

## COVER SHEET

TITLE: Overexpression of the *rnf* operon of *Rhodobacter sphaeroides* to increase hydrogen production

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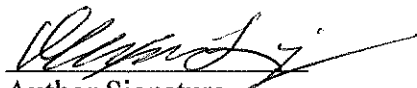
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**ABSTRACT**

**Overexpression of the *rnf* operon in *Rhodobacter sphaeroides* to increase hydrogen production**

Hydrogen has the potential to be a renewable fuel when produced by photosynthetic bacteria. *Rhodobacter sphaeroides* can produce hydrogen via the nitrogenase enzyme. In this research, several methods are used to attempt to create *R. sphaeroides* strains that overexpress the *rnf* operon. This operon codes for a protein complex that is proposed to have a role in providing nitrogenase with electrons to produce hydrogen, and its overexpression is expected to increase hydrogen yield. However, no effect on hydrogen production is observed in the overexpression strains. Future research will assay for transcripts of the *rnf* operon and for the Rnf complex in the experimental strains compared to their controls to determine if our strains do in fact produce higher amounts of the Rnf complex. If these strains are overexpressing the Rnf complex, then this may indicate that the Rnf complex functions somewhat differently in *R. sphaeroides* than has previously been suggested.

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# **Overexpression of the *rnf* operon in *Rhodobacter sphaeroides* to increase hydrogen production**

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## **Abstract**

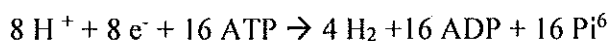
Hydrogen has the potential to be a clean, renewable fuel source when produced by photosynthetic bacteria. *Rhodobacter sphaeroides*, a purple non-sulfur alphaproteobacterium, can produce hydrogen via the nitrogenase enzyme. In this research, several methods are used to attempt to create *R. sphaeroides* strains that overexpress the *rnf* operon. This operon codes for a protein complex that is proposed to have a role in providing nitrogenase with electrons to produce hydrogen, and its overexpression is expected to increase hydrogen yield. However, no effect on hydrogen production is observed in any of the strains we generated. Future research will assay the amount of transcript of the *rnf* operon and the amount of Rnf complex in the experimental strains compared to their controls to determine if our strains do in fact produce higher amounts of the Rnf complex. If these strains are overexpressing the Rnf complex, then the fact that no effect on hydrogen production is observed might indicate that the Rnf complex functions somewhat differently in *R. sphaeroides* than has previously been suggested.

## **Introduction**

Hydrogen gas is a promising alternative to fossil fuels. Hydrogen has the highest energy density by weight of any currently used fuel, and the only product of its combustion is water<sup>7</sup>. However, the most common method of producing hydrogen, steam reformation, relies on the combustion of fossil fuels. In addition to its potential as a biofuel, 9 million tons of hydrogen are produced in the United States each year for use in fertilizer production and other industrial processes; 95% of this hydrogen is produced by steam reformation, which uses natural gas<sup>2</sup>. Microbial production of hydrogen gas could provide an efficient, inexpensive, and clean source of energy. Photosynthetic bacteria can use indirect biophotolysis to produce hydrogen as a natural byproduct of their metabolism. These bacteria use electrons harvested from a carbon

source and light energy to produce hydrogen. The carbon sources used could include wastewater, waste feedstock, or byproducts of industrial processes<sup>5</sup>. By increasing the amount of hydrogen that bacteria can produce from waste products, water, and sunlight, microbes can become a viable and economical source of hydrogen.

The purple non-sulfur bacterium *Rhodobacter sphaeroides* produces hydrogen via the nitrogenase enzyme. Nitrogenase fixes dinitrogen gas from the atmosphere into ammonia, a form of nitrogen the cell can assimilate into its biomass. However, in the absence of N<sub>2</sub> and in the presence of a fixed source of nitrogen other than ammonia, nitrogenase is still active, forming hydrogen using the following equation:



As nitrogenase is highly sensitive to oxygen, this reaction must take place when the cell is growing anaerobically. The ATP and reducing power used by this enzyme are provided by photosynthesis and the oxidation of a provided carbon source. Therefore, increasing the amount of reductant directed towards nitrogenase should increase the amount of hydrogen produced.

