EFFECT OF ENVIRONMENTAL FACTORS ON SUCCINATE PRODUCTION BY METABOLICALLY ENGINEERED *ESCHERICHIA COLI*

by

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ABSTRACT

Succinate has a wide range of applications in the chemical, food, and pharmaceutical industries, and its production by the metabolically engineering *Escherichia coli* AFP111 was studied. The principal aims of this research were to understand the effects of pH, base counter-ion, CO_2 concentration and flue gas components in two-phase fermentations using a defined medium. A pH of 6.4 yielded the highest specific succinate productivity, and the metabolic flux determined with ^{13}C-labeled glucose showed that 61% of the PEP partitioned to oxaloacetate and 39% partitioned to pyruvate. Although a pH of 6.4 was optimal, the flux distribution at a pH of 6.8 was not significantly different. $Ca(OH)_2$ was superior to NaOH or KOH as the base for controlling the pH.

A series of dual-phase fermentations showed that CO_2 concentration influenced succinate production. The succinate specific productivity was increased from 1.89 mg/gh at 0% CO_2 to 224.50 mg/gh at 50% CO_2, and the yield was increased from 0.04 g/g to 0.75 g/g. Above 50% CO_2, succinate production did not increase with increasing CO_2 gas concentration. Using ^{13}C-labeled glucose, the fraction of flux into the pentose phosphate pathway increased from 0.04 at

3% CO_2 to 0.17 at 50% CO_2. Also, the fractional flux through carboxylation at the PEP node increased slightly from 0.53 at 3% CO_2 to 0.63 at 50% CO_2. The increased flux into the pentose phosphate pathway is attributed to an increased demand for NADH with elevated CO_2. A four-process explicit model to describe the CO_2 transfer and utilization was proposed. The model predicted that at CO_2 concentrations below about 40% the system becomes limited by gas phase CO_2, while at higher CO_2 concentrations the system is limited by PPC enzyme kinetics.

Finally, each of the other components of flue gas, oxygen (O_2), nitrogen dioxide (NO_2), sulfur dioxide (SO_2) or carbon monoxide (CO), was studied for succinate production. Following aerobic cell growth, cells were exposed to 50% CO_2 and 3-10% O_2, and 50-300 ppm NO_2, SO_2 or CO during a succinate production phase. Although 3% O_2 did not significantly affect succinate formation, 10% O_2 reduced the final succinate concentration from 33 g/L to 17 g/L, specific succinate productivity from 1.90 to 1.13 mmol/g·h and the succinate yield from 1.15 to 0.81 mol/mol glucose. The effect of O_2 correlated with the culture redox potential (ORP) with more reducing conditions favoring succinate production. The trace gases NO_2 and SO_2 also reduced the rate of succinate formation by as much as 50%, but led to a greater than two-fold increase in pyruvate formation. 100-500 ppm CO showed no effect on succinate production rate or yield. Using synthetic flue gas AFP111 could generate 12 g/L succinate with a succinate specific productivity of 0.73 mmol/g·h and a yield of 0.65 mol/mol.

INDEX WORDS: Succinic acid, *Escherichia coli*, pH, Base counterion, Metabolic flux analysis, ^{13}C-labeling, Tricarboxylic acid cycle, Glyoxylate shunt, Carbon dioxide, Flue gas, Oxygen,

Nitrogen dioxide, Sulfur dioxide, Carbon monoxide, Redox potential, Pentose phosphate pathway.

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DEDICATION

To my family

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CHAPTER I

INTRODUCTION

Succinate is a four-carbon dicarboxylic acid which serves as a precursor for many commercial chemicals, such as 1,4-butanediol, tetrahydrofuran, and γ-butyrolactone (McKinlay et al., 2007). Traditionally, succinate is produced through petrochemical processes. Rising prices and concerns over the environmental impact of oil-based industries have elevated interest in microbial fermentation as a viable alternative for succinate production. Moreover, microbial succinate production can have the environmental benefit of CO_2 fixation. Some anaerobic bacteria such as *Anaerobiospirillum succiniciproducens* (Lee et al., 2000) and *Actinobacillus succinogenes* (Guettler et al., 1999) produce succinate as the major fermentation product. During anaerobic fermentation, these organisms fix the greenhouse gas CO_2 via carboxylation reactions and convert C3 to C4 metabolites. As a model system, recombinant *Escherichia coli* can generate succinate through native PPC (Matsumura et al., 2002), PCK (Matte et al., 1997), or heterologous PYC (Modak and Kelly, 1995). *E. coli* AFP111 that contains *ldhA*, *pfl* and *ptsG* mutations could generate high concentration of succinate (Vemuri et al., 2002).

To understand the metabolic regulation of *E. coli* AFP111 in response to environmental conditions three studies were undertaken. In the first study, the effect of pH and base counterion were investigated and optimal pH and base counterion were selected.

To elucidate the cellular requirement for CO_2, the effect of CO_2 concentration in the gas phase on succinate formation was studied in a second study. Metabolic flux analysis coupled with ^{13}C-labeled tracing was applied to determine the carbon distribution at varied CO_2 concentrations. Possible rate limiting factors of CO_2 transport and utilization by *E. coli* were discussed.

One readily available industrial source of CO_2 is flue gas, which often contains several other gaseous components such as O_2, NO_2, SO_2, and CO. In a third study, the influence of these gas components on the anaerobic formation of succinate was examined.

These studies provide a detailed understanding of the effect of pH, base counterion, and gaseous components on the physiology of *E. coli* AFP111, and bring to light how microbial metabolism responds to environmental conditions. This information increases our understanding of metabolic regulation in central metabolism. Such knowledge may provide guidance for other products of central metabolism and their derivatives.

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CHAPTER II

LITERATURE REVIEW

Introduction

Succinic acid (the salt is succinate) is a common metabolite formed by plants, animals and microorganisms. A linear saturated dicarboxylic acid (Figure II-1), it can be chemically converted into N-methylpyrrolidone, 1,4-butanediol, tetrahydrofuran, γ-butyrolactone, and adipic acid. It can also be easily esterified to dimethyl succinate, which is regarded as an environmentally friendly solvent (Tsao et al., 1999; Zeikus et al., 1999; McKinlay et al., 2007a). Succinate and its derivatives are now widely used as specialty chemicals with applications in polymers, foods, pharmaceuticals, and cosmetics. There are four major existing markets for succinate: surfactant/detergent extender/foaming agent; ion chelator; acidulant/pH modifier in food industry; and health-related agents including pharmaceuticals, antibiotics, amino acids and vitamins (Sriram and Dennis, 1999; Zeikus et al., 1999; McKinlay et al., 2007a). Figure II-2 illustrates the routes leading to succinate-based intermediates and chemicals.

Succinate is currently manufactured by the hydrogenation of maleic anhydride to succinic anhydride, followed by hydration to succinate (Tsao et al., 1999). As the cost for the chemical route increases in the coming years while improvements in the biological process are attained, a biological route may become economic. This review examines the literature on microbial

production of succinate regarding microorganisms, metabolic pathways involved and the metabolic flux analysis.

Microbes that produce succinate

Succinate is an intermediate in the central metabolic pathway of all organisms. It also accumulates in several anaerobic and facultative microorganisms. For example, typical gastrointestinal bacteria such as *Escherichia coli*, *Pectinatus* sp., and *Bacteroides* sp. can form succinate. Propionate-producing bacteria such as *Propionibacterium* species have also been reported to produce succinate from sugars or amino acids. Another source of succinate-producing microbes is rumen bacteria such as *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Actinobacillus succinogenes*, *Bacteroides amylophilus*, *Prevotella ruminicola*, *Succinimonas amylolytica*, *Succinivibrio dextrinisolvens*, *Wolinella succinogenes*, *Cytophaga succinicans* *Actinobacillus succinogenes* and *Mannheimia succiniciproducens* (Gokarn et al., 1997; Zeikus et al., 1999; McKinlay et al., 2007a). Another good succinate producer is a non-rumen anaerobic bacterium, *Anaerobiospirillum succiniciproducens,* which is capable of utilizing various sugar as a carbon source (Lee et al., 1999b). In this section some major succinate-producing microorganisms will be described, which are divided artificially into two groups: obligate anaerobes and facultative anaerobes.

Obligate anaerobes

Fibrobacter succinogenes

Anaerobic rumen bacteria are one source of succinate-producing microbes. For example, *Fibrobacter succinogenes* S85, a cellulolytic rumen bacterium, is very efficient in degrading lignocellulosic substrates (Bibollet et al., 2000) and can utilize glucose, cellobiose, microcrystalline cellulose, Walseth cellulose (acid swollen cellulose), pulped paper, and steam-exploded yellow poplar as substrates (Weimer, 1993; Gokarn et al., 1997). The major end product produced from each of these substrates was succinate (70-83%), the principal secondary product was acetate (16-30%). Maximum succinate productivity ranged from 14.1 mg/l·h for steam-exploded yellow poplar to 59.7 mg/l·h for pulped paper (Gokarn et al., 1997).

Anaerobiospirillum succiniciproducens

Another good succinate producer is a non-rumen anaerobic bacterium, *Anaerobiospirillum succiniciproducens,* which is capable of utilizing glucose, lactose, sucrose, maltose, and fructose (Lee et al., 1999b). In whey fermentation to produce a succinate-rich animal feed additive, 90% whey lactose was consumed at high CO_2 level (35 g/L $MgCO_3$ supplying CO_2) in batch fermentation resulting in a succinate yield of 84% and a succinate to acetate ratio of 4.3 g/g (Samuelov et al., 1999). Continuous fermentation of untreated whey by *A. succiniciproducens* at a dilution rate of 0.11 h^{-1} resulted in the production of succinate with high productivity (1.35 g/l·h), high conversion yield (93%), and high ratio of succinic acid to acetic acid (5:1) (Lee et al., 2000). In a wood hydrolysate media (equivalent to 27 g/L glucose), *A.*

succiniciproducens produced 24 g/L succinate with the yield of 88% when supplemented with 10 g/L corn steep as a complex nitrogen source (Lee et al., 2003a). Using 6.5 g/L glycerol as a carbon source, a succinate yield of 133% was obtained, and the mass ratio of succinate to acetic acid was 26:1 (Lee et al., 2001). In a cell recycle bioreactor of continuous *A. succiniciproducens* fermentation, the biomass concentration and succinate volumetric productivity reached values of 42 g/L and 14.8 g/l·h, respectively (Meynial-Salles et al., 2008). To limit end-products inhibition on growth, a mono-polar electrodialysis pilot was coupled to the cell recycle bioreactor. This system allowed continuous removal of succinate and acetate from the permeate and recycle an organic acids depleted solution in the reactor. The integrated membrane-bioreactor-electrodialysis process produced a five times concentrated succinate solution (83 g/L) compared to the cell recycle reactor system, at a high average succinate yield of 1.35 mol/mol and a slightly lower volumetric productivity of 10.4 g/l·h (Meynial-Salles et al., 2008).

Facultative anaerobes

Actinobacillus succinogenes and *Mannheimia succiniciproducens*

Actinobacillus succinogenes and *Mannheimia succiniciproducens* are facultatively anaerobic, and both were isolated from the bovine rumen. *A. succinogenes* was isolated from a Michigan State University cow, whereas *M. succiniciproducens* was isolated from a Korean cow. Their genome sequences are more similar to each other than to any other sequenced genome (Hong et al., 2004). Not surprisingly, the two species share many metabolic traits. Both produce

succinate, formate, and acetate as major fermentation products. When consuming glucose, *M. succiniciproducens* also produces lactate, whereas *A. succinogenes* produces ethanol (van der Werf et al., 1997; Lee et al., 2002).

Both species have been characterized in industrially relevant conditions. *A. succinogenes* strain 130Z could produce more than 30 g/L succinate in media containing 20 g/L corn steep liquor (Guettler et al., 1999). The ratio of succinate to acetate was between 5:1 and 8:1. Formate was also produced along with small amounts of pyruvate, oxaloacetate, and ethanol. In a medium containing corn steep liquor, yeast extract, and glucose, *A. succinogenes* produced 80 g/L succinate (Guettler et al., 1996), a remarkable titer for a wild-type strain. *A. succinogenes* was also used in a reactor with plastic composite supports designed to increase succinate productivities through biofilm formation and high cell densities (Urbance et al., 2003, 2004). Using the reactor for a continuous fermentation with industrial quality yeast extract and corn steep liquor, the highest final succinate concentration (10.4 g/L) and percentage yields (71.6%) occurred at the dilution rate of 0.2 h^{-1} with an agitation rate of 150 rpm (Urbance et al., 2004). However, with high cell densities estimated at 160 g DCW/L, specific productivity was poor (~55 mg/g·h).

M. succiniciproducens was grown in batch and continuous cultures on whey plus corn steep liquor. Succinate yields and productivities were 71% at 1.2 g/l·h and 69% at 3.9 g/l·h in batch and continuous cultures, respectively (Lee et al., 2003b). Batch cultures of *M. succiniciproducens* using a medium containing glucose and yeast extract produced 10.4 g/L

succinate with a yield of 0.45 g/g and the mass ratio of succinate to acetate of 2:1 (Lee et al., 2006). In batch fermentation using glucose and xylose, the yield and productivity of 63% and 1.19 g/l·h were achieved. In comparison, wood hydrolysate was found to be as good as refined carbohydrates for the growth of *M. succiniciproducens* and succinic acid production resulting in the yield and productivity of 56% and 1.17 g/l·h under the same condition. By the continuous cultivation of *M. succiniciproducens* using glucose and xylose mixture, a succinic acid productivity of 3.29 g/l·h could be achieved (Kim et al., 2004).

Escherichia coli

Escherichia coli is a facultative bacterium which under anaerobic conditions converts sugars to a mixture of organic acids. The major products are lactate, acetate, ethanol and formate with smaller amounts of succinate (Clark, 1989). Though *E. coli* forms succinate only as a minor fermentation product (Wood, 1981), there has been considerable effort to engineer *E. coli* for succinate production since it could be genetically engineered with relative ease. The recombinant *E. coli* will be covered in the later section.

Pathways involved for succinate formation

The pathways to form succinate are for convenience divided into three parts: glycolysis, pentose phosphate pathway, and the pathway leading to succinate from pyruvate or phosphoenolpyruvate (PEP). The following are the details for these pathways and the principal enzymes.

Glycolysis

Glycolysis is the sequence of reactions that converts glucose into pyruvate with the concomitant production of a relatively small amount of ATP. Glycolysis can be carried out anaerobically and is thus an especially important pathway for organisms that can ferment sugars. An overview of the glycolytic pathway is presented in Figure II-3. Glycolysis can be considered to have two parts. First, glucose is converted to two moles of glyceraldehyde-3-phosphate via a process that consumes two moles of ATP. Second, five subsequent reactions convert the two moles of glyceraldehyde-3-phosphate into two molecules of pyruvate, and this sequence generates four molecules of ATP. The net result of glycolysis is 2 moles of ATP produced per mole of glucose. In addition to ATP, the products of glycolysis are NADH and pyruvate. NADH must be recycled to NAD^+, lest NAD^+ become limiting for glycolysis.

Phosphoenolpyruvate:sugar phosphotransferase system

The bacterial phosphotransferase system (PTS) is the major transport system for many carbohydrates that are phosphorylated concomitantly with the translocation step through the membrane (group translocation). The unique features of this PTS include the use of phosphoenolpyruvate (PEP) as the phosphoryl donor for sugar phosphorylation and the presence of three essential catalytic entities: Enzyme I (EI); histidine protein HPr; and a number of carbohydrate-specific enzymes collectively called Enzyme II (EII). PTS proteins are phosphoproteins in which the phospho group is attached to either a histidine residue or a cysteine residue. After phosphorylation of EI by PEP, the phospho group is transferred to HPr. The EII

proteins are required for the transport of the carbohydrates across the membrane and the transfer of the phospho group from phospho-HPr to the carbohydrates (Postma et al., 1993).

Glucokinase

Glucokinase (EC 2.7.1.2) phosphorylates glucose using ATP as a donor to give glucose-6-phosphate and ADP. In *E. coli* and most other bacteria, glucose is transported by the PTS as glucose-6-phosphate, thus eliminating the need for glucokinase in the utilization of glucose. Glucokinase from many sources requires a metal ion for its activity. For example, in *Aeropyrum pernix*, *Bacillus stearothermophilus*, and *Dictyostelium discoideum* the activity completely depends on the present of Mg^{2+} (Baumann, 1969; Goward et al., 1986; Hansen et al., 2002); and in *Thiobacillus versutus* Mn^{2+} is most effective to activate the activity of glucokinase though other divalent cation can partially replace it (Klein and Harles, 1986). Kinetic values for this enzyme have been studied (Scopes and Bannon, 1995; Meyer et al., 1997; Sakuraba et al., 2003), and the representative Km values in *E. coli* for glucose and ATP are 0.15 mM and 0.50 mM, respectively (Arora and Pedersen, 1995). The optimal pH of glucokinase is between 7 and 9 (Baumann, 1969; Hengartner and Zuber, 1973; Porter et al., 1982; Goward et al., 1986; Sakuraba et al., 2003).

Phosphofructokinase

Phosphofructokinase (PFK, EC 2.7.1.11) is a glycolytic enzyme that catalyzes the irreversible transfer of a phosphate from ATP to fructose-6-phosphate. PFK is the key regulatory enzyme for glycolysis. When ATP levels are high in the cell, the cell no longer needs metabolic

energy production to occur. In this case, PFK activity is inhibited allosterically by ATP itself, closing the valve on the flow of carbohydrates through glycolysis (Zheng and Kemp, 1992; Garrett and Grisham, 1999; Wang and Kemp, 2001). PFK is also inhibited by abundant cellular concentrations of citrate; therefore glycolysis and tricarboxylic acid cycle (TCA cycle, also named citric acid cycle) are coupled via PFK. When citrate levels are high, cells presumably obtain sufficient energy from the TCA cycle and do not need glycolysis to provide more carbon into the TCA pathway (Kotlarz and Buc, 1982; Garrett and Grisham, 1999). Another modulator of PFK is fructose-2,6-bisphosphate, an allosteric activator that increases the affinity of PFK for fructose-6-phosphate. Stimulation of PFK is also achieved by decreasing the inhibitory effects of ATP as well as inhibiting fructose-1,6-bisphosphatase, the enzyme that catalyzes the opposite reaction (Garrett and Grisham, 1999).

Pyruvate kinase

Pyruvate kinase (EC 2.7.1.40) mediates the transfer of a phosphoryl group from PEP to ADP to generate ATP and pyruvate. The reaction requires Mg^{2+} and is stimulated by K^+ and certain other monovalent cations such as Rb^+, Cs^+, Na^+, NH_4^+, Li^+ (De Medicis et al., 1982; Mesecar and Nowak, 1997; Garrett and Grisham, 1999). Pyruvate kinase is the third regulated enzyme of glycolysis. It is activated by fructose-1,6-bisphosphate (Malcovati and Valentini, 1982) and inhibited by ATP, acetyl-CoA and alanine (a biosynthetic product of pyruvate) (Garrett and Grisham, 1999).

Pentose phosphate pathway

The pentose phosphate pathway begins with glucose 6-phosphate, a six-carbon sugar, and produce three-, four-, five-, six-, ans seven-carbon sugar (Figure II-3). It produces NADPH for reductive reactions vital to biosynthetic purposes and ribose-5-phosphate esstential for nucleic acid synthesis (Garrett and Grisham, 1999). The pentose phosphate pathway was active during cell growth coupled with succinate production (Lee et al., 2006; McKinlayet al., 2007b). The pentose phosphate pathway may also be active during the non-cell-growth succinate production phase of the dual-phase fermentation.

Glucose 6-phosphate dehydrogenase

The pentose phosphate pathway begins with glucose 6-phoaphate. The products of the reactions are cyclic ester (the lactone of phosphogluconic acid) and NADPH. As the first step of a major pathway, the reaction is irreversible and highly regulated. It is strongly inhibited by NADPH, and the inhibition depends on the cytosolic $NADP^+$/NADPH ratio (Garrett and Grisham, 1999). The enzyme from *E. coli* was activated by bivalent cations as chlorides with Mg^{2+} and Ca^{2+} most active at about 2×10^{-2} M concentration. However, in systems containing calcium or magnesium ions the addition of other bivalent cations, copper, cadmium and zinc especially, caused marked inhibition. Michaelis constants for the enzyme-substrate complex were of the order of 3×10^{-4} for glucose 6-phosphate dehydrogenase. The optimal pH was 7.7-8.6 (Scott and Cohen, 1953).

Phosphogluconate dehydrogenase

The 6-phosphogluconate dehydrogenase catalyzes the decarboxylation of 6-phosphogluconate to yield D-ribulose 5-phosphate. This oxidation leads to reduction of $NADP^+$ to NADPH and the release of CO_2 (Garrett and Grisham, 1999). Similary to glucose 6-phosphate dehydrogenase, the *E. coli* 6-phosphogluconate dehydrogenase was activated by bivalent cations as chlorides, with Mg^{2+} and Ca^{2+} most active at about 2×10^{-2} M concentration. Michaelis constants for this enzyme-substrate complex were of the order of 3×10^{-5} M, and the optimal pH was 6.6-7.7 (Scott and Cohen, 1953).

Pathways that lead to succinate formation from PEP/pyruvate

PEP or pyruvate produced from glycolysis can generate succinate through either of two pathways: the reductive branch of the TCA cyclye and glyoxylate shunt (Figure II-4). The key enzymes involved are described in the subsection below.

PEP carboxylase

PEP carboxylase (PPC; EC 4.1.1.31) catalyzes the carboxylation of phosphoenolpyruvate (PEP) to form oxaloacetate using Mn^{2+} or Mg^{2+} as a cofactor. The reaction can be divided into three steps: first, bicarbonate ion nucleophilic attacks the phosphoryl group of PEP forming a carboxyphosphate intermediate and the enolate of pyruvate; second, carboxyphosphate intermediate decomposes to enzyme-bound CO_2 and phosphate; and finally the enzyme-bound CO_2 combines with the metal-stabilized enolate to form OAA (Matsumura et al., 2002). This

enzyme is widely present in higher plants, algae, photosynthetic and cyanobacteria bacteria, and most nonphotosynthetic bacteria; however it is not found in yeast, fungi, and animals. The PPCs from different sources have similar characteristics both on functions and structures.

The optimal pH of PPC varies with the effectors. For example, with only Mg^{2+} the optimal pH of *E.coli* PPC is between 9-10; while with the addition of acetyl-CoA or Mn^{2+}, the high activity range is pH 7-10 (Sanwal and Maeba, 1966; Corwin and Fanning, 1968). Other reports of optimal pH of *E. coli* PPC is 8-9 (Yoshinaga et al., 1970; Wohl and Markus, 1972). The optimal pH of plant PPC is 7.5-8.5 (Lepiniec et al., 1994). Most PPCs are subject to allosteric regulation; however, the metabolite effectors vary among the bacteria, alga, and higher plants. *E. coli* PPC is allosterically regulated by multiple effectors, and Table II-1 lists these effectors as well as their mechanisms. The PPC of *Chlamydomonas reinhardtii*, a green alga, is activated by glutamine (Gln) and dihydroxyacetone phosphate and inhibited by glutamic acid (Glu) (Rivoal et al., 1998). Higher plant PPCs are usually activated by glucose-6-phosphate and inhibited by malate (Walker and Edwards, 1991; Tovar-Mendez et al., 2000), aspartate (Tovar-Mendez et al., 2000), pyruvate and oxaloacetate (Walker and Edwards, 1991).

Kinetics studies for PPCs have been completed; however, most of the reported kinetic data concern a poorly defined protein since no consensus procedures for purification and assay were followed. The apparent Km values for PEP and HCO_3^- are of the same order of magnitude (Lepiniec et al., 1994). The Vmax was 93.7 μmol per min per μg *E. coli* enzyme (Smith, 1971). The Vmax of *E. coli* PPC in another report was in 0.7 nmoles ml^{-1} min^{-1} in the absence of

activators; while Vmax varied between 1.05 nmoles ml^{-1} min^{-1} with 0.006 mM acetyl-CoA and 50.0 nmoles ml^{-1} min^{-1} with 10 mM fructose bisphosphate and 0.3 mM acetyl-CoA (Silverstein and Willis, 1973).

Pyruvate carboxylase

Pyruvate carboxylase (PC; EC 6.4.1.1), a biotin-dependent enzyme has been found widely among eukaryotic tissues and in many prokaryotic species. It is a ligase that catalyzes the irreversible carboxylation of pyruvate to oxaloacetate, providing oxaloacetate for gluconeogenesis and replenishing tricarboxylic acid cycle intermediates. The overall reaction catalyzed by PC involves two partial reactions that occur at spatially separate subsites within the active site, with the covalently bound biotin acting as a mobile carboxyl group carrier. In the first partial reaction, biotin is carboxylated using ATP and HCO_3^- as substrates whilst in the second partial reaction, the carboxyl group from carboxybiotin is transferred to pyruvate. In some organisms this enzyme may require acetyl-CoA as a cofactor. For example, in absence of acetyl-CoA the maximal rate of oxaloacetate synthesis by *Bacillus* sp. is 20% of that obtained in presence of saturating concentrations of acetyl-CoA (Libor et al., 1978); however in yeast this enzyme is quite active in the absence of acetyl-CoA (Attwood, 1995). In *Pseudomonas citronellolis* and *Pseudomonas fluorescens* no obvious effect was caused by acetyl-CoA (Milrad de Forchetti and Cazzulo, 1976). No acetyl-CoA is required for the PC of *Methanobacterium thermoautotrophicum* (Mukhopadhyay et al., 1998). There are variety of activators and inhibitors for PC from different sources. K^+, NH_4^+, Mg^{2+}, and Mn^{2+} are usually metal activators in

microorganisms while ADP (competition by ATP) and 2-oxoglutarate are inhibitors (Milrad de Forchetti and Cazzulo, 1976; Gurr and Jones, 1977; Charles and Willer, 1984; Modak and Kelly, 1995).

The Km for ATP varies remarkably in different sources: from 0.04 mM in *Thiobacillus novellas* (Charles and Willer, 1984) to 0.38 mM in *Bacillus stearothermophilus* (Cazzulo et al., 1970). The Km value for HCO_3^- , $MgATP^{2-}$, and pyruvate are about 0.3 mM, 0.08 mM and 0.25 mM, respectively (Milrad de Forchetti and Cazzulo, 1976; Gurr and Jones, 1977; Charles and Willer, 1984; Modak and Kelly, 1995).

