Epsilon-Poly-L-Lysine Produced by S. albulus

A Major Qualifying Project Report
Submitted to the Faculty
Of the
WORCESTER POLYTECHNIC INSTITUTE
In partial fulfillment of the requirements for the
Degree of Bachelor of Science
by

Maureen E. Ryder
2010

Approved:

_____
Michael A. Buckholt

_____
Allison Hunter

Contents

Abstract

Since its discovery in 1977, ε-Poly-L-Lysine (ε-PL) has been used in a variety of applications. The primary usage of ε-PL currently is as an anti-microbial food additive, though it can be found as a drug delivery carrier, hydrogel, endotoxin remover, biosensor, or disinfectant, among other possibilities. The goal of this project was primarily to investigate whether a particular strain of *Streptomyces albulus* that allegedly produces ε-Poly-L-Lysine could be used as a future positive control for ε-Poly-L-Lysine production.

1 What is ε-Poly-L-Lysine?

1.1 Uses of ε-Poly-L-Lysine

Potential applications of poly-ε-lysine (ε-PL) and its derivatives

Fields of application	Application examples
Biomedical applications (drug delivery carrier, endotoxin remover, biosensor)	1. Complex with synthetic double-stranded RNA polyriboinosinic–polyribocytidylic acid (poly I · poly C) as an endogenous interferon inducer 2. Conjugate with antifolate agent methotrexate (MTX) or other anticancer drug for the treatment of human leukemias, sarcomas, and other forms of neoplastic diseases 3. Non-viral gene delivery vectors 4. Lipopolysaccharides (LPS)-Endotoxin removal 5. Immobilization of enzymes for the biosensor
Food industry (food preservative, emulsifying agent, dietary agent)	1. Natural and safe preservatives 2. Complex with dextran for the use as a bifunctional emulsifier and antibacterial agent 3. Suppress dietary fat absorption from the small intestine by inhibiting pancreatic lipase activity for the preventing obesity
Others applications (disinfectant, hydrogels, superabsorbent, biochip, bioelectronics)	1. Bactericidal compositions comprising ε-PL and components extracted from citrus fruit seeds or peels are safely useable in food, washing dishes, kitchen utensils, lavatories 2. Hydrogels with high water sorption or stimuli–response capacity synthesized by cross-linking of ε-PL have various potential applications in fields such as agriculture, food and medicinal industries 3. Polylysine has been used to construct devices that led to numerous products such as multi-array sensors, optical read/write discs or nano-scale electronic/photonic circuits

Table 1. Potential Applications of Epsilon Polylysine and its Derivatives. (Shih 2006)

Since its discovery in 1977, ε-Poly-L-Lysine has been used for a variety of applications. The primary usage of ε-PL currently is as an anti-microbial food additive, though it can be found as a drug delivery carrier, hydrogel, endotoxin remover, biosensor, or disinfectant, among other possibilities. Many of the applications are particular for ε-PL and its derivatives as opposed to α-PL due to a chemical modification on the alpha amino group, which can hinder its adsorption (Yoshida 2003).

The proposed mechanism for the antimicrobial activity of ε-PL involves the molecules' adsorption onto a cell surface, leading to a stripping of the outer membrane and consequent abnormal distribution of cytoplasm, bringing about cell

death. (Georgnaras 2005, Yoshida 2003). This mechanism can also explain the differences that have been observed in antimicrobial effectiveness on various organisms; differences in cell surfaces would lead to differences in adsorption levels (Yoshida 2003). ε-Poly-L-Lysine can inhibit growth in a variety of organisms at concentrations of less than 100 μg/mL, though the amount needed for food preservation is less than 300ppm (Yoshida 2003). The anti-microbial activity of ε-PL has lead to its use as a food preservative in Japan since the 1980s, in foods such as boiled rice and sliced fish (Hiraki 2003) and in 2004 ε-PL was deemed Generally Recognized As Safe (GRAS) by the US Food and Drug Administration for use as a food preservative in amounts less than 50mg/kg (Tarantino 2004).

