UNIVERSITY OF CALIFORNIA

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Production of Bio-Based Chemicals *In vivo* and *In vitro*

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biochemistry and Molecular Biology

By:

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ABSTRACT OF THE DISSERTATION

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University of California, Los Angeles, 2015

Professor James U. Bowie, Chair

Over the last century the petro chemical industry has provided an abundant and cheap source of hydrocarbons that have impacted and transformed many facets of our lives. Petrochemicals not only provide fuel that revolutionized transportation industry but also provided cheap petrochemical feedstock molecules that form a basis of many textiles, plastics, adhesives, detergents, and lubricants that are indispensable in modern life. Our dependence on the petrochemical industry and the sheer quantity of petrochemical based products that are consumed have led to a number of adverse and unintended effects on our environment. Recent attention to unintended effects of the petrochemical industry such as climate change, air pollution, and landfill overflow have sparked renewed interest in green chemistry and research in renewables that could potentially decrease our dependence on the petrochemical industry.
Over the last two decades, with the advent of simple and cheap genetic engineering, a new field called metabolic engineering has emerged. A large focus of this field is centered around re-engineering the metabolism of simple organisms to produce bulk and specialty chemicals. We genetically engineered a lithoautotrophic organism, *Ralstonia eutropha*, to produce drop-in ready biofuels (isobutanol and 3-methyl-1-butanol) in an electro-bio reactor using CO₂ as the sole carbon source and electricity as the sole energy input. This method integrates electrochemical formate production and biological CO₂ fixation and higher alcohol synthesis. The liquid fuels generated are a relatively stable way to store energy and possess energy densities about 100 times higher than current-day batteries.

A different approach to metabolic engineering coined synthetic biochemistry seeks to reconstitute complex metabolic pathways outside of cells. The Synthetic Biochemistry approach eliminates the myriad unwanted side reactions that occur in cells and product toxicity, enabling near 100% theoretical yields. Unfortunately, eliminating the cellular environment also eliminates key regulatory networks involved in maintaining appropriate carbon flux and cofactor balance. Synthetic biochemistry seeks to replace these complex regulatory networks in vitro by creating self-balancing pathways that automatically regulate the production and consumption of cofactors while maintaining constant carbon flux through the pathway. To this end, we developed the concept of a molecular purge valve. Generally, the purge valve is made up of a NADPH dependent oxidase, a NADH dependent oxidase and a NADH specific oxidase (*noxE*). We first demonstrated this proof of concept by engineering the *G. stearothermophilus* pyruvate dehydrogenase complex to flip its cofactor specificity from NADH to NADPH and using it in the purge valve to transform pyruvate to either polyhydroxybutyrate bioplastic or isoprene at near quantitative yields. The concept of using a purge valve to maintain carbon flux in an inherently imbalanced pathway was expanded through the production of polyhydroxybutyrate from glucose using a synthetic pathway called the PBG cycle. The PBG cycle is made up of enzymes from the pentose phosphate pathway, the bifido shunt, and standard glycolysis (EMP). This pathway utilizes the
purge valve concept at the two reductase enzymes of the pentose phosphate pathway and produced the bioplastic polyhydroxybutyrate from glucose. The PBG pathway ran semi-continuously for 55 hours and produced 86% yield from 110 mM glucose. The pathway had a maximum productivity of 0.7g/L PHB and maintained 50% of its productivity over the course of the 55 hours at room temperature. The PBG cycle serves as a demonstration that synthetic biochemistry could be an alternative to in vivo metabolic engineering for the production of bulk and specialty chemicals and could potentially be one way to replace some of the petrochemicals we use today with renewable bio-based chemicals.
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Chapter 2 describes the results of an electrofuels project that was undertaken by a small group in the Liao lab. I would like to thank Han Li for her efforts on this project, her help, her discussions, her support, and the understanding that she showed me. I would also like to thank former and current members of the lab for their unwavering support and friendship including Samuel Mainguet, Fabienne Douchoud, David Wernick, Luisa Gronenberg, and Kwang Myung Cho.

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Li, H; Opgenorth, P; et. al., Integrated Electromicrobial Conversion of CO₂ to Higher Alcohols. Science, March 2012, Vol. 335 no. 6067 p. 1569
Chapter 1:

Introduction to consolidated bioprocessing for bio-based chemical production
Introduction

The petrochemical industry has powered and sustained much of the economic growth in the 20th century but our heavy reliance on petroleum based products has led to a number of adverse and unintended effects. The burning of fossil fuels has been linked to air pollution and climate change, the complicated drilling, extraction, and processing has led to the contamination of waters and oceans, and the usage of non-degradable plastics have filled our landfills. The effects of the petrochemical industry will only become more acute as the demand for commodity chemicals increases with the growing global population and growing middle class. The cumulative effect on petrochemical industry has resurrected an interest in renewable and environmentally friendly bio-based substitutes of commodity chemicals in order to meet future demand.

The first and probably most successful example of industrial production of bio-based chemicals is the production of ethanol from simple sugars. Traditionally, yeast has been used to ferment hexose sugars to produce ethanol on relatively small scales. Modern production of ethanol for use as a biofuel takes place in an almost identical process. The natural anaerobic fermentation of simple sugars to produce ethanol is accomplished through a well-known glycolytic pathway called the Embden–Meyerhof–Parnas (EMP) pathway. In this fashion sugar is relatively efficiently converted into ethanol with a 66% theoretical yield and titers around of 100 g/L.

In order for bio-based chemicals to compete and potentially replace petrochemicals they need to be produced from cheap and abundant feedstock at relatively high yield. The most abundant raw material for bio-based chemical production is sugars. Currently, refined sugars like glucose sell on the open market for around $0.50/Kg and at these prices it still requires relatively high yields depending on the specific product to produce economically viable chemicals. Additionally, the utilization of large amounts of refined sugars from corn or sugar cane that would be required to produce commodity chemicals on
an industrially relevant scale could potentially lead to an increase in food prices. The use of unrefined sugars and waste biomass such as starch, cellulose, hemicellulose, and lignocellulose would bypass many of the pitfalls of using refined sugars but is mainly limited by the challenge of cost-effectively releasing these sugars from recalcitrant biomass\textsuperscript{11}. As a comparison, coal is currently the cheapest energy source on the planet but when the additional cost of carbon capture and sequestration lignocellulosic biomass takes its place as the most economical source of energy\textsuperscript{12}. An alternative to using sugar as a substrate would be to consolidate the carbon capture and product production in a single organism or process. By engineering a lithoautotrophic organism such \textit{Ralstonia eutropha} to directly produce your product of interest from CO\textsubscript{2} you can avoid using sugar substrates altogether.

A complete solution to producing renewable bio-based chemicals at a price point that can compete with petrochemicals requires the consideration of land use, water distribution, agricultural practice, infrastructure compatibility, technological process development, and overall environmental impact. The rest of this chapter will focus on the details associated with two completely different types of biotransformation processes, the \textit{in vivo} production of biofuels and the \textit{in vitro} production of commodity chemicals.

\textbf{In vivo production of biofuels}

\textbf{Second generation Biofuels}

First generation biofuels such as ethanol have demonstrated the feasibility of biofuels on an industrial scale but its chemical properties do not compare favorably to gasoline, which has limited its use to a fuel additive and not a fuel substitute. Ethanol has a relatively low vapor pressure, it is hygroscopic (which will eventually lead to corrosion of engine parts and pipelines with the existing infrastructure), and has a lower energy density than gasoline\textsuperscript{13}. Because of the different properties of fuel replacements each fuel application has a specific requirements and standards that are outlined and must be met. Currently,
there are three ethanol/gasoline blend levels for use in internal combustion engines. E10 (10% ethanol and 90% gasoline) and E15 (10-15% ethanol and 85-90% gasoline) are substantially similar to gasoline and can be used interchangeably in automobiles manufactured in 2001 or newer\textsuperscript{13}. E85, also known as flex-fuel, is a higher blend of ethanol and gasoline containing between 51-85% ethanol but is limited by the geography/climate that it can be sold in and also requires flex-fuel vehicles that are specifically designed to run on higher blends of ethanol\textsuperscript{13}. These regulations have made first generation biofuels like bioethanol an initial success but inherently limit its widespread use as a direct substitute for gasoline. Second generation biofuels such as butanol and higher alcohols have longer carbon chains and possess fuel properties that are much more similar to gasoline. In fact, both n-butanol and isobutanol have a similar vapor pressure, energy density, octane number, and hygroscopicity that it is compatible with the current infrastructure and is potentially a direct replacement for petroleum derived gasoline. A comparison of first and second generation biofuels with gasoline is shown in Table 1-1.

**Pathways for the production of second generation biofuels**

N-butanol and isobutanol have been known to be produced as a natural product from numerous strains of clostridium. In the past, high titers (around 25 g/L)\textsuperscript{14} of these compounds have been naturally produced through fermentation of *clostridium beijerinckii* BA101. Through genetic and transcriptomic studies it was determined that these compounds were produced by a dedicated anabolic pathway that used acetyl-CoA as its building block metabolite. Although high titers of butanol have been produced in the native organism it is not an ideal production platform for the large scale production due to its slow growth, high risk of contamination, and strict anaerobic fermentation requirements. Recent studies have engineered this pathway into more amenable organisms like *E. coli* and yeast. The acetyl-CoA dependent pathway homologously expressed in these organisms for the production of n-butanol produced 0.55 mg/L and 2.5 mg/L, respectively. Additionally, the *E. coli* strain has been further
engineered by knocking out competing pathways and swapping out a ferredoxin dependent enzyme with NADH dependent enzyme which dramatically improved overall titers to almost 30g/L\textsuperscript{15}.

An alternative, non-native pathway is also known to produce higher alcohols is the keto acid pathway. The Keto acid pathway follows a similar metabolic logic to ethanol biosynthesis which does not require acetyl-CoA as a metabolite and has been shown to produce high titers without extensive metabolic engineering. The CoA-independent pathway is a natural extension of the alcohol biosynthesis where pyruvate (a α-keto acid) is decarboxylated and reduced to produce ethanol by the combined reactions of pyruvate decarboxylase and alcohol dehydrogenase. In the CoA-independent pathway higher alcohols such as isobutanol are produced via the same decarboxylation and reduction reaction scheme except that it acts on a longer chain keto acid, 3-methyl-2-oxo-butanoate\textsuperscript{16}.

The longer chain α-keto acids are a natural metabolite from branched chain amino acid biosynthesis. The first step of this pathway involves the condensation of two pyruvate molecules to form acetolactate and CO\textsubscript{2} which is performed by the enzyme acetolactate synthase. Acetolactate (a β-keto acid) is then isomerized and reduced to 2,3-dihydroxy-methyl-butanoate by the enzyme IlvC and subsequently dehydrated to the α-keto acid 3-methyl-2-oxo-butanoate by the enzyme IlvD. The 5-carbon α–keto acid is then decarboxylated into an aldehyde by a keto acid decarboxylase, such as KIVD from \textit{lactococcus lactis}. This enzyme is a close homologue of the pyruvate decarboxylase enzyme used in ethanol biosynthesis except that it has a larger pocket in its active site which can accommodate larger substrates like 3-methyl-2-oxo-butanoate. The last step in this pathway converts isobutanal to isobutanol by a reduction of the aldehyde by alcohol dehydrogenase. Many enzymes exist with the substrate promiscuity to perform this reduction on higher aldehyde substrates, but two of the most commonly used alcohol dehydrogenases are ADH2 from yeast and Yqhd from \textit{E. coli}. The α–keto acid pathway for the production of higher alcohols is very efficient and has produced more than 20 g/L of
isobutanol when genetically engineered into a host *E. coli* cell\(^\text{17}\). This extremely high yield actually exceeds the toxicity limit of isobutanol on *E. coli* and while the growth profile of this organism is inhibited the cell still continues to produce isobutanol. A diagram of this pathway and the potential higher alcohols that can be produced is shown in figure 1-2. In chapter 2, I discuss how a lithoautotrophic organism was used as a biocatalyst to transform electrical energy into liquid biofuel through introducing the isobutanol pathway in *Ralstonia eutropha*.

*In vitro production of chemicals*

An alternative approach to the production of bio-based chemicals *in vivo* is to divorce the desired biochemical transformation from the organism by constructing biochemical pathways *in vitro*. By assembling dedicated pathways *in vitro* and getting rid of any unwanted side reactions it is possible to perform complex transformations at nearly 100% of the theoretical yield. In addition, removing the complexities associated with cellular life should greatly increase the reaction speed, decrease or eliminate product toxicity, and simplify product purification\(^\text{18}\). Over the last century *in vitro* pathways have been limited to diagnostics or enzymatic pathway elucidation and have only recently been seen as a potential means for bulk chemical production. One of the main drawbacks to *in vitro* chemical production is the loss of the complex regulatory network that controls cofactor usage and carbon flow inside the cell. A new approach coined “synthetic biochemistry” seeks to replace the complex regulatory network present inside cells with simple, dedicated, auto-regulatory pathways that manage cofactor production and consumption while maintaining constant carbon flux in a cell free environment.

**Cofactor Balance**
In general, catabolic pathways like glycolysis break down molecules into simple 2 and 3 carbon building block compounds and high energy cofactors that can be fed into anabolic pathways to build more complex molecules required for life\cite{19}. Most catabolic and anabolic enzymatic pathways are not stoichiometrically balanced in terms of the cofactors they use and carbon flux. Cells get around this with a complex regulatory network that shunts the carbon flux through multiple catabolic and anabolic pathways that are functioning simultaneously\cite{20}. While this is good for \textit{in vivo} metabolic engineering because most non-natural pathways are accommodated by the cell, it also serves to decrease the yield of the desired product. Assembling pathways \textit{in vitro} that are composed of both catabolic and anabolic reactions requires the engineer to figure out how to regulate carbon flow and balance cofactor production and consumption without the aid of the cell.

