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Recovery of intracellular materials by electroporation

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ABSTRACT

RECOVERY OF INTRACELLULAR MATERIALS BY ELECTROPORATION

by

Wendy Elaine Reid

Electroporation is commonly used to transfer genetic materials such as DNA into the cell by exposing the cell to a high electric field that momentarily permeabilizes the cell membrane. This premise was utilized to use electroporation as a means of releasing intracellular proteins. A procedure has been developed for the release of intracellular proteins through the use of electroporation. This procedure is a breakthrough. In general, industrial scale techniques to recover proteins from microorganisms are inefficient (Clarkson, 1993). A benefit of this electroporation-based mechanism would be the reduction of steps in the downstream processing required to purify the desired product.

Yeast cells in suspensions were subjected to high voltage electric pulses by using an E.coli Pulser from *BIO-RAD*. The pulses permeabilized the cell and allowed the release of intracellular materials. Research has shown that the amount of released materials increased with the number of times the cells were subjected to pulses. In addition, experiments show that even more material can be released by repulsing the cells. Other parameters to affect the release of intracellular materials were optical density, growth stage, and the storage of the cell suspensions. The protein assay was found to be an effective means to determine the released intracellular product concentration.

RECOVERY OF INTRACELLULAR MATERIALS BY ELECTROPORATION

by
Wendy Elaine Reid

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This thesis is dedicated to
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CHAPTER 1

INTRODUCTION

1.1 Purpose of Cell Disruption

Cell disruption is the disintegration of the cell to retrieve a desired product, such as intracellular proteins that would not be generally secreted by the microorganism (Schaeiwitz et al., 1989). Disruption of cells on an industrial scale can be done to produce hormones and proteins for therapeutic and nutritional applications (Engler, 1979). This process takes place as a result of damaging the surrounding cell envelope and other intracellular structures such as organelles. The strength of the cell is affected by the growth environment and the genetic information within the cell (Engler, 1985). Other factors that affect cell disruption, could be the pH of the lysis buffer, and the properties of the cell wall (Schaeiwitz et al., 1989; Bauer et al., 1989).

1.2 Current Cell Disruption Technologies

There are two categories of methods for cell disruption: physical and chemical. Physical methods of disruption include the use of mechanical devices such as ball mills and homogenizers, where a high shear force is used to rip the cell wall apart; other physical methods involve the use of shock waves, or ultrasound (Schaeiwitz et al., 1989; Brodelius et al., 1988; Bauer et al., 1989). Chemical methods include osmotic shock, use of solvents, and enzyme lysis, where the chemicals are used for the permeabilization of the cell membrane. Osmotic shock is the use of the osmotic pressure to burst the cells open. Some solvents can be used to dissolve the cell membrane.

The method of cell disruption affects the following steps needed to recover the bioproduct in downstream processing. Downstream processing includes separation steps such as cell disruption, centrifugation, phase separation, adsorption and chromatography (Bauer et al., 1989). The efficiency of the method of cell disruption is dependent on how much of the desired product is recovered during the separation stage.

When using mechanical devices such as ball mills or homogenizers, the cell wall is ground into micronized bits. This is unfavorable because it affects the subsequent stages in separation and purification. Longer times and higher g forces are required as a result to sediment the bits of the cell debris (Siddiqi et al., 1996). In industry, mechanical methods are favored due to the high expense of chemical methods and the resulting chemical contamination of the product (Schaeiwitz et al., 1989).

1.3 Electroporation as a Means of Intracellular Product Release

Electroporation is the transient permeabilization of a cell membrane to genetic materials such as plasmid DNA as a result of its exposure to high electric fields (Rosemberg et al., 1994). This exposure causes transient holes to be formed in the membrane (Hofmann and Dev, 1993). This phenomenon can be utilized to use electroporation as a means of recovering cell products without cell disruption. A cell suspension is exposed to high voltage pulses for short periods of time. These pulses permeabilize the cell membrane. See Figure 1. The intracellular materials could then flow out of the cell as a result of the permeabilized cell membrane. There would be no insertion of genetic materials. The goal would be the efficient retrieval of the desired intracellular products with little or no

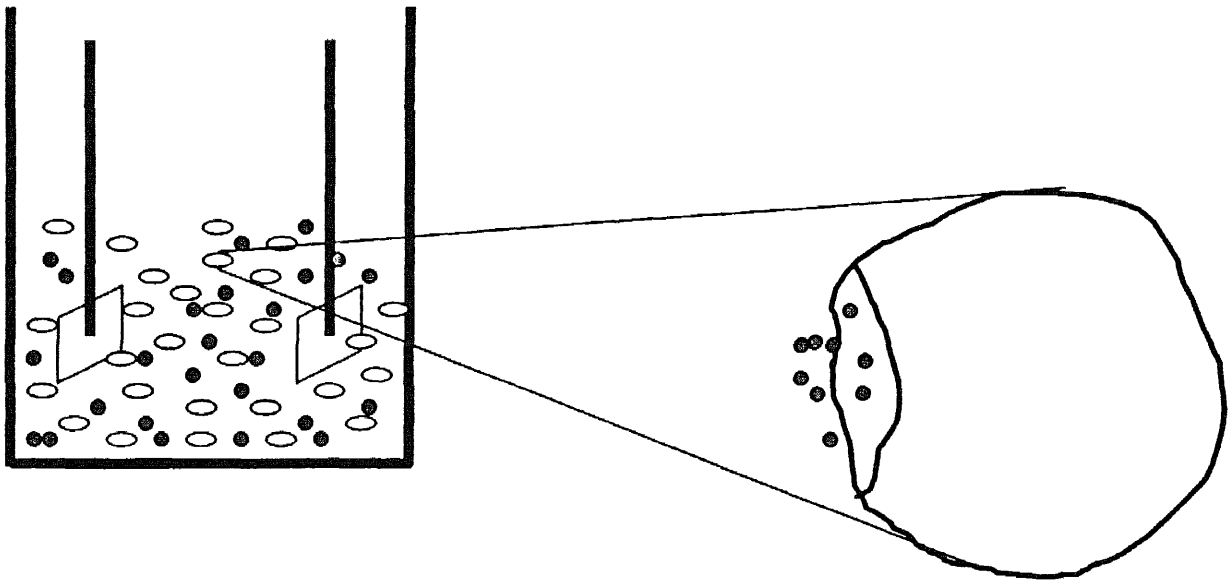

Figure 1.1 Schematic of the Electroporation of Yeast Cells: Yeast Cells (White) Intracellular Products (Gray); Picture Not Drawn to Scale.

permanent disruption of the cell membrane and the overall cell. In fact, the cell may continue to live. A benefit in this proposed mechanism is the reduction of the steps in downstream processing to purify the product, since cell debris are hardly produced.

1.4 Research Objective

The research objective is to efficiently retrieve intracellular materials through the use of electroporation; specifically the use of multiple electric pulses are employed to permeate the cells of *Saccharomyces cerevisiae*. The key is to retrieve the biomolecules with little or no damage to the cells thereby avoiding problems in downstream processing caused by the presence of cell debris.

The general objectives of this research can be divided into four categories:

a. To retrieve intracellular proteins from yeast cells via an electric pulse with little or no damage to yeast cells.

b. To study the effects of multiple pulses and cell concentration on the release of intracellular proteins.

c. To compare the amount of protein released due to total cell lysis with that obtained by way of electroporation.

d. To study the effects of cell incubation time on the amount of protein released.

1.5 Research Methodology

For this research, a yeast strain supplied by the United States Department of Agriculture has been used. Yeast has been widely used in the research of cell disruption processes. This research was carried out in three phases.

Phase I: Fermentation of Yeast Cells

a. Cells will be cultured and harvested under specific conditions.

b. Yeast cells will be washed after being harvested.

Phase II: Electroporation

a. Yeast cells will be electroporated under specified conditions. A number of studies will be done by varying the concentration and shake time.

b. Samples will be centrifuged and then analyzed using a spectrophotometer.

Phase III: Parallel Study

a. Cells will be disrupted using the methods of electroporation and total cell lysis in parallel.

b. There will be a comparative study of the released bioproducts obtained by each method.

CHAPTER 2

THEORY

2.1 Electroporation

The use of electroporation as a technique to retrieve desired intracellular materials is an extraordinary concept. The cell is subjected to multiple high voltage pulses that permeate the cell membrane. Because the concentration of the intracellular materials is greater within the interior portion of the cell, the intracellular materials flow from a region of higher concentration to a region of lower concentration. The intracellular materials flow from the cell interior into the buffer that the cells are suspended in. This can be achieved with little or no damage to the cell. It is expected that the amount of released intracellular material should increase as the number of multiple pulses increase until the release of the intracellular materials reaches a maximum. It is proposed that pulses applied beyond this point weakens the cell membrane.

2.2 Total Protein Content of Cells

For purposes of this research, the total protein content of cells is defined as the total amount of proteins released from cells as a result of a total cell lysis procedure. In this study, the cell is completely disrupted to release all intracellular materials. The retrieved protein solutions are analyzed such that the results are termed as the concentration equivalent to 100% protein content. Results from the electroporation experiments are represented as a percentage of the total amount of protein released due to total cell lysis.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Materials Used

3.1.1 Fermentation

Yeast Strains: *Saccharomyces cerevisiae* NRRL Y-12636 and NRRL Y-2034 from the United States Department of Agriculture (Peoria, IL); Dextrose (anhydrous, FW 180.16) from Fisher Scientific (Springfield, NJ); YM Nutrient Broth and Agar Components: Yeast Extract, Agar Type E, Malt Extract, Peptone (Enzymatic hydrolysate, Type 1 from Meat) from Sigma (St. Louis, MO).

3.1.2 Electroporation

Sterile Centrifugation Tubes, 15 ml from Fisher Scientific (Springfield, NJ); Transfer Pipettes from VWR Scientific Products (Bridgeport, NJ).

3.1.3 Total Cell Lysis

Potassium Acetate, FW 98.15, from Fisher Scientific (Springfield, NJ); Phosphoric Acid, (concentration 85%), EDTA (purity 99%, FW 292.2), TRIZMA Base (purity 99.9%, FW 121.1), Lauryl Sulfate (purity 95%, FW 288.4) from Sigma (St. Louis, MO); Polypropylene Centrifuge Tubes, 50 ml from Sorvall (Newtown, CT).

3.1.4 Analysis

Folin-Cioucalteu's Phenol Reagent, Bovine Serum Albumin (96% Minimum), Brillliant Blue G, FW 854.0, from Sigma (St. Louis, MO); 85% Phosphoric Acid from Merck (Rahway, NJ); Sodium Hydroxide (purity 97.1%, FW 40.0), 95% Ethyl Alcohol, Cupric Sulfate (purity 99.2%, FW 249.68) from Fisher Scientific (Springfield, NJ); Sodium Citrate, Sodium Carbonate (anhydrous, FW 105.99) from Mallinckrodt (New York, NY).

3.2 Equipment

3.2.1 Fermentation

Omni Culture Bench-Top Fermenter Model # 178657 from Virtis (Gardiner, NY); Model 25X Electric Sterilizer from Wisconsin Aluminum Foundry (Manitowoc, Wis.); Environmental Incubator Shaker Model G-24 with Digital Display from New Brunswick Scientific (Edison, NJ); Clinical Centrifuge from International Equipment (Needham Hts., Mass.).

3.2.2 Electroporation

E.coli Pulser Transformation Apparatus and E.coli Pulser/Gene Pulser Cuvettes (Cuvette Gap 0.2 cm, 400 μl) from *BIO-RAD* (Richmond, CA).

3.2.3 Total Cell Lysis

Sorvall Centrifuge, and Model 28/50 Rotor from Sorvall (Newtown, CT).

3.2.4 Analysis

HITACHI Model U-2000 Double Beam UV/Vis Spectrophotometer from HITACHI Instruments, Inc. (Danbury, CT); Quartz Cuvettes (3ml volume) from Sigma (St. Louis, MO).

3.3 Experimental Procedure

3.3.1 Fermentation

The protocol for fermentation was developed by combining procedures obtained from the United States Department of Agriculture (Peoria, IL) and The Virtis Company (Gardiner, NY) to suit the experiment specifications. YM nutrient broth was prepared by adding 3 g of yeast extract, 3 g malt extract, and 5 g of peptone, 10 g of glucose and 1 liter of distilled water. See Table 3.1.

Table 3.1 List of YM Nutrient Broth and Agar Components

Yeast Extract (g)	**3**	**1.5**	**0.75**	**0.375**	**0.3**	**0.15**
Malt Extract (g)	**3**	**1.5**	**0.75**	**0.375**	**0.3**	**0.15**
Peptone (g)	**5**	**2.5**	**1.25**	**0.625**	**0.5**	**0.25**
Glucose (g)	**10**	**5**	**2.5**	**1.25**	**1**	**0.5**
Distilled Water (ml)	**1000**	**500**	**250**	**125**	**100**	**50**
Agar (g)	**20**	**10**	**5**	**2.5**	**2**	**1**

When sterilizing the broth, the extracts were separated from glucose to prevent caramelization or the formation of toxic products between the carbohydrates and trace elements that could inhibit the growth of yeast cells. Therefore, the 10 g of glucose were

added to 500 ml of distilled water and the yeast extract, malt extract, and peptone were added to the remaining 500 ml of distilled water. The YM agar was prepared by adding 20 g of agar to the amounts of extracts (yeast extract, malt extract, and peptone), and 10 g of glucose to 1 liter of distilled water. The agar was also separated in the same manner as the YM broth, agar and extracts with 500 ml of water and glucose with remaining 500 ml of water. Each mixture was sterilized by use of a Model 25X Electric Sterilizer (Wisconsin Aluminum Foundry, Wis.). The temperature and duration for sterilization were 121 °C for 15 to 30 minutes.

The fermentation vessel (The Virtis Company) and its components, the culture vessel, and head plate were sterilized at 121° C for at least 30 minutes at 15 psig. The cartridge heater and temperature sensing probe were removed. The terminal connections were wrapped loosely with nonabsorbent cotton and foil. This was important in order to prevent condensation from accumulating in the temperature sensing probe and cartridge heater thermowells, since contact with liquid will result in damage of these probes. The vessel assembly was vented before autoclaving. This was done by attaching the air sparger and exhaust air outlet to the air filters, where the non-sterile side of the air filter was left open for pressure equalization. The vessel assembly was not autoclaved dry. It was sterilized with the medium, the YM broth.

After sterilizing the needed equipment, the yeast strain (USDA, Peoria, IL) was obtained and a deep file mark was made on the middle of the tube. The tube was then wiped with cotton moistened with alcohol. Next the tube was broken and the pellet was poured into a 10X75 mm tube with 0.5 to 0.7 ml of YM broth. The tube was allowed to stand for a few minutes to allow the pellet to dissolve. The tube was gently shaken. A

loopful of the suspension was streaked on YM agar plates to grow cells. The plates were incubated at 25°C for 2 to 4 days. The broth tube was stored in a refrigerator at a temperature of 0 to 5°C. The broth culture was restreaked if no colonies were obtained. After sufficient growth of cells, the cells were transferred to a shake flask with 500 ml of YM broth. In early stages of experimental studies, the entire fermentation procedure was done. After reducing the quantities needed for experimental studies, it was decided to eliminate the fermenter stage of the process and simply harvest the cells following the shake flask stage.

The shake flask was incubated for 12 to 30 hours at a temperature of 30°C. The YM broth was initially added to the fermenter. Later the shake flask contents were added to the fermenter. The time of fermentation varied from 24 to 36. The yeast cell suspensions were then prepared for the electroporation stage of the experiment.

3.3.2 Cell Suspension Preparation and Storage

3.3.2.1 Methods of Preparation: The yeast cell suspensions were divided into several 15 ml sterile centrifuge tubes (Fisher Scientific). These samples were centrifuged (International Equipment). The supernatant was discarded, and half the cell pellets were stored at -20 °C for 1 day. The remaining tubes were used fresh. The pellets were washed 1 to 2 times with sterile deionized water that was filtered by a 4 Module Cartridge (Fisher Scientific). When the cells were washed, the tubes were gently inverted to distribute the cells throughout the suspension. After each wash, the suspension was centrifuged for 5 minutes at 3000xg. Finally, after the last wash, sterile deionized water was added to the cell pellet and the cell concentration was adjusted to an optical density

of 1.3 to 1.5 at a wavelength of 600 nm which corresponded to a cell concentration of 1 x 10^8 cells/ml (*BIO-RAD* (b)).

Three methods were followed when preparing cell suspensions. These methods were developed as a result of the ongoing challenge to conduct experiments and generate reasonable data. After washing cells and making the specified suspension, the suspension was centrifuged for 5 minutes at 3000xg. The supernatant was poured off and labeled as the "before-pulse" sample. The cells were then resuspended in an amount of sterile water equal to the amount poured off. The optical density was checked to see if it was close to the previous concentration. The concentration should always be approximately the same. The suspension was then pulsed according to the electroporation procedure.

In the second method, the batch of cells were divided into equal amounts corresponding to the number of pulse sets after washing the cells. For example, if the cells were pulsed 0, 10, and 20 times there were 3 pulse sets. Therefore, the batch of cells were divided into three equal amounts; the volume in each was at least 3 ml.

The third method was similar to the second. The volume of the samples were reduced. Instead of the samples being at least 3 ml in volume, these samples were 400 µl in volume and diluted to 4 ml with deionized water. This method was quickly discarded due to an extraordinary variation in results.

3.3.2.2 Cell Washing Protocols: A study of the release of proteins as a result of washing the yeast cell suspension was done to find out how the optical density changes as a result of multiple washes. The search was done in an attempt to have an optical density of zero (0) after the washes. The study was done before and after a few

modifications. Initial studies consisted of first centrifuging a batch of cells for 5 minutes at a speed of 3000xg. The broth was poured off and the cell pellet was resuspended in the same amount of sterile water. The tube was inverted several times to wash the cells. Centrifugation of these cells at the same speed and time followed. The water was poured off. The pellet was resuspended in sterile water again. This was the second wash. The tube was inverted again and later centrifuged. Each of the supernatant solutions was poured into clean sterile tubes and labeled the "first wash" and "second wash'' respectively. The pellet was resuspended and centrifuged again. This supernatant solution was labeled "before-pulse". Each of the supernatants was measured for Protein Concentration at 280 nm with the HITACHI U-2000 spectrophotometer.

Modified protocols consisted of combining samples. This was done to prepare samples for electroporation. Because five samples with 3 ml per sample were needed for electroporation, one tube was not sufficient to make up this volume. The use of five samples was a means to deal with oscillations that occurred when analyzing individual samples after pulsing. Although the five samples may oscillate the statistical average should increase as the pulses increase. Therefore, 2 batches of cells were combined and divided into five sterile tubes. The next cell washing protocol incorporated this change in the previously described protocol. After obtaining the cell broth suspension from the shake flask upon cell culturing, the cell suspension was added to several sterile tubes, a volume of 10 ml each. The tubes were labeled A, B, and C; here A indicates the batch that will not be divided, and B and C are divided and combined among 5 tubes.

Tubes A, B, C were washed according to the protocol initially explained. However B and C were divided and combined among the 5 tubes. Each of the 5 tubes

was labeled B and C(1) , B and C(2) etc. Each of the 5 tubes was centrifuged for 5 minutes at 3000xg. The supernatant was then poured off. There was also a study where the before-pulse sample was centrifuged at 6000xg for 6 minutes. The results of 3000xg and 6000xg were compared. The study was perfected as certain observations were made. The reasons for these will be discussed in the section on results and discussion.

3.3.3 Electroporation

3.3.3.1 Repetitive Pulse Study: The repetitive pulse study was done to study the effects of multiple pulses on the electroporation cuvettes. The E. coli Pulser (*BIO-RAD*) was set for 1.5 kV. An electroporation cuvette was first filled with 400 µl of sterile water. The cuvette was then pulsed several times. The time constant and actual voltage applied were recorded. Figure 3.1 shows the equipment used to conduct the electroporation experiments (*BIO-RAD*).

3.3.3.2 Multiple Pulse Study: The electroporation procedure recommended by *BIO-RAD* was modified. Each tube of cell suspension was electroporated using an E. coli Pulser. Each suspension was divided among eight (8) clean electroporation cuvettes also from *BIO-RAD*. The dimension of the cuvette gap was 0.2 cm wide. The volume of the cuvette was 400 µl. The E. coli pulser was set to 1.5 kV and the average time constant was 5.0 msec. The cell samples were pulsed numerous times and compared. They were pulsed 10, 20, 40, and 60 times. For each pulse set, there were (4) four

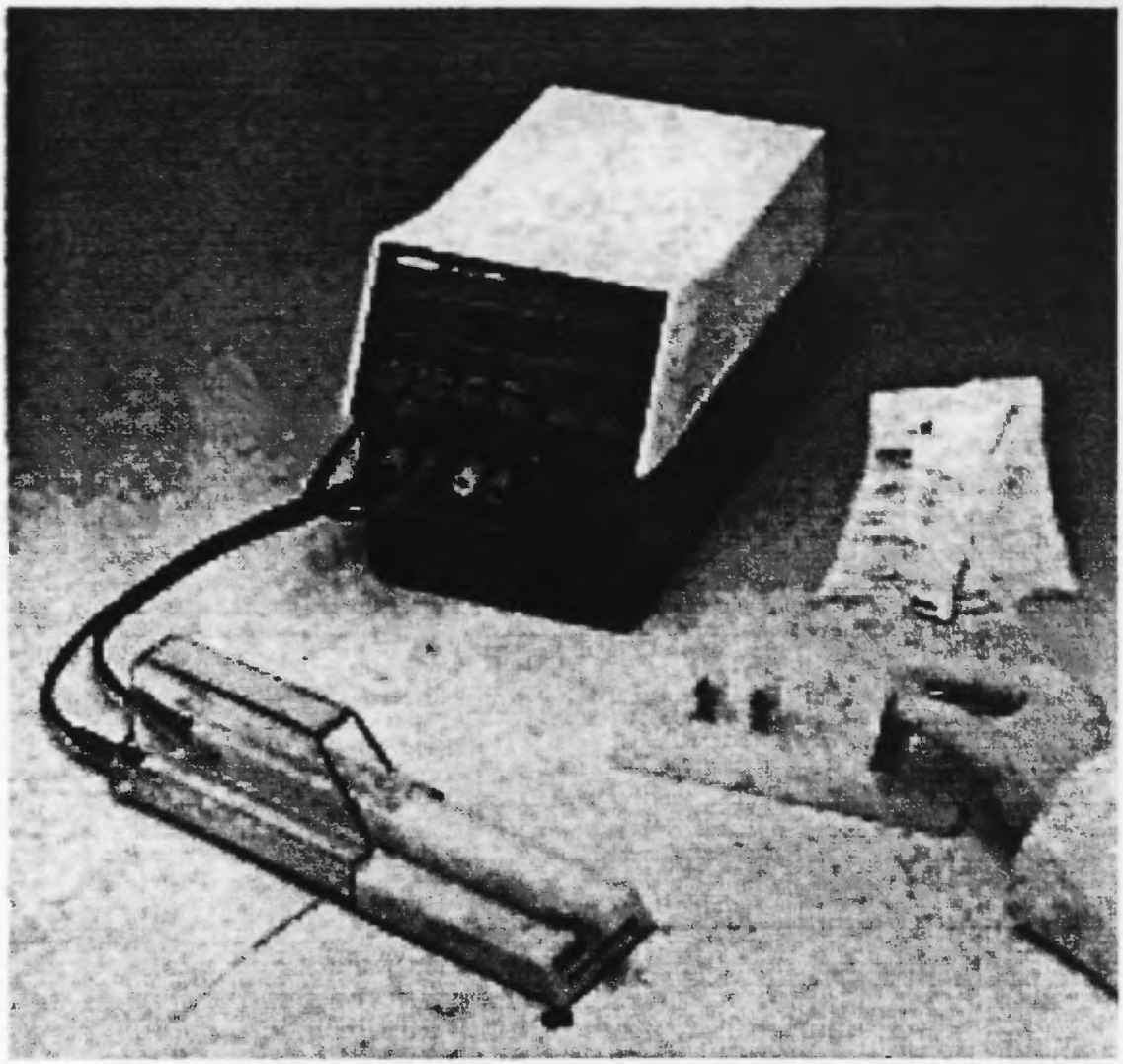

Figure 3.1 E.coli Pulser Transformation Apparatus. Taken from the Cover of the "E.coli Pulser Transformation Apparatus Operation Instructions and Applications Guide" from *BIO-RAD*.

samples to represent each set of pulses that were made from each pair of cell batches. Initially, the experiment was done with only one sample for a single pulse set that corresponded to 1 pulse. The procedure was then modified to multiple pulses to study the trend of the released intracellular materials.

A pipette was used to transfer 400 µl of cell suspension to the electroporation cuvettes. The electroporation cuvette was loaded on to the apparatus. The sample was then pulsed the specified number of times. Eight 400 µl samples were pulsed for each cell suspension. The pulsed samples were transferred using a VWR pipette into a sterile centrifuge tube (Fisher Scientific).

The sample was centrifuged at room temperature for 5 minutes at 3000xg. The supernatant was poured off into a new sterile tube. This was done after pulsing all the samples. The supernatant tubes were labeled with the number of pulses and with the sample number ranging from 1 to 5. The cell pellet was resuspended in sterile water and the entire procedure was repeated.

3.3.4 Analysis of Intracellular Proteins

3.3.4.1 Coomassie Blue

Reagent Preparation

100 g of Brilliant Blue G (Sigma) was dissolved in a mixture of 100 ml 85% phosphoric acid (Merck) and 50 ml of 95% ethanol (Fisher Scientific). After the dye was completely dissolved, the volume was diluted to 1 liter with deionized water (Bradford, 1976).

Table 3.2 List of Reagent Components for Coomassie Blue Assay (Bradford, 1976)

Brilliant Blue G (g)	**100**
Phosphoric Acid (85%), (ml)	**100**
Ethanol (95%), (ml)	**50**
Deionized Water (ml)	**1000**

Procedure

First, the spectrophotometer (HITACHI) was warmed for 15 minutes. In a clean tube, 20 µl of sample and 50 µl of sodium hydroxide were added. 1 ml of dye reagent was then added and the mixture was incubated for 5 minutes. The absorbance was measured at a wavelength of 590 nm in a polystyrene cuvette (Fisher Scientific). This procedure was scaled down to fit the experiment (Bradford, 1976).

3.3.4.2 Bradford Assay

This assay coupled with the Coomassie Blue Assay previously discussed is essentially the same procedure with a few modifications. It is included because both procedures were used to determine the protein concentration.

Preparation Needed Solutions

Bradford Stock Solution

100 ml of 95% ethanol (Fisher Scientific), 200 ml of 88% phosphoric acid (Merck), and 350 mg of Brilliant Blue G (Sigma) were combined and stored at room temperature (Bollag, 1996).

Table 3.3 List of Volume Components for the Bradford Stock Solution (Bollag, 1996)

Ethanol	**100 ml**
Phosphoric Acid	**200 ml**
Brilliant Blue G	**350 g**

Bradford Working Buffer

425 ml of distilled water, 15 ml of 95% ethanol, 30 ml of 88% phosphoric acid, and 30 ml Bradford Stock Solution were mixed and filtered through Whatman No. 1 filter paper.

Table 3.4 List of Components for the Bradford Working Buffer (Bollag, 1996)

Ethanol	**15 ml**
Phosphoric Acid	**30 ml**
Stock Solution	**30 ml**
Distilled Water	**425 ml**

Procedure

A protein solution with a maximum volume of 100 µl was pipetted into a tube. The experimental buffer was then added to make the total volume 100 µl. 1 ml of the Bradford Working Buffer was added and vortexed. The absorbance was measured at a wavelength of 595 nm and was read after 2 minutes but before an hour.

3.3.4.3 Lowry Assay

Preparation of Solutions

Solution A

0.5 g of cupric sulfate (Fisher Scientific), 1g of sodium citrate, and 100 ml of distilled water were mixed and stored at room temperature.

Table 3.5 List of Components for Solution A for the Lowry Assay (Bollag, 1996)

Cupric Sulfate	**0.5 g**
Sodium Citrate (hydrated)	**1 g**
Distilled Water	**100 ml**

Solution B

20 g of sodium carbonate, 4 g sodium hydroxide, and distilled water up to 1 liter were mixed and stored at room temperature.

Table 3.6 List of Components for Solution B of the Lowry Assay (Bollag, 1996)

Sodium Carbonate	**20 g**
Sodium Hydroxide	**4 g**
Distilled Water	**Dilute up to a liter**

Solution C

1 ml of Solution A and 50 ml of Solution B were mixed.

Table 3.7 List of Components for Solution C of the Lowry Assay (Bollag, 1996)

Solution A	**1 ml**
Solution B	**50 ml**

Solution D

10 ml of Folin-Cioucalteu phenol reagent (Sigma) and 10 ml of distilled water were mixed.

Table 3.8 List of Components for Solution D of the Lowry Assay (Bollag, 1996)

Folin-Cioucalteu	**10 ml**
Distilled Water	**10 ml**

Procedure

A protein sample solution was brought to a volume of 0.5 ml with distilled water. Then 2.5 ml of Solution C was added. The mixture was vortexed and allowed to stand at room temperature for 5 to 10 minutes. 0.25 ml of Solution D was added and vortexed. The absorbance was measured at a wavelength of 750 nm after 20 to 30 minutes.

3.3.4.4 UV Absorbance Assay: A 3 ml volume of the protein solution was put into a clean quartz cuvette. The absorbance was measured at a wavelength of 280 nm. The analysis of the intracellular proteins was carried out. The absorbance of the supernatant was measured at a wavelength of 280 nm using the HITACHI spectrophotometer. The Quartz cuvettes (Sigma) were properly cleaned. The absorbance of each supernatant was

then measured one by one. The absorbance values were compared to the absorbance of Bovine Serum Albumin (BSA) solutions at various concentrations. The supernatant absorbance was located on the calibration curve and the corresponding BSA concentration was found. This information provided the absorbance of the release of intracellular materials and the equivalent BSA concentration. The challenge that lay in the future will be the identification of released proteins.

3.3.4.5 Generation of Calibration Curves: For each of the above methods of protein determination, a calibration curve was generated. Bovine Serum Albumin (BSA) was dissolved in sterile water. First, four tubes were obtained. The first tube contained 5 mg of BSA dissolved in 5 ml of water where the concentration was 1 mg/ml. The second tube contained 2.5 ml taken from the first tube and 2.5 ml of water making the concentration of tube 2, 0.5 mg/ml. The third tube had 2.5 ml of water with 2.5 ml of the protein solution from tube 2. The concentration of this tube was 0.25 mg/ml. The final tube contained 2.5 ml of water with 2.5 ml of solution 3 making the final concentration 0.125 mg/ml. The solution concentrations were then measured spectrophotometrically by following the procedure for the UV absorbance method. Lowry and Bradford methods have specific ways to produce the calibration curves. For each, the absorbance was plotted versus the concentration.

3.3.5 Total Cell Lysis Procedure

Preparation of Solutions

Solution P1

To make a solution of 50 mM Tris-HCl, 10 mM EDTA with a pH of 8, 6.055 g of Tris Base and 3.722 g EDTA.2 H_2O was dissolved in water. The solution was allowed to cool before adjusting the pH with HCl. The volume was adjusted to 1 liter. It was stored at 4 ° C.

Solution P2

8.0 g of sodium hydroxide pellets dissolved in 950 ml of water was mixed with 50 ml 20% lauryl sulfate solution. The final volume was 1 liter with a concentration of 200 mM sodium hydroxide with 1% lauryl sulfate. It was stored at room temperature.

