ISOLATION AND ANTIMICROBIAL POTENTIAL OF EPSILON-POLY-L-LYSINE

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By

Elisabeth Curylo, Shi Chian Khor, Christopher Mayo, Mary Gwendolyn Ruda, Gregory Parks

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Approved:

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Professor Michael A. Buckholt

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Professor Jill Rulfs

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Professor Theodore C. Crusberg

ABSTRACT

ε-Poly-L-lysine (εPL) is a biopolymer that has potential uses in a variety of fields including medicine, bioelectronics, and the food industry (as a food preservative). The goal of this project was to isolate εPL producing bacteria or fungi from soils suspected to be polluted by heavy metals, including chromates. The project concentrated on assaying the soil samples for chromium content, testing the specificity of the method of Itzhaki (to measure concentrations of εPL), and testing the antimicrobial effectiveness of εPL.

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Table of Contents

Table of Tables

Table of Figures

Authorship

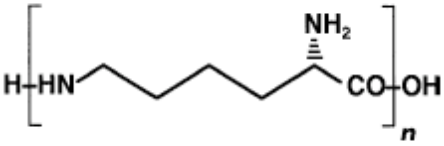
Section	Title	Author
*****	Acknowledgements	Everyone
*****	Abstract	Everyone
1	Introduction	*****
1.1	What is ε-poly-L-lysine?	Chris Mayo
1.2	Uses of ε-poly-L-lysine	Greg Parks, Mary Ruda
1.3	Purpose and Goals of the Experiment	Everyone
1.4	Antimicrobial Properties of ε-poly-L-lysine	Elisabeth Curylo
2	Materials and Methods	*****
2.1	Soil Sample Collection	Everyone
2.2	Screening Method for Detection of ε-poly-L-lysine Producing Bacteria	Greg Parks, Chris Mayo
2.3	Preparation of Soil Samples of the Chromium Assay	Shi Chian Khor
2.4	Preparation of Chromium Solution	Shi Chian Khor
2.5	Spectrophotometric Determination of Chromium in Soil Samples	Shi Chian Khor, Chris Mayo, Mary Ruda
2.6	Method of Itzhaki for Analysis and Determination of ε-poly-L-lysine	Shi Chian Khor, Chris Mayo, Mary Ruda
2.7	Fermentation of Streptomyces albulus routin	Chris Mayo
2.8	Measuring Production of ε-poly-L-lysine by Streptomyces albulus routine	Gregory Parks
2.9	Fermentation of Putative Positives	Chris Mayo
2.10	Filter Disk Assay for ε-poly-L-lysine Activity	Greg Parks
2.11	Antimicrobial Effectiveness of ε-poly-L-lysine MIC Test	Chris Mayo, Greg Parks
3	Results	*****
3.1	Chromium Assay Standard Curve	Shi Chian Khor
3.2	Concentration of Chromium in the Collected Soil Samples	Shi Chian Khor
3.3	Method of Itzhaki for ε-poly-L-lysine	Shi Chian Khor
3.4	Method of Itzhaki for poly-L-lysine	Shi Chian Khor
3.5	Method of Itzhaki for L-lysine	Shi Chian Khor
3.6	Determination of εPL in Streptomyce albulus routien	Shi Chian Khor
3.7	Screening for ε-poly-L-lysine Producing Bacteria	Elisabeth Curylo
3.8	Filter Disk Assay	Chris Mayo
3.9	Minimum Inhibitory Concentration Test	Greg Parks
4	Discussion	*****
4.1	Presence of Chromium in Soil Samples	Shi Chian Khor
4.2	Method of Itzhaki to Detect and Quantify ε-poly-L-lysine	Greg Parks, Chris Mayo
4.3	Antimicrobial Effectiveness of ε-poly-L-lysine	Elisabeth Curylo, Greg Parks, Chris Mayo
4.4	Summary of Results	Elisabeth Curylo
*** Revising and editing completed by Elisabeth Curylo, Christopher Mayo, Gregory Parks, and Mary Gwendolyn Ruda		

INTRODUCTION

1.1 What is ε-Poly-L-lysine?

ε-Poly-L-lysine (εPL) is a linear homopolymeric compound created through the peptide bond linkage of lysine monomers at the α-carboxyl and ε-amino groups (Shima and Sakai, 1981b). It is naturally secreted by various *Streptomycetaceae* bacteria and some filamentous fungi and is more commonly found than its isomer α-Poly-L-lysine (Nishikawa and Ogawa 2002; Shima and Sakai, 1981a,b; Skozan et al., 1997; Takehara et al., 1999). Unlike α-poly-L-lysine and many other proteins, the amide linkage in ε-poly-L-lysine is between the ε-amino carbon and carboxyl group (Figure 1). In α-poly-L-lysine the amide linkage is between the α-amino carbon and carboxyl group. Synthetic manufacture can only produce α-poly-L-lysine and so ε-poly-L-lysine is produced by organisms and extracted. Industrial production of ε-poly-L-lysine is carried out by aerobic fermentation of *Streptomyces albulus* strain 346 which was isolated in Japan (Chisso 2007).

Figure 1. Chemical formula of e-poly-L-lysine (Yoshida and Nagasawa, 2003)



1.2 Uses of ε-poly-L-lysine

Lysine is one of one of three basic amino acids. The amino acid has a positively charged ε-amino group. A polymer of lysine residues therefore is highly positively

charged. In polar solutions, the εPL polymer forms a compound with hydrophilic carboxyl and amino groups on the outside and hydrophobic methylene groups on the inside. The cationic nature of εPL allows it to inhibit the formation of cell membranes of a wide range of microbes, including yeasts, fungi, and both gram-positive and gram-negative bacteria by stripping the outer membrane and abnormal distribution of cytoplasm (Yoshida and Nagasawa, 2003). Since εPL is non-toxic to humans even at high doses and exhibits antimicrobial activity (Shima and Sakai, 1977; Shima et al., 1982, 1984), the molecule has been utilized as a food preservative in Japan for many years (Hiraki et al., 2003).

α-Poly-L-lysine is not antimicrobial because a chemical modification on the alpha amino group hinders the adsorption of the molecule to the cell's surfaces (Yoshida and Nagasawa, 2003). The proposed mechanism for εPL antimicrobial activity is its electrostatic adsorption onto the cell surface of microorganisms leading to stripping of the outer membrane and abnormal distribution of cytoplasm (Shima et al. 1984). Differences observed in the antimicrobial effectiveness on various organisms may stem from a difference in the cell surfaces among the organisms (Yoshida and Nagasawa, 2003).

To achieve maximum antimicrobial activity, at least 10 monomeric L-lysine residues are needed (Shima et al. 1984). εPL produced by *Streptomyces albulus* consists of 25-35 monomeric L-lysine residues. Taste becomes an issue when εPL is used as a food preservative: long εPL polymers and high concentrations of εPL cause a bitter taste in food. However, εPL exhibits a high antimicrobial activity in low concentrations. Rats given ε-poly-L-lysine at 3000, 10,000 and 30,000 suffered no toxicity in reproductive,

neurological, and immunological functions for two generations (Neda et al. 1999). ε-Poly-L-lysine has been shown to inhibit growth in a wide variety of organisms at concentrations of less than 100 μg/mL (Yoshida and Nagasawa, 2003). The amount of εPL needed in food preservation is much lower than even 300 ppm. εPL also has Generally Recognized As Safe (GRAS) status for certain food applications (Anon CFSAN/Office of Food Additive Safety 2004).

εPL has an isoelectric point of 9.0. Since antimicrobial action of εPL is an electrostatic interaction with the cell surface, pH can greatly affect antimicrobial activity. In alkaline conditions, greater amounts of εPL are needed as antimicrobial activity is lowered. A pH range of 5.0-8.0 is best for εPL antimicrobial activity. The addition of anionic molecules decreases εPL activity since the cationic charge is partially lost (Yoshida and Nagasawa, 2003).

Studies have also been performed to investigate the use of εPL as an emulsifying agent (Ho et al., 2000), a dietary agent (Kido et al., 2003), drug-delivery carrier (Shen and Ryser, 1978, 1979, 1981), a gene delivery carrier (Chiou et al., 1994; Dorudi et al., 1993), in the development of hydrogels (Kunioka, 1995; Kunioka and Choi, 1995), and as a coating material for biochips and bioelectronics (Ostuni et al. 1999; Cai et al., 2002; Wallace et al., 2000).

ε –Poly-L-lysine holds potential for not only an antimicrobial agent but also a bioremediator. Heavy metal binding of εPL has not been sufficiently explored and holds a lot of potential. If εPL proves to be useful in removing heavy-metals from water sources, the cheap mass production of the polymer would be an extraordinary resource

for locations where heavy-metal pollution is a problem. The properties of εPL make it an ideal molecule to use in bioremediation. Due to the cationic nature of εPL it is believed that it will have the ability to bind to heavy metals. Additionally, since εPL is a biopolymer it is also biodegradable, which would mean that the method would also be environmentally friendly and safe. Presently, there is a lot of heavy-metal pollution in water and an inexpensive process is needed to reduce heavy metal release from industries, which are legally allowed to discharge certain amounts of heavy metals into water (APHA).

1.3 Purpose and Goals of the Experiment

The goals of the project were the following:

1. To collect soil samples from areas suspected and/or known to contain high concentrations of heavy metals (specifically chromium).
2. To test all soil samples for the presence of chromium.
3. To isolate ε-poly-L-lysine producing bacteria from collected soil samples.
4. To test the specificity of the method of Itzhaki in measuring εPL.
5. To ferment *Streptomyces albulus* Routien (ATCC, Manassas, VA) and putative ε-poly-L-lysine producing bacteria.
6. To determine if *Streptomyces albulus* Routien produces εPL and can serve as a positive control for comparison to other organisms grown from the soil samples.

