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 the control technology of pullulan fermentation

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

Completed: student of group BEBT-20
Zhang TIANYI
Scientific supervisor
Olga ANDREYEVA, Dr. Sc., Prof.
Reviewer
Olena OKHMAT, Ph.D., As. prof.

Kyiv 2024

KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN

Faculty: Chemical and Biopharmaceutical Technologies

Department: Biotechnology, Leather and Fur

First (Bachelor's) level of higher education

Specialty: 162 Biotechnology and Bioengineering

Educational and professional program Biotechnology

APPROVE

Head of Department of Biotechnology,

Leather and Fur, Professor,

Doctor of Technical Science

Olena MOKROUSOVA

_____

«___»_____2024

ASSIGNMENTS

FOR THE QUALIFICATION THESIS

Zhang Tianyi

1. Thesis topic Study on the control technology of pullulan fermentation

Scientific supervisor Olga Andreyeva, Dr. Sc., 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	Chapter 1. Literature review	From 06 April 2024 to 20 April 2024	
3	Chapter 2. Object, purpose, and methods of the study	From 21 April 2024 to 30 April 2024	
4	Chapter 3. Experimental part	From 01 May 2024 to 10 May 2024	
5	Conclusions	From 07 May 2024 to 12 May 2024	
6	Draw up a bachelor's thesis (final version)	From 20 May 2024 to 25 May 2024	
7	Submission of qualification work to the supervisor for feedback	From 25 May 2024 to 27 May 2024	
8	Submission of bachelor's thesis to the department for review (14 days before the defense)	27 May 2024	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	31 May 2024	
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	03 June 2024	

I am familiar with the task:
Student _____ Zhang TIANYI

Scientific supervisor. _____Olga ANDREYEVA

SUMMARY

Zhang Tianyi. Study on the control technology of pullulan fermentation. – Manuscript.

Qualification thesis on the specialty 162 «Biotechnology and Bioengineering». – Kyiv National University of Technologies and Design, Kyiv, 2024.

Pullulan, as an important linear extracellular polysaccharide, is mainly produced by Aureobasidium pullulans through a specific biosynthetic pathway. Its structure is based on the tight connection of the repeat unit of maltotriose through α-(1-6) glycosidic bonds. this unique chemical structure gives pullulan a series of remarkable physical and chemical properties, such as high-water solubility, excellent film-forming ability, excellent oxygen resistance, good plasticity, low viscosity and easy biodegradation. In this paper, the carbon source of pullulan produced by Aureobasidium pullulans was studied, and the effects of various metal ions on pullulan biosynthesis were compared through a large number of experiments in order to obtain a better medium formula. finally, the effects of continuous fermentation and fermenter fermentation on pullulan production by Aureobasidium pullulans were studied.

In the experiment of studying the effects of continuous fermentation and fermenter fermentation on the production of pullulan by Aureobasidium pullulans, the changes of sugar production by Aureobasidium pullulans under different conditions were systematically explored through shaking table and fermenter experiments. The results showed that 30 ℃ was favorable for the growth of Aureobasidium pullulans, while 28 ℃ was more suitable for pullulan synthesis. In batch culture, pullulan production and cell growth rate reached the peak at 400 rpm speed, while 220 rpm speed was the best for shake flask culture.

In the experiment of the effect of different carbon source combinations on pullulan production by Aureobasidium pullulans, the effects of sucrose, glucose and fructose as carbon sources on pullulan production by Aureobasidium pullulans were deeply compared. combined with the combination of the three carbon sources, the

corresponding fermentation parameters were obtained. The experimental data showed that although sucrose as a carbon source achieved the highest yield in the process of pullulan biosynthesis, the carbon source combination of fructose and glucose at 1:1 showed significant economic advantages and potential for development.

In the experiment of studying the effect of different metal ions on pullulan production by Aureobasidium pullulans, the optimal concentration of each metal ion was obtained by adding magnesium, iron, copper, zinc, calcium, manganese and other metal ions to the culture medium. The results showed that the types and concentrations of trace elements had significant effects on the polysaccharide yield of Aureobasidium pullulans, and the yield was significantly different under different combinations and concentrations, and the best concentrations were 0.05 g/L $MnCl_2$, 0.05 g/L $ZnSO_4$, 0.15 g/L $CaCl_2$, 0.1 g/L $CuSO_4$, 0.06 g/L $FeSO_4$ and 0.2 g/L $MgSO_4$.

Keywords: Aureobasidium pullulans, Pullulan, Optimization of fermentation conditions, medium optimization.

TABLE OF CONTENTS

INTRODUCTION

The carbon content of microbial cells accounts for nearly half of its dry weight, so carbon source is very important for microbial growth, and it is the most needed nutrient for microorganisms except water. Different carbon sources have different effects on the growth and metabolism of microorganisms. In monosaccharides, disaccharides and polysaccharides, microorganisms are usually more inclined to use monosaccharides because of their simple structure and easy to be absorbed and utilized by microorganisms. Among monosaccharides, hexoses such as glucose and fructose are usually more popular with microbes than pentose such as mannose and galactose.

For polysaccharides, the utilization ability of microorganisms varies according to the type and structure of polysaccharides. For example, starch, as a common polysaccharide, is usually more popular than pure polysaccharides such as cellulose or chitin because it is easily degraded into monosaccharides by microorganisms. However, heteropolysaccharides, such as Agar, have relatively weak utilization ability of microorganisms because of their complex structure. Text Aureobasidium pullulans is a special microorganism. In the process of synthesizing pullulan, sucrose as its carbon source substrate has the highest yield. Sucrose has become the best carbon source for pullulan synthesis by Aureobasidium pullulans because sucrose is first degraded into fructose and glucose when it is used. This degradation process not only provides energy and carbon source for microorganisms, but more importantly, it can activate the synthase system of pullulan. Under the stimulation of fructose isomerization, the synthesis of pullulan was further promoted.

Aureobasidium pullulans is a special microorganism. In the process of synthesizing pullulan, sucrose as its carbon source substrate has the highest yield. Sucrose has become the best carbon source for pullulan synthesis by Aureobasidium pullulans because sucrose is first degraded into fructose and glucose when it is used. This degradation process not only provides energy and carbon source for microorganisms, but more importantly, it can activate the synthase system of pullulan.

Under the stimulation of fructose isomerization, the synthesis of pullulan was further promoted.

Although sucrose is composed of glucose and fructose as disaccharides, there may be more metabolic steps to convert pullulan into pullulan than glucose and fructose, Aureobasidium pullulans seems to have the ability to utilize sucrose efficiently. In order to further understand how Aureobasidium pullulans can be synthesized by sucrose, we carried out further research. By exploring the metabolic pathway and regulation mechanism of sucrose in Aureobasidium pullulans, we hope to optimize the production process of pullulan, improve the yield and efficiency, and contribute to the development of organic industry.

In this experiment, the effects of sucrose, glucose and fructose as carbon sources on pullulan production by Aureobasidium pullulans were compared, and a low-cost and superior carbon source was obtained by combining these three carbon sources.

In this experiment, the effects of magnesium, calcium, zinc, manganese and copper ions on the production of pullulan by Aureobasidium pullulans were studied. The sweat wiping yield was compared by setting different concentration gradients to find a suitable concentration to improve the culture medium.

The effects of culture medium with sucrose, fructose, glucose and xylose as carbon sources on the production of pullulan by Aureobasidium pullulans were studied. By changing the ratio of sucrose, fructose and glucose in the carbon source, we try to obtain a carbon source with low cost and good effect.

The changes of sugar production of Aureobasidium pullulans under different processes were studied by shaking table and fermenter. A better fermentation environment was obtained by changing the rate, temperature and inoculation amount of the shaker. Change the aeration rate of the fermenter and the rotational speed of the stirring rod to find an environmental condition suitable for pullulan production by Aureobasidium pullulans.

The relevance of the topic is *Aureobasidium pullulans;* Pullulan; Optimization of fermentation conditions; Medium optimization.

The purpose of the study is to investigate the impact of controlled fermentation process on the production of pullulan polysaccharide by Aureobasidium pullulans.

The objectives of the study: acquire and analyze the corresponding experimental data for discussion.

The object of the study: Aureobasidium pullulans

The subject of the study: Prulan fermentation process.

Research methods: fermentation experiments.

The scientific novelty: There are still many uncertainties surrounding the research on pullulan fermentation control.

The practical significance of the results obtained is helps optimize existing culture media and fermentation conditions.

CHATPER 1
LITERATURE REVIEW

1.1 Overview of microbial polysaccharides

Microbial polysaccharides are polysaccharides synthesized by microorganisms such as molds and yeasts. It has a complex branching structure, and the location and number of its branching points are different, which leads to the diversity of physical and chemical properties of microbial polysaccharides [**Error! Reference source not found.**]. At the same time, the spatial structure of microbial polysaccharides is usually irregular three-dimensional network, which makes microbial polysaccharides have good viscosity and stability. Microbial polysaccharides are mainly divided into extracellular, cell wall and intracellular polysaccharides [**Error! Reference source not found.**]. As a kind of biological macromolecule, microbial extracellular polysaccharides have been paid more and more attention in many fields, especially in medicine, food, cosmetics and other fields. in order to give full play to the wide use of microbial extracellular polysaccharides.

1.2 Introduction of pullulan polysaccharides

Pullulan (Pullulan) is a natural water-soluble polysaccharide produced by Aureobasidium pullulans fermentation. Its molecular weight is formed by the combination of maltotriose through α-1,6-glycosidic bond, and the average molecular weight is 2×10^5 Da [**Error! Reference source not found.**]. Because this polysaccharide can be degraded and utilized by microorganisms in nature, it will never cause environmental pollution and is known as pollution-free plastic.

Pullulan polysaccharides have been widely used in many fields because of their unique physical and chemical properties and physiological functions. In the pharmaceutical industry, it can be used as capsule forming agent, thickener, adhesive and so on. In the food industry, pullulan polysaccharide is used as antiskating agent, forming agent, quality improver, emulsifier, thickener and so on. Its aqueous solution has a smooth and refreshing feeling, which can improve the taste and improve the

quality of food. In addition, pullulan polysaccharide can also be used in cosmetics, chemical industry, environmental protection, pesticides and other fields.

