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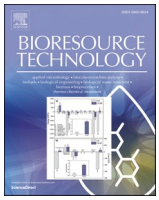


3D printed polylactide scaffolding for laccase immobilization to improve enzyme stability and estrogen removal from wastewater

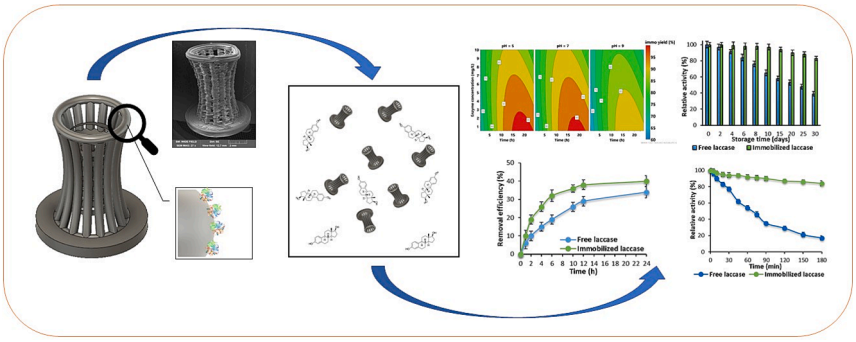
Agnieszka Rybarczyk [a], Wojciech Smułek [a], Adam Grzywaczyk [a], Ewa Kaczorek [a], Teofil Jesionowski [a], Long D. Nghiem [b], Jakub Zdarta [a,*]

[a] Institute of Chemical Technology and Engineering, Faculty of Chemical Technology, Poznan University of Technology, Berdychowo 4, PL-60965 Poznan, Poland
[b] Centre for Technology in Water and Wastewater, University of Technology Sydney, Ultimo, NSW 2007, Australia

HIGHLIGHTS

- Production of stable biocatalytic system using open-structure 3D printed scaffolds.
- 24 h, pH 5 and 5 mg/mL enzyme solution were optimal immobilization conditions.
- High thermal and chemical stability of system with immobilized laccase.
- Removal of over 30% of E2 and 40% of EE2 from wastewater using produced systems.
- Wastewater matrix constituents affect enzymatic treatment of micropollutants.

GRAPHICAL ABSTRACT



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ABSTRACT

This study reports a biocatalytic system of immobilized laccase and 3D printed open-structure biopolymer scaffoldings. The scaffoldings were computer-designed and 3D printed using polylactide (PLA) filament. The immobilization of laccase onto the 3D printed PLA scaffolds were optimized with regard to pH, enzyme concentration, and immobilization time. Laccase immobilization resulted in a small reduction in reactivity (in terms of Michaelis constant and maximum reaction rate) but led to significant improvement in chemical and thermal stability. After 20 days of storage, the immobilized and free laccase showed 80% and 35% retention of the initial enzymatic activity, respectively. The immobilized laccase on 3D printed PLA scaffolds achieved 10% improvement in the removal of estrogens from real wastewater as compared to free laccase and showed the significant reusability potential. Results here are promising but also highlight the need for further study to improve enzymatic activity and reusability.

1. Introduction

Over the past few years, enzyme immobilization has emerged as a potential approach to improve enzyme stability and reusability (Zdarta et al., 2022a; Madhavan et al., 2017). In an immobilized enzymatic system, the biocatalyst is attached or bound to a carrier for protection

* Corresponding author.
E-mail address: jakub.zdarta@put.poznan.pl (J. Zdarta).

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and recovery. The efficiency of enzyme immobilization depends on several factors, among them carrier design, functions, and properties are the most important (Gonçalves et al., 2019).

Important considerations when designing the carrier for enzyme immobilization include shape, geometry, and the number and type of functional groups as they govern the effectiveness of enzyme immobilization such as stability, enzyme activity, and reusability. In recent years, 3D printing has emerged as a modern and versatile method for the direct fabrication of carrier materials to immobilize enzymes (Shao et al., 2022). Using 3D printing, complex support structures can be precisely produced in a short time without harmful solvents (Ye et al., 2019). Considering the characteristic, properties, and wide possibilities of use, common materials for 3D printing for enzyme immobilization are polylactide (PLA), acrylonitrile–butadienestyrene copolymer, and nylon. While all of these polymers can offer good mechanical stability, PLA stands out primarily for its biodegradability and biocompatibility. Advantages also include ease of molding and a wide range of applications (Blanco, 2020). Furthermore, PLA is a biodegradable polymer that can be made from starch and waste biomass. PLA is readily degradable under suitable environmental conditions by hydrolysis of ester bonds (Vu et al., 2020).