ε-Poly-L-Lysine is not only useful as an antimicrobial agent; it has potential to be an efficient, environmentally friendly bioremediator. Its cationic properties bring up the potential ability for heavy metal binding. Additionally, it is a biodegradable non-toxic polymer, which means it would be safe for the environment. Due to its cationic nature, it is hypothesized that εPL may have the ability to reduce chromate and other anionic heavy metal wastes from industrial discharge through biosorption. Biosorption is a possibly cost effective way of removing heavy metals from industrial wastewaters (Rao Popuri 2007). This has not been sufficiently explored, though if ε-Poly-L-Lysine were able to remove heavy metals from water sources it would be an invaluable resource.

1.2 Structure and Properties of ε-Poly-L-Lysine

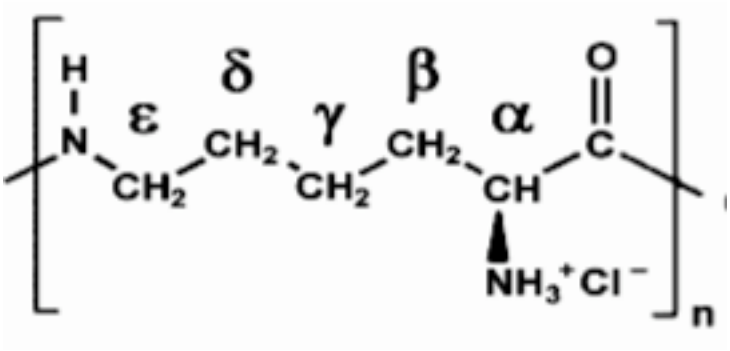

Figure 1. Structure of ε-Poly-L-Lysine (Hirohara 2007)

ε-Poly-L-Lysine (ε-PL) is a homo-polymeric compound of 25-30 lysine monomers linked at the ε-amino groups (Shima 1981). It is a biodegradable, non-toxic, and water soluble substance secreted in varying amounts by the filamentous bacteria *Streptomycetaceae* (Shih 2006, Shima 1981). ε-PL in water contains a positively charged hydrophilic amino group and a hydrophobic methylene group that leads it to have interesting and useful cationic properties, discussed later in this section. ε-PL is unlike proteins and α-poly-L-Lysine (α-PL) in that the amide linkage in ε-PL is between the ε-amino carbon and the carbonyl group (see Figure 1) while the amide linkage in α-PL is found between the α-amino carbon and the carboxyl group (Shima 1981). The amino acid Lysine has a positively charged ε- amino group, which causes a polymer of lysine residues to be highly positively charged. In a hydrophilic environment, the carboxyl and amino groups arrange themselves towards the outside of a globular structure, while the hydrophobic methylene groups are arranged towards the inside of the globular structure.

In 1977, Shima and Sakai were the first to discover ε-PL as a result of screening for Dragendorff's positive substances produced by *Streptomyces albulus*

ssp. *Lysinopolymerus* strain 346 (Shima 1997). Dragendorff's reagent activates in the presence of alkaloid substances, alerting the researchers that a molecule of interest was present. Because of this, many early papers refer to ε-PL as the DP (Dragendorff's Positive) substance, before the structure was determined and it was properly named. Conventional industrial polypeptide and poly-amino acid synthesis regimes cannot properly form the necessary ε bonds, leading to the practice of ε-Poly-L-Lysine being produced through fermentation with bacteria and subsequently extracted. Prihardi Kahar has established a protocol for the efficient fermentation of *Streptomyces albulus* strain 410 using pH control, and has subsequently refined her data through investigation of production of ε-PL in and Airlift Bioreactor (Kahar 2001, 2002).

