Effects of Different Pulsing Characteristics on Transformation Efficiency Via Electroporation

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Abstract: This study will focus on how the different characteristics of the applied pulse, including voltage, pulse length, frequency and multiple pulsing affect transformation efficiency of Top10 E-coli cells. It will be shown that transformation efficiency will be maximal at optimum settings different from that suggested by electroporator manufacturer. Experimental results will also verify pore formation model developed by Schoenbach et al.

I. Introduction

Electroporation(EP) is a phenomenon in eukaryotic and prokaryotic cells involving the rearrangement of the phospholipid bilayer membrane in response to an external electric field. This rearrangement of the membrane leads to the formation of aqueous pores, which increases the conductivity of the cell. The aqueous pores are channels in the membrane that aid the transport of water-soluble molecules. As a result, the membrane becomes permeable to otherwise impermeable molecules. This has many significant biological applications including direct transfer of genes, proteins, and drugs into the cell interior. EP also has potentially useful applications in medicine such as transdermal drug delivery. Specifically, skin melanomas, a type of skin cancer, have been successfully treated with low doses of bleomycin delivered to the interior of cancerous cells via EP. Bleomycin is a powerful drug that binds to the DNA of the tumor cell, preventing DNA replication and thus leading to the eventual death of the cell without any further cell divisions (Neuman et al., 1998). Another application of EP is the selective killing of cells using pulsed electric field (Schoenbach et al., 1999). This can be used in cancer treatment or decontamination of food and water.

II. Background

The actual mechanism of pore formation is not well understood. Numerous experimental studies have been aimed at revealing the mechanism of pore formation in various types of membranes ranging from artificial lipid bilayers to red blood cells to chick myocyte monolayers. These studies investigated the properties of pore formation and resealing using pulse charge techniques. The majority of the studies have focused on lipid bilayer membranes since they are more common in living organisms. The unique bilayer structure of the membrane is a consequence of the way lipid molecules behave in an aqueous environment (Alberts et al,. 1998). The most abundant membrane lipids are phospholipids. Specifically, phosphatidylcholine, found in most cell membrane, consists of a polar hydrophilic head made up of a choline group, a phosphate group, and a glycerol group. Attached to the polar head are two nonpolar hydrophobic tails made up of one saturated and one unsaturated hydrocarbon chain. In an aqueous environment, the polar heads form electrostatic and hydrogen bonds with the polar water molecules while the nonpolar tails repel water molecules. To minimize chemical energy of the system, the two lipid layers rearrange so that the hydrophobic tails cluster together in the inner space between the two layers, away from the water molecules. The polar heads are faced outward from both sides, chemically interacting with water molecules, effectively shielding the hydrocarbon tails from the water. This arrangement gives the membrane a unique self-sealing property. A tear in the membrane creates an open edge that exposes the hydrophobic region to water molecules resulting in an unfavorable energy state of the system. If the opening is small, the membrane will quickly rearrange to reseal the tear eliminating the free edge. This is the underlying concept for reversible electrical breakdown (REB) in an EP process. The membrane also acts as a barrier

to charged particles due to a difference in the dielectric constant of water and lipid. There is an energy associated with moving charged particles from a high dielectric medium (water) to a low dielectric medium (lipid) known as the Born energy (Weaver & Chismadzhev, 1996). In a numerical approximation in which the lipid layer is represented as a planar sheet and an ion as a point charge, the Born energy (energy required to move the point charge to the center of the plane) is about 2.8×10^{-19} J. This suggests that under normal conditions, spontaneous membrane transport of ions is negligible.

EP in a lipid bilayer membrane occurs under relatively mild biochemical conditions, often with a small change in temperature. It is believed that EP has a nonlinear dependence on transmembrane voltage, denoted as U(t). If this voltage is maintained in the range $0.2 < U < 1$ V during a pulse, the cell undergoes REB and molecular transport occurs. Although the most prominent effect of EP is a significant increase in permeability of the membrane, other effects include fusion of cells and insertion of protein into the membrane itself.

For simplicity, treating the cell as a spherical entity is often desired for modeling purposes. For a spherical cell subjected to a small electric field, the transmembrane potential U(t), can be analytically estimated as follows (Weaver & Chismadzhev, 1996):

$$U(t) = \frac{1.5 E r_{cell}}{1 + r_{cell} G(\rho_{in} + 0.5\rho_{out})}\left[1 - \exp(-t/\tau_{cell})\right]\cos\theta \qquad (1)$$

where r_{cell} is the radius of the cell, ρ_{in} and ρ_{out} are the resistivities of the intracellular and intercellular medium respectively, G is the constant average membrane conductance, θ is angle between the electric field E and the site of interest on the cell membrane and τ is the time constant related to the charging of the cell. For a mammalian cell, $r_{cell} \approx 10^{-5}$ m and $\tau \approx$

10^{-6} s assuming that conductance is uniform and constant throughout the cell (Weaver & Chismadzhev, 1996). This transmembrane potential is largely responsible for the size and number of pores that form during application of an electric field.

There are several competing theories for the mechanism of EP. The most prominent one is the transient aqueous pore theory. This theory suggests that pore formation is caused by a combination of thermal fluctuations and local electric fields across the membrane. This theory involves the estimation of the free energy change, $\Delta W(r,U)$, due to the formation of aqueous pores (large dielectric constant) within a lipid membrane (small dielectric constant);

$$\Delta W(r,U) = 2\pi\gamma r - \pi\Gamma r^2 - C_{LW}U^2\pi r^2 \qquad (2)$$

in which γ is the linear energy density at the circumference of the pore, r is the radius of the pore, Γ is the surface energy density of the lipid bilayer membrane, and C_{LW} is the difference in specific capacitance when water replaces the lipid at the pores. When the transmembrane voltage is zero, the free energy equation reduces to the first two terms. The first term ($2\pi\gamma r$) represents the energy gain due to edge formation of the pore and the second term ($\pi\Gamma r^2$) is associated with the energy lost by cutting out a lipid area the size of the pore. In living cells, the presence of a transmembrane voltage brings the third term into the free energy; the bilayer membrane acts like a capacitor. When a pore is formed, water fills the cylindrical region formerly occupied by lipid. Exchanging the dielectric constant of the membrane for that of water effectively changes the specific capacitance in the pore region and yields the change in electrical energy as indicated by the third term. When compared to experimental behaviors, this model performed fairly well, but was not able to yield completely accurate values of transmembrane voltage and membrane conductance. This maybe due to failure to account for membrane recovery during a reversible electrical breakdown.

