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ORIGINAL PAPER

pH and base counterion affect succinate production in dual-phase *Escherichia coli* fermentations

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Abstract Succinate production was studied in *Escherichia coli* AFP111, which contains mutations in pyruvate formate lyase (*pfl*), lactate dehydrogenase (*ldhA*) and the phosphotransferase system glucosephosphotransferase enzyme II (*ptsG*). Two-phase fermentations using a defined medium at several controlled levels of pH were conducted in which an aerobic cell growth phase was followed by an anaerobic succinate production phase using 100% (v/v) CO_2. A pH of 6.4 yielded the highest specific succinate productivity. A metabolic flux analysis at a pH of 6.4 using ^{13}C-labeled glucose showed that 61% of the PEP partitioned to oxaloacetate and 39% partitioned to pyruvate, while 93% of the succinate was formed via the reductive arm of the TCA cycle. The flux distribution at a pH of 6.8 was also analyzed and was not significantly different compared to that at a pH of 6.4. $Ca(OH)_2$ was superior to NaOH or KOH as the base for controlling the pH. By maintaining the pH at 6.4 using 25% (w/v) $Ca(OH)_2$, the process achieved an average succinate productivity of 1.42 g/l h with a yield of 0.61 g/g.

Keywords Succinic acid · CO_2 · Calcium

Introduction

Succinic acid (succinate) and its derivatives are widely used as specialty chemicals in foods, pharmaceuticals and cosmetics [10], and it can serve as a starting material for many commercially important products [37]. Some anaerobic bacteria, such as *Anaerobiospirillum succiniciproducens* [13, 14], *Actinobacillus succinogenes* [11, 31] and *Mannheimia succiniciproducens* [15], produce succinate as the major fermentation product. During anaerobic fermentation, these organisms fix the greenhouse gas CO_2 via carboxylation reactions and convert C_3 to C_4 metabolites. Recombinant *Escherichia coli* can also generate a high concentration of succinate. For example, an anaerobic process generates 15.6 g/l succinate with a yield of 0.85 g/g glucose through overexpression of pyruvate carboxylase in an alcohol dehydrogenase and lactate dehydrogenase mutant [25]. Aerobically, *E. coli* generates 6.7 g/l succinate at a yield of 0.71 g/g glucose, although in this case CO_2 is not fixed [16, 17]). A dual-phase *E. coli* process (aerobic growth followed by an anaerobic succinate production phase) generates nearly 100 g/l succinate at a productivity of 1.3 g/l h and a yield of 1.1 g/g glucose [34].

A few studies have investigated the effect of pH on succinate production. For example, Van der Werf et al. [32] found that succinate was produced in the pH range of 6.0–7.4 by *Actinobacillus sp.,* but pH was not controlled. Samuelov et al. [24] reported that for *A. succiniciproducens* a pH of 6.2 was better than a pH of 7.2 using NaOH. Optimal anaerobic succinate production by *E. coli* was reported to occur with a 0.2 M sodium phosphate buffer having a pH of 6.5 at the beginning of the fermentation [1], though pH was not controlled. Similarly, a 0.2 M sodium phosphate buffer at an initial pH of 6.5 was found to provide the optimal initial conditions for succinate production by *Bacteroides fragilis* [12]. Other studies select a pH rather than optimize it, and universally use either Na_2CO_3 or NaOH for pH control, resulting in sodium ion accumulation.

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The highest reported succinate concentration has been generated by *E. coli* AFP111 and its derivatives using a dual-phase process [34]. We selected this strain to study the influence of pH, and also compared its metabolism at two different values of pH using flux analysis with ^{13}C-labeled glucose. The formation of succinate requires the use of base in order to maintain the pH. When NaOH was used in initial experiments, we observed a decrease in the rate of succinate formation during the course of the anaerobic phase. To determine whether the Na^+ specifically is detrimental to succinate formation, we compared three common bases [NaOH, KOH and $Ca(OH)_2$] and elucidated their effects as base counterions.

Materials and methods

Bacterial strain

Escherichia coli AFP111 (F^+ λ^- *rpoS*396 (Am) *rph*-1 Δ*pflAB::*Cam *ldhA::*Kan *ptsG*) was used in this study [3, 5].

Media and fermentation conditions

All fermentations used a defined medium containing (per liter): 40.0 g glucose, 3.0 g citric acid, 3.0 g $Na_2HPO_4{\cdot}7H_2O$, 8.00 g KH_2PO_4, 8.00 g $(NH_4)_2HPO_4$, 0.20 g NH_4Cl, 0.75 g $(NH_4)_2SO_4$, 0.84 g $NaHCO_3$, 1.00 g $MgSO_4{\cdot}7H_2O$, 10.0 mg $CaCl_2{\cdot}2H_2O$, 0.5 mg $ZnSO_4{\cdot}7H_2O$, 0.25 mg $CuCl_2{\cdot}2H_2O$, 2.5 mg $MnSO_4{\cdot}H_2O$, 1.75 mg $CoCl_2$ $6H_2O$, 0.12 mg H_3BO_3, 1.77 mg $Al_2(SO_{4)3}{\cdot}xH_2O$, 0.5 mg $Na_2MoO_4{\cdot}2H_2O$, 16.1 mg Fe(III) citrate, 20 mg thiamine HCl and 2 mg biotin.

