METABOLIC ENGINEERING OF SUGAR PATHWAYS IN *ESCHERICHIA COLI*

by

TIAN XIA

(Under the Direction of Mark A. Eiteman)

ABSTRACT

The primary goal of this research is to engineer sugar pathways in *Escherichia coli* and generate high amounts of particular pathway intermediates for the production of desired products. A consortium of two strains of *Escherichia coli* was used to convert a mixture of xylose and glucose to succinate in a dual phase aerobic/anaerobic process. By implementing a consortium approach for the conversion of sugar mixtures, the pathway for each sugar-to-product conversion was able to be optimized independently. The approach eliminated carbon catabolite repression and is flexible to the utilization of feedstocks with variable sugar compositions. *Escherichia coli* unable to metabolize D-glucose accumulates a small amount of D-glucose from D-xylose or L-arabinose: deletion of genes in glycolysis and pentose phosphate pathway increased accumulation of D-glucose. The results establish a direct link between pentoses and hexoses, and provide a novel strategy to increase carbon backbone length from five to six carbons by directing flux through the pentose phosphate pathway. Finally, deletion of phosphoglucose isomerase in the glycolytic pathway successfully diverted the carbon flux toward sugar donor UDP-glucose accumulation to glycosylate quercetin.

INDEX WORDS: metabolic engineering, pathway, succinic acid, xylose, lignocellulosic hydrolysate, pentose phosphate pathway, 6-phosphofructokinase, phosphoglucose isomerase, glycosylation, glucose-6-phosphate 1-dehydrogenase

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DEDICATION

To my family

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Central carbon metabolism is critical for metabolic engineering as it is tightly connected with overall cell function (Nielsen, 2011a). Among various bacteria, metabolic engineering of the central carbon metabolism has been most extensively studied in *Escherichia coli*. Metabolic networks in *Escherichia coli* comprise hundreds of metabolites that are interconnected through a large number of biochemical and regulatory reactions (Perrenoud and Sauer, 2005). These metabolites are involved in the degradation of nutrients and biosynthesis of cellular constituents such as proteins, lipids, carbohydrates, DNA and RNA. The cellular constituents are formed by polymerization of key building blocks such as amino acids, fatty acids, nucleotides, and sugar moieties. Amazingly, all of the more than 50 building blocks are formed from essentially 12 precursor metabolites including glucose 6-phosphate, fructose 6-phosphate, ribose 5-phosphate, erythrose 4-phosphate, glyceraldehyde 3-phosphate, 3-phosphoglyceric acid, phosphoenolpyruvate, pyruvate, oxaloacetate, acetyl-CoA, α-ketoglutarate, and succinyl-CoA, which are each themselves generated in central carbon metabolism from the available carbon sources (Varma and Palsson, 1993; Nielsen, 2003). The biosynthesis of these 12 precursor metabolites is remarkably conserved among living organisms because these precursors form all organic chemicals found in nature (Nielsen, 2011b). Central carbon metabolism not only generates the 12 precursor metabolites, but also captures the Gibbs free energy (primarily ATP), and electron acceptors/donors (primarily NADH and

NADPH), that are required for biosynthesis of building blocks and macromolecules. In *Escherichia coli* and most heterotrophic bacteria, the main pathways of the central carbon metabolism consists of the phosphotransferase system (PTS), glycolytic pathway, pentose phosphate (PP) pathway, gluconeogenesis and the tricarboxylic acid cycle (TCA) with the glyoxylate bypass. Knowledge about the regulation of central carbon metabolism allows the engineering of selected metabolic pathways to redirect carbon fluxes toward precursors for industrially important metabolites (Keasling, 2010; Nielsen, 2011b). Efforts have been made to increase metabolite production by metabolic engineering of *E. coli* sugar pathways, especially glycolysis and glyconeogenesis.

Glucose is the most widely utilized carbon source in bioprocesses with *E. coli*. *E. coli* metabolizes glucose by the Embden-Meyerhof-Parnas pathway to produce numerous intermediary metabolites and energy. First, the phosphoenolpyruvate:sugar phosphotransferase system (PTS) internalizes glucose into the cytoplasm and phosphorylates glucose. The soluble and nonsugar-specific protein components Enzyme I (EI) and the phosphor histidine carrier protein (HPr) relay a phosphoryl group from phosphoenolpyruvate (PEP) to the glucose-specific enzymes IIA and integral membrane permease IIBC (Stock et al., 1982; Misset et al., 1983). The dependence of PTS on PEP makes PTS important in determining the PEP/PYR ratio and carbon flux distribution. Under both aerobic and anaerobic conditions, glucose is converted into pyruvate by a sequence of 10 enzymatic reactions. The first four consecutive reactions split the glucose molecule (C6) into two phosphoglyceraldehyde (C3) molecules. The subsequent five consecutive reactions oxidize the phosphoglyceraldehyde to pyruvate, with a net yield of two ATP molecules. Flux control may be broadly distributed among most of the enzymes

in the glycolytic pathway. For instance, pyruvate kinase (PykF) is activated by fructose1,6-bisphosphate, which enables it to keep pace with high incoming flux, while PykF is allosterically inhibited by ATP to reduce flux when the energy state is high. Phosphofructokinase (Pfk) is inhibited by ATP, but the inhibitory effect is reversed by ADP and other phosphonucleosides (Blangly et al., 1968; Fenton et al., 2003). Changes in PEP concentration also affect glycolytic and TCA cycle flux in *E. coli* (Yang et al., 2003; Peng et al., 2004). PEP acts as an inhibitor of the enzymes of the initial reactions of glycolysis. It inhibits glucokinase, phosphoglucoisomerase (Pgi), Pfk and aldolase (Fba) (Ogawa et al., 2007). The internal redox state also plays a central role in the allosteric regulation of glycolysis. For example, the flow through glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is regulated by the NADH/NAD ratio (Garrigues et al., 1997). Moreover, NADH is a competitive inhibitor of GAPDH (Zhu and Shimizu, 2005).

The pentose phosphate pathway (PPP) is an alternative route for glucose degradation and enables *E. coli* to utilize pentoses such as D-xylose and L-arabinose, which are primary monosaccharide components in lignocellulosic biomass. PPP is the only pathway by which these sugars can be catabolized (Fraenkel, 1987; Lin, 1996). PPP is composed of an oxidative branch and a nonoxidative branch. The oxidative branch converts glucose-6-phosphate to 6-phosphogluconate, generating NADPH as the reducing equivalent for biosynthesis and ribulose-5-phosphate as the precursor for nucleic acids. The nonoxidative branch produces fructose-6-phosphate, glyceraldehyde-3-P, and erythrose-4-P, interconnecting the glycolytic and aromatic biosynthetic pathways with PPP. The PPP provides intermediates to the cell for the biosynthesis of amino acids, vitamins, nucleotides and cell wall constituents. To enter the PPP, xylose

can be transported by two different systems of inducible permeases. One is a high affinity ABC transporter (encoded by *xylFGH*) system (K_M= 0.2 – 4.0 μM for xylose) driven by ATP. The other is a low affinity xylose-proton symport system (K_M= 63 – 169 μM for xylose) encoded by *xylE* gene (Sumiya et al., 1995). After being internalized, xylose is converted to D-xylulose and phosphorylated to D-xylulose-5-P by xylose isomerase (*xylA*) and xylulosekinase (*xylB*) (Gonzalez et al., 2002). L-Arabinose is also transported by two inducible systems in *E. coli* K12. The low-affinity permease (*araE*) (K_M = 100 μM) is energized by proton motive force (Lin, 1996). The high affinity system (*araFG*) (K_M = 1 – 3 μM) is energized by a high-energy covalent bond (Macpherson et al., 1981). L-Arabinose is sequentially metabolized by L-arabinose isomerase (AraA), which converts arabinose to ribulose; ribulokinase (AraB), which converts ribulose to ribulose-5-phosphate; and L-ribulose-5-phosphate-4-epimerase (AraD), which converts ribulose-5-phosphate to xylulose-5-phosphate (Lin, 1996). Metabolic flow through the pentose phosphate pathway is controlled mainly by the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which depends on the energy and redox potential of the cell. ATP, NADH, and NADPH repress these enzymes. Most of the enzymes in the pentose phosphate pathway of *E. coli* are repressed by their final products, i.e., glucose-6-phosphate, fructose-6-phosphate, ribose-5-phosphate, glycerdehyde-3-phosphate, and ribulose-5-phosphate (Sprenger, 1995; Ratushny et al., 2006).

A key problem in the metabolism of more than one sugar is carbon-catabolite repression (CCR). From the perspective of evolutionary outcome, CCR facilitates microorganisms to survive and dominate in ever-changing nutrient conditions. The

universal principle of CCR in all microbes is that the cells choose the most energy efficient cognate substrate as the most preferred carbon source, which help optimize the total macromolecular content of a cell and allow faster growth in unpredictable conditions (Beg et al., 2007; Portnoy et al., 2011). Many microorganisms, such as *E. coli*, prefer glucose as a primary carbon source. However, the preferred cognate substrate differs among different organisms. For example, rather than glucose and cellobiose, cellodextrins are the preferred carbon source for *Clostridium thermocellum* (Zhang et al., 2005). The most common mechanism for CCR is global regulation. Four components play the central role in the global regulation mechanism. They are the signal metabolite cyclic AMP (cAMP), the transcription activator CRP (cAMP receptor protein), adenylate cyclase (Cya) and the IIA component of the glucose-specific PTS ($EIIA^{Glc}$; also called catabolite repression resistance or Crr). When *E. coli* grows rapidly by metabolizing carbon sources such as glucose, the phosphate on $EIIA^{Glc}$ would be drained towards the sugars, which means $EIIA^{Glc}$ is preferentially dephosphorylated (Hogema et al., 1998; Bettenbrock et al., 2007). Whereas when glucose is absent, the phosphorylated form of $EIIA^{Glc}$ activates adenylate cyclase activity (Park et al., 2006). Adenylate cyclase synthesizes cAMP, which binds CRP, its receptor protein, and the cAMP–CRP complex activates the promoters of many catabolic genes and operons. The promoters have to be activated to facilitate the binding of RNA polymerase or the formation of the open transcription complex (Malan et al., 1984; Tagami and Aiba, 1998; Busby and Ebright, 1999). The cAMP-CRP complex also mediates CCR of non-protein-coding genes, such as regulatory RNAs (sRNA) Spot 42, CyaR, and SgrS 31,32 (Gorke and Vogel, 2008). For example, CRP represses the *spf* gene encoding the Spot 42 (base-pairing sRNA), that

pairs with *galK* mRNA and suppresses the expression of the galactose catabolic (*gal*) operon (Beisel and Storz, 2011). The PEP to pyruvate ratio actually controls $EIIA^{Glc}$ phosphorylation. If the concentration ratio between PEP and pyruvate is high, $EIIA^{Glc}$ exist mainly as phosphorylated form. In contrast, if the concentration ratio between PEP and pyruvate is low, then $EIIA^{Glc}$ is predominantly dephosphorylated (Hogema et al., 1998; Bettenbrock et al., 2007). Besides global regulation, there are also operon-specific mechanisms for CCR. Inducer exclusion is the main reason for the glucose–lactose diauxie in *E. coli* (Inada et al., 1996). Specifically, cAMP levels during growth on glucose or lactose are similarly low (Inada et al., 1996), and the low amounts of cAMP are essentially sufficient for the *lac* operon to be expressed. The *E. coli* needs to metabolize some lactose to generate allolactose (a lactose isomer formed by β‑galactosidase), which binds and inactivates the *lac* repressor, allowing the expression of *lac* operon. However, the presence of glucose inactivates the lactose permease, LacY (Winkler and Wilson, 1967). In the absence of glucose, predominantly phosphorylated $EIIA^{Glc}$ does not interact with LacY, whereas in the presence of glucose, non-phosphorylated $EIIA^{Glc}$ binds LacY to form a complex so that LacY is inactivated (Nelson et al., 1983; Hogema et al., 1999). The same inducer exclusion mechanism also applies to the transport of other secondary carbon sources, such as maltose, melibiose, raffinose, maltose, glycerol and galactose (Misko et al., 1987; Postma *et al*., 1993; Titgemeyer et al., 1994). Unphosphorylated $EIIA^{Glc}$ can bind to a number of enzymes involved in the metabolism of non-PTS carbon sources and thereby inhibits the utilization of these carbon sources. $EIIA^{Glc}$ plays an important role in both global regulation and inducer exclusion mechanisms of CCR.

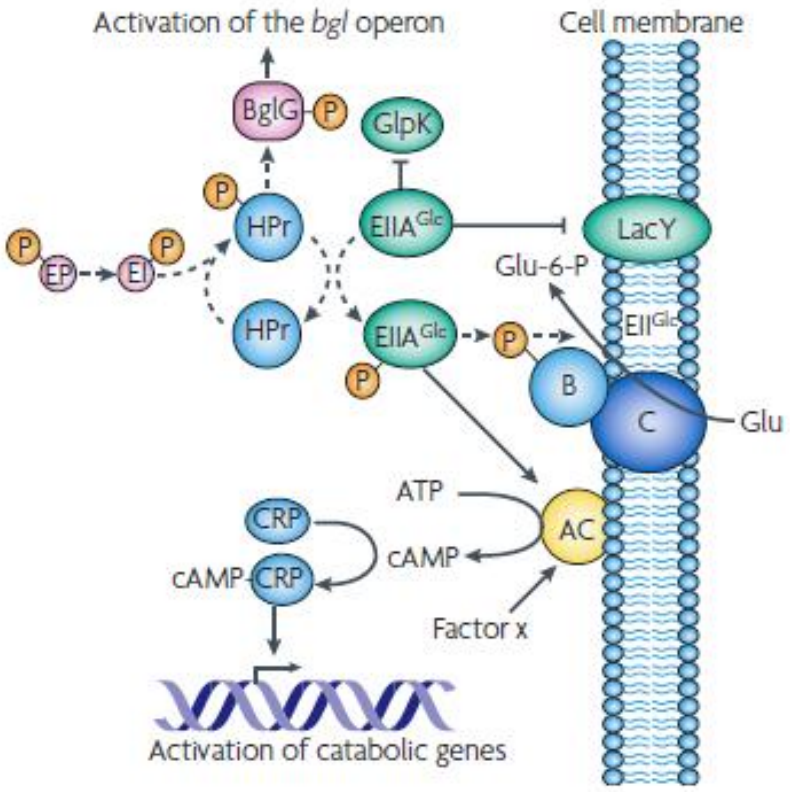

Figure 1.1 Carbon catabolite repression (CCR) in *Escherichia coli*. (Adapted from Görke and Stülke, 2008).

Metabolic engineering of glucose pathway could contribute to eliminating CCR. In industrial bioprocesses, eliminating CCR would be advantageous to reduce operating time and increase productivity. In particular, hydrolysis of lignocellulose, which is the most abundant source of renewable energy, yields a sugar mixture containing mainly glucose, arabinose and xylose (Sheehan et al., 1999). CCR causes *E. coli* and other microbes to consume this mixture of sugars sequentially. In order to utilize all sugars in a mixture simultaneously, CCR could be disrupted by inactivating PTS components. *E. coli* strains with inactivated PTS enzyme I, HPr, and IIA^{Glc} (PTS^{-} $Glucose^{-}$ phenotype) have

been evolved using an aerobic continuous culture selection process to generate strains that are able to grow on glucose at a lower growth rate (μ=0.4 h^{-1}) compared to wild-type (μ=0.7 h^{-1}) under aerobic conditions (Flores, 1996). A PTS^- $Glucose^+$ strain is capable of simultaneously consuming glucose and other carbon sources like arabinose, but glucose still exerts a partial repressive effect on xylose consumption (Hernández-Montalvo et al., 2001; Flores et al., 2005a; Flores et al., 2005b). Another *E. coli* PTS^- $Glucose^+$ strain with inactivated enzyme I, HPr, and IIA^{Glc} has been evolved by continuous culture under anaerobic conditions, and is able to co-metabolize glucose and xylose anaerobically and produce ethanol (Balderas-Hernández et al., 2011). The inactivation of $IIBC^{Glc}$ (*ptsG)* also has an important impact on eliminating CCR. In minimal medium containing 2 g/L each of either glucose-arabinose mixture or glucose-xylose mixture, a wild type strain utilized glucose and pentose sequentially, whereas the *E. coli ptsG* mutant consumed these sugars simultaneously in about half the time compared to the wild type strain (Nichols et al., 2001). *E. coli* strains constructed for the production of lactic acid were cultured in medium containing 50 g/L each of glucose and xylose, where a *ptsG* mutant utilized 75% of the xylose but the isogenic $ptsG^+$ strains only consumed 18–20% of xylose (Dien et al., 2002). Directed evolution on L-arabinose combined with a *ptsG* mutation led to an *E. coli* MC4100 derivative able to consume simultaneously glucose, xylose and arabinose (Jarmander et al., 2014). Deletion of *ptsG* gene together with overexpression of the endogenous *xylR* gene, which activates the *xyl* operon as a transcriptional enhancer (Ni et al., 2013), rendered engineered *E. coli* able to utilize glucose and xylose simultaneously. Apart from cellular level engineering, CCR can also be regulated at reactor level. For example, *Candida shehatae* cells consume D-xylose

approximately three times more slowly than D-glucose on a 50% D-glucose:50% D-xylose mixture. A two-phase process of rapid aerobic growth on D-xylose to high cell densities then add D-glucose in ethanol production phase increased D-xylose utilization, reduced process time, and increased final ethanol levels. In separate studies, CCR on xylose and maltose was eliminated by keeping glucose levels below 25 g/L in lactic acid fermentation with *Enterococcus mundtii* (Abdel-Rahman et al., 2015) and *Enterococcus faecalis* (Yun et al., 2001).

Fast sugar uptake can also cause overflow metabolism, a metabolic imbalance that exists in both eukaryotic cells and prokaryotic cells. High glycolytic fluxes in *Saccharomyces cerevisiae* lead to the formation of byproducts ethanol and glycerol (De Deken, 1966; Fiechter et al., 1981; Postma et al., 1989; van Hoek et al., 1998). In *E. coli*, the formation of acetate is the consequence of overflow metabolism. Although typically considered a fermentation product when *E. coli* grows under oxygen-limited conditions, *E. coli* also synthesizes a significant amount of acetate under aerobic conditions (Majewski and Domach, 1990; Han et al., 1992). In *E. coli*, pyruvate formate lyase (Pfl) catalyzes the conversion of pyruvate and coenzyme A (CoA) into acetyl-CoA (AcCoA) and formate. Acetate is generated from acetyl-CoA (AcCoA) via phosphotransacetylase (Pta) and acetate kinase (Ack). Another pathway is from pyruvate via pyruvate oxidase (Pox) (Wolfe, 2005). Acetate can be metabolized back to AcCoA by either the reversed reactions of Pta-Ack or by acetyl-CoA synthetase (ACS). With high glycolytic flux, the rate of AcCoA synthesis surpasses the capacity of the tricarboxylic acid (TCA) cycle, and part of the excess AcCoA is diverted to generate acetate (Delgado and Liao, 1997). The TCA cycle is repressed by several global regulators (Ishii et al., 2007; Valgepea et al.,

2010; Yao et al., 2011). As the glycolytic flux or the specific glucose consumption rate increases, and fructose 1,6-bisphosphate (FBP) concentration increases (Schaub and Reuss, 2008; Kochanowski et al., 2013). The increased FBP allosterically enhances the activity of Pyk and Ppc, thus decreasing the PEP/PYR ratio. As mentioned above, a low PEP/PYR ratio leads $EIIA^{Glc}$-P to be dephosphorylated and in turn decreases cAMP–Crp level, which therefore represses the expression of the TCA cycle genes as well as ACS (Fig. 1.2). In addition to cAMP–Crp, the catabolite repressor/activator protein (Cra) and anoxic respiration control system (ArcA/B) also play important roles in the control of carbon flow in *E. coli* (Shimada et al., 2011). High glucose uptake rates inhibit Cra activity (Saier and Ramseier, 1996), which in turn represses TCA cycle genes such as *icdA* and *aceA* (Fig. 1.2). In the ArcA/B system, ArcA/B responds to the redox state of the membrane-associated redox carriers such as quinones in the respiratory chain (Georgellis et al., 2001; Malpica et al., 2004). The decreased quinone pool causes ArcB to be self-phosphorylated (ArcB-P), and then ArcB-P transphosphorylates ArcA, which represses TCA cycle genes. From the point view of redox balance and oxidative stress regulation, TCA cycle activity must be repressed in accordance with the increased activity of glycolysis. In fact, fast sugar uptake means that respiration is activated with the increased production of NADH, and more reactive oxygen species (ROS) would be generated in the respiratory chain, which causes damage to the cell. Since the flux from acetyl-CoA to acetate does not generate any NADH while the flux from acetyl-CoA through the TCA cycle generates 8NAD(P)H and $2FADH_2$, carbon flow diversion to acetate could be viewed as a means to reduce or prevent further NAD(P)H accumulation (El-Mansi and Holms, 1989; Holms 2001; Vemuri et al., 2006). Nevertheless, the

