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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 **Application of Biological Enzymes in Purification of Sulfate Lignin from Broad leaved Wood**

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Completed: student of group BEBT-20
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Faculty: Chemical and Biopharmaceutical Technologies
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ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Chen Zhuo

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SUMMARY

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Sulfate pulping is a chemical pulping method that uses alkaline solution to cook plant fiber raw materials, and the black liquor produced by this method contains a large amount of organic compounds such as lignin and sugars. Lignin is a renewable natural polymer compound with great development and utilization value.

Black liquor has a complex composition, in which lignin will combine with various organic compounds through chemical bonds or other forces, which is not conducive to its further modification and utilization. Therefore, how to maximize the purity of lignin is of great significance for lignin development research.

In this project, one of the lignin purification methods, enzymatic hydrolysis, was mainly investigated, using biological enzymes to hydrolyze off the sugars connected to lignin, together with H+ to efficiently break the LCC bond on the lignin structure, thus improving its purity, and analyzing and comparing the purification effect of this method with that of the traditional acid precipitation method from different perspectives, to prove its applicability.

The main purpose of this project is to compare the advantages and disadvantages of different treatments for the purification of lignin by exploring the optimal conditions and dosage of alkali-resistant cellulase and xylanase, and by applying the characterization means of ion chromatography for the determination of sugar content, Fourier infrared spectroscopy for the determination of the change of functional groups, permeation gel chromatography for the determination of molecular weight size, and UV spectrophotometry for the determination of UV absorbance strength and weakness. The results showed that the cellulase dosage of 100 U/L and the reaction at pH=9 and 45 ℃

for 80 min was optimal, and the xylanase dosage of 100 U/L and the reaction at pH=9 and 40 ℃ for 100 min was the best; moreover, it was found that the biological enzyme could better destroy the structure of LCC after characterization, and the carbohydrates therein were degraded into monosaccharides, such as xylose, which greatly improved the purity of lignin; the purity of lignin obtained from the purification of black liquor using cellulase and xylanase was approximate, 93.8% and 92.9%, respectively, which were higher than that of 86.6% in the traditional acid precipitation method.

Keywords: Black liquor，lignin, bio-enzyme, separation and purification, LCC, xylanase, cellulase

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INTRODUCTION

Relevance of the topic. Separation and recovery of lignin and other polymers from cooking black liquor is one of the effective ways to effectively use cooking black liquor and one of the important steps in biomass processing technology. Among them, lignin extraction and purification by biorefinery is an environmentally friendly technology with simple operation, low energy consumption, no secondary pollution and mild reaction conditions. At present, many raw materials for lignin purification by biological method have been researched by many domestic scientists, and some of them have been found to be effective.

The purpose of the study is to purify sulfate lignin from broadleaf wood by using a green bioenzymatic method to break the strong bonds between lignin and carbohydrate complex.

The objectives of the research are to optimize the technology for producing lignin from black liquor in order to save natural resources and reduce environmental pollution. To solve these problems, it is necessary:

1. Perform an analysis of the components of black liquor pulp using the sulfate method;
2. To study the effect of cellulase purification on sulfate lignin by determining the sugar content, molecular weight and ultraviolet absorbance after the enzymatic reaction;
3. Investigate the effect of xylanase purification on sulfated lignin by determining the sugar content, molecular weight and ultraviolet absorbance after the enzymatic reaction.

The object of the study is a bioenzymatic method for the purification of sulfate lignin, as well as black liquor, sulfate lignin of broad-leaved wood, biological enzymes in the form of xylanase and cellulase.

The subject of the study is the influence of the conditions of the bioenzymatic purification method on the structure and properties of sulfate lignin of broad-leaved wood.

Research methods. In the work we used:

1. Methods of liquid chromatography, ultraviolet spectroscopy and ion chromatography to determine the content of a number of components in black liquor (solids, ash, sugar, lignin);

2. One-way method for analyzing cellulase and xylanase (determining the optimal pH and temperature, reaction time and enzyme dosage;

3. Infrared spectroscopy method to compare the structure (functional groups) of enzymatically purified lignin and acid deposition lignin;

4. Liquid chromatography method to compare the molecular weight of enzymatically purified lignin and acid precipitation lignin;

5. Ultraviolet spectroscopy method to compare the UV absorption of lignin from different purification methods.

Scientific novelty is to substantiate the feasibility of a new method for purifying lignin from broad-leaved wood using bioenzymes to eliminate the shortcomings of the traditional acid deposition method. At the same time, to avoid the influence of pH on the activity of bioenzymes in black liquor, the bioenzymes used in the work were replaced with alkali-tolerant bioenzymes; this improved the effect of alkaline treatment on LCC and lignin purity.

The practical significance of the results obtained lies in finding a rational way to utilize black liquor in order to transform it into a valuable resource.

Scope and structure of work

The qualifying work consists of an introduction, three chapters, a conclusion, and a list of references (60 titles in total).

CHAPTER 1
LITERATURE REVIEW

1.1 Overview and basic properties of black liquor

1.1.1 Source of black liquor

Paper industry for the national economic development and social civilization enhancement has a strong role in promoting the development of the paper industry in many countries around the world and the growth rate of gross domestic product has a close link. 2014 China surpassed the United States to become the largest country in the production of paper and paperboard products, with a total output of up to 1.04 billion tons [1]. The plant raw materials used in papermaking are mainly cellulose, hemicellulose (polysaccharides) and lignin, of which cellulose is the active ingredient in papermaking and pulping, while lignin, hemicellulose, and caustic soda added in the pulping become the main components of black liquor [2]. The source of black liquor is very wide, but most of them are discharged into rivers and lakes in the form of wastewater. At present, except for a few paper mills with large production scale using concentrated combustion method to recover alkali and energy, the black liquor of more than 5000 mills with an annual production capacity of less than 5000t in China is discharged directly without treatment, and the pollution problem brought by it should not be ignored [3]. Therefore, to find a reasonable way to properly treat the black liquor so that its waste into treasure has become one of the priorities of environmental protection, and is of great significance to the realization of sustainable development.

1.1.2 Ingredients in black liquor

In the pulp and paper making process, part of the ash in the wood will react with NaOH to generate Na_2SiO_3, and the pigment in the raw material, pectin, etc. will also react with alkali to generate color-bearing substances, which will deepen the color of the pulp. Therefore, the black liquor contains a variety of substances, of which 1/3 is inorganic, mainly KCl, $CaSO_4$, Na_2SiO_3, etc., and 2/3 is organic, mainly including

lignin, hemicellulose, sugars, etc. [4, 5], and lignin accounts for the majority of the content of organic matter [6, 7, 8].

1.1.3 Treatment of black liquor in papermaking

The lignin and cellulose, as well as xylose, nitrogen, phosphorus and potassium contained in black liquor are highly utilized in agriculture and industry. However, traditionally, black liquor is usually incinerated to generate steam for energy recovery [9, 10]. This treatment method is relatively simple, but it wastes fuel and causes atmospheric pollution, which cannot solve the problem fundamentally. In addition, the common traditional black liquor treatment methods such as acid precipitation method, alkali recovery method [11], precipitation filtration method, coagulation precipitation method, electrodialysis technology [12], not only have the disadvantages of poor filtration effect, high requirement of conditions, high cost and difficult to be popularized, but also cause a waste of useful substances in black liquor.

In addition, the emerging methods such as membrane treatment [13] and persulfate advanced oxidation treatment technology [14, 15] in recent years, although they can remove pollutants efficiently and are environmentally friendly, have the shortcomings of high investment and operation costs and high energy demand. Therefore, the harmless treatment and high-value utilization of pulping black liquor can not only solve the current black liquor treatment problem, but also provide a green and sustainable solution to the energy saving and emission reduction target of paper enterprises [16], which meets the national "dual-carbon" strategic policy [17]. The bio-enzymatic method used in this project has the advantages of strong specificity, green and non-polluting, which is very suitable for the theme of sustainable development.

1.2 Lignin characterization and resource utilization

1.2.1 Overview of lignin

Lignin is a renewable aromatic ring-containing macromolecular organic polymer in nature [18]. It is widely found in nature, especially in herbaceous and woody plants. It enhances the capriciousness of plant cell walls, increases resistance to water erosion and external damage, and is the strongest chemical component of plant cell walls [19]. For a long time, lignin, as a by-product of sulfate-based pulping, is often discarded as a residue in the biomass refining process or used as a heat source in the alkali recovery combustion section to be directly incinerated [20], and only a very small portion of it is used to prepare low-value chemicals by modification or compounding [21].

Trapped by technical difficulties such as biosynthesis, molecular structure, purification and separation, and chemical modification, lignin has been difficult to be utilized at high value and unable to produce high economic value. Therefore, exploring the ways of high value utilization of lignin to enhance the economic competitiveness is an important issue that needs to be solved urgently for the utilization of lignin at present [22].

