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 **Screening, identification, and fermentation optimization of endophytic bacteria in the root system of IAA producing corn**

First (Bachelor's) level of higher education

Specialty 162 "Biotechnology and Bioengineering"

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Completed: student of group BEBT-20
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APPROVE
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Doctor of Technical Science
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ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Jinshuo Wei

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Scientific supervisor Olga Iungin, Ph.D., Assoc. Prof.

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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	
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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 _____ Jinshuo WEI

Scientific supervisor _____Olga IUNGIN

SUMMARY

Jinshuo Wei. Screening, identification, and fermentation optimization of endophytic bacteria in the root system of IAA producing corn – Manuscript.

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Corn is one of the grain crops with the longest history of cultivation in the world, and China, as a large agricultural country, has seen rapid development of the corn planting industry, with the increased production of corn accounting for about 50 per cent of the growth in grain production. As people's demand for corn increases year by year, people use a lot of chemical fertilizers, resulting in environmental pollution, the fertility of the soil decreases, the nutritional value of corn decreases and other problems, microbial fertilizers made of PGPR is an important way to solve the above problems. In this experiment, we screened out the high IAA-producing strains from the inter-root soil of maize and identified their species, and then selected the strains with the highest IAA production to optimise the fermentation conditions, and the main results were as follows: 15 strains of IAA-promoting bacteria in the maize rhizosphere soil were screened to obtain the strains with the high capacity of IAA production, and a total of 5 strains of bacteria were obtained with the strong capacity of IAA production, of which Chang 7-6 was one of them secreting IAA. Among them, Chang 7-6 was the strain with the strongest ability to secrete IAA, with an IAA production of 30.06 mg/L at 24 h. The biological identification of the five strains was carried out through morphological observation, physiological and biochemical tests, and homology analysis of 16S rDNA gene sequences, etc., and it was determined that Chang 7-6 belonged to the Bacillus mobilis, and Chang 7-4 belonged to the Bacillusmobilis. Strain Chang 7-4 belongs to Bacillusaltitudinis, strains 6WC-2 and ZH58-6 have the highest similarity to Bacillusp. and strain 6WC-3 belongs to Bacillusstratosphericus. Finally, strain Chang 7-6 was used to optimise the fermentation conditions for its IAA-producing ability through one-way test and

response surface analysis. The results showed that the theoretical fermentation conditions for strain Chang 7-6 to produce the largest IAA concentration were: tryptophan concentration 0.22 g/L, pH 8.4, temperature 30.8 ℃ and time 24.2h, and its IAA yield could reach 40.2 mg/L. Validation experiments were carried out under the above experimental conditions, and the IAA yield of 38.891±0.056 mg/L was gained, which differed from the theoretically optimised value by 3.27%, and the error was within the reliable range, proving that the parameters were stable and reliable.

Keywords: Maize inter-root soils; Phytophthora infestans; Screening; Identification; Optimisation of fermentation conditions

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INTRODUCTION

Maize is one of the oldest food crops in the world, ranking third after wheat and rice in terms of area under cultivation. According to the survey, the global demand for corn is growing every year. China as a large agricultural country, corn planting industry is developing rapidly, the increase in corn production accounted for about 50 per cent of the growth in food production. In order to increase corn production and meet people's demand for corn, in the process of corn planting and growth, people use a lot of chemical fertilizers, although it can improve the crop yield and get the short-term benefits such as prevention of crop diseases, but in the long run, not only caused by soil compaction, production costs, soil fertility decline outside and other issues, but also caused a decline in the nutritional value of the crop, food safety risks increase. Safety risks increase. At present, the urgent problem facing China's agriculture is to repair the land damaged and polluted by long-term irregular application of chemical fertilizers, and the microbial fungicide made of PGPR is the key to solve the above problems. A large number of studies have shown that PGPR can promote plant nutrient uptake, improve the structure of soil microbial communities, and enhance the vitality of plant inter-root soil. In order to increase maize yield safely and effectively, research and development of green and safe biological agents and microbial fertilizers are the most effective methods at present. In this study, we screened PGPR strains with strong IAA-producing ability from maize inter-root soil, identified their species through identification, and optimized the IAA-producing conditions of the strains through single-factor and response surface analyses in order to obtain the fermentation conditions that maximize the yield of IAA, which provides a theoretical and practical basis for the research and development of high-quality microbial fertilizers.

Object of study – strains 4CV-1, 4CV-2, 4CV-3, Chang 7-3, Chang 7-4, Chang 7-6, Chang 7-7, ZH58-1, ZH58-3, ZH58-4, ZH58-6, 6WC-2, 6WC-3, 6WC-6,

6WC-8 were isolated and preserved by the Microbiology Laboratory of the Qilu University of Technology (Shandong Academy of Sciences, Shandong Province).

Subject of study – screening of IAA-producing strains, identification of strains, determination of IAA-producing capacity and optimization of culture conditions to improve IAA production.

Purpose of the study. As a large agricultural country, China has a rapidly growing maize industry, with maize accounting for 50 per cent of total grain production. Due to the excessive application of chemical fertilizers and pesticides in agriculture in China, not only does it cause problems such as lower agricultural yields, soil crusting and higher costs, but it also pollutes the soil, water and the environment. High-quality microbial agents can be a good solution to the above problems. In this study, we identified IAA-producing bacteria by screening them from the inter-root soil of maize, and improved the IAA production by optimizing the cultivation conditions and medium composition. The screening of high-yielding IAA strains can provide excellent strains for the development of microbial agents with high-yielding IAA function, which can promote the development of agriculture.

CHAPTER 1

LITERATURE REVIEW

1.1 Plant inter-root biotrophic bacteria and major species

Inter-root biotrophic bacteria refer to the beneficial effects of living in the inter-root zone of plants, which can promote plant growth, improve the physical and chemical properties of the soil, and protect plants from abiotic and biotic stresses. They can directly promote plant growth and biomass and enhance seed germination through nitrogen fixation, phosphorus solubilisation and potassium solubilisation, production of phytohormones, and indirectly promote plant growth through antagonism with pathogenic bacteria and competition with them for binding sites [1]. PGPR are very commonly distributed in nature, and most of the microorganisms are capable of producing IAA, and to a certain extent, the species of PGPR vary depending on the type of plant, of which Most of the plant inter-root-promoting bacteria belong to the bacterial group. Upwards of 20 genera of PGPR strains have been isolated and identified [2]. The common PGPR species are Acetobacter, Fusobacterium, Alkaloid-producing Bacillus, Arthrobacter, Nitrogen-fixing Spirochetes, Bacillus, Bayerinckerella, Burkholderia, Dexteria, Pseudomonas, Rhizobium, etc., and these strains are originated from many types of plants, such as food crops, cash crops, medicinal plants, and so on. Inter-root microorganisms provide a good microenvironment for plant growth and development, which can maintain the ecological balance of the root system and is important for plant growth [3].

1.2 Facilitation mechanisms of plant inter-root biotrophs

Bacteria that promote plant root growth are mainly soil-derived, they are found in higher densities in the plant root system, in decreasing order from roots to stems

and leaves, and they live in symbiosis with the plant root system for most of the plant life cycle. Endophytes can promote plant growth in both direct and indirect ways [4].

① Direct growth promotion:

Certain PGPR act as nitrogen-fixing bacteria that convert unavailable nitrogen into available ammonia, thus providing a source of nitrogen for plants. Nitrogen exists in nature mainly in the form of nitrogen gas in the atmosphere, and nitrogen is an essential nutrient element for plant growth, but atmospheric nitrogen cannot be directly utilized for plants. Through the catalysis of nitrogen-fixing enzymes, nitrogen is converted into absorbable nitrogen compounds such as ammonia or nitrite, which provides a source of nitrogen for plants. This process not only increases the nitrogen content in the soil and improves the fertility of the soil, but also promotes the growth and development of plants and improves crop yields. Wang Huiqiao, Chen Weifeng and others showed that treating the soil with autochthonous nitrogen-fixing bacteria can effectively increase the effective nitrogen content in it, which in turn can effectively increase the yield of corn [5].

Some PGPR can produce phytohormones such as growth hormone and ACC deaminase, which can help plant growth and metabolism, thus directly achieving the purpose of promoting plant growth. Growth hormone i.e. IAA is an essential hormone in the plant growth process, it is the most common natural plant hormone and the first hormone discovered that can promote plant growth. IAA is involved in almost all the cellular processes in the plant body and influences the development of the plant, IAA can affect the division and expansion of plant cells, control the development of the root system and the formation of structures. Low concentrations of IAA can promote the elongation of primary roots, while high concentrations of IAA inhibit the growth of primary roots, but can promote the formation of lateral roots and the increase of root hairs, and the presence of IAA can also increase the area and length of the roots, helping the plant to better utilise the soil nutrients [6]. Li Fuyan et al. showed that Bacillus gorillaensis YC9L and YC3172 and Bacillus chestnutii YC506, which have efficient IAA secretion, have good root promotion

effects on maize and lettuce [7]. Certain PGPR can produce ACC deaminase, which synthesises ethylene under the action of ACC deaminase and breaks down the precursor ACC into ammonia and α-butyronic acid, which are the main carbon and nitrogen sources for microbial growth and reproduction, and at the same time reduces the accumulation of ACC, which effectively reduces the ethylene content in the plant and promotes the elongation of the plant roots, as well as promoting the microorganisms' own growth and Han et al. showed that Pyrrolobacter burkholderia P10, which has the ability to produce ACC deaminase, can effectively promote plant growth through the production of IAA, iron carriers and so on [8].

Certain PGPR have the ability to solubilise phosphorus. Phosphorus is a necessary element for plant growth and development, plant photosynthesis, cellular respiration, cell division and other biochemical reaction processes are closely related to phosphorus, up to 95% of the phosphorus in the soil is insoluble phosphate form of ineffective phosphorus, can not be directly absorbed and used by the plant, phosphorus-solubilising bacteria are able to decompose the insoluble phosphorus in the soil, and convert it into soluble phosphorus-containing compounds that can be absorbed by the plant. It can help plants to absorb phosphorus effectively. Li Sisi et al. showed that the phosphorus solubilising bacteria can effectively increase the soil quick-acting phosphorus content and promote the growth of maize seedlings [9]. In addition, PGPR also have the ability to alleviate soil mineralisation pollution, promote plant root development, enhance plant disease resistance and improve crop yield, which has good application value and ecological benefits [10-11].