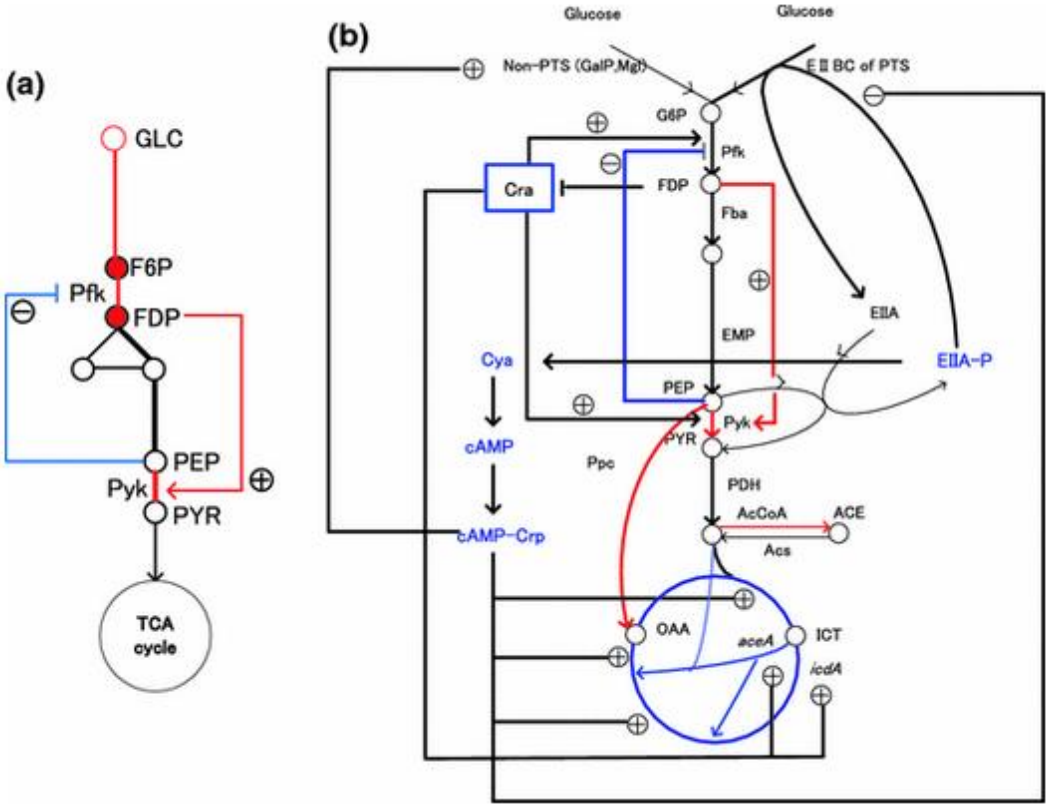

Figure 1.2 Catabolite regulation of the central metabolism: a) enzyme-level regulation, b) overall enzyme-level and transcriptional regulations (Adapted from Shimizu., 2015).

accumulation of acetate by this complex overflow metabolism is a crucial problem in commercial scale production since this by-product retards cell growth, inhibits recombinant protein production and wastes carbon source (Shiloach et al., 1996; Wong et al., 2008).

Reduced glycolysis flux can alleviate overflow metabolism. The conventional approach to avoid overflow metabolism is to reduce the glycolytic flux by implementing feeding strategies to maintain the substrate concentration at low level (Jensen and Carlsen, 1990; Vidal et al., 2005). Another approach to reduce the glucose uptake rate is to modulate the metabolic pathway. Modification of glucose transport capacity successfully reduced acetate production under aerobic conditions. Addition of the metabolically inert glucose analog methyl α-glucoside (α-MG), which serves as a competitive inhibitor of PTS glucose transport, to *E. coli* cultures growing in complex medium supplemented with glucose reduced acetate concentration by 54%, and increased the specific activity of a recombinant protein product and the final biomass concentration (Chou et al, 1994). Since sugar transport with PTS influences the PEP/PYR ratio and carbon flux distribution, elimination of PTS components significantly impacts carbon flux distribution in central metabolism (Flores et al., 2002). Inactivation of *ptsG* resulted in significant reduction of acetate secretion and was able to increase plasmid DNA vaccine synthesis, recombinant protein synthesis or produce integral membrane spanning proteins that were not possible to produce with the corresponding wild type (Chou et al., 1994; Bäcklund et al., 2011; Fuentes et al., 2013). Similar results have been reported with *E. coli* strains having deletion of the *ptsHI-crr* operon. The engineered strains accumulate 80% less acetate than their isogenic wild type strains when growing in either minimal or

rich media with glucose as a carbon source (Flores et al., 1996; Sigüenza et al., 1999; Lara et al., 2008; Fuentes et al., 2013), and the protein yield of the mutant strain was twice that of the wild type (Lara et al., 2008). A subtler method is overexpressing the regulatory protein Mlc, encoded by *mlc* gene, to repress the *ptsHI* and *ptsG* genes. It also reduced acetate accumulation and increased capacity to consume this acid (Hosono et al., 1995; Plumbridge, 2002). In addition to the modification of sugar transport, engineering of upper glycolysis steps is also fairly effective to cope with overflow metabolism. Redirecting carbon flux to the pentose phosphate pathway by phosphoglucose isomerase (*pgi*) or phosphofructokinase (*pfkA*) deletions has significantly decreased acetate secretion in comparison with the parent strains (Hua et al., 2003; Toya et al., 2010; Yao et al., 2011; Xie et al., 2014).

Succinate production with *E. coli* is a good example that employs comprehensive metabolic engineering of sugar pathways to produce a TCA cycle intermediate. In particular, an optimal production strategy needs potentially to include the co-assimilation of carbon sources, the elimination of by-product formation, and the reconciliation of energy and redox balance with carbon metabolism. Succinate is an important C4 building-block chemical, for which new applications recently have been reported, such as the production of 1,4-butanediol based polymers. Multinational corporations, such as DuPont, BASF, and Mitsubishi, have commercialized the polymer produced from biobased succinate (Cukalovic and Stevens, 2008; Beauprez et al., 2010; Xu et al., 2010). To debottleneck the limitations in succinate production, metabolic engineering strategies applied to *E. coli* cell factories can be classified as: a) improvement of substrate utilization and eliminate CCR; b) enhancement of pathways directly involved in the

succinate production; c) deletion of competing pathways toward by-product accumulation (Zhang et al., 2009; Cao et al., 2011; Du et al., 2011; Wang et al., 2011). One of the first approaches with respect to elimination of competing pathways is to inactivate the pyruvate–formate lyase (*pflB*) and lactate dehydrogenase (*ldhA*) genes. However, a *pflB ldhA* double mutant strain produced minor amounts of succinate and showed poor growth on glucose in anaerobic conditions (Bunch et al., 1997; Donnelly et al., 1998). The problem was probably caused by a redox imbalance and the accumulation intermediate metabolites such as pyruvate (Vemuri et al., 2002). A spontaneous *ptsG* mutant was able to produce succinate, acetate, and ethanol with molar yields of 1.0, 0.5, and 0.5 mol/mol glucose, respectively (Donnelly et al., 1998). Based on this phenomenon, inactivation of *ptsG* in several *E. coli* mutant strains improved succinate molar yield and specific productivity (Chatterjee et al., 2001; Lin et al., 2005). For succinate production, *ptsG* gene deletion leads to a lower rate of glucose consumption, which not only benefits redox balance and reduces acetate accumulation but also helps to overcome CCR if using mixed sugar carbon source.

Glycosylation is one of the most important and prevalent modifications in nature as it plays a central role in cellular communication and the orchestration of life in general (De Bruyn et al., 2015). Sugar residues can be attached to a variety of molecules ranging from macromolecules (proteins, lipids, cell wall glycans) to small molecules (secondary metabolites and oligosaccharides) (Bowles et al., 2005). Glycosylation of small molecules enhances their solubility, stability or bioactivity (Yamamoto et al., 1990; Kren and Martinkova, 2001; Zhao et al., 2014). In addition, different sugar residues alter the pharmacological properties of the same compound (Olthof et al., 2000; Terao et al., 2001;

Choi et al., 2009; Sanchez-del-Campo et al., 2009; Kim et al., 2011). The UDP-sugars and corresponding UGTs are capable of efficiently glycosylating various compounds from diverse chemical classes in a regio- and stereo selective way (Bowles et al., 2005, 2006). The traditional ways to obtain naturally occurring glycosides are: a) extraction from plants; b) organic synthesis. However, the extraction from plants is low-yielding, laborious and highly dependent on geographical and seasonal factors. Although in vitro plant cell cultures could help to tackle these problems, it is not cost effective and thus restricted towards relatively highly priced metabolites (Rao and Ravishankar, 2002; Verpoorte and Memelink, 2002; Bertoli et al., 2010; Hussain et al., 2012). Organic synthesis of natural glycosides needs various protecting and deprotecting steps to achieve the stereoselective formation of specific glycosidic linkages (Demchenko, 2008; Seebergeret al., 2009). It generates toxic waste and consumes very expensive catalysts (de Roode et al., 2003; Zhu and Schmidt, 2009). Biotechnological solutions to the above mentioned problems include *in vitro* (enzymatic) and *in vivo* (bioconversion) production of glycosides. Glycosyltransferases (GTs) are the most common carbohydrate active enzyme for glycosylation since it displays superior conversion efficiencies (up to 100%) towards an enormous variety of small molecules (Desmet et al., 2012; De Winter et al., 2014). The main constraint to use GTs for *in vitro* conversion is the need for extremely expensive UDP-sugars, which are rarely available in large quantities (Ruffing and Chen, 2006).

The *in vivo* UDP-sugar based glycosylation eliminates the extensive enzyme purification and the addition of expensive cofactors. Overall, glycosyltransferases are a well-studied enzymes with extensive information available on the active site or amino

acid boxes, and more glycosyltransferases become available through functional screening of metagenome and genome libraries (Rabausch et al., 2013; Han et al., 2014). Currently glycosides derived from specialized metabolites are produced with titers below of 0.1 - 0.2 g/L. Research efforts have predominantly focused on the screening and expression of UGTs, while generally little effort has been put into the efficient generation and regeneration of UDP-sugars. *In vivo* routes, typically *E. coli* sugar metabolism pathways, naturally generate a sugar 6-phosphate pool, which can be converted to glucose 1-phosphate by phosphoglucomutase (Pgm) and subsequently to UDP-glucose by glucose 1-phosphate uridylyltransferase GalU. Predominant UDP-sugars in nature to glycosylate specialized metabolites are UDP-glucose, UDP-glucuronate, UDP-xylose, UDP-arabinose and UDP-rhamnose. These UDP-sugars can be elegantly formed through the combined reactions of a large variety of epimerases, dehydrogenases or decarboxylases, which means UDP-glucose could be considered as a central pivot and starting point to produce various UDP-sugars. Reduced glycolysis flux by metabolic engineering will potentially benefit the accumulation of sugar 6-phosphate pool, as well as prevent the by-product formation, diverting all the carbon to generate UDP-sugars, biomass and energy.

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CHAPTER 2

SUCCINATE PRODUCTION FROM XYLOSE-GLUCOSE MIXTURES USING A CONSORTIUM OF ENGINEERED *ESCHERICHIA COLI*[1]

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Abstract

A consortium of two strains of *Escherichia coli* was used to convert a mixture of xylose and glucose to succinate in a dual phase aerobic/anaerobic process. First, succinate production from xylose or glucose alone was compared using *E. coli* expressing either heterologous pyruvate carboxylase or heterologous ATP-forming PEP carboxykinase. Expression of PEP carboxykinase resulted in higher succinate yield (0.86 g/g) and specific productivity (155 mg/gh) for xylose conversion, while expression of pyruvate carboxylase resulted in higher succinate productivity (76 mg/gh) for glucose conversion although the succinate yield (0.91-0.99 g/g) was about the same whether pyruvate carboxylase or PEP carboxykinase was expressed. Then, processes using the xylose-selective strain TXXP pTrc99A-*pck* and the glucose-selective strain TXG0 pTrc99A-*pyc* as a consortium were designed for two different feed ratios of glucose:xylose. For either feed ratio, the consortium generated over 40 g/L succinate efficiently with yields greater than 0.90 g succinate/g total sugar. This study demonstrates two advantages of the consortium approach for the conversion of sugar mixtures: the ability to optimize the pathway for each sugar-to-product conversion independently, and the ability to adjust the consumption rate for each sugar independently, for example, by altering the biomass concentration of each consortium member strain.

Key Words: succinic acid, xylose, lignocellulosic hydrolysate

Introduction

There has long been an interest in the use of sugars liberated from lignocellulose feedstocks to produce biofuels and other industrially important biochemicals by fermentation. Unfortunately, unlike starch-containing crops such as corn which can be hydrolyzed to yield the single sugar glucose, lignocellulosic hydrolysates yield a mixture of sugars which can include the hexoses galactose, glucose and mannose as well as the pentoses arabinose and xylose. Significant research efforts have focused on developing a single microorganism that can convert all of the sugars found in lignocellulosic hydrolysates to useful products with limited success.

An alternative approach involves the use of a consortium of isogenic microorganisms that act together to convert the sugars contained in lignocellulosic hydrolysates into biochemical products (Eiteman et al., 2009). In this approach each consortium member can be engineered to metabolize only one target sugar in the mixture. Because each member of such a consortium metabolizes only a single substrate, each can carry out a desired conversion while effectively ignoring other substrates. Moreover, since consortium members are the same species, growth incompatibilities which might exist from pH, temperature, nutritional requirements or negative cell-to-cell interactions are avoided (Chen, 2011). Microbial consortia have been used to metabolize glucose, xylose and arabinose simultaneously as well as remove the inhibitor acetic acid selectively (Lakshmanaswamy et al., 2011; Xia et al., 2012). The approach has also been used to generate lactic acid from a mixture of glucose and xylose (Eiteman et al., 2009). One unique advantage of consortia for the production of a biochemical product is that the entire pathway between a target substrate and the desired product can be optimized.

Because the consortium strains are independent, a metabolic engineering strategy to maximize each sugar-to-product conversion can be implemented independently. Of course, consortia could also be designed to generate multiple products from sugar mixtures, or intermediates which are themselves chemically combined in a separate process for the ultimate product. Another advantage is that the relative cell densities of each member of a consortium can be tuned to match the substrate concentrations and the rate of product formation (Eiteman et al., 2009). The increasing interest and applications of microbial consortia have been reviewed (Wael et al., 2010).

Succinate has long been of interest as a biochemical product and precursor for several industrial chemicals (see review by Thakker et al., 2012). Several companies have recently brought succinate derived through fermentation processes onto the marketplace (Erickson, 2013; Harmsen et al., 2014). As a mixed-acid fermenter, wild-type *E. coli* anaerobically generates a mixture of formate, lactate, succinate, acetate and ethanol (Blackwood et al., 1956; Clark, 1989), and this microbe serves as one commercially viable microbial platform for succinate formation (Alonso et al., 2014). Key aspects of succinate formation from glucose include inactivation of pyruvate formate lyase (*pflB*), lactate dehydrogenase (*ldhA*) and the glucose-specific IIB component of the phosphotransferase system (*ptsG*) (Gupta and Clark, 1989; Bunch et al., 1997; Stols and Donnelly, 1997; Donnelly et al., 1998; Chatterjee et al., 2001). Succinate yield and productivity from glucose can be further increased by strategies which elevate anaplerotic fluxes: overexpression of malic enzyme (Stols and Donnelly, 1997), PEP carboxylase (Millard et al., 1996), pyruvate carboxylase (Gokarn et al., 1998, 2000; Vemuri et al., 2002a, b), both PEP carboxylase and pyruvate carboxylase (Lin et al., 2005a), PEP

carboxykinase (Kim et al., 2004), and introducing targeted mutations in the promoter region for PEP carboxykinase (Jantama et al., 2008; Zhang et al., 2009). Interestingly, overexpression of PEP carboxykinase enhances succinate production in *E. coli* only in a PEP carboxylase mutant, not in the wild-type (Millard et al., 1996; Kim et al., 2004). Of all these approaches using *E. coli*, the highest succinate titer from glucose of nearly 100 g/L was achieved by overexpressing pyruvate carboxylase (Vemuri et al., 2002b). A common scheme for succinate production involves an initial aerobic growth phase followed by an anaerobic production phase (Nghiem et al., 1999; Vemuri et al., 2002b). In such a dual phase process specific operational conditions can be applied to each phase, and the biotransformation enzymes expressed in the growth phase can remain active during the anaerobic production phase (Vemuri et al., 2002b).

Although glucose is the typical substrate used to study succinate formation by *E. coli*, xylose can also be converted to succinate. For example, a *ptsG pflB ldhA* triple knockout metabolized 80% of the xylose added to corn steep liquor medium, resulting in a succinate yield of 0.5 g/g, about 40% less than the succinate yield from glucose (Andersson et al., 2007), while a *ackA pta ldhA* triple knockout overexpressing PEP carboxylase (*ppc* gene) attained a succinate yield of 0.34 g/g (Lin et al., 2005b). Succinate formation from pentoses is generally expected to achieve lower yields than succinate formation from hexoses because of different ATP yields and redox balances between these substrates. In particular, the conversion of xylose to PEP results in the net consumption of ATP whereas the formation of PEP from glucose is ATP neutral. Based on this recognized difference, an ATP-forming PEP carboxykinase was recently overexpressed in *E. coli ldhA pflB ppc*, resulting in a succinate yield of 0.87 g/g from

xylose (Liu et al., 2012). The PEP to OAA step could generate ATP at elevated extracellular CO_2 concentrations, and higher biomass and succinate yields are predicted with greater ATP formation (Singh et al., 2011). These numerous previous studies on succinate production do not establish whether overexpression of pyruvate carboxylase, or PEP carboxykinase in an *E. coli ppc* knockout, would lead to the greater productivity and yield of succinate from either glucose or xylose (Figure 2.1).

The objective of this study was to use a consortium of two *E. coli* strains for the conversion of a xylose-glucose mixture to succinate. The use of microbial consortia permits the independent comparison and optimization of xylose-to-succinate and glucose-to-succinate pathways. In particular, we compared the overexpression of pyruvate carboxylase and PEP carboxykinase in two *E. coli* strains growing on either glucose or xylose, and then selected the best combination of these strains to design a synchronized process for the optimal conversion of these two substrates to succinate in a dual-phase fermentation.

Materials and Methods

Strains and Plasmids

Four *E. coli* strains designated TXX0, TXXP , TXG0, and TXGP (Table 2.1) were derived from wild-type *E. coli* C (ATCC8739). TXX0 and TXXP are able to use xylose as a sole carbon source but because of the *ptsG*, *glk*, *manZ* and *crr* knockouts are unable to metabolize glucose (Xia et al., 2012). TXG0 and TXGP are able to use glucose as a sole carbon source but because of the *xylA* deletion are unable to metabolize xylose (Xia et al., 2012). These strains were constructed by transducing ATCC8739 with the corresponding Keio (FRT)Kan deletions (Baba et al., 2006), and when necessary for the

generation of additional knockout strains, curing the Kan(R) using the pCP20 plasmid, which contains a temperature-inducible FLP recombinase as well as a temperature-sensitive replicon (Datsenko and Wanner, 2000).

The *pck* gene encoding PEP carboxykinase was PCR amplified with primers 5′-CGAGCTCATGAACTCAGTTGATTTGACCG-3′ (forward) and 5′-GCTCTAGAGCATTCCGTCAATTAAAACAAG-3′ (reverse) using *Bacillus subtilis* subsp. *subtilis* strain 168 genomic DNA as the template as described by Liu et al., 2012. The 1,653 bp PCR product was purified, restricted with *Sac*I and *Xba*I and ligated into the regulable expression vector pTrc99A that had also been restricted with *Sac*I and *Xba*I to yield the plasmid pTrc99A-*pck*, which was subsequently transformed into TXXP and TXGP. The plasmid pTrc99A-*pyc* containing the *pyc* gene from *Rhizobium etli* encoding pyruvate carboxylase (Gokarn et al., 2000, 2001) was transformed into strain TXX0 and TXG0 (Table 2.1).

Growth Conditions

The defined basal medium contained (per L): 13.3 g KH_2PO_4, 4.0 g $(NH_4)_2HPO_4$, 1.2 g $MgSO_4 \cdot 7H_2O$, 13.0 mg $Zn(CH_3COO)_2 \cdot 2H_2O$, 1.5 mg $CuCl_2 \cdot 2H_2O$, 15.0 mg $MnCl_2 \cdot 4H_2O$, 2.5 mg $CoCl_2 \cdot 6H_2O$, 3.0 mg H_3BO_3, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, 100 mg Fe(III) citrate, 8.4 mg $Na_2EDTA \cdot 2H_2O$, 1.7 g citric acid, 4.5 mg thiamine·HCl, and 50 mg ampicillin. The medium was adjusted to a pH of 7.0 with 30% (w/v) NaOH.

