Bioremediation of Dyes Using Ultrafine Membrane Prepared from the Waste Culture of *Ganoderma lucidum* with *in-situ* Immobilization of Laccase

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A one-step method for laccase production and immobilization using the waste culture of *Ganoderma lucidum* (*G. lucidum*) was developed, and the laccase was immobilized by the mycelium, which was in the form of a white membrane consisting of superfine fibers. This medical and edible membrane was characterized by scanning electron microscope (SEM), and the fiber diameter was found to be between 1 and 3 μm, with a porous structure formed in the membrane. Fourier transform infrared spectroscopy (FT-IR) and thermogravimetric analysis (TGA) showed that the membrane contained polysaccharide groups and had good thermal resistivity. The membrane was used for the decolorization of methyl violet and malachite green, while the MTT test showed that the membrane had good biocompatibility. The experimental results indicated that the membrane might be applicable for other environmental protection applications in the future.

Keywords: Ultrafine fibrous membrane; Polysaccharide; Fungi; Ganoderma lucidum; Laccase

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INTRODUCTION

Microbes play an essential role in our lives and in nature. They have a high surface area to volume ratio, are easy to adapt to large scale production, have a wide diversity, and are easy to manipulate genetically, such that they can be used to produce organic acids (Yu *et al.* 2011), enzymes (Kasana *et al.* 2011), antibiotics (Chiang *et al.* 2011), and other things using the large scale fermentations. However, the microbial industry has for a long time tended to produce high quantities of wastewater with high Chemical Oxygen Demand (COD) and high-level nutrients, which impedes the development of the industry, even if some measures such as flocculation, coagulation, adsorption, membrane filtration, and also the microbial decolorization and degradation have been employed to deal with this problem (Chandra *et al.* 2008).

Ganoderma lucidum is a well-known fungal species in China. The fruiting body of *G. lucidum* was considered as a panacea, which could bring people back to life according to many old tales. *G. lucidum* also has been widely used as a traditional Chinese medicine. Nowadays *G. lucidum* has been used to treat and prevent cancer,

immunological disorders, hepatitis, hypertension, and other diseases. Many studies have revealed that *G. lucidum* extracts can inhibit the growth of many tumor cells such as lung cancer cell, colorectal prostate cancer cells, and breast cancer cells (Li *et al.* 2007; Liu and Zhang 2007; Xu *et al.* 2011). The fruiting body of *G. lucidum* is made of polysaccharides, terpenoids, amino acids, alkaloids, and proteins, *etc*. Polysaccharides are regarded as the most important type of compound among these components, and they have exhibited all kinds of pharmacological effects: anti-tumor, anti-oxidation, activation of the immune system (Zhang *et al.* 2011), *etc*. Outstandingly, the *G. lucidum* polysaccharide has a unique metabolic pathway, such that it can be absorbed by the immune system, and thus makes the body produce immunoreactive substances to react with the tumor cells (Wang *et al.* 1997; Zhang and Lin 1999).

G. lucidum has been used to produce the polysaccharides (Li *et al.* 2007; Liu and Zhang 2007; Ding *et al.* 2012a), ganoderic acid (Xu *et al.* 2008), enzyme (Ding *et al.* 2012b) and so on in the laboratories. However, the culture of *G. lucidum* is usually drained after its use, no matter what valuable things it may contain or no matter whether it would contaminate the ground water, or affect the microbiology diversity in the water. In particular, the mycelium of *G. lucidum* is also rich in polysaccharides and laccase in the waste culture, and it would be very useful if it can be reused, especially so that the laccase could be used to decolorize industrial dyes (Zilly *et al.* 2002) and it was also immobilized on polyacrylonitrile (PAN) nanofibrous membrane to treat the wastewater (Wang *et al.* 2014). We herein report a one-step method of laccase production and laccase immobilization using the waste culture of *G. lucidum* for dyes bioremediation.

EXPERIMENTAL

Organism and Medium

The *G. lucidum* was obtained from our own laboratory, and it was saved using potato dextrose agar (PDA) slants containing 200 g potato, 20 g dextrose, and 20 g agar for each liter. The slants were inoculated and cultured at 30 ºC for 5 days, then kept at 4 ºC. The composition of the seed medium was 20 g dextrose, 10 g wheat bran, 3 g KH_2PO_4, and 2 g $MgSO_4$. The fermentation medium composition was same as that of the seed medium. All medium was autoclaved at 121 ºC for 30 min, and the dextrose was sterilized separately to avoid the Maillard reaction.

