

Full Length Research Paper

High-efficiency conversion of glycerol to D-lactic acid with metabolically engineered *Escherichia coli*

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***Escherichia coli* strain CICIM B0013 was genetically engineered to efficiently produce optically pure D-lactate (higher than 99.9%) from glycerol with a minimum of by-products. When *E. coli* B0013-070 ($\Delta ackA$, Δpta , Δpps , $\Delta pflB$, Δdld , $\Delta poxB$, $\Delta adhE$, $\Delta frdA$) was cultivated aerobically for 9 h followed by 27 h under microaerobic fermentation, it produced 98.5 g l⁻¹ of D-lactate with no more than 2 g l⁻¹ total by-products from glycerol. During the microaerobic phase, the average D-lactate productivity and yield were 3.45 g l⁻¹ h⁻¹ and 64 g/100 g glycerol, respectively. Elevated expression of the lactate dehydrogenase gene (*ldhA*) in strain B0013-070 improved conversion of glycerol to D-lactate resulting in a yield and productivity of 78 g/100 g glycerol and 3.65 g l⁻¹ h⁻¹, respectively. The metabolically engineered *E. coli* strain B0013-070 (pTH-*ldhA*) efficiently converted glycerol to D-lactate with a 2.1-fold higher D-lactate productivity than previously reported. It is concluded that overexpression of *ldhA* at an appropriate level is important for the balance between cell growth and D-lactate synthesis. Furthermore, biodiesel-based glycerol can be an appropriate substrate for industrial scale D-lactate production.**

Key words: D-Lactate, glycerol, *Escherichia coli*, metabolic engineering, overexpression of *ldhA*.

INTRODUCTION

Currently, glycerol has become an inexpensive and an abundant carbon source due to its generation as a by-product by approximately 10% of the total biodiesel fuel production process (Durnin et al., 2009; Hong et al., 2009). It has been used for the production of many valuable chemicals such as 1,3-propanediol, dihydroxyacetone, ethanol and succinate (Yazdani and Gonzalez, 2008; Zhang et al., 2010). Lactic acid is another valuable chemical which could be largely produced from crude glycerol (Hong et al., 2009; Mazumdar et al., 2010). If lactate production could be integrated into existing

biodiesel production facilities, then the cost of lactate and biodiesel would be expected to decrease and will assist in reducing the large amount of glycerol by-product from the biodiesel manufacturing process.

When compared with other lactic acid producers, the simple nutritional requirements and rapid growth with high product yield by *Escherichia coli* are the desirable properties for an industrial process. Furthermore, the ability to ferment glycerol to D-lactate and the ease of genetic modification might make *E. coli* to be the most suitable strain for industrial production of D-lactate in the future (Hong et al., 2009). Another advantage is that D-lactate could be produced from glycerol by *E. coli* at higher yield than from common sugars due to the different average degree of reduction per carbon (Nielsen et al., 2003; Yazdani and Gonzalez, 2007).

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Table 1. *Escherichia coli* strains, plasmids and primers.

Strain	Relevant characteristics	Source
<i>E. coli</i> JM109	<i>endA1</i> , <i>recA1</i> , <i>gyrA96</i> , <i>thi1</i> , <i>hsdR17</i> (<i>rk</i> -, <i>mk</i> +) , <i>relA1</i> , <i>supE44</i> , Δ (<i>proAB</i> , <i>lac</i> , F'(<i>proAB</i> , <i>lac</i> ^f , <i>lacZ</i> Δ M15, <i>traD</i>	CICIM-CU
<i>E. coli</i> CICIM B0013	Wild type	CICIM-CU
<i>E. coli</i> CICIM B0013-070	B0013, Δ <i>ack-pta</i> , Δ <i>pps</i> , Δ <i>pf1B</i> , Δ <i>dld</i> , Δ <i>poxB</i> , Δ <i>adhE</i> , Δ <i>frdA</i>	This study
<i>E. coli</i> CICIM B0013-070(pTH- <i>ldhA</i>)	B0013-070 / pTH- <i>ldhA</i>	This study
<i>E. coli</i> CICIM B0013-070(pUC- <i>ldhA</i>)	B0013-070 / pUC- <i>ldhA</i>	This study
Plasmids		
pTH18Kr	Km ^r	Hashimoto-Gotoh et al., 2000
pTH- <i>ldhA</i>	pTH18Kr containing D-lactate dehydrogenase gene (<i>ldhA</i>), Km ^r	This study
pUC- <i>ldhA</i>	pUC-19 containing D-lactate dehydrogenase gene (<i>ldhA</i>), Ap ^r	This study
Primers		
LDHa1	5'-GGGCAGCCCGAGCGTCATCAG-3'	This study
LDHa2	5'-CTTGAATTCAAGCTTGCTGCCGGAATCATCATTITTTT-3'	This study