Ideally, one *R. sphaeroides* cell could produce 12 moles of hydrogen from one mole of glucose, disregarding biomass generation<sup>7</sup>. To maximize reductant flow and approach this ideal yield, several tactics can be used. One is to decrease the amount of biomass produced by deleting non-essential structures from the cell. Another method is to eliminate pathways that compete with nitrogenase for reductant, such as the Calvin-Benson-Bassham pathway and polyhydroxybutyrate synthesis<sup>13</sup>. Previous research has shown that inhibiting these pathways results in increased hydrogen production<sup>13</sup>. A third tactic would be to increase the expression of

cellular components known to be involved in the flow of reductant towards nitrogenase, which is examined in this research.

One cellular system involved in transporting reductant to nitrogenase in *Rhodobacter* species is the Rnf complex (RnfABCDEFGH). The seven subunits are transcribed from a single operon and the complex functions as a membrane-bound oxidoreductase. This operon is transcribed under conditions where nitrogenase is active<sup>10</sup>. Genetic analysis suggests that the Rnf complex contains iron-sulfur clusters, electron transport systems, and ion channels<sup>1</sup>. This complex is found in a variety of bacteria, as well as some archaea. In many organisms, the complex is used to harness the energy released in transferring electrons from ferredoxin to NAD<sup>+</sup> to create a proton or sodium ion gradient, which can then be used for such purposes as ATP generation<sup>1</sup>. However, the reverse process is thought to occur in *Rhodobacter* species, where the Rnf complex uses a proton gradient generated via photosynthesis to transfer electrons from NADH to a specialized ferredoxin, which then reduces nitrogenase<sup>1,10</sup>.

Overexpression of the *rnf* operon, resulting in higher expression of the Rnf, has been shown to increase both the amount of Rnf proteins in the cell and nitrogenase activity proteins in *R. capsulatus*, presumably by increasing the reduced ferredoxin pool responsible for passing electrons to nitrogenase<sup>4</sup>. In the *R. capsulatus* study, expression of the *rnf* operon in *R. sphaeroides* is increased by placing the operon under the control of the *nifH* promoter, which naturally controls expression of the nitrogenase structural proteins, and is one of the most active promoters in the cell under hydrogen-producing conditions<sup>4</sup>. We hypothesize that increased nitrogenase activity as a result of overexpression of the *rnf* operon will result in increased hydrogen production in *R. sphaeroides*.

## Methods

In this research, attempts are made to increase the amount of the Rnf complex in the cell by overexpression of the *rnf* operon. The parent strains used to create the overexpression strains are wild type 2.4.1<sup>3</sup> and B22-1.2 15, a derivative of 2.4.1 that is unable to fix carbon and produces more hydrogen gas than the wild type under photoheterotrophic conditions (unpublished data). The vectors used are pIND5, which is maintained and replicated within the cell in the presence of kanamycin and contains an IPTG (Isopropyl  $\beta$ -D-1-thiogalactopyranoside)-inducible promoter<sup>11</sup>, and pk18mobsacB, a suicide vector containing a kanamycin resistance cassette and a sucrose sensitivity cassette which is used to facilitate recombination events<sup>9</sup>. Each plasmid made in this project was conjugated into both *R. sphaeroides* parent strains.

## **Plasmids**

Several plasmids were constructed for this study (Figure 1). Construct 1 consists of the *rnf* operon downstream of the IPTG-inducible promoter in pIND5. This plasmid was constructed by amplifying the *rnf* operon from 2.4.1 genomic DNA via PCR. A version named “rnf short” containing only the *rnf* operon (*rnfABCDEFGHI*) and a version named “rnf long” containing the *rnf* operon and the gene *dnaK*, located immediately downstream of the *rnf* operon, were both amplified and used throughout the cloning process in an identical manner. The primers used to amplify the *rnf* regions contained recognition sites for the restriction enzymes NdeI and BamHI. The amplified *rnf* regions and pIND5 were digested with these enzymes and ligated together

Construct 2 consists of pIND5 with the *rnf* operon downstream of the 2.4.1 *nifH* promoter, which controls regulation of the nitrogenase structural genes, one of the most highly

expressed operons in the 2.4.1 genome under photoheterotrophic, anaerobic conditions when ammonia is absent<sup>4</sup>. Construct 2 was created by removing the IPTG-inducible promoter from Construct 1 via restriction digests using NdeI and PciI. The *nifH* promoter was then amplified from 2.4.1 genomic DNA using primers containing these same restriction sites, and the two pieces of DNA were ligated together.