Solution P3

294.45 g of potassium acetate was dissolved in 500 ml water. The pH was adjusted to 5.5 with glacial acetic acid (approximately 110 ml). The volume was then adjusted to 1 liter with water. The final concentration should be 3 M potassium acetate. It was stored at 4 ° C.

Procedure

This total cell lysis protocol was taken from Qiagen Midi/Maxi Plasmid/Cosmid Purification Protocol (Qiagen, 1993) and modified to suit the specified experimental objectives. First, freshly grown yeast cells were adjusted to an optical density of 1.3 to 1.5, which corresponded to a cell concentration of 1 x 10^8 cells/ml (*BIO-RAD* (b)). There were 3 samples having this concentration. These samples were combined in a 50 ml polypropylene Sorvall Centrifuge tube. The yeast cells were harvested at 4°C for 15

minutes in a Sorvall centrifuge at a speed of 6000xg in a Sorvall 28/50 rotor. The supernatant was then removed and the cell pellet was suspended in 12 ml of buffer P1, where it was 4 ml/sample. 12 ml of buffer P2 was then added and the solution was mixed gently. It was incubated at room temperature for 5 minutes. Then 12 ml of chilled buffer P3 was added and incubated on ice for 20 minutes. When the solution was mixed, it was gently inverted 5 to 6 times. Lastly, the solution was centrifuged at a speed of 30,000xg (15,000 rpm). The supernatant was poured off and the absorbance was measured. The blank contained 12 ml of P1, P2, and P3.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

This chapter focuses on the presentation of the results obtained from the experimental studies. Some results are as expected, others are not. However, all of the results aided in the development of efficient electroporation-based protein extraction procedures.

The results of the experimental study will be presented in the following order: (1) the results and findings of fermentation procedures; (2) the search for improved ways to prepare yeast cell suspensions; (3) the electroporation procedures and the manner in which they evolved; these procedures were done in parallel with the total cell lysis procedures; and (4) the determination of the protein concentration via an array of protein determination methods such as Lowry, Bradford, and UV Absorbance assays.

4.2 Fermentation

In this section, the findings made as a result of growing the cells for fermentation will be discussed. The yeast strains used were obtained from the United States Department of Agriculture (Peoria, IL); they were NRRL Y-12636 and NRRL Y-2034, wine and beer yeasts respectively. The Y-2034 strain was used during the period 9/26/96-12/4/96 and the Y-12636 strain was used from 1/24/97 to 6/98.

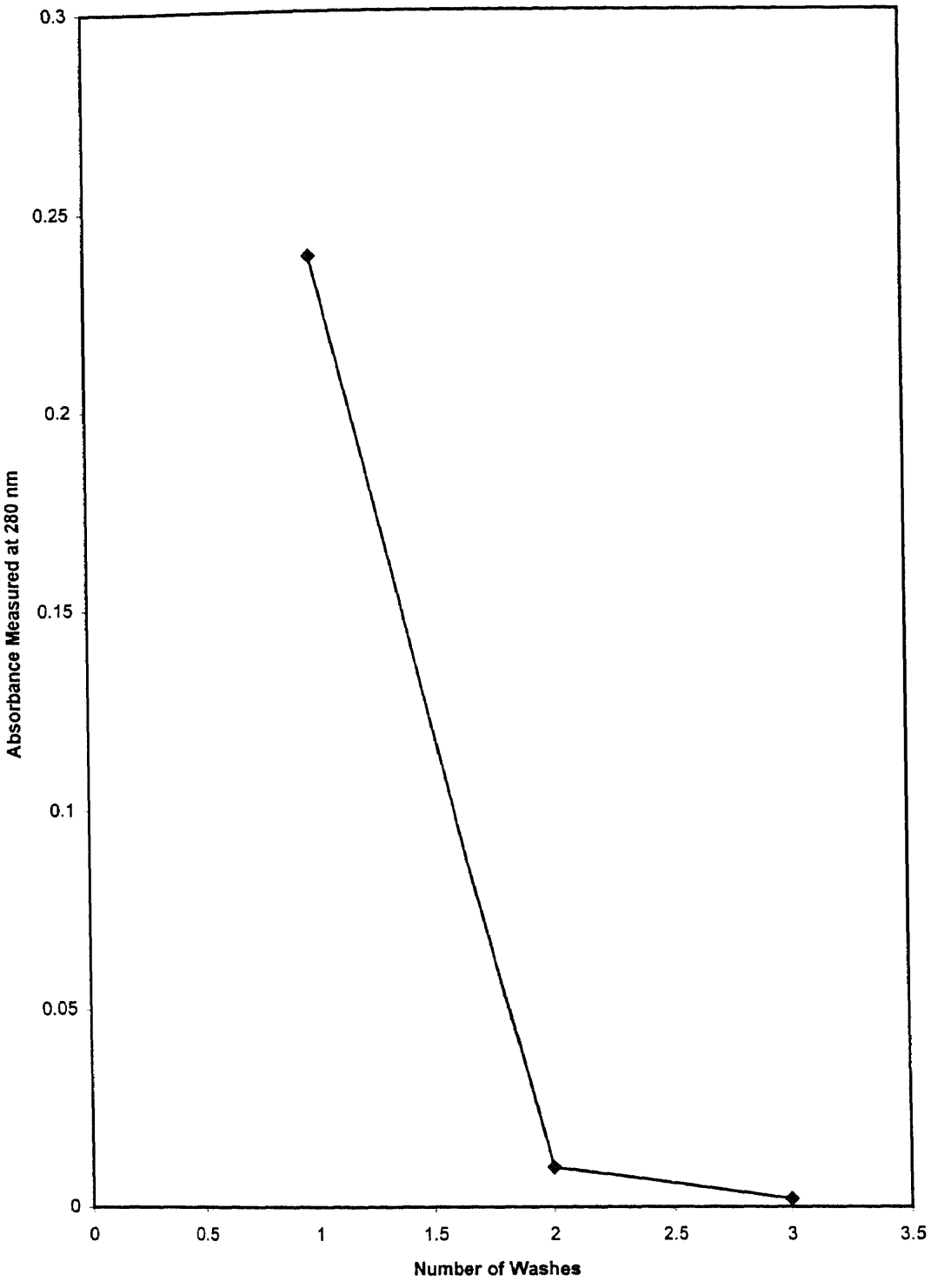

Figure 4.1 Optical Density of Supernatant After 2 Washes; Yeast Strain: Y-12636; Measured at 280 nm using Quartz Cuvettes; Volume (11.5 ml)

The entire protocol indicated in Chapter 3 was followed when culturing *Saccharomyces cerevisiae*. As time progressed, shake time and size of sample became major concerns. The object was to minimize the time needed to grow the cells and to decrease the volume of the batch of cells prepared, since the samples under study were extremely small as compared to the batch of cells harvested from fermentation. To achieve this, the fermentation step was eliminated and the cells were grown in the shake flask stage.

4.3 Cell Suspension Preparation

Cell suspension preparation became an important concern in the electroporation studies. It was expected that the before-pulse (0 pulse) product concentration from the cells would be zero. Since the cells have not yet been pulsed, no intracellular product should have been released. Another concern was the change in cell suspension absorbance as a result of washing the cells. Table 4.1 and Figure 4.1 illustrate the results of washing at room temperature and centrifuging for 5 minutes at 3000xg between each wash. The data are for the initial cell washing procedure with a single batch of cells that was not divided.

Table 4.1 Optical Density of Supernatant After 2 Washes; Yeast Strain: Y-12636 Measured at 280 nm using Quartz Cuvettes; Volume (11.5 ml)

After Wash Sample	*Absorbance*
First	**0.239**
Second	**0.010**
Before Pulse	**0.002**

The results indicate that there was a decrease in the absorbance as the number of washes increased and the optical density was nearly zero for the before-pulse sample. The reader will notice that this result does not resemble the data collected in electroporation studies discussed in section 4.4. None of the data tables indicate an absorbance of 0 for the before-pulse samples. This discrepancy then led to the modification of the cell washing protocol, where the cell suspensions were divided as done when preparing the samples to be electroporated. The single batch was compared to the divided batch. The initial absorbance values for samples A, B, and C are given below in Table 4.2.

Table 4.2 Initial Absorbance Values for Samples A, B, and C; Yeast Strain: Y-12636 Suspended in Sterile Deionized Water Measured at 280 nm

Samples	*Absorbance*
A	**2.078**
B	**2.130**
C	**1.987**
Average of B and C	**2.028**

Table 4.3 Optical Density of Supernatant After 2 Washes for Samples A, B, and C; Yeast Strain: Y-12636 Measured at 280 nm

After Wash	*A*	*B*	*C*
First	**0.183**	**0.228**	**0.165**
Second	**0.020**	**0.023**	**0.033**

The results in Table 4.3 show how the absorbance decreases after the first two washes.

Table 4.4 illustrates the results for the before-pulse samples.

Table 4.4 Optical Density of Supernatant for Before-Pulse Samples for Batch A and Combined Batches B and C; Yeast Strain: Y-12636 Measured at 280 nm

Samples	*Before Pulse Absorbance*
A	**0.04**
B and C (1)	**0.038**
B and C (2)	**0.032**
B and C (3)	**0.031**
B and C (4)	**0.031**
B and C (5)	**0.034**

Samples B and C are the absorbance values for the combined samples. Sample A is for the sample that was a single batch. The samples for B and C increased from the second wash samples. The sample A also increases. The fact that the concentrations increased

was a concern. However, the B and C samples were very close. This led to the conclusion that sample size was a factor in conducting the experiments. This result prompted the omission of the 400 µl sample preparation procedure. This small sample size caused absorbance values to fluctuate. All studies that followed this study was done with at least 3 ml volume conditions. This size sample was initially disregarded due to the large amount of time to pulse the samples. The use of small samples was an effort to save time, to be more efficient.

Another problem associated with the 400 µl sample occurred while centrifuging the 400 µl sample. A speed of 3000xg was not strong enough to sediment the cells. Thus, the speed was increased to 6000xg. So, there was a concern to find the effects of centrifuging at 3000xg and 6000xg for the before-pulse samples at a constant five (5) minutes. Results for 6000xg were always higher than those for 3000xg. See Tables 4.5 and 4.6. It appears that centrifuging at 6000xg causes some additional intracellular products to be released. Also, 3000xg may cause it to be released. However, the amount released appears to be less when comparing the absorbance of the samples after each wash.

Table 4.5 Intracellular Product Absorbance for Before-Pulse Samples; Measured at 280 nm; Centrifugation Speed and Time = 3000xg and 5 minutes

Batch	*Before Pulse*
A	**0.027**
D	**0.023**
E	**0.023**
B and C (1)	**0.046**
B and C (2)	**0.044**
B and C (3)	**0.069**
B and C (4)	**0.054**
B and C (5)	**0.054**

Table 4.6 Intracellular Product Absorbance for Before-Pulse Samples; Measured at 280 nm; Centrifugation Speed and Time = 6000xg and 5 minutes

Batch	*Before Pulse*
A1	**0.028**
D1	**0.029**
E1	**0.032**
B1 and C1 (1)	**0.113**
B1 and C1 (2)	**0.083**
B1 and C1 (3)	**0.102**
B1 and C1 (4)	**0.083**
B1 and C1 (5)	**0.052**

It was concluded from this study that centrifuging samples might have caused stress to cells by forcing the release of intracellular materials. Also, the following modifications were made. Future samples were to be centrifuged at 3000xg for all washes and before-pulse samples; pulsed samples were to be at least 3 ml in volume to decrease the oscillation that occurs at smaller volumes.

4.4 Electroporation

This section focuses on the electroporation of yeast cells to retrieve the intracellular products. Cells were electroporated at room temperature using an E.coli Pulser (*BIO-RAD*). Before pulsing the cells, a repetitive pulse test was done to examine the effects of multiple pulses on the electroporation cuvettes. Next, the effect of pulsing the

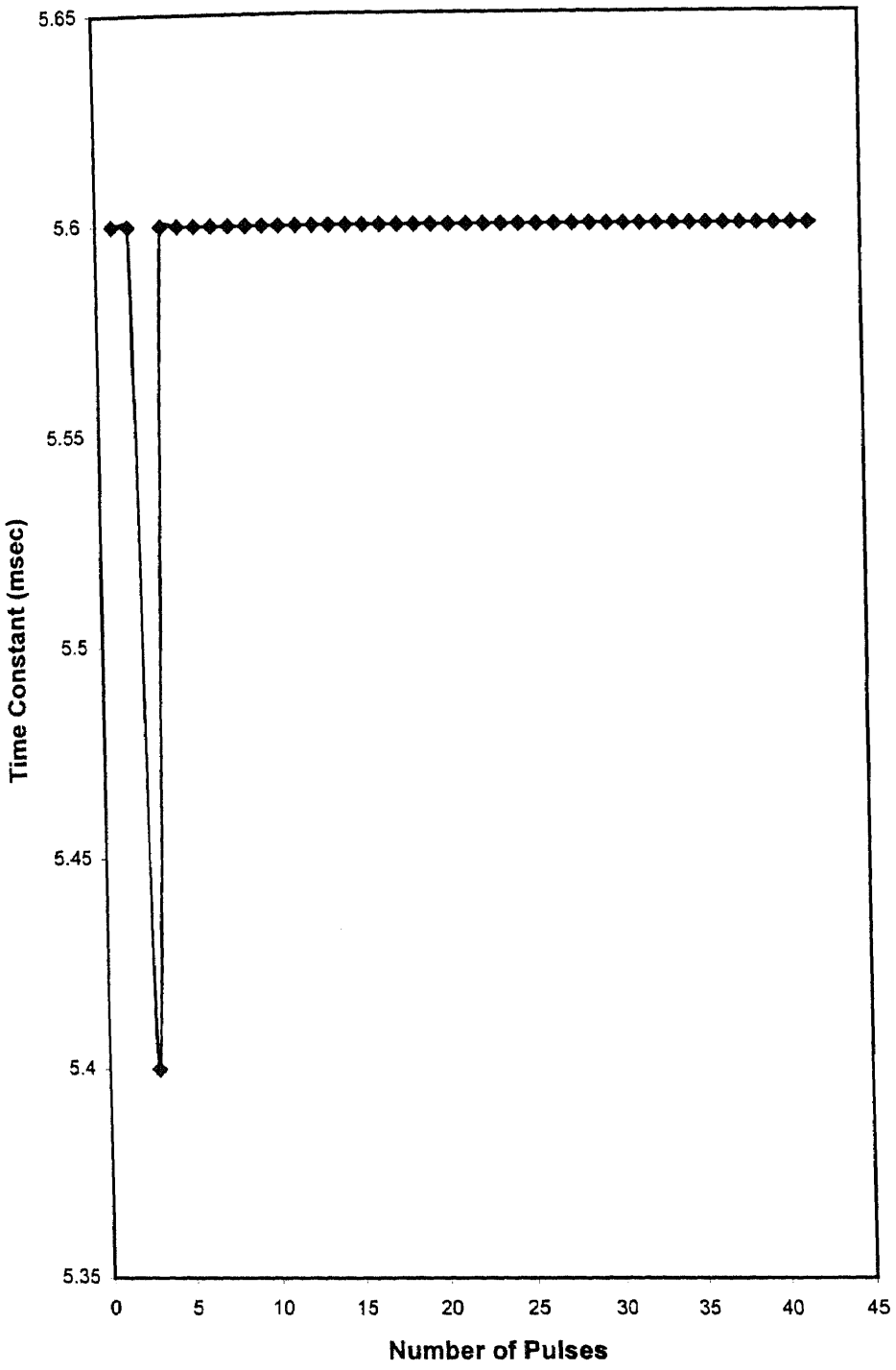

Figure 4.2 Time Constant of Electroporation Cuvettes Filled with Sterile Deionized Water; Cuvettes Pulsed 43 Times; Time constant (msec) and Set Voltage (1.5 kV); Cuvette gap (0.2 cm)

suspension one time was done. This then led to a search to find what would happen in the presence of multiple pulses and then to a multiple sample study where several samples were pulsed the same amount of times and the average of the sample absorbances were taken. Finally, a repulsing study was implemented where samples were pulsed then the cell pellet was resuspended and the entire electroporation procedure repeated a second time.

4.5 Repetitive Pulse Test

The repetitive pulse test gave results that were constant as a result of pulsing the cuvette 43 times. Figure 4.2 illustrates this. There appears to be a sharp decrease in the time constant data points. In actuality, this decrease was not as great as it seems; the difference was 0.2 msec. The set voltage was 1.5 kV and the time constant was approximately 5.6 msec. The cuvette was filled with 400 μl of sterile deionized water.

4.6 Single Pulse Study and Analysis

Initially there were problems with adjusting the baseline of the spectrophotometer (HITACHI) when analyzing the single pulse samples. The baseline should have been zero; in Table 4.7 the shift of the baseline is illustrated. The cell solution was analyzed using the Coomassie Blue Assay. The wavelength used was 590 nm.

Table 4.7 Nonzero Baseline when Measuring the Released Intracellular Material; Yeast Strain: Y-2034 Cell Suspension Optical Density (1.410)

Sample		*Baseline*
UV Absorbance	**Before Pulse**	**0.010**
	After Pulse	**0.010**
Coomassie Blue	**Before Pulse**	**-0.601**
	After Pulse	**0.612**

The pulsed sample was also analyzed using the UV Absorbance Assay at 280 nm using quartz cuvettes. These methods were done in parallel to detect if there were released intracellular material in the supernatant at 280 nm then at 590 nm to check if the released materials were proteins. One tube with the yeast cell suspension was pulsed once in 400 microliter portions. The results are given below in Table 4.8.

Table 4.8 Intracellular Product Absorbance Before and After One Pulse Analyzed using Coomassie Blue and UV Absorbance Assay; Yeast Strain: Y-2034 Coomassie Blue Measured at 590 nm and UV Absorbance Measured at 280 nm Optical Density (1.417)

Assay	*Before Pulse*	*After Pulse*
UV Absorbance	**0.158**	**0.003**
Coomassie Blue	**3.612**	**0.265**

The results were inconsistent and show no sign of intracellular proteins being released. The cell supernatant was then analyzed after pulsing the sample two times, although the

results were inconsistent. The intracellular product concentration is provided in Table 4.9 These are the data for the first multiple pulse study.

Table 4.9 Intracellular Product Absorbance Before and After 2 Pulses Analyzed using Coomassie Blue and UV Absorbance Assay; Yeast Strain: Y-2034; Coomassie Blue Measured at 590 nm and UV Absorbance Measured at 280 nm; Optical Density (1.480)

Assay	*Before Pulse*	*After 2 Pulses*
UV Absorbance	**0.425**	**0.143**
Coomassie Blue	**0.043**	**0.047**

Again results were inconsistent. Next, samples were pulsed over a short range of 1 to 10 pulses. Data are provided in Table 4.10.

Table 4.10 Intracellular Product Absorbance Before and After 1, 2, and 10 Pulses; Analyzed using Coomassie Blue; Yeast Strain: Y-2034; Coomassie Blue Measured at 590 nm; Optical Density (1.115) Before Pulse (BP) After Pulse (AP)

Assay	*1*		*2*		*10*	
	BP	**AP**	**BP**	**AP**	**BP**	**AP**
Coomassie Blue	**0.054**	**0.045**	**0.043**	**0.034**	**0.123**	**0.110**

This was done to study the trend by varying the number of pulses. It was analyzed by Coomassie Blue only. Results were again inconsistent; also the baseline of the spectrophotometer was not zero.

These inconsistent results led to the use of the Bradford Assay as a method of determining the protein concentration. The calibration curve for Bradford was generated

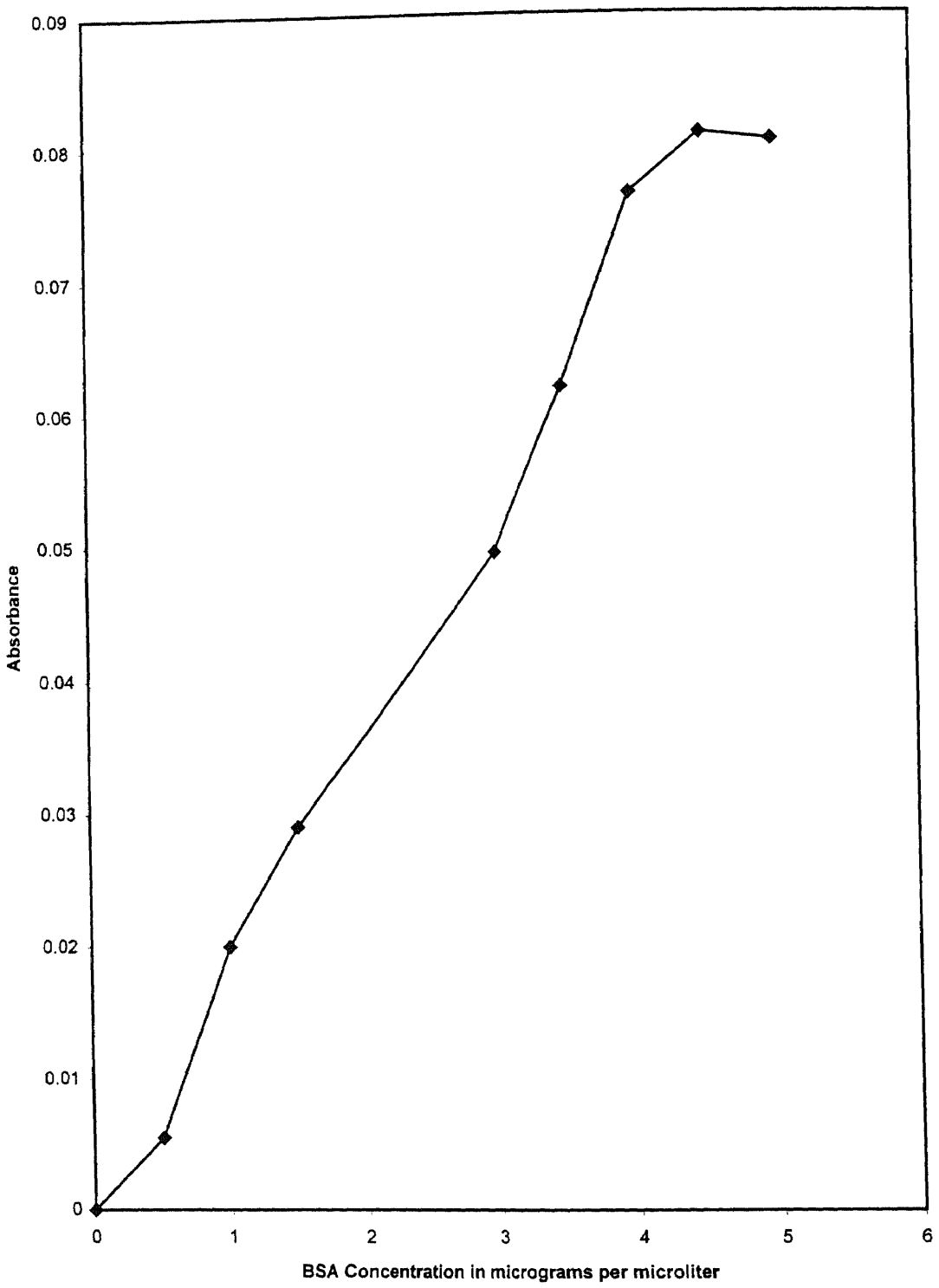

Figure 4.3 Calibration Curve for Bradford Assay with Bovine Serum Albumin

and is shown in Figure 4.3. The multiple pulsed samples were analyzed by following the procedure for the Bradford assay. There were two methods: one for 10 µl of protein sample and the other for 50 µl of protein sample. Initially, the 10 µl protocol was used. The sample was pulsed 1 to 20 times. Table 4.11 illustrates the results of pulsing the samples.

Table 4.11 Intracellular Product Absorbance Before and After 1, 10, and 20 Pulses; Analyzed using Bradford Assay; Yeast Strain: Y-12636 Bradford Assay Measured at 595 nm; Optical Density (1.264) Before Pulse (BP) After Pulse (AP); 10 µl Procedure

Pulses	*1*		*10*		*20*	
	BP	**AP**	**BP**	**AP**	**BP**	**AP**
Absorbance	**0.004**	**0.003**	**-0.010**	**0.005**	**-0.014**	**0.004**

The absorbances were again inconsistent. As a result, the 50 µl procedure assay was used instead. Possibly the size of the sample was the problem. Table 4.12 illustrates results of the 50 µl study.

Table 4.12 Intracellular Product Absorbance Before and After 1, 10, 20, and 40 Pulses; Analyzed using Bradford Assay 50 µl Procedure Measured at 595 nm; Yeast Strain: Y-12636; Before Pulse (BP) After Pulse (AP) Optical Density (1.242)

Pulses	*1*		*10*		*20*		*40*	
	BP	**AP**	**BP**	**AP**	**BP**	**AP**	**BP**	**AP**
Absorbance	**-0.012**	**-0.014**	**-0.018**	**-0.018**	**-0.020**	**-0.020**	**-0.011**	**-0.011**

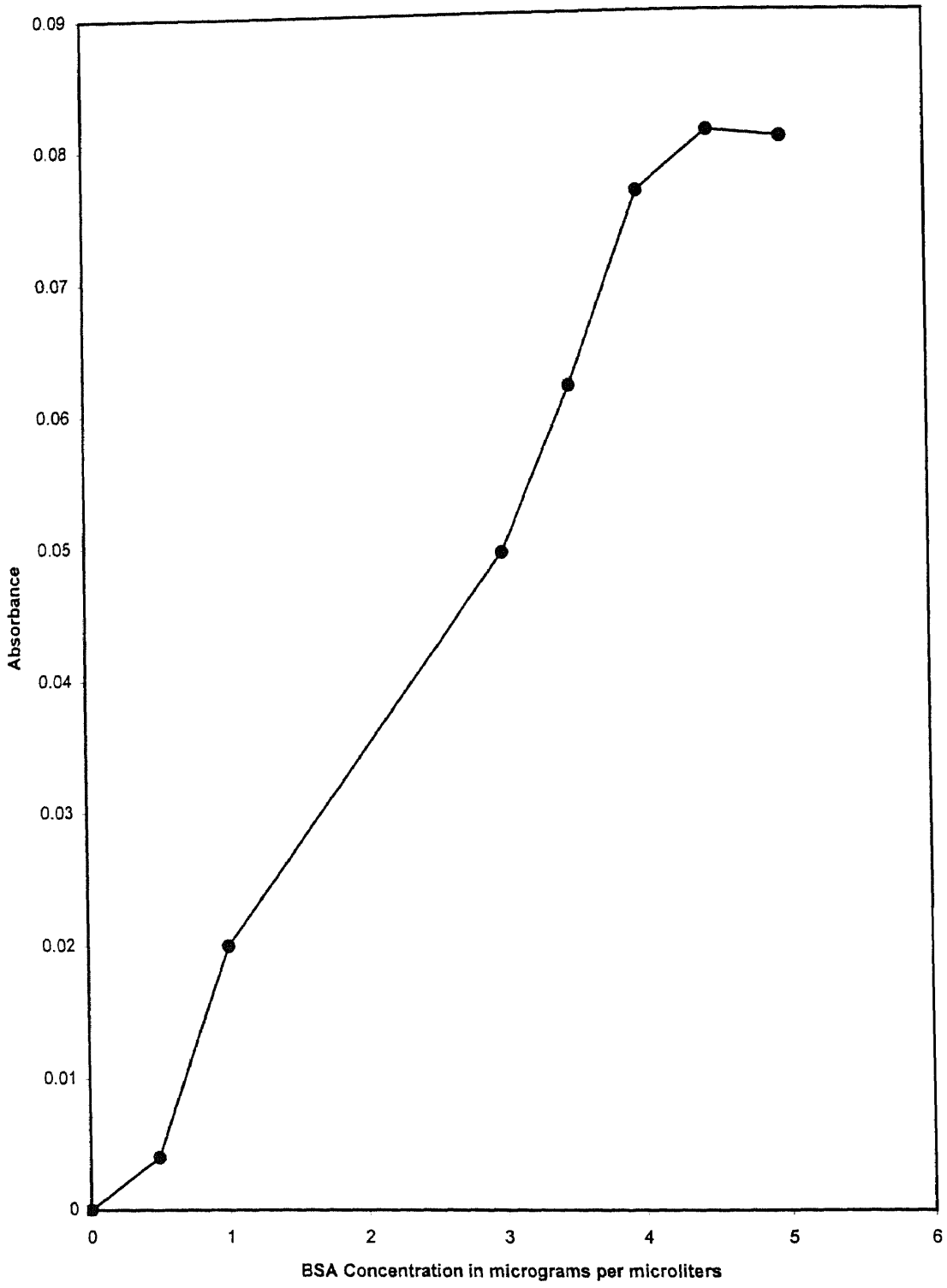

Figure 4.4 Calibration Curve of Bradford Assay with a BSA Concentration Ranging from 0 to 5 Micrograms per Microliters

This experimental run showed no consistency in results. A solution to the problem was to generate a calibration curve for very small protein concentrations from 0 to 5 μg/ml. This was to check if the Bradford assay could detect proteins at such small concentrations. Figure 4.4 shows the calibration curve for 0 to 5 μg/ml. There was no oscillation in the absorbance values, therefore this proves the technique to be valid for small concentrations of pure proteins. Next, electroporated samples were washed. This was done to see how the Bradford Assay would be affected. This time the UV absorbance assay was done in parallel. Tables 4.13 and 4.14 show the results.

Table 4.13 Intracellular Product Absorbance Before and After 1, 10, and 20 Pulses to Washed Cells; Analyzed using Bradford Assay Measured at 595 nm; Yeast Strain: Y-12636; Before Pulse (BP) and After Pulse (AP) Optical Density (1.47)

Pulses	***1***		***10***		***20***	
	BP	**AP**	**BP**	**AP**	**BP**	**AP**
Absorbance	**-0.011**	**-0.009**	**-0.011**	**-0.010**	**0.011**	**-0.008**

Table 4.14 Intracellular Product Absorbance Before and After 1 and 10 Pulses of Washed Cells; Analyzed using UV Absorbance Assay Measured at 280 nm; Optical Density (1.3) Measured at 600 nm

Pulses	***0***	***1***	***10***
Absorbance	**0.041**	**0.036**	**0.055**

The UV Absobance assay showed that something was being released. The Bradford Assay continued to yield oscillating results. However, a new problem arose. The zero (0) absorbance was higher than the ten (10) pulse absorbance. See Figure 4.5.

