Biology Internal Assessment

Gene Induction:
Does the presence of lactose in E.coli increase the rate of activity of the enzyme β-galactosidase?

Personal Engagement:

My interest in the role of genetics peaked when covering Unit 7 (Nucleic acids and proteins), sub-topic 7.4 (Translation) in which I learned of the role of tRNA-activating enzymes on tRNA molecules, used for DNA translation. I have always been interested in how microscopic entities can function based on triggers and carry out the functions that our bodies depend on. After further reading, I stumbled across a similar concept that some genes are inactive, triggered by the presence of an inducer molecule. I then found a feasible but still intriguing investigation for my internal assessment to do on my newly acquired knowledge.

Research Question:

In this investigation I will be considering the following research question:
Does the presence of lactose in E.coli increase the rate of activity of the enzyme β-galactosidase?

Hypothesis:

For this investigation, I expect to find that with the presence of lactose, the rate of enzymatic will be higher as opposed to when no lactose is present. This is because the presence of lactose switches on the gene lacZ in E.coli, which triggers the production of the enzyme β-galactosidase. This enzyme normally breaks down lactose into galactose and glucose, however in this practical ortho-nitrophenyl-β-D-galactosidase (ONPG) will be used as a substrate. ONPG will be broken down into galactose and ortho-nitrophenyl, a yellow product, which will function as an indicator. Below is the chemical equation for the breakdown of ONPG:

$$\underset{\text{(ONPG)}}{C^{12}H^{15}NO^{8}} \xrightarrow{\beta\text{-galactosidase}} \underset{\text{(galactose)}}{C^{6}H^{12}O^{6}} + \underset{\text{(ortho-nitrophenyl)}}{C^{12}H^{15}NO^{8}}$$

Expectation: the value of absorbance will increase with time. The graph would look similar to the one sketched below, the line representing the E.coli culture containing lactose will be steeper than that representing the E.coli without the presence of lactose.

— E.coli containing lactose
— E. coli without the presence of lactose

Background Information:

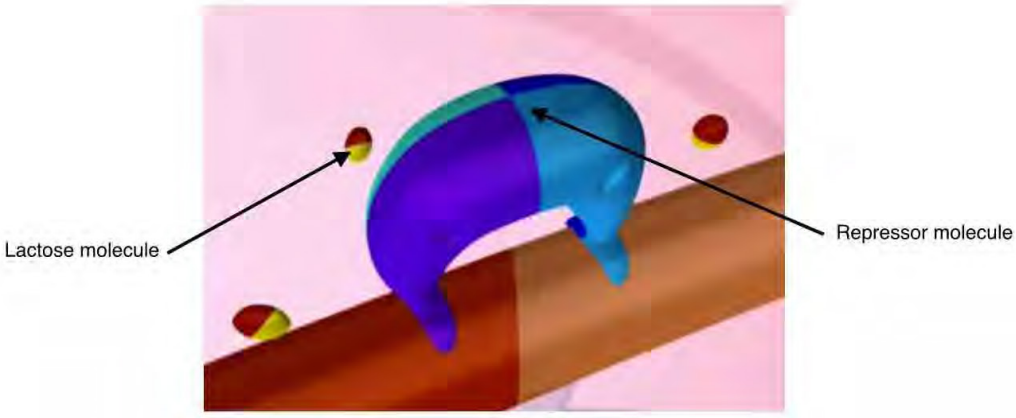
Both the disaccharide lactose and monosaccharide glucose can be used as sources of carbon and energy for the bacteria *Escherichia coli* (E. coli) (Lodish et al, 2000). When E.coli cells are grown in a medium containing lactose instead of glucose, activity of the enzyme β-galactosidase, which metabolises lactose, increases. This means that when E.coli is grown in a medium lacking lactose, β-galactosidase is likely to be absent or only present in very small quantities. The cause of the increase in enzymatic activity was shown by early studies to be due to induction: "the synthesis of new enzyme molecules" (Lodish et al, 2000). More specifically, these new enzymes are encoded by a system known as the lac operon. An operon is "a group of genes which are transcribed together as a single mRNA" (Carr, n.d.). The lac operon includes the genes lacZ, lacY and lacA, which are all correspondingly regulated, and is responsible for the breakdown of lactose with the important gene in this case being the gene lacZ, which codes for the enzyme β-galactosidase that (as previously mentioned) initiates the metabolism of lactose and other compounds of similar structure (Lodish et al, 2000). The equation below summarises the breakdown of lactose by β-galactosidase:

$$\underset{\text{(lactose)}}{C^{12}H^{22}O^{11}} \xrightarrow{\beta\text{-galactosidase}} \underset{\text{(galactose)}}{C^{6}H^{12}O^{6}} + \underset{\text{(glucose)}}{C^{6}H^{12}O^{6}}$$