Construct 3 contains the *nifH* promoter flanked by one kilobase of DNA from either side of the native *rnf* promoter on the plasmid pk18mobsacB. The *nifH* promoter and the *rnf* downstream region were amplified via PCR from Construct 2 with primers containing restriction sites for XbaI and AseI. This amplicon was then inserted into pk18mobsacB using these restriction enzymes. The *rnf* upstream region was PCR amplified from the 2.4.1 genome using primers containing restriction sites for XbaI and HindIII, which were used to insert this amplicon into pk18mobsacB directly upstream of the inserted *nifH* promoter.

Once each construct was completed, it was transformed into *Escherichia coli* strain DH5 $\alpha$ <sup>13</sup>. The plasmids were prepped from DH5 $\alpha$  using a Qiagen Mini-Prep Kit. The identity of each plasmid was confirmed via restriction digests and analysis of the cut plasmid via gel electrophoresis. The verified plasmids were then transformed into *E. coli* strain S17-1<sup>11</sup>, which can conjugate with *R. sphaeroides*. Constructs 1 and 2 were conjugated into strains 2.4.1 and B22-1.2 15, and were then prepped using a Qiagen mini-prep kit and confirmed via Sanger sequencing. pIND5 and Construct 2 without the *rnf* operon were conjugated into the *R. sphaeroides* strains to generate the control strains. Each strain was given a unique ID, which can be found in Table 1.

The *R. sphaeroides* strains containing Construct 3 were grown in the presence of kanamycin to select for cells in which Construct 3 had been incorporated into the genome via homologous recombination. The culture was then plated onto kanamycin containing plates and patched onto both kanamycin plates and 10% sucrose plates. Colonies that grew on kanamycin but were sensitive to sucrose were inoculated into Siström's Minimal Media<sup>12</sup> (Sis) with no selective factor to allow the plasmid to excise from the genome via a second recombination event. These cultures were plated onto Sis with sucrose to select for cells from which the plasmid had been excised. Genomic DNA was prepped from these colonies, and the identity of the promoter at the start of the *rnf* operon was confirmed via Sanger sequencing. The parent strains were used as controls. Each derived strain was given a unique ID (Table 1).

### **Hydrogen Production**

All strains were grown photoheterotrophically and anaerobically in modified Sis in which the ammonia had been replaced by additional glutamate to keep the molar concentration of nitrogen constant. Strains containing Constructs 1 and 2 were grown in the presence of kanamycin. Varying concentrations of IPTG (0, 10, and 100  $\mu$ M) were added to strains containing Construct 1 to induce expression of the *rnf* operon. All strains were tested for gas production using an AER-200 respirometer (Challenge Technology, Springdale, AR) to measure the amount of gas produced in comparison to a control. The chemical composition of the gas produced was quantified using gas chromatography. Measures of cell density were taken concurrently with gas production measurements using a Klett-Summerson photoelectric colorimeter (Klett MFG Co., NY). Statistical analysis of data was performed using the program R (r-project.org). Significance is defined as  $p \leq 0.05$ .

## Results

The 2.4.1 strains containing Construct 1 showed no difference in hydrogen production compared to 2.4.1 containing only pIND5 at any IPTG concentration. Increasing concentrations of IPTG were not correlated with increased hydrogen production. No trends were observed between experimental strains or IPTG concentrations in the metrics of both total gas production and rate of production normalized per cell (Figure 2, Table 2). However, more data points are needed to draw conclusions about the hydrogen production of these strains. H<sub>2</sub> production from the B22-1.2 15 strains containing Construct 1 has yet to be characterized.

The 2.4.1 strains containing Construct 2 also showed no significant differences in hydrogen production compared to their control in either total gas production or rate per cell. (Figure 3, Table 3). However, the total hydrogen production of AML 9 compared to AML 7 was nearly significant ( $p = 0.06477$ ) and showed higher hydrogen yield than AML 7 and AML 8. A larger sample size may confirm this trend. The B22-12 15 strains containing Construct 2 have yet to be characterized.

