ESTABLISHING SYNTHETIC PATHWAYS IN *ESCHERICHIA COLI* FOR BIOSYNTHESIS OF C3 AND C4 HIGH VALUE COMMODITY CHEMICALS

by

Rachit Jain

(Under the Direction of Yajun Yan)

ABSTRACT

The rapid consumption of oil for fuel and chemicals is leading to carbon dioxide emission at vast quantities each year. At this hour the need is to gradually reduce the reliance on oil for large scale manufacture of fuels and chemicals. The development of renewable/ alternative energy technologies and the production of "green chemicals" provide a means to address these concerns. The biological manufacture of C3-C6 commodity chemicals has gained much attention over the past decade due to their large annual market and wide industrial applications. Metabolic engineering has enabled the biological manufacture of these chemicals via construction of novel metabolic pathways or enhancement of existing ones.

In this work, we engineer *Escherichia coli* for the production high value bulk chemicals – 1,2-propanediol, 1-propanol and 1,4-butanediol. We systematically engineer *E. coli* to enhance the production of 1,2-propanediol to maximize yield from glucose. Building on this work, we

identify an optimal diol dehydratase and expand the 1,2-propanediol pathway to establish a novel platform for 1-propanol production from glucose. Deriving inspiration from rational design approaches we then engineer the diol dehydratase to enhance activity toward a non-native substrate. We demonstrate an alliance of protein engineering strategies and metabolic engineering strategies to achieve the production of 1,4-butanediol from xylose via a novel pathway.

Overall, by establishing novel synthetic pathways and engineering the cell's native metabolism we demonstrate efficient and reliable production of C3 and C4 bulk chemicals in a prokaryotic system. In doing so, we intend to reduce the overdependence on oil for manufacture of fuels and chemicals.

INDEX WORDS: 1,2-Propanediol, 1-propanol, 1,4-butanediol, *Escherichia coli*,
diol dehydratase, metabolic engineering, protein engineering, bulk chemicals

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DEDICATION

To my dear wife, Deepti for her love, support and patience. For being with me every step of the way and for sharing this aspiration with me 5 years ago. To my loving parents, for always supporting me in my endeavors. For always believing in me and guiding me, even through my eccentricities. To my brother, Akshett, for being a wonderful sibling. To my family, for all the wonderful memories and support during my school days and after. To Dr. Basavaraj Girennavar, for never giving up and setting an example for all young entrepreneurs in India. To all those who take a chance and chase their dreams.

I dedicate my dissertation to all of you.

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"*The true sign of intelligence is not knowledge but imagination*"

- Albert Einstein

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INTRODUCTION

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1.1. Microbial Synthesis of Chemicals

The production of a wide range of commodity chemicals relies on petrochemical based approaches. These chemicals have an estimated annual market of over $2 trillion (Burk, 2010). Generally, the production of chemicals via chemical approaches has advantages of being a well-established production platform associated with low production costs. However, some severe drawbacks exist in petroleum-based manufacture approaches, such as: (a) use of toxic/ environmentally harmful or expensive catalysts (b) generation of toxic intermediates (c) requirement of high temperature/ pressure processes (d) high energy inputs (e) production of stereo-specific chemicals is difficult. The dependence on these manufacturing approaches contributes to the release of over 30 billion tonnes of carbon dioxide into the atmosphere annually (Burk, 2010). The concerns of oil crisis and environment deterioration compel us to look for alternative ways to produce petroleum-based fuels and chemicals. By channeling efforts toward manufacture of "green chemicals", the reliance on oil based chemicals and fuels can be greatly reduced. Several reports of establishing biological platforms for manufacture of current petrochemical based commodities have surfaced over the last decade. Metabolic engineering of microbes provides a promising alternative for the production of these petroleum-derived compounds.

Metabolic engineering is a technique that first emerged in the early 1990s. Since then, this technology has been developing rapidly, which has been greatly dependent on the significant advances in its contiguous fields. DNA sequencing and bioinformatics analysis reveals new metabolic pathways and enzyme variants; enrichment in protein structure information provides foundation for rational protein engineering; advanced analytical tools identify pathway bottlenecks from RNA, protein or metabolite levels; the availability of a series of genetic tools

facilitates pathway optimization; advancement in fermentation technology enables scale up for industrial scale production.(Lee et al., 2012)

Metabolic engineering efforts has enabled the biological production of a large number of commodity chemicals (Jang et al., 2012b) including C3 and C4 alcohols such as 1,3-propanediol (Raynaud et al., 2003a), 1,2-propanediol (Cameron et al., 1998), 1-propanol (Shen and Liao, 2008c), 2,3-butanediol (Yan et al., 2009b), n-butanol (Atsumi et al., 2008a), isobutanol (Atsumi et al., 2008e), 1,4-butanediol (Yim et al., 2011b). Although a large number of chemicals have been produced biologically, the commercial manufacture of only a handful of these chemicals has become a reality. Challenges remain in enhancing the production of chemicals whose metabolic routes are known and to achieve the production of chemicals whose microbial production has never been identified/ established.

Microbes (eukaryotic or prokaryotic) that have a simple genetic background and fast growth rate are usually used as hosts to produce various compounds. This strategy serves both metabolic logic as well as industrial scale process economics. Some successful examples include production of 1,3-propanediol in *Escherichia coli* (Nakamura and Whited, 2003a), engineering *Saccharomyces cerevisiae* for the production of antimalarial drug precursor artemisinic acid (Ro et al., 2006). While *E. coli* and *S. cerevisiae* are the two most commonly used hosts for metabolic engineering, other non-conventional hosts have also been explored for their distinctiveness.

Here, we review the microbial production of C3 and C4 high value commodity chemicals, emphasizing on metabolic engineering efforts to enhance their production. We review various alcohols (including ethanol, 1-propanol, isopropanol, 1-butanol, isobutanol) and diols (2,3-butanediol, 1,4-butanediol, 1,3-propanediol, 1,2-propanediol).

1.2. Microbial Production of Alcohols

1.2.1. Ethanol

Currently, ethanol is the most widely used biofuel. In 2013, approximately 1040 million m^3 of ethanol was produced worldwide, over 80% of which was consumed for application as a fuel additive. The largest producer of bioethanol is USA with 518 million m^3, followed by Brazil with 277 million m^3. It is reported that annually over 98% of bioethanol is made from corn (RFA, 2014). As the concerns of fuel and food competition increase, the production of biofuels from non-edible feedstocks has gained increasing attention. Here we summarize the recent progress on the metabolic engineering of microorganisms to use non-edible feedstocks, such as lignocellulosics and brown macroalgae for ethanol production.

Lignocellulosics have been long considered as a good alternative carbon source for biofuel production. However, due to its recalcitrant nature, lignocellulosic biomass usually needs to be pretreated and hydrolyzed before it can be used by microorganisms for biofuel production. Lignocellulosic biomass contains several hexoses and pentoses, with glucose and xylose as the two most abundant components. *S. cerevisiae* can efficiently produce ethanol from glucose but is unable to natively use pentose sugars such as xylose as a carbon source (van Maris et al., 2007). To enable D-xylose fermentation the conversion of D-xylose to D-xylulose is necessary, for which heterologous enzymes such as xylose reductase (XR) and xylitol dehydrogenase (XDH) or just xylose isomerase (XI) are required. The expression XI has the advantage of overcoming redox imbalance, which is commonly an issue while expressing the XR-XDH system. However,

bacterial XI genes are not well expressed in yeast. To surpass this hurdle, a fungal XI gene was identified and shown to have high activity in yeast. When it was overexpressed along with the genes of the non-oxidative pentose phosphate pathway, the resulting yeast strain grew anaerobically on D-xylose and produced ethanol with the yield comparable with that on glucose (van Maris et al., 2007). Transportation of D-xylose into the cell is another factor that limits xylose utilization. To further improve ethanol production from *S. cerevisiae*, a sugar transporter gene was expressed in a xylose-assimilating yeast strain. Xylose uptake ability and ethanol productivity were significantly improved via both xylose fermentation and xylose-glucose co-fermentation (Katahira et al., 2008). Another difficulty that hinders successful utilization of mixed sugars in cellulosic hydrolysates is the sequential consumption of xylose after glucose depletion. To address this difficulty, an engineered *S. cerevisiae* strain was constructed to co-ferment xylose and cellobiose. Cellobiose was transported into yeast cells by a high-affinity cellodextrin transporter, and then hydrolyzed by an intracellular β-glucosidase. It was found that the intracellular hydrolysis of cellobiose minimizes glucose repression on xylose fermentation. The resulting yeast strains utilized xylose and cellobiose simultaneously, leading to an increase in ethanol titer of about 20% compared to that of glucose and xylose co-fermentation (Ha et al., 2011).

The above-mentioned process requires pretreatment and lignocellulosic hydrolyzation to fermentable sugars before large scale production. Consolidated bioprocessing (CBP) combines cellulase secretary expression, cellulose hydrolysis, and biofuel production into a single step and represents a more effective technology for biofuel production (Lynd et al., 2008). To achieve CBP, ethanologenic microorganisms have been engineered to express either non-complexed or

complexed cellulase (cellulosomes) systems. A non-complexed cellulase system was constructed by expressing an endoglucanase and β-glucosidase in *S. cerevisiae*. The resulting strain was enabled to grow on amorphous cellulose and produced up to 1.0 g/L of ethanol under anaerobic conditions (Den Haan et al., 2007). Compared to non-complexed cellulase systems, cellulosomes exhibit much higher degradation efficiency because of their highly ordered structural organization. Tsai and coworkers assembled a functional mini cellulosome on the yeast surface by using a synthetic yeast consortium (Tsai et al., 2010). One yeast strain displayed a trifunctional scaffoldin carrying three divergent cohesin domains. The others three strains secreted one of the three corresponding dockerin-tagged cellulases. The secreted cellulases were docked onto the displayed scaffoldin in a highly organized manner. By adjusting the ratios of these four strains, 1.87 g/L ethanol was produced with a yield of 93% theoretical maximum (Tsai et al., 2010).

To simplify this system, researchers co-expressed scaffoldin and dockerin-tagged cellulases in one yeast cell. Wen and coworkers displayed a series of uni-, bi-, and trifunctional mini cellulosomes. Compared with the uni- and bifunctional mini cellulosomes, the trifunctional complexes showed better cellulose hydrolysis efficiency. The engineered yeast strain produced 1.8 g/L ethanol using phosphoric acid-swollen cellulose as the carbon source (Wen et al., 2010). To further increase the display level, Fan and coworkers displayed cellulosomes using two individual mini scaffoldins. The engineered *S. cerevisiae* was able to hydrolyze microcrystalline cellulose efficiently, and produce ethanol with a titer of 1.4 g/L (Fan et al., 2012). Sakamoto and coworkers constructed a *S. cerevisiae* strain that not only hydrolyzed hemicelluloses by co-displaying endoxylanase, β-xylosidase, and β-glucosidase but that also assimilated xylose by

expressing XR, XDH and xylulokinase. The recombinant strain produced 8.2 g/L of ethanol directly from rice straw hydrolysate with the yield of 0.41 g/g (Sakamoto et al., 2012).

Besides lignocellulosics, brown macroalgae has also been investigated as feedstock for bioethanol production. Its cultivation requires no arable land, fresh water or fertilizer. Alginate, mannitol and glucan are the main sugars in brown macroalgae and these sugars can be easily separated by simple biorefinery processes such as milling, leaching and extraction. A yeast strain was engineered to assimilate major sugars in brown macroalgae for ethanol production. An alginate monomer (4-deoxy-L-erythro-5-hexoseulose uronate, or DEHU) transporter was discovered from *Asteromyces cruciatus*. The expression of this transporter gene together with other alginate and mannitol catabolism genes enabled a *S. cerevisiae* strain to efficiently metabolize DEHU and mannitol. Under anaerobic conditions, 36.2 g/L of ethanol was produced, which corresponds to 83% of theoretical maximum yield (Enquist-Newman et al., 2014). *E. coli* has also been engineered to use brown macroalgae as the sustainable feedstock for ethanol production. First, a secretable alginate lyase system was introduced into *E. coli* to enable efficient and rapid degradation of alginate. Coupling this system with alginate uptake and metabolic pathway generated a microbial platform that can produce ethanol directly from macroalgal biomass with a titer of 4.7 % v/v and a yield of 0.281 g/g macroalgae (80% of theoretical maximum yield) (Wargacki et al., 2012).

1.2.2. 1-Propanol

Compared to ethanol, 1-propanol has a higher octane number and is considered to be a better biofuel. 1-Propanol is also an important feed stock in chemical industry and its annual market is over 130,000 tonnes. It can be dehydrated to produce propylene, a starting material for polypropylene plastics. It can also replace methanol during the production of biodiesel fuel to avoid using organic solvents.(Atsumi and Liao, 2008a)

1-Propanol can be biosynthesized via different precursors, such as 2-ketobutyrate and propionyl-coenzyme A (CoA). 1-Propanol production from 2-ketobutyrate is an example of synthesis of higher alcohols using keto-acid pathways (Atsumi et al., 2008d). Through these pathways, various alcohols can be synthesized from the corresponding keto acids, which are catalyzed by promiscuous keto-acid decarboxylase (KDC) and alcohol dehydrogenase (ADH) (Figure 1.1).

Shen and Liao reported the production of 2-ketobutyrate by extending the *E. coli* threonine biosynthetic pathway (Shen and Liao, 2008b). The production of 1-propanol was systematically optimized through elimination of competing pathways and deregulation of amino-acid biosynthesis, leading to a final titer of about 1 g/L. Atsumi and Liao designed a shorter route which bypasses threonine biosynthesis and directly converts pyruvate to 2-ketobutyrate (Atsumi and Liao, 2008a). This pathway relies on a foreign enzyme, citramalate synthase (CimA). CimA variants with improved activity and resistance to feedback inhibition were obtained by directed evolution. These efforts led to 1-propanol production at 3.5 g/L after 92 hours.(Atsumi and Liao, 2008a)

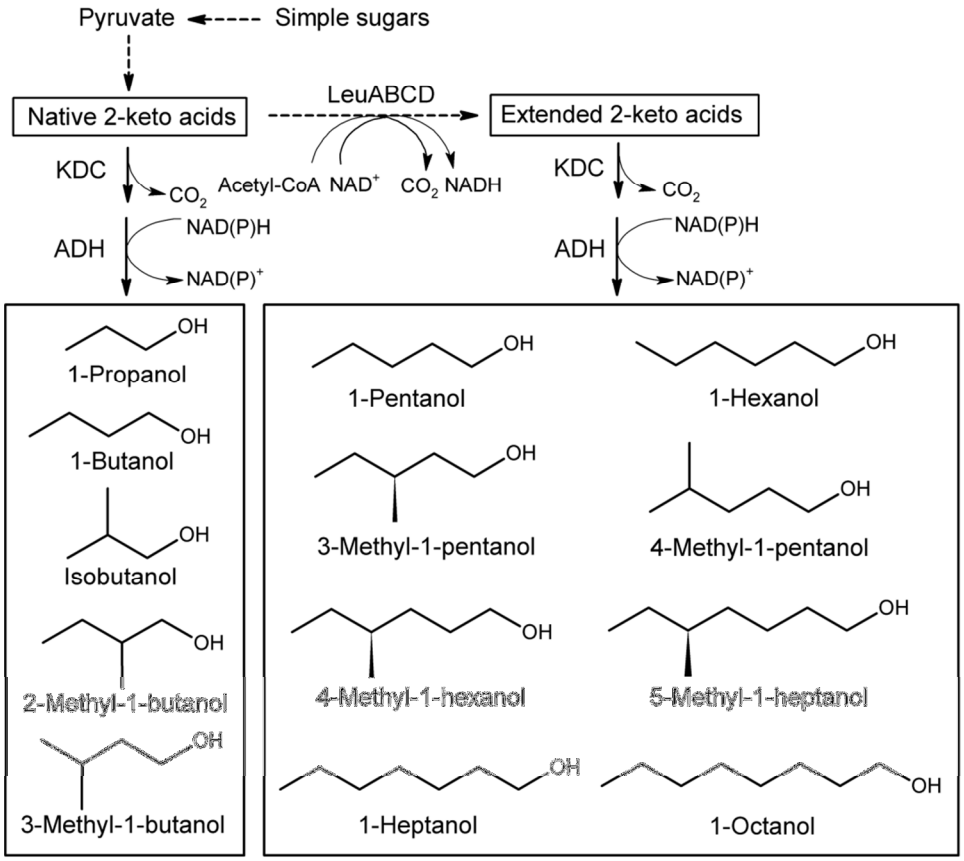

Figure 1.1. Microbial production of higher alcohols by keto acid pathways. ADH, alcohol dehydrogenase; KDC, keto acid decarboxylase; LeuABCD: leucine biosynthesis operon.

1-Propanol production via propionyl-CoA was first achieved in *Thermobifida fusca* (Deng and Fong, 2011). Based upon computational analysis of the metabolic network, two distinct pathways leading to the synthesis of propionyl-CoA were identified by extending the threonine biosynthesis pathway or succinyl-CoA pathway (Figure 1.2). The introduction of a bifunctional butyraldehyde/alcohol dehydrogenase led to 1-propanol production from various carbon sources. The highest 1-propanol titer (0.48 g/L) was obtained using switchgrass as the carbon source. These two propionyl-CoA based pathways have been reconstituted in *E. coli*, resulting 1-

propanol production at 10.8 g/L and 150 mg/L, respectively (Choi et al., 2012a; Srirangan et al., 2013).

1.2.3. Isopropanol

Isopropanol is natively produced by several species of *Clostridium*. It can also be used as a biofuel. In chemical industry, isopropanol can be dehydrated to yield propylene, a monomer for manufacturing polypropylene. Isopropanol is also used as an alternative to methanol to produce biodiesel (Hanai et al., 2007). Currently, the only reported isopropanol pathway is the extension of *Clostridium* acetone pathway. Acetyl-CoA is converted to isopropanol by the sequential catalysis of acetyl-CoA acetyltransferase (AtoB), acetoacetyl-CoA transferase (AcoAT), acetoacetate decarboxylase (ADC), and secondary alcohol dehydrogenase (ADH) (Figure 1.2). Hanai and coworkers screened these enzymes from different microorganisms to identify the most suitable candidates (Hanai et al., 2007). The expression of the most suitable set of enzymes resulted in the production of 81.6 mM isopropanol with a yield of 43.5% (mol/mol) in shake flask studies (Hanai et al., 2007). In a fed-batch process incorporated with a gas stripping recovery method, 143 g/L of isopropanol was produced after 240 hours with a yield of 67.4% (mol/mol) (Inokuma et al., 2010b).

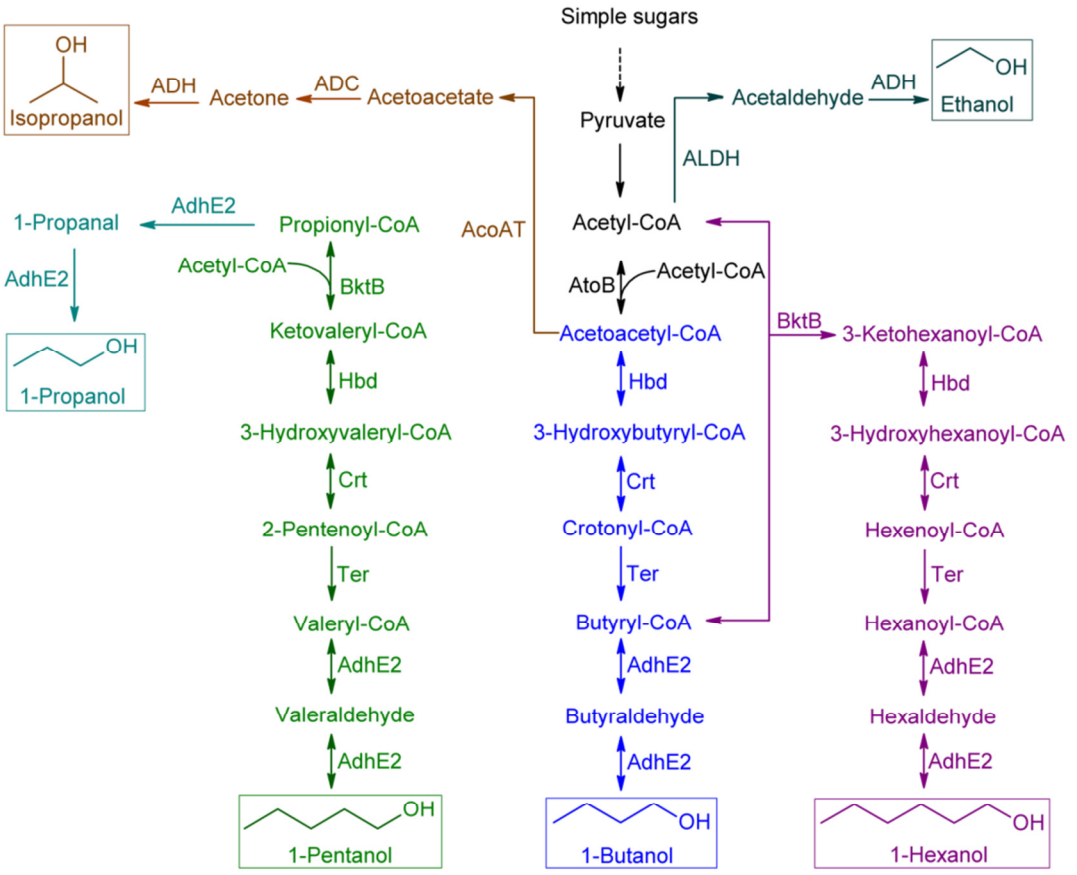

Figure 1.2. Microbial production of alcohols using CoA-dependent pathways. AcoAT, acetoacetyl-CoA transferase; ADC, acetoacetate decarboxylase; ADH, alcohol dehydrogenase; AdhE2, aldehyde/alcohol dehydrogenase; ALDH, acetaldehyde dehydrogenase; AtoB, acetyl-CoA acetyltransferase; BktB, β-ketothiolase; Crt, crotonase; Hbd, acetoacetyl-CoA thiolase; Ter, *trans*-enoyl-coenzyme A reductase.

Soma and coworkers investigated isopropanol production from lignocellulosic biomass. In their work, a recombinant *E. coli* strain displaying β-glucosidase on the cell surface and expressing the isopropanol synthetic pathway produced 69 mM isopropanol after 21 hours of fermentation from cellobiose (Soma et al., 2012). Kusakabe and coworkers constructed the isopropanol pathway in cyanobacteria *Synechococcus elongatus*. The enzyme-coding genes were integrated into the

genome. Under optimized conditions, the engineered cyanobacteria produced 26.5 mg/L of isopropanol directly from solar energy and carbon dioxide (Kusakabe et al., 2013). This pathway was also introduced into *Cupriavidus necator* strain Re2133. The synthetic production pathway was rationally designed through codon optimization, gene replacement, and manipulating gene expression levels in order to efficiently divert carbon flux toward isopropanol. These efforts led to 3.44 g/L isopropanol from fructose (Grousseau et al., 2014).

1.2.4. 1-Butanol

1-Butanol has been considered as an excellent biofuel due to hydrophobicity and similar energy density to gasoline. 1-Butanol can be mixed with gasoline at any ratio or completely replace gasoline. It is not corrosive and is compatible with existing pipeline infrastructure. In addition, the vapor pressure of 1-butanol (4mm Hg at 20ºC) is much lower than that of ethanol (45 mm Hg at 20ºC), which makes its separation more cost-effective (Atsumi et al., 2008c). 1-Butanol can be produced by two distinct biosynthetic pathways: the keto-acid pathway and the CoA-dependent pathway. In the keto-acid pathway, the precursor 2-ketovalerate is synthesized from 2-ketobutyrate by the action of LeuABCD (leucine biosynthesis operon) and then 2-ketovalerate is converted to 1-butanol by the two broad substrate enzymes KDC and ADH. Using this pathway, about 1 g/L of 1-butanol was produced from *E. coli* (Shen and Liao, 2008a) and 242.8 mg/L from *S. cerevisiae* (Si et al., 2014).

1-Butanol can be produced natively via CoA-dependent pathway by various species of *Clostridium*. This pathway starts from acetyl-CoA, and the enzymes involved include acetyl-

CoA acetyltransferase (Thl), acetoacetyl-CoA thiolase (Hbd), 3-hydroxybutyryl-CoA dehydrogenase (Crt), butyryl-CoA dehydrogenase (Bcd), electron transfer flavoprotein (Etf), aldehyde/alcohol dehydrogenase (AdhE2) (Figure 1.2). However, as *Clostridium* species are strict anaerobes and the growth rate is slower than *E. coli*, metabolic engineering strategies have been adopted to construct recombinant strains of aerobic microorganisms for 1-butanol production with high titer and productivity (Atsumi et al., 2008b; Inui et al., 2008). Atsumi and coworkers reconstituted *Clostridium* 1-butanol pathway in *E. coli* and the initial strain produced only 13.9 mg/L of 1-butanol (Atsumi et al., 2008b). The pathway was further optimized by identification of alternative enzymes from other organisms, deletion of competing pathways and optimization of culture medium. These efforts led to improving 1-butanol titer up to 552 mg/L in rich medium supplemented with glycerol (Atsumi et al., 2008b).

Three major hurdles were identified with the *Clostridium* 1-butanol pathway: firstly, all the reactions are reversible; secondly, a total of four NADH molecules were consumed to produce one 1-butanol molecule; thirdly, the condensation reaction of acetyl-CoA to produce acetoacetyl-CoA was determined to be rate limiting. The first concern was addressed by introducing an irreversible *trans*-enoyl-CoA reductase (Ter). To address the second difficulty, (competing) NADH-consuming pathways were disrupted and a formate dehydrogenase was expressed to generate more NADH from formate. In order to overcome the third hurdle, the *Clostridium* Thl was replaced with *E. coli* native AtoB due to its higher specific activity. These modifications resulted in NADH and acetyl-CoA driving forces and enabled high-titer (30 g/L) and high-yield production of 1-butanol anaerobically (Shen et al., 2011).

1.2.5. Isobutanol

Isobutanol is another potential biofuel with high octane value and energy density. It can mix with gasoline at any proportion and is also known to be compatible with existing combustion engines and fuel transportation infrastructure (Bastian et al., 2011). As an important building block, it is used as the feedstock for the production of p-xylene, isobutyl acetate and isobutyl esters. Isobutanol production has been reported from 2-keto-isovalerate, the intermediate of valine biosynthesis. The byproduct generating pathways that consume pyruvate and acetyl-CoA were disrupted. The resultant strain JCL260 achieved isobutanol production at 22 g/L from shake flasks under micro-aerobic conditions (Atsumi et al., 2008d). In a 1-L bioreactor this strain achieved over 50 g/L isobutanol within 72 hours with *in situ* isobutanol removal using gas stripping (Baez et al., 2011).

Desai and coworkers engineered *E. coli* to produce isobutanol from cellobiose. A beta-glucosidase was expressed extracellularly by either excretion into the medium or anchoring to the cell membrane. The excretion system allowed *E. coli* to grow with cellobiose as a sole carbon source at rates comparable to those with glucose. The genes expressing isobutanol production pathway enzymes were then introduced to this system. The most productive strain converted cellobiose to isobutanol with a titer of 7.64 g/L and a productivity of 0.16 g/L/h (Desai et al., 2014).

Unlike carbohydrates and lipids, proteins are not commonly used as feedstocks for biofuel production. Recently, Huo and coworkers engineered *E. coli* to assimilate protein hydrolysates

by introducing three heterologous transamination and deamination cycles for isobutanol production. Isobutanol was produced from different protein sources, such as the biomass generated from *S. cerevisiae*, *E. coli* and *Bacillus subtilis.* Isobutanol titer of 4 g/L was achieved, which corresponds to about 56% of theoretical yield (Huo et al., 2011a).

Isobutanol production from *S. cerevisiae* has also been investigated. Overall, the titers are lower than those obtained in *E. coli*. Matsuda and coworkers improved isobutanol production from *S. cerevisiae* by eliminating competing pathways and resolving cofactor imbalance (Matsuda et al., 2013). The precursor pyruvate pool was increased by disrupting genes related to pyruvate metabolism. NADPH supply was improved by introducing transhydrogenase-like shunts. This resulted in 1.62 g/L of isobutanol production with a yield of 0.016 g/g glucose via batch fermentation (Matsuda et al., 2013). It was determined that in yeast, the upstream pathway is confined to mitochondria, whereas the downstream pathway is located in the cytoplasm. The transport of the intermediate 2-keto-isovalerate across membranes decreases productivity. To improve isobutanol production, the valine biosynthesis enzymes were relocated from the mitochondrial matrix into the cytosol (Brat et al., 2012). To this end, the N-terminal mitochondrial targeting sequences of Ilv2, Ilv3 and Ilv5 were deleted. About 0.63 g/L of isobutanol was produced at a yield of 15 mg/g glucose. In another work, the isobutanol pathway was completely assembled in the mitochondria. Compared with the control, compartmentalization of the pathway into mitochondria improved isobutanol production by 260%, whereas over-expression of the same pathway in the cytoplasm only increased yields by 10% (Avalos et al., 2013b).

1.2.6. Others alcohols

Besides the above mentioned alcohols, other alcohols with even longer chains have also drawn increasing attention. Both 2-methyl-1-butanol (2MB) and 3-methyl-1-butanol (3MB) were produced via the keto-acid pathways. 2-Keto-3-methylvalerate (KMV), an intermediate in isoleucine biosynthetic pathway, is converted to 2MB by KDC and ADH. Over-expressing the key enzymes in the upper pathway and deleting competing pathways led to an *E. coli* strain that produces 1.25 g/L 2MB in 24 hours with yields of up to 0.17 g/g glucose (Cann and Liao, 2008). Similarly, 3-methyl-1-butanol (3MB) was synthesized from 2-ketoisocaproate (KIC), the direct precursor to leucine. With pathway optimization, 1.3 g/L 3MB was produced (Connor and Liao, 2008). Marcheschi and coworkers engineered *E. coli* LeuA by structure-based protein engineering to carry out recursive chain elongation reactions of keto-acids (Marcheschi et al., 2012). This enzyme has been successfully used for the production of a series of long chain alcohols, such as 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, directly from glucose (Figure 1.1) (Marcheschi et al., 2012).

Production of 1-hexanol and 1-pentanol was also achieved by extending the CoA-dependent 1-butanol pathway (Figure 1.2). β-Ketothiolase (BktB) catalyzed the condensation of acetyl-CoA and butyryl-CoA to 3-ketohexanoyl-CoA. The subsequent reactions were catalyzed by Hbd, Crt, Ter, AdhE2, leading to 1-hexanol production. Under anaerobic conditions 1-hexanol was produced at a titer of 47 mg/L from glucose (Tseng and Prather, 2012). Similarly, to achieve 1-pentanol production, BktB was used to catalyze the condensation of acetyl-CoA and propionyl-CoA to form 3-ketovaleryl-CoA. Through several reduction and dehydration reactions, 3-

ketovaleryl-CoA was converted to 1-pentanol. After systematic pathway optimization and strain engineering, the engineered *E. coli* strain achieved 19 mg/L of 1-pentanol production from glucose and 109 mg/L of 1-pentanol from glycerol (Dekishima et al., 2011).

