Increasing laccase activity of white rot fungi by mutagenesis and treating papermaking wastewater

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Abstract. The aim of the work was to apply ultraviolet mutagenesis on white rot fungus *Pleurotus ostreatus* for enhancement of laccase production. The mutant strain UV-6 with higher laccase activity was obtained by screening and the highest laccase activity was 135U/L, which was 77% higher than that of the wild strain. The papermaking wastewater was treated by mutant strain UV-6 and the COD removal rate reached 85%, which was 21% above the degradation rate (70%) by the wild strains. It was proved that ultraviolet mutagenesis is the effective method to improve laccase activity of *Pleurotus ostreatus*, and the results of our work are significant for the potential application of laccase.

1. Introduction

Papermaking wastewater contains a large number of refractory organic pollutants, such as cellulose, hemicellulose and lignin, etc. The waste water is discharged into water body, which will cause serious pollution to the water body. The papermaking black liquor is the main pollution source, accounting for about 90% of the total quality of pollutants. Lignin and its degradation are the main components of the black liquor, and its quality can reach 50% of the total COD [1]. Therefore, the key to solving the effects of papermaking industry on water pollution is the degradation and removal of lignin.

White-rot fungus is considered a significant degradation of lignin, cellulose and hemicellulose in the biosphere. It can make the substances completely transformed into carbon dioxide and water [2]. They usually secrete three main lignin degrading enzymes, which are lignin peroxidase (LiP), manganese peroxidise (MnP) and laccase [3,4]. Laccase has shown greater research value and application potential in pulp bleaching, industrial wastewater treatment, dye decolorization, biological sensing and degradation of environmental pollutants, etc. Some breakthroughs and remarkable achievements have been made. Singh *et al* stated that the biobleaching of wheat straw-rich soda pulp by alkali-tolerant laccase from γ-proteobacterium JB [5]. Asadgol *et al* reported that the purified laccase from *Paraconiothyrium variabile* (*PvL*) was an efficient biocatalyst for elimination of phenol and the endocrine disrupting chemical bisphenol A [6]. Rodríguez-Couto S reported that Remazol Brilliant Blue R was decolorized efficiently by *T. pubencens* grown on sunflower-seed shells under solid-state fermentation conditions [7]. Senthilvelan *et al* reported biodegradation of lignin of 91–98% could be reached of different concentrations laccase in the pH value of 7.0 and 5.0 mM HOBT in 32°C for 24 h. Chemical oxygen demand and total organic carbon were reduced 84 and 80%, respectively [8].

Laccases are multicopper oxidases, which can be found in fungi, bacteria, plants, and insects.

Laccase can oxidize a broad range of aromatic compounds in the mild and extreme environmental conditions. Many researchers have conducted extensive research on laccase and its producing strain. It mainly involves the growth characteristics of the strains, the separation and purification of laccase, synthesis control of laccase, enzymology properties and so on [9-11]. But most white-rot fungus belongs to the basidiomycete fungi, aerobic, slower growth, enzyme production cycle is long, and enzyme production rate is not reached the requirements of industrial production. Thus there is an urgent need to adopt some new ways and means to improve laccase production. Breeding new laccase strains, improving laccase production is still a hot topic in future study.

A mutagen is a physical or chemical agent which causes mutations. Ultraviolet (UV) radiation is 260 nanometers, inducing a dimer of the neighboring pyrimidine, especially if both of these are thymine, a cyclobutyl dimer is produced. UV-induced dimerization usually causes a missing mutation when the modified chain is copied [12]. Recent research shows that applying UV mutagenesis can enhance the production of laccase quantitatively [13,14].

In the paper, a mutant strain with higher laccase production was achieved from white-rot fungus *Pleurotus ostreatus* Z1 by UV mutagenesis. And papermaking wastewater treatment was researched.

2. Materials and methods

2.1. Strains and culture medium

P. ostreatus Z1 was get from the microbiology laboratory at the School of Civil and Architectural Engineering College, Liaoning University of Technology, Jinzhou, China. Strain Z1 was incubated on the potato dextrose agar (PDA) plates aseptically at 30°C for 5 days. When the PDA was completely covered by mycelium, the mycelium plug (10 mm in diameter) was used for inoculum. Four mycelia plugs were transferred to a conical flask. The flask was incubated on a rotating shaker (150 rpm) at 30°C. After 5 days, the strain Z1 were collected and then stored at 4°C. For the study of papermaking waste water bioremediation, basal liquid culture medium was used: glucose 20 gL^{-1}, $(NH_4)_2SO_4$ 0.5 gL^{-1}, KH_2PO_4 2 gL^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 gL^{-1} and thiamine hydrochloride (VB_1) 0.02 gL^{-1}; pH of the liquid medium was adjusted to 6.5 with diluted hydrochloric acid before sterilization by autoclaving [15]. Primary screening medium was prepared as PDA medium supplemented with 0.2% guaiacol. Re-screening medium was basal liquid culture medium.