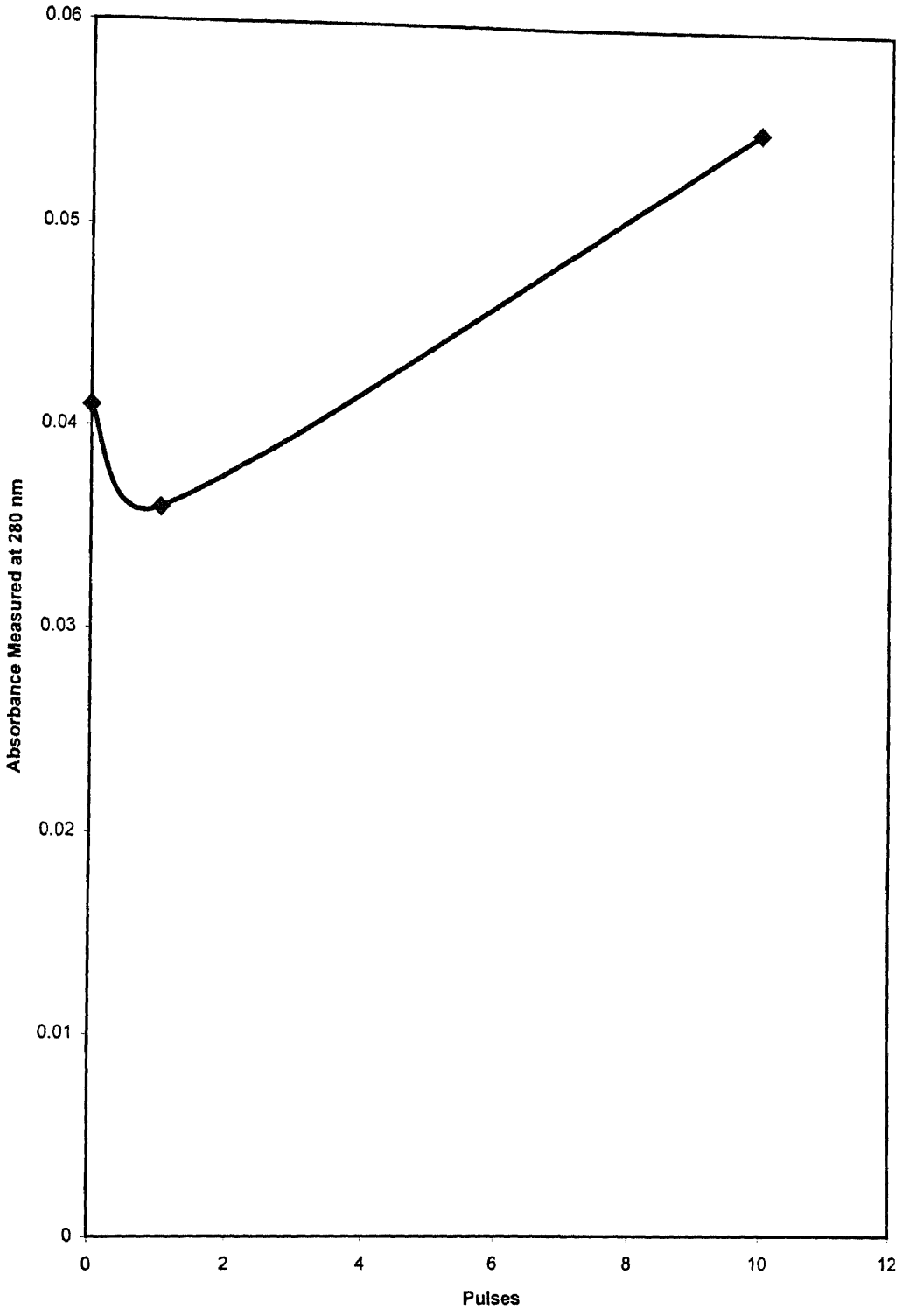

Figure 4.5 Intracellular Product Absorbance Before and After 1 and 10 Pulses of Washed Cells; Analyzed using UV Absorbance Assay Measured at 280 nm; Optical Density (1.3) Measured at 600 nm

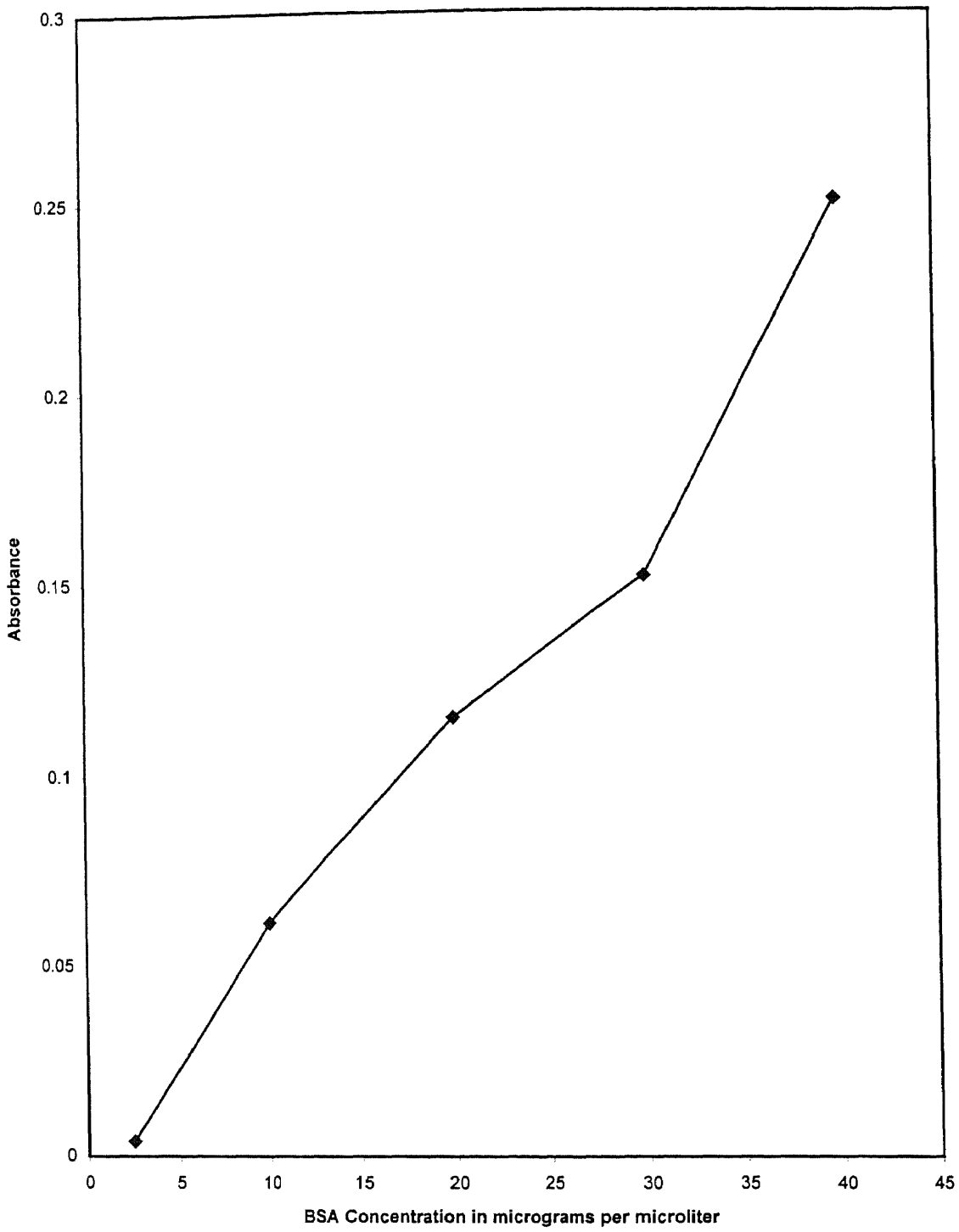

Figure 4.6 Calibration Curve for Lowry Method with BSA

Next, the Lowry Assay was then used as a protein determination method. Figure 4.6 shows the calibration curve. Table 4.15 shows the results of using the Lowry assay as a protein determination method. The data were inconsistent and oscillating. There is only a single absorbance value listed for the 0-pulse column. The procedure suggested that one 0-pulse sample was to be assayed. Usually the values are averaged, however they were all listed to illustrate the disparity.

Table 4.15 Intracellular Product Absorbance Before and After Pulsing Yeast Suspension 1, 10, 20, 40, and 60 Times; Analyzed using Lowry Assay at 750 nm; Yeast Strain: Y-12636

Pulses	*0*	*1*	*10*	*20*	*40*	*60*
Abs.1	**0.042**	**0.028**	**0.009**	**-0.012**	**-0.003**	**0.032**
Abs. 2		**0.001**	**0.001**	**0.032**	**0.008**	**0.013**

The UV Absorbance assay was then repeated with some modifications. The method of preparing the cell sample was altered. Originally, when preparing the sample the natural protein concentration was removed when washing the cells. Upon washing, the sample was then divided into the appropriate number of samples to be pulsed multiple times. The method was altered by first dividing the sample then washing each sample. So each sample will begin with the same concentration. Refer to the experimental section for preparing the cell solutions. Table 4.16 and Figure 4.7 show the results of this new method. The cell samples were pulsed 0 to 20 times.

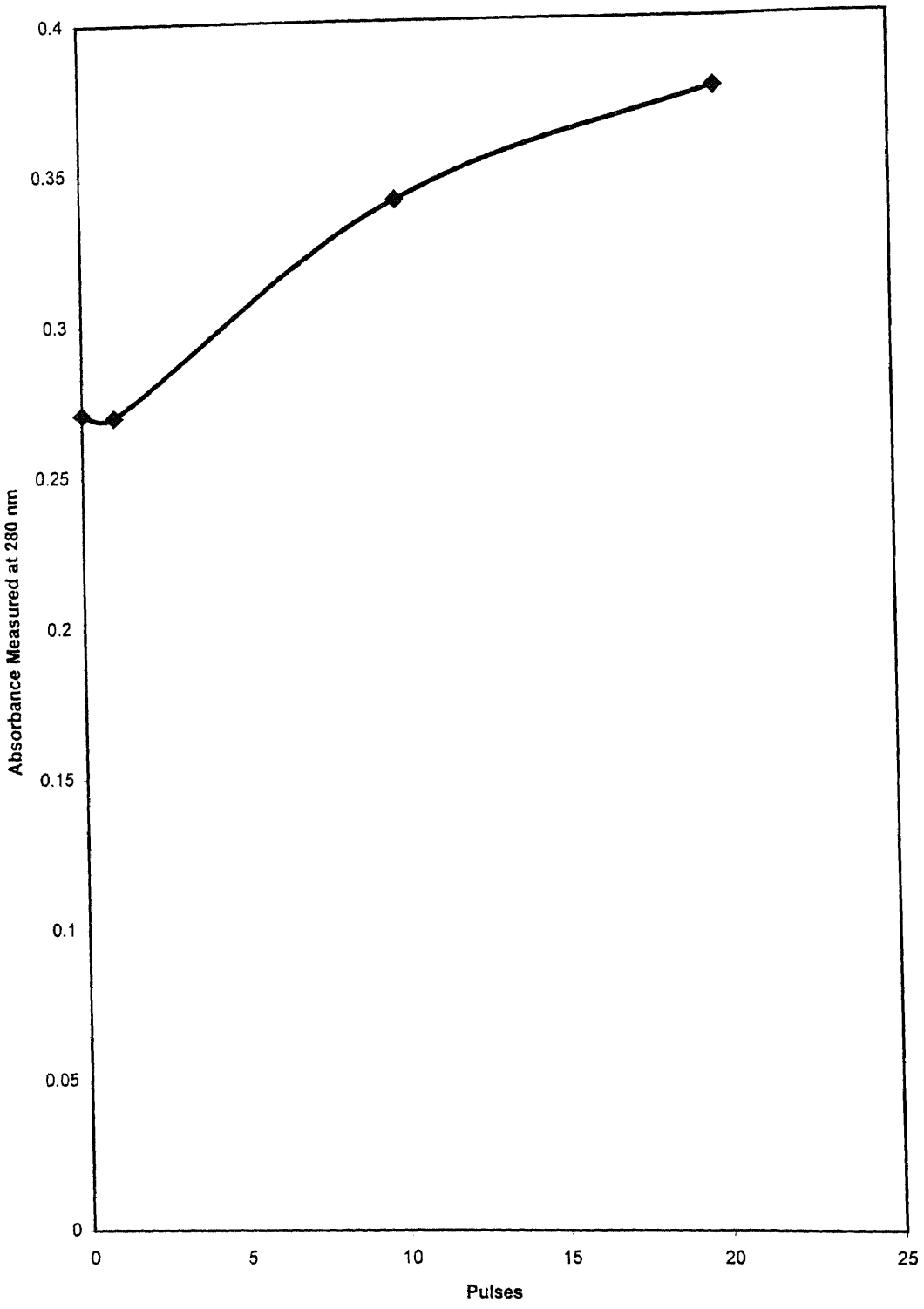

Figure 4.7 Intracellular Product Absorbance Before and After Pulsing the Yeast Suspension 1,10, and 20 Times; Analyzed using UV Absorbance Assay Measured at 280 nm; Yeast Strain: Y-12636 Optical Density (1.665 and 1.684)

Table 4.16 Intracellular Product Absorbance Before and After Pulsing Yeast Suspension 1,10, and 20 Times; Analyzed using UV Absorbance Assay Measured at 280 nm; Yeast Strain: Y-12636 Optical Density of Batches (1.665 and 1.684)

Pulses	*0*	*1*	*10*	*20*
Absorbance	**0.271**	**0.270**	**0.341**	**0.377**

There is a relationship between the number of pulses and released material. The absorbance increases from 0.271 to 0.377.

The next concern was to find out if the presence of the cells could be detected at 280 nm. This was done to see if the centrifugation step was to be deleted from the procedure. A tube with 3 ml of 1 mg/ml BSA and 3 ml water was solution 1 and another one with 3 ml of 1 mg/ml BSA and 3 ml cell suspension was solution 2. They were compared by measuring the absorbance at 280 nm.

Table 4.17 Comparison of Absorbance Values for Bovine Serum Albumin (BSA) and Water with BSA and Cell Suspension; Yeast Strain: Y-12636

Solution	*Absorbance*
3 ml BSA and Water	**0.249**
3ml BSA and Cell Suspension	**2.009**

The presence of the cells was detected at 280 nm, therefore the centrifugation step remained. Cell suspensions were then pulsed 0 to 60 times. In these studies the absorbance of the released material decreased as samples were pulsed from 0 to 60 times. See Table 4.18 and Figure 4.8.

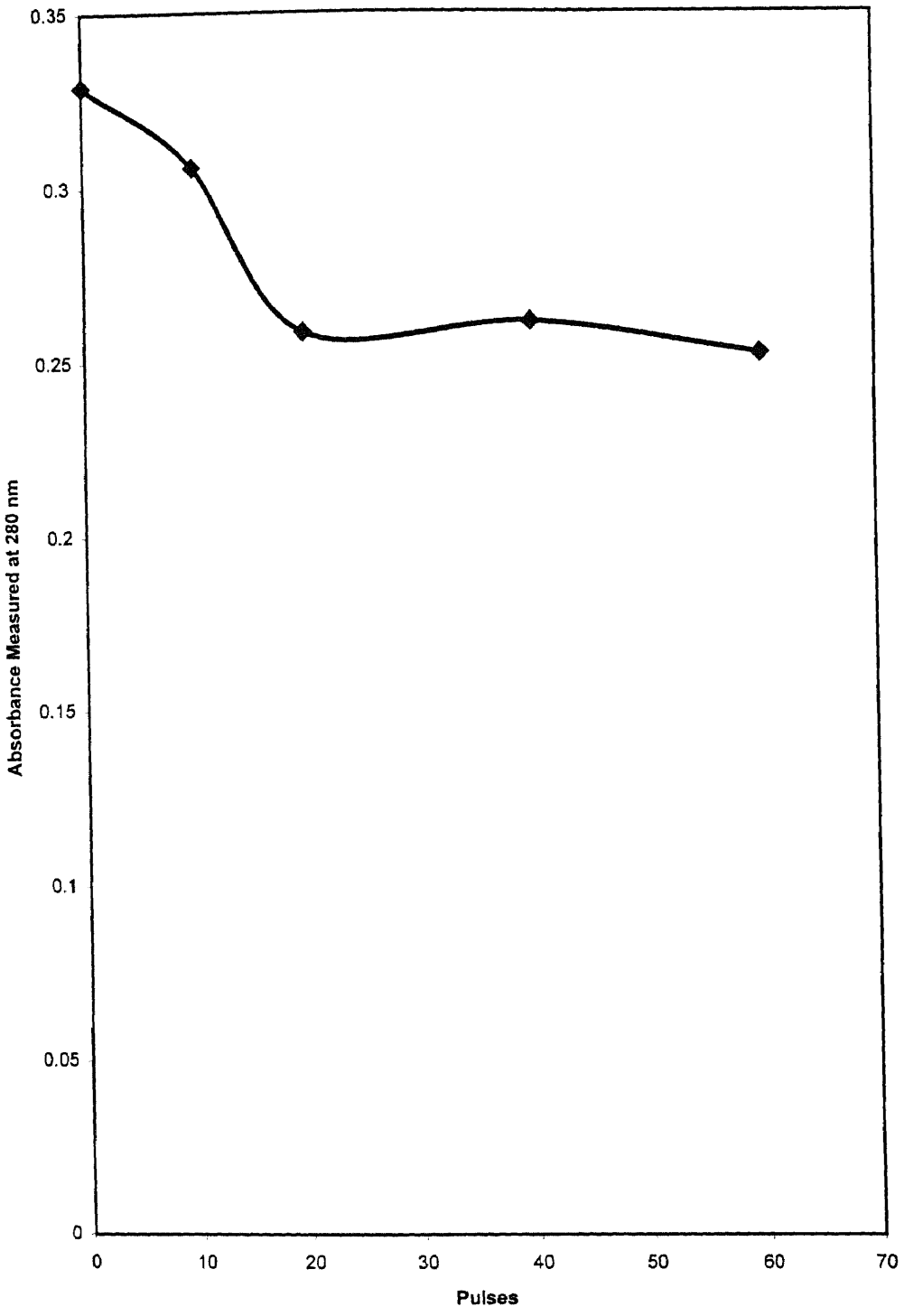

Figure 4.8 Intracellular Product Absorbance Before and After Pulsing the Yeast Suspension 0 to 60 Times; Analyzed using the UV Absorbance Assay Measured at 280 nm; Yeast Strain: Y-12636 Optical Density (1.507 and 1.560)

Table 4.18 Intracellular Product Absorbance Before and After Pulsing the Yeast Suspension 0 to 60 Times; Analyzed using the UV Absorbance Assay Measured at 280 nm; Yeast Strain: Y-12636 Optical Density (1.507 and 1.560)

Pulses	*0*	*10*	*20*	*40*	*60*
Absorbance	**0.329**	**0.306**	**0.259**	**0.262**	**0.253**

Another problem that existed when conducting such experiments was the time consumed. When pulsing a sample, there would be a large gap in time between pulsing and analyzing 40 and 60 pulse samples. In an effort to alleviate this problem the cell suspension preparation was modified again. The cell suspension was prepared using second method discussed in section 3.3.2.1. Instead of pulsing an entire sample of cells at least 3 ml in volume, a 400 μl sample was used instead. This 400 μl sample was diluted to 4 ml upon pulsing then analyzed using the UV absorbance assay.

Table 4.19 Intracellular Product Absorbance Before and After 0 to 60 Pulses; 400 μl of Pulsed Suspension Diluted to 4 ml; Analyzed using UV Absorbance Assay at 280 nm; Yeast Strain: Y-12636 Optical Density (1.791 and 1.790)

Pulses	*0*	*10*	*20*	*40*	*60*
Absorbance	**0.072**	**0.058**	**0.043**	**0.027**	**0.040**

In Figure 4.9 and Table 4.19, the results were not as expected. The absorbance values decreased as pulses increased. The volumes that were diluted were small. In instances where the number of pulses were greater than 20, there were very little cells settled after the sample was centrifuged. Therefore the centrifugation time was increased to 6 minutes.

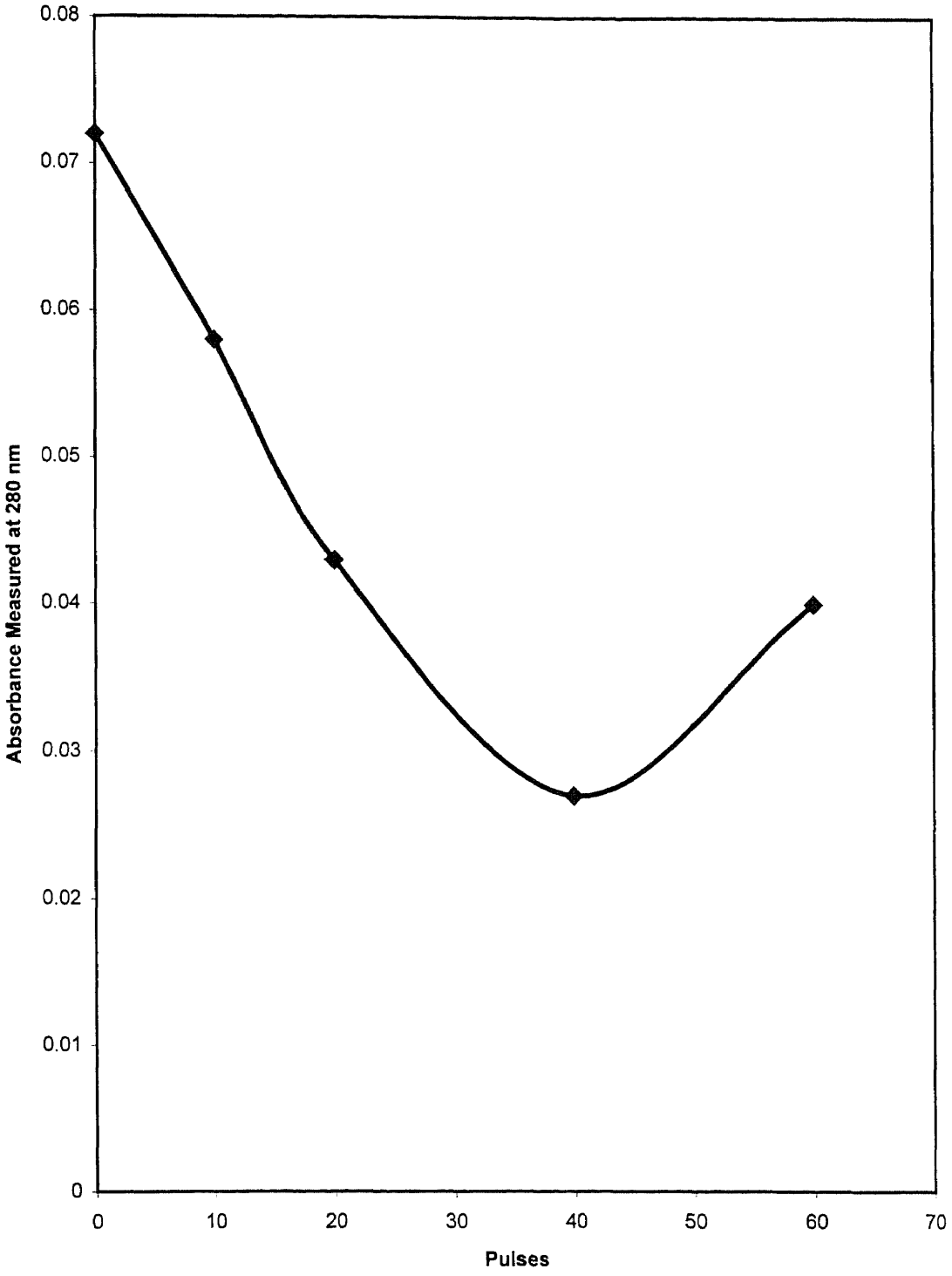

Figure 4.9 Intracellular Product Absorbance Before and After 0 to 60 Pulses; 400 Microliters of Pulsed Suspension Diluted to 4 ml; Analyzed using UV Absorbance Assay Measured at 280 nm; Yeast Strain: Y-12636 Optical Density (1.791 and 1.790)

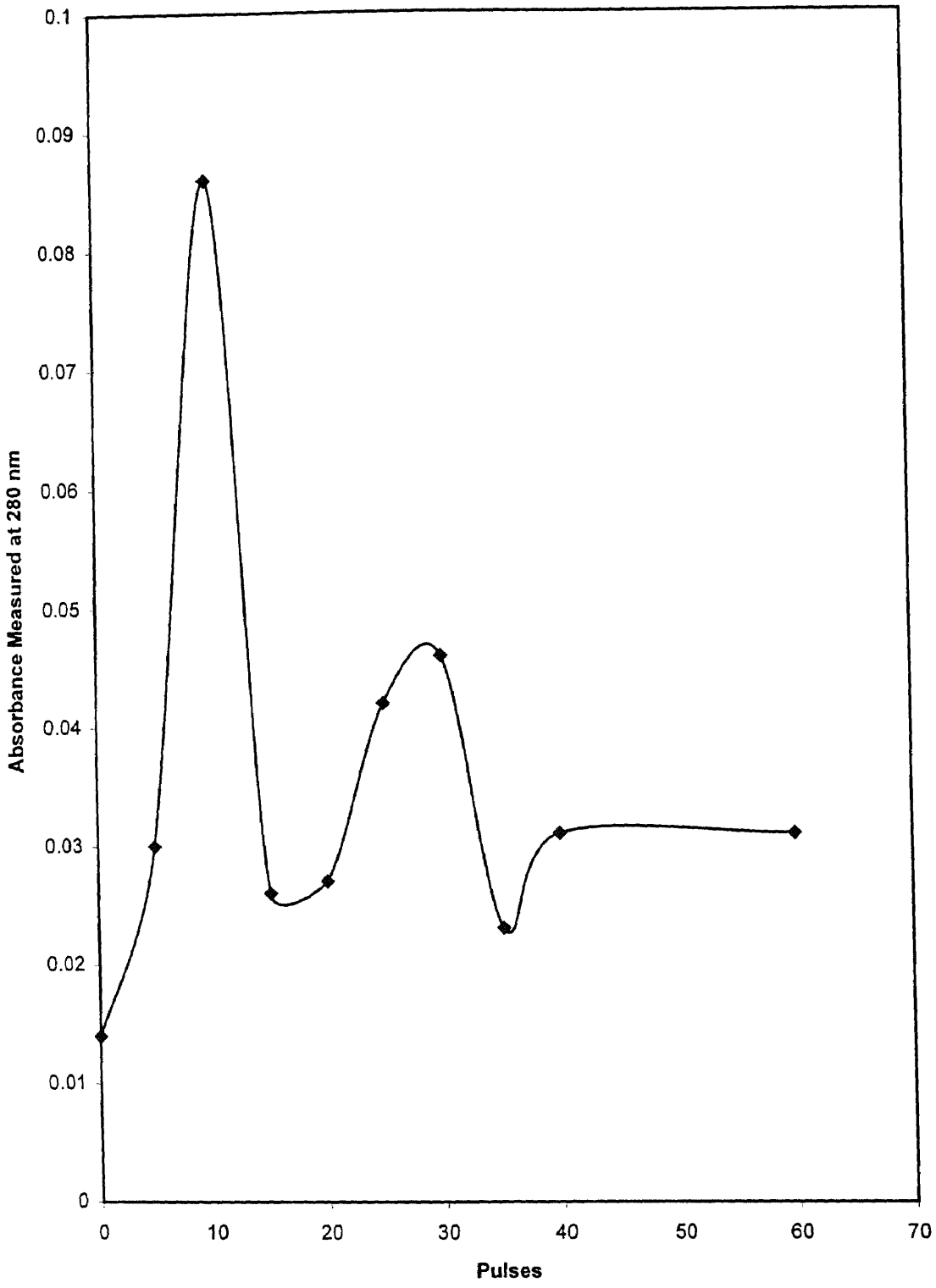

Figure 4.10 Intracellular Product Absorbance Before and After 0 to 60 Pulses; 400 Micoliters of Pulsed Suspension Diluted to 4 ml; Analyzed using UV Absorbance Assay at 280 nm; Yeast Strain: Y-12636 Optical Density (0.930)

The oscillation of the results raised questions as to what was happening at intermediate pulses. A study was then done at 0, 5, 10, 15, 20, 25, 30, 35, 40 and 60 pulses. These samples were prepared using 400 µl of cell suspension diluted to 4 ml. These results were oscillating even more. At 15 pulses the centrifugation speed was adjusted to 5000xg to sediment and separate the cells from the supernatant. Table 4.20 and Figure 4.10 illustrates the results.

Table 4.20 Intracellular Product Absorbance Before and After 0 to 60 Pulses; 400 µl of Pulsed Suspension Diluted to 4 ml; Analyzed using UV Absorbance Assay at 280 nm; Yeast Strain: Y-12636 Optical Density (0.930)

Pulse	**0**	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**60**
Abs.	**0.014**	**0.030**	**0.086**	**0.026**	**0.027**	**0.042**	**0.046**	**0.023**	**0.031**	**0.031**

The next phase of studies focused on multiple samples, where each pulse set had anywhere from 2 to 5 samples. Because of the oscillations of independent samples, it was hypothesized that the statistical average of the absorbances would increase. Table 4.21 illustrates the absorbance values of a batch of cells divided into 5 samples.

Table 4.21 Optical Density Variability Test Before Centrifugation (BC) and After Centrifugation (AC); BC Measured at 600 nm; After Centrifugation Measured at 280 nm

Before Centrifugation	**1.814**	**1.806**	**1.783**	**1.807**	**1.789**
After Centrifugation	**0.028**	**0.026**	**0.016**	**0.030**	**0.022**

The absorbances of the 5 samples were changing. This means the samples were beginning from different baselines.

Before reporting the results of the various multiple sample studies the problems that have occurred up to this point are summarized. These problems include : (1) the inability to get reproducible results; (2) Bradford and Lowry protein methods were not reliable assays for this electroporation study; (3) UV Absorbance assay was the only one to give results that have shown something was being released; (4) the centrifugation of the small volume samples needed higher speeds and times; (5) the possibility that cell washing was an important factor in the experiment.

The next set of experiments will look at multiple samples. These experiments will focus on parameters such as shake time, cell freshness, multiple pulsing, and cell washing.

Five samples (5) will be used for each of the pulse sets. There will be 5 before-pulse samples and 5 for each pulse set thereafter (10, 20, 40, etc.). Although the value of the absorbance may oscillate the average of these values should increase as the number of pulses increase. Table 4.22 shows the results of incorporation the statistical average. Samples in this study were prepared using 400 μl of sample diluted with sterile deionized water.

