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Spores and Germination

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Vanessa E. Doto

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Professor Theodore Crusberg, PhD, Major Advisor

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ABSTRACT:

In order for spores, which are metabolically dormant, to return to life they must go through the process of germination. Germination is a complex and vital step in the generation of growing cells from spores. It is known that spore germination is initiated by specific nutrients. The purpose of this Major Qualifying Project was to identify certain initiators that will begin the germination process. Hydrogen Peroxide, uv treated spores and hypochlorite were used to investigate the effect of these lethal agents in the germination process.

Acknowledgments

I would like to thank Professor Crusburg Ph.D, for all of his guidance and assistance throughout the duration of this project.

INTRODUCTION:

Spore germination is an extremely complex process. Following germination spores become vegetatively growing cells, which are much less resistant to chemical and physical agents. Due to the increasing terrorist threats using biological weapons as attack methods, it is becoming more of an issue to find ways to stop spores before they have the opportunity to cause any damage. It is important to know the biochemical signals that start the germination process which then allow us to figure a way to end this complex process.

In this MQP I tested three agents that are known to have an effect on the germination of spores. To carry out the experiments, mixtures of spores along with different reagents were tested using a spectrophotometric assay to measure the absorbance of the spore suspensions over a one to two hour time period.

The scattering of light from vegetative cells versus germinating spores was used to view the differences in absorbance between the spore and the germinating spore. Spores have a higher absorbance compared to vegetative cells, since they scatter light more effectively than vegetative cells. The absorbance will decrease when the spore is germinating or changing from its typical spore form. These recordings allowed us to conclude which of the experiments have the greatest effect on spores/cells.

Reagents that were used in this MQP to test for their affect on the spore germination process were hydrogen peroxide, hypochlorite and UV treated spores. The aim was to see if the spores still had the ability to germinate after such treatments. These experiments will help identify solutions that will stop the spores from germinating into viable vegetative cells and then being able to multiply.

BACKGROUND ON GERMINATION

The Spore

The Bacillus family's distinguishing feature is the production of endospores, which are highly refractile resting structures formed within the bacterial cells. The genus Bacillus is distinguished from the other endospore-forming bacteria on the basis of being a strict or facultative aerobe, rod-shaped, and catalase-positive [9].

Figure 1 Bacillius subtilis in the spore formation. Oval Structure in the center is the spore- resistant form of the bacteria.
[12] http://europa.eu.int/comm/research/success/en/pur/0291e.html

The bacterial endospore is a highly-evolved structure capable of maintaining the bacterial genome in a protected, viable state for extended periods [1]. Bacterial spores can survive in the environment for incredibly long time periods even at physical extremes for any other life forms on earth [2]. Their ability to survive is mainly due to their tough outer coat which protects them from any outside harm. This is one of the key reasons that makes it such a desirable weapon for biological warfare attacks.

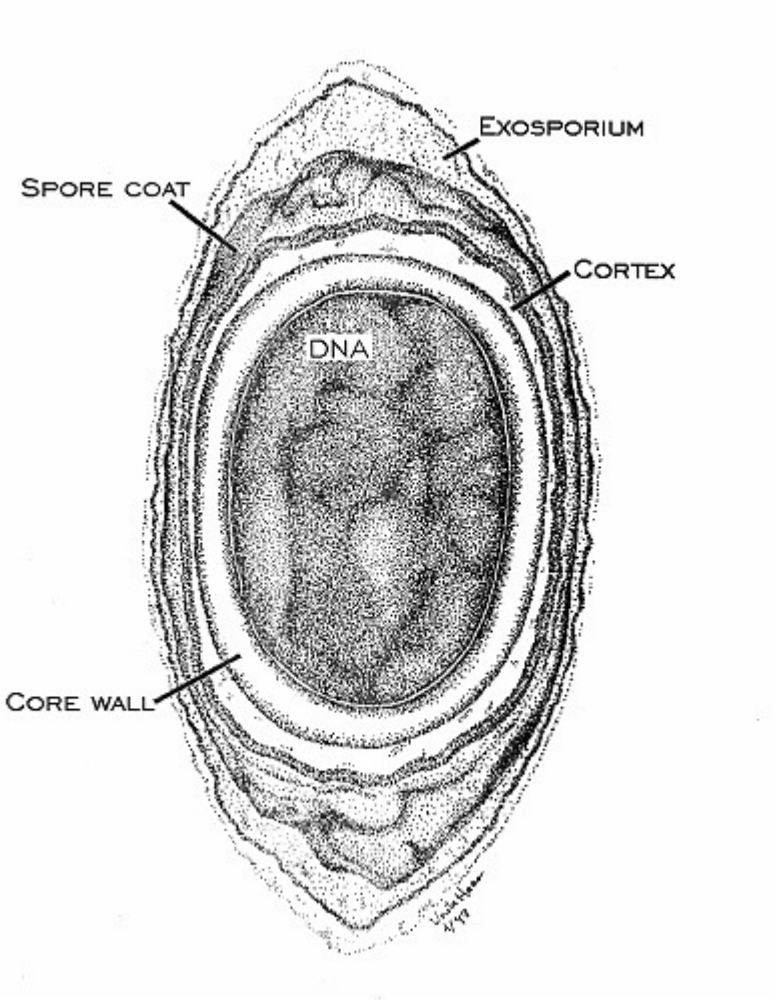


Figure 2 Major components of a spore.
[9] http://textbookofbacteriology.net/Bacillus.html

Many characteristics aid in the spore's ability to withstand extreme conditions. Spores contain a protoplast, protoplast membrane, cortex and three coat layers. The coat is a particularly thick protein coat. It is a highly ordered structure consisting of the following three distinct layers: an electron-dense outer coat, a thinner inner coat, and an electron-diffuse undercoat. This outer coat helps protect the dormant spore from enzymes, such as lysozyme, and from mechanical disruption. Resistance to organic solvents and heat seems to be a function of the peptidoglycan cortex which underlies the spores' coat [6].

The spore coat also protects the spore from some chemicals, such as hydrogen peroxide. We observed this process in the experiment that was conducted for this MQP. Hydrogen peroxide, along with UV treated spores and hypochlorite were all used to

observe the spores' germination process using the spectrophotometer. Since the coat is protecting the spore we observed whether or not the absorbance would decrease after the spore had been exposed to this reagent, indicating a change in the spore's composition.

All these layers of the coat acting together aid in the spores' ability to linger around long enough to then be used as biological weapons to inflict harm. Spores can be left alone for an extremely long period of time and then under ideal germination conditions they can begin to grow and produce the harmful, sometimes deadly proteins, which cause a number of infections and sicknesses. [7]

The formation of a spore involves the original cell replicating its genetic material, and then one copy of this becomes surrounded by the tough coating. The outer cell then disintegrates, releasing the spore which is now well protected against a variety of trauma, including extremes of heat and cold, and an absence of nutrients, water, or air.[4]

Under these protective layers of the outer cell lies the spore's core. It is inside the core where dipicolinic acid, salt calcium dipicolinate are all found. These materials are what greatly aid in the spores' longevity [7]. Once the spore's outer layers begin to break down in the presents of certain disinfectants, such as the ones being tested in this MQP, it is these inner components of the core that will spill out of the spore and alter the solution's make-up; thus allowing us to see a change in absorbance.

It is of much interest to try and extract all the coat proteins of the spore's core, but it is an extremely hard process to try and obtain these proteins from an intact spore. One spore specific organic chemical, dipicolinic acid (DPA) which is only found in spores, is synthesized via the lysine biosynthetic pathway. [5] The major role of the calcium DPA complex seems to be the removal of water during the germination process. Calcium dipicolinate also contributes to about 17% of a spore's dry weight.[7] Not only does the DPA help aid in the spore's indestructibility but it is also only observed in endospores, making it an indicator of the spore. [8].

Germination

Germination of a spore is the process by which a dormant spore goes through a number of degradative events in order to become a viable cell. This is a process of

interrelated biochemical events occurs in the spore. Spore germination has been difficult to study because of the extremely rapid physiological responses in a cell whose structure is biochemically intractable [14].

A number of nutrients such as, amino acids, sugars, dodecylamine, exogenous Ca^{2+} -DPA among others are all inducers of the germination process.

Once a spore is influenced by these extra-cellular compounds it will then trigger the production of an intracellular signal, which signals the next step in germination. When the spores are exposed to the ideal conditions or specific nutrients they break the dormancy period and begin to take on all the features associated with a vegetative cell [15] Throughout this MQP I observed spores that were undergoing a number of changes induced by the agents I was adding to the spore suspension. This allowed me to view a change in the absorbance indicating that the agent had some affect on a part of the germination process.

Heating spores to a designed temperature, around 70^{o}C, can also affect the germination of the spore. Since DPA is such a big part of the spores' make-up, if the spore is heated to a certain point the DPA will be lost and the spore will no longer germinate. This MQP was designed to find out which of the several disinfectants had the most effect on the components involved in the germination process. The absorbance we observed was continuously changing when the spore became affected by the reagent. This is due to the fact that spores scatter light more effectively then the cells do. A change in the absorbance indicates that there is a change in the enzymatic activity in the spore.

The germination process triggers many nutrient-receptor interactions, including the release of DPA as well as Ca^{2+}. It is the release of DPA which allows the uptake of water into the spores' core. The spore then swells to a much larger size than it is while it is germinating. [10] This indicates the spore is coming out of its dormancy stage. For this MQP it meant a lower absorbency would be observed since the larger the cell the less scattering of light, indicating a vegetative cell is being formed and the spore is coming out of its distinctive spore form.