1.2.2 Structure of lignin

Natural lignin is an amorphous, three-dimensional aromatic polymer formed by the dehydrogenation and polymerization of three alcohol monomers (coniferyl alcohol, erucyl alcohol, and p-coumarol), which correspond to the three basic phenylpropane unit structures constituting lignin, i.e., the guaiacylphenylpropane unit (G), the silylpropylphenylpropane unit (S), and the p-hydroxyphenylpropane unit (H), respectively. The three alcohol monomers of lignin with their respective corresponding three basic unit structures are shown in Figure 1.1.

In addition, the composition of lignin varies depending on the type of biomass, softwoods such as conifers are more primitive plants with a more homogeneous lignin type consisting mainly of G (90%-95%) monomer, broadleaves are hardwoods and belong to the post-evolutionary plants with an increase in the lignin type over the

former, with G and S monomers accounting for about 50% each [23], and herbaceous plants have a richer lignin type, with H, G and S monomers are present [24]. The guaiacylphenylpropane unit has a number of positions on the benzene ring where it can react, generating a large number of C-C bonds and interconnecting them, so that the average molecular weight of coniferous lignin tends to be very high, and broadleaf lignin is usually more readily decomposed [25].

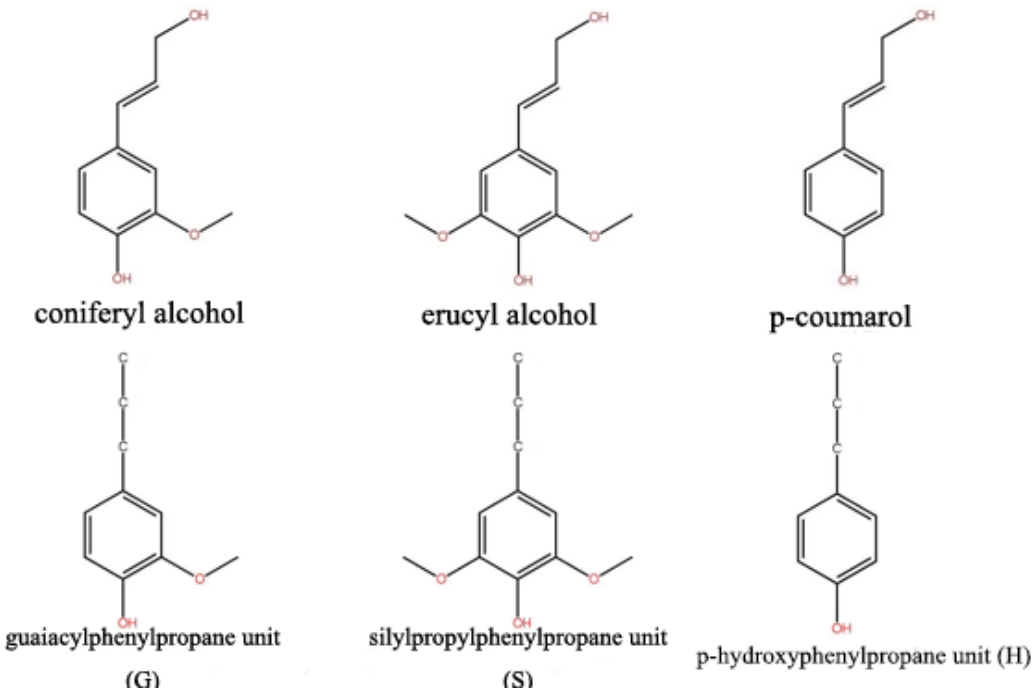

Figure 1.1 – **Alcohol monomers of lignin with their corresponding basic unit structures** [26]

The basic structural unit of lignin is mainly composed of C-C bonds (β–5, 5–5, β–1, β– β) and C–O-–C bonds (β–O–4, 4–O–5, α–O–4), which account for 30%~40% and 60%~70% of lignin, respectively, and the aryl ether bonds of β–O–4 have the highest number of occurrences, and it is related to the types of plants, usually the frequency of β–O–4 bonds is the highest in broad-leaved plants, followed by coniferous plants, and the lowest is herbaceous plants [27]. O–4 bond is the most frequent in

broad-leaved plants, followed by coniferous plants, and the lowest is herbaceous plants [27]. In addition, the content of β–O–4 bonds is related to lignin reactivity, the higher the content, the higher the lignin activity [28]. As shown in Figure 1.2, the molecular structure of lignin monomers contains a variety of reactive functional groups, such as hydroxyl, carboxyl, carbonyl, methyl, and side chain structures. Among them, the hydroxyl groups are categorized into phenolic and alcoholic hydroxyl groups, which can exist either in free form or connected to alkyl and aromatic groups through ether bonds [29].

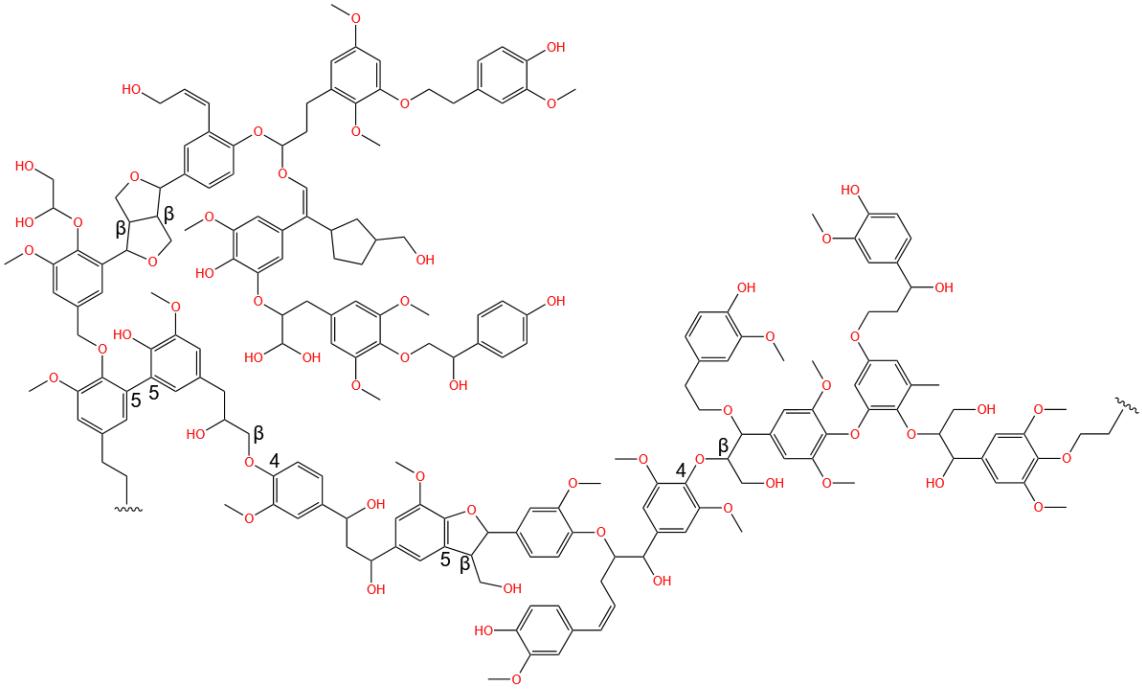

Figure 1.2 – **Bonding structure and functional groups of lignin** [31]

Due to the diversity of groups, lignin can undergo a variety of chemical reactions such as oxidation, reduction, halogenation, alcoholysis, nitration, acid demethoxylation, condensation, hydrolysis, sulfonation, alkylation, photolysis, or graft copolymerization. The phenolic-type structural unit in lignin determines its hydrophilicity, which makes it difficult to hydrolyze, and at the same time measures the solubility properties and reactivity of lignin. Ester-type structures such as vanillic acid, ferulic acid, p-hydroxybenzoic acid, lilac acid, and p-coumaric acid are present at the α-position or γ-

position of the side chain [30]. Meanwhile, lignin has a three-dimensional space structure that gives it a certain compressive strength and rigidity [25].

1.2.3 Resource utilization of lignin

As the second largest natural polymer material, lignin contains a variety of active functional groups in the molecule, but over the years, due to its high value utilization has not been found, 95% of the lignin is directly discharged with the pulping waste stream or concentrated and incinerated, and less than 2% of the lignin is converted into high value lignin sulfate and lignin sulfonate and other industrial products every year [32]. This leads to both environmental pollution and waste of resources [33]. It can be seen that the resource utilization of lignin is of great significance in promoting economic development and environmental protection. Some studies have shown that lignin can be applied to polymers such as carbon fiber, wood adhesives, furfural and polyurethane foam [34, 35, 36]. In recent years, researchers have attempted to prepare lignin nanoparticles (LNPs) with uniform size and morphology from complex lignin feedstocks; Zhongshan Wang et al. prepared LNPs using different methods, such as mechanical and self-assembly methods, and explored their application in hydrogel materials [37]; and Mang Zhang prepared LNPs based on ultrasonically synergistic antisolvent method and applied lignin/surfactant nanofluidics to improve crude oil recovery [38]. In addition, lignin can be prepared as potassium, sodium, and ammonium salts by modifying it through chemical reactions such as hydroxymethylation, amination, sulfomethylation, condensation, or graft copolymerization, which can endow it with good chelating, adhesion, holdout, and surface activity and adsorption [39]. Modified lignin can be used as adsorbents [40, 41], catalysts [42], fertilizer slow-release materials [43], etc. Lignin-based surfactants are also widely used as asphalt emulsifiers, concrete water reducing agents, etc [44]. If all biomass resources in nature, including lignin, can be fully utilized, their economic

benefits will be greatly improved, and they can also promote environmentally sustainable development.

