OVERCOMING ACETATE IN RECOMBINNAT PROTEIN PRODUCTION BY METABOLIC ENGINEERING OF *ESCHERICHIA COLI*

by

YINGJIE MA

(Under the Direction of Mark A. Eiteman)

ABSTRACT

The primary goal of this research is to understand the physiological cause for overflow metabolism of *E. coli* in order to improve the yield and productivity of protein production. *E. coli* strains with genetic modifications (pyc^+, pps^+ and $poxB^-$) at pyruvate node were examined. All these strains were grown in glucose-limited fed-batch with defined medium at two different growth rates: 0.15 h^{-1} and 0.35h^{-1}. More protein and less acetate were produced at low growth rate. Overexpression *pyc* improves protein yield at low growth rate. Overexpression of *pps* did not improve protein production but increased glucose uptake rate. Knockout *poxB* has a detrimental impact on protein production. Protein production in $poxB^-$ strain containing *pyc* or *pps* decreased significantly compared to the strain containing *pyc* or *pps* alone. The intracellular pyruvate/PEP ratio was essentially unchanged between low and high growth rate.

INDEX WORDS: Protein production, Acetate, *Escherichia coli*, Pyruvate carboxylase, PEP synthase, Pyruvate oxidase, Fed-batch

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DEDICATION

Dedicated to my parents, Zhigang Ma & Jian Zhou for their love.

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Chapter 1

Introduction

Recombinant DNA technology has made the production of large quantities of recombinant protein feasible, efficient and environmentally friendly. Recombinant proteins produced by fermentation have a wide application in both analyses and therapeutics. Examples of commercialized analytical enzymes include creatinase, hexokinase and urease. More than 100 recombinant proteins are used in therapy such as insulin, somatotropin (human growth hormone), interferon-α and several monoclonal antibodies. The therapeutic market is expected to increase at a rate of 5-15% annually (Pavlou and Reichert 2004). Both prokaryotic and eukaryotic cells can serve as hosts for recombinant protein expression system.

Among the bacterial expression systems, *Escherichia coli* species is one of the most widely used hosts for the expression of recombinant protein because of the well understanding of its genetics and metabolism. *E. coli* cells are usually grown to a high cell density by fed-batch culture to achieve the maximum yield of recombinant proteins (Lee 1996). However, *E. coli* can secrete acetic acid, which has detrimental effects on both cell growth and protein production. A number of methods including metabolic engineering and process modification have been adopted to reduce the acetate accumulation and enhance recombinant protein yield.

The long-term objective of this research is to improve the yield and productivity of protein production processes by reducing acetate formation. Since acetate is directly generated from pyruvate, pyruvate level in cells has a close relationship to acetate generation. In this thesis, genetic modification was made to pathways at the node of pyruvate metabolism in *E. coli*, and

the effect of these modifications on protein production, acetate accumulation and intercellular metabolite levels were examined in *E. coli* in glucose-limited fed-batch under different controlled growth rate. This study increases our understanding of the underlying physiological causes for overflow metabolism, and the consequences of metabolic and process modifications.

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Chapter 2

Literature Review

Production of recombinant protein by *E. coli*

Bacteria, yeast and mammalian cells are the most commonly used hosts for recombinant protein expression. In contrast with yeast and mammalian cells, the disadvantage of bacterial cells as host of expression system is that they are not able to process many eukaryotic proteins through post-translational modification including glycosylation or disulphide bond formation. However, an advantage of bacteria is these cells' ability to grow to high cell densities at large scale. As a result, bacteria remain common hosts for recombinant protein production.

Among bacterial expression systems, the gram-negative bacterium *Escherichia coli* is an attractive organism for recombinant protein production, especially for those proteins for which post-translational processing is not required. Advantages of using *E. coli* for protein expression systems include its rapid growth, well-characterized genetics, the availability of numerous cloning vectors and host, an understanding of the fermentation process, and the ability to produce large quantities of recombinant protein inexpensively. Recombinant protein products can accumulate in either the cytoplasm or periplasmic space. Recent developments of secreting protein to the extracellular space make *E. coli* even more attractive as a host system (Shin and Chen 2008), and many products from *E. coli* are in the development or are being produced (Table 1. (Reichert and Paquette 2003; Walsh 2003; Graumann and Premstaller 2006; Walsh 2006). Despite the wide use and success of *E. coli*, for protein production, research continues to

focus on improving *E. coli* to increase the rate of recombinant protein production and increase final product concentration.

Table 1. Examples of recombinant proteins commercially produced.

Recombinant protein [a)]	Companies
Tissue Plasminogen activator (r-tPA) mutant	Roche
rh Insulin and analogs	Eli Lilly, Aventis
rh Hormone (rhGH)	Genentech, Eli Lilly, Pfizer, Schwartz Pharma, Novo Nordisk
rh Granulocyte-colony stimulating factor (rh G-CSF), pegylated rh G-CSF	Amgen
Interferon alfacon-1	Valeant
r Intergeron β-1b	Schering AG, Chiron
r Interferon γ-1b	Genentec
r Pertussis toxin	Chiron
Asparaginase	Merck
r IL-2-diphtheria toxin fusion	Seragen/Ligand

a) r, recombinant; h human; rh recombinant human

Recombinant protein production in *E. coli*

Recombinant DNA technology has made it possible to clone any gene of interest and to manipulate bacteria cells to overproduce the desired protein. To produce a particular recombinant protein in *E. coli*, the cells must be transformed with DNA encoding for that protein. Often this "transformation" is achieved by inserting the DNA into a vector construct (i.e., plasmid). Then such DNA becomes part of *E. coli* cells' genetic makeup and is replicated. The host cells will usually be transformed with a high copy number plasmid in order to generate a high concentration of the desired product. Host strain, gene dosage, promoter system and efficiency of transcription strongly affect the yield and quality of recombinant proteins (Baneyx 1999).

Many challenges exist is expressing high concentrations of protein in *E. coli* and other cells. The yield can be impacted by protein toxicity to the host cells (Bird et al. 2004), loss of ribosomes and cell viability (Dong et al. 1995), and difference in the condon usage between the foreign gene and native *E. coli* (Chen and Inouye 1994; Wu et al. 2004). A common problem with expressing a recombinant protein in *E. coli* is metabolic overload and stress in host strain. Generating foreign proteins at unnaturally high concentrations leads to a metabolic burden in *E. coli* caused by competing with the cellular synthesis and drawing major resources to the foreign products (Bentley et al. 1990). Strong expression of recombinant protein inhibits the glucose uptake rate and reduces respiratory capacity resulting in growth inhibition and acetate formation (Neubauer et al. 2003). The synthesis of recombinant protein is affected by the physiological character of host cell as well. The energy, metabolites and cofactors used for the synthesis of the foreign protein must be obtained from native *E. coli* metabolism. Thus, modifying the host cell for more efficient metabolism will broadly improve the effectiveness and stability of protein recombinant production in *E. coli*.

Metabolism of *E. coli*

Many protein production processes of *E. coli* use a defined medium with glucose as the carbon and energy source. Like most other microorganisms, *E. coli* metabolizes glucose via the biochemical pathways of glycolysis and the TCA cycle (Figure 1).

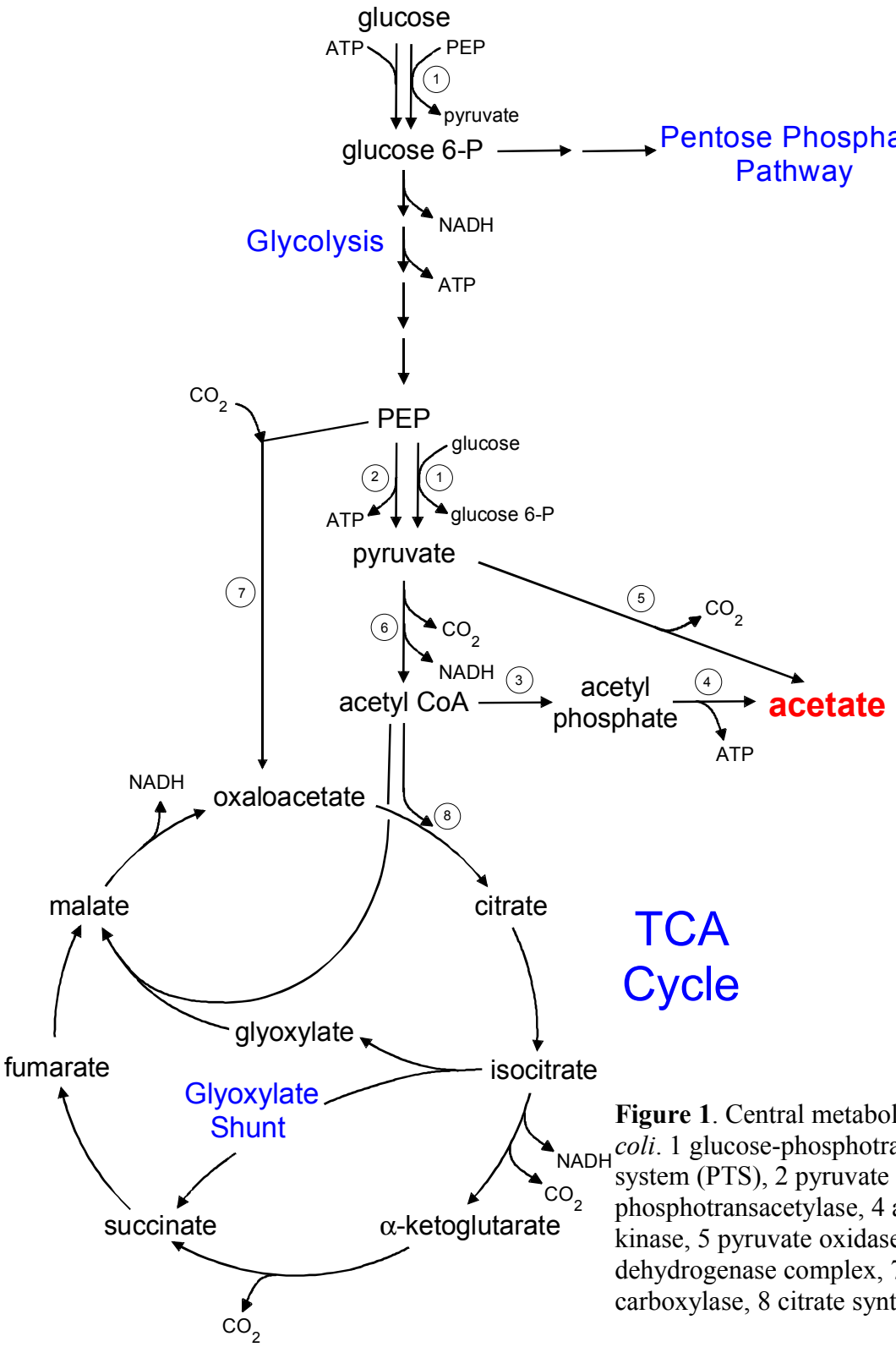

Figure 1. Central metabolism in *E. coli*. 1 glucose-phosphotransferase system (PTS), 2 pyruvate kinase, 3 phosphotransacetylase, 4 acetate kinase, 5 pyruvate oxidase, 6 pyruvate dehydrogenase complex, 7 PEP carboxylase, 8 citrate synthase.

Glucose metabolism starts by the transport of glucose across the cell membrane in the form of glucose 6-phosphate through the phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS) (Kundig et al. 1964). In this process, the phosphate is first transferred from PEP to histidine protein (HPr) by enzyme I (EI, encoded by *ptsI*), and PEP is converted to pyruvate. Then the high energy phosphate on HPr is transferred to glucose by a carbohydrate specific enzyme II (EII^{Glc} for the glucose PTS). EII^{Glc} consists of two proteins, the soluble IIA^{Glc} (encoded by *crr*) and the $IICB^{Glc}$ protein (encoded by *pts*G) (Postma et al. 1993). IIA^{Glc} transfers the phosphoryl group from HPr to the membrane-bound glucose transporter, $IICB^{Glc}$. Glucose transport leads to dephosphorylation of IIA^{Glc}, and the dephosphorylation of protein IIA^{Glc} is also considered the main mechanism causing catabolite repression (Saier et al. 1996). The ratio of PEP and pyruvate, the driving force for the phosphorylation of the PTS protein, plays a role in the regulation of the PTS activity (Weigel et al. 1982). The phosphorylation state of enzyme IIA^{Glc} is decreased at a high pyruvate/PEP ratio (Hogema et al. 1998).

By being transported into the cell and transformed into glucose 6-phosphate, glucose has been activated for the subsequent reactions. Two metabolic pathways are involved in the glucose metabolism: the principal pathway is the Embden-Meyerhof pathway (EMP); a second pathway is the pentose phosphate pathway (PPP). In the EMP pathway, glucose generates two moles of pyruvate, two moles of adenosine triphosphate (ATP) and 2 moles of reduced nicotinamide adenine dinucleotide (NADH). Phosphofructokinase catalyzes the irreversible phosphorylation of fructose 6-phosphate. Phosphofructokinase is a key enzyme in the control of glycolysis, inhibited by both ATP and citrate (Passonneau and Lowry 1964). However, the glycolytic flux in *E. coli* is controlled mainly by the ATP demand of the cell rather than by

glycolytic enzymes (Koebmann et al. 2002). By lowering ATP level in cells, the carbon flux in glycolysis increases significantly (Koebmann et al. 2002; Noda et al. 2006).

Another major route of glucose utilization is the pentose phosphate pathway (PPP). Two distinct phases occur in this pathway. The first phase is the oxidative phase, in which glucose 6-phosphate is converted to ribulose 5-phosphate, a precursor for nucleotide and nucleic acid synthesis. Nicotinamide adenine dinucleotide phosphate is reduced to NADPH, and this compound is used in many biosynthetic reactions. The second phase comprises nonoxidative steps that convert pentose phosphates to glucose 6-phosphate, which begin the cycle again. In this phase, transaldolase and transketolase catalyze the interconversion of three-, four- five-, six-, and seven-carbon sugars such as erythrose-4-phosphate which will serve in anabolic processes (Wood 1986). Ultimately, pyruvate is generated from glucose.

Under aerobic conditions, pyruvate is further oxidized via the tricarboxylic acid (TCA) cycle. Pyruvate enters the cycle through acetyl-CoA by the enzyme complex pyruvate dehydrogenase (PDH) which is composed of multiple copies of three enzymes: pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase. In the reaction, one mole of NADH is generated per mole of pyruvate (Harris et al. 2002). Acetyl-CoA derived from pyruvate enters the TCA cycle by undergoing condensation with oxaloacetate mediated by citrate synthase. In seven sequential reactions, citrate is converted to oxaloacetate and two moles of CO_2. TCA cycle is important for the production of reducing equivalents that are used by the respiratory complexes to produce ATP (Zeng and Deckwer 1994). During one cycle, three moles of NADH, one mole of oxidized flavin adenine ($FADH_2$), and one mole of nucleoside triphosphate (either ATP or GTP) are formed. Under aerobic conditions, $FADH_2$ and NADH are

oxidized through oxidative phosphorylation respiratory chain with the simultaneous reduction of O_2 to H_2O. The energy of oxidation is captured through the synthesis of ATP (Campbell 2004).

The TCA cycle, glycolysis, and pentose phosphate pathway play important roles in the biosynthesis of amino acids. All twenty amino acids involved in protein synthesis are derived from intermediates of these three pathways and can be grouped into six families corresponding to their metabolic intermediates in central metabolism (Table 2) (Nelson and Cox 2004). Additionally, glycolysis, pentose phosphate pathway and the TCA cycle also supply reducing power (NADH and NADPH) and energy (ATP) for amino acid biosynthesis.

Table 2. Amino acid biosynthetic groups according to metabolic intermediate.

Metabolic intermediate	Pathway	Amino Acid
α -ketoglutarate	TCA cycle	Glu, Gln, Pro, Arg
Oxaloacetate	TCA cycle	Asp, Asn, Met, Thr, Lys
3-phosphoglycerate	Glycolysis	Ser, Gly, Cys
Pyruvate	Glycolysis	Ala, Val, Leu, Ile
Erythrose 4-phosphate and PEP	PPP and Glycolysis	Try, Phe, Tyr
Ribose 5-phosphate	PPP	His

Synthesis of proteins at a high rate depletes amino acid pools and affects the specific cellular yield of recombinant protein at high cell concentrations (Panda 2003). As the carbon compounds are withdrawn from central metabolism, *E. coli* requires anaplerotic pathways to replenish those intermediates. One important anaplerotic pathway is PEP carboxylase, in which PEP and CO_2 generate oxaloacetate (Matte et al. 1997). This reaction is highly regulated, and PEP carboxylase is activated by fructose-1,6-diphosphate, acetyl-CoA, GTP and is inhibited by aspartate and malate (Morikawa et al. 1980).

