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Decolorization of Remazol Brilliant Blue R with immobilized laccase from *Megasporoporia* sp. onto spent coffee ground biochar

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Abstract

In this study, biochar was synthesized from spent coffee grounds and subsequently treated with glutaraldehyde for the immobilization of laccase, which was used in the decolorization of an azo dye. The optimal conditions for immobilizing *Megasporoporia* sp. laccase onto activated biochar (10 mg) were investigated by varying the times, enzyme activities, and glutaraldehyde concentrations. The maximum enzyme loading (68.80±2.89 mg/g) with a laccase activity of 16.39±1.35 U/g was achieved by using 1% (v/w) glutaraldehyde as the crosslinking agent, with an initial laccase activity of 40 U/g support after performing the incubation reaction for 2 hrs. This immobilized enzyme achieved a maximum decolorization rate of 95.00% when incubated with 100 ppm Remazol Brilliant Blue R in sodium acetate buffer (pH 5.0) at 50°C for 3 hrs. The thermal stability of immobilized laccase with spent coffee ground biochar (SCGB-laccase) after incubation at 50°C for 6 hrs was significantly higher than that of the crude enzyme with decolorization rates of 79.21% and 53.70%, respectively. Additionally, the residual activity of immobilized SCGB-laccase was 57.64% after being used for six cycles. The degradation product was tested for its phytotoxicity, and it was found that the germination index values of mung bean and sweet corn were significantly higher in the treated dye (77.66% and 64.50%, respectively) compared to those in the non-treated dye (51.88% and 34.89%, respectively). Therefore, the efficiency of immobilized laccase onto activated spent coffee ground biochar for dye decolorization was noted, and this technique could be further applied for wastewater treatment on an industrial scale.

Keywords: Dye decolorization, Immobilization, Laccase, *Megasporoporia* sp., Spent coffee ground biochar

1. Introduction

Coffee is one of the most popular beverages worldwide, and global coffee consumption has been increasing annually. During coffee processing, from the coffee cherry to the final drinkable coffee, significant amounts of solid waste are generated. Among these waste materials, spent coffee grounds (SCGs), which refers to the non-edible solid waste produced after milling and brewing coffee, account for approximately 91% of the initial weight of ground coffee, making them the most abundant waste generated from coffee processing [1]. Therefore, rather than disposing of them by burning, which can contribute to air pollution, there have been several attempts to utilize SCGs as a feedstock to produce various valuable products. Biochar is one of the valuable products obtained from SCGs through pyrolysis [2], which has been utilized for the remediation of various pollutants, such as antibiotics, heavy metals, and toxic chemicals, in soil and water bodies [3]. Although biochar has demonstrated high efficiency in absorbing pollutants, the substances adsorbed onto it will persist without further processing. To enhance the efficacy of biochar in remediation, immobilization of enzymes that are capable of degrading pollutants onto its surface could introduce a new mechanism with desirable applications.

In our previous study, white rot fungi were isolated from different areas in Thailand, and their ability to produce ligninolytic enzymes was screened. One of the potential isolates was *Megasporoporia* sp., which could produce

laccase with an activity of 4.24 U/mL in a liquid culture medium after cultivation at room temperature for 4 days [4]. This crude laccase efficiently degraded 50 ppm Reactive Black 5, with over 80% degradation achieved. However, the practical application of this enzyme is still limited due to a significant decrease in its activity at high temperatures (> 60°C) [5]. To rectify this issue, an investigation into the appropriate technique for immobilizing *Megasporoporia* sp. crude laccase onto spent coffee ground biochar (SCGB) has been performed in this study to enhance enzyme stability and enable its reusability.

The structure and physicochemical properties of biochar are crucial factors that directly affect the yield of enzyme immobilization. Therefore, modifying the biochar structure to increase surface area and porosity is necessary to enhance its capability regarding adsorption and enzyme immobilization [6, 7]. In the previous report, it was shown that walnut shell biochar treated with 30% (w/w) KOH resulted in a higher specific surface area (712.07 m^2/g) compared to acid treatments (H_2SO_4 or H_3PO_4, 114.38 m^2/g and 117.64 m^2/g, respectively) and $ZnCl_2$ treatment (534.40 m^2/g). This outcome was attributed to the conducive formation of pores in biochar through the addition of oxygen-containing functional groups in an alkaline environment. Ahmadpour and Rashidi [8] reported a comparison of alkali treatments for biochar activation, wherein higher micropore volume and surface area (2864 m^2/g and 1.48 cm^3/g, respectively) were found in KOH treatment compared to NaOH treatment (1695 m^2/g and 0.88 cm^3/g, respectively). Therefore, in this study, SCGs were treated with KOH and pyrolyzed to produce biochar. The obtained SCGB was then employed as a supporting material to optimize the conditions for the immobilizing *Megasporoporia* sp. crude laccase using glutaraldehyde as a crosslinker. Subsequently, the properties of the immobilized SCGB-laccase were investigated based on the decolorization activity with Remazol Brilliant Blue R (RBBR), serving as a model anthraquinone dye, to assess its efficiency for potential industrial wastewater treatment applications.

2. Materials and methods

2.1 Preparation of activated biochar from spent coffee ground

The SCG of *Coffea arabica* L. used in this study were collected from a coffee shop in Bangkok, Thailand, washed with tap water, oven-dried at 60°C until a constant weight was reached, and filtered through a 60-mesh sieve. The preparation of activated biochar from SCGB was conducted according to a previous method of Nguyen et al. [9] with slight modifications. First, 50 g of dried SCG was soaked in 200 mL of 2M KOH for overnight (16 h) under stirring condition at 50 rpm. The mixture was then filtrated through Whatman No.1 filter paper and subsequently oven-dried at 60°C before undergoing pyrolysis at 550°C for 2 hrs in a muffle furnace. After pyrolysis, the SCGB was washed with 0.1M HCl and then distilled water until it reached a neutral pH. It was subsequently oven-dried and stored in a desiccator before use. The primary structure of the SCGB was determined by the Fourier transform infrared (FTIR) technique using a Vertex 70 spectrophotometer (Bruker, Ettlingen, Germany) within the wave number range of 500 to 4000 cm^{-1}. The surface morphology and porosity of sample were investigated using field emission scanning electron microscopy (FE-SEM) (S-4800, Hitachi, Tokyo, Japan) and Brunauer-Emmett-Teller (BET) technique (Belsorp-mini II, BEL Japan, Inc., Japan), respectively.