2.2. Analytical method

Laccase activity was confirmed as represented by Niku-Paavola *et al* [16]. Chemical oxygen demand (COD) was determined by standard methods. The color was represented in terms of the CPPA standard method [1].

2.3. Preparation of spore suspension

The suspension of spore was prepared from PDA slant, a slop of biomass from slant agar was suspended in distilled water, diluted appropriately and then counted to 10^8 conidia /mL putting the cell counter under the microscope. A stock of each strain of mutants and wild type in microtubes were maintained at 4°C for any purpose of inoculation and treatment [17].

2.4. Mutagenesis by UV irradiation

Spore suspension (5 mL) of *P. ostreatus* Z1 was transferred to a sterile plate with a rotor. The plate was removed and exposed to UV radiation of 254 nm at different time from 0 s to 300 s with a distance of 30 cm and the UV lamp at 15 W. A non-irradiated plate was maintained as control. After the irradiation, the sample on the plate was plated onto PDA plates with fully dilution for measuring survival rate. The culture was permitted to grow in the dark to avoid photoreactivation at 30°C for 5–6 days until the fungal colonies were observed.

2.5. Selection of mutans producing high laccase activity

The surviving colonies were inoculated in a screening medium PDA – guaiacol medium plates. The mutants were selected based on the intensity of color produced on screening medium plates after 5-6 days of incubation at 30°C. The fatality rate of spore was counted giving percentage of survival among spores. The qualitative laccase activity was judged by the intensity of color and the mutant strain colony diameter. The mutant strains producing the larger diameter and deeper color ring were selected. The mutants obtained were inoculated on PDA slant agar where after sporulation were conserved in 4°C.

The mutants obtained were then introduced into re-screening medium mentioned above. The single colony was transferred to a 250mL conical flask with 50 mL basal liquid culture medium. After incubation, Erlenmeyer flasks containing 50 mL of medium were cultured on a rotary shaker at 150 rpm and 30°C for 5-6 days. After incubation laccase produced by the mutants was assayed spectrophotometrically. The genetic stability of high laccase production of mutants was evaluated by 5 successive secondary culture experiments.

2.6. Mutants on papermaking wastewater treatment

The papermaking wastewater was collected from Jincheng paper mill, located in Jinzhou, China. Mutant was incubated on the PDA plates aseptically at 30°C for 5-6 days. When the PDA was completely covered by mycelium, the mycelium plug (10 mm in diameter) was used for inoculum. Four mycelia plugs were transferred to a conical flask. The flask was incubated on a rotating shaker (150 rpm) at 30°C. After 5-6 days, the pellets of mutant strain were collected and then transferred into 1000 mL conical flask. The flask was supplemented with 400 mL papermaking wastewater. The wastewater was diluted by tap water. Glucose, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$ and VB_1 were mixed into the wastewater. The ultimate pH was set to 7.0. On a rotating vibrator, the temperature was kept at 30°C and at 150 rpm. Before the analysis, samples were extracted from the flask at an interval of 24 h and each sample was centrifuged at 10,000 g for 10 min to remove the biomass. All the experiments were done at least three times.

3. Results and discussion

3.1. Mutants selected

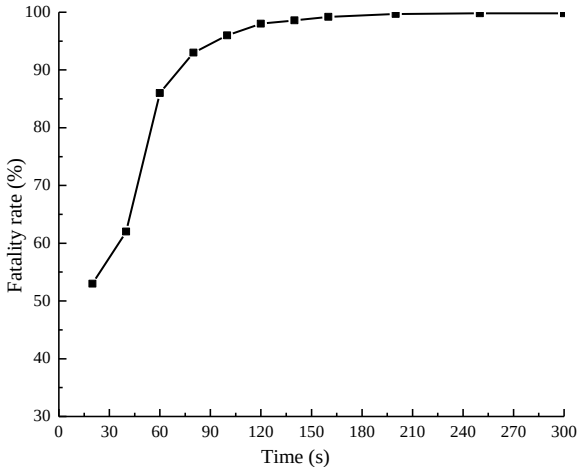

Figure 1. Fatality rate curve of UV mutagenesis.