Dual-phase fermentations operating in batch mode with an initial volume of 1.2 l in 2.5-l fermentors (Bioflow III, New Brunswick Scientific, Edison, NJ) were inoculated using 50 ml grown for 10–12 h to an optical density (OD_{600}) of about 2–4 in the same medium in 250-ml shake flasks. Oxygen-enriched air as necessary was sparged at 1.0 l/min with an agitation of 200–500 rpm to maintain the dissolved oxygen (DO) above 40% as measured by an on-line probe (Mettler-Toledo Process Analytical Instruments, Wilmington, MA). During growth, the pH was controlled at 7.0 with 20% (w/v) NaOH, and the temperature was maintained at 37°C. When the OD_{600} reached about 20, the aerobic growth phase was terminated by switching the inlet gas composition to 100% (v/v) CO_2. Simultaneously, the total flow rate was reduced to 500 ml/min (dry basis, 0°C and 1 atm), the agitation reduced to 200 rpm, and 120 ml of 550 g/l glucose was added. During this anaerobic phase, the pH was controlled with either 25% (w/v) NaOH, 25% (w/v) KOH or 25% (w/v) $Ca(OH)_2$ as indicated.

Analysis of glucose and products

Samples were diluted by 1% H_2SO_4 as necessary and centrifuged (10,000×*g* for 10 min at 4°C) and the supernatant analyzed for glucose, succinate, pyruvate, acetate and ethanol by high performance liquid chromatography (HPLC) as previously described [6]. When calcium succinate was generated, the total succinate (dissolved plus precipitate) was determined by diluting the sample with 1% H_2SO_4 and sonicating prior to analysis.

For metabolic flux analysis involving [1-^{13}C] glucose, dual-phase fermentations were repeated as described above except the initial volume was 0.6 l in 1.0-L fermentors (Bioflow III, New Brunswick Scientific, Edison, NJ), the inoculum volume was reduced to 25 ml, and the initial glucose concentration was 30 g/l. The glucose concentration was monitored using a glucose analyzer (YSI 2700 SELECT^{TM}, Yellow Springs Instrument, Inc, Yellow Springs, OH). When the glucose concentration reached about 2 g/l, the system was switched to anaerobic conditions by sparging CO_2. When the glucose concentration reached about 1 g/l, 10 ml of 450 g/l [1-^{13}C] glucose (99%, Cambridge Isotope Laboratories, Andover, MA) was added into the fermentor, and the pH was reduced within a couple minutes to the desired value with 20% (v/v) H_2SO_4. Samples were then collected every 30 min, centrifuged at 0°C (10,000×*g* for 10 min) and the supernatants stored at −4°C for later LC-MS and NMR analyses.

LC-MS analysis of succinate

The supernatant was filtered by a 0.2-μM syringe filter (Cole-Parmer Instrument Co., Vernon Hills, IL) prior to LC-MS analysis. The liquid chromatography was performed at 25°C (140B Solvent Delivery System, Applied Biosystems, Foster City, CA) using a C18 column (Kromasil, 250 × 1 mm, 5-μm particles, 100-Å pore space, Keystone Scientific, Inc., Bellefonte, PA). The mobile phase at 50 μl/min was a gradient of aqueous acetic acid (0.1% w/v, pH 3.23) and methanol. Electrospray ionization mass spectrometry was performed using a single quadrupole instrument with an electrospray ion source (PE Sciex API 1 Plus, Concord, ON, Canada). The operating conditions were: nebulizer gas (N_2) at a flow rate of 0.6 l/min and pressure of 30 psi, curtain gas (N_2) at a flow rate of 0.8 l/min and interface temperature 50°C. Data were acquired in negative ion mode with a capillary voltage of 3,500 V. Mass peak heights were determined using the BioTool Box Version B software package (Applied Biosystems/PE Sciex, Foster City, CA). The relative concentrations of M + 0, M + 1, M + 2 and M + 3 (M + 0 represents succinate without ^{13}C label having a m/z of 117,

etc.) were calculated by the fractions of each peak heights after correcting for naturally occurring ^{13}C (1.109%) and ^{18}O (0.20%) [22].

Metabolic modeling and flux analysis

An overall flux balance was developed using stoichiometric analysis and the pseudo-steady-state condition for intracellular metabolites [27]. The balance equations for *E. coli* AFP111 metabolism have been developed previously [34], and this model was further extended by including the pentose phosphate pathway (Fig. 1). Flux partitions (ϕ) were defined as fractional fluxes (v) into one of two key branches. The fraction of flux into the pentose phosphate pathway (ϕ_{PPP}) is defined as

$$\phi_{PPP} = \frac{v_3}{v_2 + v_3}$$

The fraction of flux through anaplerotic carboxylation at the PEP node (ϕ_{PPC}) is defined as:

$$\phi_{PPC} = \frac{v_{13}}{v_{13} + v_{14}}$$

The model contained 27 fluxes and 21 metabolites (Fig. 1 and Appendix A). Since insignificant growth occurs during the anaerobic process [34], glucose consumption and product formation rates were essentially constant (e.g., Fig. 2); thus, the process exhibited a metabolic pseudo-steady state. Although five fluxes are known, the balance equations represent an underdetermined set. In order to calculate the optimal solution for the flux model, LC-MS results with 1-^{13}C-glucose were used. Specifically, we wrote analogous balance equations for isotopomers of each metabolite [28]. We calculated the distribution of succinate isotopomers for a given set of fluxes and calculated the mole fraction (X_{calc}) of each isotopomer i. These calculated values were compared with the mole fractions of each isotopomer observed from the LC-MS results (X_{obs}). The optimal solution for the flux model was then determined by minimizing the weighted sum of squared residuals over the four mass peaks [33]:

$$\text{error} = \sqrt{\sum_{i=0}^{3} \frac{\left(X_{calc,i} - X_{obs,i}\right)^2}{s_i^2}}$$

where s is the standard deviation of the observed mole fraction determined by three LC-MS analyses of each sample. The isotopomer analysis cannot determine whether flux occurred through the malic enzyme and/or pyruvate oxidase because these pathways do not alter the ^{13}C-labeled distribution compared, respectively, with PEP carboxylase and acetate formation via acetyl CoA. Therefore, the model did not include these two pathways.

NMR analysis of succinate

The sample supernatant was filtered by a 0.2-μM syringe filter, and then the filtered supernatant was mixed with 15% volume of deuterium dioxide (D_2O) for NMR analysis. Proton-coupled ^{13}C NMR spectra (500 NMR spectroscopy; Varian Inc., Palo Alto, CA) at 125.7 MHz were obtained with the following spectral parameters: 45° pulses, 31.4-kHz spectral width and 45-s relaxation delay. Field stabilization was achieved by locking on the D_2O frequency. ^{13}C chemical shift assignments for succinate were determined by comparison with the natural abundance standard.

NMR was not used to calculate fluxes, but was employed to complement the LC-MS results. Specifically, since the LC-MS results did not provide information about the position of the label, we were able to use NMR to observe the total ^{13}C enrichment at C-1 position (S1) and that at C-2 position of succinate (S2). The enrichment ratio of S2/S1 was calculated from the ratio of the peak area of the methylene group to the carboxyl group of succinate. This observed enrichment ratio was compared to the enrichment ratio calculated from the optimized metabolic flux model. We also calculated the redox ratio as the fluxes through NAD(P)H formation steps in the pathways divided by fluxes through NAD(P)H consumption steps.

We chose for all metabolic flux analyses the interval between the time 1-^{13}C-glucose was added and the time that the succinate concentration reached about 4 g/l (approximately 2 h).

Results

Effect of pH on succinate production

E. coli AFP111 accumulates significant amounts of succinate during an anaerobic non-growth phase after growing to a high cell density under aerobic conditions at a pH of 7.0. During succinate generation, CO_2 is incorporated into the central metabolism through the action of the enzyme PEP carboxylase (PPC) [8]. We first examined the effect of pH on succinate formation. We controlled the pH at a constant level in the range of 5.8–7.0 using 25% $Ca(OH)_2$ as the neutralizing agent. Figure 2 shows the generation of several products during the anaerobic process when the pH was maintained at 6.4. Over the course of 14–16 h, succinate was generated to 25–30 g/l, pyruvate to 7–10 g/l, ethanol to about 1 g/l and acetate to less than 1 g/l. For each pH studied, the glucose consumption rate (q_G), the volumetric succinate productivity (Qs), specific succinate productivity (q_S) and mass product yields of succinate, acetate, ethanol and pyruvate were calculated during the 14 h of the anaerobic phase, and the values reported are the means of