Culture Conditions

Four pieces of hyphae from the PDA slant were inoculated to the seed culture containing 100 mL medium prepared in a 250 mL flask for 5 days at 30 ºC on a rotator (150 rpm), then 3 mL of the seed culture was taken out to inoculate into the fermentation medium and cultured in a 250 mL flask at 30 ºC which contained 100 mL medium.

Ultrafine Fibrous Membrane

When the experiments (enzyme assay, measurement of polysaccharides and terpenoids, *etc*.) were done, the left fermentation culture was incubated at 30 ºC under static condition for 4 to 7 days to produce the ultrafine fibrous membrane. And total sugar content was assayed after the formation of the membrane (Kim *et al.* 2006).

Membrane Morphology

After 4 days culture, the membranes were taken out, and a scanning electron microscopy instrument (SEM) was used to examine the surface morphology. Prior to SEM observation, the membrane samples were frozen at -80 ºC and lyophilized to keep their shape, and then they were mounted on an aluminum stub using carbon tab and coated with gold.

FTIR Analysis

FTIR spectra were acquired using the Nicolet iS10 FT–IR spectrometer to identify the chemical structure of the membranes, and the sample was cut into thin films and then was laid on the top of a diamond attenuated total reflection (ATR) and was scanned between 4000 to 500 cm^{-1}.

Thermogravimetric Analysis

A Mettler Toledo 1100SF was used to carry out the thermogravimetric analysis of the ultrafine fibrous membranes, and the samples were ground to powder form and approximately 10 mg of the sample was placed in the pans under a dynamic nitrogen atmosphere between 30 to 950 ºC. The scanning rate of the experiment was 20 K/min.

Preparation of Film Extract

After sterilization with 75% alcohol for 15 min, the films were placed in plain medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), according to the ratio of 6 cm^2 area of the film to 1 mL volume of plain medium. The extraction was conducted at 37 ˚C for 72 h. The obtained film extract had to be used within a 24 h period.

Cell Culture

The mouse fibroblast cells (L-929) were grown at 37 °C in a humidified atmosphere of 5% CO_2 in DMEM. The culture medium was supplemented with 10% FBS, 100 μg/mL streptomycin, 100 U/mL penicillin, and 4×10^{-3} M L-glutamine.

According to ISO-10993, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium romide] assay was performed to determine the *in-vitro* cytotoxicity of the film extracts. L929 cells were seeded into a 96-well plate at a density of 5000 cells per well and incubated with 100 μL culture medium containing either in the DMEM medium or the film extract fluid at 37 °C for various time periods. After the incubation, the culture medium in each well was removed, and the cells were washed three times with phosphate buffer saline (PBS). 20 μL of MTT solution (5 mg/mL) was added to each well and cells were cultured for another 4 h. The supernatant was discarded and then 100 μL of dimethyl sulphoxide (DMSO) was added to each well. The optical density (OD) values of the plate were measured on an EIX-800 Microelisa reader at 570 nm (Bio-Tek Inc., USA).

The Laccase Immobilized on the Membrane

Enzyme concentration was determined by the method of Bradford, with bovine serum albumin as the protein standard (Bradford 1976). The activity of free and immobilized laccase was determined at 30 ºC using ABTS as a substrate. For free laccase, the reaction mixture contained 2.9 mL ABTS (0.5 mM/L, dissolved in HAc–NaAc buffer), and 0.1 mL laccase solution (3 g/L, dissolved in HAc–NaAc buffer). After

incubating 4 min at 30 ºC under constant shaking, the absorbance of the solution was measured in a UV–vis spectrophotometer at a wavelength of 420 nm.

In the case of immobilized laccase, the *G. lucidum* membranes were cut into pieces (2*2 cm). Then the membranes were immersed into 2.9 mL of 0.5 mmol/L ABTS solution. The following steps were the same as the free laccase.

Unit activity was defined as $1000AV/t\varepsilon M_0$, where A is the absorbance at time t, V is the volume of reaction solution, t is the reaction time, ε is the molar extinction coefficient for the oxidation of ABTS at 420 nm, and M_0 is the mass of laccase.

The decolorization of the dyes by the laccase on the membrane was investigated. The *G. lucidum* membranes (2*2 cm) were separately added into the 10 mg/L methyl violet and malachite green dissolved in the seed medium contained in 5 mL tube, and the experiments was conducted on a rotator with the speed of 100 rpm at the room temperature. The decolorization of dyes was calculated using the following formula,

$$\text{Decolorization efficiency} = (A_0 - A)/A_0 \quad (1)$$

where A_0 and A are the absorbency of the initial and remaining dyes, respectively. The absorbencies was measured with a UV–vis spectrophotometer at 583 nm and 617 nm for methyl violet and malachite green separately (λ_{max} for each dye).

