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 **Optimizing the production of *Escherichia coli* MG1655 ε-Fermentation conditions of polylysine**
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Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
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Specialty: 162 Biotechnology and Bioengineering
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**ASSIGNMENTS**
**FOR THE QUALIFICATION THESIS**
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Scientific supervisor Ihor Hretskyi, Ph.D., As. prof

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# SUMMARY

**Jiage Zhang. Optimizing the production of Escherichia coli MG1655 ε-Fermentation conditions of polylysine. – Manuscript.**

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In recent years, with the improvement of people's material living standards, the public health concept has been gradually enhanced, and food preservatives, as a key factor in food safety, have been highly valued by the state and society. Compared with chemical preservatives, more green, more efficient, non-toxic natural preservatives are favored by more and more consumers.ε -Polylysine (ε -poly-L-lysine, ε -PL), as a natural preservative made of lysine, has the advantages of excellent antibacterial effect, soluble in water, strong thermal stability, biodegradable, and safety and high efficiency.

In this study, using univariate and response surface analysis to explore the fermentation conditions of ε -PL, the optimal fermentation conditions for ε -polylysine production were 60.29 g glucose, 7.49 g yeast powder, 12.08 g ammonium sulfate, and the predicted value of ε -polylysine was 0.2953 g / L. To test the accuracy of this response surface method, a shake flask fermentation experiment with ε -polylysine was performed to obtain an average yield of ε -polylysine of 0.286 ± 0.016 g / L. The result varies slightly from the predicted value of the model, which proves the accuracy of the model and has some practical value.

*Key words:ε-Polylysine,Escherichia coli,fermentation optimization,response surface experiment.*

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# INTRODUCTION

In recent years, with the improvement of people's material living standards, the public health concept has been gradually enhanced, and food preservatives, as a key factor in food safety, have been highly valued by the state and society. Compared with chemical preservatives, more green, more efficient, non-toxic natural preservatives are favored by more and more consumers. ε -Pollysine (ε -poly-L-lysine, ε -PL) as a natural preservative of lysine polymerization, with excellent antibacterial effect, soluble in water, strong thermal stability, biodegradable, safe and efficient, has a wide development prospect and huge business opportunities, but because of ε -polylysine research compared with other developed countries started late, the high production cost, fermentation efficiency and low yield become an important problem, so improve the production of microbial fermentation ε -polylysine production is still of great significance. The purpose of this paper is to increase the production of ε -PL by optimizing the conditions for ε -PL production in E. coli MG1655 and to provide a basis for the industrial production and application of ε -PL.

ε -Polylysine (ε -poly-L-lysine, ε -PL) is a homopeptide polymer consisting of 25 – 35 L-lysine residues linked by the ε -amino group, it has a wide antibacterial spectrum and high safety, with good antibacterial effects on various microorganisms such as Gram positive bacteria, Gram negative bacteria, fungi, etc. Dry ε-Polylysine is a light yellow powder solid, slightly bitter, highly hygroscopic, and therefore requires sealed storage. ε- PL as a natural preservative of lysine polymerization, with excellent antibacterial effect, soluble in water, strong thermal stability, biodegradable, safe and efficient, has a wide development prospect and huge business opportunities.

This experiment first optimized the growth of *Escherichia coli* MG1655 strain, and then explored the effects of three single factors: glucose, yeast powder, and ammonium sulfate on the impact of ε-PL. The concentration of ε - polylysine in the fermentation broth was measured and the common determination methods of ε-

polylysine mainly include chromatography, colorimetric methods and fluorescence methods. On the basis of single factor experiments, ε - PL response surface experiment was conducted on the ratio of glucose, yeast powder, and ammonium sulfate using the content of ε - polylysine as the indicator. By constructing a response surface graph, the trend of the influence of various factors and their interactions on response variables can be visually displayed, and then the culture medium formula can be optimized.

In this study, univariate and response surface analyses were employed to investigate the fermentation conditions of ε -PL. The optimal fermentation conditions for ε -polylysine production were determined as follows: 60.29 g glucose, 7.49 g yeast powder, and 12.08 g ammonium sulfate. The predicted value of ε -polylysine was found to be 0.2953 g/L. The accuracy of the response surface method was evaluated by conducting a shake flask fermentation experiment with ε-polylysine, resulting in an average yield of 0.286 ± 0.016 g/L for ε-polylysine. The obtained result exhibits slight deviation from the predicted value of the model, thereby validating its accuracy and demonstrating practical significance. To lay the foundation for evaluating the economic feasibility of optimizing production processes (including raw material costs, energy consumption, equipment investment, etc.) and applying research results to future practical production.

**Purpose of the study** - compared with chemical preservatives, ε -PL is increasingly favored by consumers as a more environmentally friendly, efficient, and non-toxic natural preservative.The purpose of this paper is to increase the production of ε -PL by optimizing the conditions for ε -PL production in E. coli MG1655 and to provide a basis for the industrial production and application of ε -PL.

**Object of study -** ε -Polylysine (ε -poly-L-lysine, ε -PL) is a homopeptide polymer consisting of 25 – 35 L-lysine residues linked by the ε -amino group, it has a wide antibacterial spectrum and high safety, with good antibacterial effects on various microorganisms such as Gram positive bacteria, Gram negative bacteria, fungi, etc. Dry ε - Polylysine is a light yellow powder solid, slightly bitter, highly

hygroscopic, and therefore requires sealed storage. ε- PL as a natural preservative of lysine polymerization, with excellent antibacterial effect, soluble in water, strong thermal stability, biodegradable, safe and efficient, has a wide development prospect and huge business opportunities.

**Subject of study** – *Escherichia coli* MG1655 is a model strain widely used in molecular biology research and biotechnology. It is a derivative strain of the Escherichia coli K-12 series 12, known for its genetic stability, clear genome sequence, good biochemical characteristics, and ease of genetic manipulation. It has been widely used in various fields such as protein expression, metabolic engineering, gene regulation, microbial physiology, and synthetic biology.

# CHAPTER 1
# LITERATURE REVIEW

## 1.1 Study of the ε -polylysine amino acid protein

ε-Polylysine (ε -poly-L-lysine, ε -PL) is an antibacterial polymeric polypeptide formed from dehydration and condensation of L-lysine monomer (usually 25 to 30), which can be decomposed into lysine in the human body and is widely recognized in the field of food safety and production.ε -Polylysine was first discovered in 1977 by Japanese scientists Shima and Sakai [1-3] in the fermentation broth of Streptomyces white (Stre8ikinptomyces albulus 346). The molecular weight range of ε-PL is mostly between 3600 and 4300, and this molecular weight range can show superior antibacterial activity, while the molecular weight below 1300 will lose the antibacterial capacity.

