

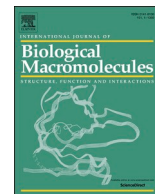


Title	Immobilization of laccases on mechanically ground silk fibroin nanofibers for enhanced stability
Author(s)	Miyawaki, Ayari; Sakai, Shinji
Citation	International Journal of Biological Macromolecules. 2024, 282(1), p. 136745
Version Type	VoR
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Immobilization of laccases on mechanically ground silk fibroin nanofibers for enhanced stability

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ARTICLE INFO

Keywords:
Silk fibroin nanofiber
Laccase
Enzyme immobilization

ABSTRACT

Azo dyes in textile industry effluents pose significant health and environmental risks. Laccase is an enzyme capable of degrading azo dyes, offering an environmentally friendly solution for treating textile wastewater. However, laccases need to be immobilized on specific carriers to enable effective reuse in batch reactors and continuous operation in flow-through reactors. This study employed silk fibroin nanofibers (SFNFs) obtained by mechanically grinding degummed silkworm silk as sustainable carriers to immobilize laccases through carbodiimide-mediated crosslinking. The immobilized laccases (SFNF-laccases) exhibited improved pH tolerance in the range of pH 3.0–8.0 with a smaller reduction in activity compared to free laccases (SFNF-laccases: 32.9 %, free laccases: 50.4 %). The thermal stability of immobilized laccases was also improved, showing 19, 13, and 9 % higher activities than those of free laccases at 40, 50, and 60 °C, respectively. After 8 days of storage, the activity of SFNF-laccases was 79 % of their activity immediately after immobilization, whereas free laccases retained only 29 % of their initial activity. In addition, SFNF-laccases maintained 73 % of their original operational activity in a flow-through reactor after 8 days. These results demonstrate the great potential of mechanically ground SFNFs as carriers of laccase and the resulting SFNF-laccases in industrial wastewater treatment.

1. Introduction

Wastewater from the textile industry contains 280,000 tons of dyes per year, and more than 70 % of them are azo dyes [1,2]. Azo dyes have carcinogenic, mutagenic, and teratogenic toxicity and can bioaccumulate in aquatic organisms, leading to potential risks for human health through the consumption of contaminated products [3]. Discharging azo dyes into water sources also has severe environmental consequences, including bioaccumulation, toxicity, ecosystem disruption, and long-term ecological impacts. Therefore, developing sustainable and efficient treatment methods to remove azo dyes from industrial wastewater is critical for human well-being and environmental protection.

Physical and chemical methods, such as absorption and photocatalytic degradation, have been used to degrade azo dyes [4,5]. However, these methods can cause secondary pollution by emitting by-products and may not be efficient in treating industrial wastewater due to its wide pH range and the presence of heat, color, and metals [6,7]. Enzymes have been primarily used to degrade azo dyes in

industrial wastewater because they do not encounter the aforementioned issues. Misal et al. reported the use of the enzyme azoreductase to degrade azo dyes [8]. Soybean peroxidase can also be used to degrade azo dyes, as reported by Ali et al. [9]. Among them, laccase is an enzyme that oxidizes phenol moieties, anilines, and aromatic thiols to the corresponding radicals and has demonstrated high specificity and high reaction efficiency for the degradation of azo dyes [10]. To enable continuous operation and reuse of the catalyst, laccase needs to be immobilized on carriers [11–13]. This study pioneers the use of mechanically ground silk fibroin nanofibers (SFNFs) to immobilize laccases, providing a novel approach to enhance the stability of laccases.

In this study, we successfully immobilized laccases on SFNFs through carbodiimide-mediated crosslinking. The SFNFs with a diameter of 100–300 nm and a length of 4–7 μm were obtained through mechanically grinding degummed silkworm (*Bombyx mori*) silk fibroin fibers generated by the sericultural industry. Compared to synthetic fibers, the silk fibers used in this study present significant advantages, such as abundance, natural origin, and low environmental impacts [14–16]. Furthermore, recycling silk waste and old garments into SFNFs can

Abbreviations: SFNF, silk fibroin nanofiber; ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid ammonium salt).

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<https://doi.org/10.1016/j.ijbiomac.2024.136745>

Received 15 August 2024; Received in revised form 15 October 2024; Accepted 18 October 2024

Available online 19 October 2024

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effectively reuse biodegradable resources, reduce reliance on synthetic materials, and promote sustainability.

Nanofibrous carriers possess a large surface area to volume ratio and numerous reactive groups on the surface [17,18]. These favorable properties can offer abundant active sites for covalent bonding, thereby reducing the conformational flexibility and enhancing the resistance to denaturation, which is particularly beneficial for stabilizing enzymes [19–22]. In addition to the environmental benefits of SFNFs, they offer easy operation and versatile functionalities. Their large surface area and abundant reactive groups, such as carboxyl and primary amine, provide an excellent matrix for enzyme immobilization. An electrospinning method has been employed to prepare SFNFs after dissolving the native degummed silk fibers to form a silk fibroin solution [23]. Compared to electrospinning, the mechanical grinding method has advantages in terms of simple process, low energy consumption, and time efficiency [24].

Various enzymes, such as peroxidase, glucose oxidase, and uricase, have been immobilized on silk fibroin membranes prepared from silk fibroin solutions through chemical crosslinking [25–27]. Enzymes have also been immobilized on SFNFs prepared through electrospinning [28,29]. To the best of our knowledge, this is the first report of using SFNFs obtained by mechanically grinding silk fibroin fibers for enzyme immobilization. The objectives of this work are to optimize the preparation of SFNF-laccases and to evaluate their catalytic properties. The enzymes immobilized on SFNFs showed higher stability than free enzymes, consistent with previous studies [19–22]. Then, the effects of pH, temperature, and storage period on the stability of the immobilized laccases were evaluated using 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid ammonium salt) (ABTS) as a model of azo dyes. Additionally, the functionality of the laccase-immobilized SFNF nonwoven membrane was studied as a reaction component of an enzymatic flow-through reactor (Fig. 1).

2. Materials and methods

2.1. Materials

An SFNF dispersion in water (1.0 % w/w) was obtained by defibrillating silk fibroin fibers using an ultra-fine friction grinder (Super Masscolloider MKCA 6–3, Masuko Sangyo Co., Saitama, Japan) according to a reported method [24]. The diameter and length of the SFNFs were 100–300 nm and 4–7 μm , respectively. *N*-hydroxysulfosuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide monohydrochloride (EDC), and ABTS were purchased from

Fujifilm Wako Pure Chemical Industries (Osaka, Japan), Peptide Institute (Osaka, Japan), and Tokyo Chemical Industry (Tokyo, Japan), respectively. Food-grade laccase powder (referred to as crude laccase) was purchased from Enzymes.bio (Wellington, New Zealand), which contains 40 % w/w active laccase enzyme from *Aspergillus oryzae* (10,000 U/g) and 60 % w/w other impurities (supplier's data). Crude laccase was used without any further purification, and the amount of laccase described in the later text was based on the active laccase protein content.

