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Enhanced succinate production from glycerol by engineered

***Escherichia coli* strains**

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Abstract

In this study, an engineered strain *Escherichia coli* MLB (*ldhA⁻ pflB⁻*) was constructed for production of succinate from glycerol. The succinate yield was 0.37 mol/mol in anaerobic culture, however, the growth and glycerol consumption rates were very slow, resulting in a low succinate level. Two-stage fermentation was performed in flasks, and the succinate yield reached 0.93 mol/mol, but the succinate titer was still low. Hence, overexpression of malate dehydrogenase, malic enzyme, phosphoenolpyruvate (PEP) carboxylase and PEP carboxykinase (PCK) from *E. coli*, and pyruvate carboxylase from *Corynebacterium glutamicum* in MLB was investigated for improving succinate production. Overexpression of PCK resulted in remarkable enhancement of glycerol consumption and succinate production. In flask experiments, the succinate concentration reached 118.1 mM, and in a 1.5-L bioreactor the succinate concentration further increased to 360.2 mM. The highest succinate yield achieved 0.93 mol/mol, which was 93% of the theoretical yield, in the anaerobic stage.

Key words: succinate, *Escherichia coli*, glycerol, phosphoenolpyruvate carboxykinase, two-stage fermentation

1. Introduction

Glycerol is a by-product of biodiesel industry. With the increased production of biodiesel, crude glycerol is generated in a large quantity, and for every 9 kg of biodiesel produced, about 1 kg of crude glycerol by-product is formed (Dasari et al., 2005). There is an urgent need to develop effective methods to convert glycerol to higher value products, and conversion of glycerol to 1,3-propanediol and 3-hydroxypropionic acid (Huang et al., 2012), ethanol (Nikel et al., 2010), succinate (Lee et al., 2001), fumarate (Li et al., 2014), free fatty acids (Wu et al., 2014) and so on, has been explored. Glycerol is a more reductive carbon source compared to glucose, and conversion of 1 mol glycerol to pyruvate generates 2 mol of NADH. Because of its higher reduction degree, it is evident that fermentation of glycerol is favorable to generating more reduced products like succinate and 1,3-propanediol whose production from sugars is limited by the availability of reducing equivalents.

Succinate is one of the key platform chemicals, and it is widely used in production of foods, pharmaceuticals, and biodegradable plastics (Zeikus et al., 1999). It was evaluated as one of the top building block chemicals produced from biomass (Werpy et al., 2004). Traditionally, succinate is synthesized from petroleum-derived maleic anhydride, which is unrenewable. Production of succinate from fermentation can alleviate the dependence on oil supply in the future (Werpy et al., 2004), and the bioprocess has many advantages over the chemical processes owing to its simplicity and environment friendly nature. Some bacteria strains, including *Actinobacillus*

succinogenes (Jiang et al., 2014; Zhao et al., 2016), *Anaerobiospirillum succiniciproducens* (Lee et al., 2001), *Mannheimia succiniciproducens* (Lee et al., 2003), and metabolically engineered *Escherichia coli* (Vemuri et al., 2002), are capable of producing succinate efficiently.

Though *E. coli* cannot grow anaerobically on glycerol without an exogenous electron acceptor, recent studies have indicated that it can ferment glycerol as the sole carbon source under particular anaerobic conditions. Dharmadi et al. (2006) found that *E. coli* was capable of anaerobically fermenting glycerol in a pH-dependent manner. Zhang et al. (2010) constructed an engineered strain *E. coli* XZ721 which could convert glycerol to succinate anaerobically by inactivating *pflB* and *ptsI* and introducing a mutation on promoter of *pck* to increase its expression. Microaerobic condition was also used for converting glycerol to succinate by *E. coli*, in which the pathways for the synthesis of competing by-products were blocked and a gene encoding the pyruvate carboxylase of *Lactococcus lactis* was expressed (Blankschien et al., 2010). Supplementary Table S1 summarizes the performances of succinate production with glycerol as the carbon source reported by various investigators.

Glycerol is metabolized in *E. coli* through three ways to form dihydroxyacetone (DHA) phosphate (DHAP): the glycerol kinase (GlpK) and aerobic glycerol 3-phosphate dehydrogenase (GlpD) route; the GlpK and anaerobic glycerol 3-phosphate dehydrogenase (GlpABC) route; the fermentative glycerol dehydrogenase (GldA) and DHA kinase (DhaKLM) route (Fig. S1). The GlpK-GlpD and GlpK-GlpABC routes reduce flavin in glycerol dissimilation (Tran et al., 1997).

