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 PHA-producing strains**
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
Liu XUECHUAN

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Reviewer
Olena OKHMAT, 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
Liu Xuechuan

1. Thesis topic **Optimization of fermentation conditions for PHA-producing strains**
Scientific supervisor Iryna Voloshyna, 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

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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	
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I am familiar with the task:

Student _____ Liu XUECHUAN

Scientific supervisor _____ Iryna VOLOSHYNA

SUMMARY

Xuechuan Liu. Optimization of fermentation conditions for PHA-producing strains – Manuscript.

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Over the years, as more and more attention has been paid to environmental problems, the state has promulgated a number of plastic restriction orders, intensifying efforts to rectify the "white pollution" problem and promote "sustainable development". Under this background, among many degradable biomaterials, PHA, also known as polyhydroxyalkanoates, is considered to be a substitute for plastic products. The physical properties of PHA molecules are similar to those of common plastics, Monomer molecules with different lengths can be used to produce materials with different properties such as stretchability and hardness. Although PHA molecules can be produced by a variety of microorganisms through their internal metabolic pathways and the corresponding materials can be produced in the laboratory, there are still some problems in making them applicable to large-scale production and practice. In this study, the fermentation conditions of PHA were optimized by single factor screening experiment, using *E. coli* JM109 as the chassis engineering strain, using cheap recycled glycerol as carbon source, and using indoor shake flask culture method. Without considering the interaction of each component, it was found that the optimal fermentation temperature was 37 ℃, the yield was 3.45 G/L, the optimal fermentation time was 48 H, the yield was 3.32 G/L, the optimal pH value was 7.0, the higher yield was 3.82 G/L, and the optimal rotation speed was 200 rpm. The yield can reach 4.11 G/L. This study not only reveals the internal relationship between microbial growth and PHA synthesis in the fermentation process, but also provides a strong theoretical and practical basis for the industrial production of biodegradable plastics. This study deeply understood the key factors found in PHA fermentation production, which has guiding and reference significance for the further transformation of microbial strains, the optimization of fermentation

process and the expansion of PHA application fields in the future, such as medical materials and packaging materials.

Keywords: Polyhydroxyalkanoate (PHA); optimization of fermentation conditions; Single factor; glycerin

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INTRODUCTION

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The relevance of the topic is Optimization of fermentation conditions for PHA production strains.

The purpose of the study is The yield of PHA was improved by optimizing the

growth and development of PHA producing strains and the nutrition and environmental conditions for PHA production.

The objectives of the study The yield of PHA was improved by optimizing the growth and development of PHA producing strains and the nutrition and environmental conditions for PHA production. The object of the study.

The subject of the study P.a. Zhuang

Research methods is Single factor screening optimization experiment

The scientific novelty The PHA strain produced by *E. coli* JM109 was selected, and the fermentation culture, composition and fermentation conditions were optimized

The practical significance of the results obtained is Increase PHA output and provide data support for reducing its production cost.

The task of the study is The optimum temperature, time, pH and rotational speed of *E. coli* JM109 fermentation to produce PHA molecules were determined by single factor experiment, and the fermentation conditions were optimized.

The goal of the study is Through the optimization of fermentation conditions, the high yield of PHA strains was achieved, which provided a certain theoretical reference for the follow-up research.

CHAPTER 1
LITERATURE REVIEW

1.1 Reasons for topic selection

From 2007 to 2021, China has issued a series of plastic restrictions, which reflects China's determination to vigorously address the "white pollution" problem and achieve sustainable development. In this context, biodegradable material technology came into being. Since the discovery of the first poly (3-hydroxybutyrate) (PHB, one of the most common polyhydroxyalkanoates) in microbial cells, the research in this field has gradually deepened.

Polyhydroxyalkanoate (PHA) is a kind of fatty acid copolyester with various structures and functions, which is formed by microorganisms using various carbon sources through *in vivo* metabolic pathways. It is widely used in medicine, agriculture, environmental protection, packaging, cosmetics, food and disposable goods. Among them, PHB, as a kind of PHA, is considered to be a potentially valuable biomaterial because of its strong biodegradability and biocompatibility.PHB is a new polymer material with high mechanical strength, good thermal stability and biodegradability. Therefore, PHB has broad application prospects in packaging, medical devices, agricultural films and fibers.

With the development of biotechnology and genetic engineering, researchers are also exploring ways to improve the yield and quality of PHB by genetically engineered microorganisms. This is expected to provide a more economical and efficient method for the production of PHA, thus promoting the application and development of PHA in more fields.

Polyhydroxyalkanoate (PHA) is a new biodegradable bio-based material with good biodegradability and biocompatibility. In addition, the recovery and disposal of PHA is unique due to its non-toxic side effects, and it can be recycled as animal feed. Traditional waste of old plastics is usually disposed of by landfill or incineration, resulting in land, air and other pollution problems.On the other hand, PHA can be processed through many processes, and as a feed, it can produce economic benefits

again, realizing the double cycle of feed and economy. There have been many industrialized and commercialized cases of PHA production process since 1959, but the problems of low efficiency and high cost of PHA synthesis and purification have seriously affected its application and promotion in the market. According to relevant reports, the ideal cost of PHA is about $4 per kilogram. But even this price is 1 times higher than the commonly used petroleum-based plastic products [1].In addition to this, population growth has led to an increase in global demand for plastic products. Petroleum-based plastics (PBP) are still the main form of plastic products. However, the large oil consumption of plastic production will exacerbate the energy shortage. All kinds of toxic and harmful gases emitted by petrochemical industry also cause damage to the environment and human health. According to relevant statistics, the annual plastic recycling can only reach 13% -15% of the total amount of waste plastics. Polyhydroxyalkanoates (PHAs)As a representative of bioplastics, it is usually used as an energy storage substance in microbial cells. Producing 1 kg of PHA instead of PBP reduces CO2 emissions by 2 kg and saves 30 megajoules of fossil energy. Data show that plastic waste accumulates in the natural environment at a rate of 2500 tons worldwide.

Generally speaking, in addition to pyrolysis, in nature, the rate of photolysis and biodegradation of plastics is very slow, generally 200-400 years. In order to deal with this "white pollution", many countries have begun to implement the "reduction, reuse, reuse" three R plan. However, 3 R projects are still difficult to realize in some places that are difficult to regenerate or are difficult to regenerate. Therefore, many research units at home and abroad are conducting research and development of biodegradable materials[2] .

In a word, the development of biodegradable materials technology provides new ideas and methods to solve the problem of "white pollution". With the deepening of research and the expansion of application fields, biodegradable materials such as PHA are expected to play a more important role in the future and contribute to the realization of sustainable development goals.

In this study, *E. coli* JM109 was selected as the engineering strain to produce PHA, and the gene pTBBC was transformed into *E. coli*, and then the strain was activated, and the PCR verification was carried out to determine that it could be applied to the production of laboratory fermentation, and the most commonly used PHA molecule β-hydroxybutyrate (PHB) was produced by using waste glycerol as the substrate. PHA is the most frequently used, discovered and studied PHA molecule. Subsequently, the fermentation conditions, medium components and other fermentation parameters were optimized by single factor optimization and screening experiments, which provided a reference for large-scale application and practice of PHA fermentation production.

1.2 PHA Overview

1.2.1 Introduction to PHA

Polyhydroxyalkanoates (PHAs) are important biomaterials with biodegradability, biocompatibility and low toxicity, which are widely used in drug delivery, tissue engineering, gene therapy and other fields. The structure of polyhydroxyalkanoates is mainly composed of poly (lactic acid) (PDLA), poly (glycolic acid) (PGA), poly (caprolactone) (PCL), poly (D-lactic acid-co-glycolic acid) (PDLA-co-PGA) and other monomers. The monomer structure is shown in the figure.

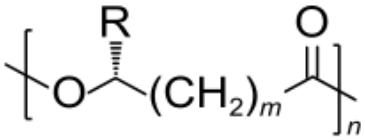

Figure 1.1 – PHA Monomer Structure

Microbiologists, molecular biologists, biochemists, chemical engineers, chemists, polymer specialists, and medical researchers have studied the biopolyester polyhydroxyalkanoates produced by many bacteria[3].

1.2.2 Classification of PHA

The monomer structure of PHA is different, so there are many kinds. With the

deepening of PHA research, more than 150 kinds of PHA have been identified so far [4].Based on the number of carbon atoms that make up the monomer, PHA can be divided into three types: short-chain PHA, medium-chain and long-chain PHA, and short-chain medium-chain and long-chain co-recombinant PHA. SCL-PHA contains only 3-hydroxybutyric acid, 3-3-hydroxybutyrate-3-pentanoic acid (PHBV), and only 3-5 C. Medium-chain and long-chain PHA contain 6 to 16 C. PHA are short, medium and long chain copolymers, short and medium chain respectively. Polyhydroxyalkanes (PHA) can be divided into homopolymers and copolymers according to their monomer types. Single monomers, such as polyhydroxybutyric acid (PHB), poly3-hydroxyvaleric acid (PHV), etc., can be copolymerized with various monomers to improve their softness and plasticity. Therefore, the chemical modification of PHAs varies depending on the composition, size, and monomer copolymerization type of the side chain substitute[5].

