SELECTIVE CONSUMPTION OF ACETATE WITHOUT SUGAR DEGRADATION BY METABOLICALLY ENGINEERED *ESCHERICHIA COLI*

by

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(Under the direction of Mark A. Eiteman)

ABSTRACT

The removal of acetate from biomass hydrolysate is essential for improving microbial production of biochemicals. This research focuses on microbial removal of acetate using the concept of substrate selective degradation. Acetate is selectively removed from glucose-xylose mixtures by metabolically engineered *Escherichia coli* strain KD840 with mutations in phosphotransferase system (PTS) genes of glucose (*ptsG, manZ, crr*), glucokinase (*glk*) and xylose (*xylA*). In batch cultures, KD840 consumed acetate exclusively at first, and no sugars were consumed up to 30 hours after acetate was exhausted. Six *Escherichia coli* strains were compared for maximum specific growth rate using 5 g/L acetate as the sole carbon source. MC4100 showed the greatest μ_{MAX} at 0.368 h^{-1} while MG1655 showed the lowest μ_{MAX} at 0.244 h^{-1}. Interestingly, MC4100 does not have a functional *acs-yjcH-yjcG* operon. ALS1126 (MG1655 *acs-yjcH-yjcG*) attained a μ_{MAX} of 0.262 h^{-1}, indicating *acs-yjcH-yjcG* operon does not affect the growth rate of *E. coli* on acetate.

INDEX WORDS: Acetate, Biomass hydrolysate, *Escherichia coli*, Metabolic Engineering, Operon, Phosphotransferase system, Substrate selective degradation

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DEDICATION

To my family

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Arun Shivkumar Lakshmanaswamy

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CHAPTER 1

INTRODUCTION

Introduction and Literature

Lignocellulosic biomass

Lignocellulose is the major structural component of plants, and is composed of the carbohydrate polymers cellulose and hemicellulose (up to 70% of the total dry matter) and lignin. The carbohydrate polymers are held together by lignin and by hydrogen and covalent bonds. Lignocellulosic biomass constitutes up to 90% of the plant biomass, equivalent to about 200×10^9 tons per year, of which $8\text{-}20\times10^9$ tons is accessible (Lin and Tanaka, 2006). Lignocellulose can potentially serve as a resource for conversion into different products such as biofuels, chemicals, improved animal feeds and human nutrients. In the U. S., ethanol from starch-based biomass is currently mixed with gasoline to as great as 10% v/v (Klinke et al., 2004).

Lignocellulosic biomass must be pretreated to make the polymeric carbohydrates available as monomers for microbial fermentation. The pretreatment primarily involves two steps. Delignification is initially carried out to release the carbohydrate polymers from lignin, and depolymerization is performed to reduce the complex carbohydrate polymers to simple free sugars (Lin and Tanaka, 2006). While cellulose is a high molecular weight glucose polymer, hemicelluloses are polymers of pentoses (xylose and arabinose) and hexoses, largely mannose or glucose (Fan et al., 1982; Bobleter 1994). Lignin mainly consists of polymerized aromatic alcohols such as coniferyl alcohol, sinapyl and p-coumaryl alcohol (Klinke et al., 2004).

Pre-treatment methods

Acid hydrolysis, enzymatic degradation, steaming or steam explosion (STEX), ammonia freeze explosion (AFEX) and wet oxidation (WO) are a few pre-treatment methods available to release carbohydrates from the lignocellulosic biomass. Acids and alkaline catalysts are often used during pretreatment at high temperatures. Weak acids are efficient in removing lignin but do not hydrolyze cellulose very well (Grethlein and Converse, 1991; Nguyen, 1993). The crystalline structure of cellulose makes it resistant to hydrolysis. When aqueous acid (0.3 mol/l sulphuric acid) is used to hydrolyze cellulose, much of the glucose obtained is also destroyed (Yu and Zhang, 2004). Enzymatic hydrolysis can be carried out using a mixture of cellulases, hemicellulases and lignases, although a process using enzymes remains very expensive on large scale due to high cellulase cost (Walker and Wilson, 1991).

In addition to monomeric sugar units, various other end products are formed during the pretreatment of lignocellulosic biomass. These products include phenols, furans and carboxylic acids. Products such as 2-furfural or 2-furoic acid are formed from the dehydration of monosaccharides. Phenolic compounds like 4-hydroxybenzoic acid, vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, syringaldehyde, and syringic acid are formed from the hydrolytic cleavage of lignin (Klinke et al., 2002). Acetic acid is generated because of the acetylation of hemicellulose and lignin (Sarkanen and Ludwig, 1971; Fengel and Wegener, 1989; Torssell, 1997). Glycolic acid and lactic acid are generated from alkaline degradation of carbohydrates (Alén et al., 1990; Sjöström, 1991). Formic acid is a product of sugar and lignin degradation (Klinke et al., 2002).

Inhibitory effect of acids, furans and phenol on microbial fermentation

Carboxylic acids, phenols and furan compounds are strong fermentation inhibitors by negatively affecting cell membrane function, growth and glycolysis of ethanol-producing bacteria (Klinke et al., 2002). During glucose fermentation to ethanol by *Saccharomyces cerevisiae* or *Zymomonas mobilis*, furans and phenol inhibit growth and reduce ethanol production rate (Delgenes et al. 1996). Ethanol production by *Z. mobilis* CP4 (pZB5) from xylose was reduced even at 0.5 mM vanillic acid (Ranatunga et al., 1997).

The presence of acetic acid in the medium causes several inhibitory effects on the growth of bacteria. Acetic acid has a negative effect on the stability of intracellular proteins (Stephanopoulos, 1998), and this molecule enters the cell and dissociates in the higher cellular pH to cause a reduction in proton motive force (Diaz-Ricci et al., 1990). In the absence of base for pH control, acetate acidifies the growth medium to a pH of 5 or less, at which point cell lysis occurs as a result of irreversible denaturation of proteins and DNA (Cherrington et al., 1991). Finally, the presence of acetate can impact the rate and yield of product formation. For example, *Escherichia coli* LY01 showed poor ethanol production and yield when acetate was present at 283 mM (Zaldivar and Ingram, 1999). Acetic acid in combination with furfural and furfuryl alcohol showed even greater growth inhibition of *E. coli* and lower ethanol yield (Zaldivar and Ingram, 1999, Zaldivar et al., 2000).

Microorganisms producing ethanol

Several bacteria, yeasts and fungi can produce ethanol from lignocellulosic biomass. *S. cerevisiae* is the preferred microbe, as it can produce ethanol to a final concentration of 18% (v/v) from glucose. *Z. mobilis* is able to generate ethanol at a very high yield because this organism uses the Entner-Doudoroff (ED) pathway, which is a unique characteristic of some

ethanol-producing organisms (Lin and Tanaka, 2006). *Z. mobilis* has a homoethanol fermentation pathway and tolerates up to 120 g/L ethanol (Sprenger, 1996). *E. coli* and *Clostridium* spp. also produce ethanol as a fermentation product.

E. coli possess several advantages over other microbes for the production of ethanol. *E. coli* can ferment a wide spectrum of sugars and does not require any complex growth factors. Industrially, *E. coli* have been extensively used to produce a variety of products such as recombinant proteins. *E. coli* strains can readily be altered by metabolic engineering to produce ethanol selectively (Ingram et al., 1987; Millichip and Doelle, 1989).

Ethanol producing pathways in bacteria

Many organisms consume simple sugars through the Embden-Meyer-Parnas pathway (glycolysis) to generate pyruvate, NADH and ATP. Under anaerobic conditions, oxidative phosphorylation does not occur, and 95% of the pyruvate is directed into pathways which regenerate NAD and form an organic end product. NAD must be regenerated for glycolysis and additional ATP production (Ingram et al., 1987). The conversion of pyruvate to ethanol is one such pathway to regenerate NAD. In yeast and *Z. mobilis*, pyruvate is initially converted to acetaldehyde and carbon dioxide by pyruvate decarboxylase (EC 4.1.1.1). This step is followed by the reduction of acetaldehyde to ethanol by alcohol dehydrogenase isoenzymes (EC 1.1.1.1), which is accompanied by the oxidation of NADH to NAD (Wills et al., 1981; Neale et al., 1986). In many bacteria, including *E. coli*, pyruvate is converted to acetyl CoA and formate by pyruvate-formate lyase. The acetyl CoA is then converted to acetaldehyde and ethanol by a two-step process via alcohol dehydrogenase which generates 2 moles of NAD.

Acetate assimilation in Escherichia coli

In *E. coli* acetate in its undissociated form (i.e., acetic acid) does not require a specific transport system to enter the cell (Repaske and Adler, 1981). However, the recent identification of the *actP* gene which encodes acetate permease, a membrane carrier for acetate, has confirmed the presence of an acetate transport system in *E. coli*. This transport system appears to play an important role when *E. coli* are grown on acetate and this substrate becomes scarce (Gimenez et al., 2003).

Once acetate is transported into the cell, acetate is converted into acetyl-CoA which in turn is metabolized through the glyoxylate pathway. The organism possesses two distinct enzymatic mechanisms by which acetate is converted to acetyl-CoA.

One mechanism to convert acetate into acetyl-CoA (the "PTA-ACK pathway") is via the sequential action of acetate kinase (ACK) and phosphotransacetylase (PTA). Acetate kinase (EC 2.7.2.1) mediates the reaction of acetate with one ATP molecule to form acetyl phosphate and ADP. Phosphotransacetylase (EC 2.3.1.8) then mediates the reaction of acetyl phosphate with CoA to form acetyl-CoA. This second step also results in the liberation of inorganic phosphate (Rose et al., 1954). ACK is encoded by the *ackA* gene while the *pta* gene encodes PTA. The *pta* and *ackA* genes map at about min 50 on the *E. coli* chromosome (Brown et al., 1977). ACK and PTA enzymes are active during aerobic and anaerobic conditions. ACK and PTA mediate reversible steps, and hence these enzymes are also involved in secretion of acetate during growth on sugars, in which case the order is first PTA and then ACK (Brown et al., 1977; Kwan et al., 1988). Acetate kinase has an optimal pH of 7.4 (Brown et al., 1977). ACK and PTA activities do not vary significantly among wild type strains of *E. coli* grown using different carbon sources (Brown et al., 1977).

The second mechanism for the cellular metabolism of acetate (the "AMP-ACS pathway") involves the enzyme acetyl Coenzyme A synthetase (ACS, EC 6.2.1.1) encoded by the *acs* gene, and which converts acetate to acetyl CoA by a two-step irreversible process. ACS initially converts acetate with ATP to an enzyme-bound intermediate acetyl-AMP. This reaction results in the formation of pyrophosphate. The enzyme then converts acetyl-AMP with a molecule of CoA to form acetyl-CoA and release AMP (Chou and Lipmann, 1952; Berg., 1956). ACS has a very high affinity towards acetate (K_M of 200 μM), and therefore its role is to scavenge acetate at very low concentrations (Brown et al., 1977; Kumari et al., 1995). Two promoter regions *acs*P2 and *acs*P1 are associated with the transcription of the *acs* gene. *acs*P2 is the principal promoter, while *acs*P1 is a much weaker promoter located almost 200 bp upstream of *acs*P2 (Kumari et al., 2000; Browning et al., 2002; Beatty et al., 2003). The *acs* gene is also regulated by transcription factors cyclic AMP receptor protein (CRP) (Kumari et al., 2000), nucleotide associated proteins IHF and FIS (Browning et al., 2004) and two sigma factors (Shin et al., 1997). Acetate kinase mutants are able to produce acetyl-CoA from acetate, ATP and Coenzyme A due to ACS activity (Brown et al., 1977). ACS cannot substitute GTP for ATP and requires Mg^{2+} as a cofactor. The enzyme has optimal activity at a pH of 8.5. ACS mediates acetate uptake from the medium with high affinity and a lower V_{MAX} value than ACK. The enzyme is not synthesized in the absence of acetate in the growth medium (Brown et al., 1977).

E. coli require the AMP-ACS pathway for growth at low concentrations of acetate, and appear to use the ACK-PTA pathway when acetate is available at comparatively high concentrations. Double mutant strains lacking both pathways are not able to utilize acetate (Kumari et al., 1995). Other acetate activation enzymes have been identified in bacteria, including as propionyl-CoA synthetase encoded by *prpE* gene of *Salmonella enterica* serovar

typhimurium (Horswill and Escalante-Semerena., 1999) and propionate kinases encoded by the *tdcD* gene in *E. coli* (Hesslinger et al., 1998).

