Production of biohydrogen in metabolically engineered

*Escherichia coli* strains

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ABSTRACT

Microbes have diverse biosynthetic pathways to produce molecular hydrogen and potentially hold the key to the viable macroscale utilization and production of hydrogen from renewable sources. However, low production yield has been a major limiting factor for large-scale biohydrogen production because of various metabolic bottlenecks. Dark fermentations seem to hold the best promise for biohydrogen production due to low costs and relatively high production yields. Because of the relative ease associated with the metabolic engineering of *E. coli*, this organism was chosen for this study. *E. coli* strains were engineered for greater production of hydrogen by using a combinatorial strategy of over-expressing the components of the hydrogen-evolving complex, and the interruption of uptake hydrogenases. This multiple-step approach, which has never been reported in *E. coli*, could shed light on possible bottlenecks in the hydrogen production pathway and eventually result in the engineering of a very efficient hydrogen-producing *E. coli* strain.

Uptake hydrogenases 1 and 2 were deleted in the *E. coli* genome along with *hycA*, which is responsible for repressing the hydrogen-evolving complex. However, the deletion of *hycA* did not increase hydrogen yield compared to wildtype. This result was inconsistent with previous studies and may have been caused by an incorrect construction. Deletion of the uptake hydrogenases in conjunction with an over-expression of *hycEG*, which encodes for the large and small subunits of the hydrogen-evolving enzyme hydrogenase 3, produced about 4.5% more hydrogen than wild-type *E. coli* during anaerobic batch fermentations. Maximum hydrogen yield was obtained at a pH range of 5-6 for all strains. This work provides for the possible application of this
knowledge towards developing a commercially efficient hydrogen-producing strain of *E. coli*
ACKNOWLEDGEMENTS

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INTRODUCTION

In recent years there has been an increasing interest in alternative fuels due to rising petroleum costs, escalating diplomatic tensions with oil producing countries, and the rising levels of greenhouse gases in the atmosphere[1]. The use of hydrogen as a fuel only gives off pure water as a byproduct, making it an attractive non-polluting energy carrier[2, 3]. Hydrogen has the largest energy content per weight of any known fuel, and can be produced by various means[4, 5]. Unfortunately, both of the main methods for hydrogen production, electrolysis of water and thermocatalytic reformation of hydrogen-rich compounds, usually require high energy inputs obtained from non-renewable resources[5]. Known as biohydrogen production, biological production of hydrogen solves this problem by using microorganisms to convert biomass into hydrogen gas[4, 6].

1.1 Biohydrogen production methods

There are several means of biohydrogen production, including direct biophotolysis, indirect biophotolysis, photo-fermentation, water-gas shift reaction, and dark fermentation[5, 7]. Each of these methods uses different microorganisms to produce hydrogen gas. Direct biophotolysis is the most extensively studied method and is usually carried out using photosynthetic microalgae or cyanobacteria[8, 9]. The photosynthetic organisms in this system produce hydrogen and oxygen from water using solar energy. However, this method utilizes expensive and complicated photo-bioreactors that must be supplied with CO₂ and suffers from low solar energy to hydrogen conversion efficiencies[4-6, 8].
Indirect biophotolysis gets around the problems of direct biophotolysis through combining strategies[4]. There are many different ways that indirect biophotolysis is done, but in most strategies, the first step involves growing photosynthetic algae in large ponds to obtain biomass. The second step varies, but usually involves using the biomass in a hydrogen-producing fermentation[4, 5, 8].

One of those hydrogen-producing fermentations is called photo-fermentation. This type of fermentation is carried out by photosynthetic bacteria which use sunlight and biomass to produce hydrogen[8]. However, this production strategy is also plagued by expensive photo-bioreactors and has not been extensively studied [4].

In addition, the water-gas shift reaction of photoheterotrophic bacteria is another way to produce hydrogen. Certain members of the superfamily Rhodospirillaceae can grow in the dark using CO as their sole carbon source and produce CO₂ and H₂ as byproducts. Although this method of production is very efficient, the major obstacle is the need for the CO to be in solution and at a sufficient concentration for the bacteria. Overcoming this problem would involve incorporating new technologies and designing new bioreactors.[5]

Dark fermentations offer a solution to many of the problems that other biohydrogen production methods have. Unlike the water-gas shift reaction or direct biophotolysis, dark fermentations do not require an input of gas to produce hydrogen. All fermentations are done with microorganisms that utilize organic substrates as their carbon sources[5]. This method also solves the problem of expensive photo-bioreactors which are necessary for direct biophotolysis and photo-fermentations. The fermentations utilized in dark fermentations are anaerobic and do not require sunlight[8]. These key
features of dark fermentation keep the bioreactor design simple and reduce production cost by lowering the amount of maintenance and area required[4, 5].

1.2 Hydrogen-producing enzymes

Each one of these biohydrogen production methods is solely dependent on the hydrogen-producing enzymes found inside the microorganisms. These enzymes catalyze a very simple redox reaction: $2H^+ + 2e^- \rightarrow H_2$. In order to perform this reaction, hydrogen evolving enzymes have complex metallo-clusters as active sites and require special maturation proteins[4, 10, 11]. The three main hydrogen-evolving enzymes are nitrogenases, Ni-Fe hydrogenases, and Fe-hydrogenases[12]. Nitrogenases are the most inefficient of the enzymes and require ATP to function. Ni-Fe hydrogenases work about 15 times better than nitrogenases at producing hydrogen and do not require ATP. Not only can Ni-Fe hydrogenases produce hydrogen, but they can also work as uptake-hydrogenases, utilizing the electrons from hydrogen and using them to reduce NAD(P)[13-15]. The most efficient hydrogen producing enzymes are Fe-hydrogenases, which can have an activity 1000 times better then nitrogenases and about 10-100 times better then Ni-Fe hydrogenases[11]. Although their activity is high, Fe-hydrogenases are extremely sensitive to oxygen.[4]

Direct photolysis, the most extensively studied method of biohydrogen production, utilizes Fe-hydrogenases found in photosynthetic organisms to convert solar energy into hydrogen[8, 9]. However, the oxygen produced by photosynthesis interferes with the efficiency of the Fe-hydrogenases and leads to low yields[5].
Dark fermentation seems to hold the best promise for biohydrogen production due to its low cost, a relatively high production efficiency, and stable hydrogen evolving enzymes\[^4\, 5\, 8\]. Ni-Fe hydrogenases and Fe-hydrogenases can be utilized in dark fermentations by using pure cultures or a mixture of anaerobic microorganisms\[^16\]. Since no oxygen is produced or consumed in these reactions, both types of hydrogenases are less likely to be inactivated by oxygen\[^8\]. Organic wastes from agriculture or sewage can be fed into large anaerobic bioreactors, achieving the dual goals of waste management and hydrogen production \[^17\, 18\]. However, several obstacles such as hydrogen consumption by uptake hydrogenases and overall low production yields due to inefficient metabolic pathways limit the viable macroscale production of hydrogen from dark fermentation \[^4\, 6\, 9\].