1.3 The main connections of lignin to cellulose and hemicellulose in black liquor

1.3.1 Lignin-hemicellulose linkages

There are more complex interactions between lignin and hemicellulose, including covalent and noncovalent bonds, in which the lignin-carbohydrate complex (LCC) formed by covalent bonding connection possesses both the mechanical strength and hydrophilicity of lignin, biocompatibility of polysaccharides, hydrophobicity, and resistance to pathogenicity, which lead to the recalcitrant anti-degradation of lignin [45], which seriously hinders the separation of lignin and cellulose and hemicellulose separation. Hemicellulose is a multibranched low molecular polymer composed of non-crystalline complex glycans, while lignin is an aromatic polymer, and the two are crosslinked together through component deposition, biosynthesis and other processes during the differentiation of the plant cell wall, and they form covalent bonds of the LCC to a different extent and in a different way. The researchers used wet chemistry to isolate and characterize the LCCs and found that lignin and hemicellulose are mainly connected by ester and ether bonds.

1.3.2 Lignin-cellulose linkages

Due to the large number of hydroxyl groups on the surface of cellulose, it can bind to lignin precursors through electrostatic interactions (mainly including hydrogen bonding, spatial interactions between 2 dipole molecules, and London dispersion forces between atomic molecules), so that the aryl ring structure of lignin macromolecules can be located parallel to the surface of cellulose [46].

It was found that the larger the binding area of cellulose and lignin, the stronger the interaction force between the two, in which the methoxyl contained in lignin can increase the contact with the hydroxyl group of cellulose and form ether bonds, thus

increasing the interaction force between the two and enhancing the resistance of cell wall to degradation [47].

1.4 Methods of lignin isolation and purification

In plants, lignin is intertwined with cellulose and hemicellulose, which often need to be separated in order to be able to better study lignin. The separation and purification methods of lignin are generally divided into two kinds: the first is to dissolve cellulose and hemicellulose and other components, and the remaining insoluble component is lignin. The other method is to dissolve lignin as a soluble substance, separating cellulose and other insoluble components. However, the lignin yield of this method is low [48]. Due to the different mechanisms used to isolate lignin, biomass may have different degrees of chemical bond cleavage or generation during the reaction process, which can lead to different structural compositions, contents and chemical properties of lignin [49].

1.4.1 Enzymatic purification

Enzymatic method mainly uses biological enzymes to break the LCC barrier, destroy the connecting bond between lignin and carbohydrates, and promote the hydrolysis of hemicellulose and cellulose, so as to realize the efficient purification of lignin. Bio-enzymes are known for their high efficiency in trace amounts, and their advantages of specific reactants, non-corrosive to equipment, green and non-polluting are obvious. Commonly used biological enzymes mainly include cellulase and xylanase. For example, Song [50] prepared alkaline sulfite black liquor (ASBL) and soda pulping black liquor (SPBL) from corn stover as raw material in the laboratory, and purified the lignin in the two kinds of black liquor raw materials by using composite enzymes. The results showed that after 100 mL of black liquor reacted with 20 mL of composite enzyme at 50 ° C for 6 h, the purity of lignin in the two kinds of black liquor and precipitates was greatly increased, and the final ratios of lignin to

organics in ASBL and precipitates were 79.56% and 81.23%, and the ratios of lignin to organics in SPBL and precipitates were 79.52% and 84.23%, respectively. Enzymatic digestion in combination with other methods also produces good results in the purification of lignin. For example, Wu [51] et al. purified lignin from spruce wood and poplar wood using enzymatic digestion + mild acid digestion. The results showed that cellulase could hydrolyze nearly 90% of the carbohydrates in the samples, and when another 0.01 mol/L acid was added, the carbohydrates in the samples were basically hydrolyzed, and the purification effect was remarkable [52]. The basic principle is that the biological enzyme cleaves the cross-linking structures (e.g., β-1, 4-glucosidic bonds in cellulose and β-1, 4-xylosidic bonds in hemicellulose) in the black liquor, which enables the acid to act better on the LCC structure on lignin, resulting in better detachment of small molecular weight lignin and reduction of the dosage of acid.

1.4.1.1 Xylanase

Xylan, the second most abundant renewable resource, is the main component of hemicellulose, and its basic structure consists of a main chain of β-D xylose tandemly linked to a relatively short and varied glycosyl group in the side chain. Xylan is heterogeneous, so its hydrolase is not one enzyme, but a group of enzymes. They mainly include endonuclease β-1, 4-xylanase, which cleaves the main chain, α-D glucuronidase, α-L-arabinofuranosidase, acetyl xylan esterase, and β-D-xylosidase, which acts on xylo-oligosaccharides, for example, to break the side-chain moiety [53]. Among them, endonuclease β-1, 4-xylanase mainly cuts off the xylan glycosidic bonds on the main chain randomly, thus fragmenting the macromolecular hemicellulose into oligosaccharides with different molecular weights, and therefore the content of this enzyme determines the effect of xylanase [54]. It has been shown that xylanase is highly effective in hydrolyzing both cellulose and hemicellulose [55]. Xylanase can separate hemicellulose from lignin, and its mechanism is mainly by destroying the LCC

structure, so as to achieve the effect of lignin purification. For example, Jiang Zhengqiang [56] and others found that xylanase can degrade hemicellulose in raw materials and destroy the LCC structure in it through the process of wheatgrass pulping; De Alencar et al. also proved in their study that the action of xylanase can destroy the connecting bonds in lignin-carbohydrates [57].

1.4.1.2 Cellulase

Cellulase is a complex enzyme consisting of β-glucosidase, endoglucanase, exoglucanase and other enzymes with a single function [58]. Under the combined action of multiple enzymes, cellulases hydrolyze cellulose into oligosaccharides and small molecules of glucose of varying lengths. Leschine S B. [59] found that cellulases efficiently hydrolyze β-1,4-glucosidic bonds in cellulose, in which endoglucanase releases new ends by breaking internal bonds in the amorphous region of cellulose, and fibridibioside hydrolases can act on the endoglucanase generated or existing ends to degrade the crystalline region of cellulose.

Conclusions to chapter 1

1. Black liquor comes from a wide range of sources and is not yet well utilised, but it contains a variety of substances that have potential for use.

2. Lignin is highly utilised and purification methods are very diverse, but each has its own advantages and disadvantages.

3. Enzymatic purification of lignin is more efficient and less polluting.

CHAPTER 2
OBJECT, PURPOSE AND METHODS OF THE STUDY

2.1 Object, purpose and methods of the study

The purpose of the study is to purify sulfate lignin from broadleaf wood by using a green bioenzymatic method to break the strong bonds between lignin and carbohydrate complex.

The object of the study is a bioenzymatic method for the purification of sulfate lignin, as well as black liquor, sulfate lignin of broad-leaved wood, biological enzymes in the form of xylanase and cellulase.

The subject of the study is the influence of the conditions of the bioenzymatic purification method on the structure and properties of sulfate lignin of broad-leaved wood.

2.2 Experimental preparation

2.2.1 Raw materials for experiments

The black liquor of sulfate method pulp used in this experiment was purchased from Huatai Paper (Shandong, China).

2.2.2 Experimental Drugs

The experimental drugs were alkali-resistant cellulase, xylanase, pyridine and tetrahydrofuran with specifications detailed in Table 2.1.

Table 2.1 – **Main experimental reagents**

№	Experimental reagents	Norm	Manufacturer
1	*2*	*3*	*4*
1	HCl	99%	Tianjin Guangfu Fine Chemical Research Institute
2	H_2SO_4	99%	Tianjin Guangfu Fine Chemical Research Institute

Continuation of Table 2.1

1	*2*	*3*	*4*
3	Alkali-resistant cellulase	3000 μ/g	Xia Sheng Biological (Beijing, China)
4	Alkali-resistant xylanase	2364 μ/g	Xia Sheng Biological (Beijing, China)
5	Pyridine	AR grade	McLean Reagent Co.
6	Acetic anhydride	AR grade	McLean Reagent Co.
7	Tetrahydrofuran	AR grade	McLean Reagent Co.
8	Potassium Bromide	Spectral Grade	Aladdin's Reagent

2.3 Laboratory Instruments

The experimental instruments include electric constant temperature blast drying oven, ion chromatography, ultraviolet spectrophotometer, gel permeation chromatography, Fourier transform infrared spectrometer. Specific specifications are detailed in Table 2.2.

