Enhanced Butanol Production in *Clostridium acetobutylicum* Using Small Regulatory RNAs

for Metabolic Engineering

HONORS THESIS

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Abstract

This project uses an RNA-based gene expression control system for metabolic engineering of *Clostridium acetobutylicum*. The primary focus is enhancing production of *n*-butanol, a green alternative to fossil fuels. Butanol is an attractive alternative fuel with higher energy density than other biofuels, and can directly replace gasoline. To improve the production of butanol using *Clostridium,* a small RNA (sRNA) platform is utilized. RNA is useful as a genetic regulatory tool because it provides flexible expression tuning compared to the on/off DNA knockout method. sRNAs are used by bacteria for regulating gene expression, as they bind to protein-coding mRNA sequences. Through binding, sRNA can enhance or reduce mRNA translation and thus protein expression. Two genes in the metabolic pathway, *buk* and *hydA*, will be down regulated using sRNA, potentially increasing butanol titer and yield without compromising cell growth. Down-regulation of these genes presents a novel opportunity to modify *Clostridium*, as *hydA* is essential to cellular function and cannot be completely turned off. Furthermore, using sRNA allows for simultaneous targeting of both genes. This is done using genetic engineering techniques to transform wild type cells with the desired genes. The recombinant plasmid for sRNA production is derived from *E. coli* and then ported over. Mutants are then screened and tested to determine performance as compared to the original strain. One mutant and a control plasmid have been successfully transformed. Preliminary results show that the mutant targeting *hydA* successfully down regulates the gene and reduces production of butyric acid, while also increasing butanol production. This project could have significant contribution to improving economic viability of biologically derived *n*-butanol. Furthermore, the sRNA platform has potential for broad applications in metabolically engineering various bacterial species.

Acknowledgments

This project would not have been possible if not for the guidance of my advisors, and graduate students who I worked closely with. I would like to thank Dr. David Wood and Dr. Richard Lease for advising me during my research, and Ashwin Lahiry for instructing me on laboratory procedures. Thanks also to Sam Stimple for support and providing an engaging work environment. Thanks to Dr. Yang's research group for their insight and assistance. And, thanks to the College of Engineering for their generous undergraduate research scholarship.

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F. Liu, C. Xue, K. Xiao, H. Yang, S.T. Yang, High performance PDMS (mixed matrix) membrane for n-butanol recovery from aqueous solution by pervaporation, Bioresour. Technol., in review (2016).

M. Lin, Z. Wang, H. Yang, S.T. Yang, Production of propionic acid from soy molasses by high-cell density fermentation with *Propionibacterium acidipropionici*, AIChE annual meeting, San Francisco, Nov. 13-18, 2016.

Fields of Study

Major Field: Chemical and Biomolecular Engineering

Minor Field: Biology

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Chapter 1: Introduction

1.1 Backgrounds and Motivation

Consumer consumption of gasoline in the United States totals 384.74 million gallons per day. Due to its nonrenewable status, damaging environmental impacts, and trade requirements, alternative fuels are under active development. One such fuel is biobutanol. This four-carbon alcohol is fermentatively derived from biological carbon sources like sugarcane, cassava, and wheat [1]. Biologically produced *n*-butanol (bio-butanol) displays potential as a gasoline alternative, sharing many key traits and being directly usable in cars and pipelines without infrastructure changes. Currently, ethanol can be blended at up to 85% volume as a gasoline substitute, but butanol can fully replace gasoline with less performance trade-offs in conventional combustion engines [1]. Butanol has 87% of the energy content of gasoline, compared to only 67% for ethanol [14, 16]. Furthermore, bio-butanol has industrial applications as a chemical feedstock, solvent, lubricant and diluent [1, 2, 4].

The foremost microbe used in fermentative butanol production is *Clostridium acetobutylicum.* This is a gram-positive, spore-forming, rod-shaped, obligate anaerobe. Furthermore, it is capable of utilizing a wide variety of carbon sources to grow, and produce metabolites. Fermentation of butanol using the Acetone-Butanol-Ethanol (ABE) metabolic pathway in *C. acetobutylicum* is a well-understood process. This fermentation has been used industrially since 1916, and was first discovered in the late 1800's by Louis Pasteur [14]. For example, in the Second World War, *C. acetobutylicum* was used to produce acetone for use in explosives [2]. Other microbial systems are being researched for potentials use in producing this biofuel, but thus far *Clostridium* remains the most promising means of doing so [1, 2]. The ABE fermentation pathway in *C. acetobutylicum* is shown in **Figure 1**. This pathway consists of two

phases: acidogenic and solventogenic. The acidogenic phase has acid production (acetate and butyrate), with ATP formation and hydrogen evolution. Acid production reduces external pH, and triggers the solventogenic phase. This phase produces the pathway's namesake solvents acetone, butanol, and ethanol in a 3:6:1 ratio [1,5,7]. Regulation of this pathway is complex. *C. acetobutylicum* produces butanol through the alcohol-aldehyde dehydrogenase (*AAD)* and *AAD2* enzymes, expressed by the genes *adhE* and *adhE2* [7]. In the wild type microbe, *C. acetobutylicum* ATCC 824, butanol production reaches about 10.3 g/L via the solventogenic phase, while acidogenic fermentation can produce up to 5.5 g/L [7, 8]. Butanol is produced by enzymatic transformation of butyryl-CoA into butanol, as ethanol is transformed from acetyl-CoA. Metabolite production is found primarily in the stationary phase. Butyrate, is produced via expression of the *buk* gene, which directly competes for carbon flux with butanol production [3].

While the *Clostridia* butanol fermentation pathway is well understood, there are several barriers to the economic feasibility of bio-butanol: substrate costs, titer/selectivity, and recovery costs [1, 2, 4]. Currently, biobutanol has a market price of $7.94 per gallon vs. ~ $2.00 per gallon gasoline. Production costs for biobutanol range from one to three dollars per gallon [15]. To reduce production costs, yield and selectivity must be increased. Conventional metabolic engineering strategies have shown successful application towards this goal [6-12]. Metabolic engineering typically involves gene manipulation through addition, deletion, or over-expression in a pathway. While metabolic engineering techniques are easily applied in well-understood bacteria like *E. coli*, *Clostridia* lack the genomic background for easy genetic manipulation. Attempts to knock out acidogenic pathways that produce byproducts have not fully succeeded. While single knock out attempts have succeeded [2], double knockouts targeting multiple genes have failed, since the pathways are important to ATP production and cell health [11, 12]. This is

especially relevant in concerning the *hydA* gene, which is expressed in hydrogenase production, and is essential to microbe survival. Previous attempts to knock-out this gene have proven unsuccessful [11, 12].

Small regulatory RNA (sRNA) is universal in bacteria for regulation of gene expression. These are complex polymers that base pair with messenger RNA (mRNA). Normally, DNA encodes genes that are transcribed to mRNA. This is then translated to proteins. But, when sRNA binds mRNA, protein synthesis can be prevented. In *E. coli*, DsrA sRNA (DsrA) has tertiary structure in the form of stem-loops, and is used in activation of *rpoS* and *hns* translation [3]. See more about the DsrA in **Figure 2**. The DsrA has three loops in its stem, with the relevant sequences on the first two. It is dual acting; modifying the code of one of the stem loops can produce synthetic sRNA variants, which are re-targeted as desired. Since we can modify two loops, we can thus target two genes: *hydA* and *buk*. As mentioned, these genes regulate the hydrogen and butyrate production pathways, respectively, as seen in **Figure 1**.

sRNA regulation provides a more flexible and tunable alternative to knocking out DNA genes. Advantages of using this tool include portable and cost efficient control of the pathways, as well as circumvention of tampering with the host chromosomal DNA. The sRNA can be used to target mRNA used for translating the fermentative enzymes used in a metabolic pathway of interest. Thus, down-regulation presents an intriguing alternative to completely eliminating undesirable side reactions or components of a metabolic pathway [3]. Post-transcriptional tuning of gene expression for *hydA* and *buk* can enhance titer by directing carbon flux through the ATP and butanol production pathways. This has a dual effect of reducing side reactions and increasing the primary reaction.

1.2 Problem Statement

Improving yield and selectivity of the fermentative process is a challenging problem. The goal of this project is to increase these in *C. acetobutylicum* by targeting the *hydA* and *buk* genes using an sRNA based metabolic engineering tool. This project also seeks to demonstrate the effectiveness of this tool as a platform that can be easily used across differing microbes. The platform can circumvent the present conundrum of deactivating vital genes, and also provide a flexible, simpler method to act on dual-targets within a microbe. The effectiveness of retargeting the DsrA stem loop will be tested for three different targets: *hydA* expression, *buk* expression, and both *hydA* and *buk* gene expression. Each of these will be tested for enhanced butanol production at different concentrations of this sRNA in *C. acetobutylicum*, as compared to the ATCC 824 wild type, using the native *E. coli* DsrA, and current standards of butanol production.