After UV irradiation, the sample on the plate was plated onto PDA plates with fully dilution for measuring survival rate. The culture was allowed to grow in the dark at 30°C for 5–6 days until the

fungal colonies were observed. From figure 1, with the extension of UV irradiation time, the fatality rate increased rapidly. The fatality rate reached almost 100% for 120 s. It is not conducive to the selection of laccase high yield strains by too low and too high fatality rate. So the final choice between 75% and 90% fatality rate of exposure to the ultraviolet rays irradiation time is the best time. We choose 60 s for the best time to UV mutagenesis. Morphological of the wild fungus Z1 and mutant fungus for 40s and 60s on PDA plate were seen from figures 2-4.

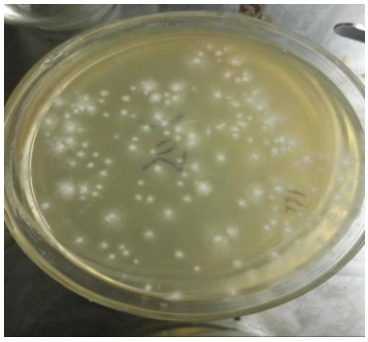

Figure 2. Morphological of the wild fungus Z1 on PDA plate.

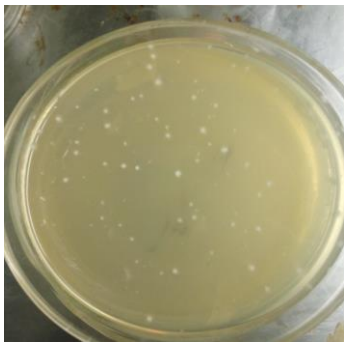

Figure 3. Morphological of the mutant fungus for 40s on PDA plate.

Figure 4. Morphological of the mutant fungus for 60s on PDA plate.

The mutant fungi for UV irradiation 60s colonies were inoculated in a screening medium PDA – guaiacol medium plates. Guaiacol is not only having good induction effect on white rot fungi secreting laccase, but also a kind of color agent of laccase [18]. The mutants were selected based on the intensity of color produced on screening medium plates after 5-6 days of incubation at 30°C. The qualitative laccase activity was judged by the intensity of color and the mutant strain colony diameter. The mutant strains producing the larger diameter and deeper color ring were selected (figures 5 and 6). Eight mutants were screened, from UV-1 to UV-8, which had higher laccase activity.

Figure 5. Morphological of the wild fungus Z1 on PDA-guaiacol medium plate.

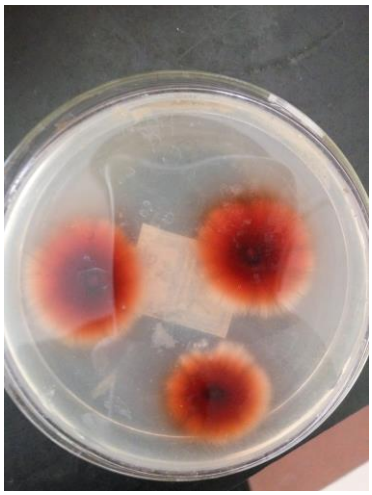

Figure 6. Morphological of the mutant fungus UV-6 on PDA-guaiacol medium plate.

The primary screening mutants obtained were inoculated on re-screening medium. After incubation, Erlenmeyer flasks were incubated on a rotating shaker at 150 rpm and 30°C for 5-6 days. After incubation laccase produced by the mutants and wild strain were assayed. A higher laccase activity

producer UV-6 was chosen. The laccase activity was shown in figure 7. In the first 5 days, in medium the concentration of glucose dropped sharply and was almost completely utilized during the fifth day of cultivation, and at the same time, the largest biomass was produced. In depleted glucose, the biomass stopped enhancing, and a large number of laccase secretions were observed, indicating a change in secondary metabolism. This is according to Galhaup *et al* [19]. The laccase secretion was relatively low in the glucose rich environment (the first 5 days), with a yield of 10.37 and 32.2 U/l, while the increased laccase secretions were found in the following days. The maximum laccase activity obtained on the day 11 was 76 and 115.7 U/L, followed by a decrease. This phenomenon can be explained by the failure of a large number of genes in glucose, leading to enzyme synthesis associated with enzyme secretion [20]. Improving laccase production by continuously providing low, uninhibited glucose in the medium can reduce the inhibition of glucose [19].

