MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN
Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

# QUALIFICATION THESIS

on the topic **Using *Bacillus subtilis* 168 for ε- Research on Optimization of Polylysine Fermentation Conditions**

First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
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APPROVE
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Doctor of Technical Science
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**ASSIGNMENTS**
**FOR THE QUALIFICATION THESIS**
**Qi Xiaohan**

1. Thesis topic **Using *Bacillus subtilis* 168 for ε- Research on Optimization of Polylysine Fermentation Conditions**
Scientific supervisor Olga Andreyeva, Dr. Sc., Prof.

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## EXECUTION SCHEDULE

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| 1 | Introduction | From 01 April 2024 to 11 April 2024 | |
| 2 | Chapter 1. Literature review | From 06 April 2024 to 20 April 2024 | |
| 3 | Chapter 2. Object, purpose, and methods of the study | From 21 April 2024 to 30 April 2024 | |
| 4 | Chapter 3. Experimental part | From 01 May 2024 to 10 May 2024 | |
| 5 | Conclusions | From 07 May 2024 to 12 May 2024 | |
| 6 | Draw up a bachelor's thesis (final version) | From 20 May 2024 to 25 May 2024 | |
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I am familiar with the task:

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Scientific supervisor _____Olga ANDREYEVA

# SUMMARY

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In this experiment, the optimization of ε-polylysine fermentation conditions using the fermentation method was performed by first determining the content of the relevant components in the culture medium through a one-way experiment to observe the effect on the experiment. In the one-way experiment, the optimal values of the factors affecting the medium were determined under different conditions. Since fermentation optimization through one-way experiments alone is a time-consuming and laborious process, and the variables that produce the best response are often not identified. Based on the experimental data obtained from single-factor experiments, after comprehensive consideration of optimization, it can be investigated by response surface methodology. Response surface methodology (RSM) is a statistical technique used to empirically model and assess the impact of variables by varying one independent variable while fixing all other variables at certain levels. By using statistical methods to optimize all impact parameters, the limitations of the optimization process in single-factor experiments can be eliminated and many factors can be quickly screened to capture the effects of interactions between these factors. In this study, we attempted to isolate a strain of Bacillus that can produce ε-PL and optimize the experimental data for the production of ε-PL using statistical methods to achieve the goal of increasing the yield of ε-PL through reasonable culture conditions.

The optimization of M3G medium in this study was primarily conducted through one-way experiments and response surface methodology to identify the key factors influencing the yield of ε-polylysine based on experimental data, aiming to determine optimal ratios for enhancing ε-polylysine production.

By investigating the impact of carbon source, nitrogen source, and inorganic salt on ε-polylysine production through Bacillus subtilis 168 fermentations, we have successfully developed an optimized culture formulation for efficient ε-polylysine production: glucose

addition at a quantity of 60.3 g/L, yeast powder addition at a quantity of 7.49 g/L, ammonium sulfate addition at a quantity of 12.1 g/L. The predicted yield of ε-polylysine reached 0.3365 g/L. The optimal conditions were carefully selected for the shake flask fermentation experiment of ε-polylysine in order to ensure the accuracy of the experiments conducted in this response surface method. Three replications were 0.306±0.006 g/L. These Findings provide a solid experimental foundation for further fermentation production.

*Key words:* ε-polylysine, Bacillus subtilis 168, Response surface methodology

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# INTRODUCTION

With the continuous development of the economy and society, people's living standards are improving. Consequently, there is an increasing focus on food safety issues, with one of the most prominent concerns being food preservation. As a typical aerobic bacterium, Bacillus subtilis 168 is widely distributed in soil and decaying organic matter. This strain not only grows rapidly, but also has low nutritional requirements and can efficiently produce many proteins and metabolites. Bacillus subtilis 168 does not produce toxins and is therefore classified as a safe and non-pathogenic microbe. The ε-Polylysine (ε-PL) is an L-lysine homomeric polymer isolated from actinomycete culture medium. ε-Polylysine is the best choice to replace traditional chemical preservatives because of its safety, non-toxic, biodegradable and wide antibacterial spectrum. At present, the research on ε-Polylysine in our country is still in the experimental research stage, the main problem is that the yield of ε-Polylysine production strains is not high, so increasing the yield of ε-Polylysine to meet the market demand is of great significance to protect human health. The main research method of this experiment was carried out on the basis of M3G medium, through one-way experiments, then Box-Behnken experimental design, according to the principle of Plackett-Burman experimental design, and formulated each group of medium components, shaking flasks to measure the ε-polylysine yield after fermentation for 96h, and comparing the actual yield with the predicted value, so as to carry out the optimization accordingly.

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medium components, shaking flasks to measure the ε-polylysine yield after fermentation for 96 h, and comparing the actual yield with the predicted value, so as to carry out the optimization accordingly.

This thesis will firstly introduce the definition, origin and use of polylysine in detail, as well as the structural properties, catalytic properties and specific applications of polylysine, and at the same time, it will also give a comprehensive description of the characteristics of Bacillus subtilis 168 and the characteristics of polylysine production by Bacillus subtilis 168. Secondly, the experimental materials and methods will be introduced, mainly on the experimental reagents, equipment and experimental principles, experimental methods and so on. This topic will investigate the maximum yield of polylysine and its changing law under different conditions with different influencing factors. Finally, the experimental results and analysis.

**The relevance of the topic** is to optimize the fermentation production of ε-polylysine and improve the fermentation yield.

**The objectives of the study** is to learn the experimental principles of single factor experiment and response surface experiment, to obtain the optimal content affecting polylysine yield through experiments, and to allocate appropriate medium.

**The purpose of the study** is the study is to actively carry out research on the production of ε-polylysine by fermentation, especially the cultivation of high-yielding ε-polylysine strains by fermentation, which has an important economic and applied value to increase the yield of ε-polylysine, in order to realize the industrial production of ε-polylysine at an early date to satisfy the demand for ε-polylysine in the fields of food, medicine, and electronics, etc., by utilizing the studies such as the response surface experiments.

**The object of the study**: ε-Polylysine, Response surface methodology

ε-polylysine (ε-PL) is a lysine homomeric polymer isolated from actinomycetes culture-medium by S. Shima and H. Sakai in Japan. ε-PL has the characteristics of safety, non-toxicity, high temperature resistance, wide antibacterial spectrum, etc. It has great advantages in the preservation of beverages, foods and health drugs, etc., and can be metabolized into the necessary amino acid L-lysine to be further absorbed and utilized in

the human body, and is a nutritional preservative. Response surface methodology (RSM) is a statistical technique used to empirically model and assess the impact of variables by varying one independent variable while fixing all other variables at certain levels.

**The subject of the study**: Bacillus subtilis 168.

Bacillus subtilis 168 is a class of aerobic bacteria, widely distributed in soil and decaying organic matter. Bacillus subtilis is a non-pathogenic and safe microorganism with fast growth rate, low nutritional requirements, high efficiency secretion of many proteins and metabolites, and does not produce toxins.

**Research methods**: Single-factor experiment, Response Surface Analysis experiment.

**The scientific novelty** is to explore the influencing factors of polylysine, combine them organically, and configure media to optimize fermentation conditions and increase polylysine yield.

**The practical significance** of the results obtained is that the natural antiseptics produced by microbial fermentation not only have low production cost, but also can be obtained by strain modification and optimization of fermentation conditions with excellent properties. The production of ε-polylysine by microbial fermentation has excellent antiseptic properties and great commercial potential. ε-PL has a wide spectrum of bacterial inhibition, high safety, good thermal stability and convenient processing. It has been widely used for food preservation in developed countries since it was licensed by FDA in 2003. In the production of ε-PL, Japan has realized the industrialized large-scale production by fermentation method, compared with which, the domestic production of ε-PL by fermentation method is still in the stage of experimental pilot study due to the gap between the yield of strains and the fermentation process. Therefore, in order to realize the industrial production of ε-PL as soon as possible and to meet the demand for ε-PL in the fields of food, medicine, chemical industry and electronics in China, it is of great economic and applied value to actively carry out the research on the production of ε-PL by fermentation method.

# CHAPTER 1
# LITERATURE REVIEW

## 1.1 ε-Polylysine Research Overview

### 1.1.1 Source, properties of ε-polylysine

ε-Polylysine (ε-PL) is a stable and large amount of DP substance found in a strain of Actinobacillus sp. No. 346 in 1977 by Japanese scholars S. Shima and H. Sakai in the process of screening Dragendo~Positive (abbreviated as DP) substances in microorganisms. After careful analysis of the acid hydrolysis products and structural identification, it was confirmed that this DP substance is actually a homomonomeric polymer consisting of 25-30 lysine residues. ε-Polylysine has the characteristics of safety and non-toxicity, high-temperature resistance, and a wide range of bacterial inhibition [0], and therefore shows significant advantages in the field of preservation and conservation of food and healthcare medicines [0]. ε-Polylysine is able to be converted into essential L-lysine in the body, which is a very important component in the preservation of food. In the human body, ε-polylysine can be converted into essential L-lysine, which can be absorbed and utilized by the body, and thus it is classified as a nutritive preservative [0]. In view of its unique properties and functions, the future development of ε-polylysine is promising.

In terms of physical properties, ε-polylysine presents a light-yellow color with strong hygroscopicity and a slightly bitter taste, and its structure is a straight chain polymer polymerized from L-lysine. Under physiological conditions, ε-polylysine is positively charged, soluble in water, insoluble in organic solvents such as ether and ethanol, and has good thermal stability [0]. Since ε-polylysine is a mixture, it does not have a fixed melting point, but begins to soften and decompose at temperatures above 250 °C [0].

