PRODUCTION OF PYRUVATE AND LACTATE BY METABOLICALLY ENGINEERING *ESCHERICHIA COLI*

by

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(Under the Direction of Mark A. Eiteman)

ABSTRACT

Pyruvic acid and lactic acid have a wide range of applications in the food, chemical, and pharmaceutical industries. The primary goal of this research is to improve pyruvate and lactate production through bacterial strain engineering and fermentation process development.

Lactate production was studied using *E. coli* strain YYC202 (*aceEF pfl poxB pps*), which generated 90 g/L lactate in a two-phase fermentation (aerobic growth phase followed by anaerobic phase for lactate production). This process also generated over 7 g/L succinate. $Ca(OH)_2$ was found to be superior to NaOH for pH control. Strain ALS961 (YYC202 *ppc*) prevented succinate accumulation but growth was very poor. Strain ALS974 (YYC202 *frdABCD*) reduced succinate formation by 70% to less than 3 g/L. ^{13}C-NMR using uniformly labeled acetate demonstrated that succinate formation by ALS974 was biochemically derived from acetate in the medium. The two-phase process with ALS974 achieved 138 g/L lactate (1.55 M, 97% of carbon products) with a yield of 0.99 g/g and productivity of 6.3 g/L·h during the anaerobic phase. By using the dissolved oxygen (DO) during the initial cell-growth phase to

monitor residual acetate, and timing the switch to the anaerobic production phase with acetate depletion, succinate production was reduced to less than 1 g/L, and ethanol production was eliminated. Furthermore, by using a cell-recycle fermentation with ultrafiltration, the anaerobic lactate production phase was prolonged from 22 h to 34 h, and an overall lactate productivity of 4.2 g/L·h was achieved. The productivity of this process was nearly 20% greater than the productivity of the fed-batch process.

Pyruvate was studied using *E. coli* strain ALS929 (*pflB aceEF poxB pps ldhA*). First, a series of steady-state (chemostat) experiments were conducted to evaluate pyruvate formation under four different nutrient-limited conditions: glucose, acetate, nitrogen (ammonia), and phosphorus (phosphate). The greatest specific glucose consumption rate of 1.60 g/g·h and specific pyruvate formation rate of 1.10 g/g·h were found under conditions of acetate-limited growth. The specific glucose consumption rate and therefore pyruvate productivity were further increased to 2.67 g/g·h and 2.01 g/g·h respectively by introducing ATP synthase knockout (*atpFH*), an *arcA* knockout and introducing heterologous NADH oxidase. A fed-batch process at a constant specific growth rate of 0.15 h^{-1} using ALS1059 (*pflB aceEF poxB pps ldhA atpFH arcA*) resulted in the highest pyruvate yield and productivity. In a defined medium with 5mM betaine, a final pyruvate concentration of 89.7 g/L, a yield of 0.68 g/g and a productivity of 2.04 g/L·h were achieved.

INDEX WORDS: Pyruvate, Lactate, *Escherichia coli*, Fumarate reductase, Glyoxylate shunt, Succinate, Ultrafiltration, Dissolved oxygen, Glycolysis, $F_1F_0(H^+)$-ATP synthase, NADH oxidase, *arcA*, Betaine, Chemostat, Fed-batch

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Dedicated to my family

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CHAPTER I

INTRODUCTION

Pyruvic acid and lactic acid are two of the most widely used organic acids in the food, chemical, and pharmaceutical industries (Li et al. 2001, Wasewar et al. 2004, Narayanan et al. 2004). Pyruvic acid, also known as 2-oxopropanoic acid, α-ketopropionic acid or acetylformic acid, is one of the most important α-oxocarboxylic acids. It plays a key role in central metabolism of living organisms. Lactic acid, also known as 2-hydroxy propionic acid, is the most widely occurring carboxylic acid in nature (Narayanan et al. 2004). Pyruvic acid and lactic acid are both three-carbon organic acids and have similar structures (Fig. I-1). At one terminal carbon atom (position 1) on either molecule is an acid or carboxyl group; at the other terminal carbon atom (position 3) is a methyl group. The only difference between the two compounds is that the central carbon atom of pyruvic acid is a keto group while the central carbon atom of lactic acid is a hydroxyl group. Because of this similarity, pyruvic acid and lactic acid can be interconverted by an oxidation (lactate to pyruvate) or reduction (pyruvate to lactate). In microorganisms, this redox reaction is catalyzed by the enzyme lactate dehydrogenase (LDH). A strategy for microbial production of pyruvic acid and lactic acid could have some similarities. If pyruvic acid is generated significantly in a microbial process, then it might be possible that lactic acid could also be generated through a similar process having process or genetic changes.

High costs of chemical synthesis have prevented the extensive use of pyruvate and lactate for many applications (Li et al. 2001). Fermentation processes using microorganisms have been developed to produce high concentrations of pyruvate and lactate. Metabolic engineering and traditional strategies for mutation and selection have also been applied to microorganisms to enhance their ability to produce pyruvate and lactate.

One of the goals of this research is to develop metabolically engineered strains and microbial fermentation processes to produce pyruvate and lactate in high yield and productivity. Although strategies for pyruvate and lactate production have similarities, strains and fermentation conditions required for pyruvate and lactate generation have key differences and must be investigated separately. In this dissertation, Chapter III and IV focus on lactate production. Genetic modifications prevented strains from forming by-products and increased the yield of lactate from glucose. A two-phase process was developed and optimized to prolong lactate production at high productivity. Chapter V focuses on pyruvate production. Pyruvate productivity and yield were improved by genetic modifications and a substrate-limited fed-batch process. These studies provide insights into pathways used to accumulate pyruvate or lactate, and to how microbial metabolism responds to genetic perturbations and environmental conditions. This information increases our understanding of gene and enzyme regulation in central metabolism may provide guidance for other products of central metabolism and their derivatives.

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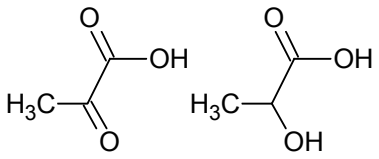

Pyruvic acid Lactic acid

Figure I-1: Structure of pyruvic acid and lactic acid

CHAPTER II

LITERATURE REVIEW

Application of pyruvic acid

In addition to playing a key role in central metabolism of living organisms, pyruvic acid (pyruvate) is a substrate for the enzymatic production of L-tryptophan, L-tyrosine, D-/L-alanine and L-dihydroxyphenylalanine (L-DOPA) (Li et al. 2001). Pyruvate has also been used in the production of crop protection agents, polymers, cosmetics and food additives (Li et al. 2001). Calcium pyruvate finds use in the food industry as a fat burner because it accelerates the metabolism of fatty acids in the human body (Roufs 1996). Clinical studies have demonstrated that pyruvate promotes fat and weight loss significantly (Stanko et al. 1992a, Stanko et al. 1992b), improves exercise endurance capacity (Stanko et al. 1990), and reduces cholesterol levels effectively (Stanko et al. 1994). Recently an open-label study concluded that peeling with pyruvate is an effective, safe and well-tolerated procedure in the treatment of acne (Cotellessa 2004). The commercial demand for pyruvate has greatly increased as this chemical is widely used in drug, agrochemical, chemical and food industries.

Application of lactic acid

Lactic acid (lactate) and its derivatives have a wide range of applications in the food,

pharmaceutical, leather and textile industries (Vickroy 1985, Hofvendahl and Hahn-Hagerdal 2000). Recently, polylactic acid (PLA) has been developed as a renewable, biodegradable and environmentally friendly polymer (Wehrenberg 1981, Kharas et al. 1994). An advantage of a biological process over a chemical process for the production of lactate is the prospect of generating optically pure lactate (Wehrenberg 1981, Blomqvist 2001, Tsuji 2002), which is an important characteristic for many of its end uses (Blomqvist 2001, Jong et al. 2001, Tsuji 2002).

General methods of pyruvic acid production

Chemical method

The first method for producing pyruvate at an industrial scale was a chemical process. This process involved dehydration and decarboxylation of tartaric acid in the presence of saturating concentrations of potassium hydrogen sulfates at 220°C (Howard and Fraser 1932). The final product was obtained by vacuum distillation. Although this method was simple, it was not cost-effective. The total cost was about $8,000-9,000 / ton based on the market price of raw materials. This high cost prevented pyruvic acid from being widely used (Li et al. 2001).

Biotechnological methods

Compared to a chemical approach for pyruvate production, biotechnological methods, including enzymatic, resting cell and fermentative processes, offer a promising alternative for cost-efficient process development.

Enzymatic processes

Enzymatic processes for producing pyruvate involve a single enzymatic step catalyzed by raw or purified enzyme (or whole cells) using lactate as substrate. *Acetobacter sp.* has been used to produce about 20 g/L pyruvate by oxidizing D-lactate at a high conversion rate (Cooper 1989). However, this process is difficult to commercialize due to the higher cost of D-lactate compared to L-lactate. Another enzymatic process for producing pyruvate was by oxidizing L-lactate to pyruvate by glycolate oxidase from *Hansenula polymorpha* (Anoton et al. 1995). But pyruvate can be further oxidized to acetate or alanine by hydrogen peroxide which is a byproduct of the enzymatic process catalyzed by glycolate oxidase. Catalase from *Pichia pastoris* has been used to remove hydrogen peroxide and prevent pyruvate from oxidation (Anton et al. 1996, Eisenberg et al. 1997).

Resting cell processes

Resting cell processes involve a series of enzymatic steps in non-growing microbial cells using a substrate such as glucose. *Acinetobacter sp.* and *Debaryomyces coudertii* have been used to produce pyruvate by resting cell method (Izumi et al. 1982, Moriguchi et al. 1984). Although the culture times of resting cell processes were shorter than direct fermentative methods, cells still require cultivation, separation from growth medium, and washing before the production phase. Some resting cell methods do not require that the cells be separated from the growth medium. Cells can be prevented from growing by limiting nitrogen source (Behrens and Fiedler 1979) or by altering pH (Besnainou et al. 1989, Besnainou et al. 1990). Problems of contamination and strain stability still prevent prolonged resting cell processes.

Fermentation processes

Direct fermentation processes have also been used to produce pyruvate. Many recombinant microorganisms including yeast and *Escherichia coli* have been developed to accumulate pyruvate from different carbon sources. These strains will be discussed in detail in following sections.

General methods of lactic acid production

Chemical method

The commercial chemical synthesis of lactate is based on a series of chemical reactions involving lactonitrile (Narayanan et al. 2004). In the first step, lactonitrile is produced by reaction of hydrogen cyanide and acetaldehyde in the presence of a base. This reaction occurs in liquid phase and requires high pressure. Lactonitrile is recovered and purified by distillation and then hydrolyzed to lactic acid and the corresponding ammonium salt either by concentrated HCl or by H_2SO_4. For recovery and purify, this crude lactic acid is esterifed by methanol to produce methyl lactate. Methyl lactate is then removed and purified by distillation. Finally, lactic acid is generated by hydrolyzation of methyl lactate by water under acid catalyst. This process is shown in Fig II-1.

Other chemical methods may be also used for lactate production, including base catalyzed degradation of sugars, oxidation of propylene glycol, reaction of acetaldehyde, carbon monoxide and water at elevated temperature and pressures, hydrolysis of chloropropionic acid, carbohydrate fermentation, and nitric acid oxidation of propylene (Narayanan et al. 2004).

The chemical synthesis method produces a racemic mixture of lactate, which is undesirable for industrial uses. The need for optically pure isomers favors the production of lactate by microbiological production rather than chemical processes.

Microbiological production

Microbiological production of lactate has the advantages that an optically pure product can be obtained by choosing a microorganism producing only one of the isomers. Also, microorganisms are often able to use cheap raw materials, such as starch, xylose, and cheese whey produced by the dairy industry.

Although many microorganisms produce lactate, lactic acid bacteria (LAB) have been particularly selected for commercial production of lactate (Litchfield 1996). Most lactic acid bacteria are Gram-positive, including *Carnobacterium, Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Hofvendahl and Hahn-Hagerdal 2000). Generally, LABs are facultatively anaerobic or microaerophilic, nonmotile, and nonspore forming. Their competitive advantages over other microorganisms are their high pH and lactate tolerance. Strain selection for lactate production is normally based on the desired lactate enantiomer, substrate, temperature, pH and lactic acid tolerances, and yields and lactate productivity (Litchfield 1996). L-lactate has been produced by *Lactobacillus helveticus*, *Lactobacillus amylophilus*, and *Lactobacillus delbruekii*. Highly pure D-lactate has been obtained by using *Lactobacillus bulgaricus*. The ability to utilize various carbohydrates is another important consideration in selection of strain.

For example, *L. delbreuckii* subspecies *delbreuckii* is able to ferment sucrose, *L. delbreuckii* subspecies *bulgaricus* is able to use lactose, while *L. helveticus* is able to use both lactose and galactose. *L. amylophilus* and *Lactobacillus amylovirus* are able to ferment starch. *Lactobacillus lactis* can ferment glucose, sucrose and galactose. *Lactobacillus pentosus* has been used to ferment sulfite waste liquor (Hofvendahl and Hahn-Hagerdal 2000).

Although lactic acid bacteria have many advantages, they also have undesirable traits, such as very low growth rates, difficulty in achieving high density, a requirement for complex nutrients which complicates product recovery, and incomplete or negligible pentose utilization (Narayanan et al. 2004). Due to these disadvantages of lactic acid bacteria, other microorganisms have been proposed as alternative producers of lactate, including strains of *Bacillus*, *Rhizopus*, *Escherichia*, *Saccharomyces*, and *Kluyveromyces* (Lockwood et al. 1936, Litchfield 1996, Hofvendahl and Hahn-Hagerdal 2000, Bianchi et al. 2001, Ishida et al. 2005).

Rhizopus oryzae converts glucose to large amounts of L-lactate aerobically (Lockwood et al. 1936). This microorganism grows and produces lactate in a chemically defined medium containing inorganic nitrogen sources, such as ammonium salts or nitrates and mineral salts, without supplements of amino acids and vitamins required by the lactic acid bacteria (Lockwood et al. 1936, Litchfield 1996). *R. oryzae* utilizes both complex carbohydrates and pentose sugars for lactate production. Many studies have been carried on to increase the yield and productivity of lactate by *R. oryzae*, especially on decreasing accumulation of fumaric acid, the main by-product (Wang et al. 2005). A final lactate concentration of 80 g/L, a productivity of 1.34 g/L·h, a mass yield of 0.75, and a low fumaric acid concentration of 0.22 g/L were obtained

using *R. oryzae* (CGMCC 3.1263) in the optimal conditions (Wang et al. 2005).

Metabolic engineering and traditional strategies for mutation and selection have been applied to *Escherichia*, *Saccharomyces*, and *Kluyveromyces* to enhance their ability of producing lactate (Bianchi et al. 2001, Ishida et al. 2005). These recombinant strains will be discussed in detail in following sections.

Biochemical pathways involved in pyruvic acid and lactic acid production

Pyruvate is a key metabolite synthesized via glycolysis, and it occupies an important position in the central pathways. The biochemical pathways directly involved in the formation and assimilation of pyruvate are shown in Fig. II-2. During growth of *E. coli* on glucose, pyruvate is generated mainly from PEP by pyruvate kinase and by the phosphotransferase system (PTS) of glucose uptake. Pyruvate is primarily converted into acetyl CoA by pyruvate dehydrogenase (PDH) aerobically or pyruvate formate lyase (PFL) anaerobically. Pyruvate can also be converted into acetate, malate and PEP by pyruvate oxidase (POX), malic enzyme and PEP synthase (PPS), respectively. In addition to these directly related pathways, many adjacent pathways and regulatory proteins are likely to impact the accumulation of pyruvate, such as PEP carboxylase (PPC), acetyl CoA synthetase, phosphotransacetylase, acetate kinase, and the glyoxylate shunt (Fig. II-2).

Lactate is synthesized from pyruvate in a single reaction step by the enzyme lactate dehydrogenase (LDH) (Fig. II-2). Other pathways related to production of lactate are also shown in Fig. 2.

A more detailed description for each enzyme will be provided below.

Biochemical pathways directly impacting pyruvate and lactate formation

PEP:sugars phosphotransferase system (PTS) and pyruvate kinase (PYK)

PEP:sugars phosphotransferase system (PTS) is the major pathway in which a large number of sugars are phosphorylated and transported into the cell (Postma et al. 1993, Ginsburg and Peterkofsky 2002). This system is also involved in the regulation of a number of other metabolic pathways (Postma et al. 1993). Translocation of carbohydrates is coupled to their phosphorylation with the energy provided by the glycolytic intermediate PEP. Pyruvate is the product of the reaction. The overall reaction of PTS can be described by the following equation for numerous microorganisms and carbohydrates:

$$\text{PEP} + \text{Carbohydrate}_{(ext)} \xrightarrow{PTS} \text{Pyruvate} + \text{Carbohydrate-P}_{(int)}$$

The PTS is composed of two soluble, cytoplasmic, sugar-nonspecific proteins, enzyme I and HPr, and sugar specific protein(s), enzyme II (Postma et al. 1993, Dimitrova et al. 2003). Enzyme I and HPr participate in the phosphorylation of all PTS carbohydrates in a given microorganism and thus have been called the general PTS proteins. Enzymes II are carbohydrate specific and may consist of a single membrane-bound protein composed of three domains (A, B, and C) or of two or more proteins, at least one of which is membrane bound (e.g., B and C) and one of which is soluble (Postma et al. 1993). There is a phosphotransfer cascade in PTS. Enzyme I is a ~64 kDa protein, which can be phosphorylated on a histidine residue (His189) by PEP in the presence of Mg^{2+} (Ginsburg and Peterkofsky 2002). HPr (~ 9 kDa) is able to accept a

phosphoryl group from phosphorylated enzyme I on its His15. Then the phosphorylated HPr is capable of phosphorylating a variety of sugar-specific enzyme IIs. In the case of glucose, a soluble, cytoplasmic protein, IIA^{Glc} accepts the phosphoryl group on His90. Then, a membrane-associated protein, enzyme IIB-IIC, transfers phosphoryl group from phosphorylated IIA^{Glc} to glucose and transports it into cells (Ginsburg and Peterkofsky 2002). There are several proposed feedback associated regulation mechanisms for PTS, including the membrane potential, energy-dependent efflux of substrates, competition for HPr-P, and regulation by intracellular phosphor-compounds (Postma et al. 1993).

After glucose is converted into glucose-6-phosphate and transported into the cell, it enters glycolysis, the stepwise degradation of glucose into pyruvate. Glycolysis plays important roles in providing precursor compounds, energy and reducing power for other pathways. There are 9 reactions and 9 enzymes involved in glycolysis starting from glucose-6-phosphate. The final enzyme involved in glycolysis is pyruvate kinase (PYK). Pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase; EC 2.7.1.40) catalyzes the conversion of PEP and ADP to pyruvate and ATP (Mattevi et al. 1995):

$$\text{PEP} + \text{Mg·ADP} + \text{H}^+ \xrightarrow{K^+} \text{Mg·ATP} + \text{pyruvate}$$

This reaction is irreversible under physiological conditions.

There are two pyruvate kinase isoenzymes expressed in *E. coli*, PykF (type I) and PykA (type II), encoded by the *pykF* and *pykA* genes, respectively (Ponce et al. 1995). Both pyruvate kinase isoenzymes have an active role in pyruvate biosynthesis when glucose is the only carbon source, but the PykF isoenzyme contributes to a greater extent (Ponce et al. 1995). PYK is the

major regulatory enzyme of glycolysis. The catalytic activity of PYK is controlled by the substrate PEP and by one or more allosteric effectors (Mattevi et al. 1995). PykF is allosterically activated by fructose-1,6-bisphosphate (FBP) and inhibited by ATP. PykA is activated by AMP and monophosphorylated sugars such as glucose-6-phosphate and ribose-6-phosphate (Mattevi et al. 1995).

Pyruvate dehydrogenase (PDH)

The conversion of pyruvate to acetyl-CoA is the connecting link between glycolysis and the TCA cycle:

$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \longrightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

This reaction is catalyzed by a multi-enzyme complex called the pyruvate dehydrogenase complex, a sixty subunit complex involving three enzymes: 24 pyruvate dehydrogenase (E1, EC 1.2.4.1), 24 dihydrolipoyl transacetylase (E2, EC 2.3.1.12), and 12 dihydrolipoyl dehydrogenase (E3, EC 1.8.1.4) (Garrett and Grisham 1998). These enzymes are associated noncovalently. The first step of the reaction is catalyzed by pyruvate dehydrogenase (E1): pyruvate releases CO_2 and binds to thiamine pyrophosphate (TPP), forming hydroxyethyl TPP (HETPP). The pyruvate dehydrogenase (E1) is a heterotetramer ($\alpha 2\beta 2$) with a total molecular weight of 154 kDa, and is encoded by the gene *aceE*. The second step is catalyzed by dihydrolipoyl transacetylase (E2): the hydroxyethyl group is transferred to lipoic acid and oxidized to form acetyl dihydrolipoamide. Subsequently, the acetyl group is transferred to CoA. E2 is encoded by the *aceF* gene. Finally, the reduced dihydrolipoamide is reoxidized by NAD^+ to active lipoic acid and NADH. This step

is catalyzed by dihydrolipoyl dehydrogenase (E3) encoded by *lpd*. Several coenzymes are also involved in the PDH reaction, including thiamine pyrophosphate, coenzyme A, lipoic acid, NAD^+, and FAD.

The complex is highly regulated by its products acetyl-CoA and NADH (Garrett and Grisham 1998). High levels of either product allosterically inhibit the activity of PDH. PDH is also sensitive to the energy status of the cell. The activity of PDH is activated by AMP and inhibited by GTP (Garrett and Grisham 1998).

Pyruvate-formate lyase (PFL)

Pyruvate-formate lyase (PFL, EC 2.3.1.54) encoded by *pflB* catalyzes the nonoxidative conversion of pyruvate and coenzyme A (CoA) to acetyl-CoA and formate under anaerobic conditions (Lehtio et al. 2002):

$$\text{Pyruvate} + \text{CoA} \longrightarrow \text{Acetyl-CoA} + \text{Formate}$$

This reaction plays an important role in the anaerobic carbon metabolism in *E. coli* and other prokaryotes (Guo and Himo 2004). PFL is a homodimer of 85 kDa subunits. It displays half-of-sites activity so that only one of the subunits is active at a time (Lehtio et al. 2002). PFL is present in its inactive form under aerobic conditions, but in anaerobic conditions is activated by an activating enzyme (EC 1.97.1.4) containing an iron-sulfur cluster, which introduces a free radical into the inactive form of PFL. Studies have shown that there are approximately 10-fold higher levels of PFL protein present in cells grown anaerobically with glucose compared with in cells grown aerobically with glucose (Sawers and Bock 1988). These induced levels could be

elevated a further 1.5- to 2.0-fold by the addition of pyruvate to the growth medium (Sawers and Bock 1988).

Pyruvate oxidase (PoxB)

Pyruvate oxidase (PoxB, EC 1.2.2.2) is a peripheral membrane flavoprotein that catalyzes the decarboxylation of pyruvate to acetate and CO_2, coupled to the reduction of flavin adenine dinucleotide (FAD) (Gennis and Hager, 1976):

$$\text{Pyruvate} + H_2O + \text{FAD} \longrightarrow \text{Acetate} + CO_2 + FADH_2$$

Although PoxB was regarded as a non-essential and potentially wasteful enzyme of uncertain metabolic function, recent studies suggest that PoxB makes a significant contribution to the aerobic growth efficiency in glucose minimal medium (Abdel-Hamid et al. 2001). Generally, acetyl units for biosynthetic purposes are provided by PDH aerobically and PFL anaerobically. PoxB might be important during the transition between exponential and stationary phases (through Acetyl-CoA synthetase) under microaerobic conditions where both PDH and PFL function poorly (Abdel-Hamid et al. 2001).

The enzyme is a homotetramer consisting of four identical 62 kDa subunits, encoded by the *poxB* gene. Each subunit contains a tightly, but non-covalently bound FAD and loosely bound TPP through Mg^{2+} (Abdel-Hamid et al. 2001). PoxB is strongly activated by a variety of phospholipids which increase the maximum velocity about 20-fold and decrease K_m for pyruvate about 10-fold (Abdel-Hamid et al. 2001).