Two pathways were reported for glycerol metabolism in *E. coli*. After glycerol has entered the cell via the facilitated diffusion GlpF permease, the pathways involved in glycerol metabolism of *E. coli* diverge depending on the degree of aerobiosis. Under aerobic conditions, glycerol is initially phosphorylated using ATP as the phosphoryl donor (GlpK), and subsequently oxidized to dihydroxyacetone phosphate (DHAP) by GlpD (Durnin et al. 2009) with quinones as electron acceptors. In the absence of oxygen, glycerol is oxidized to dihydroxyacetone (DHA) by GldA using NAD⁺ as the electron acceptor and then phosphorylated to dihydroxyacetone phosphate (DHAP) by Dhak (Durnin et al., 2009). The dihydroxyacetone phosphate is then incorporated into EMP glycolysis pathway and finally to pyruvate. Since lactate production from glycerol results in excess NADH, small amounts of dissolved oxygen are possibly required to oxidize the NADH and restore the redox balance.

Wild type *E. coli* can produce lactate from glycerol together with by-products such as acetate, succinate, formate, pyruvate and ethanol, especially in the presence of oxygen. To minimize by-products production, genes encoding pyruvate-formate lyase (encoded by *pf1B*), phosphotransacetylase (*pta*), fumarate reductase (*frdA*), alcohol/acetaldehyde dehydrogenase (*adhE*) and aerobic D-lactate dehydrogenase (*dld*) were deleted (Mazumdar et al., 2010). Apart from these five genes, genes encoding

pyruvate oxidase (*poxB*) and PEP synthase (*pps*) were also deleted to construct a homolactatic fermentative strain for D-lactate production in our previous research (Zhou et al., 2011). However, the volumetric lactic acid production rate is still much lower for glycerol than for glucose, which is far from commercial benefit.

In this report, appropriate oxygen supply strategy and weak strength overexpression of *ldhA* resulted in higher D-lactate productivity and yield from glycerol.

MATERIALS AND METHODS

Strains and plasmids used in this study are listed in Table 1. All strains and plasmids were stored at CICIM-CU (Culture and Information Center of Industrial Microorganism of China Universities at Jiangnan University, <http://CICIM-CU.jiangnan.edu.cn>).

Growth condition

During strain construction, cultures were grown aerobically at 37°C in LB medium (10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl, pH 7). Ampicillin (50 mg/L), kanamycin (50 mg/L) or gentamycin (30 mg/L) were added as needed during strain construction.

Modified M9 medium contained (L⁻¹): 15.11 g Na₂HPO₄·12H₂O, 3 g KH₂PO₄, 1 g NH₄Cl, 0.5 g NaCl to which 0.1 % MgSO₄ solution (1 M) and 0.1% trace metal stock solution were added. The trace metal stock solution contained (L⁻¹): 2.4 g FeCl₃·6H₂O, 0.3 g CoCl₂·6H₂O, 0.15 g CuCl₂·2H₂O, 0.3 g ZnCl₂, 0.3 g Na₂MoO₄·2H₂O, 0.075 g

H₃BO₃, 0.495 g MnCl₂·4H₂O. Both LB medium and the modified M9 medium were sterilized at 121°C for 20 min. The trace metal stock solution was filter-sterilized through a 0.22 µm pore-size filter.

Cells were grown in 50 ml of LB medium in a 250 ml flask at 37°C with shaking (200 rpm) for 8 to 10 h until an OD₆₀₀ of 2.0 to 2.5 (early to mid-log phase of growth) was reached. Cells were collected by centrifugation (4300 xg, 10 min) and resuspended in M9 medium. These cells were inoculated into 50 ml of modified M9 medium in a 250 ml flask to attain an initial OD₆₀₀ of 0.05 and cultivated at 37°C with shaking (200 rpm).

Genetic methods

Standard molecular biology protocols (Joseph and Russell, 2001) were used for DNA manipulation unless otherwise specifically described. PCR was performed using *Pfu* Turbo DNA polymerase (Stratagene, Santa Clara, CA). The PCR product was purified with a BioEev-Tech DNA purification kit (BioEev-Tech, Scientific and Technical Co., Ltd, Peking, China). Manufacturer's protocols were followed for restriction endonuclease digestion and DNA ligation (TaKaRa, Dalian, China). Plasmids from *E. coli* JM109 were isolated with a Qiagen plasmid isolation kit (Qiagen, Valencia, CA) as indicated in the manufacturer's protocol.