One of the first instances where \textit{in vitro} metabolism was used to make chemicals was the production of ethanol from glucose using whole cell extracts\cite{21,22}. This pathway is inherently unbalanced; the breakdown of glucose by glycolysis makes more ATP than what is used to build ethanol from pyruvate. Because of this, the only way to produce a significant amount of ethanol was by adding a specific amount of ATPase to hydrolyze the excess ATP that builds up. The ATPase activity could be mimicked by adding arsenate, which uncoupled ATP synthesis from glycolysis at the GAPDH enzyme. The GAPDH reaction creates 1-arseno-3-phosphoglycerate instead of the typical 1,3-bisphosphoglycerate, which has a half-life of no more than a few milliseconds and effectively balances the ATP production in glycolysis. While this pathway was efficient at converting glucose to ethanol, titrating the ATPase activity for carbon flux would be difficult to perform in a sustained reaction and the use of an extremely toxic compound like arsenate is not ideal. More modern attempts at \textit{in vitro} production of chemicals typically rely on sacrificial substrates to regenerate cofactors and drive the reaction forward or rely on perfect stoichiometry between the catabolic and anabolic phases of the pathway\cite{23-25}. In chapter 3, I discuss an entirely new concept of maintaining NAD(P)H balance in an inherently unbalance pathways
called a molecular purge valve. In this initial proof of concept we produce polyhydroxybutyrate bioplastic or isoprene from pyruvate in an inherently unbalanced pathway with high yield and high productivity. Chapter 4 is an extension of this concept and utilized the molecular purge valve in an entirely synthetic pathway for the conversion of glucose to polyhydroxybutyrate. In this example, the in \textit{vitro} pathway produces 9.8 g/L of polyhydroxybutyrate with a maximum productivity of 0.7 g/L/h and 85.6 % yield. Based on experience from the ethanol industry, these values are already close to generally accepted thresholds needed for industrial production\textsuperscript{8} (90 % yields with a productivity of 1 g/L/h and titers of 40 g/L), yet we made little effort to maximize production. Taken together, these results suggest that the in \textit{vitro} production of chemicals may be a viable alternative to \textit{in vivo} metabolic engineering of commodity chemicals and even certain segments of the petrochemical industry.
<table>
<thead>
<tr>
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<th>Ethanol</th>
<th>Isobutanol</th>
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<td>29</td>
<td>29</td>
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<tr>
<td>Hydrosopicity</td>
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</tr>
<tr>
<td>Compatibility with current infrastructure</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 1-1. list of the fuel properties associated with ethanol (1\textsuperscript{st} generation biofuel), isobutanol (2\textsuperscript{nd} generation biofuel), and gasoline.
Figure 1-1. (A) The general α-keto acid (CoA independent) pathway for the production of higher alcohols (figure adapted from Atsumi, 2008). This shows the diversity of Alcohols that can be made from this pathway given the corresponding Keto acid precursor. (B) The CoA independent pathway for the production of isobutanol. The first three enzymatic steps make the precursor 2-ketoisovalerate and are part of the branched chain amino acid pathway. The last two enzymatic steps are the general decarboxylase and dehydrogenase steps to turn the α-keto acid precursor 2-ketoisovalerate into isobutanol.
References


Chapter 2:

Electrical conversion of CO$_2$ to higher alcohols in an integrated electro-microbial process
Abstract:

One of the major challenges in utilizing intermittent electrical energy is its storage. Current methods of storage, such as chemical batteries, hydraulic pumping, and water splitting, suffer from low energy density or incompatibility with current transportation applications. Here we report a method to store electric energy as chemical energy in higher alcohols, which can be used as liquid transportation fuels. We genetically engineered a lithoautotrophic organism, *Ralstonia eutropha*, to produce isobutanol and 3-methyl-1-butanol in an electro-bio reactor using CO$_2$ as the sole carbon source and electricity as the sole energy input. The method integrates electrochemical formate production and biological CO$_2$ fixation and higher alcohol synthesis. The liquid fuels generated possess energy densities about 100 times higher than current-day batteries.
Introduction:

Photovoltaic cells harvest energy from sunlight and generate electricity with relatively high energy efficiencies, typically ranging from 10 to 20\%\textsuperscript{1,2}. However, due to the diffuse and intermittent nature of solar energy, the electricity produced by these means needs to be efficiently stored. The current methods of electricity storage via batteries suffer from low energy density, which generally ranges between 0.1-0.7MJ/kg (or 0.5-2.0 MJ/L)\textsuperscript{3,4}. Alternatively, electrolytic water splitting stores electrical energy in chemical bonds in \( \text{H}_2 \) molecules with high efficiencies\textsuperscript{2}. However, \( \text{H}_2 \) utilization in the transportation sector faces many engineering challenges.

The solar electricity-powered water splitting in effect achieves the “light reaction” of biological photosynthesis in that they both convert solar energy to chemical reducing energy, in the form of either \( \text{H}_2 \) or biological reducing equivalent, \( \text{NADPH} \)\textsuperscript{5,6}. Some lithoautotrophic microorganism can utilize \( \text{H}_2 \) to generate \( \text{NADH} \) and \( \text{ATP} \) and to power \( \text{CO}_2 \) fixation in the Calvin-Benson-Bassham (CBB) cycle, the same series of reactions in the “dark reaction” of photosynthesis. Using the product of CBB cycle as a precursor, carbon chains with various lengths, conformations, and functionalities can be synthesized\textsuperscript{7,8}. Therefore, a hybrid process comprised of the man-made “light reaction” and the biological “dark reaction” to store electricity in the C-C bonds of liquid fuels could be possible. However, the low solubility and mass transfer rate of \( \text{H}_2 \) in microbial culture limits the efficiency and scalability of such processes. In addition, the explosive gas mixture (\( \text{H}_2:\text{CO}_2:O_2=8:1:1 \)) required for microbial culture presents a major safety issue.

Compared to \( \text{H}_2 \), formic acid would be a favorable energy carrier at the interface between electrolysis and microbial cells. Electrochemical production of formic acid from \( \text{CO}_2 \) and \( \text{H}_2\text{O} \) has been extensively studied and can achieve relatively high current efficiencies\textsuperscript{9,10}. Formate is highly soluble and is readily
converted to both carbon dioxide and NADH in a stoichiometric ratio by formate dehydrogenase in the cells, circumventing the poor mass transfer issue of both CO$_2$ and H$_2$ as gas substrates. However, the high solubility of formic acid increases the cost of product separation from electrochemical process. If not separated effectively, accumulated formate can be decomposed at the anode, reducing the yield of the process(9). As such, an integrated process featuring simultaneous electrochemical formate production and biological formate utilization is desirable, since the costly product separation could be circumvented and no formate accumulation would occur. When producing compounds more reduced than formate, such as higher alcohols, more reducing power than CO$_2$ is required. Thus, excess CO$_2$ will be released by the microbes, which provide dissolved CO$_2$ in the vicinity of the working electrode to be reduced electrochemically. However, the adverse effect of the electrochemical process on microbial cells needs to be addressed$^{11}$.

As such, an integrated process for production of liquid fuel from electricity requires 1) metabolic engineering of a lithoautotrophic organism to produce liquid fuels, 2) electrochemical production of formate from CO$_2$, and 3) eliminating the adverse effect of electrolysis on microbial cells. In this work, we chose lithoautotrophic microorganism _Ralstonia eutropha_ as the production organism, which can fix CO$_2$ in the dark using H$_2$ or formate as the energy source, and branched-chain alcohols, isobutanol and 3-methyl-1-butanol (3MB), as the target products. Isobutanol and 3MB have energy densities of 36.1 and 37.7MJ/kg (or 29.0 and 30.5MJ/L), respectively, which are two orders of magnitude higher than that of batteries. During lithotrophic growth, molecular H$_2$ is oxidized by a membrane-bound hydrogenase (MBH) and a soluble hydrogenase (SH), and formate is metabolized by a soluble formate dehydrogenase (FDH) to provide _R. eutropha_ with the reducing power$^{12-14}$, which then drives the CBB cycle and other metabolic pathways (Figure 1A).
One special metabolic feature of *R. entropha* is that it is one of the best-known natural polyhydroxyalkanoate (PHA) hyper-producers. PHA such as poly[R-(−)-3-hydroxybutyrate] (PHB) is produced as a storage compound and also as the metabolic sink for carbon and reducing equivalents\textsuperscript{15–17}. When PHB synthesis is disrupted, large amounts of pyruvate (the upstream substrate of PHB biosynthetic pathway) is secreted out of the cells\textsuperscript{18}, suggesting that the overall metabolic network is well-suited for pushing carbon and reducing power through this pathway at the pyruvate node. Thus, the keto acid pathways for isobutanol and 3MB production\textsuperscript{19} are well-positioned to channel both pyruvate and NADPH into biofuel production as the new metabolic sink.
Results:

We first used a multiple-copy plasmid to overexpress the keto acid decarboxylase (KDC) kivd along with one of the three different alcohol dehydrogenases (ADH): adhA from Lactococcus lactis, adh2 from Saccharomyces cerevisiae, and yqhD from Escherichia coli\textsuperscript{19,20}. Among these ADH’s, YqhD is NADPH-dependent, while the others are NADH-dependent. The Ralstonia strain with kivd and yqhD overexpression produced the highest amount of isobutanol from 2-keto isovalerate (KIV) with the lowest amount isobutyraldehyde accumulated (Figure 2A). This result is consistent with the fact that the highly efficient polyhydroxyalkanoate (PHA) production pathway in this organism uses NADPH as the reducing cofactor, suggesting that there is an abundant NADPH supply in the cell.

These results pinpointed the availability of different reducing cofactors in the cell under heterotrophic growth on fructose. In the lithoautotrophic biofuel production scenario, the oxidation of H\textsubscript{2} or formate directly yields NADH. But R. eutropha is equipped with an unusually high number of transhydrogenase isoenzymes that convert NADH to NADPH\textsuperscript{13} (Figure 1A). Indeed, previous studies have shown that NADPH/NADP\textsuperscript{+} ratio is much higher than that of NADH/NAD\textsuperscript{+} under autotrophic condition\textsuperscript{18}, suggesting that the NADPH dependent aldehyde reduction catalyzed by YqhD may also be favorable for biofuel production from CO\textsubscript{2}.

Without keto acids added to the medium, biofuel production from fructose by the wildtype strain H16 with kivd and yqhD overexpression reached only ~1.7 mg/L of isobutanol and ~3.8mg/L of 3MB (Figure 2B). These data suggest the necessity for the enhancement of the native keto acid chain elongation pathway. To do so, the strong phaC1 promoter that drives the expression of the
host’s PHA synthesis operon (*phaC1AB1*) was knocked-in in front of the *ilvBHC* operon and the *ilvD* gene in *R. eutropha* genome, which encode the enzymes responsible for the branched-chain amino acid biosynthesis (Figure 2C). The resulting strain LH75 showed significantly higher levels of acetohydroxy-acid synthase (AHAS), IlvC, and IlvD enzyme activities compared to the wildtype when assayed *in vitro* using cell lysate (Figure 2E,F,G). Unfortunately, when the *kivd* and *yqhD* cassette was introduced to LH75 to form strain LH106, the isobutanol and 3MB productivities on fructose were similar to the wildtype strain H16 transformed with the same Ehrlich cassette but without enhancement of the amino acid pathway (Figure 2B).

The high enzymatic activity *in vitro* and low productivity *in vivo* suggests that post-translational regulations on the native enzymes may control the flux. In fact, the anabolic AHAS enzymes that catalyzed the first-committed step of the keto acid chain elongation are well-known for their strict feedback inhibition by pathway end products and intermediates. To disrupt the post-translational regulation, a catabolic AHAS encoded by *alsS* from *Bacillus subtilis* was used\(^{20}\), which has high specificity to pyruvate and is not subjected to feedback inhibition. The *alsS* gene together with *ilvC* and *ilvD* genes from *E. coli* were cloned to form a synthetic operon driven by the Ralstonia *phaCl* promoter, which was then integrated into the *R. eutropha* genome to replace the native *phaB2C2* operon (Figure 2D). The resulting strain LH67, although only showing marginally elevated enzymatic activities *in vitro* (Figure 2E,F,G) compared to LH75, did provide more keto acid intermediates for biofuel production *in vivo*: when *kivd* and *yqhD* were introduced to LH67, the resulting strain LH74 produced ~155mg/L isobutanol and ~142mg/L 3MB under the same conditions as described above (Figure 2B). The isobutanol and 3MB titer was about 30-fold higher than that of LH106 (described above). To integrate the fuel production pathways with host metabolism, the PHB biosynthesis genes *phaC1AB1* in strain LH74 were
disrupted by a chloramphenicol acetyltransferase (CAT) cassette to give rise to the production strain LH74D (Figure 3A), which produced isobutanol and 3MB to ~176mg/L and ~160mg/L from fructose.

After demonstrating its isobutanol and 3MB productivity heterotrophically, LH74D was tested for autotrophic biofuel production on CO₂ and H₂. The O₂/CO₂ flow rate was adjusted accordingly to keep the ratio of H₂:CO₂:O₂=8:1:1. Under these conditions, the strain LH74D was able to produce a final titer exceeding 1 g/L of fuels (~536mg/L isobutanol and ~520mg/L 3MB) in 5 days in the J minimal medium (Figure 3B). Notably, the maximal production rate was reached at ~380mg L⁻¹/day⁻¹ and ~400mg L⁻¹/day⁻¹ for isobutanol and 3MB, respectively, when the cells entered the stationary phase, indicating high metabolic flux through the engineered biofuel production pathway. This result demonstrates the feasibility of using hydrogen to drive CO₂ reduction to isobutanol and 3MB. However, the low solubility and mass transfer of hydrogen limits the efficiency of its utilization by the cells.

We then tested the feasibility of using formic acid as the diffusible and soluble reducing power. Formic acid, or formate, is toxic to microbial cells at high concentrations because the protonated acid molecules penetrate the cell membrane and acidify the cytoplasm upon proton dissociation. As a result, the proton motive force across the membrane is reduced. To keep a constant low formate concentration in cell culture, pH-coupled formic acid feeding was used to add formic acid in small increments. These conditions enabled normal cell growth and relatively high biofuel productivity (Figure 3C) in the J minimal medium. The final titer of fuels was over 1.4 g/L (~846mg/L isobutanol and ~570mg/L 3MB) in around 5 days. Also, the specific productivity of fuels from formate (87.9 mg L⁻¹/day/OD) was much higher than that from hydrogen and CO₂ (9.2 mg L⁻¹/day/OD). Although the peak productivity from formate to fuels (25 mg/L/h) is about
10-fold less than that demonstrated from glucose to isobutanol using *E. coli* in un-optimized shake flasks\textsuperscript{19}, further improvement in productivity can be expected using existing technologies.

As discussed previously, supplying formate by in-situ electrochemical CO\textsubscript{2} reduction in culture medium may eventually increase efficiency and avoid product purification (Figure 4A). To test the feasibility of an integrated electro-microbial process, we tested Pb, In, Zn and other metals as a cathode to reduce CO\textsubscript{2} to formic acid with H\textsubscript{2}O as the proton source. At the anode (Pt mesh), O\textsubscript{2} is produced from H\textsubscript{2}O, and is conveniently utilized by Ralstonia in the integrated process. By voltammetry study and the Faradaic yield measurement, we determined that the optimal potential is around -1.6V against the Ag/AgCl reference electrode for the formate production reaction using an In plate cathode in the German minimal medium\textsuperscript{22} bubbled with air containing 15% CO\textsubscript{2} (data not shown). Under these conditions, formate can be generated at a relatively high rate, with hydrogen generated as a by-product. Both formate and hydrogen can serve as the energy source to support cell growth and biofuel production (Figures. 3B, C). Since electrolysis produces fine H\textsubscript{2} bubbles, mass transfer rate can be increased without mechanically dispersing large volume of gas substrate\textsuperscript{23}, which is a significant energy cost in the conventional fermentation processes. Thus, hydrogen by-product will not be wasted.

