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Novel Synthesis of Oxidoreductase Immobilized Biocatalyst for Effective Anthraquinone Dye Treatment in Bioreactor

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Over the past few decades, biotransformations of compounds with complicated and reinforced structures, especially some poorly biodegradable organic pollutants, have attracted extensive research attention. Benefiting from recently developed techniques of protein engineering, oxidoreductase industrial applications such as laccases, tyrosinases, and various oxygenases have been recognized as a promising alternative technique as compared with the conventional treatment processes of industrial textile effluents. However, the lack of long-term operational stability and reusability of the above-mentioned enzymes may limit their further large-scale industrialization. To overcome this, a novel biocatalyst was developed by immobilizing laccase from *Trametes versicolor* onto ultraporous gamma-alumina powders (laccase@UPA(*γ*)), followed by transferring it into a portable and easy-to-carry bioreactor for Remazol Brilliant Blue R (RBBR) dye biodegradation. The obtained results showed that the treatment capacity of laccase@UPA(*γ*) towards RBBR reached about 60 mg/g after 24 h of contact time at pH 5. These preliminary results highlight the potentials of bio-based inorganic materials in industrial wastewater treatment, which can broaden our understanding of their practical applications in the environmental field.

1. Introduction

Nowadays, as synthetic dye applications on the printing and dyeing processes of fabrics have been extensively developed worldwide, about 50000 tons of dyes get discharged into the environment every year, and nearly 20% of industrial water pollution results from dyestuff related industries (Bilal et al., 2017; Routoula and Patwardhan, 2020). Among the various dye compounds, Remazol Brilliant Blue R (RBBR) poses a series of environmental problems as its complicated and reinforced anthraquinone structure makes it difficult to be degraded naturally in the environment (Moussavi and Mahmoudi, 2009; Gök et al., 2010). In the meantime, government legislation is becoming stricter than before regarding dye treatment in industrial textile effluents, especially in the more developed countries (Osma et al., 2010). Therefore, in the context of green and sustainable chemistry, proper treatments regarding purifications and remediations of industrial textile effluents are necessary and urgent.

Generally, the most common methods in terms of dye treatment can be classified into physical, chemical/electrochemical, biological methods, and/or emerging combination of several above-mentioned techniques with the purpose of synergistic effects (Cicek et al., 2007; Madrakian et al., 2013). Among these conventional methods, enzyme-based biocatalysis which is widely characterized by high chemo-, regio-, and stereoselectivity has many advantages. Thanks to the recent advances in modern biotechnology and protein engineering, biotransformations of poorly biodegradable dyes into non-hazardous or less-hazardous substances have been recognized as a key strategy to control the level of contaminations in water and soil (Sheldon and Woodley, 2018; Zdarta et al., 2018). Laccase (oxidoreductase, EC 1.30.3.2), which is secreted by white-rot fungi, has received numerous research attention because it only needs molecular oxygen (air) as a co-substrate and can catalyze the oxidation of a great variety of phenolic and non-phenolic compounds

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(Osma et al., 2010; Hou et al., 2014). In addition, laccase applications in the biodegradation of endocrine disrupting chemicals (EDCs) and dye treatments have also been extensively studied in the last decades (Hou et al., 2014; Zdarta et al., 2018). The potential use of enzyme in industrial applications normally requires enzyme immobilization, in which proper selection of supporting carrier (organic origin, inorganic origin, and hybrid materials) is of great importance regarding enhanced enzyme stability against harsh conditions of pH, temperature, and pressure (Torres-Salas et al., 2011; Zdarta et al., 2018). More recently, broad research interests have been focused on inorganic materials which can be obtained relatively cheaply and usually by uncomplicated synthesis procedures. For example, Lassouane et al. (2019) synthesized crude laccase immobilized Ca-alginate beads for Bisphenol A (BPA) degradation from aqueous solutions.
In this study, laccase from *Trametes versicolor* (laccase *T.*) was cross-linked immobilized by ultraporous gamma-alumina powders (UPA(*γ*)) with glutaraldehyde as the bifunctional cross-linker, and the obtained biocatalyst (laccase@UPA(*γ*)) was transferred into a disposable 2 mL polystyrene column for RBBR dye biodegradation. The present study highlights the UPA potentials in terms of oxidoreductase immobilization, which can broaden the practical applicability of aluminium materials in industrial wastewater treatment.