The GldA-DhaKLM fermentative route is reported to enable efficient utilization of glycerol under both anaerobic and microaerobic conditions (Gonzalez et al., 2008; Durnin et al., 2009). However, DhaKLM uses phosphoenolpyruvate (PEP) as the phosphoryl donor, impacting succinate synthesis because *E. coli* does not have pyruvate carboxylase to provide oxaloacetate (OAA), the precursor of succinate, from pyruvate.

PCK catalyzes the reaction to interconversion between OAA and PEP, and it plays an important role in gluconeogenesis. ATP is generated when PCK catalyzes the reaction to form OAA from PEP, and at the same time CO₂ is fixed (Wu et al., 2012). It was reported that the *pck* gene of a natural succinate producer, *A. succinogenes*, was overexpressed in *E. coli*, however, it showed little effect on succinate production when glucose was used as the carbon source (Kim et al., 2004). Some researchers have found PCK is important for succinate production because of ATP formation (Wu et al., 2007; Zhang et al., 2009). In this study, the pathways that synthesize competing metabolic by-products were blocked (Fig. S1), and the resulted strains were used to ferment glycerol in two-stage cultures, in which an aerobic growth stage was followed by an anaerobic production stage. The *pck* gene from *E. coli* was overexpressed in the *E. coli* mutants, and the effects on glycerol consumption and succinate production were investigated. Malate dehydrogenase (MDH), malic enzyme (ME) and PEP carboxylase (PPC) from *E. coli*, and pyruvate carboxylase (PYC) from *C. glutamicum* were also overexpressed to examine their effects on succinate production in two-stage fermentation.

2. Materials and methods

2.1 Strains and plasmids

E. coli K12 strain MG1655 was obtained from Coli Genetic Stock Center (CGSC) and was used to construct strains for succinate production. The strains and plasmids used in this study are listed in Table 1. Primers for gene cloning and verification are listed in Table 2. The one-step gene knockout method (Datsenko and Wanner, 2000) was used to delete various genes to suppress by-products formation. Genes were cloned and inserted into pTrc99a, and were expressed in various mutant strains to facilitate glycerol utilization and succinate production. All the strains were stored in 25% (w/w) glycerol at -20°C.

2.2 Media and culture conditions

For strain construction and primary inoculum development, *E. coli* cells were grown at 37°C in Luria-Bertani broth (LB, per liter: tryptone 10 g, yeast extract 5 g, and sodium chloride 10 g), in which appropriate antibiotics were included when needed at the following concentrations: kanamycin, 30 mg/L; ampicillin, 100 mg/L.

The salt medium (SM) that was based on M9 contained (per liter) $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 15.12 g, KH_2PO_4 3.0 g, NaCl 0.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, CaCl_2 0.011 g, NH_4Cl 1.0 g, 1% (w/v) vitamin B1 0.2 mL, and trace elements solution 0.1 mL. The stock solution of trace elements contained the following (per liter) in 3 M HCl: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 80 g, $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ 10 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 2.0 g, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 1.0 g,

NaMoO₄·2H₂O 2.0 g, MnSO₄·H₂O 10 g, CoCl₂ 4.0 g, and H₃BO₄ 0.5 g.

Anaerobic fermentation was carried out in 50-mL bottles (Schott) containing 40 mL SM with 15 g/L glycerol and 15 g/L NaHCO₃, and was inoculated with 1 mL primary preculture (see below). The headspace of the anaerobic bottle was filled with CO₂ and the cells were incubated at 37°C and 220 rpm for 6 days.

For two-stage fermentation in flasks, cells were initially grown aerobically in the growth medium (GM) prepared by supplementing SM with 5 g/L of glycerol, and 100 mg/L of ampicillin was added for the strains carrying the recombinant plasmids. The GM was supplemented with biotin 1.0 mg/L for the strains expressing PYC. The medium for the anaerobic culture (MA) of the two-stage fermentation was SM supplemented with 15 g/L of glycerol and 15 g/L of NaHCO₃ but without addition of NH₄Cl.

