April 2007

Extraction of Chlorophyll using Dimethyl sulfoxide and Acetone

Kurtis R. Allain
Worcester Polytechnic Institute

Extraction of Chlorophyll using Dimethylsulfoxide and Acetone

A Major Qualifying Project Report:

submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

by

_____

Kurtis Allain

Date: April 26, 2007

Approved:

_____

Professor Ted Crusberg, Advisor

TABLE OF CONTENTS

ABSTRACT

Chlorophyll samples were extracted from Nostoc using various concentrations of dimethyl sulfoxide (DMSO) and acetone. The samples were exposed to a range of light and heat conditions and analyzed using a spectrophotometer. Visible spectra Plots were generated to show the most effective extraction method, which was then used as the standard for experiments using secondary models. Acidification experiments were then conducted to quantify the conversion transfer from chlorophyll to pheophytin.

ACKNOWLEDGMENTS

I would like to firs and foremost thank my advisor Ted Crusberg Ph.D for his guidance and support throughout this project. I would also like to thank him for allowing the use of his lab. I would like to thank the chemistry department for letting me use their spectrophotometer. And finally, I would like to thank my family and friends for all the support they have given me here at WPI.

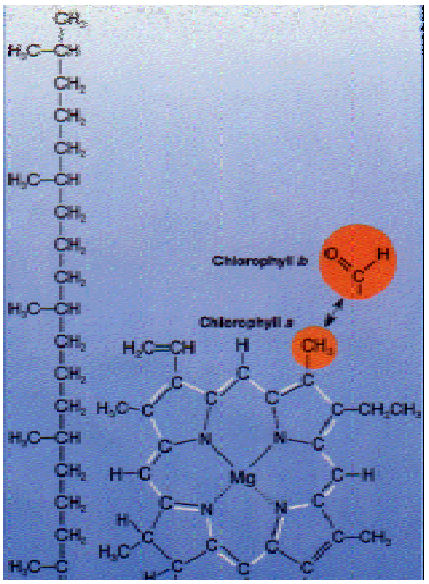
LIST OF FIGURES

Introduction

Chlorophyll is a green photosynthetic molecule found in most plants, algae and cyanobacteria. Chlorophyll absorbs sunlight and uses the energy to "fix" carbon dioxide to yield carbohydrates and oxygen. This process is known as photosynthesis, and is the driving force of life in plants. Chlorophyll is a cyclic tetrapyrolle, which is similar in structure to that of hemoglobin with the exception that the central metal is magnesium versus iron.

There are two main types of chlorophyll found in most plants and most cyanobacteria, chlorophyll a and chlorophyll b in the ratio of 3:1 respectively. The difference between chlorophyll a and b is the methyl side chain in a, is replaced by a formyl group in b.

Figure 1: Structures of chlorophyll a and b



http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/Chlorophyll.html

Located within most photosynthetic organisms, in contrast to chlorophyll a and b are several non-chlorophyll accessory pigments such as carotenoids. Carotenoids also absorb light and transfer the energy to an alternate photosystem. Carotenoids also serve as antioxidants by way of dissipating excess light energy. Most of the chlorophyll and non - chlorophyll pigments have different spectra for one of two reasons. First, the spectra of the different pigments are modified by their local protein environment or the intrinsic properties of the accessory pigments are different from those of chlorophyll. This is important when performing analysis on the spectra of the various pigments located within plants.

PHOTOSYSTEMS

Chlorophyll molecules are arranged in a specific manor within and around protein complexes called photosystems. A photosystem, also called a reaction center, is an enzyme (located within the chloroplasts) that uses the light it receives from an alternate source to reduce molecules within the membrane. Within this photosynthetic membrane, photosystems provide the driving force for the bioenergetic proton and electron transfer chain. There are two different types of reaction centers; photosystem 1 and photosystem 2. Photosystem 1 generally works in conjunction with Photosystem 2, which causes an oxidation reaction of water generating O_2 and H^+ (through several intermediates). Therefore photosystem 1 is usually reduced, also through several intermediates within the thykaloid membrane by the electrons generated from photosystem 2. The flow of electrons produced by the reaction center is used to shuttle H^+ across the thykaloid membrane which allows for a chemiosmotic potential used to generate ATP. $NADP^+$ is ultimately reduced to NADPH, which is used to reduce CO_2 into sugars along with other reactions.

