ε-POLY-L-LYSINE PRODUCTION IN STATIC AND AGITATED CULTURES WITH DIFFERENT OPERATION MODES

by
Mert Yıldırım

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University
2019

ε-POLY-L-LYSINE PRODUCTION IN STATIC AND AGITATED CULTURES WITH DIFFERENT OPERATION MODES

APPROVED BY:

Assoc. Prof. Dr. Ali Özhan Aytekin .....
(Thesis Supervisor)
(Yeditepe University)

Assoc. Prof Dr. Özlem Aytekin .....
(Sağlık Bilimleri University)

Assist. Prof. Dr. İlkem Emrah Nikerel .....
(Yeditepe University)

DATE OF APPROVAL: ..../..../2019

ACKNOWLEDGEMENTS

I thank to Assoc. Prof. Ali Özhan Aytekin from Yeditepe University for helping me in this study, to support and direct me and for trusting me. I thank to Neslihan Kayra and Yaprak Petek Koraltan from Yeditepe University for their innovative thoughts about project.

I thank to Assoc. Prof. Özlem Aytekin from Sağlık Bilimleri University and her student Sevda Arısoy for their contributions.

I thank to Assistant Prof. İlkem Emrah Nikerel from Yeditepe University and his students Gizem Banger and Burcu Şirin for their help on using HPLC.

I thank to Postdoctoral researchers Hüseyin Apdik and Ezgi Apdik from Yeditepe University for their encouragement and guidance.

I thank to TUBITAK for supporting 118M602 numbered “Production of ε-Poly-L-Lysine in continuous bioprocessing” project and also for providing the financial resources for material supply.

ABSTRACT

ε-POLY-L-LYSINE PRODUCTION IN STATIC AND AGITATED CULTURES WITH DIFFERENT OPERATION MODES

Biopolymers have expanded in the global industry over the past two decades, because of its usage areas, durability, and price. Most of the biopolymers can be collected and/or extracted from natural sources. However, some of them are produced by microorganisms. Homopolymers are polymeric molecules in which one type of chemical repeat unit or monomer is repeated many times. Homopolymers are the most difficult to produce and due to high cost. ε-Poly-L-Lysine (ε-PL) is a natural food preservative and high yield antimicrobial agent produced from a fermentation process using *Streptomyces albulus*. ε-PL is a homopolymer of L-lysine, one of the basic amino acids and is a lysine straight chain polymer. ε-PL contains 25-35 L-lysine monomer which is linked by ε-amino bond coupled with a ε-amine to L-lysine. In this study, it was aimed to compare ε-PL production using *Streptomyces albulus* under static and agitated cultures. Beyond that a different operation mode with 3 different strategies were studied. In semi-continuous operation mode experiments glucose feeding, pH control under sugar free media and glucose feeding with pH control strategies were studied. High Performance Liquid Chromatography (HPLC) was used for determination of ε-PL. Also, biomass, sugar, and nitrogen contents were followed by cell dry weight, sugar analysis based on DNS method, and Nessler's reagent, respectively. The antimicrobial activity of the produced ε-PL was tested. Based on results, it was found out that the production with agitation was more efficient than static one. According to the results of the study, the amount of ε-PL produced by agitated culture is 3 times higher than the static culture. According to semi-continuous operation mode results, glucose feeding strategies production result was 0.33g/L, in second strategy which was pH control with sugar free media, the production amount was 0.51g/L for the third strategy which was glucose feeding with pH control, the production amount was 0.45g/L at most. The novel result of the study is that production with static culture can be increased with different surface area/volume ratio.

ÖZET

ε-POLY-L-LİZİN ÜRETİMİNİN STATİK VE KARIŞTIRMALI KÜLTÜRLERDE FARKLI OPERASYON MODLARI İLE YAPILMASI

Biyopolimerler kullanım alanlarından, dayanıklılıklarından ve fiyatlarından dolayı küresel endüstrideki son yirmi yılda büyüme göstermiştir. Biyopolimerlerin çoğu doğal kaynaklardan toplanabilir ve/veya ekstrakte edilebilir. Ancak, bazıları mikroorganizmalar tarafından üretilir. Homopolimerler, bir tür kimyasal tekrar ünitesinin veya monomerinin birçok kez tekrarlandığı polimerik moleküllerdir. Homopolimerler üretilmesi en zor olanıdır ve maliyeti yüksektir. ε-Poly-L-Lisin (ε-PL), *Streptomyces albulus* kullanılarak yapılan bir fermantasyon işleminden üretilen doğal bir gıda koruyucusu ve yüksek verimli antimikrobiyal maddedir. ε-PL, bazik amino asitlerden biri olan L-lisinin bir homopolimeridir ve bir lisin düz zincir polimeridir. ε-PL, bir ε-amin ile L-lisine, ε-amino bağı ile bağlanmış 25-35 L-lisin monomeri içerir. Bu çalışmada, *Streptomyces albulus* kullanılarak ε-PL üretiminin statik ve ajite edilmiş kültürler altında karşılaştırılması amaçlanmıştır. Bunun ötesinde, 3 farklı stratejiyle farklı bir operasyon modu incelenmiştir. Yarı sürekli çalışma modunda, glukoz beslemesi, şekersiz ortamlarda pH kontrolü ve glukoz beslemesi ile pH kontrol stratejisi incelenmiştir. ε-PL tayini için Yüksek Performanslı Sıvı Kromatografisi (HPLC) kullanıldı. Ayrıca, biyokütle, şeker ve azot içeriğini sırasıyla hücre kuru ağırlığı, DNS yöntemine göre şeker analizi ve Nessler reaktifi ile takip edildi. Üretilen ε-PL'nin antimikrobiyal aktivitesi test edildi. Elde edilen sonuçlara göre, ajitasyonlu üretimin statik üretimden daha verimli olduğu tespit edildi. Çalışmanın sonuçlarına göre, ajite edilmiş kültürün ürettiği ε-PL miktarı statik kültürden 3 kat daha fazladır. Yarı-sürekli çalışma modu sonuçlarına göre, glukoz besleme stratejisi ile yapılan üretim sonucu 0.33 g/L, şekersiz ortam ile pH kontrolü olan ikinci stratejide 0.51 g/L, pH kontrol ile glikoz beslemeli üçüncü strateji için üretim miktarı 0.45 g/L'dir. Çalışmanın özgün sonucu, statik yüzeyli üretimin farklı yüzey alanı/hacim oranıyla arttırılabiliyor olmasıdır.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS III

ABSTRACT IV

ÖZET V

LIST OF FIGURES VIII

LIST OF TABLES X

LIST OF SYMBOLS/ABBREVIATIONS XI

1. INTRODUCTION 1

1.1. ε-POLY-L-LYSINE 1

1.1.1. Structure of ε-PL 2

1.1.2. Production of ε-PL 3

1.1.3. Purification And Characterization 5

1.1.4. Applications 6

1.1.5. Microorganisms Used In The Production of ε-PL 9

1.1.6. Production Technologies 12

1.2. AIM OF THE STUDY 16

2. MATERIALS AND METHODS 17

2.1. CHEMICALS AND MICROORGANISMS 17

2.2. CELL PREPARATIONS 17

2.3. EXPERIMENTAL METHOD 17

2.3.1. Agitated Culture Experiments 17

2.3.2. Static Culture Experiments 18

2.3.3. HPLC Measurements 19

2.3.4. Semi-Continuous Operational Mode and Production Strategies 20

2.3.5. Antimicrobial Activity Assay 21

2.3.6. Statistical Analysis 21

3. RESULTS 22

3.1. BATCH MODE PRODUCTION FOR ε-POLY-L-LYSINE 22

3.1.1. Agitated Culture Production For ε-Poly-L-Lysine 22

3.1.2. Static Culture Production For ε-Poly-L-Lysine 26
3.2. PRODUCTION OF ε-PL WITH SEMI-CONTINUOUS OPERATION MODE INCLUDING STRATEGIES 31
4. DISCUSSION 40
5. CONCLUSION 44
REFERENCES 45
APPENDIX A 60

LIST OF FIGURES

Figure 1.1. Chemical structure of ε-PL.....3

Figure 2.1. ε-PL standard (P8920-Sigma) prepared at different concentrations measured with HPLC at 215nm. 20

Figure 3.1. Optical density measurement of *S.albulus* for ε-PL production.....23

Figure 3.2. Dry cell weight measurement of *S.albulus* for ε-PL production. 23

Figure 3.3. pH measurement of *S.albulus* for ε-PL production. 24

Figure 3.4. Ammonia concentration of *S.albulus* for ε-PL production. 24

Figure 3.5. Glucose concentration of *S.albulus* for ε-PL production. 25

Figure 3.6. ε-PL production measurement for *S.albulus* in agitated culture experiment. ...25

Figure 3.7. Results of agitated cultured ε-PL production. 26

Figure 3.8. Optical density measurements for *Streptomyces albulus* in static culture experiment using three groups with different S/V ratio. 27

Figure 3.9. pH measurements of *Streptomyces albulus* in static culture experiment using three groups with different S/V ratio. 28

Figure 3.10. Dry cell weight measurements of *Streptomyces albulus* in static culture experiment using three groups with different S/V ratio. 28

Figure 3.11. Measurement of ε-Poly-L-Lysine with static culture production.....29

Figure 3.12. Optical density measurement of ε-Poly-L-Lysine with static culture production. 30

Figure 3.13. Glucose concentration of ε-Poly-L-Lysine with static culture production. 30

Figure 3.14. Ammonium concentration of ε-Poly-L-Lysine with static culture production. 31

Figure 3.15. Optical density measurements for semi-continuous production of ε-PL. 32

Figure 3.16. Dry cell weight measurements for semi-continuous production of ε-PL.....32

Figure 3.17. pH measurements for semi-continuous production of ε-PL.....33

Figure 3.18. Ammonia concentration for semi-continuous production of ε-PL.....33

Figure 3.19. Glucose concentration for semi-continuous production of ε-PL.....34

Figure 3.20. ε-PL measurement for semi-continuous production.....35

Figure 3.21. Results of 1st strategy in semi-continuous production of ε-PL.....36

Figure 3.22. Results of 2nd strategy in semi-continuous production of ε-PL.....37

Figure 3.23. Results of 3rd strategy in semi-continuous production of ε-PL. 38

LIST OF TABLES

Table 1.1. Efficiency comparison of microorganisms that are ε-PL producer 1

Table 2.1. Surface area/volume ratios of erlenmeyer flasks for ε-PL production 19

Table 2.2. Semi-continuous operational mode strategies. 20

Table 3.1. Purity results of ε-PL measurements. 39

LIST OF SYMBOLS/ABBREVIATIONS

ε-PL	ε-Poly-L-Lysine
MTX	Methotrexate
G	Gram
L	Liter
mL	Milliliter
μL	Microliter
°C	Celsius
GAE	Gallic acid equivalent
VVM	Volumes of gas per culture volume and minute
MeO	Methyl orange
B.subtilis	*Bacillus subtilis*
B.cereus	*Bacillus cereus*
SSF	Simultaneous saccharification and fermentation
ADME	Absorption, distribution, metabolism, excretion
MIC	Minimum inhibitory concentration
HPLC	High performance liquid chromatogram

1. INTRODUCTION

1.1. ε-POLY-L-LYSINE

Today, polymers are produced by microorganisms with high efficiency. Homopoly (aminoacid) and homooligo (aminoacid) are the most difficult to produce and are the most rare species that deserve the most attention. Subspecies of homopoly can be sorted the into poly(ε-L-lysine) (ε-PL), poly(c-glutamicacid) (c-PGA), poly(c-L-diaminobutanoic acid), and poly (L-diaminopropionic acid) (PDAP) [1–3]. ε-PL is a homopolymer, represented by peptide bonds between the alpha-carboxyl and ε-amino groups of L-Lysine. Today, ε-PL is useful for many fields and is an interesting research topic. These fields can be listed mainly as food industry, medicine, material engineering and electronics. The practices in these areas are continuing for every living thing and increasing our quality of life day by day [4–6]. ε-PL can be produced easily by many microorganisms. The microorganism which obtained the title of specific producer by providing the most efficient production results is *Streptomyces albulus*. The production results of the studies collected were shown in Table 1.1.

Due to the antibacterial and antimicrobial properties of ε-PL, it has been preferred by many researchers. As an example it has been a novel discovery by experimenting with rats made by Hiraki in 1995 and 2000. Experiment has been shown us the food preservative and food additive properties of ε-PL [7,8].

Table 1.1. Efficiency comparison of microorganisms that are ε-PL producer

Strain	Production (g/L)	Molecular Weight (kDa)	Key Process	References
S. albulus S410	48.30	3.2-4.5	pH control strategy	Kahar et al. (2001)[9]

S. albulus TUST2	20.2	1.5-4.5	Pulsed feeding strategy	Jia et al. (2009)[10]
Streptomyces sp.M-Z 18	35.24	4.21	Agro-industrial by-products applications	Ren et al. (2014)[11]
Kitasataspora sp. MY 5-36	34.10	5.05	Continuous immobilization	Zhang et al. (2010)[12]
Bacillus subtilis sp.	0.0763	-	Medium optimization	El-sersy et al. (2012)[13]
S. noursei NRRL. 5126	1.99	<10	DO control strategy	Bankar and Singhal (2011)[4]

In previous studies, researchers have tried several methods to increase production. pH control, cheap raw material application, bioreactor trial and cell immobilization are among those methods.

1.1.1. Structure of ε-PL

ε-Poly-L-Lysine (ε-PL) is a homo-polymeric compound which contains 25-30 lysine monomers linked at the ε-amino groups [7,14,15]. It is a biodegradable, nontoxic, and water soluble substance. It is secreted in different amounts by the filamentous bacteria *Streptomycetaceae* [5,16]. A positively charged hydrophilic amino group and a hydrophobic methylene group is present in ε-PL in water. This leads it to have unusual and useful cationic properties. ε-PL is not like 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 while the amide linkage in α-PL is found between the α-amino carbon and the carboxyl group [17,18]. Lysine, which is an

amino acid, has a positively charged ε-amino group, which results in a polymer of lysine residues to be positively charged in considerable amounts. In a hydrophilic environment, the carboxyl and amino groups realign themselves in the direction of the outside of a globular structure, while the hydrophobic methylene groups are aligned to the inside of the globular complex. In 1977, Shima and Sakai (1977) realized ε-PL produced by *Streptomyces albulus* ssp. *Lysinopolymerus* strain 346 [18]. Dragendorff's reagent activates in the existence of alkaloid substances, alerting the researchers that a molecule of interest was existent. Due to this, many early researches refer to ε-PL as the DP (Dragendorff's Positive) substance, before the structure was determined and 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 [9,19].

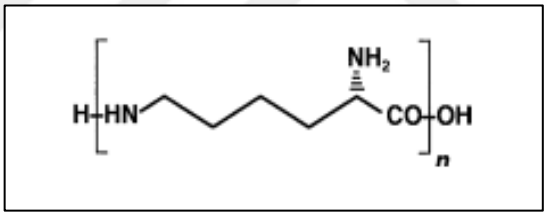

Figure 1.1. Chemical Structure of ε-PL

1.1.2. Production of ε-PL

After the discovery of the ε-PL, the active work became more active and determined by Shima and Sakai in 1977. Shima and Sakai (1977) have an important place in the formation of new production paths with the ε-PL aerobic fermentation they have isolated [18]. In these trials, wild type of *Streptomyces albulus 346* was used. Shima and Sakai (1977) carried the development even further with their experiments, and they observed ε-PL with fermentation at pH 5.9. ε-PL build-up was realized on the culture medium when cell growth reached the stationary phase and the pH of the media decreased to 3.0-5.0 which was prerequisite for ε-PL production. The extension of 1-lysine to the culture medium was not improve the

production of ε-PL in fermentation. Then they examined the production substrates with resting cells using ε-PL, $C_6H_{12}O_6$ and $(NH_4)_2SO_4$ [20–22]. ε-PL deposition was observed under acidic pH values and 4-5 g of ε-PL was formed. When cultured with culture cells, ε-PL has an ε-PL-degrading enzyme in the pH range of 4.9-5.9, showing *S. albulus*, which is rapidly degraded.

These studies were accelerated by Hiraki's work in 1999 [23]. To enhance ε-PL efficiency, S-(2-aminoethyl)-1'cysteine plus glycine-resistant mutants were acquired, which improved productivity. Kahar brought a different point of view to this field in 2001 [9]. Kahar (2001) argued that the most important point of ε-PL production is pH control mechanism. He divided the optimized fermentation into 2 phases. Increased ε-PL production at pH 2 with pH 3.9. An expansion in the pH of the culture medium was observed due to glucose consumption resulting in degradation of the produced ε-PL. In this structure, the ε-PL efficiency in a fed batch culture was increased from 5.8 g/L to 49.2 g/L. In another study, the ε-PL production was achieved in the air lift bioreactor and compared with the jar fermenter to decrease the cost of production counting down-processing of ε-PL. The production amount of ε-PL was comparable in both fermenter but the power utilization was higher in the jar fermenter. In addition, infiltration of intracellular nucleic acid-related substances into the culture medium in the air-lift bioreactor was fewer than that of the jar fermenter and therefore lowered the cost of downstream processing [14,24,25].

Banker and Singhal (2010) reported that the aeration and agglomeration of fermentation fluid enhanced the production of ε-PL, cell mass formation and glycerol use. From literature it could be understandable that agitation at 300rpm and 2.0 vvm results in producing larger amounts of ε-PL as it is related to the growth[4]. Alternative ε-PL producers, such as *Streptomyces griseofuscus* [26–28], *Streptomyces aureofaciens* [29–31], *Kitasatospora sp. PL614* [10,32,33], were isolated from soil. In order to increase productivity, several statistical methods were used to enhance the environment for the production of ε-PL. In 2006, Shih and Shen used the response surface method to optimize the production of ε-PL. In another experimental design, the Plackett-Burman design was used to determine and choose the most important cultural variables, and more advanced processes were used for further optimization [13,34]. For better production of ε-PL, metabolic precursors such as amino acids, TCA acid cycling intermediates and cofactors have been investigated. After 24 hours of citric acid addition and 48 hours of medium, L-aspartate has a notable effect on the

production of ε-PL [35,36]. Other components such as manganese, iron and cobalt increase ε-PL efficiency and are the most effective metal iron among them [37,38]. ε-PL production with immobilized *Kitasatospora sp. 5-36* cells using inert supports such as bagasse, synthetic sponge, macro-porous silica gel and loofah sponge was examined. Immobilized cells can be utilized 6 times, and therefore this approach has been established to be a encouraging tool for industrial applications [13,39].

In 2014, Chheda introduced a unique invention and gave a new perspective to ε-PL production. In his study, Chheda ε-PL production using *Bacillus cereus* has put a lot of microorganisms into production path [40]. Chheda isolating *Bacillus cereus* from the soil, using 25 mL medium in 100 mL Erlenmeyer flasks, produced at 32°C at pH of 6.8. In 2012, El-Sersy successfully managed the production of ε-PL using M3G medium in 100 mL Erlenmeyer flasks by isolating *Bacillus subtilis* from seawater [13]. Xia and Xu brought a new point of view and achieved successful co-production trials [3].

1.1.3. Purification and Characterization

ε-PL is a cationic homopoly(amino acid). Shima and Sakai in 1991, Lee in 1998 were isolated and purified from the culture medium by ion exchange chromatography on an Amberlite IRC-50 (H + form) column at pH 7.4 [16,41]. Medium was centrifuged then supernatant was administered to the Amberlite IRC-50 column and washed successively with 0.2 N acetic acid and water. Afterwards eluting with 0.1 N hydrochloric acid, the eluate was neutralized with 6 M sodium hydroxide, colored with activated charcoal and evaporated to a small volume. Finally, pure material (hydrochloride) was collected by precipitation like an off white powder by the addition of a mixture of ethanol/diethyl-ether (2:1). This material was further purified to a homogeneous homogeneity by column chromatography on CM-cellulose and gel filtration on Sephadex column [42,43]. As a result of studies, ε-PL could be further characterized by amino acid analysis [28,44]. In 2002, Nishikawa and Ogawa also Saimura's works identified the molecular weight of ε-PL by gel filtration on a Sephadex column. Even though shorter lengths of ε-PL have been reported, it has been shown to consist of 25-30 components, mostly with a molecular mass of about 4000 g/mol [24]. In 2000 Hiraki's work proved us, the ε-PL showed high water solubility and stability, the ε-PL

solution was boiled at 100°C for 30 minutes, or autoclaved (DAIHAN Scientific MaXterile 60, Korea) at 120°C for 20 minutes [23].

1.1.4. Applications

ε-PL is used in many field. It has proved its usability in food preservative [45], emulsifying agent [46], dietary agent [22], Interferon Inducer [17], drug delivery carrier [47], gene delivery carrier [27,39,43], hydrogels [48,49], bioelectronics and biochip [17], hydrogels and water absorbing materials [22,50].