1.3 Organization

Chapter two of this thesis discusses the materials and methods used in executing the project. In Chapter three, results and discussion, as well as setbacks and are discussed. Chapter four provides a summary and conclusion, as well as suggestions for future work. Figures are placed at the end of each appropriate section.
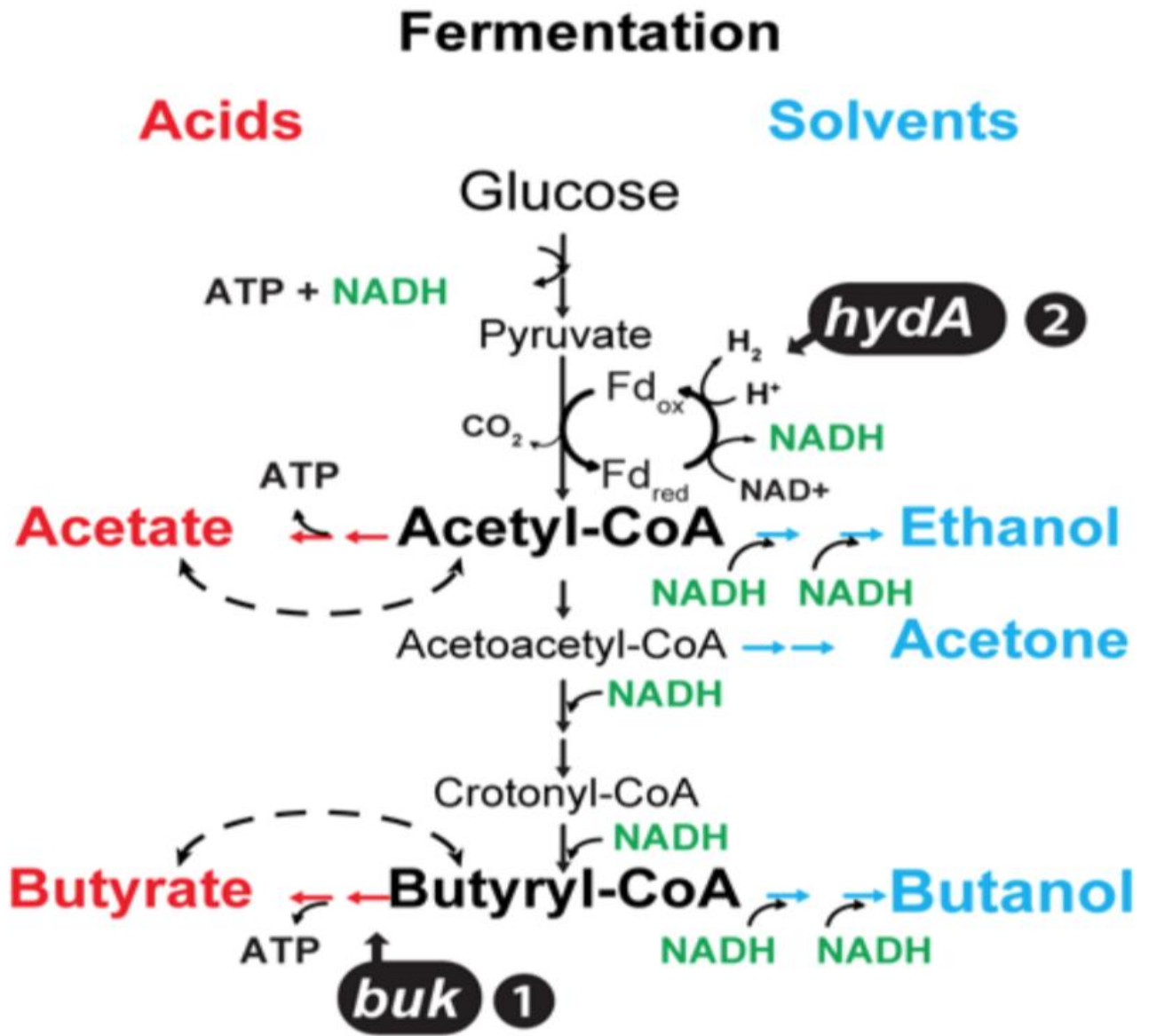
Figure 1: *C. acetobutylicum* Metabolic Pathway [19]

Enzymes are shown with arrows. Acid and solvent fermentations are shown in red and blue respectively. Acetate and butyrate pathways comprise the acid production. The hydrogen pathway is regulated by *hydA* (essential). An enzyme generates NADH from NAD, which is favored when this pathway is blocked. Acid production detracts from butanol synthesis and selectivity. The hydrogen pathway also consumes NADH required for butanol production.
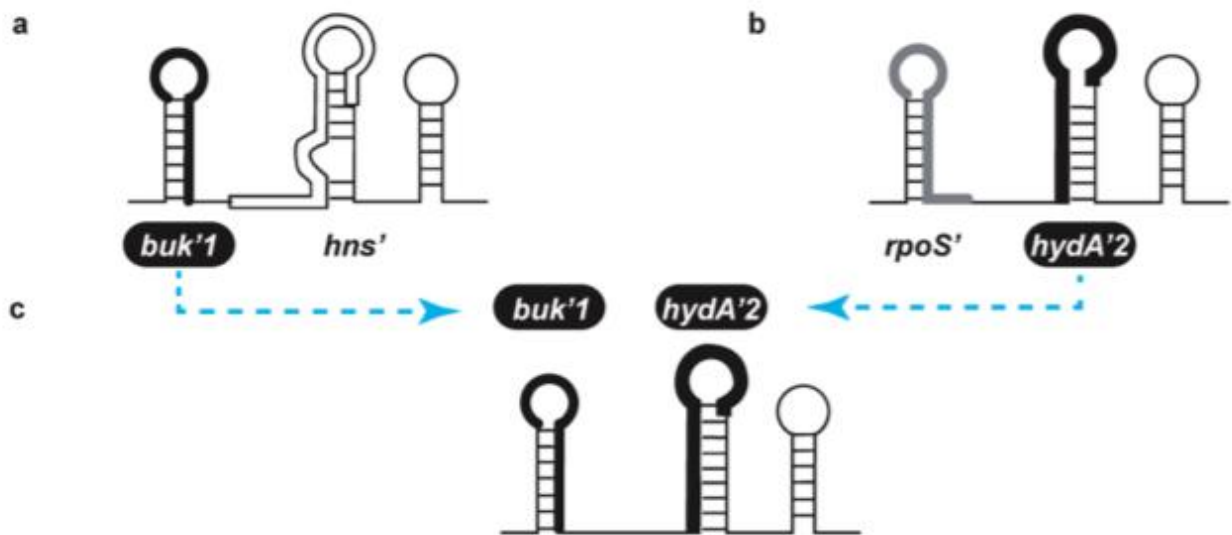
Figure 2: sRNA Scaffold Design [18]

The RNA loops on itself three times, due to complementary sequences. Here are shown three

variants with a) being retargeted for *buk,* b) being retargeted for *hydA,* and c) having both targets.

Chapter 2: Methodology

2.1 Bacterial Strains and Plasmids

In order to perform this experiment, two strains of *E. coli* were required, and *Clostridium acetobutylicum* ATCC 824 was used. Plasmid amplification was performed using the *E. coli* strain CA434, graciously provided by Dr. Yang's laboratory. Constructed vectors were transformed into CA434 using heat shock transformation. Subsequent methylation of the plasmid was undertaken in *E. coli* strain pAN2, also provided by Dr. Yang's laboratory. This was then used to transform the final vector in *C. acetobutylicum* ATCC 824. All *E. coli* cells were grown aerobically in LB medium (LB Broth, Miller R453642) with shaking in a rotary shaker, at 37°C. *Clostridium acetobutylicum* was grown in clostridial growth medium (CGM). Strains were retained via 15% Glycerol stocks constructed and stored at -80°C for up to eight months.

Furthermore, the construction of several plasmids was necessary. Plasmid construction will not be discussed in this work, as Ashwin Lahiry undertook this. Several initial plasmids have been constructed for use in proof of concept work. Proof-of-concept plasmids are pAL001 (containing a *hydA* targeting DsrA), pALDsrA (containing the native *E. coli* DsrA), and have pMTL85151 as the control plasmid for transformation into *Clostridium*. These vectors were designed for use in either electroporation or conjugation into *C. acetobutylicum*. Features of interest include the *ORF H* gene for plasmid replication within *C. acetobutylicum*, *ColE1* for plasmid replication in *E. coli*, *catP* for thiamphenicol resistance, and *traJ* for *E. coli* conjugation. Plasmid maps can be found in **Figure 3**. A list of strains and plasmids is provided in **Table 1, Appendix A.**

2.2 Transformation Methods

2.21 Heat Shock

Heat shock transformation was executed using 50 μL of CA434 cells, and 10 μL of ligation product. These were cooled to 4°C, mixed and held on ice for 30 minutes. Subsequently, the mixture was placed in a 42°C water bath for one minute, and then returned to ice for five minutes. The mixture was then regenerated in 240 μL of SOC medium (Invitrogen 15544-034) and held for 60 minutes at 37°C and 250 rpm. The mixture was then spread onto plates having cycloserine as a selective agent. All cell exposure to air was performed near a flame to ensure sterility.

2.22 Electroporation

C. acetobutylicum ATCC 824 cells were grown in 50 mL serum bottles in CGM to mid-log phase, based on an OD_{600} of 0.6. These were inoculated using a 2.5 mL seed culture grown overnight in an anaerobic test tube containing 5 mL CGM, inoculated with 250 μL of a culture regenerated from 15% glycerol stock and grown overnight to OD_{600} of 2-3. Cells were harvested via centrifugation at 2200g, for 10 minutes at 4°C. The cells were then resuspended and washed in a total of 60 mL ETM buffer (270 mM saccharose, 0.6 mM Na_2HPO_4, 4.4 mM NaH_2PO_4, 10 mM $MgCl_2$). This suspension was centrifuged for 10 minutes at 2200g, again at 4°C. The resulting pellet was resuspended in total 1.5 mL ET buffer (270 mM saccharose, 0.6 mM Na_2HPO_4, 4.4 mM NaH_2PO_4). Electroporation cuvettes (0.1 cm inter-electrode distance) were placed on ice for 15 minutes, before adding 400 μL cells, and 10 μL (400 ng/μL) of recombinant DNA plasmid. Cuvettes were held on ice for another 15 minutes, before performing electroporation. Electroporation was performed using 2kV voltage, 200 Ohms resistance, and 25 μF capacitance (Bio-Rad Gene Pulser). Resulting time constant values were kept under 3 msec.