The soil samples were collected from known areas of high pollutants in order to find high yielding εPL producing strains of bacteria or fungi that have been

environmentally challenged with chromium. Once the soil samples were collected, a chromium assay was performed in order to determine the amount of chromium present in the soil. In preparation for a larger project, which would involve searching for other εPL producing strains of bacteria or fungi other than *Streptomyces albulus*, the specificity of the method of Itzhaki was tested. The purpose of that was to determine how useful the method of Itzhaki would be in measuring the amount of εPL in the organisms isolated by this project. Lastly, a positive control stain, *Streptomyces albulus* Routien, was fermented and tested for any εPL production.

It is hypothesized that the highest εPL producing bacterial strains will be found in soils containing higher concentrations of chromium. It is also hypothesized that εPL isolated from the bacterial strains collected in the soil samples will have antimicrobial potential and the ability to bind to heavy metals. This project will test the effectiveness of ε-poly-L-lysine against some bacterial strains that it is known to inhibit; and will also examine whether or not the collected soil samples contained bacteria which produce the polymer. Finally those bacteria will be fermented and the fermentation broth will be tested for the presence of an active antimicrobial properties.

Collection of soil samples from areas historically challenged with heavy metals was an initial screening method for εPL producers. These sites were chosen because if they were at one time polluted with heavy metals, the bacteria in the soil would have been challenged to overcome that exposure. So, these bacteria would have to find a way to protect themselves from a rising concentration of heavy metal. If they survived, it may have been because they were able to produce a polymer (i.e. ε –poly-L-lysine) that would

neutralize the heavy metal contamination. To determine if ε-poly-L-lysine was being produced by bacteria in the collected samples, two dyes, Methylene blue and Remazol blue, were utilized for screening. The dyes detected polylysine based on electrostatic interactions with the polymer.

MATERIALS AND METHODS

2.1 Soil Sample Collection

Soil samples were obtained from areas that were either known or suspected to be polluted with heavy metals by common knowledge of historical industrial dumping sites (Ji, Hamrick, and Pagenkopf, 2001). These sites include Singing Dam (Sutton, MA, Lat. 42.29776N, Long. 71.289744W), West Hill Dam (Uxbridge, MA, Lat. 42.14979N, Long. 71.608546), West River (Uxbridge, MA, Lat. 42.103811N, Long. 71.60854W), Williston Pond (Easthampton, MA, Lat. 42.265155N, Long. 72.66919W), and Paint Shop Pond (Wellesley, MA, Lat. 42.29776N, Long. 71.289744W). The Singing Dam in Sutton, MA was reported to be the area of highest heavy metal deposit concentration in the Blackstone River (Hamrick et al, 2002). Paint Shop Pond was once a waste dump for a paint factory in Wellesley, MA and to this day, is still suspected to contain elevated levels of lead and chromium among other metals (Wellesley College, 2001). All the samples were taken at about 6 inches below the surface of soil along the water's edge. Multiple samples were taken from each site at different arbitrary locations. The collected soil samples were stored in a 4°C refrigerator until use.

The collected samples were named according to the location from which they were obtained. Since multiple samples were taken at each site, numbers were used to represent each sample. Table 1 lists all the locations of the collected samples, as well as, their corresponding abbreviations.

Table 1. A List of All Soil Sample Locations

Soil Sample Location	Soil Sample Abbreviation

Singing Dam (Site 1)	SD1
Singing Dam (Site 2)	SD2
Singing Dam (Site 3)	SD3
Singing Dam (Site 4)	SD4
Paint Shop Pond (Site 1)	PSP1
Paint Shop Pond (Site 2)	PSP2
Paint Shop Pond (Site 3)	PSP3
Paint Shop Pond (Site 4)	PSP4
Wilson Pond (Site 1)	WP1
Wilson Pond (Site 2)	WP2
Wilson Pond (Site 3)	WP3
West River (Site 1)	WR1
West River (Site 2)	WR2
West River (Site 3)	WR3
West Hill Dam (Site 1)	WHD1
West Hill Dam (Site 2)	WHD2
West Hill Dam (Site 3)	WHD3
West Hill Dam (Site 4)	WHD4
West Hill Dam (Site 5)	WHD5
West Hill Dam (Site 6)	WHD6

2.2 Preparation of Soil Samples for the Chromium Assay

Concentrations of chromium in each of the soil samples were determined using the diphenylcarbazide method from *Standard Methods for the Examination of Water and Wastewater* (APHA). The reaction between hexavalent chromium and diphenylcarbazide produces a red-violet product which is measured spectrophotometrically at a wavelength of 540nm.

Briefly, portions of each the collected samples were placed in individual tubes and then left in a drying oven uncapped at 65°C for three days. One gram of dry weight of each of the samples was mixed with 10mL of 1M HCl and placed on shakers for at least 24 hours at room temperature. The collected samples were centrifuged at 2,500 rpm

for 5 minutes to remove any particles in the mixture and the supernatant removed for assay.

2.3 Preparation of Chromium Solution

Analytical grade $K_2Cr_2O_7$ (ACROS Organics, St. Louis, MO) was used to make all chromium standard solutions used in the experiments. A stock solution of 1 g/L was prepared by dissolving the powder in reagent grade water. The stock solution was diluted in water to make the standards for the assay, which ranged from 0.50 mg/L to 5.00 mg/L of $K_2Cr_2O_7$.

The molecular weight of $K_2Cr_2O_7$ is 294.18 g/mol, of which 35.35% of the powder is actually chromium. Therefore, in 1000.00 mg/L of stock solution there is 353.50 mg/L of chromium. Similarly, the prepared standard solutions represent concentrations of chromium that range from 0.18 mg/L to 1.77 mg/L.

2.4 Spectrophotometric Determination of Chromium in Soil Samples

The concentrations of the collected samples were determined by mixing 0.5 mL of sample with 1.0 mL of 0.2 N H_2SO_4 and 200 µL of 0.5% diphenylcarbazide (in acetone). This was diluted to 10.0 mL with reagent grade water in a 15.0 mL conical tube and left at room temperature 2-3 minutes before measuring the optical density at 540 nm.

Note: For the standard solutions reagent grade water was used as a blank. However, for each of the collected samples, 0.5 mL of sample with 9.5 mL of reagent grade water was used.

2.5 Screening Method for Detection of ε –Poly-L-lysine Producing Bacteria

After soil samples were collected from sites determined to be high in heavy metal pollution, 1 g of soil was suspended in 50 mL of water. Then, 100 µL of this suspension was put onto minimal media plates (agar + glycerol) and any colonies that grew were then transferred to plates containing an inclusion or exclusion dye (Remazol blue or Methylene blue respectively). These plates were prepared with the following media components: (per L H_2O) 1 ml Kirk's Mineral Solution (0.00760 M Nitrilotriacetate * H_2O, 0.0122 M $MgSO_4$ * 7 H_2O, 0.00296 M $MnSO_4$ * H_2O, 0.0171 M NaCl, 0.000360 M $FeSO_4$ * 7 H_2O, 0.000466 M $CoSO_4$ * 7 H_2O, 0.000739 M $CaCl_2$, 0.000452 M $ZnSO_4$ * 7 H_2O, 0.0000401 M $CuSO_4$ * 5 H_2O, 0.0000274 M $AlK(SO_4)_2$ * 12 H_2O, 0.000162 M H_3BO_3, 0.0000455 M $NaMoO_4$ * 2 H_2O), 0.0877 M Glycerol, 0.02% Dye (Methylene blue or Remazol blue), 0.00185 M $MgPO_4$ * 3 H_2O, 0.00499 M Ammonium Sulfate, 0.00567 M Sodium Dihydrogen Phosphate, 0.1g Yeast Extract, and 47.75% Agar(1.5%). If the colonies took up the Remazol blue dye they were suspected to be ε-polylysine producers (i.e. putative positives): this hypothesis was the same if the colonies excluded the Methylene blue dye. Because εPL is cationic, these dyes would either be attracted or repelled by the polymer resulting in either a colony coloration or halo around the colony, respectively. These putative positives were then plated onto a plate containing Poly-R 478 dye (polyanthraquinone), which is another cationic dye, to further indicate any ε-polylysine production (Shima, Sakai 1977).

2.5 Method of Itzhaki for Analysis and Determination of εPL

The purpose of collecting soil samples was to obtain εPL producing bacteria that were challenged environmentally in the presence of chromium. In order to screen for those εPL producing bacteria, the method of Itzhaki was used to detect and quantify the amount of εPL produced (Itzhaki 1972).

However, it was important to establish the specificity of the assay before it could be used as a definitive method in verifying an organism's ability to produce εPL. For this, the assay was performed several times for the following: ε-poly-L-lysine (provided by Chisso Corporation, Tokyo, Japan as a powder of 1:1 εPL and dextrin), monomeric L-lysine (Sigma-Aldrich, St. Louis, MO), and α-poly-L-lysine (Sigma-Aldrich, St. Louis, MO).

Solutions were prepared for εPL, monomeric L-lysine, and α-poly-L-lysine starting at a concentration of 1000.00 mg/L. By using a 2-fold dilution, a range of concentrations was made from 7.81 mg/L to 1000.00 mg/L.

For each (εPL, α-poly-L-lysine, and monomeric L-lysine) 0.1 mL of the sample was added to 1.9 mL 0.1mM phosphate buffer (pH 6.6) and 2.0 mL methyl orange solution (0.1 mM methyl orange solution (Sigma-Aldrich, St. Louis, MO)) in a 15.0 mL conical tube. Then, the mixture was placed on a shaker at 30°C for 30 minutes and centrifuged at 2,500 rpm for 5 minutes. The resulting supernatant was then measured in a spectrophotometer at a wavelength of 465 nm using two blanks: a methyl orange blank and a phosphate buffer blank.

