PRODUCTION OF PYRUVATE BY *ESCHERICHIA COLI*

USING METABOLIC ENGINEERING

by

Fei Yang

(Under the Direction of Dr. Mark A. Eiteman)

Pyruvate is a three-carbon ketoacid produced at the end of glycolysis. This research involves the metabolic engineering of the bacterium *Escherichia coli* in order to generate pyruvic acid (pyruvate). The Plackett-Burman design was used for a media optimization. Feeding strategies were also optimized. The addition of uncoupler 2,4-dinitrophenol increased pyruvate production and decreased lactate accumulation for CGSC6162 and CGSC6162 *ppc*, but the improvement is not significant. Using the "optimal media" for four strains CGSC6162, CGSC6162 *ppc,* CGSC6162 *poxB,* and CGSC6162 *ldhA* showed no significant difference in cell growth or on final pyruvate concentration. Fermentations at 37°C generally produced more pyruvate and less acetate than at 40°C. 37°C is the optimal temperature compared to 34°C and 40°C.

INDEX WORDS: Pyruvate, *Escherichia coli*, Pyruvate dehydrogenase complex, Phosphoenolpyruvate carboxylase, Pyruvate oxidase, NADH oxidase, 2,4-dinitrophenol, Plackett-Burman design

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FEI YANG

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FEI YANG

Approved:

Major Professor: Mark A. Eiteman

Committee: Guigen Zhang

Joy Peterson

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2004

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Fei Yang

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CHAPTER 1

INTRODUCTION

Microbial fermentation processes are used to produce a wide variety of biochemicals. Fermentations may be improved by genetically modifying microbial metabolism. Metabolic engineering is the rational alteration of metabolism in order for cells to generate a new product or to generate a product at a higher yield or rate. This research involves the metabolic engineering of the bacterium *Escherichia coli* in order to generate pyruvic acid (pyruvate).

Pyruvate

Pyruvate is a three-carbon ketoacid produced at the end of glycolysis. There is an increasing demand for pyruvate since it can be used to synthesize many drugs and biochemicals. For example, pyruvate is a raw material to produce the amino acids L-tryptophan (Nakazawa et al., 1972), L-tyrosine, and 3,4-dihydroxyphenyl-L-alanine (Yamada et al., 1972). Pyruvate finds applications in the chemical industry and in cosmetics. Animal studies suggest that pyruvate leads to weight loss by increasing the resting metabolic rate (Ivy et al., 1994). A few clinical trials also indicate that pyruvate supplements may improve exercise endurance (Stanko, 1990a; Stanko, 1990b). Calcium pyruvate is used as a dietary supplement for both sedentary and active populations due to its ability to accelerate the metabolism of fatty acids (Stanko et al., 1992, Roufs et al., 1996). In addition, pyruvate appears to function as an antioxidant to inhibit the production of harmful free radicals (Deboer et al., 1993), and it has been shown to inhibit the growth of cancer tumors in animals (Stanko et al., 1994). However, this effect has not been confirmed in human studies. Pyruvate

is also employed in the production of crop protection agents, polymers and food additives (Li et al., 2001). As pyruvate has been widely used in pharmacy, agrochemical, biochemical, food industries and cosmetics, the commercial demand for pyruvate has been increasing (Yonehara et al., 1994).

Biological production of pyruvate

Three biological methods exist to produce pyruvate: using resting cells, an enzymatic route and direct fermentation (Li et al., 2001). In the resting cell method a substrate (such as lactate) is converted to pyruvate by cells separated from the growth medium. Microorganisms such as *Acinetobacter* sp. and *Debaryomyces coudertii* have been used to produce pyruvate in the resting cell method (Li et al., 2001). Cells are cultivated, separated and washed before being used in the biosynthesis of pyruvate. Such multiple steps seem to be the great drawback for the application of the resting cell method. In the enzymatic method, pyruvate is synthesized from a substrate (such as lactate) by a single enzyme in microbial cells instead of a series of enzymes as in the resting cell method. For example, *Acetobacter* sp. can oxidize D-(-)-lactate to pyruvate with a high conversion rate (Cooper, 1989). However, this process is not economical since D-(-)-lactate is relatively expensive. Similarly, glycolate oxidase will oxidize L-lactate to pyruvate with a high substrate conversion rate in *Hansenula polymorpha* (Anton et al., 1995). The principal shortcoming of the glycolate oxidase process is that hydrogen peroxide is produced during the conversion, and it must be removed or transformed to avoid the pyruvate from being further oxidized to acetate. Direct fermentation has advantages of comparatively low cost and potentially high product purity. However, a

potential bottleneck of direct fermentation is that only a small amount of pyruvate is generally secreted into the culture broth from wild type microorganisms since pyruvate is a key metabolic intermediate. Efforts have therefore focused on finding a microorganism generating pyruvate at a high yield and rate.

Yeasts are the most widely studied microorganisms used to produce pyruvate from glucose or other carbon sources. The yeasts *D. coudertii* (Moriguchi, 1982) and *Saccharomyces exiguus* (Yokota et al., 1984) are known to accumulate pyruvate, as well as the basidiomycetes *Schizophyllum commune* (Takao et al., 1982) and *Agricus campestris* (Yokota et al., 1984). Yeast strains belonging to the genus *Torulopsis* isolated by a Japanese company accumulate more than 50 g/L pyruvate (Miyata et al., 1989). For example, *Torulopsis glabrata* IFO 0005, having multi-vitamin auxotrophy (thiamine hydrochloride, nicotinic acid, biotin and pyridoxine hydrochloride), accumulated 67.8 g/L pyruvate in 63 hours (yield of 0.494) in a fed-batch fermentation with successive additions of glucose (Miyata et al., 1996). *T. glabrata* ACII-33, a mutant with decreased pyruvate decarboxylase activity, accumulated 60.3 g/L pyruvate in 47 hours (yield of 0.678) in a 3 L fermenter (Miyata et al., 1999). *T. glabrata* WSH-IP303 using ammonium chloride as the sole nitrogen source accumulated 69 g/L pyruvate in 56 hours (yield of 0.620) in a 5 L batch fermenter (Li et al., 2001a). The thiamine concentration significantly affected pyruvate dehydrogenase and pyruvate decarboxylase activities, and played an important role in cell growth and pyruvate production. With addition of thiamine (30 μg/L initially and 10 μg/L during fermentation), the overall pyruvate yield was improved by 15% due to the decrease of ethanol production (Hua et al., 1999).

Although yeasts are commonly used for pyruvate production, prokaryotes can also accumulate pyruvate, including bacteria of the genera *Corynebacterium* (Yokota et al., 1984), *Acinetobacter* (Izumi et al., 1982), *Enterobacter aerogenes* (Yokota et al., 1989). *Corynebacterium* accumulated 4.7 g/L pyruvic acid in four days (Yokota et al., 1984), and *Acinetobacter* accumulated 11.6 g/L in 96 hours (Izumi et al., 1982). *E. coli* mutants also accumulate pyruvate from glucose. A lipoic acid auxotroph of *E. coli*, W1485lip2, accumulated 25.5 g/L pyruvate in 32 hours (yield of 0.51) in polypeptone (4 g/L) supplemented media (Yokota et al., 1994). With *E. coli* W1485lip2 as the parent, an F_1-ATPase-defective mutant strain, TBLA-1, produced more than 30 g/L pyruvate (yield of 0.600) in 24 hours (Yokota et al., 1994).

Another approach for *E. coli* to generate pyruvate from glucose is by using mutants in the pyruvate dehydrogenase complex. CGSC6162 (an *aceF* mutant) and CGSC6162 *Δppc* accumulated greater than 35 g/L pyruvate in a medium supplemented with acetate (Tomar et al., 2002). Pyruvate mass yield from glucose was 0.72 in CGSC6162 with volumetric productivities above 1.5 g/Lh. For CGSC6162 *Δppc*, pyruvate yields of 0.78 and volumetric productivities above 1.2 g/Lh were attained (Tomar et al., 2002).

The pH of the media appears to affect pyruvate production. For the lipoic acid auxotroph of *E. coli*, W1485lip2, the effect of pH on pyruvate production was examined by controlling the pH of the medium at 5.5, 6.0 and 6.5. A controlled pH at 6.0 was optimal and resulted in 25.5 g/L pyruvate from 50 g/L glucose after 32 h at pH 6.0 (Yokota et al., 1994).

Organic nutrients are necessary for the production of pyruvate (Yokota et al., 1994). For the lipoic acid auxotroph of *E. coli*, W1485lip2, growth can be supported by L-lysine plus

L-methionine instead of lipoic acid. Thus, polypeptone, which contains these two amino acids, enhances the cell growth and glucose consumption markedly (Yokota et al., 1994).

Metabolic Pathways of Pyruvate in E. coli

E. coli metabolizes glucose via the Embden-Meyerhof-Parnas (EMP) pathway to a branch point at phosphoenolpyruvate (PEP). The majority of carbon flux from PEP leads to pyruvate via the enzyme pyruvate kinase or via the PEP-dependent transport of glucose into the cell (Clark, 1989). The minority of carbon flux from PEP forms oxaloacetate via the anaplerotic enzyme PEP carboxylase (PPC) to replenish tricarboxylic acid (TCA) cycle intermediates used for cellular components (Gottschalk, 1985). A small fraction of PEP is consumed for the syntheses of aromatic amino acids. In the TCA cycle, reduction potential in the form of three molecules of NADH and one molecule of $FADH_2$ is yielded. A high-energy phosphate bond is also formed with the phosphorylation of GDP to GTP.

Pyruvate is a major metabolic junction linking carbohydrate or amino acid utilization to energy generation and biosynthetic pathways. Some amino acids can enter TCA cycle through conversion to pyruvate followed by anaplerotic sequences which produce cycle intermediates. Likewise, the synthesis of alanine, leucine, isoleucine and valine requires pyruvate. Metabolism of pyruvate in *E. coli* depends on the presence of oxygen in the environment. Under anaerobic conditions, pyruvate is converted to lactate by fermentative lactate dehydrogenase (Clark, 1989), and to formate and acetyl CoA by pyruvate formate lyase. These metabolic routes are necessary as means for the organism to regenerate NAD^+ required in glycolysis. However, under aerobic conditions, lactate dehydrogenase and

pyruvate formate lyase are commonly not active (Clark, 1989). Anaerobic conditions are required to shift the inactive form of pyruvate formate lyase to the active enzyme. During conditions of high pyruvate accumulation even under aerobic conditions *E. coli* converts pyruvate to lactate by the fermentative lactate dehydrogenase (Clark, 1989; Snoep et al., 1991). Under aerobic conditions pyruvate primarily is metabolized to acetyl CoA which enters the TCA cycle or forms acetate. This decarboxylation of pyruvate to acetyl CoA is catalyzed by the multienzyme system called the pyruvate dehydrogenase complex (PDH complex). Acetyl CoA commonly couples with oxaloacetate to form citrate as the initial step of the tricarboxylic acid (TCA) cycle. Acetyl CoA can also go through a two-step process to generate ATP and acetate via the enzymes phosphotransacetylase and acetate kinase.

Pyruvate can be converted directly into acetate and carbon dioxide by pyruvate oxidase (POX). The activity of POX is growth-phase dependent, with the maximum activity occurring at early stationary phase (Chang et al., 1994), and POX makes a significant contribution to growth efficiency in glucose minimal medium (Abdel-Hamid et al., 2001).

Pyruvate oxidase

Pyruvate oxidase (pyruvate: cytochrome b_1 oxidoreductase, EC 1.2.2.2) is a peripheral membrane enzyme which catalyzes the oxidative decarboxylation of pyruvate to yield acetate plus CO_2. (This enzyme is a dehydrogenase and is misnamed.) Pyruvate oxidase is activated by pyruvate and 2-oxobutanoate (α-ketobutyrate), although pyruvate is the preferred substrate. Pyruvate oxidase may function as a safety valve to convert excess pyruvate to acetate rather than to acetyl-CoA, thus maintaining the intracellular CoA pool for other metabolic functions

(Abdel-Hamid et al., 2001).

The oxidase is a slightly acidic protein with a pI of 5.6, near the optimal pH for activity. This enzyme is a homotetramer of 62,000 dalton subunits. Each subunit carries a tightly (but non-covalently) bound molecule of flavin adenine dinucleotide (FAD) and a loosely bound molecule of thiamine pyrophosphate (TPP) which requires Mg^{2+} for binding (Mather et al., 1982; Gennis and Hager, 1976). In other words, thiamine pyrophosphate (TPP) and flavin adenine dinucleotide (FAD) are the cofactors of pyruvate oxidase.

Pyruvate oxidase displays unusual activation phenomena. A wide variety of lipids (phospholipids, neutral lipids, or synthetic detergents) serve as activators of pyruvate oxidase (Mather, et al., 1982; Gennis and Hager, 1976). Hydrophobic interaction between the lipid and protein is critical, and furthermore, no electric charge is required on the lipid for successful activation. Lipid activation is a relatively slow process and depends on the presence of TPP, Mg^{2+}, and pyruvate. Lipid binding increases enzyme affinity for pyruvate and TPP/ Mg^{2+} (Chang and Cronan, 1984). Most of the activating ability of lipid is due to the phospholipid fraction. For example, incubation of the enzyme with phospholipids (phosphatidylethanolamine, PE) followed by a second preincubation period in the presence of TTP, Mg^{2+}, and pyruvate results in a considerably lowered specific activity (55%) compared to that observed when all the components are added to the enzyme simultaneously (Cunningham and Hager, 1971).