After electroporation, cuvettes were immediately returned to ice for 10 minutes. Subsequent regeneration in 1 mL of liquid CGM was performed at 37°C until bubbles indicated growth (4 – 12 hours). Then, 3 mL of liquid CGM with thiamphenical (15 µL/mL final concentration) was added. This was grown at 37°C for 12 hours, or until substantial growth was observed. Resulting cells were confirmed as described in 2.3, before being stored at -80°C in 15% glycerol stock solutions. All electroporation steps were performed anaerobically using an anaerobic chamber.

2.23 Conjugation

C. acetobutylicum ATCC 824 cells were grown in 5 mL liquid CGM at 37°C using a anaerobic tubes at OD_{600} of 2-3, inoculated with 250 µL of a culture regenerated from 15% glycerol stock and grown overnight to OD_{600} of 2-3. *E. coli* CA434 cells harboring recombinant plasmids were grown in liquid LB medium containing 30 mg/L chloramphenicol at 37°C and 250 rpm to reach OD_{600} of 1.5–2.0. 3 mL of donor CA434 cells were centrifuged at 4000f doe 2 minutes before being resuspended and washed using 1 mL of sterile phosphate-buffered saline (PBS). This as mixed with 0.4 mL of recipient ATCC 824 cells, and spotted onto well-dried CGM agar plates using a pipette. Plates were then incubated at 37°C for 24 hours to allow conjugation to occur. After 24 hours, 1 mL CGM was applied to each plate to harvest cells. Harvested cells were resuspended and spread onto CGM plates containing 45 mg/mL thiamphenicol and 250 mg/mL cycloserine as selective agents. Plates were incubated for 2-3 days to allow colony growth, and positive transformants were then confirmed as described in 2.3, before being stored at -80°C in 15% glycerol stock solutions. All conjugation steps were also performed in an anaerobic chamber.

2.3 Mutant Confirmation

Mutant confirmation was done using colony PCR screening and plasmid extraction. Colonies were added to a PCR reaction using the appropriate *Taq* polymerase and primers. Resulting DNA products were confirmed using gel electrophoresis.

DNA was extracted by mini prep, as follows. 2 mL culture was centrifuged at 10,000g for 10 minutes. The pellet was resuspended in 200 µL P1 buffer, and 10 µL lysozyme added. The mixture was held at 37°C for 20 minutes. Then 250 µL P2 buffer was added, the solution gently mixed and let sit for 5 minutes. 350 µL N3 buffer was added and solution centrifuged at 21,000g for 5 minutes. The solution was then transferred to a filter, and centrifuged for 2 minutes at 6,000g. A 700 µL silica wash was added, and centrifuged at 10,000g for 1 minute. The pass through was discarded and filter centrifuged again at the same settings. 30 µL de-ionized water was added and let stand for 1 minute before centrifuging at 10,000g for 5 minutes. The pass through was recycled and centrifuged again at the same settings. Resulting DNA was then sent for final confirmation via sequencing.

2.4 Fermentation

Fermentation kinetics were studied using serum bottles. 125 mL serum bottles were prepared with 50 mL CGM, and purged to anaerobic condition using N_2 for a time period of 10 minutes. These were inoculated using 2.5 mL of a seed culture grown overnight in an anaerobic test tube having 5 mL of CGM, inoculated with 250 µL stock culture regenerated from 15% glycerol and grown overnight to OD_{600} of 2-3. Fermentation broth was sampled at 12 hour intervals, using 1-mL syringes, checked for OD_{600} and pH and centrifuged for 15 minutes at

12,800 rpm. The supernatant was collected and stored at -20°C for future analysis. All fermentative runs were performed in triplicate.

2.5 Analytical Methods

Cell density was measured using a spectrophotometer (UV-16-1, Shimadzu, Columbia, MD), at 600 nm. Glucose values were analyzed using a high-performance liquid chromatograph (HPLC, LC-20AD, Shimadzu, Columbia, MD) equipped with an HPC-87H organic acid analysis column (Bio-Rad) at 65°C, with 0.6 mL/min, 5mM H_2SO_4 as the mobile phase, as described elsewhere [2]. A gas chromatograph (GC-2014 Shimadzu, Columbia, MD) equipped with a flame ionization detector (FID) and a 30.0 m fused silica column (Stabilwax-DA, 0.25 mm film thickness and 0.25 mm ID, Restek, Bellefonte, PA) was used to analyze butanol, ethanol, acetone, acetate, and butyrate values. The GC was run at an injection temperature of 200°C with 1 μL of sample injected using an auto injector (AOC-20i, Shimadzu). Column temperature was held at 80°C for 3 minutes, increased at 30°C/min to 150°C, and held at 150°C for 3.7 minutes. Furthermore, enzyme activity assays were used to determine level of sRNA expression within fermentation cultures.

2.6 Statistical Analysis

Average values from triplicate bottles were calculated and reported.

Chapter 3: Results and Discussion

3.1 Roadblocks and Troubleshooting

Although transformation of *C. acetobutylicum* has a demonstrated history of success and should be trivial, initial attempts to transform our plasmids into *Clostridium* were unsuccessful. There have been many unexpected challenges. First, electroporation results were showing odd sized DNA results from colony PCR, not appropriate to the sizes expected for our recombinant DNA plasmids. Eventually, it was found that our ATCC 824 stock was impure, having some plasmid already within it. This also had the unfortunate effect of conferring antibiotic resistance to our control/recipient strain. Upon determination of this, a fresh strain was ordered from ATCC. The first strain we received was nonproductive and had to be replaced. Having fixed our stock ATCC 824 issue, we determined that the agar present in reinforced clostridial media (RCM) was preventing proper purification of cells from medium components during electroporation preparation steps. This caused electroporation cuvettes to be burst, and reduced successfulness of the transformation. Upon switching to CGM media, initial attempts at *C. acetobutylicum* culture were met with perplexing unsuccessfulness, and we determined that the type of tryptone and yeast extract used were not appropriate quality for use in CGM. Issues with anaerobic chamber integrity have also set back progress and complicated transformation.

3.2 Metabolic Engineering Results

Plasmids have been constructed, and verified in *E. coli.* The pAL001, pALDsrA, and pMTL85151 plasmid maps are shown in **Figures 3 - 5.** Currently, pAL001 and pMTL85151 plasmids have been successfully transformed into *C. acetobutylicum* ATCC 824. These mutants are referred to as *C. acetobutylicum* pAL001 and *C. acetobutylicum* pMTL85151, respectively.

Sequencing has shown that the pAL001 mutants are a mixed population, with some having lost part or all of their synthetic sRNA sequence conferring *hydA* regulation.

3.3 Fermentation Results

Exploratory fermentation was performed to compare *C. acetobutylicum* pAL001 to ATCC 824, using serum bottles run in triplicate for 60 hours. Fermentation data from the first run differed from those of the second two, so all data is reported based on the duplicates. Data tables are attached in **Tables 2 and 3**, in **Appendix B**. As seen in **Figures 6 & 7**, the pAL001 mutant significantly outperforms ATCC 824 in regards to both butanol and butyrate production. pAL001 produces ~0.45 g/L butanol vs. 0.05 /L in ATCC 824. This is almost 10-fold improvement. Furthermore, butyrate production is severely reduced: 0.01 g/L vs. 0.27 g/L. Reduction is 27-fold. Data must be considered in light of ATCC 824 having acid crashed during these studies. That is, pH values were below optimal for solventogenesis. Comparison of other solvents production also produced interesting results. While ethanol production was approximately equal between both strains, acetone production was improved four fold in pAL001. It produced ~0.2 g/L acetone compared to ~0.05 g/L in ATCC 824. Acetic acid production was unchanged from the wild type strain.

Both strains reached OD_{600} of 5.5, but the wild type strain had a longer lag time before the log phase: 20 hours vs. 10 hours in the mutant strain. Figures showing discussed comparisons are given in **Appendix C**.