Gene over-expression was achieved by cloning the D-lactate dehydrogenase gene (*ldhA*) in a low-copy vector pTH18Kr (Hashimoto-Gotoh et al., 2000) and a high-copy vector pUC19 (Lee et al., 2006) in *E. coli* JM109 in order to investigate the effect of increasing copy numbers of *ldhA* on microbial growth and fermentation properties. Both plasmids were transformed into B0013-070 by electroporation (Joseph and Russell 2001). To construct plasmid pUC-*ldhA*, the *ldhA* gene was PCR amplified using genomic DNA from strain B0013 and primers LDHa1 and LDHa2 (Table 1). The PCR product was cloned within the *Sma*I site of pUC19. To construct plasmid pTH-*ldhA*, the *ldhA* fragment was recovered from plasmid pUC-*ldhA* with *Hind*III digestion, and cloned into the *Hind*III site of pTH18Kr.

Fed-batch fermentation

Prior to use, the cultures (stored as glycerol stocks at -70°C) were streaked onto LB plates and incubated overnight at 37°C. A single colony was used to inoculate 250 ml flasks containing 30 ml LB medium. After 10 h, cells were harvested by centrifugation (4300 xg, 10 min), resuspended in 15 ml M9 medium and this suspension was used to inoculate 150 ml fresh modified M9 medium containing 5 g/L glycerol (in a 500 ml flask) resulting in an OD₆₀₀ of about 0.255. After incubation for 9 h, this seed culture was used to inoculate a jar-fermentor containing modified M9 medium.

A modified two-phase fed-batch process was used similar to that reported by Zhu et al. (2007) except that an anaerobic phase generated by nitrogen sparging was not applied. A 7-L bioreactor (Bioflo110; New Brunswick Scientific Co., Inc., Edison, NJ), initially containing 3 L of medium, was used for D-lactate production from glycerol. The modified two-phase fed-batch process was initiated by permitting aerobic cell growth until the OD₆₀₀ of about 30 (corresponding to about 11.4 g/L dry cell weight [DCW]) was reached and the microaerobic fermentation phase commenced. During the aerobic phase, the culture was grown at pH 7 controlled by the addition of 25% (wt/vol) NH₄OH and the dissolved oxygen tension (DOT; Mettler-Toledo AutoChem Inc., Columbia, MD) was controlled above 40% of saturation by automatic control of the air and O₂ flow between 3 to 7 L/min and agitation between 200 and 1000 rpm. During the microaerobic phase, the culture was grown at pH 7 controlled by the addition of 25% (wt/vol) Ca(OH)₂ and DOT was controlled under 1% of saturation by controlling the air

and O₂ flow at 5 L/min and agitation at 600 rpm. Glycerol was added at an initial concentration of 30 g/L during the aerobic phase and glycerol at a concentration of 600 g/L was automatically fed into the bioreactor to maintain the concentration above 10 g/L during the microaerobic phase. The fermentations were run for 36 h (unless otherwise stated), time at which the final product concentrations were measured.

Analytical methods

The total biomass concentration was determined by measuring the OD₆₀₀ of the culture and after centrifuging, washing the cells and drying to constant weight, a standard curve relating the OD to biomass was constructed (1 OD₆₀₀ was equivalent to 0.38 g/L DCW). The glycerol concentration was estimated using a glycerol determination kit (FG0100-1KT; Sigma-Aldrich, Inc., St. Louis, Mo). Samples for HPLC analysis were acidified by the addition of 5 mM H₂SO₄, the proteins in the samples were precipitated with 10% trichloroacetic acid and removed by centrifugation at 10,625 xg for 5 min. The supernatant was analyzed for D-lactate and by-products by a HPLC system, equipped with a Dionex p680 pump (Dionex Corporation, Sunnyvale, CA), a Shodex RSpak KC-811 column (Shodex RSpak KC-811 F6378030; Showa Denko K.K., Kawasaki, Japan) and a UV (210 nm) detector (Dionex UVD170U). Samples were eluted with 0.1% (wt/vol) H₃PO₄ at a flow rate of 1 ml/min. The separation temperature was maintained at 50°C. Lactic acid isomeric purity was measured by HPLC using a chiral column (CLC-L; Advanced Separation Technologies Inc., city NJ, USA), at room temperature, equilibrated with 1 ml/min of 5 mM CuSO₄ as the mobile phase and detected at 254-nm with a UV detector. The growth rates of the strains were calculated with Origin 8.0 software, and the nonlinear curve fitting was carried out with the formula: $y = A2 + (A1 - A2) / (1 + \exp((x - x_0) / dx))$, where A1 and A2 are the biomass concentration (g-cell dry weight L⁻¹).

RESULTS

A modified two-phase fermentation system for optically pure D-lactate (higher than 99.9%) production from glycerol by *E. coli* CICIM B0013 and its derivative strains was set up to exploit the two glycerol metabolic pathways (Figure 1). In order to maintain a redox balance between glycerol catalysis and D-lactate production during the microaerobic phase, a mixture of air and O₂ was supplied at a constant flow rate.