2.2 Laccase production

Laccase was produced from white rot fungus, *Megasporoporia* sp., which was provided by the Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University. The fungus was cultivated on potato dextrose agar (PDA) at room temperature (28 ± 2°C). After 5 days, the mycelium were harvested, homogenized, and transferred into laccase production medium [10]. The cultivation was performed at room temperature (28 ± 2°C) under shaking condition at 150 rpm for 7 days. The supernatant was collected as a crude enzyme by centrifugation at 18,000 x g for 10 mins, concentrated by ultrafiltration (10 kDa MW membrane cut–off, Amicon, USA) and freeze–dried for further experiments.

2.3 Determination of laccase activity

Laccase activity is assayed based on the oxidation reaction of 2,2'–azinobis–3–ethylbenzothiazoline–6–sulfonic acid (ABTS) which was monitored by the increase in absorbance at 420 nm. The reaction mixture (1 mL) contained 400 µL of 0.25 mM ABTS, 500 µL of 0.1 M sodium acetate buffer of pH 3.0, and 100 µL of culture supernatant [11]. One unit of enzyme activity (U) is defined as the amount of enzyme required to oxidize 1 µmol of ABTS per minute. Laccase activity (U/mL) was calculated based on equation (1).

$$\text{Laccase activity} = \left(\frac{U}{mL}\right) = \frac{\Delta Abs \times V_{total} \times 10^3}{\varepsilon \times d \times t \times V_{enzyme}} \quad (1)$$

Where ΔAbs is the difference between final and initial absorbances; V_{total} is total volume of the reaction; ε is molar absorptivity (3.6×10^4 $M^{-1}.cm^{-1}$); t is time (min); d is optical path (1 cm); and V_{enzyme} is enzyme volume that added to the reaction. The surface morphology of immobilized SCGB-laccase was investigated using FE-SEM (S-4800; Hitachi, Tokyo, Japan) and BET technique (Belsorp-mini II, BEL Japan, Inc., Japan).

2.4 Optimization of laccase immobilization

For the immobilization of laccase by the covalent binding method, the SCGB (10 mg) was immersed into 3% (v/v) glutaraldehyde solution (5 mL) and incubated overnight at room temperature under the stirring at 150 rpm [12]. Then, the modified SCGB was harvested by centrifugation at 18,000 x g for 10 min, washed three times to remove the excess chemical reagent and air dried at room temperature. For enzyme immobilization, the modified SCGB was suspended in crude laccase with 50 mM citrate buffer, pH 3.0 (5 mL), and incubated at room temperature under the stirring condition for 1 h. After that, the obtained product was collected by centrifugation, washed three times with citrate buffer, and freeze-dried at -50°C. The enzyme solution recovered after immobilization and the washed buffers were collected to determine the total protein content according to Lowry's method [13] with bovine serum albumin as a standard. The enzyme loading in obtained sample was calculated as follow equation (2):

$$G = (Ci - Cf) / m \quad (2)$$

where G is the enzyme loading (mg/g), Ci is the total protein in enzyme solution before immobilization (mg), Cf *is* the total protein in the supernatant and wash solution (mg), and m is the dry weight of obtained sample (g). The activity of immobilized SCGB-laccase (U/g dry weight of obtained sample) was assayed as previously described using 10 mg of obtained sample in each reaction. The optimal condition for laccase immobilization was determined by varying the glutaraldehyde concentration (1–9% v/w), the laccase activity (10–60 U/g dry weight of modified SCGB) and incubation time of enzyme (1-6 hrs), respectively. Each experiment was conducted as one factor at the time and the variable which presented the highest laccase activity was selected for the subsequent experiment. The primary structure, surface morphology, and porosity of the immobilized SCGB-laccase at the optimal condition were determined as previously described.

2.5 Characteristics of immobilized SCGB-laccase for RBBR decolorization

2.5.1 Effect of pH and temperature

RBBR was used as a substrate to determine the efficiency of immobilized laccase for dye decolorization. The effect of pH and temperature on RBBR decolorization was determined by incubation of the reaction mixtures consisting of immobilized or free enzymes (at the same laccase activity; 2 U) with 100 ppm of RBBR in the 50 mM buffer, including sodium citrate buffer (pH 3.0 to 5.0) or phosphate buffer (pH 5.0 to 7.0), at different temperatures ranging from 30 to 70°C. The aliquots were taken after incubation for 3 hrs and the absorbances at λmax (592 nm) were observed compared with those of controls, including untreated and treated dyes with the denatured enzyme. The percentage of dye decolorization was calculated as fellow equation (3).

$$\text{Dye decolorization (\%)} = [(\text{Abs initial} - \text{Abs final}) / \text{Abs initial}] \times 100 \quad (3)$$

Where Abs initial is the initial dye absorbance and Abs final is the final dye absorbance.

2.5.2 Thermostability

The thermostabilities of free and immobilized laccase were determined by incubating the enzymes at the optimum pH and temperature without the addition of substrate. Every 60-min interval, the aliquots were taken to determine their decolorization abilities as described above**.** The experiment was conducted in triplicates to ensure the accuracy and reliability of the results.

2.5.3 Reusability

The reusability of the immobilized enzyme was evaluated under the optimized condition for 3 hours. After being used, the immobilized enzymes were recovered by centrifugation at 18,000 x g for 10 mins, followed by washing with 50 mM acetate buffer, pH 5.0. The recovered enzymes were then used in the subsequent cycle with fresh dye solutions. Decolorization was determined as a percentage of dye removal. Controls in this study included SCGB

without enzyme and SCGB immobilized with denatured laccase. The experiment was performed in triplicates for accuracy and reproducibility.