In terms of biological properties, ε-polylysine is a peptide with bacteriostatic properties. Compared to other chemical preservatives, ε-polylysine has a higher safety profile, with an acute oral toxicity of 5 g/kg [0]. ε-polylysine has a fairly wide range of bacteriostatic properties, significantly inhibiting and killing a wide range of microorganisms, including, but not limited to, Pseudohyphomyces acnes and Pseudomonas

fargesii in the genus Saccharomyces; heat-resistant Bacillus adipocarcinosus, Bacillus coagulans, and Bacillus cereus in the genus Gram-positive bacteria; and Aerobacter aerogenes and Escherichia coli among Gram-negative bacteria [0]. This broad-spectrum bacteriostatic ability makes ε-polylysine potentially valuable for food preservation and medical applications.

### 1.1.2 Structure and mechanism of bacterial inhibition of ε-polylysine

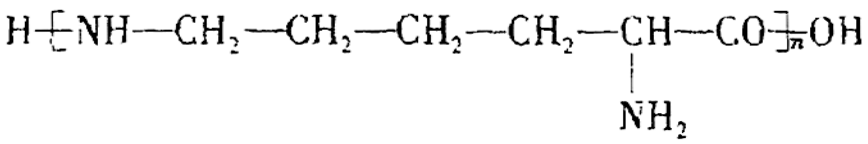

Figure 1.1 - **Structural formula of ε-polylysine**

ε-Polylysine is a homopolymer formed by the polymerization of 25-30 L-lysines, with a relative molecular weight of 3500-4500[0], whose amide bond is formed by the ε-amino group of lysine and the α-carboxy group of another lysine, and whose chemical structure formula is shown in Figure 1.1.

ε-Polylysine has a wide range of bacterial inhibitory ability and significant effect, including against Gram-negative bacteria, Gram-positive bacteria and some viruses, etc.; in addition, it also shows some inhibitory effect on yeasts and some fungi. Compared with α-polylysine, ε-polylysine is more prominent in bacteriostatic properties [0]. It was found that the bacteriostatic activity of ε-polylysine is susceptible to chemical modification of the amino group. When α-amino or ε-amino groups are chemically modified, their bacteriostatic activity decreases significantly [0]. In view of the broad-spectrum inhibitory properties of ε-polylysine, its production strains themselves face certain inhibitory effects. Studies have shown that ε-polylysine-producing strains have ε-polylysine-degrading enzymes on the cell membrane, which can protect the strains from the damage of self-produced ε-polylysine. In 2006, Hamano et al. isolated and purified ε-polylysine-degrading enzyme from S. albulus, and carried out the molecular cloning and functional analysis of

the enzyme. By constructing an ε-polylysine-degrading enzyme-inactivated knockout strain, they investigated the degrading enzyme activity, sensitivity to ε-polylysine, and yield of the mutant strain. This study showed that ε-polylysine degrading enzyme plays a key role in preventing the toxic effects of ε-polylysine [11].

### 1.1.3 Factors affecting the production of ε-polylysine

(1) Temperature

The effect of temperature on the yield of ε-polylysine was not significant, which was mainly due to the high thermal stability of ε-polylysine, which was neither easily decomposed nor inactivated under higher temperature conditions. Liu Wei of South China Agricultural University observed in his research experiment on the relationship between temperature and ε-polylysine yield that the yield of ε-polylysine was higher when the incubation temperature was set at 30 °C. Therefore, from the point of view of practical production, 30 °C can be used as the optimum temperature for fermentation [0].

(2) Rotation speed

The cultured seed solution was inoculated into the fermentation medium and separately placed in shakers with different rotational speeds but all other conditions were the same to determine the yield of ε-polylysine in the medium after fermentation. According to the currently known experimental data, 200 r/min can be selected as the culture shaker speed, and the yield of ε-polylysine is higher [0].

(3) pH value

pH plays an important role in the synthesis of ε-polylysine. During the production of ε-polylysine by shake flask fermentation, the pH of the fermentation broth decreases rapidly. This decrease in pH provides an ideal acidic and alkaline environment for the bacteria, which promotes the synthesis of ε-polylysine, allowing the bacteria to produce this compound efficiently. As the fermentation process continues, the pH of the fermentation broth decreases further, and this decreasing trend is often accompanied by a decrease in the efficiency of ε-polylysine synthesis. In view of this, in order to enhance the production of ε-polylysine, effective regulation of pH

during the fermentation process becomes a key strategy. This regulation not only helps to maintain the bacterial activity, but also ensures that the synthesis environment is in an optimal state, thus enhancing ε-polylysine production [0].

### 1.1.4 Common Applications of ε-Polylysine

(1) Application of ε-polylysine in foods

As a class of microbial preservatives with broad development prospects in natural preservatives, ε-polylysine mainly functions as a preservative in food [0]. ε-polylysine has excellent performance, with biodegradable, water-soluble, wide spectrum of bacterial inhibition, thermal stability, etc., and it still has good bacterial inhibition and activity under the conditions of heating treatment at 80 °C for 60 min or 120 °C for 20 min [0], so it can be sterilized with food raw materials at the same time to avoid secondary contamination.

Using electrostatic force, ε-polylysine is adsorbed on the cell membrane of microorganisms, and then binds with membrane proteins to damage the membrane structure. In this process, ε-polylysine induces the rupture of lysosomal membranes in cells, releasing enzymes such as proteases, which ultimately leads to cell lysis and death [0]. In food preservation applications, ε-polylysine can be used to form a composite bacteriostatic agent with other reagents such as alcohols, glycerol fats, and tea polyphenols to enhance its inhibition of microbial growth through synergistic effects [0].

(2) Application of ε-polylysine in medicine

In the field of pharmaceuticals, ε-polylysine has a unique application value, especially in the slow release and targeting carrier of drugs. Due to its cation-rich property, ε-polylysine can effectively electrostatically interact with anionic substances, a property that makes it easier to cross biological membranes, which in turn reduces the resistance to drug transport and improves the efficiency of drug transport. ε-polylysine was used to wrap T2 toxin and accurately detect the amount of antibody through a highly specific antigen-antibody reaction by Fan-Tsl et al. This study not only broadened the application scope of ε-polylysine, but also brought new ideas and methods to the field of pharmaceutical detection [0].

In the field of gene therapy, the solution to the targeting challenge of target gene transfer can be achieved by constructing composite vectors in order to mediate gene transfer. In this process, polylysosomes, with their unique advantages, are ideal for such composite vectors with significant advantages and potentials. Such materials are not only biocompatible, but also capable of efficiently delivering target genes to target cells, providing new possibilities for gene therapy research and applications. The study revealed that the adsorption and transfection efficiency of the composite vector constructed using polylysine was significantly improved. Yuan Xiaoyan et al. prepared a nucleic acid vector consisting of dextran, ε-polylysine and the peptide VAPG targeting smooth muscle cells, which could protect the nucleic acid drug targeting into smooth muscle cells with a transfection efficiency as high as 63.2 %, which has a great application prospect in the field of biomedical gene therapy.

(3) Application of ε-polylysine in highly absorbent resin

Highly absorbent resins are favored for their excellent water-absorbing ability and water-holding properties in many fields such as hygiene and environmental protection. Water-soluble ε-polylysine can be converted into highly efficient highly absorbent polylysine by a specific radiation cross-linking technique. In the study by Choi et al. γ-rays were utilized to cross-link ε-polylysine, and cross-linkers with up to 200-fold water absorption were successfully produced [0].

## 1.2 Bacillus subtilis 168 and its ε-polylysine production

### 1.2.1 Overview of Bacillus subtilis 168

Bacillus subtilis belongs to the genus Bacillus, whose colonies have a rough surface and a dirty white or yellowish color. As an aerobic bacterium, Bacillus subtilis is able to effectively utilize proteins and various sugars as a source of nutrition. In view of the fast growth rate of B. subtilis, low requirements for nutritional conditions, and can efficiently produce a variety of proteins and metabolites; B. subtilis does not have toxicity, and is therefore recognized as a safe and non-pathogenic microorganisms. This characteristic makes it show a broad application potential in many fields such as medicine and food.

### 1.2.2 Overview of common fermentation production processes for Bacillus subtilis 168

In order to overcome the problem of low yield of Bacillus subtilis 168 in industrial production, Miaomiao Wang et al. constructed Bacillus subtilis THBS-2 and THBS-8 with high expression of surfactant by overexpressing key genes in the fatty acid synthesis pathway. In order to further increase the yield, IPTG, leucine and concentrated medium were added at the fermentation time of 3 h and 24 h respectively, and the fermentation expression level of Bacillus subtilis THBS-2 reached 24 g/L after 48 h shaker fermentation [0]. In addition to genetically modifying strains, screening of strain sources is also a way to obtain a source of high-yielding strains, and obtaining strains with relatively superior performance also requires further optimization of their fermentation and production conditions in order to maximize their production. Monia Blibech et al. used the Plackett-Burman design and evaluated the effect of a set of 14 culture variables on the production of β-mannanase production and subsequently optimized the experimental conditions by response surface methodology using Box-Behnken design, which ultimately reduced the production cost and led to a 5.91-fold increase in β-mannanase production by Bacillus subtilis US191 [0].