Lactate dehydrogenase (LDH)

Under anaerobic conditions and in the absence of alternative electron acceptors, *E. coli* converts sugars to a mixture of reduced organic compounds such as lactate and ethanol to reach redox balance (Clark 1989). There are three lactate dehydrogenases (LDHs) in *E. coli* which reduce pyruvate to lactate (Jiang et al. 2001). Two of them are membrane bound flavoproteins (one specific for L-lactate and the other for D-lactate) which couple to the respiration chain and are required for aerobic growth on lactate. These two LDHs are better described as lactate oxidases (Jiang et al. 2001). The fermentative lactate dehydrogenase (LDH, EC 1.1.1.28) encoded by *ldhA* is a soluble enzyme which catalyzes the conversion of pyruvate to D-lactic acid (Tarmy and Kaplan 1968a,b), coupled to the consumption of NADH:

$$\text{Pyruvate} + \text{NADH} \longrightarrow \text{Lactate} + \text{NAD}^+$$

The fermentative LDH of *E. coli* is induced approximately ten-fold in anaerobically grown cultures at acidic pH (Clark 1989) and is allosterically activated by its substrate, pyruvate (Tarmy and Kaplan, 1968b).

PEP Synthase (PPS)

Phosphoenolpyruvate synthase (PPS, EC 2.7.9.2) encoded by *pps* gene is a gluconeogenic enzyme which catalyzes the direct conversion of pyruvate to PEP, coupled to the breakage of both of the phosphoanhydride bonds of ATP (Patnaik et al. 1992):

$$\text{ATP} + \text{Pyruvate} + \text{H}_2\text{O} \longrightarrow \text{PEP} + \text{AMP} + \text{phosphate}$$

This enzyme is required for growth on single three-carbon substrates such as pyruvate and lactate

(Patnaik et al. 1992). This reaction is a ping-pong reaction in which PPS is phosphorylated by ATP and then the phosphoryl group is transferred to pyruvate (Narindrasorasak and Bridger 1977).

Biochemical pathways and regulatory proteins indirectly impacting pyruvate and lactate formation

Acetyl-CoA Synthetase (ACS)

Acetyl-CoenzymeA (acetyl-CoA) is an important metabolite in a variety of important physiological process that link anabolism and catabolism (Andenberg et al. 1996, Gulick et al. 2003). In addition to serving as the key substrate for TCA cycle, acetyl-CoA is an essential building block for the synthesis of fatty acids, amino acids and biomass (Andenberg et al., 1996, Gulick et al. 2003). During growth on glucose, direct formation of acetyl-CoA from pyruvate is catalyzed by PDH aerobically or PFL anaerobically (Clark 1989, Andenberg et al. 1996). Another pathway which converts acetate to acetyl-CoA is catalyzed by acetyl-CoA synthetase (ACS, EC 6.2.1.1) encoded by *acs*. This enzyme is important to maintain adequate levels of acetyl-CoA, especially when the organisms grow on acetate or become unable to generate acetyl-CoA directly from pyruvate (Gulick et al. 2003, Zelic et al. 2003):

$$\text{ATP} + \text{acetate} + \text{CoA} \longrightarrow \text{AMP} + \text{diphosphate} + \text{acetyl-CoA}$$

ACS belongs to a family of AMP-forming enzymes catalyzing a two-step reaction. The crystal structures of ACS and the kinetics data of the family of AMP-forming enzymes suggest

the reaction mechanism of acetyl-CoA synthetase is a Bi-Uni-Uni-Bi ping-pong reaction (Horswill and Escalante-Semerena 2002, Gulick et al. 2003). ATP and acetate bind to the enzyme in the first half-reaction to release the first product, pyrophosphate, and form the intermediate, acetyl-AMP. In the second half-reaction, the third substrate, coenzyme A, binds to the enzyme to allow the formation of the thioester and then release the two products, acetyl-CoA and AMP (Horswill and Escalante-Semerena 2002, Gulick et al. 2003).

PEP carboxylase (PPC)

Phosphoenolpyruvate carboxylase (PPC or PEPC; EC 4.1.1.31) encoded by *ppc* gene is a CO_2-fixing enzyme which catalyzes the conversion of PEP to oxaloacetate in the presence of Mg^{2+} (Izui et al. 2004):

$$\text{PEP} + \text{HCO}_3^- \xrightarrow{Mg^{2+}} \text{Oxaloacetate} + \text{Phosphate}$$

PPC exists widely in a number of organisms, including bacteria, plants, and algae, but it is not found in animals, fungi, and yeasts (Kai et al. 2003, Izui et al. 2004). PPCs from various sources are usually composed of four identical subunits with molecular weight of 95–110 kDa.

During cell growth, four-carbon compounds such as oxaloacetate are withdrawn from TCA cycle for biomass biosynthesis. These four carbon compounds need to be fed back into the TCA cycle from other reactions. PPC plays primarily an anaplerotic role by replenishing C4-dicarboxylic acids to the TCA cycle for both energy and biosynthetic metabolism. Most PPCs are allosteric enzymes with a wide variety of allosteric effectors depending on the species (Kai et al. 2003, Izui et al. 2004). *E. coli* PPC is activated by acetyl-CoA,

fructose-1,6-bisphosphate, GTP, fatty acids and their CoA derivatives, and the enzyme is inhibited by L-aspartate, L-malate, citrate, succinate, and fumarate (Kai et al. 2003, Izui et al. 2004).

Energy and $(F_1F_0)H^+$-ATP synthase complex

Many studies have been conducted to address the question of what controls the flux through glycolysis and how this flux can be increased. In yeast, results suggest that none of the glycolytic enzymes exert significant control on the pathway flux (Schaaff et al. 1989). In *E. coli*, overexpression of the proteins that catalyze the uptake and phosphorylation of the glucose did not increase the flux (Ruyter 1991). Recent results demonstrated that the majority of flux control of glycolysis resides not inside but outside the pathway, i.e., in enzymes that consumed ATP. Specifically, the flow of the glycolytic pathway is enhanced by a decrease in the energy level of the cell (Koebmann et al. 2002). Phosphofructokinase-I and pyruvate kinase-II in *E. coli* are activated by ADP and AMP, respectively (Kotiarz et al. 1975, Babul et al. 1978).

The proton-translocating $(F_1F_0)H^+$-ATP synthase (EC 3.6.1.34) is responsible for oxidative phosphorylation and is located in the membrane in *E. coli* (Sorgen et al., 1998). It catalyzes ATP synthesis by utilizing the energy of a transmembrane electrochemical potential of proton (Ono et al., 2004). The enzyme can also work in opposite direction at the expense of ATP when necessary to generate proton gradient when required for locomotion, nutrient uptake, and other functions (Weber and Senior, 2003).

In *E. coli*, the $(F_1F_0)H^+$-ATP synthase consists of two sectors, a membrane integral sector F_0 and cytoplasmic sector F_1 (Figure II-3) (Weber and Senior, 2003). The F_0 sector consists of three integral membrane subunits (*a, b_2, c_{10}*) which form a proton channel to carry out proton conduction. The F_1 sector consists of five dissimilar subunits (α_3, β_3, γ, δ, ε) and houses catalytic sites for ATP synthesis or ATP hydrolysis. The F_0 and F_1 sectors are joined by two narrow stalks and functions as a pair of rotary motors. The central stalk complex, the "rotor", comprises the c_{10} subunit in F_0 and $\gamma\varepsilon$ subunits in F_1, and the peripheral second stalk, the "stator", consists of the b_2 subunit in F_0 and δ subunit in F_1 (Figure II-3). Downhill protons, being carried by mid-membrane carboxylates in *c* subunit (*c*Asp61 in *E. coli*), flow through the subunit-*a* channels that lead to opposite sides of the membrane and drive rotation of the *c*-ring (Figure II-3) (Boyer, 1997; Cross and Muller, 2004). Because there is a stable interaction between $\gamma\varepsilon$ and the top of the *c*-subunit oligomer and the *a*-subunits are anchored through $b_2\delta$ to the $\alpha_3\beta_3$ hexamer ring of F_1, rotation of the *c*-ring forces γ to rotate in the center of the F_1 domain. This rotation induces conformational changes at the catalytic sites on the cytoplasmic β-subunits, promoting the tight binding of substrates, ADP and Pi, and release of product, ATP (Boyer, 1997; Cross and Muller, 2004). Conversely, ATP hydrolysis in F_1 drives the reverse rotation of the rotor and causes F_0 to pump protons in the reverse direction (Ono et al., 2004).

The soluble F_1 subunit of $(F_1F_0)H^+$-ATP synthase can hydrolyze ATP in vitro independently of the F_0 subunit. Several plasmids which express the *atpAGD* genes constitutively and to different extents were introduced into wild-type *E. coli* BOE270 and *Δatp E. coli* LM3115 to

study the effect of energy level on glycolysis (Koebmann et al. 2002). The *E. coli* genes *atpAGD* encode the α, β and γ subunits of the F_1 subunit of $(F_1F_0)H^+$-ATP synthase which exert the strongest ATPase activity. The concentration of ATP in BOE270 was 25% lower at the highest ATPase expression, and the concentration of ADP was increased by 65%. The flux of glycolytic pathway increased gradually with increase of the ATPase expression and reached 170% of the wild-type flux at the highest expression level. At the highest expression level of ATPase, the growth rate was decreased to 76% of the parental strain, and the growth yield (biomass per mole of glucose) was decreased to 45% of the parental strain.

E. coli strain TC36 (*adhE atpFH ldhA frdBC pfl sucA*) has previously been constructed for homoacetate production (Causey et al., 2003). To reduce growth during oxidative metabolism, mutations in $(F_1F_0)H^+$-ATP synthase were introduced which disrupted oxidative phosphorylation. Deletion of subunits *b* and *c* of F_0 (encoded by *atpFH*) resulted in the separation of F_1 sector from the membrane. Therefore, the ATP synthesis function of $(F_1F_0)H^+$-ATP synthase was disrupted although the hydrolytic activity of F_1-ATPase in the cytoplasm was preserved. The mutations resulted in a small reduction in cell yield and growth rate compared to the wild-type and parent strains. Two-fold increase (9 $mmol \cdot h^{-1} \cdot g^{-1}$ to 18 $mmol \cdot h^{-1} \cdot g^{-1}$) in glycolytic flux was observed compared with W3110 (wild type). This flux increase is due to the increase in the ADP available for glycolysis and a decrease of allosteric inhibition of oxidative phosphorylation by ATP.

Production of pyruvate and lactate by recombinant *Escherichia coli*

Although several microorganisms produce significant quantities of pyruvate and lactate from glucose and other renewable resources (Hofvendahl and Hahn-Hagerdal 2000), *E. coli* has many advantages including rapid growth and simple nutritional requirements. Moreover, the ease of genetic manipulation of *E. coli* makes possible metabolic engineering strategies for the improvement of pyruvate and lactate accumulation in *E. coli* (Chang et al. 1999).

Although wild-type *E. coli* itself is not a commercially relevant producer of pyruvate or lactate, many strategies have been applied to modify *E. coli* strains metabolically to enhance their ability to accumulate these two compounds. The strategies have aimed to enhance the flux flowing into pyruvate and lactate (e.g., glycolysis and LDH), and/or to repress or knock-out pathways which consume these compounds (e.g., PDH and PoxB). A more detailed description of the various strategies applied will now be provided.

Production of pyruvate by recombinant *E. coli*

Several *E. coli* strains have been studied for pyruvate production (Strains summarized in Table II-1).

A lipoic acid auxotroph of *E. coli*, W1485*lip2* (*lipA2*), was used to produce pyruvate aerobically from glucose under lipoic acid-deficient conditions (Yokota et al. 1994a). The decrease in the activity of PDH under the conditions of lipoic acid deficiency impaired the oxidative decarboxylation of pyruvate to acetyl coenzyme A. Under optimal culture conditions, 25.5 g/L pyruvic acid was obtained from 50 g/L glucose in 32-40 h at pH 6.0.

A *lipA2 bgl*$^{+}$ *atpA401* mutant (TBLA-1) was constructed to produce pyruvate aerobically from glucose (Yokota et al. 1994b). This F_1-ATPase-defective mutant was constructed by the transduction of a defective gene for the α subunit of F_1-ATPase, *atpA401*, into strain W1485*lip2*. TBLA-1 produced more than 30 g/L of pyruvic acid from 50 g/L glucose in 24 h (Yokota et al. 1994b, Yokota et al. 1997). A decrease in the energy level of TBLA-1 strain enhanced the flow of the glycolytic pathway and then pyruvate production (Yokota et al. 1997). TBLA-1 had a higher rate of oxygen consumption and higher b-type cytochromes content than its parent strain, W1485*lip2*. The activities of the PTS for glucose uptake were higher in TBLA-1 than in W1485*lip2*. Phosphoglycerate kinase and pyruvate kinase-I were also found to be higher in TBLA-1 than in W1485*lip2* during the exponential growth phase.

An *aceF* mutant (CGSC6162) and an *aceF ppc* mutant (CGSC7916) were studied in complex media using glucose and acetate as carbon sources. More than 30 g/L pyruvate was obtained with both CGSC6162 and CGSC7916 (Tomar et al. 2003). High mass yields were achieved in CGSC6162 (0.72) and CGSC7916 (0.78). The volumetric productivities for CGSC6162 and CGSC7916 were 1.5g/L·h and 1.2 g/L·h, respectively. The greatest pyruvate production rate occurred early in fermentations when cell growth rate was the greatest. The studies of effect of pH and temperature on production of pyruvate showed that a pH of 7.0 and the lowest temperature studied (32°C) favored the greatest pyruvate generation.

An *E. coli* strain YYC202 *ldhA::Kan* (Hfr *zbi*::Tn10 *poxB*1 *Δ*(*aceEF*) *rpsL pps*-4 *pfl*-1 *ldhA*) was used for the production of pyruvate in fed-batch fermentation (Zelic et al. 2003). This auxotroph strain needed acetate for growth in glucose minimal medium due to the complete

block in its ability to convert pyruvate into acetyl-CoA or acetate. A final pyruvate concentration higher than 62 g/L, a productivity of 1.75 g/L·h and a pyruvate/glucose molar yield of 1.11 mol/mol (0.56 g/g) were obtained. At the optimal process conditions, the acetate feeding rate was regulated by an open-loop acetate control system based on a correlation between volume-specific CO_2 transfer rate and acetate consumption rate. In a subsequent study, continuous experiments with cell retention, repetitive fed-batch, and an in situ product recovery process with fully intergrated electrodialysis were applied and achieved a volumetric productivity of 4.5 g/L·h (Zelic et al. 2004). However, due to apparent strain instabilities, the fermentation time was limited to about 40 h. Using a repetitive fed-batch process, a yield of 0.89 g/g and a productivity of 6.04 g/L·h were obtained. By a fully integrated approach, a (calculated) final pyruvate concentration of 79 g/L was achieved.

A strain TC44 (*ΔfocA-pflB ΔfrdBC ΔldhA ΔatpFH ΔadhE ΔsucA poxB::FRT ΔackA*) was used to produce pyruvate in mineral salts medium containing glucose as the sole carbon source (Causey et al. 2004). PFL, LDH, PoxB, alcohol dehydrogenase, acetate kinase, and fumarate reductase were knocked-out to prevent pyruvate from being converting into other fermentation products. In this strain, glycolytic flux exceeded that of strain W3110, the parental strain, by more than 50%. This increase in flux is due to the increase of the availability of ADP for glycolysis and decrease of allosteric inhibition by ATP accumulation by introducing the ATPase mutation (*ΔatpFH*). The oxidative phosphorylation is disrupted in this strain while the hydrolytic activity of F_1-ATPase is preserved. A principal difference between this strain and other pyruvate production strains is the presence of PDH. The TCA cycle was interrupted by repressing *sucAB*

encoding 2-ketoglutarate dehydrogenase instead of PDH knockout to prevent pyruvate from being completely oxidized to CO_2. This approach achieved 67.4 g/L with a yield of 0.75 g/g and a productivity of 1.2 g/L·h in a glucose defined medium.

Production of lactate by recombinant *Escherichia coli*

Several *E. coli* strains have been studied for lactate production (Strains summarized in Table II-2).

A *pta ppc* mutant (JP203) was constructed to produce D-lactate in complex media (Chang et al. 1999). When JP203 was grown to 10 g/L DCW under aerobic conditions and then shifted to anaerobic conditions, 62.2 g/L of D-lactate was generated in 60 h, a volumetric productivity of 1.04 g/L·h. The lactate yield was about 0.9 g/g glucose. From this strain *ldhA* was knocked out, and the resulting strain (JP204) transformed with pL565 encoding for the L-LDH from *Lactobacillus casei*. The resulting strain JP204 (pL565) was grown aerobically for 12 h to 7.2 g/L DCW (Chang et al. 1999). After a shift to anaerobic conditions, 45 g/L L-lactate was produced from 65 g/L glucose in 67 h. Succinate accumulated as the major by-product at concentrations up to 12% of L-lactate.

A *pfl ldh*A mutant (FBR11) with a plasmid containing the *ldh* gene encoding L-lactate dehydrogenase (L-LDH) from *Streptococcus bovis* was used to produce of L-lactic acid anaerobically on complex media composed of tryptone, yeast extract and sugar(s) (Dien et al. 2001). This plasmid pVALDH1 was stable under anaerobic condition without antibiotics presumably because *E. coli* required LDH for fermentative growth. The strain showed faster

growth on glucose (μ_{max} = 0.94 h^{-1}) compared to xylose (μ_{max} = 0.62 h^{-1}). A high temperature favored lactate production slightly (35°C versus 30°C). Low pH (5.5) decreased lactate production significantly (compared to 6.5 and 7.5) probably because pH 5.5 is far below the optimal growth pH for *E. coli*, and lactic acid becomes more inhibitory with decreasing pH (Dien et al. 2001). A final concentration of 73.2 g/L was obtained with a yield of 0.93 g/g and a productivity of 2.33 g/L·h. A small amount of succinate was also generated (0.9-2.2 g/L).

Several *E. coli* strains, SZ40 (*pflB frdBC*), SZ58 (*pflB frdBC adhE*) and SZ63 (*pflB frdBC adhE ackA*), were constructed to produce D-lactate (Zhou et al. 2003a). These strains required only mineral salts as nutrients and lacked plasmids and antibiotic resistance genes. The yields of D-lactate of these strains were close to the theoretical maximum yield (1 g/g), and the optical purity exceeded 99%. The final biomass concentrations of these three strains were low (0.5 - 2.5 g/L), and the time required to complete the fermentation of 5% glucose was long (120-168 h).

A strain was constructed from the D-lactate producing strain SZ63 by replacing part of the chromosomal *ldhA* coding region with *Pediococcus acidilactici ldhL* encoding an L-lactate dehydrogenase (Zhou et al. 2003b). Although the initial strain grew and fermented poorly, a mutant (SZ85) was readily isolated by selecting for improved growth. Sequencing revealed mutations in the upstream, coding, and terminator regions of *ldhL* in SZ85, which were proposed to be responsible for increased L-lactate dehydrogenase activity. SZ85 produced up to 50 g/L L-lactate in M9 medium containing glucose or xylose with a yield of 93 to 95%, and an optical purity greater than 99%, in 120 h (glucose) or 312 h (xylose). SZ85 remained prototrophic and was devoid of plasmids and antibiotic resistance genes.

Production of pyruvate and lactate by other recombinant organisms

Production of pyruvate

Yeast is the most commonly used microorganism for producing pyruvate using glucose or other carbon sources as the substrate. For example, *Candida lipolytica* AJ 14353 accumulated 43.6 g/L pyruvate at a yield of 0.44 g/g in 72 h (Uchio et al. 1976) and *Saccharomyces cerevisiae* accumulated 36.9 g/L pyruvate at a yield of 0.37 g/g in 48 h (Yonehhara and Yomoto, 1987) under conditions of thiamine limitation. These two strains are thiamine auxotrophs. Thiamine is a cofactor of the PDH complex and pyruvate decarboxylase. A multi-vitamin auxotroph of *Torulopsis glabrata* accumulated about 68 g/L in 63 h at a yield of 0.494 g/g (Miyata and Yonehara 1996). Nicotinic acid, thiamine, pyridoxine and biotin were required in the medium for growing this strain. These vitamins are co-factors of PDH, pyruvate decarboxylase, pyruvate carboxylase, and transaminase, respectively. Accumulation of pyruvate by these strains was caused by the reduced activity of these enzymes under conditions of deficiency of these vitamins.

Production of lactate

Recombinant yeast has also been used for lactate production (Hofvendahl and Hahn-Hagerdal 2000, Bianchi et al. 2001, Ishida et al. 2005). Yeasts are more tolerant to low pH than lactic acid bacteria. Industrial lactate production by using lactic acid bacteria requires large amounts of $CaCO_3$, NaOH, or other neutralizing agents, which complicates downstream

processing and yields large amounts of gypsum as a by-product (Benninga 1990). High pH tolerance and its genetic accessibility make yeast an interesting microorganism for the production of lactate. Recombinant yeast can be used fro the production of lactate by transformation with heterologous LDH genes. However, the yield of lactate of recombinant yeast is low because of the production of ethanol which is generated from pyruvate by pyruvate decarboxylase. To increase the metabolic flow from pyruvate to lactate, a mutation of pyruvate decarboxylase should be introduced into recombinant yeast.

The yeast *Kluyveromyces lactic* only has a single gene, *KlPDC1*, encoding for pyruvate decarboxylase. The strains in which *KlPDC1* is deleted do not produce ethanol. A *Kluyveromyces lactic* BM3-12D (pLAZ10) was constructed by introducing mutations in pyruvate decarboxylase and PDH and contained the pLAZ10 carrying the bovine *LDH* gene (Bianchi et al. 2001). Without the ability to generate acetyl-CoA, this strain was unable to grow on a minimal medium containing only six-carbon sugars or only three-carbon compounds. Ethanol and glucose were together provided for the cell growth and lactate production. A final lactate concentration of 60 g/L and a mass yield of 0.85 (from glucose) were obtained in a 500 h fermentation.

Metabolically engineered yeast *S. cerevisiae* has also been used for lactate production (Ishida et al. 2005). *S. cerevisiae* has two active structural pyruvate decarboxylase genes, *PDC1* and *PDC5*, and an inactive gene, *PDC6*. Although PDC activity in *S. cerevisiae* is mainly due to *PDC1*, *PDC5* was observed to compensate for a *PDC1* deletion because the deletion led to a great increase in *PDC5* promoter-driven mRNA expression (Hohmann and Cederberg 1990). The amount of ethanol could only be slightly decreased by using single *PDC1* or *PDC5* mutant

strains. A double mutant strain (i.e., both *PDC1* and *PDC5*) is strongly impaired for growth on glucose medium (Flikweert et al. 1999). *S. cerevisiae* YIBO-7A was constructed for lactate production by introducing *PDC1* mutant and the bovine *LDH* gene (Ishida et al. 2005). A final lactate concentration of 55.6 g/L, a final ethanol concentration 16.9 g/L and a lactate mass yield 0.62 were obtained in 72 h. Under nonneutralizing conditions (final pH = 2.8), 50.2 g/L of lactate and 16.7 g/L ethanol were obtained by strain YIBO-7A.

A comparison of pyruvate and lactate production

As previously discussed, pyruvic acid and lactic acid can be interconverted by a single oxidation (lactate to pyruvate) or reduction (pyruvate to lactate) step catalyzed by the enzyme lactate dehydrogenase (LDH) due to their structure similarity. The strategies applied in the metabolic engineered strains listed in Table II-1 and Table II-2 are similar: fluxes to pyruvate and lactate were enhanced (e.g., glycolysis and LDH), and/or pathways which consume these two compounds (e.g., PDH, PoxB, and PPC) were repressed or knocked-out. But there are also several key differences between the strains for pyruvate and lactate production.

The first difference between the strains is, of course, the activity of LDH. For pyruvate production, LDH is an enzyme which catalyzes a reaction consuming pyruvate. LDH therefore must be repressed or knocked-out to increase the yield of pyruvate. For lactate production, LDH is the key enzyme catalyzing the final lactate-generating step. High activity of LDH is required for high yield and productivity of lactate production.