2.2. Immobilization of laccases on SFNFs

The dispersion of SFNFs was mixed with 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES)-buffered solution (pH 6.0) to obtain a 0.5 % w/v SFNF dispersion. Then, EDC (0–80 mg/mL) and NHS (0–8 mg/mL) were added sequentially at 20 °C. After 1 h of stirring, the resulting NHS-bearing SFNFs were washed twice with a 0.1 M MES-buffered solution (pH 6.0) via centrifugation (3500 rpm for 2 min) to remove residual EDC and NHS. The solution was then mixed with a 0.1 M MES-buffered solution containing crude laccases and stirred for 1–23 h at 20 °C. To remove residual laccases and impurities, the resulting SFNFs were washed sequentially with purified water and a phosphate-buffered solution (pH 7.0) via centrifugation (3500 rpm for 3 min). Then, the resultant laccase-immobilized SFNFs (SFNF-laccases) were dispersed in purified water to obtain an SFNF concentration of 1.0 mg/mL.

2.3. Laccase activity measurement

The catalytic activity of laccase was determined by monitoring the time course of ABTS oxidation [30,31]. In a 96-well plate, 10 μL of sample (SFNF-laccases or free laccases) was mixed with 150 μL of an ABTS solution (0.02–1.0 mM) in a succinate-buffered solution (pH 5.0). The selection of pH 5.0 was based on the limited conversion of ABTS to ABTS^{+} at higher pH levels, as shown in Fig. S1, which illustrates the conversion efficiency of ABTS^{+} under different pH conditions. The absorbance changes at 420 nm were measured using a plate reader (SpectraMax iD3, Molecular Devices, LLC, California, USA). The catalytic activity was calculated using Eq. (1) [32]. In this equation, the molar absorbance of ABTS (ϵ) is $36 \text{ mM}^{-1} \text{ cm}^{-1}$, $\Delta A_{420\text{nm}}$ is the absorbance change at 420 nm wavelength, V is the sample volume, and S is the base area. The catalytic activities of SFNF-laccases and free laccases were calculated using Eqs. (2) and (3) and expressed as mmol/s/mg of SFNF and laccase, respectively.

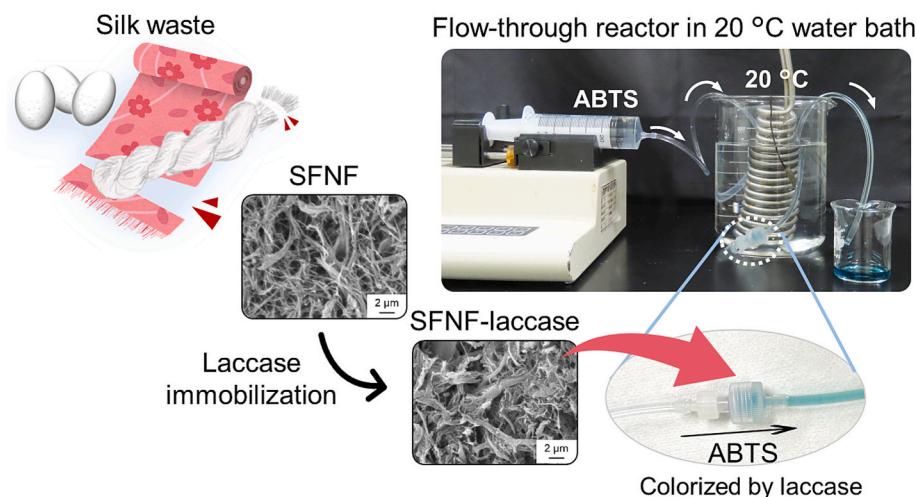


Fig. 1. Schematic of the immobilization of laccases on SFNFs and the operational stability test of SFNF-laccases using ABTS as a model of azo dyes in a flow-through reactor.

$$\text{Catalytic activity (mmol/s/mL)} = \frac{\Delta A_{420 \text{ nm}} (\text{s}^{-1}) \times S (\text{cm}^2) \times 1000}{\epsilon (\text{mM}^{-1} \text{cm}^{-1}) \times V (\text{mL})} \quad (1)$$

$$\begin{aligned} \text{Catalytic activity (mmol/s/mg}_{\text{SFNF}}) \\ = \frac{\text{Catalytic activity (mmol/s/mL)}}{\text{Concentration of SFNF - laccase (mg/mL)}} \end{aligned} \quad (2)$$

$$\begin{aligned} \text{Catalytic activity (mmol/s/mg}_{\text{laccase}}) \\ = \frac{\text{Catalytic activity (mmol/s/mL)}}{\text{Concentration of free laccase (mg/mL)}} \end{aligned} \quad (3)$$

2.4. Effects of pH, temperature, and storage period on stability

An SFNF-laccase dispersion (SFNF: 2.0 mg/mL) was incubated with a 0.1 M citrate-buffered solution (pH 3.0, 4.0), a succinate-buffered solution (pH 5.0), or a phosphate-buffered solution (pH 6.0, 7.0, 8.0) at 20 °C to achieve an SFNF concentration of 1.0 mg/mL. As a control, a solution containing 40 mg/mL laccase was mixed with the buffered solutions under the same conditions to obtain a laccase concentration of 4.0 mg/mL. After incubation for 8 h, the catalytic activity of SFNF-laccases was measured. The effect of temperature on stability was evaluated using the same method. Purified water containing SFNF-laccases (1.0 mg/mL SFNF) or 4.0 mg/mL free laccases was maintained at 4–70 °C for 8 h, then their catalytic activities were measured. The effect of storage period was evaluated after the SFNF-laccases (1.0 mg/mL SFNF) and free laccases (4.0 mg/mL) were stored in purified water at 20 °C for 0–8 days. The relative activity was expressed as the ratio of the activity of SFNF-laccases or free laccases at a certain time point during storage to that at the start of storage.

