Bachelor Degree Project

UNIVERSITY OF SKÖVDE

GROWTH CONDITIONS AND EXPERIMENTAL SETUP FOR BACTERIAL GROWTH AND THE FADING OF PHENOLPHTHALEIN IN ALKALINE SOLUTION

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Abstract

The whole project consisted of two different parts. The first subproject were running optical density measurements to assist in finding optimal growth conditions and experimental setup for use of *E.coli* BL21 (DE3) bacteria with the PYCARD gene transformed into them. The transformed bacteria will be used for generating data for modeling. Previous laboratory attempts had problems with timing of the exponential phase when several tests were performed simultaneously. The optimizations of a method for displaying growth include trying out using different medium but also different inoculation ratios between culture and medium to see the effects on growth rate. Results have shown that lysogeny broth together with an inoculation ratio of 1:25 results in rapid absorbance increase in the bacterial growth curves.

The second part of the thesis project was to study the kinetics of fading of phenolphthalein in sodium hydroxide solution in order to provide a basis for experiments illustrating this in laboratory environment. Phenolphthalein is used in many different applications such as an active ingredient in some laxatives but the perhaps most common use is as an acid-base indicator of pH. The experiments were performed by mixing alkaline solutions of varying sodium hydroxide concentration together with sodium chloride, then after addition of phenolphthalein to study absorbance variations over time to illustrate the rate law which correlates the rate of color fade to sodium hydroxide concentration. The results have proven that they correspond directly so that increasing the concentration of sodium hydroxide raised the color fading rate just as much in percent.

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List of Abbreviations

OD	Optical density
LB	Lysogeny broth
TB	Terrific broth
NaOH	Sodium hydroxide
NaCl	Sodium chloride
Ln	Natural logarithm
OH^-	Hydroxide ion

1. Introduction

This report includes two different projects. The overall aim with these two sub-projects was to develop relatively simple laborations that can be used to generate data for systems biology modeling. The first project was to study and develop methods for using transformed OneShot *E.coli* BL21 (DE3) bacterial cells with the PYCARD gene inserted into them to set up and create curves for display of the different phases of bacterial growth. The second project of the thesis work was about the kinetics of the color-fading of phenolphthalein when in alkaline solution, which was to be used as an illustration of the rate law which explains how reaction rate is affected by the concentration of reactants together with a constant called rate coefficient (Blauch, 2009).

1.1 Growth Conditions

Bacterial growth rate depends on several factors including medium, temperature, pH and so on. A generation time under optimal conditions is specified for each type of bacteria which mean the shortest time it takes for the bacteria to duplicate. Growth can be studied by measuring optical density (OD) which enables a way of counting the amount of bacteria in a sample. The method works by sending light through at a specific wavelength, in this case 600 nm (OD_{600}). The optical density obtained is a number based on the amount of diffracted light. The number is directly correlated to the amount of bacteria (Nassif, 2004).

Bacteria are usually grown in batch samples, which mean that they are in a controlled environment. Growth is started by a lag phase where growth progress is very slow or zero as seen in the following Figure 1. A new milieu in a new medium after incubation requires some time for the bacteria to react and adapt before a new phase can start. The bacteria is not completely inactive in the lag phase, they grow in size and develop primary metabolites. This can be production of proteins, enzymes and RNA but also coenzymes and division factors required for making new cells. The adaption is also important for the bacteria to be able to metabolize the new growth medium.

The second phase is known as log phase, seen as the inclining slope of the graph following the lag phase in Figure 1. It is symbolized by logarithmic or exponential growth of the number of bacteria but also maturing of the cells. It begins with accelerating growth phase, where the speed increases exponentially. Log phase starts with accelerating growth phase which greatly improves cell division from the previous lag phase. The end of the log phase is called decelerating phase where the slope on the growth curve flattens out and connects to the following stationary growth phase, see Figure 1. Dividing occurs on constant basis through log phase with speed depending on cell-type, medium and incubation conditions. The growth rate in the log phase is described as generation- or doubling time which is the time taken for each cell

cycle. An optimal generation time is only possible in an environment with lots of nutrients and sufficient biological space. Generation time for *E.coli* can vary from 15-20 minutes in an ideal laboratory environment to 12 hours in the human colon (Seckbach, 2006).