Production of D-lactate from glycerol by wild type *E. coli* CICIM B0013

Although *E. coli* B0013 grew rapidly on glycerol during the aerobic phase (about 8 h), only 6.7 g/L D-lactate was produced. At the start of the microaerobic phase, the D-lactate concentration increased to 9.3 g/L but no D-lactate was detected at the end of the fermentation (Figure 2). The pyruvate and succinate concentrations increased rapidly as lactate concentration decreased, and the final concentration of pyruvate and succinate were 5.3 and 15.9 g/L, respectively after 36 h. Little acetate was produced but increased to 4.0 g/L at the end of the fermentation. No formate was detected.

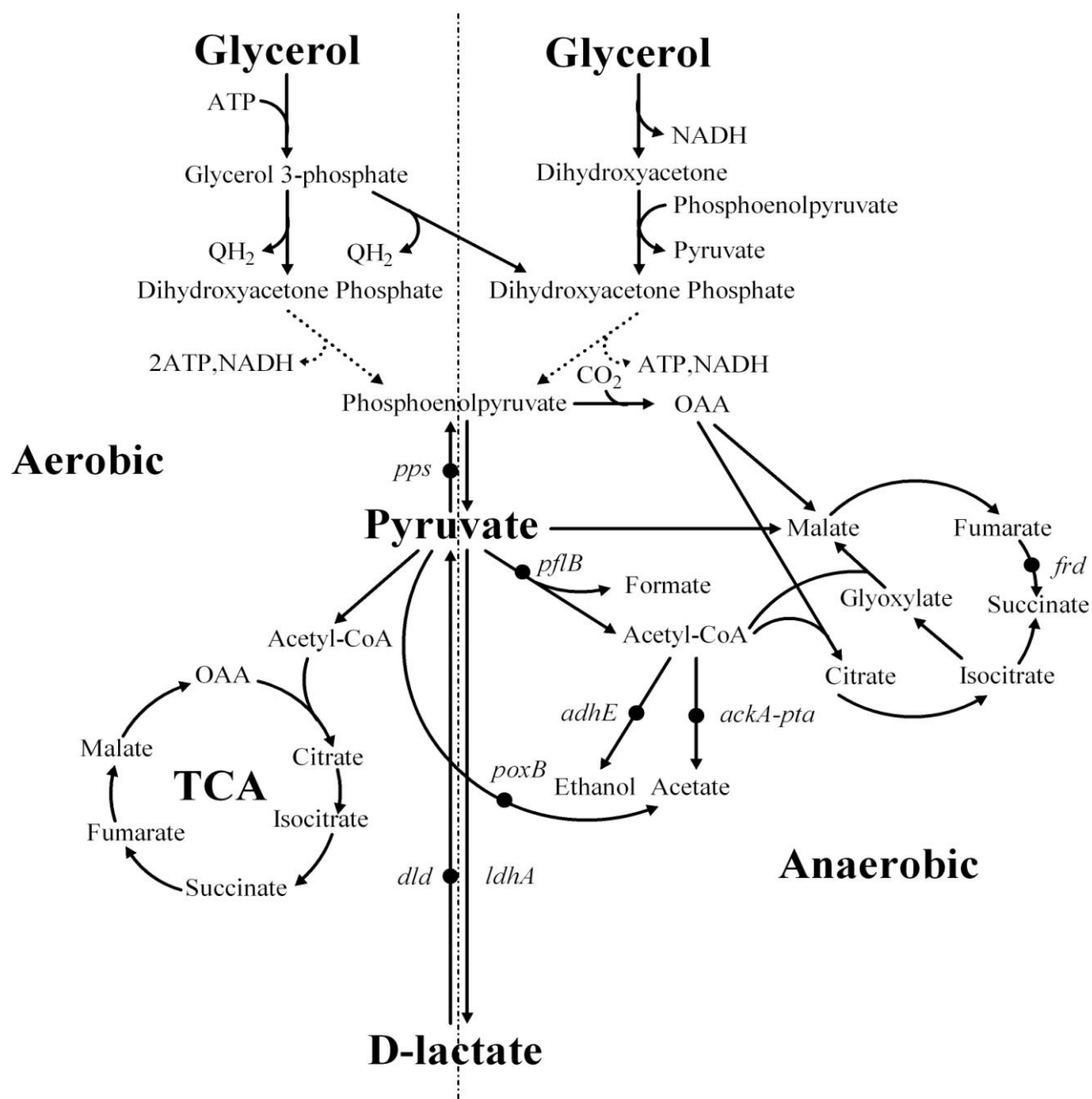


Figure 1. General metabolic pathway in the production of D-lactate by *E. coli* strains from glycerol. Relevant enzymes and genes are shown. Broken lines represent multiple steps. *pps*, PEP synthase; *pflB*, pyruvate formate lyase; *ldhA*, fermentative D-lactate dehydrogenase; *dld*, D-lactate dehydrogenase; *poxB*, pyruvate oxidase; *pta*, phosphotransacetylase; *ackA*, acetate kinase; *adhE*, alcohol dehydrogenase; *frd*, fumarate reductase; QH₂, reduced quinones; OAA, oxaloacetic acid; TCA, tricarboxylic acid cycle; Filled in circles represent the gene deletions performed in this study.