Pyruvate oxidase is a type of a “shuttle” enzyme between the cytosol and inner membrane depending on the intracellular pyruvate concentration. When the intracellular pyruvate concentration is low, the enzyme is inactive and located in the cytosol, whereas high

concentrations of pyruvate trigger a conformational change that exposes the C-terminal lipid-binding domain. The enzyme then becomes activated and transfers electrons to the electron transport chain via ubiquinone dissolved in the lipid bilayer (Chang and Cronan, 1984; Carter and Cennis, 1985).

The activity of *E. coli* pyruvate oxidase is growth-phase dependent with activity increasing during growth and reaching a maximum at early stationary phase (Abdel-Hamid et al., 2001). Pyruvate oxidase also makes a significant contribution to growth efficiency in glucose minimal medium (Abdel-Hamid et al., 2001). The expression of *poxB* is regulated by the product of the *rpoS* gene (Chang et al., 1994), a regulatory gene that controls a number of stationary-phase-induced genes and which has been shown to encode a novel σ factor (σ^{38}) preferentially synthesized in the stationary phase (Tanaka et al., 1993).

As a consequence of replacing the PDH complex by pyruvate oxidase, the growth rate, growth yield and the carbon conversion efficiency (flux to biomass) of *E. coli* were lowered, indicating that more carbon has to be oxidized to CO_2 for energy generation (Abdel-Hamid et al., 2001). The *poxB* gene was also shown to be expressed (albeit at a lower rate) during anaerobic growth. Pyruvate oxidase might serve as a source of acetyl units under microaerobic conditions when both the PDH complex and pyruvate formate lyase would function poorly (Chang et al., 1994).

Pyruvate dehydrogenase (PDH) complex

The pyruvate dehydrogenase (PDH) complex of *E. coli* catalyzes the NAD-dependent oxidative decarboxylation of pyruvate to acetyl CoA and CO_2 by three enzymes: pyruvate

decarboxylase (E1), dihydrolipoyl transacetylase (E2), and dihydrolipoyl dehydrogenase (E3). Three coenzymes are also required in this multienzyme system: thiamine pyrophosphate (TTP), lipoic acid, and NAD^+ (Zubay, 1993). The oxidative decarboxylation of pyruvate follows several steps:

$$\text{Pyruvate} + \text{TPP-E1} \rightarrow CO_2 + \text{hydroxyethyl-TPP-E1}$$

$$\text{Hydroxyethyl-TPP-E1} + \text{lipoyl-E2} \rightarrow \text{S-acetyldihydrolipoyl-E2} + \text{TPP-E1}$$

$$\text{S-acetyldihydrolipoyl-E2} + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{dihydrolipoyl-E2}$$

$$\text{Dihydrolipoyl-E2} + \text{FAD-E3} \rightarrow \text{lipoyl-E2} + \text{FAD (red.)-E3}$$

$$\text{FAD (red.)-E3} + NAD^+ \rightarrow \text{FAD-E3} + \text{NADH} + H^+$$

The structure of PDH complex has been identified. Pyruvate decarboxylase and dihydrolipoyl dehydrogenase are assembled on a 24-meric dihydrolipoyl transacetylase core with an approximate polypeptide stoichiometry of 24:24:12 (E1:E2:E3) (Dave et al., 1995). The covalently bound lipoyl cofactors of the E2 play an important role in the catalytic mechanism since they are reductively acetylated, transacetylated and reoxidized in turn at the respective E1, E2 and E3 active sites. The investigation of the substrate specificity of the PDH complex from *E. coli* demonstrated that binding of pyruvate to the PDH complex must be mediated by two distinct points on the binding center, one for the carbonyl and the other for the carboxyl function.

The PDH complex is expressed from the *pdhR-aceEF-lpd* operon which encodes a pyruvate-responsive repressor, PdhP (*pdhR*), and the three enzymic subunits, E1 (*aceE*), E2 (*aceF*) and E3 (*lpd*) (Quail et al., 1994). Mutations in the *aceE* and *aceF* genes lead to a requirement for acetate plus succinate for best aerobic growth, and *pdhR* mutants synthesize

the PDH complex at a constitutive (pyruvate non-inducible) level (Dave et al., 1995).

The regulation of the PDH complex activity is very complex. In *E. coli*, it is subject to allosteric control. Phosphate (Schwartz and Reed, 1970), phosphoenolpyruvate (Schwartz et al., 1968) and a low energy charge of the adenylate nucleotides (Shen et al., 1968) are found to be the positive affectors. In addition, pyruvate exerts a positive cooperative effect upon the overall reaction, and acetyl CoA is a feedback inhibitor. That is, the PDH complex is inhibited by acetyl-CoA, though this inhibition can be overcome by pyruvate (Schwartz et al., 1968). This cooperativity is also influenced by the co-factor thiamin diphosphate. In addition, a series of metabolites from the glycolytic pathway have an activating influence on the enzyme reaction while TCA cycle intermediates are inhibitors, and the target for all these effectors seems to be the pyruvate dehydrogenase component of the complex (Bisswanger, 1981). In *E. coli*, the activity of the PDH complex *in vivo* under anaerobic condition is inhibited by the high NADH/NAD ratio (Clark, 1989; Snoep et al., 1991). In *Enterococcus faecalis,* the PDH complex is also strongly affected by the NADH/NAD ratio (Snoep et al., 1990), though the inhibiting effect of NADH is mediated by the redox potential (Snoep et al., 1990).

PEP carboxylase

Phosphoenolpyruvate (PEP), the metabolic precursor of pyruvate, is principally converted either to pyruvate or to oxaloacetate. For oxaloacetate synthesis, *E. coli* uses the allosteric enzyme PEP carboxylase (Gottschalk, 1985), a tetramer composed of identical subunits of about 100,000 molecular weight. The optimum pH of PEP carboxylase is 8-9

(Wohl and Markus, 1972; Yoshinaga et al., 1970).

PEP carboxylase catalyzes the reaction that fixes HCO_3^- on phosphoenolpyruvate (PEP) to form oxaloacetate (OAA) and inorganic phosphate with Mg^+ as a cofactor (Utter and Kolenbrander, 1972):

$$PEP + CO_2 + ATP \rightarrow OAA + ADP + P_i.$$

This path is generally the only means for *E. coli* to replenish TCA cycle intermediates used in cell growth on glucose. Although necessary for cell growth when glucose is the sole carbon source, this path also reduces the carbon flux to pyruvate.

Studies on the control mechanisms for the synthesis and activity of the enzyme have shown that the enzyme is an allosteric enzyme regulated by multiple affectors: the enzyme of *E. coli* is activated by acetyl coenzyme A (co-precursor switching activation), fructose 1,6-bisphosphate (precursor activation) (Izui, 1970a), guanosine 5'-diphosphate 3'-diphosphate (ppGpp) (stringent control) (Taguchi, 1977), GTP (compensatory feedback activation) (Taguchi, 1977), and long-chain fatty acids and their CoA derivatives (end-product switching activation) (Izui, 1970b). The *E. coli* enzyme is inhibited by L-aspartate and L-malate as a result of end-product feedback inhibition (Nishikido et al., 1965; Nishikido et al., 1968). In *Corynebacterium* and *Brevibacterium* amino acid production strains, the activity of PEP carboxylase is strongly inhibited by aspartate, which has led to the conclusion that PEP carboxylase regulates the carbon flow for the synthesis of the aspartate family of amino acids (Cremer et al., 1991).

PEP carboxylase is encoded in *E. coli* by the *ppc* gene. The expression of the *ppc* gene is repressed by aspartate and induced by acetyl CoA and a high ATP/ADP ratio (Zubay, 1993).

Overexpression of *ppc* has been used to enhance the carbon flow towards lysine biosynthesis in *Corynebacterium* (Cremer et al., 1991). Also, in order to make conversion of PEP to OAA constitutive, attempts have been made to overcome feedback regulation of PEP carboxylase (Yokota and Shiio, 1988). Gokarn et al. (2000) suggested that in the absence of PEP carboxylase, carbon from glucose flows under anaerobic conditions exclusively from phosphoenolpyruvate to pyruvate, increasing the pool of intracellular pyruvate available to activate the allosteric enzymes lactate dehydrogenase and increase flux towards lactate. A process to generate pyruvate might benefit from a mutation in the *ppc* gene, provided that the cells had some means to replenish TCA cycle intermediates.

NADH oxidase

NADH oxidase was initially characterized from *Streptococcus faecalis* 10C1 (Hoskins et al., 1962). Subsequently, NADH oxidase was purified to homogeneity showing that the enzyme contained one FAD molecule per subunit (M_r=51000) (Schmidt et al., 1986). The enzyme is dimeric and each subunit contains one FAD molecule and one non-flavin redox center (Ross and Claiborne, 1992). NADH oxidase catalyzes the following reaction:

$$2NADH + 2H^+ + O_2 \rightarrow 2NAD^+ + 2H_2O$$

Input of oxygen into metabolism via NADH oxidase is important for controlling competence development throughout growth. The presence of NADH oxidase allows *Streptococcus pneumoniae* to reoxidize a fraction of the glycolytic NADH, using O_2 instead of pyruvate, thus improving the efficiency of glucose catabolism (Condon, 1987) and allowing the high ATP level required for the uptake of transforming DNA to be reached

(Clave and Trombe, 1989). Interestingly, overexpresstion of NADH oxidase causes a shift from homolactic to mixed-acid fermentation in *Lactococcus lactis* (Lopez de Felipe et al., 1998).

Regeneration of NAD^+ is vital for all organisms. Glycolysis is possible only if NADH can be reoxidized since NAD is a necessary participant in the oxidation of glyceraldehyde-3-phosphate (Zubay, 1993). Under anaerobic conditions, pyruvate is converted to lactate, succinate and ethanol in order to regenerate NAD^+ (Clark, 1989). Under aerobic conditions NADH is typically reoxidized by oxidative phosphorylation, a membrane-bound process which generates ATP. *E. coli* can thus balance the cofactors NAD^+ and NADH (Gokarn et al., 2000). When *E. coli aceF* mutants were cultured in glucose and acetate under aerobic conditions, lactate surprisingly appeared as a co-product with the concentration of pyruvate diminishing (Tomar et al., 2002). The appearance of lactate suggested that the synthesis of lactate dehydrogenase, normally observed in *E. coli* only under anaerobic conditions, is induced under these aerobic cultures. Lactate dehydrogenase is activated by high concentration of NADH in the cultures (Clark, 1989). Thus, one explanation is that NAD^+ could not be regenerated from NADH quickly enough via oxidative phosphorylation to meet the demand of glucose uptake through the EMP pathway.

Enhancing reoxidation of NADH should therefore be accompanied by a reduction in the formation of lactate. For the production of pyruvate under aerobic conditions, less pyruvate should be converted to lactate if reoxidation of NADH is facilitated. Also, the addition of flavin adenine dinucleotide (FAD), a coenzyme required for NADH oxidase activity, to the medium can increase the activity of the overproduced NADH oxidase (Lopez De Felipe et al.,

1998).

The nucleotide sequence of *nox*, the gene which encodes NADH oxidase, was characterized at the functional and physiological level. The complex phenotype of *nox* mutants suggests that NADH oxidase could have a crucial role in the sensing of O_2 and in transducing this environmental signal into metabolic changes that affect the global physiology (Auzat et al., 1999). Lopez De Felipe et al. (1998) constructed an NADH oxidase-overproducing *L. lactis* strain by cloning the *Streptococcus mutans nox-2* gene. This engineered system allowed a nisin-controlled 150-fold overproduction of NADH oxidase resulting in decreased NADH/NAD^+ ratio under aerobic conditions. The addition of FAD to the induced cultures resulted in increased activity of the overproduced NADH oxidase, and consequently, a more efficient pyruvate flux redistribution was observed compared to that in the same medium lacking FAD. Under these conditions lactate production was abolished though lactate dehydrogenase activity was still found, indicating that efficient NADH oxidation was solely responsible for the abolished flux through lactate dehydrogenase. In other words, overexpression of NADH oxidase resulted in diminished flux through lactate dehydrogenase because of the reduced concentration of this enzyme's cosubstrate, NADH (Lopez De Felipe et al., 1998).

Uncouplers

Uncouplers have little in common chemically except that all are lipid-soluble weak acids. Both their protonated and conjugate base retain lipid solubility because the negative charge is delocalized. A typical uncoupler is 2,4-dinitrophenol, which has conjugate acid and conjugate

base forms. The dinitrophenolate anion is resonance stable, and its negative ionic charge is broadly distributed over the ring structure of the molecule. Because the negative charge is delocalized, both the acid and base forms of dinitrophenol are sufficiently hydrophobic to dissolve in the membrane (Horton et al., 1996).