2.6 Fermentation of Streptomyces albulus Routien

The *Streptomyces albulus* Routien (ATCC 12757) was streaked onto plates containing LB medium and were placed in a 28°C incubator for 5 days. M3G medium (0.275 M glucose, 0.0757 M $(NH_4)_2SO_4$, 5 g yeast extract, 0.00999 M KH_2PO_4, 0.00361 M $MgSO_4 \cdot 7H_2O$, 0.000177 M $FeSO_4 \cdot 7H_2O$, 0.000223 M $ZnSO_4 \cdot 7H_2O$) was prepared and autoclaved for 20 minutes at 120°C. Four loops from the *S. albulus* Routien plate were suspended in 2 mL of M3G medium. Since growth was a problem, four loops were used to increase the probability of growth in the fermentation broth. 0.5 mL of that suspension was placed into a 300 mL baffle flask (sterilized by autoclaving for 20 minutes at 120°C) containing 50 mL of M3G medium. The baffle flask was placed in a shaking water bath at for 96 hours at 26°C and shaking at 250 rpm.

2.7 Measuring Production of εPL by Streptomyces albulus Routien

The method of Itzhaki was also used to determine whether any εPL was produced by *S. albulus* Routien. At the end of the fermentation, 1 mL of the liquid was placed into a 1.5 mL microfuge tube and centrifuged for 5 minutes at 2,500 rpm. Then 0.1 mL of the resulting supernatant was added to 1.9 mL phosphate buffer and 2.0 mL methyl orange solution into a 15.0 mL conical tube. The mixtures were placed on a shaker at 30°C for 30 minutes and then centrifuged at 2,500 rpm for 5 minutes. The resulting supernatant was measured in a spectrophotometer at a wavelength of 465 nm.

2.8 Fermentation of Putative Positives

After screening the soil samples for possible ε-polylysine producing bacteria, the colonies which were selected were re-streaked on minimal media plates. Then, four loops of the bacteria were placed in 2 mL of M3G Media. This solution was mixed then 0.5 mL was removed and placed in 300 mL of M3G Media in a baffle flask. The flask was incubated in a 30°C water bath shaking at 250 rpm for 96 hours.

2.8 Filter Disk Assay for ε-Polylysine Activity

In order to determine the antimicrobial activity of the provided ε-polylysine sample (Chisso Corporation, Tokyo, Japan) and the possible production of an antimicrobial polymer by our bacterial samples, a filter disk assay was performed. Four bacteria were selected to serve as a target for the polymer, *E. coli*, *B. cereus*, *S. marcescens*, and *M. luteus*. The *B. cereus* and *M. luteus* species are both gram-positive bacteria, while *S. marcescens* and *E. coli* are gram-negative bacteria. These species were chosen to test a wide spectrum of possible antimicrobial activity that ε-polylysine has previously been shown to exhibit (Yoshida and Nagasawa, 2003).

Plates were prepared using LB media and 100 µL of an overnight culture of the individual bacteria. The plates were poured with 20 ml of LB after which 100 µL of bacteria was added to the poured plate. Once the plates solidified, a filter disk was placed on the plate containing 100 µL of each solution listed in Table 2. Disks 1-11 contained supernatants from the putative positive fermentations. The negative control was distilled H_2O while ampicillin served as a positive control. A two-fold serial dilution was

performed with 400 µg/mL of ε-polylysine and the disks were labeled according to concentration.

The samples that were used in this assay were the supernatants of putative positive fermentations. The fermentation broths were centrifuged at 2,500 rpm for 5 minutes. Plates were incubated at 37°C for 48 hours. After the 48 hour incubation period, they were removed and examined for presence of a halo for inhibition. Pictures were taken of each plate, shown in the results section (Figure 9).

2.9 Antimicrobial Effectiveness of εPL - Minimum Inhibitory Concentration Test

The minimum inhibitory concentration of an antimicrobial agent is defined as the lowest concentration at which visible growth of an organism is inhibited. Four strains of bacteria, *Escherichia coli, Serratia marcescens, Micrococcus luteus,* and *Bacillus cereus*, were grown in the presence of two fold serially diluted concentrations of ε-polylysine ranging in concentration from 3.125 to 100 µg/mL. The strains were grown for 48 hours at 30ºC in LB media. To quantify the growth of bacteria, the optical density of the samples were measured in a spectrophotometer at 600 nm.

A second experiment was performed using α-poly-L-lysine as the antimicrobial agent. Again, four strains of bacteria were used as target organisms and a serial dilution of the α-poly-L-lysine concentration was performed giving a range of dilutions from 225 µg/mL to 3 µg/mL. The strains were grown for 24 hours at 30ºC in LB Media. Optical density analysis was done at 600 nm to determine the amount of bacteria in each tube.

RESULTS

3.1 Chromium Assay Standard Curve

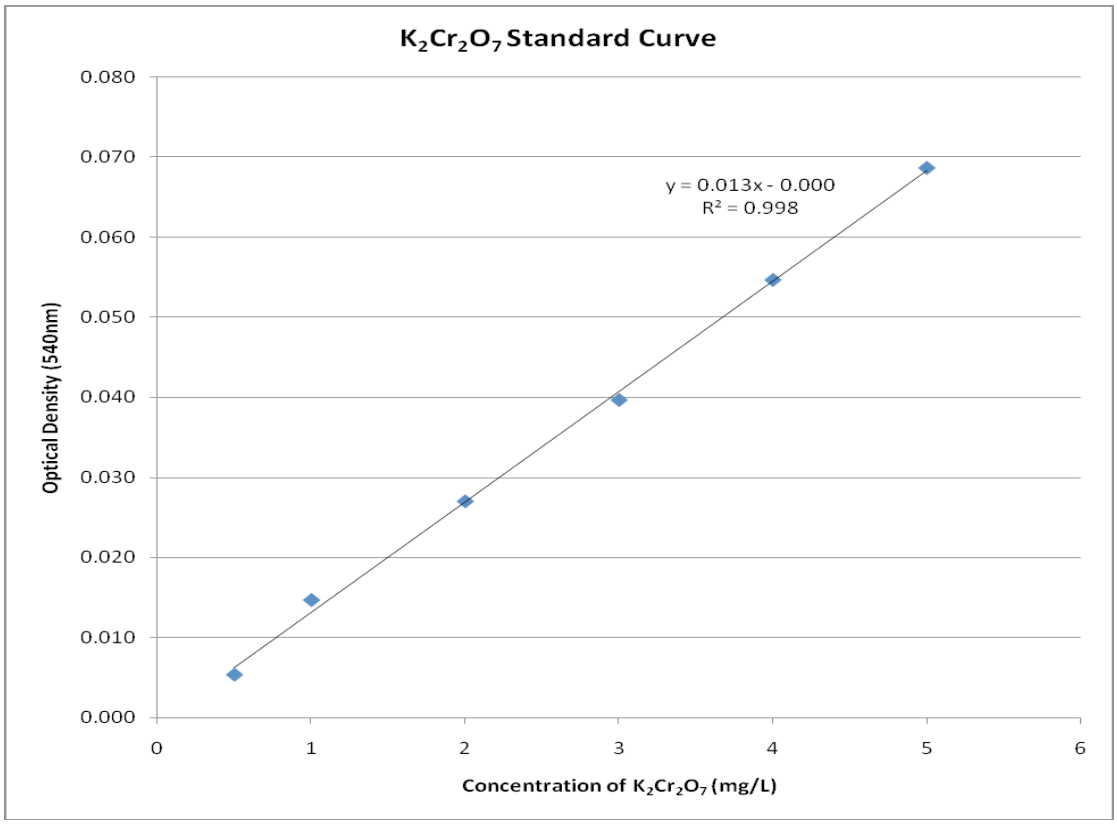
The chromium assay was established using a series of 6 standards ranging from 0.5 mg/L to 5 mg/L of $K_2Cr_2O_7$ (which corresponds to 0.18 mg/L to 1.77 mg/L of chromium). Since 0.5 mL of standard is used in the assay, this represents

Table 2. Chromium Assay

Concentration of $K_2Cr_2O_7$	Trial 1	Trial 2	Trial 3	Average	Standard Error
5 mg/L	0.069	0.069	0.068	0.069	0.000
4 mg/L	0.054	0.055	0.055	0.055	0.000
3 mg/L	0.039	0.040	0.040	0.040	0.000
2 mg/L	0.027	0.027	0.027	0.027	0.000
1 mg/L	0.015	0.015	0.014	0.015	0.000
0.5 mg/L	0.005	0.006	0.005	0.005	0.000

Table 2. Optical density values of the $K_2Cr_2O_7$ standard solutions at a wavelength of 540 nm. For each standard solution, the experiment was performed three times and the average and standard error was obtained for those solutions, this data is shown plotted in Figure 2.

Figure 2. $K_2Cr_2O_7$ Standard Curve



The plot was created from the averaged optical density values of the standard solutions from Table 2. The standard curve yields a straight line that runs through almost all the points with equation $\boldsymbol{y = 0.013x - 0.000}$, where

$\boldsymbol{y = absorbance\ value\ at\ 540\ nm}$ and $\boldsymbol{x = concentration\ of}$ $K_2Cr_2O_7$.

The $K_2Cr_2O_7$ standard curve was obtained by using the average values obtained from the triplicate samples (see Table 2 and Figure 2) and has an R-value of 0.998. The assay was then used to determine the amount of chromium present in each soil sample. The samples were prepared as described in the materials and methods section following extraction in 1M HCl and measured using the diphenylcarbazide method (see Table 3). Since it is known that 35.35% of $K_2Cr_2O_7$ consists of chromium and from the $K_2Cr_2O_7$

standard curve, the amount of chromium for each soil sample was calculated accordingly (see Table 4).

