

# Research progress on the isolation and purification of ligninase

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**Abstract:** Lignin is a natural polymer substance with a phenolic structure, widely present in woody plants, herbs, vascular plants, is the second largest natural polymer material in nature after cellulose in quantity, and is the only aromatic compound that can be obtained from renewable resources in industry. Lignin is widely used in building materials industry, petroleum industry, light industry, and agriculture. Lignin is also an important part of human dietary fibres (DF), which can change the activity of microorganisms in the intestinal system, reduce the content of cholesterol and blood sugar in the blood, and have the effect of preventing cardiovascular disease; Lignin also has antioxidant activity and inhibition of cancer cell activity. As a natural antioxidant active substance, the anti-free radical activity of lignin in wood and crop straw has been extensively studied, while lignin in dietary fiber such as vegetables and fruits has not been studied much. Extracting lignin from carrots and studying its structure and antioxidant activity is expected to obtain a natural, non-toxic side effect antioxidant active substance, which has a wide application prospect in the food industry, cosmetics, health products and other aspects.

**Keywords:** Lignin; Fibres; Ligninase.

## 1. Heterologous expression of ligninase

Enzymatic treatment of dye wastewater is gaining increasing attention as an environmentally friendly and sustainable bioremediation method. However, industrial applications catalyzed by biological enzymes still have some limitations, such as economic efficiency, mass production of ligninase systems, and tolerance of ligninase activity. Therefore, researchers have used genetic engineering to increase the production of enzymes, enhancing their activity and stability. In recent years, researchers have further overcome these problems by selecting appropriate hosts, such as *E. coli* or *Pichia pastoris*, for heterologous expression and optimizing the composition of the medium. As a single-celled microorganism, yeast has unique advantages in the field of expression systems. Its well-established heterologous protein folding pathway and its rapid growth make it one of the more commonly used expression systems in genetic engineering, which is an ideal choice for researchers. The advantages of *Pichia* yeast mainly include the following 3 points:

1) The expression is relatively high. Using *Pichia* yeast as an expression system, the yield of foreign proteins expressed is often ideal.

2) Good stability. *Pichia* yeast is integrated by homologous recombination, so the recombinant strains constructed are relatively stable, and foreign genes are not easy to lose.

3) Fast, easy, low cost.

*Pichia* yeast has the biological characteristics mentioned above, so it is very effective for the expression of foreign genes and proteins. For example, Fangfang Fan et al. reported a sample from the strain *Trametes* sp. The laccase gene of 48424 was expressed in *Pichia pichia* to obtain a high yield of recombinant laccase, which can decolor dyes with different structures. In addition, the expression of laccase gene from strain trogi in *Pichia* yeast can obtain heat-resistant recombinant laccase that can maintain half of its activity after 45 minutes at 70 °C, and can decolor azo dyes such as acid

red 26. Studies have shown that through protein engineering, not only can its expression be increased, but also the stability of recombinant ligninase can be further improved, which is an effective means to increase the participation of ligninase systems in industrial applications. In addition, many researchers have tried to further overcome the problems of industrial application of lignin enzyme biocatalysis by optimizing the composition of the medium. For example, relevant studies have found that metal ions and aromatic compounds can effectively stimulate the production of ligninase by white rot fungi, so the appropriate addition of metal ions and aromatic compounds in the medium can achieve the purpose of increasing the expression of enzymes and their activities. Rui Zhuo et al. reported that metal ions ( $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ ) and aromatic compounds (vanillic acid, cinnamic acid, ferulic acid) have certain effects on laccase activity and transcription, which indicates that metal ions and aromatic compounds can play a role in further synergistic stimulation and increase enzyme production.

## 2. Separation and purification

### 2.1. Salinization

Ammonium sulfate precipitation method refers to the addition of high concentrations of salt ions to an untreated protein solution, and the use of competing water molecules between salt ions and proteins to destroy the hydration membrane on the surface of the protein, resulting in a decrease in protein solubility and achieving the goal of protein separation and purification. Different concentrations of saturated ammonium sulfate are used to fractionate different types of proteins into precipitation, a process called salting. This process allows the protein to precipitate without destroying its own structure, so it is widely used. For example, white rot fungal laccase is initially purified by fractional precipitation of ammonium sulfate to remove some heteroproteins; The laccase secreted by *Stropharia*

rugosoannulata can reach a purification factor of 1.94 after precipitation of ammonium sulfate saturated with 30% to 70% saturated ammonium sulfate; Ganoderma sugae Murr laccase crude enzyme solution was precipitated by fractionation of ammonium sulfate at 30% to 70% saturation to complete the preliminary purification. For protein samples after salting, dialysis concentration is a necessary selection process. Dialysis bags can usually be used to separate proteins from salt ions in a salted sample solution, and the dialysis bag can be immersed in water or a buffer to achieve the role of desalting.

