OPTIMIZING TRANSFORMATION OF KLUYVEROMYCES MARXIANUS: ELECTROPORATION VS. CHEMICAL TRANSFORMATION

By

Darian McMillan

A capstone project submitted for
Graduation with University Honors

May 9, 2019

University Honors
University of California, Riverside

APPROVED

_____
Dr. Ian Wheeldon
Department of Chemical and Environmental Engineering

_____
Dr. Richard Cardullo, Howard H Hays Jr. Chair, University Honors

Abstract

The yeast species Kluyveromyces Marxianus, along with other species of yeasts, are traditionally used as research models for cell biology, biochemistry, and biotechnology. However, Saccharomyces Cerevisiae has long been the dominant yeast in all of these areas. There is large amounts in diversity between the different yeasts but recently there has been a focus on the industrial potential of yeasts. This is where the dairy yeast, Kluyveromyces Marxianus, becomes particularly interesting and valuable for industry. Kluyveromyces Marxianus has the fastest growth rate, it is thermotolerant, and is able to utilize a wide range of carbon sources. These traits make it particularly useful for industrial application. The thermotolerance greatly reduces cooling costs and facilitates in situ product removal. Other traits include its flexible substrates as well as its ability to maintain a low pH and withstand high temperatures for non-aseptic cultures. Therefore, we are interested in constructing a whole genome-wide library for sgRNA in Kluyveromyces Marxianus. We want to use this industrially valuable yeast to produce a functional genetic screen. In order to do this, a high transformation efficiency is necessary or else hundreds of different plasmid transformations will need to be performed. The goal is to reach a minimum transformation efficiency of 1x10^6. To reach this goal, two different methods of transforming a plasmid were tested and optimized - chemical transformation and electroporation. To optimize the protocols, a certain variable in each protocol was altered. In the chemical transformations, dithiothreitol (DTT) concentration was varied. In the electroporation experiments, voltage of the electroporator was altered. Using electroporation revealed a higher transformation efficiency than a chemical transformation, but the results from the electroporation were inconsistent. More research has to be done on how to optimize the electroporation protocol and generate consistent results.

Acknowledgements

I would like to acknowledge my capstone faculty mentor Ian Wheeldon, for his guidance and support during all four years that I was a part of his lab. I would also like to thank Mengwan Li for the large amounts of her knowledge, advice, and time that she shared with me.

Table of Contents

List of Figures

Introduction

The yeast species *Kluyveromyces Marxianus*, along with other species of yeast, are traditionally used as research models for cell biology, biochemistry, and biotechnology. However, *Saccharomyces Cerevisiae* has long been the dominant yeast in all of these areas. There is a large amount of diversity between the different yeasts and the areas of research in which they are useful, but recently there has been a focus on the industrial potential of yeasts. This is where the dairy yeast, *Kluyveromyces Marxianus*, becomes particularly interesting and valuable for industry. *Kluyveromyces Marxianus* has the fastest growth rate, it is thermotolerant, and is able to utilize a wide range of carbon sources. These traits make it particularly useful for industrial application. In Figure 1, it is shown how well *Kluyveromyces Marxianus* is able to grow on a variety of substrates. This is useful because this flexibility makes this yeast very versatile and thus useful in different fields of research. In addition, the thermotolerance greatly reduces cooling costs and facilitates *in situ* product removal. In Figure 2, *Kluyveromyces Marxianus'* ability to grow not only faster but at a higher temperature is displayed in comparison to the abilities of the commonly used yeast, *Saccharomyces Cerevisiae.* The ability to withstand high

