lee, H.J., Seung, D., Park, S., 2014. Production of 1,3-propanediol from glycerol using the newly isolated *Klebsiella pneumoniae* J2B. Bioresour. Technol. 159, 223–231.
Edwards, R.A., Keller, L.H., Schifferli, D.M., 1998. Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. Gene 207, 149–157.
Fukushima, K., Chang, Y.H., Kimura, Y., 2007. Enhanced stereocomplex formation of poly(L-lactic acid) and poly(D-lactic acid) in the presence of stereoblock poly(lactic acid). Macromol. Biosci. 7, 829–835.
Huang, Y., Li, Z., Shimizu, K., Ye, Q., 2012. Simultaneous production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol by a recombinant strain of *Klebsiella pneumoniae*. Bioresour. Technol. 103, 351–359.
Huang, Y., Li, Z., Shimizu, K., Ye, Q., 2013. Co-production of 3-hydroxypropionic acid and 1,3-propanediol by *Klebseilla pneumoniae* expressing *aldH* under microaerobic conditions. Bioresour. Technol. 128, 505–512.
Ishida, N., Suzuki, T., Tokuhiro, K., Nagamori, E., Onishi, T., Saitoh, S., Kitamoto, K., Takahashi, H., 2006. D-lactic acid production by metabolically engineered *Saccharomyces cerevisiae*. J. Biosci. Bioeng. 101, 172–177.
Jung, W.S., Kang, J.H., Chu, H.S., Choi, I.S., Cho, K.S., 2014. Elevated production of 3-hydroxypropionic acid by metabolic engineering of the glycerol metabolism in *Escherichia coli*. Metab. Eng. 23, 116–122.
Kumar, V., Sankaranarayanan, M., Durgapal, M., Zhou, S., Ko, Y., Ashok, S., Sarkar, R., Park, S., 2013. Simultaneous production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol using resting cells of the lactate dehydrogenase-deficient recombinant *Klebsiella pneumoniae* overexpressing an aldehyde dehydrogenase. Bioresour. Technol. 135, 555–563.
Kwak, S., Park, Y., Seo, J., 2013. Biosynthesis of 3-hydroxypropionic acid from glycerol in recombinant *Escherichia coli* expressing *Lactobacillus brevis dhaB* and *dhaR* gene clusters and *E. coli* K-12 *aldH*. Bioresour. Technol. 135, 432–439.
Luo, L.H., Kim, C.H., Heo, S., Oh, B., Hong, W., Kim, S., Kim, D., Seo, J., 2012. Production of 3-hydroxypropionic acid through propionaldehyde dehydrogenase PduP mediated biosynthetic pathway in *Klebsiella pneumoniae*. Bioresour. Technol. 103, 1–6.
Mazumdar, S., Clomburg, J.M., Gonzalez, R., 2010. *Escherichia coli* strains engineered for homofermentative production of D-lactic acid from glycerol. Appl. Environ. Microbiol. 76, 4327–4336.
Menzel, K., Zeng, A.P., Deckwer, W.D., 1997. High concentration and productivity of 1,3-propanediol from continuous fermentation of glycerol by *Klebsiella pneumoniae*. Enzyme Microb. Technol. 20, 82–86.
Nguyen, C.M., Choi, G.J., Choi, Y.H., Jang, K.S., Kim, J.C., 2013. D- and L-lactic acid production from fresh sweet potato through simultaneous saccharification and fermentation. Biochem. Eng. J. 81, 40–46.
Okano, K., Zhang, Q., Shinkawa, S., Yoshida, S., Tanaka, T., Fukuda, H., Kondo, A., 2009. Efficient production of optically pure D-lactic acid from raw corn starch by using a genetically modified L-lactate dehydrogenase gene-deficient and α-amylase-secreting *Lactobacillus plantarum* strain. Appl. Environ. Microbiol. 75, 462–467.
Okino, S., Suda, M., Fujikura, K., Inui, M., Yukawa, H., 2008. Production of D-lactic acid by *Corynebacterium glutamicum* under oxygen deprivation. Appl. Microbiol. Biotechnol. 78, 449–454.
Posada, J.A., Cardona, C.A., Gonzalez, R., 2012. Analysis of the production process of optically pure D-lactic acid from raw glycerol using engineered *Escherichia coli* strains. Appl. Biochem. Biotechnol. 166, 680–699.
Roland, K., Curtiss, R., Sizemore, D., 1999. Construction and evaluation of a Δ*cya* Δ*crp Salmonella typhimurium* strain expressing avian pathogenic *Escherichia coli* O78 LPS as a vaccine to prevent airsacculitis in chickens. Avian Dis. 43, 429–441.
Rossi, D.M., de Souza, E.A., Ayub, M.A., 2013. Biodiesel residual glycerol metabolism by *Klebsiella pneumoniae*: pool of metabolites under anaerobiosis and oxygen limitation as a function of feeding rates. Appl. Biochem. Biotechnol. 169, 1952–1964.
Seo, J.W., Seo, M.Y., Oh, B.R., Heo, S.Y., Baek, J.O., Rairakhwada, D., Luo, L.H., Hong, W.K., Kim, C.H., 2010. Identification and utilization of a 1,3-propanediol oxidoreductase isoenzyme for production of 1,3-propanediol from glycerol in *Klebsiella pneumoniae*. Appl. Microbiol. Biotechnol. 85, 659–666.
Song, Z., Sun, Y., Wei, B., Xiu, Z., 2013. Two-step salting-out extraction of 1,3-propanediol and lactic acid from the fermentation broth of *Klebsiella pneumoniae* on biodiesel-derived crude glycerol. Eng. Life Sci. 13, 487–495.
Tarmy, E.M., Kaplan, N.O., 1968. Chemical characterization of D-lactate dehydrogenase from *Escherichia coli* B. J. Biol. Chem. 243, 2579–2586.
Wang, F.M., Meng, Q.Q., 2012. Effects of *budC* gene knockout and *ldhA* overexpression on D-lactic acid production by *Klebsiella pneumoniae*. J. Beijing Univ. Chem. Technol. (Natl. Sci.) 39, 84–89.
Wang, W., Sun, J., Hartlep, M., Deckwer, W.D., Zeng, A.P., 2003. Combined use of proteomic analysis and enzyme activity assays for metabolic pathway analysis of glycerol fermentation by *Klebsiella pneumoniae*. Biotechnol. Bioeng. 83, 525–536.
Wang, Q., Yang, P., Liu, C., Xue, Y., Xian, M., Zhao, G., 2013. Biosynthesis of poly(3-hydroxypropionate) from glycerol by recombinant *Escherichia coli*. Bioresour. Technol. 138, 548–551.
Zhou, L., Zuo, Z.R., Chen, X.Z., Niu, D.D., Tian, K.M., Prior, B.A., Shen, W., Shi, G.Y., Singh, S., Wang, Z.X., 2011. Evaluation of genetic manipulation strategies on D-Lactate production by *Escherichia coli*. Curr. Microbiol. 62, 981–989.
Zhu, J., Shimizu, K., 2004. The effect of *pfl* gene knockout on the metabolism for optically pure D-lactate production by *Escherichia coli*. Appl. Microbiol. Biotechnol. 64, 367–375.
Zou, H., Wu, Z., Xian, M., Liu, H., Cheng, T., Cao, Y., 2013. Not only osmoprotectant: betaine increased lactate dehydrogenase activity and L-lactate production in *lactobacilli*. Bioresour. Technol. 148, 591–595.