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Immobilization of *Trametes versicolor* cultures for improving laccase production in bubble column reactor intensified by sonication

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Abstract The mycelia of *Trametes versicolor* immobilized in alginate beads provided higher laccase production than that in pelleted form. An efficient ultrasonic treatment enhanced laccase production from the immobilized *T. versicolor* cultures. The optimized treatment process consisted of exposing 36-h-old bead cultures to 7-min ultrasonic treatments twice with a 12-h interval using a fixed ultrasonic power and frequency (120 W, 40 kHz). Using the intensification strategy with sonication, laccase production increased by more than 2.1-fold greater than the untreated control in both flasks and bubble column reactors. The enhancement of laccase production by ultrasonic treatment is related to the improved mass transfer of nutrients and product between the liquid medium and the gel matrix. These results provide a basis for the large-scale and highly-efficient production of laccase using sonobioreactors.

Keywords Alginate beads · *Trametes versicolor* · Laccase · Mass transfer · Ultrasonic stimulation

Abbreviations

C_0	Initial crystal violet concentration, μg/mL
C_t	Dynamic (after adsorption for t time) crystal violet concentration, μg/mL
m	Weight of alginate beads, g
V	Solution volume of crystal violet, mL
Q_t	Mass of crystal violet adsorbed at time t, μg/g wet gel
Q_e	Mass of crystal violet adsorbed at equilibrium, μg/g wet gel
K_d	Overall rate constant of adsorption, min^{-1}
t	Reaction time, min
TBA	2-thiobarbituric acid
TBARS	TBA-reactive substances

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Introduction

Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) belongs to a group of copper-containing blue oxidases found in higher plants, fungi, insects, and bacteria [2]. These enzymes catalyze the one-electron oxidation of a reducing substrate coupled to the four-electron reduction of molecular oxygen to water [42]. Laccases can oxidize a wide variety of phenolic compounds, aromatic amines, and even nonphenolic substrates in the presence of redox mediators [35]. Due to their broad substrate spectrum, laccases are used for many different applications including dye decolorization, pulp delignification and bleaching, bioremediation of environmental pollutants, food processing, biopolymer modification, and biosensors [25, 44]. However, a long fermentation process and low laccase yield in fungal cultures cannot meet the high demand for laccase, and therefore its use in industrial applications is currently limited [25, 29]. Different strategies have been employed to improve the production of laccase, such as medium optimization, isolation and breeding of high-producing strains, the utilization of inducers, and the heterologous expression of laccase genes [29, 47, 52]. The enzyme yields obtained by these methods are still low relative to the high demand for the enzyme. Therefore,

the search for novel, highly-efficient strategies for laccase production continues to be of interest.

Cell immobilization has many advantages, including simplified cell separation from liquid, reuse of immobilized cells, enhanced product yield, and improved process control [13, 31]. Cell entrapment in polymeric matrices is a widely used immobilization method and calcium alginate is one of the most suitable carriers [39]. However, one of the critical factors affecting the growth and metabolic efficiency of immobilized cells is mass transfer resistance [16, 31, 39, 53]. To address this issue, research has focused on optimizing the cell-immobilization system, including parameters such as carrier type, immobilization method, particle size, and cell loading [16, 31, 39]. Studies on laccase production using immobilized cells have tested different supports such as alginate gel beads [5, 11], polyurethane foam [45], stainless steel sponges [9], textile strips, and plastic materials [22, 34]. However, most studies have focused on carrier comparison and optimizing culture conditions. Process intensification can be one important strategy to enhance mass transfer in immobilization system, such as flow control, and reactor design. For an immobilized cell reactor, external mass transfer resistance can be reduced by increasing the velocity of the bulk solution relative to the gel beads [15]. Design of the impeller geometric in an immobilized enzyme reactor could also facilitate the diffusion process between the alginate beads and the solution [18]. Therefore, there is still interest in the use of process intensification with immobilized cells to improve laccase production.

Low-energy ultrasound has the potential to increase the productivity of biological processes [8]. Sonobioreactors are derived from the conventionally configured bioreactors by certain modifications to supply ultrasound to the fluid with large volume. There are different types of sonobioreactors, such as a bioreactor equipped within a cylindrical rod-shaped probe, a bioreactor using an alloy transducer outside the vessel, and a bioreactor with its vessel immersed in an ultrasonic bath [8]. They provide the possibility to apply the ultrasonic strategy to the culture in large scale. Recent study has proved that suitable ultrasound treatment was beneficial for laccase production from pelleted mycelia of *Trametes versicolor* [50]. In this study, *T. versicolor* was entrapped in calcium alginate beads and ultrasonic stimulation was optimized for the improvement of laccase production. The repeat-batch system was also tested to demonstrate the advantages of using an ultrasonic strategy for laccase production by immobilized cells. In addition, the process intensification strategy with ultrasound was further investigated for the laccase fermentation in the bubble column reactor.

