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 **Optimization of fermentation conditions for PLA strains**
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
Ni MING XIAO

Scientific supervisor
Ihor HRETSKYI, Ph.D., As. prof.

Reviewer
Liubov ZELENA, Ph.D., As. prof.

Kyiv 2024

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APPROVE
Head of Department of Biotechnology, Leather and Fur, Professor,
Doctor of Technical Science
Olena MOKROUSOVA
_____
«___»_____2024

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Ni Ming Xiao

1. Thesis topic **Optimization of fermentation conditions for PLA strains**
Scientific supervisor Ihor Hretskyi, Ph.D., As. prof

approved by the order of KNUTD "__"_____2024, №____

2. Initial data for work: assignments for qualification thesis, scientific literature on the topic of qualification thesis, materials of Pre-graduation practice

3. Content of the thesis (list of questions to be developed): literature review; object, purpose, and methods of the study; experimental part; conclusions

4. Date of issuance of the assignments_____

EXECUTION SCHEDULE

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	From 01 April 2024 to 11 April 2024	
2	Section 1 Literature review	From 06 April 2024 to 20 April 2024	
3	Section 2 Object, purpose, and methods of the research	From 21 April 2024 to 30 April 2024	
4	Section 3 Experimental part	From 01 May 2024 to 10 May 2024	
5	Conclusions	From 07 May 2024 to 12 May 2024	
6	Draw up a bachelor's thesis (final version)	From 12 May 2024 to 24 May 2024	
7	Submission of qualification work to the supervisor for feedback (14 days before the defense)	From 24 May 2024 to 10 June 2024	
8	Submission of bachelor's thesis to the department for review (12 days before the defense)	13 June 2024	
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10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	17 June 2024	

I am familiar with the task:

Student _____ Ni MING XIAO

Scientific supervisor _____ Ihor HRETSKYI

SUMMARY

Ni Ming Xiao. Optimization of fermentation conditions for PLA strains.– Manuscript.

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Since the world entered the 21st century, the rapid development of science and technology and economy, environmental problems and energy depletion have become increasingly serious, and with the promulgation of a series of national plastic restrictions, people have begun to pay more and more attention to degradable materials, the so-called degradable materials, that is, materials that can be completely decomposed by bacteria and other microorganisms in a suitable natural environment, can be a good alternative to plastic products. One of the most popular materials is polylactic acid (PLA), a polymer polymerized from lactic acid as the main raw material, which is made from renewable plant resources (such as straw) from a synthetic point of view. There are several methods for the production of PLA, such as direct polycondensation method, two-step method, which not only has the characteristics of existing plastic polyethylene, polypropylene, polystyrene propylene and other materials, but also has degradability. It is clear that there is a plenty of advantages and applications of PLA, the use of it has many limitations, for example high cost etc. This paper aims to construct a suitable fermentation strain by collating relevant research papers at home and abroad, and then conduct a single factor experiment to find the most suitable fermentation conditions, in order to achieve the improvement of yield and quality.

Keywords: PLA, Fermentation strain, fermentation conditions.

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INTRODUCTION

Purpose of the study – there are few studies on optimizing the fermentation conditions of polylactic acid strains to improve the yield of polylactic acid. Therefore, in-depth construction and fermentation of high-yielding PLA strains and establishment of a stable, efficient and practical polylactic acid fermentation environment can lay a theoretical foundation for the industrial production of polylactic acid.

Object of study – PLA;

Bacterial strain: E .coli JW3169;

Carrier: pCAT204-IdhA-pCT540.

Subject of study – (1) Suitable plasmids, vectors and strains were selected by synthetic biological methods to construct strains that could produce PLA efficiently.

(2) The effects of different temperature, pH, rotational speed and fermentation time on polylactic acid (PLA) fermentation were investigated by shaker fermentation method and single factor method, so as to obtain the most suitable experimental conditions for polylactic acid (PLA) fermentation

CHAPTER 1
LITERATURE REVIEW

1.1 Overview of polylactic acid

1.1.1 Structure of polylactic acid

Polylactic acid (PLA) is formed by the polymerization of lactic acid, belongs to poly-A-amino acid, is a linear aliphatic polyester. The basic chemical structure and the structure of two optically active enantiomers (L-lactic acid and D-lactic acid) are shown in Figure 1.1 and 1.2.

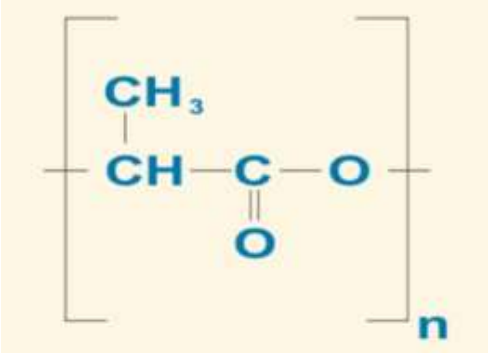

Figure 1.1 – Molecular structure formula of polylactic acid

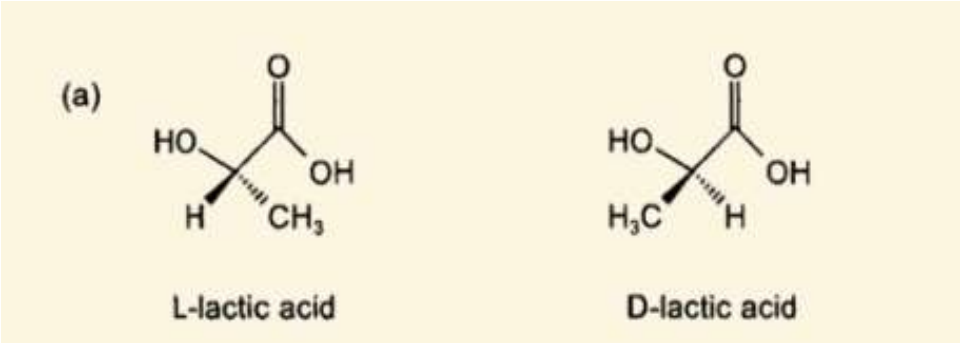

Figure 1.2 – Structure diagram of two optically active enantiomers of polylactic acid

Left-handed polylactic acid (PLLA) is a polymer from the polymerization of polylactic acid, while right-handed polylactic acid (PDLA) is a polymer from the polymerization of right-handed lactic acid, and the polymer from the polymerization

of equal proportions of D-lactic acid and polylactic acid = is called racemic polylactic acid (PDLLA).

1.1.2 The fermentation of polylactic acid

To obtain polylactic acid, you must first undergo lactic fermentation. That is, once the sugar enters the cell, it is first converted into pyruvate through several enzymatic stages. This conversion produces chemical energy in the form of ATP (adenosine triphosphate) and reductive equivalent (NADH), and in order to recover these reducers, the bacteria further convert pyruvate into lactic acid, releasing NAD+. In other words, lactic acid is produced primarily to keep cellular processes going. The chemical energy obtained can be used for several processes elsewhere in the body, for example, the growth, maintenance, and movement of the body [1].

The current methods are mostly to treat crops such as straw in advance through various industrial processes (such as crushing, etc.), which can separate cellulose, hemicellulose and lignocellulose, thus making cellulose yield larger. The above three substances are hydrolyzed, enzymatic degradation and other steps to obtain carbohydrate compounds, and then transferred to the shaker or fermenter to ferment lactic acid, the detailed process of the process is shown in Figure 1.3 [2].