For single strain processes, TXX0 pTrc99A-*pyc*, TXXP pTrc99A-*pck*, TXG0 pTrc99A-*pyc*, or TXGP pTrc99A-*pck* were first grown for 7-9 h (2 mL in 15 mL test tubes) using Lysogeny Broth (LB) at 37 ℃, then transferred into 250 mL shake flasks containing 50 mL basal medium with 5 g/L yeast extract and 15 g/L D-(+)-xylose or 15

Table 2.1. Strains and plasmids used in the study of succinate production.

Strain or Plasmid	Relevant Characteristics	Reference
Strains		
ATCC8739	*E. coli* C	wild-type
TXX0	ATCC8739 *ptsG glk manZ crr pflB ldhA*::[FRT]Kan	This study
TXXP	ATCC8739 *ptsG glk manZ crr pflB ldhA ppc*::[FRT]Kan	This study
TXG0	ATCC8739 *ptsG xylA pflB ldhA*::[FRT]Kan	This study
TXGP	ATCC8739 *ptsG xylA pflB ldhA ppc*::[FRT]Kan	This study
Plasmids		
pTrc99A-*pck*	*pck* gene from *Bacillus subtilis* subsp. *subtilis* strain 168	This study, Liu et al., 2012
pTrc99A-*pyc*	*pyc* gene from *Rhizobium etli*	Gokarn et al., 2000, 2001

g/L D-(+)-glucose and having an agitation of 250 rpm (19 mm pitch). When the OD reached 3.0, 0.5 mM IPTG was added, and when the OD reached 4.0, the flask contents were used to inoculate the bioreactor containing basal medium, 0.5 mM IPTG, and a single sugar at 15 g/L. These batch processes were carried out in duplicate at 1.0 L volume in a 2.5 L bioreactor with a constant agitation of 500 rpm (Bioflo 2000, New Brunswick Scientific Co. Edison, NJ, USA). During an initial aerobic phase, 1.0 L/min air or oxygen-enriched air as necessary was sparged with 0.2 L/min CO_2 into the bioreactor to maintain the dissolved oxygen above 40% saturation. At the onset of the anaerobic phase when the OD reached about 10, the air flow was halted (but CO_2 flow was maintained), the agitation was reduced to 300 rpm, and 15 g xylose or 15 g glucose in 30 mL was added to the culture. During the anaerobic phase, two additional 15 g/30 mL solutions of glucose or xylose were added to the culture. The pH was controlled at 7.0 (aerobic phase) or 6.3 (anaerobic phase) using a mixture of 10% (w/v) NaOH and 15% (w/v) KOH. For both phases the temperature was controlled at 37 ℃, and Antifoam C (Sigma) was used as necessary to control foaming. *E. coli* requires anaplerotic flux, either through PPC or PPCK, for growth in a defined medium. Moreover, these enzymes and PYC use CO_2 or bicarbonate as a substrate. Therefore, in order for growth to occur and for methodological consistency, cells were necessarily induced in the shake flask inoculum, and the bioreactor experiments were supplied with CO_2.

For processes using two strains as a consortium, two different feed mixtures of glucose:xylose added periodically batch-wise during the anaerobic phase were examined: 10 g glucose + 10 g xylose (referred to as a 10:10 feed) and 15 g glucose + 5 g xylose (15:5 feed). In these studies, the selected xylose-consuming and glucose-consuming

strains were first grown individually for 7-9 h using LB as described above, and 1 mL of each culture was transferred separately into two 250 mL shake flasks containing 50 mL basal medium and 5 g/L yeast extract with glucose or xylose. When the OD reached 3.0 for each individual culture, 0.5 mM IPTG was added, and then when each OD reached 4.0, the flasks were used to inoculate a single bioreactor. These processes also used 1.0 L initial volume in the bioreactor as described above containing basal medium with initial concentrations of 15 g/L glucose + 8.0 g/L xylose (for 10:10 feed) or 30 g/L glucose + 10 g/L xylose (15:5 feed). Moreover, because the total biomass concentration was greater compared to the single strain processes, 4.0 g $(NH_4)_2SO_4$ was added during the aerobic phase for the 10:10 feed once when the OD reached 10, or three times for the 15:5 feed when the OD reached 7, 15 and 25. At the onset of an anaerobic phase, and again periodically during the phase, a mixture of 10 g glucose + 10 g xylose in 30 mL (10:10 feed) or 15 g glucose + 5 g xylose in 40 mL (15:5 feed) was added to the bioreactor. Other conditions were identical to the single-strain processes.

Analyses

The optical density (OD) measured at 600 nm was used to monitor cell growth (DU-650 spectrophotometer, Beckman Instruments, San Jose, CA) and frozen at -20 ℃ until further analysis. Thawed samples were centrifuged (4 ℃, 6800 × g for 15 min), and filtered (0.45 μm nylon, Acrodisc, Pall Corporation, Port Washington, NY). Liquid chromatography was used to quantify xylose, glucose and organic products (Eiteman and Chastain, 1997).

Results

Single Strain Batch Processes

The objective of this study was to examine the conversion of glucose-xylose mixtures into succinate using a consortium of two *E. coli* strains which independently metabolize these sugars. Using two strains permits metabolic engineering of each strain independently to optimize the target conversion, xylose-to-succinate or glucose-to-succinate. Thus, we first compared two strategies for each sugar-to-succinate conversion to allow selection of the best set of strains to compose the consortium. In particular, we compared the conversion of these sugars to succinate via the heterologous PEP carboxykinase or pyruvate carboxylase enzymes (Figure 2.1).

Four strains were therefore constructed to examine succinate production from the appropriate sugar in aerobic-anaerobic dual-phase fermentations. TXX0 and TXXP are unable to metabolize glucose as a result of the knockouts in *ptsG*, *manZ*, *glk* and *crr* (and thus selectively metabolize xylose), while TXG0 and TXGP are unable to metabolize xylose as a result of the *xylA* knockout (Eiteman et al., 2008). All four strains have knockouts in the *pflB, ldhA* and *ptsG* genes which have been previously shown to direct more PEP or pyruvate to succinate (Chatterjee et al., 2001). The two strains TXXP and TXGP additionally contain knockouts of the *ppc* gene which enhances succinate formation in a strain overproducing ATP-generating PEP carboxykinase (Figure 2.1, Liu et al., 2012). Thus, we compared TXG0 pTrc99A-*pyc* with TXGP pTrc99A-*pck* for the conversion of glucose to succinate, and TXX0 pTrc99A-*pyc* with TXXP pTrc99A-*pck* for the conversion of xylose to succinate.

Representative fermentations converting xylose to succinate and glucose to succinate are shown respectively in Figures 2.2-2.3. TXXP pTrc99A-*pck* metabolized 11 g/L xylose at a growth rate of 0.33 h^{-1} during the aerobic phase (Figure 2.2), and then

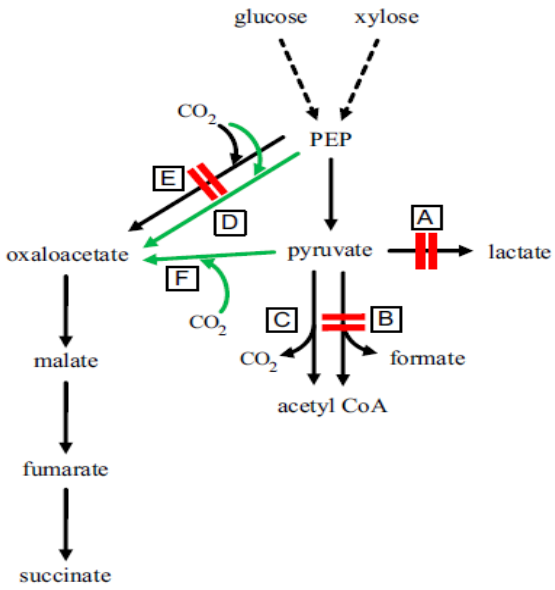

Figure 2.1: Two pathways for succinate production from xylose or glucose in *E. coli*. Gene knockouts are indicated in red, and overexpressed genes are indicated in green. Glucose or xylose is metabolized via the Embden-Meyerhof-Parnas and Pentose Phosphate Pathways to PEP and pyruvate. As demonstrated by numerous studies previously, anaerobic succinate formation can be increased by knocking out lactate dehydrogenase (A) and pyruvate formate lyase (B), which permits *E. coli* growth aerobically via the pyruvate dehydrogenase complex (C). Two approaches to elevate succinate formation further include 1) overexpression of ATP-forming pyruvate carboxykinase (D) in a PEP carboxylase (E) knockout, and 2) overexpression of heterologous pyruvate carboxylase (F). Oxaloacetate formed by either process is converted anaerobically by native enzymes to succinate.

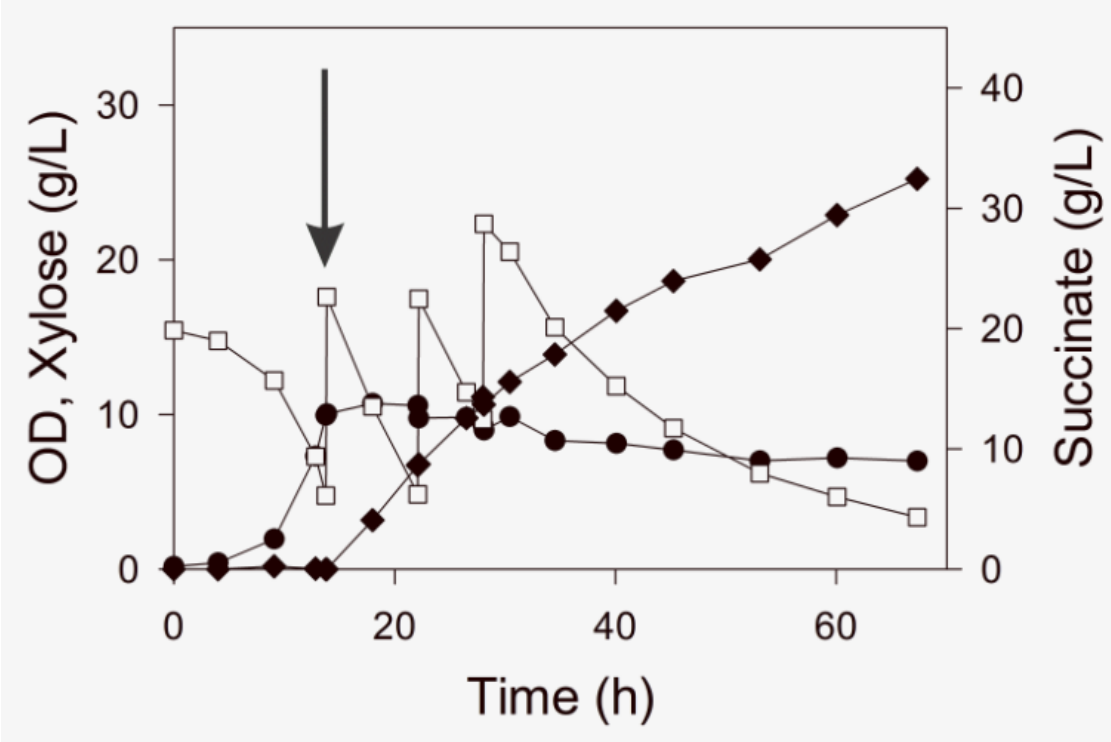

Figure 2.2: Dual-phase growth and succinate production on xylose. *E. coli* TXXP/pTrc99A-*pck*. OD: ●; xylose: □; succinate: ◆. The vertical arrow indicates the change from aerobic to aerobic conditions.

during the subsequent anaerobic phase of 53 h, 32 g/L succinate accumulated. For either strain growing on xylose, acetate was the primary byproduct, reaching a final concentration of 1.0-1.4 g/L (data not shown). Similarly, TXG0 pTrc99A-*pyc* metabolized about 10 g/L glucose at a growth rate of 0.21 h^{-1}, and then during an anaerobic phase this strain generated about 37 g/L succinate (Figure 2.3). In the case of glucose conversion, acetate accumulated as the major byproduct to a concentration of 2-3 g/L. For all strains, succinate productivity decreased gradually as the anaerobic phase progressed, so that after the third addition of either sugar, the rate of succinate formation was typically 50-70% lower than at the onset of anaerobic conditions.

Figure 2.4 summarizes key results from these comparative fermentations. For the case of xylose consumption, both the succinate yield (0.86 g/g) and productivity (155 mg/gh) were significantly greater using TXXP pTrc99A-*pck* compared to TXX0 pTrc99A-*pyc*. For glucose consumption, succinate productivity was significantly greater using TXG0 pTrc99A-*pyc* (76 mg/gh) whereas there was no significant difference between the strains for succinate yield (0.91 – 0.99 g/g). A carbon recovery calculation cannot be completed because of the presence of multiple pathways to succinate (Vemuri et al., 2002a).

Consortium process

The sets of strains examined were able to metabolize either glucose or xylose, but not both of these sugars. In order to utilize a xylose-glucose mixture, a consortium of two strains is necessary. Based on the dual criteria of anaerobic yield and productivity, the single strain processes demonstrated that TXXP pTrc99A-*pck* and TXG0 pTrc99A-*pyc* performed the best in converting xylose and glucose, respectively, and therefore these

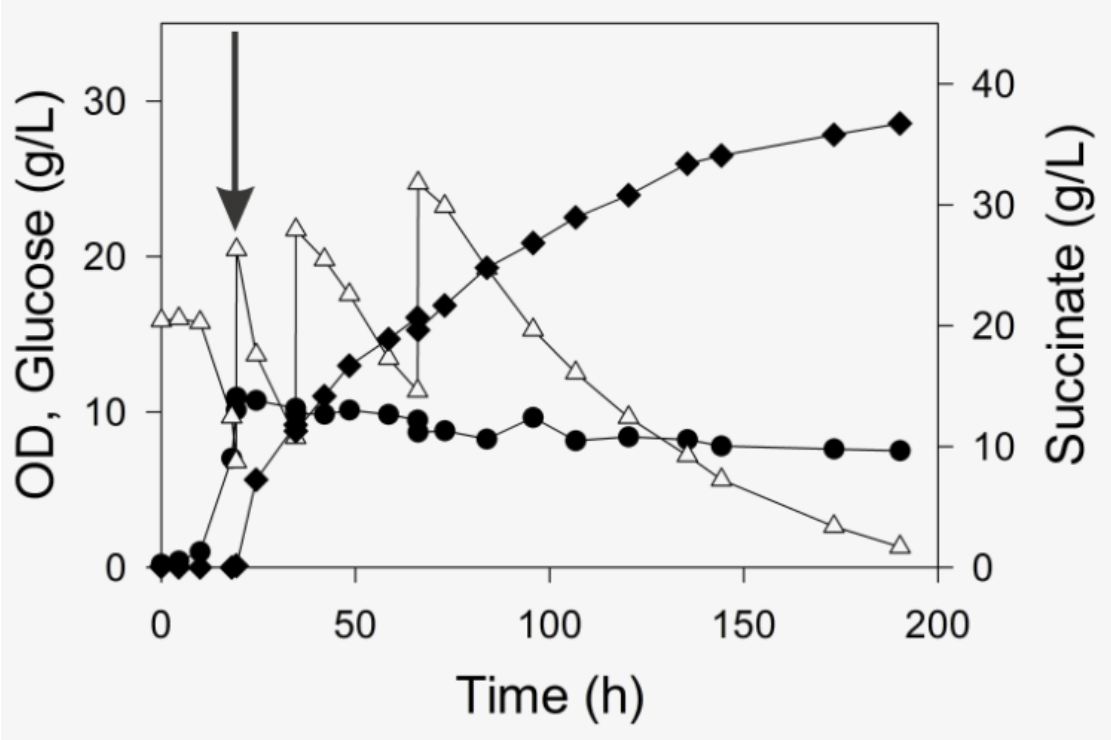

Figure 2.3: Dual-phase growth and succinate production on glucose. *E. coli* TXG0/pTrc99A-*pyc*. OD: ●; glucose: △; succinate: ◆. The vertical arrow indicates the change from aerobic to aerobic conditions.

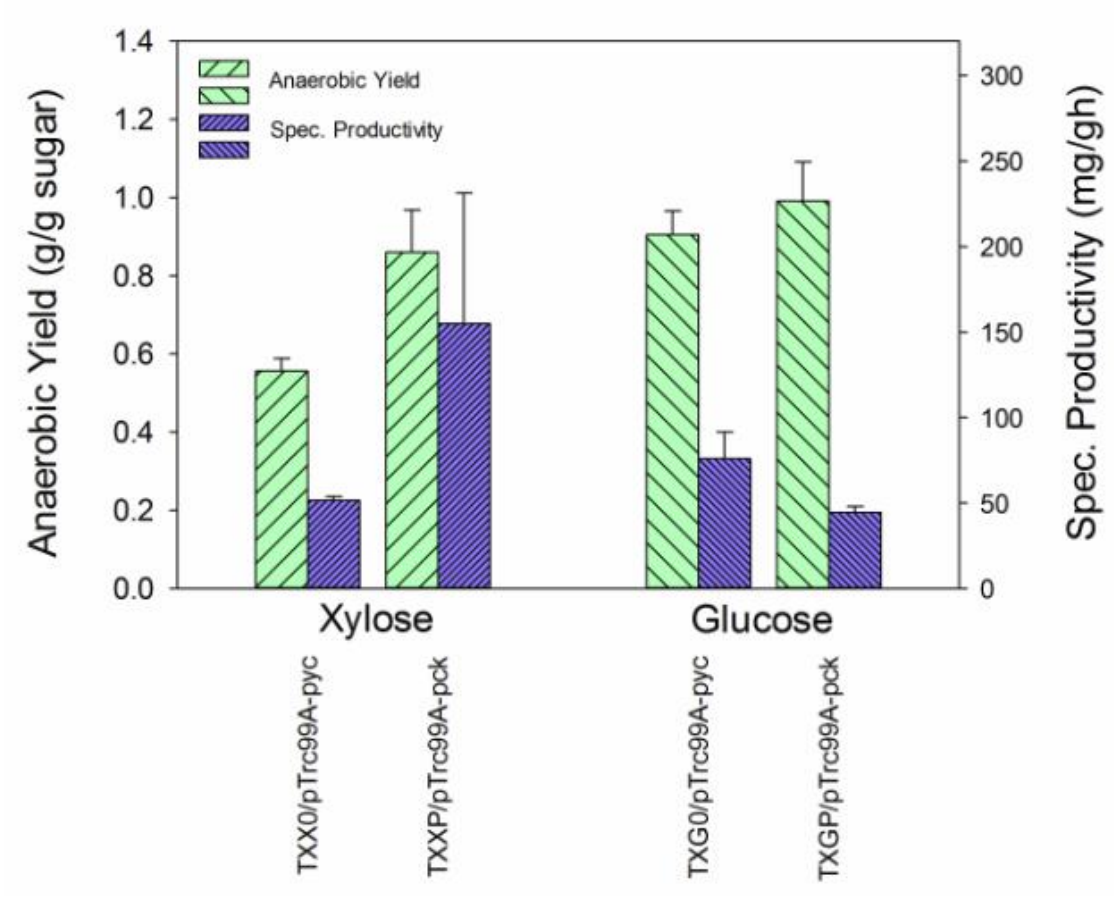

Figure 2.4: Anaerobic mass yield (g/g) and specific productivity (mg/gh) of succinate. For *E. coli* strains converting xylose or glucose to succinate. The anaerobic mass yield is calculated as the mass of succinate generated divided by the total mass of sugars (glucose + xylose) consumed.

strains were selected for the consortium. Furthermore, two different sugar compositions for the feed stream added batch-wise during the anaerobic phase were investigated: a feed composed of a 10:10 ratio of glucose:xylose and a feed composed of a 15:5 ratio of glucose:xylose. These glucose and xylose mixtures exemplify potential sugars combinations which can be obtained from lignocellulosic hydrolysates prepared from different feed stocks. In these different cases, to maintain effective conversion of both sugars to succinate during the non-growth anaerobic phase, the aerobic growth phase of the two strains needed to be aligned to provide the correct ratio of biomass concentrations for the anaerobic phase. Simply put, more TXXP pTrc99A-*pck* is needed as the concentration of xylose increases in the medium. Using the specific productivities for each strain determined during the single-strain/single-sugar processes (Figure 2.2), we estimated that the biomass ratio of these two strains necessary for the anaerobic phase was approximately 2:1 TXG0 pTrc99A-*pyc*:TXXP pTrc99A-*pck* for the 10:10 feed and 6:1 TXG0 pTrc99A-*pyc*:TXXP pTrc99A-*pck* for the 15:5 glucose:xylose feed. In order to achieve this desired ratio for the two strains, the inoculation of the culture with each strain was aligned so that at the time of the switch to anaerobic conditions the appropriate biomass ratio was achieved.