Acetate metabolism

Acetyl-CoA is an important intermediate that enters the tricarboxylic acid (TCA) cycle by combining with oxaloacetate to form citrate and subsequently isocitrate. *E. coli* carry out an anaplerotic pathway known as the glyoxylate pathway, "shunt" or "cycle" when grown on acetate as the sole carbon source. The glyoxylate pathway differs from the TCA cycle because it bypasses the two oxidative steps in which carbon dioxide is generated. The glyoxylate pathway enzymes isocitrate lyase and malate synthase redirect isocitrate from the TCA cycle and prevent the loss of acetate carbon. This pathway is a mechanism to form a 4-carbon precursor molecule from two 2-carbon substrates like acetate (Kornberg et al., 1966). Isocitrate lyase mediates the reaction of isocitrate (6-carbons) to succinate (4-carbons) and glyoxylate (2-carbon). Glyoxylate then reacts with acetyl CoA via the action of malate synthase to form malate (4-carbon).

Isocitrate lyase (ICL, EC 4.1.3.1) catalyzes the conversion of isocitrate by an aldol cleavage to form glyoxylate and succinate (Campbell et al., 1953). ICL is a tetramer with each subunit encoded by the *aceA* gene (Diehl and McFadden, 1994). ICL requires an exogenous cation, and Mg^{2+} is the more effective than Mn^{2+}, Ni^{2+} or Sr^{2+} (Hoyt et al., 1988). ICL has low affinity towards its substrate and requires a large intracellular pool of isocitrate to function (Holmes, 1987). The optimum pH for activity is 7.3 (Robertson and Reeves, 1987). Mutant *E. coli* strains lacking ICL activity are unable to grow when acetate is the only available carbon source. However, these strains are able to grow well on glucose (Kornberg et al., 1966).

Malate synthase (MS, EC 2.3.3.9) catalyzes the condensation of acetyl-CoA with glyoxylate to produce malate, an intermediate in the TCA cycle (Wong et al., 1956; Ornston and Ornston, 1969). MS requires Mg^{2+} as a cofactor (Howard et al., 2000).

Isocitrate dehydrogenase (ICDH, EC 1.1.1.41) catalyzes the conversion of isocitrate to α-ketoglutarate. Bacteria that possess an NADP-dependent ICDH are able to grow on acetate, while strains having an NAD-dependent ICDH, or which lack a complete TCA cycle or respiratory pathway are incapable of growing on acetate (Chen and Gadal, 1990). An NADP-dependent ICDH is required when acetate is the sole carbon source because cells use this enzymatic step to synthesize more than 90% of the NADPH required by the cell for biosynthesis (Vasquez and Reeves, 1979; Walsh and Koshland, 1984). *E. coli* have one NADP-linked ICDH consisting of two identical subunits encoded by the *icd* gene (Thorsness and Koshland, 1988).

The TCA cycle enzyme ICDH competes with the first glyoxylate pathway enzyme ICL for the common substrate isocitrate. The reversible phosphorylation of ICDH regulates the flow of carbon between the TCA cycle and glyoxylate cycle. When acetate is the sole carbon source, phosphorylation of active ICDH occurs by the binding of NADP in a reaction that is catalyzed by the bifunctional enzyme isocitrate dehydrogenase kinase/phosphatase encoded by *aceB*. Phosphorylated ICDH is unable to bind to isocitrate as a result of disturbed hydrogen bonds between serine-113 and isocitrate. Also, electrostatic repulsion is created between phosphate of the phosphoserine and the γ-carboxylate of isocitrate (Dean et al., 1989; Dean and Koshland, 1990; Hurley et al., 1990). Almost 75% of the ICDH in bacteria growing on acetate remains inactive due to its phosphorylation (Edlin and Sundaram, 1989), and thus in *E. coli* the pathway flux from isocitrate to α-ketoglutarate diminishes (Borthwik et al., 1984). The majority of isocitrate is thereby forced into the glyoxylate cycle. Because isocitrate lyase has a much lower

affinity (K_M of 604 μM) for isocitrate than ICDH (K_M of 8 μM), a high concentration of intracellular isocitrate is required to sustain the carbon flux through isocitrate lyase (El-Mansi et al., 1985; Holmes et al., 1986). ICDH phosphorylation maintains a consistent ICDH activity although the cellular levels of ICDH vary among *E. coli* strains (LaPorte et al., 1985). The specific activity of ICDH decreases while adapting and utilizing acetate but increases when acetate is exhausted (Lowry et al., 1971). Mutant strains unable to phosphorylate ICDH are incapable of growing on acetate (LaPorte et al., 1985).

Alternative pathway

In the absence of oxygen, the glyoxylate pathway cannot function (Iuchi and Lin, 1988; Iuchi et al., 1989). However, acetate utilization can occur in anaerobes which use the cofactor ferredoxin. In these cases, ferredoxin forms pyruvate and α-ketoglutarate through reductive carboxylation. The low redox potential of ferredoxin prevents the oxidative decarboxylation of pyruvate to acetyl CoA and CO_2 and of α-ketoglutarate to succinyl CoA and CO_2. Ferredoxin production is observed in many anaerobes and some photosynthetic bacteria, but is not present in *E. coli* (Buchanan and Arnon, 1970; Adman et al., 1973; Cozzone, 1998).

Gene regulation when acetate is carbon source

Gene expression in *E. coli* is vastly different when the organism grows on acetate compared to other substrates like glucose. For example, the *aceK* and *aceA* genes were up-regulated by more than 10 times in MC4100 with acetate was the sole carbon source compared to glucose as sole carbon source (Oh et al., 2001). Other related genes such as *mdh* which encodes for malate dehydrogenase, *fumA*, *fumC* and *fumB* genes which together encode for fumarase (Oh et al., 2002), and the *gtlA* and *acnB* genes which encode respectively for citrate synthase and aconitase were also up-regulated. MC4100 grown on M9 medium containing acetate generally

down-regulated glycolytic genes (such as *pfkA, fba, gapA, epd, pgk, eno, pykF* and *ppc*) and also pentose pathway genes *zwf* and *gnd*. The *aceEF* operon encoding for pyruvate dehydrogenase was also considerably down-regulated during growth on acetate (Oh et al., 2002).

Glucose and xylose intake mechanisms

In *E. coli*, carbohydrates have to be phosphorylated before they can be metabolized. The phosphotransferase system (PTS) is a carbohydrate-specific transport system consisting of two proteins EI and EII, involved in phosphorylation and transport of a carbohydrate into the bacterial cell. While a single EI and the carrier protein HPr are common to most PTSs, EII proteins are carbohydrate-specific. The EII enzyme specific for glucose phosphorylation in *E. coli* is glucosephosphotransferase (EC 2.7.1.69): a protein with two membrane bound domains B and C ($EIICB^{glc}$) and one soluble protein ($EIIA^{glc}$). $EIICB^{glc}$ is coded by the *ptsG* (*gpt*) gene and $EIIA^{glc}$ is encoded by the *crr* gene. The phospho group required for phosphorylation is acquired from phosphoenolpyruvate and is transferred to glucose through the phosphorylated intermediates of EI, HPr and EII domains (Postma et al., 1993). The EII enzyme "specific" for mannose phosphorylation, mannosephosphotransferase (EC 2.7.1.69) is encoded by the *manZ* (*mpt*) gene and it can also transport and phosphorylate glucose. Glucokinase (EC 2.7.1.2), an ATP dependent enzyme encoded by the *glk* gene is also known to phosphorylate glucose intracellularly (Curtis and Epstein, 1975). Hence, *E. coli* are believed to possess three enzymes which phosphorylate glucose molecules. Curtis and Epstein (1975) reported that all three knockouts (*ptsG manZ glk*) were required to prevent *E. coli* from growing on glucose as the sole carbon source.

Due to considerable sequence homology between EII proteins in the PTS systems, genes corresponding to other carbohydrate PTS systems besides glucose might be able to transfer

glucose into the cell. The *malX* gene of the maltose PTS system codes for a protein whose amino acid sequence displays nearly 35% identity to the enzyme coded by *ptsG*. This protein has a molecular weight of 56,654 Daltons and is able to recognize glucose although glucose is not the primary substrate (Reidl and Boos, 1991). EII^{fru} protein of the fructose specific PTS system which catalyses the intake and phosphorylation of fructose to fructose 1-phosphate also showed homology with EII^{glu}, particularly in regions surrounding histidine residues. The EII^{fru} protein is expressed by the gene *fruA* (Prior and Kornberg, 1988). The *bgl* operon which enables *E. coli* to grow on β-glucosides, consists of the *bglF* gene which encodes the specific transport protein enzyme phospho-β-glucosidase (EII^{bgl}) (Schnetz et al., 1987; Schnetz and Rak, 1988). The enzyme EII^{bgl} also shows significant sequence homology to the carboxyl-terminal of the EII^{glu} protein. The His-547 region of the amino acid sequence of EII^{bgl} obtains a phosphate group from the HPr protein and transfers it to His-306 located in its amino terminal region. The His-306 region is highly conserved in the EII^{bgl} and EII^{glu} proteins (Bramley and Kornberg, 1987).

$EIIA^{glu}$ coded by *crr* is a well-characterized protein in *E. coli*. This 169 amino acid sequence has a molecular weight of 18,251 Daltons. P-HPr phosphorylates $EIIA^{glu}$ at His-90 as an intermediate during PEP-phosphotransfer of glucose. Phosphorylation of sucrose and sugar intake by maltose PTS system requires a functional $EIIA^{glu}$ in *E. coli* (Lengeler et al., 1982; Dills and Seno, 1983; Schmid et al., 1988; Presper et al., 1989; Reidl and Boos, 1991). Strains lacking the *crr* gene expressed normal levels of all PTS proteins except for $EIIA^{glu}$ whose activity was found to be extremely low (Saier and Roseman, 1976).

The xylose transport system of *E. coli* consists of two key enzymes which transport xylose into the cell and convert it to xylulose 5-phosphate before it is catabolized in the pentose phosphate pathway (Schellenberg et al., 1984). Within the cell, xylose isomerase (EC 5.3.1.5)

isomerizes xylose to xylulose which is subsequently phosphorylated to xylulose-phosphate by xylose kinase (EC 2.7.1.17). The genes which code for xylose isomerase and xylose kinase are *xylA* and *xylB* respectively (David and Wiesmeyer, 1970).

Acetate degradation and accumulation in the presence of glucose

Although *E. coli* can grow on acetate as the sole carbon source, it does not do so in the presence of glucose from which the cells derive more energy (El-Mansi et al., 2006). Another problem with acetate degradation in the presence of glucose is the enzymes involved in the glyoxylate shunt are repressed in the presence of glucose (Shiloach and Fass, 2005). Acetate is also secreted by *E. coli* when grown in higher concentrations of sugars because of the saturation of TCA cycle by fast oxidation that takes place in glycolysis (Majewski and Domach, 1990; Han et al., 1992). Numerous researchers have studied the phenomenon of acetate excretion and on overcoming the inhibition caused when acetate is secreted. For example, the *Bacillus subtilis* enzyme acetolactate synthase was cloned in *E. coli* to redirect the flow of carbon from pyruvate to acetoin and acetolactate (Aristidou et al., 1994). *E. coli* K cloned with the clostridial constitutive acetone operon was able to convert acetate to acetone which could be removed by aeration and agitation because of its high volatility (Bermejo et al., 1998). Other strategies to reduce acetate formation are reducing growth rate of *E. coli* by controlling the glucose supply (Riesenberg. 1991), or removal of acetate from the medium by dialysis fermentation of *E. coli* cultures (Portner and Markl, 1998). Overall, the presence of even 2 g/L of acetate in the growth medium slows *E. coli* growth and affects biomass formation (Shiloach and Fass, 2005).

Objectives

This study is one facet of a project whose ultimate objective is to increase the yield and productivity of biochemicals such as ethanol from lignocellulosic hydrolysate using metabolically engineered *E. coli*. As an important part of this project, this study focuses on an approach to use metabolically engineered *E. coli* for the complete consumption of acetate in the hydrolysate but avoid the consumption of any sugar.

The three main objectives are

1. Comprehensively compare *E. coli* strains growing on acetate for growth rate, biomass yield and key enzyme activities.
2. Select and construct an *E. coli* strain that selectively consumes acetate in the presence of sugars.
3. Demonstrate continuous selective acetate consumption in simulated lignocellulosic hydrolysates.

Hypotheses

1. The comprehensive comparison of *E. coli* strains on acetate would help identify a fast growing strain which will be used for constructing an acetate-specific strain.
2. The constructed strain will grow on acetate but not on xylose and glucose. These characteristics will be maintained on simulated lignocellulosic hydrolysate.