2. Experimental procedure

2.1 Materials and chemicals

The raw laminated metallic aluminium plate (100×100 mm, 1.0 mm of thickness, 99.99% of purity) and disposable 2 mL polystyrene column were supplied by Goodfellow Cambridge Ltd. and ThermoFisher Scientific Inc., respectively. The chemicals including acetone, mercury(II) nitrate monohydrate ($Hg(NO_3)_2 \cdot H_2O$, ≥ 98.5%), silver nitrate ($AgNO_3$, ≥ 99.0%), laccase *T.* (≥ 0.5 $U \cdot mg^{-1}$), RBBR (also called RB19, representative anthraquinone dye), (3-aminopropyl)triethoxysilane (APTES, 99%), glutaraldehyde (25% of *v*/*v*), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, HPLC), sodium acetate (CH_3COONa, ≥ 99.0%), sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$, ≥ 99.0%, phosphate buffer), glacial acetic acid (CH_3CO_2H, ≥ 99.5%), and sodium hydroxide (NaOH, ≥ 97.0%) were purchased from Sigma-Aldrich, Inc.. All the chemicals used in this study were of analytical grade and used as received directly without further purification. Milli-Q water (Millipore Corp.) with a specific resistivity of 18.2 $M\Omega \cdot cm^{-1}$ at 25 °C was used to prepare solutions throughout the experiments.

2.2 UPA(*γ*) powders and laccase@UPA(*γ*) biocatalyst syntheses

The UPA(*γ*) monolith sample was synthesized via facile oxidation process according to the previously reported studies (Vignes et al., 2008; Bouslama et al., 2012; Khodan et al., 2018). Briefly, high purity but fragile UPA monolith sample was obtained with a growth rate of ~1 $cm \cdot h^{-1}$ at room temperature in a humid atmosphere (70–80% RH) by the oxidation of metallic aluminium plate through a liquid mercury-silver layer. Anhydrous UPA(*γ*) monolith can be obtained under 4 h of isochronous annealing treatment in air at 950 °C. After rigorous grinding process, UPA(*γ*) powders can be accordingly obtained.
Prior to the immobilization procedure, UPA(*γ*) powders were silanized with 2.5% (*v*/*v*) APTES in acetone at 45 °C and 100 rpm for 24 h, followed by washing the obtained powders with phosphate buffer for three times to remove any residual organics. Subsequently, a given amount of APTES silanized UPA(*γ*) powders were dispersed in 0.5% (*v*/*v*) glutaraldehyde solution under neutral pH conditions. The mixture was kept under stirring at 20 °C and 100 rpm for 6 h, followed by the washing procedure as mentioned above to remove unreacted glutaraldehyde molecules. After glutaraldehyde functionalization, the obtained powders were suspended in laccase *T.* solution at 20 °C and 100 rpm for 24 h, resulting in Schiff base formation and subsequent cross-linking of laccase *T.* with functionalized UPA(*γ*) powders (laccase@UPA(*γ*)).

2.3 Characterizations

The obtained samples were characterized by using scanning electron microscopy (SEM, Zeiss Supra 40 VP) and transmission electron microscopy (TEM, JEOL 2011) techniques. The XRD pattern was carried out by using an Inel Equinox 1000 X-ray diffractometer (Inel) with Co K*α* radiation source (*λ* = 1.7902 Å), and the analysis was performed at 2*θ* diffraction angles from 20° to 95° at a speed of 2°/min. The FTIR spectra were recorded by using a PerkinElmer Spectrum 100 system spectrometer in pressed KBr pellets (Sigma-Aldrich, 99%, analytical reagent) and in the 400–4000 cm^{-1} region.