Another difference is that normally pyruvate production is an aerobic process whereas

lactate production is an anaerobic process (at least for a lactate production phase). The reason for this difference is due to the role of NADH in these two processes. In glycolysis, 2 moles of NADH are generated for degradation of each mole of glucose along with generation of 2 moles of pyruvate:

$$\text{Glucose} + 2\,NAD^+ + 2\,ADP \longrightarrow 2\,\text{Pyruvate} + 2\,\text{NADH} + 2\,\text{ATP}$$

To maintain pyruvate production, NADH must be reoxidized back to NAD^+. NAD^+ is of course required to accept electrons generated from oxidative degradation of glucose, and low NAD^+ limits glycolysis (Garrett and Grisham 1998). Therefore, pyruvate production requires oxygen (or perhaps another oxidizing agent) as the final electron acceptor to prevent NADH accumulation:

$$\text{NADH} + H^+ + 1/2\,O_2 + 2.5\,\text{ADP} \longrightarrow NAD^+ + H_2O + 2.5\,\text{ATP}$$

For lactate production, NADH is needed to oxidize pyruvate to lactate by LDH. As noted above, two moles of NADH are generated by degradation of one mole of glucose to two moles of pyruvate. Two moles of NADH are required for the conversion of two moles of pyruvate to two mole lactate:

$$2\,\text{Pyruvate} + 2\,\text{NADH} \longrightarrow 2\,\text{Lactate} + 2\,NAD^+$$

The carbon balance and redox balance status can be achieved by homofermentative lactate fermentation (Clark 1989). Moreover, oxygen is not required to regenerate NAD^+, and indeed oxygen and othere oxidizing agents should be avoided in a lactate production process to prevent the consumption of the NADH needed as a cofactor for LDH.

The main consuming pathways of pyruvate (to acetyl-CoA) are different under aerobic

(PDH) or anaerobic (PFL) conditions. Therefore strategies for preventing the conversion of pyruvate to acetyl-CoA are different aerobically and anaerobically. PDH must be knocked-out or repressed in pyruvate production whereas PFL must be knocked-out or repressed in lactate production.

For lactate production, high pyruvate concentration and a reductive environment in cells enhance the conversion of pyruvate to succinate through PPC or malic enzyme. To prevent from producing succinate under anaerobic conditions, PPC and/or malic enzyme should be repressed or knocked-out. In reports of aerobic pyruvate production processes, succinate was not found as a by-product. Thus for pyruvate, PPC and/or malic enzyme knock-outs might not be necessary.

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Table II-1. Recombinant *E. coli* strains used for pyruvate production

Strain	Mutations	Auxotroph	Yield (g/g)	Pyruvate (g/L)	Productivity (g/L·h)	Media	Reference
W1485*lip2*		Lipoic acid	0.51	25.5	0.64	Complex	Yokota et al. 1994a
TBLA-1	*atpA401*	Lipoic acid	0.60	30.0	1.25	Complex	Yokota et al. 1994b
CGSC6162	*aceF*		0.72	> 30.0	1.50	Complex	Tomar et al. 2003
CGSC7916	*aceF, ppc*		0.78	> 30.0	1.20	Complex	Tomar et al. 2003
YYC202 *ldhA::Kan*	*aceEF, ldhA, pps, pfl, poxB*		0.56	62.0	1.75	Minimal	Zelic et al. 2003
TC44	*adhE, atpFH, ackA, ldhA, frdBC, pfl, poxB, sucA*		0.75	67.4	1.20	Minimal	Causey et al. 2004

Table II-2. Recombinant *E. coli* strains used for lactate production

Strain	Mutations	Overexpression	Isomer	Yield (g/g)	Lactate (g/L)	Productivity (g/L·h)	Media	Reference
JP203	*Pta, ppc*		D	0.9	62.2	1.04	Complex	Chang et al. 1999
JP204	*pta, ppc, ldhA*	*ldh* from *Lactobacillus casei*	L	0.69	45.0	0.67	Complex	Chang et al. 1999
FBR11	*pfl, ldhA*	*ldh* from *Streptococcus bovis*	L	0.93	73.2	2.33	Complex	Dien et al. 2001
SZ85	*pflB, frdBC, adhE, ackA, ldhA*	*ldhL* from *Pediococcus acidilactici*	L	0.93	50.0	0.42	Minimal	Zhou et al. 2003b

(a) Addition of hydrogen cyanide

$$CH_3CHO + HCN \longrightarrow CH_3CHOHCN$$

(b) Hydrolysis by H_2SO_4

$$CH_3CHOHCN + 1/2\ H_2SO_4 \longrightarrow CH_3CHOHCOOH + 1/2\ (NH_4)_2SO_4$$

(c) Esterification

$$CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O$$

(d) Hydrolysis by H_2O

$$CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH$$

Figure II-1. Chemical synthesis of lactate

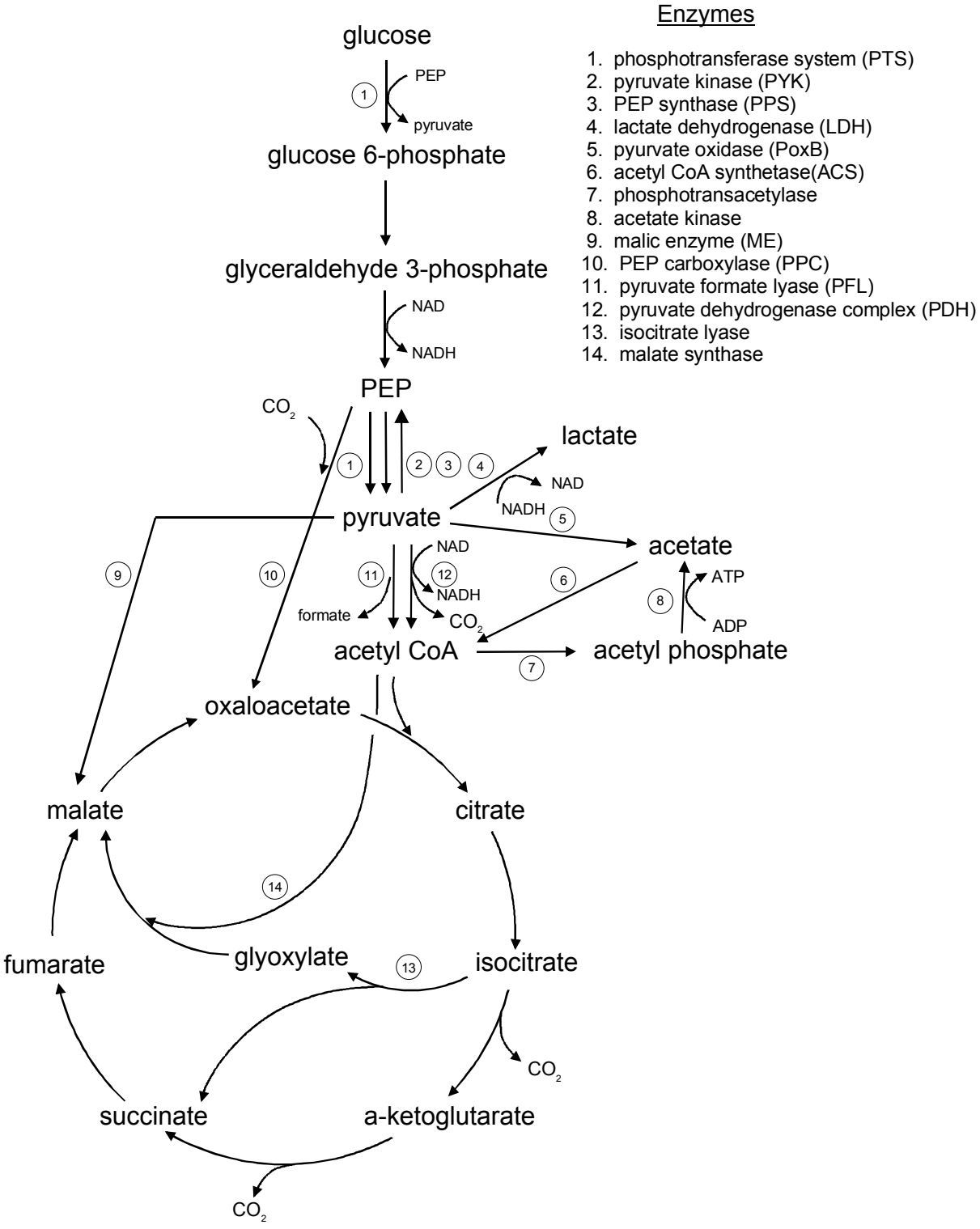

Figure II-2. Metabolic pathways of *E. coli* related to pyruvate and lactate production

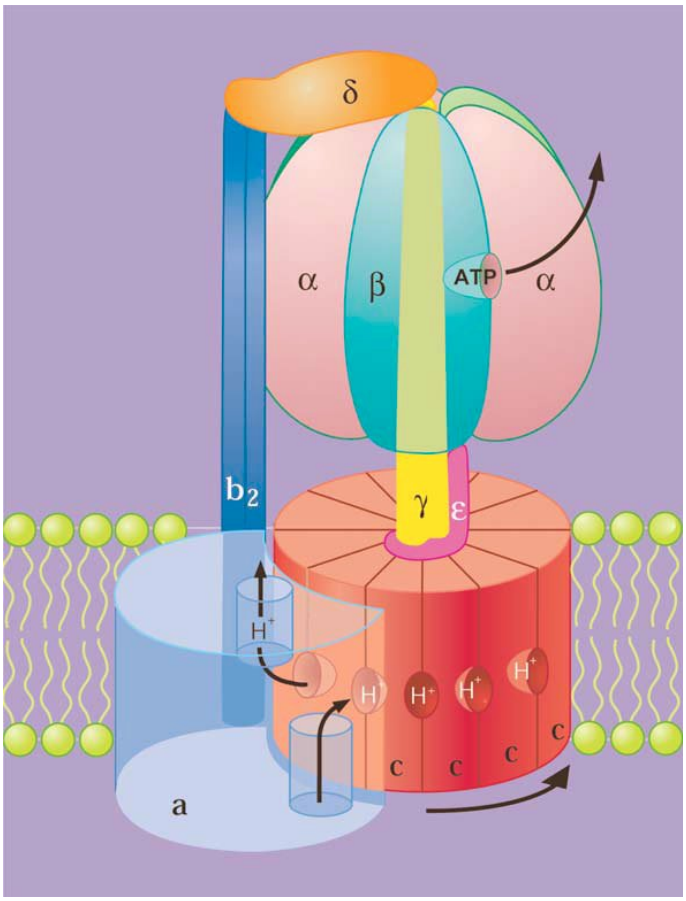

Figure II-3. A rotary binding-change model for the *E. coli* $(F_1F_0)H^+$-ATP synthase. (adapted from Cross and Muller, 2004)

CHAPTER III

HOMOLACTATE FERMENTATION BY METABOLICALLY ENGINEERED *ESCHERICHIA COLI* [1]

[1] Zhu Y, Eiteman MA, DeWitt K, Altman E (2007) Appl Environ Microbiol 73(2):456-64. Reprinted here with permission of publisher.

Abstract

We report the homofermentative production of lactate in *Escherichia coli* strains containing mutations in the *aceEF*, *pfl*, *poxB* and *pps* genes which encode respectively the pyruvate dehydrogenase complex, pyruvate formate lyase, pyruvate oxidase, and PEP synthase. The process uses a defined medium and a two fermentation phases: aerobic growth to an OD of about 30, followed by anaerobic production. Strain YYC202 (*aceEF*, *pfl*, *poxB*, *pps*) generated 90 g/L lactate in 16 h during the anaerobic phase (yield of 0.95 g/g and a productivity of 5.6 g/L·h). $Ca(OH)_2$ was found to be superior to NaOH for pH control, and interestingly, significant succinate also accumulated (over 7 g/L) despite the use of N_2 for maintaining anaerobic conditions. Strain ALS961 (YYC202 *ppc*) prevented succinate accumulation but growth was very poor. Strain ALS974 (YYC202 *frdABCD*) reduced succinate formation by 70% to less than 3 g/L. ^{13}C-NMR using uniformly labeled acetate demonstrated that succinate formation by ALS974 was biochemically derived from acetate in the medium. The absence of uniformly labeled succinate, however, demonstrated that glyoxylate did not reenter the TCA cycle via oxaloacetate. By minimizing the residual acetate at the time the production phase commenced, the process with ALS974 achieved 138 g/L lactate (1.55 M, 97% of carbon products) with a yield of 0.99 g/g and productivity of 6.3 g/L·h during the anaerobic phase.

Introduction

Lactic acid (lactate) and its derivatives have a wide range of applications in the food, pharmaceutical, leather and textile industries (13, 27). Recently, polylactic acid (PLA) has been developed as a renewable, biodegradable and environmentally friendly polymer (16, 28). An advantage of a biological process over a chemical process for the production of lactate is the prospect of generating optically pure lactate (2, 26, 28), which is an important characteristic for many of its end uses (2, 10, 26).

Although several microorganisms can produce lactate by fermentation of glucose and other renewable resources (13), *Escherichia coli* has many advantages including rapid growth and simple nutritional requirements. Moreover, the ease of genetic manipulation of *E. coli* makes possible metabolic engineering strategies for improving lactate accumulation in *E. coli* (6). Several *E. coli* strains have been studied for lactate production. For example, a *pfl ldhA* mutant (FBR11) containing a plasmid with the *ldh* gene from *Streptococcus bovis* (L-lactate dehydrogenase, L-LDH) produced L-lactic acid anaerobically on complex media (11). Using glucose, a concentration of 73.2 g/L was obtained with a yield of 0.93 g/g and a productivity of 2.33 g/L·h. A small amount of succinate was also generated (0.9-2.2 g/L). A *pta ppc* mutant (JP203) first grown aerobically in complex media to 10 g/L dry cell weight (DCW) generated 62.2 g/L of D-lactate under subsequent anaerobic conditions with a yield of about 0.9 g/g and a volumetric productivity of 1.0 g/L·h (6). Similarly, the *ldhA* mutant of JP203 containing pLS65 encoding for *Lactobacillus casei* L-LDH was grown aerobically to 7.2 g/L DCW, and generated 45 g/L L-lactate with a 0.7 g/g yield and a productivity of 0.67 g/L·h after an anaerobic switch (6). A *pflB frdBC adhE ackA* mutant (SZ63) grown on minimal media generated 48.5 g/L D-lactate of 99% optical purity close to the theoretical maximum yield of 1.0 g/g (33). After

replacing part of the chromosomal *ldhA* coding region with *Pediococcus acidilactici ldhL* encoding L-LDH, the new strain (SZ85) generated 50 g/L L-lactate at 95% yield with a productivity of 0.42 g/L·h (34). Recent results indicate that some characteristics of *E. coli* compare favorably with lactic acid bacteria, which often attain yields surpassing 90% (13).

E. coli normally generates a mixture of reduced end-products anaerobically from carbohydrates (7) as a result of reducing pyruvate to achieve a redox balance. Although lactate is one possible product, pyruvate is principally metabolized to acetyl CoA by pyruvate formate lyase (anaerobic) or by the pyruvate dehydrogenase complex (aerobic). To maximize production of lactate, genes encoding these two enzymes should be removed. However, a consequence of preventing metabolic flux from pyruvate to acetyl CoA is that the organism must obtain acetyl CoA from another carbon source such as acetate. In addition to removing genes involved in acetyl CoA synthesis from pyruvate, genes encoding other enzymes which compete with LDH for the substrate pyruvate also are candidates for deletion.

E. coli YYC202 which contains mutations in *pflB, aceEF, poxB,* and *pps* genes was previously used to produce 70 g/L pyruvate aerobically (32), but also generated 26 g/L lactate. The objective of this current study was to achieve high lactate yields and productivities by a two-phase (aerobic then anaerobic) fermentation of YYC202 in a defined medium. Moreover, we sought to reduce formation of the by-product succinate by removing the *ppc* or *frdABCD* genes which encode PEP carboxylase or fumarate reductase, respectively. Fig. III-1 shows the principal enzymes involved in the consumption of acetate and glucose relevant to the production of lactate. The simplicity of fermentation conditions and nutritional requirements are the advantages of this approach for lactate production.

Materials and Methods

Strains

Escherichia coli YYC202 (DSM 14335, Hfr *zbi*::Tn10 *poxB*1 Δ(*aceEF*) *rpsL pps*-4 *pfl*-1) was generously provided by J. E. Cronan, Jr., Univ. Illinois. Strains ALS961 (YYC202 *ppc*) and ALS974 (YYC202 *frdABCD*) were constructed during this study.

To construct ALS961, the *ppc* gene which encodes PEP carboxylase was knocked out using the lambda Red recombination system (31). Primers were designed which could amplify the chloramphenicol acetyltransferase gene and promoter from pACYC184 (5) bracketed by the first and last 50 bases of the *ppc* coding sequence. The forward primer 5' ATGAACGAACAATATTCCGCATTGCGTAGTAATGTCAGTATGCTCGGCAATTGAGAAG CACACGGTCACA 3' contained the first 50 bases of the *ppc* coding sequence followed by bases 3601 – 3620 of pACYC184 while the reverse primer 5' TTAGCCGGTATTACGCATACCTGCCGCAATCCCGGCAATAGTGACCATTATACCTGTGA CGGAAGATCAC 3' contained the last 50 bases of the *ppc* coding sequence followed by bases 400 – 419 of pACYC184. The bases homologous to pACYC184 are underlined in the primers. The two primers were used to amplify a 1,143 bp fragment from pACYC184 DNA using the polymerase chain reaction (PCR) with *Pfu* polymerase. The resulting DNA was gel-isolated and electroporated into DY330 electrocompetent cells which were prepared as described (31). Cam(R) colonies were then selected. The presence of the *ppc*::Cam knockout was confirmed by performing PCR with the following two primer pairs which could amplify the *ppc* coding sequence. The forward primer 5' CGAACAATATTCCGCATTGCG 3' contains bases 6-26 of the *ppc* gene while the reverse primer 5' TATTACGCATACCTGCCGCAA 3' contains bases 2624-2644 of the *ppc* gene. PCR amplification with these two primers yields a 2,639 bp

fragment from the wild-type *ppc* gene and a 1,130 bp fragment from the Δ*ppc*::Cam knockout.

The *frdABCD* genes of the fumarase reductase complex were knocked out to construct ALS974 using the lambda Red recombination system. Primers were designed which could amplify the chloramphenicol acetyltransferase gene and promoter from pACYC184 bracketed by the first 50 bases of the *frdA* gene and last 50 bases of the *frdD* gene. The forward primer 5' GTGCAAACCTTTCAAGCCGATCTTGCCATTGTAGGCGCCGGTGGCGCGGGTTGAGAA GCACACGGTCACA 3' contains the first 50 bases of the *frdA* coding sequence followed by bases 3601 – 3620 of pACYC184 while the reverse primer 5' TTAGATTGTAACGACACCAATCAGCGTGACAACTGTCAGGATAGCAGCCATACCTGT GACGGAAGATCAC 3' contains the last 50 bases of the *frdD* coding sequence followed by bases 400 – 419 of pACYC184 (bases homologous to pACYC184 are underlined). The two primers were used to amplify a 1,143 bp fragment from pACYC184 DNA using PCR with *Pfu* polymerase. The resulting DNA was gel-isolated and electroporated into DY330 electrocompetent cells. Cam(R) colonies were then selected. The presence of the Δ*frdABCD*::Cam knockout was confirmed by performing PCR with the following two primer pairs which could amplify the *frdABCD* genes. The forward primer 5' GTGCAAACCTTTCAAGCCGA 3' contains the first 20 bases of the *frdA* gene while the reverse primer 5' TGTAACGACACCAATCAGCG 3' contains bases 335 - 354 of the *frdD* gene. PCR amplification with these two primers yielded a 3,306 bp fragment from the wild-type *frdABCD* gene and a 1,137 bp fragment from the Δ*frdABCD*::Cam knockout.

Growth conditions

For each bioreactor experiment, cells were first grown in a 250 mL shake flask containing 30 mL TYA medium for about 6 h, before transferring 5 mL to 50 mL of SF medium in a 250 mL

shake flask. After 10 h of growth, the contents of this shake flask were used to inoculate a fermentor containing GAM medium. All flasks were incubated at 37°C and 250 rpm (19 mm pitch). TYA medium contained (per L): 10.0 g tryptone, 5.0 g NaCl, 1.0 g yeast extract, 1.36 g $Na(CH_3COO)\cdot 3H_2O$. SF medium contained (per L): 10.0 g glucose, 2.3 g $Na(CH_3COO)\cdot 3H_2O$, 5.66 g $Na_2HPO_4\cdot 7H_2O$, 1.5 g KH_2PO_4, 0.25 g NaCl, 0.5 g NH_4Cl, 0.1 g $MgSO_4\cdot 7H_2O$, 0.013 g $CaCl_2\cdot 2H_2O$, 0.02 g thiamine·HCl, 0.5 g L-isoleucine. GAM medium contained (per L): 20.0 g glucose, 11.52 g $Na(CH_3COO)\cdot 3H_2O$, 1.5 g $NaH_2PO_4\cdot H_2O$, 3.25 g KH_2PO_4, 3.275 g $K_2HPO_4\cdot 3H_2O$, 0.2 g NH_4Cl, 2.0 g $(NH_4)_2SO_4$, 1.024 g $MgSO_4\cdot 7H_2O$, 0.01 g $CaCl_2\cdot 2H_2O$, 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, 1.772 mg $Al_2(SO_4)_3$, 0.5 mg $Na_2MoO_4\cdot 2H_2O$, 18.29 mg $FeSO_4\cdot 7H_2O$, 0.02 g thiamine·HCl, 0.75 g L-isoleucine.

Fed-batch fermentation

Two-phase fed-batch experiments were carried out in a 2.5 L bioreactor (Bioflow 2000, New Brunswick Scientific Co. Edison, NJ, USA) initially containing 1.5 L medium. In the first phase, air and O_2 were mixed as necessary at 1.0 L/min total flow rate and 400 rpm agitation to maintain a dissolved oxygen concentration (DO) above 40% of saturation. During this aerobic phase, the pH was controlled at 7.0 using 30% (w/v) NH_4OH, and the temperature was controlled at 37°C. A feed solution of 400 g/L glucose and 150 g/L $Na(CH_3COO)\cdot 3H_2O$ was automatically added to maintain the glucose concentration above 10 g/L (YSI 7200 Select Glucose Analyzer, YSI Inc., Yellow Springs, OH, USA). After the cell concentration reached an optical density (OD) of 30 (corresponding to about 11 g/L DCW), an anaerobic phase was initiated. In this second phase, N_2 was sparged into fermentor at 0.15 L/min, the pH was controlled at 7.0 using 20% (w/v) NaOH or 25% (w/v) $Ca(OH)_2$, and the temperature was

controlled at 37°C unless otherwise noted. During the anaerobic phase, a 600 g/L glucose feed solution was added to maintain glucose above 10 g/L.

Several baffled shake flask studies at 37°C using an agitation of 250 rpm were accomplished to determine whether ALS961 grew in the presence of specific carbon sources. For these studies, cells were grown for 6 h in 250 mL shake flasks containing 30 mL TYA medium, and 2 mL used to inoculate 20 mL GAM medium supplemented with either casamino acids (5 g/L), a single amino acid (0.1 g/L), citrate (0.1 g/L), succinate (0.1 g/L), or fumarate (0.1 g/L). After 12 h of growth, 2 mL was again used to inoculated 20 mL of an identical medium, and cell growth was monitored for 24 h. Additionally in separate experiments, glucose in GAM medium was replaced with 1 g/L succinate or fumarate.

Analyses

The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, Calif.) was used to monitor cell growth, and this value was correlated to dry cell mass. The final concentrations of soluble organic compounds were determined by liquid chromatography as previous described (12). When $Ca(OH)_2$ was used for pH control, a homogeneous sample was diluted by a factor of 10 to dissolve any calcium lactate prior to analysis. Thus, in these cases the concentrations reported reflect total lactate formed, both dissolved and precipitated.

