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Efficient anaerobic production of succinate from glycerol in engineered *Escherichia coli* by using dual carbon sources and limiting oxygen supply in preceding aerobic culture

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Abstract

Glycerol is an important resource for production of value-added bioproducts due to its large availability from the biodiesel industry as a by-product. In this study, two metabolic regulation strategies were applied in the aerobic stage of a two-stage fermentation to achieve high metabolic capacities of the *pflB ldhA* double mutant *Escherichia coli* strain overexpressing phosphoenolpyruvate carboxykinase (PCK) in the subsequent anaerobic stage: use of acetate as a co-carbon source of glycerol and restriction of oxygen supply in the PCK induction period. The succinate concentration achieved 926.7 mM with a yield of 0.91 mol/mol during the anaerobic stage of fermentation in a 1.5-L reactor. qRT-PCR indicated that the two strategies enhanced transcription of genes related with glycerol metabolism and succinate production. Our results showed this metabolically engineered *E. coli* strain has a great potential in producing succinate using glycerol as carbon source.

Keywords: succinate; glycerol; acetate; *Escherichia coli*; two-stage fermentation

1. Introduction

Non-food based renewable substrates are attractive feedstock to replace sugars to produce bio-based bulk chemicals, and is the new trend in bio-manufacturing. Biodiesel is one of the most promising alternate and renewable fuels, and its production generates about 10% (w/w) of glycerol as a byproduct (Yang et al., 2012). The fast growth of biodiesel production has generated large quantities of crude glycerol, leading to a drastic decrease in its price. Great attentions have been paid to development of new technologies to convert glycerol to value-added chemicals. Previous studies have shown that glycerol could be biologically converted to higher value products, such as 1,3-propanediol (Zhong et al., 2014), 3-hydroxypropionic acid (Huang et al., 2012), ethanol (Nikel et al., 2010), succinate (Choi et al., 2016), free fatty acids (Wu et al., 2014), and etc.

Succinate was evaluated by the U.S. Department of Energy as one of the top 12 building block chemicals produced from biomass (Werpy and Petersen, 2004). It is widely used in production of foods, pharmaceuticals, and biodegradable plastics (Zeikus et al., 1999). Traditionally, succinate is produced from petroleum-derived maleic anhydride, which, however, is unrenewable. A green bioprocess is urgently required to produce succinate from renewable resources. Many previous investigations focused on converting sugars to succinate by bacteria, including *Actinobacillus succinogenes* (Mckinlay et al., 2005; Zhao et al., 2016), *Anaerobiospirillum succiniciproducens* (Lee et al., 2008), *Mannheimia succiniciproducens* (Choi et al., 2016), and metabolically engineered *Escherichia coli* (Vemuri et al., 2002). In this study, glycerol was chosen as the substrate for succinate production. Biosynthesis of succinate, a C_4-dicarboxylic acid, from glycerol needs fixation of CO_2, a major greenhouse gas which leads to the global warming. Succinate

is synthesized aerobically as an intermediate of TCA cycle; it also can be synthesized from PEP through PCK or PPC enzymes under anaerobic fermentation conditions. The processes of succinate production could be combined with ethanol fermentation to reduce the emission of CO_2 as shown in our previous study (Wu et al., 2012). Theoretically, through anaerobic fermentation of glycerol, production of 1 mol succinate consumes 1 mol CO_2 (Fig S1), indicating succinate production from glycerol can be an effective process for CO_2 fixation. In addition, the process to convert glycerol to succinate is redox balanced, and thus is superior to that using glucose. Therefore, it has great potential to improve the microbial succinate biosynthesis from glycerol.

E. coli does not naturally accumulate large amount of succinate in fermentation. To improve succinate production in *E. coli*, competitive pathways were disrupted, such as pyruvate:formate lyase (*pflB*), lactate dehydrogenase (*ldhA*) (Vemuri et al., 2002), alcohol dehydrogenase (*adhE*) (Sanchez et al., 2005a), and phosphate acetyltransferase-acetate kinase (Sanchez et al., 2005b). Another strategy to improve succinate production is to overexpress genes directly involved in the succinate synthesis pathway, including those coding for native or exogenous CO_2-fixation enzymes: phosphoenolpyruvate (PEP) carboxykinase (PCK) (Liu et al., 2012), PEP carboxylase (PPC) (Millard et al., 1996), pyruvate carboxylase (PYC) (Lin et al., 2004), and malic enzyme (ME) (Stols et al., 1997). Simplified metabolic pathways of succinate production form glycerol are shown in Fig. S1. *E. coli* can hardly grow under anaerobic conditions using glycerol as the carbon source without existence of exogenous electron acceptor (Lin, 1976). Recently, researchers reported the consumption of glycerol under particular anaerobic conditions (Dharmadi et al., 2006), but the glycerol consumption rate remained low. In order to increase production of

succinate from glycerol, a series of genes, including *atpE*, *fdoH* and so on, were knocked out individually based on the results of simulation, and the fermentation results showed that disruption of these genes had positive effects on succinate produced from glycerol (Mienda et al., 2016a; Mienda et al., 2016b). The highest succinate concentration of these engineered strains increased to about 2 g/L after 7 days of fermentation. An engineered *E. coli* was constructed by interrupting *pflB* and *ptsI* together with mutation on the promoter of *pck* to increase its expression. As a result, 102 mM of succinate was accumulated in 144 h (Zhang et al., 2010). PYC from *Lactococcus lactis* was also overexpressed to convert glycerol to succinate under microaerobic condition by engineered *E. coli* strain, whose pathways of byproducts were blocked. The concentration of succinate reached to 118 mM in this case (Blankschien et al., 2010). Aerobic production of succinate from glycerol was also examined by using the engineered strain *E. coli* E2-*Δsdh-ppc-sucAB* (Li et al., 2013). Although the final concentration and productivity of succinate were higher than those obtained under anaerobic and micro-aerobic conditions, the succinate yield dropped dramatically. Previously, two-stage fermentation was used to enhance succinate production from sugars (Vemuri et al., 2002; Wu et al., 2007). We have constructed an *E. coli* strain MLB/pTrc99A-*pck* by deleting *pflB* and *ldhA* and overexpressing *pck*. This strain produced 118.1 and 360.2 mM of succinate in flasks and a 1.5-L bioreactor, respectively, in two-stage fermentation (Li et al., 2016a). Supplementary Table S1 summarizes the fermentation of *E. coli* to produce succinate with glycerol as the carbon source by various investigators.