Materials and methods

Microorganisms

T. versicolor CICC 14001 was obtained from the China Center of Industrial Culture Collection. This strain was maintained at 4 °C after subculturing at 30 °C every 2–3 weeks on potato dextrose agar slants.

Culture media and conditions

The *T. versicolor* mycelia were incubated at 30 °C on potato dextrose agar slants. After 1 week, three mycelia sections ($\sim$0.5 $\times$ 1.0 cm) were excised from the slant and used to inoculate the culture medium. Fungi were precultured in 250 mL flasks containing 100 mL of potato dextrose broth for 7 days and the precultured broth was used for cell immobilization. Immobilized mycelia were cultured in a 250 mL flask with 60 mL of working volume. The loading particle volume was measured using the drainage method, and the particle loading rate was calculated as a ratio of the loading particle volume to the flask working volume. The liquid medium for laccase production was modified from Birhanli's method [4], and consisted of the following (per liter): KH_2PO_4, 0.2 g; $CaCl_2{\cdot}2H_2O$, 0.1 g; $MgSO_4{\cdot}7H_2O$, 0.05 g; $NH_4H_2PO_4$, 0.5 g; $FeSO_4{\cdot}7H_2O$, 0.035 g; glucose, 2 g; NH_4Cl, 0.27 g; $CuSO_4{\cdot}5H_2O$, 0.02 g. The pH of the culture broth for laccase production was adjusted with 1 mol/L HCl or NaOH to 4.0 during the fermentation process. All the flasks were cultured on a rotary shaker at 150 r/min, 26 °C.

Immobilization of mycelia in alginate bead

The precultured broth was gently homogenized and centrifuged at 6,869$\times g$ for 20 min. The supernatant was decanted and the residual mycelia were washed three times with distilled water. Sodium alginate (Mannuronate/Guluronate: 0.54; 3.0 %, w/v) in distilled water was sterilized at 120 °C for 20 min. After cooling, the mycelia were added to the alginate solution and mixed thoroughly and the biomass concentration was 6.0 g dry weight of mycelia pre liter. Beads were prepared by dropping droplets of the solution into a sterilized $CaCl_2$ solution (2.0 %, w/v). The $CaCl_2$ concentration was selected according to previously reported results [26]. The beads remained in the $CaCl_2$ solution for 6 h to allow for complete gelation. The beads were uniform and had an average diameter of 5.3 mm. Prior to using the beads for laccase production, the immobilized mycelia were precultured in potato dextrose broth for 3 days. The final biomass was 0.9 g (dry weight of mycelia) per 100 mL of beads. The precultured beads

were washed with distilled water before they were used for laccase production.

Ultrasonic stimulation

For the ultrasonic stimulation, the flasks were placed in the center of an ultrasonic cleaning bath (NINGBO SCIENTZ Biotechnology CO. LTD., China) supported by a stainless-steel basket. The distance between the bottom of the flasks and the bottom of ultrasonic bath was set at 10 mm, and each flask was placed at the same position in the ultrasonic bath for the ultrasonic treatment. The water temperature in the ultrasonic bath was kept at 26 °C using a water circulation cooling device (Beijing Changliu Scientific Instruments CO. LTD., China). The ultrasonic frequency (40 kHz) and power (120 W) were selected based on results previously reported in the literature [27, 50]. The ultrasonic treatment was conducted using the "duty cycle" control, which consisted of 10 s working time and 10 s stop time. To test the effect of ultrasound on laccase production at different culture phases, the beads (9 mL beads per flask) were exposed to a 5-min ultrasonic treatment at different culture stages (12, 24, 36, 48, 60, and 72 h). To test the effect of treatment duration, the beads were exposed to an ultrasonic treatment for a given time period (3, 5, 7, 9, and 11 min) after they had been cultured for 36 h. To test the effect of repeated exposure, the beads were repeatedly exposed to 7-min ultrasonic treatments at different intervals (6, 12, 18, and 24 h) after they had been cultured for 36 h. Following the ultrasonic treatments, the flasks with the immobilized mycelia were returned to the shaker for continued cultivation. All experiments were conducted in triplicate.