2.6 Phytotoxicity studies

The phytotoxicity test was carried out using mung beans (*Vigna radiata L.*), and sweet corn (*Zea mays L.*) seeds as model plants. A total of 100 seeds for each plant species were placed on sterilized filter paper in sterilized petri plates, with 10 seeds per plate and 10 replicates in total and kept at room temperature throughout the experiment [14]. During the test, the seeds were watered with 5 mL of a solution containing either RBBR (100 ppm) or the decolorized products. A control set included the watering of tap water instead of dye solution. The germination index (GI) was measured from the relation between radical lengths in the sample and control after cultivation on 2nd day for mung bean and 3rd day for sweet corn as shown in equation (5).

$$\text{Germination index (\%)} = (Ns / Nc) \times (Ls / Lc) \times 100 \quad (5)$$

Where Ns and Nc represent the number of germinated seeds in the sample and control, respectively, and La and Lc denote the average root length (cm) in the sample and control, respectively.

2.7 Statistical analysis

All experiments were performed in triplicate. Data were presented as an average ± standard deviation. One–way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) was employed to compare any significant differences among average values at the level of $p \leq 0.05$ (IBM SPSS Statistics 29; IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1 Activated biochar from spent coffee ground

In this study, the activated biochar was synthesized by impregnating SCGs with 2M KOH and subjecting them to pyrolysis at 550°C for 1 hr, resulting in a yield of 47.12±2.85 % activated carbon product from dry starting material to the activated carbon product. This yield is slightly higher than those reported for KOH treatment of SCG biochar at the weight ratios of 2:1 (KOH:SCG) with pyrolysis at 800°C for 2 hrs (42.5% w/w) [15], 2:1 with pyrolysis at 400°C for 2 hrs (16% w/w) [16], and 4:1 with pyrolysis at 600°C for 1 hr (23% w/w) [17]. These different KOH concentrations and pyrolysis conditions not only affect the yield but also the quality of activated biochar [18]. Therefore, the morphological structure and surface area of SCGB were analyzed using SEM and BET, respectively. The average pore diameter of SCGB was 2.74 nm which indicated the presence of micro- (< 2 nm) and meso- (2–50 nm) pores in its structure as shown in Figure 1(A), 1(B) [19]. These micropore and mesopore structures have been reported to be more favorable for enzyme immobilization compared to macropores [20]. Therefore, the SCGB in this study might be well-suited for applications in laccase immobilization. Additionally, the surface area of SCGB was measured as 81.68 m^2/g, falling within the average range of 50-100 m^2/g for biochar produced at 500-550°C [17]. However, it was approximately two-fold higher than the values reported in previous studies on KOH-treated biochar from SCGs, which were within the range of 40-46 m^2/g [9, 16, 21].

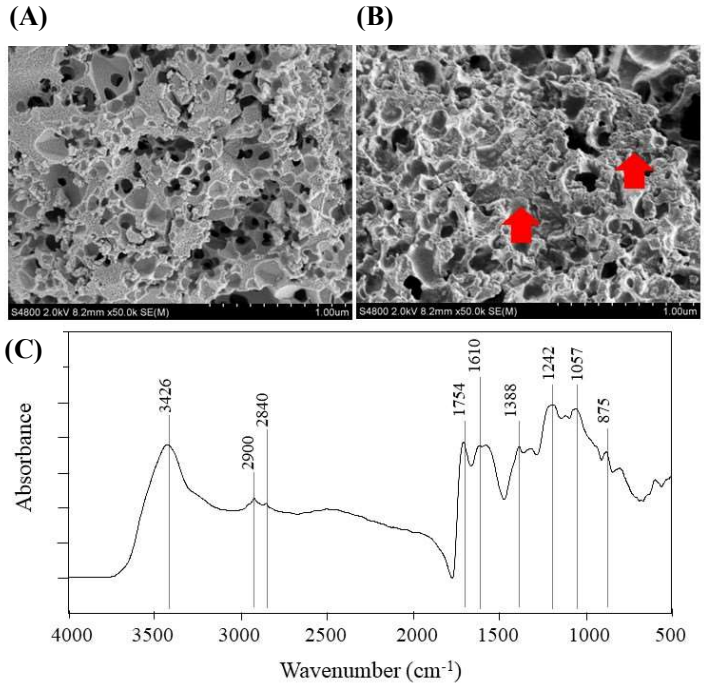

Figure 1 SEM images of spent coffee ground biochar (A) before and (B) after immobilized with *Megasporoporia* laccase, and (C) the FTIR spectra of spent coffee ground biochar.

The functional groups of SCGB were further investigated from FT-IR spectra, as shown in Figure 1(C). In general, the broad peak between 3600 to 3000 cm^{-1}, associated with the hydroxyl group (O-H) in the biochar structure, is expected to significantly decrease after pyrolysis due to the dehydration of holocellulose and the breaking of hydrogen bonds [22]. However, an intensive peak at 3426 cm^{-1} was observed in this study due to the structural modification of SCGB with KOH that is intended for the further application of enzyme immobilization [9]. A peak at 1754 cm^{-1}, corresponding to C=O stretching of carbonyl or carboxylic groups, which are oxygen-containing functional groups, was also observed. These oxygen-containing groups are known to provide the active sites that enhance the adsorption ability of biochar [23]. Bond stretching of functional groups commonly observed in biochar generated from pyrolysis at temperatures below 600°C, including C–H stretching (2900, 2840, and 1057 cm^{-1}) in aliphatic and aromatic structures, C=C stretching (1610 cm^{-1}) of the carbonyl group from lignin, and the C–O stretching of ester groups (1388 and 1242 cm^{-1}), was also present in the SCGB structure [17].