2.4 Data processing

The concentration of RBBR and laccase *T.* in supernatant (C_t, mg·L^{-1}) were determined by spectrophotometry method at the wavelength of 590 and 420 nm, respectively (UviLine 9400 UV-Visible spectrophotometer, Secomam). C_0 (mg·L^{-1}) is the initial concentration of corresponding substances in suspension.

3. Results and discussion

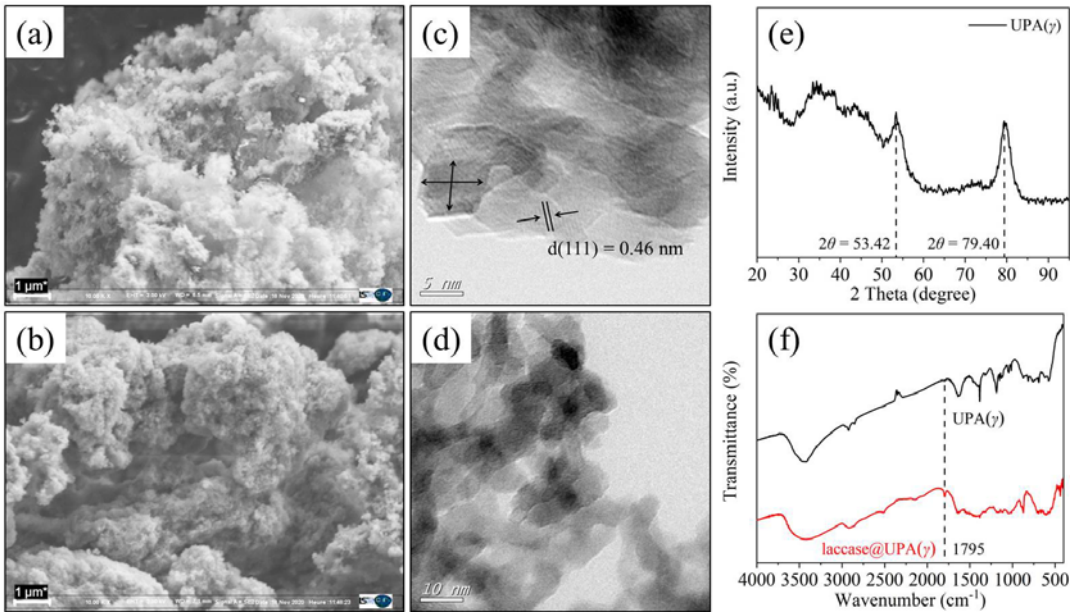


Figure 1: SEM images of (a) UPA(γ) powders and (b) laccase@UPA(γ) biocatalyst, TEM images of (c) UPA(γ) powders and (d) laccase@UPA(γ) biocatalyst, (e) XRD pattern of UPA(γ) powders, and (f) FTIR spectra of UPA(γ) powders and laccase@UPA(γ) biocatalyst.

Figure 1a–1d showed the SEM and TEM images of UPA(γ) powders and laccase@UPA(γ) biocatalyst, which evidences the ultraporous morphology of obtained samples. TEM image at higher magnification (Figure 1c) revealed the clear lattice fringe with a *d*-spacing of 0.46 nm, which corresponds to the (111) plane of gamma-alumina (JCPDS 10-0425) (Souza Santos et al., 2000). By comparing Figure 1d with Figure 1c, the UPA(γ) border became smoother after laccase *T.* immobilization. Based on the calculation from Bragg equation ($2d\sin\theta = n\lambda$, n = 1, 2, 3, etc.) (Figure 1e), the two typical diffraction peaks at 2θ = 53.42° (d = 1.99 nm) and 79.40° (d = 0.14 nm) correspond to the (400) and (440) planes of gamma-alumina, respectively (JCPDS 29-0063) (Souza Santos et al., 2000). After laccase *T.* immobilization, the absorption band at 1795 cm^{-1} was assigned to C=O stretching vibration (Figure 1f). Moreover, a broader absorption band in the range of 3400–3500 cm^{-1} indicated the presence of carboxyl and amino groups distributed on the UPA(γ) surface after laccase *T.* immobilization. The above-mentioned discussions confirm that laccase *T.* was successfully immobilized on the UPA(γ) surface with glutaraldehyde as the cross-linker.