However, when *Ralstonia* cells were inoculated in the electrochemical reactor, no growth was observed. Growth study using the fast-growing microorganism *E. coli* showed transient inhibition of electrolysis on cell growth (Figure 4B). One possibility is that unstable toxic compounds might be produced in the electrolysis reaction. When electricity is turned off, the inhibitory compounds decay quickly and the cell growth is resumed. We hypothesized that
reactive oxygen species and reactive nitrogen species may be generated by the anode, thus causing growth inhibition. To test this hypothesis, three plasmid-based reporter constructs were assembled. Each of the plasmids contains a lacZ gene driven by the promoter of the Ralstonia genes \textit{katG} (encoding a catalase), \textit{sodC} (encoding a copper-zinc superoxide dismutase), or \textit{NorA} (encoding an iron-sulfur cluster repair di-iron protein). The promoters of \textit{katG}, \textit{sodC} and \textit{NorA} have been shown to be activated by hydrogen peroxide (H$_2$O$_2$), superoxide free radicals (O$_2^-$) and nitric oxide (NO), respectively\textsuperscript{24-26}. The plasmids were then transformed into the wild type Ralstonia strain H16. When the plasmid-bearing strains were exposed to electrolysis, expression of $\beta$-galactosidase from both \textit{sodC} and \textit{NorA} promoters were greatly induced, but not for \textit{katG} promoter (Figure 4C). These results were consistent with the arguments that O$_2^-$ and NO might be generated on the Pt anode, and suggested that these unstable reactive compounds trigger stress responses in Ralstonia cells and may be responsible for the transient growth inhibition.

To circumvent this toxicity problem, a porous ceramic cup was used to separate the cathode and the anode (Figure 4D). The porous ceramic material provides a tortuous diffusion path for chemicals. Therefore, the reactive compounds produced on the anode inside the cup may be decomposed before reaching the cells growing outside the cup. This strategy is more economical compared to the use of ion-exchange membranes to separate the electrodes. Using this approach, healthy growth of \textit{Ralstonia} biofuel production strain LH74D on electricity and CO$_2$ was achieved. Over 140mg/L biofuels were produced in 4 days (Figure 4E). Further optimization of the culture condition is needed to achieve high productivity over a prolonged time period.
Conclusion and Discussion:

In summary, we demonstrate the feasibility of conversion of electricity to high-energy-density liquid fuels in an integrated process using an engineered *R. eutropha* strain as the biocatalyst and CO$_2$ as the carbon source. The electro-microbial process first generates formate or hydrogen as the diffusible reducing intermediates, which then drive the microbial reduction of CO$_2$ to isobutanol and 3MB. This process does not depend on the biological “light reaction”; and the electricity generated from photovoltaic cells or wind turbines, or off-peak grid power can be used to drive CO$_2$ fixation and fuel production. Thus, it provides a way to store intermittent renewable energy in liquid transportation fuel with high energy density.

The separation of the “light” and “dark” reactions avoids the simultaneous demand of light-exposing surface area and culture containing volume in typical photo-bioreactors. Electricity can be generated and transmitted to remotely power fuel synthesis in the vicinity of a CO$_2$ source. The use of diffusible reducing intermediates minimizes the dependence on electrode surface area. The use of formate provides further advantages in large scale operations. Upon entering the cell, formate is converted to CO$_2$ and NADH by formate dehydrogenase, thus providing an inexpensive way to deliver both CO$_2$ and reducing power into the cell. The high solubility of formate and its safety features are highly attractive. Furthermore, since formate is the major byproduct of biomass processing, transformation of formate into liquid fuel compatible with transportation needs using this technology will also play an important role in the biomass refinery process. The approach demonstrated here can also be applicable to produce other chemicals, thus opening the possibility of electricity-driven bioconversion of CO$_2$ to a variety of chemicals.
To realize the potential of this process, both the electrochemical production of formate and the microbial production of higher alcohols needs to be optimized. The theoretical energy efficiency from H₂ or formate to isobutanol is about 50% (supplementary information). In mature microbial processes, 40-90% of theoretical efficiency can be achieved. If the energy efficiency of electrochemical production of formate or H₂ can be as high as 50-80%, then the overall energy efficiency of electricity to higher alcohols can be 10-36%. Currently, the photovoltaic solar cells commonly achieve 10-20% of energy efficiency. Taken together, the overall solar-to-fuel efficiency by coupling photovoltaic-energy generation to the integrated electro-microbial fuel production can be 1-7.2%. If such efficiencies are achieved, the electro-microbial process compares favorably to the biological photosynthesis-derived fuels or chemicals.

Methods:

Cloning procedure
The genes *kivd* (*Lactococcus lactis*), *adhA* (*Lactococcus lactis*), *adh2* (*Saccharomyces cerevisiae*), and *yqhD* (*Escherichia. coli*) were amplified using genomic DNA of appropriate organisms. The *kivd-adhA*, *kivd-adh2*, and *kivd-yqhD* artificial operons were then made by SOE (splicing by overlap extension) PCR with ribosome binding site sequence AGGAG in front of each gene. The operons were inserted digested with BspEI and NcoI and inserted into the broad-host-range vector pBHR 1(MoBiTec, Göttingen, Germany).

The 500bp DNA fragments upstream of *Ralstonia eutropha phaB2* gene and downstream of *phaC2* gene were amplified from genomic DNA and assembled with SOE with a linker region containing NotI and NcoI enzyme sites in between. The assembly product was digested with MluI and Xbal and inserted into the conjugation vector pNHG 1 to form pLH50. The artificial operon containing *alsS* (*Bacillus subtilis*), *ilvC* (*E.coli*), and *ilvD* (*E.coli*) was amplified from plasmid pSA 69 and assembled with the 836bp *phaC1* promoter region amplified from *R. eutropha* genomic DNA by SOE. This fragment was then inserted into the SacI site of pLH50.

The 1000bp DNA fragments upstream of *R. eutropha ilvB* gene and from 1-1000bp of *ilvB* gene open reading frame were amplified from genomic DNA and assembled with the *phaC1* promoter region by SOE. The assembly product was inserted into Ndel and Xbal sites of pNHG 1. The *phaC1* promoter knock-in plasmid for *ilvD* gene was constructed similarly.

The 1000bp DNA fragments upstream of *R.eutropha phaC1* gene and downstream of *phaB1* gene were amplified from genomic DNA and assembled with the chloramphenicol acetyltransferase (CAT) gene by SOE. The assembly product was inserted into MluI and Xbal sites of pNHG 1.

The DNA fragments from -500bp to 150bp relative to the *katG*, *sodC*, and *NorA* gene open reading frame of *R.eutropha* were amplified from the genomic DNA and assembled with the *lacZ* (β-galactosidase) gene using SOE. The resulting products were then inserted into the BspEI and Ncol sites of broad-host-
range vector pBHR 1. The transcription direction of lacZ genes was the opposite of the CAT promoter in the plasmid.

**Electroporation and Conjugation procedure**

The broad-host-range plasmids were transformed into *R.eutropha* cells by electroporation. Overnight culture of *R.eutropha* strains was inoculated into 20ml rich medium (16g/L nutrient broth, 10g/L Yeast extract, 5g/L (NH₄)₂SO₄) and allowed to grow to OD600=0.8 in 30°C. The cells were harvested by centrifugation, washed twice with ice-cold 0.3M sucrose, and then resuspended in 1ml of ice-cold 0.3M sucrose solution. 0.1ml of this resuspended cells were mixed with 50ng plasmid DNA and electroporated with 11.5kV/cm, 5.0ms, followed by rescuing with 0.2ml rich medium in 30°C for 2 hours and plated on rich medium plates containing 200mg/L kanamycin. Conjugation and double-crossover counter selection were performed as described previously²⁹.

**Enzyme assays**

*R.eutropha* cells were cultured under autotrophic condition with H₂:CO₂:O₂=8:1:1 in minimal medium{Schlegel, 1961 #68} for 48 hours in 30°C. 20ml of culture was harvested by centrifugation, washed twice with ice-cold lysis buffer (5mM MgSO₄, 50 mM Tris-Cl, pH 8.0), and resuspended with 1ml lysis buffer. After bead beating, the lysate was then centrifuged at 13,200 rpm for 20 minutes at 4°C. The supernatant was then retrieved for subsequent enzyme assays. Acetohydroxy-acid synthase (AHAS), ilvC, and ilvD assays were performed as described previously⁷.

The β-galactosidase assays were performed as follows: After incubated overnight in rich medium (10 g/l peptone, 10 g/l yeast extract, 5 g/l beef extract, and 5 g/l (NH₄)₂SO₄), Ralstonia cells were harvest and inoculate into the electro-microbial bioreactors with 300mL German minimal medium supplemented with 10g/L Na₂SO₄ and 4g/L fructose. Gas flow rate for the bioreactors was 200mL/min for air and 30-
40mL/min for CO$_2$. Electrolysis was performed using a platinum mesh as the anode and an Indium foil as the cathode. Electricity was provided by the DC power supply. The voltage between two electrodes was around 4V and current was around 250mA. For the control, no electrolysis was performed. After 3 hours, cells were harvested and concentrated by 100 fold. The reactions were started by added appropriate amount of cells into a reaction mixture containing 100 ul chloroform, 50 ul 0.1% SDS, 200 ul ONPG (4mg/ml), 950 ul Z buffer (Z buffer per 50 mL: 0.80g Na$_2$HPO$_4$·7H$_2$O, 0.28g NaH$_2$PO$_4$·H$_2$O, 0.5mL 1M KCl, 0.05 mL 1M MgSO$_4$, 0.135 mL β-mercaptoethanol). Vortex tubes for 10-15 sec. Let assay proceed for appropriate time. Stop assay by addition of 500 ul Na$_2$CO$_3$. Centrifuge tubes at max speed for 1 min to separate chloroform. Remove 1 ml of aqueous layer and record A$_{420}$ (or A$_{405}$ for non-ideal case) and A$_{550}$ for each sample. Calculate units of B-gal activity as follows:

\[
\text{B-gal units (Miller)} = 1000 \times \left( A_{420} - 1.75 \times A_{550} \right) / (\text{time} \times \text{vol} \times \text{OD}_{600})
\]

Miller units are in ΔA$_{420}$ min$^{-1}$ ml$^{-1}$.

**Conditions of keto-acid feeding experiments**

The *R.eutropha* cells were cultured in 20ml minimal medium{Schlegel, 1961 #68} containing 5g/L fructose in 250ml screw-cap shake flasks. When cell density reached OD$_{600}$=0.3, 3g/L 2-ketoisovalerate (KIV) was added. After 48 hours of incubation at 30°C, isobutanol and isobutyraldehyde were quantified using gas chromatography (GC)$^{19}$.

**Heterotrophic production conditions**

*R.eutropha* cells were cultured in German minimal medium$^{22}$ containing 4g/L fructose for 48 hours. Appropriate amount of cells were then washed and inoculated in 20ml of the same medium in 250ml screw-cap shake flasks to obtain initial OD$_{600}$ of 0.3. After 48 hours of incubation at 30°C, alcohols were quantified using gas chromatography (GC)$^{19}$.
Autotrophic production conditions

*R*.eutropha cells were cultured in German minimal medium with the volume of 1.8L in a 5L fermentor with the gas flow rates were as follows: H$_2$ 200mL/min, O$_2$/CO$_2$ mixture (1:1 ratio) 50 mL/min. The initial OD600 was around 1.0. H$_2$ was provided by an electricity-powered hydrogen generator (No-Maintenace H$_2$ Generator 500, PerkinElmer Inc., CA) and fed directly to the fermentor without purification or compression. Evaporated alcohols in venting gas were condensed with a Graham condenser and collected. Daily, samples of culture broth and condensation liquid were taken and alcohols were quantified using gas chromatography (GC).

For the formate-based fermentation, *R*.eutropha cells were cultured in J minimal medium with the volume of 1.8L in a 5L fermentor. J minimal medium was prepared by autoclaving 1 g/L (NH$_4$)$_2$SO$_4$, 0.5 g/L KH$_2$PO$_4$, and 6.8 g/L NaHPO$_4$ in MilliQ ddH$_2$O and aseptically adding 0.2 g/L MgSO$_4$-7H$_2$O, 20 mg/L FeSO$_4$-7H$_2$O, 4mg/L CaSO$_4$-2H$_2$O, 100 ug/L thiamine hydrochloride, and 1ml/L SL7 metals solution (1%v/v 5M HCl (aq), 1.5 g/L FeCl$_2$-4H$_2$O, 0.19 g/L CoCl$_2$-6H$_2$O, 0.1 g/L MnCl$_2$-4H$_2$O, 0.07 g/L ZnCl$_2$, 0.062 g/L H$_3$BO$_3$, 0.036 g/L Na$_2$MoO$_4$-2H$_2$O, 0.025 g/L NiCl$_2$-6H$_2$O, and 0.017 g/L CuCl$_2$·2H$_2$O). Control set points for agitation, temperature, pH, DO, air flow % and O$_2$ flow % were 300 rpm, 30°C, 7.2, 5%, 100%, and 0%, respectively. Gas flow was controlled by a dynamic-control cascade driven by DO with a gas flow of 0.5 SLPM at 0% out and 2.5 SLPM at 100% out. To control pH, 50% v/v formic acid with 2 g/l KH$_2$PO$_4$ was fed in following a pH-driven control cascade set to no flow with 0% out and 1 second pulses every 10 seconds at -100% out by the controller. This feed thereby serves to lower the pH and replenish the carbon supply as formate is consumed by the cells. Evaporated alcohols in venting gas were condensed with a Graham condenser and collected. Samples of culture broth and condensation liquid were taken and alcohols were quantified using gas chromatography (GC).
Integrated electro-microbial fuel production was performed as follows: Ralstonia cells were inoculated into the electro-microbial bioreactors with 350mL German minimal medium supplemented with 10g/L Na₂SO₄. Gas flow rate for the bioreactors was 200mL/min for air and 30-40mL/min for CO₂. Electrolysis was performed using a platinum mesh as the anode and an Indium foil as the cathode. A porous ceramic cup was used to separate the cathode and the anode. Electricity was provided by the DC power supply. The voltage between two electrodes was around 4V and current was around 250mA. Evaporated alcohols in venting gas were condensed with a Graham condenser and collected. Samples of culture broth and condensation liquid were taken and alcohols were quantified using gas chromatography (GC).