C-13 NMR Analysis

In order to determine how acetate was being metabolized by the cells, one additional fed-batch experiment was conducted during which approximately 1 g/L of uniformly ^{13}C labeled acetate was added 1 h after the beginning of the anaerobic phase. Samples were centrifuged (twice at 10000×g for 10 min), the supernatant mixed with 33% volume deuterium dioxide (D_2O)

and filtered for NMR analysis. Proton-decoupled ^{13}C-NMR spectra (300 NMR Spectroscopy, Varian Inc., Palo Alto, CA) at 75.4 MHz were obtained with the following spectral parameters: 45° pulses, 18.1 kHz spectral width and 4.5 s relaxation delay. Field stabilization was achieved by locking on the D_2O frequency. ^{13}C chemical shift assignments for glucose, acetate, lactate, pyruvate, succinate, and glyoxylate were determined by comparing with natural abundance standards. The ratio of peak area to labeled acetate concentration was used to calculate concentrations of other labeled compounds. Corrections were made for different compounds when necessary. The concentration of labeled acetate added was obtained from HPLC. Concentrations of other labeled compounds were calculated using the following equation:

$$Cont_S = \frac{Area_S}{CF_S \cdot R_A}$$

where $Cont_S$ is the labeled compound concentration, $Area_S$ is the integral area of the peak corresponding to labeled carbon atom of compound, CF_S is the correction factor obtained from standard spectra for the compound due to difference in relaxation time, and R_A is the ratio of peak area to labeled acetate concentration.

Results

Production of Lactate

The two-phase fermentation of *E. coli* YYC202 involved an aerobic growth phase to an OD of about 30 followed by an anaerobic non-growth, lactate production phase. The deletion of both pyruvate dehydrogenase complex genes (*aceEF*) and *pfl* in YYC202 necessitated the use of acetate in addition to glucose as carbon sources during the growth phase. Using NaOH during the anaerobic phase to control the pH at 7.0 (in replicate fermentations), 75.1 g/L lactate was generated in 32 h (Fig. III-2) for an overall process productivity of about 2.4 g/L·h. Lactate

was produced only during the 16 h anaerobic phase, and during this anaerobic phase alone the average productivity was about 4.3 g/L·h. However, the productivity decreased substantially during the course of the anaerobic phase, with a volumetric productivity of 9 g/L·h at the onset of anaerobic conditions decreasing to about 2 g/L·h. This decline was greater than could be accounted for merely as a result of diluting the fermentor contents from about 1.5L to 2.5 L. Interestingly, at the end of an experiment 9 g/L pyruvate remained, and over 4 g/L succinate despite using 100% N_2 to maintain anaerobic conditions (Table III-1). During the anaerobic phase the OD decreases merely because of the dilution effect and because the volume of individual cells decreases when cells are no longer growing.

We next examined lactate production by YYC202 at a temperature of 40°C or using 10% H_2 in N_2 during the anaerobic phase. In both cases, we observed an insignificant change in lactate concentrations, yields or productivities. A study using a pH of 6.0, however, resulted in a 50% decrease in final concentration and productivity, with a 20% reduction in lactate yield (data not shown). Therefore, for subsequent studies we maintained the pH at 7.0, the temperature at 37°C and used 100% N_2 to maintain anaerobic conditions.

Selection of Base for pH Control

The formation of lactate requires the use of a significant quantity of base to maintain the pH at 7.0. When NaOH was used in our initial experiments, the Na^+ concentration by the end of the process was about 0.60 mol/L. Since we observed a significant decrease in the rate of lactate formation during the course of the anaerobic phase, we completed two experiments to determine whether the Na^+ ion contributed to this decline in productivity. Because $Ca(OH)_2$ is used in some commercial lactate fermentations (8), the first experiment we conducted used $Ca(OH)_2$ instead of NaOH as the base for pH control (Fig. III-3). Using this base, the

volumetric lactate production rate remained high throughout the course of the anaerobic production phase (about 5-8 g/L·h, with an average of 5.6 g/L·h), resulting in a final lactate concentration of 90.0 g/L (Table 1). Since the reduction in the lactate productivity over the course of the anaerobic phase can largely be attributed to a dilution of fermentor contents (i.e., adding base and glucose solution to a non-growing culture), this result suggests that the presence of Ca^{2+} did not reduce the rate of lactate production. Note that OD cannot be measured accurately after the addition of a slurry of $Ca(OH)_2$.

To confirm whether the Na^+ was reducing lactate productivity, we conducted a second experiment in which the pH was controlled with a mixture of NaOH and Na_2SO_4, thereby adding more Na^+ than necessary merely to control pH. In this case, the final concentration of lactate achieved was 41 g/L, at which point lactate production essentially ceased (data not shown).

In order to quantify the relationship between Na^+, Ca^{2+} and the rate of lactate production, we next calculated the *specific* productivity using the lactate concentrations observed during the course of the anaerobic phase and the cell mass concentration at the time of transition between the two phases (Fig. III-4). The consistent decline in specific productivity with increasing Na^+ regardless of how that ion was added (i.e., NaOH and/or Na_2SO_4) demonstrates an inhibition of this ion toward lactate formation to a much greater extent than with Ca^{2+}. This inhibition in lactate formation occurs in the absence of any cell growth.

Effect of a ppc knockout

Despite the absence of CO_2 in the inlet gas and the presumed insignificant quantity of CO_2 generated when the cells were not growing, succinate accumulated to 7 g/L during the anaerobic phase of YYC202 fermentations (Table III-1). This surprising result led us to conclude that succinate production during the anaerobic phase might best be prevented by the

deletion in one or more of the genes in the succinate pathway (see Fig. III-1).

One strategy proposed to prevent succinate generation was to knock out *ppc* in YYC202 and thereby prevent the formation of oxaloacetate from PEP via PEP carboxylase (Fig. III-1). Previous reports indicate *E. coli* with a *ppc* knockout does not grow on glucose as the sole carbon source (1, 17, 22, 24, 33). In our current study, ALS961 (YYC202 *ppc*) similarly did not grow aerobically in shake flasks on GAM medium. Furthermore, ALS961 did not grow on GAM medium supplemented with citrate or fumarate, and growth was poor on GAM medium supplemented with succinate. However, ALS961 grew in GAM medium supplemented with 5 g/L casamino acids or on GAM medium when glucose was substituted by succinate or fumarate. We also examined growth when GAM medium was supplemented with a single amino acid (all but isoleucine, which was present in GAM medium). Of the 19 individual amino acids examined, growth was observed only in the presence of glutamate, aspartate, or glutamine, and no significant difference was observed when the concentration was 0.1 g/L or 1.0 g/L.

A two-phase fermentation of ALS961 was completed on GAM medium supplemented with 1 g/L glutamate. The maximum specific growth rate was about 60% less than observed for YYC202. Furthermore, growth stalled when the OD reached about 4.5, and at this time the conditions were switched to the anaerobic phase. As expected, during the 8 h of anaerobic conditions, succinate did not accumulate (Table III-1). Despite the low OD and thus low volumetric lactate formation rate, the *specific* lactate formation rate was 1.0 g/g·h, about the same as observed in the YYC202 anaerobic phase.

Effect of an frdABCD knockout

Although YYC202 containing the *ppc* mutation showed no growth in GAM medium, the strain successfully prevented the formation of the by-product succinate when grown in GAM

medium supplemented with glutamate. Since the growth was still unacceptably slow and eventually stalled altogether, we next constructed and examined a YYC202 *frdABCD* mutant, ALS974. Unlike ALS961, ALS974 did not show a reduced growth rate compared to YYC202 in GAM medium. Using $Ca(OH)_2$ for pH control, 92 g/L lactate was generated in 15 h of a production phase, or about 32 h total fermentation time, results very similar to those obtained using YYC202 (Table III-1). The yield during the anaerobic production phase was over 0.99 g/g (0.81 g/g for both phases). The volumetric productivity decreased from about 9 g/L·h to 6 g/L·h during the anaerobic phase, a change attributable to volume increase due to glucose feeding and base control. The specific lactate productivity was 0.9 – 1.1 g/g·h for the duration of the production phase. Surprisingly, succinate was also formed in ALS974 during the anaerobic phase, although its final concentration was always less than 3 g/L. Furthermore, the accumulation of succinate correlated with the consumption of acetate, some residual amount of which remained in the culture at the end of the growth phase. In those experiments in which the acetate concentration happened to be low at the end of the growth phase, that remaining acetate was slowly consumed during the anaerobic phase at about 0.03 - 0.05 g/g·h, and the accumulation of succinate ceased when acetate was depleted. In those experiments in which the residual acetate concentration was comparatively high, acetate was also slowly consumed during the anaerobic phase. However, succinate generation ceased when the concentration reached 3 g/L. Then, while acetate consumption continued, ethanol accumulated instead of succinate. The consumption of acetate and formation of either succinate or ethanol did not appear to influence the production of lactate.

^{13}C NMR analysis

Since succinate accumulation in ALS974 correlated with acetate consumption during the

anaerobic phase, we speculated that succinate was formed from acetate through the glyoxylate shunt. To test this hypothesis, we repeated the fed-batch fermentation with ALS974 but introduced uniformly ^{13}C-labeled acetate into the bioreactor one hour after the beginning of the anaerobic phase. If the glyoxylate shunt was the route from uniformly labeled acetate to generate succinate, then doublets for the C1 and C2 carbons of succinate would be observed in the NMR spectrum as a result of spin-spin coupling.

After the labeled acetate was added, the expected doublet peaks were observed at the positions of the acetate methyl (C1) and carboxylic (C2) carbons, and the integral areas of these peaks decreased as the anaerobic phase progressed. Moreover, doublet peaks appeared at the position of the succinate methylene (C2/C3) and carboxylic (C1/C4) carbons, and these peak areas increased as the anaerobic phase progressed (Fig. III-5). Doublet peaks of the doublet methylene carbons were not observed, indicating the absence of a significant quantity of succinate labeled at each of the four carbons. Doublet peaks also appeared at positions for ethanol methyl (C1) and hydroxyl (C2) carbons two hours after acetate addition which increased as the anaerobic phase progressed. Doublet peaks were not observed for glucose, lactate, or pyruvate. No other unidentified peaks were observed in any of the NMR studies.

We next estimated the quantity of succinate and ethanol generated using the integral areas of the doublets peaks. At the beginning of anaerobic phase (1-2 h after start of phase), about 50% of the labeled acetate was recovered in succinate, and the balance was unaccounted. Later in the anaerobic phase (5-6 h), about 50% of labeled acetate was converted to succinate and about 50% was converted to ethanol.

Prolonged Fermentation

In the previous fermentations using ALS974, the volume changed from 1.5 L to about 2.4

L during the course of the fed-batch process due to glucose feeding and base control, both of which served to dilute the non-growing cells. Since the fermentor capacity was about 2.4L, the experiments did not address the question of the maximum concentration of (calcium) lactate which could be produced. We therefore repeated the ALS974 fermentation using more concentrated $Ca(OH)_2$ as base. In this case, a final lactate concentration of 138 g/L (1.55 M, 97% of the total carbon products) was achieved in 39 h (Fig. III-6) in a final volume of 2.0 L, while other products (3%) included pyruvate (1.5 g/L), succinate (3 g/L) and ethanol (0.3 g/L). The average anaerobic phase productivity was 6.3 g/L·h (Table III-1).

Discussion

Lactate is produced by *E. coli* to achieve redox balance under anaerobic conditions and in the absence of external electron acceptors (7). Lactate is generated from pyruvate by lactate dehydrogenase (LDH, EC 1.1.1.28) encoded by *ldhA*, which is coupled to the oxidation of NADH to NAD^+. Due to the prospect for both a carbon and a redox balance in the conversion of glucose exclusively to lactate, a homolactate fermentation can theoretically be achieved in *E. coli* with a yield of lactate of 2 mol/mol glucose (1 g/g). Achieving high yield homolactate production requires the absence of competing pathways which would serve to oxidize NADH and/or direct carbon to other products. Of course, oxygen and other electron acceptors must also be avoided to prevent oxidation of NADH by any means other than via lactate dehydrogenase.

E. coli YYC202 with mutations in the *pflB, aceEF, poxB*, and *pps* genes was previously constructed and observed to accumulate nearly 70 g/L pyruvate and 26 g/L lactate aerobically (32). This strain has knockouts in genes which encode for the pyruvate dehydrogenase

complex (PDH), and the enzymes pyruvate formate lyase (PFL), pyruvate oxidase (POX) and PEP synthase, and is therefore ideally suited to study the potential for a homolactate fermentation by *E. coli*. Consequences of these multiple mutations include an inability to grow anaerobically and a growth requirement for acetate. The absence of growth under anaerobic conditions necessitates a two-phase aerobic-anaerobic process, but it also provides a means to achieve high lactate yield by preventing glucose (or other carbon sources) from being diverted to biomass. Furthermore, pyruvate accumulation during growth may increase the flux toward lactate due to the allosteric activation of LDH (30).

Using YYC202, we observed lactate concentrations consistently over 70 g/L with overall productivities (i.e., including aerobic growth phase) greater than 2.5 g/L·h and yields greater than 0.7 g/g. Two important results were observed in these fermentations. First, Ca^{2+} is superior to Na^{+} as a base counterion. Although the specific cause for this difference remains unknown, *E. coli* growth rate has been demonstrated to be much more sensitive to Na^{+} under anaerobic conditions than under aerobic conditions apparently as a result of the cell's reduced extrusion activity for this specific ion (25). The effect of Na^{+} is particularly intriguing in this study because it occurs in cells which are not growing, demonstrating that elevated Na^{+} not only affects growth rate (25), but it also affects product formation independently. The results do not distinguish whether the observed Na^{+} "inhibition" is related to cell energetics or whether Na^{+} merely inhibits a key enzyme specifically. Additional studies with other cations such as K^{+} (not examined in this study) would help clarify the inhibitory effects of Na^{+}. The difference observed between Ca^{2+} and Na^{+} suggests $Ca(OH)_2$ may benefit other *E. coli* fermentation processes which require a high level of base addition. Second, despite using N_2 at low flowrate to maintain anaerobic conditions, as great as 7 g/L succinate was observed at the end of the

fermentations. Assuming that a portion of this succinate was derived via PEP carboxylase (Fig. III-1), this observation led us to question the source of the CO_2 for this conversion. In order to improve on the goal of a homolactate fermentation by preventing succinate accumulation two strategies were employed: knock-outs of either *ppc* or *frdABCD* were introduced into YYC202.

In ALS961 (YYC202 containing a *ppc* knockout), we observed only limited growth when the medium was supplemented with glutamate. Although lactate accumulated only slowly at the low cell density achieved, the specific rate equaled the rate observed with YYC202. Moreover, accumulation of succinate was indeed prevented. Thus, a homolactate fermentation by ALS961 might be quite successful if the growth characteristics of this strain were improved, for example, by allowing co-metabolism of a 4-carbon substrate.

Our studies further clarify the growth requirements of *E. coli* lacking the enzyme PEP carboxylase. The TCA cycle is an important source of intermediates necessary for biosynthesis (22, 24), and as they are withdrawn these biochemicals must be replenished using anaplerotic pathways. PEP carboxylase is the primary anaplerotic pathway in *E. coli*, and as expected a *ppc* mutation prevents growth on glucose minimal medium (1, 18, 22, 24, 33). Although PEP carboxykinase (encoded by *pckA* gene) and malic enzymes (*maeB* and *sfcA*) also catalyze reversible C3-carboxylation/C4-decarboxylation reactions, these two pathways appear to be responsible for decarboxylation in *E. coli* rather than for carboxylation (22). Moreover, the *pckA*, *maeB*, and *sfcA* genes are subject to catabolite repression in the presence of glucose (19, 21, 22). Theoretically, the glyoxylate shunt could compensate for a *ppc* mutation by generating TCA cycle intermediates from acetate. Despite reports of the shunt also being subject to catabolite repression in the presence of glucose (19, 21, 22), our NMR results demonstrate that this shunt is at least able to generate succinate from acetate. Why was this succinate unable to support

growth in the absence of PEP carboxylase? The slow rate of acetate consumption (only 0.03 g/g·h) may have been insufficient to support the anaplerotic requirement for growth. That ALS961 grew on medium supplemented with any one of the three amino acids which are metabolically linked to the TCA cycle demonstrates that these amino acids could, at least to some degree, fulfill the anaplerotic demand in the absence of PEP carboxylase.

Growth of ALS961 was not observed in medium supplemented with TCA cycle intermediates citrate or fumarate, and growth was very poor with succinate. Most *E. coli* strains do not consume citrate aerobically due to the lack of a functional transport system (20). Also, the main aerobic C4-dicarboxylate transport gene (*dctA*) is subject to cyclic AMP receptor protein (CRP)–mediated catabolite repression by glucose (9, 15). The slight growth observed with succinate implicates a DctA-independent carrier that is able to transport succinate but not fumarate (14). Growth of ALS961 when glucose was substituted by fumarate or succinate (but not when glucose and either one was present) further supports the hypothesis that glucose repression prevented the growth of ALS961 on a medium supplemented with fumarate or succinate compared to a medium supplemented with a key amino acid.

The second strategy examined to prevent succinate accumulation was a knock-out of *frdABCD* (3, 33). In *E. coli*, two distinct enzymes catalyze succinate oxidation and fumarate reduction (4): succinate-ubiquinone oxidoreductase (SQR) functions aerobically as part of TCA cycle and menaquinol-fumarate oxidoreductase (QFR) is mainly used for anaerobic respiration and succinate production. Under anaerobic conditions, *E. coli* accumulates succinate from PEP via PPC, transaminase and aspartase (or malate dehydrogenase and fumarate hydratase) and QFR. Therefore, a mutation in *frdABCD* encoding for QFR should not affect aerobic growth but would prevent anaerobic succinate by this route. Using ALS974 (YYC202 containing an *frdABCD*

knockout) reduced the final succinate concentration from 7 g/L to about 2 g/L, with residual succinate linked by NMR to acetate consumption. As noted above, the activity of the glyoxylate shunt was sufficient to convert the remaining acetate to succinate anaerobically but seems to have been insufficient to allow aerobic growth in the absence of PPC. By precise control of the acetate concentration to ensure a near zero concentration at the transition between aerobic growth and anaerobic production, the process might achieve a further reduction in the formation of by-product succinate.

Our NMR results shed additional light on the metabolic pathways used by *E. coli* during the anaerobic non-growth phase. Normally, ^{13}C labeled experiments are conducted to provide information for stoichiometric metabolic flux analysis (MFA) (23, 29). In an MFA experiment, metabolic and isotopic steady-state should be achieved using either a chemostat or a well-controlled fed-batch (23). In this current study, a chemostat could not be applied since ALS974 does not grow under anaerobic conditions. Instead, we used uniformly labeled acetate to determine merely if acetate was the source of succinate or other products during the anaerobic non-growth phase. The ^{13}C - ^{13}C structure of uniformly labeled acetate has doublet peaks for both carbons in the ^{13}C-NMR spectrum due to ^{13}C - ^{13}C spin coupling (23). By virtue of the presence of doublets, we are able to determine the products of acetate metabolism for those pathways in which the ^{13}C - ^{13}C bond of acetate is not broken. That no doublet was observed for glucose, lactate and pyruvate indicates that acetate was not converted into these compounds, at least not without the breaking of the C-C acetate bond. Acetate was confirmed to be a source of both succinate and ethanol since doublet peaks corresponding to these compounds appeared soon after the addition of labeled acetate. We conclude therefore that the production of succinate from acetate is the result of the enzymatic sequence converting acetate to acetyl CoA,

acetyl CoA and oxaloacetate to citrate then isocitrate, and isocitrate to succinate and glyoxylate via the enzyme isocitrate lyase (Fig. III-7). Although some CO_2 could be generated by the oxidation of malate to pyruvate, the source of the CO_2 for the conversion of PEP to oxaloacetate during the N_2-supplied, non-growth phase remains unexplained. Additional studies are needed to identify whether this small amount of CO_2 comes from the pentose phosphate pathway, remaining activity of TCA cycle enzymes or from another pathway.

The glyoxylate resulting from the enzymatic generation of succinate via isocitrate lyase would not contain a doublet from uniformly labeled acetate. The action of the second enzyme of the glyoxylate shunt, malate synthase, would subsequently convert glyoxylate and additional acetyl CoA into malate. Because a portion of the acetyl CoA is uniformly labeled, an elevated portion of the malate would be labeled simultaneously at C1 and C2. If it were converted into oxaloacetate, malate labeled at C1 and C2 would lead to a small but readily detectable concentration of uniformly labeled succinate via the TCA cycle. However, we observed only one doublet pattern at the methylene carbon of succinate, consistent with the absence of uniformly labeled succinate. This result indicates either that the glyoxylate shunt is incomplete during the anaerobic non-growth phase, or that the malate generated from the glyoxylate shunt is not converted to oxaloacetate. We discard the first explanation because we did not observe accumulation of glyoxylate or malate. We also did not observe the accumulation of any other labeled compound and therefore speculate that malate generated from glyoxylate was converted into a compound that we could not measure, such as pyruvate via malic enzyme (Fig. III-7). This pathway would have broken the ^{13}C-^{13}C bond introduced into malate from uniformly labeled acetate and generated $^{13}CO_2$ and $^{13}C1$-pyruvate. Interestingly, this proposed route would lead to a small quantity of lactate generated from acetate. The high concentration of

naturally labeled lactate and pyruvate relative to the amount of acetate consumed prevented accurate confirmation of this pathway using ^{13}C NMR.

During the first 3 h of the anaerobic phase, we observed ^{13}C labeling patterns consistent with succinate generation occurring via the glyoxylate shunt, and with glyoxylate being converted to pyruvate via malate. Not considering ATP, the metabolism of acetate early in the anaerobic phase thus may be summarized as (see Fig. III-7):

$$\text{oxaloacetate} + 2\ \text{acetate} + \text{NAD(P)} \rightarrow \text{succinate} + CO_2 + \text{pyruvate} + \text{NAD(P)H}$$

The fraction of ethanol generated slowly increased so that by the 3-6 h after beginning of the anaerobic phase, we observed approximately equimolar succinate and ethanol production from acetate, with the latter being presumably formed directly:

$$\text{acetate} + 2\text{NADH} \rightarrow \text{ethanol} + 2\text{NAD}$$

The first reaction generates reduced cofactors while the second consumes reduced cofactors, although together they are not redox balanced. So why does the organism initially generate succinate and then increasingly ethanol from acetate? First, in comparison to the flux through glycolysis and towards lactate, the fluxes toward these two products is very small (less than 5%). Thus, the presence of these two pathways for acetate assimilation does not significantly impact the overall redox balance and may merely offer the cells a means to fine tune the redox environment as the culture enters and during anaerobic conditions. Second, previous research with wild-type *E. coli* indicates that the NADH/NAD ratio under aerobic conditions is less than half of the value under anaerobic conditions (17). The sudden change from aerobic growth to anaerobic conditions could therefore result in a temporary deficit in NADH until a new, higher steady-state NADH/NAD ratio is attained. During such transient conditions, the cell may actually prefer reactions which generate NADH compared to those which consume NADH. If

this is the case, it is reasonable that initially more succinate was generated from acetate than ethanol, as we observed. Accumulation of succinate in a homolactate fermentation might be avoided by the careful control of acetate so that this substrate is not present at the onset of the anaerobic phase.

With ALS974 we report the highest levels of lactate achieved for *E. coli* in a defined medium (Table III-1): a final lactate concentration of 138 g/L (97% of the total carbon products), overall productivity of 3.5 g/L·h, anaerobic productivity of 6.3 g/L·h, and overall yield of 0.86 g/g glucose.

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Table III-1. Comparison of *E. coli* strains for homolactate fermentation. Fermentor volumes were initially 1L. The two substrates glucose and acetate were fed until the OD reached about 30, and then 600 g/L glucose was fed until the volume reached about 2L. Data represent means of two experimental runs.