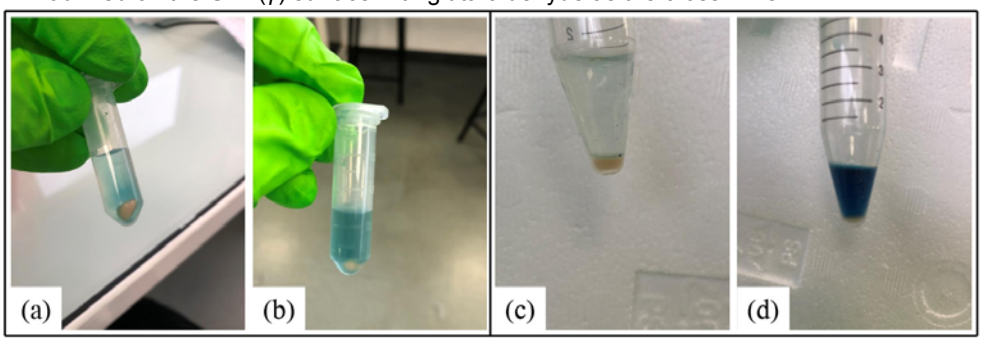


Figure 2: Photos of two parallel experiments regarding ABTS oxidation monitoring: (a) to (b) 0.5 mg, and (c) to (d) 2.0 mg of laccase@UPA(γ) biocatalyst.

In Figure 2, two parallel experiments regarding ABTS oxidation by laccase@UPA(*γ*) biocatalyst were conducted, and positive results related to active laccase *T*. on UPA(*γ*) surface can be observed obviously. In Figures 2a and 2c, the green coloured $ABTS^{\bullet+}$ radical was initially diffused from the surface of biocatalyst.