Free energy conservation in aerobically respiring microbial cells is accomplished principally via oxidative phosphorylation (Mitchell, 1972). Substrate oxidation creates a proton motive force across the intracellular cytoplasm membrane, and this provides the driving force for the phosphorylation of ADP to form high energy covalent bonds in ATP. The oxidative phosphorylation can be uncoupled using protonphores (uncouplers) which carry protons through cells' intracellular cytoplasm membrane and dissipate the driving force of oxidative phosphorylation. Oxidation of the substrate still occurs but the phosphorylation of ADP to ATP is reduced and consequently there is less energy available for the formation of biomass.

Uncouplers are widely used in wastewater treatment. The goal of wastewater treatment is mineralization of organic matter to carbon dioxide. To achieve this goal, the production of excess sludge should be minimized. Uncouplers can be used in wastewater treatment since they can uncouple intracelluar phosphorylation from the oxidation of organic matter and limit a cell's ability to capture energy from substrate oxidation, so that cell growth is inhibited (Okey and Stensel, 1993). Although the addition of chemical uncouplers can significantly reduce sludge production, long-term bioacclimation can eventually negate the effects of uncoupler addition (Strand et al., 1999).

One of the first oxidative uncouplers studied was 2,4-dinitrophenol. In 1955,

2,4-dinitrophenol was reported to stimulate the respiration rate of activated sludge at a concentration of 4 mg/L (Rich and Yates, 1955). 2,4-dinitrophenol is toxic to microorganisms (Gage and Neihardt, 1993), and has been used to reduce biomass production during biodegradation. The efficiency of biomass production was reduced by 62% with a simultaneous increase in the specific substrate uptake rate in *Pseudomonas putida* when the feed was supplemented with 100 mg/L 2,4-dinitrophenol (Low and Chase, 1998). Decreases in pH alone had no effect on biomass production, but caused additional protonphore induced reduction of biomass production (Low and Chase, 1998). The effects of chlorinated phenols on respiratory activities and cell yields have also been reported. For example, pentachlorophenol can lower cell yields (Klecka and Maier, 1985), and inconsistent oxygen uptake was observed with 2,4,5-trichlorophenol (Karns et al., 1983) and pentachlorophenol (Steiert et al., 1987). Okey and Stensel (1993) tested several chlorinated and nitrated phenols and benzoates for their short-term effects on cell yield, COD consumption, and respiration of activated sludge. The results showed that 2,4-dichlorophenol at a concentration of 30 mg/L caused 50% less cell synthesis compared in the absence of an uncoupler, and chlorophenols had clear uncoupler effects at concentrations greater than 8 to 20 μg/mg mixed liquor volatile suspended solids.

Uncouplers of electron transport can reduce the glucose molar growth yields (Y_{gluc}) of *Bacteroides ruminicola, Bacteroides succinogenes, and Butyrivibrio fibrisolvens* compared with those obtained without uncouplers (Dawson, 1979). Among these organisms 2,4-dinitrophenol appears to be more effective than dicyclohexylcarbodiimide as a growth modifier. In most cases, relatively low uncoupler concentrations (≤10 μM) did not affect

growth rates, while growth was significantly reduced or even completely inhibited at a concentration of 100 μM (Dawson, 1979).

CHAPTER 2

OBJECTIVES AND HYPOTHESES

This study employs a metabolic engineering approach with the objective of generating pyruvate at a yield and rate higher than previously reported. Studies will be performed in two *E. coli* strains: CGSC6162 (an *aceF* mutant) and CGSC6162 *ppc*.

1. Media selection is important in pyruvate accumulation.

Although supplementing the media with tryptone improves cell growth, it also hastens the induction of pyruvate oxidase. Therefore, a media composition which balances growth and *poxB* expression will result in optimal pyruvate production.

2. Temperature is important in pyruvate accumulation.

Previous research has demonstrated that reduced temperature prolongs the commencing of pyruvate oxidase induction. Therefore, a temperature which balances growth rate and *poxB* induction will result in optimal pyruvate production.

3. Addition of uncouplers will benefit pyruvate production.

Uncouplers can uncouple the oxidative phosphorylation so that oxidation of NADH occurs but the phosphorylation of ADP to ATP is reduced. Consequently less energy is available for the cell. If, as evidence suggests, pyruvate accummulation results in excess NADH and ATP, the addition of uncouples will provide the cells the means to generate more pyruvate.

4. A poxB knockout will increase the accumulation of pyruvate.

As noted previously, pyruvate oxidase catalyzes the conversion of pyruvate to acetate. Significant pyruvate oxidase activity was observed when acetate was depleted, and pyruvate accumulation slowed in fed-batch fermentations. This observation suggests reducing or eliminating the activity of pyruvate oxidase will reduce pyruvate conversion to acetate. More accumulation of pyruvate is expected in a *poxB* mutant of *E. coli* already lacking activity in the PDH complex.

5. Addition of NADH oxidase will increase the accumulation of pyruvate.

The accumulation of lactate during aerobic conditions suggests that NADH is being converted into NAD to keep pace with the demand of glycolysis. One way to enhance the regeneration of NAD is by introducing the *nox* gene into the strains. The *nox* gene encodes NADH oxidase which converts NADH and oxygen directly into NAD and water without the generation of ATP. More accumulation of pyruvate is expected in a *nox* mutant of *E. coli* already lacking activity in the PDH complex.

CHAPTER 3

PRODUCTION OF PYRUVATE BY ESCHERICHIA COLI USING METABOLIC ENGINEERING

MATERIALS AND METHODS

Strains and Plasmids

The strains used were CGSC6162 (also known as ALS789, F+ *ace*F10 *fadR*200 *tyr* T58(AS) *adhE*80 *mel*-1), ALS799 (CGSC6162 *ppc*::Kan), ALS843 (CGSC6162 *poxB*15::*lacZ*::Cam) and ALS887 (CGSC6162 *ldhA*::Kan). The strain CGSC6162 was also transformed with the pTrc99A-*nox* plasmid expressing NADH oxidase from *Streptococcus mutants*.

Media and Growth Conditions

Shake flask media

The medium contains (g/L): glucose, 40.0; acetic acid, 1.0; succinic acid, 5.0; Maxarome, 5.0; tryptone, 5.0; KH_2PO_4, 0.6; $(NH_4)_2HPO_4 \cdot 7H_2O$, 2.28; NH_4Cl, 6.0. Note: Maxarome is a low salt Baker's yeast extract.

All shake flasks were cultured at 37 °C with 350 rpm agitation and an initial pH of 7.0.

Fermentation media

Media optimization:

The first step in the study was to optimize the media using the Plackett-Burman design. Thus, for several media of the components the concentration used in a single experiment occurred within a range of concentrations. Media contained (ranges, g/L): glucose, 40; acetic

acid, 1.0–5.0; succinic acid, 1.0–5.0; Maxarome, 1.0–5.0; tryptone, 1.0–5.0; Mg^{2+}, 0.004–0.04; Ca^{2+}, 0.004–0.04; Cu^{2+}, 0.0001–0.001; KH_2PO_4, 0.3; $(NH_4)_2HPO_4$, 1.13; NH_4Cl, 2.0.

Strain comparison and addition of uncoupler:

For an experiment using an uncoupler, 2,4-dinitrophenol was added to a concentration of 100 μmol/L.

For fermenter studies 1.5L (initial volume) were used in 2.5L computer-controlled fermenters (Bioflow III, New Brunswick Scientific Scientific Co., Edison, NJ) at 37°C (or 32°C, 40°C) and 750 rpm with 1.5 L/min constant air flowrate. 20% NaOH and 20% HCl were used to control pH. For fed batch fermentations, acetic acid was fed at 0.2 g/Lh during the fermentation. The fermentations lasted for 24 hours, and samples were taken periodically. The samples were stored at –20°C for subsequent analysis.

Analytical Methods

Cell growth was monitored by measuring the optical density (OD) at 600 nm (DU-650 UV-Vis spectrophotometer, Beckman Instruments), and this value was correlated to the dry cell mass.

The concentrations of pyruvate, glucose, acetate, succinate and lactate in the samples were measured using HPLC (Eiteman and Chastain, 1997).

Enzyme Assays

Cells were harvested at the end of the stationary growth phase. 10 ml of cell-containing media was first centrifuged (7500 ×g for 10 minutes at 4°C), supernatant decanted, and the cells re-suspended in buffer. The cell material was again centrifuged (7500 × g for 10 minutes

at 4°C) and suspended and then the cell was disrupted using French Press. The cell extract was centrifuged (12000 × g for 10 minutes at 4°C), and the supernatant (cell free extract) used for enzyme assays.

NADH oxidase activity was assayed spectrophotometrically at 37°C in a total volume of 1 mL containing 50 mM sodium phosphate buffer (pH 7.0), 0.29 mM NADH, and 0.3 mM EDTA. The reaction was initiated by the addition of a suitable amount of extract (50 to 150 μL) and monitored by the decrease in absorbance at 340 nm. A unit of NADH oxidase activity was defined as the amount which catalyzed the oxidation of 1 μmol of NADH to NAD per min at 37°C. Lactate dehydrogenase (Bunch et al., 1997) was measured spectrophotometrically (DU-650 UV-Vis spectrophotometer, Beckman Instrument). One unit of lactate dehydrogenase activity is defined as the amount of enzyme catalyzing the conversion of 1 μmol of substrate per minute. Protein concentrations were determined according to a BCA-Protein Assay method (Stoscheck, 1990).

RESULTS

1. Media selection is important in pyruvate accumulation.

There are a large number of reports on optimization of carbon and nitrogen source on classical method of medium optimization by changing one independent variable while fixing all the others at a certain level. This can be extremely time consuming and expensive for a larger number of variables. Conventional practice of single factor optimization by maintaining other factors at an unspecified constant level does not depict the combined effect of all the factors involved. The method requires a large number of experiments to determine

optimum levels. Optimizing all the effecting parameters can eliminate these limitations of a single factor optimization process collectively by statistical experimental design using Plackett-Burman. There are many other techniques available for screening and optimization of process parameters including non-statistical self-optimization technique. Plackett-Burman design is a well-established and widely used statistical design technique for the screening of the medium components.

The Plackett-Burman design was chosen for a media optimization study. The Plackett-Burman design (Plackett and Burman, 1946) allows the investigation of many factors using relatively few measurements, fewer than a comparable fractional factorial design. Plackett-Burman design is very useful for determining the one or two most important factors. In this research, the main effects were assumed to be much larger than two-factor interactions.

All the experiments were carried out according to a design matrix (Table 1). Each row represents a different experiment (a "run") and each column represents a different independent variable. Each independent variable was tested at two levels, a high concentration (+) and a low concentration (-). One variable was assigned as a "dummy variable" without a level change in order to estimate the experimental error. A dummy factor is an imaginary variable, incorporated in a design, but for which the change between the levels does not cause a physical change. The following variables were designated for analysis: Maxarome, tryptone, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, succinate, and acetate. The effects of glucose on cell growth and pyruvate production were not examined in this research. Therefore the glucose concentration was unchanged, and glucose was assigned as the dummy

Table 1: Plackett-Burman matrix for the study of 7 variables with 12 experiments.

Experiment	Maxarome	Tryptone	$MgSO_4 \cdot 7H_2O$	$CaCl_2 \cdot 2H_2O$	$CuSO_4 \cdot 5H_2O$	Succinate	Acetate	Glucose
1	1	-1	1	-1	-1	-1	1	1
2	1	1	-1	1	-1	-1	-1	1
3	-1	1	1	-1	1	-1	-1	-1
4	1	-1	1	1	-1	1	-1	-1
5	1	1	-1	1	1	-1	1	-1
6	1	1	1	-1	1	1	-1	1
7	-1	1	1	1	-1	1	1	-1
8	-1	-1	1	1	1	-1	1	1
9	-1	-1	-1	1	1	1	-1	1
10	1	-1	-1	-1	1	1	1	-1
11	-1	1	-1	-1	-1	1	1	1
12	-1	-1	-1	-1	-1	-1	-1	-1

+1, high level of a particular variable; -1, low level of the same variable. Glucose is the dummy variable, and the concentration is 40g/L. No duplicates in this design.

variable.

All the experiments were performed in fermenters according to the design matrix, and for each experiment 7 "results" were measured or calculated: 1) maximum pyruvate concentration, 2) maximum OD, 3) maximum pyruvate productivity, 4) pyruvate yield when glucose is depleted, 5) pyruvate yield calculated at time of maximum OD, 6) pyruvate productivity calculated at time of maximum OD, and 7) specific productivity calculated at the time of maximum OD. Thus, seven different variables were tested in only 12 experiments in randomized order according to the matrix shown in Table 1. The variables under study and the specific corresponding concentrations used are shown in Table 2. In the plackett-Burman design, the most important factors are preferred to be put into the left positions of the matrix. The effects of Maxarome and tryptone on cell growth and pyruvate production are deemed more important in our study, and we put Maxarome and tryptone in the left positions of the matrix.