The lac operon in E. coli, a lot of which was researched by Francois Jacob and Jacques Monod in the 1960s, is one of the earliest examples of gene control and remains one of the most understood examples (Lodish et al, 2000). The lac operon is an example of an inducible system in which the interaction of the repressor and inducer molecules mediates gene expression (http://vcell.ndsu.nodak.edu/animations/lacOperon/movie-flash.htm). In general terms, a repressor is "a protein produced by a regulator gene which binds to a site on the operon to prevent transcription of structural genes" and an inducer "a metabolite which prevents the repressor from binding to the DNA so that the structural genes can be transcribed (Carr, n.d.).

In other words, genes belonging to an inducible system are inactive unless in the presence of an inducer molecule, such as with the lac operon in the presence of lactose, and the repressor molecule prevents transcription of the lac operon genes by binding to the control region on the operon (http://vcell.ndsu.nodak.edu/animations/lacOperon/movie-flash.htm). Jacob and Monod predicted that the binding site for the lac operon was a specific DNA sequence near the transcription start site of the lac operon, their model of repressor control of transcription in the lac operon proposes that an inducer molecule causes an increase in transcription of the lac operon (Lodish et al, 2000).

The lactose (acting as an inducer) triggers gene expression by passively entering the E.coli cell, binding to the repressor molecule and releasing it from the control region, as can be seen in the diagram below (http://vcell.ndsu.nodak.edu/animations/lacOperon/movie-flash.htm).

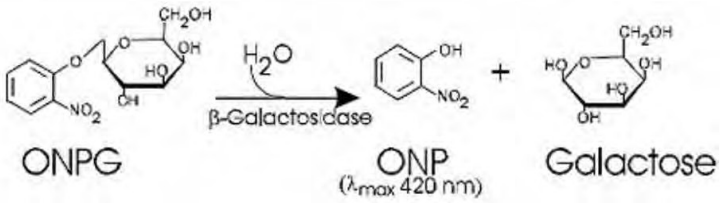


Retrieved October 8, 2015 from http://vcell.ndsu.nodak.edu/animations/lacOperon/movie-flash.htm

RNA polymerase then begins transcription of the molecule, while ribosomes bind to the messenger RNA (mRNA) resulting in the proteins/enzymes beta-galactosidase and permease being translated. While beta-galactosidase breaks down lactose, permease, when embedded in the cell membrane, provides a direct route for the lactose outside the cell to be imported into the cell. Once lactose concentration in the E.coli cell is greatly reduced, the lactose unbinds from the repressor molecule, allowing it to bind again with the control region, halting gene expression.

The standard method of assessing β-galactosidase activity involves the colorimetric measurement of the yellow colour formed by Ortho-nitrophenyl-β-D-galactosidase (ONPG), a chemical analog of lactose. This will be used as the substrate (i.e. the assay) for the following experiment in order to give a quantitative measure of enzyme activity.

When ONPG comes into contact with beta galactosidase the oxygen bridge between 'ONP' and galactose is broken down through hydrolysis (in the same way lactose is broken down) and galactose and o-Nitrophenyl, a yellow alkaline compound, are produced as can be seen in the diagram below. The intensity of the yellow colour determines the rate of enzymatic activity.



Retrieved May 29, 2015 from http://www.biotek.com/assets/tech_resources/52/prwfig25.jpg

Variables

Independent and Dependent Variables

The Independent Variable is the presence or absence of lactose, as it is as a result of a change in this variable that leads to a change in my dependent variable β-galactosidase activity, which will be measured through the intensity in colour of the yellow alkaline compound. With the presence of lactose, I expect to find the rate of enzymatic activity to be higher than when lactose is not present.

Due to my limited amount of ONPG, which could not be bought locally but instead had to be ordered online, I will use two E.coli cultures, one comprised of E.coli grown in a medium containing lactose and the other culture comprised of only E.coli.
Test tubes will be filled with $2cm^3$ of ONPG solution and $0.3cm^3$ of E.coli.