2.5. Continuous operation in a flow-through reactor

To pack SFNF-laccases in a flow-through reactor, an SFNF-laccase dispersion was injected into a membrane holder (13 mm diameter) equipped with a membrane filter (0.8 μm pore diameter). The amount of SFNF-laccases deposited onto each membrane filter was set to be 0.1, 0.2, and 0.4 mg of SFNFs. Then, the deposited SFNF-laccases were covered with a membrane filter (0.8 μm pore diameter) to produce an SFNF-laccase layer sandwiched by the membrane filters. After inserting the sandwiched filters in a membrane holder, it was immersed in a water bath at 20 °C, allowing a succinate-buffered solution (pH 5.0) containing 0.2 mM ABTS to pass through the membrane holder. The flow rate was varied in the range of 0.074–2.23 cm/min. The stability of the SFNF-laccases was determined by flowing the solution at 0.74 cm/min for 8 days. The relative activity was expressed as the ratio of the activity of SFNF-laccases at a certain time point to that at the start of the flow.

3. Results and discussion

3.1. Laccases immobilized on SFNFs

The catalytic performance of SFNF-laccases obtained through crosslinking using 12 mg/mL EDC for 3 h at 20 °C in 0.04 mM ABTS was studied. As shown in Fig. 2A, the immobilized laccases on SFNFs catalyzed the oxidation of ABTS to form ABTS^{•+}, and the achieved maximum ABTS^{•+} concentration was comparable to that of the free laccases. When the concentration of ABTS was 1.0 mM (Fig. 2B), the catalytic activities of SFNF-laccases and free laccases were 1.15 ± 0.03 mmol/s/mg_{SFNF} and 0.53 ± 0.01 mmol/s/mg_{laccase}, respectively (mean ± S.D., n = 3). These results demonstrate that the SFNF-laccases derived from 1.0 mg SFNFs exhibit a catalytic activity comparable to that of 2.18 mg laccases. The immobilization amount was also confirmed by measuring the change in the dry weight of SFNFs before and after laccase immobilization: The dry weight increased 3.32 ± 0.09-fold, implying that 2.32 mg laccases were immobilized on 1.0 mg SFNFs (mean ± S.D., n = 3).

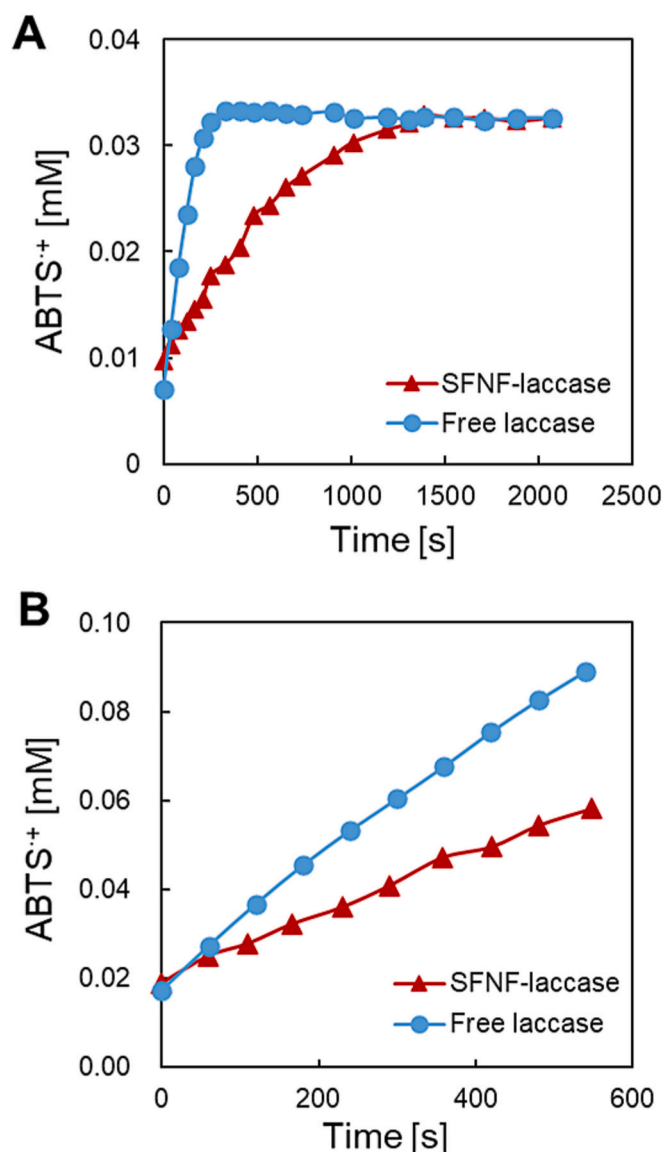


Fig. 2. Time-dependent ABTS^{•+} generation from A) 0.04 mM and B) 1.0 mM ABTS in the presence of SFNF-laccases (1.0 mg/mL SFNF) and free laccases (4.0 mg/mL) in a succinate-buffered solution (pH 5.0) at 20 °C. The SFNF-laccases were prepared using 12 mg/mL EDC and 1 mg/mL NHS with an immobilization time of 3 h.

The specific activity of immobilized laccases was determined by dividing the amount of immobilized laccases derived from the catalytic activity by the amount derived from the dry weight. The calculated specific activity was 94.0 %, suggesting that laccases can be immobilized on SFNFs without losing their catalytic activity. Similarly, Silva et al. reported a 99 ± 1 % specific activity of laccases after being immobilized on functionalized spent grains [33].

Next, the effect of the concentration of the crosslinking agent, EDC, on the catalytic performance of the resulting SFNF-laccases was investigated. As shown in Fig. 3A, for all SFNF-laccases, the rate of the conversion from ABTS to ABTS^{•+} increased proportionally with increasing oxidation time. At a given time, the conversion increased with increasing EDC concentration up to 12 mg/mL and became stable above this concentration. The catalytic activities of SFNF-laccases prepared using 4, 6, 8, 12, 24, 48, and 80 mg/mL EDC were 0.37, 0.51, 0.81, 1.15, 1.24, 1.19, and 1.12 mmol/s/mg_{SFNF}, respectively (Fig. 3B). A similar trend of increasing activity with increasing EDC concentration was reported by Naghdi et al. for the immobilization of laccases on pinewood