Statistical analyses were made to determine those medium variables that had a significant effect on the 7 results. The effect for each variable (E_A) was determined as the difference between the mean response (R_+) for the n_+ runs (e.g., 6) at the high level (+1) and the mean response (R_-) for the n_- runs (e.g., 6) at the low level (-1). Thus,

$$E_A = R_+/ n_+ - R_-/ n_- \qquad \text{Equation (1)}$$

The experimental error was determined as the mean square of the dummy effects (E_d):

$$V_E = [\Sigma E_d]^2/n \qquad \text{Equation (2)}$$

In equation 2, V_E is the variance of the effects, and n is the number of dummy variables. Since only one dummy variable occurs in our experiments, n is equal to 1.

Table 2: Nutritional variables under study and their corresponding concentrations

Variable	Low level (mg/L)	High level (mg/L)
Maxarome	1000	5000
Tryptone	1000	5000
Mg^{2+}	4	40
Ca^{2+}	4	40
Cu^{2+}	0.1	1.0
Succinate	1000	5000
Acetate	1000	5000

The standard error of the effects (SE_E) was determined as the square root of the variance:

$$SE_E = \text{sqrt}(V_E) \qquad \text{Equation (3)}$$

Finally, the significance level of each variable effect was determined using Student's *t*-test:

$$t\text{-value} = E_A / SE_E \qquad \text{Equation (4)}$$

These analyses allow evaluation of the probability of finding the observed effect purely by chance. Each variable may significantly affect the system, either positively or negatively. Statistical analyses of the experimental results were completed, and the effect of each variable on the value of each parameter together with its associate p-value and the level of significance were determined (Table 3).

The effect of the dummy variable (glucose) should ideally be zero. A departure of this effect from zero is assumed to indicate the degree of the lack of experimental precision and analytical error in measuring the response. However, the effect of the dummy variable could also reflect interaction between variables, which is not accounted for by the Plackett-Burman approach.

In these results, the effect of the dummy variable was small compared with the other effects (Table 3), which indicated that glucose can be used for the calculation of the variance, as a measure of the uncertainty of the results.

Tryptone and Maxarome were identified as the most important components for pyruvate productivity, in terms of maximum pyruvate concentration, maximum OD, maximum productivity and productivity at maximum OD. $MgSO_4 \cdot 7H_2O$ had a significant negative effect on productivity at maximum OD. $CaCl_2 \cdot 2H_2O$ had a significant negative effect on maximum productivity. $CuSO_4 \cdot 5H_2O$ had a significant negative effect on productivity at

Table 3: Media variables and their effects on Maximum pyruvate concentration, Maximum OD, Maximum productivity and Productivity at maximum OD.

variables	Maximum Pyruvate Conc. (g/L)			Maximum OD			Maximum Productivity			Productivity at Max OD		
	Effect	p-value	sign. level	Effect	p-value	sign. level	Effect	p-value	sign. level	Effect	p-value	sign. level
Maxarome	8.67	0.07	90%	4.04	0.09	90%	0.53	0.04	95%	0.61	0.022	95%
Tryptone	8.52	0.07	90%	2.84	0.13	85%	0.56	0.04	95%	0.43	0.031	95%
$MgSO_4 \cdot 7H_2O$	0.67	0.62		-0.11	0.88		-0.00	0.97		-0.14	0.095	90%
$CaCl_2 \cdot 2H_2O$	-1.28	0.42		-0.07	0.92		-0.13	0.17	80%	0.18	0.075	90%
$CuSO_4 \cdot 5H_2O$	0.08	0.95		-0.80	0.40		0.06	0.35		-0.20	0.067	90%
Succinate	1.84	0.31	70%	-0.57	0.51		0.16	0.14	85%	0.02	0.47	
Acetate	-1.21	0.44		1.67	0.21		-0.12	0.18	80%	0.31	0.043	95%
Glucose	-0.98			-0.58			-0.03			0.02		

“sign. level” means significant level. The larger significant level is, the more significantly that factor affects the result.

maximum OD. Succinate had a significant positive effect on maximum pyruvate and maximum productivity. Acetate had a significant negative effect on maximum productivity and yield at glucose depletion (which is not listed in table 3). Previous research also showed that complex media components like tryptone andMaxarome were important for pyruvate production. Specifically, the addition of yeast extract and tryptone resulted in improved growth and pyruvate accumulation (Tomar, 2002).

The magnesium ion, Mg^{2+}, did not show any significant effects on the pyruvate production or cell growth, except that Mg^{2+} had significant negative effect on the pyruvate productivity at maximum OD. One explanation is that Mg^{2+} is required for the activity of pyruvate oxidase since each pyruvate oxidase subunit carries a loosely bound molecule of thiamine pyrophosphate (TTP) which requires Mg^{2+} for binding. Furthermore, the activity of pyruvate oxidase is growth-phase dependent with activity increasing during growth and reaching a maximum at early stationary phase. Since pyruvate oxidase is expressed principally at the time of maximum OD, the presence of excess Mg^{2+} might elevate the flux from pyruvate to acetate via this enzyme at the time of maximum OD. Nevertheless, the medium subsequently selected contained the lower concentration of $MgSO_4 \cdot 7H_2O$ (4mg/L).
The results showed that succinate had a significant positive effect on the maximum pyruvate concentration and maximum productivity. The results showed that succinate had a significant positive effect on the maximum pyruvate concentration and maximum productivity. This result is not surprising since succinate is an intermediate of the TCA cycle. Because CGSC6162 has a mutation in the aceF gene expressing a component of the PDH complex, this strain has a reduced ability compared to a wild-type to generate TCA cycle intermediates

via acetyl CoA. The biochemical pathways available to generate TCA cycle components necessary for biomass generation are PEP carboxylase, malic enzyme and the glyoxylate shunt. Either of the first two would consume PEP and pyruvate and therefore directly supply TCA cycle intermediates at the direct expense of pyruvate accumulation. As acetyl CoA is the key substrate in the glyoxylate shunt, this pathway may be operating at a reduced rate in CGSC6162, and be insufficient to meet the cell's demand for TCA cycle intermediates. In any case, supplying the cell with a TCA cycle intermediate such as succinate would relieve the burden of generating this intermediate needed for biomass formation, and would understandably improve growth and pyruvate formation.

Acetate has significant positive effects on the maximum OD and the productivity at maximum OD. However, acetate also has significant negative effects on maximum productivity and yield at zero glucose concentration. Usually, different feeding strategy may have different effect on cell growth and pyruvate production. For example, if the acetate was continuously added into the media, the pyruvate will continuously accumulate. In a previous study, CGSC6162 was observed to consume all the acetate supplied, and the maximum pyruvate concentration corresponded with the time of acetate depletion. After this pyruvate maximum, the CGSC6162 generated both acetate and lactate (Tomar, 2002). These results suggested a strategy of starting the fermentation at a low concentration of acetate and then feeding acetate during the fermentation. Our results suggest that as long as acetate is not depleted, the pyruvate will continuously accumulate. After the Plackett-Burman study, the initial concentration of acetic acid was selected to be 0.5 g/L. The acetic acid consumption rate was calculated from the previous fermentations. To keep acetic acid at 0.5 g/L for the

first 6 hours, acetic acid was fed at 0.3 g/Lh according to the acetic acid consumption of *E. coli*.

The media selected was: glucose, 60.0 g/L; acetic acid, 0.5 g/L; succinic acid, 7.5 g/L; Maxarome, 5.0 g/L; tryptone, 5.0 g/L; KH_2PO_4, 0.3 g/L; $(NH_4)_2HPO_4$, 1.13 g/L; NH_4Cl, 2.0 g/L, acetic acid was fed at 0.3 g/Lh.

2. Addition of uncouplers will increase pyruvate production.

Uncouplers are compounds which uncouple the oxidative phosphorylation so that oxidation of NADH occurs but the phosphorylation of ADP to ATP is reduced. Consequently in the presence of the uncoupler less energy is available for the cell. Since the biochemical pathway from glucose to pyruvate generates both ATP and NADH which might accumulate, it is possible that the addition of an uncoupler with provide the cell with a mean to reduce their potential accumulation.

A study was completed in which the uncoupler 2,4-dinitrophenol ($C_6H_4O_5N_2$) was added into the media. First, a range of different concentrations of 2,4-dinitrophenol (range 0 μM-160 μM) was examined with strain CGSC6162 to determine the effect on cell growth. All fermentations were performed in shake flasks in duplicate, and the OD was measured with time (Figure 1). The results indicate that 2,4-dinitrophenol in the range of 0 μM - 160 μM did not affect the cell growth significantly. In other studies relatively low uncoupler concentrations (≤10 μM) did not affect growth rates, while growth was significantly reduced or even completely inhibited at a concentration of 100 μM (Dawson, 1979). We decided to add 2,4-dinitrophenol in the fermenter at 100 μM.

FIGURE 1:

The effects of 2,4-dinitrophenol on cell growth of *E. coli* CGSC6162 in shake flasks.

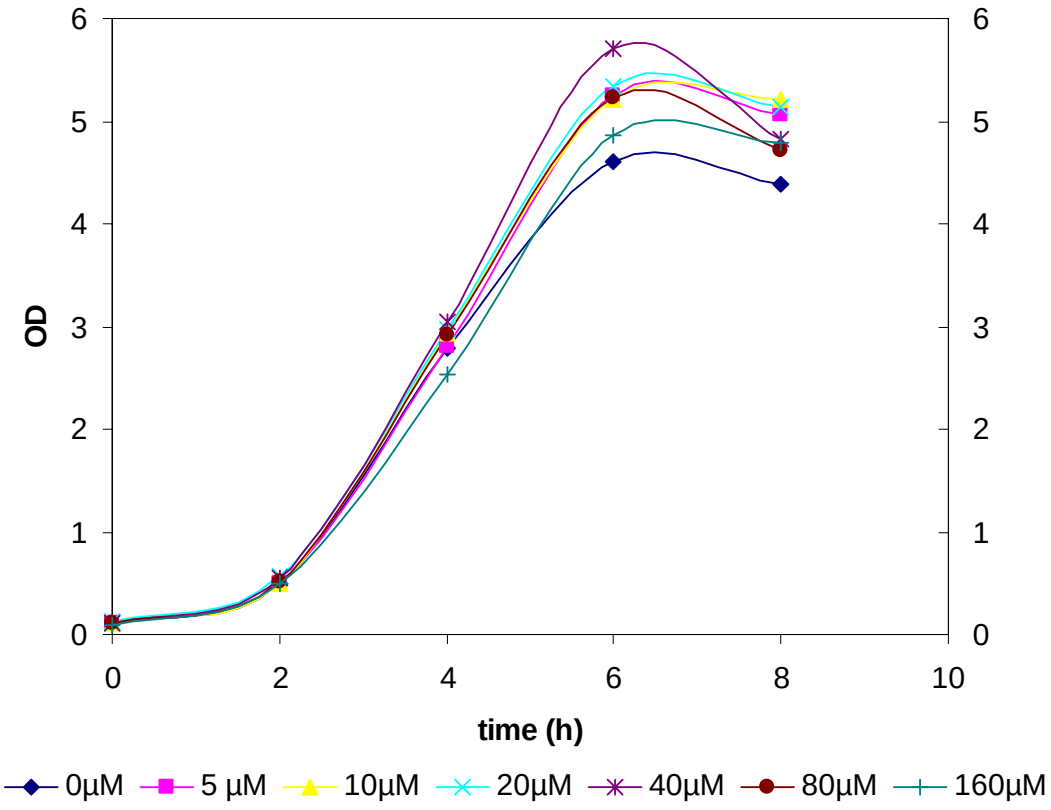
OD
time (h)
0μM
5 μM
10μM
20μM
40μM
80μM
160μM

Table 4: The effects of 100μM 2,4-dinitrophenol on pyruvate and lactate production of CGSC6162 and CGSC6162 *ppc*.

Strains	Dinitrophenol	Max Pyruvate (g/L)	Max Productivity (g/Lh)	Max Lactate (g/L)
CGSC6162	-	46.9	2.42	3.95
CGSC6162	-	17.7	2.22	2.72
CGSC6162	-	21.0	2.57	3.96
CGSC6162	-	23.2	1.48	12.10
CGSC6162	+	34.4	1.94	4.65
CGSC6162	+	35.3	1.75	3.67
CGSC6162 *ppc*	-	32.1	1.79	6.90
CGSC6162 *ppc*	-	34.2	1.70	2.71
CGSC6162 *ppc*	+	38.5	2.37	3.31
CGSC6162 *ppc*	+	32.0	2.11	5.25

Fermentations were conducted in duplicate using CGSC6162 and CGSC6162 *ppc* with the addition of the uncoupler 2,4-dinitrophenol (Table 4). For CGSC6162, the average concentration of pyruvate at 24 hours increased from 27.2 g/L to 34.9 g/L, and lactate production at 24 hours decreased from 5.7 g/L to 4.2 g/L. For CGSC6162 *Δppc*, the average concentration of pyruvate at 24 hours increased from 33.2 g/L to 35.3 g/L, while lactate production did not change at the end of the 24 hour fermentation. Statistical analysis showed that 2,4-dinitrophenol did not significantly affect maximum pyruvate concentration, maximum productivity or maximum lactate for strain CGSC6162, with p-values of 0.49, 0.36, and 0.66, respectively. However, the first fermentation of CGSC6162 is the very different from the others. The maximum pyruvate concentration in this fermentation is 46.9 g/L, which is much different from the rest of three (17.7 g/L, 21.0 g/L, and 23.2 g/L). We therefore have reason to doubt this fermentation. The statistical analysis was applied again after deleting this fermentation. The results showed that 2,4-dinitrophenol significantly affected maximum pyruvate concentration (p-value of 0.0065), but not maximum productivity or maximum lactate for strain CGSC6162 (p-values of 0.53 and 0.62, respectively). Even though the maximum pyruvate of first fermentation is much higher than the rest three fermentations, there is no mistake in the fermentation process, and we cannot simple ignore the data. For CGSC6162 *ppc*, 2,4-dinitrophenol did not significantly affect maximum pyruvate concentration, maximum productivity or maximum lactate, (p-values of 0.60, 0.087 and 0.84, respectively). In summary, the addition of uncoupler 2,4-dinitrophenol increased pyruvate and decreased lactate accumulation for these two strains, but the improvement is not significant, and we cannot conclude that uncoupler 2,4-dinitrophenol can significantly

improve pyruvate production.