The Dependent Variable will therefore be the activity of β-galactosidase, measured in the intensity (absorbance) of the visible yellow colour using a colorimeter, as according to my hypothesis, it is affected by a change in my independent variable - the presence of lactose. As the Independent Variable varies, enzymatic activity is required to respond similarly.

Control variable	Why it needs to be controlled	How it will be controlled
E. coli strain	The process of making E.coli involves a number of different processes, including the use of agar jelly and a specific growing process for the E.coli.	The two E.coli cultures that will be used will come from the same original E.coli strain as this reduces variations in the contents of the E.coli.
Type of agar	Agar is a medium often used for bacterial cultures. Depending on the type used the nutrients of the agar can vary, which would affect the growth process of the E.coli.	When the E.coli strain is made in the lab the same type of agar will be used, melted and spread on to a large tray for the E.col cultures to be produced.
Temperature of the room	Temperature is a limiting factor in terms of enzymatic activity as enzymes work best at an optimum temperature. If the temperature is too low/high enzymes can work less efficiently or denature. For B-galactosidase, 37°C is the optimum temperature.	Prior to the experiment, air conditioners/ fans will be turned off to reduce potential air flow in the room. A thermometer will also be placed in each test tube to monitor temperature and a water bath will be used, set at 37°C in order to reduce random

Control Variables

Measurement of the yellow colour of o-nitrophenyl	Measuring using the naked eye is a qualitative measurement and is therefore less accurate as the intensity of the colour is open to human error and bias.	A colorimeter will be used instead in order to record a quantitative measure (absorption) of the intensity of the colour.
pH	pH can be a limiting factor for enzyme activity as the further it deviates from the optimum pH, the lower the enzyme activity.	Phosphate buffer (set at pH 7) will be mixed with the ONPG powder to make ONPG solution, keeping the pH of both sol in test tubes A and B constant.
Volumes	The amount of product produced is proportional to the volume of ONPG used. Therefore if more ONPG is used, there will be more broken down product, resulting in a more intense yellow colour.	For each test tube, the same amount of ONPG solution - 2cm^3 - will be measured out using a disposable syringe as well as the same amount of E.coli - 0.3cm^3, which will be measured using a transfer pipette (plastic pasteur pipette).

Preliminary experiment

I was unsure which wavelength setting to set my colorimeter at in order to record the colour of the o-nitrophenyl. When conducting second hand research, I found the range of wavelength for yellow light to be between 570-590nm. However instructions for a colorimeter indicated that the wavelength should be set at 430nm for yellow light (https://www.omega.com/manuals/manualpdf/M3780.pdf). I carried out my practical twice, with the setting set first at 430nm and then at 470nm. For both I placed a cuvette containing the solution into the colorimeter, I found that at 430nm there was little detection of absorbance in comparison to the colorimeter was set at 470nm. Therefore for my official practical I decided to use 470nm

Method

Apparatus:

- 50cm^3 E. coli culture (A) contains lactose
- 40cm^3 ONPG powder
- 20cm^3 measuring cylinder (± 0.5cm^3)
- X2 Test tubes
- Toluene (Methylbenzene)
- Digital miligram balance (± 0.001g)
- Glass thermometer (± 0.5°C)
- Labquest (v2)
- 50cm^3 E.coli culture (B) no lactose
- ph 7 Phosphate buffer
- 10cm^3 plastic pasteur pipettes (± 0.01cm^3)
- 100cm^3 glass beaker (± 0.5cm^3)
- X2 Rubber stoppers/bungs
- Stopwatch (± 0.01s)
- Colorimeter (± 0.001) and cuvettes
- Water bath (± 0.25°C), metal test tube rack

- Disposable syringes (no needle)
- Biohazardous waste disposal bags

Preparation

24-48 hours prior to the experiment prepare two cultures of nutrient broth (melted agar) containing E.coli (at least 100cm^3 in volume each) from which samples will be taken. One culture (A) will be induced with lactose, having been grown in a medium containing lactose, while the other (B) will not.

Health and Safety

- Before and after the practical clean the workspace using disinfectant solution in order to reduce the risk of spreading contamination (always working under the assumption that the E.coli culture is contaminated, even if it isn't).
- When handling the Toluene, the environment should be ventilated if possible as toluene evaporates relatively quickly and may contaminate the surrounding air. Safety goggles, gloves and a face mask should also be worn as it can cause health problems when inhaled or if it comes in contact with the eyes or skin.
- Although safety gloves should be worn throughout, it should be ensured particularly when handling toluene and E.coli. Most E.coli cultures used in labs are harmless, however these bacterial cultures can become contaminated with pathogenic bacteria.
- Dispose of the samples, gloves and any other material that may have come in contact with either the toluene or E.coli into a biohazard bag.
- Any apparatus used should be rinsed and submerged and left in a dilute bleach solution.