Unfortunately, neither strain performed as expected, faring far worse than literature values show the wild type being capable of achieving. Furthermore, pH shift experienced was inconsistent between the two strains, with the wild type seeming to have remained in the

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acidogenic phase, causing acid crash and preventing entry into solventogenesis. The mutant

strain demonstrated superior pH regulation, switching from acid production to solvent production

earlier relative to the control. Furthermore, neither strain seemed to consume much glucose,

having only used 20 g/L equivalent over the course of a 60-hour fermentation. These

complications can be attributed to the serum bottle fermentations being performed in CGM,

rather than one designed for metabolite production like P2 medium. This was done due to a lack

of the necessary medium components to produce P2. Even so, the total productivity of carbon

conversion to metabolites was much lower than expected.
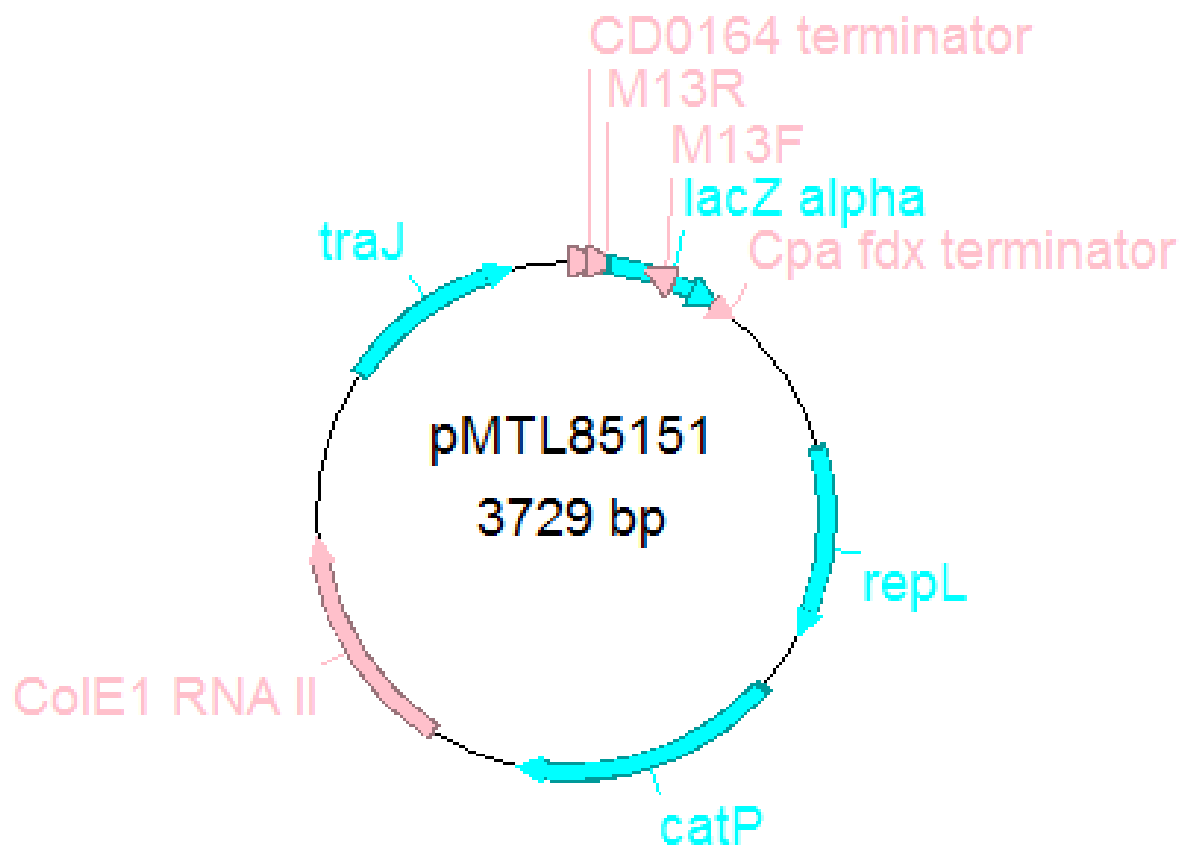
Figure 3: pMTL85151 Plasmid Map.
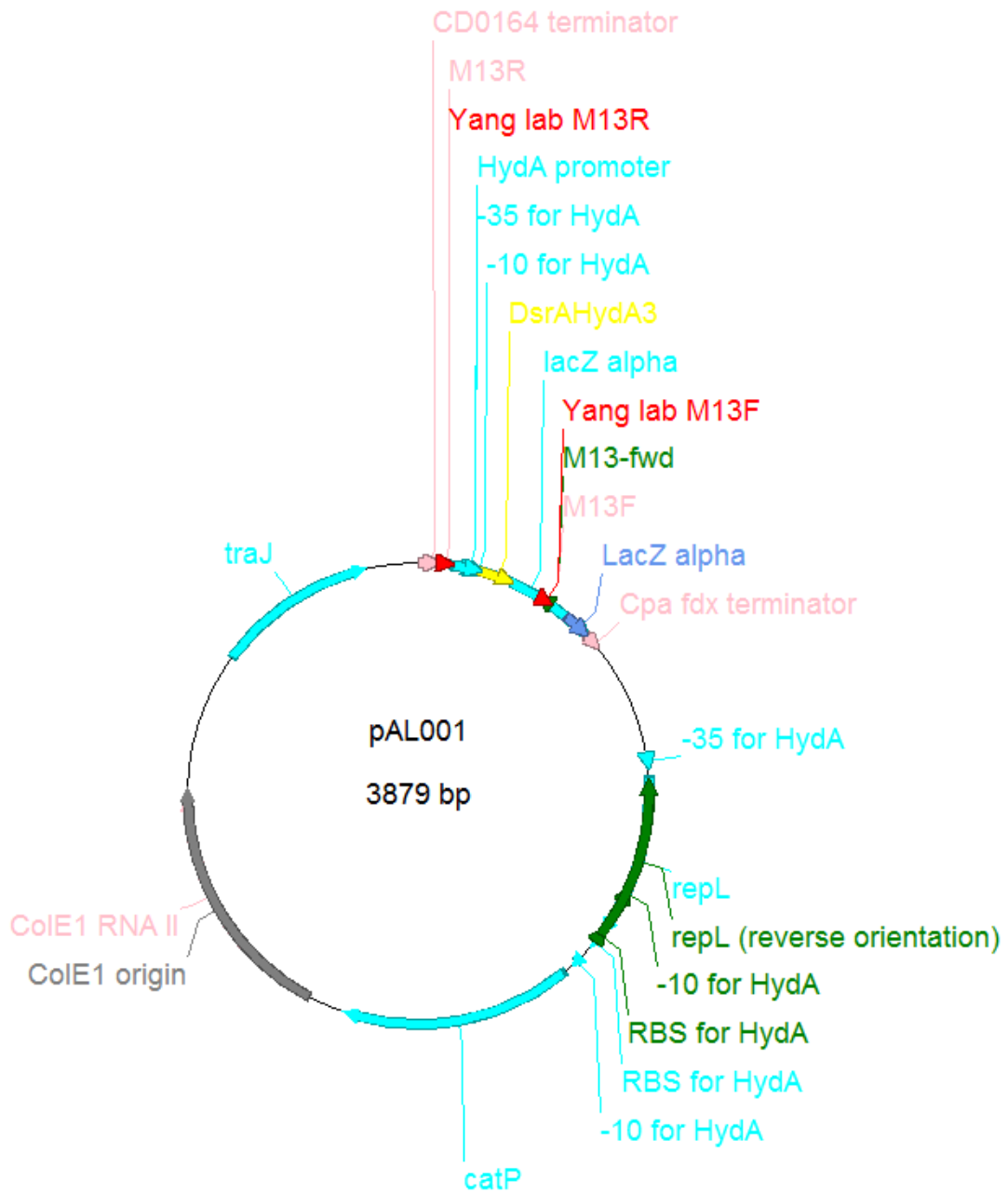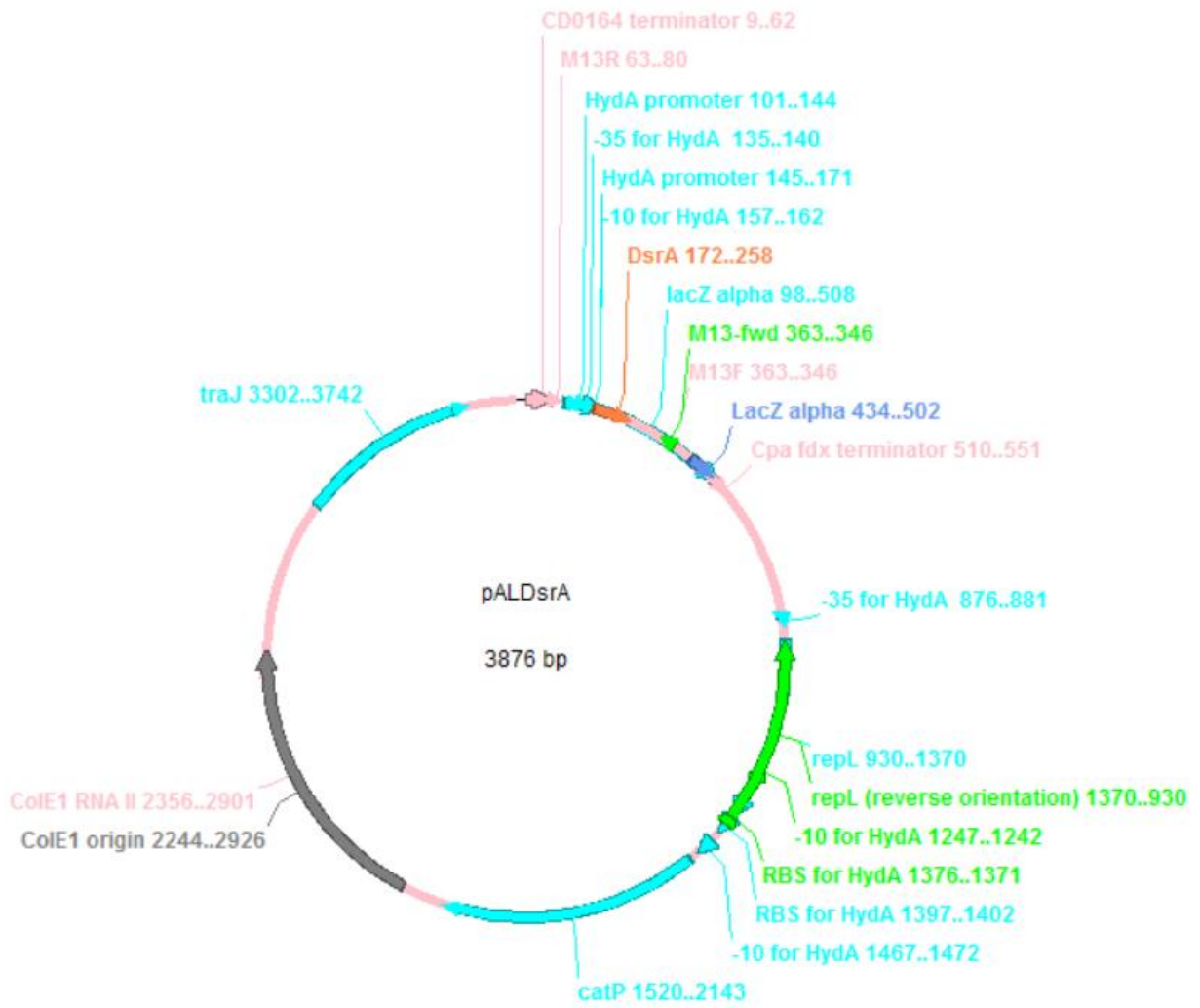
Figure 4: pAL001 Plasmid Map.
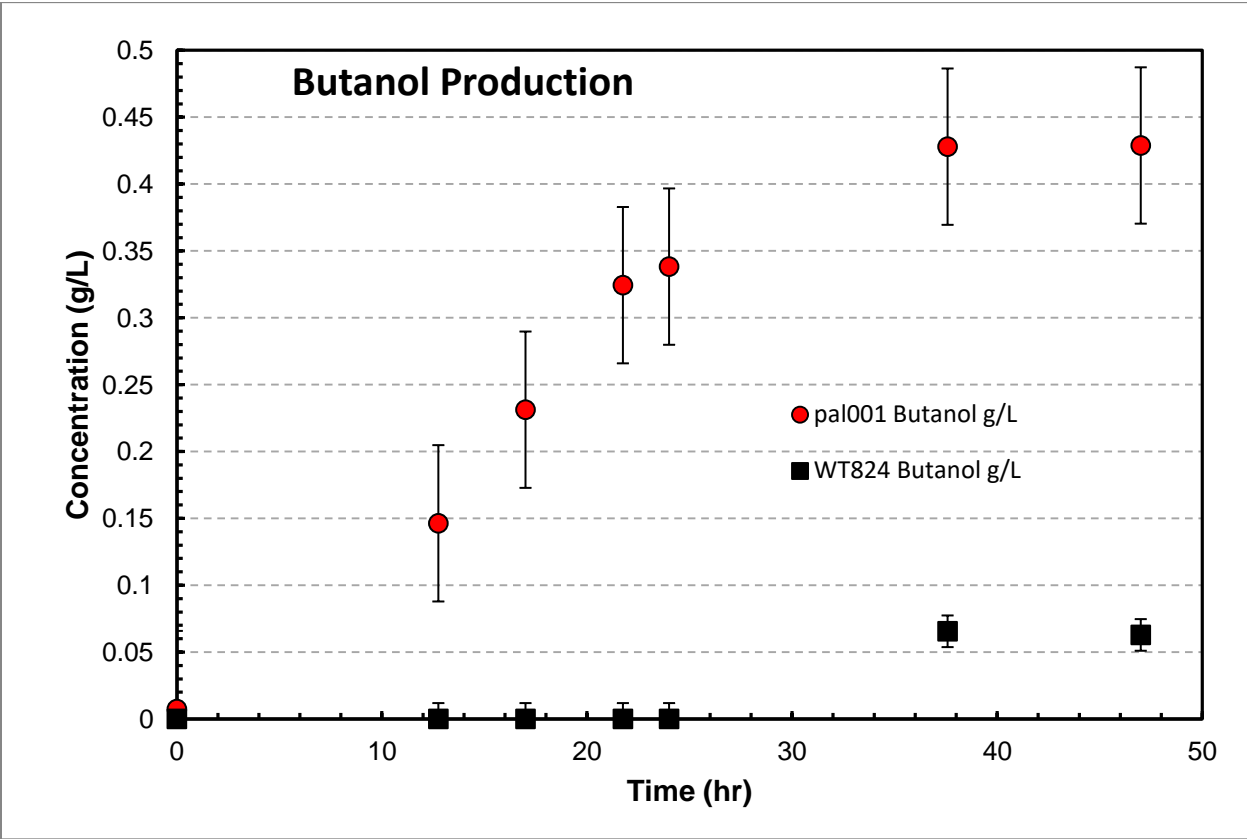
Figure 5: pALDsrA Plasmid Map.