3. Additional genetic engineering manipulation can increase the accumulation of pyruvate.

1. A poxB knockout will increase the accumulation of pyruvate.

Pyruvate oxidase functions as a safety valve to convert excess pyruvate to acetate rather than to acetyl-CoA, thus maintaining the intracellular CoA pool for other metabolic functions. Reducing or eliminating the activity of pyruvate oxidase may reduce pyruvate conversion to acetate. More accumulation of pyruvate is expected in a *poxB* mutant of *E. coli* already lacking activity in the PDH complex.

CGSC6162 was compared with CGSC6162 *poxB*. The two strains were studied using an initial volume of 1.5L in a 2.5L fermenter in duplicate using the "optimal" media. Samples were taken periodically over the course of a 24h fermentation. The following three measurements were compared: a) maximum pyruvate concentration, b) maximum lactate concentration, and c) maximum OD (Table 5).

The results were unexpected. CGSC6162 *poxB* accumulated acetic acid during the fermentation, and indeed produced more acetic acid than CGSC6162. CGSC6162 consumed a total of 8.4 g acetic acid in 24 hours; while CGSC6162 *poxB* produced 6.4 g acetic acid in 24 hours. CGSC6162 *poxB* also produced less pyruvate than CGSC6162. The average concentration of pyruvate produced after 24 hours was 27.2 g/L for CGSC6162 but was only 18.6 g/L for CGSC6162 *poxB*. Lactate production also dramatically decreased from 5.7 g/L (CGSC6162) to 1.4 g/L (CGSC6162 *poxB*) at the end of the 24 hour fermentation. One

Table 5: The effect of a *poxB* knockout and *ldhA* knockout on pyruvate, lactate and acetate production for CGSC6162 and CGSC6162 *poxB*, and CGSC6162 *ldhA*.

Strains	Max Pyruvate (g/L)	Max Productivity (g/Lh)	Max Lactate (g/L)	Acetate consumption (g)
CGSC6162	46.9	2.42	3.95	9.45
CGSC6162	17.7	2.22	2.72	8.61
CGSC6162	21.1	2.57	3.96	7.19
CGSC6162	23.2	1.49	12.10	8.24
CGSC6162 *poxB*	22.7	1.27	1.04	-7.45
CGSC6162 *poxB*	11.0	0.62	1.31	-4.73
CGSC6162 *poxB*	21.2	0.93	1.43	-6.87
CGSC6162 *ldhA*	21.6	1.87	1.55	5.85
CGSC6162 *ldhA*	24.9	1.91	0.71	7.89
CGSC6162 *ldhA*	22.1	0.92	1.75	1.72

explanation is that even though pyruvate cannot be converted directly to acetate by pyruvate oxidase, cells managed to induce others metabolic pathways to produced acetate. Also, the reduction of lactate production for CGSC6162 *poxB* indicated that this strain has another means to balance the cofactors NADH and NAD.

2. A ldhA knockout will increase the accumulation of pyruvate.

Lactate surprisingly appeared as a product during the aerobic accumulation of pyruvate. Lactate generation from pyruvate by lactate dehydrogenase is known to be used to by *E. coli* as a means to balance the cofactors NADH and NAD. An *ldhA* knockout should prevent the conversion of pyruvate to lactate.

CGSC6162 was compared with CGSC6162 *ldhA*. The two strains were studied at 1.5L (initial volume) in a 2.5L fermenter in duplicate using the "optimal" media as determined from the plackett-Burman study. Samples were taken periodically for 24h. The following three measurements were compared: a) maximum pyruvate concentration, b) maximum lactate concentration, and c) maximum OD.

Pyruvate production was not improved in CGSC6162 *ldhA* compared to CGSC6162 (Table 5), but lactate generation at 24 hours was reduced from 5.68 g/L (CGSC6162) to 1.34 g/L (CGSC6162 *ldhA*). Interestingly, lactate production still remained despite the absence of *ldhA*. Moreover, the *ldhA* mutant showed greater lactate generation than the *poxB* mutant. In CGSC6162 *ldhA* the concentrations of acetic acid and succinic acid essentially remained unchanged.

A comparison of pyruvate accumulation by CGSC6162, CGSC6162 *ppc,* CGSC6162

poxB, and CGSC6162 *ldhA* using the "optimal" media, demonstrated that none of the three mutations made a significant difference on cell growth or on final pyruvate concentration. The highest concentration of pyruvate obtained was 47 g/L for CGSC6162, but on other occasions for this strain the final pyruvate concentration was lower. The *ldhA* and *poxB* mutants showed substantially lower production of lactate than the other strains.

3. Addition of NADH oxidase will increase the accumulation of pyruvate.

Three NADH oxidase-overproducing strains (ALS225 pTrc99A-*nox*, CGSC6162 pTrc99A-*nox* and CGSC6162 *ppc* pTrc99A-*nox*) were constructed by cloning the *nox* gene from *Streptococcus pneumoniae* in CGSC6162 and CGSC6162 *ppc*. NADH oxidase activities were measured for these three strains and their parent strains (Table 6).

ALS225 pTrc99A-*nox* showed higher NADH oxidase activity than its parent strain ALS225. However, CGSC6162 pTrc99A-*nox* and CGSC6162 *ppc* pTrc99A-*nox* have lower NADH oxidase activity compared to their parent strains. This result offers an explanation for why both CGSC6162 pTrc99A-*nox* and CGSC6162 *ppc* pTrc99A-*nox* strains produced less pyruvate compared to their parent strains under similar conditions in a previous study (Tomar, 2002).

4. Temperature is important in pyruvate accumulation.

Previous research demonstrated that reduced temperature prolonged the time point at which of pyruvate oxidase activity increased. Therefore, a reduced temperature which still produces high growth rate while reducing pyruvate oxidase activity should improve pyruvate

Table 6: Specific activity (U/mg) of pyruvate oxidase for ALS225 and ALS225 pTrc99A-*nox*, CGSC6162 pTrc99A and CGSC6162 pTrc99A-*nox*, CGSC6162 *ppc* and CGSC6162 *ppc* pTrc99A-*nox*

Strains	Specific activity (U/mg)
ALS225	0.01
ALS225 pTrc99A-*nox*	0.21
CGSC6162	0.11
CGSC6162 pTrc99A-*nox*	0.10
CGSC6162 *ppc*	0.10
CGSC6162 *ppc* pTrc99A-*nox*	0.06

production.

Fermentations were performed in duplicate using three different temperatures (34°C, 37°C, and 40°C) (Table 5). The enzyme activities of NADH oxidase and lactate dehydrogenase were determined at 16, 20, and 24 hours.

Maximum lactate accumulation appeared when lactate dehydrogenase activity was the highest, and low lactate dehydrogenase activity correlated with low lactate accumulation. Pyruvate production was highly correlated with the lactate accumulation, with high pyruvate concentration companying low lactate concentration.

The fermentations at 34°C were inconsistent. The specific enzyme activity of lactate dehydrogenase was 0.09 U/mg in one experiment, and 0.31 U/mg in the second trial. One explanation is that the lactate dehydrogenase was not stable at comparative low temperature (34°C). Alternatively, the LDH activity measured by the spectrophotometric method was not the true indication of the activity inside the cell as loss of activity may have occurred during the preparation of cell free extract.

Also, the LDH specific activities in the study ranged from 0.06 U/mg to 0.31 U/mg, which were not low since LDH activities without conferring plasmids ranged from 0.01 to 0.23 under aerobic conditions in *E. coli* (Bunch et al., 1997). Lactate dehydrogenase enzyme has a tendency to undergo conformational change or the induced fit in the presence of substrate (Gottschalk, 1985). Lactate dehydrogenase may have undergone conformational changes when the pyruvate concentration was very high during the fermentation and attained the high-affinity state to generate lactate. Also, lactate could be formed via the enzyme lactate-malate transhydrogenase that converts pyruvate and malate into lactate and

oxaloacetate. Presence of this enzyme under high pyruvate concentration could result in lactate generation despite low LDH activity.

High NADH oxidase activity correlated with low lactate concentration, and low NADH oxidase activity correlated with high lactate concentration for 37°C fermentations. High NADH oxidase may have resulted in diminished flux through lactate dehydrogenase (LDH) because of the reduction of this enzyme's cofactor, NADH. This result was not observed for the 40°C fermentations.

Fermentations at 37°C generally produced more pyruvate and less acetate than at 40°C. The specific activity of NADH oxidase was also measured to be greater at 37°C than at 34°C and 40°C, suggesting 37°C is the optimal temperature. In most of the fermentations, final pyruvate concentration reached about 20 g/L before the rate of pyruvate production slowed, and the concentration never reached 30 g/L. This observation was consistent in all the fermentations, irrespective of temperature. Cell growth ceased after a certain time even when acetate was present in the media. Cell growth could have stopped as a result of the accumulation of a inhibitory metabolite or as a result of a limitation in an essential nutrient. As already noted, both NADH and ATP could conceivably accumulate during pyruvate production. Although these compounds would not themselves likely be inhibitory, the relative absence of NAD and ADP could cause the cessation of cell growth. α-ketobutyrate is a precursor for the generation of isoleucine in bacterial metabolism. Since pyruvate only relative absence of NAD and ADP could cause the cessation of cell growth. α-ketobutyrate is a precursor for the generation of isoleucine in bacterial metabolism. Since pyruvate only differs by one C from α-ketobutyrate, it acts a competitive inhibitor for the conversion of

Table 7: Comparison of pyruvate production using three different fermentation temperatures.

Temperature	Max. pyruvate (g/L)	Max. lactate (g/L)	Max. acetate (g/L)	NADH oxidase (U/mg)	Lactate dehydrogenase (U/mg)
34°C	27.0	3.49	2.43	0.07	0.09
34°C	12.2	13.40	3.75	0.14	0.31
37°C	23.2	12.10	1.12	0.10	0.17
37°C	27.7	6.13	2.01	0.21	0.17
40°C	22.8	9.00	2.44	0.06	0.11
40°C	19.4	9.29	2.14	0.13	0.06

α-ketobutyrate to isoleucine (Gollop et al., 1989). The high concentration of pyruvate formed could inhibit isoleucine generation, an essential amino acid, causing growth rate to slow or cease. Exogenously adding isoleucine under high pyruvate concentration could make up for the inhibition and resume cell growth and pyruvate generation.

CHAPTER 4

CONCLUSIONS

In this study the Plackett-Burman design was used for a media optimization. Succinate and complex media tryptone and Maxarome had positive effects on cell growth and pyruvate production, and were used at high concentrations in the subsequent fermentations. The ions Mg^{2+} and Cu^{2+} had negative effects, and were therefore set at low concentrations in the media. Feeding strategies were also optimized. The initial concentration of acetic acid was 0.5 g/L. To keep acetic acid at about 0.5 g/L for the first 6 hours, acetic acid was fed at 0.3 g/Lh according to the acetic acid consumption of *E. coli*.

The addition of uncoupler 2,4-dinitrophenol increased pyruvate production and decreased lactate accumulation for CGSC6162 and CGSC6162 *ppc*. Since the improvement is not significant, the uncoupler 2,4-dinitrophenol does not conclusively improve pyruvate production.

Using the "optimal media" for four strains CGSC6162, CGSC6162 *ppc,* CGSC6162 *poxB,* and CGSC6162 *ldhA* showed no significant difference in cell growth or on final pyruvate concentration. The greatest concentration of pyruvate obtained was 47 g/L for CGSC6162, but on other occasions for this strain the final pyruvate concentration was much lower. The *ldhA* and *poxB* mutants showed substantially lower production of lactate than the other strains.

CGSC6162 *p*Trc99A-*nox* and CGSC6162 *ppc p*Trc99A-*nox* have less NADH oxidase activity compared to their parent strains. This result explains the reason that both CGSC6162 *p*Trc99A-*nox* and CGSC6162 *ppc p*Trc99A-*nox* strains produced less pyruvate compared to

their parent strains under similar conditions in the previous study (Tomar, 2002).

The fermentations at 34°C were inconsistent. The LDH activity measured by the spectrophotometric method was not the true indication of the activity inside the cell as loss of activity may have caused during the preparation of cell free extract. NADH oxidase activity correlated with lactate concentration at 37°C fermentations. High NADH oxidase may have resulted in diminished flux through lactate dehydrogenase (LDH) because of the reduction of this enzyme's cofactor, NADH. This result was not observed for the 40°C fermentations.