1.3. Microbial Production of Diols

The biological manufacture of attractive petrochemical derived diols has gained significant commercial interest over the past decade due to their diverse applications and increasing annual global demand. The commercial scale biological manufacture of 1,3-propanediol (1,3-PDO) has been undertaken by DuPont (Celinska, 2010), Tate & Lyle (Kraus, 2008) and Genencor (Nakamura and Whited, 2003b); while the commercial scale bio-manufacturing of 1,4-butanediol (1,4-BDO) is established by Genomatica (Burk, 2010). In following section, the microbial production of C4 diols (2,3-butanediol and 1,4-butanediol) and C3 diols (1,3-propanediol and 1,2-propanediol) will be highlighted.

1.3.1. 2,3-Butanediol

The three stereo isomers of 2,3-butanediol (2,3-BDO) find application in various industries and their derivatives have an annual combined market of over 32 million tons (Shen et al., 2012a). (R,R)-2,3-BDO in particular is used as an antifreeze; while 2,3-BDOs in general are used for the production of various chemicals like methyl ethyl ketone (MEK), 1,3-butadiene, acetoin, diacetyl, etc. Additionally, 2,3-BDOs find application in the manufacture of perfumes, printing ink, food supplements, pharmaceuticals, fumigants, etc.(Shen et al., 2012a).

The microbial production of 2,3-butanediol has been pursued by both native producers and heterologous hosts (Ji and Huang, 2014). The metabolism of native producers generally results in generating a mixture of 2,3-BDO stereoisomers. For instance, *Klebsiella* and *Enterobacter* species are known to produce both (S,S)- and meso-2,3-BDO, while *Bacillus* species are known to produce both (R,R)- and meso-2,3-BDO (Ji and Huang, 2014). By engineering *Klebsiella pneumoniae*, 2,3-BDO titer of 150 g/L has already been achieved via fed-batch fermentation (Ma et al., 2009). The production of enantio-pure 2,3-BDO has been largely achieved by introducing stereo-specific 2,3-BDO pathways in heterologous microbes such as *E. coli*. Due to the large market of 2,3-BDO and its derivatives, significant progress in its biological production from a variety of carbon sources has been achieved (Ji and Huang, 2014). In this section, the general biochemical scheme of its microbial production, followed by metabolic engineering strategies implemented to achieve enhanced production in both native producers and in *E. coli* will be discussed.

The biosynthesis of meso/ (R,R)-2,3-BDO is a 3 step metabolic process from an endogenous precursor metabolite produced in nearly all microbes – pyruvate. The enzymes involved in its bio-catalysis include acetolactate synthase (ALS), acetolactate decarboxylase (ALDC) and a stereospecific secondary alcohol dehydrogenase (sADH) leading to the formation of acetolactate, acetoin and 2,3-BDO respectively (Figure 1.3). It has been determined that the expression of *K. pneumoniae* meso-dehydrogenase as the sADH leads to the formation of meso-2,3-BDO, while the expression of *B. subtilis*/ *C. beijerinckii*/ *T. brockii* sADH leads to the formation of (R,R)-2,3-BDO from acetoin (Yan et al., 2009a). The biosynthesis of (S,S)-2,3-BDO can be achieved from diacetyl as the target precursor, contrary to pyruvate. The expression of diacetyl reductase

leads to the generation of (S)-acetoin from diacetyl, while the subsequent expression of (S)-2,3-butanediol dehydrogenase catalyzes the formation of (S,S)-2,3-BDO (Ui et al., 2004).

In 1993, high titer production of 2,3-BDO was achieved at 118 g/L from *K. oxytoca* using molasses, where the biomass generated from a batch process was recycled for use in subsequent batch processes. This strategy overcame growth inhibition in the presence of high initial substrate concentrations and achieved a productivity of 2.4 g/L/h (Afschar et al., 1993). Recently, further engineering of *K. oxytoca* resulted in achieving a 2,3-BDO titer of 130 g/L with a yield of 0.48 g/g glucose in fed-batch studies. In their work, *K. oxytoca* was engineered to eliminate the production of a dominant by-product – ethanol via disruption of *aldA*. This resulted in diverting the carbon flux into the 2,3-BDO pathway (Ji et al., 2010).

In another metabolic engineering work, the production of 2,3-BDO by *K. pneumoniae* achieved a titer of 150 g/L with an impressive productivity of 4.21 g/L/h. In order to achieve this, first shake flask studies were performed to identify significant factors influencing nutritional requirements. Following the identification of corn steep liquor powder and $(NH_4)_2HPO_4$ as significant factors, an optimal medium was designed to promote 2,3-BDO production. Finally, the parameters influencing fed-batch studies were optimized (Ma et al., 2009). Recently, an engineered *Serratia marcescens* strain growing on sucrose reported the highest 2,3-BDO titer of 152 g/L via fed-batch studies. In their work, the disruption of *swrW* encoding serrawettin W1 synthase significantly lowered the production of serrawettin W1 (a harmful exolipid that causes excessive foaming). Fed-batch culture of the engineered strain resulted in 2,3-BDO productivity of 2.67 g/L/h (Zhang et al., 2010).

The production of enantio-pure 2,3-BDO has been demonstrated by engineering *E. coli*. The production enantio-pure meso-2,3-BDO from glucose was reported by expressing *K. pneumoniae* ALS, ALDC, and meso-2,3-BDO dehydrogenase in *E. coli* achieving a titer of 17.7 g/L (Ui et al., 1997). In another work, the production of enantio-pure meso- or (R,R)-2,3-BDO was achieved from *E. coli* growing on glucose. In order to achieve stereo-specific production, the activity of sADH from *K. pneumoniae*, *B. subtilis*/ *C. beijerinckii*/ *T. brockii* were characterized. By expressing the complete pathway using the stereo-specific sADH, production of either meso-2,3-BDO or (R,R)-2,3-BDO was reported (Yan et al., 2009a). Based on this work, the production of enantio-pure (R,R)-2,3-BDO from glycerol was also reported by engineering *E. coli*. It was determined that the disruption of pathways leading to acetate accumulation (*ackA*/ *poxB*) prevented its accumulation and also the accumulation of acetoin. The engineered strain achieved (R,R)-2,3-BDO biosynthesis at 9.56 g/L from 10 mL shake flask studies (Shen et al., 2012a).

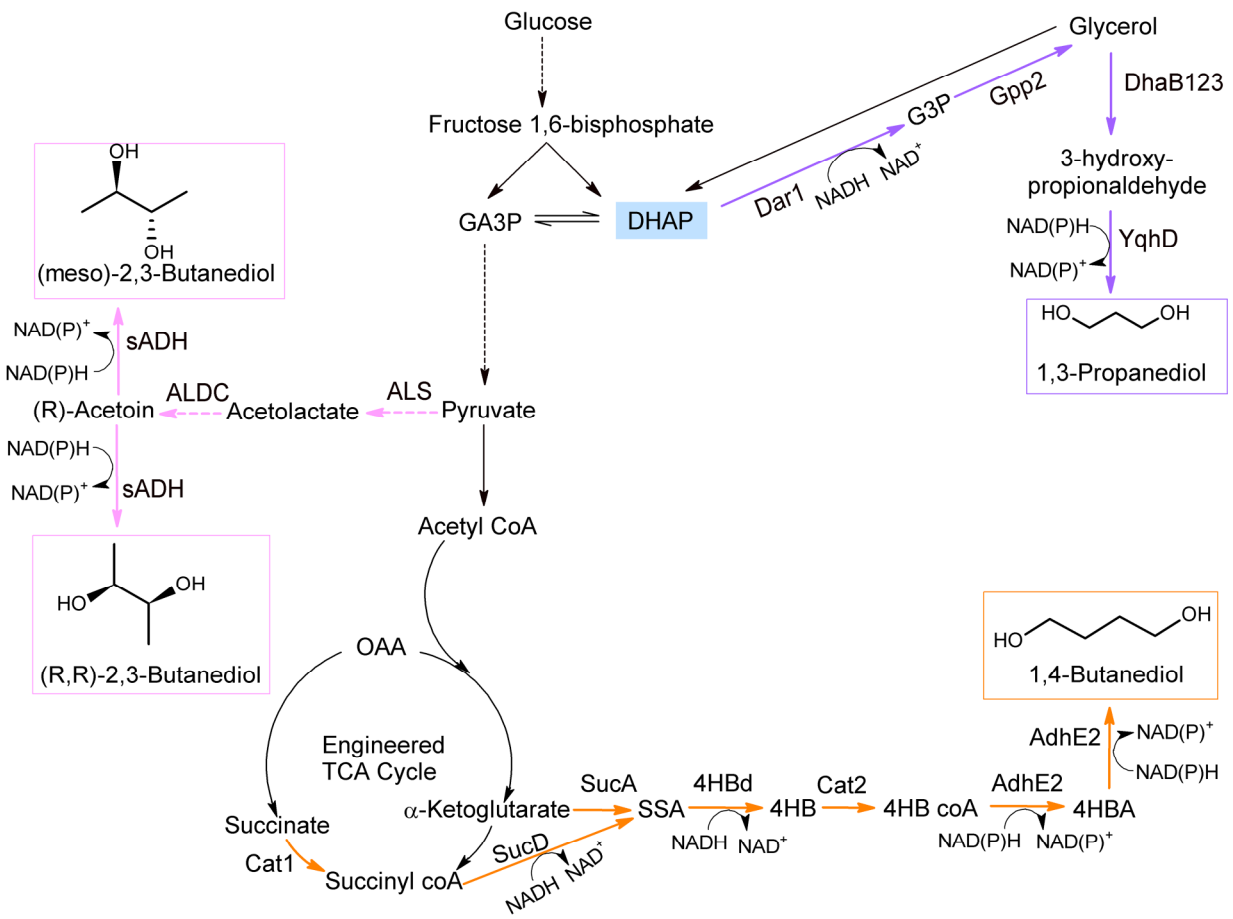

Figure 1.3. Microbial routes for the production of 2,3-butanediol, 1,4-butanediol and 1,3-propanediol. Intermediates: 4HB, 4-hydroxybutyrate; 4HB-CoA, 4-hydroxybutyrl-CoA; 4HBA, 4-hydroxybutyraldehyde; DHAP, dihydroxyacetone- phosphate; G3P, glycerol 3-phosphate; GA3P, glyceraldehyde 3-phosphate; OAA, oxaloacetate; SSA, succinyl semialdehyde. Enzymes: 4HBd, 4-hydroxybutyrate dehydrogenase; ALDC, acetolactate decarboxylase; ALS, acetolactate synthase, AdhE2, aldehyde/alcohol dehydrogenase; Cat1, succinate-CoA transferase; Cat2, 4-hydroxybutyryl-CoA transferase; Dar1, glycerol 3-phosphate dehydrogenase; DhaB123, glycerol dehydratase; Gpp2, glycerol 3-phosphate phosphatase; sADH, stereospecific secondary alcohol dehydrogenase; SucA, 2-oxoglutarate decarboxylase; SucD, succinate semialdehyde dehydrogenase; YqhD, 1,3-PDO oxidoreductase.

The production of enantio-pure (S,S)-2,3-BDO from *E. coli* was achieved at 73% conversion efficiency by feeding diacetyl to cultures (Ui et al., 2004). In their work, the expression of *K. pneumoniae* diacetyl reductase (also known as meso-2,3-butanediol dehydrogenase) and *Brevibacterium saccharolyticum* (S)-2,3-butanediol dehydrogenase led to the production of (S,S)-2,3-BDO via the formation of (S)-acetoin (Ui et al., 2004). Recently, highly pure (S,S)-2,3-BDO production at 26.8 g/L was achieved by feeding 40 g/L diacetyl. In their work, 2,3-butanediol dehydrogenase from an *Enterobacter cloacae* species was expressed in *E. coli*. Using optimal fed-batch strategies to feed diacetyl at regular time intervals, (S,S)-2,3-BDO production was achieved (Li et al., 2012).

The production of 2,3-BDO from carbon sources such as xylose, corn-corb hydrolysate, molasses, acid hydrolysates of Jatropha hulls, Jerusalem artichoke stalk and tuber, sucrose, even steel industry gas wastes and syngas has been reviewed elsewhere (Ji and Huang, 2014).

1.3.2. 1,4-Butanediol

Among butanediols, the biological manufacture of 1,4-butanediol (1,4-BDO) was achieved most recently (Yim et al., 2011a). 1,4-BDO has an annual market of over 2.5 million tons worldwide and finds applications in the manufacture of plastics, solvents, fibers, polyesters, etc.(Yim et al., 2011a). Until 2011, the production of 1,4-butanediol was solely dependent on petrochemical derivatives due to the lack of any identified natural plant/ microbial routes. In 2011, the *de novo* biosynthesis of 1,4-BDO via a biological platform was reported for the first time (Yim et al., 2011a). In their work, two heterologous pathways led to the production of 1,4-BDO in *E. coli* by

channeling carbon from the tricarboxylic acid cycle (TCA cycle) intermediates – succinate and α-ketoglutarate via 6 and 5 steps respectively (Figure 1.3). In both routes, the production of common precursor 4-hydroxybutyrate (4-HB) was first achieved followed by establishment of downstream pathway leading to 1,4-BDO production. The production of 4-HB from succinate required the expression of two heterologous enzymes – succinate semialdehyde dehydrogenase and 4-hydroxybutyrate dehydrogenase. Whereas, the production of 4-HB from α-ketoglutarate required the expression of heterologous enzyme 2-oxoglutarate decarboxylase in addition to 4-hydroxybutyrate dehydrogenase. With the successful production of key intermediate 4-HB, an efficient bio-catalytic method for conversion of 4-HB to 1,4-BDO was then pursued. 4-HB was catalyzed to 4-hydroxybutyrl-CoA, via the action of 4-hydroxybutyryl-CoA transferase. In order to catalyze the final two steps leading to 1,4-BDO, a bifunctional alcohol dehydrogenase was expressed. For *de novo* biosynthesis, the upstream and downstream pathways were co-expressed in *E. coli*. Furthermore, in order to establish an efficient production platform, a metabolic model based approach was utilized as a guide to engineer *E. coli*'s native metabolism, promoting a more thermodynamically favorable process. The metabolic routes leading to production of native fermentative products (lactate, succinate, ethanol, and formate) were disrupted; additionally, the carbon flux towards reductive TCA cycle was disrupted to promote oxidative TCA cycle metabolism. With this, more reducing power was endogenously available to drive desired biocatalysis leading to efficient 1,4-BDO biosynthesis from renewable feedstocks such as glucose, xylose. Their work demonstrates effective utilization of metabolic engineering strategies to optimize synthetic metabolic routes, exemplified by achieving over 18 g/L 1,4-BDO in 2 L bioreactors.(Yim et al., 2011a)

Although 18 g/L of 1,4-BDO was achieved, the final two steps leading to 1,4-BDO production was determined to be inefficient. It was shown that the bifunctional alcohol dehydrogenase (AdhE2) catalyzing 4-hydroxybutyryl-CoA and 4-hydroxybutyrlaldehyde to 1,4-BDO displayed low specificity (Hwang et al., 2014; Yim et al., 2011a). Recently, another approach to efficiently catalyze these two reactions was reported with the use of two substrate specific dehydrogenases. In their work, *Clostridium saccharoperbutylacetonicum* butyraldehyde dehydrogenase (Bld) was engineered to enhance specificity towards 4-hydroxybutyryl-CoA, while *C. saccharoperbutylacetonicum* butanol dehydrogenase (Bdh) was used for the catalysis of the final reaction (Hwang et al., 2014). In order to enhance activity of Bld towards 4-hydroxybutyryl-CoA, random mutagenesis was performed, which led to the identification of a critical amino acid site (L273) influencing the cofactor binding affinity and structural stability. A four-fold increase in titer of 1,4-BDO biosynthesis was achieved with the use of Bld (L273T)- Bdh module compared with the module utilizing AdhE2 for the final two catalytic steps (Hwang et al., 2014).

1.3.3. 1,3-Propanediol

Among the C3 diols, 1,3-propanediol (1,3-PDO) is regarded as an important platform chemical with an annual demand of over one million tons; widely used for the manufacture of various polymers with desirable properties, for production of drugs, cosmetics, lubricants, etc. (Celinska, 2010; Tang et al., 2009). The first report of microbial production of 1,3-propanediol was in 1881 by *Clostridium pasteurianum* growing on glycerol anaerobically (Saxena et al., 2009). The biological production of 1,3-PDO has since been achieved from a wide range of microorganisms using glucose or glycerol as the carbon source. Some of the microbes known to natively produce

1,3-PDO include *Clostridium*, *Lactobacilli*, *Klebsiella*, *Enterobacter*, and *Citrobacter* (Jang et al., 2012c; Saxena et al., 2009). Among these, the fermentation of glycerol led to achieving 83.6 g/L of 1,3-PDO with a yield of 0.54 g/g glycerol using an engineered *C. acetobutylicum* strain as reported by Metabolic Explorer researchers (Gonzalez-Pajuelo et al., 2005). Furthermore, the highest reported titer of 1,3-PDO at 135 g/L was achieved using glucose fermentation from an engineered *E. coli* strain (Nakamura and Whited, 2003a). The most successful metabolic engineering strategies to enhance the production of 1,3-PDO will be discussed in this section.

While utilizing glycerol as the carbon source, 1,3-PDO production is achieved by engineering its reductive branch via two steps (Figure 1.3). In the first step, 3-hydroxypropionaldehyde (3-HPA) is generated by the action of glycerol dehydratase by dehydrating glycerol. Following subsequent reduction by a 1,3-PDO oxidoreductase, 1,3-PDO is produced. However, the oxidative branch of glycerol dissimilation pathway is known to compete for glycerol via the action of glycerol dehydrogenase and dihydroxyacetone kinase, leading to the formation of DHAP (Saxena et al., 2009). The major bottlenecks to improve 1,3-PDO include oxygen sensitive nature of dehydratase, NADH availability and harmful by-products accumulation.

It was observed that under aerobic conditions, when the substrate is absent, coenzyme B12-dependent glycerol dehydratase undergoes inactivation (Tobimatsu et al., 2000). In order to overcome this limitation, strategies such as addition of ATP, Mg^{+2}, expression of B12-independent dehydratase, and reactivating factors have been used (Honda et al., 1980; Xu et al., 2009a). The production of 1,3-PDO at high titers also results in toxic accumulation of 3-HPA, which is detrimental to achieving industrial scale production. In order to overcome this hurdle, a

two stage fed-batch fermentation strategy was utilized by sequentially feeding glycerol to achieve 38.1 g/L 1,3-PDO without 3-HPA toxification by a *K. pneumoniae* strain (Zhang et al., 2007). Several studies have since been conducted to identify *Klebsiella* strains with improved tolerance to high 1,3-PDO titers. In 2009, *K. pneumoniae* strain HR526 enabled 1,3-PDO production at 102.06 g/L via fed-batch fermentation using glycerol as carbon source. In order to achieve this, *K. pneumoniae* strain was engineered to disrupt lactate dehydrogenase activity in order to prevent the accumulation of lactate (Xu et al., 2009b).

Metabolic engineering of *C. acetobutylicum* with the introduction of a B12-independent pathway of *C. butyricum* led to 1,3-PDO production at 1104 mM (83.6 g/L). Fed-batch studies resulted in high volumetric productivity (3 g/L/h) of 1,3-PDO, emphasizing the advantage of an alternative dehydratase (Gonzalez-Pajuelo et al., 2005; Raynaud et al., 2003b). In order to establish a more efficient production platform, an *E. coli* strain was engineered to generate glycerol from glucose. The *S. cerevisiae* glycerol 3-phosphate dehydrogenase (*dar1*) and glycerol 3-phosphate phosphatase (*gpp2*) enzymes were expressed in *E. coli* in addition to the expression of *K. pneumoniae* glycerol dehydratase and its reactivating factors (*dhaB1*, *dhaB2*, *dhaB3, dhaBX* and *orfX*). *E. coli* strains natively expressing NADPH dependent YqhD for 1,3-PDO oxido-reductase activity was found to be more efficient in 1,3-PDO production, as compared to a strain expressing NADH dependent heterologous DhaT. Further metabolic engineering strategies include down-regulation of glyceraldehyde 3-phosphate dehydrogenase to limit the carbon flux towards TCA cycle; replacement of phosphoenolpyruvate (PEP)-dependent glucose uptake system with ATP-dependent glucose transport system to enhance glucose uptake efficiency. This was achieved via replacement of PTS with galactose permease and glucokinase. Additionally, in

order to prevent the loss of glycerol to native metabolism, major competing pathways (*glpK*, *gldA*) were disrupted. These efforts resulted in achieving 1,3-PDO at 135 g/L from 10 L fed batch fermentations with a high yield of 0.62 mol/mol (at a rate of 3.5 g/L/h) (Celinska, 2010; Maervoet et al., 2011; Nakamura and Whited, 2003a). In another work, the improvement of YqhD activity towards 3-HPA was pursued in order to overcome the limitation of 1,3-PDO oxidoreductase activity via error-prone PCR. This led to the identification of a mutant (D99QN147H) with 4 four-fold higher activity towards 3-HPA. The expression of this mutant YqhD in *E. coli* led to an increase in 1,3-PDO conversion from 3-HPA (Li et al., 2008).

Detailed reviews encompassing metabolic engineering efforts to engineer the glycerol dissimilation pathways, use of two stage fermentations, improvement of strain tolerance, use of glucose to produce glycerol, and engineering *Clostridia*, *S. cerevisiae*, *Klebsiella* microbes for 1,3-PDO production can be found elsewhere (Celinska, 2010; Maervoet et al., 2011; Nakamura and Whited, 2003a; Raynaud et al., 2003b; Saxena et al., 2009).

1.3.4. 1,2-Propanediol

1,2-Propanediol (1,2-PDO) has a reported annual market of over 1 billion pounds and is primarily used as an antifreeze; while also finding applications in textile industry for the manufacture of ink, in the pharmaceutical industry, and also for the manufacture of detergents, cosmetics (Altaras and Cameron, 1999b; Berrios-Rivera et al., 2003b). The biosynthesis of 1,2-PDO was first reported in 1954, when a *Clostridium* species was identified to be capable of its production natively (Saxena et al., 2010). Since then several bacterial and yeast species have

been identified with the ability of natively producing 1,2-PDO. Initially, the production of 1,2-PDO from *E. coli* was reported via the fermentation of uncommon sugars like fucose and rhamnose (Boronat and Aguilar, 1981). Additionally, it was reported that under phosphate limiting conditions, *C. sphenoides* was capable producing of 1,2-PDO from fructose, mannose and cellobiose as well. In their work, it was reported that the maximum amount of 1,2-PDO (72.6 mM) was produced with the fermentation of rhamnose (Tran-Din and Gottaschalk, 1985). The highest reported titer of 1,2-PDO till date was achieved at 9.1 g/L with a yield of 0.20 g/g glucose using *C. thermosaccharolyticum* via fermentation (Sanchez Rivera et al., 1987). Recently, metabolic engineering efforts have enabled the anaerobic production of 1,2-PDO in *E. coli* using glucose (Altaras and Cameron, 1999b; Altaras and Cameron, 2000a; Cameron et al., 1998). It was also reported that utilization of more reduced carbon sources like sorbitol and gluconate did not improve 1,2-PDO production in *E. coli* (Berrios-Rivera et al., 2003b). Furthermore, the production of 1,2-PDO using *S. cerevisiae* has been demonstrated by metabolic engineering using glucose and glycerol (Joon-Young et al., 2008; Joon-Young et al., 2011). Here, the biochemical pathways leading to 1,2-propanediol using fucose/rhamnose or glucose/ glycerol will be highlighted with an emphasis on important metabolic engineering efforts to enhance its production depending on the microorganism (*E. coli*/ *S. cerevisiae*/ cyanobacteria).

The two major biochemical pathways leading to 1,2-PDO production involves the production of lactaldehyde as its immediate precursor. It was initially found that L-rhamnose is first isomerized to L-rhamnulose followed by subsequent phosphorylation to L-rhamnulose-1-phosphate. Similarly, L-fucolose-1-phosphate is produced via isomerization and phosphorylation of L-fucose. Following cleavage of these two phosphorylated sugars by aldolase, DHAP and L-

lactaldehyde are generated simultaneously. L-lactaldehyde is then reduced to L-1,2-PDO via the action of propanediol oxidoreductase (Saxena et al., 2010). Although the metabolic routes from rhamnose and fucose involve only 4 steps, the high cost of these sugars prevents commercial scale manufacture (Saxena et al., 2010). Due to this, majority of metabolic engineering efforts for its production have focused on utilization of cheaper carbon sources like glucose and glycerol.

The production of 1,2-PDO from glucose/glycerol involves the production of methylglyoxal from the glycolytic intermediate DHAP via the action of methylglyoxal synthase. The production of 1,2-PDO from methylglyoxal can be achieved either through the formation of lactaldehyde or through the formation of hydroxyacetone (acetol) via the action of secondary alcohol dehydrogenase or methylglyoxal reductase respectively. The subsequent action of alcohol dehydrogenase on lactaldehyde/ hydroxyacetone leads to the formation of 1,2-PDO (Jain and Yan, 2011a). It was initially determined that just the over-expression of either methylglyoxal synthase or alcohol dehydrogenase led to the production of 1,2-PDO in *E. coli* using glucose (Altaras and Cameron, 1999b). Enhanced production of 1,2-PDO was then achieved using glucose, by expressing the complete pathway via lactaldehyde (*E. coli* methylglyoxal synthase, glycerol dehydrogenase and 1,2-propanediol oxidoreductase) in an engineered *E. coli* strain lacking lactate dehydrogenase activity. About 4.5 g/L of 1,2-PDO was produced with a yield of 0.19 g/g glucose in 2 L fed-batch anaerobic fermentations (Altaras and Cameron, 2000a). The highest reported titer of 1,2-PDO (5.6 g/L) achieved in *E. coli* was reported by engineering its glycerol dissimilation pathway. In their work, DHAP availability was increased by replacing *E. coli*'s native PEP-dependent dihydroxyacetone kinase with *C. freundii* ATP- dependent

dihydroxyacetone kinase. By over-expressing *E. coli* methylglyoxal synthase, glycerol dehydrogenase and aldehyde oxidoreductase, 1,2-PDO production was achieved. Combining these strategies with the disruption of major fermentative by-product pathways (lactate and acetate) led to 1,2-propanediol production at 5.6 g/L in a fermenter with 400 mL medium containing glycerol (Clomburg and Gonzalez, 2011b).

The production of 1,2-PDO from *S. cerevisiae* was achieved by engineering the glycolysis pathway along with the expression of *E. coli* methylglyoxal synthase and glycerol dehydrogenase enzymes. With the disruption of the gene encoding triose phosphate isomerase, carbon flux to DHAP was increased in the engineered *S. cerevisiae* strain which led to 1,2-PDO production at 1.11 g/L (Joon-Young et al., 2008). In another report, *S. cerevisiae* was engineered to increase glycerol uptake rate as a strategy to enhance 1,2-PDO production. With the over-expression of the glycerol dissimilation pathways along with *E. coli* methylglyoxal synthase and glycerol dehydrogenase enzymes, 1,2-PDO was produced at 2.19 g/L using a modified YEPD medium containing 1% glycerol and 0.1% galactose (Joon-Young et al., 2011).

While the production of 1,2-PDO has been largely pursued utilizing a range of carbon sources and metabolic engineering of *E. coli* and *S. cerevisiae*, in 2013, its production was reported from CO_2, using an engineered cyanobacterial strain. In their work, the *E. coli* 1,2-PDO pathway was introduced in cyanobacteria, however, with the replacement of NADH-specific secondary alcohol dehydrogenase with a *C. beijerinckii* NADPH-specific secondary alcohol dehydrogenase to enhance its production. This strategy resulted in 0.15 g/L of 1,2-PDO production with the consumption of CO_2 (Li and Liao, 2013).

1.4. Summary of Doctoral Dissertation

In this work, a prokaryotic system – *Escherichia coli* grown on simple carbon sources will be used as a "cell factory" for the production of target chemicals (1,2-propanediol, 1-propanol, 1,4-butanediol) via the manipulation of its native metabolism.

In the second chapter we establish a novel microbial platform for the production of 1-propanol from glucose by expanding the 1,2-propanediol pathway. To do this we first characterize different enzymes involved the 1,2-propanediol pathway by *in vitro* assays. We then establish a dual pathway for the production of 1,2-propanediol. Following this, we screen different diol dehydratases and identify the dehydratase from *Klebsiella oxytoca* as the optimal candidate to dehydrate 1,2-propanediol. With the expression of the 1,2-propanediol pathway we achieve 0.8 g/L of 1,2-propanediol from 20 mL anaerobic shake flask studies. Furthermore, with the expression of the complete pathway we achieve 1-propanol biosynthesis at 0.25 g/L from *E. coli* growing on glucose. (Jain and Yan, 2011)

In the third chapter we pursue the enhancement in production of 1,2-propanediol and 1-propanol from glucose. We first systematically engineer *E. coli* to address various limitations of the introduced 1,2-propanediol pathway via: (a) expression of optimal minimal set of enzymes for 1,2-propanediol production (b) preventing loss of carbon to competing pathways (c) improving intracellular NADH availability (d) improving growth of the engineered strain (Jain et al., 2014). These efforts enable 1,2-propanediol production at enhanced titer without sacrificing yield for the first time. We then channel our efforts to enhance the activity of diol dehydratase to maximize the conversion of 1,2-propanediol to 1-propanol. For this, we construct a fusion diol dehydratase and develop a dual strain strategy, which results in near complete conversion of 1,2-

propanediol to 1-propanol. This work represents over 11 fold increase in *de novo* biosynthesis of 1-propanol from glucose as compared to our previous work in chapter 2. (Jain et al., 2014)

In the fourth chapter we derive inspiration from our previous work to utilize a diol dehydratase for the production of a high value bulk chemical via the expansion of native metabolism. Specifically, we engineer the *Klebsiella oxytoca* diol dehydratase via structure based redesign to enhance activity toward a non-native C4 triol (1,2,4-butanetriol). We perform *in silico* analysis to identify candidate mutants via docking simulations. We then characterize the activity of these mutants via feeding experiments and *in vitro* assays. Following this, we express the 1,2,4-butanetriol pathway in an engineered *E. coli* strain to prevent loss of carbon toward its native xylose catabolism pathway(s). With the expression of the complete pathway we achieve *de novo* biosynthesis of 1,4-butanediol from xylose via a novel metabolic route.

CHAPTER 2

DEHYDRATASE MEDIATED 1-PROPANOL PRODUCTION IN METABOLICALLY ENGINEERED *ESCHERICHIA COLI*

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Abstract

Background: With the increasing consumption of fossil fuels, the question of meeting the global energy demand is of great importance in the near future. As an effective solution, production of higher alcohols from renewable sources by microorganisms has been proposed to address both energy crisis and environmental concerns. Higher alcohols contain more than two carbon atoms and have better physiochemical properties than ethanol as fuel substitutes.