PEP carboxykinase

Phosphoenolpyruvate (PEP) carboxykinase (PCK; GTP/ATP: oxaloacetate carboxylyase, EC 4.1.1.32/49) catalyzes the nucleoside triphosphate-dependent reversible carboxylation of OAA from PEP, CO_2, and the corresponding nucleoside diphosphate. The conversion from PEP to OAA is the first committed step of gluconeogenesis in *E. coli* and is part of the gluconeogenic pathway in virtually all organisms. PCK is also an important enzyme in the glycolytic pathways of some organisms, such as *Ascaris suum* and *Trypanosoma cruzi*, where it forms OAA from PEP, which in turn enters the TCA cycle. In humans and other mammals, PCK is a central enzyme in carbohydrate metabolism, helping to regulate the blood glucose level (Matte et al., 1997; Jabalquinto et al., 2004).

A. succiniciproducens PCK structure has been reported (Cotelesage et al., 2005). The sequence of the *A. succiniciproducens* PCK was similar to those of all known ATP/ADP-

dependent PCK. In particular, the *A. succiniciproducens* enzyme was 67.3% identical and 79.2% similar to the *E. coli* enzyme (Laivenieks et al., 1997; Delbaere et al., 2004).

A. succiniciproducens PCK is oxygen stable, has a pH optimum of 6.7-7.1, and is stable from pH 5.0 to 9.0. The Km values for the substrate PEP, the cosubstrates bicarbonate, and ADP are 0.54 mM, 17 mM and 0.42 mM, respectively. The enzyme required Mn^{2+} or Co^{2+} in addition to Mg^{2+} to exhibit maximum activity (Podkovyrov et al., 1993). At physiological concentrations of Mn^{2+} and Mg^{2+}, the affinity for CO_2 increases as the ATP/ADP ratio is increased in the assay medium, which indicated that a high ATP/ADP ratio favors CO_2 fixation by the PEP carboxykinase from *A. succiniciproducens* (Bazaes et al., 2007). Similiarly, *E.coli* PCK is activated by Mg^{2+} and Mn^{2+}, and the Km values for PEP, bicarbonate, and ADP are 0.07 mM, 13 mM, and 0.05 mM, respectively (Krebs and Bridger, 1980).

Glyoxylate shunt

The glyoxylate cycle or glyoxylate shunt bypasses the two decarboxylation of the TCA cycle that converts isocitrate to succinyl-CoA (decarboxylation of isocitrate to α-ketoglutarate and decarboxylation of α-ketoglutarate to succinyl-CoA), and instead routes isocitrate through the isocitrate lyase and malate synthase reactions (Fig.4). Isocitrate lyase (ICL, isocitrate glyoxylate lyase, EC 4.1.3.1), the first enzyme unique to the glyoxylate shunt functions at a branch point of carbon metabolism and diverts isocitrate to glyoxylate and succinate. The glyoxylate produced by ICL reacts with a second molecule of acetyl-CoA through malate synthase (EC 2.3.3.9) to form malate. Glyoxylate cycle thus enables the synthesis of a mole of

succinate from two moles of acetate (as acetyl-CoA), being the overall net reaction:

2 acetyl-CoA + 2 H_2O + NAD^+ = succinate + 2 CoA + NADH + H^+

In this way, the glyoxylate shunt replenishes intermediates of the TCA cycle and conserves carbon that would otherwise be oxidized and lost to biosynthetic pathways. This replenishing function has been termed anaplerotic (Kornberg, 1966).

In *E. coli* ICL has no detectable activity in absence of exogenous cation (Hoyt et al., 1988). Of the cations needed for ICL activity, Mg^{2+} was the most effective cation tested, while Mn^{2+}, Co^{2+}, Ni^{2+} and Sr^{2+} were 54%, 17%, 7% and 3%, respectively, as effective as Mg^{2+}. Several chemicals inhibit ICL (Hoyt et al., 1988). Oxalate and malonate, analogs of glyoxylate, are linear competitive inhibitors of ICL with respect to isocitrate and their Ki values are 5.1 μM and 580 μM, respectively. Succinate, a reaction product, is a linear noncompetitive inhibitor of ICL with respect to isocitrate (Ki value of 290 μM). Itaconate, a succinate analog, is an uncompetitive inhibitor of ICL with respect to isocitrate with a Ki value of 5.3 μM. Chloride, phosphate and sulphate ions are competitive inhibitors with respect to isocitrate, and PEP inhibits non-competitively (MacKintosh and Nimmo, 1988). The optimal pH of ICL is 7.3 in *E. coli* and the Km for isocitrate is 63 μM at physiological pH and in the absence of competing anions.

Malate synthase, in the second step of the glyoxylate shunt, catalyzes the condensation of glyoxylate and acetyl-CoA and hydrolysis of the intermediate to yield malate and CoA. Almost all malate synthases from different sources require Mg^{2+} though the optimal concentration varies, from 0.005 mM in *Streptomyces coelicolor* A3 (Loke et al., 2002) to 40 mM in *Corynebacterium*

glutamicum (Reinscheid et al., 1994). Other metal ions support significant malate synthase activity: Co^{2+} replaces about 30% of the activity obtained with Mg^{2+} (Reinscheid et al., 1994; Watanabe et al., 2001); Mn^{2+} yields 15-40% of the activation of Mg^{2+} (Dixon et al., 1960; Reinscheid et al., 1994; Watanabe et al., 2001; Smith et al., 2003). The Km of malate synthase for acetyl-CoA is between about 0.01 mM and 0.08 mM whilst the Km for glyoxylate varies between around 0.0006 and 0.1 mM (Dixon et al., 1960; Woodcock and Merrett, 1978; Sundaram et al., 1980; Durchschlag et al., 1981; Bruinenberg et al., 1989; Reinscheid et al., 1994; Beeckmans et al., 1994; Watanabe et al., 2001; Loke et al., 2002; Smith et al., 2003).

Other enzymes

There are several other enzymes involved in succinate production. Malic enzymes have been found in most living organisms. These enzymes catalyze the reversible oxidative decarboxylation of malate to pyruvate and CO_2, with the concomitant reduction of the cofactor $NAD(P)^+$ to NAD(P)H. A divalent cation is required for this catalysis. The Km values determined in the presence of saturating cofactor and Mn^{2+} were 0.26 mM for malate (physiological direction) and 16 mM for pyruvate (reverse direction) (Stols and Donnelly, 1997).

Fumarate reductase (EC 1.3.1.6) catalyzes the reduction of fumarate to succinate with NADH as a cofactor. There are few reports on the Km value for fumarate and NADH. Wetzstein and Gottschalk (1985) found that the Km values for fumarate and NADH in *Bacteroides amylophilus* were 0.025 mM and 0.004 mM, respectively. The optimal pH is close to the neutral pH: 7.4, 6.5, and 6.0 in *Streptococcus faecalis*, *Streptococcus lactis*, and *Fibrobacter*

succinogenes, respectively. Succinate has about 15% inhibition on fumarate reductase, whilst Zn^{2+} has strong inhibition (Aue and Deiel, 1967; Wetzstein and Gottschalk, 1985; Chen et al., 2001).

Pathways that lead to by-products

E. coli and other facultative organisms respire oxygen or alternative electron acceptors (e.g. nitrate) but can also grow in the absence of external electron acceptors by coupling reduction of metabolic intermediates to NADH oxidation, a process known as fermentation. In mixed acid fermenters, pyruvate is reduced to a mixture of fermentation products including lactate, succinate, acetate, and ethanol. The formation of ethanol from pyruvate consumes 2 moles of NADH and generates CO_2. Ethanol formation therefore represents an undesirable use of NADH in competition with its requirement in succinate production. Moreover, ethanol production is counterproductive toward an underlying goal of CO_2 sequestration. The generation of lactate similarly consumes one mole of NADH, and therefore in addition to representing a diversion of carbon lactate formation wastes redox potential.

Pyruvate formate-lyase

Pyruvate formate-lyase (PFL, EC 2.3.1.54) is central enzyme in bacterial anaerobic metabolism catalysing the reversible reaction of pyruvate and coenzyme A into acetyl-CoA and formate (Garrett and Grisham, 1999). PFL is thus the anaerobic counterpart of pyruvate

dehydrogenase. This pathway competes with PYC for pyruvate and therefore reduces succinate synthesis directly.

Alcohol dehydrogenase

Alcohol dehydrogenase (ADH, E.C.1.1.1.1) converts acetyl-CoA to acetaldehyde and then to ethanol in a two-step reduction that is coupled to oxidation of two moles of NADH. Actually there are two enzymes involved in this process, the fermentative acetaldehyde dehydrogenase (ACDH) and alcohol dehydrogenase (ADH), which are expressed anaerobically and are repressed both by air and by nitrate in wild-type *E. coli* (Clark and Cronan, 1980). These two activities are dual functions of a single protein encoded by the *adhE* gene at 27.5 min on the *E. coli* chromosome. The transcription of *adhE* is induced only under anaerobic conditions in response to elevated levels of NADH (Leonardo et al., 1993; Leonardo et al., 1996). Interestingly, *adhE* mutants of *E. coli* cannot grow under fermentative conditions even when the cells have alternative means to oxidize NADH (e.g. via lactate dehydrogenase) (Clark, 1989).

Lactate dehydrogenase

There are three lactate dehydrogenases (LDH) in *E. coli* which catalyze the interconversion of pyruvate and lactate. Two LDHs are membrane-bound flavoproteins, which couple to the respiratory chain and are better described as lactate oxidases. These enzymes, one specific for the D-isomer and the other specific for the L-isomer, are required for aerobic growth on lactate (Kline and Mahler, 1965). The single fermentative lactate dehydrogenase (LDH, E.C.1.1.1.28) is a soluble NAD-linked enzyme that converts pyruvate to D-lactate (Tarmy and

Kaplan, 1968a; Tarmy and Kaplan, 1968b). This reaction consumes one NADH per pyruvate, recycling the NADH generated during glycolysis. The fermentative LDH is induced approximately tenfold in anaerobically grown cultures at acidic pH (Clark and Cronan, 1980; Mat-Jan et al., 1989, Jiang et al., 2001). Pyruvate caused a two to four fold increase in expression of *ldhA* gene encoding LDH (Jiang et al., 2001). LDH has several inhibitors: 0.3 mM oxamate inhibits *E. coli* LDH; 1.0 mM ATP has 50% inhibition on *Leuconostoc lactis* LDH (Garvie, 1980); and ADP and pyruvate also show inhibition in several organisms (Garland and Kaplan, 1967; Erwin and Gotschlich, 1993). Depending on the organism, the Km value is 0.07-1.5 mM for pyruvate and 0.00021-0.16 mM for NADH (Garvie, 1980; Le Bras and Garel, 1991; Kochhar et al., 1992; Dartois et al., 1995).

Recombinant approach to form succinate

To increase succinate production there are two goals: 1) prevent the accumulation of undesired products; and 2) enhance the succinate formation. Several gene knockouts and pathway modification have been studied to improve the production of succinate. This section focuses on *E. coli* recombinants.

Decreasing by-product formation

This section will focus on gene knockouts to reduce by-product formation during succinate production. The strategy of combining knockout with gene transformation will be

described in the next section. Deletions in lactate and formate-forming steps can improve succinate accumulation in *E. coli*. A mutation in lactate dehydrogenase (*ldh*) had little effect on anaerobic growth (Mat-Jan et al., 1989). However, *E. coli* NZN111 lacking both pyruvate-formate lyase (*pfl*) and *ldh* genes exhibited marginal anaerobic growth on glucose (Bunch et al., 1997). When NZN111 was aerobically cultured on acetate, it regained the ability to ferment glucose with succinic acid as the major product in subsequent anaerobic culture. In dual-phase culture (comprise an initial aerobic growth phase followed by an anaerobic production phase) carried out in flasks, succinic acid was produced at a level of 11.26 g/L from 13.4 g/L of glucose with a succinic acid yield of 1.28 mol/mol glucose and a productivity of 1.13 g/l·h in the anaerobic phase (Wu et al., 2007). *E. coli* AFP111, a spontaneous mutant of NZN111, had restored ability to grow anaerobically (Donnelly et al., 1998). The causative mutation in AFP111 was eventually mapped to the *ptsG* gene, which encodes glucose-specific permease of the PTS. The loss of function of this gene product greatly increased the PEP pool and favored the formation of succinate (Chatterjee et al., 2001). AFP111 grown anaerobically under 5% H_2–95% CO_2 resulted in a succinate yield of 0.70 g/g and a molar succinate-to-acetate ratio of 1.97 (Donnelly et al., 1998). Moreover, under dual-phase fermentation this strain resulted in a succinate productivity and yield as high as 0.87 g/l·h and 0.99 g/g, respectively (Nghiem et al., 1999).

Jantama et al. (2008a) combined gene deletions with metabolic evolution to develop a *Escherichia coli* C that produce succinate and malate. After deletion of the central anaerobic

fermentation genes (*ldhA*, *adhE*, *ackA*), the pathway for malate and succinate production remained as the primary route for the regeneration of NAD^+. Additional deletions (*focA*, *pflB*, *poxB*, *mgsA*) were introduced as further improvements. The best succinate biocatalysts, strains KJ060 (*ldhA*, *adhE*, *ackA*, *focA*, *pflB*) and KJ073 (*ldhA*, *adhE*, *ackA*, *focA*, *pflB*, *mgsA*, *poxB*), produce 622-733 mM of succinate with molar yields of 1.2-1.6 per mole of metabolized glucose. Strain KJ073 was further engineered for improvements in succinate production (Jantama et al., 2008b). Deletion of the threonine decarboxylase (*tdcD*; acetate kinase homologue) and 2-ketobutyrate formate-lyase (*tdcE*; pyruvate formate-lyase homologue) reduced the acetate level by 50% and increased succinate yield (1.3 mol/mol glucose) by almost 10% compared to KJ073. Deletion of two genes involved in oxaloacetate metabolism, aspartate aminotransferase (*aspC*) and the NAD^+-linked malic enzyme (*sfcA*) (Strain KJ122) significantly increased succinate yield (1.5 mol/mol glucose), succinate titer (700 mM), and average volumetric productivity (0.9 g/l·h). Residual pyruvate and acetate were substantially reduced by further deletion of *pta* encoding phosphotransacetylase to obtain a strain KJ134 (*ΔldhA*, *ΔadhE*, *ΔfocA-pflB*, *ΔmgsA*, *ΔpoxB*, *ΔtdcDE*, *ΔcitF*, *ΔaspC*, *ΔsfcA*, *Δpta-ackA*). Strains KJ122 and KJ134 produced near theoretical yields of succinate during simple, anaerobic, batch fermentations using mineral salts medium.

Cultures of *E. coli* GNB10578 (*ndh*) wtih inactivated NADH dehydrogenase produced more fermentation metabolites than wide type *E. coli* MG1655; the additional inactivation of *pta-ackA* reduced the level of acetate and ethanol, and the yield of succinate and lactate was

increased 5.6- and 14.8-fold compared with those values of the culture of the wild type strain, however, D-lactate was the primary product (Yun et al., 2005).

Knocking out isocitrate lyase regulator encoded by *iclR* reduced the by-products, acetate and ethanol (Wang et al., 2006). Recently, a system was reported that allows *E. coli* to produce and accumulate succinate under aerobic conditions (Lin et al., 2005c). In this system, *sdhAB* gene encoding succinate dehydrogenase and *icd* gene encoding isocitrate dehydrogenase were inactivated to redirect the fluxes toward succinate. The glyoxylate operon aceBAK repressor (*iclR*) was inactivated to activate the glyoxylate bypass as a detour for succinate production. Moreover, pyruvate oxidase (*poxB*) and acetate kinase-phosphotransacetylase (*ackA-pta*) were knocked out to reduce the accumulation of acetate from pyruvate and acetyl-CoA. Experiments in shake flasks showed that 1.7 g/L succinate could be produced aerobically in 24 h with a yield of 0.17 g/g. In a batch reactor, the succinate production rate was faster, reaching 0.33 g/g in 24 h with a concentration of 2.6 g/L; further cultivation (83 h) showed that succinate production reached 5.1 g/L with a yield of 0.46 g/g. However, substantial pyruvate and the TCA cycle intermediates citrate and isocitrate also accumulated.

Enhancing succinate formation

In addition to using gene knockouts to reduce by-product formation, additional carbon can be directed to succinate through gene transformation. One approach is to increase the flux at the PEP node by overexpressing PCK and PPC. In wild type *E. coli* K-12, *A. succinogenes* PCK

overexpression had no effect on succinate fermentation (Kim et al., 2004). In contrast, in a PPC mutant, PCK overexpression increased succinate production from 0.36 g/L to 2.38 g/L and reduced lactate from 4.39 g/L to 3.15 g/L. Overexpression of native PCK had no effect on succinate formation despite a 66-fold higher activity of PCK (Millard et al., 1996).

PPC overexpression increased succinate from 3.27 g/L in host strain JCL1208 (lacks the *lac* operon but contains a chromosomally inserted $lacI^q$ gene) to 4.44 g/L (Millard et al., 1996). Because the fermentations required 18 h to complete and lowered the pH of the medium, the ampicillin initially present may have been destroyed and selective pressure for maintaining the plasmid lost. Therefore, the researchers subsequently used the antibiotic carbenicillin, more stable at lower pH, and periodically added carbenicillin to the medium. With 37 g/L glucose and 50 g/L $MgCO_3$, PPC increased the production of succinate from 3.0 g/L in the control culture to 10.7 g/L in PPC-overexpressing strain, making succinate the major fermentation product by weight.

Inactivation of *ptsG* with overexpression of a *Sorghum ppc* on succinate production has been studied (Lin et al., 2005d). One mutant named PTSG1 contained five knockedouts (*ΔsdhAB, Δicd, ΔiclR, ΔpoxB*, *Δ*(*ackA-pta*)); while another named PTSG2 contained four knockouts (*ΔsdhAB*, *ΔiclR, ΔpoxB*, *Δ*(*ackA-pta*)). PTSG1 has glyoxylate pathway for aerobic succinate production, while PTSG2 has two possible routes: the glyoxylate cycle and the oxidative branch of the TCA cycle. The additional inactivation of *ptsG* did improve succinate production. At approximately 48 h, PTSG1 *ptsG* produced 5.2 g/L succinate while PTSG1 produced 3.7 g/L

succinate. Similarly PTSG2 *ptsG* produced 5.8 g/L succinate compared to PTSG2, which produced 4.7 g/L. In both cases, the yield and the volumetric producitivity was higher when *ptsG* was inactivated. The overexpression of *Sorghum* PPC (on plasmid pKK313) was next examined on these strains. Overexpression of *Sorghum* PPC was effective in increasing succinate production. For example, the succinate production using PTSG1 *ptsG* (pKK313) was 8.0 g/L, 116% higher than using PTSG1. The specific succinate productivity was also higher in all the strains overexpressing PPC. However PPC overexpression only slightly increased succinate yield, particularly for PTSG2. Finally PTSG2 *ptsG* (pKK313) was selected as the best strain for high succinate production, and a fed-batch fermentation was performed under aerobic conditions (Lin et al., 2005a). The process produced 58.3 g/L succinate in 59 h. During the fermentation the succinate yield was 0.62 ± 0.05 g/g glucose, the productivity was 1.00 ± 0.06 g/l·h, and the specific productivity was 89.77 ± 3.40 mg/g·h.

Another approach to direct pyruvate to succinate is by using PYC. PYC expression caused increased carbon flow towards OAA in wild-type *E. coli* cells without affecting the glucose uptake rate or the growth rate (Gokarn et al., 1998). A wild-type *E. coli* transformed with plasmid pTrc99A-*pyc* expressing *Rhizobium etli* PYC resulted in a succinate yield of 0.17 g/g and a productivity of 0.17 g/l·h (Gokarn et al., 1998). *R. etli* PYC was able to restore the growth of an *E. coli ppc* null mutant in minimal glucose medium (Gokarn et al., 2001). During aerobic glucose metabolism, expression of PYC resulted in a 56% increase in biomass yield and a 43% decrease in acetate yield. During anaerobic glucose metabolism, expression of PYC caused a 2.7-

fold increase from 1.57 g/L to 4.36 g/L, thereby making succinate the major product by mass. The increase in succinate came mainly at the expense of lactate formation. Fermentation patterns of *E. coli* with and without PPC and PYC were compared under anaerobic conditions with glucose as a carbon source. Compared with the strain having a single chromosomal copy of *ppc* (VJS676), the fermentation with the *ppc* null mutant (JCL1242) resulted in a significant decrease in the final succinate concentration (1.0 g/L versus 0.2 g/L). Succinate formation was restored by the expression of either PPC or PYC in *ppc* mutant. For example, the fermentations using JCL1242/pPC201 (*ppc*+) surpassed by 44% the succinate yield obtained using VJS676. The *pyc* gene from *R. etli* similarly compensated for the lack of PPC activity. Fermentations using JCL1242/pTrc99A-pyc resulted in a 66% increase in succinate yield compared to that using VJS676. The strain JCL1242 with the *pyc* gene showed a succinate yield 19% greater than that of the same strain with multiple copies of the *ppc* gene.

Dual-phase fermentations of *E. coli* AFP111 transformed with *pyc* gene (AFP111/pTrc99A-*pyc*) also demonstrated that PYC overexpression enhanced succinate accumulation (Vemuri et al., 2002). A final succinate concentration of 99.2 g/L with an overall yield of 110% and a productivity of 1.3 g/l·h was reported. Growth, substrate consumption, product formation, and activities of key enzymes from glucose fermentation were compared for NZN111, NZN111/pTrc99A-*pyc*, AFP111, and AFP111/pTrc99A-*pyc* under both exclusively anaerobic and dual-phase conditions (Vemuri et al., 2002).

In a *ptsG* mutant with galactose permease overexpressed, succinate yield was not significantly different (0.76 mol/mol) though the cell growth rate and glucose consumption rate was increased. Coexpression of PYC and galactose permease in the *ptsG* mutant showed higher succinate yield (1.2 mol/mol glucose) (Wang et al., 2006a). The *ptsG* and *iclR* double knockout strain plus *Bacillus subtilis pyc* expression can produce higher amount of succinate (1.29 mol/mol glucose) than a strain having *ptsG* single knockout plus *pyc* overexpression. The acetate and ethanol yields dropped below 0.2 mol/mol at the same time (Wang et al., 2006b).

Overexpression of another PYC (*L. lactis*) has been studied for succinate production in *E. coli* (Lin et al., 2005e). Plasmid pHL413 bearing *L. lactis pyc* was transformed into the parental wild-type strain, GJT001. Results showed that PYC from *L. lactis* was effective in increasing succinate production and yield by 95%. Then the researchers studied the coexpression of *Sorghum vulgare* PPC and *L. lactis* PYC. By overexpressing the *S. vulgare* PPC only, succinate production increased from 0.5 g/L to 1.7 g/L, and the yield increased from 2.6% to 9.2%. Coexpression of PYC and PPC resulted in a final succinate concentration of 2.0 g/L with a yield of 11.1%. The effect of PYC from *L. lactis* on an *adhE*, *ldhA* double mutant *E. coli* strain, SBS110MG has been investigated (Sanchez et al., 2005). Compared to the control strain succinate concentration in transformant increased 25-fold from 0.6 g/L to 15.6 g/L using an initial glucose of 18.7 g/L. The succinate yield increased from 0.13 to 0.83 g/g, while the acetate yield dropped from 72 mg/L to 48 mg/L. Additionally, *ptsG* inactivation in this transformant increased the succinate yield to 0.92 g/g with a reduction in glucose consumption rate of 33%.

A strategy to increase acetyl-CoA to increase succinate production in *E. coli* was described (Lin et al., 2004). This approach relies on the increased intracellular acetyl-CoA and CoA levels by overexpressing *E. coli* pantothenate kinase (PANK). The results showed that coexpression of PANK and *Sorghum* PPC resulted in the highest succinate final concentration of 2.8 g/L in shake flasks for 24 h at 37°C at 250 rpm. This strain had an intracellular acetyl-CoA concentration of 0.48 mg/L at 24 h compared to the control (having PPC but no PANK) with only 0.014 mg/L. Lactate production decreased significantly from 4.5 g/L to 1.8 g/L when both PANK and PPC were overexpressed. The results of *L. lactis* PYC and PANK coexpression showed similar trends as coexpression of PPC and PANK for succinate, lactate, acetyl-CoA, and CoA, and the succinate concentration increased from 1.18 g/L to 2.36 g/L. These similar trends are expected since acetyl-CoA is a potential allosteric activator for both PPC and PYC. Enhanced PPC or PYC activity would presumably increase conversion of PEP or pyruvate to OAA and subsequently succinate.

Overexpressing the *E. coli* malic enzyme was also studied for succinate synthesis. A *pfl ldhA* double mutant NZN111 was used to produce succinate. NZN111 harboring pTrcML that encodes *E. coli* malic enzyme produced 6 and 8 g/L of succinate from 20 g/L of glucose in flask culture at 37°C and 30°C, respectively. When NZN111 (pTrcML) was cultured at 30°C with intermittent glucose feeding the final succinate concentration was 9.5 g/L and the ratio of succinate to acetate was 13:1 (Hong and Lee, 2001). In another similar study (Stols and

Donnelly, 1997), the induction of malic enzyme in NZN111 increased succinate production from 2 g/L to 10 g/L after over 40 h fermentation with the initial glucose concentration of 20 g/L.

Succinate could also be produced efficiently from fumarate by a recombinant *E. coli* strain DH5α/pGC1002 containing the multicopy plasmid with fumarate reductase gene (*frd*) (Wang et al., 1998). Succinate reached over 60 g/L in 48.5 h starting with 64 g/L fumarate. Significant substrate inhibition occurred when the initial fumarate concentration was 90 g/L, and L-malate became the major fermentation product (Wang et al., 1998). The conversion of fumarate to succinate and malate by *E. coli* strains initially at low cell density ($<6.25 \times 10^7$ cells/mL) has also been studied (Goldberg et al., 1983). The rate of fumarate utilization was higher in transformant JRG1346 which overexpressed *frd* gene than in the wild type. The yield of succinate was increased from 0.26 g/g to 0.60 g/g after one day of incubation, and the rate of succinate production about four times greater than that obtained with the wild type. At high cell densities (7.5×10^8-1.9×10^9 cells/mL), after 4 days of incubation the yield of succinate by the wild type and JRS1346 was 0.12 g/g and 0.82 g/g, respectively. The rate of succinate production by strains JRG1346 was 10 times higher than that obtained with the wild type. Malate was formed by the wild type, but not detected in cultures of JRG1346.

Environmental impact on succinate production

The remarkable metabolic flexibility in microorganisms is tightly regulated in response to factors such as pH, redox potential, carbon source, and the availability of oxygen or other

electron acceptors (Clark and Cronan, 1980; Unden and Bongaerts, 1997). In this section the effects of some environmental factors on the succinate formation are reviewed.