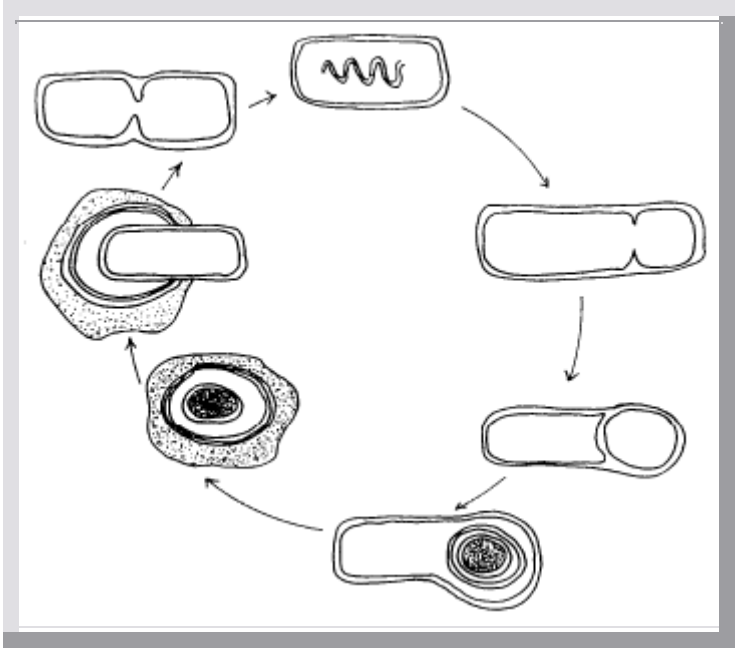


Figure 3 The cycle of a spore formation and germination
[13]
http://www.bmb.leeds.ac.uk/mbiology/ug/ugteach/icu8/introduction/bacteria.html

In one study that was conducted to examine which enzymes have the most importance in the germination process, it was concluded that agents had one of two possible effects on the spore. Exogenous compounds either repressed the response to one of the nutrient receptors, or these exogenous components blocked the response to several of the nutrient receptors. [11] Knowing these interactions then can allow one to control the germination process by altering the treatment of the spores using one of the agents that were used in this MQP. For the purpose of this MQP the interactions allowed us to measure the amount of light being scattered or not being scattered when the spore suspension was treated with one of three known initiators of germination.

Once the spores had been pre-treated by one of the methods their light absorbance were measured at 600nm and will be plotted versus time. Loss of rigidness of the spores reduces their light-scattering properties. This then permits more light to pass through the spore suspension and reach the detector, giving us a lower optical density. After germination, the spore core, polar tubes and other contents of a spore are all free in the suspension which reduces the optical density.

Germination Process:

In *B.subtilis* spores, once the DPA is released from the core it triggers the hydrolysis of the spore's peptidoglycan cortex. Hydrolysis of the spore's cortex is essential for the removal of outside strain so that outgrowth of the core is possible. Hydrolysis is initiated by germination-specific lytic enzymes (GSLEs). [29] It is acidification of the core during endospore formation that is responsible for the accumulation of storage compounds inside the core prior to germination event [30]

After the spore has released amino acids and sugars to begin to break down the spore's outer coat, receptor proteins on the spore's membrane bind to aromatic structures found on amino acids and ribonucleotides. Three known examples that trigger germination are the response to L-alanine in *Bacillus subtilis*, L-proline in *Bacillus megaterium*, and inosine in *Bacillus cereus* [16]. Since we are working with *Bacillus subtilis* the germinant we will be using is L-alanine.

L-alanine acts as an activator of certain enzymes which produce germination substances needed to initiate the germination process. After the addition of L-alanine many peptidoglycan structural changes are observed enabling the spore to respond to the germinant [31]

L-alanine is recognized by receptors encoded by homologous tricistronic operons, the *gerA, gerB*, and *gerK* in *B. subtilis*. These operons are encoded by proteins found in the spore's inner membrane. It is the gerA receptor that triggers the spore to germinate when the addition of L-alanine has occurred. [11]

Once the cortex hydrolysis process has finished, the core will have swelled and become fully hydrated. The completion of the core hydration allows for enzymatic activity in the core. This enzymatic activity initiates metabolism, allowing the spores to finish germination [32]

Reagents

Hydrogen Peroxide

Fenton's Reagent is a solution of hydrogen peroxide and an iron catalyst that is used to oxidize contaminants.[17] Ionic catalysts plus the hydrogen peroxide, which involves the conversion of hydrogen peroxide to highly reactive hydroxyl radicals. It is the dissolving of O_2 that results in a lethal action. [18] The reactivity of hydroxyl radicals means that they are most effective when they are produced in the immediate area of their target, often times DNA. [17]

$$Fe^{2+} + H_2O_2 \text{ ----> } Fe^{3+} + OH^- + {}^{\cdot}OH$$
$$Fe^{3+} + H_2O_2 \text{ ----> } Fe^{2+} + {}^{\cdot}OOH + H^+$$

Figure 4 Fenton Reagent equation
[22] http://www.h2o2.com/applications/industrialwastewater/fentonsreagent.html

Hydroxyl radical is one of the strongest oxidants known. Since it is highly reactive it attacks membrane lipids, DNA, and other essential cell components which is why it is often used in water waste clean-up because of its ability to kill many microbes. Transition metals are often added to catalyze the formation of the hydroxyl radicals, which is why in the Fenton reagent iron increases the efficacy of hydrogen peroxide [20]

The optimal concentration of H_2O_2 to germinate spores is usually between 2 to 5% but varies between species. [19] For my experiments we used a solution that was 3% H_2O_2.

Hydrogen peroxide is used widely as a method of sterilization. H_2O_2 is both bactericidal and sporicidal but does not leave toxic residues that could adversely affect the product being sterilized. It is well known that hydrogen peroxide's strength increases noticeably with increasing temperatures. At very high concentrations, H_2O_2 can cause a major break up of the spores coat structure, cortex as well as the core. At lower concentrations, H_2O_2 can kill spores as well, just without evident cytological changes.

This shows that low concentrations are sufficient to kill spores and that lytic responses are secondary to the initial killing. [24]

The Fenton reagent combines an iron catalyst with hydrogen peroxide to kill spores of *Bacillus* species. This killing is increased by the addition of a surfactant which will allow the liquid to directly come in contact with many more of the spores.

There are many different hypotheses of why the Fenton Reagent kills spores. One of the many ways is that this reagent kills spores by DNA damage. Another idea is that it acts in the spore core or on an external spore layer to destroy the spore. [17]

Many toxic chemicals or events, such as heat, oxidizing agents, and UV and gamma radiation that are usually used to damage spore DNA are not quite successful. This is due to the fact that the DNA is highly protected by the spore coats, the low permeability of the spore's inner membrane, the core's low water content, and the saturation of spore DNA. The DNA is generally not damaged when the spore is killed by many of these toxic chemicals.[17]

In one study that was done to see the killing time of hydrogen peroxide, it was found that it has a CT time of about five logs in ten minutes. This means virtually all of the spores would be killed (not viable) after just ten minutes.[33] Knowing this information allowed me to conclude that if in that time period hydrogen peroxide kills bacteria it will have an effect on the germination process of spores, and how great of an effect.

Hypochlorite

Sodium hypochlorite (NaOCl) is a compound that is often times used in water purification and tends to be an unstable compound. Sodium hypochlorite is available commercially as a liquid and comes in different concentrations depending on its usage. For domestic use, the commercial products usually contain 5% sodium hypochlorite and have a pH around 11. For more industrial usages it is more concentrated and contains 10-15% sodium hypochlorite with a pH of 13. [23]

Sodium hypochlorite is mainly used as a disinfectant and is effective against bacteria, viruses and fungi. Often times after an anthrax contamination/scare, healthcare workers use standard precautions to thoroughly disinfect all instruments used in an

invasive procedure or autopsy. This involves the use of hypochlorite as a sporicidal agent. Besides anthrax, hypochlorite is also effective in destroying Brucellosis, cholera, as well as inactivating, Ricin-Toxin.[25]

In one study that was conducted to find the mechanisms of killing *B.subtilis* spores by use of hypochlorite it was discovered that hypochlorite does not kill *B. subtilis* spores by DNA damage. This is because of the protective spore coat, which is major factor in a spore's resistance to hypochlorite. Spore killing by hypochlorite appears to make the germination process defective, which could be due to the severe damage to the spore's inner membrane which was observed in the study published in the *Journal of Applied Microbiology.* [26]

The concentration of disinfectant x time(in minutes); is the CT, to achieve a certain level of kill for hypochlorite was measured to be four logs in about seventeen minutes.[34] This also confirms the fact mentioned above, that the line of attack hypochlorite follows on killing spores does in fact make the germination process defective.

UV

Ultraviolet (UV) radiation is often used as a method for inactivating viruses, mycoplasma, bacteria and fungi. It is also successfully used in the destruction of airborne microorganisms. [27]

One study showed the combination of UV and hydrogen peroxide used to kill spores synergistically. Spores that are sensitized to ultraviolet killing by H_2O_2 treatment followed by drying are much less able to absorb and then degrade H_2O_2. The combined effort requires that both agents be present at the same time. The interactions between UV and H_2O_2 most likely occurred close to or within the spore body and enhanced production of hydroxyl radical. [28]

METHODOLOGY

WAYS TO OBSERVE GERMINATION:

When heat activated B. *subtilis* spores lose their dipicolinic acid and become vegetative cells they will not scatter light as efficiently as do spores. As a suspension of spores undergoes germination, an increase in the transmission of light and a decrease in the absorbance is observed. This allowed us to know that all the spores had become vegetative cells and were no longer germinating or vice versa, depending on the results we observed.

Germination of spores can be monitored in a number of other ways as well. In this MQP we observed the increase or decrease in the OD for suspensions of germinating spores.

After conducting these three experiments to record the absorbance I was able to see what effect each of the disinfectants had on the germination process. By viewing a change in the absorbance over time, it is evident that some metabolically related process was occurring in the spore, causing it to change from the characteristic spore shape into another form that does not scatter light as effectively.

The experiments included (a) native spores in water only, (b) spores treated with 3% concentration of just H_2O_2 and same incubation time, (c) spores treated to hypochlorite as well as spore exposed to UV light. (*See below for treatment methods and volumes of each used)*

Materials used:

To conduct the spectrophotometirc assay the spectrophotomer used was *Milton Roy-Spectronic 601*. Set to 600nm throughout the whole experiment.