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CHAPTER 2

SELECTIVE MICROBIAL REMOVAL OF ACETATE FROM SUGAR MIXTURES

[1]Lakshmanaswamy, A., Eiteman, M.A., Altman, E. To be submitted to *Biotechnol. Bioeng.*

Abstract

Acetic acid is an unavoidable constituent of the biomass hydrolysate generated from acetylated hemicellulose and lignin. The removal of acetate from hydrolysate is necessary for improving the microbial production of biochemicals. In this study, acetate is selectively removed from mixtures of glucose and xylose by metabolically engineered *Escherichia coli* strain ALS1060 with mutations in the phosphotransferase system (PTS) genes of glucose (*ptsG*, *manZ*), glucokinase (*glk*) and xylose (*xylA*). In batch culture, ALS1060 consumed exclusively acetate, and then consumed the two sugars only at a very slow rate after the acetate in the medium had been exhausted. In order to eliminate all sugar consumption, we also examined the effects of knockouts in the *crr* gene and glucose non-specific PTS genes *malX*, *fruA*, *fruB*, and *bglF* in ALS1060. Batch and fed-batch process carried out using the *crr* knockout strain KD840 demonstrated that KD840 did not utilize the sugars for more than 30 hours after acetate was selectively consumed from a glucose-xylose mixture.

Introduction and Literature Review

Conversion of lignocellulosic biomass to fuels and chemicals by microbial fermentation is a promising alternative to petroleum-based processes (Zaldivar et al., 2001). Lignocellulosic materials are inexpensive and readily available, and are largely carbohydrates in the form of cellulose and hemicellulose (Klinke et al., 2004). However, several challenges remain which limit the wide use of lignocellulosic biomass. One challenge is that biomass hydrolysates contain inhibitors such as acetic acid (acetate). Acetate is an unavoidable product of hemicellulose depolymerization since xylose is acetylated in lignocellulose (Timmel, 1967; Sarkanen and Ludwig, 1971; Fengel and Wegener, 1989; Chesson et al., 1993; Torssell, 1997). Xylose conversion appears particularly sensitive to acetate, with a 1.5 g/L concentration at a pH of 5.0 reducing 50% of the yield of ethanol using *S. cerevisiae* 259ST (Helle et al., 2003). The membrane of *S. cerevisiae* is highly permeable to acetate, and therefore this yeast is particularly susceptible to acetate inhibition (Casal et al., 1998). Acetate also exacerbates other inhibitory effects: for example, furfuryl alcohol and 2-furfural reduce ethanol yield by *E. coli* more in the presence of acetate (Zaldivar and Ingram, 1999; Zaldivar et al., 1999, 2000). Although the generation of some inhibitors might be reduced by judicious design of the hydrolysis process or by genetic improvements in the biomass itself, elimination of all acetate in a lignocellulosic hydrolysate does not currently seem feasible.

A wide variety of strategies have been proposed to ameliorate the effect of acetate on fermentation (Lasko et al., 2000). For example, ion exchange (Horváth et al., 2004; Chandel et al., 2007) or activated carbon (Benson et al., 2005) can remove acetate from solutions. Similarly, extraction with ethyl acetate reduces acetic acid (and furfural, vanillin and 4-hydroxybenzoic acid) and results in a 93% improvement in ethanol yield using *Pichia stipitis*

(Wilson et al., 1989). These approaches involve an additional processing step which significantly affects overall costs (Von Sivers et al., 1994). Preferably, an approach should not only leave the production microorganisms unaffected but remove acetate completely at very low cost.

We have previously reported a biological strategy for selectively removing components from a mixture (Eiteman et al., 2008). The approach involves the genetic construction of a single strain that will utilize *only* one component in a mixture. Since many organisms including *E. coli* readily consume acetate when this compound is the sole carbon source (Holms 1986), acetate might be removed from a mixture of xylose, glucose and acetate (for example) with a strain that is genetically prevented from consuming xylose and glucose. Glucose uptake primarily is mediated by glucosephosphotransferase (EC 2.7.1.69) for which the $EIICB^{glc}$ component is encoded by *ptsG* (Postma et al., 1993), mannosephosphotransferase (EC 2.7.1.69) for which the EII component is encoded by *manZ* (Curtis and Epstein, 1975) and ATP-dependent glucokinase (EC 2.7.1.2) encoded by *glk* (Curtis and Epstein, 1975). Knocking out the *ptsG*, *manZ* and *glk* genes prevents *E. coli* from consuming glucose in a short batch process, while a *xylA* mutant is unable to consume xylose (Eiteman et al., 2008). Thus, a strain with the four knockouts (i.e., *ptsG manZ glk xylA*) might prevent consumption of both sugars but allow normal acetate metabolism.

Other cellular processes could also be involved in glucose transport. Due to considerable sequence homology between EII proteins, genes corresponding to other carbohydrate PTS systems might also transfer glucose into the cell. For example, the *malX* gene of the maltose PTS system encodes a protein that binds to glucose and displays nearly 35% sequence identity to the protein encoded by *ptsG* (Reidl and Boos, 1991), and the EII^{fru} protein of the fructose

specific PTS system (encoded by the *fruA* gene) shows similarity with EII^{glu} (Prior and Kornberg, 1988). The *bglF* gene in *E. coli* encodes the specific transport protein phospho-β-glucosidase (EII^{bgl}) (Schnetz et al., 1987; Schnetz and Rak, 1988) and shows significant sequence homology to the carboxyl-terminal of the EII^{glu} protein (Bramley and Kornberg, 1987). $EIIA^{glu}$ is phosphorylated during PEP-phosphotransfer of glucose, and strains lacking the *crr* gene express normal levels of all PTS proteins except for $EIIA^{glu}$ (Saier and Roseman, 1976). Since none of these carbohydrate transport genes is known to be involved in acetate metabolism, knockouts of these genes could eliminate any residual glucose uptake without affecting acetate consumption.

Using a mixture of xylose, glucose and acetate as a model for biomass hydrolysate containing an inhibitor, the goals of this study were to construct an acetate-selective strain which does not consume xylose and glucose, and to demonstrate that this strain could effectively remove acetate from a mixture containing these sugars.

Materials and Methods

Bacterial Strains

The *Escherichia coli* strains studied are shown in Table 2.1. All of the strains used in this research were either taken from the ALS or KD laboratory collection. Strains containing Δ*ptsG*763::FRT, Δ*manZ*743::FRT, Δ*glk*-726::FRT, Δ*xylA*748::FRT, Δ*crr*-746::FRT, Δ*fruA*723::FRT, Δ*fruB*725::FRT, or Δ*bglF*753::FRT deletions were generated by transducing MG1655 with the corresponding Keio (FRT)Kan deletion (Baba et al., 2006) and then curing the Kan(R) using the pCP20 plasmid, which contains a temperature-inducible FLP recombinase as well as a temperature-sensitive replicon (Datsenko and Wanner, 2000). The Δ*xylA*748::(FRT)kan, Δ*crr*-746::(FRT)Kan and Δ*malX*769::(FRT)Kan deletions that are

contained in ALS1072, KD840 and KD857, respectively, are Keio (FRT)Kan deletions in which the Kan(R) has not been cured.

Shake Flask Growth Conditions

Basal medium (BA10) contained (per L): 13.3 g KH_2PO_4, 4.0 g $(NH_4)_2HPO_4$, 1.2 g $MgSO_4 \cdot 7H_2O$, 13.0 mg $Zn(CH_3COO)_2 \cdot 2H_2O$, 1.5 mg $CuCl_2 \cdot 2H_2O$, 15.0 mg $MnCl_2 \cdot 4H_2O$, 2.5 mg $CoCl_2 \cdot 6H_2O$, 3.0 mg H_3BO_3, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, 100 mg Fe(III)citrate, 8.4 mg $Na_2EDTA \cdot 2H_2O$, 1.7 g citric acid, 0.0045 g thiamine·HCl, and 10 g/L acetate using $Na(CH_3COO) \cdot 3H_2O$. BA2 medium was identical except it contained 2 g/L acetate. Both BA2 and BA10 media were supplemented with xylose and/or glucose as described in the text. Acetate, xylose and glucose were autoclaved separately, sterily combined, and neutralized with NaOH to a pH of 7.0. Concentrations are reported as acetate as the monovalent anion.

Table 2.1 *E. coli* strains used in this study.[*]

Strain	Genotype
MG1655	F-λ- *rph*-1 (wild type)
ALS1060	MG1655 Δ*ptsG*763::FRT Δ*manZ*743::FRT Δ*glk*-726::FRT Δ*xylA*748::FRT
ALS1072	MG1655 Δ*ptsG*763::FRT Δ*manZ*743::FRT Δ*glk*-726::FRT Δ*xylA*748::(FRT)Kan
ALS1122	MG1655 Δ*ptsG*763::FRT Δ*manZ*743::FRT Δ*glk*-726::FRT Δ*xylA*748::FRT Δ*crr*-746::FRT
ALS1123	MG1655 Δ*ptsG*763::FRT Δ*manZ*743::FRT Δ*glk*-726::FRT Δ*xylA*748::FRT Δ*fruA*723::FRT
ALS1124	MG1655 Δ*ptsG*763::FRT Δ*manZ*743::FRT Δ*glk*-726::FRT Δ*xylA*748::FRT Δ*fruB*725::FRT
ALS1125	MG1655 Δ*ptsG*763::FRT Δ*manZ*743::FRT Δ*glk*-726::FRT Δ*xylA*748::FRT Δ*bglF*753::FRT
KD840	MG1655 Δ*ptsG*763::FRT Δ*manZ*743::FRT Δ*glk*-726::FRT Δ*xylA*748::FRT Δ*crr*-746::(FRT)Kan
KD857	MG1655 Δ*ptsG*763::FRT Δ*manZ*743::FRT Δ*glk*-726::FRT Δ*xylA*748::FRT Δ*malX*769::(FRT)Kan

[*] Arun Lakshmanaswamy assisted Ronni Altman in the construction of *E.coli* strains except ALS1060

Growth Conditions

For shake flask experiments, 50 mL BA2 medium contained 2 g/L glucose in 250 mL baffled shake flasks at 37°C and 350 rpm (19 mm pitch).

For bioreactor experiments, the selected strain was first grown (5 mL) in a 30 mL shaking test tube containing 5 g/L tryptone, 2.5 g/L yeast extract, 5 g/L NaCl and 2.5 g/L acetate, then transferred to a baffled 250 mL shake flask containing 50 mL BA10 medium incubated at 37°C and 250 rpm (19 mm pitch). When the OD of the culture reached 2.0 - 2.5, the contents of the shake flask were transferred to a bioreactor.

Batch processes using 1.0 L BA10 medium were carried in a 2.5 L bioreactor (Bioflo 2000, New Brunswick Scientific Co. Edison, NJ, USA). Air was sparged into the fermenter at a flowrate of 1.0 L/min, and the agitation was 500 rpm to ensure no oxygen limitation. The pH was controlled at 7.0 using 20% (w/v) NaOH or 20% (v/v) H_2SO_4, and the temperature was controlled at 37°C.

Fed-batch processes initially operated in batch mode and contained the BA medium with 5 g/L acetate. When the OD reached 3.0 - 3.5, BA10 medium supplemented with xylose and/or glucose was fed at an exponentially increasing rate to achieve a constant growth rate of 0.07 h^{-1}. Concentrated NH_4OH was used for base control, and the feed solution contained 100 mg/L kanamycin.

Assays

The optical density measured at 600 nm absorbance (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, Calif.) was used to monitor cell growth. Glucose, xylose, acetate, and other organic by-products were quantified as previously described (Eiteman and Chastain, 1997).

Results

Batch Growth on Acetate

Escherichia coli MG1655 is a common wild-type strain (Jensen 1993) which is able to consume acetate as the sole carbon source. We first sought to quantify this growth in an aerobic batch culture, and Figure 2.1 shows the results in BA10 medium (i.e., initial concentration of 10 g/L acetate). MG1655 formed approximately 2.5 g/L cells (OD = 7.1) at a specific growth rate of 0.23 h^{-1}.