Carbon dioxide and pH

Succinate production requires CO_2 by PPC, PYC, or PCK to form the key intermediate OAA. High level of CO_2 benefits succinate formation. In whey fermentation for succinate-rich product by *A. succiniciproducens*, the CO_2 was supplied as $MgCO_3$, which dissolved during the course of the fermentation. At the highest level of CO_2 (corresponding to 35 g/L $MgCO_3$), more than 90% of the lactose was consumed and the succinate yield was 84%. At the lowest level of CO_2 48% of the lactose was consumed, and the succinate yield was less than 4% (Samuelov et al., 1999).

External CO_2 supply had a negative effect on *A. succiniciproducens* growth at both pH 6.2 and pH 6.5, while it had a somewhat positive effect on succinate production. The effect of CO_2 on succinate yield was higher at pH 6.5 than at pH 6.2 (Lee et al., 1999a). Without the supply of CO_2, the maximum cell densities reached were OD_{660} of 5.3 and 5.6 at the culture pH of 6.2 and 6.5, respectively. Cell growth was severely suppressed by the external CO_2 supply at both pH (OD_{660} of < 3.6). Succinate yields were similar at 82-83% in the absence or presence of CO_2 supply at the culture pH of 6.2. At pH 6.5, supplying CO_2 increased succinate yield slightly, from 84% without CO_2 to 88% with CO_2. Acetate production was similar at the culture pH of 6.2 and 6.5 with or without CO_2 supply.

High ratio of CO_2-HCO_3^- to glucose favors succinate formation. In *A. succiniciproducens* fermentation, when the molar ratio of CO_2-HCO_3^- to glucose was between 1.0 and 0.5, about 15% of the available carbon (from glucose plus CO_2-HCO_3^-) was incorporated into cell mass, 90% of the glucose was consumed and 65% of the carbon was converted to succinate. At low ratio of CO_2-HCO_3^- to glucose (0.065), 45-60% of the glucose was consumed and only 8% of the carbon was converted into cell mass. Approximately 50% of the carbon was fermented into lactate, only 30% was converted into succinate (Samuelov et al., 1991). pH also impact on the levels of fermentative enzyme activities responsible for end product formation. At low pH (6.2) and high CO_2-HCO_3^- concentrations, the major product was succinate, whereas at pH 7.2 and low CO_2-HCO_3^- concentrations, the highest levels of lactate, ethanol, and acetate were observed. In cells grown at pH 6.2 and high CO_2 concentrations, PCK activity was increased from 10 U/mg to 356U/mg, 35-fold higher than the activity at pH 7.2 and low CO_2-HCO_3^- concentrations. LDH and ADH were not detected under high CO_2-HCO_3^- concentration, while under low CO_2-HCO_3^- concentration the activities of those were 27 U/mg and 44 U/mg, respectively. Low levels of pyruvate kinase and high levels of malate dehydrogenase, fumarate reductase, and acetate kinase were found in cell extracts under either condition.

In glucose fermentation by *Actinobacillus* sp. 130Z, higher CO_2 resulted in more succinate production (Van der Werf et al., 1997). Four levels of CO_2 (10, 25, 50, 100 mol of CO_2 per 100 mol of glucose) were investigated. With the CO_2 level increased the succinate yield was increased from 0.12 g/g to 0.45 g/g glucose, while ethanol reduced from 0.18 g/g to 0.05 g/g

glucose. The acetate and formate production did not significantly change. The ^{13}C-labelling experiments (McKinlay et al., 2008) showed that high $NaHCO_3$ concentrations decreased the amount of flux shunted by OAA decarboxylation in *Actinobacillus succinogenes*. In addition, pyruvate carboxylating flux increased in response to high $NaHCO_3$ concentrations.

The metabolic responses of *Mannheimia succiniciproducens* to the different dissolved CO_2 has been investigated (Song et al., 2007). Cell growth was severely suppressed when the dissolved CO_2 concentration was below 8.74 mM. On the other hand, cell growth and succinic acid production increased proportionally as the dissolved CO_2 concentration increased from 8.74 to 141 mM. The yields of biomass and succinic acid on glucose obtained at the dissolved CO_2 concentration of 141 mM were 1.49 and 1.52 times higher, respectively, than those obtained at the dissolved CO_2 concentration of 8.74 mM. Other CO_2 sources such as $NaHCO_3$, $MgCO_3$, or $CaCO_3$ had positive effects on cell growth and succinic acid production. However, growth inhibition was observed when excessive bicarbonate salts were added.

Redox potential/Hydrogen

Redox potential is an important fermentation parameter, and the theory is described in detail (Srinivas et al., 1988). The extracellular oxidoreduction potential (ORP) can affect the forms of NAD^+, the intermediary metabolites, the enzyme activities and the end product distribution. For example, at moderate reducing conditions (-100 mV), the production of formate, acetate, ethanol, and lactate by *E. coli* was in molar proportions of approximately 2.5:1:1:0.3,

while at highly reducing conditions (-320 mV), this ratio was 2:0.6:1:2 (Riondet et al., 2000). In *C. thermosuccinogenes* different controlled culture redox potentials (-225, -250, -275, and -310 mV) similarly affected the metabolic carbon flux at a constant pH of 7.25. Lower values of culture redox potential were correlated with increased succinate, acetate, and formate fluxes and decreased ethanol and hydrogen fluxes (Sridhar and Eiteman, 2001).

The intracellular potential in bacteria is assumed to be a function of either the NADH concentration or the NADH/NAD ratio. Succinate is a highly reduced fermentation product consuming reducing equavalents NADH (Clark, 1989). Therefore, the change of redox potential may alter the succinate formation. One strategy to change the culture redox potential is the addition of an extra electron donor (e.g., hydrogen).

External supply of H_2 enhanced *A. succiniciproducens* cell growth rate and succinate yield (Lee et al., 1999a). The optimum ratio of H_2/CO_2 was 5:95 (v/v) when 20 g/L glucose was used. Under this condition, the maximum specific growth rate was 0.61 h^{-1}; and succinic acid yield and productivity were 0.91 g/g and 1.8 g/l·h, respectively. If pure CO_2 was used, the maximum specific growth rate was 0.55 h^{-1}; and the succinate yield and productivity decreased to 0.86 g/g and 1.0 g/l·h, respectively.

Gas compositions of 100% N_2, 5% H_2 with 95% N_2, and 100% H_2 were compared to investigate the effect of hydrogen on the succinate production by *Actinobacillus* sp. 130Z (Van der Werf et al., 1997). The addition of hydrogen to the gas phase resulted in a significant increase in the succinate production. Under the three conditions, the succinate yields based on glucose

were 0.52, 0.64, and 0.73 g/g, respectively. In agreement with these observations, growing *A. succinogenes* with H_2 increased the flux to succinate, but more so at high $NaHCO_3$ concentrations than at low ones (McKinlay et al., 2008). The presence of H_2 also significantly decreased fumarate formation rates at both $NaHCO_3$ concentrations suggesting that fumarate reductase flux is limited by reductant availability in the absence of additional electron sources. NADH-consuming flux to ethanol increased in the presence of H_2. NADH-producing flux through formate dehydrogenase and/or pyruvate dehydrogenase decreased in the presence of H_2 at both high and low $NaHCO_3$ concentrations.

Hydrogen also affects succinate fermentation by an *E. coli* mutant which overexpressed the NAD^+-dependent malic enzyme (Stols and Donnelly, 1997). Using air-CO_2-H_2 (1:1:2) the apparent yield of succinate during the anaerobic portion of the fermentation was 1.2 g/g glucose, compared to 0.65 g/g in the absence of hydrogen.

Not all studies showed that H_2 enhances succinate formation. In the fermentation by *E. coli* NZN111 (pTrcML) which overexpresses malic enzyme a CO_2:H_2 (1:1) gas mixture resulted in a lower succinate concentration than when pure CO_2 was used (Hong and Lee, 2002). A possible reason was that the reduction in CO_2 limited the carboxylation by amplified malic enzyme.

Potentially, formate can act as a source of both electrons and carbon dioxide. However, the addition of formate to the growth medium did not affect the fermentation balance, nor was it shown to be consumed by glucose-fermenting cultures (Van der Werf et al., 1997).

The NADH/NAD ratio can also be changed by using different carbon substrates (Zeikus, 1980). For example, more-reduced carbohydrates such as sorbitol and mannitol lead to the production of more reduced end products in *Actinobacillus* sp. 130Z (Van der Werf et al., 1997). Using recombinant *E. coli* sorbitol led to higher succinate and ethanol productivity, while lactate and acetate production decreased substantially (Lin et al., 2005b).

Metabolic flux analysis

Methods for quantifying intracellular fluxes are important for understanding the interactions of the pathways in metabolic networks, and such methods are also essential for the metabolic engineering to redirecting the metabolism towards production of desired metabolites. In recent years, metabolic flux analysis has become one of the major tools in metabolic engineering (Nielsen, 2003). The metabolic flux is defined as the rate at which the input metabolites are utilized to form output metabolites. The aim of metabolic flux analysis is to quantify all metabolic fluxes in the central metabolism of a microorganism. The result is a flux map that shows the distribution of anabolic and catabolic fluxes over the metabolic network. Based on such a flux map or a comparison of different flux maps, possible targets for genetic modifications might be identified, the result of an already performed genetic manipulation can be judged or conclusions about the cellular energy metabolism can be drawn (Stephanopoulos et al., 1998).

Metabolic flux analysis has been used to estimate flux distributions to achieve the maximum yield of succinate in *E. coli* (Lee et al., 2002). The actual maximum yield of succinate obtained was only 83% of its theoretical one due to the insufficient reducing power. By supplying more reducing power the yield could be increased to its theoretical yield. Furthermore, the optimal metabolic pathways for the production of succinate could be proposed based on the results of metabolic flux analysis. The pyruvate carboxylation pathway should be used rather than PEP carboxylation pathway for optimal succinate production in *E. coli*. Similarly, Wang et al. (2006b) found three potential improvement target sites by metabolic flux analysis, the glucose phosphotransferase transport system, the pyruvate carboxylase, and the glyoxylate shunt. All the three genetic modified strains showed increased succinate yield. The final strain TUQ19/pQZ6 had a high yield of 1.29 mol succinate/mol glucose and high productivity.

The metabolic flux analysis could determine the flux split fractions to the glyoxylate pathway and to the reductive arm of the TCA cycle at the oxaloacetate (OAA) node. The most favorable split ratio to obtain the highest succinate yield was the fractional partition of OAA to glyoxylate of 0.32 and 0.68 to the reductive arm of the TCA cycle in reconstructed *E. coli*. The succinate yield achieved was 1.7 mol/mol (Sanchez et al., 2006).

The metabolism of a transformed *E. coli* NZN111 which overexpressed malic enzyme was investigate using metabolic flux analysis (Hong and Lee, 2000). The result showed that the anaerobic cultivation could be divided into two phases. During the first phase, most of the malate produced was excreted to the medium, while only a small amount converted to succinate. In the

second phase, the glucose consumption rate decreased significantly, and so did almost all of the intracellular metabolic fluxes. However, this system is not accurate since some pyruvate and succinate accumulated intracellularly. Later, the authors proposed a modified flux analysis method by introducing intracellular pyruvate and succinate pools (Hong and Lee, 2001). By this new method the concentrations of intracellular metabolites were successfully predicted and the differences between the measured and calculated reaction rates could be considerably reduced.

A metabolic flux analysis was performed under continuous fermentations (Oh et al., 2008). The glucose uptake rate, glycolysis flux, and pentose phosphate pathway flux all increased when increasing the dilution rate, although the fraction of glucose to the glycolysis and to the pentose phosphate pathway remained constant at about 30% and 70%, respectively. The biosynthesis fluxes in the glycolysis and in the pentose phosphate pathway increased when the dilution rate was increased, plus the ratio of biosynthesis flux to glucose flux increased. Moreover, the metabolite secretion fluxes increased at higher dilution rate. However, the ratio of byproduct secretion fluxes to glucose flux decreased, indicating that more carbon sources were consumed for biosynthesis rather than metabolite secretion when increasing the dilution rate.

Flux estimation is often combined with labeling experiments. A specifically ^{13}C labeled substrate like [1-^{13}C] glucose is fed to the biological system, and then the labeled carbon atoms are distributed through the metabolic network until finally the isotopic enrichment in the intracellular metabolite pools can be measured by nuclear magnetic resonance (NMR) or mass

spectroscopy (MS). The resulting data provide a large amount of information to measure the intracellular fluxes.

The pathways for succinate formation in *Saccharomyces cerevisiae* during anaerobic fermentation have been studied by the introduction of ^{13}C aspartate into the metabolic network (Camarasa et al., 2003). The assimilation of [3-^{13}C] aspartate led to the formation of [2,3-^{13}C] malate and [2,3-^{13}C] succinate. This non-random distribution of ^{13}C labeling in malate and succinate demonstrates that the TCA pathway operates during yeast fermentation as both an oxidative and a reductive branch. Further experiments indicated that the reductive branch of the TCA cycle was the main pathway for succinate production if aspartate was used as the nitrogen source.

The ^{13}C-labelled experiments have been used to understand carbon flux distribution to succinate and alternative products in *Actinobacillus succinogenes* (McKinlay et al., 2007b). [1-^{13}C] glucose was fed to the cells and the resulting isotopomers of excreted organic acids, proteinaceous amino acids, and glycogen monomers were analyzed by GC-MS and NMR. The isotopomer data, together with the glucose consumption and product formation rates and the *A. succinogenes* biomass composition, were applied to a metabolic flux model. The optimal flux distribution of *Actinobacillus succinogenes* was obtained by minimizing the error between the measured data and the model predicted data. The same method has been applied to determine the metabolism at different $NaHCO_3$ and H_2 concentrations using a substrate mixture of [1-^{13}C] glucose, [U-^{13}C] glucose, and unlabeled $NaHCO_3$ (McKinlay et al., 2008). Pyruvate

carboxylating flux increased in response to high $NaHCO_3$ concentrations. Formate-, acetate-, and ethanol-producing pathway fluxes increased or decreased appropriately in response to the different redox demands imposed by the different $NaHCO_3$ and H_2 concentrations.

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Table II-1. Activators and inhibitors of *E. coli* PPC

Effectors	**Mechanism**	**Reference**
Positive effectors		
Acetyl-CoA	Co-precursor switching activation	(Izui et al., 1970a)
Fructose 1,6-bisphosphate	Precursor activation	(Izui et al., 1970a)
Fatty acids and their CoA derivatives	End-product switching activation	(Izui et al., 1970b)
Guanosine 3', 5' - diphosphate (ppGpp)	Stringent control	(Taguchi et al., 1977)
GTP	Compensatory feedback activation	(Taguchi et al., 1977)
Negative effectors		
L-aspartate	End-product feedback inhibition	(Nishikido et al., 1965; Nishikido et al., 1968; Izui et al., 1981)
L-malate	End-product feedback inhibition	(Nishikido et al., 1965; Nishikido et al., 1968; Izui et al., 1981)
Citrate	End-product feedback inhibition	(Gold and Smith, 1974)
Succinate	End-product feedback inhibition	(Corwin and Fanning, 1968)
Fumarate	End-product feedback inhibition	(Gold and Smith, 1974)
Cys	--	(Gold and Smith, 1974)
NEM	--	(Wohl and Markus, 1972)

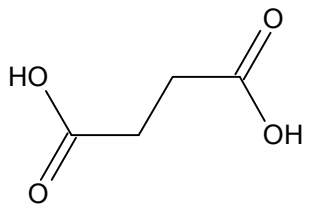

Figure II-1. Structure of succinic acid

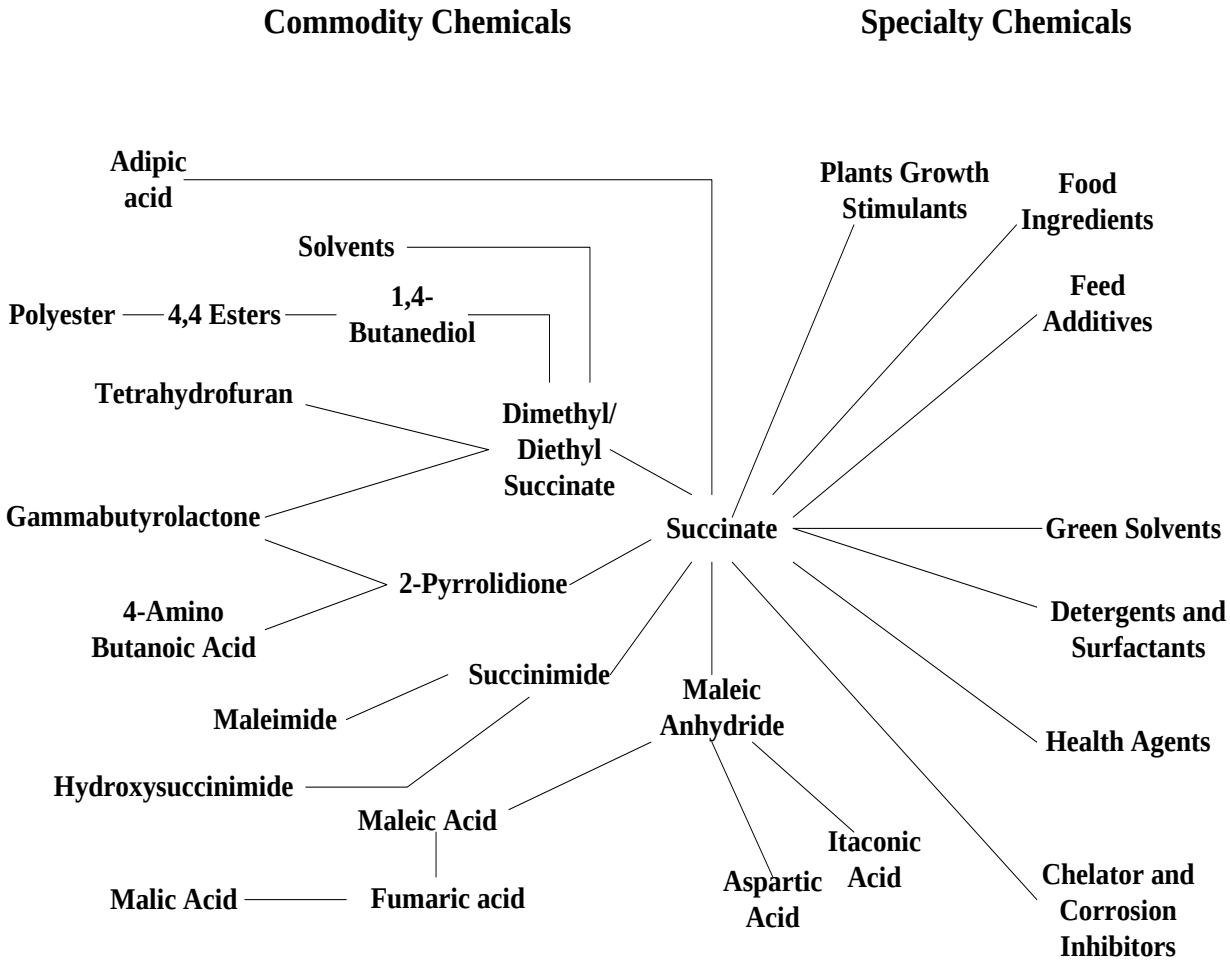

Figure II-2. Succinate-based products (adapted from Zeikus et al., 1999)

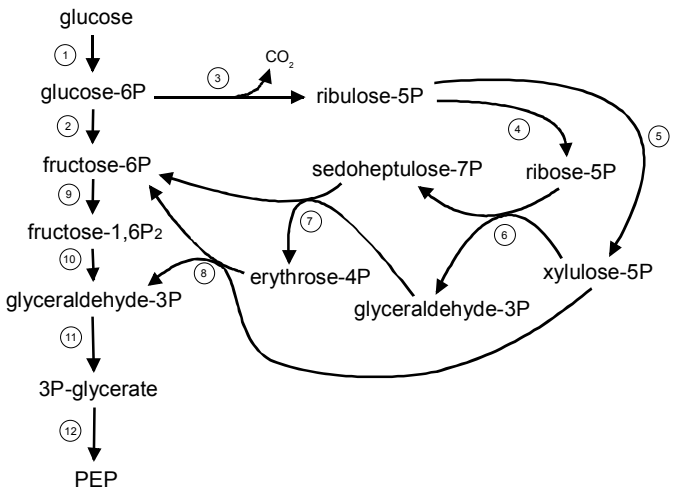

Figure II-3. Glycolytic pathway and pentose phosphate pathway. Not all enzymatic steps or intermediates are shown. Key enzymes in the pathways are as follows: 1, glucokinase/phosphotransferase system (PTS); 2, phosphoglucoisomerase; 3, 6-phosphogluconate dehydrogenase; 4, phosphopentose epimerase; 5, phosphopentose epimerase; 6, transketolase; 7, transaldolase; 8, transketolase; 9, phosphofructokinase; 10, fructose biphosphate aldolase; 11, glyceraldehyde 3-phosphate dehydrogenase and hosphoglycerate kinase; and 12, phosphoglycerate mutase.

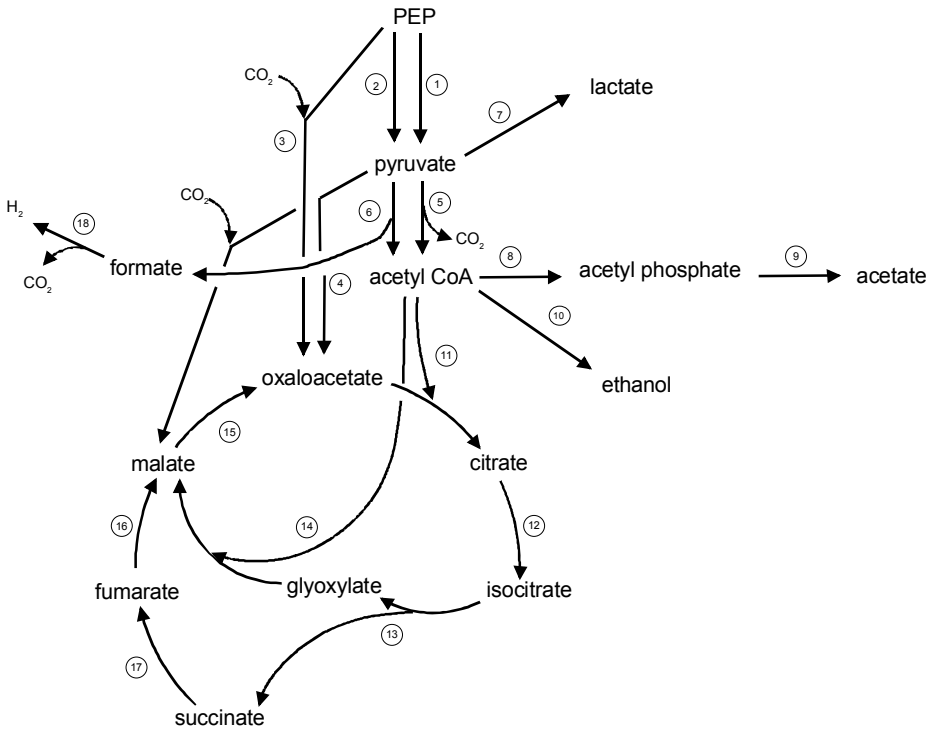

Figure II-4. The metabolic pathway to form succinate from PEP and/or pyruvate. Not all enzymatic steps or intermediates are shown. Key enzymes in the pathways are as follows: 1, PTS; 2, pyruvate kinase; 3, PEP carboxylase; 4, pyruvate carboxylase; 5, pyruvate dehydrogenase complex; 6, pyruvate formate lyase; 7, lactate dehydrogenase; 8, phosphoacetyltransferase; 9, acetate kinase; 10, acetaldehyde dehydrogenase and alcohol dehydrogenase; 11, citrate synthase; 12, aconitase; 13, isocitrate lyase; 14, malate synthase; 15, malate dehydrogenase; 16, fumarase; 17, fumarate reductase; and 18, formate dehydrogenase.

CHAPTER III

PH AND BASE COUNTERION AFFECT SUCCINATE PRODUCTION IN DUAL-PHASE *ESCHERICHIA COLI* FERMENTATIONS[1]

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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 anaerobic succinate production phase using 100% (v/v) CO_2. A pH of 6.4 yielded the highest specific succinate productivity. The metabolic flux at a pH of 6.4 was determined by metabolic flux analysis with ^{13}C-labeled glucose. 61% of the PEP partitioned to oxaloacetate and 39% partitioned to pyruvate, and 93% of the succinate was formed via the reductive arm of the TCA cycle. The flux distribution at a pH of 6.8 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.

Introduction

Succinic acid (succinate) and its derivatives are widely used as specialty chemicals for application in foods, pharmaceuticals, and cosmetics (Guettler et al. 1998), and it can serve as a starting material for many commercially important products (Zeikus et al. 1999). Some anaerobic bacteria such as *Anaerobiospirillum succiniciproducens* (Lee et al. 2000; 2001), *Actinobacillus succinogenes* (Guettler et al. 1999; Urbance et al. 2004) and *Mannheimia succiniciproducens* (Lee et al. 2006), 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 (Sanchez et al. 2005). 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 (Lin et al. 2005a, b). 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 (Vemuri et al. 2002a).

A few studies have investigated the effect of pH on succinate production. For example, Van der Werf et al. (1997) found that succinate was produced in the pH range of 6.0 to 7.4 by *Actinobacillus* sp., but pH was not controlled. Samuelov et al. (1991) 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 (Agarwal et al. 2006), 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* (Isar et al. 2006). 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.

The highest reported succinate concentration has been generated by *E. coli* AFP111 and its derivatives using a dual-phase process (Vemuri et al. 2002a). We selected this strain to study the influence of pH as well as the base counterion on the anaerobic formation of succinate in a defined medium. We also compared the metabolism of this strain at two pH level with metabolic flux analysis using ^{13}C-labeled glucose.

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 (Donnelly et al. 1998; Chatterjee et al. 2001).

Media and fermentation conditions

All fermentations used a defined medium containing (per L): 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 $CoCl_2 \cdot 6H_2O$, 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 with an initial volume of 1.2 L in 2.5-L Bioflow III fermenters (New Brunswick Scientific, Edison, NJ, U.S.A.) were inoculated from 50 ml grown for 10-12 h 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 culture optical density (OD) reached about 20, the aerobic growth phase was terminated by switching the inlet gas composition to 100% (v/v) CO_2. Simultaneously, the total flowrate 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.