Recent studies have focused more on the resealing process of the membrane after pulsing. This process has a significant effect on the number of molecules exchanged between the intracellular fluid and the surrounding medium. The viability of the cell is also dependent on the membrane's ability to reseal following EP. Cells that experience irreversible electrical breakdown can burst from a sudden influx of external fluid into intracellular space. Experimental data have shown that irreversible breakdowns are frequently caused by longer pulses or high voltage pulses. At high voltage, pore density increases significantly. Therefore interaction between different pores has to be taken into account in mathematical modeling of the cell membrane.

The Joshi and Schoenbach model investigates both expansion and resealing of pores by studying the distribution of radii pore and the influence of transmembrane potential on this distribution. The Joshi and Schoenbach equation for the transmembrane potential is similar to Equation 2 with an additional term to account for the steric repulsion between the polar lipid heads along the pores edge (Joshi & Schoenbach, 2000):

$$E(r,t) = 2\pi l r - \pi s r^2 + \left(\frac{C}{r}\right)^2 - \pi a_p V^2 r^2 \qquad (3)$$

The third term is the steric repulsion term and C is experimentally determined to be 9.67 X 10^{-15} $J^{1/2}$m. Furthermore, a_p is a coefficient representing the property of the membrane in water and is expressed in terms of membrane thickness h and permitivity ε as $a_p = \varepsilon/2h$. The transmembrane potential was calculated using a lumped equivalent circuit (Appendix A Fig.1). In the lumped circuit, R_m and C_m are the resistance and the capacitance of the membrane, while R_s and C_s are the resistance and capacitance of the cell suspension

respectively. The variable R_c denotes the resistance of intracellular fluid. This lumped circuit disregards the nucleus on the grounds that the nucleus is significantly smaller than that of the cell. It is found that the membrane resistance is inversely proportional to the size of the pore as expected since a larger pore leads to a higher flow of ions through the membrane, thus decreasing resistance.

The Joshi and Schoenbach model provides insight into pore dynamics, specifically the behavior of r. The model shows the existence of a critical pore radius r_{crit} (18 nm for the case of zero transmembrane voltage). Energetically, pores with radius below the critical radius will tend to drift to a smaller size over time, while those with a radius above the critical value will increase in size indefinitely. This provides a testable criteria for irreversible electrical breakdown. Although the model does not yield detailed dynamics, Joshi and Shchoenbach argues that a high voltage pulse may not be sufficient to cause irreversible electrical break down if the duration of the pulse is short. These short pulse lengths do not allow enough time to reach the threshold radius beyond which the pores will grow indefinitely.

This project is mainly concerned with transporting molecular DNA into bacterial cells via electrical pulsing. This process is known as electrotransfection. This is a relatively new field in science. The first successful electrotransfection (ET) was reported in the early 1980s. Researchers felt that ET had great potential in human gene therapy because it was mainly a physical phenomenon, which meant the procedure would not be sensitive to the usual problems, which attend biochemical selectivity. There are two ways that ET can be applied in gene therapy namely, in vitro and in vivo. In vitro ET requires that the cell be isolated from the organism prior to transformation while in vivo ET enables the cell to remain in its natural environment during transformation. The mechanism by which the DNA travels through the

cell membrane is still unclear. Studies have eliminated passive diffusion and osmotic pressure as possible driving forces for the transfer of DNA. Experiment with plasmid DNA containing the B-galactosidase gene has suggested that electrophoresis may be the driving force behind DNA flow. It is believed that local electric fields drive the negatively charged DNA particle through the pores, widening the pores in the process (Weaver & Chismadzhev, 1996).

There have been recent studies, primarily done on mice, regarding in vivo ET. One study on electrochemotherapy of tumor cells on mice showed a correlation between field distribution and growth of tumors (Miklavcic et al. 1998). Tumors cells that were exposed to stronger electric field experienced a longer delay in growth than other tumor cells.

Most studies on electroporation have focused on transforming different types of cells ranging from mammalian cells to plant cells (Lin 1994). The aim of these studies have been mainly biological consequences such as getting cells to express different types of gene. In this study of electroporation, the focus will be placed on the physical aspects of EP. The goal is to optimize transformation efficiency by varying physical factors such as applied voltage, pulse length, number of pulses, and frequency of pulses. The response of the cells to variation in these factors will provide valuable insights into the mechanism of EP.

III. Experimental designs and procedures

The central experiment of this project is the transformation of Escherichia-coli bacteria with Ampicillin-resistant DNA plasmid. In the initial stage of this experiment DH5a, a safe strand of E-coli was used for transformation. The cells have to undergo a series of preparation steps in order for them to be ready for electroporation. An overnight culture is

prepared from a stock supply of DH5a. The cells are washed with a solution if HEPES-buffer and resuspended in glycerol stabilizing solution. A detailed protocol for this procedure is included in Appendix B (protocol 1). E-competent cells are divided into 50 µl aliquots and stored at –80°C ready for electroporation.

A solution of Amp-resistant plasmid DNA was prepared using the Quantum Prep plasmid midiprep kit. Essentially, plasmid DNA is isolated from plasmid containing bacteria by lysing the cells and through a series of centrifugation, separating plasmid DNA via molecular weights (Appendix B: Protocol 2). The amount and size of DNA is determined using a procedure called agarose gel electrophoresis. The basic principle of electrophoresis is that when DNA, a negatively charged molecule, is placed in an electric field, it will move towards the anode. In the agarose medium, the rate of migration of the DNA molecule is determined by its size, assuming DNA molecules have equal charge. The DNA is stained with ethidium bromide and is imaged by its fluorescence under ultraviolet light. The band of migrated DNA in the gel is compared to a standard 1 Kilobase ladder to yield a quantitative measurement as a function of size (number of nucleic acid base pairing in the double helix strand).

The transformation is induced by electroporation using a BTX Square-wave electroporator (model ECM 830), which generates square-wave pulses of varying voltage and pulse length. This electroporator has a voltage range of 5-500 Volts (LV mode) or 30-3000 Volts (HV mode). Pulse length ranges from 10 ìs – 10 s (LV mode) and 10 ìs – 600 ìs (HV mode). The ECM 830 is also capable of multiple pulsing (1-99 pulses) at adjustable intervals from 100 ms – 10 s. The ECM 830 consists of a central pulsing unit and an external electroporating chamber containing the anode and cathode connected to the central unit.