Carbon flux in *E. coli* can also be redirected from central metabolism to acetate at the pyruvate node. Two major pathways in *E. coli* lead to acetate from pyruvate (Figure 2). One

pathway converts acetate to pyruvate directly by pyruvate oxidase (*poxB*) (Blake et al. 1982), and the other pathway involves three sequential steps: 1) the formation of acetyl CoA by the pyruvate dehydrogenase complex, 2) the conversion of acetyl CoA to acetyl phosphate by phosphotransacetylase (*pta*), and 3) the formation of acetate by acetate kinase (*ackA*) (el-Mansi and Holms 1989).

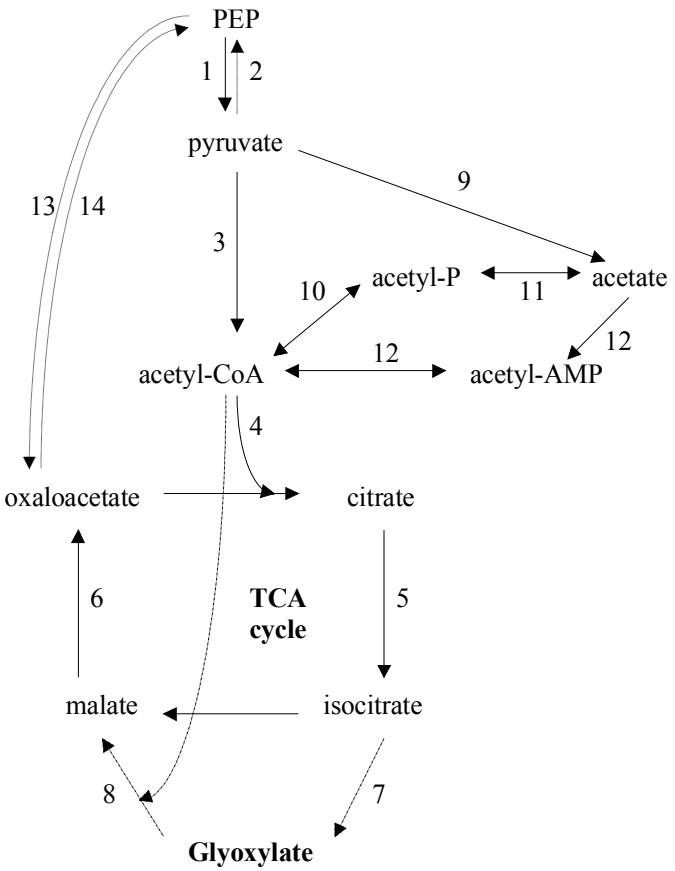

Figure 2. The main metabolism pathways of acetate in *E. coli* including TCA cycle, glyoxylate shunt, and gluconeogenesis. 1 pyruvate kinase (*pykA*), 2 PEP synthase (*ppsA*), 3 pyruvate dehydrogenase (*aceE*), 4 citrate synthase (*gltA*), 5 aconitase (*acn*), 6 malate dehydrogenase (*mdh*), 7 isocitrate lyase (*aceA*), 8 malate synthase (*aceB*), 9 pyruvate oxidase B (*poxB*), 10 phospotransacetylase (*pta*), 11 acetate kinase (*ackA*), 12 acetyl-CoA synthetase (*acs*), 13 PEP carboxylase (*ppc*), 14. PEP carboxykinase (*pckA*)

E. coli can produce acetate under anaerobic or oxygen-limiting conditions when the maximum oxygen transfer capacity of a reactor is reached. However, under aerobic conditions cells can still accumulate acetate. Aerobic acetate formation typically occurs when cells exceed a threshold of specific growth rate or glucose consumption rate (Ko et al. 1995; Johnston et al. 2003). This phenomenon is referred to as "overflow metabolism" (Doelle et al. 1982). The threshold for *E. coli* in growth rate is 0.2 h^{-1} for complex media and 0.35 h^{-1} for defined medium (Paalme et al. 1990). However, *E. coli* will still generate acetate at low specific growth rates when limited by a nutrient other than carbon. Therefore, specific glucose consumption rate is the critical variable for determining acetate formation (Eiteman and Altman 2006).

The reasons for overflow metabolism have been studied extensively. The onset of acetate formation is correlated with a maximum (plateau) in the specific oxygen uptake rate, an observation which suggests a limitation in the capacity of respiration (Andersen and von Meyenburg 1980; Paalme et al. 1997). In respiration, NADH is reoxidized to NAD. When excess glucose exists in the medium, cells will uptake glucose at their maximum rate. As the carbon flux to the TCA cycle results in NADH formation while the flux to the acetate production does not, cells tend to redirect acetyl-CoA flux to acetate in order to avoid NADH accumulation. Another explanation suggests that the capacity of the TCA cycle will be reached before that of respiration (Majewski and Domach 1990). When the glucose consumption rate reaches the capacity of the TCA cycle, the excess carbon will form acetate. In this case, NADH production and respiration increase until either the maximum rate of respiration or the maximum glucose consumption rate is reached. However, the study of the growth rate control in *E. coli* at near to maximum growth rate indicates that a respiratory limitation is more likely (Paalme et al. 1997). The production of

acetate depends on many factors such as bacterial strain, growth condition, and glucose supply strategy (Luli and Strohl 1990; Kleman and Strohl 1994; van de Walle and Shiloach 1998).

Acetate also can serve as a carbon source through the glyoxylate shunt (*aceBAK*). To enter this shunt, acetate first must be activated to acetyl-CoA (Clark and Cronan 1996). This activation can be achieved by acetyl-CoA synthetase (*acs*) or the reverse action of Pta-AckA (Brown et al. 1977) (Figure 2). In the glyoxylate shunt, the enzyme isocitrate lyase converts isocitrate into succinate and glyoxylate (Diehl and McFadden 1994). Glyoxylate and acetyl CoA then are converted to malate by the enzyme malate synthase (Ornston and Ornston 1969). The cycle is completed by the TCA cycle steps which convert malate to oxaloacetate then with a second acetyl CoA to citrate and then to isocitrate. The glyoxylate shunt is another anaplerotic pathway resulting in the incorporation of carbon from acetyl-CoA. Glyoxylate shunt is induced when *E. coli* grows on acetate or another fatty acid as the sole carbon source (Vanderwinkel et al. 1968). The shunt cannot be effectively induced in the presence of glucose (Kornberg 1966).

Acetate formation can also be affected by gluconeogenesis. Two important reactions in gluconeogenesis are the conversion of oxaloacetate directly to PEP through PEP carboxykinase (*pck*A) and conversion of pyruvate to PEP through PEP synthase (*pps*A) (Phue and Shiloach 2005). These reactions cause the pyruvate and acetyl-CoA concentrations to decrease (Krebs and Bridger 1980; Phue et al. 2005) and hence less acetate accumulates (Phue and Shiloach 2005).

PEP synthase plays an important role in *E. coli* mediating the reverse of pyruvate kinase (*pyk*A) and couples the formation of PEP with the cleavage of two mole of ATP. This enzyme is inhibited by PEP as well as AMP (Chulavatnatol and Atkinson 1973). PEP synthase is essential for growth of *E. coli* on three-carbon sources such as pyruvate and lactate (Cooper and Kornberg

1967). A slight increase in PEP synthase activity in the presence of glucose stimulates oxygen consumption (Patnaik et al. 1992).

Acetate affects protein production

The ideal process for recombinant protein production with *E. coli* is high levels of gene expression for the target protein in high cell density culture. However, the simultaneous formation of by-product acetate is unfavorable to the high cell density culture process (Lee 1996).

Accumulation of acetate in the medium is harmful to recombinant protein production, because acetate inhibits cell growth as well as protein generation (Kleman and Strohl 1994). Acetate retards cell growth rate even at a concentration as low as 0.5 g/L (Nakano et al. 1997). The formation of acetate diverts carbon and energy to an undesired direction instead of biomass accumulation and protein synthesis (March et al. 2002). Several proteins (Blankenhorn et al. 1999) and genes (Arnold et al. 2001) are influenced by acetate, especially those involved in the *E. coli* transcription-translation system, the general stress response and regulation. Short-chain acids also repress the rate of RNA and DNA protein synthesis (Cherrington et al. 1990) which are essential in recombinant protein production. Moreover, in the fermentation medium, the protonated form of acetate (acetic acid) interferes with ATP production by reducing the ΔpH contribution to the proton-motive force (Jensen and Carlsen 1990; Luli and Strohl 1990). The threshold growth rate for the onset of acetate formation is lower for cells induced to generate a recombinant protein (Akesson et al. 1999). By alleviating accumulation of acetate, protein production can be improved. Acetate formation can in general be ameliorated by designing a process in such a way as to avoid acetate formation, or by altering the metabolism of *E. coli*.

Process modification

As noted previously, *E. coli* will generate acetate when the cells grow at a high growth/glucose consumption rate even without oxygen limitation (Ko et al. 1995; Eiteman and Altman 2006). However, limiting the glucose uptake rate by using a substrate-limited fed-batch culture can be used to control acetate (Akesson et al. 1999). Several feeding strategies are applied to fed-batch fermentation. Exponentially increasing a glucose feed can be used to maintain a constant specific growth rate of cells (Hellmuth et al. 1994). In an exponential fed-batch process, the glucose is fed exponentially to match the growing population, and the glucose concentration remains zero (Babu et al. 2000). However, the optimal feed strategy requires the glucose addition rate be maintained at levels just below the threshold for acetate production (Johnston et al. 2002). Since the threshold varies from strains and production conditions, strategies to track the glucose consumption rate threshold allow cell growth and protein production to occur at the maximum rates without overflow metabolism (Johnston et al. 2003). Thus, feedback control can maintain the feeding rate of glucose efficiently through online process variables such as pH (Robbins and Taylor 1989), dissolved oxygen (DO) (Zawada and Swartz 2005) and glucose concentration (Kleman et al. 1991b). Based on the assumption that acetate formation is linked to a respiratory limitation, standard dissolved oxygen sensors with short pulses to the substrate feed rate, the onset of acetate can be detected and the flow rate of feed is adjusted (Akesson et al. 1999; Akesson et al. 2001).

Instead of controlling the growth rate, another approach of reducing the inhibitory effects of acetate is to remove acetate from culture during fermentation process. Removal of acetate from the medium by dialysis has prolonged the exponential growth phase and allowed *E. coli* cell grows to a very high density in fermentation (Nakano et al. 1997). Dialysis fermentation also

increases the volumetric protein production (Fuchs et al. 2002) Another method to remove acetate from a fermentor is macroporus ion-exchange resins (Chen et al. 2005). However, this method will remove nutrients for cell growth while removing acetate (De Mey et al. 2007).

Growth conditions including pH, dissolved oxygen and the medium affect cell behavior and product formation. Lower pH leads *E. coli* to form less acetate (Kleman and Strohl 1994), while *E. coli* produces more organic acids such as acetate under an anaerobic environment (Berrios-Rivera et al. 2002). In the latter case, oxygen limitation represses the constitutive expression of the glyoxylate shunt and gluconeogenesis which are involved in acetate consumption and regulation. With the dissolved oxygen level above 30%, even when grown at the maximum rate on glucose, the strain *E. coli* B does not accumulate acetate (Phue and Shiloach 2005). However, in a high cell density culture, with the increased oxygen demand as well as viscosity of the medium (Riesenberg and Guthke 1999), temporary oxygen limitation will occur especially in large bioreactors (Enfors et al. 2001). Membrane cyclone reactor has been designed to improve bioreactor performance in oxygen transfer rate during fermentation process (Hartbrich et al. 1996). By adding chemical compounds serving as oxygen carriers will reduce the oxygen limitation in medium (Galaction et al. 2004).

Finally, the medium composition will affect acetate production. Because acetate formation is specifically caused by rapid glucose consumption, changing the carbon source will reduce acetate formation. For example, the replacement of glucose by fructose will decrease acetate production, and increase protein formation in batch culture (Aristidou et al. 1999). Glycerol also can be used as the carbon source to reduce acetate (Nakano et al. 1997). By supplying glycerol in the medium after induction, protein production is increased by 1.6 fold compared to the use of glucose (Luo et al. 2006). Adding supplements directly to the medium

serves to replenish intermediates consumed during protein production. For example, glycine, methioine, aspartate or yeast extract in the medium also can relieve the negative effect of acetate on recombinant cell growth (Han et al. 1993; Losen et al. 2004).

Metabolic engineering

In addition to changing the protein production process, acetate formation also can be affected by modifying the pathways or (genetic) regulatory systems which generate acetate. One metabolic approach to reduce acetate accumulation is by deleting or blocking the biochemical pathway leading to acetate. Since acetate is mainly generated by the *pta-ack*A and *pox*B pathways, most studies have focused on these two pathways. With the elimination of the *pta-ack*A and/or the *pox*B pathway, acetate production can be reduced and more carbon can be directed to central metabolism (Chang et al. 1999; Dittrich et al. 2005b). The absence of pyruvate oxidase activity reduces nongrowth-related energy metabolism (maintenance coefficient) (Vemuri et al. 2005). Knocking out *poxB* (and *ldh*A and *pfl*B gene encoding, respectively, for the anaerobic enzymes lactate dehydrogenase and pyruvate formate lyase) results in strains that are more tolerant to dissolved oxygen gradients, and a significant reduction in acetate formation is observed (Lara et al. 2006). Similarly, employing an antisense RNA to block partially the biosynthesis of phosphotransacetylase and acetate kinase will increase protein production (Kim and Cha 2003). However, several problems arise. First of all, deletion of *pta-ack*A pathway causes pyruvate excretion (Diaz-Ricci et al. 1991). Additionally, because both pathways have important roles in the overall fitness of *E .coli*, absence of the *pta-ack*A and/or the *pox*B pathway decreases cell density (Abdel-Hamid et al. 2001; Dittrich et al. 2005b), which is unfavorable to commercial production of recombinant proteins. The *pta-ack*A pathway generates ATP necessary for cell growth, prevents acetyl-CoA from saturating the TCA cycle (Pruss and Wolfe

1994), and provides a sufficient level of acetyl phosphate, a compound which is especially important under starvation conditions (Nystrom and Gustavsson 1998). The *poxB* gene is induced during the stationary phase and contributes to aerobic growth efficiency when *E. coli* is growing on minimal medium (Abdel-Hamid et al. 2001). The protein encoded by *poxB* gene has an important role in resistance to carbon and oxidative stress (Flores et al. 2004; King and Ferenci 2005).

Since acetate accumulation is caused by an imbalance between glucose uptake and the demand of energy and biosynthesis, acetate generation can be avoided by reducing glucose uptake via metabolic engineering in *E. coli*. For example, disabling PTS system by deleting the *ptsHI* operon in *E. coli* improved recombinant protein production significantly and reduced acetate production in batch bioreactors with complex medium (Wong et al. 2007). Knocking out the Glucose PTS $IICB^{Glc}$ protein inactivates the PTS system, reduces glucose uptake and acetate secretion, but does not affect protein production (Picon et al. 2005). Under aerobic conditions, in an *E. coli* strain which lacks the PTS and uses the galactose-proton symport system for glucose uptake, no acetate is detected and biomass increases significantly. However, these methods decrease the maximum growth rate (Chen et al. 1997; Ponce 1999). The Mlc protein is a global regulator of carbohydrate consumption and represses the transcription of *ptsG* (Kimata et al. 1998). Thus, overexpressing Mlc results in a slower fermentation rate (Kim et al. 1999) but increases protein yield substantially while reducing acetate by more than 50% in a batch shake-flask culture using complex medium (Cho et al. 2005). By using a stronger promoter for galactose permease gene *gal*P in a PTS^- strain, an alternate glucose transport system which does not directly generate pyruvate is activated which will alleviate acetate accumulation without sacrificing the growth rate (De Anda et al. 2006).

In addition to modifying the glucose uptake system, modifications in central metabolism also can indirectly reduce acetate. Since acetate generation is related to the saturation of TCA cycle, increasing capacity of TCA cycle at rate-limiting step will help increase the carbon flux to central metabolism instead of acetate. Overexpression of the gene encoding citrate synthase (*gltA*) which convert acetyl CoA and oxaloacetate to citrate increased cell dry weight, and no acetate excretion was detected (De Maeseneire et al. 2006).

Not only can the acetate concentration be altered by modulating specific carbon-metabolic pathways, but it can also be affected by modulating concentrations of cofactors or regulatory systems in central metabolism. The expression of several genes in TCA cycle is repressed by a global regulatory Arc system (Iuchi and Lin 1988). Deletion of *arcA* gene resulted in a 10% increase in the threshold specific glucose consumption rate for acetate formation (Vemuri et al. 2006a), and a 30% increased production of a model protein in batch fermentation (Vemuri et al. 2006b). Recent evidence indicates that the Arc regulon is triggered by an increased NADH/NAD ratio (Georgellis et al. 2001; Malpica et al. 2004). NADH is a specific and strong allosteric inhibitor of the enzyme citrate synthase (Molgat et al. 1992). An accumulation of NADH caused by elevated glycolytic flux would therefore tend to drive the carbon flux from acetyl CoA to the acetate pathways. NADH formation can be reduced by expressing water-forming NADH oxidase (NOX) from *Streptococcus pneumoniae* (Vemuri et al. 2006a). Using NADH oxidase increased the model protein β-galactosidase yield and reduced the final acetate concentration more than 40%. Deletion of *arcA* gene with *nox* overexpression almost eliminated acetate accumulation even at a high growth rate ($> 0.7\ h^{-1}$) and increased the protein yield more than 2 fold (Vemuri et al. 2006b).