Figure 6: Comparison of Butanol Production between pAL001 Mutant and ATCC 824 Control
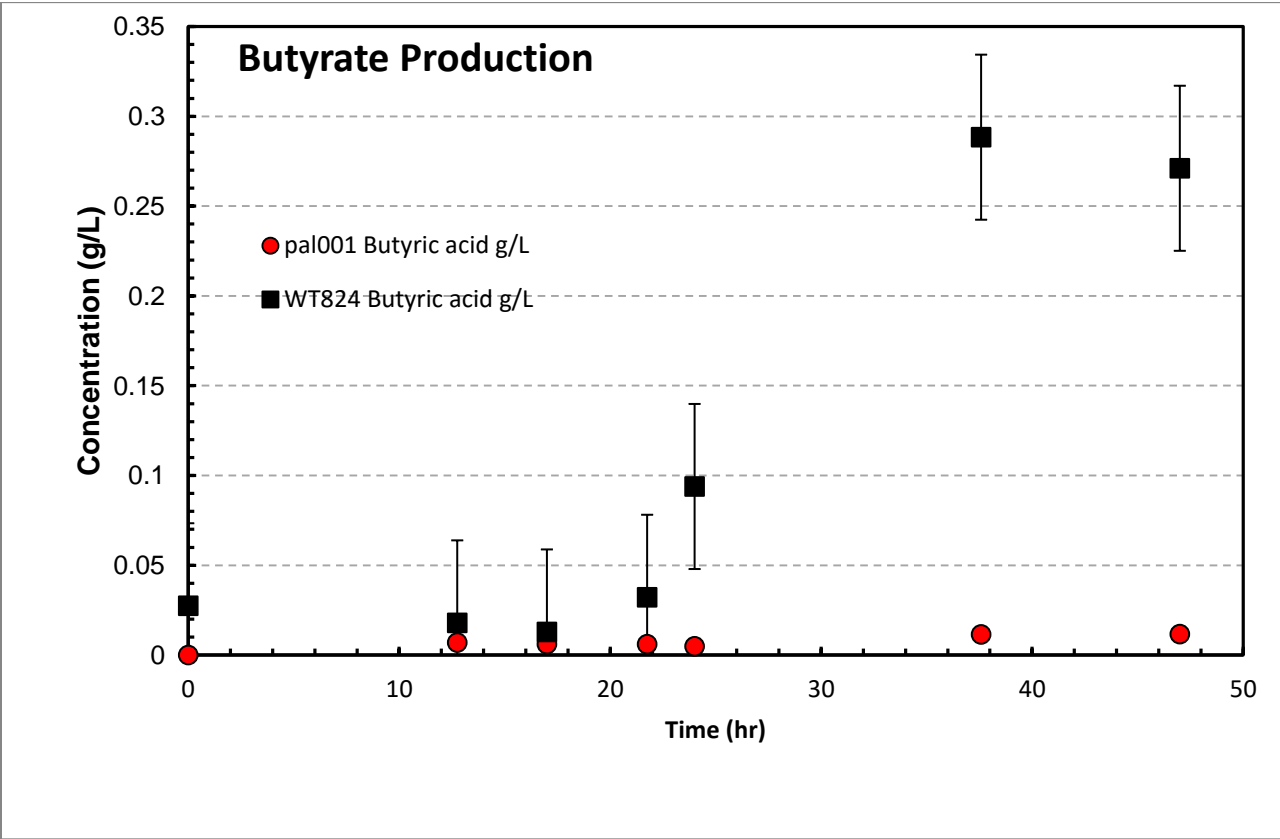
Strain.

Figure 7: Comparison of Butyrate Production between pAL001 Mutant and ATCC 824 Control

Strain.

Chapter 4: Conclusions & Recommendations

4.1 Conclusions

Butanol is a promising alternative fuel that can directly replace gasoline in current infrastructure. The major impediment to butanol utilization is high production costs, primarily stemming from low yield and selectivity in fermentation. Standard metabolic engineering practices have demonstrated some effectiveness in improving the *C. acetobutylicum* production of butanol, but have not managed to achieve economic feasibleness. One major problem is the targeting of organism essential genes, such as *hydA*. Since these cannot be knocked out, there is little that can be done to redirect carbon flux to desirable pathways. Furthermore, construction of double knock-outs is a time consuming and nontrivial process. We have developed an sRNA gene expression control system is a promising metabolic engineering tool, which provides a finessed alternative to the classical knockout technique. This both allows targeting of essential genes, previously inaccessible for metabolic tuning, and also an easy platform to construct dual-targeting mutants.

Preliminary data shows that our single target mutant strain outperforms the wild type, albeit both strains performed quite poorly in the initial fermentation.

4.2 Recommendations

The mutant pAL001 strain is currently being purified to ensure a pure population before moving forward with further fermentation and assays to quantify its performance. In light of poor performance from both pAL001 and ATCC 824 in CG, fermentation should be repeated in the appropriate P2 medium. Furthermore, all constructed mutants are being ported over to the pMTL82151 plasmid, which has better compatibility and ease of insertion into ATCC 824.