				Final Concentrations (g/L)				
Strain	Base	OD[c]	Acetate (g/L)[c]	Lactate	Pyruvate	Acetate	Succinate	Ethanol
YYC202	NaOH	32.3	1.4	75.1	9.1		4.4	0
YYC202	$Ca(OH)_2$	22.9	5.1	90.0	2.6		7.3	0
ALS961[a]	$Ca(OH)_2$	4.5	7.2	14.1	7.1		0	0
ALS974	$Ca(OH)_2$	26.0	5.1	92.0	2.1		2.6	0.6
ALS974[b]	$Ca(OH)_2$	30.6	5.0	138.1	1.5		2.3	0.3

a medium supplemented with 1.0 g/L glutamate

b more concentrated base and feed used

c At end of aerobic phase

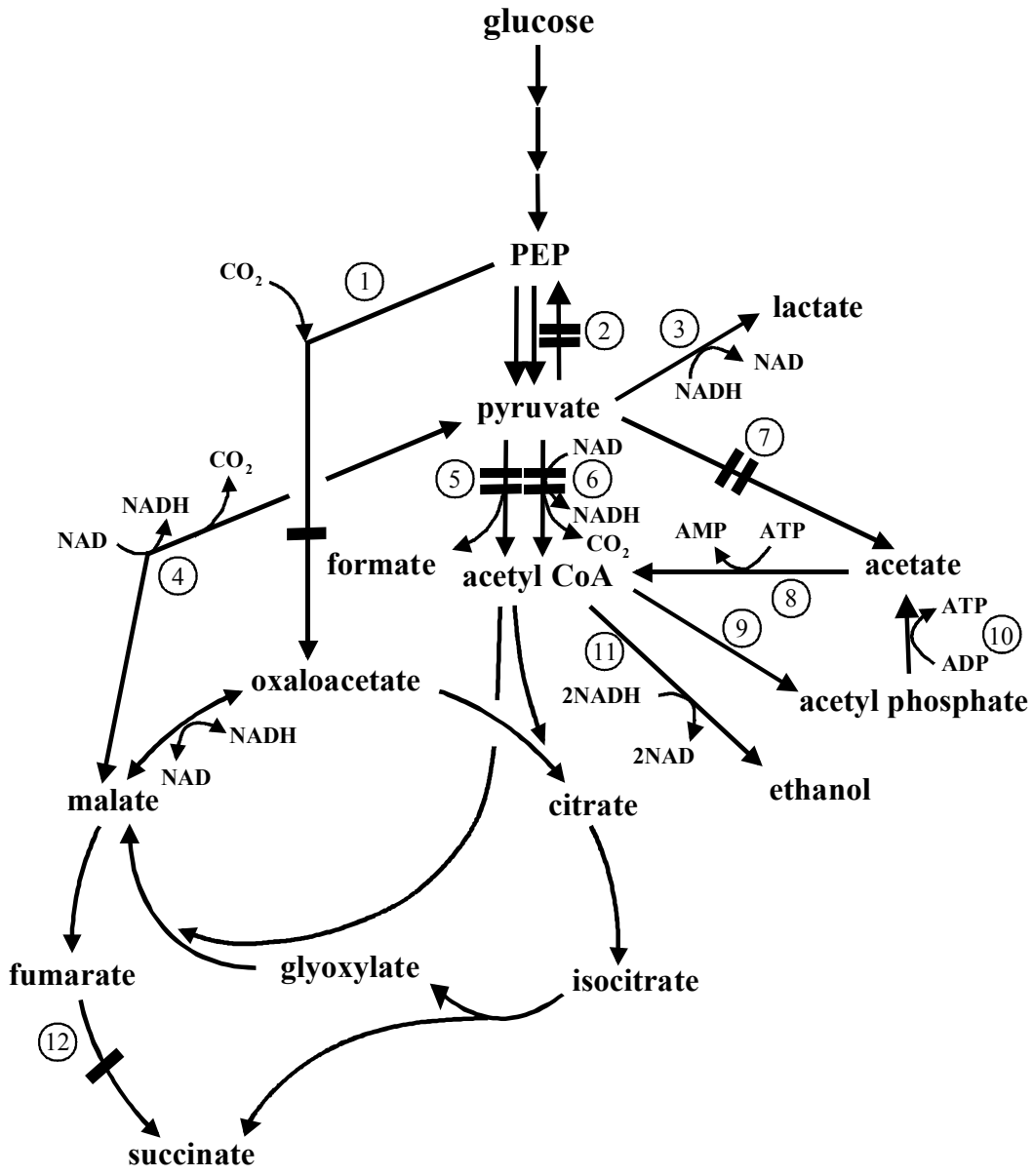

Figure III-1: Key enzymatic reactions in the production of lactate by *Escherichia coli* strains. Enzymes: 1) PEP carboxylase, 2) PEP synthase, 3) lactate dehydrogenase, 4) malic enzyme, 5) pyruvate formate lyase, 6) pyruvate dehydrogenase complex, 7) pyruvate oxidase, 8) acetyl CoA synthetase, 9) phosphotransacetylase, 10) acetate kinase, 11) alcohol dehydrogenase, 12) fumarate reductase. Double lines indicate mutations present in YYC202. Single lines indicate additional mutations studied.

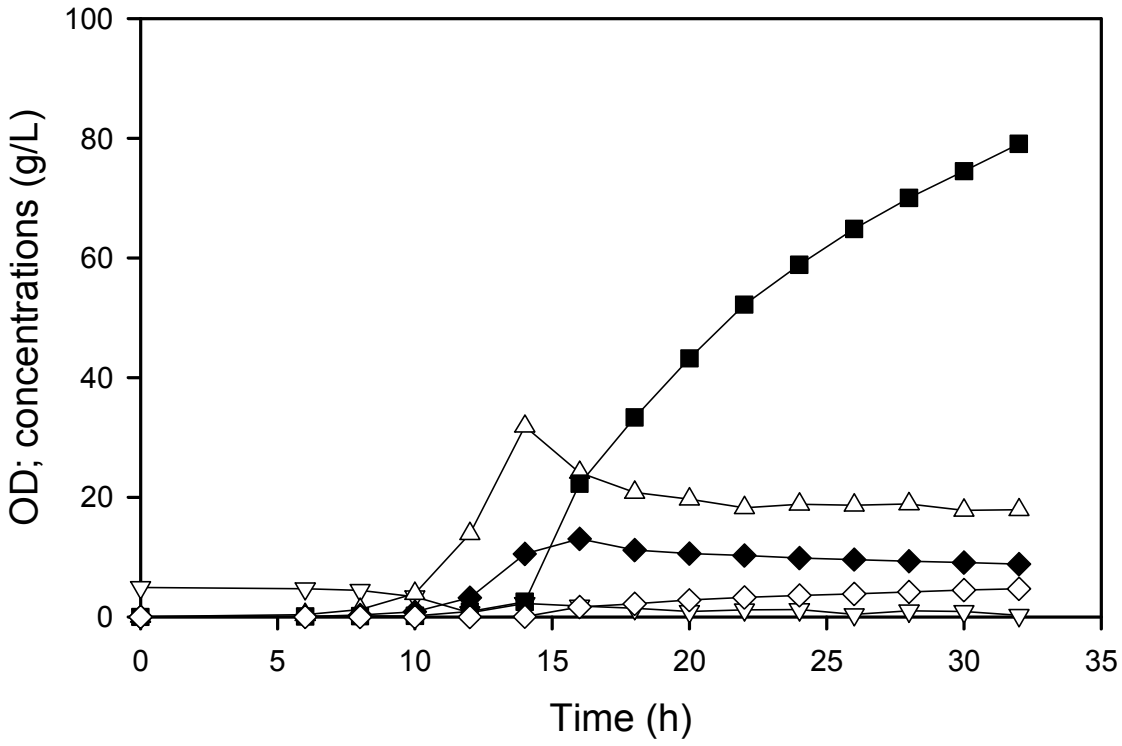

Figure III-2: Production of lactate during two phase fermentation of YYC202. Cells were grown aerobically to an OD of 30 with NH_4OH for pH control and then switched to anaerobic conditions (at approximately 14 h) with NaOH for pH control. After the initial 20 g/L glucose was reduced to 10 g/L, glucose was automatically fed to maintain a concentration above 10 g/L. The figure shows concentrations of lactate (■), OD (△), acetate (▽), pyruvate (◆), and succinate (◇).

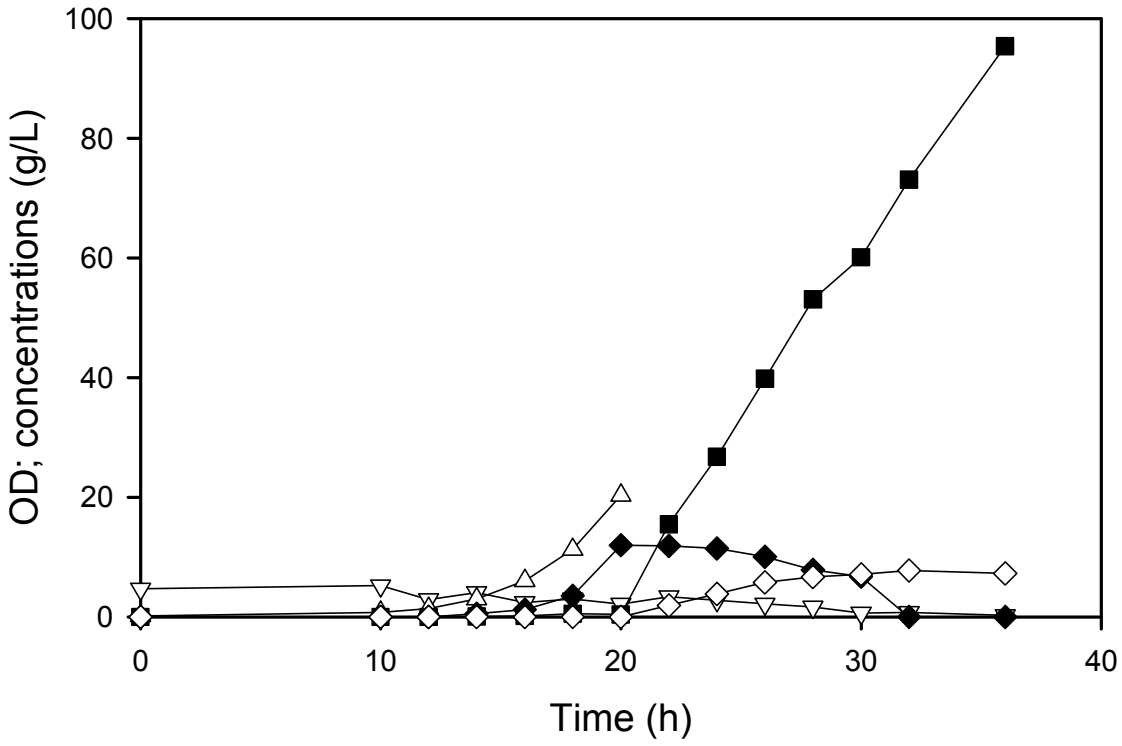

Figure III-3: Production of lactate during two phase fermentation of YYC202. Cells were grown aerobically to an OD of 30 with NH_4OH for pH control and then switched to anaerobic conditions (at approximately 14 h) with $Ca(OH)_2$ for pH control. After the initial 20 g/L glucose was reduced to 10 g/L, glucose was automatically fed to maintain a concentration above 10 g/L. The figure shows concentrations of lactate (■), OD (△), acetate (▽), pyruvate (◆), and succinate (◇).

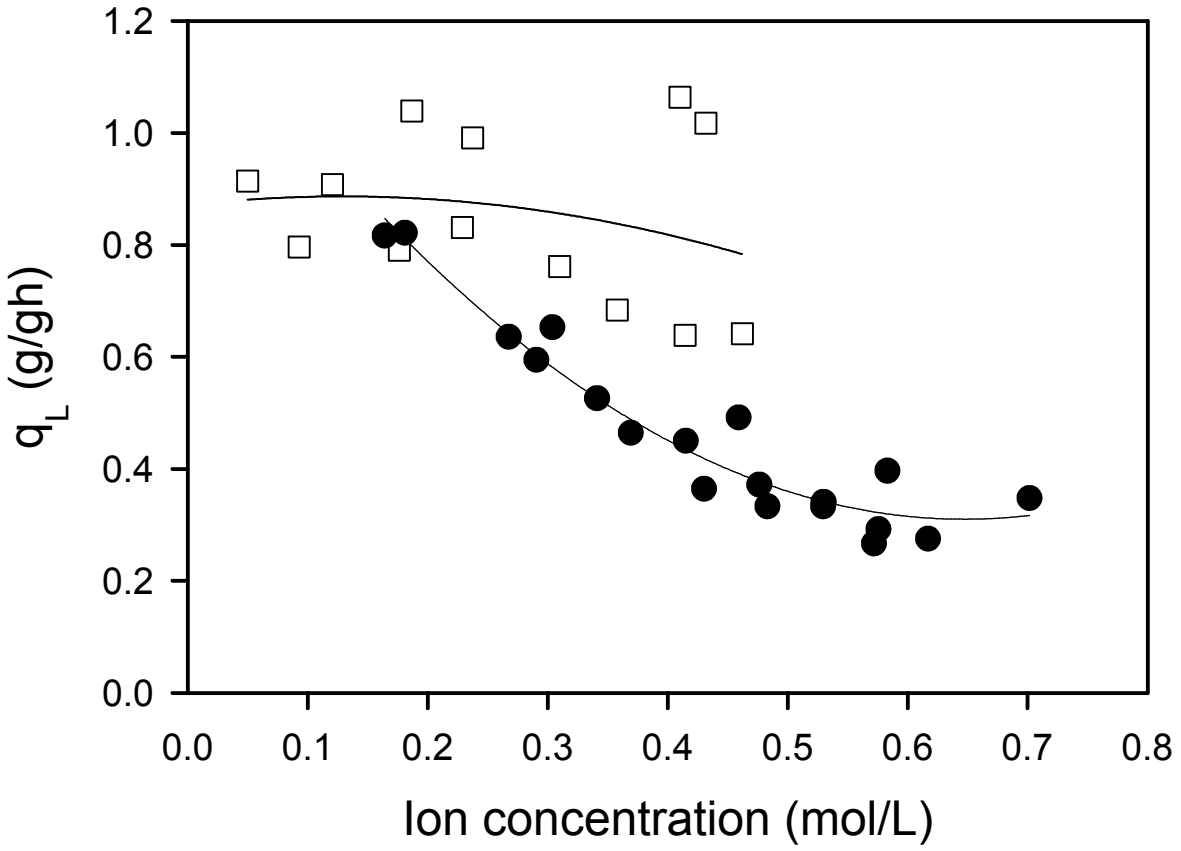

Figure III-4: The effect of ion concentrations on the specific rate of lactate production over the course of the anaerobic production of lactate. The figure shows specific rate of lactate production (g/g DCW·h) in the presence of Na^+ (●) and Ca^{2+} (□).

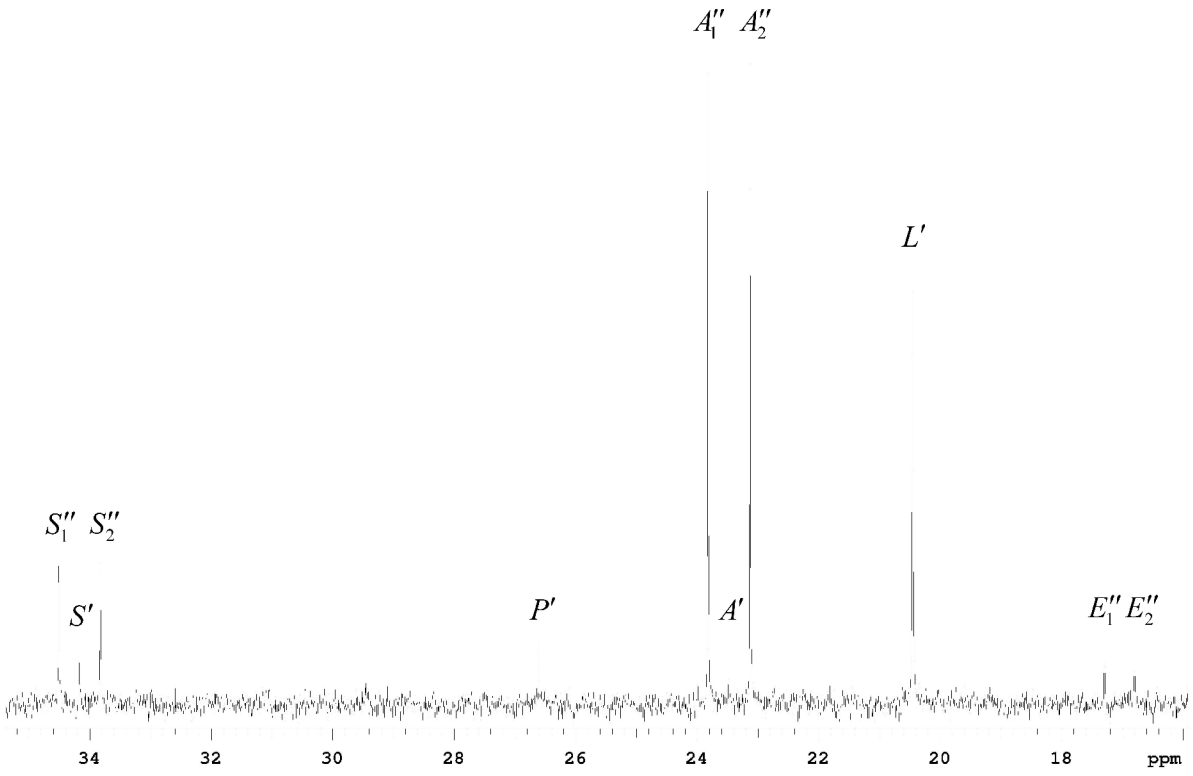

Figure III-5: Portion of ^{13}C-NMR spectrum of fermentation medium two hours after the addition of uniformly labeled acetate into the anaerobic phase of ALS974 fermentation. S_1'' and S_2'' correspond to the doublet of the $-CH_2-$ group of succinate; S' corresponds to the singlet of the $-CH_2-$ group of succinate; P' corresponds to the singlet of the CH_3- group of pyruvate; A_1'' and A_2'' correspond to the doublet of the CH_3- group of acetate; A' corresponds to the doublet of the CH_3- group of acetate; L' corresponds to the singlet of the CH_3- group of lactate; E_1'' and E_2'' correspond to the doublet of the CH_3- group of ethanol.

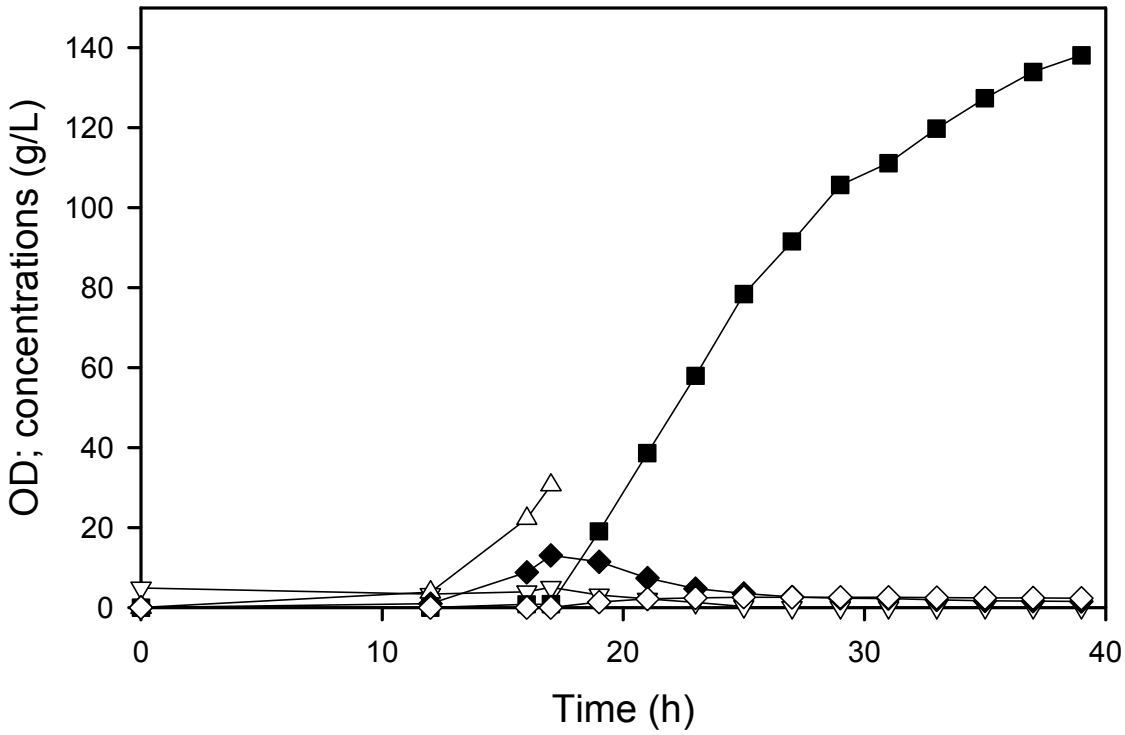

Figure III-6: Production of lactate during two phase fermentation of ALS974. Cells were grown aerobically to an OD of 30 with NH_4OH for pH control and then switched to anaerobic conditions (at approximately 14 h) with $Ca(OH)_2$ for pH control. After the initial 20 g/L glucose was reduced to 10 g/L, glucose was automatically fed to maintain a concentration above 10 g/L. The figure shows concentrations of lactate (■), OD (△), acetate (▽), pyruvate (◆), and succinate (◇).

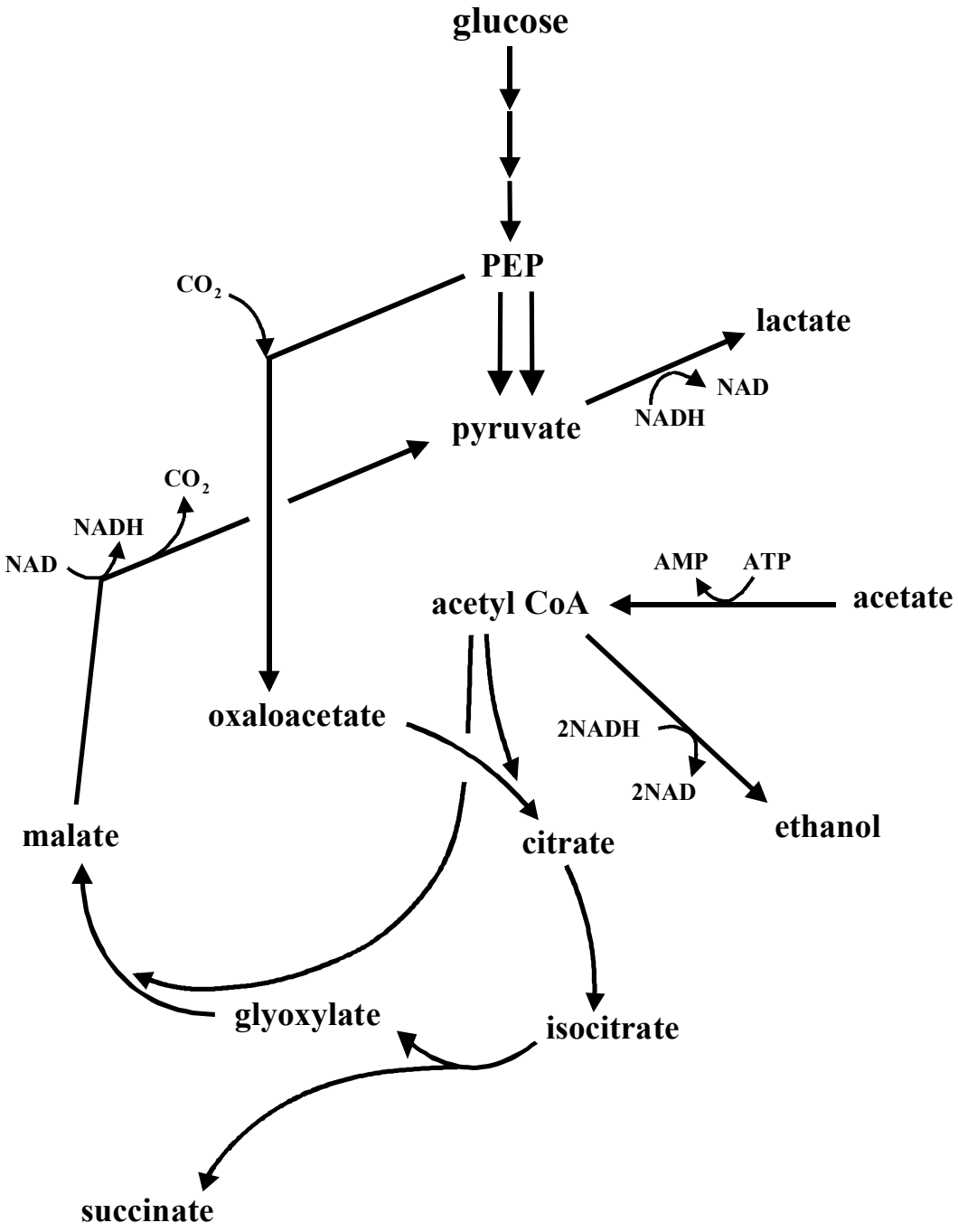

Figure III-7: Proposed pathways used by *E. coli* ALS974 during anaerobic conversion of glucose and acetate to principally lactate, and some ethanol and succinate. ^{13}C NMR results demonstrate that malate is not significantly converted into oxaloacetate.

