DETECTION AND IDENTIFICATION OF LIVE AND DEAD BACTERIA BY SPECTROSCOPIC ANALYSIS AND DESIGNING A NOVEL INSTRUMENT FOR IN SITU APPLICATION

A Thesis

by

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ABSTRACT

Most of the illnesses in this world are caused by bacterial infections. Thus, their in situ detection and identification is a matter of worldwide concern in human pathology. This MS thesis research presents the in situ detection of the number of live and dead bacteria in an infected area, by means of absorption and fluorescence emission of the present analysis, as well as the flux of UV radiation needed to inactivate or kill 80-90% of the live bacteria. The effects of UV radiations upon various components of bacteria by spectroscopic analysis are discussed. With the help of this spectroscopic analysis, the design of hand-held synchronous spectrometer is proposed to detect and identify bacteria instantly and in situ. Fluorescence and absorption spectra of two different bacterial species (*Escherichia coli*, *Rhodococcus opacus*), two different bacteria proteins (tryptophan and tyrosine) and bacteria DNA have also been studied. The technique discussed in this research and proposed design of a novel hand-held instrument is expected to be able to perform live and dead bacteria identification in a much shorter period of time than the standard methods and in addition provide in situ detection of bacteria in wounds which is not possible with present methods. A nano-second time resolved spectroscopy system has been designed and implemented on an optical table and tested for proper operation, in order to do further research on DNA dimerization mechanism and singlet oxygen formation mechanism after illuminating methylene blue.

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NOMENCLATURE

E. coli	*Escherichia coli*
Rhodococcus	*Rhodococcus opacus*
MB	Methylene Blue
Xe	Xenon
OD	Optical Density

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Bacterial infections cause most of the common diseases and illness in this world. Thus, their in situ detection and identification is a matter of worldwide concern in human pathology. The detection and identification of live and dead bacteria by culture and incubation techniques are very time (~24hrs) and personnel consuming, as well as needs a lot of high-technology expertise and consumes a large amount of energy. The long time required for detection could lead to consequent worsening of the patient's condition. Rapid and in situ detection are very important in maximum of the patient's cases. To that effect, in situ detection of the bacteria with a hand-held device is proposed in this thesis.

Our experimental results have shown that the initial detection of the number of bacteria and the inactivation of live bacteria as a function of UV irradiation time is very efficient. The technique discussed in this research and proposed design of a novel hand-held instrument is expected to be able to perform live and dead bacteria identification in a much shorter period of time than the standard methods and in addition provide in situ detection of bacteria in wounds which is not possible with present methods. In addition, a nano-second time resolved spectroscopy system has been designed and implemented on an optical table and tested for proper operation, in order to do further research on

bacteria DNA dimerizing mechanism and singlet oxygen formation mechanism after illuminating methylene blue, which can also inactivate bacteria.

Previous studies have proposed the identification and classification of bacteria by absorption and fluorescence spectroscopy with different excitation and emission wavelengths. Giana *et al.* [1] described the identification of three different bacteria, those are *Escherichia coli*, *Enterococcus faecalis*, and *Staphylococcus aureus*, by two different excitation wavelength (at 410 nm and 430 nm). The identification of Pseudomonas species were reported by Shelly *et al.* [2], [3] using an excitation–emission matrix of their fluorescent pigments. In this method, an incubation procedure of bacteria in a particular medium was required to produce a characteristic profile. Leblance *et al.* [4] described a classification of 25 strains of bacteria with the excitation wavelength at 250 nm. Also Sohn *et al.* [5] reported the identification and classification of *Escherichia coli (E. coli)*, *Campylobacter*, and *Salmonella* by fluorescence spectroscopy and multivariate analysis. They used a synchronous scan technique to determine the optimum excitation–emission wavelengths and then they categorized the bacteria according to the genus and determined their concentrations via principal component analysis (PCA).

In addition, Nelson [6] reported a method to select the best excitation wavelength and also the selective excitation of biological molecular groups for best bacteria species identification by using multi-excitation fluorescence spectroscopy. Maquelin *et al.* [7]

reported rapid detection and identification of bacteria directly in culture plate by vibrational spectroscopy (Raman and infrared). Also vibrational spectroscopy has been used for rapid detection and identification of bacterial species in liquid suspension [8] and bacterial contamination in liquids [9]. Roselle *et al.* [10] described the changes in the auto-fluorescence spectrum of *E. coli* after 210 days kept in saline solution, and also studied the differences in the spectra of viable bacteria, comparing culturable and unculturable bacteria (which do not grow in culture medium).

In this research, absorption and fluorescence spectroscopy has been used for the analysis live and dead bacteria, and a hand-held spectrometer is designed for in situ application of these analyses. In **Chapter II**, some methodological review is discussed related to my research and the main objectives of my research. In the methodological review section, some basic and important information have been discussed about general basic bacteria, the studied bacteria and their proteins. Also basic spectroscopy techniques, such absorption, fluorescence and synchronous scan fluorescence have been explained. In the research objective section, we have explained the main objectives of the analysis.

Chapter III focuses on the main spectroscopic analysis of our research with the techniques, results and discussion. In the first section, the spectroscopic analysis of bacteria are discussed. In the first part, the experiments on bacteria concentration vs. the peak intensity of emission spectrum is presented and discussed. In the second part of the first section, the effects of UV radiation on bacteria, our analysis and conclusions have

been explained. In the second section of this chapter, additional spectroscopic methods for the analysis of bacterial proteins and bacteria DNA are used for further bacteria studies. Four different experimental results and discussion of spectroscopic analysis represented in this section, including, differentiation between bacteria and their DNA, differentiation between bacterial proteins and DNA, further radiation effects on bacteria and differences between *E. coli* (gram-negative bacteria) and *Rhodococcus* (gram-positive bacteria).

Chapter IV focuses on our proposed design of the hand-held synchronous spectrometer and detail discussion of its operations for in situ applications of our analysis of this research. Here two different designs for hand-held synchronous spectrometer with some proposed specifications are disclosed.

In **Chapter V**, the basic concept of time-resolved spectroscopy has been discussed and a design of nano-second time-resolved spectroscopic system is proposed and implemented in an optical table. The operation principle and a time resolution diagram of that design have also been discussed in some detail. The purpose of this design is its use for further research on DNA dimerization mechanism and measure the formation and decay of singlet oxygen formed by irradiating methylene blue with 661nm LED light.

In **Chapter VI**, the conclusions of the experimental results of this research are presented and summarizes the spectroscopic analysis on bacteria, design and application of the

hand-held synchronous spectrometer. It also contains the design and implementation of the nano-second time-resolved spectroscopy system and describes further time resolved research plans with this system.

CHAPTER II

METHODOLOGY OVERVIEW AND RESEARCH OBJECTIVE

2.1 Methodology Overview

In conventional methods, the detection and identification of live bacteria depends on the culture and incubation techniques, which takes around 24 hrs. As bacteria reproduce every 6-20min, this long time for detection could be dangerous for patients. So for rapid detection, we have used spectroscopic analysis of bacteria and also in the later chapter (chapter IV) we will discuss about our proposed design for hand-held spectrometer which can be used for in situ detection of live bacteria. In this research, absorption, conventional fluorescence and synchronous fluorescence spectroscopy have been used for the analysis.

2.1.1 A Review of Studied Bacteria

Generally Bacteria are composed of ribosomes, a chromosome, plasmids, pili, plasma membrane cytoplasm, capsule etc. Bacteria have an outer cell wall that gives them shape. The two most important components of bacteria are DNA and bacteria flagellum. DNA helps them to reproduce and bacteria flagellum helps them to move from one place to another, find food and sense enemy (antibiotics). In Figure 1, different parts of bacteria body have been shown (left side), and the complex structure of bacteria

flagellum has also been shown in the right side. The base part of the flagellum helps to rotate the hook and the filament part.

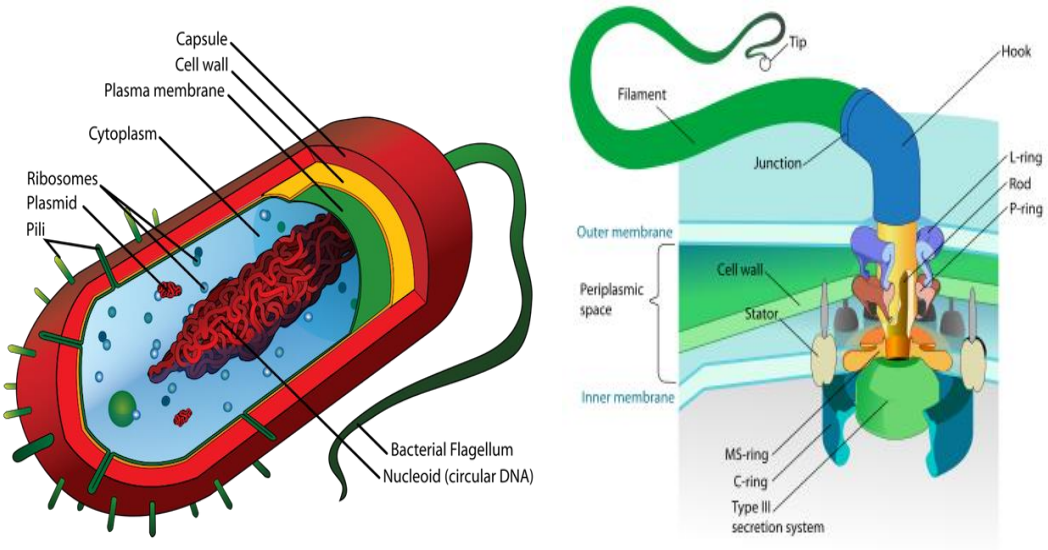

Figure 1: General Components of Bacteria (Left), Structure of Flagellum (Right) [11]

2.1.1.1 Some Interesting Facts about Bacteria

- Bacteria have a single cell and lack of a true nucleus, the cell surrounded by a membrane.
- It has tolerance at extreme conditions: temperatures above 100 °C and below -30 °C; They can be found from bottom of the sea, to upper atmosphere, to soil, plants and animals.
- They can “eat" everything sunlight, sulfur, iron, etc.
- Bacteria can withstand of radiation 1,000 times greater than humans. [12]

- Bacteria are only a few microns in length (0.3 to 700 microns).
- Bacteria are also vital in recycling nutrients and also important for nitrogen and carbon fixation.

2.1.1.2 Reproduction

Bacteria are asexual in nature; they multiply themselves every 6-20 mins. When they have food, the mother cell starts growing and after the cell enlarges to a certain volume, it starts to make replica of its own DNA. In the meantime the whole cell of the bacterium starts to split in two identical part and thus from one bacteria it turns into two spate bacteria with identical DNA and body part. In one sense, it can be said that as long as bacteria have food and not killed by external source, a bacteria cell does not die rather they just multiply themselves again and again. In Figure 2, the general procedure of bacteria reproduction has been shown.

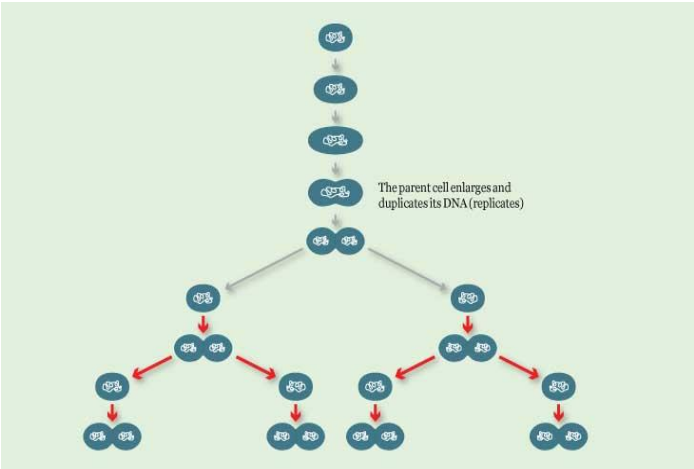

Figure 2: Bacteria Reproduction Technique [13]

Here, mainly two bacteria are studied namely *Escherichia coli* and *Rhodococcus opacus*. *Escherichia coli (E. coli)* is a gram negative bacterium and *Rhodococcus opacus* is a gram positive bacterium. Most of the bacteria usually fall under one of these two categories (Gram-positive and Gram-negative). The major difference between these two types of bacteria is their cell wall structure. Gram-positive bacteria have a cell wall consisting of a thick layer of polymer surrounding the cytoplasmic membrane and Gram-negative bacteria have a very complex multilayered cell wall, as shown in Figure 3.

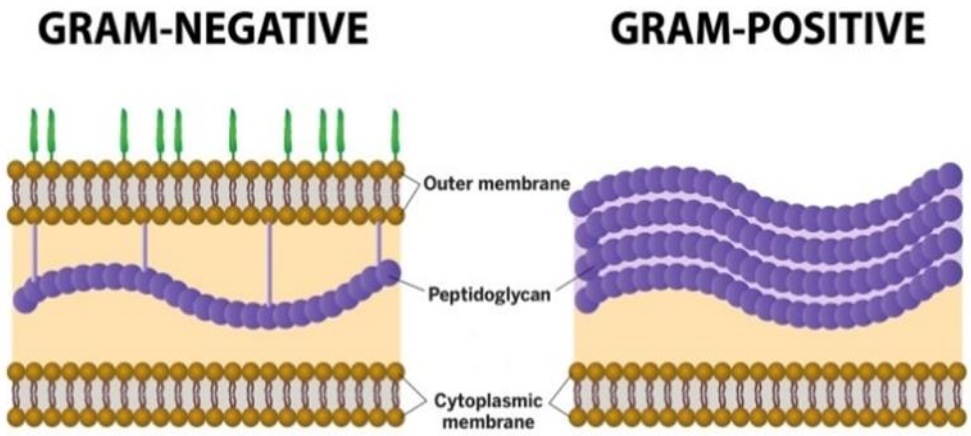

Figure 3: Difference between Gram-Positive and Gram-Negative Cell Structure [14]

2.1.2 A Review of Spectroscopy

2.1.2.1 Absorption Spectroscopy

Absorption spectroscopy is an analytical technique that measures the amount of light absorbed by a sample at a particular wavelength. The sample absorbs energy from the radiating field. The wavelength region generally used is from 200 to about 800nm. The

absorbance A, is defined as: $A = \log_{10} (I_0/I_t)$, where I_0 denotes to the intensity of light incident on the sample and I_t denotes intensity of light transmitted by the sample. [15] The absorbance of a sample is related to the concentration of the absorbing species and the path length of the sample by the Beer-Lambert Law; $A = \varepsilon cl$, where ε is the molar extinction coefficient (mol^{-1} dm^3 cm^{-1}), c is the concentration (mol dm^{-3}) and l is the path length (cm). [15]

2.1.2.2 Fluorescence (Emission) Spectroscopy

Fluorescence spectroscopy measures the intensity of the emitted photons from a sample after the absorption of photons by the sample from an excitation beam at a specific wavelength, which passes through the solution. In fluorescence spectroscopy both and/or the emission spectrum of a sample can be measured. The excitation spectrum is based on the light which is absorbed by the sample and the emission spectrum is based on the light which is emitted from the sample after absorption. The concentration (c) of the sample is proportional to the intensity value of the emission spectrum. [16]

An excitation spectrum is achieved by keeping the emission monochromator at a constant wavelength, and the excitation spectrum is recorded as a function of the wavelength, as shown in Figure 4 [17]. A fluorescence emission spectrum is achieved by keeping the excitation monochromator at a constant wavelength and the emission spectrum is recorded as a function of wavelength, as shown in Figure 4 [17]. The

excitation spectrum is usually a similar shape as the absorbance spectrum and almost mirror image to the emission spectrum.