The additional metabolic burden resulting from recombinant protein production diminishes the carbon flow in the TCA cycle, and therefore diminishes the availability of oxaloacetate which serves as a precursor biochemical of amino acids, which would lead to additional acetate formation from acetyl-CoA (March et al. 2002). As a result, replenishment of central metabolism by anaplerotic routes is an approach to reduce acetate formation (Farmer and Liao 1997) and increase protein production (March et al. 2002). As noted previously, a major anaplerotic path in *E. coli* is from PEP to the TCA cycle intermediate oxaloacetate via PEP carboxylase (encoded by *ppc*). Overexpression of the *ppc* gene results in a 60% reduction in acetate formation in shake-flask cultures grown to low cell density (Farmer and Liao 1997). However, PEP carboxylase overexpression diminishes the supply of PEP needed for PTS-mediated glucose uptake and decreases the growth rate (Gokarn et al. 2000). In contrast to the overexpression of PEP carboxylase, expressing heterogenous pyruvate carboxylase in *E. coli* directs pyruvate to oxaloacetate without affecting glucose uptake rate or growth rate (Gokarn et al. 2001). In a *pyc*-containing strain, the model protein β-galactosidase increased more than 60% while the acetate production decreased about 57% under a fed-batch fermentation (March et al. 2002). With the additional knockout of *poxB* gene, a *pyc*-containing strain resulted in an 80% reduction in acetate (Vemuri et al. 2005).

An alternative approach to minimize acetate accumulation is to enhance acetate consumption by the activation of the glyoxylate shunt, another anaplerotic pathway involved in acetate utilization (van de Walle and Shiloach 1998). With a higher activity of the glyoxylate shunt, *E. coli* B produces less acetate than *E. coli* K-12 strains even at high glucose concentration. The glyoxylate bypass is regulated by several factors, such as negative regulation factor FadR which is encoded by *fadR* gene (Maloy and Nunn 1981). An fadR mutant with PPC

overexpression resulted in a 70% decrease in acetate in the shake-flask culture with only a slightly decrease in biomass yield (Farmer and Liao 1997).

Many studies have been completed to understand and reduce acetate formation with the ultimate goal of enhanced protein production. While genetic approaches are focusing on the metabolism in acetate formation, process approaches study the process conditions which can match these genetic characteristics. All approaches including both process and genetic modification are not mutually exclusive and can complement each other in reducing acetate formation.

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Chapter 3

Overcoming acetate in protein production

Introduction

E. coli is one of the most attractive organisms for the production of recombinant proteins because of its well-established genetics and ability to grow rapidly to high cell density at large scale with simple nutritional requirements. Recombinant proteins produced by *E. coli* serve a wide variety of markets (Arbabi-Ghahroudi et al. 2005; Graumann and Premstaller 2006; Walsh 2006). However, the secretion of the metabolic by-product acetate during the growth of *E. coli* is a major constraint for high protein productivity.

The accumulation of acetic acid (acetate) in fermentation process inhibits cell growth (Luli and Strohl 1990; Chou et al. 1994) and the production of recombinant proteins (Jensen and Carlsen 1990; Turner et al. 1994). The generation of acetate not only diverts carbon and energy from protein production (March et al. 2002), but also represses the rate of RNA and DNA protein synthesis (Cherrington et al. 1990). The presence of acetate induces stress response even at low concentrations (Gschaedler et al. 1999). Moreover, acetate acts as an uncoupler, reducing the ΔpH contribution to proton motive force (Axe and Bailey 1995). The detrimental effect of acetate is particularly relevant when *E. coli* is cultured at high cell density for recombinant protein (Lee 1996).

Acetate is generated directly from pyruvate through pyruvate oxidase (*poxB*) or a 3-step pathway (el-Mansi and Holms 1989) via pyruvate dehydrogenase (*aceEF*, *lpd*), phosphotransacetylase (*pta*) and acetate kinase (*ack*). The phenomenon of *E. coli* accumulating

acetate when grown at a high glucose consumption even in the presence of sufficient oxygen (Andersen and von Meyenburg 1980) is referred to as overflow metabolism (Doelle et al. 1982). Overflow metabolism is attributed to 1) the limited capacity of the tricarboxylic acid (TCA) cycle which causes excess carbon flux to form acetate instead of entering central metabolic pathway (Holms 1986; Majewski and Domach 1990), and 2) the saturation of the electron transport in respiration in which NADH is reoxidized (Andersen and von Meyenburg 1980; Paalme et al. 1997). Since the TCA cycle generates NADH while acetate formation does not, cells might form acetate as a means to avoid additional NADH generation (Vemuri et al. 2005).

Since acetate accumulation occurs at a high glucose consumption rate, control of glucose feeding rate can be used to reduce acetate formation (Kleman et al. 1991a; Akesson et al. 1999; Riesenberg and Guthke 1999; Johnston et al. 2003). Fed-batch culture with exponential feeding allows cells to grow at a constant specific growth rate (Hellmuth et al. 1994), and acetate formation can be minimized by controlling the specific growth rate below the critical value at which acetate starts to accumulate (Kleman and Strohl 1994). In an exponential fed-batch process, the glucose is fed exponentially to match the desired (constant) specific growth rate, and the glucose concentration remains near zero (Babu et al. 2000).

Pyruvate is at a key position in glucose metabolism and is an important branch point between catabolism and anabolism. This biochemical participates in over 50 biochemical reactions in *E. coli* (Kanehisa et al. 2002). Acetate accumulation is correlated with intracellular concentration of pyruvate (Vemuri et al. 2005). As glucose consumption rate is increased, the intracellular pyruvate concentration increases while the PEP concentration decreases, most significantly at the onset of acetate formation. The ratio between pyruvate and PEP therefore increases from under 1 to 25 (Vemuri et al. 2005). Since pyruvate is a biochemical precursor to

acetate, elevated intracellular pyruvate could indicate increased fluxes in the pathways leading to the formation of acetate. The regulation at pyruvate node will have an impact on acetate generation.

Genetic modification at pyruvate node is known to affect acetate formation. An elevated intracellular pyruvate concentration activates pyruvate oxidase (POX, EC1.2.2.2, encoded by *poxB*) (Chang and Cronan 1984; Carter and Gennis 1985) which directs carbon flux away from acetyl-CoA and maintains the intracellular pool of CoA for other metabolic functions (Dittrich et al. 2005a). Deleting *poxB* in *E. coli* reduces acetate generation (Chang et al. 1999). Diverting pyruvate to oxaloacetate through an additional anaplerotic pathway by overexpression of heterologous pyruvate carboxylase (PYC, EC6.4.4.1, encoded by *pyc*) also decreases acetate production (Gokarn et al. 2001) and improves biomass production (March et al. 2002; Vemuri et al. 2005). Pyruvate also can be converted to PEP by PEP synthase, which is essential for growth of *E. coli* on three-carbon sources such as pyruvate and lactate (Cooper and Kornberg 1967), and therefore, may change the pyruvate and PEP ratio. Moreover, PEP can be converted to oxaloacetate by pyruvate carboxylase. Since oxaloacetate is an important precursor for amino acid synthesis, increasing PEP pool in *E. coli* might increase the carbon flux to oxaloacetate, and therefore, increase protein production.

Many research studied protein production in the metabolic engineered *E. coli* strains in batch culture in which cells grow at its maximum growth rate. In this study, we investigated the physiological response of metabolic modifications at the pyruvate node (Figure 1) in *E. coli* grown under a constant specific growth rate in fed-batch. By overexpressing PEP synthase and pyruvate carboxylase and delelting pyruvate oxidase, cell will use carbon more efficiently, and thus less acetate will be accumulated. Understanding the underlying physiological causes for

overflow metabolism and how the cell responds to genetic perturbations will help improve the yield and productivity of protein production processes.

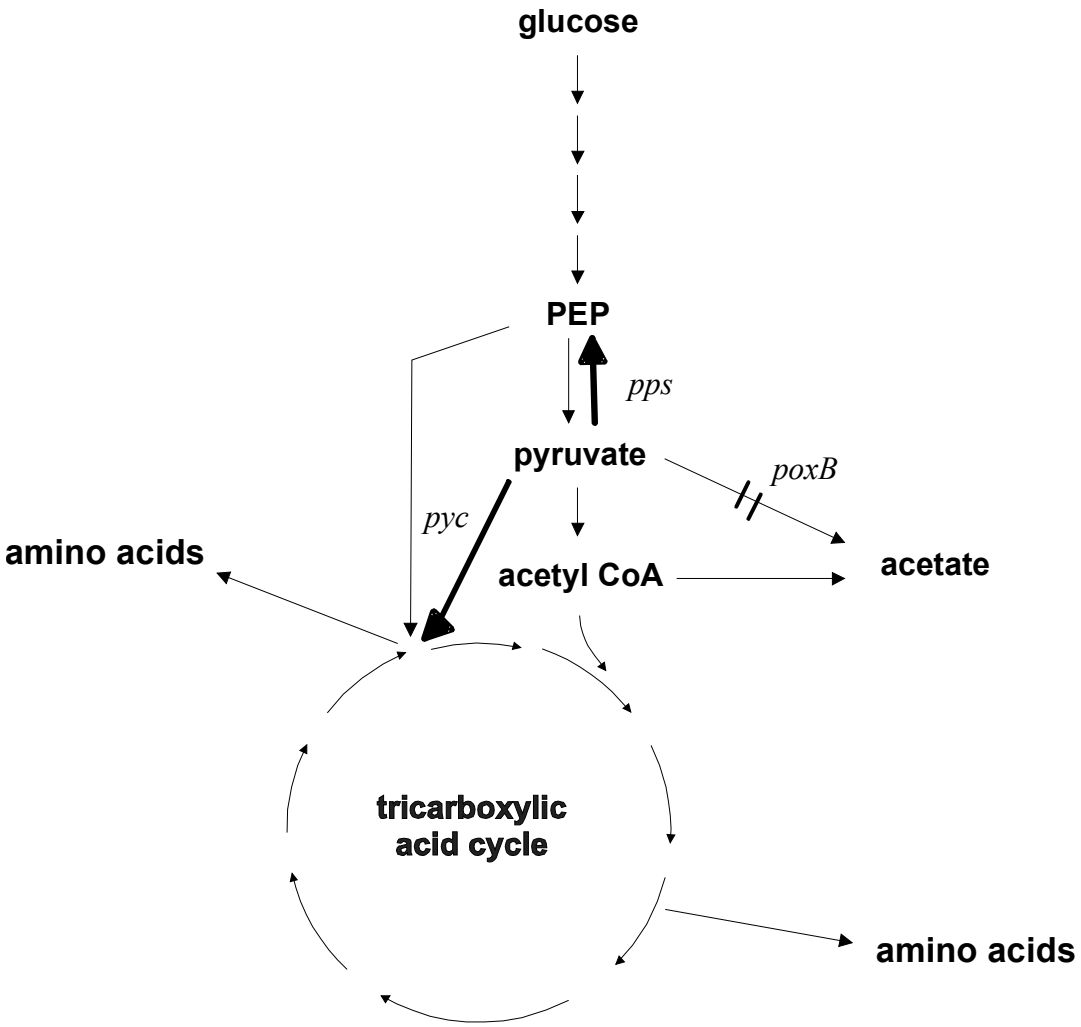

Figure 3. Genetic modifications studied in *E. coli*: *pps*: PEP synthase, *pyc*: pyruvate carboxylase, *poxB*: pyruvate oxidase B. Double lines indicate a gene knockout while a thickened line indicates overexpression of genes associated with that pathway.

Materials and Methods

Strains

Table 3 lists the strains used in this study. *E. coli* MG1655 was the host strain, and β-galactosidase encoded by the *Escherichia coli lacZ* gene was expressed via the pTrc99A-*lacZ* ampicillin-resistant plasmid under the control of the *trc* promoter. Some constructs contained pyruvate carboxylase encoded by the *pyc* gene or PEP synthase encoded by the *pps* gene expressed via the plasmid pACYC. The pACYC184-*trc*-*pyc* chloramphenicol-resistant plasmid contains the *Rhizobium etli pyc* gene under the control of the *trc* promoter. The pACYC184-*trc*-*ppsA* chloramphenicol-resistant plasmid contains the *E. coli ppsA* gene under the control of the trc promoter. The pACYC184-*trc* plasmid is a derivative of the pACYC184 plasmid and contains the *trc* promoter and multiple cloning sites from pTrc99A. Because the pACYC184-*trc* plasmid contains the P15A origin of replication, it is compatible with the pTrc99A plasmid. Both pTrc99A- and pACYC-derived plasmids are induced by isopropyl-β-thiogalactopyranoside. Two strains contained a knockout of the *poxB* gene.

Table 3. Strains used in this study.

Strain	Genotype	Plasmid †	*pyc*	*pps*	*poxB*
ALS1100	MG1655		–	+	+
ALS1102	MG1655	pACYC-*pyc*	++	+	+
ALS1108	MG1655 *poxB*	pACYC-*pyc*	++	+	–
ALS1109	MG1655	pACYC-*pps*	–	++	+
ALS1110	MG1655 *poxB*	pACYC-*pps*	–	++	–

† All strains additionally contained pTrc99A-*lacZ*

Gene expression levels: no activity (–); natural wild-type expression (+); overexpression (++)

Growth conditions

For each bioreactor experiment, cells were first grown in a capped tube containing 5 mL defined medium for about 11 h, before transferring to 50 mL of defined medium in a 250 mL

shake flask. When this preculture reached an optical density (OD) of about 2, the preculture was diluted with defined medium to a total volume of 100 mL and an OD of 1.0 before inoculation of the bioreactor. Both tube and flasks were incubated at 37°C and 250 rpm. The defined medium contained (per liter of distilled water): glucose, 3 g; citric acid, 1.70 g; KH_2PO_4, 13.30 g; $(NH_4)_2HPO_4$, 4.50 g; $MgSO_4 \cdot 7H_2O$, 1.2 g; $Zn(CH_3COO)_2 \cdot 2H_2O$, 13 mg; $CuCl_2 \cdot 2H_2O$, 1.5 mg; $MnCl_2 \cdot 4H_2O$, 15 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. 50 mg/L ampicillin and/or 5 mg/L chloramphenicol were used for selective pressure to maintain the plasmid(s).

Fed-batch fermentation

All fermentations of 1 L volume were conducted in duplicate in a bench-top bioreactor (Bioflow II, New Brunswick Scientific, New Brunswick NJ) operating at 500 rpm, 37°C and with a flow air/O_2 flowrate maintained at 1.0 L/min. The dissolved oxygen (DO) concentration was maintained above 30% of saturation for the duration of processes by the addition of pure oxygen. A pH of 6.8 was maintained with concentrated NH_4OH. Exponential feeding maintained a constant growth rate at either 0.15 h^{-1} or 0.35 h^{-1} commenced when the initial 3 g/L glucose was nearly depleted. Cultures were induced by 0.5 mM IPTG when the OD reached 7. The feed contained 250 g/L glucose, 30 g/L $(NH_4)_2SO_4$, 2 mL antifoam C and antibiotic(s) appropriate for the particular strain.

Analytical methods

Samples were stored at -20°C for subsequent analyses. Cell density was measured using OD at 600 nm (UV-650 spectrophotometer, Beckman Instruments, San Jose, Calif.), and this measurement was correlated to dry cell mass. Concentrations of glucose and acetate were

determined by high-pressure liquid chromatography (HPLC) as previously described (Eiteman and Chastain 1997).

Enzyme assays

Cells were washed, resuspended in 0.1 M Tris-HCl buffer (pH 8.0), ruptured with a French pressure cell (15000 psi), and centrifuged (7600×g for 20 min at 4°C) to remove cell debris. The cell extract was analyzed for pyruvate carboxylase (Payne and Morris 1969) and PEP synthase (Cooper and Kornberg 1969). For β-galactosidase activity, aliquots were thawed and diluted to an OD of 0.02 with DI water. Diluted samples were analyzed for β-galactosidase activity by measuring the rate of reaction with *o*-nitrophenyl-β-galactoside (ONPG) (Pardee et al. 1959).

Intracellular PEP/pyruvate ratio

When the OD was between 25 and 30, one 5 mL sample was rapidly quenched into 25 mL of 60% (v/v) methanol at -60°C. Intracellular PEP and pyruvate were extracted from the cell pellet by adding 500 μL of 50% (v/v) methanol and 2 mL of 35% perchloric acid. Cells were lysed after two freeze-thaw cycles. Protein and cell fragments were removed by centrifugation (7600×g for 30 min at 0ºC). The supernatant was neutralized with 5 M K_2CO_3, and recentrifuged (7600×g for 10 min). Samples were measured for intracellular pyruvate and PEP by enzymatic analysis (Schaefer et al. 1999).