Ethical Considerations

- The fact that the E.coli is still a live organism should be kept in mind. Samples should not be wasted and the solution should be used sparingly, or in a way that rules out any surplus material.
- Some equipment was manufactured by a company that endorsed child labour.

Procedure

1. Switch on the water bath and set it at 37°C, placing the metal test tube rack inside.
2. Make ONPG solution with a concentration of 4mg of ONPG to 10cm^3 of phosphate buffer.
   - Using the digital milligram balance (± 0.001g) measure 4mg (0.004cm^3) of ONPG powder.
   - Measure out 10cm^3 of the phosphate buffer using a 20cm^3 measuring cylinder (± 0.5cm^3
   - Combine the two in a 100cm^3 beaker (± 0.5cm^3) and mix together using a stirring rod.
3. Set up and label two test tubes, A and B.
4. Using a measuring pipette (± 0.01cm^3) add 0.3cm^3 of E.coli from culture A into test tube A.
5. Using a measuring pipette (± 0.01cm^3) add 0.3cm^{3}of E.coli from culture B into test tube B.

6. Using a measuring pipette (± 0.01cm^3) add one drop of toluene to each test tube (the toluene causes the cell membrane to be disrupted, allowing the ONPG to combine with the solutions and therefore be catalysed).
7. Place a bung over each test tube and shake them vigorously.
8. Using the disposable syringe, add 2cm^3 of the ONPG solution to each test tube, A and B.
9. Place the test tubes in the water bath (± 0.25°C) at 37°C and begin the stopwatch (± 0.01s).
10. Using a colorimeter (± 0.001), with the filter set at 470nm, measure the absorbance of the tw samples. This is done by pouring the sample (from either test tube A or B) into a cuvette, placing it inside the colorimeter and attaching the BT (British Telecom) plug to the Labquest in order to display the readings. A reference mark should be put on one of the clear sides of the cuvettes so they are always oriented the same way inside the cuvette slot of the colorimeter.
11. Continue to record the level of absorbance at 10 minute intervals for 60 minutes (1 hour) using the colorimeter (± 0.001)
12. Repeat the practical a further four times (so there are five sets of results for both Cultures).

Plot a line graph of absorbance (y axis) against time (x axis) as both pieces of data are measured across equal increments of time and are therefore continuous (rather than discrete).

Results

Data Analysis:

The data is continuous for each variable and will therefore be plotted as two separate lines (one for Culture A and one for Culture B) on a line graph in order for comparisons to be drawn between the two sets of data. From the raw data[1] that I collected, I calculated the average (mean) level of absorbance out of the five trials for every ten minute interval.

In terms of statistical analysis, the data has a normal distribution, therefore the Paired T-Test will be calculated using the excel spreadsheet function[2]. This will give me a value that determines whether the two sets of data differ from each other in a significant way. The closer the value of T is to 0, the more likely there is to be little to no significant difference in the two sets of data, conversely the further it deviates from 0 (i.e. the larger the number) the more significantly different the two pieces of data are.