Some PGPRs have potassium-solubilising effects. Potassium is one of the plant growth regulators, which is involved in cell division and nutrient production, can improve the photosynthetic efficiency of leaves, regulate the water balance inside and outside plant cells, maintain stable osmotic properties, and interact with nitrogen to affect protein synthesis and utilization, in addition to regulating the movement of leaf stomata to help plants adapt to different environmental conditions. Potassium absorption and utilization is directly related to plant growth and development, but the

amount of potassium in the soil that can be directly utilized by plants is very limited. Potassium solubilising bacteria can effectively increase the amount of potassium in the soil that can be directly used by plants, and the potassium solubilising mechanism of potassium solubilising bacteria mainly includes acidolysis, production of extracellular polysaccharides, and enzymatic solubilisation, etc. [12]. Acidolysis is mainly the potassium solubilising process of B. solubilis can produce certain acidic products, which can reduce the pH value of the surrounding environment and thus achieve the purpose of increasing the solubility of potassium ions [13]. The extracellular polysaccharides produced by the depotassium bacteria in the process of metabolism form a complex with potassium minerals, and the complex has the function of strongly adsorbing organic acids and inorganic ions through the action of ionic bonding, hydrogen bonding and covalent bonding, which leads to changes in its microregion, thus releasing potassium ions from minerals. The main component of enzymatic action is that some potassium-solubilising bacteria can secrete specific potassium-solubilising enzymes that affect the dynamic balance of mineral dissolution and crystallisation, thus promoting the release of potassium ions [14].

Certain PGPR can synthesise ferrophiles to promote iron uptake in plants, which is beneficial to plant growth and development [15]. Iron exists in nature mainly in the form of trivalent iron, and its utilization by plants is extremely low. Ferrophiles are low molecular weight organic compounds capable of binding trivalent iron in the soil environment, which can improve the solubility and effectiveness of iron. Microorganisms synthesize and secrete ferritin to chelate iron in the soil to improve its utilization, which not only meets their own metabolism, but also helps to alleviate iron deficiency in plants, and promotes the increase of their biomass [16].

② Indirect growth stimulation

Indirect stimulation of plant growth is mainly related to biological control, PGPR can stimulate plant growth by shaping the plant microbial community through the secretion of certain biologically active molecules, such as antibiotics, hydrolytic enzymes, nutrient-limiting enzymes and so on [17]. In addition, PGPR can protect

plants from pathogens by competing for pathogen nutrients and living space, thus effectively restraining the number of pathogens. Zhang Yiling et al. found that the marine bacterium Hao 2018 indirectly helped plant growth by increasing the pH and activity of several enzymes and regulating the composition of the microbiome in rhizobial soils, reducing the uptake of lead and Bacillus Pb available in the soil [18].

PGPR protects plants from pathogens or initiates plant response mechanisms to stress by stimulating plant defense mechanisms. Plants will encounter various abiotic stresses such as cold and drought during the life process, and when plants are subjected to certain external stimuli, some studies have proved that PGPR will help plants to produce a series of stress responses to cope with external abiotic stresses as well as self-repairing, which is the plant's stress response to external stimuli. For example, Zhang Jing et al. isolated the soil-dwelling Echinococcus DYM7 and mouth protruding umbilical helminth DYM11 from the root system of maize, which had a significant effect on the germination of maize seeds under the stress of 0.15 mol/L NaCl, with a germination rate of 90% [19]. Habib et al. showed that after inoculation with PGPR under stress, the germination rate, growth parameters, and chlorophyll contents of okra plants were all increased [20]. Ansari et al. showed that the growth attributes, photosynthetic pigment efficiency and other physiological attributes of wheat plants inoculated with nitrogen producing Pseudomonas aeruginosa FAP5 incubated under drought stress were found to be significantly improved, which effectively alleviated the abiotic stress pressure on wheat plants. The above studies indicate that the rational application of PGPR is an effective way to alleviate abiotic stress in plants [21].

1.3 Current status of the application of inter-root biotrophic bacteria in plants

Inter-root Probiotics (PGPR) are widely used, and PGPR are well known for their ability to contribute to sustainable agriculture. Diseases are very common in crops and are an important cause of reduced crop yields, and PGPR can be used as a

substitute for pesticides to protect plants from a wide range of pathogens. In recent years, there have been many reports on the application of antagonistic bacteria to soil-borne diseases both at home and abroad, and a variety of PGPR have been used to inhibit or control diseases in plants, for example, *Pseudomonas fluorescens* screened by Saravanan et al. could well inhibit banana wilt [22]. In addition, there are many researches on the application of PGPR in growth promotion. PGPR can effectively increase the activities of catalase, sucrase and urease in soil, improve soil quality and soil fertility, which effectively improves the nutrient supply of crops and achieves the purpose of promoting plant growth. In addition, certain PGPR, as plant growth stimulants, are able to produce plant hormones such as growth hormone, gibberellin, cytokinin or directly stimulate crop growth by regulating the content of plant ethylene [23].

Currently, PGPR can be made into microbial fertilizers including microbial inoculants and composite microbial fertilizers, which have a wide range of applications in improving soil fertility, providing the nutrients needed by plants, and increasing food production [24]. Currently, a wide range of microbial fertilizers made from PGPR are used for commercial production worldwide, especially concentrated in developed countries. Studies have shown that most of the PGPR in various cash crops such as rice, maize, wheat, tomato, etc. have properties that enhance plant growth including plant height, seed germination, crop yield and nutrient content. With the increasing emphasis on agro-ecological development, the use of biopesticides made from PGPR as an alternative to traditional chemical pesticides is greener, more effective and sustainable, and is the main direction for the development of fertilizers in the future.

1.4 Factors affecting the production of IAA by plant inter-root biotrophic bacteria

The main factor affecting the synthesis of IAA by PGPR is the environment. PGPR regulates IAA biosynthesis under different environmental conditions.

Tryptophan is the precursor substance for microbial IAA synthesis, and the concentration of tryptophan directly affects the IAA production, within a certain range, microbial IAA production is proportional to the concentration of tryptophan, Shah Rushabh et al. investigated the effect of different concentrations of tryptophan on the production of IAA by strain 7MM11, and found that at tryptophan concentrations of 1g/L or less, the IAA production increased with the increase of tryptophan concentration and increased, and the highest IAA production was achieved at a tryptophan concentration of 1 g/L [25]. Temperature, pH, carbon source, nitrogen source, etc. are all determinants of IAA synthesis regulated by PGPR. Chandra et al. found that strain CA1001 could produce 91.7 μg/mL of IAA at pH 9, and when 1% dextrose was used as a carbon source, strain CA1001 synthesized IAA at a high level of 104 μg/mL; however, the presence of nitrogen in the culture medium Chandra et al. found that strain CA2004 synthesized the highest amount of IAA, 81.7 μg/mL, at 37°C [26]. The optimal pH and temperature for IAA production varied greatly among different strains, and it is important to optimize the optimal pH and temperature conditions for the strains.

1.5 Current status of research on screening of IAA-producing strains and optimisation of fermentation conditions

At present, the IAA-producing strains screened in China produce low levels of IAA, such as Liu Bui et al. screened a high and stable IAA-producing strain B16 from endophytic bacteria in the roots of *Astragalus membranaceus* in Mongolia, which could reach 40.658 mg/L after optimization of the cultivation conditions [27]. Chang Luyin et al. experimentally screened and obtained a multifunctional inter-root facultative bacterium named YM3, Bacillus subtilis, with an IAA-producing capacity of 59.21 mg/L [28]. Cheng Xinyu et al. isolated IAA-producing strains from the inter-root soil of the salt-tolerant plant Salt Claw Claw, and screened a total of 67 PGPR strains with IAA-secreting capacity in the range of 16.65-71.63 mg-L-1 [29]. High IAA-producing strains screened abroad had high IAA-producing capacity

compared to domestic ones, e.g., Shah Rushabh et al [30]. A strain of *Phytophthora* spp. from tomato rhizosphere soil was screened for IAA production up to 89.22 mg/L. Edi Husen et al. screened 14 strains of *Phytophthora* spp. from soil bacteria and 12 strains of these strains had IAA production up to 3501.85-5829.99 mg/L [31]. Walpola et al. screened a strain of endophytic bacteria PSB-X, a genus of endophytic bacteria, from a bed of waste cultured bacteria [32]. Walpola et al. screened the endophytic strain PSB-13 from the abandoned beds, which produced up to 229 mg/L of IAA.

At present, the research on PGPR with IAA secretion ability has become a popular field, and the methods to improve IAA production at home and abroad mainly include physical mutation of strains, medium optimization, application of additional PGPR fertilizer and optimization of fermentation culture conditions. For example, Dongyun Wang et al. optimized the composition of fermentation medium and fermentation culture conditions for IAA production by Bacillus subtilis strain 8-32, and its IAA production capacity was increased from 8.48 mg L-1 before optimization to 65.06 mg L-1 after optimization after fermentation [33]. Jasibe et al. isolated and screened a promastigote strain BG-5 from the inter-root soil of alkali ponceau, and then optimized its fermentation conditions and medium composition, and the IAA production of strain BG-5 after optimization was 87.86 μg/mL, which was about twice as much as that before optimization [34]. Zhang Zhen et al. screened Salt-tolerant *Arthrobacter* sp. with the ability to secrete IAA from plant inter-root soil, and after one-factor test and optimization of fermentation conditions, the strain was able to produce IAA up to 92.31 mg/L [35]. In foreign studies, most of the strains were optimized by fermentation and the level of IAA production increased dramatically, which was significantly higher than the level of IAA production by the strains before optimization.Apine et al. isolated strain PVM from agricultural soil, and after optimization of the fermentation culture, its IAA production reached 2191 mg/L within 2d [36]. Nutaratat et al. used Saccharomyces cerevisiae red yeast for IAA production by fermentation. Nutaratat et al. used Saccharomyces cerevisiae red

yeast to produce IAA, and the IAA production was as high as 2743.9 mg/L after the optimization of supplemented batch fermentation [37].