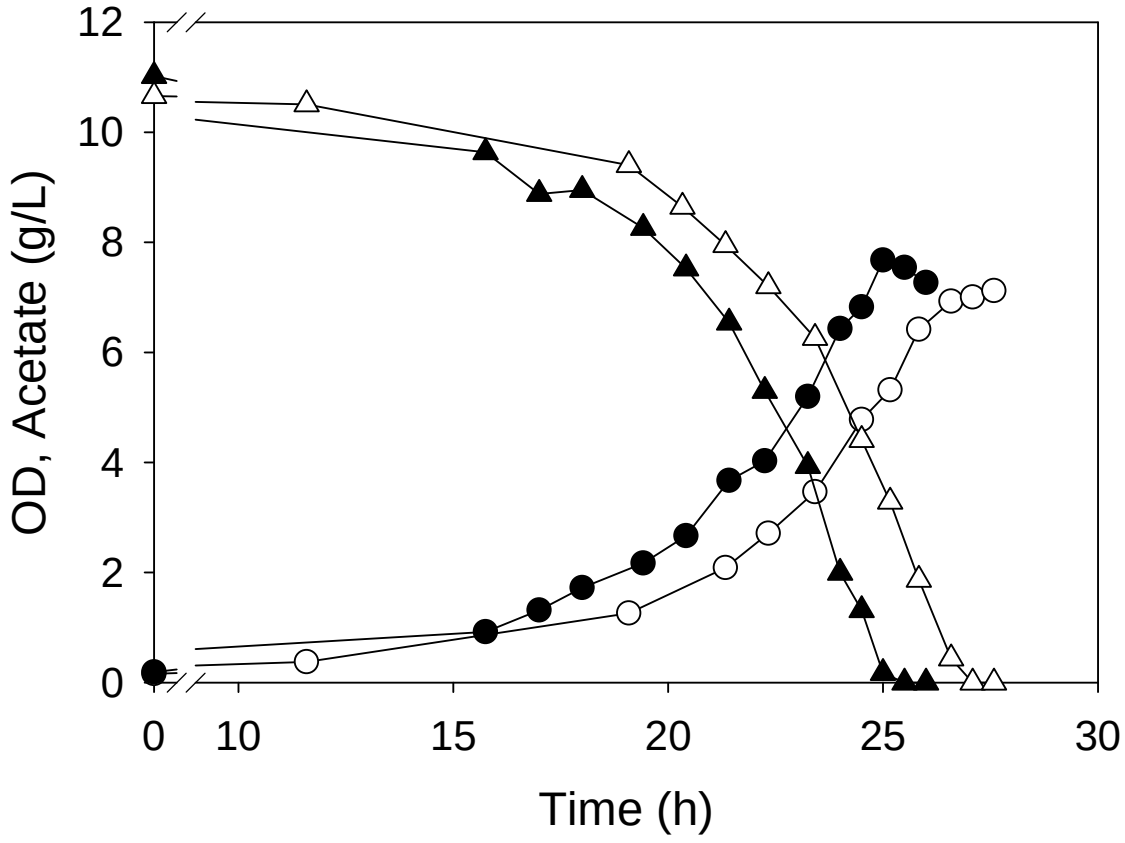

Figure 2.1: Aerobic batch culture of *E. coli* MG1655 (hollow symbols) and ALS1060 (solid symbols) using BA10 medium which initially contained 10 g/L acetate (▲, △) as the sole carbon source. The OD (●, ○) was measured over the course of the processes.

E. coli ALS1060 has four knockouts of genes coding for proteins involved in the utilization of xylose and glucose: *ptsG* encodes the $IICB^{Glc}$ domain of the phosphotransferase system (PTS) for carbohydrate transport (Postma et al., 1993), *manZ* encodes the IID^{Man} domain of the mannose PTS permease (Huber and Erni, 1996), *glk* encodes glucokinase (Curtis and Epstein, 1975) while *xylA* encodes xylose isomerase. We hypothesized that these four mutations would prevent the utilization of both xylose and glucose. In order to determine whether these mutations had any effect on the growth on acetate, we first grew ALS1060 in the same medium (Figure 2.1). Similar to MG1655, ALS1060 formed 2.5 g/L cells (OD = 7.7), and attained a specific growth rate of 0.22 h^{-1}.

Batch Growth on Acetate in the Presence of Sugars

Our next objective was to determine whether acetate could be exclusively consumed from a mixture of sugars. Since ALS1060 contains knockouts involved in the consumption of xylose or glucose, the growth of this strain in a medium containing xylose, glucose and acetate is expected to identical to growth in the medium containing acetate alone. In order to test this prediction, we grew ALS1060 in batch culture over an extended period of time in BA10 medium in the presence of either 20 g/L glucose, 10 g/L xylose or in a mixture of 10 g/L xylose and 20 g/L glucose

Batch culture using ALS1060 in BA10 medium containing 20 g/L glucose did result in exclusive acetate consumption during the first 20 h of the process (Figure 2.2). Moreover, during growth on acetate the specific growth rate was 0.21 h^{-1}, essentially identical to the growth rate observed in medium without glucose (Figure 2.1). Interestingly, slow glucose consumption commenced about the time acetate was nearly exhausted (Figure 2.2), and the specific growth rate of ALS1060 after acetate was exhausted was found to be 0.014 h^{-1}.

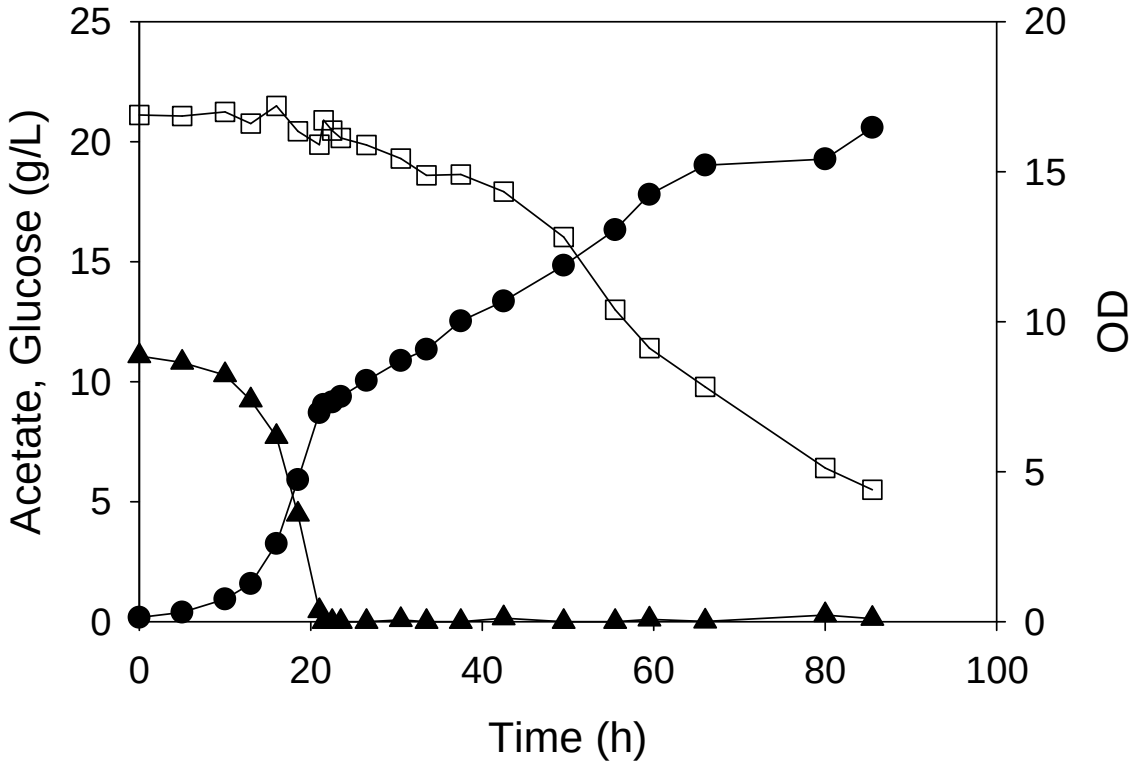

Figure 2.2: Aerobic batch culture of *E. coli* ALS1060 using BA10 medium plus 20 g/L glucose. Glucose (□), acetate (▲) and the OD (●) were measured over the course of the process.

During batch culture using BA10 medium containing 10 g/L xylose ALS1060 consumed exclusively acetate, and the concentration of xylose remained unaltered throughout the process (Figure 2.3). During the 24 h of acetate consumption, the specific growth rate was approximately 0.15 h^{-1}.

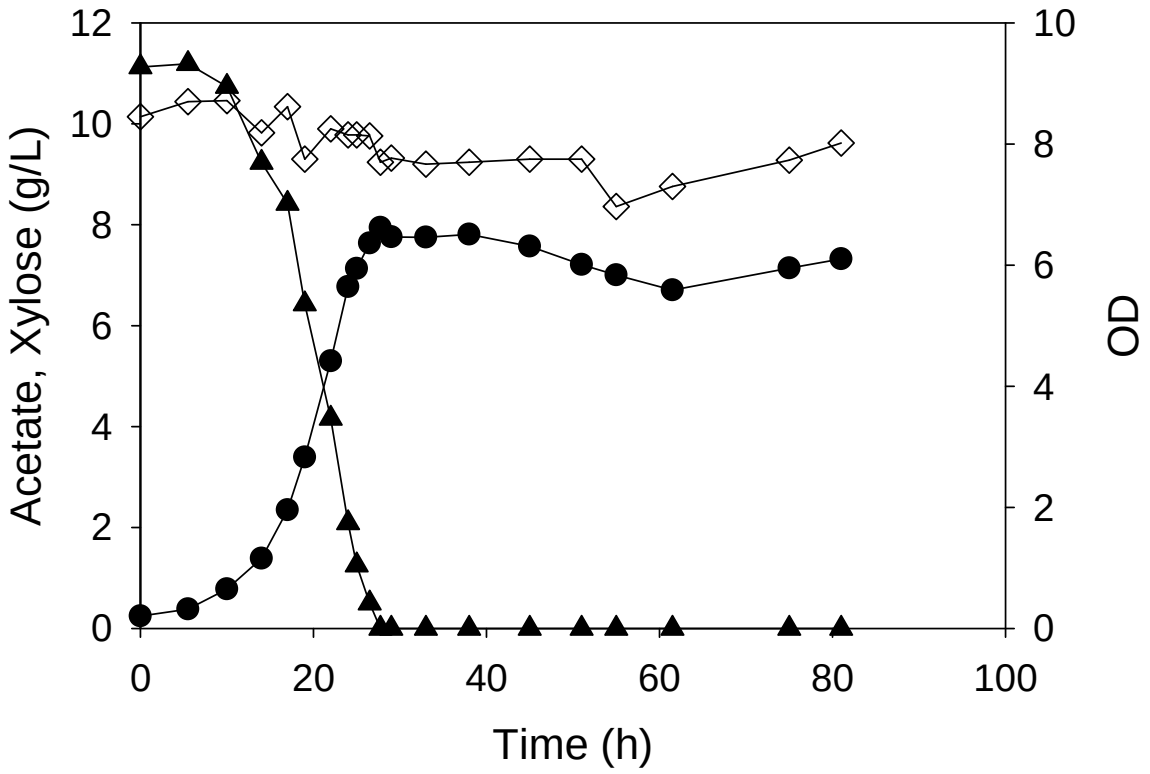

Figure 2.3: Aerobic batch culture of *E. coli* ALS1060 using BA10 medium plus 10 g/L xylose. Xylose (◇), acetate (▲) and the OD (●) were measured over the course of the process.

In order to study how the presence of both sugars influenced acetate utilization, ALS1060 was inoculated into BA10 medium with 20 g/L glucose and 10 g/L xylose. In this case, ALS1060 consumed the acetate in 30 h, during which time less than 2 g/L glucose and 0.6 g/L xylose was consumed. During this initial period, the cell growth rate was approximately 0.13 h^{-1}. Over the next 40 h, about 7 g/L glucose and 3 g/L xylose were slowly consumed (Figure 2.4).