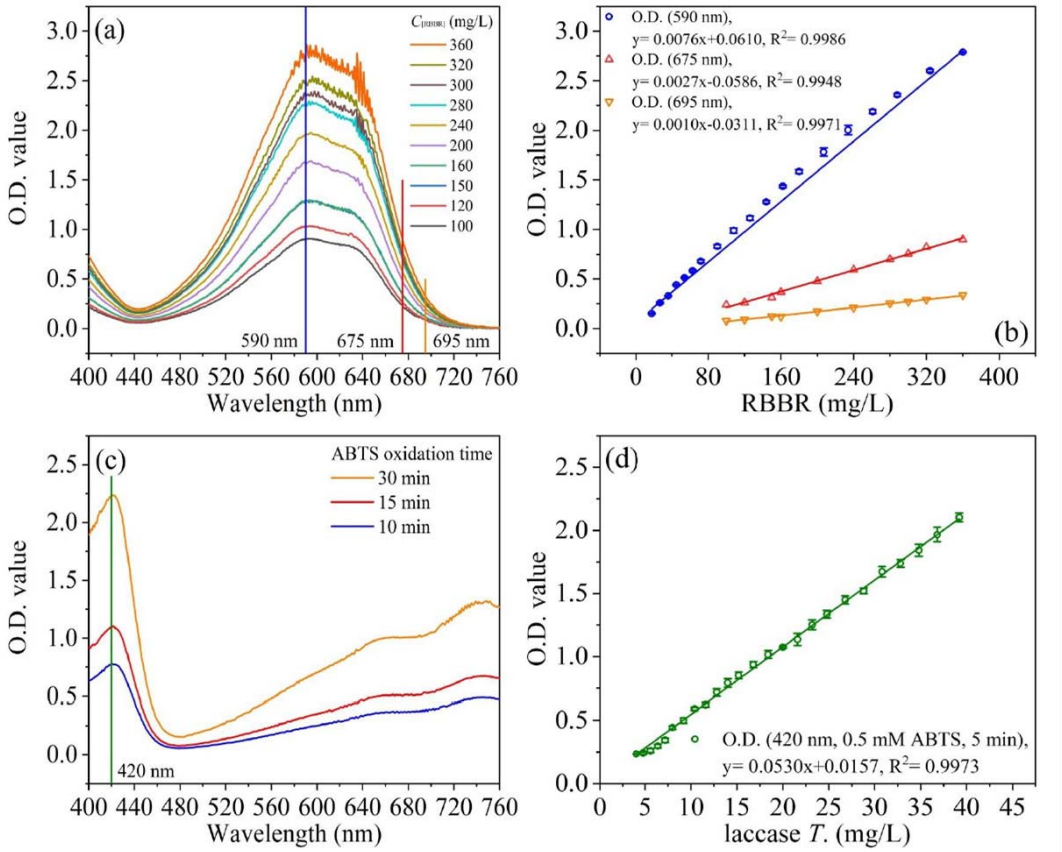


Figure 3: (a) Visible spectral curves of RBBR at different concentrations, (b) working curves of RBBR with optical density (O.D.) values at different wavelengths (λ = 590, 675, and 695 nm), (c) visible spectral curves of laccase T. assay by using ABTS as the substrate at different oxidation time (t = 10, 15, and 30 min), and (d) working curves of laccase T. assay by using ABTS as the substrate at 5 min of oxidation time (λ = 420 nm).

Figure 3 showed the working curves of RBBR and laccase *T*. (ABTS as the substrate) stoichiometrically determined by the spectrophotometry method. As shown in Figure 3b, the linear relationships between optical density (O.D.) value and RBBR concentration can also be obtained at higher spectral positions (i.e., 675 and 695 nm). Therefore, for the determination of high RBBR concentration which may exceed the detection limit of the visible spectrophotometer, a higher spectral position for measurement (e.g., 675 and 695 nm) other than one more step dilution operation at 590 nm of spectrum peak can also be applicable.

In Figure 3c, the O.D. value regarding ABTS oxidation kept increasing as oxidation time increased. Generally, the oxidation of colourless ABTS can undergo a fast one-electron transfer process, and the reaction product $ABTS^{\bullet+}$ is a stable green coloured radical (Pinkernell et al., 2000). According to Fernando Bautista et al. (2010), laccase *T*. possesses higher oxidation capacity because it can catalyze the four 1-electron oxidations of electron-rich compounds with the simultaneous 4-electron reduction of molecular dioxygen to water. Therefore, in terms of laccase *T*. working curve, the ABTS oxidation time was fixed at 5 min, in which the corresponding determination coefficient (R^2) of linear equation fitting and relative error bar of triplicate measurements were 0.997 and below 5%, respectively (Figure 3d). In addition, since the measurement accuracy is highly dependent on the oxidation time, it is also suggested to separately measure the corresponding O.D. value rather than unify the measurements altogether.

Figure 4a showed the schematic diagram of a disposable polystyrene column, in which 2 mL of RBBR suspension can be contained and shaken with laccase@UPA(*γ*) biocatalyst inside after different time intervals. The sponge support inside the polystyrene column played the role of a holder without obvious RBBR dilution after even up to 5 cycles of dilution (Figure 4b). In Figure 4c, it is worth mentioning that after RBBR dilution, a little amount of RBBR molecules retained but distributed uniformly on the surface of sponge support.

