How is induced expression of LacZ affected by different pHs?

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Abstract:

The expression of the *lacZ* gene was studied to see how different pHs affect its expression. Understanding this will help future *lacZ* studies in their applications in molecular biology, medicine, and biotechnology. *LacZ* cloned into the pET (AMP) vector was transformed by heat shock into Sigma Aldrich's Sig10 chemically competent *E. coli* cells. Then, Isopropyl-β-D-thiogalactoside (IPTG) was used to induce the expression of *lacZ*. The gene was then expressed at the following pH values: 3, 4, 7, 10, and 11 to investigate how pH affects the expression of *lacZ*. Lastly, X-gal, a soluble colorless compound, was hydrolyzed by the beta-galactosidase enzyme to create a blue substrate, and a 10% triton detergent was used to lyse the cells and so that the difference in color could be recorded by a spectrophotometer. Although the goal of this experiment was to produce direct and clear results to indicate a relationship, unfortunately a standard curve wasn't generated before measuring the optical density of the cultures and therefore the exact molar concentrations of blue from the X-gal cannot be calculated with the data from the spectrophotometry. Additionally, the results of the spectrophotometer seem to indicate that the higher absorbances may not be due to the blue precipitate, as control cultures that did not react with the X-gal also had absorbance values. However, given that the optical densities of each well was approximately the same, the trend that is seen in the absorbance values also cannot be the result of higher pHs killing off cells either.

Keywords: lacZ gene, gene expression, IPTG, pH, X-gal, spectrophotometry

Introduction:

Determining the pH values that optimize *lacZ* gene expression is important for future quantitative assay measurements. Enzymes are sensitive to changes in pH, as non-optimal pHs can cause denaturing, so it's important to find an appropriate pH range where the gene is expressed without killing off cells or denaturing the enzyme [1, 2, 3]. As beta-galactosidase is becoming a more popularized method for cancer detection, it has become useful in marking cell senescence and identifying cancer at earlier stages [3, 4, 5]. It then is essential to identify the specific pHs that produce the best results, as *lacZ* gene expression can potentially save or risk someone's life. This investigation of expression at various pHs will therefore not only help future *lacZ* gene studies, but also other assays performed on beta-galactosidase in relation to fields such as molecular biology, medicine, and biotechnology.

After confirming the successful transformation of the *lacZ* gene, an *E. coli* culture was then grown and *lacZ* gene expression was analyzed at different pHs, with analysis being based on X-gal staining. A study by William R Schwan and colleagues investigating osmolarity and pH growth conditions of the *fim* promoter in *E. coli* observed beta-galatosidase units at pH ranges 5 to 8, indicating that activity was best at a neutral pH of 7 [4]. Similarly, a study by James A Coker and colleagues investigating the characteristics of beta-galatosidase at a low temperature and pH measured activity from pH 4.0 to 10.0, and found beta-galactosidase activity detectable from pH 6.0 to 9.5, with a peak at pH 7.0 [6]. Both studies used *E. coli* cells. However, in a study by Xinxin Xu and colleagues identifying the optimization of an active beta-galactosidase in heterologous expression systems, the optimal pH for induction of *lacZ* expression as measured through beta-galactosidase formation in *E. coli* was found to be in the 5.5 - 6.5 pH range after testing pHs 3 to 11 [7]. The goal of the study was to investigate *lacZ* gene expression at various pHs as an attempt to reproduce these older studies.

Methods

Research Design and Participants

The overall research design for this study is an experiment, with a sample size of 20 wells of bacteria.

Horizontal Gene Transfer and Bacterial Transformation

One of the first steps required to investigate the effect of pH in the expression of *lacZ* is bacterial transformation of the vector containing *lacZ*, to generate a strain where *lacZ* gene expression can be induced. Bacterial transformation is a type of horizontal gene transfer,a method that microbes use to integrate genetic material from other organisms [8, 9]. This method has allowed bacteria to survive by constantly changing microbial genomes and undergoing gene evolution that makes them better adapted to the environment. However, this integration does not occur easily, as the bacterial genome is located in cytoplasm, meaning DNA can't passively diffuse through the cell wall into the genome [8, 9]. Bacterial transformation, which refers to the third method of horizontal gene transfer, is a technique in molecular cloning to produce multiple copies of a recombinant DNA molecule [8, 9]. The process introduces foreign DNA into bacterial

cells, allowing the acquisition of new genetic traits. Recombinant plasmids, the products of bacterial transformation, are constructed by inserting the desired DNA sequence into a vector. In transformation, DNA (usually as a plasmid), is introduced into a bacterial cell so the bacteria can make more copies of the desired sequence for further analysis and or manipulation [8, 9].