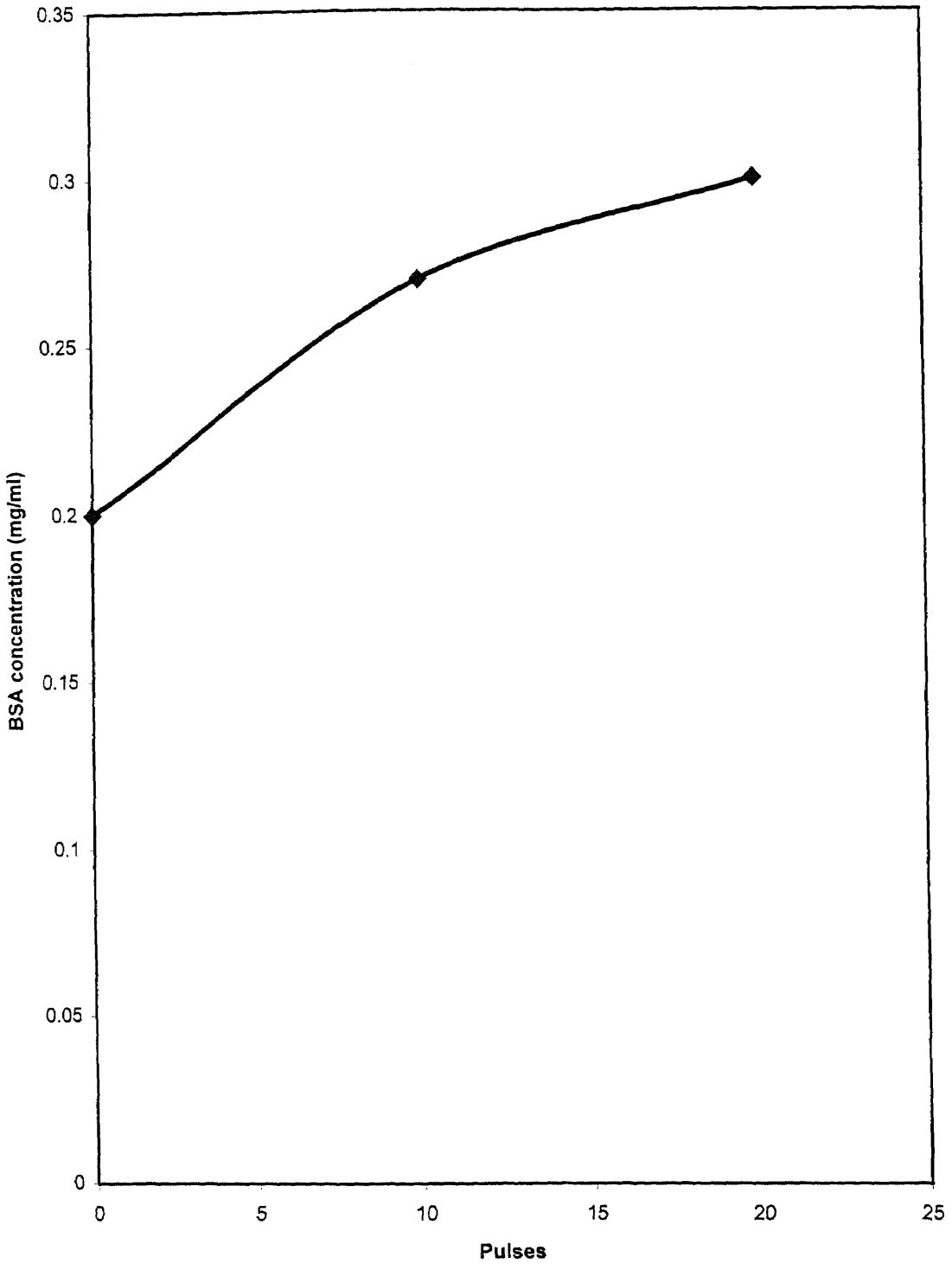

Figure 4.11 Equivalent BSA Concentration; Yeast Strain: Y-12636; Analyzed using UV Absorbance Assay at 280 nm

Table 4.22 Intracellular Product Absorbance Before and After 10 and 20 Pulses; Analyzed using UV Absorbance Assay Measured at 280 nm; Yeast Strain: Y-12636; 400 µl Samples Diluted to 4 ml

Pulses	*0*	*10*	*20*
Abs. 1	**0.007**	**0.020**	**0.024**
Abs. 2	**0.016**	**0.018**	**0.018**
Abs. 3	**0.008**	**0.014**	**0.020**
Abs. 4	**0.056**	**0.014**	**0.016**
Abs. 5	**0.007**	**0.013**	**0.016**

The absorbance values for 10 and 20 pulses indicate the desired direction. At 0 pulses, one of the values were extremely high. Table 4.23 and Figure 4.11 indicate the equivalent BSA concentration. The average absorbance was calculated based on absorbance values of 1, 2, 3 and 5 due to the high value of Abs. 4 for 0 pulses. The samples used to produce the calibration curve were based on 200 µl, whereas the cell solution was 400 µl. Therefore, the average absorbance values were divided by 2. See Figure 4.12.

Table 4.23 Average Absorbance and Corresponding BSA Concentration; Yeast Strain: Y-12636; Analyzed using UV Absorbance Assay at 280 nm

Pulses	*Average Absorbance*	*BSA Concentration*
0	**0.005**	**0.2 mg/ml**
10	**0.008**	**0.27 mg/ml**
20	**0.010**	**0.3 mg/ml**

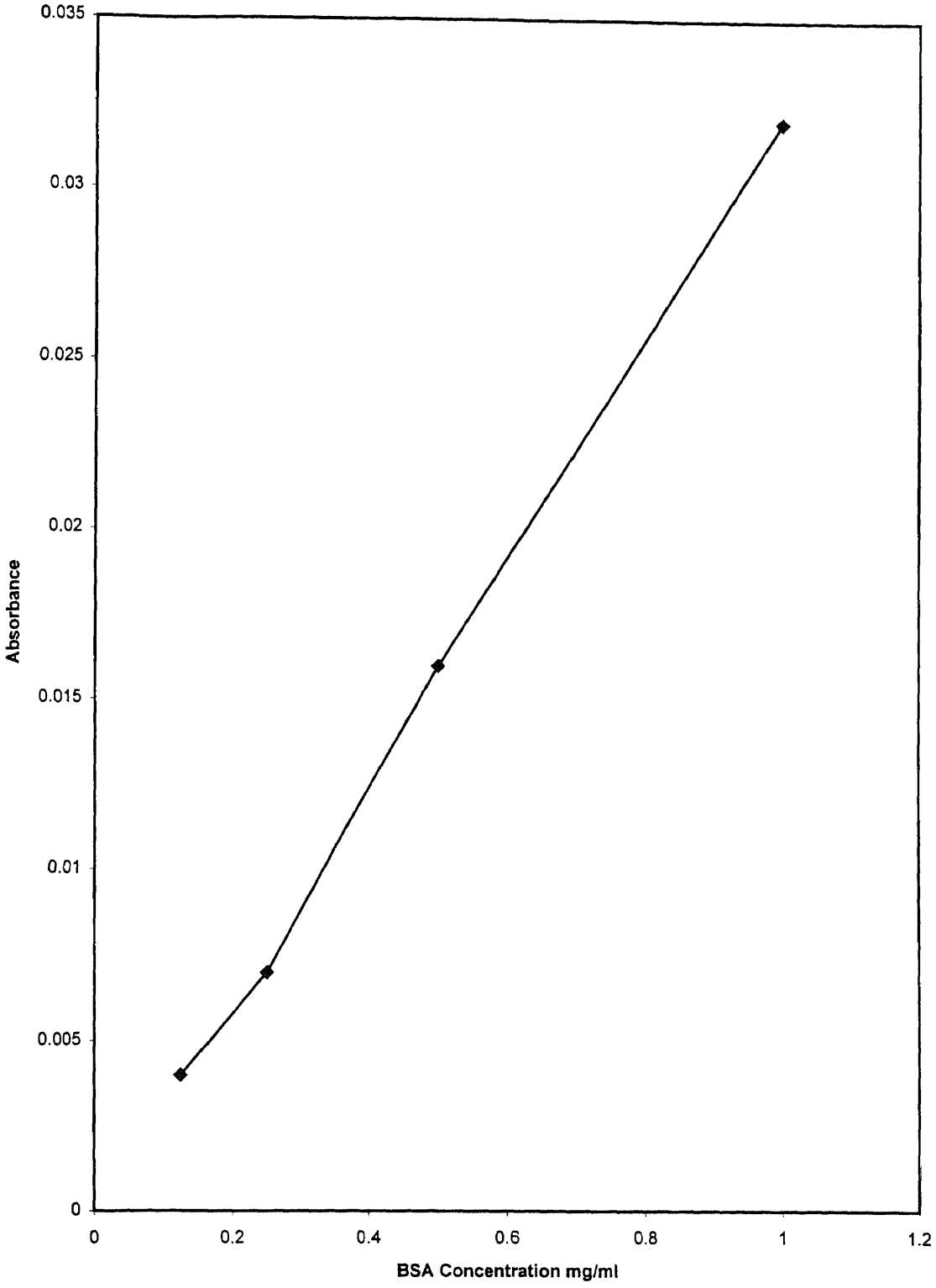

Figure 4.12 Calibration Data for UV Absorbance Assay where 200 Microliters of the Specified BSA Concentration is Diluted in 4 ml Sterile Water

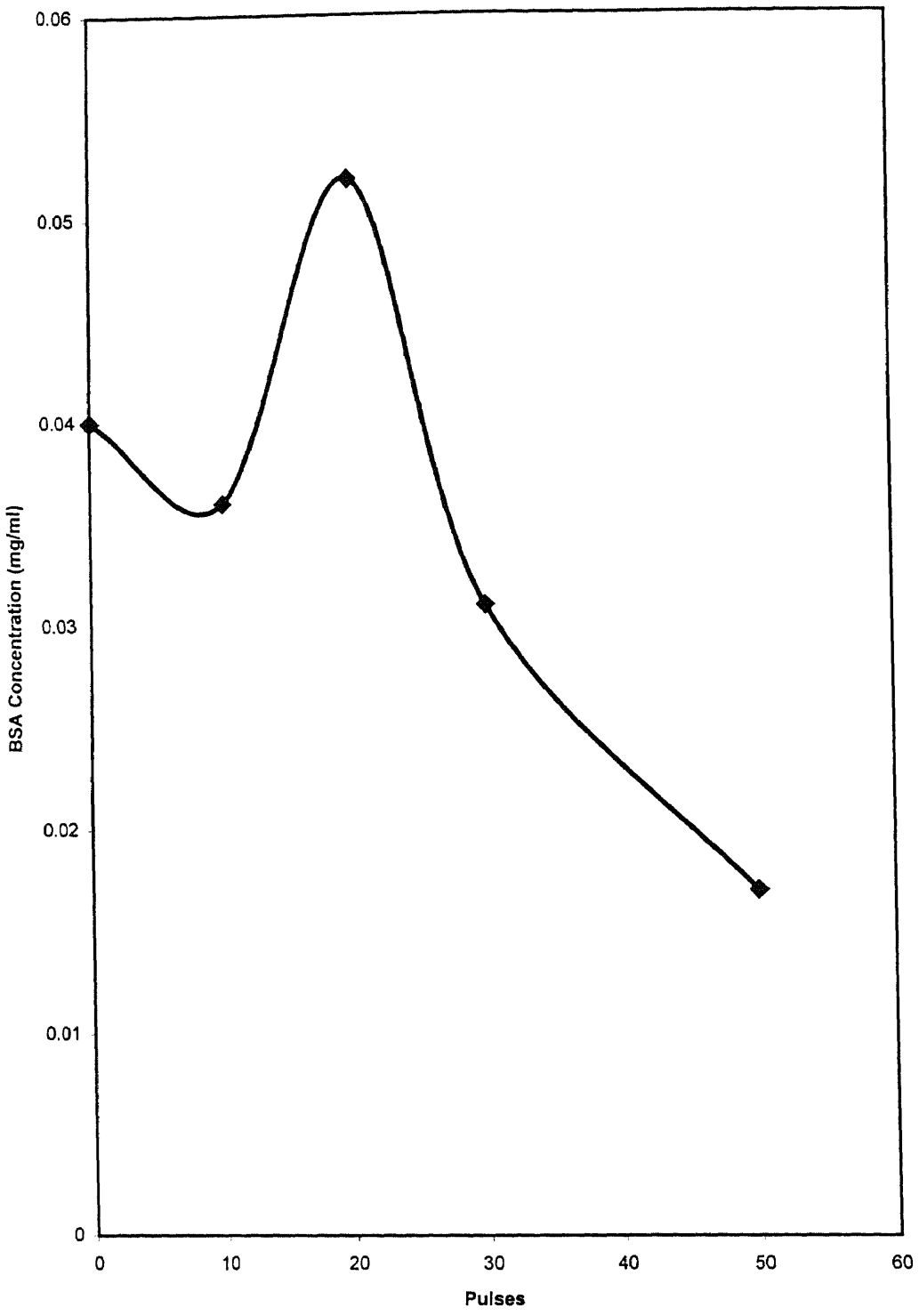

Figure 4.13 Equivalent BSA Concentration; Yeast Strain: Y-12636; Analyzed using UV Absorbance Assay

The major problem at this point was reproducing the results at pulses greater than twenty (20). Thus far this was the second time the values were as expected. The study was repeated including 30 and 50 pulse samples. The average absorbance data for pulsing 5 samples of 0 to 50 pulse sets are given in Table 4.24 and Figure 4.13.

Table 4.24 Average Absorbance Corresponding to BSA Concentration; Yeast Strain: Y-12636; Analyzed using UV Absorbance Assay at 280 nm

Pulses	*Average Absorbance*	*BSA Concentration (mg/ml)*
0	0.024	0.04
10	0.0216	0.036
20	0.0328	0.052
30	0.0182	0.031
50	0.0098	0.017

These cells were stored in the freezer in 10 % glycerol solution. The cells used were 3 weeks old. The average absorbance was then expressed in terms of the BSA concentration. The calibration curve was generated using the procedure discussed in the experimental section. Figure 4.14 illustrates the calibration curve. In this study, 400 µl of each suspension was diluted to 4 ml and the absorbance was measured. The results were oscillating again. This prompted a shift in electroporation studies. It was an effort to find what was the cause of the results not being reproducible. This prompted the study of the effects of cell washing and centrifugation speed that was discussed earlier.

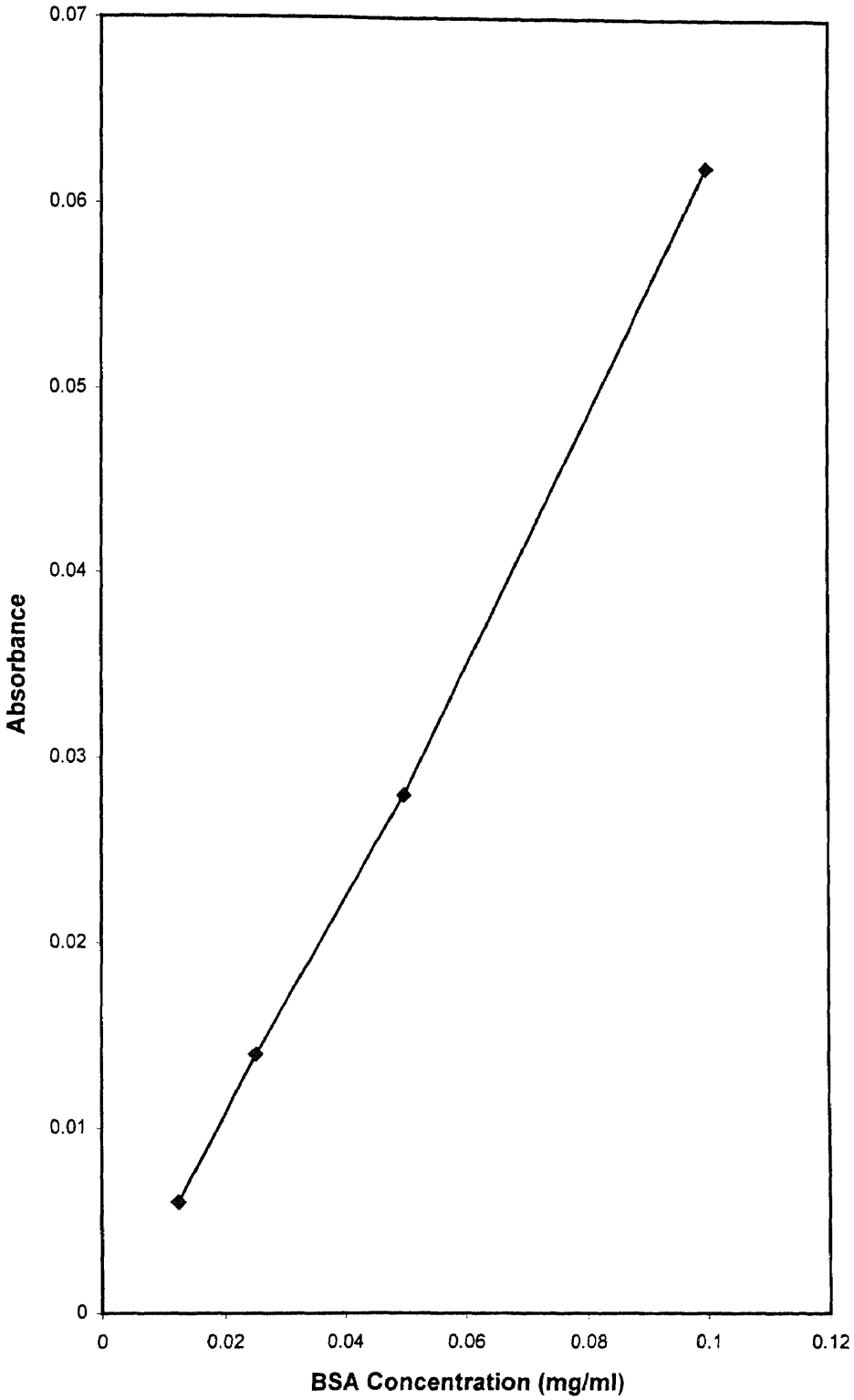

Figure 4.14 Calibration Curve for 400 Microliters of BSA Diluted to 4 ml

The next set of experiments dealt with: (1) a pulse study for 0 to 60 pulses (2) a pulse study for 0 to 40 pulses with an additional 40 pulse study electroporated in intervals of 20 pulses with a 20 minute pause; (3) a parallel total cell lysis study and (4) a study focusing on the effects of shake time on the released material as a result of pulsing and repulsing the cell pellet.

All cell suspensions in the indicated experiments were at least 3 ml in volume and the centrifugation speed upon pulsing was 3000xg. The results of electroporating Yeast Strain: Y-12636 0 to 60 times while suspended in sterile deionized water were as expected. The fresh cells were not washed. The optical density of the suspensions was approximately 1.6. The cells were electroporated over a two-day period: two samples, the first day and two samples, the second day. The experiment was done in parts due to the large amount of time needed to pulse the cell samples. The prepared samples were indicated by the pulse set and a letter A or B. For example, 0-A denotes the A sample of the 0 pulse set. There are 2 samples used to prepare the cell suspension, A and B. Table 4.25 indicates this. There are A and B samples for pulse sets ranging from 0 to 60. The purpose of this table is to illustrate the absorbance of the suspension prior to dividing the cells to be electroporated.

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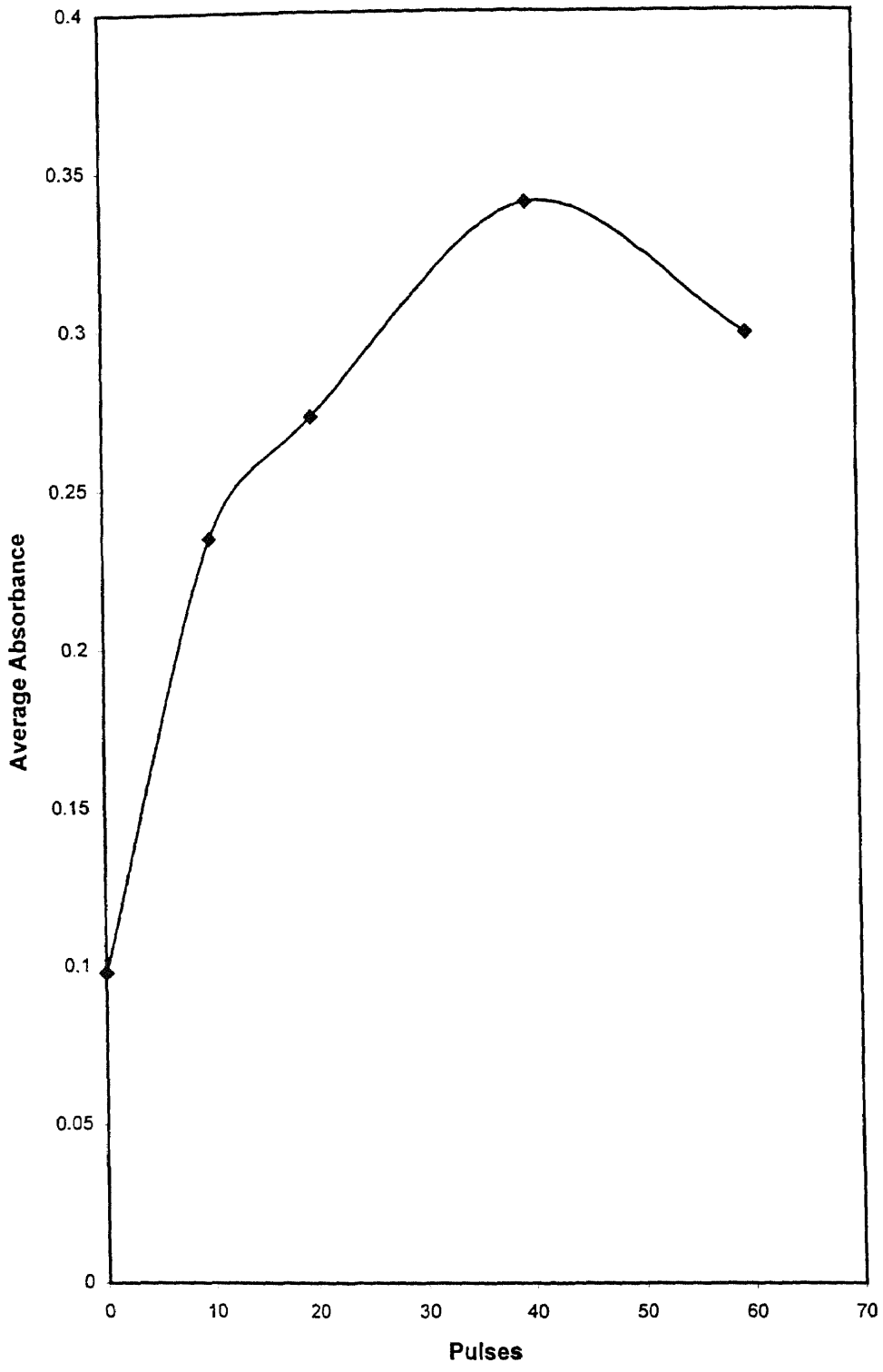

Figure 4.15 Intracellular Product Absorbance of Fresh Yeast Cells; Strain: Y-12636; Measured at 280 nm

Table 4.25 Optical Density Measured at 600 nm; Volume (12 ml); Yeast Strain: Y-12636

Batch	*Absorbance*
0-A	**1.638**
0-B	**1.628**
10-A	**1.673**
10-B	**1.665**
20-A	**1.632**
20-B	**1.656**
40-A	**1.664**
40-B	**1.652**
60-A	**1.664**
60-B	**1.682**

The samples A and B of each pulse set was combined and divided into 4 samples for each pulse set. The results from the electroporation of the samples are below. The data were as expected. The absorbance of the supernatant increased as the pulses increased. This shows that upon pulsing there were intracellular materials being released. See Figure 4.15 and Table 4.26.

Table 4.26 Intracellular Product Absorbance of Fresh Yeast Cells; Strain: Y-12636 Measured at 280 nm

Pulses	*0*	*10*	*20*	*40*	*60*
Average Absorbance	**0.098**	**0.235**	**0.273**	**0.340**	**0.299**

Pulsed samples had a thin layer of white bubbles across the surface. The cells that were pulsed 60 times appeared to have been damaged; instead of a pellet at the bottom, there were cell debris along the side of the tube after centrifuging. Before centrifuging, the suspension was cloudy. The supernatant was clear with the cell pellet concentrated at the bottom of the tube after centrifuging. The absorbance of the released intracellular products increased as the pulses increased from 0 to 40. At 60, the absorbance decreased. At this point, there is no reasonable explanation for this observation.

In the next study, the fresh cells were shaken for 14 hours. The purpose of this study was to observe the effects of pulsing the cells in 20 pulse intervals versus pulsing the cells 40 times straight as well as, to observe the overall trend of the absorbance values after pulsing the cells. The cells were not washed. The absorbances of the prepared suspensions are below. The prepared samples were labeled the same as in the previous study.

Table 4.27 Optical Density Measured at 600 nm; Volume (12 ml); Yeast Strain: Y-12636

Batch	*Absorbance*
0-A	**1.320**
0-B	**1.372**
10-A	**1.356**
10-B	**1.354**
20-A	**1.359**
20-B	**1.364**
40-A	**1.322**
40-B	**1.388**

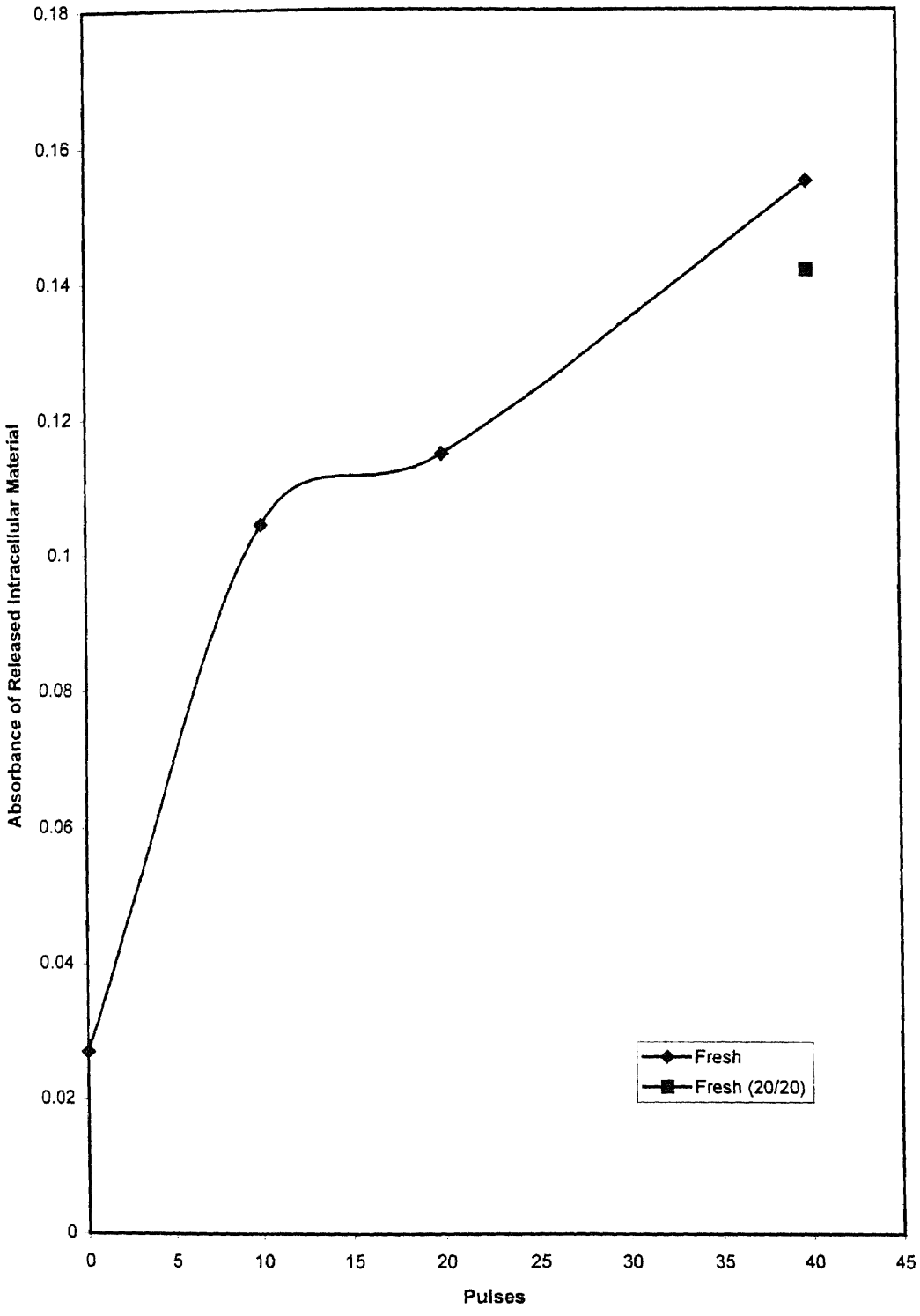

Figure 4.16 Intracellular Product Absorbance of Fresh Yeast Cells Measured at 280 nm

Table 4.28 Intracellular Product Absorbance of Fresh Yeast Cells; Strain: Y-12636 Measured at 280 nm

Pulses	*0*	*10*	*20*	*40*	*40-20/20*
Average Absorbance	**0.027**	**0.1045**	**0.115**	**0.155**	**0.142**

The 20/20 samples were pulsed 20 times then left to sit for 20 minutes and then pulsed the remaining 20 times again. The physical characteristics remained the same. Figure 4.16 illustrates the increase in absorbance from 0 to 40 pulses. The absorbance of the 20/20 sample was slightly smaller than that for the 40 pulse sample. However, both were greater than the 20 pulse values. The 40 pulse and 20/20 pulse samples were analyzed on day two. Samples for pulse sets 0 to 20 were based on 2 samples whereas samples for 40 and 20/20 were based on one sample. The results were as expected. However, later experiments were done under controlled conditions. The samples analyzed on day 2 were stored in the refrigerator for one night at 4° C.

The above experiment was repeated. The cells were pulsed 0 to 40 times with a 20/20 sample. There were 4 total samples for each pulse set. In two samples the cells were fresh, the remaining two were pulsed 2 days later. The optical density of the prepared cell suspensions was measured at a wavelength of 600 nm. The values are shown in Table 4.29.

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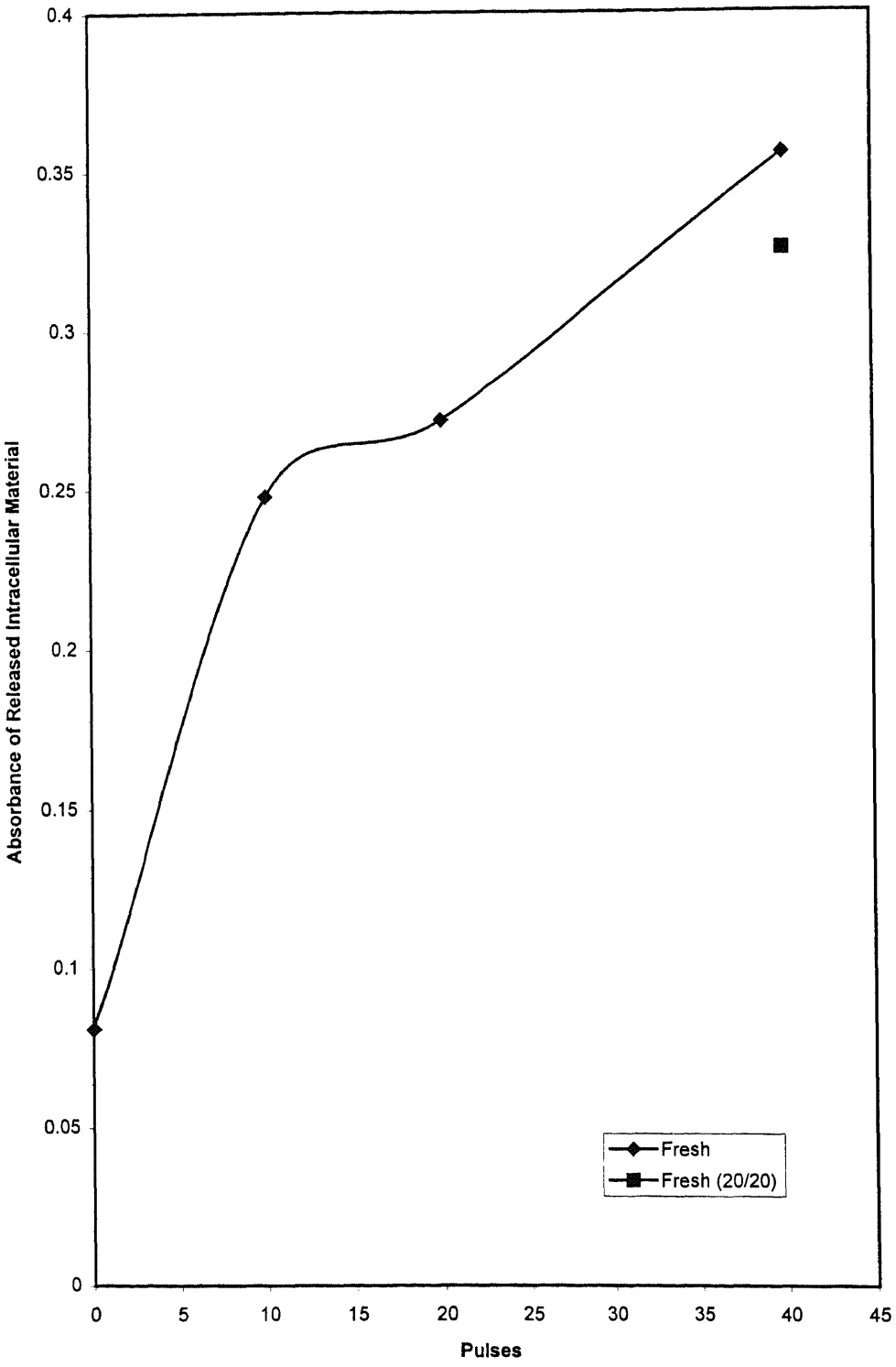

Figure 4.17 Intracellular Product Absorbance of Fresh Yeast Cells Measured at 280 nm

Table 4.29 Optical Density Measured at 600 nm; Volume (12 ml); Yeast Strain: Y-12636

Batch	*Absorbance*
0-A	1.769
0-B	1.798
10-A	1.750
10-B	1.759
20-A	1.735
20-B	1.761
40-A	1.813
40-B	1.798
40-C	1.782
40-D	1.784

The absorbance of the prepared samples range from 1.735 to 1.813. Table 4.30 and Figure 4.17 show the results of the electroporated fresh cells.