CHAPTER IV

INDIRECT MONITORING OF ACETATE EXHAUSTION AND CELL RECYCLE IMPROVE LACTATE PRODUCTION BY NON-GROWING *ESCHERICHIA COLI* [1]

[1] Zhu Y, Eiteman MA, Altman E. 2008. Submitted to Biotechnology Letters.

Abstract

A two-phase lactate fermentation by metabolically engineered *Escherichia coli* ALS974 generates as the primary by-products succinate and ethanol anaerobically from acetate. The dissolved oxygen (DO) during the initial cell-growth phase can be used to monitor residual acetate. By using the disappearance of acetate to time the switch to the anaerobic production phase, succinate and ethanol production can be minimized. Without DO monitoring, succinate and ethanol are generated to about 3 g/L and 1 g/L, respectively. With DO monitoring, the final succinate concentration was less than 1 g/L, and ethanol was not detected during a process that generated more than 130 g/L lactate. Furthermore, by using a cell-recycle fermentation with ultrafiltration, the anaerobic lactate production phase was prolonged from 22 h to 34 h, and an overall lactate productivity of 4.2 g/L·h was achieved. The productivity of this process was nearly 20% greater than the productivity of the fed-batch process.

Introduction

Lactic acid (lactate) is a widely used organic acid generated by microorganisms (Narayanan et al 2004; Wasewar et al 2004). Although lactic acid bacteria naturally accumulate lactate (Konings et al 2000), metabolically engineered *Escherichia coli* strains are also able to accumulate substantial lactate with high productivities (Dien et al 2001; Zhou et al 2006; Zhu et al 2007). For example, a two-phase aerobic-anaerobic fermentation with *E. coli aceEF pfl poxB pps frdABCD* generated 138 g/L with an overall productivity of 3.5 g/L·h (Zhu et al 2007). Moreover, considering the non-growth phase alone, the process generated lactate at a specific rate of 0.90 g/g·h, which corresponded to a volumetric rate of 6.3 g/L·h at a cell density of 12 g/L. This particular two-phase process has two characteristics which offer opportunities for further improvement.

One aspect of this process is that both glucose and acetate are required to support microbial growth in an initial aerobic phase. During a subsequent anaerobic non-growth phase about 3 g/L succinate also accumulates despite the deletion of the *frdABCD* genes which encode fumarate reductase. ^{13}C-NMR analysis demonstrated that succinate is biochemically derived from acetate which remains at the end of the aerobic growth phase (Zhu et al 2007). This observation suggests that succinate might be reduced as a by-product if acetate were exhausted at the moment of transitioning between aerobic growth and anaerobic production. A method is needed to monitor the presence of acetate either directly or indirectly, and use that "signal" as the switch to the production phase.

A second aspect of this process is that the lactate is generated only during a non-growth phase which has followed a growth phase. Since this initial growth phase is unproductive for lactate accumulation, the overall productivity could be substantially increased by prolonging the

relative duration of the non-growth phase. Lactate formation appears to be limited by the presence of high counterion (e.g., K^+, Na^+) concentrations (Zhu et al 2007). Therefore, the process may be prolonged by removing the lactate and counterion during the production phase.

In the present study, we propose a practical means to monitor acetate indirectly by taking advantage of the growth requirement for this nutrient. Specifically, the disappearance of acetate should be accompanied by an increase in dissolved oxygen. By ensuring that the acetate is nearly exhausted at the time of switching to the production phase, by-products generated from acetate should be minimized. Moreover, we investigate two methods to recycle cells during the anaerobic phase as means to prolong lactate generation.

Materials and Methods

Strain. *Escherichia coli* ALS974 (Hfr *zbi*::Tn10 *poxB*1 Δ(*aceEF*) *rpsL pps*-4 *pfl*-1 *frdABCD*) was used in this study (Zhu et al 2007).

Growth conditions. For each bioreactor experiment, cells were first grown for 6 h in a 250 mL shake flask containing 30 mL TYA medium, before transferring 5 mL to a 250 mL shake flask containing 50 mL SF medium. After 10 h of growth, the contents of this shake flask were used to inoculate a bioreactor containing GAM medium. All flasks were incubated at 37°C and 250 rpm (19 mm pitch). TYA medium contained (per L): 10.0 g tryptone, 5.0 g NaCl, 1.0 g yeast extract, 1.36 g $Na(CH_3COO)\cdot 3H_2O$. SF medium contained (per L): 10.0 g glucose, 2.3 g $Na(CH_3COO)\cdot 3H_2O$, 5.66 g $Na_2HPO_4\cdot 7H_2O$, 1.5 g KH_2PO_4, 0.25 g NaCl, 0.5 g NH_4Cl, 0.1 g $MgSO_4\cdot 7H_2O$, 0.013 g $CaCl_2\cdot 2H_2O$, 0.02 g thiamine·HCl, 0.5 g L-isoleucine. GAM medium contained (per L): 20.0 g glucose, 11.52 g $Na(CH_3COO)\cdot 3H_2O$, 1.5 g $NaH_2PO_4\cdot H_2O$, 3.25 g KH_2PO_4, 3.275 g $K_2HPO_4\cdot 3H_2O$, 0.2 g NH_4Cl, 2.0 g $(NH_4)_2SO_4$, 1.024 g $MgSO_4\cdot 7H_2O$, 0.01 g $CaCl_2\cdot 2H_2O$, 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, 1.772 mg $Al_2(SO_4)_3$, 0.5 mg $Na_2MoO_4 \cdot 2H_2O$, 18.29 mg $FeSO_4 \cdot 7H_2O$, 0.02 g thiamine·HCl, 0.75 g L-isoleucine. The resuspension buffer used for cells after centrifugation contained (per L): 20.0 g glucose, 1.5 g $NaH_2PO_4 \cdot H_2O$, 3.25 g KH_2PO_4, 3.275 g $K_2HPO_4 \cdot 3H_2O$. The buffered glucose solution used in ultrafiltration process contained (per L): 30.0 g glucose, 1.5 g $NaH_2PO_4 \cdot H_2O$, 3.25 g KH_2PO_4, 3.275 g $K_2HPO_4 \cdot 3H_2O$.

Fed-batch process. Two-phase fed-batch processes were carried out in a 2.5 L bioreactor (Bioflow 2000, New Brunswick Scientific Co. Edison, NJ, USA) initially containing 1.0 L medium. Oxygen enriched air was used to maintain a dissolved oxygen (DO) above 50% saturation. The feeding of the solution was automatically controlled by a glucose analyzer (YSI 7200 Select Glucose Analyzer, YSI Inc., Yellow Springs, OH, USA) to maintain the glucose concentration nominally above 10 g/L. After the cell concentration reached an OD of 25 (corresponding to about 10 g/L DCW), the feeding solution was changed from a solution of 400 g/L glucose and 150 g/L $Na(CH_3COO) \cdot 3H_2O$ to a 600 g/L glucose solution. The anaerobic phase was initiated as described in the results, and subsequently the pH was controlled at 7.0 using 30% (wt/vol) $Ca(OH)_2$.

Cell recycling. Two methods were examined to recycle cells during the anaerobic lactate production phase. Centrifugation/resuspension was conducted by removing the contents of the bioreactor at three 8 h intervals during the anaerobic phase. This broth was centrifuged at 10°C at 5,000 × g for 10 minutes, cell pellets resuspended in 1 L resuspension buffer and then transferred back to the bioreactor, a process that required about 45 minutes. Ultrafiltration was conducted by pumping enough of the bioreactor contents out through a membrane filtration cartridge (UFP-500-E-4A, 500,000 NMWC, 420 cm^2, Amersham Biosciences Corp., Westborough, MA USA) using a transmembrane pressure of 8-10 Psi to decrease the volume to

500 mL from an initial volume of about 1.5 L. Then, 800 mL buffered glucose solution buffer was pumped into the bioreactor and another lactate production cycle started. Three cycles of ultrafiltration/solution addition were used, varying in duration from 6 – 10 h.

Analyses. The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, Calif.) was used to monitor cell growth. The final concentrations of soluble organic compounds were determined by liquid chromatography (Eiteman and Chastain 1997).

Results and Discussion

Controlling acetate concentration at the end of aerobic phase

Acetate is required for growth of *E. coli* ALS974 due to deletions of both the pyruvate dehydrogenase complex (*aceEF*) and pyruvate formate lyase (*pfl*). During aerobic growth, the ratio of the glucose consumption rate to the acetate consumption rate was found previously to be about 2.5 (Zhu et al 2007). In order to prevent acetate limitation during this growth phase when glucose was being monitored, the feed contained more acetate than necessary. However, because the acetate concentration is not controlled by this simple strategy, acetate would unavoidably remain when the process is switched to the anaerobic phase in which lactate is generated. Thus, some residual acetate would be converted to succinate anaerobically through the glyoxylate shunt (Zhu et al 2007). Ideally to minimize succinate production, the switch between aerobic and anaerobic conditions should occur when the acetate concentration is near zero.

Since acetate is required for aerobic growth, we reasoned that the dissolved oxygen (DO) concentration might be a useful indicator of acetate depletion. Specifically, when the acetate

concentration decreases to zero, cell growth should cease and the oxygen uptake rate should fall markedly over a short period of time. This transition in oxygen uptake rate should be observed by a sudden increase in the measured DO (Ruottinen et al 2007). In turn, the absence of acetate during the anaerobic production phase should significantly reduce the formation of the by-product succinic acid.

A fed-batch process was conducted to determine if DO could be an indicator for acetate depletion. When the cell density (OD) reached about 25, the acetate-glucose feed solution was changed to one containing only glucose. By maintaining a constant agitation and gas flow rate and composition, the DO remained about 70-80 (Figure IV-1). When the acetate concentration (as subsequently measured) decreased to about 0.5 g/L, the DO increased to 128% over the course of 10 minutes (20.10 – 20.24 h). Note that at this time, the air was being supplemented with O_2, which accounts for the DO measurement being above 100%. At the end of this interval, the process was switched to the anaerobic phase by sparging N_2 at 0.15 L/min. The acetate concentration at this switch time was subsequently determined to be less than 0.1 g/L. After an additional 26 h, a final lactate concentration of 133 g/L was achieved, similar to previous results (Zhu et al 2007). More importantly, the final succinate concentration was less than 1 g/L and ethanol was not detected. These concentrations are less than the 3 g/L succinate and 0.3 g/L ethanol observed when the DO increase was not used as an indicator of acetate exhaustion (Zhu et al., 2007). Replicate fermentations confirmed that DO begins to increase when the acetate concentration falls below 0.5 g/L, with acetate depletion occurring within ten minutes under these conditions. Ethanol and succinate by-product formation can be substantially reduced by careful control of acetate concentration at the time of the switch to anaerobic conditions.

Cell recycling

Although ALS974 does not grow anaerobically, the specific lactate productivity remains high (above 0.8 g/g·h) until the lactate concentration reaches 120 g/L (Zhu et al 2007). Reduced productivity beyond 120 g/L is likely due to build up of the Ca^{2+} ion, lactate, or calcium lactate precipitate. To prolong a high rate of lactate production, cells could be recovered and resuspended in fresh medium (i.e. with reduced lactate and Ca^{2+}, Na^{+} or K^{+}). Since cells do not grow during anaerobic lactate production, only glucose is essential in a resuspension medium. Under such a process the cells would essentially become purely a biocatalyst converting glucose into lactate with minimal by-products. We examined two different means to recycle cells, centrifugation/resuspension and ultrafiltration.

The bioprocesses which included cell recycling were initiated with the same conditions as described above. For centrifugation, the process was paused at 8 h intervals during the anaerobic phase, the medium removed and centrifuged. The cells where then resuspended and the new medium returned to the bioreactor for another cycle. Figure IV-2 shows the lactate concentration in the bioreactor for an example process. In this case during the first 8 h period, 76 g/L of lactate was generated for a productivity of 9.4 g/L·h (Figure IV-2). During a second 8h period, 69.6 g/L of lactate was generated for a productivity of 8.7 g/L·h, and then subsequent cycles generated lactate at rates of 5.8 g/L·h and 4.2 g/L·h, respectively. By prolonging the overall process to 52 h (and the production phase alone to 34 h), an overall lactate productivity of 4.0 g/L·h was achieved. Furthermore, because the switch from aerobic to anaerobic conditions coincided with acetate exhaustion (by using the DO) and acetate was absent in the resuspension solution, less than 0.5 g/L succinate and no ethanol were detected in these three cycles.

Although centrifugation with resuspension provides fresh medium to almost the entire culture, this manual approach contributed to some cell loss and could potentially cause contamination. The centrifugation/resuspension process is also cumbersome in its time requirement (Kim et al 2004; Kwon et al 2006).

Ultrafiltration offers another means to recycle cells and prolong the formation of products (Choi et al 2000; von Weymarn et al 2002). In these experiments the growth phase was conducted as already described and the cells were processed using ultrafiltration at 6 – 10 h intervals during the anaerobic phase. Ultrafiltration reduced the volume by a factor of about three from 1.5 L to 0.5 L, and then the feed solution was supplied based on demand for glucose. Using ultrafiltration, the lactate productivity was 8.6 g/L·h for the first 8h (Figure IV-2), and then 7.5 g/L·h, 7.2 g/L·h and 4.9 g/L·h for subsequent, respective cycles. Like the centrifugation process described above, the anaerobic phase was prolonged to about 34 h. Prolonging the anaerobic production phase increased the overall productivity to 4.2 g/L·h, similar to the centrifugation and 20% greater than the fed-batch process without any recycling (3.5 g/L·h, Zhu et al 2007). Again, less than 0.5 g/L succinate and no ethanol were observed. Compared to the centrifugation/resuspension process, the ultrafiltration process did not expose the cells to potential contamination, and by being continuous in operation permitted the simultaneous control of the bioreactor contents for pH and temperature. These reasons may explain the greater specific lactate productivity (above 0.9 g/g·h) for a longer time compared to the centrifugation/resuspension process.

In summary, the by-products succinate and ethanol can be minimized by monitoring the increase of DO during aerobic growth which is an indirect indicator of acetate depletion. By prolonging the anaerobic phase by 50% from 22 h to 34 h, we were able to attain an overall

lactate productivity of 4.2 g/L·h by the ultrafiltration process, a productivity that was nearly 20% greater than the fed-batch process. Moreover, less than 0.5 g/L succinate and no ethanol occurred after the first cycle, since no acetate was available in the fresh medium.

Acknowledgments

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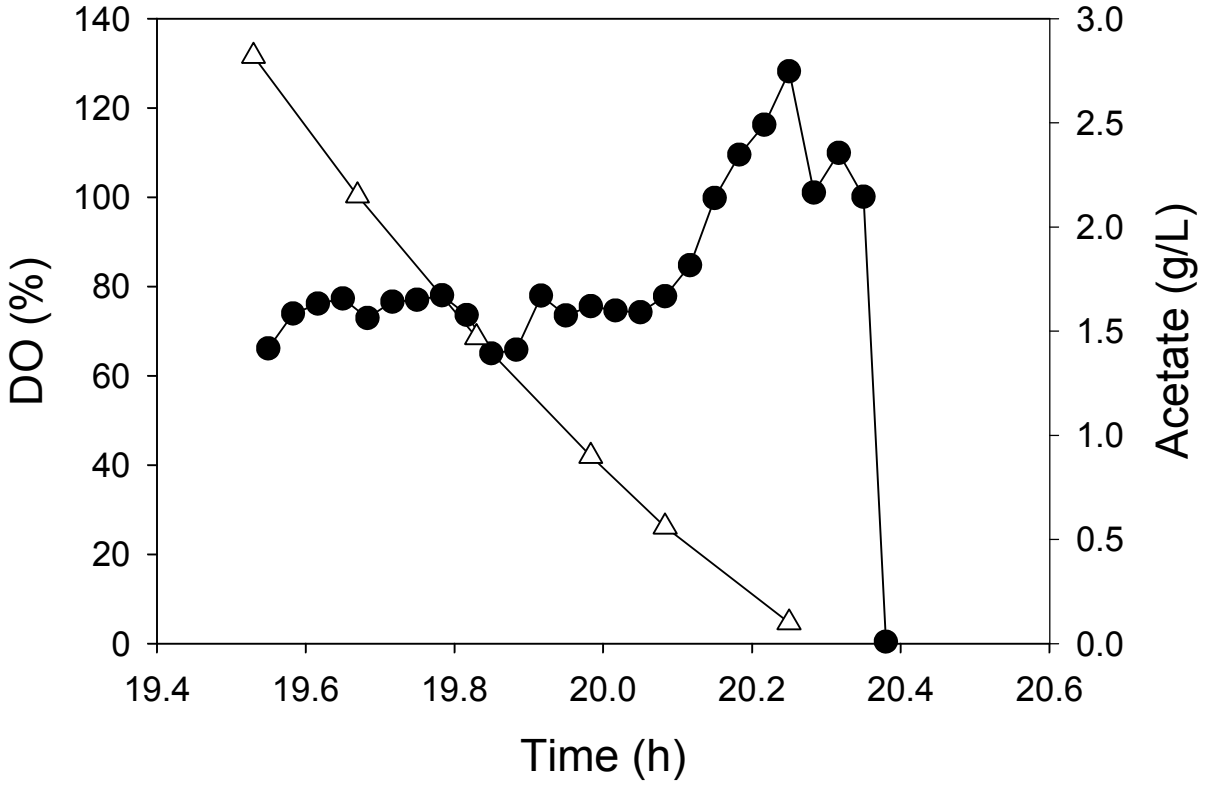

Figure IV-1: Dissolved oxygen as a percentage of air saturation (DO, ●) and acetate concentration (△) during the aerobic growth phase. The agitation and gas flowrate were fixed prior to the initiation of the anaerobic production phase at 20.37 h. The optical density (OD) of the culture was approximately 25.

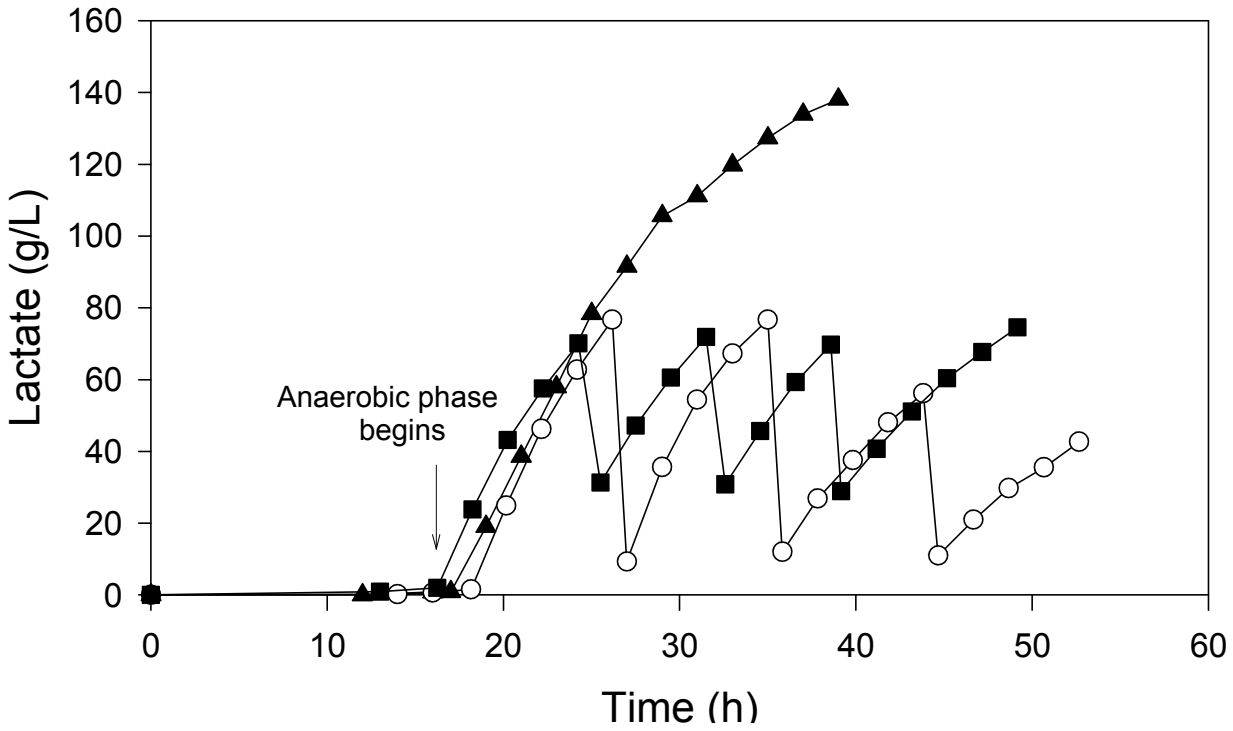

Figure IV-2: Comparison of lactate production by ALS974 in two-phase fed-batch fermentation with and without cell recycling. The figure shows concentration of lactate in a fed-batch process without cell recycling (▲), the process with intermittent centrifugation/resuspension (O) and the process with intermittent ultrafiltration (■).

CHAPTER V

HIGH GLYCOLYTIC FLUX IMPROVES PYRUVATE PRODUCTION BY METABOLICALLY ENGINEERED *ESCHERICHIA COLI* [1]

[1] Zhu Y, Eiteman MA, Altman E. 2008. To be submitted to Applied and Environmental Microbiology

Abstract

We report pyruvate formation in *Escherichia coli* ALS929 containing mutations in the *aceEF*, *pfl*, *poxB, pps* and *ldhA* genes which encode respectively the pyruvate dehydrogenase complex, pyruvate formate lyase, pyruvate oxidase, PEP synthase and lactate dehydrogenase. The glycolytic rate and pyruvate productivity was compared using glucose, acetate, nitrogen or phosphorus-limited chemostats at a growth rate of 0.15 h^{-1}. Of these four nutrients, acetate-limitation attained the highest glycolytic flux (1.60 g/g·h), pyruvate formation rate (1.11 g/g·h) and pyruvate yield (0.70 g/g). Additional mutations in *atpFH* and *arcA* (ALS1059) further elevated steady-state glycolytic flux to 2.38 g/g·h, with heterologous NADH oxidase expression causing only modest additional improvement. A fed-batch process using defined medium and 5 mM betaine as osmoprotectant with an exponential feeding rate of 0.15 h^{-1} achieved 90 g/L pyruvate with an overall productivity of 2.1 g/L·h and yield of 0.68 g pyruvate/g glucose.

Introduction

Pyruvic acid (pyruvate) is widely used in food, chemicals, and pharmaceuticals. The chemical is a precursor for the enzymatic production of L-tryptophan, L-tyrosine, D-/L-alanine and L-dihydroxyphenylalanine (Li et al. 2001), and it also serves in several health-related roles, including for weight loss (Stanko et al. 1992a, Stanko et al. 1992b, Roufs 1996), exercise endurance (Stanko et al. 1990), cholesterol reduction (Stanko et al. 1994) and acne treatment (Cotellessa et al. 2004). By applying metabolic engineering strategies, microorganisms such as *Escherichia coli* and yeasts can produce significant quantities of pyruvate from glucose and other renewable resources (Li et al. 2001). In general, such approaches must delete or repress pathways which metabolize pyruvate. For example, pyruvate accumulates readily in *E. coli* strains having mutations in *aceEF* encoding for components of the pyruvate dehydrogenase complex (PDH, Tomar et al. 2002). Additional mutations of *ldhA*, *poxB*, *pfl* and *pps* genes further improve pyruvate formation in YYC202 *ldhA* (Zelic et al. 2003). Figure V-1 shows the principal metabolic pathways and enzymes involved in the production of pyruvate.