Conclusions to chapter 1

1. Inter-root biotrophic bacteria are widely present in the inter-root zone of plants and are able to promote plant growth, improve soil physico-chemical properties, and protect plants from abiotic and biotic stresses, among other beneficial effects. More than 20 genera of PGPR strains have been isolated and identified.
2. Endophytic bacteria can promote plant growth in both direct and indirect ways, and the direct growth-promoting effects include nitrogen fixation, production of phytohormones such as growth hormone, phosphorus solubilisation, potassium solubilisation and promotion of iron uptake in plants.
3. The indirect growth-promoting effects of PGPR include shaping the plant microbial community through the secretion of certain bioactive molecules to stimulate plant growth, and PGPR can protect plants from pathogens by competing for pathogen nutrients and living space, effectively restraining the population of pathogens.
4. PGPR has a wide range of applications, PGPR can be used as a substitute for pesticides to protect plants, and PGPR can also be made into microbial fertilizer to promote plant growth.
5. There are many factors affecting the production of IAA by PGPR, including tryptophan concentration, temperature, pH, carbon source, nitrogen source and so on.
6. In this study, we screened PGPR strains with strong ability to produce IAA from the inter-root soil of maize, identified their species through identification, and then optimized the conditions for the production of IAA

by the strains to obtain the best fermentation conditions, so as to maximize the production of IAA, and to provide theoretical and practical basis for the research and development of high-quality microbial fertilizers.

CHAPTER 2

OBJECT, PURPOSE AND METHODS OF THE STUDY

2.1 Object, subject and purpose of the study

Object of study – strains 4CV-1, 4CV-2, 4CV-3, Chang 7-3, Chang 7-4, Chang 7-6, Chang 7-7, ZH58-1, ZH58-3, ZH58-4, ZH58-6, 6WC-2, 6WC-3, 6WC-6, 6WC-8 were isolated and preserved by the Microbiology Laboratory of the Qilu University of Technology (Shandong Academy of Sciences, Shandong Province).

Subject of study – screening of IAA-producing strains, identification of strains, determination of IAA-producing capacity and optimization of culture conditions to improve IAA production.

Purpose of the study. As a large agricultural country, China has a rapidly growing maize industry, with maize accounting for 50 per cent of total grain production. Due to the excessive application of chemical fertilizers and pesticides in agriculture in China, not only does it cause problems such as lower agricultural yields, soil crusting and higher costs, but it also pollutes the soil, water and the environment. High-quality microbial agents can be a good solution to the above problems. In this study, we identified IAA-producing bacteria by screening them from the inter-root soil of maize, and improved the IAA production by optimizing the cultivation conditions and medium composition. The screening of high-yielding IAA strains can provide excellent strains for the development of microbial agents with high-yielding IAA function, which can promote the development of agriculture.

2.2 Screening of IAA-producing bacteria

2.2.1 Qualitative determination of IAA-producing bacteria

The frozen strains were inoculated in LB medium containing L-tryptophan (100 mg/L), and three sets of replicates were set up for each strain. After incubation in a constant temperature incubator (37 °C, 180 rpm) for 1 day, 1 mL of the bacterial

solution was taken, and an equal amount of Salkowski's colorimetric solution was added and dropped onto a white ceramic plate for the color development reaction. At the same time, 1 mL of 50 mg/L indoleacetic acid standard solution was used as positive control, and the medium without bacteria was used as negative control. It was placed away from light for 30 min, and the color change was observed, the color change to pink indicated that IAA was produced the darker the color, the stronger the separation effect; the unchanged color was negative, indicating that IAA could not be separated [38].

2.2.2 Preparation of IAA standard curves

Take 0.05g of IAA analytical standard and make 1000mg/L high-purity masterbatch in a 50mL volumetric flask. Take 500, 1000, 1500, 2000, 2500mL of mother liquor to 50mL to form 10, 20, 30, 40, 50mg/L. Take 2.5mL of the above solution and add Salkowski's colorimetric solution in equal volume, and use 5mL volumetric flask to make the solution. It was allowed to stand for 30 min at room temperature and protected from light, and then taken out and immediately measured its OD535 value by spectrophotometer, and adjusted to zero with the medium to which the colorimetric solution had been added, and repeated three times. The standard curve was plotted with IAA concentration as the horizontal coordinate and absorbance as the vertical coordinate, and the IAA content in the bacterial solution was calculated according to the standard curve.

2.2.3 Quantification of IAA

Determination of IAA content in the culture medium of the strains. The initial screening strains were re-screened. The IAA content was detected by UV spectrophotometry: 5mL of bacterial liquid was taken, centrifuged at 8000r/min for 5min, 2.5mL of supernatant was added with an equal amount of Salkowski's

colorimetric liquid reagent and mixed, and the absorbance value of the fermentation broth and the absorbance of the bacterial broth at 600nm were determined by a UV spectrophotometer at a wavelength of 535 at room temperature, protected from light for 30min. The IAA content per unit volume was calculated against the standard curve when the value of the bacterial solution at 600nm was 1. Three sets of replicates were done for each strain, and the strains with strong IAA production ability were screened and kept in glycerol tubes for storage.

2.3 Identification of IAA-producing bacteria

2.3.1 Morphological observations

① Observation of colony morphology

The preserved IAA-producing bacteria were inoculated into LB liquid medium, and after 24h of incubation, they were inoculated into LB solid medium and placed into the 37℃ constant temperature incubator for 24h of inverted incubation, and then the morphological characteristics of the colonies on the medium were observed, including morphology, color, edge, surface, transparency, etc., and this information was recorded.

② Gram stain

Gram type is an important physiological and biochemical characteristic of bacteria, which can be classified into two groups according to the differences in the composition and structure of their cell walls: Gram-positive and Gram-negative bacteria. The specific staining procedure is as follows:

Smear: The slide was slightly heated over a flame and placed on a tabletop, two drops of saline were taken and placed on the slide using an inoculation ring. Then use the alcohol lamp to cauterize the inoculation ring to sterilize, after cooling, take 1-2 drops of the bacterial fluid to be tested and E. coli bacterial fluid to be added on the saline respectively, and mix well.

Drying: Holding the slide at one end with the specimen facing upwards, pass the

slide quickly over the flame to the point where the slide does not feel hot to the touch of the skin, so that the moisture on the smear slowly evaporates and fixes the strain.

Initial staining: add drops of crystal violet solution to cover the specimen, stain for one minute, and rinse slowly with running water until no color flows down.

Mordanting: add Lugol's iodine solution to cover the specimen, stain for one minute, rinse slowly with running water until no color comes out.

Decolorization: cover the specimen with drops of 95% alcohol and decolorize for 30 seconds, then immediately wash off the ethanol with running water.

Restaining: Absorb water from the slide with absorbent paper, then stain with Senna Restaining Solution for one minute, rinse with water and absorb the water.

Microscopic examination: observation with an oil mirror.

2.3.2 Physiological and biochemical characterization

The isolated strains were subjected to conventional physiological and biochemical reaction tests, such as starch hydrolysis test, V-P test, methyl red test, gelatin liquefaction test, nitrate reduction test, etc., with reference to the "Manual of Systematic Identification of Common Bacteria".

① Starch hydrolysis test

Starch produces a blue color when exposed to iodine solution, but the area where the bacteria hydrolyze starch no longer produces a blue color when measured with iodine solution, indicating that the bacteria produce amylase, as follows:

The starch hydrolysis medium was cooled and aseptically manipulated to make plates. Inoculate the strains separately on the medium plate by line, and label the name of bacteria on the reverse side of the plate. Cultivate the plate in the incubator at 37℃ for 24 h. Observe the results: open the lid of the dish, add a small amount of iodine solution on the medium, gently rotate the dish so that the iodine solution is evenly spread over the whole plate. If a colorless transparent circle appears around the colony, it indicates that the starch is hydrolyzed and is positive.

② V-P test

Certain bacterial growth in glucose protein Chen water medium can decompose glucose to produce pyruvate, and pyruvate can be condensed, decarboxylated and converted into acetyl methyl methanol, such as adding a strong alkali, that is, with the role of oxygen in the air to produce diacetyl, diacetyl and guanidinium peptone in the role of the components to generate red compounds, known as the positive reaction, there is no red compounds produced is called the negative reaction, the specific operating procedures are as follows:

The strains were inoculated in glucose peptone water medium (5 ml of culture solution in each test tube) and placed in the incubator at 37 degrees Celsius for 24 h. After 24 h of incubation, 1 ml of culture solution was removed by adding an equal amount of 40% NaOH and 0.5 ml of α-naphthol, shaking vigorously, and the color 23 changes were observed after 15 to 30 min. Red color was positive and yellow color was negative.

③ Methyl red test

The methyl red test is used to detect organic acids produced from glucose. Certain bacteria can produce large amounts of acid in glucose peptone water medium, lowering the pH to 4~5. Acid production can be indicated by the discoloration of the methyl red indicator added. The color change range of methyl red is pH 4.2 (red) to 6.3 (yellow). When the bacteria decompose glucose to produce acid, the culture solution changes from the original orange color to red, which is the positive reaction of methyl red. The specific operation steps are as follows:

Inoculate the strains in glucose peptone water medium (5 ml of culture solution in each test tube), and place it in the incubator at 37 degrees Celsius for 24 h. Add 1 or 2 drops of methyl red reagent to the bacterial solution; red color is a positive reaction, and yellow color is a negative reaction.