## 1.2 Structure and function of the ε -polylysine

ε -Polylysine is a homopeptide polymer consisting of 25 – 35 L-lysine residues linked by the ε -amino group with a molecular weight range of approximately 3600 – 4700 Da. Its structure is shown in Figure 1-1. It is a linear polymer, molecules contain multiple free amino group, under physiological conditions with positive electricity, can through electrostatic interaction with bacterial cell wall and the negative charge on the membrane, this special structure gives its good water solubility and stability, in a wide range of pH (pH 2-9) can maintain strong sterilization and antibacterial ability. The dried ε-polylysine is a pale yellow powder solid, slightly bitter, highly hygroscopic and sealed. ε -Polylysine antibacterial spectrum, high safety, soluble in water but insoluble in ethanol, its maximum solubility is not less than 500g / L, and in high temperature (such as 121℃, 30 minutes) treatment remains stable, which makes it suitable for all kinds of food processing conditions, and harmless to organisms and the environment, become a

highly watched natural biological preservative, in the pursuit of healthy and safe modern food industry has important application value.(Fig. 1.1).

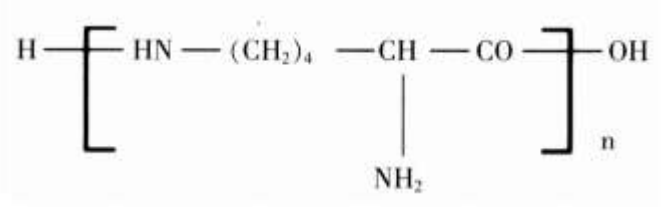



Figure 1.1 – Chemical structure of the ε -polylysine

### 1.3 Common applications of ε- Polylysine

ε- Polylysine(ε- PL), as a natural, safe, efficient, and commercially promising microbial preservative, can effectively inhibit the growth of harmful microorganisms by adding trace amounts, and does not have too much impact on the taste of food. It meets the health needs of consumers and is widely used in various processed foods. ε- PL has a wide antibacterial spectrum and high safety, with good antibacterial effects on various microorganisms such as Gram positive bacteria, Gram negative bacteria, fungi, etc., especially *Escherichia coli* and *Salmonella* that cannot be effectively inhibited by other natural preservatives. Research by Zhang Quanjing and others found that ε- PL has a good preservation and antibacterial effect on fresh meat, which will mixing ε- PL with acetic acid can enhance its freshness and quality[7]. ε-PL, as a new type of food preservative, can not only be used alone, but can also be mixed with other food additives, such as when ε-PL is mixed with aminoacetic acid, a significant synergistic promotion effect is produced, greatly prolonging the preservation and preservation time of concentrated milk [8].

In addition, in the field of medicine ε- Polylysine also has broad application prospects. ε- Polylysine is a cationic polymer that can interact with anionic substances, generate strong electrostatic forces, and can penetrate biofilms well, making it a good carrier for certain drugs to exert their effects. He Limin et al. conducted research on the APA carrier composed of ε-PL is used to alleviate pain in rats and cancer patients: APA microencapsulated BBC (bovine chromaffin cells) can

produce significant analgesic effects, indicating that APA plays a very effective carrier role. In addition, ε- Polylysine can also be used for wound healing materials, surface coatings on medical devices, and anti-corrosion of drug formulations, especially for products suitable for sensitive populations [9].

With the deepening of domestic and international research, ε- PL also has important applications in agriculture and plant protection, weight loss and health care, electronic materials, cosmetics, and other fields [10, 11].

### 1.4 Overview of *Escherichia coli* MG1655

*Escherichia coli* MG1655 is a model strain widely used in molecular biology research and biotechnology. It is a derivative strain of the Escherichia coli K-12 series [12], known for its genetic stability, clear genome sequence, good biochemical characteristics, and ease of genetic manipulation. It has been widely used in various fields such as protein expression, metabolic engineering, gene regulation, microbial physiology, and synthetic biology.

*Escherichia coli* MG1655 originates from W1485 [13] and is one of the relatively wild type *Escherichia coli* strains that have not undergone extensive genetic modification, thus maintaining many natural characteristics and possessing good genetic operability. The genome sequence of *Escherichia coli* MG1655 was first determined in 1997, with a genome size of approximately 4.6 Mb and containing approximately 4200 genes. It is one of the earliest organisms to be fully sequenced, laying the foundation for subsequent gene function research. *Escherichia coli* MG1655 and its various modified strains not only help to reveal basic life processes, but also play important roles in cutting-edge fields such as metabolic engineering and synthetic biology.

### 1.5 Overview of Common Fermentation Production Processes of Escherichia coli MG1655

The key factors in the fermentation process of *Escherichia coli* MG1655 are mainly manifested in two aspects: the composition of the fermentation medium and the control of fermentation conditions. The difference in the proportion of each component in the fermentation medium can affect the enzyme activity in the microbial synthesis pathway, thereby affecting the synthesis and accumulation of metabolites; Suitable fermentation conditions can provide an ideal environment for the growth and metabolic processes of microorganisms, which can shorten the fermentation cycle and increase the yield of the target product.

(1) Composition of fermentation medium

Carbon source is an important source of energy for organisms, which can be divided into fast carbon source and slow-release carbon source according to the utilization characteristics of microorganisms. Microorganisms exhibit different performance in different carbon sources. Glucose, as one of the common fast carbon sources, can quickly participate in the growth and metabolism of microorganisms, providing energy and carbon skeleton. Escherichia coli has a fast growth rate and may produce by-products such as acetic acid during the cultivation process, which not only inhibits cell growth but also affects the expression of exogenous proteins; Slow carbon source decomposition, long continuous energy supply time, suitable for the synthesis of antibiotics and other products. Therefore, it is necessary to choose appropriate carbon sources to increase the yield of the target product. The concentration of carbon sources also plays a crucial role in fermentation production, and can be controlled by optimizing feeding methods. Tang et al. used the DO stat method to control the addition of substrates and optimize the type and concentration of carbon sources, resulting in a significant increase in the biomass and enzyme activity of the recombinant strains. Nitrogen sources can also be divided into quick acting nitrogen sources and slow acting nitrogen sources. Quick acting nitrogen sources have a negative regulatory effect on the synthesis of some metabolic

products, such as increasing the supply of ammonium salts in the spiramycin fermentation of Streptomyces, which increases the growth rate of the cell while reducing biomass and yield. The nutrients contained in organic nitrogen sources can increase the concentration of bacterial cells, but excessive input can also lead to premature cell apoptosis, and the accumulation of nitrogen-containing intermediate byproducts can also increase the metabolic burden of bacterial cells, which is not conducive to protein synthesis and product transformation. In addition, different metabolites require different nitrogen source concentrations. To regulate bacterial growth, nitrogen source concentrations can be adjusted by adding nitrogen sources during the fermentation process. According to the metabolic characteristics of microorganisms, an appropriate amount of inorganic salt ions are usually added to optimize the fermentation production of microorganisms. In addition to nitrogen-containing inorganic salts, inorganic salts containing elements such as sodium, calcium, potassium, sulfur, phosphorus, and magnesium are also important nutrients in the cell growth process. Trace elements such as zinc, iron, and copper are generally involved in microbial metabolism as coenzyme factors.