The two reaction centers can be differentiated by the wavelength of light to which it is most reactive. Photosystem 1 tends to react more around 700nm and photosystem 2 is more reactive around 680nm. The difference between the two systems can also be determined by the type of terminal electron acceptor located on the molecule. Photosystems that are type 1 use ferredoxin-like sulfur clusters as electron acceptors and type 2 systems use a quinine terminal electron acceptor. Although each system functions differently; they are both found in chloroplasts, cyanobacteria and exhaust oxygen as a byproduct. This is how the oxygen in the earth's atmosphere is generated.

Within the crusts of the desert are several different cyanobacteria, all of which work in conjunction with each other in order to survive. Nostoc, Microcoleus and Scytonem are three examples located within these crusts.

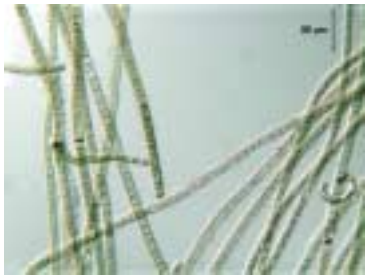
Nostoc is a single cell, fresh water, non-filamentous cyanobacteria that forms spherical colonies, which are easily disassociated when vigorously mixed. The organism is often found at the bottom of lakes and springs, on moist rocks, occasionally in marine habitats and oddly enough, desert crusts. Nostoc also has the ability to grow symbiotically with other organisms often providing nitrogen to the host.

Figure 2: Nostoc microscopy

http://www-cyanosite.bio.purdue.edu/images/images.html

Scytonema is a filamentous blue green that is cylindrical in shape, and occasionally contains tight cross walls and gas vesicles. This organism forms “chains” of cells that are remarkably difficult to separate into single cells. Scytonema is vital in desert crusts because it obtains the ability to produce sunscreens which protect itself as well as other blue greens within the crust from harmful Ultraviolet (UV) rays.

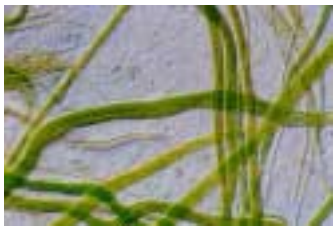
Figure 3: Scytonema microscopy



http://silicasecchidisk.conncoll.edu/LucidKeys/Carolina_Key/html/Scytonema_Main.html

Microcoleus is a filamentous cyanobacteria also found in desert crusts. These organisms provide a polysaccharide sheath that surrounds adjacent biological life and intertwines with the surrounding soil particles. Belnap and Gardner (1993) found that Microcoleus is a very important constituent in the formation of desert crusts which greatly decrease the velocity of surface water and hinder erosion. Belnap and Gardner (1993) also found that the sheaths generated by the organism obtain the ability to absorb around eight times its weight in water. This is an incredibly important feature due to the fact that water is very limited in the desert. It was also observed that when the sheath was exposed to water, it would swell and cover surfaces more extensively. These findings show that this organism plays a vital role in the formation of crusts in the dessert. Hendrickson and Kubly (1984)

Figure 4: Microcoleus microscopy



http://microbes.arc.nasa.gov/gallery/lightms.html

These three organisms combined provide the necessary tools to thrive in the desert. The Nostoc provides the nitrogen to be "fixed" by other organisms. The Scytonema provides the UV blocking sunscreens needed to survive the extreme conditions in the desert; and the Microcoleus generates the scaffold needed to stabilize the soil.