1.3 Sources of pullulan polysaccharides

Pullulan polysaccharides come from a wide range of sources. Since the strain *Aureobasidium* which can produce this polysaccharide was first reported by Bauer in 1938, researchers have found a variety of strains capable of producing pullulan [**Error! Reference source not found.**]. These strains include red yeast (*Rhodotorula bacarum*) isolated from leaves, chestnut blight fungus (*Cryphonectria parasitica*) isolated from the edge bark of decaying chestnut trees, and polysaccharides extracted from ascomycetes licheniformis (*Teloschistes flavicans*) by hot alkali [**Error! Reference source not found.**]. Among all the strains that can produce pullulan, Aureobasidium pullulans (*Aureobasidium pullulans*) is favored because of its stability in fermentation and high yield of pullulan [**Error! Reference source not found.**]. This microorganism has five different cell forms, such as yeast-like cells, budding spores, swollen spores, chlamydospores and mycelia, showing its morphological diversity [**Error! Reference source not found.**].

Aureobasidium pullulans is a kind of microorganism widely found in nature. It is commonly found in rocks, woody surfaces and fungi in humid temperate regions. It can also be isolated from leaves and decaying substances of different plants. More surprisingly, the strain can survive and produce even in extreme conditions such as glaciers and high concentrations of salt water [**Error! Reference source not found.**]. This extensive adaptability enables Aureobasidium pullulans to survive in a variety of complex environments and produce a variety of valuable metabolites, such as poly malic acid, β-glucan, melanin and antibiotics. It shows its important value as a biological resource [**Error! Reference source not found.**].

1.4 Research progress of pullulan polysaccharides

1.4.1 Progress in the study of Synthesis Mechanism

Pullulan produced by Aureobasidium pullulans is an important linear extracellular polysaccharide, which is widely used in food, medicine and other industrial fields, and its unique chemical structure gives it excellent properties. Although researchers have conducted extensive and in-depth studies on the synthesis mechanism of pullulan polysaccharides, there are still many unsolved mysteries.

The metabolic pathway of Aureobasidium pullulans is similar to that of other yeasts, mainly by carbohydrate metabolism.

The researchers speculated the possible synthetic pathway of pullulan through a series of experiments. In this process, structural regions such as urinary glucose diphosphate content changes, amylase domain, glycogen synthase domain and transmembrane transport domain all play a key role. In particular, the short α-1,4-Glucosyl chain is elongated by the glycogen synthase domain to form a long α-1,4-Glucosyl chain, then these precursors are transported outside the plasma membrane through the transmembrane transport domain, and then hydrolyzed by the amylase domain. Finally, pullulan polysaccharides were synthesized [**Error! Reference source not found.**].

In addition, the researchers also found some key factors affecting the synthesis of pullulan polysaccharides. For example, Yang et al have found that the activity of cAMP-PKA can regulate the expression of UGP1 gene, which in turn affects the synthesis of pullulan. When the activity of cAMP-PKA is low, the expression of UGP1 gene is high, which is beneficial to the synthesis of pullulan, while when it is high, the expression of UGP1 gene is disabled and the synthesis of pullulan is restricted [**Error! Reference source not found.**].

On the other hand, multi-domain AmAgs2 is considered to be the key enzyme and primer for pullulan biosynthesis, and its activity directly affects the yield of pullulan [**Error! Reference source not found.**]. In addition, the biosynthesis of pullulan is also

inhibited by glucose and regulated by signal pathway, which constitute a complex network of pullulan biosynthesis mechanism.

Although we have made some understanding about the synthesis mechanism of pullulan polysaccharides, there are still many details and regulatory mechanisms that need to be further studied and explored. With the progress of science and technology and the deepening of research, it is believed that we will have a more comprehensive and in-depth understanding of the synthesis mechanism of pullulan, so as to provide a more solid foundation for its application in various fields.

1.4.2 Progress in fermentation production

In 1976, Lin Yuan Co., Ltd. took the lead in the commercial production of pullulan. Because Linyuan Company has the patent of pullulan production, the company monopolizes the pullulan industry, thus restricting the industrial production of pullulan. Until 2000, after Lin Yuan's patent expired, other related companies also began to commercialize the production of pullulan. Linyuan biochemical Laboratory authorized Pfizer to commercialize and sell membrane oral care products containing patented pullulan polysaccharides from the laboratory, and to supply pullulan powder to Warner Lambert, a subsidiary of Pfizer Pharmaceuticals. Due to the unique properties and potential applications of pullulan, the demand for pullulan is increasing day by day. Linyuan has made great efforts to develop its own industry, gradually expanding the market of pullulan polysaccharides, and opening branches in Europe, Asia, Southeast Asia, Oceania, North and South America. In China, some enterprises such as Shandong Kangnaxin Biotechnology Co., Ltd and Tianjin Beiyang Baichuan Biotechnology Co., Ltd. have also begun the R & D and commercial production of pullulan polysaccharides. Pullulan, as a commercially important microbial extracellular polysaccharide with development value and good industrial prospect, has been studied extensively and deeply by a wide range of researchers. It is mainly through

the optimization of microbial culture medium and fermentation process to achieve the purpose of high yield and high quality of pullulan polysaccharides.

1.4.3 Research Progress on the effect of medium composition on the Synthesis of pullulan Polysaccharide

(1) Carbon source

In the production process of pullulan polysaccharide, the selection of carbon source is very important. Aureobasidium pullulans can be fermented with a variety of carbon sources, such as glucose, sucrose, xylose and fructose. Although monosaccharide carbon source usually has advantages in microbial fermentation, sucrose shows its particularity in pullulan production. Many studies have shown that sucrose as a carbon source can significantly increase the yield of pullulan. This may be due to the synthesis of pullulan through a specific metabolic pathway after sucrose is degraded into glucose and fructose in cells [**Error! Reference source not found.**].

However, the cost of sucrose is relatively high, which limits its application in the industrial production of pullulan polysaccharide. As a result, researchers began to explore the possibility of using cheap carbon sources, such as agricultural and food industry waste. These wastes are rich in nutrients and can provide carbon sources for microorganisms while reducing production costs.

(2) Nitrogen source

Nitrogen source is another key component in microbial fermentation medium, which has a significant effect on the synthesis of pullulan polysaccharide. The type and concentration of nitrogen sources will affect the yield of pullulan polysaccharide. Inorganic nitrogen sources such as NH_4NO_3, $(NH_4)_2CO_3$, NH_4Cl, $NaNO_3$, $(NH_4)_2SO_4$, and organic nitrogen sources such as peptone, corn pulp and yeast powder are all nitrogen sources that can be used by Aureobasidium pullulans.

Studies have shown that specific types and concentrations of nitrogen sources can promote the synthesis of pullulan polysaccharides. For example, when NH_4NO_3

and $(NH_4)_2SO_4$ were used as nitrogen sources, the yield of pullulan was higher. In addition, the use of compound nitrogen sources has also proved beneficial because they can provide more comprehensive nutrition to meet the needs of microbial growth and metabolism [**Error! Reference source not found.**].

However, too high nitrogen concentration may have a negative effect on the yield of pullulan polysaccharides. This is because excessive nitrogen sources may change the ratio of carbon to nitrogen, causing carbon flux to flow to other metabolic pathways rather than pullulan synthesis. Therefore, in the production process of pullulan polysaccharides, it is necessary to carefully control the concentration of nitrogen source to optimize the yield. In addition, nitrogen sources also affected the activities of enzymes related to pullulan synthesis in Aureobasidium pullulans. For example, urinary glucose diphosphate phosphorylase is a key enzyme in pullulan biosynthesis pathway, and its activity is regulated by the type and concentration of nitrogen sources.

Therefore, the synthetic pathway of pullulan can be indirectly regulated by adjusting the type and concentration of nitrogen sources, and its yield and quality can be further optimized [**Error! Reference source not found.**].

1.5 Fermentation mode and bioreactor design of pullulan

1.5.1 Batch fermentation

Batch fermentation is a culture method in which specific live production bacteria are inoculated in the sterilized medium and no matter is added or removed from the fermentation broth in the process (except for the continuous supply of oxygen to aerobic microorganisms). The characteristic of this approach is that the environment of microorganisms changes significantly over time, which leads to continuous changes in the chemical and physical state in the culture tank. Therefore, the whole fermentation process is not in a stable state, but unsteady state.

In practice, a challenge often encountered is that the optimal conditions for cell growth are not completely consistent with those for the production of metabolites. In order to solve this problem, these conditions can be artificially adjusted during the culture process, such as changing temperature, pH or dissolved oxygen (DO) levels, to optimize the growth environment of microorganisms, so as to maximize the yield of the target products.

By carefully controlling these fermentation conditions, the yield and quality of the target products can be significantly improved and the production cost can be reduced at the same time. Therefore, it is a very effective strategy to artificially change the conditions in the batch fermentation process to optimize the production process.

1.5.2 Fed-batch fermentation

Fed-batch culture technology, as an advanced microbial culture technology, has shown its unique application value and broad prospects in the fields of bioengineering, food industry and pharmaceutical industry. Its core idea is to realize the sustainable growth of microorganisms and the effective accumulation of metabolites by constantly adding new medium solution to the culture medium.

The core advantage of this process lies in its high flexibility and controllability. By accurately controlling the feed rate and the dilution of the medium, we can effectively maintain the balance of microbial growth. The maintenance of this equilibrium state is very important for the growth of microorganisms, it can not only ensure the healthy growth of microorganisms, but also promote the stable accumulation of metabolites. It has a wide application prospect in bioengineering, food industry, pharmaceutical industry and other fields.

1.5.3 Continuous culture and fermentation

Continuous fermentation, also known as continuous culture, is an efficient microbial culture and fermentation technology. In the later stage of logarithmic growth

of microorganisms, the system will continuously inject fresh disinfection medium into the fermenter at a stable rate, and discharge the fermentation liquid at the same rate at the same time to maintain the continuous culture and fermentation of microorganisms. This method has a series of remarkable characteristics and advantages.

First, continuous fermentation is a method of culture and fermentation in a steady state. In this state, the environmental conditions of culture and fermentation, such as temperature, pH value, culture concentration, product concentration, dissolved oxygen and redox potential, remain constant. As a result, the logarithmic growth period of microorganisms can be extended indefinitely, and the growth rate and specific growth rate of microorganisms remain stable. at the same time, the cell concentration, the total amount of bacteria and the volume of culture medium in the fermenter are also constant.