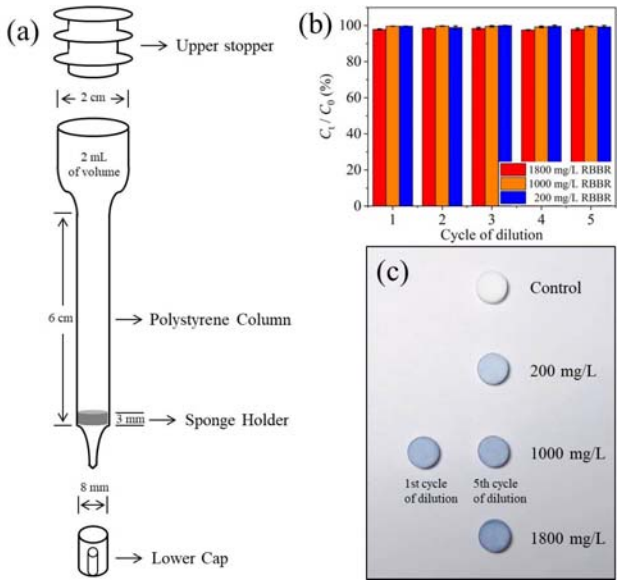


Figure 4: (a) Schematic diagram of disposable 2 mL polystyrene column, (b) residual RBBR (C_t / C_0, %) in suspension and (c) corresponding photos of sponge supports inside the polystyrene column after different cycles of dilution. $C_{[RBBR]initial}$ = 200, 1000, and 1800 mg·L^{-1}.

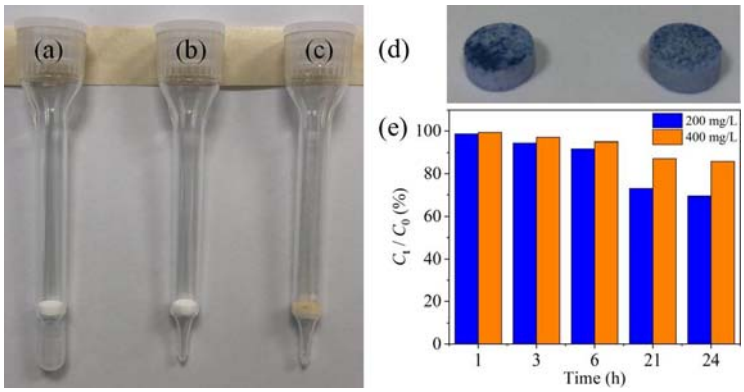


Figure 5: Photos of polystyrene column (a) with and (b) without the lower cap to avoid liquid leakage, (c) photo of sponge support holding laccase@UPA(γ) biocatalyst in polystyrene column, (d) photos of sponge support surface after RBBR dye biodegradation, and (e) residual RBBR (C_t / C_0, %) after different time intervals of laccase@UPA(γ) biocatalyst treatment in polystyrene column bioreactor at pH = 5.0.

In terms of catalytic performances of obtained laccase@UPA(γ) biocatalyst, three parameters including laccase *T*. immobilization yield (%), immobilization efficiency (%), and activity recovery (%) were calculated by using ABTS as the substrate (Sheldon and Pelt, 2013). Accordingly, the corresponding results were determined as 90.5%, 19.3%, and 17.5%, respectively. Furthermore, by using glutaraldehyde as the cross-linker, the laccase *T*. immobilization capacity by UPA(γ) powders was 244.4 mg·g^{-1} at pH = 7.5.
As shown in Figure 5d, after corresponding experiments and excluding the suspension by using the upper stopper of the polystyrene column, the surface of sponge support became coarser, and some blue coloured laccase@UPA(γ) samples distributed non-uniformly on its surface as compared with the results discussed above (Figure 4c). By transferring the obtained laccase@UPA(γ) biocatalyst into polystyrene column for RBBR dye biodegradation, approximately 25% and 10% of RBBR with 200 and 400 mg·L^{-1} of initial dye concentrations can be degraded with 2 mg of biocatalyst after 24 h of incubation time, respectively (Figure 5e).

4. Conslusions

In the context of green and sustainable chemistry, this communication is devoted to the preliminary experimental results regarding the synthesis of laccase *T*. immobilized biocatalyst (laccase@UPA(γ)), in which the efficient immobilization capacity of laccase *T*. by UPA(γ) powders was 244.4 mg·g^{-1} at pH = 7.5. The

polystyrene column was applied as a portable and easy-to-carry bioreactor for RBBR dye biodegradation, which can be used as the first prototype of an enzymatic bioreactor for dye treatment in industrial textile effluents. Inspired by these results, the optimization of this process is underway.

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