Table 4.30 Intracellular Product Absorbance of Fresh Yeast Cells; Strain: Y-12636 Measured at 280 nm

Pulses	*0*	*10*	*20*	*40*	*40-20/20*
Average Absorbance	0.081	0.248	0.272	0.356	0.326

Sample 20/20(1) was not used for 30 minutes and the 20/20(2) was not used for 25 minutes. The cell pellet appeared to have decreased in size as the pulses increased. The suspension and the supernatant were cloudy and clear respectively before and after centrifuging. Also, the samples pulsed 20 times or more had a thin layer of bubbles across the top. The absorbance values increased as the pulses increased. Again, the 20/20 pulse set absorbance was slightly lower than the 40 pulse set, as indicated in the

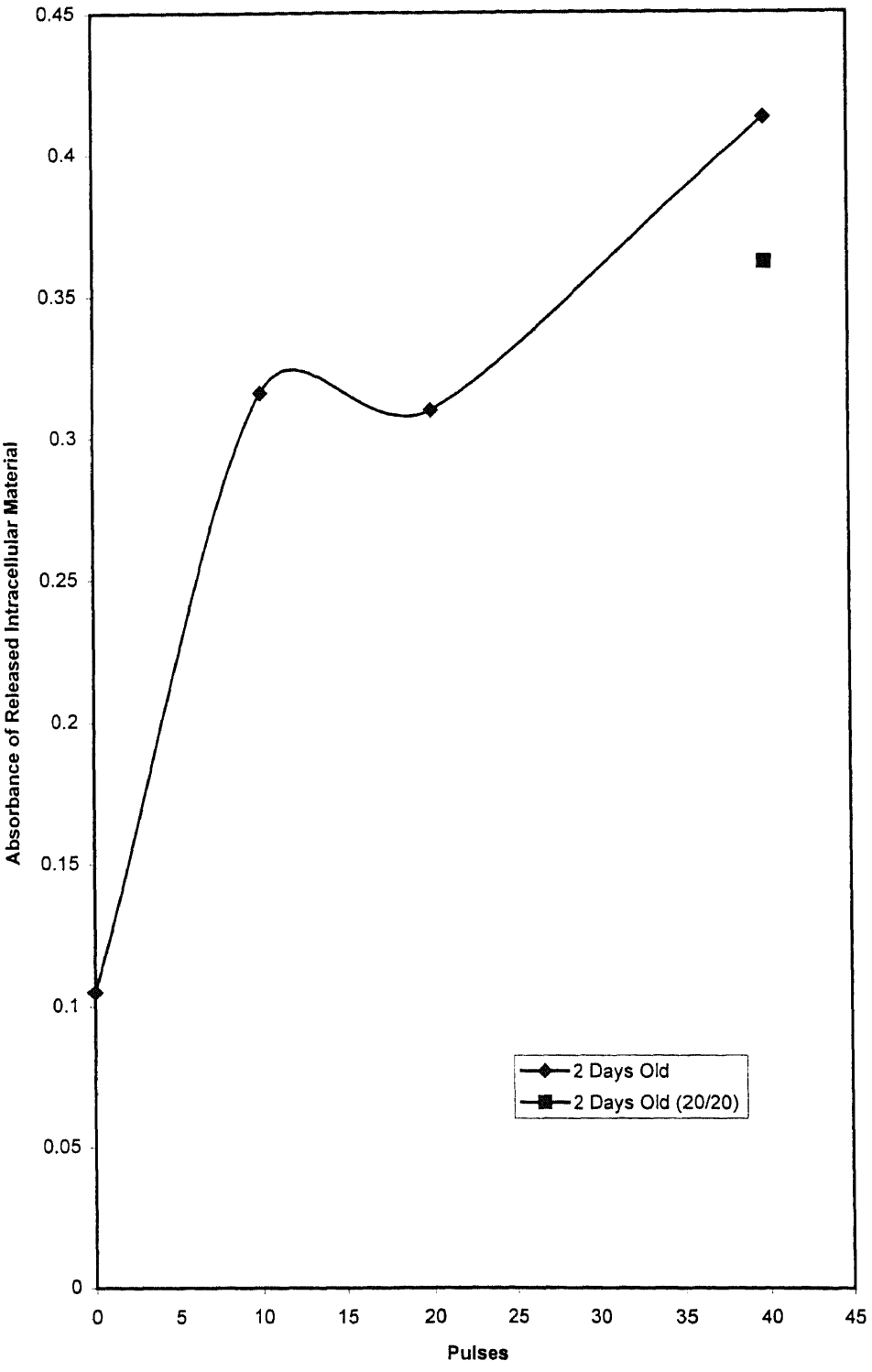

Figure 4.18 Intracellular Product Absorbance of 2 Days Old Yeast Cells; Strain: Y-12636; Measured at 280 nm

graph. This may have been due to the strength of the cell membrane as a result of pulsing. When pulsing the cells continuously for 20 times with a pause then 20 times again, the cell membrane was believed to be momentarily permeabilized. Following each pulse the holes formed and then they close. When pulsing the cells continuously for 40 times, the strength of the cell membrane was believed to have been weakened not allowing it to open and close; causing temporary openings. Because these holes are not able to close, more intracellular materials were released for samples pulsed 40 times straight. This claim is supported when comparing 20/20 absorbance to that of the 40 pulse sample, which is significantly higher. The 2-day old samples showed an overall increase from 0 to 40 pulses, but for 20 pulses there was a decrease in the average absorbance. The results are provided in Table 4.31 and Figure 4.18.

Table 4.31 Intracellular Product Absorbance of 2 Days Old Yeast Cells; Strain: Y-12636 Measured at 280 nm

Pulses	***0***	***10***	***20***	***40***	***40-20/20***
Average Absorbance	**0.105**	**0.316**	**0.310**	**0.413**	**0.362**

The sample pulsed 40 times in 20 pulse intervals had a lower absorbance than the sample pulsed 40 times continuously. Again, this may also be attributed to the strength of the cell membrane.

4.7 Parallel Studies: Electroporation and Total Cell Lysis

The total cell lysis procedure was incorporated in the experimental program. This was needed to estimate how effective was the electroporation procedure in retrieving intracellular materials from yeast cells. The total cell lysis method was taken from Qiagen (1993). The procedure was followed as indicated in Chapter 3. There were 2 samples of cell suspensions that were lysed. The lysed absorbance corresponded to the total protein content of the cells. The optical density of the prepared samples are provided below:

Table 4.32 Optical Density Measured at 600 nm; Volume (12 ml); Yeast Strain: Y-12636

Batch	*Absorbance*
1	1.874
2	1.708
3	1.823
4	1.771
5	1.747
6	1.810
7	1.844
8	1.800
9	1.783
10	1.807

Table 4.33 Total Cell Lysis of Fresh Yeast Cells; Strain: Y-12636; Intracellular Product Absorbance at 280 nm; Volume (50 ml)

Sample	*Absorbance*
1	0.759
2	0.762

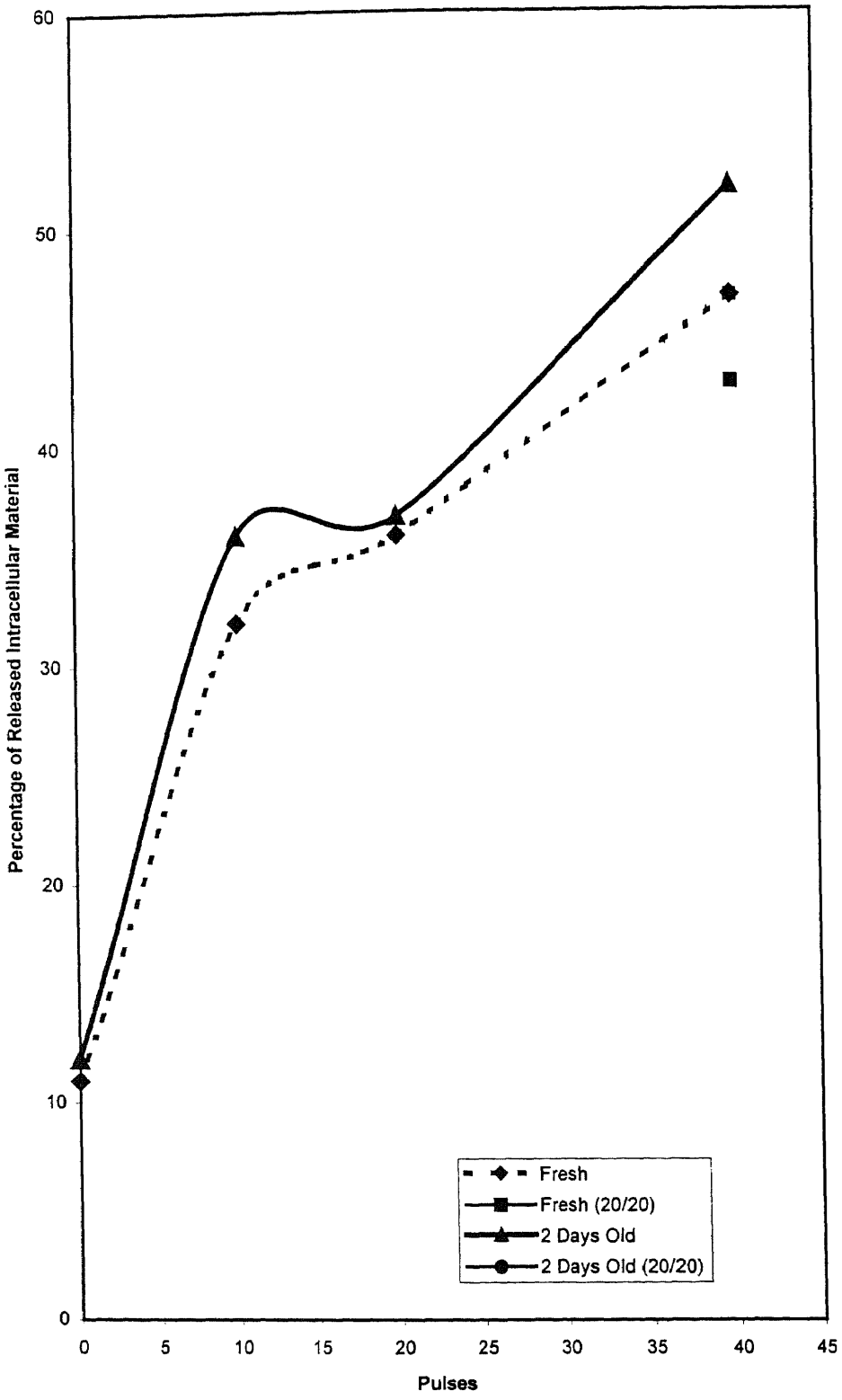

Figure 4.19 Percentage of Released Intracellular Material; Strain: Y-12636; Measured at 280 nm

Some observations were made regarding the total cell lysis samples. The supernatant was clear after centrifuging at 4 ° C to harvest the cells. Buffers P1 and P2 mixed with the cell pellet made a cloudy mixture. The addition of buffer P3 caused a precipitate to form. In preparing the blank, the precipitate also was formed. After centrifuging the buffer suspension, the precipitate and the cell debris were concentrated at the bottom.

The total cell lysis results were compared with the results of an earlier electroporation study listed in Table 4.30 and 4.31. The absorbance obtained in samples subjected to electroporation was divided by the average absorbance obtained via total cell lysis, then multiplied by 100 to get a value for the percent released. The results are given below in Table 4.34 and Figure 4.19. Later studies were done in parallel, where electroporation and total cell lysis were done on the same day.

Table 4.34 Electroporation Data for Fresh and 2 Day Old Cells; Strain: Y-12636; Intracellular Product Absorbance Measured at 280 nm and Percentage of Intracellular Product Released

Pulses	*0*	*10*	*20*	*40*	*40-20/20*
Average Absorbance (fresh)	**0.081**	**0.248**	**0.272**	**0.336**	**0.326**
% Released (fresh)	**11%**	**32%**	**36%**	**47%**	**43%**
Average Absorbance (2 days old)	**0.105**	**0.316**	**0.310**	**0.413**	**0.362**
% Released (2 days old)	**12%**	**36%**	**36.9%**	**52%**	**47%**

The amount of released intracellular materials from the total cell lysis protocol averages to a value of 0.760. When comparing these values to a previous electroporation experiment, it turns out that the amount of intracellular product released ranges from 11 % to 52 % for 0 to 40 pulses.

The parallel study was then conducted. The cells were cultured for 2 days and shaken for approximately 12 hours. The electroporation of the cells was done in 2 parts. Four samples were electroporated for each set of pulses ranging from 0 to 40. The first two samples were electroporated on the first day. The second two, on the next day. The purpose was to see how the absorbance of the released intracellular products compare for fresh and 1 day old cells. The remaining samples were stored in the refrigerator at 4 ° C for 1 day.

The goal of the two studies was to compare the amount of the intracellular product released when the cells were totally disrupted to what was released when the cells were permeated with an electric pulse. Two batches of YM broth were inoculated with the colonies of the same agar slope and incubated and grown under the same conditions. They were shaken for 12 hours at 30 °C. The optical density of yeast cells was approximately 1.4. The cell suspensions were not washed in this study. Tables 4.35 and 4.36 show the optical densities for each sample.

Table 4.35 Optical Density of Total Cell Lysis Samples Measured at 600 nm; Yeast Strain: Y-12636; Volume (30 ml)

Sample	*Absorbance*
1	**1.439**
2	**1.458**
3	**1.453**

Table 4.36 Optical Density of Electroporation Samples Measured at 600 nm; Volume (10 ml)

Sample	*Absorbance*
0	**1.410**
10	**1.422**
20	**1.434**
40	**1.430**

The absorbance of the supernatant as a result of lysing the cells are given in Table 4.37.

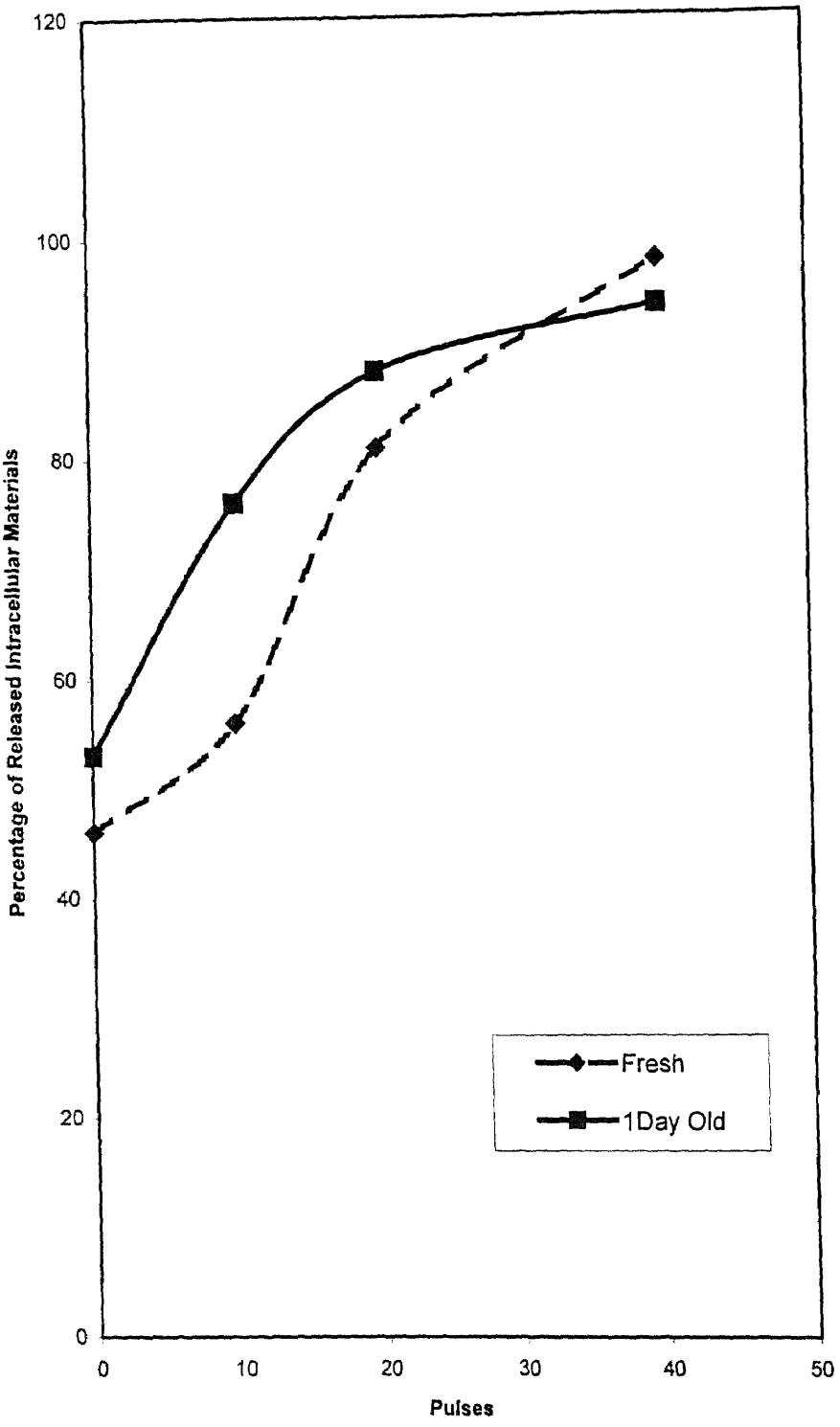

Figure 4.20 Percentage of Released Intracellular Material; Strain: Y-12636; Measured at 280 nm

Table 4.37 Total Cell Lysis of Fresh Yeast Cells; Strain: Y-12636; Intracellular Product Absorbance at 280 nm; Volume (50 ml)

Sample	*Absorbance*
1	0.384
2	0.409
3	0.373
Average	0.389

Table 4.38 Electroporation Data for Yeast Cells; Strain: Y-12636; Intracellular Product Absorbance at 280 nm and Percentage of Intracellular Product Released

Pulses	*0*	*10*	*20*	*40*
Average Absorbance	0.179	0.220	0.317	0.380
% Released (fresh)	46%	56%	81%	98%
Average Absorbance	0.206	0.294	0.342	0.367
% Released (1 day old)	53%	76%	88%	94%

Table 4.38 and Figure 4.20 illustrate that the total cell lysis absorbance was quite low, therefore making the percent released as a result of pulsing appear very high. What

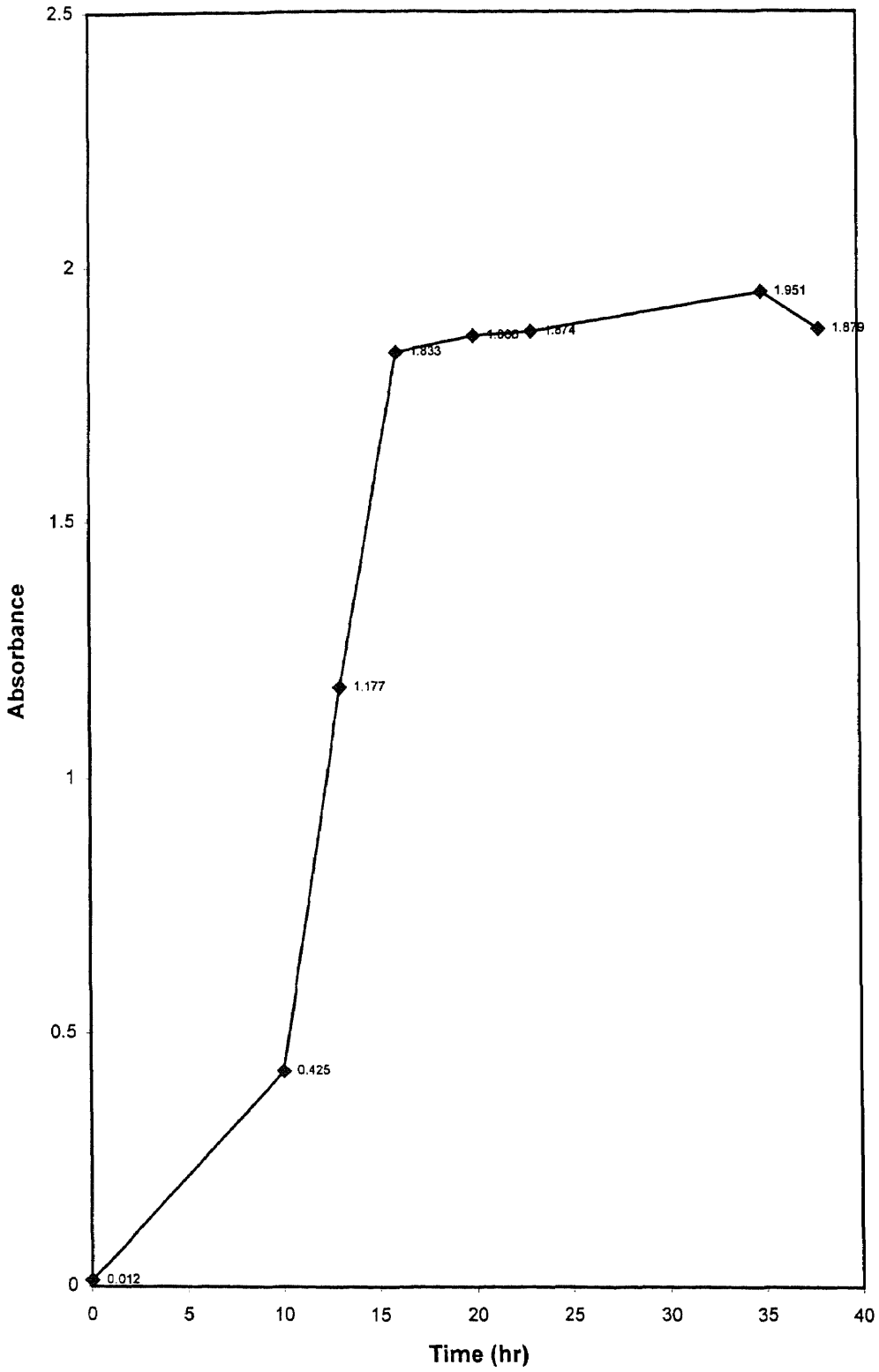

Figure 4.21 Growth Curve for Yeast Strain: Y-12636

happened was that the cell suspensions were prepared then the cells were first lysed. The entire process took approximately 3 hours. During this time, the electroporation samples were sitting and possibly growing. Consequently, the electroporation samples were beginning to be pulsed 15 hours after the agar stage, as opposed to 12 hours. At which time, the cells were still in their logarithmic phase of growth. Figure 4.21 illustrates this.

The average of the released intracellular product due to cell lysis was 0.389, whereas the average of the released product due to electroporation ranged from 0.179 to 0.380, which yields to a release of 48 % to 98 %. This was extremely high.

While conducting the experiment, a spark appeared in the black portion of the connection to the cuvette holder of the apparatus. The spark appeared twice on the second day the cells were pulsed. Once, when pulsing the fifth sample of the 40 pulse set, and the other when the eighth sample was pulsed. A sample was divided into eight cuvettes. The appearance of the spark was coupled with a popping sound. This malfunction caused one half of the cell suspension to shoot out of the cuvette.

There were a few suggestions for solutions: (1) the arcing was possibly due to the cells not being washed; a solution would be to wash the cells. (2) If the problem persisted after washing the cells, then the equipment was probably damaged. (3) If the problem did not exist after washing the cells then it would be concluded that the unwashed sample caused the apparatus to malfunction.

The previous experiment was repeated with a few changes. The cells were washed once to see if the washed cells contributed to the arcing. Two samples were electroporated on the first day and the remaining two on the second day. Also, there were no 20/20 samples electroporated.

The purpose of this experiment was to address the arcing problem. The total cell lysis study was done in parallel with the electroporation experiment. The previous study was repeated. The only change was that the cells were shaken for 15 hours. The optical density of the yeast cells was approximately 1.5. The total cell lysis was based on unwashed cells; and the electroporation was based on washed cells. Table 4.39 and Table 4.40 show how the optical densities changed as a result of washing.

Table 4.39 Optical Density of Total Cell Lysis Samples Measured at 600 nm; Volume (30 ml) No Washing

Sample	*Average Absorbance*
1	1.671
2	1.734
3	1.746

Table 4.40 Optical Density of Electroporation Samples Measured at 600 nm; Volume (10 ml) After Washing; Strain: Y-12636

Sample	*Average Absorbance*
0	1.542
10	1.522
20	1.541
40	1.586

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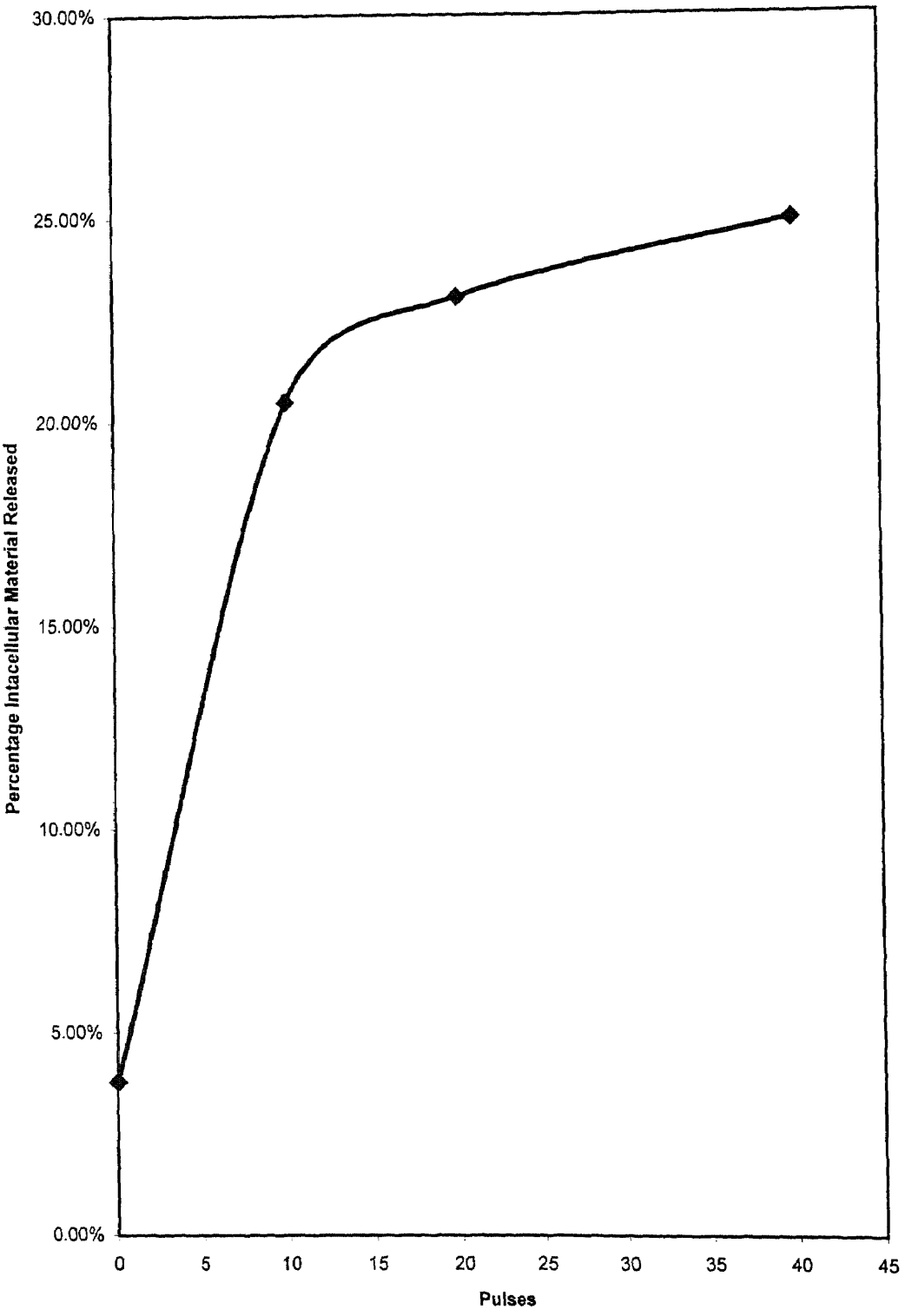


Figure 4.22 Percentage of Released Intracellular Material

Table 4.41 Optical Density of Intracellular Product Absorbance from Total Cell Lysis Measured at 280 nm; Strain: Y-12636

Sample	*Absorbance*
1	0.718
2	0.711
3	0.740
Average	0.723

Table 4.42 Electroporation Data for Fresh Yeast Cells; Strain: Y-12636; Intracellular Product Absorbance Measured at 280 nm; Percentage of Intracellular Product Released

Pulses	*0*	*10*	*20*	*40*
Average Absorbance	*0.0275*	*0.148*	*0.167*	*0.183*
% Released	*3.8%*	*20.5%*	*23.1%*	*25%*

Figure 4.22 shows that the absorbance increased as the pulses increased from 0 to 40 pulses for the fresh cells. A spark appeared again when pulsing the one day old cells. The average of the released intracellular product as a result of total cell lysis was 0.723. This was a major difference from the value obtained in the previous study in Table 4.37 that was equal to 0.389. However, it does agree with previous cell lysis data in Table 4.33, which was 0.760. The discrepancy could be due to the difference in the time allotted to shake the inoculated broth. Data for Table 4.36 was shaken for 12 hours. This study's data was generated from cells shaken for 15 hours and data for Table 4.33 was shaken for 24 hours. Later, further research will be presented to study the effects of shake time and intracellular product release.

The released intracellular product as a result of electroporation ranged from 0.02 to 0.183. This was very low when compared to values for the previous study in Table 4.38 that range from 0.179 to 0.380. This discrepancy was a direct result of the cells being washed once in this study. Therefore, the natural protein concentration of the cell suspension was removed.

The day 2 results could not be obtained. After seeing the spark the experiment was terminated. The arcing appeared on day 2 for both experiments. Possible solutions were only doing the experiments with fresh cells since the cells were probably releasing intracellular products that had a high salt concentration. It could also have been alleviated by washing cells on the second day. Another option was to freeze the cell pellet to prevent the excretion of any intracellular products. Also, it was possible that the apparatus could have been damaged.