Enhancement of D-lactate production by elimination of by-product synthesis pathways

To minimize by-products production (acetate, succinate, formate, pyruvate and ethanol), a mutant derivative strain B0013-070 was constructed in our previous research (Zhou et al., 2011). This engineered B0013-070 was proved to be able to mainly homo-ferment conversion of

glycerol to D-lactate. After 36 h, 98.5 g/L D-lactate was obtained with less than 2 g/L total by-products. The average D-lactate productivity during the microaerobic phase (over 27 h) was 3.45 g/L/h, and the D-lactate yield was 64 g/100 g glycerol (Figure 3a and Table 3).

The effect of the time allowed for aerobic growth of the cells to continue before switching to the microaerobic fermentation phase was investigated. In the fermentation

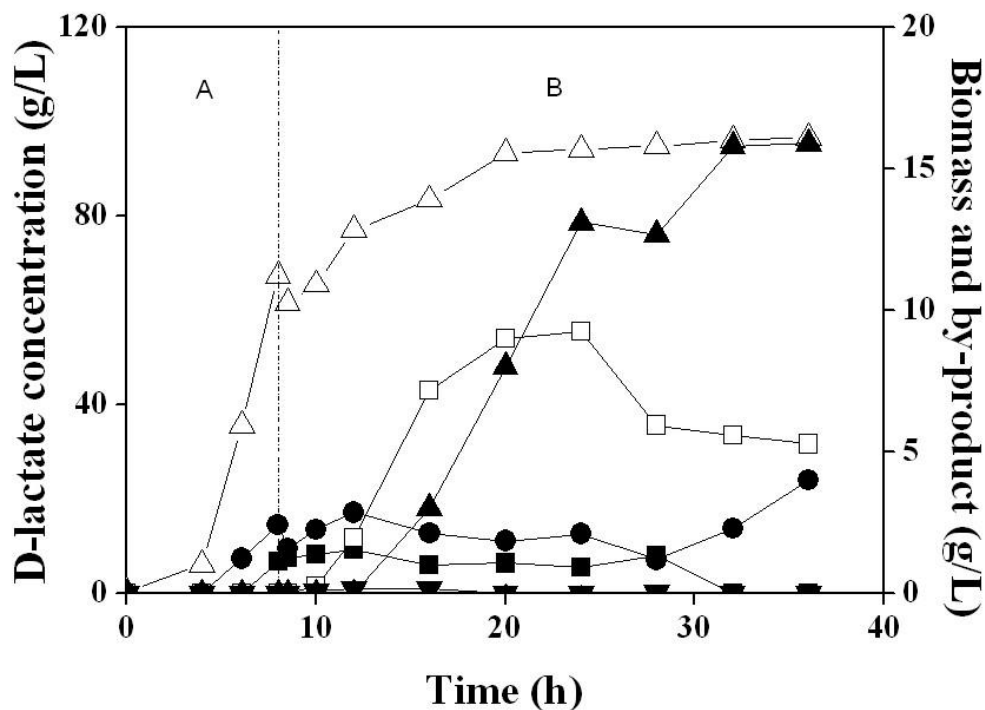


Figure 2. Product concentrations and cell mass during the two-phase lactate fermentation of wild type strain *E. coli* CICIM B0013. The figure shows concentrations of lactate (■), acetate (●), succinate (▲), formate (▼), pyruvate (□) and cell mass (Δ). A: aerobic phase; B: microaerobic phase.

process described above (Figure 3), the OD_{600} was allowed to reach about 30 before the microaerobic fermentation phase was commenced. Switching to the microaerobic fermentation when cells were allowed to reach a lower cell density (5.7 g/L) had a negative effect on the production of D-lactate (Figure 3b). The D-lactate concentration achieved in this fermentation process was 32 g/L, only about one third of the concentration produced when the cells were allowed to grow further before switching to microaerobic conditions. The D-lactate volumetric productivity during the microaerobic phase also decreased to 1.13 g/L/h, and the D-lactate yield was 63 g/100 g glycerol (Tables 2 and 3). However, by-product yields were also much lesser than those observed in the fermentation with a higher biomass concentration (Figure 3b and Table 3). The effect of allowing growth to proceed to a higher biomass than 11.4 g/L before switching to microaerobic conditions was also tested, and glycerol was added with an initial concentration of about 50 g/L. However, growth was slower above cell density 11.4 g/L and in an experiment where 17.1 g/L biomass was attained, the D-lactate concentration and productivity declined as compared to the fermentation where the switch was made at cell density of 11.4 g/L (data not shown). These results point to the need to ensure that an optimal biomass concentration is present in the bioreactor before commencing the microaerobic phase.