Results

Production of β-galactosidase in MG1655 at different growth rates

All strains were grown at two different growth rates using a fed-batch process by controlling the exponential feeding rate of a 250 g/L glucose solution. ALS1100 can be considered the "experimental control", the wild-type MG1655 containing the plasmid pTrc99A-*lacZ* expressing the model protein β-galactosidase. Pyruvate carboxylase activity was not present in this strain, and the level of PEP synthase activity was not elevated. At the higher specific growth rate ($\mu = 0.35\ h^{-1}$), ALS1100 grew to an OD of about 53 in 12.5 h and generated 115 kU/ L β-galactosidase (Fig. 4). During the process, the β-galactosidase concentration increased with cell mass, except during the last 2 h when no additional protein was generated. Interestingly, the time when protein production ceased corresponded with the onset of acetate accumulation. By the end of fermentation, about 2.1 g/L acetate was generated and less than 0.1 g/L glucose accumulated in the medium.

At the lower specific growth rate ($\mu = 0.15\ h^{-1}$), ALS1100 reached an OD about 53, but this OD required 19 h. β-galactosidase production almost doubled compared to the higher growth rate, reaching 214 kU/L by the end of the process (Fig. 4). Although the rate of β-galactosidase production slowed at the end of process, it did not essentially stop as was observed at the higher growth rate. Less than 0.1 g/L acetate was observed during the process. In duplicate experiments, low growth rate consistently resulted in greater recombinant protein production and reduced acetate formation.

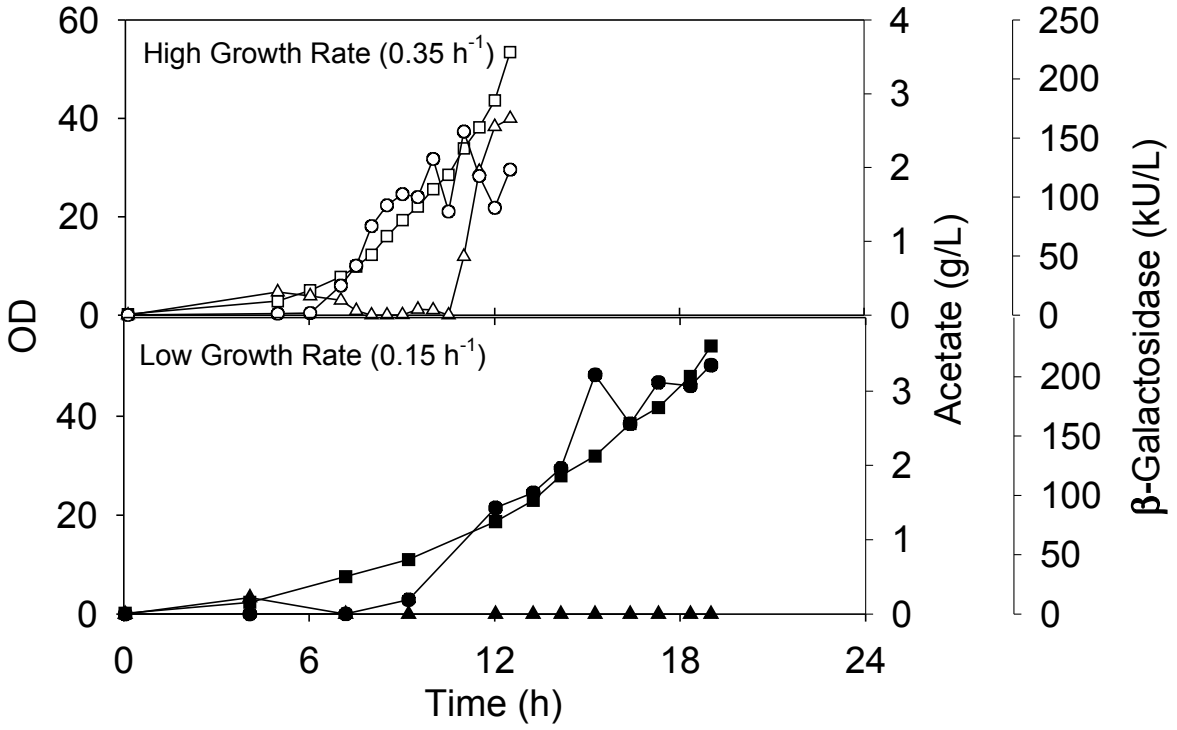

Figure 4: Fed-batch process using ALS1100 at two different growth rates. The culture at high growth rate (hollow symbols) and culture at low growth rate (solid symbols) were induced with 0.5 mM IPTG at 6.5 h and 8 h, respectively. Samples were measured for culture OD: (□,■), β-galactosidase (○,●), acetate (Δ,▲).

Effect of pyc

The production of recombinant β-galactosidase and acetate was next studied using the same fed-batch processes with *E. coli* ALS1102 containing the pACYC-*pyc* plasmid expressing heterologous pyruvate carboxylase (*pyc*) from *Rhizobium etli*. More than 20 U/g cell pyruvate carboxylase activity was measured which confirmed the overexpression of *pyc* gene in ALS1102. At the low specific growth rate (μ=0.15 h^{-1}), ALS1102 reached an OD of 54 and generated 372 kU/L β-galactosidase (Fig. 5), 73% more protein than was observed using the control strain ALS1100 at the low growth rate. The production rate of β-galactosidase again slowed during the last 4 h. No acetate was detected in the medium.

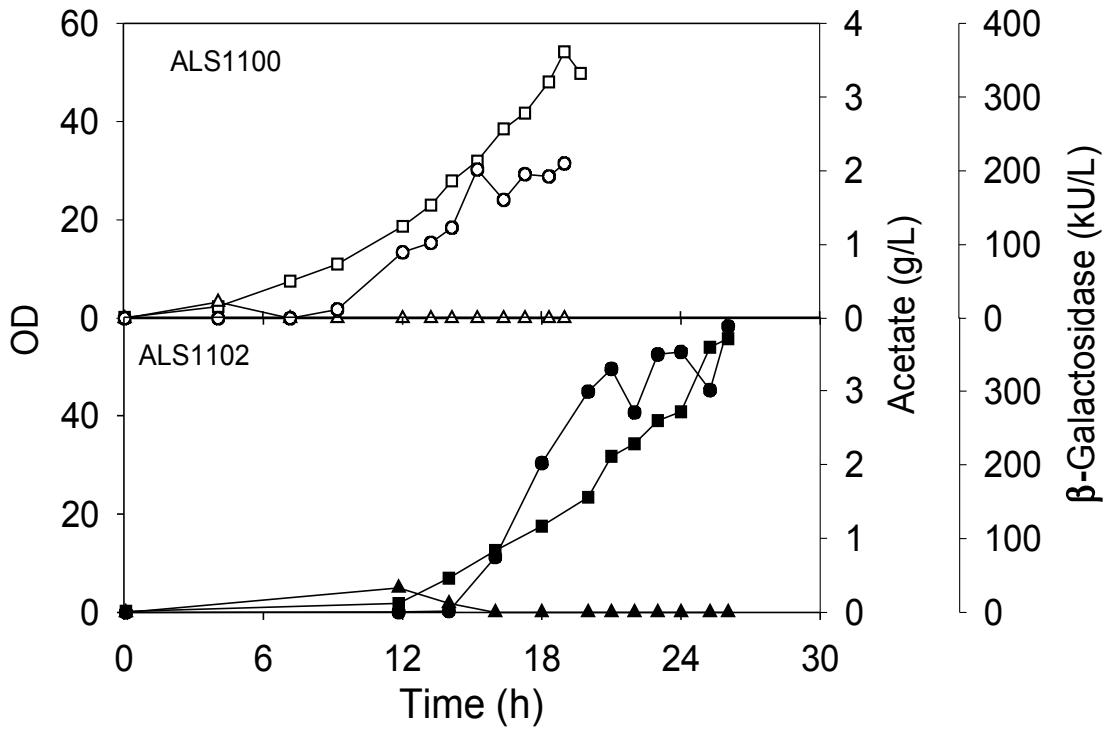

Figure 5: Low growth rate fed-batch processes comparing ALS1102 (solid symbols) and ALS1100 (hollow symbols). ALS1100 was induced with 0.5 mM IPTG at 8 h, while ALS1102 was induced with 0.5 mM IPTG at 14.4 h. Samples were measured for culture OD: (□,■), β-galactosidase (○,●), acetate (Δ,▲).

In contrast, at the high specific growth rate ($\mu = 0.35\ h^{-1}$), ALS1102 only generated 8.5 kU/L β-galactosidase and 1.1 g/L acetate by the end of the process (OD 45) (Fig. 6). Corresponding to the time that the acetate concentration reached 0.5 g/L, the β-galactosidase decreased 50% from about 16 kU/L to about 8 kU/L, and remained at this lower level for the remainder of the fermentation. Glucose also accumulated to the concentration of 27 g/L glucose during the last 2-3 h, indicating that the cells were no longer able to consume glucose at the rate supplied. The specific glucose consumption rate was 0.68 g/g/h which was 13% lower than that in control strain. Not only was the quantity of β-galactosidase generated during the high growth rate process lower than the β-galactosidase generated during low growth rate for the same strain, but it was lower than the β-galactosidase generated during high growth rate for the control strain ALS1100. However, the acetate concentration from using ALS1102 was about half of the acetate concentration from using ALS1100. The overexpression of *pyc* gene in *E. coli* improved protein production at the low growth rate, but it reduced protein production at the high growth rate.

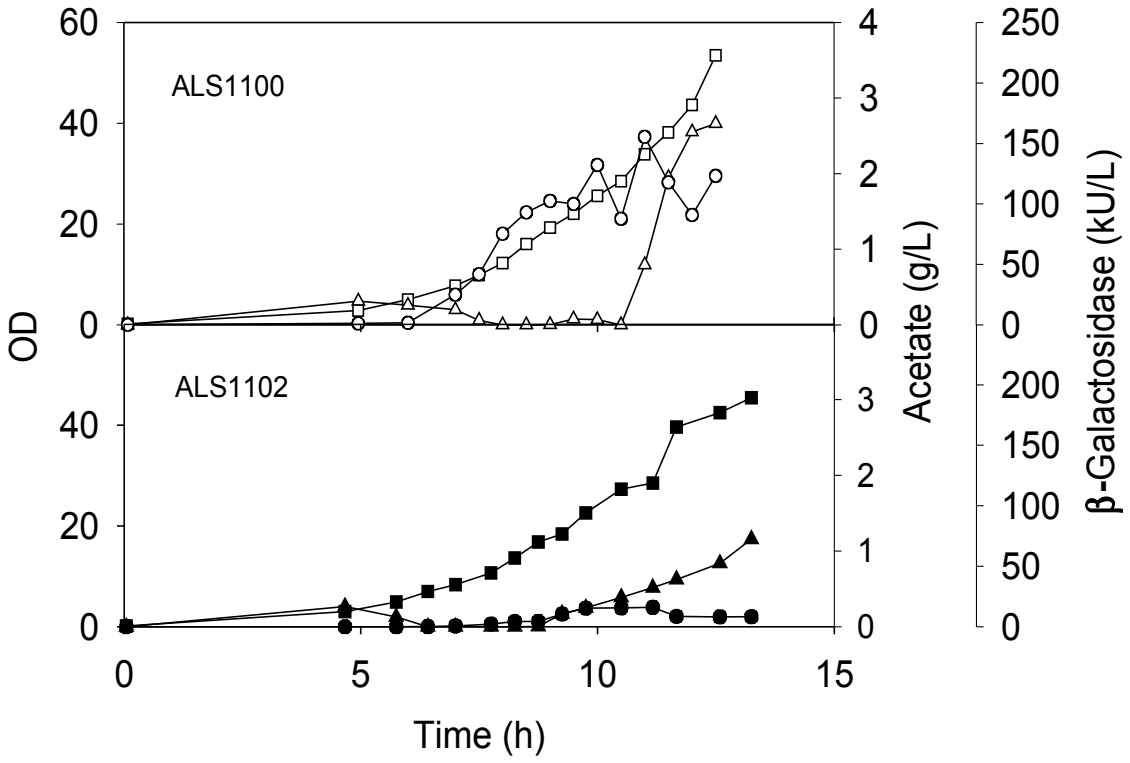

Figure 6: High growth rate fed-batch processes comparing ALS1102 (solid symbols) and ALS1100 (hollow symbols). ALS1100 was induced with 0.5 mM IPTG at 6 h, while ALS1102 was induced at 7.1 h. Samples were measured for culture OD: (□,■), β-galactosidase (○,●), acetate (Δ,▲).

Effect of pps

Next ALS1109 containing the pACYC-*pps* plasmid was studied, in which PEP synthase was overexpressed. Enzyme activity of PEP synthase activity was 64 U/g cell (compared to 3.4 U/g cell in ALS1100) confirming the overexpression of *pps* gene in ALS1109. At the lower growth rate, ALS1109 produced 168 kU/L β-galactosidase, identical to the protein production in ALS1100 (Fig. 7). Acetate was not detected in the medium. At the higher growth rate, cells only generated 24 kU/L β-galactosidase. This result is consistent with results observed using ALS1100 and ALS1102 that the higher growth rate of 0.35 h^{-1} yields lower recombinant protein than the lower growth rate of 0.15 h^{-1}. Moreover, although recombinant protein production in ALS1109 was similar to ALS1100 at the lower growth rate, the protein production in ALS1100 was five times greater than ALS1109 at the higher growth rate. Of course, ALS1109 contains two plasmids whereas ALS1100 only contains one. However, in contrast with ALS1100 and ALS1102, no glucose was observed when ALS1109 was grown at the higher growth rate. Glucose consumption rate was maintained at a higher level of 0.96 g/g/h in the presence of PEP synthase. Nevertheless, the overexpression of *pps* gene did not improve protein production.

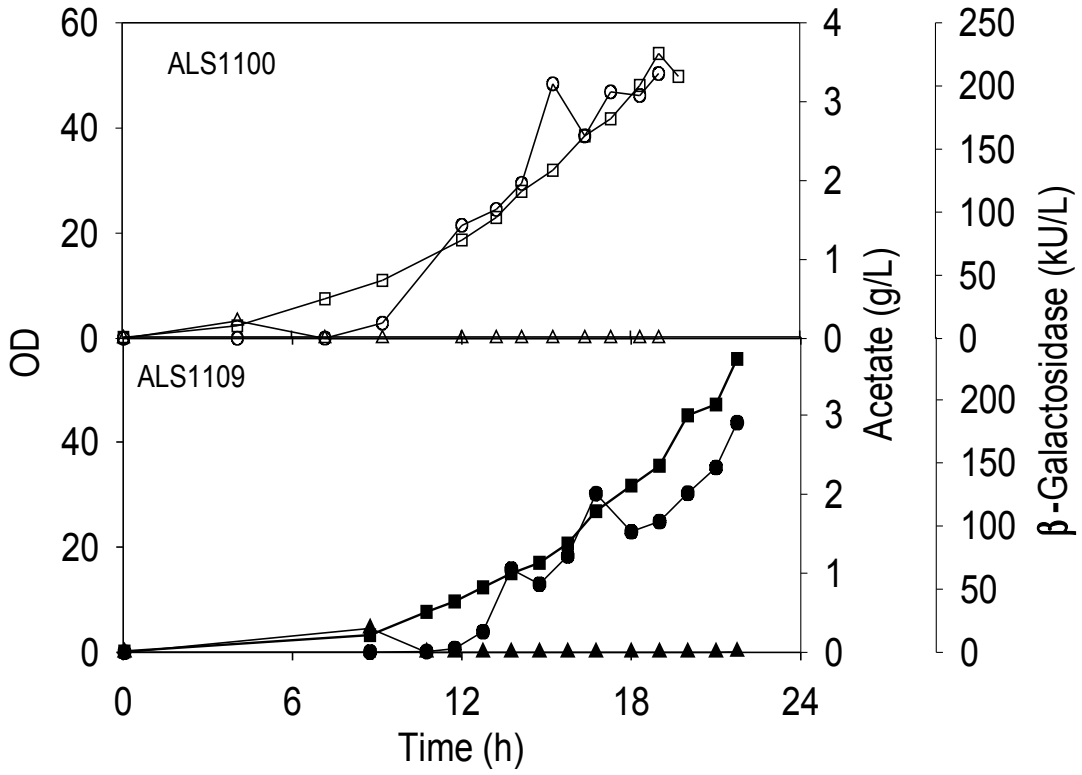

Figure 7: Low growth rate fed-batch processes comparing ALS1109 (solid symbols) and ALS1100 (hollow symbols). ALS1109 was induced with 0.5 mM IPTG at 10.8 h, while ALS1100 was induced at 6 h. Samples were measured for culture OD: (□,■), β-galactosidase (○,●), acetate (Δ,▲).