The medium for fermentation carried out in a 1.5-L bioreactor (BIOTECH-1.5; Baoxing Co., Shanghai, China) was SM supplemented with 52 g/L (565 mM) of glycerol, and the concentrations of Na₂HPO₄·12H₂O, KH₂PO₄ and NH₄Cl were changed to 3.78, 0.75 and 10 g/L, respectively. Ampicillin was included at the concentration of 100 mg/L.

The primary preculture was prepared by transfer of 1 mL stock culture to 30 mL LB medium in a 250-mL flask, in which the cells were aerobically incubated at 37°C and 220 rpm for 8 h. For two-stage fermentation experiments carried out in flasks, 2-mL aliquots of the primary preculture were transferred to 500-ml flasks containing 100 mL GM, in which the cells were incubated under the same conditions for 8 h.

Then IPTG was added to a final concentration of 0.1 mM to induce overexpression of the cloned gene, and the cells were incubated for another 4 h. The cells were harvested aseptically by centrifugation at 4°C and 6300×g for 5 min, and then were resuspended in fresh MA to an optical density at 600 nm (OD₆₀₀) of around 15. Anaerobic culture was performed in 50 mL anaerobic bottles containing 40 mL of the cell suspension, and the headspace was filled with CO₂. The anaerobic culture was performed at 220 rpm and 37°C for 72 hours.

For the two-stage fermentation experiments carried out in the 1.5-L bioreactor, the primary preculture was transferred to 1 L of fermentation medium in the 1.5-L bioreactor. Fed-batch experiments were carried out at 37 °C. Cell growth was initiated by sparging air into bioreactor at the rate of 1.5 vvm and the dissolved oxygen tension was maintained above 10% saturation by agitation at 300 to 900 rpm. During the induction of PCK expression, the aeration rate was 1.5 vvm and the maximum agitation rate was 900 rpm. The pH value was controlled automatically at 7.0 in the aerobic stage by addition of 2 M NaOH. When the dry cell weight (DCW) reached 4.4 g/L, 0.5 mM IPTG was added to the culture. The cells were incubated for another 4 hours, and then the process was shifted to the anaerobic stage by stopping aeration and filling the headspace of bioreactor with CO₂, and pH was kept above 6.3 by adding basic magnesium carbonate in the anaerobic stage.

2.3 Analytical methods

Cell growth was monitored by measuring the OD₆₀₀, which was converted to

DCW according to the relationship between OD₆₀₀ and DCW (1 OD₆₀₀ was equivalent to 0.33 g DCW/L). Culture samples were centrifuged for 10 min at 4°C and 13,000×g. The supernatant was filtered through a 0.22 µm nylon syringe filter. The concentrations of glycerol, succinate and other organic acids were determined by using a high-pressure liquid chromatograph system (LC-20AT; Shimadzu, Japan) equipped with a cation-exchange column (HPX-87H; Bio-Rad, USA), a differential refractive index detector (RID-10A; Shimadzu), a UV-VIS detector (SPD-20A; Shimadzu) at 210 nm, and an on-line degasser system (DGU-20A3; Shimadzu). The column was operated at 65°C, and the mobile phase was 5 mM H₂SO₄ at 0.6 mL/min.

For measurement of intracellular enzyme activities, cells were harvested by centrifugation (13,000×g for 10 min at 4°C) and washed twice with cold 100 mM Tris-HCl (pH 7.5) at 4°C. The cell pellets were resuspended in the same buffer containing 0.1 mM EDTA. Then the cells were disrupted on ice with an ultrasonic disrupter (JY92-II; Scientz Biotechnology Co., Ningbo, China) for 99 cycles (a working period of 3 seconds with a 10-second interval for each cycle) at a power output of 200 W. The cell debris was removed by centrifugation at 13,000×g and 4°C for 30 min, and the supernatant was used for enzyme activity determination.

The activities of MDH, ME, isocitrate lyase (ICL), PCK, PPC and pyruvate kinase (PK) were measured by monitoring the formation or disappearance of NADH. The assay conditions were described previously (Wu et al., 2007). One unit of activity was defined as the amount of enzyme to oxidize 1 µmol of NADH or to reduce 1 µmol of NAD⁺ per min. The total protein concentration in crude cell extract was

measured by the method of Bradford (1976) with bovine serum albumin as a standard.

2.4 Statistical analysis

Statistical analyses were carried out using Microsoft Excel 2007. Unpaired two-tailed Student's t-test was used to analyze the data. Statistical significance was defined as $P < 0.05$.