3.2 Concentration of Chromium in the Collected Soil Samples

The mean optical density was obtained from the triplicate samples (see Table 3) for each soil sample. During the preparation of the soil samples for the chromium assay, it was noted that all the soil samples were of different yellow hues (some darker or lighter between each sample). Due to this inconsistency, for each soil sample, 0.5 mL of sample with 9.5 mL of reagent grade water was used as a blank (as noted in the materials and methods section).

Table 3. Optical density values of collected soil samples

Soil Samples	Trial 1	Trial 2	Trial 3	Average Optical density
SD1	0.009	0.011	0.010	0.010
SD2	0.009	0.009	0.009	0.009
SD3	0.020	0.021	0.021	0.021
SD4	0.014	0.013	0.014	0.014
PSP1*	0.001	0.004	0.005	0.003
PSP2*	0.008	0.006	0.007	0.007
PSP3*	0.005	0.005	0.005	0.005
PSP4*	0.007	0.007	0.006	0.007
WP1	0.029	0.029	0.028	0.029
WP2	0.014	0.014	0.014	0.014
WP3	0.005	0.004	0.006	0.005
WR1	0.009	0.009	0.007	0.008
WR2	0.015	0.016	0.015	0.015
WR3	0.014	0.012	0.013	0.013
WHD1	0.014	0.014	0.014	0.014
WHD2	0.009	0.009	0.008	0.009
WHD3	0.007	0.006	0.006	0.006
WHD4	0.004	0.004	0.005	0.004
WHD5	0.019	0.020	0.019	0.019

WHD6	0.006	0.005	0.005	0.005

Note:
* The soil samples from Paint Shop Pond contained a large quantity of leaves and very little soil.

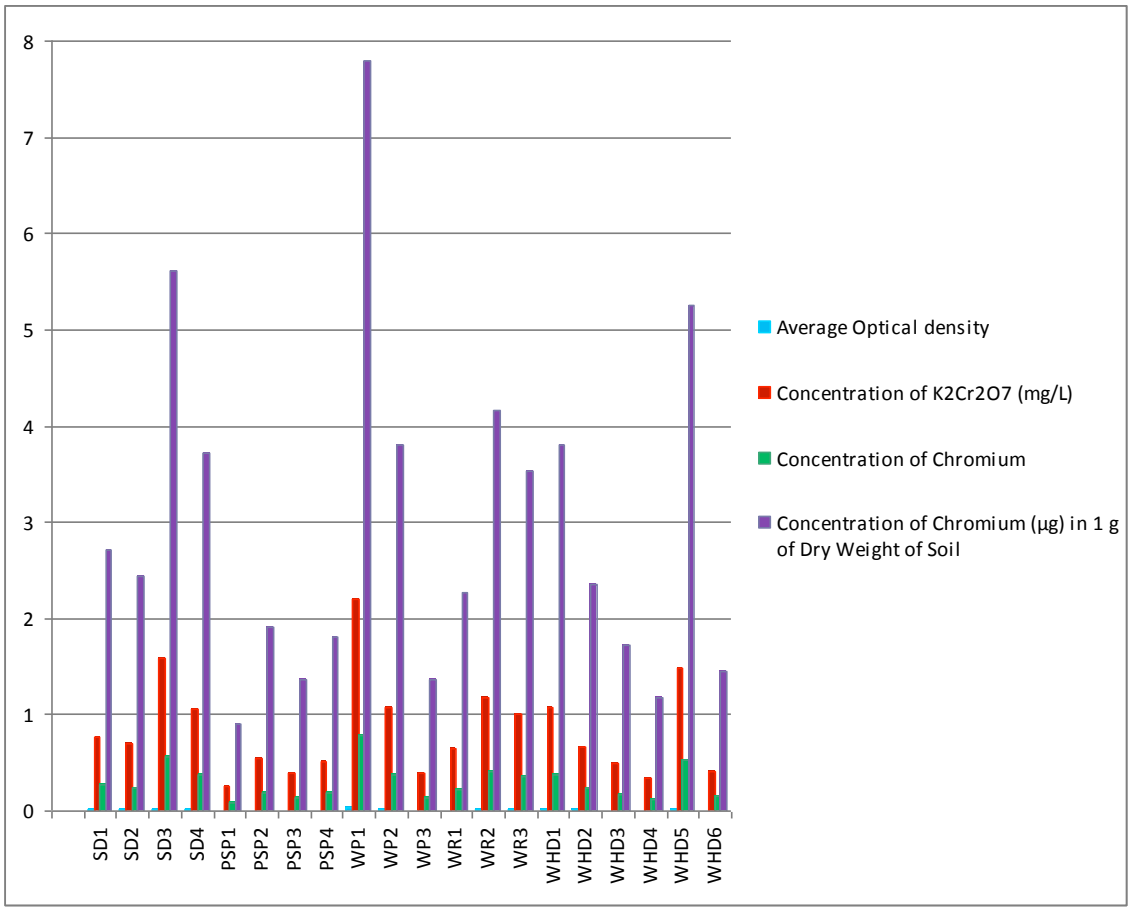
The concentration of $K_2Cr_2O_7$ of each soil sample was calculated from the equation obtained from the standard curve (see Figure 2) using the mean absorbance. The concentration of chromium in the sample solution was calculated by multiplying the value for the concentration of $K_2Cr_2O_7$ by 0.3535 (since 35.35% of the compound is chromium). Further calculations were made to determine the amount of chromium (μg) in 1 g of dry weight of soil. The data shown in Table 4 is shown graphically in Figure 3.

Table 4. Determination of the Concentration of Chromium in the Collected Soil Samples

Soil Samples	Average Optical density	Concentration of $K_2Cr_2O_7$ (mg/L)	Concentration of Chromium (mg/L)	Concentration of Chromium (μg) in 1 g of Dry Weight of Soil
SD1	0.010	0.769	0.272	2.719
SD2	0.009	0.692	0.245	2.447
SD3	0.021	1.590	0.562	5.620
SD4	0.014	1.051	0.372	3.716
PSP1	0.003	0.256	0.091	0.906
PSP2	0.007	0.538	0.190	1.903
PSP3	0.005	0.385	0.136	1.360
PSP4	0.007	0.513	0.181	1.813
WP1	0.029	2.205	0.780	7.795
WP2	0.014	1.077	0.381	3.807
WP3	0.005	0.385	0.136	1.360
WR1	0.008	0.641	0.227	2.266
WR2	0.015	1.179	0.417	4.169
WR3	0.013	1.000	0.354	3.535
WHD1	0.014	1.077	0.381	3.807
WHD2	0.009	0.667	0.236	2.357

WHD3	0.006	0.487	0.172	1.722
WHD4	0.004	0.333	0.118	1.178
WHD5	0.019	1.487	0.526	5.257
WHD6	0.005	0.410	0.145	1.450

Figure 3. Chromium Concentrations of Collected Samples



3.3 Method of Itzhaki for εPL

The optical density of the resulting supernatant for all concentrations of εPL was measured twice, once with a 0.1mM phosphate buffer blank and once with a methyl orange blank (2mL of methyl orange solution with 2mL of phosphate buffer). Since we

are looking for a decrease in absorbance, the phosphate buffer blank is the best method. The method of Itzhaki allows a water-insoluble complex to be formed from the interaction between the cationic εPL and the anionic methyl orange, this allows the complex to fall out of solution. Following centrifugation, the amount of εPL in the supernatant is measured spectrophotometrically at a wavelength of 465 nm. It is assumed that with higher concentrations of εPL, the amount of methyl orange remaining in the supernatant would decrease (lower optical density value).

Table 5. Optical density of εPL at 465nm with a methyl orange blank

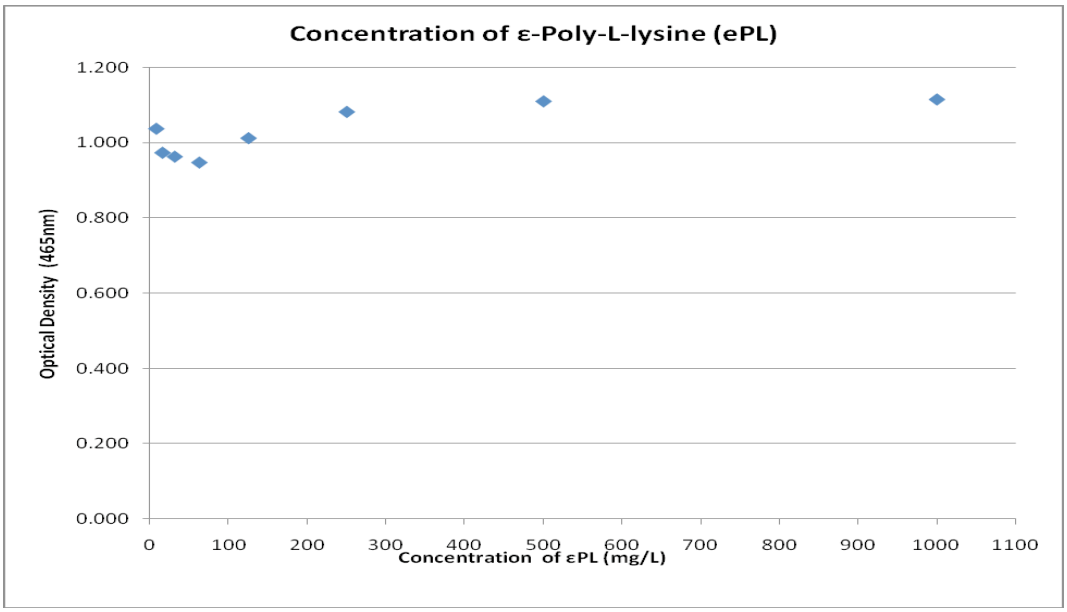
Concentration of ε-poly-L-lysine (εPL)	Trial 1	Trial 2	Trial 3	Average	Standard Error
1000.00 mg/L	-0.040	-0.043	-0.040	-0.041	0.001
500.00 mg/L	-0.043	-0.038	-0.042	-0.041	0.002
250.00 mg/L	-0.067	-0.075	-0.060	-0.067	0.004
125.00 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
62.50 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
31.25 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
15.63 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
7.81 mg/L	-0.079	-0.071	-0.078	-0.076	0.003
Phosphate Buffer + Methyl Orange	0.000	0.006	-0.002	-0.003	0.002

Table 6. Optical density of εPL at 465nm with a phosphate buffer blank

Concentration of ε-Poly-L-lysine (εPL)	Trial 1	Trial 2	Trial 3	Average	Standard Error
1000.00 mg/L	1.116	1.114	1.116	1.115	0.001
500.00 mg/L	1.109	1.113	1.109	1.110	0.001
250.00 mg/L	1.081	1.081	1.085	1.082	0.001
125.00 mg/L	1.045	0.995	0.998	1.013	0.016
62.50 mg/L	0.874	0.989	0.981	0.948	0.037
31.25 mg/L	0.965	0.970	0.956	0.964	0.004
15.63 mg/L	0.987	0.973	0.962	0.974	0.007
7.81 mg/L	1.046	1.029	1.038	1.038	0.005
Phosphate Buffer + Methyl Orange	1.148	1.155	1.152	1.152	0.002

In Figure 4, the average optical densities from Table 6 were plotted versus concentration of εPL (see Table 6) from concentrations 1000.00 mg/L to 62.50 mg/L the optical density decreases.