④ Gelatin liquefaction test

Microorganisms can utilize various proteins and amino acids as nitrogen sources, and when there is a lack of saccharides, gelatin and other macromolecules can also be used as a source of energy. Gelatin is a protein produced by the hydrolysis

of collagen, which can be maintained in a gel state and exist in solid form below 25°C, while gelatin will liquefy above 25°C. Some microorganisms can produce an extracellular enzyme called gelatinase to hydrolyze this gelatin. Some microorganisms can produce an extracellular enzyme called gelatinase, which hydrolyzes this gelatin protein, thus liquefying the gelatin, and it can even remain in a liquefied state at 4℃. Specific procedures are as follows: take 3 gelatin medium test tubes with a marker pen to mark the name of each tube to be inoculated with bacteria. With aseptic operation, puncture the strain in the gelatin medium, the inoculated test tubes in 20 ℃ incubator culture 48 h. The inoculated test tubes in 20 ℃ incubator culture 48 h to observe the gelatin liquefaction.

⑤ Nitrate reduction test

Some bacteria have a nitrate-reducing capacity, which reduces nitrate to nitrite or ammonia and nitrogen, among others. When Grice's reagent is added, nitrite interacts with the acetic acid in it to form nitrite, which interacts with p-aminobenzene nitrate to form diazobenzene sulfuric acid, which combines with α-naphthylamine to form the red color N-α-naphthylamine azobenzene mineral acid, which is a positive reaction.

There are two possibilities for the cause of a negative reaction: the bacteria cannot reduce nitrate, then nitrate is still present in the culture medium after incubation. Or nitrite continues to decompose to produce ammonia and nitrogen, then there is no nitrate present in the culture medium. The presence or absence of nitrate can be checked with diphenylamine reagent. If there is nitrate, when the solution is added to 1~2 drops of diphenylamine reagent, the culture solution, if it is blue, it means that there is still nitrate in the culture, and there is no nitrite reaction, indicating that there is no nitrate reduction; if it is not blue, it means that nitrate and newly generated nitrite have been reduced to other substances, so it should still be dealt with according to the nitrate reduction of positive.

2.3.3 Molecular biology identification

The molecular biology of the strains was identified using 16SrDNA sequence analysis. The steps were:

① DNA extraction: follow the extraction steps of the kit for strain DNA ② PCR amplification: Bacterial 16SrDNA universal primer 27F/1492R was utilized for amplification, (forward primer: 5'-AGAGTTTGATCCTGGGCTCAG-3'; reverse primer: 5'-GGTTACCTTGTTACGACTT-3'). The amplification system was 25 μL: 12.5 μL of MIX, 0.5 μL of each upstream and downstream primer, 1 μL of template, and 10.5 μL of ddH2O. The PCR reaction program was as follows: pre-denaturation at 95 ˚C for 5 min, denaturation at 94 ˚C, annealing at 56 ˚C for 1 min, extension at 72 ˚C for 1.5 min for 30 cycles, and post-extension at 72 ˚C for 10 min. The PCR amplified products were detected by agarose gel electrophoresis.

The 16SrDNA sequence of the strain was amplified.

③ Sequencing: The amplified sequences were sequenced by Shanghai Bioengineering, and the sequencing results were compared in NCBI to find the similar sequences and downloaded, and the phylogenetic tree was constructed by MEGA7.0 software.

2.4 Optimization of fermentation conditions

2.4.1 Determination of IAA in the fermentation broth of the strain

The same as Chapter 2, the Salkowski's method and UV spectrophotometry were used to detect the IAA content: 5mL of fermentation broth of the strain was taken and centrifuged at 8000r/min for 5min. 2.5mL of supernatant was taken and added with an equal amount of Salkowski's colorimetric solution reagent and then volume was fixed. The absorbance value of the fermentation broth was measured by UV spectrophotometer at the wavelength of 535 after 30min of room temperature and light protection. The content of IAA in the fermentation broth was calculated per unit volume against the standard curve. Three replications were done for each group to screen fermentation conditions that gave the strain the strongest ability to produce

IAA.

2.4.2 Optimization of medium carbon source

The activated bacterial solution (activated by LB liquid medium) was inoculated with 2% inoculum in 10g/L glucose, soluble starch, sucrose, maltose as different carbon sources, other culture conditions remained unchanged, and the absorbance at 535nm was measured by UV spectrophotometer for 24h of continuous cultivation, to determine the content of IAA (3 replicates were set for each treatment), and the highest amount of IAA was selected as the best carbon source. The group with the highest IAA production was selected as the best carbon source.

2.4.3 Optimization of medium nitrogen source

Ammonium sulfate, beef paste, peptone and urea at 20g/L were used as different nitrogen sources, and their IAA contents were determined by continuous cultivation for 24h with the rest of the cultivation conditions unchanged (three replicates were set up for each treatment), and the group with the highest amount of IAA produced was selected as the best nitrogen source.

2.4.4 Optimization of medium tryptophan concentration

The gradient of tryptophan concentration was set at 2, 4 and 6 g/L, and the IAA production under different tryptophan concentrations was determined after 24 h of incubation with the rest of the incubation conditions unchanged (three replicates were set for each treatment), and the group with the highest IAA production was selected as the optimal tryptophan concentration.

2.4.5 Optimization of incubation temperature

The strains were set to be cultured at different temperatures of 27, 32, 37, and

42°C, and the IAA production of the strains was determined after 24 h under the rest of the culture conditions unchanged (three replicates were set for each treatment), and the group with the highest amount of IAA production was selected as the optimal temperature.

2.4.6 Optimization of incubation time

Other culture conditions were the same, and the fermentation broth was taken at intervals of 12, 24, 36 and 48 h to determine the IAA content of the strain (3 replicates were set up for each treatment), and to determine the optimal culture time of the strain.

2.4.7 Initial pH optimization

The pH of the medium was adjusted to 5, 7 and 9, respectively, and the IAA production of the strains was determined after 24 h of incubation with the rest of the incubation conditions unchanged (three replicates were set for each treatment) to determine the optimal pH for IAA production by the strains.

2.4.8 Response surface optimization for IAA production conditions of the strain

Through the results of the previous one-way experiments, tryptophan concentration (a), pH (b), temperature (c), and time (d) were set as the independent variables of the experiment, and IAA concentration Y was used as the model response value, and the levels of each factor are shown in Table2.1. According to the Box-Benhnken design of experiments, 29 groups of experimental protocols were obtained, and the regression fitting analysis of the table experimental results was carried out using Design-Expert 13 software.

Table 2.1- Strain Chang 7-6 Factor Levels

considerations	level (of achievement etc)

	-1	0	1
a	0	0.2	0.4
b	7	8	9
c	27	32	37
d	12	24	36

Conclusions to Chapter 2

1. The strains used in this experiment were 4CV-1, 4CV-2, 4CV-3, Chang 7-3, Chang 7-4, Chang 7-6, Chang 7-7, ZH58-1, ZH58-3, ZH58-4, ZH58-6, 6WC-2, 6WC-3, 6WC-6, and 6WC-8, all of which were isolated and preserved by the Microbiology Laboratory of the Qilu University of Technology (Shandong Provincial Academy of Sciences).
2. Screening of IAA-producing bacteria: Salkowski's colorimetric method was used to screen the IAA-producing ability of the bacteria in the first stage and then in the second stage, in order to screen the high IAA-producing strains.
3. The identification of IAA-producing bacteria included morphological identification, physiological and biochemical identification, and molecular biological identification.
4. The optimization of fermentation conditions included single-factor experiments: to study the effects of temperature, pH, carbon source and nitrogen source on IAA production; orthogonal experiments: to design orthogonal experiments based on the results of single-factor experiments to find out the optimal combination of fermentation conditions; and finally validation to confirm the validity.

CHAPTER 3

EXPERIMENTAL PART

3.1 Screening results of IAA-producing endophytes

When 15 strains of bacterial strains were qualitatively screened initially, the colour was changed to pink indicating that they all had the ability to produce IAA, 15 strains of bacterial maize inter-root endophytes were re-screened by Salkowski's colorimetric method with spectrophotometer, and the results of the test are shown in Table 3.1 .It can be found in Table 2. 3 that strains of Chang 7-6, 6WC-3, 6WC-2, Chang 7-4 and ZH58-6 had a higher ability to produce IAA, and the amount of IAA they produced reached 30.0% and 30.0% respectively. IAA reached 30.06 mg/L, 28.95 mg/L, 27.58 mg/L, 27.39 mg/L and 26.59 mg/L, respectively. These five strains with high IAA production capacity were selected for the subsequent experiments.

Table 3.1 –Amount of IAA produced by strains of bacteria

serial number	strains	IAA (mg/L)	serial number	strains	IAA (mg/L)
1	Chang 7-6	30.06±0.03	9	Chang 7-3	19.50±0.09
2	6WC-3	28.95±0.05	10	6WC-6	15.72±0.07
3	6WC-2	27.58±0.04	11	4CV-1	11.74±0.05
4	Chang 7-4	27.39±0.03	12	4CV-2	10.81±0.09
5	ZH58-6	26.59±0.02	13	4CV-3	10.62±0.06
6	ZH58-4	21.80±0.08	14	6WC-8	9.01±0.07
7	ZH58-1	21.43±0.11	15	Chang 7-7	6.40±0.07
8	ZH58-3	20.75±0.04			

3.2 Identification of IAA-producing endophytes

3.2.1 Morphological and physiological and biochemical characterization results

Strains Chang7-6, Chang7-4, 6WC-3, 6WC-2 and ZH58-6 were inoculated into LB solid medium and cultured for 24h to observe the colony morphology, in which the colonies were mainly rounded, creamy-white in color, with rounded edges, and the surfaces of the colonies were bulging and opaque, and the Gram staining was observed under the microscope, and the five strains Chang7-6, 6WC-3, 6WC-2, Chang7-4 and ZH58-6 were all G bacteria. -4 and ZH58-6 were all G^+ bacteria with short rods. The colony morphology of the five strains as well as the Gram results are shown in Figures 3.1 through 3.5.