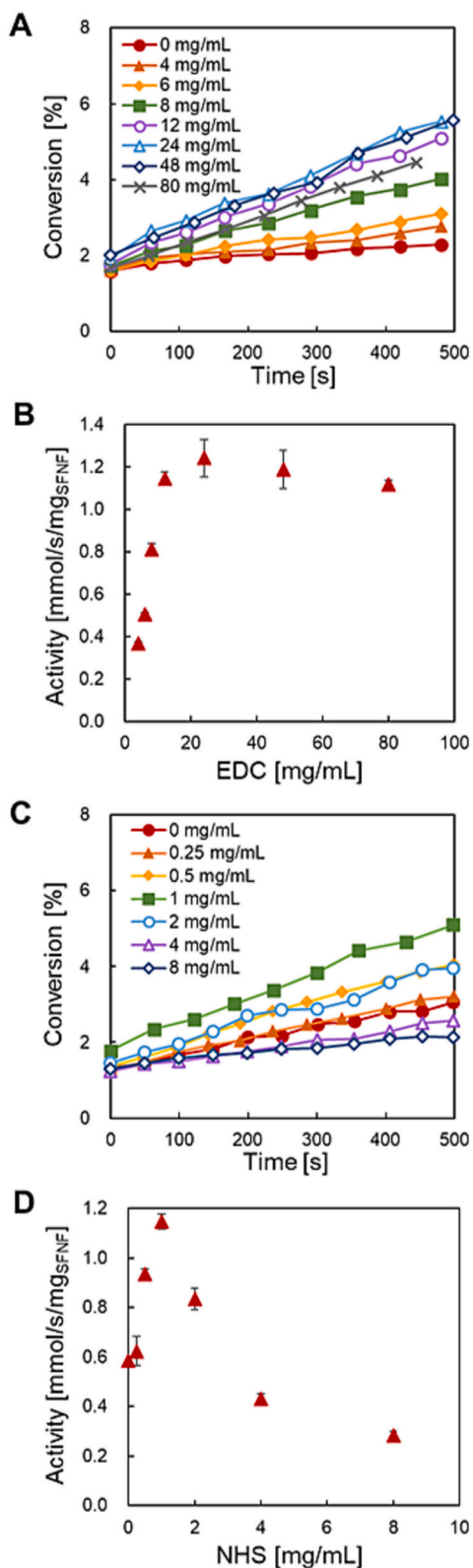


Fig. 3. Effects of EDC and NHS concentrations on the catalytic activity of SFNF-laccases. A, C) Time-dependent conversion from 1.0 mM ABTS to $\text{ABTS}^{+\cdot}$ in the presence of SFNF-laccases (1.0 mg/mL SFNF) prepared under different A) EDC and C) NHS concentrations in a succinate-buffered solution (pH 5.0) at 20 °C. B, D) Catalytic activities calculated by Eqs. (1) and (2) from the data shown in B) Fig. 3A and D) Fig. 3C. The SFNF-laccases were prepared with a reaction time of 3 h.

nanobiochar [34]. EDC binds to the carboxyl groups on the enzyme surface or immobilization matrix. When combined with NHS, EDC activates these groups to form stable NHS esters, which then react with amine groups to create stable amide bonds. Thus, a high EDC concentration may promote crosslinking, allowing more enzyme molecules to be securely attached to the carrier material. All NHS were most likely consumed at 12 mg/mL EDC, indicating that the observed activity did not change beyond this concentration. In the sample obtained by mixing SFNFs and laccases without EDC (0 mg/mL in Fig. 3B), the formation of $\text{ABTS}^{+\cdot}$ increased over time, probably due to the laccases being physically adsorbed on SFNFs.

The effect of the NHS concentration on the catalytic activity of SFNF-laccases was also investigated at 12 mg/mL EDC. The conversion rates of $\text{ABTS}^{+\cdot}$ shown in Fig. 3C were used to calculate the catalytic activities shown in Fig. 3D. The catalytic activity of SFNF-laccases peaked at 1 mg/mL NHS. Below this concentration, the increasing catalytic activity with increasing NHS concentration would be caused by the increasing formation of NHS esters at the carboxyl groups of SFNFs. Above 1 mg/mL, the significant decrease in catalytic activity with increasing NHS concentration may have been caused by denaturation triggered by a conformational change due to the increasing crosslinking point per laccase molecule. Sun et al. immobilized trypsin on magnetic nanoparticles via carbodiimide crosslinking with EDC at different concentrations in the presence of sufficient NHS [22]. They mentioned that the catalytic activity increased and then decreased with increasing EDC concentration because of the increasing number of immobilized enzyme molecules and altered structure of the enzyme due to the high number of crosslinking points. Thus, enzymes denature because of the high crosslinking density in carbodiimide crosslinking.

The catalytic performances of the SFNF-laccases prepared under different reaction times were evaluated. The catalytic activities were derived from the conversion rates of $\text{ABTS}^{+\cdot}$ (Fig. 4A). As shown in Fig. 4B, the catalytic activity increased as the reaction time was extended from 1 to 3 h but decreased upon further extending the reaction time; the activity of SFNF-laccases obtained in 23 h of reaction was 0.85 ± 0.03 mmol/s/mg_{SFNF} (mean \pm S.D., $n = 3$). The initial increase in catalytic activity (within 1–3 h) could be explained by the increase in the amount of immobilized laccases. The subsequent decrease in catalytic activity (from 3 to 23 h) was supposed to be related to the denaturation of the immobilized laccases, which occurred due to the increasing steric hindrance between the laccases as the amount of immobilized laccases increased [35]. This phenomenon was also reported by Zhou et al. [36] in a study on the immobilization of adenylate cyclase (AC) on graphene oxide via 8-arm polyethylene glycol amine (8arm-PEG-NH₂). As the concentration of 8arm-PEG-NH₂ increased, the activity of the immobilized AC first increased and then decreased because the steric hindrance between ACs caused denaturation. Similar results have been reported by Silva et al. and Naghdi et al. for the immobilization of laccases on spent grains and pinewood nanobiochar, which achieved maximum catalytic activity at reaction times of approximately 3 and 3.5 h, respectively [33,34]. It was assumed that the enzymes adsorbed on the carriers formed a layer-by-layer structure, and then the activity per mass of the enzyme-immobilized carrier decreased due to the decreasing availability of the enzymes. In our research, the mass change in the enzyme-immobilized carrier was normalized as the activity was expressed as “per mass of carrier”.

Based on the above results, SFNF-laccases prepared using 12 mg/mL EDC and 1 mg/mL NHS with a reaction time of 3 h had the best catalytic activity and were used in the following experiments.

3.2. Effects of pH, temperature, and storage period on the stability

Immobilization has been reported to have a positive effect on the stability of a variety of enzymes on different carriers [18,20–22]. The effects of pH, temperature, and storage period on the stability of laccases immobilized on SFNFs were studied.