Bacillus subtilis spores were used from a stock solution of $3.56x10^8$ mL. Agents used were H_2O_2 at a 3% concentration. The sulfur fixer (Rapid Fixer A) was also used in some of the experiments. L-alanine (Sigma) was used to induce germination in the spores. Sodium hypochlorite (commercial bleach) was used in a 1% diluted form to mix into the spore suspention.

A UV light system was used in the UV experiments. UV-transparent cuvettes were used to mix and illuminate the spores.

Techniques and volumes for each of the experiments:

Spores + H_20 + L-alanine

1. Pipet 30 uL of *B. subtilis* spore suspension (3.56 x 10^8/mL) into 1.00 mL of dH_20 into a cuvette.
2. Add 1.25 mL of 20 mM L-alanine to get a final concentration of 10 mM. (for water and spores only do not add L-alanine)
3. Next place the in the spectrophotometer which was set to 600 nm. Zero using a cuvette filled with dH_2O. This is the initial reading.
4. Once the first reading is taken place the cuvette in the 40°C heat block and incubate over a two hour period; taking readings every 10/20 minutes.
5. Repeat the above steps 3 times.
6. Dispose cuvettes in the Biohazards disposal.

(See Figures 6-11- for results)

Spores + H_2O_2

- 35uL spores
- 2.267 H_2O
- 33.3uL H_2O_2

1. Pipeted 35 uL of *B. subtilis* spore suspension (3.56 x 10^8/mL) into 2.267 mL of dH_20 into a cuvette.
2. Add 33.3 uL of 30% H_2O_2.
3. Heat for 10 minutes in the 40°C heat block.
4. Place the cuvette in the spectrophotometer, which was set to 600 nm and take a reading. (Always zero using a cuvette filled with dH_2O)
5. Now incubate in the heat block and take readings every 10/20 minutes over a two hour period.
6. Repeat the above steps three times.
7. Dispose cuvettes in the Biohazards disposal.

(See Figures 23-25 for results)

Spores + H_2O_2, and fixer

- 35uL spores
- 967 dH_2O
- 33.3uL of 3% H_2O_2

1. Pipet 35 uL of *B. subtilis* spore suspension (3.56 x 10^8/mL) into .967 mL of dH_20 into a cuvette.
2. Add 33.3 uL of 30% H_2O_2.
3. Heat for 30 minutes in the 40°C heat block.
3a. Heat for 10minutes.
4. Now add in 1.3mL of Rapid Fixer A to the cuvette. Place this in the spectrophotometer, which was set to 600 nm and take a reading. (Always zero using a cuvette filled with dH_2O)
5. After this initial reading incubate in the heat block and take readings every 10/20 minutes.
6. Repeat the above steps three times. Do for both of the times stated in step 3.
7. For a control: using the Fixer and H_20_2 do the same steps except *do not add spores*. (This was to see the reaction between the two and if the sulfur precipitate will affect absorbance). So in a cuvette only mix 1.3 fixer + 1.0 dH_2O + 33.3uL H_2O_2 and then incubate in the heat block and take readings every 10/20 minutes for a two hour period.
8. Dispose cuvettes in the Biohazards disposal.
(See Figures 16-22 for results)

Spores + hypochlorite

- 1.15 H_2O
- 35uL spores
- 23uL NaOCl

1. Pipet 35 uL of *B. subtilis* spore suspension (3.56 x 10^8/mL) into 1.15 mL of dH_20 into a cuvette.
2. Add 23 uL of NaOCl.
3. Heat for 10 minutes in the 40°C heat block.
4. To the heated mixture, now add 1.15 L-alanine.
5. Place the cuvette in the spectrophotometer, which was set to 600 nm and take a reading. (Always zero using a cuvette filled with dH_2O)
6. Now incubate cuvette in the heat block and take readings every 10/20 minutes for a two hour interlude.
7. Repeat the above steps three times.
8. Dispose cuvettes in the Biohazards disposal.
(See Figures 26-31 for results)

Spores irradiated in UV light

- 35uL spores
- 1.25mL dH20
- Irradiated for 20 mins
- For the control add 1.25mL dH_2O

Or add in 1.25 mL L-alanine

1. Pipet 35 uL of *B. subtilis* spore suspension (3.56 x 10^8/mL) into 1.25 mL of dH_20 into a cuvette.
2. Place on the UV block for 20 min.
3. Add 1.25 mL of dH_2O (*for control*)
3a. Add 1.25mL of L-alanine for another experiment.
4. Place the cuvette in the spectrophotometer, which was set to 600 nm and take a reading. (Always zero using a cuvette filled with dH_2O)
5. Now incubate in the heat block and take readings every 10/20 minutes for a two hour interlude.
6. Repeat the above steps three times for each mixture 3 and 3a.
7. Dispose cuvettes in the Biohazards disposal.

(See table 32-37 for results)

Results

SPORES and L-alanine

Figure 5 Spores and L-alanine

Time	Absorbance
0	0.96
10	0.95
20	0.94
30	0.936
45	0.931
60	0.925
75	0.929
90	0.927
105	0.931
120	0.931
135	0.93

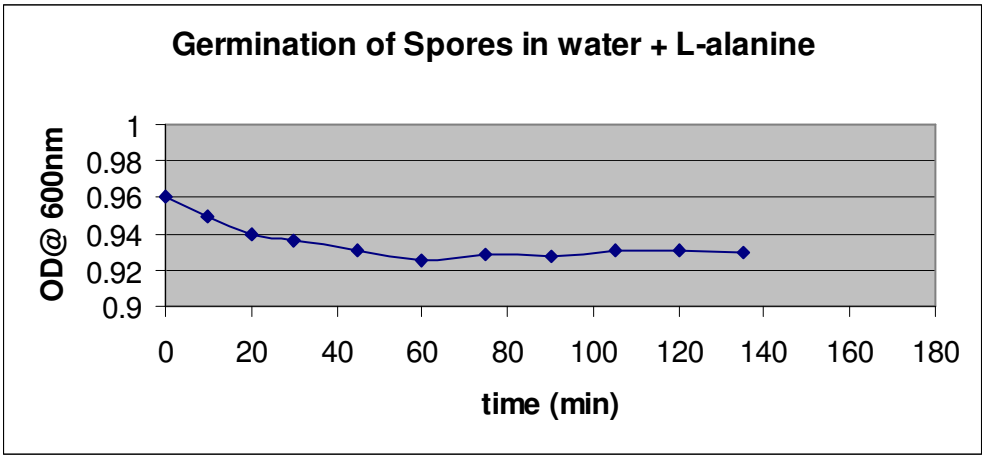


Figure 6 Spores and L-alanine

Time	Absrobance
0	0.958
10	0.968
20	0.959
30	0.947
45	0.945
60	0.934
75	0.933
90	0.937
105	0.939
120	0.946
135	0.944

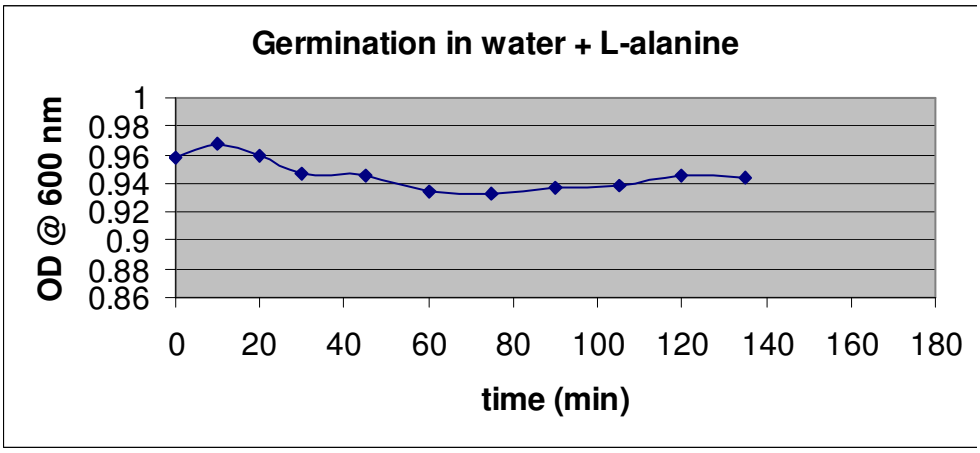
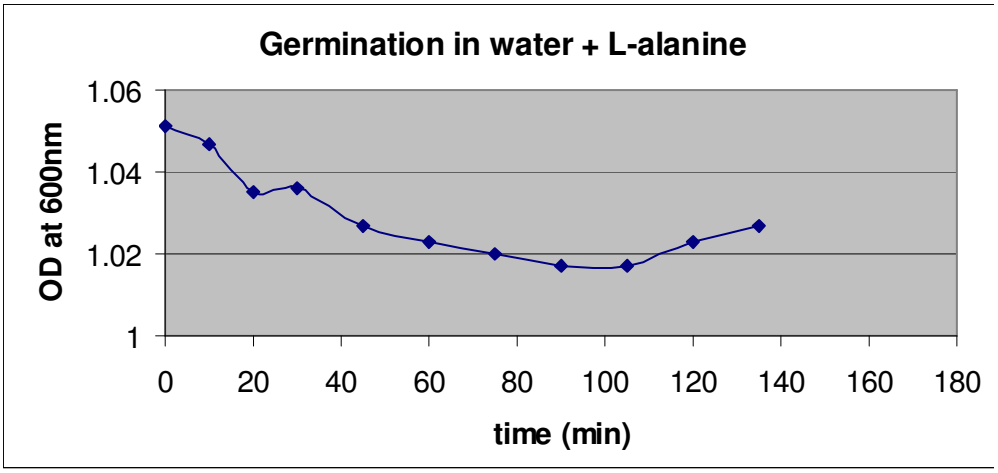


Figure 7 Spores and L-alanine

Time	Absorbance
0	1.051
10	1.047
20	1.035
30	1.036
45	1.027
60	1.023
75	1.02
90	1.017
105	1.017
120	1.023
135	1.027