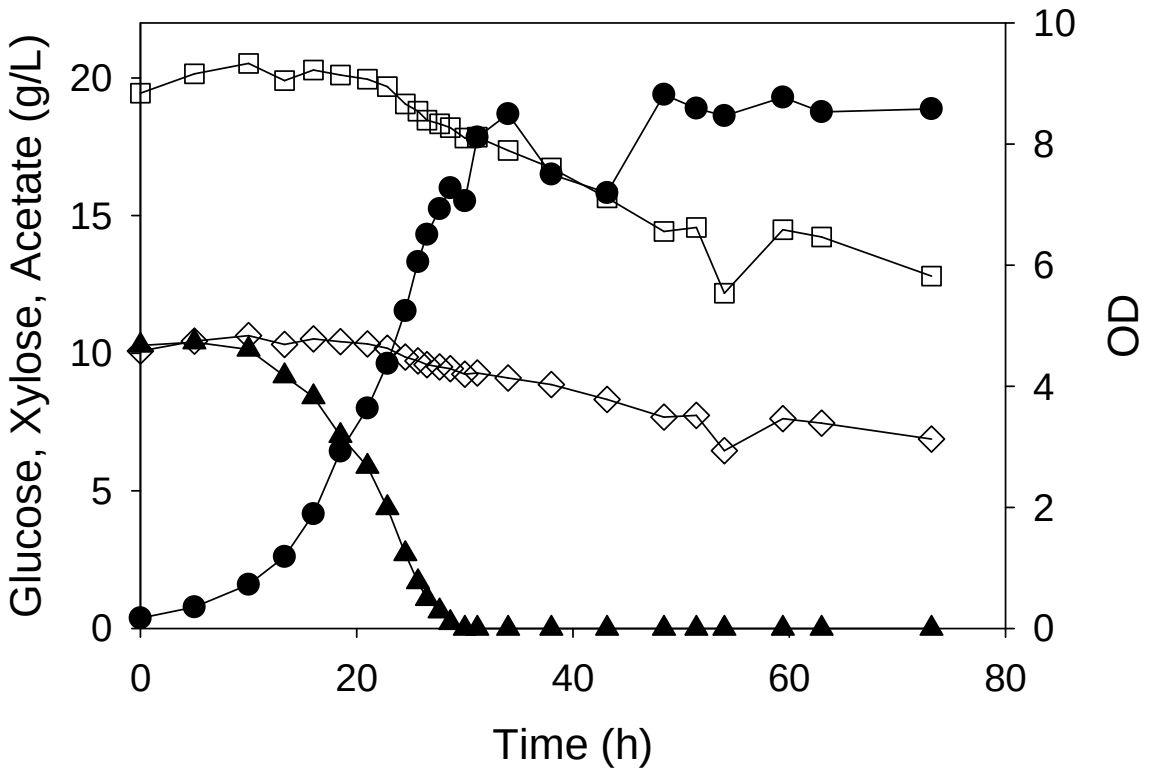

Figure 2.4: Aerobic batch culture of *E. coli* ALS1060 using BA10 medium plus 20 g/L glucose and 10 g/L xylose. Glucose (□), xylose (◇), acetate (▲), and the OD (●) were measured over the course of the process.

Additional knockouts to eliminate glucose consumption

Because ALS1060 grew in the presence of glucose albeit slowly, *E. coli* must have another means to transport and utilize glucose. Mutations in the *ptsG manZ glk* genes are insufficient to prevent glucose consumption. With the goal of completely excluding glucose consumption, we next examined growth on BA2 medium with 2 g/L glucose of strains having mutations additionally in one of several other genes encoding non-specific PTS proteins: *malX* encoding a protein of the maltose-specific PTS (KD857), *fruA* or *fruB* encoding proteins of the fructose-specific PTS (ALS1123 and ALS1124, respectively); the *bglF* gene involved in the PTS

of β-glucosides (ALS1125), or *crr* which encodes the $EIIA^{glu}$ (ALS1122). To compare growth and glucose consumption, two parameters were measured: the rate of glucose uptake for approximately 30 h beyond the time that acetate was exhausted ($\Delta G/\Delta t$), and the change in the optical density for approximately 30 h beyond the time that acetate was exhausted ($\Delta OD/\Delta T$). Compared to ALS1060, only ALS1122 (with the *crr* mutation) showed a significantly reduced rate of glucose consumption at only 8.4 mg/L·h (Table 2.2). This strain also showed essentially no change in the optical density over the 30 h period, in contrast to the other strains.

Table 2.2 Growth characteristics of strain in BAG media

Name of strain	ΔOD/Δt (AU/h)	ΔG/Δt (mg/L·h)
ALS1060	0.018	18.0
ALS1122	-0.003	8.4
ALS1123	0.017	16.9
ALS1124	0.016	20.3
ALS1125	0.035	28.7
KD857	0.023	21.4

Batch Growth of crr Knockout on Acetate in the Presence of Sugars

Our next goal was to determine if the *crr* knockout prevented the consumption of glucose and xylose compared to the *ptsG manZ glk* strain, ALS1060. For this first study we used KD840 (ALS1122 with kanamycin resistance) growing in BA10 medium with 20 g/L glucose. The 10 g/L acetate was consumed during the first 35 h (Figure 2.5), and cells attained a slower specific growth rate of 0.13 h^{-1} on acetate. During the 35 h of acetate consumption and for another 35 h

after acetate consumption, glucose was not consumed. However, after 70 h glucose was consumed rather quickly, with 12 g/L consumed over the next 17 h.

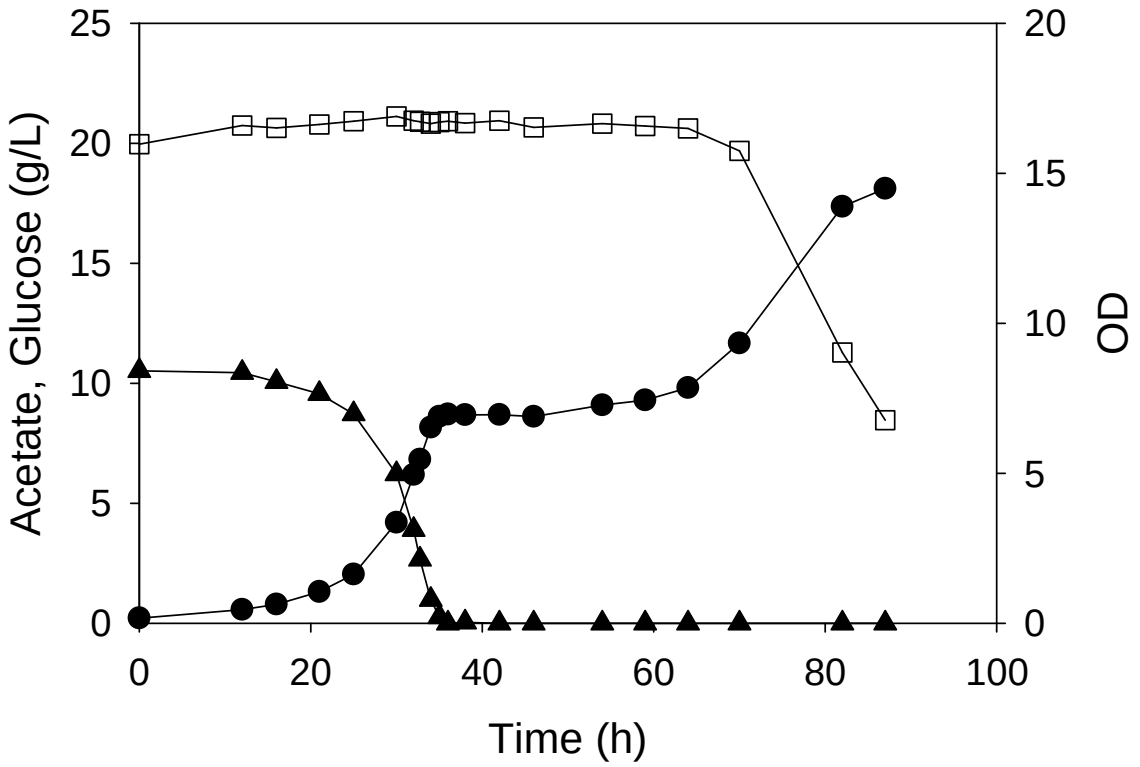

Figure 2.5: Aerobic batch culture of *E. coli* KD840 on BA10 medium with 20 g/L glucose. Glucose (□), acetate (▲) and the OD (●) were measured over the course of the process.

We also examined batch growth of KD840 in BA10 medium with 20 g/L glucose and 10 g/L xylose. Similar to the growth of KD840 in the mixture of acetate and glucose, acetate was completely consumed by 45 h with a specific growth rate of 0.12 h^{-1}, and the concentrations of xylose and glucose remained unchanged over the next 45 h (Figure 2.6). After about 90 h, the concentration of glucose decreased comparatively quickly.

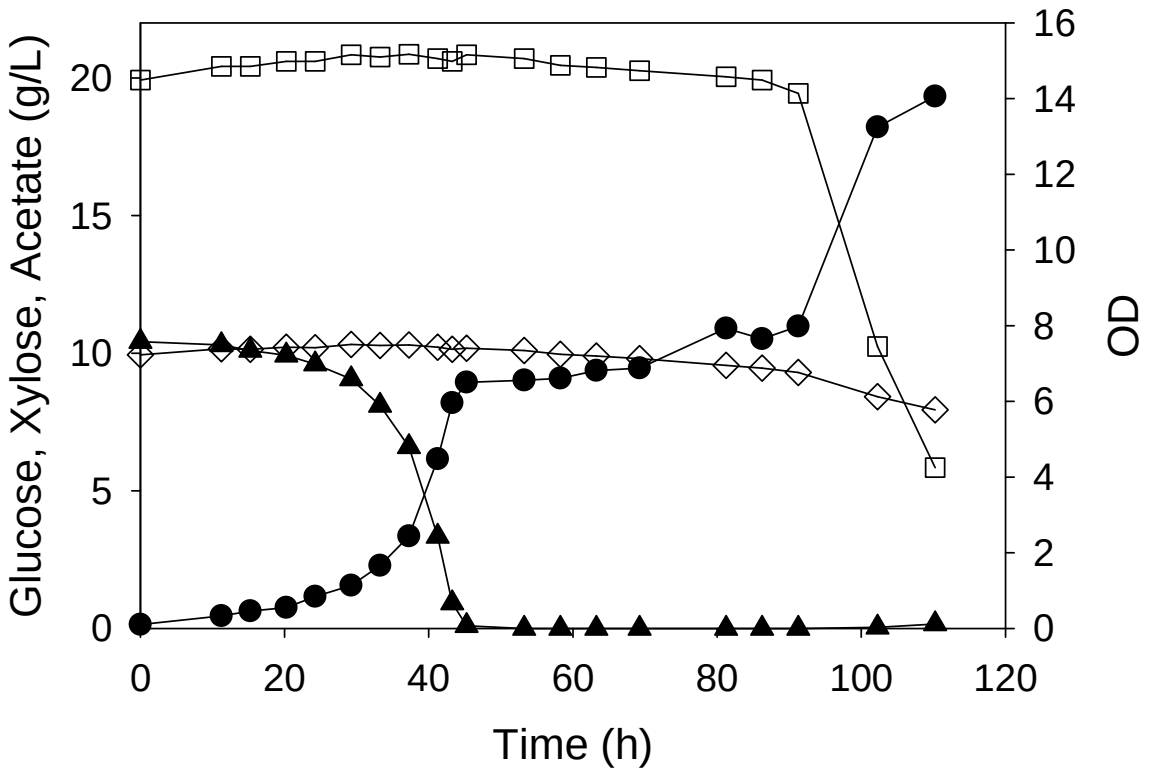

Figure 2.6: Aerobic batch culture of *E. coli* KD840 using BA10 medium plus 20 g/L glucose and 10 g/L xylose. Glucose (□), xylose (◇), acetate (▲), and the OD (●) were measured over the course of the process.

Fed-Batch Growth on Acetate in the Presence of Sugars

To determine whether the acetate-selective strains could continuously remove acetate, fed-batch processes were carried out using ALS1072 and KD840. ALS1072 is identical to ALS1060, but also is resistant to kanamycin, which permitted this antibiotic to be included in the medium to prevent contamination. Fed-batch processes are different than batch processes in that during the latter process the cell growth rate is maximal since the cells are not limited by the availability of the carbon source acetate. In contrast, an acetate-limited fed-batch process controls the growth rate below its maximum rate and maintains the substrate concentration near

zero throughout the process. After an initial batch phase of about 20 h, xylose, glucose and acetate were fed at a constant rate for an additional 70 h.

ALS1072 consumed acetate during the batch phase and attained a specific growth rate of 0.13 h^{-1}. Glucose and xylose were not consumed during the batch phase, but their concentrations decreased at slowly during the fed-batch phase (Figure 2.7). Succinate was generated as an end product ultimately to a concentration of 5.5 g/L. Succinate had not been detected in any other prior experiment.