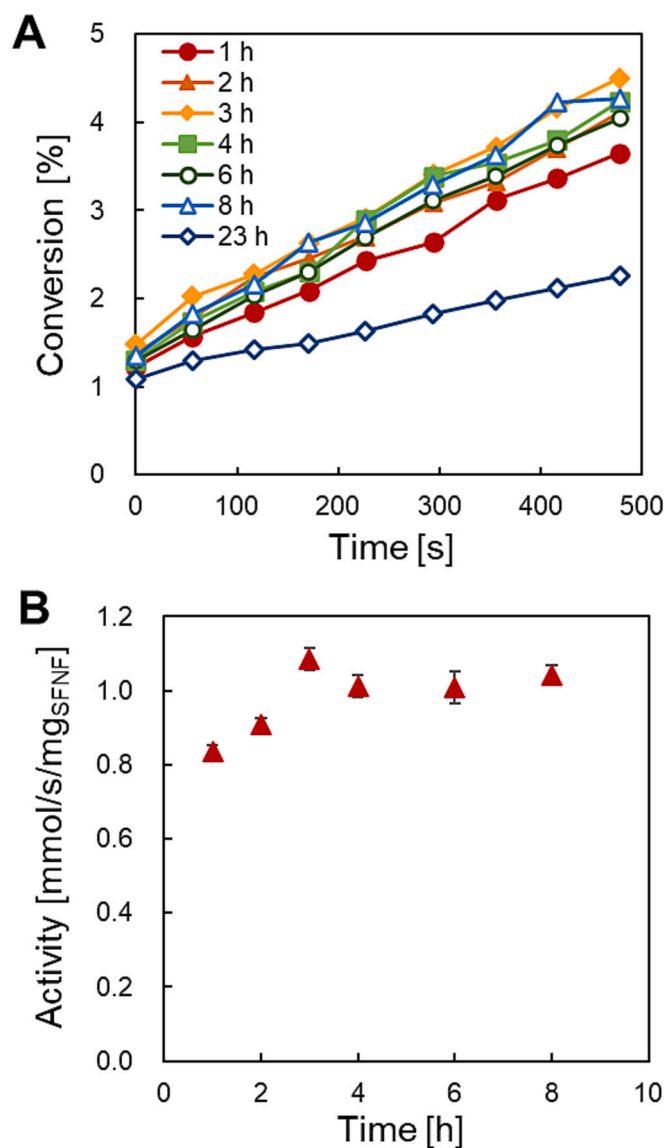


Fig. 4. Effect of reaction time for immobilization on the catalytic activity of SFNF-laccases. A) Time-dependent conversion from 1.0 mM ABTS to ABTS⁺ in the presence of SFNF-laccases (1.0 mg/mL SFNF) prepared under different reaction times in a succinate-buffered solution (pH 5.0) at 20 °C. B) Catalytic activities calculated by Eqs. (1) and (2) from the data shown in Fig. 4A. Bars represent mean \pm S.D. ($n = 3$). The SFNF-laccases were prepared using 12 mg/mL EDC and 1 mg/mL NHS.

Fig. 5A and B show the conversion rates of ABTS⁺ in the presence of immobilized and free laccases incubated in buffered solutions with pH 3.0–8.0. The catalytic activities (Fig. 5C) were derived from the conversion rates shown in Fig. 5A and B and expressed as relative values to the initial activity before storage (Fig. S2). Both free and immobilized laccases exhibited the highest activity at pH 6.0, and their activities decreased as pH changed. This phenomenon was explained by Toledo-Núñez et al.; unfavorable pH conditions cause conformational changes, which, in turn, lead to denaturation [37]. SFNF-laccases showed smaller fluctuations in activity than free laccases (SFNF-laccases: 32.9 %, free laccases: 50.4 %). This is because the laccases in SFNF-laccases are crosslinked at multiple points on the SFNFs, including in the areas close to the active sites, which makes the conformational structure more rigid and resistant to pH changes. Wang et al., Dehghanifard et al., Lassouane et al., and Chen et al. [18,20,21,38] also reported that enzymes, including laccases, immobilized on carriers showed higher pH tolerance

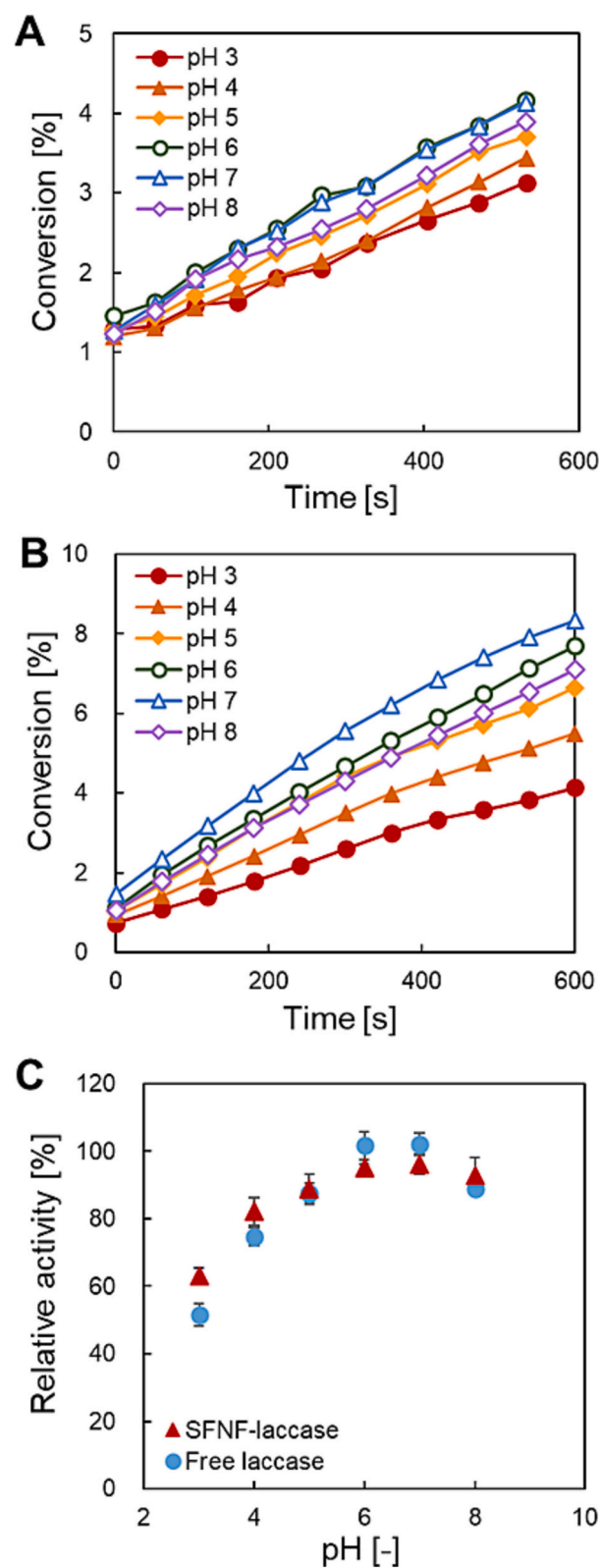


Fig. 5. Effect of pH on the catalytic activity of SFNF-laccases for 8 h of storage in solutions. Time-dependent conversion of 1.0 mM ABTS to ABTS⁺ in the presence of A) SFNF-laccases (1.0 mg/mL) and B) free laccases (4 mg/mL) in a succinate-buffered solution (pH 5.0) at 20 °C. C) Relative activities calculated by Eqs. (1)–(3) from the data in Fig. 5A and B and expressed as a percentage relative to the activity before storage (100 %). Bars represent mean \pm S.D. ($n = 3$). The SFNF-laccases were prepared using 12 mg/mL EDC and 1 mg/mL NHS with a reaction time of 3 h.

than free enzymes. Chen et al. evaluated the pH tolerance of laccases immobilized on magnetic particles for 0.5 h and showed that the immobilized laccases were hard to denature upon pH change. Thus, the relative activity difference of immobilized laccases between the maximum and the minimum was 20 % smaller than that of free laccases.