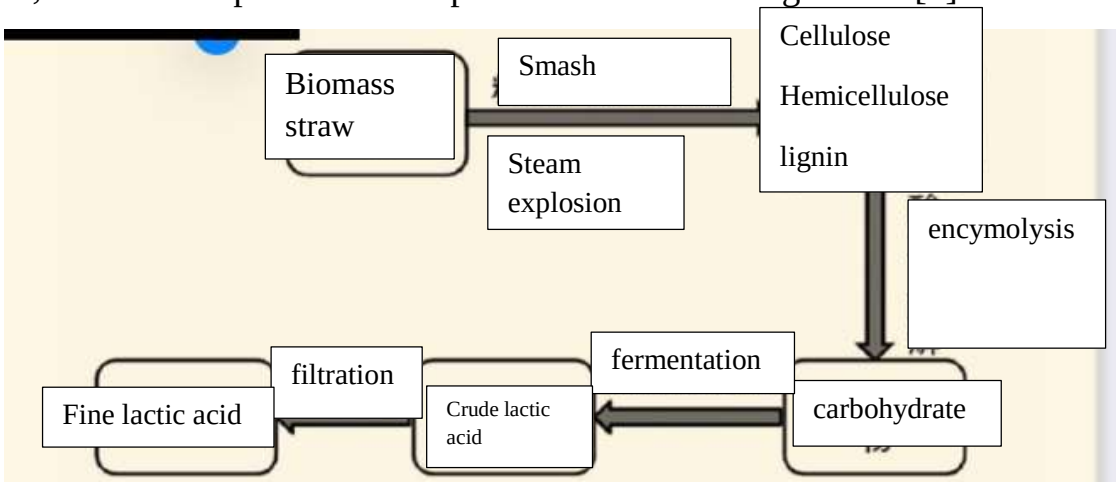

Figure1.3 – Industrial production of polylactic acid process

1.1.3 Synthesis of polylactic acid

Polylactic acid has a variety of synthesis methods, the more commonly used are mainly direct polycondensation method and open loop polymerization method, and the former can be divided into solution polycondensation method and melt polycondensation method two, these two methods have large defects, solution polycondensation method is the disadvantage of the need to consume more solvents, not only economically not applicable, but also cause greater pollution. (See Figure 1-4 for relevant equations)

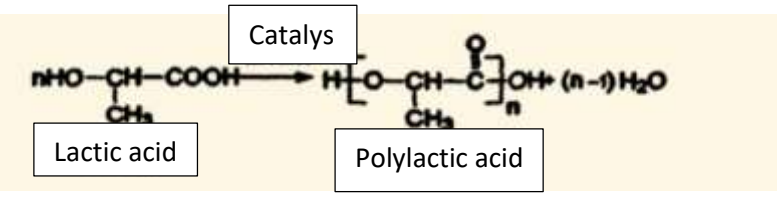

Figure1.4 – Direct polycondensation reaction equationss

The other synthesis method is ring-opening polymerization method, which first forms lactide through dehydration between lactic acid raw materials, then obtains lactide with higher purity through a series of purification methods, and then carries out ring-opening polymerization under the action of catalyst. In this way, the molecular weight of polylactic acid will be increased, and the molecular weight distribution range is narrow, and the controllability is good. Therefore, this method is used as the main method for commercial polylactic acid synthesis. (Fig. 1-5) [3].

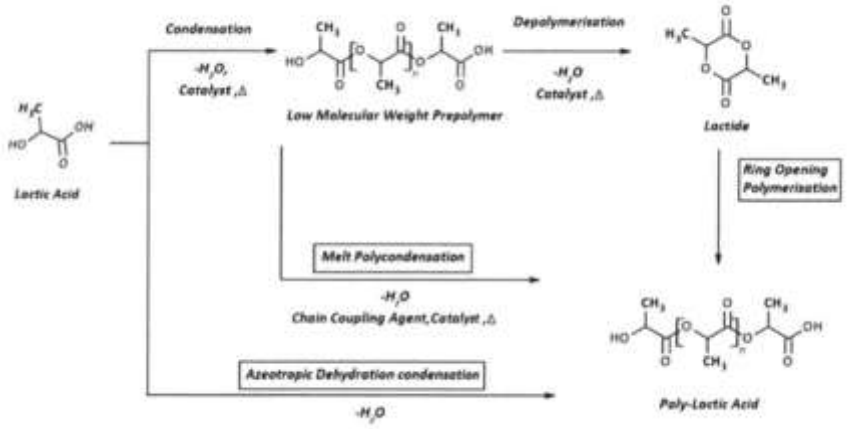

Figure1.5 – Synthetic route of polylactic acid [4]

There is also a less common method of high molecular weight synthesis by chain extension, which has the advantage of increasing its molecular weight relative to previous synthesis methods. The chain extender used is a low molecular weight compound or oligomer containing a bifunctive group (epoxy group, isocyanate group, umazoline group, amide group, acid anhydride, etc.). They can react with the hydroxyl or carboxyl group of PLA in the melting process, linking the two PLA chains, increasing the molecular weight of the polymer but also reducing hydrolysis and degradation reactions. (Fig. 1-6) [5].

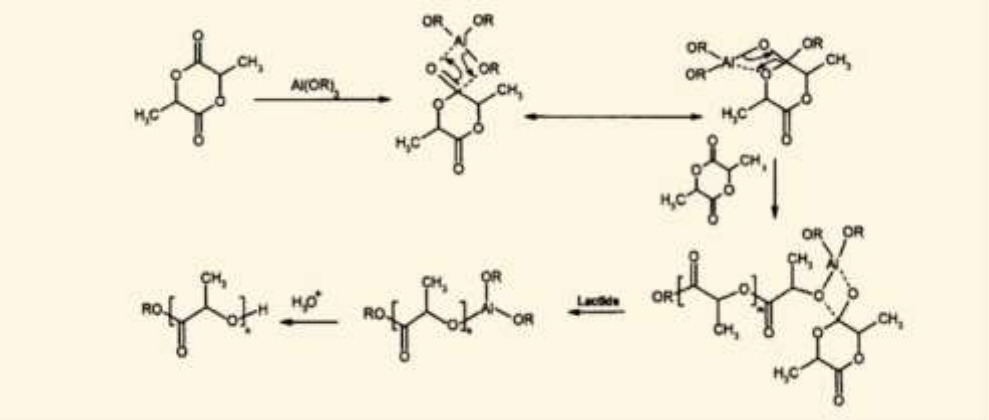

Figure1.6 – Third synthetic method of polylactic acid [6].

1.1.4 Advantages and disadvantages of polylactic acid

The main advantage of using PLA is that it is environmentally friendly. Since this material can be obtained from common crops, it can be used without worrying about stock. In addition, after full use, it can also be transformed into environmentally friendly substances through industrial treatment (such as used to make chemical fertilizers), indicating that it is environmentally friendly at disposal. Another advantage is that PLA is biocompatible. This property can be used for biomedical applications. The material does not have toxic and carcinogenic effects on humans. When PLA degrades, it breaks down into H_2O and CO_2 without interfering with tissue healing [7,8].

In addition, in terms of processability, PLA has better thermal processability compared with other biopolymers such as PHA, PEG and PCL. This can be achieved through a variety of processes that can be applied or used to form PLA into

bioplastics. These processes include drying, film extrusion, casting film and sheet, blow molding and thermoforming. Finally, the energy required to produce PLA-based bioplastics is 25 to 55 percent less than that of conventional petroleum-based plastics, and may even be less than 10 percent in special cases.

However, although PLA is considered by many to be one of the best materials in the industry, it has some disadvantages. First, its elongation at break is less than 10%, indicating that it is very brittle and its use in various applications is limited [7,8]. Especially in the medical field, it is difficult to make products that require plastic deformation from polylactic acid products.

It has a strong hydrophobic, static water contact Angle of about 80˚, direct contact with biological fluids, can cause tissue inflammation to the living host. It has been used as a soft cell-friendly tissue engineering material [7,8]. Thus, blends of polymers with other polymers, functionalization, and the addition of nanofillers [1] have been used.

1.1.5 Application of polylactic acid

PLA has good biocompatibility and degradability, so it can be used in environmental protection, textile, construction, food, medical and other fields.

1.1.5.1 Medical field

The biomedical industry is one of the industries adding polylactic acid bioplastics to their products. Some interesting properties make polylactic acid an ideal material for biomedical applications. One of them is its hydrolysis mechanism, where polylactic acid is naturally degraded in situ. Therefore, no additional surgery is required to remove the implanted device. As a result, recovery rates for patients can be improved and health system costs minimized. The natural biocompatibility of polylactic acid also contributes to the achievement of a lower critical immune response. Since the degradation products consist of lactic acid and short polymers, these familiar substances can be metabolized by the body. However, pure PLA may face some difficulties in meeting all the requirements needed in the field. Therefore, over the years, polylactic acid-based nanocomposites have been extensively studied

as alternative materials. Polylactic acid based nanocomposites can be used as matrix with copolymers and nanocomposites [10]. The improvement of nanomaterials and their appropriate applications make nanomedicine an emerging field of medical therapy today.

1.1.5.2 Packaging/food packaging field

The next industrial application is in packaging. Polylactic acid, derived from lactic acid, is a thermoplastic, biodegradable aliphatic polyester with great potential for packaging applications. Lactic acid monomer has two optical isomers, and the ratio of these two isomers determines the quality of polylactic acid as a packaging material. For example, when 100%L-PLA monomer is used, high melting and crystallization points can be obtained. At the same time, when 90/10% D/L copolymer is used, it will produce a polymerizable melt higher than its Tg, meeting the bulk packaging conditions. As a packaging material, polylactic acid is a good alternative and replacement for traditional plastics such as low density polyethylene (LDPE), high density polyethylene (HDPE), polystyrene (PS) and polyethylene terephthalate (PET).