No significant differences were observed between the control and experimental strains created using Construct 3 (Figure 4, Table 4). However, the total hydrogen production of AML 12 compared to its parent strain, B22-1.2 15, was nearly significant ( $p = 0.0690$ ). A larger sample size may confirm this trend, but without further experimentation, we must at this point conclude that these attempts to overexpress the *rnf* operon do not result in increased hydrogen yield in *R. sphaeroides*.

## Discussion

Although not all strains have yet been characterized, and larger sample sizes are needed to confirm any trends, the data seems to indicate that these methods of overexpression of the *rnf* operon do not result in increased hydrogen production. There are several reasons why this may be. For Constructs 1 and 2, a variable number of plasmid copies may be maintained in the cell, as there is no way to control for copy number. However, this effect was likely averaged out over an entire culture of cells and may not have been observable.

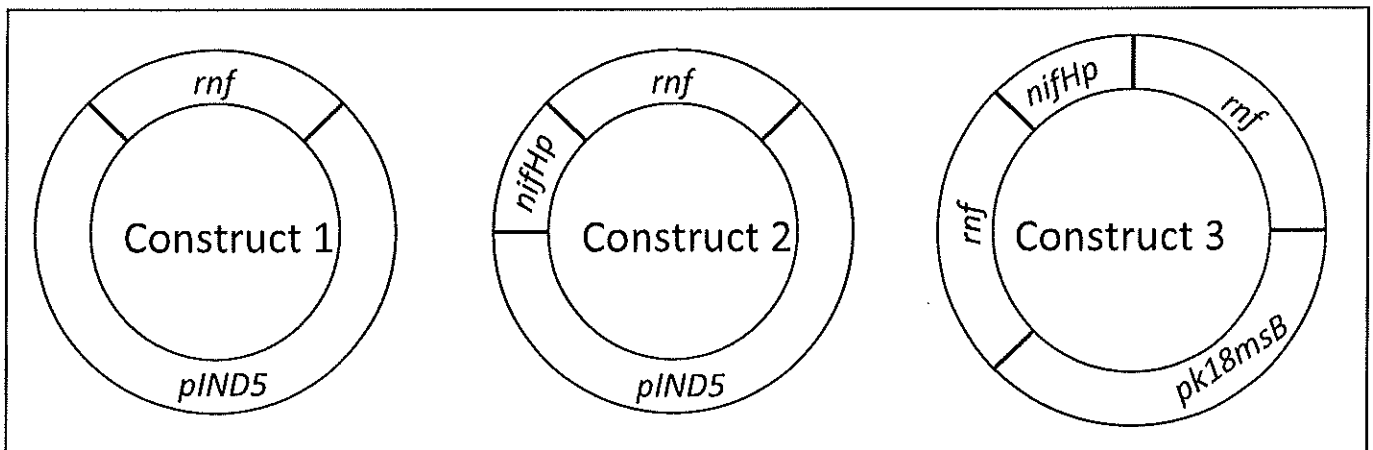
Another issue is that the Rnf complex may not be overexpressed at the translational level. Although our attempted methods of overexpression utilize promoters that would be expected to result in higher transcript production, it does not necessarily result in higher levels of mRNA transcripts or of translated proteins. A regulatory mechanism may be preventing additional Rnf proteins from being made, which would prevent an overexpression of the complex. It can be determined if post-transcriptional regulation is occurring by measuring the amount of mRNA transcripts of the *rnf* operon exist in the cell, and by measuring the amount of translated Rnf protein in the cell.

Further data is needed to confirm whether there are any real differences in hydrogen production between the experimental and control strains. AML 12 showed a larger increase in hydrogen yield compared to its parent strain, B22-1.2 15, than AML 10 did to 2.4.1, although this difference was not significant. Characterization of the remaining B22-1.2 15-derived strains may reveal a trend.