3. Results and Discussion

3.1 Anaerobic culture of different mutant strains

Glycerol can be effectively metabolized by the rumen bacteria such as *A. succiniciproducens* (Lee et al., 2001), but hardly by *E. coli*. Wild-type *E. coli* MG1655 grew very slowly on glycerol anaerobically, and the DCW increased from 0.05 to only 0.10 g/L in 6 days. The major products were formate and ethanol, with small amounts of succinate and acetate, and no lactate was detected (Table 3). Disrupting the competing pathways of succinate production is a way to improve succinate production from glycerol. One mol lactate formation needs one mol glycerol together with 1 mol NADH net production. Hence, the formation of lactate is redox unbalanced when glycerol is used as carbon source. The *ldhA* single deletion in MG1655 had little effect on succinate production and glycerol consumption (Table 3). To reduce production of formate and ethanol, the *pflB* gene encoding pyruvate formate-lyase was deleted. Deletion of *pflB* (strain MB) greatly reduced the consumption of glycerol, but the yield of succinate was improved from 0.05 to 0.29

mol/mol and lactate became the major byproduct. The double mutant strain MLB was constructed by deletion of *ldhA* in MB. The concentrations of ethanol and acetate formed by MLB were decreased and no lactate was produced. The yield of succinate was further improved to 0.37 mol/mol. However, MLB only utilized 4.4 mM glycerol in 6 days under anaerobic fermentation condition. Because of the low cell mass and slow utilization of glycerol under anaerobic condition, a two-stage fermentation strategy was adopted for further improving succinate production.

3.2 Two-stage fermentation using different mutant strains

Two-stage fermentation was carried out in flasks using different strains and the results are summarized in Table 4. The wildtype strain MG1655 consumed 48.1 mM glycerol in 72 h and the yield of succinate was 0.12 mol/mol. Single deletion of *ldhA* gene encoding lactate dehydrogenase had little effect on the yield of succinate, while the glycerol consumption rate decreased. Interestingly, deletion of *pflB* increased the yield of succinate from 0.12 to 0.68 mol/mol in the anaerobic stage of two-stage fermentation. At the same time, small amount of lactate was produced. The double mutant MLB remarkably reduced the formation of byproducts, and the yield of succinate increased from 0.12 to 0.93 mol/mol, approaching the theoretical yield (1 mol/mol). The glycerol consumption was improved because of the higher cell density. Therefore, two-stage fermentation was more suitable than anaerobic fermentation for succinate production from glycerol by strain MLB.

3.3 Effects of *mdh*, *pyc*, *ppc* and *maeA* overexpression

It is well known that MDH, ME, PPC and PYC play important roles in succinate production from glucose in *E. coli* (Millard et al.,1996; Stols et al.,1997; Vemuri et al.,2002; Wang et al.,2009). Therefore, overexpression of the genes coding for these enzymes in MLB was investigated, respectively.

Overexpression of MDH, ME and PPC could not improve glycerol utilization and succinate production (Table 5) compared to MLB (Table 4). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is inhibited by accumulated NADH (Zhu et al., 2004). Wang et al. (2009) showed that overexpression of MDH in NZN111 improved the rates of glucose consumption and succinate formation due to the decrease of intracellular NADH/NAD⁺ ratio. This in turn could result in elimination of the inhibition of GAPDH. However, overexpression of MDH in MLB did not increase the glycerol consumption and succinate production. Since glycerol is a more reduced carbon source than glucose, the NADH/NAD⁺ ratio in the glycerol-grown cells is expected to be higher than that of glucose-grown cells under anaerobic condition, and the activity of GAPDH could still be inhibited. The K_m values of NAD⁺-dependent malic enzyme are 0.26 mM for malate and 16 mM for pyruvate (Stols et al., 1997). Thus, the favorite direction of the reaction catalyzed by ME should be from malate to pyruvate, and we speculated this might be the reason for the ineffectiveness of *maeA* overexpression. Liu et al. (2012) found that the enhancement of ATP supply could improve succinate production. The reaction catalyzed by PPC does not generate ATP, therefore, the performance of the strain

MLB overexpressing *ppc* was not satisfactory.

PYC catalyzes the irreversible carboxylation of pyruvate to form OAA, however, *E. coli* does not have PYC. Hence, the *pyc* of *C. glutamicum* was cloned and overexpressed in MLB. The constructed strain consumed 44.6 mM glycerol and produced 41.3 mM succinate, two times those of MLB/pTrc99a (Table 6). The succinate yield was 0.92 mol/mol. Blankschien et al. (2010) expressed pyruvate carboxylase from *L. lactis* to enhance the production of succinate from the pyruvate node under microaerobic condition. Thus, overexpression of PYC had positive effects on succinate production and glycerol consumption.