Bubble column reactor

A bubble column reactor with 2 L working volume was used for the laccase production with immobilized cells. The reactor was constructed from a flanged glass column (Inner diameter = 8 cm, Height = 45 cm) containing an air sparger (compressed stainless steel particles) at the bottom of the bioreactor for air supply (Fig. 1). The bead loading rate was 15 % (v/v, 300 mL) and the pH of the culture broth was controlled automatically by the pH controlling system (pH-800, East Biotech Equipment and Technology Co., Ltd., ZhenJiang, China). Fermentation was conducted at 26 °C and an aeration rate of 200 mL/min. When ultrasonic treatment was conducted at the optimized condition, the bioreactor was put into the centre of the ultrasonic bath and each bioreactor was put at the same position for the ultrasonic treatment. Following the ultrasonic treatments, the culture of the immobilized cells in bioreactors continued. All experiments were conducted in triplicate.

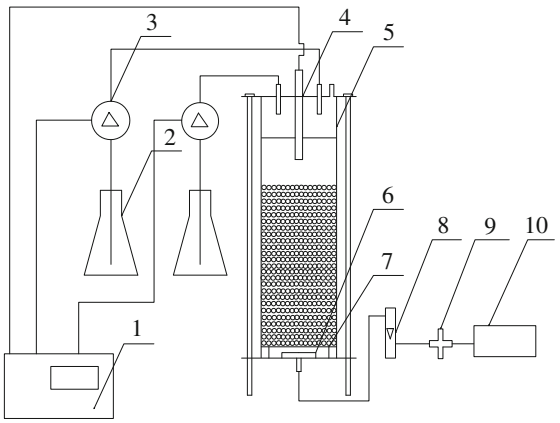

Fig. 1 Schematic diagram of the bubble column reactor. *1*-pH controller, *2*-acid/alkali container, *3*-peristaltic pump, *4*-pH probe, *5*-glass column, *6*-air sparger, *7*-particle supporter, *8*-rotameter, *9*-air filter, *10*-air pump

Analysis

The cell-free supernatants were used to quantify the proteins and sugars in the media. The proteins were determined using the Bradford assay with bovine serum albumin as the standard [6], and reducing sugars remaining in the medium were quantified using Miller's method with glucose as the standard [32]. Laccase activity in the medium was quantified spectrophotometrically in a reaction medium containing a 100 mM tartaric acid buffer (pH 3.0) with 0.1 % (w/v) catechol as the substrate at 25 °C [17]. A suitable amount of the cell-free supernatant was added to the substrate solution for 5 min, and the increase in the absorbance at 450 nm was measured using a UV-2100 spectrophotometer (Shanghai, China). The molar absorption coefficient of catechol is 2,211 L/(mol cm) [21]. One unit of activity is defined as the amount of laccase required to oxidize one μmol of catechol per minute.

To determine the effect of ultrasound on the mass transfer of substrate, we evaluated the absorption of crystal violet by the alginate beads (without mycelia) before and after ultrasonic treatment. Batch adsorption studies were carried out in 250 mL flasks containing 6 μg/mL crystal violet and 150 g/L alginate beads with 60 mL of working volume. The flasks were agitated at 150 r/min for 75 min in a rotary shaker at 26 °C. Samples were taken from the reaction medium for the analysis of the residual dye concentration in the solution, which was determined using a UV/VIS spectrophotometer [30]. The amount of crystal violet adsorbed per unit of beads was calculated according to the following equation:

$$Q_t = \frac{(C_0 - C_t)V}{m} \quad (1)$$

where C_0 and C_t represent the initial and dynamic (after adsorption for t time) crystal violet concentrations (μg/mL); m and V represent the weight of the beads (g) and the solution volume of crystal violet (mL); Q_t is the mass (μg/g wet gel) of crystal violet adsorbed.

The adsorption kinetic of crystal violet from the liquid phase to the gel beads was analyzed based on the Lagergren rate equation [1]:

$$K_d t = -\ln\left(1 - \frac{Q_t}{Q_e}\right) \quad (2)$$

where Q_t and Q_e are the mass of crystal violet adsorbed at time t and at equilibrium respectively (μg/g wet gel); K_d is overall rate constant of adsorption (min^{-1}); and t is the reaction time (min).

To test the effect of ultrasound on laccase stability, the cell-free supernatants were exposed to the same intermittent ultrasound after it had been cultured for 36 and 48 h, respectively. Samples were withdrawn during the sonication process and the laccase activity was determined. All the supernatants were obtained from the cultures with ultrasonic strategy.