Additionally, we will need to test for overexpression of the Rnf complex in these experimental strains before conclusions can be drawn about its effects on hydrogen production in

*R. sphaeroides*. If no overexpression is found, this would indicate either a flaw in the methods used or a potential difference in regulation of the Rnf complex between *R. sphaeroides* and *R. capsulatus*, since similar methods were shown to result in overexpression of the Rnf complex in this species. If the experimental strains do overexpress the Rnf complex, this could suggest that the complex has a somewhat different function in *R. sphaeroides* than in *R. capsulatus*.

### Tables and Figures



**Figure 1. Vectors used to induce overexpression of the *rnf* operon in *R. sphaeroides* strains.** Construct 1, containing *pIND5* and the *rnf* operon, is intended to be maintained within the cell and expressed via an IPTG-inducible promoter. Construct 2 is identical to Construct 1 except that the *nifH* promoter replaces the IPTG-inducible promoter. Construct 3 consists of the *nifH* promoter flanked by 1 kb regions of DNA located upstream and downstream of the *rnf* promoter in the 2.4.1 genome. This construct was used to replace the native *rnf* promoter with *nifH* promoter via homologous recombination (see Methods).

**Table 1. Strains constructed to overexpress the *rnf* operon.**

Strain ID	Parent Strain	Plasmid construct used to generate strain
AML 1	2.4.1	pIND5
AML 2	2.4.1	Construct 1: rnf short
AML 3	2.4.1	Construct 1: rnf long
AML 4	B22-1.2 15	pIND5
AML 5	B22-1.2 15	Construct 1: rnf short
AML 6	B22-1.2 15	Construct 1: rnf long
AML 7	2.4.1	Construct 2: no rnf
AML 8	2.4.1	Construct 2: rnf short
AML 9	2.4.1	Construct 2: rnf long
AML 11	B22-1.2 15	Construct 2: no rnf
AML 13	B22-1.2 15	Construct 2: rnf short
AML 14	B22-1.2 15	Construct 2: rnf long
AML 10	2.4.1	Construct 3
AML 12	B22-1.2 15	Construct 3

**Table 2. Hydrogen production from strains containing Construct 1.** Data presented in this table represents the average of all cultures of a strain tested. Total hydrogen production is 90% of the total amount of gas produced by a single 19 mL respirometer culture. Rate of hydrogen production per Klett unit was measured by dividing the instantaneous rate of hydrogen production by its Klett unit reading at that time (1 Klett Unit =  $1 \times 10^7$  cells/mL). All values of rate per Klett unit from growing cells were averaged to give the reported values for each culture. Standard error of the mean is given for each data point where multiple cultures were tested.

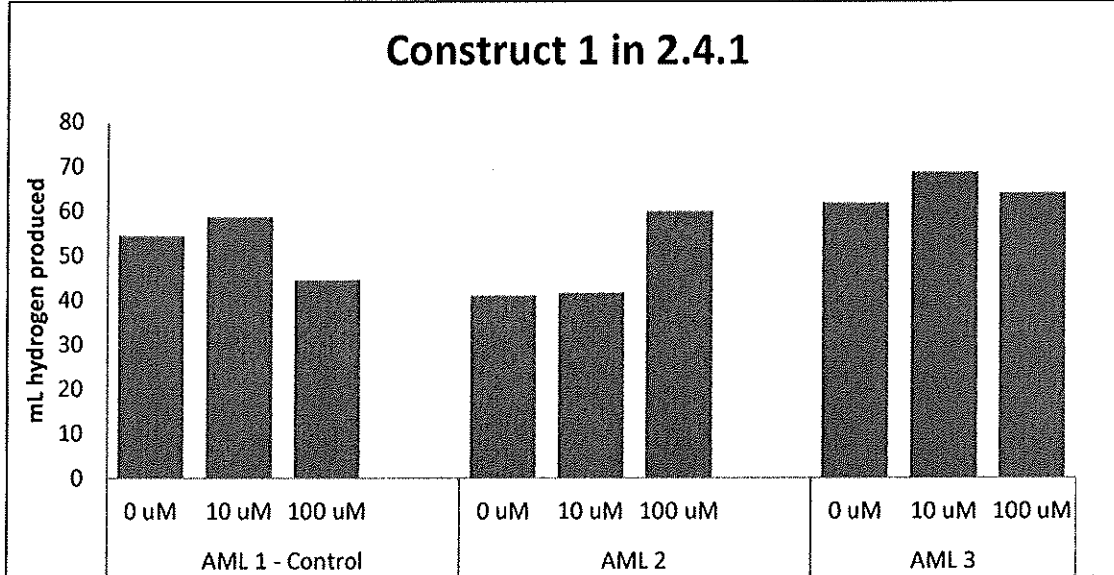
Strain and IPTG concentration	Total hydrogen production	Rate of hydrogen production per Klett unit
AML 1 – 0 $\mu$ M	54.486	0.003290
AML 1 – 10 $\mu$ M	58.761	0.005134
AML 1 – 100 $\mu$ M	44.622 $\pm$ 10.377	0.003520 $\pm$ 0.000692
AML 2 – 0 $\mu$ M	41.058	0.002929
AML 2 – 10 $\mu$ M	41.706 $\pm$ 3.429	0.003805 $\pm$ 0.000241
AML 2 – 100 $\mu$ M	53.938 $\pm$ 2.997	0.002753 $\pm$ 0.000176
AML 3 – 0 $\mu$ M	55.643 $\pm$ 0.0495	0.004288 $\pm$ 0.000067
AML 3 – 10 $\mu$ M	61.767	0.004722
AML 3 – 100 $\mu$ M	57.690 $\pm$ 0.09	0.004677 $\pm$ 0.000092