Competent Cells and Heat Shock

Sig10 chemically competent *E. coli* cells were used for transformation. After thawing, they are then mixed with plasmid DNA on ice for up to 30 minutes. Then heat shock is performed– the process of suddenly increasing and decreasing the temperature around cells to allow the cell membrane to become more permeable– to help the uptaking of exogenous DNA [10]. Thermo Fisher's protocol suggests that the cells are repeatedly submerged into a heated water bath before being returned to ice.

Following heat shock, the transformed cells enter a recovery period to express the antibiotic resistance genes from the plasmid. Pre-warmed SOC media, a bacterial growth broth, is also added, as it helps improve the transformation efficiency of the cells. After growing in the liquid SOC media, the cells are then plated on solid media with antibiotics so that when they are examined the next day after incubation, successfully transformed colonies can be identified. Once identified, the colonies are screened– using restriction enzymes and gel electrophoresis– for the presence of the desired DNA and confirm the sequence [10].

Restriction Enzymes and Gel Electrophoresis

Restriction enzymes are proteins that cleave DNA at specific sites along a molecule. They are commonly used to manipulate fragments of DNA as a tool for recombinant DNA technology [11, 12, 13]. Restriction enzymes recognize short, specific sequences of nucleotide bases. When it recognizes a sequence, it cuts through the DNA molecule by catalyzing the hydrolysis of the bond between adjacent nucleotides. In molecular cloning, restriction enzymes are used to insert DNA into the vector by digesting– using an endonuclease to cut at a specific base in the middle or end of a sequence– both the insert and vector DNA molecules with the same restriction enzymes. Then, an enzyme called DNA ligase or recombinase ligates the fragment into the plasmid backbone by catalyzing the phosphodiester linkages between adjacent nucleotides of the plasmid and the fragment of interest. For a specific cloning reaction, restriction enzymes are appropriately chosen to produce compatible "sticky ends" between the RE-digested vector and the insert, so that a re-circularized plasmid with the fragment of interest can be created [11, 12, 13].

To verify whether cloning was successful, restriction enzymes are used again to cleave the transformed DNA at different sites into various fragments. These fragments are then analyzed through gel electrophoresis, a process that depends on the negative charge of DNA. DNA is first loaded into wells within an agarose gel, and a current is run through the gel. The negatively charged DNA fragments will then migrate towards the positively charged anode away from the wells, separating it by size based on the distance it travels along the gel, with farther distances corresponding to shorter fragments. To analyze the lengths of the fragments, a control ladder– a set of fragments with known size– will be run with the experimental DNA to estimate their size.

After separating, the DNA is then stained with a dye and visualized under UV light to confirm the presence and size of the inserted DNA [11, 12, 13].

X-gal and Spectrophotometry

In this particular project, the gene of interest is the *lacZ* gene. The *lacZ* gene of *E. coli* produces the enzyme beta-galactosidase, also referred to as beta-gal. It is an enzyme of the lactose operon that requires 21 amino acids essential to its catalytic activity. This gene is significant in molecular biology due to its role in Jacob and Monod's development of the inducible operon model [14, 15, 16]. *lacZ* is also significant in recombinant DNA through blue-white screening, one of the most commonly used methods to visually select *E. coli* with the desired recombinant plasmid [14, 15, 16]. Other uses of *lacZ* include in the food, medical, and biotechnological industries, such as with Hamed and colleagues' research on the activation of the *lacZ* gene in the DH5a strain can be a promising candidate for the dairy industry and Li and colleagues' research using the *lacZ* gene and beta-galactosidase enzyme as a biomarker to aid primary ovarian cancer diagnosis and treatment [14, 15]. The produced enzyme, beta-galactosidase, is best known for its reaction with X-gal (5-bromo-4-chloro-3-indoyl-β-d-galactopyranoside). X-gal is a soluble colorless compound with galactosidase linked to a substituted indole. The beta-galatosidase enzyme is highly specific for the galactose part of the substrate, but not specific for the remainder. It hydrolyzes X-gal, releasing the substituted indole in the reaction which spontaneously dimerizes to produce an insoluble blue product. This reaction causes colonies of *E. coli* with the *lacZ* gene growing on a medium with X-gal to become blue [14, 15, 16].

