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

QUALIFICATION THESIS

on the topic **Study on optimizing the fermentation of putrescine using *Bacillus subtilis* 168 engineering strain**
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Specialty 162 "Biotechnology and Bioengineering"
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Completed: student of group BEBT-21
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APPROVE
Head of Biotechnology, Leather and Fur Department, Professor,
Dr. Sc., Prof.
_____Olena MOKROUSOVA
«___»_____2025

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Zhao Yanming

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Scientific supervisor Ph.D., Assoc. Prof. Olena Okhmat

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1	Introduction	until 11 April 2025	
2	Chapter 1. Literature review	until 20 April 2025	
3	Chapter 2. Object, purpose, and methods of the study	until 30 April 2025	
4	Chapter 3. Experimental part	until 11 May 2025	
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Abstract

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1,4-Butanediamine (1,4-diaminobutane), an important chemical intermediate, finds extensive application in high-performance nylon, pharmaceutical intermediates, and bio-based materials, among others. As a result, its market demand has been continuously growing. Traditional chemical synthesis methods depend heavily on non-renewable resources and result in significant environmental pollution. Consequently, the advancement of green and efficient biological synthesis approaches has emerged as a prominent research focus. *Bacillus subtilis* 168, owing to its well-characterized genetic background, diverse molecular modification techniques, and excellent biosafety profile, serves as an ideal platform for the biosynthesis of 1,4-butanediamine. In this study, the fermentation process of the engineered *Bacillus subtilis* 168 strain was optimized using single-factor experiments and response surface methodology. The results indicated that the optimal fermentation conditions were 8.5 g/L glucose, 12.6 g/L L-arginine, and a fermentation time of 31.8 h, achieving a 1,4-butanediamine yield of 0.782 ± 0.028 g/L. This value closely matched the model prediction, thereby validating the reliability and accuracy of the model. This study offers a solid theoretical and experimental foundation for the industrial production of 1,4-butanediamine.

Key words: 1,4-butanediamine, Bacillus subtilis 168, fermentation optimization, Response Surface Methodology, single factor analysis

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INTRODUCTION

In this study, through single-factor experiments, Box-Behnken response surface methodology, and fermentation process validation, we systematically optimized the metabolic efficiency of an engineered *Bacillus subtilis* 168 strain for 1,4-butanediamine biosynthesis. Experimental results demonstrated that under optimized conditions (8.5 g/L glucose, 12.6 g/L L-arginine, and 31.8 h fermentation time), the 1,4-butanediamine yield reached 0.782 ± 0.028 g/L, representing a 3.13-fold increase compared to pre-optimization levels. Notably, traditional chemical synthesis methods for 1,4-butanediamine are energy-intensive and environmentally detrimental, whereas microbial-based biosynthesis aligns with global sustainability goals. This study provides a robust methodological framework for metabolic engineering in microbial cell factories, bridging the gap between laboratory-scale research and industrial applications.

The relevance of the topic is Green biosynthesis process development and metabolic engineering optimization.

The purpose of the study is to optimize the fermentation process of an engineered *Bacillus subtilis* 168, overcome metabolic bottlenecks, and enhance 1,4-butanediamine synthesis efficiency for industrial applications.

The objectives of the study are to investigate the independent regulatory effects of glucose concentration, L-arginine concentration, and fermentation time on 1,4-butanediamine production, establish a three-factor, three-level quadratic regression model to analyze variable interactions, and ultimately determine optimal process parameters while validating the model's engineering applicability for industrial scalability.

The object of the study Engineered *Bacillus subtilis* 168

The subject of the study 1,4-Butanediamine fermentation process optimization

Research methods single-factor experiments, Box-Behnken response surface design, model validation

The scientific novelty first systematic application of response surface methodology to optimize 1,4-butanediamine synthesis in *Bacillus subtilis* 168, elucidating mechanisms of key variable interactions.

The practical significance of the results obtained is the optimized process significantly improves yield, providing technical support for green production in bio-based polyamide materials and pharmaceuticals, and advancing progress toward the "Dual Carbon" goals (carbon peaking and carbon neutrality).

CHAPTER 1
LITERATURE REVIEW

1.1 OVERVIEW OF THE STUDIES ON PUTRESCINE

Putrescine, also known as 1, 4-diaminbutane or putrescine, is an aliphatic diamine compound ($C_4H_{12}N_2$) with a four-carbon chain linked to an amino group. It is one of the common binary amines found in prokaryotes and eukaryotes. Its molecular weight is small (about 88.15 g/mol), volatile and strong moisture absorption, need to be sealed and stored in the dark to prevent decomposition or moisture absorption. This compound was first identified as a metabolic marker of protein spoilage. It is a colorless liquid at room temperature with a strong ammonia taste, a boiling point of 158-160 °C, easily soluble in water, and strongly basic ($pKa_1 \approx 10.8$)[1, 2] .

1.1.1 SOURCES AND TYPES OF PUTAMYLDIAMINE

Butanediamine is an important biogenic amine and chemical intermediate. Its sources can be divided into two categories: chemical synthesis and biosynthesis. In industry, chemical synthesis is the dominant method, and its core process relies on petroleum-based raw materials through multi-step catalytic reactions. For example, Wanhua Chemical uses acrylonitrile, etc. as raw materials to synthesize cyano-propionaldehyde, and coproduces 1, 3-and 1, 4-butanediamine through ammonification and hydrogenation, with a total yield of over 95%, which has the advantages of high catalyst efficiency and low cost. Another typical method is to produce succinonitrile with acrylinitrile and hydrocyanic acid under basic catalyst, and then convert to the target product by hydrogenation[3] . Although the chemical method is mature and efficient, its dependence on non-renewable resources, strict reaction conditions and environmental pollution have prompted the research

direction to turn to green alternative technologies. The biosynthetic method uses metabolic engineering to modify microorganisms to achieve efficient synthesis of putamide. The biosynthetic pathway mainly includes three metabolic pathways, ornithine decarboxylase (ODC), arginine decarboxylase (ADC) and arginase (ARG), among which the ODC pathway is the main one in microbial fermentation, that is, the direct production of putamen by decarboxylation of L-ornithine catalyzed by SpeC/SpeF enzyme[5. 6.] . In addition, the biosynthesis of putenediamine is closely related to the urea cycle, and the metabolic abnormalities of its precursors arginine and ornithine have been found to be related to astrocytic dysfunction in Alzheimer's disease, revealing the natural existence and metabolic complexity of this substance in organisms[7.] . The former improves substrate conversion efficiency by optimizing the genetic characteristics of strains and culture conditions, while the latter relies on specific enzymes to modify the substrate structure. Both methods replace petrochemical resources with renewable raw materials such as glucose.

The types of butanediamine mainly involve its structural isomers and derivatives. Chemically, butanediamine is a derivative of butane containing two amino groups, the most common of which is 1, 4-butanediamine ($C_4H_{12}N_2$). This compound is colorless crystalline or liquid, has a pungent odor, is easily soluble in water, and is widely used in the synthesis of polyamide materials (such as PA46 and PA4T)[8.] , dye intermediates, and biomedical research, such as as a precursor of γ-aminobutyric acid (GABA) in Alzheimer's disease[7.] . Although the chemical formula is $C_4H_{12}N_2$, the other isomer, 1, 3-butanediamine, is less industrially used and mostly occurs as a co-product of chemical synthesis pathways, which needs to be further utilized by separation and purification.

