Laccase Cross-Linked Ultraporous Aluminas for Sustainable Biodegradation of Remazol Brilliant Blue R

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Abstract: Over the past few decades, enzyme-based green and sustainable chemistry has attracted extensive research attention, which provides a promising alternative to the conventional treatment methods of recalcitrant micropollutants. However, enzyme denaturation and stability loss remain critical challenges for its potential applications in industrial wastewater treatment. In this study, laccase from *Trametes versicolor* (laccase *T.* ) was cross-linked immobilized by ultraporous alumina (UPA) for the sustainable biodegradation of Remazol Brilliant Blue R (RBBR). Through sequential use of an aminosilane coupling agent (3-aminopropyl)triethoxysilane (APTES) and bifunctional cross-linker glutaraldehyde (GA), the synthesized biocatalysts showed better immobilization performances (about 4-fold to physical adsorption). The GA concentration considerably affected the laccase *T.* cross-linking degree, while the GA post-treatment protocol showed the highest laccase *T.* immobilization yield with lower activity recovery. Moreover, the biocatalyst stabilities including pH stability, thermal stability, storage stability, and reusability were also studied. Tolerance to broader pH and temperature ranges, better storage stability, good reusability of laccase *T.* cross-linked UPA(γ) biocatalysts, and their continuous RBBR biodegradation efficiency highlight the potentials of enzyme-based inorganic materials in industrial wastewater treatment, which can broaden our understanding of their practical applications in environmental fields.

Keywords: laccase *T.* immobilization; UPA; cross-linking; RBBR; kinetic model fittings

1. Introduction

Throughout organic synthesis development, the first in vitro synthesis of natural urea product (1828, by Friedrich Wöhler) and the serendipitous discovery of the first synthetic dye (mauveine, also known as aniline purple; 1856, by William Perkin) are generally considered as two historical landmarks [1,2]. In recent decades, the toxic properties of many reagents and solvents have been well identified. Meanwhile, growing concerns have also focused on the synthetic chemical compounds and/or waste-related by-products generated by modern pharmaceutical and fine chemical industries. Most of these organics, especially the compounds with complicated and reinforced structures (such as hormones, pesticides, endocrine disrupting chemicals (EDCs), and synthetic dyes, etc.), are persistent and poorly biodegradable in the environment [3–5]. Due to their potential for long-range
transport, resistance to environmental degradation, and ability to bio-magnify and bio-accumulate in the ecosystem, researchers have long recognized the necessity to consider the transition of these organic chemistry-based industries to greener and more sustainable manufacturing processes that can minimize or preferably avoid the release of pollutants and the use of hazardous materials [1,6–8].

In the context of green and sustainable chemistry, e.g., the slogan “reduce–reuse–recycle” (3Rs), enzyme-related biocatalysis has many benefits to offer. Enabled by recent advances in modern biotechnology and protein engineering, bioconversion of persistent organic pollutants (POPs) into non-hazardous or less-hazardous substances has been recognized as a key strategy to control the level of contamination in water and soil [3,7]. These biologically mediated chemical reactions can be generally performed under mild conditions by using a biocompatible and biodegradable catalyst that can be derived from renewable resources [1,6–8]. Furthermore, reactions involving multifunctional enzyme molecules characterized by high chemo-, regio-, and stereoselectivity can integrate multiple steps into enzymatic cascade processes, which affords more step-economical routes without the conventional requirements of complex functional group activation, protection, and deprotection steps [6,9]. According to the literature, the global market for industrial applications of enzymes is projected to reach USD 8.7 billion in 2026 at a compound annual growth rate (CAGR) of 6.3% from 2021 to 2026 [10]. Despite all of the above-mentioned advantages, the industrial applications of enzymes are often hampered by the lack of long-term operational stability and difficult enzyme recovery [1,6–8]. To expand the commercial viability and sustainability of an enzyme, immobilization by converting it from a homogeneous catalyst to a heterogeneous catalyst, which generally exhibits enhanced stability under both operational and storage conditions than free enzymes, has been extensively studied [3,4,7,11–14].

Generally, conventional immobilization methods of enzymes, notably oxidoreductases (such as laccase, tyrosinase, lignin, and manganese peroxidases, etc., all of Enzyme Commission class 1), can be divided into five main categories, i.e., (i) covalent bonding, (ii) adsorption (non-covalent), (iii) encapsulation, (iv) entrapment, and (v) cross-linking [3,6,15]. Among the existing immobilization methods, the covalent bonding and cross-linking techniques are characterized with strong and stable interactions between enzyme molecules and supporting materials [3]. Based on this, the proper selection of supporting materials of different origins (such as organic, inorganic, and hybrid materials) usually ensures enhanced enzyme stability against harsh conditions of pH, temperature, and pressure [3,4,12,13]. In comparison with the materials of organic origin, broad research interests also focused on inorganic materials that can be synthesized relatively cheaply and usually by uncomplicated synthesis procedures [3,16]. Moreover, the abundant presence of many functional groups, large specific surface area, controllable nanometer size similar to that of enzyme molecules, and exceptional mechanical and chemical stabilities may facilitate the versatility of inorganic materials in the case of enzyme immobilization [3,17]. For example, Hou et al. synthesized laccase-immobilized TiO\(_2\) nanoparticles and TiO\(_2\) functionalized polyether-sulfone (PES) membranes by different approaches, and their biocatalytic performances were compared in terms of laccase activity, loading, activity recovery, stability, and kinetic parameters [4]. Sheldon et al. summarized the existing literature devoted to enzyme immobilization, and three important terminologies, i.e., enzyme immobilization yield (IY, %), immobilization efficiency (IE, %), and activity recovery (AR, %), were introduced in detail [8].