Gene expression can be regulated by the carbon source under aerobic conditions in *E. coli* (Kao et al., 2005). When *E. coli* uses acetate as the carbon source for growth, the enzymes benefitting succinate production are up-regulated, including those in

glyoxylate shunt, reductive TCA cycle, and gluconeogenesis (Oh et al., 2002; Wu et al., 2007). Meanwhile, *E. coli* has three pathways involved in the respiratory (aerobic respiratory, GlpK-GlpD; anaerobic respiratory, GlpK-GlpABC) and fermentative utilization of glycerol (fermentative route, GldA-DhaKLM) (Fig. S1) (Durnin et al., 2009). During the induction stage of Pck, changing the level of aeration, which affect the dissolved oxygen level of the culture, also can affect the gene expression of glycerol consumption and metabolism.

In this study, the metabolic regulation of the engineered strain, which was affected by the different aerobic cultivation strategies of the two-stage fermentation, was investigated for further increasing its succinate production from glycerol in the subsequent anaerobic stage. Acetate was used as a co-substrate in the aerobic stage to facilitate the consumption of glycerol in the subsequent anaerobic stage. Furthermore, it was found that limiting aeration during the induction of PCK was also critical to the improvement of glycerol consumption and succinate production.

2. Materials and Methods

2.1 Strain and Media

E. coli strain MLB (Δ*ldhA*::FRT Δ*pflB*::FRT) over-expressing *pck* (MLB/pTrc99a-*pck*) (Li et al., 2016a) was used exclusively in this study. This strain was stored in 25% (w/w) glycerol at -20 °C.

Luria-Bertani broth (LB), which contained (per liter) tryptone 10 g, yeast extract 5 g, and sodium chloride 10 g, was used for primary inoculum preparation. Ampicillin (100 mg/L) was included when needed.

Salt medium (SM) that was based on M9 contained (per liter) $Na_2HPO_4 \cdot 12H_2O$ 15.12 g, KH_2PO_4 3.0 g, NaCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 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: $FeSO_4 \cdot 7H_2O$ 80 g, $AlCl_3 \cdot 6H_2O$ 10 g, $ZnSO_4 \cdot 7H_2O$ 2.0 g, $CuCl_2 \cdot 2H_2O$ 1.0 g, $NaMoO_4 \cdot 2H_2O$ 2.0 g, $MnSO_4 \cdot H_2O$ 10 g, $CoCl_2$ 4.0 g, and H_3BO_4 0.5 g.

For the two-stage fermentation in shake flasks, media GSM, GASM and ASM, which were prepared by supplementing SM with 54.3 mM (5 g/L) of glycerol, 32.6 mM (3 g/L) of glycerol plus 24.4 mM (2 g/L) of sodium acetate, and 61.0 mM (5 g/L) of sodium acetate, respectively. The medium for anaerobic fermentation in flasks was SM supplemented with 163.0 mM (15 g/L) of glycerol and 20 g/L of basic magnesium carbonate but without NH_4Cl.

The medium of fermentation carried out in a 1.5-L bioreactor was SM containing 543.3 mM (50 g/L) glycerol and ammonium acetate [64.8 mM (5 g/L) or 129.7 mM (10 g/L)]. In addition, the concentrations of $Na_2HPO_4 \cdot 12H_2O$ and KH_2PO_4 were changed to 3.78 and 0.75 g/L, respectively. Ampicillin was included at 100 mg/L.

2.2 Culture Conditions

For two-stage fermentation carried out in flasks, the preculture was prepared by transferring 1mL of the stock culture to 30 mL of LB in a 250 mL flask and aerobically incubated at 37 °C and 220 rpm for 8 h. 2-mL aliquots of the preculture were transferred to 500 mL flasks containing 100 mL GSM, GASM or ASM, in which the cells were incubated at 37 °C and 220 rpm for 8 (GSM, GASM) or 10 h (ASM). Then, IPTG was added to a final concentration of 0.1 mM, and the cells were incubated for another 4 h. The cells were harvested by centrifugation at 4 °C and 6300×*g* for 5 min, and were resuspended in the anaerobic fermentation medium at a cell density around 15 (OD_{600}). 20-mL aliquots of the cells suspension were dispensed

to 50 mL schott bottles, and the headspace was filled with CO_2 to start the anaerobic culture at 37 °C and 220 rpm for 72 h. All the experiments were performed in triplicates.

For the experiments carried out in the bioreactor, the preculture was also prepared by transferring 1 mL of the stock culture to 30 mL of LB in a 250 mL flask and aerobically incubated at 37 °C and 220 rpm for 8 h. All the preculture was transferred to 1 L of medium in a 1.5-L bioreactor (BIOTECH-1.5; Baoxing Co., Shanghai, China) and incubated at 37 °C. The initial aeration and the agitation rates were 1 L/min and 300 rpm, respectively. The pH was controlled automatically at 7.0 in the aerobic stage by 1 M H_2SO_4 or 2 M NaOH, and it was kept above 6.3 by adding basic magnesium carbonate in the anaerobic stage. The aeration rate was 1 L/min and the agitation speed was 300 rpm at the beginning of fermentation. The dissolved oxygen (DO) was kept above 10% of air saturation by changing the agitation speed until the agitation reached maximal 900 rpm. To induce *pck* overexpression, 0.5 mM IPTG was added and the cells were further cultured for another 4 h. To investigate the effects of aeration during the PCK induction period, four different aeration rates (5, 2, 1.5 and 1 L/min) were examined, and these four cultivations were named as B1, B2, B3, and B4. During the anaerobic stage, anaerobic condition was obtained by stopping aeration and filling the headspace with CO_2 and the agitation speed was kept at 300 rpm. A bag full of CO_2 was connected to bioreactor to provide part of CO_2 used for succinate production and maintained anaerobic condition. Basic magnesium carbonate was added to 20 g/L at the commencement of anaerobic stage. To maintain the pH above 6.3, additional basic magnesium carbonate was added into the bioreactor depending on the acid production rate.

2.3 Analytical Methods

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}), which was converted to dry cell weight (DCW) according to a relationship between OD_{600} and DCW (1 OD_{600} was equivalent to 0.333 g DCW/L). Culture samples were centrifuged for 10 min at 4°C and 13,000×*g* in a microcentrifuge to remove the cells. The resulting supernatant was filtered through a 0.22 μm nylon syringe filter. Glycerol, ethanol, succinate and acetate 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 H_2SO_4 at 0.6 mL/min.

2.4 Quantitative Real-time PCR (qRT-PCR)

At the end of the aerobic growth stage cells were taken for extracting total RNA. Total cellular mRNA was extracted with RNAeasy Mini Kit (Tiangen, China) according to the instruction of the manufacturer. The quantity and purity of RNA were determined by comparison of the optical densities measured at 260 nm and 280 nm using NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). 1 % formaldehyde agarose-gel electrophoresis was also carried out to confirm satisfaction of the requirements of RT-PCR experiment.