### 1.2.3 Fermentation process for the production of ε-polylysine by Bacillus subtilis 168

(1)ε-polylysine generating bacteria

In 1947, Eprain synthesized α-type polylysine using a chemical method, which is a polylysine linked by an amide bond formed by α-amino and α-carboxy groups. Because of the lower bacteriostatic activity of α-polylysine compared to ε-polylysine, α-polylysine has some toxicity and has been replaced by ε-polylysine [0]. According to the current known research results, the production of ε-polylysine is limited to the microbial fermentation route. By analyzing the current studies in depth, we found that ε-polylysine can only be produced by microbial fermentation techniques. Masanobu N et al. added the acidic dye polyR-478 to the culture medium and carried out a detailed investigation of more than 300

ε-polylysine-producing strains present in the soil. They succeeded in discovering the species relationships among these strains with the help of 16S rRNA sequence analysis. The results showed that most of the ε-polylysine-producing strains were clustered in two major groups of microorganisms, Sireptomycetaceae and Ergot fungi. This finding not only enriches our understanding of the mechanism of ε-polylysine production, but also provides a valuable reference for future microbial resource mining. In addition, the degree of polymerization of ε-polylysine in different strains also differed significantly depending on the strain [0]. In recent years, it has also been reported in the literature that some strains of the genus Ribes norvegicus are also capable of producing ε-polylysine. Zhou Jun and Xu Hong et al. intensively studied the fermentation broth of Ribes norvegicus and isolated and purified ε-polylysine from it. Firstly, the researchers treated the fermentation broth by ion exchange technology and initially extracted the crude product of ε-polylysine. Subsequently, the chromatographically pure ε-polylysine product was obtained by Sephadex G-25 column chromatography using gel filtration technique, and the overall yield of the whole separation process was as high as 81.2 %. In determining the relative molecular mass of ε-polylysine, the research team chose both SDS-PAGE electrophoresis and gel permeation chromatography (GPC). After precise measurements, the relative molecular masses of ε-polylysine were 5.01 × 103 and 5.05 × 103, respectively, a value that is slightly higher compared to the relative molecular masses of ε-polylysine produced by other strains reported in the literature. This finding not only demonstrates the advantages of R. norvegicus in ε-polylysine production, but also provides an important reference for further research and application [0].

(2) Mutagenesis breeding of ε-polylysine-producing bacteria

In the pursuit of large-scale fermentation efficiency, strain improvement has become a key step in the development of fermentation technology. Physical and chemical mutagenesis play an important role in the mutation breeding of ε-polylysine-producing bacteria. At present, the yield obtained by mutation breeding in China has been improved to a certain extent, for example, Zhang Chao utilized $^{60}$Coy-ray irradiation mutagenesis to obtain a mutant strain with a yield 131.6 % higher than that of the original strain[0]; Zhang

Chao utilized a large number of ultraviolet mutagenesis to obtain a mutant strain with a yield 42.9 % higher than that of the original strain [0].

(3) Biosynthesis pathway and regulation of ε-polylysine

Shoji Shima and his team used $^{14}C$ labeling technology to trace and analyze the synthesis mechanism of ε-polylysine. In the process, they observed the key role of L-lysine in the synthesis of ε-polylysine, which is an indispensable precursor substance for ε-polylysine biosynthesis. This finding provides a new possibility for the synthetic pathway of ε-polylysine: firstly, monomeric lysine is generated through the lysine pathway, and then, catalyzed by ε-polylysine synthetase, monomeric lysine is gradually polymerized, and ultimately ε-polylysine is formed [0]. In the study of Takahiro Kawai, et al. the more catalytically active components, were extracted from the cytosol of Sireptomyces albulus with the help of technical means such as ion exchange column chromatography. In an extracellular environment, they mixed this component with lysine in culture, and after a period of interaction, they found that ε-polylysine was generated in the mixture. This important finding not only verifies the previous hypothesis that microbial synthesis of ε-polylysine does not follow the traditional DNA-mRNA-protein synthesis pathway, but relies on the catalytic action of a specific intracellular enzyme or enzymes, i.e., a non-ribosomal synthesis system [0].

(4) Fermentation production process

Shaking flask fermentation process: the strain was preserved in the slant (Gao's No. 1 medium), and a ring of aerial spores was picked precisely using an inoculation ring, and then transferred to the liquid seed medium (20 mL medium/250 mL triangular flask). Under the condition of 30°C, oscillation culture was carried out for a duration of 24-36 h; then 5 mL of the bacterial liquid was taken out from the above seed medium and further transferred to the fermentation medium (100ml/500mL), and continued to be oscillated under the condition of 30 °C for a period of time extended to 36-48 h, and after the completion of the above cultivation process, the collection of fermentation products could be carried out. Under optimal conditions, the accumulation of ε-polylysine could reach 0.4 g/L [0].

Flow-added replenishment medium (g/L): glucose 800 g/L and ammonium sulfate 80 g/L, respectively, were sterilized at 120 °C for 20 min, followed by cooling and mixing. During the preparation of the liquid seed, we removed a loop from the S410 slant seed and inoculated it into a 500 mL triangular flask containing 100 mL of M3G medium. Subsequently, this culture was shaken in a rotary shaker at 30 °C and 220 r/min overnight for about 15 h. The replenishment-batch fermentation technique is widely used for ε-polylysine production [0].

Flow-addition fermentation process in a 5L self-controlled fermenter: first, 200 mL of liquid seeds were inoculated into a 5L self-controlled fermenter containing 1.8 L of M3G medium. The airflow ratio was adjusted from 1:0.5 to 1:2.5 (V/V/m), and the rotational speed was intelligently regulated by automatic monitoring of dissolved oxygen (maintained at 30 %). The initial pH was set at 3.8 and the fermentation was carried out at a constant temperature of 30 °C. The fermentation was carried out at a constant temperature of 30 °C. The pH of the fermentation was set at 3.8. In terms of pH control, there are two main phases: in the first phase, i.e. the bacterial growth phase, the pH is strictly monitored and maintained above 5.0. Once it was lower than 5.0, $NH_4OH$ was used to adjust the pH value; in the second stage, product accumulation stage, the pH value was adjusted to 4.0 to optimize the accumulation of ε-polylysine; meanwhile, in order to ensure the stability of the fermentation process, the glucose concentration in the fermentation broth was closely monitored and maintained at 10 g/L.

When the concentration was lower than this threshold, flow-through replenishment was used to ensure that the glucose concentration is always maintained at 10 g/L (usually starting after 48 h). After 4-7 days of fermentation, the yield of ε-polylysine could reach up to 483 g/L. In a follow-up study, Prihandi Kahar et al. used an air-lift fermenter and successfully reduced the power consumption to 0.3 $kW/m^3$ by adjusting the fermentation parameters appropriately. Although the yield of ε-polylysine was reduced to some extent, this strategy is more suitable for large-scale fermentation production can significantly improve the economy and sustainability of the whole process [0].

### 1.3 Significance of the subject

Natural preservatives produced by microbial fermentation not only have low production cost, but also can be obtained by strain modification and optimization of fermentation conditions. The production of ε-polylysine by microbial fermentation has excellent antiseptic properties and great commercial potential. ε-PL has a wide spectrum of bacterial inhibition, high safety, good thermal stability and convenient processing. It has been widely used for food preservation in developed countries since it was licensed by FDA in 2003. In the production of ε-PL, Japan has realized the industrialized large-scale production by fermentation method, compared with which, the domestic production of ε-PL by fermentation method is still in the stage of experimental pilot study due to the gap between the yield of strains and the fermentation process. Therefore, in order to realize the industrial production of ε-PL as soon as possible and to meet the demand for ε-PL in the fields of food, medicine, chemical industry and electronics in China, it is of great economic and applied value to actively carry out the research on the production of ε-PL by fermentation method.

### Conclusions to chapter 1

1. ε-Polylysine is safe, non-toxic, heat-resistant, and has a broad spectrum of bacterial inhibition, and has demonstrated significant advantages in a variety of fields. ε-Polylysine is also converted to essential substances in the human body, and is therefore categorized as a nutritive preservative. ε-Polylysine has tremendous application value.

2. Bacillus subtilis 168 is characterized by its fast growth rate and its ability to efficiently produce a wide range of proteins and metabolites; it is non-toxic and thus recognized as a safe and non-pathogenic microorganism. This characteristic has developmental advantages in experimental research.

3. There are different research methods for fermentation production using Bacillus subtilis 168, and the appropriate production process can be selected according to the experimental conditions and experimental requirements.

# CHAPTER 2
# OBJECT, PURPOSE AND METHODS OF THE STUDY

## 2.1 Object and purpose of the study

**The objectives of the study** are to learn the experimental principles of single factor experiment and response surface experiment, to obtain the optimal content affecting polylysine yield through experiments, and to allocate appropriate medium.

**The purpose of the study** is the study is to actively carry out research on the production of ε-polylysine by fermentation, especially the cultivation of high-yielding ε-polylysine strains by fermentation, which has an important economic and applied value to increase the yield of ε-polylysine, in order to realize the industrial production of ε-polylysine at an early date to satisfy the demand for ε-polylysine in the fields of food, medicine, and electronics, etc., by utilizing the studies such as the response surface experiments.

**The object of the study**: ε-Polylysine, Response surface methodology. ε-polylysine (ε-PL) is a lysine homomeric polymer isolated from actinomycetes culture-medium by S. Shima and H. Sakai in Japan. ε-PL has the characteristics of safety, non-toxicity, high temperature resistance, wide antibacterial spectrum, etc. It has great advantages in the preservation of beverages, foods and health drugs, etc., and can be metabolized into the necessary amino acid L-lysine to be further absorbed and utilized in the human body, and is a nutritional preservative. Response surface methodology (RSM) is a statistical technique used to empirically model and assess the impact of variables by varying one independent variable while fixing all other variables at certain levels.

**The subject of the study**: Bacillus subtilis 168. Bacillus subtilis 168 is a class of aerobic bacteria, widely distributed in soil and decaying organic matter. Bacillus subtilis is a non-pathogenic and safe microorganism with fast growth rate, low nutritional requirements, high efficiency secretion of many proteins and metabolites, and does not produce toxins.

## 2.2 Experimental materials

### 2.2.1 Strain and Reagents

Bacillus subtilis 168 basic production strain, preserved in the Faculty of Bioengineering, Qilu University of Technology.