3.4 Effects of PCK overexpression

PCK, a key enzyme in gluconeogenesis, catalyzes the reaction of forming PEP from OAA. Use of this enzyme in the reverse direction to form OAA from PEP is advantageous to succinate production (Wu et al., 2007, Zhang et al., 2009), because ATP is formed rather than pyrophosphate. Therefore, expression of the *pck* gene from *E. coli* was investigated in different mutants, and the strains carrying the empty plasmid pTrc99a were used as the control strains. The glycerol utilization, succinate and by-products production are shown in Table 6.

In MG1655 and ML, *pck* expression resulted in improvement of the succinate yield from 0.12 to around 0.6 mol/mol. However, the amount of consumed glycerol and produced succinate were only slightly increased compared with the controls carrying pTrc99a (Table 6). Overexpression of PCK in MB enhanced the yield of

succinate from 0.68 to 0.83 mol/mol, and the amount of consumed glycerol increased by four-fold. At the same time, lactate was produced. Deletion of LDH further improved succinate production. The succinate yield of MLB/pTrc99a-*pck* achieved 0.92 mol/mol, which was similar to that of MLB/pTrc99a (0.93 mol/mol), but the concentration of succinate produced by MLB/pTrc99a-*pck* reached 118.1 mM, about 6 times that produced by MLB/pTrc99a (21.5 mM). During the anaerobic stage, the cell density of all strains decreased, however, *pck* overexpression lessened the decrease. This phenomenon could be attributed to more ATP produced in the reaction catalyzed by PCK.

3.5 Key enzyme activities in MLB/pTrc99a-pck related with succinate production

The above experiments indicated that overexpression of PCK in all the strains used greatly enhanced glycerol utilization and succinate production. To understand the effect of PCK overexpression, the activities of six enzymes in the MLB strains at the onset of the anaerobic culture were assayed. These enzymes included the anaplerotic enzymes ME, PCK and PPC, the first enzyme in the glyoxylate shunt ICL, the first enzyme in the reductive TCA cycle MDH, and the enzyme PK converting PEP to pyruvate.

The differences in specific activities of these enzymes in MLB and MLB/pTrc99a were not statistically significant, while the differences in the specific activities of ICL, PCK, ME, and MDH in the strain expressing PCK were significant: 5.9, 4.0, 1.5, and 1.5 times, respectively, those without the overexpression of PCK

(Fig. 1). The specific activities of PK were almost the same in these strains, and no pyruvate was detected. PPC is regulated by multiple effectors. When cells were grown on glycerol, the enzyme activity of PPC was depressed (Morikawa et al., 1980). Millard et al. (1996) found overexpression of PCK had no effect on succinate production using glucose as carbon source, and the enzyme activity of PPC was decreased. However, previous studies by other groups showed that increased expression of PCK could facilitate the flux through PCK (Zhang et al., 2009; Zhang et al., 2010). The K_m value for HCO_3^- of PCK (13.0 mM) was higher than that of PPC (0.15 mM). Deok et al. (2006) assumed that glyconeogenic PCK is more suitable for succinate production at high concentration of HCO_3^- . In our study, the specific activity of PPC of these three strains changed little. The great enhancement of succinate production in the *pck* overexpression strain indicated that the anaplerotic activity and ATP supply were critical to succinate production from glycerol.

3.6 Two-stage fermentation in the 1.5-L bioreactor

It was reported that the anaerobic growth of *E. coli* was very slow in minimal medium with glycerol as the carbon source (Murarka et al., 2008), and the low cell density caused a long fermentation time. In order to increase the cell density, MLB/pTrc99a-*pck* was grown in the medium containing 565 mM of glycerol under aerobic condition; and 0.5 mM IPTG was added at 12 h for inducing *pck* overexpression when the DCW reached 4.4 g/L. At 16 h, the DCW achieved 9.4 g/L, and the concentration of glycerol decreased to 166 mM. Then, the anaerobic