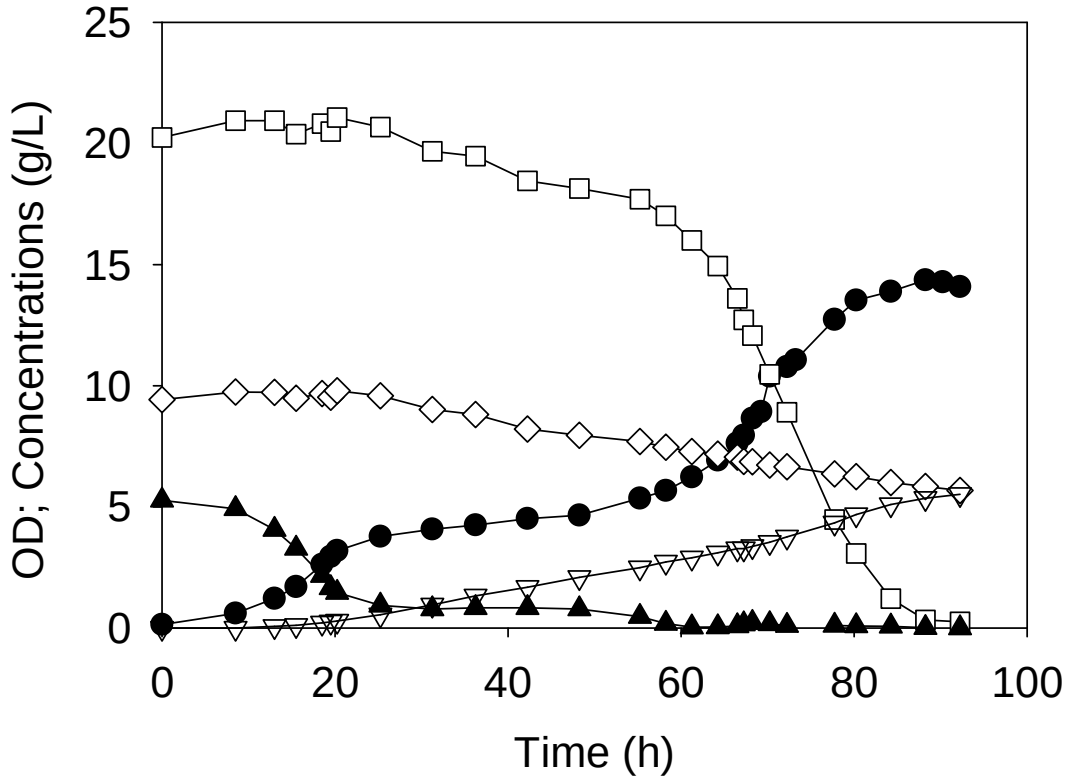

Figure 2.7: Fed-batch aerobic fermentation of *E. coli* ALS1072 using a mixture of glucose (□), xylose (◇) and acetate (▲). The OD (●) and succinate (▽) were also measured over the course of the fermentation.

KD840 also consumed acetate during the initial batch phase and attained a specific growth rate of 0.13 h^{-1}. The concentrations of both sugars were unchanged during the batch phase, and also during the first 31 h of the fed-batch process (Figure 2.8). The glucose concentration decreased rather quickly after about 62 h, becoming exhausted at about 95 h. Succinate was again generated as an end product ultimately to a concentration of 3.5 g/L.

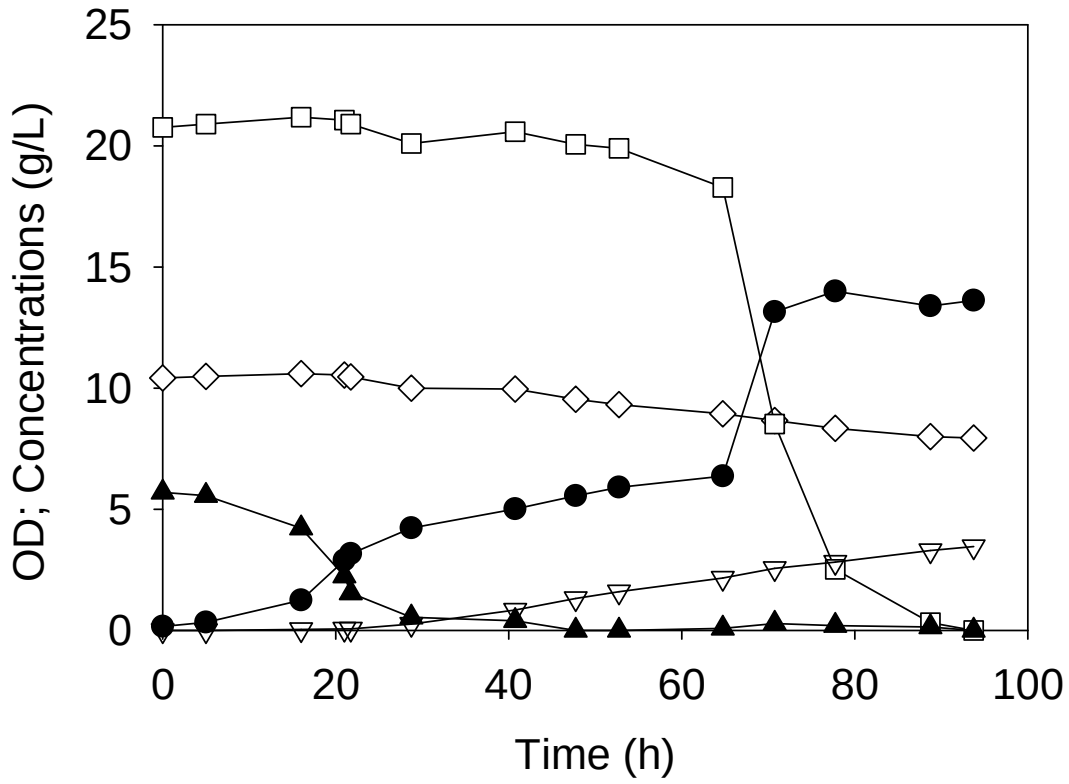

Figure 2.8: Fed-batch aerobic fermentation of *E. coli* KD840 using a mixture of glucose (□), xylose (◇) and acetate (▲). The OD (●) and succinate (▽) were also measured over the course of the fermentation.

Discussion

Substrate selective degradation of acetate from mixed sugars using metabolically engineered *E. coli* is a novel strategy that can be applied for conversion of lignocellulosic hydrolysate to biofuels. Although *E. coli* ALS1060 with mutations in the phosphotransferase system (PTS) genes of glucose (*ptsG*, *manZ*), glucokinase (*glk*) and xylose (*xylA*) preferably degraded acetate, this strain consumed glucose slowly after acetate was exhausted. Furthermore, ALS1060 consumed xylose, but only in the presence of glucose.

With the goal of eliminating the consumption of glucose, strains containing single mutations in the *crr* gene and glucose non-specific PTS genes *malX*, *fruA*, *fruB*, and *bglF* were compared. Based on these results KD840 was constructed with a knockout of the *crr* gene in addition to the *ptsG*, *manZ*, *glk* and *xylA* knockouts. The *crr* gene encodes the $EIIA^{glu}$ protein which is required for the PEP-phosphotransfer of glucose. The $EIIA^{glu}$ protein is also required for the transport of sucrose or maltose (Lengeler et al., 1982). The absence of the *crr* gene should therefore prevent not only glucose but also sucrose and maltose uptake by the cells.

The additional knockout of the *crr* gene significantly reduced the cells' ability to consume glucose (and xylose). Fed-batch experiments further demonstrated that the *crr* knockout significantly curtailed the cells' ability to consume glucose. Nevertheless, batch fermentations demonstrated that KD840 was unable to consume glucose and xylose for at least 35 h after acetate was exhausted. However, the glucose concentration afterwards decreased slowly (Figures 2.5, 2.6). These results could be interpreted merely to be the result of microbial contamination, as maintaining sterility would likely be difficult in an aerated glucose/xylose-rich solution, despite the presence of the antibiotic kanamycin. Of course, another possibility is that mutations ultimately provide the cells an ability to consume the glucose. Interestingly, succinate

was observed in the fed-batch processes but not in the batch processes. In the fed-batch process acetate was continually fed whereas in the batch process the cells were not exposed to acetate again after the medium was initially exhausted. Succinate is therefore likely to be derived from acetate. Indeed, *E. coli* has been shown synthesize succinate from acetate as a result of the glyoxylate shunt (Zhu et al., 2007). The presence of succinate does suggest that under these growth conditions *E. coli* is unable to oxidize NADH fast enough to prevent succinate from being excreted.

While several PTS gene mutations were studied to prevent the consumption of glucose, acetate utilization remained largely unaffected as PTS systems in *E. coli* are known to mediate the transfer of hexose sugars only (Postma et al., 1993). Acetate freely diffuses across the *E. coli* cell membrane, is phosphorylated by PTA-ACK and AMP-ACS pathways (Chou and Lipmann, 1952; Rose et al., 1954; Berg, 1956) and metabolized through the glyoxylate shunt. Although ALS1060 showed specific growth rate identical to MG1655 when grown on acetate in the presence of sugars, KD840 had almost a 50% reduction in growth rate on acetate indicating that *crr* gene might play an unknown role in acetate utilization.

The concept of using strains which can only metabolize a single carbon source in a mixture of carbon sources has several advantages over the use of a single organism which degrades all the substrates (Eiteman et al., 2008). For the upgrading of lignocellulosic hydrolysates, this approach offers the prospect of selectively removing inhibitors such as acetate which are generated during the pretreatment process. Thus, a subsequent process to convert the sugar mixture (exemplified by xylose and glucose) could be developed without the concern of reduced productivity resulting from the presence of the inhibitor. Furthermore, if implemented as a fed-batch process, this approach could be relatively independent of the concentrations of the

inhibitor and sugars, and therefore could be used for any lignocellulosic hydrolysate. Specifically, in a fed-batch process the fermenter conditions (that is, what the cells are exposed to) depend on the rate of feeding and not on the concentrations of the feed stream. More generally, this approach could be used in other circumstances as a separation method to removed one compound selectively from a mixture.

Acknowledgments

This project was supported by the U.S. Department of Energy SBIR-STTR Grants Program, the Georgia Research Alliance, the Consortium for Plant Biotechnology Research (CPBR), the National Science Foundation (NSF), and the Georgia Experiment Station. We also acknowledge S. A. Lee and R. Altman for technical assistance.

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CHAPTER 3

COMPARISON OF ESCHERICHIA COLI STRAINS GROWN EXCLUSIVELY ON ACETATE

[1]Lakshmanaswamy, A., Eiteman, M.A., Altman, E. To be submitted to *Appl. Microbiol*.

Abstract

Six *Escherichia coli* strains were compared for maximum specific growth rate, biomass yield, dry cell weight and isocitrate lyase activity using a medium containing 5 g/L acetate as the sole carbon source. Among the strains tested, MC4100 showed the greatest specific growth rate (μ_{MAX}) at 0.368 h^{-1} while MG1655 showed the lowest specific growth rate at 0.244 h^{-1}. Because MC4100 does not have a functional *acs* operon, ALS1126 (MG1655 *acs*) was also examined to determine if the high maximum specific growth rate could be attributed to the absence of acetyl CoA synthetase activity. ALS1126 attained a maximum specific growth rate on the acetate medium of 0.262 h^{-1}, indicating that the absence of the *acs* operon in MC4100 does not by itself account for this strain's comparatively high growth rate on acetate. The reasons for the high growth rate of MC4100 on acetate remain unknown.

Introduction and Literature Review

Like many organisms, *Escherichia coli* are able to grow on acetate as their sole source of carbon and energy (Holms. 1986). Under this growth condition, *E. coli* enzymatically convert acetate to acetyl-CoA which then enters the tricarboxylic acid (TCA) cycle. Although acetate assimilation in *E. coli* does not require a specific transport system because undissociated acetate can diffuse across the cell membrane (Repaske and Adler, 1981), an acetate permease encoded by *actP* does play a role in acetate transport (Gimenez et al., 2003).

E. coli possess two enzymatic mechanisms to convert acetate to acetyl-CoA. The PTA-ACK pathway involves the sequential action of acetate kinase (ACK) and phosphotransacetylase (PTA). Acetate kinase (EC 2.7.2.1) mediates the conversion of acetate and ATP to acetyl phosphate and ADP; phosphotransacetylase (EC 2.3.1.8) mediates the conversion of acetyl phosphate and CoA to form acetyl-CoA and inorganic phosphate (Rose et al., 1954). The AMP-ACS pathway involves acetyl CoA synthetase (ACS, EC 6.2.1.1) using a two-step irreversible process: first, acetate and ATP form an enzyme-bound acetyl-AMP accompanied by the liberation of pyrophosphate; second, acetyl-AMP and CoA form acetyl-CoA and AMP (Chou and Lipmann, 1952; Berg., 1956). ACS has a very high affinity towards acetate (K_M of 200 μM), and therefore is able to scavenge acetate at very low concentrations (Brown et al., 1977; Kumari et al., 1995). An *acs* mutation leads to poor growth in acetate concentrations less than 10 mM (0.6 g/L), while mutations in *ack* or *pta* lead to poor growth in acetate concentrations greater than 25 mM. Double mutant strains lacking both pathways are unable to utilize acetate (Kumari et al., 1995).

Acetyl-CoA generated from acetate is metabolized via the glyoxylate shunt, a pathway which circumvents the oxidative steps of the TCA cycle. The two enzymes isocitrate lyase

(Campbell et al., 1953) and malate synthase (Ornston and Ornston, 1969) are unique to the glyoxylate shunt, and are necessary for the growth of *E. coli* on acetate (Cozzone, 1998). The overall result of this pathway is the formation of one 4-carbon TCA cycle intermediate from two 2-carbon acetate molecules (Kornberg et al., 1966).