During application of a pulse, a cuvette containing E-coli cells and the DNA mixture is placed in contact between the anode and cathode. The cuvette has a 1 mm gap between electrodes to provide liner fields of 5×10^3 Volts/m – 3×10^6 Volts/m.

Prior to electroporation, a liquid LB growth medium must be prepared and kept at 37°C. The LB growth medium consists of tryptone (10 g), yeast extract (5 g), and sodium chloride (10 g) diluted to 1 L with deionized water. The medium is autoclaved at high temperature for twenty minutes to sterilize the medium. To create an ampicillin plate, one adds agar (10 g) prior to autoclaving and 2 ml of pre-mixed ampicillin dilute (20 mg/ml) after autoclaving.

The electroporation step is described in the (Appendix B: Protocol 3). The voltage and pulse length are set to the desired values, which are displayed on the central pulsing unit. 1ìl of diluted plasmid DNA, of a predetermined concentration, is added to 50 ìl of cell solution. The mixture is transferred to a 1 mm gap in the cuvette. Once the cuvette is secured in the EP chamber between the electrodes, pulsing is initiated. Immediately following pulsing, 1ml of liquid LB growth medium is added to the mixture to to promote growth of viable cells. The solution is incubated at 37°C with moderate shaking for one hour. Approximately 100 ìl of the cell solution is spread uniformly on LB AMP-plate and incubate at 37°C for 15 hours. Transformation efficiency is determined by colony counts. If AMP resistant plasmid DNA has been successfully transferred into the cell and is expressed, the cells will show resistivity to the antibiotic ampicillin. Such cells will grow into visible colonies on the AMP plate.

This study sought to examine the effectiveness of various choices of pulse conditions. To provide a benchmark, and to determine baseline standards for our protocols, several

experiments were conducted at the BTX factory-suggested settings of 500 V and 8 ms pulse length. Several different dilutions of DNA also were used to determine the optimal amount. For the purpose of this project, it was determined that 1ng of plasmid DNA is optimum, yielding a statistically significant number of cell colonies, without creating so many that counting them would have been too difficult. The same type of experiment was performed to determine the optimum number of electroporated cells to be plated. AMP-plates where plated with different amount of cell solution ranging from 10 ìl to 500 ìl. It was determined that 100 ìl yielded an acceptable number of colonies.

In this project, experimental designs were developed with the aid of a computer module known as Design of Experiments (DOE) (Ref. Statsoft). DOE offers a wide range of experimental designs that are aimed at reducing the number of runs, saving time and costs, yet at the same time optimizing data for analysis yielded by these runs. For the purpose of this project, which requires the variation of the two independent variables, voltage and pulse length, the central composite design was used. The central composite design ensures reliable representation of the independent contributions of the two factors (voltage and pulse length) to the dependent factor, which in our case is the transformation efficiency. This is achieved by making the columns of the design matrix orthogonal (Box, Hunter & Hunter, 1978), meaning that the sum of the products of their elements within each row is equal to zero. In central composite designs, the elements of the design matrix are either 1 or -1 corresponding to high levels and low levels of both factors respectively. It is generally true that the closer to orthogonal the columns are, the more independent information can be extracted from the design, and thus, the better the design (Statsoft, 1984).

The central composite designs were carried out using carefully controlled culture of cells known as the "Top10 electrocomp kit" prepared by Invitrogen (Ref. Invitrogen). This kit consisted of 20 X 50 µl aliquots of highly electrocompetent E-coli cells. The kit also included 100 ng of supercoiled pUC18 DNA strand. Each such strand contains about 2.6 kilo-base pairs weighing 660 Daltons each. Due to the super electrocompetent nature of the Top10 cells, some slight changes in the electroporation protocol were necessary (Appendix B: Protocol 4). Specifically, the amount of DNA used was decreased from 1 ng to either 10 pg or 20 pg. This is ensured that bacterial colonies were not too dense to be counted. With sufficient Top10 cells for forty runs, we decided that two central composite design experiments (10 runs each), determining the independent contributions of voltage and pulse length to transformation efficiency, would be performed with increasing resolution selected by varying the range of variation. The resolution of the designs would be 1/10 of the difference between the maximum and minimum values specified for each independent factor.

For central composite design 1, the specified voltage range was 200 V(min)-500 V(max) and the pulse length range was 5 ms – 15 ms. In the second central composite design the voltage range was from 350 V – 500 V and the pulse length range was 7 ms – 17 ms. Analysis of results for the first design determined that 10 pg of DNA (as suggested by Invitrogen Corp.) did not yield as many colonies as expected, making it necessary to double the amount of DNA to achieve a sufficient number of bacterial colonies.

The third central composite design was geared toward testing transformation efficiency at high voltage and short pulse length. The voltage range for this design was 600 V – 700 V and the pulse length range was 10 us – 20 us. The fourth and final experiment was designed without using the DOE module. This design tested the behavior of transformation

efficiency due to multiple pulsing and variation in the frequency of the pulses. The voltage and pulse length setting for all ten runs in this experiment were set at V = 500 Volts, Pulse Length = 8 ms as suggested by BTX corp. Five runs were made using 2 pulses separated by different intervals namely 100 us, 300 ms, 500 ms, 700 ms and 900 ms. Variation in intervals corresponds to variation in frequencies of the pulses. The time intervals for the other five runs were held constant at 500 ms with the number of pulses changing from 1 – 5.