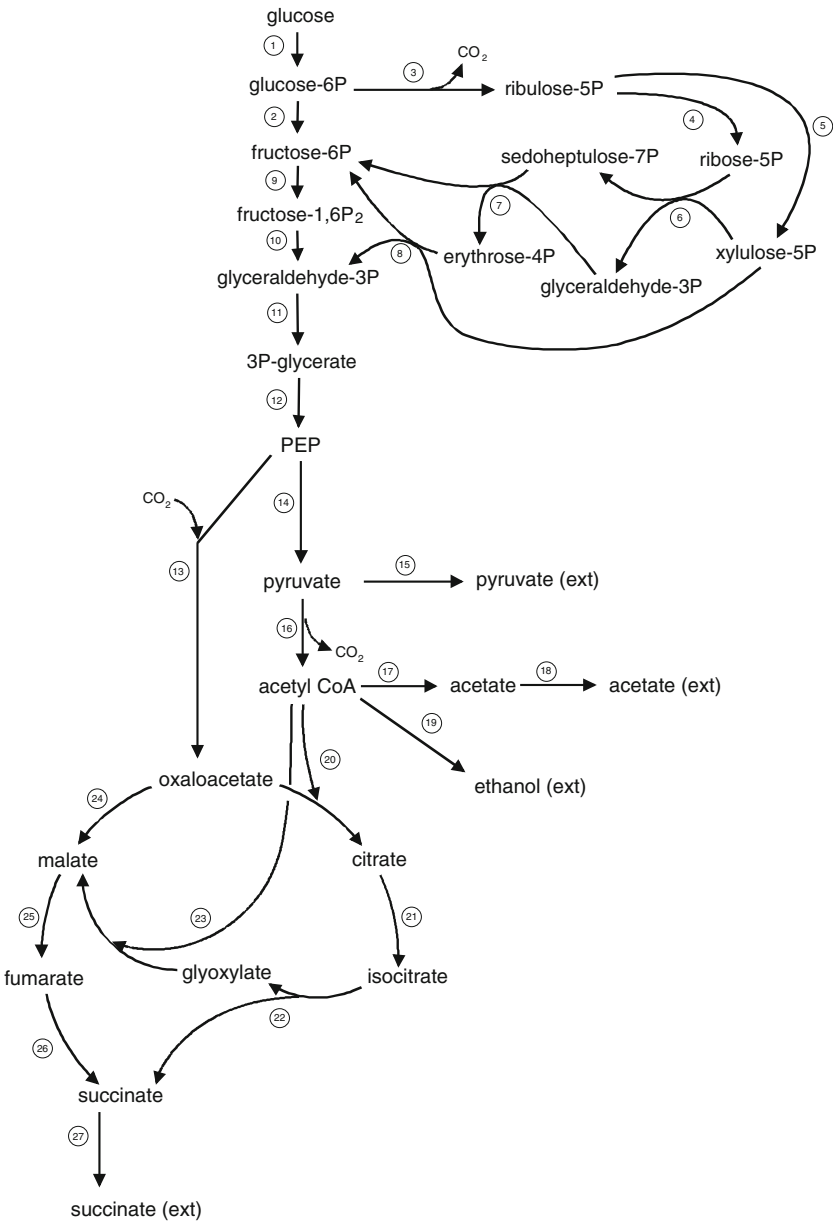

Fig. 1 Biochemical pathways for the synthesis of succinate from glucose in *E. coli*. Not all enzymatic steps or intermediates are shown. Key enzymes in the pathways are as follows: (*1*) glucokinase, (*2*) phosphoglucoisomerase; (*3*) 6-phosphogluconate dehydrogenase; (*4*) phosphopentose epimerase; (*5*) phosphopentose epimerase; (*6*) transketolase; (*7*) transaldolase; (*8*) transketolase; (*9*) phosphofructokinase; (*10*) fructose biphosphate aldolase; (*11*) glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase; (*12*) phosphoglycerate mutase and enolase; (*13*) PEP carboxylase; (*14*) pyruvate kinase; (*16*) pyruvate dehydrogenase complex; (*17*) phosphoacetyltransferase; (*18*) acetate kinase; (*19*) acetaldehyde dehydrogenase and alcohol dehydrogenase; (*20*) citrate synthase; (*21*) aconitase; (*22*) isocitrate lyase; (*23*) malate synthase; (*24*) malate dehydrogenase; (*25*) fumarase; (*26*) fumarate reductase

two to three experiments (Table 1). In the pH range of 5.8–6.4, q_G and q_S increased with increasing pH, but the yields of succinate and other products did not significantly change. When controlled pH was greater than 6.6, q_G and q_S decreased. Fermentations in which the pH was controlled at 6.4 resulted in the highest specific succinate productivity. Moreover, the volumetric succinate productivity at a pH of 6.4 or 6.6 remained high throughout the course of the anaerobic production phase (about 1.2–1.8 g/l h), resulting in the highest mean succinate productivity (Table 1). For a pH above 6.6, the productivity declined over the course of the anaerobic phase (from about 2.0 to 0.5 g/l h). Since pure (acidic) CO_2 was sparged into the fermenter during the anaerobic phase and three acid products were formed, base was required to maintain the pH (Fig. 3). Indeed, an unacceptably large quantity of base was needed above a pH of about 6.6. The observed reduction in specific productivity, which would account for any "dilution effect," demonstrates that the cells generated a maximal rate of succinate at the intermediate pH. Because of these results, a pH controlled at 6.4 during the anaerobic production phase was selected for the subsequent study.

Metabolic flux analysis

Succinate is formed through two pathways: the reductive arm of the tricarboxylic acid cycle (TCA) via the anaplerotic enzyme PPC and the glyoxylate shunt [35]. To

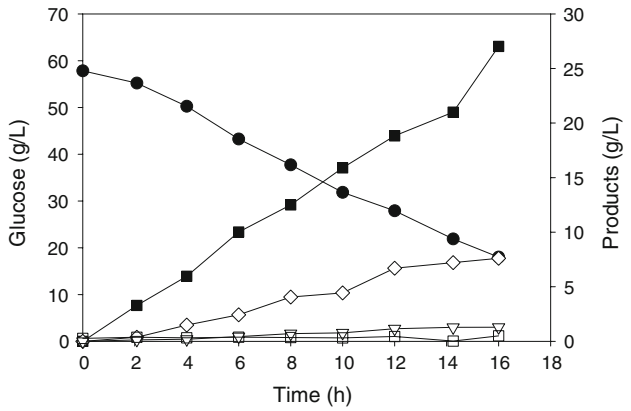

Fig. 2 Production of succinate (*filled square*), pyruvate (*open diamond*), ethanol (*open triangle*) and acetate (*open square*) from glucose (*filled circle*) during the anaerobic non-growth production phase for *E. coli* AFP111 using 25% $Ca(OH)_2$ to control pH at 6.4