3.2 Optimization of laccase immobilization on biochar

Enzyme immobilization onto biochar can be achieved through both physical (adsorption) and chemical (covalent bonding) methods; however, the covalent immobilization is particularly advantageous as it contributes to high catalytic efficiency, stability, and reusability [24]. Therefore, in this study, the immobilization of *Megasporoporia* laccase was conducted through covalent bonding between the OH groups in KOH-activated SCGB and the NH/NH_2 groups in laccase, using glutaraldehyde as a crosslinking agent [25]. The SCGB was pretreated with glutaraldehyde and washed before enzyme immobilization to prevent the enzyme inactivation caused by glutaraldehyde-induced alteration in enzyme configuration [26]. However, it is crucial to optimize the concentration of glutaraldehyde during enzyme immobilization. The experiments were conducted by restricting the initial laccase activity of 30 U/g with the reaction time set at 3 hrs. The results presented in Figure 2(A) demonstrate a significant decrease in laccase activities of the immobilized enzyme and the values of enzyme loading when concentrations of glutaraldehyde exceed 3% (v/w). This decrease may be attributed to the interaction of glutaraldehyde with itself rather than with laccase, since glutaraldehyde is a bifunctional reagent [20, 27]. The highest laccase activity (13.40 ± 0.67 U/g) was achieved with the immobilized enzyme when SCGB was pretreated with 3% (v/w) glutaraldehyde. However, this activity was not significantly different to those obtained with addition of 1% (13.39 ± 1.35 U/g) and 2% (13.32 ± 0.48 U/g) glutaraldehyde addition. Comparable findings have been reported for laccase immobilization on chitosan beads [28] and carbon nanotubes [29], where efficient recovery activities from 79% to 95% were observed within a glutaraldehyde concentration range of 1% to 3%. Therefore, the lowest concentration (1% v/w) of glutaraldehyde was selected for the further optimization of the initial enzyme concentration.

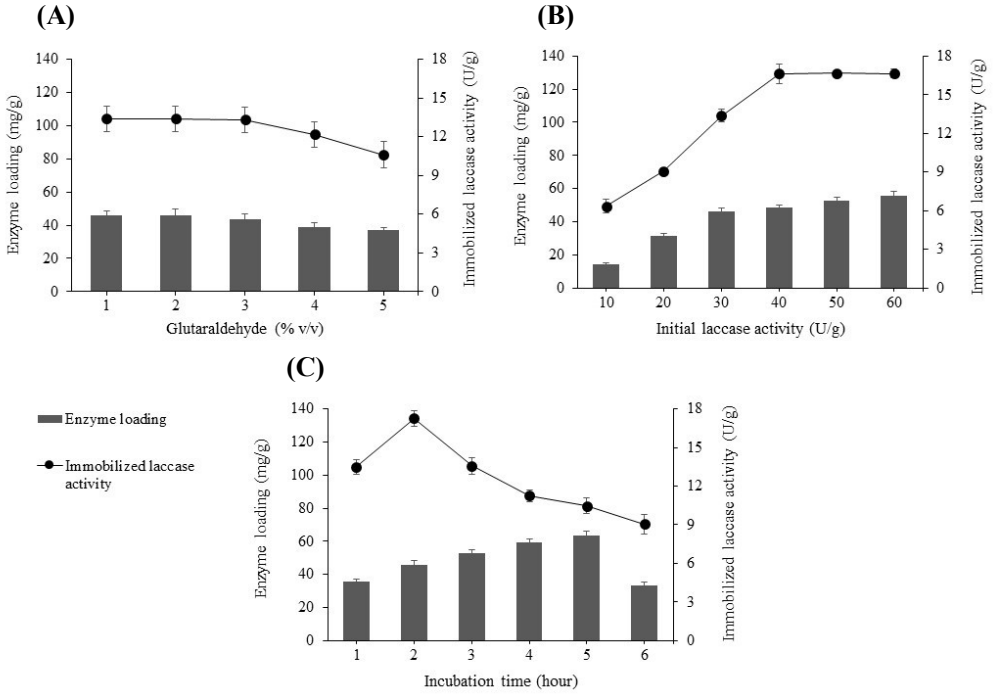

Figure 2 Optimization of laccase immobilization by varying (A) glutaraldehyde concentrations, (B) initial laccase concentrations, and (C) times. Data are shown as the mean ± SD (error bars), derived from triplicate experiments.

As shown in Figure 2(B), higher enzyme concentrations during the initial stages resulted in a significant increase in both immobilized SCGB-laccase activities and enzyme loading values. Subsequently, the activity of immobilized SCGB-laccase reached a maximum value of 16.63 ± 0.75 U/g at an enzyme concentration of 40 U/g and remained constant at higher enzyme concentrations, while the enzyme loading values continued to increase. These results relate to previous reports in which higher enzyme concentrations led to a decline or constancy in immobilized enzyme activities. This phenomenon is attributed to the overlapping of enzyme molecules in the same pore space of the support, which consequently limits substrate accessibility [30, 31]. Based on the obtained results, the optimum enzyme concentration in this study was 40 U/g and this concentration was then used to optimize the time for the crosslinking reaction. A similar increasing trend of enzyme loading values was observed with an increase in crosslinking time, as shown in Figure 2(C). However, the activity of immobilized SCGB-laccase appeared to decline with further increases in reaction time beyond 2 hrs due to the overload of enzyme molecules as described previously. It could be concluded that the optimal condition for enzyme immobilization with the maximum laccase activity of 17.25 ± 2.47 U/g was achieved by using 1% (v/w) glutaraldehyde, with an initial laccase activity of 40 U/g support after performing the incubation reaction for 2 hrs. Under these conditions, the immobilization efficiency of SCGB-laccase was about 43%, as determined by comparing the initial and immobilized activities. The activity of immobilized SCGB-laccase under optimal conditions was 1.3 times higher than that of immobilized SCGB-laccase (13.32 ± 0.92 U/g) under the reference conditions (4% glutaraldehyde and an initial laccase activity of 30 U/g) [26], resulting in a reduction in the total processing time of at least 2 hrs. The morphological structure of immobilized SCGB-laccase was observed using SEM, as shown in Figure 1(B). SCGB with an enzyme layer covering the surface of SCGB was investigated in comparison to SCGB without enzyme immobilization.