The generation of H_2O_2, $O_2^{\cdot-}$ and $^{\cdot}OH$ was monitored in the culture medium during the culture process and after ultrasound exposure. H_2O_2 was measured based on a specific complex formed by hydroperoxides with titanium (Ti^{4+}), which can be determined by colorimetry. 0.2 mL of a titanium reagent (20 % titanic tetrachloride in concentrated HCl, v/v) was added to 2 mL of cell-free supernatants, followed by the addition of 0.4 mL concentrated NH_4OH to precipitate the peroxide-titanium complex. After centrifugation for 5 min at 1,0000×g, the supernatant was discarded and the precipitate was solubilized in 3 mL of 2 mol/L H_2SO_4 and filtered prior to measurement of absorbance at 415 nm against a water blank which had been carried through the same procedure [7]. The concentration of the peroxide in the culture medium was determined by comparing the absorbance against a standard curve representing titanium-H_2O_2 complex at different concentrations. The $O_2^{\cdot-}$ concentration was determined by a modified method of Elstner and Heupel [12]. 1 mL of hydroxylammonium chloride (1 mol/L) was added to 1 mL of cell-free supernatants. The mixture was incubated at 25 °C for 20 min for nitrite formation. After that, 1 mL of sulfanilic acid (17 mmol/L) and 1 mL (7 mmol/L) α-naphthylamine were added to the mixture. The reaction was conducted at 25 °C for 20 min, and then the absorbance of the mixture was determined at 530 nm in the spectrophotometer. The amount of produced nitrite was obtained by comparing the absorbance against a standard curve representing different NO_2^- concentration. The amount of $O_2^{\cdot-}$ was calculated taking into account the stoichiometry of the nitrite-formation reaction (2 $O_2^{\cdot-}$:1 NO_2^-). The concentration of $^{\cdot}OH$ was estimated as TBA-reactive substances (TBARS) production from 2-deoxyribosethe. 0.5 mL of 2.8 % (wt/vol) trichloroacetic acid and 0.5 mL of 1 % (wt/vol) 2-thiobarbituric acid (TBA) in 50 mM NaOH were added to 1-ml cell-free supernatants and heated for 10 min at 100 °C. After the mixture cooled, the absorbance was read at 532 nm [14].

Results and discussion

Laccase production by *T. versicolor* immobilized in alginate beads

Laccase production by the immobilized *T. versicolor* at various bead loading rates (5, 10, 15, 20, 25, and 30 %, v/v) increased as the particle loading rate increased from 5 to 15 % (Fig. 2). When the bead loading rate was further increased, laccase production decreased due to nutrient deficiency and the limitation of mass transfer [26]. At the optimal loading rate (15 %), more than 90 % of the reducing sugars in the broth were consumed after 24 h of culture (Fig. 3). At that point, laccase production increased sharply and reached a maximum of 503.3 U/L after 72 h of culture (protein concentration was 0.112 mg/mL). A similar phenomenon was reported in the immobilized cell culture of *Bjerkandera adusta*, in which the laccase production increased sharply once the sugars were nearly consumed [33]. The protein concentration increased as the laccase activity increased. The decrease in both protein and enzyme activity beyond 72 h occurred due to the degradation of laccase by proteases [33, 41]. The laccase production obtained in this study was much higher than those obtained by Dominguez et al. using alginate as a carrier for

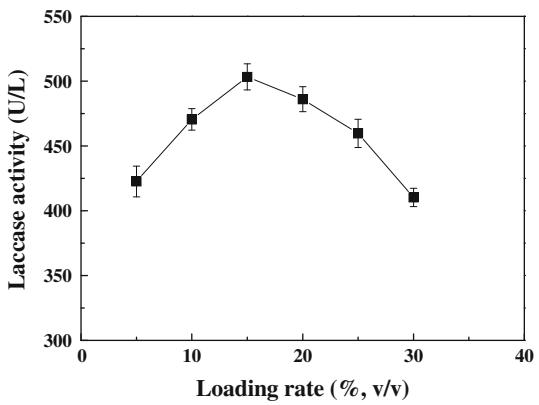

Fig. 2 Effect of bead loading rate on laccase production by immobilized *T. versicolor*