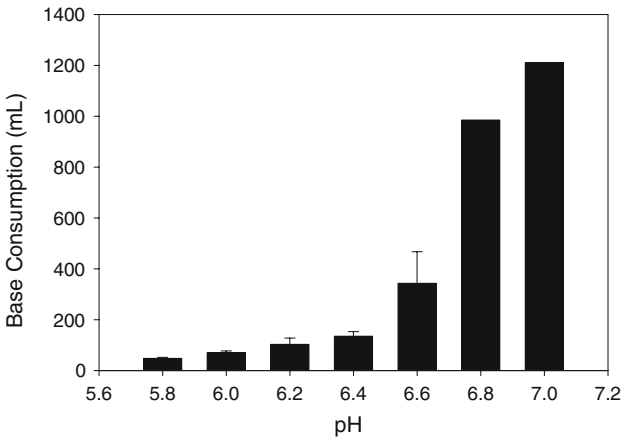

Fig. 3 Volume of 25% $Ca(OH)_2$ consumption during 14 h of an anaerobic non-growth production phase for *E. coli* AFP111 under different levels of controlled pH

understand whether pH affects the distribution of these two pathways, we compared the metabolic fluxes at a pH of 6.4 to that at a pH of 6.8. We first determined the flux distribution at a pH of 6.4. Table 3 compares the observed mass distribution of succinate and the calculated mass distribution by the optimal metabolic model (i.e., least error as defined in "Materials and methods"). Two methods were used to validate the metabolic model. First, we compared the enrichment ratio S2/S1 observed in the NMR results with that ratio predicted from the metabolic model obtained from independent LC-MS results (Table 2). In addition, we calculated the redox balance based on the metabolic model, and the value was very close to 1 (Table 2). The resulting metabolic fluxes for the process operating at a pH of 6.4 showed that 93% of the 1.25 mol succinate formed per mole glucose was generated via the reductive arm of the TCA cycle and 7% via the glyoxylate shunt (Fig. 4a). At the PEP node, ϕ_{PPC} was 0.61 (i.e., about 61% of PEP partitioned to the reductive branch of the TCA cycle). Although 39% of the PEP formed pyruvate, most of the pyruvate ultimately became by-products (external pyruvate, acetate and ethanol), and only 12% of the pyruvate became succinate via the glyoxylate shunt (either directly or from malate).

During succinate accumulation CO_2 was released via 6-phosphogluconate dehydrogenase and pyruvate dehydrogenase (or pyruvate oxidase) but sequestered via PPC (Fig. 1). At a pH of 6.4, the net CO_2 consumption rate was 1.10 mmol/g h. The overall stoichiometric coefficient for CO_2 (i.e., the ratio of net CO_2 consumption to glucose consumption) was 0.62 (Fig. 4a).

We similarly determined the metabolic fluxes at a pH of 6.8 (Table 2). Although the higher pH significantly reduced the glucose consumption rate and succinate production rate (Fig. 4b), it did not alter the carbon partitioning compared to a pH of 6.4: succinate yield was 1.24 mol/mol glucose, ϕ_{PPP} of 0.15 and ϕ_{PPC} of 0.62 (Table 2). Also, 38% of the intermediate PEP formed pyruvate, and about 10% of the pyruvate became succinate via the glyoxylate shunt; the CO_2 stoichiometric coefficient was 0.59 (Fig. 4b).

Table 1 The volumetric productivity (Q), specific consumption or production rates (q) and product mass yields (Y) of *E. coli* AFP111 during 14 h of an anaerobic non-growth phase using $Ca(OH)_2$ as the base to control pH

pH	q_G (mg/g h)	Q_S (g/l h)	q_S (mg/g h)	Y_S (g/g)	Y_A (g/g)	Y_P (g/g)	Y_E (g/g)
5.8	108.7^{a}	0.67^{a}	76.5^{a}	0.71^{a}	0.01^{a}	0.24^{a}	0.00^{a}
6.0	189.3^{b}	1.02^{b}	126.0^{b}	0.67^{a}	0.01^{a}	0.21^{a}	0.02^{a}
6.2	192.6^{b}	1.18^{bc}	125.7^{b}	0.65^{a}	0.02^{a}	0.24^{a}	0.02^{a}
6.4	284.6^{c}	1.42^{c}	174.3^{c}	0.61^{a}	-0.01^{a}	0.24^{a}	0.03^{a}
6.6	257.4^{bc}	1.49^{c}	169.3^{c}	0.66^{a}	0.00^{a}	0.16^{a}	0.03^{a}
6.8	166.3^{b}	0.99^{b}	119.4^{ab}	0.72^{a}	0.01^{a}	0.19^{a}	0.03^{a}
7.0	85.7^{d}	0.36^{d}	47.4^{d}	0.55^{b}	0.06^{b}	0.05^{b}	0.02^{a}

Different letters were statistically significantly different at the 90% confidence level

G glucose, *S* succinate, *A* acetate, *P* pyruvate, *E* ethanol

Table 2 Comparison of the mass distributions of succinate formed by *E. coli* AFP111 as observed by mass spectrometry and as calculated by the optimal metabolic model

pH	φ_{PPP}	φ_{PPC}		M + 0	M + 1	M + 2	M + 3	(S2/S1)[a]	R/O
6.4	0.12	0.61	Observed	0.585 ± 0.006	0.399 ± 0.009	0.014 ± 0.008	0.002 ± 0.002	19.56	
			Calculated	0.591	0.384	0.022	0.003	18.32	0.97
6.8	0.15	0.62	Observed	0.580 ± 0.008	0.395 ± 0.009	0.018 ± 0.005	0.009 ± 0.006	18.76	
			Calculated	0.576	0.398	0.022	0.003	18.79	1.06