However, X-gal stains in vitro, and the method chosen for analysis of the produced color blue was spectrophotometry, which measures how much a substance absorbs light by measuring the intensity of light that passes through a sample. Using spectrophotometry in combination with Beer-Lambert's Law, the molar concentration of the substance can then be calculated with the absorbance values gathered in the data [16]. A standard curve of known molar concentrations of blue must be set up first, so that the molar absorption coefficient can be determined. Then, the unknown concentration is analyzed and plotted against the gradient, so that its molar concentration can be calculated [17]. However, in vitro staining cannot be detected by spectrophotometers, so a 10% Triton X-100 was used to lyse the cells. Triton X-100 is a non-ionic, non-denaturing surface-active chemical agent that lowers the surface tension of liquids. It is used in molecular biology, biochemistry, and cell biology. Triton X-100 is also important in cell lysis, by breaking down lipid layers without denaturing proteins. This allows the in vitro staining to enter the solution and be correctly measured through spectrophotometry [18].

Transformation of the plasmid

Sigma Aldrich's Sig10 Chemically Competent Cells were taken out of -80 C and thawed on ice (approximately 20-30 min). Two agar plates were removed from storage at 4 C and warmed up to room temperature and then incubated in 37 C incubator. One agar plate was labeled as *lacZ* gene while the other as the control gene. 60 microliters of *lacZ* DNA was then reconstituted at a ratio of 50 ng per microliter and then diluted at a 1:50 ratio. A control DNA of the same amount was also prepared to ensure that the cells are growing properly. 1-5 ul of both DNA was then

mixed into 20-50 ul of competent cells in a microcentrifuge or Falcon tube. The competent cell/DNA mixture was then incubated on ice for 30 mins. Heat shock was then administered on each transformation tube by placing the bottom ½ of the tube into a 42 C water bath for 45 seconds. The tubes were then back on ice for 2 min. 1,000 ul LB liquid media without antibiotic was then added to the bacteria and grown in 37 C shaking incubator for 1 hour. All of the transformations were then plated onto two 10 cm LB agar plate with ampicillin, one for the *lacZ* gene and one with the control gene. These plates were then also incubated at 37 C overnight. The next day, one large colony was picked from the *lacZ* plate and inoculated to make 5mL of culture. This culture was then grown at 37 C overnight with shaking.

DNA Extraction

The next day, 1.5ul of DNA was extracted using Monarch's Plasmid Miniprep Kit and Protocol [19]. First, the culture was spun down through centrifugation and the supernatant was discarded. Then, the pellet was resuspended with Monarch's Plasmid Resuspension Buffer and pipetted to ensure a completed resuspension. The cells were then lysed with Monarch's Plasmid Lysis Buffer and the tube was inverted until the solution became dark pink and clear. After a one minute incubation, Monarch's Plasmid Neutralization Buffer was added, and the tube was inverted again until uniformly yellow and a precipitate formed. Following a 2 minute incubation, the solution was centrifuged once again for 2 minutes before the supernatant was transferred to the spin column.

The column was centrifuged for another minute, flow-through was discarded, and washed twice with Monarch's Plasmid Wash Buffer 1. Finally, the solution was transferred into a clean microfuge tube with Monarch's DNA Elutin Buffer. After waiting for a minute, the new solution was spun for a minute to elute the DNA. Following this standard protocol, a nanodrop was used to confirm that the miniprep was done well, resulting in high DNA concentrations [19].

Diagnostic Restriction Digest

Restriction digestion was then performed on the 2uL of the mini-prepped DNA, which was inserted into 2 tubes for digestion with restriction enzymes, one with just XbaI and one with both XbaI and SspI. The XbaI digest would result in two fragments, whereas the XbaI and SspI digest would result in three fragments.

Each reaction mixture included deionized water, restriction enzyme 10X buffer, acetylated BSA, and the desired DNA in a sterile tube. After mixing with a pipette, the restriction enzymes XbaI and SspI were added. The solution was mixed again by pipetting, before being centrifuged for a few seconds in a microfuge.

The samples were then incubated for an hour to complete digestion. After this incubation period, a 6X loading buffer was added to each reaction to prepare the DNA solution for gel analysis.