RESULTS AND DISCUSSION

Culture of *G. Lucidum* Membrane

The *G. lucidum* in this research had been used in the authors' lab for many years. The production of ganoderic acid (Fang and Zhong 2002), polysaccharide (Li *et al.* 2007; Liu and Zhang 2007; Ding *et al.* 2012a), enzyme (Ding *et al.* 2012b), and its medical applications (Zhang *et al.* 2011) all have been thoroughly studied. However, all the fermentation culture after each study would be poured out to the sewer, which not only would waste the useful components, but also pollute the environment with its high COD, and the microorganism community in the sewer also would be affected at the same time.

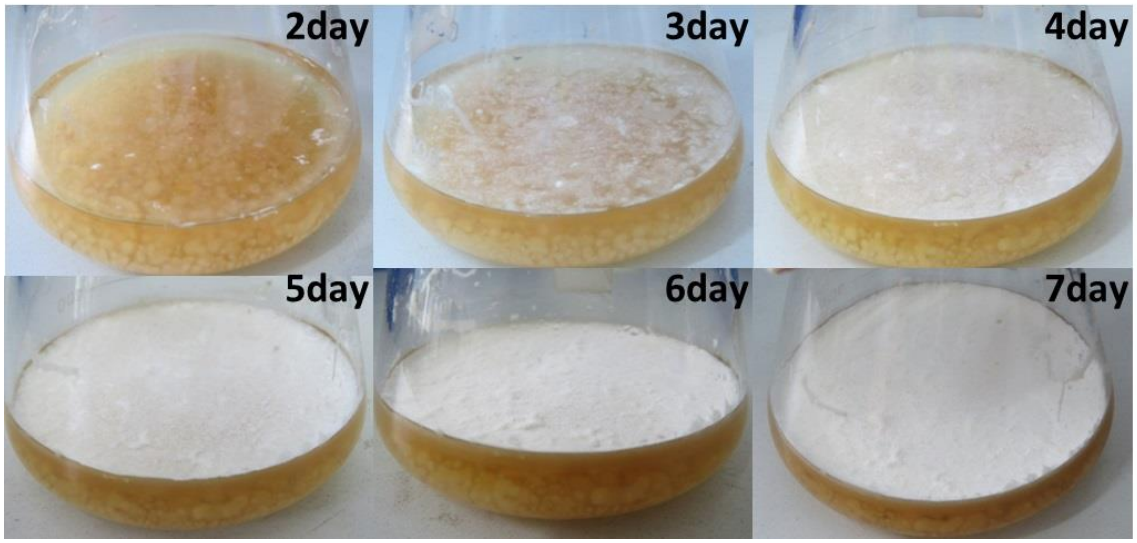


Fig. 1. The growth process of *G. lucidum* membrane under static culture condition

In the present work, when the experiments (enzyme assay, measurement of polysaccharides, and terpenoids, *etc*.) were completed, the flasks that contained the fermentation culture would be placed in a static condition. Little mycelium appeared on the surface of the culture after 1 day; however, the colonies expanded dramatically on the third day, such that the material almost covered the entire surface, and an ultrafine fibrous membrane appeared on the fourth day. The membrane would continue to grow during the next three days, as indicated in Fig. 1. Even after 5 to 7 days of culture for other useful substrate production, such as the polysaccharides and terpenoids, the *G. lucidum* would still grow vigorously to produce the membrane. Notably, when the membrane was taken out, another membrane would come out soon at the air-liquid interface.

The mycelium itself contained lots of useful components, such as polysaccharide, chitin, and so on, and the total sugar content of the membrane was 28 wt%. Also, when the fungi began to grow to form the membrane, the mycelium was exposed to the culture medium that contained laccase, tyrosinase, polysaccharide, and ganoderic acid, *etc*. Then all these bioactive products might be attached to the membranes through absorption/ hydrogen bonding or other intermolecular forces. Thus, a simultaneously laccase production and laccase immobilization was developed. However, Fang and Zhong (2002) found the ganoderic acid production was obviously enhanced using the two-stage fermentation (a 4-day shaking condition followed by a 12-day static culture), which was similar to our work; this implied that more ganoderic acid would be produced and stick to the membranes.