The cDNA was obtained by GoScript Reverse Transcription System Kit (Promega, USA). The reaction was carried out in a MJ Mini PCR system (Bio-Rad, USA). The reaction mixture was incubated for 5 min at 25°C for primer extension, 15 min at 42 °C for reverse transcription, and then 15 min at 70°C for inactivation of the

reverse transcriptase. Then, the synthesized cDNA was diluted 10-fold with nuclease-free water and stored at -20°C until further use. The genes and their primers used for qRT-PCR analysis are listed in Table S2. The transcriptional level of 16s RNA is relatively constant, and it was selected as the internal standard. qRT-PCR was carried out using the Bio-Rad CFX96 Real-Time PCR system (Bio-Rad, USA) with GoTaq qPCR Master Mix (Promega, USA). The reaction mixture was incubated for 10 min at 95°C for Taq activation, followed by 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/extension). The qRT-PCR measurement of each gene was performed in triplicate.

The comparative C_T method was used for relative quantification of gene expression. The *rrsA* gene encoding 16S ribosomal RNA was used as a housekeeping gene to standardize the amount of template added to the reaction (Martínez et al., 2010). The difference between the C_T of the gene of interest and that of the reference gene (ΔC_T) was calculated for all the genes measured. The comparative expression level was calculated using the formula $2^{-\Delta\Delta CT}$ as previously described (Li et al., 2016b).

2.5 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

Since *E. coli* metabolizes glycerol slowly even under aerobic conditions and the *pflB ldhA* double mutant can hardly metabolize glycerol anaerobically, two-stage

fermentation which contained an aerobic growth stage and a subsequent anaerobic production stage was adopted to enhance glycerol consumption and succinate production at a higher cell density (Li et al., 2016a). Even though this strategy was effective for succinate production from glucose (Wu et al., 2007), the aerobically GSM-grown MLB cells consumed glycerol and produced succinate very slowly in the anaerobic stage (Li et al., 2016a). To solve these problems, different cultivation strategies based on metabolic regulation were investigated in this study.

3.1 Effects of acetate supplementation in the aerobic stage

Bacterial metabolism is regulated by the carbon source in the culture medium. For example, the aerobically acetate-grown NZN111 cells restored the abilities of quick glucose consumption and succinate production in subsequent anaerobic fermentation (Wu et al., 2007). In this study, glycerol, glycerol plus acetate, and acetate were used as carbon sources in the aerobic growth stage, respectively. The results in the anaerobic stage using these aerobically grown cells on different carbon sources during the aerobic stage are shown in Table 1.

No matter what carbon source was used during the aerobic stage, MLB could hardly convert glycerol to succinate in the anaerobic stage effectively. Although the succinate produced by the ASM-grown MLB cells (grown on acetate) was lower than the GSM-grown (grown on glycerol) cells, the succinate yield was the highest. In contrast, the cells grown on GASM (glycerol plus acetate) produced 19.23 mM succinate, which was 1.58 and 3.31 times that produced by the GSM-grown and ASM-grown cells, respectively. Without induction of PCK in MLB/pTrc99a-*pck*, the succinate concentration and yield were slightly increased, about 23.8% and 24.3% higher than that of GASM-grown MLB, respectively. The enhancement of succinate

production could be attributed to the leakage expression of PCK under the control of *trc* promoter in a glucose free medium. Induction of PCK by addition of IPTG resulted in significantly increased glycerol consumption and succinate production in both of GASM-grown and GSM-grown MLB/pTrc99a-*pck*. ATP gained in the reaction catalyzed by PCK can meet the ATP requirement for transporters and maintenance. The concentration of succinate produced by GASM-grown MLB/pTrc99a-*pck* was 46.4% higher than that of the GSM-grown MLB/pTrc99a-*pck*. The specific succinate production rate obtained by the GASM-grown cells (459.88 μmol/g DCW·h) was 1.80 times that of the GSM-grown MLB/pTrc99a-*pck* (255.49 μmol/g DCW·h). Furthermore, during the anaerobic fermentation, very little acetate and ethanol were detected. Therefore, a high succinate yield about 0.93 mol succinate/mol glycerol was reached.

Interestingly, all of the ASM-grown cells could not consume glycerol quickly in the subsequent anaerobic stage, even though previous study indicated that the acetate-grown NZN111 cells could efficiently convert glucose to succinate a similar process (Wu et al., 2007). A feature of the aerobic metabolism of MLB or MLB/pTrc99a-*pck* in the GASM medium was that acetate and glycerol were metabolized simultaneously. The fact that enhanced glycerol consumption and succinate production by the cells grown on GASM implied that the enzymes in glycerol metabolism pathway should be up regulated by the presence of glycerol in the aerobic stage, especially during the PCK induction period. It is indeed desirable that a series of glycerol assimilation pathway enzymes should be expressed during aerobic phase before anaerobic fermentation for the efficient glycerol consumption in the subsequent anaerobic stage. In the flask experiments, the transcriptional levels of genes for anaerobic glycerol dissimilation were up regulated in GASM as compared to that in ASM (Fig. 1B). This

might be caused by the higher cell density obtained in the former medium that resulted in lower dissolved oxygen level. The ratio of GlpD to GlpABC is high when oxygen or nitrate serves as a terminal electron acceptor and low when fumarate serves this role (Freedberg and Lin, 1973). Some of the glycerol dissimilation genes are induced by glycerol-3-phosphate (Lin, 1976) which is higher when glycerol is used as carbon source. Hence, the genes expression of glycerol dissimilation pathway are decided by carbon source and oxygen level. During the stage of inducing PCK expression, DO might be low because of cell growth. Therefore, it could also be considered as a microaerobic stage. The fermentative pathway might be activated for anaerobic glycerol dissimilation.

3.2 Comparison of gene transcription in cells grown on different carbon sources

Since the MLB/pTrc99a-*pck* cells aerobically grown in GASM and inducted with IPTG could convert glycerol to succinate efficiently, the transcriptional levels of key enzymes related with glycerol consumption and succinate production were investigated by using qRT-PCR. The GSM-grown MLB/pTrc99a-*pck* was used as a control. Samples of cells grown on different carbon sources with induction by IPTG were taken at the end of the aerobic growth stage for extracting total RNA. The qRT-PCR results of genes encoding the key enzymes are shown in Fig. 1.