Figure 2.5 shows a representative fermentation using the feed ratio of 10:10 glucose:xylose, while Figure 2.6 shows the use of a 15:5 glucose:xylose feed. As expected, the two strains both grew during the aerobic phase and in a subsequent anaerobic phase converted xylose and glucose independently and simultaneously to over 40 g/L during within 100 h. During the processes the contributions of each strain to the biomass concentration are unknown; however, these values can be estimated by the

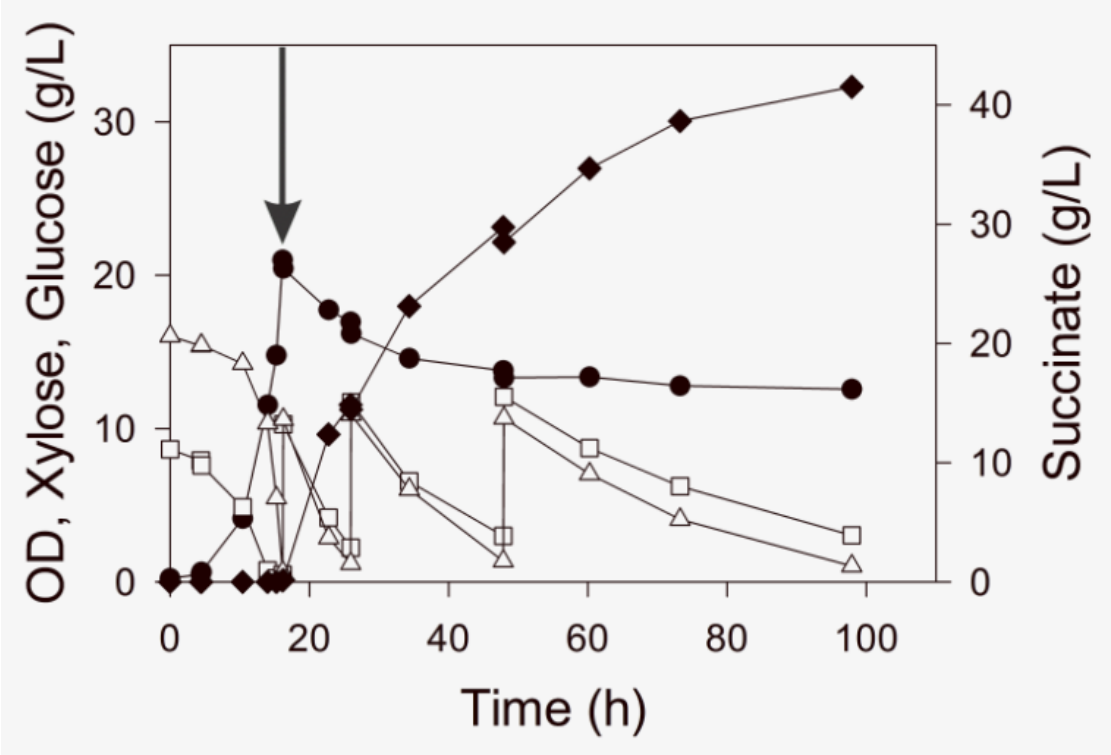

Figure 2.5: Dual-phase growth and succinate production on 1:1 glucose:xylose mixture. By a consortium of *E. coli* TXXP/pTrc99A-*pck* and TXG0/pTrc99A-*pyc*. OD: ●; xylose: □; glucose: △; succinate: ◆. The vertical arrow indicates the change from aerobic to aerobic conditions.

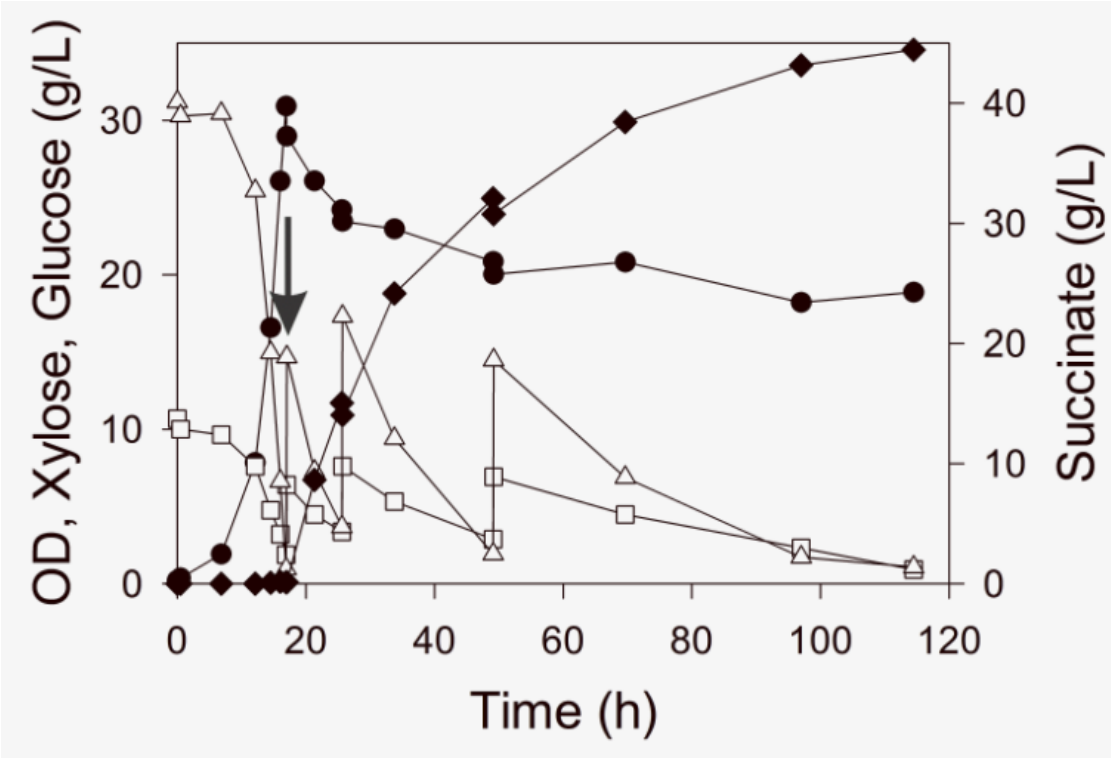

Figure 2.6: Dual-phase growth and succinate production on 3:1 glucose:xylose mixture. By a consortium of *E. coli* TXXP/pTrc99A-*pck* and TXG0/pTrc99A-*pyc*. OD: ●; xylose: □; glucose: △; succinate: ◆. The vertical arrow indicates the change from aerobic to aerobic conditions.

sugars consumed during the aerobic phase. In order to convert the 15:5 glucose:xylose feed into succinate effectively (Figure 2.6), the biomass concentration of TXG0 pTrc99A-*pyc* was 5-6 times greater than the concentration of TXXP pTrc99A-*pck*, a requirement that was accomplished by inoculating the xylose-consuming strain first. In each case the overall volumetric productivities (i.e., succinate from both sugars) was 1.7-2.0 g/L ħ at the onset of the anaerobic phase, but slowed to about 0.3-0.4 g/L ħ by the end of the processes. During the anaerobic phase the succinate mass yield on (total) sugars was 0.92 g/g for the 10:10 feed and 0.97 g/g for the 15:5 feed, values which agree closely with the yields predicted from single-sugar results of 0.93 g/g and 0.96 g/g, respectively.

Discussion

In this study, two engineered strains were used simultaneously to accumulate succinate from a mixture of xylose and glucose. Previous research has shown that either the overexpression of pyruvate carboxylase (Vemuri et al., 2002a,b) or overexpression of ATP-forming PEP carboxykinase in a *ppc* mutant (Kim et al., 2004; Liu et al., 2012) increases the anaplerotic flux to succinate during an anaerobic product formation phase, and we examined both of these approaches independently for each conversion using the same plasmid expression system. An explanation proposed for the need to knockout *ppc* to obtain the beneficial effect of PEP carboxykinase has been the 100-fold lower K_M toward bicarbonate of PEP carboxylase compared to PEP carboxykinase (Podkovyrov and Zeikus, 1993; Millard et al., 1996; Kim et al., 2004). Our result show that PEP carboxykinase from *B. subtilis* leads to greater succinate yield and productivity when xylose is the carbon source (Liu et al., 2012), while pyruvate carboxylase leads to greater

glucose-to-succinate conversion. The unique benefit of using microbial consortia to carry out a conversion of mixtures is the ability to engineer each member strain independently for a targeted conversion without compromising the performance of another conversion. Thus, we were able to overexpress the *pck* gene from *B. subtilis* for the xylose-consumer and overexpress the *pyc* gene from *R. etli* for the glucose-consumer, each in a background genotype also appropriate for that conversion, and therefore have greater flexibility to optimize the conversion of the mixture.

A second benefit of the consortium approach for the conversion of sugar mixtures is that a process can be designed for any given composition of substrates. For the present work, two different ratios of glucose/xylose were examined (10:10 and 15:5), and in each case an initial target biomass composition was achieved to ensure efficient conversion of the substrate mixture in a subsequent production phase. That is, both substrates could be consumed at a rate which matched the composition of a feed stream so that neither substrate was exhausted by itself to yield an unproductive one-substrate process. Such an alignment of rates is difficult in a classic one-strain conversion. For example, *E. coli* AFP184 depleted 30 g/L xylose during a 32 h anaerobic phase, though during that time almost half of the 40 g/L glucose remained (Andersson et al., 2007). Similarly, the small amount of glucose consumed by *E. coli* K12 *ldhA pflB ppc* pTrc-*Bspck* generated largely biomass, and there was no ability to direct the utilization of each substrate independently (Liu et al., 2012). In this study we simply adjusted the inoculum time for the two strains. More elegant methods to control the proportion of members in a consortium, including genetic circuits based on quorum sensing compounds (You et al., 2004) and complimentary auxotrophic amino acid exchange (Shou et al., 2007; Kerner et al., 2012).

In a nutrient-limited process the proportion of consortium members is self-correcting (Eiteman et al., 2008).

A defined medium was used for these studies to insure that each strain in the consortium consumed only the one corresponding carbon and energy source. Use of a defined medium contrasts with several other succinate-production processes which used a complex medium. This more stringent but industrially practical medium also causes reduced recombinant protein expression and reduced glucose uptake in the *ptsG* knockouts. Other approaches are available to increase xylose or glucose utilization, including overexpression of malate dehydrogenase (Wang et al., 2009) and expression of the *Zymomonas mobilis* glucose facilitator Glf (Li et al., 2013). Indeed, an important benefit of the consortium approach is that any additional metabolic engineering used to enhance xylose-to-succinate or glucose-to-succinate conversion can be implemented independently in the target strains, without impacting the conversion in the other member of the consortium. The approach thus does not lose its value as new, future improvements are made in the conversion of either sugar to a product.

Our results do not provide insight as to why PEP carboxykinase is superior for xylose conversion while pyruvate carboxylase is superior for glucose conversion. The two substrates have vastly different means of transport and thus differ in their resulting ATP yields. Moreover, the two substrates lead to differential expression levels of genes coding for regulatory and key metabolic enzymes involved in central metabolism. For example, many of the genes encoding enzymes in the Embden-Meyerhof-Parnas pathway show a significantly higher expression level when *E. coli* grows on glucose compared to xylose (Gonzalez et al., 2002). One possibility is simply that during growth on glucose,

partial activation of some auxiliary phosphotransferase system such that encoded by *manZ* (Curtis and Epstein, 1975), leads to a comparatively greater rate of pyruvate formation, the substrate for pyruvate carboxylase, compared to strains growing on xylose.

When xylose serves as the carbon source, 1 mol xylose requires the expenditure of 2 mol ATP for transport by the high-affinity ABC transporter and phosphorylation (Lin, 1996; Linton and Higgins, 1998). Overexpression of ATP-generating PEP-carboxykinase results in a net ATP yield for the conversion into succinate of 0.67 mol/mol xylose (Liu et al., 2012). In contrast, when glucose serves as the carbon source, because 1 mol ATP is expended to transport and phosphorylate 1 mol glucose through the galactose ABC transporter or galactose permease plus glucokinase (Death and Ferenci, 1993; Ferenci, 1996; McDonald et al., 1997; Hua et al., 2004), and pyruvate carboxylation also uses ATP, the net ATP yield for succinate formation is 0 mol/mol glucose. Although for both strains inactivation of pyruvate formate lyase impairs acetate generation and thus additional ATP generation (Gonzalez et al., 2002), we noted approximately three times greater acetate formation during glucose consumption compared to xylose consumption despite the slower glucose consumption rate in the *ptsG* knockouts. The anaerobic production of this byproduct, even in strains lacking pyruvate formate lyase, may reflect the different ATP demand to fulfill maintenance requirements under non-growth conditions. Additional independent metabolic engineering of the xylose-to-succinate and glucose-to-succinate pathways and associated regulatory control could be completed to improve upon the results obtained in this study.

We consistently observed the gradual slowing of succinate formation during the course of the anaerobic phase, a typical result which may be due to product inhibition

and/or cell viability. The general inhibitory effects of organic acids on *E. coli* growth and productivity have been previously investigated (Luli and Strohl, 1990; Zaldivar and Ingram, 1999; Warnecke and Gill, 2005). The productivity of succinate formation has previously been observed to decrease during the anaerobic phase of a dual-phase process when the succinate concentration exceeds about 40 g/L (Andersson et al., 2007; 2009; 2010). Additionally, the accumulation of counterions Na+ and K+ necessary to control the pH may inhibit cellular functions (Wu et al., 2013). In our study no attempt was made to minimize counterion toxicity, and the total counterion (Na+ plus K+) concentration reached about 1.0 M at the end of the processes. The productivity we achieved could be further improved by removing the succinate salt, and then resuspending cells in fresh medium (Andersson et al., 2010).

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CHAPTER 3

ACCUMULATION OF D-GLUCOSE FROM PENTOSES BY METABOLICALLY ENGINEERED *ESCHERICHIA COLI*[2]

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[2]Xia T, Han Q, Costanzo WV, Zhu Y, Urbauer JL, Eiteman MA. 2015. Applied and environmental microbiology. 81(10): 3387-94.

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Abstract

Escherichia coli unable to metabolize D-glucose (knockouts in *ptsG*, *manZ*, *glk*) accumulates a small amount of D-glucose (yield of about 0.01 g/g) during growth on the pentoses D-xylose or L-arabinose as a sole carbon source. Additional knockouts in *zwf* and *pfkA* genes encoding respectively D-glucose-6-phosphate 1-dehydrogenase and 6-phosphofructokinase I (*E. coli* MEC143) increased accumulation to greater than 1 g/L D-glucose and about 100 mg/L D-mannose from 5 g/L D-xylose or L-arabinose. Knockouts of other genes associated with interconversions of D-glucose-phosphates demonstrate that D-glucose is formed primarily by the dephosphorylation of D-glucose-6P. Under controlled batch conditions with 20 g/L D-xylose, MEC143 generated 4.4 g/L D-glucose and 0.6 g/L D-mannose. The results establish a direct link between pentoses and hexoses, and provide a novel strategy to increase carbon backbone length from five to six carbons by directing flux through the pentose phosphate pathway.

Key words: L-arabinose; D-glucose-6-phosphate 1-dehydrogenase; pentose phosphate pathway; 6-phosphofructokinase; phosphoglucose isomerase; D-xylose

Introduction

The pentose phosphate (PP) pathway interconverts phospho-sugars having 3-7 carbon atoms principally by the action of the reversible enzymes transketolase and transaldolase. During the consumption of hexoses such as D-glucose or D-fructose, entry of carbon into this pathway provides many microorganisms including *Escherichia coli* the means to generate the reduced co-factor NADPH and to synthesize specific building-block compounds derived from intermediates of this pathway (e.g., phenylalanine, histidine, ribose). For microorganisms having the requisite kinases and sugar transport mechanisms, the PP pathway also provides convenient entry points for the catabolism of many other sugars including D-xylose and L-arabinose.

We have previously studied D-xylose and L-arabinose metabolism in *E. coli* that lacks the ability to metabolize D-glucose due to knockouts in the *ptsG*, *manZ* and *glk* genes (15, 16, 48). Recently, small but consistent amounts (about 50 mg/L) of D-glucose were observed as the accumulated end-product when *E. coli ptsG manZ glk* was grown on 5 g/L of either pentose in a defined medium (unpublished). How might D-glucose be derived from these pentoses?

Both D-xylose and L-arabinose are converted to the common intermediate D-xylulose-5P (Figure 1), which via the PP pathway partitions to 67% D-fructose-6P and 33% D-glyceraldehyde-3P without the involvement of ATP:

$$3\ \text{D-xylulose-5P} \rightarrow 2\ \text{D-fructose-6P} + \text{D-glyceraldehyde-3P} \qquad [1]$$

During growth of cells having a complete glycolytic pathway, the 2 moles of D-fructose-6P formed via Eq. 1 readily generates 4 moles of D-glyceraldehyde-3P. For D-glucose to accumulate from pentoses in cells prevented from metabolizing D-glucose, we reasoned

that some D-fructose-6P generated from these pentoses (i.e., by Eq. 1) is converted "back" to D-glucose, and that once formed, the D-glucose was unable to reenter metabolism in the triple knockout strain. We furthermore hypothesized that even more D-glucose would accumulate from pentoses in cells that were further constrained from metabolizing D-fructose-6P or D-glucose-6P.

Because D-fructose-6P conversion to D-glyceraldehyde-3P is ubiquitous in wild-type organisms, D-glucose is not typically considered a product of D-xylose or L-arabinose metabolism, and the conversion of these pentoses to readily available D-glucose would in itself not seem to be an economically viable process. However, if the yields and rates were sufficiently large, the accumulation of hexoses directly from pentoses might advance the use of lignocellulosic hydrolysates with organisms such as *Saccharomyces cerevisiae* which metabolize D-glucose readily but are natively unable to consume pentoses. Moreover, conversion of 5-carbon saccharides into 6-carbon saccharides derived from D-fructose-6P offers a unique platform both to build carbon length and potentially to generate compounds in industrially relevant organisms such as *E. coli* that might not be possible under typical conditions in which products of D-fructose-6P do not accumulate.

The objectives of this study were to examine D-glucose formation from the pentoses D-xylose and L-arabinose. Specifically, we sought to identify the pathway involved in the formation of D-glucose from pentoses and to increase further the formation of D-glucose by preventing D-fructose-6P and D-glucose-6P metabolism. Finally, under the controlled conditions of a bioreactor we examined if elevated

concentrations of D-glucose could be synthesized from either D-xylose or L-arabinose as sole carbon sources.

Materials and Methods

Bacterial Strains

Escherichia coli ALS1048 (MG1655 Δ*ptsG*763::(FRT) Δ*manZ*743::(FRT) Δ*glk*-726::(FRT)) was used to construct additional strains as listed in Table 3.1 (16). These strains were constructed by transducing ALS1048 with the corresponding Keio (FRT)Kan deletions (2), and if necessary, curing the Kan(R) using the pCP20 plasmid, which contains a temperature-inducible FLP recombinase as well as a temperature-sensitive replicon (12). All strains were verified by PCR.

In one experiment the *pgi* gene encoding *E. coli* phosphoglucose isomerase was overexpressed. To construct the pTrc99A-*pgi* plasmid, the *pgi* gene was PCR amplified with primers 5′- GGGAAAGAATTCAAAAACATCAATCCAACGCAGACCGC -3′ (forward) and 5′- GGGAAAGGATCCTTAACCGCGCCACGCTTTATAGCG-3′ (reverse) using *E.coli* BW25113 genomic DNA as the template. The 1,671 bp PCR product was purified, restricted with *Eco*RI and *BamH*I and ligated into the regulable expression vector pTrc99A that had also been restricted with *Eco*RI and *BamH*I to yield the plasmid pTrc99A-*pgi*, which was subsequently transformed into MEC320 and MEC321.

Growth medium and conditions

The defined medium used for the shake flask experiments contained (per liter): 1.70 g citric acid, 13.30 g KH_2PO_4, 4.50 g $(NH_4)_2HPO_4$, 1.2 g $MgSO_4 \cdot 7H_2O$, 13 mg $Zn(CH_3COO)_2 \cdot 2H_2O$, 1.5 mg $CuCl_2 \cdot 2H_2O$, 15 mg $MnCl_2 \cdot 4H_2O$, 2.5 mg $CoCl_2 \cdot 6H_2O$,

Table 3.1. *E. coli* strains used in the study of D-glucose accumulation.