Table 2.2 – **Main Experimental Instruments**

№	Name of Instrument	Norm	Manufacturer
1	Ion Chromatography	ICS5000+DC	Thermo Fisher Scientific
2	UV Spectrophotometer	Shimadzu UV-2600	Shimadzu, Japan
3	Gel Permeation Chromatography	Waters	Shimadzu, Japan
4	Fourier Transform Infrared Spectrometer	ALPHA	Thermo Fisher Scientific

2.4 Experimental content

2.4.1 Exploring the optimal reaction conditions for two enzymes

In this experiment, three single-factor variables were set up by the controlled variable method, which were the degradation rate of substrate by three different factors,

namely, enzyme digestion temperature, enzyme digestion time and pH, and the design of the single-factor experiments is shown in Table 2.3. The pH gradients were set up as follows: 8.0, 8.5, 9.0, 9.5, and 10.0, respectively; the enzyme digestion temperatures were set up as follows: 35 °C, 40 °C, 45 °C, 50 °C, and 55 °C, respectively; and the enzyme digestion times were set up as follows: 40 min, 60 min, 80 min, 100 min, and 120 min, and a total of 15 sets of experiments were performed, one for each of two bio-enzymes. The enzyme digestion temperature was set at 35 ℃, 40 ℃, 45 ℃, 50 ℃ and 55 ℃, and the enzyme digestion time was set at 40 min, 60 min, 80 min, 100 min and 120 min, with a total of 15 groups of experiments.

The specific steps of the experiment were as follows: weigh 50 mL of black liquor in a 100 mL conical flask, add 20 U/L of different biological enzymes after adjusting the pH and put them into the shaking table under different conditions for digestion, and then put them into the 85 ℃ water bath for inactivation after digestion. The black liquor at the end of the reaction was taken and diluted 200 times, and the sugar content in the black liquor was determined by ion chromatography, reflecting the optimal reaction conditions for the two enzymes respectively.

Table 2.3 – **Single-factor reaction condition setting**

№	pH	Temperature（℃）	Time（min）
1	8.0	35	40
2	8.5	40	60
3	9.0	45	80
4	9.5	50	100
5	10.0	55	120

2.4.2 Determination of the optimum amount of both enzymes

Through the optimal reaction conditions for enzymatic digestion determined in step 2.4.1, a one-way experiment was designed to investigate the changes in the

degradation rate of the substrate by the dosage of the two enzymes, and the gradient of the enzyme dosage was set at 20 U/L, 40 U/L, 60 U/L, 80 U/L, 100 U/L, 120 U/L, 140 U/L, 160 U/L and 180 U/L, with a total of nine sets of experiments.

The specific experimental steps were as follows: weigh 50 mL of black liquor treated with cellulase under optimal conditions, put it into a 100 mL conical flask, and add different dosages of alkaline-resistant cellulase with gradient setting with the optimal pH, temperature, and reaction time as the experimental conditions determined in step 2.4.1, and then put it into a water bath at 85 ℃ to inactivate the black liquor for 15 min after the reaction in a shaking bed. cool it down to room temperature and then take the two kinds of enzymes respectively. After cooling to room temperature, the two enzymes were taken and diluted 200 times each in the black liquor treated with different dosages, and the sugar content in the black liquor was determined by ion chromatography, reflecting the optimum amount of the enzymes.

Weigh 50mL of black liquor treated with xylanase under the optimal conditions, put it into a 100mL conical flask, take the optimal conditions in step 2.4.1 as the reaction conditions, add different dosages of alkali-resistant xylanase under the gradient setting, and the subsequent operation is the same as above.

2.4.3 Determination of lignin purity

0.1 g of lignin prepared by each of the three methods at pH=4 [60] in step 2.2.3 was taken into a digestion tube, and 8.7 ml of H2SO4 solution at a concentration of 4% was added and held at 121 °C for 1 h.

The supernatant was taken at the end of the digestion and diluted 200-fold, and filtered through a 0.22 μm inorganic needle filter into a sample vial for determination of the sugar content by ion chromatography. The amount of heterosaccharides on lignin was determined using a column CarboPac PA20 (3 mm×150 mm), an EC detector (Au electrode for the working electrode and Ag/AgCl electrode for the reference electrode) with an injection volume of 25 μL, a column temperature of 30 ℃, and a gradient

drenching of the mobile phases of 250 mm/L NaOH and distilled water at a flow rate of 0.4 mL/min. The amount of heterosaccharides on lignin was then calculated. purity of lignin.

2.4.4 Determination of the molecular weight of the resulting lignin

The black liquor treated and untreated under the two groups of optimal conditions and optimal enzyme dosage were taken, and the pH was adjusted to 6, 5, 4, 3 and 2 with 72% H_2SO_4 sulfuric acid in a graded manner, respectively, and the black liquor was centrifuged for each grade, and the supernatant was poured off after centrifugation twice at each grade, and the precipitate was removed after washing with deionized water for 2~3 times. The precipitated lignin was lyophilized to obtain lignin powder.

A certain mass of lignin powder (50~100 mg) was weighed in a vial, and 2 mL of pyridine and 2 mL of acetic anhydride were added to the vial, and the reaction was carried out for 48 h at room temperature, protected from light, and shaken several times to fully react the lignin. At the end of the reaction, the lignin was ice-sedimented with hydrochloric acid at 4 °C, pH=2.

After ice-sedimentation, centrifugation was carried out, and the supernatant was poured off, and the acetylated lignin was lyophilized. A certain amount of acetylated lignin powder (5-10 mg) was weighed in a vial, and the lignin was dissolved at a concentration of 1 g/L using tetrahydrofuran as a solvent, and the molecular weights of different samples were measured by gel permeation chromatography (GPC).

A Waters 2695 chromatograph equipped with a series of columns of PLgel MIXED-E and a UV detector (set at 254 nm) was used for the determination. Tetrahydrofuran (THF) was used as the mobile phase at a flow rate of 0.6 mL min-1. The columns were calibrated using a range of polystyrene standards in the 2-100 kDa range. The column temperature was maintained at 40 ± 0.1 °C. The sample volume was 10μm per injection. The sample volume was 10 μL per injection.

2.4.5 Infrared spectral analysis

The lignin extracted by the three methods in step 2.4.3 was weighed 2mg each and 200mg of pure KBr in an onyx mortar, quickly finely ground and mixed well, and then placed in a mold to be pressed by a powder tablet press with a pressure control of 6 MPa and a time of 5 s. The pressed tablets were used to detect the functional groups of lignin using an ALPHA infrared spectrometer with a test wavelength of 400~4000cm^{-1}, and to compare the differences in lignin functional groups between the enzymatic and acid precipitation methods. Comparison of lignin functional groups between enzymatic method and acid precipitation method.

2.4.6 Ultraviolet spectral analysis

Weigh a certain mass of lignin powder purified by the three methods in 2.4.3 (5~10mg each) in a vial respectively, add dimethyl sulfoxide according to the ratio of 1g:1L, dissolve it fully and then dilute it 50-fold using dimethyl sulfoxide, and determine the difference in UV absorbance between lignin of the enzyme digestion method and the acid precipitation method by using an ultraviolet spectrophotometer. The wavelength of the UV spectrophotometer was 280~400 cm-1, and the scanning speed was 4 nm/s. The UV spectrophotometer was used to measure the difference in UV absorbance between the enzymatic method and the acid precipitation method.

Conclusions to chapter 2

1. Cellulase and xylanase were explored to determine the optimum pH, temperature, reaction time and enzyme dosage using a one-factor method.

2. Comparison of molecular weight differences between enzymatically purified lignin and acid precipitated lignin using liquid chromatography and other methods.

3. Comparison of the structural (functional group) differences between enzymatically purified lignin and acid precipitated lignin using infrared spectroscopy and other methods.

4. Comparison of UV absorbance changes between enzyme purified lignin and acid precipitated lignin

CHAPTER 3
EXPERIMENTAL PART

3.1 Optimal reaction conditions for biological enzymes

As can be seen from Figure 3.1, the enzymatic efficiency of alkali-resistant xylanase and cellulase is similar under different pH conditions, when the pH is 8.0~9.0 the reaction activity is greatly increased, and when the pH is 9~10 and shows a large decline trend, we can think that the optimal pH of the two enzymes is 9.