The third phase is called stationary phase where the amount of bacteria is constant since bacterial divisions and deaths are equal. The phase is working this way because the medium is insufficient in nutrients to provide further growth (Monod, 1949). There can also be limitations of space or inhibitory metabolites/end products are preventing further growth. The bacteria produce secondary metabolites to protect it from various hazards such as dehydration, vermin or oxidization. Antibiotics are secondary metabolites that are used by humans to get rid of bacterial infections and they are both used clinically and in labs.

The final death phase occurs when more bacteria are dying than dividing. The total amounts of bacteria are decreasing because they are lacking survival conditions, most often nutrients or toxic products are accumulating (Todar, 2011).

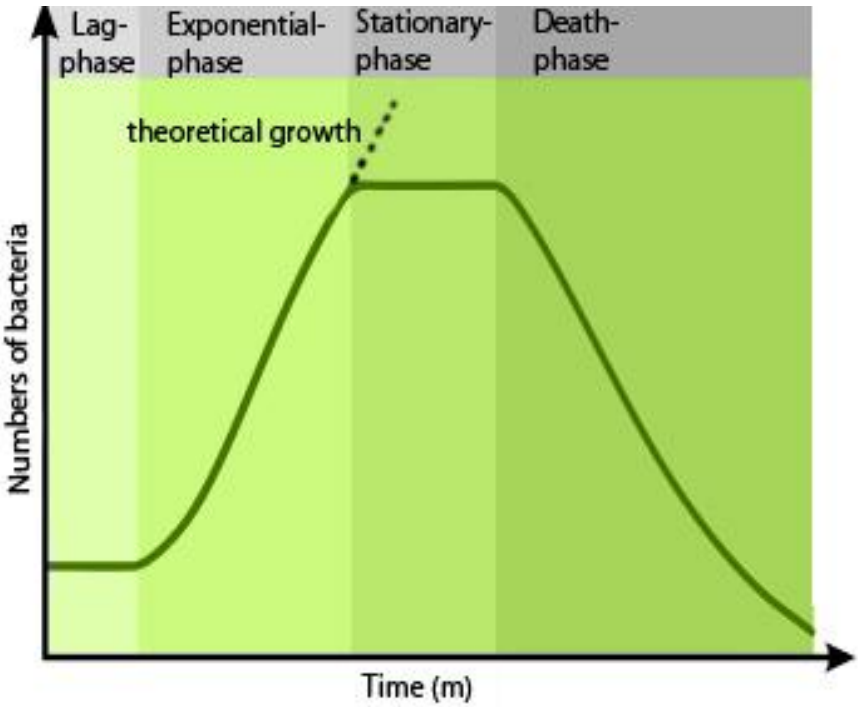

Figure 1. Displayed in order in the curve are all mentioned phases of growth which characterizes growth of any bacterial culture in a sample. Theoretical growth is explained as if more medium or biological space had been available allowing further growth expansion in exponential phase.

A half-day laboration creates a need for a method that enables a fast bacterial growth to reach stationary phase quickly and being able to illustrate it on a short time. The aim of this part was therefore to optimize growth conditions for this laboration. OneShot *E.coli* BL21 (DE3) bacteria containing the PYCARD-gene were grown based on different parameters such as growth medium and

starting concentration. A set up of a laboration to be used by students to illustrate bacterial growth is proposed. No induction of the PYCARD-gene to remove the normal repressor was used in any of the samples.

1.2 Fading of Phenolphthalein

A rate law is a mathematical equation that describes the progression of a reaction. The equation defines how the concentration of participating reactants affects reaction speed. The present reaction is observed by studying the fading colors of alkaline sodium hydroxide solution after addition of the indicator phenolphthalein. Phenolphthalein is a common acid-base indicator providing color indication of different pH. The color varies from orange in strong acidic solution, colorless in acidic or near neutral solution to red-pink in basic and then fade into colorless again after time at even higher pH. The given color will fade back to colorless after some time independent of pH. When phenolphthalein is below pH 7 it is colorless due to the form of structure **1**or H_2P as seen in the figure 2 below. When in higher pH the two phenolic protons are removed, opening up the lactone ring that gives the characteristic red-pink color. In this solution, between pH 8 and 12, structure is in quinoid form named **2** or P^{2-}. When pH gets even higher than 12 the red-pink color slowly fade to colorless when benzenoid structure **3** or POH^{3-} occur. The different conformations of phenolphthalein are displayed in the figure below. All color changes are reversible but the rate of reaction is different; H_2P to P^{2-} is extremely rapid but the conversion from red-colored P^{2-} to colorless POH^{3-} take more time allowing for it to be measured by for example absorbance readings (Nicholson, 1989).