3.3 Characteristic of Immobilized SCGB-laccase for RBBR Decolorization

3.3.1 Effect of pH and temperature on laccase activities

The efficiency of crude and immobilized SCGB-laccase for RBBR decolorization was investigated at different pH levels and temperatures as shown in Figure 3. The immobilized SCGB-laccase exhibited higher efficiency in dye decolorization across all conditions compared to the crude enzyme at the same level of laccase activity. This enhancement may be attributed to the combined efficiency of enzyme activity and the ability of SCGB to adsorb organic contaminants [3]. At room temperature, the optimal pH of crude laccase for RBBR decolorization (66.94

± 1.62%) was achieved at pH 4, while the immobilized SCGB-laccase showed maximum activity (89.84 ± 2.45%) at pH 5. The shift in optimal temperature after immobilization was also previously observed in laccase immobilized on pine needle biochar (from pH 3 to 4) [32], and cetyltrimethylammonium bromide (CTAB)-KOH modified biochar (from pH 4 to 5) [33]. Several factors may be responsible for these results, particularly the alteration of the three-dimensional enzyme structure during the immobilization procedure. However, the immobilized SCGB-laccase exhibited activity with over 80% dye decolorization over a wide pH range from 3 to 7.

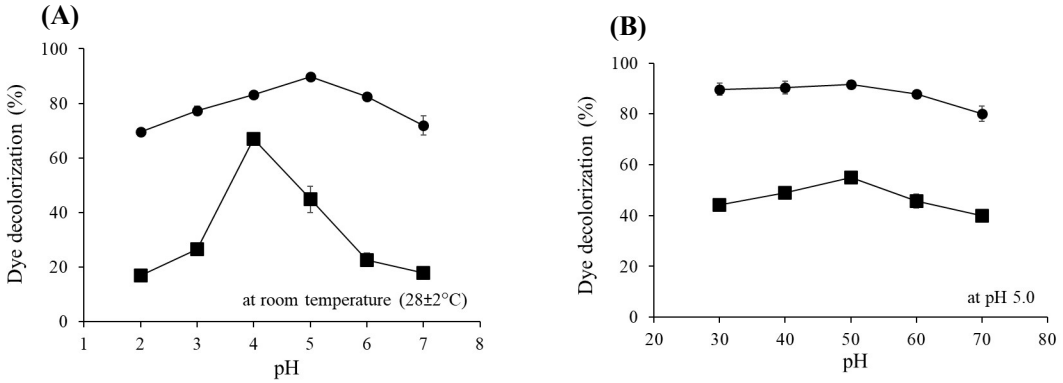

Figure 3 Effect of (A) pHs and (B) temperatures on decolorization of RBBR (100 ppm) by (■) crude and (●) immobilized SCGB-laccase after incubation for 3 hrs.

At the optimal pH, the effect of temperature on dye decolorization led to a similar trend for both crude and immobilized SCGB-laccase in which the highest dye decolorization of crude (54.96 ± 2.09%) and immobilized SCGB-laccase (91.52 ± 1.67%) was found at 50°C. However, at the higher temperatures of 60°C and 70°C, the activity of crude laccase significantly dropped to 45.72 ± 2.83% and 40.00 ± 2.09%, respectively, while the immobilized SCGB-laccase continued to present efficient activity with RBBR decolorization of 87.72 ± 1.45% and 80.06 ± 1.67%, respectively. This ability is noteworthy for the application of immobilized SCGB-laccase on SCGB in the wastewater treatment of the textile industry, where effluent temperatures after the dyeing process typically range from 50 to 60°C [34].

3.3.2 Thermostability

Thermal stability is a key characteristic of enzymes for industrial applications since several processes across different industries are conducted at elevated temperatures. Both crude and immobilized SCGB-laccase were separately incubated at optimal temperature (50°C), and aliquots of each enzyme were then taken every hour for 7 hrs to investigate the activity of dye decolorization as shown in Figure 4. The immobilized SCGB-laccase retained 79.21 ± 4.02% of its initial activity for dye decolorization after incubation at 50°C for 7 hrs. In contrast, under the same conditions, the crude laccase exhibited a greater reduction in activity by retaining only half (53.70 ± 7.44%) of its initial decolorization activity. The better thermostability of immobilized SCGB-laccase can be attributed to the interactions between laccase molecules and SCGB during immobilization, which may alter and stabilize the conformation of the enzyme to resist heat denaturation [35, 36].

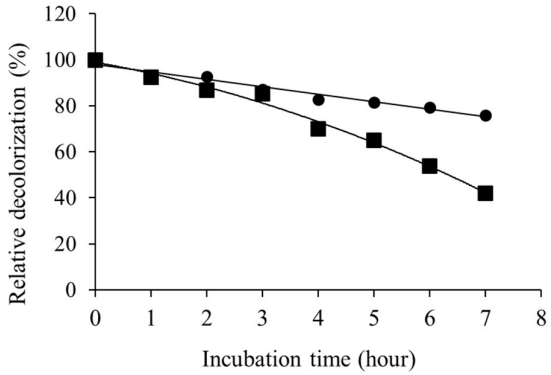

Figure 4 The relative activity of (■) crude and (●) immobilized SCGB-laccase on RBBR (100 ppm) decolorization after incubation at optimal pH (4 and 5, respectively) and temperature (50°C) for difference times.

3.3.3 Reusability

In this study, SCGB with immobilized denatured laccase (prepared under optimal conditions) and plain SCGB (without enzyme immobilization) were used as controls since several studies have reported the ability of biochar to absorb some pollutants [3]. In the first cycle of RBBR decolorization, the control of SCGB with/without denatured laccase exhibited decolorization abilities of 30.80 ± 2.96% and 42.68 ± 1.58%, respectively, whereas the immobilized SCGB-laccase achieved a significantly higher dye decolorization of 95.00 ± 2.58% (Figure 5). The dye absorption capacity of both control samples gradually decreased during the fourth cycle and stabilized at a lower level (10%-7%) during the fifth and sixth cycles. The immobilized SCGB-laccase maintained dye decolorization at more than 80% after three cycles and then retained dye decolorization at around 57.64 ± 1.28% after six cycles. The decrease in decolorization can be caused by enzyme denaturation and leakage from the carrier during utilization [35]. The retained activity of immobilized SCGB-laccase on SCGB in this study was slightly higher than those of immobilized SCGB-laccases on CTAB-KOH modified biochar [20], pine needle biochar [32], and rice straw biochar [37], which retained around 40% to 50% activity after the sixth cycle. The immobilization of laccase on SCGB not only enhances enzyme thermostability but also enables its reuse, thereby reducing overall processing costs.