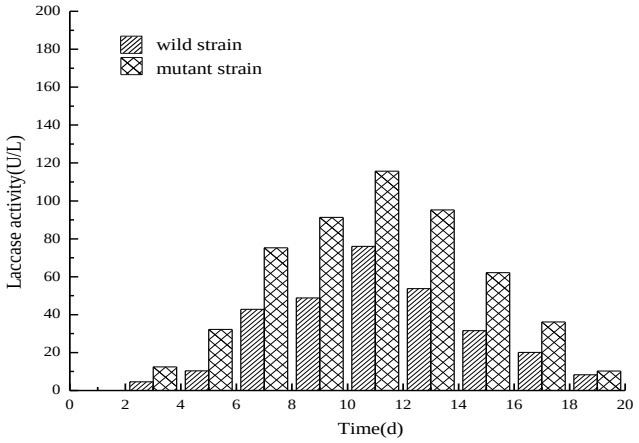

Figure 7. Laccase activity of wild strain and mutant strain.

3.2. Genetical stability of the mutant strain UV-6

To ascertain whether mutant strain UV-6 was genetically stable, we inherited 5 generations and measured the laccase activity of each generation. All generations have shown the same activity and productivity as the original UV-6, indicating that the mutant gene is genetically stable and appropriate for industrial production. The fermentation condition of this strain was optimized (data not shown). The laccase activity reached 135 U/L.

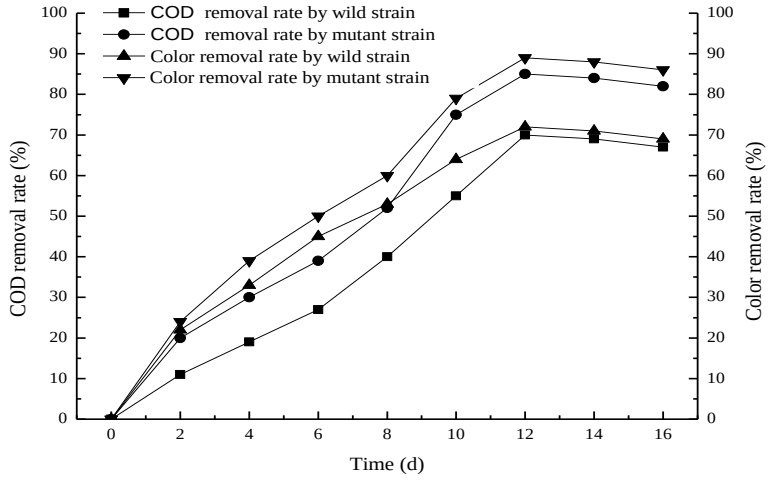

Figure 8. Color and COD removal by the wild strain and mutant strain.

3.3. Mutants on papermaking wastewater treatment

The pollution index of papermaking wastewater was 1000 mg/L of COD_{Cr} and 800 times of colority. The pollution index of papermaking wastewater decreased to 150 mg/L of COD_{Cr} and 88 times of the colority by the mutant strain. As can be seen from figure 8, the COD_{Cr} removal rates were 70% and 85% by the wild strain and mutant strain, respectively. The chroma removing rates were 72% and 89% by the wild strain and mutant strain, respectively. Therefore, it can be concluded that the mutant fungi have great potential and can be applied to the treatment of papermaking wastewater.

4. Conclusion

Using UV mutagenesis method, we have successfully produced a mutant strain UV-6. The current research results clearly demonstrate the effective of UV mutagenesis and improve laccase production capacity of *P. ostreatus*. The mutant is genetically stable. Mutant UV-6 of *P. ostreatus* strain was optimized, and the laccase yield increases by 77% compared with that of the parent strain which is a good result. The papermaking wastewater was treated by mutant UV-6 and the COD_{Cr} removal rates reached 85%, which was 21% above the COD_{Cr} removal rates (70%) by the wild strains. Therefore, for industrial applications, this is a very promising strain. Finally, UV mutagenesis proves that it is an efficient tool for constructing the second generation, which has the characteristics of better efficiency and economic application.

Acknowledgments

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