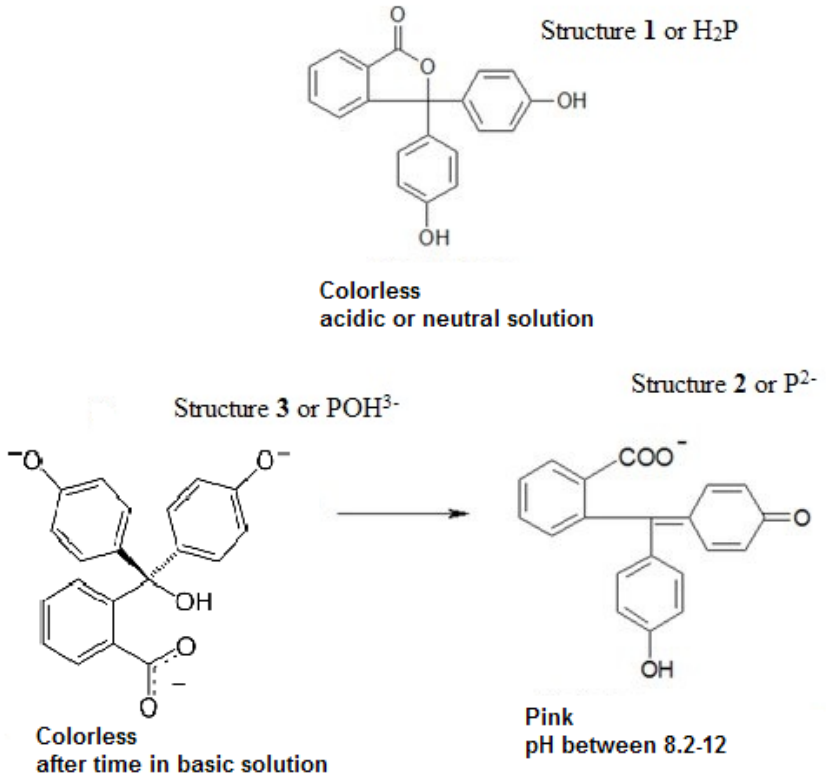

Figure 2. Showing the different structural forms of phenolphthalein which changes color from colorless to pink when passing from a pH below 8 up to 10. The change from colorless to pink is very fast but then color slowly reverses back to colorless again at higher pH. This is because phenolphthalein is entering another colorless structural form which formation rate can be studied by measuring the rate of color fade.

In this project the color fade from red to colorless of different concentrations of sodium hydroxide in alkaline solution mixed with phenolphthalein will be studied. The results are used to set up a laboration for a basic pharmacology course where the goal is to let the students learn about rate law and also how to illustrate it.

2. Methods

2.1 Growth Conditions

One vial of OneShot *E.coli* BL21 (DE3) was transformed with 5ng DNA containing the full length PYCARD gene according to manual (InvitroGen Life Science, 2010). The bacteria were plated on lysogeny broth (LB)-plates containing 100 mg/L ampicillin. The reason for transforming the bacteria with insertion of the PYCARD gene is to enable future research with interest in that gene.

The following day the plate was used to set up an overnight culture where a single colony was put in 3 ml LB-media, for recipe see appendix I, containing 100 mg ampicillin/L and put on an oscillating shaker at 225 RPM at 37°C in the incubator.

The next day the overnight culture was used for four different dilutions of the culture were made with fresh LB-media and incubated in the same incubator. Cell density at the specific wavelength 600 nm, in this case optical density$_{600}$ ($OD_{600)}$ was measured at certain points through use of a WPA Biowave CO8000 Cell Density Meter in order to see the effects of the different dilutions 1:25, 1:50, 1:100 and 1:200. OD_{600} was measured in the 3 ml overnight culture before addition of new LB and also after dilution into the 50 ml culture at time zero. Before reaching exponential growth phase where growth speed increases, measurements do not have to be made more than once each hour. As the growth increase, measurements have to be made more often. Each 30 minutes for OD_{600} between 0.1 and 0.4 and after that each 10-15 minutes to be able to clearly show the growth as a curve reaching exponential phase.

A secondary dilution was made in one experiment to see what effect on OD_{600} levels a secondary dilution would have. For those, the same amount of sample as new medium were mixed and analyzed to make the OD_{600} reading more accurate.

A media called terrific broth (TB), for recipe see appendix II, containing more nutrients was also tried out to see if a change to a more nutritious media would affect the speed of growth. The 3 ml overnight cultures were tried both in TB and LB.