The purpose of the next study was to investigate the effects of shake time; 13 hours was compared to 16 hours. This was done to compare the amount of released intracellular products at each growth time. The problems with arcing were also addressed. At each of these growth stages a sample of each of the pulse sets 0, 10, and 40 pulses were electroporated.

Table 4.43 Optical Density of Total Cell Lysis Samples Measured at 600 nm; Stain: Y-12636; Volume (30 ml) No Washing

Samples	*Absorbance (13 hours)*	*Absorbance (16 hours)*
1	1.145	1.771
2	1.151	1.770
3	1.157	1.745

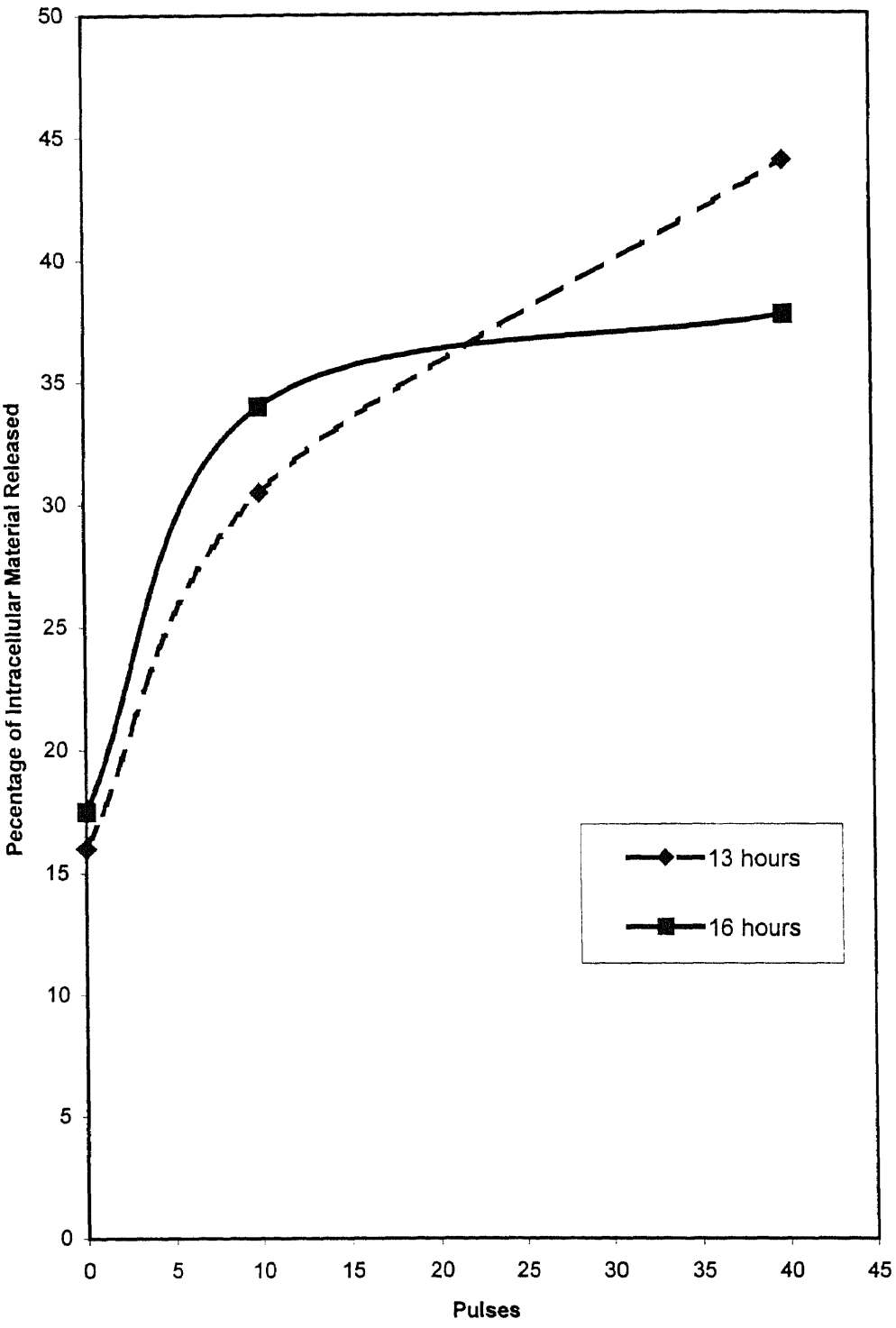


Figure 4.23 Percentage of Intracellular Materials Released Data for 1 Day Old Yeast Cells; Strain: Y-12636; Measured at 280 nm

Table 4.44 Intracellular Product Absorbance for 1 Day Old Yeast Cells from Total Cell Lysis Measured at 280 nm; Strain: Y-12636

Samples	*Absorbance (13 hours)*	*Absorbance (16 hours)*
1	**0.249**	**0.615**
2	**0.275**	**0.598**
3	**0.261**	**0.618**
Average	**0.262**	**0.610**

Table 4.45 Electroporation Data for 1 Day Old Yeast Cells; Strain: Y-12636; Intracellular Product Absorbance at 280 nm; Percentage Released

Pulses	*0*		*10*		*40*	
Shake Time	*13 hrs.*	*16 hrs.*	*13 hrs.*	*16 hrs.*	*13 hrs.*	*16 hrs.*
Average Absorbance	**0.042**	**0.107**	**0.080**	**0.208**	**0.115**	**0.230**
% Released	**16%**	**17.5%**	**30.5%**	**34%**	**44%**	**37.7%**

Growth time did affect the amount of released intracellular product. However, this impact appeared to have been proportional. See Table 4.45 and Figure 4.23. In Table 4.45 the percentages of the 13 and 16 hour samples for each pulse set were approximately the same. Also, the total lysis studies at these times agreed with previous experiments. For example, in Table 4.37 the absorbance of the total lysis equals 0.389. This agreed with 0.262 in Table 4.45 from this study in that it is significantly lower than those of the values of the absorbance at 15 and 24 hour periods. Both are significantly lower than 0.6 and 0.7 that was generated from the lysis studies done at 15 and 24 hours illustrated in Table 4.41 and 4.33 respectively.

The effects of repulsing the cell suspensions were studied in the next experiment. The cells were shaken for 15 hours at 30 °C. The cells were initially pulsed 0, 10, 20, and 40 times. After pulsing, the samples were centrifuged and the supernatant was poured off to have the absorbance measured. The cell pellet that was left was resuspended in 3 ml of sterile deionized water. The experimental procedure was then repeated. The cell suspensions were pulsed 0, 10, 20, and 40 times again. This study was done to see how the absorbance values compare after the repeated pulsing of the cells. Figure 4.24 and Table 4.47 illustrates the results.

Table 4.46 Optical Density of Electroporation Samples Measured at 600 nm; Strain: Y-12636; Volume (3 ml)

Sample	*0*	*10*	*20*	*40*
1	1.575	1.550	1.581	1.529
2	1.589	1.562	1.570	1.568
Average	1.582	1.556	1.576	1.549

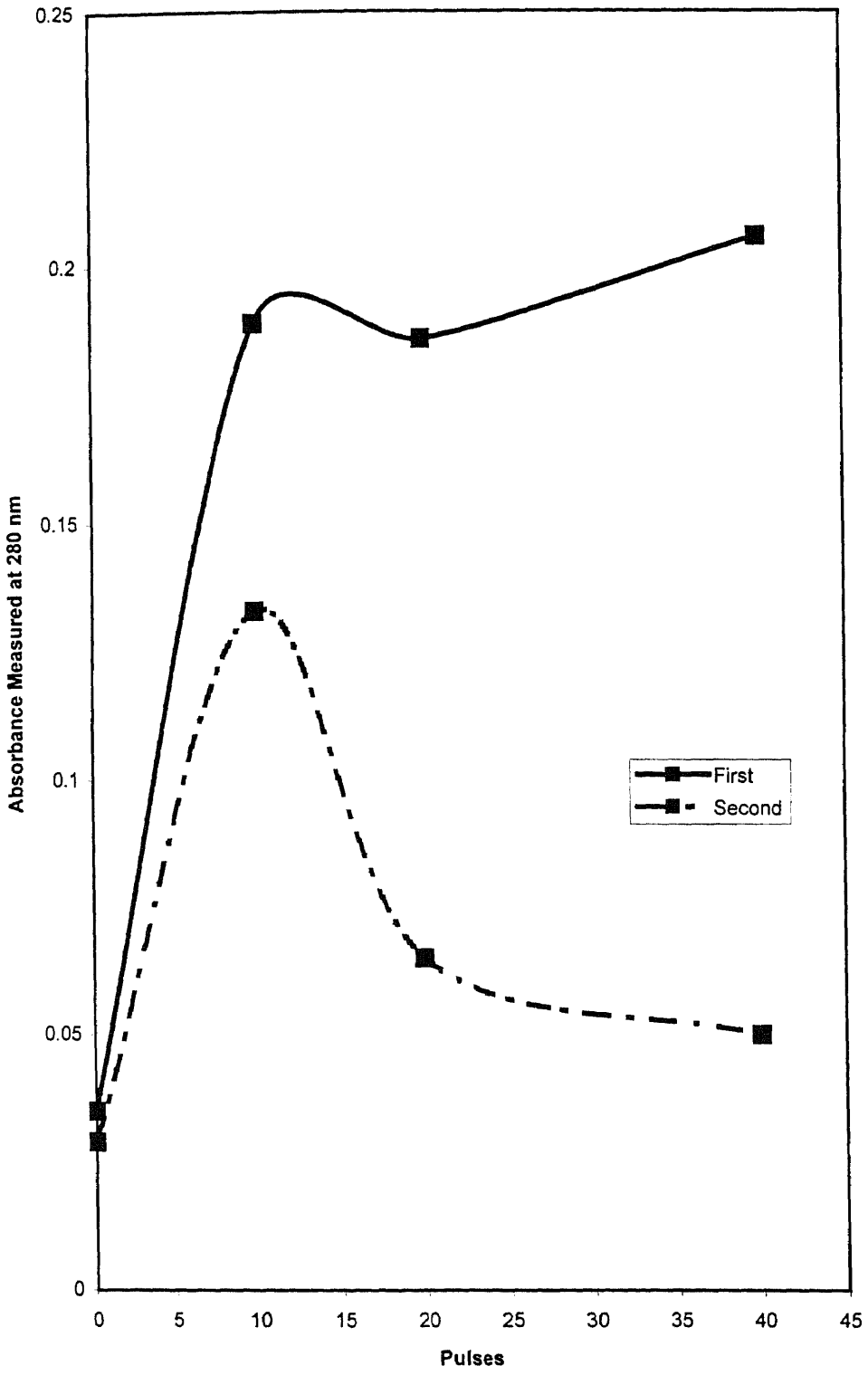


Figure 4.24 Electroporation Data for First and Second Round Pulsing of Yeast Cells; Strain: Y-12636; Intracellular Product Absorbance at 280 nm

Table 4.47 Electroporation Data for First and Second Round Pulsing of Yeast Cells; Strain: Y-12636; Intracellular Product Absorbance at 280 nm

Pulses	*0*	*10*	*20*	*40*
Average Absorbance (first round)	**0.035**	**0.189**	**0.186**	**0.0206**
Average Absorbance (second round)	**0.029**	**0.133**	**0.065**	**0.050**

The supernatant of repulsed samples appeared to be cloudy. Possibly a speed of 3000xg was too low. The following observations were made when pulsing the samples a second time: (1) cuvettes did not get warm; (2) the time constant remained constant; and (3) no froth appeared across the surface.

There was additional release of intracellular product when repulsing the cells. The absorbance values for the repulsed samples decreased as expected. However, there was a discrepancy with 20 and 40 pulse samples that were pulsed the second time. The value at 20 was a little higher. Also, the 10 pulse sample released approximately the same amount twice.

The total intracellular product concentration had an absorbance of 0.110. Comparing this absorbance to what was released via electroporation would give results that surpass 100%. This was an unrealistic and impossible result. The cells were stored for 2 nights in the freezer at -18° C. No arcing appeared. The experiment was conducted

using new electroporation cuvettes. This is believed to have avoided the arcing problem.

The experiment was repeated with the following changes: (1) the statistical average was based on three (3) samples; (2) the repulsed cell pellet was stored in the freezer at $-18\ ^{\circ}C$ for one night; and (3) the cells were shaken for 17 hours.

Tables 4.48 and 4.49 show the optical densities of the electroporation and total cell lysis samples. Table 4.50 shows the tabulated total cell lysis data for fresh cells. Again, the purpose of presenting the optical density data is to show that the baseline concentrations were approximately the same.

Table 4.48 Optical Density of the Electroporation Samples Measured at 600 nm

Sample	*0*	*10*	*20*	*40*
1	**1.884**	**1.906**	**1.835**	**1.781**
2	**1.826**	**1.876**	**1.874**	**1.898**
3	**1.913**	**1.849**	**1.848**	**1.857**

Table 4.49 Optical Density of the Total Cell Lysis Samples Measured at 600 nm

Sample	*Absorbance*
1	**1.801**
2	**1.869**
3	**1.838**

Table 4.50 Optical Density of Intracellular Product Released from Total Lysis Measured at 280 nm; Product Absorbance of Yeast Strain: Y-12636

Sample	*Absorbance*
1	**0.808**
2	**0.781**
3	**0.736**
Avg. Abs.	**0.775**

Similar observations were made in this repulsing study. The cuvettes did not get warm, the time constant remained constant, and no froth appeared when the second round of pulsing was conducted. The total cell lysis solution was centrifuged at 16,000 rpm instead of 15, 000 rpm, which is specified in section 3.3.5. Table 4.51 and Figure 4.25 illustrate the results after electroporating the yeast cells.

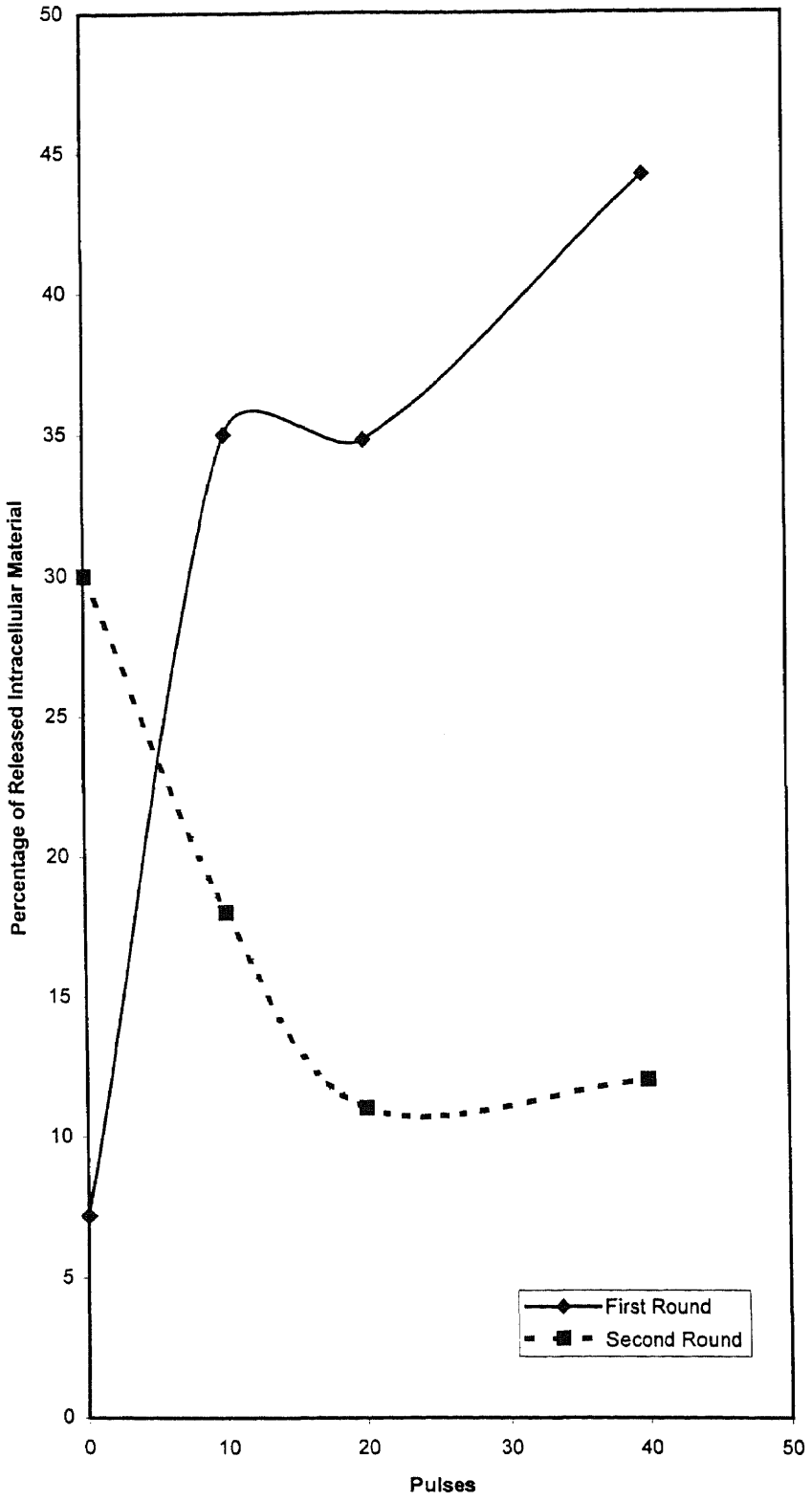


Figure 4.25 First and Second RoundPulsing Data Analyzed using UV Absorbance Assay Measured at 280 nm

Table 4.51 Electroporation Data and Percentage of Released Intracellular Product for First and Second Round Pulsing of Yeast Cells based on a Total Cell Lysis Absorbance of 0.775; Strain: Y-12636; Intracellular Product Absorbance Measured at 280 nm

Pulses	*0*	*10*	*20*	*40*
Avg. Abs.	**0.056**	**0.272**	**0.270**	**0.343**
% Released *(First Round)*	**7.2 %**	**35 %**	**34.8 %**	**44.2 %**
Avg. Abs.	**0.232**	**0.139**	**0.086**	**0.092**
% Released *(Second Round)*	**30 %**	**18 %**	**11 %**	**12 %**

The absorbance of the released intracellular material increased as the number of pulses increased during the first round of pulsing. The trend for the repulsed samples showed a decrease in the absorbances from 0 to 20 pulses, then a slight increase at 40 pulses. The unusually high value of the 0 pulse sample during the second round was due to two factors: the pellet being suspended in 3 ml of sterile water instead of 7 ml; and the freezing and thawing of the cell pellet which contributed to additional intracellular material being released. This differs from the 0 pulse data points in Table 4.47, which were very close. The repulsing portion of the experiment presented in Table 4.47 was performed on the first day while the cells were still fresh.

The study was repeated with cells having a shake time equal to 15 hours. The baseline absorbances of the electroporation and total cell lysis samples were significantly

different. Here the electroporation data are presented without the percentage of released intracellular material due to the difference in the baseline absorbances. Tables 4.52 and 4.53 show the optical densities of the electroporation and total cell lysis samples respectively.

Table 4.52 Optical Density of the Electroporation Samples Measured at 600 nm

Sample	*0*	*10*	*20*	*40*
1	1.776	1.730	1.776	1.843
2	1.844	1.792	1.803	1.845
3	1.775	1.830	1.874	1.867

Table 4.53 Optical Density of the Total Cell Lysis Samples Measured at 600 nm.

Sample	*Absorbance*
1	1.471
2	1.450
3	1.486

The absorbance values of the tables above differ by approximately 0.4. The trend of the first round of pulsing increased as the pulses increased. The second round of pulsing showed a decrease from the 0 pulse to the 10 pulse value followed by a gradual increase from 10 to 40 pulses. The electroporation data are illustrated in Table 4.54 and Figure 4.26.

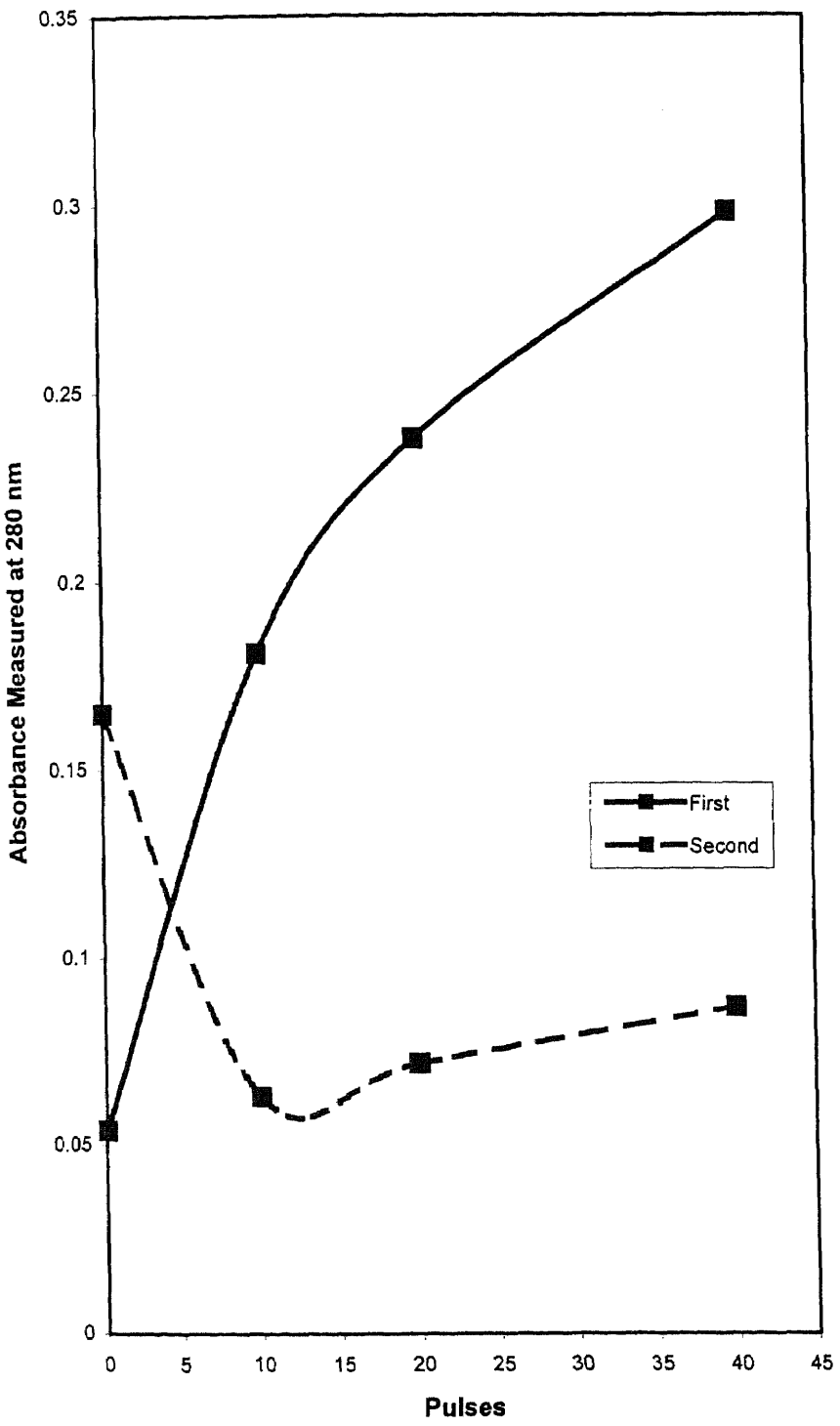


Figure 4.26 First and Second Round Pulsing Data Analyzed using UV Absorbance Assay Measured at 280 nm

Table 4.54 Electroporation Data for Yeast Cells; Strain: Y-12636; Intracellular Product Absorbance at 280 nm

Sample	*0*	*10*	*20*	*40*
First Round ***(Fresh cells)*** *Avg. Abs*	**0.054**	**0.181**	**0.238**	**0.298**
Second Round ***(Repulsed Cells)*** *Avg. Abs.*	**0.165**	**0.063**	**0.072**	**0.087**

The average absorbance for the released intracellular material due to total cell lysis was 0.499. See Table 4.55. This value was surprisingly low.

Table 4.55 Optical Density of Intracellular Product Released from Total Lysis Measured at 280 nm; Product Absorbance of Strain: Y-12636

Sample	*Absorbance*
1	**0.473**
2	**0.494**
3	**0.530**
Avg. Abs.	**0.499**

The next study focuses on the electroporation of yeast cells that were shaken for 33 hours. The repulsed cells were stored for 5 days at – 18 ° C. The optical densities of the total cell lysis and the electroporation samples were very close. This was due to the fact

that the cells were harvested at 33 hours, at which time the cells were well into their stationary phase. Tables 4.56 and 4.57 illustrate this.

Table 4.56 Optical Density of the Electroporation Samples Measured at 600 nm

Sample	*0*	*10*	*20*	*40*
1	1.926	1.899	1.916	1.936
2	1.917	1.897	1.868	1.854
3	1.951	1.911	1.902	1.911

Table 4.57 Optical Density of the Total Cell Lysis Samples Measured at 600 nm

Sample	*Absorbance*
1	1.912
2	1.927
3	1.890

Below the product concentration as a result of total cell lysis is presented in Table 58. The average absorbance is 0.830. This was the largest absorbance of released intracellular material due to the total cell lysis. It appears as the time the cells were shaken increased the amount released due to total cell lysis also increased. Table 4.59 and Figure 4.27 show the electroporation data. Symbols "I" and "II" were used to distinguish between the two 33 hour studies that are discussed.

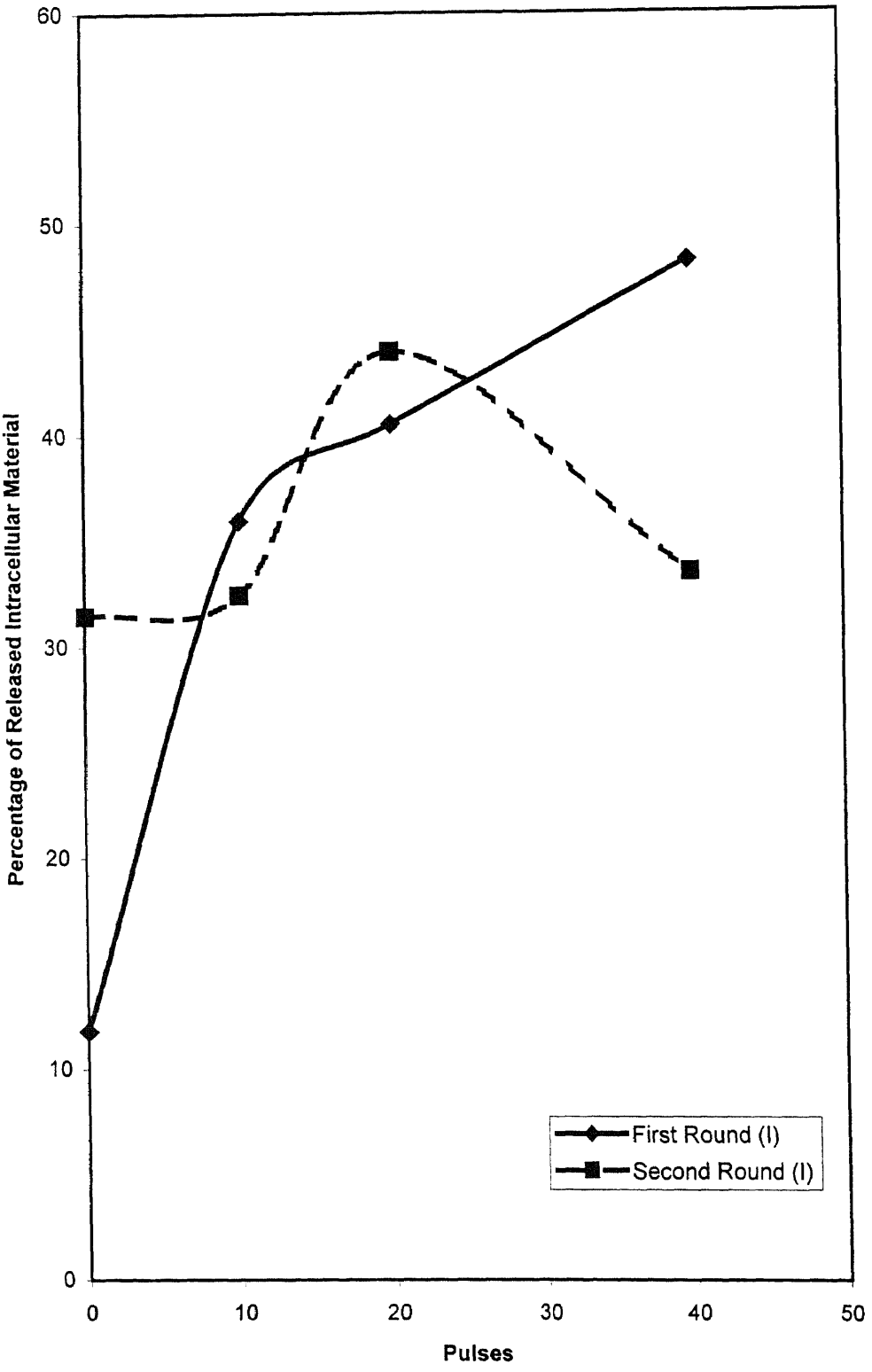


Figure 4.27 Percentage of Released Intracellular Material

Table 4.58 Optical Density of Intracellular Product Concentration from Total Cell Lysis Measured at 280 nm; Product Absorbance of Strain: Y-12636

Sample	*Absorbance*
1	0.823
2	0.850
3	0.818
Avg. Abs.	0.830

Table 4.59 Electroporation Data for Yeast Cells; Strain: Y-12636; Intracellular Product Absorbance at 280 nm; Percentage of Released Intracellular Material based on a Total Cell Lysis Absorbance of 0.830

Sample	*0*	*10*	*20*	*40*
First Round ***(Fresh cells)*** *Avg. Abs*	0.098	0.299	0.337	0.401
% Released *(First Round)*	11.8 %	36 %	40.6 %	48.3 %
Second Round ***(Repulsed Cells)*** *Avg. Abs.*	0.262	0.270	0.365	0.279
% Released *(Second Round)*	31.5 %	32.5 %	44 %	33.6 %

The 33 hour study was conducted again to reproduce the previously explained results. Tables 4.60 and 4.61 illustrate the starting absorbances of the total cell lysis and electroporation samples. The values were constant because the yeast cells were in the stationary phase of growth.

Table 4.60 Optical Density of the Electroporation Samples Measured at 600 nm

Sample	*0*	*10*	*20*	*40*
1	1.846	1.843	1.785	1.845
2	1.829	1.849	1.812	1.866
3	1.819	1.863	1.831	1.814

Table 4.61 Optical Density of the Total Cell Lysis Samples Measured at 600 nm

Sample	*Absorbance*
1	1.862
2	1.847
3	1.739

Tables 4.62 and 4.63 present the release due to electroporation and total cell lysis. The average release due to total cell lysis was 0.678. This value was lower than that of the previous study which was 0.830. There is no concrete explanation as to why the value was unusually low. In previous studies there were instances where the total cell lysis procedure gave results that were unusual and out of the ordinary.