2 Bacteria Used

2.1 Streptomyces albulus

Figure 2. Irregular form of *Streptomyces albus,* a close relative of *Streptomyces albulus* (Bryan MacDonald, Christopher Adams, and Kyle Smith, Brigham Young University, Provo, UT)

The primary source for ε-Poly-L-Lysine across available literature proves to be *Streptomyces albulus* (Hirohara 2007). Pictured at left, *S. albus*, a closely related species, can be seen showing an undulate margin in a 10-day culture on nutrient agar. The genus *Streptomyces* is of the phylum Actinobacteria, which

implies that *S. albulus* is gram positive. *Streptomyces* bacteria are typically found in soil or decaying vegetation, and have a characteristically complex secondary metabolism, which makes them an invaluable resource for pharmaceutical manipulation for drug production(Shirling 1972).

The strain of *Streptomyces albulus* used in this project is a known producer of ε-Poly-L-Lysine. It is slower growing than a lot of common laboratory bacteria, often needing 3-5 days incubation for sufficient growth.

2.2 Pediococcus acidilactici

Pediococcus bacteria are gram-positive bacteria that generally appear in pairs or triads. This species is considered a Lactic Acid Bacteria, so it can tolerate lower pH ranges, is non-respirating, and creates lactic acid as the metabolic end product of carbohydrate fermentation. The particular strain used in this project is L-Lysine sensitive; it cannot synthesize lysine *de-novo*, and must take it up from the surrounding environment.

3 Goals

The goals of this project are:

1.) To obtain bacteria able to produce ε-Poly-L-Lysine from an alternate research establishment or research supply source.

2.) To find an adequate method of detection of presence of ε-Poly-L-lysine (positive control needed) and use this to determine presence of ε-Poly-L-lysine produced by bacteria.

3.) Hypothesis: If the obtained bacterial strain produces ε-Poly-L-lysine, then a significant abundance of lysine monomers will be shown to be present in the strain of bacteria.

4 Methods and Materials

There were various techniques used in this project to try and determine the presence of ε-Poly-L-lysine and prove its abundance in *S. albulus*.

4.1 Reconstitution and Creation of Freezer Stock

The vial containing *S. albulus* was heated and cracked and 5.0 mL of sterilized LB broth was added to reconstitute the dried bacteria. This mixture was transferred to 5.0 mL LB broth and M3G media in conical tubes, and allowed to incubate for 5 days. After 5 days, 3 vials of 1.0 mL LB incubated *S. albulus* were added to 40% glycerol in H_2O to a cryogenic vial and placed into the -80° C freezer.

4.2 Thin Layer Chromatography

Thin Layer Chromatography was used to separate solutions of different types of lysine, to determine if these would travel differently and thereby be able to

be differentiated from each other. The solution used to separate the compounds was a 4:1:1:2 ratio of n-butanol: glacial acetic acid (17.4 M): pyridine: dH_2O. The ε-Poly-L-lysine that was ordered did not easily dissolve in either H_2O or ethyl alcohol as originally expected. Upon further investigation and contacting the supplier, it was discovered that the side chains were protected using Carboxybenzyl, which begs the question of its usefulness if the side chains are unable to interact with stationary or mobile phase of the TLC. The ε-Poly-L-lysine was finally found to dissolve in dichloromethane.

The silica gel plates were spotted with the following lanes:

Table 2. Lane Designation for Silica Gel Chromatography

Plate 1		
Lane	Compound	Volume
1	α-Poly-L-lysine	2 μL
2	α-Poly-L-lysine	20 μL
3	ε-Poly-L-lysine	2 μL
4	ε-Poly-L-lysine	20 μL
5	*S. albulus* in LB broth	10 μL
6	*S. albulus* in LB broth	20 μL
7	*S. albulus* in M3G media	10 μL
Plate 2		
Lane	Compound	Volume
1	*S. albulus* in LB broth	20 μL
2	LB broth	20 μL
3	*S. albulus* in M3G media	20 μL
4	M3G media	20 μL

After placing the plate in a TLC tank and allowing the chromatography to run for about 4 hours, the plate was removed from the mobile solution and allowed to dry. Upon drying, the plate was sprayed with a ninhydrin solution (0.5mL ninhydrin stock + 15 mL ethanol), which was used to visualize the presence of amino acids.