The enrichment ratio (S2/S1) was observed from NMR results. The redox ratio R/O was calculated from the optimal metabolic model

Observed values are shown as mean ± standard deviation from three analyses

[a] The standard deviation for NMR measurements was 4–10%

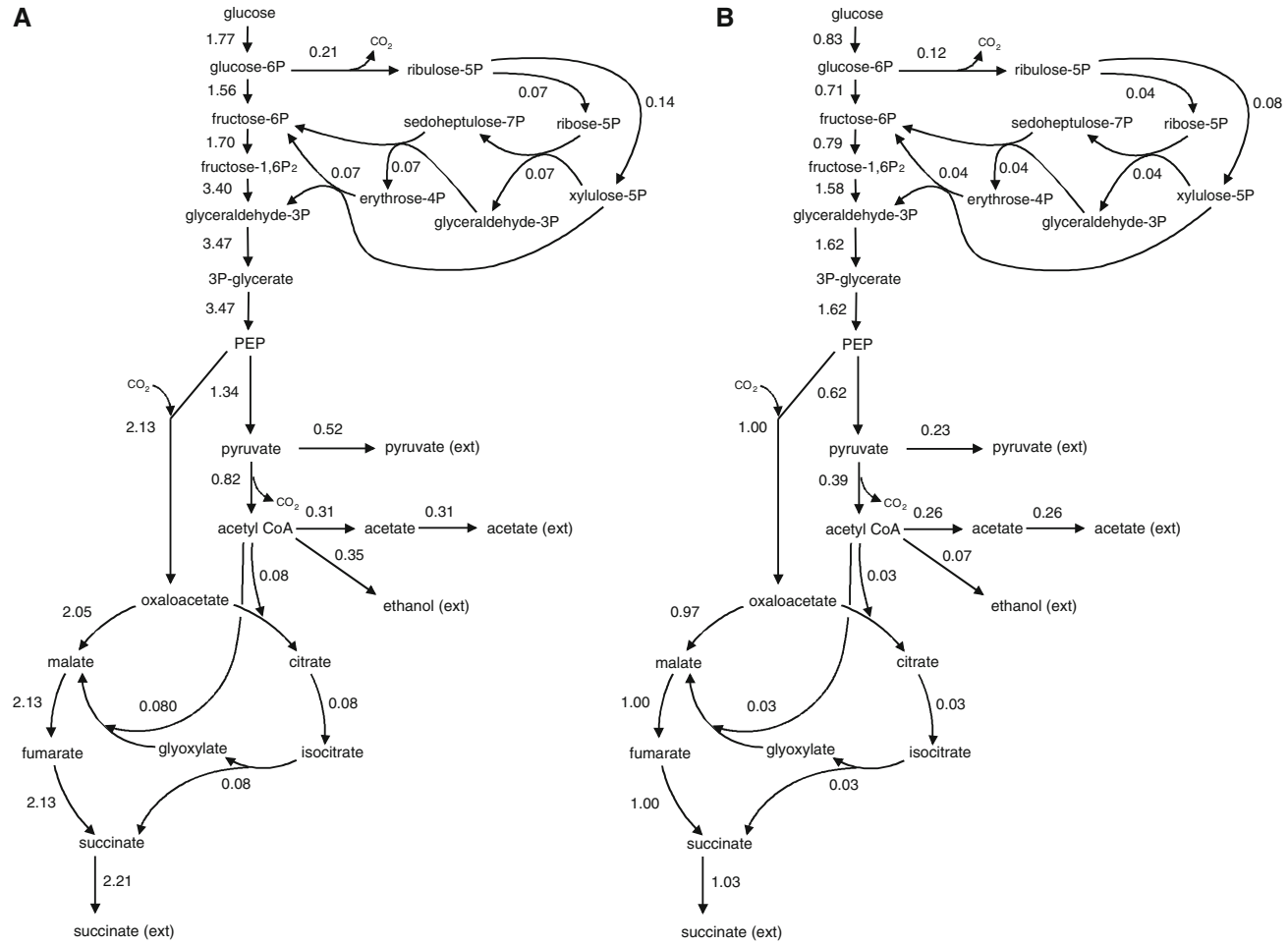

Fig. 4 Metabolic fluxes (mmol/g h) during an anaerobic non-growth production phase of *E. coli* AFP111 at a pH of 6.4 (**a**) and a pH of 6.8 (**b**)

Effect of base counterion

Using a pH of 6.4 and 100% CO_2 in the gas phase, we next examined the effect of the type of neutralizing agent used on succinate production. Three different bases were compared: 25% KOH, 25% NaOH or 25% $Ca(OH)_2$. The effects of K^+ and Na^+ appeared to be similar, with the succinate productivity declining during the anaerobic phase (from about 1.7 to 0.3 g/l h). The difference in volume used (Table 3) was due to the unequal molar base concentrations, and the total moles of KOH or NaOH added during the 14-h anaerobic phase were similar. When $Ca(OH)_2$ was used for pH control, some calcium succinate precipitated, and the volumetric succinate productivity remained high during 14 h of an anaerobic production phase (about 1.2–1.8 g/l h), resulting in the highest mean