Results: We designed a novel 1-propanol metabolic pathway by expanding the well-known 1,2-propanediol pathway with two more enzymatic steps catalyzed by a 1,2-propanediol dehydratase and an alcohol dehydrogenase. In order to engineer the pathway into *E. coli*, we evaluated the activities of eight different methylglyoxal synthases which play crucial roles in shunting carbon flux from glycolysis towards 1-propanol biosynthesis, as well as two secondary alcohol dehydrogenases of different origins that reduce both methylglyoxal and hydroxyacetone. It is evident from our results that the most active enzymes are the methylglyoxal synthase from *Bacillus subtilis* and the secondary alcohol dehydrogenase from *Klebsiella pneumoniae*, encoded by *mgsA* and *budC* respectively. With the expression of these two genes and the *E. coli ydjG* encoding methylglyoxal reductase, we achieved the production of 1,2-propanediol at 0.8 g/L in shake flask experiments. We then characterized the catalytic efficiency of three different diol-dehydratases on 1,2-propanediol and identified the optimal one as the 1,2-propanediol dehydratase from *Klebsiella oxytoca*, encoded by the operon *ppdABC*. Co-expressing this enzyme with the above 1,2-propanediol pathway in wild type *E. coli* resulted in the production of 1-propanol at a titer of 0.25 g/L.

Conclusions: We have successfully established a new pathway for 1-propanol production by shunting the carbon flux from glycolysis. To our knowledge, it is the first time that this pathway has been utilized to produce 1-propanol in *E. coli*. The work presented here forms a basis for further improvement in production. We speculate that dragging more carbon flux towards methylglyoxal by manipulating glycolytic pathway and eliminating competing pathways such as lactate generation can further enhance the production of 1-propanol.

2.1. Introduction

The excessive utilization of petroleum plays a major role in the release of the greenhouse gas-carbon dioxide contributing to global warming. Renewable energy sources provide a wide platform of resources to address the problem of increasing energy demand. The manufacture of biofuels such as higher chain alcohols from renewable sources provides an alternative energy source which possesses the advantage of having desirable fuel properties and uncomplicated transportability (Atsumi et al., 2008e; Atsumi and Liao, 2008b; Connor and Atsumi, 2010). The synthesis of various higher chain alcohols has been achieved by constructing biosynthetic pathways in *E. coli* and other micro-organisms (Atsumi et al., 2008a; Atsumi et al., 2008e; Atsumi and Liao, 2008b; Connor and Atsumi, 2010; Inokuma et al., 2010a; Shen and Liao, 2008c; WHO). Here, we describe the design of a new pathway for 1-propanol synthesis and its validation in *E. coli*.

In petrochemical industry, 1-propanol is produced from ethene by a reaction with carbon monoxide and hydrogen to give propionaldehyde, which is then hydrogenated (WHO). 1-propanol is also produced as a by-product when potatoes or grains are fermented during the commercial manufacture of ethanol (CLC; WHO). The general use of 1-propanol is in the manufacture of drugs and cosmetics such as lotions, soaps, and nail polishes. It also finds applications in the manufacture of flexographic printing ink and textiles (CLC; WHO).

Recently, the use of 1-propanol as a potential fuel substitute to petroleum has promoted the interest in its production via biological approaches. In 2008, Atsumi *et al.* and Shen *and Liao* reported the production of 1-propanol from glucose by metabolic engineering of *E. coli*. Their work relied on the keto-acid pathway in *E. coli* with 2-ketobutyrate as a key intermediate

(Atsumi et al., 2008e; Shen and Liao, 2008c). The 2-ketobutyrate was converted to 1-propanol by the action of a keto acid decarboxylase and an alcohol dehydrogenase. Wild type *E. coli* carrying this pathway was able to produce around 0.15 g/L of 1-propanol. With the elimination of the genes *metA*, *tdh*, *ilvB*, *ilvl* and *adhE* encoding the enzymes *o*-succinyltransferase, threonine dehydrogenase, acetohydroxy acid synthase and alcohol dehydrogenase respectively, the production of 1-propanol achieved was 1 g/L. Atsumi *et al.* reported higher levels of 1-propanol production in *E. coli* using *cimA* encoding a citramalate synthase from *Methanococcus jannaschii* (Atsumi et al., 2008a). They established a direct route for the conversion of pyruvate to 2-ketobutyrate. With the utilization of citramalate pathway and incorporating an evolutionary strategy based on growth they were able to overcome feedback inhibition by isoleucine. Using wild type *cimA* they achieved 0.3 g/L of 1-propanol production. With the development of *cimA* variants, the production of 1-propanol was 9 times higher compared to the wild type *cimA*.

We developed a new approach for the biosynthesis of 1-propanol by extending the well-known 1,2-propanediol pathway. As the pathway scheme shown in Figure 2.1, the intermediate of glycolysis dihydroxyacetone phosphate is converted to methylglyoxal by the action of the enzyme methylglyoxal synthase. The methylglyoxal generated is further reduced to either hydroxyacetone or lactaldehyde via two different routes. The formation of hydroxyacetone is catalyzed by the enzyme methylglyoxal reductase which is a primary alcohol dehydrogenase, while a secondary alcohol dehydrogenase such as glycerol dehydrogenase reduces methylglyoxal into lactaldehyde. Both hydroxyacetone and lactaldehyde can be further reduced to 1,2-propanediol by either a secondary alcohol dehydrogenase or a primary alcohol dehydrogenase. The dehydration of 1,2-propanediol into 1-propanal can be achieved by a diol dehydratase. The conversion of 1-propanal to 1-propanol is also catalyzed by a primary alcohol dehydrogenase.

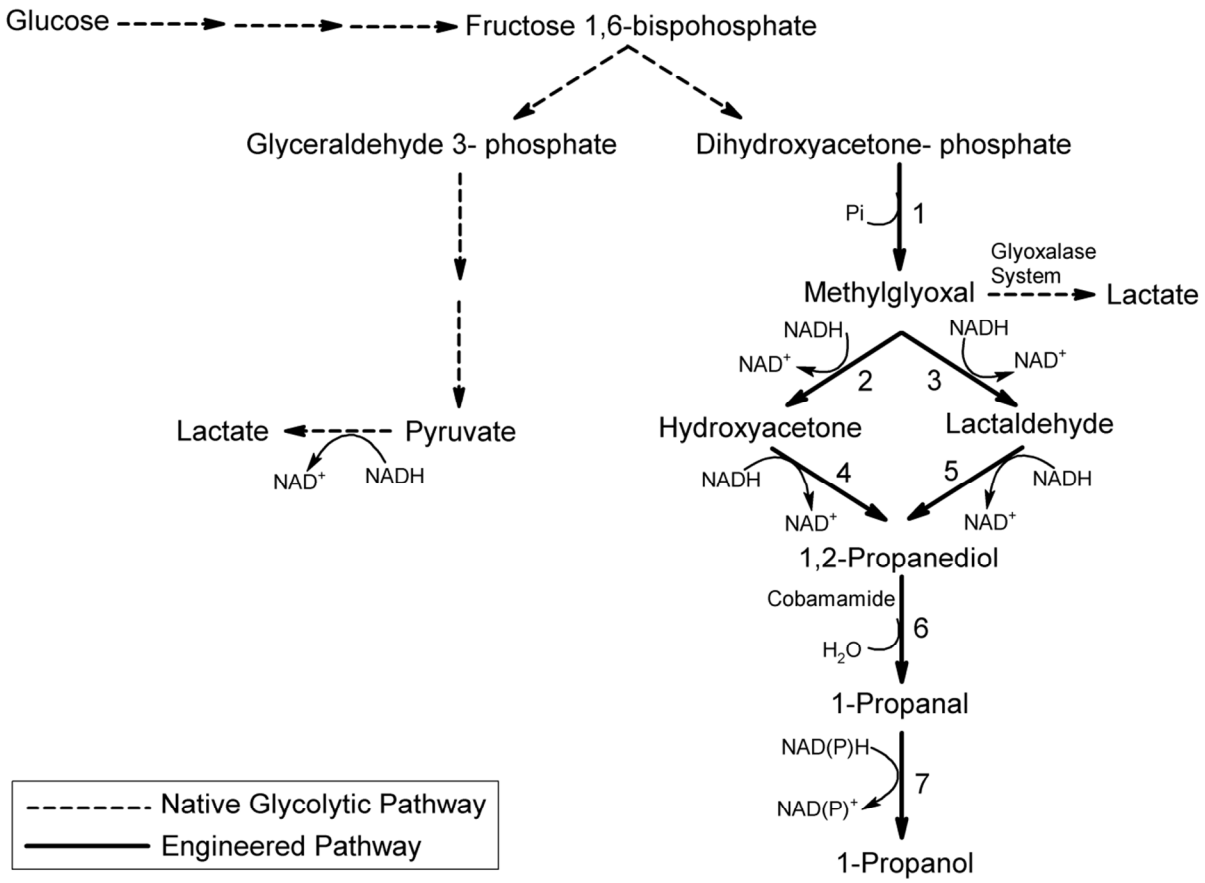


Figure 2.1. Designed metabolic pathway for 1,2-propanediol and 1-propanol production. Key enzymes 1: methylglyoxal synthase (*mgsA*); 2: methylglyoxal reductase (*ydjG*); 3, 4: secondary alcohol dehydrogenase (*gldA/ budC*); 5: primary alcohol dehydrogenase (*fucO*); 6: diol dehydratase (*ppdABC/ gldABC/ dhaB12*); 7: primary alcohol dehydrogenase (*yqhD*).

The pathway that leads to the synthesis of 1,2-propanediol has been introduced into both *E. coli* and *Saccharomyces cerevisiae*. By overexpressing the *E. coli* genes *mgsA* and *gldA* and relying on the native expression of other enzymes, Altaras *et al.* achieved the production of 0.7 g/L of 1,2-propanediol in *E. coli* (Altaras and Cameron, 1999a). 1.08 g/L 1,2-propanediol production in *E. coli* was reported by Berrios-Rivera *et al.* by utlizing *Clostridium acetobutylicum mgsA* and *E.*

coli gldA in a strain defecient in lactate production and using an initial glucose concentration of 101.68 mM (Berrios-Rivera et al., 2003a). Enhanced production of 1,2-propanediol in *E. coli* was also reported by Altaras *et al.* (Altaras and Cameron, 2000b). The study involved expression of more complete pathway by addition of *fucO* gene (1,2-propanediol oxidoreductase) responsible for the conversion of lactaldehyde to 1,2-propanediol and deletion of the competing pathway for lactate which involves the gene *ldhA*. Shake flask fermentation with the *ldhA*- strain carrying the pathway led to the production of 1,2-propanediol at a titer of 1.27 g/L , while fed-batch fermentation gave a result of 4.5 g/L of 1,2-propanediol. 1,2-propanediol production in *S. cerevisiae* was achieved by Joon-Young *et al.* (Joon-Young et al., 2008). Their strategy was based on the idea of channeling the carbon flux towards dihydroxyacetone phosphate with the deletion of triosphoshate isomerase in *S. cerevisiae* via triple homologous recombination. With the introduction of 1,2-propanediol pathways consisting of the *E. coli* genes *mgsA* and *gldA,* the engineered *S. cerevisiae* produced 1.11 g/L of 1,2-propanediol compared to 0.89 g/L produced from the strain lacking the gene *tpiI*.

In this study, we first constructed the 1,2-propanediol pathway in the wild type *E. coli* strain BW25113 by expressing the genes, *mgsA* from *B. subtilis*, *budC* from *K. pneumoniae*, and native *E. coli ydjG*, which resulted in the production of 1,2-propanediol at a titer of 0.8 g/L in shake flasks. We further achieved the conversion of 1,2-propanediol to 1-propanol via two successive enzymatic steps by expressing the operon *ppdABC* from *K. oxytoca* and using the native activity of *E. coli* alcohol dehydrogenases (Jeter, 1990; O' Brien et al., 2004; Roth et al., 1996). This established a new pathway for 1-propanol production by engineering the glycolytic pathway in *E. coli*.

2.2. Results and Discussion

2.2.1. Methylglyoxal Synthase Assay

1,2-Propanediol pathway branches from glycolysis and competes for the intermediate dihydroxyacetone phosphate, with the glycolytic pathway. The first enzyme of 1,2-propanediol pathway, methylglyoxal synthase catalyzing irreversible conversion of dihydroxyacetone-phosphate to methylglyoxal holds paramount importance in channeling carbon flux towards 1,2-propanediol biosynthesis (Altaras and Cameron, 1999a; Berrios-Rivera et al., 2003a). Highly active methylglyoxal synthase is therefore desirable. We screened the activity of methylglyoxal synthase from eight different sources. We amplified the *mgsA* genes from the microorganisms: *C. acetobutylicum* (ATCC# 824), *B. subtilis* 168, *C. difficile* R20291, *E. coli* MG1655, *T. thermophilus* HB27, *K. pneumoniae* MGH78578, *P. fluorescens* Pf-5, and *R. eutropha* H16 respectively. These genes were cloned and expressed in wild type *E. coli* BW25113 using eight plasmids pRJ1-pRJ8. Each gene was under the control of the IPTG-inducible pLlacO1 promoter. Using dihydroxyacetone- phosphate as the substrate, we successfully detected the functional expression of all *mgsA* genes *in vitro*, where the specific activities varied from 0.0052 U/mg to 0.1242 U/mg (Table 2.1). We identified most suitable methylglyoxal synthase as the *mgsA* from *B. subtilis* demonstrating the highest ratio of specific activity/K_m (0.1186) and having a specific activity of 0.0561 U/mg. Without the overexpression of *mgsA* gene, we also detected the native expression of *E. coli mgsA,* which gave a specific activity of only 0.0008 U/mg, much lower than that of any overexpression.

Table 2.1. Methylglyoxal synthase assay results. Substrate dihydroxyacetone phosphate concentration was varied from 0.15 mM to 1.5 mM for all reactions. 1 unit (U) was defined as the amount (μmoles) of methylglyoxal formed per unit time (min).

***mgsA* source**	**Specific Activity (U/mg)**	**K_m (mM)**	**Specific Activity/ K_m (U/mg/mM)**
C. acetobutylicum	0.0541 ± 0.0042	0.7763 ± 0.005	0.0697
B. subtilis	**0.0561 ± 0.0031**	**0.473 ± 0.07**	**0.1186**
C. difficile	0.0597 ± 0.0039	1.439 ± 0.06	0.0415
E. coli	0.1242 ± 0.0069	1.418 ± 0.12	0.0876
T. thermophilus	0.0161 ± 0.0004	2.118 ± 0.07	0.0076
K. pneumoniae	0.0165 ± 0.0009	2.82 ± 0.3	0.0058
P. fluorescens	0.0133 ± 0.0082	1.56 ± 0.02	0.0085
R. eutropha	0.0052 ± 0.0004	0.7 ± 0.03	0.00743

2.2.2. Methylglyoxal Reductase Assay

We examined the activity of *E. coli* methylglyoxal reductase encoded by the gene *ydjG* by using plasmid pRJ10. As a part of aldo-keto reductase family, the product of *ydjG* executes a catalytic activity of reduction on methylglyoxal using NADH to generate hydroxyacetone (Luccio et al., 2006). We determined both the specific activity and substrate affinity of *E. coli* methylglyoxal reductase on methylglyoxal. When the gene is overexpressed by pRJ10, the specific activity was determined to be 1.62 ± 0.012 U/mg. The enzyme also showed sufficient substrate specificity with a K_m value of 3.31 ± 0.02 mM.

2.2.3. Secondary Alcohol Dehydrogenase Assay

The synthesis of 1,2-propanediol from methylglyoxal occurs through two different pathways. For the pathway via lactaldehyde leading to 1, 2-propanediol formation we evaluated the activities of two NADH dependent secondary alcohol dehydrogenases: *E. coli* glycerol dehydrogenase (*gldA*) and *K. pneumoniae* diol dehydrogenase (*budC*) on methylglyoxal. We also tested the catalytic properties of these two secondary alcohol dehydrogenases on hydroxyacetone for the completion of the other pathway.

The genes *gldA* and *budC* were cloned and expressed in *E. coli* using the plasmid pRJ9 and pYY109. The specific activity and K_m value of glycerol dehydrogenase and diol dehydrogenase were determined for the substrates methylglyoxal and hydroxyacetone. Both enzymes showed dehydrogenation activity leading to the conversion of methylglyoxal to lactaldehyde and hydroxyacetone to 1,-2-propanediol. Table 2.2 provides the results of this assay. The diol dehydrogenase and glycerol dehydrogenase reduced both methylglyoxal and hydroxyacetone. When methylglyoxal was used as the substrate, the diol dehydrogenase demonstrated a specific activity of 3.718 U/mg with a K_m value of 0.78 mM; while the glycerol dehydrogenase showed both lower specific activity (2.456 U/mg) and substrate affinity (K_m = 68.24 mM). Similar results were observed when hydroxyacetone was tested as a substrate, the diol dehydrogenase more efficiently reduced hydroxyacetone into 1,2-propanediol (specific activity= 4.97 U/mg; K_m= 1.83 mM) compared with the glycerol dehydrogenase (specific activity= 0.912 U/mg; K_m= 10.47 mM).

Table 2.2. Specific activity and K_m determination of the secondary alcohol dehydrogenases. The decrease in absorbance of NADH at 340 nm was recorded and used for calculations using the substrates methylglyoxal and hydroxyacetone. Substrate concentration was varied from 20 mM – 120 mM. 1 unit (U) was defined as the amount (µmoles) of product formed per unit time (min).

Gene	Methylglyoxal		Hydroxyacetone	
	Specific Activity (U/mg)	**K_m (mM)**	**Specific Activity (U/mg)**	**K_m (mM)**
gldA	2.456 ± 0.001	68.24 ± 0.05	0.912 ± 0.008	10.47 ± 0.55
budC	3.718 ± 0.066	0.78 ± 0.03	4.97 ± 0.007	1.83 ± 0.63

2.2.4. Propanediol Dehydratase *in vivo* Assay

The diol dehydratases we tested included a propanediol dehydratase (PPD) originating in *K. oxytoca*, a glycerol dehydratase (GLD) from *K. pneumoniae*, and a glycerol dehydratase (GLD) from *C. butyricum*. The PPD of *K. oxytoca* and the GLD of *K. pneumoniae* are iso-functional enzymes which catalyze the coenzyme B12-depedent conversion of 1,2-propanediol or glycerol to the corresponding aldehyde (Honda et al., 1980; Luccio et al., 2006; Tobimatsu et al., 1996; Tobimatsu et al., 1997). These enzymes have been utilized to develop a biological process to produce 1,3-propanediol from glycerol (Raynaud et al., 2003b). Each of these enzymes consists of three subunits encoded by three structural genes (*ppdABC* or *gldABC*). Although the catalytic site is hosted by subunit A, the presence of subunits B and C are obligatory for enzyme activity (Tobimatsu et al., 1996). In order to evaluate their catalytic efficiency towards 1,2-propanediol, all three subunits were co-expressed in *E. coli* to reconstitute the enzymes using the plasmids pYY93 and pYY134. The GLD from *C. butyricum* is a coenzyme B12-independent diol

dehydratase comprised of two subunits encoded by *dhaB12*, which only demonstrates activity under strict anaerobic conditions (Raynaud et al., 2003b). To evaluate its catalytic efficiency, we constructed the plasmid pYY167 to co-express these two units.

The formation of 1-propanol from 1,2-propanediol involves two enzymatic steps. For the first step we evaluated the dehydration activity of three different diol dehydratases for the generation of 1-propanal. For the second step we relied on the native alcohol dehydrogenase activity of *E. coli* to convert the generated 1-propanal to 1-propanol. An experiment was designed and conducted as we described to perform the *in vivo* enzyme assay of propanediol dehydratase and also to evaluate the native activity of *E. coli* for the final step. Whole-cell bioconversion studies using wild type *E. coli* strain BW25113 carrying pYY93, pYY134, and pYY167 respectively were conducted in shake flasks by feeding 5 g/L (65.7 mM) 1,2-propanediol as the substrate. The samples were collected after 24 h and analyzed by HPLC-RID. The results are presented in Figure 2.2.

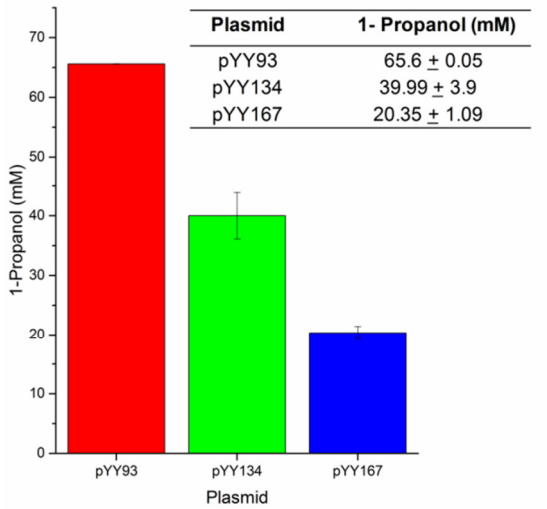


Figure 2.2. The results of *in vivo* enzyme assay of diol dehydratase using 5 g/L (65.7 mM) 1,2-propanediol as the substrate.

As seen from the results, the catalytic efficiency of *K. oxytoca* PPD was the highest among all, producing 65.6 mM 1-propanol amounting to nearly 100% conversion. This result also indicated that the native expression of alcohol dehydrogenases in *E. coli* is sufficient to convert 1-propanal to 1-propanol completely. Overexpression of the alcohol dehydrogenases will not be necessary for 1-propanol production in *E. coli*. The *K. pneumoniae* GLD and *C. butyricum* GLD only demonstrated about 60.9% and 30.9% of the catalytic efficiency of *K. oxytoca* PPD, producing 39.99 mM and 20.35 mM 1-propanol, respectively.

2.2.5. Production of 1,2-Propanediol and 1-Propanol in *E. coli*

In order to introduce the 1-propanol pathway into wild type *E. coli* strain BW25113, we constructed two plasmids. The first plasmid pRJ11 carries the genes encoding the most active enzymes for 1,2-propanediol biosynthesis. Specifically, *mgsA* from *B. subtilis*, *ydjG* from *E. coli,* and *budC* from *K. pneumoniae* were organized as a synthetic operon under the control of IPTG-inducible pLlacO1 promoter in a high-copy number plasmid. The second plasmid pYY93 contains only the structural genes *ppdABC* encoded by *K. oxytoca* PPD. For the enzymatic steps of lactaldehyde to 1,2-propanediol and 1-propanal to 1-propanol, we completely relied on the native expression of alcohol dehydrogenases in *E. coli*, as is indicated to be sufficient (Altaras and Cameron, 1999a; Raynaud et al., 2003b). We also evaluated M9 medium in comparison to low-phosphate medium for the production of 1,2-propnaediol (Data not provided). The results of an initial experiment show significant increase in production using low-phosphate medium. Using M9 medium resulted in only about 0.1 g/L of 1,2- propanediol generation after 48 h of anaerobic fermentation compared to about 0.8 g/L from low-phosphate medium. Hence low-phosphate medium was used for all fermentation studies. The culture conditions were the same as described in methods and materials.

We first transformed the plasmid pRJ11 into wild type *E. coli* strain BW25113 to achieve 1,2-propanediol production. It has been reported that the enzyme methylglyoxal synthase is inhibited by phosphate ion (Hopper and Cooper, 1971; Hopper and Cooper, 1972). The inhibition of methylglyoxal synthase would result in the carbon flux being diverted to glyceraldehyde-3-phosphate instead of methylglyoxal by the conversion action of triose phosphate isomerase. Therefore a low- phosphate medium was employed to avoid this problem. The fermentation experiments were conducted in 20 mL cultures as described in the methods and materials. The fermentation samples were collected after 24 h and 48 h and analyzed by HPLC-RID. As the results shown in Table 2.3, after 24 h 0.66 g/L 1,2-propanediol was produced. The production reached 0.80 g/L after 48 h. Lactate was detected as the dominant by-product and was accumulated at over 7 g/L. We also conducted the experiments aerobically. However, only a trace amount (<0.01 g/L) of 1,2-propanediol was produced and the cell growth was much better than in anaerobic conditions, which indicates that glycolysis is very active in aerobic condition and drags almost all carbon flux towards pyruvate for cell growth or other cell activities.

With the successful establishment of the pathways for 1, 2-propanediol production using pRJ11, we hypothesized that the co-expression of pRJ11 with pYY93 would result in the production of 1-propanol. To test this, wild type *E. coli* BW25113 was transformed with both pRJ11 and pYY93 for 1-propanol production by electroporation. The fermentation condition was similar to that used for 1,2-propanediol production with the addition of 10μM coenzyme B-12 to the culture along with IPTG (0.1 mM) after 6 h. After 24 h, the double transformed strain produced 0.11 g/L 1-propanol with 0.44 g/L 1, 2-propanediol remaining unconverted (Table 2.3). After 48 h, 1-propanol was produced at 0.25 g/L with 0.46 g/L 1,2-propanediol remaining unconverted. The major by-product was again lactate at over 7 g/L.

Table 2.3. 1,2-Propanediol and 1-propanol production in low- phosphate medium using *E. coli* strain *BW25113* transformed with the appropriate plasmid(s).

Plasmid	1, 2-Propanediol (g/L)		1- Propanol (g/L)	
	24h	48h	24h	48h
pRJ11	0.66 ± 0.01	0.80 ± 0.01	-----	-----
pRJ11 and pYY93	0.44 ± 0.01	0.46 ± 0.02	0.11 ± 0.01	0.25 ± 0.07

2.3. Conclusions

We have successfully established a new pathway for 1- propanol production by shunting the native glycolytic pathway in *E. coli*. The addition of the coenzyme B-12 dependent propanediol dehydratase from *K. oxytoca* resulted in the conversion of 1,2-propanediol to 1-propanal which was then dehydrogenated by *E. coli* native activity to 1- propanol.

From the assay of methylglyoxal synthase it was determined that the *mgsA* from *B. subtilis* was the most active. Since the accumulation of methylglyoxal in high quantities is toxic to the cell (Ackerman et al., 1974), it is important that the generated methylglyoxal is immediately converted to another metabolite by the downstream enzymes in the pathway. To address this problem we screened the activity of methylglyoxal reductase and two secondary alcohol dehydrogenases.

To evaluate 1, 2-propanediol formation, methylglyoxal synthase (*mgsA*) from *B. subtilis* and propanediol dehydratase (*budC*) from *K. pneumoniae* were expressed leading to the conversion of dihydroxyacetone phosphate to 1,2-propanediol via the formation of methylglyoxal and

lactaldehyde. To strengthen our constructed pathway the introduction of *E. coli* methylglyoxal reductase (*ydjG*), a dual metabolic route for production of 1, 2-propanediol was established for the first time. This resulted in channeling the carbon flux from methylglyoxal to hydroxyacetone and 1,2-propanediol. Fermentation with *E. coli* BW25113 transformed with pRJ11 carrying the three above mentioned genes produced 0.8 g/L 1,2-propanediol after 48 h of anaerobic fermentation.

We also evaluated the use of a medium copy number vector (pRJ12) for 1,2-propanediol production (data not provided). This was done using the same genes used for the construction of high copy number plasmid pRJ11 but in the backbone of a medium copy number vector pCS27. However, the production of 1,2-propanediol from a medium copy number vector (pRJ12) was found to be significantly lower than the production by high copy number vector (pRJ11). Hence the medium copy number vector was not selected for 1, 2-propanediol and 1-propanol production.

The result of the *in vivo* enzyme assay (Figure 2.2) shows almost 100% conversion of 1, 2-propanediol to 1-propanol indicating that the conversion of 1,2-propanediol to 1-propanal was very efficient and that the native expression of alcohol dehydrogenases in *E. coli* is sufficient in converting 1-propanal to 1-propanol. However, it was not the case for the strain carrying the plasmids pRJ11 and pYY93 which showed much lower conversion of 1,2-propanediol to 1-propanol as about 0.46 g/L of 1,2-propanediol was left unconverted. We speculate that the reason for this could be the expression issue of *ppdABC*. The optimal expression of these three subunits can be successfully achieved in aerobic conditions as we did in *in vivo* assay (Tobimatsu et al., 1996). However, in anaerobic conditions which is required for 1,2-propanediol production, the protein expression might be negatively affected due to low cellular energy and nutrients. Such a

problem could be resolved in a more controlled environment such as in a bench scale fermenter by the delicate adjustment of oxygen level during fermentation course.

The accumulation of 7 g/L lactate indicates that the carbon flux towards pyruvate is still strong in anaerobic conditions. The main branch of glycolysis plays the major role. Theoretically, one molecule of fructose-1,6-bisphosphate is broken down into one molecule of glyceraldehyde-3-phosphate and one molecule of dihydroxyacetone phosphate (Stribling and Perham, 1973). However, the presence of triose phosphate isomerase seems to channel the carbon flux back to the main branch toward pyruvate biosynthesis (Stribling and Perham, 1973). In addition, the pentose phosphate pathway is also very active in low phosphate conditions (Kruger and Schaewen, 2003). This pathway does not generate dihydroxyacetone phosphate as an intermediate, but directly goes to pyruvate. The pyruvate generated is acted upon by lactate dehydrogenase (*ldhA*) resulting in the production of lactate (Jiang et al., 2001). Another minor route of lactate formation is via the glyoxalase pathway where methylglyoxal is converted to lactate by the native expression of *gloA* (Clugston et al., 1998).

Overall, the work presented here represents 1-propanol production in a wild type *E. coli* strain and forms a basis for further enhancement in production. The effect of competing pathways is significant and the deletion of the same has not been explored in this study. We speculate that by the knock-out of genes encoding for lactate dehydrogenase (*ldhA)*, glyoxalase I (*gloA*) and other competing pathways (*tpiA* and *zwf*) the production of 1- propanol can be further enhanced, which will be pursued in the near future.

2.4. Methods and Materials

2.4.1. Chemicals and Reagents

Hydroxyacetone was bought from Acros Organics (New Jersey, USA); methylglyoxal and 1,2-propanediol were purchased from Sigma Aldrich (St. Louis, Mo); 1-propanol was obtained from Fisher Scientific (Atlanta, GA). KOD DNA polymerase was obtained from EMD Chemicals Inc., NJ. All restriction enzymes were bought from New England Biolabs (Beverly, MA). The rapid DNA ligase was obtained from Roche Applied Science (Indianapolis, IN).All the enzymes were used according to the instructions of the manufacturer.

2.4.2. Plasmids and Strains

E. coli strain XL1-Blue (Stratagene, CA) was used for DNA manipulations; while wild type *E. coli* strain BW25113 (*E. coli* Genetic Resource Center, CT) and *E. coli* strain BL21* (Invitrogen) were employed for enzyme assays and shake flask experiments. Plasmids pZE12-luc (Lutz and Bujard, 1997), pCS27 (Shen and Liao, 2008c) and pCDF-Duet1 (EMD Chemicals Inc., NJ) were used for DNA cloning. The features and descriptions of the used strains and plasmids are listed in Table 2.4.

2.4.3. DNA manipulations

All DNA manipulations were performed according to the standard procedures as described previously (Ausubel et al., 1994). The primers involved in DNA manipulations are listed in Table 2.5. The plasmids listed in Table 2.4 were constructed as described below.