EXPERIMENTAL CONDITIONS

In order to conduct lab experiments that accurately reflect the conditions in which these organisms are found, the organisms must be able to interact with one another in appropriate proportions of Nostoc, Microcoleus and Scytonemia, 1:8:1 respectively. There are several ways to quantitate the amount of cells to be added to an experimental culture, some of which are easier than others. One possible way to achieve this is cell counting. Since Nostoc is a single celled non-filamentous organism that separates easily, this process can easily be done. However, Microcoleous and Scytonema are fillamentous organisms that string together and barely break apart in a Waring blender, counting individual cells is unrealistic. An alternate solution would be to quantitate the protein content within each sample, although this requires specialized techniques and is not easily done. The mass of cells could also be an alternative form of measurment for Nostoc but again, Microcoleous and Scytonemia are very difficult to separate and therefore quantitate accurately. It was found that the easiest, shortest, most inexpensive and most reasonable way to quantitate the amount of cells for these cultures would be to normalize a chlorophyll extraction assay that can quickly be performed and therefore determine the amount of cells, which is directly corilated to the amount of chlorophyll within the sample.

Chlorophyll a was extracted from a primary model Nostoc and later two other genera of cyanobacteria, Microcoleous and Scytonema. The chlorophyll was extracted from a 1 mL alloquot of cell suspension using a 1.5mL to 8.5mL ratio of dimethylsulfoxide (DMSO) and acetone, respectively. The samples were then incubated for thirty minutes (to facilitate the chlorophyll extraction) and then put in a tissue grinder to sheer the cell walls. The resulting solution was then analyzed by way of spectrophotometry.

Because chlorophyll, along with Scytonemia and Microcoleous are sensitive to various "environmental" influences; each sample was exposed to a variety of light conditions which include exposure to natural light, artificial light and the absence of white lite. This experiment was conducted in order to determine and account for any and all affects incurred by the samples due to light within each model. The chlorophyll may turn into pheophytin and the Scytonema may produce UV sunscreens that may interfere with the spectrophotometric analysis. The spectrophotometry results were then entered into excell and plotted into graph form.

There are various conditions that may cause chlorophyll to degrade into pheophytin, (which absorbs light in the same region of the spectrum as chlorophyll) which may cause error in the absorption spectrum. Because this situation is not conducive to accurate results, the concentration of chlorophyll and pheophytin must be determined. This can be done by determining the ratio of chlorophyll to pheophytin, which requires the process of acidification. During acidification HCL was added to a chlorophyll sample, which forces the transfer to pheophytin. When this is completed, the absorbance prior to acidification (Rb) is related to the absorbance after acidification (Ra) to form the acid ratio Rb/Ra.

The purpose of these experiments was not necessarily obtain a complete extraction of chlorophyll a from all three models, but to normalize a method which would provide a standard approach to defining the initial composition of cyanobacteria added to each culture.

MATERIALS AND METHODS

The three genre of cyanobacteria (Nostoc, Microcoleus and Scytonema) were grown for 4-6 weeks in the Bioprocess Lab under low light. Roughly ½ of each culture was transferred from their respective growth flasks and homogenized using a 20mL Warring Blender for 1 minute at room temperature. Using a solution of DMSO and acetone in a respective ratio of 1.5 to 8.5, chlorophyll was extracted from all three models.

A 1mL suspension of primary model Nostoc was placed into a 15mL plastic conical centrifuge tube and centrifuged for ten minutes. The supernatant was poured off and the pellet was washed 2X in 10mL dH_2O. 1.5mL DMSO was added and the pellet was put back into suspension. The mixture was then transferred into a tissue grinder which was operated 20 times. This was performed to aid the extraction of chlorophyll. Once grinded, the solution was incubated at 50°C for 30 minutes. The liquid was then removed from the tissue grinder by pipetting into a 15 mL conical centrifuge tube and the tissue grinder was washed 3X with a total of 8.5 mL of spectroscopic grade acetone. The solution was allowed to stand for a short period of time prior to an additional 10 minutes of centrifugation in a swinging bucket clinical centrifuge. The supernatant was then transferred into 1 cm quartz cuvettes and analyzed by way of spectrophotometry (using a

Beckman DU650) at wavelengths between 640nm and 675nm, one of the two prominent peaks of chlorophyll a. The results were then recorded in excel.

This process was then performed on two additional samples of Nostoc which were exposed to either natural or artificial light for a period of 1 hour prior to incubation. The results were recorded into excel.

For comparison reasons, 1mg of chlorophyll a was ordered from sigma and was put into a 100mL solution of one part DMSO and 9 parts acetone. This was used as the control. The diluted sample from sigma was analyzed by spectrophotometry and the results were also recorded into excel.