Second, the continuous fermentation method significantly reduces the cleaning, loading, disinfection, inoculation, canning and other operation time needed in batch culture and fermentation. This not only saves manpower and material resources, reduces costs, but also greatly improves production efficiency.

Third, continuous fermentation also reduces the size of the equipment and reduces the investment. At the same time, through the use of multi-stage continuous culture and fermentation technology, the equipment configuration is more perfect and reasonable, which is conducive to the realization of mechanization and automatic control.

Fourth, the performance of the products produced by continuous culture and fermentation is stable, which provides a strong guarantee for the quality control of products.

Fifth, continuous fermentation provides a good environment for high-speed growth of microorganisms in a constant state, which makes it more convenient to study the physiological, biochemical and genetic characteristics of microorganisms.

Sixth, continuous fermentation also has some potential shortcomings. Due to continuous culture and fermentation for a long time, it is easy to have the problems of strain variation, degradation and miscellaneous bacteria pollution. In addition, if the operation is improper, the newly added medium may not be easily mixed with the original medium, which will affect the stability and efficiency of the fermentation process.

Conclusions to chapter 1

With the rapid progress of modern society, the state pays more and more attention to the organic industry, and consumers' in-depth understanding of the value of organic products has also promoted the continuous growth of the market demand for organic products. Pullulan, as a high molecular extracellular polysaccharide produced by Aureobasidium pullulans in the fermentation process, has been widely used in medicine, environmental protection and other fields because of its unique physical and chemical properties.

Under the background of increasingly mature fungal fermentation and purification technology, the cost-effectiveness of pullulan polysaccharides is largely affected by the cost of carbon sources. At present, the industrial production of pullulan mainly depends on sucrose as the fermentation substrate. However, the market price of organic sucrose is about 14 ¥/kg, glucose is about 4 ¥/kg, and the price of fructose is about 10 ¥/kg. The high price of sucrose limits its application in organic production. Therefore, it is particularly urgent to develop pullulan fermentation process with non-sucrose carbon source.

As a universal carbon source with more friendly price, although the efficiency of glucose in pullulan fermentation is relatively low, its potential alternative value can not be ignored. In order to improve the yield and production efficiency of pullulan, and to achieve its efficient and organic production, it is particularly important to optimize the conditions for the synthesis of pullulan.

This study will explore the composition of pullulan fermentation medium and the optimization strategy of culture conditions. First of all, we will try to change the ratio of carbon sources to explore the effects of different carbon source combinations on the yield of pullulan polysaccharides. Secondly, the promoting effect of metal ions on the fermentation process of pullulan was studied. In addition, we will also compare different fermentation processes to find the most suitable fermentation method for pullulan production.

Through these optimization measures, we expect to reduce the production cost of pullulan, improve its production efficiency, and promote the wide application of pullulan in organic industry. This will not only help to meet consumers' demand for organic products, but also inject new impetus into the sustainable development of the organic industry.

CHAPTER 2
OBJECT, PURPOSE AND METHODS OF THE STUDY

2.1 Object and purpose of the study

The relevance of the topic is Aureobasidium pullulans; Pullulan; Optimization of fermentation conditions; Medium optimization.

The purpose of the study is to investigate the impact of controlled fermentation process on the production of pullulan polysaccharide by Aureobasidium pullulans.

The objectives of the study: acquire and analyze the corresponding experimental data for discussion. The object of the study: Aureobasidium pullulans. The subject of the study: Prulan fermentation process.

2.2 Materials and methods

2.2.1 Strain and culture medium

Aureobasidium pullulans, preserved in the State key Laboratory of Biomaterials and Green Papermaking, Qilu University of Technology.

Seed medium (g/L): sucrose 80, ammonium sulfate 0.5, yeast extract 2, natural pH. Fermentation medium (g/L): yeast extract 3; ammonium sulfate 0.6, sodium chloride 2, dipotassium hydrogen phosphate 6; carbon source and alignment ratio are shown in Table 2.1.

Table 2.1 - Ratio and content of different carbon sources

No	Carbon source	Proportion	Concentration, g/L
1	2	3	4
5	Glucose	1	100
2	Sucrose: glucose	3:7	30:70
3	Sucrose: glucose	1:1	50:50

Continuation of Table 2.1

1	2	3	4
4	Sucrose: glucose	7:3	70:30
5	Glucose	1	100
6	Fructose	1	100
7	Fructose: glucose	3:7	30:70
8	Fructose: glucose	1:1	50:50
9	Fructose: glucose	7:3	70:30
10	Xylose	1	100

Table 2.2 - Reagents

Reagent	Manufacturer
Sucrose	Shanghai Sijie Biotechnology Co., Ltd.
$(NH_4)_2SO_4$	Beijing Oboxing Biotechnology Co., Ltd.
NaCl	National Pharmaceutical Group Chemical Reagent Co., Ltd.
K_2HPO_4	National Pharmaceutical Group Chemical Reagent Co., Ltd.
Glucose	National Pharmaceutical Group Chemical Reagent Co., Ltd.
Fructose	Shanghai Sijie Biotechnology Co., Ltd.
Xylose	Shanghai Sijie Biotechnology Co., Ltd.

2.2.2 Instruments

Table 2.3 - Instruments

Instrument name	Manufacturer
1	2
Centrifuge	Qingdao Jingcheng Instrument Co., Ltd.
Shaker	Shanghai Zhichu Instrument Co., Ltd.
UV spectrophotometer	Shanghai Shengke Instrument Equipment Co., Ltd.

Continuation of Table 2.3

1	2
Clean bench	Dongguan Yaning Purification Technology Co., Ltd.
Autoclave	Jinan Aibolai Instrument Equipment Co., Ltd.
Glass fermentation tank	Shanghai Bailun Biotechnology Co., Ltd.
Ultrasonic cell disruptor	Shanghai Zhengqiao Scientific Instruments Co., Ltd.

2.2.3 Cultivation method

Seed culture: Take out the strains from -70°C, thaw and activate them, inoculate them into 50 ml seed culture medium, and culture them in a shake flask at 28 °C and 220 rpm for 8 hours. Shake flask fermentation culture: Inoculate 10 ml of activated seed liquid into 50 ml of fermentation medium, and culture in shake flask for 72 hours at 28 °C and 220 rpm.

2.2.4 Preparation of cell-free extract solution

Quickly freeze 5 mL of fermentation broth in liquid nitrogen for 10 min to stop the intracellular reaction, collect wet cells, and centrifuge at 40°C and 12,000 rpm for 20 min. The cells were resuspended in 5 mL of 0.2 mol/L pre-cooled phosphate buffer (pH 70), and then sonicated in an ultrasonic bath in an ultrasonic cell disrupter at 20 kHz. The duration of ultrasonic disruption was 10 min, and there was an interval between active disruption and passive disruption. The time is all 10s. Then centrifuge at 40°C and 12,000 rpm for 20 min to remove cell debris, and the supernatant is used as a cell-free extract for measuring key enzyme activities.

2.2.5 Enzyme activity assay

(1) Determination of pullulan synthesis ability

The 5 ml fermentation broth was centrifuged at 12000 rpm for 10 minutes, and the wet cells were re-suspended in 5 ml conversion solution (5 g glucose, 0.2 g

$MgS0_4 \cdot 7H_20$, pH 6.0), then transformed at 30 ℃ for 3.5 h in a 200-rpm shaker. After transformation, the content of pullulan in the transformation solution was determined.

(2) determination of pullulan-degrading enzyme activity.

The 5 ml fermentation broth was centrifuged by 10min (4 ℃, 12000 rpm). The supernatant was mixed with pullulan standard, and the mixture was incubated in a water bath at 50 ℃ for 3 h. After the end of the reaction, the reaction was immediately heated at 100 ℃, and the concentration of reducing sugar in the supernatant before and after the reaction was determined. A unit of pullulan-degrading enzyme activity is defined as releasing 1 μ mol of glucose equivalent per minute at 50 ℃.

(3) determination of key enzymes in pullulan synthesis.

GTF enzyme activity assay:

The p-nitrophenyl-α-D-glucopyranoside substrate solution of 0.1mol/L and pH4.0 was prepared with sodium acetate buffer of 0.2mL and 10mmol/L, and the substrate solution was balanced in a water bath at 40 ℃ for 5 minutes. The properly diluted enzyme solution of 0.2mL was quickly added to the temperature-balanced substrate solution and quickly mixed to start the enzymatic hydrolysis reaction, and the reaction time was set at 5 minutes. At the end of the reaction, the 0.4mol/L glycine-NaOH buffer of pH10.5 of 3mL was added to stop the enzymatic hydrolysis. The absorbance of the solution was determined at 410nm wavelength. The reaction solution mixed with diluted enzyme solution was heated at 100 ℃ for 5 minutes as a blank control. The enzyme activity was calculated by comparing the standard curve (which was drawn by measuring the absorbance of p-nitrobenzene solution of different concentrations under 410nm). A unit of enzyme activity is defined as the amount of enzyme required to release 1 μ mol p-nitrophenol per minute [**Error! Reference source not found.**].

PMG enzyme activity assay:

Tris-HCl (50 umol / L pH 7.5), 5 umol / L magnesium chloride, 50 umol / L potassium chloride, 0.15 umol / L NADH, 1.25 umol / LATP, 50 μ g fructose diphosphate aldolase, 20 μ g glycerol phosphate dehydrogenase and appropriate

sample solution to be tested. The reaction was initiated by adding 1 μ mol fructose phosphate, and the absorbance was measured at 340 nm. A unit of enzyme activity is defined as the amount of enzyme required to release 1 μ mol substrate per minute.

2.2.6 Analytical method

Determination of cell dry weight and pullulan yield:

The fermentation broth or bioconversion liquid of 20 ml was placed in a water bath at 80 ℃ and 15min was continuously heated to make it inactive. Subsequently, the treated liquid was centrifuged at the speed of 12000r/min for continuous 20min, thus the supernatant and precipitate were separated. The precipitate is washed three times with distilled water to collect cells. After collection, the cells were dried in an oven at 70 ℃ until their mass reached a constant state. The supernatant of 10ml was removed and anhydrous ethanol of 20 ml was added. After vibrating and mixing, it was placed at 4 ℃ for 12 hours. Then, the 10min was centrifuged again at the speed of 12000 r/min, and the supernatant was discarded. Finally, the remaining sediment is dried in an oven at 70 ℃ until its quality is constant.