Table 3 The volumetric productivity (Q), specific consumption or production rates (q) and product mass yields (Y) of *E. coli* AFP111 during 14 h of an anaerobic non-growth phase using three different bases to control pH at 6.4

Base (25% w/v)	q_G (mg/g h)	Q_S (g/l h)	q_S (mg/g h)	Y_S (g/g)	Y_P (g/g)	Y_A (g/g)	Y_E (g/g)	V_{base} (ml)
$Ca(OH)_2$	248.6 ± 31.8	1.42 ± 0.19	174.3 ± 40.2	0.61 ± 0.13	0.24 ± 0.04	−0.01 ± 0.02	0.03 ± 0.02	135 ± 18
KOH	177.9 ± 19.0	0.88 ± 0.07	95.3 ± 5.8	0.54 ± 0.08	0.37 ± 0.01	−0.01 ± 0.02	0.01 ± 0.03	113 ± 7
NaOH	176.2 ± 28.2	0.99 ± 0.19	101.6 ± 23.9	0.57 ± 0.04	0.21 ± 0.08	0.02 ± 0.01	0.00 ± 0.02	66 ± 15

Data were presented as mean ± standard deviation from two to three replications

G glucose, *S* succinate, *A* acetate, *P* pyruvate, *E* ethanol

succinate productivity (q_S) (Table 3). The small reduction in succinate productivity over the course of the anaerobic phase for $Ca(OH)_2$ can be attributed to a dilution of fermenter contents (i.e., adding base to a non-growing population). Twenty-five percent $Ca(OH)_2$ and 25% NaOH have similar OH^- molar concentrations (0.67 M for $Ca(OH)_2$ and 0.63 M for NaOH). However, during the 14-h anaerobic phase about twice the amount of $Ca(OH)_2$ was consumed compared to NaOH (Table 3), which is consistent with the higher q_S for $Ca(OH)_2$. The values of q_G and Y_S using $Ca(OH)_2$ were also higher than those at NaOH or KOH as a base counterion (Table 3).

Discussion

Between the pH range of 5.8–6.8, the specific succinate formation and glucose consumption rates achieved their maximum values, although the fermentation product yields were not significantly affected by the external pH (Table 1). Most aerobic and facultatively anaerobic bacteria stringently regulate the cytoplasmic pH (pH_i), and the pH_i of *E. coli* is unaffected by large variations in the medium pH (pH_{ex}) [2]. Olsen et al. [21] found that the pH_i of *E. coli* (7.0–8.0) did not vary significantly over the pH_{ex} ranging from 5.5 to 8.0. However, the pH gradient ($\Delta pH = pH_i - pH_{ex}$) is approximately 1.5 at a pH_{ex} of 5.5 and decreases with increasing pH_{ex}, ultimately reaching 0 at a pH_{ex} of 8.0 [21]. A large ΔpH is associated with more active transport of H^+ and other ions across the membrane to maintain pH homeostasis [2]. One might anticipate that the increased maintenance requirement resulting from reduced pH might reduce the product yield. Although no product yield reduction was observed, the burden of maintaining ΔpH at low pH may be responsible for the reduced rates of substrate utilization and succinate formation.

The succinate formation rate did not increase indefinitely with increasing pH; it decreased above a pH of 6.4. The quantity of buffer required to maintain the pH increased with increasing pH (Fig. 3). Thus, when the pH was above 6.4, the ionic strength increased more quickly during the succinate formation phase, an effect that may have been detrimental to the rate of succinate formation. Two mechanisms may exist that result in the observed optimum pH: a ΔpH effect that reduces succinate formation at low pH and an ionic strength effect that reduces the succinate formation rate at higher pH. Interestingly, the optimal pH for succinate productivity observed in this study (6.4) corresponds closely with the pK_a of the carbonic acid/bicarbonate equilibrium (6.35). Our optimal pH for succinate production by *E. coli* AFP111 near 6.4 is also consistent with other succinate-producing bacteria, including *A. succiniciproducens* at a pH of 6.2 [24] and *Bacteroides fragilis* at a pH of 6.5 [12].

The results demonstrate that ^{13}C-labeling can be successfully applied to calculate metabolic fluxes during non-growth succinate production. For this process, the reductive branch of the TCA cycle was the most important pathway for succinate formation. This result is consistent with other succinate-producing *E. coli* strains, including a *ptsG* mutant TUQ2 [36] and *E. coli* strains that overexpress *Lactococcus lactis* pyruvate carboxylase [26]. Surprisingly, the flux distribution/carbon distribution was not significantly different between a pH of 6.4 and 6.8. In particular, the pH of the medium, which in this pH range would have a large impact on the CO_2/HCO_3^- ratio [7, 29], did not affect the flux partition at the PEP node (Fig. 4). Similar to our results with *E. coli*, the distribution of fermentation products by *Actinobacillus sp.* 130z [32] did not differ in the pH range of 6.0–7.4. The key enzyme PPC uses bicarbonate as the form of CO_2 [20], and flux through this pathway could be expected to depend on the external availability of CO_2/HCO_3^-. Indeed, in the presence of limiting concentrations of CO_2, *E. coli* AFP111 formed less succinate (data not shown); however, in this study 100% CO_2 was used. Under these circumstances, the yield and flux results support the conclusion that CO_2 was not limiting despite the dissociation between the dissolved gas and bicarbonate in this pH range. Consequently, under these non-limiting conditions, increasing the total quantity of CO_2 (i.e., CO_2 plus HCO_3^-) as a result of increasing the pH would not lead to an increase in the PPC flux and associated succinate formation. Furthermore, as the previous research described above would suggest, pH_i is likely unchanged over the pH range studied, and pH would therefore not affect the

activities of the various enzymes in the glucose to succinate metabolic pathways.