1.1.2 STRUCTURE AND FUNCTION OF BUTYLENE DIAMINE

Butanediamine is a linear aliphatic binary amine compound composed of four carbon atoms with the molecular formula $H_2N(CH_2)_4NH_2$ and contains two isomers, 1, 3-butanediamine and 1, 4-butanediamine. Its two amino groups ($-NH_2$) are highly alkaline and reactive. Among them, 1, 4-butanediamine (putamine) is a polyamine precursor involved in the regulation of cell metabolism in vivo, while 1, 3-butanediamine is mainly used in industrial applications. Butyric diamine is a colorless transparent liquid at room temperature with a pungent ammonia taste. It is easily soluble in polar solvents such as water and ethanol. Under acidic conditions, it can produce polyamide materials by condensation reaction with carboxylic acid compounds, while maintaining chemical stability at high temperature (such as above 120°C)[9.] . Putamine is a common biogenic amine, which can combine with various biological macromolecules (proteins, nucleic acids, phospholipids, etc.) through hydrogen bonds and ionic bonds in the organism, participate in the metabolic regulation of cells, and play a role in the anti-tumor reaction with other biogenic amines in the organism[10.] . In addition, butanediamine is also of great value in the production of engineering plastics, pharmaceuticals, agrochemicals, and surfactants[11. 12.] . As a plant growth regulator, putamine plays an important role in the regulation of plant growth and development, and is also a commonly used agricultural chemical[13.] . The structure is shown in Figure 1.1.

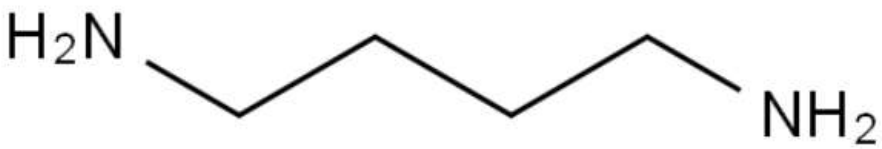

Figure 1.1 – Chemical structure of putamyldiamine

1.1.3 FACTORS INFLUENCING THE PRODUCTION OF PUTRESCINE

The production of putonediamine is affected by multiple factors. The research on the influencing factors of putonediamine fermentation mainly focuses on the following aspects: medium composition, pH, temperature, dissolved oxygen, fermentation mode, etc. By optimizing these parameters, the butanediamine synthesis ability of the strain could be significantly improved.

Medium is the material basis for the metabolism of bacteria, its distribution than directly decided to yield limit. The selection of carbon source is particularly critical. Although glucose can supply energy rapidly, its high concentration is easy to cause catabolite inhibition, so it is necessary to balance cell growth and product synthesis by feeding in batch. Slow-acting carbon sources such as glycerol were more conducive to the accumulation of secondary metabolites. Ammonium salt or combined nitrogen source should form an appropriate C/N ratio with carbon source. The addition of precursor substances such as glutamate can significantly improve the efficiency of putamine synthesis. In addition, trace elements such as Mg^{2+} and Fe^{2+} have a regulatory effect on glutamate decarboxylase activity, and deficiency can lead to the blocking of metabolic pathways.

Precise control of the same key environmental parameters. For example, the biomass accumulation of some strains is faster at 37°C, but the synthesis stage of butandiamine should be reduced to 30-33°C to reduce the competitive accumulation of by-products. The pH of fermentation broth should be stabilized between 6.5 and 7.5 to maintain the optimal activity of lysine decarboxylase. The level of dissolved oxygen is particularly important for aerobic fermentation. Sufficient oxygen can promote TCA cycle and energy metabolism, but excessive dissolved oxygen may induce oxidative stress, so it is necessary to adjust stirring rate or ventilation to achieve dynamic balance.

Fermentation mode choice directly affect the production cycle and cost efficiency. Fed-batch fermentation is a key strategy to prolong the fermentation cycle by adding limiting substrates (such as glucose or nitrogen), reduce the substrate inhibition effect, and finally increase the production of putamide by more than 40%.

1.1.4 COMMON APPLICATIONS OF PUTAMYLDIAMINE

As an important chemical intermediate, butylene diamine has shown diversified application value in the industrial field. Its industrial application is centered on the synthesis of polyamide materials, and extends to rubber modification, bio-based materials development and other fields. In the polyamide material system, the most important application of putamine is the synthesis of polyamide 46 (PA46)[8.] . The material was prepared by the condensation reaction of 1, 4-butanediamine (putreamine) and adipic acid. The uniform arrangement of amide groups in the molecular chain gives the material a high melting point of 295 ° C, more than 70% of the degree of crystallization, and excellent thermal mechanical properties [14. ~16.] . These characteristics make PA46 the preferred engineering plastic for heat-resistant components of automotive engines, turbocharger pipe fittings, transmission gears, as well as electronic and electrical packaging materials and heat dissipation components[17. 18.]. At the same time, the high-strength fiber made of PA46 by melt spinning plays an important role in the textile field such as special protective equipment and industrial press cloth. The annual global demand for 1, 4-butylene diamine has reached 10,000 tons, and the market price in Europe has exceeded 1,600 Euros/ton. With the expansion of downstream industries such as new energy vehicles and 5G equipment, the demand shows a continuous growth trend[19.] .

The application diversity of putamine is also reflected in the expansion of high temperature nylon materials. By copolymerization with monomers such as terylene

acid, aromatic polyamides with high thermal deformation temperature and chemical corrosion resistance can be prepared. Such materials show unique advantages in extreme working conditions such as chemical equipment lining and oil and gas seals[20.] . In the rubber industry, butanediamine derivatives, as an important precursor of vulcanization promoters, can significantly improve the wear resistance and anti-aging ability of tires, conveyor belts and other products by regulating the crosslinking density of rubber molecules. Although this application is not the mainstream direction, it has formed a stable market segment demand.

Under the trend of sustainable development, the bio-based properties of putrescine have attracted much attention. Compared with petroleum-based products, butanediamine prepared by biological fermentation has significant advantages in carbon footprint control, which is highly consistent with the current green transformation trend of the global chemical industry. It is worth noting that 1, 4-butanediol, a derivative compound of butanediamine, as a key monomer of polyester material, supports the industries such as polybutylene terepthalate (PBT) and polybutylene succinate (PBS) with an annual output of 2.5 million tons and a market value of 6.19 billion US dollars[21.] , further expanding the application dimension of butanediamine compounds. With the tightening of environmental protection regulations and the upgrading of low-carbon materials demand, the strategic value of butylene diamine in the field of bio-based polymer materials will continue to be highlighted, becoming a key node connecting traditional chemical industry and green manufacturing.

1.2 *BACILLUS SUBTILIS* 168 AND ITS BUTANEDIAMINE PRODUCTION

1.2.1 OVERVIEW OF *BACILLUS SUBTILIS* 168

Bacillus subtilis 168 is a model engineering strain of Gram-positive bacteria in Bacillus genus. It is known for its clear genetic background, perfect genetic

operation tools, and high safety (GRAS certification), and is widely used in the research fields of genetic engineering, industrial enzyme production, and synthetic biology.

The whole genome of this strain was sequenced in 1997, which was about 4.2 Mb in length and contained 4106 genes (271 of which were essential genes), laying the foundation for metabolic engineering and functional research[22]. It has the advantages of rapid division (about 30 minutes/passage), efficient secretion (such as Sec and Tat pathway) and absence of endotoxin, but the wild type has problems such as fermentation autolysis and protein degradation by extracellular protease[23]. Researchers constructed protease deficient hosts such as WB600 and WB800 by gene knockout technology, and knocked out 6 and 8 protease genes respectively, which significantly inhibited 99% of the extracellular protease activity[24, 25]. Westers et al. deleted 332 non-essential genes through genome simplification strategy, and the ability of the mutant strain to express cellulase and protease increased by 1.7 times and 2.5 times, respectively[26]. As an FDA-certified safe strain, it is widely used in food grade products (γ-polyglutamic acid, antimicrobial peptides), pharmaceutical proteins (lactoferritins), and agricultural resources (straw degradation). The introduction of dynamic regulatory systems, such as IPTG-inducible promoter[27] and mazF reverse selection marker plasmid[28], further optimized the precision and controllability of gene expression. Despite the challenges of limited promoter compatibility and insufficient plasmid stability, the potential of *Bacillus subtilis* 168 as an efficient microbial cell factory continues to be expanded by strengthening the robustness of chassis cells and optimizing metabolic network.