2.1.2.3 Synchronous Fluorescence Spectroscopy

Synchronous fluorescence spectra are obtained by scanning both the excitation and emission monochromators synchronously instead of only one monochromator at a time during scanning as used in conventional fluorescence spectroscopy. There is always a fixed wavelength interval ($\Delta\lambda$) between the excitation and emission wavelengths while scanning the synchronous spectrum, as shown in Figure 4(c) [17]. This wavelength interval ($\Delta\lambda$) is very important to have a good resolution from a mixture of components.

Synchronous fluorescence spectra at adjusted wavelength interval ($\Delta\lambda$) and other parameters can be effectively used to obtain data from a sample composed of numerous compounds in a single scan with better peak resolution. The synchronous fluorescence spectrum depends on the excitation and emission spectra and also on the wavelength difference, $\Delta\lambda$, between the excitation, λ_{ex}, and emission, λ_{em} wavelengths.

For getting an excitation spectrum, a sample needs to be excited at a range of wavelength (such as A_1, A_2,….., A_8, A_9) and the fluorescence will be recorded only at the maximum emission wavelength (e.g. F_5). Similarly, for getting the fluorescence emission spectrum, a sample is usually excited only at a maximum absorption

wavelength (e.g. A_5) and fluorescence is recorded at a range of emission wavelength (such as F_1, F_2,…., F_8, F_9) as shown in Figure 5 [18].

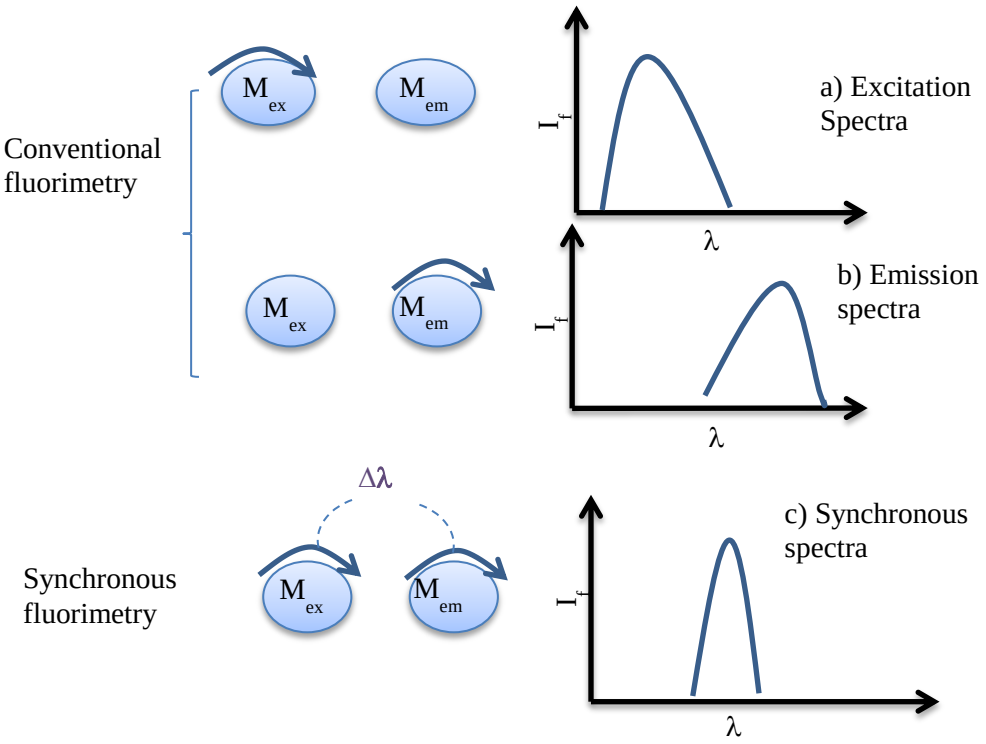

Figure 4: Difference between Conventional (A&B) and Synchronous Fluorimetry (C), (M_{ex} = Excitation Monochromator, M_{em} = Emission Monochromator); [17]

In the case of synchronous fluorescence spectrum, a wavelength difference between absorption and emission band $\Delta\lambda$ is defined, so that the spectrum will be recorded only when the wavelength difference get matched. If we take $\Delta\lambda = A_4 \sim F_4$, the spectrum will start recording when the excitation monochromator is at A_4 and emission monochromator is at F_4. Later on, the sample will be excited at A_5, A_6, ….., A_8, A_9 and

corresponding fluorescence will be recorded at F_5, F_6 ……. F_8, F_9, respectively as shown in Figure 5 [18].

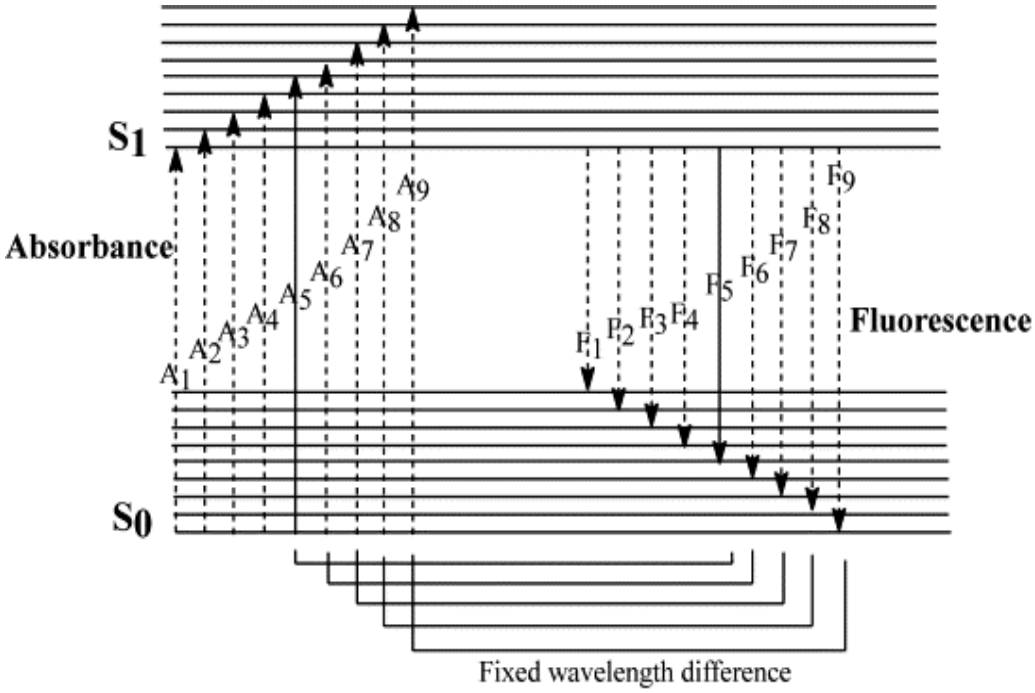

Figure 5: Jablonski Diagram of Synchronous Fluorescence Scan [18]

2.2 Research Objective

Now-a-days, millions of dollar**s** are spent for the detection and identification of live bacteria and as bacteria reproduce in every 6-20 minute intervals, the rapid detection became more important in recent years. Thus the aim of this research is to find an effective and in situ way for rapid analysis of bacteria. The research objectives of this MS thesis are:

- Development of an analytic technique for instant detection and identification of the concentration of live bacteria (bacteria/mL) of the sample in an infected area. The methods that have been used for this research are absorption spectra, fluorescence spectra and synchronous spectroscopy. We have performed these experiments both for gram-negative bacteria (*E. coli*) and gram-positive (*Rhodococcus*) bacteria.

- Detection of the percentage of live and dead bacteria was achieved by analyzing the fluorescence and synchronous spectra of bacteria before and after treatment with UV radiation. With the help of this spectroscopic analysis of the bacteria spectra before and after irradiating at various amount of time, the percentage of live bacteria is determined. We have also calculated the average flux and corresponding time for inactivating or killing specific percentage of bacteria.

- Spectroscopic analysis of different bacterial components such as bacterial proteins (tryptophan, tyrosine) and bacteria DNA, and identification and differentiation between them. Also analysis of bacteria spectrum for further radiation after their inactivation or killing have been studied and discussed.

- Design of a novel hand-held instrument for in situ diagnosis by incorporating all the spectral analysis of identification and detection techniques proposed in this research. The proposed spectrometer can perform both conventional fluorescence

and synchronous fluorescence spectroscopy. This instrument can be used in remote area as well as in the hospitals for rapid detection.

- Design of a nano-second time resolved spectroscopy system and implementing it on an optical table for further research on DNA dimerizing mechanism and singlet oxygen formation mechanism after illuminating methylene blue, which can also inactivate bacteria.

CHAPTER III

SPECTROSCOPIC ANALYSIS, RESULTS AND DISCUSSION

3.1 Spectroscopic Analysis of Bacteria

Gram-negative bacteria *Escherichia coli (E. coli)*, which are very well-known bacteria, have been used to perform the spectroscopic analysis of these bacteria. The absorption spectra of bacteria have been taken to identify the wavelengths where bacteria show higher absorption. This wavelength is important to know, because to get the highest emission peak from the emission spectra we need to excite the bacteria with the wavelength where it absorbs more. As it can be seen from absorption spectra in Figure 6, the bacteria absorb more at 218nm, 260nm and 280nm wavelengths. After exciting the bacteria sample with 220nm, 260nm and 280nm (The range of the excitation and emission grating of the spectrofluorophotometer starts from 220nm wavelength, so we could not excite at 218nm), we found out that the emission spectra with excitation wavelength 280nm gives the highest emission peak.

To calculate further the concentration of bacteria at different dilutions, the absorbance (OD=optical density) of 600nm had also been recorded. It is common wavelength for measuring the concentration of bacteria cell in a liquid, because most of the light is scattered by the bacteria cells and gives a weaker electric signal than a cuvette without any cell. As scattering of longer wavelengths is less than that of shorter wavelengths, the

intensity of light scattering and the OD of bacteria sample depend on the wavelength. [19] From the Beer-Lambert Law, we know that the OD (absorbance) is related to the concentration of the sample. The Beer-Lambert Law is:

$OD = -\log I / (I_o) = \varepsilon \times l \times c$

Here in this equation, I_o is the intensity of the incident light, I is the intensity of the transmitted light which is measured by the photo detector, l is the pathlength of the transmitted light through the sample, ε is the extinction cross-section and c is the concentration of the sample. Here in our application, the extinction cross-section and the path length are constant. So OD is proportional to the concentration of the bacterial sample. It has been estimated that for OD = 1.0 at 600 nm wavelength, the concentration of bacteria is 8.0×10^8 CFU/mL for *E. coli*. [20]]

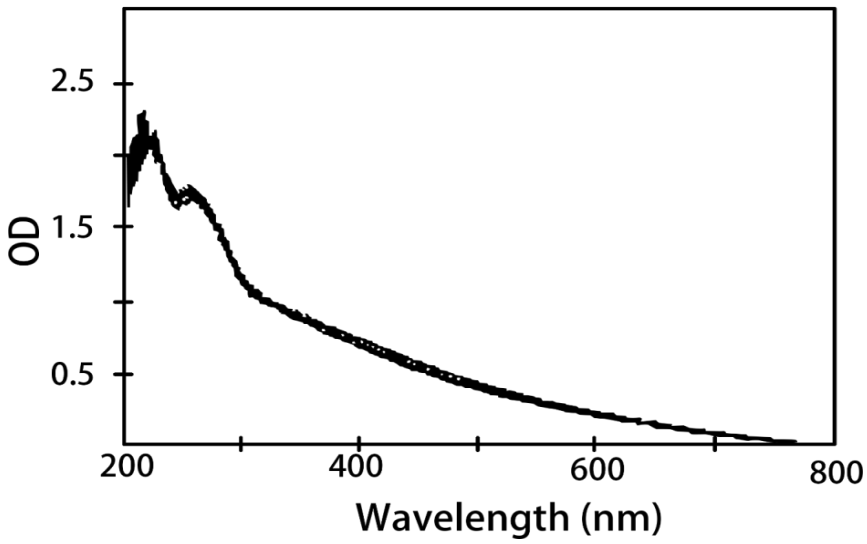

Figure 6: Absorption Spectrum of *E. coli*

For our analysis with fluorescence and synchronous fluorescence spectroscopy, we have used RF-5301PC Spectrofluorophotometer from Shimadzu. For radiating bacteria with UV light, we have used Oriel Lamp housing and U-330 optical filter. The preparation of bacteria and their strain information has been given in Appendix A.

3.1.1 Experiment and Result 1: Bacteria Emission Peak vs. Concentration

We have done several experiments with different concentrations of *E. coli* bacteria and recorded the emission peak for each concentration. One of the experimental results i**s** shown in Figure 7. For this absorption spectra experiment initially 10 µL of moderate concentrated *E. coli* was diluted by 20 mL water and a part of this sample was taken in a 3 cm long and 1 cm optical path cuvette.

From the absorption spectrum, the OD of 600 nm wavelength was recorded and used to calculate the initial concentration of the bacteria taking into account that the concentration of bacteria is 8.0×10^8 CFU/mL when OD = 1.0 at 600 nm wavelength. Then the fluorescence and synchronous emission were taken, where the $\Delta\lambda$ of the synchronous spectrum was 55, this spectrum is referred as 'Sol1' in Figure 7. Then this initial solution was diluted 50% and again absorption, fluorescence and synchronous emission were taken same as before. In the figure, synchronous spectra of several dilutions have been shown.

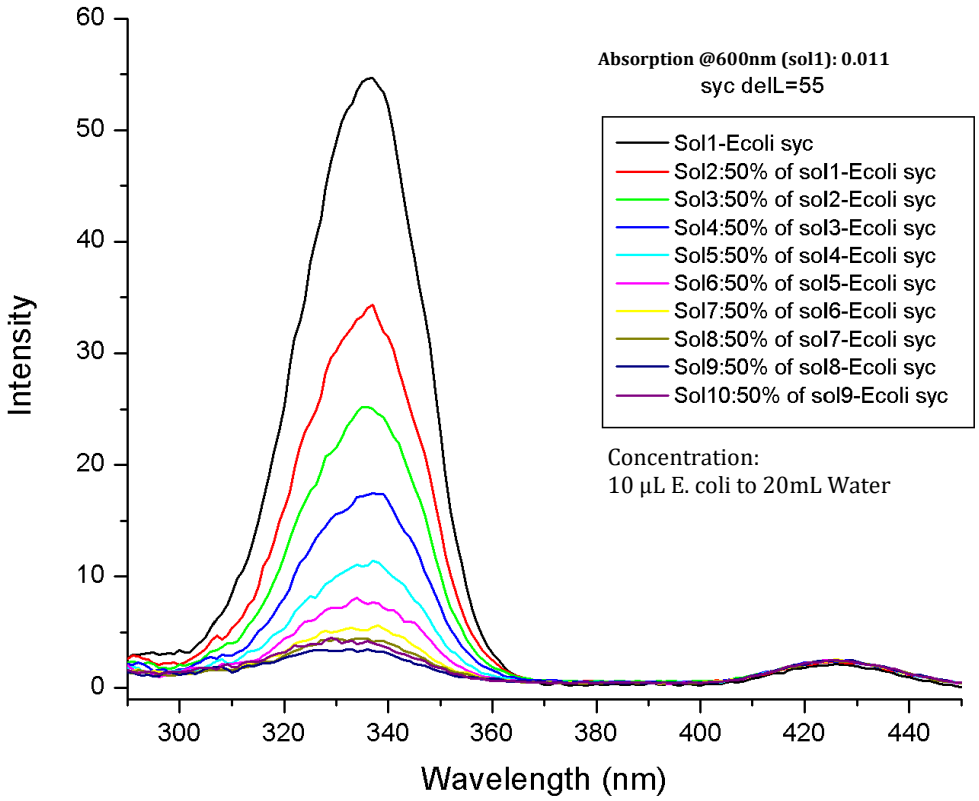

Figure 7: *E. coli* Synchronous Spectrum for Different Concentration

After repeating this experiment several times, we found out that the gradual decrease of emission intensity with respect to concentration actually follows similar exponential decay curve shown in Figure 8. So from this curve, we can conclude that gram-negative *E. coli* have specific peak intensity for a specific range of concentration (bacteria/mL). With the help of this curve and our proposed handheld spectrometer (discussed in chapter IV), we can diagnose the concentration of bacteria in an infected area in situ and instantly.

