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

QUALIFICATION THESIS

on the topic **Optimization of Laccase Fermentation Conditions Using *Escherichia coli* BL21 (DE3)**

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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FOR THE QUALIFICATION THESIS

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SUMMARY

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Laccase is a kind of blue polycopper oxidase, which has been widely used in many fields of industry, such as green environmental protection, paper bleaching industry, pharmaceutical industry, sewage treatment, food industry and other fields have made a very broad application prospect. However, the low yield of laccase seriously limits the actual production and use of laccase, which cannot meet the increasing actual market demand of laccase. Therefore, this paper adopts the method of combining single factor experiment and response surface experiment to optimize the laccase fermentation conditions of *Escherichia coli* BL21. The software was used to analyze the experimental data and model, and the optimal fermentation process conditions for laccase fermentation production were obtained as follows: The added amount of glucose was 9.95 g, the added amount of ammonium chloride was 10.97 g, the pH was 7.52, and the predicted value of laccase activity was 7.4525 U/mL. The actual laccase activity was 7.43U/mL according to the fermentation conditions predicted by the software, which was not much different from the predicted value, proving the reliability of the software analysis. In this experiment, the laccase fermentation conditions were optimized by response surface method, which effectively improved the laccase yield, and was applied to the industrial field, which reduced the production cost and improved the economic benefits, and also provided a certain experimental basis for the subsequent use of *Escherichia coli* laccase fermentation.

Key words: Escherichia coli BL21; lacquerase; fermentation condition optimization

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INTRODUCTION

Laccase is a kind of blue polycopper oxidase, which has been widely used in many fields of industry, such as green environmental protection, paper bleaching industry, pharmaceutical industry, sewage treatment, food industry and other fields have made a very broad application prospect. However, the low yield of laccase seriously limits the actual production and use of laccase, which cannot meet the increasing actual market demand of laccase. Therefore, this paper adopts the method of combining single factor experiment and response surface experiment to optimize the laccase fermentation conditions of *Escherichia coli* BL21. The software was used to analyze the experimental data and model, and the optimal fermentation process conditions for laccase fermentation production were obtained as follows: The added amount of glucose was 9.95 g, the added amount of ammonium chloride was 10.97 g, the pH was 7.52, and the predicted value of laccase activity was 7.4525 U/mL. The actual laccase activity was 7.43 U/mL according to the fermentation conditions predicted by the software, which was not much different from the predicted value, proving the reliability of the software analysis. In this experiment, the laccase fermentation conditions were optimized by response surface method, which effectively improved the laccase yield, and was applied to the industrial field, which reduced the production cost and improved the economic benefits, and also provided a certain experimental basis for the subsequent use of *Escherichia coli* laccase fermentation.

The relevance of the topic is the optimization of laccase fermentation conditions was studied by using *Escherichia coli* BL21(DE3).

The purpose of the study is to increase the laccase production

The objectives of increasing the laccase production by using a facile method

The object of the study is the laccase produced by *Escherichia coli*.

The subject of the study is Single-factor experiments and response surface experiments were used to optimize the fermentation conditions of laccase.

Research methods is One-factor experiments and response surface experiments

The scientific novelty is exploring the optimal fermentation process conditions to improve the laccase yield and reduce the application cost.

The practical significance of the results obtained is the best fermentation conditions are obtained, and the experiments, the results are not different from the expected effect.

CHAPTER 1
LITERATURE REVIEW

1.1 Overview of the laccase studies

1.1.1 Source and type of laccase

Laccase (EC1.10.3.2) is a copper-containing polyphenol oxidase, belonging to the blue polycopper oxidase family [0], which can oxidize a variety of substances, such as polyphenols, lignin, aniline, polycyclic aromatic hydrocarbons and even inorganic substances, using molecular oxygen as the electron acceptor, and the only by-product produced in the reaction process is water [2]. Studies have shown that laccase can promote oxidative coupling of monomers in natural phenolic substances, thus affecting the formation of lignin. For example, Pleuropleura fungus laccase, which plays an important role in lignin degradation, can be called lignin modifying enzyme [3]. In addition, laccase can also be polymerized to form dimer or trimer forms [4].

Laccase comes from various sources and is widely distributed. In 1883, Japanese scientist Yoshida isolated and extracted laccase from Rhus vernacular for the first time. In 1894, Bertrand also discovered this protein from fungi and named it Laccase [5]. Since then, scientists have gone on to isolate and identify laccase from more than 20 species of bacteria [6], most white rot fungi [7], some higher plants [8], and lichens [9]. In addition, polyphenol oxidase with laccase-like activity has been found in metagenomic libraries in insect cuticle[10], oyster [11], and bovine rumen [12].

Laccases can be roughly divided into four categories according to their kinship [13]: animal laccase, plant laccase, fungal laccase and bacterial laccase.

Relatively few laccases are found in animal bodies, and there are known [14] such as pig kidney, tobacco day moth (Manduca sextan), mosquito, hemp fly, and migratory insects of phoenix butterfly and dipteral.

Plant laccase is the earliest kind of studied, lacquer species belong to angiosperms subphylum dicotyledonous plant class Rossana iced end run (Anacardiaceae) lacquer family (ANA Ca RD IA CEA E) originated in our country, it is a plant treasure in our country. At present, the plants that produce laccase are: sumac, Burmese Lien gas, Khmer paint, lemon fruit, Palestinian Chinese Pistache, California pepper tree, etc. Vegetable laccase and fruit laccase types are also more, such as cabbage laccase, potato laccase, beet laccase, apple laccase. These laccases mainly come from the cell wall of plant cells, catalyze the free radical polymerization of lignin structural monomers, and participate in the synthesis of lignin [15,16].