The glyoxylate shunt gene *aceA* encoding isocitrate lyase was greatly up-regulated in MLB/pTrc99a-*pck* grown in GASM, compared to that of the GSM-grown one, while the ASM-grown cells showed a much higher transcriptional level (Fig 1A). The *aceBAK* operon is under control of IclR and FadR. It has been reported that when *E. coli* is grown on acetate, *aceA* gene is significantly up-regulated (Oh et al., 2002). The glycolytic pathway provides PEP which can be converted to oxaloacetate (OAA),

the precursor of succinate. The up-regulation of *aceA* could be due to down-regulation of IclR and FadR. Triosephosphate isomerase (encoded by *tpiA* gene) catalyzes isomerization between glyceraldehyde 3-phosphate and didhydroxyacetone phosphate, a reaction involved in both glycolysis and gluconeogenesis. The gene *tpiA* was up-regulated in cells grown on glycerol plus acetate, while it was down-regulated in cells grown on acetate (Fig. 1A). This phenomenon indicated that presence of glycerol is necessary in the aerobic stage for effective metabolism of glycerol in the subsequent anaerobic stage.

Three pathways are involved in glycerol consumption: GldA-DhaKLM, GlpK-GlpABC and GlpK-GlpD (Durnin et al., 2009). The first route involves glycerol metabolism without the presence of electron acceptor, while the latter two routes with the presence of electron acceptor; *glpD* encodes an aerobic dehydrogenase while *glpABC* encodes an anaerobic dehydrogenase. The transcription of *gldA* and *dhaK* in the route GldA-DhaKLM was up-regulated in cells grown in GASM, and thus could be favorable to anaerobic glycerol metabolism. The genes of *glpF* and *glpK*, encoding glycerol facilitator and glycerol kinase, respectively, are on the same operon (Berman-Kurtz et al., 1971), and both genes were up-regulated in cells grown in GASM (Fig. 1B). In previous study, it was shown that the strain of *glpK* deletion could not grow in mineral salt medium, and indicated that GldA-DhaKLM of the *glpK* mutant strain might not support anaerobic growth and succinate production (Zhang et al., 2010). The transcription of respiratory *glpA* and *glpD* in GASM-grown cells was down-regulated compared to that of GSM-grown cells. It has been shown that in microaerobic fermentation of glycerol, GldA-DhaKLM and GlpK-GlpD pathways all contribute to the conversion of glycerol to DHAP (Durnin et al., 2009). A recent study showed that the deletion of *glpC* could improve succinate from glycerol under

anaerobic condition, and the researchers hypothesized that the deletion of *glpC* activated the fermentative pathway of GldA-DhaKLM (Mienda and Shamsir, 2015). According to the results of qRT-PCR, we assumed that fermentative GldA-DhaKLM played the main role for glycerol consumption in the anaerobic stage of two-stage fermentation condition.

Fumarate reductase catalyzes the reduction of fumarate to succinate under anaerobic conditions. Malate dehydrogenase catalyzes malate to generate oxaloacetate, using NAD^+ as an electron acceptor. NAD^+-dependent malic enzyme catalyzes the decarboxylation of malate to form pyruvate which is a reversible reaction. These enzymes are favorable to succinate production. In this study, the related genes encoded these enzymes were *frdA*, *mdh* and *maeA*, and which were all up-regulated in GASM (Fig. 1C and Fig. 1D). These up-regulated genes, using MLB/pTrc99a-*pck* strain in GASM medium, are per se an advantage for the metabolic regulation strategy in order to improve succinate synthesis in *E. coli* by the reductive TCA pathway.

Lots of researches focused on the effects of *ppc* and *pck* overexpression on succinate production (Liu et al., 2012; Millard et al., 1996), because they both catalyze the reaction to convert pyruvate to OAA. In the reaction catalyzed by PCK, ATP is formed with the formation of OAA, and is favorable for succinate production (Zhang et al., 2009). Interestingly, compared with GSM-grown cells, the expression of *ppc* in the GASM-grown was slightly up-regulated (1.1-fold). The global transcription factor, Cra, activated the transcription of *pck* gene, while repressed the *ppc* gene, and Cra is inhibited by FBP (Matsuoka and Shimizu, 2011; Kochanowskiet et al., 2013). Overexpression of Cra in *E. coli* caused the down-regulation of *ppc* gene (Zhu et al. 2016). In the ASM-grown cells, lower FBP level activated Cra, and thus the transcription of *ppc* is repressed. Hence, in the ASM-grown cells *ppc* was sharply

down-regulated. The transcriptional levels of *pck* were both down-regulated in cells grown in GASM and ASM compared with that of GSM when IPTG were not added into these media. The existence of acetate in the culture medium inhibited the leakage of *pck* transcription in the metabolically engineered strains. The transcriptional level of *pck* was greatly improved in MLB/pTrc99a-*pck* (168.9-fold) grown in GASM when induced with IPTG as compared to MLB (Fig. 2). Hence, we speculated that PCK might not be the restriction factor for enhancing succinate production in the PCK overexpressed MLB/pTrc99a-*pck*.

The qRT-PCR data indicated that addition of acetate as a co-carbon source of the aerobic stage increased the expression of genes encoding enzymes related with anaerobic succinate production. In addition, the presence of glycerol was necessary, because the glycerol consumption pathways needed the existing of glycerol in the aerobic stage which can induce the transcription of these enzymes favorable to the subsequent anaerobic glycerol metabolism.

3.3 Effects of air supply on subsequent anaerobic glycerol metabolism

Oxygen level is quite important for the metabolic regulation in *E. coli* (Shimizu, 2013). In the aerobic stage of two-stage culture system, oxygen supply is expected to affect glycerol metabolism in the subsequent anaerobic stage. Hence, MLB/pTrc99a-*pck* was aerobically cultured in 500-mL flasks containing 50, 100 or 150 mL of GASM to provide the cells with different levels of aeration, and then the cells were harvested and transferred to the anaerobic medium for succinate production. The results of anaerobic fermentation are shown in Table 2.

The cells grown in 100 mL medium consumed 146 mM glycerol in the sequential anaerobic stage, 1.28 and 1.57 times that consumed by the cells grown in

50 mL and 150 mL medium, respectively. At the same time, 135 mM succinate was accumulated at the end of fermentation, which was the highest among these three conditions. The specific production rate of succinate reached 406 μmol/g DCW·h, about 1.3 times that of the cells grown in 50 mL and 150 mL medium. The succinate yields for the cultures grown in 50 mL and 100 mL medium were the same, but for the culture in 150 mL medium dropped a little because more acetate and ethanol were accumulated. These results showed that oxygen supply in the aerobic stage was very important for the glycerol utilization and succinate production in the subsequent anaerobic stage; excessive or insufficient oxygen supply could reduce succinate production.