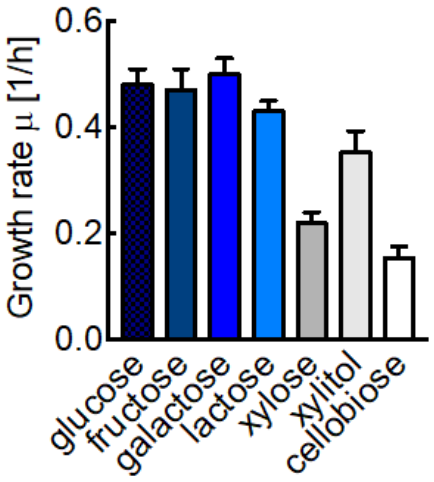


Figure 1 – Growth on C_5, C_6, and C_{12} sugars

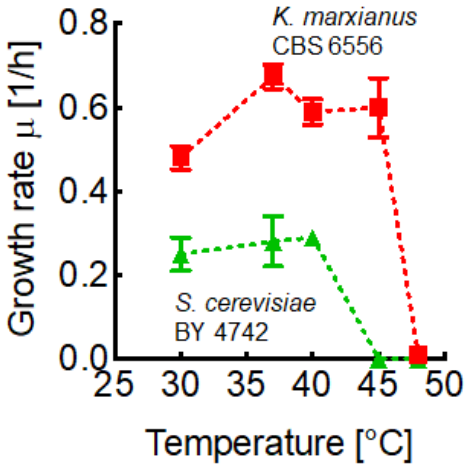


Figure 2 - Fast Growth at 45 °C and low pH

temperatures during growth helps to deal with non-aseptic cultures and reducing the chance of contamination.

Therefore, due to the unique and valuable qualities that *Kluyveromyces Marxianus* offers, we are interested in constructing a whole genome-wide library for sgRNA in *Kluyveromyces Marxianus*. We want to use this industrially valuable yeast to produce a functional genetic screen. In order to do this, a large number of transformations will have to be done in which several different plasmids are transformed into the yeast cell. Transformations are often used to introduce foreign DNA into a cell. Often, transformations are useful because it allows for the manipulation and amplification of a plasmid. However, for constructing a genome-wide library several hundreds of transformations would need to be done. So, to save time and increase effectiveness, a high transformation efficiency is necessary. Transformation efficiency is defined as the number of colony-forming units that are produced from the transformation of 1 µg of plasmid into a certain volume of competent cells (NEB, 2019). That is where the importance of optimizing a transformation protocol comes into play. The goal transformation efficiency is $1x10^6$. Two different methods of transformation, chemical transformation and electroporation, will be tested in producing the best protocol for conducting transformations and receiving high transformation efficiencies.

Methods

A transformation is when foreign DNA is introduced into competent cells. There are different ways to conduct a transformation, but the two being used in this experiment are

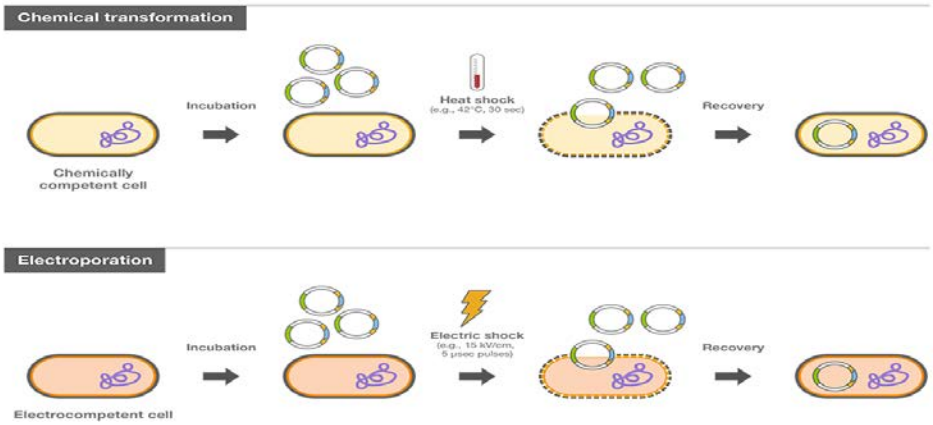
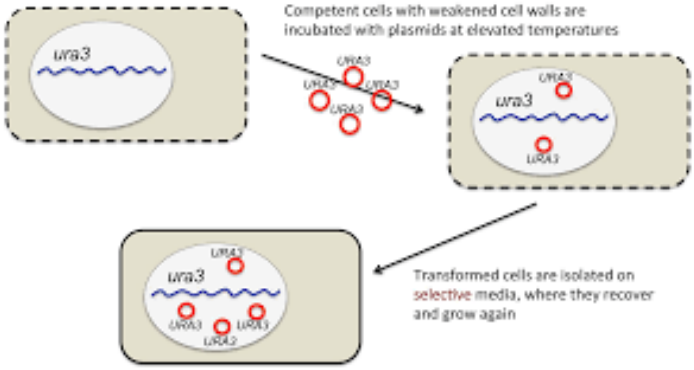