fermentation stage was initiated by sparging CO₂ into the bioreactor. When the concentration of glycerol dropped below 72 mM (at 24.5 h), a concentrated glycerol solution was added and the glycerol concentration increased to 365 mM. The total anaerobic stage lasted 114 h, and 360.2 mM succinate and 25.5 mM acetate were produced at the end of anaerobic fermentation. The profiles of cell density, residual glycerol and metabolites concentrations in the two-stage culture are shown in Fig. 2. Because insoluble basic magnesium carbonate was added in the anaerobic stage, the cell density was not measured in this stage. At the beginning of anaerobic stage, glycerol was consumed rapidly at a rate of 6.2 mM/h, and the productivity of succinate reached 5.8 mM/h. However, after 68 h, the glycerol consumption rate and succinate productivity dropped significantly, which were 1.0 and 0.97 mM/h, respectively. Compared with the experiments carried out in flasks, the productivity and final titer of succinate in the 1.5-L bioreactor were significant increased due to the high cell density (Table 6). The overall succinate yields in the whole two-stage fermentation and the anaerobic stage were 0.46 and 0.93 mol/mol, respectively, 46% and 93% of the theoretically yield (1 mol/mol).

Different strategies of fermentation, such as, anaerobic (Zhang et al., 2010), microaerobic (Blankschien et al., 2010) and aerobic (Li et al., 2013) cultures have been carried out for succinate production using glycerol as the carbon source, but two-stage fermentation for succinate production from glycerol has been scarcely applied. The yield of succinate in two-stage fermentation obtained in this study was higher than that of aerobic condition (Li et al., 2013), and the final titer of succinate

produced in this work was much higher than microaerobic or anaerobic fermentation (Blankschien et al., 2010; Zhang et al., 2010). Thus, two-stage fermentation is more suitable for succinate production from glycerol to reach higher yield and titer. This process should be further adapted to utilization of crude glycerol, a byproduct of biodiesel production, to reduce the cost of fermentation.

4. Conclusions

In this study, overexpression of native PCK in the double mutant MLB (deficient of both *pflB* and *ldhA*) greatly improved glycerol utilization and succinate production. Overexpression of *pck* also resulted in significant increases in the activities of MDH, ME and ICL. To improve the succinate production, two-stage fermentation was performed in shake flasks and the titer of succinate reached 118.1 mM with a yield of 0.93 mol/mol. Two-stage fermentation was also carried out in a 1.5-L bioreactor, 360.2 mM succinate was produced with a yield of 0.93 mol/mol in the anaerobic stage.

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Table 1. Strains and plasmids used in this study.

Strains and plasmids	Relevant characteristics	Sources
Strains		
MG1655	<i>F⁻ lambda⁻ ilvG⁻ rfb⁻ rph⁻</i>	CGSC
ML	MG1655 Δ <i>ldhA</i> ::FRT	This study
MB	MG1655 Δ <i>pflB</i> ::FRT	This study
MLB	MG1655 Δ <i>ldhA</i> ::FRT Δ <i>pflB</i> ::FRT	This study
Plasmids		
pKD4	Km ^R , oriR6K γ , <i>rgnB</i> (Ter)	CGSC
pKD46	Ap ^R , <i>araBp-gam-bet-exo</i> , repA101 (ts), oriR101	CGSC
pCP20	Ap ^R , Cm ^R , FLP recombination	CGSC
pTrc99a	Cloning vector, Ap ^R , ColE1 origin vector	Lab stock
pTrc99a- <i>pck</i>	pTrc99a carrying PEP carboxykinase (<i>pck</i>) from <i>E. coli</i>	This study
pTrc99a- <i>ppc</i>	pTrc99a carrying PEP carboxylase (<i>ppc</i>) from <i>E. coli</i>	This study
pTrc99a- <i>pyc</i>	pTrc99a carrying pyruvate carboxylase (<i>pyc</i>) from <i>C. glutamicum</i>	This study
pTrc99a- <i>maeA</i>	pTrc99a carrying malic enzyme (<i>maeA</i>) from <i>E. coli</i>	Lab stock
pTrc99a- <i>mdh</i>	pTrc99a carrying malate dehydrogenase (<i>mdh</i>) from <i>E. coli</i>	Wang et al., 2009