Gel Electrophoresis

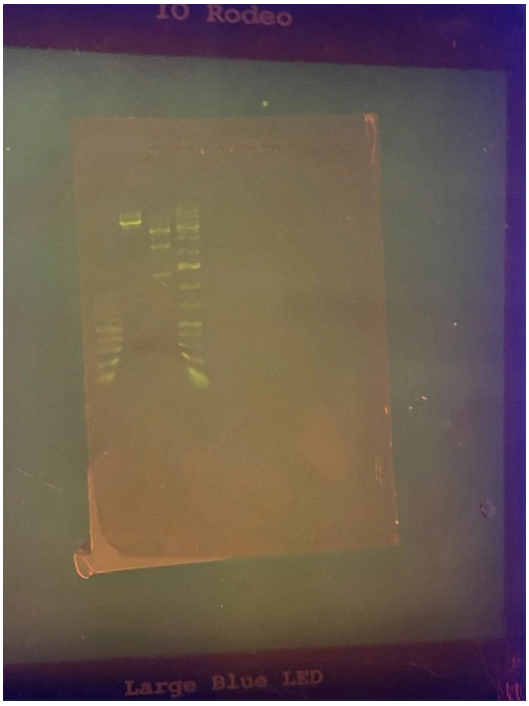

Figure 1. *The agarose gel electrophoresis analysis of the cloned construct. The rightmost well corresponds to the 1kb DNA ladder fragment, the second well was run with only the XbaI digest, and the third well was run with both the XbaI and SspI digest. The first well was a test well to practice injecting the DNA into the agarose wells.*

After digesting successfully, an agarose gel was run with a 1kb DNA ladder fragment to separate out the digested fragments and confirm the 8,000 base pairs of the cloned construct [20]. After verifying the cloned construct, the remaining 0.5mL of culture left was used to make a glycerol stock. A 50% glycerol solution was prepared by diluting an 100% glycerol solution with deionized water. Then, 0.5ml of 50% glycerol solution was added and mixed through inversion. Finally, the stock was stored in a -80 C freezer for long term storage.

pH treatment

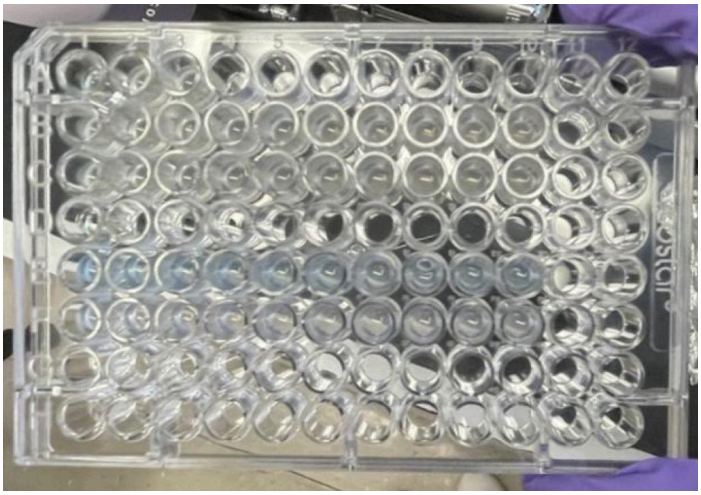

Figure 2. *The well plate prior to insertion and measurement of the absorbance values. Row E corresponds to all cultures with IPTG, and Row F corresponds to all cultures without IPTG. The blue precipitate is the result of the beta-galactosidase and Xgal reaction. Columns 1 & 2 correspond with pH 3, columns 3 & 4 correspond with pH 4, columns 5 & 6 correspond with pH 7, columns 7 & 8 correspond with pH 10, and columns 9 & 10 correspond with pH 11.*

From the glycerol stock, bacteria was streaked out onto agar plates. A large colony was picked, and 5ml cultures were inoculated and grown overnight at 37C. The next day, 2 larger 50 mL cultures were inoculated and grown into larger cultures at 37C. After inoculation, the optical density of the cultures were measured, ensuring they reached 0.6. Once the optical density (OD) was 0.6, IPTG and Xgal were added to confirm that gene expression was working based on blue color appearing. Once gene expression was confirmed with b-galactosidase expression turning Xgal into a blue precipitate, the treatment portion began. To prepare the acids and bases, 6M HCl was diluted into 1M HCl, which was then diluted again 10,000 times to get pH 4. 3 ul of HCL was additionally added to make pH 3, meaning it was diluted 1,000 times. 2M NaOH was also diluted into 1M NaOH, which was then diluted again 10,000 times to get pH 10. 3 ul of NaOH was also additionally added to make pH 11, meaning it was diluted 1,000 times.