**Table 3. Hydrogen production of the overexpression strains containing Construct 2.** Data presented in this table represents the average of all cultures of a strain tested. Total hydrogen production is 90% of the total amount of gas produced by a single 19 mL respirometer culture. Rate of hydrogen production per Klett unit was measured by dividing the rate of hydrogen production over 30 minutes by its Klett unit reading during that time period. All measures of rate per Klett unit taken during exponential phase of the culture were averaged. A one-way ANOVA test was used to generate p-values as a measure of significance ( $p \leq 0.05$ ). Experimental cultures (AML 8, AML 9) were compared to AML 7. Standard error of the mean is given for each data point.

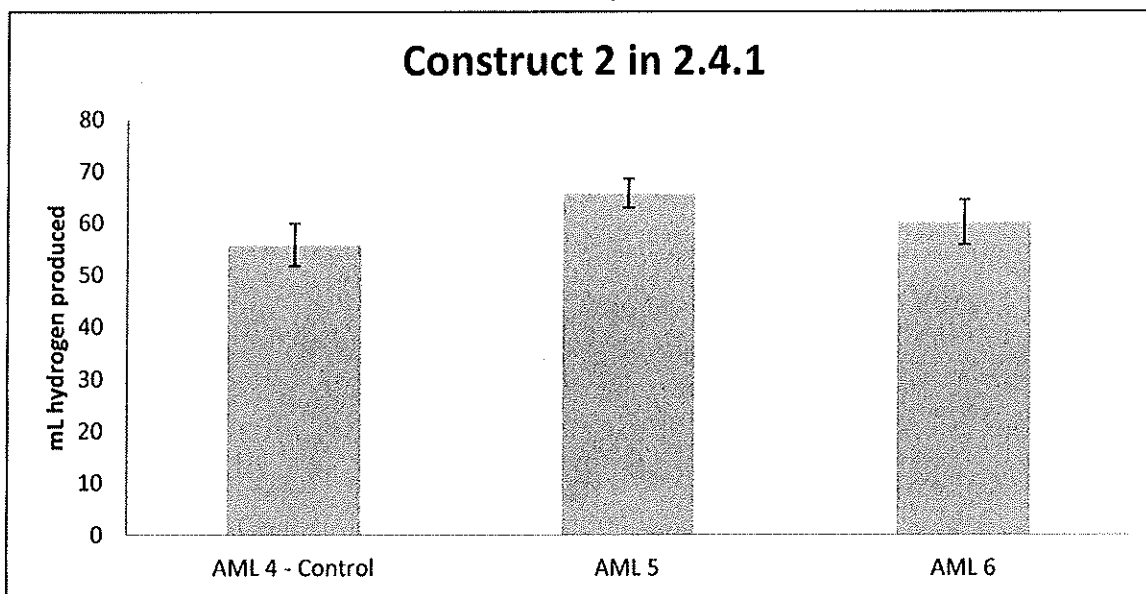
Strain	Total hydrogen production	Rate of hydrogen production per Klett unit	p-value (total hydrogen, rate/KU)
AML 7	54.761 ± 4.089	0.003383 ± 0.000184	NA
AML 8	54.050 ± 10.325	0.003809 ± 0.000364	0.5059, 0.4151
AML 9	62.400 ± 1.420	0.004053 ± 0.000316	0.06477, 0.2114