Enhancement of D-lactate production by elevating expression of D-lactate dehydrogenase

Two expression strategies for expressing different copy numbers of *ldhA* were investigated to evaluate their effect on growth and fermentation profiles. A low copy vector pTH18kr and a high copy vector pUC19 were used for *ldhA* overexpression in B0013-070. As shown in Figure 3a and d, the strain B0013-070 (pTH-*ldhA*) showed similar growth characteristics as B0013-070, but a greater lactate concentration and volumetric and specific productivity was achieved; and an improved lactate yield was observed by B0013-070 (pTH-*ldhA*) (Table 2). Furthermore, less D-lactate was accumulated by B0013-070 (pTH-*ldhA*) than B0013-070 during the aerobic phase. However, the strain B0013-070 harboring the high copy number of pUC-*ldhA* vector produced 111.5 g/L D-lactate but only after 56 h fermentation as compared to 36 h for the other fermentations required for completion (Figure 3c). This result points to an inhibitory effect of the high copy number plasmid. Furthermore, the specific growth rate during the aerobic growth phase for strain B0013-070 (pUC-*ldhA*) was much lower than the other strains (Table 3). Both the specific and volumetric D-lactate productivity of B0013-070 (pTH-*ldhA*) was greater than B0013-070 (pUC-*ldhA*) again indicating that the high copy number might negatively affect productivity