Table 4.62 Optical Density of Intracellular Product Released from Total Lysis Measured at 280 nm; Product Absorbance of Strain: Y-12636

Sample	*Absorbance*
1	**0.721**
2	**0.679**
3	**0.635**
Avg. Abs.	**0.678**

Figure 4.28 shows an increase in absorbance values from 0 to 40 pulses for the first round of pulsing. In the second round there was an increase in absorbance between 0 and 20 pulses then there was a decrease at 40. Figure 4.29 shows a combination of the results of both 33 hour studies. Again, symbols "I" and "II" were used to distinguish between the two 33 hour studies that are discussed. First round pulsing of both studies follow identical trends. There was a sharp increase from 0 to 10 pulses then a slight increase from 10 to 20. It continues to slope upward to 40 pulses. Each study illustrates a significant decrease at 40 pulses during the second round of pulsing. The results for the 33 hour stationary phase studies show a greater release of intracellular material than previous studies with cells within the log phase of growth from 12 to 18 hours.

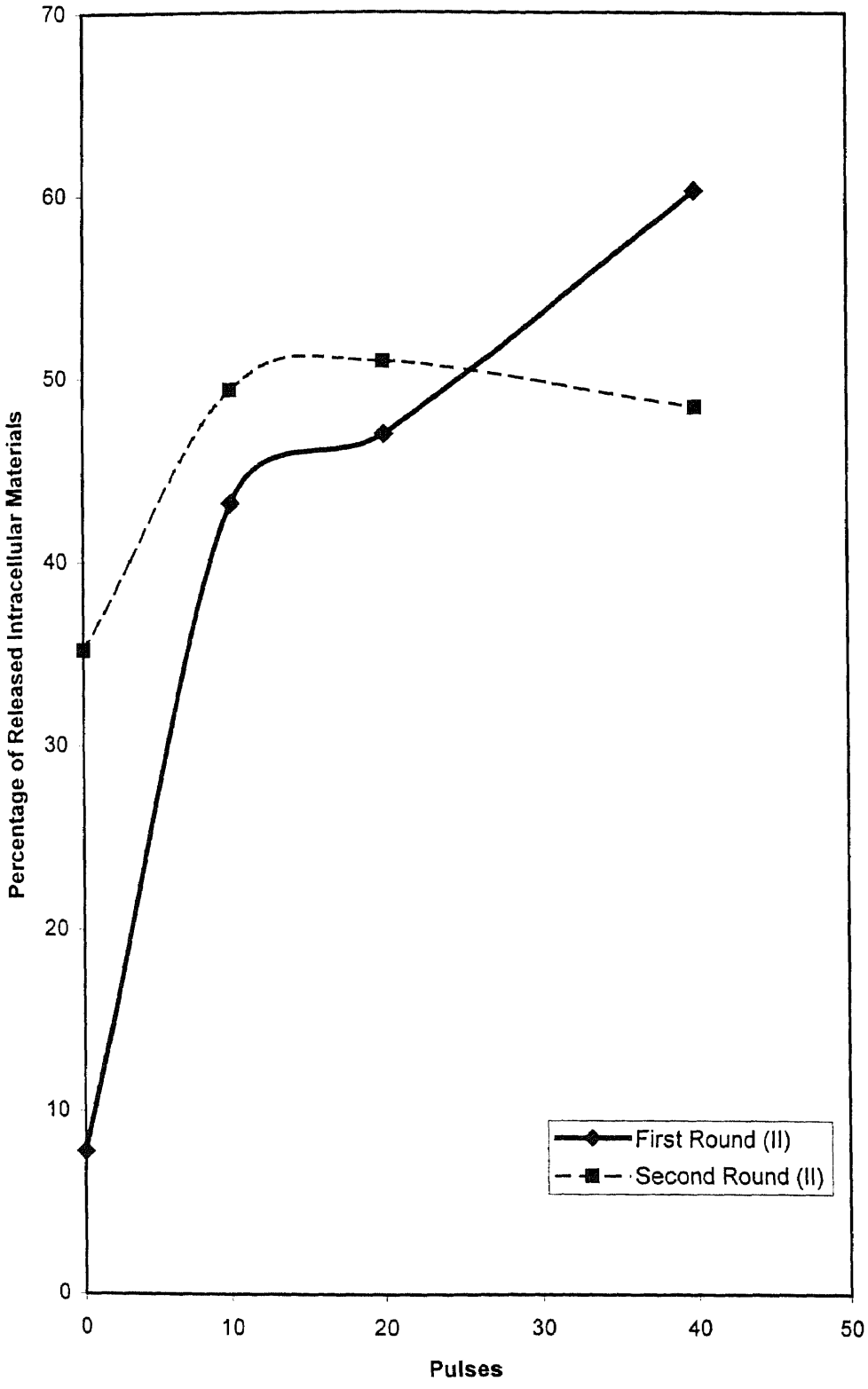


Figure 4.28 Percentage of Released Intracellular Materials

Table 4.63 Electroporation Data for Yeast Cells; Strain: Y-12636; Intracellular Product Concentration at 280 nm; Percentage of Released Intracellular Material based on a Total Cell Lysis Absorbance of 0.678

Sample	*0*	*10*	*20*	*40*
First Round ***(Fresh cells)*** *Avg. Abs*	0.053	0.293	0.319	0.409
% Released *(First Round)*	7.8 %	43.2 %	47 %	60.3 %
Second Round ***(Repulsed Cells)*** *Avg. Abs.*	0.239	0.335	0.346	0.329
% Released *(Second Round)*	35.2 %	49.4 %	51 %	48.5 %

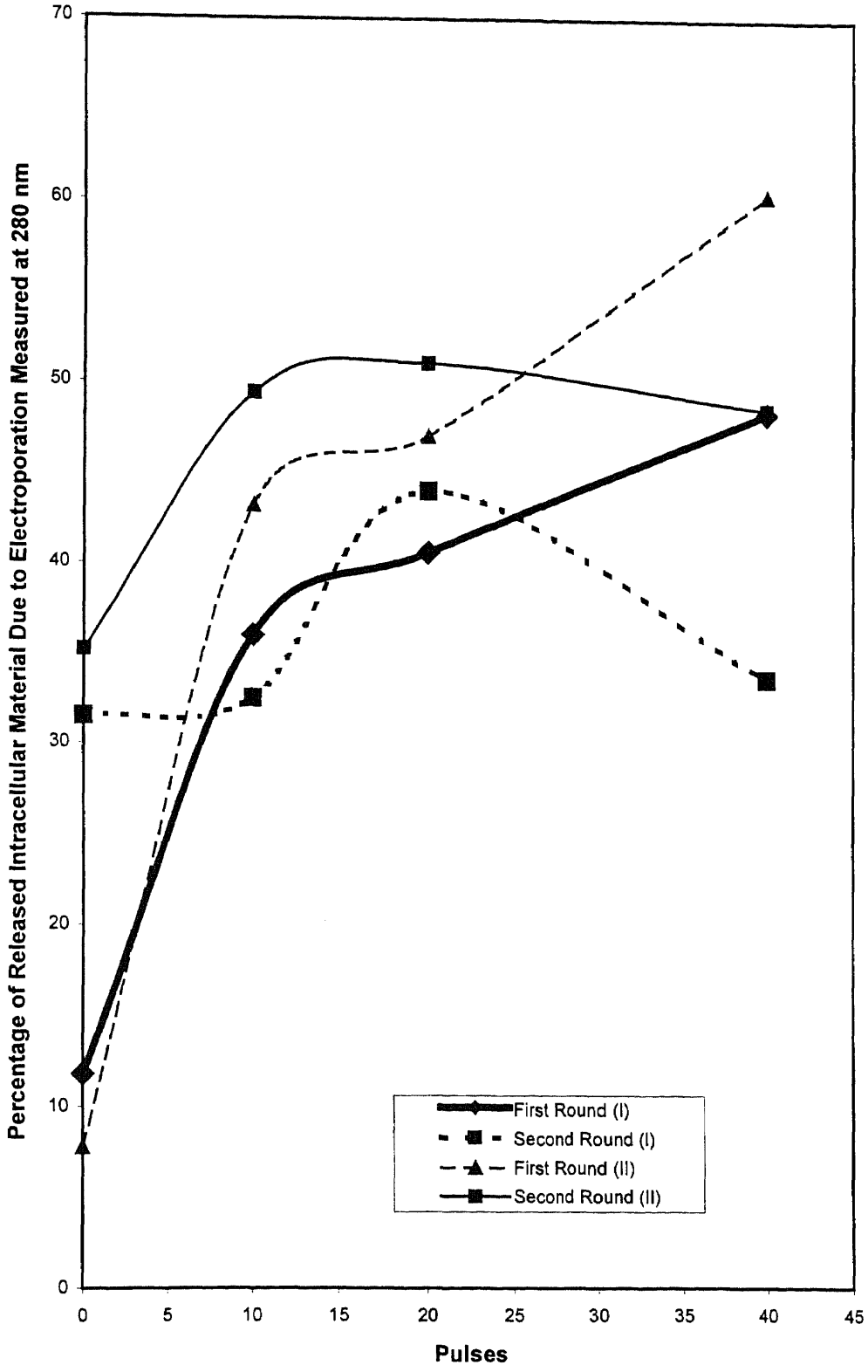


Figure 4.29 Percentage of Released Intracellular Materials; Measured at 280 nm for the 33 Hour Studies

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

A procedure for the electroporation of yeast cells to release intracellular proteins has been developed. Factors such cell storage, phase of cell growth, sample volume, cell freshness, multiple pulsing, and cell washing have affected the release of the intracellular materials. Use of a statistical average is a good way to measure the absorbance of released material. Sample volumes of at least 3 ml should be used in electroporation studies; samples of this size give results that are able to be reproduced and are as speculated. The study of minute samples in microliter volumes yield oscillating results. When the sample size is extremely small, large experimental error is introduced, resulting in fluctuating absorbances.

Cell suspensions stored in the refrigerator overnight at 4 ° C yield absorbances that are slightly higher than those obtained when the cells are fresh. Also, the freezing at – 18 ° C and later thawing of cell pellet affects the osmotic pressure of the cell by aiding in the release of intracellular material.

Yeast Strain: NRRL Y-12636 demonstrates the log phase of growth between 12 and 18 hours. The stationary phase of growth is obtained from 18 hours and on. Cells harvested within the log growth phase should not be used due to fact that during this time the concentration of the cell suspension is rapidly changing. The duration of the experiment is extremely time consuming; therefore if cells are harvested at such a time the concentration will change from the start of the experiment to the end of the

experiment. The use of cells within the stationary phase will alleviate this problem as a result of the time consuming nature of the entire experimental study. The growth time of the cell suspension is directly related to the concentration up until the sample reaches the stationary phase.

The electroporation cuvettes have been found to be able to be used after multiple pulsing. Fresh yeast cell suspensions subjected to multiple pulses increasingly release intracellular materials as a function of the number of pulses. However, the absorbance for 60 pulses is always less than that for 40 pulses. Therefore, release as a result of electroporation reaches a plateau somewhere between 20 and 60 pulses. Further experiments needs to be done to find the exact point at which this happens. Suspensions pulsed 40 times straight versus those pulsed in intervals show that the absorbances were greater for the continuously pulsed samples.

The search to find a means to determine the protein concentration was a difficult one. The protein assay based on UV Absorbance proved to be the most effective and reliable means to determine the protein concentration. Bradford and Lowry assays were not effective in determining the protein concentration in this study. Perhaps, these assays could be used in conjunction with larger sample volumes. The procedure could be scaled up to use protein samples that are at least 3 ml in volume.

The incorporation of the above mentioned findings has aided in the development of a new and improved means to extract desired products from intracellular components.

5.2 Recommendations

There are a number of recommendations regarding the ongoing development of efficient, reliable, and reproducible procedures to use electroporation as a technique to release desired intracellular materials. First, solutions to problems such as arcing, and finding the maximum number of pulses to release the greatest amount of intracellular material need to be researched further. The arcing problem seems to have been avoided through the use of new electroporation cuvettes.

Next, experiments should be introduced to further qualify the claims regarding the extent of cell disruption through the use of a microscope and the identification of the released materials by using electrophoresis. The efficiency can be improved by using an electroporation apparatus that pulses larger volumes of at least 3 ml in order to analyze more samples in less time. Also, the increase in sample size could possibly be the solution to using other protein determination methods. The larger volumes remove the oscillatory results that are demonstrated when using minute volumes of microliter portions.

The apparatus should be comfortable for the user. The user should research vendors to find an apparatus that is more user friendly. The continuous pressing to apply the multiple pulses wears out the key pads, as a result causes the user to exert more energy to apply a pulse. The increased force by the user causes severe pain and discomfort to the fingers of the user. The user should look for an apparatus with a hand held pulsing mechanism that can be depressed using the thumb. The user could also research equipment that can be computer programmed to deliver the desired number of

pulses to a given number of samples. These setups would be more comfortable for the user and would increase the overall efficiency of the experiment.

The effects of changing parameters such as the set voltage, yeast strain, time constant, and the electroporation buffer can also be studied. A given yeast strain could be subjected to pulses that may vary from 1.5 to 2.5 kV. The experiment could be repeated using different electroporation buffers such as polyethyleneglycol (PEG) or sorbitol, and different yeast strains or other types of cells, such as those of plants or animals.

These changes and improvements will aid in the continuous development of techniques to recover the desired intracellular materials.

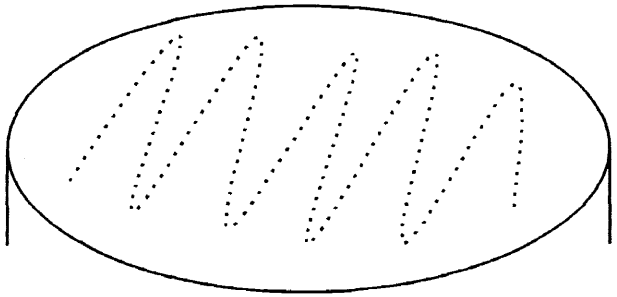
APPENDIX

SCHEMATICS OF INTEGRATED PROCEDURES

The following schematics give illustrative descriptions of procedures done to achieve reproducible data from electroporation experiments. The schematics discuss fermentation, cell solution preparation, electroporation, and total cell lysis procedures. They are to be used in conjunction with the experimental methods discussed in Chapter 3. The purpose of the schematics is to provide the reader with a deeper understanding of experimental procedures.

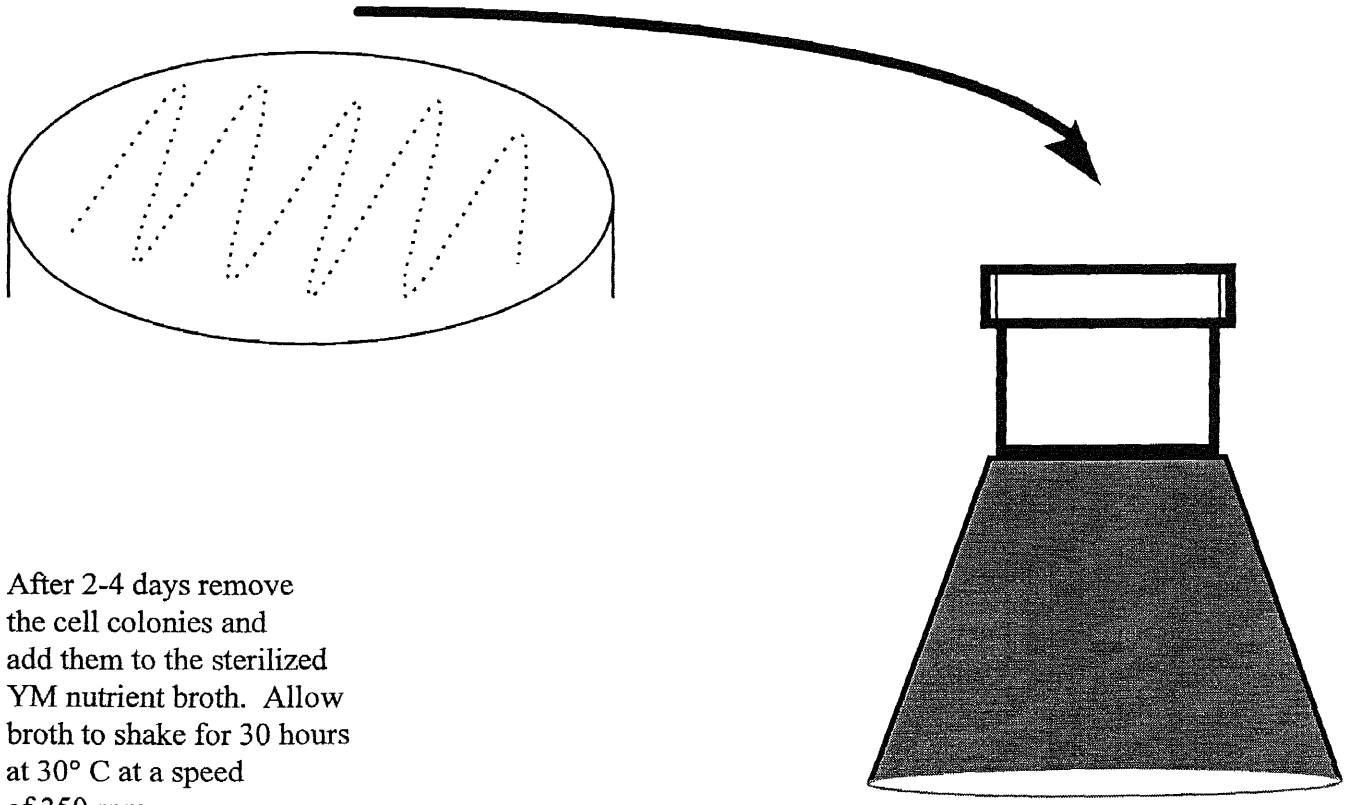
Fermentation Protocol

1.



Streak cells on agar. Allow cells to grow 2 to 4 days. Store at room temperature approximately 25° C.

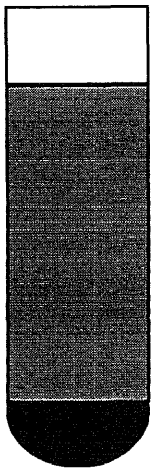
2.



After 2-4 days remove the cell colonies and add them to the sterilized YM nutrient broth. Allow broth to shake for 30 hours at 30° C at a speed of 350 rpm.

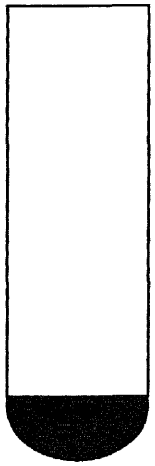
CELL SOLUTION PREPARATION
Method A

1.



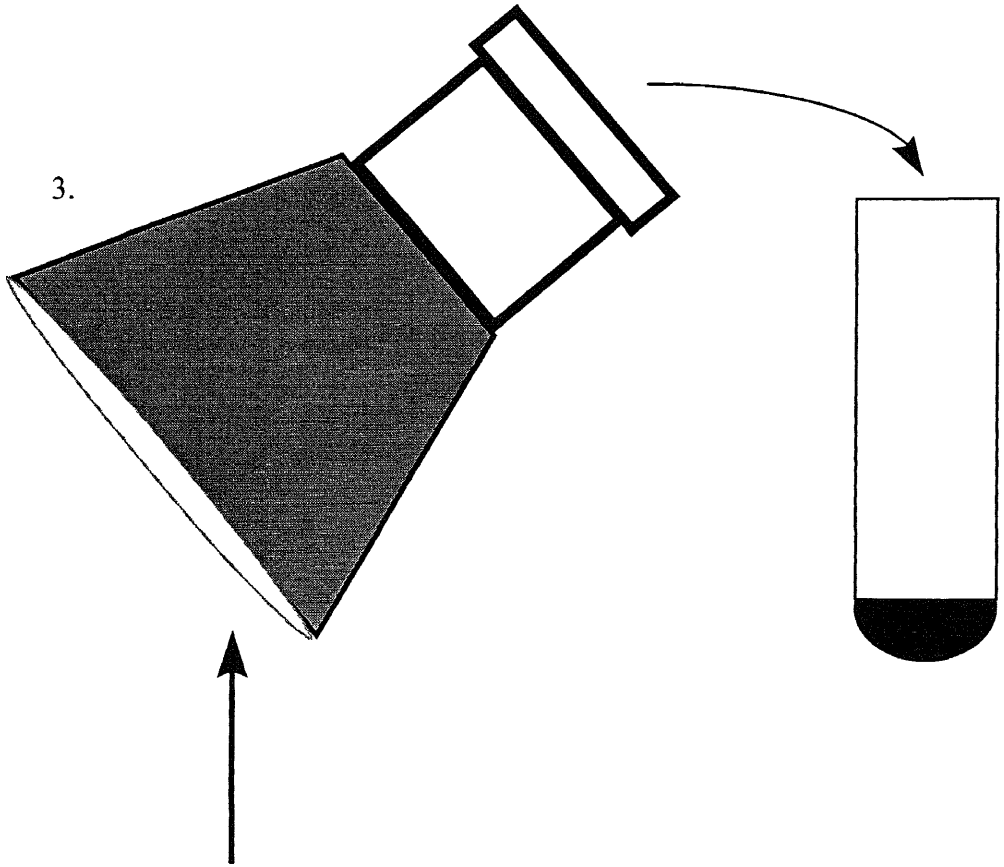
Cells and nutrient broth after centrifuging to harvest cells after centrifuging.

2.



Nutrient broth poured off leaving the cell pellet at bottom.

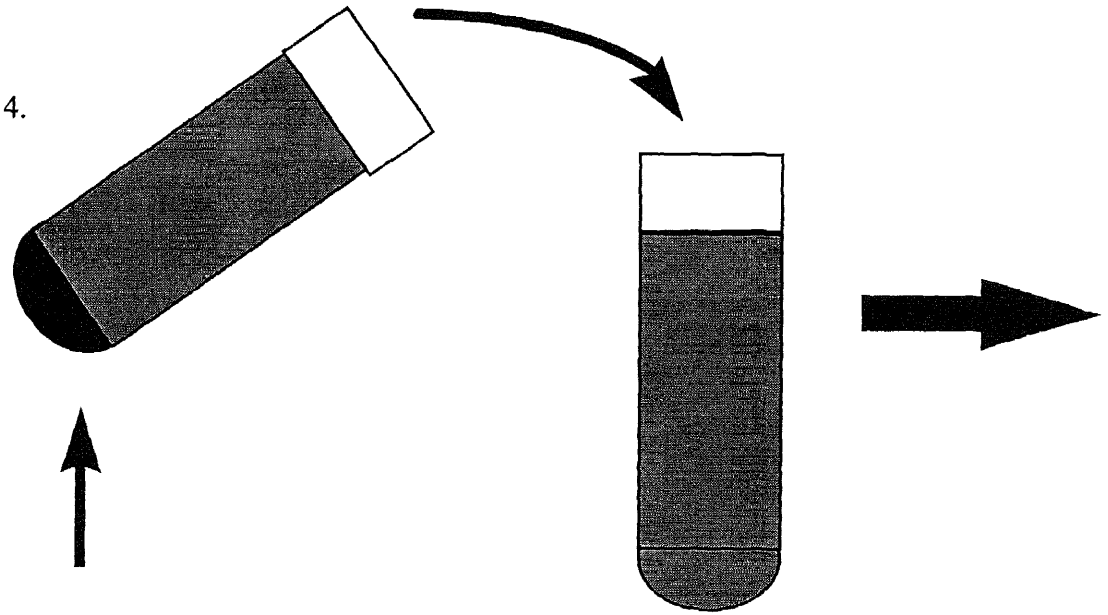
3.



Sterile Deionized Water

Sterile water added to cell pellet. This mixture is inverted gently to form a suspension. The suspension is then centrifuged at 3000xg for 5 minutes.

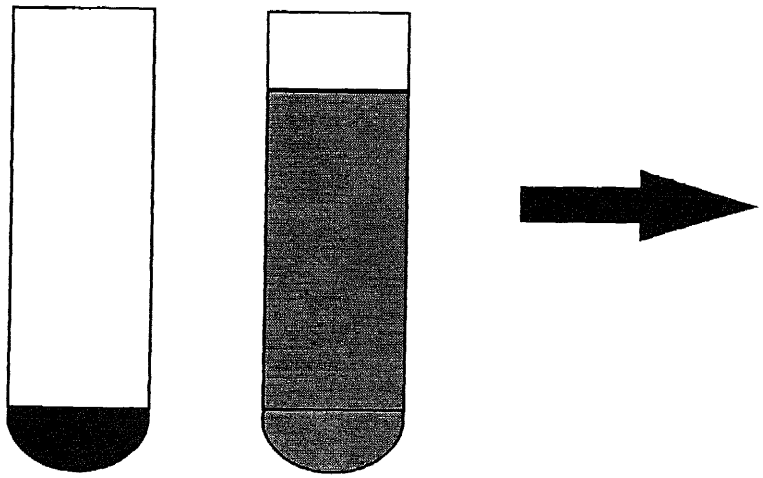
4.



Cell pellet and supernatant, where the supernatent is being poured off.

This is the before pulse (BP) solution. It will be assayed later to determine the initial protein concentration prior to pulsing.

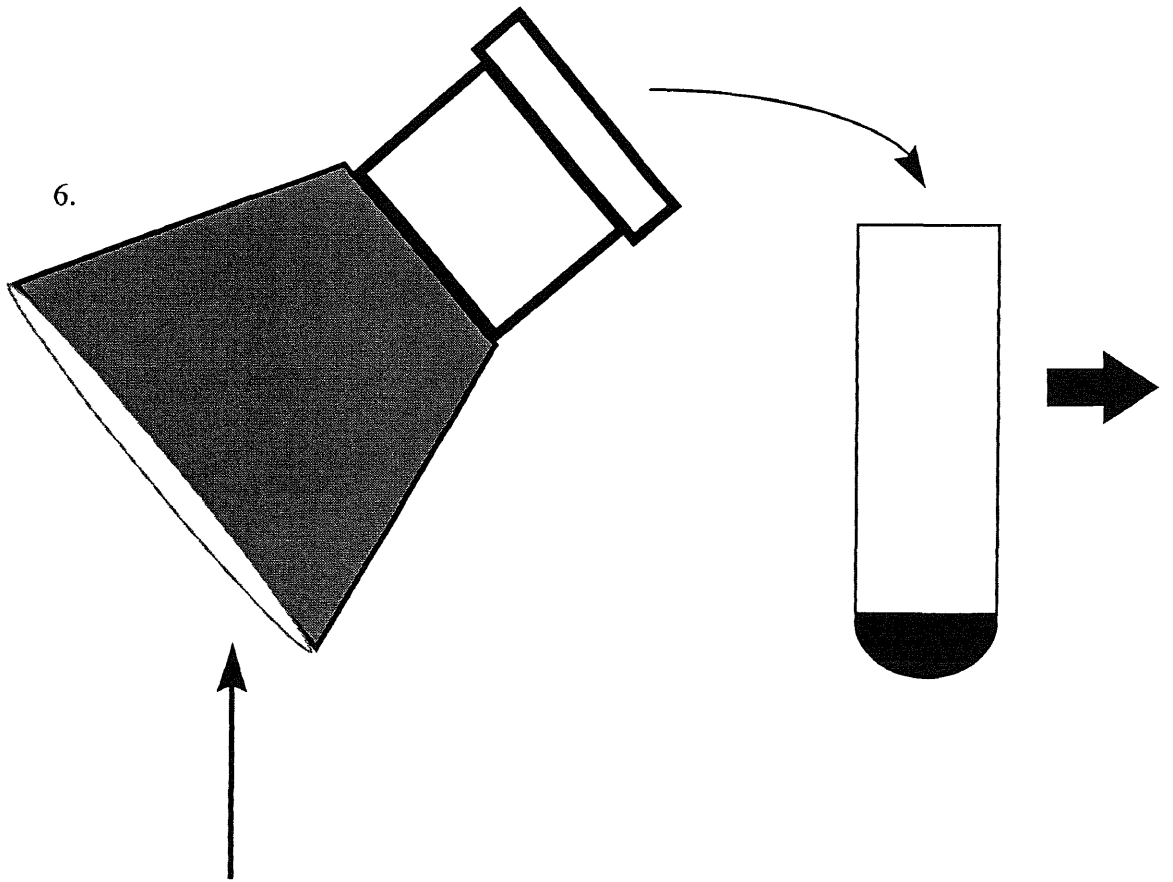
5.



A: Cell pellet left behind

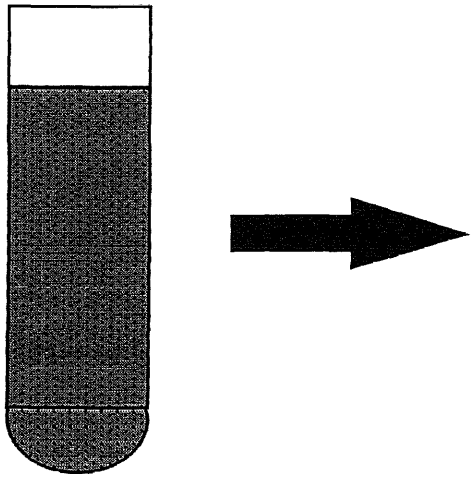
B: Supernatant to be assayed to find the before pulse concentration

6.



Sterile Deionized Water

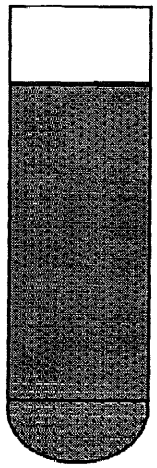
Cell pellet resuspended in sterile water. After adding the water, gently invert tube several times to make it homogeneous.

7. 

Cell suspensions are then pulsed according to the electroporation protocol.

Method B

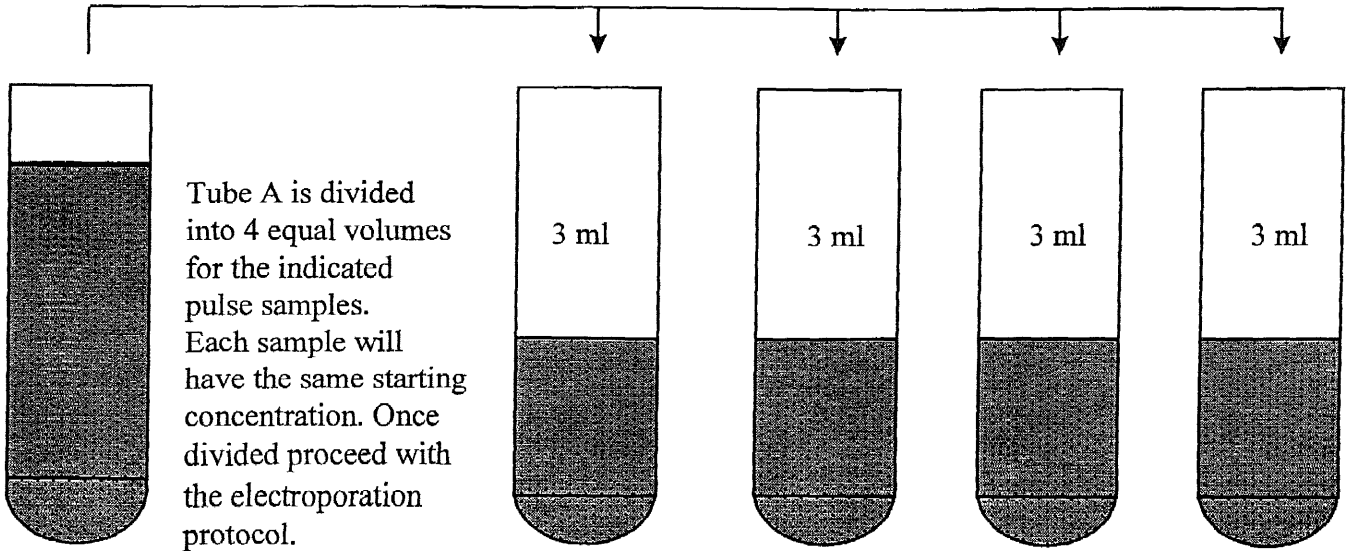
1.