In this study, laccase from _Trametes versicolor_ (laccase T.) was cross-linked immobilized by ultraporous alumina (i.e., UPA(γ) and UPA(θ) powders) synthesized via a facile oxidation process. Glutaraldehyde (GA) was employed as the bifunctional cross-linker that primarily bridges free amino groups (usually lysine residues) on the surface of neighboring enzyme molecules and/or amino groups from the supporting materials [6,7]. The sequential immobilization and GA post-treatment protocols were compared based on the obtained laccase T. AR (%) (Figure 1). Subsequently, the synthesized biocatalysts were applied to the
biodegradation of Remazol Brilliant Blue R (RBBR), which widely exists in industrial textile effluents [18]. The present study highlights the UPA potentials in terms of oxidoreductase immobilization, which can broaden the practical applicability of aluminum materials to industrial wastewater treatment.

**Figure 1.** Schematic illustrations of synthetic mechanisms underlying (a) APTES-silanized UPA powders and different protocols for laccase T. cross-linked UPA biocatalysts: (b) sequential immobilization, and (c) GA post-treatment.

### 2. Results and Discussion

#### 2.1. Effects of GA Concentration and Immobilization Protocols

GA is generally the cross-linker of choice as it is inexpensive and readily available in commercial quantities, and the cross-linking of proteins with GA has been designated as generally regarded as safe (GRAS) for use, especially in food and beverage processing [6,7,19]. According to Torres-Salas et al., predicting and rationalizing the orientation of enzymes that covalently attach to the supporting materials may affect the AR (%) of biocatalysts [11]. Moreover, the cross-linker ratio to the enzyme is also crucial for enzyme immobilization as the low cross-linker ratio cannot induce sufficient Schiff base formation; while on the other hand, excessive cross-linking may lead to the activ-
ity loss of enzyme molecules [4,6,12]. Figure 1 shows the synthetic mechanisms of (3-aminopropyl)triethoxyxilane (APTES)-silanized UPA powders (Figure 1a) and two different protocols for laccase T. immobilization on UPA surfaces (i.e., sequential immobilization, Figure 1b; and GA post-treatment, Figure 1c). In addition, the GA concentration effect on sequential laccase T. immobilization by UPA powders is shown in Table 1. Compared with physical adsorption, higher laccase T. immobilization performances can be observed in the cross-linking process, and the highest laccase T. AR (%) was obtained with a GA concentration of 0.50% (v/v) (Table 1). The UPA powders in different polycrystalline phases showed different laccase T. immobilization performances, while negligible immobilization capacity was found for UPA(α) powders. Moreover, the amount of GA considerably affected the laccase T. cross-linking degree, which is consistent with a previous study [12].

Table 1. Effect of GA concentration (v/v) on IY (%), IE (%), and AR (% or mg g\(^{-1}\) carrier) of laccase T./UPA (physical adsorption) and laccase T. cross-linked UPA (sequential immobilization) biocatalysts in different UPA polycrystalline phases.

| GA Concentration (v/v) | Laccase T./UPA(γ) Powders | Laccase T. Cross-Linked UPA(γ) Biocatalysts |
|------------------------|--------------------------|------------------------------------------|
| IY (%)                 | 68.8 ± 0.5               | 88.5 ± 0.5                               |
| 0.25%                  | 90.5 ± 0.4               | 89.7 ± 0.6                               |
| 0.50%                  | 19.3 ± 0.4               | 19.2 ± 0.2                               |
| 1.00%                  | 17.5 ± 0.2               | 17.2 ± 0.1                               |
| 1.50%                  | 244.4 ± 2.4              | 241.3 ± 1.4                              |
| 3.00%                  | 70.0 ± 2.2               | 203.7 ± 4.3                              |

| AR (%)                 | 5.0 ± 0.2                | 14.6 ± 0.3                               |
| 0.25%                  | 14.7 ± 0.3               | 16.8 ± 0.5                               |
| 0.50%                  | 17.2 ± 0.1               | 14.7 ± 0.3                               |
| 1.00%                  | 203.7 ± 2.4              | 14.3 ± 0.2                               |
| 1.50%                  | 3.4 172.7                | 199.6 ± 2.5                              |
| 3.00%                  | 3.5 166.8                |                                         |

| AR (mg g\(^{-1}\) carrier) | 70.0 ± 2.2               | 203.7 ± 4.3                              |
| 0.25%                     | 244.4 ± 2.4              | 241.3 ± 1.4                              |
| 0.50%                     | 203.7 ± 4.3              | 241.3 ± 1.4                              |
| 1.00%                     | 3.4 172.7                | 199.6 ± 2.5                              |
| 1.50%                     | 3.5 166.8                |                                         |
| 3.00%                     | 3.6 160.0                |                                         |

\(^a\) AR (%) mathematically equals the value of IY (%) multiplied by the IE (%). \(^b\) AR (mg g\(^{-1}\) carrier) is the obtained enzyme activity immobilized by supporting materials.