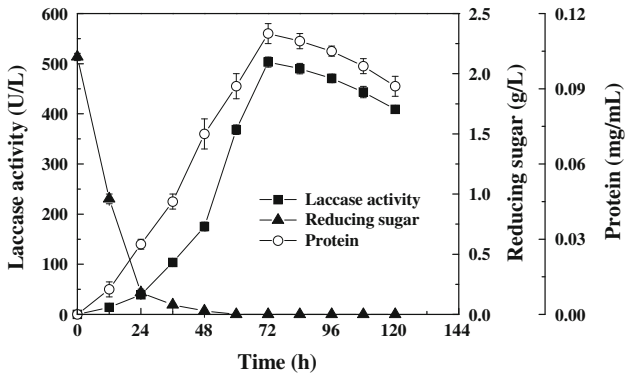

Fig. 3 Time course of laccase fermentation by immobilized *T. versicolor*

T. versicolor immobilization (Table 1) [11]; this could be due to differences in the fungal strain, the immobilization process, or culture conditions. In our previous research, the pelleted mycelia were reported to produce laccase of 328.9 U/L with the initial biomass of 0.84 g/L in the flask culture [50]. The immobilized mycelia in this study provided much higher laccase production (470.5 U/L) with similar initial biomass (10 % loading rate, 0.9 g/L). This phenomenon was in agreement with the report by Papagianni et al. [37], which stated that glucoamylase production in the immobilized culture of *Aspergillus niger* was significantly higher than that from the mycelium in the pelleted form. The mechanism for the increased enzyme production in immobilized cultures was related to the physiological differences between immobilized microbial cells growing at a solid–liquid interface and free cells growing in liquid medium [37]. Comparisons between free and immobilized cells have illustrated phenotypic differences, indicating that cell immobilization is a process of fundamental importance, influencing the composition of cell wall or plasma membrane and stimulating responses that may affect the expression of a differentiated state [19, 20]. To investigate the potential of the enzyme in future applications, laccase was further separated from the broth according to a simple and efficient protocol [49]. The purified laccase exhibited 99.5 % of phenol degradation efficiency at pH 5.0, 25 °C in the buffer system.

Effect of ultrasound on laccase production by immobilized *T. versicolor*

The immobilized mycelia were exposed to a 5 min ultrasound treatment at various cultivation times (Table 2). When the mycelia cultures were exposed to an ultrasonic treatment after 36 h of culture, the laccase production significantly increased to 758.4 U/L. Therefore, the effect of the duration of the ultrasonic treatment on laccase production was tested after 36 h culture. The 7-min ultrasonic treatment provided the better laccase level, while prolonged ultrasonic treatments resulted in a decrease in laccase production (Table 2). This could be due to cell damage resulting from excessive ultrasonic stimulation [43]. Compared with the optimal duration of ultrasonic treatment for the pelleted mycelia culture of *T. versicolor* (5 min) [50], treatment is longer for the immobilized culture due to the obstructing effect of alginate gel. The culture was also subjected to multiple 7-min ultrasonic treatments after 36 h culture, and the highest laccase activity was obtained when the two treatments were separated by a 12-h interval (Table 3). Increasing the number of ultrasonic treatments resulted in decreased laccase activity. Similar results were also observed in *T. versicolor* pelleted mycelia and *E. purpurea* hairy roots stimulated by repeated ultrasonic treatments: excessive ultrasonic stimulation resulted in severe mechanical stress [27, 50].

The highest laccase production was obtained after 72 h culture using the optimized ultrasonic strategy (Fig. 4). Using the optimized strategy, the protein concentration increased to 0.22 mg/mL, and laccase activity in the culture broth increased to 116 % higher than the untreated control. In the pelleted culture of *T. versicolor* with suitable ultrasonic treatment, laccase production increased to 79.1 % greater than the untreated control [50]. The greater increase of laccase production in immobilized cultures occurred because the alginate gel may protect the

Table 1 Laccase production from immobilized cells in batch liquid cultures

Strain	Carrier	Laccase yield (U L^{-1})	Culture time (day)	Reference
Agaricus sp.	Textile strip	3,950	18	[22]
Trametes hirsuta	Stainless steel sponges	5,000	9	[9]
Trametes hirsuta	Stainless steel sponges	2,206	8	[40]
Trametes versicolor	Alginate	4,000	14	[11]
Trametes versicolor	Alginate	10,915[a]	3	Current work
Trametes versicolor + ultrasonic treatment	Alginate	23,140[a]	3	Current work

[a] Laccase activity was assayed spectrophotometrically by measuring the oxidation of ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] at 420 nm. One unit of activity is defined as the amount of laccase required to oxidize one μmol of ABTS per minute at 25 °C. The method of enzyme assay and the definition of enzyme activity were the same as in References 7, 9, 16, 33

Table 2 Effect of ultrasound timing and duration on laccase production by immobilized *T. versicolor*

Ultrasound condition	Ultrasound timing (h)					Ultrasound duration (min)				
	12	24	36	48	60	3	5	7	9	11
Laccase activity (U/L)[a]	432.1 ± 10.7	483.3 ± 8.4	758.4 ± 15.1	633.2 ± 9.9	535.2 ± 11.2	583.2 ± 12.6	758.4 ± 15.1	842.5 ± 16.8	774.3 ± 14.3	670.3 ± 10.1

[a] The maximal laccase activity in the culture broth achieved during the culture process

Table 3 Effect of the multiple ultrasonic treatments on laccase production by immobilized *T. versicolor*

[a] The maximal laccase activity in the culture broth achieved during the culture process

Ultrasound times	Laccase activity (U/L)[a]			
	6-h Interval	12-h Interval	18-h Interval	24-h Interval
1	842.5 ± 16.8	842.5 ± 16.8	842.5 ± 16.8	842.5 ± 16.8
2	879.9 ± 10.7	1,087.4 ± 21.4	898.4 ± 14.9	879.2 ± 17.6
3	723.6 ± 15.2	915.4 ± 13.9	898.4 ± 14.9	879.2 ± 17.6