4.3 Determination of Lysine levels in *S. albulus* cells

The test organism, *Pediococcus acidilactici* was reconstituted and subsequently subcultured into 10mL Difco Micro Assay Culture Broth (inoculum broth) and incubated at 37° C for 24 hours. The resultant suspension was centrifuged at 600g for 3 minutes to separate cell pellet from the liquid supernatant. The supernatant was removed and cells were re-suspended in 10 mL of 0.85% (w/v) sterile saline solution to wash them, and then centrifuged and washed two more times in 10 mL of 0.85% (w/v) sterile saline solution to remove lingering inoculum broth. 1.0 mL of resuspended cell solution in saline was diluted with 19.0 mL 0.85% (w/v) sterile saline solution to create the diluted inoculum suspension.

The stock solution of L-lysine monomers was prepared fresh to prevent degradation. 0.001g L-Lysine was dissolved into 20.0 mL purified H_2O to create the L-Lysine stock solution. 0.5 mL of Stock Solution was diluted with 416 mL purified H_2O to create the Standard Working Solution with a lysine concentration of 6.0 mg/mL. Nine tubes were used for the standard curve

determination. Each tube contained 5.0 mL Assay broth with the following concentrations of purified water and Standard Working Solution:

Table 3. Standard Curve Solutions

Tube	Standard Working Solution (mL/10mL tube)	Purified H2O (mL/10mL tube)	Final Amino Acid Concentration (mg/mL)
Blank	0.0	5.0	0
2	0.5	4.5	3
3	1.0	4.0	6
4	1.5	3.5	9
5	2.0	3.0	12
6	2.5	2.5	15
7	3.0	2.0	18
8	4.0	1.0	24
9	5.0	0.0	30

Each tube was inoculated with 100 μL Diluted Inoculum Suspension and allowed to incubate for 20 hours at 37 C. Growth response was measured turbidometrically via spectrophotometry at 660 nm.

The experimental organism, *S. albulus* was subcultured in inoculum broth at 37 C for five days. Similar to the test organism, these cells were centrifuged and washed in 10 mL of 0.85% (w/v) sterile saline solution three times. The cellular pellet was removed and weighed by subtracting the weight of a sterile conical tube from the weight of the tube with the pellet. 0.02 g of cellular pellet was lysed using a mechanical-blending technique of placing a sterile

toothpick into the tube and vortexing for 2 minutes. This lysate suspension was diluted to a total volume of 20mL. The Diluted Cellular Lysate was added in increasing amounts to a series of tubes each containing 5.0 mL of Assay medium as well as the following concentrations of purified water:

Table 4. Cellular Lysate Solutions

Tube	Diluted Cellular Lysate (mL/10mL tube)	Purified H2O (mL/10mL tube)
Blank	0	5.0
2	0.5	4.5
3	1	4.0
4	1.5	3.5
5	2	3.0
6	2.5	2.5
7	3	2.0
8	4	1.0
9	5	0.0

Each tube was inoculated with 100 μL Diluted Inoculum Suspension and allowed to incubate for 20 hours at 37 C. Growth response was measured turbidometrically via spectrophotometry at 660 nm.

5 Results

5.1 Reconstitution and Creation of Freezer Stock

The *S. albulus* obtained was successfully grown in LB broth and M3G minimal media. The freezer stocks were successfully reconstituted thus showing the effective long-term storage of the -80° C freezer.

5.2 Thin Layer Chromatography

The TLC plates were inconclusive, as amino acids in the bacteria and growth media caused all of the spots from *S. albulus* to show a positive result in the ninhydrin stain.

5.3 Determination of Lysine levels in *S. albulus* cells

As expected, the absorbance at 660nm increased along with the increasing amount of lysine monomers in solution. At low levels of lysine no absorbance was seen; this implies that if there was any experimental organism growth in these tubes, the levels were too low to be calculated by the spectrophotometer. These data points were left out of the standard curve trendline calculation, as they do not accurately reflect growth. As seen in Figure 3, the Standard Curve determined by the known levels of lysine solution resulted in the calculated trendline showing that Absorbance = 0.0006(Lysine [μg/mL]) – 0.0053. This information was later compared to unknown levels of lysine solutions to determine the average lysine level per mL.