Determination of molecular weight of pullulan:

Pullulan obtained by drying was crushed in a pulverizer. The 5 mg sample was dissolved in 1ml ultra-pure water and the sample concentration was 5 mg $\cdot mL^{-1}$. At the same time, the standard sample of 10mg pullulan polysaccharide (342~894000 Da) was dissolved in 1ml ultra-pure water, and the concentration of the standard sample was 10mg $\cdot mL^{-1}$. The standard and sample solutions were filtered with a 0.22 μ m filter membrane, and the filtered liquid was placed in a liquid phase vial for determination. The molecular weight of pullulan was determined by Shimadzu liquid chromatograph, equipped with differential detector and laser detector, gel chromatographic column (TSKgelGMPWXL column), and the temperature of differential detector was 30 ℃. The mobile phase was 0.1mol/L sodium nitrate solution, and the flow rate was 0.5 ml $\cdot min^{-1}$.

Conclusions to chapter 2

Chapter 2 includes a description of the object, subject, purpose and methods of the study. The materials used in the work (Sucrose, ammonium sulfate, sodium chloride, potassium hydrogen phosphate, glucose, fructose, xylose, etc.) and equipment

(Centrifuge, Shaker, Ultrasonic cell disruptor, UV spectrophotometer, clean bench, Autoclave Glass fermentation tank) are given.

As research methods we selected the Cultivation method. Preparation of cell-free extract solution. Preparation of cell-free extract solution. GTF enzyme activity assay. GTF enzyme activity assay, Determination of molecular weight of pullulan, Determination of cell dry weight and pullulan yield.

CHAPTER 3
EXPERIMENTAL PART

3.1 STUDY OF THE INFLUENCE OF VARIOUS CARBON SOURCES ON PULLULAN BIOSYNTHESIS

3.1.1 Effects of different carbon sources on the synthesis of pullulan by Aureobasidium pullulans

In order to investigate the effects of different carbon sources on the biosynthesis of pullulan by Aureobasidium pullulans, sucrose, glucose, fructose and xylose were selected and cultured in shake flask with sucrose and glucose, glucose and fructose (see Figure 3.1).

Figure 3.1A shows that in addition to xylose, changing the ratio of basic sucrose, glucose and fructose has little effect on cell biomass, but xylose has obvious disadvantages.

Figure 3.1B shows that sucrose is still the most suitable carbon source for pullulan production by Aureobasidium pullulans, but the addition of a small amount of glucose has little effect on it, and the ratio of fructose and glucose at 1:1 has a good result.

Figure 3.1C shows that changing the ratio of carbon sources has little effect on the molecular weight of pullulan polysaccharides during the whole fermentation stage. If you want to change the molecular weight of pullulan polysaccharides by changing carbon sources, you should try other carbon sources.

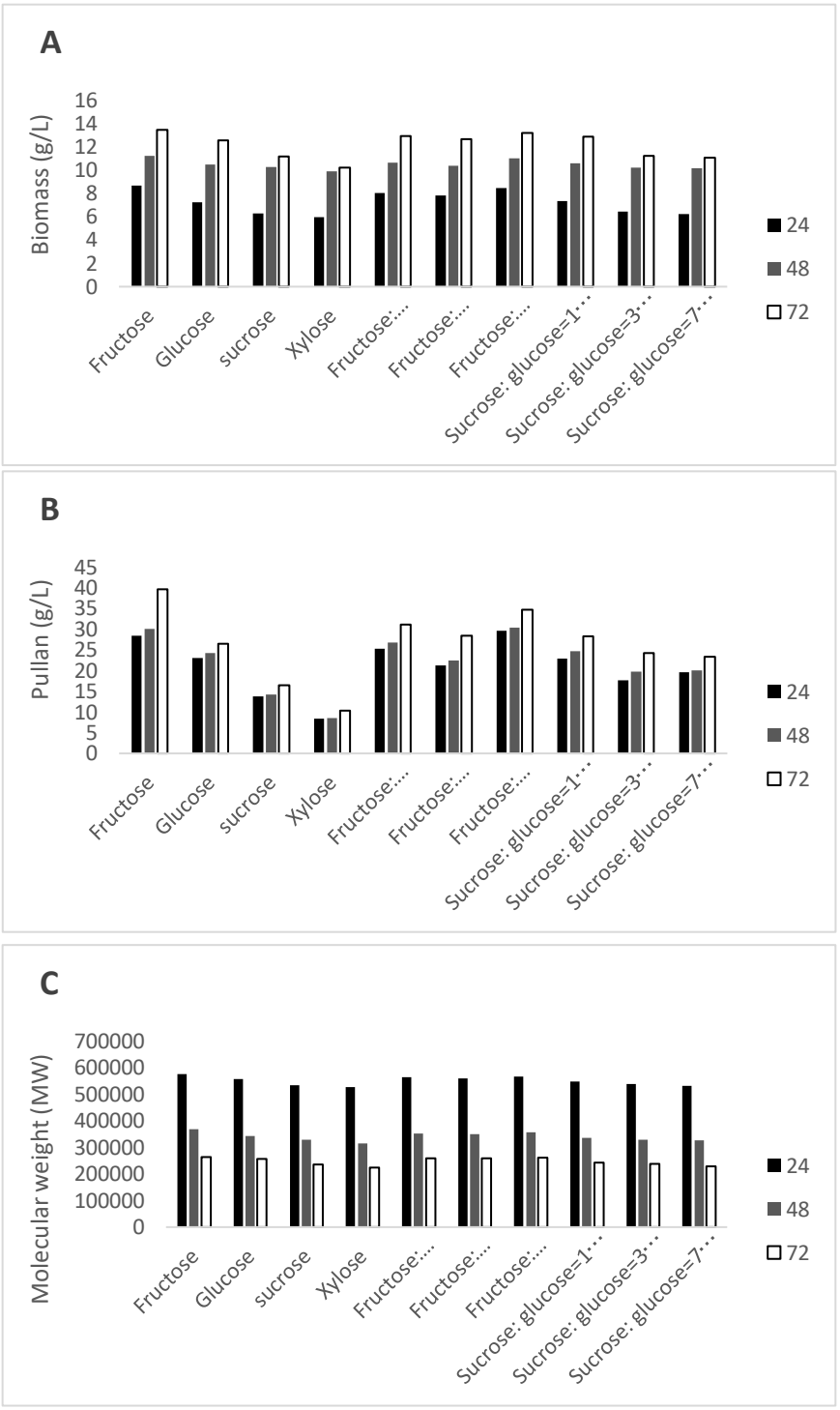

Fig. 3-1 Effects of different carbon sources on pullulan biosynthesis

3.1.2 Synthesis ability and enzyme activity of pullulan

In the fermentation process of biosynthesis of pullulan from different carbon sources, the yield and molecular weight of pullulan will change obviously, in which the molecular weight of pullulan is higher at the initial stage of fermentation culture, and then decreases gradually. Therefore, the ability of cells to synthesize pullulan and the activity of pullulan-degrading enzymes were determined. Sucrose was used as the control group, and the results were shown in Figure 3-2.

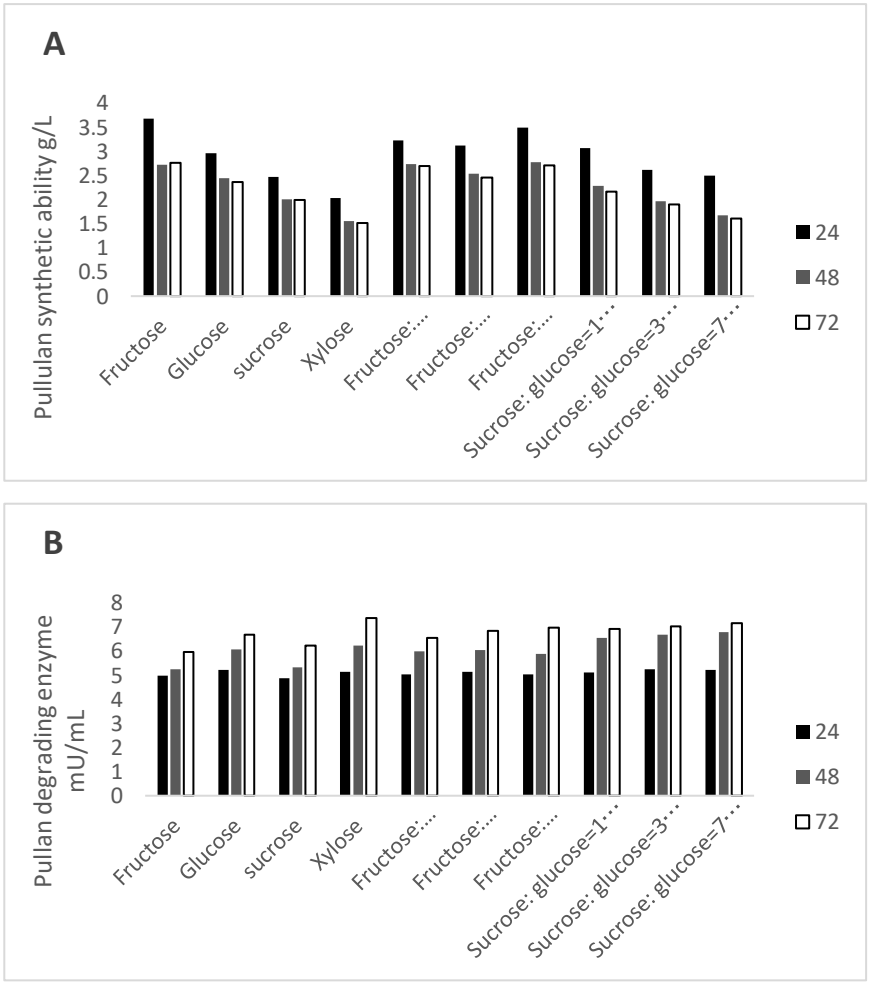

Fig. 3-2 Synthetic ability and enzyme activity of pullulan under different carbon sources

As can be seen from Figure 3-2A, when using different carbon sources as substrate, the synthesis ability of pullulan was higher than that of the control group during the whole fermentation period, and pullulan could be synthesized efficiently,

but when the ratio of fructose to glucose was 1: 1, the synthesis ability of pullulan in fermentation was significantly better than that of single fructose or fructose glucose (consistent with the results of Figure 3-2B). As can be seen from Figure 3-3B, compared with sucrose as the control group, the activity of pullulan-degrading enzymes in cells using other carbon sources was higher than that in the control group. Among them, the group using xylose had the highest activity of degrading enzymes, so when using starch as substrate, the molecular weight of pullulan was lower than that of the control group.