For the methylglyoxal synthase assay, the plasmids pRJ1-pRJ8 were constructed by cloning *mgsA* genes from eight different sources into the vector pZE12-luc separately. Using the primers

listed in Table 2.5, the *mgsA* genes were PCR amplified from the genomic DNA of *C. acetobutylicum* (ATCC824), *B. subtilis* 168, *Clostridium difficile* R20291, *E. coli* MG1655, *Thermus thermophilus* HB27, *K. pneumoniae* MGH78578, *Pseudomonas fluorescens* Pf-5, and *Ralstonia eutropha* H16 respectively. The DNA fragments obtained were digested with restriction enzymes for 3 h. *Acc65I* and *SphI* restriction enzymes were used to digest *mgsA* genes from *C. acetobutylicum* (ATCC824), *B. subtilis* 168, *C. difficile* R20291, *E. coli* MG1655, *T. thermophilus* HB27, *K. pneumoniae* MGH78578, *BsrGI* and *SphI* for the *mgsA* from *P. fluorescens* Pf-5, and *Acc65I* and *XbaI* for the *mgsA* from *R. eutropha* H16. The vector pZE12-luc was also digested with the appropriate restriction enzymes for the above mentioned eight genes. The digested genes were then inserted into the vector pZE12-luc separately.

In order to determine the activity of methylglyoxal reductase, the plasmid pRJ10 was constructed. The *ydjG* gene PCR amplified from *E. coli* MG1655 was cloned into pZE-12luc with restriction enzymes *Acc65I* and *XbaI* generating pRJ10. For the assay of secondary alcohol dehydrogenases, plasmids pRJ9 and pYY109 were constructed. The *gldA* gene from *E. coli* MG1655 was inserted into pZE12-luc vector using restriction enzymes *SphI* and *XbaI* for the construction of plasmid pRJ9. We created pYY109 by inserting the *budC* gene from *K. pneumoniae* MGH78578 into pCDF-Duet1 vector. The restriction enzymes used for the construction of plasmid pYY109 were *BamHI* and *SalI.*

For diol dehydratase assay, plasmids pYY93, pYY134, and pYY167 were constructed. The *ppdABC* operon obtained via PCR from the genomic DNA of *K. oxytoca* was digested with restriction enzymes *BsiWI* and *HindIII* and inserted into plasmid pCS27 digested by *BsiWI* and *HindIII*. Similarly, the *gldABC* operon was PCR amplified from genomic DNA of *K. pneumoniae* MGH 78578 and inserted into pCS27 using restriction enzymes *Acc65I* and *HindIII*.

To construct pYY167, we first synthesized the operon *dhaB12* from *Clostridium butyricum* by a PCR assembly of 50 bp oligonucleotides designed from Helix Systems (NIH). The codons were optimized for *E. coli* standard expression. The operon was cloned into pCS27 using *Acc65I* and *HindIII*, forming pYY167.

Following the enzyme assays, plasmid pRJ11 was constructed using the most active enzymes in order to produce 1, 2-propanediol. The plasmid pRJ11 was generated via the ligation of three genes on the backbone of pZE12-luc vector. The genes *ydjG*, *budC* and *mgsA* were PCR amplified using the primers listed in Table 2.5 from the genomic DNA of *E. coli* MG1655, *K. pneumoniae* MGH78578 and *B. subtilis* 168, respectively. Following this, the PCR amplified *ydjG* gene product was digested with *Acc65I* and *SalI*. The PCR amplified *budC* gene product was digested with *SalI* and *PstI*, and the PCR amplified *mgsA* gene was digested with *PstI* and *XbaI*. Vector pZE12-luc was digested with *Acc65I* and *XbaI*. After digestion for 3 h, the three gene fragments and the vector were ligated simultaneously, creating pRJ11. It should be noted that RBS sequence (AGGAGA) was inserted upstream of each structure gene with 6-8 nucleotides in between to facilitate protein translations.

2.4.4. Culture Medium and Fermentation Conditions

M9 minimum was used for the in-vivo assay of propanediol dehydratase and low-phosphate minimum medium was used for shake flask fermentations. M9 minimum medium consisted of (per liter): 20 g glucose, 5 g yeast extract, 12.8 g $Na_2HPO_4.7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.5 mM $MgSO_4$, and 0.05 mM $CaCl_2$. The low-phosphate medium consisted of (per liter): 20 g glucose, 5 g NaCl, 5 g yeast extract, 1.5 g KCl, 1 g NH_4Cl, 0.2 g $MgCl_2$, 0.07 g Na_2SO_4, and 0.00 5g $FeCl_3$, which was buffered to pH 6.8 with 13.3 g of $NaHCO_3$ and 10 g of 3-

[N-morpholino] propanesulfonic acid (MOPS) (Altaras and Cameron, 1999a; Altaras and Cameron, 2000b).

For the shake flask fermentations, 1 mL of seed culture was prepared in LB medium containing necessary antibiotics and grown overnight at 37°C in a shaker set at 250 rpm. After overnight incubation, the culture was inoculated into 20 mL of M9 or low-phosphate medium containing appropriate antibiotics in 150 mL serum bottles. After growing at 37°C for 3 h, the cultures were switched to an anaerobic condition by sparging nitrogen gas. IPTG was added into the culture to a final concentration of 0.1 mM 6 h after inoculation to induce protein expression. Then the fermentation was carried out at 30°C at 250 rpm. Samples were taken after 24 and 48 h and analyzed with HPLC-RID.

2.4.5. HPLC-RID Analysis

The analysis of fermentation products was done via HPLC (Shimadzu) equipped with a Coregel-64H column (Transgenomic). 1 mL sample was collected and centrifuged at 15,000 rpm for 10 minutes and the supernatant was filtered and used for analysis. The mobile phase used was 4mN H_2SO_4 having a flow rate of 0.6 mL/min and an oven temperature set at 60°C (Eiteman and Chastain, 1997).

2.4.6. Enzyme Crude Extract Preparation

E. coli strain BL21* was employed to express *budC* carried by pYY109. Expression of other individual enzymes was conducted in the wild type *E. coli* strain BW25113 harboring the corresponding plasmids. Generally, the transformed strains were pre-inoculated into LB liquid medium containing appropriate antibiotics and grown at 37°C overnight with shaking. The following day, 1 mL of pre-inoculum was added to 50 mL of fresh LB medium containing

necessary antibiotics. The culture was left to grow at 37°C with shaking until the OD_{600} reached approximately 0.6. At that point, IPTG was added to a final concentration of 1 mM and the protein expression was conducted at 30°C for 3 h. The cells were collected by centrifugation at 5000 rpm for 10 min at 4°C. The cell pellets were resuspended in 2 mL of 50 mM imidazole-HCl buffer (pH 7.0). Cell disruption was performed using French Press, and soluble protein was obtained by ultra-centrifugation for enzyme assays. Total protein concentration was estimated using the BCA kit (Pierce Chemicals).

2.4.7. Methylglyoxal Synthase Assay

Methylglyoxal synthase assay was performed as described previously with minor revisions (Altaras and Cameron, 1999a; Berrios-Rivera et al., 2003a; Hopper and Cooper, 1971). The assay was carried out using a two-step procedure. The reaction mixture (500 µL) consisted of 50 mM imidazole-HCl buffer (pH 7.0), 0.15-1.5 mM dihydroxyacetone phosphate, and 25 µL crude extract. Reaction was started with the addition of 25 µL crude extract to the reaction mixture and incubated in a water bath at 30°C for 30 seconds. Followed by this, the reaction was immediately stopped by the addition of 30 µL sample of the reaction mixture to the detection mixture and incubated in a water bath at 30°C for 15 minutes. The detection mixture consisted of 300 µL of DI water, 110 µL of 0.1 % 2,4-dinitrophenylhydrazine dissolved in 2 N HCl. After the completion of 15 minutes 550 µL of 10% NaOH was added to the detection mixture and then incubated at room temperature for another 15 minutes. (Final volume of detection mixture = 990µl). The samples were diluted 10 times before measuring the absorbance at 550 nm. 1 µmol of methylglyoxal has an absorbance value of 16.4 at 550 nm (Berrios-Rivera et al., 2003a).

2.4.8. Methylglyoxal reductase assay

The reaction mixture contained 20-120 mM methylglyoxal and 0.25 mM NADH, in imidazole-HCl buffer (pH 7.0) having a final volume of 970 μL. The assay was begun with the addition of 30 μL crude extract to the reaction mixture. The reaction was allowed to proceed for 60 seconds at 37^{o}C. We measured the decrease in absorbance of NADH at 340 nm to calculate the specific activity (Altaras and Cameron, 1999a; Berrios-Rivera et al., 2003a).

2.4.9. Secondary Alcohol Dehydrogenase Assay

The enzyme crude extracts prepared from *gldA* and *budC* expression were used for this assay. The reaction mixture consisted of 20-120 mM of methylglyoxal or hydroxyacetone and 0.25 mM NADH in imidazole-HCl buffer at (pH 7.0) with a final volume of 970 μL. The assay was begun with the addition of 30 μL crude extract to the reaction mixture. The reaction was allowed to proceed for 60 seconds at 37^{o}C. We measured the decrease in absorbance of NADH at 340 nm to calculate the specific activity (Altaras and Cameron, 1999a; Berrios-Rivera et al., 2003a).

2.4.10. Propanediol Dehydratase *in vivo* Assay

The assay was carried out to evaluate the activities of three diol dehydratases on 1,2-propanediol. Three *E. coli* strains generated by transforming the wild type *E. coli* BW25113 with pYY93, pYY134, and pYY167 respectively were used for this purpose. Pre-inoculum from an overnight culture was added to 10 mL of M9 medium (1:100 V/V) and grown at 37^{o}C. IPTG was added to the cultures to a final concentration of 0.1 mM and 1,2-propanediol was added to the cultures as the substrate to a final concentration of 5 g/L (65.7 mM) after 4 h. The cell cultures carrying pYY167 was grown anaerobically; while the cell cultures carrying pYY93 or pYY134 were grown micro-aerobically. Coenzyme-B12 (cobamamide) was also added to the cell cultures

having pYY93 and pYY134 to a final concentration of 10 μM after 4 h. Samples were collected after 24 h and analyzed for 1-propanol generation using HPLC-RID as described above. The enzyme activities were reflected by the formation of 1-propanol.

Competing Interests

The University of Georgia has filed a United States patent on this technology.

Table 2.4. List of strains and plasmids used in this study.

Strain	**Genotype**	**Reference**
E. coli BW25113	*rrnBT14 DlacZWJ16 hsdR514 DaraBADAH33 DrhaBADLD78*	(CGSC)
E. coli BL21*	*F^- ompT $hsdS_B$ (r_B^- m_B^-) gal dcm (DE3)*	Invitrogen
E. coli XL-1 Blue	*recA1 endA1gyrA96thi-1hsdR17supE44relA1lac [F' proAB lacIqZDM15Tn10 (TetR)]*	Stratagene
Plasmid	**Description**	**Reference**
pZE12- luc	pLlacO1::luc(VF); *ColE1 ori*; *Amp^R*	(Lutz and Bujard, 1997)
pCS27	pLlacO1:: MCS; *p15A ori; Kan^R*	(Shen and Liao, 2008c)
pCDF-Duet1	pT7lac::MCS;*CDF ori; Sm^R*	EMD Chemicals Inc., NJ
pYY93	*ppdABC* from *K. oxytoca* cloned into pCS27	This study
pYY109	*budC* from *K. pneumoniae* cloned into pCDF-Duet1	This study
pYY134	*gldA*BC from *K. pneumoniae* cloned into pCS27	This study
pYY167	*dhab12* from *C. butyricum* cloned into pCS27	This study
pRJ1	*mgsA* from *C. acetobutylicum* cloned into pZE12-luc	This study
pRJ2	*mgsA* from *B. subtilis* cloned into pZE12-luc	This study
pRJ3	*mgsA* from *C. difficile* cloned into pZE12-luc	This study
pRJ4	*mgsA* from *E. coli* cloned into pZE12-luc	This study
pRJ5	*mgsA* from *T. thermophilus* cloned into pZE12-luc	This study
pRJ6	*mgsA* from *K. pneumoniae* cloned into pZE12-luc	This study
pRJ7	*mgsA* from *P. fluorescens* cloned into pZE12-luc	This study
pRJ8	*mgsA* from *R. eutropha* cloned into pZE12-luc	This study
pRJ9	*gldA* from *E. coli* cloned into pZE12-luc	This study
pRJ10	*ydjG* from *E. coli* cloned into pZE12-luc	This study

pRJ11	*ydjG* from *E. coli, budC* from *K. pneumoniae*, and *mgsA* from *B. subtilis* cloned in pZE12-luc	This study

Table 2.5. Primers used in this study. Underlined nucleotides represent restriction sites. Italicized nucleotides represent ribosome binding sites inserted in the primer.

Plasmid	Gene	Primer Sequence (5'-3')
pRJ1	*mgsA*	F: GGGAAAGGTACCATGGCACTTATAATGAATAGTAAAAAAAAGATAGC
		R: GGGAAAGCATGCTTAAAAATTGTCTTTTCTAATTTTTTGGTAATAAT
pRJ2	*mgsA*	F: GGGAAAGGTACCATGAAAATTGCTTTGATCGCGCATG
		R: GGGAAAGCATGCTTATACATTCGGCTCTTCTCCCCGA
pRJ3	*mgsA*	F: GGGAAAGGTACCATGAATATAGCATTAGTAGCACATGACCAAATGAA
		R: GGGAAAGCATGCTTAAATACGTTGACTTTTGCTTTTTCTAACTTCTC
pRJ4	*mgsA*	F: GGGAAAGGTACCATGGAACTGACGACTCGCAC
		R: GGGAAAGCATGCTTACTTCAGACGGTCCGCGA
pRJ5	*mgsA*	F: GGGAAAGGTACCATGCCCATGAAGGCCCTGGC
		R: GGGAAAGCATGCCTATTGGGGGGTTCCCTTGC
pRJ6	*mgsA*	F: GGGAAAGGTACCATGTGGAATGAAAATATGGAACTGACAACACGTAC
		R: GGGAAAGCATGCTTATTTCAGGCGCTCGGCAA
pRJ7	*mgsA*	F: GGGAAATGTACAATGATCGGTATCAGTTTCACCC
		R: GGGAAAGCATGCTTATCCTCGGCCGGCCAGGTA
pRJ8	*mgsA*	F: GGGAAAGGTACCATGACTCGCCCCCGCATCGCGTTGAT
		R: GGGAAATCTAGATCAGCTGGCCGCCGCTTCGT
pRJ9	*gldA*	F: GGGAAAGCATGC*AGGAGATATACC*ATGGACCGCATTATTCAATCACCGG
		R: GGGAAATCTAGATTATTCCCACTCTTGCAGGAAACGC
pRJ10	*ydjG*	F: GGGAAAGGTACCATGAAAAAGATACCTTTAGGCACAACGG
		R: GGGAAATCTAGATTAACGCTCCAGGGCCTCTGCCATTTCC
pRJ11	*ydjG*	F: GGGAAAGGTACCATGAAAAAGATACCTTTAGGCACAACGG

		R: GGGAAAGTCGACTTAACGCTCCAGGGCCTCTGCCATT
	budC	F: GGGAAAGTCGAC*AGGAGATATACC*ATGAAAAAAGTCGCACTTGTTACCGG
	mgsA	R: GGGAAACTGCAGTTAGTTAAACACCATCCCGCCGTCG
		F: GGGAAACTGCAG*AGGAGATATACC*ATGAAAATTGCTTTGATCGCGCATGAC
		R: GGGAAATCTAGATTATACATTCGGCTCTTCTCCCCGA
pYY93	*ppdABC*	F: GGGAAACGTACGATGAGATCGAAAAGATTTGAAGCACTGGCGAAACG
		R: GGGAAAAAGCTTTTAATCGTCGCCTTTGAGTTTTTTACGCTCGACG
pYY109	*budC*	F: GGGAAAGGATCCGAAAAAAGTCGCACTTGTTACCGGCG
		R: GGGAAAGTCGACTTAGTTAAACACCATCCCGCCGTCG
pYY134	*gldABC*	F: GGGCCCGGTACCATGAAAAGATCAAAACGATTTGCAGTACTGGCCCA
		R: GGGCCCAAGCTTTTAGCTTCCTTTACGCAGCTTATGCCGCTGCTGAT
pYY167	*dhaB12*	F: GGGAAAGGTACCATGATCAGCAAAGGGTTCAGCACCCAG
		R: GGGAAAAAGCTTTTATTCCGCGCCTATAGTACACGGAATGCCCATAA

CHAPTER 3

SYSTEMATICALLY ENGINEERING *ESCHERICHIA COLI* FOR EFFICIENT PRODUCTION OF 1,2-PROPANEDIOL AND 1-PROPANOL

Abstract

The biological production of high value commodity 1,2-propanediol has been established by engineering the glycolysis pathway. However, the simultaneous achievement of high titer and high yield has not been reported yet, as all efforts in increasing the titer have resulted in low yields. In this work, we overcome this limitation by employing an optimal minimal set of enzymes, channeling the carbon flux into the 1,2-propanediol pathway, increasing NADH availability, and improving the anaerobic growth of the engineered *Escherichia coli* strain by developing a cell adaptation method. These efforts lead to 1,2-propanediol production at a titer of 5.13 g/L with a yield of 0.48 g/g glucose in 20 mL shake flask studies. On this basis, we pursue the enhancement of 1-propanol production from the 1,2-propanediol platform. By constructing a fusion diol dehydratase and developing a dual strain process, we achieve a 1-propanol titer of 2.91 g/L in 20 mL shake flask studies. To summarize, we report the production of 1,2-propanediol at enhanced titer and enhanced yield simultaneously in *E. coli* for the first time. Furthermore, we establish an efficient system for the production of biofuel 1-propanol biologically.

Keywords: 1,2-Propanediol, 1-propanol, metabolic engineering, *Escherichia coli*, biofuels

3.1. Introduction

A vast number of high value chemicals, fine chemicals, and other commodity products rely on the petrochemical industry for large-scale manufacture. It is reported that currently about 3 billion barrels of oil are consumed annually for the production of industrial chemicals (Burk, 2010). These processes are responsible for releasing about 30 billion tons of carbon dioxide into the atmosphere, as reported for the year 2008 (Burk, 2010). The ongoing depletion of fossil fuels will lead to the increase in the cost of manufacturing these chemicals in the near future and also pose a threat to the environment by releasing vast quantities of carbon dioxide. An alternative to this is the biological manufacture through the development of synthetic metabolic routes via metabolic engineering (Huo et al., 2011b) and fermentation, which reduces the dependence on oil by using renewable sources as raw materials, and decreases the release of greenhouse gases into the atmosphere (Burk, 2010; Jang et al., 2012a).

Metabolic engineering efforts via fermentation optimization, utilization of different carbon sources, strain engineering and growth improvement strategies have led to the establishment of biological platforms for the manufacturing of ethanol (Alterthum and Ingram, 1989; Ohta et al., 1991), succinic acid (Vemuri et al., 2002), lactic acid (Mazumdar et al., 2010; Zhou et al., 2003), etc. These approaches have proven to be efficient for the production of indigenous metabolites. However, the manufacturing of non-native high-value chemicals or commodities requires the construction of novel synthetic pathways by the expansion of native metabolism via combinatorial biosynthesis and manipulation of cellular metabolism. These strategies have led to the establishment of novel biosynthetic pathways for the production of advanced biofuels such as longer chain alcohols (Atsumi et al., 2008e; Avalos et al., 2013a), various C2- C6 platform

chemicals (Jang et al., 2012a; Lan et al., 2013; Shen et al., 2012c), polymers (Jung et al., 2010; Jung and Lee, 2011), antioxidants (Lin et al., 2014; Yan et al., 2008) and pharmaceuticals (Lin et al., 2013; Ro et al., 2006).

Although the biological manufacture of 1,2-propanediol (propylene glycol) and 1-propanol has been achieved, at present, they are manufactured from the petrochemical industry at a large scale (Altaras and Cameron, 1999a; Shen and Liao, 2008c). 1,2-Propanediol has an annual market of over 1 billion pounds in U.S.A. alone and is primarily used in the manufacturing of antifreeze and deicers, and also in the manufacture of pharmaceuticals, polyester resin, laundry detergent, cosmetics, and so on (Berrios-Rivera et al., 2003a). 1-Propanol has been proposed as a potential biofuel and is currently used in the following industries: pharmaceuticals, textiles, printing ink, cosmetics, etc. (Atsumi et al., 2008e; Shen and Liao, 2008c).

The biosynthesis of 1,2-propanediol has been achieved in *E. coli* and *Saccharomyces cerevisiae*, by engineering the glycolysis pathway (Altaras and Cameron, 1999a; Altaras and Cameron, 2000b; Berrios-Rivera et al., 2003a; Clomburg and Gonzalez, 2011a; Jain and Yan, 2011b; Joon-Young et al., 2008; Joon-Young et al., 2011; Li and Liao, 2013). Metabolic engineering efforts such as selection of optimal carbon source (Berrios-Rivera et al., 2003a), optimization of fermentation medium and conditions (Altaras and Cameron, 2000b), scale up to 400 mL or 2000 mL fed batch fermentation (Altaras and Cameron, 2000b; Clomburg and Gonzalez, 2011a) and strain engineering have resulted in 1,2-propanediol production at low titers and yields (Supporting Information Table S3.1). To achieve both high titer and high yield is challenging and has not been reported yet. For instance, the highest reported titer of 1,2-propanediol of 5.6

g/L was achieved at a low yield of 21.3% (w/w) from engineered *E. coli* in a fermenter with 400 mL medium using glycerol as the carbon source (Clomburg and Gonzalez, 2011a). Whereas, the highest reported yield of 0.20 g/g using glucose as the carbon source was achieved at a low titer of 1.4 g/L in 150 mL shake flask studies (Altaras and Cameron, 2000b). A major limitation in these established methods is the dominance of the native metabolism in dragging the carbon flux toward by-product formation, thereby undermining the synthetic pathway. In order to promote industrial interest, it is necessary to achieve high titer without sacrificing yield, utilize inexpensive medium and prevent the loss of carbon to harmful byproduct accumulation.

The biosynthesis of 1-propanol has been well established from the keto-acid pathway (Shen and Liao, 2013) and the citramalate pathway (Atsumi et al., 2008e). By engineering the amino acid biosynthetic pathway, 1-propanol was produced from a key keto-acid intermediate- 2-ketobutyrate at about 1 g/L in an engineered *E. coli* strain (Shen and Liao, 2008c). Further enhancement of 1-propanol production in *E. coli* was achieved (3.5 g/L) by constructing a citramalate pathway, utilizing an evolved citramalate synthase capable of preventing feedback inhibition by isoleucine (Atsumi et al., 2008e). By engineering an $_{L}$-threonine overproducing strain, 1-propanol production was achieved at 10.8 g/L using glucose and at 10.3 g/L using glycerol in bioreactors with 2 L medium (Choi et al., 2012b). Recently, it was demonstrated that the synergy between the threonine pathway and citramalate pathway can be modulated, proving to be more efficient than the individual pathways. This resulted in a 1-propanol yield of 0.15 g/g glucose at a titer of 8 g/L (Shen and Liao, 2013).

In our previous work, we expanded the 1,2-propanediol pathway to establish a novel 1-propanol biosynthesis approach anaerobically in wild type *E. coli* (Figure 3.1). In doing so, the 1,2-propanediol (about 0.8 g/L) was dehydrated by the action of *Klebsiella oxytoca* diol dehydratase to 1-propanal, following which native reduction resulted in 1-propanol production at 0.25 g/L using wild type *E. coli* (Jain and Yan, 2011b). Our previous results suggested two major limitations: (a) dominance of native metabolism toward byproduct formation, thereby limiting 1,2-propanediol titer (b) low conversion efficiency of 1,2-propanediol to 1-propanol.

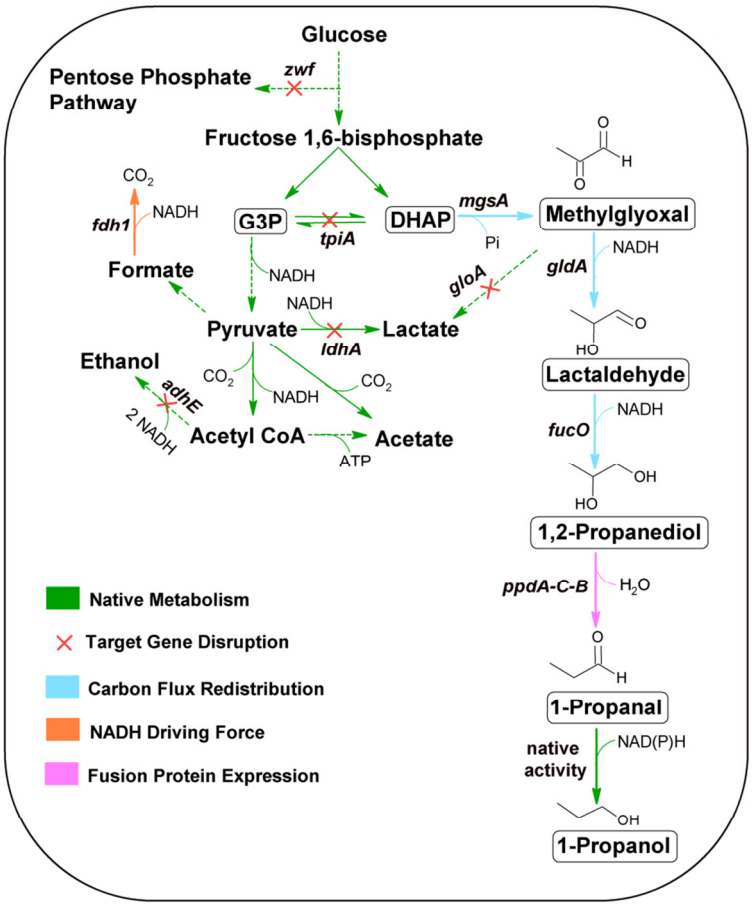


Figure 3.1. Schematic representation of 1,2-propanediol pathway, 1-propanol pathway and major native competing pathways in *E. coli*. G3P: glyceraldehyde 3-phosphate; DHAP: dihydroxyacetone phosphate.

In this work, we overcome both of the above mentioned limitations, resulting in boosting 1,2-propanediol titer without sacrificing yield and also enhancing 1,2-propanediol conversion efficiency to 1-propanol. We first pursue the enhancement of 1,2-propanediol production by systematically engineering *E. coli*. By overexpressing the optimal set of enzymes, redirecting the carbon flux, utilizing a system for intracellular NADH regeneration and adapting the cells to improve growth, we accomplish 1,2-propanediol production at the highest reported titer and yield simultaneously in shake flask studies. We achieve 1,2-propanediol production at 5.13 g/L in 20 mL shake flask studies, which is comparable to the highest reported titer (5.6 g/L) using a fermenter with 400 mL medium (Clomburg and Gonzalez, 2011a). Our process also achieved a yield of 0.48 g/g glucose, which is more than double the yields reported until date. Furthermore, we demonstrate the improvement in 1-propanol production from the established 1,2-propanediol platform. The combined effect of creating a fusion diol dehydratase and a dual strain strategy leads to1-propanol production at 2.91 g/L in 20 mL shake flask studies. This work represents a step forward in making the biological manufacture of 1,2-propanediol and 1-propanol more economically viable.

3.2. Results and Discussion

3.2.1. Identification of the Optimal Minimal Set of Enzymes for 1,2-Propanediol Production

The production of 1,2-propanediol from glycolytic intermediate dihydroxyacetone phosphate has been well established (Altaras and Cameron, 1999a; Altaras and Cameron, 2000b; Berrios-

Rivera et al., 2003a). The expression of either methylglyoxal synthase or secondary alcohol dehydrogenase has been shown to be sufficient for 1,2-propanediol production (Altaras and Cameron, 1999a). It has also been demonstrated that the coexpression of methylglyoxal synthase with a secondary alcohol dehydrogenase (Altaras and Cameron, 1999a) or the expression of the complete pathway (methylglyoxal synthase, secondary alcohol dehydrogenase, 1,2-propanediol oxidoreductase) (Altaras and Cameron, 2000b) leads to the production of 1,2-propanediol at various levels. The overexpression of too many enzymes in *E. coli* may lead to metabolic burdens (Ajikumar et al., 2010; Alper et al., 2005; Xu et al., 2013) which may affect the cell growth and product yield. Hence, our objective was to identify the optimal minimal set of enzymes required for efficient production of 1,2-propanediol.

As several candidate enzymes are available for each step of the pathway and different combinations of their expression is possible, we performed a systematic study by overexpressing candidate enzymes in a stepwise manner. We had previously characterized methylglyoxal synthase from eight different bacterial sources and identified the methylglyoxal synthase from *Clostridium acetobutylicum* and *Bacillus subtilis* as the most proficient. We first overexpressed the two synthases using plasmids pRJ1 and pRJ2 (Jain and Yan, 2011b) in wild type *E. coli* individually. Fermentations were carried out according to the method described in the Methods as “shake flask fermentations”.

After 48 h of fermentation, it was found that 1,2-propanediol was produced at a low amount. As shown in Figure 3.2, the overexpression of methylglyoxal synthase from *B. subtilis* resulted in

1,2-propanediol production at 0.46 g/L. Whereas, the overexpression of methylglyoxal synthase from *C. acetobutylicum* resulted in 1,2-propanediol production at 0.29 g/L.

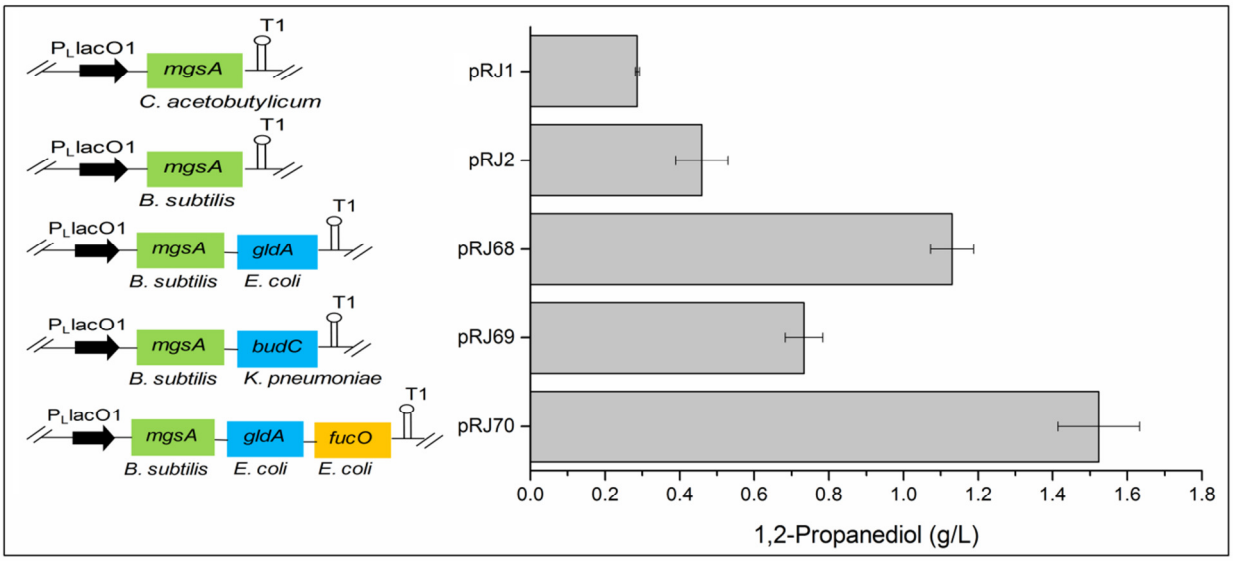


Figure 3.2. Identification of optimal minimal set of enzymes for 1,2-propanediol production in 20 mL anaerobic shake flask studies using wild type *E. coli* BW25113 transformed with different plasmids. The data were generated from three independent experiments.