A

Cells suspended in sterile water after washing and recording the optical density

2.

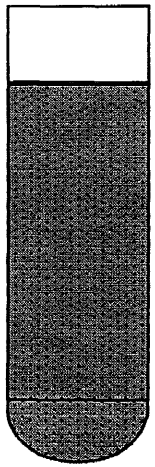


Tube A is divided into 4 equal volumes for the indicated pulse samples. Each sample will have the same starting concentration. Once divided proceed with the electroporation protocol.

A 0 10 20 40

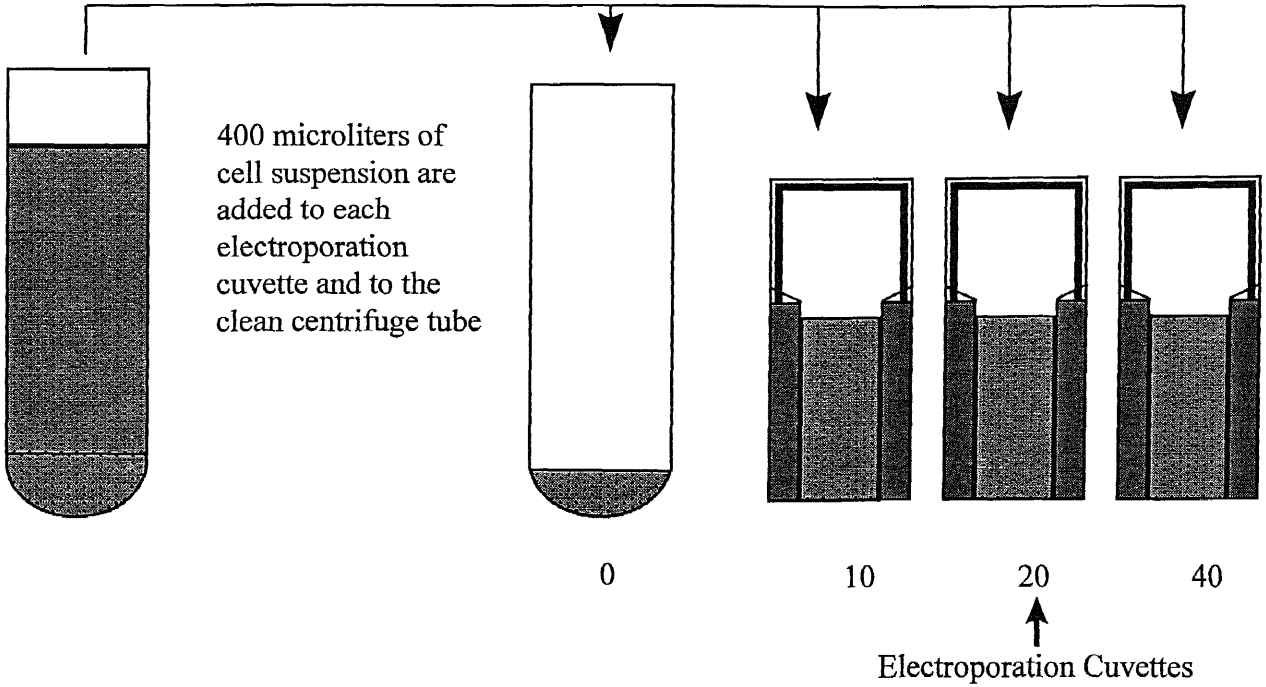
Method C

1.



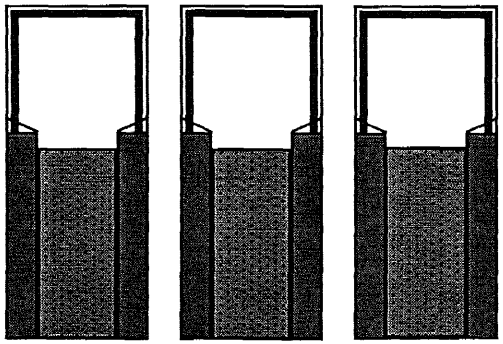
Cells suspended in sterile water after washing and recording the optical density

2.



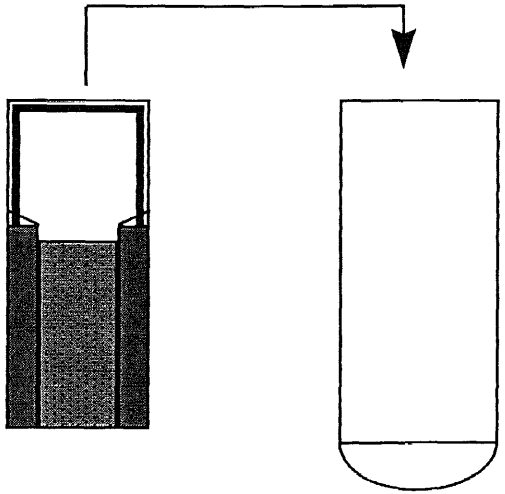
400 microliters of cell suspension are added to each electroporation cuvette and to the clean centrifuge tube

3.



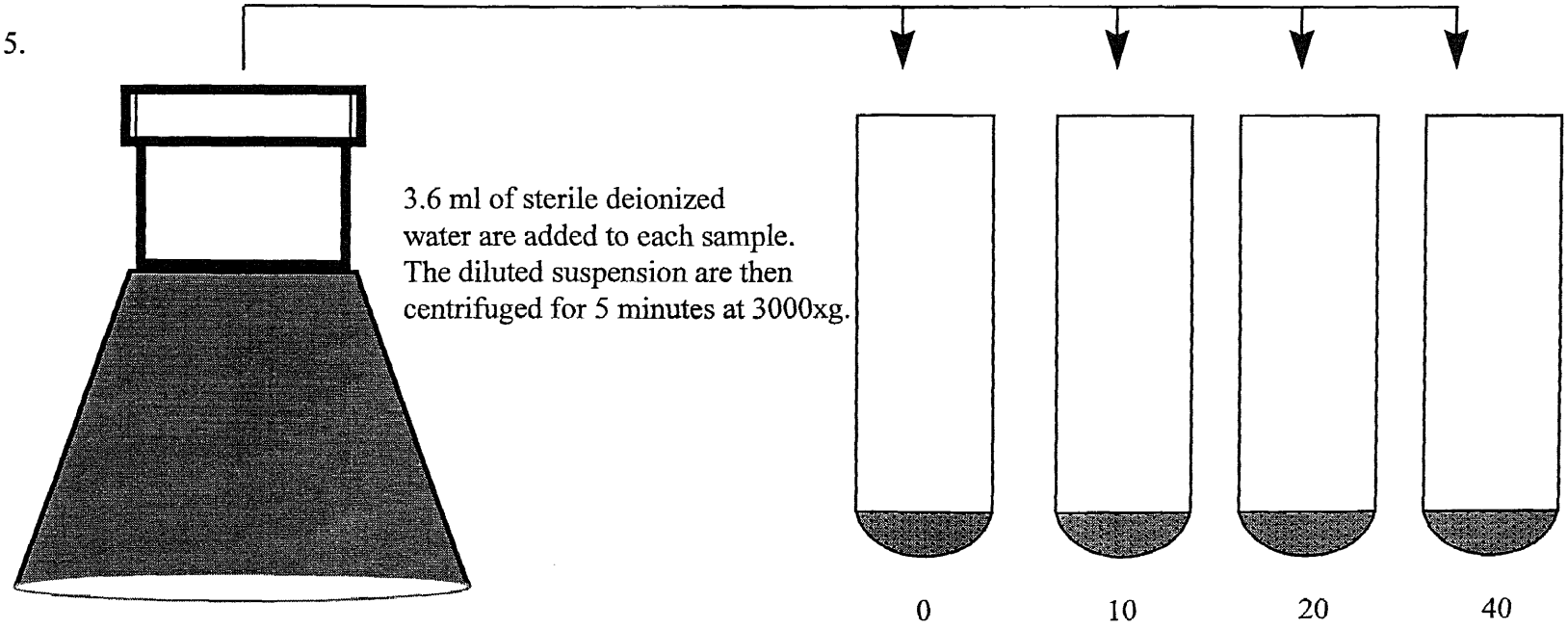
Cell suspension pulsed the indicated amounts

4.



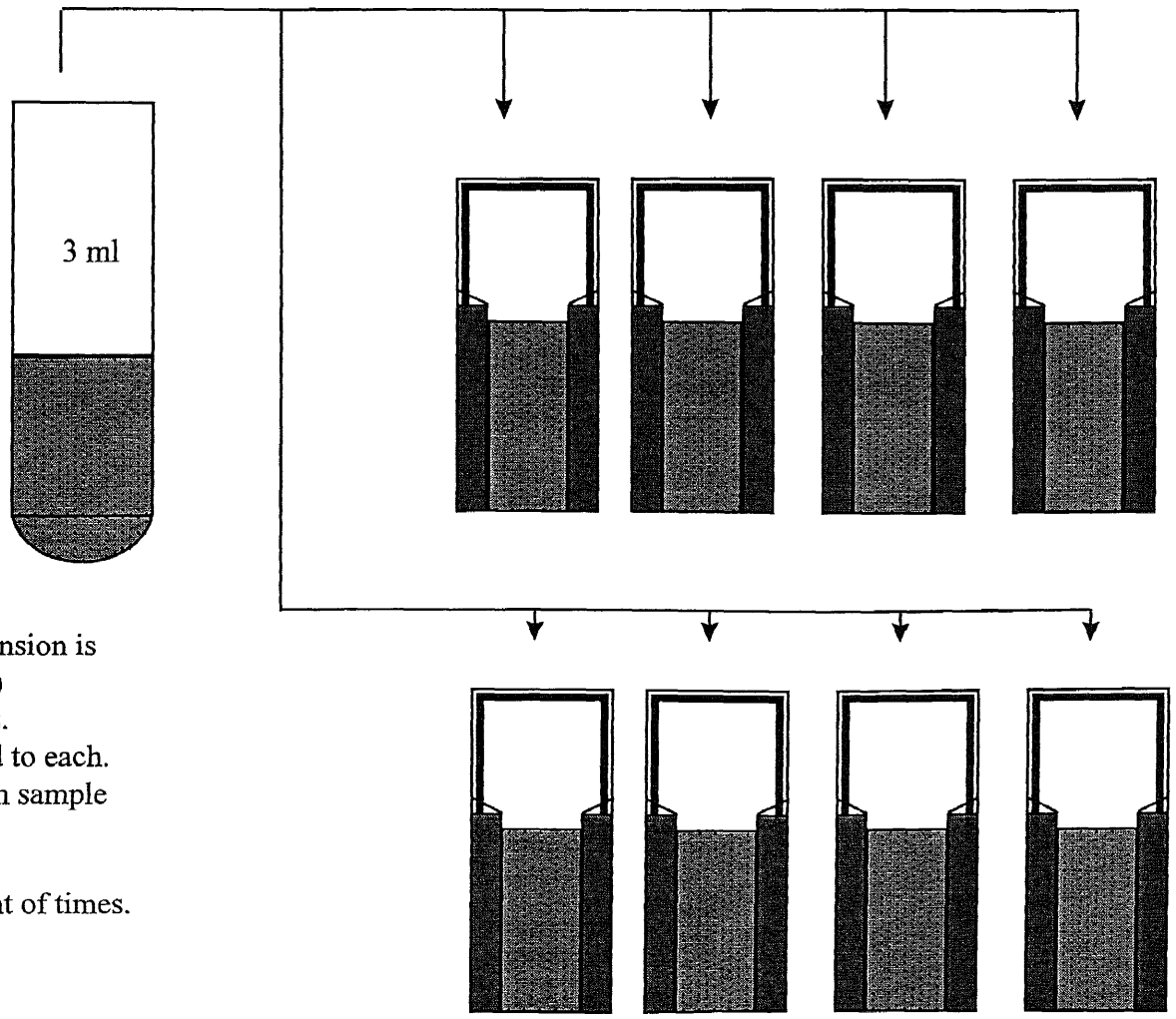
Each pulsed sample is added to a new sterile tube

5.


3.6 ml of sterile deionized
water are added to each sample.
The diluted suspension are then
centrifuged for 5 minutes at 3000xg.
0
10
20
40

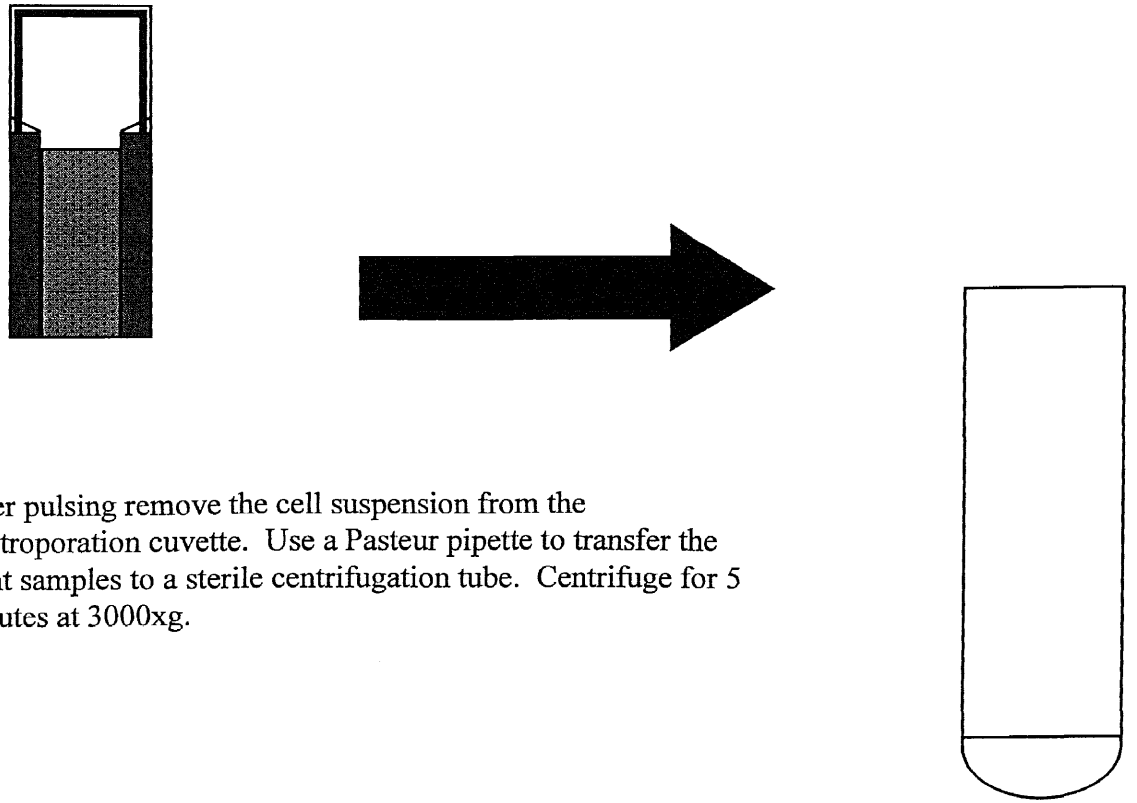
Electroporation Protocol

1.



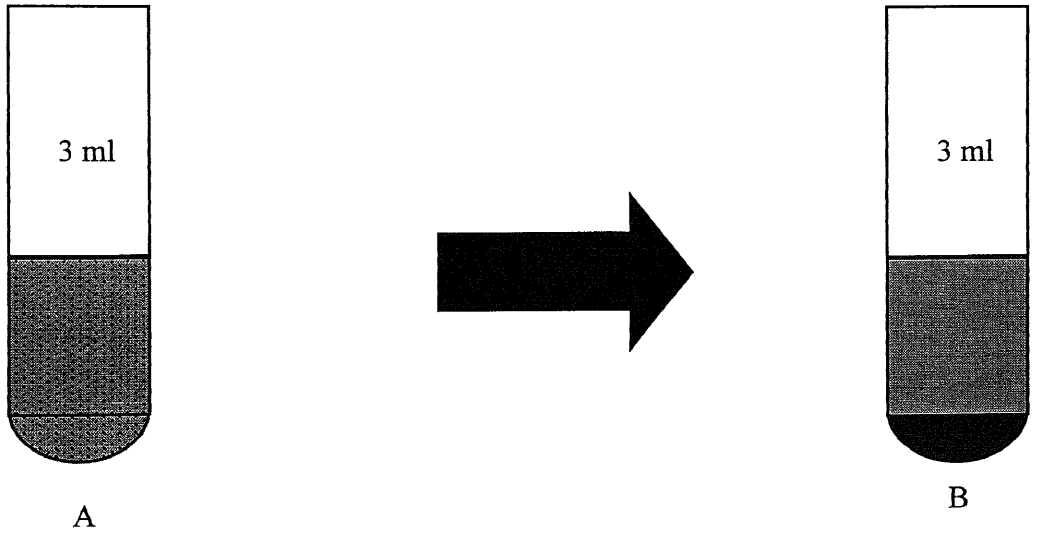
The prepared cell suspension is divided among eight (8) electroporation cuvettes. 400 microliters is added to each. This is repeated for each sample of each pulse set. Each electroporation cuvette is pulsed a given amount of times.

2.



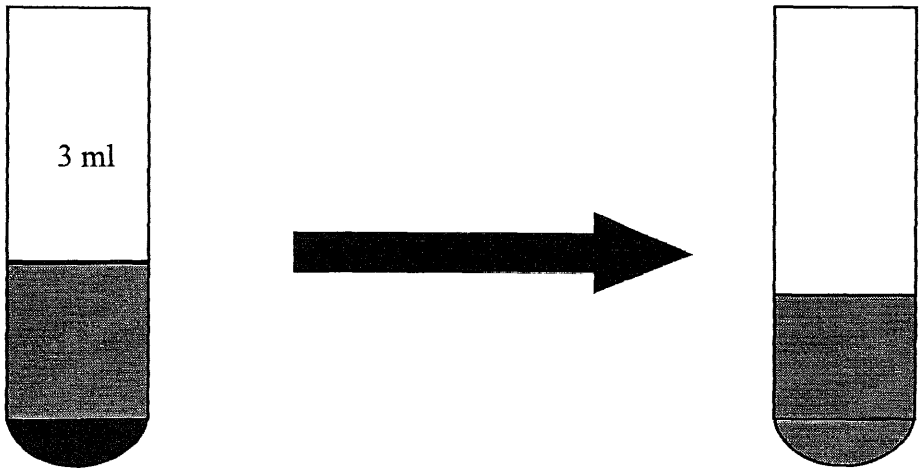
After pulsing remove the cell suspension from the electroporation cuvette. Use a Pasteur pipette to transfer the eight samples to a sterile centrifugation tube. Centrifuge for 5 minutes at 3000xg.

3.



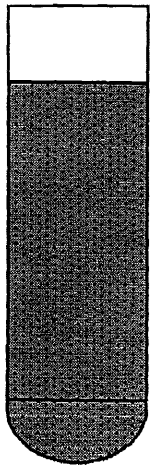
A represents the cell suspension prior to centrifugation. B represents the cell suspension after centrifugation.

4.



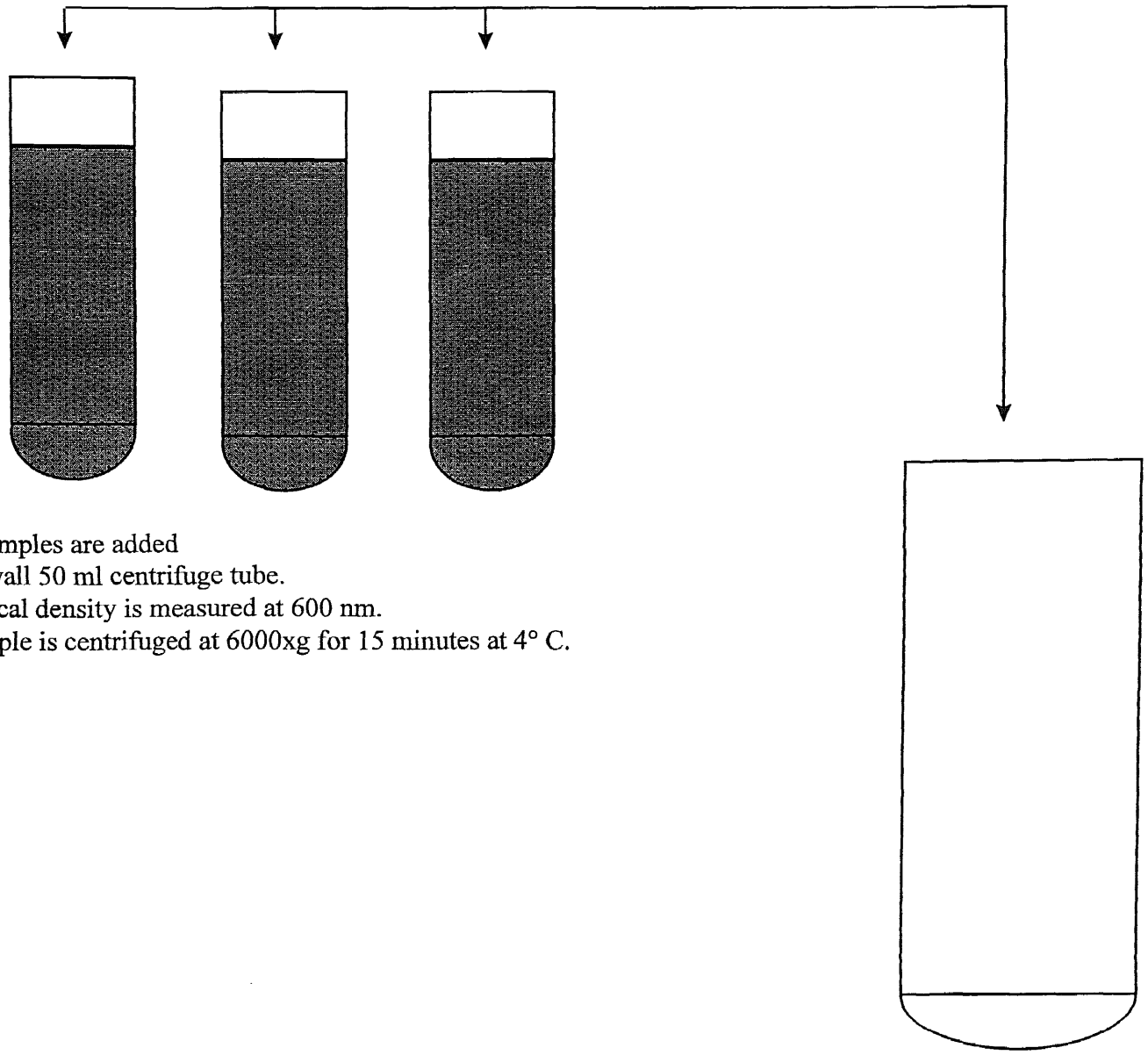
The supernatant is poured off and labeled by the number of pulses it was subjected to. The supernatant is assayed using the UV Absorbance assay measured at 280 nm.

Total Cell Lysis Protocol

1. 

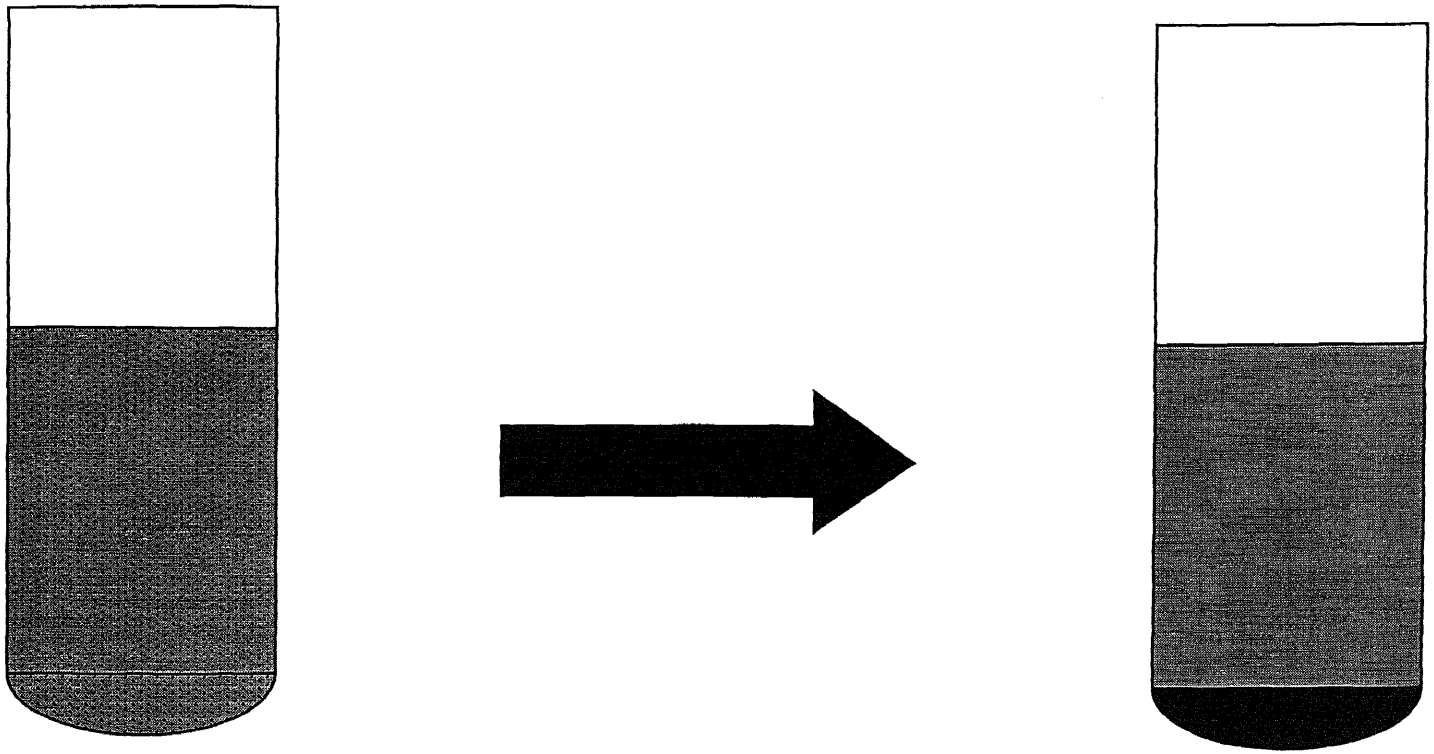
Cell suspension after washing. Volume of suspension is 10 ml. Each total cell lysis sample will consist of three, 10 ml samples.

2.



The 3 samples are added
to a Sorvall 50 ml centrifuge tube.
The optical density is measured at 600 nm.
The sample is centrifuged at 6000xg for 15 minutes at 4° C.

3.



Centrifuge tube with cell pellet concentrated at bottom after centrifuging. The supernatant is discarded. 12 ml of buffers P1, P2, and P3 are added to the pellet accordingly following the protocol. The final mixture is centrifuged at 30,000xg (15,000 rpm) for 30 minutes at 4° C.

REFERENCES

Bauer, E., I. Hones, H.-E. Jacob, and H. Berg. 1989. "Electrolesion of Yeast Cells for Release of Proteins." *Studia Biophysica.* 130: 180-192.

BIO-RAD (a). "E.coli Pulser Transformation Apparatus Operation Instructions and Applications Guide." Richmond, California.

BIO-RAD (b). "Genetic Systems News." Richmond, California.

Bollag, D. M. 1996. *Protein Methods.* New York: Wiley-Liss.

Bradford, M. M. 1976. "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding." *Anal. Biochem.* 72: 248.

Brodelius, P. E., C. Funk, and R. Shillito. 1988. "Permeabilization of Cultivated Plant Cells by Electroporation for Release of Intracellularly Stored Secondary Products." *Plant Cell Reports.* 7: 186-188.

Clarkson, Andrew I., Patrick Lefevre, and Nigel J. Titchener-Hooker. 1993. "A Study of Process Interactions between Cell Disruption and Debris Clarification Stages in the Recovery of Yeast Intracellular Products." *Biotechnology Progress.* 9: 462-467.

Engler, C. R., and Campbell W. Stewart. 1979. "New Method of Measuring Cell Wall Rupture." *Biotechnology and Bioengineering.* 21: 1861-1869.

Engler, C. R. 1985. "Disruption of Microbial Cells." *Comprehensive Biotechnology.* 2: 305-324.

Estruch, Juan Jose, Stephen A. Goff, and Lyle Crossland.. 1994. "Transformation of Yeast Cells by Electroporation in Combination with a Screening Method That Avoids Catabolic Repression." *Biotechniques.* 16: 610-612.

Hall, J. L. and A. L. Moore. 1983. *Isolation of Membranes and Organelles from Plant Cells.* New York: Academic Press.

Hofmann, G. A., and S. B. Dev. 1993. "Electroporation: From Research Laboratories To Clinical Practice." *IEEE.* 1420-1421.

Hughes, D. E., J. W. T. Wimpenny, and D. Lloyd. 1971. "The Disintegration of Micro-organisms." *Methods in Microbiology.* 5B: 2-53.

Ishiguro, J. and W. Kobayashi. 1995. "A Practical Method for Fission Yeast Transformation by Electroporation." *Jon. J. Genet.* 70: 1-6.

Kilbane II, J. J., and B. A. Bielaga. 1991. "Instantaneous Gene Transfer from Donor to Recipient Microorganisms *via* Electroporation." *Biotechniques.* 3: 354-365.

Marcil, Robert, and D. Higgins. 1992. "Direct Transfer of Plasmid DNA from Yeast to E.coli by Electroporation." *Nucleic Acids Research.* 20: 917.

Meilhoc, Eliane, Jean-Michel Masson, and Justin Teissie. 1990. "High Efficiency Transformation of Intact Yeast Cells by Electric Field Pulses." *Biotechnology.* 8: 223-227.

Mosqueira, F. G., J. J. Higgins, P. Dunnill, and M. D. Lilly.. 1981. "Characteristics of Mechanically Disrupted Baker's Yeast in Relation to its Separation in Industrial Centrifuges." *Biotechnology and Bioengineering.* 23: 335-343.

Qiagen Inc. 1993. *Qiagen Plasmid Handbook.* Chatsworth, California.

Rosemberg, Yosef, Michal Rotemberg, and Rafi Korenstein. 1994. "Electroporation of the Photosynthetic Membrane: Stuctural Changes in Protein and Lipid-Protein "Domains." *Biophysical Journal.* 67: 1060-1066.

Shaeiwitz, J. A., J. B. Blair, and R.-C. Ruaan. 1989. "Evidence that Yeast Cell Wall Debris Can Separate Proteins by Ion-Exchange During Cell Lysis." *Biotechnology and Bioengineering.* 34: 137-140.

Siddiqi, S. F., N. J. Tichener-Hooker, and P. Ayazi Shamlou. 1996. "Simulation of Particle Size Distribution Changes Occurring During High-Pressure Disruption of Baker's Yeast." *Biotechnology and Bioengineering.* 50: 145-150.

Sixou, S., N. Eynard, J. M. Escoubas, E. Werner, and J. Teissie. 1991. "Optimized Conditions for Electrotransformation of Bacteria are Related to the Extent of Electropermeabilization." *Biochimica et Biophisica Acta.* 1088: 135-138.

USDA. YM Broth Protocol. Peoria, IL.

The Virtis Company. OPERATING INSTRUCTIONS FOR: Omni-Culture Bench-Top Fermenter. Gardiner, NY.