1.3 Metabolic engineering as a strategy

The theoretical yields of hydrogen from dark fermentations depend largely on the type of anaerobic organisms that are used in the fermentation. Although glucose can theoretically provide 12 mol of hydrogen per mol glucose, there are no metabolic pathways existing in nature that would allow this, since cell growth would not be possible\[^19\]. Facultative anaerobes evolve 2 mol of hydrogen from each mole of glucose consumed, whereas strict anaerobes evolve 4 mol\[^20\]. However, these theoretical yields are based on known metabolism and can be increased by engineering the metabolic pathways that convert glucose into hydrogen\[^8\, 21-23\].

The redirection of metabolic pathways for enhanced production of existing natural products, production of unnatural products, or degradation of unwanted molecules (e.g.
environmental contaminants) is referred to as metabolic engineering. Metabolic engineering joins systematic and quantitative analysis of pathways using molecular biology, modern analytical techniques, and genomic approaches. Since Bailey coined this definition for the emerging discipline of metabolic engineering, tremendous progress has been made in numerous fields [24-28]. Likewise, metabolic engineering could also hold the answer to biohydrogen production problems, by providing a way to eliminate bottle necks, increasing carbon flow to the hydrogen producing pathway, and engineer more efficient and/or oxygen-resistant hydrogen-evolving enzymes[23, 29]. The model organism and facultative anaerobe, *Escherichia coli*, was chosen to undergo engineering for increased hydrogen production due to its well known metabolic pathways, its relative flexibility for genetic manipulation, and the availability of an established genetic engineering tool kit[8, 30-32].

1.4 *Escherichia coli* and mixed acid fermentation

*E. coli* is a facultative anaerobe, which means that it is able to grow in anaerobic and aerobic conditions. When growing in aerobic conditions, pyruvate generated from the break down of sugars is mainly used by pyruvate dehydrogenase (PDH) and then further converted through the TCA cycle or into acetate[33]. In contrast, most pyruvate generated during anaerobic conditions is converted by pyruvate formate lyase (PFL) and produces formate and acetyl coenzyme A (AcCoA). The AcCoA is further broken down into acetate and ethanol. A portion of the pyruvate is converted into lactate depending on conditions. In addition, the cell produces succinate. This anaerobic production of organic acids is referred to as mixed acid fermentation.[34]
Absence of exogenous electron acceptors, such as oxygen and nitrate, requires an alternative way to regenerate NAD\(^+\) in \textit{E. coli} cells. In anaerobic conditions, the excreted products from the fermentation are in reduced form and help cells maintain a constant supply of NAD\(^+\) for glycolysis\cite{35}. The formation of lactate, ethanol, and succinate generates NAD\(^+\), while the production of acetate yields ATP from substrate phosphorylation. The resulting ATP is essential because an incomplete TCA cycle under anaerobic conditions does not generate ATP\cite{33}.

Formate is produced in anaerobic conditions in order to get rid of extra reducing equivalents that would have been lost through the reduction of NAD\(^+\) under aerobic conditions\cite{36}. In addition, formate can be further broken down into hydrogen gas and CO\(_2\) during acidic conditions to maintain pH of the fermentation broth and to lower the concentration of formate in the cell\cite{15, 36}.

\textbf{1.5 Hydrogen metabolism in \textit{E. coli}}

\textit{E. coli} utilizes two modes of hydrogen metabolism: respiratory hydrogen oxidation (uptake) linked to quinine reduction and non-energy conserving hydrogen evolution during fermentative growth \cite{37, 38}. Four hydrogenase isoenzymes have been identified in the \textit{E. coli} genome\cite{39}. Two hydrogenases (hydrogenase 1 and 2) are involved in periplasmic hydrogen uptake, while the others (hydrogenase 3 and 4) are part of cytoplasmically oriented formate hydrogenase complexes\cite{12, 36, 37, 39}. The uptake hydrogenases 1 and 2 are multi-subunit, membrane-bound, nickel-containing Fe/S proteins encoded by the \textit{hya} and \textit{hyb} operons, respectively \cite{12}. Hydrogenase 3, located on the \textit{hyc} operon, produces hydrogen from formate as a part of the formate
hydrogenlyase complex (FHL-1), which is active during mixed-acid fermentation at slightly acidic pH. Hydrogenase 4, located on the hyf operon has been shown to produce hydrogen as part of the FHL-2 complex at slightly alkaline pH[40, 41]. However expression of hydrogenase 4 is not significant in the wild-type strain[39].

The FHL complex of E. coli is a multi-enzyme complex that catalyses the reversible formation of equimolar amounts of H₂ and CO₂ from the oxidation of formate as a response to the acidic conditions under anaerobic fermentations[22]. Formate dehydrogenase (FDH-H), which is encoded by the fdlhF gene is the only protein in the FHL complex that is not encoded by the hyc operon. This gene is regulated by the presence of hydrogenase 3 (Hyd-3), and four polypeptides which are all encoded on the hyc operon[39]. Hydrogenase 3 (Hyd-3) is composed of a cytoplasmically-oriented large subunit, encoded by hycE and a small subunit encoded by hycG. The remaining four polypeptides along with the product of hycG are membrane integral electron transfer components.[36]

Transcription of the FHL complex is activated by the gene product of fhlA, called the FHL activator protein (FHLA) [39]. The FHL repressor protein (HycA) is encoded by hycA, which is found on the same operon as Hyd-3[22]. This repressor binds to FHLA and stops FHL transcription[42].

1.6 Metabolic engineering methods in E. coli

The engineering and regulation of these hydrogenases has the potential to lead to an E. coli strain that is capable of producing large quantities of hydrogen[8]. This
potential has led to multiple studies which have tried to optimize hydrogen production through metabolic engineering[20-22, 43].

One of the most commonly used methods for performing metabolic engineering is the knock-out of particular chromosomal genes[30, 44, 45]. Originally this was performed by creating mutations either by chemical methods or by ultraviolet radiation and then screening them[31]. This method however is very broad, results in many nonspecific mutations, and can be very time-consuming. Fortunately, with the advent of modern molecular microbiology and the availability of entire genome sequences, this method has been replaced by the directed disruption of chromosomal genes[30, 46].