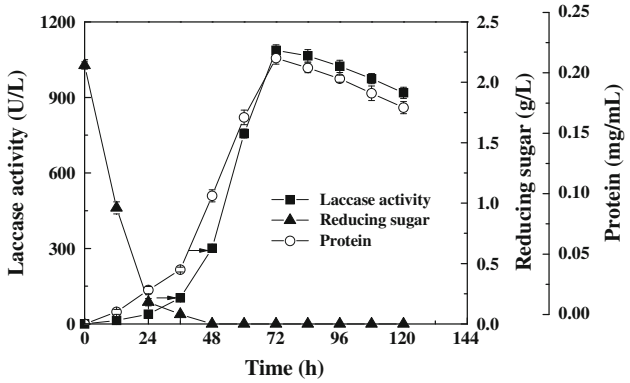

Fig. 4 Time course of laccase production by immobilized *T. versicolor* with the optimized ultrasonic treatment strategy. *Arrow* indicates the ultrasonic treatment timepoint

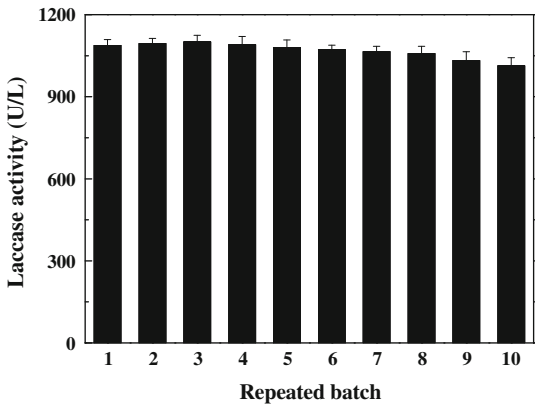

Fig. 5 Repeat batch use of immobilized mycelia for laccase production with the optimized ultrasonic treatment strategy

immobilized mycelia from mechanical damage caused by ultrasound. The potential to reuse the immobilized mycelia with the ultrasonic strategy was also tested, and the immobilized mycelia were able to continue producing significant levels of laccase activity (>1,000 U/L) with the ultrasonic treatment for 10 cycles (Fig. 5).

To evaluate the effect of ultrasound on mass diffusion using the alginate beads, the adsorption kinetics of crystal violet by the beads was compared with and without ultrasonic treatment. The 7-min ultrasound treatment did not affect the adsorption capacity of the gel beads (Fig. 6). Based on the adsorption kinetic data and the Lagergren rate equation, the K_d values were 0.072 min^{-1} ($R^2 = 0.997$) and 0.112 min^{-1} ($R^2 = 0.992$) for the sonicated and untreated beads, respectively. These results demonstrated that the ultrasonic treatment was beneficial for the mass diffusion from the liquid phase to the gel matrix; therefore, the improvement in the diffusion of nutrients and products is one of the reasons for the increase in laccase production by immobilized cells. The K_d is the overall rate constant of adsorption, and it is dependent upon both external and internal mass transfer. The external mass transfer refers to the transfer of substrates from the bulk liquid medium to the carrier surface through the external liquid film surrounding the immobilized cell bead [38]. This film can impede mass transfer of nutrients and products, and therefore, be a rate-controlling factor [8]. Ultrasonic treatment is an efficient method to thin the fluid boundary layer around the beads to enhance mass transfer [23]. Internal mass transfer relates to the transfer of substrates and products within the carrier solid phase and is influenced by the immobilization matrix size, texture, and porosity [38]. In this study, the porosity of the gel beads did not change significantly following the ultrasonic treatment (data not shown); however, cavitation generated at a low ultrasound frequency can create an increase in cell membrane porosity, which can change the texture of the surface layer of the beads and partially improve internal mass transfer [10, 46]. Therefore, the increase in mass transfer for the alginate bead culture system was due to the

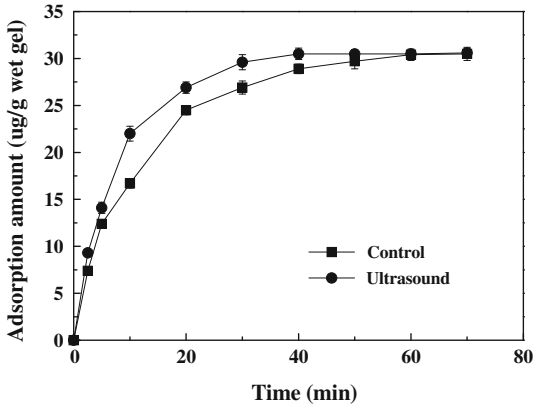

Fig. 6 Effect of ultrasonic treatment on the crystal violet adsorption of alginate gel beads