Because pyruvate resides biochemically at the end of glycolysis, pyruvate production is directly related to the glycolytic flux. Therefore, metabolic engineering strategies to form pyruvate also aim to enhance glycolysis (Yokota et al. 1994a, Yokota et al. 1994b, Causey et al. 2004). Glycolysis is not transcriptionally limited, and control principally resides outside the pathway by cellular demand for global cofactors such as ATP and NADH (Koebmann et al. 2002, Oliver 2002, Vemuri et al. 2006). Glycolytic flux is substantially increased by disrupting oxidative phosphorylation or by increasing ATP hydrolysis (Koebmann et al. 2002, Causey et al. 2004). Increased glycolytic flux assists pyruvate accumulation: for example, a F_1-ATPase-defective mutant (*E. coli lipA2 bgl*$^+$ *atpA401*) generated pyruvate more quickly than

its parent (Yokota et al. 1994a, Yokota et al. 1994b). Similarly, *E. coli atpFH* (strain TC44) deficient in oxidative phosphorylation but having hydrolytic F_1-ATPase activity showed elevated glycolytic flux and generated 64.9 g/L pyruvate with a yield of 0.75 g/g and a volumetric productivity of 1.2 g/L·h (Causey et al. 2004). In addition to reducing oxidative phosphorylation, reducing NADH availability directly also increases glycolytic flux. For example, glycolytic flux is increased 70% by introducing water-forming NADH oxidase from *Streptococcus pneumoniae* into *E. coli* and in an *arcA* mutant (Vemuri et al. 2006).

Another goal in a pyruvate production process is of course high yield. Since the principal by-product of pyruvate formation is biomass, high glycolytic flux is sought relative to low biomass generation. Glycolytic flux will achieve a maximum by supplying glucose in excess and limiting cell growth by another substrate, for example during a chemostat or fed-batch culture. With mutations in the pathways leading to acetyl CoA from pyruvate, *E. coli* YYC202 *ldhA* requires both glucose and acetate as carbon sources for cell growth (Zelic et al. 2003), making this strain useful to examine nutrient limitation using acetate or non-carbon compounds. In a previous study, 62 g/L pyruvate was generated with a yield from glucose of 0.56 g/g at a rate of 1.75 g/L·h (Zelic et al. 2003) but glycolytic flux was not maximized, and cell growth was not limited. A fed-batch process limited by the availability of a substrate other than glucose would limit cell growth and would simultaneously maximize glycolytic flux, pyruvate yield and productivity. The objective of the present study was to use chemostat and fed-batch experiments to study the affect of limiting substrate and genetic perturbations on glycolytic flux and pyruvate generation. Specifically, using *E. coli* YYC202 *ldhA* (strain ALS929) we compared glucose, acetate, nitrogen and phosphorus as growth limiting nutrients, and using the best conditions compared the affects of mutations in *atpFH*, *arcA* and the result of introducing

heterologous NADH oxidase activity.

Materials and Methods:

Strains

Table V-1 lists the strains used in this study. Derivatives of YYC202 that contained *ldhA*::Kan, Δ*arcA*726::(FRT)Kan, or Δ*atpFH*::Cam were constructed using P1 transduction. Kan(R) was deleted from Δ*arcA*726::(FRT)Kan transductants using the curable pCP20 plasmid which overproduces FLP recombinase (Cherepanov and Wackernagel, 1995). The ptrc99A-*nox* plasmid which overproduces *Streptococcus pneumoniae* NADH oxidase has been previously described (Vemuri et al., 2006). All manipulations that involved YYC202 were performed using TYA medium.

The F_1F_0 proton-translocating ATPase complex of *E. coli*, which catalyzes the synthesis of ATP from inorganic phosphate, is encoded by the *atpIBEFHAGDC* operon. Because the *atpFH* genes are transcriptionally coupled, a deletion that encompassed both *atpF* and *atpH* could easily be constructed using the lambda Red recombination system (Yu et al., 2000; Datsenko and Wanner, 2000). Primers were designed which could amplify the chloramphenicol acetyltransferase gene and promoter from pACYC184 bracketed by the first 50 bases of the *atpF* gene and the last 50 bases of the *atpH* gene. The forward primer 5′ GTGAATCTTAACGCAACAATCCTCGGCCAGGCCATCGCGTTTGTCCTGTTTTGAGAAG CACACGGTCACA 3′ contains the first 50 bases of the *atpF* coding sequence followed by bases 3601 – 3620 of pACYC184 while the reverse primer 5′ TTAAGACTGCAAGACGTCTGCAAGGCGCTCAAGACGACCGCGTACGCTGCTACCTGT GACGGAAGATCAC 3′ contains the last 50 bases of the *atpH* coding sequence followed by

bases 400 – 419 of pACYC184. The bases homologous to pACYC184 are underlined in the primers. The two primers were used to amplify a 1,162 bp fragment from pACYC184 DNA using the polymerase chain reaction (PCR) and *Pfu* polymerase. The resulting DNA was gel-isolated, electroporated into DY330 electrocompetent cells and Cam(R) colonies were then selected. The presence of the Δ*atpFH*::Cam knockout was confirmed by performing PCR with the following two primer pairs which could amplify the *atpFH* genes. The forward primer 5′ CTTAACGCAACAATCCTCGG 3′ contains bases 7 – 26 of the *atpF* gene while the reverse primer 5′ TAAGACTGCAAGACGTCTGC 3′ contains bases 514 – 533 of the *atpH* gene. PCR amplification with these two primers yields a 1,012 bp fragment from the wild-type *atpFH* gene and a 1,155 bp fragment from the Δ*atpFH*::Cam knockout.

Growth conditions

For all experiments cells were first grown in a 250 mL shake flask containing 30 mL TYA medium for about 8 h, before transferring 5 mL to 50 mL of SF medium in a 250 mL shake flask. After 12 h of growth, the contents of this shake flask were used to inoculate a bioreactor containing GAM medium. TYA medium contained (per L): 10.0 g tryptone, 5.0 g NaCl, 1.0 g yeast extract, 1.36 g $Na(CH_3COO)\cdot 3H_2O$. SF medium contained (per L): 10.0 g glucose, 2.3 g $Na(CH_3COO)\cdot 3H_2O$, 5.66 g $Na_2HPO_4\cdot 7H_2O$, 1.5 g KH_2PO_4, 0.25 g NaCl, 0.5 g NH_4Cl, 0.1 g $MgSO_4\cdot 7H_2O$, 0.013 g $CaCl_2\cdot 2H_2O$, 0.02 g thiamine·HCl, 0.5 g L-isoleucine. GAM medium contained (per L): 30.0 g glucose, 2.75 g $Na(CH_3COO)\cdot 3H_2O$, 1.5 g $NaH_2PO_4\cdot H_2O$, 3.25 g KH_2PO_4, 3.275 g $K_2HPO_4\cdot 3H_2O$, 0.2 g NH_4Cl, 2.0 g $(NH_4)_2SO_4$, 1.024 g $MgSO_4\cdot 7H_2O$, 0.01 g $CaCl_2\cdot 2H_2O$, 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, 1.772 mg $Al_2(SO_4)_3$, 0.5 mg $Na_2MoO_4\cdot 2H_2O$, 18.29 mg $FeSO_4\cdot 7H_2O$, 0.02 g thiamine·HCl, 0.75 g L-isoleucine. CGAM medium contained (per L): 35.0

g glucose, 9.22 g $Na(CH_3COO)\cdot 3H_2O$, 2.51 g K_2HPO_4, 1.44 g KH_2PO_4, 0.4 g NH_4Cl, 4.0 g $(NH_4)_2SO_4$, 0.15 g $MgSO_4\cdot 7H_2O$, 0.01 g $CaCl_2\cdot 2H_2O$, 0.05 g $Na_2EDTA\cdot 2H_2O$, 0.25 mg $ZnSO_4\cdot 7H_2O$, 0.125 mg $CuCl_2\cdot 2H_2O$, 1.25 mg $MnSO_4\cdot H_2O$, 0.875 mg $CoCl_2\cdot 6H_2O$, 0.06 mg H_3BO_3, 0.8859 mg $Al_2(SO_4)_3$, 0.25 mg $Na_2MoO_4\cdot 2H_2O$, 5.50 mg $FeSO_4\cdot 7H_2O$, 0.02 g thiamine·HCl, 0.2 g L-isoleucine.

Flasks were incubated at 37°C and 250 rpm (19 mm pitch). In bioreactors, the pH was maintained at 7.0 with 20% (w/v) NaOH, the temperature at 37°C, and the agitation at 400 rpm. An air flowrate of 1.0 L/min ensured that the dissolved oxygen concentration remained above 40% saturation (Unit Instruments mass flow controllers, Orange, CA).

Chemostat

Continuous fermentations of 1.0 L volume operated as chemostats and were initiated in batch mode in a 2.5 L bioreactor (Bioflow 2000, New Brunswick Scientific Co. Inc. Edison, N.J., USA). A steady-state condition was assumed after five residence times at which time the oxygen and CO_2 concentrations in the effluent gas remained unchanged. Several nutrient-limited chemostats were examined by altering the concentration of one nutrient in CGAM medium while leaving others unchanged. Specifically, for glucose-limitation the feed glucose concentration was 5.0 g/L, for acetate-limitation the feed contained 2.30 g/L $Na(CH_3COO)\cdot 3H_2O$, for nitrogen-limitation the feed contained 0.1 g/L NH_4Cl and 1.0 g/L $(NH_4)_2SO_4$, while for a phosphate-limited chemostat the feed contained 0.08 g/L K_2HPO_4 and 0.04 g/L KH_2PO_4.

Fed-batch processes

Fed-batch processes were carried out in a 2.5 L bioreactor (Bioflow 2000, New Brunswick Scientific Co. Edison, NJ, USA) initially containing 1.0 L GAM medium. Cells grew

at maximum specific growth rate until the initial acetate was nearly exhausted (OD about 3.0). At this time, the fed-batch mode commenced by exponentially feeding a solution containing 600 g/L glucose and 30 g/L acetate so that cell growth was controlled at a constant specific rate of about 0.15 h^{-1}. The pH was controlled at 7.0 using 5% (w/v) NH_4OH/25% KOH, the temperature was controlled at 37°C, and the agitation maintained at 400 rpm. Air and O_2 were mixed as necessary at 1.0 L/min total flow rate to maintain a dissolved oxygen concentration (DO) above 40% of saturation.

Analyses

The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, Calif.) was used to monitor cell growth, and this value was correlated to dry cell mass. Concentrations of soluble organic compounds were determined by liquid chromatography (Eiteman and Chastain, 1997). Concentrations of oxygen and CO_2 in the off-gas were measured (Ultramat 23 gas analyzer, Siemens, Germany).

Results

Selection of limiting substrate

Escherichia coli containing gene mutations corresponding to major pathways involved in pyruvate metabolism (i.e., *aceEF ldhA pfl poxB pps*) will accumulate that product under aerobic conditions in a medium containing both glucose and acetate (Zelic et al., 2003). We propose that the highest productivity and pyruvate yield from glucose will occur when cell growth is limited and under conditions of excess glucose to maximize glucose consumption rate and hence glycolytic flux. To test this hypothesis, we conducted chemostats using ALS929 to compare four different nutrient limitations: glucose, acetate, nitrogen (ammonium), and phosphorus

(phosphate). Table V-2 summarizes key parameters determined from these steady-state experiments at the dilution rate of 0.15 h^{-1}.

The greatest specific glucose consumption rate q_G was observed when acetate limited growth. This maximum of 1.60 g/g·h is about twice as great as the value observed when either nitrogen or phosphorus limited growth and over 4 times greater than the q_G attained when glucose limited growth. The value of q_G was indeed related to pyruvate formation, with acetate-limited growth also achieving the greatest rate of pyruvate formation at 1.10 g/g·h. Acetate-limited growth thereby resulted in a pyruvate mass yield based on glucose consumed of 0.70 g/g. Nitrogen-limited conditions generated pyruvate at about 50% of this maximal rate, and achieved a similar mass yield of 0.67 g/g. However, phosphorus-limited conditions accumulated pyruvate at a much lower rate: the rate of pyruvate formation was only 0.20 g/g·h with a yield of 0.26 g/g. The pyruvate yield was similar for glucose- and phosphorus-limited conditions.

Since acetate is required for the growth of ALS929, the molar ratio of glucose-to-acetate consumed should be an indicator of the excess glucose which is used by the cell but does not generate biomass. Values for this molar G:A ratio were 0.41, 1.4, 0.70 and 8.1 respectively for glucose-, nitrogen-, phosphorus- and acetate-limited growth. Thus, under acetate-limited conditions the cell consumed 8 glucose molecules for every acetate, while under glucose-limited conditions the cells consumed almost 2.5 acetate molecules for every glucose.

The specific nutrients which limited growth also affected respiration and carbon dioxide evolution. Phosphorus-limited cells consumed 58% more oxygen than acetate-limited cells, and generated over 7 times more CO_2 than acetate-limited cells. The very low carbon dioxide evolution rate when acetate limited growth (RQ was 0.127) provides further evidence that

glucose very effectively was converted to pyruvate with minimal "loss" of carbon, and therefore for subsequent studies we used acetate as the limiting substrate.

Genetic approaches to elevate glycolytic flux

With mutations in major metabolic pathways from pyruvate, ALS929 generates pyruvate and has limited ability to generate other soluble by-products from glucose. The acetate-limited chemostat achieved a molar carbon recovery of 82%, though no other product was detected in the effluent using liquid chromatograph with refractive index detection. (We analyzed for all the tricarboxylic acid cycle intermediates and other common products of *E. coli* bioprocesses). In order to increase yield particularly, the yield of biomass itself would have to be reduced. We therefore explored several genetic modifications to increase glycolytic flux and/or reduce the formation of biomass. Three genetic modifications were examined: knockouts in *arcA* and *atpFH*, and the overexpression of heterologous water-forming NADH oxidase. We first conducted acetate-limited chemostats at the same dilution rate of 0.15 h^{-1} using strains with one or more of these genetic modifications.

Previous reports suggest that the ArcA regulator system is controlled by the redox environment or specifically a high $NADH/NAD^+$ ratio, and NADH accumulates in cells which have high glycolytic flux (Iuchi et al., 1994; Georgellis et al., 2001; Vemuri et al., 2006). The expression levels of several respiratory genes repressed by ArcA are increased over seven-fold in an *arcA* knockout (Vemuri et al. 2006). In order to test whether glycolytic flux and pyruvate generation could be increased by relieving this respiratory repression, we knocked out *arcA* in ALS929 to construct ALS1054 and characterized this strain in an acetate-limited chemostat (Table V-3). ALS1054 had similar biomass and pyruvate yield on glucose as ALS929, and the specific rates q_G and q_{Pyr} were slightly lower, suggesting that respiratory processes and other

enzymes under the control of the ArcA system did not limit glycolysis in ALS929 under these conditions.

Membrane bound (F_1F_0) H^+-ATP synthase (EC 3.6.1.34) is responsible for oxidative phosphorylation (Sorgen et al., 1998) and catalyzes ATP synthesis by capturing the energy of the transmembrane electrochemical potential (Ono et al., 2004). In previous studies knockouts in the subunits *b* and *c* of F_0 encoded by *atpFH* reduced cell yield and growth rates slightly but increased by two-fold the glycolytic flux compared to a wild-type (Causey et al., 2004). In our study, we similarly eliminated ATP synthesis via oxidative phosphorylation by constructing the *atpFH* knockout ALS1058. With ALS1058 under steady-state conditions, the specific glucose consumption rate was increased 36%, and the specific pyruvate productivity was increased 49% compared to the parent strain ALS929 (Table V-3). An *atpFH* deletion also reduced cell yield on glucose by 30% and increased pyruvate yield to 0.76. These factors resulted in a 49% increase in the yield of pyruvate on biomass: 10.7 g pyruvate was generated for every 1 g of ALS1058 biomass generated. Under acetate-limited *steady-state* conditions ALS1058 consumed 9.2 glucose molecules for every acetate consumed. The vast majority of glucose has been diverted to the product pyruvate instead of biomass.

We next examined the combination of an *atpFH* and the *arcA* knockout by constructing strain ALS1059. Whereas the *arcA* mutation alone did not improve glucose consumption and pyruvate formation, the *arcA* knockout in the *atpFH* strain did improve these parameters beyond the levels observed with the *atpFH* knockout alone (Table V-3). ALS1059 showed a specific rate q_G of 2.38 g/g·h, 49% greater than the parent ALS929, and a pyruvate yield of 0.78 g/g. Interestingly, ALS1059 generated almost three times as much CO_2 as ALS929, and 59% more than ALS1058 containing only the *atpFH* knockout. Despite this increase in CO_2 generation,

CO_2 remains a very small fraction of the total carbon products. Only 3.7% of the total carbon consumed by ALS1059 leads to CO_2 formation, compared to 2.0% of the total carbon consumed by ALS929 in an acetate-limited chemostat.

During elevated glucose consumption rate, respiration reaches a capacity and cells accumulate NADH (Vemuri et al., 2006). Introduction of water-forming NADH oxidase provides cells with another means to reoxidize NADH to NAD^+ without energy generation. To determine whether providing cells with another outlet for NADH, we transformed ALS929, ALS1054 and ALS1059 with pTrc99A-*nox* expressing the water-forming NADH oxidase from *S. pneumoniae*. Introduction of NADH oxidase activity into ALS929 and ALS1054 resulted in slight decreases in specific glucose consumption and pyruvate formation rates (Table V-2). However, for ALS1059/pTrc99A-*nox* these rates were increased by about 10% compared to ALS1059. Furthermore, 12.9 g of pyruvate were generated for every 1 g of ALS1059/pTrc99A-*nox*.

Fed-batch processes

Although chemostat experiments provide steady-state information and high volumetric rates, such an operational mode does not permit the product pyruvate to accumulate to a significant final concentration. We therefore next examined a fed-batch process using an exponential feed in order to maintain a constant specific growth rate of 0.15 h^{-1}. Similar to the chemostat experiments, the growth was limited by acetate (except for a short initial portion of the process).

One disadvantage of fed-batch processes is that numerous substances accumulate, not only the desired product. Because the accumulation of pyruvate in our current study would simultaneously require the accumulation of K^+ (or another counterion) to maintain the pH, we

conducted an experiment to determine whether the addition of the known osmoprotectant betaine (Underwood et al. 2004; Zhou et al. 2006) would improve pyruvate accumulation. Without betaine present in the medium under acetate-limited conditions, ALS929 was able to accumulate over 56 g/L pyruvate in about 28 h (Figure V-2). At this time, cell growth ceased at an OD of 17, even though acetate was still being consumed. In the presence of 5 mM betaine, ALS929 accumulated over 70 g/L pyruvate in 34 h with the OD achieving 24. Because growth was limited by the feed rate, the presence of betaine did not affect the growth rate or the rate of pyruvate formation. Similar to the chemostat results (Table 3), the maximum specific rate of pyruvate formation q_{Pyr} was about 1.2 g/g·h. The average productivity over the course of the process was 2.1 g/L·h, although the maximum rate was about 5.8 g/L·h. Betaine did not improve pyruvate yield, but merely allowed the process to continue to a 25% higher final pyruvate concentration. We therefore used 5 mM betaine in subsequent fed-batch experiments.

We next examined pyruvate formation in a fed-batch process with ALS1059, the strain which attained a 67% higher steady-state rate of pyruvate formation than ALS929 (Table V-3). In the presence of 5 mM betaine, ALS1059 accumulated about 90 g/L pyruvate in 44 h with a yield of 0.68 g/g (Figure V-3). As expected, the biomass generation with ALS1059 was lower than observed for ALS929. Interestingly, the higher specific rate was compensated by lower biomass concentration, resulting in an average volumetric productivity of about 2.1 g/L·h, identical to ALS929. With a higher final pyruvate concentration and lower biomass concentration, 14.4 g pyruvate was generated for each 1 g of biomass.

Discussion

E. coli generates pyruvate aerobically primarily through glycolysis and the

PEP:carbohydrate phosphotransferase system (PTS). Pyruvate is metabolized by numerous pathways, including pyruvate dehydrogenase, pyruvate formate lyase, pyruvate oxidase, PEP synthase and lactate dehydrogenase. A strain with knockouts in these pathways accumulates pyruvate as the main product (Zelic et al. 2003). In order to increase pyruvate yield from glucose, cell growth must be limited and excess glucose supplied. An exponential fed-batch process is ideal to limit growth at a constant rate, reducing aeration demand and offering controlled growth conditions (Lee et al., 1999; Babaeipour et al., 2008).

In the present study we found that acetate limitation resulted in the highest rate of pyruvate accumulation among four limiting nutrients examined. Glucose limitation would be expected to limit glycolysis directly, and in our study this operational mode resulted in the lowest rate of pyruvate formation. Acetate-limited growth led to a nearly two-fold greater pyruvate accumulation than phosphorus or nitrogen limitation. Of course, ALS929 is unable to synthesize the essential biomass precursor acetyl-CoA from pyruvate, and requires acetate for the synthesis of acetyl-coA via acetyl-CoA synthase (Figure V-1). One possible explanation for the high rate of pyruvate formation under acetate limitation is that pyruvate kinase, which directly forms pyruvate, is allosterically inhibited by acetyl-CoA (Garrett and Grisham 1998). Therefore, when ALS929 is limited for growth by acetate compared to limitation by any other nutrient, the cells would presumably be limited in acetyl-CoA. When the strain is limited by another nutrient such as nitrogen or phosphorus, acetate and acetyl CoA would be in excess. Under conditions of limited acetyl CoA, allosteric inhibition of pyruvate kinase by acetyl-CoA should be minimized, allowing glycolysis to operate maximally.

We speculate that the reduced pyruvate yield under phosphorus-limited conditions (compared to nitrogen-limited conditions) may be a result of very strong uncoupling of

anabolism and catabolism in excess carbon cultures which limits the glycolysis in favor of less energy-efficient pathways, such as pentose phosphate pathway (Dauner et al., 2001). For *Bacillus subtilis* the oxidative pentose phosphate pathway flux exceeded 50% of the total glucose flux in a slow-growing (0.10 h^{-1}), phosphorus-limited chemostat (Dauner et al., 2001). In *E. coli* the *gnd* gene encoding the pentose phosphate pathway enzyme phosphogluconate dehydrogenase which releases CO_2, was overexpressed 5-fold under phosphorus-limited conditions (VanBogelen et al. 1996). In the current study, the high CO_2 evolution rate and low yield observed during phosphorus-limited conditions is consistent with a higher carbon flux through the pentose phosphate pathway.

Given acetate-limited conditions, genetic modifications can also increase glycolytic flux while minimizing biomass formation. The general conclusion from previous is that glycolytic flux control primarily resides outside the glycolytic pathway. For example, glycolytic flux is enhanced by decreasing the cell's energy level through overexpressing F_1 subunit or disrupting F_0 subunit of (F_1F_0) H^+-ATP synthase complex (Koebmann et al. 2002; Oliver 2002; Causey et al. 2004). Moreover, overexpression of enzyme II^{Glc} of the PTS did not increase the flux in *E. coli* (Ruyter 1991). The (F_1F_0) H^+-ATP synthase complex consists of two subunits, the membrane bound F_0 which forms a proton channel, and the cytoplasmic F_1 which contains the catalytic site for ATP formation (Koebmann et al. 2002). The whole enzyme complex catalyzes ATP synthesis by utilizing the energy of a transmembrane electrochemical potential of proton, while the soluble F_1 subunit can hydrolyze ATP in vitro independently of the F_0 subunit (Ono et al., 2004). Deletion of subunits *b* and *c* of F_0 (encoded by *atpFH*) inactivated F_0 and resulted in the separation of F_1 sector from the membrane (Causey et al. 2003; Sorgen et al. 1998). Therefore, ATP synthesis by oxidative phosphorylation through F_0 is disrupted while the hydrolytic activity

of F_1 subunit in the cytoplasm was preserved. Deletion of *atpFH* increases the availability of ADP and AMP (Causey et al., 2004), which respectively activate phosphofructokinase-I and pyruvate kinase-II in *E. coli* (Kotiarz et al. 1975; Babul et al. 1978), and minimizes the allosteric inhibition of these enzymes by ATP (Garrett and Grisham 1998). Consistent with the previous studies (Koebmann et al. 2002; Causey et al. 2004), our results show that (F_1F_0) H^+-ATP synthase complex mutation (Δ*atpFH*) significantly increased the steady-state specific glucose consumption and pyruvate production rates while reducing the cell yield (Table V-3).