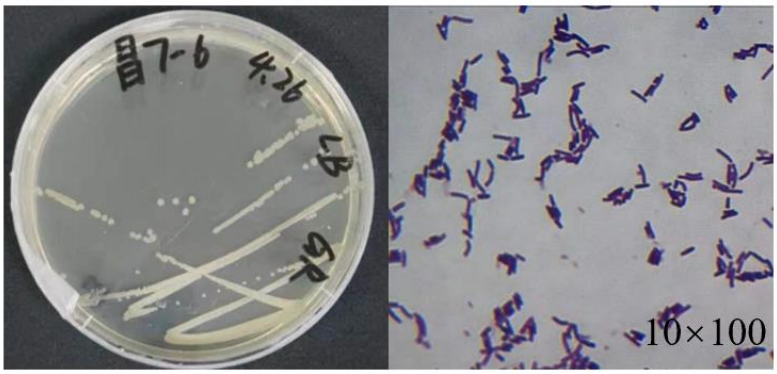

Figure 3.1- Colony morphology and Gram staining results of Chang 7-6

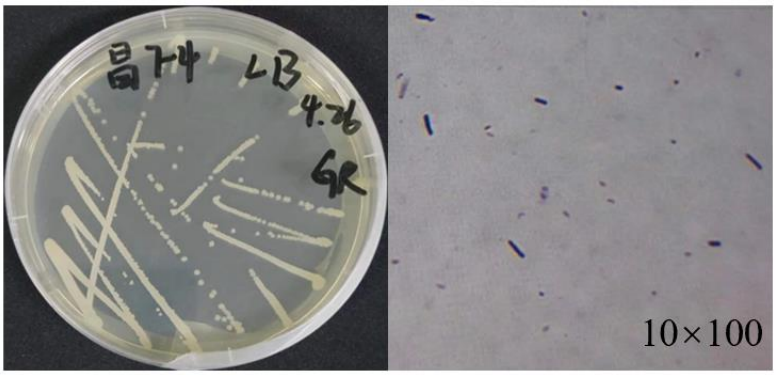

Figure 3.2- Colony morphology and Gram staining results of Chang 7-4

Figure 3.3- Colony morphology and Gram staining result of 6WC-2

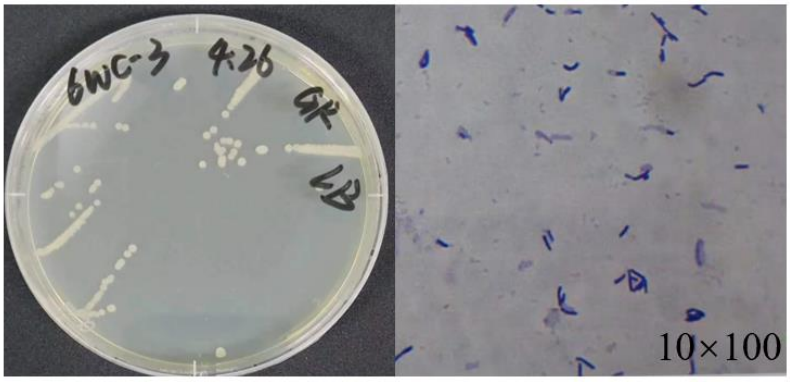

Figure 3.4- Graph of colony morphology and Gram staining results of 6WC-3

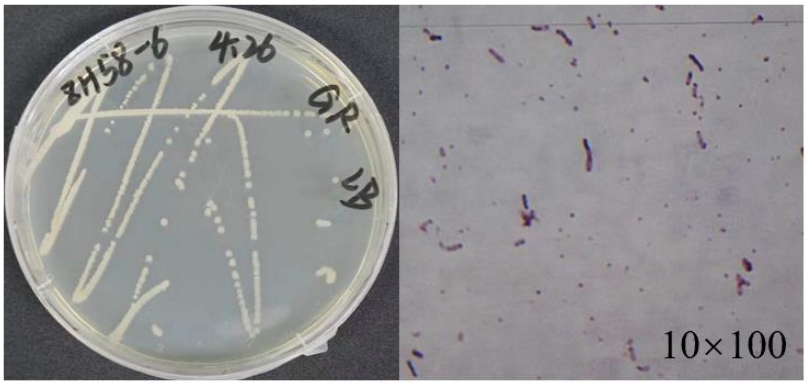

Figure 3.5- Colony morphology and Gram staining results of ZH58-6

Table 3.2- Morphological characterization of strains

colony morphology						Gram stain (used distinguished two different kinds of bacteria)	
strains	color	verge	geometry	open (non-	swell	in the	fungal

				secretive)		end	
Chang 7-6	opaline	round and neat	orbicular	non-transparent	swell	G^+	rod-shaped
Chang 7-4	opaline	round and neat	approximately circular	non-transparent	microstructure	G^+	rod-shaped
6WC-2	spoken lines in opera	round and neat	approximately circular	non-transparent	microstructure	G^+	rod-shaped
6WC-3	spoken lines in opera	round and neat	orbicular	non-transparent	swell	G^+	rod-shaped
ZH58-6	opaline	round and neat	orbicular	non-transparent	swell	G^+	rod-shaped

3.2.2 Physiological and biochemical characterization results

Table 3.3- Physiological and biochemical characteristics of the strains

Test metrics	Chang 7-4	Chang 7-6	6WC-2	6WC-3	ZH58-6
V-P test	-	-	-	-	-
Methyl Red Test	+	-	+	-	-
Gelatin liquefaction test	+	-	+	+	+
starch hydrolysis test	+	+	+	+	+
nitrate reduction	-	-	-	-	-

Note: "+" is positive; "-" is negative.

3.2.3 Molecular Biological Characterization Results

The 16SrDNA sequences of strains Chang7-6, Chang7-4, 6WC-2, 6WC-3, and ZH58-6 were obtained by 16SrDNA sequencing, and NCBI database was performed

for sequence comparison, and the phylogenetic tree was drawn using MEGA-X software. The 16SrDNA gene phylogenetic tree of the strains is shown in Figure 3-6. Combined with the physiological and biochemical tests and 16SrDNA sequence comparison, it can be determined that strain Chang 7-6 belongs to *Bacillus mobilis*, strain Chang 7-4 belongs to *Bacillus altitudinis*, and strains 6WC-2 and ZH58-6 belong to *Bacillus* sp. with the highest similarity, and strain 6WC-3 belongs to *Bacillus stratosphericus*.

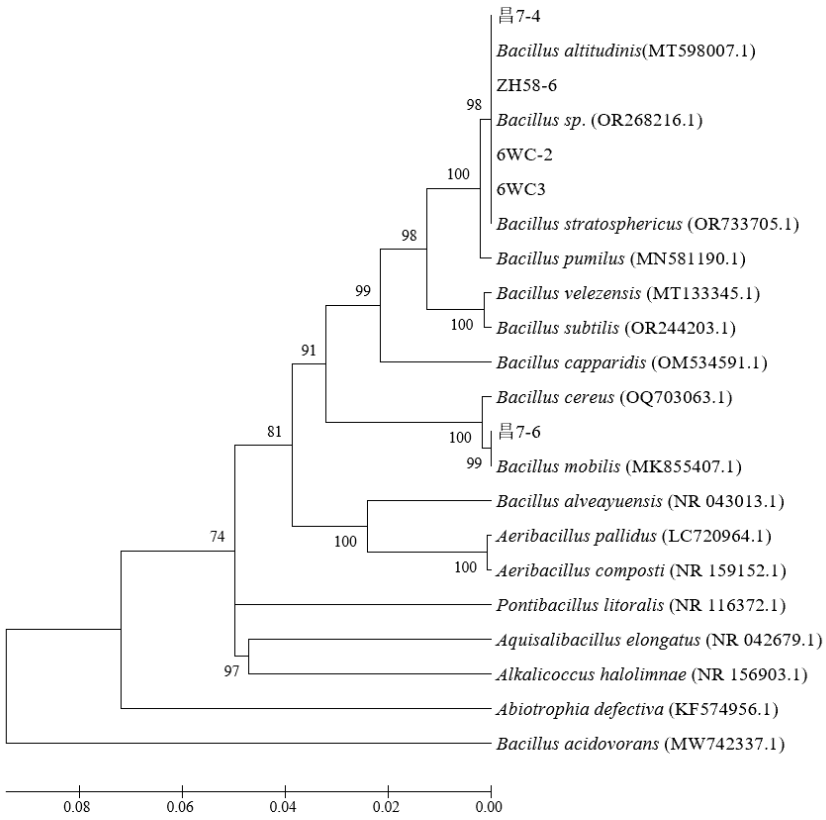

Figure 3.6- Phylogenetic tree of 16SrDNA genes of the strain

3.3 Experimental results of optimization of fermentation conditions

3.3.1 IAA standard curve

The standard curve was made using OD535 absorbance value as the horizontal coordinate (x) and the content of IAA as the vertical coordinate (y) and the linear equation y=0.0161x-0.0021, R^2=0.9956 was obtained.

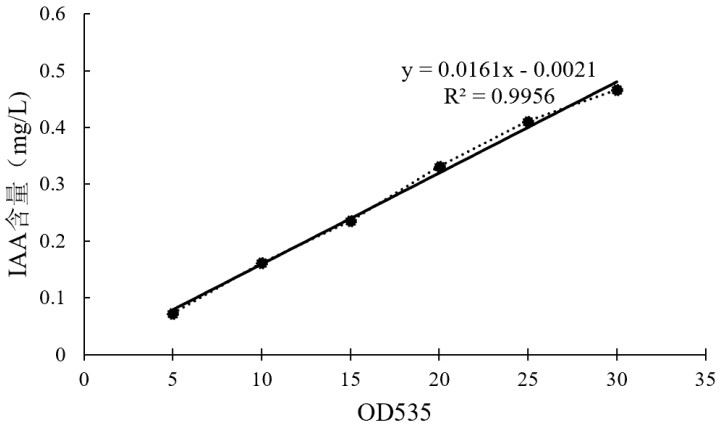

Figure 3.7- IAA Standard Curve

3.3.2 Results of the one-way test

① Effects of different carbon and nitrogen sources on the amount of IAA production by strain Chang7-6

From (a), it can be found that the type of carbon source has a significant effect on the amount of IAA production by Chang 7-6. The IAA production capacity of strain Chang 7-6 was glucose > sucrose > maltose > starch in descending order. When the carbon source was glucose, the IAA production of strain Chang 7-6 reached the maximum of 25.04 mg/L; when sucrose, maltose and starch were used as the carbon source, the IAA production of strain Chang 7-6 was 21.19 mg/L, 19.70 mg/L and 18.64 mg/L, respectively.