Next, we overexpressed secondary alcohol dehydrogenases in addition to the *B. subtilis* methylglyoxal synthase to evaluate if their overexpression is necessary to enhance the production of 1,2-propanediol. The plasmids pRJ68 carrying *B. subtilis mgsA*, *E. coli gldA* and pRJ69 carrying *B. subtilis mgsA*, *Klebsiella pneumoniae budC* were transferred into wild type *E. coli* independently for fermentation studies. After 48 h of fermentation, the *E. coli* strain transformed with pRJ68 increased the 1,2-propanediol titer to 1.13 g/L (Figure 3.2). We had previously determined that the diol dehydrogenase (*budC*) from *K. pneumoniae* had a higher *in vitro* catalytic efficiency than *E. coli gldA* toward methylglyoxal (Jain and Yan, 2011b). Contrary to our expectation, the coexpression of *budC* from *K. pneumoniae* with *B. subtilis mgsA* (pRJ69)

led to lower levels of 1,2-propanediol production (0.73 g/L) as compared to when *E. coli gldA* was coexpressed. This result demonstrated the beneficial effect of overexpressing *E. coli* native dehydrogenase toward 1,2-propanediol production over the heterologous dehydrogenase despite having a lower catalytic efficiency *in vitro*.

Finally, we proceeded to evaluate if the overexpression of 1,2-propanediol oxidoreductase (*fucO*) can further facilitate the increase of 1,2-propanediol production. To test this, plasmid pRJ70 carrying *mgsA* from *B. subtilis*, *gldA*, and *fucO* from *E. coli* was transferred into wild type *E. coli* for fermentation studies. After 48 h of fermentation, the *E. coli* strain carrying pRJ70 further increased the 1,2-propanediol titer to 1.52 g/L (yield = 0.13 g/g glucose). Thus, we identified the optimal minimal set of enzymes to be overexpressed for 1,2-propanediol production. This stepwise approach resulted in increasing the titer from 0.29 g/L to 1.52 g/L (Figure 3.2). The plasmid pRJ70 was selected for all further studies as it resulted in the highest titer. However, the carbon flux toward the glycolytic pathway remained dominant in the wild type strain overexpressing the 1,2-propanediol pathway. The major competing pathway was found to be lactate, which was produced at 6.21 g/L (Supporting Information Table S3.2). No pyruvate accumulation was observed, suggesting that most of it was channeled toward lactate generation due to the possible increase in lactate dehydrogenase activity (*ldhA*) (Jiang et al., 2001). Ethanol, acetate, formate and succinate were also produced at 0.53 g/L, 2.03 g/L, 0.89 g/L and 0.26 g/L respectively (Supporting Information Table S3.2).

3.2.2. Disruption of Lactate Producing Pathways

As seen from the above results, the dominant byproduct lactate was accumulated as a result of the carbon flux being dominant toward glycolysis. We speculated that the elimination of lactate generation would result in making more carbon available for utilization into the 1,2-propanediol pathway. In order to study the effect of disrupting both the lactate generating pathways (through glyoxalase system and lactate dehydrogenase), we constructed an *E. coli* strain RJ31 with *gloA* and *ldhA* gene disruptions. We then tested this strain transformed with pRJ70 for 1,2-propanediol production via 48 h fermentation studies. As expected, lactate production decreased from 6.21 g/L to 0.54 g/L (Supporting Information Table S3.2). Surprisingly, this strain resulted in reducing 1,2-propanediol titer by nearly 41% of the wild type to 0.90 g/L (yield = 0.07 g/g glucose). Interestingly, pyruvate was accumulated at 7.18 g/L and ethanol generation increased from 0.53 g/L to 2.77 g/L. Succinate generation increased from 0.26 g/L to 1.41 g/L and formate from 0.89 g/L to 2.45 g/L. Acetate production decreased from 2.03 g/L to 0.99 g/L.

Although lactate production was drastically lowered, a significant amount of pyruvate was accumulated along with an increase in other fermentative byproducts. This result indicated the overriding capability of glycolysis to redirect the carbon flux toward production of indigenous metabolites. This suggested that merely the deletion of the major fermentative byproduct pathway was not an efficient strategy to tap into the carbon flux for the production of 1,2-propanediol.

3.2.3. Carbon Flux Redistribution

In glycolysis, the three-carbon intermediate dihydroxyacetone phosphate is converted into glyceraldehyde 3-phosphate by the action of triose phosphate isomerase (*tpiA*) (Jain and Yan, 2011b). This native activity results in dragging the carbon flux away from the 1,2-propanediol pathway's starter molecule – dihydroxyacetone phosphate as indicated by the above results. So, we discerned that disrupting this major competing pathway for carbon flux is essential in order to conserve the carbon for the 1,2-propanediol pathway. We also speculated that the action of glucose 6-phosphate dehydrogenase (*zwf*) leading to pentose phosphate pathway may further result in the loss of carbon. With this cognition, we also hypothesized that in addition to *zwf* and *tpiA* disruptions, the disruption of other major competing pathways for the carbon flux under anaerobic conditions would also reduce byproduct formation. To test these hypotheses, we constructed *E. coli* strain RJ57 with the following gene disruptions: *zwf* encoding glucose 6-phosphate dehydrogenase, *tpiA* encoding triose phosphate isomerase, *ldhA* encoding lactate dehydrogenase, *gloA* encoding glyoxalase I, and *adhE* encoding alcohol dehydrogenase (ethanol generating pathway).

E. coli strain RJ57 was transformed with pRJ70 and fermentation was carried out for 48 h. As expected, this strain generated the least amount of byproducts, with lactate at 0.14 g/L, succinate at 0.22 g/L, formate at 0.33 g/L, acetate at 0.65 g/L, and ethanol at 0.05 g/L. Interestingly, this strain did not accumulate any pyruvate. However, 1,2-propanediol was produced at merely 0.35 g/L with a yield of 0.22 g/g glucose. As seen from Table 3.1, the amount of glucose consumed was drastically lower in the engineered strain (1.64 g/L) as compared to the wild type strain

(11.75 g/L) after the fermentation study. We reasoned that this led to a decrease in the rate of glycolysis thereby diminishing the cell's capacity to generate carbon flux and NADH. It was also observed that at the end of the fermentation study that the optical density of the engineered strain was dramatically lower (OD_{600} = 0.38) than the wild type strain after fermentation (OD_{600} = 1.89) (Supporting Information Table S3.2). We then pursued to surmount these limitations by increasing NADH availability and enhancing cell growth.

Table 3.1. 1,2-Propanediol titer and yield achieved after 48 h of IPTG induction from 20 mL anaerobic shake flask studies. (A): regular strategy; (B): cell adaptation strategy; (C): optimized cell adaptation strategy; OD_{600}: optical density measured at 600 nm; S: grams of substrate consumed (glucose); 1,2-PD: 1,2-propanediol. The data were generated from three independent experiments.

Strain/ Plasmid	**Optical density** (OD_{600})	**Glucose consumed** (g/L)	**1,2-PD titer** (g/L)	**Yield** (1,2-PD titer/ S) (g/g)
BW25113/ pRJ70	1.89 ± 0.06	11.75 ± 0.40	1.52 ± 0.11	0.13 ± 0.01
RJ31/ pRJ70	2.41 ± 0.13	12.91 ± 0.60	0.90 ± 0.04	0.07 ± 0.01
RJ57/ pRJ70	0.38 ± 0.03	1.64 ± 0.29	0.35 ± 0.00	0.22 ± 0.04
BW25113/ pRJ70 + pRJ58	1.07 ± 0.08	14.94 ± 0.30	2.14 ± 0.07	0.14 ± 0.01
RJ57/ pRJ70 + pRJ58 (A-48hr)	0.41 ± 0.04	1.75 ± 0.44	0.59 ± 0.02	0.34 ± 0.07
RJ57/ pRJ70 + pRJ58 (B-48hr)	0.51 ± 0.18	5.35 ± 0.20	2.12 ± 0.20	0.40 ± 0.02
RJ57/ pRJ70 + pRJ58 (C-48hr)	0.57 ± 0.02	5.52 ± 0.88	2.39 ± 0.27	0.44 ± 0.02
RJ57/ pRJ70 + pRJ58 (C-120hr)	0.80 ± 0.02	10.60 ± 0.33	5.13 ± 0.14	0.48 ± 0.01

3.2.4. Increasing NADH Availability as a Driving Force

We analyze that with the disruption of *zwf* and *tpiA* genes (strain RJ57), the carbon flux will be split equally among glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. As a consequence, with every cycle of glycolysis only one molecule of NADH can be generated in the engineered strain. The production of acetate via acetyl CoA results in the production of an additional NADH molecule per molecule of glucose (Figure 3.1). Furthermore, with the disruption of *ldhA* and *adhE*, major NADH competing pathways were disrupted. Since two molecules of NADH are consumed by the 1,2-propanediol pathway, this system can be regarded as NADH balanced at steady state conditions. Although NADH balance is achieved, this system may not favor the production of 1,2-propanediol as NADH may also be utilized by the cell for other anabolic reactions in the form of NADPH.

We then hypothesized that increasing the NADH availability would serve as a driving force for 1,2-propanediol production. We aimed at increasing the cellular NADH levels by overexpressing *Candida boidinii* formate dehydrogenase (*fdh1*), which has previously been shown to regenerate intracellular NADH (Berrios-Rivera et al., 2002). An *in vitro* enzyme assay confirmed that the enzyme had sufficient catalytic efficiency to generate NADH from formate (k_m =12.69 mM, k_{cat} = 26.33 min^{-1}).

To test this hypothesis, strain RJ57 was transformed with plasmids pRJ70 and pRJ58 (plasmid carrying *Candida boidinii fdh1*), and fermentations were carried out for 48 h. It was seen that the titer of 1,2-propanediol was increased to 0.59 g/L as compared to 0.35 g/L from the engineered

strain without NADH regeneration system (Table 3.1). This result indicates that the NADH regeneration system facilitates in driving 1,2-propanediol production. As expected the yield showed a dramatic increase, from 0.22 g/g to 0.34 g/g glucose. The optical density (OD_{600}) however, remained low at 0.41 at the end of the study.

Additionally, we tested this NADH regeneration system in wild type *E. coli*. As expected, after 48 h of fermentation, 1,2-propanediol production was increased to 2.14 g/L as compared to 1.52 g/L from the wild type strain without NADH regeneration system. This strain accomplished a yield of 0.14 g/g glucose and accumulated the maximum amount of byproducts, with lactate at 8.22 g/L (Supporting Information Table S3.2). This positioned our process wherein high titer (2.14 g/L) was achieved in the wild type strain at a low yield (0.14 g/g glucose) or a high yield (0.34 g/g glucose) was achieved in an engineered strain at a low titer (0.59 g/L) (Figure 3.3A).

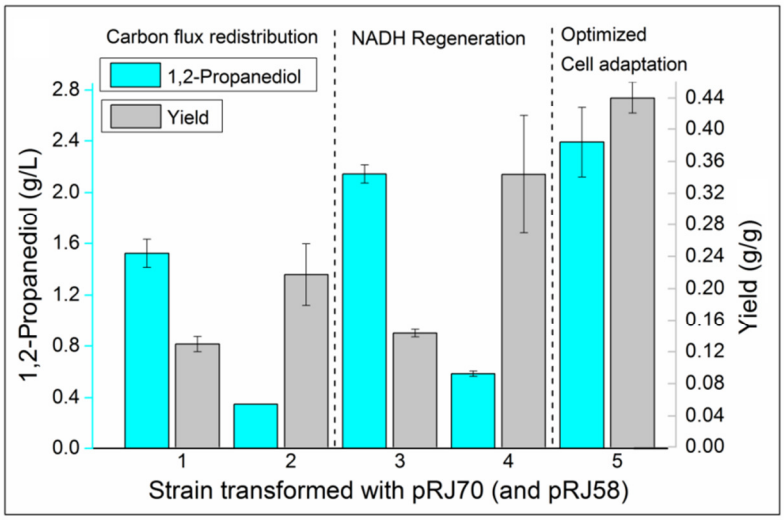


Figure 3.3A. Comparison of 1,2-propanediol titers (g/L) and yields (g/g glucose consumed) using different strategies after 48 h of study. 1: BW25113/pRJ70; 2: RJ57/pRJ70; 3: BW25113/pRJ70 + pRJ58; 4: RJ57/pRJ70 + pRJ58; 5: RJ57/pRJ70 + pRJ58.

3.2.5. Cell Adaptation Studies

As seen from the above results, when the optical density of the engineered strain remained low (OD_{600} = 0.41), the 1,2-propanediol titer was also low (0.59 g/L) even with the NADH regeneration system. Since this strain achieved higher yield (0.34 g/g glucose), we speculated that improving the cell growth may result in increasing the 1,2-propanediol titer without sacrificing yield. We then channeled our efforts toward increasing the cell growth of the engineered strain (RJ57 transformed with plasmids pRJ70 and pRJ58) with the objective of achieving both high titer and high yield simultaneously. Preliminary experiments were conducted to test the effect of increasing the inoculum volume by various amounts, changing IPTG induction time, and changing fermentation temperature. However, these experiments failed to recover the cell growth (data not provided). We then hypothesized that by adapting the engineered strain we could emulate the fermentation environment prior to commencement of study, which would recover cell growth. To test this hypothesis, this strain was then subjected to "cell adaptation", by growing the overnight culture anaerobically in the low phosphate formate medium followed by fermentation studies as described in methods and materials.

As a result, cell growth (OD_{600}) was increased to 0.51 after 48 h. Furthermore, the amount of glucose consumed was also increased to 5.35 g/L after 48 h as compared to 1.75 g/L from this strain without cell adaptation (Table 3.1). Due to this effect, the titer of 1,2-propanediol was dramatically increased to 2.12 g/L after 48 h with a yield of 0.40 g/g glucose. Thus, the cell adaptation strategy successfully increased the cell growth of this strain and resulted in achieving both high titer and high yield simultaneously.

Furthermore, by adjusting the IPTG induction time even higher titers and yield of 1,2-propanediol was achieved as described below. Using this "optimized cell adaptation strategy" a 120 h fermentation study was carried out where IPTG was added to the cultures when the optical density reached 0.46. As seen from Table 3.1, this strain consumed 5.52 g/L of glucose, achieved an optical density of 0.57 and produced 2.39 g/L of 1,2-propanediol after 48 h (after IPTG induction) with a yield of 0.44 g/g glucose. This represents a more than 3 fold increase in glucose consumption as compared to this strain without cell adaptation. Also, this result indicated a higher titer of 2.39 g/L and a higher yield (0.44 g/g glucose) as compared to the wild type strain carrying plasmids pRJ70 and pRJ58 (2.14 g/L with a yield of 0.14 g/g glucose) after 48 h of fermentation (Table 3.1). At the end of the fermentation study (120 h after IPTG induction), 1,2-propanediol was produced at 5.13 g/L with a yield of 0.48 g/g glucose (Figure 3.3B). This strain achieved a final optical density of 0.8 and consumed a total of 10.6 g/L of glucose. The major byproducts (lactate, ethanol, and pyruvate) were nearly eliminated (<0.1 g/L) and the minor byproduct succinate was accumulated at 0.71 g/L. The dominant byproduct for this case was acetate which was produced at 3.84 g/L. Thus, the optimized cell adaptation strategy achieved both high titer and high yield of 1,2-propanediol simultaneously. Interestingly, this strain produced 1.16 g/L of 1,2-propanediol prior to IPTG induction. This result indicated that the leaky expression of the pathway enzymes stimulated the production of 1,2-propanediol. This conclusion is also supported by various studies confirming that the transcription of operons may occur even with the absence of a derepressor (IPTG/ lactose) in the environment of pLac promoter (Penumetcha et al., 2010).

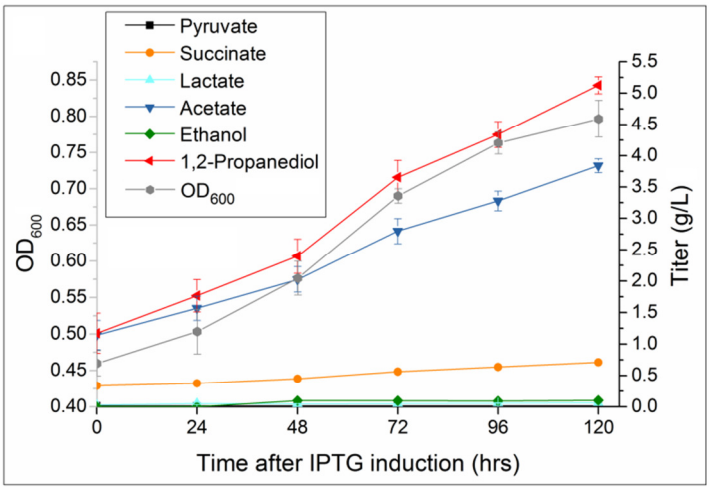


Figure 3.3B. Time course of 1,2-propanediol production, major cellular byproducts and cell growth from a 120 h shake flask study using strain RJ57/pRJ70 + pRJ58 grown with optimized cell adaptation strategy. The data were generated from three independent experiments.

3.2.6. 1-Propanol Production

In our previous work, we had established a novel metabolic route for the production of 1-propanol in wild type *E. coli* by expanding the 1,2-propanediol pathway (Figure 3.1). A whole cell conversion study showed 100% conversion of 1,2-propanediol (5 g/L) to 1-propanol. However, during fermentation studies, most of the 1,2-propanediol remained unconverted (0.46 g/L), producing only 0.25 g/L of 1-propanol (Jain and Yan, 2011b). In this work, we pursued the enhancement of 1-propanol production building on our efficient 1,2-propanediol platform. In order to identify any potential hurdles, we first tested 1-propanol production in a wild type strain instead of directly utilizing the enhanced 1,2-propanediol production system. We first transferred plasmids pRJ70 and pYY93 (a medium copy number plasmid carrying *ppdABC* operon) in wild type *E. coli*, following which fermentations were carried out for 48 h. As a result, 0.11 g/L of 1-

propanol was produced with 0.95 g/L of 1,2-propanediol remaining unconverted. Similar to our previous work, this result depicted lower catalytic efficiency of the diol dehydratase during fermentation. We speculated that this could be due to pathway intermediate accumulation, resulting in inhibition of diol dehydratase activity. However, after thorough HPLC analysis, no pathway intermediates were detected from the shake flask fermentations. Hence, we concluded that the effect of pathway intermediates accumulation on dehydratase activity was not of concern.

We then speculated that the use of a high copy number plasmid (pZE12-luc) to express the 1,2-propanediol pathway may have led to plasmid incompatibility with pYY93, thereby affecting the diol dehydratase activity. To test this hypothesis, a 24 h study was performed as described in methods and materials as "whole cell conversion studies". Wild type *E. coli* transformed with pYY93 served as the control for diol dehydratase activity resulting in 100% conversion of 1,2-propanediol to 1-propanol, which was consistent with our previous study (Jain and Yan, 2011b). As seen from Figure 3.4A, the *E. coli* strain transformed with plasmids pYY93 and pZE12-luc (without 1,2-propanediol pathway operon) also achieved 100% conversion to 1-propanol. Hence, we concluded that there was no incompatibility between plasmids pYY93 and pZE12-luc.

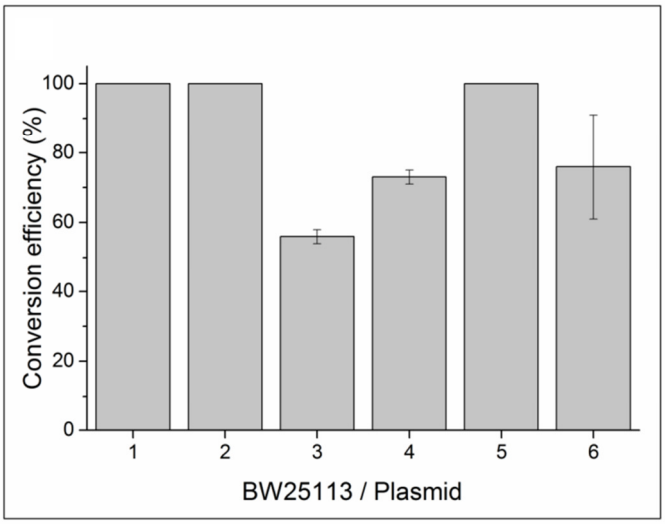


Figure 3.4A. Whole cell conversion studies for 1-propanol production with 1,2-propanediol feeding.1: BW25113/pYY93; 2:BW25113/pYY93 + pZE12-luc; 3: BW25113/pYY93 + pRJ70; 4: BW25113/pRJ49; 5: BW25113/pRJ79; 6: BW25113/pRJ81.

Since there were no pathway intermediates detected and plasmid incompatibility was not of concern, we speculated that the overexpression of too many enzymes (1,2-propanediol pathway) may affect the diol dehydratase activity by interfering with its native enzyme complex formation. The *K. oxytoca* diol dehydratase enzyme consists of a complex of three subunits (*ppdA*, *ppdB*, *ppdC*). The crystal structure had determined that the enzyme complex exists as a dimer of the three subunits (Shibata et al., 2002). Therefore, any perturbation to this complex formation may hinder its catalytic efficiency. To test this hypothesis, plasmids pRJ70 and pYY93 were transferred into *E. coli* for another whole cell conversion study. It was seen that the *E. coli* strain transformed with pYY93 and pRJ70 resulted in a 44% decrease in conversion efficiency to 1-propanol as compared to the *E. coli* strain transformed with only pYY93 (Figure 3.4A). Hence,

we inferred that the overexpression of the 1,2-propanediol pathway operon reduced the diol dehydratase catalytic efficiency by possibly interfering with the enzyme complex formation.

Interestingly, when the *ppdABC* operon was inserted into high copy number plasmid pZE12-luc (pRJ49) and was transferred into *E. coli* for a whole cell conversion study, it resulted in a conversion efficiency of 73% (Figure 3.4A), even without the overexpression of the 1,2-propanediol pathway operon. This result depicts a decrease in conversion efficiency by 27% compared to its expression via a medium copy number plasmid (pYY93) as shown above. Hence, it indicated that too much expression of the diol dehydratase subunits itself may reduce the catalytic efficiency possibly due to interference with the enzyme subunits' recognition and assembly.

To address this concern, we developed an approach which would facilitate the enzyme complex formation as well as achieve an equimolar level of the three subunits even with higher levels of expression. To achieve this, all three subunits were expressed as a fusion protein under the control of a common RBS as described below. By carefully examining the crystal structure of the diol dehydratase in both substrate free (PDBid: 1IWB) (Shibata et al., 2002) and substrate bound (PDBid: 1UC5) (Shibata et al., 2003) forms, we deduced the distances between the C and N terminuses of each subunit using Pymol software. By evaluating different subunit alignment possibilities we inferred that in order to facilitate the native enzyme complex formation a suitable approach was to link the C terminus of *ppdA* with N terminus of *ppdC* and the C terminus of *ppdC* with N terminus of *ppdB* (*ppdA-C-B*). To achieve this, a 13 amino acid linker between C terminus of *ppdA* and the N terminus of *ppdC* and a 9 amino acid linker between C terminus of

ppdC and the N terminus of *ppdB* were inserted as described in methods and materials ("DNA manipulations"). The length of the amino acid linkers were chosen so as to ensure flexibility among the subunits. Similarly, another possible approach to facilitate the native enzyme complex formation was also tested (*ppdC-B-A*). The sequence of *ppdA-C-B* and the *ppdC-B-A* reading frames have been provided in Supporting Information.

Plasmids pRJ79 and pRJ81carrying the *ppdA-C-B* and the *ppdC-B-A* reading frames respectively were transferred into *E. coli* individually for a whole cell conversion study. From Figure 3.4A it was seen that the *E. coli* strain transformed with pRJ79 showed 100% conversion of 1,2-propanediol to 1-propanol, whereas the *E. coli* strain transformed with pRJ81 showed a lower conversion efficiency of 76%. Thus, the optimal fusion diol dehydratase (*ppdA-C-B*) restored the conversion efficiency to 100% even with a high level expression of the subunits using a high copy number plasmid (pZE12-luc). Furthermore, the catalytic activity of this fusion diol dehydratase was found to be superior to the native one in a whole cell conversion study (Figure 3.4B). As seen from Figure 3.4B, the native and fusion diol dehydratases showed similar activity up to 9 h, however, after 9 h the activity of the native diol dehydratase became lower than that of the fusion diol dehydratase. We then hypothesized that the coexpression of this fusion diol dehydratase with the 1,2-propanediol pathway operon would enhance the production of 1-propanol.

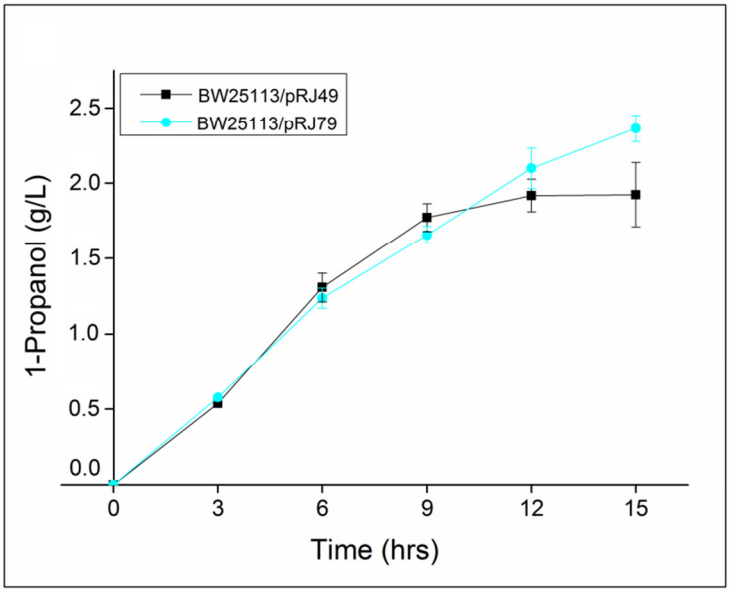


Figure 3.4B. Comparison of 1-propanol production using superior fusion diol dehydratase (pRJ79) as compared to natural diol dehydratase (pRJ49) using wild type *E. coli* BW25113 via whole cell conversion studies. The data were generated from three independent experiments.

To test this hypothesis, a high copy number plasmid pRJ93 carrying the *ppdA-C-B* reading frame and the 1,2-propanediol pathway genes was transferred into wild type *E. coli* for a 48 h fermentation study. Similarly, 1-propanol production was also tested using medium (pRJ99) and low copy number (pRJ100) plasmids. It was seen that when the high copy number plasmid was used to express the complete pathway, 1-propanol was produced at 0.62 g/L with 1.51 g/L of 1,2-propanediol remaining unconverted at the end of the study. Whereas, when the medium and low copy number plasmids were used to express the complete pathway, 0.29 g/L and 0.30 g/L of 1-propanol were produced with 1.14 g/L and 0.31 g/L of 1,2-propanediol remaining unconverted, respectively. Thus, even with the utilization of a fusion diol dehydratase, the conversion efficiency to 1-propanol could not be enhanced during fermentation. We reasoned that since a large portion of 1,2-propanediol is generated during the later stages of fermentation (Figure

3.3B) where the catalytic activity of diol dehydratase might be lower, the conversion of 1,2-propanediol to 1-propanol might be hampered. This inference is also supported by the observation from the whole cell conversion study (Figure 3.4B), where the diol dehydratases showed higher catalytic efficiency during the initial phase of the study with a gradual decrease in the efficiency with time.

3.2.7. Dual Strain Strategies for Improvement of 1-Propanol Production

To address the above- mentioned concern, we hypothesized that using the "optimized cell adaptation strategy", 1,2-propanediol production can be achieved prior to the addition of the strain expressing the downstream 1,2-propanediol conversion pathway. This would make a large portion of 1,2-propanediol available when the diol dehydratase activity would be at its highest, thus resulting in enhanced 1-propanol production.

To test this hypothesis, the strain expressing the 1,2-propanediol pathway (strain RJ57 transformed with plasmids pRJ70 and pRJ58) was grown utilizing "optimized cell adaptation strategy" for 72 h. Following this, the strain expressing the fusion diol dehydratase (wild type *E. coli* strain transformed with plasmids pRJ79 and pRJ58) was added into the cultures via resuspension strategy or direct addition (as described in methods and materials as "dual strain strategies for 1-propanol production"). It was seen that after 72 h, 3.66 g/L of 1,2-propanediol was produced (prior to the addition of the strain expressing the downstream pathway). After the addition of the strain expressing the downstream pathway to the culture via the resuspension strategy, 0.89 g/L of 1-propanol was produced with 2.61 g/L of 1,2-propanediol remaining

unconverted at the end of the study. Whereas, when the strain expressing the downstream pathway was added to the culture via the direct addition strategy, 1.5 g/L of 1-propanol was produced with 2.1 g/L of 1,2-propanediol remaining unconverted. Thus, by expressing the two portions of the pathway in different strains, 1-propanol production was enhanced to 1.5 g/L. The addition of the downstream strain directly proved to be more efficient than the resuspension strategy. However, most of the 1,2-propanediol still remained unconverted.

Alternatively, we utilized another approach for 1-propanol production by availing the leaky expression of the 1,2-propanediol pathway and coupling it with the dual strain strategy. In this approach, 1,2-propanediol production was carried out via "optimized cell adaptation strategy", however without IPTG induction. It was seen that even without IPTG induction, 3.36 g/L of 1,2-propanediol was produced after 120 h (prior to the addition of the strain expressing the downstream pathway) due to leaky expression. After 120 h of fermentation, 1 mL of the strain expressing the downstream pathway was added to the cultures directly and the study was then carried out as described in methods and materials ("dual strain strategies for 1-propanol production"). As seen from Figure 3.5, 2.91 g/L of 1-propanol was produced with 0.20 g/L of 1,2-propanediol remaining unconverted at the end of the study. This result showed that nearly all of the 1,2-propanediol was converted to 1-propanol using this method. Thus, the utilization of an optimized cell adaptation strategy with leaky operon expression for 1,2-propanediol production coupled with a dual strain strategy resulted in enhancing 1-propanol production to 2.91 g/L. This result represents over 11-fold increase in 1-propanol production compared with our previous work (0.25 g/L) (Jain and Yan, 2011b).