People's awareness of food safety and requirements has increased gradually. The use of chemicals as preservatives has become very difficult and natural preservatives have increased in recent years both at home and abroad. The interest in antibacterial and safe new products has increased. Non-toxic natural preservatives have now become a hot research topic and are not only harmless to humans but also to nature, and there has been a tendency towards the naturalization of conservatives. ε-poly-l-lysine (ε-PL) is a kind of peptide bond that binds the carboxyl group and the amino group [51]. ε-PL has a wide range of antibacterial properties, requiring only a small amount. Poly-L-lysine has broad spectrum antibacterial properties and inhibits gram-positive bacteria [52]. It has an inhibitory effect on fungi even when its concentration is less than 100 μg/ml. Stable under hot, acid and alkali conditions. It does not affect the taste of the food and can be used in food industry as a preservative and the production scale has increased considerably. It can be used in cakes and bread products because it effectively inhibits heat resistant *Bacillus* [53]. In addition, it can act as a preservative in cold consumption products such as milk and cream, and it can be used in combination with other substances to achieve high efficiency and preservation. ADME study findings and no determined toxicity in safety studies makes ε-PL usable as a food additive [45]. Studies in rats did not show a toxicity to neurological functions, reproduction system and immunological functions. For two generations no ill effects were observed in embryos. Due to these finding and its antibacterial properties, ε-PL is now industrialized as a food additive. For example spraying or dipping the fish with a concentration of 1000-5000 ppm results in a preservative effect. It is also used in Japan in staple foods such as rice and noodles as a preservative [51].

One of the issues of ε-PL is its interaction with proteins and acidic polysaccharides which can lead to decrease in antimicrobial activity. Due to its bad emulsifying effects it has a limited application in starchy foods but its emulsifying effects can increase when it is conjugated with dextran with Maillard reaction. This can result in a emulsifier with a better functionality than commercially used emulsifiers [54]. The conjugated PL-dextran is not effected by high salt concentrations and high pH making it useful as a food additive, an emulsifier and antibacterial agent.

Obesity is one of the serious diseases in the world than can lead to health issues such as diabetes, hypertension and atherosclerosis. A way to treat this disease is limiting the absorption of dietary fat in the intestines by ingesting natural products that accomplishes the mentioned effect. ε-PL is found to have a lipase inhibitory effect and suppressive roles on postprandial hypertriacylglycerides [15,55]. At a concentration range of 10-1100 mg/L ε-PL can inhibit lipase activity and work as a breaker in emulsions and interaction of ε-PL with bile salts is responsible for lipase inhibitory effects. The observed effects of ε-PL on rats on postprandial hypertriacylglycerides showed that when it is administered intragastrically at an amount of 15 mg/kg it showed a significantly lower plasma triacylglycerol. This suggests that ε-PL can decrease dietary fat absorption from the small intestines with its lipase inhibitory activity.

Irradiation of ε-PL with γ beams can change it into a hydrogel. This hydrogel can be produced from 10 percent (by weight) ε-PL (liquid) when 85 kGy or more irradiation was applied. The water amount of the produced hydrogel is related to the dosage of the rays as the increasing doses of γ radiations decreased the water content. The swelling of the ε-PL hydrogel is also related to the pH [56,57]. Low pH causes the gel to swell due to the ionic interactions with the amino groups and high-pH causes gel to collapse. High water binding capacity of these gels can be achieved by cross-linking ε-PL with polysaccharides resulting in a huge water absorbing capacity, gel power and swelling rates. Usage of the hydrogels with these properties are thought to have many applications in agriculture, food and medical industries.

Due to its polycationic properties in alpha and epsilon form, due to its water solubility, biodegradability and biocompatibility, PL has pioneered great innovations in many areas. In the area of health, efficacy of some interferon inducers, antiviral and antitumor agents can be boosted using a-PL. In addition, it increases drug transport by decreasing resistance of

drugs and hematocytes, liposomes and so on. It has been observed that organelle fusion increases its efficacy[58]. To this end, the use of α-PL drug delivery systems in human, in particular for the gene have been studied by various scientists. Recently, ε-PL has been utilized in enzyme immobilization. Enzyme sensors or enzyme reactors was used for clinical analysis in this area. Synthetic double-stranded RNA polyriboinosynic-polydigidic acid was effective as an endogenous interferon activator. The antifolate agent is among the common drugs used for the treatment of methotrexate (MTX), sarcomas, human leukemia and other disease. Nonetheless, due to the insufficiency of MTX transport, resistance to methotrexate has been encountered in many cases. Cellular uptake was increased by the conjugation of MTX to α-PL and offers a new way of dealing with drug resistance associated with insufficient transport. MTX conjugated 60,000 Mr fragments of a poly-α-(D-lysine) and α-PL having molecular weights of 3100 to 130000 and assesed their cellular uptake and growth [59]. The data also showed that, the intracellular release rate of the active drug after conjugate uptake in methotrexate-resistant cells was the comparable in size as the rate of uptake of the free drug by competent cells. Thus, resistance to drugs can be reduced when PL was used as a method of transport as a result of its poor transporting properties. Some researches have shown that α-PL is a potentially flexible drug carrier due to that cells are readily taken, drug carries many functional amino groups to which it can be covalently bound, that has a wide molecular size which may be adjusted to specific demands, target cells are readily degradable and non-toxic. In addition, a covalently conjugated drug to the a-PL carrier may leave a pharmacologically active additive which has a higher efficiency in the cells than the free drug. Since hopes for human gene therapy was expressed, the use of genetic materials as therapeutic agents for the modification of the somatic cellular genotype has been made for a wide range of diseases [28,60]. Various techniques have been discovered for introducing genes to mammalian cultured cells, but in vitro transfection activities have not been successfully applied in vivo. The use of retroviral or adenoviral vectors for in vivo transfection have various disadvantages, with inadequate success rate. Repeated use of the delivery system have been hampered by adenoviral vectors that have a large immunogenicity and have possible viral associated toxicity, including oncogenic effects through retroviral vectors, endogenous virus recombination and splicing mutagenesis, including viral replication via oncogenic effects can be given as an example for these disadvantages.. Because of these, non-immunogenic non-viral vectors consisting of self-assembled components are interesting alternatives to viral processes for gene therapy [61–63]. The

investigation of cationic polymer-based gene delivery vectors came as a result of the speeding development of non-viral gene delivery vectors. Cationic polymers have been shown to complex with plasmid DNA via ionic interactions, thus DNA was defended from nuclease degradation and has worked as a platform to gain better the cellular yield of DNA. It is commonly accepted that α-PL is strongly bound to DNA to ensure that the DNA molecule is compressed [64–66]. However, there are some down sides that causes DNA to experience problems that restricts its use as a gene carrier in vivo due to the stoichiometric complex consisting of α-PL. Therefore, the α-PL is customized with varying substances to control and improve complex features. Recently, lactose bound to the PEG-PL block polymer and specifically formed a Lac-PEG-PL carrier to target hepatoma cells. The complex that is formed among novel Lac-PEG-PL carrier with plasmid DNA and act as an efficient gene carrier with better solubility and less toxicity than that of α-PL. Various modifications of α-PL have been reported in the literature for precise cell targeting and better delivery of genes in vivo. Modified PEG-PL copolymer as well as gene distribution. In addition to gene distribution, the modified PEG-PL copolymer has also been used efficiently as a carrier for cis-diamindyloroplatin, an antineoplastic agent frequently utilized in treating tumors of head, ovaries and neck. Preparation of PL-PL adsorbents and the utilization of endotoxins in the selective removal of cell products employed as drugs in the immobilization of glucose oxidase on a PL-modified polycarbonate membrane are among the other uses of PL or its derivates in biomedical fields [40,67] to be used as a glucose sensor. Recently, Micro or Nano-capsules that consists of α-PL or its derivatives have been tailored to encapsulate cell lines that are convenient for delivery of ocular drug or in vivo delivery of bioactive molecules. Since these capsules are semi-permeable and biocompatible, the continuous secretion of the required therapeutic agents by the encapsulated cells can be very important.

1.1.5. Microorganisms Used In The Production of ε-PL

Bacillus sp. are flat or even close to the rod and they are resistant to rough conditions [68]. They are generally gram positive and have mobile peritric flagella [69]. They are aerobic and facultative anaerobes. In a lot of them oxygen is an electron acceptor and endospore form. Vegetative cells are 0.5 x 1.2 to 2.5 x 10 μm in diameter[70]. *Bacillus* Colony morphology of the genus is diverse. Generally they are cream or white colored colonies and

a few types of yellow, orange, pink and black color pigmented colonies are also present [71]. *B. mycoides* colonies grown in rhizoid agar spread on the medium, *Bacillus*' s thermophilic, mesophilic and psychrophilic species are found. They can survive at very high temperatures and their optimum growth conditions are around 35-37ºC and pH 7 [72]. They grow quite well on the medium and utilize carbon as source of organic acid, sugar and containing alcohol; as a source of nitrogen they develop very well [73]. The *Bacillus* genus is capable of forming spores under inappropriate conditions. Spores can be cylindrical, oval, round or kidney shaped. In addition, spores can settle in the cell centrally or sub terminally [74]. Cell wall of *Bacillus*, surface layer that completely covers the cell surface it forms paracrystalline. *Bacillus*' usually contain carbohydrate capsules [54]. Typical habitats are soil despite being in nature as wide as milk and can be obtained from a variety of media such as water and food. They produce catalase and acid but do not form gas [75]. Some types of *Bacillus* are important as proteolytic enzymes and some *Bacillus* can be used in cheese making [76]. Some species are also pathogenic to insects. Several species of *Bacillus* produce antibiotics from the polypeptide class. Antibiotic formation was observed when there was a sporulation stage in cultures [77].

According to a study by Turnbull, the diagnosis of *Bacillus* species and for the determination of differences between species, sports and sporangium morphologies are essential [78]. Accordingly, the *Bacillus* were grouped in 3 groups. The first group is divided into two as *Bacillus* A and B in themselves. It's both sporangia was not swollen in the group. Spores are elliptical or cylindrical shape and centrally or terminally located. The difference between group A and group B is that in group A cell width is less than 1 μm and in B subgroup it is greater than 1 μm. To A subgroup *B. megaterium* [79] and *B. cereus*, for example; *B. licheniformis*[80], *B. subtilis*, *B. pumilus*, *B. firmus* and *B. coagulans* may be given. The second group of *Bacillus* species sporangia swollen. Spores are elliptical, centric or terminal. Examples of *Bacillus* species in this group include *B.* polymyxa, *B. adventures*, *B. circulans*, *B. stearothermophilus*, *B. alvei*, B *laterosporus* and *B. Brevis* [69,70,81–83]. In the third group of *Bacillus* species are in subterminal or terminal position. For example, *B. sphaericus* is an example of this group. Most bacteria in this genus are not pathogens. Two in humans and animals *bacillus* These are *B. antracis* and *B. cereus*. *Bacillus anthracis* called anthrax, shepherd in humans and animals is the causative agent of the disease [20,67,70,84,85]. Disease of *B. anthracis* spores damages skin, mucous membranes and enters the lungs

through respiratory tract to cause damage. *B. anthracis*' spread from there to meninges through the bloodstream and they tend to multiply there. According to the entrance location, *B. anthrac* can cause skin anthrax, lung anthrax, intestinal anthrax and causes septicemia. *B. subtilis* powder are found in soil, fertilizers, plants and animals with milk. It is effective in the breakdown of milk beverages, bread, vegetables and fruits. Some of this substance, called subtilin is obtained from the culture supernatants of *B. Subtilis* and it has been shown that it has an inhibitory effect against bacteria. Bacteria are actually saprophytes as a result of direct penetration into the tissue and especially the eye Panophthalmia can cause eye inflammations such as piridoxilate [86–89]. *B. subtilis*'s nutrients are also suspected to cause poisoning. Factors such as nitrogen source, carbon source, oxygen level, milk components affects the development of *B. cereus* in milk. At the same time the development of *B. cereus* milk also depends on the pH and temperature during processing. Because their spores stay alive in pasteurized milk, they cause milk to degrade at room temperature. *B. cereus* can lead to diarrhea, nausea-vomiting, different kinds of food poisoning [73,90–92].

The genus *Streptomyces* is part of the phylum actinobacteria (or actinomycetes) which includes other genera such as Mycobacterium, Corynebacterium, Nocardia, Rhodococcus and Frankia. They are gram-positive, sporulating and filamentous bacteria. At the ecological level, they are ubiquitous of the soil and are often recognized as part of consortia bacteria involved in the decomposition of organic components such as chitin or cellulose. As such, they have a prominent role in composts [93] and in forest ecosystems [62] for the realization of the carbon cycle. This bacterial genus is mainly known and studied for its capability to produce a large amount of secondary metabolites. It has been estimated that 53 percent of known antibiotics are produced by actinomycetes, of which 40 percent by species of *Streptomyces* [62,66]. Along with the production of antibiotics, *Streptomyces* bacteria are responsible for producing a broad enzymatic repertoire. The production metabolites are regulated during their development cycle. The life cycle of *Streptomyces* is atypical with different cell stages compared most bacteria [94–96]. Its cell cycle begins with a spore that sprouts and gives hyphae. The hyphae develop radially and form a branched network called vegetative mycelium. Many stimuli and in particular a limiting nutrient concentration induce a difference morphological union of vegetative mycelium to aerial mycelium. This mycelium is composed unbranched hyphae [4,97]. The differentiation of the latter ends with an asynchronous septation and by the formation of spore chains that are the form of

resistance and dissemination bacteria. [98–100]. Along with the morphological differentiation is set up a biochemical differentiation, with a high activity of secondary metabolism and in particular the production of antibiotics during differentiation of vegetative mycelium into aerial mycelium[66,101–104].

1.1.6. Production Technologies

To understand E-PL production, it is necessary to know the concept of fermentation. Fermentation technologies should be handled on a subject and production basis. Alcohol, acetic acid and lactic acid fermentation are the basis of this subject. Then it would be correct to examine in terms of production strategies. Alcohol fermentation is defined as the decomposition of ethyl alcohol and carbon dioxide in the anaerobic environment by the yeasts of sugars. Alcohol fermentation includes ethyl alcohol and carbon dioxide as well as substances such as glycerin, acetaldehyde and acetic acid [105–107]. García-Parrilla et al. (2011) states that gluconic acid bacteria, such as gluconobacter japonicus, returned the glucose in the fruit to gluconic acid, followed by the preservation of the sweetener fructose gluconic[108]. Composition of investigations showed that 11 anthocyanin compounds have been identified and the amounts of these compounds are present and the main compounds of pelargonidine 3-glucoside and derivatives thereof have been identified. In addition, during the gluconic fermentation, the anthocyanin composition must be protected by the preservation of the anthocyanin, the gluconic fermentation and the beverages having higher antioxidant activity than the beverages associated with alcohol fermentation. Ohya et al. (2005) aimed at improving the alcohol producing power of Saccharomyces cerevisiae [109,110]. The functionality protocol that appeared in the RIM15 gene was inserted into the unidentifiable region of 50 of the RIM15 promoter of the glycogenogenic gene (PCK1). At the end of the study, the strain of PCK1 promoter was shown to show a better alcohol fermentation in the area where the RIM15 gene was deleted from repeating water at repetitive and excessive times. Soto-Cruz et al. (2013) Agave duranguensis's alcoholic fermentation is obtained from the selection of domestic yeast and of the agave plant in the inside of the juice of agave plant [111,112].

Acetic acid fermentation is defined as the conversion of fermentable sugars by yeasts into ethanol and conversion with acetic acid bacteria into acetic acid and water. In order to

increase the efficiency of acetic acid production, Gullo et al. (2012) aimed to perform acetic acid fermentation by working in combination with static and immersion fermentation with acetic acid bacteria which are not capable of producing cellulose with alcoholic and sugary substrates[113]. At the end of the study, 8.00-9.00 percent (w/v) acetic acid concentration was obtained and the medium suitable for the development of the selected starter culture was determined prior to submerged fermentation provided by small scale batch fermentation and static fermentation before submerged fermentation. Mounir et al. who researched to increase the efficiency of acetic acid, isolated the acetic acid bacteria from the fruits of apple and cactus, aimed at the simultaneous production of heat resistant acetobacter strains and gluconic and acetic acids during the fermentation of the bioreactor acetic acid [114]. As a result of their research, acetic acid bacteria isolated from cactus fruits were obtained with 7.64 percent (w/v) acetic acid bacteria isolated from the apple, and 10.08 percent (w/v) acetic acid concentration was obtained from cactus. It has also been found that acetic acid bacteria isolated from cactus fruit can simultaneously produce acetic acid and gluconic acid and are less susceptible to ethanol depletion.

Lactic acid fermentation is a type of fermentation in which the glucose is converted into acetic acid, lactic acid, ethyl alcohol and CO_2 by heterofermentative lactic acid bacteria in an environment without oxygen. In a study, it was aimed to identify the lactic acid bacteria obtained from the cherry plant, the selection of the mixed endogenous starter culture and the preparation of the protocol for the processing of cherry puree added from the roots by infusion [53,114]. The research was finalized by determination that the selection of autochthonous lactic acid bacteria and the addition of root infusion to provide acceptable product, preservation of the nutritional, antioxidant and sensory properties of the product and an alternative technological option in the processing of sweet cherries.

The batch culture is known as a closed system under the aseptic conditions at the beginning of the medium, which is usually added under aseptic conditions to the bioreactor in which inoculum, the medium and nutrients are added. During cultivation, it is theoretically stable, practically insignificant changes in the culture volume, a low base/acid solution to keep the pH at an aimed level from the feed rate and culture by sampling or sampling by air/gas sampling, usually due to the small values of the complete working volume of the bioreactor are ignored [34,115]. Generally, a certain amount of live cells are added to the bioreactor which has sterile media at the start of batch cultivation. Following the inoculation, the cell

growth follows the regular growth curve that was divided into four main phases [116]. Since the delay phase is an weak phase of the culture, although cells are metabolically active, they adapt their enzymatic devices to a new habitat, there is no notable increase in biomass amounts, substrate utilization, or product synthesis, it is desired to decrease this phase it as much as possible. Concentration of microorganisms in the inoculum, the status they are in physiologically, inoculation composition and medium used for growth are the main factors that increase the time of delay phase. The exponential (logarithmic growth) stage, swift cell proliferation, is characterized by a fixed specific growth rate that is identical to the maximum specific growth rate of the culture under conditions without growth restriction. At the finish of the exponential phase (when aerobically grown cultures results in a quick increase in dissolved oxygen concentration) nutrients are used up by the cells which leads to a continuous decrease in specific growth rate and a passage to a stationary phase where growth is stopped and endogenous carbon are used and energy reserves. This step is crucial for the synthesis of secondary metabolites. Because of the simplicity of the operations in batch mode, many industrial bioreactors uses this method. [4,117–119].

The fed batch group culture can be explained by a half-open system. In that system single or added feeders are aseptically and consecutively blended into the tank when being contained in the product. Thus, the volume of water in the bioreactor increases during the process [34,61,109,115]. The capability to lengthen product synthesis, to obtain superior cell densities are among the advantages that fed batch cultures have over batch cultures. These advantages result in increased concentration of the produced biomass, the ability to result in a higher yield or fertility by the extension of controlled successive nutrients. The fed series is effectively utilized in processes where substrate inhibition or catabolic printing is wanted. The effect which this situation to be expected to beaten is called Crabtree effect (suppression of high concentrations of yeast respiratory enzymes) with glucose, feeding the remaining substrate in the batch process, using a safe concentration in batch mode [120]. By feeding the substrate progressively, the production of ethanol with yeasts can be eradicate under aerobic conditions where large amounts of cell density is needed. An increased production rate can be reached with a high and constant specific growth rate by controlling exponential feeding of the substrate. The cell metabolism may be improved by consecutive provide of the nutrients and in situations where a increased culture water viscosity is desired and continuous dilution of the media can beat the problems related to infuse and oxygen transfer.

In the world of science there are a number of protocols for adding a substrate to the bioreactor. The correct selection of nutrient feed rate could cause significantly increase culture performance as it affects cellular growth rate, cell physiology and product formation odd.

The continuous system which the nutrients are aseptically and continuously combined to system and the culture medium is removed simultaneously is called a continuous culture system. In this process due to a constant feed and feed rate the volume of the culture medium constant. [23,110,121]. Frequently, continuous culture is used synonymously for a chemostat described by a constant specific growth rate of cells, which is controlled by the presence of the limiting feeder, even though there are other types of continuous processes such as a particular turbid state, which are equal to the dilution rate or nutristat could be used. The capability to adapt the optimum setting for a long term and a high yield synthesis, the capability to assure constant quality, and decreasing the unprofitable reactor operation periods are among the leverages that continuous culture have over batch mode. Despite these leverages, there are a number of issues that prevent the widespread use of continuous process. Those problems are the risk of increased contamination in the medium due to the pumped in and out of the reactor, the risk of genetic mutations in the production type in a long-term action and extra financial aids that may be wanted for technical expectations [19,63,110].