(2) Control of fermentation conditions

Temperature has a significant impact on enzyme activity, cell growth, protein properties, and the production of specific metabolites. In general, high temperature can cause enzyme denaturation, while low temperature can delay metabolic rate. Both can lead to a decrease in the yield of the target product. Therefore, choosing an appropriate temperature is beneficial for the growth of microorganisms and the synthesis of fermentation products. The pH of the fermentation broth is one of the important parameters in the fermentation process of Escherichia coli, which can reflect the metabolic activity of microorganisms. The growth and metabolism of microorganisms can affect the hydrogen ion balance in the culture medium, thereby altering pH. During the fermentation process, the pH of the culture medium constantly changes. Maintaining the pH within a certain range not only ensures the growth and metabolism of microorganisms, but also inhibits the proliferation of

miscellaneous bacteria. Researchers often use ammonia to regulate the pH of fermentation broth. Ammonia can also supplement nitrogen sources, but attention should be paid to the concentration of $NH_4^+$. If the concentration is too high, it will have an inhibitory effect on the growth of Escherichia coli. The growth and metabolism process of *Escherichia coli* MG1655 cannot be separated from the participation of oxygen, so the concentration of dissolved oxygen is also an important factor affecting cell growth and product synthesis. In the actual fermentation process, the level of dissolved oxygen varies, and the expression level of genes also varies. The level of dissolved oxygen needs to be determined based on the specific needs of product synthesis. For example, the fermentation of phenylalanine can only achieve maximum phenylalanine production when oxygen supply is limited. If oxygen supply is sufficient, the synthesis of the product is actually limited; Hao et al. increased the regeneration rate of cofactors through two-stage fermentation, thereby increasing the production of L-valine. Recombinant strains are usually divided into two stages during fermentation, namely the growth stage and the production stage. In the first stage, it is necessary to accumulate as much bacterial mass as possible, and in the second stage, the target product is synthesized. Sriubolmas et al. induced recombinant Escherichia coli to produce penicillin G acylase at different IPTG concentrations and found that enzyme activity increased with increasing IPTG concentration at low IPTG concentrations (0.025-0.1 mM), while enzyme activity gradually decreased at higher IPTG concentrations (0.2-0.5 mM). Therefore, it is necessary to choose the inducer and induction time.

*Escherichia coli* MG1655, as a popular laboratory strain, is commonly used in the production of protein drugs, recombinant enzymes, vaccines, biofuels, and other biological products. The fermentation production of its specific metabolites follows a precise standardized process. Common fermentation processes include shaking bottle fermentation culture method and fermentation tank culture method. The shake flask fermentation culture method usually involves transferring 3mL of seed culture to 30mL of fermentation medium with a 6% inoculation amount and placing it in a

500mL standard shake flask. The base sugar is 10g ·$L^{-1}$, and 400 g ·$L^{-1}$ glucose solution is added dropwise to maintain the residual sugar concentration of the fermentation broth. The fermentation is carried out at 37 ℃ and a speed of 100r · $min^{-1}$, and the culture is carried out for 36 hours. The fermentation tank cultivation method generally requires taking an appropriate amount of seed liquid and transferring it to the fermentation tank. The base sugar is 10g · $L^{-1}$. The residual sugar concentration of the fermentation liquid is controlled at 5-10g ·$L^{-1}$ by adding 700g · $L^{-1}$ glucose solution, adding 10% polyether defoamer, and adding concentrated ammonia water to control the pH at around 7.3; By adjusting the stirring speed and ventilation rate, the relative dissolved oxygen (DO) of the culture medium in the fermentation tank is controlled at around 25%, the cultivation temperature is 30 ℃, and the fermentation tank is cultured for 36 hours. To ensure the smooth progress of the fermentation experiment, samples are taken every 2 hours during the fermentation process to measure OD600, pH, and glucose concentration.

The specific operation of the fermentation production process of *Escherichia coli* MG1655 generally requires the first recovery of Escherichia coli MG1655 from a stored strain library, and then inoculation into LB liquid culture medium or other suitable basic culture media for *Escherichia coli* growth for seed cultivation. During this period, the temperature is controlled at 37 ℃ and the rotation speed is 200 rpm, and the cultivation is carried out for several hours until overnight, so that the bacterial body reaches a certain concentration. Based on the requirements of the target product, select a fermentation medium containing suitable carbon sources, nitrogen sources, inorganic salts, trace elements, growth factors, and possible inducers. Inoculate the expanded seed liquid into a fermentation tank in a certain proportion (such as 1%~10%), and the tank needs to be thoroughly cleaned and disinfected in advance, and filled with sterilized culture medium. During the fermentation process, strict monitoring and management should be implemented, with regular analysis of growth and product indicators and flexible optimization. Temperature (usually 37 ℃), pH (such as around 7.0, depending on specific strain and product requirements),

dissolved oxygen (DO), and tank pressure should be precisely controlled to maintain optimal physiological status and metabolic activity. For long-term or high-density fermentation, dynamic feeding strategies should also be implemented to maintain growth rate, prevent substrate inhibition effects, and promote product accumulation. Effective methods are used to isolate bacterial cells during harvest, followed by the use of various purification techniques to obtain the target product. Finally, chemical, physical, and biological activity analyses are conducted on the product to verify purity and activity, ensuring efficient, stable, and compliant production of the target product.

### 1.6 Common fermentation processes for ε -polylysine

With the development of biotechnology, the fermentation process of ε-polylysine has also been continuously optimized and improved, and advanced technologies such as genetic modification and metabolic engineering have been continuously applied in fermentation production, further improving production efficiency and product characteristics of ε-polylysine. Common fermentation processes include medium optimization, pH regulation, and dissolved oxygen control.