3.1.3 Activities of key enzymes in pullulan synthesis

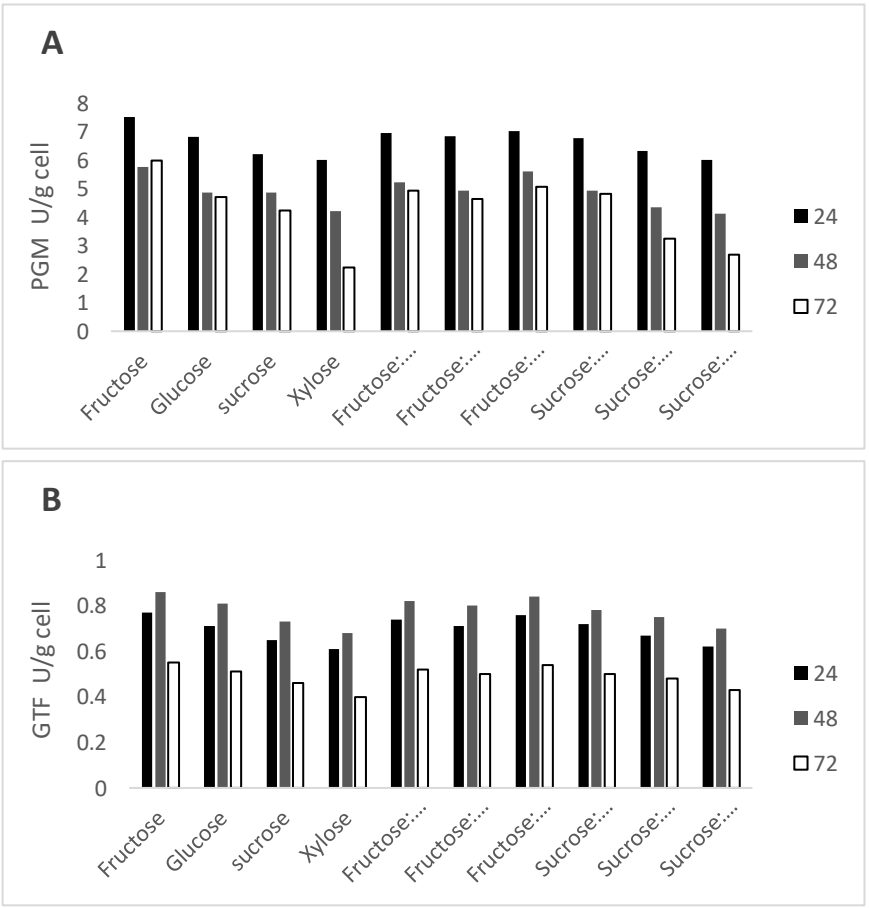

Fig. 3-3 activities of key enzymes in pullulan synthesis under different carbon sources

Previous studies have shown that the synthetic ability of pullulan is affected by the activities of key enzymes PGM and GTF [Error! Reference source not found.]. For this reason, the activities of the key enzymes of pullulan synthesis in different fermentation stages were determined, and sucrose was used as the control group. The results are shown in Figure 2-3. As can be seen from the Figure, in the early stage of fermentation (24 h), the use of other carbon sources and sucrose kept a high activity of the key enzyme PGM, and then the activity decreased gradually. No matter what kind of carbon source, the activity of the key enzyme GTF remains at a similar level, although there are fluctuations, but the numerical difference is not significant.

Summary to Part 3.1

This Part aims to explore the feasibility of replacing sucrose by deeply studying the combination effect of carbon sources such as fructose, glucose and xylose in pullulan biosynthesis.

The experimental results showed that although sucrose showed the best yield in pullulan biosynthesis as a carbon source, the carbon source combination of fructose and glucose at the ratio of 1:1 showed unique economic advantages considering the cost factor. and has the potential for further development. Based on the fermentation kinetic parameters, this chapter further discussed the internal physiological mechanism of pullulan biosynthesis by different carbon sources. By detecting the activities of key enzymes, the specific ways and mechanisms of different carbon sources affecting pullulan biosynthesis were revealed, which provided a strong theoretical support for other carbon sources to replace sucrose. This study not only helps to optimize the technological conditions of pullulan biosynthesis and reduce the production cost, but also provides a useful reference for exploring the application of new carbon sources in microbial fermentation industry.

3.2 EFFECTS OF DIFFERENT METAL IONS ON PULLULAN PRODUCTION BY AUREOBASIDIUM PULLULANS

3.2.1 Introduction

Trace elements are indispensable to maintain the normal metabolism of organisms. Although microorganisms have little demand for them, once there is a lack, excess or imbalance of these trace elements, they will have a profound impact on the metabolic activities of microorganisms. Among these trace elements, metal ions play an important role, and they are indispensable to the growth of microorganisms.

Aureobasidium pullulans need a lot of trace elements, and many metal ions play an important role in the metabolic process of Aureobasidium pullulans. For example, metal ions such as iron, magnesium and calcium play a vital role in the construction of core structures such as microbial cell walls, nucleic acids and proteins. These metal ions are not only the basic components of microbial cell structure, but also play an indispensable role in maintaining the stability and functional integrity of cell structure.

Magnesium ions play an important role in the growth and metabolism of microorganisms. It is not only the activator of hexose phosphorylase, isocitrate dehydrogenase, peptidase, carboxylase and other enzymes, these enzymes play an important role in microbial metabolism; moreover, magnesium ions also play a role in stabilizing ribosome, plasma membrane and nucleic acid. Once magnesium ion deficiency, it may lead to ribosome and plasma membrane damage, which in turn hinders the growth of microorganisms.

Iron, as another important metal ion, is a key component of many enzymes. It can combine with the amino acid residues of the enzyme to form a complex and participate in the catalytic reaction. At the same time, iron is also involved in the synthesis of DNA in microorganisms, in which DNA deoxyribonuclease and several DNA polymerases need iron ion catalysis. In addition, iron is essential for the synthesis of cell walls, proteins and nucleic acids.

Zinc ion, as one of the necessary trace elements in the process of microbial growth and metabolism, participates in and stabilizes the folding conformation of metalloproteins and plays a key role in maintaining the structure and function of proteins. In addition, zinc ions are also involved in important intracellular biochemical reactions at the global level, such as the synthesis of RNA and DNA, enzyme catalysis and so on. Zinc ion can affect the core processes such as ATP enzyme activity, DNA repair mechanism, antibiotic resistance and stress response of microorganisms, showing its comprehensiveness and importance in microbial metabolism.

In order to better explore the metabolic pathway and regulation mechanism of various metal ions in Aureobasidium pullulans, we hope to optimize the production process of pullulan polysaccharides, improve the yield and efficiency, and contribute to the development of organic industry. In this experiment, several metal ions such as magnesium, iron, copper, zinc, calcium and manganese were added to the culture medium to shake flask fermentation and compare their effects on pullulan production by Aureobasidium pullulans, and try to optimize the existing medium.

3.2.2 Materials and methods

3.2.2.1 Strain and medium

Aureobasidium pullulans, preserved in the State key Laboratory of Biomaterials and Green Papermaking, Qilu University of Technology.

Seed medium(g/L): sucrose 80, ammonium sulfate 0.5, yeast extract 2, natural PH fermentation medium(g/L): sucrose 100, yeast extract 3, ammonium sulfate 0.6, sodium chloride 2, dipotassium hydrogen phosphate 6 various metal ions ($MnCl_2$, $ZnSO_4$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $MgSO_4$) are detailed in 3.2.2.

Table 3-1 Reagents

Reagent	Manufacturer
$MnCl_2$	National Pharmaceutical Group Chemical Reagent Co., Ltd.
$ZnSO_4$	National Pharmaceutical Group Chemical Reagent Co., Ltd.
$CaCl_2$	National Pharmaceutical Group Chemical Reagent Co., Ltd.
$CuSO_4$	National Pharmaceutical Group Chemical Reagent Co., Ltd.
$FeSO_4$	National Pharmaceutical Group Chemical Reagent Co., Ltd.
$MgSO_4$	National Pharmaceutical Group Chemical Reagent Co., Ltd.

3.2.2.2 Training method

On the basis of the basic fermentation medium, the concentrations of all kinds of metal ions ($MnCl_2$, $ZnSO_4$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $MgSO_4$) were designed as 0.04g / L, 0.05g / L, 0.06g / L, 0.1g / L, 0.15g / L and 0.2g / L respectively, and MgSO4 designed an extra 0.25g / L for 37 groups. And cultivated according to the method shown in the second chapter.

3.2.2.3 Analytical method

The method is the same as in Chapter 2.

3.2.3 Results and discussion

Other medium components and culture conditions in the fermentation medium remained unchanged. Different concentrations of $MnCl_2$, $ZnSO_4$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $MgSO_4$ were added to determine the yield of polysaccharides. The results are shown in Figure 3-4.

Figure 3-4 shows that the yield of polysaccharides increases with the increase of $MnCl_2$ concentration. When the concentration of $MnCl_2$ was lower than that of 0.05g/L, the polysaccharide yield increased with the increase of $MnCl_2$ concentration, but when the concentration of $MnCl_2$ was higher than that of 0.05g/L, the polysaccharide yield

decreased with the increase of $MnCl_2$ concentration. Considering the yield and biomass of polysaccharides, the best $MnCl_2$ concentration for fermentation was 0.05 g/L.