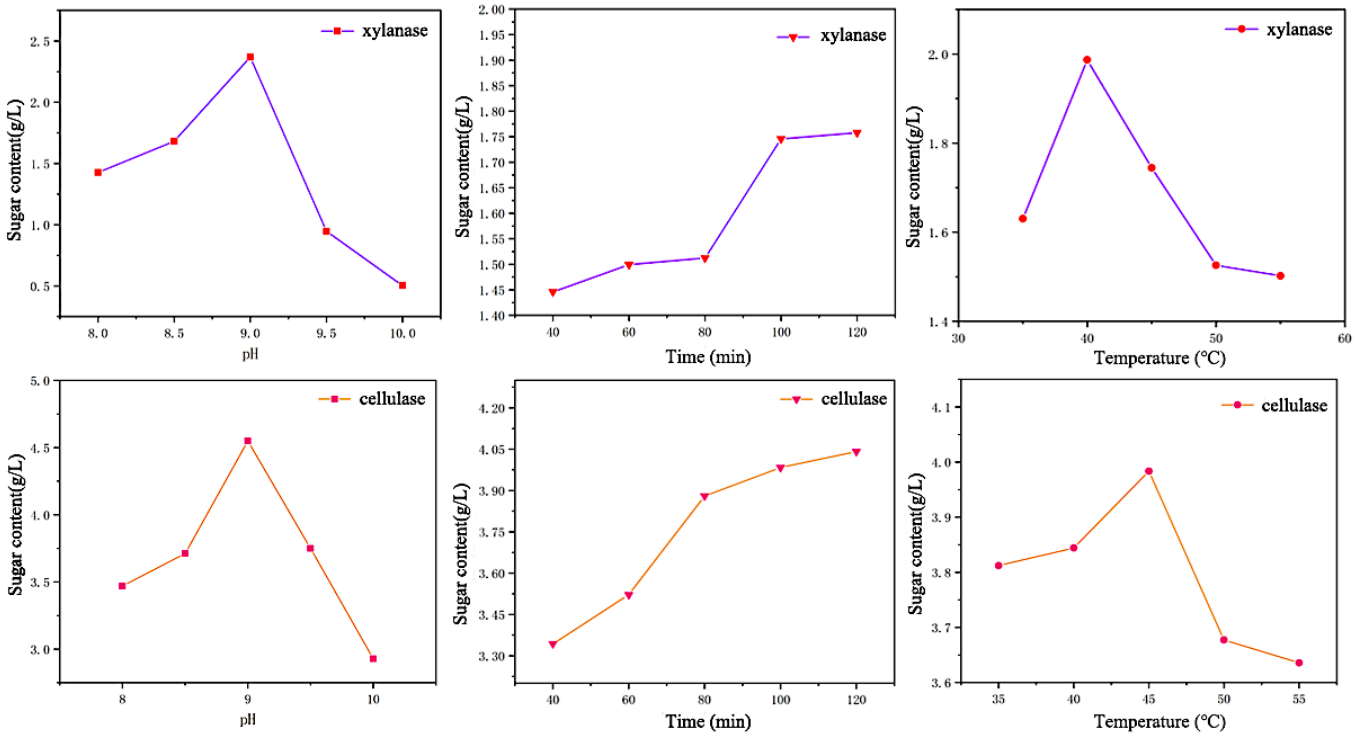

Figure 3.1 – **Changes in sugar content after reaction of two enzymes under different conditions**

However, the enzyme activity is different in the reaction of the other two conditions. The enzymatic efficiency of xylanase peaked after 100 min of reaction, and the efficiency gradually stabilized with the prolongation of time, while cellulase already showed the trend when the reaction time reached 80 min, so we think that the optimal reaction time of xylanase and cellulase are 100 min and 80 min, respectively.

similarly, xylanase has the highest reaction efficiency at 40°C, while the optimal reaction temperature of cellulase is 45°C. reaction temperature is 45°C.

In summary, the optimal conditions for alkali-resistant xylanase reaction were pH=9 and 100 min at 40°C, while the optimal conditions for alkaline-resistant cellulase reaction were pH=9 and 80 min at 45°C.

3.2 Optimal dosage of biological enzymes

Through Figure 3.2, it was found that the dosage of both enzymes was the same at 100 U/L when the sugar content in the black liquor was the highest, indicating that the reaction was the most effective, while the enzymatic efficiency was basically stable after exceeding 100 U/L. It can be seen that the optimal dosage of the two enzymes is 100 U/L.

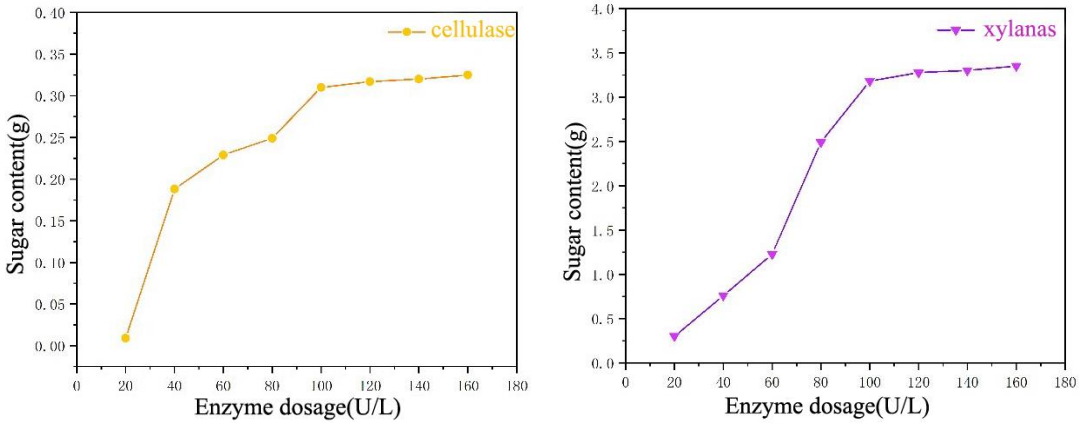

Figure 3.2 – **Reaction efficiency of two enzymes in different amounts**

3.3 Determination of lignin purity

According to Figure 3.3, it can be seen that the sugar content in the lignin obtained from the black liquor after enzymatic hydrolysis at pH=4 are less than that obtained from the acid precipitation method. This indicates that the effect of the bio-enzyme on the breaking of LCC bonds is more complete. The purity of lignin was further calculated, and it was found that the purity of lignin obtained from the lignin

stock solution of acid precipitation method was 86.6%, whereas the purity of lignin obtained from the reaction of cellulase and xylanase reached 92.9% and 93.8%, respectively, which proved the superiority of the purification of lignin by bio-enzymes.

The lignin purity calculation formula is shown in (3.1)

$$Lignin\ purity\ X = \frac{1-\sum total\ sugar\ content}{1} \times 100\% \qquad (3.1)$$

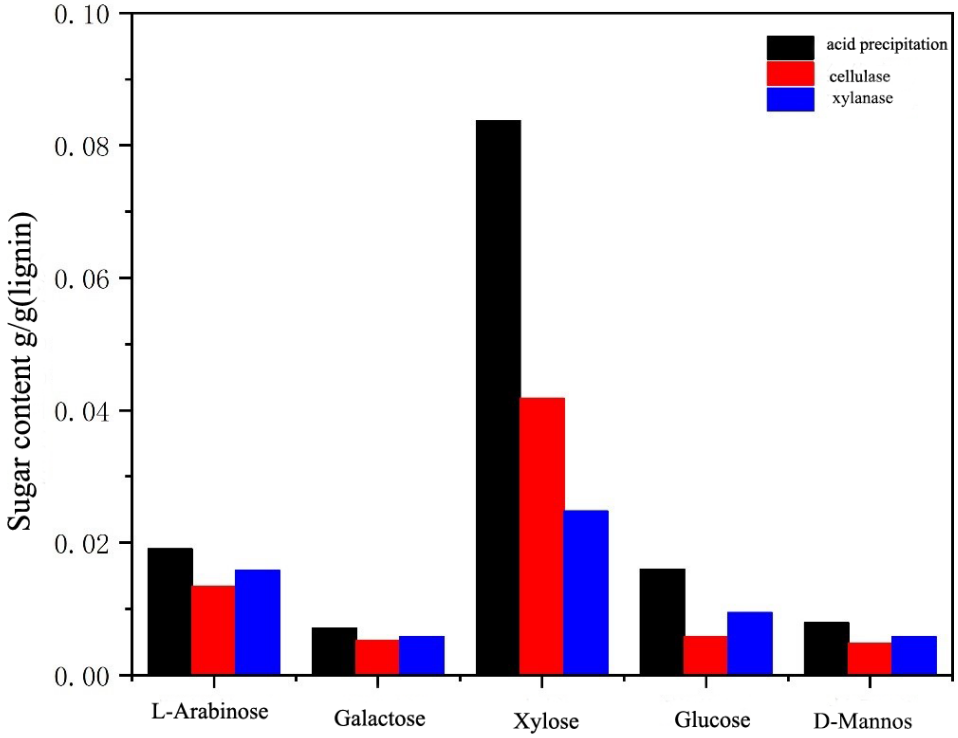

Figure 3.3 – **Comparison of sugar content in lignin obtained from three black liquors**

3.4 Molecular weight of the resulting lignin

The relative molecular masses of the components of the three black liquors and their distributions are shown in Figure 3.4 and Table 3.1. Compared with the lignin powder obtained from the untreated black liquor (Y), the Mw of the lignin obtained from the black liquor reacted by cellulase (X) and xylanase (M) under the same pH

conditions was generally lower and more homogeneous (small dispersion coefficient). This indicates that the molecular weight of lignin obtained after enzyme treatment is generally low, further proving that the addition of enzyme makes it easier for small molecular weight lignin to be precipitated.