The composition of the culture medium has a significant impact on microbial growth and product metabolism. In 2006, SHIH et al. developed a culture medium suitable for *S.Albulus* IFO 14147 using response surface methodology, greatly increasing the Production of ε- PL; CHHEDA et al. used orthogonal design method to determine the new type the optimal composition of the culture medium for ε- PL producer Bacillus cereus enables the ε-PL yield significantly increased from 36.29mg/L to 83.49mg/L. M3G medium was first reported to be used for the culture medium for ε-PL fermentation and widely used in production of the ε-PL, single factor method, orthogonal array method, response surface method, etc. can be used to optimize this original culture medium.

Meanwhile, researchers have found that certain industrial by-products can also replace commonly used carbon and nitrogen sources in ε-PL production，it can not

only reduce the production cost of ε-PL can also convert waste materials into high-value products. For example, Fujiaolong and others use cassava residue hydrolysate as a carbon source in fermentation culture medium for production ε- PL. REN et al. used glycerol as a carbon source in the ε-PL fermentation medium has been further improved based on this medium. Ultimately the yield of ε- PL increased from the initial 2.27g/L to 62.36g/L. It can be seen from this that low-cost industrial and agricultural waste can be used as a substitute carbon source required in ε- PL fermentation is not only economical and environmentally friendly, but also has broad development prospects.

The experiments show that the optimal pH for accumulating ε -PL is 4.0 and the optimal pH for cell growth is 6.0, while the pH control strategy can ensure sufficient accumulation of ε -PL high biomass by automated fed base solution. REN et al. improved this classic pH control strategy by combining it with acidic pH shock, and the fermentation process can be divided into three stages, namely, pre-acid shock adaptation stage at pH is 5.0, acidic pH shock at pH3.0, and pH recovery to 4.0 to produce ε -PL. Eventually, the maximum yield of ε -PL in the fermentation broth was up to 54.70g/L. In contrast to the classical two-segment pH control method, the metabolic activity of the production strain is severely inhibited during the pH shock period. When pH values were at 4.0, cell growth and ε -PL production were significantly enhanced, and were more favorable for cell growth when pH> 4.5.

Dissolved oxygen level is another key parameter in ε -PL production. The fermentation broth with high dissolved oxygen levels favors cell growth and ε -PL biosynthesis, however high oxygen consumption and cell density often limit the oxygen supply in the ε -PL fermentation broth. BANKAR et al. established a two-stage DO control strategy suitable for the fermentation kinetics of S. Noursei NRRL 5126 by studying different aeration and stirring conditions. XU and others respectively use two strategies to improve the oxygen limitation problem, one is to add an oxygen carrier to the fermentation broth, adding 0.5% n-dodecane to the fermentation broth could effectively keep the DO content greater than 32%

saturation. Under the condition of increasing oxygen, the ε -PL yield increased from 23.4g/L to 30.8g/L. The second is the introduction of the hyalinto hemoglobin gene into the S.albulus PD-1 chromosome to deliver oxygen to terminal respiratory oxidase, which greatly enhances its ability to bind oxygen and increases the production of ε -PL from 22.7g/L to 34.2g/L.

Optimizing the bioreactors may also improve the overall fermentation process efficiency. The most commonly used bioreactors in the ε -PL are the reactors with mechanical agitation and conventional ventilation. However, however and others innovatively used loofah as green fixation material for ε -PL production, combined ISPR with cell fixation technology, and the final yield and bacteria volume of ε -PL reached 34.1 and 26.5g/L respectively, which can also greatly shorten the fermentation cycle. This novel solid-state fermentation has promise for application in producing ε -PL in the near future.

In addition, there are many new strategies to produce ε -PL, such as CHEEN, which improve the purification efficiency of ε -PL for the first time by means of optimizing the ion form of ion exchange resin. The results show that NH4 + is the best ionic form for IRC-50 resin to extract ε -PL. In addition, the researchers also extracted ε -PL with the environmentally friendly alcohol / salt dual water system (ATPS). The product purity reaches 92.39% and the recovery rate is 87.72%, providing more possibilities for the purification of ε -PL. YAN and other experiments found that reducing the oxidative stress in cells is a new strategy to improve ε -PL production. Adding the antioxidant glutathione (glutathione, GSH) could increase the highest increase of ε -PL fermentation level to 68.8% in the fermentation shake flask, and the highest ε -PL level of fed batch fermentation was 46.5g/L. Thus the addition of reducing agents to improve excessive intracellular oxidative stress helps to maintain a high rate of ε -PL production during fermentation.

ε-PL production using *Escherichia coli* MG1655 as the starting strain is a precision engineering project, which includes inoculating the recovered Escherichia coli MG1655 into a culture medium for seed cultivation, optimizing the preparation

of the culture medium, and strictly controlling the fermentation conditions to ensure healthy growth of the bacteria and efficient accumulation of products. In order to improve ε-PL requires detailed optimization of fermentation conditions, including adjustments to the composition of the culture medium (carbon source, nitrogen source, minerals, growth factors, etc.), pH value, temperature, dissolved oxygen level, fermentation time, and inoculation amount. On the basis of single factor experiments, ε − PL content as the standard, response surface experiments were conducted on the total amount of composite carbon sources, carbon source ratio, total amount of composite nitrogen sources, and the ratio of organic and inorganic nitrogen sources,the purified ε -polylysine requires further treatment, such as concentration, drying, to meet the required quality standards and ensure its efficacy and safety as a food preservative or other application areas.

### 1.7 Research ideas for the project

*Escherichia coli* MG1655 is a widely used industrial fermentation strain, through optimized the E. coli MG1655 strain fermentation of ε -PL, such as medium composition, each distribution ratio , using single factor and response surface analysis to obtain the optimal fermentation conditions, in order to get the most suitable for E. coli MG1655 production ε -PL fermentation conditions, to lay some experimental foundation for further industrial production.