**Table 4. Hydrogen production of the overexpression strains with the *nifH* promoter in place of the native *rnf* promoter in the genome.** Data presented in this table represents the average of all cultures of a strain tested. Total hydrogen production is 90% of the total amount of gas produced by a single 19 mL respirometer culture. Rate of hydrogen production per Klett unit was measured by dividing the rate of hydrogen production over 30 minutes by its Klett unit reading during that time period. All measures of rate per Klett unit taken during exponential phase of the culture were averaged. A one-way ANOVA test was used to generate p-values as a measure of significance ( $p \leq 0.05$ ). Experimental cultures (AML 10, AML 12) were compared to their parent strains. Standard error of the mean is given for each data point.

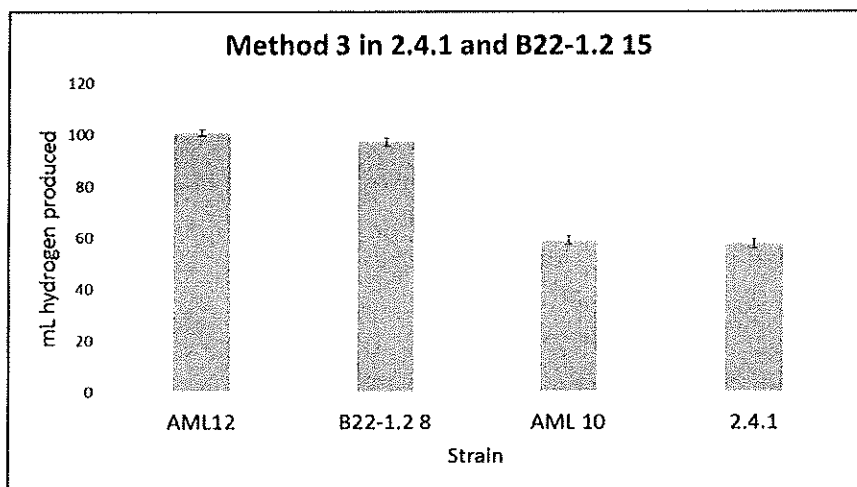
Strain	Total hydrogen production	Rate of hydrogen production per Klett unit	p-value (total hydrogen, rate/KU)
AML 10	59.085 ± 1.738	0.004600 ± 0.000595	0.6324, 0.1978
2.4.1	57.750 ± 1.645	0.005037 ± 0.000678	NA
AML 12	100.766 ± 1.143	0.005398 ± 0.000698	0.0690, 0.1292
B22-1.2 15	97.067 ± 1.572	0.004575 ± 0.000302	NA



**Figure 2. Hydrogen production of 2.4.1 cultures containing pIND5 with the *rnf* operon insert (Construct 1).** Cultures of AML 1, 2 and 3 containing varying concentrations (0, 10, and 100  $\mu$ M) of IPTG were tested, and no effects were found. AML 1 acted as a control strain, as did the samples containing 0  $\mu$ M of IPTG. No significant differences were found in hydrogen production between different strains or between different IPTG concentrations in total hydrogen production or in rate of hydrogen produced per klett unit during log phase.



**Figure 3. Hydrogen production of 2.4.1 cultures containing Construct 2.** Total hydrogen production and rate per Klett unit were measured for cultures of AML 4, 5, and 6. AML 4 acted as the control strain. No significant differences were found in total gas production between the experimental strains and the control, and a large amount of variability was observed between duplicates. No significant differences were observed in rate per Klett units.



**Figure 4. Hydrogen production of cultures containing the nitrogenase promoter in place of the native genomic rnf promoter.** The total hydrogen production and rate of hydrogen production per Klett unit were measured from cultures AML 10 and AML 12, with 2.4.1 and B22-1.2 15 acting as control strains. No significant difference or trend was observed in either metric.

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