The classification as “dynamic” culture systems distinguishes them from “static” culture systems without fluid movement. An example of a "static" culture is a standard culture flask or plate. The static culture condition requires more space and labor. Different culture circumstances, like shaking flasks, mixers, air lift reactors, rotational disc reactors and modified air lift or gas lift bioreactors, have been used for the production of ε-PL under agitation conditions. The most widely used bioreactors are the stirred tank reactor and the air lift reactor. Bioreactors carry out synthetic action including livings and biochemical active substances under different oxygen conditions. Airlift bioreactors were identical to bubble column reactors, a small diversity in that it includes a drawing tube. An air lift bioreactor comprising an inner draw tube is referred to as an air lift bioreactor comprising an inner loop, while an air lift bioreactor comprising an outer draw tube is announced an air lift bioreactor comprising an outer loop.

By selecting the culture conditions, the substances to be added and the species of microorganism to be used, it is possible to control and increase the important properties of biopolymers and biosynthesis such as molecular weight and structure. Microorganisms can synthesize ε-PL extracellularly in two seperate ways. The first is the static culture used to obtain cellulose films or membranes on the interface between air and liquid medium. The other one is agitated culture in which the chemical ε-PL is distributed as fibrous structures throughout the culture medium. The agitated culture may be carried out in an orbital shaker, electromagnetic shaker or bioreactors.

Seeding in static medium does not provide high ε-PL yields compared to cultivation in agitated culture. There are some disadvantages in ε-PL production that hinder parameter control to make yields more difficult in static cultures. Therefore, it is difficult to control pH, cell growth and control of the inclusion of reagents during seeding, and these parameters are known to be critical for determining optimum yield conditions. In static culture, ε-PL production is straight forward and inexpensive and can be grown in bottles and containers. Nonetheless, due to the biosynthesis time of up to 7 days, it is not suitable for commercial scale manufacturing. However, growing in agitated culture has increased yielding efficiency and good capability to be manufactured on a industrial rate.

1.2. AIM OF THE STUDY

In this study, it was aimed to compare ε-Poly-L-Lysine production using *Streptomyces albulus* under static culture and agitated culture. Beyond that a different operation mode with 3 different strategies were studied. In semi-continuous operation mode experiments glucose feeding, pH control under sugar free media and glucose feeding with pH control strategies were studied Techniques used in this study were chosen among the production techniques that are present in the literature. Sugar, ammonia determination and biomass contents were followed by cell dry weight, sugar analysis based on DNS method, and Nessler's reagent, respectively. Concentrations and purity was analyzed with reversed phase HPLC method.

2. MATERIALS AND METHODS

2.1. CHEMICALS AND MICROORGANISMS

All chemicals were purchased from Sigma Aldrich St. Louis, Missouri, USA. *Streptomyces albulus* DSMZ40492 was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen).

2.2. CELL PREPARATIONS

Streptomyces albulus DSMZ40492 were cultivated on Nutrient Agar and inventories were used for the experiment. The cell is used as a result of two activation. In first activation liquid culture was prepared by inoculation at a ratio of 1:10, 2 days later inoculation is done again. The stock was thawed prior to using. Frozen stock was generated with DMSO (Dimethyl sulfoxide) in certain proportions. The culture was prepared before the experiments.

2.3. EXPERIMENTAL METHOD

2.3.1. Agitated Culture Experiments

After several trial experiments, agitated culture experiments began when the method was fixed. In the 144-hour experiment, ε-PL, biomass, ammonia concentration, glucose concentration and pH measurements were followed. In this experiment M3G media was used. M3G Medium (g/L) contains: Glucose, 50; yeast extract, 5; $(NH_4)2SO_4$, 10; KH_2PO_4, 1.36; K_2HPO_4, 0.8; $MgSO_4+7H_2O$, 0.5; ZnSO4+$7H_2O$, 0.04; and $FeSO_4+7H_2O$, 0.03. pH 6.8. pH adjusted with 6M NaOH. After sterilization, inoculation was performed at 5 percent. In the rotating shaker, a measurement was taken every 6 hours in the experiment at 30°C for 144 hours. The experiment was repeated following specific standards. In repeating experiments amount of Erlenmeyer flasks and parallel groups was increased. In new experiment, *Streptomyces albulus* was used. In total, 24 pieces 250 mL Erlenmeyer flask

was used and M3G medium was used for *Streptomyces albulus*. Incubation was left at 30°C in the rotating shaker at 150 rpm.

Optical density measurement was performed at 600nm. The protocol followed in this study was as follows. Samples taken from Erlenmeyer flasks were placed in the cuvettes (IsoLAB, Germany) and measured. Blank was used as dH_2O. Cell dry weight was measured according to the protocol. The filter paper which has 0.2µm pore size (Sartorium Stedim) was left to dry for 2 nights and passed through the vacuum filtration system (ISOLAB, Germany) with 5ml sample. The full and empty weight of the filter paper was measured. The filter papers were dried at 60°C. In order to measure pH, 5 mL sample was taken to 15mL falcon and measured by pH meter (Mettler Toledo S220).

Intermittent measurements were made for 144 hour in agitated culture experiment. pH measurements, cell dry weight measurement, optical density measurement and ε-poly-L-Lysine measurement were performed. A total of 18 pieces of 250 mL Erlenmeyer flask for 5 percent *Streptomyces albulus* inoculation was made and was left in rotating shaker at 150 rpm 30°C for 144 hour. At last standard ε-poly-L-Lysine measurements were done. ε-poly-L-Lysine concentrations determined in the experiment are as follows; 0.1 g/L, 0.075 g/L, 0.05 g/L, 0.025 g/L and 0.01 g/L. After preparation, the concentrations were measured with HPLC.

2.3.2. Static Culture Experiments

In static culture experiments, experimental setup was prepared according to Surface area/volume ratios of Erlenmeyer flasks. Measurement method of Erlenmeyer flask is given in Table 2. After the measurement, the results of the Surface area/volume formula are in the Table 2. Surface area/volume ratio of 3 rates were selected by the result of trial experiments. In new experiment, 0.2 S/V, 0.7 S/V, and 1.2 S/V, was used and measured for 144 hours. In this experiment, *Streptomyces albulus* was used with 5 percent inoculation rate. ε-PL, pH, biomass, sugar, and nitrogen contents were followed. The number of measuring cups used in the experiment was 207 in total with 3 parallel groups. The M3G media was used for this experiment.

Table 2.1. Surface area/volume ratios of erlenmeyer flasks for ε-PL production

	250mL-Erlenmeyer Flask				500mL-Erlenmeyer Flask			
Added Volume (mL)	**Diameter (mm)**	**Height (mm)**	**S.Area (mm^2)**	**S/V Ratio**	**Diameter (mm)**	**Height (mm)**	**S.Area (mm^2)**	**S/V Ratio**
50	82	18	5281,017	1,056203	104	14	8494,867	1,698973
100	77	28	4656,626	0,465663	101	20	8011,847	0,801185
150					98	27	7542,964	0,502864

For glucose determination assay, sodium potassium tartrate solution was prepared. Afterwards, 96 mM 3,5-Dinitrosalicylic Acid Solution was prepared. Then, the samples were diluted and mixed at 1:1 ratio. The results were measured at 540nm with UV-visible spectroscopy. These protocols were based on Enzymatic Assay of α-AMYLASE (Sigma)[122]. For determination of ammonia concentration, Nesslerization spectrophotometric method followed. For ammonia concentration determination, Rochelle salt solution was prepared according to protocol. 5ml sample diluted with 5ml dH_20 afterwards 100µl Nessler reagent and 100µl Rochelle salt solution was added. Mixture was waited at room temperature for 15 minutes and absorbance was measured at 425nm.

2.3.3. HPLC Measurements

ε-PL measurement was performed by HPLC system (Shimadzu, Japan). Sample was taken with syringe and filtered with 0.20µm filter (Sartorius Stedim Minisart) to glass vials (ISOlab,Germany). ODS-120-T 25 cm × 4.6 mm was used as the column. The mobile phase was prepared as a mixed phase. 6.80 mL of H_3PO_4 per 1000 mL of water and 23:2 ratio of acetonitrile. Flow rate was 0.8 mL/min and measurment done at 215nm in UV detector.

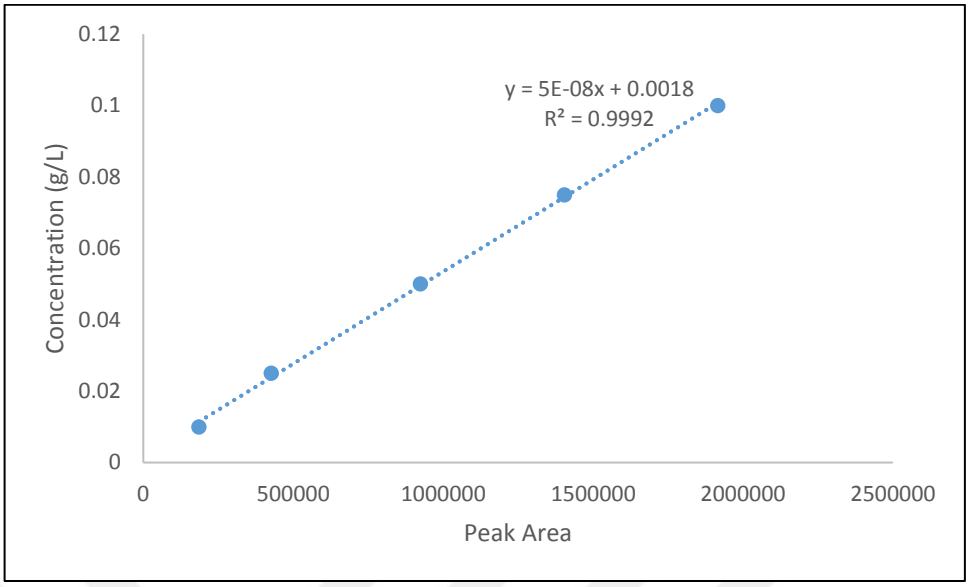

Figure 2.1. ε-PL standard (P8920-Sigma) prepared at different concentrations measured with HPLC at 215nm.

2.3.4. Semi-Continuous Operational Mode and Production Strategies

In semi-continuous operational mode experiments 3 different strategies was determined and *Streptomyces albulus* was used. In the first strategy, glucose feeding was done with 50 percent volume change. Every 12 hour 25ml sample was collected and 25ml M3G media was added. In the second strategy, sugar-free medium was used to control pH. In this strategy, the pH was kept about 3. Also this was done with 50 percent volume change like first strategy. In the third strategy, the pH was increased from 1 to 3. They were fed with normal medium. These experiments were conducted with 3 parallel and control groups of each strategy. M3G medium was used in this study. Samples was incubated at 30°C at 150rpm. Experiments lasted for 144 hour.

Table 2.2. Semi-continuous operational mode strategies.

Semi-continuous operational mode strategies;
1- Glucose Feeding in every 12 hours with 50 percent volume change.
2- pH control strategy with sugar-free media. Every 12 hours with 50 percent volume change.
3- Glucose Feeding with pH contol. Every 12 hours with 50 percent volume change.

2.3.5. Antimicrobial Activity Assay

The antimicrobial activity of the produced ε-PL was tested by the antimicrobial activity assay. The lethal dose from the generated ε-PL was administered by dilution and as in one. Selected cells are *Pseudomonas aeruginosa*(ATCC 15442), *Listeria monocytogenes*(ATCC 19115), *Escherichia coli*(ATCC 10536) and *Yersinia enterocolitica*(ATCC 27729). Cells were brought to 10^7 cfu/mL with saline solution and the experiment was performed.

2.3.6. Statistical Analysis

The experiments were carried out in 3 parallel and 2 repetitions. Student t-test was used for statistical analysis.

3. RESULTS

In this study, ε-PL production was performed by using static and agitated culture methods. *Streptomyces albulus* microorganism was used for production. Measurement methods were determined based on previous studies. Beyond that a different operation mode with 3 different strategies were studied. In semi-continuous operation mode experiments glucose feeding, pH control under sugar free media and glucose feeding with pH control strategies were studied. HPLC was used to measure ε-PL. In the follow-up study, Biomass measurement, pH measurement, sugar concentration determination, ammonia and cell growth were monitored. Further ε-PL measurements was performed with HPLC due to accurate results.

3.1. BATCH MODE PRODUCTION FOR ε-POLY-L-LYSINE

In batch mode production, agitated culture and static culture production were divided into two categories.

3.1.1. Agitated Culture Production For ε-Poly-L-Lysine

Optical Density measurement was given in Figure 3.1. At the 144th hour OD measured as 8.5. This means that *S.albulus* did not enter the stationary phase.

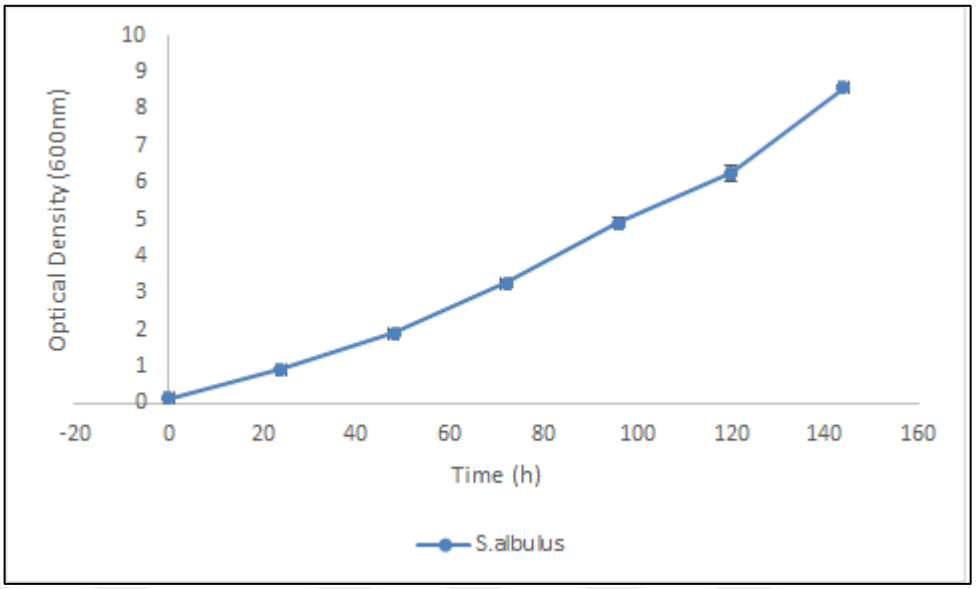

Figure 3.1. Optical density measurement of *S.albulus* for ε-PL production.

Figure 3.2 shows the Dry cell weight measurement of Control group. At the 144th hour CDW measured as 3.17g/L. During 144 hours DCW increased by 2.5g/L.

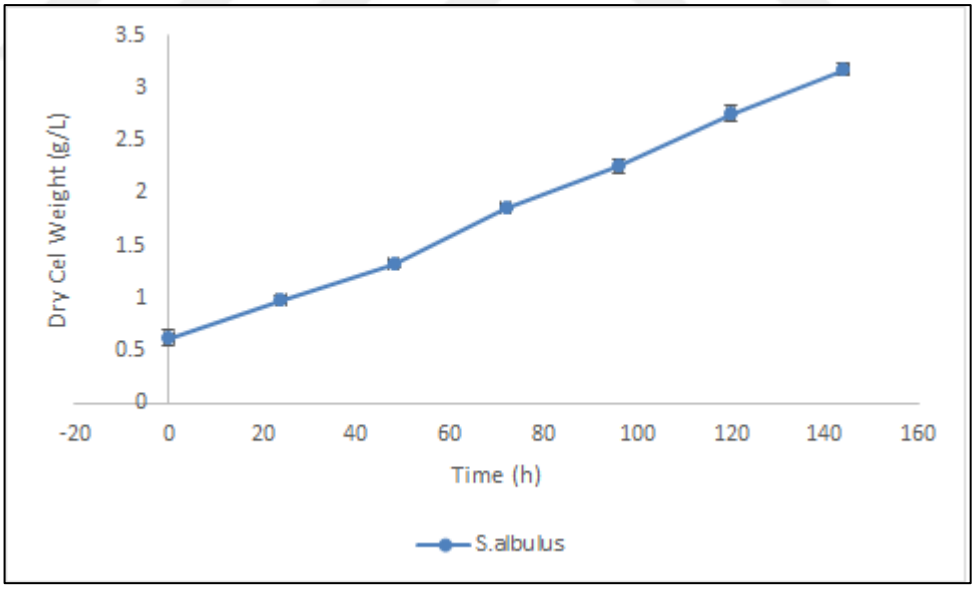

Figure 3.2. Dry cell weight measurement of *S.albulus* for ε-PL production.

As shown in Figure 3.3 experiment started with 6.8 pH. At the 144th hour pH was around 3.5. Ongoing decline is associated with increased biomass.

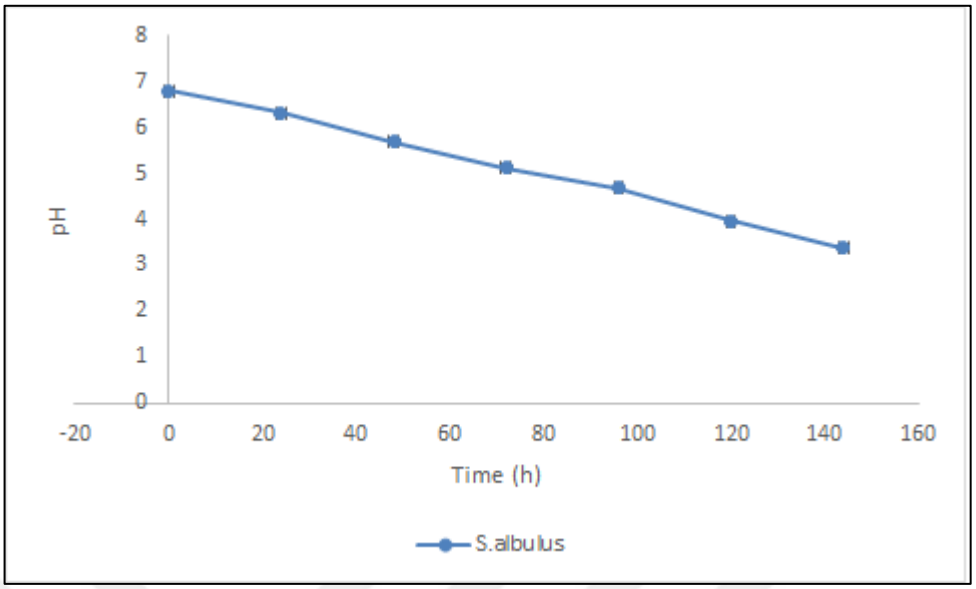

Figure 3.3. pH measurement of *S.albulus* for ε-PL production.

Ammonia concentration was shown in Figure 3.4. Experiment started with 0.34g/L. At 144th hour ammonia concentration was measured as 0.06g/L.

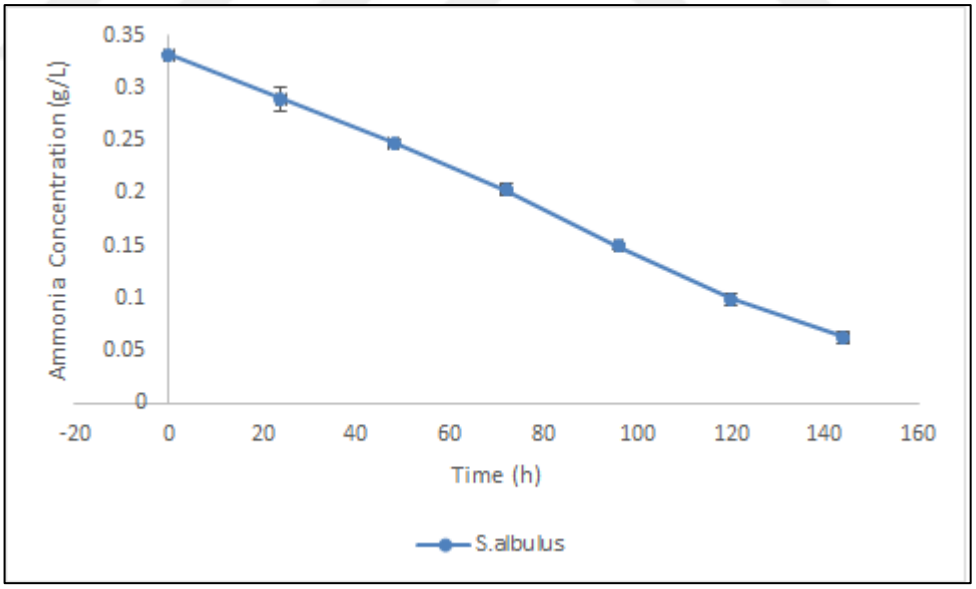

Figure 3.4. Ammonia concentration of *S.albulus* for ε-PL production.

