BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Effect of culture operating conditions on succinate production in a multiphase fed-batch bioreactor using an engineered *Escherichia coli* strain

Jiangfeng Zhu · Chandresh Thakker · Ka-Yiu San · George Bennett

Abstract A metabolically engineered *Escherichia coli* strain SBS550MG (pHL413) was used in this study to investigate the impact of various culture operating conditions for improving the specific succinate production rate for better final titer while maintaining the theoretical succinate yield on glucose in multiphase fed-batch cultures. Previously, we reported that changes in the level of aeration during the cell growth phase significantly modified gene expression profiles and metabolic fluxes in this system (Martinez et al. 2010). Based on these observations, the examination of culture conditions was mainly focused on the aerobic growth phase. It was found that 2–5 h of low dissolved oxygen culture during the aerobic phase improves cell productivity, but pH control during the aerobic phase was not favorable for the system. Cell viability has been identified as a major limiting factor for succinate production. Supplementing LB medium and betaine, an anti-osmotic stress reagent, did not improve cell activity. A higher succinate titer (537.8 mM) using the current metabolic engineering *E. coli* strain was achieved, which can potentially be improved further by increasing cell viability.

Keywords Succinate production · *Escherichia coli* · Culture conditions · Multiphase fermentation

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Introduction

Chemical production using bioprocesses are becoming increasingly competitive as environmental concerns and fossil raw material cost increases. The US Department of Energy has identified 12 sugar-derived chemicals as top value-added chemicals from biomass, and, among them, succinic acid was considered as one of the most promising chemicals (Werpy and Petersen 2004). Succinic acid is an intermediate of the tricarboxylic acid cycle and one of the fermentation end-products of anaerobic metabolism. It can serve as a precursor of numerous commercial chemicals including pharmaceuticals and biodegradable polymers (Hong and Lee 2002).

There is a significant market opportunity for the development of biobased industrial production of succinic acid. In the past few years, some natural microbial strains such as *Actinobacillus succinogenes*, *Anaerobiospirillum succiniproducens*, and *Mannheima succiniproducens* were found to be able to produce succinate as a major metabolite along with ethanol, acetate and lactate as cometabolites (Lee et al. 2003, 2001, 2006; McKinlay et al. 2005; Oh et

al. 2008). Since the cultures of these natural succinate producing strains are more difficult to operate as compared with the popular genetic engineering strains, a variety of metabolic engineered *Escherichia coli* strains (Jantama et al. 2008; Sanchez et al. 2005; Vemuri et al. 2002) and *Saccharamyces cerevisiae* strains (Otero et al. 2007) were developed for succinic acid bioproduction. The succinate yields in these *E. coli* systems were reported to be between 1.16 and 1.7 mol/mol glucose in the cultures. The major challenges to make the succinate bioproduction economically viable are primarily associated with reducing the overall cost of the fermentation. Three main issues, such as using a low cost medium, a high succinate yield on the carbon source, and volumetric productivity above 2.5 g L^{-1} h^{-1}, must be addressed so that the biobased succinate production can be commercialized (Werpy and Petersen 2004).

The strain used in this work was SBS550MG (pHL413), created by Sánchez and et al. (2005). This strain has the following genes deleted, *adh*E, *ldh*A, *icl*R, and *ackA-pta* and overexpresses the gene *pyc* encoding for pyruvate carboxylase from *Lactococcus lactis*. This strain has shown to produce succinate at high yield, 1.6 mol succinate/mol glucose, in a dual-phase system. The first phase was aerobic and the cells were grown in shake flasks followed by an anaerobic phase where succinate was produced in shake flasks or batch bioreactor (Sanchez et al. 2005, 2006). This yield value is also the theoretical maximum for this strain (Cox et al. 2006 and Sanchez et al. 2006). Recently, Martinez et al. (2010) have reported the metabolic effect of two different aeration conditions during the growth phase of SBS550MG (pHL413) on metabolite and gene expression profiles, as well as the metabolic fluxes, during the production phase. The metabolic flux distributions and product yields during the anaerobic phase were significantly affected by the levels of aeration during the growth phase. The low aeration experiment showed better succinate production with respect to yield, titer, and volumetric productivity (Martinez et al. 2010).

The metabolic behavior of a cell is highly influenced by the operation conditions of the process. Appropriate process conditions are crucial for the metabolic engineered strains to reach their maximum potential, as they may allow the modified metabolic routes to behave as predicted. The physiological state of the cells is critical in a dual-phase system where the enzymes produced during the first phase (aerobic-growth phase) will influence the performance of the cells in the anaerobic-production phase. In the present study, we examined the influence of growth phase operating conditions, such as dissolved oxygen (DO), culture temperature, additional nutrient feeding, and pH control on succinate production during the anaerobic phase using *E. coli* strain SBS550MG (pHL413) in a bioreactor.

The objective is to improve the strain performance for better succinate production rates and increasing the final titer while maintaining the theoretical succinate yield on glucose.