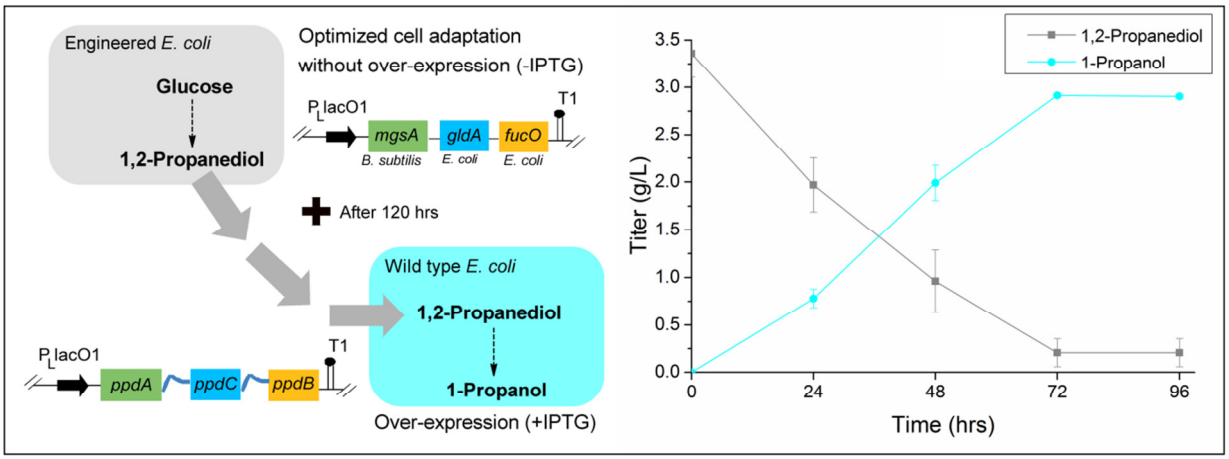


Figure 3.5. Schematic representation of a dual strain strategy and time course of 1-propanol production. The 1,2-propanediol producing strain lacked IPTG induction; the 1-propanol producing strain was added to the cultures after 120 h. The data were generated from three independent experiments.

3.3. Discussion

In order to conserve more carbon and NADH for 1,2-propanediol production, we first disrupted the major byproduct pathway (lactate). Interestingly, the expression of 1,2-propanediol pathway in this strain (RJ31) resulted in lowering 1,2-propanediol production by nearly 40% of the wild type strain and accumulated pyruvate at 7.18 g/L (Supporting Information Table S3.2). This led to the understanding that since the carbon flux was dominant toward the glycolytic pathway, the disruption of competing pathways for carbon is vital. In order to direct the carbon effectively into the 1,2-propanediol pathway, the major carbon competing pathways were disrupted (strain RJ57). However, the expression of the 1,2-propanediol pathway in this strain resulted in lowering the titer to just 0.35 g/L. We then hypothesized that the development of a NADH

regeneration system would serve as a driving force for 1,2-propanediol production. It is seen that with the introduction of this NADH regeneration system, 1,2-propanediol production was increased in both the engineered (RJ57) and wild type strains (Table 3.1). With the development of cell adaptation strategy, the growth of the engineered strain was improved and 1,2-propanediol production was achieved at a high titer (5.13 g/L) with a high yield of 0.48 g/g glucose.

The theoretical maximum yield for anaerobic production of 1,2-propanediol was previously calculated to be 1.2 mol/mol glucose (0.51 g/g glucose) (Cameron et al., 1998). It should be noted that we used yeast extract in the culture medium to facilitate cell growth, which has been extensively used in metabolic engineering. The contribution of yeast extract toward target product yield was evaluated using yeast extract as the sole carbon source, following which it was determined that the contribution of yeast extract toward product yield was minor or even negligible (Atsumi et al., 2008e). Therefore, we investigated the contribution of yeast extract toward 1,2-propanediol yield using the same approach. Control experiments were then carried out using yeast extract as the sole carbon source. At the end of fermentation study it was determined that no 1,2-propanediol was produced in both the wild type and the engineered strains. Hence, the contribution of yeast extract toward product yield was considered to be negligible. Thus, we concluded that strain RJ57 expressing the 1,2-propanediol pathway grown with cell adaptation strategy achieved 94% of theoretical maximum yield (0.48 g/g glucose).

With a highly efficient system established for 1,2-propanediol, we then channeled our efforts toward resolving its low conversion efficiency to 1-propanol. As seen from Figure 3.4A, with the

creation of a fusion diol dehydratase, the conversion efficiency was recovered to 100% in whole cell conversion studies. However, during fermentation, most of the 1,2-propanediol remained unconverted. We reasoned that since the catalytic activity of the diol dehydratases showed gradual decrease with time, the production of 1-propanol was hampered when 1,2-propanediol is generated gradually. To circumvent this predicament, a dual strain strategy was utilized where 1,2-propanediol production was first achieved, following which the strain expressing the fusion diol dehydratase was added to the cultures. By relying on the leaky expression of the upstream pathway for 1,2-propanediol production, this dual strategy resulted in converting nearly all of the 1,2-propanediol to 1-propanol, boosting the titer to 2.91 g/L (Figure 3.5).

3.4. Conclusions

Majority of the world's demand for high value/ bulk chemicals and energy is met by utilization of fossil fuels. With the emergence of metabolic engineering, better solutions are emerging to meet the global demand. Here, we provide effective steps toward the future scale up and industrial scale biological manufacture of current petrochemical based compounds 1,2-propanediol and 1-propanol. In this work, the production of 1,2-propanediol is pursued followed by the production of 1-propanol by adapting different metabolic engineering strategies to surpass the hurdles in enhancing their production.

3.5. Methods and Materials

3.5.1. Chemicals and Reagents

1,2-Propanediol and methylglyoxal standards were procured from Sigma Aldrich (St. Louis, MO); acetol (hydroxyacetone) was procured from Acros Organics (New Jersey, U.S.A.) and 1-propanol was purchased from Fisher Scientific (Atlanta, GA). For amplifying DNA in order for cloning, phusion high fidelity polymerase (New England Biolabs, Beverly, MA) and KOD DNA polymerase (EMD Chemicals Inc., NJ) were used. In order to verify knockout strains, GoTaq DNA polymerase (Promega, Madison, WI) was utilized. Restriction enzymes were procured from New England Biolabs (Beverly, MA). For ligation reactions either Rapid DNA ligase (Roche Applied Science, Indianapolis, IN) or Quick DNA ligase (New England Biolabs, Beverly, MA) were used.

3.5.2. Bacterial Strains and Plasmids

E. coli strain BW25113 (*E. coli* Genetic Resource Center) as wild type and its knockout derivatives were used for shake flask experiments (Baba et al., 2006). Using P1 transduction, target genes were disrupted to construct engineered strains with more than one gene disruption (Thomason et al., 2007). Plasmid pCP20 was used to remove kanamycin resistance gene from target strains (Datsenko and Wanner, 2000). Verification of gene disruption/ antibiotic resistance loss was done via colony PCR. For DNA manipulations, *E. coli* strain XL1-Blue (Stratagene, CA) was used. For enzyme assays, *E. coli* BL21 Star (DE3) was used. Plasmids pZE12-luc (Lutz

and Bujard, 1997), pCS27 (Shen and Liao, 2008c), pSA74 (Huo et al., 2011b) and pETDuet-1 (EMD Chemicals Inc., NJ) were used for DNA cloning. The characteristics of strains and plasmids are described in Table 3.2.

3.5.3. DNA Manipulations

The procedures used for DNA manipulations were performed according to the methods described previously (Ausubel et al., 1994). Plasmids pRJ1, pRJ2, and pYY93 were constructed previously (Jain and Yan, 2011b). To create pRJ68, the *E. coli gldA* gene was inserted downstream to the *mgsA* gene in pRJ2 using SphI and XbaI. Similarly to create pRJ69, the *budC* gene from *K. pneumoniae* was inserted downstream to the *mgsA* gene in pRJ2 using SphI and XbaI. To create pRJ70, the genes *gldA* and *fucO* were inserted simultaneously downstream to the *mgsA* gene in pRJ2 using SphI, BamHI and XbaI. The *Candida boidinii fdh1* gene encoding formate dehydrogenase was codon optimized for expression in *E. coli*. The sequence is provided in the Supporting Information. To create pRJ58, the *fdh1* gene was synthesized via PCR based oligonucleotide assembly and inserted into pCS27 using Acc65I and BamHI. In order to express his-tagged FDH protein, pRJ98 was constructed with the *fdh1* gene inserted into pET-Duet-1 using BamHI and SalI.

To construct pRJ49, the *ppdABC* operon was digested with BsrGI and XbaI and inserted into plasmid pZE12-luc digested with Acc65I and XbaI. In order to create fusion diol dehydratase proteins, we first linked the *ppdA*, *ppdB*, and *ppdC* genes into single reading frames (*ppdA-C-B* or *ppdC-B-A*). Overlap PCR was used achieve these, with the removal of corresponding start and

stop codons using the designed primers. Plasmid pRJ79 was constructed, with the *ppdA-C-B* reading frame inserted into pZE12-luc using EcoRI and XbaI. Similarly, pRJ81 was constructed by inserting *ppdC-B-A* reading frame into pZE12-luc using EcoRI and XbaI. To create pRJ93, the *ppdA-C-B* reading frame and the *mgsA-gldA-fucO* operon were cloned simultaneously intopZE12-luc using EcoRI, BsrGI, and XbaI. To construct plasmid pRJ99, the *ppdA-C-B* and the *mgsA-gldA-fucO* operon (obtained using pRJ93 as the template) as a single DNA fragment were digested with BsiWI and XhoI. Following digestion, the fragment was inserted into the plasmid pCS27 digested with Acc65I and SalI. Similarly pRJ100 was constructed by cloning this fragment into plasmid pSA74.

3.5.4. Formate Dehydrogenase Enzyme Assay

E. coli BL21 Star (DE3) harboring plasmid pRJ98 was used to evaluate the activity of formate dehydrogenase. 500 µL of the overnight cultures was added to 50 mL fresh LB and was grown for 3 h at 37^{o}C. IPTG was added to a final concentration of 1 mM to induce protein expression and the cultures were then grown for another 3 h at 30^{o}C. Next, the cultures were centrifuged and the cells were lysed using Mini Bead Beater (Biospec). Following another round of centrifugation, the resulting supernatant was collected. His-Spin Protein Miniprep kit (Zymo Research, Irvine, CA) was used to obtain the purified protein from the supernatant. Protein concentration estimation was done using the Pierce BCA protein assay kit (Thermo Scientific, Atlanta, GA). The formate dehydrogenase enzyme assay using the purified protein was done with modifications to the method described previously (Berrios-Rivera et al., 2002). The reaction system (1 mL) consisted of 50 mM sodium phosphate buffer (pH 7.0), 0.25 mM NAD^{+}, 50 µL of

purified enzyme and substrate sodium formate. The concentration of substrate sodium formate was varied from 0 mM to 75 mM. The molecular weight of the protein along with his-tag was estimated to be 41748 Da. The reaction rates were monitored for 30 s at 37^{o}C by measuring the change in absorbance at 340 nm.

3.5.5. Culture Medium

Modified M9 medium was used for the whole cell conversion study of propanediol dehydratase, which consisted of (per liter): 20 g glucose, 5 g yeast extract, 12.8 g $Na_2HPO_4.7H_2O$, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 0.5 mM $MgSO_4$, and 0.05 mM $CaCl_2$. The modified low phosphate medium was used for shake flask fermentations and consisted of (per liter): 40 g glucose, 5 g NaCl, 5 g yeast extract, 1 g NH_4Cl, 1.5 g KCl, 0.2 g $MgCl_2$, 0.07 g Na_2SO_4, and 0.005 g $FeCl_3$. The medium was then buffered to pH 6.8 with 13.3 g of $NaHCO_3$ and 10 g of 3-[N-morpholino] propanesulfonic acid (MOPS) (Altaras and Cameron, 2000b; Jain and Yan, 2011b). For experiments with the expression of formate dehydrogenase, in order to regenerate cellular NADH, 50 mM sodium formate was added to the modified low phosphate medium to make low phosphate formate medium (Berrios-Rivera et al., 2002).

3.5.6. Whole Cell Conversion Studies

For whole cell conversion studies, 2 mL of seed cultures were prepared in LB containing necessary antibiotics and grown overnight at 37^{o}C in a shaker set at 250 rpm. After overnight incubation, 100 µL of the cultures were inoculated into 10 mL of modified M9 medium

containing appropriate antibiotics in 125 mL screw cap bottles and grown at 37°C for 4 h. After this, 0.1 mM IPTG, 10 µM coenzyme B12, and 5 g/L 1,2-propanediol were added to the medium and grown at 30°C for 24 h. Samples (1 mL) were collected at the end of the study for HPLC analysis.

3.5.7. Shake Flask Fermentations

For shake flask fermentation studies, 200 µL of the overnight cultures (grown in LB) were inoculated into 20 mL modified low phosphate medium or low phosphate formate medium in 150 mL serum bottles. The cultures were made anaerobic by sparging with a mixture of nitrogen and carbon dioxide gas and grown in a shaker at 37°C, 250 rpm. IPTG was added into the cultures to a final concentration of 0.1 mM, after 3 h (unless mentioned otherwise) to induce protein expression. For experiments involving 1-propanol production, 10 µM coenzyme B12 was added to the cultures along with IPTG. Fermentations were then carried out at 30°C in a shaker set at 250 rpm. Samples were collected and analyzed for cell growth and products.

3.5.8. Cell Adaptation

The overnight cultures of strain RJ57 harboring plasmids pRJ70 and pRJ58 were initially made in 3 mL LB and grown until exponential growth phase (until OD_{600} = 0.7). Following this, 1 mL of the cultures were added to 10 mL of fresh low phosphate formate medium in vacutainer glass tubes (BD) and made anaerobic immediately. The cultures were then grown until OD_{600} reached

about 0.2. Next, 1 mL of these subcultures were added into 20 mL of fresh low phosphate formate medium for shake flask fermentation studies as described above.

3.5.9. Dual Strain Strategies for 1-Propanol Production

We used a dual strain strategy to express the upstream 1,2-propanediol pathway and the downstream 1-propanol pathway in different strains. The strain RJ57 harboring plasmids pRJ70 and pRJ58 was used to express the upstream 1,2-propanediol pathway and the wild type strain harboring plasmids pRJ79 and pRJ58 was used to express the downstream 1-propanol pathway. First, 1,2-propanediol production was achieved, following which the strain expressing 1-propanol pathway was prepared and added to the cultures as described below. The strain RJ57 harboring plasmids pRJ70 and pRJ58 was grown using the methods described in "cell adaptation", following which, fermentations were carried out for 72 or 120 h as described in "shake flask fermentations", with the addition of IPTG when the optical density reached 0.46. Meanwhile, the wild type strain harboring plasmids pRJ79 and pRJ58 was prepared for sequential inoculation. The overnight cultures (60 µL of 2 mL) of the strain expressing 1-propanol pathway was used to inoculate 6 mL of fresh LB, which was grown at 37ºC for 3 h. Next, IPTG (0.1 mM) and 10 µM coenzyme B12 were added in order to facilitate protein expression and the cultures were grown at 30ºC for another 5 h (OD_{600} was approximately 2.0). These cultures were then added to the 72 or 120 h cultures (OD_{600} = 0.69 or OD_{600} = 0.79 after 72 or 120 h respectively) of the strain expressing the upstream 1,2-propanediol pathway in two different strategies. In the first strategy, the cultures (6 mL) of the strain expressing the downstream 1-propanol pathway was centrifuged and resuspended in 1 mL of fresh low

phosphate formate medium. This 1 mL resuspended cultures were added to the 72 h cultures along with 0.1 mM IPTG and 10 µM coenzyme B12. In the second strategy, 1 mL cultures of the strain expressing the downstream pathway was directly added to the 72 or 120 h cultures along with 0.1 mM IPTG and 10 µM coenzyme B12. Fermentations were then carried out at 30ºC and samples were collected for analysis.

3.5.10. Analytical Procedures

Cell growth was analyzed by measuring the optical density at 600 nm using a UV-650 spectrophotometer (Beckman Instruments, San Jose, CA, U.S.A.). The samples from fermentation studies and whole cell conversion studies were analyzed via HPLC-RID. First, the samples were prepared via centrifugation at 15,000 rpm for 15 min. Following this, the resulting supernatants were filtered and used for analysis in a HPLC (Shimadzu) equipped with a Coregel-64H column (Transgenomic) with 4 mN H_2SO_4 as the mobile phase. The flow rate of mobile phase was 0.6 mL/min and the oven temperature was set at 60^{o}C (Eiteman and Chastain, 1997). For the separation of 1,2-propanediol and acetol and also for the separation of ethanol and MOPS, the oven temperature was set at 40^{o}C.

Competing Interests

The University of Georgia has filed a patent application on this technology.

Supporting Information

This material is provided in Appendix A.

Table 3.2. List of strains and plasmids used in this study.

Strain	**Genotype**	**Reference**
E. coli BW25113	*F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ⁻, rph-1, Δ(rhaD-rhaB)568, hsdR514*	Yale CGSC
E. coli XL-1 Blue	*recA1 endA1gyrA96thi-1hsdR17supE44relA1lac [F' proAB lacIqZDM15Tn10 (TetR)]*	Stratagene
E. coli BL21 Star (DE3)	*F⁻ompT hsdS$_B$ ($r_B^-m_B^-$) gal dcm (DE3)*	Invitrogen
JW1375-1	BW25113 Δ*ldhA::kan*	Yale CGSC
JW1643-2	BW25113 Δ*gloA::kan*	Yale CGSC
JW3890-2	BW25113 Δ*tpiA::kan*	Yale CGSC
JW1841-1	BW25113 Δ*zwf::kan*	Yale CGSC
JW1228-1	BW25113 Δ*adhE::kan*	Yale CGSC
RJ31	BW25113 Δ*gloA* Δ*ldhA*	This study
RJ57	BW25113 Δ*zwf* Δ*tpiA* Δ*ldhA* Δ*gloA* Δ*adhE*	This study
Plasmid	**Description**	**Reference**
pZE12- luc	pLlacO1; luc; *ColE1 ori*; *Amp^R*	(Lutz and Bujard, 1997)
pCS27	pLlacO1; *p15A ori; Kan^R*	(Shen and Liao, 2008c)
pSA74	pLlacO1; *pSC101ori*; *Cm^R*	(Huo et al., 2011b)
pETDuet-1	two T7 promoters; two MCS; *pBR322 ori*; *Amp^R*	Novagen
pYY93	*ppdABC* from *K. oxytoca* cloned into pCS27	(Jain and Yan,

		2011b)
pRJ1	*mgsA* from *C. acetobutylicum* cloned into pZE12-luc	(Jain and Yan, 2011b)
pRJ2	*mgsA* from *B. subtilis* cloned into pZE12-luc	(Jain and Yan, 2011b)
pRJ49	*ppdABC* from *K. oxytoca* cloned into pZE12-luc	This study
pRJ58	*fdh1* from *C. boidinii* cloned into pCS27	This study
pRJ68	*mgsA* from *B. subtilis* and *gldA* from *E. coli* cloned into pZE12-luc	This study
pRJ69	*mgsA* from *B. subtilis* and *budC* from *K. pneumoniae* cloned into pZE12-luc	This study
pRJ70	*mgsA* from *B. subtilis*, *gldA* from *E. coli* and *fucO* from *E. coli* cloned into pZE12-luc	This study
pRJ79	Fusion *ppdA-C-B* cloned into pZE12-luc	This study
pRJ81	Fusion *ppdC-B-A* cloned into pZE12-luc	This study
pRJ93	Fusion *ppdA-C-B*, *mgsA* from *B. subtilis*, *gldA* from *E. coli* and *fucO* from *E. coli* cloned into pZE12-luc	This study
pRJ98	*fdh1* from *C. boidinii* cloned into pET-Duet-1	This study
pRJ99	Fusion *ppdA-C-B*, *mgsA* from *B. subtilis*, *gldA* from *E. coli* and *fucO* from *E. coli* cloned into pCS27	This study
pRJ100	Fusion *ppdA-C-B*, *mgsA* from *B. subtilis*, *gldA* from *E. coli* and *fucO* from *E. coli* cloned into pSA74	This study

CHAPTER 4

RATIONALLY REDESIGNED DIOL DEHYDRATASE ENABLES BIOSYNTHESIS OF 1,4-BUTANEDIOL FROM XYLOSE

To be submitted to Proceedings of the National Academy of Sciences of the United States of America.
Rachit Jain, Xinxiao Sun, Mengyin Cheng, Qipeng Yuan, James C. Liao and Yajun Yan: Rational Engineering of Diol Dehydratase Enables 1,4-Butanediol Biosynthesis From Xylose.

Abstract

Establishing novel synthetic routes for microbial production of chemicals often requires overcoming pathway bottlenecks by tailoring enzymes to enhance biocatalysis or even achieve non-native catalysis. Diol dehydratases have been extensively studied for their interactions with C2 and C3 diols. However, attempts on utilizing these insights to enable catalysis on non-native substrates with more than two hydroxyl groups have been plagued with low efficiencies. Here, we rationally redesign the *Klebsiella oxytoca* diol dehydratase to enable and enhance catalytic activity toward a non-native C4 triol (1,2,4-butanetriol). We analyze dehydratase's interaction with 1,2-propanediol and glycerol, which leads us to develop rationally conceived hypotheses. An *in silico* approach is then developed to identify and screen candidate mutants with desired activity. This leads to an engineered diol dehydratase with nearly 5 fold higher catalytic activity toward 1,2,4-butanetriol as determined by *in vitro* assays. Based on this, we then expand the 1,2,4-butanetriol pathway to establish a novel 1,4-butanediol production platform. We first engineer *E. coli*'s xylose catabolism to enhance the biosynthesis of 1,2,4-butanetriol from 150 mg/L to 874 mg/L. By introducing the complete pathway in the engineered strain we achieve *de novo* biosynthesis of 1,4-butanediol at 218 mg/L from xylose. This work expands the repertoire of substrates catalyzed by diol dehydratases and serves as an elucidation to establish novel biosynthetic pathways involving dehydratase based biocatalysis.

Keywords: 1,4-Butanediol, Diol dehydratase, Xylose, 1,2,4-Butanetriol, *Escherichia coli*

4.1. Introduction

Biological platform for production of various industrially attractive chemicals including pharmaceuticals (Ajikumar et al., 2010; Lin et al., 2014; Ro et al., 2006; Yan et al., 2008), biofuels (Atsumi et al., 2008e; Avalos et al., 2013a; Huo et al., 2011b; Lan et al., 2013; Xu et al., 2013), polymers (Jung and Lee, 2011; Nikel et al., 2010; Xiong et al., 2014) and bulk chemicals (Jang et al., 2012a; Raynaud et al., 2003b; Shen et al., 2012b) has been established by introducing synthetic routes or enhancing native routes (Ohta et al., 1991; Vemuri et al., 2002) in microbes. In addition to manipulation of cellular metabolism, protein engineering provides powerful tools to overcome bottlenecks in the biosynthesis of chemicals.

The most common approach to engineer proteins is to create a library of mutants, either rationally designed or random. Generally, creating a large library of mutants (commonly achieved via random mutagenesis) is considered desirable as it heightens the possibility of identifying an optimal mutant (Dietrich et al., 2010). However, validating a large library of mutants requires a high throughput screening platform, which may not be amenable to all catalytic reactions. Alternatively, semi-rational/ rational approaches may prove to be advantageous, by screening a large number of mutants *in silico*, thus obviating the need of a high throughput screening platform (Lutz, 2010).

Rational approaches to engineer protein activity have been largely pursued via manipulation of amino acid residues in the catalytic pocket achieved via site directed mutagenesis (Marcheschi et al., 2013). In order to promote non-native catalysis toward larger substrates, increasing the catalytic pocket has been the predominant strategy, generally achieved via replacement of target residues with smaller ones (Marcheschi et al., 2013). Although substrate accommodation is achieved, merely substituting with a smaller amino acid may not always promote efficient

catalysis, which is influenced by enzyme- substrate complex stability, local electrostatic environment, steric hindrance, other interactions (hydrogen bonds, hydrophobic interactions, van der Waals interactions, etc.) and substrate orientation. Hence, the benefits of rational approaches that merely rely on accommodating the substrate are undermined as they tend to overlook critical interactions influencing catalysis. As such, substituting target residues via saturation mutagenesis (Savile et al., 2010) and/ or directed evolution has been shown to improve catalysis possibly due to changes in the above mentioned interactions altering catalysis. The utility of these semi-rational approaches are in turn contingent on sensitivity of high-throughput screening platforms for identification of optimal mutant(s) which generally involves laborious experimental procedures (Dietrich et al., 2010).

In this work, we engineer the *Klebsiella oxytoca* diol dehydratase to achieve non-native catalysis via a two-step rational design approach taking into account critical interactions influencing catalysis. The *K. oxytoca* diol dehydratase has a complex coenzyme B12 dependent reaction mechanism, and is known to dehydrate its native substrate – 1,2-propanediol (1,2-PD) efficiently and to undergo suicide inactivation in the presence of a C3 triol – glycerol (Doitomi et al., 2012). Since the catalytic mechanism of *K. oxytoca* diol dehydratase has been well studied with 1,2-PD and also with glycerol, we chose this enzyme as a candidate for rational engineering approaches to promote catalysis toward a non-natural C4 triol – 1,2,4-butanetriol (1,2,4-BTO).

We first adapt predominant strategies of rational design via preventing interaction with an undesired residue and increasing the size of the catalytic pocket to enable the accommodation of a larger substrate. In the second step of rational design, we consider other factors influencing catalysis which are commonly overlooked. Specifically, we study the effect of mutating target amino acids in the catalytic pocket on substrate position based upon steric effects while

maintaining a relatively similar local hydrophobic environment. Prudent application of this approach leads to increasing dehydratase activity by nearly 5 fold toward 1,2,4-BTO in comparison to the native protein. These efforts enable the production of 1,4-butanediol (1,4-BDO) via a novel metabolic route (Figure 4.1) from xylose (the most abundant C5 sugar in lignocellulosic biomass) (Zhou et al., 2012), which is different from the previous artificial pathway commencing from succinate and/ or α-ketoglutarate (Hwang et al., 2014; Yim et al., 2011c). 1,4-BDO is not known to be natively produced by any microbe, and is a valuable commodity chemical (annual market of over 2.5 million tons worldwide) used in the manufacture of plastics, solvents, fibers, other chemicals whose combined market is in excess of $4 billion (Ji and Huang, 2014; Yim et al., 2011c).

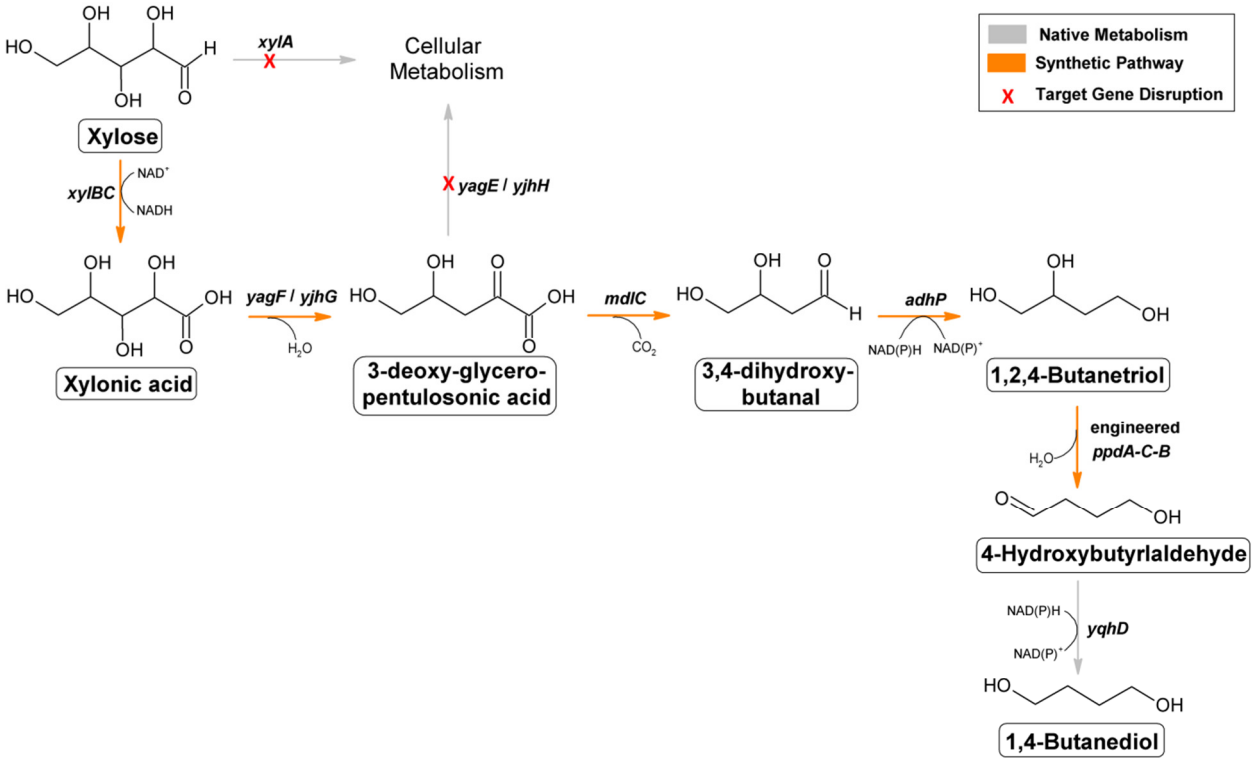


Figure 4.1. Schematic representation of novel 1,4-butanediol pathway from xylose and native competing pathways in *E. coli*. Genes: *xylA*: encoding xylose isomerase; *xylBC*: encoding xylose dehydrogenase; *yagF* or *yjhG*: encoding xylonate dehydratase; *mdlC*: encoding benzoylformate decarboxylase; *adhP*: encoding alcohol dehydrogenase; *ppdA-C-B*: encoding diol dehydratase; *yqhD*: encoding alcohol dehydrogenase; *yagE* or *yjhH*: encoding aldolase.

4.2. Results and discussion

4.2.1. Native dehydratase possesses nominal activity toward 1,2,4-butanetriol

Diol dehydratases are commercially important enzymes which have been successfully utilized for the production of high value commodities like 1,3-propanediol from glycerol (Raynaud et al., 2003b), 1-propanol from 1,2-PD (Jain and Yan, 2011), 2-butanone from meso-2,3-butanediol

(Yoneda et al., 2014). In our previous work we improved the biocatalytic efficiency of *K. oxytoca* diol dehydratase toward its native substrate (1,2-PD) by linking its 3 subunits to create a fusion protein (*ppdA-C-B*) (Jain et al., 2014). Here, we selected this fusion dehydratase for rational engineering approaches to dehydrate a non-native C4 triol, as it would be congenial to purification and characterization studies. We performed *in vitro* enzyme assays by coupling the dehydration activity of this native fusion dehydratase with the subsequent reduction activity of alcohol dehydrogenase (encoded by *yqhD*).

Native diol dehydratase's catalytic activity was determined to be 32 fold lower toward 1,2,4-BTO (k_{cat} = 17.08 min^{-1}) as compared to its catalytic activity toward 1,2-PD (k_{cat} = 556.99 min^{-1}). In order to then evaluate its catalytic efficiency for *in vivo* production of 1,4-BDO we performed whole cell bioconversion studies. As seen from Figure 4.2A, no detectable production of 4-hydroxybutrylaldehyde or 1,4-BDO was observed from cultures fed with 1,2,4-BTO, even though activity toward 1,2,4-BTO was detected *in vitro*. The native dehydratase completely converted 1,2-PD in whole cell conversion studies, as indicated by 1-propanol production (Jain et al., 2014), validating our *in vitro* studies (Figure 4.2B). Hence, to promote 1,4-BDO biosynthesis we pursued the enhancement in dehydratase's activity toward 1,2,4-BTO via rational engineering approaches encouraged by its inherent nominal activity.