Further mutants are also currently being transformed into *Clostridium* to test their effect when targeting the *buk* gene and using the native *E. coli* variant. As well, work is being done to transform the dual acting sRNA into *Clostridium*, as this is the best outcome mutant being explored by this project.

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Appendix A: List of Strains and Plasmids.

Table 1: List of Strains and Plasmids.

STRAINS	
C. acetobutylicum	*E. coli*
ATCC 824 (Wild Type)	CA434 (plasmid amplification strain)
pAL001 (*hydA* targeting mutant)	pAN2 (methylation strain)
pALDsrA (native *E. coli* DsrA mutant)	
pMTL85151 (control transformed mutant)	
PLASMIDS	
pAL001 (*hydA* targeting plasmid constructed using pMTL85151)	
pALDsrA (native *E. coli* targeting plasmid constructed using pMTL85151)	
pMTL85151 (Original Base Plasmid)	
pMTL82151 (New Base Plasmid)	

Appendix B: Serum Bottle Data Tables.

Table 2: Fermentation Data for pAL001 Mutant.

Time (hrs)	0	12.75	17	21.75	24	37.58	47
Acetone (g/L)	0.008065	0.063641	0.10206	0.121275	0.159715	0.198425	0.198155
Ethanol (g/L)	0.0103	0.014575	0.01443	0.01729	0.000215	0.00025	0.000245
Butanol (g/L)	0.007355	0.14628	0.23123	0.324365	0.338245	0.427935	0.428815
Acetate (g/L)	0.210295	0.188035	0.17876	0.1661	0.138325	0.126115	0.122915
Butyrate (g/L)	0	0.00693	0.00606	0.00603	0.00486	0.01147	0.01164
OD	1.045	3.4	4.875	5.4	5.81	5.69	5.31
pH	6.22	4.925	4.93	5.15	4.85	4.83	4.805
Glucose (g/L)	62.74583647	39.88660139	38.70951544	34.10620136	32.03950121	29.54347644	28.59067704

Table 3: Fermentation Data for ATCC 824 Control.

Time (hrs)	0	12.75	17	21.75	24	37.58	47
Acetone (g/L)	0.00508	0.00422	0.00584	0.00315	0.00508	0.05134	0.04972
Ethanol (g/L)	0.01363	0.01037	0.00565	0.00693	0.00011	0.00016	0.00016
Butanol (g/L)	0	0	0	0	0	0.06554	0.06281
Acetate (g/L)	0.22784	0.23455	0.17786	0.19898	0.15484	0.15761	0.14945
Butyrate (g/L)	0.02742	0.0179	0.01284	0.03215	0.09385	0.28836	0.27104
OD	0.51	0.53	0.55	1.01	2.14	5.41	4.9
pH	5.6	6.08	6.16	5.17	4.28	3.72	3.75
Glucose (g/L)	62.76675872	56.32270483	45.00795046	34.76441543	34.87237426	32.62197673	27.32697297
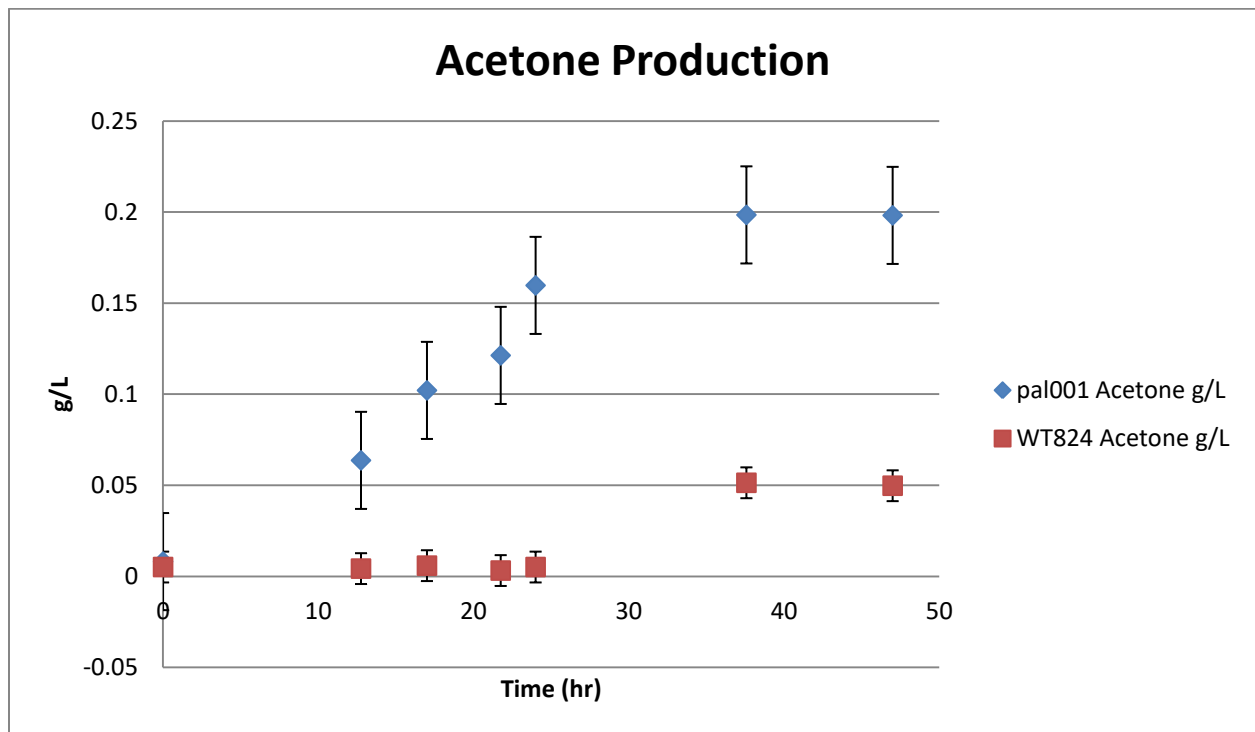
Figure 8: Comparison of Acetone Production.
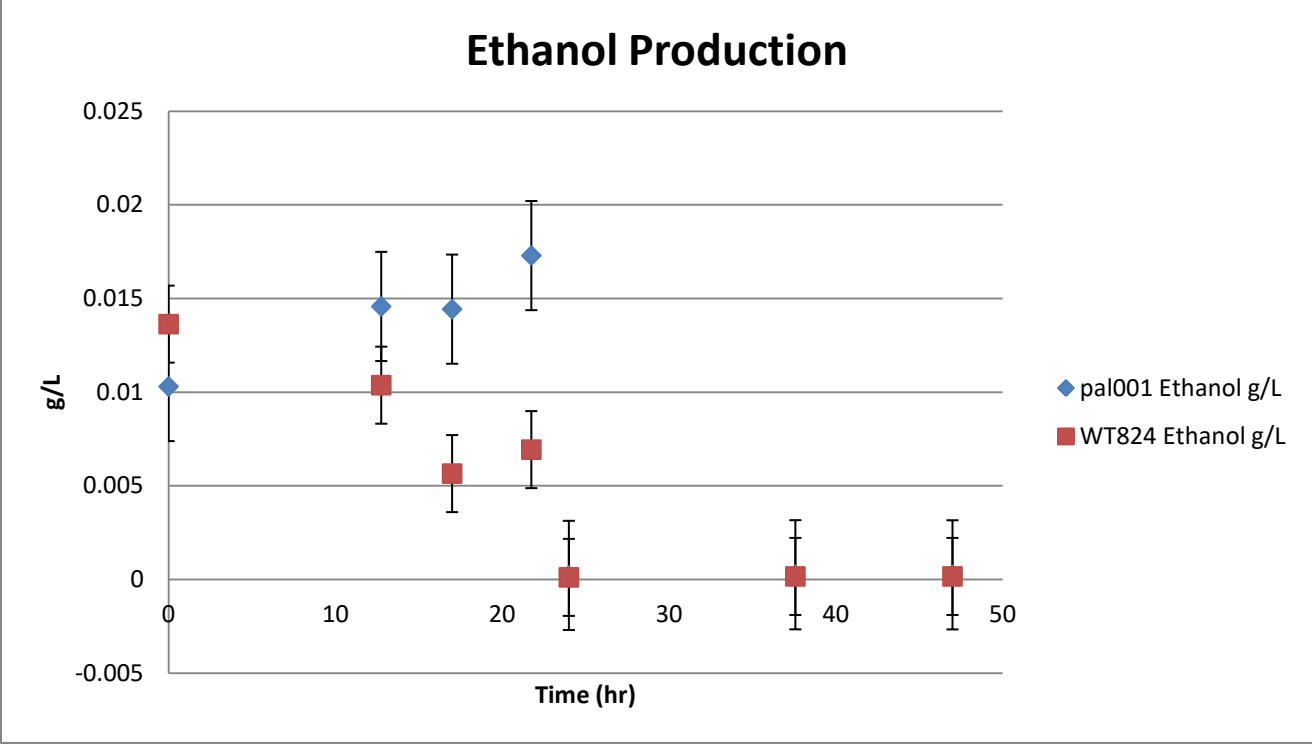
Figure 9: Comparison of Ethanol Production.
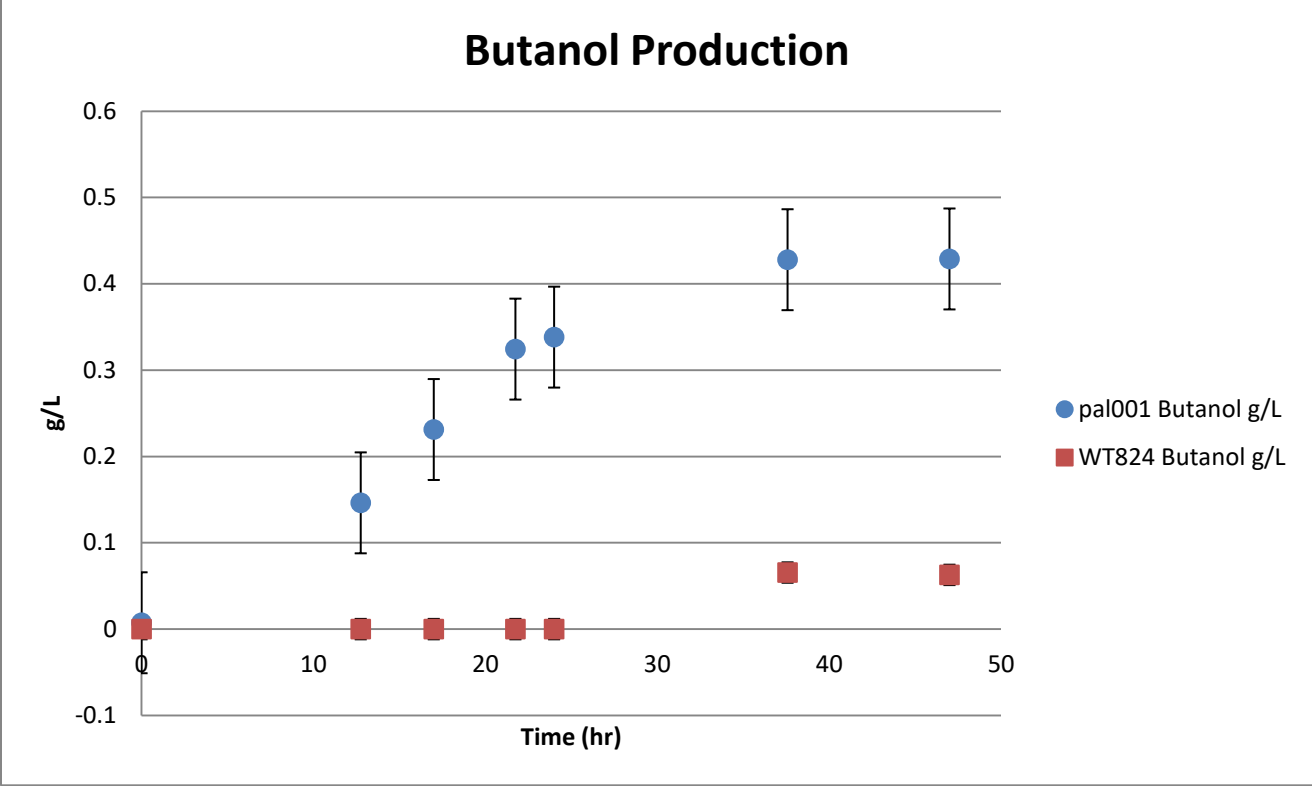
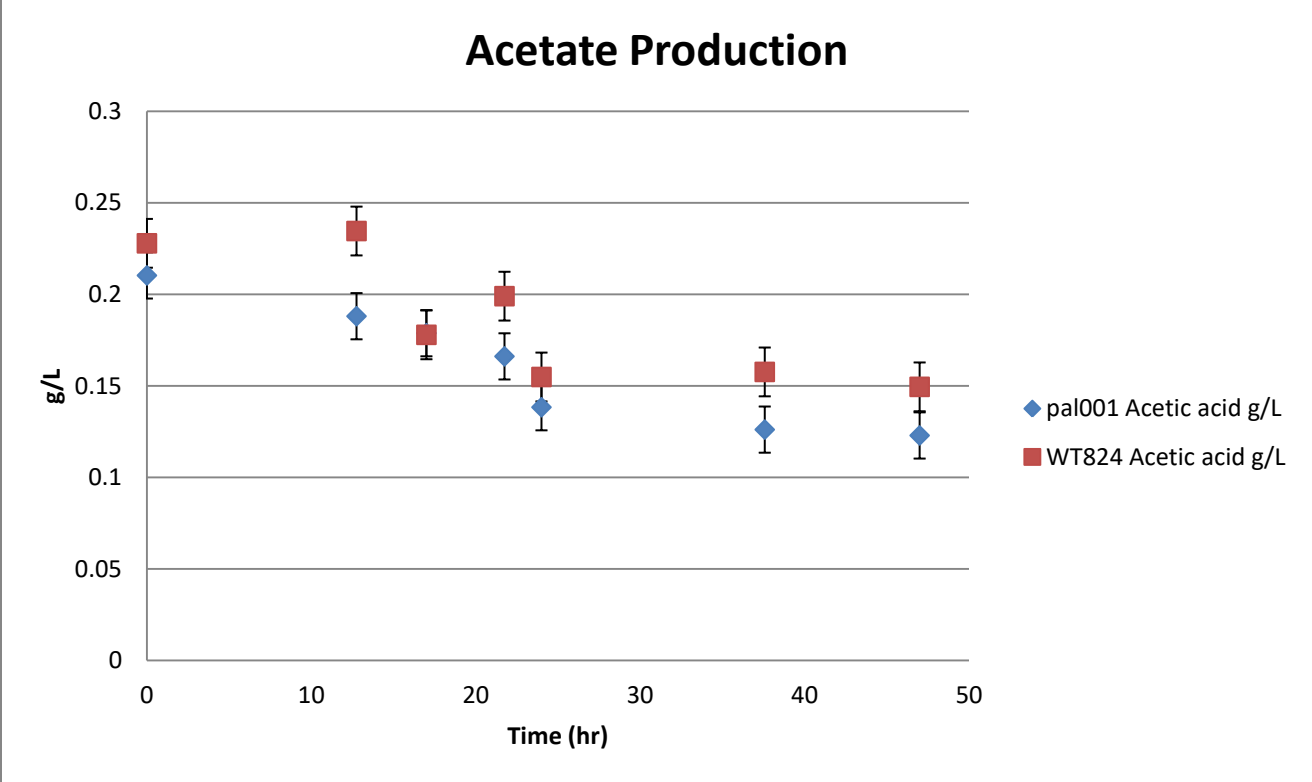**Figure 10:** Comparison of Butanol Production.