Materials and methods

Microorganism and culture conditions

The succinate-producing *E. coli* strain SBS550MG (pHL413) was previously constructed from MG1655 wild type. The mutant strain lacks the *icl*R, *adh*E, *ldh*A, and *ackA-pta* genes, while bearing a plasmid expressing the *pyc* gene from *L. lactis*, which encodes the pyruvate carboxylase enzyme for CO_2 fixation (Sanchez et al. 2005).

The culture medium contains (1 L): 20 g of tryptone, 10 g of yeast extract, 2.4 mmol of K_2HPO_4, 5.1 mmol of KH_2PO_4, and 13.8 mmol of $(NH_4)_2SO_4$. A small amount of antifoam reagent 204 (Sigma) was added to the medium (30 μl/L) and the medium pH was adjusted to 7.5 before autoclaving. The solutions of 1 mmol $CaCl_2$, 1.2 mmol $MgSO_4$, 0.6 mg of thiamine, and 2 g of glucose were filter sterilized separately and added to the medium after autoclaving.

A 1-L bioreactor (BioFlo 110, New Brunswick Scientific, Edison, NJ, USA) was used for the cultures and the initial working volume was kept at 600–800 ml depending on the DO conditions. The temperature was maintained at 37 °C unless otherwise mentioned. The fed-batch cultures were started with aerobic cultures with the agitation speed controlled between 500 and 800 rpm depending on the DO conditions and glucose was fed into the medium in a batch-wise mode so that the culture pH would not exceed the range between 6.5 and 7.5. The reaction was purged with air at a flow rate of 1–1.5 L/min during the aerobic phase and the purge gas was changed into CO_2 at a flow rate of 0.2 L/min when switching into anaerobic culture conditions. The agitation speed was changed to 250 rpm when switching to anaerobic conditions. A batch of glucose was fed into the culture when switching to anaerobic phase so that the glucose concentration in the culture will be about 100–150 mM. About 4.0 g of $MgCO_3$ suspension in sterile water was also fed into the bioreactor to control the pH.

Analytical procedures

Cell growth was monitored by optical density measurements at 600 nm using a UV/visible spectrophotometer (Spectronic 1001, Bausch and Lomb). Samples were diluted appropriately with 0.9% NaCl to keep the OD_{600} value between 0.1 and 0.5.

To measure the extracellular metabolite concentrations, 1 ml of sample was centrifuged and then the supernatant was filtered through a 0.45-μm pore size filter for analysis by high-performance liquid chromatography (HPLC). The HPLC system (Shimadzu-10A Systems, Shimadzu, Columbia, MD, USA) was equipped with a cation-exchange column (Aminex HPX-87 H, BioRad Labs, Hercules, CA, USA), a UV detector (Shimadzu SPD-10A), and a differential refractive index detector (Waters 2410, Waters, Milford, MA, USA). The mobile phase was 2.5 mM H_2SO_4 at a steady flow rate 0.5 ml/min. The column temperature was maintained at 55 °C (Sanchez et al. 2005; Yang et al. 1999).

Enzyme assay

Cell extracts of the *E. coli* strain which is equivalent to 100 OD_{600} units were prepared by washing the cell pellets with phosphate buffered saline (PBS) buffer (pH 7.4) and the cell wall was broken by 10 sonication steps in the ultrasonic disrupter with 30-s intervals between each step. Cell debris was removed by centrifugation (10,000×*g* for 30 min at 4 °C), and cell extracts were used to measure enzyme activities. The enzyme activity of pyruvate carboxylase (Pyc) was measured in a 1 ml mixture in PBS buffer (pH 7.4) containing 0.05 M $NaHCO_3$, 0.05 M $MgCl_2$, 5 mM AcCoA, 5 mM ATP, 0.1 g/L DNTB, 10 mM pyruvate, and five units of citrate synthase. The mixture was incubated at 37 °C and the absorbance changes at 412 nm were measured using a spectrophotometer (Thermo Fischer UNICAM UV1). The total protein in the cell extracts was determined with Bradford's reagent (Sigma) and bovine serum albumin as the standard.

Cell viability measurement using flow cytometry

Cell viability was measured using cell pellets collected by spinning down 1 ml of culture broth followed by washing the cells twice with PBS buffer (pH 7.4). Then the cell pellets were resuspended in PBS buffer to a final OD_{600} value between 0.01 and 0.05. As propidium iodide (PI, Sigma) can traverse the membrane of cells with compromised membranes and bind to the DNA (fluorescing red), the cells were stained with PI at a concentration of 2 mg/L and left at room temperature for 5 min to identify dead or injured cells (Portle et al. 2007). The flow cytometer used was a FACScalibur (BD Biosciences), with a 15 mW, 488 nm, air-cooled argon-ion laser. All parameters were logarithmically amplified, with the following settings: FSC E01, SSC 381 V, FL1 601 V, FL2 500 V, FL3 575 V. A side scatter threshold was applied to gate out much of the noise (at channel 130). Twenty thousand to 40,000 events were collected for each sample. The flow cytometer was calibrated with EGFP calibration beads (Clonetech).