Polylysine standard, etc. (Qilu University of Technology)

Methyl orange, methylene blue (Chengdu Kelon Chemical Company)

### 2.2.2 Medium

M3G medium (1L): glucose: 50 g, yeast powder: 5 g, $(NH_4)_2SO_4$: 10 g, $KH_2PO_4$-$2H_2O$: 1.4 g, $MgSO_4 \cdot 7H_2O$: 0.5 g, $K_2HPO_4 \cdot 2H_2O$: 0.8 g, $FeSO_4 \cdot 7H_2O$: 0.03 g, $ZnSO_4 \cdot 7H_2O$: 0.03 g.

### 2.2.3 Laboratory instruments

Electronic balance (Beijing Sartorius Instruments Co.)

Biochemical incubator (Shanghai Jinghong Experimental Equipment Co.)

Ultra-clean bench (Suzhou Bozhao Scientific Instrument Co.)

Vertical automatic electric pressure sterilizer (Guangzhou Wondi Instrument Co.)

Multi-function Combination Shaker (Shanghai Minquan Instrument Co.)

Ultraviolet-visible (Spectrophotometer HITAGHI Co.)

Digital Blst Drying Oven (Shanghai Boxun Industry Co.)

## 2.3 Experimental purpose

With the development of society and economy, people's living standard is getting higher and higher, and the food safety problems related to it have also attracted more and more attention, in which the preservation of food is one of the key issues of food safety. Bacillus subtilis is a kind of aerobic bacteria, widely distributed in soil and decayed organic matter. Bacillus subtilis is a kind of aerobic bacteria widely distributed in soil and decayed organic matter. ε-Polylysine (ε-PL) is a lysine homomonomer polymer isolated and extracted from actinomycetes culture medium by Japanese scholars S. Shima and H. Sakai.

ε-PL is safe and non-toxic, high temperature resistant, and has wide inhibitory spectrum, ε-PL is safe, non-toxic, high-temperature resistant, and has a wide spectrum of bacterial inhibition, which has great advantages in the preservation of beverages, food, and health care drugs. ε-PL can be metabolized into the essential amino acid L-lysine in the body to be further absorbed and utilized, and it is a kind of nutritious antiseptic, so ε-PL has a very broad development prospect. This thesis focuses on the optimization of fermentation conditions of ε-Polylysine by Bacillus subtilis in detail.

## 2.4 Experimental method

### 2.4.1 Determination of ε-polylysine content

The methyl orange colorimetric method of Shima and Itzhaki is currently used to measure ε-polylysine. The method centers on the ability of the sulfonate anion in methyl orange to interact with the abundant amino cation in ε-polylysine in the pH range of 3-10, resulting in the formation of a specific precipitate. The formation of this precipitate showed a linear correlation with the concentration of ε-polylysine over a specific range.

Determination of the standard curve of ε-polylysine: Take 10 mL of standard ε-polylysine dilution of 0, 20, 40, 60, 80, 100 ug/mL and 10 mL of methyl orange solution (1 mM) respectively (0 ug/mL with buffer as standard), 30 °C 200 r/min shaking reaction for 30 min, and then centrifuged at 4000 r/min for 15 min to remove the resulting ε-polylysine-methyl orange complex. The supernatant was diluted 50-fold with phosphate buffer (0.1 M, pH 6.6), and the absorbance value at 465 nm was measured spectrophotometrically using the reaction of phosphate buffer with methyl orange as a blank control. The measured OD value as the x-axis, the standard ε-polylysine content as the y-axis to do the standard curve, can be obtained ε-polylysine standard curve.

Determination of ε-polylysine content in the samples: under aseptic conditions, the target strains were selected from the slant medium, inoculated and cultured to obtain the initial culture medium, i.e., the seed solution; the seed solution was inoculated into the medium at an inoculation ratio of 2 %. When the fermentation process was completed, an appropriate amount of fermentation broth was selected and centrifuged at a rotational speed

of 6000 r/min for 10 minutes; 2 mL of the supernatant was taken and mixed with an equal amount of 1 mmol/L methyl orange solution; the mixture was put into a shaker and shaken at a constant speed for 30 minutes for the reaction, and after the completion of the reaction, centrifugation was carried out again at a rotational speed of 6000 r/min for a duration of After centrifugation, 1 mL of the supernatant was taken and diluted to 20 mL using distilled water. Finally, the absorbance value of the diluted solution was determined using a spectrophotometer. The exact amount of ε-polylysine in the sample can be calculated from the pre-drawn standard curve.

### 2.4.2 Isolation and screening of ε-polylysine-producing bacteria

Soil was collected from Qilu University of Technology, naturally airborne for 3-10 d, crushed and sieved, and baked at 50 °C for 1 h. 1 g of soil sample was added to a triangular flask containing glass beads and 10 mL of 0.85 % saline, and incubated for 10 min at 30° C with shaking, and then left to stand for 30 min, and then the supernatant was appropriately diluted and coated with a screening medium containing $K_2Cr_2O_7$ and incubated at 30 °C for 7 d. According to the morphology of colonies, single colonies similar to actinobacteria were excavated and placed horizontally in a methylene blue screening plate and continued to be incubated for 2-3 d. Single colonies were picked out to produce hyaline rings. According to the colony morphology, single colonies similar to Actinomyces were excavated and placed horizontally in the methylene blue screening plate to continue to cultivate for 2-3 d, and single colonies producing hyaline circles were picked and preserved in slant.

The strains obtained from slant preservation were inoculated into M3G medium, incubated at 30 °C, 200 r/min in shake flasks for 72 h, centrifuged at 4000 r/min for 5min, and the supernatant was taken and detected dropwise with Dragendorff reagent and Methylene Orange reagent, and strains showing precipitation were initially identified as ε-polylysine-producing bacteria.

### 2.4.3 Qualitative Analysis of Fermentation Products

#### 2.4.3.1 Extraction and purification of fermentation products

The spores of ε-polylysine-producing strains were picked into triangular flasks with fermentation medium, incubated at 30 °C and 200 r/min for 72 h. The fermentation broth was centrifuged at 8000 r/min for 10 min, and the supernatant was adjusted the pH to 8.5 with NaOH, and centrifuged at 12000 r/min for 10 min. The supernatant was adsorbed with cation ($H^+$) exchange resin (D152), and then adsorbed with 0.2 mol/L acetic acid and 0.1 mol/L acetic acid, respectively. After adsorption with cation ($H^+$) exchange resin (D152), the supernatant was rinsed and eluted with 0.2 mol/L acetic acid and 0.1 mol/hydrochloric acid, respectively. The eluent was detected by Dragendorff's reagent, and the collection of eluents was started when precipitation appeared. The eluate was neutralized with NaOH to pH 6.5, centrifuged at 12,000 r/min for 10 min, and the supernatant was transferred to a dialysis bag (3000) for 1-2 d of dialysis.

#### 2.4.3.2 Bacterial inhibition experiment

Pour the sterilized agar medium into the petri dish, 15 mL per dish (lower layer), wait until it is half solidified with aseptic operation on the surface of the medium directly and vertically on the Oxford cup, the melted medium cooled down to about 50 °C to add the test bacteria to mix, 5 mL per dish to add to the solidified medium (upper layer). After the plate is solidified, 200 μL of sample solution is added to the Oxford cup and incubated overnight in an incubator at a suitable temperature to observe the growth of the products on the plate.

#### 2.4.3.3 Determination of product composition

A certain amount of ε-polylysine standard and product samples were put into ampoules and hydrolyzed with 4 mL 6M HCL at 121 °C for 20 h. 10 μL of ε-polylysine standard, product samples, hydrolyzed ε-polylysine standard and product samples, and L-lysine standard were taken and analyzed for their constituents by thin-layer chromatography.

Thin layer: cellulose, silica gel GF254;

Spreading agent: n-butanol: glacial acetic acid: pyridine: water = 4:1:1:2

(V/V);

Color developer: 0.2 % n-butanol solution of ninhydrin.

### 2.4.4 Strain characterization

#### 2.4.4.1 Morphological characterization of strains

Pick the spores from the slant of the strain and line them on the Bennett plate, incubate at 30°C and observe the morphological changes of the strain on the plate.

#### 2.4.4.2 Physiological and biochemical experiments

(1) Starch hydrolysis experiment: pick the spores of the strain and connect them on the plate, incubate at 30 °C for 3d, add drops of Lu's iodine solution around the colony, observe whether there is a transparent circle around the colony, if there is, then it means that the starch is hydrolyzed and it is positive, and vice versa is negative.

(2) Carbon source utilization experiment: pick the spores of the strain inoculated in the medium shaking bottle culture 1d, take 100 uL of bacterial solution evenly coated in the carbon source utilization medium plate, and then the sterilized filter paper flat on the plate, respectively, sucked 100 uL of different carbon source solution added to the different filter paper, 30 °C incubation for 3 d, and observe whether the colonies appear around the filter paper.

### 2.4.5 Experiments on the promotion of ε-polylysine production by yeast powder

In exploring the effect of yeast powder content on the fermentation effect, six different gradients were designed around the yeast powder content based on the base medium formulation. In this process, all the medium components except yeast powder content were kept consistent with the base medium formulation to formulate the desired experimental medium.

The experiments were carried out with yeast powder at 2.5 g/L, 5.0 g/L, 7.5 g/L, 10.0 g/L, 12.5 g/L and 15.0 g/L content under the rotational speed of 180 r/min and the incubation temperature of 30 °C incubation, and the ε-polylysine yield was determined at the end of fermentation, with three replicates set up for each content.

### 2.4.6 Experiments on ammonium sulfate promotion of ε-polylysine production

In exploring the effect of ammonium sulfate content on the fermentation effect, six different gradients were designed around the ammonium sulfate content based on the formulation of the base medium. In this process, all other medium components except ammonium sulfate content were kept consistent with the base medium formulation to formulate the desired experimental medium. The experiments were carried out with ammonium sulfate at 8 g/L, 10 g/L, 12 g/L, 14 g/L, 16 g/L, and 18 g/L content under the rotational speed of 180r/min and incubation temperature of 30 ℃ culture conditions, and the ε-polylysine yield was determined at the end of fermentation, with three replicates set up for each content.