Membrane Morphology

The SEM observation revealed the membrane structures and shapes, as illustrated in Fig. 2. After 4 days the *G. lucidum* produced the membranes with a cross-linking fibrous network, and the fibers had a diameter between 1 and 3 μm. Also, pores with different sizes were also found in the membrane. At the very beginning, the fibers of the hypha just exhibited growth of radicals (Scheme 1). Then the bifurcations turned out to form small cross-linking networks when fibers grew to sufficient length, and the small networks would grow in three dimensions to connect with other small networks to form the ultrafine fibrous membrane. The membrane would have the same fibers and pores if all the small networks on the surface of the flasks grew uniformly; however, they were always different from sections to sections. The growing time was a key factor to form a good membrane. Zhang and Zhong (2010) have found that the *G. lucidum* would produce the spores on the 6^{th} day under liquid static culture conditions, which would form another structure that might affect the ultrafine fibrous membrane. The culture condition was another key element, and the smooth pellets were the main existing forms of *G. lucidum* under shaking conditions.

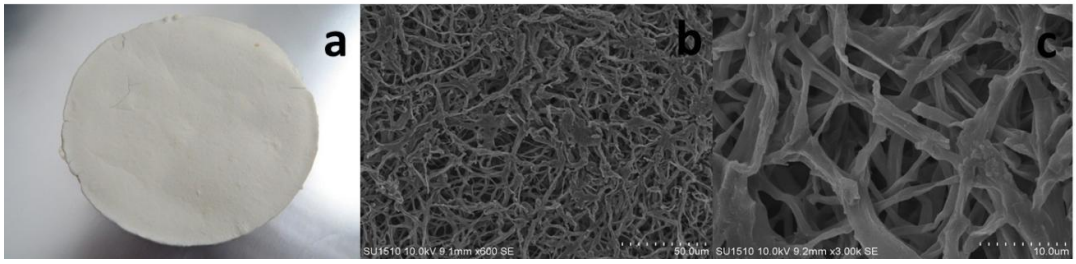
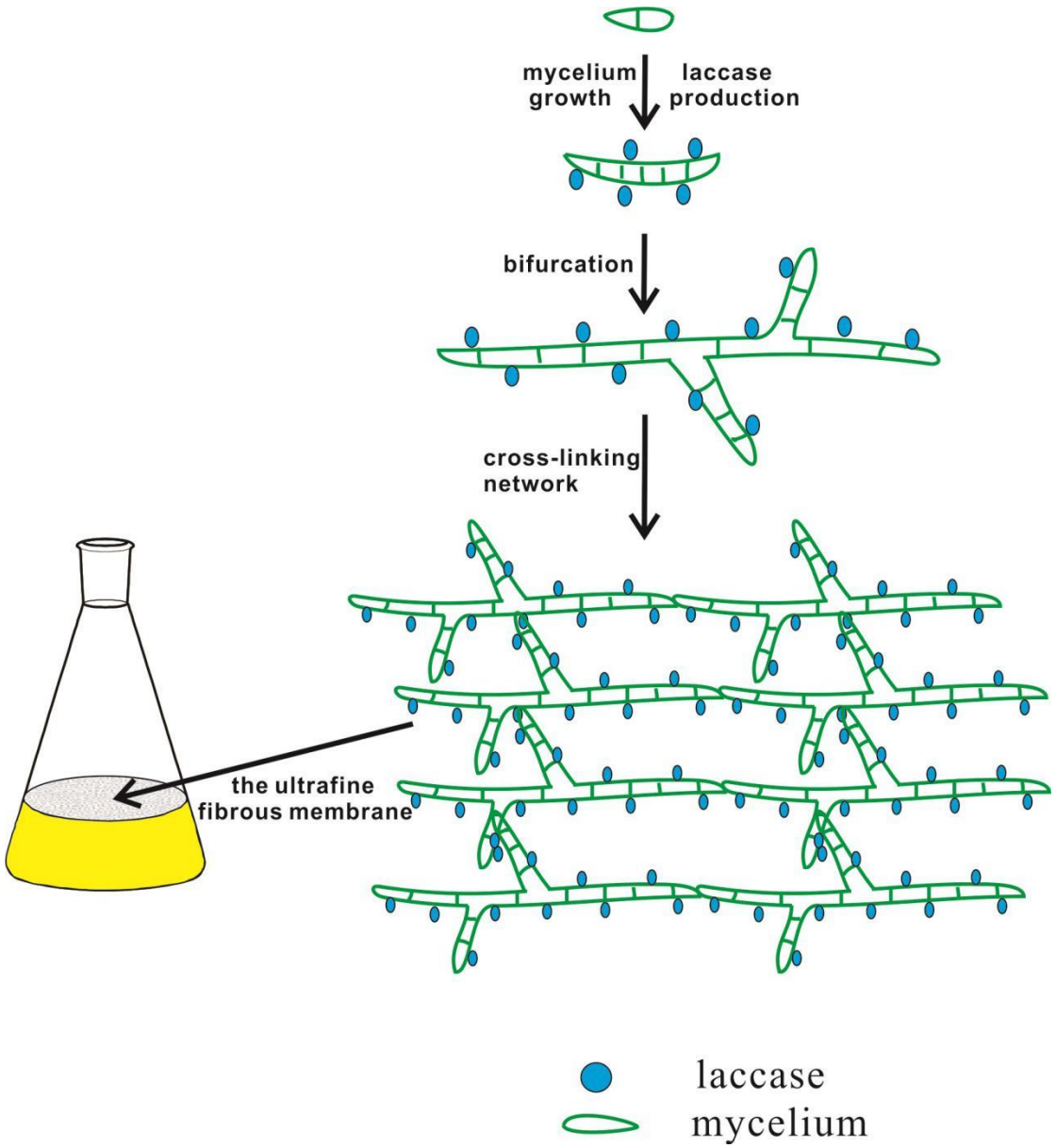