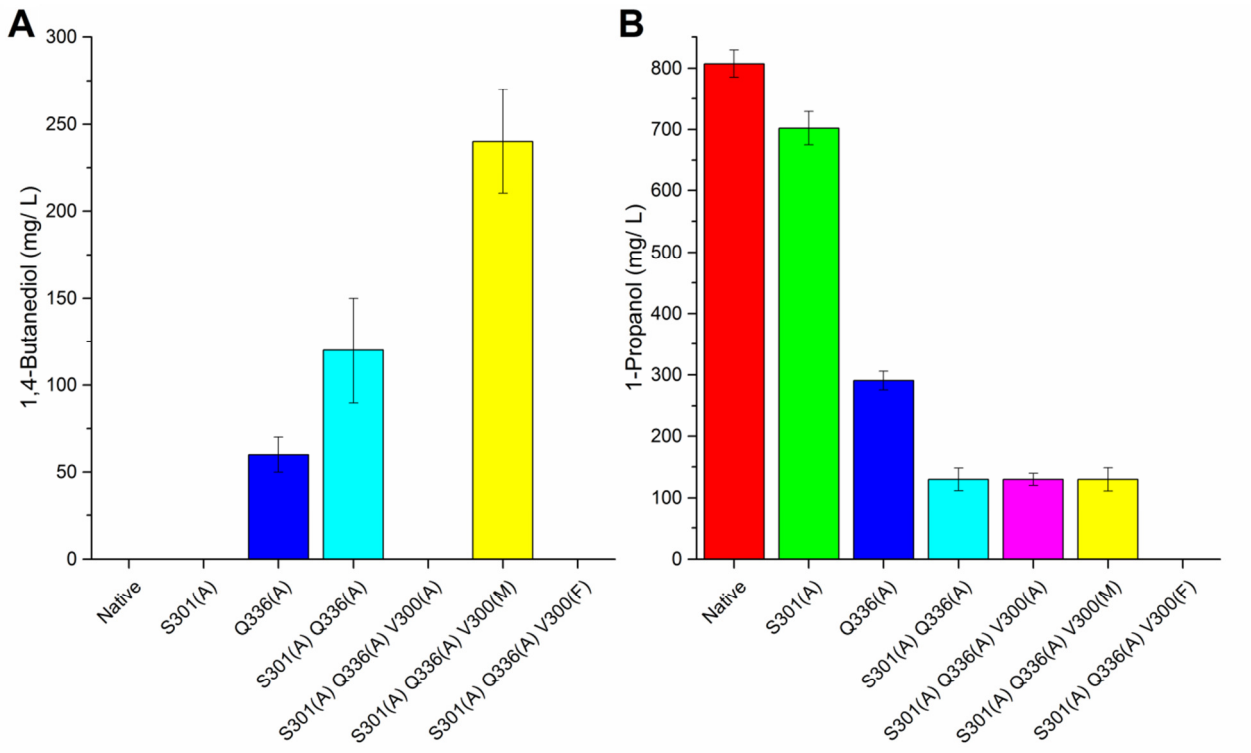


Figure 4.2. Results of whole cell biocatalysis experiments using native and mutant dehydratases. (A) 1,4-Butanediol produced from whole cell biocatalysis experiments by feeding 1,2,4-butanetriol; (B) 1-Propanol produced from whole cell biocatalysis experiments by feeding 1,2-propanediol. The data were generated from three independent experiments.

4.2.2. Improving resistance to substrate inhibition alone does not facilitate biocatalysis

The activity of *K. oxytoca* diol dehydratase is known to be influenced by the number of hydroxyl groups present in the substrate. High catalytic efficiency is exhibited toward 1,2-PD (a C3 diol), while limited catalytic efficiency is observed toward glycerol (a C3 triol) due to substrate inhibition (Yamanishi et al., 2012). An approach to address this inhibition has been developed from studying the protein-substrate complex, where it was inferred that the interaction of the C3 hydroxyl group of glycerol with S301 leads to inhibition of catalysis (Doitomi et al., 2012). Recently, it has been shown that the mutation of S301 to A301 prevents this interaction with

glycerol, thereby making it more resistant to substrate inhibition (Yamanishi et al., 2012). Based on these findings we speculated that a similar substrate inhibition by a C4 triol (1,2,4-BTO) was plausible. Hence, we hypothesized that by improving substrate inhibition resistance toward 1,2,4-BTO, desired biocatalysis would be promoted. We then tested this hypothesis via whole cell biocatalysis experiments.

The biocatalytic efficiency of S301A mutant toward 1,2,4-BTO, glycerol and 1,2-PD were tested. Contrary to our expectation, no detectable quantities of products were observed from cultures fed with 1,2,4-BTO (Figure 4.2A). Furthermore, it was seen that the S301A mutant dehydratase showed a noticeable increase in resistance toward substrate inhibition by glycerol, consistent with previous studies (Yamanishi et al., 2012); leading to 1,3-propanediol production at 0.13 g/L as compared to 0.04 g/L from the native dehydratase (Supporting Information Figure S4.1). As seen from Figure 4.2B, the native dehydratase completely converted all of the 1,2-PD, while the S301A mutant dehydratase showed a minor decrease in conversion efficiency toward 1,2-PD leading to 1-propanol production at 0.70 g/L as compared to 0.81 g/L from native dehydratase.

We then speculated that although improvement in resistance toward a C3 triol substrate inhibition was achieved, it may be difficult to accommodate a C4 triol in the catalytic site of diol dehydratase, since 1,2,4-BTO is a larger substrate.

4.2.3. Promoting substrate accommodation enhances biocatalysis toward 1,2,4-butanetriol

We then calculated the space in the catalytic pocket by measuring the distance between Q336 and Q296. We chose Q336 as a candidate for mutation to a smaller residue. Alanine was chosen

as the substitute over glycine, as alanine's side chain is comparatively less reactive (Betts and Russel, 2003; Robinson and Sauer, 1998). By mutating Q336 to A336 we noticed an increase in this distance from 7.93 Å to 8.47 Å via *in silico* analysis (Figure 4.3). We speculated that this would allow more space for the larger substrate to be accommodated. Such an increase in the space of the catalytic pocket due to mutation of Q336 to A336 has also been observed in previous studies (Yamanishi et al., 2012).

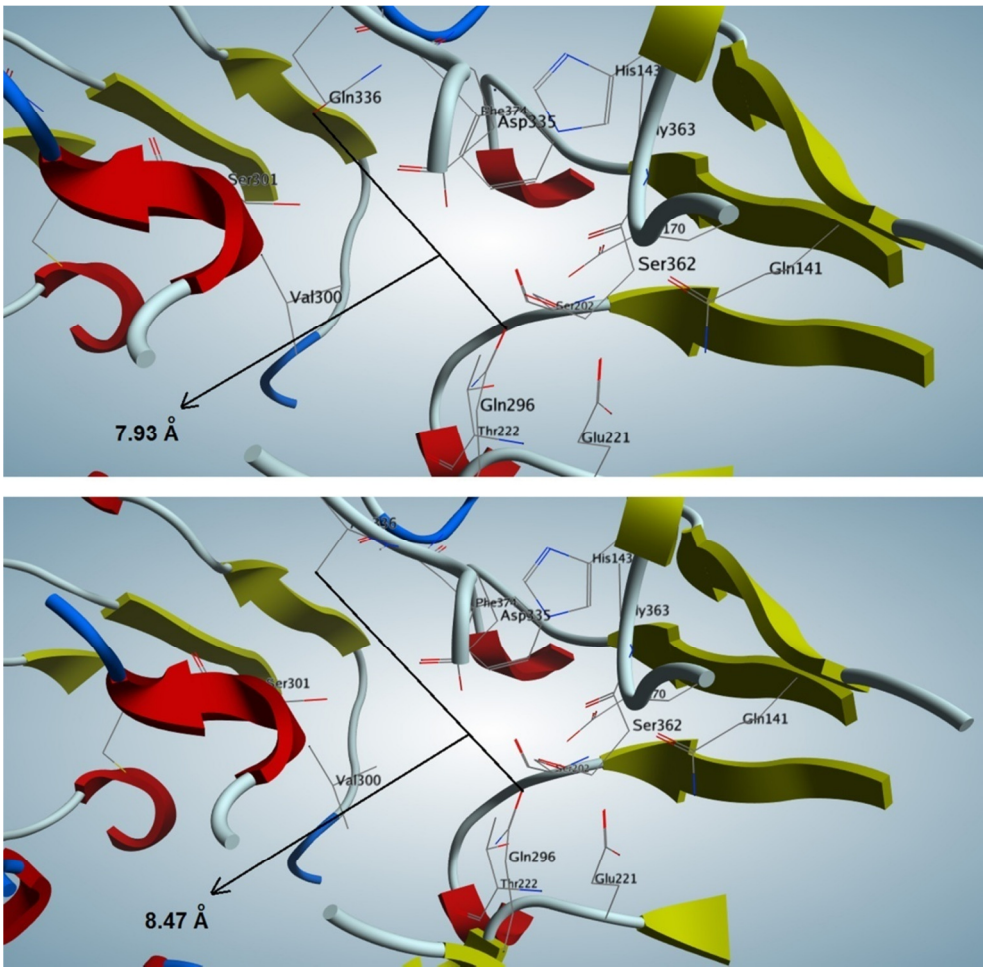


Figure 4.3. Representation of the catalytic pocket of native and Q336A mutant dehydratase. Distance calculated in Å between Q296 and Q336/ A336 using existing diol dehydratase crystal structure (PDBid: 1UC5).

Hence, we hypothesized that mutating Q336 to A336 would result in the desired dehydration activity due to accommodation of 1,2,4-BTO in the active site. In order to test this hypothesis, we created Q336A mutant dehydratase and performed whole cell biocatalysis experiments. As seen from Figure 4.2A, the mutation of Q336 to A336 resulted in achieving detectable biocatalysis toward 1,2,4-BTO, producing 1,4-BDO at 60 mg/L. However, majority of the 1,2,4-BTO remained unconverted. Since no 4-hydroxybutrylaldehyde was detected, this result indicated that the native activity of *E. coli* was sufficient to reduce 4-hydroxybutrylaldehyde to 1,4-BDO. Hence, we concluded that overexpression of a dehydrogenase was not necessary for the reduction of 4-hydroxybutrylaldehyde to 1,4-BDO.

Interestingly, *E. coli* cultures expressing the Q336A mutant dehydratase exhibited a further reduction in conversion efficiency toward the native substrate – 1,2-PD, indicated by a decrease in 1-propanol production to 0.29 g/L (Figure 4.2B). We also studied the activity of Q336A mutant dehydratase toward glycerol, and found a marginal increase in 1,3-propanediol production to 0.06 g/L as compared to the native dehydratase (0.04 g/L), while lower than that of S301A mutant (0.13 g/L) (Supporting Information Figure S4.1).

Next, we performed docking simulations to further analyze the interaction of 1,2,4-BTO with the Q336A mutant dehydratase. Docking simulations with Q336A mutant revealed that an interaction between S301 and 1,2,4-BTO was highly likely. We then hypothesized that by creating a S301A Q336A double mutant, the catalytic efficiency toward 1,2,4-BTO may be further increased due to: (a) prevention of interaction between the C4 hydroxyl group of 1,2,4-BTO with S301 and (b) promotion of substrate accommodation.

In order to test this hypothesis, we constructed S301A Q336A mutant diol dehydratase and performed whole cell biocatalysis experiments. As seen from Figure 4.2A, biocatalytic efficiency toward 1,2,4-BTO was further improved as indicated by increase in 1,4-BDO production to 120 mg/L. Furthermore, we observed an even greater decrease in conversion efficiency toward 1,2-PD, indicated by a decrease in 1-propanol production to 0.13 g/L. While, improvement in resistance to substrate inhibition toward the C3 triol (glycerol) was found to be similar (0.05 g/L 1,3-propanediol) to the native dehydratase (0.04 g/L 1,3-propanediol) (Supporting Information Figure S4.1).

We then proceeded to characterize the *in vitro* catalytic efficiency of S301A Q336A mutant dehydratase via coupled enzyme assays using the purified protein. As seen from Table 4.1, activity toward 1,2,4-BTO was increased due to S301A Q336A mutations (K_{cat} = 76.21 min^{-1}), as compared to the native dehydratase (K_{cat} = 17.08 min^{-1}). Consistent with our observations from whole cell biocatalysis experiments, activity (K_{cat}) toward native substrate 1,2-PD was decreased from 556.99 min^{-1} to 39.49 min^{-1}. Overall, predominant rational design strategies (substrate accommodation and preventing undesired interaction) improved catalysis toward 1,2,4-butanetriol, however at low biocatalytic efficiency.

Table 4.1. Results of *in vitro* characterization of native and mutant dehydratases via coupled enzyme assay.

Dehydratase	**Substrate**	K_{cat} **(min^{-1})**	K_m **(mM)**
Native	1,2,4-BTO	17.08 ± 0.09	2.61 ± 0.05
S301A Q336A	1,2,4-BTO	76.21 ± 7.62	7.42 ± 1.77
S301A Q336A V300M	1,2,4-BTO	83.14 ± 3.46	7.34 ± 0.80
Native	1,2-PD	556.99 ± 81.69	7.90 ± 2.74
S301A Q336A	1,2-PD	39.49 ± 0.69	3.16 ± 0.32
S301A Q336A V300M	1,2-PD	38.11 ± 1.39	3.50 ± 0.40

4.2.4. Promoting the maintenance of O1-M ion distance improves activity toward 1,2,4-butanetriol

In order to further improve catalytic activity toward 1,2,4-BTO, we used the knowledge of its reaction mechanism as a guiding principle to enhance non-native dehydratase based catalysis. The complex catalytic mechanism of diol dehydratase with 1,2-PD has been studied, where it was found that the binding of the substrate in coordination with the metal ion initiates catalysis via the homolytic cleavage of Co-C bond (of adenosylcobalamin) (Shibata et al., 2002). The resultant adenosyl radical abstracts a hydrogen atom from the C1 position of the substrate, thus creating a 1,2 diol radical. Next, the OH group from C2 of the substrate migrates to the C1 position, creating a 1,1 diol radical. With this, the 1,1 diol radical back abstracts the hydrogen

atom from 5'-deoxyadenosine, producing a 1,1 diol product and regenerating the adenosyl radical. With this, the loss of a water molecule leads to the product aldehyde.(Shibata et al., 2002)

The metal ion plays an essential role in catalysis by (a) holding the substrate in the favorable orientation leading to initiation of catalysis (b) allowing hydrogen abstraction from the substrate and OH group migration (Kamachi et al., 2011). It was determined that the distance between the metal ion and O1 of 1,2-PD was 2.5 Å, representative of an optimal distance for catalysis to proceed (Kamachi et al., 2011). From these studies we inferred that a distance close to 2.5 Å between the metal ion in the active site and O1 of the substrate is critical to catalysis, as an increased distance may not be thermodynamically favorable to catalysis (hydrogen abstraction, hydrogen back-abstraction or OH group migration) leading to possible substrate inhibition. Hence, we hypothesized that rational engineering approaches may help promote a substrate orientation and position which is thermodynamically more favorable to catalysis (O1 of substrate being closer to 2.5 Å from metal ion).

It is commonly known that a substrate's orientation in the catalytic pocket can be influenced by residues in close proximity; we hypothesized that these interactions can be availed to persuade desired catalysis (by altering the distance between O1 of substrate and metal ion). We developed an *in silico* screening approach to (a) identify prospective targets within the 5 Å radius that would influence substrate orientation (b) serve as a screening platform to study the influence of substitutions on substrate (1,2,4-BTO) orientation and distance from metal ion. Residues directly involved in catalysis were first eliminated as candidates (Q141, E170, E221, H143, Q296, D335 and S362) (Kamachi et al., 2011). T222, V300 and F374, were identified as a potential targets initially, as they are within the 5 Å radius, and not involved in catalysis directly. By performing

docking studies, we evaluated the effect of substituting the three target residues to other amino acids and determined that the mutation of V300 led to a more pronounced effect on substrate position. This observation is also supported by previous studies speculating that V300 may play a role in distinguishing substrate conformations due to steric repulsion (Doitomi et al., 2014).

Next, we performed a systematic study *in silico*, by mutating V300 to different amino acids with hydrophobic side chains based upon the S301A Q336A mutant dehydratase. This was done in order to (a) maintain a relatively similar hydrophobic environment in the catalytic pocket, (b) promote the effects of steric hindrance on substrate position. Docking simulations were carried out for each case, and the distance between the metal ion and O1 of 1,2,4-BTO were calculated for enzyme substrate complexes with the lowest energy; serving as a screening criteria for identification of candidate mutants (Table 4.2).

Table 4.2. Results of docking simulations with O1 (1,2,4-BTO)- M ion distance calculated as a result of influence on substrate position based upon amino acid substitutions.

Dehydratase	**Substrate**	**O1-M Distance (Å)**	**Reference**
S301A Q336A	1,2,4-BTO	7.19	Docking/ This study
S301A Q336A V300A	1,2,4-BTO	7.61	Docking/ This study
S301A Q336A V300M	1,2,4-BTO	2.63	Docking/ This study
S301A Q336A V300F	1,2,4-BTO	8.32	Docking/ This study

This study indicated a strong correlation between the choice of amino acid substitution and the distance between O1 of 1,2,4-BTO and metal ion of the lowest energy complexes. It was seen

that substitution of V300 with A300, M300 or F300 greatly influenced this distance, possibly due to steric repulsion (Table 4.2). It was observed that the substitution of V300 with M300 resulted in maintaining a distance of 2.63 Å between O1 of 1,2,4-BTO and the metal ion (Figure 4.4), while substitution with A300 or F300 increased this distance to 7.61 Å or 8.32 Å respectively (Table 4.2). We speculate that substitution with methionine maybe more likely to favor catalysis as (a) it's side chain is relatively more flexible than phenylalanine's, (b) methionine possesses a relatively less reactive side chain like alanine, (c) may promote a more pronounced steric hindrance effect toward 1,2,4-BTO, as it is bulkier than alanine (Betts and Russel, 2003). We also validated our docking studies by superimposing 1,2-PD obtained from crystal structure (PDBid:1UC5) with the docked 1,2,4-BTO (Supporting Information Figure S4.2).

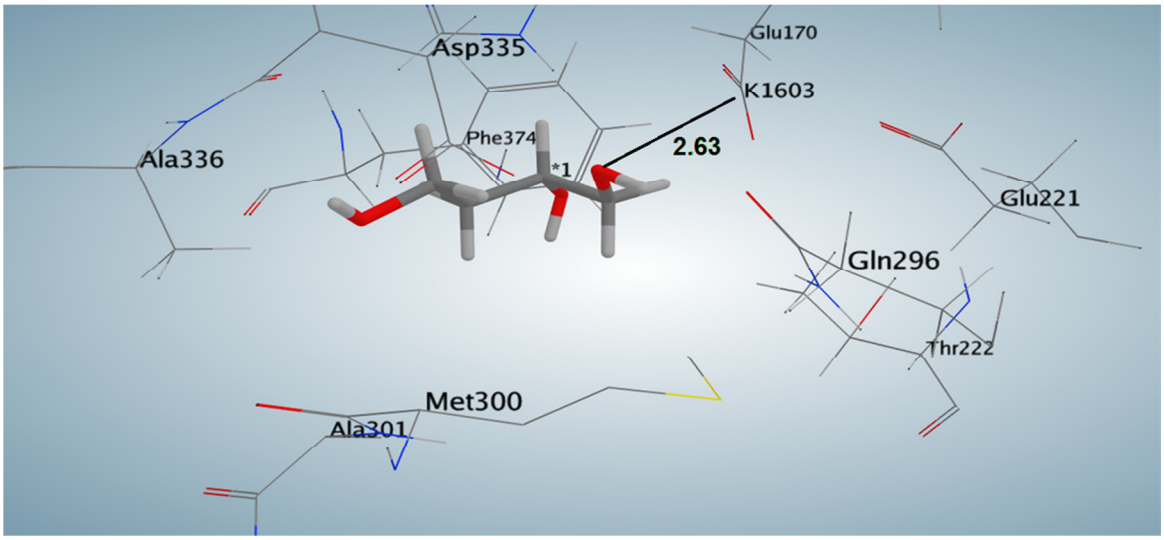


Figure 4.4. Representation of 1,2,4-butanetriol's interaction in the catalytic pocket of Q336A S301A V300M mutant diol dehydratase. Distance calculated in Å between the metal ion and C1OH of 1,2,4-butanetriol as obtained from docking studies.

Next, we created dehydratase S301A Q336A V300A, S301A Q336A V300M, S301A Q336A V300F mutants and tested their activity toward 1,2,4-BTO via whole cell biocatalysis studies. As

seen from Figure 4.2A, the S301A Q336A V300M mutant further promoted catalysis as expected, and increased 1,4-BDO titer to nearly 250 mg/L. No detectable amounts of 1,4-BDO was observed from cultures expressing the S301A Q336A V300A or S301A Q336A F300 mutants. Interestingly, the S301A Q336A V300A and S301A Q336A V300M mutants showed similar activity toward 1,2-PD and glycerol as the S301A Q336A mutant. However, no products were detected from cultures expressing S301A Q336A V300F mutant dehydratase when fed with either 1,2-PD or glycerol (Figure 4.2B/ Supporting Information Figure S4.1). Hence, the combinatorial mutation of Q336A, S301A and V300M resulted in promoting catalysis toward the desired C4 triol.

We then characterized the *in vitro* catalytic efficiency of this S301A Q336A V300M mutant dehydratase via coupled enzyme assays. As seen from Table 4.1, activity of S301A Q336A V300M dehydratase toward 1,2,4-BTO (K_{cat} = 83.14 min^{-1}) was nearly 5 fold higher as compared to the native dehydratase (K_{cat} = 17.08 min^{-1}). Similar to the S301A Q336A mutant, the activity of S301A Q336A V300M mutant dehydratase toward 1,2-PD was decreased (K_{cat} = 38.11 min^{-1}).

Additionally, we tested T222 or F374 mutations as control for 1,4-BDO production, via whole cell biocatalysis studies. As expected, we could not identify any additional mutant leading to improved production of 1,4-BDO as compared to S301A Gln336A mutant dehydratase (Supporting Information Figure S4.3). Furthermore, we evaluated if the coexpression of reactivation factors (*ddrAB*) would improve 1,4-BDO production, since its coexpression has been shown to improve dehydratase's resistance toward substrate inhibition by glycerol (Shibata et al., 2005). We performed whole cell conversion studies by feeding 1,2,4-BTO or glycerol as a control. It was observed that dehydratase's resistance toward substrate inhibition by glycerol was

improved (indicated by improvement in 1,3-propanediol production), consistent with previous studies (Shibata et al., 2005). However, no noticeable improvement in 1,4-BDO production was observed from native or mutant dehydratases when coexpressed with reactivation factors.

4.2.5. De novo biosynthesis of 1,4-butanediol from xylose

After *in vitro* characterization of dehydratase and its mutants we pursued the *de novo* biosynthesis of 1,4-BDO from xylose. To achieve this, we first adapted and engineered the 1,2,4-BTO biosynthesis pathway from xylose (Abdel-Ghany et al., 2013; Niu et al., 2003; Valdehuesaa et al., 2014). It has been previously shown that the introduction of a *Caulobacter crescentus* xylose dehydrogenase (encoded by *xylBC*) and *Pseudomonas putida* keto acid decarboxylase (encoded by *mdlC*) in *E. coli* enables the production of 1,2,4-BTO from xylose (Niu et al., 2003). In this pathway, xylose is catalyzed to xylonic acid by the action of *C. crescentus* xylose dehydrogenase, following which the activity of native xylonic acid dehydratase (encoded by *yjhG* or *yagF*) results in the formation of 3-deoxy-glycero-pentulosonic acid. Next, 3-deoxy-glycero-pentulosonic acid is decarboxylated to 3,4-dihydroxy-butanal by the action of *P. putida* keto acid decarboxylase. 1,2,4-BTO is then produced by the action of native alcohol dehydrogenase (*adhP*) on 3,4-dihydroxy-butanal (Niu et al., 2003; Valdehuesaa et al., 2014) (Figure 4.1). We calculated the theoretical maximum yield of 1,2,4-BTO from this pathway to be 1 mol/ mol xylose (0.71 g/g xylose).

We initially performed a systematic approach to improve the production of 1,2,4-butanetriol. *E. coli* strain RJ171 was first constructed, with the disruption of major carbon competing pathway for xylose utilization (pentose phosphate pathway via the action of xylose isomerase, encoded by

xylA). We studied the effect of overexpressing just the heterologous enzymes (*xylBC* and *mdlC*) in comparison to overexpressing the complete 1,2,4-BTO pathway (*xylBC*, *mdlC*, *yjhG*, *yagF*, *adhP*) in this strain. As seen from Supporting Information Figure S4.4, 1,2,4-BTO production was achieved at 150 mg/L or 170 mg/L from strain RJ171 expressing the partial or complete 1,2,4-BTO pathway respectively. In order to further prevent the loss of carbon to major competing pathways, we constructed *E. coli* strain RJ173 with *xylA* and *yjhH* gene disruptions and *E. coli* strain RJ175 with *xylA* and *yagE* gene disruptions (Figure 4.1). As seen from Supporting Information Figure S4.4, 1,2,4-BTO production was improved to 520 mg/L or 550 mg/L from strains RJ173 and RJ175 expressing the complete 1,2,4-BTO pathway respectively. However, majority of carbon was not utilized for 1,2,4-BTO production as indicated by the yield (0.08 g/g xylose), as observed for strain RJ175 expressing the complete 1,2,4-BTO pathway.

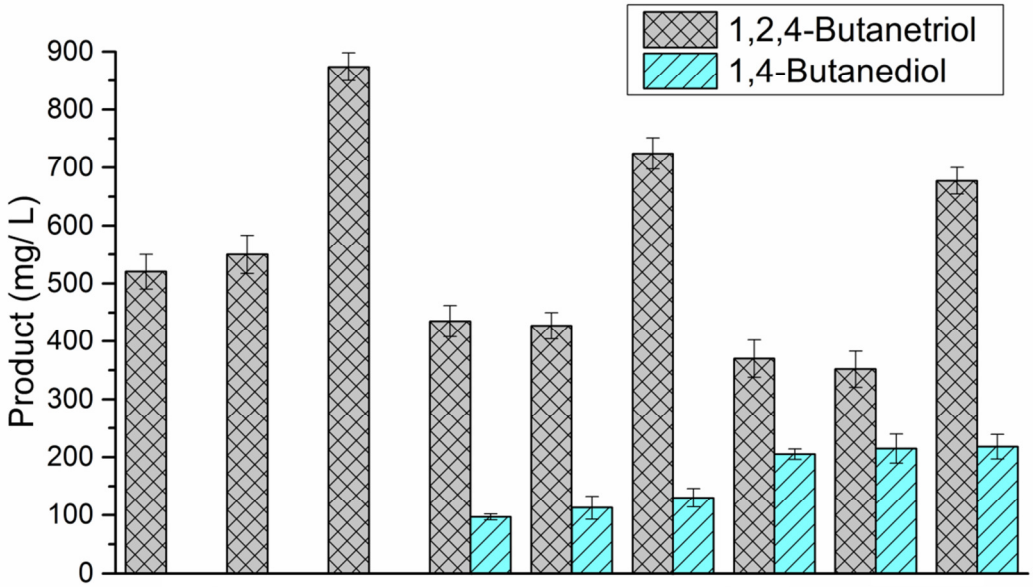
Next, to maximize the utilization of carbon toward 1,2,4-BTO production, we constructed an *E. coli* triple mutant strain with *xylA*, *yjhH* and *yagE* gene disruptions (RJ177). We hypothesized that in doing so, nearly all of the xylose would be utilized for 1,2,4-butanetriol production. We then performed shake flask experiments using M9 xylose medium using strain RJ177 transformed with plasmids expressing the complete 1,2,4-BTO pathway. Contrary to our expectation, the production of 1,2,4-BTO from this strain expressing the complete 1,2,4-BTO pathway was reduced to merely 188 mg/L. It was observed that this strain consumed only 0.33 g/L xylose, as compared to 6.53 g/L from strain RJ175 expressing the 1,2,4-BTO pathway. Additionally, it was observed that the growth of strain RJ177 expressing the 1,2,4-BTO pathway was greatly reduced, reaching an optical density of 0.51 after 48 hours, as compared to 2.65 from strain RJ175 expressing the 1,2,4-BTO pathway. No cell growth was observed in the absence of

yeast extract in the medium. This observation is consistent with previous studies, where the disruption of *xylA*, *yjhH* and *yagE* eliminated growth on xylose alone (Valdehuesaa et al., 2014).

In order to improve the cell growth and 1,2,4-BTO production, we then utilized LB xylose medium and performed shake flask experiments. It was seen that strain RJ177 expressing the complete 1,2,4-BTO pathway further improved 1,2,4-BTO production to 874 mg/L (Supporting Information Figure S4.4), with a yield of 0.66 g/g xylose. This result represents a yield of about 93% theoretical maximum. Additionally, it was observed that the cell growth of this strain was improved, indicated by an increase in optical density from 0.51 to 2.2 after 48 hours, while xylose consumption was improved from 0.33 g/L to 1.32 g/L. Hence, by reducing loss of carbon to native metabolism and using LB xylose medium, the production of 1,2,4-BTO was enhanced.

After achieving 1,2,4-BTO biosynthesis at 874 mg/L, we proceeded to over-express diol dehydratase in addition to 1,2,4-BTO pathway for 1,4-BDO production. To do this, we used the engineered strains and transformed them with pRJ182 and pRJ186 or pRJ188 or pRJ189 (plasmids carrying *xylBC*, *mdlC* along with the native or S301 Q336 mutant or S301 Q336 V300M mutant diol dehydratase respectively). After 48 hours of shake flask studies it was seen that the overexpression of native dehydratase along with the 1,2,4-BTO pathway did not produce any detectable quantities of 1,4-BDO (Figure 4.5). However, the overexpression of S301 Q336 mutant dehydratase along with the 1,2,4-BTO pathway resulted in the *de novo* biosynthesis of 1,4-BDO at 97 mg/L, 113 mg/L and 130 mg/L from strains RJ173, RJ175 and RJ177 respectively. About 455 mg/L, 427 mg/L and 724 mg/L of 1,2,4-BTO was left unconverted from these strains respectively.

We then tested the effect of overexpressing the S301 Q336 V300M mutant diol dehydratase along with the 1,2,4-BTO pathway on 1,4-BDO biosynthesis. As expected, 1,4-BDO production was further improved, achieving 205 mg/L, 215 mg/L and 218 mg/L from strains RJ173, RJ175 and RJ177 respectively. About 370 mg/L, 352 mg/L and 677 mg/L of 1,2,4-BTO was left unconverted at the end of the study from these strains respectively (Figure 4.5). These results supported the observed activity of diol dehydratase and its mutants as inferred from whole cell biocatalysis and *in vitro* assay experiments. Hence, *de novo* biosynthesis of 1,4-BDO was achieved directly from xylose at 218 mg/L.