In order to compare different protocols regarding laccase T. immobilization, the GA concentration that led to the highest laccase T. AR (%) was applied, and the comparison results are shown in Table 2. Compared with other protocols, the sequential immobilization protocol showed the highest laccase T. AR (%) (about 4-fold compared to physical adsorption). However, the GA post-treatment protocol showed the highest laccase T. IY (%) with lower laccase T. AR (%) (Table 2). By using GA as the cross-linking agent, strong covalent linkages could be formed, not only between the laccase T. and UPA surfaces but also between the laccase T. molecules, resulting in the formation of a stable network structure [4,6]. However, this structure could also change the laccase T. conformation and lead to the unfavorable orientation of laccase T. active sites (Figure 1c) [11]. On the contrary, during the sequential immobilization process, the covalent linkages were mainly formed between the laccase T. and UPA surfaces, which can provide higher mobility of the immobilized laccase T. molecules. Similar results can be found in the study conducted by Hou et al., in which different procedures regarding laccase immobilization were compared, and the sequential procedure exhibited the highest activity and AR (%) [4]. Therefore, higher laccase T. AR (%) can be observed in the sequential immobilization protocol, and the laccase T. cross-linked UPA(γ) biocatalysts synthesized by this protocol were further studied in terms of the biocatalyst stability.
Table 2. Comparison of different protocols regarding laccase *T.* immobilization by UPA(γ) powders (0.50% (v/v) of GA concentration).

|                      | Sequential Immobilization | GA Post-Treatment | Physical Adsorption |
|----------------------|---------------------------|-------------------|---------------------|
| IY (%)               | 90.5 ± 0.4                | 90.8 ± 0.4        | 68.8 ± 0.5          |
| IE (%)               | 19.3 ± 0.4                | 12.9 ± 0.3        | 7.3 ± 0.4           |
| AR (%)               | 17.5 ± 0.2                | 11.7 ± 0.1        | 5.0 ± 0.2           |
| AR (mg·g⁻¹ carrier)  | 244.4 ± 2.4               | 164.3 ± 1.5       | 70.0 ± 2.2          |

2.2. Characterizations

Figure 2a,b shows the scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images of UPA(γ) powders, which evidenced the ultraporous morphology of the obtained samples. Based on calculation from the Bragg equation (2dsinθ = nλ, n = 1, 2, 3, etc.), the two typical X-ray diffraction (XRD) patterns of UPA(γ) powders 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) (Figure 2c) [20]. The nitrogen adsorption–desorption isotherm of UPA(γ) powders followed the typical characteristics of type IV isotherm and H3 hysteresis (IUPAC), which indicates the mesoporous property of UPA(γ) powders (Figure 2d) [21,22]. Moreover, the specific surface area of UPA(γ) powders was 201.7 m²·g⁻¹ with an average pore diameter of 15.3 nm (Figure 2d).

Figure 2. (a) SEM and (b) TEM images, (c) XRD pattern, and (d) nitrogen adsorption–desorption isotherm of UPA(γ) powders (inset: corresponding pore size distribution).
Figure 3a shows the Fourier transform infrared (FTIR) spectra of free laccase T. molecules, UPA(γ) powders, APTES-silanized UPA(γ) powders, and laccase T. cross-linked UPA(γ) biocatalysts. According to the FTIR spectrum of UPA(γ) powders, the broad absorption band in the range of 3400–3500 cm\(^{-1}\) and the weak absorption band at 1642 cm\(^{-1}\) were assigned to the –OH and Al–OH stretching vibrations of alumina, respectively [23,24]. The symmetric vibrations at 755 and 568 cm\(^{-1}\) were assigned to the bending vibrations of Al–O–Al bonds [24]. In the FTIR spectrum of the laccase T. molecules, the absorption band at 2923 cm\(^{-1}\), amide I band at 1641 cm\(^{-1}\), amide III band in the range of 1420–1210 cm\(^{-1}\), amide V and VI bands in the range of 800–500 cm\(^{-1}\), and the band in the range of 1165–948 cm\(^{-1}\) were assigned to the C–H stretching vibration in the CH\(_2\) group present on the laccase T. surfaces, C=O stretching vibration, CN stretching and NH bending vibrations, NH and C=O bending vibrations, and the characteristics of proteins that laccase T. possesses in its molecular structure, respectively [25,26].

Figure 3b shows the FTIR spectra comparison of UPA(γ) powders and APTES-silanized UPA(γ) powders, APTES-silanized UPA(γ) powders, and laccase T. cross-linked UPA(γ) biocatalysts, and free laccase T. molecules, FTIR spectra comparison of UPA(γ) powders and APTES-silanized UPA(γ) powders, and laccase T. cross-linked UPA(γ) biocatalysts. According to the FTIR spectrum of UPA(γ) powders, the broad absorption band in the range of 3400–3500 cm\(^{-1}\) and the weak absorption band at 1642 cm\(^{-1}\) were assigned to the –OH and Al–OH stretching vibrations of alumina, respectively [23,24]. The symmetric vibrations at 755 and 568 cm\(^{-1}\) were assigned to the bending vibrations of Al–O–Al bonds [24]. In the FTIR spectrum of the laccase T. molecules, the absorption band at 2923 cm\(^{-1}\), amide I band at 1641 cm\(^{-1}\), amide III band in the range of 1420–1210 cm\(^{-1}\), amide V and VI bands in the range of 800–500 cm\(^{-1}\), and the band in the range of 1165–948 cm\(^{-1}\) were assigned to the C–H stretching vibration in the CH\(_2\) group present on the laccase T. surfaces, C=O stretching vibration, CN stretching and NH bending vibrations, NH and C=O bending vibrations, and the characteristics of proteins that laccase T. possesses in its molecular structure, respectively [25,26].