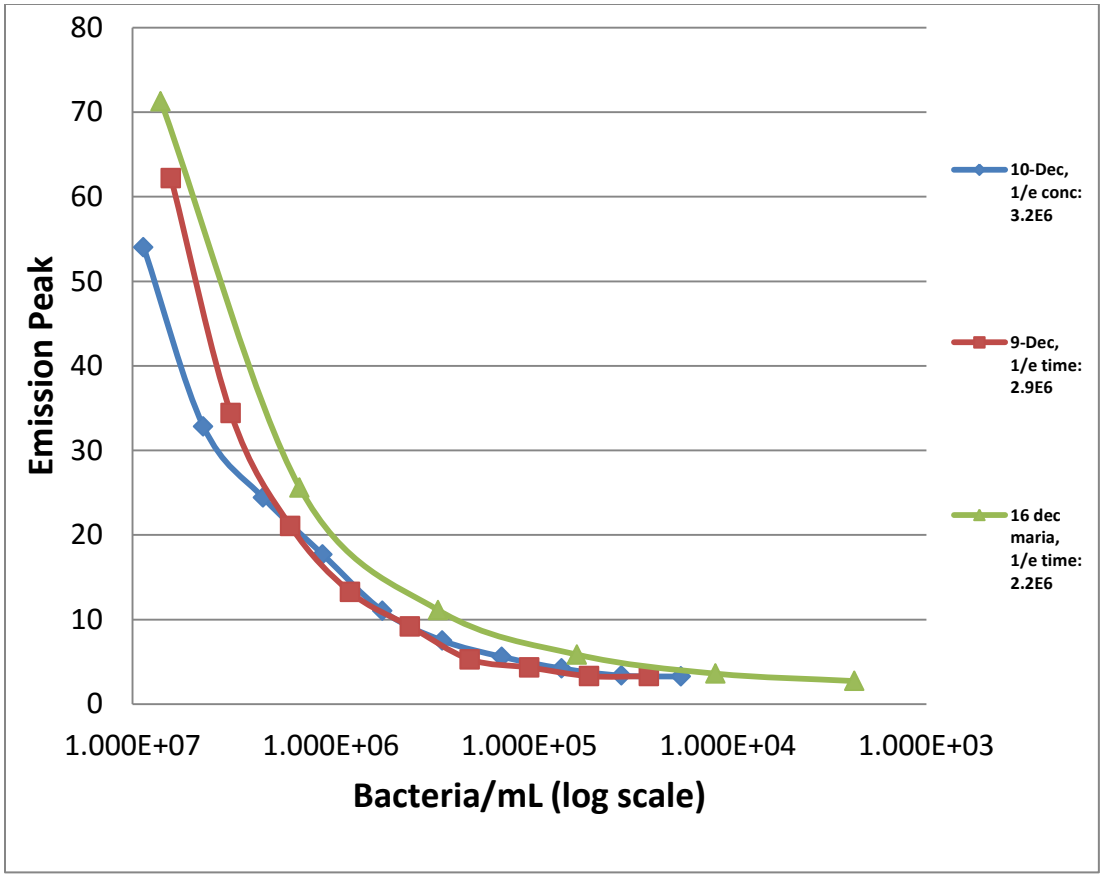

Figure 8: *E. coli* Emission Peak vs. Concentration (Bacteria/mL)

Similarly, several experiments have been done with a gram-positive bacteria *Rhodococcus opacus*. One of the experimental results is shown in Figure 9. For this experiment initially 10 µL moderate concentrated *E. coli* was diluted by 20 mL water and a part of this sample was taken in a 3 cm long and 1 cm wide cuvette to take the absorption spectrum. From the absorption spectrum, the OD of 600 nm wavelength was recorded to calculate the initial concentration of the bacteria taking into account that the concentration of bacteria is 8.0×10^8 CFU/mL when OD = 1.0 at 600 nm wavelength. Then the fluorescence and synchronous emission were taken, where the $\Delta\lambda$ of the

synchronous spectrum was 55 and this spectrum refers to the 'Sol1' in Figure 9. Then the initial solution was diluted 50% and the absorption, fluorescence and synchronous emission spectra were recorded as previously. In the figure, synchronous spectra of several dilutions have been shown.

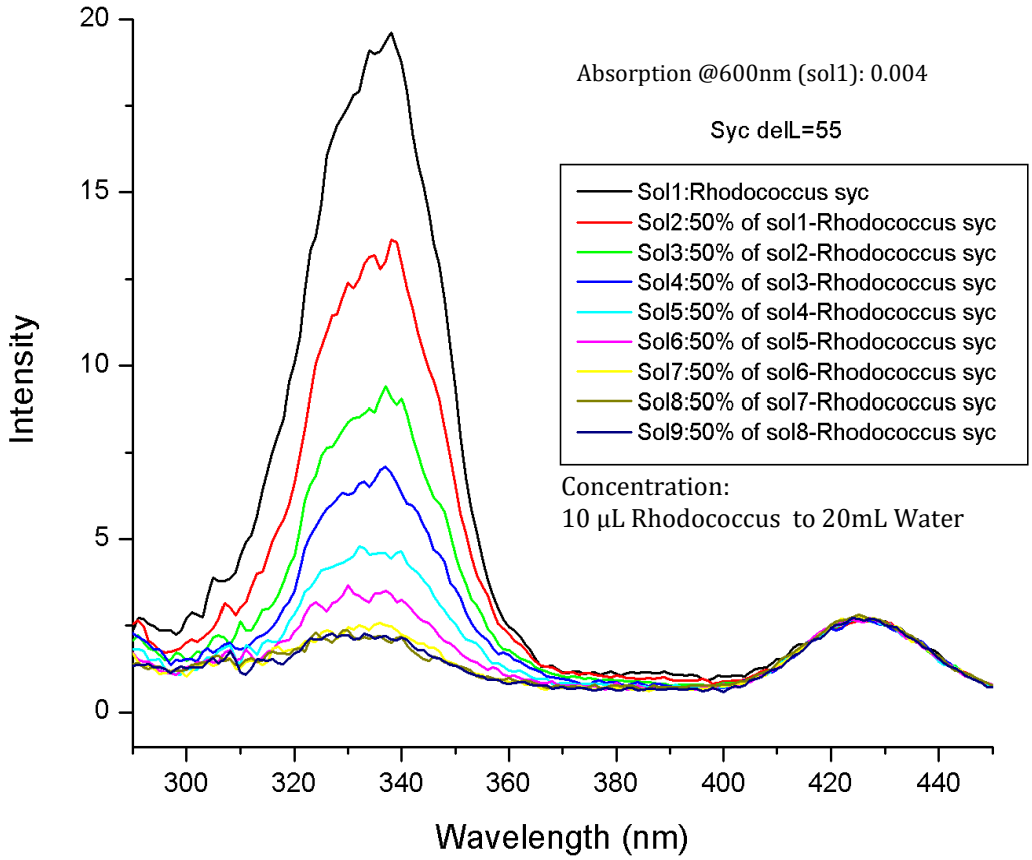

Figure 9: *Rhodococcus* Synchronous Spectra for Different Concentration

After repeating this experiment several times with several samples, we found that there was a gradual decrease of emission intensity with respect to concentration, which

follows the exponential decay curve shown in Figure 10. This exponential decay curve for gram-positive *Rhodococcus* is different from gram-negative *E. coli*. So from this curve, we can conclude that gram-positive *Rhodococcus* has a specific peak intensity for a specific range of concentration (bacteria/mL). With the help of this curve and our proposed handheld spectrometer (discussed in chapter IV), we can diagnose the concentration of *Rhodococcus* bacteria in an infected area in situ, instantly.

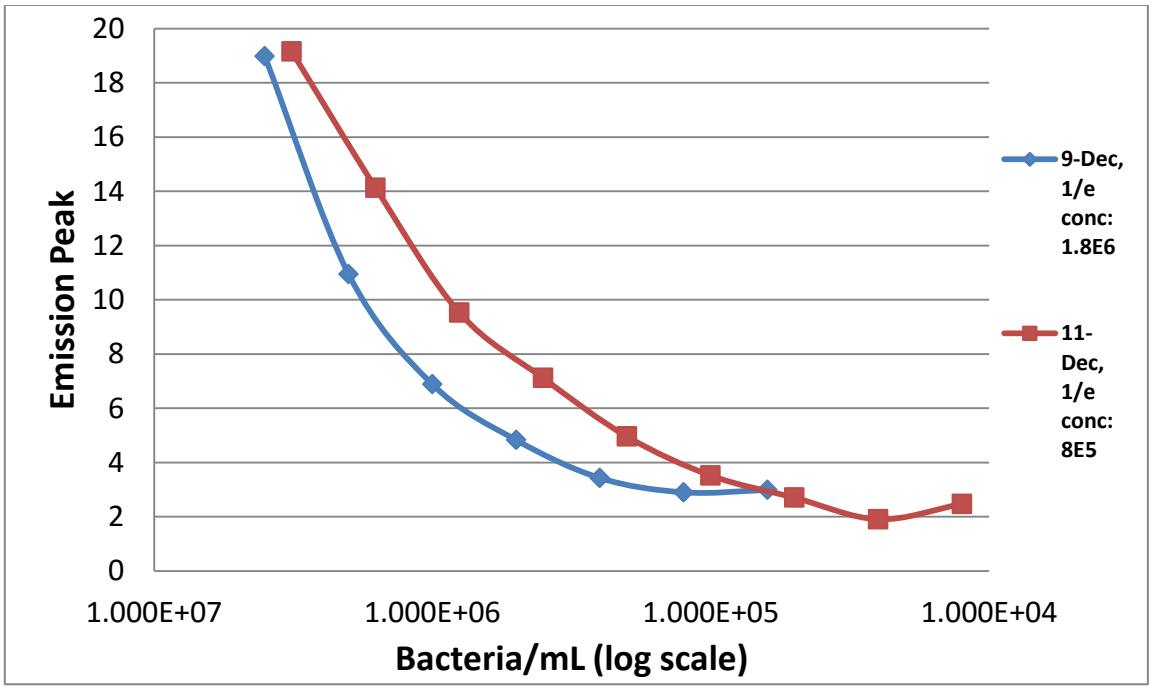

Figure 10: *Rhodococcus* Emission Peak vs. Concentration (Bacteria/mL)

3.1.2 Experiment and Result 2: Bacteria UV Irradiation

The next target of our research was to identify a way to detect the percentage of dead bacteria by spectroscopic analysis. For this experiment, we irradiated bacteria with UV

light as it has already been known that UV light can effectively kill bacteria and took the absorption and emission spectrum for different time and different amount of irradiation. In order to proceed with this research target; we performed several experiments with different power of UV light and using different optical filter. We started from 5min irradiation without any optical filter for our 1st few experiments and found out that the emission peak intensity is decreasing so rapidly. Then we started to decrease the irradiation time interval and decrease the power of UV light in order to determine the gradual change rate.

3.1.2.1 Experiment with *E. coli*

The best values that we acquired, were at 30 second irradiation time interval with 1.1-1.3 mW power of UV light and in order to make the UV light pure enough we used U-330 optical filter which has a transmission range from 230-440 nm wavelength. For one of the experiment that is shown in the Figure 11, we irradiated the bacteria sample at 30 seconds intervals and for each irradiation period we recorded the absorption and emission spectra. At each step, we also recorded the OD at 600 nm wavelength to calculate the concentration, that is OD_{600} of 1.0 = 8.0×10^{8} CFU/mL (we can also calculate the concentration from emission peak intensity vs concentration curve that we found in the previous section) and as well as put aside a sample of each step irradiation for manual culture in the Plant Pathology and Microbiology Department, TAMU, (we also repeated this experiment with Bio-Chem Air Quality Laboratory, TAMU). In the manual culture, they put those samples in the petri dishes and let grow colonies for

around 24 hrs. Then by counting the number of colonies, they can calculate the concentration of bacteria in that sample. In Figure 11, the emission spectra of *E. coli* bacteria of different irradiation time interval have been shown.

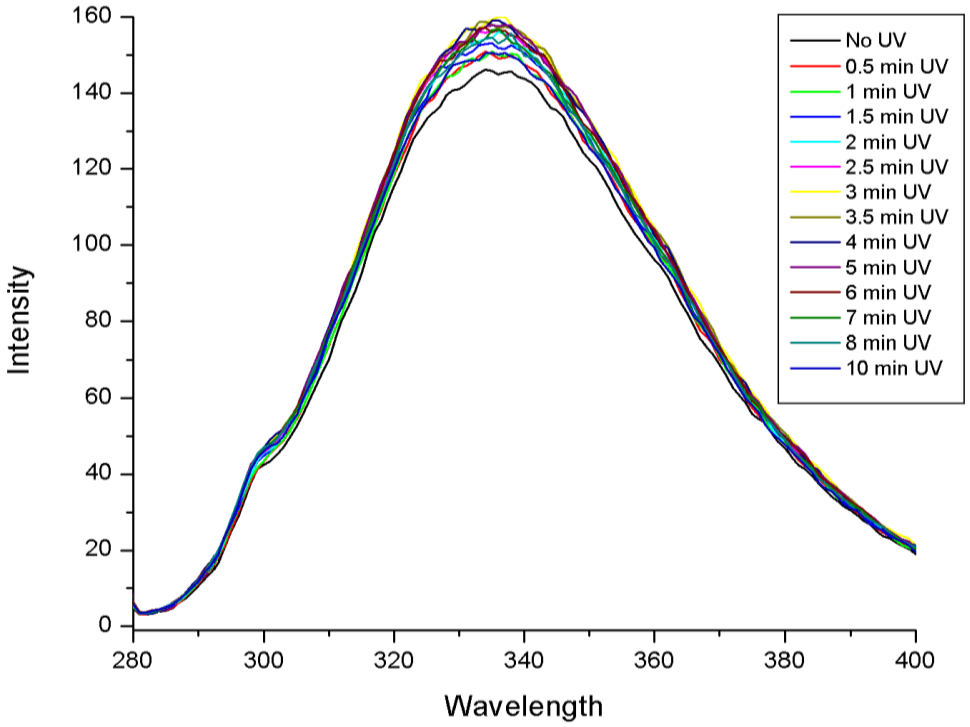

Figure 11: *E. coli* Emission Spectrum for Different UV Irradiation Time

From the emission spectrum, Figure 11, we have determined that the wavelength of the peak intensity is around 335nm. We plotted the gradual change in intensity of emission peak at 335 nm wavelength for different irradiation time interval, as shown in the Figure 12. As we can observe in the figure that the values of 335nm intensity keep increasing for certain time and then start to decrease and become stable. We compared our

spectroscopic analysis data with manually cultured bacteria concentration data as shown in Figure 13; and after several repetition of this same experiment, we can conclude that when the emission peak is increased up to 6-10% of its initial value, 80-90% bacteria of the initial sample is dead. So we can identify the percentage of live bacteria from the change of the intensity in emission peak after irradiating with UV light and with the help of the proposed hand held spectrometer we can diagnose this in situ.