### 2.4.7 Experiments on glucose-promoted ε-polylysine production

In exploring the effect of glucose content on the fermentation effect, six different gradients were designed around the glucose content based on the base medium formulation. In this process, all other medium components except glucose content were kept consistent with the base medium formulation to formulate the desired experimental medium. Under the rotational speed of 180 r/min and incubation temperature of 30 ℃ culture conditions, the glucose was experimented at 30 g/L, 40 g/L, 50 g/L, 60 g/L, 70 g/L and 80 g/L content, and the ε-polylysine yield was determined at the end of the fermentation, and three replicates were set up for each content.

### 2.4.8 Response surface experimental design

Response surface methodology (RSM) is a statistical technique used for empirical modeling and assessing the effects of variables by varying one independent variable while fixing all other variables at a certain level. By using statistical methods to optimize all impact parameters, the limitations of the optimization process in a single-factor experiment can be eliminated and many factors can be quickly screened to capture the effects of the interactions between these factors. There are two main stages in this experiment. First, on the basis of the single-factor experiment, we screened the media components required for ε-polylysine production with the help of

Plackett-Burman (P-B) design. The screening process was mainly based on the main effects of the components rather than the interaction effects among them. Setting the yield of ε-polylysine as a judgment index and the additions of glucose, yeast powder and $(NH_4)_2SO_4$ as influencing factors, a three-factor, three-level Box Behnken test was designed by applying the principle of Response Surface Analysis (RSM) with the aim of optimizing the medium formulation in order to obtain the optimal medium combination. In this test, two levels with more obvious gaps, i.e., low level (-1) and high level (+1), were set for each factor to ensure that the results of the test were broad and representative. Table 2.1 details the factor level design of the response surface test.

Table 2.1-**Test factor level table of response surface**

| Level | Glucose (g/L) | Yeast powder (g/L) | $(NH_4)_2SO_4$ (g/L) |
|---|---|---|---|
| -1 | 50 | 5 | 10 |
| 0 | 60 | 7.5 | 12 |
| 1 | 70 | 10 | 14 |

The key components of the Plackett-Burman design were optimized in depth using Box Behnken Design (BBD). The experimental data were analyzed by multiple regression fitting through the statistical software MINTAB Version 15 (Minitab Inc, Pennsylvania, USA) and response curves were plotted based on the resulting regression model equations. In addition to this, analysis of variance (ANOVA) was applied to estimate the statistical parameters, the experimental data were exhaustively analyzed, and intuitive response surface plots were drawn.

**Conclusions to chapter 2**

1. The ε-polylysine standard curve y = -0.331x + 0.197 was measured by ltzhaki method.

2. The fermentation product of Bacillus subtilis 168 was qualitatively analyzed by bacteriostatic experiment and thin layer chromatography, and the fermentation product of this strain was ε-polylysine, and its bacteriostatic range was roughly similar to ε-polylysine.

# CHAPTER 3
# EXPERIMENTAL PART

## 3.1 Preparation of ε-polylysine standard curve

According to the method described by Itzhaki, the standard solution of ε-polylysine at different concentrations was reacted with methylene orange, and then the absorbance value was measured by spectrophotometer at 465 nm, and the measured light absorption value was taken as the x-axis, and the concentration of ε-polylysine was taken as the y-axis, and the data were processed by Microsoft Excel to make the standard curve and standard equation. The standard curve is shown in Figure 3.1. The standard equation is y = -0.331x + 0.197, and the correlation coefficient is $R^2$=0.9995, which meets the requirement of the accuracy of the standard equation, and can be used to calculate the yield of ε-polylysine.

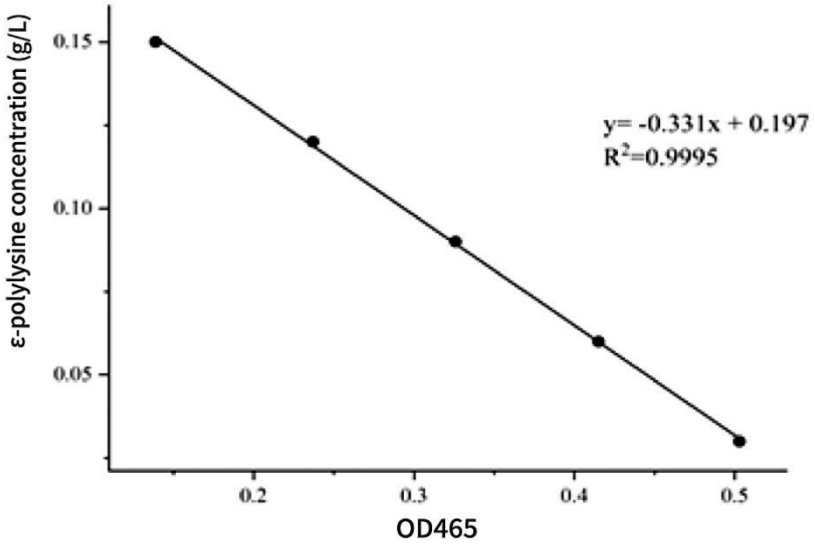


Figure 3.1- **Effect of different content of yeast powder on ε-polylysine yield**

## 3.2 Screening of ε-polylysine producing strains

ε-polylysine-producing bacteria are mainly actinomyces, adding potassium dichromate to the separation plate can significantly inhibit the growth of fungi and bacteria in soil, but the inhibitory effect on actinomyces is relatively small, so adding potassium chromate can effectively improve the screening efficiency. The treated soil solution was coated in Gao's synthetic medium No. 1 containing 50 mg/L $K_2Cr_2O_7$, and a single colony

was selected after culture in the incubator. A total of 198 strains of actinomycetes were isolated and screened.

The isolated single colonies were cut off together with AGAR blocks and placed flat on a plate containing 0.002 % methylene blue for further culture. Since ε-polylysine is an alkaline substance, it can produce a transparent ring by electrostatic repulsion with methylene blue, according to which the alkali-producing strains can be screened out. A total of 103 alkali-producing strains were screened from 198 soil isolates by this method.

The selected alkali-producing strains were cultured in shake flask, 200 μL supernatant was taken after fermentation solution centrifugation, and a few drops of Dragendorff reagent and methyl orange reagent were added, respectively. The fermentation supernatant of 5 strains could undergo orange-red precipitation reaction with Dragendorff reagent and methyl orange reagent at the same time. According to the characteristic precipitation reaction of ε-polylysine with Dragendorff reagent and methyl orange reagent, it was preliminarily inferred that the five isolates produced ε-polylysine. Itzhaki method was used to determine the ε-polylysine production of the 5 strains, and the highest concentration of the product was found in Bacillus subtilis 168.

### 3.3 Qualitative analysis of fermentation products

#### 3.3.1 Bacteriostatic assay of fermentation products

Escherichia coli, Staphylococcus aureus and Candida albicans, representing Gram-positive bacteria, Gram-negative bacteria and fungi, respectively, were used as indicator bacteria to test the bacterial inhibition effect of the fermentation product of strain Bacillus subtilis 168. The results were shown in Figures 3.2, in which the bacterial inhibition circle was produced around the oxford cups in the three kinds of indicator bacterial plates, which indicated that the fermentation product of strain Bacillus subtilis 168 was able to inhibit the growth and proliferation of Gram-positive bacteria, negative bacteria and fungal growth and proliferation, similar to the range of ε-polylysine inhibition.

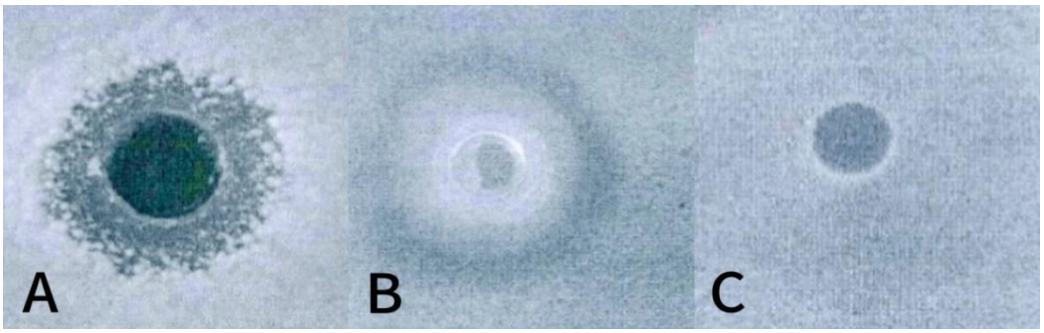


Figure 3.2 - **Results of bacteriostatic test of fermentation broth of strain Bacillus subtilis 168: A - Candida albicans; B - Escherichia coli; C - Staphylococcus aureus**

### 3.3.2 Identification of product constituents

The ε-polylysine standard, the fermentation product sample, the hydrolysate of the ε-polylysine standard, the hydrolysate of the fermentation product and the L-lysine standard were subjected to thin-layer chromatography, respectively, and the results are shown in Figure 3.3. ε-polylysine standard and the sample of the fermentation product of Bacillus subtilis 168 formed a spot at the starting point, and the samples of the fermentation product of Bacillus subtilis 168 and ε-polylysine standard and the hydrolysis product of ε-polylysine formed similar spots on thin plates with equal Rf values. The hydrolyzed product and lysine standard formed a similar spot on the thin plate, and the Rf values were equal. Combined with the results of bacterial inhibition experiments, it can be concluded that the fermentation product of Bacillus subtilis 168 is a homopolymer of lysine, ε-polylysine.