Strain	Genotype	Reference
ALS1048	MG1655 Δ*ptsG*763::(FRT) Δ*manZ*743::(FRT) Δ*glk*-726::(FRT)	16
MEC132	ALS1048 Δ*pfkA*775::Kan	This study
MEC143	ALS1048 Δ*pfkA*775::(FRT) Δ*zwf*-777::Kan	This study
MEC144	ALS1048 Δ*zwf*-777::Kan	This study
MEC151	ALS1048 Δ*pfkA*775::(FRT) Δ*zwf*-777::(FRT) Δ*mak*-759::Kan	This study
MEC152	ALS1048 Δ*pfkA*775::(FRT) Δ*zwf*-777::(FRT) Δ*agp*-746::Kan	This study
MEC178	ALS1048 Δ*pfkA*775::(FRT) Δ*zwf*-777::(FRT) Δ*gcd*-742::Kan	This study
MEC180	ALS1048 Δ*pfkA*775::(FRT) Δ*zwf*-777::(FRT) Δ*xylA*748::Kan	This study
MEC319	ALS1048 Δ*pfkA*775::(FRT) Δ*zwf*-777::(FRT) Δ*pgm*-736::Kan	This study
MEC320	ALS1048 Δ*pfkA*775::(FRT) Δ*pgi*-721::Kan	This study
MEC321	ALS1048 Δ*pfkA*775::(FRT) Δ*zwf*-777::(FRT) Δ*pgi*-721::Kan	This study

3.0 mg H_3BO_3, 2.5 mg Na_2MoO_4 $2H_2O$, 100 mg Fe(III) citrate, 4.5 mg thiamine HCl, 8.4 mg Na_2(EDTA) $2H_2O$, and 5.0 g D-xylose, L-arabinose, glycerol or D-fructose. The pH was adjusted to 7.0 with 30% (w/v) NaOH. Cells were routinely stored on lysogeny broth (LB) agar plates and transferred to 20 ml defined medium in a 125-ml shake flask, from which 2 ml was transferred to the 50 mL defined medium in a 250 mL shake flask used for these studies. Shake flask studies were replicated 3-6 times for each strain and pentose when D-glucose was detected. Statistical analyses were completed using Student's t-test (two-tailed, equal variance), and $p<0.10$ was considered the criterion for significance. For larger scale studies in a controlled bioreactor, the sequence of transfers was identical, and the 50 mL from the final shake flask was used to inoculate the larger vessel. The flasks were incubated at 37 ℃ with an agitation of 250 rpm. Samples were stored at -20 ℃ for subsequent analysis.

A single controlled batch process at 1.0 L volume was carried out using D-xylose in a 2.5 L bioreactor (Bioflo 2000, New Brunswick Scientific Co. Edison, NJ, USA). The same defined medium was used except the concentration of D-xylose was 20 g/L. Air or oxygen as necessary was sparged into the fermenter with the agitation set at 500 rpm to maintain the dissolved oxygen above 40% saturation. The pH was controlled at 7.0 using 20% NaOH, and the temperature was controlled at 37 ℃. Antifoam C (Sigma) was used as necessary to control foaming.

Continuous processes using MEC143 operated as nitrogen(N)-limited chemostats at 1.0 L volume were conducted in the same 2.5 L fermenter. To ensure N-limitation and prevent contamination, the medium contained (per L) 1.0 g $(NH_4)_2HPO_4$ (15 mM N), 8.0 g D-xylose and 40 mg kanamycin, but otherwise remained unchanged. Four dilution

rates (growth rates) were examined in the range 0.08 – 0.15 h^{-1}, and a steady-state condition was assumed after four residence times at which time the oxygen and CO_2 concentrations in the effluent gas appeared constant. These processes were conducted at 37 ℃ with an air flowrate of 0.5 L/min, an agitation of 400 rpm and a pH of 7.0. The dissolved oxygen remained above 40% saturation. A carbon balance was completed using a unit carbon formula weight for *E. coli* cell mass of 24.6 g/mol (5).

Analytical Methods

The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, Calif.) was used to monitor cell growth. Liquid chromatography with a refractive index detector and a Coregel 64-H ion-exclusion column (Transgenomic Ltd., Glasgow, United Kingdom) using a mobile phase of 4 mN H_2SO_4 was used for analysis of sugars and acetic acid as described previously (14). For dry cell weight measurement, three 25.0 mL samples were centrifuged (8400 × *g*, 10 min), the pellets washed by vortex mixing with 30 mL 0.9% saline solution and then centrifuged again. After repeating the washing step twice using DI water, the cell pellets were dried at 60 ℃ for 24 h and weighed. The concentrations of oxygen and CO_2 in the off-gas were measured using a gas analyzer (Innova 1313 gas monitor, Lumasense Technologies, Ballerup, Denmark).

The presence of sugars was confirmed by comparing samples with standards using a derivatization protocol with a GC-MS (6). The GC-MS method was used only for identification and not quantification, in particular those cases in which the analytes eluted closely by HPLC (D-mannose and D-xylose) or to confirm the absence of a sugar (e.g., D-fructose). Briefly, samples were centrifuged, the supernatant evaporated to

dryness, and then derivatized with 700 µL hexamethyldisilazane, 200 µL anhydrous pyridine, and 10 µL trifluoroacetic acid at 60 ℃ for 3 h. Detection of derivatized analytes was accomplished with a GC-MS (HP6890/HP5973, electron ionization energy of 70 eV, Agilent Technologies, Inc., Santa Clara, CA USA). One microliter (1 µL) was injected onto a 30 m HP-5MS column (Agilent Technologies, Inc.) in the split flow mode, 30:1 with 1 mL/min flow rate. The temperature profile began at 50 ℃ for 1 min, increased at 2 ℃/min to 100 ℃, increased at 5 ℃/min to 250 ℃, and held for 5 min. Injector temperature was 250 ℃, MS source was 230 ℃, MS Quad was 150 ℃, and the GC-MS interface was 280 ℃. For the N-limited chemostats, the ammonia nitrogen (NH_4-N) in feed and effluent was analyzed using the colorimetric EPA Method 350.1 (44).

We used NMR to demonstrate that D-glucose was formed biologically and accumulated in the medium. Four samples were analyzed: a glucose standard, a D-glucose-6P standard, a sample from a shake flask experiment (i.e., containing D-glucose), and a D-glucose-6P standard incubated in sterile medium for 24 h at 37 ℃. NMR data were acquired using a Varian INOVA instrument with a cryogenic probe system at 14.1 T (600 MHz ^{1}H). The sample temperature was maintained at 25 ℃. Standard, natural abundance, two-dimensional ^{1}H, ^{13}C-HSQC spectra were acquired in the constant-time (^{13}C decoupled) mode. Chemical shift assignments were made by reference to database entries and published works (3, 9, 38, 39, 40, 43). The ^{1}H chemical shifts were referenced with respect to external Na^+DSS^- (sodium 4,4-dimethyl-4-silapentane-1-sulfonate) in D_2O at 25 ℃ (0.0 ppm). The ^{13}C chemical shifts were referenced indirectly assuming the absolute frequency ratio: $^{13}C/^1H = 0.251449530$ (47). D_2O was added to samples to a

final concentration of approximately 7% for instrumental lock. NMR data were processed and signal intensities measured using Felix (Accelrys, San Diego, CA).

Results

Formation of glucose from xylose or arabinose

E. coli ALS1048 contains knockouts in the *ptsG*, *manZ* and *glk* genes and is unable to metabolize D-glucose (16). Using this strain as a baseline for comparison, we first sought to determine whether additional D-glucose would accumulate from either D-xylose or L-arabinose if D-fructose-6P and D-glucose-6P were prevented from entering glycolysis and the PP pathway. Specifically, we first constructed strains with additional knockouts in *pfkA* encoding 6P-fructokinase I (EC 2.7.1.11) and/or *zwf* encoding D-glucose-6P 1-dehydrogenase (EC 1.1.1.49). During growth in shake flasks using either 5 g/L D-xylose or 5 g/L L-arabinose, ALS1048 and ALS1048 *zwf* (MEC144) accumulated D-glucose at a yield of about 0.01 g/g from either pentose (Figure 3.2), while ALS1048 *pfkA* (MEC132) generated D-glucose at yields of 0.13 g/g from D-xylose and 0.17 g/g from L-arabinose. Eliminating both pathways in ALS1048 *pfkA zwf* (MEC143) resulted in the accumulation of D-glucose at yields of 0.26 – 0.29 g/g. For both *pfkA*-knockout strains MEC132 and MEC143, we also observed the formation of 60-180 mg/L D-mannose using HPLC and confirmed by GC-MS (see Materials and Methods). No other product such as D-fructose was identified by GC-MS. Moreover, the D-glucose and D-mannose were not metabolized within several hours after the pentose was exhausted. These results clearly show that *E. coli* can generate D-glucose from pentoses through D-fructose-6P, suggesting a route for the formation of 6-carbon products from 5-carbon substrates by preventing the intermediate D-fructose-6P from entering glycolysis and the

PP pathway (Figure 3.1). We also repeated the identical shake flask experiments using MEC143 with 5 g/L glycerol or 5 g/L D-fructose, and observed D-glucose as a final product at a concentration of about 60 mg/L or 75 mg/L, respectively (yield of about 0.01 g/g).

Identification of key enzymes involved in glucose formation

By preventing D-glucose utilization in *E. coli* while simultaneously blocking entry of D-fructose-6P into glycolysis and re-entry into the PP pathway, significant D-glucose formed from D-xylose or L-arabinose (Figure 3.2). We therefore sought next to clarify the pathway *E. coli* uses to convert D-fructose-6P to D-glucose by constructing additional knockout strains.

The formation of some D-mannose during the accumulation of D-glucose suggests the involvement of D-mannose as a pathway intermediate. Also, one possible route for D-glucose formation from D-fructose-6P would be via D-fructose. The enzyme mannokinase (EC 2.7.1.4) encoded by *mak* is known to phosphorylate D-mannose and D-fructose (42). We therefore hypothesized that mannokinase might be involved in the accumulation of D-glucose from pentoses via the conversion of D-fructose-6P to D-fructose. However, *E. coli* ALS1048 *pfkA zwf mak* (MEC151) did not show any difference in D-glucose formation from either pentose compared to MEC143 (Figure 3.2). Also, D-mannose was formed as before (230-270 mg/L), supporting the conclusion that mannokinase is not involved in the formation of either D-mannose or D-glucose from pentoses.

Another pathway that potentially could serve to form D-glucose is via the enzyme xylose isomerase (EC 5.3.1.5). In addition to interconverting D-xylose and D-xylulose, the

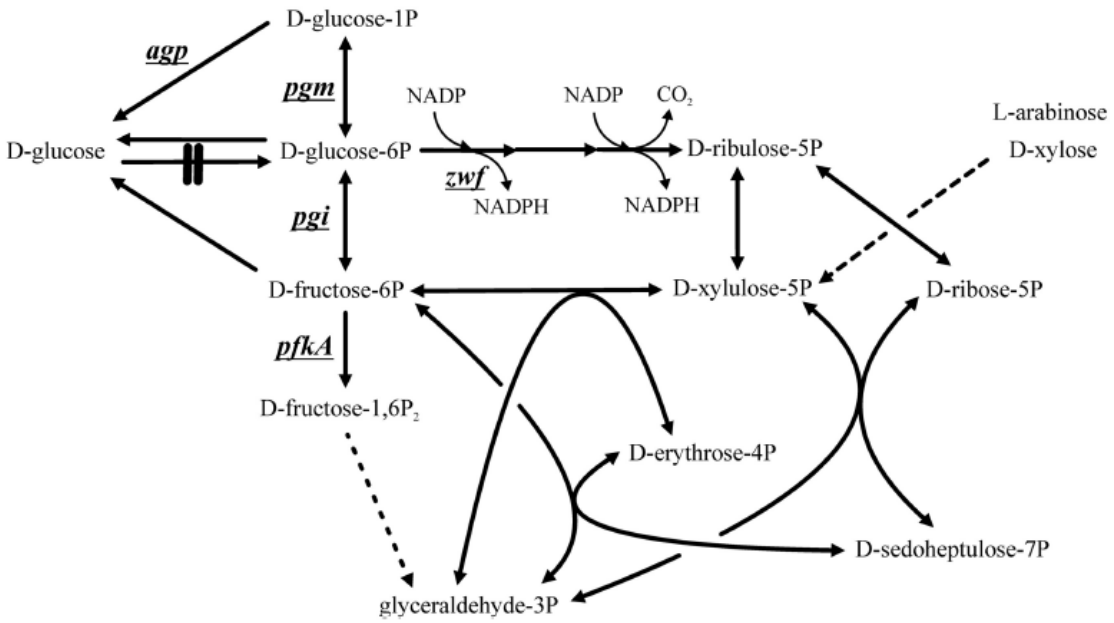

Figure 3.1: The pentose phosphate pathway and upper glycolysis of *Escherichia coli.* Consider either L-arabinose or D-xylose as carbon sources and which form D-xylulose-5P as a common intermediate (dashed line). Genes (underlined) and the enzymes they code are: glucose-1-phosphatase (EC 3.1.3.10, encoded by *agp*), phosphoglucomutase (EC 5.4.2.2, *pgm*), D-glucose-6P isomerase (EC 5.3.1.9; encoded by *pgi*), D-glucose-6P 1-dehydrogenase (EC 1.1.1.49; *zwf*), and 6-phosphofructokinase I (EC 2.7.1.11; *pfkA*). *E. coli ptsG manZ glk* (strain ALS1048) has gene deletions which prevent the microbe from metabolizing D-glucose (knockout indicated by double line). The conversion of D-fructose-1,6P_2 to glyceraldehyde-3P is mediated by enzymes in several steps (dotted line).

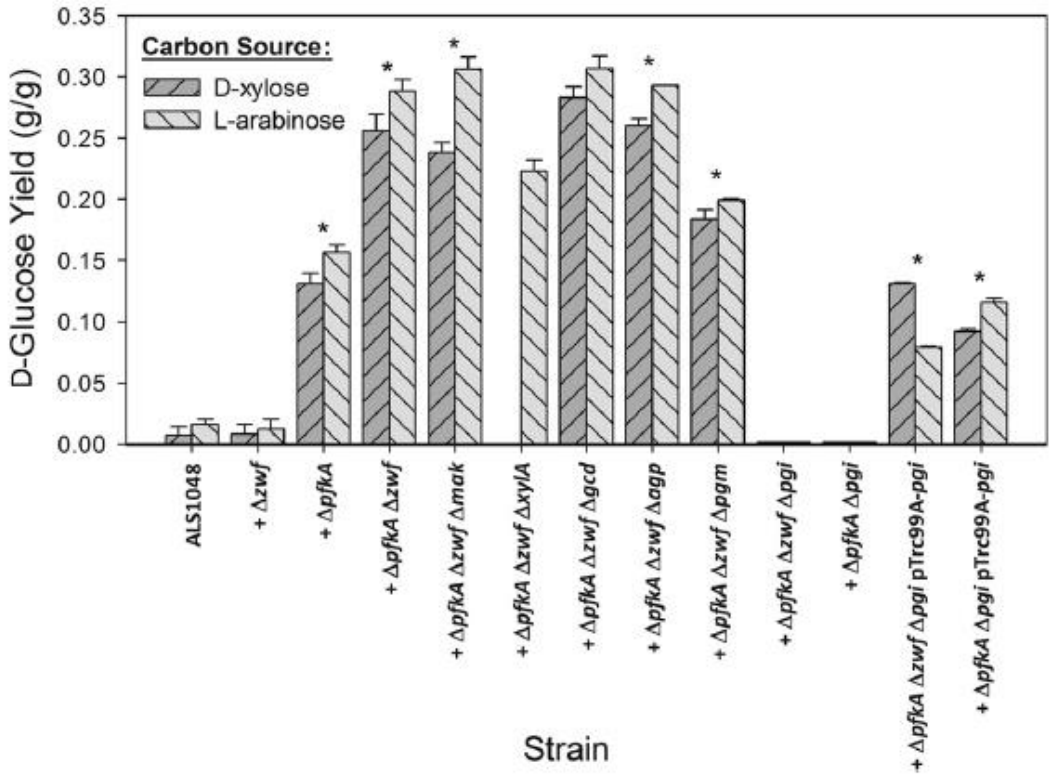

Figure 3.2: Comparison of *E. coli* strains for the production of D-glucose. 5 g/L L-arabinose or 5 g/L D-xylose was utilized. All strains are derived from ALS1048 (MG1655 *ptsG manZ glk*) and have additional gene knockouts as indicated. Strains which accumulated D-glucose were studied in 3-6 replicate cultures grown at 50 mL in a 250 mL shake flask, and error bars show the standard error of the measurements from these replicate samples. An asterisk (*) indicates a significant difference ($P<0.10$) in yield of D-glucose from D-xylose compared to from L-arabinose.

E. coli xylose isomerase interconverts D-fructose and D-glucose (7, 17, 46), but less efficiently (41). Though the K_M values for D-fructose, D-glucose and D-xylose have not been reported for the *E. coli* enzyme, the k_{CAT} values for D-glucose and D-fructose are similar for the enzyme from other organisms (29), suggesting that D-fructose and D-glucose both readily serve as substrates for this isomerization. We therefore knocked out the *xylA* gene to form strain ALS1048 *pfkA zwf xylA* (MEC180). Of course, the *xylA* knockout also renders this strain unable to consume D-xylose, and therefore only the conversion of L-arabinose to D-glucose could be examined. The deletion of xylose isomerase reduced D-glucose yield only slightly to 0.23 g/g (Figure 3.2), and D-mannose was detected at 170-190 mg/L, corresponding to a yield of about 0.03 g/g. These results suggest xylose isomerase does not play a significant role in the formation of both D-glucose and D-mannose from pentoses.

We next examined one possible route through which D-glucose could be utilized. The *gcd* gene encoding glucose dehydrogenase (EC 1.1.5.2) is able to convert D-glucose into D-glucono-1,5-lactone which can then spontaneously form gluconate (45), although pyrroloquinoline quinone appears to be necessary for this conversion in *E. coli* (31). In order to determine whether D-glucose accumulation is influenced by glucose dehydrogenase, we constructed ALS1048 *pfkA zwf gcd* (MEC178). MEC178 formed D-glucose from D-xylose (yield of 0.28 g/g) or from L-arabinose (0.30 g/g), and also formed about 110-150 mg/L D-mannose from either pentose (0.03 g/g), indicating that this route does not significantly affect hexose formation (Figure 3.2).

We next examined whether the formation of D-glucose was the result of the hydrolysis of either D-glucose-1P or D-glucose-6P. D-Fructose-6P is converted to D-

glucose-6P by glucose-6P isomerase encoded by *pgi*, D-glucose-6P is converted to D-glucose-1P by phosphoglucomutase encoded by *pgm*, and D-glucose-1P can be dephosphorylated by D-glucose 1-phosphatase encoded by *agp* (Figure 3.1). The D-glucose yield was unchanged as a result of the *agp* knockout (ALS1048 *pfkA zwf agp*, MEC152), and was 0.18-0.20 g/g with a *pgm* knockout (ALS1048 *pfkA zwf pgm*, MEC319). Both MEC152 and MEC319 accumulated 130-170 mg/L D-mannose from 5 g/L of either pentose. However, D-glucose and D-mannose formation were completely eliminated as a result of the *pgi* knockout (ALS1048 *pfkA zwf pgi*, MEC321, Figure 2). Because MEC319 showed a slight reduction in D-glucose yield, the results do not exclude the possibility of some D-glucose-1P hydrolysis resulting in D-glucose formation, although the lower observed glucose yield in MEC319 compared to MEC143 could also be simply due to the cells' reduced ability to form necessary metabolites from D-glucose-1P and UDP-D-glucose. The complete elimination of D-glucose formation as a result of a *pgi* deletion supports the conclusion that the hydrolysis of D-glucose-6P is the principal final step by which D-glucose is formed from pentoses.

Two additional experiments were conducted to confirm the role of *pgi* in D-glucose formation. First, because the *pgi* knockout blocks D-glucose-6P formation (Figure 1), the *zwf* knockout should not affect D-glucose formation in a *pgi* knockout. In other words, the *ptsG manZ glk pfkA pgi* strain should also be unable to accumulate D-glucose. We therefore examined ALS1048 *pfkA pgi* (MEC320), and indeed observed no D-glucose formation from either D-xylose or L-arabinose (Figure 3.2). Second, we transformed both MEC320 and MEC321 with pTrc99A-*pgi* expressing native glucose-6P isomerase, and these strains regained the ability to accumulate D-glucose from either D-

xylose or L-arabinose. MEC320 pTrc99A-*pgi* attained a yield of 0.09 g/g and MEC321 pTrc99A-*pgi* attained a yield of 0.13 g/g.

Interestingly, knockout strains which generated more than 0.05 g/g D-glucose accumulated significantly more D-glucose from L-arabinose than from D-xylose ($p<0.10$, Figure 3.2) except MEC178 (ALS1048 *pfkA zwf gcd*) for which there was no significant difference. For example, MEC143 (ALS1048 *pfkA zwf*) generated 27% more D-glucose from L-arabinose than from D-xylose.

Finally, we confirmed D-glucose was the biological product from both pentoses by comparing the NMR spectra of D-glucose and D-glucose-6P, and also by demonstrating that D-glucose could not have formed from D-glucose-6P by chemical hydrolysis within the medium nor during the HPLC method at the temperatures used (Figure 3.3). The NMR results confirm that extracellular D-glucose and not D-glucose-6P was the biological product of D-xylose or L-arabinose metabolism in these knockout strains.