Acidification of the control was performed in order to force the conversion to pheophytin and to obtain the acid ratio. This was performed by the addition of 1mL HCL into the centrifuge tube.

These experiments were then precisely performed on the two secondary models of Microcoleus and Scytonema.

RESULTS

In figure 5 below, the absorption spectrum of chlorophyll a from Sigma shows two very distinct peaks located at 430nm and 660nm.

Figure 5: Absorption spectrum of chlorophyll a from Sigma

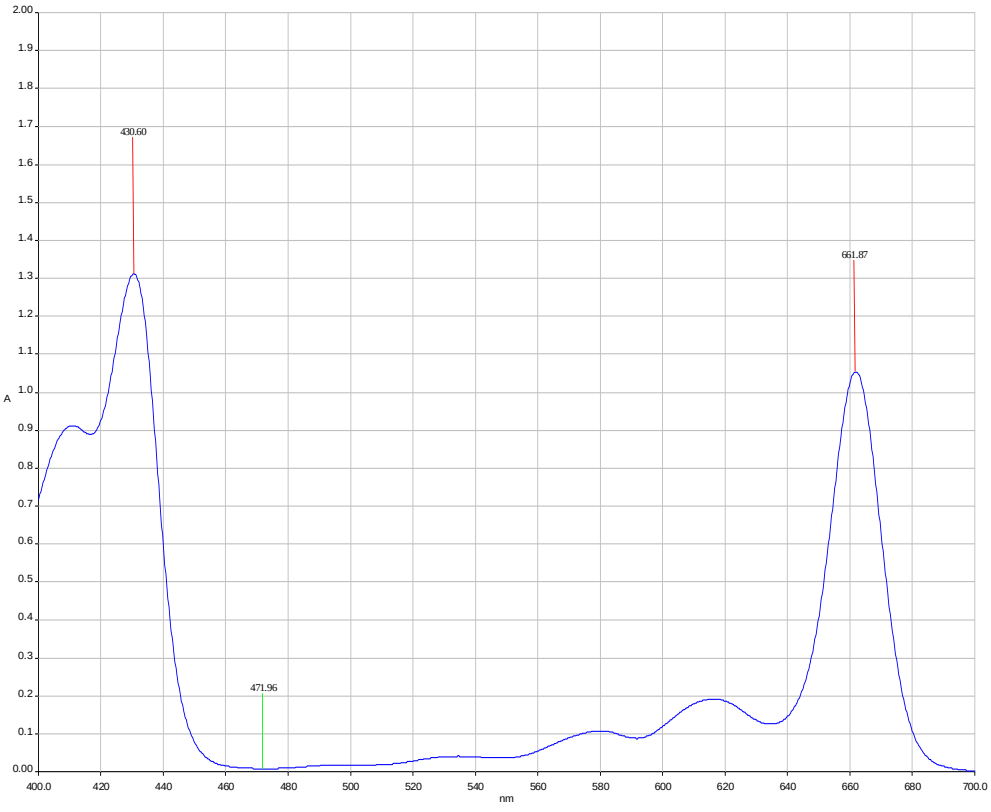


Figure 6: Absorption spectrum of Scytonemin

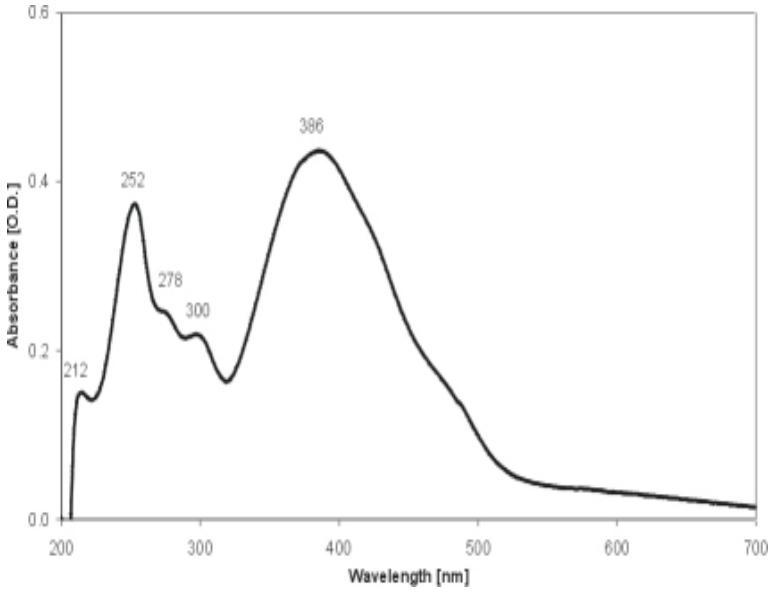


Figure 6 depicts the two prominent absorbance peaks at 250nm and at 386nm.