2.2 Fading of Phenolphthalein

The experimental setup for showing kinetics of the fading of phenolphthalein in alkaline solution were set up using a Hitachi U-2010 spectrophotometer at 550 nm along with different Sodium hydroxide (NaOH) solutions in concentrations between 0.05 M and 0.3 M. Standard 0.3 M Sodium chloride-(NaCl) and 0.3 M NaOH-solutions were prepared. The less concentrated NaOH-solutions were prepared by mixing the standard 0.3 M NaOH with 0.3 M NaCl-solution into the right NaOH-concentration.

NaOH concentrations used were 0.3 M, 0.2 M, 0.1 M and 0.05 M. Phenolphthalein was prepared by making 0.20% w/v phenolphthalein in ethanol. Different type of cuvettes both plastic and quarts of different volumes were tried out in the experiments. The 3 ml quarts cuvettes were chosen because of reliable results by effective mixing of NaOH and phenolphthalein with the pipette-tip and by inverting the cuvette with parafilm as cover. At least two half-lives of the color fading were measured before ending each experiment to be able to make graphic curves, which means reaching 1/4 from the starting value of absorbance. This also explains the different time measurement ranges since 0.3 M NaOH was much faster to fade in color than lower concentrations of NaOH so time ranges had to be tried out. Samples were zeroed with dH_2O and after that NaOH were added to the cuvette. Varying amounts of phenolphthalein were tried out to get an absorbance possible to read and this was then added to the sample which was instantly mixed before reading absorbance each 30 s, timed by the spectrophotometer. All material was kept at room temperature since temperature changes can

affect the rate of reaction. Finding a starting absorbance of ~1 was tried out in early experiments and decided amounts to use were 2 ml NaOH and 4µl 0.2 w/v % phenolphthalein.

3. Results and Discussion

3.1 Growth Conditions

The first task was to try to find a suitable dilution (inoculation ratio) between overnight culture and medium. Three dilutions to start with were chosen, 1:200 ratios between culture and medium, 1:100 and 1:50. The results are displayed in Figure 3.

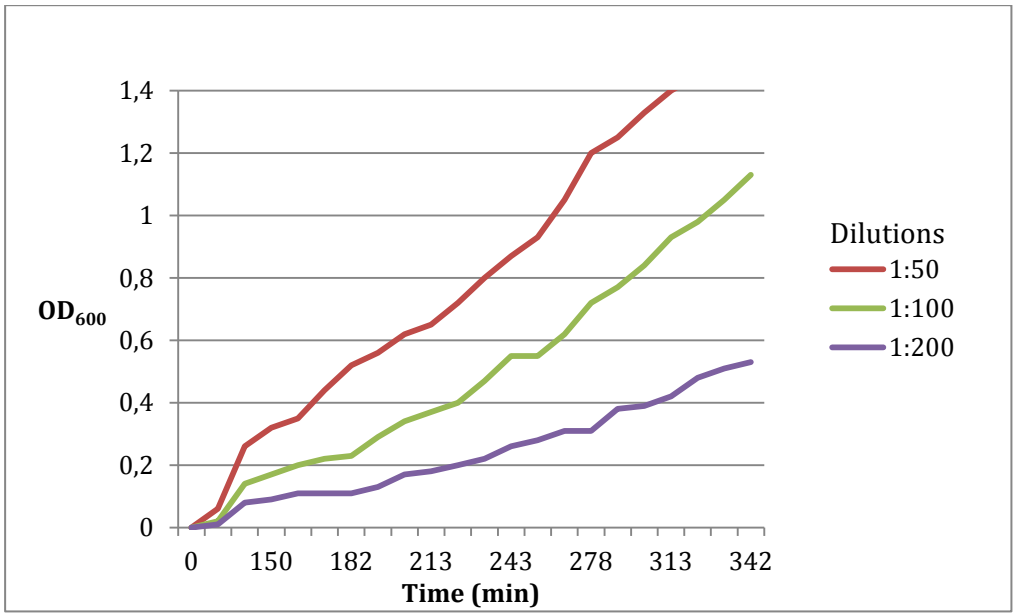

Figure 3. Showing the growth curves of *E. coli* BL21 (DE3). It is clear that the higher the concentration of bacteria the faster the growth is so for a fast illustration of growth and creating a curve 1:50 is best suited of the three alternatives. Growth is still not well suited to create a growth curve during a half-day laboration since no sign of stationary phase can be seen yet.