IV. Results and Discussion

Standard Run	Voltage (Volts)	Pulse Length (ms)	Bacterial Colonies
1	200	5	0
2	200	15	0
3	450	5	4
4	450	15	32
5	325	10	3
6	148	10	0
7	501	10	67
8	325	3	0
9	325	17	3
10	325	10	0

Table 1: Bacterial colony count for various electroporation pulses with pulse setting values determined from Central Composite Design 1 (Voltage: 200 – 500 Volts, Pulse length: 5 – 15 ms)

The purpose of Experiments 1 and 2 was to obtain an optimum setting, specifically the voltage and the duration of the pulse, to achieve the highest transformation efficiency of Top10 E-coli cells by electroporation. The transformation efficiency will be directly correlated to the number of bacterial colonies that thrive on the AMP plates. Results for experiment one is reported in Table 1. The plate with the largest number of bacterial colonies, 67, had a pulse voltage of 501 Volts and a pulse length of 10 ms. These values were

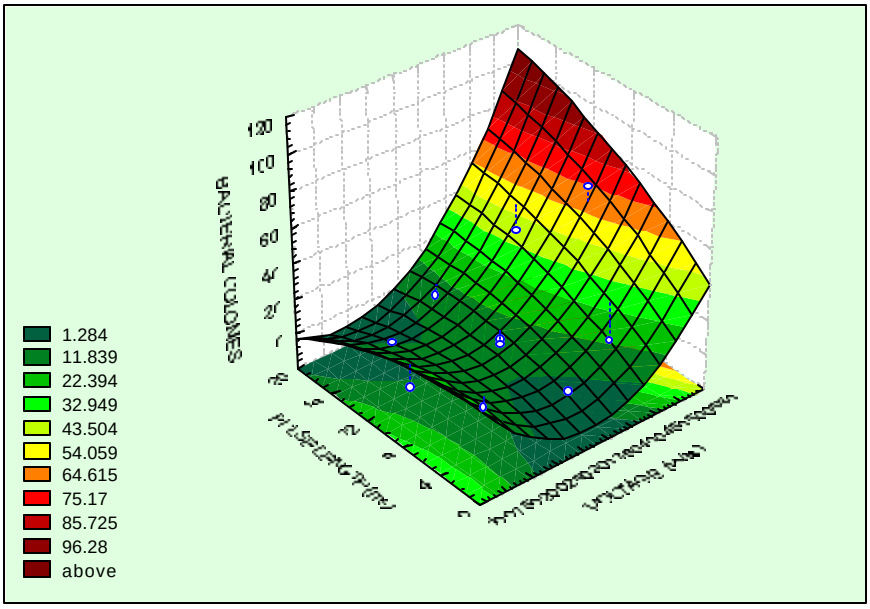

FIGURE 1 A response surface fit to data from Table 1 using Equation 4. This shows the effects of voltage and pulse length on colony count in the electroporation of Top10 E-coli cells.

very close to the factory suggested optimum setting of 500 Volts and 8 ms. The drop off in transformation efficiency as voltages or pulse lengths decreased was dramatic. To get a better picture of the decline in transformation efficiency as well as to quantify its peak value, the data from experiment one was fitted to a response surface following the general quadratic surface equation:

$$y = b_0 + b_1 x_1 + \ldots + b_k x_k + b_{12} x_1 x_2 + b_{13} x_1 x_3 + \ldots + b_{k-1,k} x_{k-1} x_k + b_{11} x_1^{\,2} + \ldots + b_{kk} x_k^{\,2} \qquad (4)$$

where y is the dependent variable, which in this case is bacterial count, $x_1 \ldots x_k$ are the independent factors (for this experiment k = 2, and the independent factors are voltage and pulse length), $x_1 * x_2 \ldots x_{k-1} * x_k$ are the interaction between the independent factors and $x_1^{\,2} \ldots x_k^{\,2}$ are the quadratic components of those factors. The fitted response surface (Figure 1) shows the decline as both the voltage and pulse length decrease. Large decreases in pulse length at constant high voltage also have a significant effect on transformation efficiency.

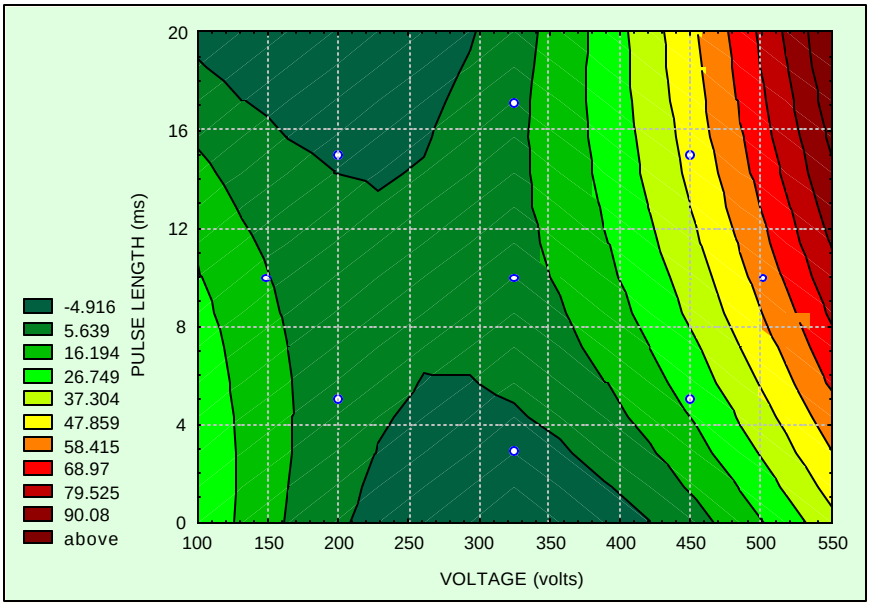

FIGURE 2 A contour plot of colony counts for different settings of voltage and pulse length. Colony count increases dramatically as voltage increases. The plot predicts that a maximum colony count occurs in the upper right region of the contour.

This is evident in the 450 Volts setting. At a pulse length of 15 ms, the bacterial colony count was 32, approximately half the maximum value. However, a 10 ms decrease in pulse length caused the colony count to drop to approximately 1/8 the maximum value, consistent with the behavior of pore radius with respect to time predicted by the Joshi and Schoenbach model (Joshi & Schoenbach, 2000). Even though 450 Volts is sufficient to initiate pore formation, the pore radius and thus the its size will only grow at a specific rate. Therefore, the duration of the applied pulse must provide enough time for the pores to increase in size. If the pulse is shut down prematurely, pores size will not be adequate for plasmid DNA transport across the membrane. Pore radii will not have reached the threshold value and thus will begin to reseal after the applied pulse is discontinued. The minimum and maximum setting values were obtained from the contour plot of Experiment 1 (Figure 2). The observed minimum for the transformation efficiency occurs at a pulse setting of V ~ 148 Volts and pulse length ~ 3 ms.