**Calculation of formate or H₂-to-isobutanol energy efficiency**

The maximum efficiencies for the production of isobutanol and 3-methyl-butanol while using hydrogen as sole source of energy were calculated. Each problem was defined as the optimization of the respective product flux while constrained to a mass balance and a given input flux; this is described by:

\[
\min(f^Tv) \text{ such that } Sv = 0 \text{ and } v_{H_2} = 1
\]

Here \(S\) is the stoichiometric matrix of the system, \(v\) is the vector of fluxes through each reaction in the system, and \(f\) is a vector such that \(f^Tv\) is our objective function. In our calculation, the system is defined by the reactions in the Calvin-Benson cycle, the reactions involved in glycolysis, the reactions in the valine and leucine biosynthesis pathway, the alcohol production reactions (KDC and ADH), \(H_2\) and CO₂ import reactions, the alcohol outlet reactions and a reaction through which ATP is obtained through the oxidation of NAD(P)H (this reaction can have varying stoichiometry or P/O ratio ranging from 1.5 to 3.0). Additionally, the elements of vector \(f\) were set to zero for all elements except those corresponding to the flux of the alcohol being optimized (set to -1). Performing the optimization as described maximizes the amount of product obtained from 1 mole of formate or \(H_2\); the amount of formate or \(H_2\) needed to obtain 1 mole of product is therefore given by \(v_{alcohol}^{-1}\).
The results were summarized as follows:

Table S1. Summary of theoretical yield for higher alcohol production from H₂

<table>
<thead>
<tr>
<th>P/O ratio (ATP/NAD(P)H)</th>
<th>Formate or H₂ needed to produce 1mole alcohol (mole)</th>
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<tbody>
<tr>
<td></td>
<td>isobutanol</td>
<td>21.33</td>
<td>28.99</td>
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<td>1.5</td>
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<td>3.0</td>
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<td>16.66</td>
<td>21.98</td>
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According to the calculation above, we assumed that 18-19 mole H₂ are needed to form 1 mole isobutanol. Given that the energy densities of H₂ and isobutanol are 143 MJ/kg and 36.1 MJ/kg, respectively, the energy efficiency from H₂ to isobutanol is 51.9-49.1%. The efficiency of 50% is used.

Figures:
Figure 2-1 Designing *Ralstonia eutropha* cells as the biocatalyst in the process of electricity storage. (a) Schematic presentation of the energy conversion and carbon flow route of the overall process. CBB cycle, Calvin-Benson-Bassham cycle;ETC, electron transportation chain;MBH, membrane-bound hydrogenase;SH, soluble hydrogenase; FDH, formate dehydrogenase.(b)Engineered metabolic pathways from CO₂ to fuels in the context of the host’s metabolic network. RuBP, Ribulose-1,5-bisphosphate;3PGA, 3-phospho-D-glycerate;2PGA, 2-phospho-D-glycerate; PEP, phosphoenolpyruvate; PHB, poly[R-(−)-3-hydroxybutyrate];AHAS, acetohydroxy-acid synthase;KDC, 2-keto-acid decarboxylase;ADH, alcohol dehydrogenase.
Figure 2-2 Construction of the synthetic isobutanol and 3-methyl-1-butanol production pathway in *Ralstonia eutropha*. (a) isobutanol and isobutyraldehyde formation by the synthetic Ehrlich cassette. The 2-keto-acid decarboxylase (KDC) encoded by *kivd* of *Lactococcus lactis* was overexpressed in combination with different alcohol dehydrogenases (ADHs) encoded by *adhA* (*L.lactis*), *adh2* (*Saccharomyces cerevisiae*), and *yqhD* (*Escherichia coli*), respectively. 3g of 2-ketoisovalerate was added in the culture as the immediate precursor of the synthetic pathway. The culture medium is German minimal medium (supplementary information) with 4g/L fructose. (b) Heterotrophic isobutanol and 3-methyl-1-butanol (3MB) production from 4g/L fructose in German minimal medium using H16, LH75, and LH67 strains transformed with a plasmid harboring the *kivd* and *yqhD* overexpression cassette. LH106 is the strain resulted from LH75 transformed with the *kivd* and *yqhD* plasmid. LH74 is the strain
resulted from LH67 transformed with the kivd and yqhD plasmid. (c) Construction of LH75 strain. Integration of the phaC1 promoter in front of the R. eutropha ilvBHC operon and ilvD gene to enhance branched-chaineamino acid biosynthesis. (d) Construction of LH67 strain. Integration of alsS (Bacillus subtilis), ilvC (E.coli), and ilvD (E.coli) in R. eutropha genome. The AHAS (acetohydroxy-acid synthase, encoded by ilvBH or alsS) (e), IlvC (f), and IlvD (g) specific activities in vitro as measured using cell extract of wildtype H16, LH75 and LH67. Error bars indicate standard deviation (n=3).
Fig3 Autotrophic higher alcohol production by the engineered Ralstonia strain. (a) Construction of the production strain LH74D. (b) Biofuel production performance by LH74D from CO$_2$ using electrolysis-generated H$_2$ as the sole energy source. (c) Biofuel production performance by LH74D using formic acid as the sole carbon and energy source. Detailed conditions and methods are described in supplementary information. Error bars indicate standard deviation (n=3).
Figure 2-4 The integrated electro-microbial process for biofuel production from electricity and CO₂ (a) Schematic presentation showing the in-situ electrochemical CO₂ reduction (and H₂O splitting) coupled with biofuel production by the engineered *Ralstonia eutropha* strain. (b) Transient inhibitory effect of in-situ electrolysis on the growth of *E.coli* cells. (c) The induction of *Ralstonia* katG, sodC, and NorA promoters in electrolysis conditions. The katG, sodC, and NorA promoters are induced by hydrogen peroxide (H₂O₂), superoxide free radicals (O₂⁻) and nitric oxide (NO), respectively. The promoters are used to drive the expression of the *lacZ* reporter gene. And the promoter activities are measured by the β-galactosidase assay. Error bars indicate standard deviation (n=3). (d) The configuration of the electro-microbial bioreactor. The cathode and the anode form concentric cylinders. The porous ceramic cup separates the two electrodes. (e) Biofuel production by the LH74 strain (described in the text) in the integrated electro-microbial process. Error bars indicate standard deviation (n=3).
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Table 2-1. Summary of theoretical yield for higher alcohol production from H₂
References:


Chapter 3:

A Synthetic Biochemistry Molecular Purge Valve Module that Maintains Redox Balance
Abstract:

The greatest potential environmental benefit of metabolic engineering would be the production of low value/high volume commodity chemicals, such as biofuels. Yet the high yields required for the economic viability of low-value chemicals is particularly hard to achieve in microbes due to the myriad competing biochemical pathways needed for cell viability. An alternative approach, which we call synthetic biochemistry, is to divorce the desired biochemical transformation from the organism by constructing biochemical pathways in vitro. Viable synthetic biochemistry, however, will require simple methods to replace the cellular circuitry that maintains cofactor balance. Here we design a simple purge valve module for maintaining NADP⁺/NADPH balance while allowing constant carbon flux. We test the purge valve in two systems where cofactor and carbon usage are unbalanced: the polyhydroxybutyryl bioplastic and the isoprene biosynthesis pathways. We find that the system is highly robust to variations in cofactor levels and readily transportable. The molecular purge valve provides a step toward developing continuously operating, sustainable synthetic biochemistry systems.
Introduction:

Metabolic engineering and synthetic biology have been employed for the production of high value chemicals but has not been as successful in meeting the stringent economics of large scale commodity chemical manufacturing. Microbial systems are often hampered by a variety of technical challenges that make it hard to achieve cost competitiveness, including poor yields due to competing pathways; low productivity caused by slow growth rates or difficulties in pathway optimization; contaminating microbial growth; product toxicity; and expensive product isolation.

Another approach that is beginning to receive attention is to perform complex biochemical transformations using mixtures of enzymes in a reaction vessel rather than within a cell. Building single, dedicated pathways *in vitro* can eliminate side reactions that occur in the cell, so that nearly 100% yields and fast reaction times are possible. *In vitro* biochemical systems also allow for more precise control over optimization and product toxicity problems can be more easily diagnosed and mitigated. Moreover, product extraction can be more facile.

Traditionally, *in vitro* pathway construction has been relegated to use as a research tool or in applications that require only 1-3 enzymes for the production of chiral compounds and other high value chemicals. Improvements in protein expression and access to stable enzymes have made more complex systems possible, however. *In vitro* biotransformation systems have been reported in recent years involving systems of over thirty enzymes. One of the first modern studies in this area was an artificial pathway that produced hydrogen from starch. The concept was recently advanced with a creative system that generated hydrogen from cellobiose at nearly 100% yields. In another effort, hyper-thermophilic glycolysis enzymes were heterologously expressed, heat purified, and assembled to convert glucose to n-butanol in 82% yield. In another study, an elegantly simplified non-
phosphorylative Entner-Doudoroff pathway from hyper thermophilic archaea was constructed to produce ethanol and isobutanol in ~55% yields\textsuperscript{19}. These pioneering studies illustrate the flexibility of synthetic biochemistry and the potential for high yields.

A limiting feature of earlier work in synthetic biochemistry systems is the need to either feed the systems with high energy cofactors, or if they are generated by the pathway \textit{in situ}, balance their use to ensure regeneration. For example, in the Sieber pathway to generate ethanol and isopropanol from glucose\textsuperscript{19}, the pathway was beautifully designed to reduce only two moles of NAD\textsuperscript{+} to NADH during the catabolic phase and to reoxidize two moles of NADH in the anabolic phase. Thus, the utilization of NAD\textsuperscript{+} was perfectly balanced allowing multiple passes through the pathway. In other systems, the pathways were also designed to maintain cofactor balance\textsuperscript{18}.

The requirement for perfect balance limits synthetic biochemistry in two ways, however. First, it places stringent constraints on pathway design, potentially limiting the types of chemicals that can be made. Second, it may ultimately limit the number of cycles the system can perform without adding additional, expensive high energy co-factors. Why? If there is any spontaneous oxidation of NADH, it effectively creates an unintended cofactor imbalance because more NADH is required than would be expected based on the stoichiometry of the system. Thus, over time the levels of NADH will dissipate and the system will wind down. As the economic viability of a synthetic biochemistry system will likely depend on the ability to run the systems in a self-sustaining manner for long periods of time, we need to develop methods that can maintain high energy cofactor levels without requiring perfect pathway stoichiometry.
Ideally, we believe that a synthetic biochemistry system should be able to generate more high energy cofactors in the catabolic phase of the pathway, than is needed in the anabolic phase of the pathway so that gradual losses by spontaneous ATP hydrolysis or NAD(P)H oxidation can be tolerated. On the other hand, if an excess of high energy cofactors is produced, it is necessary to also find a way to dissipate excess production so that the reactions can proceed (i.e., carbon flux can be maintained). In short, we need a way to maintain cofactor balance. If it were possible to regulate cofactor balance without affecting carbon flux, it would also allow for much more flexibility in pathway design. As a step toward this goal, we describe a synthetic biochemistry purge valve module to maintain the proper balance of NADPH and test if this general approach can be applied for the production of the bioplastic polyhydroxybutyrate (PHB) and isoprene.

PHB and other polyhydroxyalkanoates (PHA’s) are biodegradable thermoplastics. PHA’s can have characteristics similar to many popular petrochemical derived polymers, but are nontoxic and biodegradable, so they are attracting increased attention as a possible green alternative to petroleum based polymers in a wide range of applications\textsuperscript{20,21,22}. The best characterized and most abundant PHA polymer is polyhydroxybutyrate (PHB) that is naturally produced from Acetyl-CoA as a carbon and energy storage mechanism in many organisms\textsuperscript{22}. Currently, industrial production of PHB is done by \textit{in vivo} batch culture processes under nutrient starvation. This process is typically very time consuming, requires large fermentation volumes, and requires expensive methods for the extraction of PHB\textsuperscript{20}. Prior attempts to produce bioplastic \textit{in vitro} have required the addition of sacrificial substrates and a molar excess of cofactors to convert acetate to PHB\textsuperscript{23}.

Isoprene is a platform chemical for a variety of products, but it is mostly employed in the production of synthetic rubber\textsuperscript{24-26}. The isoprenoid pathway also provides precursors for over 25,000
known biomolecules including drugs such as taxol and potential biofuels. There have been a number of efforts to produce isoprene in microorganisms and the best reported yield is 28% from glucose. We recently showed that a synthetic biochemistry system could produce isoprene in >95% yield from pyruvate as long as high energy cofactors were added.

Here we employ a purge valve system in a pathway to convert pyruvate into PHB that maintains sustainable reducing cofactor balance, without the requirement for perfect stoichiometric matching of cofactor generation and usage to carbon usage. Further, we show that our purge valve module can be used as the basis for the production of other Acetyl-CoA derived products by applying it to the production of isoprene from pyruvate via the mevalonate pathway. Regulatory modules like this can free us from having to perfectly balance cofactor utilization when designing synthetic biochemistry systems.
**Results:**

The biotransformation of pyruvate into PHB, illustrates a basic co-factor imbalance problem that is encountered in biochemical systems. In particular, conversion of pyruvate to Acetyl-CoA by pyruvate dehydrogenase (PDH) yields one molecule of NADH. However, the three enzyme pathway (phaA, B, and C) to PHB from Acetyl-CoA utilizes only one half a molecule of NADPH per Acetyl-CoA. Thus, the canonical pathway produces an excess of reducing equivalents. Moreover, the reducing equivalents are of the wrong type (NADH rather than NADPH). We therefore designed a pathway, shown in Fig. 1A that can generate the correct cofactor and regulate its production.

In our design, we create a synthetic biochemistry “purge valve” that effectively decouples the stoichiometric production of NAD(P)H from Acetyl-CoA (Fig. 1). To this end we employ a mixture of both an NAD⁺-utilizing wild-type PDH (PDH_{NADH}), a mutant PDH that utilizes NADP⁺ (PDH_{NADPH}), and a water generating NADH oxidase (NoxE) that specifically oxidizes NADH, but not NADPH. By employing this metabolic node, we generate NADPH needed for PHB production from pyruvate, but also dissipate excess reducing equivalents in an auto-regulatory manner. As illustrated in Fig. 1B, under low NADPH, high NADP⁺ conditions, the mutant PDH_{NADPH} can operate to generate Acetyl-CoA and restore NADPH levels. Under high NADPH, low NADP⁺ conditions, the PDH_{NADPH} activity will automatically be choked off, and the wild-type PDH_{NADH} will be used preferentially to produce Acetyl-CoA and NADH. In this high NADPH condition, the reducing equivalents are not needed. Because the reducing equivalents are produced in the form of NADH and not NADPH, they are eliminated by the oxidase, NoxE. The presence of NoxE ensures that NADH never builds up and the PDH_{NADH} can always operate to generate carbon for the PHB pathway in the form of Acetyl-CoA. The PDH_{NADH} / PDH_{NADPH} /NoxE system acts like a purge valve that opens under conditions of high NADPH to relieve the excess reducing equivalent
“pressure” (i.e. buildup of NADH) and allow carbon flux to be maintained. An engineering schematic of the purge valve system is shown in Fig. 1C.