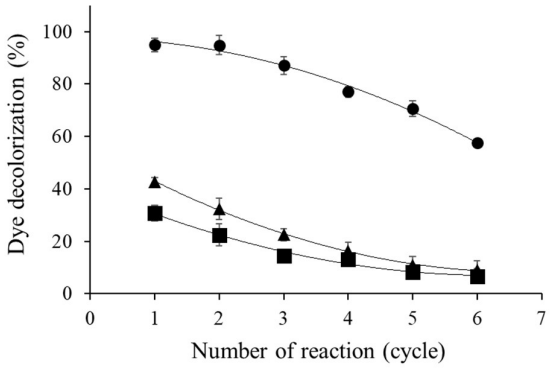

Figure 5 Reusability of immobilized SCGB-laccase (●) for RBBR decolorization. Each reaction was conducted with 100 ppm RBBR in 50 mM sodium citrate buffer at pH 5 and incubated at 50°C for 3 hours. Controls include SCGB without enzyme (◆) and SCGB immobilized with denatured laccase (■) to illustrate dye absorption during repeated cycles.

3.4 Phytotoxicity study

Wastewater generated from textile industries, when discharged into water bodies and subsequently used for agriculture, may cause serious environmental and health problems if it still contains some toxic compounds. Therefore, this study conducted a phytotoxicity analysis of dye before and after treatment with immobilized SCGB-laccase. The analysis used a GI calculated from the percentage of seed germination and root elongation of mung beans (*V. radiata* L.), and sweet corn (*Z. mays* L.), both of which are used as recognized models for evaluating wastewater toxicity [38, 39]. *V. radiata* L. and *Z. mays* L. seeds grown with 100 ppm of RBBR dye exhibited significantly lower GI values (52.7% and 34.9%, respectively) compared to tap water controls (100%), as shown in Table 1. These results support previous reports that azo dyes can reduce seed germination rate and root elongation, potentially by the inhibition of ATPase activity, a crucial enzyme for photosynthesis [40]. A higher inherent ability to tolerate toxic azo dye was found by *V. radiata* L. seeds compared to those of *Z. mays* L. This difference in tolerance might be linked to the difference between the C3-photosynthetic pathway of *V. radiata* L. and the C4 pathway of *Z. mays* L., as observed in chromium toxicity studies [41]. Additionally, *V. radiata* L. has been reported to employ various enzymatic and non-enzymatic antioxidant systems to combat the oxidative stress induced by azo dyes [42].

Table 1 Phytotoxicity effect of RBBR before and after treatment with immobilized SCGB-laccase on mung beans (*Vigna radiata L.*) and sweet corn (*Zea mays L.*) seeds.

Treatments*	Seed germinations (%)**		Root elongations (cm)		GI (%)	
	V. radiata L.	*Z. mays* L.	*V. radiata* L.	*Z. mays* L.	*V. radiata* L.	*Z. mays* L.
Control	100.8 ± 7.6^{a}	100.0 ± 7.1^{a}	2.0 ± 0.2^{a}	2.1 ± 0.2^{a}	100.7 ± 6.2^{a}	100.0 ± 3.4^{a}
RBBR	86.7 ± 5.8^{b}	63.7 ± 7.2^{b}	1.0 ± 0.2^{b}	1.0 ± 0.1^{b}	52.7 ± 3.4^{c}	34.9 ± 5.8^{c}
Laccase-treated RBBR	93.3 ± 5.8^{ab}	73.8 ± 2.1^{b}	1.8 ± 0.3^{a}	1.9 ± 0.2^{a}	83.5 ± 4.9^{b}	64.5 ± 3.7^{b}

*The seeds were irrigated with 5 mL of different solutions, including tap water as a control, RBBR (100 ppm), and the decolorized products obtained by treating RBBR (100 ppm) with immobilized SCGB-laccase for 3 hours (resulting in 95% decolorization). Each treatment was maintained at room temperature (28 ± 3 °C). The number of germinated seeds and root lengths in the samples and control were measured after cultivation on the 2nd day for mung bean and 3rd day for sweet corn to calculate the germination indexes (GI; %).

**Data are presented as mean ± standard deviation derived from ten replicates (N=10). The different letters in the same column indicated significant differences at $p \leq 0.05$ (ANOVA and DMRT).

From Table 1, it can be observed that the product from dye decolorization with immobilized SCGB-laccase exhibited an increase in GI values, reaching 83.5% for *V. radiata* L. and 64.5% for *Z. mays* L. According to Zucconi et al. [43], the toxicity can be categorized into three levels: GI values lower than 50% indicate high phytotoxicity, values between 50% and 80% suggest moderate phytotoxicity, and values exceeding 80% imply the absence of phytotoxicity. Therefore, the treatment of RBBR with immobilized SCGB-laccase resulted in a reduction of toxicity from moderate toxicity to no toxicity with *V. radiata* L. and from high toxicity to moderate toxicity with *Z. mays* L. This result is consistent with a previous report on the toxicity reduction of RBBR (100 ppm) in *V. radiata* L. seeds after treatment with *Pleurotus ostreatus* laccase, where the GI value increased from 54% (untreated dye) to 78% [44]. In addition, a reduction in toxicity of RBBR (133 ppm) was also observed in ryegrass (*Lolium perenne*) seeds after treatment with immobilized SCGB-laccase on alumina spherical pellets, leading to a shift from high to moderate toxicity with GI values ranging from 26% (untreated dye) to 69% (treated dye) [34]. Based on the results of this study, the treatment of dye with immobilized SCGB-laccase can be considered as an eco-friendly process, as it effectively reduces dye toxicity without producing any toxic by-products.