Although several research groups have studied growth of *E. coli* on acetate and completed detailed flux analyses (Holms 1986; Strohl and Kleman, 1994; El-Mansi et al, 2006), to our knowledge there has not been a comparison of the growth rate of *E. coli* strains using acetate as the sole carbon source. *E. coli* strains having a high growth rate on acetate, and understanding what characteristics cause this high growth rate, are of interest for the selective biological removal of acetate from lignocellulose hydrolysates (chapter 2).

Materials and Methods

Bacterial Strains

The *Escherichia coli* strains studied are shown in Table 3.1. All of the strains used in this research were taken from the ALS laboratory collection. The Δ(*acs yjcH yjcG*)::Cam operon deletion was generated as follows: Primers were designed which could amplify the chloramphenicol acetyltransferase gene and promoter from pACYC184 bracketed by the first 50 bases of the *acs* gene and last 50 bases of the *yjcG* gene. The forward primer 5' ATGAGCCAAATTCACAAACACACCATTCCTGCCAACATCGCAGACCGTTGTTGAGA AGCACACGGTCACA 3' contains the first 50 bases of the *acs* coding sequence followed by bases 3601 – 3620 of pACYC184 while the reverse primer 5' TTAATGCGCGCGGCCTTGCTCAACGCCAAAGCCGGTCTGGGAGCGGATAATACCTGT GACGGAAGATCAC 3' contains the last 50 bases of the *yjcG* coding sequence followed by bases 400 – 419 of pACYC184. The bases homologous to pACYC184 are underlined in the

primers. The two primers were used to amplify a 1,143 bp fragment from pACYC184 DNA using the polymerase chain reaction (PCR) with Pfu polymease. The resulting DNA was gel-isolated and electroporated into DY330 electrocompetent cells. Cam(R) colonies were then selected. The presence of the Δ(*acs yjcH yjcG*)::Cam deletion was confirmed by performing PCR with the following two primer pairs which could amplify the *acs yjcH yjcG* genes. The forward primer 5' TCCTGCCAACATCGCAGACC 3' contains bases 27 – 46 downstream of the acs start codon while the reverse primer 5' CCTTGCTCAACGCCAAAGCC 3' contains bases 14 – 33 upstream of the TAA stop of *yjcG*. PCR amplification with these two primers yields a 4,119 bp fragment from the wild-type *acs yjcH yjcG* operon and a 1,162 bp fragment from the Δ(*acs yjcH yjcG*)::Cam deletion. The resulting Δ(*acs yjcH yjcG*)::Cam deletion was then transduced into MG1655.

Shake Flask Growth Conditions

Basal medium (BA5) contained (per L): 13.3 g KH_2PO_4, 4.0 g $(NH_4)_2HPO_4$, 1.2 g $MgSO_4 \cdot 7H_2O$, 13.0 mg $Zn(CH_3COO)_2 \cdot 2H_2O$, 1.5 mg $CuCl_2 \cdot 2H_2O$, 15.0 mg $MnCl_2 \cdot 4H_2O$, 2.5 mg $CoCl_2 \cdot 6H_2O$, 3.0 mg H_3BO_3, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, 100 mg Fe(III)citrate, 8.4 mg $Na_2EDTA \cdot 2H_2O$, 1.7 g citric acid, 0.0045 g thiamine·HCl, and 5 g/L acetate using $Na(CH_3COO) \cdot 3H_2O$. (Mass concentrations are reported as the acetate ion, with 5 g/L = 84.7 mM) Acetate was autoclaved separately, sterily combined with other medium components and neutralized with NaOH to a pH of 7.0. Strains were inoculated from a Luria-Bertani broth plate into 50 mL of BA5 medium in 250 mL baffled shake flasks at 37°C and 350 rpm (19 mm pitch).

Table 3.1 *E. coli* strains used in this study.*

Strain	Genotype
MG1655	F-λ- *rph*-1 (wild type)
MC4100	F- *araD139* Δ(*lac*)*U169 rpsL150 thi f1bB5301 deoC7 ptsF25 hsr-hsm+*
W	Wt
JM101	F' *traD36 proA+ proB+ lacIq* Δ(*lacZ*)M15 Δ(*lac-proAB*) *supE thi*-1
BL21	F- *dcm ompT hsdS* (R-M-) *gal*
W3110	F- λ- *mcrA mcrB* in(*rrnD-rrnE*)1
ALS1126	MG1655 *acs yjcH yjcG*::Cam

When the OD reached 2.5 in a first shake flask, 5 mL of culture was used to inoculate two identical 250 mL shake flasks from which samples were taken.

Assays

The optical density measured at 600 nm absorbance (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, Calif.) was used to monitor cell growth. Acetate was quantified as previously described (Eiteman and Chastain, 1997). When the OD reached 2.5, ICL activity (Dixon and Kornberg, 1959) was measured in duplicate.

* Arun Lakshmanaswamy assisted Ronnie Altman in the construction of *E.coli* stain ASL1126

Results

Growth Rate and Enzyme Activity

Six strains of *E. coli* were examined for growth using acetate as the sole carbon source (BA5 medium). Of these, MC4100 had the greatest specific growth rate of 0.368 h^{-1} while MG1655 showed the lowest specific growth rate of 0.244 h^{-1} (Table 3.2). The strains can be categorized based on their growth rate values: MC4100 and W are "fast growers" with growth rates about 0.35 h^{-1} while W3110 and MG1655 are "slow growers" with specific growth rates less than 0.25 h^{-1}.

The ICL activity also varied significantly among the strains, from 0.43 to 2.0 (U/mg). Interestingly, the two slowest growers, MG1655 and W3110, showed the greatest ICL activity among the strains (Table 3.2).

Table 3.2 Specific growth rate and ICL activity of *E. coli* strains used in study. The values are means from the two shake flasks.

Strain	Specific Growth Rate (h^{-1})	ICL Activity (U/mg protein)
MC4100	0.368	0.59
W	0.346	0.43
JM101	0.317	0.61
BL21	0.290	0.48
W3110	0.246	2.00
MG1655	0.244	1.73

The growth rate of MC4100 on acetate was 50% greater than the growth rate of MG1655. A recent genome-wide array comparing MC4100 and MG1655 indicated a genetic alteration in the region of the *acs* gene (Peters et al., 2002); specifically, MC4100 contains an IS2 fragment at the 898 position of the *acs* gene (Giminez et al., 2003). Recent studies have also characterized

the *actP* gene (previously *yjcG*) proposed to facilitate the transport of acetate, and which is cotranscribed with *acs*, and the uncharacterized gene *yjcH*. The proposed acetate operon (*acs-yjcH-yjcG*) is transcribed from the *acs* promoters present at the 5' end of the *acs* gene (Giminez et al., 2003).

Influence of acetate operon on growth rate in acetate medium

In order to determine the effect of the acetate operon on growth rate, ALS1126 was constructed by knocking out the *acs-yjcH-yjcG* genes in MG1655. On BA5 medium ALS1126 showed a specific growth rate of 0.262 h^{-1}, insignificantly different from the specific growth rate observed with MG1655. These results demonstrate that the *acs* mutation of MC4100 is not by itself responsible for the greater growth rate observed with MC4100 compared to MG1655.

Discussion

E. coli strains show a large range of maximum specific growth rates on acetate. MC4100, which lacks a functional ACS due to an insertion fragment (IS2) in the *acs* gene of the proposed *acs-yjcH-yjcG* operon, was surprisingly the fastest growing strain. However, knocking out the *acs-yjcH-yjcG* operon in MG1655 had no effect on growth in a medium containing 5 g/L acetate. The result indicates that the mutation in *acs-yjcH-yjcG* operon is not responsible for the high growth rate observed in MC4100 on acetate.

MC4100 would be a good candidate for the selective consumption of acetate from a mixture containing glucose and xylose. Previously, MG1655 derivative ALS1060 with knockouts in phosphotransferase system (PTS) genes (*ptsG*, *manZ*, *crr*), glucokinase (*glk*) and xylose (*xylA*) selectively removed acetate from a mixture of glucose and xylose. Because MC4100 has a higher growth rate than MG1655 on acetate, MC4100 could utilize acetate at a

much faster rate under the same conditions of the batch and fed batch processes carried out using ALS1060 (Chapter 2).

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CHAPTER 4

CONCLUSIONS

The long term vision of this project is to increase the potential for using lignocellulosic hydrolysate for the production of biochemicals and fuels by fermentation. One aspect of the project has been the construction of microbial strains (*E. coli*) which can only metabolize a single carbon source in a mixture of carbon sources. In particular, this research focused on strains of *E. coli* which can exclusively consume acetate from a mixture containing glucose and xylose. In this direction two main goals were set. The first goal was to test the concept of selective acetate consumption from a glucose-xylose mixture. *E. coli* MG1655 was chosen as the model strain to test the concept because it is widely studied, and substantial in-house experience is available. Knocking out the *ptsG*, *manZ* and *glk* genes of the phosphotransferase system (PTS) prevents *E. coli* from consuming glucose in a short batch process, while a *xylA* mutant is unable to consume xylose (Eiteman et al., 2008). Our first hypothesis was that a strain (ALS1060) with these four knockouts (i.e., *ptsG manZ glk xylA*) would not be able to consume these sugars but would have normal acetate metabolism. ALS1060 (containing these four knockouts) successfully metabolized acetate at approximately the same rate as the parent strain but utilized the two sugars (primarily glucose) slowly after acetate was exhausted. We interpreted this result to indicate other PTS genes existed which could facilitate the intake of glucose at a very slow rate. Indeed, a strain containing an additional knockout of the *crr* gene (KD840) did not utilize the sugars after more than 35 h in batch culture. However, the culture did show a reduction in

glucose, an observation which could be a consequence of mutations in KD840 or merely contamination.

The second goal was to compare *E. coli* strains for maximum specific growth rate when acetate is provided as the sole carbon source. The basis of this study was to select the fastest growing strain and metabolically engineer it to selectively consume acetate from a glucose-xylose mixture. This strain was then planned to be used in experiments conducted in our first goal to improve the overall process. MC4100 was the fastest growing strain, and MG1655 grew very poorly on acetate. MC4100 is derived from MG1655 and does not express acetyl-CoA synthetase (ACS) enzyme. ACS enzyme is involved in one the two known mechanisms by which *E. coli* consume acetate. The *acs* gene of the *acs-yjcH-yjcG* operon in MC4100 has an insertion fragment which prevents the expression of the operon (Giminez et al., 2003). However, knocking out *acs-yjcH-yjcG* operon in MG1655 did not improve its growth rate on acetate.

The results of this research demonstrate that acetate can indeed be selectively removed from this mixture with minimal loss of the carbohydrates. Future investigations could include studying other genes for the complete removal of glucose consumption, as well as microarray and enzyme activity studies to reveal the genetic reasons why certain strains of *E. coli* grow more quickly on acetate than others.

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APPENDIX

Basal Medium (BA) Composition (per liter)

Citric acid	1.70 g
KH_2PO_4	13.30 g
$(NH_4)_2HPO_4$	4.00 g
$MgSO_4 \cdot 7H_2O$	1.20 g
$Zn(CH_3COO)_2 \cdot 2H_2O$	13.0 mg
$CuCl_2 \cdot 2H_2O$	1.5 mg
$MnCl_2 \cdot 4H_2O$	15.0 mg
$CoCl_2 \cdot 6H_2O$	2.5 mg
H_3BO_3	3.0 mg
$Na_2MoO_4 \cdot 2H_2O$	2.5 mg
Fe(III) citrate	100 mg
thiamine·HCl	4.5 mg
$Na_2(EDTA) \cdot 2H_2O$	8.4 mg

Fermentation experimental conditions

The data collected for the fermentation experiments are provided in the following pages. All fermentations (Batch and Fed Batch) were carried out under the following experimental conditions.