Spores and Water

Figure 8 Spores and water

Time	Absorbance
0	1.215
10	1.198
20	1.189
40	1.182
60	1.176
80	1.167
100	1.173

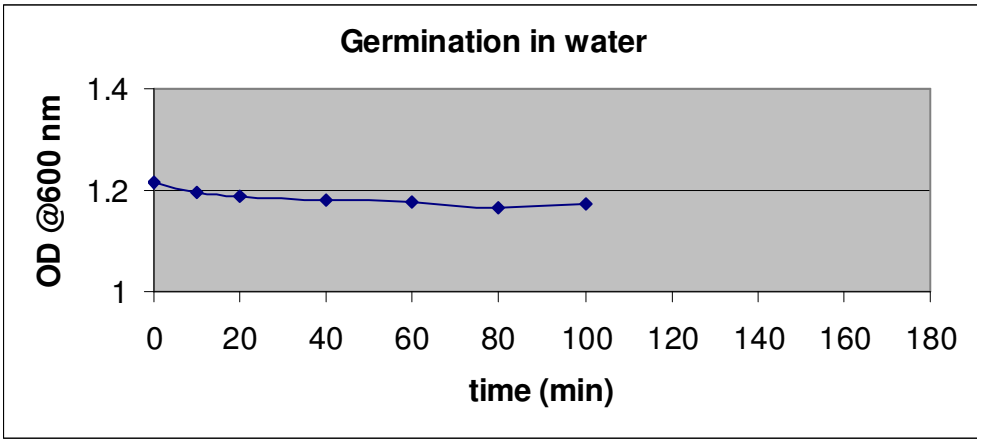


Figure 9 Spores and water

Time	Absorbance
0	1.024
10	0.995
20	0.984
40	0.982
60	0.972
80	0.967
100	0.954

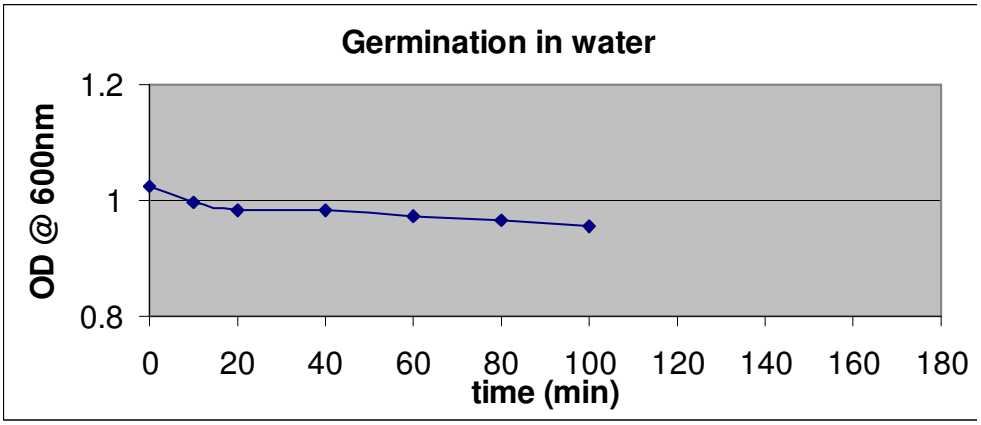
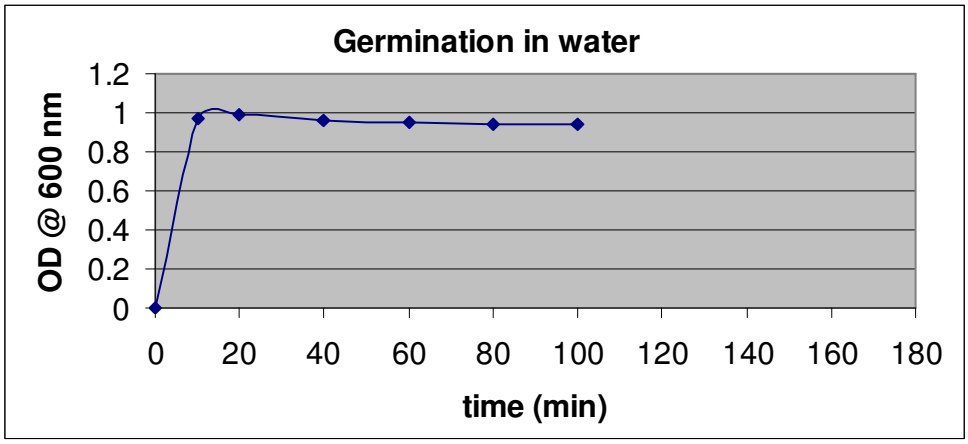


Figure 10 Spores and water

Time	Absorbance
0	0
10	0.973
20	0.986
40	0.962
60	0.955
80	0.945
100	0.939



Hydrogen Peroxide Test runs- H_2O_2 (incubated for X min) + Fixer

Figure 11 Hydrogen Peroxide test runs (10 min)

Time	Absorbance
0(prefixer)	1.7
10	2.907
20	2.726
40	2.050
60	1.469
80	1.117
100	.976
120	.861

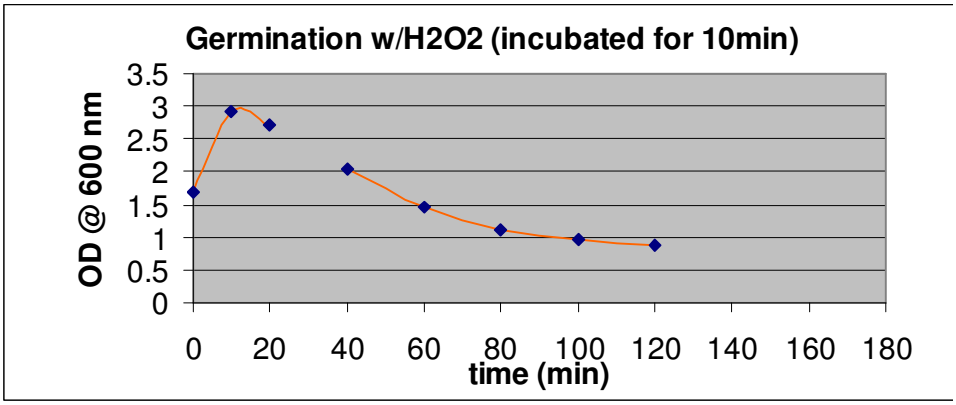


Figure 12 Hydrogen Peroxide test runs (20 min)

Time	Absorbance
0(prefixer)	1.69
10	2.685
20	2.149
40	1.591
60	1.286
80	1.230
100	1.110
120	.861

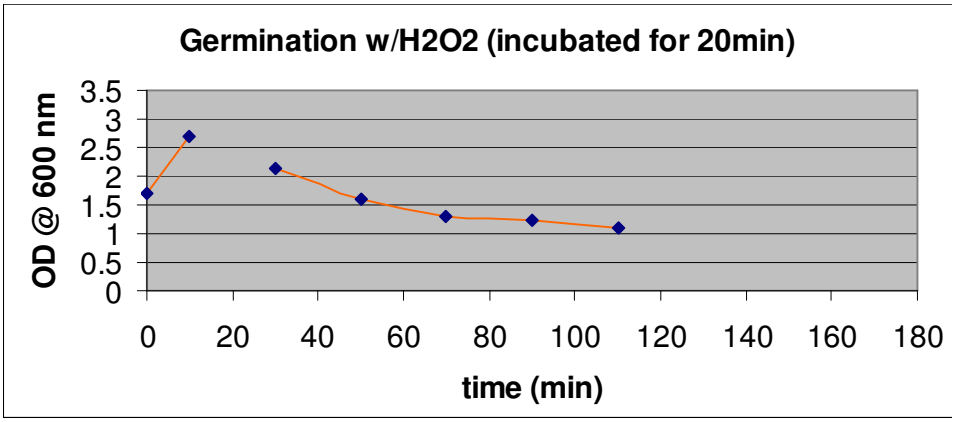


Figure 13 Hydrogen Peroxide test runs (30 min)

Time	Absorbance
0(prefixer)	1.698
10	2.695
20	2.402
40	1.649
60	1.385
80	1.232
100	1.040
120	577

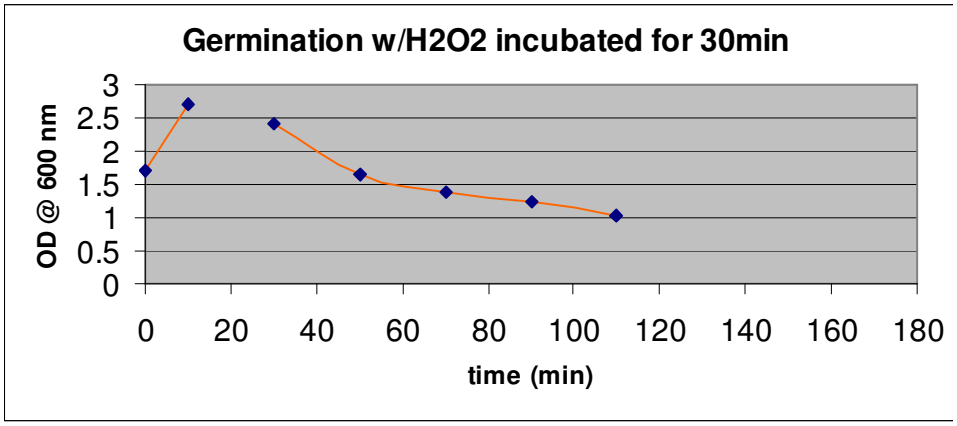
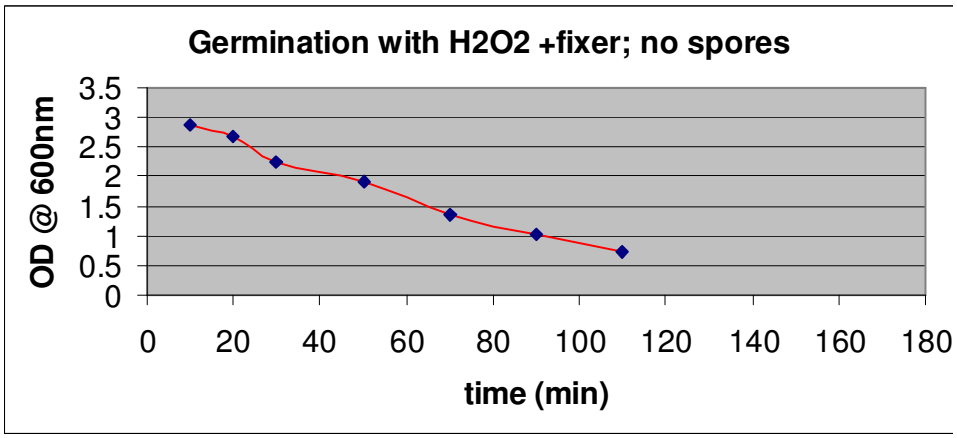