At Figure 3.5 Glucose concentration was shown. Consumed glucose concentration is near 25g/L.

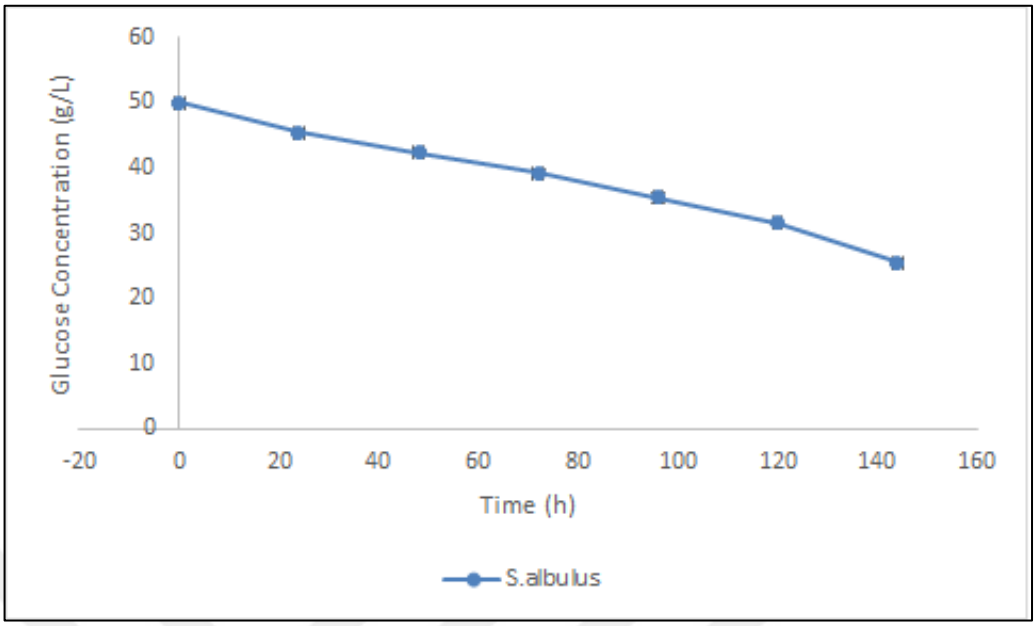

Figure 3.5. Glucose concentration of *S.albulus* for ε-PL production.

Production of ε-PL was given in Figure 3.6. Production of ε-PL was measured as 1.06g/L at 120th hour. At the 0th hour there was 0.1g/L ε-PL. At the 144th hour production decreased to 1.01g/L. Significant increase occurred between 24 and 36 hours. After 120th hour decrease was observed.

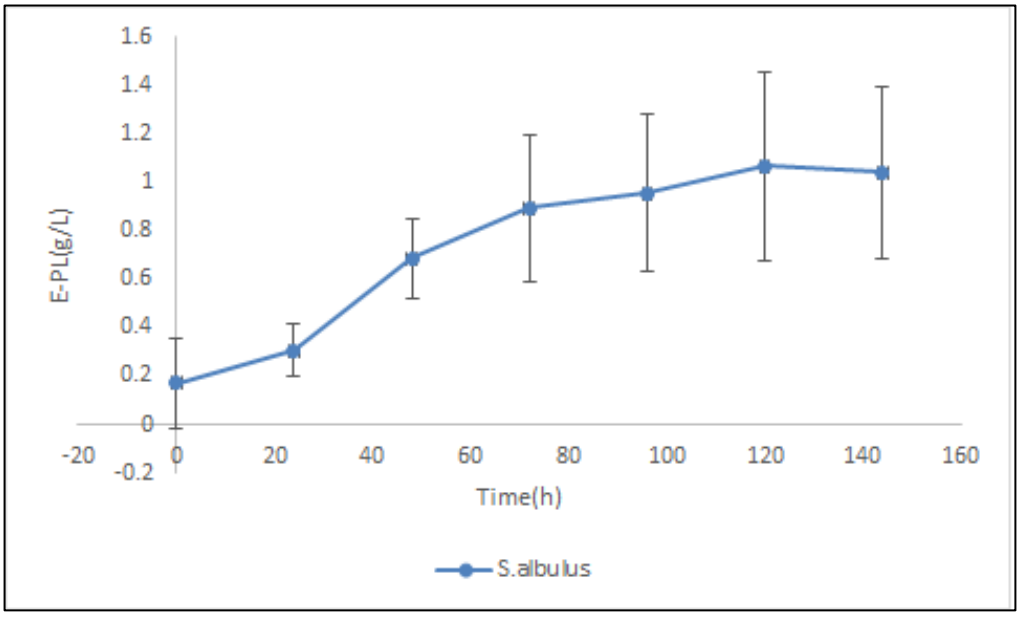

Figure 3.6. ε-PL production measurement for *S.albulus* in agitated culture experiment.

All results affecting production factors are given in Figure 3.7. The results are interrelated. E-PL production increased while ammonia concentration decreased. Cell dry weight and optical density with pH drop are inversely proportional. This result confirms the increase in biomass. The amount of sugar used and the increase in production are proportional as well.

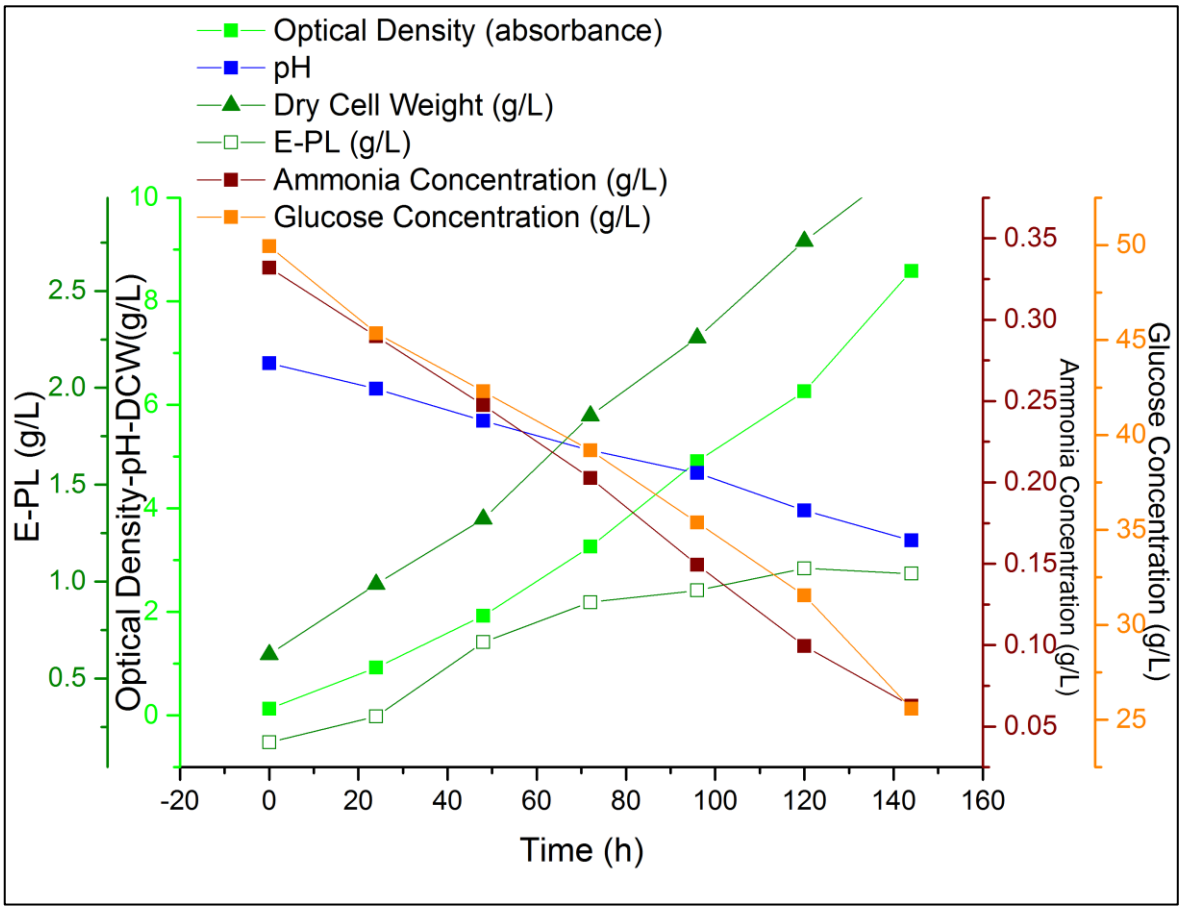

Figure 3.7. Results of agitated cultured ε-PL production.

3.1.2. Static Culture Production For ε-Poly-L-Lysine

ε-PL production was carried out in static culture with 144 hour measurements in this part of the experiment. Optical density, pH measurement and dry cell weight measurement were performed. Results are available in Figure 3.8., Figure 3.9., Figure 3.10. and Figure 3.11..

The first static culture experiment can be seen in Figure 3.8. Three different S/V ratios were used. 0.2, 0.7 and 1.2 S/V ratios were selected based on statistical data. Cell growth showed an uniform appearance. The cell line measurements were finalized with 0.4 for 0.2 S/V ratio,

0.6 for the cell line with 0.7 S/V ratio and 0.7 for the cell line with 1.2 S/V ratio was. The measurement was made with UV-visible Spectroscopy at 600 nm. At the end of 144 hours, the results were accurate.

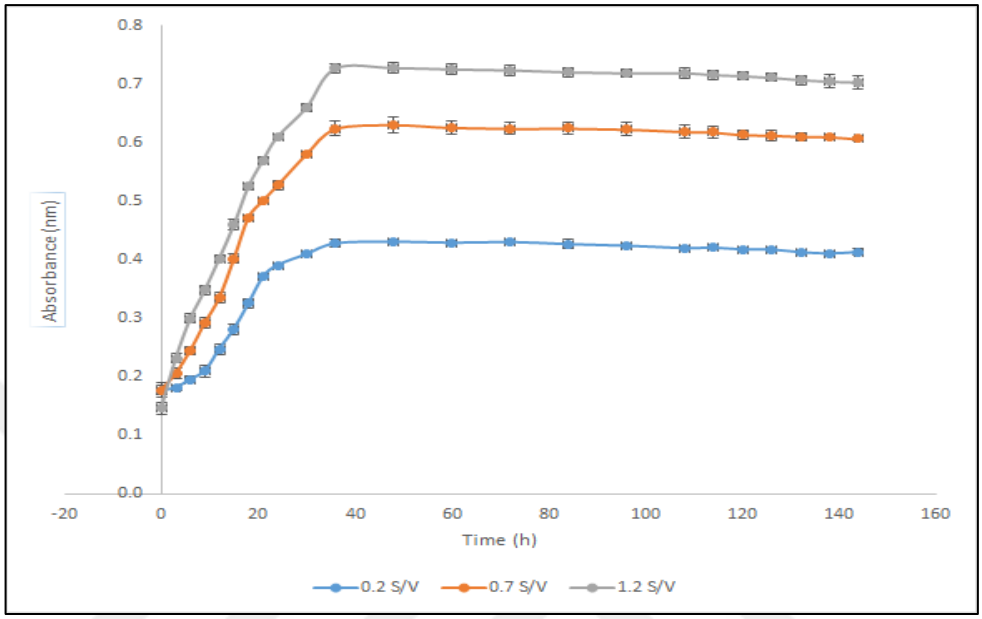

Figure 3.8. Optical density measurements for *Streptomyces albulus* in static culture experiment using three groups with different S/V ratio.

Beginning of production and microorganism growth in relation to the changing pH graph is seen at Figure 3.9. At the end of production, the amount of pH, which is about 6, decreased to 5.6, 5.1 and 4.9 respectively.

Dry cell weight, cell growth and ε-PL production results were parallel and confirms the obtained results. The cell dry weight was performed for 2 g/L to 6 g/L for the 1.2 S/V cell line at Figure 3.10. *Streptomyces albulus* was used in this experiment.

ε-PL production by static culture was measured by HPLC. Three different S/V ratio cell lines were used in this experiment. These ratios are 0.2, 0.7 and 1.2. In this part, ε-PL production, optical density measurement and sugar determination were performed. The microorganism used was *Streptomyces albulus*.

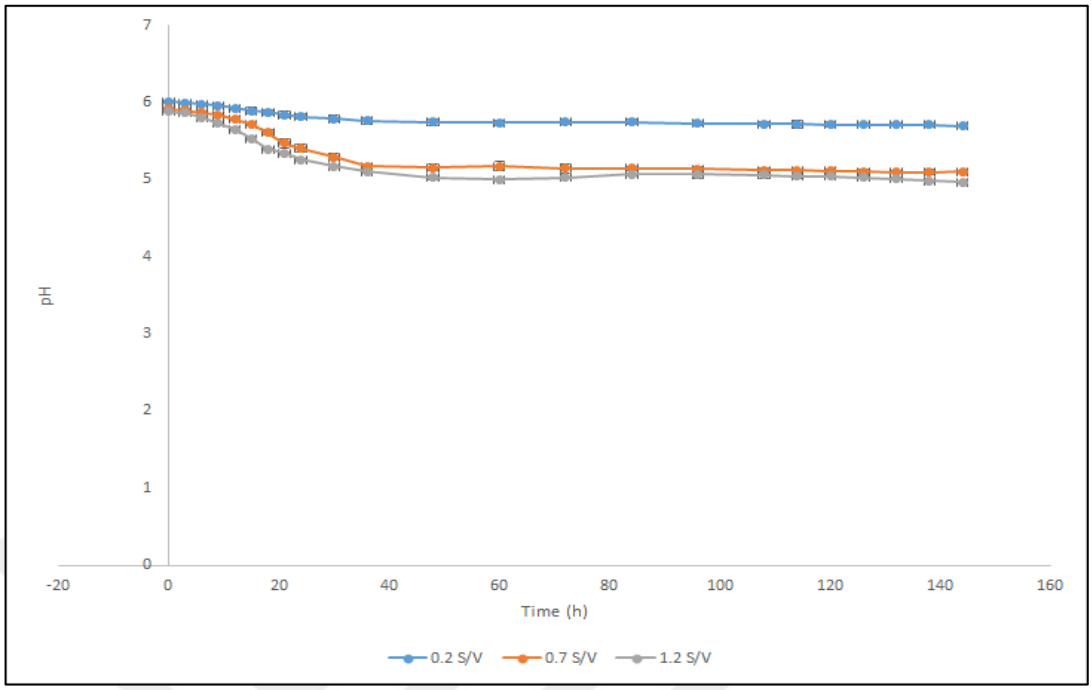

Figure 3.9. pH measurements of *Streptomyces albulus* in static culture experiment using three groups with different S/V ratio.

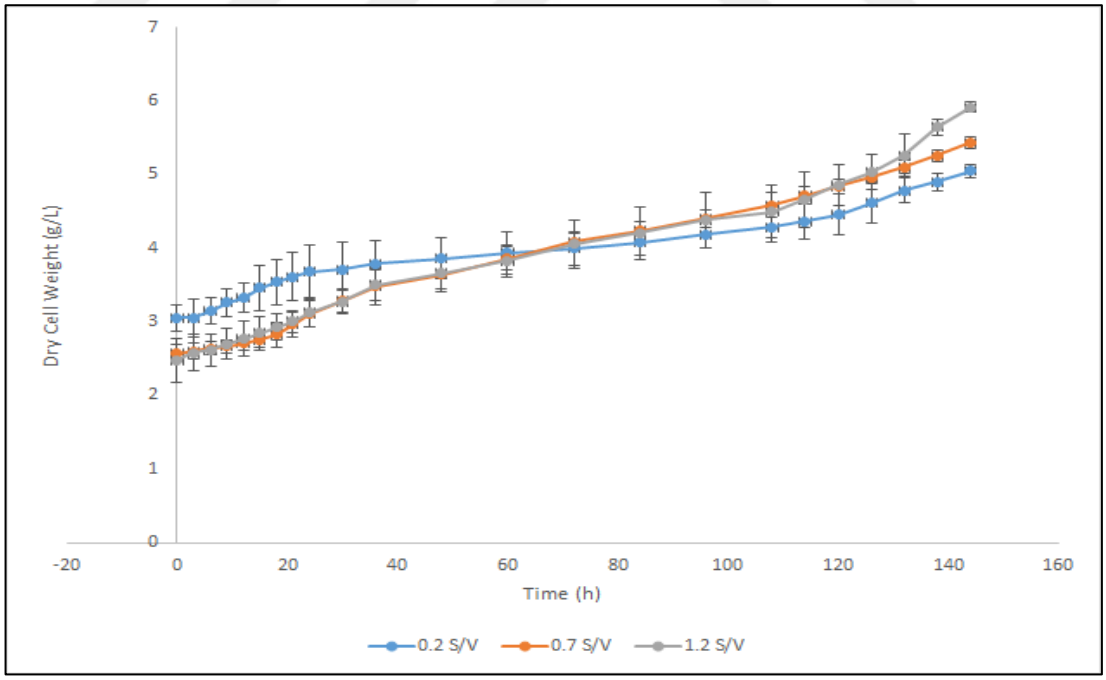

Figure 3.10. Dry cell weight measurements of *Streptomyces albulus* in static culture experiment using three groups with different S/V ratio.

As can be seen in Figure 3.11, ε-PL production in static culture yielded similar results at 0.2 and 0.7 S/V ratios. When the S/V ratio increased to 1.2, the highest amount of ε-PL was observed. When compared statistically, error rate of 1.2 S/V was higher than parallel groups. HPLC was used in the final analysis of this experiment.

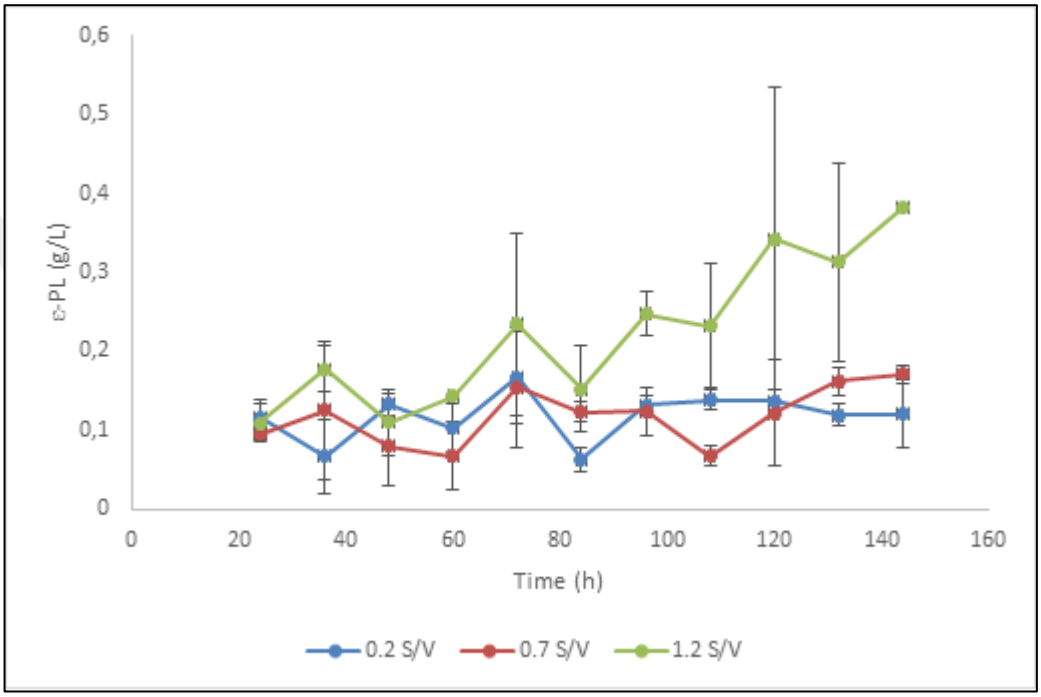

Figure 3.11. Measurement of ε-Poly-L-Lysine with static culture production.

As shown in Figure 3.12, optical density measurements at 600nm were shown. The results showed uniform growth for all cell lines and showed the highest cell growth for 1.2 S/V. Following this, 0.7 S/V ratio cell line and 0.2 S/V ratio line showed growth.

Figure 3.13 shows the results of the determination of sugar. The production has changed in the right proportion. The results are statistically acceptable. Compared to the production, 0.2 S/V cell line showed the least variation in sugar determination.