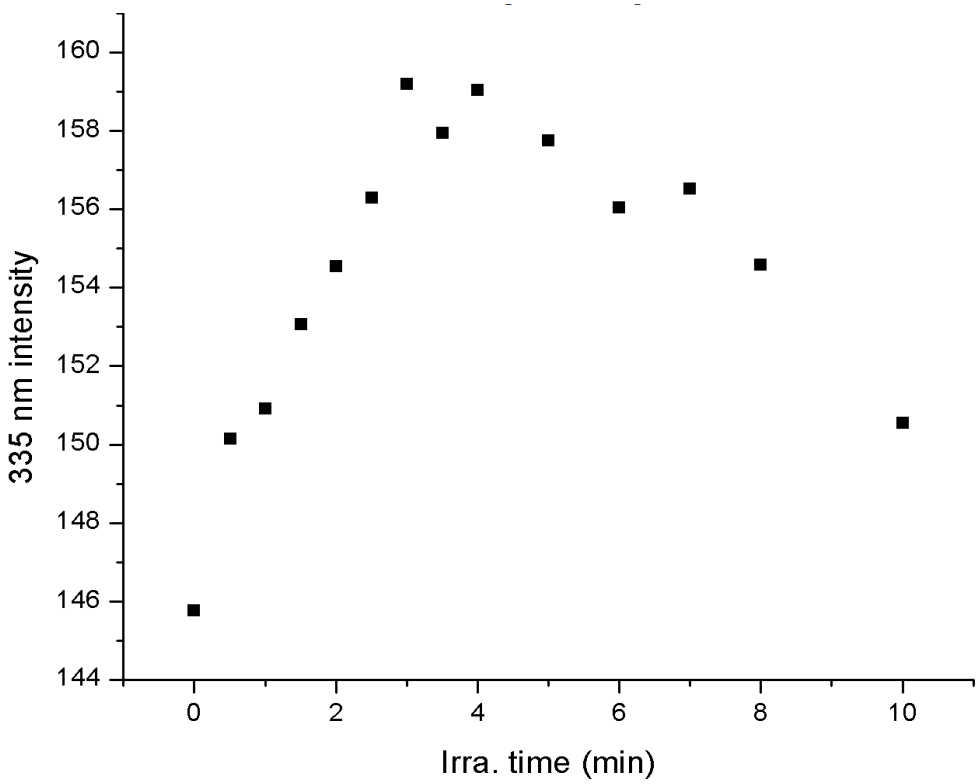

Figure 12: 335nm Peak Intensity Decay with Time by UV Irradiation

In addition, when we analyzed our several repeated result of the manually cultured bacteria and their concentration as shown in Figure 13, we found out that every time 80-90% bacteria is dead when we irradiate the bacteria around 1-3mins by UV light with 1.3mW power and U-330 filter. As we all know, human skin can get burned if it is kept in front of UV light for a long time, but with 1-3mins of UV light with this much low power (1.1-1.3mW) it would not hurt human skin rather it will kill bacteria.

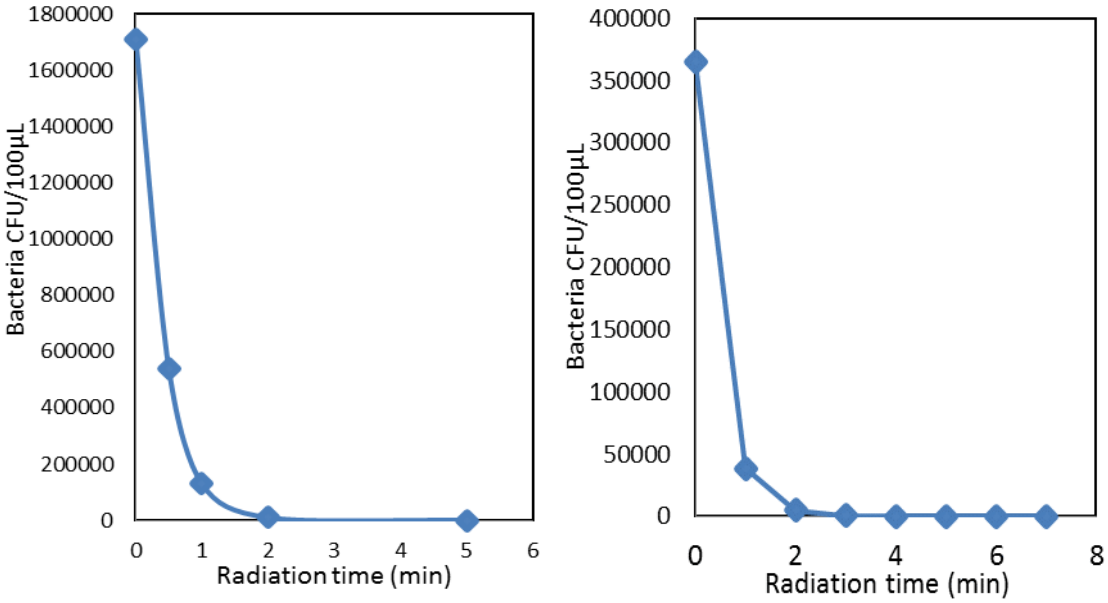

Figure 13: Manually Cultured; Concentration vs Irradiation Time Plot

3.1.2.2 Experiment with *Rhodococcus*

After performing this experiment with gram-negative bacteria, we repeated this experiment with gram-positive *Rhodococcus* bacteria. We repeated with same 30 second

irradiation time interval with 1.1-1.3 mW power of UV light and with U-330 optical filter which has a transmission range from 230-440nm wavelength. For one of the experiment that is shown in Figure 14, we irradiated the bacteria sample every 30 second and for each irradiation interval we recorded the absorption and emission spectra. At each step, we also recorded the OD at 600nm wavelength to calculate the concentration (we can also calculate the concentration from emission peak intensity vs concentration curve that we found in the previous section). In Figure 14, the emission spectra of *Rhodococcus* bacteria of different irradiation time interval have shown.

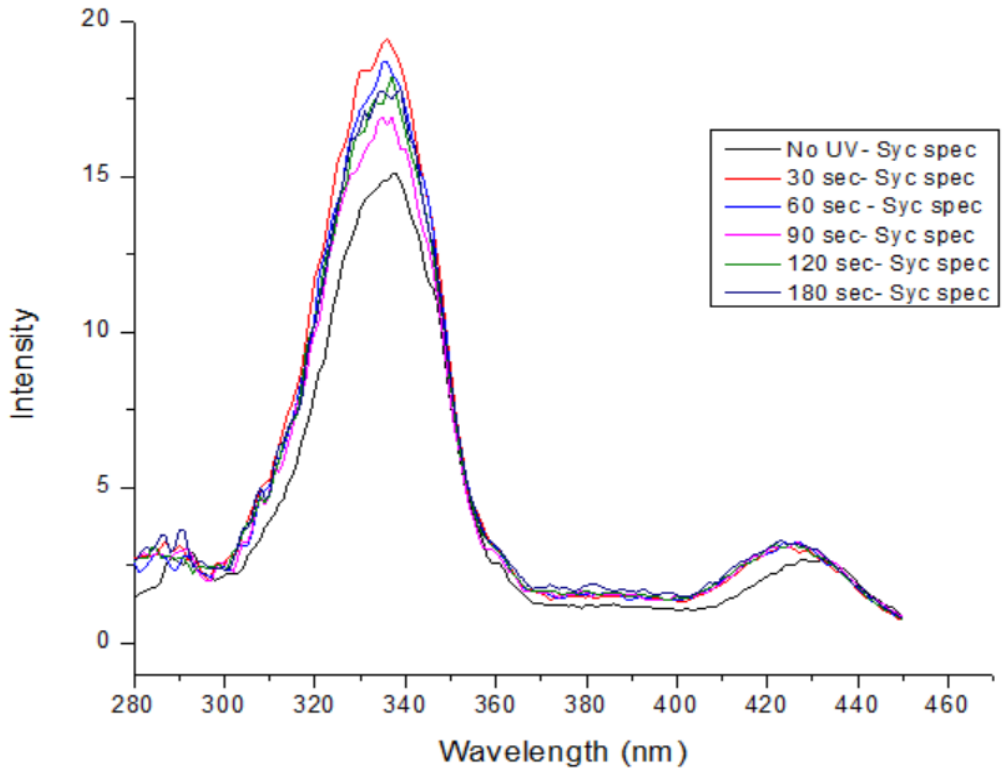

Figure 14: *Rhodococcus* Emission Spectrum for Different UV Irradiation Time

From the emission spectrum in Figure 14, we have found the wavelength with peak intensity is around 340nm. We plotted the gradual change in intensity of emission peak at 340 nm wavelength for different irradiation time interval, as shown in Figure 15. As we can observe in the figure that the values of 340nm intensity keep increasing for certain time and then start to decrease and become stable, which follows the similar pattern like gram-negative *E. coli*.

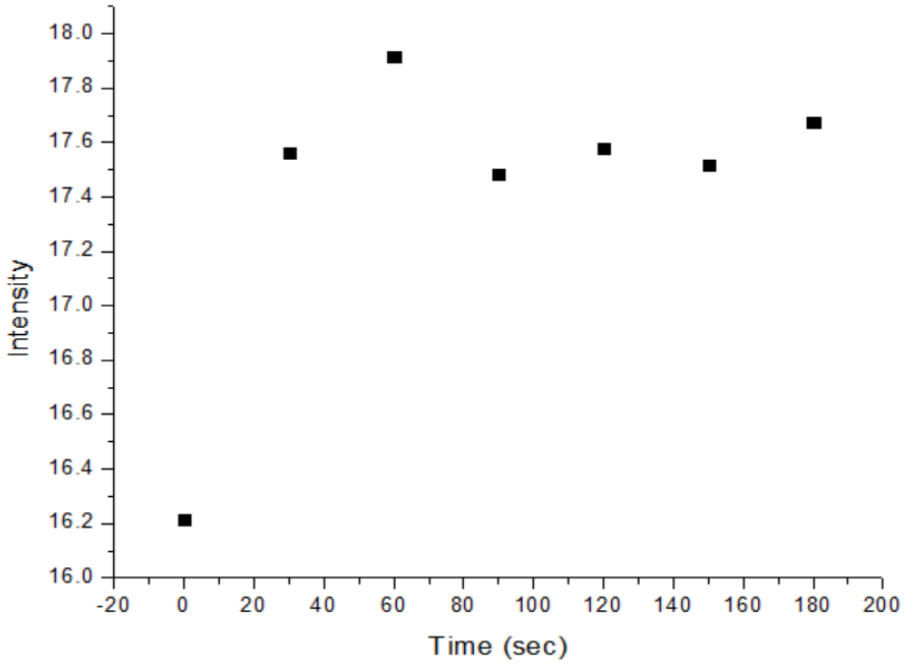

Figure 15: 340nm Peak Intensity Decay with Time by UV Irradiation

3.2 Spectroscopic Analysis of Bacteria Proteins and DNA

In addition, the spectroscopic analysis of the bacteria DNA and their two important proteins (tryptophan and tyrosine) were also performed. Our main objective of this part of the research was to find a way to differentiate DNA from the bacteria itself and from bacterial proteins for further research on 'pure' DNA.

3.2.1 Experiment and Result 1: Difference between Bacteria and DNA

For differentiating between pure bacteria DNA from bacteria spectrum, we have chosen synchronous fluorescence emission spectrum. As we discussed previously in Chapter II, synchronous emission spectrum is better for good resolution and mixture of component.

As we can see in the Figure 16 that the fluorescence emission spectrum of bacteria and bacteria DNA have almost identical shape of the spectrum, so differentiating between them is hard. The emission spectrum of bacteria was excited at 281nm and the emission spectrum of DNA was excited at 260nm in the Figure 16.

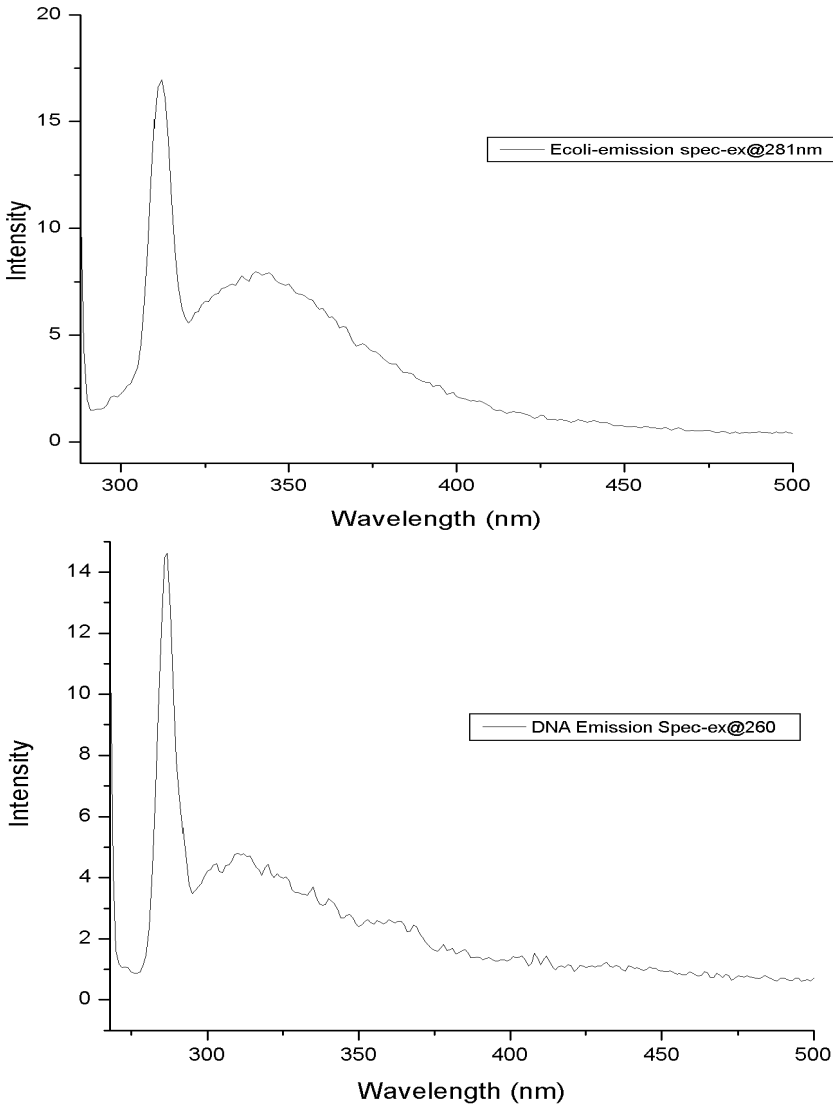

Figure 16: Fluorescence Emission of Bacteria (Top) and DNA (Bottom)

Now if we take the synchronous fluorescence emission of both the bacteria and DNA separately as shown in the Figure 17, we can observe two peaks at 325nm and 360nm wavelength for each spectrum.