Figure 4. Plot of the mean optical density values versus concentration of ε-Poly-L-lysine



3.4 Method of Itzhaki for α-poly-L-lysine

The optical density of the resulting supernatant for all concentrations of α-poly-L-lysine was measured twice, once with a 0.1mM phosphate buffer blank and once with a methyl orange blank (2mL of methyl orange solution with 2mL of phosphate buffer). α-Poly-L-lysine was used to determine whether the method of Itzhaki would react to other polymers of lysine and would produce the expected result (higher concentrations of the polymer, lower optical density). Using the procedure described in the materials and methods, the optical densities using varying concentrations of poly-L-lysine were measured (see Table 7 and Table 8).

Table 7. Optical density values of α-poly-L-lysine at 465nm with a methyl orange blank

Concentration of Poly-L-lysine	Trial 1	Trial 2	Trial 3	Average	Standard Error
1000.00 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
500.00 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
250.00 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
125.00 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
62.50 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
31.25 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
15.63 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
7.81 mg/L	-0.079	-0.071	-0.078	-0.076	-0.079
Phosphate Buffer + Methyl Orange	-0.002	0.000	-0.003	-0.002	-0.002

Table 8. Optical Density of α-poly-L-lysine with Phosphate Buffer Blank

Concentration of α-Poly-L-lysine	Trial 1	Trial 2	Trial 3	Average	Standard Error
1000.00 mg/L	0.330	0.327	0.327	0.328	0.001
500.00 mg/L	0.371	0.376	0.377	0.375	0.002
250.00 mg/L	0.370	0.368	0.364	0.367	0.002
125.00 mg/L	0.403	0.399	0.417	0.406	0.005
62.50 mg/L	0.527	0.533	0.534	0.531	0.002
31.25 mg/L	0.768	0.783	0.777	0.776	0.004
15.63 mg/L	0.959	0.964	0.982	0.968	0.007
7.81 mg/L	1.067	1.077	1.069	1.071	0.003
Phosphate Buffer + Methyl Orange	1.148	1.155	1.152	1.152	0.002

Using methyl orange as a blank (see Table 7) meant that most of the optical density values were less than -0.100 since the spectrophotometer could not read values below that. However, when phosphate buffer was used as a blank, the optical density exhibited the expected results, indicating that the method of Itzhaki works for polymers of α-poly-L-lysine. At concentrations of greater than 125.00 mg/L of α-poly-L-lysine the

optical density values do not decrease suggesting that the assay for α-poly-L-lysine is limited by the dye used.

In Figure 5, the optical densities from Table 7 were plotted versus concentration of α-poly-L-lysine and the leveling off can be observed for concentrations higher than 62 mg/L. In Figure 6, the optical from Table 8 densities were plotted versus concentration of α-poly-L-lysine (7.81 mg/L to 125.00 mg/L) and demonstrate a straight line curve with an R-value of 0.868.

Figure 5. Plot of the mean opitical density values versus concentration of α-poly-L-lysine

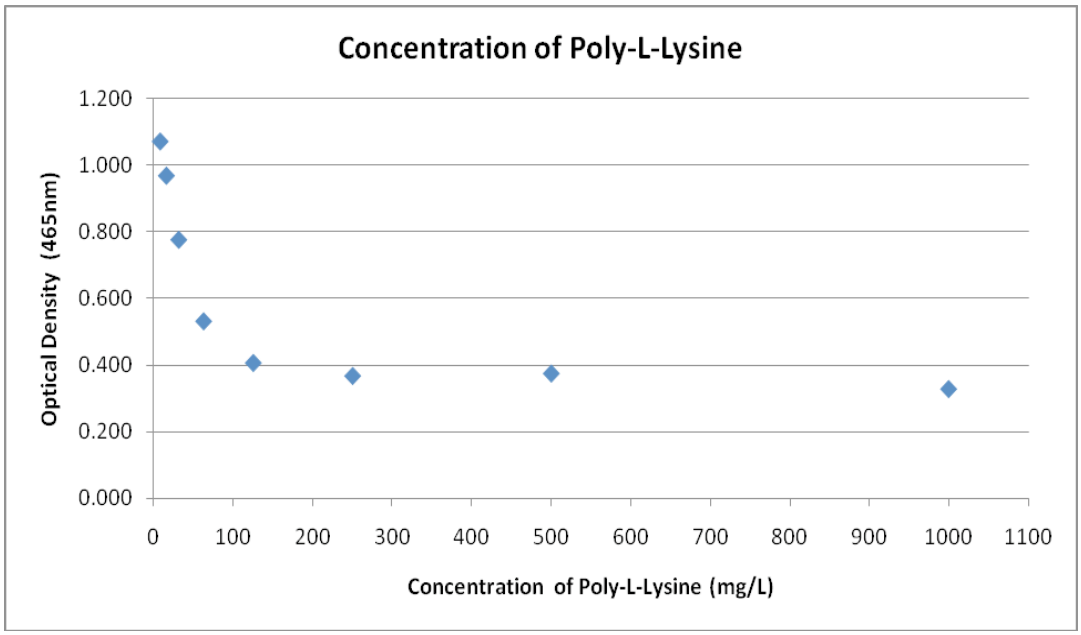
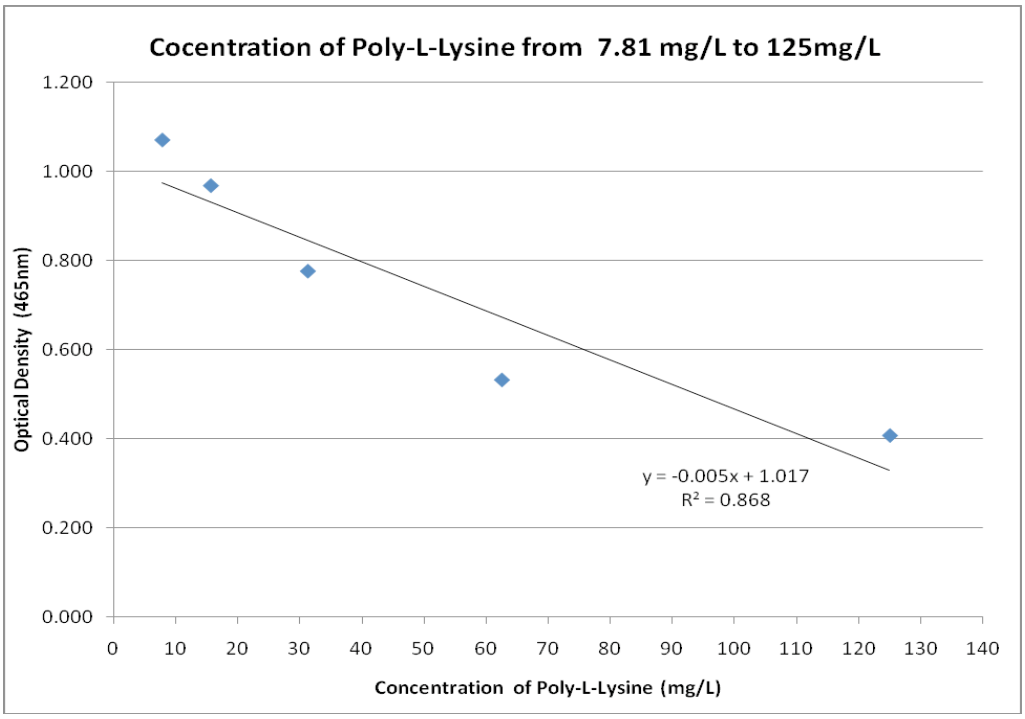


Figure 6. Plot of the mean optical density versus concentration of α-poly-L-lysine (Only values ranging from 7.81 mg/L to 125.00 mg/L were used, see Table 9).



3.5 Method of Itzhaki for monomeric L-lysine

The optical density of the samples, after centrifuging, for all concentrations of monomeric L-lysine was measured twice, once with a 0.1 mM phosphate buffer blank and once with a methyl orange blank (2 mL of methyl orange solution with 2 mL of phosphate buffer). Using methyl orange as the blank (see Table 9), meant that most of the optical density values were less than -0.100 since the spectrophotometer could not read values below that.