1.2.2 OVERVIEW OF COMMON FERMENTATION PROCESSES OF *BACILLUS SUBTILIS* 168

The core of *Bacillus subtilis* 168 fermentation technology lies in two key elements, namely the composition of fermentation medium and the control of fermentation conditions. Changes in the ratio of medium components directly affect the synthesis efficiency of target products by regulating the activities of key enzymes in the metabolic pathways of bacteria. At the same time, appropriate fermentation conditions (such as temperature, pH, dissolved oxygen, etc.) can build a stable environment for the growth and metabolism of bacteria, which can not only effectively shorten the fermentation cycle, but also significantly enhance the product accumulation ability.

(1) The composition of fermentation medium

Fermentation medium is the basis for the growth and metabolism of *Bacillus subtilis* 168, and its composition should be optimized according to strain characteristics and target product requirements. The medium usually contains carbon sources, nitrogen sources, inorganic salts, growth factors and trace elements. Carbon source is the main source of energy and carbon skeleton of bacteria. The type and concentration of carbon source (such as glucose, sucrose or starch) significantly regulate the growth rate of bacteria and the accumulation efficiency of metabolites. For example, as a simple carbohydrate, glucose at 40 g/L can significantly improve the growth rate and spore formation rate due to its direct metabolic path and high energy efficiency[29.] . Nitrogen sources provide amino acids and nucleotides through substances such as peptone, urea or ammonium sulfate, which lay the foundation for the propagation of bacteria and the synthesis of metabolites[30.] . Studies have shown that precise regulation of carbon-nitrogen ratio is crucial to product synthesis. For example, Yin Mengmeng et al.[31.] increased the protease yield of Bacillus brevus SCU11 by 100% by optimizing the ratio of carbon-nitrogen. In addition, inorganic salts such as NaCl, $MgSO_4$, K_2SO_4

and $CaCO_3$ play a key role in regulating osmotic pressure, maintaining enzyme activity and promoting sporulation. Among them, Ca^{2+} and Mg^{2+} significantly promoted bacterial proliferation and exogenous protein expression by activating key enzymatic reactions. However, $CaCO_3$ enhanced the sporulation rate by buffering the pH value of fermentation broth[32.] . Huang Fujia et al.[33.] further pointed out that the addition of an appropriate amount of phosphate can improve the biological activity of proteins. Through optimization methods such as response surface method and orthogonal experiment, medium ratio can be significantly improved. For example, KILANI-FEKI et al.[34.] increased the yield of antifungal metabolites by 93% by adjusting the medium composition of *Bacillus subtilis* M13, and Xu Rui Ping[35.] Achieved efficient growth by optimizing the formula of totally soluble medium.

(2) Control of fermentation conditions

Fermentation conditions directly affect the metabolic pathways of bacteria and the accumulation of target products, so it is necessary to optimize the process through the collaborative regulation of multiple parameters. Temperature has a significant effect on the growth and metabolism of *Bacillus subtilis* 168. Most studies have optimized 37℃ to maximize the number of bacteria and the accumulation of antibacterial substances[36. 37.] , while Mu Chang-ching[38.] cultivated *Bacillus subtilis* B-332 at 28℃ and 190 r·min-1 in a shaker, and found that its antibacterial activity against magnaporthe oryzae reached the peak. These results indicated that the different demand of target products should correspond to the specific temperature strategy. The initial pH regulation should be maintained in the neutral to weak alkaline range (such as pH 8.04) to ensure enzyme activity and product synthesis efficiency. Zhang Zhi et al.[39.] improved protease activity by 8% through pH optimization, which verified the directional regulation of environmental pH on metabolic pathways. The selection of inoculum should take into account the balance between efficiency and metabolism, usually controlled at 2%-4% (volume fraction), too high is easy to cause the accumulation of metabolic

waste, too low will prolong the fermentation cycle. Xu Yaying et al.[40.] used the single-factor experimental method to optimize the fermentation process parameters of *Bacillus subtilis*, and finally determined 4% as the optimal inoculum amount. The synergistic regulation of liquid loading and fermentation speed directly affects dissolved oxygen coefficient (KLa). Reducing liquid loading can improve dissolved oxygen efficiency, but the evaporation problem of fermentation broth needs to be balanced. High rotational speed (e.g., 200-250 $r \cdot min^{-1}$) can enhance the mixing degree of bacterial fluid and improve the contact efficiency of bacteria with nutrients and oxygen, thereby promoting growth and sporulation[41.] . Huang Fan et al.[42.] optimized the yield of neutral protease by dynamically adjusting the speed, reflecting the dynamic regulation of dissolved oxygen level on product synthesis. In addition, fed-batch fermentation strategy can effectively avoid nutrient deficiency and accumulation of metabolic by-products (such as acetic acid) by real-time addition of carbon sources, nitrogen sources and inorganic salts and intermittent discharge of part of the fermentation broth. MEENA et al.[43.] used the response surface method to optimize the fed-parameters of Bacillus subsp. KLP2015, and increased the lipopeptide yield by 1.8 times. It further confirmed the universal value of dynamic feeding to industrial production.

The fermentation production of *Bacillus subtilis* 168 needs to start from the activation of the strain. First, the stored strain is inoculated into the activation medium (such as LB medium) and cultured at 37℃ and 180 rpm for 18-24 hours to restore the activity. Then the activated strains were transferred to the optimized seed medium (containing cassava starch, sodium nitrite and other components), expanded to a cell density of more than 1.0×108 cfu/ml at 33℃ and 150-200 rpm to form the secondary seed solution. According to the metabolic characteristics and synthesis requirements of the target products, fermentation media containing suitable carbon sources (such as glucose), efficient nitrogen sources (such as soybean meal meal), inorganic salts, trace elements, growth factors, and specific inducers were optimized. During inoculation, the secondary seed liquid was added

into the fermenter in a certain proportion, and the metabolism of the bacteria was optimized by dynamic regulation of temperature (mainly 37℃), pH (7.2-8.0) and dissolved oxygen (DO, maintained by stirring at 200 rpm and ventilating at 0.5-1.3 m^3/h). In order to avoid nutrient deficiency, carbon, nitrogen, and trace elements were added in real time. The cell density and product concentration were monitored by online sensors, and the feeding parameters were optimized by response surface method. For high viscosity fermentation broth, heat shock treatment (short time heating up 5-10℃ and then cooling down) is implemented regularly to improve the yield. At the end of fermentation, the bacteria or extracellular products were separated by centrifugation (4000-8000 rpm), and the target products were obtained by spray drying or chromatography purification. Finally, the efficacy of the products was verified by antibacterial activity or enzyme activity detection.

1.2.3 FERMENTATION PROCESS OF BUTYLENE DIAMINE PRODUCTION BY *BACILLUS SUBTILIS* 168

The production of butanediamine by engineered *Bacillus subtilis* strain 168 is a highly systematic bioengineering practice. Its core goal is to improve the density of bacteria and the efficiency of product synthesis through multi-dimensional collaborative optimization of medium composition and fermentation kinetic parameters. As a Gram-positive model strain, *Bacillus subtilis* has attracted much attention in the field of butanediamine biosynthesis due to its clear genetic background, strong plasticity of metabolic network and excellent tolerance. As an important platform compound, the optimization of butanediamine fermentation process needs to take into account the balance of physiological characteristics and metabolic flux.