**Enzyme engineering and cofactor specificity**

To implement our purge valve module, we needed an NADPH-utilizing PDH. A mutant of *E. coli* PDH has been engineered to have NADP⁺ specificity by introducing mutations into the E3 enzyme (EcE3)\(^3\). The *E. coli* PDH proved too unstable for our system, however. We therefore engineered a mutant of the thermophilic *G. stearothermophilus* PDH that preferentially accepts NADP⁺ with increased enzyme stability.

Similar to design of the *E. coli* PDH mutant, the *G. stearothermophilus* PDH mutant was designed by overlaying the known structure of the *G. stearothermophilus* E3 subunit (GsE3)\(^3\) with the known structure of the related *E. coli* glutathione reductase, which utilizes NADP⁺ (REF or PDBID:1GET). The structural superposition allowed us to position the additional phosphate moiety in the active site of the GsE3, based on how it was placed in glutathione reductase (see Fig. 2). We could then design side chain substitutions in GsE3 that might allow acceptance of the phosphate. We were guided by the prior successful design of the EcE3 enzyme which shares 47% sequence identity with the GsE3\(^3,34\). The mutations introduced into EcE3 were E206V, G207R, A208K, G209H and S213R (GsE3 numbering). After examining the changes in the context of the GsE3 structure, we decided to introduce all but G209H, because it appeared that the new His side chain might create steric clashes with nearby K224 and N237 residues.

The kinetic properties of the engineered and wild-type enzymes reveal that the mutations alter specificity as desired. The kinetic parameters are listed in Supplemental Table I. For the wild-type *G.
The GsPDH\textsuperscript{NADH} and GsPDH\textsuperscript{NADPH} enzymes (henceforth designated PDH\textsuperscript{NADH} and PDH\textsuperscript{NADPH}) were much more stable than their *E. coli* counterparts. As shown in Supplemental Figure 1, the *G. stearothermophilus* enzymes retained ~50% activity after one hour incubation at 67°C whereas the *E. coli* PDH enzymes were completely inactivated at 50 °C.

A second key requirement of the purge valve design is the use of an NADH oxidase with high cofactor specificity. We chose NoxE from *L. lactis* as it is a water forming NADH oxidase so it doesn’t generate any toxic products such as hydrogen peroxide\textsuperscript{31,32,35}. As shown in Table 1, the $K_{cat}$ of NoxE is 248.8 times greater with NADH than NADPH and $k_{cat}/K_m$ is 9900 times greater.

**Enzyme catalyzed production of PHB from pyruvate**

The enzymes chosen for use in this work are listed in Table I. In the initial tests we only employed the wild type, PDH\textsuperscript{NADH} complex and NoxE to generate Acetyl-CoA and supplied NADPH exogenously. After optimizing enzyme ratios in this simple system, we then added the mutant PDH\textsuperscript{NADPH} to test *in situ* generation of NADPH. Finally, the amount of PDH\textsuperscript{NADPH} was optimized, keeping the other enzymes fixed.

The progress of the optimized pyruvate to PHB reaction is shown in Fig. 3 along with a control lacking the last enzyme, phaC. Both reactions had a PDH\textsuperscript{NADPH}:PDH\textsuperscript{NADH} ratio of 40:1. At this ratio, the NADPH levels rise rapidly ($A_{340}$) and are maintained throughout the time course (NoxE rapidly oxidizes NADH so
changes in $A_{340}$ reflect only changes in NADPH levels). At the same time, PHB granules are produced as monitored by $A_{600}$.

We assayed the PHB production using a gas chromatography method and found that the optimized reaction produced $2.45 \pm 0.5 \text{ mg/mL}$ of PHB from 50 mM pyruvate which represents nearly complete conversion ($94 \pm 20 \%$) of pyruvate to plastic. In the optimized system, we started with 0.5 mM NADP$^+$, so achieving 94% yield requires over 90 turnovers of the NADP$^+$ cofactor, indicating a high level of system sustainability.

The stability of the full system was assessed by mixing components together and then initiating the reaction at various time delays. The decrease in extent of the reaction is shown in Supplemental Fig. 2. We find that the extent of reaction remains $\sim 50\%$ after two days.

**Autoregulatory behavior of purge valve**

The regulatory behavior of the purge valve is better seen at sub-optimal enzyme concentrations and ratios of PDH$^{NADPH}$ to PDH$^{NADH}$ that slow down the response time. In the optimized assay (40:1 mole ratio of PDH$^{NADPH}$:PDH$^{NADH}$), we observed a rapid rise in NADPH levels which was sustained throughout. In the non-optimal systems shown in Fig. 4, the purge valve cannot respond as rapidly to drops in NADPH concentrations so we can observe variations in NADPH levels as the system develops. We still observe a rapid initial rise in NADPH levels, but as intermediates build up, the consumption starts to outstrip NADPH production. Eventually, the system compensates by generating higher levels of NADPH. The restoration of NADPH levels would be impossible without the proper operation of the purge valve system.
The system is robust to cofactor levels

To test whether the system was robust to changes in cofactor levels, we varied the initial cofactor concentrations in the reactions and measured the yields of PHB. Each reaction was constructed with combinations of NAD\(^+\), NADH, NADP\(^+\) or NADPH at either 0.1 mM or 1 mM and the production of PHB was monitored by the final OD at 600 nm. All of the reaction conditions were compared to the optimized reaction that produced nearly complete conversion of pyruvate to PHB and were within random variation. This result indicates that the purge valve can compensate readily for changes in cofactor concentrations and reduction states.

Porting the purge valve system to isoprene production

To test the versatility of the molecular purge valve and whether it can be applied as a general platform for the production of a diverse array Acetyl-CoA derived compounds we used the PDH purge valve to produce isoprene via the Acetyl-CoA dependent mevalonate pathway. We have previously described the \textit{in vitro} production of isoprene from pyruvate, which required the use of exogenously added NADPH\(^{30}\). Similar to the PHB pathway the mevalonate pathway has an inherently different carbon and cofactor stoichiometry. In particular, the mevalonate pathway requires 3 Acetyl-CoA and 2 NADPH for the production of isoprene (see Fig. 6A). Thus, system sustainability requires generation and regulation of NADPH levels.

We tested whether the purge valve system can replace exogenously added NADPH in the production of isoprene. As shown in Fig. 6B, the full purge valve system produces an 88.2±8.4% yield from 3 mM pyruvate. This yield is even higher than the 81.4 ± 2.0 % yield obtained if we add NADPH exogenously.
(Fig. 6B). If we leave out any of the purge valve components (PDH$^{\text{NADPH}}$, PDH$^{\text{NADH}}$ or NoxE), yields are dramatically reduced. Thus, the purge valve system is clearly transportable to other synthetic biochemistry systems.
Discussion

Maintaining proper cofactor balance is an essential part of generating flux and providing a driving force through an enzymatic pathway. *In vivo*, the enzymatic specificity for the cofactors NADH and NADPH are typically used to control the carbon flux through catabolic and anabolic pathways respectively. Organisms typically sense the reduction state of these cofactors and use this information to up-regulate or down-regulate catabolic and anabolic pathways to cope with environmental changes. *In vitro* systems, however, do not have the myriad peripheral pathways that facilitate this fine control. Moreover, the natural anabolic and catabolic specificities for NADH and NADPH complicate *in vitro* biotransformations. Synthetic biochemistry systems have often dealt with these problems by careful considerations of cofactor stoichiometry in pathway design, through the use of expensive sacrificial metabolites, reengineering enzymes so that only a single cofactor type is needed, adding excess cofactors, or constantly adding intermediates to the reaction mix to sustain the process.

In this work we created a robust node of control to balance the production and consumption of NADPH and NADH in a self-regulating and self-balancing manner. To our knowledge, this is the first *in vitro* pathway that maintains cofactor balance without requiring adherence to stoichiometry in the generation and utilization of cofactors to ensure carbon flux. In part because the system can sustain high levels of NADPH, we can drive the transformation to near completion, converting pyruvate to either PHB or isoprene at nearly 100% of the theoretical yield. Moreover, the high yields in our system are robust to 10-fold variations in cofactor levels.

Ultimately it will be necessary to expand this pathway to incorporate the conversion of low cost substrates such as glucose or other sugars into pyruvate, which would involve the glycolysis pathway or parts of the glycolysis pathway. Indeed a synthetic biochemistry system employing glycolysis has been
demonstrated previously\textsuperscript{5}. Building more complex compounds from Acetyl-CoA such as fatty acids, polyketides, and other isoprenoids will also require the incorporation and recycling of ATP. Thus, it would be desirable to develop simple methods to regulate the ATP/ADP levels within the context of synthetic biochemistry. ATP regulation will be an interesting challenge for future synthetic biochemistry pathway designs. Ultimately, developing regulatory systems like the purge valve employed here, will free synthetic biochemistry system design from having to consume the high energy cofactors during the anabolic phase in perfect stoichiometric balance. Thus, our approach can help diversify the chemical targets of synthetic biochemistry.
Methods

Materials

Miller LB media or Miller LB-agar (BD Difco) was used for growth of bacterial strains in liquid or solid media. *E. coli* BL21Gold(DE3) [B, F-, *ompT*, *hsdS*<sub>B</sub>, (*r<sub>B</sub>−,m<sub>B</sub>−*), *dcm*,*Tet*,*gal*,(DE3) *endA* Hte] (Agilent) was used as host for both cloning and expression of recombinant proteins using pET vectors. *E. coli* TOP10(DE3) [F- *mcrA* Δ(*mrr*-*hsdRMS*-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(*ara*-leu)7697 galE15 galK16 rpsL(*Str<sup>R</sup>) endA1 <sup>+</sup>] was used for expression of recombinant proteins from the pBAD/p15A vector<sup>37</sup>. Plasmids pET28a<sup>+</sup> and pET22b<sup>+</sup> were purchased from Novagen. HotStart Taq Mastermix (Denville) was used for gene amplification from genomic or plasmid DNA. Phusion DNA polymerase (Finnzymes), Taq DNA ligase (MCLab), and T5 Exonuclease (Epicenter) were purchased separately and used to make the assembly master mix (AMM) used for cloning (ref). ATP, (±)-α-lipoic acid, pyruvate, Coenzyme A, and NAD<sup>+</sup> were from Sigma.

Plasmid Construction

The expression plasmids for the PHB enzymes were constructed from the pET28a plasmid backbone using the Nde1 and Sac1 cut sites to produce constructs with an N-terminal 6xHis tag for purification. The genes encoding acetyl CoA acetyltransferase (phaA; YP_725941) and acetoAcetyl-CoA reductase (phaB; YP_725942)<sup>23</sup> were amplified and cloned from *R. eutropha* genomic DNA. The gene encoding polyhydroxybutyrate synthase<sup>38,39</sup> (phaC; HE_610111) was synthesized and codon optimized for expression in an *E. coli* host at Life Technologies before being subcloned into the pET28a expression vector. For the isoprene pathway, the constructs were the same as described in reference<sup>30</sup>.
*E. coli* BL21-Gold cells were used as the host strain for enzyme expression. All enzymes were expressed in Luria-Bertani (LB) media supplemented with 50 μg/mL kanamycin and were induced with 0.2 mM isopropyl-β-D-1-thiogalactopyranoside added to the culture at the end of log phase growth. The phaA, phaB, MVK, PMVK, and IspS were induced at 37 °C overnight and phaB, THL/HMGR, HMGS, and IDI were induced at 18 °C overnight. The phaC was induced at 25 °C for 5 hours before harvesting.

**Enzyme purification**

Cells from 0.5 L of culture were harvested by centrifugation and resuspended in 150mM Tris pH 7.5, 100 mM NaCl. The cells were lysed on ice with sonication and the cell debris was removed by 12,000 x g centrifugation at 4 °C. The supernatant was then mixed with 5 mL nickel-nitrilotriacetic acid (NTA) agarose and after 30 minutes, the agarose slurry was loaded onto a gravity column and washed with five column volumes of 100 mM Tris pH 7.5, 100 mM NaCl, 15 mM imidazole. The enzyme was then eluted with 250 mM imidazole, 100 mM Tris pH 7.5. The resulting enzyme was dialyzed into 50 mM Tris pH 7.5, 50 mM NaCl and stored at 4 °C.

**Expression vectors for PDH Subunits E1αβ, E2, and E3, and *E. coli* LplA**

The E1, E2, and E3 domains were all amplified separately from *G. stearothermophilus* genomic DNA (ATCC) using primers that contained 15-20 bp complementary to the gene and 15-20 bp complementary to the multiple cloning site in the vector where the gene would be placed. The genes encoding E1α and E1β were amplified together from *G. stearothermophilus* genomic DNA and cloned into pET28a(+) that had been digested with NcoI and XhoI. This created a tag-less construct for E1 expression under control of the T7 promoter where E1α translation uses the RBS from the pET28 vector while E1β uses the endogenous RBS from *G. stearothermophilus*. The E2 and E3 domains were amplified separately and cloned into pET22b(+) digested with NdeI and XhoI or pET28a(+) digested with NcoI and XhoI.
respectively to create tag-less E2 and E3 constructs. The *E. coli* lipoate protein ligase, LplA, was amplified from *E. coli* K12 genomic DNA and assembled into pBAD/p15A digested with XhoI and EcoRI to create 6xHis-LplA.

**Overexpression and purification of *G. stearothermophilus* PDH subunits and *E. coli* LplA.**

All *E. coli* strains were grown at 37°C in LB-media supplemented with appropriate antibiotic (100 µg/mL ampicillin, 50 µg/mL kanamycin, or 34 µg/mL chloramphenicol). For all constructs, 5 mL of overnight starter culture was used to inoculate 1 L of LB-media. Once the OD_{600} reached 0.6, 0.3 mM IPTG (pET vectors) or 0.02% arabinose (pBAD/p15A) was added to induce protein expression. After 16 hours, cells were harvested, resuspended (4 mL/g wet cells) in 50 mM Tris-Cl pH 7.5, 0.1 M NaCl (Buffer A), lysed by sonication, and cell debris removed by centrifugation at 30,000xg for 20 min.

25mL of the *E. coli* lysate containing 6xHis-LplA was loaded onto a 3 mL Ni-NTA resin (Qiagen), washed with 25 mL Buffer A containing 10 mM imidazole, and eluted with 5 mL Buffer A containing 250 mM imidazole. Pure 6xHis-LplA was then stored at 4°C until use.