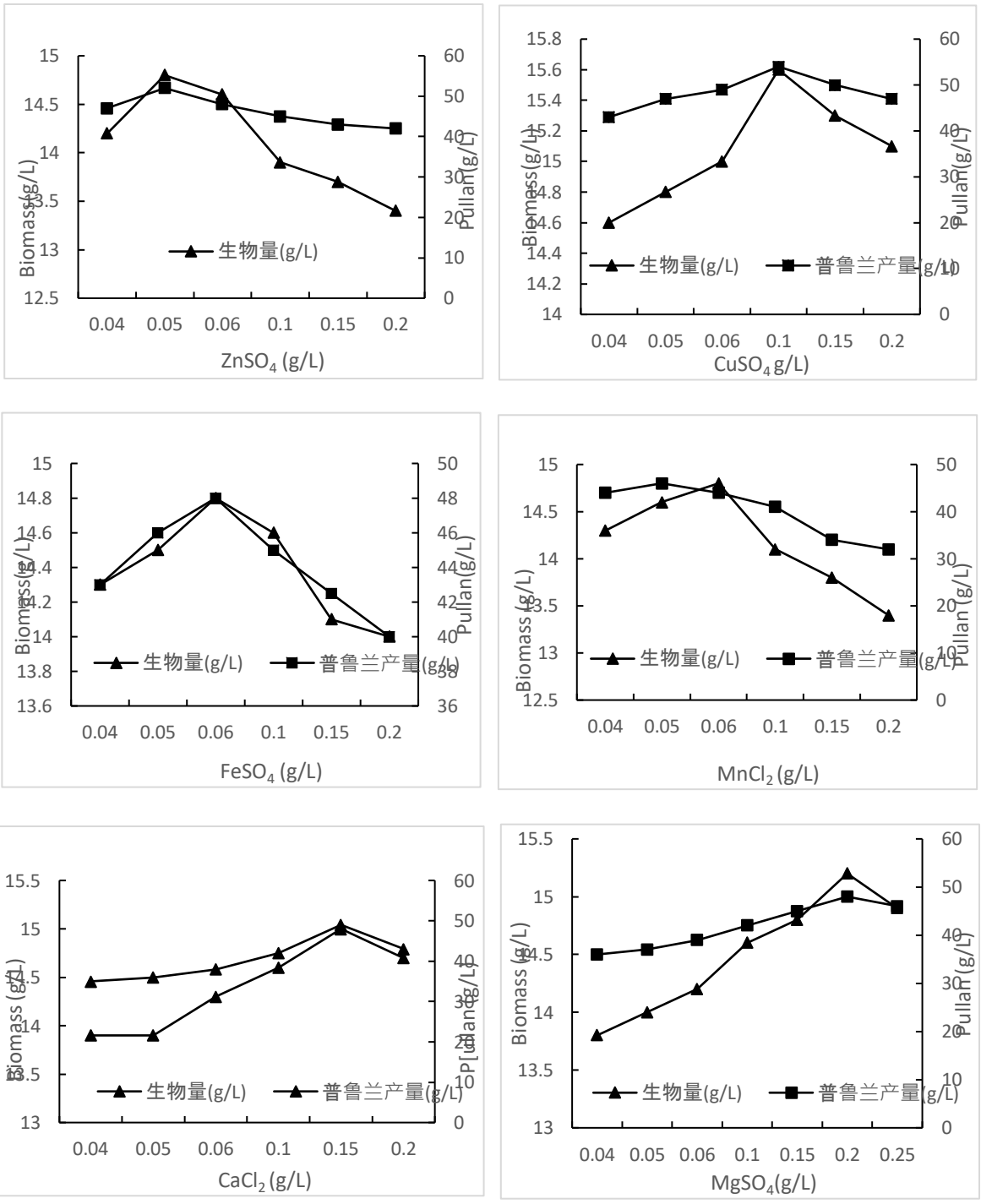

Fig. 3-4 Effect of metal ions concentration on fermentation

Figure 3-4 shows that the yield of polysaccharides increases with the increase of $ZnSO_4$ concentration. When the concentration of $ZnSO_4$ was lower than that of 0.05g/L,

the polysaccharide yield increased with the increase of $ZnSO_4$ concentration, but when the concentration of $ZnSO_4$ was higher than that of 0.05g/L, the polysaccharide yield decreased with the increase of $ZnSO_4$ concentration. Considering the yield and biomass of polysaccharides, the best ZnSO4 concentration for fermentation was 0.05g/L.

Figure 3-4 shows that the yield of polysaccharides increases with the increase of $CaCl_2$ concentration. When the concentration of $CaCl_2$ was lower than that of 0.15g/L, the polysaccharide yield increased with the increase of $CaCl_2$ concentration, but when the concentration of $CaCl_2$ was higher than that of 0.15g/L, the polysaccharide yield decreased with the increase of $CaCl_2$ concentration. Considering the yield and biomass of polysaccharides, the best $CaCl_2$ concentration for fermentation was 0.15g/L.

Figure 3-4 shows that the yield of polysaccharides increases with the increase of $CuSO_4$ concentration. When the concentration of $CuSO_4$ was lower than that of 0.1g/L, the polysaccharide yield increased with the increase of $CuSO_4$ concentration, but when the concentration of $CuSO_4$ was higher than that of 0.1g/L, the polysaccharide yield decreased with the increase of $CuSO_4$ concentration. Considering the yield and biomass of polysaccharides, the best $CuSO_4$ concentration for fermentation was 0.1g/L.

Figure 3-4 shows that the yield of polysaccharides increases with the increase of $FeSO_4$ concentration. When the concentration of $FeSO_4$ was lower than that of 0.06g/L, the polysaccharide yield increased with the increase of $FeSO_4$ concentration, but when the concentration of $FeSO_4$ was higher than that of 0.06g/L, the polysaccharide yield decreased with the increase of $FeSO_4$ concentration. Considering the yield and biomass of polysaccharides, the best $FeSO_4$ concentration for fermentation was 0.06g/L.

Figure 3-4 shows that the yield of polysaccharides increases with the increase of $MgSO_4$ concentration. When the concentration of $MgSO_4$ was lower than that of 0.2g/L, the polysaccharide yield increased with the increase of $MgSO_4$ concentration, but when the concentration of $MgSO_4$ was higher than that of 0.2g/L, the polysaccharide yield decreased with the increase of $MgSO_4$ concentration. Considering the yield and biomass of polysaccharides, the best $MgSO_4$ concentration for fermentation was 0.2g/L.

Summary to Part 3.2

In this Part, the specific effects of micro elements such as $MnCl_2$, $ZnSO_4$, $CaCl_2$, $CuSO_4$, $FeSO_4$ and $MgSO_4$ on the polysaccharide yield and biomass of Aureobasidium pullulans during liquid fermentation were studied. The results showed that there were significant differences in the effects of different trace elements and their respective concentrations on the yield of polysaccharide from Aureobasidium pullulans. The best concentration was 0.05g/L$MnCl_2$,0.05g/L$ZnSO_4$, 0.15 g/LCaCl2, 0.1 g/L$CuSO_4$, 0.06 g/L$FeSO_4$, 0.2 g/L$MgSO_4$.

Although the current study has not yet determined the optimal combination of trace elements through orthogonal experiments, a large number of experimental data accumulated in this experiment undoubtedly provide a strong support for further optimizing the fermentation process of pullulans. By comparing and analyzing the differences among different trace element treatment groups, we can preliminarily infer that some trace elements may have potential advantages in promoting the synthesis of pullulans. At the same time, the experimental results also revealed that the effect of trace element concentration on the polysaccharide yield of Aureobasidium pullulans was not a simple linear relationship, but showed a complex nonlinear characteristic. In this experiment, the effects of trace elements on the yield and biomass of Aureobasidium pullulans polysaccharides during liquid fermentation were studied, which provided important experimental data and theoretical basis for further optimizing the fermentation process of pullulans. On this basis, the future research can further explore the optimal combination of trace elements through orthogonal experiments, and explore the mechanism of the utilization of trace elements by Aureobasidium pullulans, in order to maximize the yield of polysaccharides.

3.3 EFFECTS OF BATCH FERMENTATION AND SHAKE FLASK FERMENTATION ON PULLULAN PRODUCTION BY AUREOBASIDIUM PULLULANS

3.3.1 Introduction

Fermentation is a complex process, and its core is to use the growth and metabolic activities of microorganisms to synthesize the products we need. This process mainly focuses on two aspects: the growth status of microorganisms and the formation of the final product.

Shake flask fermentation is the most commonly used technology in fermentation experiments. According to the physiological characteristics of different bacteria, it is necessary to fine regulate all kinds of culture conditions. This culture method, often referred to as shaking culture or shaking flask culture, involves inoculating microbial cells into a liquid medium and then placing them on a shaker or oscillator to oscillate continuously. This oscillation not only makes the medium fully mixed with oxygen, increases the supply of dissolved oxygen, but also makes the cell reproduction more uniform, thus improving the culture efficiency. Among them, the regulation of oxygen supply capacity is particularly important, mainly by adjusting the amount of shake flask medium and the rotational speed of the shaker.

Shake flask fermentation technology is widely used in many fields, such as strain screening, microbial expansion culture and so on. it is the basic culture method in microbial physiology, biochemistry, fermentation technology and other life science research. Because of its efficient and uniform cultivation, this technology plays an important role in scientific research and industrial production.

Batch fermentation, also known as batch culture, is a method of microbial culture in a closed system. Its core is that after putting a limited amount of nutrients into the system, a small number of microbial strains are introduced to make it complete a complete growth cycle under specific conditions. In this process, except for aeration and acid-based solution addition to maintain oxygen supply (for aerobic fermentation)

and adjust the pH value of fermentation broth, the system does not exchange any other materials with the outside world. The culture medium is one-time input, and the final product is also harvested at one time, which is very common in the fermentation process.

Specifically, batch fermentation involves inoculating specific active producing bacteria into the sterilized medium and no longer adding or removing any substance to the fermentation broth during fermentation (except for a continuous supply of oxygen required by aerobic microorganisms). In the process of batch fermentation, the environment of microorganisms is dynamic, that is, the chemical and physical states in the culture tank constantly evolve with the passage of time, so the whole fermentation process is unsteady. However, when the optimal conditions for cell growth (such as temperature, pH and dissolved oxygen) are not consistent with the optimal conditions for the formation of metabolites, these conditions can be artificially adjusted to achieve the highest product yield. This flexibility and controllability make batch fermentation a popular technology in microbial culture. The purpose of this experiment was to compare the effects of batch fermentation and shake flask fermentation on pullulan production by Aureobasidium pullulans by changing the environmental data such as temperature, rotational speed, ventilation and so on.

3.3.2 Materials and methods

3.3.2.1 Strain and medium

Aureobasidium pullulans (A. Pullulans), preserved in the State key Laboratory of Biomaterials and Green Papermaking, Qilu University of Technology.