The fermentation process first focused on the fine design of the medium, and the optimal matching ratio of key ingredients was screened by orthogonal test and

response surface method. The synergistic effects of carbon sources (such as glucose, fructose, glycerol), nitrogen sources (yeast powder, peptone, amino acid) and inorganic salt (magnesium ion, phosphate) on the growth of the cell and the yield of putamide were investigated. On this basis, multi-parameters were adjusted according to the fermentation conditions, including temperature, pH value, dissolved oxygen content, stirring speed, inoculum amount and fermentation cycle. The optimal interval of each parameter was determined by combining the shake flask pre-experiment with the fermenter amplification experiment. The reference ranges of temperature (37℃), pH (6.8-7.2) and dissolved oxygen (30%-40%) were initially determined by shaking flask experiment, and then amplified and verified in a 5L fermenter. Online sensors were used to monitor the accumulation trend of metabolic by-products (such as acetic acid and lactic acid) in real time, and the stirring speed (400-800 rpm) and feeding strategy were dynamically adjusted. Substrate inhibition effects were avoided. Then Box-Behnken three-factor three-level response surface method was used to analyze the interaction between glucose concentration, L-arginine concentration and fermentation time. Design Expert 8.0.6 software was used to construct a quadratic regression model. The nonlinear relationship and significant influence between variables were analyzed, and the optimization path was visualized by response surface plot and contour plot. The optimization protocol was further validated by batch cultivation, such as dynamic monitoring of putamide accumulation at different fermentation times (12-36 h) under fixed glucose (8 g/L) and L-arginine (12 g/L) conditions, combined with statistical repeated experiments to ensure data reliability. Finally, the optimal combination of fermentation cycle and process parameters was determined by integrating medium component optimization, fermentation parameter precise control and model-driven condition prediction, which provided theoretical basis and technical support for efficient industrial production of putamide.

1.3 PROJECT IDEA AND DESIGN

1.3.1 PURPOSE AND SIGNIFICANCE OF THE PROJECT

As an important organic chemical raw material, the market demand for putamine continues to grow. However, the traditional production method of putamine mainly relies on chemical synthesis, which not only requires expensive catalysts, but also harsh reaction conditions, and most of the raw materials are non-renewable petroleum products, which is contrary to the global green and sustainable development concept. Therefore, it is particularly important to develop an environmentally friendly and efficient production method for putamyldiamine using renewable resources as raw materials. As a common probiotic, *Bacillus subtilis* has the advantages of rapid growth, vigorous metabolism, and easy cultivation. In recent years, its wide application in the field of biosynthesis has shown great potential. Through metabolic engineering of *Bacillus subtilis*, it is expected to achieve efficient production of putamide, thus opening up a new path for its industrial manufacturing.

The purpose of this study is to improve the yield and reduce the production cost of N-ethylenediamine by optimizing the fermentation process of N-ethylenediamine produced by *Bacillus subtilis*, and to provide theoretical basis and technical support for industrial production of N-ethylenediamine. Compared with traditional chemical synthesis, microbial fermentation has the advantages of easy availability of raw materials, mild reaction conditions, and less environmental pollution, which is in line with the concept of green production. The objectives of this study were to screen the key factors affecting the production of N-ethylenediamine by B. subtilis, such as medium composition, fermentation temperature, pH value, and dissolved oxygen content, and to improve the production efficiency of N-ethylenediamine by optimizing fermentation conditions.

1.3.2 RESEARCH IDEAS OF THE PROJECT

In this study, the growth condition and fermentation conditions of the engineering strain of *Bacillus subtilis* 168 were optimized, and the cultivation conditions of the engineering strain of *Bacillus subtilis* 168 for the production of butylene diamine were optimized, including the composition of the medium and the ratio of each component. The batch culture method was used to improve the density of the cell, so as to improve the production efficiency of butylene diamine.

Conclusions to chapter 1

1. Applications and Production Challenges of 1,4-Butanediamine： 1,4-Butanediamine is a critical chemical intermediate. Traditional chemical synthesis relies on petroleum resources and causes severe pollution, driving biosynthesis as a green alternative.

2. Advantages of *Bacillus subtilis* 168：The strain features a well-characterized genetic background and high biosafety but requires genetic modification to address autolysis and protease interference, making it an ideal host for biosynthesis.

3. Fermentation Process Challenges:Substrate inhibition, byproduct accumulation, and bacterial stability are key bottlenecks, necessitating multifactor collaborative optimization to enhance yield.

4. Research Objectives and Methodology:Optimize key parameters (glucose, arginine, fermentation time) via single-factor experiments and response surface methodology to establish an efficient fermentation process model.

CHAPTER 2
OBJECT, PURPOSE, AND METHODS OF THE STUDY

Purpose of the study. To optimize the production process of butylene diamine by Bacillus subtilis, improve the yield and reduce the production cost, and provide theoretical basis and technical support for the industrial production of butylene diamine.

Object of study – *Bacillus subtilis* 168 engineered strain.

2.1 EXPERIMENTAL MATERIAL

2.1.1 EXPERIMENTAL STRAINS AND INSTRUMENTS

The engineered *Bacillus subtilis* 168 fermentation strain was obtained from the laboratory of Bioengineering Department of Qilu University of Technology (Tab. 2.1).

Table 2.1 – Main instruments and equipment

Instrument Name	Company
Bechtop	Suzhou BAizhao Scientific Instrument Co. LTD
Electronic balance	Mettler Toledo Instruments LTD
Precision pH meter	Thermo Fisher Orion
Water-proof electric thermostatic incubator	Shanghai Jinghong Experimental Equipment Co., LTD
Numerical control ultrasonic cleaner	Kunshan Ultrasonic Instrument Co., LTD
Digital display air drying oven	Shanghai Boxun Industrial Co., LTD. Medical equipment factory
Dual beam UV-visible spectrophotometer	HITACHI, Japan
Multi-function combined shaker	Shanghai MINQUAN Instrument Co., LTD

2.1.2 REAGENTS AND MEDIA

SOB medium (1 L) : 5 g yeast powder, 20 g peptone, 0.5 g NaCl, 0.95 g MgCl2, 0.186 g KCl, pH 7.0.

2.2 EXPERIMENTAL METHODS

2.2.1 DETERMINATION OF THE CONCENTRATION OF PUTAMYLDIAMINE

The common methods for the determination of putamide concentration mainly include high performance liquid chromatography (HPLC), spectrophotometry, fluorescence method and gas chromatography (GC). Among them, high performance liquid chromatography combined with pre-column derivatization is the most widely used method, especially for the detection of complex substrates such as fermentation broth. This method reacts with dansyl chloride and other derivative reagents to form stable derivatives of putamyldiamine. After separation by C18 reversed-phase chromatographic column, it is combined with fluorescence detector for quantitative analysis, with high sensitivity and accuracy. "In addition, spectrophotometric methods, which are based on chromogenic reactions such as the formation of purple compounds from hydrhydrin, calculate concentrations from absorbance values and are easy to operate but susceptible to interference from other amines." The fluorescence method achieves high sensitivity detection by forming fluorescent products with phthalaldehyde (OPA) and other reagents and putrescine, but the reaction conditions need to be strictly controlled. All the above methods need to rely on sophisticated equipment or complex steps, and the choice needs to be weighed according to the needs in practical application.

Spectrophotometry is commonly used to determine the concentration of putamide in fermentation broth, which has a simple operation process and low cost. The specific steps were as follows: After the fermentation broth to be tested was

centrifuged to remove impurities, it was mixed with the chromogenic agent ninhydrin hydrate, and the reaction was heated in pH 9.0 buffer at 80 ° C for 20 minutes to generate purple products. After cooling to room temperature, absorbance was measured with a spectrophotometer at a wavelength of 570 nm and zeroed with blank solution. The actual concentration was calculated by a pre-made standard curve (concentration range 0.1-0.6 g/L, R^2=0.9995). This method requires the average of three repeated measurements to improve accuracy. This method has shown a high correlation coefficient (R2=0.9995) and sample recovery (> 98%), which is suitable for rapid detection needs of routine laboratories.