1.1.5.3 Agriculture

Polylactic acid is also used in agriculture. The incorporation of plastics into agricultural applications, known as plastic cultivation, began in the 1950s with the aim of improving and increasing yields. There are several good reasons to use plastics in agriculture :(1) mulch can protect agricultural soil from erosion and crops from insects, birds and weeds; (2) Tunnel shielding greenhouse.

1.1.5.4 Automobile industry

In addition, over the years, the automotive industry has also shown interest in using PLA in components. These materials exhibit excellent properties and help improve fuel efficiency as the weight of the vehicle may be reduced. The reduction of greenhouse gas (GHG) emissions into the atmosphere also reached 100. 23% of global carbon emissions are related to automobiles, and 80% of environmental pollution comes from total emissions from automobiles. For every 10% reduction in vehicle weight, fuel efficiency can be improved by 7%. For every kilogram of mass

lost in a vehicle, about 20 kilograms of carbon dioxide emissions are reduced. Therefore, the use of biocomposites in this industry can achieve dual benefits; Improve fuel efficiency by reducing total vehicle weight and CO_2 emissions to the environment. In addition, if nanocomposites are used instead of steel and aluminum housings, the weight of the car can be reduced by about 40-55%. Mass reduction in automotive applications is crucial for future electric vehicles to optimize the ratio of vehicle weight to battery capacity [11].

1.1.6 Research status of polylactic acid

The rapid development of human society in recent years has brought many problems, among which, environmental and resource problems are particularly serious, such as white pollution, plastic products made of polyethylene, polypropylene, polyvinyl chloride, etc. used by human beings in the process of industrialization have a short-term indelible negative impact on nature. Therefore, a new degradable material - polylactic acid came into being, it has good biocompatibility and biodegradability, and the product after degradation is harmless to nature, and has increasingly become a substitute for plastics. By searching relevant literature, the author collected the following research status at home and abroad:

The study of polylactic acid in foreign countries began earlier and its development was relatively mature. Since the 1930s, many scientists have devoted themselves to polylactic acid research.

In 2019, Liu et al. isolated three strains (Enterococcus faecalis B101, Acinetobacter calc acetate C1, and Pseudomonas aeruginosa CS) that were able to utilize some phenolic substances (vanillin, 4-hydroxybenzaldehyde, or acetaldehyde) in ammonia-pretreated corn stalks as the sole carbon source. The lactic acid yield of corn stalks pretreated with the above-mentioned polyphenol-degrading bacteria and Lactobacillus pentosa FL 0421,50 g/L ammonia was nearly tripled [from 16.98 g/L to 31.35 g/L LA (0.63 g/g corn straw)]. In another paper, Liu et al. (2020) cultured Trichoderma viridis R16 on alkali/peroxide pretreated corncob as a substrate in a feed-batch SSF process and used the resulting enzyme for LA production of Bacillus

coagulans LA 204. Due to the high ability of Trichoderma viridis R16 enzyme to degrade inhibitors compared to some commercial enzymes, lactic acid production is increased by 24% [12].

In 2020, Nor Fasihah Zaaba discussed the explanation mechanism of various PLA in his paper. He first discussed the different optical isomers of PLA (PLLA, PDLA, PDLLA, etc.) and their physical and chemical properties. He then discussed various methods of PLA degradation, such as hydrolytic degradation (hydrolytic degradation involves the diffusion of water molecules, which start in amorphous regions and subsequently trigger ester bond breakage). Hydrolytic degradation continued along the crystal boundary), photodegradation (the authors proposed; The photodegradation mechanism is initiated by the conversion of the polymer through photoionization (Norrish type 1), followed by polymer chain breaking (Norrish type II), which can lead to the Norrish reaction, crosslinking reaction or oxidation process. Microbial degradation (Microbial degradation is described as microorganisms that convert organic compounds into less toxic or more useful forms in the laboratory or natural environment. The microbial degradation mechanism is activated after hydrolysis of high molecular weight PLA) [13].

In 2021, Daninle Rigott of the American Chemical Society in his research by preparing a mixture of polylactic acid and new biological research polymer (PPeF) with different concentrations, the UV shielding performance of traditional PLA was significantly improved, and the transmission rate at 275 nm was reduced from 47.3% of pure PLA to 0.77%. The PPeF is only 1 wt %, and the decrease in transmittance in the visible region of these PPeF parts is small, allowing the production of optically transparent films. At the same time, the authors found that although the PLA/PPeF blend is completely immiscible, PPeF effectively enhances the ductility of PLA, as the tensile fracture strain increases from 7% of pure PLA to 200% of the blend, and the weight of PPeF is 30 wt%. This composition is also the most promising from a gas barrier point of view, as the gas permeability of CO_2 and O_2 drops to a quarter of that of pure PLA, comparable to polyethylene terephthalate. These results indicate that PLA/PPeF blends with a PPeF fraction of 30 wt % are very promising for food

packaging applications, and their performance can be further enhanced by the use of suitable compatibents [14].

In 2022, Garrido, Ricardo Soberon et al used cellulose in cow manure as fermentation raw material to produce lactic acid and synthesize PLA. They found that Enterococcus faecalis produced 144g/L of lactic acid in cow manure, which was 121.8g/L more productive than Lactobacillus valerate (21.8g/L for Lactobacillus valerate). It is 69.5g/L more productive than Bacillus (74.5g/L for Bacillus). At the same time, the productivity of lactic acid production by Enterococcus faecalis (5.1g/(L·h)) was significantly higher than that of Lactobacillus valerate (0.8g/(L·h)) and Bacillus hoensis (0.38g/(L·h)) [15].

Compared with foreign countries, China's research on PLA started late, since the establishment of the PLA project team in 2005, domestic scholars have gradually increased the research on PLA synthesis, modification and degradation.

In 2013, Luo Yufen used easily available D-L-lactic acid as raw material and cheap SnO as catalyst to synthesize the star-shaped polylactic acid (SPDLLA) with pentaerythritol as core by direct fusion polymerization. Although the molecular weight is relatively low, the average weight of SPDLLA can reach 20100, which can meet the needs of drug sustained release [16].

In 2021, Xia Yiwei et al. combined wood flour (WF) with PLA by melt blending, and then used TMC-328 as a nucleating agent to prepare high-heat resistant PLA/WF composites. Finally, it was found that both of them had a positive effect on the crystallization behavior of PLA, and could significantly accelerate the crystallization rate of PLA. The addition of WF and TMC-328 can effectively increase the crystallization rate of PLA, significantly increase the heat resistance (146.5℃), and also increase the tensile strength [17].

In 2023, Cui Zhaoning uses the biological fermentation technology of Lactoballus rhamnosus, whose storage number is CGMCC No.19507. After fermentation, the bacteria are filtered out to obtain calcium lactate, lactic acid and calcium sulfate are hydrolyzed with sulfuric acid, calcium sulfate is filtered to obtain Shi Yin by-product, and the clear liquid is crude lactic acid. Polylactic acid with 99%

chemical purity and 99% optical purity was obtained through ion exchange, membrane filtration, molecular shrinkage and crystallization [18]. In the same year, Li Xinfang selected polybutylene succinate (PBS), a biodegradable flexible material with low cost, as the toughening agent, acetyl-tributyl citrate (ATBC) with solvation and plasticization as the interphase solubility, and diisopropylbenzene peroxide (DCP), which was polymerized by free radicals, as the compatibilizing agent. The effects of PBS, ATBC and DCP contents on the mechanical properties of PLA/PBS/ATBC/TMC blends were comprehensively analyzed. The optimal ratio of each component in PLA/PBS/ATBC/TMC blends was 80:20:10:0.4:0.3, and the elongation at break of the blends was 416.5%. Compared with pure PLA, the impact strength is 4.22vkJ/m2, which is 165% higher than that of pure PLA, the tensile strength is 44.3 MPa, and the light transmittion is 82%. Obviously, PLA has the best toughening and permeability performance. At the same time, they found that adding PBS, ATBC and TMC to PLA matrix improved the crystallization capacity of PLA. When DCP is added to PLA/PBS/ATBC/TMC blend, the glass transition temperature of the blend is reduced. When DCP content is 0.3%, the glass transition temperature of the blend is reduced by 10.8℃ compared with pure PLA. It is obvious that the compatibility of PLA/PBS blend is the best, and the toughening and reflection effect of the blend is significant [19].