# CHAPTER 2

# OBJECT, PURPOSE, AND METHODS OF THE STUDY

## 2.1 Experimental strains and instruments

*Escherichia coli* MG1655 K-12 was provided by the laboratory;The main instruments are shown in Table 2.1.(Tab. 2.1)

Table 2.1 – **Main instruments**

| **Instrument name** | **company** |
|---|---|
| Purification workbench | Suzhou Baizhao Scientific Instrument Co., Ltd |
| Electronic balance | Mettler Toledo Instruments Co., Ltd |
| Precision pH meter | Thermo Flyer Orion |
| Waterproof electric constant temperature incubator | Shanghai Jinghong Experimental Equipment Co., Ltd |
| CNC ultrasonic cleaner | Kunshan Ultrasound Instrument Co., Ltd |
| Digital blast drying oven | Shanghai Boxun Industrial Co., Ltd. Medical Equipment Factory |
| Dual beam UV visible spectrophotometer | HITACHI, Japan |
| Multi functional combination shaker | Shanghai Minquan Instrument Co., Ltd |

## 2.2 Reagent and medium

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

This experiment adopts a Box-Behnken design with three factors and three levels, including glucose content, yeast powder content, and $(NH_4)_2SO_4$, based on the results of a single factor experiment. The experimental factor levels and codes are shown in Table 2.2.(Tab. 2.2)

Table 2.2 – **Box Behnken experimental factor level and coding**

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

## 2.3 Experimental methods

### 2.3.1 Determination of the ε-PL concentration

The common determination methods of ε -polylysine mainly include chromatography, colorimetric methods and fluorescence methods. High performance liquid chromatography (HPLC) is a common analytical method, especially molecular exclusion chromatography (SEC) is suitable for determining the content and molecular weight of high molecular weight substances such as ε-polylysine. The fermentation broth containing ε-polylysine was appropriately treated and dissolved in organic solvents, separated using a column according to the molecular size, followed by quantitative analysis. In addition, the concentration of ε-polyfluorescence can be quantified by the quenching or enhancement effect of poly-lysine. For example, the fluorescence signal change was measured by the interaction of a specific fluorescent

probe with ε-polylysine. This method is sensitive and highly specific, and it is suitable for the analysis of trace samples. The fluorescence quenching method, similar to the fluorescence method, is also based on the quenching effect of ε -polylysine on the fluorescence emitted from fluorescent molecules, and its concentration was determined by measuring the change in the fluorescence intensity before and after the addition of ε -polylysine. The above methods need to accurately control the experimental conditions to ensure the accuracy of the results, but the experimental equipment has high requirements and high cost, which is difficult to meet the simple and rapid analysis requirements of research and development and production.

However, the methyl orange method is sensitive and simple, which is often used to determine the content of ε -polylysine content in the fermentation broth. 2 mL of the tested solution is mixed with 2 mL of methyl orange solution (1 mmo / lL) for 30℃ oscillation reaction for 30min, and centrifuged at 4000 r/min for 15min. The supernatant was diluted 10 times with potassium phosphate buffer (1 mmo/lL pH6.9), using potassium phosphate buffer and methyl orange as a blank control, and the absorptive value of 465nm was determined using a spectrophotometer, which has showed a high correlation coefficient ($R^2$=0.9993) and sample recovery (greater than 98%).

Can also absorb 1 mL of samples containing different concentrations of ε polylysine, place in a 5-mL centrifuge tube, added 1 mL of the DR reagent, shake up, schlammbildung, centrifugation at 7,000 r/min for 10 min, discard the supernatant, the precipitates were washed with 2 mL of absolute ethanol, centrifugation at 7,000 r/min for 10 min, discard the supernatant, add to 2 mL $Na_2S$, to duce a black precipitate, by centrifugation at 12,000 r/min for 10 min, discard the supernatant, 2 mL of concentrated nitric acid was added to the precipitate, after 20 min, With the precipitate being completely dissolved, diluted to 10 mL with deionized water, remove the 1 ml, add 5 mL of thiourea solution. At 20% nitric acid solution was

added to thiourea, and the absorbance value was measured at λ =435 nm, and then, the concentration of ε -polylysine was analyzed.

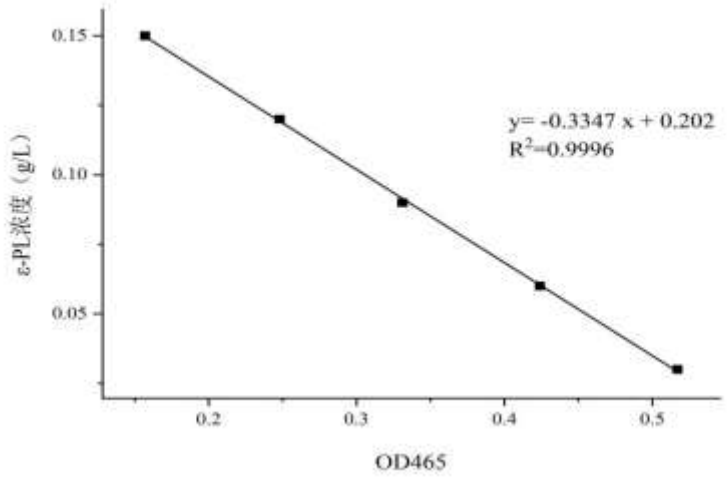



Figure 2.1 - Standard curve as determined by the ε-polylysine concentration

### 2.3.2 Yeast powder promotion ε - Polylysine production experiment

Select 6 gradients with yeast powder content ranging from 2.5g to 15.0g, and set three repeated groups for each gradient to reduce the impact of errors on the experimental results. The fermentation medium contains glucose as a carbon source, as well as other necessary inorganic salts and trace elements. Under the same conditions mentioned above, maintaining a temperature of 30 ℃ and adjusting the pH value above 5.0 promotes bacterial growth, and later adjusting it to around 4.0 promotes bacterial growth ε- PL synthesis, culture for 5 days, and measure the fermentation broth separately ε－ Polylysine concentration.

### 2.3.3 Ammonium sulfate promotes ε - Polylysine production experiment

Design a series of experimental groups, and add different concentrations of yeast powder (such as 8g/L, 10g/L, 12g/L, 14g/L, etc.) to each group. The other culture medium components and ratios are the same as the above experiments. Set up multiple repeated experiments to increase the statistical accuracy of the data. Control

the temperature at around 37 ℃, adjust the pH value to neutral to slightly alkaline, and maintain sufficient oxygen supply during the fermentation process to support bacterial growth and product synthesis. Cultivate for 5 days and measure the fermentation broth separately ε－ Polylysine concentration.

### 2.3.4 Glucose promotion ε - Polylysine production experiment

Select glucose with different concentrations of 30g, 40g, 50g, 60g, 70g, and 80g, and repeat multiple groups to improve the reliability of the experiment. Take fermentation broth separately for measurement ε－ Polylysine concentration.