The individual domains of *G. stearothermophilus* PDH were partially purified from *E. coli* lysates by heat prior to modification and reconstitution of the PDH complex. E1αβ, E2, or E3 containing lysates were incubated at 65°C for 35 minutes to heat denature *E. coli* proteins followed by centrifugation at 30,000xg for 20 min to pellet the precipitated proteins. Nearly all of the PDH domains remain in the supernatant. Next, the E2 domain was lipoated in the heated extract by the addition of 1 mM (±)-α-lipoic acid, 2 mM ATP, 3 mM MgCl₂, and 50 µg of purified 6xHis-LplA. The lipoation reaction was then allowed to proceed with gentle mixing overnight at 25 ºC, yielding lipoated E2 (E2lip). After lipoation, E1αβ, E2lip, and E3 were mixed in a 3:1:3 molar ratio and incubated for at least 1 hour at 25 ºC to form
the active GsPDH complex. The GsPDH complex was then isolated by ultracentrifugation (Beckman) for 4 hours at 95,000xg. The resulting yellow pellet was resuspended in 20 mM Tris-Cl, pH 7.5 in 1/50 the starting volume and assayed for activity. SDS-PAGE analysis confirmed the presence of all 4 domains and indicated that the preparation was >90% pure. The reconstituted complex was stored at 4°C until use.

**Enzyme activity and optimization.**

NoxE was assayed by monitoring the oxidation of NAD(P)H at 340 nm. The assay was carried out in 100 mM tris-HCl pH 7.5, 5 mM MgCl₂, 5 mM KCl, and 0.2 mM NAD(P)H.

WT and mutant PDH were assayed by monitoring the reduction of NAD(P)⁺ at 340 nm. The assay was carried out in 50 mM Tris pH 7.5, 5 mM MgCl₂, 5 mM pyruvate, 1 mM CoA, and 0.5 mM of NAD(P)⁺.

PhaC was assayed by monitoring the absorbance of the hydrolysis of the thioester bond of the substrate 3HBCoA at 235 nm. The assay was carried out in 100 mM Tris pH 7.5, 5 mM MgCl₂ and 0.15 mM 3HBCoA.

Activity of isoprene pathway enzymes were measured as reported previously. The amount of each enzyme in the reconstituted isoprene pathway described below is show in Supplementary Table 2.

**Final PHB reaction conditions and analysis**

The optimized self-sustaining reaction for the biotransformation of pyruvate to PHB was composed of 250 mM Tris pH 7.5, 5 mM MgCl₂, 5 mM KCl, 0.5 mM CoA, 0.1 mM NAD⁺, 0.5 mM NADP⁺, 50 mM pyruvate, 2 µg PDH NAD⁺, 76 µg PDH NADPH⁺, 23 µg phaA, 14 µgphaB, and 32 µg phaC in a final reaction...
volume of 200 µL. The reactions were initiated with the addition of pyruvate, which was left out of the initial mixture. All PHB reactions were performed at room temperature. For testing the autoregulatory behavior of the purge valve, some enzyme concentrations were suboptimal: 5 µg phaA, 2.5 µg phaB, and 1.9 µg phaC.

To assay for PHB, the reactions were lyophilized and incubated with 1 mL chloroform, 0.45 mL methanol, and 0.05 mL H₂SO₄ to hydrolyze the polymer and generate methyl 3-hydroxybutyrate. The reactions were extracted with 0.5 ml water and 1 µL of the chloroform layer was applied to a 0.25 micron HP-Innowax column using a HP 5890 Series II gas chromatogram. The GC method used an injection temperature that was held at 35°C for 5 minutes before it was increased to 275°C over 40 minutes. The peak intensities were compared to an authentic standard to assess concentrations.

**Isoprene Reaction Conditions and Analysis**

*In vitro* production of isoprene was performed as described previously with the following changes. 200 µL reactions were set up in 2 mL gas tight vials containing enzymes, 3 mM pyruvate, 15 mM ATP, 0.6 mM CoA, 0.2 mM NAD+, 0.4 mM NADP+ (or 5 mM NADPH), 10 mM MgCl₂, 20 mM KCl, 0.1 mM thiamine pyrophosphate in 100 mM tris-Cl pH 8.5 and incubated at 32 ºC for 18 hours. Isoprene production was monitored by direct sampling of 100 µL the headspace using a 100 µL gas-tight syringe. The headspace was analyzed by GC-FID (HP5980II) equipped with a GS-GasPro column (0.32 mm x 30 m, Agilent) as described previously. The amount of isoprene produced was quantified by comparison to a standard curve of various isoprene concentrations sampled in the same manner.
Figures:

**Table I.** Enzymes used in this work

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<td>HMGR</td>
<td>HMG-CoA Reductase</td>
<td>WP_002357755</td>
<td>pET28</td>
<td>N-His</td>
<td>E. faecalis</td>
</tr>
<tr>
<td>9</td>
<td>MVK</td>
<td>Mevalonate kinase</td>
<td>BAA24409</td>
<td>pET28</td>
<td>N-His</td>
<td>S. cerevisiae</td>
</tr>
<tr>
<td>10</td>
<td>PMVK</td>
<td>Phosphomevalonate kinase</td>
<td>NP_344303</td>
<td>pET28</td>
<td>N-His</td>
<td>S. solfataricus</td>
</tr>
<tr>
<td>11</td>
<td>MDC</td>
<td>Diphosphomevalonate decarboxylase</td>
<td>NP_014441</td>
<td>pET28</td>
<td>N-His</td>
<td>S. cerevisiae</td>
</tr>
<tr>
<td>12</td>
<td>Idi</td>
<td>Isopentenyl diphosphate isomerase</td>
<td>NP_417365</td>
<td>pET22</td>
<td>C-His</td>
<td>E. coli</td>
</tr>
<tr>
<td>13</td>
<td>IspS</td>
<td>Isoprene Synthase</td>
<td>Q50L36</td>
<td>pET28</td>
<td>N-His</td>
<td>P. alba</td>
</tr>
</tbody>
</table>
Figure 1. A synthetic biochemistry purge valve system for the production of PHB. (A) The *in vitro* metabolic pathway for the conversion of pyruvate to PHB. The pathway consists of 6 separate reactions: reaction 1 (PDH\textsubscript{NADH}), reaction 2 (PDH\textsubscript{NADPH}), reaction 3 (NoxE), reaction 4 (PhaA), reaction 5 (PhaB), reaction 6 (PhaC). The purge valve is highlighted in the red box. (B) How the purge valve is designed to function. At low NADPH (high NADP\textsuperscript{+}), PDH\textsubscript{NADPH} reaction dominates, generating Acetyl-CoA and NADPH from pyruvate and NADP\textsuperscript{+}. The purge valve is effectively “off”. In high NADPH (low NADP\textsuperscript{+}) conditions, the PHD\textsubscript{NADPH} enzyme is starved for oxidized cofactor, shutting this pathway to Acetyl-CoA down. In this situation, the PDH\textsubscript{NADH}/NoxE system takes over, producing only Acetyl-CoA. In
other words, the purge valve is effectively “on”. (C) A chemical engineering schematic of the purge valve system used in the production of PHB from pyruvate, involving a cofactor recycle loop.
Figure 2. Design of the PDH$^{\text{NADPH}}$ enzyme. The structures of the wild type G. stearothermophilus E3 subunit (E3, green backbone trace) is shown overlaid on the structure of E. coli glutathione reductase (GTX-NADPH, gray backbone trace). The NADPH substrate from glutathione reductase is shown in stick representation, showing the placement of the phosphate moiety that needs to be accommodated. The residues changed to accept the phosphate (E206V, G207R, A208K, and S213R) are shown in purple.
Figure 3. Production of PHB using an optimized system. In this reaction the production of PHB is monitored by an increase $A_{600}$ (blue) caused by precipitation of the PHB granules. No increase is seen in the absence of the PHB polymerase, PhaC. The production of PHB is confirmed by a gas chromatography assay (see text). The $A_{340}$ (red) monitors the level of NADPH because no NADH is allowed to build up because of the presence of NoxE. The purge valve system maintains a high level of NADPH throughout the reaction.
Figure 4. *Time course of pyruvate to PHB optimization reaction using sub-optimal ratios of PDH\textsuperscript{NADPH} and PDH\textsuperscript{NADH}*. The $A_{340}$ traces (red), monitoring NADPH levels fall into three distinct phases. A fast initial reduction of NADPH by the PDH\textsuperscript{NADPH} is followed by a slow oxidation of NADPH by PhaB as the intermediate levels rise. As the reaction proceeds, the purge valve effectively turns off and NADPH levels rise again. The evolution of the system coincides with the increase at $A_{600}$ (blue) which represents the precipitation of the PHB granules from solution.
Figure 5. The purge valve system is robust. A) The graph shows relative yield of PHB upon starting with different amounts of each of the cofactors. The relative yields represent the ratio of the final $A_{600}$ for the reaction, relative to the final $A_{600}$ for the optimized reaction. All reactions show a relatively robust yield in comparison to the negative control lacking the final phaC enzyme (orange bar). The error bars reflect the standard deviation of three independent reactions. B) Table of the numbers reflected in the graph in part A.
Figure 6. Employing the purge valve for the production of isoprene. (A) The in vitro metabolic pathway for the conversion of pyruvate to isoprene. The purge valve highlighted in the red box consists of the same enzymes/reactions as in Fig. 1. In the mevalonate pathway, 3 Acetyl-CoA are used to make HMG-CoA (enzymes 6 and 7). HMG-CoA is reduced by HMGR (enzyme 8) with 2 NADPH to give mevalonate. 3 ATP are then used to convert mevalonate to isopentenyl pyrophosphate followed by production of isoprene (enzymes 9-12). (B) The graph shows the dependence of isoprene production on the purge valve composition.
valve. No purge valve was used in the first reaction \( \text{PDH}^{\text{NADH}}, \text{NADPH}, \text{NoxE} \). NADPH was simply added and NADH recycled using NoxE. The final experiment \( \text{PDH}^{\text{NADH}}, \text{PDH}^{\text{NADPH}}, \text{NoxE} \) shows results employing the full purge valve system. Leaving out any component of the purge valve system resulted in dramatic decreases in isoprene production. Each reaction was performed in duplicate.
**Supplemental Table 1. List of the catalytic properties of the purge valve enzymes.**

<table>
<thead>
<tr>
<th>Enzyme Name</th>
<th>substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (µM/min/mg)</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ecPDH - NAD</td>
<td>NAD+</td>
<td>0.68111 ± 0.062</td>
<td>9.125 ± 0.28</td>
<td>13.38</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>0.12976 ± 0.030</td>
<td>16.98 ± 1.04</td>
<td>131.01</td>
</tr>
<tr>
<td>ecPDH - NADP</td>
<td>NADP+</td>
<td>0.3281 ± 0.10</td>
<td>2.9795 ± 0.026</td>
<td>9.08</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>0.048101 ± 0.0046</td>
<td>7.242 ± 0.20</td>
<td>149.69</td>
</tr>
<tr>
<td>gsPDH - NAD+</td>
<td>NAD+</td>
<td>0.013381 ± 0.00083</td>
<td>82.651 ± 0.66</td>
<td>6167.91</td>
</tr>
<tr>
<td></td>
<td>NADP+</td>
<td>1.381 ± 0.41</td>
<td>7.3954 ± 0.85</td>
<td>5.36</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>0.52736 ± 0.049</td>
<td>91.976 ± 1.9</td>
<td>174.40</td>
</tr>
<tr>
<td>gsPDH - NADP</td>
<td>NADP+</td>
<td>0.157 ± 0.013</td>
<td>117.75 ± 8.1</td>
<td>750.00</td>
</tr>
<tr>
<td></td>
<td>NAD+</td>
<td>0.45269 ± 0.178</td>
<td>16.104 ± 1.8</td>
<td>35.56</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>0.42328 ± 0.068</td>
<td>174.3 ± 2.7</td>
<td>411.76</td>
</tr>
<tr>
<td>NoxE</td>
<td>NADH</td>
<td>0.074925 ± 0.026</td>
<td>348.57 ± 36.5</td>
<td>4653.81</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>2.9515 ± 0.77</td>
<td>1.4009 ± 0.32</td>
<td>0.47</td>
</tr>
</tbody>
</table>
Figure 3-7. *Stability of the pyruvate to PHB reaction at room temp.*
Table 3-3. *List of the enzymes and activities used in the production of PHB or Isoprene from pyruvate.*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Units/mg</th>
<th>mg added</th>
<th>Units added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  GsPDH$^{NAD}$</td>
<td>82.651±0.66</td>
<td>0.003/0.0013</td>
<td>0.25/0.11</td>
</tr>
<tr>
<td>1' GsPDH$^{NADP}$</td>
<td>117.75 ± 8.1</td>
<td>0.076/0.0095</td>
<td>8.95/1.12</td>
</tr>
<tr>
<td>2  LlNoxE</td>
<td>348.57 ± 36.5</td>
<td>0.020/0.00625</td>
<td>6.97/2.18</td>
</tr>
<tr>
<td>3  PhaA</td>
<td>51.04</td>
<td>0.023</td>
<td>2.17</td>
</tr>
<tr>
<td>4  PhaB</td>
<td>8.07</td>
<td>0.014</td>
<td>0.113</td>
</tr>
<tr>
<td>5  PhaC</td>
<td>142.7 *</td>
<td>0.032</td>
<td>4.57</td>
</tr>
<tr>
<td>6  EfTHL-HMGR</td>
<td>0.06 ± 0.002$^a$</td>
<td>0.003</td>
<td>0.00018</td>
</tr>
<tr>
<td>7  EfHMGS$^b$</td>
<td>0.6 ± 0.01</td>
<td>0.041</td>
<td>0.025</td>
</tr>
<tr>
<td>8  EfHMGR</td>
<td>0.06 ± 0.002$^a$</td>
<td>0.023</td>
<td>0.0014</td>
</tr>
<tr>
<td>9  ScMVK</td>
<td>47.0 ± 0.9</td>
<td>0.008</td>
<td>0.38</td>
</tr>
<tr>
<td>10 SsPMVK</td>
<td>0.8 ± 0.02</td>
<td>0.029</td>
<td>0.023</td>
</tr>
<tr>
<td>11 ScMDC</td>
<td>4.0 ± .07</td>
<td>0.038</td>
<td>0.152</td>
</tr>
<tr>
<td>12 EclDI</td>
<td>0.035$^c$</td>
<td>0.083</td>
<td>0.003</td>
</tr>
<tr>
<td>13 PalspS</td>
<td>0.156$^d$</td>
<td>0.088</td>
<td>0.014</td>
</tr>
</tbody>
</table>

*a) Assayed in forward direction (synthesis) by coupling to MvaS and monitoring NADPH consumption; b) mutant A110G (From Ref XXX) c) From Ref. XX; d) From Ref. YY;*
References:


Chapter 4:

The PBG Pathway: A Synthetic Biochemistry System for the Efficient Production of Bioplastic from Glucose
Abstract

Synthetic Biochemistry, the design of complex biochemical transformations for the *in vitro* production of bio-based chemicals, is a potentially high-yield, flexible alternative to *in vivo* metabolic engineering. A major challenge for Synthetic Biochemistry is to limit the costs of enzymes and cofactors by designing systems that can operate continuously with only the addition of inexpensive feedstock chemicals. To achieve this goal, it will be necessary to replace the cellular networks that generate and recycle high energy cofactors. Here we describe a designed, non-biological, synthetic pathway we call the PBG pathway to convert the low cost, renewable feedstock glucose directly into the bioplastic polyhydroxybutyrate. The PBG pathway effectively generates ATP, NAD(P)H, and CoA and balances their levels so that cofactor additions are not required. The system retains 50% of maximal flux for over 50 hours, generates yields as high as 93% and has a maximum productivity of 0.7 g/L/hr. These results suggest that Synthetic Biochemistry has the potential to replace metabolic engineering for the green production of chemicals.
Background

Nature employs a number of glycolytic pathways to break down sugar and generate a variety of carbon building blocks and high energy cofactors. Various sugar breakdown pathways can be used simultaneously, with the carbon flux shunted through the different pathways depending on the environment and the organism’s metabolic requirements. The most common sugar used as an energy source is glucose, which is broken down by two main pathways: classical glycolysis (i.e., the Embden–Meyerhof–Parnas (EMP) pathway) and the pentose phosphate pathway, both of which serve a specific purpose. Generally, sugar oxidized through the EMP pathway is converted to pyruvate, producing ATP and NADH. Pyruvate is then decarboxylated to the two-carbon building block acetyl-CoA. Acetyl-CoA can then be passed to the TCA cycle to produce more NADH, powering ATP synthesis, or is used by anabolic pathways to build complex molecules. Sugar oxidized through the pentose phosphate pathway primarily produces NADPH and 5 carbon intermediates that are used to build a variety of biomolecules including DNA. Inside of a cell these two pathways run simultaneously and are balanced to supply the proper cofactors, carbon flux, and carbon building blocks to sustain life.