In addition to the above structures, there are many side chains containing functional groups such as benzene rings, halogens, unsaturated bonds, etc. These modifications make PHA have new functions and provide some high value-added properties, including high strength, pH or temperature responsiveness, shape memory, etc. The introduction of a predetermined proportion of functional groups into the PHA polymer chain has become a reality through the absorption of fatty acids by bacteria to synthesize PHA, which allows the formation of functional PHA to be further modified.

1.2.3 Application of PHA

Polyhydroxyalkanoates (PHAs) have great application potential in the field of new medical materials and disease treatment. As a biomaterial with excellent physical and chemical properties and stability, PHA can support cell growth and adhesion, promote cell ingrowth, provide nutrient supply and metabolic waste discharge channels, so it has a wide range of applicability in tissue engineering. Because of its biocompatibility and rapid degradation, PHA is also widely used in drug controlled release. The polyhydroxyalkanoate can also be used as an entrapment material of some drugs, and has good sustained-release effect. The sustained-release effect can

greatly prolong the action time of the medicament, reduce the administration times of the medicament and improve the curative effect. In addition, polyhydroxyalkanoates can also be used to prepare drug nanocarriers for effective delivery of drugs to target sites.

PHA has good toughness, hardness and processability, so it can be used as packaging bags for various articles and products in people's daily life.

Such molecules and their various modified molecules can be used in various scenarios of human society, ranging from medical equipment and synthetic implants to various products in life.

1.2.4 PHA synthesis pathway

Polyhydroxyalkanoates (PHAs) are mainly prepared by chemical methods and biosynthetic methods. Chemical synthesis methods include transesterification, ring-opening polymerization and condensation polymerization.

Biosynthesis technology refers to the production of polyhydroxy fatty acids by the metabolic pathways of microorganisms themselves or by the modification of genetic engineering technology, which can accumulate a large number of such molecules in the body and keep them degraded. It is the most widely used PHA production method with low cost at present. PHA metabolism and synthesis pathways in microorganisms are very diverse. Fig. 1-2 shows several basic pathways of PHA synthesis.

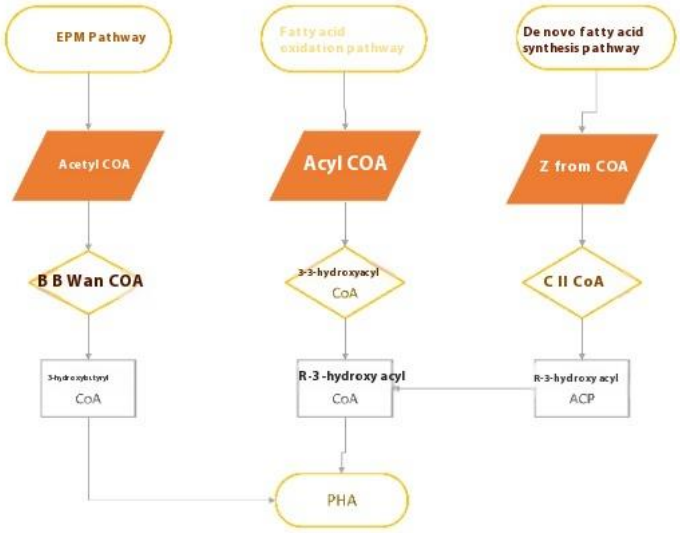

Figure 1.2 – Main synthesis routes of PHA

Biosynthesis and biodegradation are two important metabolic processes of polyhydroxyalkanoates (PHA) in microorganisms. In the aspect of biosynthesis, PHA is mainly produced by esterification of fatty acids with β-hydroxybutyric acid or β-hydroxyvaleric acid through enzyme-catalyzed reactions. In this process, it is necessary to add appropriate amount of substrate and adjust the appropriate fermentation conditions to promote the biosynthesis of PHA.

In the process of biosynthesis, the key enzyme-catalyzed reaction is the PHA synthetase catalyzed reaction. PHA synthetase I and PHA synthetase II are two main types of synthetases. Among them, PHA synthase I can directly catalyze the synthesis reaction of PHA, which can esterify fatty acids with β-hydroxybutyrate or β-hydroxyvalerate to form PHA.PHA synthase II can esterify short-chain fatty acids with β-hydroxybutyrate or β-hydroxyvalerate to further synthesize longer-chain PHA. Under the action of these two enzymes, the chain length of PHA synthesis can be adjusted to a certain extent, so as to better meet the production requirements of specific applications.

In the aspect of biodegradation, the degradation of PHA is mainly realized by PHA-degrading enzymes produced by microorganisms. PHA-degrading enzyme is the hydrolase of PHA ester bond, which can catalyze the cleavage of PHA ester bond and decompose PHA into low molecular compounds. Degradation enzymes include PHAase, Depolymerase and so on. PHA esterase is one of the most common and widely studied degradation enzymes, which usually has a high degradation efficiency and a wide range of substrate adaptability. PHA depolymerase can selectively degrade the specific site of PHA according to the structural characteristics of PHA. The existence of these two kinds of degrading enzymes provides the possibility for the effective degradation of PHA.

In addition to enzyme catalyzed reactions, a series of regulatory factors are involved in the mechanism of biosynthesis and biodegradation. In the process of biosynthesis, substrate concentration, carbon source, nitrogen source type and concentration, temperature, pH and other conditions will play a certain role in the

biosynthesis of PHA. Reasonable regulation of these conditions can improve the efficiency and yield of PHA synthesis. In the process of biodegradation, appropriate biodegradation strains and degradation environmental conditions are also needed.

In summary, in the biosynthesis process, PHA is obtained by esterification of fatty acids with precursors such as β-hydroxybutyrate or β-hydroxyvalerate through enzyme-catalyzed reactions. In the process of biodegradation, PHA is decomposed into low molecular compounds by PHA degrading enzymes. Controlling these two processes is the key to efficient PHA synthesis by microorganisms.

Microbial fermentation is often used to produce PHA in the industrial production of polyhydroxyalkanoates. First of all, high-yield chassis engineering strains were constructed by biotechnology such as genetic engineering and metabolic engineering, and then the best fermentation process parameters were obtained through laboratory research and exploration. High efficiency and high yield of PHA can be realized. In many studies of fermentation conditions, the fermentation temperature is generally controlled between 30 ℃ and 37 ℃, and the pH value is usually between 6 and 8, which can ensure the best state of enzyme activity and microbial growth in the fermentation process. Generally, the time will vary according to different strains, and generally the time should not be too long, otherwise it will lead to the decomposition of PHA molecules that have been synthesized by microorganisms through their own metabolic pathways. The rotation speed can vary greatly depending on the growth characteristics of the strains, and it is necessary to fully understand the growth environment conditions of the fungi used.

Poly (β-hydroxybutyrate) (PHB) is one of the earliest discovered and most widely studied biopolymers in the PHA family. PHB is synthesized by many bacteria under specific conditions that often include the presence of an excess carbon source, such as glycerol. Bacteria respond to nutritionally imbalanced environments by accumulating PHB as an energy store.

Unlike many traditional plastics, PHB also has significant biodegradable properties as one of the PHA molecules. Under appropriate environmental conditions, specific microbial enzymes can degrade PHB, and their structures are relatively

simple. At the same time, PHB has physical properties similar to traditional plastics, such as strength, toughness and thermoplasticity, which makes it a potential material for manufacturing various objects. PHB is also an ideal environmentally friendly material due to its degradable nature.

PHB has high stereoregularity, high crystallinity (65% -75%), characteristics similar to polypropylene, good water resistance, high optical purity, good piezoelectric property, high tensile strength, good elasticity and good heat resistance; the product has good plasticity and can form good compatibility with various copolymer resins. Because of its good biocompatibility, biodegradability, nonlinear optical activity, film-forming, gas separation, anticoagulation and other characteristics, it plays an important role in replacing traditional plastics, alleviating environmental pollution, developing new functional medical materials and new food packaging materials.

At present, in addition to chemical synthesis, the production methods of PHA are mostly single strain and mixed strain. On this basis, Wenbin[6] prepared PHA from Pseudomonas NK01 with glucose as carbon source.Pal[7]et al. utilized *Azotobacter beijerinckii* WDN01 to synthesize PHA with glucose as carbon source. A large part of the cost of these production processes comes from the consumption of substrate and energy consumption in the fermentation process, even accounting for most of the production costs. The raw material costs required to produce 1 kg of PHA are shown in Table 1-1.

Table 1-1 **Raw material cost for production of 1 kg of polyhydroxyalkanoate**

Name	Input (kg)	Unit price (yuan/kg)	Total (yuan)
Yeast powder (Oxoid)	0.5005	25	12.5125
Sodium glutamate	0.5005	48	24.024
Acid-hydrolyzed casein (Difco)	0.429	45	19.305
Glucose	0.715	3.15	2.25225

Magnesium sulfate	1.43	0.5	0.715
Potassium chloride	0.143	1.8	0.2574
Sodium chloride	14.3	0.5	13.3
Ferrous sulfate	0.0003575	0.5	0.00017875
Potassium dihydrogen phosphate	0.000775	3.5	0.0027125

Therefore, it is an effective way to reduce the cost to find a suitable, inexpensive and environmentally friendly carbon source and to optimize the fermentation conditions by using strains that can produce PHA molecules as substrates.