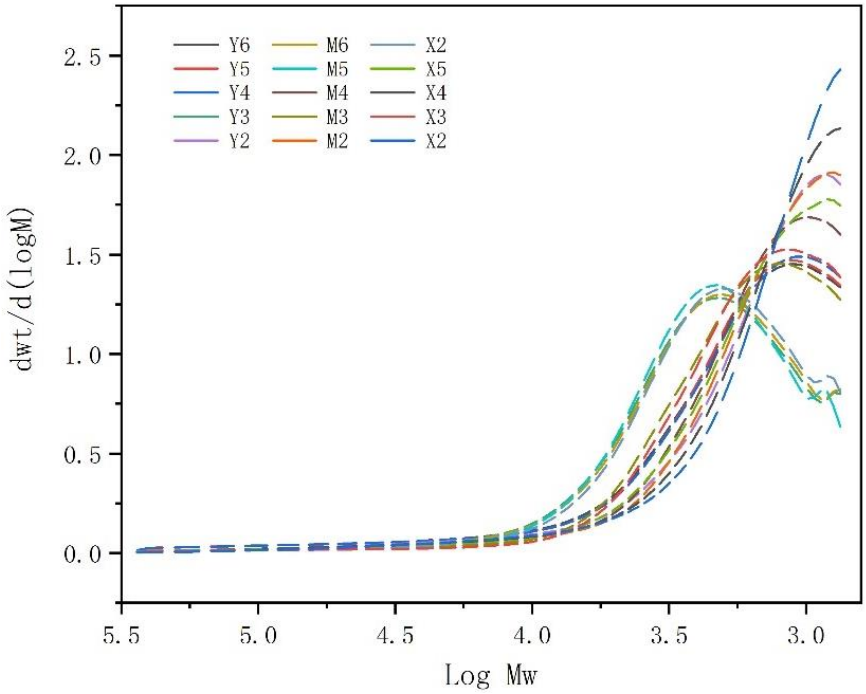

Figure 3.4 – **Relationship between logarithm of weight-averaged molecular weight and differential molecular weight distribution**

Table 3.1 – **Molecular weights of enzyme-treated lignin and acid precipitation-treated lignin at different pH conditions**

№	Mn	Mw	PDI
1	*2*	*3*	*4*
Y6	1553	6494	4.181
Y5	1540	6387	4.147
Y4	1530	6347	4.148
Y3	1894	5123	2.705
Y2	1364	4010	2.941

Continuation of Table 2.1

1	*2*	*3*	*4*
M6	1813	4947	2.729
M5	1937	4533	2.340
M4	1413	4148	2.934
M3	1528	3844	2.516
M2	1346	3683	2.734
X6	1801	4472	2.483
X5	1387	3819	2.753
X4	1304	3577	2.744
X3	1473	3559	2.416
X2	1257	3248	2.583

Y - black liquor treated by acid precipitation; M - black liquor after xylanase treatment; X - black liquor after cellulase treatment; numbers represent pH gradient; Mn: number-averaged relative molecular mass; Mw - weight-averaged relative molecular mass; PDI - dispersion coefficient.

3.5 Infrared spectral analysis

As can be seen from Figure 3.5, all the samples after enzymatic digestion showed typical lignin infrared absorption peak characteristics. For example, the strong O–H telescopic vibration absorption peak at 3328 cm-1, and the peak area is large; the characteristic absorption peak of methoxy at 2850 cm^{-1}; and the aryl ring vibration absorption peak at 1539 cm^{-1}, which indicates that the lignin extracted by enzymatic hydrolysis has obvious structural characteristics and complete structure.

The lignin obtained after enzymatic digestion at 3328 cm^{-1} on behalf of the hydroxyl group telescopic vibration peaks are weaker than the lignin obtained from the acid precipitation of the stock solution, and cellulose and hemicellulose contain a large

number of hydroxyl groups, indicating that the enzyme treatment of lignin in the impurities become less, more pure; 1160 cm-^{1} and 1008 cm-^{1} for the ether carbon-oxygen antisymmetrical telescopic vibration absorption peaks and the ester's C–O–C telescopic vibration absorption peaks, and the peaks completely disappeared after the enzyme treatment. The peak completely disappeared after the enzyme treatment, indicating that the enzyme effectively broke the ether bond, thus destroying the LCC structure; 605 cm^{-1} was the hydrocarbon bending vibration absorption peak, and the signal of this peak was not obvious in the lignin obtained after the enzyme treatment, indicating that the enzyme facilitated the full reaction between H+ and lignin, and broke a large part of the C–H bond.

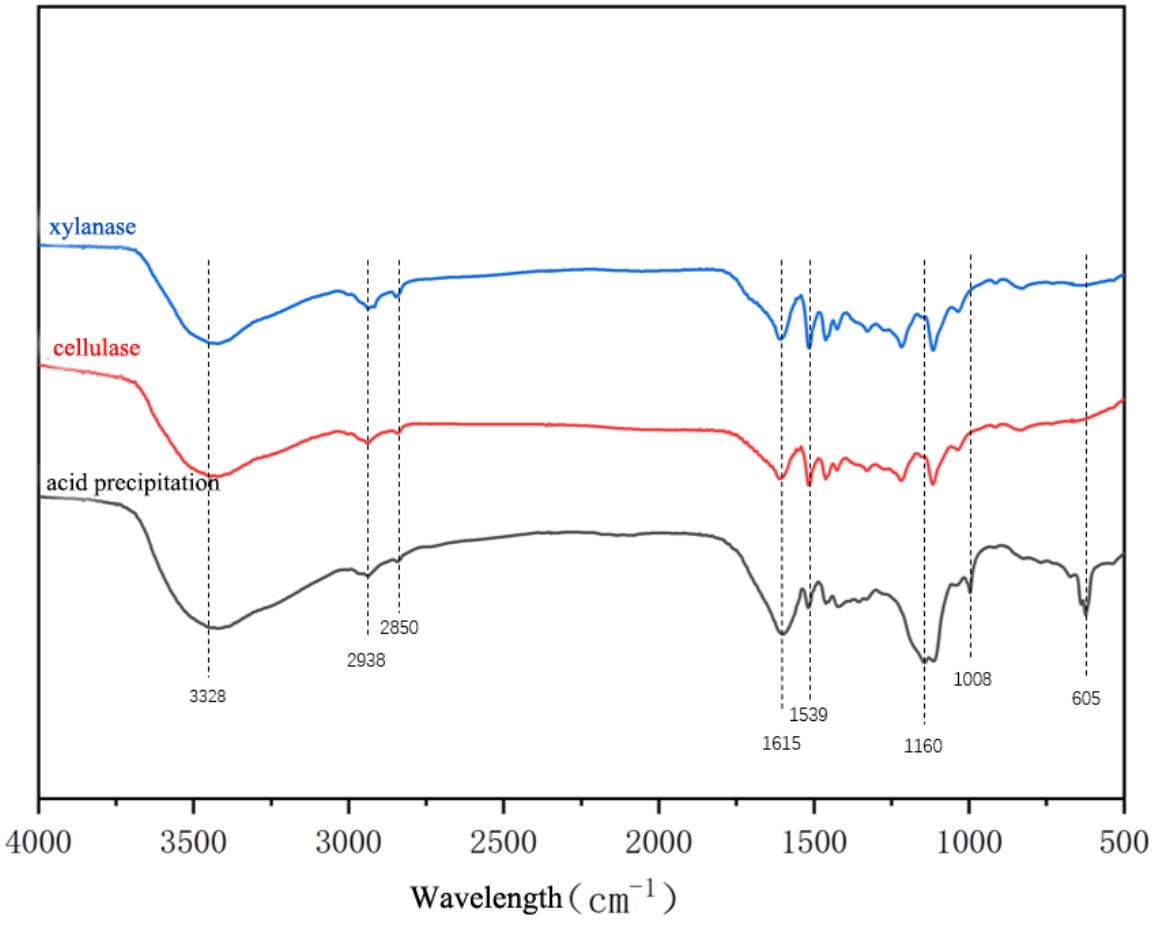

Figure 3.5 – **Infrared spectra of lignin obtained from three black liquors**

3.6 UV absorbance analysis

As can be seen in Figure 3.6, the absorbance of lignin obtained by enzymatic digestion is generally lower than that of the conventional acid precipitation method. This indicates that the cochromatic groups (such as hydroxyl groups) are less than those of lignin obtained by acid precipitation, and the fact that cellulose contains a large number of hydroxyl groups in hemicellulose further proves that lignin purified by enzymatic digestion contains less impurities (cellulose, hemicellulose and other heterosaccharides) and has a higher degree of purity, which can be mutually corroborated with the conclusions in 3.5.