In 2024, in the study of scholar Zhao Yang et al., poly (lactic acid) copolymer with special structure was prepared and synthesized by introducing the method of melt polycondensation of p-hydroxybenzoic acid, malic acid and lactic acid, and the physical and chemical properties of polylactic acid material were modified by changing the chemical structure, hoping to synthesize polylactic acid material with special structure and excellent performance. They used differential scanning calorimetry (DSC) to find that the longer the reaction time of LA: MA:p-HPA, the higher the content of p-hydroxyphenylpropionic acid (P-HPA), the higher the molecular weight of the polymer, and the higher the Tg of the polymer. At the same time, comparing the results of polymerization products with different feed ratios, the Tg of the 24h product in the 50:2:2 polymerization system was higher than that of the

other two 24h polymer groups, and the copolymerization products showed greater rigidity. In the 24h data with a feeding ratio of 50:2:10, the MA repeating unit was improved compared with 14h, resulting in a smaller increase in polymer Tg. The group with the feeding ratio of LA:MA:P-HPA=50:2:5 and the other two groups of the same type of polymer T. Compared with the small size, combined with the results of nuclear magnetic analysis, it can be inferred that the polymer branch chain of this group is longer and the degree of polymer branching is higher [20].

1.2 Overview of E. coli

1.2.1 The structure of E. coli

The cell wall of E. coli is composed of peptidoglycan, which gives it morphological stability and mechanical strength. The cell membrane is responsible for the entry and exit of substances, including the absorption of nutrients and the expulsion of metabolites. The cytoplasm contains a variety of organelles such as ribosomes and nucleic acids, and is the main place for cell metabolism. The pseudokaryotes are the genetic material of bacteria and, unlike the nuclei of eukaryotes, are not enclosed by a nuclear envelope.

Flagella are the vital motor organs of E. coli, rotating like propellers to propel the bacteria through the water. Pili are structures that E. coli adhere to the surface of host cells and help colonize the gut.

1.2.2 Application of E. coli

Genetic engineering: Escherichia coli is a commonly used recipient cell in genetic engineering. Scientists can use its characteristics of easy culture and fast reproduction to introduce foreign genes into it and obtain the required proteins through expression. This technology is widely used in biomedicine, agriculture and other fields.

Biopharmaceutical: E. coli also plays an important role in the biopharmaceutical field. By means of genetic engineering, it is possible to express protein drugs needed

by human beings in E. coli, such as insulin, growth hormone, etc. These protein drugs, when purified, can be used to treat a variety of diseases.

Sewage treatment: E. coli has certain application value in sewage treatment. They can decompose organic matter in sewage and reduce the concentration of pollutants in sewage. At the same time, Escherichia coli can also be used as an indicator organism to monitor the degree of water pollution.

Intestinal microecology research: As an important member of intestinal flora, Escherichia coli is of great significance for the study of intestinal microecology balance and intestinal diseases. The analysis of E. coli species, quantity and interaction with host cells is helpful to understand the function and regulation mechanism of intestinal microecology.

In conclusion, Escherichia coli, as a common intestinal bacteria, has unique features in terms of structure, function and application. Through in-depth study of *E. coli*, we can better understand the biological world and provide strong support for the development of biomedicine, environmental protection and other fields.

1.3 Overview of flask fermentation

Shaker fermentation is a common microbial culture method, widely used in biomedicine, agriculture, food industry and other fields. By shaker fermentation, we can effectively simulate the industrial production environment under laboratory conditions to achieve large-scale proliferation of microorganisms and accumulation of specific metabolites. The following is an overview of the common methods of flask fermentation and their advantages and disadvantages.

1.3.1 Common fermentation methods

1.3.1.1 Batch fermentation

Batch fermentation is the simplest and most commonly used fermentation process in which all carbon substrates and other components are not added during fermentation except for the neutralizer used for pH control. This closed system has advantages over other fermentation methods in terms of reducing the risk of

contamination and obtaining high lactate concentrations. Batch fermentation, on the other hand, suffers from low cell concentrations due to limited nutrient levels and is less productive mainly due to substrate and/or product inhibition. Different fermentation methods are applied in batch fermentation mode, including SSF, separate hydrolysis and fermentation (SHF), mixed culture or open fermentation.

1.3.1.2 Feed batch fermentation

Fed-batch fermentation is considered to be a better fermentation system that keeps the substrate concentration at a low level by feeding nutrients into the fermentation solution [21]. It is helpful to increase the yield of fed-batch fermentation by adding high concentration substrate without excluding fermentation liquid. However, it still does not address the inhibitory effect on yield due to the gradual accumulation of substrates. Repeated fermentation

Repeated fermentation in batch or fed-batch fermentation involves a repeated cycle by transferring some or all of the cells from a previous run into the next run [22]. Compared with other fermentation methods, repeated fermentation has obvious advantages, such as environmental friendliness, shorter time required, higher product concentrations can be obtained and no time is required for bacterial preparation.

1.3.1.3 Conventional continuous fermentation

Traditional continuous fermentation is widely used because it avoids the inhibition of the end product during batch fermentation. The common continuous fermentation system is to send the fresh culture medium into the fermenter, and at the same time the fermenter will be discharged at the same speed to ensure the continuous control of the concentration of the fermenter components. In this fermentation, the amount of products, substrates and cells in the fermentation solution can be relatively constant during the fermentation process.

1.3.2 Research status of fermentation methods

1.3.2.1 Batch fermentation

Batch fermentation is the simplest and most commonly used fermentation process in which all carbon substrates and other components are not added during

fermentation except for the neutralizer used for pH control. This closed system has advantages over other fermentation methods in terms of reducing the risk of contamination and obtaining high lactate concentrations. Batch fermentation is the simplest and most commonly used fermentation process in which all carbon substrates and other components are not added during fermentation, except for the neutralizer used for pH control. This closed system has advantages over other fermentation methods in terms of reducing the risk of contamination and obtaining high lactate concentrations. Batch fermentation, on the other hand, suffers from low cell concentrations due to limited nutrient levels and has low productivity mainly due to substrate and/or product inhibition. Studies of the dynamics of lactic acid production have shown that the final lactate concentration increases with the initial glucose concentration, up to 200 g/L. Moon et al. (2012) reported the highest lactic acid concentration in Lb batch fermentation 23. Paracarbonate CB 2121 was used to obtain 200 g/L glucose and 192 g/L lactic acid.

1.3.2.2 Feed batch fermentation

In 2023, Li Xiuping found that fed-batch fermentation was conducive to improving the fermentation efficiency of sugarcane fruit wine, and concluded through experiments that the optimal fed-batch fermentation conditions of sugarcane fruit wine were as follows: the feeding time was 2.5d and the sugar content of the feeding liquid was 29. The addition amount of Bx and filling liquid is 35% (v/v). Under the optimal conditions, the yield, alcohol content, average consumption of raw materials and average rate of alcohol production of sugarcane fruit wine were 0.405 L, 15.8% (v/v) and 1.833, respectively. Bx·L/d and 1.067% vol· L/d were 35%, 12.27%, 38.24% and 51.49% higher than that of batch fermented sugarcane fruit wine, respectively [24]. In 2024, Hassan Mohamed et al. increased lipid and biomass production of Mucor WJ11 by implementing four different feed-batch fermentation strategies (S1-S4). After 120 and 144 h, the S1 starter fermentation strategy produced the highest biomass, lipid and fatty acid contents (22 ± 0.7 g/L, 53 ± 1.2% and 28 ± 1.6%), respectively. The optimal abiotic factors were pH6.5, 25-26℃, inoculation amount 15%, 500 r/min, dissolved oxygen 20%-30% and fermentation for 120 h [25]. Batch

fermentation and feed batch fermentation can also be applied to fermentation in shaker. For example, Maria Ruottinen et al improved the production of human type II procollagen in the yeast Pichia pastoris in shaker in 2008 by using a wirelessly controlled feed batch system 26.

1.3.2.3 Repeated fermentation

In 2022, Tian Dahe et al., of Zhuhai Meda Biotech Technology Co., LTD., disclosed a method for producing PLA by repeated batch fermentation of halomonas. Compared with the previous single batch fermentation, the method of the invention leaves the fermented bacterial liquid in the fermenter according to a certain volume proportion, and re-forms a large amount of seed liquid by mixing it with LB medium. Let it ferment again. They found that the PLA content of the harvested bacterial solution was stable in the range of 70% ~ 75% after a single batch of repeated fermentation for about 50h and 15 tanks for 750h, and the molecular weight of PLA was not significantly different from that of a single batch of fermentation, and the yield was about 4.59 kg [27].