1.3 Overview of *Escherichia coli*

In 1885, *Escherichia coli* was discovered, which has a blunt, rounded end and is Gram-negative. Biochemical metabolism is very active. Glucose can be fermented to produce acid and gas, no gas can be produced by a single strain, different carbohydrates can be fermented, and different organic acids can be used. Fast growth rate, easy creation of culture conditions, and simple gene manipulation, it is widely used in the process as a more reliable chassis engineering strain. Although it can not synthesize PHA molecules in the natural state, it is still the best choice for PHA synthesis engineering strains because of its wide application range and lack of PHA degradation enzymes.

Escherichia coli is a Gram-negative *Brevibacterium* strain. Methanogens are facultative anaerobes, 0.5 × 1-3 μm in size, with flagella, swimming ability, no capsule; no spores (rarely with about 0.2 μm micropods) [11]. *E. coli* can be classified according to their specific pathogenic effects, first according to whether they are infectious or not. Secondly, it is classified according to whether it produces enterotoxin in the course of infection. Thirdly, according to its infection characteristics, we can judge whether it will produce hemolysin and whether it has the ability of hemolysis. *Escherichia coli* is a gram-negative bacterium with

individual hyphal morphology similar to that of a globular or long thread under storage conditions. Most strains of *E. coli* have a capsule structure and do not form spores. The bacterium has strong metabolic activity and can ferment glucose and various carbohydrates.

1.4 Research status of PHA synthesis from glycerol

In different PHA fermentation processes, PHB synthesis with glycerol as substrate was more likely to occur. The potential of synthesizing PHA from glycerol is much greater than that from other carbon sources, and crude glycerol, a by-product of biodiesel, has attracted much attention because of its low price and higher synthesis efficiency.

Biodiesel is a representative new clean fuel, which has attracted wide attention of the society. However, in the preparation of biodiesel, there are a large number of by-products such as glycerol, in which the high content of glycerol affects its comprehensive utilization. Therefore, the bioreactor for producing biofuel from glycerol has attracted much attention. Canadas et al.[9] used waste glycerol as the main carbon source for PHA synthesis by strain Cupriavidus necator DSM 545, and the polymer concentration could reach 9-25 G/L. The polymer was successfully prepared as an electrospun fiber scaffold for stem cell culture. Mohandas et al. [10]first reported that the marine halophilic bacterium Bacillus cereus could convert glycerol into PHA, and the content of PHA could reach 68.12% under the optimal conditions. Crude glycerol can also synthesize medium chain PHA with good uniformity by Pseudomonas, which is beneficial to its application in industry. In addition, the way of using glycerol to synthesize PHA in microorganisms is relatively simple and easy to control, which is more convenient to achieve the purpose of optimizing fermentation process parameters.

Glycerol is catalyzed by glycerol kinase (GlpK) to produce glyceraldehyde-3-phosphate (GAP). When pyruvate is decarboxylated, the pyruvate dehydrogenase complex assists in the generation of acetyl-CoA from pyruvate. The microorganism then use PhaA (Beta-ketothiolase) to polymerize that two acetyl-CoA molecules into

acetoacetate, and then PhaB (acetoacetate reductase) convert (R) 3-hydroxybutyryl-CoA into PHA. The final step in PHA synthesis is dependent on the polymerization of the 3-hydroxybutyryl CoA moiety to P (3 'HB) by PhaC (PHA synthase). The synthetic pathway is shown in figs. 1-3.

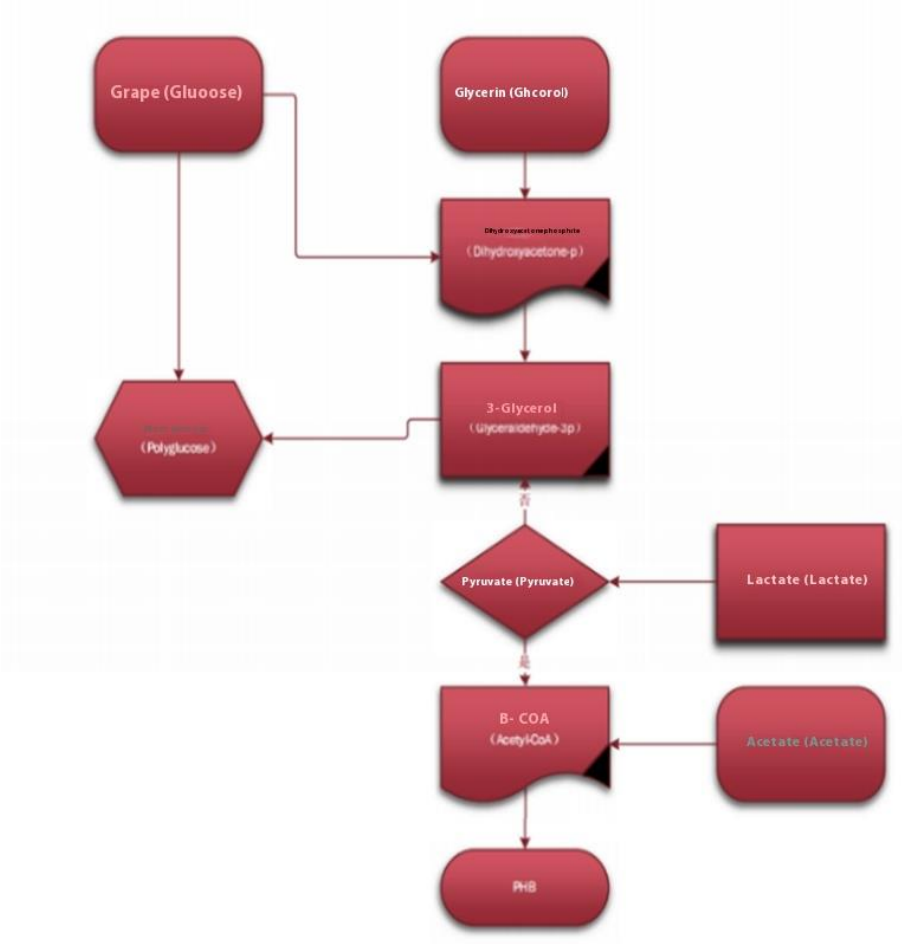

Figure 1.3 – PHA synthesis pathway of glycerol

1.5 Research status of fermentation condition optimization

1.5.1 Optimization of fermentation medium

Culture medium is a kind of compound nutrient that can make bacteria grow, proliferate and produce various substances on it, which provides the necessary carbon source, nitrogen source and water for the growth and development of microorganisms; the composition ratio difference of inorganic salts and other nutrients will not only play an important role in the growth and development of microorganisms, but also greatly affect the synthesis and extraction of products. In 2015, Wang et al. concluded by using orthogonal experiments that the primary and secondary factors affecting

PHA yield were carbon source concentration, temperature, rotation speed, liquid volume and pH, among which the carbon to nitrogen ratio had the greatest impact on PHA yield. Initial pH has minimal effect on concentration [12].

In the process of PHA production, due to the different metabolic pathways, the composition of PHA molecules produced is not the same, in addition, the different ratio of carbon to nitrogen will also affect its synthesis, studies have found that a higher ratio of carbon to nitrogen is more conducive to the synthesis of PHA molecules, but still need to add a certain amount of nitrogen source in the medium to ensure the normal development of bacteria. It is necessary to find the optimal C/N ratio between them, which can increase both the cell volume and the accumulation of PHA.

1.5.2 Optimization of fermentation conditions

The optimization of microbial fermentation conditions is one of the core contents of microbial and bioengineering research. The yield and quality of the product can be effectively improved and the cost can be reduced by adjusting the basic conditions such as the composition of the culture medium, the pH value, the temperature, the rotating speed and the like so as to achieve the maximum benefit.

In the process of optimizing the production process of polyhydroxy fatty acid esters, the biosynthetic pathway and metabolic characteristics of polyhydroxy fatty acid esters are often clarified first, and then the optimization of fermentation process is designed. Through the single factor experiment and test of each influencing factor, the factors that have significant influence on the product yield were screened out, the corresponding mathematical model was constructed, and the fermentation conditions and parameters were optimized.