Because of the exquisite control and evolutionary pressure placed on central metabolism, metabolic engineering of glycolytic pathways to divert carbon flux in vivo is extremely difficult. As a result, metabolic engineering has largely left glycolysis untouched while primarily focusing on engineering the downstream anabolic pathways that are not inextricably linked with cell survival. Due to the difficulties of reengineering metabolic pathways in living organisms, there is developing interest in an alternative approach that we call Synthetic Biochemistry, where complex metabolic pathways are reconstituted in vitro from isolated enzymes. The Synthetic Biochemistry approach eliminates both the myriad unwanted side reactions that occur in cells as well as product toxicity, enabling near 100% theoretical yields from input glucose. Moreover, by removing cellular constraints, it is easier to contemplate re-
engineering something as fundamental to cell survival as central metabolism. However, one of the key challenges of Synthetic Biochemistry is to replace the regulatory networks that maintain appropriate cofactor balance.

A number of approaches have been taken to maintain cofactor levels. One approach is to employ sacrificial substrates to generate high energy cofactors to power the pathways\textsuperscript{11-13}. For example, in a system for the production of PHB from acetate, NADPH was produced by a dead-end action of glucose dehydrogenase on glucose to produce glucanolactone\textsuperscript{14}. Unfortunately, the addition of sacrificial substrates is not only more costly but problematic because the byproducts of the sacrificial reaction eventually build up in the reaction vessel. Another approach to maintain cofactor levels is to carefully design pathways so that the stoichiometry of cofactor production and consumption is equal\textsuperscript{15,16}. For example, if the catabolic phase of the pathway generates two NADH from two NAD+, the anabolic phase in that pathway uses exactly two NADH to restore the two NAD+. However, this stoichiometric balancing approach limits flexibility in the number and type of compounds that can be produced. Indeed, most naturally occurring catabolic and anabolic pathways are unbalanced in regards to cofactor production and usage. Ideally, Synthetic Biochemistry platforms need to be developed that can auto-regulate cofactor balance, generating or eliminating high energy cofactors as needed to maintain carbon flux, rather than requiring stoichiometric recycling.

Previously, we described the development of a molecular purge valve to auto-regulate NADPH levels (described below), and demonstrated its effectiveness in the production of the bioplastic polyhydroxybutyrate (PHB) and isoprene from pyruvate\textsuperscript{17}. In the pathways starting with pyruvate, more reducing equivalents were generated than was needed for biosynthesis, but the purge valve system regulated the NADPH levels, allowing continuous flux through the pathways. However, pyruvate is too expensive to use as a feedstock for low-value commodity chemical production so we sought to develop
pathways that employ glucose to use as a cheaper feedstock. In theory, a purge valve system could be incorporated into the canonical EMP glycolytic pathway to produce PHB from glucose at the step catalyzed by glyceraldehyde-3-phosphate dehydrogenase. However, in addition to NAD(P)H reducing equivalents, the classical EMP glycolytic pathway also produces net 2 ATP per glucose where the PHB pathway requires 0 ATP. As a result, there is also an imbalance in ATP production and usage between the catabolic EMP pathway and the anabolic PHB pathway.

A solution to the ATP imbalance would be to design an ATP free pathway as has been done in the past for other cell free systems. One such example is the use of the pentose phosphate pathway for the production of H₂ from glucose, which produces NADPH but does not produce any ATP¹⁸,¹⁹. In another example, the Sieber group designed an alternative pathway for the production of ethanol and isobutanol that only generates and utilizes NADH in addition to pyruvate¹⁶. A limitation of these pathways, however, is that they do not generate acetyl-CoA or ATP which are necessary for the biosynthesis of PHB and many biochemicals beyond simple alcohol fuels. In another example, the Ohtake group modified the canonical EMP pathway to generate acetyl-CoA for the production of butanol from glucose that was net zero for ATP by employing a non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase¹⁵. However, while butanol was produced from acetyl-CoA, the system required the addition of expensive intermediates like acetyl-CoA and 3-hydroxybutyryl-CoA throughout the process to maintain flux, and it is unclear if sustainable production could be realized with this system.

In order to efficiently employ glucose to produce the widely used biochemical building block acetyl-CoA, we designed a new Synthetic Biochemistry pathway that we call the PBG cycle (Pentose/Bifido/Glycolysis) because it is built from the components of the pentose phosphate pathway, the bifidobacterium shunt, and glycolysis. Our PBG pathway advances Synthetic Biochemistry in a
number of ways: (1) It produces the key biosynthetic intermediate acetyl-CoA from glucose. (2) It generates and recycles ATP. (3) It employs two new purge valves for the auto-regulation of reducing equivalents. (4) The pathway is sufficiently robust that no addition of expensive intermediates or cofactors is required after many days of operation. The optimized process produces PHB at 86% theoretical yield from a single addition of 110 mM glucose with a maximum productivity of 0.7 g/L/h. Furthermore, the system is stable, retaining 50% of its initial activity after for 55 hours.

**Results and Discussion**

**Overview of pathway design:** Polyhydroxybutyrate (PHB) is a naturally occurring carbon and energy storage compound typically synthesized from two acetyl-CoA and one NADPH by a wide variety of prokaryotes and can be used as a biodegradable plastic\textsuperscript{20–22}. However, there are no natural glycolytic pathways that can be reconstituted \textit{in vitro} where the production of NAD(P)H, ATP, and acetyl-CoA is stoichiometric with the usage requirements of the PHB pathway. As a result, we designed a new, non-natural synthetic cycle to perform the complex conversion of glucose into PHB. In order to create this new pathway, enzymes from the pentose phosphate pathway, the bifidobacterium shunt, and glycolysis were assembled to produce what we call the PBG pathway shown in Figure 4-1. Central to PBG pathway are enzymes that cycle between C6, C5, and C3 sugar phosphates while siphoning off a C2 intermediate in the form of acetyl phosphate. Acetyl phosphate can then be converted directly to acetyl-CoA via phosphotransacetylase (pta) which is used as a building block for PHB biosynthesis. One CO\textsubscript{2} is lost in each cycle during the oxidation of glucose by 6-phosphogluconate dehydrogenase (Gnd) which provides an irreversible step, supplying a forward driving force through the pathway. To complete the cycle, glyceraldehyde-3-phosphate is eventually converted back to fructose-6-phosphate and glucose-6-phosphate, restoring ATP in the process.
The detailed metabolic logic of the PBG cycle can be explained as the combination of three sub-phases that roughly correspond to parts of the three original pathways (Pentose/Bifido/Glycolysis) comprising the cycle. Initially, 2 glucose molecules enter the cycle and are converted to glucose-6-phosphate (G6P) at the expense of 2 ATP through the action of glucokinase. The first phase of the cycle then passes through the oxidative branch of the pentose phosphate pathway which breaks down 2 G6P to 2 xylulose-5-phosphate (X5P) producing 4 NAD(P)H in the process. In the second phase of the cycle, xylulose-5-phosphate phosphoketolase (Xfp) from the bifidobacterium shunt splits the 2 X5P molecules into 2 acetyl-phosphate and 2 glyceraldehyde-3-phosphate (G3P). Acetyl-phosphate is subsequently converted into acetyl-CoA by phosphotransacetylase (Pta) and then used as a carbon building block for PHB production while G3P is eventually recycled in the third phase. In the third phase, using glycolysis enzymes, 2 G3P molecules (from 2 input G6P) are condensed into fructose-1,6-bisphosphate (FBP) by fructose bisphosphate aldolase (Fba) and then recycled back to G6P making an ATP in the process via the reverse reaction of phosphofructokinase B (PfkB). The ATP produced is then consumed by glucokinase, which allows another molecule of glucose to enter the cycle in the form of glucose-6-phosphate, thus completing the cycle. Overall, the PBG cycle produces a net of 2 acetyl-CoA, 4 NAD(P)H, and 0 ATP for each glucose molecule and 66.6% theoretical molar yield of carbon due to the production of CO₂.

**Identification of an ATP generating phosphofructokinase:** A key element of the PBG pathway is quantitative regeneration of ATP during the conversion of fructose 1,6 bisphosphate into fructose-6-phosphate. Although a similar reaction is performed in natural gluconeogenesis by fructose-1,6-bisphosphatase, it generates inorganic phosphate rather than ATP. We therefore wondered whether we could employ the glycolysis enzyme, phosphofructokinase (Pfk) in the reverse direction. Normally, Pfk catalyzes the ATP dependent phosphorylation of F16BP to FBP and is a key regulatory step in the EMP pathway. While two different isoforms of Pfk, PfkA and PfkB, have been identified and
characterized from various organisms, it is generally thought that the reverse reaction (FBP and ADP to F16BP and ATP) does not occur in vivo\(^{25}\), hence the use of a non-phosphorylating fbpase in gluconeogenesis.

Prior to implementing the PBG pathway, it was clearly necessary to see if we could find a Pfk enzyme that would efficiently generate ATP in a reverse reaction. It has been reported that PfkA and PfkB can catalyze the reverse reaction\(^{25,26}\) so we screened multiple enzymes including the *E. coli* PfkA, *E. coli* PfkB, *G. stercothermophilus* Pfk, and a regulatory mutant of *G. stercothermophilus* Pfk (R211A) that abolishes the inhibition by PEP and GDP. From this screen we found that the *E. coli* PfkB efficiently generated ATP at high enough flux to be used in the PBG cycle.

**Enzyme engineering for new purge valves:** The PBG pathway generates more reducing equivalents than is needed to generate PHB (4 NADPH generated per glucose but only 1 NADPH needed). Thus, to prevent the buildup of NADPH, we needed a way to purge the excess reducing equivalents. In prior work, we developed the concept of a molecular purge valve to regulate NADPH levels. The basic design of a dehydrogenase purge valve is described in Figure 4-2a. We employ a mixture of dehydrogenases, one that generates NADH and one that generate NADPH. Any NADH produced is immediately recycled back to NAD\(+\), while the NADPH generated can be employed in biosynthetic reactions.

In our previous work we developed a molecular purge comprised of a wild-type NAD\(+\) dependent pyruvate dehydrogenase, and a mutant NADP\(+\) dependent pyruvate dehydrogenase. The PBG pathway does not employ pyruvate dehydrogenase, however, so we needed to develop new purge valve modules. There are two reduction steps in the PBG pathway that could be modified to create a purge valve: glucose-6-phosphate dehydrogenase (Zwf) and gluconate-6-phosphate dehydrogenase (Gnd).
Both Zwf and Gnd wild-type enzymes have a strong preference for NADP+ over NAD+ so we needed to develop re-engineered enzymes to favor NAD+ rather than NADP+.

To flip the cofactor specificity of Zwf and Gnd, mutations in the NADP+ binding pocket were designed based on structural models. First, a homology model of the Zwf enzyme from Geobacillus stearothermophilus was generated based on the crystal structure of Glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides with NADPH bound (PDBID 1E7Y). A comparison of the model with the NADPH bound structure shows that A47 in GsZwf would come within 4.0 Å of the phosphate at the 2’ position of the ribose (see Figure 4-2). In many other corresponding NAD+ dependent dehydrogenases, an Asp residue at this position confers specificity for NAD+ over NADP+. As a result, the A47D mutant of GsZwf was generated and assayed for cofactor specificity. Kinetic parameters shown in Supplementary Table 1 indicate that the A47D mutation reverses the specificity of the enzyme. In particular, for the wild-type GsZwf, $k_{cat}/K_m$ is 14-fold higher for NADP+ than NAD+, while for the re-engineered mutant $k_{cat}/K_m$ is nearly 5-fold higher for NAD+.

For Gnd, there is a crystal structure of the enzyme from G. stearothermophilus (GsGnd) albeit without NADP+ bound. To identify the NADP+ binding site and residues that may be important for binding in GsGnd, the GsGnd structure was aligned with the NADP+ bound structure 1PGN from Ovis aries. Based on sequence comparison with NAD+ dependent reductases as well as proximity to the 2’ phosphate, we introduced a series of mutations (R34Y, N33D and K38L) in an effort to exclude NADP+ (see Figure 4-2). The R34Y mutation was chosen because R34 is changed to Y in the bispecific enzyme gntZ from B. subtilis while the N33D mutation was chosen because the conserved N33 is Asp in NAD+ dependent dehydrogenases. The K38L mutation was designed to sterically interfere with the phosphate NADP+. The kinetics and specificity for the GsGnd triple mutant N33D/R34Y/K38L are shown in Supplementary Table 1. The triple mutant is essentially a bispecific enzyme with only a slight preference for NAD+ ($k_{cat}/K_m =$
0.86 for NAD+ and 2.00 for NADP+), but it is now sufficiently active with NAD+ to serve in the purge valve node (see below).

**System improvement to compensate for Xfp promiscuity:** Our initial tests resulted in poor yields, so we considered the possibility of a side reaction caused by the promiscuity of Xfp\textsuperscript{27,28}. Xfp has phosphoketolase activity with both X5P as well as fructose-6-phosphate (F6P). The secondary reaction with fructose-6-phosphate would create a potentially wasteful trap by producing erythrose-4-phosphate (E4P) as a dead-end product. To solve this problem we added three additional enzymes from the non-oxidative pentose phosphate pathway, transketolase A (TktA), transaldolase B (TalB), and ribose-5-phosphate isomerase (RpiA), so that E4P is fed back into the PBG cycle without additional metabolic burden. The full PBG pathway including the salvage system is shown in Figure 4-1B. As described below, the introduction of the salvage pathway proved critical for the efficient production of PHB.