4. Conclusions

In this research, SCGs were used to synthesize the activated biochar and then employed as the carrier for *Megasporoporia* sp. laccase immobilization. The conditions for immobilizing laccase were successfully optimized, resulting in enhanced decolorization activity at higher temperatures and a wider range of pH values compared to that of crude laccase. The immobilized SCGB-laccase could be reused for up to six cycles of dye decolorization, and the decolorized products showed lower phytotoxicity compared to RBBR. The overall results of this study clearly indicate the great potential of the immobilized SCGB-laccase in textile wastewater treatment, which can be considered as a green-based and cost-effective method.

5. Conflict of Interest

The authors declare that there are no conflicts of interest.

6. Acknowledgements

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7. References

[1] Forcina A, Petrillo A, Travaglioni M, di Chiara S, De Felice F. A comparative life cycle assessment of different spent coffee ground reuse strategies and a sensitivity analysis for verifying the environmental convenience based on the location of sites. J Clean Prod. 2023;385:135727.

[2] Pandey D, Daverey A, Dutta K, Arunachalam K. Bioremoval of toxic malachite green from water through simultaneous decolorization and degradation using laccase immobilized biochar. Chemosphere. 2022;297 (134126):1-8.

[3] Nguyen VT, Vo TDH, Tran T, Nguyen TN, Le TNC, Bui XT, et al. Biochar derived from the spent coffee ground for ammonium adsorption from aqueous solution. Case Stud Chem Environ Eng. 2021;4(100141):1-7.

[4] Permpornsakul P, Prasongsuk S, Lotrakul P, Eveleigh DE, Kobayashi DY, Wu SH, Imai T, Punnapayak H. Two new records of the resupinate polypore fungi, *Ceriporia cystidiata* and *Macrohyporia dictyopora*, in Thailand. Sci Asia, 2016;42(3):171-177.

[5] Permpornsakul P, Prasongsuk S, Lotrakul P, Eveleigh DE, Kobayashi DY, Imai T, Punnapayak H. Biological treatment of reactive black 5 by resupinate white rot fungus *Phanerochaete sordida* PBU 0057. Pol J Environ Stud. 2016;25(3):1-6.

[6] Yang X, Zhang S, Ju M, Liu L. Preparation and modification of biochar materials and their application in soil remediation. Appl Sci. 2019;9(1365):1-25.

[7] Xiang J, Luo BX, Li JM, Mi Y, Tian B, Gong SJ, et al. Development of KOH and H_3PO_4-modified composite biochar from corn straw and activated sludge for removing methylene blue. Int J Environ Sci Technol. 2022;20(2):1673-88.

[8] Ahmadpour A, Rashidi H, Mahboub MJD, Farmad MR. Comparing the performance of KOH with NaOH-activated anthracites in terms of methane storage. Adsorp Sci Technol. 2013;31(8):729-745.

[9] Nguyen VT, Vo TD, Nguyen TB, Dat ND, Huu BT, Nguyen XC, et al. Adsorption of norfloxacin from aqueous solution on biochar derived from spent coffee ground: Master variables and response surface method optimized adsorption process. Chemosphere. 2022;288(2):132577.

[10] Bankeeree W, Prasongsuk S, Imai T, Lotrakul P, Punnapayak H. A novel xylan-polyvinyl alcohol hydrogel bead with laccase entrapment for decolorization of reactive black 5. Bioresources. 2016;11(3):6984-7000.

[11] Yanto DHY, Guntoro MA, Nurhayat OD, Anita SH, Oktaviani M, Ramadhan KP, et al. Biodegradation and biodetoxification of batik dye wastewater by laccase from *Trametes hirsuta* EDN 082 immobilised on light expanded clay aggregate. 3 Biotech. 2021;11(5):247.

[12] Ha NTH, Toan NC, Kajitvichyanukul P. Enhanced paraquat removal from contaminated water using cell-immobilized biochar. Clean Technol Environ Policy. 2022;24(4):1073-1085.

[13] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193(1):265–275.

[14] Osma JF, Toca-Herrera JL, Rodriguez-Couto S. Transformation pathway of Remazol Brilliant Blue R by immobilised laccase. Bioresour Technol. 2010;101(22):8509-8514.

[15] Liu X, Zhang S, Wen X, Chen X, Wen Y, Shi X, et al. High yield conversion of biowaste coffee grounds into hierarchical porous carbon for superior capacitive energy storage. Sci Rep. 2020;10(1):3518.

[16] Travis W, Gadipelli S, Guo Z. Superior CO2 adsorption from waste coffee ground derived carbons. RSC Adv. 2015;5(37):29558-29562.

[17] Muzyka R, Misztal E, Hrabak J, Banks SW, Sajdak M. Various biomass pyrolysis conditions influence the porosity and pore size distribution of biochar. Energy, 2023;263(126128):1-14.

[18] Atabani AE, Ali I, Naqvi SR, Badruddin IA, Aslam M, Mahmoud E, et al. A state-of-the-art review on spent coffee ground (SCG) pyrolysis for future biorefinery. Chemosphere. 2022;286(2):131730.

[19] Thommes M, Kaneko K, Neimark AV, Olivier JP, Rodriguez-Reinoso F, Rouquerol J, Sing KSW. Physisorption of gases, with special reference to the evaluation of surface area and pore size distribution, (IUPAC Technical Report). Pure Appl Chem. 2015;87(9-10):1051-1069.

[20] Wang Z, Ren D, Yu H, Zhang S, Zhang X, Chen W. Preparation optimization and stability comparison study of alkali-modified biochar immobilized laccase under multi-immobilization methods. Biochem Eng J. 2022;181(108401):1-11.