Liu Runze et al. Used a halophilic bacterium as raw material and screened its culture conditions to obtain the best strain for high PHB production. The researchers found that by adjusting the C/N ratio, stirring speed, temperature and pH value, the PHB production can be improved [13].In addition, fed-batch fermentation is a new microbial fermentation method, which can reduce the interference of external

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method, which can effectively control the lipase content in Escherichia coli, and optimize it to improve its production efficiency[15]. Duan Min and other researchers studied the mutant strain *E. coli* A29 The fermentation conditions of *E. coli* A29 were optimized at the shake flask level, and the relationship between the different components of the fermentation medium (carbon source, nitrogen source, phosphate) and the fermentation conditions (pH, inoculum size, fermentation time, fermentation temperature) was explored, and the optimal medium conditions were obtained.Under the optimal conditions, the PSA production was increased by 1. 9 G compared with that without optimization. After determining the optimal combination of fermentation conditions, the mutagenic strain *E. coli* A29 was cultured in a 7 L fermentor. The PSA yield of the mutant strain *E. coli* A29 reached 6. 09 G in a 7 L fermentor, which was 44. 67% higher than that of the unoptimized strain.Efficient fermentation optimization of PSA production by *E. coli* A29[16]. Zhao Guoqin et al. Used this strain as the initial strain, carried out genetic engineering modification on it, and obtained an acid-producing strain Asble with good acid-producing sophorolipids, which was cultured in shake flasks and fermentors respectively.The optimal culture condition [17] was determined by screening the culture medium of ArlpAleu3, which had been successfully established by the research group, with ArlpAleu3 as the research object.

Zhou et al. Used Bacillus subtilis YPS-32 as the fermentation strain to produce surfactin, and optimized the fermentation medium and culture conditions of Bacillus subtilis YPS-32 to produce surfactin. The fermentation process of Bacillus subtilis YPS-32 was optimized by single factor experiment and response surface methodology, which laid a foundation for its industrial development and application[18].

Yu Wenna et al. Optimized the fermentation conditions of Pediococcus acidilactici J1 to produce EPS by single factor experiment. The EPS yield of P. Acidilactici J1 was significantly improved by single factor experiment, which was 4.76 times of that before optimization[19].

Jiang Zhengri et al. Introduced SRT-ELP36 into *E. coli* to construct an expression vector containing SRT-ELP36, and carried out shake-flask culture by adding 0.5 mmol/L thiogalactoside (IPTG) at the end of logarithmic growth phase to

the medium TB, 5% of medium volume (medium volume/shake-flask volume), induction temperature of 37 ℃, Its OD600 (absorbance at 600 nm) was 22.3 and the maximum volumetric yield of protein was 0.60 g/L[20]. Mihaela [21]et al. studied Bacillus licheniformis (BL)Viability of ATCC 21424 in a shaker culture (100 ml Erlenmeyer shaker culture) and in a 7 L periodic action bioreactor (SMF).

In related studies, polyhydroxyalkanoates have been produced by glycerol fermentation, and the corresponding fermentation process system has been established by optimizing the fermentation conditions.

Zeqing Liu Laboratory [22]focused on the production of PHA using mixed microbial cultures and various wastes. In this paper, the characteristics and structure of PHA were introduced, and the research results of PHA synthesis from residual activated sludge, waste oil, glycerol, molasses and lignocellulose were summarized.The advantages and disadvantages of using PHA as carbon source were analyzed, and the mechanism was explained. Jin Dayao[23]optimized the fermentation conditions of Pseudomonas putida to realize the synthesis of PHA by mixed fermentation using glycerol as substrate.

Microbial fermentation usually refers to the process in which microorganisms use fermented raw materials for growth and metabolism, while producing targeted products, which are widely used in many fields such as chemical industry, food industry, environmental protection, pharmaceuticals, energy and agriculture. Proper culture conditions and nutrient environment are the basis and prerequisite for microbial fermentation process[24].

The optimization of fermentation conditions is the key link to improve the yield of the target product. The following are common factors that need to be controlled

Time: PHA is a substance that stores energy and has a high accumulation rate in the early and middle stages, but if it lasts too long, it will cause substrate depletion; at this time, bacteria will eat PHA, thus reducing the final output.

Temperature: Each microorganism has its own suitable growth environment, so it should be set according to the characteristics of different strains. Under different environmental conditions, temperature has a certain effect on the growth of bacteria,

enzyme activity and production of products.

PH: Under different pH conditions, it has a certain impact on the growth and development of microorganisms and the production of substances. It is generally desirable to maintain the pH at a desired value, which can be adjusted by the addition of an acid or base.

Stirring speed: The appropriate stirring speed can ensure the dispersion of nutrients in the medium, and have a certain impact on the effect of oxygen transfer.

Dissolved oxygen: The level of dissolved oxygen will directly affect the growth of aerobic bacteria and the production of products. The mixing rate and the aeration rate are usually used for adjustment.

Content of nutrients: including carbon source, nitrogen source, metal ions, etc., which are essential for the normal growth and development of microorganisms. Different fermentation strains have different requirements for nutrients, so it is necessary to optimize them.

Number of inoculations: The number of inoculations has an effect on the initial reaction rate and product yield.

After the optimization of fermentation conditions, PHA needs to be separated and purified to obtain higher purity products. In the PHA production process, multiple purification methods are often used in parallel to construct a new recovery system, which is also a major strategy to reduce the recovery cost[25].Wang Zhihui [26]intends to use dimethyl sulfoxide (DMSO) as the raw material of PHA, and conduct systematic and in-depth research on the raw materials (such as high purity, high molecular weight, thermoplasticity and thermal stability) required in the process of preparing PHA from PHA.

1.5.3 Control of fermentation parameters

By adjusting the process parameters, the yield and quality can be effectively improved. In this experiment, the optimal fermentation results were achieved by adjusting the temperature, time, pH and rotation speed.

As far as temperature is concerned, the main factor is fermentation temperature. It was found that suitable high temperature could enhance the activity of enzyme and

accelerate its metabolic process, thus increasing the yield of products. But at the same time, it should be noted that the temperature should not be too high, because too high temperature will not only inactivate the enzyme molecules, but also lead to the death of the production strain.

The fermentation time should be determined according to the growth rate of the strain and the characteristics of the molecules to be produced. If the molecules to be produced are secondary metabolites such as antibiotics, alcohols, vitamins, etc., it should not be too short, because the strain needs time to synthesize a large number of primary metabolites as the synthetic substrate of secondary metabolites. If the molecules to be produced are storage compounds, the time should not be too long. To prevent the strain from breaking down molecules that have been synthesized.

It is also important to control the pH. The pH has a great effect on the enzyme activity, and the optimum pH of each enzyme system is different. Therefore, it is necessary to find out the most suitable pH value to ensure the activity of the strain and the activity of its enzymes in vivo.

Speed control is also an important step. The stirring speed is properly adjusted, so that the fermentation liquid and the substrate are fully stirred, and the oxygen content in the fermentation liquid is higher, which is beneficial to the yield and purity of the fermentation product.

1.6 PHA Analysis Method

PHA molecules are composed of different numbers of monomers. After fermentation, simple molecular detection methods are needed to detect the molecular composition and quality of the products. Here, several simple methods will be introduced.

1.6.1 High performance liquid chromatography

HPLC is an analytical chemistry tool for the separation, identification, and quantification of liquid samples. Organic solvents with various polarities are used as the mobile phase, which is input into the stationary phase under the action of pressure, and the separation is carried out through the difference of the interaction of the components in the mobile phase. The individual elements in the sample are separated,

depending on the difference in forces between the static phase and the moving phase, and fed into the detector.

The main body comprises a high-pressure infusion pump, an ejector, a chromatographic column, a detector and a distillate collecting device. It has broad application prospects in medicine, biopharmaceuticals, environmental monitoring and other fields, and professional knowledge in food safety, chemical analysis and other fields. The method can be used for the qualitative and quantitative analysis of a single compound and a single component of a mixture. The method is characterized by high efficiency, rapidity and sensitivity. The instrument is suitable for different kinds of samples, including high temperature unstable materials and polymer materials. There are mainly liquid-solid phase adsorption chromatography, liquid-liquid partition chromatography, ion-pair chromatography, molecular exclusion chromatography and so on. When selecting different column types, the nature of the sample, the separation effect on the sample and the requirements for the determination should be considered comprehensively in order to achieve the best determination results. Generally speaking, HPLC is an extremely important analytical tool, which plays a key role in many scientific fields, especially in quality control and complex sample analysis. Because of its high efficiency and accuracy, HPLC has become one of the standard equipments in many laboratories.

1.6.2 Gas chromatography

Gas Chromatography (GC) is a chromatographic analysis technique using gas as the mobile phase. The basic principle is to use a vaporized sample driven by a carrier gas (usually an inert gas such as N2, He, Ar, or H2) through a chromatographic column containing a stationary phase. Because of the different interaction forces between different components in the sample and the stationary phase, the retention time of each component in the chromatographic column is also different, so as to achieve separation. These components then flow out of the column one by one and are detected and recorded by the attached detector to produce a chromatogram.

Gas chromatography has high separation efficiency and high sensitivity, which is especially suitable for the analysis of complex samples. It is especially suitable for

qualitative and quantitative analysis of volatile organic compounds. Nonvolatile liquids and solids can also be analyzed after pyrolysis. The stationary phase may be a solid adsorbent (gas-solid chromatography) or a liquid coated on a support (gas-liquid chromatography), which increases the flexibility and selectivity of the process.Gas chromatography can also be combined with other analytical techniques such as infrared absorption spectroscopy or mass spectrometry to improve the accuracy and reliability of analysis. Because that sample transfer speed in the gas phase is fast, the analysis speed is relatively fast, and the operation is simple and easy to master. In general, gas chromatography is a powerful and commonly used analytical technique, especially in the field of chemical analysis.