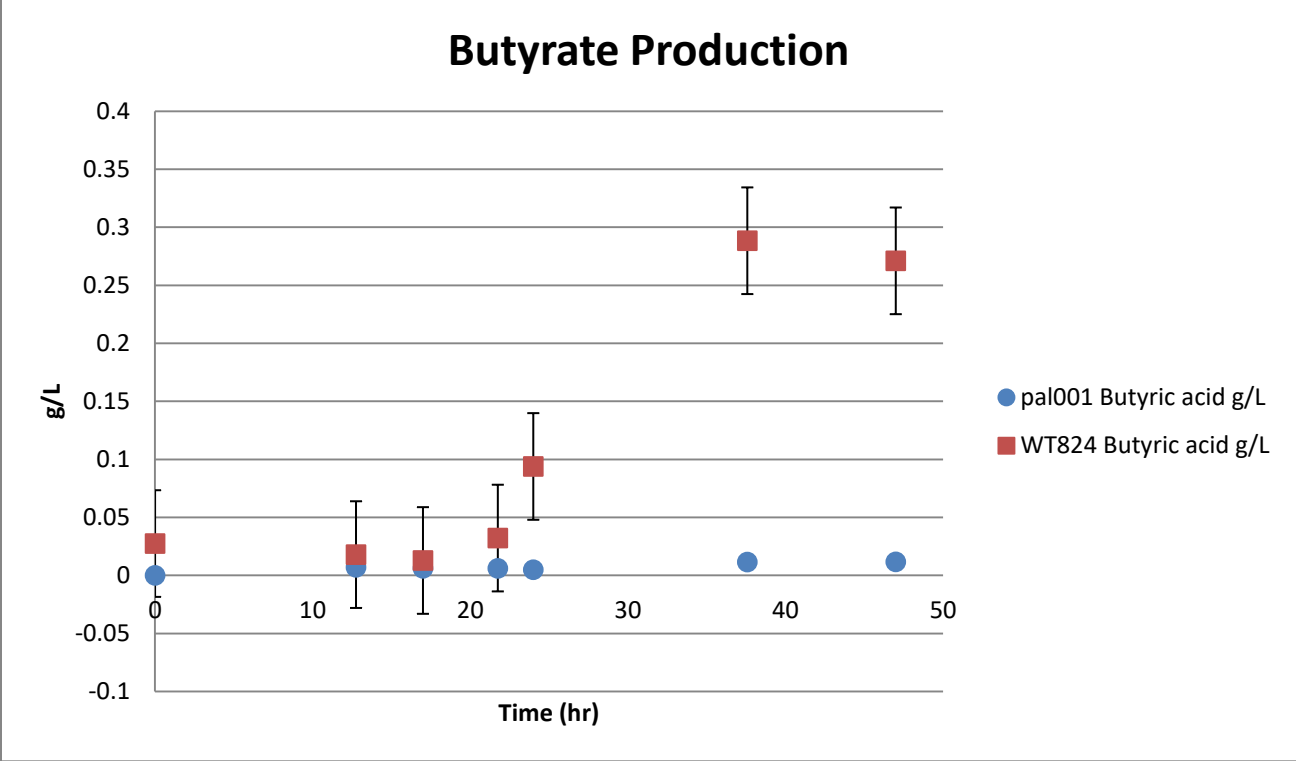**Figure 11:** Comparison of Acetate Production.