Figure 3 – A comparison between chemical transformation and electroporation

chemical transformation and electroporation. The end result of the two different methods is the same, but it is the process that is used to get the DNA into the cell that differs. As shown in figure 3, both methods begin with competent cells that are then incubated. What differs between the two methods is how the cell membrane is made permeable. In a chemical transformation, polyethylene glycol (PEG), lithium acetate, a non-specific carrier DNA (such as salmon sperm), and heat shock are necessary to make cell walls more permeable to take up the DNA. Evidence suggests that PEG promotes association of the transforming DNA with the surface of the cell and that lithium ions and heat shock promote passage of DNA into the cell. As shown in figure 4, the competent cells with the weakened cell walls are incubated with plasmids, or the foreign DNA, at elevated temperatures (the heat shock). Then the transformed cells are isolated onto selective media, where they recover and grow again. The selective media that the cells regrow on indicates whether or not the cells have taken up the plasmid. Growth on the selective media indicates that the colonies have incorporated the plasmid.

Figure 4 – An example of the process of a chemical transformation



Similarly, during electroporation the cells also have to be made competent. This is done through a number of rinses of the cell, to remove any excess salts that may interfere with the passing of electrical current through the cell. Using an electroporator, shown in figure 5, an electric current is then applied across the cell membrane which results in a temporary "pore" formation that enables the uptake of foreign DNA. Shown in figure 6, short pulses of high-voltage electric fields increase the cell's membrane potential, thereby inducing transient permeability to charged molecules like DNA.

Figure 5 – The machine used to conduct electroporation, an electroporator

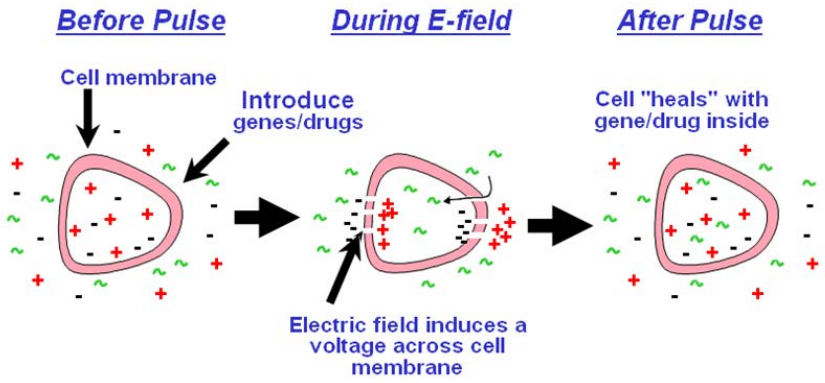


Figure 6 – An example of electroporation

Results

To optimize a protocol for chemical transformations, the reducing agent dithiothreitol (DTT) concentrations were altered. DTT has been shown to improve transformation efficiencies when used at certain concentrations. So, to figure out the optimal concentration of DTT to use, several chemical transformations were run in which 1mM, 20mM, 50mM, 70mM, and 90mM of DTT were tested. The *Kluyveromyces Marxianus* strain that was used in this series of chemical

transformations is YS402, and the plasmid that was being transformed is PIW272. The protocol is as follows:

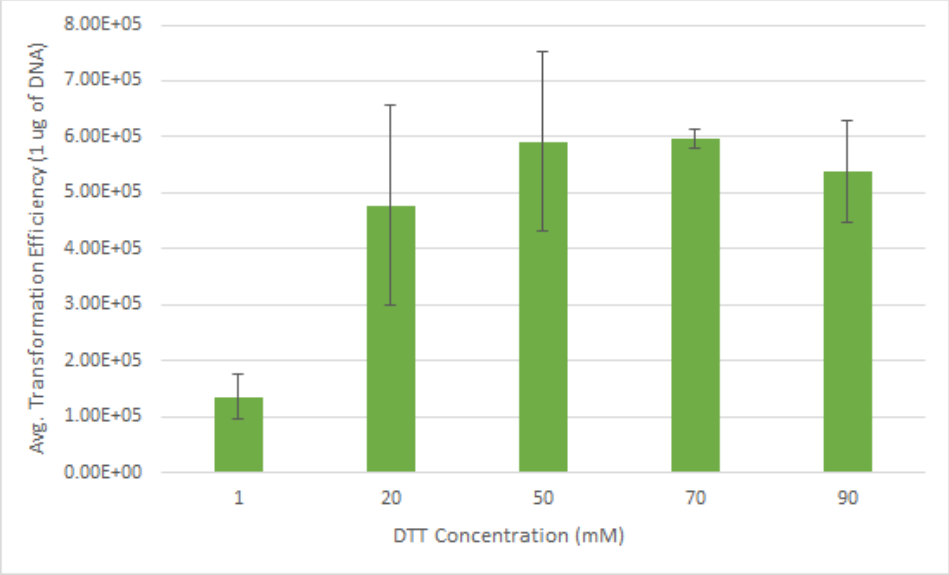
1. Inoculate colonies from YPD (yeast peptone dextrose) plate into 2.0 mL of YPD and grow overnight for 16 hours at 30℃.
2. After growth, harvest 1mL of cells by centrifugation at 5000 rpm for 1 minute.
3. Resuspend in 1 ml Transformation Mix #1 (TM1) by vortexing – repeat step 3 twice.
    a. Make TM1 the day before:
        i. 1 mL of 10xTE, 1 mL of lithium acetate, and 8 mL of double-distilled water
4. Resuspend in 10 uL carrier DNA (Salmon sperm)
    a. Boil the ssDNA 5 minutes prior to use
5. Add 1 ug of transforming DNA
6. Add 500 uL of Transformation Mix #2 (TM2) and incubate at room temperature for 15 minutes
    a. Make TM2 the day before:

	Final Concentration
50% Polyethylene Glycol (PEG)	40%
1M Lithium Acetate	0.1 M
1M Tris-HCL pH 7.5, 0.1M EDTA	10mM Tris, 1mM EDTA
1M Dithiothreitol (DTT)	*Tested: 1mM, 20mM, 50mM, 70mM, 90mM*

ddH_2O	Add last, and add whatever amount needed to reach 1000 uL

7. Mix by pipetting and incubate at room temperature for 15 minutes.
8. Heat shock at 47°C for 15 minutes.
    a. Set up the heat block at the beginning of the transformation and put in a thermometer
9. Centrifuge and resuspend with 1 mL SD-U (single dropout – ura) liquid media
    a. DO NOT vortex
10. Transfer all into a 50 mL tube. Add 9 mL of SD-U (to dilute). Mix by inverting.
11. Put 5 uL onto an SD-U plate.
12. Add 100 uL of ddH_2O onto the drop of 5 uL from the transformation. Spread the cells.
13. Put into 37°C incubator. Check on the cells in 20 hours.

Graph 1 – Average transformation efficiency (1ug of DNA) vs. DTT concentration (mM)



After running the chemical transformation multiple times, the results shown in graph 1 were obtained. From the graph it can clearly be seen that the optimal DTT concentration to use in chemical transformations with *Kluyveromyces Marxianus* is 70 mM. The average transformation efficiency for a DTT concentration of 70mM is $5.95x10^5$. Although

the goal transformation efficiency of $1x10^6$ was not obtained, the results from the chemical transformation were pretty consistent.