Agitation: 500 rpm

Acid: 20% Sulphuric acid

Base: 20% Sodium Hydroxide

Gas: Air

Gas Flow Rate: 1.0 L/m

Initial Fermenter Volume: 1.055 L

Inoculum Volume: 55 ml

pH: 7.0

Temperature: 37°C

For Fed Batch experiments only

Feed rate: 0.2 ml/min

Total feed time: 72 hours

Total feed volume: 864 ml

Experiment: Batch Controlled
Reference: 18 (A)
Strain: MG1655
Media: BA 10

Sample	Time (h)	OD	Acetate (g/L)
1	0.00	0.16	10.66
2	5.33	0.22	10.61
3	11.58	0.38	10.51
4	19.08	1.26	9.40
5	20.33		8.65
6	21.33	2.09	7.95
7	22.33	2.71	7.20
8	23.42	3.46	6.26
9	24.50	4.78	4.41
10	25.17	5.32	3.29
11	25.83	6.42	1.87
12	26.58	6.93	0.44
13	27.08	7.00	0.00
14	27.58	7.12	0.00

Experiment: Batch Controlled
Reference: 18 (B)
Strain: ALS1060
Media: BA 10

Sample	Time (h)	OD	Acetate (g/L)
1	0.00	0.19	11.02
2	15.75	0.92	9.64
3	17.00	1.32	8.88
4	18.00	1.72	8.95
5	19.42	2.17	8.26
6	20.42	2.67	7.52
7	21.42	3.67	6.54
8	22.25	4.03	5.30
9	23.25	5.20	3.94
10	24.00	6.43	1.99
11	24.50	6.83	1.31
12	25.00	7.68	0.17
13	25.50	7.54	0.00
14	26.00	7.27	0.00

Experiment: Batch Controlled
Reference: 18 (L)
Strain: ALS1060
Media: BA 10 + 20g/L Glucose

Sample Number	time (h)	OD	Glucose (g/L)	Acetate (g/L)	Succinate (g/L)
1	0.00	0.14	21.11	11.07	0.00
2	5.00	0.31	21.07	10.80	0.00
3	10.00	0.75	21.24	10.28	0.00
4	13.00	1.27	20.75	9.23	0.00
5	16.00	2.60	21.49	7.70	0.00
6	18.50	4.73	20.43	4.47	0.00
7	21.00	6.96	19.87	0.45	0.00
8	21.50	7.23	20.89	0.00	0.00
9	22.50	7.32	20.46	0.00	0.00
10	23.50	7.50	20.15	0.00	0.00
11	26.50	8.03	19.86	0.00	0.00
12	30.50	8.70	19.29	0.07	0.00
13	33.50	9.07	18.59	0.01	0.00
14	37.50	10.02	18.64	0.00	0.01
15	42.50	10.68	17.92	0.15	0.02
16	49.50	11.87	16.02	0.00	0.00
17	55.50	13.06	13.00	0.00	0.00
18	59.50	14.24	11.39	0.10	0.00
19	66.00	15.21	9.78	0.01	0.00
20	80.00	15.42	6.40	0.27	0.00
21	85.50	16.47	5.49	0.13	0.00

Experiment: Batch Controlled
Reference: 18 (M)
Strain: ALS1060
Media: BA 10 + 10g/L Xylose

Sample Number	time (h)	OD	Xylose (g/L)	Acetate (g/L)	Succinate (g/L)
1	0.00	0.20	10.14	11.12	0.00
2	5.50	0.32	10.44	11.19	0.00
3	10.00	0.65	10.46	10.74	0.00
4	14.00	1.16	9.82	9.23	0.03
5	17.00	1.96	10.34	8.42	0.12
6	19.00	2.83	9.30	6.43	0.17
7	22.00	4.42	9.90	4.16	0.34
8	24.00	5.64	9.78	2.09	0.48
9	25.00	5.94	9.78	1.25	0.54
10	26.50	6.36	9.76	0.50	0.65
11	27.75	6.62	9.24	0.00	0.74
12	29.00	6.46	9.32	0.00	0.76
13	33.00	6.46	9.20	0.00	0.77
14	38.00	6.51	9.24	0.00	0.79
15	45.00	6.31	9.30	0.00	0.82
16	51.00	6.01	9.30	0.00	0.84
17	55.00	5.84	8.36	0.00	0.77
18	61.50	5.59	8.76	0.00	0.84
19	75.00	5.95	9.28	0.00	0.96
20	81.00	6.10	9.62	0.00	1.04

Experiment: Batch Controlled
Reference: 18 (K)
Strain: ALS1060
Media: BA 10 + 20g/L Glucose + 10g/L Xylose

Sample Number	time (h)	OD	Xylose (g/L)	Glucose (g/L)	Acetate (g/L)	Succinate (g/L)
1	0.00	0.17	10.08	19.45	10.28	0.00
2	5.00	0.35	10.44	20.15	10.40	0.00
3	10.00	0.72	10.64	20.53	10.14	0.00
4	13.33	1.18	10.32	19.90	9.17	0.00
5	16.00	1.89	10.52	20.29	8.41	0.07
6	18.50	2.93	10.42	20.10	7.00	0.14
7	21.00	3.64	10.34	19.95	5.88	0.19
8	22.83	4.37	10.18	19.68	4.38	0.27
9	24.50	5.24	9.86	19.06	2.71	0.35
10	25.67	6.05	9.72	18.79	1.68	0.41
11	26.50	6.50	9.58	18.46	1.08	0.46
12	27.67	6.93	9.50	18.33	0.63	0.54
13	28.67	7.27	9.44	18.19	0.20	0.64
14	30.00	7.06	9.24	17.82	0.00	0.73
15	31.17	8.11	9.28	17.85	0.00	0.81
16	34.00	8.50	9.10	17.37	0.00	1.01
17	38.00	7.50	8.86	16.73	0.00	1.32
18	43.17	7.19	8.32	15.64	0.00	1.67
19	48.42	8.82	7.68	14.43	0.00	1.96
20	51.42	8.59	7.74	14.57	0.00	2.30
21	54.00	8.46	6.46	12.18	0.00	2.12
22	59.42	8.77	7.62	14.49	0.00	3.05
23	63.00	8.53	7.46	14.22	0.00	3.40
24	73.17	8.58	6.88	12.80	0.00	4.27

Experiment: Batch Controlled
Reference: 18 (S)
Strain: KD840
Media: BA 10 + 20g/L Glucose

Sample Number	time (h)	OD	Glucose (g/L)	Acetate (g/L)
1	0.00	0.17	20.0	10.5
2	12.00	0.46	20.7	10.4
3	16.00	0.63	20.6	10.1
4	21.00	1.05	20.8	9.6
5	25.00	1.63	20.9	8.7
6	30.00	3.36	21.1	6.2
7	32.00	4.95	20.9	3.9
8	32.75	5.46	20.9	2.7
9	34.00	6.53	20.8	1.0
10	35.00	6.89	20.9	0.3
11	36.00	6.96	20.9	0.0
12	38.00	6.95	20.8	0.0
13	42.00	6.95	20.9	0.0
14	46.00	6.89	20.7	0.0
15	54.00	7.27	20.8	0.0
16	59.00	7.44	20.7	0.0
17	64.00	7.85	20.6	0.0
18	70.00	9.34	19.7	0.0
19	82.00	13.89	11.3	0.0
20	87.00	14.49	8.5	0.0
21	92.00	14.46		
22	103.00	13.85		

Experiment: Batch Controlled
Reference: 18 (R)
Strain: KD840
Media: BA 10 + 20g/L Glucose + 10g/L Xylose

Sample Number	time (h)	OD	Xylose (g/L)	Glucose (g/L)	Acetate (g/L)
1	0.00	0.10	9.9	19.9	10.42
2	11.25	0.33	10.2	20.4	10.30
3	15.25	0.46	10.1	20.4	10.10
4	20.25	0.55	10.2	20.6	9.92
5	24.25	0.84	10.2	20.6	9.60
6	29.25	1.14	10.3	20.8	9.06
7	33.25	1.66	10.3	20.8	8.10
8	37.25	2.44	10.3	20.9	6.60
9	41.25	4.48	10.2	20.7	3.34
10	43.25	5.96	10.1	20.6	0.92
11	45.25	6.50	10.2	20.8	0.10
12	53.25	6.56	10.1	20.7	0.00
13	58.25	6.61	10.0	20.5	0.00
14	63.25	6.81	9.9	20.4	0.00
15	69.25	6.88	9.8	20.3	0.00
16	81.25	7.93	9.6	20.0	0.00
17	86.25	7.65	9.5	19.9	0.00
18	91.25	7.99	9.3	19.4	0.00
19	102.25	13.25	8.4	10.2	0.04
20	110.25	14.06	7.9	5.8	0.16

Experiment Fed-Batch (Controlled)
Reference: 18 (J)
Strain: ALS1072
Medium: BA 10 + 20g/L Glucose + 10g/L Xylose

Sample Number	time (h)	OD	Feed Amt. (mL)	Xylose (g/L)	Glucose (g/L)	Acetate (g/L)	Succinate (g/L)
1	-20.25	0.14		9.42	20.25	5.27	0.00
2	-11.75	0.61		9.74	20.94	4.91	0.00
3	-7.25	1.22		9.72	20.94	4.06	0.07
4	-4.75	1.71		9.48	20.38	3.28	0.11
5	-1.75	2.62		9.68	20.82	2.16	0.21
6	-0.75	2.95		9.53	20.49	1.66	0.26
7	0.00	3.19	0	9.79	21.07	1.46	0.29
8	5.00	3.77	60	9.59	20.68	0.93	0.54
9	11.00	4.08	132	9.02	19.68	0.78	0.95
10	16.00	4.24	192	8.81	19.48	0.83	1.32
11	22.00	4.51	264	8.21	18.46	0.83	1.68
12	28.00	4.66	336	7.96	18.14	0.78	2.09
13	35.00	5.35	420	7.69	17.70	0.47	2.49
14	38.00	5.67	456	7.46	17.01	0.18	2.72
15	41.00	6.23	492	7.28	16.00	0.03	2.90
16	44.00	6.92	528	7.18	14.94	0.04	3.10
17	46.25	7.63	555	7.06	13.61	0.08	3.27
18	47.00	7.94	564	6.88	12.72	0.16	3.27
19	48.00	8.65	576	6.86	12.07	0.22	3.37
20	50.00	10.38	600	6.73	10.47	0.17	3.54
21	52.00	10.78	624	6.65	8.90	0.10	3.76
22	57.50	12.73	690	6.36	4.48	0.11	4.34
23	60.00	13.52	720	6.24	3.08	0.08	4.68
24	64.00	13.89	768	6.01	1.21	0.07	5.09
25	68.00	14.36	816	5.84	0.33	0.01	5.36
26	72.00	14.08	864	5.68	0.25	0.00	5.52

Experiment Fed-Batch (Controlled)
Reference: 18 (J)
Strain: ALS1072
Medium: BA 10 + 20g/L Glucose + 10g/L Xylose

Feed start time: 20.3 h
Feed end time: 92.3 h

Initial Xylose:	9.9	g
Xylose Added:	8.0	g
Xylose Remaining:	10.9	g
Xylose Consumed:	7.0	g
Initial Glucose:	21.4	g
Glucose Added:	17.3	g
Glucose Remaining:	0.5	g
Glucose Consumed:	38.2	g
Initial Acetate:	5.6	g
Acetate Added:	8.9	g
Acetate Remaining:	0.0	g
Acetate Consumed:	14.4	g

Experiment Fed-Batch (Controlled)
Reference: 18 (T)
Strain: KD840
Medium: BA 10 + 20g/L Glucose + 10g/L Xylose

Sample Number	time (h)	OD	Feed Amt. (mL)	Xylose (g/L)	Glucose (g/L)	Acetate (g/L)	Succinate (g/L)
1	-21.75	0.16		10.4	20.8	5.7	0.0
2	-16.75	0.34		10.5	20.9	5.6	0.0
3	-5.75	1.25		10.6	21.2	4.2	0.0
4	-0.75	2.89		10.5	21.1	2.2	0.1
5	0.00	3.16	0	10.5	20.9	1.5	0.1
6	7.00	4.23	84	10.0	20.1	0.5	0.3
7	19.00	5.01	228	10.0	20.6	0.4	0.8
8	26.00	5.56	312	9.5	20.1	0.0	1.3
9	31.00	5.90	372	9.3	19.9	0.0	1.6
10	43.00	6.37	516	8.9	18.3	0.1	2.2
11	49.00	13.15	588	8.7	8.5	0.3	2.6
12	56.00	14.00	672	8.3	2.5	0.2	2.8
13	67.00	13.39	804	8.0	0.3	0.1	3.3
14	72.00	13.62	864	7.9	0.0	0.0	3.5

Experiment Fed-Batch (Controlled)
Reference: 18 (T)
Strain: KD840
Medium: BA 10 + 20g/L Glucose + 10g/L Xylose

Feed start time: 21.8 h
Feed end time: 93.8 h

Initial Xylose:	11.0	g
Xylose Added:	8.6	g
Xylose Remaining:	15.2	g
Xylose Consumed:	4.4	g
Initial Glucose:	20.8	g
Glucose Added:	17.3	g
Glucose Remaining:	0.0	g
Glucose Consumed:	38.1	g
Initial Acetate:	5.7	g
Acetate Added:	8.6	g
Acetate Remaining:	0.0	g
Acetate Consumed:	14.3	g