## 2.2. Ion exchange chromatography

Ion exchange chromatography is based on the difference in the charge of protein molecules themselves, and the purpose of protein molecule purification is achieved. Ion exchange chromatography includes anion exchange chromatography and cation exchange chromatography, and commonly used ion fillers include agarose, cellulose, etc. For example, the commonly used anion exchange column DEAE-sepharose FF ion chromatography column, the ion-packed agarose gel. Jia Junrui, Chen Min et al., using DEAE-sepharose FF ion chromatography column to isolate and purify the lignin-degrading enzyme of the fermentation broth of *A. parsley*, under the flow rate of 20 mmol/L, pH 5.0 sodium acetate-acetic acid buffer system, the eluate NaCl 3 mL/min flow, the target peak was collected step by step, and the target protein recovery rate reached 85%, and the purification factor could reach 2.71, so as to realize the separation and purification of protein.

## 2.3. Affinity chromatography

Affinity chromatography is a protein separation technique achieved through specific affinity between a protein and its particular ligand. For example, specific binding between antigens and antibodies; Direct and specific binding of the enzyme to the substrate.

## 2.4. Gel filtration chromatography

Gel filtration chromatography takes advantage of differences in the size of protein molecules. When eluting, larger proteins are eluted first, while smaller molecules are slower to elute.

## 3. Analysis of lignin degradation products

The methods of converting lignin into monomeric substances are physical (high temperature, high pressure), acid or base catalyzed, and biological. However, in general, the physical method is inefficient, the cost of grinding and refining or blasting is high, the chemical method has secondary pollution problems, while the biological method uses microorganisms for degradation, which can break down the lignocellulose of straw into small molecules of carbohydrates with high efficiency and no pollution, and can also achieve a certain economic value. Due to the incomplete degradation of lignin, its degradation products can be divided into two categories: low-molecular lignin degradation products and high-molecular lignin degradation products. For the separation of low molecular degradation products, solvent extraction is mainly used, firstly by ethyl acetate, and then the extracted products are subjected to graded treatment with alkaline solutions of different strengths. The lignin low

molecular degradation products can be divided into low molecular phenolics, low molecular acids and neutral fractions. The calculation of the yield shows that the neutral content is very small and low molecular phenolics and low molecular acids are the main components of the lignin degradation products. For the separation of the higher molecular degradation products, the lower molecular degradation products were removed by solvent extraction with benzyl alcohol prior to degradation, followed by extraction with ethyl acetate. It has been shown that the macromolecules produced during the initial stages of lignin degradation are quinone structures as well as  $\alpha$ -carboxy acids. Gas chromatography is used for the qualitative analysis of compounds by comparing the retention times of unknown components with those of standards under the same conditions. It is difficult to obtain satisfactory identification results when using this method for complex samples. In addition, gas chromatography has the disadvantages of high sample handling requirements and incomplete derivatization, so liquid chromatography and gas-mass spectrometry are often used for the quantitative analysis of lignin degradation products in recent years. Shiyu Fu et al. found that oxidation reactions mainly occurred during lignin degradation, and that the main components of lignin low molecular weight degradation products were low molecular phenols and low molecular acids. Further studies found that these low molecular acids mainly included purpuroic acid, vanillic acid, *p*-hydroxybenzoic acid and their derivatives, confirming that oxidative breakage of the side chain C $\alpha$ -C $\beta$  bonds occurred during lignin biodegradation. The preferential degradation of the guaiacyl and *p*-hydroxyphenyl structural units of lignin during biodegradation was also demonstrated by calculating the ratios of guaiacyl, lilacyl, and *p*-hydroxyphenyl. Deng Xun et al. studied the degradation of lignin in rice straw by strigolactone, and a large number of small molecules containing benzene rings, such as lilacyl and guaiacyl, were detected in the products, proving that the degradation of lignin aggregates first occurred in the side chains of monomers and the linkages between monomers, with breaks such as C $\alpha$ -C $\beta$  and  $\beta$ -O-4, forming monomers. During further degradation, strigolactone exhibits its own unique degradation mechanism by replacing the methoxy on the benzene ring monomer to methyl, followed by the opening of the benzene ring, which is different from the reported degradation process of white rot bacteria. A comparison of the molecular structure of lignin before and after biodegradation revealed that the increase in lignin  $\alpha$ -group content after biodegradation was accompanied by the production of an aromatic carboxylic acid structure, the formation of which was directly related to side chain oxidation and C $\alpha$ -C $\beta$  cleavage. Ellwardt et al. used <sup>13</sup>C-NMR to provide more direct evidence for the inference that benzene ring cleavage occurs during lignin biotreatment. According to their results, the biotreatment of lignin produced the following compounds:  $\alpha$ -aromatic carboxylic acid, a C $\alpha$ -C $\beta$ , oxidative cleavage product; vanillyl alcohol, possibly an  $\alpha$ -aromatic carboxylic acid reduction product or a product of other reaction pathways including C $\alpha$ -C $\beta$  oxidative cleavage; and aromatic acetic acid or aromatic ethanol, possibly from the C $\alpha$ -C $\beta$  oxidative cleavage of the  $\beta$ -aryl ether structure. The breakage of the  $\beta$ -aryl ether bond was also confirmed by Enoki et al. using the white-rot fungus *Phanerochaete chrysosporium* to treat the model compound 4-ethoxy-3-methoxyphenol propanetriol- $\beta$ -guaiacyl ether with  $\beta$ -aryl ether bonds to obtain the degradation product 2-(o-