IPTG was then added to one of the 50mL cultures, while the other was kept as a control without IPTG. Both cultures were then grown in a shaking incubator for 4 hours. The optical densities of the cultures were measured by pipetting 100 ul into wells to ensure that the pH is not killing off the cells. Based on optical density, tubes of 15mL of culture both with IPTG and without IPTG were made to ensure there was an equal amount of cells in both tubes. IPTG concentration was maintained during this process. 3mL of culture were then distributed into each tube at pHs 3, 4, 7, 10, and 11, including media and pH dilution, to ensure that each tube received an equal amount of cells. The tubes with the new pHs were incubated for 4 hours in a shaking incubator. Next, the optimal density of each culture was checked by pipetting 100 microliters of each culture into 2 wells. After ensuring that the densities were similar and that the pH wasn't killing off the bacteria, 200 microliters of each culture was pipetted into tubes and spun down. Then, the SOC media with the pH treatments were removed and 200 microliter of Xgal and Xgal buffer was added in its stead. The cultures were then incubated at 37 C for 40 min. Afterwards, the

cells were lysed with 10% triton detergent and shaken for 10 min. Finally, the cultures were inserted into an assay reader to measure the absorbance values.

Results

Results

Actual Temperature: 22.9

	1	2	3	4	5	6	7	8	9	10	11	12	
A													bacOD:600
B	0.384	0.375	0.444	0.344	0.396	0.375	0.351	0.338	0.809	0.323			bacOD:600
C	0.333	0.348	0.32	0.326	0.436	0.337	0.298	0.298	0.316	0.305			bacOD:600
D	0.045												bacOD:600
E													bacOD:600
F													bacOD:600
G													bacOD:600
H													bacOD:600

Table 1. *Measure of the optical densities of the cultures where row B corresponds to with IPTG and row C corresponds to without IPTG, with columns 1 & 2 representing pH 3, 3 & 4 representing pH 4, 5 & 6 representing pH 7, 7 & 8 representing pH 10, and 9 & 10 representing pH 11. D1 is a control with SOC media.*

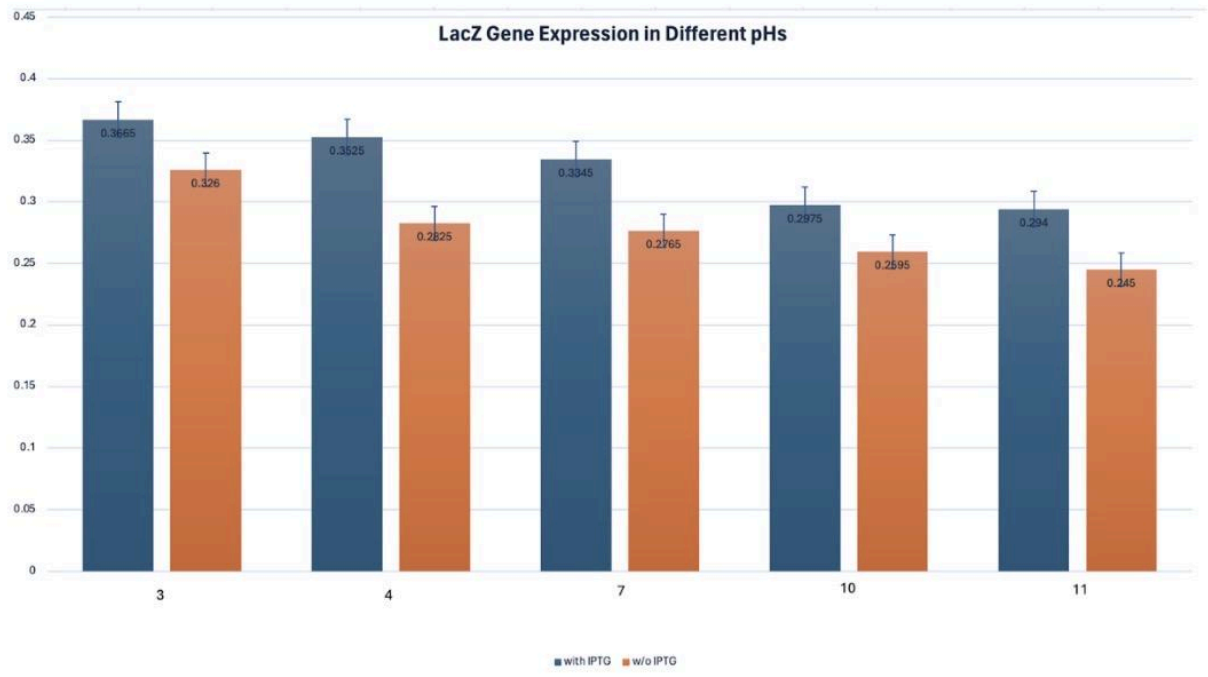

Figure 3. The graph depicts the mean absorbance values, where the blue bars represent with IPTG and the orange bars represent without IPTG. The standard error bars are included as well.