In 2023, Emine Bezirci et al used propionic acid as raw material to produce propionic acid by repeated batch fermentation and fed-batch fermentation using a two-stage fermentation method. For lactic acid obtained from whey, the maximum propionic acid concentration was 28 g/L at the 700th hour (cycle 13) and 35 g/L at the 3,600th hour (cycle 10) for lactic acid obtained from flour hydrolysates. The lactic acid and propionic acid yields of whey lactose were 0.80 g/Lh and 0.050 g/Lh, respectively, which were higher than those of flour hydrolysate (0.12 g/Lh and 0.025 g/Lh, respectively) [28].

1.3.2.4 Conventional continuous fermentation

Studies in the biological industry have shown that the productivity and production efficiency of recombinant proteins are improved by continuous culture. Sanaz Mahboudi et al. established a novel upstream fermentation process for the production of recombinant uricase from methanol-nutritive yeast *Pichia pastoris* in 2023. In the induced fermenter, the activity of recombinant uricase was 4.13, 7.2 and 0 V/mL, respectively, at the methanol feed rate of 30, 60 and 80 mL/h. In continuous

fermentation, the optimal methanol feeding method increased the yield from 0.04V /mL/h (0.0017mg /mL/h) to 0.18V /mL/h (0.0078mg /mL/h), 4.5 times that of fed-batch fermentation [29]. In 2024, Wang Zhiqi used the continuous fermentation process to optimize the high-density continuous fermentation production process of I-tyrosine, and found that in a 5 L fermenter, 30% seed inoculation amount was selected, and the substrate choline chloride concentration was 1 g/L at the beginning of fermentation. 0.2g/L choline chloride was added to the tank every 4 hours. When fermentation reached 12h, the base sugar was exhausted and glucose was supplied to the tank at a sugar supplement rate of 12g /(L·h). At this time, the liquid was discharged at a rate of 0.13 / h, making the liquid loading constant at about 20%. The highest OD_{600} reached 65 and the acid production was 55.8g/L at 35h fermentation. The conversion rate of sugar and acid was 25.4%, which provided an important reference for the industrial production of continuous L-tyrosine fermentation [30].

1.3.3 Fermentation condition optimization

1.3.3.1 Temperature

In 2023, Li Aijun et al. artificially improved the quality stability of bacterial-type tempeh in industrial production. Using four different varieties of commercial soybeans (Ls, DBL, DBS, BS) as raw materials, the modified Gompertz equation was used to fit the changes in amino acid nitrogen content of bacterial-type tempeh under three different post-fermentation temperatures (15℃, 25℃, and 40℃). The content of free amino acids at the end of fermentation was determined. The results showed that the Gompenz equation could well fit the kinetics of amino acid nitrogen formation (decision coefficient >0.96), and the predicted fermentation end points at three different post-fermentation temperatures (25℃, 15℃ and 40℃) were about 9d, 10d and 4d of fermentation, respectively. The maximum amino acid nitrogen generation of the four bacterial types of fermented fermented beans at the fermentation temperature after 15℃ is DBL>LS>DBS>BS, and the maximum amino acid nitrogen generation at the fermentation temperature after 25℃ and 40℃ is LS>DBL>DBS>BS [31].

1.3.3.2 pH

In 2023, Taking the residual sludge of a municipal sewage treatment plant as the research object, Bi et al. used batch tests to investigate the effects of constant pH adjustment of 5,7,9,10 and phased pH adjustment on the dissolution of organic matter during anaerobic fermentation, and used high-throughput sequencing technology to analyze the microbial community structure of fermentation sludge under different pH regulation methods. The results showed that more organic matter could be dissolved under alkaline and acidic conditions under constant pH, and the accumulation reached the maximum when pH=10, when the soluble organic matter (SCOD) concentration was 323.77 mg/g VSS. The production of VFAs is 183.38 mg COD/g VSS [32].

In 2014, Lei Ping et al. studied the effects of rotational speed and ventilation on mycelial biomass, exopolysaccharide yield, dissolved oxygen and mycelial morphology in a 20 L stirred fermenter. The results showed that in the experimental range, when the rotating speed was 150 r/min, the mycelial biomass was the highest (12.65 g/L), the extracellular polysaccharide yield was the highest (2.99 g/L), the relative dissolved oxygen was the fastest, the microspheres were small, compact and the proportion of filaments was small. The mycelial biomass was the largest (12.69 g/L) and exopolysaccharide yield was the highest (3.00 g/L) when the ventilation volume was 1∶ 0.65 vvm [33].

1.3.3.3 Time

In 2022, Chen Guangyin et al. added NaOH to the fermenter during the traditional two fermentation processes, and studied the effect of fermentation time on the biogas fermentation of rice straw using rice straw as raw material. They found that rice straw could still produce gas after the first fermentation, and the longer the fermentation time, the smaller the gas production (dry matter (TS) gas production was 28.11 ~ 50.73 mL/g TS). After adding NAOH to these straw, the substance cell wall of rice straw was dissolved, resulting in a large amount of organic matter spilling out, and the concentration of COD, total nitrogen, ammonium nitrogen and nitrate nitrogen extracted from rice straw was higher than that of rice straw without NaOH

treatment. At the same time, they also found that NaOH did not destroy its skeleton structure but changed the relative content of its functional groups, which aggravated the destruction of the cell structure. The rice straw treated with NaOH for 15d, 25d and 35d was used for biogas fermentation, and the gas production increased by 77.37%,119.41% and 159.94% compared with the corresponding controls, respectively. The longer the fermentation time, the better the effect of NaOH treatment on straw gas production [34].

1.4 The research idea of this topic

1.4.1.1 The research significance of this topic

Biodegradable polymers have attracted much attention for their potential in addressing environmental issues, and in many traditional sectors, such as agriculture, packaging and clothing, they have gradually phased out traditional non-degradable fossil-based commercial polymers. However, the synthesis of this biodegradable polymer is usually difficult, so the yield is small, and has not been greatly developed.

Polylactic acid (PLA), as recognized as the most promising new biodegradable polymer in the 21st century, has the advantages of being better than plastic polyethylene and polypropylene, and has excellent mechanical properties (such as high strength, high modulus, good barrier property, etc.) and good biocompatibility, non-toxic properties and degradability, in many fields such as environmental protection, medical health, etc. Has played a growing role.

However, there are few studies on optimizing the fermentation conditions of polylactic acid strains to improve the yield of polylactic acid. Therefore, in-depth construction and fermentation of high-yielding PLA strains and establishment of a stable, efficient and practical polylactic acid fermentation environment can lay a theoretical foundation for the industrial production of polylactic acid.

1.4.1.2 Main research content

(1) Suitable plasmids, vectors and strains were selected by synthetic biological methods to construct strains that could produce PLA efficiently.

(2) The effects of different temperature, pH, rotational speed and fermentation time on polylactic acid (PLA) fermentation were investigated by shaker fermentation method and single factor method, so as to obtain the most suitable experimental conditions for polylactic acid (PLA) fermentation.

1.5 conclusion of chapter 1

The first chapter mainly discusses the review and research status of PLA and its related fermentation methods. However, it is found that few fermentation methods of PLA produced by Escherichia coli are used through reviewing these literatures, so this paper aims to optimize the fermentation conditions of PLA by this method.

CHAPTER 2

OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Construction and activation of vector and PCR technique

2.1.1 Desired strain

E.coli *JW3169*

2.1.2 Required carrie**r**

pCAT204-IdhA-pCT540

2.1.3 Desired engineered bacteria

Restriction endonuclease, T4 DNA ligase, Ex TaqTM polymerase DL 2,000 DNA Marker

2.1.4 Experimental instrument

2.1.4.1 Laboratory medium

Luria-Bertani(LB) medium: yeast powder 5 g/L, peptone 10 g/L, NaCl 10 g/L. As needed, 50μg/mL kanamycin sulfate was added to the medium.