3.4 Gene expression in cells grown at different aeration level

The medium volume in a flask directly influences the specific gas-liquid area and in turn the dissolved oxygen tension. The above experiments indicated that the medium volume remarkably affected the capabilities of glycerol consumption and succinate production in the subsequent anaerobic stage. To understand the reasons of the phenomenon, cells grown in medium with different volumes were harvested at the end of aerobic stage for qRT-PCR, and the relative transcriptional levels of genes coding for some key enzymes were compared and are shown in Fig. 3. The cells grown in 50 mL medium were used as a control.

The transcription of glyoxylate shunt gene *aceA* did not change in cells grown in 100 mL and 150 mL medium compared with that in 50 mL medium, and the glycolytic gene *tpiA* was slightly up-regulated when cells were grown in both 100 mL and 150 mL medium (Fig. 3A). The glycerol consumption genes *gldA* and *dhaK* were up-regulated when cells were grown in 100 mL medium. However, the *dhaK* gene in

cells grown in 150 mL medium was significantly up-regulated while *gldA* gene was slightly down-regulated (Fig. 3B). The genes, *glpA*, *glpK* and *glpF* were all down-regulated when cells grown in both 100 mL and 150 mL medium compared with that of 50 mL medium, but the change of the transcription of *glpD* was not statistically significant. We speculated that the GlpK-GlpD played the main role in glycerol metabolism for cell growth in the aerobic stage. The high transcription level of GldA-DhaKLM was benefit for succinate production in the anaerobic stage. The TCA cycle genes *frdA* and *mdh* were all up-regulated when cells were grown in 100 mL medium, while in cells grown in 150 mL medium, *frdA* was up-regulated but *mdh* was down-regulated (Fig. 3C). The anaplerotic genes, *maeA*, *ppc* and *pck* were also up-regulated when cells were grown in 100 mL medium. However, when cells grown in 150 mL medium, only *pck* was up-regulated significantly, whereas the transcriptional levels of the other two anaplerotic genes, *maeA* and *ppc*, dropped slightly. The total anaplerotic activity seemed to be changed little between cells grown in 50 mL medium and 150 mL medium (Fig. 3D).

The qRT-PCR data indicated that different air supply levels during cells growth resulted in significant changes of transcriptional levels of the genes related to glycerol consumption and succinate production. Hence, suitable oxygen supply in the aerobic stage was very important for glycerol consumption and succinate production during the anaerobic stage.

3.5 Two-stage fermentation in a 1.5-L bioreactor with different aeration in the induction phase

The above experiments carried out in flasks indicated that control of oxygen transfer in the aerobic stage was very important for subsequent anaerobic glycerol

consumption and succinate production. To be able to more precisely control aeration, two-stage fermentation was carried out in a 1.5-L bioreactor filled with 1 L of medium. After the addition of IPTG, the reactor was aerated at different aeration rates (1, 1.5, 2, and 5 L/min) but at the same agitation speed of 900 rpm. During the induction phase, DO was controlled below 1% at the aeration rate of 1, 1.5 and 2 L/min; and was above 10% at the aeration rate of 5 L/min. The profiles of glycerol, acetate, cell, and succinate concentrations are shown in Fig. 4.

For the aeration at 5 L/min, the overall two-stage fermentation lasted 134.5 h, in which the anaerobic stage lasted 116 h. During the anaerobic stage, 104.1 mM glycerol was consumed and the final succinate concentration was only 76.1 mM. The high aeration rate resulted in high growth rate of MLB/pTrc99a-*pck* in the PCK induction period. All the initial added acetate was completely consumed but then accumulated again due to the fast growth under substrate-excess conditions. At the beginning of the anaerobic stage, the concentration of acetate reached 47.5 mM and the final concentration of acetate was 49.3 mM at the end of the anaerobic stage (Fig. 4A). The yields of succinate were 0.16 mol/mol in the whole two-stage fermentation and 0.73 mol/mol in the anaerobic stage.

Succinate production was significantly improved when the aeration rate was reduced. At the aeration rate of 2, 1.5, and 1 L/min, the concentration of succinate achieved 405.3 (Fig. 4B), 486.9 (Fig. 4C), and 241.5 mM (Fig. 4D), which were 5.33, 6.40, and 3.17 times that produced by the cells induced at the aeration rate of 5 L/min, respectively. The yields of succinate in the anaerobic stage were 0.93, 0.91, and 0.82 mol/mol, respectively. The highest average specific succinate productivity achieved 511.5 μmol/g DCW·h at the aeration rate of 1.5 L/min. At the beginning of aerobic stage, acetate and glycerol were metabolized simultaneously, and then the initially

added acetate was consumed completely. Then the microaerobic condition achieved due to the high cell density in the bioreactor, and acetate started to be produced again. Under microaerobic conditions, ArcA will be activated and plays essential roles for redox regulation (Matsuoka and Shimizu, 2011; Shimizu, 2013), where *ackA* gene is also activated by ArcA for acetate production. At the lower aeration rates, acetate could be slightly consumed at the anaerobic stage, and it might be further involved in the synthesis of succinate through the glycolate shunt. When the concentration of glycerol dropped below 70 mM (at 55-60 h), a concentrated glycerol solution was added to increase glycerol concentration. Glycerol consumption was related to the succinate production. More glycerol was consumed at the aeration rate of 1.5 L/min (Table 3). The aeration rate of 1.5 L/min in the induction phase was most suitable for the subsequent succinate production form glycerol.

3.6 Further improvement of the anaerobic succinic acid production in the 1.5-L bioreactor

The above experiments showed the importance of co-metabolism of glycerol and acetate during the aerobic stage. However, in the above experiments performed in the 1.5-L reactor, the initial added acetate was completely consumed and then acetate was produced in the aerobic stage. Hence, acetate was accumulated before the commencement of anaerobic stage. In order to maintain the existence of acetate and glycerol for cell growth in the aerobic stage, we increased the initial concentration of ammonium acetate to 129.7 mM (10 g/L). After addition of IPTG, the aeration rate was adjusted to 1.5 L/min. This two-stage fermentation (named as B5) lasted 208 h, and the final succinate achieved 926.7 mM (Fig. 5). The overall yield of succinate was 0.74 mol/mol in two-stage fermentation (calculated according to the total glycerol

consumption in the whole two-stage fermentation) and 0.91 mol/mol in the anaerobic stage. The productivity of succinate increased to 5.2 mM/h. Thus, increasing the initial concentration of ammonium acetate could enhance the productivity of succinate.

The fermentation results of different culture in 1.5-L bioreactor are summarized in Table 3. In our previous study, MLB/pTrc99a-*pck* produced 360.2 mM succinate in 130 h with glycerol as the sole carbon source in both the aerobic and anaerobic stages, and the productivity of succinate was only 3.2 mM/h (Li et al., 2016a). Addition of acetate increased anaerobic consumption of glycerol and production of succinate. The final titer of succinate obtained in the present study was the highest ever reported with glycerol as the carbon source by *E. coli* strains.