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 Bioflow III fermenters (New Brunswick Scientific, Edison, NJ, USA), the inoculum volume was reduced to 25 mL and the initial glucose concentration was 30 g/L. The glucose concentration was monitored using a YSI 2700 SELECT™ glucose analyzer (Yellow Springs Instrument, Inc, Yellow Springs, OH, USA). 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 250 g/L [1-^{13}C] glucose (99%, Cambridge Isotope Laboratories, Andover, MA, USA) was added into the fermenter, 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 minutes, 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 0.2 μM syringe filter (Cole-Parmer Instrument Co., Vernon Hills, IL, USA) prior to LC-MS analysis. The liquid chromatography was performed at 25°C using Applied Biosystems 140B Solvent Delivery System (Applied Biosystems, Foster City, CA, USA) and a Kromasil C18 column (250 mm × 1 mm, 5 μm particles, 100Å pore space, Keystone Scientific, Inc., Bellefonte, PA, USA). 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 PE Sciex API 1 Plus single quadrupole instrument with electrospray ion source (PE Sciex, 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, interface temperature 50°C. Data were acquired in negative ion mode with a capillary voltage of 3500 V. Mass peak heights were determined using the BioTool Box Version B software package (Applied Biosystems/PE Sciex, Foster City, CA, USA). 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%) (Rosman and Taylor 1998).

Metabolic modeling and flux analysis

An overall flux balance was developed using stoichiometric analysis and the pseudo-steady-state condition for intracellular metabolites (Savinell and Palsson 1992). The balance equations for *E. coli* AFP111 metabolism have been developed previously (Vemuri et al. 2002a), and this model was further extended by including the pentose phosphate pathway (Figure III-1). Flux partitions (φ) were defined as fractional fluxes (ν) into one of two key branches. The fraction of flux into the pentose phosphate pathway (φ_{PPP}) is defined as

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

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

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

The model contained 27 fluxes and 21 metabolites (Figure III-1 and Table III-1). Since insignificant growth occurs during the process phase of interest, glucose consumption and product formation rates were essentially constant; 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. Figure III-2 shows the complete labeling patterns of succinate obtained when 1-^{13}C glucose is the tracer substrate. The fraction of each succinate isotopomer is

very sensitive to a set of fluxes. Specifically, we wrote analogous balance equations for isotopomers of each metabolite (Schmidt et al. 1997). 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 (Van Dien et al. 2003):

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

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 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 (Varian Unity Inova 500; Varian Inc., Palo Alto, CA, USA) at 125.7 MHz were obtained with the following spectral parameters: 45° pulses, 31.4-kHz spectral width, and 45 second 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 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 peak area of methylene group to 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.

HPLC 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 (Eiteman and Chastain 1997).

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 central metabolism through the action of the

enzyme PEP carboxylase (PPC) (Gokarn et al. 2000). 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 III-3 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 14 h of the anaerobic phase, and the values reported are the means of 2-3 experiments (Table III-2). In the pH range of 5.8 to 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 III-2). 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 (Figure III-4). Indeed, an unacceptably large quantity of base was needed above a pH of about 6.6. 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 of PPC; and the glyoxylate shunt (Vemuri et al. 2002b). To 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. 1-^{13}C glucose was used as tracer substrate to determine the metabolic fluxes, and Figure III-5 shows an example anaerobic phase process with 1-^{13}C-glucose tracing. The glucose consumption and succinate formation rates were constant (Figure III-5); thus the process exhibited a metabolic pseudo-steady state. We first determined the flux distribution at a pH of 6.4. Table III-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 III-3). In addition, we calculated the redox balance based on the metabolic model, and the value was very close to 1 (Table III-3). 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 (Figure III-6A). 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 (Figure III-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 (Figure III-6A).

We similarly determined the metabolic fluxes at a pH of 6.8 (Table III-3). Although the higher pH significantly reduced the glucose consumption rate and succinate production rate (Figure III-6B), 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 III-3). Also, 38% of the intermediate PEP formed pyruvate, and about 10% of the pyruvate became succinate via the glyoxylate shunt; and the CO_2 stiochiometric coefficient was 0.59 (Figure III-6B).

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% $Ca(OH)_2$. When $Ca(OH)_2$ was used for pH control, 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 succinate productivity and yield (Table III-4). For both KOH and NaOH the succinate productivity declined during the anaerobic phase (from

about 1.7 to 0.3 g/L·h). 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). 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 III-4).

Discussion

In the pH range of 5.8–6.8, the specific succinate formation and glucose consumption rates achieved maxima, although the fermentation product yields were not affected by the external pH (Table III-2). 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}) (Booth 1985). Olsen et al. (2001) 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 decrease with increasing pH_{ex} ultimately to reach 0 at a pH_{ex} of 8.0 (Olsen et al. 2001). A large ΔpH is associated with more active transport of H^+ and other ions across the membrane to maintain pH homeostasis (Booth 1985). One might anticipate that the increased maintenance requirement resulting from reduced pH might reduce the product yield. Although not 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, but it decreased significantly above a pH of 6.6. The quantity of buffer required to maintain the pH increased with increasing pH (Figure III-4). Thus, when the pH was above 6.4, the ionic strength

increased more quickly during the succinate formation phase, an effect which may have been detrimental to the rate of succinate formation. So, two mechanisms may exist which result in the observed optimum pH: a ΔpH effect which reduces succinate formation at low pH, and an ionic strength effect which 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 (Samuelov et al. 1991) and *Bacteroides fragilis* at a pH of 6.5 (Isar et al. 2006).

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 (Wang et al. 2006) and other *E. coli* strains that overexpress *Lactococcus lactis* pyruvate carboxylase (Sanchez et al. 2006). 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 (Stumm and Morgan 1996; Frahm et al. 2002), did not affect the flux partition at PEP node (Figure III-6). Like our results with *E. coli*, the distribution of fermentation products by *Actinobacillus* sp. 130z (Van der Werf et al. 1997) did not differ in the pH range of 6.0–7.4. The key enzyme PPC uses bicarbonate as the form of

CO_2 (O'Leary 1982), 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 (Lu et al. in preparation). 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 biocarbonate 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 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 (Miczynski 1886) 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 removal of succinate by the precipitation of calcium succinate could relieve the inhibition of PPC (Corwin and Fanning 1968) and isocitrate lyase (MacKintosh and Nimmo 1988), two key enzymes in succinate formation (Vemuri et al. 2002b). 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 concentration of dissociated Ca^{2+} would have been only about 0.18 M. Such high

ion strength leads to osmotic stress, and under aerobic conditions *E. coli* exports some Na^+ as a response (Sakuma et al. 1998). However, under anaerobic conditions, Na^+ extrusion activity may decrease (Trchounian and Kobayashi 1999). 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+} (Zhu et al. 2007). Gouesbet et al. (1993) 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, pH within the range of 5.8 – 6.8 does not affect yield nor 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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Table III-1. Metabolic reactions for succinate production.

	Reaction
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)

Table III-2. 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_P (g/g)	Y_A (g/g)	Y_E (g/g)
5.8	108.7^{a}	0.67^{a}	76.5^{a}	0.71^{a}	0.24^{a}	0.01^{a}	0.00^{a}
6.0	189.3^{b}	1.02^{b}	126.0^{b}	0.67^{a}	0.21^{a}	0.01^{a}	0.02^{a}
6.2	192.6^{b}	1.18^{bc}	125.7^{b}	0.65^{a}	0.24^{a}	0.02^{a}	0.02^{a}
6.4	284.6^{c}	1.42^{c}	174.3^{c}	0.61^{a}	0.24^{a}	-0.01^{a}	0.03^{a}
6.6	257.4^{bc}	1.49^{c}	169.3^{c}	0.66^{a}	0.16^{a}	0.00^{a}	0.03^{a}
6.8	166.3^{b}	0.99^{b}	119.4^{ab}	0.72^{a}	0.19^{a}	0.01^{a}	0.03^{a}
7.0	85.7^{d}	0.36^{d}	47.4^{d}	0.55^{b}	0.05^{b}	0.06^{b}	0.02^{a}

Subscripts: G: glucose, S: succinate, P: pyruvate, A: acetate, E: ethanol

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

Table III-3. 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. The enrichment ratio (S2/S1) was observed from NMR results. The redox ratio R/O was calculated from 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

Observed values are shown as mean ± standard deviation from 3 analyses

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

Table III-4. 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/gh)	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)
$Ca(OH)_2$	248.6[a]	1.42[a]	174.3[a]	0.61[a]	0.24[a]	-0.01[a]	0.03[a]
KOH	177.9[b]	0.88[b]	95.3[b]	0.54[a]	0.37[b]	-0.01[a]	0.01[a]
NaOH	176.2[b]	0.99[b]	101.6[b]	0.57[a]	0.21[a]	0.02[b]	0.00[a]

Subscripts: G: glucose, S: succinate, P: pyruvate, A: acetate, E: ethanol

Different letters were statistically significant difference at the 90% confidence level

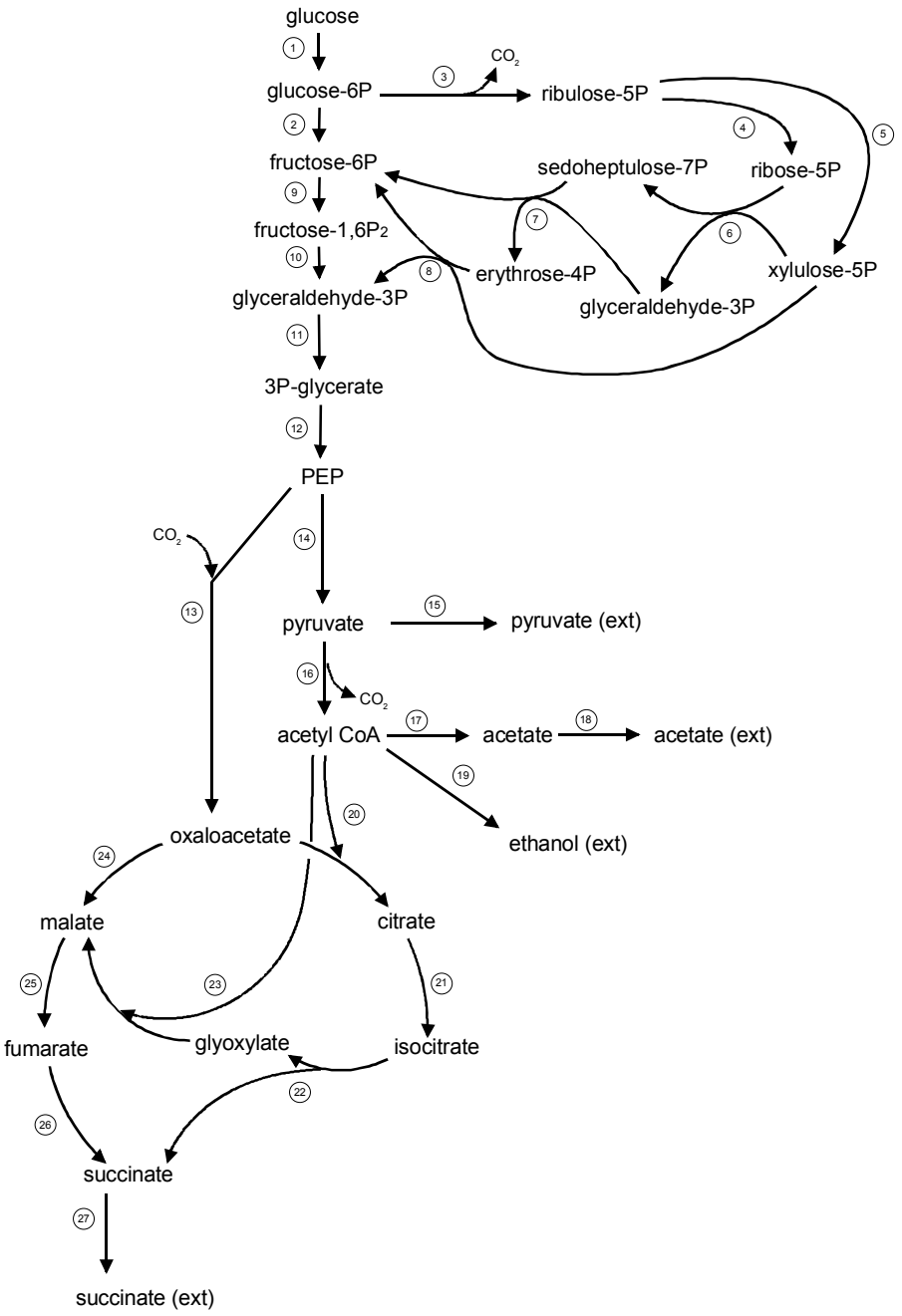


Figure III-1. Biochemical pathways for the synthesis of succisnate from glucose in *E. coli*. Not all enzymatic steps or intermediates are shown. Key enzymes in the pathways are as follows: 1) glucokinase; 2) phosphoglucoisomerase; 3) 6-phosphogluconate dehydrogenase; 4) phosphopentose epimerase; 5) 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; and 26) fumarate reductase.

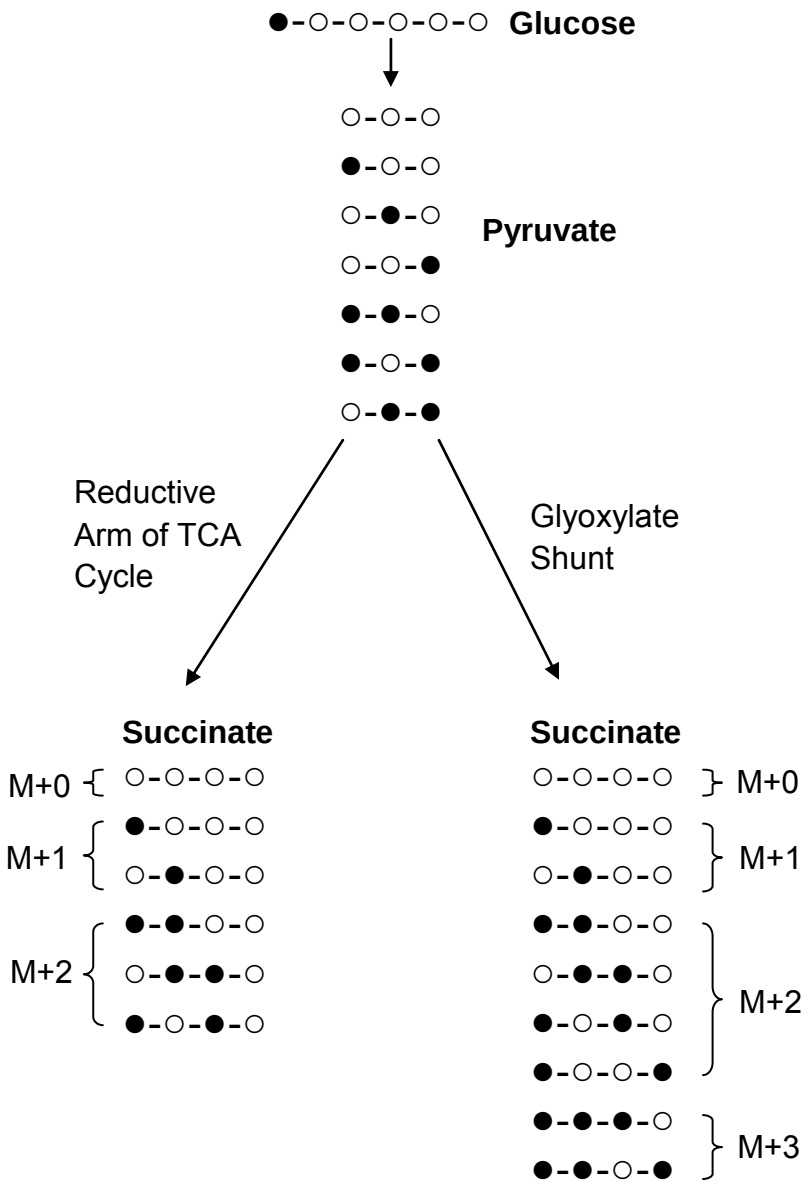


Figure III-2. Labeling patterns of succinate with 1-^{13}C glucose as tracer substrate with ^{13}C-atoms displayed solid and ^{12}C-atoms displayed hollow. Note that succinate is a symmetric molecule.

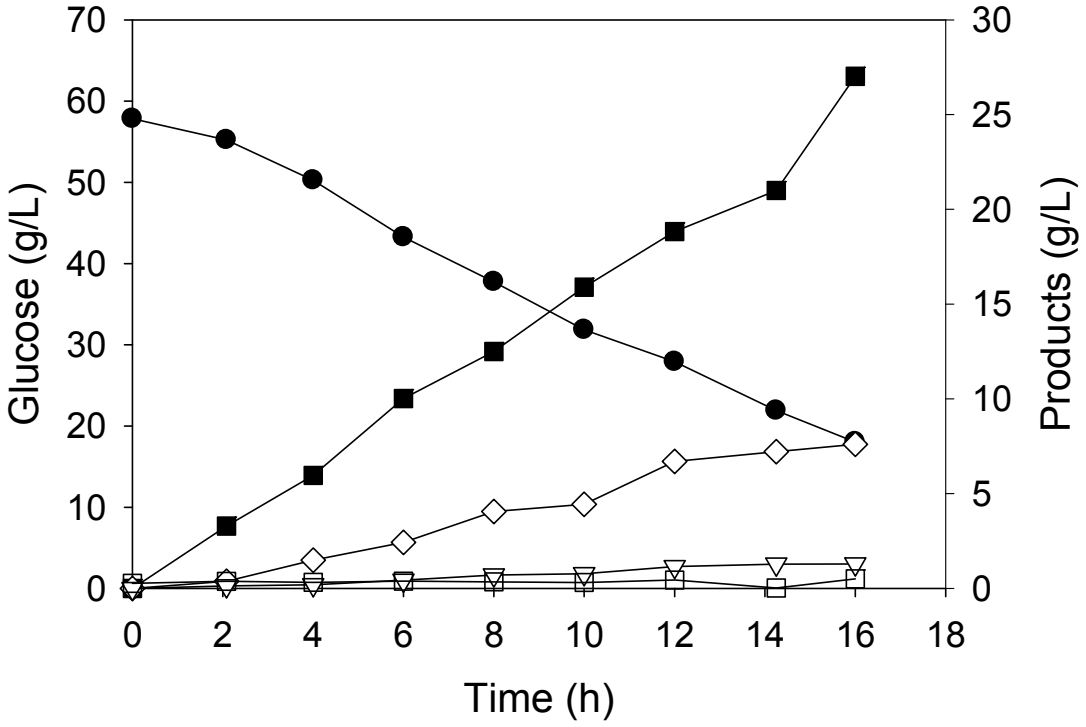


Figure III-3. Production of succinate (■), pyruvate (◇), ethanol (▽) and acetate (□) from glucose (●) during the anaerobic non-growth production phase for *E. coli* AFP111 using 25% $Ca(OH)_2$ to control pH at 6.4.

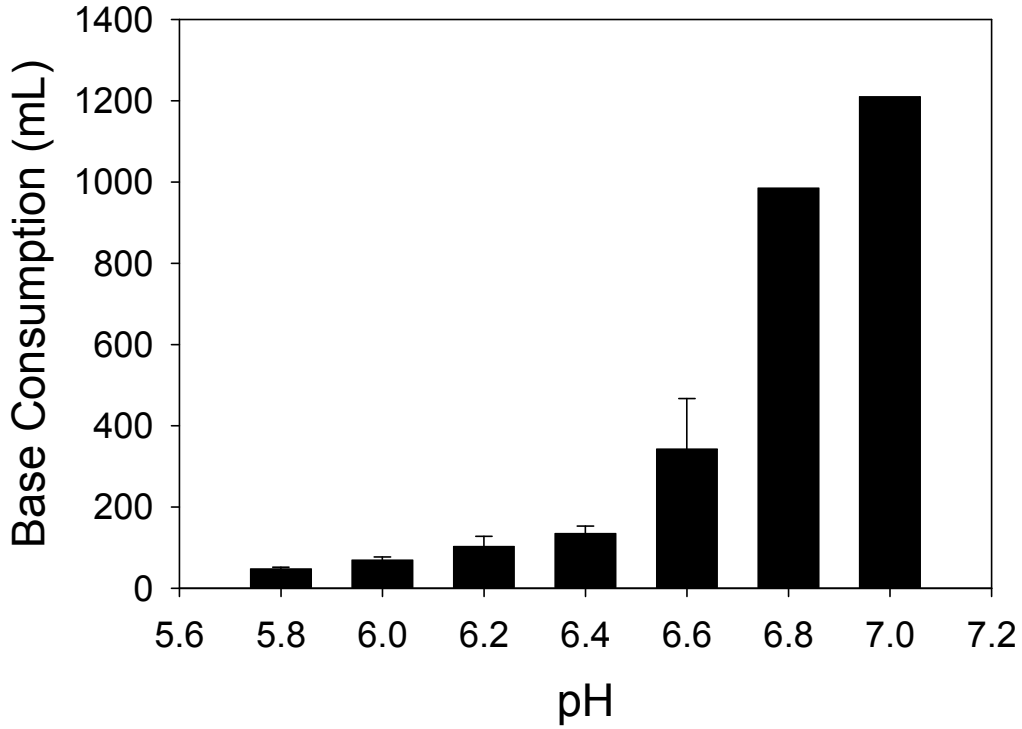


Figure III-4. Volume of 25% $Ca(OH)_2$ consumption during 14 h of an anaerobic non-growth production phase for *E. coli* AFP111 under different levels of controlled pH.

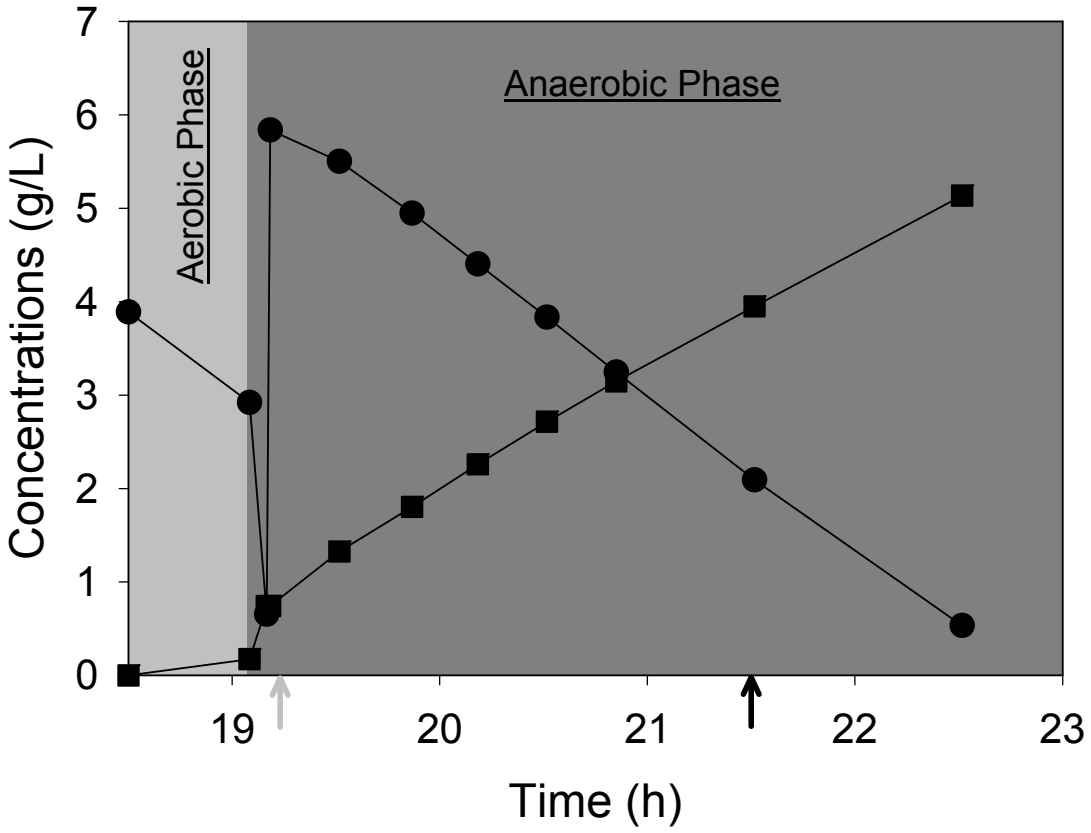
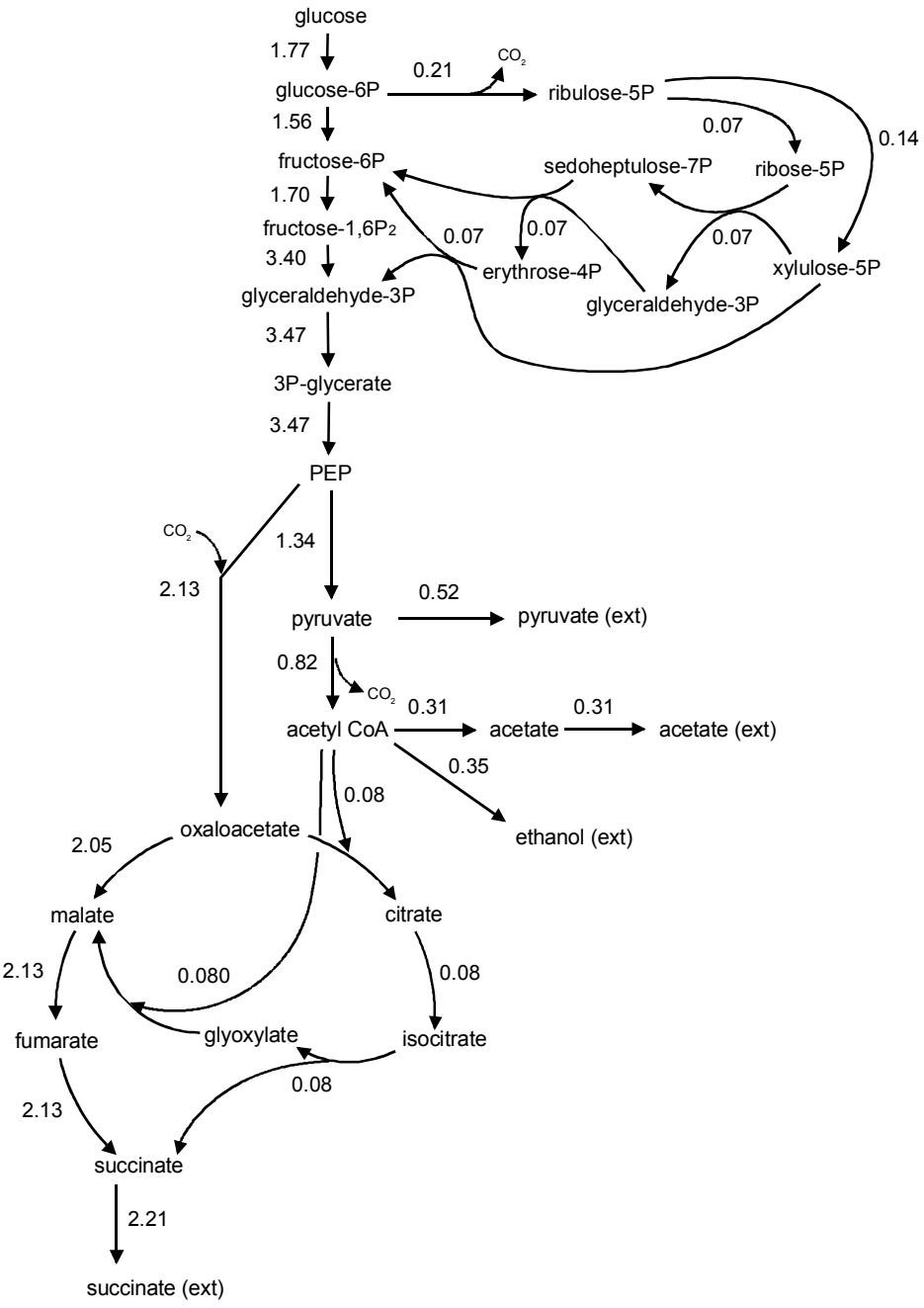


Figure III-5. Example of succinate (■) and glucose (●) during a 1-^{13}C-glucose tracing experiment. The initiation of the anaerobic phase occurred at 19.17 h as represented by the change in grayscale. A gray arrow indicates the time of 1-^{13}C glucose addition. Gray and black arrows indicate two samples used with LC-MS and NMR to calculate flux distribution.