Seed culture medium(g/L): sucrose 80, ammonium sulfate 0.5, yeast extract 2, natural pH fermentation medium (g/L): sucrose 100; yeast extract 3; magnesium sulfate 0.2,0.6 ammonium sulfate, sodium chloride 2, dipotassium hydrogen phosphate 6.

3.3.2.2 Training method

Seed culture is the same as in Chapter 2.

Shake flask culture: the seed liquid activated by 10ml was inoculated into 50ml fermentation medium, the temperature and rotational speed are shown in Table 4-1, and the fermentation time is 72 hours.

Batch culture: the secondary seeds were connected to the 5L range-mixing fermenter with 3L fermentation medium according to 10% (Viand V) inoculum. The temperature, rotational speed and aeration are shown in Table 3-2. The level of dissolved oxygen (pO2) in the fermentation broth was monitored by Mettler DO electrode. Fermented for 72 hours.

Table 3-2 temperature and rotational speed of shake flask culture

No	Temperature (℃)	Rotational speed (rmp)
1	26	200
2	28	200
3	30	200
4	32	200
5	34	200
6	30	150
7	30	180
8	30	200
9	30	220
10	30	250

Table 3-3Temperature, rotational speed and ventilation of batch culture

No	Temperature (℃)	Rotational speed(rmp)	Ventilation capacity(L/min)
1	26	400	4
2	28	400	4
3	30	400	4
4	32	400	4

5	34	400	4
6	30	200	4
7	30	300	4
8	30	400	4
9	30	500	4
10	30	600	4

3.3.2.3 Analytical method.

Same as Chapter 2.

3.3.3 Results

3.3.3.1 Effect of temperature on pullulan biosynthesis

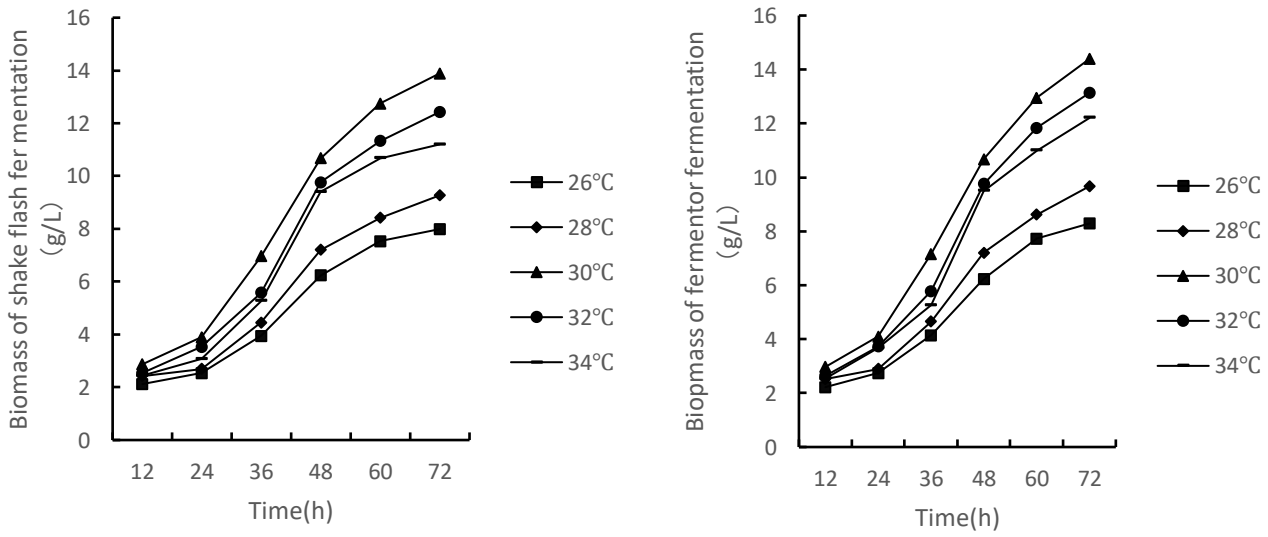

Fig. 3-5 Effect of temperature on biomass

Temperature is one of the important control conditions in the process of microbial fermentation, and its change directly affects the quality of fermentation results. The selection of optimum temperature will be different for different starting strains in the process of fermentation. According to the literature, the optimum temperature for Aureobasidium pullulans is between 26 ℃ and 34 ℃. In this experiment, the optimum temperature of the fermentation process of Aureobasidium

pullulans was studied by selecting five temperature points: 26 ℃, 28 ℃, 30 ℃, 32 ℃ and 34 ℃. The experimental results are shown in Figure 3-5 and Figure 3-6.

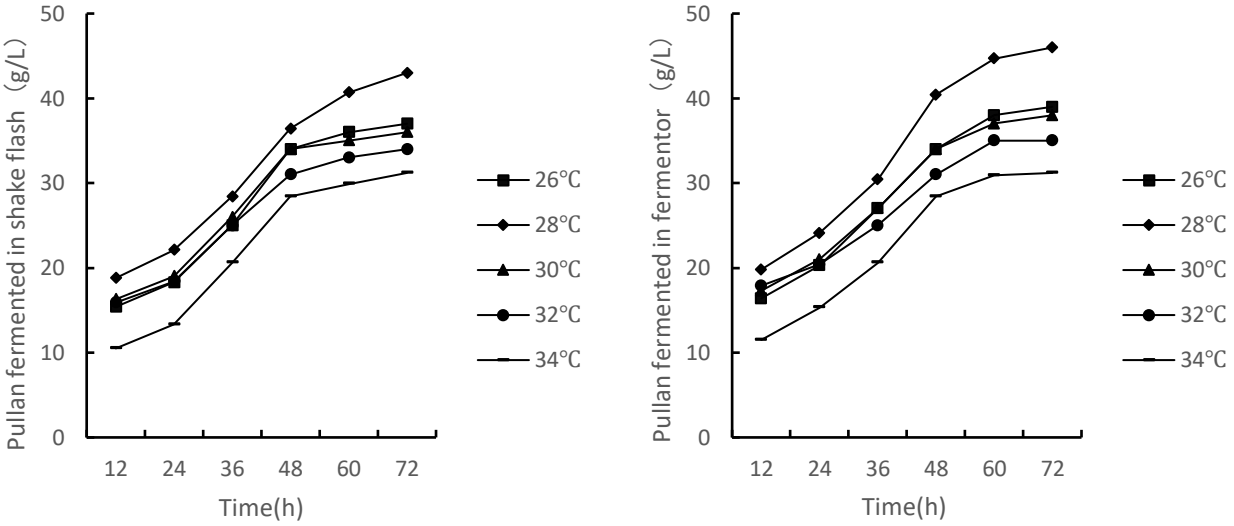

Fig. 3-6 Effect of temperature on pullulan yield

It can be seen from Figure 3-5 that different temperatures have great effects on the growth of Aureobasidium pullulans, but the change trend of the overall growth curve is similar. When the temperature is controlled between 26 ℃ and 30 ℃, it can be seen that the slope of the cell adjustment period increases gradually with the increase of temperature, that is, the adjustment period time is gradually shortened. This shows that in the suitable temperature range, Aureobasidium pullulans has strong adaptability and can enter the logarithmic growth phase more quickly. This shows that the suitable temperature is helpful to promote the growth and reproduction of Aureobasidium pullulans. However, when the culture temperature was too high to 32 ℃, the cell growth was inhibited to a certain extent. This shows that too high temperature has an adverse effect on the growth of Aureobasidium pullulans, which may slow down or even stop its growth.

It can be seen from Figure 3-6 that the final yield of pullulan increases gradually with the increase of temperature in a certain temperature range, that is, from 26 ℃ to 28 ℃. This shows that the increase of temperature is beneficial to the synthesis of pullulan polysaccharides in this temperature range. However, when the culture

temperature was too high, the synthesis of pullulan was inhibited, and the higher the culture temperature was, the more obvious the inhibition was.

Comparing Figure 3-5 and Figure 3-6, it is not difficult to find that Aureobasidium pullulans has the maximum biomass at 30 ℃ but the highest yield of pullulan at 28 ℃. This may be because high temperature affects the activities of key enzymes in the process of pullulan synthesis, thus affecting the synthesis of polysaccharides. The synthesis of pullulan is a complex biological process, which needs the participation and regulation of a variety of enzymes. When the temperature is too high, the activity of these enzymes may be destroyed or decreased, resulting in the synthesis of pullulan polysaccharides blocked.

Comparing Figure 3-5 and Figure 3-6, we can see that shaking flask culture has a numerical disadvantage compared with batch culture at the same temperature, but the overall change trend is similar, generally speaking, batch culture has better advantages than shaking flask culture.

3.3.3.2 Effect of rotational speed on pullulan biosynthesis

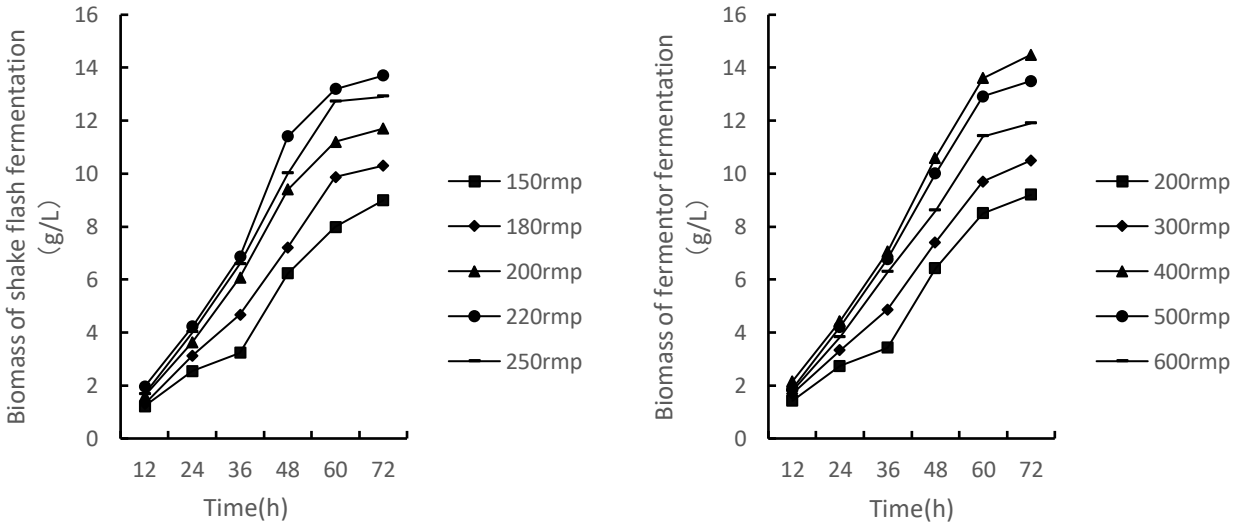

Fig. 3-7 Effect of rotational speed on biomass

The yield of pullulan polysaccharides at different rotational speeds was determined, and the experimental results were shown in Figure 3-7 and Figure 3-8.