Effect of poxB knockout

In addition to overexpression of *pyc* or *pps* gene, we also studied the impact of a *poxB* (encoding pyruvate oxidase) knockout in ALS1102 and ALS1109. ALS1108 contains the *poxB* knockout and the pACYC-*pyc* plasmid, while ALS1110 contains the *poxB* knockout and the pACYC-*pps* plasmid.

At the low growth rate, ALS1108 generated 80 kU/L β-galactosidase which is only 20% of the level generated by ALS1102 at that growth rate (Fig. 8). No acetate was detected in the medium. At the higher growth rate, ALS1108 generated 12 kU/L β-galactosidase, about the same as the level generated by ALS1102. At these conditions, the final acetate concentration was 2 g/L and glucose accumulated to a concentration of 15 g/L. The knockout of an acetate-generating pathway did not improve protein production, nor did this knockout prevent acetate formation.

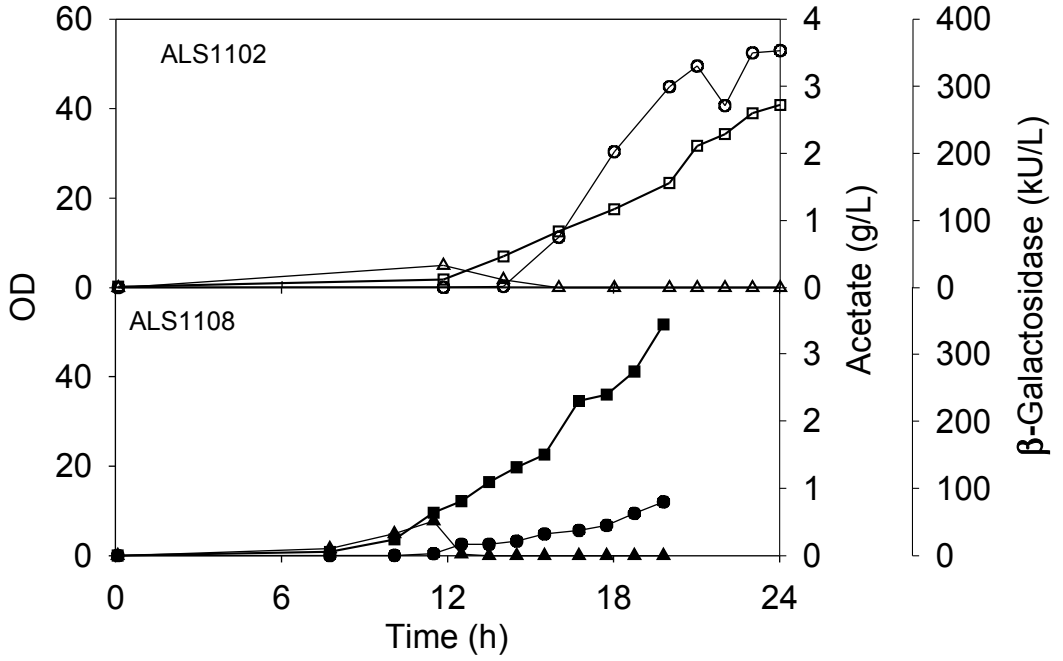

Figure 8: Low growth rate fed-batch processes comparing ALS1108 (solid symbols) and ALS1102 (hollow symbols). ALS1108 was induced with 0.5 mM IPTG at 10.6 h, while ALS1102 was induced at 7.1 h. Samples were measured for culture OD: (□,■), β-galactosidase (○,●), acetate (Δ,▲).

At the low growth rate ALS1110 achieved an OD of 51 and generated 90 kU/L β-galactosidase, about 50% of the protein concentration achieved by ALS1109 (Fig. 9). Acetate accumulated in the culture of ALS1110 to 0.2 g/L in the last 3 h, a time which correlated to the decrease in protein production decrease. At high growth rate, ALS1110 generated 28 kU/L β-galactosidase and 0.38 g/L acetate. Like ALS1109, glucose did not accumulate in the culture of ALS1110.

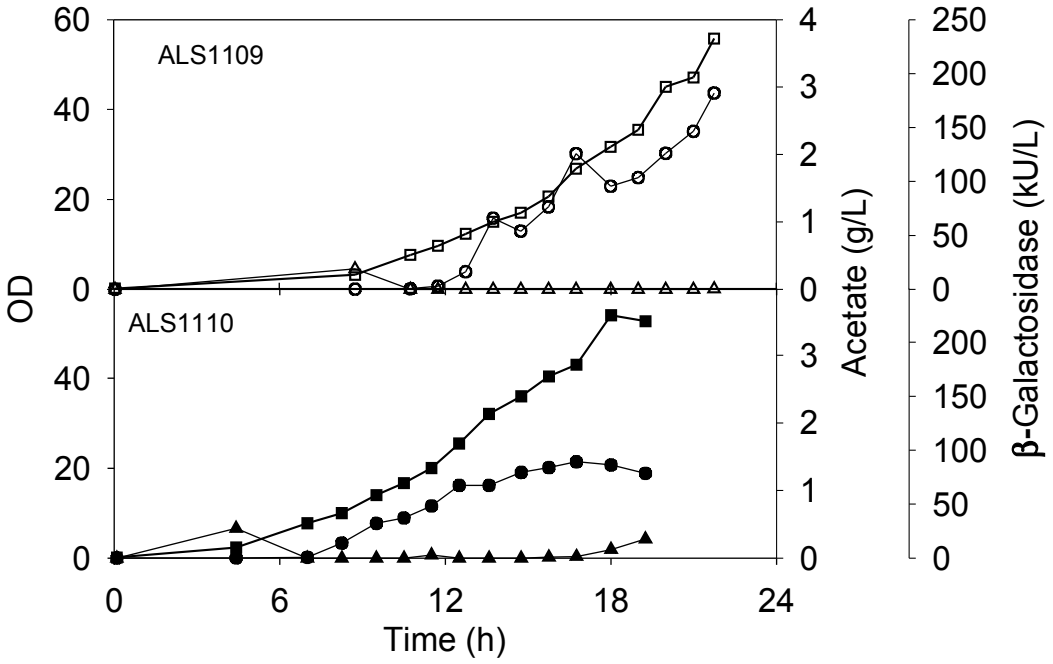

Figure 9: Low growth rate fed-batch processes comparing ALS1110 (solid symbols) and ALS1109 (hollow symbols). ALS1108 was induced with 0.5 mM IPTG at 7 h, while ALS1109 was induced at 10.8 h. Samples were measured for culture OD: (□,■), β-galactosidase (○,●), acetate (Δ,▲).

All the results including β-galactosidase activity, acetate concentration, enzyme activity and specific glucose consumption rate for all strains at different growth rates are summarized in the Table 4. In summary, the *pyc*-containing strain (with no other genetic perturbation) grown at low growth rate had the highest β-galactosidase activity among all the conditions. The β-galactosidase activity for cultures grown at a high growth rate was consistently much lower than the activity observed for the same strains grown at a low growth rate.

Table 4. Comparison of different *E. coli* strains during fed-batch processes for the production of recombinant protein. The β-galactosidase activity and acetate concentrations were measured at the end of each process when the OD was approximately 50. The intracellular pyruvate/PEP ratio, PYC activity and PPS activity were measured when the OD was 25–30. Data represent means from two experimental runs.

Strain	μ (h^{-1})	Parameter[a]					
		β-galactosidase activity (kU/L)	Acetate concentration (g/L)	Pyruvate /PEP	PYC activity (U/g cell)	PPS activity (U/g cell)	q_s (g/g/h)
ALS1100	0.15	214	0.04	1.36		3.4	0.41
	0.35	115	2.14	1.82		12	0.78
ALS1102	0.15	371	0	1.28	15	12	0.39
	0.35	9	1.15	1.34	29	-2.5	0.68
ALS1108	0.15	80	0	1.59	21	8.7	0.47
	0.35	12	2.02	1.92	24	16	0.60
ALS1109	0.15	168	0	1.22		53	0.52
	0.35	24	2.48	0.98		64	0.96
ALS1110	0.15	90	0.14	0.91		79	0.43
	0.35	28	0.38	1.07		56	0.90

[a] PEP: phosphoenolpyruvate; PYC: pyruvate carboxylase; PPS: PEP synthase; q_s: specific glucose consumption rate

Intracellular concentrations

Since genetic modifications at the pyruvate node could affect the intracellular concentration of pyruvate and its precursor PEP, these intracellular concentrations were also measured during the bioprocesses (Table 4). ALS1108 (the *poxB* knockout and the pACYC-*pyc* plasmid) had the highest pyruvate/PEP ratio at both high growth rate (ratio = 1.92) and low growth rate (1.59). Strains with *pps* overexpression showed the lowest ratios: 0.98 for ALS1109 at a high growth rate and 0.91 for ALS1110 at a low growth rate. Given the methodology for measuring these concentrations, and the fact that one observed value is divided by another observed value in the calculation of a ratio, most of these ratios are likely not significantly different. The pyruvate/PEP ratio was close to 1 in all processes.

Discussion

In this study, we performed genetic perturbations at the pyruvate node by introducing the *pyc* gene, *pps* gene and/or deleting the *poxB* gene in *E. coli*. In addition, we also studied the impact of different growth rates on those genetic modified strains. Cell growth was controlled at one of two constant specific growth rates by a glucose-limited exponential feeding strategy.

Cells consistently produced more β-galactosidase and less acetate at low growth rates (0.15 h^{-1}) than high growth rates (0.35 h^{-1}). Since glucose was the limiting nutrient for cell growth, low growth rate corresponds with low glucose uptake rate. Therefore, our results with different strains provide further evidence that acetate formation can be reduced by keeping specific glucose uptake rate less than the critical value of specific glucose consumption rate at which acetate starts to accumulate. This observation is consistent with studies on feeding strategy to reduce acetate (Akesson et al. 2001; Johnston et al. 2002). Reduced glucose consumption rate means slower glycolysis which will alleviate by-product generation in *E. coli* (Luli and Strohl 1990; Shimizu et al. 1991; Shiloach et al. 1996). In contrast, cells accumulated acetate when the glucose consumption rate was greater than the critical value (Paalme et al. 1990). Moreover, production of recombinant protein lowers the critical value of specific glucose consumption rate for acetate formation (Neubauer et al. 2003). Although cell growth and protein production are inhibited by acetate, the direct inhibitory effects occur in the range of 3 to 5 g/L acetate (Luli and Strohl 1990; Aristidou et al. 1994). However, in our study protein production decreased significantly at high growth rates even when less than 3 g/L acetate was present in the medium. Thus, acetate concentration might not be the *cause* of decreased protein production, but only a symptom, and protein production could be more directly affected by other factors. For example,

increased growth rate is known to decrease copy number of plasmids (Zabriskie and Arcurie 1986).

At low growth rate, the expression of *pyc* gene (alone) reduced acetate production and increased β-galactosidase production, similar to previous results with aerobic batch studies (March et al. 2002). The increase of protein production in the *pyc*-containing strain can be attributed to several factors. First, pyruvate carboxylase diverts carbon from pyruvate and therefore reduces the carbon flux which is available for acetate generation. Second, pyruvate carboxylase increases carbon flux from acetate to oxaloacetate (Gokarn et al. 2001) which is an important metabolic precursor for amino acid synthesis (Eiteman and Altman 2006). Third, overexpression of the *pyc* gene elevates the expression of several amino acid biosynthesis genes (Vemuri et al. 2005). At high growth rate these differences diminish, which appears to be inconsistent with March (2002). In these previous batch studies, protein yield increased in the presence of *pyc* compared to the control even when cells grew at their maximum growth rate (> 0.35 h^{-1}). Several differences could account for these observations: March used a complex medium which would provide the amino acids required for protein synthesis, while a defined medium was used in our fed-batch studies (thus, cells had to synthesis all the amino acids from building block compounds). Also, as all strains were grown at their maximum growth rate, the presence of *pyc* may have itself reduced the growth rate and glucose consumption rate compared to the control lacking the *pyc* gene, whereas in the current study growth rate was the same for the two strains.

Another factor which affects protein production is the glucose uptake rate via PTS is the pyruvate:PEP ratio (Weigel et al. 1982). The slightly lower pyruvate:PEP ratio in ALS1102 at both growth rates suggests a higher phosphorylation state of the enzyme involved in PTS which

may increase glucose uptake. Previously the expression of pyruvate carboxylase was shown to increase the expression of PTS and glycolytic genes (Vemuri et al. 2005). However, the fact that glucose accumulated in the medium at the high growth rate whereas it did not in the control strain and the calculated low specific glucose consumption rate indicate the specific glucose uptake rate was not increased by the expression of *pyc* gene. A decrease in glucose consumption rate of *E. coli* with *pyc* gene was also observed in batch fermentation with a complex medium (March et al. 2002). The reason for the apparent contradiction between *pyc* upregulating glucose-uptake genes (observed by Vemuri et al. 2005) and the reduction in glucose uptake in the presence of *pyc* is unclear.

Overexpression of PEP synthase in *E. coli* did not increase protein production. Nevertheless glucose was absent during the prolonged exponential feed when PEP synthase was overexpressed, even when strains were grown at the higher growth rate. This observation is in contrast to both the control strain ALS1100 and the *pyc*-containing strain ALS1102 in which some glucose remained unconsumed. Thus, PEP synthase increases overall glucose consumption rate. A previous study using a batch process with a defined medium demonstrated that overexpression of *pps* stimulated the glucose consumption rate (Patnaik et al. 1992). These authors hypothesized that the increased glucose uptake rate resulted from an increased PEP pool in cells, although the intracellular PEP and pyruvate concentrations were not actually measured. In our results, we indeed did observed a slightly reduced pyruvate:PEP ratio when PEP synthase was overexpressed.

PEP synthase might also impact by-product formation. Patnaik (1992) reported a significant increase in the excretion of fermentation products in *pps*+ strains, including acetate and pyruvate, and these products were proposed to be a consequence of the higher glucose

consumption rate. However, in our study the final acetate concentration was not significantly different in the *pps*+ strain (ALS1109) compared to the control strain (ALS1100). One reason that acetate generation may have been unaffected by the overexpression of PEP synthase in the current study is that the experiments were conducted in a glucose-limited fed-batch. Therefore, the comparison here is made between two strains at the *same* glucose consumption rate. In contrast, Patnaik et al. (1992) permitted the two strains to grow at each one's maximum growth rate, and, two different glucose consumption rates. As has been observed in numerous studies (including this one with ALS1100), increased glucose consumption rate (whether by feeding at a higher rate or by overexpressing PEP synthase) results in a higher rate of acetate generation. Another difference between this study and the work of Patnaik et al. (1992) is that these earlier researchers did not overexpress another protein. The overexpression of β-galactosidase in this study would tend to withdraw PEP to replenish metabolic precursors for amino acid synthesis. In another study involving PEP synthase overexpression, accumulation of acetate and pyruvate was significantly reduced when the PEP was directed to DAHP synthesis (Patnaik and Liao 1994).

The knockout of the *poxB* gene also affected acetate generation and protein production. The *poxB* mutation downregulates the *ptsG* and *crr* genes of the PTS (Vemuri et al. 2005), and the absence of pyruvate oxidase reduces glucose uptake as well as growth rate (Flores et al. 2004). Of course, in our study the growth rate was controlled at two levels below the maximum growth rate of wild-type *E. coli*, and we would not have observed reduced growth rate in *poxB* knockout strains. Pyruvate oxidase plays an important role in converting pyruvate to acetate especially at low growth rate (Chang et al. 1994). Although some authors assumed a perturbation of pyruvate oxidase would not influence carbon flux to acetate (Emmerling et al. 2002), we observed the level of acetate accumulation changed when a *poxB* knockout was combined with

the overexpression of other genes. For example, at the growth rate of 0.35 h^{-1}, the *poxB* strain showed increased acetate formation compared to the *pyc*-containing (alone) strain, whereas this knockout decreased acetate formation by 87% compared to the *pps*-containing (alone) strain. In another study using a batch process, the strain without *poxB* gene alone accumulated more acetate in the medium than the wild type (Abdel-Hamid et al. 2001). Thus, the impact of a *poxB* mutation on acetate generation might be related to the other genetic modifications. With essentially the same level of acetate accumulation at the growth rate of 0.15h^{-1}, the *poxB* mutation reduced protein production by 78% in the presence of the pACYC-*pyc* plasmid (ALS1108 vs. ALS1102), while the *poxB* mutation reduced protein production by 46% in the presence of the pACYC-*pps* plasmid (ALS1110 vs. ALS1109). This result not only provides evidence that acetate accumulation is not the essential reason for reduced protein production, but also indicates that a *poxB* knockout is detrimental to protein production. Finally, because the protein encoded by *poxB* gene participates in nutrient and respiration stress responses (Flores et al. 2004), the absence of *poxB* gene might affect the stress response of cells, which in turn affects protein production.