Figure 14 Hydrogen Peroxide + fixer-no spores

Time	Absorbance
0(prefixer)	
10	2.883
20	2.688
40	2.230
60	1.914
80	1.342
100	1.034
120	.731



HYDROGEN PEROXIDE (30MINS INCUBATION) + FIXER

Figure 15 Hydrogen Peroxide (30 min) + Fixer

Time	Absorbance
0	1.678
10	2.889
30	2.757
50	2.208
70	1.569
90	0.862
110	0.744

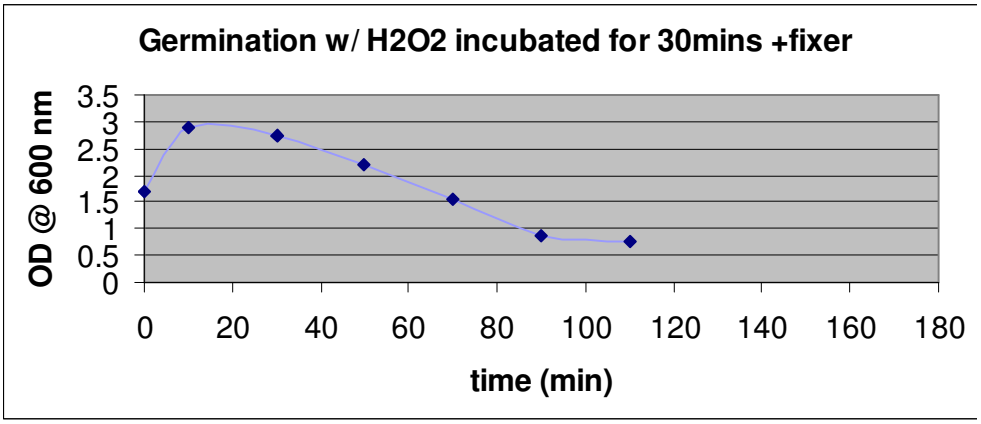


Figure 16 Hydrogen Peroxide (30 min) + Fixer

Time	Absorbance
0	1.643
10	2.937
30	2.765
50	2.27
70	1.649
90	0.941
110	0.753

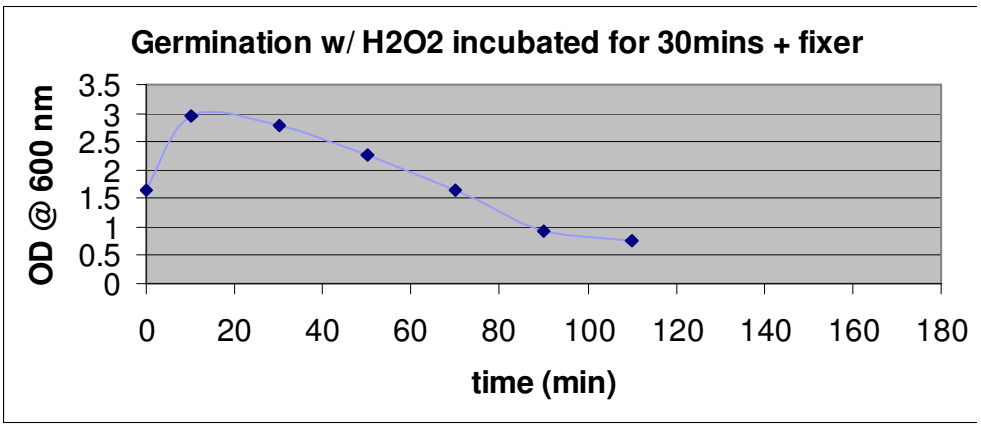


Figure 17 Hydrogen Peroxide (30 min) + Fixer

Time	Absorbance
0	1.722
10	2.867
30	2.763
50	2.095
70	1.165
90	0.78
110	0.636

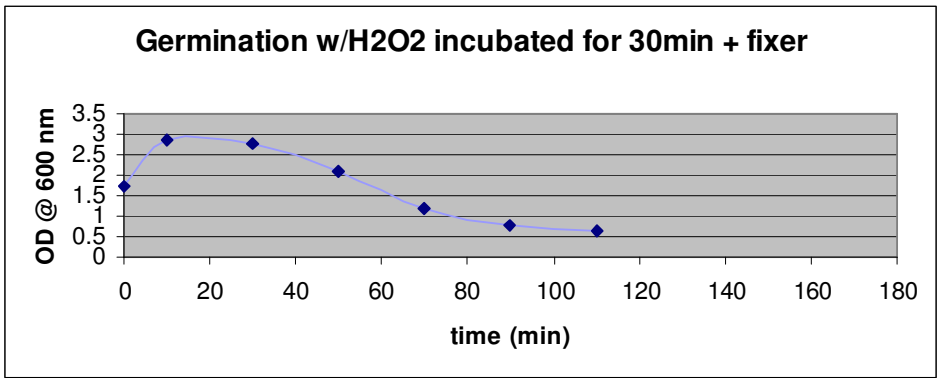
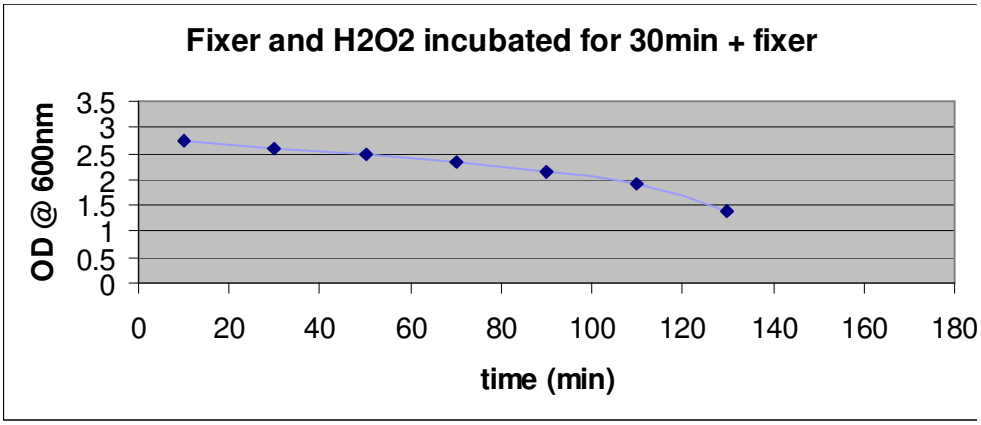


Figure 18 Hydrogen Peroxide (30 min) + Fixer

Time	Absorbance
0	
10	2.738
30	2.593
50	2.48
70	2.34
90	2.13
110	1.914
130	1.388



HYDROGEN PEROXIDE (10 MINS INCUBATION) + Fixer

Figure 19 Hydrogen Peroxide (10 min) + Fixer

Time	Absorbance
0	1.845
10	2.967
20	2.877
40	2.432
60	1.6
80	1.224
100	1.202
120	1.145
200	1.41

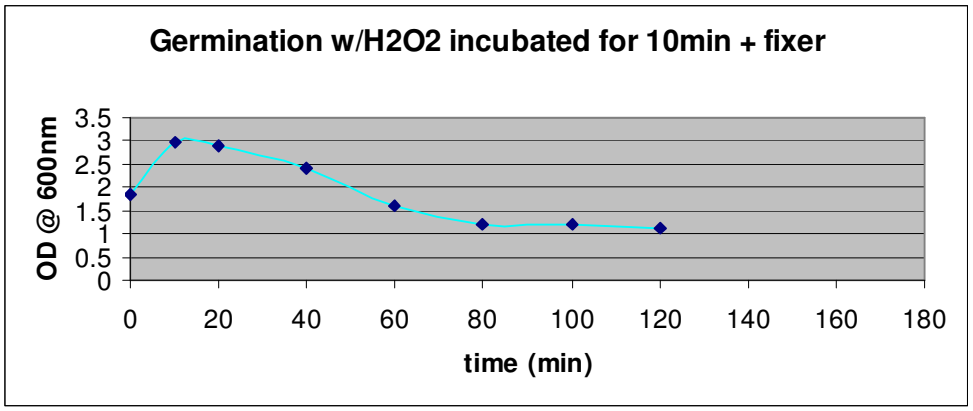


Figure 20 Hydrogen Peroxide (10 min) + Fixer

Time	Absorbance
0	1.704
10	2.921
20	2.831
40	2.593
60	1.915
80	1.343
100	1.094
120	0.881
200	1.378