Table 9. Optical density values of monomeric L-lysine at 465 nm with a phosphate buffer blank

Concentration of L-Lysine	Trial 1	Trial 2	Trial 3	Average	Standard Error
1000.00 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a

500.00 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
250.00 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
125.00 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
62.50 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
31.25 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
15.63 mg/L	< -0.100	< -0.100	< -0.100	< -0.100	n/a
7.81 mg/L	-0.079	-0.071	-0.078	-0.076	0.003
Phosphate Buffer + Methyl Orange	-0.002	0.000	-0.003	-0.002	0.001

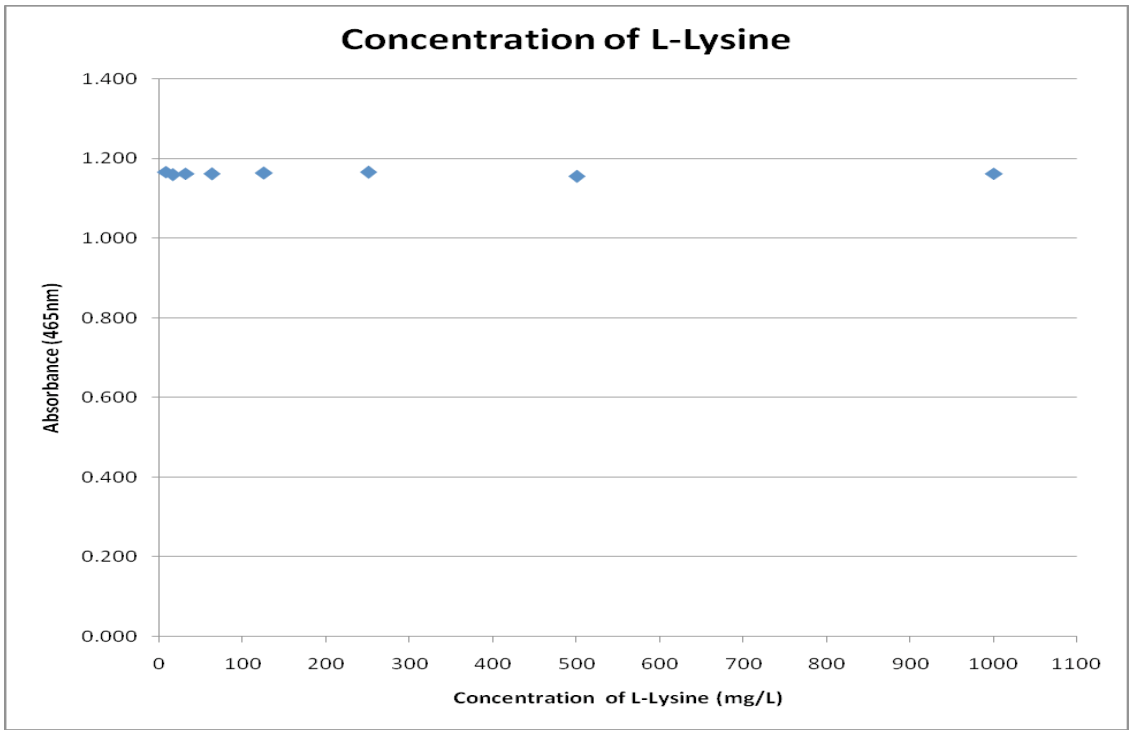
When the optical density values for monomeric L-lysine were measured against a phosphate buffer blank, the results (see Table 10) indicate that the values do not follow the expected pattern (higher concentrations of the molecules, lower optical density). In fact, all the optical density values of monomeric L-lysine were between the range 1.100-1.200.

Table 10. Optical density values of monomeric L-lysine at 465 nm with a phosphate buffer blank

Concentration of Monomeric L-Lysine	Trial 1	Trial 2	Trial 3	Average	Standard Error
1000.00 mg/L	1.150	1.165	1.173	1.163	0.007
500.00 mg/L	1.155	1.151	1.159	1.155	0.002
250.00 mg/L	1.170	1.167	1.160	1.166	0.003
125.00 mg/L	1.163	1.166	1.160	1.163	0.002
62.50 mg/L	1.158	1.166	1.160	1.161	0.002
31.25 mg/L	1.164	1.159	1.164	1.162	0.002
15.63 mg/L	1.159	1.157	1.163	1.160	0.002
7.81 mg/L	1.170	1.170	1.161	1.167	0.003
Phosphate Buffer + Methyl Orange	1.159	1.145	1.149	1.151	0.004

For the results obtained in Table 11, a plot of optical density values versus concentration of monomeric L-lysine was drawn.

Figure 7. Plot of the mean optical density values versus concentration of monomeric L-lysine (See Table 10)



3.6 Determination of εPL in Streptomyces albulus Routien

Using the procedure described in the materials and methods section, the *Streptomyces albulus* Routien strain was cultured and the culture supernatant analyzed for εPL or α-poly-L-lysine molecules. The purpose of this was to see whether the positive control strain would produce any εPL or any α-poly-L-lysine molecules. Since the data (see Table 6) for the analysis and determination of the εPL powder did not yield any conclusive results it was also not possible to estimate how much εPL the strain may have produced.

Table 11. Method of Itzhaki for *S. albulus*

	Blank with Phosphate Buffer	Blank with Phosphate Buffer + Methyl Orange
Trial 1	1.074	-0.039
Trial 2	1.080	-0.040
Trial 3	1.069	-0.026
Average	1.074	-0.035
Standard Error	0.003	0.005
2 mL Phosphate Buffer + 2 mL Methyl Orange	1.107	0.000

Optical density values of the resulting supernatant from *Streptomyces albulus Routien* strain using the method of Itzhaki.

3.7 Screening for ε-Polylysine Producing Bacteria

The data in Table 12 shows the results from the screening assays that were performed on the bacteria found in the collected soil samples. For the Methylene blue dye, a Y indicates that the dye was excluded from the colony and an N indicates that it was taken up. For the Remazol blue dye, a Y indicates that dye was taken up while an N indicates exclusion. This screening method allowed us to choose bacteria that would be fermented and assayed for ε-poly-L-lysine. Many of the samples that appeared to be producing ε-poly-L-lysine were taken from soil samples that contained high amounts of chromium (Table 4).

Table 12. Screening for ε-Poly-L-lysine producers

Location	Plate ID	Col. ID	1st Methylene	1st Remazol	2nd Methylene	2nd Remazol	Fermented?

West River 2	WR2	C	Y	Y	Y	Y	No
West River 2	WR2	A	Y	Y	Y	Y	No
Paint Shop Pond 1	PSP1	A	Y	Y	Y	Y	Yes
Paint Shop Pond 3	PSP3	A	N	Y	Y	Y	Yes
Paint Shop Pond 3	PSP3	B	N	Y	Y	Y	Yes
Paint Shop Pond 4	PSP4	A	?	Y	Y	Y	Yes
Wilson Pond 1	WP1	A	N	Y	Y	Y	Yes
Wilson Pond 1	WP1	B	Y	Y	Y	Y	Yes
Wilson Pond 2	WP2	A	Y	N	Y	?	Yes
Wilson Pond 3	WP3	A	Y	N	Y	N	Yes
Singing Dam 1	SD1	C	N	Y	N	Y	No
Singing Dam 1	SD1	A	N	Y	N	Y	Yes
Singing Dam 1	SD1	B	N	Y	Y	Y	Yes
Singing Dam 1	SD1	D	N	?	N	?	No
Singing Dam 2	SD2	A	N	Y	N	Y	Yes
Singing Dam 3	SD3	A	N	N	N	N	No
Singing Dam 3	SD3	C	?	Y	N	?	No

3.8 Filter Disk Assay

Table 13 shows the sample and colony from which each used filter disk contained.

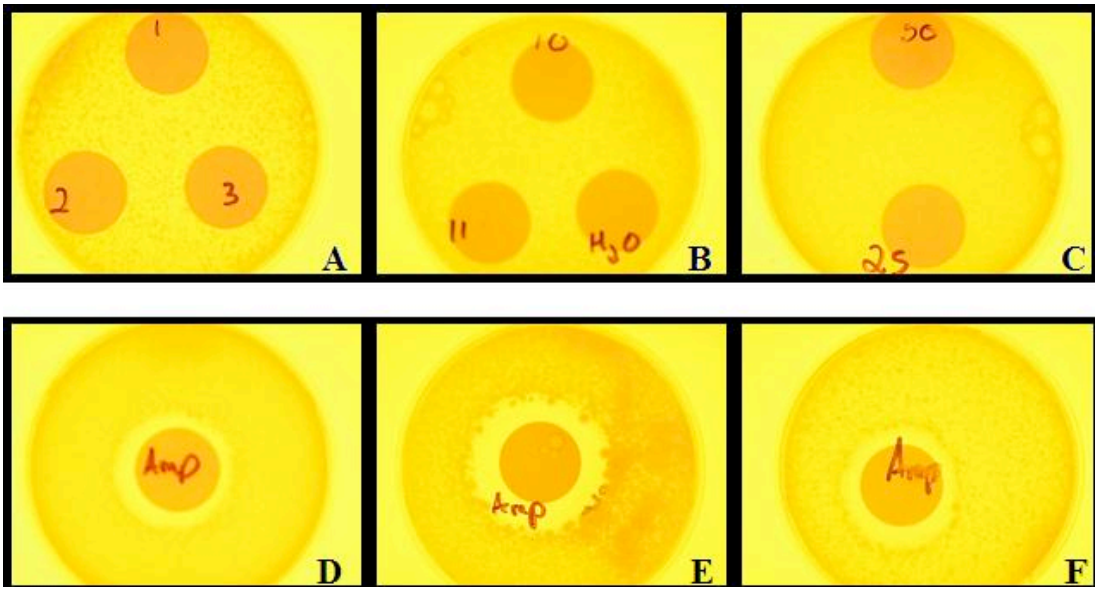
Table 13. Filter Disk Assay Guide

Disk	Sample – Colony
1	WP1 – B
2	WP1 – A
3	PSP3 – A
4	PSP4 – A
5	WP3 – A
6	SD2 – A
7	PSP3 – B

8	PSP1 – A
9	WP2 – A
10	SD1 – A
11	SD1 – B
H_2O	Water
400 µg/mL	50/50 Dextrin and ε-polylysine Sample Dilutions
200 µg/mL	
100 µg/mL	
50 µg/mL	
25 µg/mL	
12.5 µg/mL	
6.25 µg/mL	
3.125 µg/mL	
Amp	Ampicillin (1 µg/mL)

Figure 8 below shows the data from the filter disk assay that was performed using dilutions of ε-poly-L-lysine and the supernatants from the culture broth of fermented putative positives. These samples were placed on plates of *E. coli*, *B. cereus*, *M. luteus*, and S. *Marcescens*. Ampicillin was used as a positive control indicating growth inhibition.