A


glucose
1.77
glucose-6P
0.21
CO2
ribulose-5P
1.56
0.07
0.14
fructose-6P
sedoheptulose-7P
ribose-5P
1.70
fructose-1,6P2
0.07
0.07
0.07
3.40
erythrose-4P
xylulose-5P
glyceraldehyde-3P
glyceraldehyde-3P
3.47
3P-glycerate
3.47
PEP
CO2
1.34
2.13
pyruvate
0.52
pyruvate (ext)
0.82
CO2
acetyl CoA
0.31
acetate
0.31
acetate (ext)
0.35
0.08
ethanol (ext)
oxaloacetate
2.05
malate
citrate
2.13
0.080
0.08
fumarate
glyoxylate
isocitrate
0.08
2.13
succinate
2.21
succinate (ext)

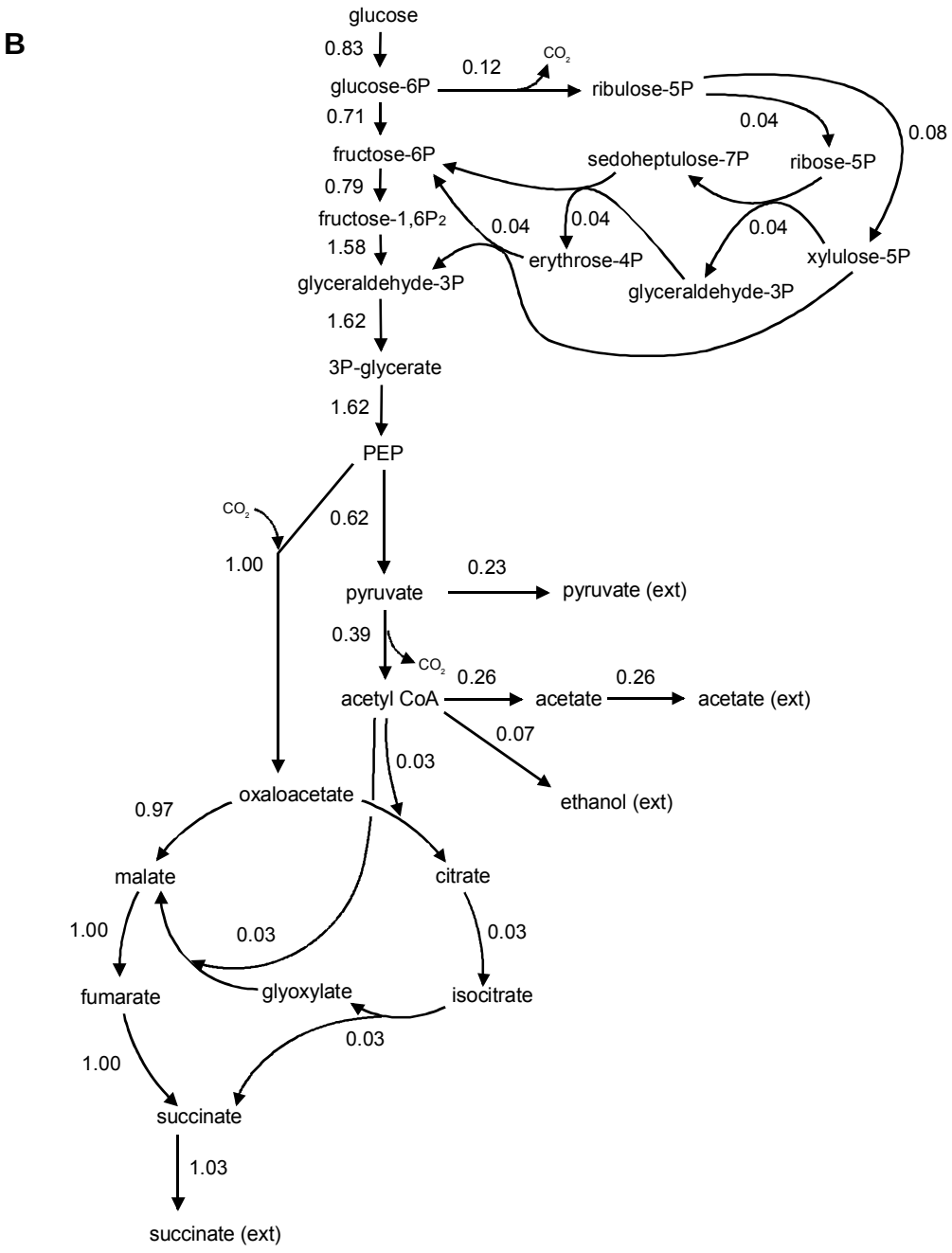


Figure III-6. Metabolic fluxes (mmol/g·h) during an anaerobic non-growth production phase of *E. coli* AFP111 at a pH of 6.4 (A) and a pH of 6.8 (B).

CHAPTER IV

EFFECT OF CARBON DIOXIDE ON SUCCINATE PRODUCTION IN DUAL-PHASE *ESCHERICHIA COLI* FERMENTATIONS[1]

[1]Shiying Lu, Mark A. Eiteman, Elliot Altman. To be submitted to J Microbiol Biotechnol

Abstract

Succinate production under different concentrations of carbon dioxide was studied in *Escherichia coli* AFP111, which contains mutations in pyruvate formate lyase (*pfl*), lactate dehydrogenase (*ldhA*) and the phosphotransferase system (*ptsG*). A series of two-phase fermentations were conducted in which an aerobic cell growth phase was followed by anaerobic succinate production phase having several constant concentrations of CO_2. As the concentration of CO_2 in the gas phase increased from 0% to 50%, the succinate specific productivity increased from 1.89 mg/g·h to 225 mg/g·h, and the succinate yield increased from 0.04 g/g to 0.75 g/g. Above 50% CO_2, succinate production did not further increase. Intracellular fluxes were determined at three CO_2 concentrations (3%, 10%, and 50%) using ^{13}C-label tracing coupled with LC-MS analysis. The fraction of carbon flux into the pentose phosphate pathway increased from 0.04 at 3% CO_2 to 0.17 at 50% CO_2. Also, the fractional flux through anaplerotic carboxylation at the PEP node increased slightly from 0.53 at 3% CO_2 to 0.63 at 50% CO_2. The increased flux into the pentose phosphate pathway is attributed to an increased demand for reduced cofactors with elevated CO_2. A four-process explicit model to describe the CO_2 transfer and utilization was proposed. The model predicted that at CO_2 concentrations below about 40% the system becomes limited by gas phase CO_2, while at higher CO_2 concentrations the system is limited by PPC enzyme kinetics.

Introduction

Microbial sequestration of carbon dioxide (CO_2) has centered on *bacteria* and *archaea* (Shively et al., 1998; Atom, 2002) in liquid suspensions or microalgal systems (Brown, 1996; Watanabe and Hall, 1996). Photosynthesis is often required for CO_2 fixation, which limits the application of biological CO_2 sequestration due to scale-up problems (Zhang et al., 2002). In addition, many microorganisms considered for CO_2 fixation have fastidious growth requirements, and have unacceptably low product yields and formation rates (Shively et al., 1998; Atom, 2002). For example, Otsuki (2001) reported a CO_2 volumetric utilization rate of only 13 mg CO_2/Lh. Substrate consumption and product formation rates are generally more than 100-fold greater in commercially relevant microbial based systems.

Another common shortcoming with many CO_2 sequestration processes is that a "product" is usually not generated. A saleable product from CO_2 would defray some costs associated with CO_2 sequestration. In the case of biological sequestration, ultimately an enzyme must incorporate CO_2 into the backbone of one organic compound (C_X) to generate another larger organic compound (C_{X+1}). Therefore, ideally CO_2 sequestration by a microbial cell would involve transforming an inexpensive C_X to a more valuable C_{X+1}. Potential examples of this strategy include the enzymes phosphoenolpyruvate (PEP) carboxylase (PPC, EC 4.1.1.31), PEP carboxykinase (PCK, EC 4.1.1.49) and pyruvate carboxylase (PYC, EC 6.4.1.1), each of which converts PEP or pyruvate (C_3) into oxaloacetate (C_4). In practice, these enzymes might convert a substrate upstream of CO_2 fixation (such as glycerol or glucose) into a product downstream of a CO_2 fixation step (such as succinic acid).

As a model system, *Escherichia coli* could sequester CO_2 through native PPC (Matsumura et al., 2002), PCK (Matte et al., 1997), or heterologous PYC (Modak and Kelly, 1995) to generate succinate. Although aerobic growth generates CO_2, a biocatalytic process would consume CO_2 under non-growth anaerobic conditions. *E. coli* with mutations in *ldhA*, *pfl* and *ptsG* genes and expressing heterologous PYC generates about 100 g/L succinate with an overall productivity of 1.3 g/L·h and based on stoichiometric considerations should consume CO_2 at a volumetric rate of at least 240 mg/L·h (Vemuri et al., 2002a; Vemuri et al., 2002b), an order of magnitude faster than comparable phototrophic processes.

Succinate is formed through two pathways: one is the reductive arm of the tricarboxylic acid cycle (TCA) through the anaplerotic enzyme PPC; and the other is the glyoxylate shunt (Vemuri et al., 2002b). The former incorporates CO_2 into central metabolism, and one objective of this study was to elucidate the cellular requirement for CO_2. Also, different CO_2 concentrations may change the fractions of succinate partitions between these and other key pathways. In this study, metabolic flux analysis coupled with ^{13}C-labeling tracing was employed to determine the metabolic fluxes at various CO_2 concentrations.

Materials and methods

Strain

Escherichia coli AFP111 (F^+ λ^- *rpoS*396 (Am) *rph*-1 Δ*pflAB::*Cam *ldhA::*Kan *ptsG*) was used in this study, which has mutations in the fermentative genes encoding for pyruvate formate lyase and lactate dehydrogenase, and has a disruption in the phosphotransferase system

of glucose uptake (Donnelly et al., 1998; Chatterjee et al., 2001). AFP111 was transformed with the *pyc* gene from *Rhizobium etli* using the pTrc99A plasmid (Gokarn et al., 2001).

Fermentation media

All fermentations used a defined medium containing (per L): 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 $CoCl_2 \cdot 6H_2O$, 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.

Fermentation conditions

Dual-phase fermentations had an initial volume of 1.2 L in 2.5-L Bioflow III fermenters (New Brunswick Scientific, Edison, NJ, USA) inoculated from 50 mL grown for 10-12 h 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% (Mettler-Toledo Process Analytical Instruments, Wilmington, MA, USA). During growth, the pH was controlled at 7.0 with 20% NaOH and 20% H_2SO_4, and the temperature was maintained at 37°C. When the culture optical density (OD_{600}) reached about 20, the aerobic growth phase was terminated by switching the inlet gas composition to the particular mixture under study. Simultaneously, the total flowrate was reduced to 500 mL/min (dry basis, 0°C and 1 atm), the pH value was controlled at 6.4, the agitation reduced to 200 rpm, 20% NaOH was replaced by 25%

$Ca(OH)_2$ as the neutralizing agent, and 120 mL of a 550 g/L glucose solution was added to increase the concentration to about 60 g/L.

The effect of CO_2 on succinate production at a pH of 6.4 was examined by controlling the proportion of CO_2 and N_2 flowrates in the influent gas using mass flow controllers (Unit Instruments, Inc., Yorba Linda, CA, USA) Gases were pre-humidified in 300 mm×50 mm glass columns (Ace Glass Inc., Vineland, NJ, USA) before entering the fermenter. Figure IV-1 shows the setup for the anaerobic production phase.

For studies 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 Bioflow III fermenters (New Brunswick Scientific, Edison, NJ, USA), the inoculum volume was reduced to 25 mL and the initial glucose concentration was 30 g/L. The glucose concentration was monitored using a YSI 2700 SELECT™ glucose analyzer (Yellow Springs Instrument, Inc, Yellow Springs, OH, USA). When the glucose concentration reached 2 g/L, the system was switched to anaerobic conditions by sparging the desired CO_2 and N_2 gas mixture. When the glucose concentration reached 1 g/L, 2.5 g [1-^{13}C] glucose (99%, Cambridge Isotope Laboratories, Andover, MA, USA) was added into the fermenter. Samples were then collected every 30 minutes, centrifuged at 0°C (10,000×g for 10 min), and the supernatants stored at -4°C for later NMR and LC-MS analysis.

LC-MS analysis of succinate

The sample supernatant was filtered by 0.2 μM syringe filter (Cole-Parmer Instrument Co., Vernon Hills, IL, USA) prior to LC-MS analysis. The liquid chromatography was performed using Applied Biosystems 140B Solvent Delivery System (Applied Biosystems, Foster City, CA,

USA) and a Kromasil C18 column (250 mm × 1 mm, 5 μm particles, 100Å pore space) (Keystone Scientific, Inc., Bellefonte, PA, USA). The mobile phase was a gradient consisting of aqueous acetic acid (0.1% w/v, pH 3.23) and methanol. The mobile phase flow rate was 50 μL/min, and the temperature was 25°C. Electrospray ionization mass spectrometry was performed using a API 1 Plus single quadrupole instrument with electrospray ion source (PE Sciex, 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, interface temperature 50°C. Data were acquired in negative ion mode with a capillary voltage of 3500 V. Mass peak heights were determined using the BioTool Box Version B software package (Applied Biosystems/PE Sciex, Foster City, CA, USA). 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%) (Rosman and Taylor, 1998).

Metabolic modeling and flux analysis

An overall flux balance was developed using stoichiometric analysis and the pseudo-steady-state condition for intracellular metabolites (Savinell and Palsson, 1992). The balance equations for *E. coli* AFP111 metabolism have been developed previously (Vemuri et al., 2002a), and this model was further extended by including the pentose phosphate pathway (Figure IV-2). Flux partitions (φ) were defined as fractional fluxes (ν) for each of two key branches. The fraction of flux into the pentose phosphate pathway (φ_{PPP}) was defined as

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

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

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

The model contained 27 fluxes and 21 metabolites (Figure IV-2 and Table IV-1). Since no growth occurred during the process phase of interest, glucose consumption and product formation rates were essentially constant; thus the process exhibited a metabolic pseudo-steady state. Although five fluxes were known, the balance equations represent an underdetermined set. The MS results using 1-^{13}C-glucose allowed us to calculate the optimal solution. Specifically, we wrote analogous balance equations for each isotopomer of each metabolite (Schmidt et al., 1997). 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 (Van Dien et al., 2003):

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

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 the malic enzyme and pyruvate oxidase were active because the ^{13}C-labeled patterns resulting from these pathways are indistinguishable respectively from the PPC and pyruvate dehydrogenase-

phosphotransacetylase-acetate kinase pathways. Therefore, the model did not include these two pathways.

NMR analysis of succinate

The sample supernatant was filtered by 0.2 μM syringe filter (Cole-Parmer Instrument Co., Vernon Hills, IL, USA), and then the filtered supernatant was mixed with 15% volume of deuterium dioxide (D_2O) for nuclear magnetic resonance (NMR) analysis. Proton-coupled ^{13}C NMR spectra (Varian Unity Inova 500; Varian Inc., Palo Alto, CA. USA) at 125.7 MHz were obtained with the following spectral parameters: 45° pulses, 31.4-kHz spectral width, and 45 second 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 a natural abundance standard.

NMR was not used to calculate fluxes, but was employed to complement the LC-MS results. Specifically, since the mass spectra did not provide information about the position of the label, we used 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 peak area of methylene group to 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 R/O ratio as the fluxes through NAD(P)H formation steps in the pathways divided by fluxes through NAD(P)H consumption steps.

HPLC Analysis of glucose and products

Samples were diluted by 1% H_2SO_4 as necessary, centrifuged (10,000×g for 10 min at 4°C), and the supernatant analyzed for glucose and organic products by high performance liquid chromatography (HPLC) as previously described (Eiteman and Chastain, 1997).

Enzyme Assays

Cell extracts of the *E. coli* strains were prepared by washing the cell pellets with an appropriate buffer and disrupting the suspended cells with an SLM-Aminco French pressure cell (Spectronic Instruments, Rochester, NY, USA) at a pressure of 14,000 lb/in^2. Cell debris were removed by centrifugation (20,000×*g* for 15 min at 4°C), and cell extracts were used to measure PPC (Maeba and Sanwal, 1969) and PYC (Payne and Morris, 1969) activities. One unit of enzyme activity is the quantity of enzyme that converts 1 μmol of substrate to product per min. Total protein in the cell extracts was determined with bovine serum albumin as the standard (Lowry et al., 1951).

Results

Effect of CO_2 concentration 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. Succinate is formed through two pathways: the reductive arm of the tricarboxylic acid cycle (TCA) via the anaplerotic enzyme PPC; and the glyoxylate shunt (Vemuri et al., 2002b). Since CO_2 is incorporated into the carbon backbone as a result of the carboxylation of PEP by PPC, we hypothesized that different CO_2 concentrations in the gas phase would impact the metabolic

fluxes and ultimately change the yield and rate of succinate generated. We therefore conducted several fermentations using a constant agitation rate and total gas flowrate but with different levels of CO_2 in the gas phase during the anaerobic phase. Figure IV-3 shows a typical dual-phase process with 50% CO_2 gas phase composition in the second anaerobic phase. Several products were generated: about 40 g/L succinate after 20 h with a yield of 0.75 ± 0.08 g/g (mean $\pm$ standard deviation) at a specific rate of 224.5 ± 27.2 mg/g·h, while the by-products pyruvate, acetate, and ethanol had final concentrations of 2-6 g/L. The concentration of CO_2 in the gas phase did affect succinate formation: between 0% and 50% CO_2, both q_S and Y_S increased with CO_2 concentration, but this trend did not extend beyond 50% CO_2 (Figure IV-4). The yield for other by-products did not change significantly with CO_2 concentration (data not shown). The activity of PPC was measured to be 0.23 U/mg protein.

Effect of CO_2 concentration on metabolic fluxes

In order to determine whether CO_2 affects the flux distribution, we conducted a series of fermentations in which 1-^{13}C-labeled glucose was added to the bioreactor during the succinate production phase. The known fluxes (glucose consumption and end-product formation rates) were calculated early during anaerobic conditions while the succinate concentration was less than 4 g/L. With these known fluxes and LC-MS results we then optimized the flux distribution. Table IV-2 compares the observed mass distribution of succinate and the calculated mass distribution by the optimal metabolic model (i.e., with error minimized as described in Materials and methods). We further validated the metabolic model by comparing the enrichment ratio S2/S1 observed by NMR with the ratio calculated from the optimal metabolic model. The

differences between the observed S2/S1 and the calculated values were less than 10% (Table IV-2). In addition, we calculated the R/O ratio based on the optimal model, and the values of this parameter for each CO_2 concentration were close to 1 (Table IV-2).

The metabolic fluxes calculated from the model indicate that CO_2 impacted the rates of substrate consumption and product formation. An increase in gas phase CO_2 concentration from 3% to 50% increased both glucose consumption rate and succinate production rate 6-fold (Figure IV-5). Also, the fraction of flux into the pentose phosphate pathway (φ_{PPP}) increased from 0.04 at 3% CO_2 to 0.17 at 50% CO_2 (Table IV-2 and Figure IV-5). The fraction of flux through the carboxylation at the PEP node (φ_{PPC}) increased from 0.53 to 0.63 between 3% CO_2 and 50% CO_2 (Table IV-2 and Figure IV-5). Only slightly more succinate was formed via the reductive arm of the TCA cycle at 50% CO_2 than at 3% CO_2 (88% versus 85%).

Some pathways generate CO_2 while one pathway consumes CO_2 during succinate formation. Specifically, CO_2 was generated via 6-phosphogluconate dehydrogenase and pyruvate dehydrogenase (or pyruvate oxidase) but consumed via PPC (Figure IV-2). A CO_2 concentration of 3% resulted in the net CO_2 consumption rate of 0.08 mmol/g·h (3.5 mg/g·h) with the overall stoichiometric coefficient for CO_2 (i.e., the molar ratio of net CO_2 consumption to glucose consumption) of 0.36. In comparison, a CO_2 concentration of 50% led to a CO_2 overall stoichiometric coefficient of 0.53 and a net consumption rate of 0.73 mmol/g·h (32 mg/g·h), corresponding to a volumetric CO_2 consumption rate of 288 mg/L·h.

Effect of agitation rate

Since low CO_2 concentrations reduced the rate of succinate formation, mass transfer could be a limiting factor. We next studied 10% CO_2 but used higher agitation rates (400 rpm and 1000 rpm) to increase the CO_2 mass transfer rate. Higher agitation rates did not alter observed values of q_S and Y_S (Figure IV-6), strong evidence that mass transfer of gaseous CO_2 was not limiting at this CO_2 concentration. The generation of all other by-products also remained unchanged (data not shown).

Effect of pyruvate carboxylase overexpression

Above 50% CO_2 succinate production did not increase with increasing CO_2 concentration, and therefore the succinate formation rate may be limited by the reaction rates involved in succinate formation. The rate of HCO_3^- consumption (and thus subsequently succinate formation) may be limited by the activity of PPC. If a similar enzyme that incorporates CO_2 is introduced into *E. coli* AFP111, the rate of succinate accumulation may be increased at CO_2 concentrations above 50%. *E. coli* AFP111/pTrc99A-*pyc* was constructed by transforming *E. coli* AFP111 with plasmid pTrc99A-*pyc*, which expresses *Rhizobium etli* pyruvate carboxylase (PYC). This enzyme catalyzes the carboxylation of pyruvate to oxaloacetate. We repeated the dual-phase processes under identical conditions (with 50% CO_2 in the gas phase) using AFP111/pTrc99A-*pyc* and compared them to those using AFP111. The q_S and Y_S by AFP111/pTrc99A-*pyc* were not increased and other by-products were not significantly different (Table IV-3). The enzyme activities of PPC and PYC in both strains were measured: AFP111 had a PPC activity of 0.23

U/mg and no detectable PYC activity; AFP111/pTrc99A-*pyc* had a PYC activity of 0.26 U/mg, however, the activity of its native PPC was reduced dramatically to 0.08 U/mg (Table IV-3).

Discussion

NMR, LC-MS and HPLC analyses using ^{13}C-labeled substrates were successfully combined to calculate metabolic fluxes during succinate production at different levels of CO_2. The LC-MS provides the numbers of labeled carbon atoms but not the labeled position, while NMR results provide information about position. For example, the MS cannot distinguish between [1-^{13}C] succinate and [2-^{13}C] succinate while NMR generates a different pattern with these two specific isotopomers. The small difference between observed and predicted enrichment ratio S2/S1 (less than 10%), as well as a calculated R/O ratio near 1 provide strong evidence for the appropriateness of our metabolic model describing succinate production during an anaerobic non-growth phase. Although the model is a simplification of metabolism, and some error may occur from anabolic reactions, these reactions are likely small because the cells are not growing significantly during the anaerobic phase. Another source of error may be alternate catabolic reactions.

Succinate is principally formed through two pathways: the reductive arm of the TCA cycle and the glyoxylate shunt (Clark, 1989; Vemuri et al., 2002b). Through the reductive arm of the TCA cycle CO_2 is incorporated into the final product succinate via the enzyme PPC. This gas is therefore necessary for succinate production, and CO_2 availability impacts substrate utilization and product accumulation rates. Not surprisingly, in our study increasing CO_2 concentration in the gas phase increased the succinate production, and ^{13}C-flux analysis demonstrated more

carbon partitioned at the PEP node into the reductive arm of the TCA cycle. As a result more CO_2 was incorporated into the carbon backbone, and the overall stoichiometric coefficient increased form 0.36 at 3% CO_2 to 0.53 at 50% CO_2. Since succinate formation through the reductive arm of the TCA cycle is not redox balanced with glucose (Vemuri et al., 2002a), a consequence of higher flux through PPC to succinate is a greater demand on reduced cofactors. The cells unexpectedly responded to an increased concentration of CO_2 in the gas phase by increasing the relative flux through the pentose phosphate pathway (Table IV-2), which paradoxically itself is a CO_2-generating pathway. Since the conversion of 1 mol glucose to PEP via glycolysis generates 2 mol NADH while the conversion of 1 mol glucose to PEP via the pentose phosphate pathway generates 3.67 moles NAD(P)H, increased pentose phosphate pathway flux is a means to meet the elevated demand for reduced cofactors. Our studies did not establish whether, under conditions of high CO_2 concentration in the gas phase, an elevated pentose phosphate pathway flux led to an elevated rate of succinate formation or whether an elevated rate of succinate formation caused an increase in the pentose phosphate pathway flux.

Although primarily NADPH is generated in the pentose phosphate pathway and NADH (and FADH) is consumed during succinate formation through the reductive arm of the TCA cycle, NADPH and NADH are interconvertible by transhydrogenases (Sauer et al., 2004). Also, FADH is generated via NAD(P)H:flavin oxidoreductase (Fontecave et al., 1987; Niviere et al., 1996). Since elevated CO_2 in the gas phase promotes additional CO_2 generation by the pentose phosphate pathway, this additional CO_2 would contain the label from 1-^{13}C-glucose as a result of the action of phosphogluconate dehydrogenase. However, we did not observe significant

incorporation of labeled CO_2 into succinate via PPC probably because the moderately high gas flowrate used in our experiments diluted the much lower quantity of labeled CO_2 generated in the pentose phosphate pathway.