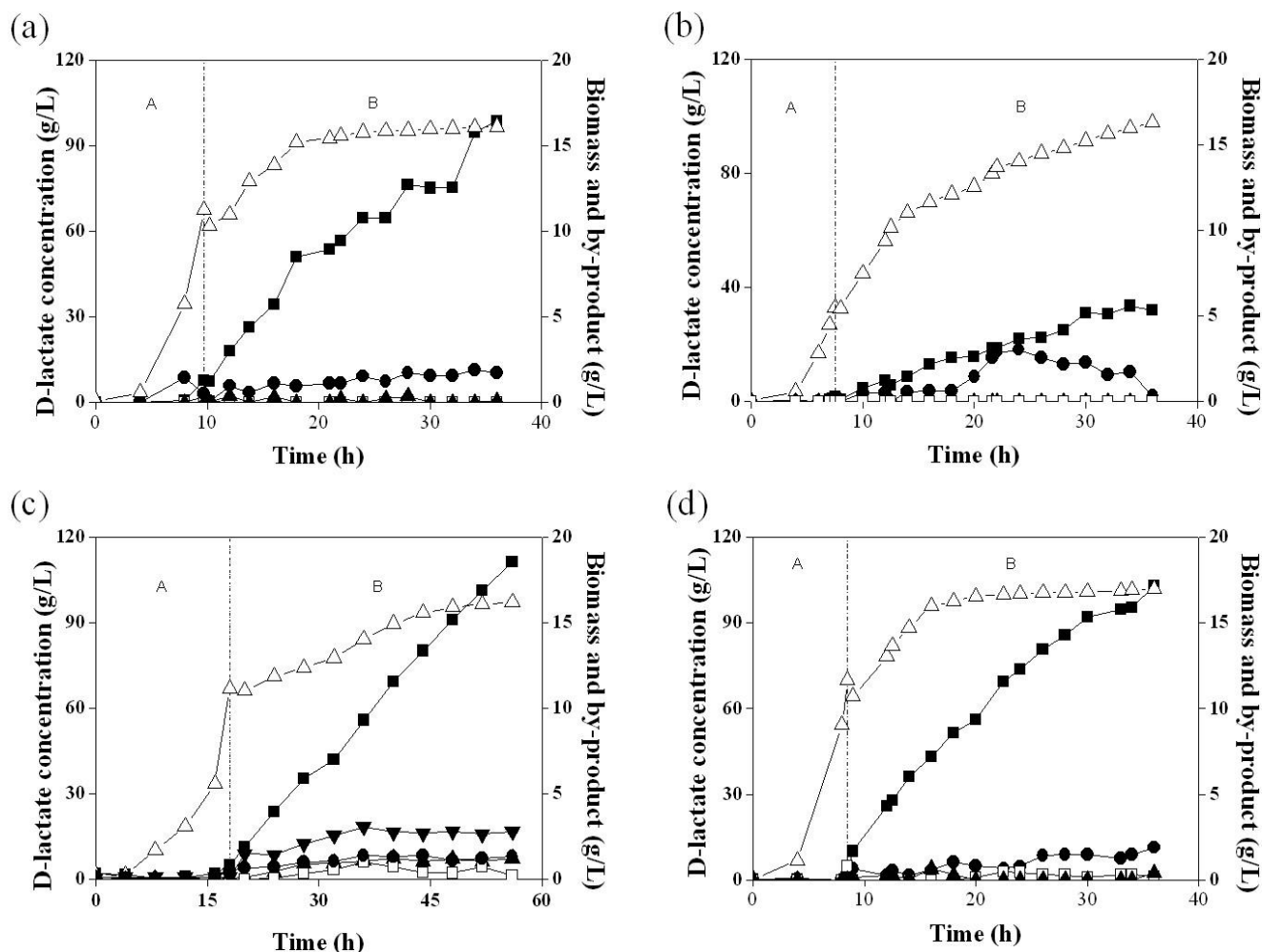


Figure 3. Product concentrations and cell mass during the two-phase lactate fermentation of the derivative strains wild type strain *E. coli* C1C1M B0013. The figure shows concentrations of lactate (■), acetate (●), succinate (▲), formate (▼), pyruvate (□) and cell mass (Δ). (a) Strain B0013-070; (b) Strain B0013-070 (with a reduced aerobic growth phase); (c) Strain B0013-070 (pUC-*ldhA*); (d) Strain B0013-070 (pTH-*ldhA*). A: aerobic phase; B: microaerobic phase.

(Table 3). The lactate yields of both strains increased to 78 g/100 g glycerol as compared to the 63 g/100 g glycerol for B0013-070 (Tables 2 and 3).

DISCUSSION

Wild-type *E. coli* usually produced lactic acid from glycerol accompanied with a large number of by-products (Hong et al., 2009; Mazumdar et al., 2010). By-products were reduced through the deletion of genes which encoded the key enzymes required for by-products synthesis. The rate of lactate production was slightly increased by elevating expression of a D-lactate dehydrogenase; but the final D-lactate concentration of 103.1 g/L with a yield of 78 g/100 g glycerol and respective volumetric and specific productivities of 3.65 g/L/h and 0.78 g/g biomass/h compares favorably with a genetically manipulated *E. coli*

strain recently reported to convert glycerol to D-lactate (Mazumdar et al., 2010). Furthermore, the experiments were conducted under conditions similar to those for industrial production and the kinetic results would be likely to reflect values obtained in practice. The major increase in D-lactate concentration and productivities was found when the oxygen supply strength was enhanced during the cell growth phase, and elevated expression of D-lactate dehydrogenase mediated a low copy plasmid.

In order to achieve an efficient fermentation of glucose to D-lactate, a two-phase fermentation process with an aerobic growth phase and an anaerobic fermentation phase was developed in our previous report (Zhou et al., 2011). Both glucose utilization rate and lactic acid synthesis rate were improved during the two-phase fermentation. Unlike glucose, more oxygen supply was needed when glycerol was used as substrate for lactate production, especially during the fermentation phase. In

Table 2. Kinetic data from fermentation of glycerol to D-lactate by various *E. coli* strains (mean \pm standard deviation of triplicate experiments).

<i>E. coli</i> strain	Relevant genetic characteristics	D-Lactate (g/L)	Yield (g lactate/100 g glycerol used)	Total glycerol Consumption (g)	Time (h)	Source or reference
CICIM B0013 ^a	Wild type	9.3 \pm 0.2 ^c	0 \pm 0	204.5 \pm 3.1	36	This study
CICIM B0013-070 ^a	$\Delta ack-pta$, Δpps , $\Delta pflB$, Δdld , $\Delta poxB$, $\Delta adhE$, $\Delta frdA$	98.5 \pm 1.5	64 \pm 0.1	615.6 \pm 5.6	36	This study
CICIM B0013-070 ^b	$\Delta ack-pta$, Δpps , $\Delta pflB$, Δdld , $\Delta poxB$, $\Delta adhE$, $\Delta frdA$	32.1 \pm 0.3	63 \pm 0.2	203.8 \pm 1.9	36	This study
CICIM B0013-070 (pTH- <i>ldhA</i>) ^a	$\Delta ack-pta$, Δpps , $\Delta pflB$, Δdld , $\Delta poxB$, $\Delta adhE$, $\Delta frdA$ With pTH- <i>ldhA</i>	103.1 \pm 1.8	78 \pm 0.3	528.7 \pm 5.3	36	This study
CICIM B0013-070 (pUC- <i>ldhA</i>) ^a	$\Delta ack-pta$, Δpps , $\Delta pflB$, Δdld , $\Delta poxB$, $\Delta adhE$, $\Delta frdA$ With pUC- <i>ldhA</i>	111.5 \pm 2.3	78 \pm 0.2	571.8 \pm 5.0	56	This study
AC-521	Wild type	85.8	88	NR ^d	90	Hong et al., 2009
LA02 Δdld (pZSgIpKgIpD)	<i>pta</i> ::FRT, <i>adhE</i> ::FRT, <i>frdA</i> ::FRT, <i>dld</i> ::FRT-Kan-FRT, With pZSgIpKgIpD	38	85	NR	72	Mazumdar et al., 2010

^aD-lactate concentration and yield produced by B0013-070 during two-phase fermentation; ^bD-lactate concentration and yield produced by B0013-070 during two-phase fermentation with a reduced aerobic growth phase; ^cThe maximum D-lactate concentration during the fermentation; ^dNR, not reported.