## Results

Batch culture phase shifts

The cell growth and metabolite production were significantly different as the culture phase shifted. As shown in Fig. 1, a typical batch culture for succinate production was started with 2 g/L of glucose under aerobic conditions using a 1–1.5 L/min of air purge and a 500–800 rpm agitation speed. The pH was not controlled during the aerobic phases. During the phase A-I, the pH decreased as glucose was consumed and then rose again as the organic acids produced by glucose metabolism were utilized by the cells in the absence of glucose. After the phase A-I, at least one batch of 2 g/L glucose was fed into the system. During the phase A-II, DO in the culture system was always at a relatively low level (0–20%). The length of phase A-II was controlled by glucose feeding. Before switching to the anaerobic phase AN, samples were taken and analyzed by HPLC which showed almost no organic acid in the system (data not shown). When switching to the phase AN, the purging gas was switched to CO_2 at a slow but steady rate (0.2 L/min), while the agitation speed was slowed down to 250 rpm. At the beginning of the phase AN, 20 g/L of glucose was feed into the system as carbon source for succinate production and pH control was turned on to control the pH at about 7.0 using NH_4OH or NaOH.

In this typical bioreactor experiment and some other trials, we observed that the succinate production rate dropped quickly after about 20 h in the production phase. Therefore, cell viability was checked by PI staining using the flow cytometer. As shown in Fig. 2a, c, a larger population of cells seems to extend their sizes after longer production phase. More significantly, the PI staining samples showed a bigger peak in the 23.5 h sample compared to the 14-h sample during the AN phase, which indicates that larger portion of cells were damaged (Fig. 2b, d).

Effect of air supply conditions in the aerobic phases

The air supply conditions were mainly controlled by changing the agitation speed during the phases A-I and AII. The 10 experiments used to compare the effect of air supply have three agitation setting patterns namely 800 rpm in both phases A-I and A-II (800+800), 800 rpm in phase A-I and 500 rpm in phase A-II (800+500), and 500 rpm in both phases A-I and A-II (500+500; Fig. 3b). The overall molar succinate yields on glucose during the production

Fig. 1 Bioreactor culture phases of a typical succinate fed-batch culture. The basic culture conditions and engineered strain are described in Methods
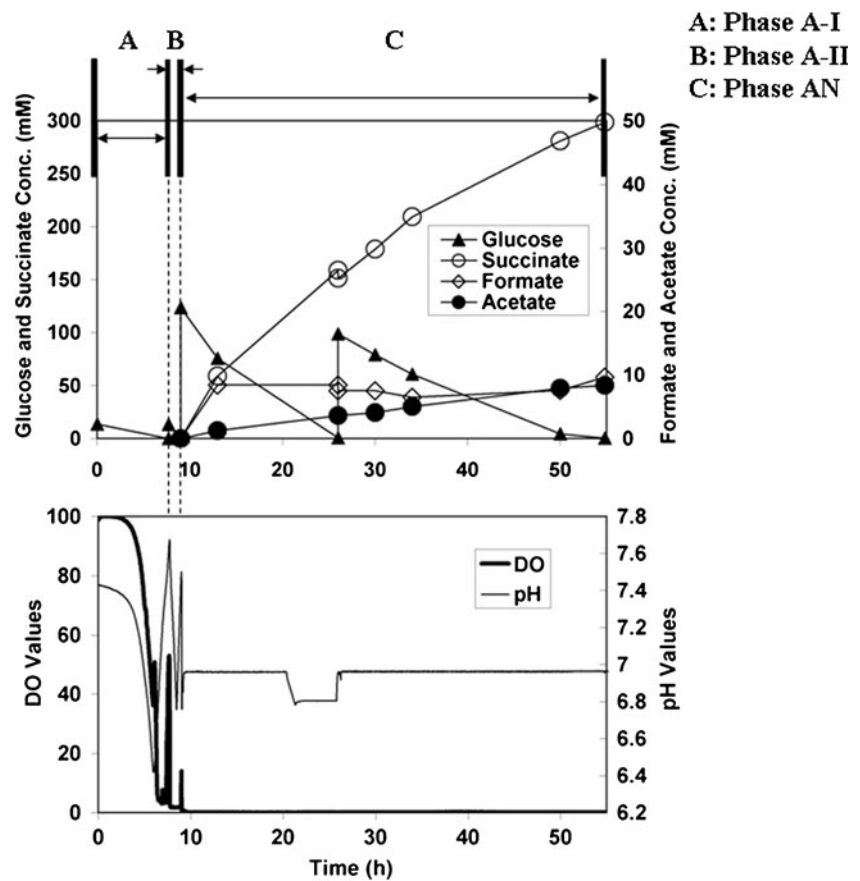
phase AN vary from 1.24 to 1.60 in these batch cultures (Fig. 3a). The specific succinate production rates vary from 0.08 to 0.12 g/L/h/OD. While high-agitation speed (800+ 800 and 800+500) runs gave relatively good succinate yields on glucose, the relatively lower agitation speed runs were more reproducible and resulted in about 1.56–1.60 mol/mol succinate yields on glucose, and the specific production rate was not much lower than higher agitation speed setups. Therefore, the stable condition (500+500) was used for further bioreactor cultures.