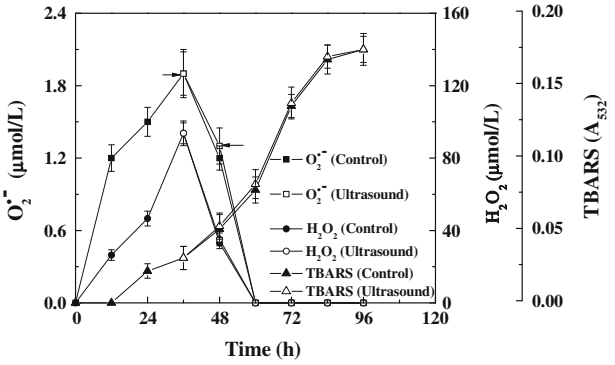

Fig. 7 Effect of ultrasonic treatment on the production of $O_2^{\cdot-}$, $^{\cdot}OH$ and H_2O_2 by immobilized *T. versicolor*. *Arrow* indicates the ultrasonic treatment timepoint

synergetic effects of the enhancement of external and internal mass transfer by ultrasound treatment.

Besides the increase in mass transfer for the alginate bead, the expression of the laccase gene (*lcc*) could also be induced by local heat and pressure stress resulted from cavitation generated in the ultrasonic treatment [50]. In addition, for the 7-min ultrasound, the laccase activity released to the medium was only 20.4 and 29.0 U/L after 36 and 48 h of the cultivation, respectively (data not shown). This indicated that the contribution of the released activity to the laccase enhancement stimulated by ultrasound was low.

Extracellular hydroxyl radical ($^{\cdot}OH$) was produced from *T. versicolor* by the quinone redox cycle [14] and the ultrasound exposure could induce transient production of the superoxide anion radicals ($O_2^{\cdot-}$) and H_2O_2 in *Taxus chinensis* cell suspension culture [51]. These free radicals are good oxidants and are beneficial for pollutant degradation. Therefore, the effect of ultrasound on the production of $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$ was also evaluated, and the results were shown in Figs. 7, 8. Ultrasonic treatment has no effect on the production of the radicals and H_2O_2 during the laccase fermentation (Fig. 7). The concentration of $O_2^{\cdot-}$ and H_2O_2 increased and reached the maximal after 36 h. Then, $O_2^{\cdot-}$ and H_2O_2 in the culture medium decreased due to the $^{\cdot}OH$ production from $O_2^{\cdot-}$ and H_2O_2 [14]. The concentration of $^{\cdot}OH$ increased and obtained the maximal after 84 h. After the ultrasound exposure at 36 h, a small rise occurred in the concentration of $O_2^{\cdot-}$ from 30 min and the level of $^{\cdot}OH$ and H_2O_2 increased significantly after 20 min and reached the peak value at 40 min (Fig. 8). All the radicals and H_2O_2 returned to their initial levels after 70 min. Similar trends were obtained for the levels of radicals and H_2O_2 after the culture was treated at 48 h. These results indicated that the transient production of $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$ after ultrasonic treatment didn't change the final levels of radicals and H_2O_2 in the supernatant of the

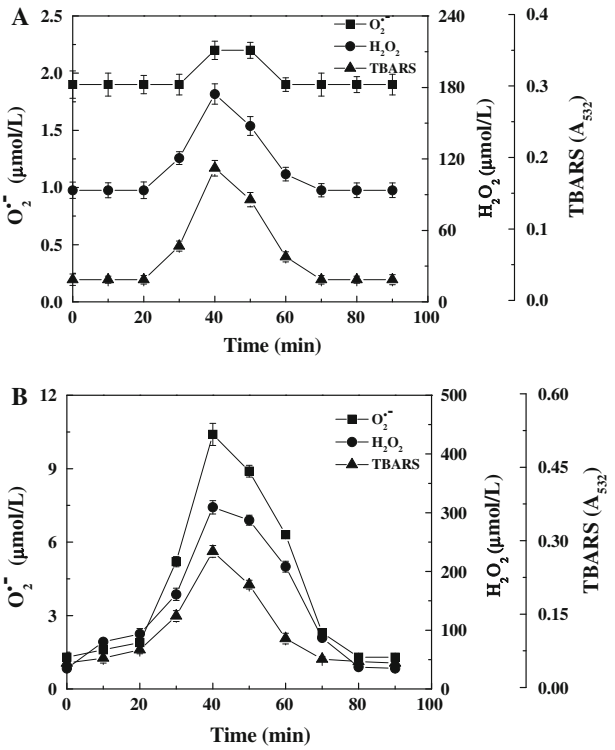

Fig. 8 Generation of $O_2^{\cdot-}$, $^{\cdot}OH$ and H_2O_2 after the ultrasound exposure to the culture of immobilized *T. versicolor* at 36 h (**a**) and 48 h (**b**) with the optimized ultrasonic treatment strategy