Our results suggest that CO_2 limited succinate formation only at low CO_2 concentrations (i.e., around 10% CO_2 in gas phase). From the metabolic flux analysis, about 90% of the succinate generated involved CO_2 incorporation by PPC. Thus, the maximum CO_2 consumption rate *per cell* (J_{CO2}^{MAX}) is 90% of the specific succinate production rate during the anaerobic phase:

$$J_{CO2}^{MAX} = \frac{0.9 q_s}{M / m_E}$$

M is molar mass of succinate (116 g/mol), and m_E is the average dry weight of an *E. coli* cell, 2.9×10^{-13} g (Neidhardt et al., 1996). Based on our experimental results at 50% CO_2, J_{CO2}^{MAX} is 1.4 $\times10^{-16}$ mmol/s. How does CO_2 limit succinate formation? Through the reductive branch of the TCA cycle, the utilization of gaseous CO_2 and its conversion ultimately to succinate can be described by four separate processes (Figure IV-7): 1) the transport of CO_2 from the gas phase to the bulk liquid phase, 2) the transport of CO_2 from the bulk across the cell membrane into the cytoplasm, 3) the intracellular conversion of dissolved CO_2 into bicarbonate, 4) the diffusion of bicarbonate and reaction catalyzed by the enzyme PPC to form oxaloacetate.

The flux of CO_2 transport from the gas (bubble) sparged into the fermenter to the liquid phase (J_{CO2}^{1}), is proportional to the mass transfer coefficient:

$$J_{CO2}^{1} = \frac{1}{(X/m_E)} \frac{d[CO_2]_{ex}}{dt} = \frac{k_L a_{(CO2)} ([CO_2^*] - [CO_2^l]_{ex})}{X/m_E} = \frac{k_L a_{(CO2)} \Delta[CO_2]}{X/m_E} \qquad (1)$$

where k_La is the volumetric CO_2 mass transfer coefficient (h^{-1}), $[CO_2^*]$ is the molar CO_2 concentration in equilibrium with the gas phase, $[CO_2^l]_{ex}$ is the molar (extracellular) liquid phase CO_2 concentration, and X is dry cell density (g/L). We measured the mass transfer coefficient for oxygen in our system at conditions identical to those used during the anaerobic phase (e.g., 200 rpm and 1.2 L). Using a correlation (Royce and Thornhill, 1991) we estimated the value of $k_La_{(CO2)}$ to be 0.0081 s^{-1}. X was 8.4 g/L in an average bioreactor during succinate production. At steady-state (with no accumulation of CO_2 or HCO_3^-), therefore,

$$J_{CO2}^1 = \frac{k_L a_{(CO2)} \Delta[CO_2]}{X/m_E} = 1.4 \times 10^{-16} \text{ mmol/s} \quad (2)$$

For our system the driving force $\Delta[CO_2]$ is at most 0.5 mmol/L. If the value of $[CO_2^*]$ is large, then the value of $[CO_2^l]_{ex}$ is relatively close to $[CO_2^*]$. For example, if the partial pressure of CO_2 is 0.10 atm, then $[CO_2^*]$ is 3.8 mM (Carroll et al., 1991) and $[CO_2^l]_{ex}$ is 3.3 mM, only 13% less than the equilibrium concentration. A gas phase CO_2 concentration of 50% (partial pressure of 0.50 atm) would result in a $[CO_2^l]_{ex}$ of about 18.5 mM, almost indistinguishable from the equilibrium concentration $[CO_2^*]$ and demonstrating no mass transfer limitation. From this analysis we conclude that at gas phase concentrations of CO_2 greater than 2-3%, mass transfer would not limit CO_2 utilization under these conditions, a conclusion consistent with our results using higher agitation rates (Figure IV-6).

The second process which could limit the succinate formation process is the diffusion of dissolved CO_2 through the cell membrane. Permeation of HCO_3^- through the lipid membrane is insignificant (Gutknecht et al., 1977). For rod-shaped cells (Berg, 1983, Neidhardt et al., 1996),

the CO_2 diffusion rate through one cell (J^2_{CO2}) is given by:

$$J^2_{CO2} = PA_E([CO^l_2]_{ex} - [CO^l_2]_{in}) \quad (3)$$

The permeability coefficient P is 3.5×10^{-3} m/s (Gutknecht et al., 1977), and A_E is the surface area of an *E. coli* cell, 6×10^{-12} m^2 (Neidhardt et al., 1996). Setting J^2_{CO2} equal to the maximum flux, $[CO^l_2]_{in}$ is essentially identical to $[CO^l_2]_{ex}$ even for low values of $[CO^l_2]_{ex}$ demonstrating diffusion of CO_2 across the cell membrane does not limit CO_2 utilization.

The third process is the intracellular hydration of CO_2 to HCO^-_3, which can occur by chemical hydration and its mechanism is described by the following equilibrium:

$$CO_2 + H_2O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HCO^-_3 + H^+$$

where k_1 is the rate constant of the forward reaction and has a value of 0.029 s^{-1}, and k_{-1} is the rate constant of reverse reaction with a value of 2.0×10^4 L/mol·s (Pocker and Bjorkquist, 1977). Accordingly, the CO_2 conversion rate is

$$J^3_{CO2} = (k_1[CO^l_2]_{in} - k_{-1}[HCO^-_3][H^+])V_E \quad (3)$$

V_E is the cytoplasm volume of an *E. coli* cell, about. 1.0×10^{-15} L (Neidhardt et al., 1996). The maximum flux occurs when there is no bicarbonate ion to act as a reactant for the reverse reaction:

$$J^3_{CO2}(max) = k_1[CO^l_2]_{in} V_E \quad (4)$$

For the example of $[CO^l_2]_{in}$ = 3.3 mM, as calculated above 10% CO_2 in the gas phase, the maximum flux $J^3_{CO2}(max)$ is about 9.6×10^{-17} mmol/s, more than 30% less than the value for J^{MAX}_{CO2} of 1.4×10^{-16} mmol/s. If the gas phase concentration is 50%, then $J^3_{CO2}(max)$ is 5.3×10^{-16} mmol/s, and this third process would be less likely to be limiting. This analysis indicates that the

third step may be rate limiting at a low concentration of CO_2 (i.e., 10%), preventing the succinate production pathway to operate at its maximum rate. This analysis does not consider the enzymatic conversion of dissolved CO_2 to bicarbonate mediated by carbonic anhydrase.

The fourth process is the utilization of HCO_3^- by the enzyme PPC, and its rate is

$$J_{CO2}^4 = \frac{V_{(PPC)}[HCO_3^-]}{(K_{M(PPC)} + [HCO_3^-])} \tag{5}$$

where $K_{M(PPC)}$ is the Michaelis-Menten constant with a value of 0.1 mM for the *E. coli* enzyme (Kai et al., 1999). $V_{(PPC)}$ is the maximum rate of HCO_3^- utilization by PPC [in mmol/s]. In order to use equation 5 to estimate the maximum value for J_{CO2}^4, a value is needed for $[HCO_3^-]$. At steady-state, the rate change of $[HCO_3^-]$ equals zero:

$$V_E \frac{d[HCO_3^-]}{dt} = (k_1[CO_2^l]_{in} - k_{-1}[HCO_3^-][H^+])V_E - \frac{V_{(PPC)}[HCO_3^-]}{(K_{M(PPC)} + [HCO_3^-])} = 0 \tag{6}$$

Using the value of 0.23 U/mg (measured at 50% CO_2) and the cell concentration of 3×10^{13} cells/L (data not shown), we find the maximum enzyme activity is 2.8×10^{-11} U/cell or 4.6×10^{-16} mmol product formed/s. Considering 10% CO_2 with $[CO_2^l]_{in}$ = 3.3 mM and an intracellular pH of 7.0 (Olsen et al., 2002), the calculated $[HCO_3^-]$ is 0.026 mM. This concentration is only one-fourth the value of K_M, so that the enzymatic reaction occurs only at 20% of the maximum $V_{(PPC)}$, resulting in a value for J_{CO2}^4 of 9.5×10^{-17} mmol/s. Considering 50% CO_2, the bicarbonate concentration far exceeds the value of K_M. Intracellular bicarbonate concentration is very sensitive to gas phase CO_2 concentration (Figure IV-8), demonstrating that the enzyme reaction is limiting at 10% CO_2 (because of low $[HCO_3^-]$), whereas at higher CO_2 concentrations, the intrinsic activity of the enzyme could limit the overall process. Indeed, at approximately

$[CO_2^l]_{in}$ equal to 15 mM, the concentration of HCO_3^- becomes much higher than $K_{M(PPC)}$ (Figure IV-8), corresponding to a $[CO_2^*]$ of 15 mM (Carroll et al., 1991); in other words the CO_2 should be about 40% in the gas phase to meet the requirement for succinate production pathway running at its maximum. Not included in this analysis are the various unknown concentrations of affectors of PPC, such as aspartate and citrate (Gold and Smith, 1974), which would further limit activity *in vivo*, particularly at high bicarbonate concentrations. Also, this simplistic analysis does not consider possible indirect effects, such as by CO_2 affecting another enzyme in the succinate pathways, or for example, the observation that succinate inhibits the activity of PPC (Gold and Smith, 1974).

The results of the analysis of the four processes involved in the incorporation of CO_2 into succinate indicate that enzymatic processes rate limiting over most of the range of gas phase CO_2 concentrations. However, the reason for the limitation may change. At very high CO_2 concentrations, the activity of the enzyme ($V_{(PPC)}$) likely limits CO_2 utilization, whereas at intermediate CO_2 concentrations the HCO_3^- concentration can limit CO_2 utilization. Only at very low CO_2 concentrations would mass transfer limit CO_2 utilization.

This analysis also suggests strategies to improve CO_2 utilization. For example mass transfer should be considered for very low CO_2 concentrations. At intermediate CO_2 concentrations, overexpression of the carbonic anhydrase catalyzing the hydration of CO_2 to HCO_3^- might be effective. If pure CO_2 were used, further improvement in succinate production should result from increased enzyme expression, as has been previously observed with pyruvate carboxlase (Vemuri et al., 2002b). Of course, elevated enzyme expression can have limited

benefit because of other affectors. For instance, in this study though PYC was expressed in AFP111/pTrc99A-*pyc*, its native PPC activity was dramatically decreased that prevented this transformant from producing more succinate. It is likely that PPC and PYC are regulated by each other. Overexpressing an enzyme that utilizes CO_2 and does not interfere with PPC at higher CO_2 gas may further increase succinate production.

This study demonstrates that CO_2 is essential to PEP carboxylation, which leads to the accumulation of succinate through the reductive branch of the TCA cycle by *E. coli* AFP111. Metabolic flux analysis with ^{13}C-labeled glucose allowed an understanding of the carbon flux partitioning of *E. coli* AFP111 in response to varied CO_2 concentrations, including the surprising observation that CO_2 affected the fraction of flux into the pentose phosphate pathway.

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Table IV-1. Metabolic reactions for succinate production.

	Reaction
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)

Table IV-2. Comparison of the mass distributions of succinate observed by mass spectrometry and those calculated by the optimal metabolic model. The enrichment ratio (S2/S1) was observed from NMR results. The redox ratio R/O was calculated from the optimal metabolic model.

CO_2 (%)	φ_{PPP}	φ_{PPC}		M+0 (%)	M+1 (%)	M+2 (%)	M+3 (%)	(S2/S1)[a]	R/O
3	0.04	0.53	Observed	0.594±0.006	0.379±0.003	0.023±0.005	0.004±0.002	17.03	1.11
			Calculated	0.591	0.379	0.027	0.003	18.53	
10	0.08	0.61	Observed	0.547±0.005	0.417±0.004	0.033±0.003	0.003±0.001	18.76	1.01
			Calculated	0.549	0.418	0.030	0.004	20.64	
50	0.17	0.63	Observed	0.586±0.005	0.377±0.011	0.031±0.009	0.005±0.005	17.53	1.03
			Calculated	0.589	0.389	0.02	0.002	18.79	

Observed values are shown as mean ± standard deviation from 3 analyses

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

Table IV-3. Comparison of AFP111 to AFP111/pTrc99A-*pyc* at 50% CO_2 in the gas phase

Strains	q_S (mg/gh)	Y_S (g/g)	Y_P (g/g)	Y_A (g/g)	Y_E (g/g)	PPC (U/mg)	PYC (U/mg)
AFP111	224.5 ± 27.2	0.75 ± 0.08	0.10 ± 0.08	0.04 ± 0.02	0.04 ± 0.01	0.23	0.00
AFP111/pTrc99A-*pyc*	188.7 ± 21.0	0.63 ± 0.02	0.08 ± 0.08	0.04 ± 0.02	0.04 ± 0.02	0.08	0.26

Data are shown as mean ± standard deviation

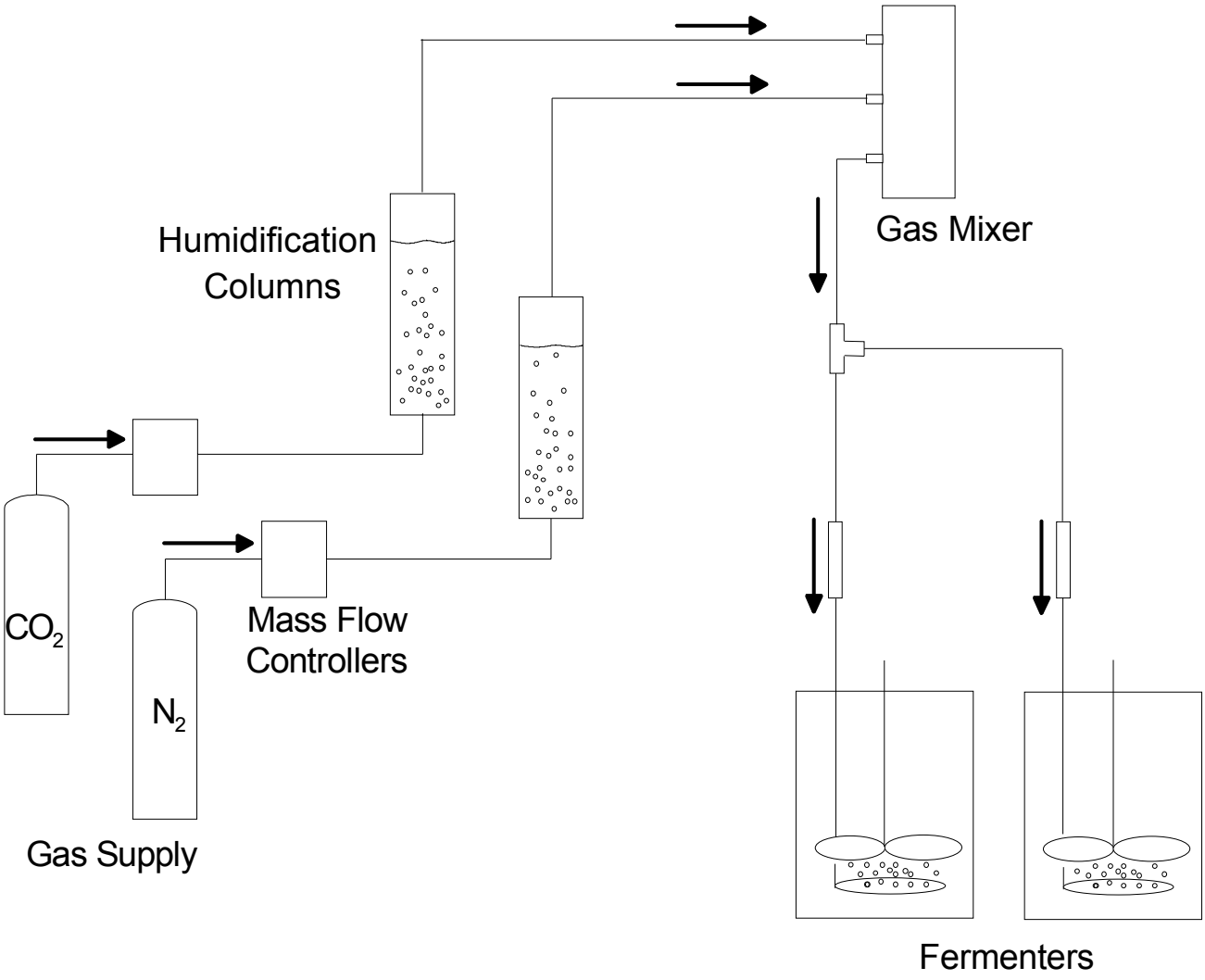


Figure IV-1. Setup of gas supply system for study of anaerobic production of succinate using *E. coli* AFP111. Nitrogen and carbon dioxide were proportioned into each 1.2 L culture volume at a total flowrate of 500 mL/min (dry basis, 1 atm, 0°C).

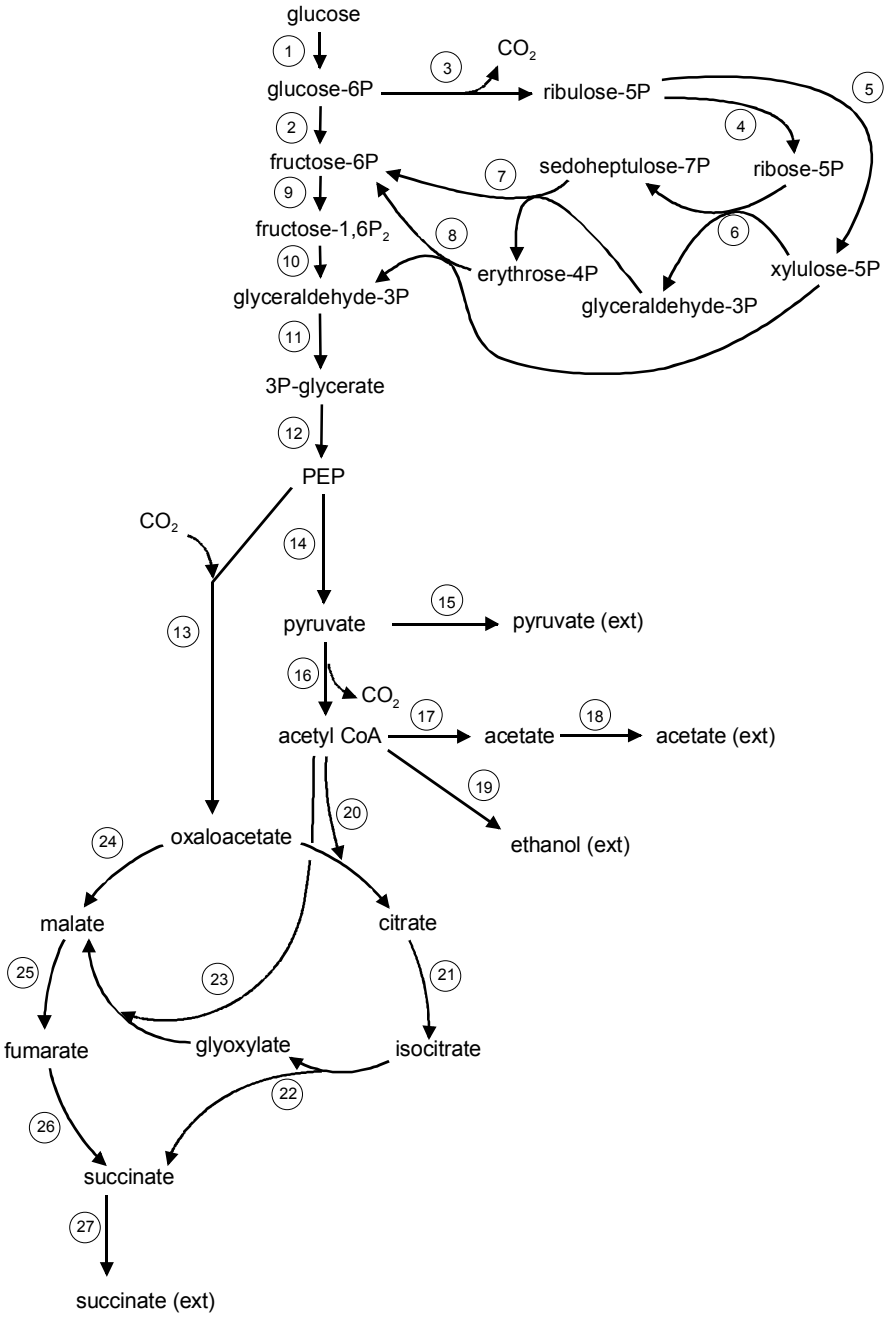


Figure IV-2. Biochemical pathways for the synthesis of succinate from glucose in *E. coli*. Not all enzymatic steps or intermediates are shown. Key enzymes in the pathways are as follows: 1, glucokinase; 2, phosphoglucoisomerase; 3, 6-phosphogluconate dehydrogenase; 4, phosphopentose epimerase; 5, phosphopentose 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; and 26, fumarate reductase.

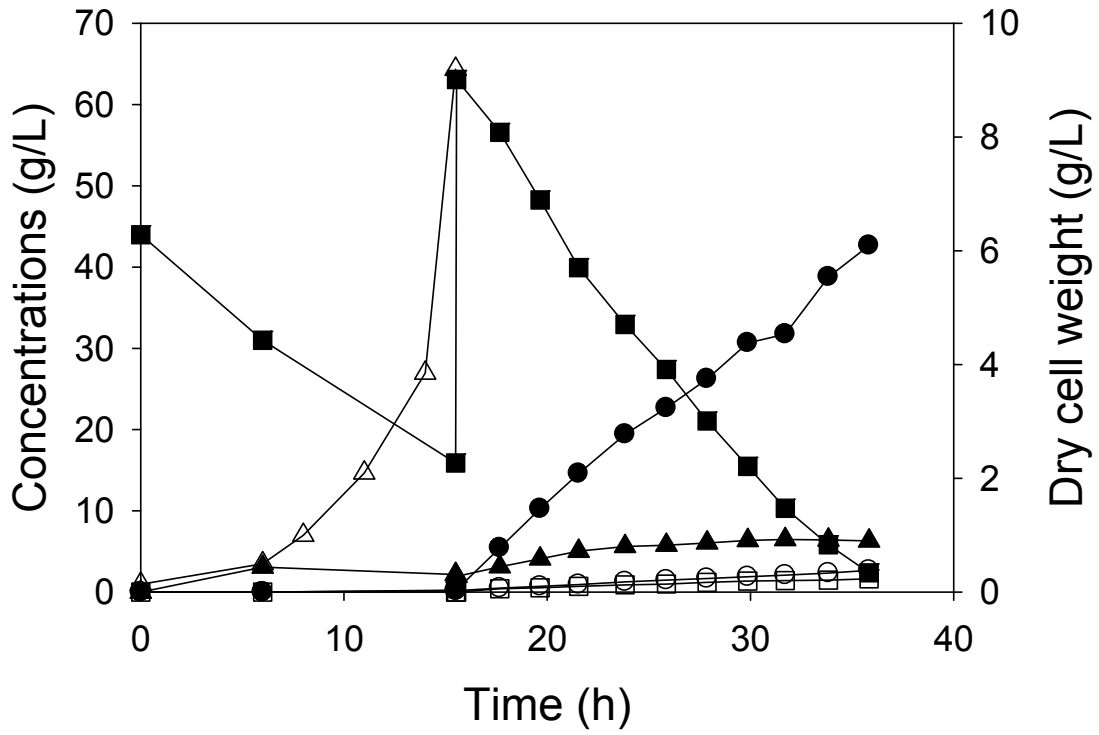


Figure IV-3. Production of succinate (●), pyruvate (▲), ethanol (□), acetate (○) and dry cell weight (△) from glucose (■) in the dual-phase fermentation of *E. coli* AFP111. The switch from aerobic conditions to the anaerobic phase occurred at approximately 16 h, when a concentrated glucose solution was added to the bioreactor.