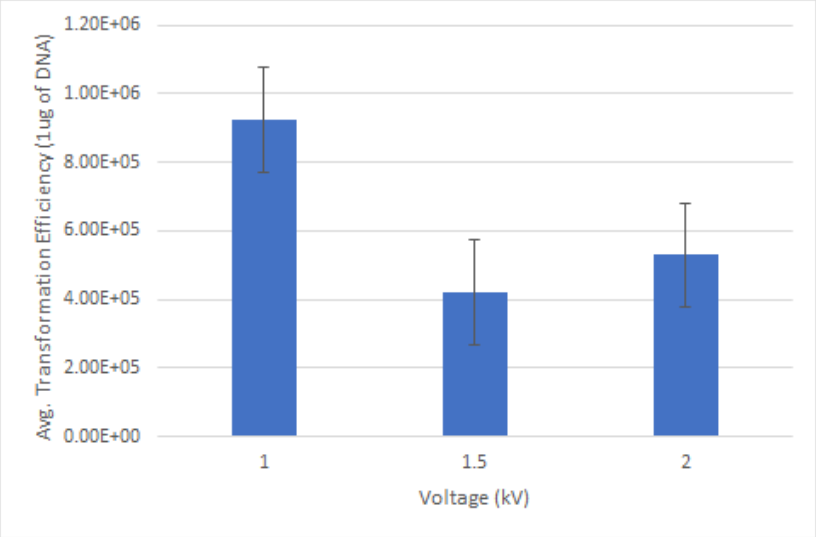
Curious as to whether or not a better transformation efficiency could be obtained from another method of transformation, electroporation was tested. In conducting the electroporation experiments, the voltage of the electroporator was varied from 1 kV, 1.5 kV, and 2 kV. The reason why voltage was chosen be the variable that is altered is because the electrical current that the cell undergoes determines the permeability of the cell wall. The *Kluyveromyces Marxianus* strain that was used during the electroporation experiments was YS626, and the plasmid was PIW1213. The two yeast strains that were used for the separate transformation experiments are very similar and would not alter the results. The two different plasmids that are used are also very similar. The electroporation protocol is as follows:

1. 2 mL of YPD culture overnight (16 hours in 30℃).
2. 1mL was centrifuged at 5000 rpm, for 1 minute
    a. All centrifugation in this protocol will be at 5000rpm for 1 minute
3. 1mL was resuspended in ice-cold water – repeated twice
4. The cells were collected by centrifugation and the pellets were resuspended in 500 uL ice cold water. This step was repeated then the pellets were suspended in 500 uL of ice cold 1M sorbitol.
5. Following centrifugation, the pellet was suspended in 40 uL of ice cold 1M sorbitol and ready to be used for transformation.
6. Plasmid DNA (1ug) was added to the cell suspension and incubated on ice for 5 minutes.

7. The mixture was transferred to a 0.2 cm electroporation cuvette and pulsed at 1kV, 1.5kV, and 2.0kV with a BioRad gene pulser. Immediately, 960 uL ice cold 1M sorbitol was added and the contents of the cuvette were gently mixed.
    a. Remove the cuvette from the electroporator and put it on ice before adding the 1M sorbitol
    b. Read the time/ms, if it is between 4.0-6.0, this process is successful. The higher the time/ms, the better.
8. Transfer 200 uL of the electroporated cell suspensions into 1800uL SD-U in a 14mL culture tube and incubate at 30℃ for 1 hour.
9. After recovery, plate 5 uL out of the 2mL onto an SD-U plate.
10. Add 100 uL of ddH_2O onto the drop of 5 uL from the transformation. Spread the cells.
11. Put into 37℃ incubator. Check on the cells in 20 hours.