### 2.3.5 Response surface experimental design

On the basis of single factor experiments,ε－PL response surface experiment was conducted on the ratio of glucose, yeast powder, and ammonium sulfate using the content of polylysine as the indicator [35]. Box-Behnken was selected as the response surface design type, and the experiment was carried out according to the designed plan. The independent variable settings and corresponding response variable values of each experimental point were recorded. The experimental data were all regression fitted using Design Expert Software Version 8.0.6 software, and the results of the variance analysis of the quadratic regression model were obtained to evaluate the significance and applicability of the model. By constructing a response surface graph, the trend of the influence of various factors and their interactions on response variables can be visually displayed, and then the culture medium formula can be optimized. Ammonium sulfate promotes ε - Polylysine production experiment.

# CHAPTER 3
# EXPERIMENTAL PART

## 3.1 Single factor experimental results

Change the content of yeast powder in the fermentation medium while keeping the remaining components unchanged. After fermentation, measure the content of yeast powder in the fermentation broth using colorimetric method ε- Polylysine content. As shown in Table 3.1, when the yeast powder content is between 2.5g and 5.0g, ε- The content of polylysine is positively correlated with the content of yeast powder; When the yeast powder content is 5.0g/L ε- The average content of polylysine reaches its maximum value, which is 0.13g/L; When the yeast powder content is between 5.0 g and 15.0 g, ε- The content of polylysine is negatively correlated with the content of yeast powder. Therefore, the optimal content of yeast powder is 5.0 g(Tab. 3.1).

Table 3.1 – **Results of the yeast powder-facilitated ε polylysine production experiments**

| The ε -Polylysine content | | | | |
|---|---|---|---|---|
| Yeast powder content (g) | Yeast powder content | The ε -Polylysine content（g/L） | Average content of ε -polylysine（g/L） | Standard deviation (error bar) |
| 2.5 | 2.5① | 0.09 | 0.08 | 0.01 |
| | 2.5② | 0.08 | | |
| | 2.5③ | 0.09 | | |
| 5.0 | 5.0① | 0.13 | 0.13 | 0.01 |
| | 5.0② | 0.14 | | |
| | 5.0③ | 0.13 | | |

| | | | | |
|---|---|---|---|---|
| 7.5 | 7.5① | 0.11 | 0.11 | 0.01 |
| | 7.5② | 0.12 | | |
| | 7.5③ | 0.10 | | |
| 10.0 | 10.0① | 0.08 | 0.08 | 0.00 |
| | 10.0② | 0.09 | | |
| | 10.0③ | 0.08 | | |
| 12.5 | 12,5① | 0.06 | 0.07 | 0.01 |
| | 12.5② | 0.07 | | |
| | 12.5③ | 0.08 | | |
| 15.0 | 15.0① | 0.05 | 0.05 | 0.01 |
| | 15.0② | 0.04 | | |
| | 15.0③ | 0.05 | | |

Change the content of $(NH_4)_2SO_4$ in the fermentation medium while keeping the remaining components unchanged. After fermentation, measure the content of $(NH_4)_2SO_4$ in the fermentation broth using colorimetric method ε- Polylysine content. As shown in Table 3.2, when the content of $(NH_4)_2SO_4$ is between 8.0 g and 10.0 g, ε- The content of polylysine is positively correlated with the content of $(NH_4)_2SO_4$; When the content of $(NH_4)_2SO_4$ is 10.0g, ε- The average content of polylysine reaches its maximum value, which is 0.13g/L; When the concentration of $(NH_4)_2SO_4$ is between 10.0 g/L and 18.0 g/L, ε- The content of polylysine is negatively correlated with the content of $(NH_4)_2SO_4$. Therefore, the optimal concentration of $(NH_4)_2SO_4$ is 10.0 g/L.(Tab. 3.2).

Table 3.2 – **Results of experiments where ammonium sulfate promoted ε polylysine production**

| The ε -Polylysine content | | | | |
|---|---|---|---|---|
| $(NH_4)_2SO_4$ content （g） | $(NH_4)_2SO_4$ content | The ε -Polylysine content (g/L) | Average content of ε –polylysine (g/L) | Standard deviation (error bar) |
| 8 | 8① | 0.11 | 0.10 | 0.01 |
| | 8② | 0.10 | | |
| | 8③ | 0.10 | | |
| 10 | 10① | 0.13 | 0.13 | 0.01 |
| | 10② | 0.14 | | |
| | 10③ | 0.13 | | |
| 12 | 12① | 0.11 | 0.11 | 0.01 |
| | 12② | 0.10 | | |
| | 12③ | 0.12 | | |
| 14 | 14① | 0.09 | 0.08 | 0.01 |
| | 14② | 0.08 | | |
| | 14③ | 0.07 | | |
| 16 | 16① | 0.06 | 0.06 | 0.01 |
| | 16② | 0.05 | | |
| | 16③ | 0.07 | | |
| 18 | 18① | 0.04 | 0.04 | 0.01 |
| | 18② | 0.04 | | |
| | 18③ | 0.05 | | |

Change the glucose content in the fermentation medium while keeping the remaining components unchanged. After fermentation, measure the glucose content in the fermentation broth using colorimetric method ε- Polylysine content. As shown in Table 3.3, when the glucose content is between 30g and 50g, ε- The content of polylysine is positively correlated with the content of glucose; When the glucose content is 50g, ε- The average content of polylysine reaches its maximum value, which is 0.13g/L; When the glucose content is between 50 g and 80 g, ε- The content of polylysine is negatively correlated with the content of glucose. Therefore, the optimal content of glucose is 50 g(Tab. 3.3).

Table 3.3 –**Results of experiments promoting glucose for ε poly-lysine production**

| The ε -Polylysine content | | | | |
|---|---|---|---|---|
| glucose content (g) | glucose content | The ε -Polylysine content（g/L） | Average content of ε -polylysine（g/L） | Standard deviation (error bar) |
| 30 | 30① | 0.08 | 0.09 | 0.00 |
| | 30② | 0.09 | | |
| | 30③ | 0.08 | | |
| 40 | 40① | 0.11 | 0.11 | 0.01 |
| | 40② | 0.09 | | |
| | 40③ | 0.11 | | |
| 50 | 50① | 0.13 | 0.13 | 0.01 |
| | 50② | 0.14 | | |
| | 50③ | 0.13 | | |
| 60 | 60① | 0.09 | 0.10 | 0.01 |
| | 60② | 0.10 | | |

| | | | | |
|---|---|---|---|---|
| | 60③ | 0.11 | | |
| 70 | 70① | 0.08 | 0.08 | 0.01 |
| | 70② | 0.08 | | |
| | 70③ | 0.07 | | |
| 80 | 80① | 0.05 | 0.06 | 0.00 |
| | 80② | 0.06 | | |
| | 80③ | 0.06 | | |