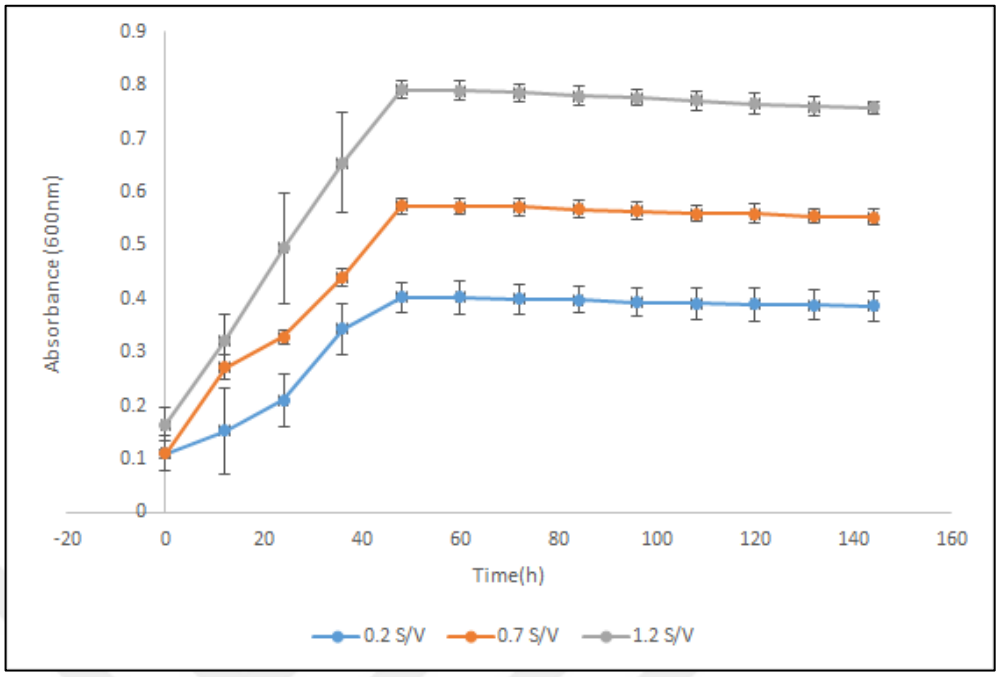

Figure 3.12. Optical density measurement of ε-Poly-L-Lysine with static culture production.

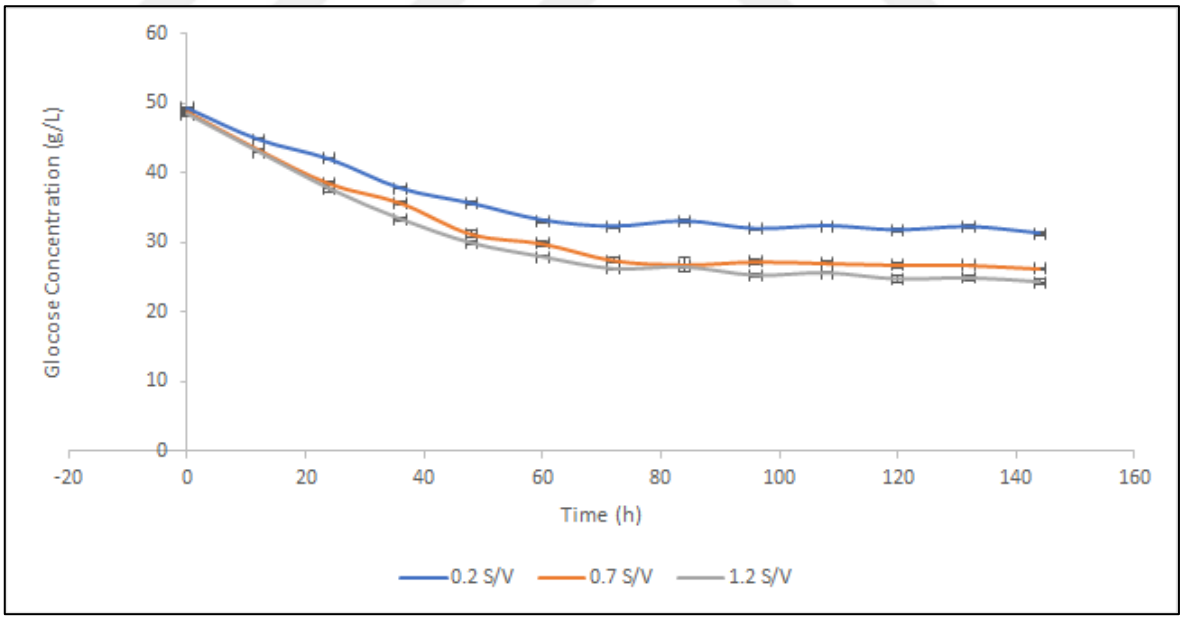

Figure 3.13. Glucose concentration of ε-Poly-L-Lysine with static culture production.

Ammonium concentration of ε-Poly-L-Lysine with static culture production was given in figure 3.14, Ammonium concentrations which measured at 425nm are shown. The largest change was observed in the group with 1.2 S/V ratio.

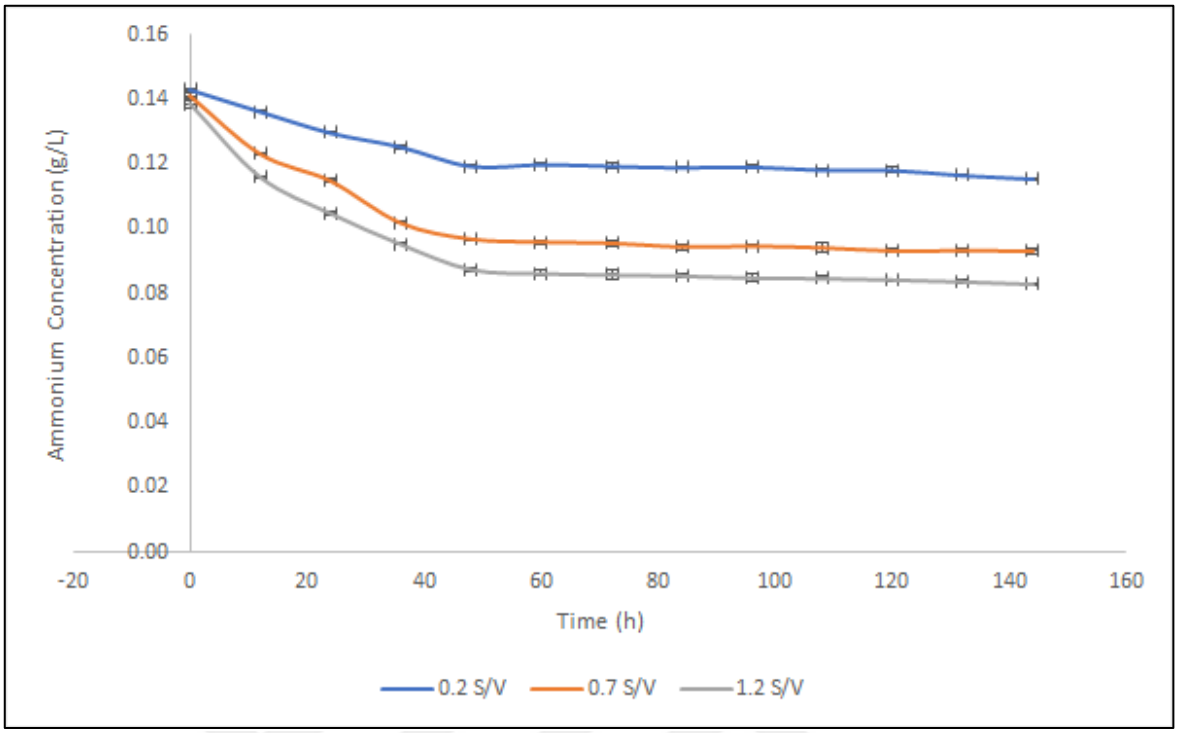

Figure 3.14. Ammonium concentration of ε-Poly-L-Lysine with static culture production.

3.2. PRODUCTION OF ε-PL WITH SEMI-CONTINUOUS OPERATION MODE INCLUDING STRATEGIES

In this part, the production of ε-PL with semi-continuous operation mode, biomass, sugar, and nitrogen contents results was shown.

Figure 3.15 shows the results for 3 different strategies. For the 1st strategy at 144th hour OD was measured as 3.2. For the 2nd strategy at 144th hour OD was measured as 0.86 and for the 3rd strategy at 144th hour OD was measured as 0.51. This result was obtained for 3 parallels at every strategy.

At Figure 3.16 Dry cell weight measurement can be seen. For the 1st strategy at 144th hour CDW was measured as 3.18g/L. For the 2nd strategy at 144th hour CDW was measured as 1.9 g/L. For the 3rd strategy at 144th hour CDW was measured as 1.6g/L.

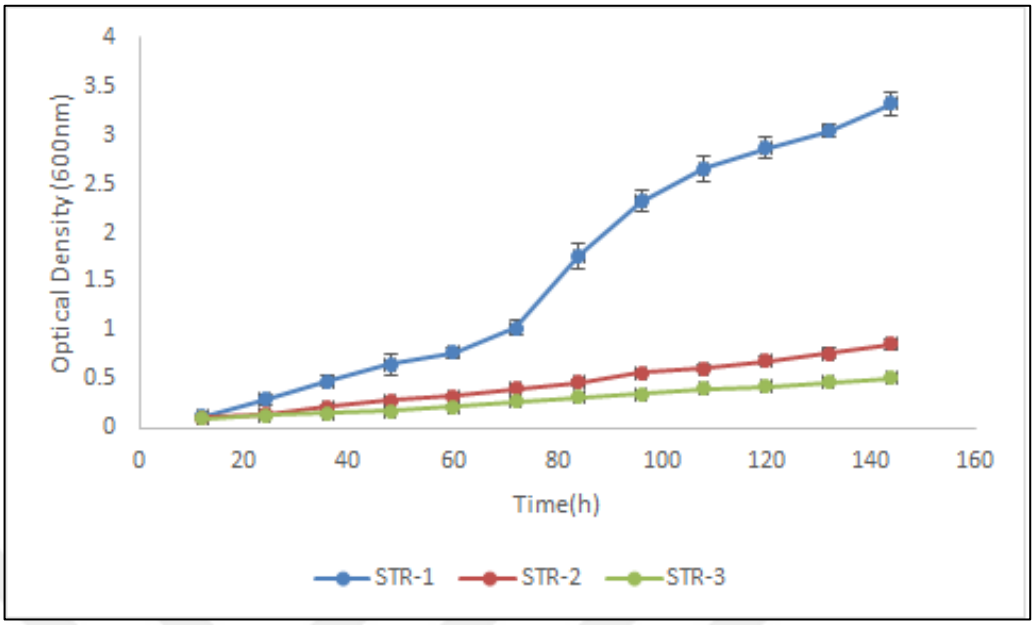

Figure 3.15. Optical density measurements for semi-continuous production of ε-PL.

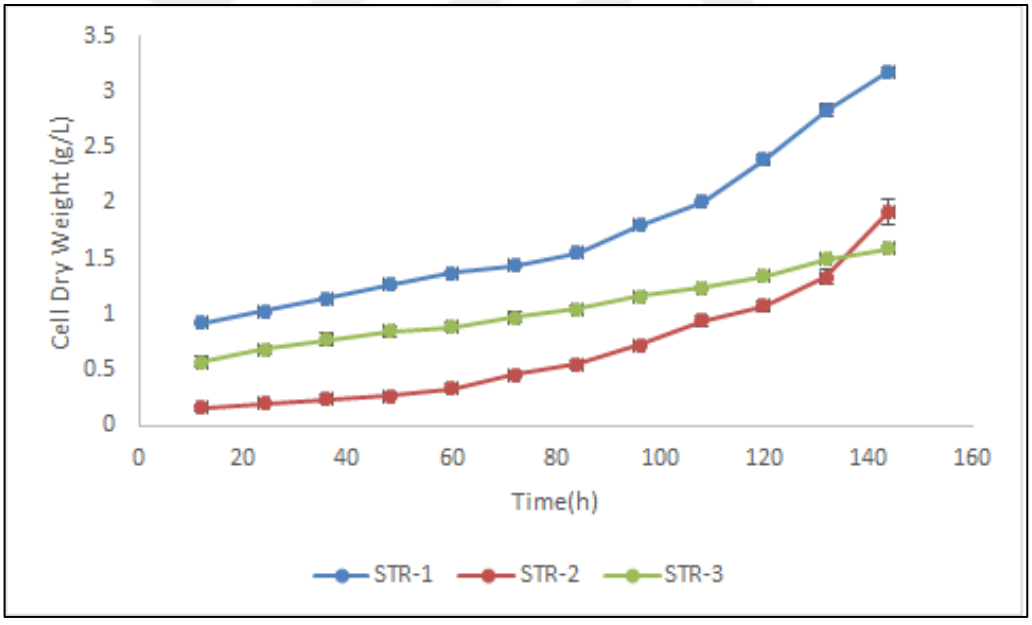

Figure 3.16. Dry cell weight measurements for semi-continuous production of ε-PL.

At Figure 3.17 pH measurements for experiment are given. At 1st strategy experiment started with 6.8 pH. At the 144th hour the pH was 5.82. For the 2nd strategy experiment stated with pH 6.8 and continued with pH 3. At the 144th hour the pH was measured as 2.92. For the 3rd strategy experiment started with pH 6.8 and continued with pH 3. At the 144th hour the pH was measured as 2.8.

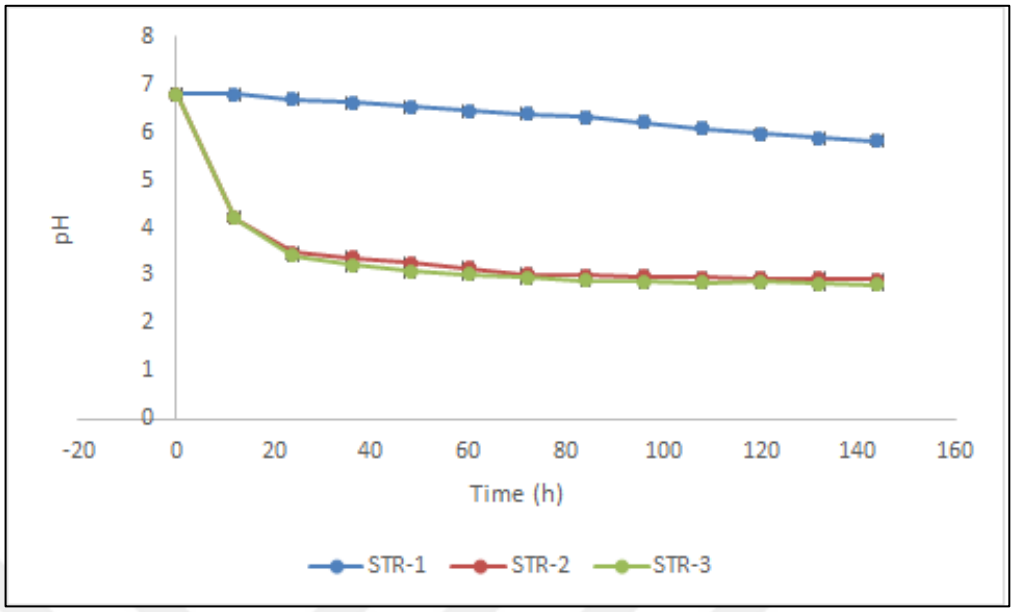

Figure 3.17. pH measurements for semi-continuous production of ε-PL.

At Figure 3.18, ammonia concentrations of 3 strategies are given. For the 1st strategy, ammonia concentration was increased slightly. At 144th hour ammonia concentration was measured as 0.22g/L. For the 2nd and 3rd strategies ammonia concentration was slightly decreased. It was measured as 0.09g/L for 2nd strategy and 0.07g/L for 3rd strategy at 144th hour.

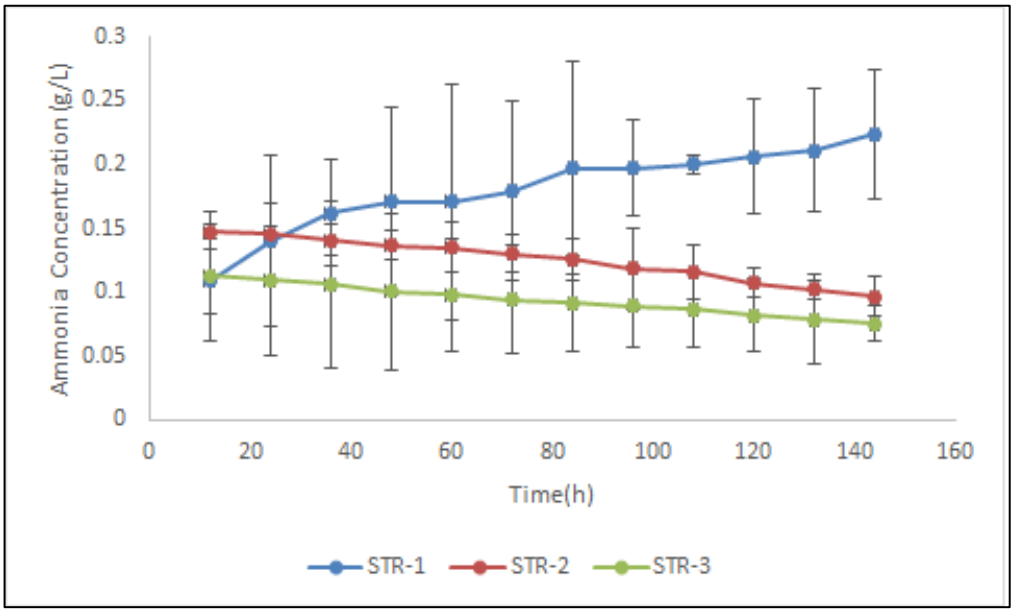

Figure 3.18. Ammonia concentration for semi-continuous production of ε-PL.

In Figure 3.19, glucose concentrations for 3 different strategies are given. For the 2nd strategy, since the experiment continued with sugar free M3G media glucose was consumed. For the 1st and 3rd strategies, experiments was continued with normal media, so at 144th hour glucose concentrations was measured as 39.25g/L for 1st strategy, 40.26g/L for 3rd strategy.

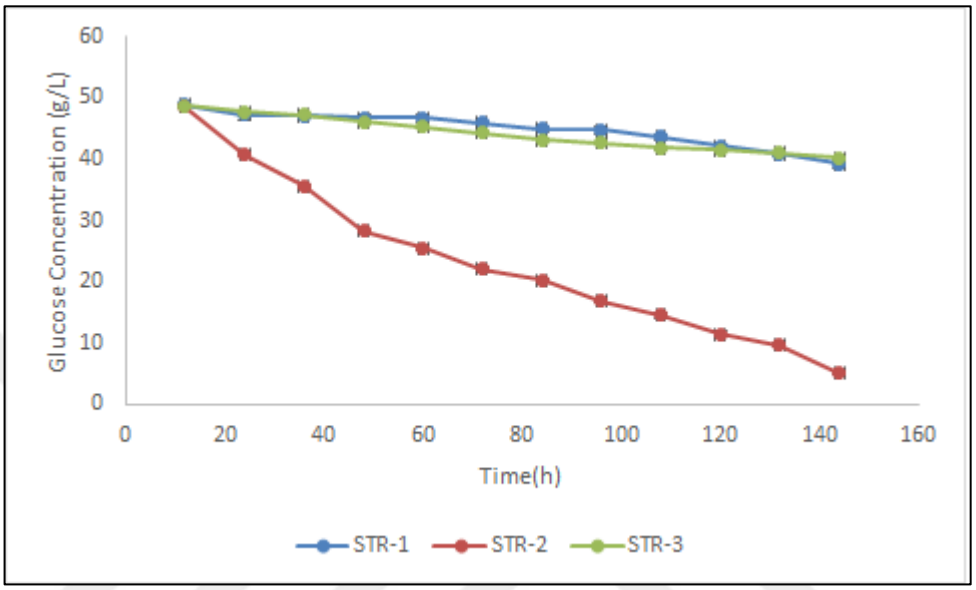

Figure 3.19. Glucose concentration for semi-continuous production of ε-PL.

In Figure 3.20, ε-PL measurement for semi-continuous production was shown. At 12th hour production amounts was similar to each. At 144th hour ε-PL production amount for 1st strategy was measured as 0.27g/L. For 2nd strategy ε-PL production amount was measured as 0.50g/L and for the 3rd strategy the amount for ε-PL production was 0.43g/L.