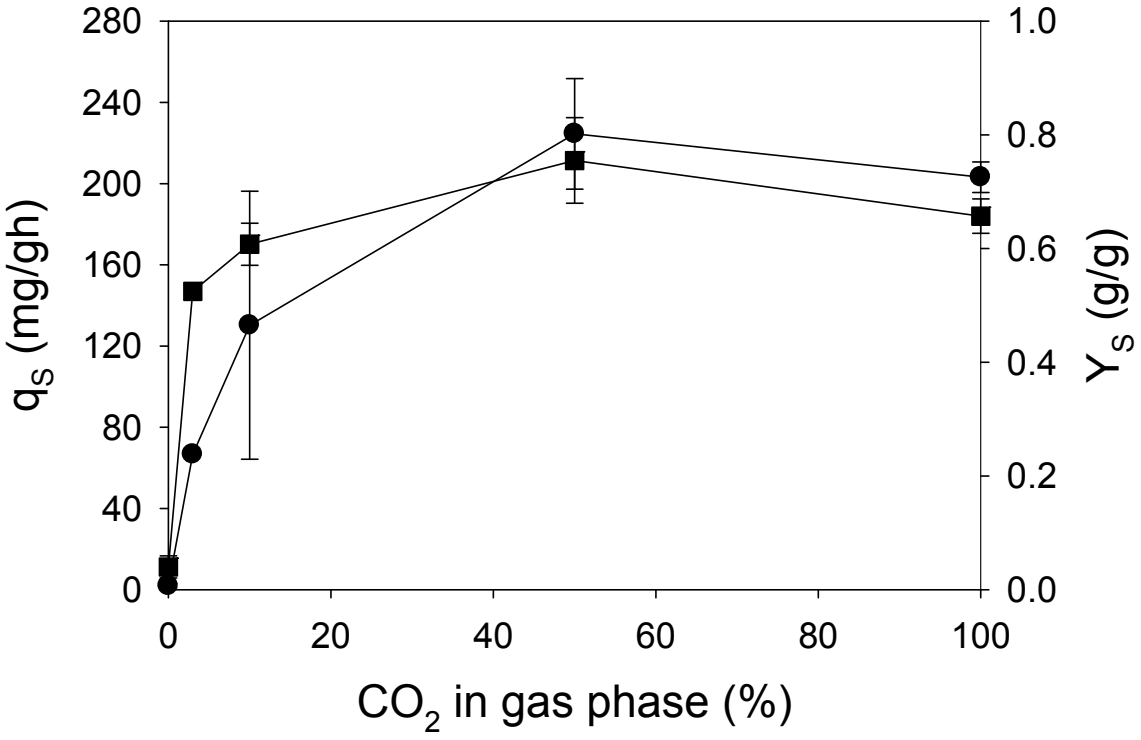
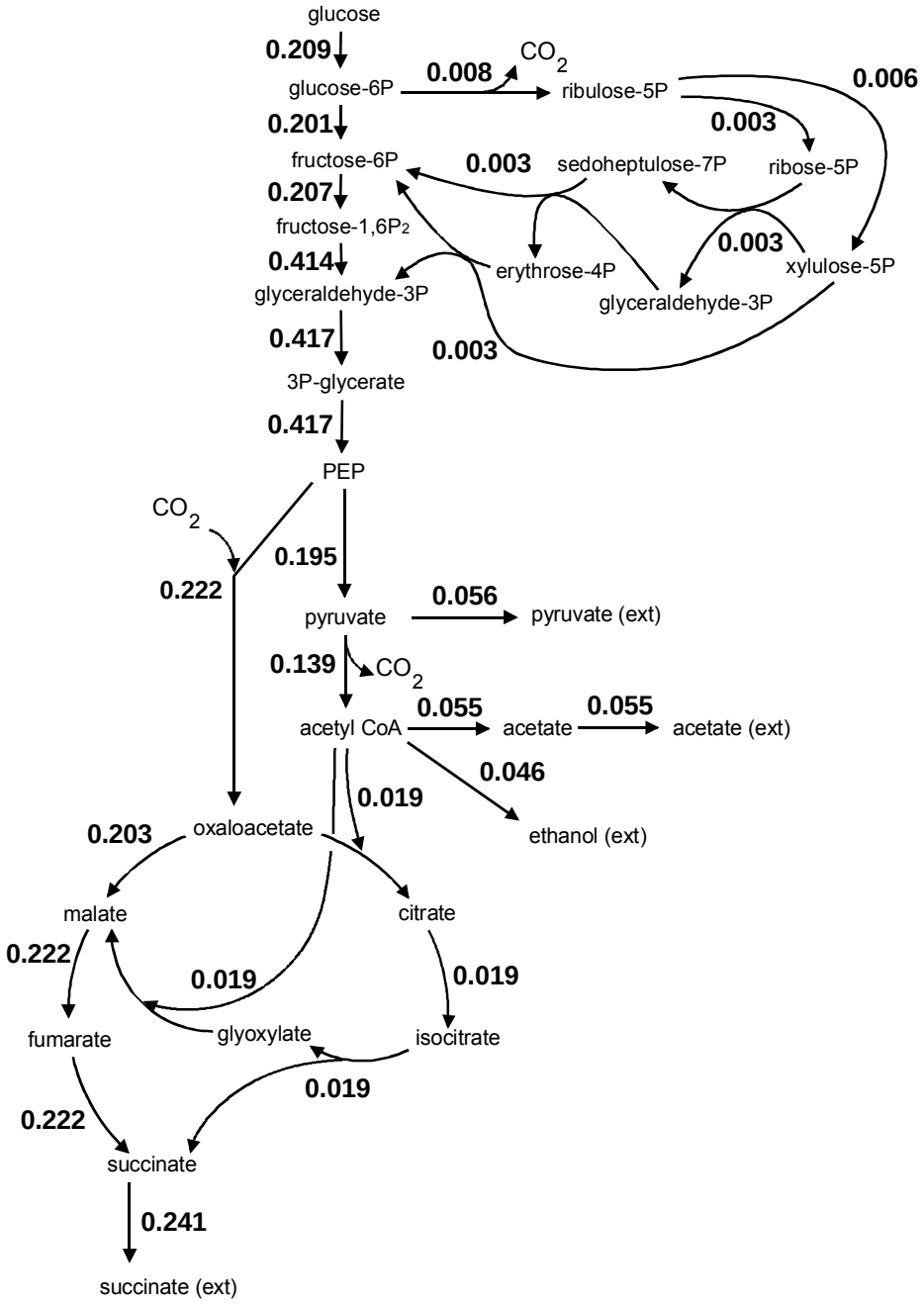
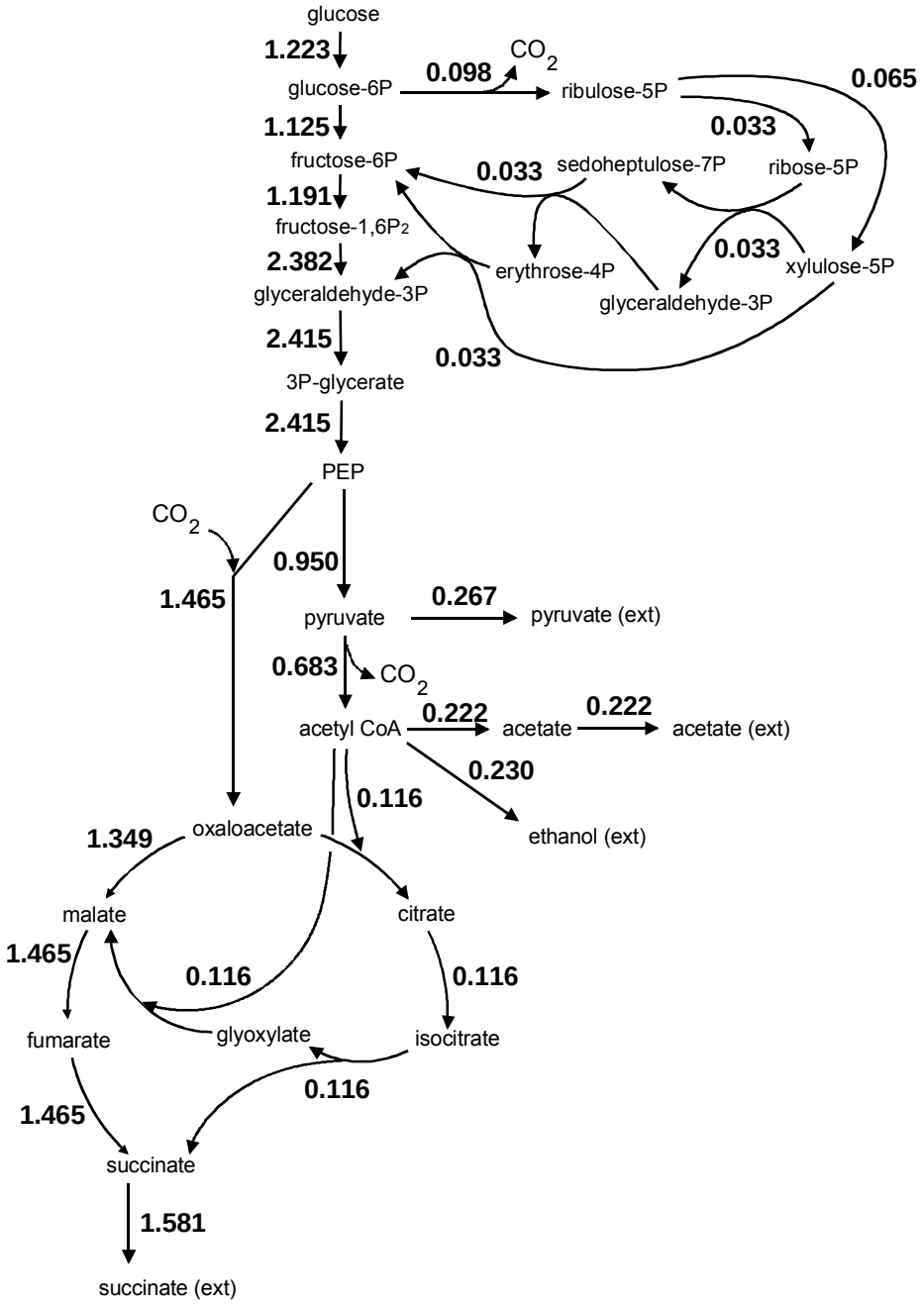


Figure IV-4. Effect of gas phase CO_2 composition on succinate production: specific rate of succinate production, q_S (●) and mass yield Y_S (■). The anaerobic portion of the process operated at a pH of 6.40.

A


glucose
0.209
glucose-6P
0.008
CO2
ribulose-5P
0.006
0.003
0.201
fructose-6P
0.003
sedoheptulose-7P
ribose-5P
0.207
fructose-1,6P2
0.003
0.414
erythrose-4P
xylulose-5P
glyceraldehyde-3P
glyceraldehyde-3P
0.417
0.003
3P-glycerate
0.417
PEP
CO2
0.195
0.222
pyruvate
0.056
pyruvate (ext)
0.139
CO2
acetyl CoA
0.055
acetate
0.055
acetate (ext)
0.046
0.019
ethanol (ext)
0.203
oxaloacetate
malate
citrate
0.222
0.019
0.019
fumarate
glyoxylate
isocitrate
0.019
0.222
succinate
0.241
succinate (ext)

B


glucose
1.223
glucose-6P
0.098
CO2
ribulose-5P
0.065
1.125
0.033
fructose-6P
0.033
sedoheptulose-7P
ribose-5P
1.191
fructose-1,6P2
2.382
0.033
xylulose-5P
erythrose-4P
glyceraldehyde-3P
glyceraldehyde-3P
2.415
0.033
3P-glycerate
2.415
PEP
CO2
0.950
1.465
pyruvate
0.267
pyruvate (ext)
0.683
CO2
acetyl CoA
0.222
acetate
0.222
acetate (ext)
0.230
0.116
ethanol (ext)
1.349
oxaloacetate
malate
citrate
1.465
0.116
0.116
fumarate
glyoxylate
isocitrate
0.116
1.465
succinate
1.581
succinate (ext)

C

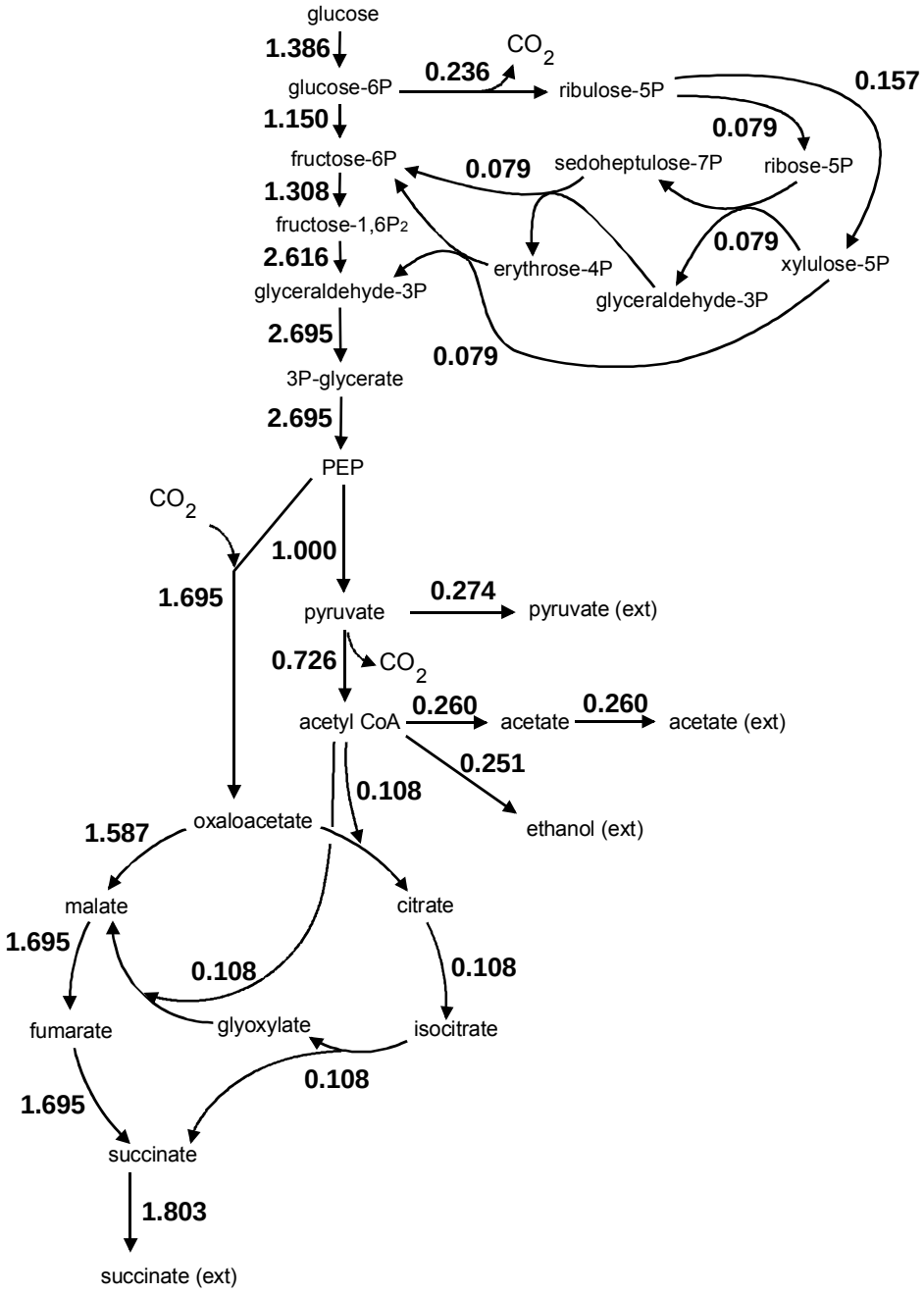


Figure IV-5. Fluxes through biochemical pathways at three CO_2 concentrations: A) 3% CO_2; B) 10% CO_2; and C) 50% CO_2. The unit of the flux is mmol/gh.

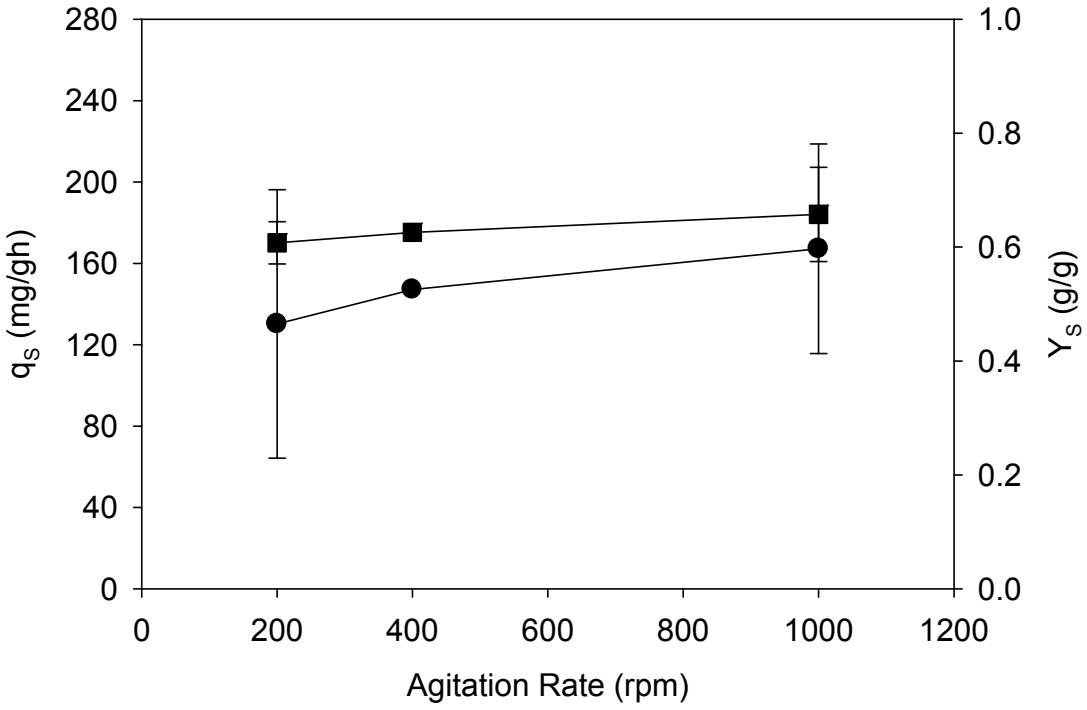


Figure IV-6. Effect of agitation on succinate production: specific rate of production, q_S (●) and mass yield Y_S (■). The anaerobic portion of the process operated at a pH of 6.4 and 10% CO_2 in the gas phase.

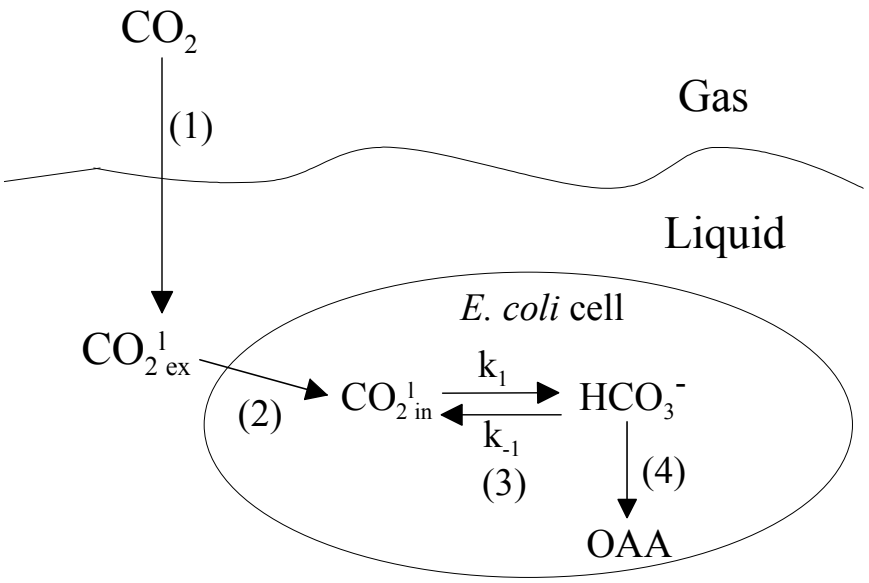


Figure IV-7. Explicit model of CO_2 transport and utilization by *E. coli* cell. OAA is oxaloacetate.

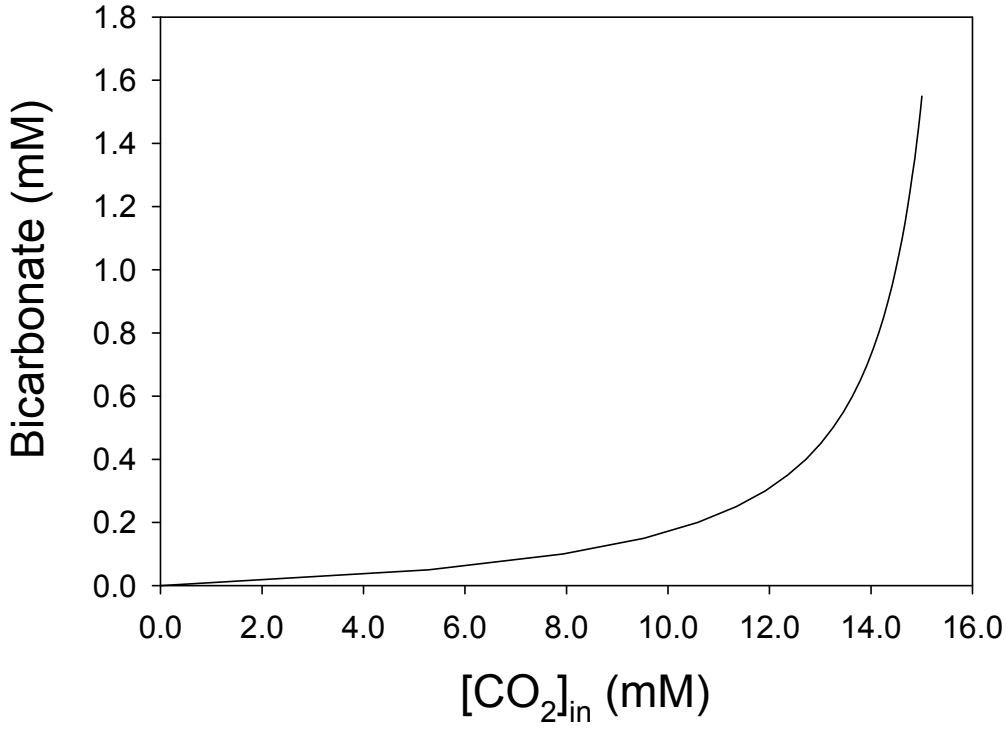


Figure IV-8. Relationship between $[CO_2^1]_{in}$ and $[HCO_3^-]$ in the succinate production system.

CHAPTER V

EFFECT OF FLUE GAS COMPONENTS ON SUCCINATE PRODUCTION IN DUAL-PHASE *ESCHERICHIA COLI* FERMENTATIONS[1]

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Abstract

Escherichia coli pfl ldhA ptsG (AFP111) was studied for succinate production in defined medium using trace gases typically found in flue gas, oxygen (O_2), nitrogen dioxide (NO_2), sulfur dioxide (SO_2) and carbon monoxide (CO). Following aerobic cell growth, cells were exposed to 50% CO_2 and 3-10% O_2, or 50-300 ppm NO_2, SO_2 or CO during a succinate production phase. Although 3% O_2 did not significantly affect succinate formation, 10% O_2 reduced the final succinate concentration from 33 g/L to 17 g/L, specific succinate productivity from 1.90 to 1.13 mmol/g·h and succinate yield from 1.15 to 0.81 mol/mol glucose. The effect of O_2 correlated with the culture redox potential (ORP) with more reducing conditions favoring succinate production. The trace gases NO_2 and SO_2 also reduced the rate of succinate formation by as much as 50%, and led to a greater than two-fold increase in pyruvate formation. Similar concentrations of CO showed no effect on succinate production rate or yield. Using synthetic flue gas AFP111 generated 12 g/L succinate with a succinate specific productivity of 0.73 mmol/g·h and a yield of 0.65 mol/mol.

Introduction

Succinate is a four-carbon dicarboxylic acid which can serve as a precursor for many commercial chemicals, such as 1,4-butanediol, tetrahydrofuran, and γ-butyrolactone (McKinlay et al., 2007). Traditionally, succinate is produced through petrochemical processes. Rising prices and concerns over the environmental impact of oil-based industries have elevated interest in microbial fermentation as a viable alternative for succinate production. Moreover, microbial succinate production can have the environmental benefit of CO_2 fixation. Although aerobic growth generates CO_2, anaerobic biochemical pathways leading to succinate incorporate CO_2 into the backbone of three-carbon compounds (e.g., PEP or pyruvate) to generate four-carbon oxaloacetate via the enzymes phosphoenolpyruvate (PEP) carboxylase (PPC, EC 4.1.1.31), PEP carboxykinase (PCK, EC 4.1.1.49) or pyruvate carboxylase (PYC, EC 6.4.1.1).

As a model system, *Escherichia coli* can generate succinate while ”sequestering” CO_2 through native PPC (Matsumura et al., 2002), PCK (Matte et al., 1997), or heterologous PYC (Modak and Kelly, 1995). *E. coli* with mutations in *ldhA*, *pfl* and *ptsG* genes and expressing heterologous PYC generates about 100 g/L succinate with an overall productivity of 1.3 g/L·h, and based on stoichiometry should consume CO_2 at a volumetric rate of at least 240 mg/L·h (Vemuri et al., 2002a; Vemuri et al., 2002b).

One readily available industrial source of CO_2 is flue gas from power plants. However, this gas often contains several other components such as O_2, NO_X, SO_2, and CO. One analysis of a boiler flue gas reported 263 ppm NO_X, 266 ppm SO_2, and 15 ppm CO (Orsini et al., 1981), while another indicated 250 ppm NO_X, 334 ppm SO_2, and 39 ppm CO (Scinto et al., 1981). A waste incineration plant reported 8-9% CO_2, 9-11% O_2, 80-150 ppm SO_2 and 150-200 ppm NO_X (Stoll et al., 2001). The components SO_2 and NO_2 are likely detrimental to microbial growth

(Robakis et al., 1983; Kosaka et al., 1986), but the effects of these gases and O_2 and CO on *E. coli* during non-growth succinate production and CO_2 utilization are unknown. The objective of this study was to examine the effect of these flue gas components (O_2, NO_2, SO_2, and CO) on the anaerobic formation of succinate by recombinant strains of *E. coli*.

Materials and methods

Strain

Escherichia coli AFP111 ($F^+\lambda^-$ *rpoS*396 (Am) *rph*-1 *ΔpflAB::*Cam *ldhA::*Kan *ptsG*) was used in this study, having mutations which direct most carbon to succinate under anaerobic conditions (Donnelly et al., 1998; Chatterjee et al., 2001, Vemuri et al., 2002b).

Fermentation medium

All fermentations used a defined medium containing (per L): 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 $CoCl_2 \cdot 6H_2O$, 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.

Fermentation conditions

Dual-phase fermentations had an initial volume of 1.2 L in 2.5 L Bioflow III fermenters (New Brunswick Scientific, Edison, NJ, USA) inoculated from 50 mL grown for 10-12 h in the same medium in 250 mL shake flasks. Oxygen-enriched air as necessary was sparged at 1.0 L/min with agitation as needed to maintain the dissolved oxygen (DO) above 40% (Mettler-Toledo Process Analytical Instruments, Wilmington, MA, USA). During this aerobic growth phase, the pH was controlled at 7.0 with 20% NaOH and 20% H_2SO_4, and the temperature was

maintained at 37°C. When the culture optical density (OD) reached about 20, the aerobic phase was terminated by switching the inlet gas composition to the particular synthetic "flue gas" mixture under study. Simultaneously, the total flowrate was reduced to 0.5 L/min (dry basis, 0°C and 1 atm), the agitation reduced to 200 rpm, 20% NaOH was replaced by 25% $Ca(OH)_2$ as neutralizing agent, and 120 mL 550 g/L glucose was added to increase the concentration to about 60 g/L.

The effect of a flue gas component was examined by controlling the influent CO_2, N_2, and the particular component being studied (e.g., NO_2) using mass flow controllers (Unit Instruments, Inc., Yorba Linda, CA, USA). All experiments used 50% CO_2, a specific concentration of one other component as indicated in the results, and the balance N_2 using pure gases and gas mixtures in N_2 (Airgas National Welders, Charlotte, NC, USA). Mixtures containing O_2 or CO were humidified in 300 mm (h) × 50 mm (d) glass columns (Ace Glass Inc., Vineland, NJ, USA) before entering the fermenter. Figure V-1 shows the experimental set-up for the anaerobic production phase. Similarly, a complete synthetic flue gas composed of 15% CO_2, 6% O_2, 79% N_2, 200 ppm NO_2, 300 ppm SO_2 and 50 ppm CO was studied.

Analyses

Cell growth was monitored by measuring the optical density (OD) at 600 nm. During succinate production, samples were centrifuged (10,000×g for 10 min at 4°C), and the supernatant analyzed for glucose and soluble products by high performance liquid chromatography (HPLC) as previously described (Eiteman and Chastain, 1997). The redox potential was monitored online using an ORP sensor and transmitter (Ingold, Mettler-Toledo, Wilmington, MA, USA).

Results

E. coli AFP111 accumulates succinic acid during an anaerobic non-growth phase after growing to a high cell density under aerobic conditions. During the anaerobic phase, CO_2 is incorporated into central metabolism through the carboxylation of phosphoenolpyruvate (PEP) by PPC. When the gas phase composition was 50% CO_2 and 50% N_2 (and in the absence of other gas components), several products were observed after about 16 h: 35 g/L succinate with a yield of 1.15 ± 0.11 mol/mol (mean $\pm$ standard deviation) at a specific rate of 1.90 ± 0.23 mmol/g·h, while the by-products pyruvate, acetate, and ethanol attained concentrations of 2-6 g/L (Figure V-2).