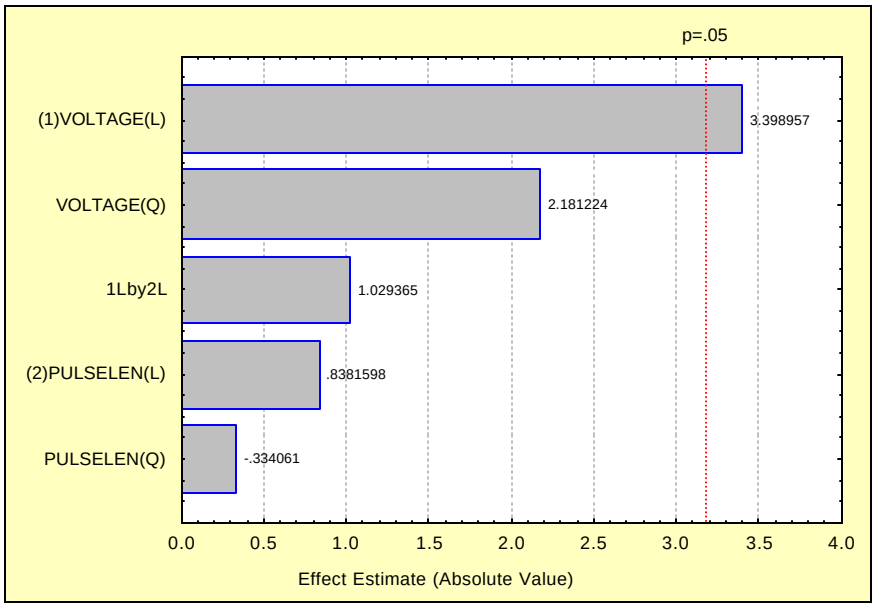

FIGURE 3 A Pareto chart of Standardized effects of voltage and pulse length on colony count in Top10 E-coli electroporation. Effects are calculated using data from Fig. 1. Here, L indicates the linear effects and Q indicates the quadratic effects. The vertical dotted line indicates the p = 0.05 threshold for statistical significance. The linear effect of voltage is the dominant effect.

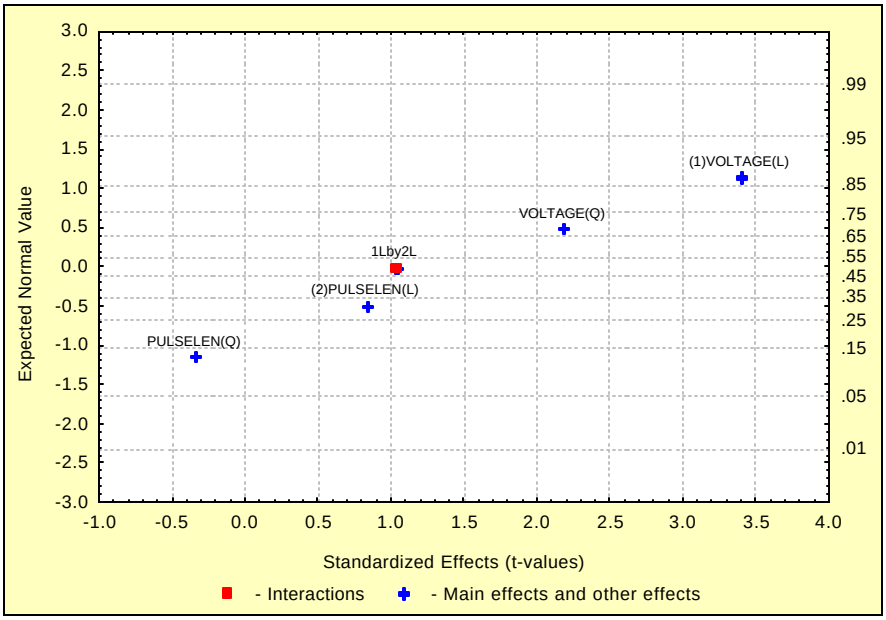

FIGURE 4 A normal probability of effects of voltage and pulse length on colony count in Top10 E-coli electroporation. Standard values of normal distribution (SVND) are computed from effect estimates. The SVND are plotted on the left Y-axis and corresponding normal probabilities on the right Y-axis. Points fall onto a straight line so actual estimates are normally distributed.

The maximum occurs at V ~ 501 Volts and pulse length ~ 17 ms. The maximum voltage value is as expected. However, there is a large discrepancy in the pulse length compared to the normal 8 ms "optimal" value. One reason for this difference is that the maximum value was obtained from general quadratic estimated response curve, therefore the approximation did not take into account the complex behavior of the pores. Specifically, if a strong pulse is applied for too long, the pore radius will exceed the threshold value and will be unable to reseal, eventually leading to cell death. This maximum value was experimentally tested in the central composite Design 2 to assess the accuracy of the approximation.

Although it is apparent that both voltage and duration of the pulse affect transformation efficiency, it is essential to study their individual effects as well as their combined effect. A Pareto chart of standardized effects indicates that the linear effect of voltage is the most significant, while the quadratic effect of pulse length is the least significant (Figure 3). In this chart, the vertical line indicates the $p = 0.05$ threshold for statistical significance. It should also be noted that the combined linear effects of voltage and pulse length are stronger than pulse length alone. A more technical graph, the normal probability plot of effects (Figure 4), gives more information regarding the effects of the independent variables. In this plot, the effect estimates are rank ordered. From these ranks, the standard values of normal distribution (SVND) is computed, assuming that the estimates come from a normal distribution with a common mean. The SVND are plotted on the left Y-axis and corresponding normal probabilities are plotted on the right Y-axis. The points on Figure 4 fall onto a straight line so it may be assumed that the actual estimates are normally distributed. Again, voltage has the strongest effect on transformation efficiency with its linear