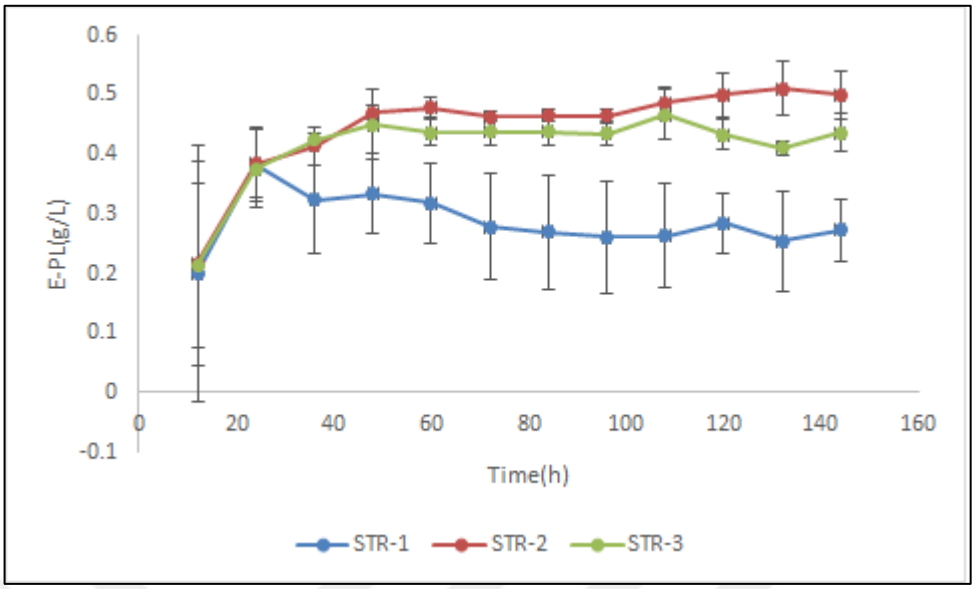

Figure 3.20. ε-PL measurement for semi-continuous production.

The production of ε-PL is 0.2 g/L at the start time of the experiment. At the end of the 24th hour, the production amount increased to 0.38g/L. Following a falling graph, the production amount at the end of 144 hours was 0.27g/L. The pH drop is directly proportional to biomass growth. Glucose and ammonia concentrations added with new medium every 12 hours were too high in this strategy(Figure 3.21).

All results affecting production factors are given in Figure 3.21. The ε-PL production amount in this strategy has reached its maximum after 132 hours. The maximum value is 0.51g/L. In 144th hour it was 0.49 g/L with a slight decrease. As it was a strategy made with sugar-free medium, glucose concentration was consumed. Optical density and dry cell weight are directly proportional. ε-PL production increased as the ammonia concentration in the medium was consumed. The pH of the environment is 2.92.

The ε-PL increased from 0.21 g/L at 12th hour to 0.37 g/L at 24th hour. Maximum production reached 0.46g/L at 108th hour. In this strategy, the pH was kept under control. Since glucose was fed every 12 hours, the amount of glucose in the medium was excessive. Cell dry weight and optical density are directly proportional. Ammonia concentration was consumed(Figure 3.23).Purity results of ε-PL production were given in Table 4, results are calculated from HPLC peaks.

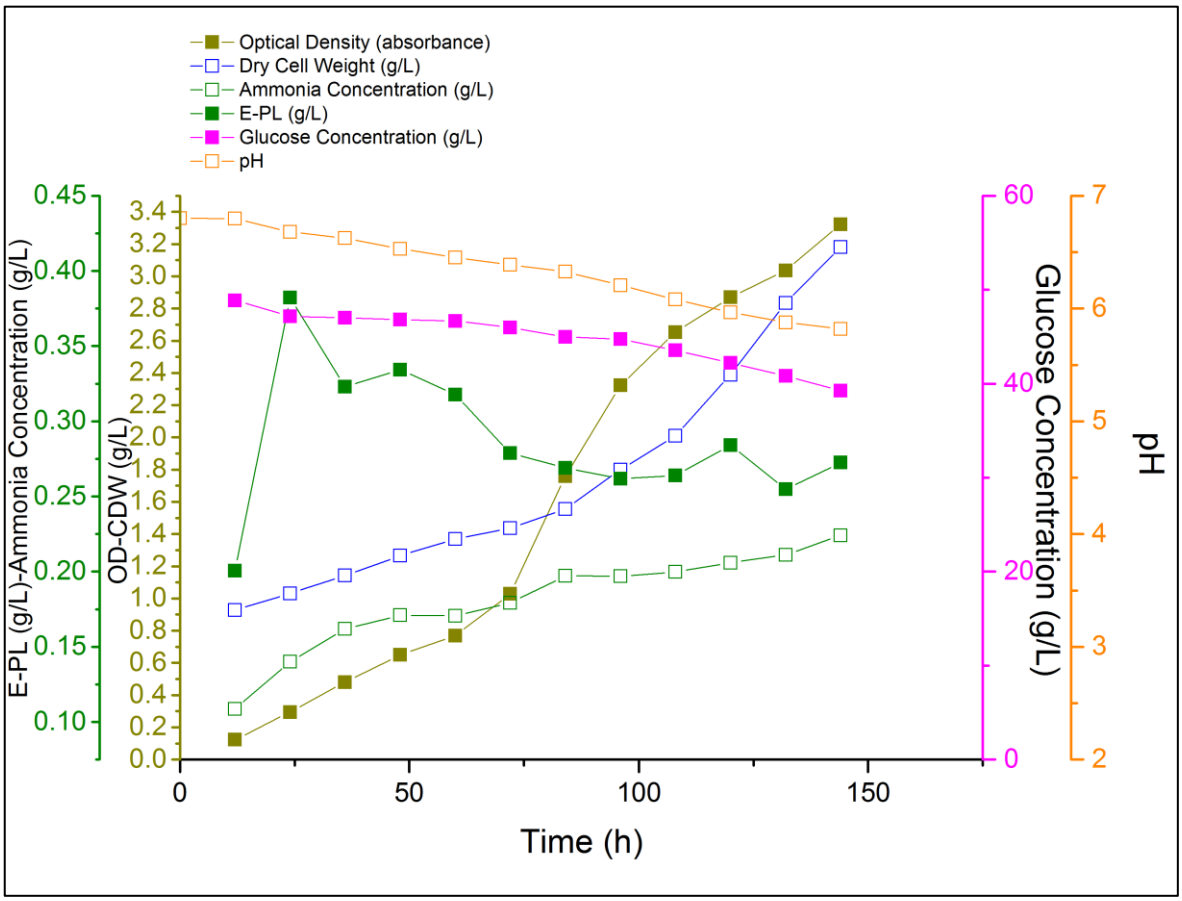

Figure 3.21. Results of 1st strategy in semi-continuous production of ε-PL.

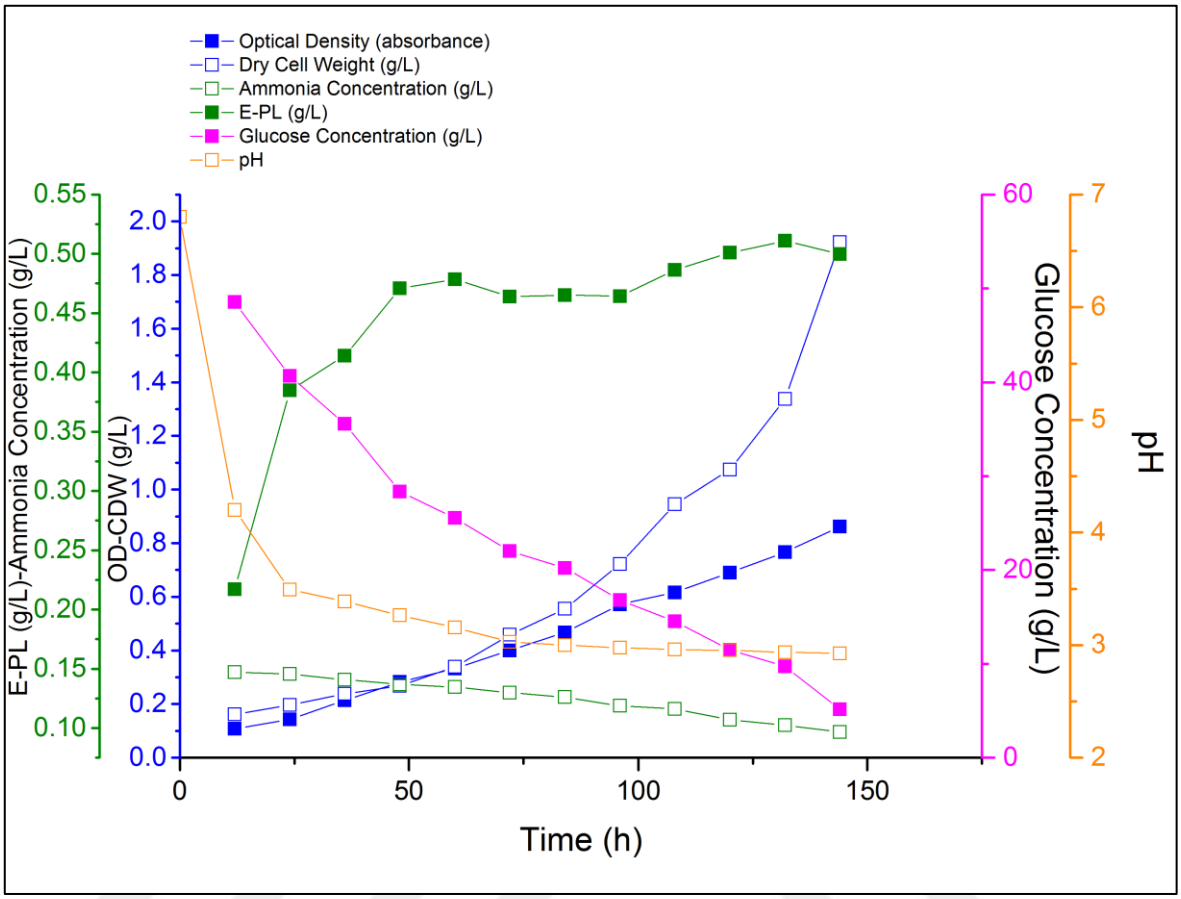

Figure 3.22. Results of 2nd strategy in semi-continuous production of ε-PL.

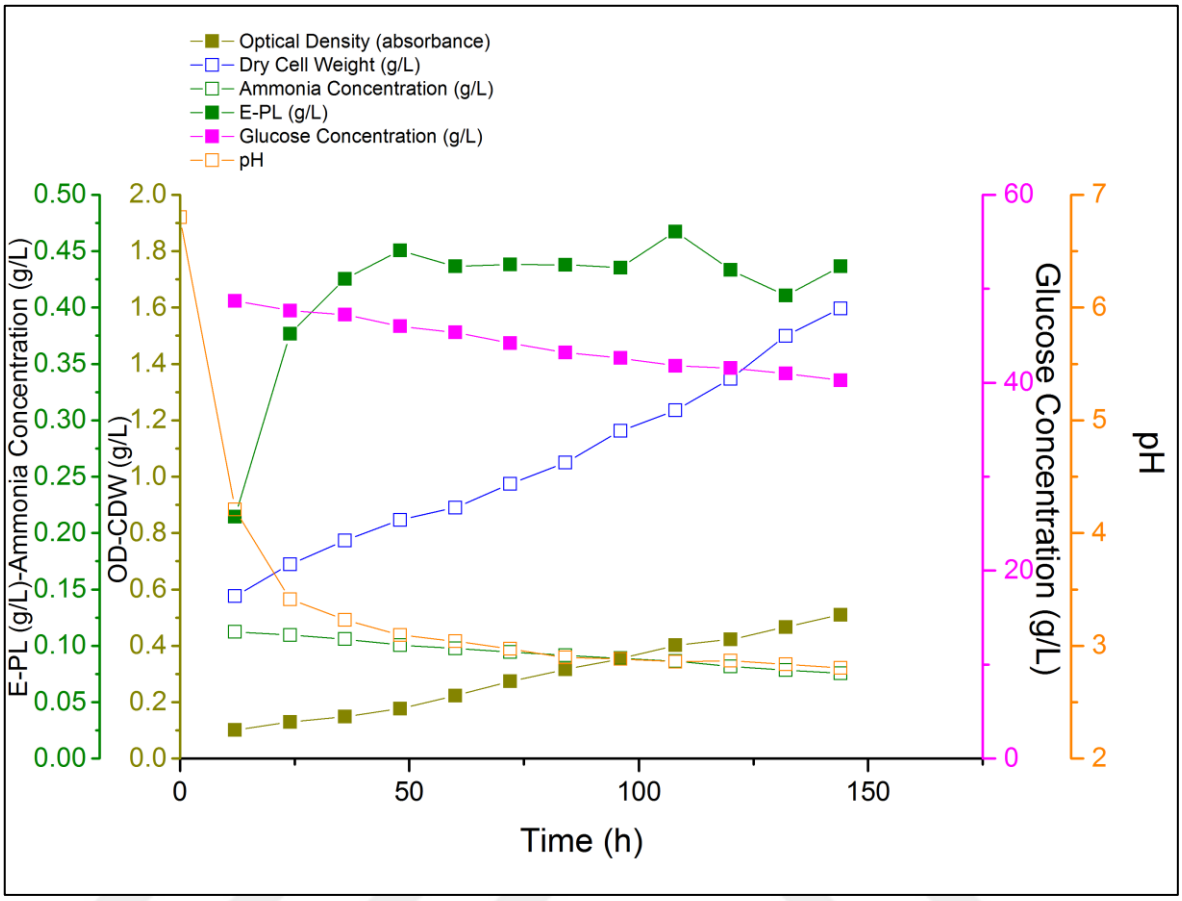

Figure 3.23. Results of 3rd strategy in semi-continuous production of ε-PL.

According to antimicrobial activity assay's results, in part A, zone measurements for *Pseudomonas aeruginosa* was measured as 1.2cm as it is and 0.9cm for diluted. In part B, zone measurements for *Escherichia coli* was measured as 1.4cm as it is and 1.2cm for diluted. In part C, zone measurements for *Yersinia enterocolitica* was measured as 1.9cm as it is and 1.5cm for diluted. In part D, zone measurements for *Listeria monocytogenes* was measured as 1.1cm as it is and 0.8cm for diluted.

Table 3.1. Purity results of ε-PL measurements.

Time(h)	Agitated Culture(g/L)	Purity (%)	STR-1 (g/L)	Purity (%)	STR-2 (g/L)	Purity (%)	STR-3 (g/L)	Purity (%)
0	0.17	46.24	-	-	-	-	-	-
12	-	-	0.20	35.26	0.22	33.48	0.21	32.19
24	0.30	47.18	0.38	36.42	0.38	34.17	0.38	32.38
36	-	-	0.32	35.73	0.41	34.42	0.43	36.24
48	0.69	45.26	0.33	34.48	0.47	33.96	0.45	33.14
60	-	-	0.32	34.51	0.48	35.11	0.44	34.25
72	0.89	44.32	0.28	33.66	0.46	34.84	0.44	35.53
84	-	-	0.27	35.21	0.47	33.18	0.44	34.72
96	0.95	41.12	0.26	34.18	0.46	32.47	0.44	32.18
108	-	-	0.26	36.24	0.49	33.46	0.47	33.26
120	1.07	42.24	0.28	37.22	0.50	35.17	0.43	31.15
132	-	-	0.25	33.26	0.51	36.49	0.41	32.48
144	1.04	41.81	0.27	35.72	0.50	34.81	0.44	34.27

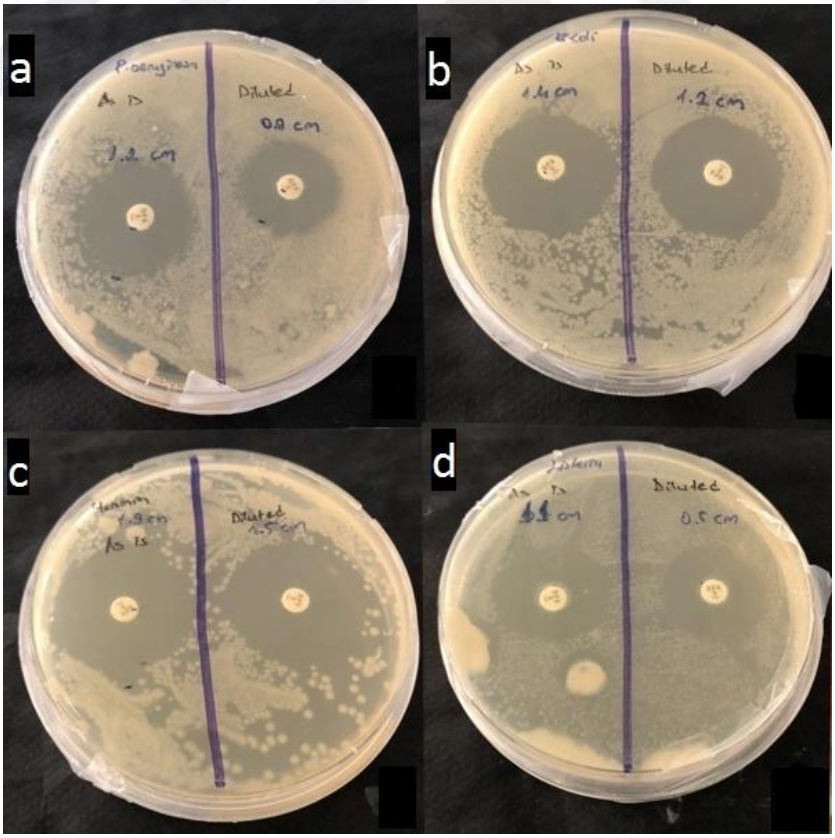

Figure 3.24. Results of antimicrobial activity assay. (a) Results for *Pseudomonas aeruginosa* was given. (b) Results for *Escherichia coli* was given. (c) Results for *Yersinia enterocolitica* was given. (d) Results for *Listeria monocytogenes* was given.

4. DISCUSSION

Literature review was performed before the studies started and 3 microorganisms to use were determined. The resulting microorganisms were; *Bacillus subtilis*, *Bacillus cereus* and *Streptomyces albulus* [13,36,119]. The common feature of 3 microorganisms is that they are ε-Poly-L-Lysine producers. Method optimization studies, which were compulsory for experiments to be carried out with microorganisms, have started. Methyl Orange was chosen as an indicator in many studies [123]. Due to its chemical structure, for ε-Poly-L-Lysine it's preferred as common [20,25]. Prior to optimization studies, a non-complex group was prepared and their measurements were performed. This group was between 3mM-0.01mM. As a result of the 3 experiments, these concentrations were reduced to 2mM, 1mM and 0.5mM. High or lower concentrations made the spectrophotometer scale difficult to measure and made the results ambiguous. After that, it was decided to make measurements with HPLC. During the study, which lasted a total of 2 years, many experiments were performed and the method was tried. The inadequacy of the results of the experiments carried out with MeO. Inconsistent MeO results and the method used are available in the Appendix.

The second stage was the choice of microorganisms. *Bacillus subtilis* and *Bacillus cereus*, which were previously determined from specific publications [20,84,88]. After that, it was decided to take the *Streptomyces albulus*, which was known as ε-Poly-L-Lysine producer. The experiments were carried out primarily with the *Bacillus* group and ε-Poly-L-Lysine was produced. The difference was that *Streptomyces albulus* proved to be the producer of ε-Poly-L-Lysine in the experiments and caused successful results in different production experiments [19,51,119]. According the production results *Streptomyces albulus* produced more ε-Poly-L-Lysine than other microorganisms. The results of the other microorganisms used in the experiments were inconsistent with the results of this study. Results are available in the Appendix.

The amount of ε-Poly-L-Lysine produced was inadequate due to literature [38,119,124]. According to Zhou production amount was measured as 1.72 g/L [125]. Factors affecting production are as follows; pH, medium, temperature, rotational speed, incubation time, surface area to volume ratio [57,84,126]. At first, different media were investigated as the medium which has been decided to be used was M3G Medium [5,127,128]. The use of these media in different experiments and different microorganisms has proved that the amount of

sugar directly affects the production. It was decided to use M3G medium after reaching this result [5].

In the experiment, production rates of ε-PL started to increase after complete method optimization was made. The first target was the pH control strategy [48,129]. For this purpose, microorganisms were observed by different experiments starting from pH measurements. This situation had a significant effect which led to different production methods. It was decided to do pH control strategy with different operational mode. This situation led to more experience about *Streptomyces albulus* microorganism. Micelle formation was observed during the experiments of *Streptomyces albulus*. In the semi-continuous operational mode experiments pH control strategy studied in 2 strategies. According the results, in 2nd strategy ε-PL production reached 0.51g/L. For 3rd strategy ε-PL production was reached to 0.46g/L. According to Kahar production amount of ε-PL with pH control strategy is 5.7g/L. Kahar did the study with Streptomyces albulus strain 410 and 5L jar fermentor[9].

As a result of researches for a different type of production, studies for static culture started. As a result of these investigations, necessary experimental setups for static culture experiment were established and trials were started [19,130]. After fixing the protocol and the experimental setup, the results were signaled by a new mode of production. After successful results, the production of ε-Poly-L-Lysine in static culture was performed.