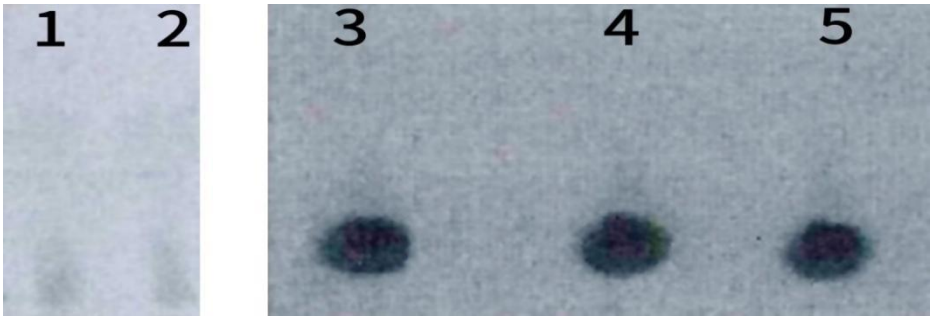


Figure 3.3 -**Thin layer chromatography results of fermentation broths:** 1 - Fermentation broth sample; 2 - ε-polylysine standard; 3 - Fermentation

sample hydrolysate; 4 - ε-polylysine standard hydrolysate; 5 - L-lysine standard

### 3.4 Strain identification

#### 3.4.1 Observation of strain morphology and morphology

Strain Bacillus subtilis 168: After 24 h of plate streaking, colonies grew on the plate. The colonies were round or irregularly shaped, with darkened, thickened, opaque and wrinkled surfaces, and could turn milky white or brown, as shown in Figure 3.4.

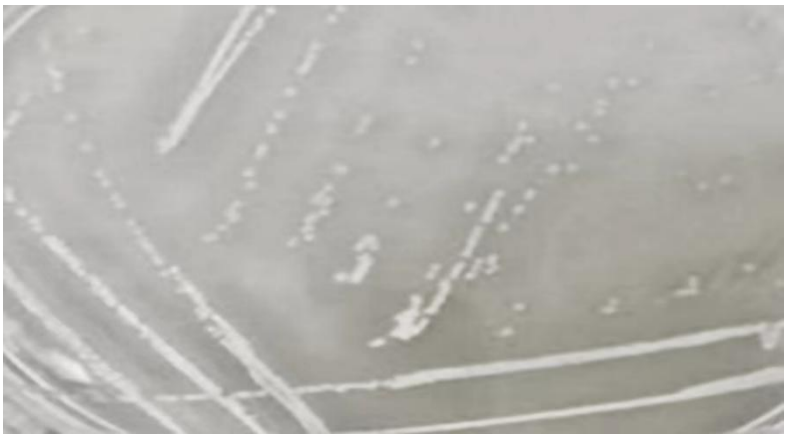

Figure 3.4 - **Growth morphology of strain Bacillus subtilis 168 on plate**

#### 3.4.2 Physiological and biochemical experiments

(1) Starch hydrolysis experiment

An important feature of Bacillus subtilis 168 is the property of producing amylase to decompose starch. Strain Bacillus subtilis 168 was inoculated into the starch hydrolysis medium plate, 30 °C incubation 2 d, the colony grew on the plate, in the colony around the drop of iodine solution of Lu, the colony around the appearance of a large and clear circle (Figure 3.5), indicating that the strain of Bacillus subtilis 168 amylolytic hydrolysis is positive, and hydrolysis of starch ability is very strong.

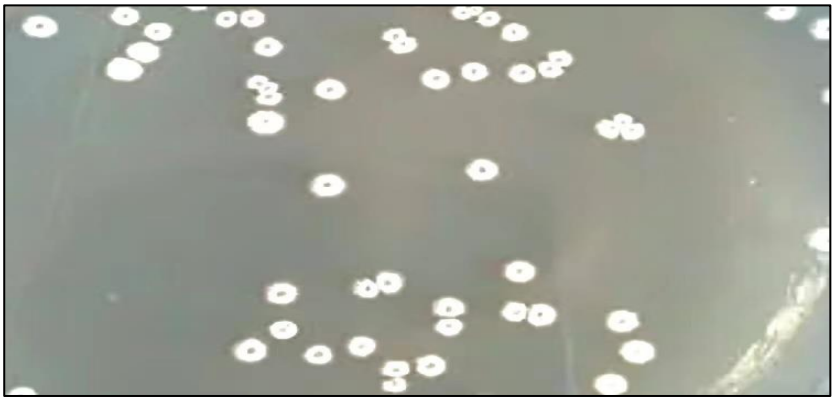

Figure 3.5 - **Starch hydrolysis experiment**

(2) Carbon source utilization experiment

Detection of strain Bacillus subtilis 168 carbon source utilization is an important indicator of strain physiological identification. Experiments will be inoculated with strains containing different carbon sources on the plate 30 ° C culture 2 d, observe the growth of colonies on the plate results shown in Table 3.1. Strain Bacillus subtilis 168 can utilize glucose, fructose, lactose, galactose and maltose.

Table 3.1 - **Utilization of sugar carbon sources by strains of bacteria**

| Saccharides | Colony growth status |
|---|---|
| Glucose | + |
| Fructose | + |
| Lactose | + |
| Galactose | + |
| Maltose | + |

Note: "+" means there is single colony growth

### 3.5 Effect of yeast powder on the yield of ε-polylysine

The effect of yeast powder content on ε-polylysine yield was examined and the experimental results are shown in Figure 3.6.

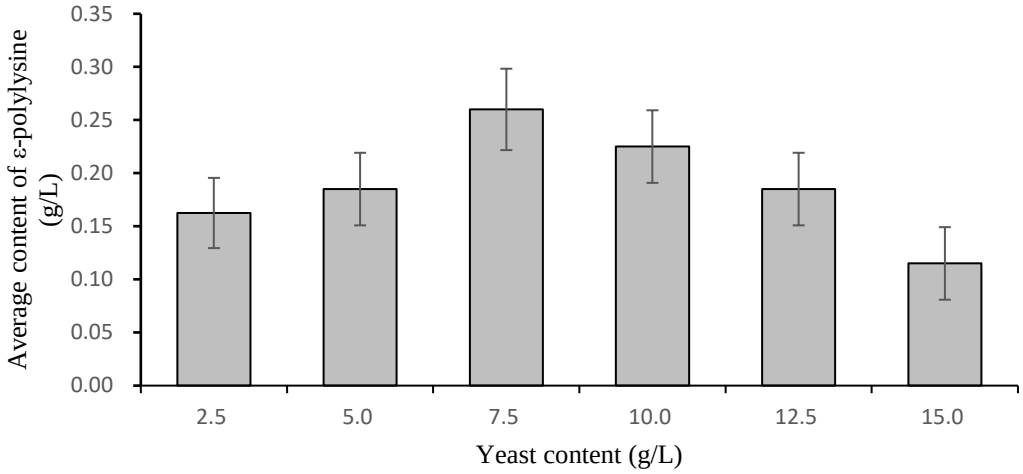


Figure 3.6 - **Effect of different content of yeast powder on ε-polylysine yield**

With the increase of yeast powder content, the ε-polylysine yield showed a trend of increasing and then decreasing. When the yeast powder content increased from 2.5 g/L to 7.5 g/L, the ε-polylysine yield increased continuously; when the yeast powder content was 7.5 g/L, the ε-polylysine yield reached the maximum value of 0.27±0.03 g/L. Continuing to increase the yeast powder content, the ε-polylysine yield began to decrease, which was presumed to be due to the fact that the higher yeast powder addition was more favorable to the growth of the bacteria and caused a significant reduction in the ε-polylysine yield. ε-polylysine yield was adversely affected.

### 3.6 Effect of ammonium sulfate on the yield of ε-polylysine

The effect of ammonium sulfate content on the yield of ε-polylysine was investigated and the experimental results are shown in Figure 3.7. With the increase of ammonium sulfate content, the ε-polylysine yield showed a trend of increasing and then decreasing. When the ammonium sulfate content increased from 8 g/L to 12 g/L, the ε-polylysine yield increased continuously; when the ammonium sulfate content was 12 g/L, the ε-polylysine yield reached the maximum value of 0.26±0.04 g/L. Continuing to increase the ammonium sulfate content, the ε-polylysine yield began to decrease, which was presumed to be

probably due to the fact that the higher ammonium sulfate additions were more conducive to the division and reproduction of the cells, thus decreased the ε-polylysine yield.

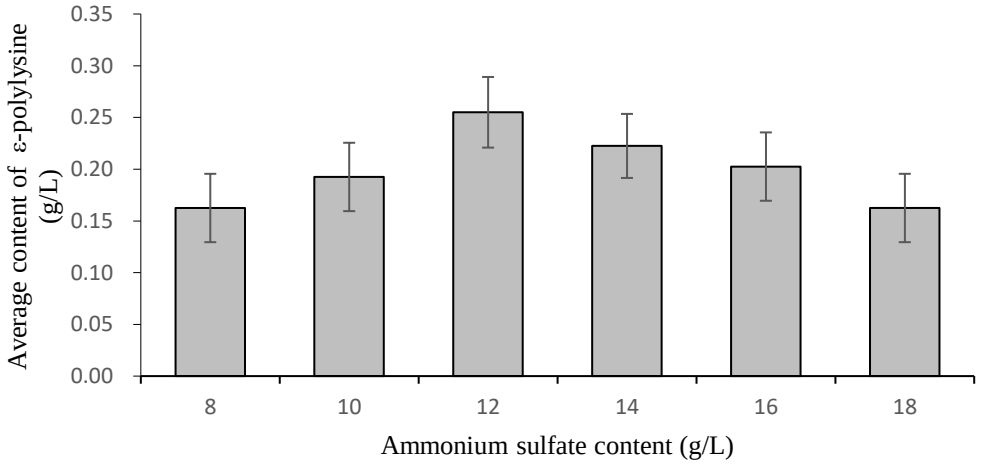


Figure 3.7 - **Effect of different content of ammonium sulfate on ε-polylysine yield**

### 3.7 Effect of glucose on ε-polylysine yield

The effect of glucose content on ε-polylysine yield was examined and the experimental results are shown in Figure 3.8. With the increase of glucose content, the ε-polylysine yield showed a trend of increasing and then decreasing. When the glucose content increased from 30 g/L to 60 g/L, the ε-polylysine yield increased continuously; when the glucose content was 60 g/L, the ε-polylysine yield reached the maximum value of 0.23±0.03 g/L. Continuing to increase the glucose content, the ε-polylysine yield began to decrease, which was presumed to be probably due to the increasing amount of glucose additions, which could produce carbon metabolism blockage to some metabolic pathways within the cell, thus resulting in the decrease of the ε-polylysine yield. pathways of carbon metabolism, thus affecting the ε-polylysine yield.