Fermentations at 37°C generally produced more pyruvate and less acetate than at 40°C. 37°C is the optimal temperature compared to 34°C and 40°C. Cell growth ceased after a certain time even when acetate was present in the media. This observation was consistent in all the fermentations, irrespective of temperature. Cell growth could have stopped as a result of the accumulation of an inhibitory metabolite or as a result of a limitation in an essential nutrient.

In the future research, a focus should be on the effects of uncouplers. Higher concentration of 2,4-dinitrophenol can be tested. Also, other kinds of uncoupler, such as 2,4,5-trichlorophenol and pentachlorophenol, can be investigated. Triplicate should be performed instead of duplicate.

Another interesting observation is that CGSC6162 *poxB* accumulated acetic acid during the fermentation, and indeed produced more acetic acid than CGSC6162. CGSC6162 *poxB* also produced less pyruvate than CGSC6162. Lactate production was dramatically decreased. All these results indicated that even though pyruvate cannot be converted directly to acetate by pyruvate oxidase, cells induced other metabolic pathways to produce acetate, and this

pathway may balance the cofactors NADH and NAD. More investigation is needed in this area.

We did not test the effect of NADH oxidase since CGSC6162 pTrc99A-*nox* and CGSC6162 *ppc* pTrc99A-*nox* have lower NADH oxidase activity compared to their parent strains. To get the higher NADH oxidase activity of CGSC6162 pTrc99A-*nox* and CGSC6162 *ppc* pTrc99A-*nox*, genetic engineering manipulation need to be improved.

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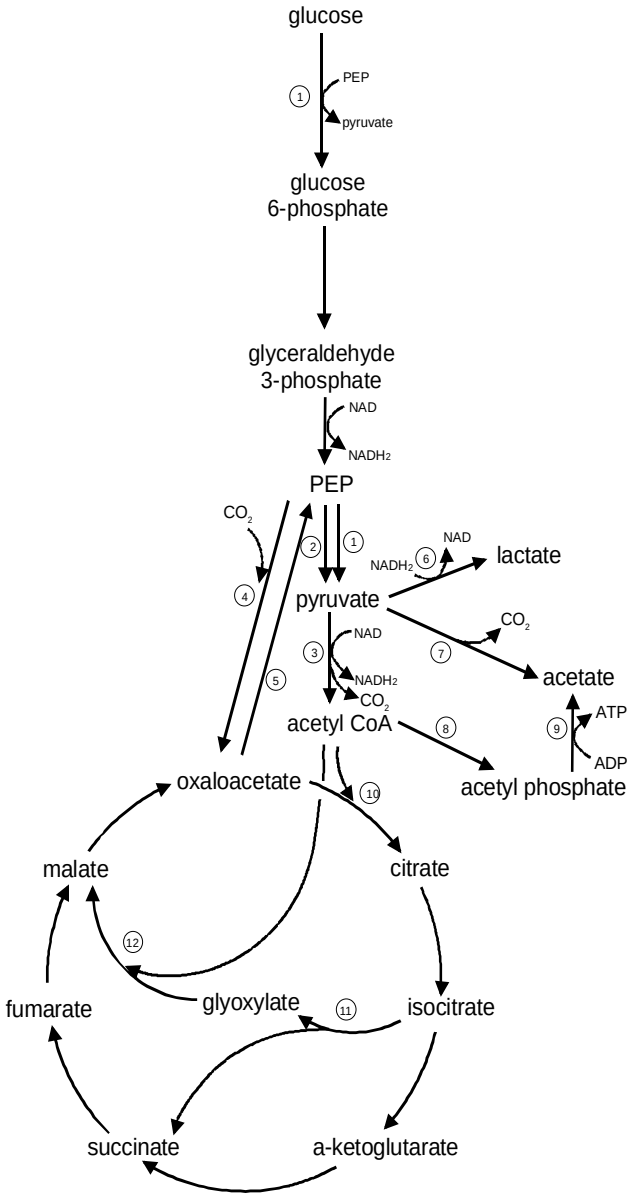
APPENDIX A

Metabolic pathway of *E. coli* under aerobic conditions when grown on glucose



Enyzmes

1. Phosphotransferase system
2. Pyruvate kinase
3. Pyruvate dehydrogenase
4. PEP carboxylase
5. PEP carboxykinase
6. Lactate dehydrogenase
7. Pyruvate oxidase
8. Phosphoacetyl transferase
9. Acetate kinase
10. Citrate synthase
11. Isocitrate lyase
12. Malate synthase

APPENDIX B

FERMENTATIONS OF PLACKETT-BURMAN DESIGN

Title: Plackett-Burman Design # 1
Date: April 17th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	pyr productivity
1	0.0	0.14	1500.0	0.000	41.888	1.128	0.000	5.524	0.000
2	2.0	0.48	1490.0	0.000	38.972	1.064	0.000	5.184	0.000
3	4.3	2.46	1484.5	0.000	29.876	0.680	0.000	3.968	0.000
4	6.0	5.06	1477.0	5.448	35.088	0.604	0.744	4.360	0.908
5	8.0	5.66	1468.0	7.172	30.228	0.348	0.640	3.332	0.897
6	10.0	6.82	1459.5	8.720	26.600	0.200	1.400	2.576	0.872
7	12.0	7.80	1454.5	11.236	22.652	0.096	1.684	1.684	0.936
8	14.0	9.76	1451.0	12.012	15.284	0.004	2.412	0.720	0.858
9	16.0	11.80	1462.0	20.128	11.320	0.000	2.824	0.204	1.258

Title: Plackett-Burman Design # 2
Date: April 11th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	pyr productivity
1	0	0.92	1500.0	0.000	35.220	0.916	0.000	0.948	0.000
2	2	1.64	1490.5	0.000	26.748	0.620	0.000	0.724	0.000
3	4	5.46	1485.5	0.000	27.324	0.164	0.000	0.336	0.000
4	6	11.00	1497.5	9.568	26.200	0.060	0.000	0.164	1.595
5	8	12.46	1503.5	10.176	11.816	0.000	0.640	0.000	1.272
6	10	12.02	1506.5	18.056	12.164	0.000	1.400	0.116	1.806
7	12	12.26	1509.5	19.400	6.816	0.000	1.684	0.080	1.617
8	14	11.96	1513.0	24.544	3.020	0.000	2.412	0.000	1.753
9	16	11.88	1513.5	27.380	0.000	0.000	2.824	0.060	1.711

Title: Plackett-Burman Design # 3
Date: April 12th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	pyr productivity
1	0	0.12	1500.0	0.000	40.124	0.964	0.000	1.128	0.000
2	2	0.72	1491.0	0.000	38.728	0.816	0.000	0.884	0.000
3	4	3.32	1486.0	3.192	28.468	0.748	0.000	0.856	0.798
4	6	6.80	1491.5	7.888	28.382	0.376	0.616	0.240	1.315
5	8	8.60	1495.5	12.496	23.160	0.108	1.128	0.136	1.562
6	10	8.66	1496.0	13.512	14.728	0.000	1.308	0.000	1.351
7	12	8.06	1497.5	14.768	9.600	0.000	1.600	0.076	1.231
8	14	9.16	1501.0	21.776	7.844	0.000	2.840	0.108	1.555
9	16	8.52	1506.5	22.556	2.368	0.000	3.680	0.208	1.410

Title: Plackett-Burman Design # 4
Date: April 12th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	pyr productivity
1	0	0.12	1500.0	0.000	34.216	4.724	0.000	0.804	0.000
2	2	1.20	1491.5	0.000	29.608	4.000	0.000	0.816	0.000
3	4	4.84	1487.5	3.180	27.104	3.652	0.000	0.760	0.795
4	6	6.66	1481.5	5.164	24.768	2.924	0.308	0.260	0.861
5	8	7.72	1480.0	10.372	27.508	2.568	0.544	0.044	1.297
6	10	7.94	1480.5	12.016	19.904	1.440	0.708	0.048	1.202
7	12	6.80	1482.5	17.017	18.312	0.000	1.148	0.000	1.418
8	14	7.72	1480.5	15.744	11.488	0.000	1.060	0.080	1.125
9	16	8.26	1480.0	23.252	10.372	0.384	2.108	0.100	1.453

Title: Plackett-Burman Design # 5
Date: April 15th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	pyr productivity
1	0.0	0.44	1500.0	0.000	39.064	1.264	0.000	5.812	0.000
2	2.0	0.60	1492.5	0.000	39.664	1.116	0.000	6.040	0.000
3	4.3	3.48	1485.5	0.000	27.900	0.708	0.000	4.368	0.000
4	6.0	8.96	1486.0	5.576	23.800	0.516	0.244	3.784	0.929
5	8.0	10.60	1486.5	12.048	20.912	0.320	0.504	3.172	1.506
6	10.0	11.46	1486.5	14.320	12.892	0.188	0.460	1.824	1.432
7	12.0	11.78	1486.5	17.592	9.400	0.000	0.424	1.132	1.466
8	14.0	12.20	1486.5	21.592	5.860	0.000	0.556	0.548	1.542
9	16.0	10.74	1489.0	27.820	2.036	0.056	0.872	0.228	1.739

Title: Plackett-Burman Design # 6
Date: April 17th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	pyr productivity
1	0.0	0.22	1500.0	0.000	39.756	5.200	0.000	1.064	0.000
2	2.0	0.34	1490.5	0.000	37.132	4.808	0.000	0.972	0.000
3	4.0	3.30	1485.0	0.000	25.952	3.324	0.000	0.576	0.000
4	6.0	6.20	1486.0	8.272	27.812	3.588	0.000	0.012	1.379
5	8.0	7.28	1496.5	12.704	15.560	1.260	0.000	0.000	1.588
6	10.0	8.34	1494.5	20.020	13.532	0.996	0.620	0.000	2.002
7	13.0	7.90	1500.0	26.676	6.716	0.584	1.248	0.000	2.052
8	14.0	7.36	1494.5	28.212	3.772	0.456	1.368	0.000	2.015
9	16.0	6.62	1490.0	31.000	0.764	0.372	2.060	0.180	1.938
10	18.0	6.42	1482.0	30.712	0.000	0.292	1.728	0.000	1.706

Title: Plackett-Burman Design # 7
Date: April 17th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	pyr productivity
1	0.0	0.22	1500.0	0.000	39.820	5.124	0.000	5.240	0.000
2	2.0	0.42	1489.0	0.000	30.240	3.940	0.000	4.060	0.000
3	4.3	3.40	1482.0	0.000	35.480	4.832	0.000	4.720	0.000
4	6.0	4.80	1475.5	3.884	30.864	4.396	0.000	4.090	0.647
5	8.0	7.92	1473.5	6.428	23.108	3.352	0.476	2.970	0.804
6	10.0	8.70	1480.0	11.992	24.904	3.672	0.956	3.080	1.199
7	12.0	9.12	1469.0	14.028	17.808	0.000	1.060	2.000	1.169
8	14.0	9.54	1466.5	14.148	11.576	0.000	0.920	1.070	1.011
9	16.0	9.20	1462.5	20.684	10.416	2.476	1.752	0.790	1.293

Title: Plackett-Burman Design # 8
Date: April 19th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	pyr productivity
1	0.0	0.24	1500.0	0.000	40.728	0.892	0.000	4.980	0.000
2	2.0	0.54	1490.0	0.000	31.844	0.756	0.000	4.290	0.000
3	4.0	1.86	1481.5	0.000	37.104	0.792	0.000	4.810	0.000
4	6.0	2.42	1475.5	2.816	30.100	0.584	0.000	3.900	0.469
5	8.0	3.00	1466.5	4.316	30.736	0.488	0.056	3.776	0.540
6	10.0	3.02	1458.0	5.920	31.644	0.380	0.152	3.768	0.592
7	12.0	3.92	1448.5	7.180	30.128	0.000	0.128	3.528	0.598
8	14.0	3.84	1440.0	8.024	27.620	0.000	0.140	3.172	0.573
9	16.0	4.08	1434.3	9.800	27.004	0.196	0.260	2.832	0.613
10	18.0	4.76	1433.3	11.904	24.164	0.236	0.800	2.512	0.661

Title: Plackett-Burman Design # 9
Date: April 19th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyr productivity
1	0.0	0.10	1500.0	0.000	41.316	4.944	0.000	1.108	0.000
2	2.0	0.46	1488.5	0.000	35.760	4.604	0.000	1.072	0.000
3	4.0	1.96	1479.0	3.736	33.024	4.152	0.000	0.588	0.934
4	6.0	2.78	1473.0	3.628	32.332	4.056	0.000	0.560	0.605
5	8.0	2.96	1464.0	5.868	33.996	3.928	0.084	0.400	0.734
6	10.0	3.02	1455.0	7.024	31.252	3.328	0.600	0.432	0.702
7	12.0	3.36	1446.5	8.956	29.428	0.000	0.460	0.136	0.746
8	14.0	4.76	1438.0	9.272	24.404	0.000	0.552	0.000	0.662
9	16.0	3.80	1428.5	12.624	26.808	2.456	0.984	0.072	0.789
10	18.0	3.84	1423.0	13.748	23.348	1.992	1.708	0.252	0.764