Several ways of performing this directed disruption in E. coli are available, but most methods involve creating the gene disruption on a plasmid and then recombining it into the chromosome. This time consuming step can be avoided by using PCR products that contain antibiotic resistance genes flanked by short upstream and downstream sequences homologous to the flanking sequences of the desired deletion. However, since E. coli naturally contain intracellular exonucleases that break down linear DNA, this recombination is not possible without assistance. Bacteriophages encode recombination systems that can overcome this problem and have been used to develop a highly efficient way to disrupt genes in E. coli.[30]

The Red recombinase method uses a temperature-sensitive plasmid containing λ Red bacteriophage recombination genes that can be transformed into E. coli and allows the recombination of linear PCR products with chromosomal DNA. The resulting transformants can be screened for the presence of antibiotic resistance, indicating that the PCR product containing the antibiotic resistance gene successfully disrupted the gene of
interest. An added feature of this method is that it can also remove the antibiotic resistance gene leaving only a small scar where the gene was. This is done by using an antibiotic resistance cassette that is flanked by FRT regions. These FRT regions are short sequences that are recognized by a temperature-sensitive FLP-recombinase which removes anything between the sites, leaving a small scar.[30]

This method allows for a relatively simple way to disrupt key genes in E. coli's metabolism and has been used to increase lactate and pyruvate production[34, 47].

1.7 Techniques for improved hydrogen production in E. coli

Several ways of improving hydrogen production in E. coli have been described[20, 22, 48, 49]. Some of these methods involve the disruption of lactate production, succinate production, or both[20, 21, 49]. While these studies show that hydrogen production is increased by changing the carbon flow that goes into making formate, they do not combine the strategy with the engineering of the hydrogenases that are involved. Other studies have focused on just the regulation of the FHL complex, but not on the uptake hydrogenases[22, 48].

Up-regulation of the FHL system in E. coli, achieved by blocking the synthesis of the FHL complex repressor, HycA, has been previously described[22]. Using glucose as a substrate, this E. coli HD701 strain evolved hydrogen at twice the rate of its counterpart wild-type strain. A combination of hycA inactivation with the over-expression of the FHL activator fhlA also managed to achieve a 2.8 fold increase in hydrogen production yield compared to the wild-type when formate was used as a substrate[22].
In order to address uptake hydrogenases, double deletions of hydrogenase 1 and hydrogenase 2 from *E. coli* have been previously made and reported on. Unfortunately the study did not focus on fermentative hydrogen production, but rather on the effect that these deletions had on proton translocation[15].

In this study *E. coli* strains constructed without hydrogenase 1 and 2 activity in conjunction with increased hydrogenase 3 activity were characterized for hydrogen production utilizing glucose as a substrate. Hydrogenase 1 activity was eliminated by the directed deletion of *hyaA* and *hyaB*, which encode the large and small subunits of this Ni-Fe hydrogenase. Hydrogenase 2 activity was eliminated by the directed deletion of *hybA*, *hybB*, and *hybC*, which encode for the small subunit, cytochrome b, and large subunits of hydrogenase 2 complex. The FHL complex was up-regulated by the directed deletion of the *hycA*, resulting in repressor loss. All of these deletions were done using the Red recombinase method.

Another method used to increase FHL activity in this study was the overexpression of the genes encoding HycE and HycG subunits of hydrogenase 3 using an IPTG-inducible expression plasmid in the hydrogenase 1 and 2 null strain. Both of the genes were combined using SOE (splicing by overlap extension) PCR. This method of PCR allows the fusion of genes that are not present directly next to one another. The 3' primer for each fragment is designed to overlap 3' sequences on the other fragment. Each gene is amplified separately using the special primers. Then the PCR products from each amplification are combined and amplified using the outer primers. This results in a PCR product containing both genes without the need for multiple restriction digests.[50]
Using these two methods, this study characterized each strain during anaerobic batch fermentations. A variety of analyses were conducted including metabolite analysis, biogas composition, and substrate consumption. This information can be used to better understand how hydrogenase repression and expression effects metabolite concentrations, and use that knowledge to develop better strategies for increasing hydrogen production.
MATERIALS AND METHODS

2.1 Media, chemicals, and cultures

Luria-Bertani (LB) Miller was used in this study and purchased from Sigma (St. Louis, MO). Ampicillin (100 \( \mu \)g/ml), chloramphenical (50 \( \mu \)g/ml), Kanamycin (50 \( \mu \)g/ml), and isopropyl-beta-D-thiogalactopyranoside (IPTG) were purchased from Fisher Scientific (Pittsburgh, PA). Enzymes were purchased from Promega (Madison WI) unless indicated otherwise. The pfu polymerase was from Stratagene (La Jolla, CA). Qiagen kits (Qiagen, Germany) were used to isolate plasmid DNAs, purify DNA fragments from gel, and clean up PCR products. Plasmids and strains used in this study are listed in Table 1.

| Table 1 Strains and plasmids used in this study |
|-----------------|-----------------|
| E. coli K12  |
| BW25113        |
| GW1            |
| GW12           |
| GW12A          |
| pHcyEG         |
| pTrc99A        |
| pTrc99A derivative containing hycEG; Amp\(^R\) |

2.2 Construction of recombinant strains

Gene deletions for the strains GW12 and GW12A were facilitated using the Red recombinase system[30]. Primers used for gene deletions and plasmid construction are listed in Table 2.
Table 2 Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hya-KO-F</td>
<td>atgaataacg agaagaaccatt ttcagggccatgcggtcagggctggttaggcttgagctgcttc</td>
<td>hya deletion</td>
</tr>
<tr>
<td>Hya-KO-R</td>
<td>agctcgctacgctgcggcaggcaacgctgcgtgtgtaacagcagaccgcatatatgtctctcttag</td>
<td>hya deletion</td>
</tr>
<tr>
<td>Hyb-KO-F</td>
<td>tgaacagacgtaatatattatagagacgctctgctgcggtcagggctggttaggcttgagctgcttc</td>
<td>hyb deletion</td>
</tr>
<tr>
<td>Hyb-KO-R</td>
<td>ttcaggaacccactgaacaccacctcgtgctgcgcatccacctatagatatatctctctcttag</td>
<td>hyb deletion</td>
</tr>
<tr>
<td>hycA-KO-F</td>
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<td>hycA deletion</td>
</tr>
<tr>
<td>hycA-KO-R</td>
<td>acacactcatgcacacgcccactccccacacacgcaacgctgcaacctgcctgtgtaaanncgtaacgctgca</td>
<td>hycA deletion</td>
</tr>
<tr>
<td>Hya2-S-F</td>
<td>gcgcagtcgtggccagtcagca</td>
<td>hya deletion confirmation</td>
</tr>
<tr>
<td>Hya2-S-R</td>
<td>cggcagcataacagctgc</td>
<td>hya deletion confirmation</td>
</tr>
<tr>
<td>Hyb2-S-F</td>
<td>gcgccttggggtgcggc</td>
<td>hyb deletion confirmation</td>
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<td>Hyb2-S-R</td>
<td>agctgtcatcttctcagtc</td>
<td>hyb deletion confirmation</td>
</tr>
<tr>
<td>hycA-S-F</td>
<td>tgcggtgaat aatgtcgatg</td>
<td>hycA deletion confirmation</td>
</tr>
<tr>
<td>hycA-S-R</td>
<td>cgcgcggcgggtgcggc</td>
<td>hycA deletion confirmation</td>
</tr>
<tr>
<td>hycE-F</td>
<td>tctcgagctcatgtctgaagaaaaaatg g</td>
<td>amplification of hycE</td>
</tr>
<tr>
<td>hycE-R</td>
<td>tcatcagcatggcgtcctcttaaatgcggtgactctttactttca</td>
<td>amplification of hycE</td>
</tr>
<tr>
<td>hycG-F</td>
<td>tgaagaggctgcaactgtaa</td>
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<tr>
<td>hycG-R</td>
<td>cctatgcatgcgcttgcgaacacacactc</td>
<td>amplification of hycG</td>
</tr>
</tbody>
</table>