Figure 8. Filter Disk Assay



Panel A above shows a plate of *B. cereus* with filter disks containing 50 and 25 µg/mL of εPL. Panel B is an *E. coli* plate with filter disks containing supernatants of fermented putative positives and water. Panel C is a plate of *M. luteus* that had filter disks containing more supernatants from putative positives. Panels D-F show the positive control disks of ampicillin applied to plates of *B.cereus*, *E. coli*, and *M. Luteus* respectively. The halo of inhibition is what would be expected in our samples had they contained εPL.

3.9 Minimum Inhibitory Concentration Test

In order to test the ability of εPL to inhibit bacterial growth, a minimum inhibitory concentration test was performed. A dilution series of εPL was added to tubes containing four different bacteria and were grown overnight. The optical density was then measured

to quantify the amount of growth in each. These experiments were repeated three times with similar results. Figure 9 and Table 14 shows the data from this experiment.

Table 14. ε-Poly-L-lysine MIC test. Sample used was from Chisso Corporation

Sample	Concentration (µg/mL)	OD 600
E. coli	100	0.002
E. coli	50	1.281
E. coli	25	1.446
E. coli	12.5	1.332
E. coli	6.25	0.361
E. coli	3.125	0.064
B. cereus	100	0.037
B. cereus	50	1.304
B. cereus	25	1.286
B. cereus	12.5	1.935
B. cereus	6.25	1.839
B. cereus	3.125	0.129
M. luteus	100	0.002
M. luteus	50	1.286
M. luteus	25	1.222
M. luteus	12.5	1.897
M. luteus	6.25	1.811
M. luteus	3.125	0.156
S. Marcescens	100	0
S. Marcescens	50	0
S. Marcescens	25	0
S. Marcescens	12.5	0
S. Marcescens	6.25	0
S. Marcescens	3.125	0.52

Figure 9. MIC Test for ε-poly-L-lysine inhibitory concentration

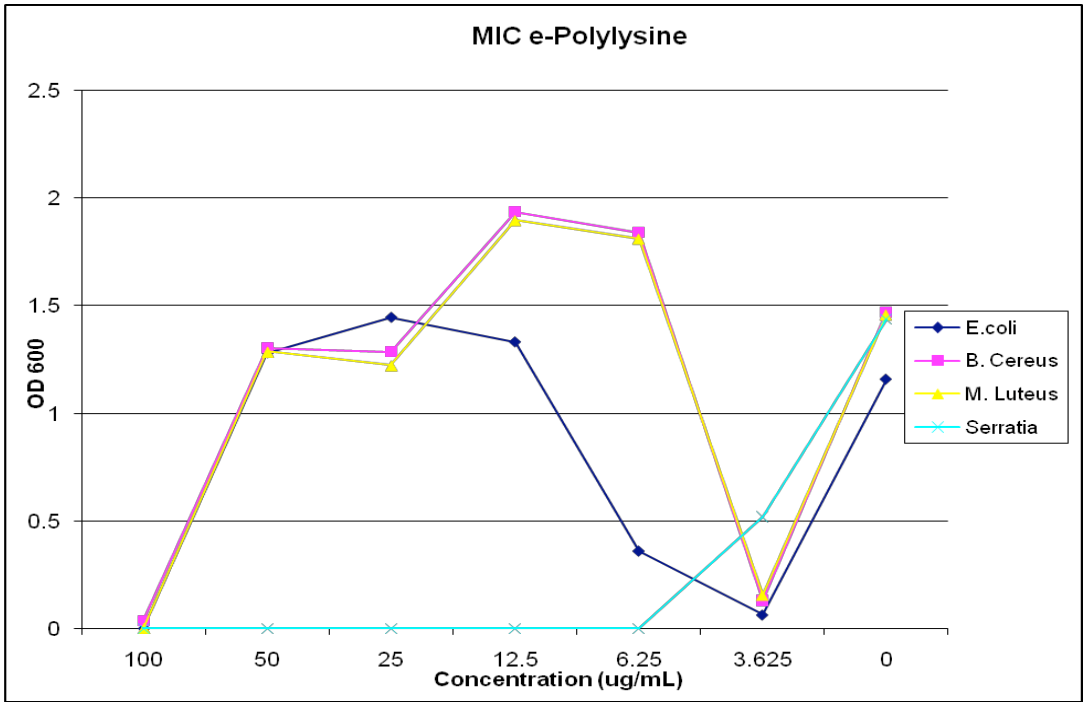


Table 15 below shows the optical densities of the tubes of bacteria after an overnight culture with α-poly-L-lysine. This data is represented graphically in Figure 10. As before, this experiment was performed three times with similar results.

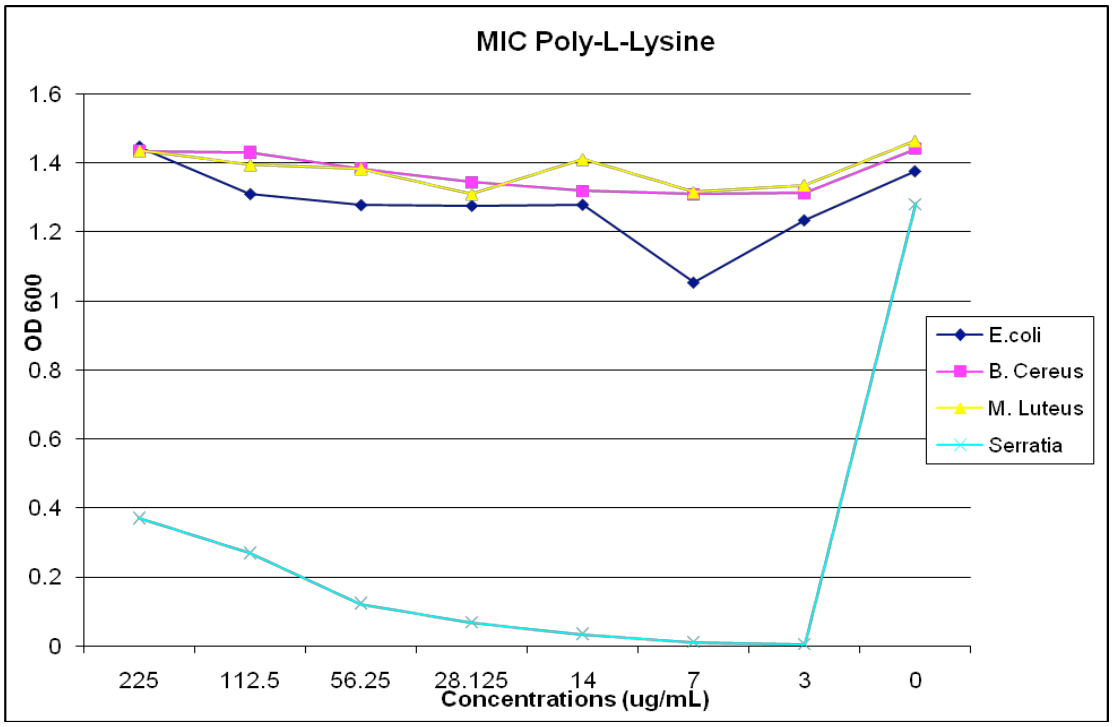
Table 15. MIC test for α-poly-L-lysine activity

Sample	Concentration (µg/mL)	OD 600
E. coli	225	1.446
E. coli	112.5	1.309
E. coli	56.25	1.277
E. coli	28.125	1.275
E. coli	14	1.278
E. coli	7	1.053
E. coli	3	1.233
E. coli	0	1.375
B. cereus	225	1.375
B. cereus	112.5	1.435
B. cereus	56.25	1.431

B. cereus	28.125	1.385
B. cereus	14	1.344
B. cereus	7	1.318
B. cereus	3	1.310
B. cereus	0	1.441
M. luteus	225	1.435
M. luteus	112.5	1.394
M. luteus	56.25	1.383
M. luteus	28.125	1.309
M. luteus	14	1.410
M. luteus	7	1.314
M. luteus	3	1.335
M. luteus	0	1.464
S. Marcescens	225	0.370
S. Marcescens	112.5	0.269
S. Marcescens	56.25	0.123
S. Marcescens	28.125	0.067
S. Marcescens	14	0.034
S. Marcescens	7	0.011
S. Marcescens	3	0.005
S. Marcescens	0	1.280

Figure 10 shows the results of the MIC test for α-poly-L-lysine. It is evident that α-poly-L-lysine did not affect the growth of *E. coli*, *B. cereus*, or *M. luteus*.

Figure 10. MIC Test for α-Poly-L-Lysine



DISCUSSION

4.1 Presence of Chromium in Soil Samples

Since our hypotheses were dependent on isolating microbes from environments with high chromium concentration it was necessary to establish that chromium was present in the soil samples collected. According to the U.S. Environmental Protection Agency (EPA), the allowable contaminant level (MCL) for chromium is 0.1 ppm (U.S. Environmental Protection Agency, 2006). This means that all the collected soil samples are above the limit set by the EPA because if the values in Table 4 are converted to ppm, where 1 ppm = 1 μg/g, then they would all be above the allowable contaminant level.