### 3.2 Box-Behnken test results

Table 3.4– **Results of the Box-Behnken experimental design**

| Experimental number | glucose/g | yeast powder /g | ammonia sulfate/g | The ε -Polylysine content（g/L） |
|---|---|---|---|---|
| 1 | 70 | 10 | 12 | 0.09 |
| 2 | 50 | 10 | 12 | 0.16 |
| 3 | 60 | 7.5 | 12 | 0.30 |
| 4 | 60 | 7.5 | 12 | 0.28 |
| 5 | 60 | 5 | 14 | 0.17 |
| 6 | 50 | 5 | 12 | 0.10 |
| 7 | 60 | 5 | 10 | 0.15 |
| 8 | 60 | 10 | 10 | 0.16 |
| 9 | 50 | 7.5 | 10 | 0.14 |
| 10 | 60 | 7.5 | 12 | 0.30 |
| 11 | 70 | 7.5 | 10 | 0.18 |
| 12 | 70 | 7.5 | 14 | 0.18 |
| 13 | 60 | 10 | 14 | 0.15 |

| | | | | |
|---|---|---|---|---|
| 14 | 60 | 7.5 | 12 | 0.28 |
| 15 | 60 | 7.5 | 12 | 0.32 |
| 16 | 50 | 7.5 | 14 | 0.16 |
| 17 | 70 | 5 | 12 | 0.15 |

### 3.3 Response surface results and analysis

All experimental data were processed by regression fitting using Design Expert Software Version 8.0.6 software, obtaining the results of secondary regression model ANOVA, Table 3.5 below, which gives the relevant data of secondary regression analysis.

Table 3.5– **ANOVA for Response Surface Quadratic Model**

| Source | Squares | df | Square | Value F | Prob > | |
|---|---|---|---|---|---|---|
| Model | 0.084 | 9 | 9.284E-003 | 31.68 | < 0.0001 | significant |
| A-gleucos | 1.825E-004 | 1 | 1.825E-004 | 0.62 | 0.4559 | |
| B- yeast powder | 2.851E-006 | 1 | 2.851E-006 | 9.730E-003 | 0.9242 | |
| C-$(NH_4)_2SO_4$ | 1.397E-004 | 1 | 1.397E-004 | 0.48 | 0.5121 | |
| AB | 3.423E-003 | 1 | 3.423E-003 | 11.68 | 0.0112 | |
| AC | 1.155E-004 | 1 | 1.155E-004 | 0.39 | 0.5501 | |
| BC | 4.120E-004 | 1 | 4.120E-004 | 1.41 | 0.2744 | |
| A^2 | 0.028 | 1 | 0.028 | 95.70 | < 0.0001 | |
| B^2 | 0.033 | 1 | 0.033 | 113.24 | < 0.0001 | |
| C^2 | 0.010 | 1 | 0.010 | 35.03 | 0.0006 | |
| Residual | 2.051E-003 | 7 | 2.930E-004 | | | |
| Lack of Fit | 9.723E-004 | 3 | 3.241E-004 | 1.20 | 0.4162 | not significant |

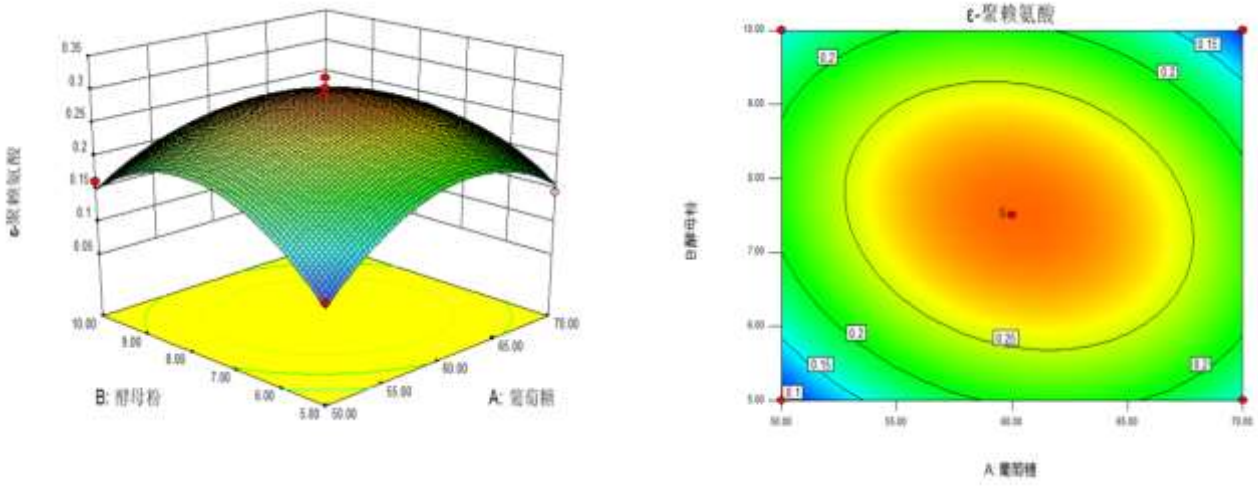
| | | | |
|---|---|---|---|
| Pure Error | 1.079E-003 | 4 | 2.697E-004 |
| Cor Total | 0.086 | 16 | |

Notes：R-Squared = 0.976；Adj R-Squared = 0.9452

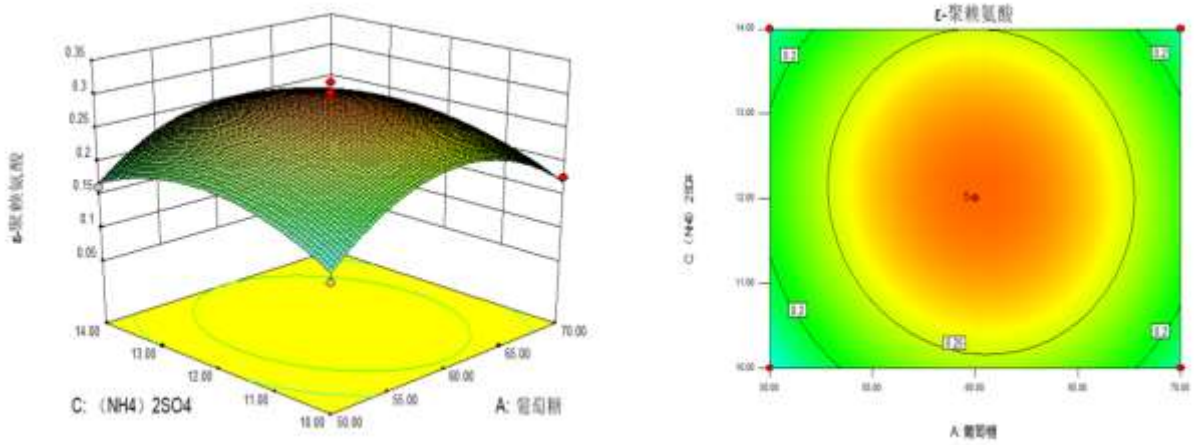
F= 31.68. P <0.05, indicating that the model is significant. The model mismatch term (lack of fit) indicates the probability that the model predicted value does not fit the actual value, P> 0.05, the mismatch term is not significant, so the model is chosen correctly. The correlation coefficient of the model, R-Squared=0.976 and AdjR-Squared=0.9452, indicates a good fit of Eq. In conclusion, the regression equation provides a suitable model for ε -poly-lysine prediction analysis.