Growth rate as shown in figure 3 is still not well suited to create a growth curve during a half-day laboration since no sign of stationary phase can be seen after more than five hours. The experiment was repeated without noticeable changes in rates of OD600 levels rising. A fourth 1:25 inoculation was performed in order to try to make the growth curve rise faster as seen in Figure 4.

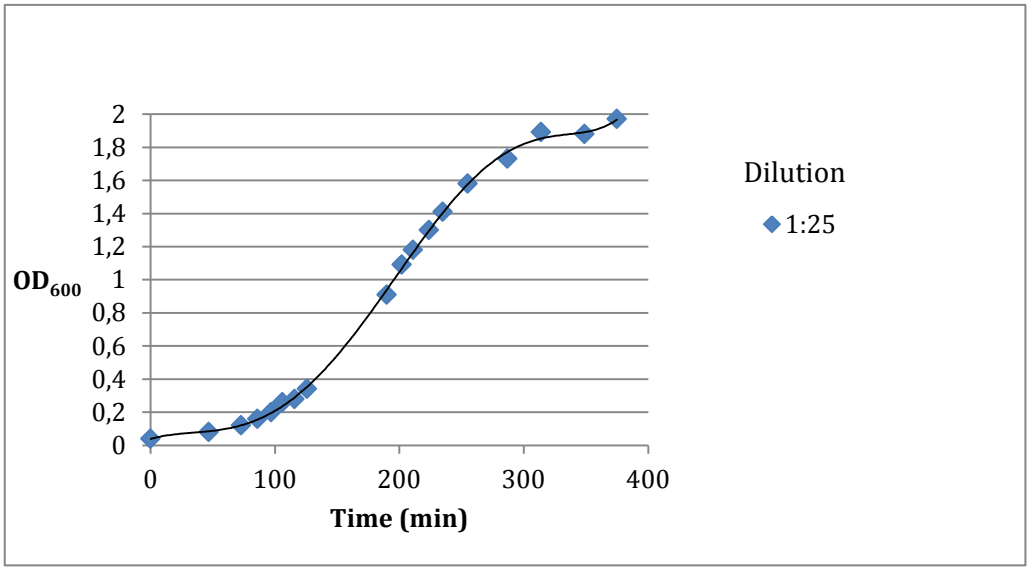

Figure 4. Showing the growth curve from a 1:25 dilution of *E. coli* BL21 (DE3). Here the growth is even faster than in previous samples with 1:50 dilution and also noticeable is the appearance of stationary phase just before the 300 minute mark.

The next experiment which is shown in figure 5 was prepared by diluting one more time by equal parts sample and fresh medium for reading of optical density when above 0.4 and then with another reading taken directly after dilution. This was made to compare the values between the original sample and after dilution to improve the accuracy of the OD_{600} readings because high-value readings are known to be inaccurate since the amount of bacteria becomes too many to effectively send light through to measure (Expression Technologies Inc, 2003).