Although the effect was not substantial, a positive impact of immobilization on storage temperature was also observed. Fig. 6A and B show the conversion rates of ABTS⁺ catalyzed by immobilized and free laccases incubated at 4–70 °C. The resulting enzyme activities derived from these data are shown in Fig. 6C. The relative activities were expressed as the ratio of the activity at a specific time point during storage to its activity at the start of storage (SFNF-laccases: 2.10 ± 0.03 mmol/s/mg_{SFNF}, free laccases: 1.00 ± 0.02 mmol/s/mg_{laccase}) (Fig. S3). In general, heat can change the enzyme's conformation and lead to denaturation. Our experiment also suggested that both the catalytic activities of SFNF-laccases and free laccases decreased with increasing temperature during storage. SFNF-laccases had slightly higher stability at higher temperatures. At 40, 50, and 60 °C, the activities of SFNF-laccases were 19, 13, and 9 % higher than those of free laccases. The enhanced thermal stability of SFNF-laccases can be attributed to the formation of covalent bonds between the enzyme and the nanofiber carrier. These bonds likely restrict the enzyme's conformational change, maintaining its active structure at high temperatures. This stabilization mechanism is supported by similar observations involving other carriers [18,20–22,39]. Patel et al. immobilized laccases on SiO₂ nanocarriers and assessed the time required for the laccase activity to decrease by half at temperatures ranging from 30 to 60 °C. The immobilized laccases showed a half-life 8.3–18.1 times longer than that of free laccases.

The storage stability of SFNF-laccases and free laccases was evaluated from preparation to day 8. Fig. 7A and B show the conversion rates of ABTS⁺ catalyzed by immobilized and free laccases. The resulting activities derived from the conversion data are shown in Fig. 7C. Both activities were expressed as 100 % on day 0. The activity of immobilized laccases was 79 % on day 8, while that of free laccases was 29 %, suggesting that the immobilized laccases were more resistant to conformational changes over time because of their more rigid conformation. Previous studies [20–22,34] have also shown that immobilized enzymes exhibit higher stability than free enzymes due to their rigid conformation. Dehghanifard et al. reported that laccases immobilized on nanoporous silica beads retained more than 85 % of their activity after 30 days, while free laccases retained only 15 %. Also, Lassouane et al. reported that laccases immobilized on calcium alginate beads with glutaraldehyde retained 96 % of their activity after 35 days. In comparison, laccases entrapped in calcium alginate beads and free laccases retained 92 % and 80 % of their activities, respectively.

3.3. Operational stability of SFNF-laccases

The stability of the immobilized laccases was evaluated in a membrane flow-through reactor (Fig. 8A). Fig. 8B illustrates the relationship between conversion and flow rate, showing that the conversion increased as the flow rate slowed. The conversions in the presence of different amounts of SFNF-laccases were also compared in Fig. 8B, wherein increasing the amount of packed SFNF-laccases resulted in increased conversion. To evaluate the operational stability, 0.1 mg SFNF-laccases and 0.74 cm/min flow rate were selected. After 8 days of operation, the remaining activity of the flow-through reactor was 73 % of its initial one (Fig. 8C), which was almost the same as the activity of SFNF-laccases (Fig. 7B). These results confirmed that the immobilized laccases were not removed by the flow and remained active despite the reaction with the substrate. A similar tendency was also reported by Zhang et al. [40], wherein laccases immobilized on a polystyrene-*block*-poly(4-vinyl pyridine) isoporous membrane exhibited 90 % storage and operational stability after 30 days. The high operational stability of SFNF-laccases in the flow-through reactor indicated that the covalent bonds formed through immobilization were robust. This stable