Table 3. Fermentation products, growth rates and D-lactate produced by *E. coli* CICIM B0013 and its derivative strains in a two-phase fermentation (mean \pm standard deviation of triplicate experiments).

Strains	Specific growth rate (1/h)	Volumetric lactate Productivity (g/l-h)	Specific lactate productivity (g/g biomass.h)	Final concentrations of products (aerobic phase; microaerobic phase) (g/L)				
				D-Lactate	Acetate	Succinate	Formate	Pyruvate
B0013 ^a	0.89 \pm 0.02	0.00 \pm 0.00	0.00 \pm 0.00	6.7 \pm 0.1; 0.0 \pm 0.0	2.4 \pm 0.2; 4.0 \pm 0.1	0.0 \pm 0.0; 15.9 \pm 1.1	0.0 \pm 0.0; 0.0 \pm 0.0	0.0 \pm 0.0; 5.3 \pm 0.2
B0013-070 ^a	0.57 \pm 0.01	3.45 \pm 0.03	0.71 \pm 0.10	7.8 \pm 0.5; 98.5 \pm 1.5	0.5 \pm 0.0; 1.7 \pm 0.2	0.0 \pm 0.0; 0.1 \pm 0.0	0.0 \pm 0.0; 0.0 \pm 0.0	0.3 \pm 0.0; 0.0 \pm 0.0
B0013-070 ^b	0.64 \pm 0.01	1.13 \pm 0.07	0.40 \pm 0.01	1.7 \pm 0.1; 32.1 \pm 0.3	0.2 \pm 0.0; 0.4 \pm 0.0	0.0 \pm 0.0; 0.0 \pm 0.0	0.0 \pm 0.0; 0.0 \pm 0.0	0.0 \pm 0.0; 0.0 \pm 0.0
B0013-070(pTH- <i>ldhA</i>) ^a	0.67 \pm 0.03	3.65 \pm 0.05	0.78 \pm 0.01	2.6 \pm 0.2; 103.1 \pm 1.8	0.8 \pm 0.1; 0.2 \pm 0.0	0.0 \pm 0.0; 0.4 \pm 0.0	0.7 \pm 0.0; 1.9 \pm 0.1	0.0 \pm 0.0; 0.0 \pm 0.0
B0013-070(pUC- <i>ldhA</i>) ^a	0.16 \pm 0.00	2.80 \pm 0.01	0.58 \pm 0.00	5.1 \pm 0.0; 111.5 \pm 2.3	0.1 \pm 0.0; 1.4 \pm 0.1	0.1 \pm 0.0; 1.3 \pm 0.2	1.5 \pm 0.1; 2.8 \pm 0.4	0.0 \pm 0.0; 0.3 \pm 0.0

^aProducts and D-lactate produced by strains during two-phase fermentation; ^bProducts and D-lactate produced by B0013-070 during two-phase fermentation with a reduced aerobic growth phase.

this study, a modified two-phase fermentation process was developed but due to the higher reducing power of glycerol, a microaerobic fermentation phase instead to the anaerobic fermentation phase was applied. Under this modified two-phase fermentation process, the engineered strain *E. coli* B0013-070 (pTH-*ldhA*) achieved a 2.1-fold higher volumetric lactic acid production rate than the best result with a recombinant *E. coli* strain LA02 Δ *dld* that overexpressed GlpK-GlpD when cultured only under microaerobic conditions (Mazumdar et al., 2010). In addition, shortening the cell growth time during the aerobic phase significantly reduced the rate of lactic acid production, indicating that enough biomass for the fermentation of lactic acid is very important. The anaerobic fermentation phase was also test for D-lactate production from glycerol without oxygen supply. However, little D-lactate was accumulated at the early fermentation phase but no D-lactate was determined at the end of the fermentation (data not shown).

Previous studies have shown that overexpression of D-lactate dehydrogenase helps to improve efficiency of lactic acid production (Mazumdar et al., 2010). In this study, the degree of overexpression of D-lactate dehydrogenase on lactate production was investigated. The excessive increase of D-lactate dehydrogenase gene copy number resulted in the delay of cell growth, although the yield of lactic acid is still high, the fermentation period was significantly prolonged. Similarly, overexpression of D-lactate dehydrogenase results in the poor growth of strains containing the multicopy plasmid in a minimal medium possibly due a reduction of the intracellular and extracellular pyruvate flux to TCA cycle (Yang et al., 1999).

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