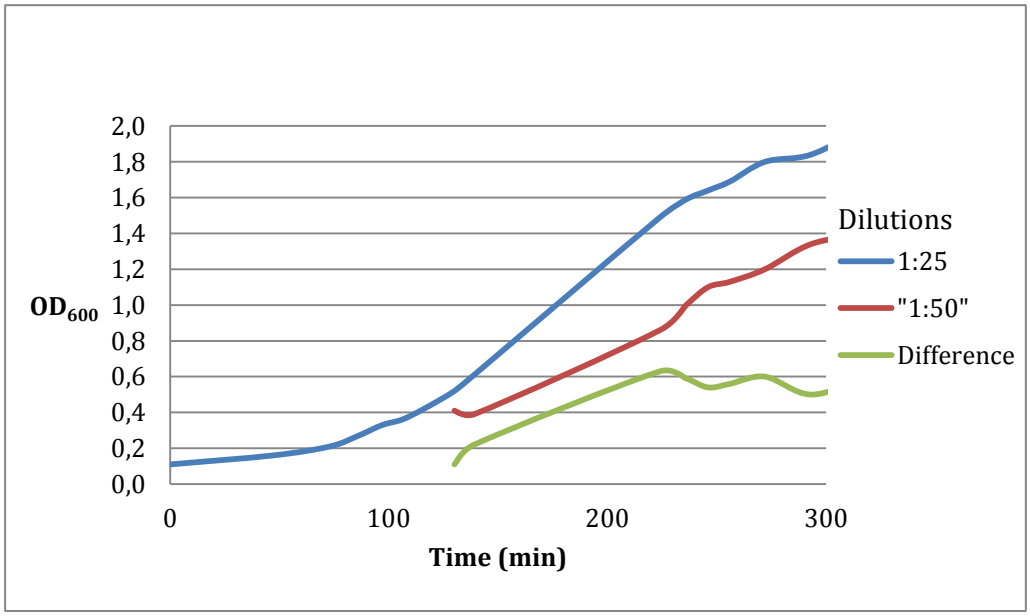

Figure 5. Growth curve of 1:25 dilution with a second dilution and comparison of values. The graph in blue represents the normal 1:25 dilution and the red line graph is the same sample further diluted with equal parts sample and fresh medium. The O.D difference between the original sample and the further diluted one is the green graph.

Starting out after passing 0.40 the difference was not that great (0.52 vs. 0.41). The difference then increased as the cell density increased and reached its peak at approximately 0.6 OD_{600} difference at OD_{600} 1.5 in the original sample. So the only possible results from this are that high OD_{600} readings are very hard to interpret and levels should probably be kept low to be accurate.

Following result shown in figure 6, is the growth curves from using two different mediums simultaneously to see if a more nutrient-filled growth medium could improve growth.

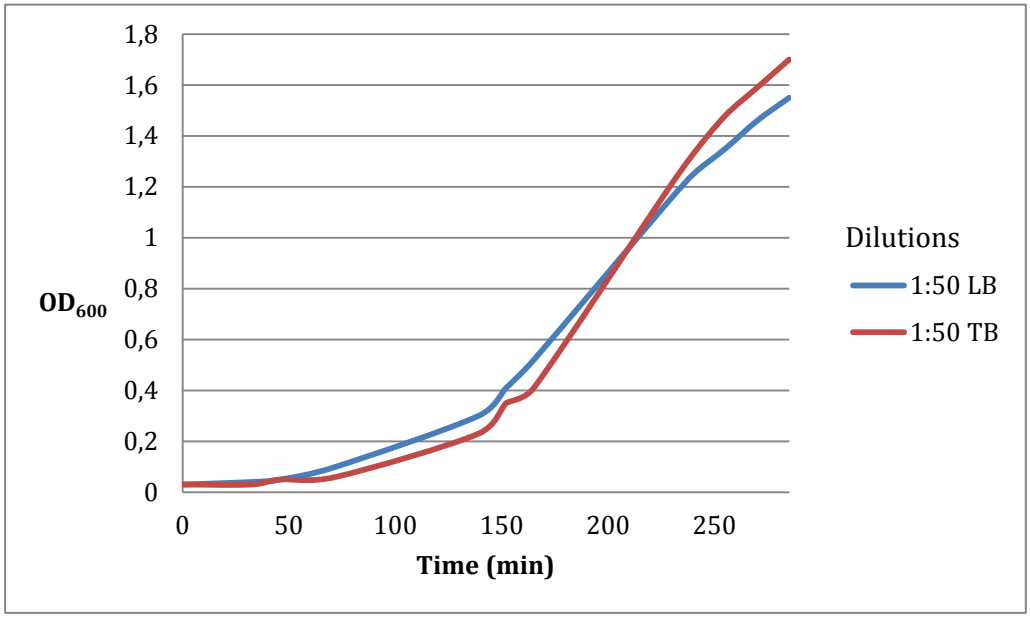

Figure 6. Growth curves comparing LB and TB medium. The graph in blue represents LB medium and the red one TB.

LB started growing a little bit faster most likely due to the fact that it was the medium used in the overnight culture. The zero used was LB and the difference between the two mediums were +0.07 in favor of TB compared to LB due to higher amounts of nutrients. The only significant difference from the graphs is that TB supports rich cultures more effectively which makes it possible to reach higher OD_{600} levels. The result is that LB is good enough to provide optimal growth conditions for the bacteria. There are also a lot of other medias that can be used with good results depending on what wants to be studied for example specific products or very high OD_{600} levels. Examples can be the super optimal broth (SOB) medium. It has twice as much peptone as LB and also richer in amino acids, peptides and magnesium to provide high density cell growth up to O.D 3-5. 2x yeast extract and tryptone broth (2YT) medium has twice the yeast amount of LB and around 60% more peptone which makes it a richer medium for supporting high cell density levels and stable for longer periods of time which allows a higher quantity of phage production to occur without exhausting nutrients (Expression Technologies Inc, 2003).