Figure 3c shows the FTIR spectra comparison of UPA(γ) powders and APTES-silanized UPA(γ) powders. The absorption band at 2933 cm\(^{-1}\) was assigned to the C–H stretching vibration of the alkane group [25]. The absorption band at 1562 cm\(^{-1}\) was assigned to the N–H bending vibration [27]. The absorption band at 750 cm\(^{-1}\) was assigned to the Si–C stretching vibration [28,29]. Moreover, the double-peak structure between 1050 and 1200 cm\(^{-1}\) was assigned to the Si–O–Si stretching vibration [28,30]. After laccase T. immobilization, the FTIR spectra comparison of APTES-silanized UPA(γ) powders and laccase T. cross-linked UPA(γ) biocatalysts is shown in Figure 3c. The twin peak at 2874 cm\(^{-1}\) was assigned to
the C–H stretching vibration of the alkane group [31]. The absorption band at 1461 cm\(^{-1}\) was assigned to the C–H bending vibration [25]. Di Bernardo et al. systematically studied different Schiff base complexes that formed over the pH range of 6–10 with the maximum formation percentage around pH 9, and the IR spectra of all the Schiff complexes showed a strong band of C=N in the range of 1649–1642 cm\(^{-1}\) [32]. According to Di Bernardo et al., Schiff base formation reached its concentration maximum approximately at pH 8.8, and a considerable amount of Schiff base can also be formed under weak alkaline conditions (e.g., pH = 7.5 in this study) [32]. In the FTIR spectrum of laccase \(T\). cross-linked UPA(γ) biocatalysts, the absorption band at 1658 cm\(^{-1}\) (with ~9 cm\(^{-1}\) of shift) was assigned to the C=N stretching vibration, which belongs to the formation of Schiff base between the amino groups of APTES-silanized UPA(γ) powders and the aldehydeic groups of GA molecules [32,33]. Moreover, no obvious absorption band of phosphate-containing and related compounds can be found in the FTIR spectra of APTES-silanized UPA(γ) powders and laccase \(T\). cross-linked UPA(γ) biocatalysts, which indicates that the phosphate molecules were completely removed after washing with Milli-Q water [34,35]. Figure 3d shows the thermogravimetric (TG) curves of UPA(γ) powders, APTES-silanized UPA(γ) powders, and laccase \(T\). cross-linked UPA(γ) biocatalysts. The corresponding mass losses occurring from 30 to 800 \(^\circ\)C were 12.1, 24.7, and 42.9 wt\%, respectively (Figure 3d). Generally, these mass losses were attributed to the release of adsorbed water, structural water related to dehydration, pyrolysis related to the methylene, C=N, carboxyl, and amino functional groups of the obtained samples [36,37]. All samples showed slight mass loss around 150 \(^\circ\)C, which can be attributed to the release of adsorbed water. Moreover, UPA(γ) powders and APTES-silanized UPA(γ) powders showed the same thermal decomposition stage around 150 \(^\circ\)C (8.0 wt\%), which indicates that only the release of adsorbed water occurred below this temperature. For the UPA(γ) powders, the further mass loss (4.1 wt\%) that occurred from 150 to 800 \(^\circ\)C can be attributed to the release of structural water related to dehydration. For the APTES-silanized UPA(γ) powders, the 12.6 wt\% (150–800 \(^\circ\)C) mass loss of the samples can be attributed to the pyrolysis related to methylene. The pyrolysis of methylene, C=N, carboxyl, and amino functional groups resulted in the 26.5 wt\% (150–800 \(^\circ\)C) mass loss of the laccase \(T\). cross-linked UPA(γ) biocatalysts.

2.3. Biocatalyst Stability Study

The effects of pH and temperature on the activity of free and immobilized laccase \(T\). are shown in Figure 4a,b, respectively. As shown in Figure 4a, the free laccase \(T\). molecules were affected more adversely by the change in solution pH than the laccase \(T\). immobilized by either the physical adsorption or cross-linking protocols. The residual activity of free laccase \(T\). molecules and laccase \(T\)./UPA(γ) powders decreased significantly at pH values higher than 5.5, while the residual activity of laccase \(T\). cross-linked UPA(γ) biocatalysts was still higher than 90%. Moreover, the immobilized laccase \(T\). showed higher pH tolerance under both acidic and alkaline conditions than the free laccase \(T\). molecules. Unlike pH effect, both the free and immobilized laccase \(T\). showed broad thermal tolerance in the temperature range from 20 to 85 \(^\circ\)C, and the residual activity of free laccase \(T\). molecules decreased more obviously after 45 min of incubation at pH 5.0 (Figure 4b). A sharp decrease in residual activity of both free and immobilized laccase \(T\). appeared at temperatures higher than 60 \(^\circ\)C, and negligible activity was observed at temperatures higher than 70 \(^\circ\)C. Similar results regarding the improved pH and thermal tolerances of immobilized enzymes can also be found in previous studies [4,12,14]. Figure 4c,d shows the effects of storage time and recycle batches on the activity of free and immobilized laccase \(T\)., respectively. As shown in Figure 4c, after 49 days of storage at 4 \(^\circ\)C, the residual activity of free laccase \(T\). molecules and immobilized laccase \(T\). exceeded 40% and 75%, respectively. For the laccase \(T\)./UPA(γ) powders, a drastic drop in activity (nearly 50%) was observed after five cycles (Figure 4d). Considering that the enzyme is linked only through non covalent electrostatic attraction without cross-linking, the pH at which these catalysts are washed after each cycle may be responsible for enzyme release. On the other hand, the laccase \(T\). cross-linked
UPA(γ) biocatalysts retained 75% of residual activity up to 10 cycles, which is consistent with previous studies [12,26].

Figure 4. (a) pH stability, (b) thermal stability, (c) storage stability, and (d) reusability profiles of free laccase T. molecules, laccase T./UPA(γ) powders, and laccase T. cross-linked UPA(γ) biocatalysts by using ABTS as the substrate. Standard experimental conditions of laccase T. activity measurement: C[ABTS]initial = 0.5 mM, pH = 5.0 ± 0.1, I = 100 mM SA, and T = 20 °C.

2.4. RBBR Kinetic Study

Figure 5 shows the comparison of UPA(γ) powders and laccase T. cross-linked UPA(γ) biocatalysts for RBBR removal. In terms of RBBR adsorption retained by UPA(γ) powders, the amount of RBBR in the suspension decreased rapidly within the first 6 h until the adsorption process achieved equilibrium (Figure 5a). In the initial step, the RBBR adsorption may result from the vacant reaction sites available on UPA(γ) surfaces. As long as the adsorption process achieved the equilibrium, approximately 25% of RBBR remained in the suspension without further removal.