Batch process to accumulate glucose

The previous experiments were all conducted in shake flasks using 5 g/L L-arabinose or D-xylose. We next conducted an experiment using MEC143 in a controlled bioreactor with approximately 20 g/L D-xylose, to determine if a proportionate increase in D-glucose (and D-mannose) accumulation would be observed. In this batch run, about 4.4 g/L D-glucose and 0.61 g/L D-mannose were formed in 25 h for an observed mass yield from D-xylose of 0.21 g D-glucose/g and 0.03 g D-mannose/g (Figure 3.4). Furthermore, neither D-glucose nor D-mannose was reassimilated 5 h after D-xylose was exhausted. A nearly proportionate increase in product formation was observed in these

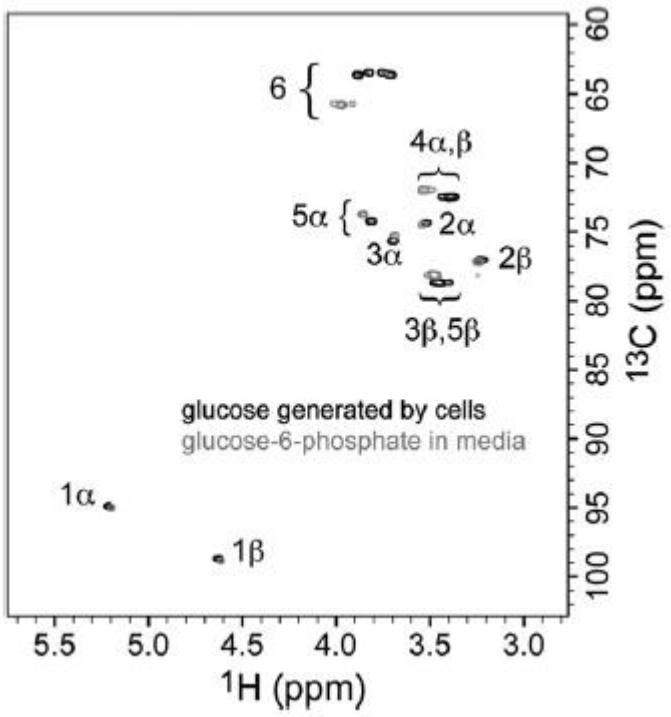

Figure 3.3: Confirmation of D-glucose production from xylose, using an overlay of the two-dimensional, ^{1}H, ^{13}C-HSQC NMR spectra of a sample of product from the pentose (black contours, medium after xylose was exhausted by cells, with D_2O added to 7%) and a sample using authentic glucose-6P in medium (gray contours, with D_2O added to 7%). The molecules are distinguished because the presence of the phosphate group on D-glucose-6P promotes characteristic ^{1}H and ^{13}C chemical shift changes, compared to D-glucose, for the nuclei at positions nearer the site of glucose attachment (positions 6, 5, and 4, with smaller changes at 3, 2 and 1). Comparison with spectra of authentic D-glucose (not shown) confirm the identities. The data demonstrate that D-glucose is the fermentation product from xylose, and that D-glucose does not form by extracellular hydrolysis of D-glucose-6P under the conditions of the experiments.

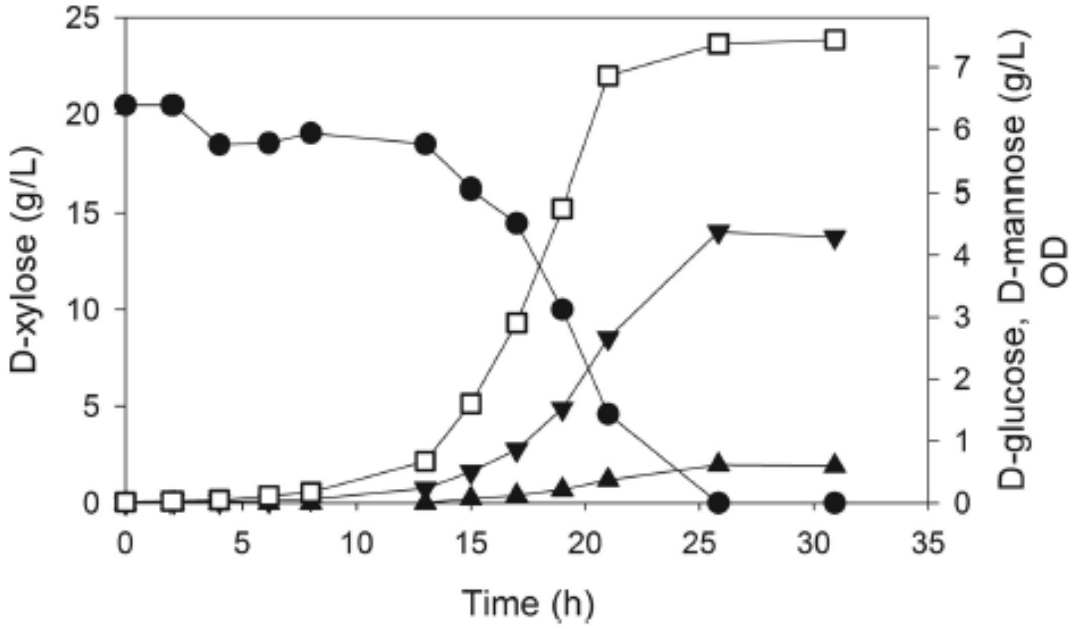

Figure 3.4: Accumulation of D-glucose and D-mannose by *E. coli* MEC143 (MG1655 *ptsG manZ glk pfkA zwf*). Cell density is measured as optical density (□), D-glucose (▼), D-mannose (▲), D-xylose (●).

controlled processes compared to the shake flask studies, suggesting that D-glucose formation is not inhibited nor repressed by D-glucose accumulation. This result also demonstrates a potential for generating substantial quantities of 6-carbon hexoses from 5-carbon pentoses.

Continuous processes to accumulate glucose

Chemostats are a convenient tool to study microbial growth and product formation under nutrient-limited conditions. During the batch process previously studied the cells were grown under nutrient-excess conditions, and we reasoned that carbon flux might be maximal if the cells were grown under conditions for which growth was limited by a nutrient other than carbon. Furthermore, being at steady-state and at a controlled growth rate, a chemostat would demonstrate whether the D-glucose observed is formed as a transient product or only during maximal cell growth. We therefore next grew MEC143 under nitrogen-limiting conditions by increasing the concentration of D-xylose and decreasing the concentration of the nitrogen source (see Materials and Methods). At four different dilution rates (D=0.08 h^{-1} to 0.15 h^{-1}), the observed yields averaged 0.26 (±0.08, standard deviation) g D-glucose/g D-xylose and 0.23 (±0.03) g dry cells/g D-xylose, and these values did not vary with dilution rate. The mean carbon recovery was 108% (±11%), 2.3-3.3 g/L D-xylose and less than 0.5 mg/L N were detected in the effluents. These results demonstrate that D-glucose formation is not a transient phenomenon. Since the yield during the nitrogen-limited chemostats where similar to yields observed in batch processes, D-glucose does appear to form as an overflow metabolite.

Discussion

During growth on D-xylose or L-arabinose, wild-type *E. coli* generates 2 moles of D-fructose-6P and 1 mole D-glyceraldehyde-3P from 3 moles of either pentose (Eq. 1). If the glycolytic pathway is complete, the 2 moles of D-fructose-6P formed via Eq. 1 readily generates 4 moles of D-glyceraldehyde-3P. Indeed, because the conversion of D-fructose-6P to D-glyceraldehyde-3P is readily accomplished in widely-studied microorganisms, D-fructose-6P is typically not thought of as an intermediate of pentose metabolism. However, our results demonstrate that *E. coli* can direct this metabolic intermediate D-fructose-6P into other 6-carbon (i.e., hexose) end-products such as D-glucose when three conditions are met.

A first condition for the accumulation of products derived from pentoses via D-fructose-6P is that glycolysis must be disrupted between D-fructose-6P and D-glyceraldehyde-3P. By blocking glycolysis, the pentose phosphate pathway essentially becomes a branched pathway during the metabolism of D-xylose or L-arabinose with two separate products, D-fructose-6P and D-glyceraldehyde-3P (Figure 3.1). That is, when D-fructose-6P cannot enter glycolysis it becomes available for the formation of other 6-carbon products, while the D-glyceraldehyde-3P remains available for the generation of ATP, NADH and the precursors that exist metabolically "below" D-glyceraldehyde-3P via the terminal steps of glycolysis and the tricarboxylic acid cycle. In *E. coli* the entry of D-fructose-6P into glycolysis can be blocked by a deletion in the *pfkA* gene.

A second condition to facilitate the accumulation of hexoses from pentoses is that metabolites should be prevented from re-entering the PP pathway, for example, from D-glucose-6P. In *E. coli* the re-entry of D-glucose-6P into the PP pathway can be prevented

by a knockout of the *zwf* gene (Figure 3.1). Finally, as a third condition the ultimate product must be excreted and should not be re-metabolized. Our results demonstrate that the knockouts in the *ptsG*, *manZ* and *glk* genes effectively block D-glucose metabolism and at least curtail its reassimilation once generated.

E. coli MEC143, which met these three conditions, accumulated significant D-glucose from either of two pentoses, L-arabinose or D-xylose. Interestingly, D-glucose was also observed, but to a much lesser extent, when glycerol or D-fructose was the sole carbon source in the same strain, probably as a result of the formation of a small quantity of D-fructose-6P generated via the non-oxidative PP pathway. Another sugar derived from D-fructose-6P, D-mannose, was also consistently observed as a by-product of D-glucose formation. D-Mannose likely accumulated as a result of the *manZ* gene deletion in all the strains studied, which prevented the uptake of not only D-glucose but also this sugar.

Of the several knockouts examined to clarify the route for D-glucose formation from the intermediate D-fructose-6P, only a deletion of the *pgi* gene coding glucose-6P isomerase eliminated D-glucose formation. Although this result implicates D-glucose-6P as the direct precursor to extracellular D-glucose, we do not establish how D-glucose-6P itself is hydrolyzed. Unfortunately *E. coli* has numerous candidate enzymes that could hydrolyze D-glucose-6P: a periplasmic acid phosphatase (37), an alkaline phosphatase (23) and eight different haloacid dehalogenase-like hydrolases (27) have all been observed to hydrolyze D-glucose-6P under various environmental conditions. Each of these enzymes might mediate the final step to D-glucose during growth on D-xylose or L-arabinose.

All strains in this study had knockouts in the *ptsG*, *manZ* and *glk* genes encoding proteins involved in the principal means for D-glucose uptake in *E. coli* (10). There is no report of these proteins being involved in D-glucose export, and our results provide no guidance to this process. *E. coli* has several known porins and permeases that can translocate D-glucose through the outer and cytoplasmic membranes (though previous studies have invariably focused on sugar *import*). For example, porins OmpF and OmpC transport D-glucose by passive diffusion across the outer membrane (35, 36). LamB functions as a broad specificity glycoporin which transports D-glucose among other mono- and polysaccharides (30). Galactose permease (GalP) readily transports D-glucose across the cytoplasmic membrane (32). In fact, for strains deficient in the PTS uptake system (*ptsH, ptsI, crr* knockouts), GalP very effectively replaces the transport functions of the $IICB^{Glc}$ PTS protein (19). Similarly, the periplasmic D-glucose/D-galactose binding receptor protein encoded by *mglB* binds D-glucose (25, 34) and contributes significantly to the growth and transport affinity for D-glucose at low extracellular D-glucose concentrations (24). Additionally, *E. coli* responds to knockouts in transport genes: the expression of the permeases *galP, mglB* and *lamB* increased as a result of a *ptsHIcrr* knockout (20). The mechanisms of D-glucose uptake under D-glucose-limiting and -excess conditions have been reviewed (18), and the various proteins involved in D-glucose uptake have recently been examined collectively in *E. coli* in the context of overflow metabolism and vaccine production (21). Interestingly, GluP has been implicated in D-glucose export in *Bacillus subtilis* (33), but we found no similar *E. coli* protein. In our current study, any of these or other proteins could also be involved in D-glucose excretion.

A consistent result was that a greater yield of D-glucose was attained from L-arabinose than from D-xylose, and this difference must result from differences in the metabolism of these two pentoses by *E. coli*. Interestingly, D-xylose and L-arabinose are transported and enter the PP pathway through different routes in *E. coli*. D-Xylose is transported by several routes: a D-xylose/proton symporter (28), an ATP-binding dependent system (1) and by promiscuous transporter activity (26). The ATP-dependent system appears to predominate under normal growth conditions (22), indicating that D-xylose uptake generally demands energy directly in the form of ATP. Cellular options for the transport of L-arabinose similarly include a high affinity ATP-dependent system and a low affinity proton symport (11), as well as promiscuous transport (13). For this pentose the low affinity, ATP-independent system appears to predominate when both systems are present and the L-arabinose concentration is relatively high (11), although this process presumably affects availability of ATP also. After cellular uptake, both of these pentoses are ultimately converted to the common intermediate D-xylulose-5P (Figure 1), through steps which require ATP for phosphorylation via D-xylulokinase or L-ribulokinase respectively for D-xylose or L-arabinose. Since the metabolism of these two pentoses would appear identical after D-xylulose-5P, one might speculate that the difference in yield D-glucose yield between the two pentoses might be due to the difference in sugar transport mechanisms. Additional studies will have to clarify this difference.

If 2 moles of D-fructose-6P generated from 3 moles of D-xylose or L-arabinose (Eq. 1) are available for D-glucose formation (corresponding to 0.67 mol/mol), the theoretical D-glucose mass yield from either pentose is 0.80 g/g. This calculation

considers the D-glyceraldehyde-3P generated from the flux-balanced PP pathway to be unavailable for D-glucose formation because additional D-glyceraldehyde-3P cannot re-enter the PP pathway without consuming D-fructose-6P. On the other hand, inclusion of the hypothetical conversion of D-glyceraldehyde-3P through the reverse Embden-Meyerhof-Parnas pathway to D-fructose-6P would result in a theoretical maximum yield 1.0 g/g. The greatest yield observed in the current study was about 0.3 g/g, a result probably due to the assimilation of some of the intermediate monosaccharides by other enzymes present in *E. coli* and not deleted in this study, and by the reversible enzymes in the PP pathway (transaldolase and transketolase) which would limit D-fructose-6P formation if these reactions approached equilibrium. Although not likely to serve as a process for generating the specific hexose D-glucose, this work demonstrates an approach to convert 5-carbon saccharides into 6-carbon saccharides, which could thereby both build carbon length and generate hexoses derived from D-fructose-6P not possible under typical conditions during growth on D-glucose.

Our results highlight two other aspects of metabolism in strains that have deletions in D-glucose uptake and other genes in upper metabolism. First, we detected no D-fructose as a product in any of the experiments, and our shake flask study with MEC143 growing on D-fructose yielded only a low concentration of D-glucose. The absence of D-fructose in the (extracellular) medium is likely because *E. coli* uses a D-fructose-specific phosphotransferase system (*fruA* and *fruB* genes) and D-fructose-1P kinase (*fruK*) to metabolize D-fructose to D-fructose-1,6P_2, bypassing D-fructose-6P. In other words, assimilation of D-fructose via this route bypasses D-fructose-6P and would also prevent D-fructose accumulation in the strains studied. In contrast, once D-glucose is

transported out of the cell, deletions of the *ptsG* and *manZ* genes prevent its uptake. A second noteworthy result from the mutants studied lies in the absence of D-glucose repression. For example, previous research has demonstrated that xylose isomerase is repressed in the presence of D-glucose (4). This effect appears to be caused specifically by D-glucose catabolite repression (8), an occurrence requiring the active catabolism of D-glucose, and which therefore is avoided in a strain unable to metabolize D-glucose. In our batch process accumulating nearly 5 g/L D-glucose (Fig. 3.3), we did not observe any deceleration of D-xylose utilization: the cells acted as though D-glucose was not present. So, *E. coli* is able to metabolize D-xylose in the presence of D-glucose when the D-glucose is not being metabolized.

In conclusion, D-glucose formation from either L-arabinose or D-xylose occurs as a result of the PP pathway leading to D-fructose-6P, which, unable to proceed into the glycolytic pathway due to a knockout in *pfkA*, equilibrates to D-glucose-6P. D-Glucose-6P likely hydrolyzes by one of several possible enzymes to D-glucose, which then accumulates when the cells are unable to metabolize it. We envision an analogous route could be used to generate similar sugars or sugar-containing compounds.

Acknowledgments

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CHAPTER 4

QUERCETIN GLUCOSIDES PRODUCTION BY ENGINEERED *ESCHERICHIA COLI*[3]

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Abstract

Quercetin is a common flavonoid present in tea, wine, apples and onions. Quercetin glucosides not only exhibit antioxidant and anticancer activity but also have much greater bioavailability than the aglycone. This study examined the accumulation of quercetin glucosides by *Escherichia coli* expressing *Arabidopsis thaliana* UDP-glycosyltransferases UGT73B3 and UGT84B1. *Escherichia coli* MG1655 expressing UGT73B3 generated 0.33 g/L quercetin-3-glucoside during growth in shake flask culture using D-glucose as a sole carbon source. In controlled bioreactors quercetin-3-glucoside concentration was increased to 0.72 g/L. An additional knockout in the gene encoding phosphoglucose isomerase successfully diverted the carbon flux toward sugar donor accumulation and elevated the glucoside production to 1.55 g/L. Under optimized controlled batch conditions with 30 g/L D-glucose and 5 g/L quercetin, MEC371 generated 3.8 g/L quercetin-3-glucoside, with yields of 0.5 mol/mol quercetin and 49 mmol/mol glucose. The results demonstrate an approach using both genetic manipulation and process optimization to glycosylate organic compounds in high yields using engineered *Escherichia coli*.

Key Words: glycosylation, glucose-6-phosphate isomerase, glucose-6-phosphate 1-dehydrogenase

Introduction

Flavonoids consist of a large group of natural polyphenols, including quercetin which is commonly found in fruits, vegetables, and cereals including apples, onions, tea and wine (Hertog et al., 1993; Picinelli et al., 1997; Price and Rhodes, 1997; Neveu et al., 2010). Quercetin possesses several beneficial biological activities including inhibition of cancer cells (Choi et al., 2008; Luo et al., 2008; Jeong et al., 2009), anti-oxidative effects (Robak and Gryglewski, 1988), anti-inflammatory activities (Erden Inal and Kahraman, 2000), as well as vasodilation and lowering of blood pressure (Duarte et al., 2001; Sanchez et al., 2006; Yamamoto and Oue, 2006). Quercetin is used in several countries for blood vessel protection and is an ingredient of some multivitamin preparations and herbal remedies (Kelly, 2011). However, the low water solubility of quercetin limits the in vivo availability and use as a food additive or dietary supplement (Gugler et al., 1975; Makino et al., 2009).

Quercetin contains five hydroxyl groups, and O-glycosylation at any of these locations increases the water solubility several-fold (Makino et al., 2009), resulting in glucosides which exhibit greater bioavailability than the aglycone quercetin (Hollman et al., 1995, 1996, 1997; Gee et al., 2000; Crespy et al., 2001). For example, 2-5 fold greater concentrations of quercetin metabolites were found in the liver, lung, heart, kidney, brain and plasma of rats fed with quercetin-3-glucoside (Q3G) compared to rats whose diets were supplemented with quercetin (Paulke et al., 2012). In addition to greater bioavailability, quercetin glucosides benefit human health similar to the aglycone. For example, Q3G suppresses growth of various colon cancer cell lines (SW480, DLD-1, and HCT116) *in vitro* by targeting the Wnt/β-catenin signaling pathway, whereas the

glucoside exerts no effect on non-tumor colon IEC-18 cells (Amado et al., 2014). Q3G reduces the proliferation of human liver cancer cells (HepG2) by inhibiting DNA topoisomerase II, while being less toxic to non-cancer cells than a reference chemotherapy drug (Sudan and Rupasinghe, 2014). Q3G furthermore improves insulin sensitivity and reduces plasma total cholesterol levels in rats (Phuwamongkolwiwat et al., 2014), blunts peroxide-induced oxidative stress in RGC-5 cells (Jung et al., 2010), attenuates lipid peroxidation (Jung et al., 2010) and inhibits growth of breast cancer cells (Yang et al., 2009). Other quercetin glucosides have similar biological activities (Day et al., 2003; Cermak et al., 2004; Song et al., 2013).