As depicted in Table 1, it can be seen that despite the various pHs in which the cultures were grown, they all had similar optical densities, with the exception of well B9, which is an outlier as a result of possible bacteria clumps. To find the accurate densities of the cultures, the optical

density of the SOC media must be subtracted from the values given by the OD 600, as the wells contained a combination of SOC media and culture. Taking the SOC media into account, this results in the rest of the wells having an average optical density of 0.304, with a minimum of 0.253 and a maximum of 0.399. The standard deviation was 0.041. The median optical density is 0.293, meaning that the distribution of the optical densities is slightly skewed to the left as the mean is greater than the median.

On the other hand, Figure 3 shows the mean and standard error of the mean bars of each of the cultures at pHs 3, 4, 7, 10, and 11, for both the with IPTG and without IPTG groups, where the with IPTG cultures express beta-galactosidase and the without IPTG cultures do not express beta-galactosidase. The expressing beta-galactosidase group is represented by the blue bars, whereas the non-expressing groups are represented with the orange bars. The x-axis shows the various pH values, and the y-axis shows the corresponding absorbance values. It can be seen that at pH 3, the expressing beta-galactosidase cultures have a mean absorbance value of 0.3665, whereas the non-expressing cultures have a mean absorbance value of 0.326. The calculated standard error of the mean bars at pH 3 also do not overlap. At pH 4, the expressing beta-galactosidase cultures have a mean absorbance value of 0.3525, whereas the nonexpressing cultures have a mean absorbance value of 0.2825. The calculated standard error of the mean bars at pH 4 also do not overlap. At pH 7, the expressing beta-galactosidase cultures have a mean absorbance value of 0.3345, whereas the non-expressing cultures have a mean absorbance value of 0.2765. The calculated standard error of the mean bars at pH 7 also do not overlap. At pH 10, the expressing beta-galactosidase cultures have a mean absorbance value of 0.2975, whereas the non-expressing cultures have a mean absorbance value of 0.2595. The calculated standard error of the mean bars at pH 10 also do not overlap. And lastly, at pH 11, the expressing beta-galactosidase cultures have a mean absorbance value of 0.294, whereas the non-expressing cultures have a mean absorbance value of 0.245. The calculated standard error of the mean bars at pH 11 also do not overlap.

Discussion

Figure 3 exhibits obvious differences in the means of the absorbance values, with standard error bars that do not overlap between the with IPTG and without IPTG groups. However, the goal of this experiment was to produce direct and clear results to indicate a relationship, and unfortunately a standard curve was not used for the spectrophotometry and therefore the exact molar concentrations X-gal could not be calculated. Additionally, the results of the spectrophotometer seem to not reveal a direct relationship between absorbance and the intensity of the color blue, as the wells in which beta-galactosidase was not being expressed, and did not produce a blue precipitate, also resulted in absorbance values. So, the direct relationship between pH and *lacZ* gene expression cannot be concluded from this data. It is then possible that the absorbance values in the non-expressing betagalactosidase cultures may be a measure of the amount of cells instead. However, given Table 1 and an analysis of the pHs, it is revealed that the optical densities of each well was approximately the same. So, the trend that is seen in the absorbance values cannot be the result of higher pHs killing off cells either.

In a future iteration of this experiment, known dilutions of the blue precipitate resulting from the X-gal being cleaved by beta galactosidase will be used to form a standard curve. Using this standard curve, the absorbance values of the stained cells at the varying pHs can then be compared and plotted along the curve, which, using Beer-Lambert's law, will lead to the determination of the molar concentrations of blue. Only then, by comparing the different concentrations of blue, can the differences in *lacZ* gene expression at the various pHs be quantified and compared.

Despite this scientific endeavor not yielding clear results, this experiment upholds the fact that science is not straightforward– it is complex, and oftentimes requires multiple trials to acquire the correct results. It is not something that can be investigated in a lab once, but instead requires rounds of troubleshooting and applying more controls to isolate and determine a cause and effect.

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