We noticed that the length of phase A-II (low DO incubation period) has some impact on the strain's performance during the production phase (AN). Therefore, a series of batch cultures were setup to evaluate the effect of the length of the low DO incubation period. As shown in Fig. 4, the A-II phase time length varies between 0.5 and 7 h. The overall succinate yields in these batches were not significantly affected. However, a short low DO incubation period seems to favor a higher specific succinate production rate. In the two cultures where the A-II phase was about 7 h, the specific succinate production rates were significantly lower, which resulted in lower overall production rate and thus lower final succinate titer. The optimal A-II phase time length seems to be between 2–5 h.

Since a too long low-DO incubation time resulted in less succinate production, we wondered if that was due to the low cell viability or low metabolic activities in these cultures. Providing more air may help cells to keep a high metabolic activity so it may help to improve the production phase performance. With that in mind, we examined a culture where we controlled DO at about 8% in the A-II phase instead of allowing the DO to be less than 5% as in normal batch cultures. The initial glucose feeding during the switch to the production phase was increased to about 40 g/L instead of 20 g/L to avoid repeated osmotic stress shock when feeding glucose during the AN phase. However, the succinate yield and the initial specific production rate of the culture were similar to the previous runs, and the final succinate concentration was not improved (data not shown).

Effect of additional nutrients feeding

We noticed that the succinate production rate decreases with time after switching to the anaerobic phase. In addition, when the low DO incubation time was too long (5–7 h), the cell performance was not as good as those incubated at low DO for shorter time periods. These

Fig. 2 Cell viability comparisons at two different time points during phase AN. **a** Cell size population distribution of AN-14-h sample. **b** Red fluorescent detection for the AN-14-h sample after PI staining. **c** Cell size population distribution of AN-23.5-h sample. **d** Red fluorescent detection for the AN-23.5-h sample after PI staining. The two light scattering properties, forward, and side scatter (FSC and SSC, respectively) are measures of cellular size and internal complexity, respectively. FL3-H represents the relative red fluorescence strength. M1 in **b** and **d** marked out the PI stained peaks, which indicates the injured or dead cells
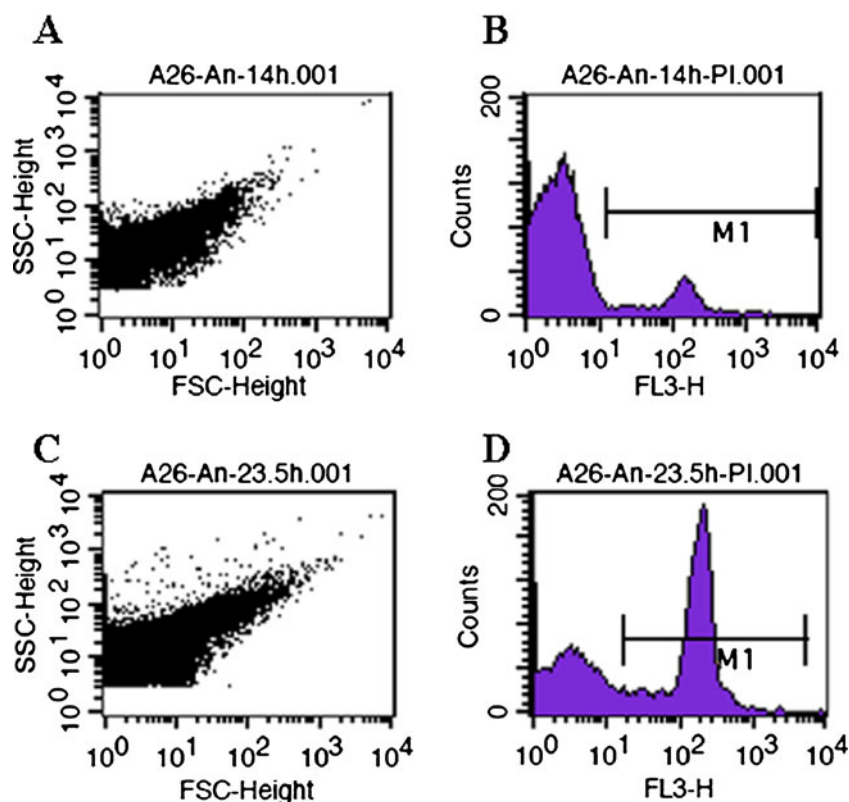
observations suggest that there might be some nutrient or energy limitation in the cultures.

To supplement nutrients so that cell can grow better and have better viability while maintaining succinate producing capacity, concentrated luria bertani broth (LB) medium (10 ml of 20×LB each time) was fed into the culture medium during the phase A-II and at the switch to the production phase AN. As shown in Fig. 5, the batch cultures with no LB supplementation (Control), one LB supplementation during the A-II phase [AII(1×)], five LB supplementations during the A-II phase [AII(5×)], and three LB supplementations during the anaerobic phase [AN(3×)] were compared for their specific succinate production rates, succinate yields on glucose, and final succinate concentrations. The final succinate titer was slightly higher in the two cultures [AII(1×) and AII(5×)] with LB supplementations during the aerobic phase, as the initial cell concentrations and initial specific succinate production rates were both relatively higher in these two cultures compared with the culture without LB supplementation. As compared to culture without LB supplementation, the succinate yield on glucose was not significantly affected in LB supplemented cultures. The LB supplementation during the anaerobic phase did not improve cell performance as observed by low specific succinate production rate (Fig. 5).