1,2,4-BTO pathway	✓	✓	✓	✓	✓	✓	✓	✓	✓
ppdA-C-B	✓	✓	✓						
ppdA-C-B Q336A S301A				✓	✓	✓			
ppdA-C-B Q336A S301A V300M							✓	✓	✓
Strain	RJ173	RJ175	RJ177	RJ173	RJ175	RJ177	RJ173	RJ175	RJ177

Figure 4.5. Results of shake flask studies for *de novo* production of 1,4-butanediol from xylose using engineered *E. coli* strains. The data were generated from three independent experiments.

4.3. Conclusions

In this study we demonstrate the prudent utilization of rational engineering approaches to engineer a diol dehydratase and achieve non-native catalysis. The use of *in silico* screening approaches via docking simulations led to the identification of critical amino acid residues, and also the influence of the substitutions without requiring a high-throughput screening platform. We demonstrate that pursuing substrate accommodation leads to enabling catalytic ability toward the non-native substrate, while, further improvement in desired catalysis is accomplished via conception of rational design approaches reliant on the reaction mechanism. This two-fold approach reinforces the utility of rationally conceived hypotheses to enhance non-native catalysis.

These efforts enabled the expansion of the 1,2,4-BTO biosynthesis pathway to 1,4-BDO. First, we enhanced 1,2,4-BTO production from 150 mg/L to 874 mg/L and achieved a yield of 93% theoretical maximum. With this, the expression of the complete pathway enabled 1,4-BDO production at 218 mg/L. It was observed that although 1,2,4-BTO production was enhanced by strain engineering, its conversion to 1,4-BDO was not improved conjointly. Hence, before proceeding to scale-up studies, further efforts to improve 1,4-BDO production may include enhancing dehydratase activity toward 1,2,4-BTO via directed evolution strategies and metabolic engineering to increase xylose uptake. Overall, we report that by rationally redesigning diol dehydratase biological production of 1,4-BDO is enabled, via a novel metabolic route from xylose.

4.4. Methods and Materials

4.4.1. Chemicals and reagents

1,2,4-BTO, 1,4-BDO, 1,3-propanediol standards were procured from Alfa Aesar (Ward Hill, MA); 1,2-PD was procured from Sigma Aldrich (St. Louis, MO); 1-propanol was procured from Fisher Scientific (Atlanta, GA). All enzymes and kits used for DNA manipulation (Restriction enzymes, Phusion high fidelity DNA polymerase and Quick DNA ligase) were procured from New England Biolabs (Beverly, MA).

4.4.2. Bacterial strains and plasmids

Whole cell biocatalysis studies and *de novo* biosynthesis studies were carried out using *E. coli* BW25113 (*E. coli* Genetic Resource Center). Knock-out strains of *E. coli* BW25113 were constructed by disrupting target gene(s) using P1 transduction as described previously (Thomason et al., 2007). Loss of antibiotic resistance was facilitated by transforming pCP20 into target strains (Datsenko and Wanner, 2000). Colony PCR was used to verify the removal of antibiotic resistance using GoTaq DNA polymerase (Promega, WI). *E. coli* BL21 Star (DE3) (Invitrogen, CA) was used for protein expression in enzyme assay experiments. *E. coli* XL1-Blue (Stratagene, CA) served as the cloning host for DNA manipulation experiments. The cloning plasmids used were pZE12-luc (Lutz and Bujard, 1997) and pCS27 (Shen and Liao, 2008c) for whole cell biocatalysis studies and shake flask experiments; pETDuet-1 (EMD Chemicals Inc., NJ) for his-tag cloning and protein purification experiments.

4.4.3. DNA manipulations

Plasmids constructed in this work are listed in Table 4.3. The *K. oxytoca* diol dehydratase reactivase factors (*ddrAB*) were cloned into the backbone of pCS27 using Acc65I, BsiWI and HindIII to create pRJ75. The *K. oxytoca* diol dehydratase (*ppdA-C-B)* reading frame was cloned into the backbone of pZE12-luc to create pRJ79 as described previously (Jain et al., 2014). The general procedure to clone diol dehydratase mutants into pZE12-luc (pRJ95-97, pRJ111-116) is described below. In order to create single amino acid mutations, overlap PCR was performed using appropriate primers and pRJ79 as the template. The resultant PCR products were purified and cloned into the backbone of pZE12-luc using EcoRI and XbaI. This procedure was repeated in order to create more than one amino acid mutations. All mutants were sequenced (Georgia Genomics Facility, University of Georgia) to verify the accuracy of mutations.

In order to purify diol dehydratase protein and its mutants, the native *ppdA-C-B* reading frame and its mutants were cloned into his-tag plasmid (pETDuet-1) using BamHI and XhoI (pRJ84, pRJ91, pRJ170). Similarly, *E. coli yqhD* was cloned into the backbone of pETDuet-1 using BamHI and PstI to create pRJ122. The genes *yjhG*, *yagF* and *adhP* were PCR amplified from *E. coli* and cloned into the backbone of pCS27 using Acc65I, EcoRI, SpeI and BamHI to create pRJ182. The genes *xylB* and *xylC* from *Caulobacter crescentus* were codon optimized for expression in *E. coli* and synthesized by GENEWIZ (South Plainfield, NJ). The codon optimized *xylB*, *xylC* genes and the *mdlC* gene from *Pseudomonas putida* were cloned into the backbone of pZE12-luc simultaneously using Acc65I, BamHI, EcoRI and XbaI to create pRJ184. The genes *xylB*, *xylC*, *mdlC* and native *ppdA-C-B* from *K. oxytoca* were cloned simultaneously into pZE12-luc using Acc65I, BamHI, EcoRI, HindIII and XbaI to create pRJ186. The plasmids pRJ188 and

pRJ189 were constructed similarly as pRJ186, however with the S301A Q336A or S301A Q336A V300M mutants of *ppdA-C-B* respectively.

4.4.4. Culture medium

Modified M9 medium was used for whole biocatalysis, while M9 xylose medium or LB xylose medium was used for *de novo* biosynthesis experiments. The modified M9 medium consisted of (per liter): 40 g glucose, 12.8 g $Na_2HPO_4.7H_2O$, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 0.5 mM $MgSO_4$, 0.05 mM $CaCl_2$ and 5 g yeast extract (Jain et al., 2014). The M9 xylose medium consisted of (per liter): 20 g xylose, 5 g yeast extract, 12.8 g $Na_2HPO_4.7H_2O$, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 0.5 mM $MgSO_4$ and 0.05 mM $CaCl_2$. LB xylose medium consisted of LB medium supplemented with 20 g/L xylose.

4.4.5. Whole cell biocatalysis

Whole cell biocatalysis experiments were performed with modifications to the method described previously (Jain and Yan, 2011). Native or mutant dehydratases were cloned into pZE12-luc and the plasmid was transformed into wild type *E. coli* BW25113. Overnight cultures (3 mL) of transformants were grown in LB (37ºC, 250 rpm) and served as the pre-inoculum. The experiments were carried out in 125 mL screw cap bottles with 10 mL of modified M9 medium. About 100 µL of the pre-inoculum was added to the screw cap bottles along with appropriate antibiotics and grown for 4 hours (37ºC, 250 rpm). Following this, 10 µM coenzyme B12, 0.1 mM IPTG and 1 g/L substrate (1,2-PD/ 1,2,4-BTO/ glycerol) were added to the cultures. The cultures were then grown for another 24 hours (37ºC, 250 rpm). HPLC analysis was done at the end of the study for product analysis by collecting 1 mL samples.

4.4.6. In vitro coupled enzyme assays

In order to determine the enzyme kinetics (K_m, K_{cat}) of diol dehydratase, *ppdA-C-B* and its mutants were first cloned into pETDuet-1 plasmid. Additionally, the *E. coli* gene encoding alcohol dehydrogenase- *yqhD* was also cloned into pETDuet-1 plasmid. The constructed plasmids were then transformed separately into *E. coli* BL21 Star (DE3). Overnight culture of transformants were made in 3 mL LB, grown overnight at 37ºC, 250 rpm. Following this, 500 µL of seed cultures were added to 125 mL shake flasks containing 50 mL LB. The cultures were then grown at 37ºC until the optical density reached 0.6, following which 1 mM IPTG was added and grown for another 5 hours at 30ºC. In order to obtain the purified protein, the cultures were first centrifuged to concentrate the cells. Using a Mini Bead Beater (Biospec), the cells were broken and the mixture was centrifuged again. The resultant supernatant was purified using His-Spin Protein Miniprep kit (Zymo Research, Irvine, CA) and the protein concentration was determined using Pierce BCA protein assay kit (Thermo Scientific, Atlanta, GA).

Diol dehydratase activity was monitored via coupling the dehydration activity with the subsequent alcohol dehydrogenase activity. The reaction mixture consisted of 0.2 mM coenzyme B12, 0.035 M K_2HPO_4 (pH 8.0), 0.025 M KCl, 0.5 mM NADPH, 5 mM $ZnCl_2$, purified diol dehydratase protein, purified alcohol dehydrogenase protein (*yqhD*) in excess. The reaction was begun with the addition of 0 – 20 mM substrate (1,2-PD/ 1,2,4-BTO). The total reaction volume was maintained at 1 mL and the rate of consumption of NADPH was monitored at 340 nm for 1 min at room temperature. Appropriate controls were used (without substrate/ without purified protein(s)/ without NADPH).

4.4.7. De novo biosynthesis

Shake flask experiments were performed using M9 xylose medium or LB xylose medium with modifications to the method described previously (Jain and Yan, 2011). Overnight cultures (3 mL) of transformants were grown in LB (37ºC, 250 rpm) and served as the pre-inoculum. Screw cap bottles (125 mL) consisting of 20 mL M9 xylose medium or LB xylose medium were used for *de novo* biosynthesis experiments. About 200 µL of the pre-inoculum was added to the screw cap bottles along with appropriate antibiotics and grown for 4 hours (37ºC, 250 rpm). Following this, 10 µM coenzyme B12 and 0.1 mM IPTG were added to the cultures. The cultures were then grown for another 48 hours (unless mentioned otherwise) at 37ºC, 250 rpm. HPLC analysis was done at the end of the study by collecting 1 mL samples.

4.4.8. Analytical procedures

Optical density was measured at 600 nm using a Genesys 10S UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). For product quantification and separation, 1 mL samples were collected at the end of whole cell biocatalysis and *de novo* biosynthesis experiments. The samples were centrifuged at 15,000 rpm for 12 min, following which the supernatant was filtered and analyzed (Jain et al., 2014). For quantification of native organic acids (Eiteman and Chastain, 1997), 1,4-BDO, 1,2,4-BTO, 1,2-PD, 1-propanol and 1,3-propanediol HPLC-RID (Shimadzu) with Coregel-64H column (Transgenomic) was used. The eluent used was 4 mN H_2SO_4 and the oven temperature was set at 60ºC for separation of xylose, 1,2-PD, 1-propanol, 1,3-propanediol and glycerol. For the separation of 1,2,4-BTO and 1,4-BDO the oven temperature was set at 40ºC and the eluent used was 16 mN H_2SO_4.

4.4.9. Computational analysis and simulations

The crystal structures used for analysis of *K. oxytoca* diol dehydratase were obtained from Protein Data Bank and included the following: substrate free form (PDBid: 1IWB) (Shibata et al., 2002), bound to 1,2-PD (PDBid: 1UC5) (Shibata et al., 2003) and bound to glycerol (PDBid: 3AUJ) (Yamanishi et al., 2012). Docking studies and protein structure analysis were done using Molecular Operating Environment (MOE) and Pymol. The structures were energy minimized prior to ligand docking simulations. All amino acids in the 5 Å radius of the catalytic residues were identified as potential targets for mutation. Mutations of specific amino acids and combinatorial mutations were constructed *in silico* and stored as separate structure files for further analysis, creating an *in silico* library. First, a docking simulation was carried out with its native substrate- 1,2-PD and critically analyzed in comparison to the known substrate bound complex (PDBid: 1UC5). This ensured that the docking simulation predicted similar ligand interactions as previously determined from crystal structures. Further docking simulations were then carried out using the native protein and the engineered proteins to study the interaction of 1,2,4-BTO with the active site residues. The orientations of substrates were analyzed, their interactions with critical active site residues were studied and distances were calculated.

Supporting Information

This material is provided in Appendix B.

Table 4.3. List of strains and plasmids used in this study.

Strain	**Genotype**	**Reference**
E. coli BW25113	*F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ⁻, rph-1, Δ(rhaD-rhaB)568, hsdR514*	Yale CGSC
E. coli XL-1 Blue	*recA1 endA1gyrA96thi-1hsdR17supE44relA1lac [F' proAB lacIqZDM15Tn10 (TetR)]*	Stratagene
E. coli BL21 Star (DE3)	*F⁻ompT hsdS$_B$ ($r_B^-m_B^-$) gal dcm (DE3)*	Invitrogen
JW3537-1	BW25113 Δ*xylA::kan*	Yale CGSC
JW5775-2	BW25113 Δ*yjhH::kan*	Yale CGSC
JW0261-1	BW25113 Δ*yagE::kan*	Yale CGSC
RJ171	BW25113 Δ*xylA*	This study
RJ173	BW25113 Δ*xylA* Δ*yjhH*	This study
RJ175	BW25113 Δ*xylA* Δ*yagE*	This study
RJ177	BW25113 Δ*xylA* Δ*yagE* Δ*yjhH*	This study
Plasmid	**Description**	**Reference**
pZE12- luc	pLlacO1; luc; *ColE1 ori*; *Amp^R*	(Lutz and Bujard, 1997)
pCS27	pLlacO1; *p15A ori; Kan^R*	(Shen and Liao, 2008c)

pETDuet-1	two T7 promoters; two MCS; *pBR322 ori*; Amp^R	Novagen
pRJ79	Fusion *ppdA-C-B* cloned into pZE12-luc	(Jain et al., 2014)
pRJ75	*ddrAB* from *K. oxytoca* cloned into pCS27	This study
pRJ95- 97, pRJ111- 116	Fusion *ppdA-C-B* mutants cloned into pZE12-luc	This study
pRJ84	Fusion *ppdA-C-B* cloned into pETDuet-1	This study
pRJ91	Fusion *ppdA-C-B* S301A Q336A cloned into pETDuet-1	This study
pRJ170	Fusion *ppdA-C-B* S301A Q336A V300M cloned into pETDuet-1	This study
pRJ122	*yqhD* from *E. coli* cloned into pETDuet-1	This study
pRJ182	*yjhG*, *yagF* and *adhP* from *E. coli* cloned into pCS27	This study
pRJ184	*xylB, xylC* from *C. crescentus* and *mdlC* from *P. putida* cloned into pZE12-luc	This study
pRJ186	*xylB, xylC* from *C. crescentus*, *mdlC* from *P. putida* and *ppdA-C-B* from *K. oxytoca* cloned into pZE12-luc	This study
pRJ188	*xylB, xylC* from *C. crescentus*, *mdlC* from *P.*	This study

	putida and *ppdA-C-B* S301A Q336A from *K.* *oxytoca* cloned into pZE12-luc	
pRJ189	*xylB, xylC* from *C. crescentus*, *mdlC* from *P.* *putida* and *ppdA-C-B* S301A Q336A V300M from *K. oxytoca* cloned into pZE12-luc	This study

CHAPTER 5

CONCLUSION

This dissertation reports the production of C3/ C4 high value commodity chemicals via establishment of novel metabolic pathways or enhancement of existing ones. First, the production of 1,2-propanediol is pursued with the goal of overcoming the challenges posed in improving its production and yield from glucose. We successfully enhance both titer and yield of 1,2-propanediol, and establish the basis of future scale-up studies. Second, we establish a novel metabolic route for the production of 1-propanol from glucose. In the third chapter we achieve near complete conversion of 1,2-propanediol to 1-propanol. Third, we engineer a diol dehydratase to achieve catalysis toward a non-native larger substrate. Based on this, we establish a novel metabolic route for the production of 1,4-butanediol from xylose.

The work in this dissertation demonstrates the hand in hand role of strain engineering via gene disruption as well as manipulating protein expression toward production of target chemicals. From an industrial perspective, processes with high titer, high yield and high productivity would enable a viable platform for manufacture of chemicals. Inferred from this, it is important to realize that simply the establishment of novel metabolic routes for production of chemicals is insufficient for large scale manufacture. It is desirable to first establish an efficient system at a shake flask level before proceeding to scale-up studies. To this end, we have established an efficient process for 1,2-propanediol production. We believe that the next steps for scale-up of 1,2-propanediol production system will be to optimize the fermentation parameters and medium.

By further reducing expensive medium components the cost of manufacture can be reduced greatly.

An important factor to consider in biological processes is the cost of downstream processing. It is desirable to have a biological process with the least amount of byproducts and biomass. Strain engineering approaches to eliminate byproduct pathways are generally utilized for this. However, it is evident from our findings (as in the case of 1,2-propanediol), that too many gene disruptions or disruption of major metabolic nodes may hamper cell growth to the extent of reducing the titer of target products. In such a case, while byproducts and biomass is reduced, the overall process is inefficient due to lower titers of the target product. Hence, it is important to realize that for each process there is an optimal amount of biomass, byproduct and target product generation. It is vital to pursue this balance during scale-up studies to meet the criteria of a commercially viable system.

Overall, we demonstrate the utility of metabolic engineering and protein engineering for the production of commodity chemicals. We utilize various metabolic engineering strategies to maximize the production of target chemicals keeping in mind that only efficient processes make large scale manufacture viable. We also demonstrate the application of rational protein engineering strategies to achieve and enhance non-native catalysis, thereby overcoming a bottleneck in the expansion of native metabolism for the production of non-native chemicals. With these efforts, we believe scale-up studies for the production of 1,2-propanediol, 1-propanol and 1,4-butanediol can be pursued in the near future using our findings.

APPENDICES

APPENDIX A

SYSTEMATICALLY ENGINEERING *ESCHERICHIA COLI* FOR EFFICIENT PRODUCTION OF 1,2-PROPANEDIOL AND 1-PROPANOL

CHAPTER 3 SUPPORTING INFORMATION

Table S3.1. Metabolic engineering efforts for biological production of 1,2-propanediol till date.

Scale	Organism	Carbon source	1,2-Propanediol titer (g/L)	Yield (g/g)	Reference
Shake flask 150 mL	*E. coli*	Glucose	0.7	N/A	(Altaras and Cameron, 1999a)
Shake flask 150 mL	*E. coli*	Glucose	1.4	0.20	(Altaras and Cameron, 2000b)
Fermenter 2000 mL	*E. coli*	Glucose	4.5	0.19	(Altaras and Cameron, 2000b)
Shake flask 35 mL	*E. coli*	Glucose	1.3	0.12	(Berrios-Rivera et al., 2003a)
Shake flask 20 mL	*S. cerevisiae*	Glucose Ethanol	1.1	N/A	(Joon-Young et al., 2008)
Shake flask N/A	*S. cerevisiae*	Glycerol Galactose	2.1	0.21	(Joon-Young et al., 2011)
Fermenter 400 mL	*E. coli*	Glycerol	5.6	N/A	(Clomburg and Gonzalez, 2011a)
Shake flask 20 mL	*E. coli*	Glucose	0.8	N/A	(Jain and Yan, 2011b)
Shake flask 50 mL	*Cyanobacteria*	CO_2	0.1	N/A	(Li and Liao, 2013)
Shake flask 10 mL	*E. coli*	Lactic acid	1.7	N/A	(Niu and Guo, 2014)
Shake flask 20 mL	*E. coli*	Glucose	5.1	0.48	**This work**

Table S3.2. 1,2-Propanediol and metabolites produced from different strains after 48 h of 20 mL anaerobic shake flask studies. 1,2-PD: 1,2-propanediol; OD_{600}: optical density measured at 600 nm. The data were generated from three independent experiments.

Strain/ Plasmid	**Pyruvate (g/L)**	**Succinate (g/L)**	**Lactate (g/L)**	**Formate (g/L)**	**Acetate (g/L)**	**Ethanol (g/L)**	**1,2-PD (g/L)**	**OD_{600}**
BW25113/ pRJ70	0.00	0.26 ± 0.05	6.21 ± 0.31	0.89 ± 0.23	2.03 ± 0.09	0.53 ± 0.03	1.52 ± 0.11	1.89 ± 0.06
RJ31/ pRJ70	7.18 ± 2.35	1.41 ± 0.41	0.54 ± 0.25	2.45 ± 0.11	0.99 ± 0.22	2.77 ± 0.01	0.90 ± 0.04	2.41 ± 0.13
RJ57/ pRJ70	0.00	0.22 ± 0.00	0.14 ± 0.11	0.33 ± 0.06	0.65 ± 0.04	0.05 ± 0.06	0.35 ± 0.00	0.38 ± 0.03
RJ57/ pRJ70+ pRJ58	0.00	0.25 ± 0.00	0.12 ± 0.08	0.00	0.67 ± 0.06	0.12 ± 0.04	0.59 ± 0.02	0.41 ± 0.04
BW25113/ pRJ70+ pRJ58	0.00	0.49 ± 0.02	8.22 ± 0.39	0.00	1.96 ± 0.19	0.77 ± 0.12	2.14 ± 0.07	1.07 ± 0.08

Codon optimized *Candida boidinii fdh1* gene sequence

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Fusion *ppdA-C-B* reading frame sequence

Linker sequences are represented in capital.

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Fusion *ppdC-B-A* reading frame sequence

Linker sequences are represented in capital.

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APPENDIX B

RATIONALLY REDESIGNED DIOL DEHYDRATASE ENABLES BIOSYNTHESIS OF 1,4-BUTANEDIOL FROM XYLOSE

CHAPTER 4 SUPPORTING INFORMATION

To be submitted to Proceedings of the National Academy of Sciences of the United States of America.
Rachit Jain, Xinxiao Sun, Mengyin Cheng, Qipeng Yuan, James C. Liao and Yajun Yan: Rational Engineering of Diol Dehydratase Enables 1,4-Butanediol Biosynthesis From Xylose.

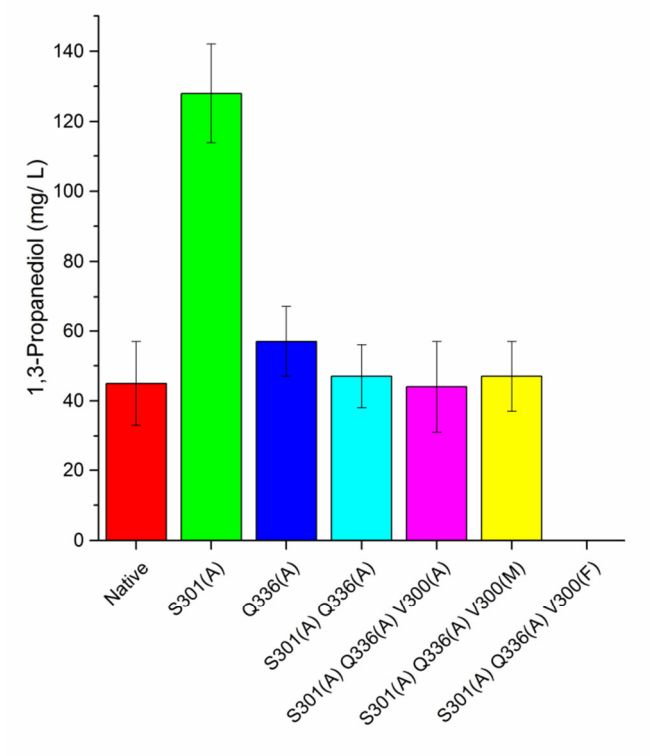

Figure S4.1. 1,3-Propanediol produced from whole cell biocatalysis experiments by feeding glycerol. The data were generated from three independent experiments.

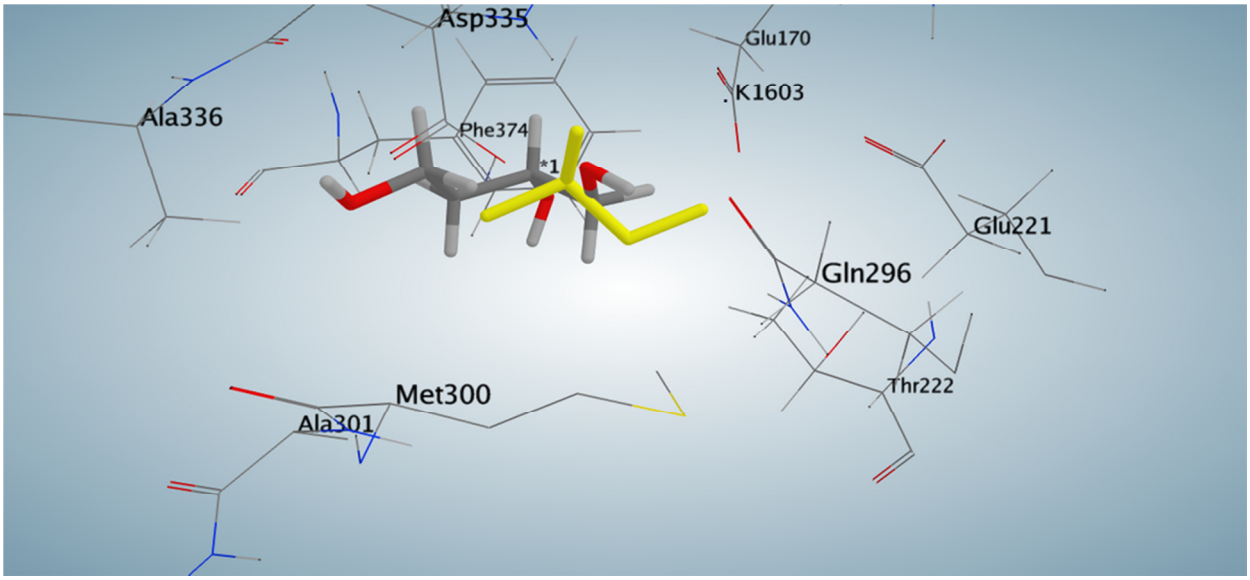

Figure S4.2. Representation of 1,2,4-butanetriol's interaction in the catalytic pocket of Q336A S301A V300M mutant diol dehydratase superimposed with 1,2-propanediol (highlighted in yellow) obtained from crystal structure (PDBid:1UC5).

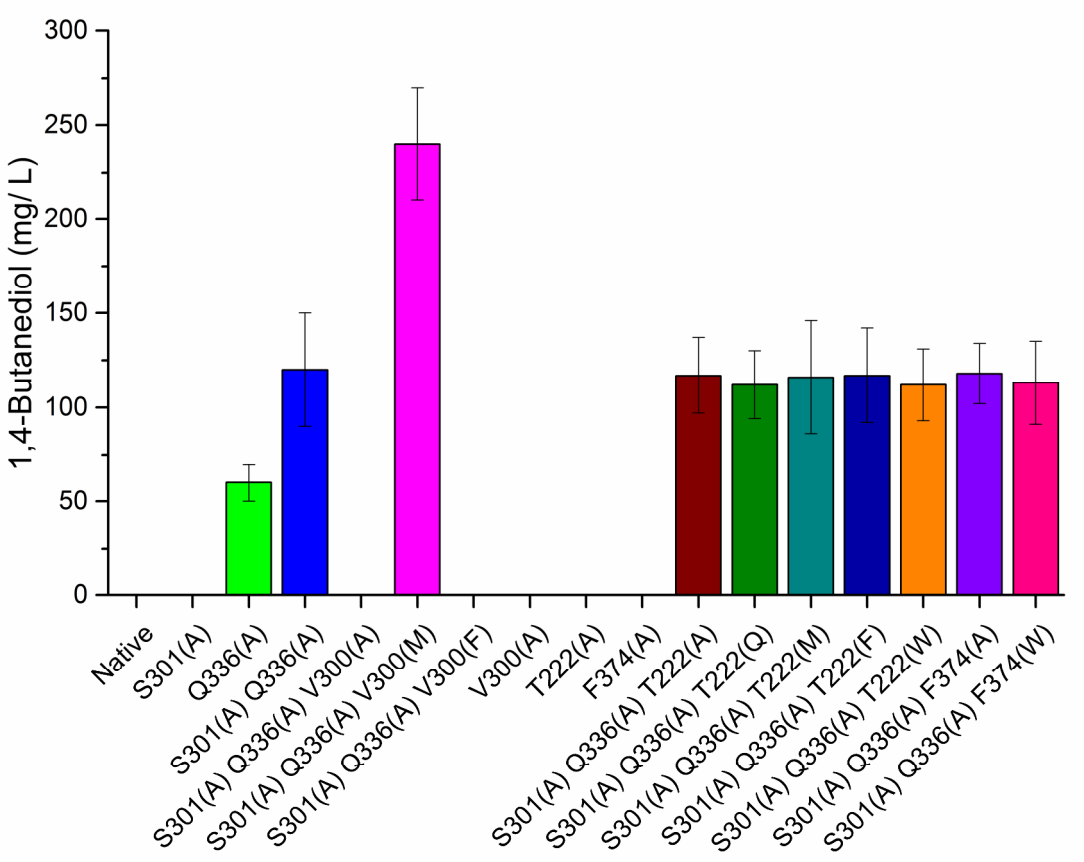

Figure S4.3. Results of 1,4-butanediol production from whole cell biocatalysis experiments by feeding 1,2,4-butanetriol in *E. coli* cultures expressing native and mutant dehydratases.

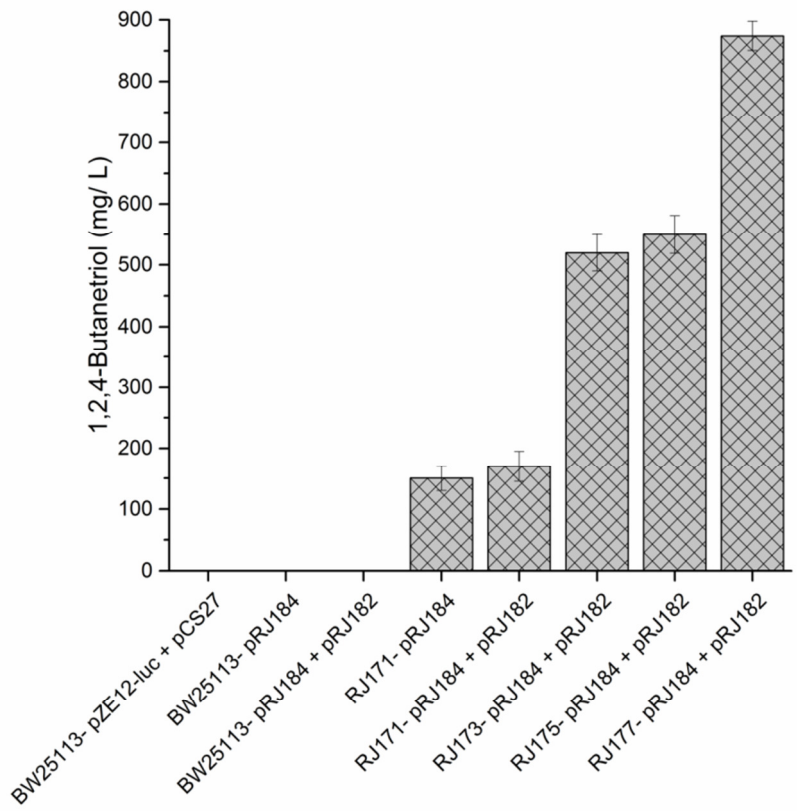

Figure S4.4. Results of shake flask studies for *de novo* production of 1,2,4-butanetriol from xylose using engineered *E. coli* strains.

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