The correlation between pyruvate:PEP ratio and acetate formation is not obvious in this study. The pyruvate:PEP ratio was essentially unchanged between low and high growth rates, although the high growth rates led to more acetate accumulation. In contrast, a recent chemostat study demonstrated that the pyruvate:PEP ratio correlated with the onset of acetate accumulation (Vemuri et al. 2006a). The difference between the observed pyruvate:PEP ratios could be due to the different growth conditions. In chemostat processes, all culture parameters are constant, and the cell density is relatively low. In the fed-batch operational mode, culture parameters are

changing as products accumulate, and cell density is comparatively high. These differences could affect the physiology of cells, and therefore the pyruvate:PEP ratio.

In summary, pyruvate occupies an important position in *E. coli* metabolism. Genetic perturbations at the pyruvate node have an impact on the intracellular concentration of pyruvate, the glucose uptake and acetate accumulation. For example, overexpressing *pyc* gene reduced both glucose consumption and acetate formation, while overexpressing *pps* gene increased glucose consumption. Different feeding rates also have an impact on acetate formation and protein production, with low growth rate favoring protein production and reducing acetate formation. Process and genetic modifications are not mutually exclusive. Process modifications must be carried out to match the affects of genetic characteristics to optimize the process.

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Appendix A
Date of fermentation process

Fermentation ALS1100 - H - 1

Fermentation Date	7/9/2008
Lab Notebook	17 (K)
Strain	ALS1100
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	44 mL
initial volume	1 L
pH	6.8
growth rate	0.35 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.16	-1.833	99.1	2.78	0.00	0	0	8.0	8.0	1.00	57	57	57	0
2	5.0	2.83	1.039	86.0	0.00	0.31	0	0	7.5	15.5	0.98	1149	1326	1237	126
3	6.0	5.01	1.612	82.1	0.00	0.26		0	7.0	22.5		1560	1925	1742	258
4	7.0	7.76	2.049	56.4	0.00	0.20	18	0	8.0	30.5	0.99	10358	39455	24906	20574
5	7.5	9.87	2.289	49.2	0.00	0.06	24	2	7.0	37.5	0.99	57296	26428	41862	21828
6	8.0	12.27	2.507	42.8	0.00	0.00	32	5	7.0	44.5	0.99	71257	79444	75351	5789
7	8.5	16.05	2.775	71.3	0.00	0.00	41	7	5.5	50.0	1.00	69153	116786	92970	33682
8	9.0	19.27	2.958	20.2	0.00	0.00	55	8	7.0	57.0	1.01	145996	58991	102493	61522
9	9.5	22.05	3.093	7.2	0.00	0.08	66	10	6.0	63.0	1.01	91979	107979	99979	11314
10	10.0	25.60	3.242	32.6	0.01	0.07	82	13	6.5	69.5	1.03	106812	157521	132166	35857
11	10.5	28.52	3.351	10.5	0.02	0.00	101	16	8.0	77.5	1.04	97457	78021	87739	13743
12	11.0	33.86	3.522	6.0	0.01	0.79	124	18	8.0	85.5	1.06	97180	213659	155420	82363
13	11.5	38.17	3.642	6.7	0.01	1.95	149	22	9.0	94.5	1.08	87692	147918	117805	42587
14	12.0	43.64	3.776	8.4	0.04	2.55	181	26	7.0	101.5	1.11	93921	87808	90864	4322
15	12.5	53.48	3.979	31.3	0.09	2.66	218	30	8.0	109.5	1.14	102667	143824	123245	29102

Fed-Batch Starting Time	5 h	Intracellular Pyruvate	0.289 mmol/L
Induction Time	6.5 h	Intracellular PEP	0.143 mmol/L
Glucose in Feed (HPLC)	247.4 g/L	PYR/PEP Ratio	2.02
Calculated Growth Rate	0.350 h^{-1}	PPS Activity	0.012 U/mg
Key Measurement Time	10 h	PYC Activity	U/mg
Dry Cell Weight	10.54 g/L		

Fermentation ALS1100 - H - 2

Fermentation Date	7/9/2008
Lab Notebook	17 (L)
Strain	ALS1100
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	44 mL
initial volume	1 L
pH	6.8
growth rate	0.35 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.17	-1.749	99.0	2.21	0.00	0	0	7.0	7.0	1.00	40	43	42	2
2	5.0	2.76	1.017	92.9	0.05	0.28	0	0	7.0	14.0	0.99	481	628	554	104
3	6.0	4.69	1.545	90.4	0.00	0.33		0	7.5	21.5		804	1168	986	258
4	7.0	7.77	2.050	69.4	0.00	0.15	18	0	9.5	31.0	0.99	21280	14127	17704	5058
5	7.5	10.02	2.305	54.7	0.00	0.00	24	2	8.5	39.5	0.99	28365	55256	41811	19015
6	8.0	12.31	2.510	46.2	0.00	0.00	32	2	7.5	47.0	0.99	41956	102200	72078	42599
7	8.5	16.34	2.794	28.3	0.00	0.00	41	4	6.5	53.5	0.99	58738	139708	99223	57255
8	9.0	19.27	2.958	52.6	0.00	0.00	55	5	7.0	60.5	1.00	90948	83883	87416	4996
9	9.5	21.92	3.087	30.0	0.01	0.00	66	6	7.0	67.5	1.00	73104	78307	75705	3679
10	10.0	26.97	3.295	40.4	0.02	0.00	82	8	7.0	74.5	1.02	99192	99008	99100	130
11	10.5	32.38	3.477	12.1	0.01	0.39	101	10	9.0	83.5	1.03	88229	90608	89419	1682
12	11.0	33.91	3.524	5.6	0.03	0.20	124	14	8.5	92.0	1.05	108978	266817	187897	111609
13	11.5	42.93	3.760	3.1	0.04	1.04	149	18	8.0	100.0	1.07	86920	111144	99032	17129
14	12.0	47.61	3.863	12.5	0.07	1.49	181	24	7.0	107.0	1.10	104044	120420	112232	11580
15	12.5	53.58	3.981	69.2	0.11	1.62	218	26	7.0	114.0	1.13	98249	111635	104942	9465

Fed-Batch Starting Time	5 h			
Induction Time	6.5 h	PPS Activity		U/mg
Glucose in Feed (HPLC)	247.4 g/L	PYC Activity		U/mg
Calculated Growth Rate	0.366 h^{-1}			
Key Measurement Time	10 h			
Dry Cell Weight	11.35 g/L			
Intracellular Pyruvate	0.247 mmol/L			
Intracellular PEP	0.151 mmol/L			
Pyruvate/PEP Ratio	1.64			

Fermentation ALS1100 - L - 1

Fermentation Date	7/23/2008
Lab Notebook	17 (M)
Strain	ALS1100
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	32 mL
initial volume	1 L
pH	6.8
growth rate	0.15 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.31	-1.178	96.2	3.01	0.00	0	0	7.0	7.0	1.00	6	7	7	1
2	4.1	2.36	0.860	79.1	1.05	0.22	0	0	7.5	14.5	0.99	45	36	41	6
3	7.2	7.73	2.045	81.5	0.00	0.00	15	3	6.0	20.5	1.00	231	271	251	28
4	9.2	13.74	2.620	69.3	0.00	0.00		4	6.0	26.5		13553	22958	18256	6650
5	12.0	17.60	2.868	64.7	0.01	0.00		8	7.0	33.5		96004	94007	95006	1412
6	13.3	23.27	3.147	65.5	0.00	0.00	72	10	7.0	40.5	1.04	114826	90663	102744	17086
7	14.2	28.11	3.336	49.6	0.00	0.00	85	11	8.0	48.5	1.05	117116	138444	127780	15081
8	15.3	32.17	3.471	46.5	0.02	0.00	104	12	7.5	56.0	1.06	203919	262747	233333	41598
9	16.4	39.77	3.683	53.9	0.00	0.00	129	14	6.5	62.5	1.08	136962	200905	168933	45214
10	17.3	38.71	3.656	51.7	0.00	0.00	151	16	6.5	69.0	1.10	241129	96682	168906	102139
11	18.3	45.65	3.821	39.8	0.00	0.00	181	20	6.0	75.0	1.13	218762	226619	222691	5556
12	19.0	49.02	3.892	53.6	0.00	0.00	201	20	7.0	82.0	1.14	280057	310093	295075	21239
13	19.7	57.57	4.053	5.6	0.00	0.08	227	23	6.5	88.5	1.16	132201	140166	136184	5632

Pump Starting Time	4.7	h
Fed-Batch Starting Time	7.5	h
Induction Time	8.1	h
Glucose in Feed (HPLC)	262.35	g/L
Calculated Growth Rate	0.150	h^{-1}
Key Measurement Time	15.4	h
Dry Cell Weight	11.35	g/L
Intracellular Pyruvate	0.237	mmol/L
Intracellular PEP	0.145	mmol/L
Pyruvate/PEP Ratio	1.63	
PPS Activity	0.003	U/mg
PYC Activity		U/mg

Fermentation ALS1100 - L - 2

Fermentation Date	7/23/2008
Lab Notebook	17 (N)
Strain	ALS1100
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	32 mL
initial volume	1 L
pH	6.8
growth rate	0.15 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.17	-1.754	98.7	3.04	0.00	0	0	8.0	8.0	1.00	10	7	9	2
2	4.1	2.40	0.874	95.9	0.98	0.22	0	2	6.0	14.0	0.99	55	31	43	17
3	7.2	7.56	2.023		0.00	0.00	15	6	7.5	21.5	1.00	188	172	180	12
4	9.2	11.03	2.400	95.0	0.00	0.00		8	6.0	27.5		11958	12085	12022	90
5	12.0	18.70	2.928	86.1	0.00	0.00		12	5.0	32.5		94993	84207	89600	7626
6	13.3	22.99	3.135	48.5	0.00	0.00	72	13	6.5	39.0	1.05	102822	101656	102239	825
7	14.2	27.96	3.331	41.2	0.03	0.00	85	14	7.0	46.0	1.05	98173	147432	122802	34832
8	15.3	31.96	3.464	61.4	0.02	0.00	104	15	7.0	53.0	1.07	248040	155442	201741	65477
9	16.4	38.52	3.651	65.2	0.00	0.00	129	18	6.0	59.0	1.09	161778	159106	160442	1889
10	17.3	41.76	3.732	36.6	0.00	0.00	151	22	7.5	66.5	1.11	176125	214557	195341	27175
11	18.3	48.08	3.873	15.6	0.03	0.00	181	25	7.0	73.5	1.13	215252	169504	192378	32348
12	19.0	54.21	3.993	13.4	0.00	0.00	201	28	6.0	79.5	1.15	178465	241139	209802	44318
13	19.7	49.81	3.908	1.8	0.00	0.00	227	28	6.0	85.5	1.17	96859	175633	136246	55701

Pump Starting Time	4.7	h
Fed-Batch Starting Time	7.5	h
Induction Time	8.1	h
Glucose in Feed (HPLC)	264.3	g/L
Calculated Growth Rate	0.158	h^{-1}
Key Measurement Time	15.4	h
Dry Cell Weight	11.14	g/L
Intracellular Pyruvate	0.183	mmol/L
Intracellular PEP	0.17	mmol/L
Pyruvate/PEP Ratio	1.08	
PPS Activity		U/mg
PYC Activity		U/mg

Fermentation ALS1102 - H - 1

Fermentation Date	8/21/2008
Lab Notebook	17 (O)
Strain	ALS1102
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	33 mL
initial volume	1 L
pH	6.8
growth rate	0.35 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C and Antiboitic

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.10	-2.293	98.3	2.80	0.00	0	0	5.5	5.5	1.00	4	4	4	0
2	4.7	3.00	1.099	88.9	0.00	0.27	0	4	6.5	12.0	0.99	32	33	32	1
3	5.8	4.76	1.561	83.9	0.00	0.13	5	6	7.0	19.0	0.99	53	54	54	1
4	6.4	6.34	1.848	77.8	0.00	0.00	9	6	7.0	26.0	0.99	68	117	93	35
5	7.0	8.25	2.110	86.2	0.00	0.00	13	6	6.5	32.5	0.99	550	717	633	118
6	7.8	12.03	2.487	52.6	0.00	0.00	19	6	6.5	39.0	0.99	2346	2399	2372	37
7	8.3	13.57	2.608	52.5	0.00	0.00	26	8	7.0	46.0	0.99	3246	3767	3507	368
8	8.8	16.16	2.782	52.0	0.00	0.00	33	9	8.5	54.5	0.99	5331	3464	4398	1320
9	9.3	18.90	2.939	44.8	0.00	0.17	44	10	8.5	63.0	0.99	15087	7425	11256	5418
10	9.8	21.76	3.080	35.5	1.09	0.25	56	10	6.5	69.5	1.00	10098	16355	13226	4425
11	10.5	26.47	3.276	53.6	4.25	0.39	79	12	7.0	76.5	1.01	9323	13971	11647	3287
12	11.2	27.21	3.304	37.9	8.41	0.52	109	12	7.5	84.0	1.04	8067	10119	9093	1451
13	11.7	36.05	3.585	63.4	14.14	0.62	146	14	8.5	92.5	1.07	8122	8547	8335	300
14	12.6	40.42	3.699	38.8	21.66	0.84	200	18	7.5	100.0	1.12	8833	6893	7863	1372
15	13.3	45.46	3.817	28.9	26.94	1.16	255	20	7.0	107.0	1.17	10164	10074	10119	63

Pump Starting Time	4.7	h
Induction Time	6.6	h
Glucose in Feed (HPLC)	268.21	g/L
Calculated Growth Rate	0.314	h^{-1}
Key Measurement Time	10.5	h
Dry Cell Weight	7.45	g/L
Intracellular Pyruvate	0.259	mmol/L
Intracellular PEP	0.18	mmol/L
Pyruvate/PEP Ratio	1.44	
PPS Activity		U/mg
PYC Activity	0.0201	U/mg

Fermentation ALS1102 - H - 2

Fermentation Date	8/21/2008
Lab Notebook	17 (P)
Strain	ALS1102
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	33 mL
initial volume	1 L
pH	6.8
growth rate	0.35 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C and Antiboitic

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.14	-1.988	99.5	2.68	0.09	0	0	7.5	7.5	1.00	5	28	16	16
2	4.7	2.99	1.096	87.5	0.00	0.07	0	4	6.5	14.0	0.99	38	20	29	13
3	5.8	4.92	1.592	85.3	0.00	0.00	5	6	7.0	21.0	0.99	62	158	110	67
4	6.4	7.02	1.948	85.4	0.00	0.00	9	6	8.0	29.0	0.99	114	145	129	22
5	7.0	8.35	2.122	82.3	0.00	0.00	13	6	6.5	35.5	0.98	688	589	638	69
6	7.8	10.66	2.366	60.9	0.00	0.00	19	6	8.0	43.5	0.98	2508	1974	2241	378
7	8.3	13.68	2.616	52.8	0.00	0.00	26	8	7.5	51.0	0.98	4992	3605	4299	981
8	8.8	16.84	2.823	48.1	0.00	0.00	33	9	7.0	58.0	0.98	5683	3314	4499	1676
9	9.3	18.41	2.913	39.1	0.00	0.00	44	10	6.0	64.0	0.99	11234	9944	10589	912
10	9.8	22.57	3.117	37.2	0.55	0.00	56	10	6.5	70.5	1.00	24694	5824	15259	13343
11	10.5	27.30	3.307	31.3	3.20	0.00	79	14	7.0	77.5	1.02	25323	5735	15529	13851
12	11.2	28.50	3.350	31.3	6.94	0.03	109	15	7.0	84.5	1.04	19831	12374	16102	5273
13	11.7	39.62	3.679	52.8	12.23	0.03	146	17	8.0	92.5	1.07	10256	6840	8548	2415
14	12.6	42.49	3.749	32.1	19.44	0.00	200	20	7.0	99.5	1.12	9076	7219	8147	1313
15	13.3	45.47	3.817	30.1	27.22	0.05	255	23	8.0	107.5	1.17	10002	6584	8293	2417

Pump Starting Time	4.7	h
Induction Time	6.6	h
Glucose in Feed (HPLC)	268.21	g/L
Calculated Growth Rate	0.318	h^{-1}
Key Measurement Time	10.5	h
Dry Cell Weight	7.1	g/L
Intracellular Pyruvate	0.201	mmol/L
Intracellular PEP	0.164	mmol/L
Pyruvate/PEP Ratio	1.23	
PPS Activity	-0.0025	U/mg
PYC Activity	0.0369	U/mg