The result for this part with growing bacteria is that LB medium is the preferred medium with 1:25 inoculation ratio between overnight culture and medium. Lag phase lasts for about one hour and 20 minutes after inoculation and measurements to time the log phase is preferably taken each 10-15 minutes after entering log phase until reaching stationary phase after about 300 minutes.

3.2 Fading of phenolphthalein

This study was interesting but before observing any good results, a lot of adjustments had to be made in order to get high enough absorbance values and also exact composition of each of the samples. The experiment was set-up using 3 ml quarts cuvettes where 2.0 ml NaOH solution were added. Then addition of 4 µl phenolphthalein solution and mixing the sample with pipette-tip and parafilm inversion were performed before starting absorbance readings.

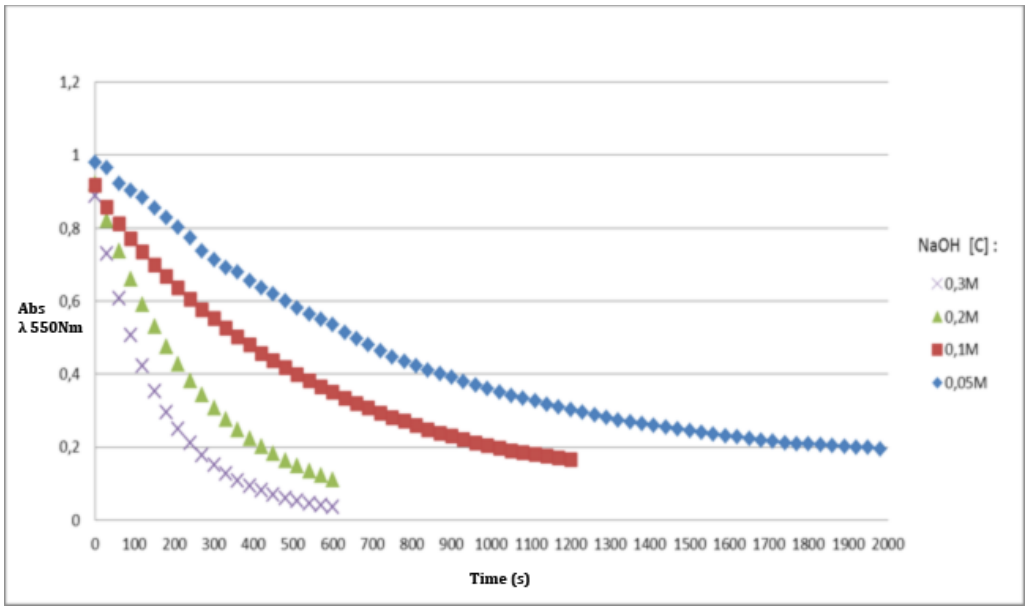

Figure 7. Graph showing fading of phenolphthalein. Fading times for each of the different NaOH concentrations are displayed.

There was some problem in obtaining correct starting values which seemed to be the almost same and not dependent on starting conditions and mixing methods. In the figure above starting values ranges from 0.888 abs for 0.3 M NaOH to 0.980 for 0.05 M NaOH probably because of unspecific concentration of the samples or mixing problems. It is still very clear that higher concentration of NaOH speeds up the color fading in corresponding fashion. To illustrate this further the half lives have been calculated from the fading curves in Figure 7. The result are presented below in Table 1.

Table 1. Half-lives of each NaOH concentration from the measurements.

0.3M (s)	0.2M (s)	0.1M (s)	0.05M (s)	Mean value(s)
110	127	140	111	122
165	190	210	166	183
330	380	420	333	366
660	760	840	665	731

The mean values are calculated using the observed half lives from the experiment in Figure 7 colored in yellow. This provides theoretic half life

values of each concentration based on a single measured half life. The mean values provide reliable information about how exact dilutions have been performed. The calculated mean values does differ a bit from the noted half life of the 0,1 M and 0,05 M samples. This is probably the result of inaccurate dilutions but could also be affected by how the samples were mixed in the cuvette. They do however correspond well enough to each other to tell that the half-life is a direct product from the concentration of NaOH so that raising the concentration to the double would impact and cut the half life in half.

Based on the values it seemed as if NaOH concentration was the important and only factor of the rate of color fading. Raising concentration to the double would impact the fading rate to half the time. This is explained by the reaction $P^{2} + OH^{-} \rightarrow POH^{3-}$. This is further visualized when the absorbance values are multiplied using the natural logarithm (Ln) as seen in Figure 8.