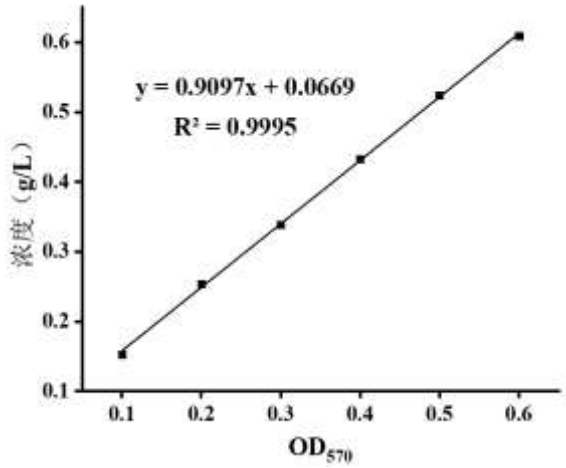

Figure 2.1 – Standard curve as determined by the putrescine concentration

2.2.2 EXPERIMENT OF ADJUSTING GLUCOSE CONCENTRATION TO PROMOTE PUTAMIDE PRODUCTION

A series of experimental groups were designed, and different concentrations of glucose (such as 2g/L, 4g/L, 8g/L, 12g/L, 16g/L, etc.) were added to each group, and multiple groups of repeated experiments were set up to increase the statistical accuracy of the data. The fermentation broth was used to measure the concentration of putamyldiamine.

2.2.3 EXPERIMENT OF ADJUSTING L-ARGININE CONCENTRATION TO PROMOTE PUTAMIDE PRODUCTION

A series of experimental groups were designed, and different concentrations of L-arginine were added to each group (such as 6g/L, 8g/L, 10g/L, 12g/L, 14g/L, etc.). The statistical accuracy of the data was increased by setting up multiple groups of repeated experiments. The fermentation broth was used to measure the concentration of putamyldiamine.

2.2.4 ADJUST THE FERMENTATION TIME TO PROMOTE PUTAMIDE PRODUCTION

A series of experimental groups were designed, and 8 g/L glucose and 12 g/L arginine were added in each group for 36 h. The concentration of putrescine in fermentation broth was measured at 12 h, 18 h, 24 h, 30 h, and 36 h, respectively.

2.2.5 RESPONSE SURFACE EXPERIMENTAL DESIGN

Based on the results of single factor screening, glucose concentration, arginine concentration and fermentation cycle were selected as the key variables, and the putamide yield was used as the evaluation index to carry out response surface method optimization. Box-Behnken experimental design was used to construct a three-factor three-level experimental matrix, and the fermentation culture was carried out according to the predetermined experimental scheme. The independent variable parameters and corresponding response values of each experimental combination were recorded in detail. The experimental data were all regression fitting processing using Design Expert Software Version 8.0.6 software, and the results of variance analysis of the quadratic regression model were obtained. The statistical significance of the model was judged by significance test. The visual

response surface map was used to analyze the influence of various experimental factors and their interactions on the product synthesis, and the optimal matching ratio of medium components was optimized.

Based on the previous single-factor optimization data, this experimental design carried out a Box-Behnken design with three factors and three levels for the three key parameters of glucose concentration, arginine concentration and fermentation time. The level gradient Settings and coding schemes of each factor are shown in Tab. 2.2.

Table 2.2 – Box-Behnken test factor levels and coding

Factor / Lever	Glucose(g/L)	L-arginine(g/L)	Time(h)
-1	6	10	24
0	8	12	30
1	10	14	36

Conclusions to chapter 2

1. Experimental equipment and materials: This chapter first introduces the layout, specific sources (such as manufacturer information or existing laboratory equipment) and function description of the equipment needed for the experiment to ensure the operability of the experimental process. At the same time, the list of raw materials required for medium preparation was detailed, including the specifications and selection basis of carbon sources, nitrogen sources, inorganic salts and other components, which provided basic material support for subsequent experiments.

2. Detection technology of putrescine concentration: For the determination of putrescine concentration, the commonly used methods such as high performance liquid chromatography (HPLC), spectrophotometry, fluorescence method and gas chromatography (GC) are listed in this paper, and their application scenarios,

advantages and disadvantages are compared. Based on the convenience, cost-effectiveness and compatibility with the experimental system, spectrophotometry was finally selected as the core detection technology in this experiment.

3. Fermentation condition optimization method: Single factor experiment method was used to screen out the key factors affecting the fermentation effect by adjusting single variables such as temperature, pH and inoculum amount. On this basis, the interaction of multiple factors was systematically analyzed by response surface method (RSM), and a mathematical model was established to optimize the medium formulation and fermentation process parameters to improve the synthesis efficiency of target products.

4. Experimental basis and objective: By integrating the configuration of experimental equipment, the selection of detection techniques, and the collaborative optimization strategy of single factor and response surface method, this chapter provides systematic data support and theoretical basis for the scientific improvement of medium formulation, aiming to lay a technical foundation for the efficient development of subsequent fermentation processes

CHAPTER 3
EXPERIMENTAL PART

3.1 RESULTS AND ANALYSIS OF SINGLE FACTOR EXPERIMENTS

3.1.1 EFFECTS OF DIFFERENT CONCENTRATIONS OF GLUCOSE ON THE YIELD OF PUTRESCINE

After adding 10 g/L arginine for 24 h, the accumulation of putrescine in fermentation broth was quantitatively detected at the end of fermentation with glucose concentration as a variable (Figure 3.1).

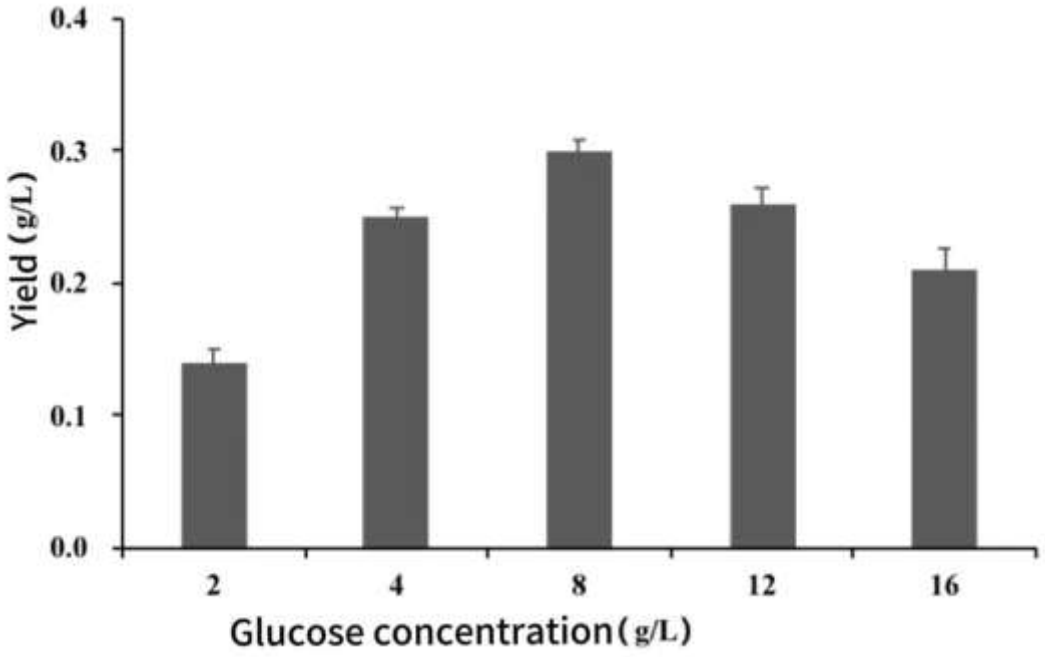

Figure 3.1 – The results of the experiment investigating the influence of glucose concentration on butanediamine production

Results As shown in Figure 3.1, when the glucose concentration was from 2g/L to 8 g/L, the yield of putrescine increased significantly with the increase of glucose concentration, from 0.14 ± 0.011 g/L to a peak of 0.30 ± 0.009 g/L. When the concentration of glucose was 8 g/L, the yield of putrescine reached the maximum (0.30±0.009g/L). When the glucose content was continuously increased from 8 g/L to 16 g/L, the yield gradually decreased to 0.26 ± 0.013 g/L at 12 g/L and further

decreased to 0.21 ± 0.016 g/L at 16 g/L. In general, the production of putamide increased first and then decreased with the change of glucose concentration. Therefore, the optimal glucose concentration was estimated to be 8 g/L, at which time the putrescine yield was the highest (0.30 g/L±0.009).