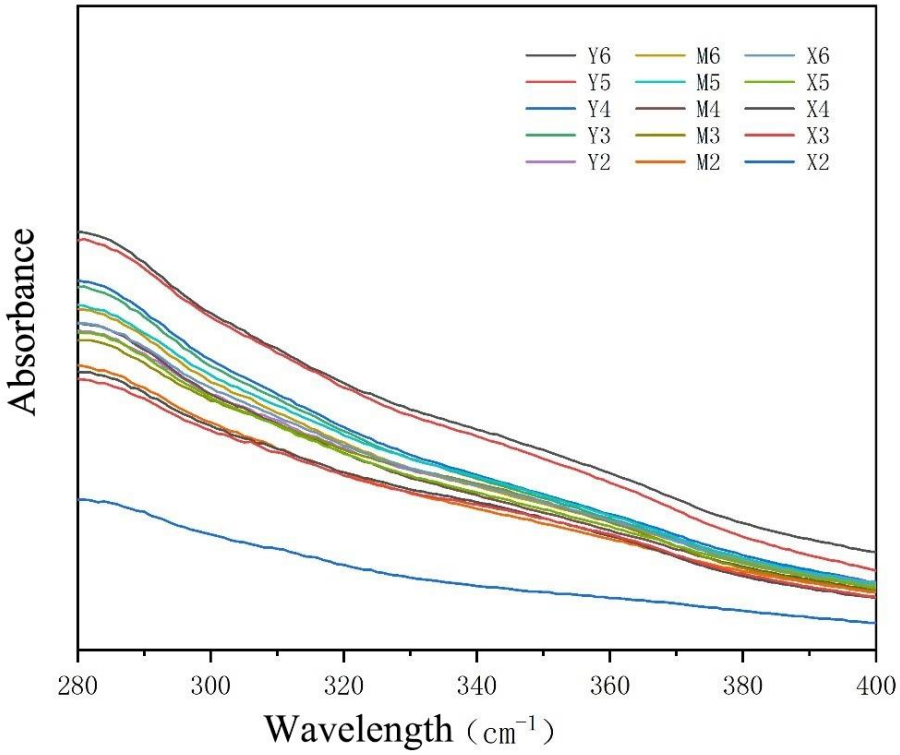

Figure 3.6 – **Ultraviolet spectra of lignin obtained from black liquor after three methods of treatment**

Conclusions to chapter 3

1. The optimum conditions and dosages for the two enzyme reactions were determined.

2. Enzymatic treatment resulted in higher purity of lignin than acid precipitation.

3. The bio-enzyme can better destroy the structure of LCC and will not change the structure of lignin for better purification.

CONCLUSIONS

This thesis mainly discusses the influence of the enzyme dosage and treatment conditions of cellulase and xylanase on the purification effect of lignin, and compares the purification effect with the traditional acid precipitation method, characterizes the obtained lignin by means of the changes in the sugar content, the molecular weight size, the changes in the functional groups, the strength of the ultraviolet absorbance, etc., and analyzes the changes in the structure and the properties of the lignin obtained by different methods. The main conclusions are as follows:

1. Cellulase dosage of 100 U/L at pH=9 and 45 ℃ for 80min resulted in the highest amount of sugar, while xylanase dosage of 100 U/L at pH=9 and 40 ℃ for 100min resulted in the best enzyme digestion.

2. The molecular weight of lignin after enzymatic treatment was significantly reduced compared with acid precipitation, and the overall molecular weight of lignin was lower than that obtained by acid precipitation, and the dispersion coefficient was smaller and more homogeneous.

3. By comparing the infrared spectra of lignin obtained from acid precipitation and enzymatic treatment of black liquor, it was found that its structure was basically unchanged, with fewer impurities, and that it could effectively destroy the LCC bond and promote the reaction between H+ and lignin.

4. By measuring the UV absorbance, it was found that the UV absorbance of lignin obtained by the enzymatic method was generally lower than that of acid precipitation lignin, indicating that the content of hemicellulose in the lignin purified by the biological enzyme was less and the purity of lignin was improved, which further proved the superiority of the enzymatic method.

5. Based on the results of the determination of the sugar content in the lignin extracted by the three treatments, the purity of lignin by acid precipitation was finally calculated to be 86.6%, the purity of lignin purified by cellulase to be 92.9%, and the

purity of lignin purified by xylanase to be 93.8%. It proved that the effect of lignin purified by biological enzymes was more obvious.

Prospect

This study focused on the isolation and purification of lignin using the strategy of biological enzyme treatment, which achieved better purification results, but the depth and breadth of the study still need to be expanded:

(1) The structure of the lignin initially isolated from the black liquor can be further analyzed in depth, such as using nuclear magnetic resonance carbon spectroscopy to more clearly characterize the functional groups.

(2) In addition to exploring the role of a single enzyme, the purification of lignin can be attempted by using multiple enzymes to synergistically treat the black liquor, or the enzymatic method working in conjunction with other methods, etc., and systematically compared with a single enzymatic method.

(3) The enzyme digestion method used in this thesis has achieved some success, which is mainly to extract lignin from black liquor by different pH levels after enzyme digestion, and similarly, we can also try to extract lignin by different molecular weights of lignin by different levels.

(4) By analyzing the infrared spectra of lignin obtained after enzymatic hydrolysis, it was found that the LCC structure of lignin was changed, but due to the complexity of the LCC structure, how many carbohydrates are there in the structure, and the mechanism of the LCC bond breaking, there is still no definite research result. At the same time, the application of biological enzymes on the lignin purification industry, lignin batch industrial processing, still need in-depth research.

It is believed that through unremitting efforts, people will eventually be able to seriously pollute the environment of the pulp black liquor into high-value utilization of the "treasure", to achieve green coordination and sustainability.

LIST OF REFERENCES

1. Tian Huashang, L I Piwu, Zhang Cong & Zhang Jie. (2024). Study on the adsorption effects of lead fromblack liquor source fulvic acids. *Journal of Qilu university of technology* (01), 67-73.
2. Leng Dongmei. (2009). Paper black liquor lignin resource treatment. *Chemical Engineering & Equipment* (12),164-167.
3. Kang Shu-xin, Liu Feng-liang. (2004). On Comprehensive Utilization Techniques of the Black Liquor from the Paper Mill. *Sci/Tech information development & Economy* (11), 206-208.
4. PEI Li-Hua, Yang Gui-Hua, Jiang Qi-Meng, Liu Xiao-Ning, Zhang Zhong-Shan & Chen Jia-Chuan. (2016). Pyrolysis Characteristics of Cotton Linter Pulping Black Liquor. *Transactions of China Pulp and Paper* (02), 29-33.
5. Liu Xian-Miao, Zhang Jun-Yi, Zhang Bo, Liu Xiu-Yun & Chen Xiu-fang. (2018). Study on the Composition and Evaporation Characteristics of Pulping Black Liquor. *Transactions of China Pulp and Paper* (02), 35-39.
6. CAI Ming-Liang, BAI Sheng-Jie & LIU Ming-Hua. (2016). The treatment of paper black liquor and the current situation of resource utilization. *East China Pulp & Paper Industry* (01), 39-42.
7. Xiao Xiang, Qi Yubo & Zhang Guangde. (2008). Processing Technology of Regenerated Papermaking Wastewater. *Environmental science and management* (02), 102-105.
8. Lu Lu. (2018). Black Liquor Treatment and Resource Utilization in Papermaking Process. *Chemical Engineering Design Communications* (07), 185.
9. Pratima Bajpai (2014), Chapter 2-Black Liquor Gasification, Amsterdam. Elsevier, 25-72.
10. Hansson J, Klugman S, Lönnqvist T, Elginoz N, Granacher J, Hasselberg P, Hedman F, Efraimsson N, Johnsson S, Poulikidou S, et al (2024). Biodiesel from

Bark and Black Liquor – A Techno-Economic, Social, and Environmental Assessment. *Energies.*17(1), 99.

11. Liu Meng. (2020). Treatment and Resource Utilization of Black Liquor in Papermaking. *Energy Conservation and Environmental Protection* (05), 38-39.
12. Wang Zhihui. (2023). Research on Traditional Treatment Methods and New Technologies of Paper Mill Wastewater. *Papermaking Equipment & Materials* (12), 4-6.
13. Wu Huan. (2022). Analysis on the Improvement of Black Liquor Treatment Technology in a Paper Pulp Company. *Hunan nonferrous metals* (01), 60-63.
14. Zhao Hongyan & Wang Jiangong. (2022). Improvement of Pulping and Papermaking Wastewater Treatment Process Based on Environmental Protection Concept. *Paper Science and Technology* (05), 13-17+32.
15. Zhang Keying. (2021). Pulp and paper wastewater treatment and reuse process of the practice of exploration. *Papermaking Equipment & Materials* (09), 92-93.
16. Fan Zhongqiu, Sha Keju, An Xingye, Song Jiayi, Li Chenxi, Cheng Zhengbai... & Liu Hongbin. (2023). Research Progress of Lignin Separation and Extraction Technologies of Black Liquor from Pulping Process. *Transactions of China Pulp and Paper* (01), 88-98.
17. Zhang Zhi, Zhang Henglai & Zhang Huiqun. (2022). Green Construction and Sustainable Development from Engineering Companies of Pulp and Paper Making Engineering Based on the "Carbon Peak and Carbon Neutrality" Goals. *China Pulp & Paper* (03), 92-96.
18. Li Zhongzheng. (2012). Research on renewable biomass resource-lignin. *Journal of Nanjing Forestry University* (Natural Science Edition) (01), 1-7.
19. Liu Ning, He Hong-Bo, Xie Hong-Tu & Zhang Xu-dong. (2011). An Overview of Studies on Lignin in Soil. *Chinese Journal of Soil Science* (04), 991-996.