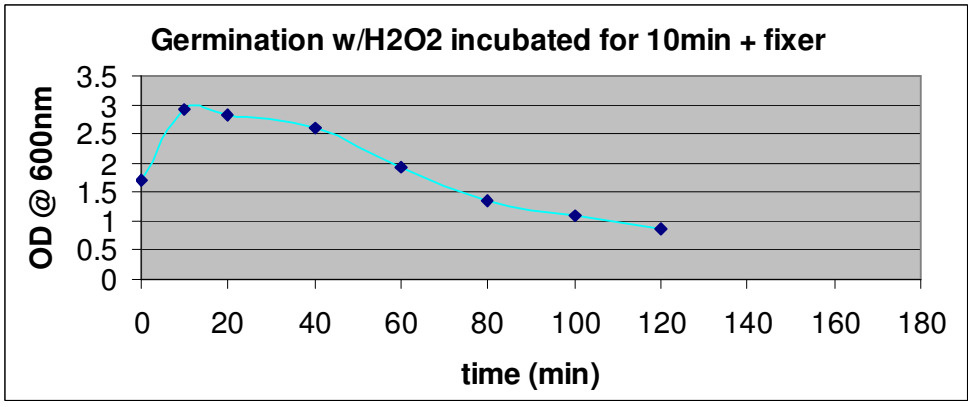
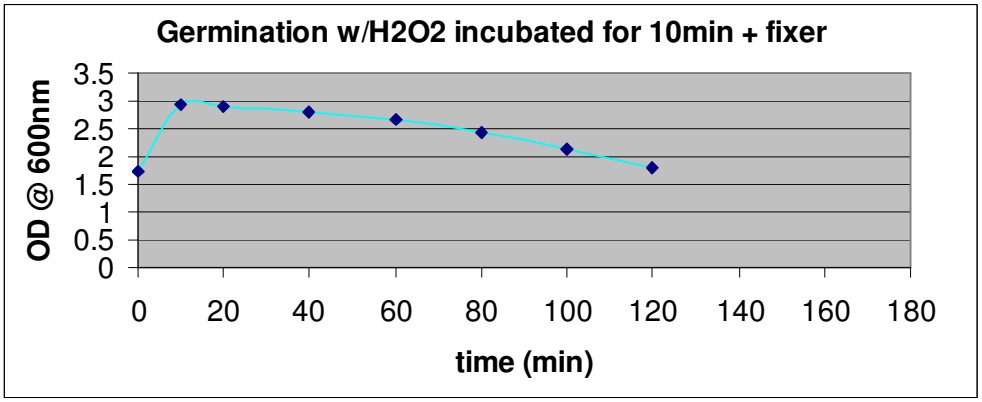


Figure 21 Hydrogen Peroxide (10 min) + Fixer

Time	Absorbance
0	1.729
10	2.949
20	2.89
40	2.816
60	2.664
80	2.441
100	2.127
120	1.801
200	0.803



HDYDROGEN PEROXIDE + SPORES

Figure 22 Hydrogen Peroxide

Time	Absorbance
0	1.027
10	1.027
20	1.034
30	1.03
40	1.025
50	1.027

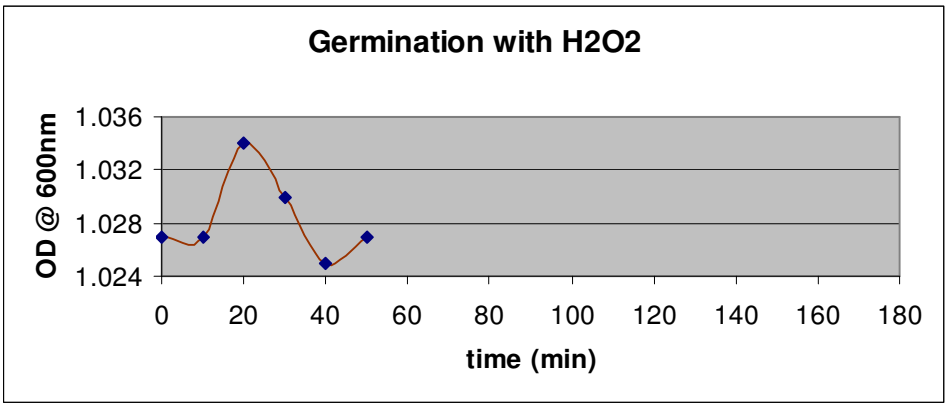


Figure 23 Hydrogen Peroxide

Time	Absorbance
0	0.957
10	0.971
20	0.969
30	0.965
40	0.963
50	0.969

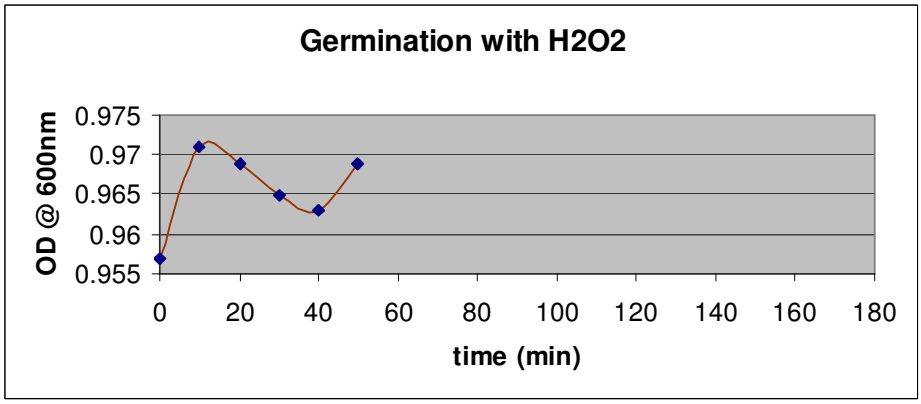
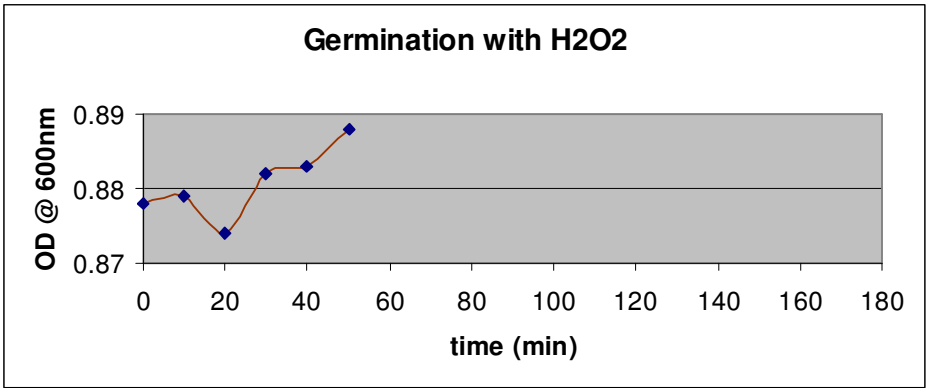


Figure 24 Hydrogen Peroxide

Time	Absorbance
0	0.878
10	0.879
20	0.874
30	0.882
40	0.883
50	0.888



HYPOCHLORITE

Figure 25 Hypochlorite

Time	Absorbance
0	0.721
10	0.713
20	0.707
40	0.711
60	0.707
80	0.703

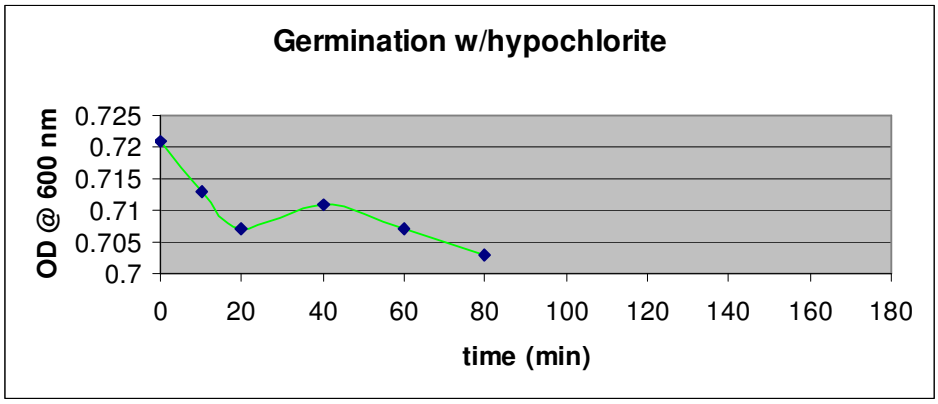


Figure 26 Hypochlorite

Time	Absorbance
0	0.712
10	0.657
20	0.571
40	0.383
60	0.319
80	0.296

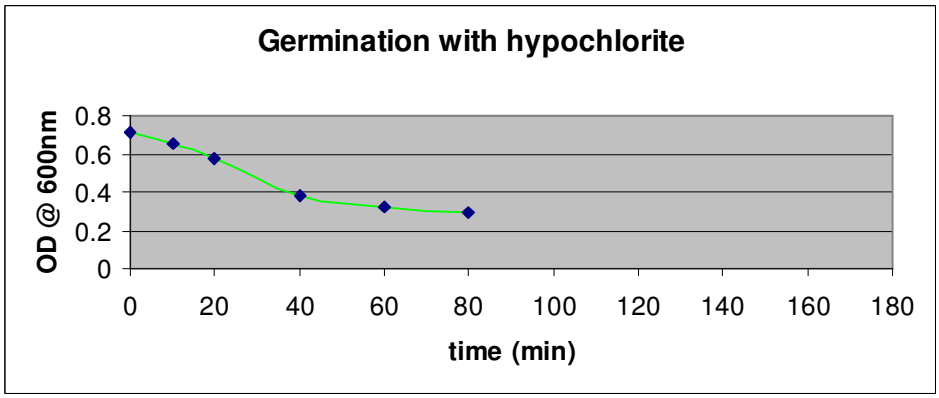


Figure 27 Hypochlorite

Time	Absorbance
0	0.787
10	0.781
20	0.77
40	0.773
60	0.768
80	0.763

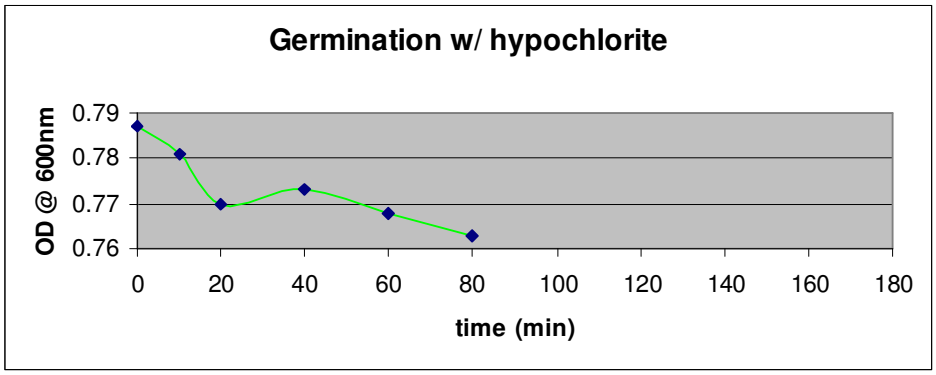


Figure 28 Hypochlorite (2nd time)