Figure 7 shows a very high absorbance peak just above 200nm and slight increases at about 280nm and again at 450nm tapering off at about 510nm.

Figure 7: Absorption spectrum of β-carotene

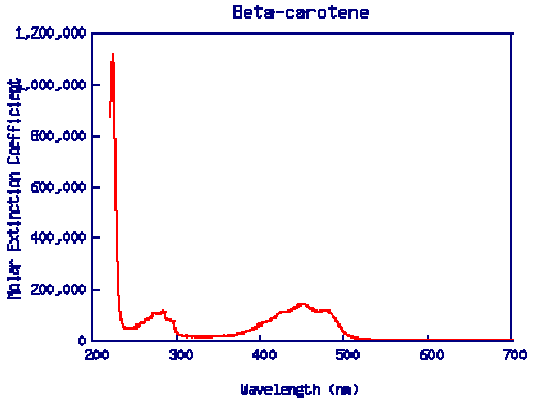


Figure 8 below shows the spectrophotometry analysis of all three cyanobacteria models after being exposed to 1 hour each of three different light conditions; artificial light, natural night and in absence of white light.

Figure 8: Spectrometry results for all three models under various light conditions

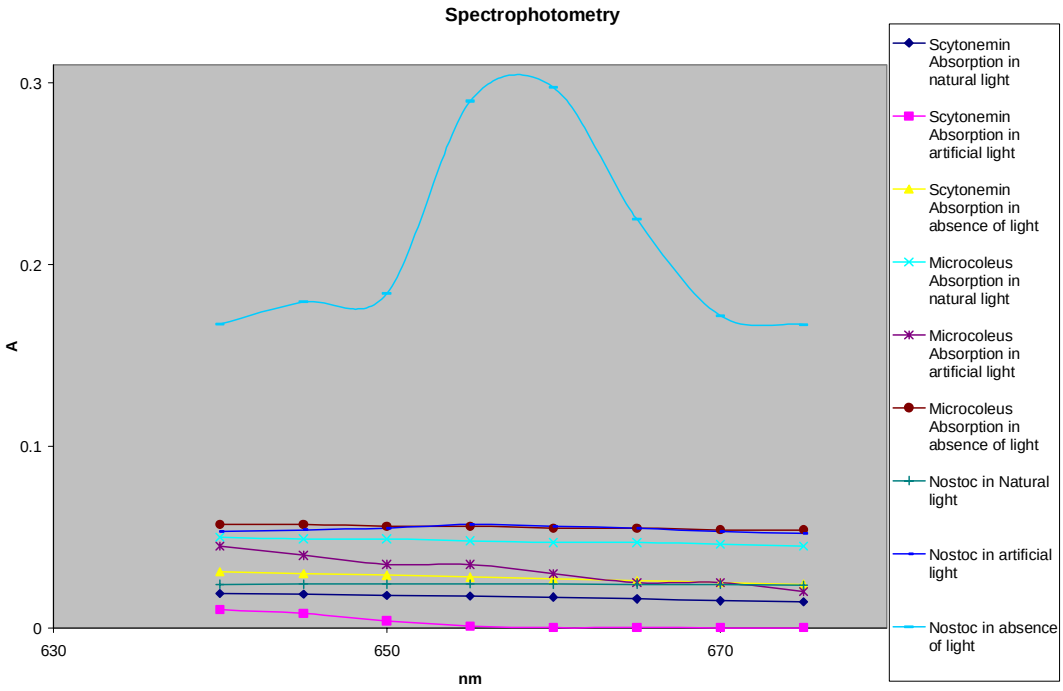


Table 1 shows the relative absorbencies of the chlorophyll a extracted from the primary model Nostoc. The chlorophyll solution was analyzed using spectrophotometry (at 660nm) before and after the addition of HCL. The acidification ratios are also stated.