2.1.6 Experimental procedure

2.1.6.1 Preparation of receptive cells

A single colony of LB AGAR plate was taken, inoculated in 2mLLB liquid medium, shaken with 3TC oscillator for 5-6 hours, transferred to 100ml saline solution, 50ml of sterile medium was filled, then incubated for about 3-4 hours, and bacteria were transferred to 50ml centrifuge tube. Pre-cooled with ice in a sterile environment and placed on ice for 30 minutes, centrifuged at 400r/min in a 4 "C centrifuge for 10 minutes, discarded the supernuant, turned the test tube into lmin, discharged from the residual medium, and added 0,1mol/l CaCl 210 ml pre-cooled ice resuspension precipitate to the precipitation. After 30 minutes in the ice bath centrifugation at 4000r/ rain, discard the supernant, add 0,1moFL CaCl 22 2ml to the

precipitate (depending on the amount of bacteria forming 1/15,1/20 cell volume), the suspended bacteria can be immediately used for conversion. When storing, sterile glycerin should be added to a final concentration of 15%, divided by 0.1mI/ tube, and stored in the refrigerator at -80°C for later use.

2.1.6.2 Transformation of plasmids

The newly prepared receptive cells were placed on the ice, 100 / ML were placed in a sterile 1.5mL EP tube, 10pL of the connection product was added, gently rotated to mix the contents, placed on the ice for 30min, the water bath was circulated at 42℃ for 90Sec, the centrifuge tube was quickly transferred to the ice bath, and the cells were cooled for 2 min. Add 400pLLB liquid culture base, culture in 3TC 200.220r/min shaker for 45min, absorb 100 / zL bacterial solution coated plate, culture at 37℃ for 12.16 h to observe the emergence of single colonies.

2.1.6.3 Colony PCR verification

The transformed simple indendrine was selected and incubated in 1.5mLEP tube containing lmLLB medium (including Amp), and incubated overnight at 37℃ with oscillations. 2009L bacterial solution was aseptically cultured in 0.5mLEP tube, centrifuge for lmin at 8,000 t/rain, discard the supernant, re-suspended precipitation with 20 / zLddH20, and then heated for 10 rain. After centrifugation at 12 000r/rain for 3min, the supernatant was used as the template, amplified by PCR, and analyzed by electrophoresis on 0.8% agarose gel.

2.1.6.4 Strain activation

Individual colonies of E. coli JW3169 were inoculated from solid medium into a 100ml triangular flask containing 10ml fresh LB medium (containing 50Ixg/ml kanamycin) and cultured overnight at 37°C, 200r/min.

2.2 Shaker fermentation and single factor experiment

2.2.1 strain

Transformed E.coli *JW3169*

2.2.2 medium

(1) LB medium: yeast extract 5g, tryptone 10g, sodium chloride 10g, NaOH to adjust the pH to 7.0, 121℃, 20min autoclave sterilization.

(2) Initial flask fermentation medium:

Shake flask fermentation medium: Glucose 20 g/L, peptone 4 g/L, yeast powder 6 g/L, anhydrous citric acid 2 g/L, potassium dihydrogen phosphate 0.15 g, methionine 0.3 g/L, KH2PO4 5.5 g/L, K2HPO4 45 g/L, MgSO4·7H2O 2 g/L, FeSO4 20 mg/L, phenol red 8 mg/L, pH 7.0 ~ 7.5.

2.2.3 Experimental instrument

Double-layer small capacity constant temperature shaking table high pressure steam sterilization; Ultra-clean workbench; AYAN-F60 Molecular Distillation Apparatus; Autoclave; Chromatograph,; SBA-40E biosensor;

2.2.4 Single factor optimization of fermentation conditions

2.2.4.1 The effect of temperature on PLA yield was determined

Experiments were carried out at 25 ℃, 30 ℃,35 ℃ and 37 ℃ respectively to explore the effects of different temperatures on the yield of experimental strains.

2.2.4.2 The effect of pH on PLA yield was determined

The effects of shaker fermentation at PH 6.0, 6.5, 7.0,7.5 and 8.0 on PLA yield were investigated.

2.2.4.3 The effect of rotational speed on PLA yield was determined

Each shaker in this group was fermented at different speeds (180 r/min, 190 r/min, 200 r/min, 210r/min, 220 r/min).

2.2.4.4 The effect of fermentation time on PLA yield was determined

The induction time is closely related to protein production and enzyme activity, and the bacteria will not grow all the time due to the restriction and influence of energy materials such as nitrogen source and carbon source. The effect of fermentation time on PLA yield was studied for 24, 36, 48 ,60 and 72 h, respectively.

2.2.5 Experimental data analysis

The experimental data in the experiment were repeated three times, and the average value and standard error of the obtained data were calculated, and then statistical analysis was carried out.

2.2.6 Detection method

(1) Bacterial concentration detection method

The fermentation liquid was placed at room temperature for 30 min, and the supernatant in the fermentation liquid was diluted 50 times with a 100 WL pipette. The OD value of the bacteria was measured at 600 nm wavelength by spectrophotometer, and the OD value was taken as the average value of three parallel shaker samples. The calculation method was shown in Formula 2-1.

The total OD value of bacteria in fermentation solution $=OD_{600} \times$ dilution ratio formula 2-1

CHAPTER 3
EXPERIMENTAL PART

3.1 Results and analysis

3.3.1 Effect of different temperature on PLA yield

Table 3.1 - **Effect of different temperature on PLA yield**

Temperature	Polylactic acid yield（g/L)	OD value of bacteria
25 ℃	25.3	6.8
30 ℃	33.1	10.1
35 ℃	28.2	7.8
37 ℃	27.5	8.3

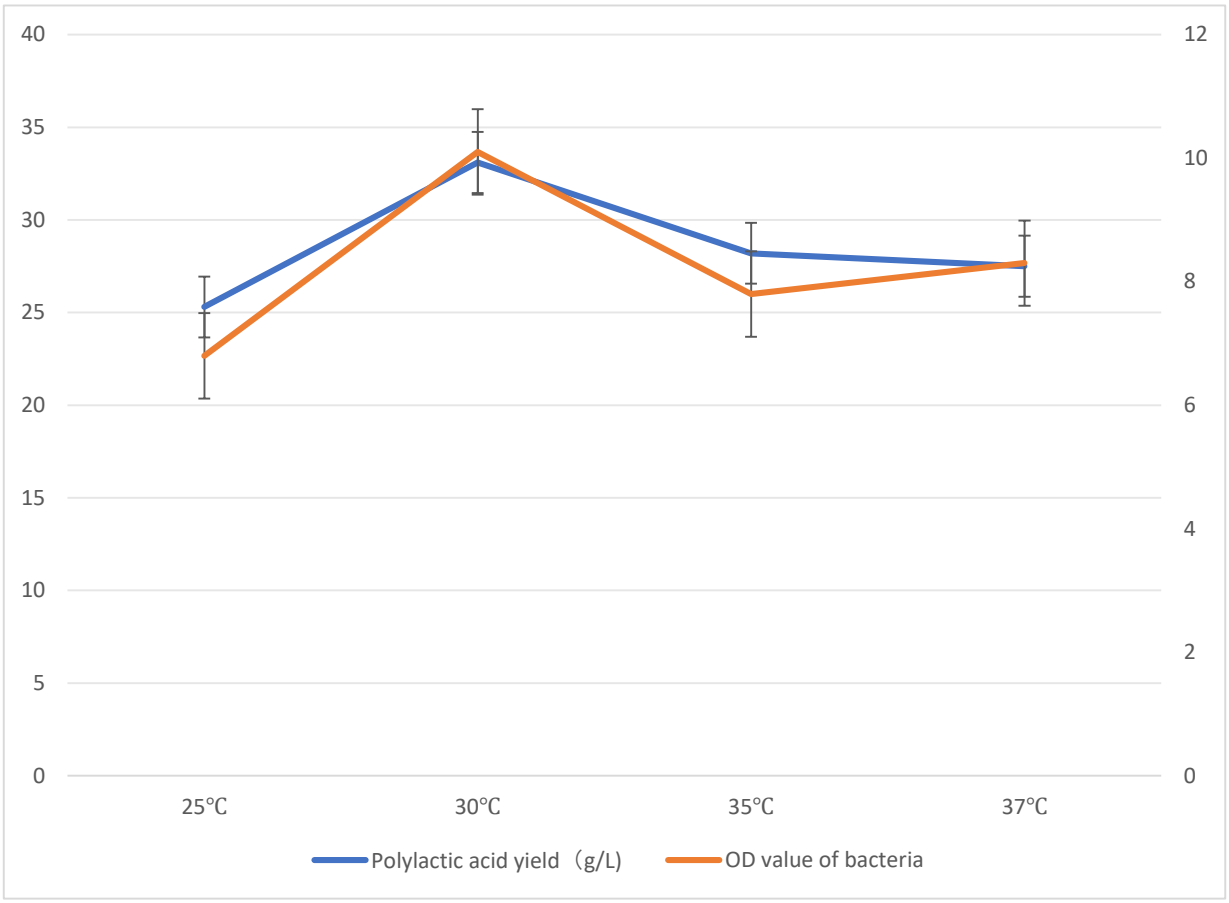

Figure 3.1 – Effect of different temperature on PLA yield

In this experiment, fermentation temperatures of 25˚C, 30˚C, 35˚C, 37˚C were used to study the effect of temperature on the activity of PLA strains and PLA yield. In addition, the optimal fermentation temperature was obtained by comparing the yield of polylactic acid at each temperature. As can be seen from Figure 2-1, the influence of temperature on polylactic acid yield and cell growth is basically similar. By observing the experimental results, it is not difficult to conclude that when the temperature is lower than 30°C, the poly-lactic acid yield is 33.1 g/L, and the cell OD value is 10.1, the poly-lactic acid yield increases with the increase of temperature and shows an upward trend. However, when the temperature continues to increase and the cell activity finally reaches 37°C, the decline of the cell activity leads to the decline of the PLA yield. From the above, we can conclude that in the appropriate temperature range 28˚C - 30˚C), the activity of the bacteria increases, which will