The Lagergren pseudo-first-order and pseudo-second-order model fittings of the RBBR adsorption kinetics and the corresponding fitting results are shown in Figure 5c and Table 3, respectively. As shown in Table 3, the low determination coefficients (R²) obtained from the Lagergren pseudo-first-order model showed that there was no significant correlation between the kinetic data and this model [38]. On the other hand, the pseudo-second-order model fitted the kinetic data better, and the calculated adsorption capacities at equilibrium (Qme) were closer to the experimental ones (Qme) [16,39]. Therefore, the RBBR adsorption retained by UPA(γ) powders followed the pseudo-second-order model.
Figure 5. Comparison of UPA(γ) powders and laccase T. cross-linked UPA(γ) biocatalysts for RBBR removal, contact time: (a) 32 h and (b) 8 h (enlargement taken from the region to the left of the dotted line in (a)). [RBBR]initial = 200 mg L\(^{-1}\), [biocatalyst]initial = 1 g L\(^{-1}\), pH = 5.0 ± 0.1, I = 100 mM SA, T = 20 °C, and stirring speed = 150 rpm. (c) Adsorption kinetic profiles of RBBR retained by UPA powders in different UPA polycrystalline phases, corresponding (d) Lagergren pseudo-first-order and (e) pseudo-second-order model fittings. [RBBR]initial = 200 mg L\(^{-1}\), [UPA]initial = 1 g L\(^{-1}\), pH = 5.0 ± 0.1, I = 100 mM SA, T = 20 °C, and stirring speed = 150 rpm.

Table 3. Parameters of RBBR adsorption kinetics fitted by the Lagergren pseudo-first-order and pseudo-second-order models at T = 20 °C.

| Kinetic Models                  | UPA(γ)     | UPA(θ)     | UPA(α)     |
|--------------------------------|------------|------------|------------|
| Lagergren pseudo-first-order   |            |            |            |
| \(k’\) (h\(^{-1}\))           | 0.127      | 0.089      | 0.034      |
| \(Q_{mc}\) (mg g\(^{-1}\))    | 7.251      | 4.594      | 1.236      |
| \(R^2\)                        | 0.565      | 0.580      | 0.265      |
| Pseudo-second-order            |            |            |            |
| \(k''\) (g mg\(^{-1}\) h\(^{-1}\)) | 0.110      | 0.194      | 1.635      |
| \(Q_{mc}\) (mg g\(^{-1}\))    | 140.845    | 77.340     | 9.823      |
| \(R^2\)                        | 0.999      | 0.999      | 0.999      |
| \(Q_{me}\) (mg g\(^{-1}\))    | 141.398    | 78.174     | 10.587     |

\(^a\) \(Q_{mc}\) (mg g\(^{-1}\)) is the adsorption capacity at equilibrium calculated from the kinetic models. \(^b\) \(Q_{me}\) (mg g\(^{-1}\)) is the experimental adsorption capacity at equilibrium.
Unlike RBBR adsorption retained by UPA(γ) powders, laccase T. cross-linked UPA(γ) biocatalysts kept continuous RBBR biodegradation even though the initial RBBR removal rate was slightly lower than that of the adsorption process (Figure 5a). After 3.2 h of the treatment, the amount of RBBR in the suspension of the biodegradation process became less than that of the adsorption process (Figure 5b). Moreover, after 30 h of the treatment, the amounts of RBBR in the suspensions of the adsorption and biodegradation processes were 25% and 10%, respectively (Figure 5).

2.5. Adsorption

For the adsorption process, two types of adsorption sites were assumed, i.e., \( a_1 \) and \( a_2 \) sites, with two adsorption constants \( k_1 \) and \( k_2 \), respectively, and the corresponding rate equations are listed as follows:

\[
\frac{dN}{dt} = -k_1(a_1 - n_1) - k_2(a_2 - n_2)
\]  
(1)

\[
\frac{dn_1}{dt} = k_1(a_1 - n_1)(N_0 - n_1 - n_2)
\]  
(2)

\[
\frac{dn_2}{dt} = k_2(a_2 - n_2)(N_0 - n_1 - n_2)
\]  
(3)

where \( N_0 \), \( n_1 \), and \( n_2 \) are the concentrations of free and adsorbed RBBR molecules on the \( a_1 \) and \( a_2 \) sites, respectively. Moreover, it was also assumed that \( k_1 >> k_2 \) and, accordingly, the RBBR molecules first occupied the \( a_1 \) sites, leaving the \( a_2 \) sites to be occupied later. Therefore, Equations (2) and (3) can be simplified to Equations (4) and (5), which are listed as follows:

\[
\frac{dn_1}{dt} = k_1(a_1 - n_1)(N_0 - n_1)
\]  
(4)

\[
\frac{dn_2}{dt} = k_2(a_2 - n_2)(N_0 - a_1 - n_2)
\]  
(5)

Their solutions are listed as follows:

\[
\frac{n_1}{N_0} = \frac{1 - \exp(-k_1t(N_0 - a_1))}{N_0 - \exp(-k_1t(N_0 - a_1))}
\]  
(6)

\[
\frac{n_2}{N_0 - a_1} = \frac{1 - \exp(-k_2t(N_0 - a_1 - a_2))}{N_0 - a_1 - \exp(-k_2t(N_0 - a_1 - a_2))}
\]  
(7)