Table 5. Standard curve data with known concentrations of Lysine.

Tube	Standard Working Solution (mL/10mL tube)	Purified H2O (mL/10mL tube)	Final Amino Acid Concentration (μg/mL)	%T at 660nm	Absorbance at 660nm
1	0.0	5.0	0	100	0
2	0.5	4.5	3	100	0
3	1.0	4.0	6	100	0
4	1.5	3.5	9	100	0
5	2.0	3.0	12	99.6	0.001740662
6	2.5	2.5	15	99.4	0.002613616
7	3.0	2.0	18	98	0.008773924
8	4.0	1.0	24	97.4	0.011441043
9	5.0	0.0	30	97.3	0.01188716

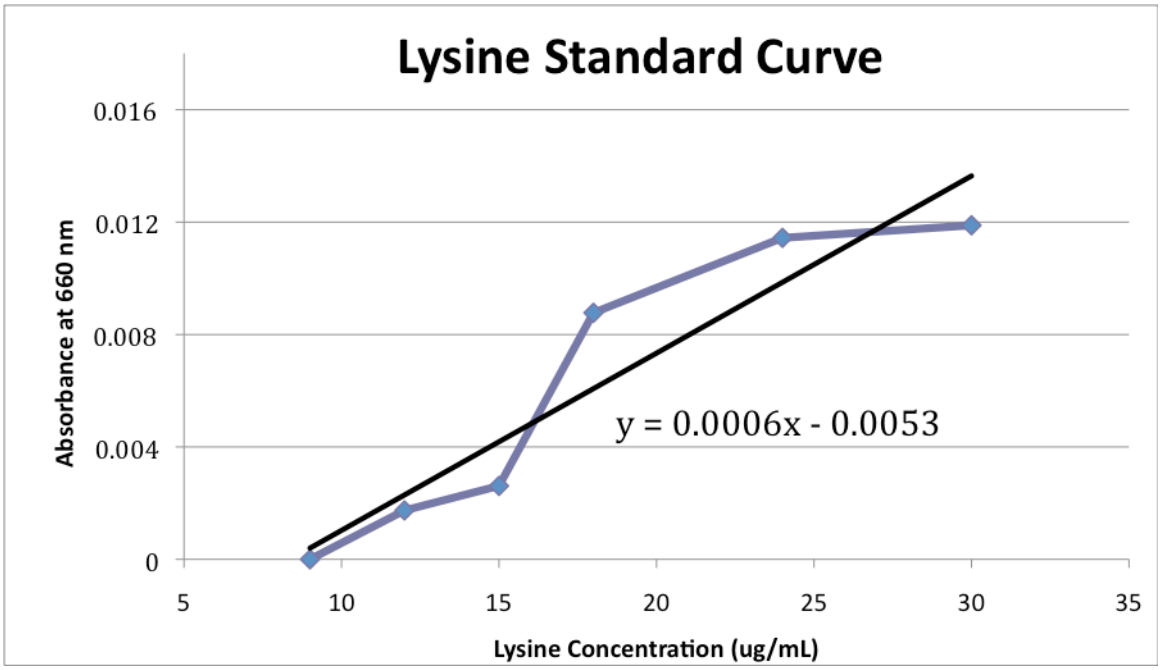

Figure 3. Lysine Standard Curve (blue) with calculated trendline (black)

Table 6. Absorbance data for test organism in unknown lysine levels from cellular lysate.