Table 2. Primers used in this study

Name	Sequence
<i>ldhA</i> -sense	5'-ATGAAACTCGCCGTTTATAGCACAAAACAGTACGACAAGAAGTACCTGCAGTGTAGGCTGGAGCTGCTTC-3'
<i>ldhA</i> -antisense	5'-TTAAACCAGTTCGTTCGGGCAGGTTTCGCCTTTTTCCAGATTGCTTAAGTCATATGAATATCCTCCTTAG-3'
<i>pflB</i> -sense	5'-ATGTCCGAGCTTAATGAAAAGTTAGCCACAGCCTGGGAAGGTTTACCAAGTGTAGGCTGGAGCTGCTTC-3'
<i>pflB</i> -antisense	5'-TTACATAGATTGAGTGAAGGTACGAGTAATAACGTCCTGCTGCTGTTCTTCATATGAATATCCTCCTTAG-3'
<i>ldhA</i> -test-F	5'-GCCTACACTAAGCATAGTTGTTGAT-3'
<i>ldhA</i> -test-R	5'-GGTTCTCAATTACAGTTTCTGACTC-3'
<i>pflB</i> -test-F	5'-ACATACTGGGTCATTTACCTGCGTG-3'
<i>pflB</i> -test-R	5'-GCTGACAAAAAAGGCTCTCGCGCTA-3'
<i>pck</i> -F	5'-GAGCGAATTCATGCGCGTTAACAATGGTTT-3' (<i>Eco</i> RI)
<i>pck</i> -R	5'-CTTCGGATCCTTACAGTTTCGGACCAGCCG-3' (<i>Bam</i> HI)
<i>ppc</i> -F	5'-CCGGAATTCATGAACGAACAATATCCGCATT-3' (<i>Eco</i> RI)
<i>ppc</i> -R	5'-AAGAAAGCTTTTAGCCGGTATTACGCATACCTG-3' (<i>Hind</i> III)
<i>pyc</i> -F	5'-CTAACCATGGTAGTGTGCGACTCACACATCTTCAAC-3' (<i>Nco</i> I)
<i>pyc</i> -R	5'-TTGCTCTAGAGTTTAGGAAACGACGACGATCAAGT-3' (<i>Xba</i> I)

Table 3. Succinate production from glycerol by *E. coli* mutant strains in anaerobic fermentation.

Strain	DCW (g/L)		Initial glycerol (mM)	Consumed glycerol (mM)	Product (mM)					Yield of succinate (mol/mol)
	Initial	Final			Succinate	Acetate	Ethanol	Formate	Lactate	
MG1655	0.05	0.10±0.02 ^a	167.2±0.8	44.2±5.4	2.3±0.2	1.3±0.1	17.6±1.7	11.5±1.9	0	0.05±0.00
ML	0.04	0.06±0.01	168.3±1.5	43.0±3.0	2.3±0.2	1.1±0.2	16.1±3.0	10.4±1.7	0	0.05±0.01
MB	0.05	0.07±0.01	169.4±2.2	7.2±0.3	2.1±0.1	0.8±0.0	0	0	1.2±0.2	0.29±0.01
MLB	0.05	0.07±0.01	165.9±0.6	4.4±0.4	1.6±0.0	0.7±0.1	0	0	0	0.37±0.04

The anaerobic fermentation lasted 6 days.

^a Data are means ± standard deviations with three replicates.

Table 4. Succinate production from glycerol by *E. coli* mutant strains in the anaerobic stage of two-stage fermentation.

Strain	DCW (g/L)		Initial glycerol (mM)	Consumed glycerol (mM)	Products (mM)					Yield of succinate (mol/mol)
	Initial	Final			Succinate	Acetate	Ethanol	Formate	Lactate	
MG1655	4.80	3.04±0.04 ^a	152.9±0.3	48.1±1.6	5.7±0.1	7.4±0.4	26.9±0.3	24.3±0.5	0	0.12±0.01
ML	4.25	2.56±0.02	155.7±1.1	29.5±1.0	3.7±0.0	1.0±0.1	17.2±0.1	17.7±0.0	0	0.12±0.01
MB	4.48	2.73±0.01	152.1±0.7	25.8±0.4	17.8±0.2	0	3.1±0.1	0	7.1±0.2	0.68±0.01
MLB	4.95	3.13±0.02	153.5±0.4	31.1±1.0	28.9±1.1	0	3.1±0.1	0	0	0.93±0.03

The anaerobic stage lasted 72 h.

^a Data are means ± standard deviations with three replicates.