4. Conclusions

In the aerobic stage, use of glycerol plus acetate as carbon source as well as suitable oxygen supply in the PCK induction period could remarkably increase glycerol consumption and succinate production in the subsequent anaerobic stage. qRT-PCR indicated that addition of acetate and limitation of oxygen supply up-regulated expression of genes related with anaerobic glycerol consumption and succinate production. The highest succinate concentration produced by the strain MLB/pTrc99a-*pck* achieved 926.7 mM with the yield of 0.91 mol/mol in the anaerobic stage. Under conditions suggested in the present investigation, our engineered *E. coli* strain can effectively produce succinate from glycerol.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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Figure captions:

Figure 1. The relative gene transcriptional levels in MLB/pTrc99a-*pck* grown in GASM and ASM compared with those in GSM at the end of aerobic growth. (A) Glycolysis and glyoxylate shunt genes; (B) Glycerol dissimilation genes; (C) TCA cycle genes; (D) Anaplerotic genes. *pck* ($P>0.05$), other genes ($P<0.05$).

Figure 2. The *pck* gene transcriptional levels in MLB/pTrc99a-*pck* (induced) compared with MLB grown in different media at the end of aerobic growth. *pck* related transcription level in GASM and ASM ($P>0.05$), comparsion of other group ($P<0.05$).

Figure 3. The relative gene transcriptional levels in MLB/pTrc99a-*pck* grown in 100 mL and 150 mL GASM compared with those in 50 mL medium at the end of aerobic growth. (A) Glycolysis and glyoxylate shunt genes; (B) Glycerol dissimilation genes; (C) TCA cycle genes; (D) Anaplerotic genes. *aceA, frdA, pck* ($P>0.05$), other genes ($P<0.05$).

Figure 4. Profiles of cell, glycerol, acetate and succinate in two-stage cultivations at different aeration rates during the induction phase in a 1.5-L ractor. Aeration rates: (A) 5 L/min (B-1); (B) 2 L/min (B-2); (C) 1.5 L/min (B-3); (D) 1 L/min (B-4).

Figure 5. Profiles of cell, glycerol, acetate and succinate in two-stage cultivation. The initial concentration of ammonium acetate of the aerobic stage was 129.7 mM and the aeration rate of the PCK induction period was 1.5 L/min (B-5).

673 Table 1. Effect of different aerobic culture medium on anaerobic glycerol consumption and succinate production

Strains	Aerobic culture medium	IPTG (mM)	Anaerobic fermentation						Specific production rate of succinate (μmol/ gDCW·h)
			Initial cell concentration (g DCW/L)	Consumed glycerol (mM)	Amt of product (mM)			Yield of succinate (mol/mol)	
					Succinate	Acetate	Ethanol		
MLB	GASM	0	5.80	27.24±0.88[a]	19.23±0.59	0.31±0.01	3.72±0.14	0.70±0.01	46.05±1.41
MLB	GSM	0	5.65	18.92±0.60	12.18±0.73	ND[b]	4.38±0.08	0.64±0.02	29.93±1.78
MLB	ASM	0	3.95	7.59±0.13	5.81±0.09	ND	1.26±0.03	0.76±0.01	20.42±0.28
MLB/pTrc99a-*pck*	GASM	0	4.43	27.49±3.69	23.81±3.22	ND	1.72±0.12	0.87±0.01	74.64±10.09
MLB/pTrc99a-*pck*	GSM	0	5.43	21.07±0.22	15.92±0.55	0.32±0.23	1.57±0..25	0.76±0.03	40.72±1.40
MLB/pTrc99a-*pck*	ASM	0	3.98	5.52±0.59	4.21±0.05	ND	ND	0.77±0.7	14.68±0.21
MLB/pTrc99a-*pck*	GASM	0.1	4.47	158.89±0.00	148.01±1.98	3.86±0.24	0.73±0.23	0.93±0.01	459.88±6.17
MLB/pTrc99a-*pck*	GSM	0.1	5.63	108.53±6.85	103.57±6.55	ND	2.34±0.09	0.95±0.01	255.49±16.15
MLB/pTrc99a-*pck*	ASM	0.1	3.93	3.42±0.42	2.92±0.01	ND	ND	0.76±0.03	10.33±0.01

674 The anaerobic stage lasted 72 h.

675 [a] Data are means ± standard deviations with three replicates.

676 [b] not detected.

Table 2. Effects of different medium volume in flask at aerobic stage on anaerobic fermentation

Medium volume in flask (mL)	Anaerobic fermentation						
	Initial cell concentration (g DCW/L)	Consumed glycerol (mM)	Amt of product (mM)			Yield of succinate (mol/mol)	Specific production rate of succinate (μmol/ g DCW·h)
			Succinate	Acetate	Ethanol		
50	5.03	113.51±0.73[a]	105.60±0.73	3.08±0.13	4.70±0.04	0.93±0.00	291.58±2.01
100	4.63	146.04±6.47	135.43±6.57	3.37±0.39	5.94±0.11	0.93±0.01	406.27±19.70
150	3.87	97.88±4.55	86.47±4.28	4.67±0.05	6.43±0.46	0.88±0.00	310.32±15.37

The anaerobic stage lasted 72 h.

[a] Data are means ± standard deviations with three replicates.

Table 3. Comparison of glycerol consumption and succinate production in different culture in 1.5L bioreactor

Culture	B-1	B-2	B-3	B-4	B-5	Ref. 43
Aerobic stage						
Carbon sources ($g \cdot L^{-1}$)	Glycerol 50; Ammonium acetate 5	Glycerol 50; Ammonium acetate 5	Glycerol 50; Ammonium acetate 5	Glycerol 50; Ammonium acetate 5	Glycerol 50; Ammonium acetate 10	Glycerol 50
Time (h)	18.6	20	20	20	30	16
Aeration rate in induction stage (vvm)	5	2	1.5	1	1.5	1.5
Maximum cell concentration ($g\ DCW \cdot L^{-1}$)	11.0	8.3	8.5	8.0	7.5	9.4
Anaerobic stage						
Time (h)	115.9	112	112	112	177.5	114
Final concentration of succinate ($mmol \cdot L^{-1}$)	76.1	405.3	486.9	241.5	926.7	360.2
Final concentration of acetate ($mmol \cdot L^{-1}$)	49.3	16.0	8.6	17.1	1.3	25.5
Glycerol consumption ($mmol \cdot L^{-1}$)	104.2	436.4	535.5	296.1	1029.1	388.5
specific succinate production rate ($\mu mol \cdot g\ DCW^{-1} \cdot h^{-1}$)	59.7	439.9	511.5	269.5	693.0	337.3
specific glycerol consumption rate ($\mu mol \cdot g\ DCW^{-1} \cdot h^{-1}$)	81.7	473.6	562.5	330.4	769.6	363.8
Yield of succinate in anaerobic stage ($mol \cdot mol^{-1}$)	0.73	0.93	0.91	0.82	0.91	0.93
Overall yield of succinate in two stage ($mol \cdot mol^{-1}$)	0.16	0.53	0.60	0.39	0.74	0.46