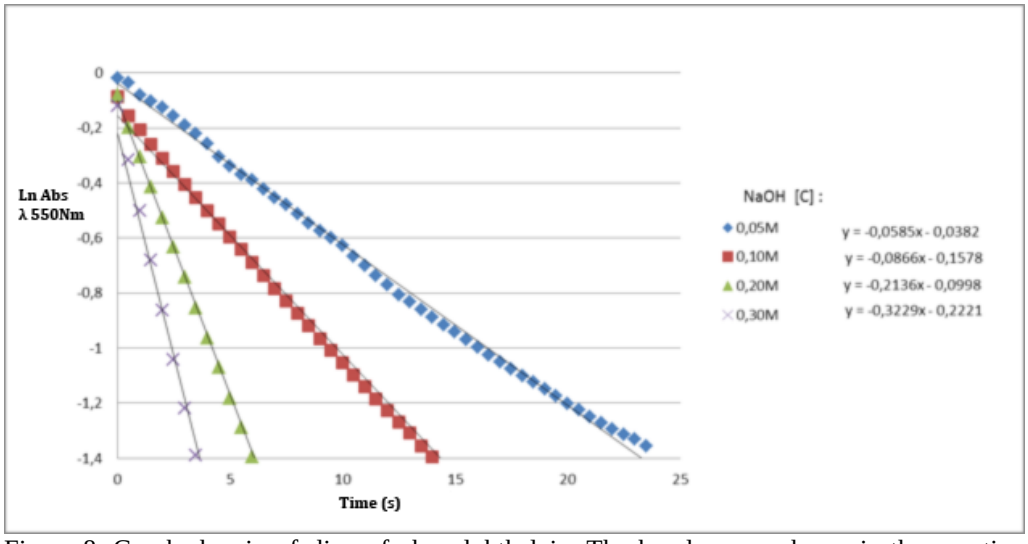

Figure 8. Graph showing fading of phenolphthalein. The k-values are shown in the equation providing further information on the relation between NaOH concentration and fading rate. Trendlines with formulas are made with the linear regression tool of Microsoft Excel.

The reason for the values not being exactly identical is probably due to concentrations not being 100% accurate as well as small differences in mixing time for samples. This can be helped by using a stop-flow instrument in the setup. Temperature control features would also improve the reproductivity of the experiments. The lines still follow each other good enough to provide a basis for stating that the rate law is first order in OH^{-} in the reaction which means that the rate of reaction is directly proportional to the concentration of OH^{-}. This states that the reaction rate of colorfading phenolphthalein is directly based on the concentration of OH^{-} ions and that it is the single factor that the half life is dependent on. This also states that phenolphthalein amounts are not affecting half life but only the starting absorbance of the samples which has been noticed in the experiments.

4. Conclusion and future experiments

4.1 Growth Conditions

The dilution should be low around 1:25 and suitable media for providing statistics for visual display of each growth phase is LB. Oscillating shaker board may be set to 250 rpm to further speed up growth processes since this was not possible in the experiments. Another way of making cell density readings since high values were hard to interpret could be further dilutions in range of 10-fold or more and then multiplying by the same number for samples with high optical density of ~0.5 or more. There might also be the possibility to use other equipment since it was noted that the OD_{600} meter often had variations in readings. TB was functioning similar but not quite as good as LB and probably only have advantages with very high amounts of cell culture. An interesting follow-up would be trying out other nutrient rich media to see if any differences can be observed. Perhaps also to study how specific products are affected by choice of medium.

4.2 Fading of phenolphthalein

This part of the project was easy to perform and illustrated the importance of OH^- concentration for the half life of the color fade of phenolpthalein. Difficulties were found in how to handle samples in the same way and to get good results it is very important for NaOH concentrations to be exact. Improvements could be possible by controlling temperature for example or by taking readings and adjusting according to temperature variations. Also a stop-flow instrument could improve the possibilities to reproduce the experiment. Accurate and exact concentrations of NaOH are very important, so perhaps scaling up the amounts could help to improve results. How exact concentrations are is easy to notice since good mixture will provide the same starting absorbance with exact concentrations of NaOH. An interesting subject could be to perform tests with known concentrations of NaOH to create curves and calculate half lifes of the reactions in order to identify the concentration of an unknown NaOH solution by using known half life times and finding the match.

5. References

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