Fungal laccase and bacterial laccase belong to microbial laccase, which are studied and reported more. In particular, fungal laccases, such as basidiomycetes, ascomycetes and other fungal laccases, are the most intensively and extensively studied category [17]. White rot fungus laccase is the most reported, there are more than 100 kinds of bacteria. For a long time, scientists found and studied laccase in plants and fungi; Until Alexandre et al. carried out further research and analysis on protein database and bacterial genome, it was found that laccase could also exist in bacteria, such as intercommons, Bacillus, Escherichia coli, Klebsiella, and pseudomonas.

1.1.2 Structure and function of common laccases

Both fungal laccase and bacterial laccase belong to extracellular enzymes and are glycoproteins in the form of single proteome, so the protein structure is relatively similar. At present, the structure of laccase is usually studied by X-ray crystal diffraction. In 1998, scientists obtained the first crystal structure of laccase, which was found in Coprinus cinereus; The research of laccase crystals did not have a great breakthrough until 2002, and people's understanding and research on the structure of laccase have gradually deepened [18].

The most typical spatial structure of the common laccase structure is

composed of three interconnected cup-shaped domains that combine together to form a spherical structure: T2Cu and T3Cu form a three-nucleated copper cluster, located between the first and third domains, and T1Cu is located in the third domain. The substrate binding site is located in the third domain. In the spherical structure, there are β-folded structure, a small amount of α-helix structure and random curling; The substrate-binding domain is located near the TCU of the third domain, where the electron cloud density is sparse and there is more space to accommodate the substrate. The three-nucleated copper cluster center is formed by the coordination of 8 histidines, and the distance between the bond lengths of T2Cu and T3Cu in bacterial laccase proteins is longer than that of other blue polycopper oxidases. In general, the trite nuclear copper cluster center in laccase communicates with the outside world to form a water channel for the transport of oxygen molecules and the reduction reaction [19]. The enzyme molecule of laccase generally contains four copper ions, which are located in the active center of laccase and play a synergic electron transfer function during the oxidation reaction: TCu exists in the form of a single electron acceptor and forms a coordination with cysteine and two histidine, and its axial coordination is a methionine amino acid residue [20]. T2-cu forms coordination bonds with two histidines and a water molecule. T3-cu is a tetrahedral structure coordinated with three histidines and an -OH-bridg [21].

1.1.3 Influencing factors of laccase production

The production of laccase is easily affected by nutritional conditions and some physicochemical factors. Fermentation conditions such as temperature, inoculation amount, ventilation, PH value, substrate concentration, etc. have a great impact on the production of laccase. In addition, the expression of exogenous laccase gene in yeast is affected by signal peptides, promoters, expression strains, heterologous expression of laccase genes and other factors [22].

Medium component [23] has a prominent influence on the production of laccase. Nitrogen source is one of the important raw materials used for the synthesis

of bacteria and enzymes, and appropriate nitrogen source type and concentration are crucial for the production of laccase: organic nitrogen sources such as yeast extract and peptone are conducive to the production of laccase; Carbon source is an important material basis for the growth, development and metabolism of microorganisms, and different carbon sources also have different impacts on the production of laccase: carbohydrates such as glucose and sucrose are commonly used carbon sources, but more complex carbon sources such as lignocellulose can also have a certain impact on the induction and production of laccase and play a role in promoting it. In addition, some metal ions such as copper and manganese also have important effects on the activity, yield and stability of laccase [24].

The presence of additives will also have a certain impact on the production of laccase, by adding phenolic substances or aromatic compounds, such as ABTS, 2, 5-dimethylaniline, ferulic acid or 3, 4-dimethoxybenzoyl alcohol can also increase the production of laccase [25].

The influence of temperature on the production of laccase is also crucial, such as most white rot fungi, the most suitable living conditions are produced at room temperature, therefore, in order to obtain a higher yield, the temperature must be controlled within the room temperature range, and laccase is a polycopper protein, extreme conditions of high temperature and high pressure usually make it inactivated.

The effect of pH value on laccase activity is mainly in the following three aspects:

(1) Too high or too low pH value will change the spatial structure of laccase, and then lead to the reduction or even loss of laccase activity;

(2) Too high or too low pH will have a certain impact on the dissociation state of the active site of laccase, so that the substrate cannot be catalyzed to decompose;

(3) Too high or too low pH will also damage the dissociation state of the substrate, so that the enzyme cannot bind to the substrate [26].

These factors together determine the level and quality of laccase production. By optimizing these factors, the production of laccase can be improved.

1.1.4 Common applications of laccase

The application of laccase mainly has the following two aspects: the first aspect is the use of fungal laccase for biological remediation or bioreactor, especially the laccase produced by white rot fungi has been widely concerned. Laccase produced by white rot fungi can oxidize olefins and degrade 2,4,6-trichlorophenol and other harmful substances, which can decolorize many industrial dyes. The second aspect is the biodegradation of lignin by laccase: laccase can oxidize phenol units in lignin to produce phenoxy groups. In environmental protection, laccase is also used to catalyze the oxidation of toxic aromatic amines and phenolic substances produced in industrial production to effectively reduce pollution [27].

Due to the large amount of wastewater produced in the paper industry and bleaching process usually contains high concentrations of lignin-containing wastes and organic chlorides, it must be effectively treated before discharge. Laccase produced by fermentation of white rot fungi is usually used to polymerize chlorophenol compounds in chlorine-containing wastewater, so as to achieve the purpose of detoxification and greatly reduce the pollution to the environment. In agriculture, it is usually necessary to spray a large number of pesticides, which will cause certain pollution when penetrated into the soil, because most of the pesticides usually contain aromatic ring compounds, and laccase can effectively catalyze the oxidation of such substances and derivatives, thereby reducing the harm of the soil of pesticides, and accelerating the degradation of harmful substances in the environment to improve soil and environmental quality.

Most importantly, laccase can also be reused and maintain a high level of activity [28]. Studies have shown that laccase can effectively remove more than 40% of the harmful substances in bleaching waste water [29].