3.1.2 EFFECTS OF DIFFERENT CONCENTRATIONS OF L-ARGININE ON THE YIELD OF PUTRESCINE

After adding 8 g/L glucose for 24 h, arginine concentration was used as a variable to quantitatively detect the accumulation of putrescine in fermentation broth at the fermentation end point (Figure 3.2).

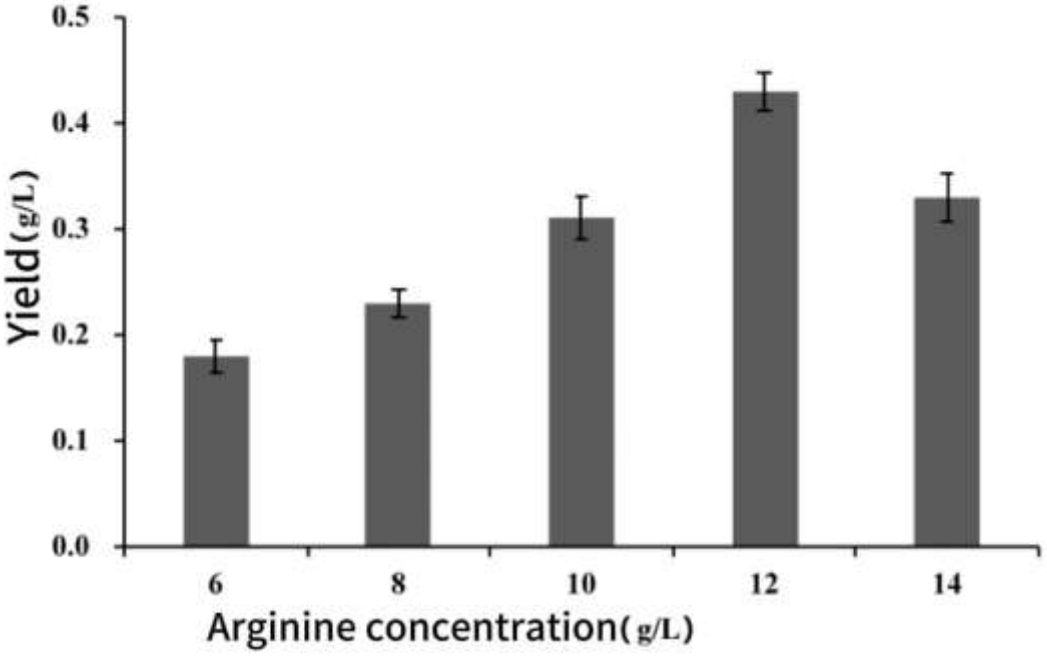

Figure 3.2 – The results of the experiment investigating the influence of arginine concentration on butanediamine production

Results As shown in Figure 3.2, when the content of arginine was 6g/L to 12g /L, the yield of putamine increased significantly with the increase of arginine concentration, from 0.18 ± 0.015g/L to the peak of 0.43± 0.018g /L. When the concentration of arginine was 12 g/L, the yield of putadine reached the maximum (0.43±0.018 g/L). When the arginine concentration increased from 12 g/L to 14

g/L, the yield of putadine decreased from 0.43±0.018 g/L to 0.33 ± 0.023 g/L with the increase of arginine concentration. In general, the yield of putadine increased first and then decreased with the change of arginine concentration. Therefore, the optimal arginine concentration was estimated to be 12 g/L, at which time the putamine yield was the highest (0.43±0.018 g/L).

3.1.3 INFLUENCE OF DIFFERENT FERMENTATION TIME ON THE YIELD OF PUTRESCINE

Under the condition of adding 8 g/L glucose and 12 g/L arginine, the accumulation of putrescine in fermentation broth was quantitatively detected at the end of fermentation with fermentation time as a variable (Figure 3.3).

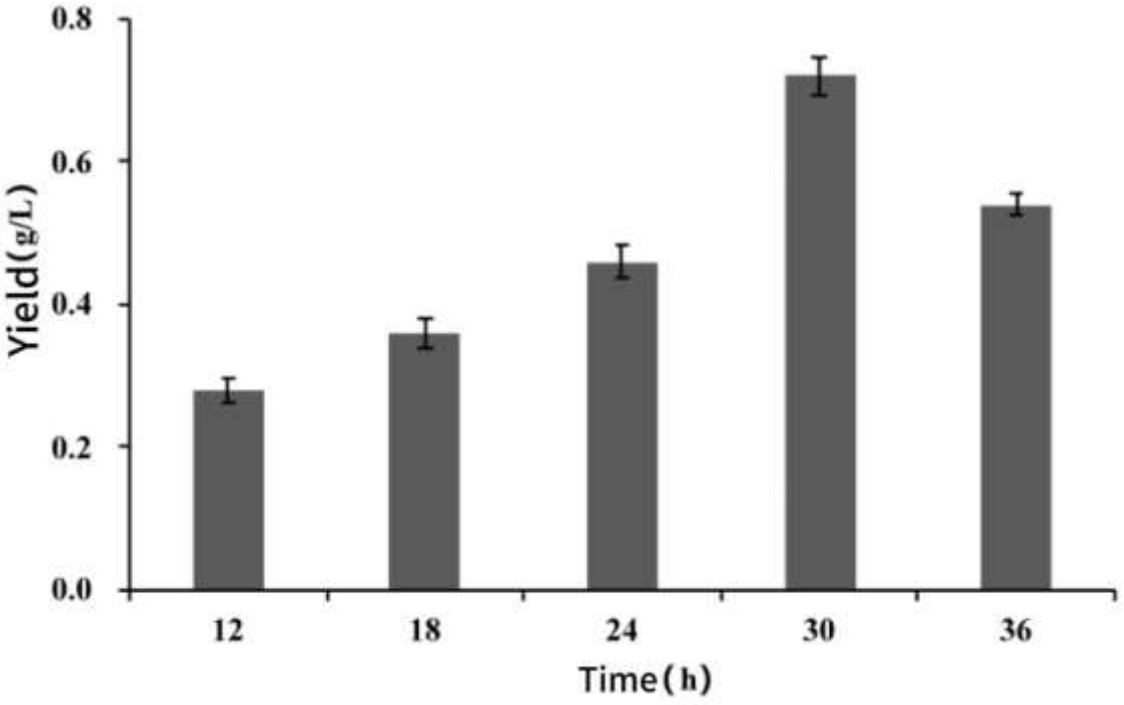

Figure 3.3 – The results of the experiment investigating the influence of fermentation time on butanediamine production

Results As shown in Figure 3.3, when the fermentation time was 12h to 30h, the yield of putamine increased significantly with the extension of fermentation time, from 0.28 ± 0.016 g/L to the peak of 0.72 ± 0.027 g/L. When the fermentation time

was 30 h, the yield of butylene diamine reached the maximum (0.72±0.027 g/L). When the fermentation time was extended to 36 h, the yield of putamide decreased from 0.72±0.027 g/L to 0.54 ± 0.015 g/L. In general, the yield of putamen increased first and then decreased with the extension of fermentation time. Therefore, the optimal fermentation time was estimated to be 30 h, at which time the yield of putamide was the highest (0.72±0.027g/L).

3.2 EXPERIMENTAL RESULTS AND ANALYSIS OF RESPONSE SURFACE

According to the experimental factor levels and codes shown in Tab. 2.2, a response surface experiment with 3 factors and 3 levels was designed using the method of Box-Behnken design (BBD). The design results are shown in Tab. 3.1.