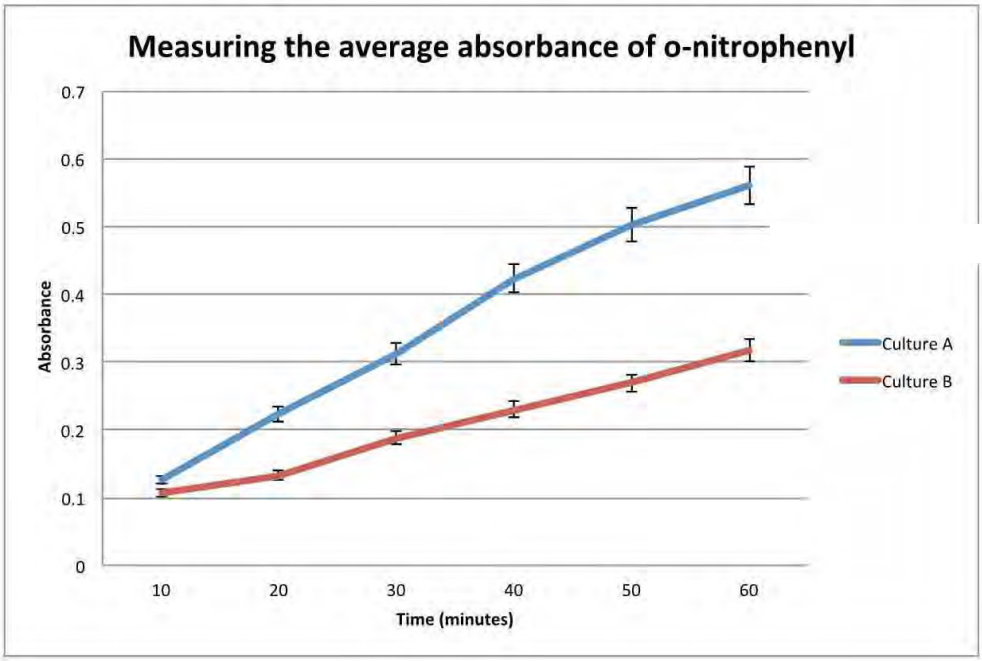
[1] See Appendix I

[2] See Appendix II

Processed Data	Time (minutes)						
	10	20	30	40	50	60	STDEV
Mean for Culture A	0.126	0.223	0.312	0.423	0.503	0.561	0.168
Mean for Culture B	0.106	0.133	0.188	0.230	0.269	0.317	0.081

Note: a reading of 0 by the colorimeter is no absorbance, while 1 is complete absorbance. Therefore, the level of absorbance depends on how close the number is to either 0 or 1. There is no specific unit used to measure absorbance as readings are calculated from a ratio of the intensity of light (of a particular wavelength) transmitted through the sample, relative to the intensity of light transmitted through a blank (i.e. the maximum possible amount of light available) (http://www.vernier.com/til/2539/).

Graph:



Graph Analysis:

The graph supports my previous hypothesis as E.coli Culture A, which included lactose shows a higher average level of absorption at every time interval in comparison to that of Culture B. For both conditions, the gradient representing the data is quite linear and the Independent and Dependent Variables show a relatively clear positive correlation. Although at 20 minutes Culture B dips slightly in value in comparison to the general slope. However this minor deviation is negligible as the difference is only small in comparison to the other points and still follows the general upward trend of the graph. With the raw data, sometimes readings did not always increase, but instead decreased slightly between a ten minute interval (as highlighted in red in Appendix I), these are likely to be anomalies but are also only minor in their deviation from their expected value.

Conclusion

To conclude, in this investigation I aimed to find out whether the presence of lactose increases the activity of the enzyme β-galactosidase in the bacterium E.coli. My hypothesis stated that 'I expect to find that with the presence of lactose, the rate of enzymatic will be higher as opposed to when no lactose is present'. Although some anomalies can be identified within the raw data (in the sense that the absorbance recording did not always continually increase), the graph and processed data strongly support this hypothesis as a general positive correlation can be identified between the two cultures. This is because the ONPG is expected to be further broken down as time passes, hence the increased absorbance recordings from the darkened colour. Moreover, absorbance measurements for Culture A are higher at all intervals of time due to the increased enzymatic activity taking place in the solution. This is because the culture was grown in a medium containing lactose and therefore less repressors prevented the lac operon from functioning and transcribing new enzymes. Visually, the two sets of data can be considered quite similar in their trend nature, however the Paired T-test value of 4.188 suggests that they are in fact different to some extent. My error bars for Culture A are wider, which shows that the data was less accurate as is deviated further from the average, the standard deviation value of 0.168 for Culture A in comparison to the value of 0.081 for Culture B also confirms this.

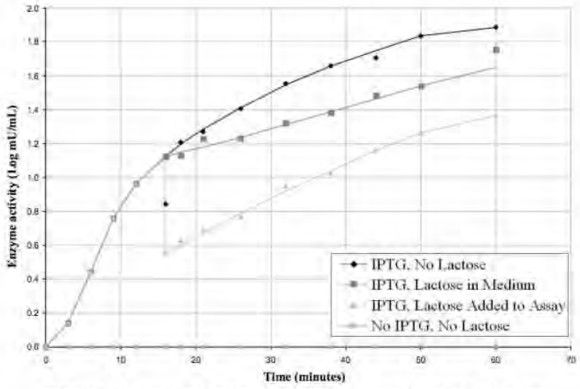


FIGURE 1. Induction of ß-galactosidase in *Escherichia coli* measured as enzyme activity vs. time.