Fermentation ALS1102- L - 1

Fermentation Date	9/30/2008
Lab Notebook	17 (Q)
Strain	ALS1102
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	28 mL
initial volume	1 L
pH	6.8
growth rate	0.15 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.22	-1.537	99.0	3.00	0.00	0	0	7.0	7.0	1.00	5	6	6	0
2	11.8	1.82	0.596	96.6	0.00	0.33	0	4	9.0	16.0	0.99	552	581	567	20
3	14.0	6.97	1.942	66.9	0.00	0.12	13	6	7.0	23.0	1.00	1968	2005	1986	26
4	16.0	12.59	2.533	69.1	0.00	0.00	24	8	7.0	30.0	1.00	50475	99575	75025	34719
5	18.0	17.54	2.864	67.7	0.00	0.00	36	10	5.0	35.0	1.01	212722	192383	202553	14382
6	20.0	23.40	3.153	55.0	0.00	0.00	50	11	5.0	40.0	1.02	136453	462058	299256	230238
7	21.0	31.71	3.457	44.0	0.00	0.00	61	11	7.0	47.0	1.02	331520	328592	330056	2071
8	22.0	34.31	3.535	57.6	0.00	0.00	72	12	6.0	53.0	1.03	323727	218296	271011	74551
9	23.0	38.98	3.663	63.9	0.00	0.00	87	14	5.0	58.0	1.04	267867	431550	349708	115742
10	24.0	40.82	3.709	40.2	0.00	0.00	108	16	7.0	65.0	1.06	309800	396533	353167	61330
11	25.3	53.93	3.988	66.3	0.00	0.00	135	20	5.5	70.5	1.08	276067	326667	301367	35780
12	26.0	55.66	4.019	74.1	0.00	0.00	156	23	7.0	77.5	1.10	346933	428444	387689	57637

Pump Starting Time	11.92	h
Fed-Batch Starting Time	14.1	h
Induction Time	14.4	h
Glucose in Feed (HPLC)	256.04	g/L
Calculated Growth Rate	0.166	h^{-1}
Key Measurement Time	21.33	h
Dry Cell Weight	8.45	g/L
Intracellular Pyruvate	0.281	mmol/L
Intracellular PEP	0.219	mmol/L
Pyruvate/PEP Ratio	1.28	
PPS Activity	0.012	U/mg
PYC Activity	0.0186	U/mg

Fermentation ALS1102- L - 2

Fermentation Date	9/30/2008
Lab Notebook	17 R
Strain	ALS1102
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	28 mL
initial volume	1 L
pH	6.8
growth rate	0.15 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.15	-1.884	97.6	2.89	0.00	0	0	8.0	8.0	1.00	8	7	8	1
2	11.8	2.50	0.917	79.2	0.00	0.36	0	4	8.0	16.0	0.99	632	639	635	5
3	14.0	6.60	1.887	65.0	0.00	0.07	13	6	6.5	22.5	1.00	1606	1484	1545	86
4	16.0	12.22	2.503	67.9	0.00	0.00	24	6	7.0	29.5	1.00	89614	131036	110325	29290
5	18.0	15.64	2.750	64.1	0.00	0.00	36	8	5.0	34.5	1.01	129063	249978	189520	85500
6	20.0	21.06	3.047	55.0	0.00	0.00	50	9	5.0	39.5	1.02	146669	135773	141221	7705
7	21.0	25.58	3.242	48.2	0.00	0.00	61	10	6.0	45.5	1.03	160964	358125	259545	139414
8	22.0	32.21	3.472	64.4	0.00	0.00	72	11	5.5	51.0	1.03	568437	335561	451999	164669
9	23.0	38.11	3.640	55.7	0.00	0.00	87	12	5.5	56.5	1.04	430033	238933	334483	135128
10	24.0	41.25	3.720	43.2	0.00	0.00	108	14	5.0	61.5	1.06	230347	393120	311733	115098
11	25.3	48.50	3.882	40.6	0.01	0.00	135	16	6.0	67.5	1.08	395733	485053	440393	63159
12	26.0	51.45	3.941	52.6	0.01	0.00	156	18	6.5	74.0	1.10	356119	503596	429857	104283

Pump Starting Time	11.92	h
Fed-Batch Starting Time	14.1	h
Induction Time	14.4	h
Glucose in Feed (HPLC)	262.14	g/L
Calculated Growth Rate	0.166	h^{-1}
Key Measurement Time	21.33	h
Dry Cell Weight	8.10	g/L
Intracellular Pyruvate	0.370	mmol/L
Intracellular PEP	0.154	mmol/L
Pyruvate/PEP Ratio	2.40	
PPS Activity		U/mg
PYC Activity	0.0112	U/mg

Fermentation ALS1108 - H - 1

Fermentation Date	11/4/2008
Lab Notebook	17 (U)
Strain	ALS1108
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	30 mL
initial volume	1 L
pH	6.8
growth rate	0.35 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C and Antiboitic

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.01	-4.804	98.2	2.68	0.00	0	0	5.0	5.0	1.00	8	5	7	2
2	4.9	3.12	1.138	98.4	0.00	0.29	0	4	8.0	13.0	0.99	33	38	35	3
3	6.0	5.26	1.659	67.1	0.00	0.12	7	4	6.5	19.5	0.99	115	93	104	16
4	7.0	8.18	2.101	66.7	0.00	0.00	12	4	7.5	27.0	0.99	2801	2512	2656	204
5	7.6	10.57	2.358	57.7	0.00	0.00	17	6	7.0	34.0	0.99	3167	4395	3781	868
6	8.1	14.22	2.654	44.2	0.00	0.00	23	7	7.0	41.0	0.99	1955	8288	5121	4478
7	8.8	18.42	2.913	49.1	0.00	0.00	33	8	6.0	47.0	0.99	4600	5326	4963	514
8	9.3	19.05	2.947	41.5	0.00	0.13	44	8	7.0	54.0	1.00	5690	13294	9492	5377
9	9.8	21.93	3.088	32.8	0.19	0.28	57	10	6.0	60.0	1.01	2868	6366	4617	2473
10	10.5	32.53	3.482	31.5	2.24	0.46	78	12	6.5	66.5	1.02	6837	5396	6116	1019
11	11.1	33.90	3.523	30.7	4.51	0.68	104	14	7.0	73.5	1.04	12643	7041	9842	3961
12	11.6	39.41	3.674	32.3	6.55	0.92	129	14	6.0	79.5	1.06	8283	8395	8339	79
13	12.3	48.77	3.887	28.6	11.57	1.34	178	20	7.5	87.0	1.11	8816	9693	9255	620
14	12.9	48.89	3.890	4.7	16.49	2.31	228	24	7.0	94.0	1.16	12421	10618	11520	1275

Pump Starting Time	4.9	h
Induction Time	6.3	h
Glucose in Feed (HPLC)	259.76	g/L
Calculated Growth Rate	0.346	h^{-1}
Key Measurement Time	8.92	h
Dry Cell Weight	7.33	g/L
Intracellular Pyruvate	0.266	mmol/L
Intracellular PEP	0.151	mmol/L
Pyruvate/PEP Ratio	1.76	
PPS Activity	0.016	U/mg
PYC Activity	0.0319	U/mg

Fermentation ALS1108 - H - 2

Fermentation Date	11/4/2008
Lab Notebook	17 (V)
Strain	ALS1108
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	30 mL
initial volume	1 L
pH	6.8
growth rate	0.35 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C and Antiboitic

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.01	-4.780	98.2	2.74	0.00	0	0	5.0	5.0	1.00	6	9	7	2
2	4.9	3.28	1.188	98.4	0.00	0.27	0	4	8.0	13.0	0.99	37	60	49	16
3	6.0	5.47	1.700	67.1	0.00	0.05	7	4	6.5	19.5	0.99	89	231	160	101
4	7.0	8.13	2.096	66.7	0.00	0.00	12	4	7.5	27.0	0.99	1067	1253	1160	132
5	7.6	10.69	2.369	57.7	0.00	0.00	17	6	7.0	34.0	0.99	3203	8041	5622	3421
6	8.1	13.84	2.627	44.2	0.00	0.00	23	7	7.0	41.0	0.99	4981	4657	4819	229
7	8.8	16.25	2.788	49.1	0.00	0.00	33	8	6.0	47.0	0.99	5165	15245	10205	7128
8	9.3	19.37	2.964	41.5	0.00	0.08	44	8	7.0	54.0	1.00	7146	6709	6927	309
9	9.8	21.90	3.086	32.8	0.14	0.22	57	10	6.0	60.0	1.01	6593	7171	6882	408
10	10.5	28.90	3.364	31.5	1.59	0.41	78	12	6.5	66.5	1.02	6993	10925	8959	2780
11	11.1	31.10	3.437	30.7	3.62	0.63	104	14	7.0	73.5	1.04	7917	14454	11185	4623
12	11.6	36.70	3.603	32.3	5.64	0.83	129	14	6.0	79.5	1.06	8840	11976	10408	2217
13	12.3	43.09	3.763	28.6	9.96	1.18	178	20	7.5	87.0	1.11	11771	11163	11467	430
14	12.9	45.77	3.824	4.7	14.38	1.61	228	24	7.0	94.0	1.16	13151	12121	12636	728

Pump Starting Time	4.9	h
Induction Time	6.3	h
Glucose in Feed (HPLC)	254.61	g/L
Calculated Growth Rate	0.328	h^{-1}
Key Measurement Time	8.92	h
Dry Cell Weight	7.36	g/L
Intracellular Pyruvate	0.245	mmol/L
Intracellular PEP	0.118	mmol/L
Pyruvate/PEP Ratio	2.08	
PPS Activity		U/mg
PYC Activity	0.0162	U/mg

Fermentation ALS1108 - L - 1

Fermentation Date	12/16/2008
Lab Notebook	25 (A)
Strain	ALS1108
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	43 mL
initial volume	1 L
pH	6.8
growth rate	0.15 h^{-1}
feed	250 g/L glucose 30g/L, $(NH_4)_2SO_4$, 2 mL 5% Antifoam C, Antibiotic

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.03	-3.576	100.0	2.72	0.00	0	0	4.5	4.5	1.00	5	7	6	1
2	7.8	2.04	0.715	89.3	0.91	0.22	0	4	6.5	11.0	0.99	81	92	86	7
3	10.1	6.59	1.886	86.5	0.00	0.00	10	4	8.0	19.0	1.00	232	254	243	16
4	11.5	9.99	2.302	80.1	0.00	0.00	19	6	6.0	25.0	1.00	6567	3617	5092	2086
5	12.5	12.23	2.504	79.3	0.00	0.00	27	8	6.5	31.5	1.00	7887	8067	7977	128
6	13.5	17.28	2.849	64.3	0.00	0.00	36	8	5.5	37.0	1.01	13774	11888	12831	1334
7	14.5	20.65	3.028	60.9	0.00	0.00	46	9	6.0	43.0	1.01	35373	36345	35859	687
8	15.5	25.97	3.257	63.6	0.00	0.00	58	10	6.0	49.0	1.02	21140	21948	21544	571
9	16.8	31.81	3.460	25.7	0.00	0.03	75	12	7.0	56.0	1.03	28023	26367	27195	1171
10	17.8	31.94	3.464	88.5	0.00	0.00	92	14	6.0	62.0	1.04	47258	31850	39554	10895
11	18.8	33.41	3.509	69.4	0.00	0.00	111	16	6.0	68.0	1.06	37660	41081	39371	2419
12	19.8	42.21	3.743	50.6	0.00	0.00	135	18	6.0	74.0	1.08	39853	43167	41510	2343
13	20.8	53.00	3.970	66.7	0.00	0.00	161	20	7.0	81.0	1.10	57680	54498	56089	2250

Pump Starting Time	8.33	h
Fed-Batch Starting Time	10.6	h
Induction Time	10.6	h
Glucose in Feed (HPLC)	260.939	g/L
Calculated Growth Rate	0.179	h^{-1}
Key Measurement Time	16.00	h
Dry Cell Weight	7.58	g/L
Intracellular Pyruvate	0.279	mmol/L
Intracellular PEP	0.165	mmol/L
Pyruvate/PEP Ratio	1.69	
PPS Activity	0.009	U/mg
PYC Activity	0.0202	U/mg

Fermentation ALS1108 - L - 2

Fermentation Date	12/16/2008
Lab Notebook	25 (B)
Strain	ALS1108
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	43 mL
initial volume	1 L
pH	6.8
growth rate	0.15 h^{-1}
feed	250 g/L glucose 30g/L, $(NH_4)_2SO_4$, 2 mL 5% Antifoam C, Antibiotic

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.03	-3.689	99.7	2.84	0.00	0	0	6.5	6.5	1.00	6	6	6	0
2	7.8	0.84	-0.178	92.0	2.16	0.10	0	2	6.0	12.5	0.99	80	87	83	5
3	10.1	3.63	1.289	76.6	2.19	0.33	10	4	5.5	18.0	1.00	241	261	251	14
4	11.5	9.58	2.260	48.1	0.00	0.51	19	6	6.0	24.0	1.00	2677	2944	2811	189
5	12.5	12.15	2.498	60.3	0.00	0.02	27	8	6.0	30.0	1.01	25258	8361	16810	11948
6	13.5	16.41	2.798	52.4	0.00	0.00	36	8	6.0	36.0	1.01	17050	16782	16916	190
7	14.5	19.74	2.982	63.2	0.00	0.00	46	10	6.0	42.0	1.01	20293	22517	21405	1572
8	15.5	22.55	3.116	47.6	0.00	0.00	58	12	6.5	48.5	1.02	30414	33986	32200	2526
9	16.8	34.53	3.542	22.0	0.00	0.00	75	14	6.5	55.0	1.03	36875	38183	37529	925
10	17.8	35.94	3.582	54.1	0.00	0.00	92	16	7.0	62.0	1.05	45074	45183	45128	77
11	18.8	41.11	3.716	48.8	0.00	0.00	111	18	6.5	68.5	1.06	59572	66217	62895	4698
12	19.8	51.60	3.944	31.1	0.00	0.17	135	20	7.5	76.0	1.08	63048	97264	80156	24194
13	20.8	39.80	3.684	13.6	0.00	0.77	161	28	7.5	83.5	1.11	47359	93226	70292	32433

Pump Starting Time	8.33	h
Fed-Batch Starting Time	10.6	h
Induction Time	10.6	h
Glucose in Feed (HPLC)	253.865	g/L
Calculated Growth Rate	0.212	h^{-1}
Key Measurement Time	16.00	h
Dry Cell Weight	7.96	g/L
Intracellular pyruvate	0.313	mmol/L
Intracellular PEP	0.213	mmol/L
Pyruvate/PEP Ratio	1.47	
PPS Activity		U/mg
PYC Activity	0.0221	U/mg

Fermentation ALS1109 - H - 1

Fermentation Date	4/14/2008
Lab Notebook	25 (G)
Strain	ALS1109
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	47 mL
initial volume	1 L
pH	6.8
growth rate	0.35 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C and Antiboitic

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.13	-2.048	99.9	2.72	0.00	0	0	4.5	4.5	1.00	5	5	5	0
2	4.7	2.60	0.955	83.7	0.36	0.22	0	1	6.0	10.5	0.99	79	39	59	28
3	5.9	5.13	1.635	72.9	0.00	0.18	8	4	5.5	16.0	1.00	117	81	99	25
4	6.5	7.66	2.036	63.7	0.00	0.09	14	6	6.0	22.0	1.00	422	181	301	171
5	7.0	9.91	2.294	65.5	0.00	0.00	20	6	5.0	27.0	1.00	1768	1916	1842	104
6	7.4	11.76	2.464	51.4	0.00	0.00	26	8	5.5	32.5	1.00	6709	3867	5288	2009
7	8.0	15.10	2.715	31.7	0.00	0.00	35	8	4.5	37.0	1.01	28098	15259	21678	9079
8	8.5	16.45	2.800	28.9	0.00	0.00	46	10	5.0	42.0	1.01	42918	48596	45757	4015
9	9.1	22.46	3.112	22.4	0.00	0.00	60	12	4.0	46.0	1.03	30084	25006	27545	3591
10	9.6	26.90	3.292	29.1	0.00	0.00	75	14	4.0	50.0	1.04	40583	27200	33892	9463
11	10.3	30.30	3.411	19.5	0.00	0.05	99	16	5.0	55.0	1.06	45173	35551	40362	6804
12	10.8	38.50	3.651	20.1	0.00	0.06	121	18	4.5	59.5	1.08	29050	35952	32501	4880
13	11.4	45.00	3.807	7.8	0.00	0.22	157	22	4.0	63.5	1.12	25188	27497	26343	1633
14	12.0	50.05	3.913	2.1	0.00	1.01	197	14	4.5	59.5	1.15	34370	27190	30780	5077

Pump Starting Time	4.8	h
Induction Time	6.6	h
Glucose in Feed (HPLC)	249.35	g/L
Calculated Growth Rate	0.363	h^{-1}
Key Measurement Time	9.67	h
Dry Cell Weight	9.69	g/L
Intracellular Pyruvate	0.234	mmol/L
Intracellular PEP	0.197	mmol/L
Pyruvate/PEP Ratio	1.19	
PPS Activity	0.076	U/mg

Fermentation ALS1109 - H - 2

Fermentation Date	4/14/2008
Lab Notebook	25 (H)
Strain	ALS1109
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	47 mL
initial volume	1 L
pH	6.8
growth rate	0.35 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C and Antiboitic