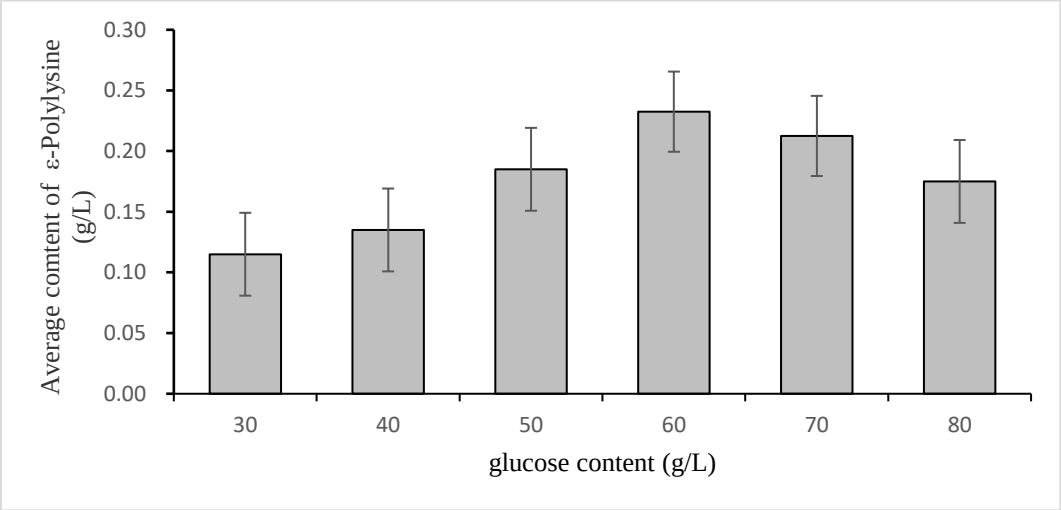


Figure 3.8 - **Effect of different content of glucose on ε-polylysine yield**

### 3.8 Culture medium optimization response surface experiment

(1) Box-Behnken experimental design results

After analyzing the Plackett-Burman experimental design, it can be concluded that glucose, inorganic salts as well as ammonium sulfate have a significant effect on the accumulation of ε-polylysine yield in the fermentation process. They inhibit ε-polylysine yield when the levels of these factors exceed a certain threshold. The central purpose of the Plackett-Burman experiment in this experiment was to establish a reference point for the Box-Behnken experimental design. The content ratios of glucose, ammonium sulfate, and yeast flour were adjusted by gradients and analyzed by measuring the yield of ε-polylysine in different experimental groups. From the experimental results, it can be seen that the highest yield of ε-polylysine was obtained when the contents of glucose, ammonium sulfate and yeast powder were 60.3 g/L, 12.1 g/L and 7.49 g/L, respectively, so this concentration was determined as the center point of Box-Behnken experimental design.

On the basis of defining the experimental center point, we can complete the Box-Behnken experimental design with the help of Design expert 8.0.6 software. In this experiment for the three variables, usually need to set up 17 groups of experiments. The software will configure the three media compositions at three different levels: +1, -1, and 0. The three media compositions will be used in the design of the Box-Behnken experiment. Based on the parameters of each set of media, we need to prepare the corresponding media

and determine the yield of ε-polylysine after 96 h of fermentation in shake flasks. During the experiment, we need to note that although our experimental design only focuses on a specific influencing factor, other trace elements are still added according to the standard ratios of the M3G medium during the configuration of the medium to ensure the completeness and accuracy of the experiment.

Table 3.2 - **Box Behnken experimental design results**

| Experiment number | Glucose (g/L) | Yeast powder (g/L) | Ammonium sulfate (g/L) | Content of ε-polylysine (g/L) |
|---|---|---|---|---|
| *1* | *2* | *3* | *4* | *5* |
| 1 | 70 | 10 | 12 | 0.11 |
| 2 | 50 | 10 | 12 | 0.20 |
| 3 | 60 | 7.5 | 12 | 0.33 |
| 4 | 60 | 7.5 | 12 | 0.34 |
| 5 | 60 | 5 | 14 | 0.20 |
| 6 | 50 | 5 | 12 | 0.12 |
| 7 | 60 | 5 | 10 | 0.18 |
| 8 | 60 | 10 | 10 | 0.20 |
| 9 | 50 | 7.5 | 10 | 0.16 |
| 10 | 60 | 7.5 | 12 | 0.34 |
| 11 | 70 | 7.5 | 10 | 0.21 |
| 12 | 70 | 7.5 | 14 | 0.22 |
| 13 | 60 | 10 | 14 | 0.17 |
| 14 | 60 | 7.5 | 12 | 0.33 |
| 15 | 60 | 7.5 | 12 | 0.35 |
| 16 | 50 | 7.5 | 14 | 0.20 |
| 17 | 70 | 5 | 12 | 0.17 |

(2) Response surface results and analysis

After measuring the experimental data, we filled them into the corresponding response values, and all the experimental data were processed by regression fitting using Design Expert Software Version 8.0.6 software to obtain the results of the quadratic

regression model ANOVA, to verify the model credibility, so as to determine the optimal culture medium ratio. The relevant data of the quadratic regression analysis were obtained in Table 3.3 below.

Table 3.3 shows the results of the regression analysis of the model, where A represents glucose, B represents ammonium sulfate, and C represents yeast flour. The model in the table has F = 44.09, P < 0.0001, indicating that the model is significant. The model loss of fit term (lack of fit) indicates the probability that the predicted values of the model do not fit the actual values, P > 0.05, the loss of fit term is not significant, so the model was chosen correctly. The correlation coefficient of the model R-Squared = 0.9827 and Adj R-Squared = 0.9604, indicating that the equation is well fitted. In conclusion, the regression equation provided a suitable model for the predictive analysis of ε-polylysine production.

Table 3.3 - **Analysis of variance of response surface quadratic model**

| Source of variation | Sum of squares | Degrees of freedom | Mean square | F value | P value | |
|---|---|---|---|---|---|---|
| Model | 0.1 | 9 | 0.011 | 44.09 | < 0.0001 | significant |
| A-glucose | 2.49E-04 | 1 | 2.49E-04 | 0.99 | 0.3529 | |
| B-yeast powder | 7.40E-06 | 1 | 7.40E-06 | 0.029 | 0.8687 | |
| C-$(NH_4)_2SO_4$ | 2.32E-04 | 1 | 2.32E-04 | 0.92 | 0.3688 | |
| AB | 4.90E-03 | 1 | 4.90E-03 | 19.49 | 0.0031 | |
| AC | 1.92E-04 | 1 | 1.92E-04 | 0.76 | 0.4115 | |
| BC | 6.06E-04 | 1 | 6.06E-04 | 2.41 | 0.1645 | |
| A^2 | 0.033 | 1 | 0.033 | 133.02 | < 0.0001 | |
| B^2 | 0.04 | 1 | 0.04 | 159.48 | < 0.0001 | |
| C^2 | 0.011 | 1 | 0.011 | 43.63 | 0.0003 | |
| Residual | 1.76E-03 | 7 | 2.51E-04 | | | |
| Lack of Fit | 1.43E-03 | 3 | 4.77E-04 | 5.79 | 0.0615 | not significant |
| Pure Error | 3.30E-04 | 4 | 8.24E-05 | | | |
| Cor Total | 0.1 | 16 | | | | |

Note: R-square = 0.9827; Adj R-square = 0.9604

(3) Response surface method to analyze the effect of factors on ε-polylysine yield

The response surface was fitted by the equation, and the response surface plot as well as the contour plot are detailed in the following figure.

Figure 3.9 a show the response surface curve of glucose addition and yeast powder addition on ε-polylysine yield. The increase of glucose addition, when other factors were optimal, resulted in a clear trend of ε-polylysine yield from low to high and then lower, and this significant magnitude of change indicates that this factor has an important effect on ε-polylysine yield. On the other hand, the yield of ε-polylysine also showed the same trend of increasing and then decreasing with the incremental increase of yeast powder content, and the magnitude of the change was also large. Therefore, it can be concluded that both factors, glucose addition and yeast powder, play a key role in ε-polylysine yield.

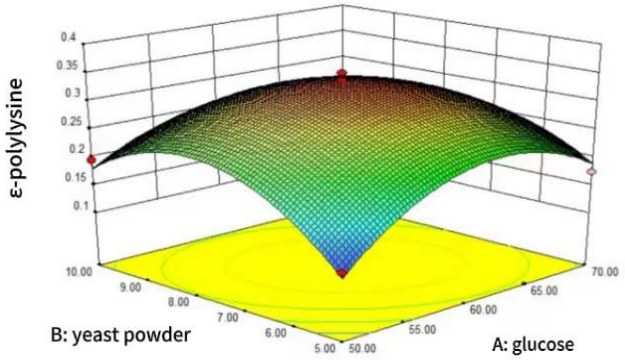


Figure 3.9 a - **Response surface curve of glucose addition and yeast powder addition**

Figure 3.9 b shows the response surface curve of glucose addition and ammonium sulfate addition on ε-polylysine yield. When the other two factors reached the optimal conditions, the enzyme activity of ε-polylysine showed a tendency of increasing and then decreasing with the increment of glucose content, and its fluctuation amplitude was significant, which demonstrated the important influence of glucose on the yield of ε-polylysine; at the same time, with the gradual increase of ammonium sulfate addition, the enzyme activity of ε-polylysine showed a similar tendency of increasing and then decreasing, and the variation amplitude was equally significant, which further proved that the concentration of glucose and ammonium sulfate addition were the most important factors for the production of ε-polylysine. This further proved the significant effect of ammonium sulfate concentration on ε-polylysine yield.