Fig. 2. The *G. lucidum* membrane (a) and its SEM image, 600× (b), 3000× (c)



Scheme 1. Formation process of *G. lucidum* ultrafine fibrous membrane and laccase immobilization under static culture condition

Ding *et al.* (2102a) discovered that the production of extracellular polysaccharides were affected apparently by *G. lucidum* pellet morphology under liquid shaking condition. At the same time, the characteristic of the ultrafine fibrous membrane with larger specific surface area would be beneficial to laccase immobilization.

FTIR Analysis

The FTIR spectra of membranes were acquired to identify the molecular structure of fibers. As can be seen from Fig. 3, all the three randomly selected zones of the membranes had the same peaks, indicating that all these zones had the same chemical composition. In the spectrum of zone 1, a wide band at 3280 cm^{-1} was attributed to the intermolecular hydrogen bond of 6-OH•••O-3′ (Zhang *et al.* 2014). Absorbance peaks at wavenumber 1030 cm^{-1} related to the C–OH bond (Dighton *et al.* 2001). Among all the

spectra, the richest C-OH band peaks and the wide peak of intermolecular hydrogen bond indicated the membrane might have much polysaccharide, and the hydrogen bonds can be further used to connect with some new functional materials during its growing process, so the peak at 1645.5 cm^{-1} of amide I band might be attributed to the laccase on the membrane (He *et al.* 2005).