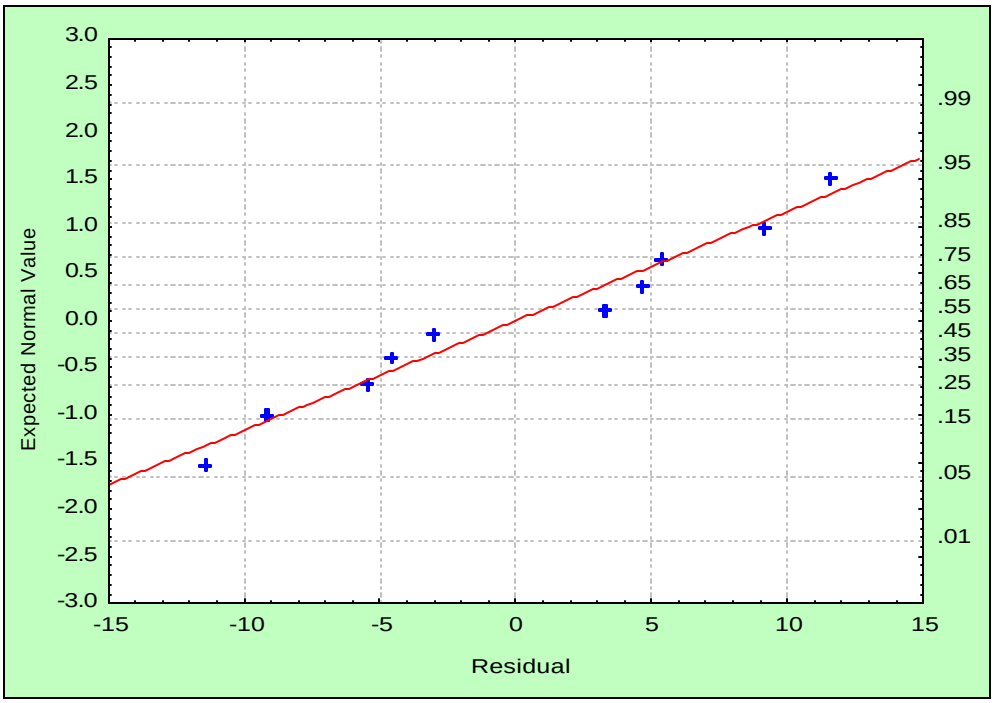

FIGURE 5 A normal probability plot of residuals for data from Fig. 1. Residuals are computed as the difference between the predicted value and the observed value of colony count. The actual residual values are on the X-axis and expected normal values are on the Y-axis. Residuals falling onto a straight line indicates a normal distribution.

effect dominating its quadratic effect. This is due to the fact that the voltage must be sufficient to initiate pore formation.

To check the adequacy of the prediction model, a normal probability plot of the residuals is provided (Figure 5). The residual values are computed as the difference between the predicted value and the observed value for transformation efficiency. The actual residual values are plotted along the X-axis and the expected normal values along the Y-axis. Since the residual values fall approximately on a straight line, one may assumed that the residuals follow the normal distribution. Therefore the prediction model used in this analysis is confirmed to be reasonable.

Standard Run	Voltage (volts)	Pulse Length (ms)	Bacterial Colonies
1	350	7	2
2	350	17	6
3	500	7	28
4	500	17	121
5	319	10	1
6	531	10	61
7	425	3	2
8	425	17	4
9	425	10	7
10	425	10	9

Table 2: Bacterial colony count for various electroporation pulses with pulse setting values determined from Central Composite Design 2 (Voltage: 350 – 500 Volts, Pulse length: 7-17 ms)

In Experiment 2, the range for voltage was decreased and the amount of plasmid DNA was doubled to obtain a higher resolution in the resulting data. Furthermore, the upper limit of the pulse length was extended to 17 ms to test the prediction from Experiment 1. The results for Experiment 2 are provided in Table 2. The result was somewhat surprising since it confirmed the prediction of the model from Experiment 1. The maximum colony count was obtained at a setting of 500 Volts and 17 ms, 7 ms longer in duration than the factory suggested pulse length. This suggests that at a voltage of 500 Volts, the pore radii do not exceed the threshold value within 17 ms, resealing is still possible and the transformation efficiency is increased because the pores are opened for a longer period of time. The fitted response surface plot of experiment 2 (Figure 6) is similar to that of Experiment 1. The contour plot (Figure 7) yielded a predicted minimum setting of 319 Volts and 3 ms and a maximum setting of 530 Volts and 17 ms. These are only estimations of the prediction model and need to be confirmed by experimental data, but further increases in voltage and pulse length are not available within the boundaries provided by the ECM830 Electroporator (see the experimental design and procedure section).

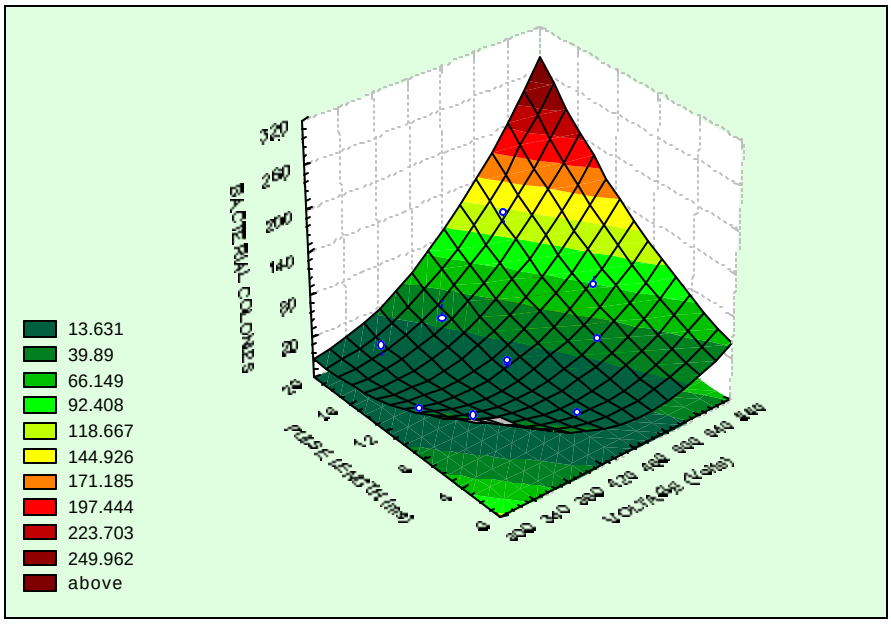

FIGURE 6 A response surface fit to data from Table 2 using Equation 4. This graph shows the effects of voltage and pulse length on colony count in the electroporation of Top10 E-coli cells.