For the electroporation protocol, it is essential that the ddH_2O and the 1M sorbitol that are used are maintained on ice. These need to be ice cold when being used because it decreases the chance of the cells overheating during the electric shock. The results from the electroporation experiments are show on graph 2. From the graph, it can be seen that the best voltage to use for the electric pulses is 1kV. While using 1kV to transform the cells, the goal transformation efficiency was obtained. The highest

Graph 2 – Average transformation efficiency (1ug of DNA) vs. Voltage (kV)



value from the three trials at 1kV was $1.7x10^6$, with the overall average of the three trials being $9.22x10^5$. Although the average of all three trials for this voltage did not reach the goal transformation efficiency, one of the trials provided promising results. The issue with the electroporation experiments was that there were fairly inconsistent. In the first attempt at electroporation the transformation mixes, that were used during the chemical transformation, were used to wash the cells instead of just ice cold water. The average transformation efficiency for the cells that were washed with the transformation mixes was $1.25x10^5$. The reason for the extremely low transformation efficiency is due to the fact that the cells were not well washed. Using the transformation mixes to wash the cells added a lot of extra salts to the cells rather than cleaned them from these unwanted salts. The cells have to be thoroughly cleaned in order for the electric current to more effectively create pores in the cell membrane. So, after realizing this, the electroporation protocol was adjusted. Instead of using transformation mixes to rinse the cells, only ice-cold water was used. This improved the results immensely, providing one of the highest averages. In hopes of obtaining such high transformation efficiencies once more, the voltages 0.5kV, 0.75kV, and 1.0kV were tested. Due to the high transformation efficiency average that was obtained from 1.0kV during the second time the electroporation protocol was tested, it was believed that the lower the voltage, the higher the transformation efficiency would be. Unfortunately, the third time that the protocol was tested with the three lower voltages, there was no growth on the plates.

Future Experiments

There are a number of future experiments that could be conducted in hopes of attaining the goal transformation efficiency. Since electroporation has shown, only once, to provide higher transformation efficiencies that those obtained from a chemical transformation, it is definitely

worth trying the electroporation transformation one more time. It is difficult to think of a reason as to why the third round of testing the electroporation protocol did not work, but one of the possibilities may be that the cells were left to grow a little longer than 16 hours. Due to this, the best time in the yeast's growth period to be transformed may have been missed. Another possibility is that the electroporation cuvette was wet before being put into the electroporator. The water residue on the outside of the cuvette may have affected the electric current and the amount that reached the cells. There are a number of potential reasons as to why no cells grew on the plate for the third round, not even the cells that were pulsed at 1kv which had provided high transformation efficiencies during the second round of experimentation. But it is worth trying the 0.5kV, 0.75kV, and 1.0kV voltages one more time and trying to minimize errors or contaminations while following the protocol. If these voltages work, and provide a minimum transformation efficiency of $1x10^6$, it will be a great help in the construction of the genome-wide library for *Kluyveromyces Marxianus*.

Discussion

Kluyveromyces Marxianus is a both a valuable and very interesting yeast. It can be very useful for industrial application because of its thermotolerance, fast growth, and ability to utilize a wide range of carbon sources. It is because of its potential in the industrial field that it is of great interest. This yeast also used to be a little less commonly used than its sister yeast *Saccharomyces Cerevisiae*. For this reason, the end goal is to construct a genome-wide library for the sgRNA of *Kluyveromyces Marxianus*. To efficiently create this genome-wide library, hundreds of plasmids will need to be transformed into the cell. To make the process as efficient and reliable as possible, a transformation protocol has to be developed that can consistently

provide a high transformation efficiency. The minimum transformation efficiency that is the goal is $1x10^6$, and this transformation efficiency looks similar to figure 7. Essentially, the more colonies that grow on the selective plate, the more cells that have taken up the plasmid, and thus the higher the transformation efficiency will be. Based on the experiments that were conducted, the chemical transformations provided consistent results. Although the transformation efficiencies were lower than that which is desired, the chemical transformation always managed to transform a decent number of cells. 70 mM of DTT seemed to be the optimal DTT concentration for providing the best transformation efficiency. Other papers have suggested that once DTT concentration is between 100-200 mM, the transformation efficiency may drastically improve. Unfortunately, there was not enough time to test 100mM or more of DTT. But based on the concentrations of DTT that were tested, 70mM provided the best results.