20. Jiang, W., Liu, S., Wu, C., Liu, Y., Yang, G., & Ni, Y. (2020). Super-stable, solvent-resistant and uniform lignin nanorods and nanospheres with a high yield in a mild and facile process. *Green Chemistry* (22), 8734-8744.
21. Yang Chi, Li Penghui, Wei Yumeng, Wang Yantign & Wu Wenjuan. (2023). Review of Lignin-based Adsorption Materials. *China Pulp & Paper* (04), 107-112.
22. Fu Chenglong, Liu Yaling, Liu Yishan & Huang Liulian. (2024). Study on the Properties of Cellulose Transparent Films Reinforced with Regenerated Lignin Particles. *China Pulp & Paper* (02), 19-25.
23. Margellou, A.G., Pappa, C.P., Psochia, E., Petala, M., & Triantafyllidis, K.S. (2023). Mild isolation and characterization of surface lignin from hydrothermally pretreated lignocellulosic forestry and agro-industrial waste biomass. *Sustainable Chemistry and Pharmacy*.
24. Liu, X., Bouxin, F. P., Fan, J., Budarin, V. L., Hu, C., & Clark, J. H. (2020). Recent Advances in the Catalytic Depolymerization of Lignin towards Phenolic Chemicals: A Review. *Chem Sus Chem*, 13(17), 4296–4317.
25. Tan Huishan. (2019). The Extraction and Purification of Lignin from Alkaline Pulping Black Liquor (MSc thesis, Tianjin University of Science & Technology)
26. Margarida Martins, M., Carvalheiro, F., & Girio, F.M. (2022). An overview of lignin pathways of valorization: from isolation to refining and conversion into value-added products. *Biomass Conversion and Biorefinery*.
27. Abdus Salam, M., Wayne Cheah, Y., Hoang Ho, P., Bernin, D., Achour, A., Nejadmoghadam, E., Öhrman, O.G., Arora, P., Olsson, L., & Creaser, D. (2022). Elucidating the role of NiMoS-USY during the hydrotreatment of Kraft lignin. *Chemical Engineering Journal.*
28. Ming Cai, N., Shuping, H., & Zhenwu, K. (2010). Research Progress of Lignin Model Compounds. *Chemistry and Industry of Forest Products* (30), 115-121.
29. Schmidt, J., Rye, C.S., & Gurnagul, N. (1995). Lignin inhibits autoxidative degradation of cellulose. *Polymer Degradation and Stability* (49), 291-297.

30. Feng Feifei, Tian Bin, Ma Pengfei, Wei Jianxin, Xu Long, Tian Yuanyu & Ma Xiaoxun. Research progress on principles and methods of lignin separation. *Chemical Industry and Engineering Progress*.
31. Li, Y., Meng, X., Meng, R., Cai, T., Pu, Y., Zhao, Z., & Ragauskas, A.J. (2023). Valorization of homogeneous linear catechyl lignin: opportunities and challenges. *RSC Advances* (13), 12750-12759.
32. Yang Jing, Jiang Jianchun, Zhang Ning, Xu Hao, Xie Jingcong & Zhao Jian. (2021). Research Progress on Lignin Degradation by Microorganism. *Biomass Chemical Engineering* (03), 62-70.
33. Chen Lei, Chen Hanping, Lu Qiang, Song Yang, Ding Xuejie, Wang Xianhua & Yang Haiping. (2014). Characterization of structure and pyrolysis behavior of lignin. *CIESC Journal* (09), 3626-3633.
34. Hoang, P. H., Dat, N. M., Cuong, T. D., & Tung, D. T. (2020). Production of 5-hydroxymethylfurfural (HMF) from rice-straw biomass using a HSO3-ZSM-5 zeolite catalyst under assistance of sonication. *RSC advances*, 10(23), 13489–13495.
35. Ji Hairui. (2019). Conversion of platform chemicals (furfuraland clavulanic acid) from lignocellulosic andtheir valorization (PhD thesis, Beijing University of Chemical Technology).
36. CHEN Ling. (2022). DES induced fabrication and functionalization of lignin/RF-based porous carbon (PhD thesis, Nanjing Forestry University).
37. Wang Zhongshan, Xiao Tianyuan, Liu Xiaodi, Cao Xinyu, Jia Wenchao & Shi Haiqiang. (2023). Preparation of Lignin Nanoparticles and Their Application in the Hydrogel Materials. *Transactions of China Pulp and Paper* (03), 69-78.
38. Zhang Meng. (2024). Preparation of lignin nanoparticles and application of nanofluids in Enhancing oil recovery field (MSc thesis, Shandong University).
39. Tang Qianqian. (2020). Progress in applied research on lignin. *Nei Jiang Ke Ji* (05), 118-119+65.

40. Heo, J. W., An, L., Chen, J., Bae, J. H., & Kim, Y. S. (2022). Preparation of amine-functionalized lignins for the selective adsorption of Methylene blue and Congo red. *Chemosphere* (295), 133815.
41. Wang Yanbao. (2021). Study on preparation of modified lignin sulfonate and adsorption for heavy metal ions. *Materials science and technology* (06),65-73.
42. Lai, S., Gao, J., Zhang, H., Cheng, L., & Xiong, X. (2020). Luffa sponge supported dendritic imidazolium ILs with high-density active sites as highly efficient and environmentally friendly catalysts for CO_2 chemical fixation. *Journal of CO_2 Utilization* (38), 148-157.
43. Yin Jing, Li Dong, Li Yangwenzhang, Duan Yali, Jiao Yangqiu, Shi Qingsheng... & Zhu Zhiqiang. (2022). Evaluation of nutrient release characteristics of modified lignin-coated slow-release fertilizers. *Journal of Agricultural Resources and Environment* (06), 1155-1163.
44. Xiong Xing-Quan, Zhang Hui & Gao Li-Zhu. (2023). Progress in Chemical Modification and Application of Lignin. *Chinese journal of applied chemistry* (06), 806-819.
45. Yong, Z., Shakeel, U., Rehman, M.A., Li, H., Xu, X., & Xu, J. (2020). Lignin-carbohydrate complexes (LCCs) and its role in biorefinery. *Journal of Cleaner Production* (253), 120076.
46. Besombes, S., & Mazeau, K. (2005). The cellulose/lignin assembly assessed by molecular modeling. Part 2: Seeking for evidence of organization of lignin molecules at the interface with cellulose. *Plant physiology and biochemistry: PPB*, 43(3), 277–286.
47. Vermaas, J.V., Crowley, M.F., & Beckham, G.T. (2019). A Quantitative Molecular Atlas for Interactions Between Lignin and Cellulose. *ACS Sustainable Chemistry & Engineering* (7), 19570-19583.
48. Jiang Tingda. (2009). Lignin (2nd ed.). Chemical Industry Press Co., Ltd.

49. Wang, H., Pu, Y., Ragauskas, A., & Yang, B. (2019). From lignin to valuable products-strategies, challenges, and prospects. *Bioresource technology*, 271, 449–461.
50. Song Wen. (2011). Purification of lignin from black liquor by biotechnology. *China Pulp & Paper Industry* (19), 59-61.
51. Liu Yu-Qi, Wu Shu-Bin, Zhuang Jun-Ping, Liu Ying, Wei Wen-Guang & Zhang Feng-Shan. (2023). Preparation of phenolic resin adhesive from softwood kraft lignin pretreated by enzyme. *Applied Chemical Industry* (08), 2342-2345+2352.
52. Holtman, K. M., Chang, H. M., & Kadla, J. F. (2004). Solution-state nuclear magnetic resonance study of the similarities between milled wood lignin and cellulolytic enzyme lignin. *Journal of agricultural and food chemistry*, 52(4), 720–726.
53. Ahmed, S., Riaz, S., & Jamil, A. (2009). Molecular cloning of fungal xylanases: an overview. *Applied microbiology and biotechnology*, 84(1), 19–35.
54. Guo Chunyan, Huang Weibin, Zhu Yi, Lu Jun & Diao Qiyu. (2007). Structural characteristics of feed xylanase and its application in production. *Feed Industry* (22), 9-11.
55. Hu Qinshu. (2012). Study on Xylanase aided bleaching (MSc thesis, Hubei University of Technology).
56. Jiang Zhengqiang, Li Lite, Li Ying. (2003). Research Progress in the Thermostable Xylanases. *China biotechnology* (08), 47-51.
57. de Alencar Guimaraes, N. C., Sorgatto, M., Peixoto-Nogueira, S. de C., Betini, J. H., Zanoelo, F. F., Marques, M. R., de Moraes Polizeli, M. de L., & Giannesi, G. C. (2013). Bioprocess and biotecnology: effect of xylanase from Aspergillus niger and Aspergillus flavus on pulp biobleaching and enzyme production using agroindustrial residues as substract. *Springer Plus* (2), 380.

58. Zhao Xin, Zhang Hong, Men Zhonghua, Ma Yong & Wen Tong. (2023). Research and Application Progress in Cellulase. *Chemistry & Bioengineering* (09), 1-9.
59. Leschine S. B. (1995). Cellulose degradation in anaerobic environments. *Annual review of microbiology* (49), 399–426.
60. Liu Quanxiao, Yang Shuhui, Zhu Lingli. (2000). Extraction of lignin from pulping black liquor by acid precipitation. *Paper Technology & Application* (04), 15-17.