Time	Absorbance	
0	0.975	
10	0.961	
20	0.949	mixed
40	0.944	1.009
60	0.985	
80	0.964	
100	0.939	

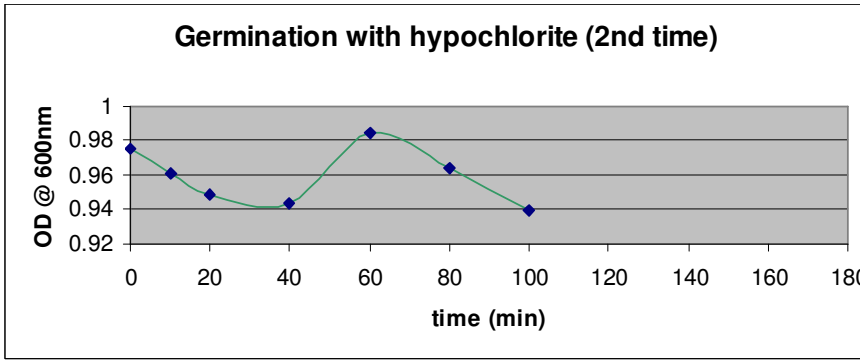


Figure 29 Hypochlorite (2nd time)

Time	Absorbance	
0	1.097	
10	1.085	
20	1.065	mixed
40	1.053	1.103
60	1.073	
80	1.052	
100	1.034	

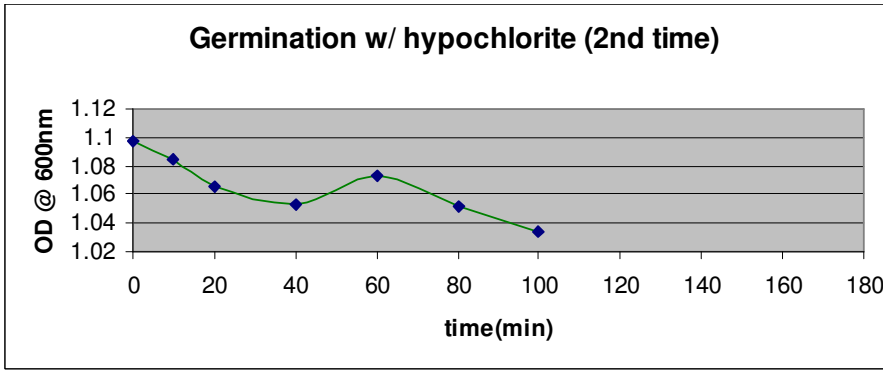
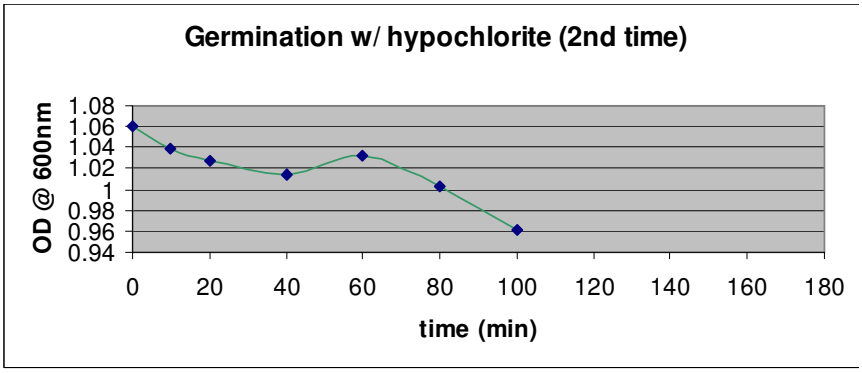


Figure 30 Hypochlorite (2nd time)

Time	Absorbance	
0	1.06	
10	1.038	
20	1.027	mixed
40	1.014	1.073
60	1.033	
80	1.002	
100	0.961	



UV WITH WATER

Figure 31 UV and water

Time	Absorbance
0	0.944
10	0.943
20	0.931
30	0.928
40	0.923
60	0.918
80	0.907
100	0.93
120	0.933

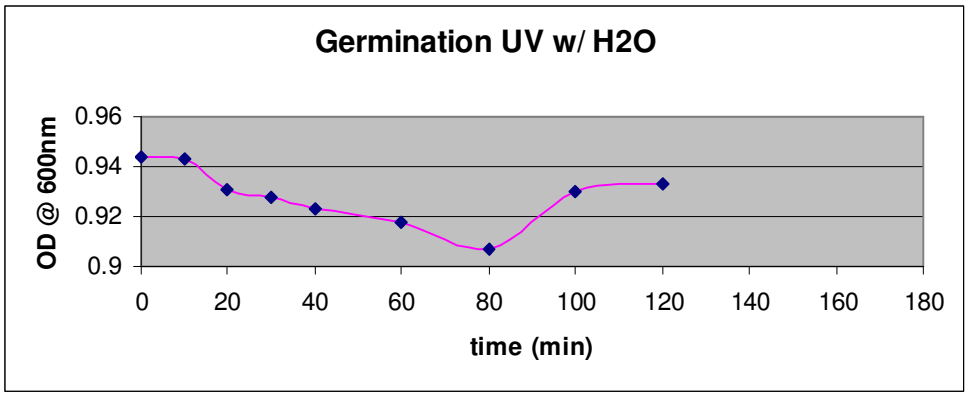


Figure 32 UV and water

Time	Absorbance
0	0.924
10	0.918
20	0.91
30	0.905
40	0.898
60	0.892
80	0.878
100	0.868
120	0.883

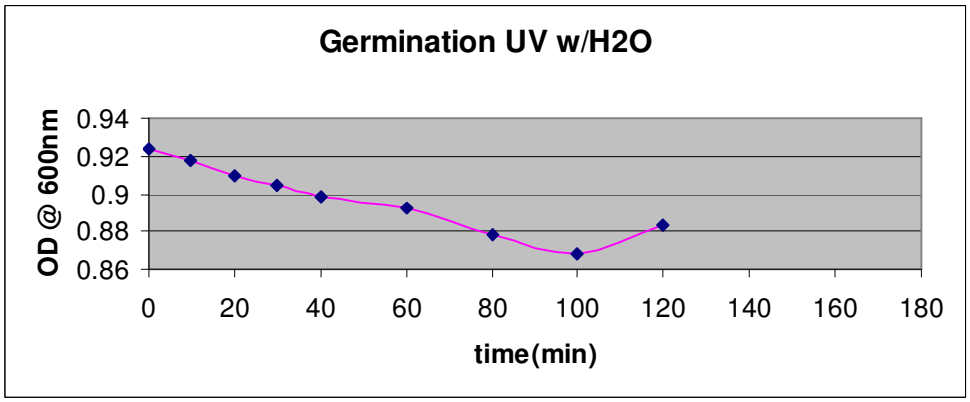
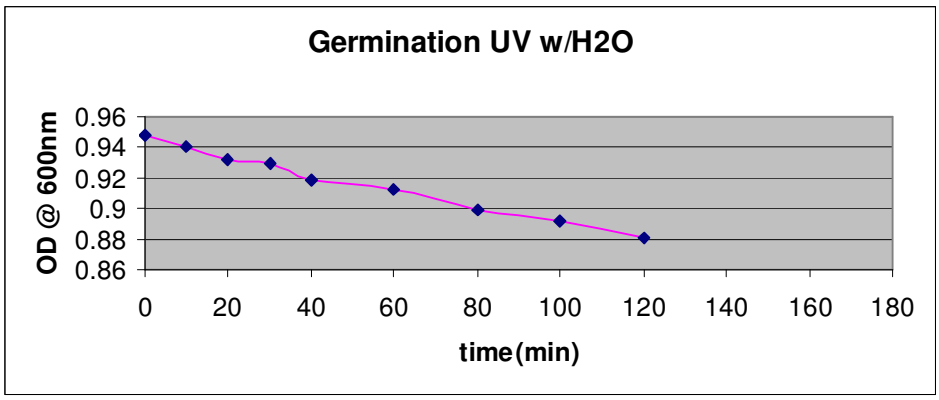


Figure 33 UV and water

Time	Absorbance
0	0.948
10	0.941
20	0.932
30	0.929
40	0.919
60	0.912
80	0.899
100	0.892
120	0.881



UV and L-Alanine

Figure 34 UV and L-alanine

Time	Absorbance
0	1.039
10	1.031
20	1.191
40	1.004
60	0.992
80	0.977
100	0.958
120	0.958

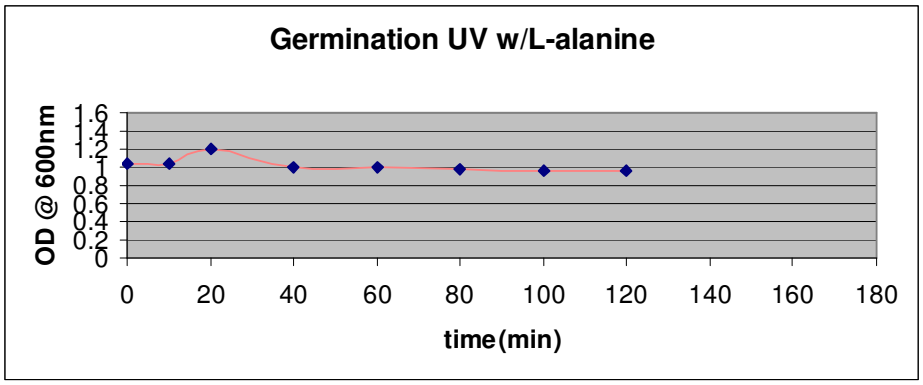


Figure 35 UV and L-alanine

Time	Absorbance
0	0.985
10	0.98
20	0.966
40	0.957
60	0.94
80	0.93
100	0.91
120	0.923