Due to the universality and special catalytic effect of laccase substrate, laccase also has a very wide application prospect in food industry, pulp and paper making, agricultural production, and pharmaceutical synthesis [30].

In the food industry, because laccase can significantly improve the quality of food at a lower cost, it is often used in the production of edible fungi and the processing

of drinks [31], such as white wine [32], apple and grape juice [33,34], tea and beer. Laccase can catalyze the oxidation of polyphenols into polyphenol oxides, and then polymerize to form larger particles, which are trapped by the filter membrane to purify the drink. In the production of edible fungi, laccase can produce some effective antibacterial substances in the process of decomposing lignin, thereby enabling the rapid growth of edible fungi and inhibiting the generation and growth of hybrid bacteria, thus improving the production efficiency of food [35]. In addition, laccase can also be used in baking bread, because laccase can catalyze the oxidation of disulfide bonds in flour, thus making bread softer and tastier [36].

One potential application of laccase in the paper industry is bioleaching of pulp. The raw material in the papermaking process contains a lot of lignin which seriously affects the color of paper. Laccase can be used to catalyze the degradation of lignin to change the traditional production process to produce high-quality pulp. In addition, laccase is more stable than other peroxidase, the reaction temperature is more appropriate [37], and it can play a more effective role, saving equipment and energy consumption, further shortening the pulp production cycle, improving production efficiency and reducing costs, and bringing higher economic benefits to the paper industry [38,39].

In agriculture, it is mainly reflected in the feeding effect of enzyme preparations. Laccase is one of the feeding enzyme preparations. With the increasing development of grain-saving feed, the level of crude fiber in grain has also been greatly improved, which has broad application prospects in agriculture and animal husbandry, such as safer and non-toxic enzyme straw storage technology. And its unique mechanism can also effectively improve the utilization and digestibility of straw [40].

In medicine, laccase can catalyze the coupling reaction of heterogeneous and homogeneous molecules, providing a way to study the synthesis of new drugs [41,42]: catalysis is conducive to the release of active ingredients in plants and the extraction of active ingredients in Chinese herbal medicine. Guo Mei et al. used recombinant laccase

from genetically engineered bacteria to extract saponins from Astragalus decoction slices, and the extraction rate was 65.6% higher than that of traditional methods [43].

In terms of organic synthesis, the catalytic oxidation conditions of laccase are relatively green and mild, the catalytic efficiency is high and the substrate is low, which has obvious advantages for catalytic synthesis of active compounds. In the process of anabolic reaction in microorganisms, laccase uses phenol hydroxyl group as substrate to catalyze oxidation to generate free radicals, and further catalyzes polymerization to form dimers, etc., after which the coupling reaction between molecules forms polymers: Kevin et al. used o-diphenol and 1,3-dicarbonyl compounds as substrates to synthesize 14 kinds of 5,6-dihydroxy-furan compounds with strong inhibitory effect on cancer cells after being catalyzed by laccase [44]； Agemate et al first acetylated penicillin X and then formed a relatively stable dimer catalyzed by laccase, thus achieving the modification of antibiotics [45]. Zhang et al. [46] used laccase for the first time to catalyze 1,3-dicarbonyl compounds and amine compounds to synthesize enamine ketone, and the yield reached 83%-95% at room temperature [47]. Laccase also has great advantages in biosensors, which can enhance the signal of the sensor, and has made great breakthroughs in environmental detection, and biosensors made of laccase are also widely used in the pharmaceutical industry.

Castrovilli et al. produced a new type of laccase sensor by electrospray deposition that is free from interference of chromium, cadmium, zinc, etc., and can be used for the detection of dithephenols in the environment [52]. The amperometry biosensor for detecting dopamine content invented by Rubio-Govea R., et al., was also made of laccase [53].

1.2 *E. coli* BL21 (DE 3) and its laccase production

1.2.1 Overview of *E. coli* BL21 (DE 3)

E. coli, also known as *Escherichia coli,* is an important group of normal bacteria in the human gut, which was discovered by the German-Austrian pediatrician Theodor Escherich in 1885. The shape of Escherichia coli is blunt at both ends, can move, spore-free Gram-negative bacteria, is a conditional pathogen, under certain conditions can cause human and animal gastrointestinal or urethra and other local tissues and organs infection. It can grow on more types of media, reproduce fast, and is generally non-toxic and harmless to the human body. *E. coli* is a population that usually includes many different species, and the differences between the species are usually detected by molecular tests, and these molecular differences also lead to differences in their pathogenicity, resistance to antibiotics, and use of carbon sources. BL21 (DE3) is one of the most commonly expressed strains in Escherichia coli, which is suitable for the expression of non-toxic proteins, and has the characteristics of low protease efficiency and insensitivity in high glucose concentration medium.

1.2.2 Characteristics of bacterial laccases

Because laccase has a very wide range of substrate types, it also has a lot of characteristics. Compared with fungal laccase, bacterial laccase has a wider pH range, thermal stability, high salt resistance and alkaline resistance, and is easier to be expressed in the host organism. Bacterial laccase can catalyze the oxidation of many phenolic substances and their derivatives, and can also degrade some phenolic substances without phenolic hydroxyl group. In addition, bacterial laccase does not need to undergo glycosylation modification after translation, and can be efficiently and mass-produced by using genetic engineering technology, which is not available for characteristic plant laccase and fungal laccase [48].

For the paper industry, the optimal pH of fungal laccase is in the acidic range,

while the pH range required by the paper industry is in the alkaline range. Therefore, the optimal pH of bacterial laccase is alkaline, which makes bacterial laccase a better choice in the paper industry and can also improve the brightness of paper to a certain extent [49].