methoxyphenol) ethanol. Alternatively, the aromatic ethanol structure can be obtained by aromatic acetic acid reduction or other routes.

## 4. Advances in the molecular biology of lignin degradation

### 4.1. Cloning of the lignin-degrading enzyme gene from the white rot fungus

In order to gain insight into the mechanism of lignin degradation by various enzymes secreted by edible bacteria, studies have been conducted from a molecular biology perspective. Since Germann et al. first cloned the laccase gene from *Chlamydomonas reinhardtii* using RT-PCR in 1986, approximately 30 different laccase genes have been cloned and sequenced from different sources, including *Chlamydomonas reinhardtii*, *P. pegmentii*, *G. parviflora*, and *Chlamydomonas stricta*. approximately 30 different sources of laccase genes have been cloned and sequenced, including *P. vulgaris*, *P. embolus*, *P. graminearum* and *P. stricta* laccase.

### 4.2. Expression of white rot degradase genes

Given the wide range of substrates that lignin-degrading enzymes can act on, they have very high potential value in many areas such as pulp and paper wastewater treatment, biological bleaching, conversion of toxic environmental pollutants, agricultural straw treatment, dye decolourisation, and energy development, making them environmentally friendly materials that have gained much attention in recent years. However, an important factor currently limiting the widespread use of these enzymes is their low yield and high price. A possible solution to this problem is to carry out cloning and expression of relevant enzyme genes with a view to obtaining high enzyme-producing strains. The process of enzyme isolation and purification should also be further investigated to improve the efficiency of isolation and purification, so as to reduce the cost of enzyme production and make it more widely used. Sun H. R. et al. overexpressed laccase in porous bacteria and found that compared to the wild type, the laccase activity, the ability to decolourise RBBR dye, the total lignin weight loss rate, and the lignin loss rate of the overexpressed strain were all much higher. The highest extracellular lignin peroxidase activity of 1997 U/L was obtained by Sun Yafan [31], who expressed the lignin peroxidase gene in *P. aeruginosa*, and the enzyme activity was determined using ricinol as a substrate, which was 122.1% higher than that of the starting strain (899 U/L). The results showed that MnP activity was several times higher than that of the wild type. However, gene expression is still influenced by factors such as N-terminal processing and glycosylation which affect protein secretion, C-terminal processing which may regulate enzyme activity, and mutation of the C-terminal processing site or truncation of the C-terminus which can affect laccase activity. Studies of laccase spectroscopy, kinetics, fixed-point mutations and recombinant laccase have shown that the level of reduction potential determines the level of activity of fungal laccase, i.e. the higher the reduction potential, the greater the laccase activity. In addition, as it is heterologous expression, different species have different codon preferences, which also affect the laccase activity. Yang et al. used recombinant genetic techniques and targeted mutagenesis to add 15 amino acid residues to the C-terminus of wild Georgian laccase to obtain a mutant of this laccase

and successfully achieved high activity in *Picea abortus*. This provides a new idea and method for future research to improve the heterologous expression of lignin degradation-related enzyme genes.

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