Although quercetin glucosides are widely distributed in nature, their concentrations in plant material are generally low, and therefore extraction of quercetin glucosides directly from plants are not a suitable source for obtaining large amounts (Lu et al., 2013). Quercetin glucoside is generated from the acidic and enzymatic hydrolysis of another compound rutin (quercetin-3-O-rutinoside), extracted from buckwheat (Kreft et al., 1999; Kim et al., 2005; Wang et al., 2011). This hydrolysis typically yields a mixture of Q3G and quercetin. Biocatalytic formation of quercetin glucosides using glucosyltransferases such as UGT73B3 and UGT84B1 requires equimolar quantities of expensive UDP-glucose (Fig. 4.1). Using whole cells is therefore a promising way to produce quercetin glucosides, because cell metabolism can provide a continual supply of UDP-glucose and the bacterium *Escherichia coli* serves as a convenient microbial cell factory to glycosylate compounds regioselectively. Previous research has established the effectiveness of using *E. coli*: expressing a *Medicago truncatula* UGT71G1 Tyr202Ala mutant in a complex medium converted 34 mg/L quercetin into 20 mg/L Q3G in 48 h (He

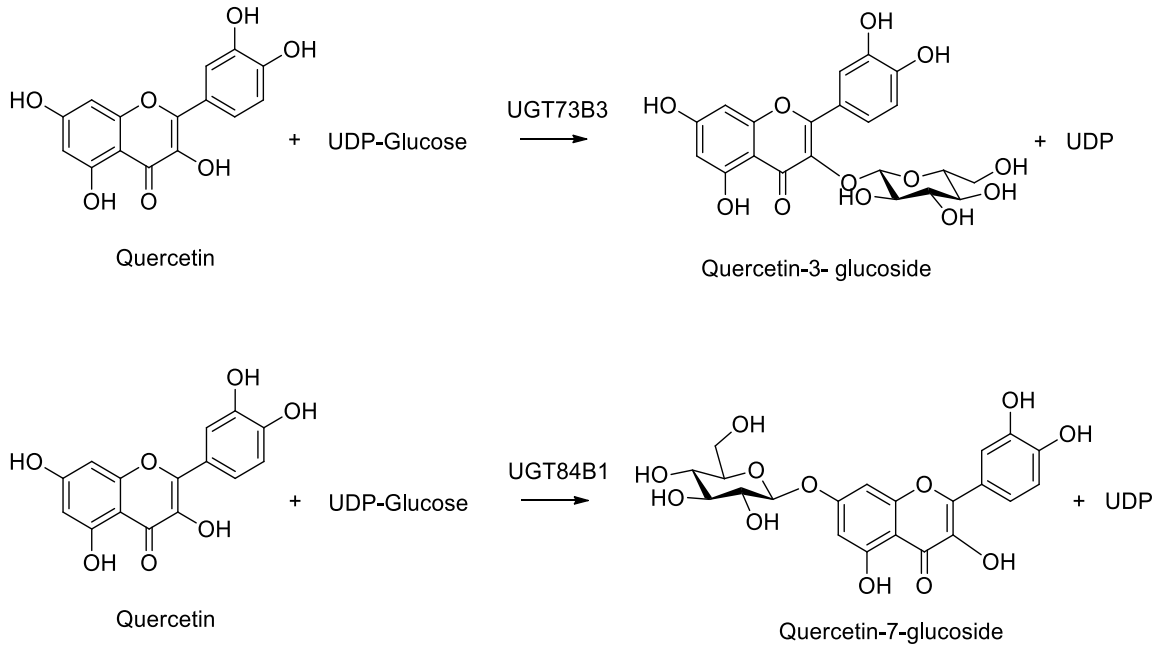

Figure 4.1: Quercetin glycosylation catalyzed by UDP-glycosyltransferase (Lim et al., 2004).

et al., 2008); expressing *Oryza sativa* glucosyltransferase cDNA RF5 converted 34 mg/L quercetin into 46.4 mg/L Q3G after 6 h (Kim et al., 2006); and expressing *Arabidopsis thaliana* UGT73B3 produced 9 mg/L Q3G in shake flasks using a complex medium, and 99 mg/L Q3G in 20 h in a 2 liter controlled bioreactor (Lim et al., 2004).

Accumulation of the sugar donor UDP-glucose metabolically generated in *E. coli* from glucose-6P (Fig. 4.2) is the key to improve the production of quercetin glucosides. Although *E. coli* synthesizes UDP-glucose, its intracellular concentration is usually 1-2 mM (Mao et al., 2006). Metabolic engineering directed at increasing the accumulation of glucose-6P, the precursor of UDP-glucose, would likely enhance the formation of the sugar donor and hence quercetin glucosides in a cell expressing glucosyltransferases. For example, *E. coli* with deletions in genes coding phosphoglucose isomerase (*pgi*) or both phosphoglucose isomerase (*pgi*) and glucose 6-phosphate dehydrogenase (*zwf*) showed substantially elevated intracellular glucose-6P (Lee and Cerami, 1987; Morita et al., 2003), and thus these mutants could elevate formation of products derived from glucose-6P. The double *pgi* and *zwf* deletions significantly increased 2-deoxy-scyllo-inosose yield and D-glucaric acid yield (Kogure et al., 2007; Shiue et al., 2015). Of course, overexpressing enzymes involved in the conversion of glucose-6P to UDP-glucose may also improve UDP-glucose formation. The co-overexpression of phosphoglucomutase (*pgm*) and UDP-glucose pyrophosphorylase (*galU*) increased the yield of UDP-sugar-derived disaccharides (Mao et al., 2006).

The objective of the current study is to use *E. coli* strain construction and process development to generate high concentrations of quercetin glucosides from quercetin. We

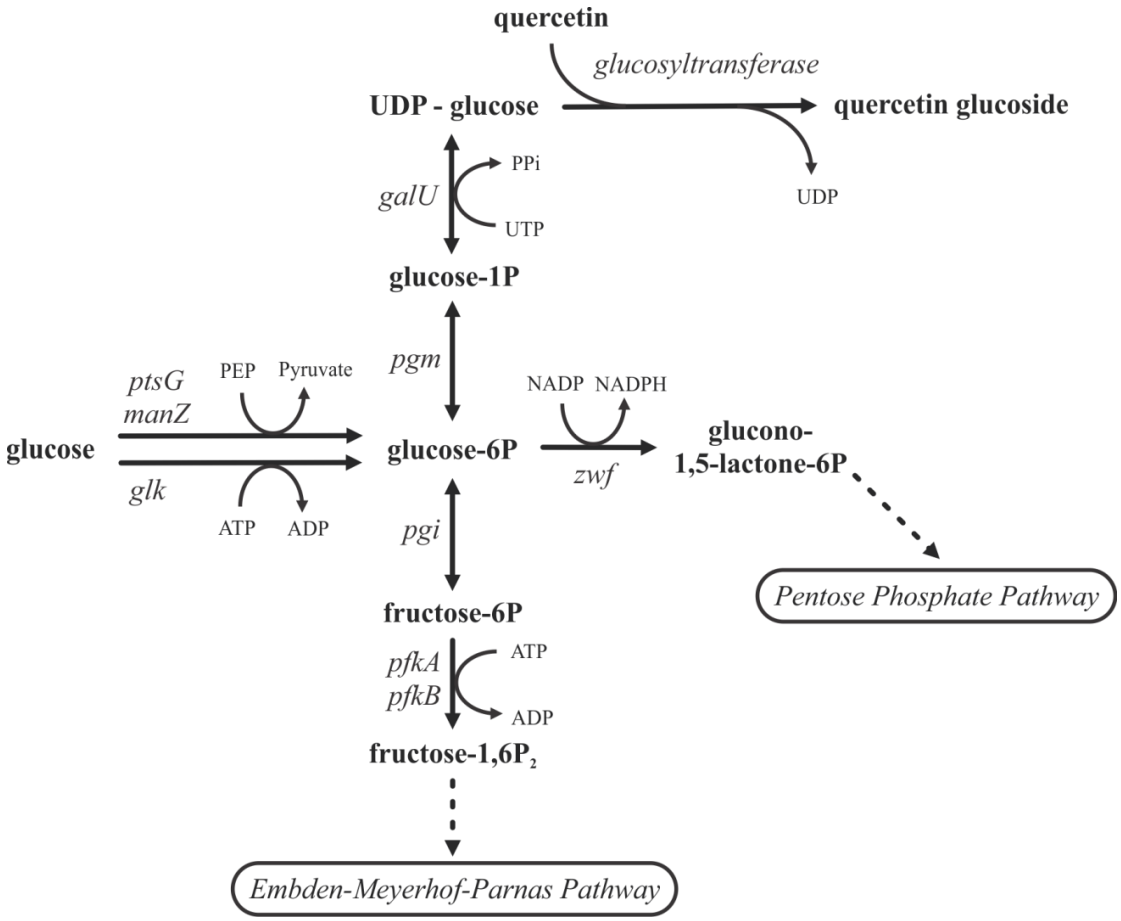

Figure 4.2: Metabolic pathways in *E. coli.* The formation of quercetin glucosides.

first studied the effect of temperature on quercetin glycosylation using two glucosyltransferases. Pathway engineering was then performed to understand the bottlenecks in quercetin glycosylation. Finally, uncontrolled and controlled processes were compared to optimize the production of quercetin glucoside.

Materials and Methods

Strains

E. coli MG1655 (wild-type, F- λ- *ilvG*- *rfb*-50 *rph*-1) was used for this study. Knockouts of *zwf* and *pgi* were constructed by transducing MG1655 with the corresponding Keio (FRT) Kan deletions (Baba et al., 2006), and if necessary to delete a second gene, curing the Kan(R) using the pCP20 plasmid, which contains a temperature-inducible FLP recombinase as well as a temperature-sensitive replicon (Datsenko and Wanner, 2000). All strains were verified by PCR.

Table 4.1. *E. coli* strains used in the study of glycosylation.

Strain	Genotype	Reference
MG1655	F- λ- *ilvG*- *rfb*-50 *rph*-1	wild-type
MEC367	MG1655 Δ*pgi-721*::Kan	This study
MEC368	MG1655 Δ*zwf*-777::Kan	This study

Construction of plasmids

The UGT73B3 and UGT84B1 genes from *Arabidopsis thaliana* were PCR cloned and digested with *EcoRI* and *KpnI*, then subsequently cloned into vector pTrc99A to yield plasmids pTrc99A-UGT73B3 and pTrc99A-UGT84B1. The UGT73B3 gene was PCR amplified with primers 5′-GGGAAAGAATTCATGAGTAGTGATCCTCATCGTAAGCTCCA-3′ (forward) and 5′-GGGAAAGGTACCTTACGAGGTAAACTCTTCTATGAAGCTGT-3′ (reverse), and the UGT84B1 gene was PCR amplified with primers 5′-GGGAAAGAATTCATGGGCAGTAGTGAGGGTCAAG-3′ (forward) and 5′-GGGAAAGGTACCTTAGGCGATTGTGATATCACTAATG-3′ (reverse). To construct the pCS27-*pgm*, pCS27-*galU*, pCS27-*glk* plasmids, the *pgm*, *galU*, and *glk* genes were PCR amplified using *E. coli* BW25113 genomic DNA as the template. The PCR fragments were digested by *KpnI* and *BamHI* and ligated into the *Kpn*I and *BamH*I sites of pCS27, generating the desired plasmids.

Growth Conditions

The defined medium used in all experiments contained (per L): 13.3 g KH_2PO_4, 4.0 g $(NH_4)_2HPO_4$, 1.2 g $MgSO_4 \cdot 7H_2O$, 13.0 mg $Zn(CH_3COO)_2 \cdot 2H_2O$, 1.5 mg $CuCl_2 \cdot 2H_2O$, 15.0 mg $MnCl_2 \cdot 4H_2O$, 2.5 mg $CoCl_2 \cdot 6H_2O$, 3.0 mg H_3BO_3, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, 100 mg Fe(III)citrate, 8.4 mg $Na_2EDTA \cdot 2H_2O$, 1.7 g citric acid, 4.5 mg thiamine·HCl, and carbon source (glucose) as indicated. As appropriate for the strain, the medium contained 100 mg/L ampicillin and 50 mg/L kanamycin.

The temperature range of 20-37 ℃ was first examined to establish the optimal temperature for glycosylation. MG1655/pTrc99A-UGT73B3 or MG1655/pTrc99A-

UGT84B1 was first cultured in a 125 mL flask containing 20 mL medium with 8 g/L glucose. When the optical density (OD) reached 3, 2 mL was used to inoculate a 250 mL shake flask containing 50 mL of the identical medium. These shake flasks were incubated at the desired temperature and 250 rpm (19 mm pitch). When the OD reached 1, 0.5 mM IPTG and 0.9 g/L quercetin dissolved in DMSO were added. All shake flasks were adjusted to an initial pH of 7.0 with 20% NaOH. This same procedure was also used to compare quercetin glycosylation in shake flasks among different strains.

Controlled Batch Fermentations

For bioreactor experiments, a strain was first grown in a 250 mL shake flask containing 50 mL medium at a pH of 7.0 and incubated at 37 ℃ and 250 rpm. When the OD reached 3, the flask contents were used to inoculate a 2.5 L bioreactor (Bioflo 2000, New Brunswick Scientific Co., Edison, NJ) containing initially 1.0 L medium with 8 g/L glucose. Air was sparged at a flow rate of 1.0 L/min, and an agitation of 500 rpm ensured the dissolved oxygen remained greater than 70% saturation. The pH was controlled at 7.0 using 20% NaOH. When OD reached 1, 0.5 mM IPTG and 2 g/L quercetin dissolved in DMSO were added. For microaerobic bioreactor experiments, the strain was cultured under aerobic condition in the first 16 h, then the dissolved oxygen level was decreased to less than 10% saturation by sparging a mixture of N_2 and air.

Analyses

The optical density at 600 nm (OD) was used to monitor cell growth (DU-650 spectrophotometer, Beckman Instruments, San Jose, CA). Liquid chromatography was used to quantify glucose, mannitol, organic acids, and ethanol (Eiteman and Chastain, 1997). To analyze quercetin and quercetin glucosides, a 1 mL sample was centrifuged to

obtain a supernatant and a cell pellet, which was resuspended in 0.2 mL DMSO to extract the flavonoids. The combined supernatant and extracted pellet fractions were centrifuged again, and the supernatant was analyzed by HPLC at 370 nm using a 5-μm C_{18} column (250×4.6mm, Whatman-Partisphere) (Lim et al., 2004). Quercetin glucosides and quercetin were analyzed using a linear gradient of 20% to 80% acetonitrile in H_2O with 0.1% trifluoroacetic acid at 1 mL/min over 60 min.

Results

Effect of Temperature on Glucoside Formation

The glucosyltransferases UGT73B3 and UGT84B1 from *A. thaliana* mediate the biotransformation of quercetin to quercetin-3-glucoside (Q3G) and quercetin-7-glucoside (Q7G), respectively (Fig. 4.1). Previous research demonstrated the product specificity of these enzymes at the low bacterial growth temperature of 20℃ but did not establish the optimal temperature for the whole cell glycosylation of quercetin by *E. coli*. We therefore first completed a set of experiments over the temperature range of 20 - 37℃ expressing these genes individually in *E. coli* MG1655 (Fig. 4.3). The results demonstrated that the optimal temperature for Q3G formation using UGT73B3 was about 33℃, while the formation of Q7G using UGT84B1 increased with increasing temperature over the entire experimental range (Fig. 4.3). With 8 g/L glucose as the sole carbon source and 0.9 g/L quercetin as available substrate for glycosylation, the maximum product concentration was 0.33 g/L for Q3G and 0.095 g/L for Q7G in the wild-type background *E. coli*. Typically, the maximum glucoside concentration was observed a few hours after glucose was exhausted, although the difference between the concentration at the time of glucose exhaustion and the maximum was only about 10%.

Since UGT73B3 consistently generated more glycosylated product, all subsequent studies were conducted at 33 ℃ with UGT73B3.

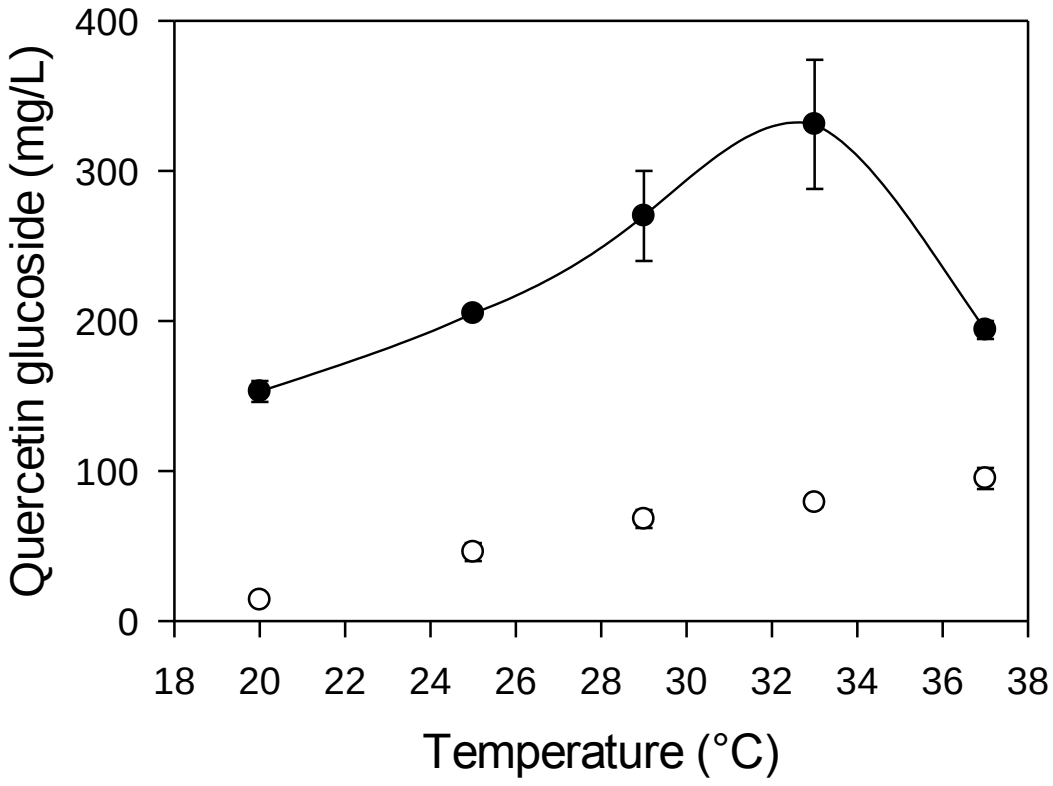

Figure 4.3: Quercetin glycosylation in shake flasks at different temperatures. MG1655/pTrc99A-UGT73B3 (●) and MG1655/pTrc99A-UGT84B1 (○).

Glucoside Formation with Engineered Strains in Shake Flask Culture

UGT73B3 requires UDP-glucose as the sugar donor for glucotransfer, and in *E. coli* UDP-glucose is formed in two enzymatic steps from glucose-6P (Fig. 4.2), a metabolite that is normally directed into the Embden–Meyerhoff–Parnas (EMP) pathway via glucose-6P isomerase (coded by the *pgi* gene) or into the Pentose Phosphate (PP) pathway via glucose-6P 1-dehydrogenase (*zwf*). In a defined medium with 5 g/L glucose as the sole carbon source, the growth rate of MG1655 was 0.73 h^{-1}, the *zwf* deletion slightly reduced the growth rate to 0.69 h^{-1}, whereas the deletion of *pgi* reduced the growth rate to 0.19 h^{-1}.

We next investigated whether reducing bacterial metabolism through each of these pathways would affect the formation of Q3G. Specifically, we compared glucoside formation in MG1655/pTrc99A-UGT73B3, MEC367/pTrc99A-UGT73B3 (*pgi* deletion), and MEC368/pTrc99A-UGT73B3 (*zwf* deletion). The glycosylations in shake flask culture were conducted with 8 g/L glucose and 0.9 g/L quercetin. Both the wild-type expressing UGT73B3 and the *zwf* knockout expressing UGT73B3 accumulated 0.33 g/L Q3G within 24 h, while the *pgi* knockout produced 0.56 g/L Q3G, a 70% increase in Q3G concentration (Fig. 4.4).

The sugar donor, UDP-glucose, is generated in three steps from glucose (Fig. 4.2). Glucose is first transported into the cell and phosphorylated to glucose-6P by a phosphotransferase system (*ptsG* or *manZ*) or by glucokinase (*glk*). Glucose-6P is then converted to glucose-1P by phosphoglucomutase (*pgm*), and glucose-1P is converted to UDP-glucose by UDP-glucose pyrophosphorylase (*galU*). We next investigated whether the overexpression of *glk*, *pgm*, or *galU* would further enhance the production of Q3G.