### 3.4 The effect of each factor on ε -polylysine production was analyzed by response surface method

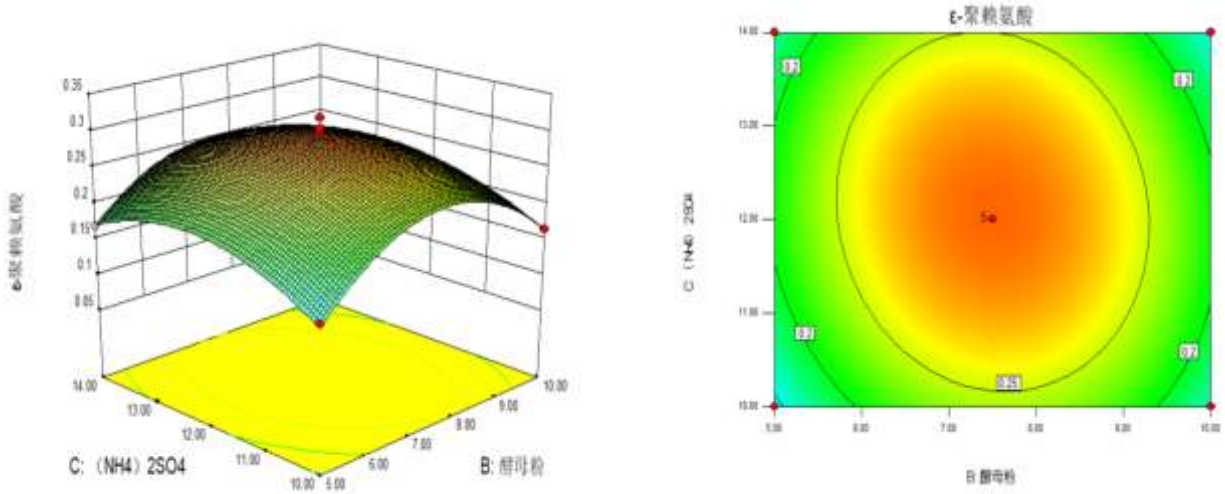
The response surface is fitted by an equation, and the response surface graph and contour map are detailed in Figure 3.1.(Fig. 3.1).




(a)




(b)




(c)

Fig.3.1– Response surface curves and contour plots for each condition

(a) Response surface curve and contour plots of glucose addition and yeast powder addition；(b) Glucose addition and ammonium sulfate response surface curves and contour plots；(c) Curve and contour plots of yeast powder addition and ammonium sulfate response

Figure (a) shows the response surface plots and contour plots of glucose addition and yeast powder to ε -polylysine production, respectively. When other factors are the best value, with the continuous increase of glucose addition, ε -polylysine production decreases from low to high, and the change range is obvious, indicating that the factor has a great influence on the production of ε -polylysine; however, with the increase of yeast powder content, the production of ε -polylysine increases first and then decreases, indicating that the effect of ammonium sulfate on ε -polylysine production is also obvious.

Figure (b) shows the response surface curve and contour plot of glucose addition and ammonium sulfate addition. When the other two factors are the best value, with the increase of glucose content, the activity of ε -polylysine showed a trend of increasing first and then decreasing, which indicates the obvious influence of glucose on the production of ε -polylysine; With the increase of ammonium sulfate, the activity of ε -polylysine also showed a trend of rising first and then decreasing, indicating that the content of ammonium sulfate has a great impact on the production of ε -polylysine.

Figure (c) shows the response surface plots and contour plots of yeast powder addition and ammonium sulfate to ε -polylysine production, respectively. When other factors were the best value, the yield of ε -polylysine increased first and then decreased, indicating that this factor affected the production of ε -polylysine. With the increase of ammonium sulfate, the yield of ε -polylysine increased first and then decreased, indicating that the factor has a significant effect on the production of ε -polylysine, which indicates the interaction between these two factors.

### 3.5 Determination and verification of optimal fermentation conditions for ε -polylysine

The experimental data and model were analyzed by software, and the best fermentation conditions for ε -polylysine fermentation were obtained: glucose addition was 60.29 g, yeast powder 7.49 g, ammonium sulfate 12.08 g, and the

predicted value of ε -polylysine was 0.2953 g / L. To test the accuracy and feasibility of the response surface method, the ε -polylysine was tested and tested in triplicate. The average yield of ε -polylysine was obtained as 0.286 ± 0.016 g / L. The result does not differ much from the predicted value of the model, which proves the accuracy of the model and has practical value.

# CONCLUSIONS

1. This study of the ε -polylysine, physicochemical properties, application, synthesis mechanism, introduced in detail, and the E. coli MG1655 morphological characteristics, preparation method, operation feasibility, followed by the experimental materials and methods, mainly for this experiment experimental reagents, equipment and experimental principle and experimental method, etc.

2. Through the growth of E. coli MG1655 strains and fermentation synthesis ε -polylysine condition optimization, the production of E. coli MG1655 fermentation ε -PL culture conditions, including the medium composition, the distribution ratio, initial sugar concentration, using batch culture method to greatly improve the body density, in order to improve the production efficiency of ε -PL.

3. The experiment explored the influence of medium ratio, pH, temperature, time and other factors on ε -PL, and conducted univariate and response surface analysis to obtain the optimal fermentation conditions: glucose addition was 60.29 g, 7.49 g yeast powder, ammonium sulfate 12.08 g, and the predicted value of ε -polylysine was 0.2953 g / L. To test the accuracy and feasibility of the response surface method, the ε -polylysine was tested and tested in triplicate. The average yield of ε -polylysine was obtained as 0.286 ± 0.016 g / L. The result does not differ much from the predicted value of the model, which proves the accuracy of the model and has practical value.

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