1.2.3 Common fermentation process for laccase production

Laccase is a kind of enzyme widely existing in nature, which has a wide range of industrial applications and important industrial value. In order to screen the strains producing laccase, solid culture or liquid culture can be used to screen. A certain number of strains were obtained from the sample environment such as soil or water for separation and purification. Organic solvents could be added to the medium to stimulate the strains and wake up the strains to produce laccase. Plate screening method could be used to screen out the strains with high laccase activity and methyl orange was used as an indicator to test the enzyme activity of laccase. However, in order to make laccase can be widely used, efficient and inexpensive fermentation process is a very important premise, the current production of laccase strains are relatively few, and the fermentation cost is slightly high, to obtain a new strain with high yield of laccase, establish and optimize the fermentation process of laccase, and then improve the production of laccase, reduce the production cost is a more important aspect of the research on laccase. The optimization of laccase fermentation conditions is mainly divided into solid fermentation conditions and liquid fermentation conditions.

Nowadays, the common fermentation methods for the production of laccase are solid fermentation and liquid fermentation. Solid state fermentation (SSF) refers to the process of growing microorganisms by using an insoluble solid culture substrate, including not only deep fermentation of solids suspended in a liquid, but also microbial culture on a medium containing no or little free water [55,56].Different from liquid fermentation, the medium of solid fermentation is in solid form. Under the condition that the water activity can meet the growth of bacteria, there is almost no free-flowing

water in the medium and on the surface. Therefore, solid fermentation takes the gas phase as the continuous phase, and the filter membrane and the covered solid phase as its fixed phase to form a three-phase system. According to investigation and research, compared with liquid fermentation technology, solid-state fermentation technology has more advantages and advantages [55,56]:

(1) The raw materials of solid-state fermentation technology not only have a wide range of sources and lower cost, but also can use recycled natural agricultural and forestry biomass or food processing waste as its substrate;

(2) Some fermented products and metabolites can better produce products under the condition of solid fermentation;

(3) Solid fermentation consumes less energy than liquid fermentation and its production process is simpler;

(4) Solid fermentation not only does not produce waste gas wastewater during the fermentation process, the concentration of its products is also higher, and the subsequent treatment process is simpler.

For the production of laccase using Escherichia coli, solid fermentation is used more frequently, and in solid fermentation, there are also many factors affecting laccase [57]:

(1) As for the influence of carbon source types on laccase, it is not necessary to add additional carbon source in the basic fermentation medium, otherwise, the enzyme activity of laccase will decrease;

(2) The added amount of yeast extract powder also has a certain effect on the enzyme activity of laccase. This is because when the yeast extract is too large, it will increase the content of nitrogen source, thus changing the ratio of carbon to nitrogen in the medium, resulting in a large number of bacteria growths, affecting the metabolism of microorganisms, and ultimately leading to a decline in the enzyme activity of laccase;

(3) For the supplemental levels of $MgSO_4 \cdot 7H_2O$ and KH_2PO_4 in the medium, the activity of laccase first increased and then decreased with the increase of the

supplemental levels;

(4) The influence of culture temperature on the activity of laccase is also more important. With the increase of culture temperature, the activity of laccase will also show a trend of first increasing and then decreasing. At 37℃, the activity of laccase is the highest, because too low temperature will cause the cell membrane to coagulate, which makes it difficult to transport substances, while too high temperature will cause the deformation and inactivation of proteins in cells.

In addition, the yield of laccase can also be improved by mutagenic breeding and recombinant expression, but in comparison, fermentation process optimization is the most effective way to improve the yield of laccase, which is also the most reported way at present. However, because *Escherichia coli* laccase is an intracellular enzyme, the yield is too low, leading to the high cost of application in the industry. Therefore, it is still an important task to improve the production efficiency of laccase and reduce the cost of industrial production.

1.3 Project Ideas and design

1.3.1 Purpose and significance of the project

The development of economy makes the production of industrial wastewater more and more serious, the impact on the environment is also more and more serious, especially the chlorine bleaching wastewater produced by the paper industry, in addition to containing extremely high BOD and COD and containing high chroma, but also contains a large number of toxic and difficult to be degraded by traditional treatment methods of organic chloride. Today, highly acclaimed biotechnological methods are mainly used for wastewater treatment to further improve environmental problems. In this paper, the production of laccase can be further improved by optimizing the yield of laccase, so as to increase the application of laccase in the treatment of industrial wastewater and other industrial fields.

1.3.2 Project research ideas

In this experiment, the effects of pH of the medium, the content of ammonium chloride in the medium and the content of glucose in the medium on the production of laccase were systematically studied through single factor experiment, and the production of laccase with different content under each condition was recorded respectively. Then the response surface experiment was optimized according to the results of the single factor experiment, so as to obtain the best conditions for laccase fermentation. The yield of laccase was increased to some extent under this fermentation condition.

Conclusions to chapter 1

Laccase is a kind of blue multi-copper oxidase, the action direction is very much, the application range is also very wide, but the yield of laccase is not high limits its application scope, so it is very important to improve the yield of laccase through some means.

CHAPTER 2
OBJECT, PURPOSE AND METHODS OF THE STUDY

2.1 Experimental materials

2.1.1 Experimental strains and instruments

Experimental strain: lab-preserved *Escherichia coli* BL21 (DE3) basic engineering strain of laccase production.

Table 2.1 - **Instruments and equipment used in the experiment**

Laboratory apparatus	Production company
Constant temperature shaking table	Changzhou Nuoji Instrument Co., LTD
High speed freezing centrifuge	Eppendorf，Germany
Low temperature refrigerator	Shanghai Senxin Experimental Instrument Co., LTD
Digital display water bath	Beijing Yongguang Medical Instrument Co., LTD
A UV-visible spectrophotometer	Shanghai Ao Yi Instrument Co., LTD
Autoclave steam sterilization cooker	Shanghai Boxun Industrial Co., LTD
Superclean bench	Sujing Group Suzhou Antai Air Technology Co., LTD
Shock incubator	Shanghai Jinghong Experimental Equipment Co., LTD
Fermenter	Zhenjiang Dongfang biological engineering equipment technology company

2.1.2 Reagents and culture media

Preparation of M9 liquid nutrient medium （1L）: glucose 30.0 g, $Na_2HPO_4 \cdot 12H_2O$ 15.2 g, KH_2PO_4 3.0 g, NaCl 0.5 g, NH_4Cl 1.0 g, $MgSO_4$ (1M) 2 mL, $CaCl_2$ (0.1M) 1 mL, Adjust the pH to 7.0 with KOH.