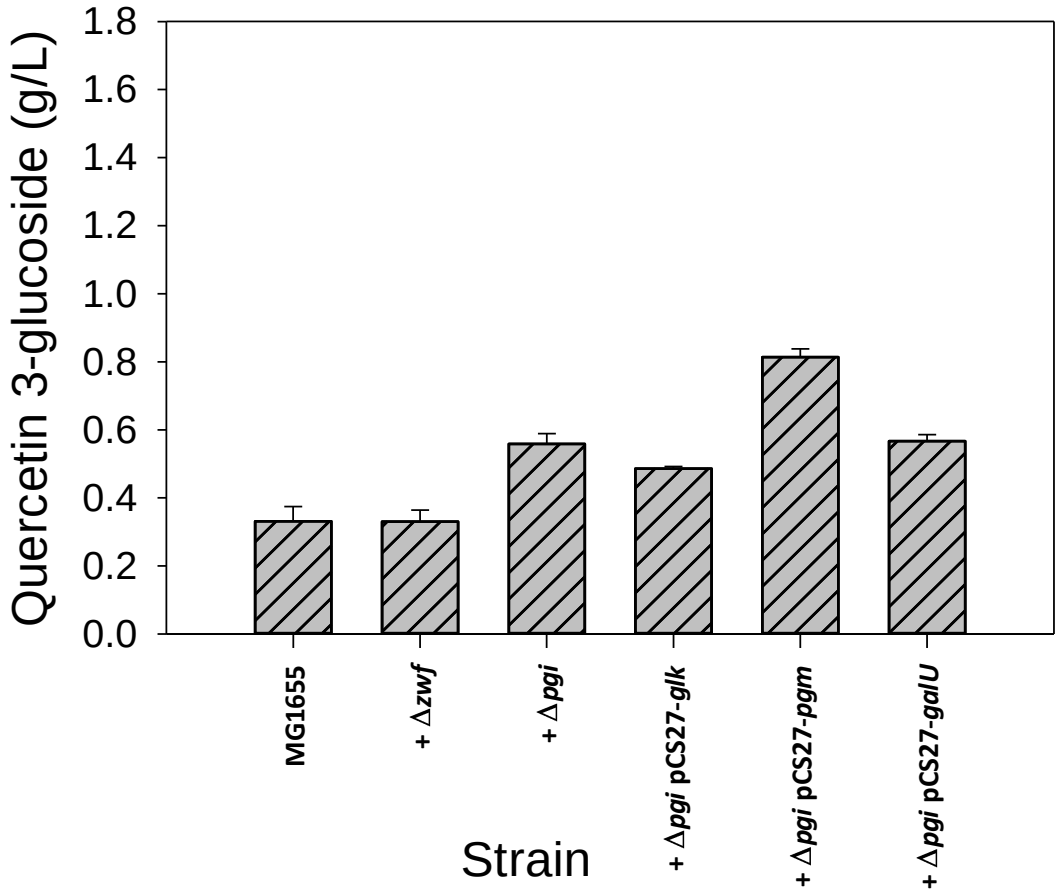

Figure 4.4: Comparison of quercetin-3-glucoside production (shake flask). Different *E. coli* strains were used. Each 50 mL culture was conducted in a shake flask with 8 g/L glucose initially, and 0.9 g/L quercetin was added when the OD reached 1.0.

The plasmids pCS27-*glk*, pCS27-*pgm*, or pCS27-*galU* were individually transformed into MEC367 in addition to pTrc99A-UGT73B3. The glycosylations were again conducted in shake flasks with 8 g/L glucose and 0.9 g/L quercetin. The strain overexpressing *pgm* gene increased Q3G production by 46%, while the overexpression of other two individual genes did not increase the formation of Q3G compared to MEC367/pTrc99A-UGT73B3 (Fig. 4.4).

Glucoside Formation in Controlled Bioreactors

We next sought to investigate whether Q3G production could be further improved in controlled bioreactors. These batch glycosylations were conducted with 8 g/L glucose and 2 g/L quercetin, and the same strain constructs were examined: MG1655/pTrc99A-UGT73B3, MEC367/pTrc99A-UGT73B3 (*pgi* deletion), and MEC368/pTrc99A-UGT73B3 (*zwf* deletion) as well as the *pgi* knockout (MEC367) overexpressing one of the genes *pgm*, *galU*, or *glk* (Fig. 4.5). The wild-type expressing UGT73B3 accumulated 0.72 g/L Q3G within 14 h. The *zwf* deletion did not alter Q3G formation, while the *pgi* deletion elevated the final concentration of Q3G to 1.55 g/L, greater than twice the concentration generated by the wild-type (Fig. 4.5). Although the oxygen was not limited during these controlled cultures, the wild type and the *zwf* knockout additionally generated 0.5 - 0.7g/L acetate as a by-product, while the *pgi* knockout did not generate acetate. In contrast to the shake flask experiments, overexpression of any of the genes *pgm*, *galU* or *glk* did not increase the formation of Q3G compared to MEC367/pTrc99A-UGT73B3 (Fig. 4.5). Most glycosylations in controlled batch cultures had over two-fold increase in Q3G concentration compared to shake flask cultures with the corresponding

strains. MEC367/pTrc99A-UGT73B3/pCS27-*pgm* is the only exception, which accumulated the same amount of Q3G in shake flasks and bioreactors.

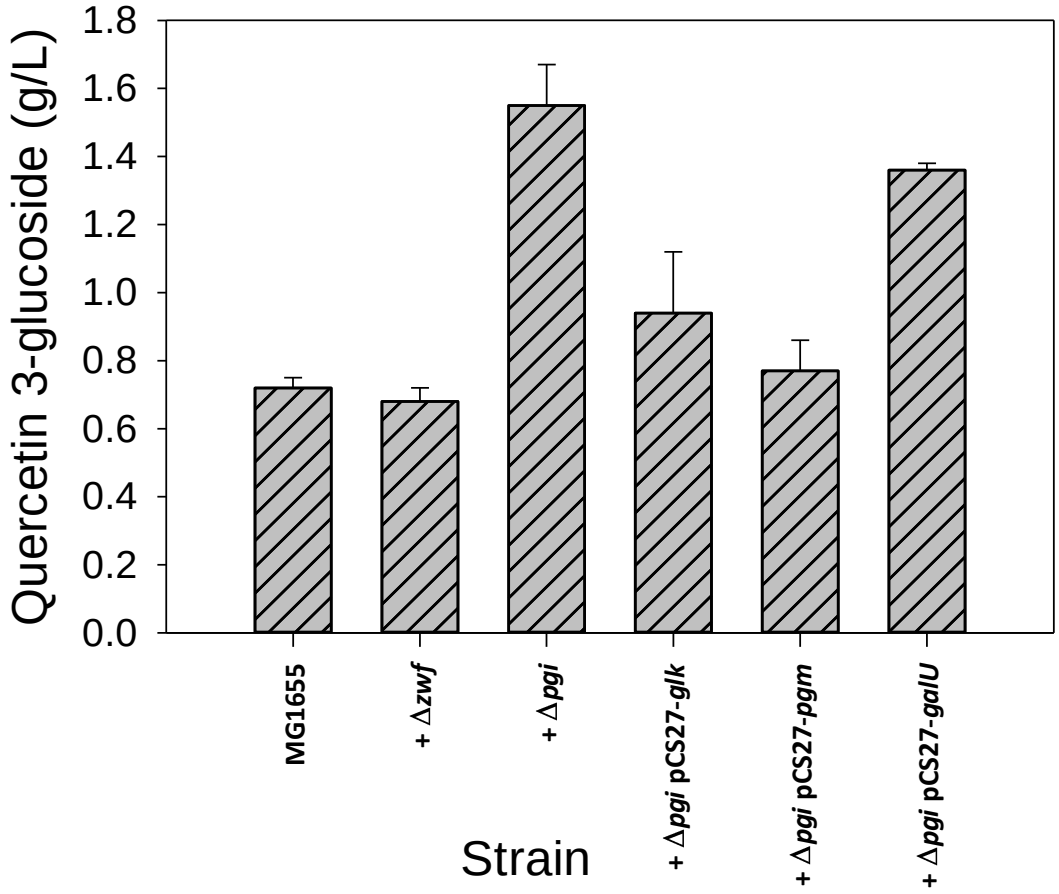

Figure 4.5: Comparison of quercetin-3-glucoside production (bioreactor). Different *E. coli* strains were used. Each 1.0 L process was conducted in a controlled bioreactor with 8 g/L glucose initially, and 2 g/L quercetin was added when the OD reached 1.0.

Since the dissolved oxygen level is a major difference between shake flasks and controlled bioreactors, we conducted a microaerobic experiment in bioreactors by maintaining the DO to less than 10% saturation after the first 16 hours of growth with MEC367/pTrc99A-UGT73B3 (*pgi* deletion). This culture generated 0.61 g/L Q3G, similar to the amount generated in the shake flask experiment. In a batch process with higher concentration of carbon source and substrate, 3.9 g/L Q3G was produced by MEC367/pTrc99A-UGT73B3 (*pgi* deletion) from 30 g/L glucose and 5 g/L quercetin (Figure 4.6).

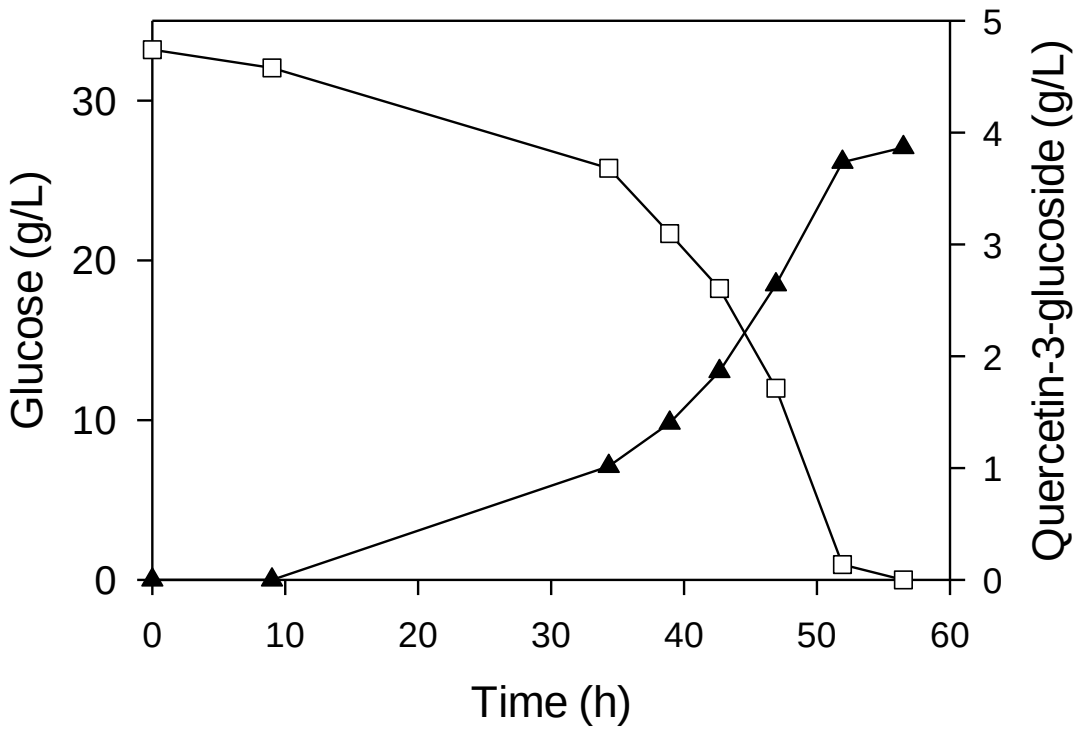

Figure 4.6: *E. coli* MEC367/pTrc99A-UGT73B3 quercetin-3-glucoside production. Quercetin-3-glucoside (▲). The 1.0 L process was conducted in a controlled bioreactor with 30 g/L glucose (□**)** initially, and 5 g/L quercetin was added when the OD reached 1.0.

Discussion

In the current study, quercetin-3-glucoside (Q3G) and quercetin-7-glucoside (Q7G) were formed from their aglycone quercetin using glucosyltransferases UGT73B3 and UGT84B1 respectively. We first examined the glucoside production in shake flasks using wild-type *E. coli* expressing these enzymes over the range of 20 ℃ – 37 ℃. Surprisingly, the results were significantly different for the two glycosylations, with UGT73B3 showing a distinct maximum in Q3G formation at about 33 ℃ while Q7G formation by UGT84B1 increased over the entire temperature range. Previous research used 20 ℃ for glucoside formation in shake flasks using a complex medium (Lim et al., 2004), and these conditions generated about 9 mg/L Q3G and 4.8 mg/L Q7G, respectively. Our shake flask studies using defined medium at this same temperature resulted in 32-fold greater Q3G (153 mg/L) and 3.5-fold greater Q7G (17 mg/L) than these previous results. Moreover, compared to results at 20 ℃, we observed 2.2-fold greater Q3G at 33 ℃ (330 mg/L) and 5.6-fold greater Q7G at 37 ℃ (95 mg/L). Our results not only demonstrate the value of temperature optimization to improve glycosylation by whole-cell catalysis but also indicate that glucosyltransferases behave differently with temperature. We can rationalize the effect of temperature by noting that glucoside formation from glucose and quercetin will not only depend on the activity and denaturation of the specific glucosyltransferase relative to other enzymes competing with substrates in this pathway, but glucoside formation will also depend on the cell growth rate, the availability of UTP to charge the sugar donor UDP-glucose, and on the equilibrium between glucose-6P and glucose-1P. Since the isomerization of glucose-6P to glucose-1P is thermodynamically unfavorable with $\Delta G^{\circ\prime} = 7.1$ kJ mol^{-1} (Berg et al.,

2002), yielding an equilibrium constant of 0.057 (at 25 ℃), increasing temperature favors glucose-1P formation slightly.

The medium and carbon source selection likely also affected glucoside formation. In previous research, LB medium was usually selected to investigate the performance of various enzymes on quercetin glycosylation (e.g., Lim, et al., 2004). By using defined medium with glucose as sole carbon source, cells were able to generate glucose-6P directly. A previous study illustrated that when *E. coli* grows on defined medium with glucose as the carbon source, enzymes of the PTS (e.g. PtsHI, Crr, and ManX) and enzymes of the upper glycolytic pathway were up-regulated compared to the cells growing in rich medium such as LB or Terrific Broth (Li et al., 2014).

Bioconversion of quercetin using various strains generally accumulated twice the amount of Q3G in controlled bioreactors as in shake flasks (Fig 4.4 and 4.5). Substrate quercetin availability was not the limiting factor. Quercetin was always excess in both the shake flask and the bioreactor experiments initiated with the same amount of glucose, although bioreactor experiments started with higher quercetin concentrations than shake flask experiments did. 0.9 g/L quercetin could theoretically generate 1.38 g/L Q3G. However, most shake flask cultures attained less than 40% of this theoretical amount. The shake flask culture of all the other strains accumulated just less than 70% of the maximal possible Q3G concentration as attained by MEC367 pCS27-*pgm*. Meanwhile, residual quercetin was detected by HPLC analysis in all shake flask cultures. MEC367 pCS27-*pgm* grew about 30% slower than MEC367, MEC367 pCS27-*glk*, and MEC367 pCS27-*galU*. Because lower glucoside yield in shake flask culture can be at least partly attributed to lower oxygenation, the lower oxygen demand in slow-growing MEC367

pCS27-*pgm* could have led to greater oxygen availability for this strain and comparatively greater glucoside formation. In a controlled bioreactor, a fully aerobic process was attainable for all strains, and under these circumstances MEC367 pCS27-*pgm* no longer outperformed the other strains.

The results demonstrate that oxygenation is important to optimal glucoside formation. The fully oxygenated process attained a greater Q3G concentration than the microaerobic process, which itself was comparable to Q3G production in shake flasks. In one experiment in which oxygen supply was inadvertently halted temporarily (data not shown), we also observed that less than 0.63 g/L of Q3G accumulated in 63 h, compared to the fully oxygenated run during which 2.8 g/L Q3G was generated in 40 h. We speculate that accumulation of UDP-glucose, which serves as the sugar donor for glucoside formation, is coupled with UTP regeneration, which is limited by ATP availability. ATP synthesis is affected by oxygen supply, because aerobic growth of *E. coli* generates about 2.5 times more ATP than anaerobic growth (Chen et al., 2011).

The glucose donor UDP-glucose is derived from glucose via glucose-6P, and blocking glucose-6P metabolism through glycolysis or the PP pathway would benefit UDP-glucose accumulation. A *pgi* knockout blocks glycolysis between glucose-6P and fructose-6P, and with this knockout the PP pathway becomes the primary pathway to metabolize glucose-6P (Fig. 4.2). A *zwf* knockout prevents glucose-6P from entering the PP pathway, and in this case glucose-6P is only metabolized through EMP pathway. We observed a significantly reduced growth rate in the *pgi* knockout, while the *zwf* knockout did not alter the specific growth rate, consistent with previous research results (Canonaco et al., 2001; Fischer and Sauer, 2003). Chemostat cultures of wild type *E. coli* on minimal

medium with glucose showed a metabolic flux distribution of 80% of the glucose entering glycolysis and 20% entering the PP pathway, and the enzyme activity of phosphoglucose isomerase was 3.6 fold higher than that of glucose 6-phosphate dehydrogenase (Zhao et al., 2004). In addition, NADPH competitively inhibits glucose 6-phosphate dehydrogenase (Olavarría et al., 2012). Thus, PP pathway probably has limited capacity to metabolize glucose-6P, and the reduced glycolytic flux for the *pgi* mutant increased the glucose-6P pool, as reported by others (Lee and Cerami, 1987; Morita et al., 2003). Furthermore, decreased glucose uptake increases phosphorylated EIIA (EIIA-P), and thus cAMP-Crp increases, which in turn activates TCA cycle; while the decreased fructose-1,6-diphosphate concentration activates Cra activity, which in turn activates glyoxylate pathway enzymes. The decreased acetate accumulation in the *pgi* knockout can thus be attributed to these regulatory changes compared to the parent strain (Hua et al., 2003; Toya et al., 2010; Yao et al., 2011; Usui et al., 2012), which is consistent with the results in this study. Obviously, the *pgi* deletion played a pivotal role in sugar donor accumulation, not only by blocking the main pathway involved in glucose-6P metabolism but also by eliminating *E. coli* overflow metabolism that affects heterologous protein expression (Eiteman and Altman, 2006).

Overexpression of phosphoglucomutase (*pgm*), UDP-glucose pyrophosphorylase (*galU*), or glucokinase (*glk*) did not improve the formation of quercetin glucoside. Individually, overexpression of *pgm* or *glk* actually decreased the final concentration of glucoside. The k_{CAT} value of phosphoglucomutase for glucose-6P and UDP-glucose pyrophosphorylase for glucose-1P have not been reported. Overexpression of *pgm* alone might have no effect simply because glucose-6P and glucose-1P are already at an

(unfavorable) equilibrium in a *pgi* knockout growing on glucose. If overexpression of *pgm* does promote glucose-1P accumulation, the glucose-1P may activate competing pathways such as its conversion to amylose and glycogen via glucose-1P adenylyltransferase and glycogen synthase. *E. coli* strains deficient in phosphoglucomutase synthesize significantly more amylose than wild type when grown on galactose or maltose, due to accumulation of glucose-1P (Brautaset et al., 1998, 2000). Overexpression of glucokinase could increase the phosphorylation of glucose, but its overexpression might also accelerate ATP consumption, which may affect UTP regeneration and coupled UDP-glucose formation. Like many prokaryotes and eukaryotes, *E. coli* naturally regenerates UTP from UDP and ATP via nucleoside diphosphate kinase (Ndk) (Lu et al., 1995). UTP regeneration may currently be the bottleneck rather than the phosphorylation of glucose. Overexpression of Ndk has previously enhanced the formation of cyanidin 3-O-glucoside in *E. coli* (Leonard et al., 2008). Therefore overexpression of Ndk could be implemented to further improve the production of quercetin glucoside.

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CHAPTER 5

CONCLUSION

In this research, metabolic engineering of sugar pathways in *Escherichia coli* was applied to design and construct more efficient fermentation/bioconversion processes that increase product yield and productivity. Cells are inclined to choose the most preferred carbon source and grow as fast as possible. However, from the engineering perspective, deceleration of the sugar metabolism can benefit the generation of a desired product.

In general, disruption of sugar transport system or upper glycolysis eliminated carbon catabolite repression and excluded overflow metabolism, directing the carbon flux to generate biomass, energy and a final product. Specifically, disruption of glucose transport could improve redox balance and reduce acetate formation, hence benefit the production of downstream glycolysis or TCA cycle intermediates such as succinate as demonstrated in chapter 2. Disruption of upper glycolysis by deleting glucose 6-phosphofructokinase or glucose-6P isomerase substantially increased fructose 6P and the glucose 6P pool, which enables the conversion from pentoses to glucose as illustrated in chapter 3 or the accumulation of glycogen synthesis pathway intermediates such as UDP-glucose, elucidated in chapter 4.

Engineering of sugar pathways is not limited to glucose transport system or glycolysis modifications. In chapter 2, glucose and xylose transport systems were partially impaired in two strains. The strategy allows the two strains grow in concert with each other, performing their own bioconversion efficiently, each with only one specific

sugar. In this way, carbon catabolite repression was completely eliminated, and the sugar to product pathways could be optimized independently. The consortium approach may potentially be combined with glycosylation, which means each strain fulfills a specific step in the formation of the glycosides. For example, the first strain produces an expensive small molecule like a flavonoid from a cheap precursor, a second strain produces the UDP-sugar, and both are utlimately converted by another strain to perform the glycosylation via a glycosyltransferase.