The results demonstrate that Ca^{2+} is superior to Na^+ or K^+ as a base counterion to control the pH during the fermentation. Although the specific cause for this difference is unknown, the calcium succinate has a solubility of only 11.8 g/l at 40°C [19], which is far lower than sodium succinate or potassium succinate. Thus, the use of $Ca(OH)_2$ would have the double benefit of removing both the cation Ca^{2+} and the anion succinate from the solution. The observed removal of succinate by the precipitation of calcium succinate could relieve the inhibition of PPC [4] and isocitrate lyase [18], two key enzymes in succinate formation [35]. Removal of the cation would reduce osmotic stress. At the end of the processes studied, the concentration of Na^+ or K^+ was about 0.5 M, while the remaining concentration of dissociated Ca^{2+} would have been only about 0.18 M. High ion strength leads to osmotic stress, and under aerobic conditions *E. coli* exports some Na^+ as a response [23]. However, under anaerobic conditions, Na^+ extrusion activity may decrease [30]. Moreover, in our study the high ionic strength only occurred under anaerobic non-growth conditions, potentially limiting the cellular ability to respond to that stress. A recent study on lactate generation similarly demonstrated that monovalent cations reduce acid formation by *E. coli* compared to Ca^{2+} [38]. Gouesbet et al. [9] found that about 0.24 M KCl (osmolarity of 820 mOsm) had inhibitory and repressive effects on anaerobic enzymes and the corresponding genes in *E. coli* due to osmotic stress.

In summary, although the pH does affect the production rate, the pH within the range of 5.8–6.8 does not affect yield or the flux distribution of products in a two-phase succinate production process using *E. coli*. The negative impact of osmotic stress during succinate accumulation can at least partly be alleviated by using calcium as the product counterion.

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Appendix A: Metabolic reactions for succinate production

v_1	Glucose + ATP ⇆ glucose 6-phosphate + ADP
v_2	Glucose 6-phosphate ⇆ fructose 6-phosphate
v_3	Glucose 6-phosphate + 2 $NADP^+$ + H_2O ⇆ ribulose 5-phosphate + CO_2 + 2 NADPH + 2 H^+
v_4	Ribulose 5-phosphate ⇆ ribose-5-phosphate
v_5	Ribulose 5-phosphate ⇆ xylulose 5-phosphate
v_6	Ribose 5-phosphate + xylulose 5-phosphate ⇆ glyceraldehyde 3-phosphate + sedoheptulose 7-phosphate
v_7	Glyceraldehyde 3-phosphate + sedoheptulose-7-phosphate ⇆ fructose 6-phosphate + erythrose 4-phosphate
v_8	Xylulose 5-phosphate + erythrose 4-phosphate ⇆ fructose 6-phosphate + glyceraldehydes 3-phosphate
v_9	Fructose 6-phosphate + ATP ⇆ fructose 1,6-bisphosphate + ADP
v_{10}	Fructose 1,6-bisphosphate ⇆ dihydroxyacetone phosphate + glyceraldehyde 3-phosphate
v_{11}	Glyceraldehyde 3-phosphate + NAD^+ + ADP + Pi ⇆ 3-phosphoglycerate + NADH + H^+ + ATP
v_{12}	3-Phosphoglycerate ⇆ PEP + H_2O
v_{13}	PEP + CO_2 + H_2O ⇆ oxaloacetate + Pi
v_{14}	Phosphoenolpyruvate + ADP ⇆ pyruvate + ATP
v_{15}	Pyruvate (intracellular) ⇆ pyruvate (extracellular)
v_{16}	Pyruvate + NAD^+ + CoA ⇆ acetyl-CoA + CO_2 + NADH + H^+
v_{17}	Acetyl-CoA + ADP + Pi ⇆ acetate + CoA + ATP
v_{18}	Acetate (intracellular) ⇆ acetate (extracellular)
v_{19}	Acetyl-CoA + 2 NADH + 2 H^+ ⇆ ethanol + 2 NAD^+
v_{20}	Oxaloacetate + acetyl-CoA + H_2O ⇆ citrate + CoA
v_{21}	Citrate ⇆ isocitrate
v_{22}	Isocitrate ⇆ glyoxylate + succinate
v_{23}	Glyoxylate + acetyl-CoA + H_2O ⇆ malate + CoA
v_{24}	Oxaloacetate + NADH + H^+ ⇆ malate + NAD^+
v_{25}	Malate ⇆ fumarate + H_2O
v_{26}	Fumarate + NADH + H^+ ⇆ succinate + NAD^+
v_{27}	Succinate (intracellular) ⇆ succinate (extracellular)

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