gradually increase the yield of polylactic acid. However, if the temperature is too high, it will inhibit bacterial metabolism, resulting in reduced production of polylactic acid. Therefore, based on the results of this experiment, we determined that the optimal fermentation temperature is 30°C.

3.2 Effect of different pH on PLA yield

Table 3.2 - **Effect of different pH on PLA yield**

pH	Polylactic acid yield（g/L)	OD value of bacteria
6.0	24.9	6.9
6.5	27.8	7.8
7.0	33.5	10.3
7.5	26.9	8.2
8.0	25.8	7.9

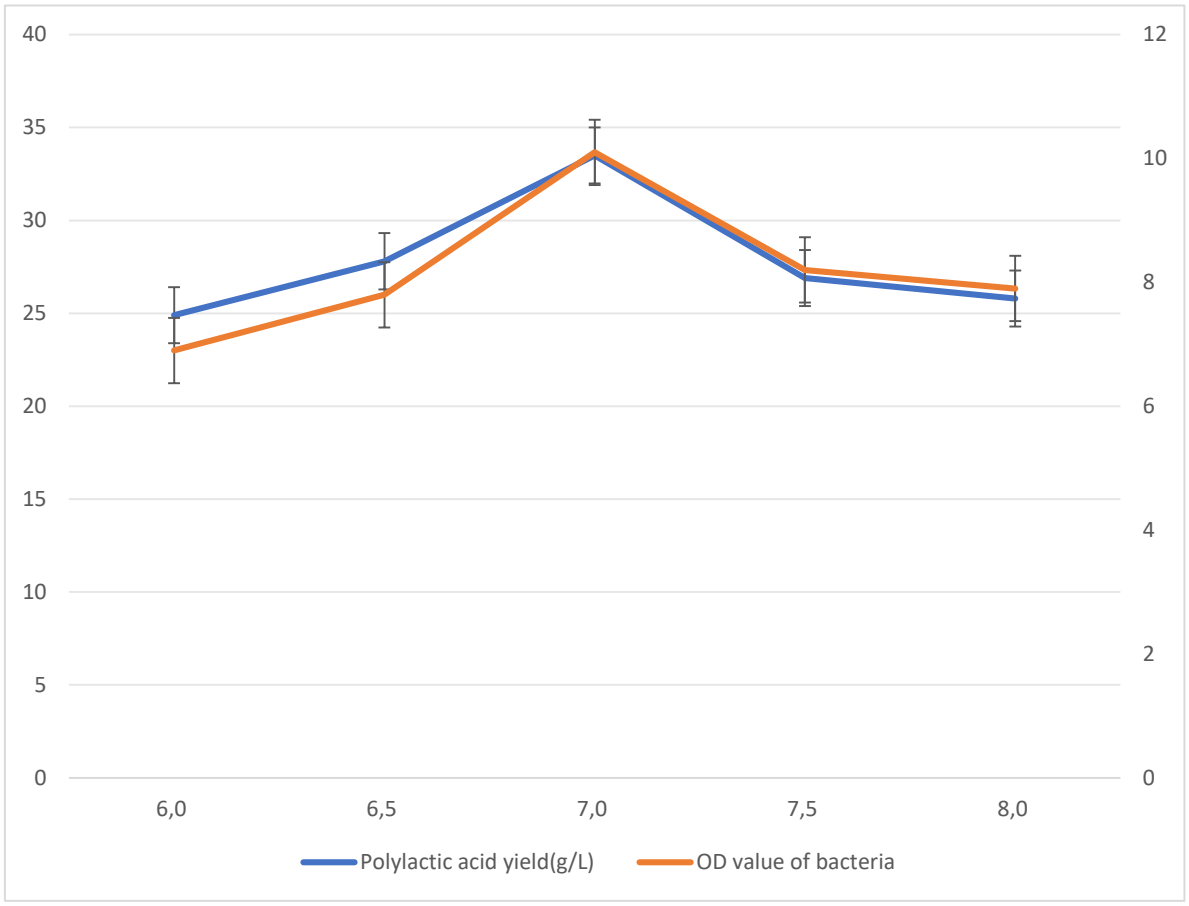

Figure 3.2 – Effect of different pH on PLA yield

In this experiment, five pH cultures of 6.0, 6.5, 7.0, 7.5, 8.0 were used to ferment Escherichia coli, respectively, to explore the effect of pH on PLA yield.

As can be seen from Figure 2-2, when pH value is 7.0, polylactic acid yield and OD value are the highest, polylactic acid yield is 33.5 g/l, and cell OD value is 10.1. When pH is 7.0, the permeability of bacterial cell membrane reaches the maximum, which can not only accelerate the discharge of waste generated by cell metabolism, but also allow the material for bacterial growth to enter the cell, but also accelerate the growth of bacterial body. Therefore, choose the optimal pH value of 7.0.

3.3 Effect of different rotational speed on PLA yield

Table 3.3 - **Effect of different rotational speed on PLA yield**

Rotational speed	Polylactic acid yield（g/L)	OD value of bacteria
180 r/min	24.1	6.3
190 r/min	25.0	7.1
200 r/min	34.2	10.4
210 r/min	24.4	7.6
220 r/min	23.2	6.5

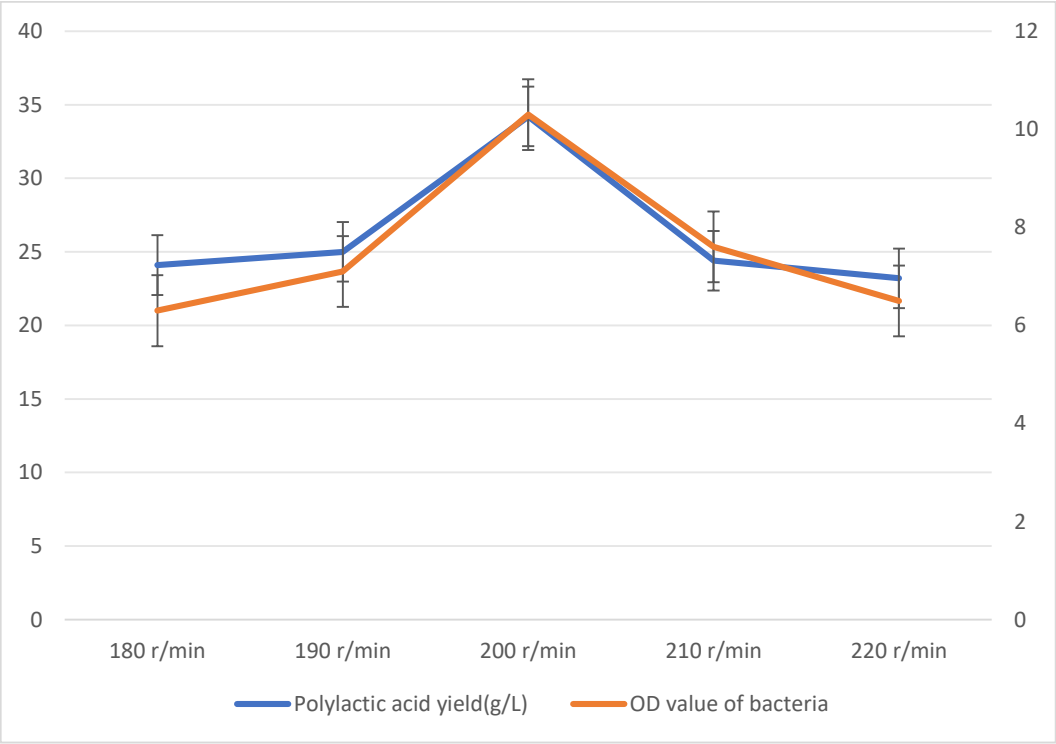

Figure 3.3 – Effect of different rotational speed on PLA yield

As can be seen from Figure 3.8, when the rotor speed is lower than 200 r/min, the polylactic acid yield and OD value of bacteria are very low, the content is only 24.1g /L, and the OD value is 6.3. And when the rotational speed reached 200 r/min, the polylactic acid yield (34.29g /L) and cell OD (10.3) value reached the best value. However, when the rotor speed was gradually increased, the PLA yield and OD value of the bacteria were gradually reduced. The optimum rotational speed for polylactic acid fermentation was 200 r/min, taking into account both bacterial condition and PLA yield.