The free RBBR molecule kinetics can be therefore expressed as follows:

\[
\frac{N}{N_0} = 1 - \frac{n_1}{N_0} - \frac{n_2}{N_0} = 1 - \frac{1 - \exp(-k_1t(N_0 - a_1))}{N_0 - \exp(-k_1t(N_0 - a_1))} - \frac{1 - \exp(-k_2t(N_0 - a_1 - a_2))}{N_0 - a_1 - \exp(-k_2t(N_0 - a_1 - a_2))}
\]  
(8)

By substituting \( k_1N_0 = a, a_1/N_0 = b, k_2N_0 = c, \) and \( a_2/N_0 = d \), Equation (8) can also be expressed as follows:

\[
\frac{N}{N_0} = 1 - e^{-a(1-b)t} - \frac{1 - e^{-c(b-d)t}}{b}. \quad (9)
\]

The solid line in Figure 6a shows the model fitting of the adsorption kinetic data by Equation (9), which can be summarized as follows:

(i) The adsorption sites (\( a_1 \)) with fast kinetics were rapidly filled (\( t \leq 0.2 \) h) with an estimated rate \( k_1N_0 = 30 \), by consuming about 50% of free RBBR molecules (dashed line, Figure 6a).

(ii) The following (almost total measured) kinetics described the adsorption of RBBR molecules on \( a_2 \) sites with a slower rate \( k_2N_0 = 1.4 \), tending to about 22% of the RBBR molecules for \( t > 10 \) h, when the adsorption process achieved equilibrium.
Figure 6. RBBR kinetics in the presence of (a) UPA(γ) powders and (b) laccase T. cross-linked UPA(γ) biocatalysts. Solid and dashed lines correspond to the model fitting of the kinetic data.

2.6. Adsorption and Biocatalysis

Since the observed differences in RBBR kinetic data in the presence of UPA(γ) powders and laccase T. cross-linked UPA(γ) biocatalysts appeared over a relatively long time ($t \geq 2$ h), the catalytic kinetics can be simply completed by a slow process of the first order, corresponding to the decomposition of the remaining RBBR molecules in the solution. At the same time, the reactions of free and adsorbed RBBR molecules cannot be distinguished easily, as the equilibrium between both populations is expected to establish faster than the decomposition, which can affect one or both populations. Taking into account Equation (9), the RBBR kinetics in the presence of laccase T. cross-linked UPA(γ) biocatalysts was expressed as follows:

$$\frac{N}{N_0} = \left[ \frac{1 - e^{-(a-b)t}}{b - e^{-(a-b)t}} \right] - \left[ \frac{1 - e^{-(c-d)t}}{a - \frac{e^{-(c-d)t}}{e^{-(c-d)t}}(1-b-d)} \right] e^{-kqN}$$

(10)

where $kq$ is the reaction constant.

The solid line in Figure 6b shows the model fitting of the adsorption and biocatalysis kinetic data by Equation (10), which can be summarized as follows:

(i) The adsorption sites (a1) with fast kinetics survived after laccase T. deposition onto UPA(γ) surfaces, and they were rapidly occupied ($t \leq 0.2$ h) with rate $k_1 N_0 = 30$ and by consuming about 50% of free RBBR molecules, which is consistent with the results mentioned above (Figure 6a).

(ii) The deposition of laccase T. onto UPA(γ) surfaces significantly affected the a2 sites: the adsorption rate decreased $k_2 N_0 = 0.7$, and the adsorption capacity increased to 35%.

(iii) The RBBR decomposition was resolved at $t > 2$ h with a rate of $k = 0.015$ h$^{-1}$.

Based on the model fittings as mentioned above, it can be suggested that the adsorption sites a1 (fast kinetics) were those lying at the outer surface of UPA(γ) grains, and the a2
sites (slow kinetics) were those belonging to the grain pores of UPA(γ) grains. Moreover, laccase T. molecules significantly modified the ultraporous structure of UPA(γ) powders: the adsorption rate decreased twice and the adsorption capacity of the obtained materials increased 50%. The proposed model validates the catalytic activity of laccase T. cross-linked UPA(γ) biocatalysts towards RBBR followed the 1st-order reaction kinetics.

3. Materials and Methods

3.1. Materials and Chemicals

The raw laminated metallic aluminum plate (100 mm × 100 mm, 1.0 mm of thickness, 99.99% of purity) was supplied by Goodfellow Cambridge Ltd. (Huntingdon, UK). The chemicals including acetone, mercury(II) nitrate monohydrate (Hg(NO$_3$)$_2$·H$_2$O, ≥98.5%), silver nitrate (AgNO$_3$, ≥99.0%), laccase T. (≥0.5 U·mg$^{-1}$, EC 1.10.3.2, polyphenoloxidase), RBBR (also called RB19, anthraquinone dye), APTES (99%, aminosilane coupling agent), GA (25% of v/v), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, HPLC), sodium acetate (CH$_3$COONa, abbreviated to SA), disodium hydrogen phosphate (Na$_2$HPO$_4$·2H$_2$O, phosphate buffer), Tris hydrochloride (Tris-HCl, ultra pure), glacial acetic acid (CH$_3$COOH), phosphoric acid (H$_3$PO$_4$), hydrochloric acid (HCl), and sodium hydroxide (NaOH), were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Laccase T. is a monomeric enzyme that contains 15 arginine and 5 lysine residues with free amino groups as the reactive anchor moieties for the cross-linking process, which is generally performed at pH 7.0–9.0 because of the high reactivity of GA in this pH range [6,40]. ABTS is commonly selected as the specific substrate because of its higher affinity to free and immobilized laccase T. than phenol-based substrates (e.g., 2,6-dimethoxyphenol (DMP), three times more sensitive) and other assay substrates (e.g., dihydroxyphenylalanine (DOPA), dimethylaminoborane (DMAB), and syringaldazine, more than 40 times more sensitive) [4]. All chemicals used in this study were of analytical grade and were used as received directly without further purification. Milli-Q water (Millipore Corp., Burlington, MA, USA) with a specific resistivity of 18.2 MΩ·cm$^{-1}$ at 25 °C was used to prepare solutions throughout the experiments.