From (b), it can be found that the type of nitrogen source had a significant effect on the amount of IAA production by Chang 7-6. The IAA production capacity of strain Chang 7-6 was urea>peptone>beef paste>ammonium sulfate in descending order. When the nitrogen source was urea, the IAA production of strain Chang 7-6 reached the maximum of 36.84 mg/L; when peptone and beef paste were used as the nitrogen source, the IAA production of strain Chang 7-6 was 25.41 and 25.66 mg/L, respectively; and the lowest IAA production of strain Chang 7-6 was 13.86 mg/L when ammonium sulfate was used as the nitrogen source.

In summary, the best carbon source for strain Chang 7-6 was glucose and the

best nitrogen source was urea.

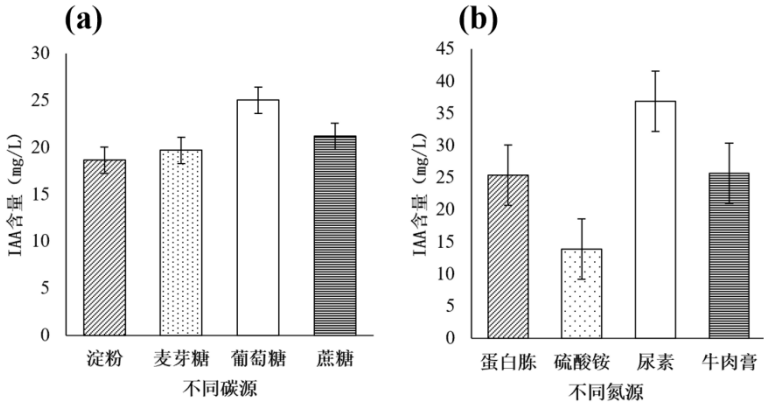

Figure 3.8- Effect of different carbon sources (a) and different nitrogen sources (b) on the amount of IAA production by strain Chang 7-6

② Effect of different pH, time, tryptophan concentration and temperature on the amount of IAA production by strain Chang 7-6

From (a), it can be found that when the pH value is 6, the IAA production is low at 14.73 mg/L; when the pH value is 8, the strain Chang 7-6 produces the maximum amount of IAA, and the content of IAA is 28.45 mg/L; when the pH value is greater than 8, the amount of IAA produced by the strain Chang 7-6 begins to decline, and when the pH value is 9, the amount of IAA produced is only 22.24 mg/L.

From (b), it can be found that when the incubation time was 12 hours, the IAA production was low at 19.70 mg/L; when the incubation time was 24 hours, the strain Chang 7-6 produced the maximum amount of IAA, and the IAA content was 25.04 mg/L; when the incubation time was greater than 24 hours, the strain Chang 7-6 began to decline in the amount of IAA production, and the incubation time was 36 and 48 hours, and the amount of IAA was 21.19 mg/L and 20.07 mg/L, respectively. The amount of IAA was 21.19mg/L and 20.07mg/L, respectively.

From (c), it can be found that when the tryptophan concentration was 2 g/L, the amount of IAA produced by strain Chang 7-6 reached the maximum value, and the IAA content was 25.04 mg/L; when the tryptophan concentration was greater than 2 g/L, the amount of IAA produced by strain Chang 7-6 began to decline, and when the

tryptophan concentration was 4 g/L and 6 g/L, the amount of IAA produced was 21.19 mg/L and 20.07 The amount of IAA produced was 21.19 mg/L and 20.07 mg/L at 4 g/L and 6 g/L, respectively.

From (d), it can be found that when the culture temperature was 22℃, the IAA production was low at 14.66mg/L; when the culture temperature was 27℃, the IAA content was 20.69mg/L; when the culture temperature was 32℃, the IAA production of strain Chang 7-6 reached the maximum value of 26.53mg/L; after the culture temperature was greater than 32℃, the IAA production of strain Chang 7-6 began to After the incubation temperature was greater than 32℃, the amount of IAA produced by strain Chang7-6 began to decline, and when the incubation temperature was 37℃, the amount of IAA produced by strain Chang7-6 was 25.16 mg/L.

In summary, the optimal incubation pH of strain Chang 7-6 was 8, the optimal incubation time was 24 h, the optimal tryptophan concentration was 2 g/L, and the optimal incubation temperature was 32 ℃.

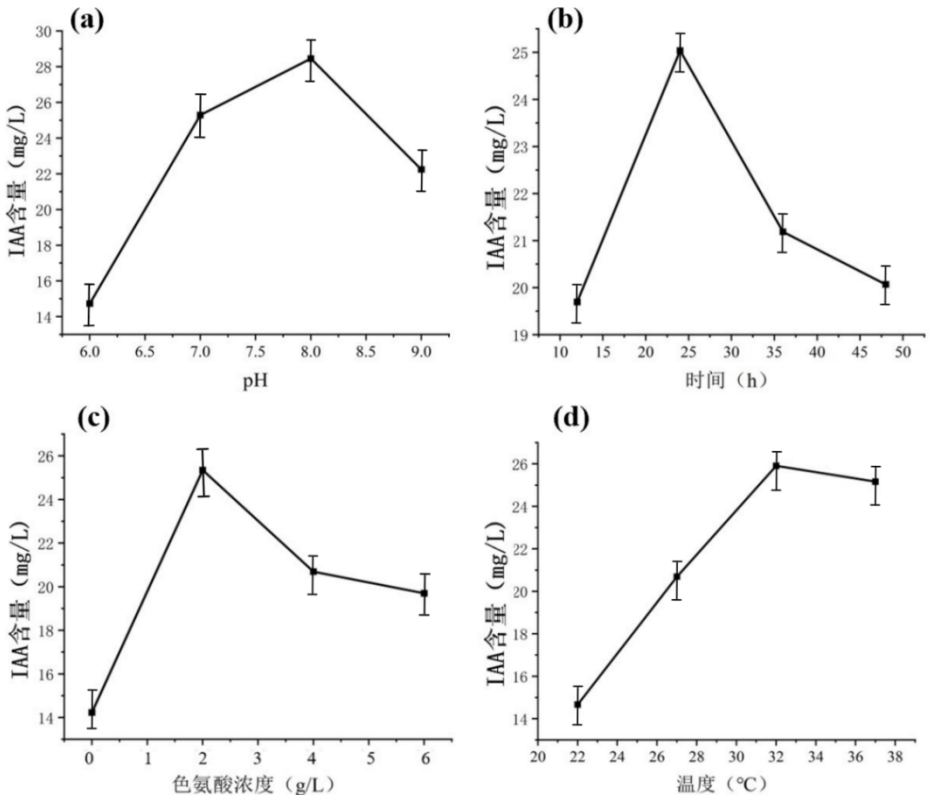

Figure 3.9- Effect of different pH (a), time (b), tryptophan concentration (c), and temperature (d) on the amount of IAA production by strain Chang 7-6

3.3.3 Results of response surface methodology to optimize the fermentation conditions for IAA production by strain Chang 7-6

Based on the results of the one-way experiment, tryptophan concentration (a), pH (b), temperature (c), and time (d) were set as the independent variables of the test to optimize the response surface test, and the specific design and results are shown in Table 3.4.

Table 3.4- Box-Benhnken Experimental Design Results

program number	a	b	c	d	IAA (mg/L)
1	0	7	32	24	32.5
2	4	7	32	24	35.2
3	0	9	32	24	36.5
4	4	9	32	24	34.4
5	2	8	27	12	37.3
6	2	8	37	12	33.8
7	2	8	27	36	35.6
8	2	8	37	36	37.2
9	0	8	32	12	32.7
10	4	8	32	12	32.9
11	0	8	32	36	33.6
12	4	8	32	36	36.7
13	2	7	27	24	35.9
14	2	9	27	24	38.7
15	2	7	37	24	37.2
16	2	9	37	24	36.8

17	0	8	27	24	31.6
18	4	8	27	24	37.8
19	0	8	37	24	34.6
20	4	8	37	24	31.9
21	2	7	32	12	33.6
22	2	9	32	12	38.3
23	2	7	32	36	38.8
24	2	9	32	36	37.6
25	2	8	32	24	40.2
26	2	8	32	24	39.8
27	2	8	32	24	39.6
28	2	8	32	24	40.3
29	2	8	32	24	40.1

Based on the principle of the least squares method, the regression analysis of the test results in Table 3.4 was performed to obtain the IAA concentration (Y) model, as shown in Equation (3.1).

$$Y = -192.60667 + 156.7a + 26.495b + 6.03167c + 0.97d - 6ab - 2.225ac + 0.302083ad - 0.16bc - 0.122917bd + 0.02125cd - 104.16667a^2 - 1.02917b^2 - 0.076667c^2 - 0.013571d^2 \quad (3.1)$$

Table 3.5- Box-Benhnken Experimental ANOVA

Source of variance	square sum (e.g. equation of squares)	(number of) degrees of freedom (physics)	mean square	F-value	P-value	significance
mould	197.16	14	14.08	47.21	<0.0001	**
a	4.56	1	4.56	15.30	0.0016	**
b	6.90	1	6.90	23.14	0.0003	**

c	2.43	1	2.43	8.15	0.0127	*
d	9.90	1	9.90	33.19	<0.0001	**
ab	5.76	1	5.76	19.31	0.0006	**
ac	19.80	1	19.80	66.39	<0.0001	**
ad	2.10	1	2.10	7.05	0.0188	*
bc	2.56	1	2.56	8.58	0.0110	*
bd	8.70	1	8.70	29.18	<0.0001	**
cd	6.50	1	6.50	21.80	0.0004	**
a^2	112.61	1	112.61	377.55	<0.0001	**
b^2	6.87	1	6.87	23.03	0.0003	**
c^2	23.83	1	23.83	79.89	<0.0001	**
d^2	24.77	1	24.77	83.05	<0.0001	**
inaccuracies	4.18	14	0.2983			
lost proposal	3.84	10	0.3836	4.51	0.0797	
pure error	0.34	4	0.0850			
add up the total	201.33	28				

From Table 3.5, it can be seen that the regression equation $P < 0.0001$, which indicates that the model is highly significant; the out-of-fit term $P = 0.0797 > 0.05$, so it indicates that the out-of-fit is not significant, and the regression model can accurately simulate the real level. The coefficient of determination $R^2 = 0.9793$ and corrected coefficient of determination $R^2_{Adj} = 0.9585$ indicate that the regression equation has a high degree of fit.