Almost all of the soil samples collected measured at least ten times the set limit of chromium in drinking water. The exception was location-1 from Paint Shop Pond even though it is reported to have high concentrations of chromium (U.S. Environmental Protection Agency, 2002). The low amount of chromium detected in the Paint Shop Pond samples could be due to the fact that during the preparation of the soil samples, there happened to be a large quantity of leaves in the soil. This means that in 1g of dry weight of soil, a large amount of leaves was weighed in the preparation process. This would lead to a smaller amount of actual soil in the sample; therefore the concentrations of chromium in these samples could be skewed slightly lower than they actually are. It is possible that the leaves in the sample led to the discrepancy in chromium concentration.

The method used to measure the chromium in the soil samples may not have been the best method to use since the protocol was slightly altered to measure soil samples and

not groundwater. It would be beneficial to conduct more tests using the diphenylcarbazide method to measure soil samples that are unlikely to contain any heavy metals or chromates. Another alternative test that could be performed would be to place a known amount of chromium in a soil sample that was found to be free of heavy metals. This would allow for another standard curve to be made and provide additional comparison to collected samples.

Since all the soil samples contained chromium, this suggests that the organisms growing in those soils would have been challenged environmentally in the presence of chromium. This would indicate that the organisms have been able to survive in an environment containing chromium, thus may indicate a higher chance of isolating εPL producing bacteria.

4.2 Method of Itzhaki to Detect and Quantify εPL

The method of Itzhaki was tested using three different molecules: εPL, α-poly-L-lysine, and monomeric L-lysine. According to the data for εPL (see Table 7) the method of Itzhaki did not appear to work as it was expected (higher concentrations of the molecules, lower optical densities). There appears to be an initial range where the εPL exhibited behavior similar to the α-poly-L-lysine (a linear range). It is assumed that the dextrin in the powder form of εPL (50% εPL and 50% dextrin) received from Chisso Corporation, may have interfered with the assay. The optical densities observed between 1000.00 mg/L of εPL with 7.81 mg/L of εPL suggest that there is almost no difference or change in the mixture (Table 6).

It is suggested that in future experiments, a purification of the sample be carried out prior to using the assay to determine the amount of εPL. Because the sample of εPL was cut with dextrin, there was no standard from which to use and measure the amount of εPL produced by the *Streptomyces albulus* Routien strain. From Table 11, it is unclear whether the *Streptomyces albulus* Routien produced any polylysine molecules. It is uncertain whether it can serve as an εPL-producing positive control strain. It is possible that this strain of *Streptomyces albulus* is not capable of producing εPL or produces far less than other strains reported.

The method of Itzhaki worked well with α-poly-L-lysine, producing a linear correlation of absorbance to concentration in the concentration range of 7.81 mg/L to 125.00 mg/L with an R value of 0.984 (see Table 9). At higher concentrations (above 125.00 mg/L) the optical density and concentration were not linearly related. This indicates that for α-poly-L-lysine, the assay is out of the linear range at concentrations above 62.50 mg/L (see Figure 9).

For monomeric L-lysine, the methyl orange dye did not seem to react with the monomeric L-lysine molecules, indicating that the method of Itzhaki does not work for this molecule (see Table 11). The optical density values did not change for different concentrations of monomeric L-lysine, rather all the values seemed to stay within the optical density range of 1.100-1.200. For the purposes of this project, this is a good indication that the molecules of interest (εPL and/or polymers) will react with the method of Itzhaki and that monomers like monomeric L-lysine will not.

The method of Itzhaki may work better if the polymer from Chisso were purified before the assay was performed. Due to the inability to find a method that would purify the sample accurately, and due to the small amount of sample, purification was not performed. Purification of the εPL powder to remove the dextrin may help yield results that should follow the expected trend (higher concentrations of the molecules, lower optical densities) as opposed to simply measuring the resulting supernatant as described in the materials and methods.

4.3 Antimicrobial Effectiveness of ε-Poly-L-lysine

To quantify the antimicrobial effectiveness of εPL, a filter disk assay and minimum inhibitory concentration test were performed. In the filter disk assay, visible halos should have formed around the filter disks if bacteria were unable to grow due to the diffusion of an antimicrobial agent from the filter disk. Instead, the filter disks showed no inhibition of growth even at the highest concentration tested (400 μg/ml). This indicates that the samples of polylysine that we used exhibited no antimicrobial activity. This could be due to the presence of dextrin in the sample and/or the sample contained little or no εPL. The subsequent minimum inhibitory concentration test showed that the bacteria were not resistant to εPL as they were unable to grow at the highest concentrations. These experiments further led us to believe that the dextrin interfered with the assay as is discussed below. The methodology used during the experiment was correct as indicated by the filter disks soaked with ampicillin (positive control). If the ε-polylysine sample that we had was pure (i.e. uncut) then this assay should work very well. Also, having a pure sample would allow us to create a standard curve relating halo

size to εPL concentration. This can then be used to estimate the production ε-poly-L-lysine in bacteria that we believed to be producers based on the screening method that was utilized.

The minimum inhibitory concentration test did not provide expected results. As shown in Figure 9 and Table 14, the bacterial growth peaked at the concentrations of 12.5 and 25 μg/ml polylysine. If the assay worked as expected, there should have been no growth of bacteria in the higher concentrations of polylysine, yet there is no discernable pattern of inhibition as the concentration increases. If a strain of bacteria was able to grow in a higher concentration, it should have been able to grow in lower concentrations; this was also not the case. Instead, three bacterial strains, *E. coli*, *B. cereus*, and *S. luteus*, all performed similarly, while *S. Marcescens* was inhibited at all the concentrations higher than 3.625. One can hypothesize that the dextrin with which the polylysine was cut inhibited the action of polylysine. For all four strains of bacteria, no growth was seen at a concentration of 100 μg/ml polylysine and for three strains no growth was seen at 3.125 μg/ml.

The second MIC test was performed with uncut α-poly-L-lysine (Table 15). In this experiment there appeared to be no inhibition at any concentration for *E. coli*, *M. luteus*, or *B. cereus* as throughout the dilution series, there was the same OD_{600} as there was with no α-poly-L-lysine present. However, *S. Marcescens* exhibited very little growth when there was even a small concentration of the polymer in the media (Figure 10). And when there was no polymer present, the bacteria were able to proliferate. Although ε-poly-L-lysine has been shown to be an effective antimicrobial agent for a

wide range of both gram positive and gram negative bacteria (Yoshida and Nagasawa, 2003), gram negative species have been found to be more susceptible to cationic polymers (Fedtke et al., 2004). Since *S. Marcescens* is a gram negative bacterium, it could have been affected by the presence of these polymers much more so than the other bacterial species.

It was difficult to determine whether or not the bacteria that were taken from the soil samples produced ε-polylysine because we were unable to acquire a sample of the strain of *S. albulus* that is a known producer. Also, the only sample of ε-polylysine that we had was cut with dextrin which we did not purify. If we had gotten a sample of either or both it would have been easier to determine not only how effective ε-Polylysine is at inhibiting bacterial growth but it would have helped us to screen for bacteria that produce the polymer.

4.4 Summary of Results

The chromium levels in the soil samples were determined by spectrophotomic analysis. After completion of this assay, the method of Itzhaki was completed to determine εPL production of the bacteria strains from each sample. A standard curve was done to assess the specificity of the assay before the samples were evaluated.

Each sample analyzed by the chromate assay was determined to have high chromate concentration, with sample one from the Williston Pond having the highest chromate concentration per one gram of sample (7.795 μg). With these results, it was hypothesized that there would be production of εPL in each sample. Because the

reproducibility of the chromate assay is poor, the results could not be reproduced. This led to random selection of samples to ferment for the antimicrobial assays.

The ampicillin plate in the filter disk assay was the only plate that demonstrated anti-microbial properties by producing a halo. This showed that the methods were completed properly, but the results obtained by the assays in this experiment did not support the hypothesis that εPL has antimicrobial potential.

The MIC results jumped randomly, but because results were similar between gram negative and gram positive pairs, there seems to be some correlation between effects of the polymer and inhibitory potential. The bacteria density readings could have been high because of the high dextrin concentration in the α-poly-L-lysine used in the assay. Dextrin could have acted as a food source for the bacteria, increasing its growth and resulting in higher OD readings. With more time and a purer strain of α-poly-L-lysine, the accuracy of these results could be optimized.

4.5 Future Directions

Based on the antimicrobial assays, the screening methods used were unreliable. It is possible that the bacteria were producing molecules similar to εPL which were interacting with the dyes producing false positives. A more reliable screening method would be beneficial.

The method of Itzhaki may work better if the molecules were purified before the assay was performed. Purification of the εPL powder to remove the dextrin may help yield results that should follow the expected trend (higher concentrations of the molecules, lower optical densities) as opposed to simply measuring the resulting

supernatant as described in the materials and methods. Thin layer chromatography could be used to purify the sample of εPL.

The *Streptomyces albulus* strain obtained from the ATCC, may not be a εPL producing bacteria and thus produced the results observed in Table 12. It is suggested that *Streptomyces albulus* Strain 410 (Kahar et al., 2001) be obtained since it has been recorded to produce εPL for further tests to use as a control. *Streptomyces albulus* Strain 346 is a mutant strain that can also be used and has been reported to produce high quantities of εPL (Chisso Corporation, 2007).

As discussed above, the sample of ε-poly-L-lysine was cut 50/50 with dextrin. It is unknown whether the dextrin may have interfered with the antimicrobial assays. Based on previous research, ε-poly-L-lysine should have exhibited antimicrobial properties with the concentrations used. Once again, a pure sample of ε-poly-L-lysine would have been beneficial in determining the antimicrobial effectiveness of the polymer.

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