Title: Plackett-Burman Design # 10
Date: June 24th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	pyr productivity
1	0.0	0.66	1500.0	0.000	58.248	7.560	0.000	0.528	0.000
2	2.0	1.04	1492.5	0.000	59.188	7.712	0.000	0.524	0.000
3	4.0	4.40	1486.0	4.396	51.160	6.764	0.000	0.104	1.099
4	6.0	5.14	1484.0	9.980	48.176	6.364	0.000	0.000	1.663
5	8.0	7.46	1487.0	16.456	39.496	3.356	0.000	0.344	2.057
6	10.5	8.80	1493.0	25.124	30.760	2.532	0.804	0.344	2.393
7	13.0	8.46	1497.5	31.508	22.552	2.068	1.404	0.292	2.424
8	15.7	8.18	1500.5	35.016	15.064	1.700	1.896	0.412	2.230
9	19.7	8.40	1508.0	40.236	7.528	1.400	2.952	0.184	2.042
10	25.8	7.94	1511.5	46.928	0.168	1.076	3.952	0.316	1.819

Title: Plackett-Burman Design # 11
Date: April 27th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	pyr productivity
1	0.0	0.24	1500.0	0.000	36.112	4.688	0.000	4.804	0.000
2	2.0	0.40	1489.5	0.000	34.904	4.520	0.000	4.664	0.000
3	4.0	1.68	1481.0	0.788	35.440	4.636	0.000	4.656	0.197
4	6.0	4.80	1477.5	3.760	29.636	3.912	0.000	3.704	0.627
5	8.0	7.48	1476.5	9.500	27.768	3.028	0.024	3.076	1.188
6	10.0	8.24	1474.0	9.628	28.200	3.060	0.024	3.116	0.963
7	12.0	8.32	1476.0	16.464	16.092	1.616	0.472	1.104	1.372
8	14.5	8.24	1477.5	20.600	10.512	1.216	0.840	0.360	1.421
9	16.0	8.00	1479.5	21.516	6.708	1.056	0.000	0.248	1.345
10	18.0	7.92	1479.0	23.140	3.120	0.848	1.472	0.160	1.286

Title: Plackett-Burman Design # 12
Date: May 23th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	pyr productivity
1	0.0	0.16	1500.0	0.000	35.888	0.836	0.000	0.808	0.000
2	2.0	0.60	1493.5	0.000	33.296	0.656	0.000	0.884	0.000
3	4.0	2.12	1486.0	1.868	32.924	0.484	0.000	0.556	0.467
4	6.0	2.60	1480.5	3.508	33.128	0.204	0.000	0.336	0.585
5	8.0	2.90	1474.5	5.724	32.752	0.100	0.000	0.148	0.716
6	10.0	3.10	1469.5	7.512	31.160	0.000	0.000	0.000	0.751
7	12.0	3.24	1467.0	9.432	29.132	0.000	0.320	0.004	0.786
8	14.0	3.26	1461.0	9.864	26.776	0.000	0.492	0.000	0.705
9	16.0	3.52	1455.0	12.204	25.516	0.000	0.000	0.000	0.763
10	18.0	3.52	1447.5	13.216	23.928	0.000	0.912	0.000	0.734

APPENDIX C

EFFECT OF DINITROPHENOL

Title: CGSC6162 Fermentation #1
Date: June 24th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetic acid feed at 0.3542g/h

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/gGlucose)
1	0.0	0.66	1500.0	0.000	58.248	7.560	0.000	0.528	0.000	0.000
2	2.0	1.04	1492.5	0.000	59.188	7.712	0.000	0.524	0.000	0.000
3	4.0	4.40	1486.0	4.396	51.160	6.764	0.000	0.104	1.099	0.620
4	6.0	5.14	1484.0	9.980	48.176	6.364	0.000	0.000	1.663	0.991
5	8.0	7.46	1487.0	16.456	39.496	3.356	0.000	0.344	2.057	0.878
6	10.5	8.80	1493.0	25.124	30.760	2.532	0.804	0.344	2.393	0.914
7	13.0	8.46	1497.5	31.508	22.552	2.068	1.404	0.292	2.424	0.883
8	15.7	8.18	1500.5	35.016	15.064	1.700	1.896	0.412	2.230	0.811
9	19.7	8.40	1508.0	40.236	7.528	1.400	2.952	0.184	2.042	0.793
10	25.8	7.94	1511.5	46.928	0.168	1.076	3.952	0.316	1.819	0.808

Title: CGSC6162 Fermentation #2
Date: September 20th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetic acid feed at 0.3542g/h

Sample	Time (h)	OD 600 (nm)	Ferm, Vol.(ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.40	1500.0	0.000	60.760	11.368	0.000	0.716	0.000	0.000
2	2.0	1.38	1492.0	0.448	59.944	11.696	0.000	0.968	0.224	0.549
3	4.3	5.36	1493.5	1.368	16.948	3.664	0.000	0.224	0.318	0.031
4	6.0	9.28	1503.5	7.188	25.024	3.432	0.172	0.308	1.198	0.201
5	8.0	9.17	1510.5	17.716	32.196	4.480	0.412	0.376	2.215	0.620
6	10.8	9.38	1517.5	17.276	17.940	2.616	1.004	0.284	1.600	0.403
7	13.0	9.49	1522.5	9.236	5.744	0.976	0.912	0.180	0.710	0.168
8	21.8	9.13	1557.5	14.664	1.052	0.876	2.148	0.624	0.673	0.246
9	24.8	8.89	1555.5	15.824	0.624	0.940	2.716	0.804	0.638	0.263

Title: CGSC6162 Fermentation #3
Date: September 20th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetic acid feed at 0.3355g/h

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.190	1500.0	0.000	58.308	10.876	0.000	0.648	0.000	0.000
2	2.0	0.846	1492.5	0.636	56.764	11.252	0.000	0.560	0.318	0.412
3	4.3	5.934	1497.0	7.616	55.904	12.168	0.000	0.520	1.771	3.168
4	6.0	8.404	1508.0							0.000
5	8.0	9.362	1511.0	20.564	34.036	4.172	1.688	0.236	2.571	0.847
6	10.8	9.916	1520.0	6.820	6.448	0.768	1.348	0.072	0.631	0.132
7	13.0	9.942	1525.0	21.048	12.396	1.908	3.964	0.364	1.619	0.458
8	21.8	8.324	1556.5	14.716	0.276	0.736	3.804	0.884	0.675	0.254
9	24.8	8.536	1546.5	14.144	0.000	0.748	2.968	1.360	0.570	0.243

Title: CGSC6162 Fermentation #4
Date:December 22th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetic acid feed at 0.3542g/h

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.16	1500.0	0.000	63.940	10.276	0.000	0.984	0.000	0.000
2	3.0	2.43	1494.0	0.000	59.464	9.276	0.312	1.248	0.000	0.000
3	6.0	9.40	1501.5	8.908	47.416	6.868	2.364	0.516	1.485	0.539
4	9.7	9.88	1523.0	8.648	18.916	2.992	2.892	0.072	0.892	0.192
5	12.0	8.95	1526.5	10.348	14.592	2.696	4.256	0.228	0.862	0.210
6	16.0	9.22	1537.0	16.600	11.628	3.124	8.272	0.384	1.038	0.317
7	20.0	8.62	1545.8	20.664	5.284	3.072	10.856	0.552	1.033	0.352
8	24.0	7.92	1541.8	23.228	0.328	0.516	12.096	1.124	0.968	0.365

Title: 1st time fermentation using CGSC6162 with dinitrophenol
Date: September 5th, 2003
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetic acid feed at 0.3542g/h
Dinitrophenol: 100μ M at time=0

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.08	1500.0	0.000	59.604	11.608	0.000	0.500	0.000	0.000
2	2.0	0.83	1496.0	0.000	60.656	12.472	0.000	0.804	0.000	0.000
3	4.0	3.02	1488.5	3.900	56.116	12.116	0.000	0.960	0.975	1.118
4	6.0	7.09	1495.5	8.832	43.184	7.060	0.296	0.652	1.472	0.538
5	8.0	9.32	1504.5	14.900	31.404	5.224	0.832	0.480	1.863	0.528
6	10.5	9.87	1511.0	15.192	17.720	3.216	1.140	0.336	1.447	0.363
7	13.0	9.25	1517.0	21.976	14.568	3.232	2.180	0.572	1.690	0.488
8	16.0	8.94	1524.0	31.076	10.964	3.656	4.072	1.216	1.942	0.639
9	24.0	9.57	1544.0	34.432	0.540	2.972	4.648	0.000	1.435	0.583

Title: 2nd time fermentation using CGSC6162 with dinitrophenol
Date: June 16th, 2004
Strain: CGSC6162
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetic acid feed at 0.3542g/h
Dinitrophenol: 100μM at time=0

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.13	1500.0	0.000	60.876	9.384	0.000	0.412	0.000	0.000
2	2.0	0.36	1499.0	0.000	58.288	8.904	0.000	1.240	0.000	0.000
3	4.0	2.41	1495.5	0.000	48.452	7.208	0.296	1.300	0.000	0.000
4	6.0	6.03	1501.5	6.612	48.692	7.148	0.248	0.344	1.102	0.689
5	8.0	8.40	1506.5	11.248	41.912	6.284	0.816	1.100	1.406	1.720
6	10.0	8.84	1519.0	17.292	32.616	5.392	1.280	1.012	1.729	0.664
7	13.0	9.00	1534.5	22.708	23.300	4.584	2.044	0.536	1.747	0.616
8	16.0	8.82	1546.0	26.092	15.376	3.940	2.372	1.464	1.631	0.510
9	20.0	9.44	1560.5	31.340	8.164	3.708	3.024	2.428	1.567	0.570
10	24.0	9.04	1573.0	35.276	1.684	3.520	3.676	3.300	1.470	0.671

Title: 1st time fermentation using CGSC6162 *ppc*::Kan with dinitrophenol
Date: September 26th, 2003
Strain: CGSC6162 *ppc*::Kan
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetate feed at 0.3542g/h
Dinitrophenol: 100μM at time=0

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.32	1500.0	0.000	51.96	10.268	0.000	0.748	0.000	0.000
2	2.0	1.15	1503.5	0.000	38.36	7.912	0.000	0.584	0.000	0.000
3	4.0	3.97	1502.0	3.808	60.02	13.084	0.000	1.108	0.952	-0.473
4	6.0	7.79	1512.0	12.104	51.29	13.420	0.000	1.372	2.017	17.905
5	8.0	8.44	1519.0	17.552	40.56	5.380	0.976	1.456	2.194	1.540
6	9.7	8.63	1520.0	22.992	36.06	4.992	1.328	1.768	2.370	1.446
7	16.5	9.65	1542.5	32.292	16.99	3.764	2.436	3.700	1.957	0.923
8	22.0	8.84	1561.0	38.532	2.96	3.180	3.312	6.876	1.751	0.786

Title: 2nd time fermentation using CGSC6162 *ppc*::Kan with dinitrophenol
Date: September 26th, 2003
Strain: CGSC6162 *ppc*::Kan Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetate feed at 0.3355g/h
Dinitrophenol: 100μM at time=0

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.96	1500.00	0.340	52.88	10.376	0.000	0.692	0.000	0.000
2	2.0	1.07	1494.00	0.500	43.30	9.108	0.000	0.656	0.250	0.017
3	4.0	4.18	1497.00	3.840	57.78	12.860	0.000	1.048	0.960	-0.715
4	6.0	8.10	1509.00	12.656	47.14	6.012	0.684	0.780	2.109	2.144
5	8.0	8.29	1514.00	11.384	23.59	2.792	1.392	0.496	1.423	0.377
6	9.7	9.44	1516.50	13.012	17.29	2.108	1.588	0.468	1.341	0.356
7	16.5	9.25	1552.00	32.004	0.11	2.864	5.252	1.924	1.940	0.600
8	22.0	8.23	1573.50	23.272	2.96	1.304	4.964	2.364	1.058	0.459

APPENDIX D.