3.2. Syntheses of UPA(γ), UPA(θ), and UPA(α) Powders

The UPA monolith samples were synthesized via a facile oxidation process according to the previous studies [41–43]. Briefly, high-purity but fragile UPA monolith samples were obtained with a growth rate of ~1 cm·h$^{-1}$ at room temperature in a humid atmosphere (70–80% RH) by the oxidation of metallic aluminum plates through a liquid layer of mercury–silver amalgam. According to Vignes et al., complete and approximately half oxidation of aluminum plates can be obtained by using plates with 99.999% and 99.99% purity, respectively [41]. Anhydrous monolithic UPA can be obtained from UPA monolith samples, converting to amorphous UPA, polycrystalline UPA(γ), UPA(θ), and UPA(α) monoliths under 4 h of isochronous annealing treatment in air at <870, 950, 1150, and 1350 °C, respectively. After the rigorous grinding process, the UPA powders in different polycrystalline phases (i.e., γ, θ, and α) can be accordingly obtained. Compared with UPA(γ) (201.7 m$^2$·g$^{-1}$) and UPA(θ) (93.4 m$^2$·g$^{-1}$) monoliths, UPA(α) monolith possesses the minimal specific surface area (5.7 m$^2$·g$^{-1}$) for laccase T. immobilization but the highest mechanical stability to be ground into powder [16]. Therefore, except where otherwise specified, the term UPA mainly refers to UPA powders in γ and θ polycrystalline phases.

3.3. Syntheses of APTES-Silanized UPA Powders, Laccase T. Cross-Linked UPA Biocatalysts, and Laccase T./UPA Powders

APTES-silanized UPA powders: In terms of the cross-linking process, APTES is one of the most frequently used organosilane agents for preparing the amino group terminated compound as anchor points for aldehyde groups [4,44]. Before the immobilization procedure, UPA 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 three times to remove any residual organics (Figure 1a).

Sequential immobilization: In this process, 2 mg of APTES-silanized UPA powders was dispersed in GA solution with different concentrations (i.e., 0.25, 0.50, 1.00, 1.50, and 3.00% of v/v) under neutral conditions (pH = 7.5). The mixture was stirred at 20 °C and 100 rpm for 6 h, followed by the washing procedure as mentioned above to remove unreacted GA molecules. After GA functionalization, the obtained powders were suspended in 7 mL of laccase T. solution (4 g·L⁻¹, oversaturated), resulting in Schiff base formation and subsequent cross-linking of laccase T. with GA functionalized UPA powders (Figure 1b). According to Matijosyte et al., the minimal cross-linking time for laccase T. should not be shorter than 19 h, and higher specific activity of laccase T. can be observed at room temperature [40]. Moreover, the cross-linking time had a negligible effect on the activity loss of free laccase T. molecules in the presence of GA (Figure 7). Consequently, to obtain the complete cross-linking equilibrium, the mixture was stirred at 20 °C and 100 rpm for 24 h.

GA post-treatment: Unlike sequential immobilization protocol, APTES-silanized UPA powders were first suspended in oversaturated laccase T. solution without the GA functionalization step, and the mixture was stirred at 20 °C and 100 rpm for 24 h. Then, the obtained powders were separated by centrifugation (4000 rpm, 5 min) and were dispersed in GA solution at 20 °C and 100 rpm for 6 h with the GA concentration that led to the highest laccase T. AR (%) (Figure 1c). For comparison purposes, the laccase T. entrapped UPA powders by non-covalent bonding (laccase T./UPA) were synthesized by following the similar protocol as mentioned above without UPA silanization or the cross-linking operation of laccase T. by using GA as the cross-linker.

3.4. Characterizations

The synthesized UPA powders, APTES-silanized UPA powders, and laccase T. cross-linked UPA biocatalysts were characterized by different techniques. The morphology of the obtained samples was characterized by SEM (Zeiss Supra 40 VP, Carl Zeiss, Jena, Germany) and TEM (JEOL 2011, JEOL Ltd., Tokyo, Japan) techniques. The powder XRD pattern of the obtained samples was determined by using an Inel Equinox 1000 X-ray diffractometer (Inel, Celje, Slovenia) with Co Kα radiation source (λ = 1.7902 Å), and the analysis was performed.
at 20 diffraction angles from 25° to 85° at a speed of 2°/min. The Brunauer–Emmett–Teller (BET) specific surface area and pore size distribution of the obtained samples were studied by nitrogen adsorption–desorption measurement (Belsorp-max, MicrotracBEL, Osaka, Japan; data analysis: MicroActive for ASAP 2460) with outgassing at 200 °C for 12 h. The mass of UPA(γ) powders and the corresponding range of points (P/P₀) used for the BET measurements were 0.0602 g and 0.0052–0.9888, respectively. The FTIR spectra were recorded by a PerkinElmer Spectrum 100 system spectrometer (PerkinElmer, Waltham, MA, USA) using pressed KBr pellets (Sigma–Aldrich, St. Louis, MO, USA, 99%, analytical reagent) in the 400–4000 cm⁻¹ region. Prior to FTIR characterization, the samples were washed with Milli-Q water, followed by the vacuum freeze-drying process to obtain completely dry samples (chemvac Combination Pump Systems, Welch Machine Inc., Germany; BTP-9ELE0X and COMBIVP-100X models, SP Scientific, Bury, UK). The TG curves were recorded by using a NETZSCH TG 209F1 Libra instrument under a nitrogen atmosphere by heating the samples from 30 to 800 °C at a heating rate of 10 °C·min⁻¹.