As another factor, it is considered that the cell may experience significant osmotic stress as succinate production goes on during the AN phase. Therefore, we added 1 mM of betaine into the culture medium at the beginning of the aerobic phase (Fig. 5 (AN-BT)). However, succinate production was not improved in the presence of betaine (Fig. 5).

Previously, it was reported that the activities of glyoxylate shunt enzymes are important for metabolic cofactor balance in the current succinate production system (Sanchez et al. 2005) and it is known that the glyoxylate shunt is activated while using acetate as a carbon source due to the phosphorylation regulation of the isocitrate dehydrogenase (Cozzone 1998; Walsh and Koshland 1985). We supplemented 10 mM of acetate at the end of the phase A-II in order to further activate the glyoxylate shunt so that the metabolic network can be further fine tuned. However, the succinate production in the AN phase was not improved (data not shown).

Effect of pH control during aerobic phase

Several pH control strategies during the aerobic phase were tested. The medium pH was controlled at 6.2 [A6 (pH 6.2)], 6.3 [A8 (pH 6.3)], and 7.0 [A17 (pH 7.0)], respectively, using NaOH and HNO_3 during the aerobic phase. As a control, another culture without pH control during the aerobic phase [A48 (no control)] was used to compare with the aforementioned ones. As shown in Fig. 6, all four batch cultures with pH control resulted in low molar succinate yield on glucose ranging from 0.56 to 1.30, and the initial
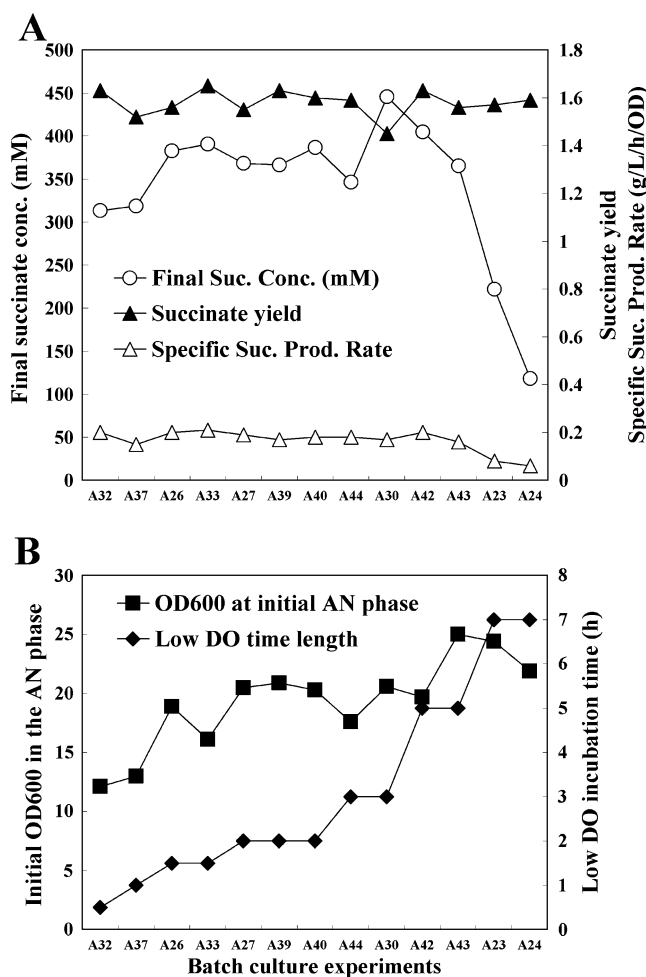
A

B
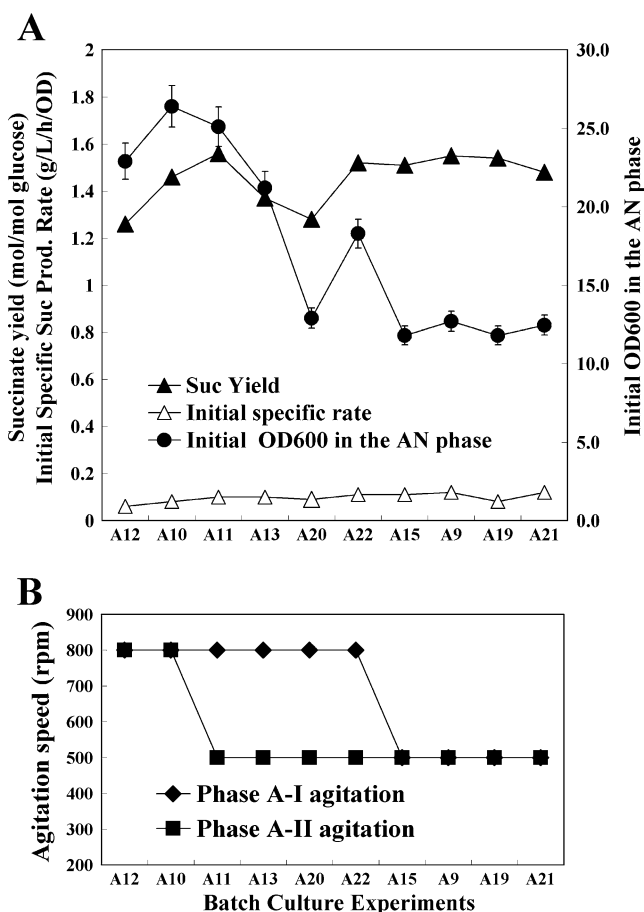
Fig. 3 Effect of DO settings in the aerobic phases. **a** Succinate yield and initial specific succinate production rate; **b** agitation speeds in the A-I and A-II phases