Table 1: Absorbance of chlorophyll a extracted from Nostoc

	Absorbance @ 660nm	Absorbance after addition of 1mL HCL	Acidification Ratio, Rb/Ra
Natural Light	0.0240	0.0043	5.63
Artificial Light	0.0560	0.017	3.29
Absence of white Light	0.2975	0.1672	1.78

Table 2 shows the absorbance of the chlorophyll extracted from Scytonema and analyzed in the spectrophotometer at 660nm. The table also shows the acidification ratio for the sample that was not exposed to light.

Table 2: Absorbance of chlorophyll a extracted from Scytonema

	Absorbance @ 660nm	Absorbance after addition of 1mL HCL	Acidification Ratio, Rb/Ra
Natural Light	0.0168	n/a	n/a
Artificial Light	0.0002	n/a	n/a
Absence of white Light	0.0207	0.0322	0.643

Table 3 shows the absorbance of the chlorophyll extracted from Microcoleus and analyzed in the spectrophotometer at 660nm. Table 3 also shows the acidification ratio for the only sample which was not exposed to light.

Table 3: Absorbance of chlorophyll a extracted from Microcoleus

	Absorbance @ 660nm	Absorbance after addition of 1mL HCL	Acidification Ratio, Rb/Ra
Natural Light	0.0467	n/a	n/a
Artificial Light	0.0300	n/a	n/a
Absence of white Light	0.0549	0.0515	1.06

Table 4 shows the absorbance of the chlorophyll a obtained from Sigma and diluted into a solution of DMSO and acetone in a 1:9 ratio respectively.

Table 4: Absorbance of chlorophyll a obtained from Sigma

	Absorbance @ 660nm
Natural Light	0.3816
Artificial Light	0.6412
Absence of white Light	0.6479

DISCUSSION

There are three main types of cyanobacteria found within the desert crusts of the Midwestern United States; Nostoc, Scytonema and Microcoleus. Each of these three organisms plays a crucial role in the generation as well as the maintenance of the desert crusts. Nostoc is very important because it is capable of living symbiotically with many other organisms and often provides Nitrogen to the host organism. Scytonema also plays an important role in the maintenance of the crusts by providing sun block pigments which helps protect against harmful UV rays. Microcoleus is the last of the three experimental models and is arguably the most important in the desert crust ecosystem. This blue green produces a polysaccharide sheath that surrounds neighboring organisms and threads in and around the soil to form the actual "crust" of the desert. This is vital because it has been shown to reduce surface water velocity, prevent erosion and even absorbs and retains large amounts of water (in respect to its size).

In order to mimic these desert conditions in the lab, each of these three organisms must be added to culture in a 1:1:8 ratio of Nostoc, Scytonema and Microcoleus respectively. In order to do this accurately, a normalized approach must be implemented. The chosen experimental design based on cost effectiveness and simplicity was to standardize the extraction of chlorophyll from each of the three cyanobacteria. This was chosen over cell counting and protein content because Scytonema as well as Microcoleus are filamentous and do not fragment into individual cells. This would make these types of experiments next to impossible.

There was deliberation on the solvent to be used for the extraction process. Acetone has been shown to extract nearly 100% of the chlorophyll a in chloroplasts as well as Nostoc;

however, it is an inadequate method to be used in conjunction with other cyanobacteria such as Microcoleus. It was proved in several journal articles such as Shoaf, Lium (1976) and Palumbo et al. (1987) that DMSO has been an efficient solvent for extraction of chlorophyll a from cyanobacteria. With the extraction method and solvents chosen, the experiments began.