The target genes were amplified using primers designed for individual target genes with genomic DNA of *E. coli* strain BW25113 cells as a template. The PCR products were gel-purified using a Qiagen Kit and assembled into a gene-deletion cassette using SOE PCR[50]. To verify the correct deletions of resulting strains, PCR primers designed using the flanking sequences of the deleted genes were used to verify the completion of each gene knock-out. The products from the PCR reactions were separated on a 1% agarose gel. For the over-expression of hydrogenase 3, the genes *hycE* and *hycG*, which encode the large subunit and small subunit of hydrogenase 3, respectively, were assembled into one expression unit using SOE PCR with the primers *hycE*-F/*hycE*-R and *hycG*-F/*hycG*-R and cloned into the plasmid pTrc99A, resulting in pThycEG (Table 1 and 2). The plasmid was transformed into the engineered strains using electroporation and grown in LB medium containing ampicillin (100 μg/ml).
2.3 Fermentations

Anaerobic batch fermentations were performed on strains BW25113 (wildtype), GW12A (ΔhyaAB, ΔhybABC, ΔhyaC), and GW12 (ΔhyaAB, ΔhybABC) with pThycEG induced, and GW12 without pThycEG induced. Each strain was characterized using a 4 port assembly Bellco 500 ml spinner flask, filled with 400 mls of LB media supplemented with 55.5 mM glucose or LB media supplemented with 55.5 mM glucose and antibiotics and grown at 37 °C in a circulating water bath. The media inside the spinner flask was sparged with nitrogen gas for 20 minutes to remove all oxygen. Media inside the spinner flask was brought to pH 6 using concentrated hydrochloric acid prior to inoculation.

Strains were grown in LB or LB with ampicillin (100 μg/ml) to an OD₆₀₀ of 1.0 at 37 °C. Multiple 1.8 ml aliquots of the cultures were stored at -80 °C with 16% glycerol and used as a seed culture for fermentation. Each of these aliquots were individually thawed and 1.5 mls were transferred to a 250 ml erlenmeyer flask containing 50 mls of LB media or LB media containing ampicillin (100 μg/ml) and allowed to incubate in a shaker at 37 °C at 216 rpm until an OD₆₀₀ of 0.6. A 30 ml disposable syringe was used to inject 20 mls of the inoculum into the spinner flask in order to start the fermentation.

The spinner flask was assembled with an autoclavable pH probe (Benson), three septums covering each of the small stainless steel openings from the angled port assembly, a rubber plug in one of the side arms, and sealing compound which was used around all of the openings to make sure that there were no air leaks. The volume of gas produced during the fermentation was measured after inoculation by attaching one end of a tube to one of the spinner flask’s headspace ports and submerging the other end in a 1
liter beaker containing 500 mls of water. A 250 ml plastic cylinder, which was fitted with a septum covered port at its base, was filled with water and inverted over the submerged end of the exhaust tube. This allowed measurement of the gas being produced by water displacement see Figure 1. Samples of trapped gas were analyzed by GC when the cylinder was full of biogas and at the end of the fermentation.

Figure 1. Schematic diagram of the experimental set-up: A- Spinner flask (400 ml media); B- Circulating water bath (37°C); C- Stir-plate; D- pH probe; E- pH meter; F- Gas sampling port; G- Media sampling port; H- Gas exhaust port; I- Gas filter (0.45 μm); J- 1000 ml beaker (500 ml water); K- Inverted 250 ml cylinder filled with water; L- Gas sampling port.

The pThycEG plasmid was induced by adding 4 mls of a filter-sterilized 100 mM IPTG stock solution at 2hrs into the bioreactor at an OD$_{600}$ ~0.5-0.6. The non-induced strain received 4 mls of di-water at 2 hrs into the fermentation to act as a control. Both strains containing plasmids had ampicillin (100 μg/ml) added to the fermentation media prior to inoculation.

2.4 Characterization of fermentations

The pH was monitored by using a Benson pH probe fitted in the spinner flask.
Cell density was recorded by taking a sample from the spinner flask and recording the OD at 600nm spectrophotometrically. Hydrogen evolution was measured using gas chromatography. A 2.5 ml volume of headspace gas was withdrawn every hour using an airtight Hamilton syringe and immediately injected into the side port of a G1540N/6890N network GC system (Agilent) set up with two TCD detectors. Nitrogen was used as the carrier gas for the HP-MolSieve column (15 m by 0.53 mm and film of 50 μm), which was used to measure hydrogen concentration. Helium was passed through a HP-PLOT U column (30 m by 0.32 mm and film of 10 μm) in tandem with a HP-MolSieve column (30 m by 0.32 mm and film of 25 μm) to measure CO₂.

A sample of the fermentation media was taken before inoculation, immediately after inoculation, and at every hour during a twelve hour period. A total of 2.5 ml of fermentation media was withdrawn every hour, with 1 ml of the sample being used for cell density measurements and the rest being filtered through a 0.45 μm PTFE syringe filter (Pall Life Sciences), flash frozen in liquid nitrogen, and stored in a -80 C° freezer for later analysis.

The frozen samples were later thawed on ice and the organic acids were analyzed by HPLC (Shimadzu 10A series). A 20 μl sample was loaded onto a BioRad HPX-87H ion exchange column (300 mm by 7.8 mm) with a mobile phase of 0.008 mM sulfuric acid. The flow rate was 0.3 ml/min with conditions that were isocratic and at a temperature of 60 C°. The organic acids were detected using a UV detector set at 210 nm.