Preparation of 10M KOH: Weigh 56 g KOH solid and dissolve it in 100 mL water before autoclaving.

1M $MgSO_4$: $MgSO_4$ 1.2037 g with double steam 10 mL dissolved, autoclave

reserve;

1M $CaCl_2$: $CaCl_2$ 1.1098 g dissolved with double steaming water 10 mL, autoclave for use;

5×M9 salt solution: $Na_2HPO_4 \cdot 12H_2O$ 15.2 g, KH_2PO_4 3.0 g, NaCl 0.5 g, NH_4Cl 1.0 g dissolved in double steaming water 200 mL;

50% glucose solution: 33.0 g glucose dissolved in double steaming water 66 mL.

Note: The above four samples were prepared separately, bottled separately, and sterilized by high pressure steam at 115 ℃ for 15 min.

Preparation of M9 medium in aseptic procedure (ready for use):

5 M9 salt solution, 200 mL,1M $MgSO_4$ 2 mL,

A 50% solution of glucose, 60 mL, 1M $CaCl_2$ 0.1 mL,

Add sterilized double steam water to 1000 mL. Add sterilized double steam water to 1000 mL.

2.2 Experimental method

2.2.1 Preparation of enzyme solution

(1) E. coli was inserted into 100 mL triangular bottle containing 5 mL M9 liquid medium and cultured at 30 ℃ and 180 rpm for 12-14 hours to obtain the culture medium;

(2) The activated culture medium was inoculated into a 100 mL triangular bottle containing 50 mL M9 liquid medium at 1% inoculation rate, and cultured at 25 ℃ and 110 rpm on a shaking table for 7 days to obtain the culture medium;

(3) The culture medium was collected and centrifuged at a speed of 8000 r/m for 15 minutes to collect the supernatant;

2.2.2 Measurement of laccase enzyme activity

The total volume of the reaction was 3 mL, which included appropriate amounts of enzyme, buffer as well as quantified substrate. The enzyme activity of laccase is defined as the amount of enzyme required to catalyze the oxidation of 1

μmol substrate per minute as 1 enzyme activity unit (U). The enzyme activity of laccase was measured by ABTS method (ε420 nm = 36000 L/mol·cm). ABTS is a phenolic substance, and the rate of oxidation determines the activity of laccase. ABTS as a substrate, has many advantages, not only good preservation, safety and non-toxic, and ABTS method is very sensitive to measure enzyme activity. Sodium acetate solution was used as its buffer solution [50]. ABTS will form ABTS free radical after reacting with laccase, and its light absorption value is much larger than that of substrate ABTS at 420nm, and with the increase of the concentration of ABTS free radical, its light absorption value will also increase, so OD value is usually detected at 420 nm [51]. In a water bath at 30 ℃, add an appropriate amount of buffer and enzyme, and then add 1mLABTS mother liquor to start the timing. The light absorption value of the reaction system at 420 nm wavelength for 3 minutes was detected by ultraviolet spectrophotometer. All reactions were repeated for 3 times.

Laccase activity (U/mL) is calculated as follows:

$$\text{CotA Enzyme live (U/mL)} = (\Delta OD \times V0 \times n \times 106) / (\Delta t \times V1 \times \varepsilon) \quad (2.1)$$

In formula: ε Is the light absorption coefficient; V0 represents the total volume of the laccase enzyme live measurement system; V1 represents the volume of the enzyme solution added to the reaction; Δt represents the reaction time; ΔOD represents the change of light absorption value before and after the reaction; n represents the dilution of the enzyme solution.

2.2.3 Experiment of optimizing glucose concentration in medium to promote laccase production

According to the different effects of different glucose concentration on laccase production, 28 g, 29 g, 30 g, 31 g, 32 g and 33 g glucose were added to M9 medium under the premise of keeping other conditions unchanged, and three parallel experiments were conducted to measure the laccase activity of each group and observe the influence of glucose concentration on laccase production and yield.

2.2.4 Experiment on promoting laccase production by optimizing the concentration of ammonium chloride in medium

According to the different concentrations of ammonium chloride in medium had different effects on laccase production, under the precondition of keeping other conditions unchanged, 0.8 g, 0.9 g, 1.0 g, 1.1 g, 1.2 g and 1.3 g ammonium chloride were added into M9 medium respectively, and three parallel experiments were conducted to determine the laccase activity of each group. The effect of ammonium chloride concentration on laccase production was observed.

2.2.5 Experiment of optimizing pH of medium to promote laccase production

According to the different effects of different pH of medium on laccase production, under the premise of keeping other conditions unchanged, equal amounts of *Escherichia coli* BL21 (DE3) were added to M9 medium with pH 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5, respectively, and three parallel experiments were conducted to determine the laccase activity of each group. The effect of pH of medium on laccase production was observed.

2.2.6 Experimental design of the response surface

In this experiment, a Box Behnken design with 3 factors and 3 levels was carried out for the 3 factors of peptone content, sodium chloride content and pH of the medium, combined with the results of the single factor experiment. The levels of test factors and their codes are shown in Table 2.2.