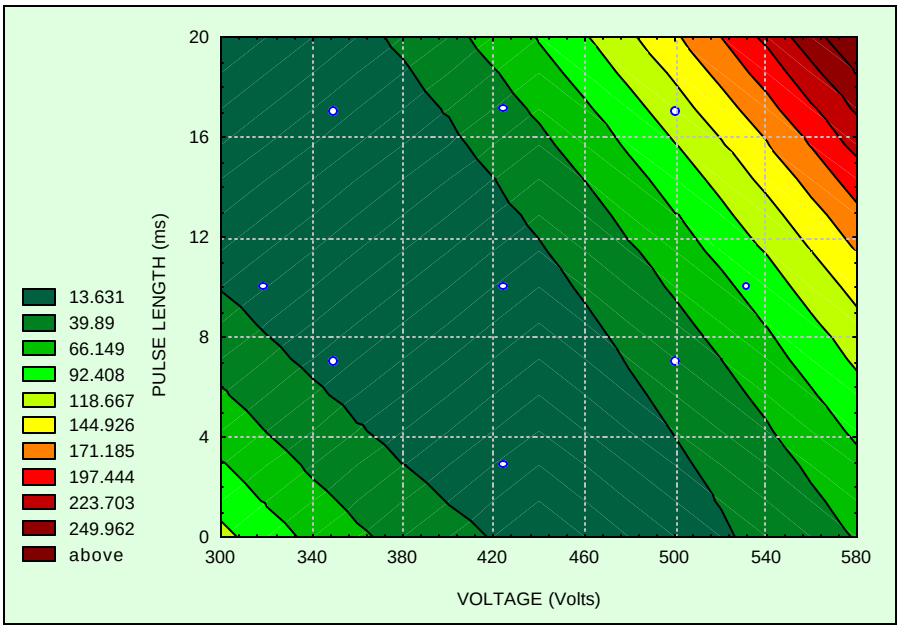

FIGURE 7 A contour plot of colony counts for different settings of voltage and pulse length. Colony count increases dramatically as voltage and pulse length increase. The plot predicts that a maximum colony count occurs in the upper right region of the contour.

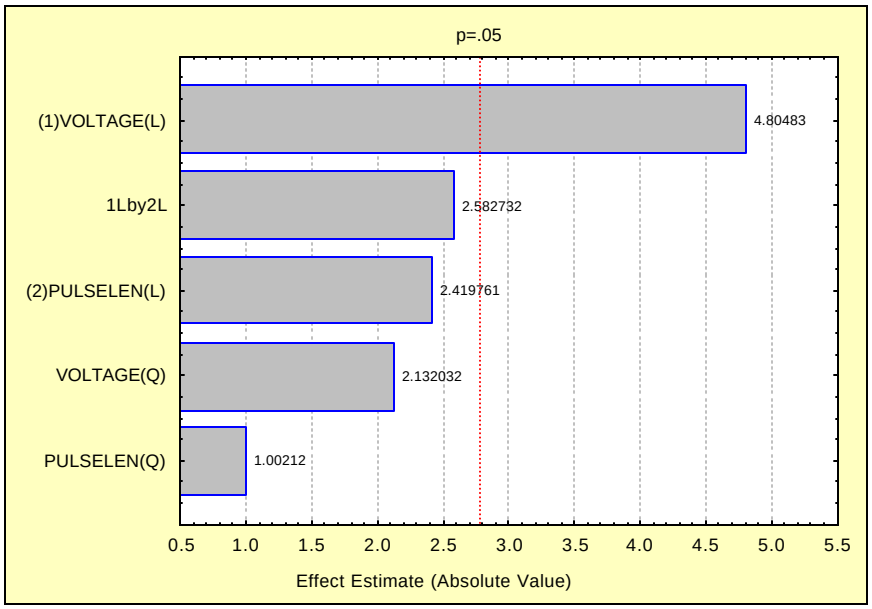

FIGURE 8 A Pareto chart of Standardized effects of voltage and pulse length on colony count in Top10 E-coli electroporation. Effects are calculated using data from Fig. 6. Here, L indicates the linear effects and Q indicates the quadratic effects. The vertical dotted line indicates the p = 0.05 threshold for statistical significance. The linear effect of voltage is the dominant effect.

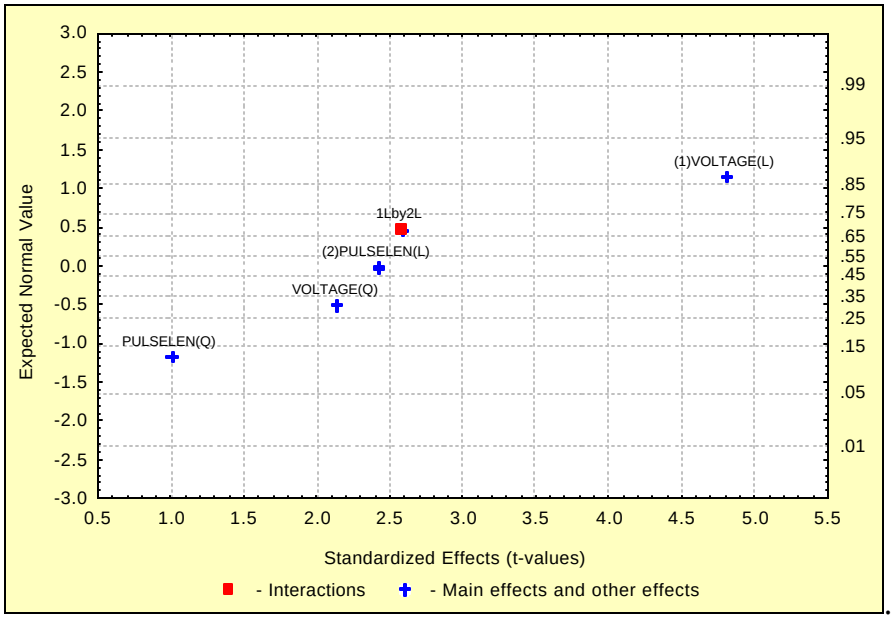

FIGURE 9 A normal probability of effects of voltage and pulse length on colony count in Top10 E-coli electroporation. Standard values of normal distribution (SVND) are computed from effect estimates. The SVND are plotted on the left Y-axis and corresponding normal probabilities on the right Y-axis. Points fall onto a straight line so actual estimates are normally distributed.

Both the Pareto chart of standardized effects (Figure 8) and the normal probability of effects (Figure 9) show that the linear effect of voltage remains the dominant independent factor. Changes in the order of the smaller effects, which would be inconsistent with results of Experiment 1, are in the Pareto chart that the standardized values for these effects to fall below the p=0.05 threshold line for statistical significance. The normal probability plot of the residuals (Figure 10) shows a fairly good fit of the residuals to the theoretical normal distribution line. It is therefore reasonable to accept the fitted model 2 as an adequate prediction model.