Glucose was measured in each sample by using a spectrophotometric enzymatic assay Kit G3293 (Sigma) by following the manufacturer’s instruction. Ethanol was
measured in each sample using a spectrophotometric enzymatic ethanol assay kit (Biovision) by following the manufacturer’s procedure.

RESULTS

3.1 Gene deletions

The Red recombinase method was used successfully to delete hydrogenase 1 and 2 in strain BW25113. This was confirmed by running a 0.8% agarose gel containing the PCR products from amplifications of the targeted regions (Fig. 2). Figure 2 shows the size of the fragment generated by upstream and downstream primers flanking the desired genes to be disrupted.

![Figure 2](image)

**Figure 2.** Confirmation of the gene knock-out of the large and small subunits of hydrogenase 1 and 2 in engineered *E. coli* strains (0.8% gel, lane A - 1 kb ladder, lane B - *hyaAB*, lane C - *hyaAB*::cat, lane D - *hyaAB*, lane E - *hybABC*, lane F - *hybABC*::cat, lane G - *hybABC*, lane .

These fragments were generated using the wildtype strain, the strain after the double homologous insertion of the antibiotic resistance cassette using the Red helper plasmid, and the strain after the antibiotic resistance is removed by the FLP recombinase plasmid. The results show that the fragments were the correct size in each subsequent
step and confirmed the correct deletion. The same confirmation was done for the *hycA* repressor (picture not shown).

### 3.2 Fermentation gas analysis

The evolution of both hydrogen and CO$_2$ was monitored every hour for a twelve hour period. Each strain was characterized for gas production using gas chromatography. Figure 3 shows that the percentage of CO$_2$ that was evolved into the headspace of the spinner flask was about the same for each strain over a 12 hr period.

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**Percent CO$_2$ in Headspace**

![Graph showing percentage of CO$_2$ in headspace over time for different strains.](image)

**Figure 3.** Percentage of carbon dioxide in headspace over time (wild type – *E. coli* BW25113, GW12 – derivative of BW25113 (*ΔhyaAB ΔhybABC*), GW12A – derivative of GW12 (*ΔhyaAB ΔhybABC ΔhycA*), and GW12 HycEG w and w/o IPTG (*ΔhyaAB ΔhybABC* containing plasmid pHycEG induced or not induced respectively). *E. coli* strains were grown anaerobically in LB supplemented with 55.5 mM glucose in spinner flasks. Carbon dioxide production was detected using a G1540N/6890N network GC system (Agilent). Carbon dioxide percentages are the average of three replicates for all strains except wildtype and GW12 HycEG w/o IPTG, which were done in duplicate. Standard deviation is indicated by bars.
Figure 4 shows that the percentage of hydrogen gas evolved into the headspace during a 12 hr period was about the same for the wildtype and GW12A. However, there was a slight increase in hydrogen percentage for GW12 HycEG with and without IPTG induction. GW12 HycEG w/IPTG showed an increase in hydrogen percentage in the headspace over wildtype between 2 and 8 hrs after inoculation, whereas GW12 HycEG w/o IPTG showed an increase over wildtype between 2 and 12 hrs after inoculation. Wildtype and GW12A again showed very similar profiles. However, this percentage of hydrogen only accounts for the headspace and does not include the hydrogen collected in the gas trap.

Figure 4. Percentage of hydrogen in headspace over time (wild type – E. coli BW25113, GW12 – derivative of BW25113 (ΔhyaAB ΔhybABC), GW12A – derivative of GW12 (ΔhyaAB ΔhybABC ΔhycA), and GW12 HycEG w and w/o IPTG (ΔhyaAB ΔhybABC containing plasmid pThycEG induced or not induced respectively). E. coli strains were grown anaerobically in LB supplemented with 55.5 mM glucose in spinner flasks. Hydrogen production was detected using a G1540N/6890N network GC system (Agilent). Hydrogen percentages are the average of three replicates for all strains except GW12 HycEG w/o IPTG, which was done in duplicate. Standard deviation is indicated by bars.
The results in Figure 5 show that GW12 HycEG with and without IPTG induction produced more total gas than GW12A and wildtype, however GW12 HycEG without IPTG maintained this increase, while the induced strain again dropped back down to wildtype levels after about 8 hrs after inoculation. Wildtype and GW12A again showed similar profiles.

![Volume of Gas Produced](image)

**Figure 5.** Volume of biogas produced over time (wild type – E. coli BW25113, GW12 – derivative of BW25113 (ΔhyaAB ΔhybABC), GW12A – derivative of GW12 (ΔhyaAB ΔhybABC ΔhycA), and GW12 HycEG w and w/o IPTG (ΔhyaAB ΔhybABC containing plasmid pThycEG induced or not induced respectively). E. coli strains were grown anaerobically in LB supplemented with 55.5 mM glucose in spinner flasks. The volume of gas evolved was measured using the water displacement method with an inverted cylinder. Volumes are the average of three replicates for all strains except GW12 HycEG w/o IPTG, which was done in duplicate. Standard deviation is indicated by bars.

The amount of hydrogen in the collected gas was calculated using percentages obtained from the sampling of the gas trap. Since this sampling was done at about 4 hr intervals, the exact amount of total hydrogen was not obtained for each hour. The results in Figure 6 show that GW12 HycEG with and without IPTG produced more total hydrogen over time then did the wildtype and GW12A.
Figure 6. Total amount of hydrogen produced over time (wild type – E. coli BW25113, GW12 – derivative of BW25113 (ΔhyaAB ΔhybABC), GW12A – derivative of GW12 (ΔhyaAB ΔhybABC ΔhycA), and GW12 HycEG w and w/o IPTG (ΔhyaAB ΔhybABC containing plasmid pThycEG induced or not induced respectively). E. coli strains were grown anaerobically in LB supplemented with 55.5 mM glucose in spinner flasks. Hydrogen production was detected using a G1540N/6890N network GC system (Agilent). Total hydrogen was calculated by adding the moles of hydrogen in the gas trap with the moles of hydrogen in the headspace. The totals are the average of three replicates for all strains except GW12 HycEG w/o IPTG, which was done in duplicate. Standard deviation is indicated by bars.