Tube	Diluted Cellular Lysate (mL/10mL tube)	Purified H2O (mL/10mL tube)	%T at 660nm	Absorbance at 660nm
1	0	5.0	100	0

2	0.5	4.5	100	0
3	1	4.0	99.8	0.000869459
4	1.5	3.5	99.8	0.000869459
5	2	3.0	99.3	0.003050752
6	2.5	2.5	98.4	0.007004902
7	3	2.0	98.5	0.00656377
8	4	1.0	98.5	0.00656377
9	5	0.0	97.7	0.010105436

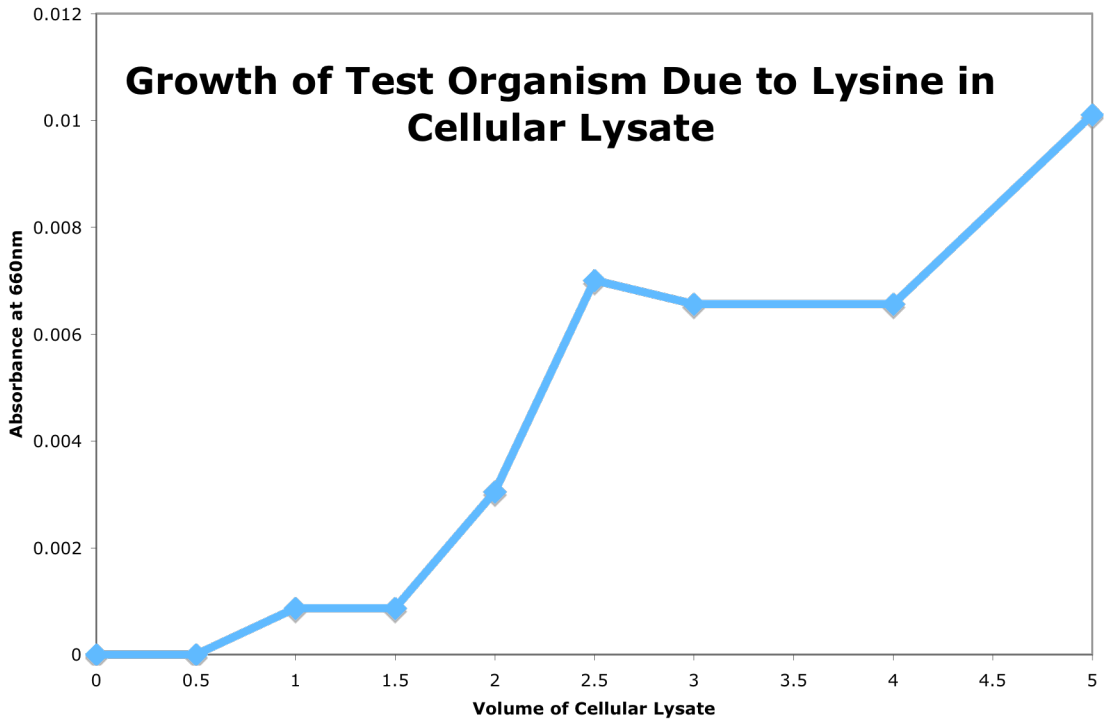

Figure 4. Growth of Test Organism

The addition of diluted *S. albulus* cellular lysate in increasing amounts allows for the calculation of the concentration of lysine in the cells via comparison to the Standard Curve seen in Figure 3. The data in Figure 4 was used to determine the mean concentration of lysine found in the diluted cellular

lysate, 5.66 μg/mL. Recalling that 0.002 g of cellular lysate was originally diluted to 20 mL, this gives the total cell lysate / lysine ratio to be 17.668 by mass.

6 Discussion

The hypothesis of this project states that if the obtained bacterial strain produced ε-Poly-L-lysine, then a significant abundance of lysine monomers would be shown to be present in the strain of bacteria. *S. albulus* was shown to contain high levels of lysine; about 1/18 of the cells' mass was lysine. This would be consistent with an organism that produces a poly-lysine; an overabundance of lysine would be necessary for the production of a lysine polymer. A non-poly-lysine producing strain of *Streptomyces griseus* contained an average of 2.13% lysine per cell by weight (Stokes, 1946), while the experimental strain in this project showed an average 5.55% lysine per cell by weight. This difference is statistically significant ($p = 0.00159$) by the Student's T-test ($p<0.05$ shows significance.) However, this experiment was only performed once, so repeated results would give more weight to these conclusions.