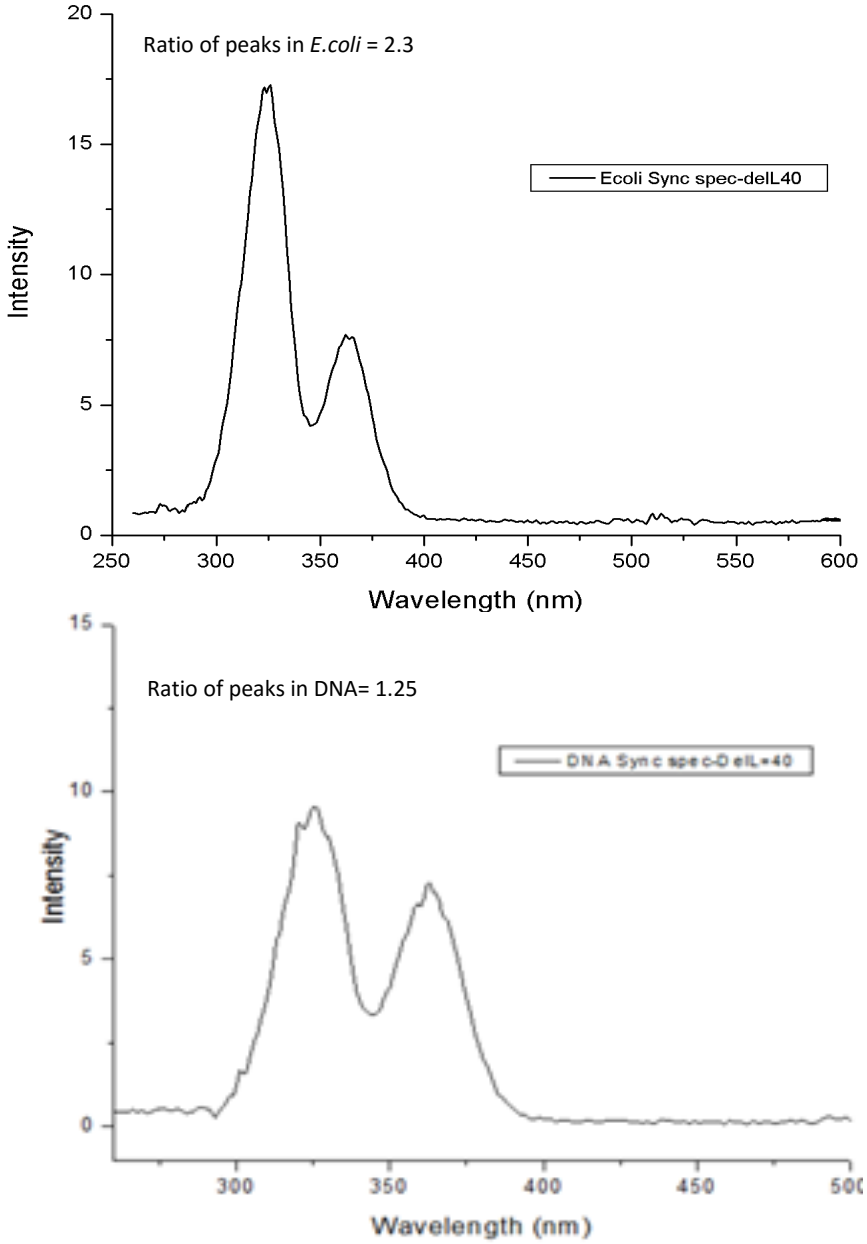

Figure 17: Synchronous Emission of Bacteria (Top) and DNA (Bottom)

The ratio of these two peaks in the synchronous emission spectrum of bacteria is much higher than the ratio of these two peaks in the synchronous emission spectrum of

bacteria DNA. Both of the synchronous emission spectrum in figure were scanned with $\Delta\lambda$=40. We have repeated this experiment for different $\Delta\lambda$ and found out $\Delta\lambda$=40 and $\Delta\lambda$=60 give significant ratio difference to easily separate bacteria spectrum and pure DNA spectrum. The synchronous emission spectrum with $\Delta\lambda$=60 for bacteria and DNA can be found in Appendix B. Thus by analyzing the synchronous spectrum, we can easily identify if one particular sample has pure DNA in it or there are some other bacteria components in it. To further perform research only on DNA of bacteria, it is very important to make sure that we are dealing with pure DNA sample.

3.2.2 Experiment and Result 2: Difference between Bacterial Proteins and DNA

Bacteria body has different proteins in it, but tryptophan and tyrosine are important ones because these two are basic protein component of the bacteria outer membrane. In the bacteria synchronous spectrum, most of the emission comes from DNA and these two proteins. If we further analyze the bacterial two important proteins, tryptophan and tyrosine; we can understand the reason behind the difference of peak ratio between bacteria and DNA synchronous spectrum. If we compare the synchronous spectrum of bacteria and DNA in Figure 17; we can observe that ratio difference between 325nm and 360nm wavelength in bacteria and DNA actually occurs, because the 325nm peak has much higher intensity in bacteria than it has in DNA.

In Figure 18, the synchronous emission spectra of tryptophan (black), tyrosine (red) and their mixture (green) have been shown and $\Delta\lambda$ was kept 40 for all of them(same as used

for DNA and bacteria analysis). We found that the synchronous emission of the mixture of tryptophan and tyrosine has a peak around 310-320nm, as shown in Figure 18. Thus from comparing the synchronous emission spectrum of bacteria, DNA and the proteins (tryptophan and tyrosine), we can conclude that the ratio difference between bacteria and DNA in the synchronous spectra occurs because in bacteria sample has huge amount of these two proteins in it and DNA sample does not have these proteins.

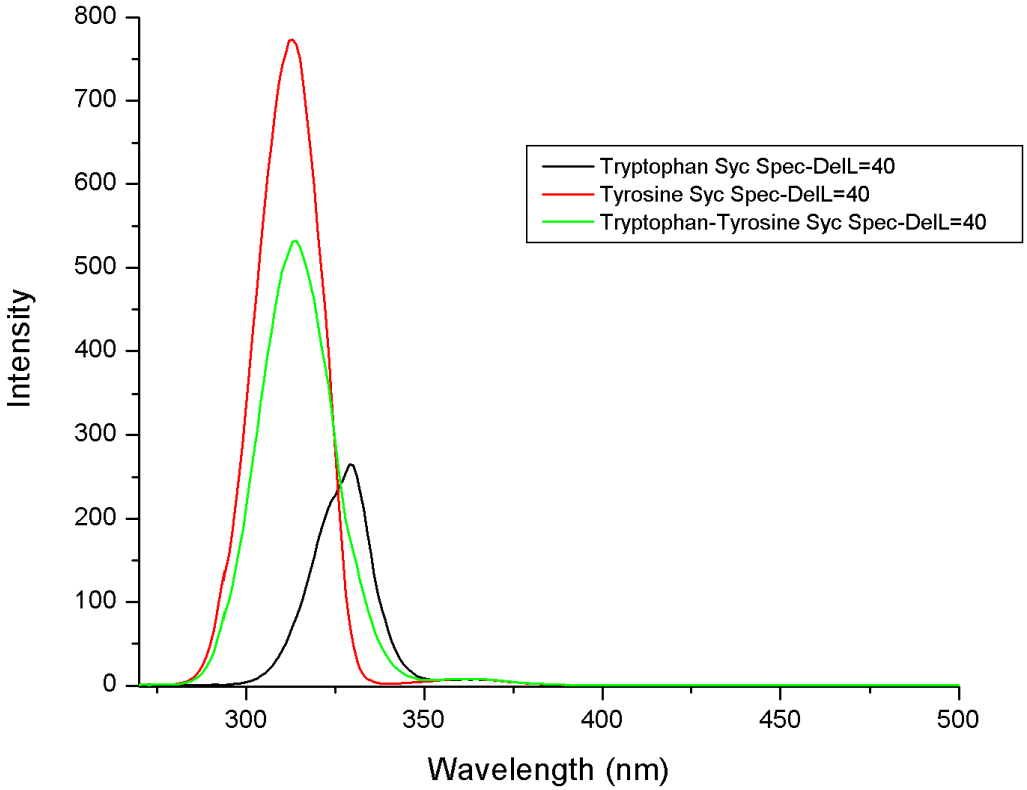

Figure 18: Synchronous Emission of Tryptophan (Black), Tyrosine (Red) and Mixture of Tryptophan and Tyrosine (Green)

From the synchronous emission spectra (with $\Delta\lambda$=40) of DNA and proteins (tryptophan and tyrosine) shown in Figure 17 and Figure 18 respectively, we can also easily differentiate between DNA and bacterial proteins (tryptophan and tyrosine). The synchronous emission spectrum (with $\Delta\lambda$=40) has 2 peaks at two different wavelengths, whereas the proteins has only one synchronous emission peak at a specific wavelength.

We did performed experiments with mixture of DNA and proteins (tryptophan and tyrosine) to find out if it can be used to differentiate proteins from this mixture. For this we scanned the synchronous spectrum with different $\Delta\lambda$ and found that $\Delta\lambda$=5 gives us good result. In the Figure 19 the result of this experiment is shown, the black synchronous fluorescence spectrum in the Figure 19a is for DNA alone and the red synchronous fluorescence spectrum is for the mixture of DNA and proteins (tryptophan and tyrosine). The green synchronous fluorescence spectrum in the Figure 19 is for the mixture of tryptophan and tyrosine. As we can observe that in the mixture of DNA and proteins, it has a sharp peak for the proteins in exactly at the same position where the green synchronous fluorescence spectrum (for the mixture of tryptophan and tyrosine) in the Figure 19 has a sharp peak. So from the mixture of bacteria DNA and proteins, we can differentiate between bacterial proteins and DNA by synchronous fluorescence emission spectra scanning with $\Delta\lambda$=5. This differentiation between bacteria DNA and proteins is important and useful, when we will do further research on “pure” bacteria DNA.

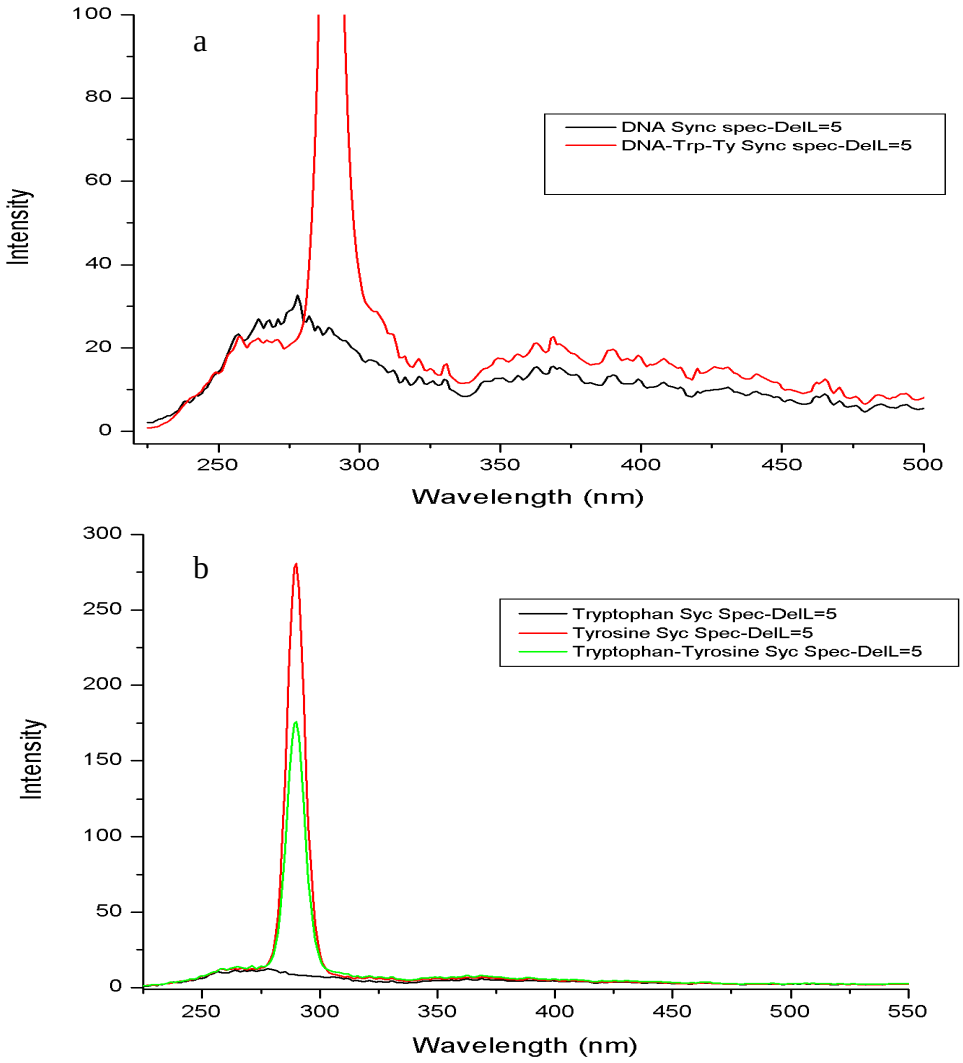

Figure 19: Synchronous Spectrum of a) DNA Alone (Black) and Mixture of DNA and Proteins (Red); b) Tryptophan (Black), Tyrosine (Red), Mixture of Tryptophan and Tyrosine (Green)

3.2.3 Experiment and Result 3: Further Irradiation of Bacteria

We have already concluded in the Chapter II that 80-90% bacteria can be killed by only 1-3mins of UV radiation with 1.1-1.3mW power. Now in this section, we will discuss about the effect of longer time irradiation of bacteria with UV light. For this experiment,

we irradiated the bacteria for 10mins and recorded the emission spectrum each step, as shown in the Figure 20. We found that peak intensity around 335 nm wavelength keep decreasing exponentially, as plotted in the Figure 20.

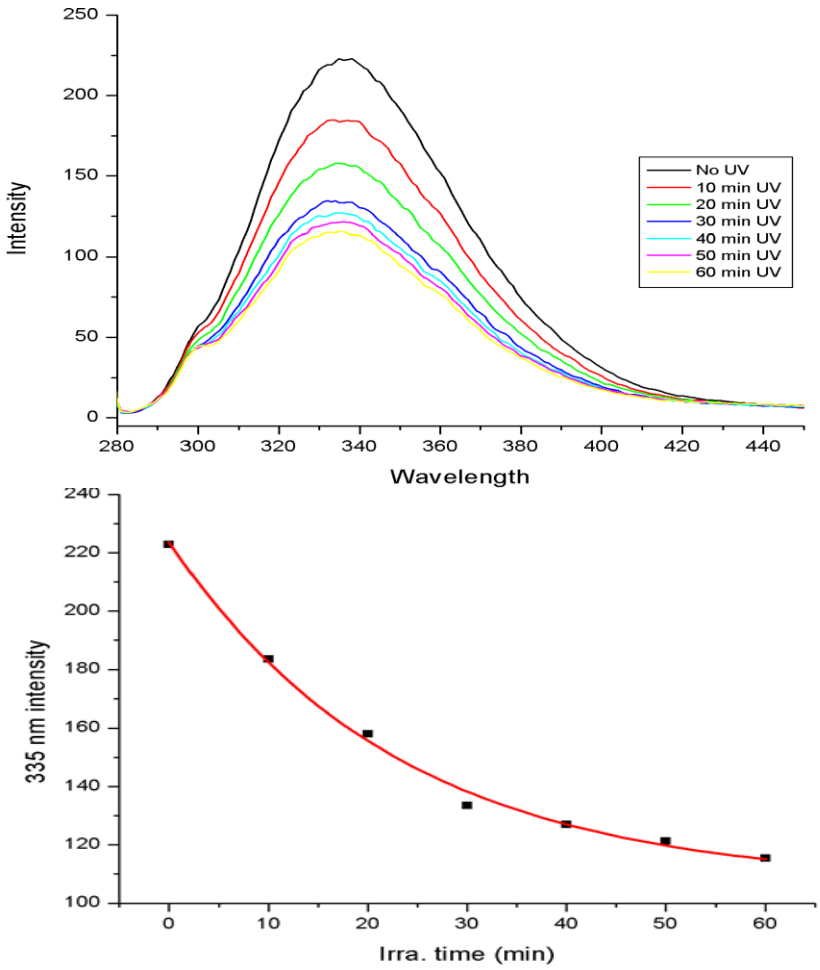

Figure 20: Longer Time Irradiation of Bacteria with UV Light

To figure out the reason for this exponential decay of the peak intensity, we performed another experiment with the bacterial proteins (tryptophan and tyrosine) as shown in

Figure 21. In this case we irradiated tryptophan and tyrosine separately, with UV light, for longer periods of time and each period of time we recorded the fluorescence spectrum. By analyzing the emission spectra, we can see in the figure that for both of the cases the peak intensity keep deceasing so rapidly as the time of UV radiation keep increasing and this rapid decay rate is almost same as the decay of bacteria for a longer time UV irradiation. So if we keep irradiating the bacteria for much longer time than 4 min, not only it kills 90% of bacteria but also it starts destroying the bacterial proteins of the outer membrane.