In order to find the rate of hydrogen production over time, the total amount of hydrogen produced was divided by the minutes after inoculation. The results in Figure 7 show that both GW12 HycEG with and without IPTG induction had higher rates of hydrogen evolution than GW12A and wildtype. However GW12 HycEG without IPTG maintained this higher rate, while the induced strain again dropped back down to wildtype levels after about 8 hrs after inoculation. Wildtype and GW12A rates of hydrogen production were about the same.
3.3 Cell density and pH measurements

The pH of the fermentation broth was sampled every hour during the anaerobic fermentations of all strains. The results in Figure 8 show that GW12 HycEG with and without IPTG induction had slightly lower pH than both wildtype and GW12A. There was also a slightly higher pH for GW12A in comparison to the wildtype.
Figure 8. pH of fermentation broth over time (wild type – E. coli BW25113, GW12 – derivative of BW25113 (ΔhyaAB ΔhybABC), GW12A – derivative of GW12 (ΔhyaAB ΔhybABC ΔhycA), and GW12 HycEG w and w/o IPTG (ΔhyaAB ΔhybABC containing plasmid pThycEG induced or not induced respectively). E. coli strains were grown anaerobically in LB supplemented with 55.5 mM glucose in spinner flasks. pH was detected using a pH probe inside the spinner flask. The pH’s are the average of three replicates for all strains except GW12 HycEG w/o IPTG, which was done in duplicate. Standard deviation is indicated by bars.

Cell density measurements were spectrophotometrically obtained at an OD$_{600}$ for each sample. The results in Figure 9 show that the cell densities over time were about the same for each strain, with only one point showing that wildtype had a lower cell density than the rest of the strains. There was a slightly higher cell density for GW12 HycEG with and without IPTG induction, but it was marginal. For this reason, cell density is not used when calculating yields of hydrogen.
Figure 9. Cell density over time (wild type – E. coli BW25113, GW12 – derivative of BW25113 (ΔhyaAB ΔhybABC), GW12A – derivative of GW12 (ΔhyaAB ΔhybABC ΔhycA), and GW12 HycEG w and w/o IPTG (ΔhyaAB ΔhybABC containing plasmid pThycEG induced or not induced respectively). E. coli strains were grown anaerobically in LB supplemented with 55.5 mM glucose in spinner flasks. Cell density was measured by sampling the spinner flask and checking the OD600 of each sample with a spectrophotometer. The OD's are the average of three replicates for all strains except GW12 HycEG w/o IPTG, which was done in duplicate. Standard deviation is indicated by bars.

3.4 Metabolite analysis

The quantity of different organic acids in the media was measured using a HPLC and an organic acids column. The results of Figure 10 show that acetate production increased over time for all strains. GW12 HycEG without IPTG showed the highest initial acetate production rates within the first 5 hours, but GW12A then showed a spike in production rate at about 6 hrs. All experimental strains showed a greater rate of acetate production when compared to wildtype in the first 8 hrs.
Figure 10. Rate of acetate produced over time (wild type – E. coli BW25113, GW12 – derivative of BW25113 (ΔhyaAB ΔhybABC), GW12A – derivative of GW12 (ΔhyaAB ΔhybABC ΔhycA), and GW12 HycEG w and w/o IPTG (ΔhyaAB ΔhybABC containing plasmid pThycEG induced or not induced respectively). E. coli strains were grown anaerobically in LB supplemented with 55.5 mM glucose in spinner flasks. Acetate was measured using an HPLC fitted with an organic acids column. The rate of acetate produced over time is the average of three replicates for all all strains except GW12 HycEG w/o IPTG, which was done in duplicate.

The results of Figure 11 show that the rate of lactate production was initially low for GW12 HycEG with and without IPTG induction, but that at about 5 hrs GW12 HycEG without IPTG starts producing lactate at a rate higher then all the other strains. On the other hand, GW12 HycEG with IPTG started producing lactate at the same rate as the wildtype and GW12A.
Rate of Lactate Produced

![Graph showing rate of lactate produced over time](image)

**Figure 11.** Rate of lactate produced over time (wild type – E. coli BW25113, GW12 – derivative of BW25113 (ΔhyaAB ΔhybABC), GW12A – derivative of GW12 (ΔhyaAB ΔhybABC ΔhyCA), and GW12 HycEG w and w/o IPTG (ΔhyaAB ΔhybABC containing plasmid pThycEG induced or not induced respectively). E. coli strains were grown anaerobically in LB supplemented with 55.5 mM glucose in spinner flasks. Lactate was measured using an HPLC fitted with an organic acids column. The rate of lactate produced over time is the average of three replicates for all strains except GW12 HycEG w/o IPTG, which was done in duplicate.

The results from the formate and succinate quantifications gave so much variability between replicates, that the data was not shown for these two metabolites.

Unfortunately the ethanol assay used also did not give quantifiable data and so that information was omitted as well.

### 3.5 Glucose assay

A spectrophotometric enzymatic assay was done in order to quantify the amount of glucose in each sample taken. The results in Figure 12 show that the percentage of glucose consumed for each strain was about the same until about 4 hrs when GW12 HycEG with and without IPTG induction starts to consume slightly more. GW12 HycEG with IPTG starts to slow down its consumption about 8 hrs after inoculation, whereas
GW12 HycEG without IPTG continues to consume glucose at a slightly higher rate than the rest of the strains. GW12A and wildtype have show very similar glucose consumption rates.

**Percent Glucose Consumed**

![Graph showing glucose consumption over time for different strains.]

**Figure 12.** Percent of glucose consumed over time (wild type - E. coli BW25113, GW12 - derivative of BW25113 (ΔhyaAB ΔhybABC), GW12A - derivative of GW12 (ΔhyaAB ΔhybABC ΔhyeA), and GW12 HycEG w and w/o IPTG (ΔhyaAB ΔhybABC containing plasmid pThycEG induced or not induced respectively). E. coli strains were grown anaerobically in LB supplemented with 55.5 mM glucose in spinner flasks. Glucose was measured using a colorometric enzyme assay. The percentage of glucose consumed is the average of duplicate runs for all strains. Standard deviation is indicated by bars.

3.6 *Hydrogen yields from glucose*

The actual yields of hydrogen from glucose were calculated using the total amount of hydrogen obtained for each time point divided by the moles of glucose consumed. The results in Figure 13 show that GW12A HycEG with and without IPTG had significantly higher rates of hydrogen yields from 0 to 4 hours. After which, the rates
look similar to GW12A and wildtype. GW12A had a slightly lower rate of hydrogen yield than wildtype.

**Figure 13.** Rate of hydrogen yield over time (wild type – *E. coli* BW25113, GW12 – derivative of BW25113 (*Δ*hya*AB Δ*hyb*ABC), GW12A – derivative of GW12 (*Δ*hya*AB Δ*hyb*ABC Δ*hyaC*), and GW12 HycEG w and w/o IPTG (*Δ*hya*AB Δ*hyb*ABC containing plasmid pThycEG induced or not induced respectively). *E. coli* strains were grown anaerobically in LB supplemented with 55.5 mM glucose in spinner flasks. Rates were calculated by dividing the total number of moles of hydrogen obtained at each time point by the moles of glucose consumed and the number of hours after inoculation that the point was taken. These rates are the average of duplicate runs for all strains.