1.6.3 Infrared spectrum analysis

Infrared spectrum analysis is mainly based on the change of molecular vibration frequency to reflect the difference of compound structure. The change of vibration frequency is not only related to the functional groups inside the molecule, but also affected by external factors, such as temperature, concentration, solvent and so on.

Infrared spectroscopy has a wide range of applications. It can be used to analyze samples in various States, including liquids, powders, solids, and thin films. At the same time, it is suitable for many types of materials, such as inorganic, organic, polymer, protein and natural products.

Infrared spectroscopy is a basic and powerful tool in the field of chemical analysis, which can provide important information about molecular structure and chemical bonds, and is of great value for qualitative analysis and structural analysis.

Conclusions to chapter 1

At the end of the paper, the author summarizes the whole paper and points out some problems and defects in the study. Finally, the author puts forward some problems for further study. On this basis, combined with the previous work, the production and quality of PHAs were discussed.

In a word, by optimizing the process parameters of PHAs, the high yield and quality of PHAs can be achieved, which lays a theoretical and experimental basis for

the efficient synthesis of PHAs. The research results of this project will lay a foundation for the industrial application of PHAs, and provide new research ideas and means for the research in this direction.

CHAPTER 2

OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 The purpose of the study

The state has implemented a series of plastic restrictions and increased efforts to regulate the "white pollution" problem in response to the growing attention to environmental issues. In this context, PHA (polyhydroxyl fatty acid ester) is considered a promising new material that can replace plastic products, attracting significant attention and research from scholars at home and abroad. The physical properties of PHA molecules are similar to those of ordinary plastics, and materials with different lengths of monomer molecules can be tailored for specific tensile properties, hardness, and other characteristics. Despite the ability to produce PHA molecules through various microorganisms' metabolic pathways and corresponding materials in laboratory settings, challenges remain in making it suitable for large-scale production and practical application.

With the continuous progress of modern technologies such as bioengineering and genetic engineering, researchers are also exploring ways to improve the yield and quality of PHB through genetically engineered microorganisms. This is expected to provide a more economical and efficient method for the production of PHA, thus promoting the application and development of PHA in more fields.

Polyhydroxyl fatty acid ester (PHA) is a new biodegradable material with good biodegradability and biocompatibility. In addition, due to the completely non-toxic side effects, the recycling and disposal of PHA is also unique, and it can be recycled as animal feed. Traditional waste of old plastic is usually disposed of by landfill or incineration, resulting in land, air and other pollution problems. On the other hand, PHA can undergo many processes after processing, as a feed can produce economic benefits again, to achieve the double cycle of material and economy. Since 1959, there have been many industrialization and commercialization cases of PHA production process. However, due to the low efficiency and high cost of PHA synthesis and purification, its application and promotion in the market have been seriously affected. According to relevant reports, the ideal cost of PHA is about $4 per kilogram. But

even this price is more than double that of commonly used petroleum-based plastics. In addition to this, population growth has led to an increase in global demand for plastic products. Petroleum-based plastics (PBP) are still the dominant form of plastic products. However, the large oil consumption of plastic production will exacerbate the energy shortage. Various toxic and harmful gases emitted by the petrochemical industry also cause damage to the environment and human health. According to relevant statistics, the annual plastic recycling can only reach 13%-15% of the total waste plastic. Polyhydroxyalkanoate (PHAs), as a representative of bioplastics, is usually used as an energy storage substance in microbial cells. Producing 1 kg of PHA instead of PBP can reduce CO_2 emissions by 2 kg and save 30 terajoules of fossil energy. According to data, plastic waste accumulates in the natural environment at a rate of 2,500 tons worldwide.

In general, in addition to high-temperature decomposition, in nature, the photolysis and biodegradation rates of plastics are very slow, generally 200-400 years. In order to deal with this "white pollution", many countries have begun to implement the "reduce, reuse, reuse" three-R plan. However, 3R projects are still difficult to achieve in some places where regeneration is difficult or difficult, so many research units at home and abroad are conducting research and development of biodegradable materials.

In short, the development of biodegradable materials technology provides a new idea and method to solve the problem of "white pollution". With the deepening of research and the expansion of application fields, biodegradable materials such as PHA are expected to play a more important role in the future, contributing to the realization of sustainable development goals.

2.2 The method of the study

In this experiment, a single factor screening optimization experiment was used to optimize PHA fermentation conditions with *E. coli* JM109 as chassis engineering strain, cheap recovered glycerol as carbon source, and indoor shaking flask culture method.

The single factor test method has important application value in the optimization

of fermentation conditions. Single-factor testing means that only one variable factor is changed during the experiment, while the other factors are kept unchanged, so as to observe the influence of the variable on the experimental results. This method is simple, direct and easy to operate and analyze.

In the optimization of fermentation conditions, single factor test is often used to preliminarily screen the main factors affecting the fermentation process, such as temperature, pH value, nutrient concentration, etc., to lay the foundation for the subsequent multi-factor optimization experiment.

2.2.1 Single factor experimental procedure

1) Determine the influencing factors: Firstly, according to the characteristics of the fermentation process and previous research experience, the factors that may affect the fermentation effect are listed, such as temperature, pH value, dissolved oxygen, types and concentrations of nutrients, etc.

2) Design the experimental scheme: design a separate experiment for each factor, each experiment only changes the level of one factor, other conditions remain unchanged, by comparing the experimental results to determine the best range of levels of each factor.

3) Conduct experiments and collect data: Conduct experiments according to the design scheme, carefully record the results of each experiment, including the growth of bacteria, product concentration and other indicators.

4) Data analysis and optimization: Through the analysis of the collected data, the influence of each factor on the fermentation effect is found out, so as to determine the optimal combination of fermentation conditions.

2.2.3 Advantages and limitations of single factor test method

Advantages: simple operation, easy to implement; The influence of individual factors on the fermentation effect can be intuitively seen, providing direction for further optimization.

Limitations: The inability to consider the interaction between factors may lead to the neglect of some important combinations of influencing factors; When there are more factors, the number of experiments will increase significantly, and the efficiency

is low.

2.2.4 Improve the efficiency of single factor test method with modern technology

Utilizing high-throughput screening techniques: Combining synthetic biology and high-throughput screening techniques, a large amount of data on microbial properties can be quickly obtained, providing more candidates for single-factor tests.

Application of artificial intelligence technology: The use of artificial intelligence technology, such as machine learning and deep learning, to analyze a large number of experimental data can more accurately predict the impact of various factors on the fermentation effect and improve optimization efficiency.

In addition to the above, there are some additional considerations and considerations to consider:

In the single factor test, the consistency of experimental conditions should be ensured to avoid the deviation of experimental results due to changes in environmental factors.

Considering the difference between the laboratory scale and industrial production, the experiment scale should be gradually enlarged in the optimization process to verify the feasibility of the optimization conditions.

Pay attention to record the detailed procedure and results of the experiment, so as to facilitate the subsequent analysis and repetition of the experiment.

Conclusions to chapter 2

Because of its simple and intuitive characteristics, the single factor test method occupies a place in the optimization of fermentation conditions. However, with the development of science and technology, especially the application of synthetic biology and artificial intelligence technology, the traditional single factor test method is also facing challenges and opportunities. By combining modern technology, the efficiency and accuracy of single factor test method can be effectively improved, and the optimization process of fermentation conditions can be accelerated. Therefore, researchers in related fields should continue to explore and try new technical methods

in order to achieve more efficient and accurate fermentation conditions optimization and promote the development of the bio-manufacturing industry. This study has an in-depth understanding of the key factors found in PHA fermentation production, and has guidance and reference significance for the further transformation of microbial strains, the optimization of fermentation process and the expansion of future application fields of PHA, such as medical materials and packaging materials.

CHAPTER 3
EXPERIMENTAL PART

3.1 Experimental materials

3.1.1 Strains

The *E. coli JM109* was preserved by our laboratory, and the plasmid pTBBC was provided by our laboratory.

3.1.2 Main instruments

Table 2.1 – **Main instruments and manufacturers**

Instrument name	Manufacturer
Electronic Balance	Mettler Group, Switzerland
Biochemical constant temperature incubator Vertical electrothermal pressure steam sterilizer LDZX-50KB	Shandong Brocade Stem Cell Application Research Institute Co., Ltd. Shanghai Shenan Medical Instrument Factory
Medical clean bench BBS-SDC	Shandong Brocade Stem Cell Application Research Institute Co., Ltd.
Ultra-pure water device	Beijing Puxi General Instrument Co., Ltd.
Shaker HZ9310K	Taicang Science and Education Equipment Factory
Freeze dryer High-speed refrigerated centrifuge 5804R	Shanghai Bilang Instrument Manufacturing Co., Ltd. Eppendorf Germany
Electric heating constant temperature incubator	Shanghai Yiheng Scientific Instrument Co., Ltd.
Ultraviolet-visible spectrophotometer	Beijing Puxi General Co., Ltd.
Lectric heat constant-temperature blast drying box	Shanghai Jinghong Test Equipment Co., Ltd.
Digital PH meter GC gas chromatograph	Shanghai Precision Scientific Instrument Co., Ltd. Agilent Corporation

3.1.3 Main reagents and manufacturers

Table 2.2 **-Main Reagents and Manufacturers**

Name of reagent or medium	Manufacturer
Trypsin Chen	Thermo Fisher Scientific Limited
Yeast extract	Thermo Fisher Scientific Limited
NaCl	Sinopharm Chemical Reagent Co., Ltd.
Deionized water	Beijing Aoboxing Biotechnology Co., Ltd.
NaOH	Beijing Aoboxing Biotechnology Co., Ltd.
Ampicillin Kanamycin	Merck-Sigma China Merck-Sigma China
Distilled water	Beijing Aoboxing Biotechnology Co., Ltd.