(1) Results of Box-Behnken test

Table 3.1 – Results of the Box-Behnken experimental design

Experiment No.	Glucose (g/L)	L-arginine (g/L)	Time (h)	Production (g/L)
1	6	10	30	0.57
2	10	10	30	0.55
3	6	14	30	0.51
4	10	14	30	0.59
5	6	12	24	0.46
6	10	12	24	0.53
7	6	12	36	0.48
8	10	12	36	0.57
9	8	10	24	0.48
10	8	14	24	0.55
11	8	10	36	0.59

Experiment No.	Glucose (g/L)	L-arginine (g/L)	Time (h)	Production (g/L)
12	8	14	36	0.64
13	8	12	30	0.76
14	8	12	30	0.71
15	8	12	30	0.73
16	8	12	30	0.74
17	8	12	30	0.72

Table 3.1 presents the data of Box-Behnken experiments with glucose concentration, arginine concentration, and fermentation time as variables. The analysis shows that:

In the repeated experiments with the combination of glucose 8 g/L, arginine 12 g/L and fermentation time 30 h (experiment 13-17), the yield of putrescine was stable at 0.71-0.76 g/L, and the yield of experiment 16 reached the highest value of 0.76 g/L. This result was significantly higher than the peak value of single factor experiment (single factor highest was 0.26 ± 0.013 g/L at arginine 12 g/L and 0.72 ± 0.027 g/L at fermentation time of 30 h), indicating that the synergistic optimization effect of multiple factors was significant.

In the single factor experiment, the highest yield (0.23 ± 0.013 g/L) was obtained at the glucose concentration of 8 g/L, and the yield (0.71-0.76 g/L) at the center of the response surface (8 g/L) was further verified to be optimal. Single factor experiments showed that the highest yield (0.43 ± 0.018 g/L) was obtained at the concentration of 12 g/L arginine, which was consistent with the yield (0.71-0.76 g/L) at the center of the response surface (12 g/L). In the single factor experiment, the fermentation time of 30 h was the highest (0.72 ± 0.027 g/L), and the fermentation time of 30 h was the optimal fermentation time (0.71-0.76 g/L).

(2) Response surface results and analysis

The experimental data were all processed by regression fitting using Design Expert Software Version 8.0.6 software, and the variance analysis and fitting statistical results of the quadratic regression model were obtained, as shown in Tab.3.2 and 3.3, and the relevant data of the quadratic regression analysis were obtained.

Table 3.2 – ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.1476	9	0.0164	15.89	0.0007	significant
A-Glucose	0.0059	1	0.0059	5.74	0.0477	
B-Arginine	0.0010	1	0.0010	1.01	0.3494	
C-Time	0.0090	1	0.0090	8.76	0.0211	
AB	0.0026	1	0.0026	2.53	0.1556	
AC	0.0000	1	0.0000	0.0191	0.8939	
BC	0.0001	1	0.0001	0.056	0.8156	
A^2	0.0550	1	0.0550	53.28	0.0002	
B^2	0.0142	1	0.0142	13.80	0.0075	
C^2	0.0473	1	0.0473	45.79	0.0003	
Residual	0.0072	7	0.0010			
Lack of Fit	0.0056	3	0.0019	4.63	0.0865	not significant
Pure Error	0.0016	4	0.0004			

Note：$R^2 = 0.9533$；Adjusted $R^2 = 0.8933$

The F value of the model was 15.89 (p = 0.0007), and the p value of the missing term was 0.0865, indicating that the model was significant as a whole. $R^2 = 0.9533$, Adjusted $R^2 = 0.8933$, Predicted $R^2 = 0.8041$, Adeq Precision = 11.3166 (> 4), indicating that the model had a strong ability to explain the variation of yield.

Glucose concentration (A) had a significant effect on the yield of putamide ($p = 0.0477$). The single factor experiment showed that the yield was the highest at 8 g/L (0.30 ± 0.009 g/L), and excessive concentration (such as 12 g/L) led to a decrease in yield (0.26 ± 0.013 g/L). Fermentation time (C) had a significant effect on the production of putamide ($p = 0.0211$), and the single factor experiment showed that 30 h was the best (0.72 ± 0.027 g/L), and the longer fermentation time (36 h 0.54 ± 0.015 g/L) might be due to aging or the accumulation of by-products. The effect of arginine concentration (B) on the yield of putamide was not significant ($p = 0.3494$), but the single factor experiment showed that the yield was the highest at 12 g/L (0.43 ± 0.018 g/L), and the yield at the center of the response surface (12 g/L) (0.74 g/L) indicated its practical importance. The p values of all interaction terms (AB, AC, BC) were greater than 0.05, indicating that there was no significant interaction between the factors. The effects of glucose ² (A^2) and time ² (C^2) on yield were both highly significant ($p = 0.0002$ and $p = 0.0003$), indicating that the nonlinear effects of glucose ² and time ² on yield were dominant and should be strictly controlled near the center. The effect of arginine ² (B^2) was significant ($p = 0.0075$), suggesting that the yield decreased when the arginine concentration deviated from 12 g/L (for example, the yield was ≤0.59 g/L at 14 g/L in experiments 3 and 4).

Table 3.3 – Fitting statistical analysis of butanediamine production by *Bacillus subtilis*

Item	Value	Item	Value
Std.Dev.	0.0312	R^2	0.9533
Mean	0.5922	Adjusted R^2	0.8933
C.V.%	5.36	Predicted R^2	0.8041
		Adeq Precision	11.3166

(3) The effects of various factors on the yield of putrescine were analyzed by response surface method

The response surface was fitted by the equation, and the response surface plot and contour plot are shown in Figure 3.4, Figure 3.5, and Figure 3.6, respectively.

Figure 3.4 show the surface plots and contour plots of the response of glucose concentration and arginine concentration to putamyldiamine production. When other factors were fixed as the optimal condition, the yield of putrescine increased rapidly first and then gradually decreased with the increase of glucose concentration, and the variation range was large. At the same time, the increase of arginine concentration also led to a significant increase and then a significant decrease in yield, and the change was equally large. The results showed that both glucose concentration and arginine concentration had significant effects on yield.

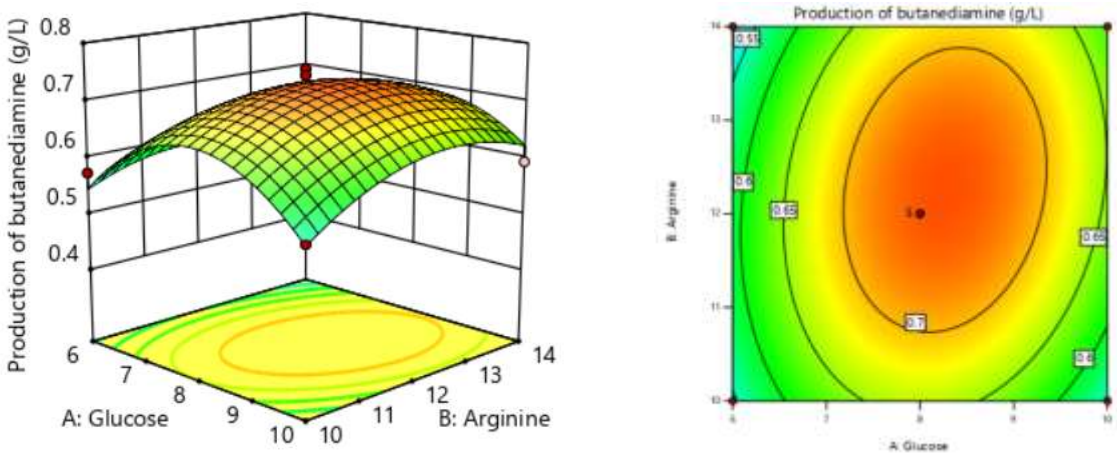

Figure 3.4 – Response surface curves and contour plots of glucose concentration and arginine concentration

Figure 3.5 show the surface plots and contour plots of the response of glucose concentration and fermentation time to putamine production. When other factors were fixed as the optimal condition, the yield of putrescine increased rapidly first and then gradually decreased with the increase of glucose concentration, and the variation range was large. At the same time, with the extension of fermentation time, the yield of butylene diamine showed a trend of first increasing and then

decreasing, and the change range was also large. The results showed that both glucose concentration and fermentation time had a significant effect on the yield.