The highest rate of hydrogen yield occurred at a pH range of about 6 to 5.25, which corresponds to about the first 3.5 hrs after inoculation. The results of Figure 14 shows that the GW12 strains with the hycEG plasmid had higher yields (~4.5%) of hydrogen per mole of glucose than wildtype. This figure also shows that both GW12 HycEG with and without induction had nearly the same yields. The wildtype strain is shown to have a yield better than or just as good as GW12A.
Figure 14. Hydrogen yield from glucose at 3 hrs after inoculation (wild type – E. coli BW25113, GW12 – derivative of BW25113 (ΔhyaAB ΔhybABC), GW12A – derivative of GW12 (ΔhyaAB ΔhybABC ΔhycA), and GW12 HycEG w and w/o IPTG (ΔhyaAB ΔhybABC containing plasmid pThycEG induced or not induced respectively). E. coli strains were grown anaerobically in LB supplemented with 55.5 mM glucose in spinner flasks. Rates were calculated by dividing the total number of moles of hydrogen obtained at three hours by the moles of glucose consumed at that time. These rates are the average of duplicate runs for all strains. Standard deviation is indicated by bars.
DISCUSSION

4.1 Gene deletions

Gene deletions of the uptake hydrogenases 1 and 2 were successfully completed in *E. coli* strain BW25311 utilizing the Red recombinase method (Fig 2). The resultant strain, GW12 (*ΔhyAB, ΔhybABC*) was used in a novel combinatorial approach which eliminated uptake hydrogenases and over-expressed key components of the formate hydrogen lyase complex (FHL). One of the strategies used to over-express this complex was the deletion of its repressor gene, *hycA*. Unfortunately, after reviewing the results of the fermentations done with this strain it was found that this deletion did not increase hydrogen production over wildtype, as was previously published (Fig 6)[22]. After careful review of the sequences used to direct the deletion of this gene, it was found that the downstream flanking region had homology to a section of the gene adjacent to *hycA*. This gene, *hycB*, codes for a membrane-integral electron transfer component of the FHL complex[36]. It has been shown that this protein is made up of four [4Fe-4S] clusters and is assumed to function as an electron carrier between formate dehydrogenase and hydrogenase[15]. Since this gene was effectively truncated in half, its ability as an electron carrier was most likely very low. This impairment of the FHL complex could explain why the *hycA* deletion did not show increased hydrogen production over the wildtype. A new downstream flanking region to *hycA* has been designed and will be tested in future studies.
4.2 Fermentation gas analysis

The biogas produced by each strain of *E. coli* in this study was quantified by water displacement and analyzed by gas chromatography. All strains studied had roughly the same percentage of CO$_2$ in the headspace (Fig 3). Although this could be attributed to equal CO$_2$ production, it could also be because CO$_2$ readily goes into solution and small increases could be masked. Dissolution into the media could be one of the reasons that the pH of the highest hydrogen producing strains in this study was slightly lower than the wildtype. Because hydrogen and CO$_2$ are both released when formate is broken down, it would follow that CO$_2$ levels would be higher for more efficient hydrogen producing strains. A very slight increase in CO$_2$ can be seen for GW12 HycEG with and without IPTG at 5 hrs, which along with the decrease in pH, suggests that CO$_2$ levels may be elevated.

Hydrogen gas production in the strains engineered to over-express the HycEG subunits of hydrogenase 3 had the highest rates of hydrogen production (Fig 7). Interestingly, both the induced and non-induced strains gave nearly identical results. It was found that the pTrc99A plasmid that was used to over-express the HycEG subunits was not only inducible by IPTG, but lactate as well[51]. The small amounts of lactate that were being generated during mixed acid fermentation were enough to induce the pThycEG expression plasmid without added IPTG. In fact these small amounts of lactate may have even served as a better inducer of the plasmid than the 1 mM amount of IPTG[51]. This could explain why GW12 HycEG without IPTG continually shows better hydrogen production than GW12 HycEG with IPTG over a long period of time. Future studies will use alternative expression plasmids such as pBAD, since it is only

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inducible by arabinose. The strain GW12A most likely had hydrogen productions similar
to wildtype because of the accidental *hycB* deletion.

### 4.3 Cell density measurements and pH

The pH of the fermentation media was measured using a pH probe fitted inside
the spinner flask. All strains had similar pH profiles during the course of the
fermentation, but there were small differences (Fig 8). As expected, both strong
hydrogen producers showed slightly lower pH than wildtype and GW12A. Again this is
partially due to the equimolar amounts of CO₂ that are produced during hydrogen
generation and partially due to the increased amounts of organic acids being produced in
these strains (Fig 11 and 12). It is interesting to note however, that GW12A had a
slightly higher pH than the wildtype. The damaged HycB protein could be lowering the
efficiency of the FHL complex. The decrease in hydrogen production would also
decrease the CO₂ levels and could be why the pH is higher than the wildtype.

By looking at both the hydrogen production and the pH of each strain, it is
apparent that the faster hydrogen production is occurring between a pH of 5 and 6. This
agrees well with the literature which states that the FHL complex utilizing hydrogenase 3
becomes active at a pH of 6.5 and below[36, 48]. Future studies using these strains
should be conducted at a constant pH of about 5 to 6. This will ensure that the maximum
hydrogen production rate is maintained throughout the fermentation.

The cell densities of each sample were measured at an OD of 600nm. All strains
showed very similar cell densities except for the wildtype at about 5 hrs (Fig 9). This
difference in cell density is most likely attributed to the fact that at about this time the
sample must be diluted to obtain an accurate $\text{OD}_{600}$. This could cause a slight difference in OD if not all the samples are diluted and measured at this time. However, there was a slight increase in final cell density when GW12 HycEG with and without induction were compared to wildtype and GW12A.

4.4 Metabolite analysis

Metabolites were analyzed using a HPLC fitted with an organic acids column. This method allowed the separation of formate, succinate, acetate, and lactate. However, the complex nature of the LB broth used in the fermentation made total separation difficult. For that reason, the results obtained from formate and succinate were not reported due to extremely high variability between replicates. Further work on complete separation must be done for future studies, or alternative assays for these organic acids must be found.

Rates of acetate production for each strain in this study were varied. GW12 HycEG without IPTG had a consistently higher acetate production rates than the same strain without induction, which could also explain the low pH observed in this strain (Fig 10). This elevated acetate production could possibly be explained by the fact that the uninduced strain was using its lactate supply, thus increasing flux towards lactate production and away from acetate production. GW12A also had higher acetate production than wildtype, which is most likely do to the double uptake hydrogenase deletions and not the $\text{hycA}$ and $\text{hycB}$ deletions. It has been shown that both hydrogenase 1 and 2 use the hydrogen that they uptake to regenerate the quinine pool[13]. This is one of the many ways that $E. \text{coli}$ can utilize hydrogen to eventually generate ATP[15].
Without this pathway available, acetate production could be increased to compensate. This could explain why all strains that contained both hydrogenase 1 and 2 deletions had higher production rates of acetate than wildtype.