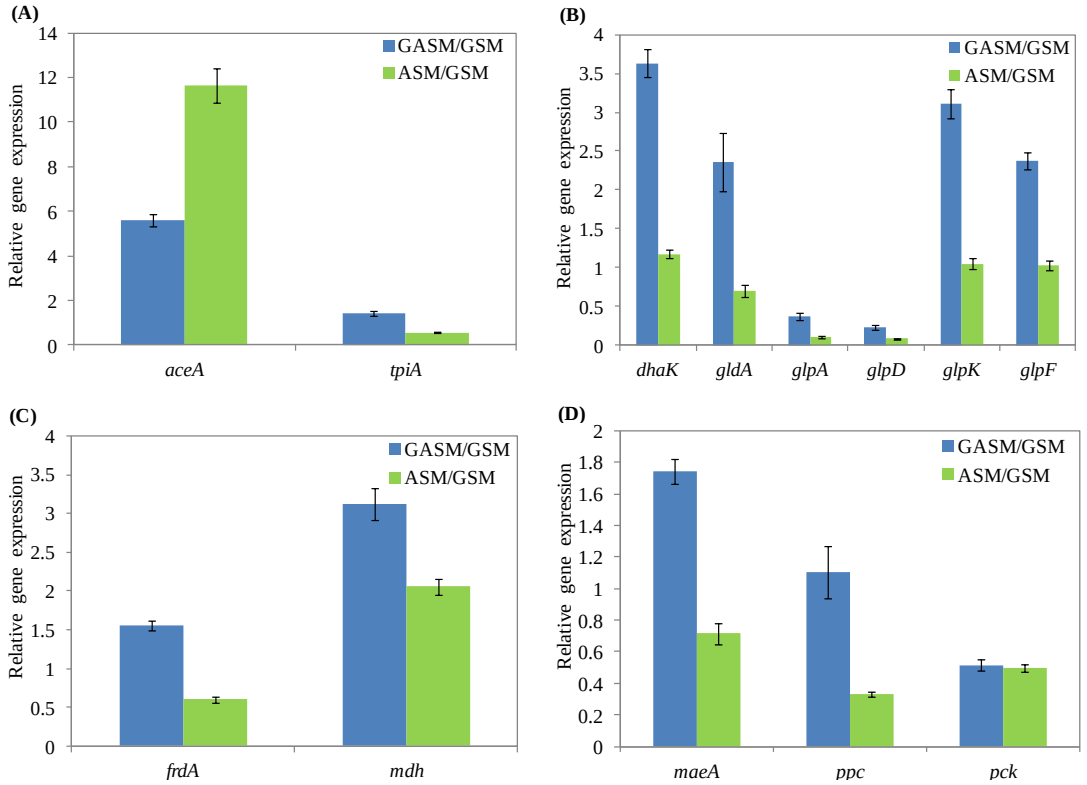
(A)
GASM/GSM
ASM/GSM
Relative gene expression
0
2
4
6
8
10
12
14
aceA
tpiA
(B)
GASM/GSM
ASM/GSM
Relative gene expression
0
0.5
1
1.5
2
2.5
3
3.5
4
dhaK
gldA
glpA
glpD
glpK
glpF
(C)
GASM/GSM
ASM/GSM
Relative gene expression
0
0.5
1
1.5
2
2.5
3
3.5
4
frdA
mdh
(D)
GASM/GSM
ASM/GSM
Relative gene expression
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
maeA
ppc
pck

Fig. 1

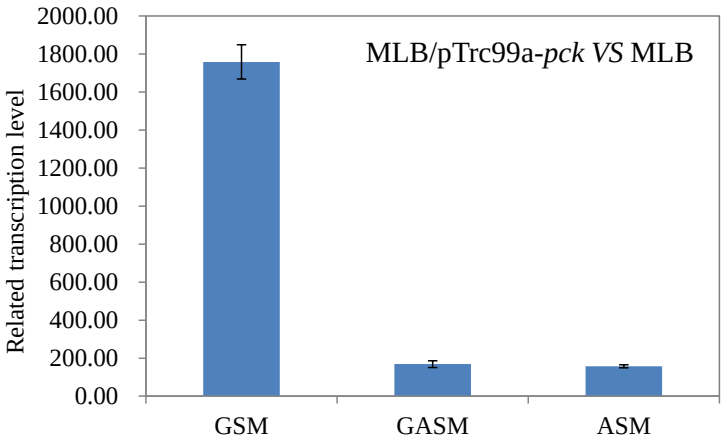
MLB/pTrc99a-*pck* *VS* MLB
Related transcription level
2000.00
1800.00
1600.00
1400.00
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
GSM
GASM
ASM