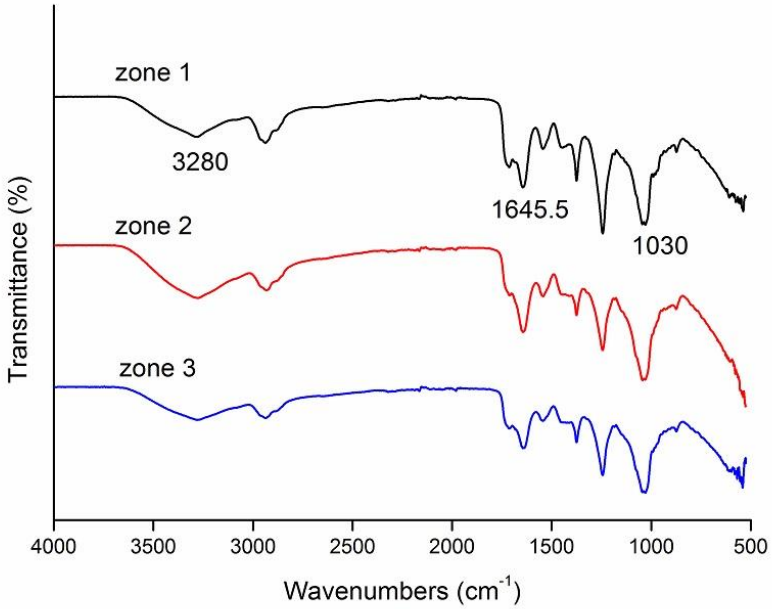


Fig. 3. FTIR analysis of *G. lucidum* membrane at different zones

Thermogravimetric Analysis

Thermogravimetric analysis is a successive process to detect the composition during the thermal decomposition of the membrane. The procedure involves measuring the sample weight in company with increasing temperature under the programmed heating, and the thermogravimetric analysis gives better understanding of the thermal stability, thermal decomposition behavior and thermal decomposition of the membrane. As can be seen from the Fig. 4, the thermogravimetric curves acquired by plotting percentage weight loss against temperature indicated that the pure membrane was unstable before the temperature reached up to 132 ºC, which revealed that there was much water in the membrane (Martins *et al*. 2009). Then it decomposed slowly till the temperature reached 660 ºC, and the percentage weight loss was found to be 63% until the temperature reached 800 ºC, then the curve quickly went down when the temperature came to 950 ºC.

The thermogravimetric analysis showed that the membrane had better thermal stability than some other materials, such as bacterial cellulose, which decomposed with an increasing rate from 200 ºC to 400 ºC, and the weight loss was about 80% when the temperature was 400 ºC (Cai and Kim 2010). However, the weight of *G. lucidum* membrane decreased slowly during the same time, and when the temperature came to 400 ºC, the loss of weight was just 52%. The reason why the membrane had better thermal stability may have been that it had complex components, while bacterial cellulose only contains glucose.

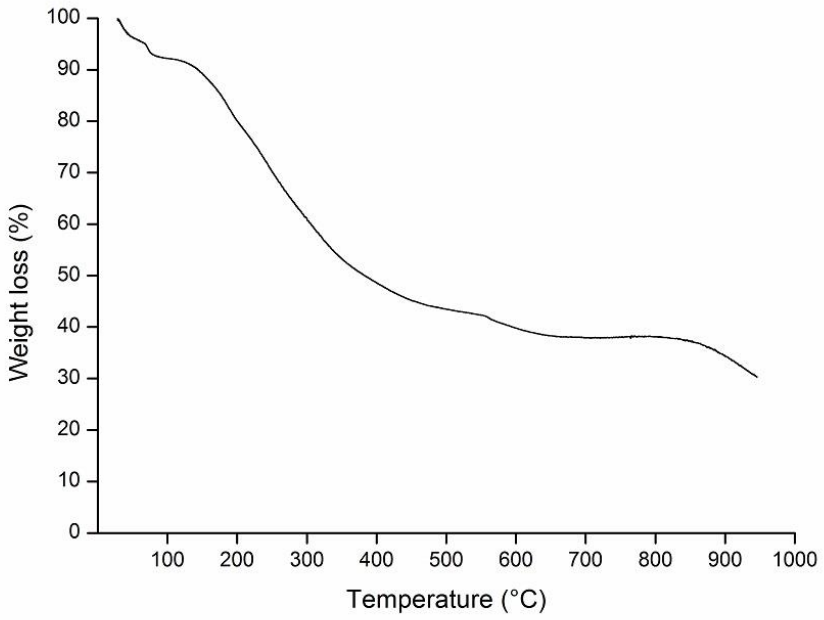


Fig. 4. The thermogravimetric analysis of *G. lucidum* membrane

MTT Test

According to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 5), the membrane extract demonstrated good biocompatibility, as indicated by good cell viabilities compared to that of the control group during the different time, and the membrane extract in 24-h culture showed the greater cell viability.