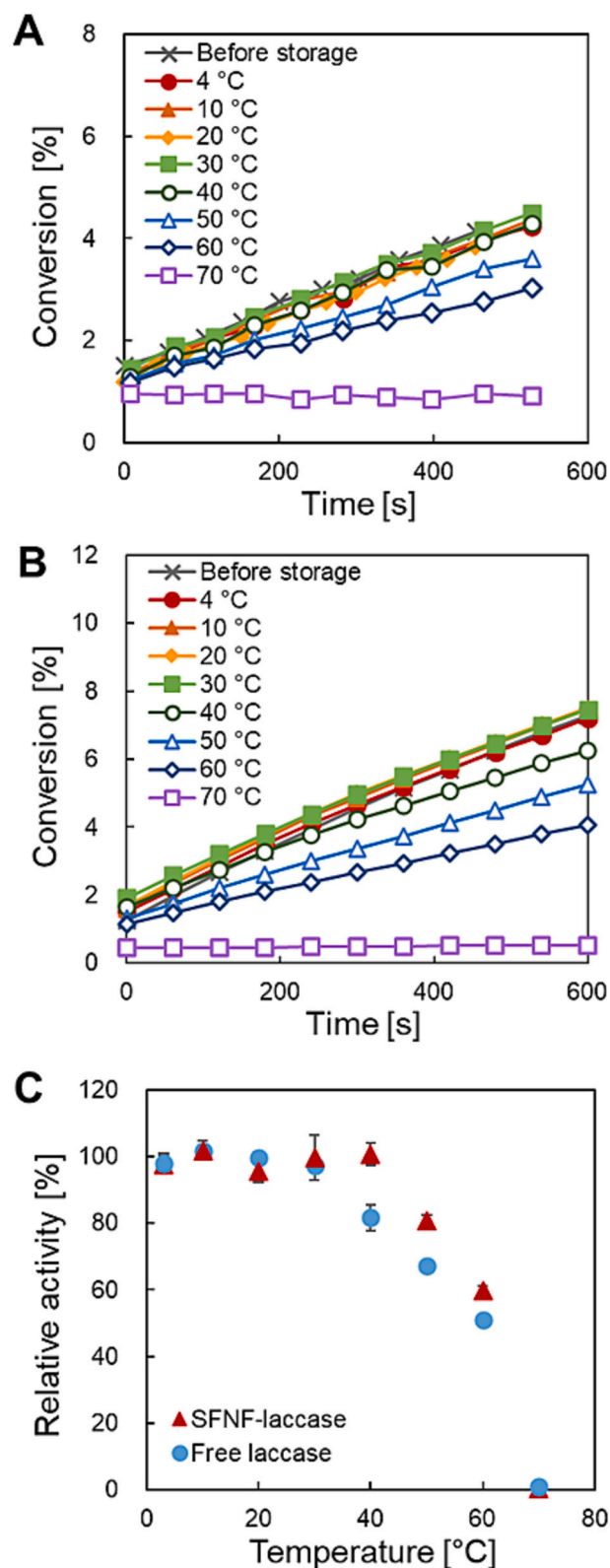


Fig. 6. Effect of temperature on the catalytic activity of SFNF-laccases for 8 h of storage in purified water. Time-dependent conversion of 1.0 mM ABTS to ABTS⁺ in the presence of A) SFNF-laccases (1.0 mg/mL SFNF) and B) 4.0 mg/mL free laccases in a succinate-buffered solution (pH 5.0) at 20 °C. C) Relative activities calculated by Eqs. (1)–(3) from the data in Fig. 6A and B and expressed as a percentage relative to the activity before storage (100 %). Bars represent mean \pm S.D. ($n = 3$). The SFNF-laccases were prepared using 12 mg/mL EDC and 1 mg/mL NHS with a reaction time of 3 h.

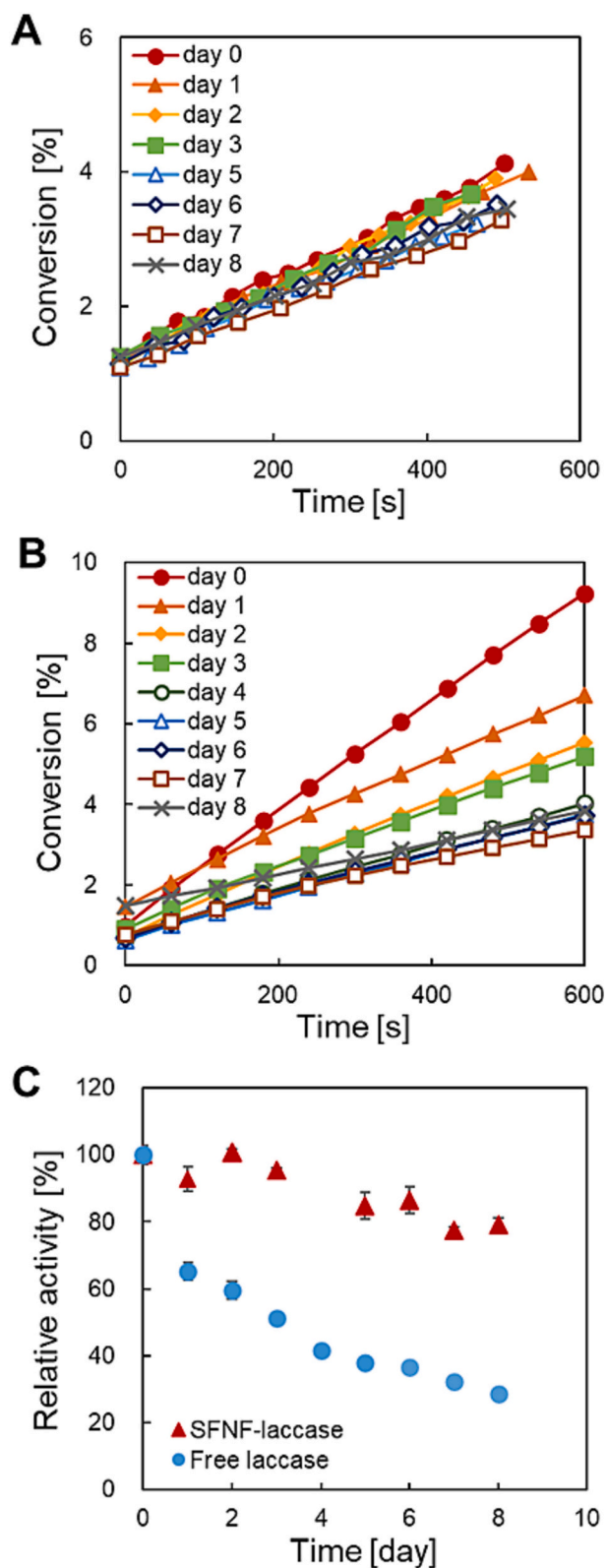


Fig. 7. Effect of the storage period in purified water on the catalytic activity of SFNF-laccases. Time-dependent conversion of 1.0 mM ABTS to ABTS^{+} in the presence of A) SFNF-laccases (1.0 mg/mL SFNF) and B) 4.0 mg/mL free laccases in a succinate-buffered solution (pH 5.0) at 20 °C. C) Relative activities of SFNF-laccases and free laccases calculated by Eqs. (1)–(3) from the data in Fig. 7A and B. Bars represent mean \pm S.D. ($n = 3$). The SFNF-laccases were prepared using 12 mg/mL EDC and 1 mg/mL NHS with a reaction time of 3 h.

attachment ensured consistent catalytic performance over extended periods, making SFNF-laccases suitable for continuous industrial applications.

Given its highly stable performance in a flow-through reactor, SFNF-laccases show significant potential for scaling up. The improved operational stability of SFNF-laccases is particularly relevant for industrial applications where continuous operation and enzyme reuse are critical. The ability to maintain high catalytic activity for extended periods underscores the potential of SFNF-laccases for large-scale, cost-effective, and sustainable industrial wastewater treatment.

3.4. Limitations and perspective

In this study, laccase from *Aspergillus oryzae* was immobilized on mechanically ground SFNFs, and the immobilized enzyme exhibited higher stability across various pH levels, temperatures, and storage periods compared to free laccase. While these results are promising, further studies using different types of laccases are necessary to fully validate the effectiveness of SFNFs as carriers for laccase immobilization. The properties of laccases depend on their origin [41,42]. For instance, laccases obtained from *Pleurotus sapidus* and *Trametes versicolor* showed the highest catalytic activities at pH 3.0 and 40 °C and pH 5.0 and 45 °C, respectively; however, after immobilization on alginate hydrogel beads, these enzymes showed the highest catalytic activities at pH 5.0 and 65 °C and pH 3.0 and 60 °C, respectively [41,42]. Once the effectiveness of SFNFs for laccases obtained from different origins is demonstrated, we anticipate that SFNF-laccases will be developed for specific reaction conditions, such as various pH and temperatures.