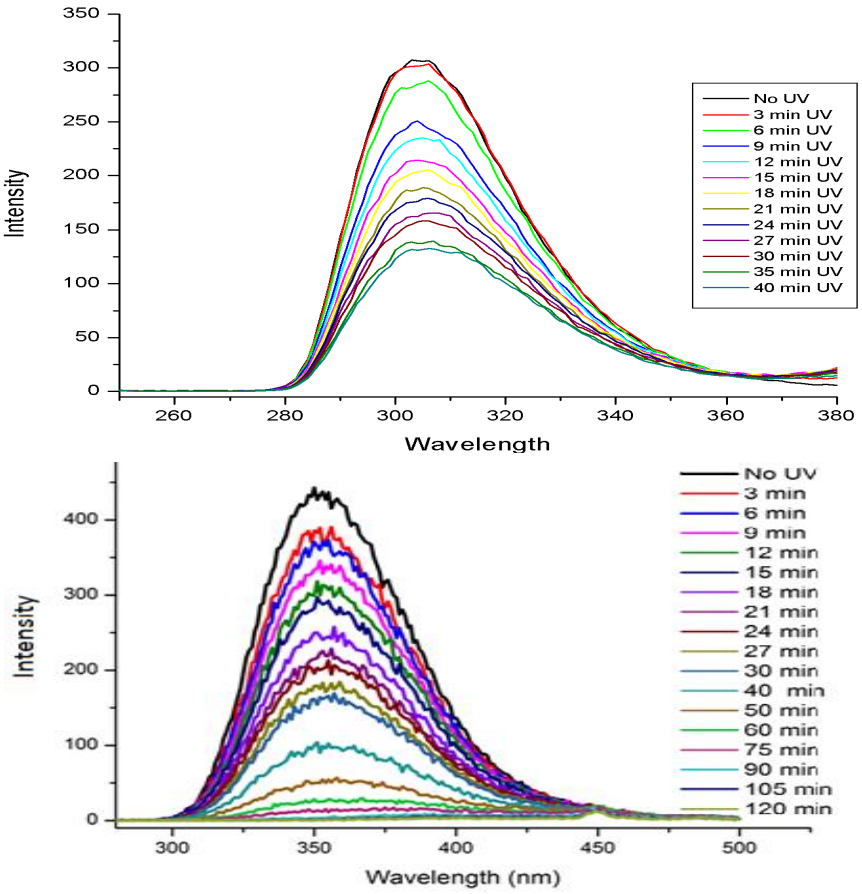

Figure 21: UV Irradiation of Tyrosine (Top) and Tryptophan (Bottom)

3.2.4 Experiment and Result 4: Difference between E. coli *and* Rhodococcus

Our next target was to find a way to differentiate between gram-negative *E. coli* and gram-positive *Rhodococcus* by spectroscopic analysis. After performing different experiment, we could differentiate gram-negative *E. coli* and gram-positive *Rhodococcus* by fluorescence emission spectrum at low concentration only. As it can be seen in the Figure 22, at low concentration, the ratio of the peak value of 310nm and 340nm are different for *E. coli* (gram negative) and *Rhodococcus* (gram positive).

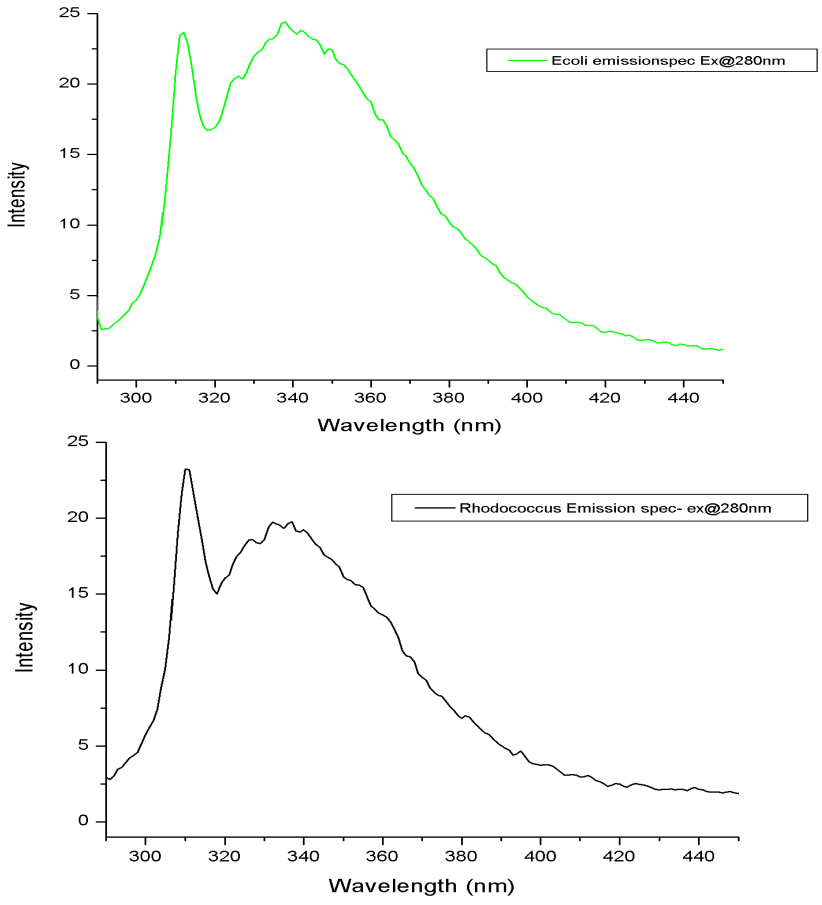

Figure 22: Fluorescence Emission Spectrum for *E. coli* (Top) and *Rhodococcus* (Bottom)

3.3 Summary

In summary, we have performed spectroscopic analysis on bacteria with different concentration and found that every time it follows a specific decay curve. From this decay curve, we can instantly identify the concentration of alive and dead bacteria in a sample or in an infected, wound area. Next we irradiated bacteria with UV light **at** different time intervals and recorded the synchronous fluorescence spectra. Later on after comparing these spectra with manually cultured data, we found that when the intensity of the emission peak increases 6-10% from its initial value, almost 80-90% bacteria are dead or inactivated.

Additionally, we did experiments on bacteria DNA and two main bacterial proteins (tryptophan and tyrosine). By spectroscopic analysis we could differentiate between bacteria and bacteria DNA, and between bacterial proteins and bacteria DNA from their mixture. We also did experiment on further UV radiation on bacteria after its inactivation and found that after inactivation if we keep irradiating bacteria, we actually start destroying its protein membrane.

CHAPTER IV

HAND-HELD SYCHRONOUS SPECTROMETER DESIGN AND DISCUSSION

In order to incorporate all of our spectroscopic analysis in a rapid and in situ application, we are designing of a hand-held synchronous spectrometer. The conventional method of bacteria analysis includes manual culture of bacteria samples in a petri-dish for around 24 hrs followed by counting the colonies generated by the live bacteria in the sample. From this number the concentration of bacteria/cm^3 can be calculated. This conventional method is very time-consuming; as bacteria reproduce every 6-20mins, this extra time can make the patient's condition worse. But with our spectroscopic analysis we can diagnose the concentration of live bacteria instantly.

Even though this spectroscopic analysis can detect bacteria concentration promptly, as we did with the spectrofluorophotometer that we used for recording fluorescence and synchronous emission spectra This system is rather large therefore not portable. When this instrument is used we have to transport the sample from the infected area and to the lab to perform the required spectroscopic analysis. For this reason, we are designing and shall construct, at a later time, a hand-held synchronous spectrometer which can scan both normal fluorescence emission and synchronous fluorescence emission spectra.

4.1 Hand-Held Synchronous Spectrometer (Design 1)

The proposed design of our hand-held synchronous spectrometer is consists of two monochromator, as shown in the Figure 23. We are proposing to use Xenon lamp as our excitation light source. The laser diode can also be used if a particular experiment needs only a few specific excitation wavelengths. The light source can be, easily, changed depending on our application. First, the excitation light source is focused into the entrance slit of the excitation monochromator; the light thus entering the spectrometer fills the entire collimating mirror (C). The slit width of the excitation monochromator will control the bandpass of light that passes through the excitation monochromator and hits the sample. Similarly, the slit width of the emission monochromator will control the width of the fluorescence that reaches and is recorded by the detector.

Bandpass (nm) = slit width (mm) × dispersion (nm/mm)

Here, dispersion depends on the grating type used in the monochromator(s) and the groove spacing of the gratings.

The collimating mirror (C) then directs the light entering to excitation monochromator and fills the entire grating (G1). The grating diffracts light and leads the diffracted light onto the focusing mirror (F). From the focusing mirror (F) the diffracted light spectrum is focused onto the exit slit of the excitation monochromator and redirected to the lens (L1). The Lens (L1) focuses the light onto the beam splitter, which is connected to the

optical fiber. The optical fiber is used to direct the light to the sample inducing excitation of the bacteria in a sample or wound.

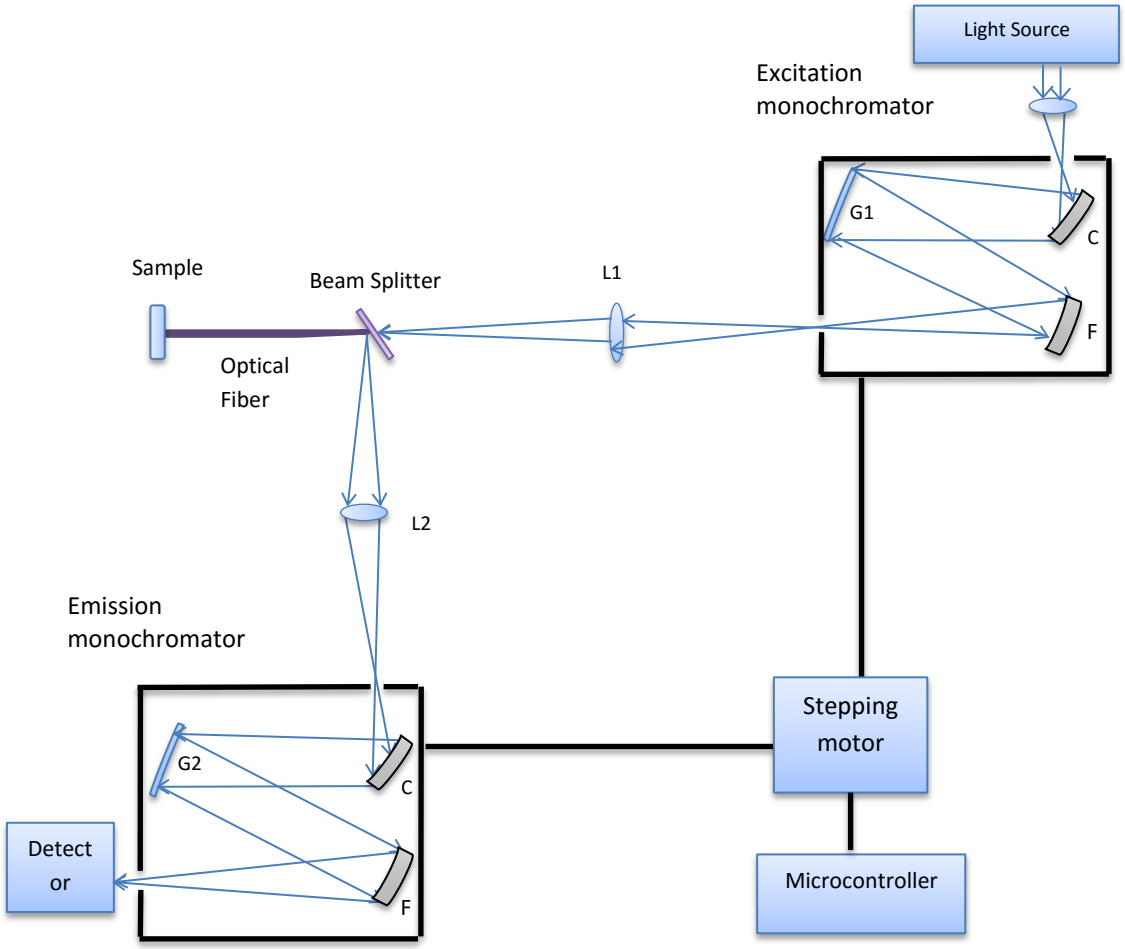

Figure 23: Hand-held Synchronous Spectrometer (Design 1)

After the sample or infected area absorbs the excitation light, the sample will emit fluorescence, which will again be redirected by the optical fiber to the beam splitter and collected at a right angle to the excitation light by the lens (L2). Here we can use the

same optical fiber to transmit the excitation light to the sample and the emission to the monochromator and subsequently to the detector or we can use different optical fibers, one for providing the excitation and another for redirecting the emission. We have to choose the right beam splitter, so that less amount fluorescence get lost because of the beam splitter.

After collecting the fluorescence light, lens (L2), will focus it to the entrance slit of the emission monochromator. The entrance slit will direct the light to the collimating mirror (C), which in turn focuses it onto the grating (G2) of the emission monochromator, where the light will diffract and lead it to the focusing mirror (F). The focusing mirror of the emission monochromator will focus the light onto the exit slit, where the light will be collected and recorded by the detector.

To control the two gratings of the two monochromator, a microcontroller stepping motor is used. For normal fluorescence spectroscopy, to record the emission spectrum we have to select an excitation wavelength and scan the emission for a range of wavelengths. To fix this excitation wavelength and control the emission grating to scan through a range of wavelength, we need a microcontroller controlled stepping motor. Also in the case of synchronous fluorescence emission, we need to move both the excitation and emission grating synchronously with a specific constant wavelength difference between them. The microcontroller based stepping motor will help to not only help to predefine the starting

wavelength of the two monochromator, also maintain the desire constant wavelength difference and help to control the synchronization between them.

4.2 Hand-Held Synchronous Spectrometer (Design 2)

Another design has been proposed in the Figure 24, which is comparatively more costly, than the first design, but results in higher resolution, better sensitivity and reduced stray-light. The reason behind the better resolution and comparatively higher price, in the second design, is the use of a dual grating monochromator. But in some of the applications, especially when we want to analyze a sample with multiple component, which have emission peaks those are very close to each other, we need better resolution to resolve those emission peaks.