Table 2.2 - **Box-Behnken test factor levels and coding**

Standard	Glucose, g	Ammonium chloride, g	Ph
-1	29	1.0	6.0
0	30	1.1	6.5
1	31	1.2	7.0

Conclusions to chapter 2

This chapter mainly describes the materials and methods required for experiments, uses *E. coli* BL21 (DE 3) stored in the laboratory, explores the influence of the concentration and glucose concentration in the medium, and designs the experiment with 3 factors based on the results of unifactor experiments, in order to obtain the best laccase fermentation conditions and improve the yield of laccase.

CHAPTER 3
EXPERIMENTAL PART

This experiment mainly uses Escherichia coli to carry out laccase fermentation. By exploring the influence of three factors on laccase yield caused by different pH of medium, different content of ammonium chloride in medium and different content of glucose in medium, it is found that laccase yield will first increase and then decrease with the increase of pH of medium. When pH of medium is 6.5, laccase yield will increase first and then decrease. The highest laccase yield was 1,19 U/mL, and too much acid and alkali were not conducive to laccase growth. The concentration of ammonium chloride in the medium also has a certain effect on the laccase yield. The experimental results show that the laccase yield increases with the increase of the concentration of ammonium chloride in the medium. When the content of ammonium chloride reaches 1.1 g, the laccase yield reaches the highest, and then decreases with the increase of the concentration of ammonium chloride. When the glucose content in the medium increased gradually, the laccase production also increased. When the glucose content was 30 g, the laccase production reached the highest, and then decreased with the increase of glucose content. These three single-factor experiments showed that the production of laccase was affected by the content of substances in the medium, and the level of enzyme production was significantly different under different conditions.

3.1 Results of single factor experiment

As shown in Table 3.1, the yield of laccase varies with the pH of the medium. Under the condition that other conditions remain unchanged, dilute acid or dilute base is used to adjust the pH of the medium to 5.0, 5.5, 6.0, 6.5, 7,7.5, respectively, and the enzyme activity is measured after being cultured at 37°C for a period of time. Because pH can affect the ionization intensity of nutrients in the medium, and then affect the

absorption of nutrients in the medium by microorganisms, it also affects the toxicity of other harmful substances to microorganisms and the enzyme activity in the metabolic process. As can be seen from the analysis in Table 3.1, with the increase of pH of the medium, the laccase yield presents a trend of first increasing and then decreasing. When the pH of the medium is about 6.5, the laccase yield is the highest, which is 1.19 U/mL. When the pH of the medium is higher or lower, the laccase yield will decline.

Table 3.1- **Effect of medium pH on laccase production**

Laccase yield		
pH	Laccase yield, U/mL	Standard deviation
5.0	0.89	0.02
5.5	1.03	0.03
6.0	1.11	0.04
6.5	1.19	0.04
7.0	1.04	0.05
7.5	0.81	0.04

As shown in Table 3.2, the content of ammonium chloride in the medium is different, and the yield of laccase is also different. The addition amount of ammonium chloride in the medium is changed to 0.8 g, 0.9g, 1.0 g, 1.1 g, 1.2 g, 1.3 g, respectively. Under the condition of other conditions being unchanged, the enzyme activity of laccase is measured after culture at 37 ℃ for a period of time. As ammonium chloride is a nitrogen source, it is an essential nutrient for the growth, development and metabolism of microorganisms in the medium. Too high or too low will change the ratio of carbon to nitrogen in the medium, resulting in a large number of bacterial growth and reproduction, affecting its metabolic process, and causing a certain impact on the growth of microorganisms in the medium. According to the analysis of Table 3.2, with the increase of ammonium chloride content in the medium, the laccase production showed a trend of first increasing and then decreasing, and when the ammonium chloride content in the medium was about 1.1 g, the laccase

production was the highest, 1.19 U/mL.

Table 3.2 - **Effect of ammonium chloride concentration in medium on laccase yield**

Laccase yield		
Ammonium chloride content, g	Laccase yield, U/mL	Standard deviation
0.8	0.84	0.04
0.9	1.01	0.03
1.0	1.14	0.03
1.1	1.19	0.04
1.2	1.07	0.05
1.3	0.92	0.04

As shown in Table 3.3, the output of laccase was different with different glucose content in the medium. The added amount of glucose in the medium was changed to 28g, 29 g, 30 g, 31 g, 32 g and 33 g, respectively. The enzyme activity of laccase was measured after being cultured at 37 ℃ for a period of time under the same other conditions. As glucose is a carbon source, it is also an essential substance in the growth, development and metabolism of microorganisms, and too high or too low glucose content will affect the ratio of carbon to nitrogen in the medium, resulting in massive growth and reproduction of bacteria, which is not conducive to the growth and development of microorganisms.

Table 3.3 shows that with the increase of glucose content in the medium. The production of laccase showed a trend of first increasing and then decreasing, and when the glucose content in the medium was about 30 g, the production of laccase was the highest, which was 1.19 U/mL. The appropriate carbon to nitrogen ratio was an indispensable factor for the growth and development of microorganisms.

Table 3.3 - **Effect of glucose content in the medium on laccase production**

Laccase yield		
Glucose content, g	Laccase production, U/mL	Standard deviation
28	0.94	0.06
29	1.06	0.05
30	1.19	0.04
31	1.03	0.04
32	0.93	0.05
33	0.73	0.03

3.2 The Box-Behnken test results

Based on the results of the single factor experiment, Box-Behnken experiment with three factors and three levels was used to explore the yield of laccase under three single factor conditions of different medium pH, ammonium chloride addition and glucose addition. The three single factor conditions were optimized. Design Expert Software Version 8.0.6 was used to perform regression fitting on all the experimental data obtained, and the results of quadratic regression model variance analysis were obtained. It was determined that the regression equation provided a suitable model for the prediction analysis of laccase production, as shown in Table 3.4, 3.5.