Located in table 1, are the relative absorbencies of the chlorophyll a extracted from the primary model Nostoc. Three different samples were exposed to three different lighting conditions all in the according solvent ratios as previously stated. The extracted chlorophyll solutions were analyzed using spectrophotometry (at 660nm). The data shows that both natural and artificial light caused some form of chemical change within the solution. The absorbencies were far less than that of the sample that was kept in the dark. This may be caused by a chemical reaction or possibly having particulate in the solution that failed to pellet during centrifugation. The absorbencies were taken again after the addition of HCL. The acidification ratios were determined and show that the reaction causing the decrease in absorbance was not due to the chlorophyll degrading to pheophytin.

Both tables 2 and 3 show similar data for the extraction of Scytonema and Microcoleus. However the absorbance values show little statistical relevance. This suggests that the extraction of the two filamentous cyanobacteria is far more difficult and has a larger margin of error. These results may be caused from in-sufficient use of the tissue grinder. When HCL was added to the samples that were not exposed to light, the Scytonema absorbance actually increased and the Microcoleus changed in significantly. This may suggest some form of inaccuracy in the machine readings. The data for the acidification

of the samples exposed to natural and artificial light conditions from both species was not statistically relevant.

Table 4 shows the absorbencies of the chlorophyll control dilution obtained through Sigma. This data not only shows that the substance obtained through Sigma is truly chlorophyll a, but the exposure to natural light caused some significant degradation of the chlorophyll a into pheophytin.

Figure 5 depicts the absorption spectrum of chlorophyll a from Sigma which was obtained through a recording spectrophotometer located in Goddard Hall. The spectrum shows two very distinct peaks located at 430nm and 660nm range.

Figure 6 shows the absorption spectrum of Scytonemin, which has two prominent absorbance peaks at 250nm and at 386nm. This spectrum is being used to show that it has little to zero overlap with the concerned absorbance range of about 660nm (chlorophyll peak). This proves that the absorption readings are actually chlorophyll and not another substance overlap.

Figure 7 shows the absorption spectrum of β-carotene, another pigment found in conjunction with chlorophyll. This spectrum is also proving that β-carotene does not interfere at all with the absorption of chlorophyll a in the wavelength range around 660nm.

Both figure 6 and 7 show that there would be a great deal of interference in the absorbance spectra of chlorophyll if it were taken in the range of 430nm.

Figure 8 shows the excel plot of the absorption spectra of all three models under various light conditions as well as the absorbance of the control sample from Sigma, ”the high flyer”. All of the cyanobacteria extraction samples show little to no change in

absorbance. This is most likely due to the very minute differences obtained throughout the experiments and excels inability to accurately portray the data. However, there was an issue that was brought to attention at the end of the experiments. This issue relates directly to the results of figure 5 and the high flyer located in figure 8. In figure 5, (which was generated by a fairly new recording spectrophotometer) the absorbance of chlorophyll a at 660nm is about 1.05. That same exact sample was then run through the spectrophotometer that was used for all other experiments and generated the high flyer in figure 8. This shows the absorbance of chlorophyll a from Sigma to read just under .3 at 660nm. The sample was then run a second and third time through the "old" machine at which point two additional, different answers were obtained. This may be a result of faulty calibration within the machine and could potentially compromise the experiment.

The ultimate goal of these experiments was to be able to grow desert crusts in vitro to enable additional experiments to be performed on the crusts more conveniently in the lab versus in the field

REFRENCES

Belnap, J. and J. S. Gardner. 1993. Soil microstructure in soils of the Colorado Plateau: the role of the cyanobacterium *Microcoleus vaginatus*. Great Basin Naturalist 53:40-47.

Palumbo, A. Mulholland, P. and Elwood,J. 1987. Extraction with DMSO to Simultaneously Measure Periphyton Photosynthesis, Chlorophyll, and ATP. Limnology and Oceanography 32:464-471.

Lium, B. and Shoaf, T. 1976. Improved Extraction of Chlorophyll a and b from Algae Using Dimethyl Sulfoxide. Limnology and Oceanography21:926-928

Hendrickson, D.A. and D.M. Kubly. 1984. Desert waters: past, present, and future. The Nature Conservancy News 34 (5): 6-12

http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/Chlorophyll.html

http://www-cyanosite.bio.purdue.edu/images/images.html

http://microbes.arc.nasa.gov/gallery/lightms.html

http://silicasecchidisk.conncoll.edu/LucidKeys/Carolina_Key/html/Scytonema_Main.html