COMPARISON OF STRAINS FOR GROWTH AND PYRUVATE PRODUCTION IN OPTIMIZED MEDIA

Title: 1st fermentation with CGSC6162 *ldhA*::Kan
Date: June 28th, 2003
Strain: CGSC6162 *ldhA*::Kan
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetate feed at 0.3355g/h

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.54	1500.0	0.000	57.824	7.172	0.000	0.344	0.000	0.000
2	1.7	0.82	1492.0	0.000	38.104	4.772	0.000	0.284	0.000	0.000
3	4.0	5.50	1490.0	2.788	36.832	4.608	0.000	0.188	0.697	0.133
4	6.0	8.84	1500.0	8.436	40.944	4.736	1.244	0.284	1.406	0.500
5	8.0	10.76	1509.0	13.092	33.008	4.048	0.168	0.208	1.637	0.528
6	10.0	10.84	1513.5	18.704	28.236	3.616	0.348	0.212	1.870	0.632
7	13.7	10.64	1533.5	21.580	19.416	2.920	0.556	0.112	1.575	0.562
8	18.0	8.32	1540.0	19.684	16.304	3.024	1.552	1.556	1.094	0.474
9	21.5	8.90	1556.0	17.136	5.120	1.880	0.512	1.852	0.797	0.325
10	24.5	10.20	1560.0	16.612	1.372	1.452	0.344	1.852	0.678	0.294

Title: 2nd fermentation with CGSC6162 *ldhA*::Kan
Date: July 11th, 2003
Strain: CGSC6162 *ldhA*::Kan
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetate feed at 0.3355g/h

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.50	1500.0	0.000	57.816	6.876	0.000	0.484	0.000	0.000
2	2.0	1.26	1496.0	0.000	31.164	3.824	0.000	0.184	0.000	0.000
3	4.0	5.86	1497.0	3.472	37.148	4.500	0.000	0.168	0.868	0.168
4	6.0	9.36	1507.0	4.880	18.776	2.064	0.000	0.040	0.813	0.125
5	8.0	8.80	1523.5	11.296	27.032	2.596	0.000	0.324	1.412	0.367
6	10.0	9.90	1517.5	12.580	19.876	1.832	0.000	0.164	1.258	0.332
7	13.0	9.18	1525.5	24.864	24.268	2.240	0.104	0.356	1.913	0.741
8	16.0	10.22	1538.0	18.948	12.080	1.184	0.068	0.132	1.184	0.414
9	20.0	8.82	1549.0	12.388	4.288	0.512	0.000	0.068	0.619	0.231
10	25.5	8.98	1557.5	18.304	0.812	1.020	0.708	0.896	0.718	0.321

Title: 3rd fermentation with CGSC6162 *ldhA*::Kan
Date: July 3rd, 2004
Strain: CGSC6162 *ldhA*::Kan
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetate feed at 0.3355g/h

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.20	1500.0	0.000	54.724	8.424	0.000	0.360	0.000	0.000
2	2.0	2.14	1503.5	0.000	39.368	6.176	0.000	0.724	0.000	0.000
3	4.0	8.06	1498.0	2.296	46.624	6.592	0.188	2.144	0.574	0.283
4	6.0	8.17	1502.5	3.296	28.068	3.996	0.280	2.200	0.549	0.124
5	8.0	9.74	1503.0	7.040	37.000	5.084	0.900	4.092	0.880	0.397
6	10.0	10.01	1506.0	5.824	18.372	2.636	0.784	3.144	0.582	0.160
7	13.0	10.54	1515.5	10.056	19.032	3.132	0.652	5.228	0.774	0.282
8	16.0	10.68	1520.5	11.184	12.444	2.540	0.776	5.452	0.699	0.265
9	20.0	11.17	1530.0	18.284	11.648	3.268	1.748	8.576	0.914	0.424
10	24.0	10.99	1541.0	22.116	2.104	3.028	1.232	4.460	0.922	0.420

Title: 1st fermentation with CGSC6162 *ppc*::Kan
Date: June 28th, 2003
Strain: CGSC6162 *ppc*::Kan
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetate feed at 0.3542g/h

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.28	1500.0	0.000	60.984	7.588	0.000	0.664	0.000	0.000
2	1.7	0.86	1491.5	0.000	65.228	8.184	0.000	0.444	0.000	0.000
3	4.0	5.32	1486.5	3.276	51.512	6.844	0.000	0.476	0.819	0.346
4	6.0	9.92	1490.0	9.700	43.764	5.484	0.000	0.372	1.617	0.563
5	8.0	11.30	1499.0	8.392	19.084	2.068	1.104	0.132	1.049	0.200
6	10.0	10.00	1507.5	11.572	17.424	1.988	1.504	0.036	1.157	0.266
7	13.7	10.26	1518.5	24.484	18.936	2.552	3.432	0.256	1.787	0.582
8	18.0	8.86	1530.0	15.048	8.640	1.636	4.748	1.116	0.836	0.287
9	21.5	9.36	1543.0	29.256	3.576	1.988	6.904	3.188	1.361	0.510
10	24.5	8.56	1538.0	32.076	0.000	2.088	6.668	4.556	1.309	0.526

Title: 2nd fermentation with CGSC6162 *ppc*::Kan
Date: June 10th, 2004
Strain: CGSC6162 *ppc*::Kan
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetate feed at 0.3542g/h

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.11	1500.0	0.000	62.564	9.280	0.000	1.644	0.000	0.000
2	2.0	0.39	1490.0	0.000	60.356	8.976	0.000	1.764	0.000	0.000
3	4.0	2.46	1494.0	1.608	54.624	8.076	0.140	2.324	0.402	0.203
4	6.0	6.64	1486.0	5.880	52.600	7.752	0.332	2.076	0.980	0.590
5	8.0	8.38	1495.5	12.440	41.428	6.192	0.744	1.548	1.555	0.589
6	10.0	9.30	1499.5	16.392	34.172	5.324	0.964	1.404	1.639	0.577
7	13.0	9.62	1513.5	22.032	25.320	4.436	1.484	1.560	1.695	0.592
8	16.0	9.56	1523.5	26.348	19.884	4.000	1.860	2.068	1.647	0.617
9	20.0	9.23	1535.0	30.516	12.660	3.428	2.284	2.920	1.526	0.611
10	24.0	8.89	1545.5	34.156	6.736	3.044	2.708	3.844	1.423	0.612

Title: 1st fermentation with CGSC6162 *poxB*15::*lacZ*::Cam
Date: June 24th, 2003
Strain: CGSC6162 *poxB*15::*lacZ*::Cam
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetate feed at 0.3355g/h

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.40	1500.0	0.000	61.116	7.792	0.000	0.588	0.000	0.000
2	2.0	3.08	1495.5	0.000	59.400	7.744	0.000	1.156	0.000	0.000
3	4.0	9.74	1499.0	4.588	51.016	7.080	0.000	2.452	1.147	0.454
4	6.0	11.08	1499.0	7.072	44.140	5.048	0.040	3.312	1.179	0.417
5	8.0	20.44	1502.0	10.172	40.016	4.476	0.256	4.712	1.272	0.482
6	10.5	12.94	1507.5	12.744	31.928	3.780	0.436	5.84	1.214	0.437
7	13.0	12.46	1510.0	15.296	25.852	3.388	0.688	7.172	1.177	0.434
8	15.7	11.52	1516.0	13.904	15.920	2.532	0.628	6.648	0.886	0.308
9	19.7	12.48	1523.5	20.040	12.428	2.880	1.036	9.600	1.017	0.412
10	25.8	12.38	1537.0	22.744	3.728	2.508	0.325	11.052	0.882	0.396

Title: 2nd fermentation with CGSC6162 *poxB*15::*lacZ*::Cam
Date: July 9th, 2003
Strain: CGSC6162 *poxB*15::*lacZ*::Cam
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetate feed at 0.3355g/h

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.44	1500.0	0.000	59.016	7.588	0.000	0.200	0.000	0.000
2	2.0	2.14	1492.5	0.000	58.956	7.660	0.000	0.216	0.000	0.000
3	4.0	10.96	1496.0	1.768	37.280	5.220	0.000	1.748	0.442	0.081
4	6.0	11.18	1501.0	4.412	41.928	6.036	0.000	3.468	0.735	0.258
5	8.0	11.88	1503.5	3.136	21.084	3.176	0.000	3.556	0.392	0.083
6	10.7	13.62	1509.5	5.412	21.300	2.924	0.536	4.120	0.506	0.143
7	13.0	11.54	1511.5	6.424	19.928	3.024	0.652	5.420	0.494	0.164
8	16.0	12.84	1515.5	9.872	21.216	3.692	1.116	8.544	0.617	0.261
9	20.0	13.68	1523.0	11.012	15.112	3.368	1.312	9.840	0.551	0.251
10	24.0	12.06	1532.0	8.968	7.076	2.444	1.128	8.540	0.374	0.173

Title: 3rd fermentation with CGSC6162 *poxB*15::*lacZ*::Cam
Date: July 3rd, 2004
Strain: CGSC6162 *poxB*15::*lacZ*::Cam
Temperature: 37°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetate feed at 0.3542g/h

Sample	Time (h)	OD 600 (nm)	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)	Pyruvate productivity (g/Lh)	Pyruvate Yield (g/g Glucose)
1	0.0	0.20	1500.0	0.000	53.540	8.136	0.000	0.808	0.000	0.000
2	2.0	0.91	1504.5	0.000	24.280	3.924	0.000	0.244	0.000	0.000
3	4.0	4.21	1495.5	2.484	36.796	5.940	0.000	0.996	0.621	0.148
4	6.0	7.49	1505.0	5.728	46.068	7.176	0.196	3.348	0.955	0.767
5	8.0	8.83	1513.5	5.444	21.324	3.440	0.240	2.020	0.681	0.169
6	10.0	10.00	1523.0	9.080	19.944	3.644	0.296	2.196	0.908	0.270
7	13.0	10.17	1540.5	11.528	15.492	3.356	0.196	2.836	0.887	0.303
8	16.0	9.66	1547.5	14.856	11.128	3.212	0.728	3.188	0.929	0.350
9	20.0	9.33	1576.5	13.888	4.768	2.328	0.796	2.708	0.694	0.285
10	24.0	9.46	1591.0	21.180	6.532	3.028	1.428	9.532	0.883	0.451

APPENDIX E

EFFECT OF TEMPERATURE

Title: 1st fermentation at 34℃ with CGSC6162
Date: December 22th, 2003
Strain: CGSC6162
Temperature: 34°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetate feed at 0.3542g/h

Sample	Time (h)	OD 600 nm	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)
1	0.0	0.16	1500.0	0.000	55.772	8.908	0.000	0.600
2	3.0	2.80	1500.5	0.000	51.624	7.908	0.000	0.488
3	6.0	7.44	1498.5	8.288	48.224	6.688	1.208	0.144
4	9.7	9.10	1516.5	12.100	33.380	4.960	1.596	0.300
5	12.0	8.33	1517.5	6.116	10.924	1.456	0.616	0.192
6	16.0	8.92	1523.0	11.612	10.340	2.192	1.468	0.440
7	20.0	8.19	1525.0	23.192	11.928	3.400	3.052	1.480
8	24.0	7.93	1529.8	26.952	4.784	2.808	3.492	2.428

Title: 2nd fermentation at 34℃ with CGSC6162
Date: January 9th, 2004
Strain: CGSC6162
Temperature: 34°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetate feed at 0.3542g/h

Sample	Time (h)	OD 600 nm	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)
1	0.0	0.40	1500.0	0.000	34.168	5.060	0.000	0.444
2	2.5	2.02	1489.5	0.000	27.448	4.372	0.000	0.340
3	6.0	8.87	1512.0	8.792	35.832	4.496	4.996	0.000
4	9.0	8.74	1526.5	6.128	11.360	1.484	4.152	0.248
5	12.0	8.27	1537.0	12.220	11.892	1.800	9.364	0.520
6	17.7	7.09	1551.5	9.560	3.756	1.084	9.184	0.636
7	20.0	7.76	1549.0	5.660	0.936	0.772	7.400	0.896
8	24.0	11.75	1531.5	4.252	0.340	1.204	13.404	3.748

Title: 1st fermentation at 40℃ with CGSC6162
Date: December 22th, 2003
Strain: CGSC6162
Temperature: 40°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetate feed at 0.3542g/h

Sample	Time (h)	OD 600 nm	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)
1	0.0	0.10	1500.0	0.000	64.476	10.288	0.000	1.624
2	3.0	1.97	1489.0	0.000	59.556	9.288	0.000	1.956
3	6.0	9.07	1494.0	6.660	46.068	7.164	1.004	1.468
4	9.7	9.64	1515.0	11.444	32.356	5.740	2.652	1.088
5	12.0	8.82	1522.0	13.880	22.836	4.856	4.308	0.980
6	16.0	9.29	1535.0	17.436	13.060	4.084	6.912	1.040
7	20.0	8.84	1541.5	19.000	6.272	3.720	8.612	1.348
8	24.0	8.36	1543.5	22.836	0.444	3.328	8.996	2.444

Title: 2nd fermentation at 40℃ with CGSC6162
Date: January 9th, 2004
Strain: CGSC6162
Temperature: 40°C
pH: 7.0
Agitation: 750 rpm
Air Flow Rate: 1.5 L/min
Fed batch: 100% acetate feed at 0.3542g/h

Sample	Time (h)	OD 600 nm	Ferm, Vol. (ml)	Pyruvate (g/L)	Glucose (g/L)	Succinate (g/L)	Lactate (g/L)	Acetate (g/L)
1	0.0	0.25	1500.0	0.000	72.852	11.216	0.000	0.928
2	2.5	1.23	1483.0	0.000	58.932	8.708	0.000	1.116
3	6.0	8.82	1501.0	8.620	44.196	6.452	2.044	0.832
4	9.0	9.51	1524.0	9.344	26.072	4.328	2.596	0.592
5	12.0	9.23	1548.0	12.196	14.148	3.052	4.152	0.580
6	17.7	8.85	1576.5	12.308	3.916	1.952	5.216	0.596
7	20.0	8.11	1574.5	17.092	1.684	2.468	8.264	0.996
8	24.0	7.67	1564.5	19.404	0.400	2.900	9.292	2.144