Retrieved October 25 t ttps://
www.microbiology.ubc.ca/sites/default/files/roles/drupal_ungrad/JEMI/2/2-117.pdf

A study was carried out to evaluate whether lactose had a biological effect on the induction of ß-galactosidase. Different methods of lactose addition were also investigated to see whether they caused an interference effect with the ONPG through competitive inhibition. Similar to this exploration, the results found a biological effect to be seen when lactose was added to the medium for growing E.coli. However, when a large concentration of lactose was added to assay samples, enzyme activity was shown to immediately decrease. The study concluded that, although a convenient enzyme assay, ONPG may be less effective at measuring ß-galactosidase activity in the presence of lactose than alternate types of enzyme assays.

Evaluation

Limitation	Why it is a limitation	How it will be improved
Range of independent variable	If the range is limited, the validity of the experiment is lower. As mine only contained two conditions, the validity was not as high.	By including a third test tube, containing a solution of just the enzyme β-galactosidase, I could have improved the validity of my results as the enzyme on its own would have acted as a control, thus improving the validity of my findings.

Limitations:

Treatment/ preservation of E.coli	Although the cultures were kept in the fridge most of the time when not being used, there were times they were left at room temperature for an extended period of time (up to 12 hours). As the E.coli is a bacteria and respires, it can be expected to divide at a rate depending on what conditions it is in. At room temperature the bacteria are prone to divide faster than if they were to have been at lower temperatures (like in the fridge). The division of bacteria cells may affect the accuracy of my results as it could cause changes in enzyme activity.	Strictly keeping the E.coli cultures in the fridge at all times they are not being used, as they only need to be taken out for the first part of the experiment. Additionally, data collection could be minimised to a less spread out period of time as this limits changes in the number of bacteria cells because, although keeping them in the fridge limits bacterial growth, it does not inhibit it completely. Therefore it should be attempted that the experiment be done within as short of a time span as possible.
Number of repeats	The more an experiment is repeated, the easier it is to find specific patterns and the higher the reliability of the findings. Additionally, anomalies become negligible the more repeats there are.	Replicating/repeating the experiment a further number of times, in the same conditions, to increase reliability of the results.
Systematic error of the colorimeter	The readings on a colorimeter can often drift, which can result in inaccurate readings of absorbance.	Calibrating the colorimeter before use. This can be done using a variety of different methods, but one of the most simple would be to use a blank cuvette and reset the colorimeter so that it reads 0 absorbance (because all light is passing through).

I would be interested in further developing this investigation by implementing more conditions as controls, such as measuring ONPG breakdown in a third solution of just the enzyme β-galactosidase, as this would be expected to have the highest enzyme activity (and therefore the most 'intense' yellow colour within a shorter period of time). When compared to the other two solutions this would help improve the validity of my findings

References

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Appendices

Appendix I (Raw data):

Raw data		Culture A (absorption ± 0.0005)					Culture B (absorption ± 0.0005)				
		Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
Time (minutes)	10	0.054	0.179	0.081	0.152	0.166	0.146	0.032	0.118	0.146	0.088
	20	0.188	0.384	0.118	0.276	0.148	0.094	0.085	0.174	0.181	0.133
	30	0.257	0.397	0.234	0.284	0.388	0.167	0.117	0.289	0.249	0.117
	40	0.363	0.546	0.392	0.387	0.425	0.228	0.151	0.331	0.271	0.168
	50	0.451	0.592	0.529	0.446	0.497	0.262	0.248	0.297	0.318	0.219
	60	0.473	0.617	0.612	0.531	0.573	0.314	0.288	0.362	0.322	0.297

Note: for time no error is included as the length of time across which measurements were made is too short and therefore including errors would be insignificant and unnecessary

Appendix II (T-test calculation spreadsheet):

	↓ category 1 data ↓	↓ category 2 data ↓	↓ difference ↓		
labels (optional) ↓	Culture A	Culture B	**Culture B - Culture A**		
10	0.126	0.106	-0.02	**average difference:**	**-0.15083**
20	0.223	0.133	-0.09	**N:**	**6**
30	0.312	0.188	-0.124		
40	0.423	0.23	-0.193	**degrees of freedom:**	**5**
50	0.503	0.269	-0.234	**t-value:**	**4.188047**
60	0.561	0.317	-0.244	**P-value:**	**0.008588**