3.2 Experimental process

3.2.1 Preparation of fermentation medium and strain

Experimental materials and pretreatment are very important parts in the optimization of fermentation conditions for the production of polyhydroxyalkanoates. Effective pretreatment and selection of appropriate experimental materials can lay the foundation for the smooth development of subsequent experiments.

(1) Preparation of fermentation strain

The prepared pTBBC plasmid was introduced into the competent cells of *E. Coli* JM109, and the recombinant *E. Coli* after transformation and colony PCR verification was stored in a refrigerator at -4 ℃.

(2) preparing a fermentation culture medium:

In this experiment, LB medium with glycerol as the substrate was used to culture the expanded strains.

Glycerol was determined as the main carbon source for the production of strains, and the optimal composition of the medium was determined according to its growth conditions and PHA production. After adding carbon source and nitrogen source, some trace elements, such as Fe, Zn, S, etc., can be added appropriately to improve the fermentation effect.

The LB medium was composed of 10 G/L peptone, 5 G/L yeast, 10 G/L NaCl, and sterilized at 121 ℃ for 20 min.

The culture medium was composed of glycerol 10 G/L, (NH4) 2SO4 7. 5 G/L, K2HPO4 · 3H2O 3 G/L, KH2PO4 2 G/L, MgSO4 · 7H2O 1 G/L, sodium citrate 1. 0 G/L and yeast extract 7 G/L.

(3) activation of fermentation strain seed

Bacteria that were removed and stored in cold storage at -4 ° C or stored at -70-80 ° C,

Melt away.

A line was drawn on the corresponding LB solid medium, and the bacteria were inoculated on the plate medium.

Place the culture substrate upside down in an incubator at 37 ° C and incubate overnight.

Incubate on LB plate for one night and observe whether there is bacterial growth.

A sterile strip or sterile needle is used to select from a single bacterium.

The selected colonies were inoculated with 50 ml of fermentation medium in 300 ml shake flasks.

Place overnight at 37 ° C in a constant temperature shaker box at 250 rpm. Through the above steps, the stored strain can be successfully activated, and a single *E. coli* colony can be obtained, which is then inoculated into the fermentation medium for fermentation.

3.2.2 Detection of cell dry weight

(1) Take 20 ml of fermented culture solution and put it into a 50 ml centrifuge tube.

(2) Set the centrifuge speed to 10000 R/min and centrifuge for 5 min. The precipitated thalli were washed once with absolute ethanol and then washed three times with distilled water.

And (3) put that thalli into an oven at the temperature of 60 deg C for drying until the quality is constant, and weigh the thalli precisely after drying to obtain the dry weight of the cells.

3.2.3 Experimental detection and analysis

(1) To prepare the sample to be tested, 40 ~ 50 mg of stem cells were placed in an esterification test tube, and 2 ml of esterification solution (containing 1 G/L benzoic acid, 3% volume ratio of concentrated sulfuric acid) and 2 ml of chloroform were added, and then it was sealed and esterified in water at 100 ℃ for 4 H. At the end of the esterification, place it at room temperature, add 1 ml of di water, mix, and leave it for a certain period of time to form a layer. The organic phase was aspirated and determined by GC.

(2) Preparation of Standards (containing different PHA monomer components) Accurately weigh, place in an esterification tube, add 2 ml of esterification solution and 2 ml of chloroform, and seal. The esterification reaction was carried out in a water bath at 100 ℃ for 4 H. At the end of the esterification, place it at room temperature, add 1 ml of di water to make it uniform, and leave it for a certain period of time to form a layer. The organic phase was aspirated and determined by GC.

(3) Gas chromatography (GC) was performed using Scion 456 GC. Rxi-1 HT (30 m × 0.32 mm × 0.25 μm, Restek, USA). Rxi-1HT (30 m × 0.32 mm × 0.2 μm, Restek, USA), FID detector 250 ℃, carrier gas N2, split ratio 10:1, injection volume 1 μL. Hold at 80 ℃ for 1.5 min, then increase to 140 ℃ at 30 ℃/min, and then increase to 240 ℃ at 40 ℃/min for 4 min.

3.2.4 Effect of Different Temperatures on PHA Production

For the study of fermentation temperature, we selected five levels of 33 ℃, 34 ℃, 35 ℃, 36 ℃ and 37 ℃. The control time was 48 H, the pH was 7.0, and the rotation speed was 250 rpm. PHA production data were obtained by fermenting the fermentation medium at different temperatures. The results showed that the PHA production was higher at 36 ℃ and 37 ℃, reaching 3. 38 G/L and 3. 45 G/L, respectively.

Table 2.3-**Data of temperature influence on experiment**

Temperature	Cell dry weight (G/L)	PHA yield (G/L)
33	4.25	2.63

34	4.95	2.99
35	5.75	3.33
36	6.33	3.38
37	6.83	3.45

The data are shown in Figure 2.1.

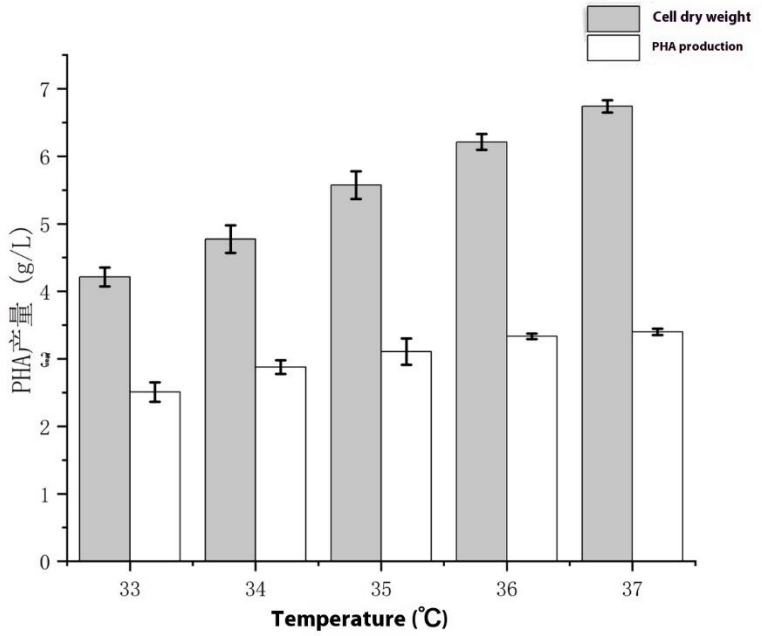

Figure 2.1 –Histogram of Influence of Temperature on PHA Production

Therefore, we can draw a preliminary conclusion that PHA production is the highest at the fermentation temperature of about 37 ℃.

3.2.5 Effect of different time on PHA production

In the study of fermentation time, five levels of 24 H, 36 H, 48 H, 60 H and 72 H were selected. The temperature was controlled at 35-37 ℃, pH was 7.0, and the rotation speed was 250 rpm. Record PHA production data. The results showed that when the pH value was 48 H and 60 H, the PHA production capacity was higher, reaching 3.32 G/L and 2.45 G/L, respectively. However, the capacity of PHA production decreased to 2.31 G/L at 72 H.

Table 2.4- **Data of Time Effect on Experiment**

Time (H)	Cell dry weight (G/L)	PHA yield (G/L)
24	3.56	2.12
36	3.77	2.35
48	5.67	3.32
60	4.43	2.45
72	4.12	2.31

The data are shown in Figure 2-2

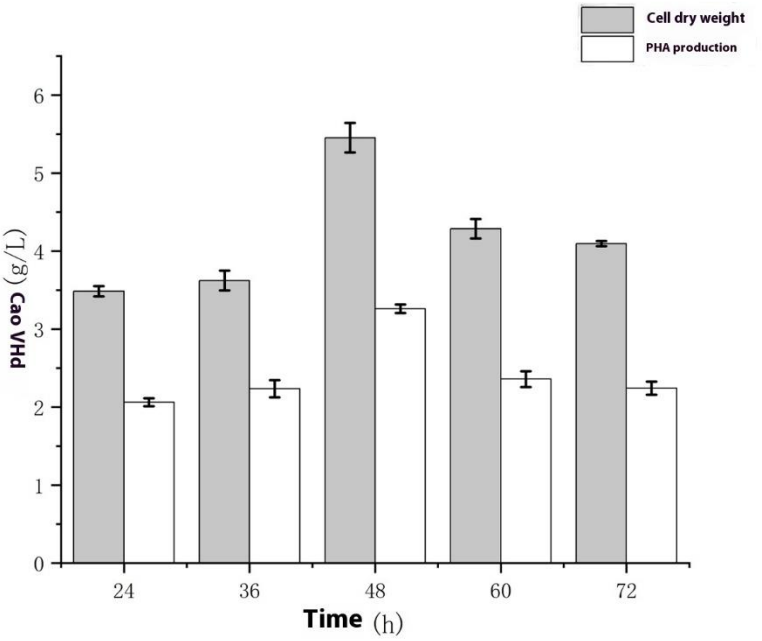

Figure 2.2 –Histogram of Influence of Time on PHA Production

Therefore, we can draw a preliminary conclusion that the strain has the highest ability to produce PHA within 48 H of fermentation.