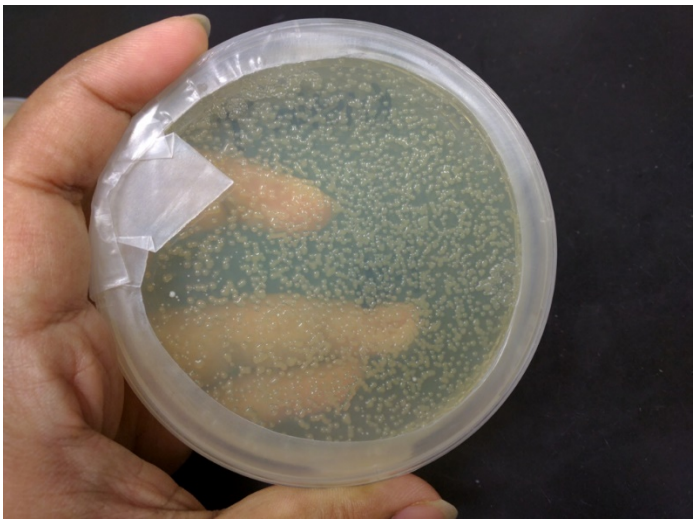


Figure 7 – A plate with a large number of transformed colonies, and thus a high transformation efficiency

On the other hand, using electroporation to transform the cells provided inconsistent results and more research definitely has to be done. In the first attempt, when a chemical transformation mix was used to rinse the cells, the transformation efficiency was extremely low. The amount of salts that the transformation mixes added to the cells had a negative effect on both the permeability of the cell membrane and thus the uptake of the plasmid. But when water and sorbitol were used to rinse the cells, the highest transformation efficiency was $1.7x10^6$ at 1kV

and the average for all three trials for this voltage was $9.22x10^5$. In attempt to reproduce such high transformation efficiencies, the electroporation protocol was tested once more but no growth was seen on the plates.

Transformation efficiencies have exceeded $1x10^6$ in other labs, but never before in this lab. Other papers have been read and other protocols have been followed but still it is difficult to consistently obtain a transformation efficiency that high. The electroporation protocol came very close to reaching the goal transformation efficiency, it just has to be done again to show that it is reliable. More experiments have to be conducted and if trying the electroporation protocol again does not yield the desired results, the protocol will have to be altered once more. Another variable or maybe even another approach will have to be tested in hopes of receiving a consistent transformation efficiency of at least $1x10^6$.

References

BioLabs, New England. “Chemical Transformation Tips.” *New England Biolabs: Reagents for the Life Sciences Industry*, 2019, www.neb.com/tools-and-resources/usage-guidelines/chemical-transformation-tips.

Bitesize Bio. “E.coli Electroporation vs Chemical Transformation.” *Bitesize Bio*, 11 July 2016, bitesizebio.com/10297/ecoli-electroporation-vs-chemical-transformation/.

Lane, Melody M., and John P. Morrissey. “Kluyveromyces Marxianus: A Yeast Emerging from Its Sister's Shadow.” *Fungal Biology Reviews*, Elsevier, 8 Feb. 2010, www.sciencedirect.com/science/article/pii/S1749461310000035.

ThermoFisher. “Bacterial Transformation and Competent Cells–A Brief Introduction.” *Thermo Fisher Scientific - US*, 2019, www.thermofisher.com/us/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/molecular-cloning/transformation/competent-cell-basics.html.

Tripp, Jennifer DeMars, et al. “Enhancement of Plasmid DNA Transformation Efficiencies in Early Stationary-Phase Yeast Cell Cultures.” *Yeast (Chichester, England)*, U.S. National Library of Medicine, May 2013, www.ncbi.nlm.nih.gov/pmc/articles/PMC3656150/.