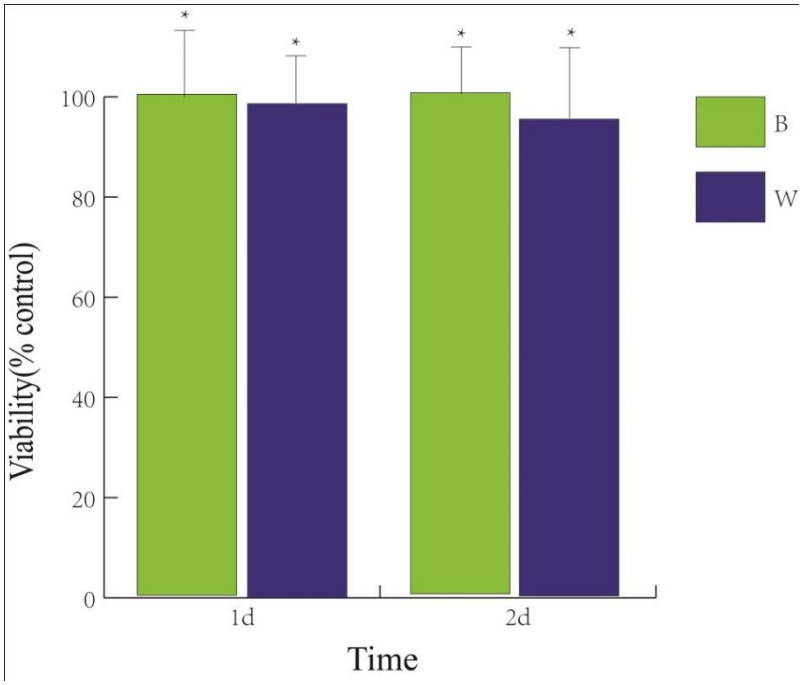


Fig. 5. The MTT test in different time (B for blank control, W for the membrane)

Based on these results, the membrane can be considered to have potential application as a potential carrier of medicinal compounds due to its good biocompatibility, edibility, and medicinal value. Moreover, some research has found that the *G. lucidum* polysaccharide has all kinds of pharmacological effects (Zhang *et al.* 2011) and it can induce the immune system to produce immunoreactive substances to react with the tumor cells (Wang *et al.* 1997; Zhang and Lin 1999). Because the *G. lucidum* polysaccharide contained in the membrane would also be introduced when the membrane was used as medicine carriers, it is meaningful to combine the both effects of the medicine and the polysaccharide to treat diseases. Further studies should be performed for assessing the interaction between them and finding out the optimum dose for different treatments.

Decolorization by the Membrane

Laccase is an important product during the cultivation of the *G. lucidum*. However, during the formation of *G. lucidum* ultrafine fibrous membrane, laccase still was present. In this experiment when a piece of membrane was exposed to the ABTS, the color of the liquid changed, and the enzyme activity of the membrane was about 5 U/g wet membrane. The mechanism of the immobilization might be that the free laccase would be attached to the surface of the *G. lucidum* fiber through van der Waals forces, and the whole membrane would be covered by the laccase; thus a single step was used to form the fibrous membrane and immobilize the laccase on the membrane simultaneously, and finally the laccase was immobilized on the membrane. This was a natural process that did not consume energy and did not waste the enzyme, compared to some other reports (Wang *et al*. 2013, 2014). Also, the process could be used to immobilize others enzymes, such as lignin peroxidase and manganese peroxidase.

Then the membrane was used to decolorize some dyes using the immobilized laccase. From Fig. 6 it can be seen that the membrane was able to decolorize the methyl violet with 85% decolorization and malachite green with 95% decolorization in 3 h.

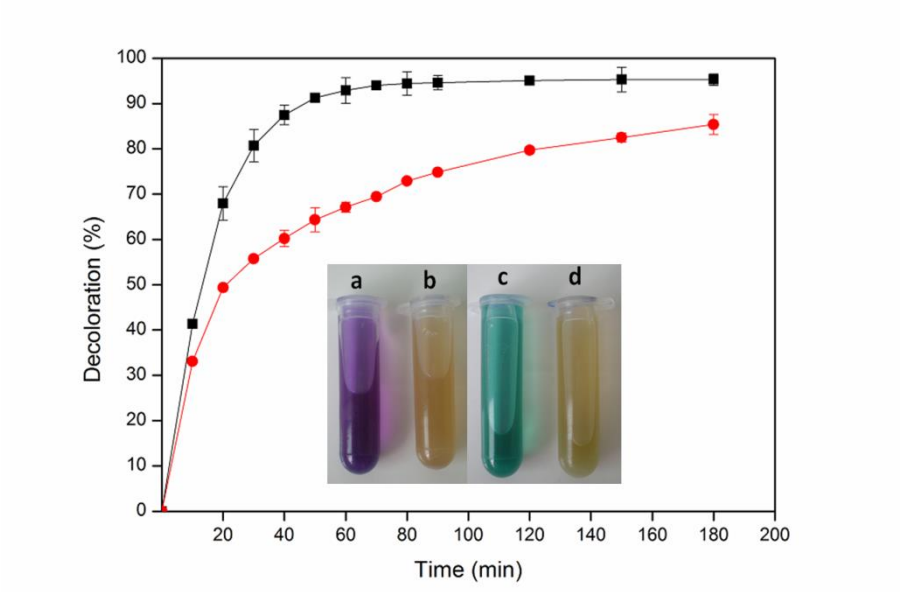


Fig. 6. Decolorization of this two dyes by the membrane: malachite green (■), methyl violet (●)

At the end of the process the membrane color was white (data not shown). Previous reports had showed that laccase can be used to decolorize the methyl violet

(Zilly *et al.* 2002) and the malachite green (Casas *et al.* 2009), and the membrane color did not change, indicating that the dyes was decolorized by the laccase immobilized on the membrane and also the functional membrane might be used in some other areas such as biosensor, biotransformation, and bio-fuel cells.