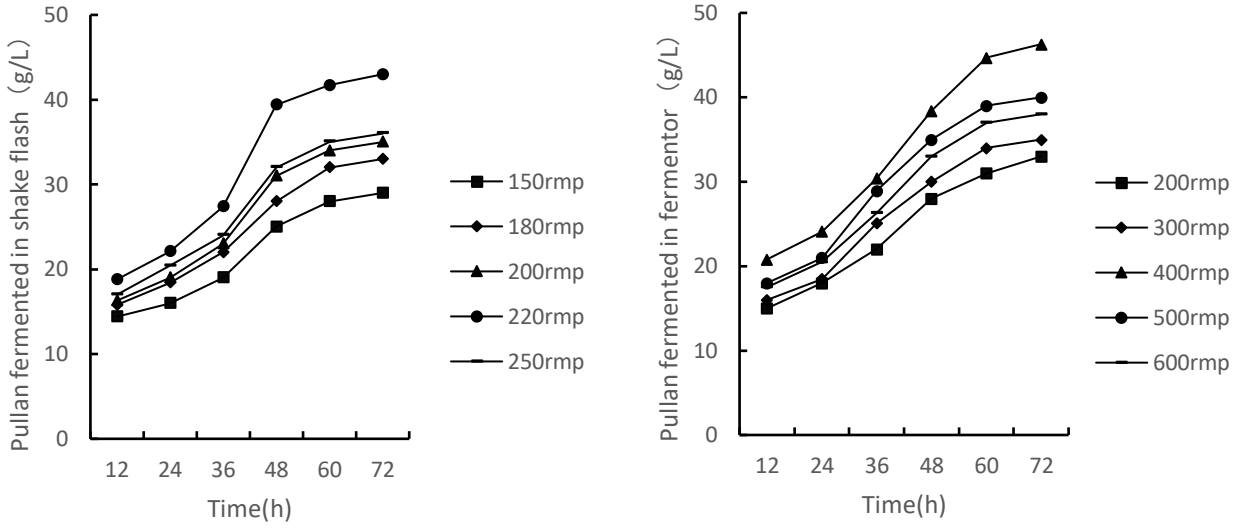

Fig. 3-8 Effect of rotational speed on pullulan yield

From Figure 3-7 and Figure 3-8, it can be seen that the rotational speed of shaking flask has a significant effect on the biomass and pullulan yield of Aureobasidium pullulans. When it is lower than 200rmp, the biomass and pullulan constant increase with the increase of rotational speed, but when it is higher than 200rmp, the cell growth is inhibited and pullulan synthesis is hindered. The reason for this may be that the rotational speed of the shaking flask determines the contact between the fermentation broth and the air, the lower rotational speed will lead to insufficient mixing of the fermentation broth with the air, and the higher rotational speed may cause the fermentation broth to move too violently.

Figure 3-7; Figure 3-8 shows that the synthesis of pullulan is inhibited when the stirring speed is 200r/min, which may be due to the fact that the lower stirring speed is not enough to provide sufficient mixing and oxygen transfer, thus affecting cell growth and metabolism. When the stirring speed is 400r/min, the synthesis rate of pullulan is higher, indicating that this speed provides suitable conditions for mixing and oxygen transfer, which is beneficial to the growth and metabolism of bacteria. When the stirring speed is too high, although high-speed stirring can provide better mixing and oxygen transfer, it may also produce excessive shear force. This shear force may

damage the bacteria and inhibit its growth, thus affecting the synthesis of pullulan polysaccharides. Therefore, too high stirring speed is not conducive to the efficient synthesis of pullulan polysaccharides.

Summary to Part 3.3

The results showed that the culture temperature had a significant effect on the growth of Aureobasidium pullulans and the synthesis of pullulan. Specifically, the growth condition of Aureobasidium pullulans was better when the culture temperature was set at 30 ℃, and the synthesis effect of pullulan polysaccharide was better at 28 ℃. This finding shows that the ability of Aureobasidium pullulans to synthesize pullulan can be effectively improved by accurately controlling the temperature in different fermentation stages, so as to optimize the fermentation process. In the experiment of discussing the effect of rotational speed on the fermentation of Aureobasidium pullulans, we found that the rotational speed parameters also had an important effect on the yield and cell growth rate of pullulan polysaccharides. In the process of batch culture, the yield and cell growth rate of pullulan reached the peak when the rotational speed was controlled at 400rpm, while under the condition of shaking flask culture, the rotational speed was the best when 220 rpm. This result is of great significance for optimizing the stirring conditions in the fermentation process of Aureobasidium pullulans and improving the production efficiency of pullulan. By adjusting the key fermentation parameters such as temperature and speed, we can optimize the fermentation process of Aureobasidium pullulans and improve the yield and quality of pullulan polysaccharides. These findings provide important theoretical basis and practical guidance for the industrial production of pullulan.

CONCLUSIONS

The main results were as follows:

(1) The culture temperature had a significant effect on the growth and pullulan synthesis of Aureobasidium pullulans. The cell growth was better at 30 ℃, and the synthesis of pullulan was better at 28 ℃. This shows that the synthetic ability of pullulan can be improved and the fermentation process can be optimized by accurately controlling the temperature of fermentation stage. At the same time, rotational speed also affected the yield and cell growth of pullulan. In batch culture, the yield and growth rate of 400 rpm were the highest, while in shake flask culture, the best speed was 220 rpm. Therefore, the regulation of temperature and rotational speed is the key to optimize the fermentation of Aureobasidium pullulans.

(2) Through a series of experiments, we found that different carbon sources had significant effects on the growth and metabolism of microorganisms. Among the combined carbon sources of fructose, glucose, sucrose and xylose, sucrose stands out as the best carbon source because of its efficient utilization and good effect. However, the carbon source mixed with fructose and glucose at 1:1 ratio also shows remarkable effect, and its potential is huge, which is worthy of further research and exploration.

(3) The effects of trace elements such as $MnCl_2$, $CaCl_2$, $ZnSO_4$, $CuSO_4$, $FeSO_4$ and $MgSO_4$ on polysaccharide yield and biomass in liquid fermentation of Aureobasidium pullulans were studied. The results showed that different trace elements and their concentrations had different effects on the yield of polysaccharide from Aureobasidium pullulans. The optimum concentration of single element was determined as follows: MnCl2 0.05 g / L, $ZnSO_4$ 0.05 g / L, $CaCl_2$ 0.15 g / L, $CuSO_4$ 0.1 g / L, $FeSO_4$ 0.06 g / L, $MgSO_4$ 0.2 g / L.

LIST OF REFERENCES

1. He, C. Y., Zhang, Z., Zhang, Y. D., Wang, G. L., Wang, C. L., Wang, D. H., & Wei, G. Y. (2021, Sep). Efficient pullulan production by Aureobasidium pullulans using cost-effective substrates. International Journal of Biological Macromolecules, 186, 544-553.

2. Wei, X., Liu, G.-L., Jia, S.-L., Chi, Z., Hu, Z., & Chi, Z.-M. (2021). Pullulan biosynthesis and its regulation in Aureobasidium spp. Carbohydrate polymers, 251, 117076.

3. Xu, L., Qiu, Z., Gong, H., Zhu, C., Sang, Q., Li, Y., & Dong, M. (2019). Synergy of microbial polysaccharides and branched-preformed particle gel on thickening and enhanced oil recovery. Chemical Engineering Science, 208, 115138.

4. Zeng, N. (2023). Study on the mechanism of regulating cell differentiation of budding stalk fungus and promoting its metabolite synthesis Shenyang Agricultural University,

5. Gao, M. (2022). Breeding and fermentation process analysis of sprouting strains with high polysaccharide yield Tianjin University of Science and Technology,

6. Hei, Y.C. (2022). Study on Pullulan biosynthesis based on substrate condition optimization Soochow University,

7. Jiang, L. (2020). Study on fermentation technology of Pullulan polysaccharide with high yield and low molecular weight by Spore stalk fungus Guangxi University,

8. Li, X.W. (2023). Study on Pullulan polysaccharide biosynthesis and targeted breeding of high-yield strains Jiangnan University,

9. Liu, F.et al. (2020). Advance on Biosynthesis and Molecular Weight Regulation of Pullan.Food and Drug,22(01),72-77.

10. Pan, Z.Q.et al. (2023). Effect of Starch Raw Materials on Pullan Polysaccharide Biosynthesis and the Underlying Physiological Mechanism. Science and Technology of Food Industry, 44(12), 124-129.

11. Sheng, L. (2015). Study on key influencing factors and mechanism of Pullulan polysaccharide biosynthesis Jiangnan University,

12. Wang, D.H.et al. (2019). Physiological mechanism of the effect of surfactants on pullulan production by bioconversion. Food Science, 40(22), 27-32.

13. Wang, Y. (2022). The role and physiological mechanism of sugar transporters RCO3 and YDB1 in Pullulan biosynthesis Soochow University,

14. Zhang, M.J. (2021). Metabolic engineering modification of Pullulan producing polysaccharide spathophyllum Tianjin University of Science and Technology,

15. Zhang, P. (2021). Effect of vitamin B5 on molecular weight and yield of Pullulan polysaccharides produced by fermentation of budding stalk fungus Tianjin University of Science and Technology,

16. Zhang, Y.F. (2022). Optimization of fermentation process of Pullulan polysaccharides produced by sprouting stalk fungus using glucose as carbon source Tianjin University of Science and Technology,

17. Zhu, C.C. (2019). Study on biosynthesis of exopolysaccharides by Rhizobium sprouting and its mechanism Soochow University.