3.4 Effect of different fermentation time on PLA yield

Table 3.4 - **Effect of different fermentation time on PLA yield**

Fermentation time	Polylactic acid yield（g/L)	OD value of bacteria
24 h	19.2	6.7
36 h	26.5	8.7
48 h	32.9	9.8
60 h	24.3	7.3
72 h	22.1	7.0

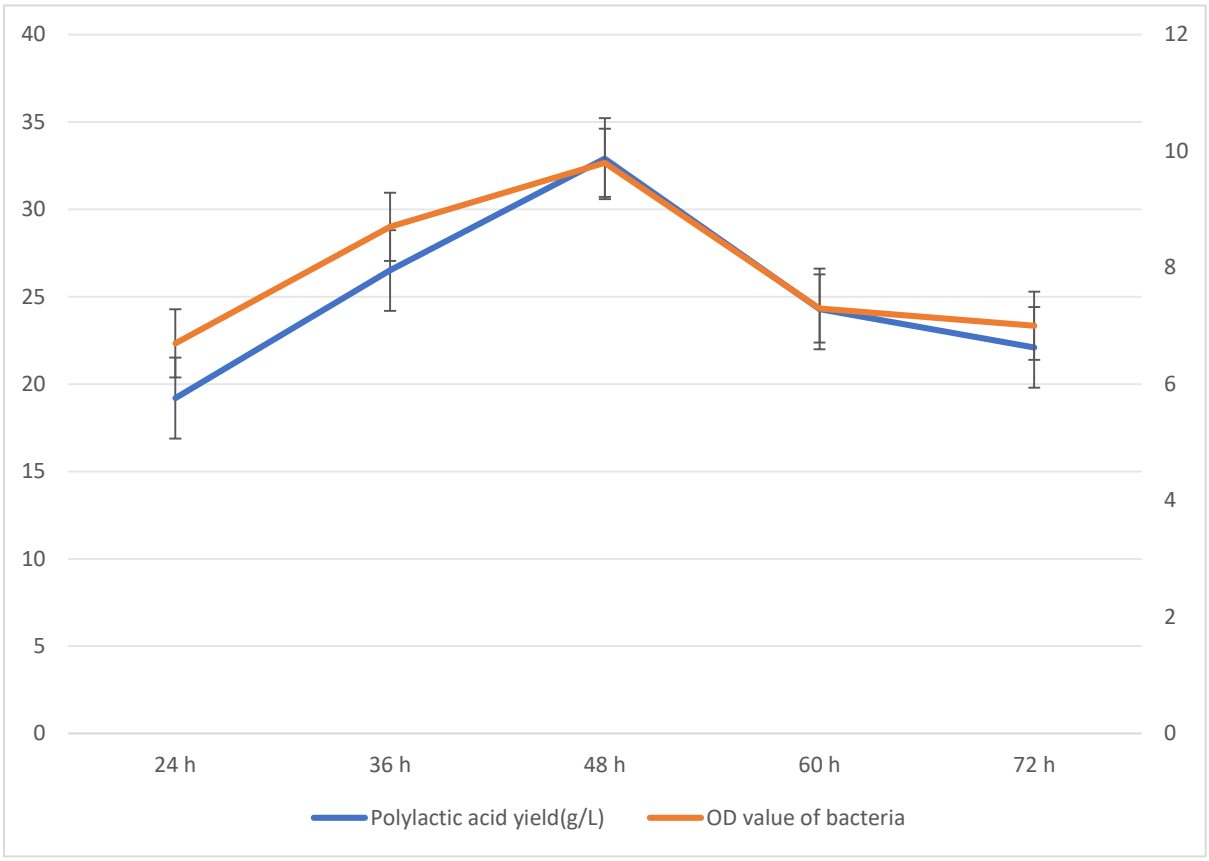

Figure 3.4 – Effect of different fermentation time on PLA yield

For the fermentation process of polylactic acid, the fermentation time is very important, and the quality and yield of the fermentation process and products are greatly affected by it. The study of Gao Ruoshan et al. showed that the best choice is that the strain is in the logarithmic stage of vigorous growth, and such a strain has strong reproduction ability and can quickly enter the logarithmic stage after inoculation of fermentation medium, thus shortening the fermentation time and increasing the acid yield [35].

In order to ensure that the strains attached to the fermentation medium have good viability and prevent cell aging and cell death, a separate medium is selected for fermentation. In this way, the optimal inoculation time can be determined by separately setting the fermentation time to affect the PLA yield and bacterial activity, so as to achieve the best fermentation effect. As shown in the figure, the maximum yield of PLA was 32.9 g/h after inoculation, and OD value reached 9.8. The effect of inoculation time on PLA yield showed a normal distribution curve. In a certain period

of time (48 h), OD values of PLA and thallus increased, but began to decrease after 36 h, which may be related to the overgrowth of thallus caused by too long fermentation time.

3.5 Conclusion of chapter 3

The optimal fermentation environment of PLA producing Escherichia coli was established by constructing a specific PLA producing strain, then shaking bottle feeding fermentation, and using single factor experiment method, that is, 30℃, pH 7, rotation speed 200 r/min and 48 h.

CONCLUSIONS

1. The research direction of this paper is to optimize the fermentation conditions of PLA strains. In previous studies, many people have optimized the fermentation conditions and methods of PLA. For example, Cui Zhaoning used rhamnebacterium to enhance the strength of PLA blends through fermentation technology [18]. Zhao Yang prepared and synthesized polylactic acid with special structure by introducing the melt polycondensation of p-hydroxybenzoic acid, malic acid and lactic acid [20], etc. However, there was limited exploration and discussion on the specific optimal fermentation conditions of PLA. Based on the research and summary of previous papers.

By using the relevant knowledge of synthetic biology, a suitable and efficient PLA producing plasmid was constructed and introduced into specific Escherichia coli to form a strain with high PLA producing efficiency. After fully PCR and activation of the strain, the feed-shaking flask fermentation was carried out. During this period, the optimal conditions for PLA production of this strain were explored by using single factor method, which was divided into four aspects: temperature, pH, rotational speed and fermentation time. Finally, the following conclusions are obtained:

(1) A strain with high PLA production efficiency was obtained, and the transformed *E.coli* JW3169 was obtained using pCAT204-IdhA-pCT540 as carrier.

(2) The optimum temperature for PLA production of this strain was 30 ℃, and the PLA yield was 33.1 g/L under the same conditions.

(3) The optimum pH for PLA production of this strain was 7.0, and the PLA yield was 33.5 g/L under the same conditions.

(4) The optimum rotational speed of this strain for PLA production was 200 r/min, and the PLA yield was 34.2 g/L under the same conditions.

(5) The optimum fermentation time for PLA production by this strain was 48 h, and the PLA yield was 32.9g /L under the same conditions.

2. outlook

In this study, only recombinant strains were used to optimize the fermentation conditions of PLA, and satisfactory results were obtained. However, it has been reported that better strains can be obtained for PLA production experiments by directly screening high-temperature and hyperosmolous strains [36], and the influence of carbon source and nitrogen source on PLA fermentation has also been studied. Therefore, future studies can further explore the effects of screened strains and more detailed fermentation conditions on the performance of PLA strains, so as to improve the fermentation efficiency of strains and obtain higher yields. In addition, the study focused on the optimization of laboratory-level fermentation conditions for producing PLA strains, and results have been reported on industrial level experiments [37]. Therefore, larger studies should be further developed in the future to more directly promote the development of the PLA industry

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