In order to verify the accuracy and significance of the multiple regression model, the established prediction model was analyzed by residual analysis, ANOVA, and comparison between predicted and actual values. Figure (a) shows the normal

probability distribution of the residuals of IAA concentration, from which it can be seen that the points are basically in a straight line; Figure (b) shows the residuals of the runtime graph, the points are scattered and there is no specific distribution, and the experimental data obeyed the normal distribution and there is no outlier; Figure (c) is the comparison of predicted and actual values, which can be seen that the predicted value of the IAA concentration is basically the same as that of the experiments, which shows that the multivariate regression model fits well with the results of the experiments and can be predicted by the regression equation. It can be seen that the predicted values of IAA concentration are basically consistent with the test, indicating that the multiple regression model fits the test results well and can be used to make predictions.

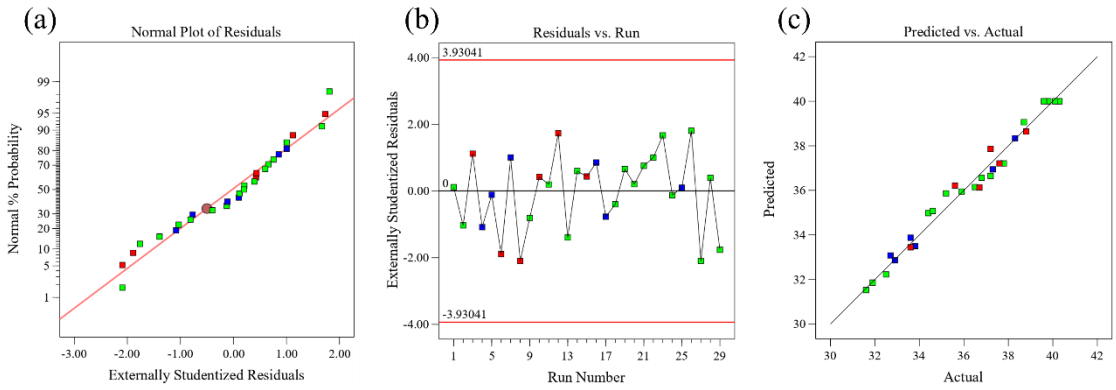

Figure 3.10- Analysis of the fit of the multiple regression model to the test results

Note: Figure (a) shows the normal probability distribution of the residuals, Figure (b) shows the residuals run chart, and Figure (c) shows the predicted values compared to the actual values.

3.3.4 Response surface interaction results

Design-Expert 13 software was analyzed to obtain response surface and contour plots as shown in Figs. (a)-(f), which visualize the effect of the interaction of the factors on the response values. The response surface plot is to keep the two independent variables at the optimum level and study the effect of the other two variables on IAA content. As can be seen from Fig. (a), the IAA content first increased and then decreased with the increase of tryptophan concentration, and the

change with pH was relatively smooth, and the larger value of its IAA content was obtained within 2 g/L~3 g/L of tryptophan content and pH 8~9. The response surface was steeper and the contour lines were elliptical, so the interaction between tryptophan concentration and pH on IAA content was stronger; from Fig. (b), the response surface was also parabolic and the contour lines were elliptical, so the interaction between tryptophan concentration and temperature on IAA content was stronger, which was consistent with the analysis of variance ; from Fig. (c), under the premise that the temperature and pH were unchanged, IAA content increased firstly and then decreased with the increase of tryptophan concentration, and also showed the trend of increasing firstly and then decreasing with the increase of time, so there existed a great value of IAA at the concentration of tryptophan of 2 g/L~3 g/L and the time of 20~26h, and at the same time, the response surface was parabolic and the contour was ellipsoidal, so the interaction between the concentration of tryptophan and the time of the up-dyeing rate was more significant; according to Fig. (d), the interaction between the concentration of IAA and temperature was more significant; according to Fig. (d), the interaction between IAA content and pH was positively correlated with the increase of pH. According to Fig. (d), IAA content was positively correlated with the increase of pH, and showed a trend of increasing and then decreasing with the increase of temperature, and its contour line was circular, so it indicated that the interaction between temperature and pH on IAA content was stronger, which was consistent with the results of ANOVA. From Figs. (e) and (f), it can be seen that the response surfaces of both showed parabolic shapes, and the great values existed at pH 8~9, temperature 29~32℃ and time 20~26h, and the contour lines were elliptical, so the interactions between pH and time and temperature and time were more significant for IAA content.

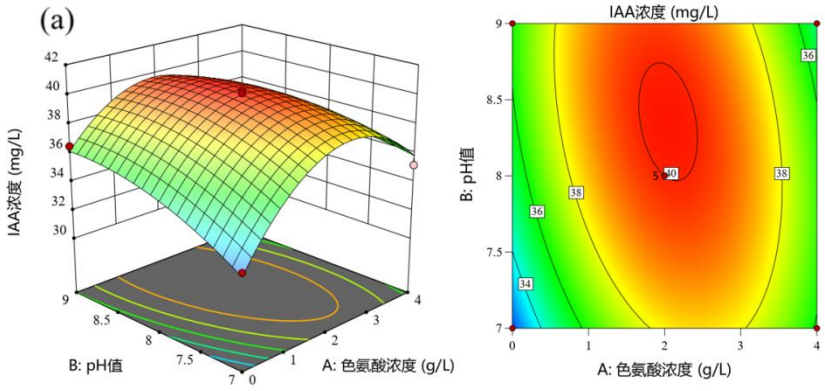

Figure 3.11- Response surface plots of tryptophan concentration interacting with pH

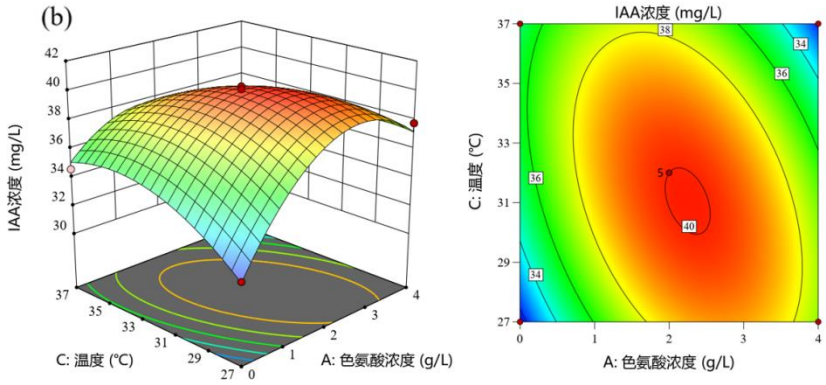

Figure 3.12- Response surface plots of tryptophan concentration interacting with temperature

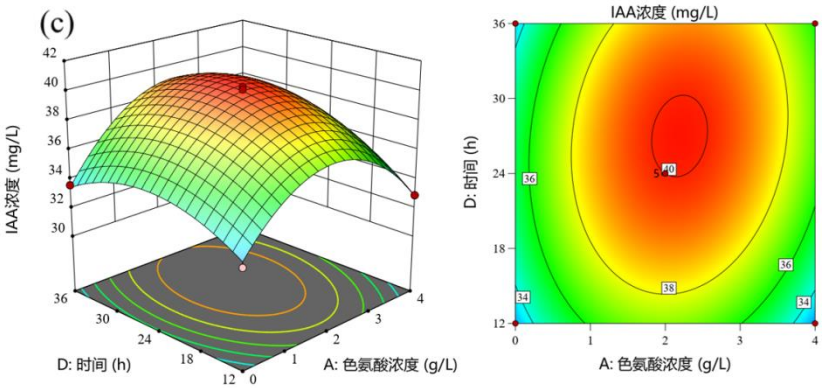

Figure 3.13- Response surface plots of tryptophan concentration interacting with time