A

B

Fig. 4 Effect of low DO incubation time lengths during phase A-II. **a** Succinate yield, initial specific succinate production rate, and final succinate titer; **b** Low DO time length during A-II phase and initial cell density in the AN phase

specific succinate production rate was significantly lower than that of the culture without pH control during the aerobic phase.

Effect of culture temperature in the aerobic phases

The expression of the heterologous pyruvate carboxylase gene *pyc* was considered to be a rate-limiting factor in this succinate-producing system (Sanchez et al. 2005; Martinez et al. 2010). The *pyc* gene size is relatively large (about 3.4 kb) and the recombinant expression of heterologous proteins in *E. coli* often results in protein aggregation (Baneyx 1999; Haacke et al. 2009). To reduce the protein synthesis rate to potentially improve the Pyc protein expression, several lower temperature cultures were tested for succinate production. As shown in Fig. 7, cell growth was not significantly affected by the temperature difference. All four cultures produced cells equivalent to 16 OD_{600} units before switching to the production phase. However, the specific succinate production rate, succinate yield, and the final succinate titer were significantly increased as the

aerobic culture temperature increased from 27 to 37 °C. We measured the Pyc activities when switching to the anaerobic phase in these cultures. As shown in Fig. 7, it seems that the lower temperature did not improve Pyc protein expression. On the other hand, the lower culture temperature still resulted in reasonable Pyc activity as compared to the culture at 37 °C.

High cell density culture

A high cell density fed-batch culture was examined for the final succinate titer using the suitable culture and medium conditions. As shown in Fig. 8, with four batches of glucose feeding, the final succinate concentration reached about 537.8 mM, along with 24 mM of formate and 52 mM of acetate as side products. It can be seen that the succinate production rate was faster in the first 10 h compared to the later production phase and the formate accumulation increased significantly after the fourth glucose feeding.
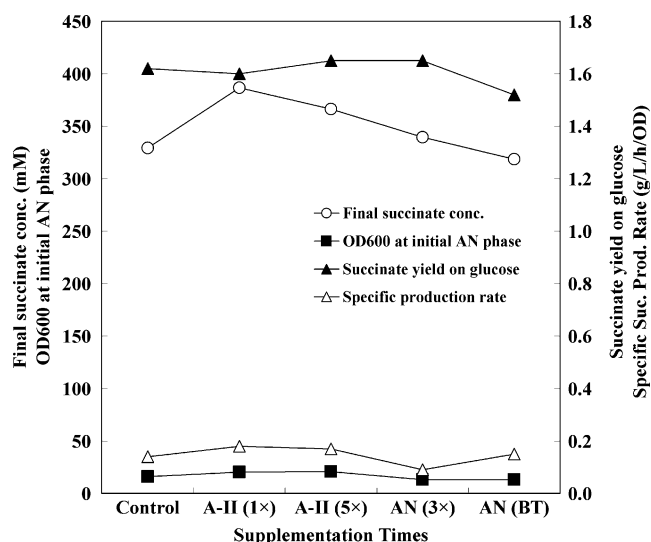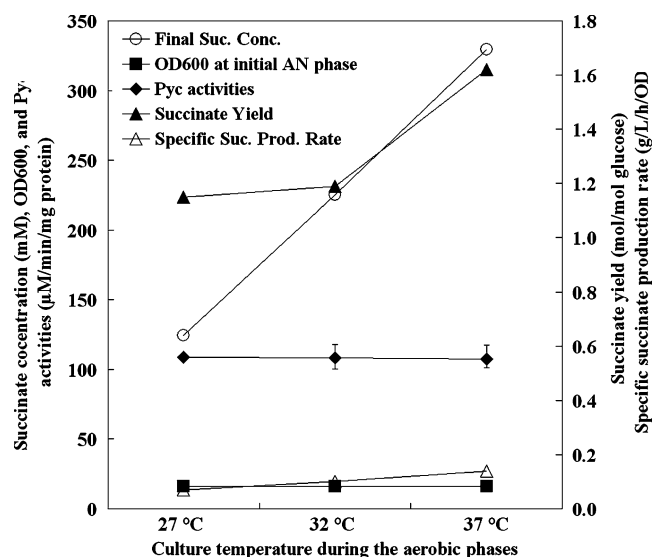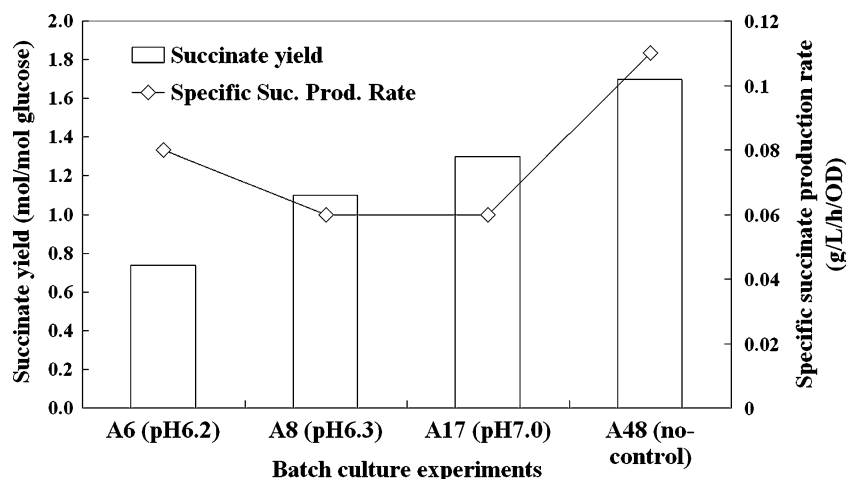
Fig. 5 Effect of LB medium supplementation. The x-axis denotes the batch cultures with no LB supplementation (control), one LB supplementation during the A-II phase [AII(1×)], five LB supplementations during the A-II phase [AII(5×)], three LB supplementations during the anaerobic phase [AN(3×)] and 1 mM of betaine at the beginning of the anaerobic phase [AN(BT)]