As a result of static culture experiments, a number of changes were made to increase production. Factors affecting production in static culture are as follows; pH, Media, Temperature and S/V ratio. After examining the effect of pH, media and temperature on production, it was decided to focus on S/V ratio. Experiments were performed repeatedly in Erlenmeyer flasks with different S/V ratio. At the end, the production was repeated with the measuring cups with 0.2, 0.7 and 1.2 S/V ratio which were useful for production. As a result, the group with the best results was found to have a 1.2 S/V ratio. The production result of the 1.2 S/V ratio group is 2.5 times higher than the 0.7 S/V ratio group and 3 times higher than the 0.2 S/V ratio group.

As it can be seen in the figures, the experiments carried out in a set affect each other. The production of ε-PL is directly proportional to cell growth and hence it is accurate to interpret the pH and depending on the experiments related to cell dry weight. Cell growth is

progressively referred to as lag phase, exponential phase, stationary phase and death phase. ε-PL production starts when the cell growth goes to the stationary phase. In pH measurement, the cell needs sugar in the environment to grow and multiply. The pH decreases as the sugar is consumed. When the cell passes into the stationary phase, the pH change stabilizes. pH plays a triggering role in cell growth. It is correct to interpret all experimental results in a connected way.

In addition to the studies, measurement results were started to be obtained by HPLC in order to increase the reliability of the results. It was understood that the results obtained by HPLC in the sets were valid. The ODS-120-T column was used for HPLC. New experimental sets were designed and measured by HPLC. The results were compared with the results of the studies and confirmed the validity. The accuracy of the test results was tested with 2 references. According to the results and references ε-PL production was measured as 1.72 in shaker and 0.42 g/L in agar [33,125].

Agitated and static culture test results compared to the results of *Streptomyces albulus* production. Cell growth is greater in the agitated culture, the pH is more variable, however, the production of ε-Poly-L-Lysine is clearer in static culture. According to these results, cell growth is important for the production of ε-Poly-L-Lysine. Static culture has proven to be an important method for the production of ε-Poly-L-Lysine in accordance with limited possibilities. S/V ratio is a production factor for static and agitated culture. Considering all the results, it was decided that the agitated culture was the most efficient for the production of ε-Poly-L-Lysine.

Antimicrobial activity assay results were compared with literature studies. The results of the study have similar results with the literature. ε-PL inhibits gram negative and gram positive bacteria. [131]

The pH control strategies applied in experiments with semi-continuous mode prove that the cell contributes to production in acidic environment. Looking at Figures 3.26 and 3.27, cell growth is proportional. When the ammonium concentrations are compared, it's obvious that the strategies with pH control was used the ammonia but it's saturated for the 1st strategy. In the sugar concentration, the 2nd strategy finished the whole carbon source in 144 hours as it was fed with sugar-free medium. It is a problem related to microorganism that the productions are lower than batch mode.

When we compare Batch and Semi-continuous modes, it is proved that batch mode gives more successful results in small scale production. When the production amounts compared, the amount of production made with semi-continuous is half of the batch mode.

5. CONCLUSION

In this study, it was aimed to compare ε-Poly-L-Lysine production with *Streptomyces albulus* under static culture and agitated culture. Beyond that a different operation mode with 3 different strategies were studied. In semi-continuous operation mode experiments glucose feeding, pH control under sugar free media and glucose feeding with pH control strategies were studied. HPLC was used to analyze the results of the production of ε-PL. Also sugar and nitrogen determination and biomass analysis were performed in this study. The techniques selected from existing production enhancing techniques have been used. As a result of the study, it was found out that the production with agitated culture was more efficient. The novel result of the study is that production with static culture can be increased with different surface ratio. ε-PL production was measured as 1.06g/L in shaker. In semi-continuous operation mode production amounts was 0.27g/L for glucose feeding strategy, 0.51g/L for pH control under sugar free media strategy and 0.44g/L for glucose feeding with pH control strategy. According to this result, production with batch mode was 3 times higher than semi-continuous mode in small scale productions. As a result of experiments conducted to increase production, it was seen that *Streptomyces albulus* had a growth rate compared to the amount of sugar. However, by changing the surface area/volume ratio, oxygen has proven to be an important factor in the growth of *Streptomyces albulus*. It will go into the history of science that it is an important production technique to be used in accordance with limited facilities. The efficiency of agitated culture is inevitable for fast and multi-production.

In future studies will continue in the field of applications.

REFERENCES

1. Hamano Y, Kito N, Kita A, Imokawa Y, Yamanaka K, Maruyama C, et al. ε-Poly-L-lysine peptide chain length regulated by the linkers connecting the transmembrane domains of ε-poly-L-lysine synthetase. *Applied and Environmental Microbiology* 2014;80(16):4993–5000.

2. Saimura M, Takehara M, Mizukami S, Kataoka K, Hirohara H. Biosynthesis of nearly monodispersed poly(ε-L-lysine) in Streptomyces species. *Biotechnology Letters* 2008;30(3):377–85.

3. Xia J, Xu Z, Xu H, Liang J, Li S, Feng X. Economical production of poly(ε-l-lysine) and poly(l-diaminopropionic acid) using cane molasses and hydrolysate of streptomyces cells by Streptomyces albulus PD-1. *Bioresource Technology* 2014;164(2):241–7.

4. Bankar SB, Singhal RS. Optimization of poly-ε-lysine production by Streptomyces noursei NRRL 5126. *Bioresource Technology* 2010;101(21):8370–5.

5. Shih IL, Shen MH. Application of response surface methodology to optimize production of poly-ε-lysine by Streptomyces albulus IFO 14147. *Enzyme and Microbial Technology* 2006;39(1):15–21.

6. Schuier M, Trentin Q, Textor M, Tosatti SGP. Biomedical interfaces: Titanium surface technology for implants and cell carriers. *Nanomedicine* 2006;1(4):449–63.

7. Kawai T, Kubota T, Hiraki J, Izumi Y. Biosynthesis of ε-poly-L-lysine in a cell-free system of Streptomyces albulus. *Biochemical and Biophysical Research Communications* 2003;311(3):635–40.

8. Hamano Y, Nicchu I, Shimizu T, Onji Y, Hiraki J, Takagi H. ε-Poly-L-lysine producer, Streptomyces albulus, has feedback-inhibition resistant aspartokinase.

Applied Microbiology and Biotechnology 2007;76(4):873–82.

9. Kahar P, Iwata T, Hiraki J, Park EY, Okabe M. Enhancement of ε-polylysine production by Streptomyces albulus strain 410 using pH control. *Journal of Bioscience and Bioengineering* 2001;91(2):190–4.

10. Chen W, Wu Q, Ouyang P, Ouyang J, Zhu H, Xu H, et al. Production of ε-poly-L-lysine by newly isolated Kitasatospora sp. PL6-3. *Biotechnology Journal* 2006;1(12):1459–63.

11. Ren XD, Chen XS, Tang L, Sun QX, Zeng X, Mao ZG. Efficient production of ε-poly-l-lysine from agro-industrial by-products by Streptomyces sp. M-Z18. *Annals of Microbiology* 2015;65(2):733–43.

12. Liao L, Mao Z, Chen X, Li S, Tang L, Zhang J. Optimization of medium for enhancement of ε-Poly-L-Lysine production by Streptomyces sp. M-Z18 with glycerol as carbon source. *Bioresource Technology* 2010;102(2):1727–32.

13. El-Sersy NA, Abdelwahab AE, Abouelkhiir SS, Abou-Zeid DM, Sabry SA. Antibacterial and anticancer activity of ε-poly-L-lysine (ε -PL) produced by a marine Bacillus subtilis sp. *Journal of Basic Microbiology* 2012;52(5):513–22.

14. Shima SS, Fukuhara Y, Sakai H. Inactivation of bacteriophages by £-poly-l-lysine produced. *Agric Biol Chem* 1982;46(7):1917–9.

15. Yoshida T, Nagasawa T. ε-Poly-l-lysine: Microbial production, biodegradation and application potential. *Applied Microbiology and Biotechnology* 2003;62(1):21–6.

16. Shima S, Sakai H. Poly-l-lysine produced by streptomyces. Part iii. chemical studies. *Agricultural and Biological Chemistry* 1981;45(11):2503–8.

17. Pandey AK, Kumar A. Improved microbial biosynthesis strategies and multifarious applications of the natural biopolymer epsilon-poly-l-lysine. *Process Biochemistry*

2014;49(3):496–505.

18. Shima S, Sakai H. Polylysine produced by Streptomyces. *Agricultural and Biological Chemistry Communication* 1977;41(9):1807–9.

19. Kahar P, Kobayashi K, Iwata T, Hiraki J, Kojima M, Okabe M. Production of ε-polylysine in an airlift bioreactor (ABR). *Journal of Bioscience and Bioengineering* 2002;93(3):274–80.

20. Jia S, Fan B, Dai Y, Wang G, Peng P, Jia Y. Fractionation and characterization of ε-Poly-L-lysine from streptomyces albulus CGMCC 1986. *Food Science and Biotechnology* 2010;19(2):361–6.

21. Zhu X, Wang P, Cui L, Yu Y, Wang Q, Fan X. Enhancement reactivity of Bombyx mori silk fibroins via genipin-mediated grafting of a tyrosine-rich polypeptide. *Journal of the Textile Institute* 2017;108(12):2115–22.

22. Shih IL, Shen MH, Van YT. Microbial synthesis of poly(ε-lysine) and its various applications. *Bioresource Technology* 2006;97(9):1148–59.

23. Hiraki J. Process for producing epsilon-poly-l-lysine with immobilized Streptomyces albulus. *Patent and Trademarkt Office* 1999(19):4–7.

24. Nishikawa M. Distribution of microbes producing antimicrobial ε-poly-l-lysine. *Applied and Environmental Microbiology* 2002;68(7):3575–81.

25. Shih I, Van Y, Shen M. Biomedical applications of chemically and microbiologically synthesized poly(Glutamic Acid) and poly(Lysine). *Mini-Reviews in Medicinal Chemistry* 2005;4(2):179–88.

26. Larson JL, Hershberger CL. Shuttle vectors for cloning recombinant DNA in Escherichia coli and Streptomyces griseofuscus C581. *Journal of Bacteriology* 1984;157(1):314–7.

27. Solenberg PJ, Baltz RH. Transposition of Tn5096 and other IS493 derivatives in Streptomyces griseofuscus. *Journal of Bacteriology* 1991;173(3):1096–104.

28. Zhang J, Marcin C, Shifflet MA, Salmon P, Brix T, Greasham R, et al. Development of a defined medium fermentation process for physostigmine production by Streptomyces griseofuscus. *Applied Microbiology and Biotechnology* 1996;44(5):568–75.

29. Sevcik J, Dauter Z, Lamzin VS, Wilson KS. Ribonuclease from Streptomyces aureofaciens at atomic resolution. *Acta Crystallographica Section D: Biological Crystallography* 1996;52(2):327–44.

30. Teruel MLA, Gontier E, Bienaime C, Saucedo JEN, Barbotin JN. Response surface analysis of chlortetracycline and tetracycline production with K-carrageenan immobilized Streptomyces aureofaciens. *Enzyme and Microbial Technology* 1997;21(5):314–20.

31. Robertson C. R. Dual aerobic hollow-fiber bioreactor for cuitivation of Streptomyces aureofaciens. *Biotechnology* 1985;XXVII(VI):1012–20.

32. Kobayashi K, Nishikawa M. Promotion of ε-poly-L-lysine production by iron in Kitasatospora kifunense. *World Journal of Microbiology and Biotechnology* 2007;23(7):1033–6.

33. Zhang Y, Feng X, Xu H, Yao Z, Ouyang P. ε-poly-l-lysine production by immobilized cells of Kitasatospora sp. MY 5-36 in repeated fed-batch cultures. *Bioresource Technology* 2010;101(14):5523–7.

34. Dong N, Mao Z, Li F, Zhao F, Li S, Zhang J-H, et al. Culture medium containing glucose and glycerol as a mixed carbon source improves ε-poly-l-lysine production by Streptomyces sp. M-Z18. *Bioprocess and Biosystems Engineering* 2011;35(3):469–75.

35. Liu YJ, Chen XS, Zhao JJ, Pan L, Mao ZG. Development of microtiter plate culture method for rapid screening of ε-poly-l-lysine-producing strains. *Applied Biochemistry and Biotechnology* 2017;183(4):1209–23.

36. Pan L, Chen XS, Liu MM, Liu YJ, Mao ZG. Efficient production of ε-poly-l-lysine from glucose by two-stage fermentation using pH shock strategy. *Process Biochemistry* 2017;63(4):8–15.

37. Liu S, Wu Q, Zhang J, Mo S. Production of ε-poly-l-lysine by Streptomyces sp. using resin-based, in situ product removal. *Biotechnology Letters* 2011;33(8):1581–5.

38. Otzen DE, Stenvang M, Meyer RL, Vad BS, Mygind T, Hyldgaard M. The antimicrobial mechanism of action of epsilon-poly-l-lysine. *Applied and Environmental Microbiology* 2014;80(24):7758–70.

39. Katare PB, Swami R, Katiyar SS, Popat A, Banerjee SK, Jain S, et al. ε-Poly-l-lysine/plasmid DNA nanoplexes for efficient gene delivery in vivo. *International Journal of Pharmaceutics* 2018;542(1–2):142–52.

40. Chheda AH, Vernekar MR. Improved production of natural food preservative ε-poly-l-lysine using a novel producer Bacillus cereus. *Food Bioscience* 2014;7(3):56–63.

41. Choi JS, Joo DK, Kim CH, Kim K, Park JS. Synthesis of a barbell-like triblock copolymer, poly(L-lysine) dendrimer-block-poly(ethylene glycol)-block-poly(L-lysine) dendrimer, and its self-assembly with plasmid DNA. *Journal of the American Chemical Society* 2000;122(3):474–80.

42. Maeda S, Kunimoto KK, Sasaki C, Kuwae A, Hanai K. Characterization of microbial poly (ε-L-lysine) by FT-IR, Raman and solid state13C NMR spectroscopies. *Journal of Molecular Structure* 2003;655(1):149–55.

43. Wagner E, Cotten M, Mechtler K, Kirlappos H, Birnstiel ML. DNA-binding transferrin conjugates as functional gene-delivery agents: synthesis by linkage of

polylysine or ethidium homodimer to the transferrin carbohydrate moiety. *Bioconjugate Chemistry* 1991;2(4):226–31.

44. Gombotz W. Protein release from alginate matrices. *Advanced Drug Delivery Reviews* 2002;31(3):267–85.

45. Ushimaru K, Hamano Y, Katano H. Antimicrobial Activity of ε-Poly-l-lysine after forming a water-insoluble complex with an anionic surfactant. *Biomacromolecules* 2017;18(4):1387–92.

46. Nojehdehian H, Moztarzadeh F, Baharvand H, Nazarian H, Tahriri M. Preparation and surface characterization of poly-l-lysine-coated PLGA microsphere scaffolds containing retinoic acid for nerve tissue engineering: In vitro study. *Colloids and Surfaces B: Biointerfaces* 2009;73(1):23–9.

47. Hudecz F, Gaál D, Kurucz I, Lányi Á, Kovács AL, Mezö G, et al. Carrier design: cytotoxicity and immunogenicity of synthetic branched polypeptides with poly(L-lysine) backbone. *Journal of Controlled Release* 1992;19(1–3):231–43.

48. Cheng C, Zhang X, Meng Y, Zhang Z, Chen J, Zhang Q. Multiresponsive and biocompatible self-healing hydrogel: Its facile synthesis in water, characterization and properties. *Soft Matter* 2017;13(16):3003–12.

49. Hyon SH, Nakajima N, Sugai H, Matsumura K. Low cytotoxic tissue adhesive based on oxidized dextran and epsilon-poly-l-lysine. *Journal of Biomedical Materials Research - Part A* 2014;102(8):2511–20.

50. Wang R, Li Q, Chi B, Wang X, Xu Z, Xu Z, et al. Enzyme-induced dual-network ϵ-poly-l-lysine-based hydrogels with robust self-healing and antibacterial performance. *Chemical Communications* 2017;53(35):4803–6.

51. Nicchu I, Takagi H, Hamano Y, Nakamori S, Hoshino Y, Kawai T. Development of gene delivery systems for the ε-poly-l-lysine producer, Streptomyces albulus. *Journal*

of Bioscience and Bioengineering 2005;99(6):636–41.

52. Ding J, Liang H, Fu S, Liu R, Deng Z, Liu T. Modification of ε-poly-l-lysine in vivo to reduce self-toxicity and enhance antibiotic overproduction. *AIChE Journal* 2018;64(12):4187–92.

53. Corsetti A, Settanni L. Lactobacilli in sourdough fermentation. *Food Research International* 2007;40(5):539–58.

54. Cooper DG, Goldenberg BG. Surface-active agents from two Bacillus species. *Applied and Environmental Microbiology* 1987;53(2):224 LP – 229.

55. Zhang X, Meng Y, Cheng C, Zhang Q, Chen L. Development of a dual drug-loaded hydrogel delivery system for enhanced cancer therapy: in situ formation, degradation and synergistic antitumor efficiency. *Journal of Materials Chemistry B* 2017;5(43):8487–97.

56. Acevedo-Fani A, Silva HD, Soliva-Fortuny R, Martín-Belloso O, Vicente AA. Formation, stability and antioxidant activity of food-grade multilayer emulsions containing resveratrol. *Food Hydrocolloids* 2017;71(2):207–15.

57. Some S, Ho SM, Dua P, Hwang E, Shin YH, Yoo H, et al. Dual functions of highly potent graphene derivative-poly-l-lysine composites to inhibit bacteria and support human cells. *ACS Nano* 2012;6(8):7151–61.

58. Shima S, Matsuoka H, Iwamoto T, Sakai H. Antimicrobial action of epsilon-poly-l-lysine. *The Journal of Antibiotics* 2012;37(11):1449–55.

59. Zou YJ, He SS, Du JZ. ε-poly(l-lysine)-based hydrogels with fast-acting and prolonged antibacterial activities. *Chinese Journal of Polymer Science (English Edition)* 2018;36(11):1239–50.

60. Callegan MC, Kane ST, Cochran DC, Gilmore MS. Molecular mechanisms of

Bacillus endophthalmitis pathogenesis . *DNA and Cell Biology* 2002;21(5–6):367–73.

61. Li S, Tang L, Chen X, Liao L, Li F, Mao Z. Isolation and characterization of a novel ε-poly-l-lysine producing strain: Streptomyces griseofuscus. *Journal of Industrial Microbiology and Biotechnology* 2011;38(4):557–63.

62. Bontemps C, Toussaint M, Revol PV, Hotel L, Jeanbille M, Uroz S, et al. Taxonomic and functional diversity of Streptomyces in a forest soil. *FEMS Microbiology Letters* 2013;342(2):157–67.

63. Skoog K, Hahn-Hägerdal B. Xylose fermentation. *Enzyme and Microbial Technology* 1988;10(2):66–80.

64. Molle V, Fujita M, Jensen ST, Eichenberger P, González-Pastor JE, Liu JS, et al. The Spo0A regulon of Bacillus subtilis. *Molecular Microbiology* 2003;50(5):1683–701.

65. Granum P. Bacillus cereus and its food poisoning toxins. *FEMS Microbiology Letters* 2002;157(2):223–8.

66. Katz E, Thompson CJ, Hopwood DA. Cloning and expression of the tyrosinase gene from Streptomyces antibioticus in Streptomyces lividans. *Microbiology* 2009;129(9):2703–14.

67. Haldenwang WG. The sigma factors of Bacillus subtilis. *Microbiological Reviews* 1995;59(1):1–30.

68. Săsărman A, Griţaenco V, Soru E. Transformation of B.subtilis 168 under the influence of desoxyribonucleic acid extracted from various Bacillus strains. *Archives Roumaines de Pathologie Expérimentales et de Microbiologie* 1965;24(1):249–54.

69. Priest FG. Extracellular enzyme synthesis in the genus Bacillus. *Bacteriological Reviews* 1977;41(3):711–53.

70. Chang S, Cohen SN. High frequency transformation of Bacillus subtilis protoplasts by plasmid DNA. *MGG Molecular & General Genetics* 1979;168(1):111–5.

71. Beveridge TJ, Murray RGE. Sites of metal deposition in the cell wall of Bacillus subtilis. *Journal of Bacteriology* 1980;141(2):876–87.

72. Dignam J, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Research* 1983;1(5):1475–89.

73. Green BD, Battisti L, Koehler TM, Thorne CB, Ivins BE. Demonstration of a capsule plasmid in Bacillus anthracis. *Infection and Immunity* 1985;49(2):291–7.

74. Aronson AI, Beckman W, Dunn P. Bacillus thuringiensis and related insect pathogens relation of spore coat synthesis and germination to parasporal body formations. *Microbiological Reviews* 1986;50(1):1–24.