The results received in the TLC experiment were able to determine that there were amino acids in the growth media and cells, but due to the non-specificity of the ninhydrin stain the presence of lysine could not be separated from the

presence of any other amino acid. The same outcome would have been observed if only the cell pellets had been run on the TLC, as cells contain many amino acids and proteins. Ninhydrin is a qualitative indicator, not quantitative, and thereby it is inadequate to show an overabundance of lysine in *S. albulus* cells. This experiment would have been more successful if a lysine specific stain could be found. A negatively charged stain that would be able to bind to positively charged molecules could work limitedly; poly-lysine would not be the only positively charged molecules in a cell, so this method would still not be specific enough to definitively determine poly-lysine production.

Even though ε-Poly-L-Lysine is secreted into the environment by *S. albulus*, the cell pellet was used in this experiment because the objective was to show an abundance of lysine monomers, not the ε-Poly-L-Lysine molecule itself. If one were to be looking for or identifying ε-Poly-L-Lysine from *S. albulus,* the growth media should be separated from the bacterial pellet after fermentation and the growth media should be used. The experiment testing lysine levels cannot determine the type of poly-lysine that might be produced by the bacteria. If *S. albulus* produced α-poly-L-lysine as opposed to ε-poly-L-lysine, these same results would still be expected. Because this distinction has not been determined in this project, this strain cannot definitively be declared a positive ε-Poly-L-Lysine producer, though evidence seems to point that way. Further investigation would be necessary to determine which type of poly-L-lysine is produced.

Additionally, high levels of ε-Poly-L-Lysine would have a quenching effect on experimental bacterial growth. In order to prevent this from happening, one would have to hydrolyze the proteins (and, in the process, any poly-lysine molecules). Doing this would still be able to show heightened levels of lysine compared to non-producers without the risk of quenching skewing the results.

Since ε-Poly-L-Lysine has been shown to be biodegradable and non toxic towards humans and the environment, (Shih, Shen, & Van, 2006) ε-Poly-L-Lysine and its derivatives have been studied for a wide range of applications including food preservation, dietary agent, creation of hydrogels, and more. Further study of ε-Poly-L-Lysine producers can help streamline the production process and bring down costs. Due to its cationic nature, it is hypothesized that εPL may have the ability to reduce chromate and other anionic heavy metal wastes from industrial discharge through biosorption. Biosorption is a potentially cost effective way of removing heavy metals from industrial wastewaters (Rao Popuri, Jammala, Reddy, & Venkata,). If an adequate producer of ε-Poly-L-Lysine were found, this application would provide an environmentally sound biosorption technique.

7 Works Cited

Curylo, Elisabeth A., et al. Isolation and Antimicrobial Potential of Epsilon Poly-L-Lysine. Ed. Jill Rulfs, Theodore C. Crusberg, and Michael Allan Buckholt. Vol. E-project-042408-104838.

Worcester Polytechnic Institute:, 2008. <http://www.wpi.edu/Pubs/E-project/Available/E-project-042408-104838/>.

Georgnaras, Ifigenia, and John N. Sofos. "Activity of ε–Polylysine Against Escherichia Coli O157:H7, *Salmonella* Typhimurium, and *Listeria Monocytogenes*." Journal of Food Science 70.9 (2005): M404-8. <http://dx.doi.org/10.1111/j.1365-2621.2005.tb08325.x>.

Hamano, Y., et al. "Ε-Poly-l -Lysine Producer, Streptomyces Albulus, has Feedback-Inhibition Resistant Aspartokinase." Applied Microbiology and Biotechnology 76.4 (2007): 873-82. <http://dx.doi.org/10.1007/s00253-007-1052-3>.

Hamano, Yoshimitsu, et al. "Development of Gene Delivery Systems for the ε-Poly-L-Lysine Producer, Streptomyces Albulus." Journal of Bioscience and Bioengineering, 99.6 (2005): 636-41. .

Hiraki, J. "Use of ADME Studies to Confirm the Safety of Epsilon-Polylysine as a Preservative in Food." Regulatory Toxicology and Pharmacology : RTP 37.2 (2003): 328. . EBSCO:.