Table 3.4 - **Results of the Box-Behnken experimental design**

Experimental number	Glucose, g	Ammonia chloride, g	Ph	Laccase enzyme activity, U/mL
1	*2*	*3*	*4*	*5*
1	30	1.1	6.5	7.21
2	29	1.1	6	4.18
3	30	1.1	6.5	7.44
4	29	1	6.5	2.84

Continuation of Table 3.4

1	*2*	*3*	*4*	*5*
5	31	1.1	7	3.59
6	30	1.2	6	3.94
7	31	1.2	6.5	1.97
8	29	1.1	7	3.60
9	30	1.2	7	3.58
10	30	1	7	4.50
11	29	1.2	6.5	3.50
12	30	1.1	6.5	7.40
13	31	1	6.5	3.50
14	31	1.1	6	3.01
15	30	1	6	3.38
16	30	1.1	6.5	7.62
17	30	1.1	6.5	7.54

3.3 Response surface results and analysis

Design Expert Software Version 8.0.6 was used to perform regression fitting for all experimental data, and the results of variance analysis of the quadratic regression model were obtained.

Table 3.5 shows the relevant data of the quadratic regression analysis.

Table 3.5 - ANOVA for Response Surface Quadratic Model

Source of variance	Quadratic sum	Degree of freedom	Mean square	F-value	P value	
1	*2*	*3*	*4*	*5*	*6*	*7*
Source	Squares	df	Square	Value	Prob > F	
Model	60.44	9	6.72	218.98	< 0.0001	significant
A-glucose	0.53	1	0.53	17.25	0.0043	
B-ammonium chloride	0.19	1	0.19	6.1	0.0429	
C-pH	0.074	1	0.074	2.41	0.1642	
AB	1.2	1	1.2	39.28	0.0004	

Continuation of Table 3.5

1	*2*	*3*	*4*	*5*	*6*	*7*
AC	0.34	1	0.34	10.94	0.013	
BC	5.40E-01	1	0.54	17.72	0.004	
A^2	23.7	1	23.7	772.78	< 0.0001	
B^2	18.91	1	18.91	616.67	< 0.0001	
C^2	9.17	1	9.17	298.86	< 0.0001	
Residual	0.21	7	0.031			
Lack of Fit	0.12	3	0.039	1.59	0.3254	not significant
Pure Error	0.098	4	2.50E-02			
Cor Total	60.66	16				

record：R-Squared = 0.9965； Adj R-Squared = 0.9919

F= 218.98 and P < 0.0001 of the model in the table indicate that the model is extremely significant. lack of fit represents the probability that the predicted value of the model does not fit the actual value (P > 0.05), and the lack of fit term is not significant, so the model is correctly selected. The correlation coefficients R-Squared= 0.9965, Adj R-Squared = 0.9919 of the models indicate that the equation is well fitted and has excellent accuracy. In summary, the regression equation provides a suitable model for the prediction and analysis of laccase production.

The effects of various factors on laccase yield were analyzed by response surface method.

The response surface is formed by fitting the equation.

Fig. 3.1 (*a*) in the figure is the response surface curve and contour diagram of the addition amount of glucose and ammonium chloride. When the other two factors were the optimal value, with the increase of glucose content, laccase activity showed a trend of first increasing and then decreasing, and the change amplitude was large, indicating that glucose had a significant effect on laccase production. With the increase of ammonium chloride content, laccase activity also showed a trend of first increasing and then decreasing, indicating that the content of ammonium chloride had a great effect on the yield of laccase.