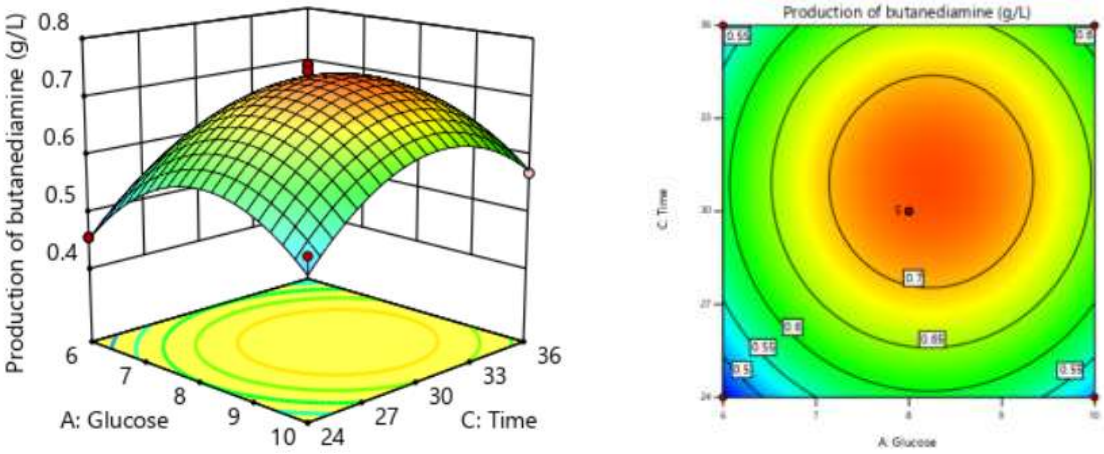

Figure 3.5 – Response surface curves and contour plots of glucose concentration and fermentation time

Figure 3.6 show the surface plots and contour plots of arginine concentration versus fermentation time in response to putamine production. When other factors were optimal, the yield increased first and then decreased with the increase of arginine concentration, which indicated that arginine concentration had a significant effect on the yield of butylene diamine. At the same time, with the extension of fermentation time, the yield of butylene diamine showed a trend of first increasing and then decreasing, indicating that fermentation time also had a certain effect on the yield of butylene diamine.

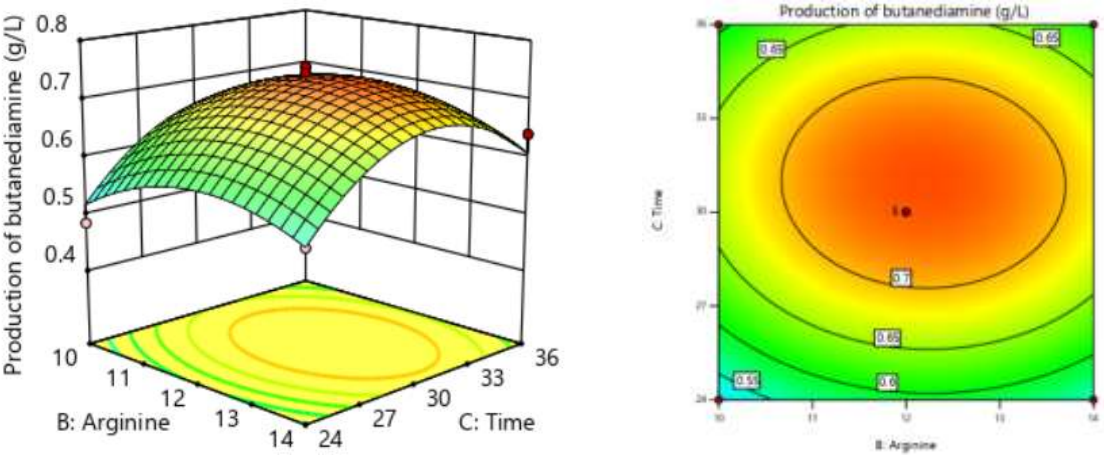

Figure 3.6 – Response surface curves and contour plots for arginine concentration and fermentation time

By software analysis, we obtained more precise optimal fermentation conditions for butanediamine production by Bacillus subtilis, as shown in Tables 3.4. Under the optimal condition of adding 8.5 g/L glucose and 12.6 g/L arginine for 31.8 h, the yield of butyric diamine reached 0.782 ± 0.028 g/L, which was 3.13 times higher than the yield before optimization (0.25 ± 0.006 g/L). The actual yield was very close to the predicted value of the software, which proved the accuracy and reliability of the model.

Table 3.4 – Optimal fermentation conditions and yields for putrescine production by *Bacillus subtilis*

Glucose(g/L)	Arginine(g/L)	Time(h)	Production(g/L)
8.5	12.6	31.8	0.782 ± 0.028

Conclusions to chapter 3

1. Single factor analysis of experimental results: glucose concentration, L-arginine concentration and fermentation time had significant effects on the yield of putamide. The highest yield was found in 8 g/L glucose (0.30 ± 0.009 g/L), and excessive glucose induced inhibition. Arginine reached its peak at 12 g/L (0.43 ± 0.018 g/L). The highest yield (0.72 ± 0.027 g/L) was obtained after 30 hours of fermentation, and decreased after 36 hours of fermentation due to the decline of bacteria.

2. Response surface design and model validation: Box-Behnken design was used to optimize three factors (glucose 6-10 g/L, arginine 10-14 g/L, time 24-36 hours) and construct a quadratic regression model (R^2=0.9533). The model was significant (p=0.0007), and glucose concentration (p=0.0477) and fermentation time (p=0.0211) had significant effects. Although arginine did not reach a significant level (p=0.3494), its actual effect was verified by the center point (12 g/L).

3. Determination and verification of the optimal fermentation conditions: the model predicted that the optimal conditions were glucose 8.5 g/L, arginine 12.6

g/L, fermentation for 31.8 hours, and the theoretical yield was 0.782 g/L. The actual yield of the verification experiment reached 0.782 ± 0.028 g/L, which was 3.13 times higher than that before optimization (0.25 ± 0.006 g/L), confirming the reliability of the model and the effectiveness of the process.

4.Interaction and parameter sensitivity analysis: glucose and arginine should be controlled at medium concentration (8-12 g/L), and their quadratic nonlinear effects were significant. The fermentation time should match the glucose concentration, too short to limit the accumulation, too long to accelerate the decline. At 12 g/L arginine, 30 h fermentation maximizes yield, but 36 h induces by-product inhibition. Glucose sensitivity was the highest, followed by fermentation time, and arginine-dependent synergistic ratio.

CONCLUSIONS

In this study, the fermentation process parameters of the engineered *Bacillus subtilis* strain 168 for the synthesis of butanediamine were systematically optimized, focusing on the key conditions such as medium components, nutrient ratio and fermentation cycle. By using the batch culture strategy, the biomass of the strain was effectively increased, and the synthesis efficiency of the target product was significantly improved. Based on the synergistic analysis of single factor experiment and response surface method, the optimal fermentation combination was obtained as follows: glucose 8.5 g/L, arginine 12.6 g/L, fermentation time 31.8 h. The yield of putamide was 0.782 ± 0.028g/L, which was in good agreement with the predicted value of the model, and fully verified the effectiveness and engineering application value of the mathematical model.

Based on the obtained optimization results of process parameters, the following work will be carried out to evaluate the process economy, which will systematically analyze the key economic parameters such as raw material cost, energy consumption index and equipment investment. It is also planned to promote the fermenter scale up test, relying on this way to verify the actual efficiency of the optimized process in the industrial production environment. These studies will provide technical support for the industrialization of this biosynthetic route.

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