Lactate is one of the major fermentation end products that compete for carbon flux with formate[21]. This study showed that the GW12A and the wildtype had very similar lactate production rates (Fig 11). GW12 HycEG with and without induction had much lower rates of production initially, but at about 5 hours GW12 HycEG without induction has a large increase in lactate production, whereas the same strain with induction has wildtype levels. This difference in lactate production is probably attributed to the fact that the uninduced strain is using the lactate that it produces to induce the plasmid pThycEG. Because the loss of lactate for the induction of the plasmid is not accounted for, it appears that the lactate production rate is low, but it could possibly be much higher. Again, care must be taken in the future to use an expression system that is not induced by or that can consume fermentation metabolites.

Due to the fact that the enzyme assay for ethanol concentration did not work, no data on ethanol concentrations was obtained. Future work for characterizing ethanol will be done by HPLC on organic acids column and detected by a Refractive Index Detector or RID. This quantification method will eliminate the need for costly enzymatic assays and will save time.

4.5 Glucose assay

Knowing how much glucose has been consumed and converted into hydrogen, is key to understanding how efficient each strain is. Glucose was measured in each sample
using an enzymatic assay. The percentage of glucose consumed did not reach higher than 40% for any of these strains (Fig 12). This was due, in large part, to the rapid decrease in pH that effectively reduced glucose consumption in the cells. However, it was noted that GW12 HycEG did consume more glucose than the other strains, with the uninduced strain coming in at a close second. This is most likely due to the increased flux to the FHL complex. Both the wildtype and GW12A have similar glucose consumption profiles.

4.5 Hydrogen yields from glucose

In order to find out how efficient each strain was at producing hydrogen from glucose, the moles of hydrogen produced were divided by the moles of glucose consumed. This yield was further divided by the hours necessary to achieve that yield, giving the rate of hydrogen yield for each strain (Fig 13). GW12 HycEG both induced and uninduced gave the highest rates of hydrogen yield. This showed that this combination of uptake suppression and hydrogenase 3 over-expression was capable of producing more hydrogen than the wild type. These rates also showed that GW12A had a slightly lower rate of hydrogen yield than the wildtype, which corresponds with what we know about the effect that the accidental HycB deletion could have on this process. This rate plotted against time also shed light on what conditions are best for optimum conversion rates. The pH of the media during peak rates is between 5 and 6.

The maximum hydrogen yield was obtained at about 3 hrs, so hydrogen yield for all strains were compared at this time point (Fig 14). GW12 HycEG with and without induction had the highest hydrogen yield at about 1.29 moles of hydrogen/mol glucose or
65% theoretical. This was about 4.5% higher than the wildtype which only had a yield of 1.23 moles of hydrogen/mol glucose or 61.5% theoretical. Although small, this difference shows that the combinatorial approach is able to increase hydrogen production. The further optimization of fermentation conditions using controlled pH may increase this percentage significantly.

4.6 Future work

This study has shown that maximum hydrogen production is obtained at a pH range of 5-6. Future work will be performed at a constant pH of about 6 in order to achieve that maximum rate throughout the fermentation.

The other factor that greatly affects hydrogen evolution rates is the partial pressures of hydrogen and CO₂ in the headspace. One way to relieve the pressure incurred by CO₂ is to remove it by passing the evolved biogas through a 30% KOH solution. This approach in conjunction with control of the overall pressure in the headspace has been shown to increase hydrogen production significantly. [52]

One of the main metabolites formed during this study was lactate. Lactate formation arises from the conversion of pyruvate by lactate dehydrogenase (LDH). This conversion utilizes pyruvate that could otherwise be used for formate production and eventual hydrogen production. A lactate dehydrogenase null *E. coli* strain has already been constructed and was found to have a 1.2 fold increase in hydrogen production[43]. By disrupting this enzyme, carbon flow should increase and provide significantly more hydrogen production than just the over-expression of hydrogenase 3 and the deletion of the uptake hydrogenases would allow.
Production of succinate, ethanol, and acetate could also be interrupted in order to see what the effect would be on hydrogen evolution. One study showed that a combination of succinate and lactate interruption increased hydrogen production by 68.5% [20].

Formate is produced from pyruvate by the pyruvate formate-lyase complex (PFL) under anaerobic conditions. Recent studies have shown that formate production can be upregulated by over-expressing PFL [21]. This technique could also be used to increase available formate for hydrogen production.

In addition to eliminating competing metabolites and enhancing formate production, more can be done to increase hydrogenase 3 activity by over-expressing the FHL activator (fhlA). This approach has been successful in the past, and when combined with other techniques may yield even higher production [48].
CONCLUSIONS

Metabolic engineering has provided a powerful tool for the construction of bacterial strains that are capable of producing a wide range of useful products[24]. This study has only begun to address the different strategies that could be used to make *E. coli* an efficient factory for the production of biohydrogen. Deletion of uptake hydrogenases and over-expression of hydrogen-evolving enzyme increased the production yield of hydrogen in the engineered *E. coli* strains by about 4.5%. However, this increase was the same for both GW12 HycEG with and without IPTG. Upon further investigation it was concluded that the pTrc99A plasmid that was used to express HycEG was also inducible by lactate[51]. The amount of lactate in the media must have been enough to induce the plasmid even without IPTG.

An error in the construction of the hycA deletion resulted in a portion of the neighboring gene, hycB to be deleted as well. This unwanted deletion most likely resulted in lowering the activity of the FHL complex and therefore strain GW12A did not show increased hydrogen production over wildtype. In fact it can be shown that GW12A had a lower rate of hydrogen yield from glucose then the wildtype at about 3.5 hours after inoculation. Accumulation of intermediate metabolites (e.g. acetate) was measured, but due to large standard deviations nothing solid could be concluded. Fermentation conditions consisting of low pH and high hydrogen and CO₂ partial pressures also limited the hydrogen production of these strains. After about 3.5 hours after inoculation, hydrogen production sharply decreased. This was due to the increasingly acidic pH that was being generated. Future studies will control the pH at about 5-6, where optimum
hydrogen production efficiencies were found. In addition to pH control, pressure control can be used to lower the partial pressure of hydrogen during fermentation. This strategy has been shown to increase hydrogen yields. To further increase the hydrogen production of these engineered strains, efforts will have to be made to increase the available formate for hydrogen evolution either through over-expressing the pyruvate formate lyase complex (PFL), through eliminating competing metabolic pathways such as lactose production, or through a combination of both.
REFERENCES