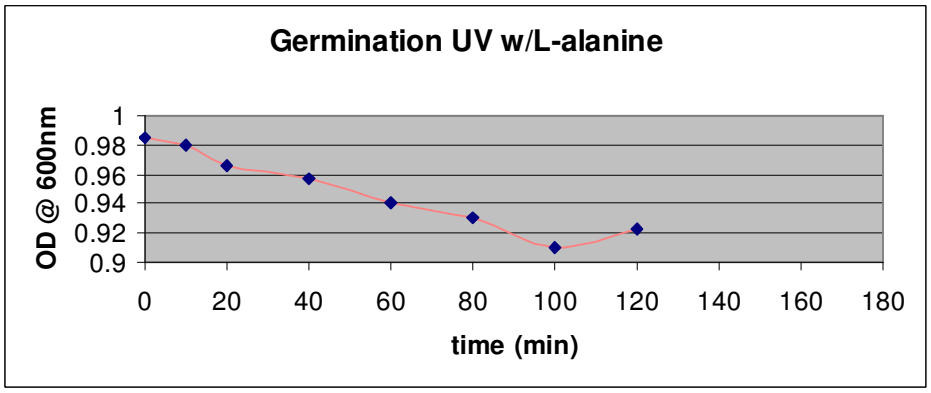
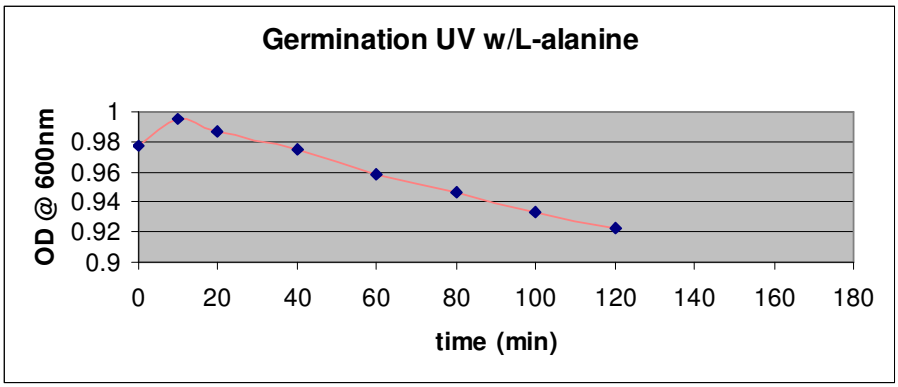


Figure 36 UV and L-alanine

Time	Absorbance
0	0.977
10	0.995
20	0.987
40	0.975
60	0.958
80	0.946
100	0.933
120	0.923



Discussion/Conclusions

Spore germination is an extremely complex process and requires a number of biochemical interactions within the spore. Many agents are known to have an affect on any number of these interactions. All of the agents used in this MQP (L-alanine, H_2O_2, hypochlorite, UV) are known to affect the spore and the germination process in a different way.

The results I got from this MQP all show that each of these disinfectants had some effect on the germination process. This indicated some activity was occurring within the spore, in response to the added disinfectant.

In Figures 6-8, in which the spores were mixed with L-alanine the absorbance remained relatively steady, demonstrating not much activity in the spores' biochemical make-up was occurring over the given time period. L-alanine is known to affect the germination process, by acting as an activator of certain enzymes to begin the germination process. This could explain the dip in some of the curves in the graphs.

In Figures 9-11 spores were only measured in water which showed no apparent affect on the germination process. Compared to when L-alanine was added into the suspension there was a lot less activity, since there was no inducer of germination to affect the germination process.

Figures 12-15 represents the 'test run' experiments done with hydrogen peroxide. These were done to see what the different heating times would have on the spores when mixed with hydrogen peroxide. After the initial heating period (of either 10, 20 or 30 minutes) spores were mixed with a Rapid Fixer A to remove all the hydrogen peroxide. This fixer seemed to cause a precipitate to form therefore not giving good results. I was not able to determine what absorbance I was reading, the precipitate caused an interference with the spores' absorbance.

The test run times selected were incubating for 30min (Figures 16-19) and 10 min (Figures 20-22). Though the incubation time was different,the graphs appear to be the same. All the graphs show an increase when the hydrogen peroxide was added, and then over time a decrease in absorbance. These decreases could have been due to the fact that a sulfur precipitate was forming once the fixer was added, so these results are unclear as

to what was being measured. In the next experiments only hydrogen peroxide was added to observe its affect with no interfering percipitate.

Figures 23-25 show the effect of hydrogen peroxide alone. Since spores were mixed with hydrogen peroxide which is a known oxidant, there was activity occurring; due to the change in absorbance recorded over the given time. Hydrogen peroxide is known to have a CT time of about 5 logs in 10mins. This means virtually all of the spores would be killed (not viable) after 10mins [33].

Figures 26-31 represents the data for the addition of hypochlorite, a strong disinfectant known to destroy the spore viability. In these figures it is much more apparent that something is occurring to the spores in the suspension. The recorded absorbencies were at a much lower number compared to the control of spores and water. The absorbance decreased over time, much more quickly then in the other experiments indicating the spore is less refractile and can not scatter light as efficiently anymore. The CT for hypochlorite was measured to be 4 logs in about 17 minutes[34]. This helps to conclude that hypochlorite acts quickly on the germination process affecting the spore and its enzymatic activities.

Figures 32-34 describes the experiment when the spores were exposed to UV in a solution of water only, and they became much less spore-like over time. This is known due the fact that UV irradiation for 30 minutes is known to kill all of the spores by DNA damage and although it was not tested for in this MQP whether or not the spores were dead, it is apparent from the graph that the UV light did have an effect on their germination process.

Figures 35-37 show spores when exposed to UV light but this time with L-alanine present. L-alanine is known as an initiator of the germination process. UV irradiation results in the formation of spore photoproduct, (SP) 5-thyminyl-5,6-dihydrothymineis which can effect the absorbance of the spore suspension [27] Since UV is acting to destroy the spores' DNA and L-alanine is initiating germination it is obvious there would be some reaction occurring with in the spore, thus affecting its appearance and ability to scatter/absorb light.

Over all conclusions

After viewing the results for each experiment, it is apparent that each reagent had some affect on the spore's form. L-alanine is known the have an affect on the germination process by initiating certain genes and biochemical pathways that begin the whole process. In the results it is apparent that there was activity occurring once it was added into the spore suspension. There is continuous activity in the absorbance indicating activity within the spore suspension.

Results from just spores and water are expected to have a steady absorbance rate, since there was no initiator present to stimulate the germination process. From the graphs this remained true, since in each one there was very little change in absorbance. This means the spore kept its distinct form throughout the entire time of observation.

For the hydrogen peroxide with fixer experiments there should have been some activity due to the fact that hydrogen peroxide is known to oxidize contaminants, which would have an effect on the spore and its form, thus affecting the absorbance measured. Once the fixer was added to help remove the H_2O_2 present in the spore suspension, it appeared that some precipitate was forming due to the increased cloudiness and build up in the cuvette. Therefore it is unclear of the effect the hydrogen/fixer combination had on the spores' germination process. In the control experiment (Figure 19), which had no spores, a change in absorbance was observed. This shows some precipitate formed and had an effect on the absorbance recorded.

Since the fixer had an effect of its own on the spore suspension, hydrogen peroxide by itself was used. Since it is such a strong oxidizing agent and often times used as a disinfectant it is known to have some effect on the spore and its germination process. At high concentrations, H_2O_2 can cause a major break up of the spores coat structure, cortex as well as the core all of which will have an effect on the spores structure. If a change in the structure has occurred this can affect the absorbance, which can be observed in the graphs. The absorbance, though not a major change, was extremely unsteady, with high and low peaks being recorded.

Hypochlorite is often times used as a sporicidal due to its ability to kill bacteria. Spore killing by hypochlorite appears to make the germination process defective, which could be due to the severe damage to the spore's inner membrane. The graphs from the experiments done using hypochlorite had the lowest absorbance and most decreased absorbance measured. This indicates that hypochlorite had an effect on the spore, leaving it non-viable since its typical spore form has been disrupted.

When the spores were irradiated on the UV block it is assumed that there will be a change in absorbance due to the fact that UV light has an effect on the germination process of spores. In the experiments done using only water and spores there was no additional agent added as in the experiments done with L-alanine. These graphs show that the UV had more of an affect on the spore and spore structure due to the change in absorbance over time. When L-alanine was added into the solution after the spores had been UV treated there was less of an obvious change. This could be due to the fact that the L-alanine might have initiated germination process; allowing the spore to maintain their distinct spore form and not affecting the absorbance as much.

Future Research

Spores of pathogens such as *Bacillius anthracis*, have been used in the past as biological weapons. It is becoming more and more of a concern of future attacks and how to detect them before they occur. The next task, after the exact pathways in which a spore germinates have been discovered, is to detect spores in a rapid and effective manner. One way to identify spores, before they have a chance to cause any harm, would be to modify a procedure that can be used to detect many spores by use of fluorescence microscopy.

The use of this technique would replace the currently used spore assays which involve a time consuming protocol and allow it to be used to monitor for the presence of *B. anthracis* spores in airborne releases.

Spores have a unique chemical that is specific to only spores, called dipicolinic acid, commonly referred to as DPA. There is currently no one simple/quick test that will allow someone to observe spores, but one would think that if there was a way to test for DPA it would be extremely easy and quick to identify and then to quickly eradicate the spores before they cause any harm.

It is known that terbium fluoresces at about 490 or 545nm when irradiated at 240nm while under a florescence microscope and is viewed in the green spectra around 540nm. It has also been shown that terbium and DPA will combine to form a [Tb(dpa)] complex. The main idea behind this future MQP is to have the terbium cation bind to the DPA anion derived from *Bacillus* spores. This MQP would allow to test and observe the DPA-terbium complex fluorescing at 540nm which is sufficient to be seen under the microscope and then use this as a way to help detect spores.

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