3.2.6 Effect of different pH values on PHA production

For the study of fermentation pH, the five levels of 6.0, 6.5, 7.0, 7.5 and 8.0 were selected, the temperature was controlled in the range of 35-37 ℃, the time was 48 H, the rotation speed was 250 rpm, and the data of PHA production was recorded. The results showed that when the pH value was 7.0, the PHA production capacity was

high, reaching 3.82 G/L. When the pH value was 8.0, the PHA production capacity decreased significantly, only 2.65 G/L.

Table 2.5- **Data of PH influence on experiment**

pH	Cell dry weight (G/L)	PHA yield (G/L)
6.0	3.82	2.13
6.5	4.56	2.94
7.0	6.88	3.82
7.5	5.45	3.46
8.0	5.25	2.65

The obtain data is plotted as a histogram as shown in figure 2.3.

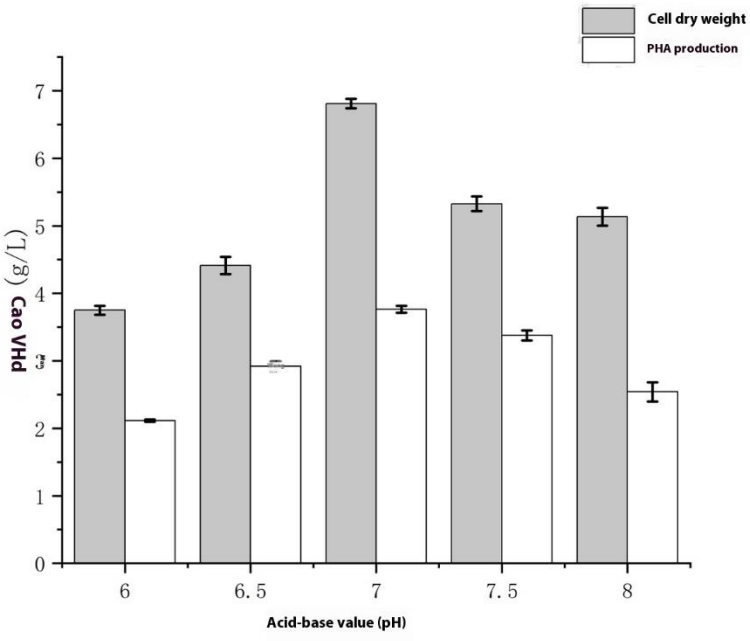

Figure 2.3 –Histogram of influence of pH on PHA production

Therefore, we can draw a preliminary conclusion that the pH value of fermentation has a significant positive effect on the ability of PHA production when the pH value is about 7.0.

3.2.7 Effect of different rotation speeds on PHA production

According to the research on the influence of the rotation speed on the yield, four levels of 100 rpm, 150 rpm, 200 rpm and 250 rpm are selected, the temperature is controlled within the range of 35-37 deg C, the time is 48 hours, the pH value is 7.0, the fermentation medium is placed at the corresponding rotation speed for fermentation, and the data is recorded. The results showed that the PHA production capacity at 200 rpm was significantly higher than that at other conditions.

Table 2.6- **Data of influence of rotating speed on experiment**

Speed (rpm)	Cell dry weight (G/L)	PHA yield (G/L)
100	4.12	2.32
150	7.31	3.24
200	7.88	4.11
250	6.59	3.21

The data are shown in Figure 2.4.

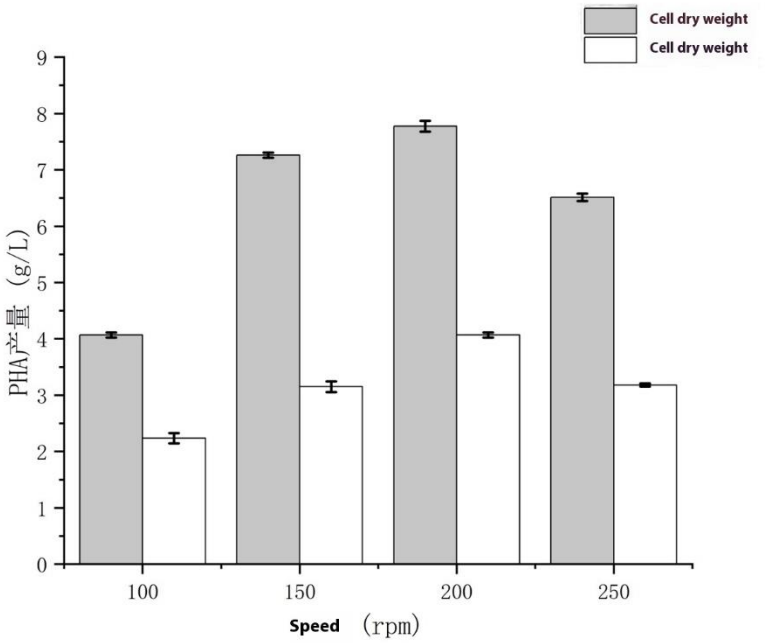

Figure 2.4 –Histogram of influence of rotation speed on PHA yield

Therefore, we can draw a preliminary conclusion that the strain has the strongest ability to produce PHA when the rotation speed is 200 rpm.

Conclusions to chapter 3

1. For the study of fermentation temperature, we selected five levels of 33 ℃, 34 ℃, 35 ℃, 36 ℃ and 37 ℃. The control time was 48 H, the pH was 7.0, and the rotation speed was 250 rpm. PHA production data were obtained by fermenting the fermentation medium at different temperatures. The results showed that the PHA production was higher at 36℃ and 37℃, reaching 3. 38 G/L and 3.45 G/L, respectively.

2. In the study of fermentation time, five levels of 24 H, 36 H, 48 H, 60 H and 72 H were selected. The temperature was controlled at 35-37℃, pH was 7.0, and the rotation speed was 250 rpm. Record PHA production data. The results showed that when the pH value was 48 H and 60 H, the PHA production capacity was higher, reaching 3.32 G/L and 2.45 G/L, respectively. However, the capacity of PHA production decreased to 2.31 G/L at 72 H.

3. For the study of fermentation pH, the five levels of 6. 0, 6. 5, 7. 0, 7. 5 and 8. 0 were selected, the temperature was controlled in the range of 35-37 ℃, the time was 48 H, the rotation speed was 250 rpm, and the data of PHA production was recorded. The results showed that when the pH value was 7. 0, the PHA production capacity was high, reaching 3. 82 G/L. When the pH value was 8. 0, the PHA production capacity decreased significantly, only 2. 65 G/L.

4. According to the research on the influence of the rotation speed on the yield, four levels of 100 rpm, 150 rpm, 200 rpm and 250 rpm are selected, the temperature is controlled within the range of 35-37 deg C, the time is 48 hours, the pH value is 7.0, the fermentation medium is placed at the corresponding rotation speed for fermentation, and the data is recorded. The results showed that the PHA production capacity at 200 rpm was significantly higher than that at other conditions.

CONCLUSIONS

In the optimization of fermentation conditions for the production of polyhydroxyalkanoates, the effects of different parameters on the yield and quality of the product were systematically studied, and the optimal conditions were determined through a series of experiments. First of all, we measured the product yield under different fermentation time and found that the fermentation time had a significant impact on the product yield, and the optimal fermentation time was 48 H. Secondly, in terms of fermentation temperature, we found that the fermentation effect at 30 ℃ was poor and the product yield was low, while the fermentation effect at 37 ℃ was good and the product yield was the highest. Therefore, the optimal fermentation temperature was 37 ℃. The study on the optimum fermentation pH showed that the yield of the product was the highest when the fermentation pH was 7. 0. In terms of the stirrer speed setting, we found that the product yield was the highest when the speed was 200 rpm.

Fermentation time, temperature, pH value and stirring speed were optimized to increase the yield of PHA. Fermentation time 48 H, temperature 37 ℃, pH 7. 0, and rotation speed 200 rpm were determined. By optimizing the reaction, the yield and quality of the product can be obviously improved.

In this experiment, the increase of PHA production was achieved by shake flask fermentation. However, it can not be really applied to large-scale production, and there are still many areas that can be improved in the experiment. We can further optimize the fermentation conditions. In this study, although we optimized the fermentation conditions under specific temperature, time, pH and stirrer speed, we can also try to optimize more conditions, such as oxygen content, substrate concentration and so on.