Fig. 7 Effect of aerobic phase culture temperatures. The basic culture conditions are as in Fig. 1 with the aerobic phase temperature adjusted as indicated

The concentration was reduced due to the dilution effect, which indicates the succinate production stopped after about 25 h.

Discussion

The operation conditions of the process greatly affect the metabolic behavior of the production strain. The genetic manipulation themselves are not enough to make a strain a high producer or achieve maximum theoretical yield. Key process conditions are important for the engineered strains to reach their maximum potential as they may allow the modified metabolic routes to behave as predicted. In the present study, we studied the influence of crucial growth
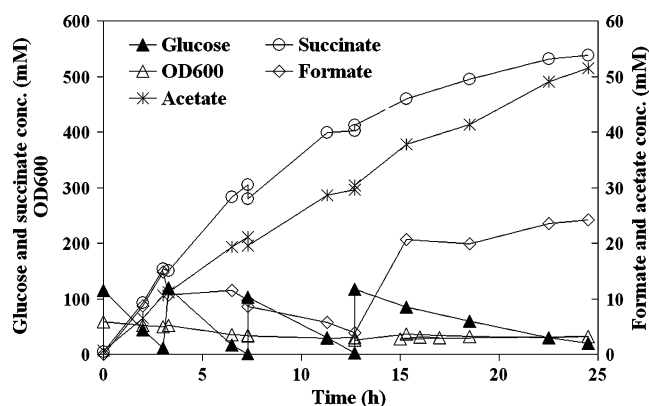
phase operating conditions, such as DO, additional nutrient feeding, pH control, and culture temperature on succinate production using *E. coli* strain SBS550MG (pHL413) in a multiphase bioreactor process. The first phase A-I was aerobic for biomass accumulation followed by second phase A-II of low DO and third phase of anaerobic succinate production. The metabolic flux distribution in the central metabolic pathways and cell viability are two important factors can affect the final succinate production rate, yield and final titer, and the metabolic flux is determined by the genetic background and operating conditions such as air supply, pH control, culture temperature, etc.

The air supply conditions in the aerobic phases A-I and A-II showed significant effects on the succinate production in the anaerobic phase AN (Fig. 3). On one hand, the air supply conditions in phases A-I and A-II affect the cell

Fig. 6 Effect of pH control during the aerobic phase. The basic culture conditions are as in Fig. 1 with the aerobic phase pH adjusted as indicated

Fig. 8 High cell density batch culture for succinate production. The culture conditions are described in "Materials and methods"

density and cell viability at the switch to phase AN, which affects the overall production rate and the final succinate titer. On the other hand, the air supply conditions also affected the pathway fine-tuning in the cell, which resulted in variant specific succinate production rates and succinate yields on glucose.

Employing higher agitation speed improves biomass yield during the aerobic phase (Fig. 3). However, succinate productivity was not stable when using 800 rpm during the AI and A-II phases. Besides, pyruvate accumulation was observed in high DO experiments. A recent extensive investigation indicated that appropriate aeration level during the aerobic phase changed the expression of the *pfl*AB, *ace*AB, and *acn*AB genes (Martinez et al. 2010). The elevated expression levels of these genes facilitate pyruvate conversion through pyruvate formate lyase and the glyoxylate shunt during the anaerobic phase. Together with the heterologous pyruvate carboxylase, this shunt balances reducing power usage and carbon processing at the pyruvate node, which resulted in good succinate yield (Cox et al. 2006).