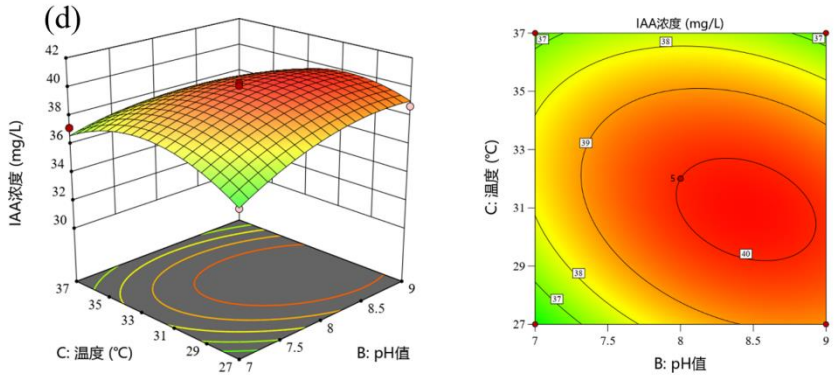

Figure 3.14- Response surface plots of pH-temperature interaction

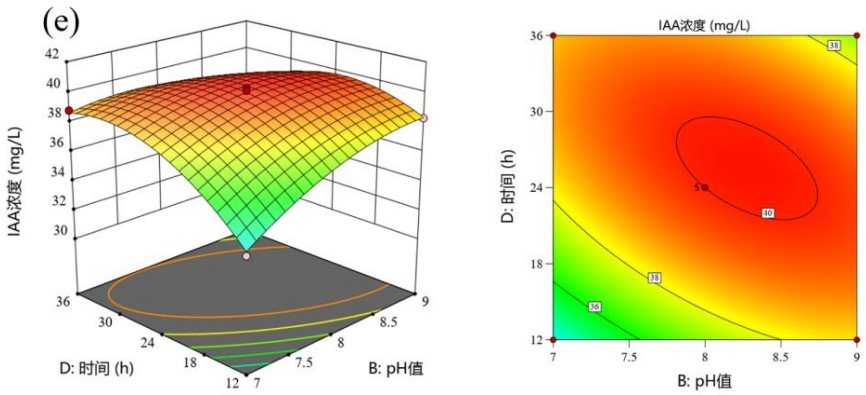

Figure 3.15- Response Surface Plot of pH Interaction with Time

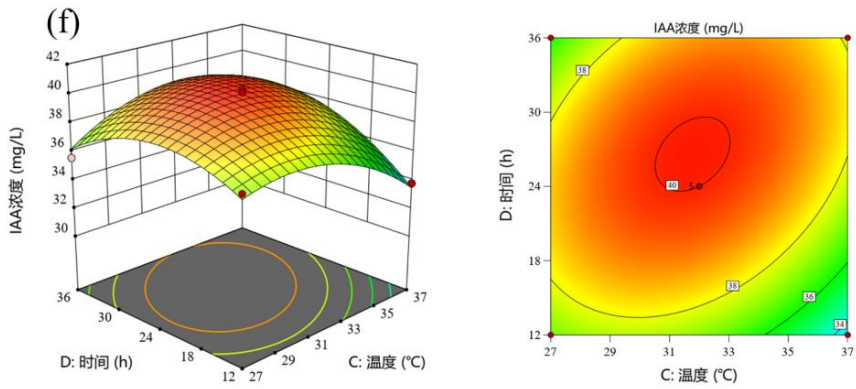

Figure 3.16- Response Surface Plot for Temperature-Time Interaction

After response surface optimization, the theoretical fermentation conditions for strain Chang 7-6 to produce the maximum concentration of IAA were obtained based on the fitted second-order model equation: tryptophan concentration 2.2 g/L, pH 8.4, temperature 30.8°C, and time 24.2h, and the yield of IAA could reach 40.2 mg/L. The fermentation conditions were as follows.

3.3.5 Validation experiments

In the actual operation, the optimal fermentation conditions predicted by the model were selected as the experimental conditions to repeat the experiment, and the average value of IAA yield was 38.891±0.056 mg/L, which differed from the theoretically optimized value by 3.27%, and the error was within the reliable range, which proved that the parameters were stable and reliable.

Conclusions to Chapter 3

1. Strains Chang 7-6, 6WC-3, 6WC-2, Chang 7-4 and ZH58-6 had higher IAA production capacity, which reached 30.06 mg/L, 28.95 mg/L, 27.58 mg/L, 27.39 mg/L26.59 mg/L, respectively.
2. Strain Chang 7-6 belonged to *Bacillus mobileus*, strain Chang 7-4 belonged to *Bacillus uplandus*, strains 6WC-2 and ZH58-6 had the highest similarity with Bacillus spp. and strain 6WC-3 belonged to *Bacillus stratophilus*.
3. The colonies of the five strains were mainly round, creamy-white in colour, with rounded edges, raised colony surfaces and opacity, and when observed under the microscope after Gram staining, strains Chang 7-6, 6WC-3, 6WC-2, Chang 7-4 and ZH58-6 were all G+ bacteria with short rod-shaped bodies.
4. After response surface optimization, the theoretical fermentation conditions for strain Chang 7-6 to produce the maximum concentration of IAA were obtained as follows: tryptophan concentration of 2.2 g/L, pH value of 8.4, temperature of 30.8 ℃, and time of 24.2h, and its IAA yield could reach 40.2 mg/L. The results showed that the fermentation conditions for strain Chang 7-6 to produce IAA were as follows.
5. The optimal fermentation conditions predicted by the model were chosen as the experimental conditions for repeating the experiments, and the average value of IAA yield was 38.891±0.056 mg/L, which was 3.27%

different from the theoretical optimised value, and the error was within the reliable range, which proved that the parameters were stable and reliable.

CONCLUSIONS

1. In this experiment, 15 strains of bacteria in the inter-root soil of maize were screened by the Salkowski colorimetric method to determine the ability of the strains to secrete IAA, and the following conclusions were drawn: all strains had the ability to produce IAA, and among them, strains Chang 7-6, 6WC-3, 6WC-2, Chang 7-4, and ZH58-6 secreted the strongest IAA, with the abilities of 30.06 mg/L, 28.95 mg/L, 27.58 mg/L, 27.39 mg/L, and 26.59 mg/L, respectively.
2. Morphological observation, physiological and biochemical identification and molecular biological identification of strains Chang7-6, Chang7-4, 6WC-2, 6WC-3 and ZH58-6 led to the following conclusions: the colonies of the five strains of bacteria were mainly rounded, with milky white color, rounded edges, elevated surface of the colonies, opaque, and Gram staining revealed that all strains were Gram-positive bacteria, with a rod-like shape; strain Strain Chang 7-4 was negative in V-P test, positive in methyl red test, positive in gelatin liquefaction test, positive in starch hydrolysis test and negative in nitrate reduction test; strain Chang 7-6 was negative in V-P test, negative in methyl red test, negative in gelatin liquefaction test, positive in starch hydrolysis test and negative in nitrate reduction test; strain 6WC-2 was negative in V-P test, positive in methyl red test and positive in gelatin liquefaction test; strain 6WC-2 was negative in V-P test, positive in methyl red test and positive in gelatin liquefaction test; strain 6WC-2 was negative in V-P test, positive in methyl red test and positive in gelatin liquefaction test. positive, gelatin liquefaction test was positive, starch hydrolysis test was positive, nitrate reduction test was negative; strain 6WC-3 V-P test was

negative, methyl red test was negative, gelatin liquefaction test was positive, starch hydrolysis test was positive, nitrate reduction test was negative; strain ZH58-6 V-P test was negative, methyl red test was negative, gelatin liquefaction test was positive, starch hydrolysis test was positive, nitrate reduction test was negative; by 16SrDNA sequencing, strain Chang 7-6 belonged to *Bacillus mobilis*, strain Chang 7-4 belonged to *Bacillus altitudinis*, and strains 6WC-2 and ZH58-6 had the highest similarity with *Bacillus sp. The* highest similarity was found between strains 6WC-2 and ZH58-6 and *Bacillu*s sp. Strain 6WC-3 belongs to *Bacillus stratosphericus*. Chang 7-6, which produced the highest amount of IAA, was selected for subsequent experiments.

3. Optimization of one-factor conditions for IAA production by strain Chang 7-6 was carried out to determine its optimal carbon source as glucose, its optimal nitrogen source as urea, and its optimal incubation temperature as 32℃, incubation time as 24h, and pH as 8.
4. Response surface optimization (RSO) analysis was carried out for the strain Chang 7-6 based on the one-way test, and the IAA yield was 40.2 mg/L after fermentation and cultivation under the optimal conditions, which was 1.3 times higher than that before optimization. After the validation experiment, the mean value of IAA yield was 38.891±0.056mg/L, and the mean value of IAA yield was 38.891±0.056mg/L, which was within the reliable range of error, proving that the parameters were stable and reliable.

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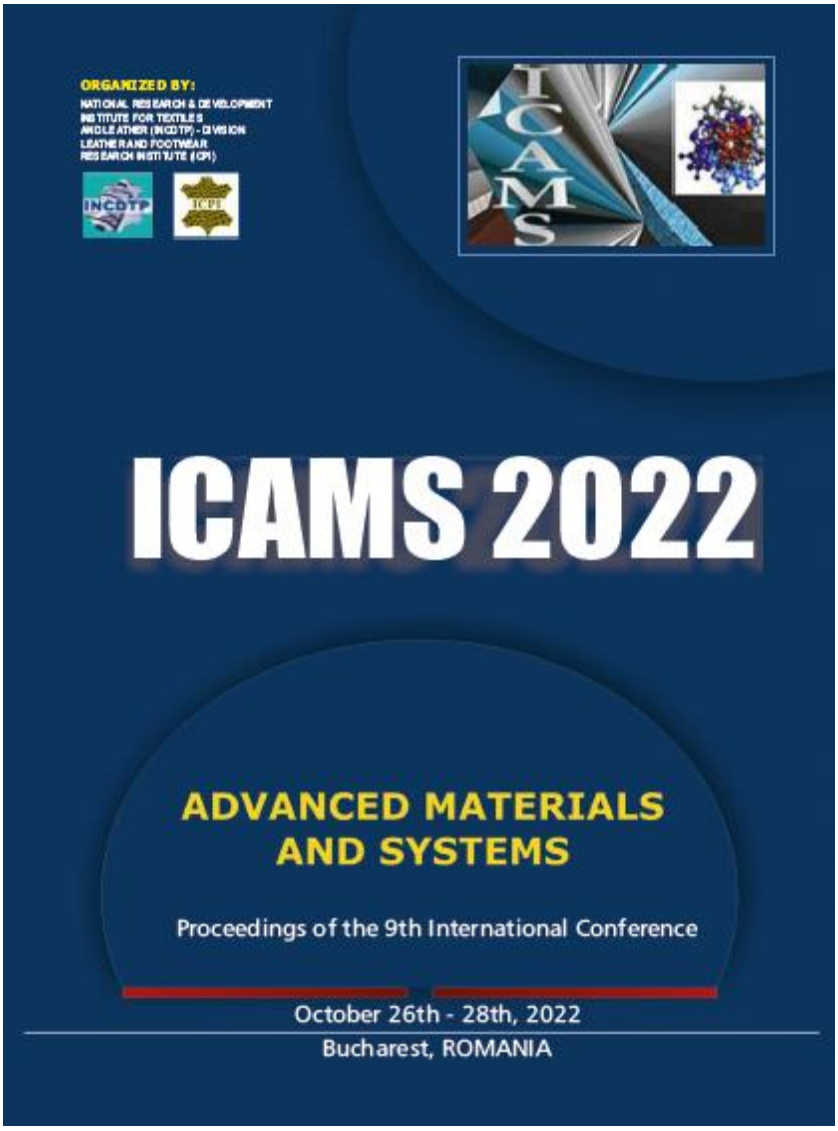
APPENDIX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NANOPIGMENTS FOR LEATHER FINISHING COATINGS

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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 dependsd 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 – $Cr_2(SO_4)_m(OH)_{6-2n}$, 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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