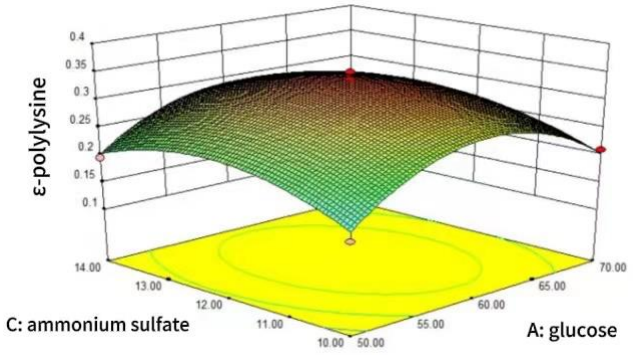


Figure 3.9 b - **Response surface curve of glucose addition and ammonium sulfate addition**

Figure 3.9 c shows the response surface curve of yeast powder addition and ammonium sulfate addition on ε-polylysine yield. When all other conditions were at the optimum, the yield of ε-polylysine showed an increasing and then decreasing trend with the increase of yeast powder addition, and the fluctuation was large, reflecting the significant effect of this factor on the yield. Similarly, the increase of ammonium sulfate caused a first increase and then decrease in the yield of ε-polylysine with the same significant change, indicating its significant effect on the yield. The above phenomena further indicated that there was a significant interaction between yeast flour addition and ammonium sulfate on ε-polylysine yield.

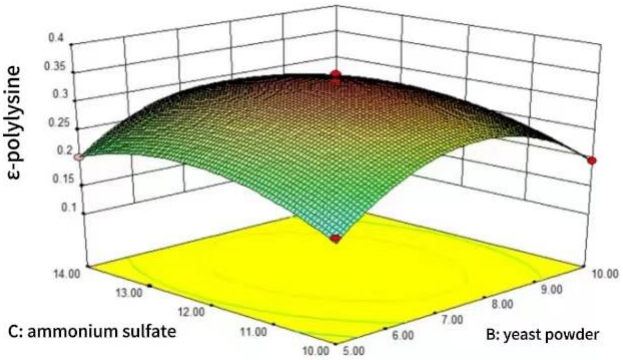


Figure 3.9 c - **Response surface curve of yeast powder addition and ammonium sulfate addition**

(4) Determination of optimal fermentation process conditions for ε-polylysine and validation experiments

With the help of professional software, the experimental data and model were analyzed in depth, and the optimal process conditions for the fermentation production of ε-polylysine were determined: the optimal addition amount of glucose was 60.3 g/L, the addition amount of yeast powder was 7.49 g/L, and the addition amount of ammonium sulphate was 12.1 g/L. The predicted yield of ε-polylysine under these conditions was 0.3365 g/L. In order to verify the accuracy and practicability of the response surface methodology, we repeated three shake flask fermentation experiments based on the above optimal conditions, and calculated and validated them. and practicality, we repeated three shake flask fermentation experiments of ε-polylysine based on the above optimal conditions, and calculated and analyzed the average ε-polylysine yield to be 0.306±0.006 g/L. This result was similar to the predicted value of the model, which strongly proved the accuracy of the model, and at the same time highlighted its application value in practice.

**Conclusions to chapter 3**

1. Standard curve of ε-polylysine measured by Itzhaki method y = -0.331x + 0.197.

2. The results showed that Bacillus subtilis 168 had antibacterial effects on gram-positive bacteria, gram-negative bacteria and fungi. The fermentation product of Bacillus subtilis 168 was ε-polylysine by TLC.

3. The experiment was designed with glucose, yeast powder and ammonium sulfate as the influencing factors, and the ε-polylysine yield was compared. The optimal ratio in the medium was determined to be 60 g/L glucose, 7.5 g/L yeast powder and 12.0 g/L ammonium sulfate.

4. Based on the above experimental results, the optimal culture formula of the strain was finally determined: glucose 60.3 g/L, yeast powder 7.49 g/L, ammonium sulfate 12.1 g/L, ε-polylysine predicted value of 0.3365 g/L. After repeated experiments with this condition for three times, the average ε-polylysine yield is

0.306±0.006 g/L, which is close to the predicted value, proving that the response surface experiment design is reasonable and reliable.

## CONCLUSIONS

1. Standard curve of ε-polylysine measured by Itzhaki method y=-0.331x + 0.197.

2. The fermentation product of Bacillus subtilis 168 was extracted and purified, and the bacteriostatic test showed that it had bacteriostatic effect on gram-positive bacteria, negative bacteria and fungi. Thin-layer chromatography confirmed that the fermentation product was ε-polylysine. The bacterial strain Bacillus subtilis 168 was identified as belonging to Bacillus genus by colony morphology observation, physiological and biochemical experiments and molecular identification of 16S rRNA.

3. The experiment was designed with glucose, yeast powder and ammonium sulfate as influencing factors. Through analysis, the optimal content of glucose, yeast powder and ammonium sulfate were determined to be 60.3 g/L, 7.49 g/L and 12.1 g/L respectively.

4. In this study, the optimal culture formula of the strain was determined by one-way and orthogonal tests: glucose 60.3 g/L, yeast 7.49 g/L, ammonium sulfate 12.1 g/L. The predicted value of ε-polylysine in the optimized fermentation medium was 0.3365 g/L. After three replications of the experiment using this condition, the average ε-polylysine yield was 0.306±0.006 g/L. The average ε-polylysine yield was 0.306±0.006 g/L. The average ε-polylysine yield was 0.006 g/L. The ε-polylysine yield was significantly increased after optimizing the medium and fermentation conditions.

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# APPENDICES

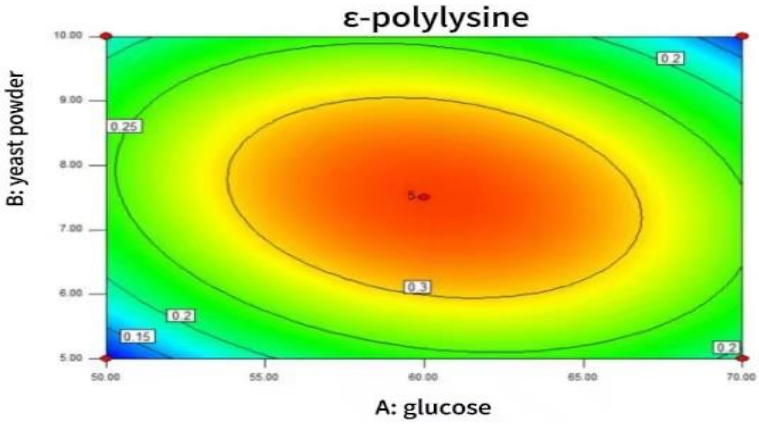


Figure A.1 - **Contour map of glucose addition and yeast powder addition on ε-polylysine yield**

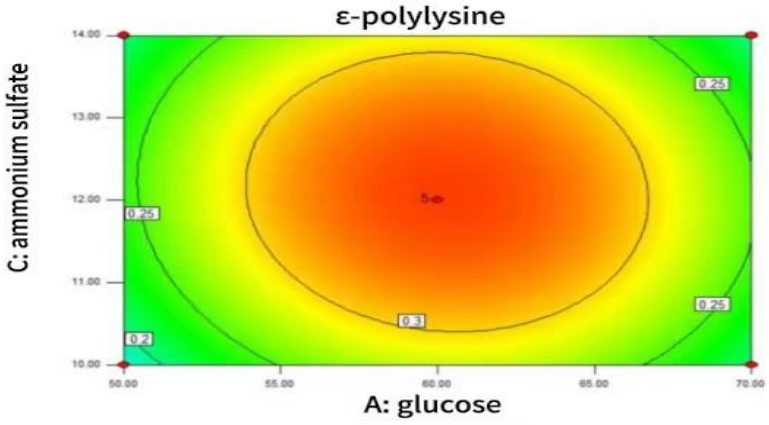


Figure A.2 - **Contour map of glucose addition and ammonium sulfate addition on ε-polylysine yield.**

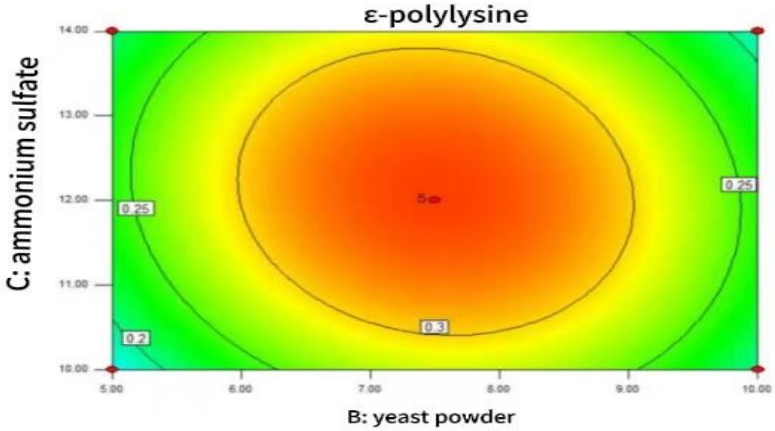


Figure A.3 - **Contour map of yeast powder addition and ammonium sulfate addition on ε-polylysine yield**