NADH is another global cofactor which impacts the physiological state. Acetate overflow metabolism commences when the NADH/NAD^+ ratio reaches a threshold value, and overflow can be reduced by increasing the NADH oxidation rate (Vemuri et al. 2006). A high NADH/NAD^+ ratio limits the availability of NAD^+ required for glycolysis since the total nicotinamide adenine dinucleotide pool is normally constant (Wimpenny and Firth 1972), and NADH inhibits several enzymes in glycolysis allosterically (Causey et al. 2004; Vemuri et al. 2006). Our chemostat results show that the introduction of NADH oxidase with or without the *arcA* deletion had little effect on specific glucose consumption rate and pyruvate productivity in ALS929, suggesting that respiratory processes under the control of the ArcA system and NADH/NAD^+ ratio did not limit glycolysis in ALS929. However, when the glycolytic rate was substantially increased as a result of the *atpFH arcA* double mutation (ALS1059), the introduction of NADH oxidase did further improve glycolytic flux. The higher rate of NADH generation in ALS1059 may cause NAD^+ availability to limit glycolytic flux.

Although the fed-batch process using ALS929 at a low constant growth rate achieved a high specific pyruvate generation rate, cell growth ceased when pyruvate reached about 40 g/L, with pyruvate ultimately 56.3 g/L. Previous studies proposed that cell growth and pyruvate

formation were inhibited by a high extracellular pyruvate concentration (Zelic et al. 2003; Zelic et al. 2004). Another explanation for the observed termination of cell growth and pyruvate formation is the high osmotic stress caused by the K^+ ion required to maintain the pH. Organic osmolytes such as betaine often naturally accumulate to protect cells from high osmotic stress (Yancey 2005). The production of ethanol (Underwood et al. 2004) and lactate (Zhou et al. 2006) have been improved by the addition of betaine to the medium. Our fed-batch results similarly demonstrated that 5 mM betaine prolonged cell growth and thereby increased the final pyruvate concentration by at least 25%. The 1M pyruvate concentration attained in this study implies that previous results were not limited as a result of pyruvate inhibition.

In summary, limiting cell growth with acetate provides a convenient means to sustain a high rate of glycolysis in a metabolically engineered *E. coli* strain blocked in pyruvate metabolism. The specific glucose consumption was further elevated by the combination of *atpFH* and *arcA* knockouts, with heterologous NADH oxidase providing only a modest further increase in glycolytic rate in the double knockout. We report the highest pyruvate concentration achieved using *E. coli* in defined medium: 90 g/L with an overall productivity of 2.1 g/L·h and yield of 0.68 g pyruvate/g glucose.

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Table V-1. Strains used in this study.

Strain	Genotype	Reference
YYC202	Hfr *zbi*::Tn*10 poxB1* Δ(*aceEF*) *rpsL pps-4 pfl-1*	John E. Cronan, Jr., Univ. Illinois
ALS929	YYC202 *ldhA*::Kan	This study
ALS1054	YYC202 *ldhA*::Kan *arcA726::FRT*	This study
ALS1058	YYC202 *ldhA*::Kan *atpFH*::Cam	This study
ALS1059	YYC202 *ldhA*::Kan *arcA726::FRT atpFH*::Cam	This study
CGSC11117 (JW4364-1)	Δ(*araD-araB*)567 Δ*lacZ*4787(::*rrnB*-3) Δ(*rhaD-rhaB*)568 *hsdR*514 *rph*-1 λ- Δ*arcA*726::(FRT)Kan	Baba et al., 2006
DY330	F- *mcrA mcrB* IN(*rrnD-rrnE*)1 Δ(lac)U169 *gal*490 λ(cI857 Δ(*cro-bioA*))	Yu et al., 2000
NZN 111	F+ λ- *rpoS*396(Am) *rph*-1 *ldhA*::Kan Δ(*pflAB*::Cam)	Bunch et al., 1997

Table V-2. Comparison of limiting nutrients during chemostats of *E. coli* ALS929 using a dilution rate of 0.15 h^{-1}.

Growth limiting nutrient	q_G (g/g·h)	q_A (g/g·h)	q_{Pyr} (g/g·h)	q_{O2} (mmol/g·h)	q_{CO2} (mmol/g·h)	$Y_{X/G}$ (g/g)	$Y_{Pyr/G}$ (g/g)	$Y_{Pyr/X}$ (g/g)
Glucose	0.36	0.28	0.085	10.97	6.27	0.43	0.24	0.56
Nitrogen	0.85	0.20	0.57	12.35	4.30	0.18	0.67	3.72
Phosphorus	0.77	0.36	0.20	13.05	8.06	0.20	0.26	1.32
Acetate	1.60	0.065	1.11	8.65	1.10	0.10	0.70	7.15

q_G: specific glucose consumption rate
q_A: specific acetate consumption rate
q_{Pyr}: specific pyruvate productivity
q_{O2}: specific oxygen consumption rate
q_{CO2}: specific carbon dioxide evolution rate
$Y_{X/G}$: mass yield coefficient of biomass/glucose
$Y_{Pyr/G}$: mass yield coefficient of pyruvate/glucose
$Y_{Pyr/X}$: mass yield coefficient of pyruvate/ biomass

Table V-3. Comparison of *E. coli* strains during acetate-limited chemostats using a dilution rate of 0.15 h^{-1}.

Strain	q_G (g/g·h)	q_A (g/g·h)	q_{Pyr} (g/g·h)	q_{O2} (mmol/g·h)	q_{CO2} (mmol/g·h)	$Y_{X/G}$ (g/g)	$Y_{Pyr/G}$ (g/g)	$Y_{Pyr/X}$ (g/g)
ALS929	1.60	0.065	1.11	8.65	1.10	0.10	0.70	7.15
ALS1054	1.46	0.054	1.04	7.30	1.29	0.11	0.72	6.72
ALS1058	2.18	0.085	1.65	10.81	1.89	0.07	0.76	10.67
ALS1059	2.38	0.091	1.86	10.60	3.00	0.07	0.78	11.97
ALS929/pTrc99A-*nox*	1.42	0.061	0.97	6.55	1.27	0.11	0.68	6.28
ALS1054/pTrc99A-*nox*	1.17	0.086	0.85	7.90	1.46	0.13	0.73	5.51
ALS1059/pTrc99A-*nox*	2.67	0.117	2.01	9.76	1.71	0.06	0.75	12.94

q_G: specific glucose consumption rate
q_A: specific acetate consumption rate
q_{Pyr}: specific pyruvate productivity
q_{O2}: specific oxygen consumption rate
q_{CO2}: specific carbon dioxide evolution rate
$Y_{X/G}$: mass yield coefficient of biomass/glucose
$Y_{Pyr/G}$: mass yield coefficient of pyruvate/glucose
$Y_{Pyr/X}$: mass yield coefficient of pyruvate/ biomass

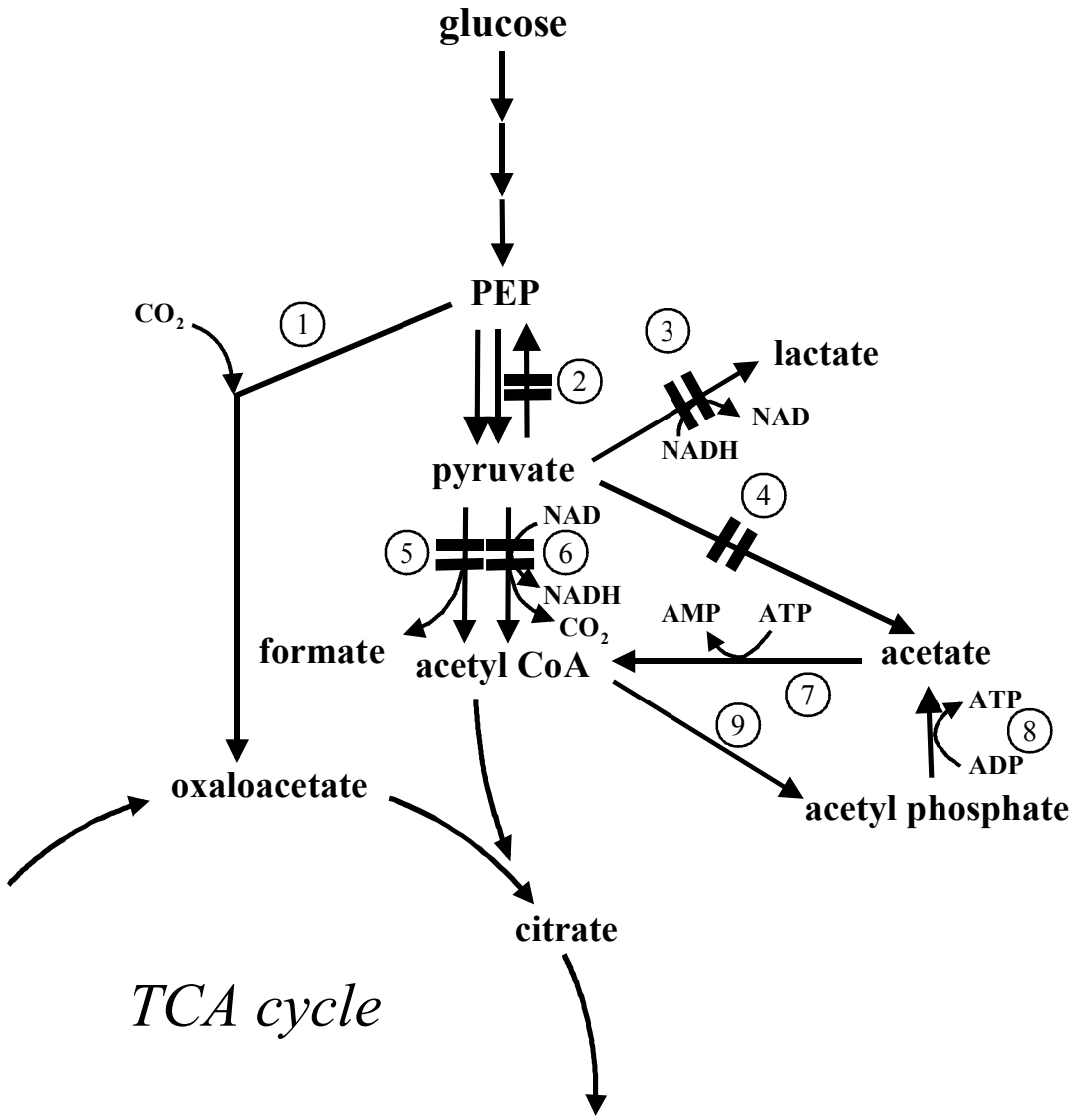

Figure V-1. Key enzymatic reactions in the production of pyruvate by *Escherichia coli* strains. Enzymes: 1) PEP carboxylase, 2) PEP synthase, 3) lactate dehydrogenase, 4) pyruvate oxidase, 5) pyruvate formate lyase, 6) pyruvate dehydrogenase complex, 7) acetyl CoA synthetase, 8) acetate kinase, 9) phosphotransacetylase.

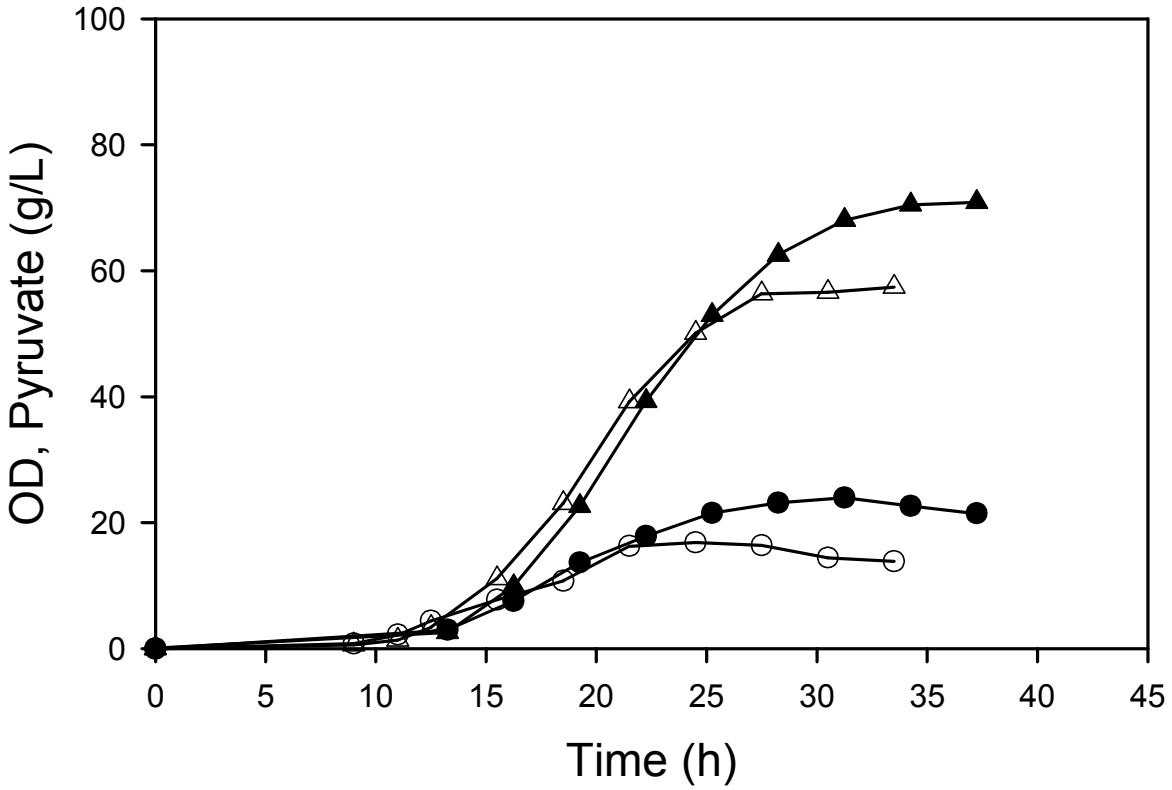

Figure V-2. The effect of 5 mM betaine (solid symbols) during a fed-batch process using *E. coli* ALS929 at a growth rate of 0.15 h^{-1}: (○,●) OD; (△,▲), pyruvate.

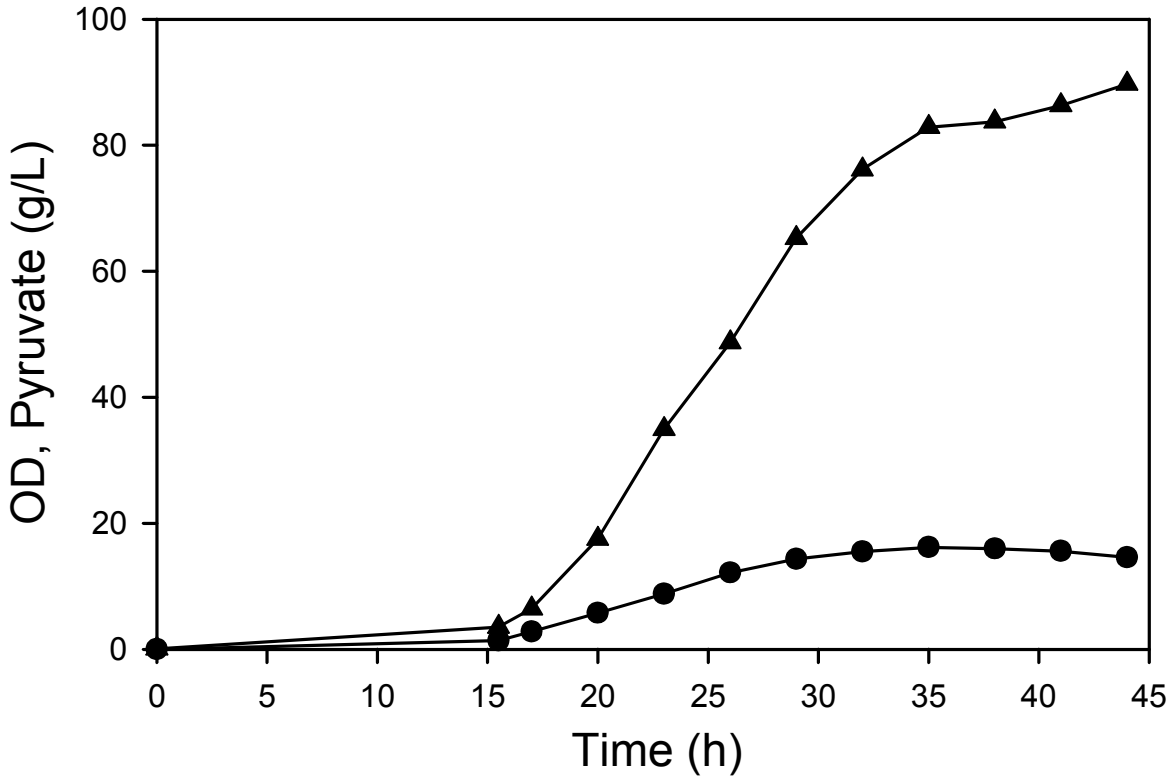

Figure V-3. A fed-batch process using *E. coli* ALS1059 at a growth rate of 0.15 h^{-1} in the presence of 5 mM betaine: (●) OD; (▲), pyruvate.

CHAPTER VI

SUMMARY

The fundamental aim of this research was to understand aspects of the regulation of microbial central metabolism and use metabolic engineering tools and fermentation process development to maximize the production of two model organic acids, pyruvate and lactate. Wild-type microorganisms normally are not able to accumulate a single compound at a high rate and to a high concentration since metabolism is tightly regulated. Development of recombinant DNA technology provides a new way for directed improvement of product yield and productivity. For example, by deleting pathways leading to by-products, flow of substrate can be redirected toward products. Fermentation process development is also essential for the improvement of product formation, and altering pathways can affect the process conditions such as pH, temperature, oxygenation, and ion strength. Pyruvate and lactate have similar structure but have different requirements for both the microorganism and the fermentation.

In *E. coli* (and many organisms), pyruvate is the terminal product of glycolysis. Lactate is mainly generated from pyruvate by a reduction catalyzed by lactate dehydrogenase (LDH). Both pyruvate and lactate processes require the elimination of key pathways which direct pyruvate to other products. *E. coli* strain YYC202 having mutations in *aceEF pfl poxB pps* genes is an ideal starting strain for both pyruvate and lactate formation since it does not permit pyruvate to flow to the TCA cycle, formate, or acetate.

The difference between pyruvate and lactate formation lies in the conversion between

these compounds by LDH. For pyruvate production, lactate is undesirable and therefore LDH must be deleted. For lactate production, LDH is the key enzyme catalyzing the final step. Another difference is that the conversion of glucose to lactate is redox balanced whereas the conversion of glucose to pyruvate generates 2 moles of NADH per mole of glucose. Therefore, a process for pyruvate formation requires the presence of an electron acceptor while the process for lactate requires the absence of electron acceptors. Oxygen is a convenient electron acceptor because the product of its reduction is water. In this research, pyruvate production therefore used aerobic conditions whereas lactate production required anaerobic conditions.

These metabolic differences impacted the strategy for the ultimate processes. For lactate production, a consequence of requiring anaerobic conditions was the formation of reduced by-products. Therefore, for the portion of the research, the focus was how to reduce the formation of succinate, which was the principal reduced product. For pyruvate production, by-product formation was not observed in aerobic conditions. The focus of pyruvate production was how to increase the mass yield and glycolytic flux.

The differences in cell-growth status also impacted the strategy for overcoming high osmotic stress or end-product inhibition. For lactate, cells are not growing in the anaerobic phase for lactate production. $Ca(OH)_2$ instead of NaOH could be used as base to neutralize pH and ultrafiltration could be used for recycling cells and alleviating lactate inhibition. However, these two methods could not be used in cell-growth associated pyruvate production due to precipitation caused by Ca^{2+} and limited oxygenation attainable in an ultrafiltration system. Instead, betaine was used as protective osmolytes to protect cells from high osmotic stress and increase cell growth and pyruvate production.

Although strategies for pyruvate and lactate production have many differences, high glucose

consumption rates and therefore high productivities have been achieved in both cases. The reason is that deletions in *aceEF*, *pfl* and *poxB* genes necessitate the use of acetate in addition to glucose as a carbon source for cell growth. Acetate is the only source for acetyl-CoA, which is essential for cell growth but also a negative feedback controller for glycolysis. Acetate is exhausted in anaerobic phase for lactate production and is the limiting substrate (in very low concentration) in the fed-batch process for pyruvate production. For both products, under conditions of low acetate and consequently low acetyl-CoA, allosteric inhibition of pyruvate kinase by acetyl-CoA should be minimized, allowing glycolysis to operate maximally. This strategy may also be used in the processes for other products requiring high glycolytic flux.

Relationship between metabolic modification and process development

Metabolic modification and process development are important *interrelated* approaches for improving yield and productivity in microbial fermentations. In this study for example, deletions in *aceEF*, *pfl* and *poxB* genes necessitated the use of isoleucine and acetate. Furthermore, an *aceEF pfl poxB* strain grows poorly under anaerobic conditions, but anaerobic conditions are required for lactate generation. Therefore, for this product a two-phase process separated cell growth from a production phase. Under aerobic conditions for pyruvate formation, the growth requirement for acetate of an *aceEF pfl poxB* strain provided an additional choice for a substrate to limit cell growth. Indeed, acetate-limitation not only increased pyruvate yield but also provided the highest glycolytic flux and pyruvate productivity.

The combination of metabolic modification and process development was required for solving specific problems. For example, to reduce the formation of succinate in lactate production, a strategy involving both metabolic modification and process development was

employed. Succinate could be generated from both substrates glucose and acetate. A deletion in *frdABCD*, which encodes the enzyme catalyzing succinate-generating reaction, reduced succinate concentration to 3 g/L from about 9 g/L. A ^{13}C-labeled fermentation process demonstrated that this 3 g/L succinate was generated from residual acetate in the medium which was added in the aerobic phase for cell growth. A process using the dissolved oxygen (DO) during the initial cell-growth phase to monitor residual acetate, and timing the switch to the anaerobic production phase with acetate depletion, further reduced succinate production to less than 1 g/L.

Special fermentation processes, such as the chemostat, were be used to evaluate metabolically engineered strains and compare fermentation conditions. For pyruvate production, a series of chemostats were conducted to select limiting substrates and also to evaluate strains and identify which global cofactor, ATP or NADH, limits the glycolytic flux.

With comprehensive consideration and proper combination of metabolic modification and process development, high yields and productivities were achieved for both pyruvate and lactate production. This strategy and the understanding of central metabolism directly related to pyruvate and lactate production could provide valuable information for improvements of other microbial fermentation products.

Future study in pyruvate and lactate production

Although high pyruvate and lactate concentrations and yields have been achieved, further improvement could be made.

For pyruvate production, only one specific growth rate was studied in both chemostats and fed-batch processes. Although biomass competes with pyruvate for glucose, biomass is of course

essential for pyruvate. At a same specific productivity, volumetric productivity increases with more biomass. Chemostats conducted to compare the effect of dilution rate (0.20 h^{-1}, 0.25 h^{-1}, 0.30 h^{-1}, etc.) on the specific pyruvate productivity, and acetate-limited fed-batch processes at different specific growth rates could further establish the relationship between yield and volumetric productivity, and optimize process conditions.

ALS1062 had the highest specific glucose consumption rate and pyruvate productivity in the chemostat. However, in the fed-batch process, the final concentration and volumetric productivity were much lower for ALS1062 than for ALS1059. Due to the high burden of plasmid reproduction and maintenance, ALS1062 has a long lag phase and grow slowly. The high specific pyruvate productivity of ALS1062 cannot compensate for the low biomass generated in fed-batch process, which resulted in low volumetric productivity. But the chemostat result of ALS1062 already demonstrated the high NADH/NAD^+ ratio is a limiting factor of glycolysis. By further understanding respiratory pathways in *E. coli*, the ability of strains to reoxidize NADH might be increased by overexpressing respiratory enzymes or regulatory proteins without introducing high copy plasmid. This might be accomplished by replacing promoters of targeted genes, or using low copy plasmid.

By further understanding the inhibition mechanism of high osmotic stress and ion strength on cell growth and pyruvate production, additional genetic modifications might be able to increase tolerance of strain to high ion strength. These strain improvements might obviate the requirement for betaine.

For lactate production, although lactate productivity was increased by ultrafiltration process, the activity of cells decreased after 3-4 cycles significantly. An immobilized cell method could be used to protect cells from dying and losing activity. An immobilized cell method could also

simplify the anaerobic process since cells can be separated from medium by a simpler way than ultrafiltration process.