Secondly, we did not try different fermentation strains. In this study, we selected a known fermentation strain to synthesize polyhydroxyalkanoates. However, there are many other microorganisms that may be more productive.

In this study, we mainly discussed the application potential of polyhydroxyalkanoates in a certain field. However, polyhydroxyalkanoates may also

have a wide range of applications in other fields. We can further study the physical and chemical properties of polyhydroxyalkanoates to determine whether they can play an important role in other fields. By optimizing the fermentation conditions, exploring different fermentation strains and expanding the application scope of polyhydroxy esters, we can further improve the synthesis efficiency and application value of polyhydroxy esters, and make important contributions to the development of related industries.

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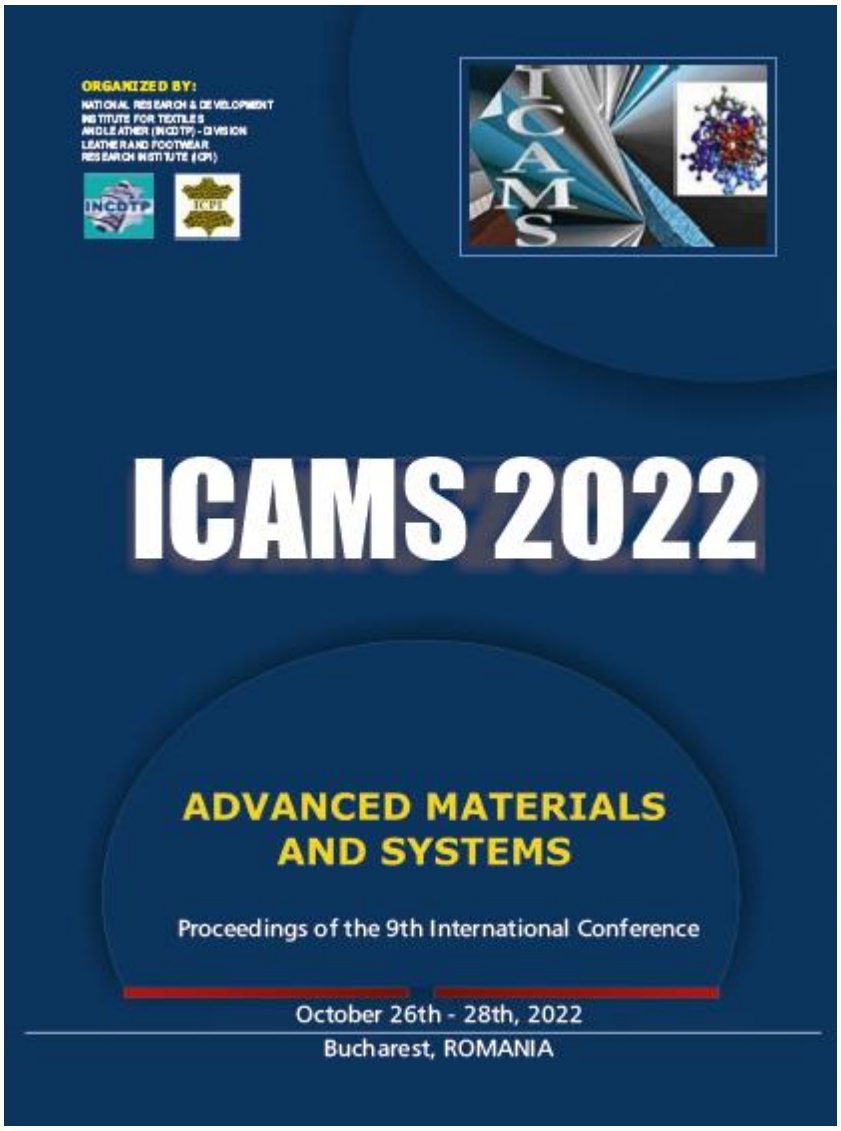
APPENDIX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NANOPIGMENTS FOR LEATHER FINISHING COATINGS

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The work is focused on obtaining nanopigments by adsorption of anionic dyes on positively charged montmorillonite. The effect of sequential modification of aqueous dispersions of montmorillonite with cationic and anionic compounds on the structural and charge characteristics of mineral dispersions was studied. The effect of chemical dispersion of agglomerates of aqueous montmorillonite dispersions after adding carbonate solutions was shown. The treatment of dispersions of original montmorillonite with sodium carbonate provides maximum dispersion of mineral aggregates by penetrating into the interstructural space of aluminosilicate packets, moving them apart and separating them. It was found that the modification of montmorillonite dispersed by sodium carbonate by adding basic chromium sulfate is accompanied by a change in the surface chemistry of the mineral and structural transformations. Structural changes are manifested by the formation of a developed structure of cationic montmorillonite. The cationic surface charge of montmorillonite and high specific surface of montmorillonite are important factors for ensuring effective adsorption of anionic dyes on the surface of the mineral. The efficiency of adsorption of anionic dyes on cationic montmorillonite is investigated. It was shown that the adsorption of dyes dependsal on the pH of the medium. The scheme of obtaining nanopigments, which were characterized by good covering power, saturated and intense colour was proposed.

Keywords: montmorillonite, pigment, leather finishing coating

INTRODUCTION

Traditional leather finishing involves applying a covering composition to the surface of leather. The finishing coating provides protection of leather from external atmospheric and mechanical impacts (Covington, 2009).

The type of leather coating depends on the content of pigments and can be (Covington, 2017; Zhuravsky *et al.*, 1996; Kasyan, 2019): aniline – a transparent coating without the use of pigments; semi-aniline – characterized by a small content of pigments to provide, mainly, a shade of color; and pigmented – with a significant content of pigments for complete coverage of the surface of leather with a colored covering layer.

Pigments provide color and covering power to the finishing coating (Winter *et al.*, 2017). Organic or inorganic pigments are used in the finishing coating of leathers. Covering compositions with organic pigments provide leather with shine, bright and intense color, but have low light fastness and heat resistance. Inorganic pigments create a high-quality coating with good light fastness and water resistance, but are characterized by a high tendency to sedimentation and are limited in color and brightness (Winter *et al.*, 2017; Osgood, 1990).

The ability of the coating to form a uniform coating stable composition with required thickness depends on the properties of the pigment, the origin of their surface, and the size of the particles.

The use of nanopigments provide improved physical and mechanical indexes of the leather finishing coating (Bondaryeva and Mokrousova, 2020; Bondaryeva *et al.*, 2021).

The aim of the work was to describe the scientific basis of patterns of anionic dyes adsorption on positively charged montmorillonite to obtain nanopigments for leather finishing coatings.

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EXPERIMENTAL

Materials

Bentonite clay from the Cherkassky deposit (Ukraine), after thorough purification and washing was used as a basis for obtaining nanopigments. The main mineral was montmorillonite, the content was 85±3 %. The value of the exchange capacity was 72 mg-eq/100 g of clay. Humidity – 27 ± 3 %.

The sodium carbonate, basic chromium sulfate (III) and anionic dyes were used to modify dispersions of montmorillonite.

Methods

The nanopigments were obtained by sequential treatment of aqueous montmorillonite dispersions (100 g/l) with sodium carbonate, basic chromium sulfate and anionic dyes.

Firstly, 6,0% of sodium carbonate from weight of dry montmorillonite was used, and then the cationic form of montmorillonite was obtained by modifying the dispersion of Na^+-montmorillonite with chromium compound. For this purpose, the basic chromium sulfate was used – $Cr_2(SO_4)_x(OH)_{6-2x}$, chromium oxide (III) content was 25.6 %. A solution of basic chromium sulfate in the amount of 10.0% Cr_2O_3 (by weight of the montmorillonite) was added to the dispersion of Na^+-montmorillonite (MMT–Na^+). Mixing was continued until a homogeneous mass of gray colour was obtained. The pH value of the modified dispersion of cationic montmorillonite (MMT-Cr^{3+}) was 4.5-5.2.

The nanopigments were prepared by gradually mixing the cationic form of montmorillonite with the anionic dyes. Mixing was performed using a mechanical mixer (30–40 min, 40–45°C) to obtain time-stable dispersions in the form of nanopigments of saturated deep colour. The consumption of anionic dyes in a ratio of 1:1 according to the mineral component. The nanopigments were obtained as the colored modified dispersions of montmorillonite.

A laser-correlation spectrometer "ZetaSizer-3" (Malvern Instrument, USA) with a Multi Computing Correlator type 7032 CE was used to study the dispersion of mineral systems.

The adsorption of dyes from aqueous solutions on the cationic form of montmorillonite was determined by measuring the light transmittance of dye solutions of different concentrations.

The electrokinetic potential was determined by microelectrophoresis.

RESULTS AND DISCUSSION

In montmorillonite modification, molecules of polar liquids (for example, sodium carbonate) can freely penetrate into the interpackets space of montmorillonite, push them apart and increase the distance between packets. As a result, montmorillonite particles disperse spontaneously in water, their number per unit volume increases significantly, and the number of direct contacts for further interactions increases.

It is shown that treatment of dispersions of native montmorillonite with sodium carbonate provides maximum dispersion of mineral aggregates by penetrating into the interstructural space of aluminosilicate packets, moving them apart and separating them.

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