culture. Ultrasound could reduce laccase activity due to the protein aggregation resulted from the radicals produced during sonication [3]. In this study, no significant decrease happened to the laccase activity in the culture medium after 1 h the ultrasonic treatment (Fig. 9). This was because that the intermittent ultrasound used in this study could reduce the formation of free radicals [24, 36]. The experimental result also indicated that there was no great change in the level of radicals in the cell-free supernatant when it was sonicated (data not shown). Thus, it can be concluded that

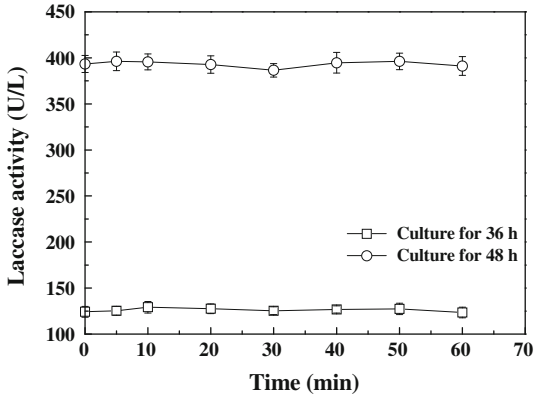

Fig. 9 Effect of ultrasound on the laccase stability in cell-free supernatants obtained from the culture of immobilized *T. versicolor* with the optimized ultrasonic treatment strategy

the reduction of laccase production with prolonged sonication was not due to the lost of laccase activity induced by ultrasound.

Laccase production by immobilized *T. versicolor* in bubble column reactor

The optimized ultrasonic strategy was applied to the immobilized *T. versicolor* in a bubble column reactor. As shown in Fig. 10, laccase production was higher in the bioreactor than in the flasks with or without the ultrasonic treatment. This may be due to the improved pH-control and aeration in the bubble column reactor [48]. With the ultrasonic treatment, the laccase activity (1,218.6 U L^{-1}) increased 112 % higher than the control (untreated culture; 574.8 U L^{-1}) Using the optimized ultrasonic strategy, the immobilized mycelia in alginate beads exhibited much higher laccase production compared to previously reported results (Table 1). The immobilized mycelia with the ultrasonic strategy also exhibited similar reusability in the bubble column reactor as that in the flasks (data not shown). To carry out the immobilized cell culture with ultrasonic strategy in large scale, a rational design of high-performance sonobioreactors should be possible. It is difficult to scale up the sonobioreactor using the ultrasonic bath. Recently, a 30-L airlift sonobioreactor was designed and established by our group [28]. It provided the potential for the scaling-up of laccase production with immobilized cells in a sonobioreactor and study on enzyme production in large-scale would be further conducted in this bioreactor.

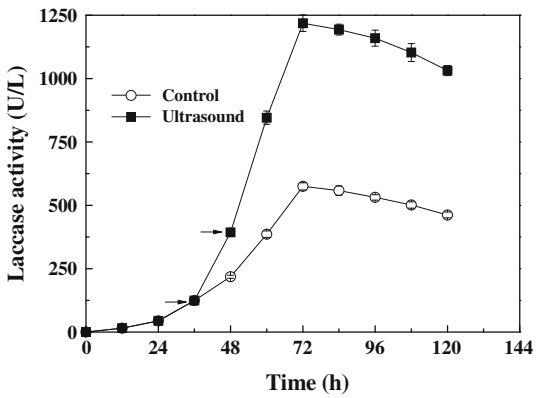

Fig. 10 Laccase production by immobilized *T. versicolor* with the optimized ultrasonic treatment strategy in the bubble column reactor. *Arrow* indicates the ultrasonic treatment timepoint

Conclusions

The immobilized mycelia of *T. versicolor* within alginate beads provided higher laccase production than that in pelleted form. Further, suitable sonication strategy improves laccase production by immobilized *T. versicolor*. Using optimized timing, duration and frequency, the ultrasonic treatment enhanced the laccase activity by more than twofold relative to the control. The increase in laccase production is related to the improvement of mass transfer from the bulk liquid medium to the gel matrix. In addition, the optimized ultrasonic strategy was succeeded in the laccase fermentation with immobilized mycelia in the bubble column reactor. This method provides a useful strategy for efficient laccase production and offers the potential for large-scale laccase production in sonobioreactors.

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