Consistently, the low DO incubation time not only fine tunes the balance between the Pyc and the Pfl–glyoxylate shunt, which are important for succinate yield, since the expressions of *pfl*AB and *ace*AB genes are affected under the low DO conditions (Böck et al. 2008; Lynch and Lin 1996), but also affects cell viability at the switch to the anaerobic production phase in the current study.

During the anaerobic phase, the specific succinate production rate drops as succinate accumulates (Figs. 1 and 8) as cell viability decrease (Fig. 2). *E. coli* subjected to osmotic stress respond by accumulating compatible solutes, such as glycine betaine, proline, and trehalose (Larsen et al. 1987). Among these osmoprotectants, glycine betaine offers the highest osmotolerance in *E. coli* (Underwood et al. 2004) and other cells (Haussinger and Lang 1991; Robinson et al. 1986). *E. coli* can only accumulate glycine

betaine or proline if supplemented in the medium or by a two-step oxidation of externally provided choline. Andersson et al. (2009) reported that addition of glycine betaine to the cultures of *E. coli* AFP184 resulted in an increased aerobic growth rate and anaerobic glucose consumption rate but decreased succinic acid yield. In contrast, studies with *E. coli* engineered for lactic acid production has shown that betaine greatly increased the volumetric lactic acid productivity (Zhou et al. 2006). In the present investigation, cell aerobic growth rate, glucose consumption, and succinate production were not affected by the addition of betaine. We are currently pursuing other approaches such as strain evolvolution under succinate pressure to improve cell viability during succinate production process.

The pH was not controlled in most of the fermentations during the aerobic phase in the current study. Instead, the pH was used as an indicator of glucose consumption to determine glucose feeding times. Less osmotic stress was introduced as an obvious benefit by allowing the pH to freely change as the cell produced or utilized organic acids during the A-I and A-II phases. However, we also observed that the succinate yield will be much lower if the pH dropped to below 5.5 during the A-I or A-II phases. Therefore, the amount of glucose feeding was carefully set at about 2 g/L each time so that acid production can be controlled at an appropriate level.

Controlling the culture pH at 7.0 was expected to give better cell growth and therefore improve overall succinate productivity during the anaerobic phase. However, both the succinate yield and specific succinate production rate were significantly lower than those in the experiments without pH control. Osmotic stress could be introduced since the pH was maintained by adding acid and base during organic acid production and absorption periods. Besides, the low DO incubation, which is recognized as a critical phase in the current study, was disturbed by the pH control since the feeding of acid and base not only changes the medium composition, but also may stress the cell and we observed DO fluctuations during the experiments. We noticed higher pyruvate accumulation in the pH controlled experiments (data not shown), which suggests that the controlled pH may affect the fine tuning of gene expression related to pyruvate metabolism. Therefore, confining the pH change range with careful glucose feeding and letting the pH freely change according to cell metabolism seems the best strategy for the current system so far.

Formate is one of the major side products during the fermentation of the current strain (Figs. 1 and 8). Besides its inhibition to cell growth, formate can also serve as a potential reducing power provider if dehydrated through formate dehydrogenase (Berrios-Rivera et al. 2002). Data

from other experiments we have conducted suggested that the *E. coli* native FDH may be activated at about pH 6.5. Therefore, two experiments were conducted by controlling the pH at 6.2 and 6.3, respectively, during the aerobic phase (Fig. 6). However, such conditions significantly reduced the succinate yield in these cultures. The low pH or the pH control reagents may have disturbed the culture conditions.

The cell viability loss during the anaerobic phase is not favorable for the succinate production (Figs. 1 and 8). The high osmotic pressure caused by succinate production and addition of neutralization reagents could be a major stress for the cell. Therefore, further strain engineering is still required for the most economical production of succinate. Since it is observed that the sizes of a large portion of cells increased during the succinate production phase (Fig. 2), one would speculate that the cell may suffer from inefficient excretion of the organic acid produced from glucose. Limiting glucose uptake rate by modifying the phosphotransferase system may help on maintaining cell viability, but it will also reduce the cell productivity, which is not preferred for succinate production. Modifying or introducing a robust heterologous succinate transport system may be helpful for future solution of the cell viability issue. Besides, the Pyc expressed using the plasmid system may also result in a high cell burden during the growth phase, which reduces cell density and viability at the switch to the anaerobic phase. Incorporating the *pyc* gene expression system on the chromosome may also help cells grow better under the stressful conditions of succinate production.

In summary, the aerobic phase culture conditions significantly affected the metabolic behavior of SBS550MG (pHL413) and therefore influence the anaerobic succinate production. To briefly conclude, we focused on the study of key operating conditions of the aerobic growth phase. It was found that 2–5 h of low DO culture during the aerobic phase improves cell productivity, and that pH control during the aerobic phase was not favorable for the fine tuning of enzymatic system of cells. It has been noticed that the cell viability decreased significantly after 20 h of succinate production, and supplying LB medium and betaine did not improve the cell activity. A higher succinate titer (537.8 mM) using the current metabolically engineered *E. coli* strain was achieved as compared to previous work (Sanchez et al. 2005; Martinez et al. 2010), and this titer apparently can still be improved by addressing the cell viability issue.

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