As discussed in the previous design, Xenon lamp(s) or laser diode(s) (for specific application) can be used as an excitation light source. Similar to the first design, this design also has two monochromator but of two different types. First the excitation light will come from the light source and will be focused at the entrance slit of excitation monochromator by a lens. Similarly, to previous design entrance slit limits the bandwidth of the excitation light. From the entrance slit the light will hit the first grating (G1) and diffracts the excitation light. The diffracted light will lead towards a curve which will focus on a middle slit (S), which will select the desired wavelength. From the middle slit (S) light will direct to the second grating (G2), where signal to noise ratio

will improve as stray light has been reduced a lot. Stray light means the light which is being detected, has some different wavelengths other than the desired wavelength. The increase of stray light can affect the measured values and often decrease the resolution. Then second grating will focus the desired excitation wavelength to the exit slit of the excitation monochromator with huge reduction in stray light.

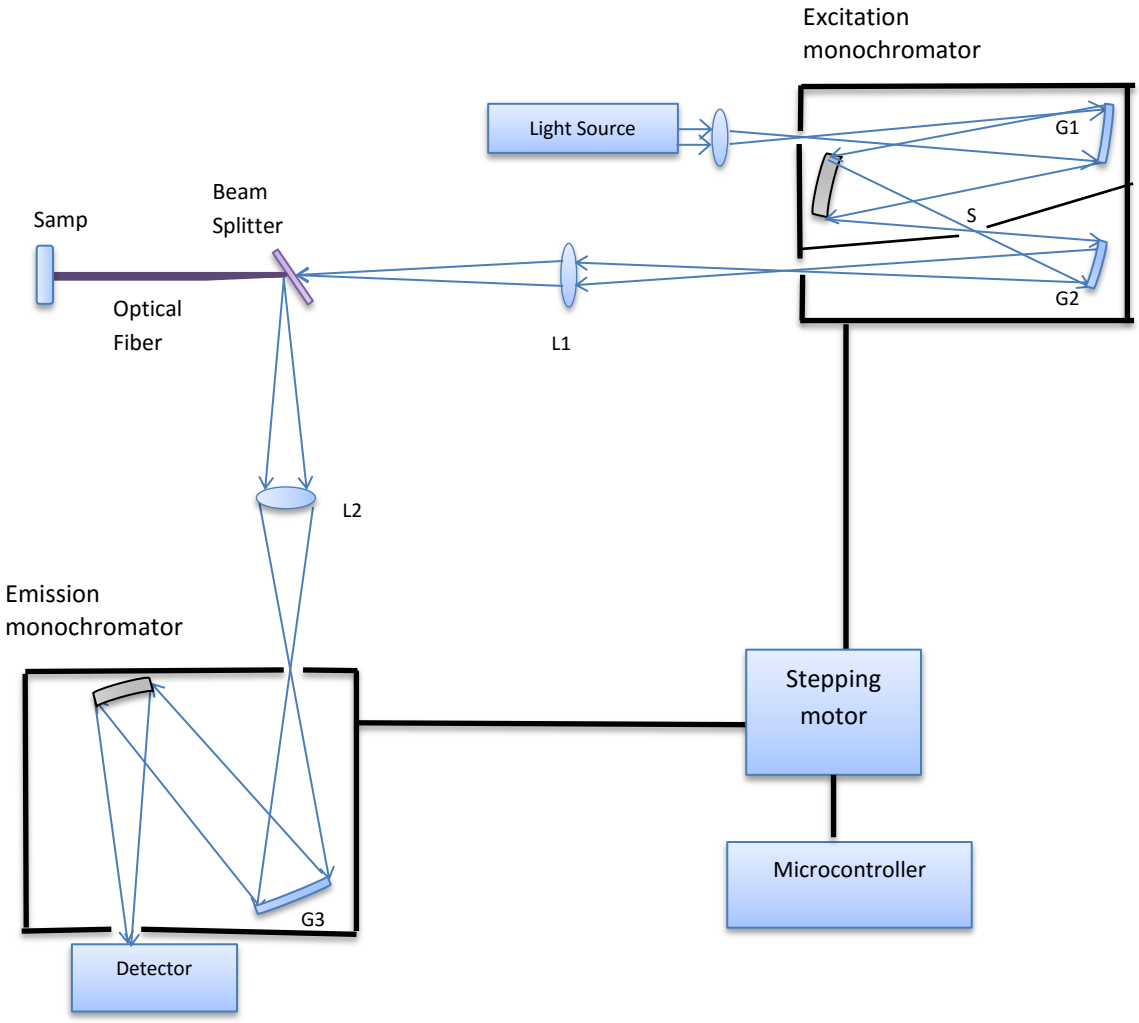

Figure 24: Hand-held Synchronous Spectrometer (Design 2)

From the exit slit of the excitation monochromator, the light will be focused onto a beam splitter by the lens (L1). The optical fiber will then direct the excitation light to the sample or infected area and collect the fluorescence emitted from the sample. The fluorescence emission will hit the beam splitter and focus to the entrance slit of emission monochromator by the lens (L2) at a right angle to the excitation light. Then grating (G3) will diffract the light and with the help of a curve mirror the light will be focused to the exit slit, from where the detector will collect it. Again similar to the previous design, we have stepping motor and microcontroller to control the two monochromator for both fluorescence spectroscopy and synchronous scan fluorescence spectroscopy.

The design can be modified further by using a dual grating structure in the emission monochromator, similar to the dual grating in the excitation monochromator. In this case the resolution will be much better than the second design, which has dual grating structure only in the excitation monochromator. Again, using dual grating structure for both of the excitation and emission monochromators, the price of this design will be a little higher than the second design.

4.3 Proposed Detail Specifications

The designs proposed for hand-held synchronous spectrometer will be operated probably by a USB/ serial port to connect with a tablet pc. Each pixel captured by the detector creates a digital respond, which corresponds to the wavelength of the light that hits it.

Then a software will be used to collect and plot this digital signal. Other, possible, specifications are listed in the Table 1.

Table 1: Proposed Specification of Hand-Held Synchronous Spectrometer

Grating Options	Different gratings can be used depending on application
Detector	Photo-multiplier
Wavelength Range	200-800 nm
Tablet Interface	USB (Universal Serial Bus)
Optical Fiber	single-strand

4.4 Summary

In summary, we are designing two different types of hand-held synchronous spectrometers. They can be used to determine concentration of live and dead bacteria in an infected area in situ, instantly. They can also be used for other spectroscopic analysis described in this thesis. This hand-held spectrometer can scan both conventional fluorescence spectra and in addition synchronous fluorescence spectra with the help of a

stepping motor and microcontroller. The two design have different grating components for the excitation monochromator. The design which includes a dual grating structure results in a better spectral resolution in exchange small additional cost.

CHAPTER V

NANO-SECOND TIME-RESOLVED SPECTROSCOPY SYSTEM

5.1 Definition of Time-Resolved Spectroscopy

Time-resolved spectroscopy allows us to measure and analyze the progressive dynamics in materials or chemical compounds and kinetics of photo-physical and physicochemical processes by means of spectroscopic techniques. In the technique, generally a laser pulse is used as a pump pulse to illuminate the sample and a flash light or the same laser pulse will use as probe pulse, hitting the photo-excited sample at a proper time delay for measuring the changes of the intermediates and study the fast interactions like intermolecular electron transfer in the nano-second to femto-second regime. The delay between this two pump and probe pulse is controlled to record the changes of the transient as a function of time by the detector, as shown in the Figure 25.

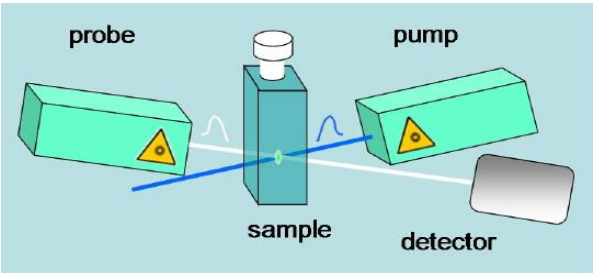

Figure 25: General Scheme for Pump and Probe Methods [21]

5.1.1 Transient-Absorption Spectroscopy

The time-resolved or transient absorption spectroscopy basically measures the changes in the absorbance of the sample at a desired range of wavelength after excitation with a pump pulse. The absorbance is measured as a function of time by controlling the delay time between pump and probe pulse. We actually do not get the measure the absorbance directly, we measure the transmittance. We calculate the absorption by this equation:

Absorbance, $A = -\log T = \log P_0/P$, [22]

Where T = transmittance, P_0= power in and P= power out.

The change in absorbance can be measured from the change of the absorbance after pump pulse and the absorbance after probe pulse. [21]

5.1.2 Time-Resolved Fluorescence Spectroscopy

Similar to transient absorption spectroscopy, time-resolved fluorescence spectroscopy basically measures the changes in the fluorescence of the sample at a desired range of wavelength after excitation with a pump pulse. Time-resolved fluorescence spectroscopy is mainly used for measuring the fluorescence life time of a molecule. After the sample is excited by pump pulse, only fluorescence light that reaches at the detector and the detector helps to get a dynamic picture of the fluorescence. Fluorescence emission is an ideal nano-range probe, because the fluorescence decay usually takes place on the nano-second time regime. One way to measure the fluorescence decay is time-correlated single-photon counting method, where it is based on the ability to detect and count individual photons by controlling the trigger time of the photodiode (detector).

5.2 Design of Time-Resolved Nanosecond Spectroscopy System

Our design of the time-resolved nanosecond spectroscopy system is shown in the Figure 26. Here the laser that is used for the pump pulse is a Nd^{3+}:YAG laser, with pulse width: 5-6 ns, and pulse energy around 200 mJ at 1064 nm. It can be operated as single pulse or continuous pulse laser at repetition rate up to 20 Hz. The laser also generates second, third and fifth harmonics, by means of KDP (potassium dihydrogen phosphate) phase-matched crystals. The other components of this system are a pulse generator, xenon lamp, mirrors, lenses, iris diaphragm, monochromator, photo-multiplier and oscilloscope.

There were two trigger pulses used with the design, one trigger originated from the laser and provides the input trigger to the pulse generator, which is used to control the delay time between the pump and probe pulses. The second trigger is the output trigger from the pulse generator which triggers the Xe lamp (probe pulse) as well the oscilloscope which is coupled to and receives the data from the photo-multiplier.

The two mirrors M1, M2 are used to redirect the laser pulse to the sample and the two lenses L1, L2 are used to focus the light of the xenon lamp on the sample, at exactly the same position where the laser pulse is striking the sample. Lens L3 is used to focus the transmitted or fluorescence light from the sample onto the monochromator slit. A photo-

multiplier is attached to the monochromator that collects the data and pass it to the oscilloscope.

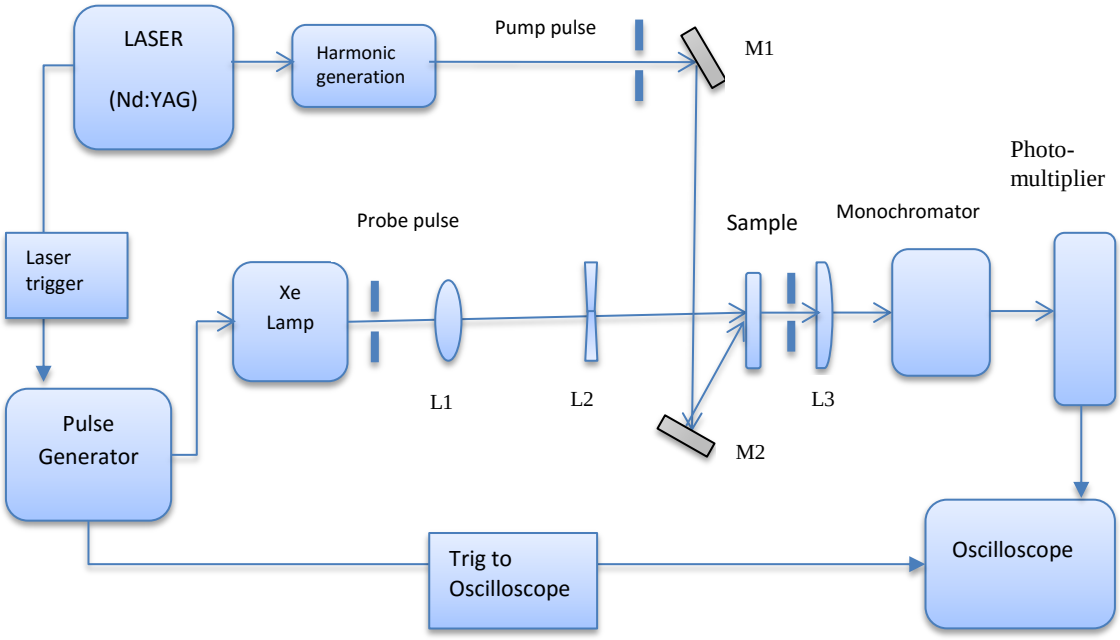

Figure 26: Design of Time-Resolved Nanosecond Spectroscopy System

The operating principle of this design is: when the pump pulse strikes the sample and excites the molecules, after a specific delay time, determined by the pulse generator, the probe pulse will impinge on the sample. The monochromator collects the corresponding emission and directs it to the photo-multiplier. By changing the angle of the grating, we will be able to take data at different wavelengths. When the oscilloscope receives an external trigger from the pulse generator, it displays the data collected from the photo-multiplier. As it can observed in the Figure 26 the pump pulse is propagating through the

sample at small angle compared to the probe pulse. The reason behind this small angle is to make certain that laser pulse does not enter the monochromator, otherwise the high intensity of this pulse might damage the monochromator, or at a minimum will induce intense scattered light which will appear as noise. The angle between the pump and probe pulse should be as small as possible in order for them to overlap in the sample as much as possible in order to generate the strongest possible signal.

5.3 Practical Implementation and Future Plan

5.3.1 Practical Implementation with Timing Diagram

After designing the optical setup of the nano-second time-resolved spectroscopy system, we implemented the design on an optical table. The optical table setup of the design is shown in the Figure 27 (a&b). The control of the laser pulse and pulse generator is shown in Figure 27 c and d respectively.

The timing diagram of the design has shown in Figure 28. Because it is a nano-second system, adjusting the timing of the triggers and the delay time is very crucial. When a laser pulse hits the sample, it gives a trigger to the pulse generator 202μs before firing the laser. The laser lamp trigger is 2.1ms long. After getting the trigger from the laser, the pulse generator will give an output trigger with such a time delay so that the probe pulse will hit the sample after our desired delay of time. As the xenon lamp, which is generating the probe pulse has its own delay time to flash after the input trigger from

delay generator, this internal delay (~9μs) of xenon lamp is needed to take into consideration when calculating the desired delay between pump and probe pulse.