Fig. 2

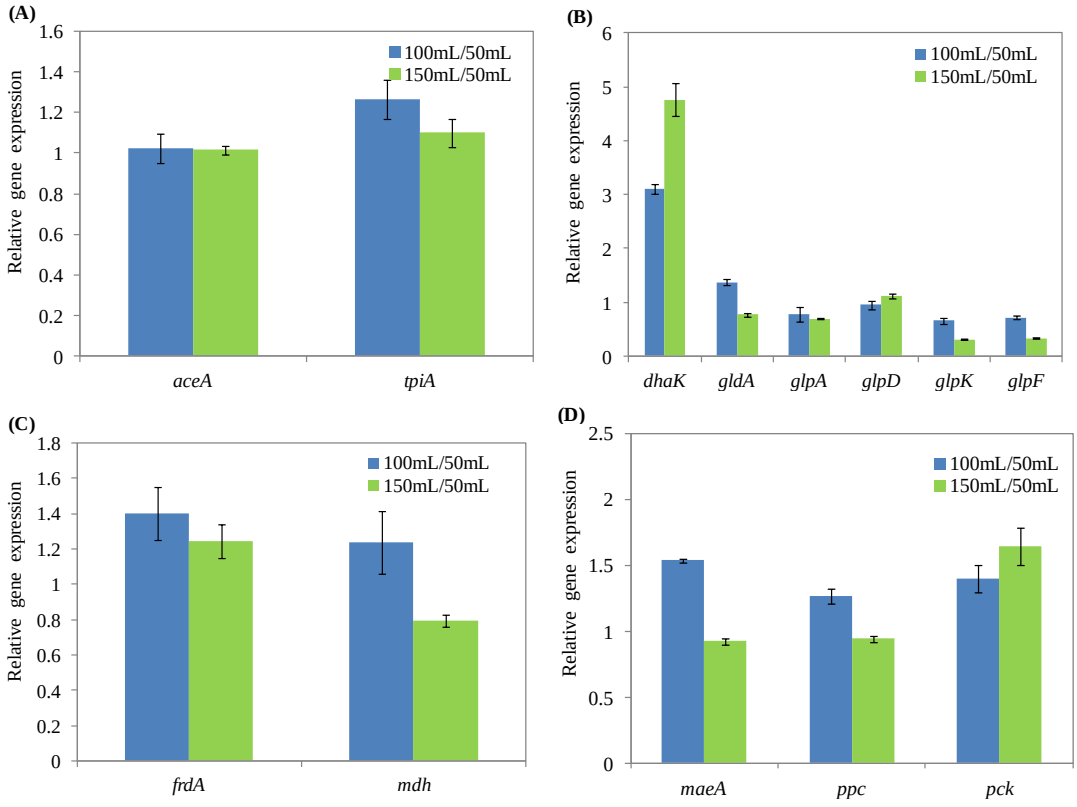
(A)
Relative gene expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
100mL/50mL
150mL/50mL
aceA
tpiA
(B)
Relative gene expression
6
5
4
3
2
1
0
100mL/50mL
150mL/50mL
dhaK
gldA
glpA
glpD
glpK
glpF
(C)
Relative gene expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
100mL/50mL
150mL/50mL
frdA
mdh
(D)
Relative gene expression
2.5
2
1.5
1
0.5
0
100mL/50mL
150mL/50mL
maeA
ppc
pck

Fig. 3

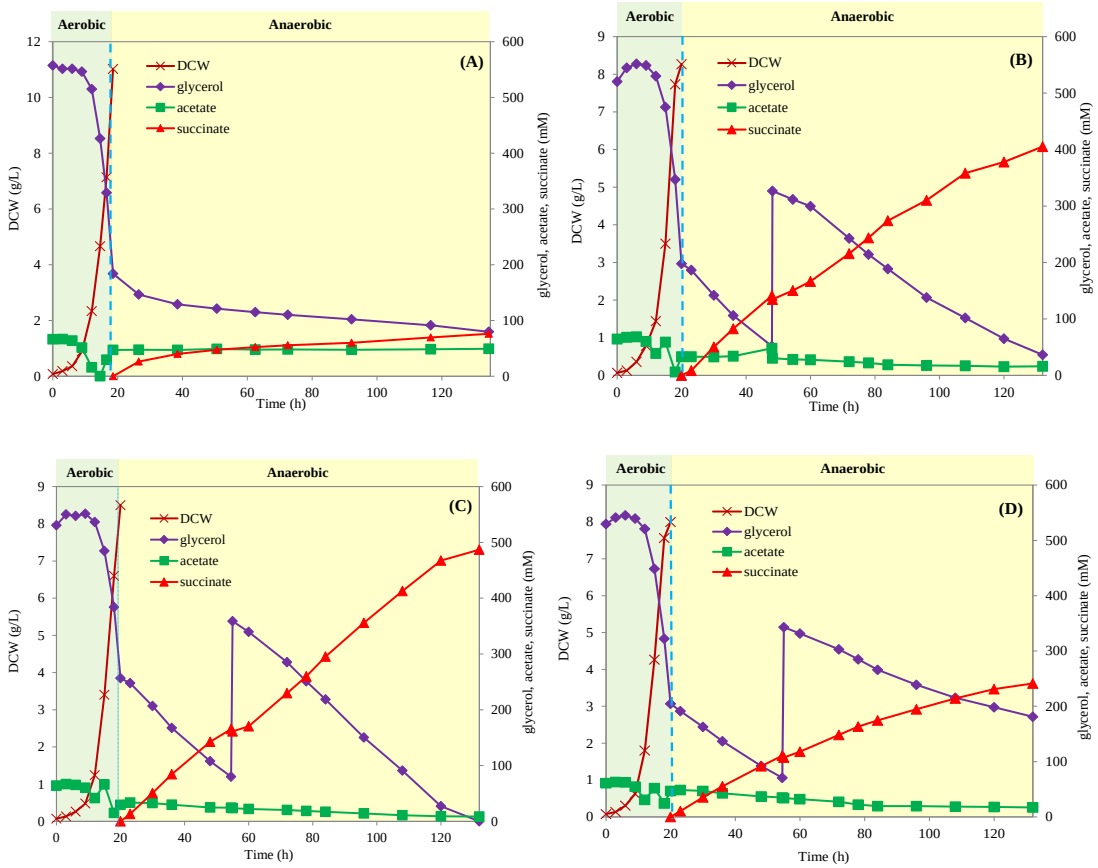
Aerobic
Anaerobic
(A)
(B)
(C)
(D)
DCW
glycerol
acetate
succinate
DCW (g/L)
glycerol, acetate, succinate (mM)
Time (h)

Fig. 4

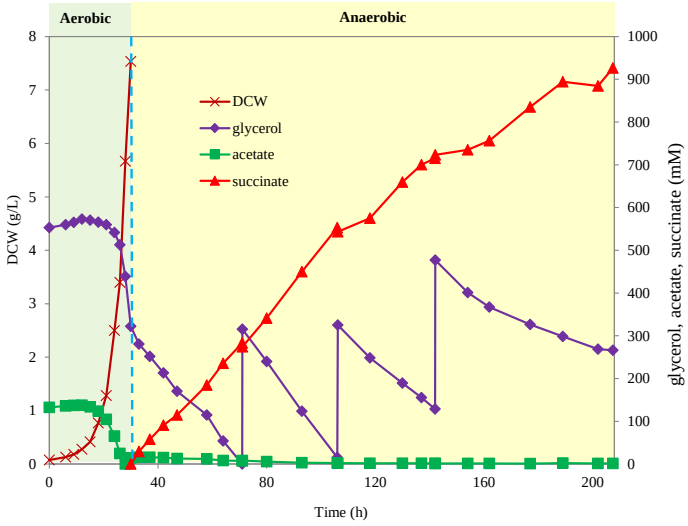
Aerobic
Anaerobic
DCW
glycerol
acetate
succinate
DCW (g/L)
glycerol, acetate, succinate (mM)
Time (h)
0
1
2
3
4
5
6
7
8
0
100
200
300
400
500
600
700
800
900
1000
0
40
80
120
160
200

Fig. 5