The effect of SFNF diameter on the catalytic performance of SFNF-laccases was not investigated in this study. SFNFs with smaller diameters could be used to immobilize more laccase molecules per unit mass. Assuming a constant reactor volume and the same mass of SFNFs, an increase in surface area may improve overall reaction efficiency by increasing the catalyst density within the reactor. The potential for faster substrate conversion and increased reaction rate makes this an attractive avenue for further investigation. However, it is important to note that reducing the diameter of SFNFs may alter their pore size and fluid flow characteristics within the reactor. These changes may affect reaction efficiency and hence should be carefully evaluated when optimizing the system in future studies.

Based on the results of this study, SFNF-laccases can most likely degrade azo dyes contained in wastewater from textile industries. In future work, we will evaluate the degradability of azo dyes by passing their solution to a flow-through reactor with an SFNF-laccase membrane. For the application of this system in actual wastewater treatment, we will also conduct azo dye degradation at various pH and temperatures to determine its stability.

The application of immobilized laccase is not limited to the degradation of azo dyes. Previous studies, such as those by Dehghanifard et al. [20] and Lassouane et al. [21], have demonstrated the capability of immobilized laccase to degrade carcinogenic phenolic compounds such as 2,4-dinitrophenol and bisphenol A, which are commonly released from industrial activities. Furthermore, laccase has established applications in the food industry, including beer and wine stabilization and fruit juice clarification [43,44]. Based on these diverse applications, the SFNF-laccase flow-through reactor could be adapted for various treatment processes. Future work will explore these applications and assess the effectiveness of the reactor under various operation conditions.

4. Conclusion

In this study, laccases were immobilized on mechanically ground SFNFs for the degradation of azo dyes in textile wastewater. Compared with free laccases, the covalently immobilized laccases on SFNFs showed an 18 % smaller decrease in activity across pH 3.0–8.0, 19 % greater thermal stability at 40 °C, and 50 % higher storage stability over

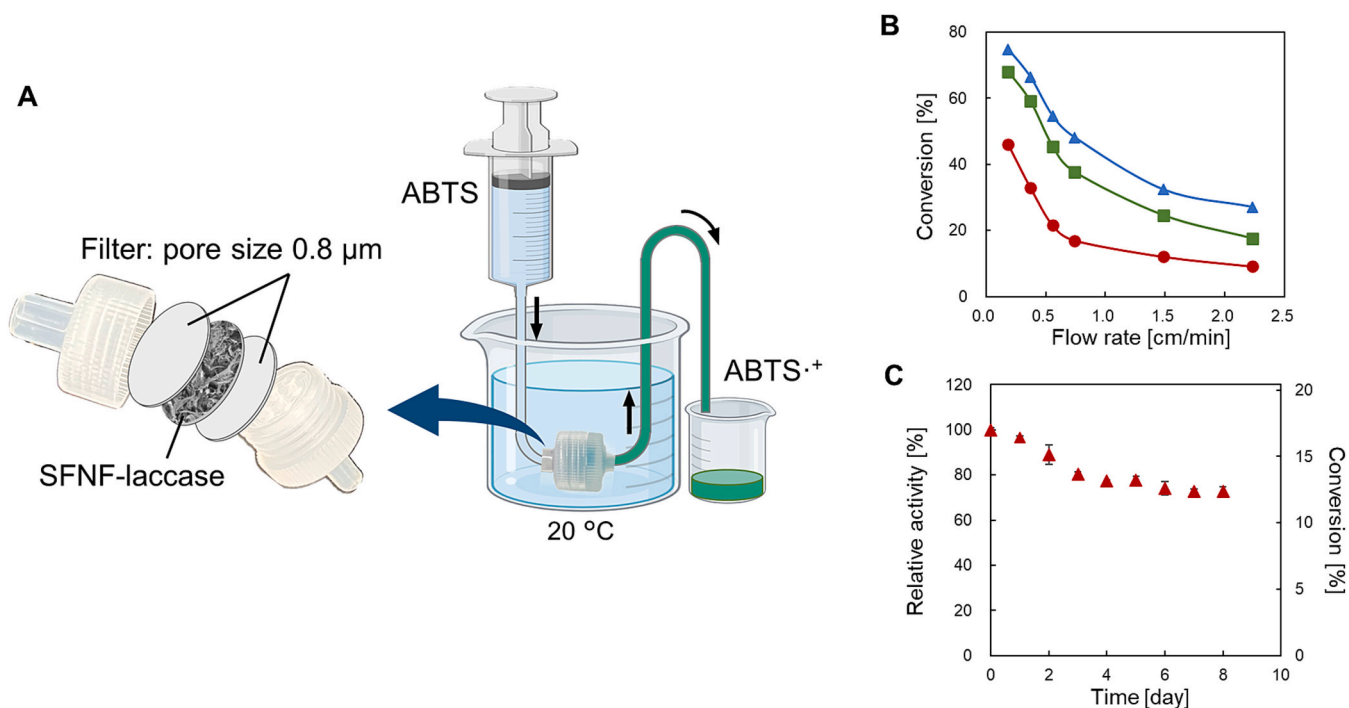


Fig. 8. A) Schematic of the experimental setup to measure the laccase activity of the SFNF-laccase membrane in a flow-through reactor under different conditions. The setup includes a membrane holder, a substrate flow system, and a temperature controller. B, C) Catalytic activity of SFNF-laccases as a membrane in a flow-through reactor at 20 °C. The substrate was 0.2 mM ABTS in a succinate-buffered solution (pH 5.0). The SFNF-laccases were prepared using 12 mg/mL EDC and 1 mg/mL NHS with a reaction time of 3 h. B) Change in conversion as a function of flow rate. The amount of SFNF-laccases in the membrane flow-through reactor was 0.1 mg (closed circles), 0.2 mg (closed squares), and 0.4 mg (closed triangles). C) Time-dependent operational stability of using 0.1 mg SFNF-laccases in a membrane flow-through reactor. Bars represent mean \pm S.D. ($n = 3$).

8 days. The activity of SFNF-laccases in a flow-through reactor increased with increasing amount of SFNF-laccases and decreasing flow rate. The activity remained at 73 % even after 8 days of continuous operation at a flow rate of 0.74 cm/min. This high level of activity retention demonstrates the long-term stability of immobilized laccases on mechanically ground SFNFs, making them a promising material for industrial wastewater treatment applications.

CRediT authorship contribution statement

Ayari Miyawaki: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Shinji Sakai:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by a Ministry of Agriculture, Forestry and Fisheries (MAFF)-commissioned project study on “Research project for sericultural bio-industry” [Grant Number JP22680575].

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2024.136745>.

Data availability

Data will be made available on request.

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