Changes in pH value will affect the enzyme conformation and the dissociation degree of substrate, thus impacting the binding and catalysis between the enzyme molecules and substrate, each enzyme has a specific pH value that the optimum catalysis occur with one substrate. In this research, pH value 5 (Fig. 7) was the best point that decolorized these two dyes quickly, which was dependent on the laccase property (Wang *et al.* 2013), and the laccase on the membrane had a bigger influence on methyl violet decolorization than malachite green.

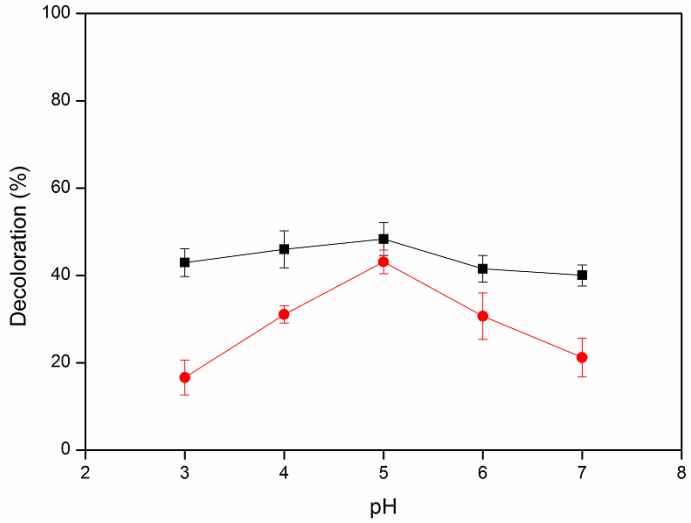


Fig. 7. Effect of pH on the decoloration rate of immobilized laccase: malachite green (■), methyl violet (●)

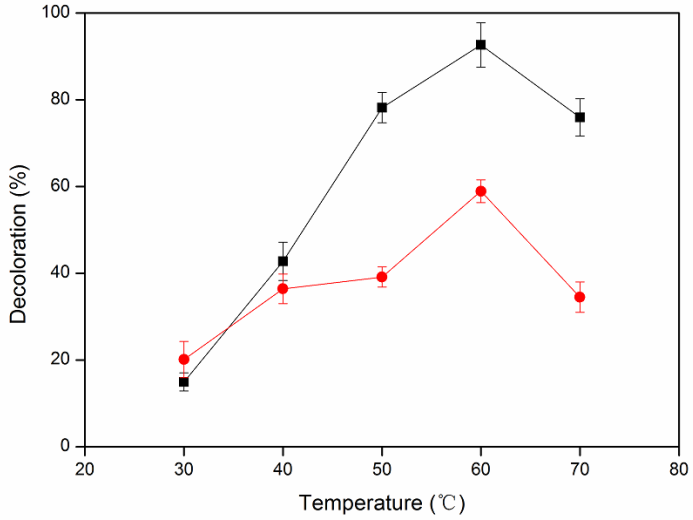


Fig. 8. Effect of temperature on the decoloration rate of immobilized laccase: malachite green (■), methyl violet (●)

The effect of temperature on decoloration of the laccase is shown in Fig. 8. The immobilized enzyme maintained a high decoloration in the temperature range of 50 to 70 ºC. However, in the lower temperature range 30 to 50 ºC, the decoloration was lower. The existing of the membrane might act as the heat resistance material to protect the enzyme.

CONCLUSIONS

1. An ultrafine fibrous membrane was produced using the waste culture of *G. lucidum* under a static condition. This approach offers a way to recycle the valuable compounds contained in the culture (such as polysaccharides) and offers a way to protect the environment.
2. The laccase produced by *G. lucidum* was immobilized on the membrane during its cultivation process. A solid membrane structure was formed during the process and the membrane showed enzyme activity, thus providing a method to immobilize the enzyme without chemical treatment.
3. The membrane was used to decolorize the methyl violet and malachite green with high efficiency, which could be used to remove other dyes, offering a promising way to decolorize and remediate real wastewater.
4. The membrane has good biocompatibility. Based on these results, the membrane can be considered to have a potential application as a carrier of medicinal agents due to its good biocompatibility, edibility, and medicinal value.

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