[21] Islam MA, Parvin MI, Dada TK, Kumar R, Antunes E. Silver adsorption on biochar produced from spent coffee grounds: validation by kinetic and isothermal modelling. Biomass Convers Biorefin. 2022;14(22): 28007-28021.

[22] Chen W, Shi S, Nguyen T, Chen M, Zhou X. Effect of temperature on the evolution of physical structure and chemical properties of bio-char derived from co-pyrolysis of lignin with high-density polyethylene. Bioresources. 2016;11(2):3923-3936.

[23] Liu C, Wang W, Wu R, Liu Y, Lin X, Kan H, Zheng Y. Preparation of acid- and alkali-modified biochar for removal of methylene blue pigment. ACS Omega, 2020;5(48):30906-30922.

[24] Kołodziejczak-Radzimska A, Nghiem LD, Jesionowski T. Functionalized materials as a versatile platform for enzyme immobilization in wastewater treatment. Curr Pollut Rep. 2021;7(3):263-276.

[25] Gęca M, Khalil AM, Tang M, Bhakta AK, Snoussi Y, Nowicki P, et al. Surface treatment of biochar-methods, surface analysis and potential applications: A comprehensive review. Surfaces. 2023;6(2):179-213.

[26] Coutinho Barcelos Costa H, Romao BB, Ribeiro E, Resende M. Glutaraldehyde effect in the immobilization process of alpha-galactosidase from Aspergillus niger in the ion exchange resin duolite A-568. Chem Eng Trans. 2013;32(2013):1105-1110.

[27] Lonappan L, Liu Y, Rouissi T, Pourcel F, Brar SK, Verma M, Surampalli RY. Covalent immobilization of laccase on citric acid functionalized micro-biochars derived from different feedstock and removal of diclofenac. Chem Eng J. 2018;351(2018):985-994.

[28] Aslam S, Asgher M, Khan NA, Bilal M. Immobilization of Pleurotus nebrodensis WC 850 laccase on glutaraldehyde cross-linked chitosan beads for enhanced biocatalytic degradation of textile dyes. J Water Proc engineering. 2021;40(101971):1-10.

[29] Habimana P, Gao J, Mwizerwa JP, Ndayambaje JB, Liu H, Luan P, Ma L, Jiang Y. Improvement of laccase activity via covalent immobilization over mesoporous silica coated magnetic multiwalled carbon nanotubes for the discoloration of synthetic dyes. ACS Omega. 2021;6(4):2777-2789.

[30] Sadeghi M, Moghimifar Z, Javadian H. Fe3O4@SiO2 nanocomposite immobilized with cellulase enzyme: Stability determination and biological activity. Chem Phys Lett. 2023;811(140161):1-12.

[31] Pandey D, Daverey A, Dutta K, Arunachalam K. Dye removal from simulated and real textile effluent using laccase immobilized on pine needle biochar. J Water Proc engineering. 2023;53(103710):1-10.

[32] Almeida LC, Barbosa AS, Fricks AT, Freitas LS, Lima ÁS, Soares CM. Use of conventional or non-conventional treatments of biochar for lipase immobilization. Process Biochem. 2017;61(2017):124-129.

[33] Wang Z, Ren D, Jiang S, Yu H, Cheng Y, Zhang S, Zhang X, Chen W. The study of laccase immobilization optimization and stability improvement on CTAB-KOH modified biochar. BMC Biotechnol. 2021;21(47):1-13.

[34] Das S, Cherwoo L, Singh R. Decoding dye degradation: Microbial remediation of textile industry effluents. Biotech Notes. 2023;4(2023):64-76.

[35] Olajuyigbe FM, Adetuyi OY, Fatokun CO. Characterization of free and immobilized laccase from Cyberlindnera fabianii and application in degradation of bisphenol A. Int J Biol Macromol. 2019;125:856-864.

[36] Li G, Nandgaonkar AG, Lu K, Krause WE, Lucia LA, Wei Q. Laccase immobilized on PAN/O-MMT composite nanofibers support for substrate bioremediation: a de novo adsorption and biocatalytic synergy. RSC Advances. 2016;6(47):41420-41427.

[37] Imam A, Suman SK, Singh R, Vempatapu BP, Ray A, Kanaujia PK. Application of laccase immobilized rice straw biochar for anthracene degradation. Environ Pollut. 2021;268(115827):1-14.

[38] Younas S, Rizvi H, Ali S, Abbas F. Irrigation of Zea mays with UASB-treated textile wastewater; effect on early irrigation of Zea mays with UASB-treated textile wastewater; effect on early growth and physiology. Environ Sci Pollut Res Int. 2020;27(13):15305-15324.

[39] Sompark C, Damrianant S, Sakkayawong N. Phytotoxicity and genotoxicity study of reactive red 141 dye on mung bean (Vigna radiata (L.) Wilczek) seedlings. Mol Biol Rep. 2024;51(51):1-11.

[40] Zhou X, Xiang X. Effect of different plants on azo-dye wastewater bio-decolorization. Procedia Environ Sci. 2013;18:540-46.

[41] Shahzad Q, Mahmood S, Javed S, Mushtaq T. Chromium stress tolerance of a C4 (*Zea mays* L.) and C3 (*Vigna radiata* L.) plants primed with UV and gamma-treated *Bacillus subtilis*. Microorganisms. 2021; 9(11): 2313.

[42] Singh HP, Mahajan P, Kaur S, Batish D, Kohli R. Chromium Toxicity and Tolerance in Plants. Environ Chem Lett. 2013; 11. doi:10.1007/s10311-013-0407-5

[43] Zucconi F, Monaco A, Forte M. Phytotoxins during the stabilization of organic matter. In: J. K. R. Gasser, Ed., Composting of agricultural and other wastes, Elsevier Applied Science Publication, New York, 1985. p. 73-86.

[44] Simões MF, Maiorano AE, dos Santos JG, Peixoto L, de Souza RFB, Neto AO, Brito AG, Ottoni CA. Microbial fuel cell-induced production of fungal laccase to degrade the anthraquinone dye Remazol Brilliant Blue R. Environ Chem Lett. 2019;17(2019):1413-1420.