**Initial PHB production and optimization of the PBG pathway:** Following the development and characterization of mutant NAD+ specific reductases, the ATP generating Pfk, and the addition of the salvage pathway as described above, we tested the ability the system to generate PHB. As an initial test, the 20 PBG enzymes (containing both purge valve nodes at Zwf and Gnd) along with the 3 enzymes of the PHB biosynthetic pathway were mixed with cofactors and the reaction initiated with the addition of 50 mM glucose (reaction components given in Supplementary Table 4-2). As shown in Figure 4-3, we see a clear increase in turbidity at 600 nm compared to a control lacking PhaC (the PHB synthase) indicating that PHB is produced.

To test the importance of the secondary activity of xfp with fructose-6-phosphate that leads to the production of erythrose-4-phosphate, we left out the three enzymes salvage pathway (tktA, talB, and
As shown in Figure 4-3, this resulted in dramatically slower accumulation of PHB compared to the entire pathway. The E4P salvage pathway is clearly a critical component of the overall system.

Since the PBG glycolytic pathway produces 4 times as many NAD(P)H than are required to build PHB, the efficiency of each purge valve node was analyzed to determine whether both purge valves were required to maintain NADPH levels and carbon flux to drive this system. The full in vitro system was reconstituted with the either the Zwf purge valve only (eliminating the NAD+ specific enzyme), the Gnd purge valve only, or both purge valves, and the rate of PHB accumulation assessed (Figure 4-3). While both individual purge valve systems maintained NADPH levels and produced PHB, leaving out the Gnd purge valve produced only about half of the carbon flux and PHB compared to using the Gnd purge valve only, or both purge valves simultaneously (Figure 4-3). The dependence of PHB production on the Gnd node suggests that Gnd is the key step, which is likely due to the fact that Gnd is the committed step of the oxidative pentose phosphate pathway because it is driven by the release of CO$_2$.

Finally, to quickly determine whether enzymatic bottlenecks were present in the system, the pathway was systematically reconstituted with 1/5$^{th}$ or 1/25$^{th}$ the starting amount of each enzyme while the other 19 enzymes remained constant and PHB accumulation was monitored at 600 nm (Supplemental Figure 4-5). Lowering the enzyme concentration 25 fold had little to no effect on PHB accumulation for the majority of the enzymes used, indicating that these enzymes were in excess in the current reaction. On the other hand, glk and xfp showed a distinct drop in PHB productivity when they were diluted and together represent potential bottlenecks that restrict carbon flux and PHB accumulation. The final optimized system with highest flux, stability, and PHB production was accomplished by increasing the concentration of enzymes glk and xfp two-fold. The final reaction components are listed in Supplementary Table 2.
Semi-continuous production of PHB from Glucose: To evaluate the efficiency and longevity of the PBG pathway, we tested the ability of the system to process a single large initial input of glucose. A practical constraint, however, is the fact that the PHB synthase (PhaC) remains bound to the end of the growing PHB chain so that when we remove PHB to quantitatively assay the amount produced, we also remove PhaC. Thus, we ran the system in a semi-continuous fashion: at regular time points we removed the produced bioplastic by centrifugation for quantitative assays, and then added new PhaC to allow the reactions to proceed. In these experiments there are no cofactor additions and no other additions of the 20 enzymes with the exception of PhaC.

To monitor the function and stability of the optimized system, the PBG pathway was used to convert either 60.7 or 109.2 mM glucose into PHB from a single addition of glucose (Figure 4-4). The reaction started with the addition of glucose and was allowed to proceed for up to 55 hours (2.3 days) without any addition of cofactors or metabolites to maintain flux in the system. The reaction could be roughly monitored continuously by absorbance at 600 nm, but for a more rigorous quantitative assessment, we employed a GC assay. After each 10 hour cycle, precipitated PHB was pelleted and removed by centrifugation and assayed.

When we start with 60.7 mM glucose, we see rapid production of PHB in the first 10 hour cycles, but then as the glucose is consumed the reaction rates diminish (see Figure 4-4). By the end of the third cycle the reaction stops because there is simply no more glucose to consume. The reaction produced 57 ± 6 mM PHB (monomer equivalents), corresponding to a 94% yield. To see if the system could process glucose for longer periods of time we increased the initial concentration of glucose to 109.2 mM glucose. The results shown in Figure 4-4 show robust activity for 5 cycles with the higher glucose starting concentrations, ultimately providing 93.8 ± 6.1 mM PHB (monomer equivalents), corresponding to an 86% yield. The maximum productivity of the PBG cycle was 0.7 g/L/h of PHB and maintained over
50% of the maximum activity over the entire 55 hour run at room temperature, with titers of 9.8 g/L of PHB from 19.7 g/L of glucose.

Conclusion

Synthetic biochemistry represents a new paradigm for the production of bulk and specialty chemicals and offers a number of distinct advantages over more traditional in vivo pathway engineering in terms of flexibility, ease of optimization, yield and productivity. In this work we were able to leverage the flexibility of synthetic biochemistry to create a completely new glycolytic pathway called the PBG cycle. The PBG pathway represents an expansion of the Synthetic Biochemistry concept through the development of a non-biological glycolytic cycle that generates the widely used biosynthetic precursor acetyl-CoA.

The effectiveness of the PBG pathway was demonstrated in the production of PHB bioplastic in >85% molar yield with a productivity of 0.7 g/L/h and a titer of 9.8 g/L. Based on experience from the ethanol industry, these values are already close to generally accepted thresholds needed for industrial production (90% yields with a productivity of 1 g/L/h and titers of 40 g/L), yet we made little effort to maximize production. Moreover, this early prototype system maintained 50% of the starting productivity after more than 2 days, even without the addition of new cofactors or enzymes other than the final PHB polymerase enzyme that remains bound to the bioplastic.

Our results suggest that Synthetic Biochemistry has the potential to rapidly reach industrially relevant production parameters. The key will be to keep enzyme costs low by finding stable enzymes so they can be used for long periods of time, methods for recycling the enzymes and inexpensive purification methods. Further expansion of Synthetic Biochemistry will require the production and regulation of ATP
as both acetyl-CoA and ATP are needed for the biosynthesis of many desirable products. Still, the PBG cycle represents a significant step forward for Synthetic Biochemistry and demonstrates that in vitro systems can reach yields and productivity required for industrial production of bio-based chemicals.

Materials and Methods

Materials.

Miller LB media or Miller LB-agar (BD Difco) was used for growth of bacterial strains in liquid or solid media cultures. E. coli BL21Gold(DE3) [B, F-, ompT, hsdS2 (rB-, mB-), dcm+, Tet', galλ, (DE3) endA Hte] (Agilent) was used as host for both cloning and expression of recombinant proteins using pET vectors. Plasmid pET28a(+) was purchased from Novagen. HotStart Taq Mastermix (Denville) was used for gene amplification from genomic or plasmid DNA. KOD Xtreme DNA polymerase (Toyobo), Taq DNA ligase (MCLab), and T5 Exonuclease (Epicenter) were purchased separately and used to make the assembly master mix (AMM) used for cloning (ref). ATP, glucose, Coenzyme A, NADP+, glutathione, and NAD+ were from Sigma.

Plasmid Construction.

The expression plasmids for the PHB enzymes were constructed from the pET28a plasmid backbone using the Nde1 and Sac1 cut sites to produce constructs with an N-terminal 6xHis tag for purification. Expression constructs for TktA, TalB, and RpiA were from the ASKA collection (RIKEN) cloned into vector pCAN2430. The genes used in this study are listed in Supplementary Table 2. The gene encoding polyhydroxybutyrate synthase31 (phaC; HE_610111) was synthesized and codon optimized for
expression in an *E. coli* host at Life Technologies before being subcloned into the pET28a expression vector.

**Enzyme purification.**

Cells from 1 L of culture were harvested by centrifugation and resuspended in 150 mM Tris pH 7.5, 100 mM NaCl. The cells were lysed on ice with sonication and the cell debris was removed by 12,000 x g centrifugation at 4 °C. The supernatant was then mixed with 5 mL nickel-nitrilotriacetic acid (NTA) agarose and after 30 minutes, the slurry was loaded onto a column and washed with five column volumes of 100 mM Tris pH 7.5, 100 mM NaCl, 15 mM imidazole. The enzymes were then eluted with 250 mM imidazole, 100 mM Tris pH 7.5. The resulting enzyme was dialyzed into 50 mM Tris pH 7.5, 50 mM NaCl and stored at 4 °C.

**Enzyme activity assays.**

All of the enzymes used in this work were assayed as outlined in Supplemental Table 2. The enzymes were assayed in 50 mM tris buffer pH 7.5, 5 mM MgCl, and 5 mM KCl which mirrors the final glucose to PHB reaction conditions. The activity of NAD(P)H producing or consuming reactions were monitored at 340nm. The activity of ATP consuming enzymes were monitored using a coupled assay with Zwf and NADP+ at 340nm.

**Initial glucose to PHB reactions and optimization.**

Initial reactions for the self-sustaining biotransformation of glucose into PHB were composed of 50 mM Tris pH 7.5, 5 mM MgCl, 5 mM KCl, 1 mM CoA, 0.5 mM NAD+, 0.5 mM NADP+, 50 mM glucose, 0.1 mM
thyamine pyrophosphate, 2 mM glutathione, and 10 mM inorganic phosphate in a final volume of 200uL. The enzyme concentrations are given in the Supplementary Table 2. The reactions were initiated with the addition of glucose and PHB production monitored by absorbance at 600 nm or using a GC assay.

To find the bottle-neck enzyme(s) and optimize the enzymatic levels in the reaction we decreased single enzymes concentrations systematically and monitored the reaction for a similar decrease in PHB. The reaction was reconstituted with the same buffers and cofactors as the initial biotransformation of glucose to PHB and 19 out of the 20 PBG pathway enzymes were held constant while a single enzyme was decreased 5 fold and 25 fold. This was systematically carried out for each of the 20 enzymes and the PHB accumulation after 10 hours was monitored at 600nm.

**Final semi-continuous glucose to PHB reactions and analysis**

Initial reactions for the self-sustaining semi-continuous biotransformation of glucose into PHB were composed of 50 mM tris pH 7.5, 5 mM MgCl, 5 mM KCl, 1 mM CoA, 0.5 mM NAD+, 0.5 mM NADP+, 50 mM glucose, 0.1 mM thyamine pyrophosphate, 2 mM glutathione, and 10 mM inorganic phosphate in a final volume of 200uL. The enzyme concentrations are given in the Supplementary Table 2. The reaction was initiated with the addition of glucose and incubated at room temperature for 10 hours. The PHB was harvested by centrifugation, the supernatant pipetted off, and additional PhaC was added to replace the PhaC that precipitated with the bioplastic. Each addition was identical to the starting amount of PhaC. The reaction was monitored at 600nm and PHB was quantified after each 10 hour cycle.
To assay for PHB the granules were harvested from the reaction mix and dried at 60˚C for 4 hours. The dry PHB was dissolved in 0.5 mL chloroform and 0.25 mL PHA solution (85 mL methanol, 15 mL H₂SO₄, and 0.7 g benzoic acid) and incubated at 95 °C for 4 hours. The reactions were extracted with 0.5 mL water and 1 uL of the organic layer was injected on a 0.25 micron HP-Innowax column in an HP5890 Series II gas chromatogram. The gas chromatography method used an injection temperature that was held at 45 °C for 5 minutes and then increased to 275 °C over 40 minutes. The peak area was compared with an authentic standard to quantify concentrations.
Figures
Figure 4-1. Synthetic PBG cycle for the production of PHB from glucose

Outline of the synthetic PBG pathway for the conversion of glucose to polyhydroxybutyrate. (a) The PBG cycle consists of the pentose phosphate pathway (red), the bifido shunt xfp enzyme and pta enzyme (orange), and the top half of gluconeogenesis (blue). The PBG cycle is connected to the PHB pathway (green) for the conversion of acetyl-CoA to polyhydroxybutyrate bioplastic. The PBG cycle employs 2 purge valves used to maintain NAD(P)H levels and are highlighted with a star. (b) Outline of the synthetic PBG pathway with the dual substrate specificity (xylulose-5-phosphate and fructose-6-phosphate). This cycle requires the use of additional pentose phosphate enzymes tktA, talB, and rpiA to transform the dead end product erythrose-4-phosphate into useful metabolites in the PBG cycle without any additional metabolic burden.
Figure 4-2. Design of Zwf and Gnd enzymes for cofactor switch

Left panel is an outline of the general purge valve concept. Each purge valve consists of a NAD dependent reductase enzyme, an NADP+ dependent reductase enzyme, and a NAD specific oxidase (noxE). The purge valves effectively decouple carbon flux from NAD(P)+ reduction and allow for non-stoichiometric pathway flux in vitro. The top right panel is a structural model of the mutant Zwf enzyme with flipped cofactor specificity. The A47D mutation effectively excluded the 5’ phosphate of NADP+ and has a preference for NAD+. The bottom right panel is a structural model of the mutant Gnd enzyme with flipped cofactor specificity. The triple mutant of N33D, R34Y, and K38L effectively excluded the 5’ phosphate of NADP+ and has a preference for NAD+. The mutations are shown as stick and ball trace and the NADP+ is highlighted in yellow.
The initial PHB production from glucose was monitored by an increase in A600 caused by precipitation of the PHB granules. The reaction was reconstituted with both purge valves (black), one purge valve at Zwf only (blue), and one purge valve at Gnd only (red). The Gnd only purge valve performed as well as using both purge valves together. The reaction was also reconstituted without PhaC (green) and without tktA, talB, or rpiA (pink) as controls.
Figure 4-4. Final semi-continuous production of PHB from 60 or 110 mM glucose

The top left and right graphs show the semi-continuous production of PHB from 60 and 110 mM glucose. Both graphs contain the trace at 600 nm and PHB yields of each 10 hour reaction in triplicate. The bottom left and right graphs show the residual glucose and total PHB produced from 60 and 110 mM glucose. These runs produced 56.8 ± 5.6 mM PHB from 60.7 mM glucose and 93.8 ± 6.1 mM PHB from 109.2 mM glucose which resulted in a 93.6% yield and 85.9% yield respectively. The maximum productivity seen in these reactions was 0.7 g/L/h of PHB.
Figure 4-5. PBG cycle enzyme optimization histogram
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<tr>
<th>Enzyme</th>
<th>Name</th>
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<th>Tag</th>
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Table 4-1. Enzymes in the PBG pathway from glucose to PHB
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Table 4-2. Enzyme activity and units added for the final reaction
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Table 4-3. Zwf and Gnd table for cofactor specificity
REFERENCES