75. Höfte H, Whiteley HR. Insecticidal crystal proteins of Bacillus thuringiensis. *Microbiological Reviews* 1989;53(2):242–55.

76. Martin PAW, Travers RS. Worldwide abundance and distribution of Bacillus thuringiensis isolates. *Applied and Environmental Microbiology* 1989;55(10):2437–42.

77. Arantes O, Lereclus D. Construction of cloning vectors for Bacillus thuringiensis. *Gene* 1991;108(2):115–9.

78. Turnbull PCB. Bacillus (Chapter 15). *Medical Microbiolgy* 1996.

79. Gill SS, Cowles EA, Patricia V. Bacillus Thuringiensis Endotoxins. *Annual Review in Entomology* 1992;37(1):615–36.

80. Takemoto S, Terasaki PI, Cecka JM, Cho YW GD. Identification of the uncultured

Bacillus of Whipple's disease. *Journal of Medicine* 1992;327(5):293–301.

81. McGaughey WH, Whalon ME. Managing insect resistance to Bacillus thuringiensis toxins. *Science* 1992;258(5087):1451–5.

82. Kloepper JW, Ryu C-M, Zhang S. Induced systemic resistance and promotion of plant growth by Bacillus spp. . *Phytopathology* 2007;94(11):1259–66.

83. Cutting SM. Bacillus probiotics. *Food Microbiology* 2011;28(2):214–20.

84. Schallmey M, Singh A, Ward OP. Developments in the use of *Bacillus* species for industrial production. *Canadian Journal of Microbiology* 2004;50(1):1–17.

85. Guérout-Fleury AM, Frandsen N, Stragier P. Plasmids for ectopic integration in Bacillus subtilis. *Gene* 1996;180(1–2):57–61.

86. Еременко ЕИ, Рязанова АГ, Писаренко СВ, Аксенова ЛЮ, Семенова ОВ, Котенева ЕА, et al. Сравнительный анализ методов генетического типирования Bacillus anthracis . *Генетика* 2019;55(1):40–51.

87. Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P. Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and Molecular Biology Reviews* 2003;64(3):548–72.

88. Vagner V, Dervyn E, Ehrlich D. A vector for systematic gene inactivation. *Gene* 1996;27(1):998.

89. Morales A, Eidinger D, Bruce AW. Intracavitary Bacillus calmette-guerin in the treatment of superficial bladder tumors. *Journal of Urology* 2017;197(2):S142–5.

90. Chen XS, Wang KF, Zheng GC, Gao Y, Mao ZG. Preparation, characterization and antimicrobial activity of ε-poly-l-lysine with short chain length produced from glycerol by Streptomyces albulus. *Process Biochemistry* 2018;68(March):22–9.

91. Velikova N, Mas N, Miguel-Romero L, Polo L, Stolte E, Zaccaria E, et al. Broadening the antibacterial spectrum of histidine kinase autophosphorylation inhibitors via the use of ε-poly-l-lysine capped mesoporous silica-based nanoparticles. *Nanomedicine: Nanotechnology, Biology, and Medicine* 2017;13(2):569–81.

92. Shih IL, Wang TC, Chou SZ, Lee G Der. Sequential production of two biopolymers-levan and poly-ε-lysine by microbial fermentation. *Bioresource Technology* 2011;102(4):3966–9.

93. Xu Z, Xu Z, Feng X, Xu D, Liang J, Xu H. Recent advances in the biotechnological production of microbial poly(ε-l-lysine) and understanding of its biosynthetic mechanism. *Applied Microbiology and Biotechnology* 2016;100(15):6619–30.

94. Long DL, Blake AJ, Champness NR, Schröder M. Lanthanide co-ordination frameworks of 4,4'-bipyridine-N,N'-dioxide. *Chemical Communications* 2000(15):1369–70.

95. Ptomyces S, Chater KF. Genetics of differentiation in introduction : Differentiation as a normal aspect of proteins. *Proteins* 1993:15(3):685–713.

96. Li S, Li F, Chen XS, Wang L, Xu J, Tang L, et al. Genome shuffling enhanced ε-poly-l-lysine production by improving glucose tolerance of Streptomyces graminearus. *Applied Biochemistry and Biotechnology* 2012;166(2):414–23.

97. Cole M. Clavulanic acid : Beta-lactamase-inhibiting beta-lactam from Streptomyces clavuligerus. *Antimicrob Agents Chemother* 1977;11(5):852–7.

98. Bierman M, Logan R, O'brien K, Seno ET, Rao RN, Schoner BE. Plasmid cloning vectors for the conjugal transfer of DNA from Escherichia coli to Streptomyces spp. (Recombinant DNA; phage 0C3 I; gene disruption; shuttle vectors; cosmids; P1 vector; mobilization; gene transplacement; SCP2*). *Gene* 1992;116(4):43–9.

99. Dorofeev YG, Naumenko AA, Radikajnen LM, Sergeenko SN. Optimizing of

thermomechanical treatment of the porous anode material. *Fizika i Khimiya Obrabotki Materialov* 1997;58(3):121–3.

100. Watve MG, Tickoo R, Jog MM, Bhole BD. How many antibiotics are produced by the genus Streptomyces. *Archives of Microbiology* 2001;176(5):386–90.

101. Bentley SD, Chater KF, Cerdeño-Tárraga A-M, Challis GL, Thomson NR, James KD, et al. Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2). *Nature* 2002;417(6885):141–7.

102. de Lima Procópio RE, da Silva IR, Martins MK, de Azevedo JL, de Araújo JM. Antibiotics produced by Streptomyces. *Brazilian Journal of Infectious Diseases* 2012;16(5):466–71.

103. Ikeda H, Ishikawa J, Hanamoto A, Shinose M, Kikuchi H, Shiba T, et al. Complete genome sequence and comparative analysis of the industrial microorganism Streptomyces avermitilis. *Nature Biotechnology* 2003;21(5):526–31.

104. Youn H, Kim E, Roe J, Hah YC, Kang S. A novel nickel-containing superoxide dismutase from Streptomyces spp. *Biochemical Journal* 2015;318(3):889–96.

105. Bai FW, Anderson WA, Moo-Young M. Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnology Advances* 2008;26(1):89–105.

106. Hahn-Hägerdal B, Palmqvist E. Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition. *Bioresource Technology* 2000;74(1):25–33.

107. Mehrez AZ, Ørskov ER, McDonald I. Rates of rumen fermentation in relation to ammonia concentration. *British Journal of Nutrition* 2005;38(03):437.

108. Rodriguez-Naranjo MI, Gil-Izquierdo A, Troncoso AM, Cantos-Villar E, Garcia-Parrilla MC. Melatonin is synthesised by yeast during alcoholic fermentation in wines. *Food Chemistry* 2011;126(4):1608–13.

109. Ohya T, Ohyama M, Kobayashi K. Optimization of human serum albumin production in methylotrophic yeast Pichia pastoris by repeated fed-batch fermentation. *Biotechnology and Bioengineering* 2005;90(7):876–87.

110. Kobayashi K, Kuwae S, Ohya T, Ohda T, Ohyama M, Tomomitsu K. High level secretion of recombinant human serum albumin by fed-batch fermentation of the methylotrophic yeast, Pichia pastoris, based on optimal methanol feeding strategy. *Journal of Bioscience and Bioengineering* 2000;90(3):280–8.

111. Páez-Lerma JB, Arias-García A, Rutiaga-Quiñones OM, Barrio E, Soto-Cruz NO. Yeasts isolated from the alcoholic fermentation of agave duranguensis during mezcal production. *Food Biotechnology* 2013;27(4):342–56.

112. Nuñez-Guerrero ME, Páez-Lerma JB, Rutiaga-Quiñones OM, González-Herrera SM, Soto-Cruz NO. Performance of mixtures of Saccharomyces and non-Saccharomyces native yeasts during alcoholic fermentation of agave duranguensis juice. *Food Microbiology* 2016;54(2):91–7.

113. Gullo VP, Zimmerman SB, Dewey RS, Hensens O, Cassidy PJ. Factumycin, a new antibiotic A40A: fermentation, isolation and antibacterial spectrum. *The Journal of Antibiotics* 2012;35(12):1705–7.

114. Paul Ross R, Morgan S, Hill C. Preservation and fermentation: Past, present and future. *International Journal of Food Microbiology* 2002;79(1–2):3–16.

115. Li F, Zhang JH, Tang L, Li S, Mao ZG, Liao LJ, et al. Production of ε-poly-l-lysine using a novel two-stage pH control strategy by Streptomyces sp. M-Z18 from glycerol. *Bioprocess and Biosystems Engineering* 2011;34(5):561–7.

116. Monod J. Remarks on the mechanism of enzyme induction. *Annual Reviews in M* 1949;3(Xl):371–94.

117. Ye R, Peng S, Xiong Y, Wang L, Xu H, Zeng Z, et al. Antibacterial activity and

mechanism of action of ε-poly-l-lysine. *Biochemical and Biophysical Research Communications* 2013;439(1):148–53.

118. Hartono SB, Gu W, Kleitz F, Liu J, He L, Middelberg APJ, et al. Poly-l-lysine functionalized large pore cubic mesostructured silica nanoparticles as biocompatible carriers for gene delivery. *ACS Nano* 2012;6(3):2104–17.

119. Chheda AH, Vernekar MR. A natural preservative ε-poly-L-lysine: Fermentative production and applications in food industry. *International Food Research Journal* 2015;22(1):23–30.

120. De Deken RH. The crabtree effect: A regulatory system in yeast. *Journal of General Microbiology* 2009;44(2):149–56.

121. Orive G, Tam SK, Pedraz JL, Hallé JP. Biocompatibility of alginate-poly-l-lysine microcapsules for cell therapy. *Biomaterials* 2006;27(20):3691–700.

122. Miller G.L. Use of dinitrosalicyclic acid reagent for determination of reducing sugar. *Analytical Chemistry* 1959;31(lll):426–8.

123. Itzhaki RF. Calorimetric method for estimating polylysine and polyarginine. *Analytical Biochemistry* 1972;574(3):569–74.

124. Chang Y, McLandsborough L, McClements DJ. Interactions of a cationic antimicrobial (ε-polylysine) with an anionic biopolymer (pectin): An isothermal titration calorimetry, microelectrophoresis, and turbidity study. *Journal of Agricultural and Food Chemistry* 2011;59(10):5579–88.

125. Zhou YP, Ren XD, Wang L, Chen XS, Mao ZG, Tang L. Enhancement of ε-poly-lysine production in ε-poly-lysine-tolerant Streptomyces sp. by genome shuffling. *Bioprocess and Biosystems Engineering* 2015;38(9):1705–13.

126. Mounir M, Ding LH, Jaffrin M, Delattre C, Michaud P, Courtois J. Extraction of

oligoglucuronans of low degrees of polymerisation from a fermentation broth by cascade filtration using a rotating disk module. *Desalination* 2006;199(1–3):207–9.

127. Wang Z, Zhuge J, Fang H, Prior BA. Glycerol production by microbial fermentation: A review. *Biotechnology Advances* 2001;19(3):201–23.

128. Shih IL, Shen MH. Optimization of cell growth and poly(ε-lysine) production in batch and fed-batch cultures by Streptomyces albulus IFO 14147. *Process Biochemistry* 2006;41(7):1644–9.

129. Karthik R, Meenakshi S. Chemical modification of chitin with polypyrrole for the uptake of Pb(II) and Cd(II) ions. *International Journal of Biological Macromolecules* 2015;78(2):157–64.

130. Krishna C. Solid-state fermentation systems - An overview. *Critical Reviews in Biotechnology* 2005;25(1–2):1–30.

131. Li YQ, Han Q, Feng JL, Tian WL, Mo HZ. Antibacterial characteristics and mechanisms of e-poly-lysine against Escherichia coli and Staphylococcus aureus. *Food Control* 2014;43(1):22–7.

APPENDIX A: MEASUREMENT OF E-PL WITH METHYL ORANGE

Experiments started with *S.albulus*. Method optimization was done before starting to experiments. For this reason, some dosages previously used in the literature were selected, then ε-Poly-L-Lysine was measured. The dosages selected were between 3mM-0.01mM. In this study, the method determined by Itzhaki in 1972 was used [123]. *S.albulus*, which prepared in 250mL Erlenmeyer flasks, samples are taken as 3mL portions to Falcons (Thermofisher Scientific Inc.) and centrifuged at 10000 rpm x 15 min (Gyrozen Co. Ltd., Korea). Measurements were made in the spectrophotometer at 465nm (Thermo Scientific, Genesys 10S UV-Vis). For *S.albulus* incubation condition was 30°C at 150 rpm with aerobic conditions. After the results were received, same experiment was performed with 1:10 dilution before measurement. Experiment repeated in terms of reliability of results with different media. The media used in this experiment was M3G. M3G Medium (g/L) contains: Glucose, 50; yeast extract, 5; $(NH_4)2SO_4$, 10; KH_2PO_4, 1.36; K_2HPO_4, 0.8; $MgSO_4+7H_2O$, 0.5; $ZnSO4+7H_2O$, 0.04; and $FeSO_4+7H_2O$, 0.03. pH 6.8. pH adjusted with 6M NaOH. It was used after being prepared and sterilized in autoclave. Inoculation was carried out with 5 percent and experiment was started. Since the results were inconsistent they excluded from this study.

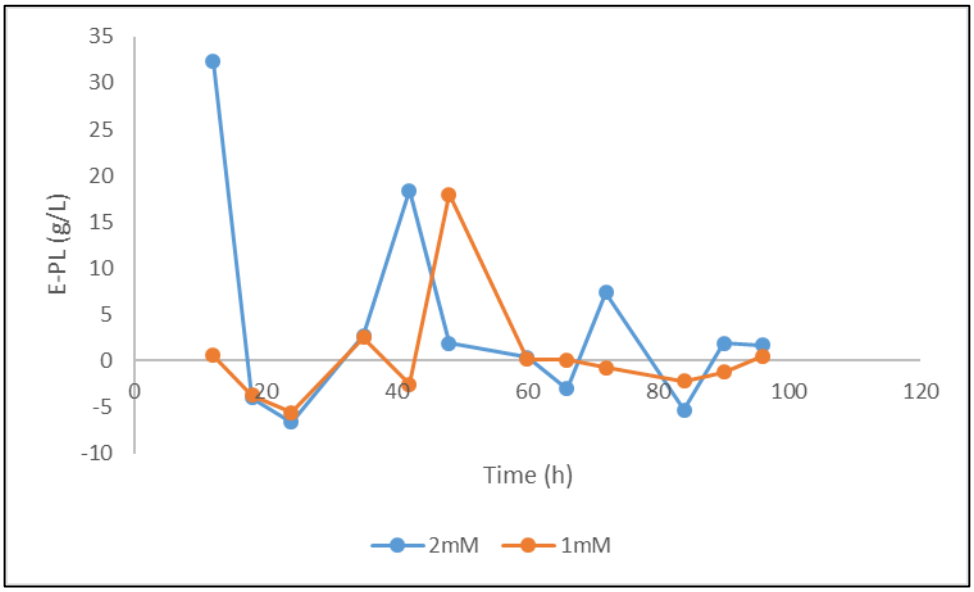

Figure A.1. Method optimization for MeO with *S.albulus.*

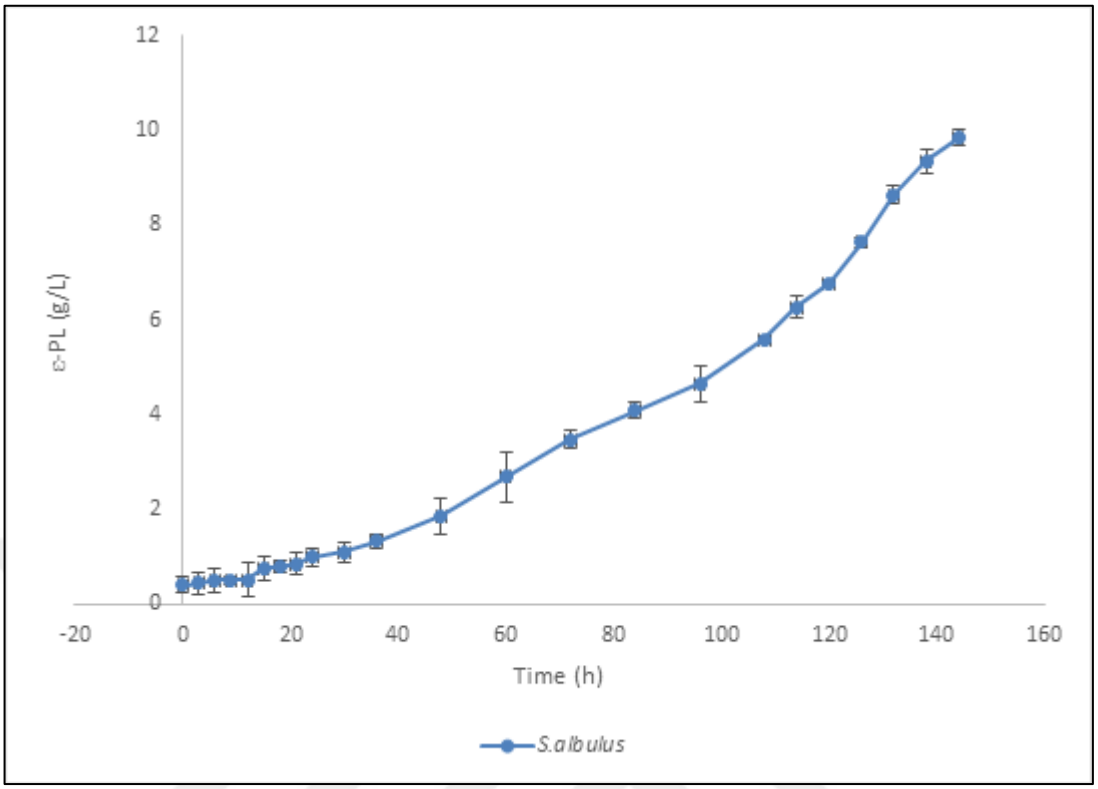

Figure A.2. ε-PL Measurement with MeO in agitated culture experiment

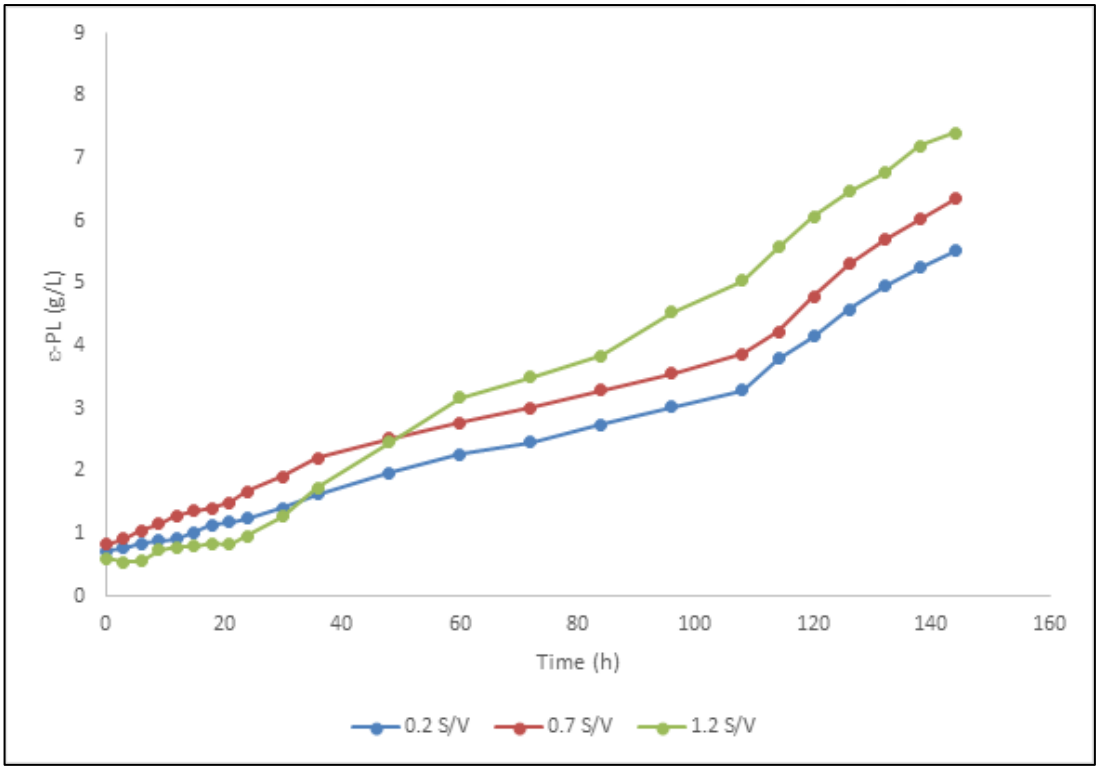

Figure A.3. ε-PL Measurement with MeO in static culture experiment