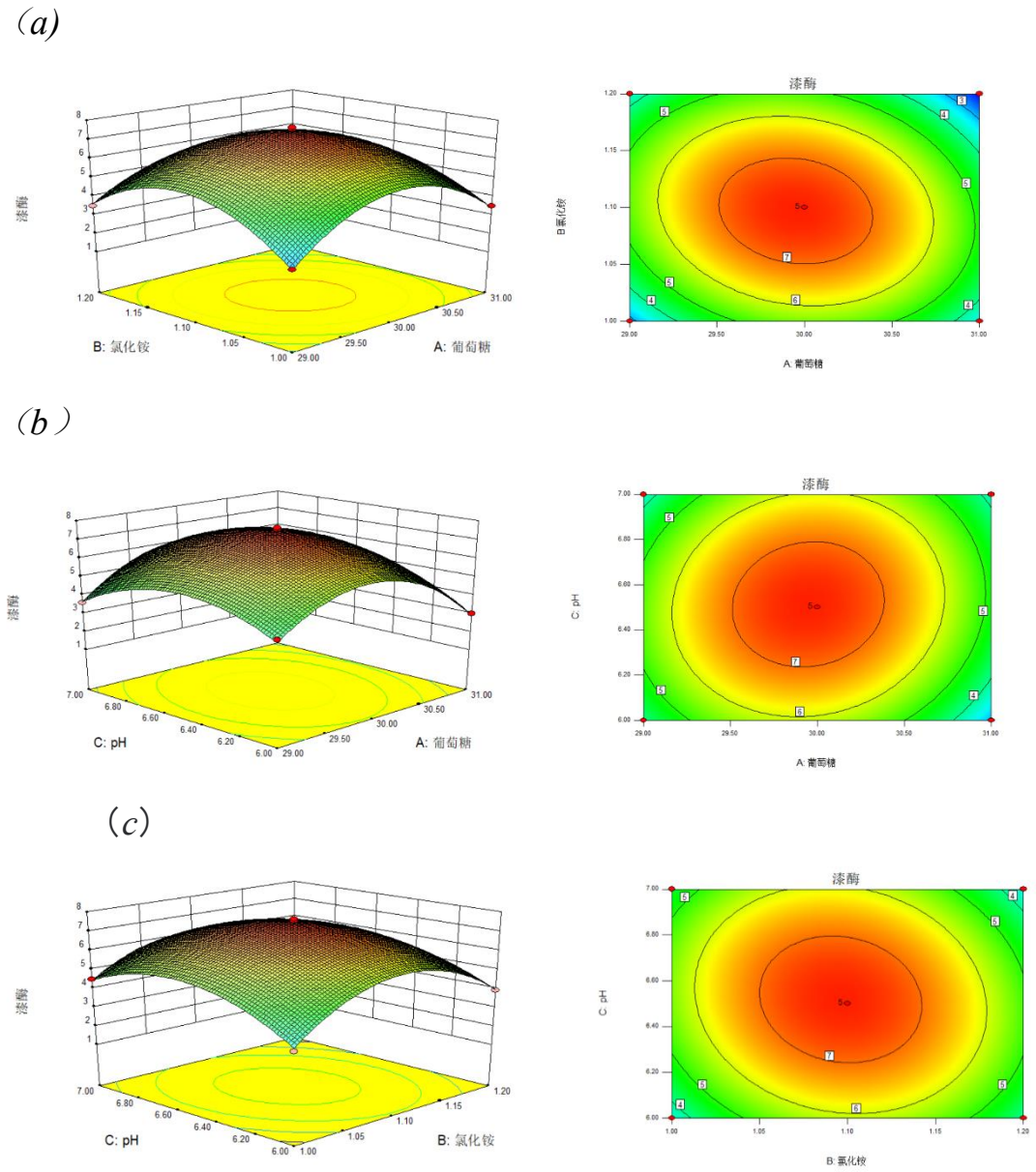

Figure 3.1. **Response surface curve and contour diagram showing the obtained dependencies**

The response surface was formed by fitting the equation. Fig. 3.1 (*b*) shows the response surface diagram and contour diagram of glucose addition and pH to laccase production, respectively. When other factors were the optimal value, the laccase yield showed a trend of decreasing from low to high with the continuous increase of glucose addition, and the change amplitude was obvious, indicating that

this factor had a great influence on the yield of laccase. With pH, the yield of laccase increased first and then decreased, and the change range was large, indicating that pH had a significant effect on the yield of laccase.

The response surface was formed by fitting the equation. Fig. 3.1 (c) shows the response surface diagram and contour diagram of the addition of ammonium chloride and pH to the yield of laccase, respectively. When the other factors were the best value, the laccase yield increased first and then decreased with the increase of ammonium chloride supplemental level, which showed that this factor had a significant effect on the yield of laccase. With the increase of pH, the yield of laccase increased first and then decreased, and the change range was large, indicating that this factor had a significant impact on the yield of laccase, indicating that the interaction between the two factors was obvious.

Conclusions to chapter 3

The experimental data and model were analyzed by software, and the optimum fermentation conditions of laccase fermentation were obtained: the addition of glucose was 9.95 g, the addition of ammonium chloride was 10.97 g, the pH was 7.52, and the predicted value of laccase activity was 7.4525 U /mL. In order to test the accuracy and feasibility of the response surface method, the best conditions were selected to carry out the shaking flask fermentation experiment of laccase, and the experiment was repeated three times. The average laccase activity was 7.381 ± 0.065 U/mL. The result is not much different from the predicted value of the model, which proves the accuracy of the model and has practical value.

CONCLUSIONS

The main research content of this paper is to use Escherichia coli for laccase fermentation, and take various inducers and pH of the medium as the main research objects. After basic passage culture of Escherichia coli, it is inoculated into the medium, and single factor experiments are carried out under different ammonium chloride content, glucose content and pH of the medium respectively. According to the properties of the reagent ABTS for measuring laccase activity, the maximum absorption wavelength of laccase activity measured was determined, and the activity size and yield of laccase under each component condition were determined. Based on the three single factor experiments, the Box-Behnken response surface method with three factors and three levels was conducted to determine the best technological conditions for laccase fermentation. The software was used to analyze the experimental data and model, and the optimal fermentation conditions for laccase fermentation production were as follows: the addition of glucose was 9.95g, the addition of ammonium chloride was 10.97g, the pH was 7.52, and the predicted value of laccase activity was 7.4525 U/mL. At this time, the yield of laccase also reached the highest.

This experiment mainly involves single factor experiment and response surface experiment. Single-factor experiment, as the name implies, is to change only one factor in each experiment while ensuring that other influencing factors remain unchanged. This experimental method requires more experiments and a longer experimental period when it involves too many factors to be examined, which costs a lot of energy and high cost. However, because this experimental method does not need to involve mathematical and statistical knowledge, and the experimental operation is simple, the experimental results are more intuitive, and the use of observation and recording is also one of the common methods for biological process optimization. However, due to the obvious defects of single-factor experiments and the difficulty in finding the best combination of influencing factors, statistical optimization method is adopted in many biological process optimization, so that the best experimental method can be selected

with fewer experiments, and the main and secondary influencing factors and the influence rules on the experiment can be quickly distinguished from the more factors.

The response surface method is most commonly used to optimize the culture of bacterial laccase, to study the influence of each influencing factor variable on a corresponding variable. In practical applications, it is mainly to obtain experimental data under different influencing factors through experiments, and then use mathematical statistics to plan tests, calculate response values, analyze and estimate variance, and find the best influence level, reduce the number of tests, and provide more intuitive graphics to facilitate research and analysis. In this experiment, the influencing factors in the production process of laccase were optimized through response surface experiment, and the best method to increase the yield of laccase was added. The optimal conditions were selected to carry out the shaking flask fermentation experiment of laccase, and the experiment was repeated for three times. The obtained results had little difference with the predicted value of the model, which proved the accuracy and feasibility of the model.

Escherichia coli laccase has broad application prospects in various fields due to the universality of its substrate, but the low production of laccase leads to its high industrial cost and limits the scope of application. Therefore, increasing the production of laccase is expected to reduce its production cost and expand the production scale of laccase, so that laccase can be widely used in major industrial production and various industries. To promote the rapid development of industry and other industries, it is also necessary to continue to find the best fermentation process conditions to improve the yield of laccase in the future research.

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