Table 5. Succinate production by MLB expressing different genes in the anaerobic stage of two-stage fermentation carried out in flasks

Plasmid in MLB	DCW (g/L)		Initial glycerol (mM)	Consumed glycerol (mM)	Product (mM)					Yield of succinate (mol/mol)
	Initial	Final			Succinate	Acetate	Ethanol	Formate	Lactate	
pTrc99a- <i>ppc</i>	4.75	3.12±0.03 ^a	161.2±0.6	19.1±0.3	16.9±0.1	0.5±0.1	1.6±0.2	0	0	0.89±0.02
pTrc99a- <i>pyc</i>	4.93	3.51±0.01	159.8±1.2	44.6±0.3	41.3±0.1	1.0±0.1	1.7±0.1	0	0	0.92±0.01
pTrc99a- <i>maeA</i>	4.79	3.15±0.05	162.1±0.9	17.1±0.7	15.5±1.1	0	0.3±0.1	0	0	0.91±0.03
pTrc99a- <i>mdh</i>	4.80	3.17±0.01	158.9±0.3	23.2±0.2	21.1±0.5	0	0.7±0.1	0	0	0.92±0.01

The anaerobic stage lasted 72 h.

^a Data are means ± standard deviations with three replicates.

Table 6. Effects of *pck* overexpression in different strains in the anaerobic stage of two-stage fermentation performed in flasks and a 1.5-L bioreactor.

Strain	Reactor	DCW (g/l)		Initial glycerol (mM)	Consumed glycerol (mM)	Product (mM)					Yield succinate (mol/mol)
		Initial	Final			Succinate	Acetate	Ethanol	Formate	Lactate	
MG1655/pTrc99a	F ^a	4.38	2.77±0.03 ^b	155.7±0.3	22.78±1.2	3.3±0.0	2.9±0.1	12.7±0.6	15.5±0.0	0	0.14±0.01
MG1655/pTrc99a- <i>pck</i>	F	4.42	3.17±0.07	153.5±0.5	36.82±0.7	23.1±1.5	0	9.5±0.5	11.7±0.2	0	0.63±0.02
ML/pTrc99a	F	4.67	2.44±0.03	154.3±1.1	23.6±1.4	2.8±0.1	1.1±0.0	12.1±0.2	14.9±0.2	0	0.12±0.01
ML/pTrc99a- <i>pck</i>	F	4.30	2.71±0.07	155.5±0.8	30.6±0.9	18.4±0.5	0.5±0.1	4.8±0.1	7.6±0.1	0	0.60±0.02
MB/pTrc99a	F	4.62	2.69±0.02	156.8±0.2	23.9±1.5	14.8±0.4	0	2.5±0.2	0	7.0±0.1	0.62±0.01
MB/pTrc99a- <i>pck</i>	F	4.54	3.23±0.02	157.2±0.1	107.3±1.2	89.5±0.6	1.1±0.2	1.1±0.1	0	10.4±0.5	0.83±0.02
MLB/pTrc99a	F	5.75	3.40±0.03	158.7±1.5	23.5±2.4	21.5±0.5	0	3.9±0.2	0	0	0.93±0.01
MLB/pTrc99a- <i>pck</i>	F	5.28	4.15±0.01	160.3±0.2	127.3±1.5	118.1±1.6	0.7±0.1	0.2±0.1	0	0	0.92±0.01
MLB/pTrc99a- <i>pck</i>	R ^c	9.37±0.14	NM ^d	165.9±10.5	388.5±21.4	360.2±20.8	25.5±2.2	0	0	0	0.93±0.01

The anaerobic stage for flasks and 1.5-L reactor lasted 72 and 114 h, respectively.

^a Fermentation carried out in flasks.

^b Data are means ± standard deviations with three replicates.

^c Fermentation carried out in 1.5-L reactor. ^d Not measured.

Figure Legends

Figure 1. Effects of overexpression of *pck* in MLB under aerobic culture on specific activities of key enzymes related with succinate production at the end of the aerobic stage in MLB with or without *pck* overexpression. ME, NAD⁺-dependent malate dehydrogenase (malic enzyme); PK, pyruvate kinase; PPC, phosphoenolpyruvate carboxylase; PCK, phosphoenolpyruvate carboxykinase; MDH, malate dehydrogenase; ICL, isocitrate lyase.

Figure 2. Profiles of DCW (●), glycerol (◆), acetate (■) and succinate (▲) in two-stage fermentation using MLB/pTrc99a-*pck* carried out in a 1.5-L bioreactor.

Fig. 1

Fig. 2