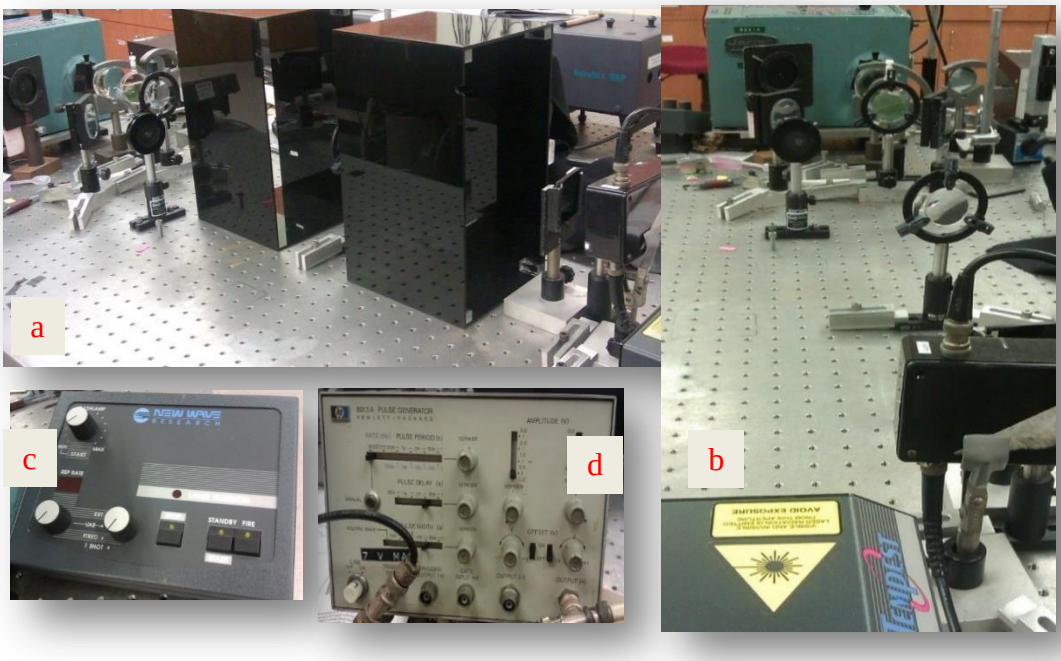

Figure 27: Practical Implementation of Nano-Sec Time-Resolved Spectroscopy System

For ensuring the proper working condition of this design and implementation, we have done some experiment with known results. The results of these experiments are shown in Appendix C.

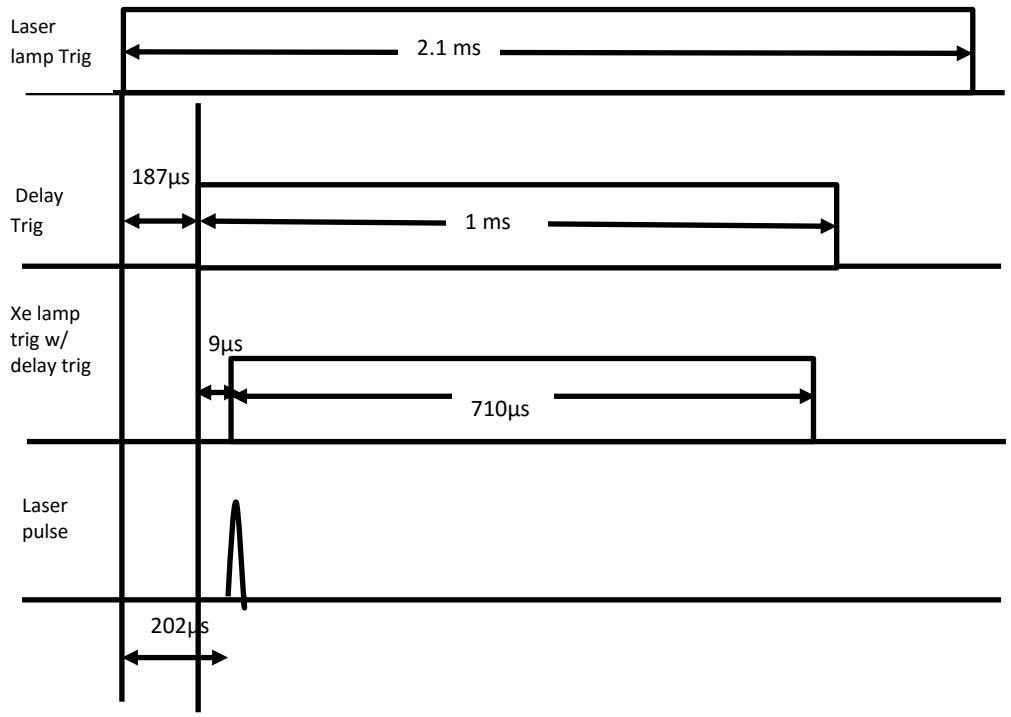

Figure 28: Timing Diagram of Time-Resolved Nanosecond Spectroscopy System

5.3.2 Applications

Time-resolved spectroscopic systems has many applications in photo-physics, materials science, nanoscience, and solar energy conversion and storage used to analyze dynamic processes of physical, chemical and biological systems. Such spectroscopic systems can also be used to measure fluorescence lifetimes.

The main purpose of our nano-second time-resolved system for further research on these two areas mentioned below.

1. The DNA of bacteria dimerizes when the bacteria become inactivated or die. This dimerization process of bacteria DNA is expected to be occurring in the

nano-second time scale. We will be able to do the analysis of the gradual process of DNA dimerizing mechanism with the help of this proposed nano-second time-resolved spectroscopy.

2. It has already been published that illuminating methylene blue with 661nm light produces singlet oxygen which can inactivate bacteria. [23] [24] The time of forming singlet oxygen from the excited singlet state of methylene blue is expected in the nano-second regime. Using our nano-second time-resolved spectroscopic system, we expect to be able to determine and derive the mechanism and the rate for the generation of singlet oxygen from the excited state of methylene blue.

5.4 Summary

In summary, we have designed and constructed a nano-second time-resolved spectroscopic system on an optical table. The purpose of this design is to use this system for further analysis of bacteria inactivation, and determine the mechanism of DNA dimerization and formation of singlet oxygen photo-generated by methylene blue.

CHAPTER VI

CONCLUSION

Rapid detection and identification of bacteria became a concern in the world recently. As the conventional, manual culture and incubation process is time (~24hrs) and personnel consuming, people are searching for faster way to detect bacteria to prevent infections. The main purpose of this research was to detect and identify bacteria concentration in situ, instantly. With our spectroscopic analysis of bacteria florescence, we are able to identify the concentration of live and dead bacteria in a sample or infected area instantly. Our UV irradiation experiment on bacteria can also pinpoint the percentage of live and dead bacteria in a sample or infected area by measuring the percent change in the intensity of the emission band maxima. This analysis also helped us to detect the amount of flux of UV radiation needed to kill or inactivate 80-90% live bacteria.

In addition, our spectroscopic analysis could distinguish between bacteria and bacteria DNA from each other, as well as between bacterial proteins (tryptophan and tyrosine) and DNA. Separating the DNA from the bacteria itself or bacterial proteins is important for further research on ‘pure’ bacteria DNA and measure of its dimerization. The excessive UV irradiation of bacteria has also been studied and compared with the irradiations of bacterial proteins (tryptophan and tyrosine); We found that irradiating bacteria for longer periods of time after its inactivation phase, the two important

bacterial proteins, tryptophan and tyrosine, which are two basic component of bacteria's outer membrane start to get destroyed.

For the application of all of our spectroscopic analysis in this research, we designed a hand-held synchronous spectrometer. This hand-held instrument is portable, therefore it can be used in remote areas as well as in hospitals. The two proposed designs of the hand-held instrument have different configurations in the excitation monochromator, one is with single grating monochromator and other one is with dual grating monochromator. The dual grating excitation monochromator will give better resolution, less stray light in exchange of a little higher price.

Additionally, a nano-second time-resolved spectroscopic system has been designed and implemented on an optical table. The operating system of this design has been explained by means of a timing diagram of the system. This system has been designed to be used for further research on bacteria inactivation, such as determining the mechanism of DNA dimerization and analyzing the formation of singlet oxygen formation from the excited state of methylene blue.

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APPENDIX A

PREPARATION OF FRESH *E.COLI* SUSPENSION

The mid-log phase (OD^{600} = 0.5) fresh cultures of *Escherichia coli* K-12 MG1655 (*E. coli* Genetic Resources at Yale CGSC, The Coli Genetic Stock Center, New Haven, NE) were grown in Luria Bertani (LB) medium [25] [26] for about 3 h at 37◦C with constant shaking at 0.102 g's. (*E. coli* is a facultative anaerobic bacterium, requiring oxygen during fermentation). The mid-log cells are uniform in age, size, and physiological characteristics, which allow them to respond more uniformly to the different stresses during further testing. They were harvested by pelletizing them in a centrifuge at 2880 g's for 7 min and resuspended in sterile MilliQ water to a cell concentration of about 109 CFU/mL (Colony Forming Units per Milliliter).

We have also used different *E. coli* strain for our analysis that is *E. coli* DH5α. For the gram-positive *Rhodococcus* bacteria, we have used *Rhodococcus* opacus PD630.

APPENDIX B

DIFFERENCE BETWEEN BACTERIA AND DNA BY SYNCHRONOUS EMISSION

As shown in Figure 29, the difference between bacteria and bacteria DNA can also be identified by the synchronous fluorescence spectrum with $\Delta\lambda=60$, similar to the identification between them discussed with $\Delta\lambda=40$ in chapter III.

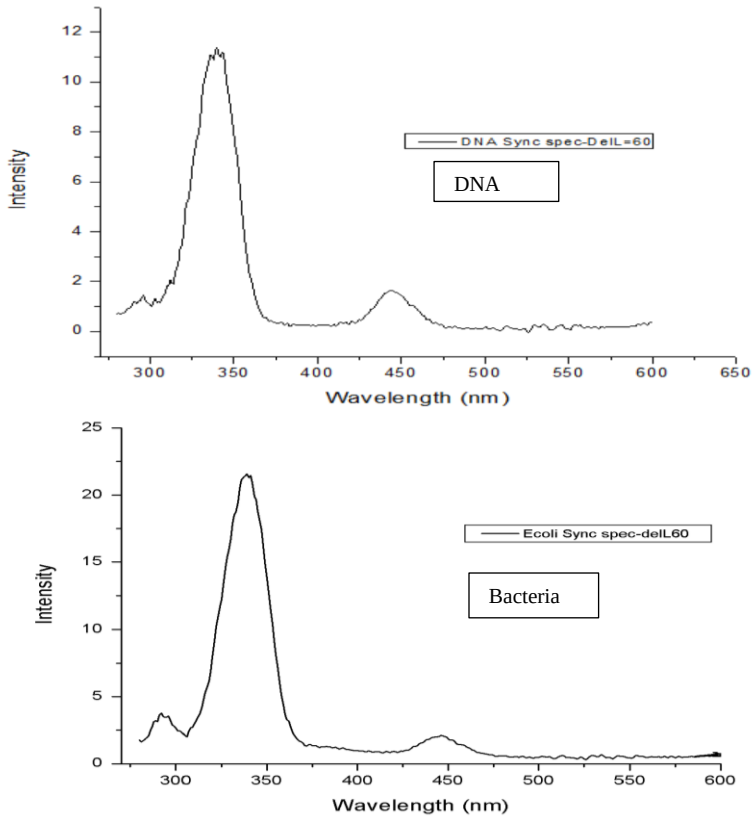

Figure 29: Difference between Bacteria and DNA by Synchronous Emission

APPENDIX C

TEST RESULT FROM NANO-SECOND TIME-RESOLVED SPECTROSCOPY

Time-Resolved Absorption (Cresyl Violet)

For ensuring proper operation of our designed of nano-second time-resolved spectroscopic system, we have measured the time-resolved absorption spectra of Cresyl violet and found that our data are identical with the literature data, as shown in Figure30.

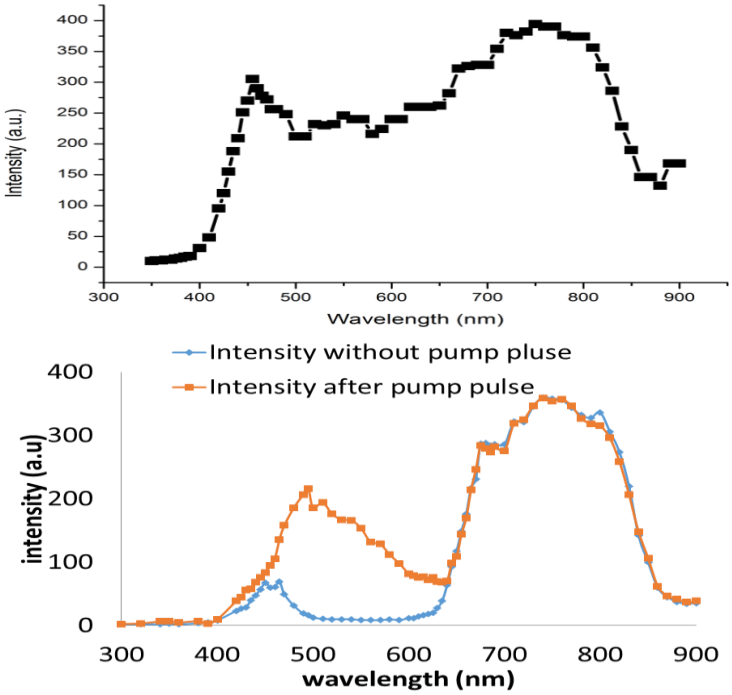

Figure 30: Transmittance Intensity: Only the Probe Pulse (Xe Lamp) without Any Sample (Top), the Probe Pulse (Xe Lamp) with Sample (Bottom)

Time-Resolved Fluorescence (Rhodamine 6)

To confirm the proper operation of our time-resolved fluorescence, designed system, we have tested it with Rhodamine 6, using the second harmonic, 532 nm pulses, for excitation. We confirmed that our result correlate with the ones listed in the literature, as shown Figure 31.

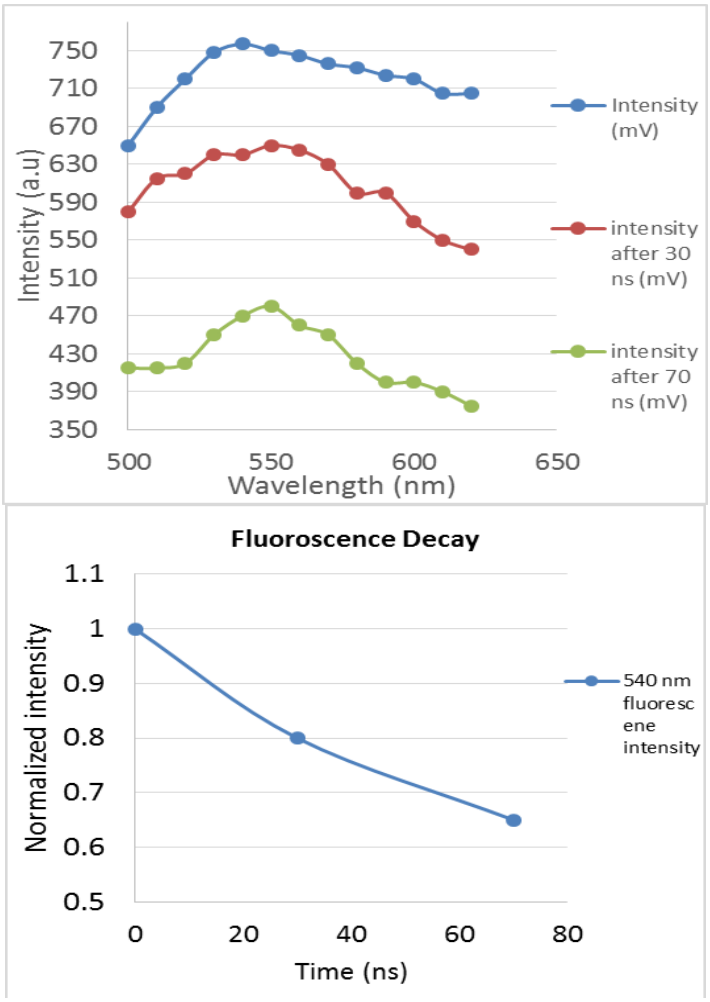

Figure 31: Time-Resolved Fluorescence of Rhodamine 6