Hirohara, Hideo, et al. "Substantially Monodispersed Poly(ε-l -Lysine)s Frequently Occurred in Newly Isolated Strains of Streptomyces Sp." Applied Microbiology and Biotechnology 76.5 (2007): 1009-16. <http://dx.doi.org/10.1007/s00253-007-1082-x>.

Kahar, Prihardi, et al. "Enhancement of ε-Polylysine Production by Streptomyces Albulus Strain 410 using pH Control." Journal of Bioscience and Bioengineering, 91.2 (2001): 190-4. .

Kahar, Prihardi, et al. "Production of ε-Polylysine in an Airlift Bioreactor (ABR)." Journal of Bioscience and Bioengineering, 93.3 (2002): 274-80. .

Kito, Mitsuaki, et al. "Purification and Characterization of an ε-Poly--Lysine-Degrading Enzyme from the ε-Poly--Lysine-Tolerant Chryseobacterium Sp. OJ7." Journal of Bioscience and Bioengineering, 96.1 (2003): 92-4. .

Kunioka, M. "Biosynthesis and Chemical Reactions of Poly(Amino Acid)s from Microorganisms." Applied Microbiology and Biotechnology 47.5 (1997): 469-75. <http://dx.doi.org/10.1007/s002530050958>.

Malachowski, Lisa Lyn. Investigation of Immobilized Biopolymers for Metal Binding [Electronic Resource]. http://www.lib.utexas.edu/etd/d/2004/malachowskil62902/malachowskil62902.pdf University of Texas, 2006 9/15/2008.

Nishikawa, Masanobu, and Ken'ichi Ogawa. "Inhibition of Epsilon-Poly-L-Lysine Biosynthesis in Streptomycetaceae Bacteria by Short-Chain Polyols." Applied and Environmental Microbiology 72.4 (2006): 2306-12. .

Rao Popuri, S., et al. "Biosorption of Hexavalent Chromium using Tamarind (Tamarindus Indica) Fruit Shell - A Comparative Study." Electronic Journal of Biotechnology Vol. 10, No. 3.July 15, 2007 (2007): 9/14/2008. . 9/14/2008.

Shih, Ing-Lung, Ming-Haw Shen, and Yi-Tsong Van. "Microbial Synthesis of Poly(ε-Lysine) and its various Applications." Bioresource Technology, 97.9 (2006): 1148-59. .

Shima, S., and H. Sakai. "Poly-L-Lysine Produced by Streptomyces. Part III. Chemical Studies." Agricultural and Biological Chemistry 45.11 (1981): 2503. . EBSCO:.

---. "Polylysine Produced by Streptomyces. Argic." The Journal of Biological Chemistry 41 (1977): 1807. . EBSCO:.

Shirling, Elwood B., and David Gottlieb. "Cooperative Description of Type Strains of Streptomyces: V. Additional Descriptions." International Journal of Systematic and Evolutionary Biology 22.4 (1972): 265-394. .

Stokes, J. L., and Marion Gunness. "The Amino Acid Composition of Microorganisms." The Journal of Bacteriology 52.2 (1946): 195-207. .

Szókán, Gy, et al. "Structure Determination and Synthesis of Lysine Isopeptides Influencing on Cell Proliferation." Biopolymers 42.3 (1997): 305-18. <http://dx.doi.org/10.1002/(SICI)1097-0282(199709)42:3<305::AID-BIP4>3.0.CO;2-Q>.

Tarantino, Laura. "FDA/CFSAN: Agency Response Letter: GRAS Notice No. GRN 000135." Office of Food Additive Safety. January 16, 2004 2004. 12/11/2008 <http://www.cfsan.fda.gov/~rdb/opa-g135.html>.

Yoshida, T., and T. Nagasawa. "Imicro-Poly-l-Lysine: Microbial Production, Biodegradation and Application Potential." Applied Microbiology and Biotechnology 62.1 (2003): 21. . EBSCO:.