3.5. RBBR Calibration Curve and Laccase T. Activity Measurement

The RBBR calibration curve was determined by spectrophotometry at the wavelength of 590 nm (Figure 8a, UviLine 9400 UV–visible spectrophotometer, Secomam, France). The laccase T. activity was determined by using ABTS as the substrate at the wavelength of 420 nm (Figure 8b). To compare different protocols regarding laccase T. immobilization by UPA powders in different polycrystalline phases, three parameters, i.e., IY (% of immobilized units divided by the total starting units), IE (% obtained active units in biocatalysts divided by the immobilized units), and AR (% or mg·g⁻¹ carrier, obtained active units in biocatalysts divided by the total starting units) were determined. The residual laccase T. activity was expressed as a relative percentage compared with the corresponding initial laccase T. activity of the immobilized amount that was taken as the control (100%). All of the experimental data are the averages of triplicate determinations and are displayed in the form of the average value ± standard deviation (S.D.).

![Figure 8](image-url)

Figure 8. Calibration curves of (a) RBBR (λ = 590 nm), and (b) laccase T. assay using ABTS as the substrate at 5-min oxidation time (λ = 420 nm). The R² values of the two calibration curves are 0.9951 and 0.9973, respectively. Standard experimental conditions of laccase T. activity measurement: C[ABTS]_initial = 0.5 mM, pH = 5.0 ± 0.1, I = 100 mM SA, and T = 20 °C.

3.6. Biocatalyst Stability Study

To compare the free laccase T. molecules, laccase T./UPA(γ) powders, and laccase T. cross-linked UPA(γ) biocatalysts, the experimental factors that can affect the biocatalyst stability including the pH, temperature, storage time, and recycle batches were studied. For the pH stability test, free and immobilized laccase T. were incubated in the pH range from 3.5 to 9.0 (20 °C, 45 min). The residual laccase T. activity was determined as mentioned above. The buffers used were SA (3.5–5.5, 100 mM), phosphate (6.0–7.5, 100 mM), and Tris-HCl (8.0–9.0, 100 mM). For the thermal stability test, the free and immobilized laccase
T. were incubated in the temperature range from 20 to 85 °C (pH = 5.0, SA buffer, 45 min). In terms of the storage stability, the free and immobilized laccase T. were stored in a refrigerator at 4 °C, and the residual laccase T. activity was determined for 49 days. For the reusability test, the residual laccase T. activity was determined through 10 consecutive batches. After each cycle, the biocatalysts were recovered by centrifugation at 4000 rpm for 5 min and washed with phosphate buffer three times.

3.7. Adsorption Kinetics Study and Data Analysis

To achieve homogeneous dispersion, a certain amount of UPA powders was used to prepare a suspension and was shaken for 1 min on the multifunctional vortex oscillator before the corresponding experiments. Appropriate volumes of RBBR (10 g·L⁻¹) and SA (400 mM) stock solutions were added to Eppendorf tubes to achieve the desired concentrations of different components. After the addition of the above components and the control of the final volume of the suspension in the Eppendorf tubes, the initial concentrations of RBBR and SA were 200 mg·L⁻¹ and 100 mM, respectively. The desired pH values of the suspensions were adjusted by adding negligible volumes of 0.1–0.01 M CH₃COOH (3.5–5.5), H₃PO₄ (6.0–7.5) or Tris-HCl (8.0–9.0) solutions. After the suspensions were shaken for the specified time interval, the solid and liquid phases were separated by centrifugation at 4000 rpm for 5 min.

The adsorption capacity at equilibrium (qe, mg·g⁻¹) and the kinetic data fitted by the Lagergren pseudo-first-order and pseudo-second-order models were determined by using the following equations [38,39]:

\[
q_e = \frac{C_0 - C_e}{m} \times V
\]  

\[
ln(Q_m - Q_t) = lnQ_m - k't
\]

\[
\frac{t}{Q_t} = \frac{1}{k''Q_m^2} + \frac{t}{Q_m}
\]

where \(C_0\) (mg·L⁻¹) is the initial adsorbate concentration in the suspension, \(C_e\) (mg·L⁻¹) is the adsorbate concentration in the supernatant at equilibrium, \(V\) (L) is the volume of the suspension, \(m\) (g) is the mass of the adsorbent. \(Q_t\) and \(Q_m\) (mg·g⁻¹) refer to the adsorption capacity at time \(t\) (h) and at equilibrium obtained from the kinetic models. \(k'\) (h⁻¹) and \(k''\) (g·mg⁻¹·h⁻¹) are the adsorption rate constants obtained from the kinetic models.

4. Conclusions

In this study, laccase T. was cross-linked immobilized on UPA(γ) powders for an effective biodegradation of RBBR. The effects of the GA concentration on the laccase T. cross-linking degree, comparison of sequential immobilization and GA post-treatment protocols, the biocatalyst stability including pH stability, thermal stability, storage stability, reusability, and the continuous RBBR biodegradation by laccase T. cross-linked UPA(γ) biocatalysts were comprehensively studied. The GA concentration considerably affected the laccase T. cross-linking degree, and the sequential immobilization showed higher laccase T. AR (%) than the GA post-treatment protocol. Moreover, the synthesized biocatalysts showed better immobilization performances (about 4-fold compared to physical adsorption). Through the application of different kinetic models, the laccase T. cross-linked UPA(γ) biocatalysts showed promising RBBR biodegradation efficiency. The findings of this study highlight the UPA potentials in industrial wastewater treatment, which can broaden our understanding of their practical applications in environmental fields.
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