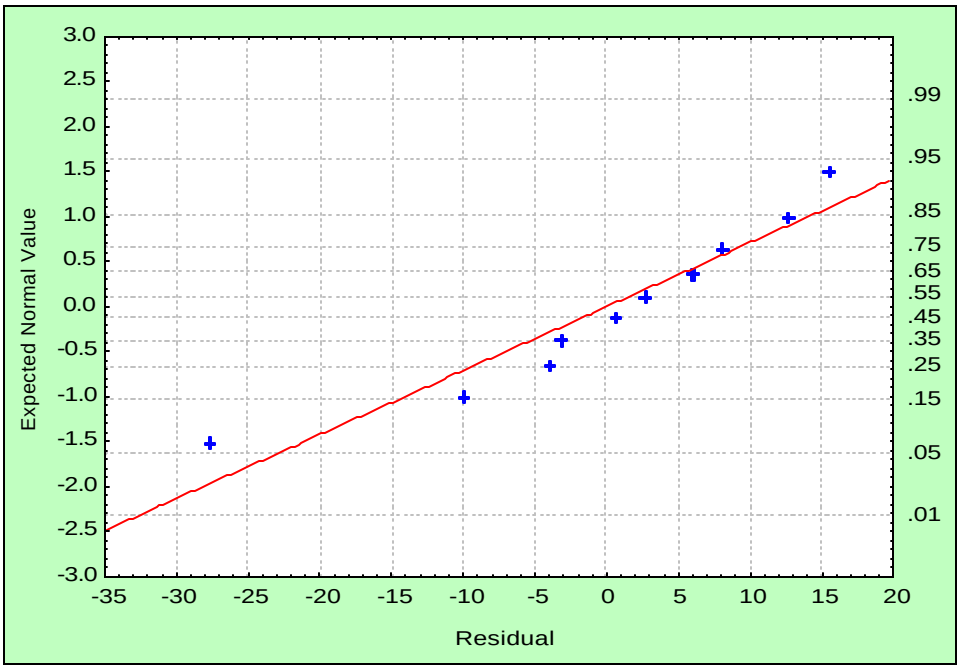

FIGURE 10 A normal probability plot of residuals for data from Fig. 6. Residuals are computed as the difference between the predicted value and the observed value of colony count. The actual residual values are on the X-axis and expected normal values are on the Y-axis. Residuals falling onto a straight line indicates a normal distribution.

Standard Run	Voltage (volts)	Pulse Length (μs)	Bacterial Colonies
1	600	10	0
2	600	20	0
3	700	10	0
4	700	20	0
5	579	15	0
6	720	15	0
7	650	8	0
8	650	22	0
9	650	15	0
10	650	15	0

Table 3: Bacterial colony count for short duration, high voltage electroporation of Top10 E-coli cells. Pulse settings were obtained from Central Composite Design 3. (Voltage: 600 – 700 Volts, Pulse length: 10 – 20 μs)

In experiment 3, none of the plates showed any sign of bacterial growth (Table 3). Note that the values for pulse length were selected to reproduce those of previous studies of high voltage pulsing (Schoenbach & Abou-Ghazala,1999). We suspect that even though the voltages are adequate, the pulse length did not provide sufficient time for the pores to grow. As a result, the transport of plasmid DNA through cell membrane did not occur. The cells are not resistant to the ampicillin antibiotic and thus died on the AMP plates. Future studies should extend the range of pulse length to obtain more compelling evidence of short duration, high voltage pulsing.

Experiment 4 yielded interesting results regarding the effects of multiple pulsing on transformation efficiency (Table 4). The first five runs tested the effects of frequency using only two pulses at standard setting of 500 Volts and 8 ms. The entire range of time interval between pulses available on the ECM 830 was tested. Results showed no cells survived two pulses. This suggests that even at 900 ms interval, the pores are not given enough time to

decrease sufficiently before the addition of a second pulse caused the radii of the pores to increase beyond the threshold value leading to cell death. With this data, the results for the second set of five runs should not come as a surprise. Only the standard single pulse setting of 500 Volts and 8 ms yielded bacterial colonies. Again, the other multiple pulse runs made the pores too large for resealing, thus causing an irreversible electrical breakdown and certain cell death. This Data will be useful in dynamic analysis of the microscopic motions of the cell membranes during EP.

Standard Run	Voltage (volts)	Pulse Length (ms)	# Pulse	Time Interval (ms)	Bacterial Colonies
1	500	8	2	100	0
2	500	8	2	300	0
3	500	8	2	500	0
4	500	8	2	700	0
5	500	8	2	900	0
6	500	8	1	500	52
7	500	8	2	500	0
8	500	8	3	500	0
9	500	8	4	500	0
10	500	8	5	500	0

Table 4: Bacterial colony count for multiple pulses electroporation of Top10 E-coli cells. Number of pulses varied from 1 – 5 pulses, while pulsing intervals varied from 100 – 900 ms.

V. Conclusion

The results from our laboratory experiments suggest that pulsing characteristics such as voltage and pulse length significantly affect colony count in E-coli cell electroporation. The pulse length and the interaction between the pulse length and the voltage have a significantly smaller effect on colony count. Analysis of the experimental data shows that voltage is the dominant effect on colony count. In our attempt to identify the optimum pulse

setting for E-coli electroporation, we found that while the voltage is as recommended by BTX (500 Volt), the pulse length should be set for a longer pulse (~ 17ms). This optimum setting is limited to E-coli electroporation using the BTX Square-wave electroporator (model ECM 830). The results for short duration, high voltage electroporation yielded zero colony count. A possible explanation for this result is that the pulse lengths, ranging from 10 – 20 μs, were too short for adequate growth of pore radii. However, further experimentation is required to test this hypothesis. The results for multiple-pulses electroporation also yielded zero colony count. Since this is only the initial stage for multiple-pulses electroporation, no definite conclusion can be drawn from the results.

VI. Future Work

In the future, studies on E-coli electroporation should focus on developing more versatile pulse generators. Specifically, electroporation should be done in the voltage range of 400 – 600 Volts with pulse length ranging from 10 – 25 ms in order to further assess optimum pulse setting as predicted by this project. Note that we used square-wave pulses exclusively in this project. Experiments with different pulse shapes may yield interesting results. Lastly, more experiments should be done with short duration, high voltage and multiple-pulses electroporation. We feel that these types of pulse setting have the potential to yield significantly higher colony counts.

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