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.13	-2.010	96.6	2.65	0.00	0	0	5.5	5.5	1.00	5	6	6	0
2	4.7	2.64	0.972	83.4	0.53	0.20	0	2	6.0	11.5	0.99	63	42	53	14
3	5.9	5.26	1.660	74.8	0.00	0.16	8	4	5.5	17.0	1.00	470	204	337	188
4	6.5	7.33	1.991	71.0	0.00	0.08	14	6	6.0	23.0	1.00	271	229	250	30
5	7.0	9.59	2.260	56.6	0.00	0.00	20	6	5.0	28.0	1.00	1444	1757	1601	222
6	7.4	12.02	2.486	36.1	0.00	0.01	26	6	5.5	33.5	1.00	3267	3999	3633	518
7	8.0	15.41	2.735	34.2	0.00	0.00	35	8	4.5	38.0	1.01	15447	5709	10578	6886
8	8.5	16.65	2.812	40.1	0.00	0.00	46	9	5.5	43.5	1.01	27526	11385	19455	11413
9	9.1	21.48	3.067	72.5	0.00	0.00	60	12	4.5	48.0	1.02	37364	15124	26244	15727
10	9.6	28.69	3.357	41.5	0.00	0.02	75	13	5.0	53.0	1.04	25993	21614	23804	3097
11	10.3	33.25	3.504	31.2	0.00	0.09	99	16	5.0	58.0	1.06	34035	19766	26900	10090
12	10.8	38.30	3.645	10.8	0.00	0.46	121	18	4.0	62.0	1.08	16652	21424	19038	3374
13	11.4	41.32	3.721	8.1	0.00	2.26	157	24	4.5	66.5	1.11	16089	19618	17854	2495
14	12.0	48.11	3.873	7.9	1.38	3.95	197	28	6.0	64.0	1.16	16545	18998	17772	1734

Pump Starting Time	4.8	h
Induction Time	6.6	h
Glucose in Feed (HPLC)	284.82	g/L
Calculated Growth Rate	0.360	h^{-1}
Key Measurement Time	9.67	h
Dry Cell Weight	9.65	g/L
Intracellular Pyruvate	0.171	mmol/L
Intracellular PEP	0.218	mmol/L
Pyruvate/PEP Ratio	0.78	
PPS Activity	0.051	U/mg

Fermentation ALS1109 - L - 1

Fermentation Date	4/29/2009
Lab Notebook	25 (I)
Strain	ALS1109
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	38 mL
initial volume	1 L
pH	6.8
growth rate	0.15 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C and Antiboitic

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.15	-1.877	100.6	2.85	0.00	0	0	4.5	4.5	1.00	5	3	4	2
2	8.8	3.25	1.180	96.2	0.00	0.30	0	2	6.5	11.0	0.99	152	111	132	29
3	10.8	7.68	2.039	81.2	0.00	0.00	12	4	5.0	16.0	1.00	864	296	580	402
4	11.8	9.68	2.270	79.8	0.00	0.00	19	5	6.0	22.0	1.00	1584	4170	2877	1829
5	12.8	12.34	2.512	72.7	0.00	0.00	26	6	6.0	28.0	1.00	21902	10824	16363	7833
6	13.8	15.01	2.708	84.0	0.00	0.00	35	7	5.5	33.5	1.01	105493	26485	65989	55867
7	14.8	17.04	2.835	72.3	0.00	0.00	46	8	5.0	38.5	1.02	75043	32937	53990	29773
8	15.8	20.64	3.027	67.1	0.00	0.00	58	10	5.5	44.0	1.02	91843	61019	76431	21795
9	16.8	26.83	3.290	68.5	0.00	0.00	71	12	5.5	49.5	1.03	182088	69319	125703	79739
10	18.0	31.69	3.456	57.3	0.00	0.00	91	14	5.5	55.0	1.05	113270	77867	95569	25034
11	19.0	35.48	3.569	53.4	0.00	0.00	110	15	5.0	60.0	1.07	118807	88293	103550	21576
12	20.0	45.06	3.808	32.2	0.00	0.00	132	17	4.5	64.5	1.08	136587	115550	126068	14875
13	21.0	47.12	3.853	31.0	0.00	0.00	165	20	5.0	69.5	1.12	178747	114082	146414	45725
14	21.8	55.77	4.021	22.6	0.00	0.01	179	22	5.5	65.5	1.14	247981	115945	181963	93364

Pump Starting Time	8.8	h
Induction Time	10.8	h
Glucose in Feed (HPLC)	260.10	g/L
Calculated Growth Rate	0.177	h^{-1}
Key Measurement Time	17.75	h
Dry Cell Weight	9.13	g/L
Intracellular Pyruvate	0.308	mmol/L
Intracellular PEP	0.242	mmol/L
Pyruvate/PEP Ratio	1.27	
PPS Activity	0.062	U/mg

Fermentation ALS1109 - L - 2

Fermentation Date	4/29/2009
Lab Notebook	25 (J)
Strain	ALS1109
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	38 mL
initial volume	1 L
pH	6.8
growth rate	0.15 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C and Antiboitic

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.10	-2.313	99.7	2.84	0.00	0	0	6.0	6.0	1.00	5	4	4	1
2	8.8	1.76	0.564	95.7	0.91	0.25	0	3	7.5	13.5	0.99	150	120	135	21
3	10.8	7.32	1.991	87.6	0.00	0.40	12	7	6.5	20.0	1.00	539	431	485	76
4	11.8	9.50	2.251	87.3	0.00	0.02	19	8	6.0	26.0	1.00	22141	6843	14492	10817
5	12.8	12.07	2.490	91.7	0.00	0.00	26	8	5.5	31.5	1.00	35944	23539	29741	8772
6	13.8	14.59	2.680	89.3	0.00	0.00	35	9	5.0	36.5	1.01	44387	35840	40113	6043
7	14.8	17.44	2.859	81.3	0.00	0.00	46	9	5.0	41.5	1.01	67613	48963	58288	13188
8	15.8	22.42	3.110	67.0	0.00	0.00	58	11	6.0	47.5	1.02	64179	88895	76537	17477
9	16.8	24.25	3.188	70.5	0.00	0.00	71	13	5.0	52.5	1.03	77358	105642	91500	19999
10	18.0	31.78	3.459	69.3	0.00	0.00	91	15	6.0	58.5	1.05	91774	118724	105249	19057
11	19.0	38.58	3.653	61.6	0.00	0.00	110	16	5.5	64.0	1.06	271662	135333	203498	96399
12	20.0	41.79	3.733	56.2	0.00	0.00	132	19	5.0	69.0	1.08	109138	241459	175298	93565
13	21.0	50.44	3.921	51.0	0.00	0.00	165	21	5.0	74.0	1.11	266976	146462	206719	85216
14	21.8	61.16	4.113	54.9	0.00	0.00	179	21	6.5	70.5	1.13	120602	186491	153546	46590

Pump Starting Time	8.8	h
Induction Time	10.8	h
Glucose in Feed (HPLC)	275.50	g/L
Calculated Growth Rate	0.184	h^{-1}
Key Measurement Time	17.75	h
Dry Cell Weight	8.64	g/L
Intracellular Pyruvate	0.252	mmol/L
Intracellular PEP	0.216	mmol/L
Pyruvate/PEP Ratio	1.17	
PPS Activity	0.043	U/mg

Fermentation ALS1110 - H - 1

Fermentation Date	3/4/2008
Lab Notebook	25 C
Strain	ALS1110
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	32 mL
initial volume	1 L
pH	6.8
growth rate	0.35 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C and Antiboitic

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.19	-1.671	101.9	2.65	0.00	0	0	5.0	5.0	1.00	8	10	9	1
2	4.5	3.10	1.130	84.6	0.28	0.31	0	2	6.0	11.0	0.99	67	89	78	15
3	5.8	5.81	1.759	68.3	0.00	0.22	9	4	5.5	16.5	1.00	182	153	168	21
4	6.5	6.40	1.857	60.9	0.00	0.00	18	4	6.0	22.5	1.00	2341	2217	2279	88
5	7.1	11.01	2.398	48.8	0.00	0.00	25	5	5.5	28.0	1.00	5857	15902	10880	7102
6	7.8	14.48	2.673	30.6	0.00	0.00	36	5	6.5	34.5	1.01	10170	14898	12534	3343
7	8.3	17.19	2.844	29.1	0.00	0.00	47	6	5.5	40.0	1.01	23905	12128	18016	8328
8	9.1	20.44	3.017	42.9	0.00	0.00	67	8	5.5	45.5	1.03	15971	13959	14965	1423
9	9.8	27.36	3.309	29.8	0.00	0.00	89	10	5.5	51.0	1.05	16108	39538	27823	16568
10	10.4	32.96	3.495	27.8	0.00	0.06	115	12	6.5	57.5	1.07	34662	44042	39352	6632
11	11.0	42.79	3.756	27.0	0.00	0.10	146	16	6.0	63.5	1.10	40455	33577	37016	4864
12	11.5	51.12	3.934	2.7	0.00	0.30	179	20	5.0	68.5	1.13	42048	27510	34779	10280

Pump Starting Time	4.6	h
Induction Time	6.0	h
Glucose in Feed (HPLC)	286.22	g/L
Calculated Growth Rate	0.392	h^{-1}
Key Measurement Time	9.83	h
Dry Cell Weight	10.99	g/L
Intracellular Pyruvate	0.224	mmol/L
Intracellular PEP	0.2	mmol/L
Pyruvate/PEP Ratio	1.12	
PPS Activity	0.040	U/mg
PYC Activity		U/mg

Fermentation ALS1110 - H - 2

Fermentation Date	3/4/2008
Lab Notebook	25 (D)
Strain	ALS1110
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	32 mL
initial volume	1 L
pH	6.8
growth rate	0.35 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C and Antiboitic

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.21	-1.556	98.9	2.94	0.00	0	0	5.0	5.0	1.00	10	9	10	1
2	4.5	3.04	1.110	86.1	0.43	0.27	0	3	6.0	11.0	0.99	71	34	52	26
3	5.8	6.07	1.803	75.5	0.00	0.31	9	5	6.5	17.5	1.00	114	21	67	65
4	6.5	7.13	1.965	64.0	0.00	0.13	18	5	7.0	24.5	1.00	2953	4188	3571	873
5	7.1	12.19	2.500	62.0	0.00	0.00	25	5	5.5	30.0	1.00	5476	5079	5277	280
6	7.8	15.60	2.747	74.5	0.00	0.00	36	7	6.0	36.0	1.01	16046	15820	15933	159
7	8.3	17.57	2.866	34.7	0.00	0.00	47	8	6.0	42.0	1.01	13405	27295	20350	9822
8	9.1	22.29	3.104	55.5	0.00	0.01	67	9	6.5	48.5	1.03	22960	30114	26537	5059
9	9.8	28.88	3.363	41.6	0.00	0.03	89	12	5.5	54.0	1.05	13331	17463	15397	2921
10	10.4	38.65	3.655	34.2	0.00	0.09	115	15	6.5	60.5	1.07	17904	36283	27094	12996
11	11.0	49.11	3.894	30.6	0.00	0.20	146	17	6.0	66.5	1.10	20308	13683	16995	4685
12	11.5	62.22	4.131	10.0	0.00	0.46	179	21	5.0	71.5	1.13	16326	27701	22013	8044

Pump Starting Time	4.6	h
Induction Time	6.0	h
Glucose in Feed (HPLC)	289.22	g/L
Calculated Growth Rate	0.413	h^{-1}
Key Measurement Time	9.83	h
Dry Cell Weight	11.49	g/L
Intracellular Pyruvate	0.199	mmol/L
Intracellular PEP	0.197	mmol/L
Pyruvate/PEP Ratio	1.01	
PPS Activity	0.072	U/mg
PYC Activity		U/mg

Fermentation ALS1110 - L - 1

Fermentation Date	3/30/2008
Lab Notebook	25 (E)
Strain	ALS1110
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	47 mL
initial volume	1 L
pH	6.8
growth rate	0.15 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C and Antiboitic

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.13	-2.056	99.3	2.96	0.00	0	0	4.5	4.5	1.00	10	18	14	5
2	4.4	2.37	0.862	85.7	0.89	0.44	0	4	6.0	10.5	0.99	132	181	156	34
3	7.0	7.74	2.047	78.8	0.00	0.00	13	8	6.0	16.5	1.00	494	832	663	240
4	8.3	10.01	2.304	76.3	0.00	0.00	22	8	6.0	22.5	1.01	11232	16605	13919	3799
5	9.5	14.02	2.640	73.1	0.00	0.00	32	9	6.5	29.0	1.01	23025	41229	32127	12872
6	10.5	16.69	2.815	66.1	0.00	0.00	42	10	6.0	35.0	1.02	27967	46428	37197	13053
7	11.5	20.02	2.996	60.6	0.00	0.04	53	12	6.5	41.5	1.02	43138	53368	48253	7233
8	12.5	25.48	3.238	49.6	0.00	0.00	66	13	6.0	47.5	1.03	53658	81226	67442	19493
9	13.6	32.09	3.469	49.5	0.00	0.00	81	14	5.5	53.0	1.04	63991	70778	67384	4799
10	14.8	35.99	3.583	20.2	0.00	0.00	102	18	5.5	58.5	1.06	87471	71400	79436	11364
11	15.8	40.42	3.699	42.3	0.00	0.02	122	18	7.0	65.5	1.07	79333	88453	83893	6448
12	16.8	43.03	3.762	31.4	0.00	0.02	145	22	5.5	71.0	1.10	88388	90183	89286	1269
13	18.0	54.02	3.989	20.2	0.00	0.13	181	24	6.0	77.0	1.13	85965	86644	86305	481
14	19.3	52.70	3.965	30.1	0.00	0.28	222	28	5.5	71.0	1.18	82117	74891	78504	5110

Pump Starting Time	4.8	h
Induction Time	6.9	h
Glucose in Feed (HPLC)	239.80	g/L
Calculated Growth Rate	0.163	h^{-1}
Key Measurement Time	12.75	h
Dry Cell Weight	8.64	g/L
Intracellular Pyruvate	0.173	mmol/L
Intracellular PEP	0.2	mmol/L
Pyruvate/PEP Ratio	0.87	
PPS Activity	0.085	U/mg

Fermentation ALS1110 - L - 2

Fermentation Date	3/30/2008
Lab Notebook	25 (F)
Strain	ALS1110
Mode	Fed-batch
Medium	Park Medium 3.0 g/L Glucose 4.0 g/L $(NH_4)_2HPO_4$
IPTG	0.5 mM
volume inoculum	47 mL
initial volume	1 L
pH	6.8
growth rate	0.15 h^{-1}
feed	250 g/L glucose 30g/L $(NH_4)_2SO_4$ 2 mL 5% Antifoam C and Antiboitic

Sample	Time (h)	OD	ln(OD)	DO%	Glucose (g/L)	Acetate (g/L)	Feed (mL)	Base (mL)	Sample (mL)	Acc Sample (mL)	Volume (L)	Activity 1 (U/L)	Activity 2 (U/L)	Mean (U/L)	Std Dev (U/L)
1	0.1	0.17	-1.796	98.8	2.52	0.07	0	0	5.0	5.0	1.00	16	15	16	1
2	4.4	2.30	0.835	86.8	0.72	0.26	0	2	6.5	11.5	0.99	191	175	183	11
3	7.0	7.50	2.015	8/.2	0.00	0.02	13	4	6.0	17.5	1.00	881	835	858	32
4	8.3	10.00	2.303	68.7	0.00	0.10	22	5	6.0	23.5	1.00	18883	19346	19115	327
5	9.5	13.97	2.637	61.5	0.00	0.00	32	6	6.5	30.0	1.01	91525	39062	65294	37097
6	10.5	17.22	2.846	62.5	0.00	0.09	42	7	5.5	35.5	1.01	129471	93715	111593	25283
7	11.5	19.92	2.991	59.2	0.00	0.00	53	8	6.0	41.5	1.02	94952	95900	95426	670
8	12.5	27.50	3.314	46.6	0.00	0.09	66	9	6.0	47.5	1.03	127677	155983	141830	20016
9	13.6	29.99	3.401	33.3	0.00	0.00	81	11	6.0	53.5	1.04	160081	188650	174365	20201
10	14.8	31.63	3.454	36.4	0.00	0.32	102	13	5.5	59.0	1.06	136889	146708	141799	6943
11	15.8	40.67	3.705	53.9	0.00	0.00	122	15	6.0	65.0	1.07	122493	113219	117856	6558
12	16.8	45.76	3.823	35.2	0.00	0.00	145	16	6.0	71.0	1.09	82331	128904	105618	32932
13	18.0	48.80	3.888	31.8	0.00	0.04	181	21	5.5	76.5	1.13	96237	93270	94753	2098
14	19.3	50.89	3.930	2.3	0.00	0.00	222	25	5.5	70.5	1.18	85711	86906	86309	845

Pump Starting Time	4.8	h
Induction Time	6.9	h
Glucose in Feed (HPLC)	263.02	g/L
Calculated Growth Rate	0.159	h^{-1}
Key Measurement Time	12.75	h
Dry Cell Weight	8.54	g/L
Intracellular Pyruvate	0.175	mmol/L
Intracellular PEP	0.185	mmol/L
Pyruvate/PEP Ratio	0.95	
PPS Activity	0.072	U/mg