We first examined the effect of O_2 on succinate formation using 50% CO_2 in the gas phase by comparing three O_2 levels (0%, 3%, and 10%). Because O_2 is an electron acceptor which is expected to affect the culture redox potential (ORP) and the net rate of NAD(P)H formation (Peguin and Soucaille, 1996), we also monitored the ORP during the anaerobic phase. Compared to oxygen-free conditions, 3% O_2 in the gas stream increased the mean ORP from -291 mV to -224 mV. However, this more oxidizing environment did not significantly affect glucose consumption or succinate production (Table V-1). In addition to resulting in a further increase in the mean ORP to -188 mV, a gas phase composition of 10% O_2 reduced the specific succinate productivity by 41% and the succinate yield by 30%, although glucose consumption was unaffected. The final succinate concentration was only about 17 g/L after 16 h using 10% O_2 as a consequence of the reduced succinate formation rate. In addition, the pyruvate yield more than doubled (from 0.21 to 0.47 mol/mol) in comparing 0% and 10% O_2. Other by-products were unaffected by 10% O_2 (Table V-1).

We next examined succinate production in the presence of 0-200 ppm NO_2, a typical range for this compound in untreated industrial flue gas. Compared to the NO_2-free gas mixture, a concentration of only 50 ppm NO_2 led to a 28% decrease in the glucose consumption rate and a 44% decrease in succinate formation rate (Table V-2). Though the succinate generation rate was fairly constant during the anaerobic phase, the final concentration of succinate similarly achieved only 17 g/L in 16 h. Lower formation of succinate in the presence of 50 ppm NO_2 (yield of 0.86 mol/mol) was associated with elevated pyruvate (0.46 mol/mol). Interestingly, the presence of 50 ppm NO_2 decreased acetate formation under anaerobic conditions although ethanol formation was unaffected. Compared to 50 ppm, the use of 200 ppm NO_2 did not further impact succinate production, but this NO_2 concentration did increase the ORP slightly to -253 mV (Table V-2).

We completed an analogous study with SO_2 in the range of 0-300 ppm, and similarly found that the presence of SO_2 reduced glucose consumption and succinate formation under anaerobic production conditions (Table V-3). Although this range of SO_2 only increased the ORP modestly, 50 ppm SO_2 reduced the glucose consumption rate by about 32%, the succinate formation rate by over 47%, and the succinate yield by 22%. Similar to the results with NO_2, the pyruvate yield increased more than two-fold, while acetate that had been generated during aerobic growth was consumed. Supplying 300 ppm SO_2 had no additional detrimental effect on the succinate production process.

Trace amounts of CO exist in flue gases, and the chemical reactivity of this reductive gas is different from the other gaseous components studied. CO in the range of 0-500 ppm was supplied with 50% CO_2 during the anaerobic succinate production phase, and the results demonstrate that this low concentration did not impact succinate production rate or yield (Table V-4). Furthermore, this low concentration of CO did not significantly affect ORP.

We also examined succinate production by *E. coli* AFP111 using a synthetic flue gas (i.e., with all flue gas components). The concentration of each component was within the typical range for this compound in untreated industrial flue gas. The glucose consumption and succinate production rates were 1.12±0.23 mmol/g·h and 0.73±0.22 mmol/g·h, respectively, similar to results observed with 200 ppm NO_2 (Table V-2) or with 300 ppm SO_2 (Table V-3). The CO_2 concentration in the synthetic flue gas was lower than when each component was studied separately (15% vs. 50%), which may cause a CO_2 limitation (Lu et al., in review). The succinate yield of 0.65±0.06 mol/mol was lower using synthetic flue gas, while the pyruvate yield of 0.51±0.03 mol/mol, acetate yield of 0.03±0.04 mol/mol, and ethanol yield of 0.15±0.04 mol/mol were similar to results using 200 ppm NO_2 (Table V-2) or 300 ppm SO_2 (Table V-3).

Discussion

E. coli AFP111 requires CO_2 during the anaerobic formation of succinate from glucose via the enzyme PPC (Vemuri et al., 2002a). One readily available source of CO_2 is industrial flue gas. However, in addition to inert N_2 this gas also can contain several other components such as SO_2, the oxidizing gases O_2, NO_2, and the reductive gas CO.

The most prevalent component of flue gas which could affect succinate formation is O_2. A high concentration of O_2 will cause the system to remain aerobic, and cells will continue to grow. At steady-state, the O_2 supply will equal the O_2 uptake rate (OUR) according to:

$$OUR = k_La\ (c_{O2}^* - c_{O2})$$

where k_La is the liquid phase mass transfer coefficient of oxygen, and c_{O2}^* is the O_2 concentration at saturation. Maximally growing *E. coli* under fully aerobic conditions have an O_2 consumption rate of 10 mmol/g·h (Vemuri et al., 2006). In the current study, cell growth was terminated when the cell concentration reached about 9 g/L (Figure V-2). Therefore, the O_2

demand to maintain (full) respiration would have been about 90 mmol/L·h. After cell growth was terminated, a synthetic flue gas mixture containing as high as 10% O_2 was supplied. Simultaneously, the total flowrate was reduced to 0.5 L/min and the agitation reduced to 200 rpm, conditions which correspond to a k_La of 36 h^{-1}. At 37°C, the liquid solubility of a 10% O_2 is about 0.1 mmol/L (Battino, 1981). Therefore, the maximum O_2 transfer rate was about 3.6 mmol/L·h, or about 24 times lower than needed to sustain fully aerobic conditions. The presence of microaerobic conditions when supplying a gas containing 10% O_2 was confirmed by the observation of a modest increase in ORP from -291 mV to -188 mV between 0% O_2 and 10% O_2. Moreover, these microaerobic conditions resulted in less succinate and ethanol (the two reduced end products). The fraction of these products ([succinate+ethanol]/[succinate+ethanol+pyruvate+acetate]) was 0.80 mol/mol, 0.77 mol/mol and 0.63 mol/mol respectively for processes having 0%, 3% and 10% O_2 in the gas phase. Because of the mass transfer resistance to microbial utilization of this gas, a greater reduction in mass transfer should further reduce the aerobicity of the culture (for example, by reducing impeller agitation or gas flowrate). However, any reduction in oxygen transport must be balanced by the requirement for CO_2 transport, since this gas is biochemically necessary to support succinate production.

Succinate is formed from glucose through two pathways: the favored reductive arm of the tricarboxylic acid (TCA) cycle or the glyoxylate shunt (Vemuri et al., 2002a). Electrons captured as NADH are consumed through the reductive branch of the TCA cycle. Of course, O_2 can serve as the terminal electron acceptor, and thereby reduce the NADH available to drive this succinate-generating pathway. In the present study, reduced NADH availability in the presence of O_2 resulted in less PEP partitioning to the reductive arm of the TCA cycle and instead a higher

portion of PEP flowed to pyruvate. In general, pyruvate could either be oxidized to acetate via pyruvate oxidase, be oxidized to acetyl-CoA via pyruvate dehydrogenase, or merely accumulate. Although a more oxidizing environment could relieve NADH inhibition of pyruvate dehydrogenase (Garrett and Grisham, 1999), we observed no increase in flux to acetate or through the glyoxylate shunt and instead pyruvate accumulated.

The soluble gas NO_2 is very reactive chemically and forms nitrate upon dissolution. In addition to being a potent mutagen (Kosaka et al., 1986), this chemical affects enzymes in the reductive arm of the TCA cycle. Nitrate inhibits fumarase and fumarate reductase while reducing succinate accumulation in a (wild-type) *E. coli* mixed acid fermentation (Wimpenny and Cole, 1967). Moreover, nitrate induces the expression of nitrate reductase which can oxidize NADH using nitrate as the terminal electron acceptor (Wimpenny and Cole, 1967; Cole and Wimpenny, 1968; Unden and Bongaerts, 1997). In our study, cells responded to the presence of NO_2 in the same way cells responded to O_2 by partitioning PEP to pyruvate which accumulated significantly. This observation suggests an analogous effect in the presence of NO_2 which could result from reduced NADH availability. However, unlike our observations when O_2 was supplied, acetate formation was reduced in presence of NO_2.

The absence of acetate is in stark contrast to the elevated formation of acetate observed by others when nitrate was the sole electron acceptor during anoxic growth of wild-type *E. coli* (Prohl et al., 1998). Growth of a *pfl* mutant was indistinguishable from the wild-type under nitrate respiratory conditions (Kaiser and Sawers, 1994), and therefore the absence of pyruvate formate lyase in APF111 does not in itself explain the lack of acetate synthesis. Mutations in both the *pfl* and *ldh* genes, which together prevent anaerobic growth (Mat-Jan et al., 1989), may be necessary to block acetate in the presence of NO_2. The cause for the absence of acetate in our

study is unclear, but it may be related to acetyl-CoA synthetase, a high-affinity enzyme encoded by the *acs* gene (Kumari et al., 1995) that allows cells to assimilate acetate. Interestingly, this enzyme's transcription promoter *acs*P1 has extensive overlap of regulatory elements with the *nrfA* operon that encodes nitrate reductase and which are both repressed by the nucleoid associated factors Fis and IHF (Browning et al., 2002). One possibility is that the presence of nitrate, which relieves repression through activators NarL and NarP (Browning et al., 2006), under the specific conditions of our experiment also relieves repression of *acs*, and thereby induces acetyl CoA synthetase expression. Such a result would rely on sufficient expression of this enzyme in the non-growth phase when the cells were first exposed to NO_2. The effect does not appear to be related solely to ORP since acetate was observed in the presence of O_2 despite the more oxidizing environment.

SO_2 is soluble gas and dissociates to bisulfite and sulfite in aqueous solution. Bisulfite serves as a potent mutagen (Mukai, et al., 1970; Singhal, 1971), an effect which may have an insignificant influence on succinate production by non-growing cells. The bisulfite or sulfite ion also inhibits PPC activity in plant tissue by nonspecific binding (Osmond and Avadhani, 1970; Mukerji and Yang, 1974), with 5-10 mM Na_2SO_3 causing 21-39% inhibition (Mukerji and Yang, 1974). Although no *E. coli* reports exist, *E. coli* PPC has similar characteristics with the plant enzyme (Matsumura et al., 2002). One possible explanation for the small reduction in succinate formation in non-growing cells is SO_2 inhibition of PPC which would reduce the carbon flux through the reductive branch of the TCA cycle. In addition, sulfite could oxidize NADPH by *E. coli* sulfite reductase (Siegel et al., 1974; Ostrowski et al., 1989), which would serve to reduce the NAD(P)H pool and lead to an effect similar to NO_2.

CO has broad biological effects due to its ability to bind heme proteins (Piantadosi, 2002), including DNA replication (Cairns and Denhardt, 1968). However, CO affects *E. coli* growing under anaerobic condition less than cells growing aerobically (Weigel and Englund, 1975). Since no heme enzymes/proteins are involved in glucose utilization and the succinate production pathways, CO would not be expected to have deleterious effects on anaerobic succinate production by non-growing cells. Furthermore, the low concentration of this insoluble gas did not change the ORP significantly, and would thus likely not alter the rate of NADH oxidation.

When all gas components were present, the glucose consumption and succinate formation rates were comparable to results using 300 ppm SO_2 or 200 ppm NO_2 in 50% CO_2 gas, although the succinate yield was lower. This result indicates that the negative effects of the oxidizing gas components are neither accumulative nor synergistic to non-growing cells. Considering the relatively low CO_2 level (15% v/v) in this synthetic flue gas, *E.coli* AFP111 has potential for utilization of actual flue gas from power plant stack exhaust. Mixing flue gas with a gas having a higher CO_2 concentration may lead to higher succinate production, as a result of higher CO_2 concentration and lower concentrations of the other undesirable gas components.

This study demonstrates that flue gas components impact succinate production by *E. coli* AFP111 in varying degrees. Those gases which increase the ORP led to the most significant reduction in succinate formation. In general, *E. coli* modified the partitioning of PEP to pyruvate in response to O_2, NO_2, and SO_2. In more oxidizing conditions, the PEP flux to pyruvate was elevated. Importantly, although growing *E. coli* is very sensitive to the mutagenic gases NO_2 and SO_2, these components have a more limited impact on succinate formation under non-growth conditions. Therefore, although maximal succinate production should exclude NO_2, SO_2, and O_2

from a CO_2-rich gas stream, these gas components can nevertheless be acceptable for succinate production and CO_2 utilization.

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Table V-1. Fermentation parameters observed for different O_2 conditions during the anaerobic succinate production phase.

O_2 (%)	q_G (mmol/gh)	q_S (mmol/gh)	Y_S (mol/mol)	Y_P (mol/mol)	Y_A (mol/mol)	Y_E (mol/mol)	ORP (mV)
0	1.65±0.05[a]	1.90±0.23[a]	1.15±0.11[a]	0.21±0.16[a]	0.11±0.05[a]	0.15±0.04[a]	-291
3	1.54±0.37[a]	1.69±0.78[a]	1.05±0.31[a]	0.26±0.30[a]	0.09±0.02[a]	0.13±0.01[a]	-224
10	1.40±0.38[a]	1.13±0.30[b]	0.81±0.01[b]	0.47±0.21[b]	0.11±0.00[a]	0.19±0.02[b]	-188

Data are shown as mean ± standard deviation

[a] Different letters were significantly different at the 90% confidence level.

Table V-2. Fermentation parameters observed for different NO_2 conditions during the anaerobic succinate production phase.

NO_2 (ppm)	q_G (mmol/gh)	q_S (mmol/gh)	Y_S (mol/mol)	Y_P (mol/mol)	Y_A (mol/mol)	Y_E (mol/mol)	ORP (mV)
0	1.65±0.05[a]	1.90±0.23[a]	1.15±0.11[a]	0.21±0.16[a]	0.11±0.05[a]	0.15±0.04[a]	-291
50	1.19±0.20[b]	1.07±0.22[b]	0.90±0.10[b]	0.46±0.17[b]	0.02±0.04[b]	0.13±0.05[a]	-269
200	1.19±0.35[b]	1.11±0.45[b]	0.91±0.10[b]	0.51±0.17[b]	0.01±0.05[b]	0.15±0.07[a]	-253

Data are shown as mean ± standard deviation

[a] Different letters were significantly different at the 90% confidence level.

Table V-3. Fermentation parameters observed for different SO_2 conditions during the anaerobic succinate production phase.

SO_2 (ppm)	q_G (mmol/gh)	q_S (mmol/gh)	Y_S (mol/mol)	Y_P (mol/mol)	Y_A (mol/mol)	Y_E (mol/mol)	ORP (mV)
0	1.65±0.05[a]	1.90±0.23[a]	1.15±0.11[a]	0.21±0.16[a]	0.11±0.05[a]	0.15±0.04[a]	-291
50	1.12±0.20[b]	1.01±0.30[b]	0.90±0.22[b]	0.45±0.14[b]	-0.02±0.05[b]	0.12±0.11[ab]	-273
300	0.94±0.22[b]	0.73±0.01[b]	0.80±0.18[b]	0.52±0.04[b]	-0.02±0.03[b]	0.04±0.06[b]	-265

Data are shown as mean ± standard deviation

[a] Different letters were significantly different at the 90% confidence level.

Table V-4. Fermentation parameters observed for different CO conditions during the anaerobic succinate production phase.

CO (ppm)	q_G (mmol/gh)	q_S (mmol/gh)	Y_S (mol/mol)	Y_P (mol/mol)	Y_A (mol/mol)	Y_E (mol/mol)	ORP (mV)
0	1.65±0.05[a]	1.90±0.23[a]	1.15±0.11[a]	0.21±0.16[a]	0.11±0.05[a]	0.15±0.04[a]	-291
100	1.55±0.36[a]	1.73±0.41[a]	1.12±0.00[a]	0.22±0.07[a]	0.09±0.02[a]	0.17±0.02[a]	-289
500	1.51±0.16[a]	1.74±0.25[a]	1.15±0.04[a]	0.16±0.00[a]	0.08±0.00[a]	0.14±0.02[a]	-300

Data are shown as mean ± standard deviation

[a] Different letters were significantly different at the 90% confidence level.

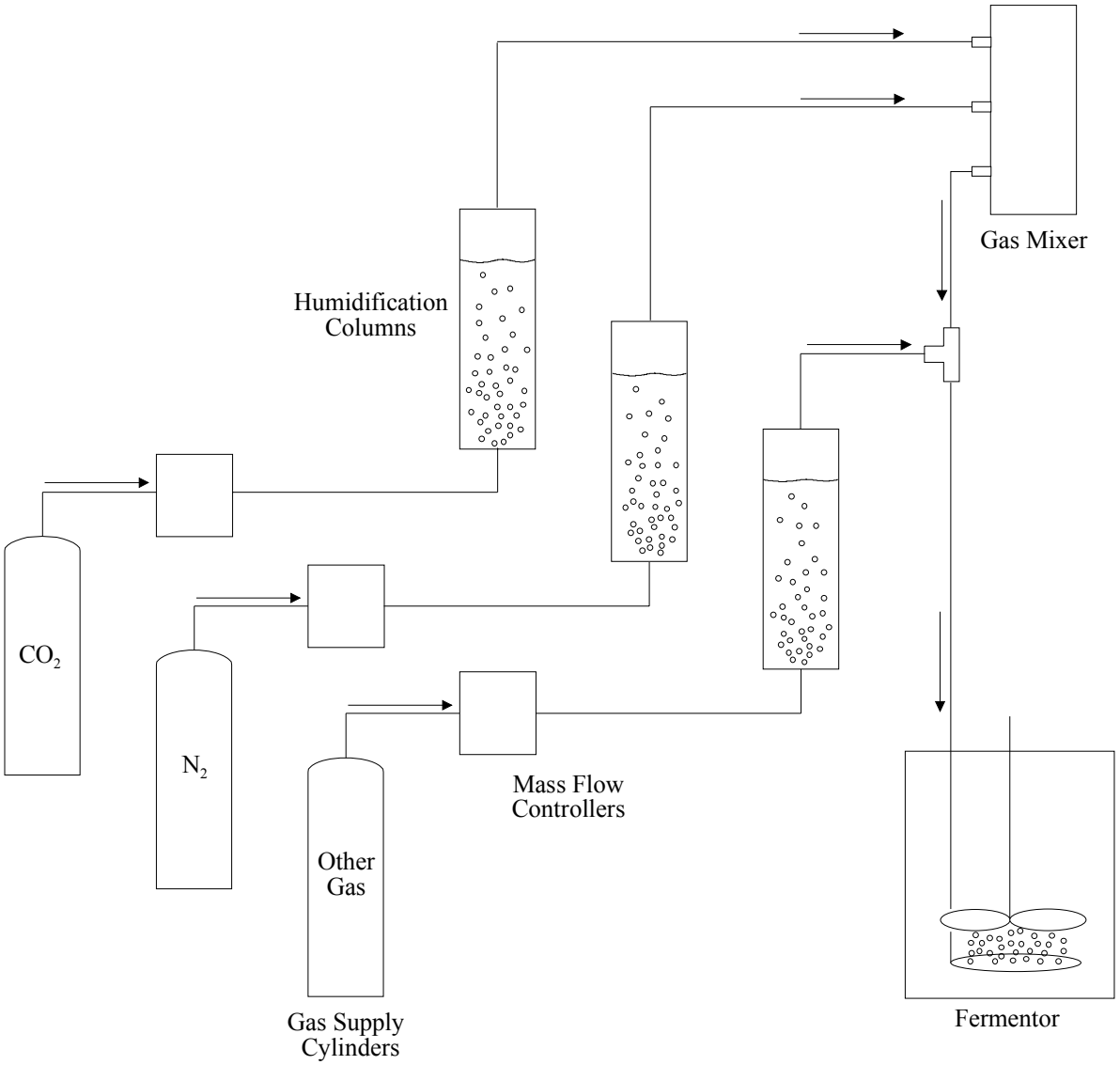

Figure V-1. Gas supply system for study of anaerobic succinate production.

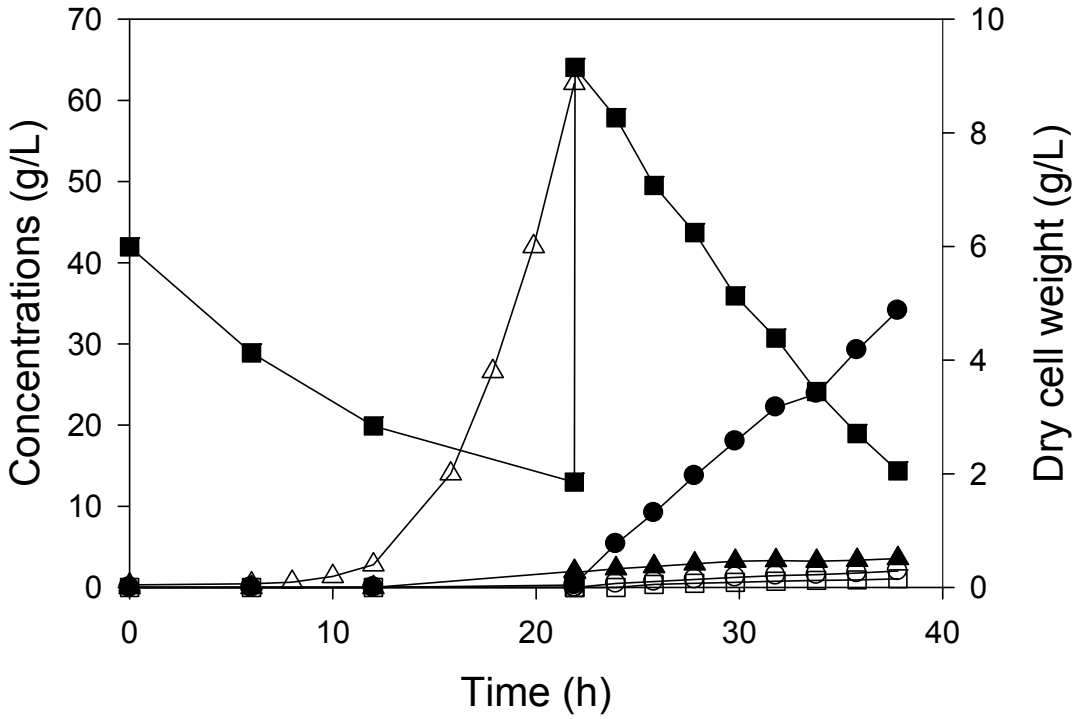

Figure V-2. Dual-phase fermentation by *E. coli* AFP111 on glucose-defined medium: dry cell weight (△), glucose (■), succinate (●), acetate (○), pyruvate (▲), and ethanol (□). The switch time from aerobic to anaerobic phase occurred at approximately 22 h, when glucose was added to increase its concentration to about 65 g/L, and the gas phase was switched to 50% CO_2 and 50% N_2.

CHAPTER VI

SUMMARY

Conclusions of this study

The fundamental aim of this research was to understand aspects of the regulation of microbial central metabolism as a result of environmental conditions. In the first part of this research, the effect of pH and base counterion on succinate formation was elucidated. Both of these environmental variables significantly affected succinate production indicating their importance in optimization of fermentation conditions. A pH slightly lower than neutral pH favoured succinate generation. The base $Ca(OH)_2$ instead of NaOH or KOH could be used to neutralize pH due to precipitation caused by Ca^{2+}. Osmotic stress may play a role in the decreased succinate formation when NaOH or KOH was a neutralizing agent.

The second part of this research demonstrated that higher CO_2 favoured succinate formation but this improvement did not occur over the entire range of CO_2 concentration. Metabolic flux analysis (MFA) coupled with ^{13}C-labeled tracing was used to understand the carbon flux partitioning in *E.coli* AFP111 in response to varied CO_2 concentrations. In particular, CO_2 affected the fraction of flux into the pentose phosphate pathway and the fraction of flux through anaplerotic carboxylation at the PEP node. In the future, ^{13}C-labeled MFA could be used to understand the consequence of perturbations by gene reconstruction and other environmental factors.

Flue gas components impact succinate production in varying degrees. Those gases which affect the culture redox potential (ORP) led to the most significant reduction in succinate

formation. ORP may be used to optimize the formation of a reductive product of interest like succinate. Although maximal succinate production should exclude NO_2, SO_2, and O_2 from a CO_2-rich gas stream, these gas components can nevertheless be acceptable for succinate formation, and *E. coli* could directly use synthetic flue gas as a source of CO_2 to generate succinate though the succinate yield was lowered.

This body of research has laid an example for the development of processes using *E. coli* AFP111 or other similar CO_2-fixing microorganisms. The environmental variables of pH, base counterion, and ORP may be used to optimize the formation of a product of interest. Furthermore, the understanding of central metabolism that is directed impacted by environmental pertubations in succinate production could provide valuable information for improvements of other microbial fermentation products.

Future study in succinate production

High osmotic stress is detrimental to succinate production; adding some osmotic protectants may keep productivity high during the course of succinate production. By further understanding the inhibition mechanism of high osmotic stress and ion strength on succinate production, additional genetic modifications might be able to increase tolerance of strain to high ion strength.

At intermediate CO_2 concentrations, succinate formation was limited by the rate of bicarbonate ion formation; overexpressing carbonic anhydrase may achieve high succinate production even with low concentration of CO_2 in the gas stream. We were unsuccessful in overexpressing the *yadF* gene which encodes *E. coli* carbonic anhydrase (CA). Understanding why overexpression was unsuccessful and constructing an efficient CA-overexpressing strain will lead to high succinate production.

The use of ^{13}C-labeling serves as a powerful tool to understand metabolism. In the current study, we measured the distribution of labeled succinate and minimized the error between observed results and the results calculated by the metabolic model. If more labeled end-products (e.g. pyruvate and acetate) can be detected simultaneously and included into the model, more accurate estimates of fluxes might be obtained, or a more detailed biochemical model could be used.

Flue gas components NO_2 or SO_2 had slight negative effect on succinate production serving as a terminal electron acceptor; however, they had different effect on product distribution compared to O_2. The acetate yield was much lower in the presence of NO_2 or SO_2. We speculated that NO_2 or SO_2 activated the acetyl-CoA synthetase (ACS) which assimilates acetate. This hypothesis needs further investigation.

Acetate is a by-product in the succinate production. Reducing acetate may redirect carbon flux toward more favorable succinate pathways. The secreted acetate could be utilized as a carbon source in the second succinate production phase. The overexpression of ACS in *Escherichia coli* may enhance the assimilation of acetate during the second phase and direct carbon to succinate formation pathway.