Figure 12: Comparison of Butyrate Production.
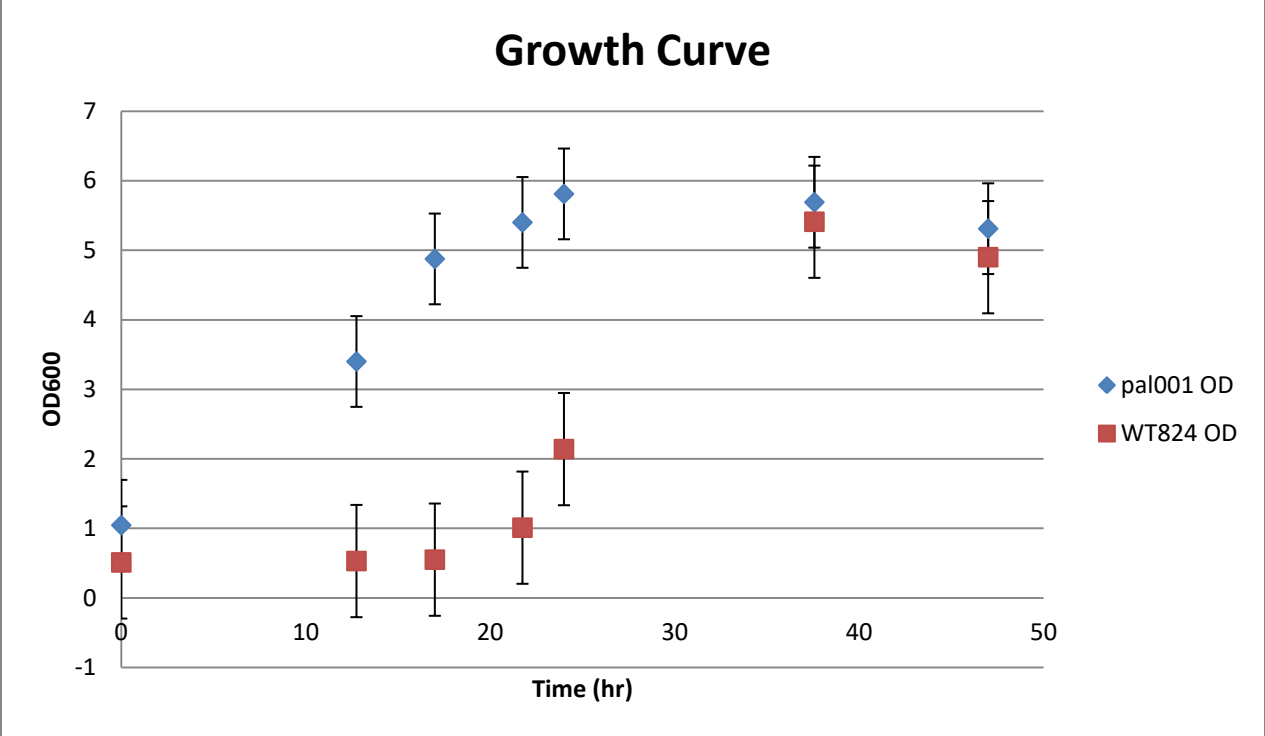
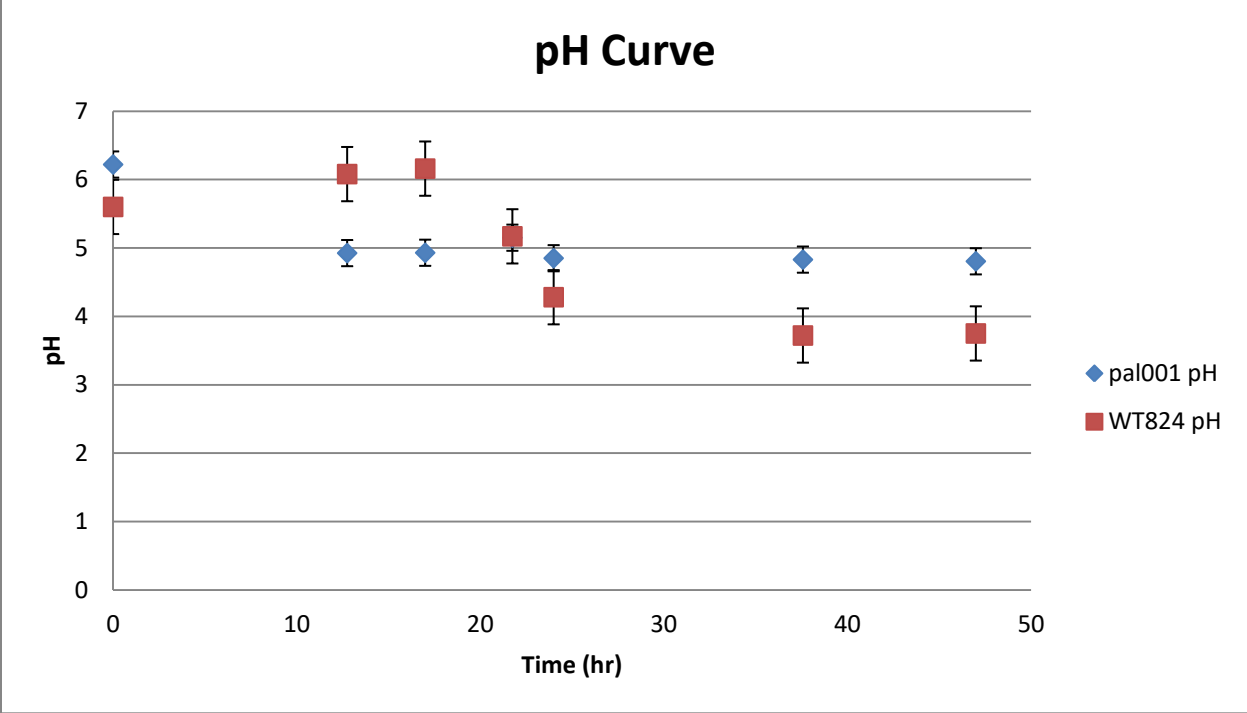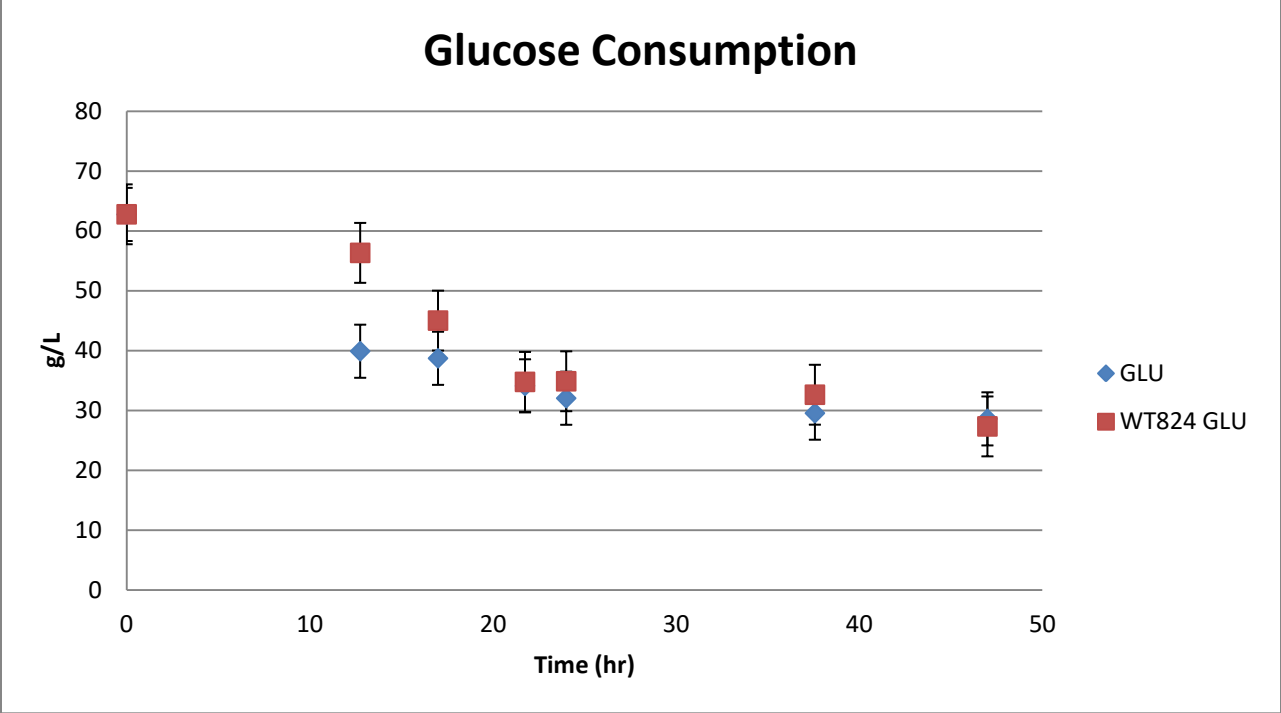**Figure 13:** Comparison of Cell Growth.

Figure 14: Comparison of Fermentation pH.

Figure 15: Comparison of Glucose Consumption.