There are several 3D printing methods, however, the Fused Filament Fabrication (FFF) technique is the most attractive as it is fast, easy-to-use and can achieve good reproducibility (when the 3D printer is calibrated correctly). To date, FFF has been widely used to 3D print PLA for enzyme immobilization. Zhang et al. (2021) used the FFF method to 3D print PLA carbon fiber reinforced scaffolding for immobilizing YCJ01 lipase. The use of carbon fiber improved the properties of the filament without changing the rheological ability to print complex structures resulting in great operational stability of immobilized enzyme over repeated usage. Singh et al. (2020) used 3D printing technology to create a flow sensor, where phosphate-degrading enzymes were encapsulated in a metal–organic framework. The resulting system was tested for the removal of copper ions, where 42% copper was adsorbed after just 40 min of running the reaction, confirming the potential of the created catalytic system for remediating metals from solutions.

To create a suitable immobilized enzyme system, it is necessary to understand the interplay among several process parameters including substrate structure, enzyme concentration, pH, and immobilization time (Bourdant et al., 2020; Wu et al., 2022). Box–Behnken experimental design is a useful tool to select optimal parameters for a given process based on the results obtained and the assumptions made using kinetic relationships from the least number of experiments (Senćanski et al., 2021; Ayinla et al., 2022). There have been several attempts to optimize enzymes via this approach (Li et al., 2022). However, there is very little information regarding the optimization of enzyme immobilization using 3D-printed scaffolds as support.

The selection of the enzyme for the process is also important in determining the practical application of the system. Belonging to the group of oxidoreductases, laccase is capable for catalyzing oxidation–reduction reactions of a wide range of phenolic compounds. This enzyme is characterized by low substrate specificity, which gives it great application potential in the degradation of contaminants present in the environment (Di Dong et al., 2023). The widespread and ubiquitous presence of pharmaceutically active compounds and estrogens in surface water has emerged as a vexing environmental problem. In addition to over-the-counter drugs and antibiotics, the most common pharmaceuticals in surface waters are hormonal agents. Estrogenic substances from both industrial and domestic sources enter the environment through many routes (Yazdan et al., 2022). Primary sources of estrogenic pollution include the pharmaceutical industry, hospitals, animal farming, and medical prescription. After administration, hormones are not fully metabolized and a portion can enter wastewater in unchanged form. Improper disposal of expired medicines can also be a source of estrogenic pollution. Pharmaceuticals also enter the environment through the release of residual compounds used in the manufacturing process. Koh et al. (2007) compiled the monitoring results of 17β-estradiol (E2) and 17α-ethynylestradiol (EE2) in wastewater and surface water. They reported the ubiquitous occurrence of these hormones in surface water across the world. Hormones, known as bioregulators, are responsible for stimulating or slowing down the work of various organs, thus controlling the functions that occur in every living organism. Special attention should be paid to estrogens, natural or synthetic steroidal compounds, which in excessive amounts can lead to organ dysfunction and the onset of many diseases. Endocrine disruptors can negatively affect sensitive hormonal pathways that regulate reproductive functions and provoke changes in offspring gene expression. The most vulnerable are aquatic organisms (fertility and egg production can decrease) and newborns. These compounds are not fully metabolized and are secreted from the organism in unchanged forms. In particular, the amount of E2 and EE2, which lead to endocrine disruption and infertility in animals, should be controlled (Gonsioroski et al., 2020). EE2 is more toxic and persistent to biodegradation than E2. EE2 can remain in the environment for several years, posing a significant environmental risk. EE2 also has a stronger tendency to absorb in organic matter and accumulate in sediments. Numerous studies have shown that even small concentrations of this hormone can lead to physiological or metabolic disorders and affect developmental abnormalities.

This work demonstrates and reports a novel 3D printing technique to immobilize laccase onto pre-designed PLA scaffolds. As a result of the modeling analyses, optimal immobilization parameters were determined, such as the concentration of the enzyme solution and the time of the process. An important part of the study was also the thorough characterization of the immobilized laccase. Finally, produced systems were tested in the degradation of 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) from real wastewater, which was a key aspect of the ongoing research and implied the environmental aspect of the study.

2. Materials and methods

2.1. Materials and reagents

Transparent plastic polylactide (PLA) from Spectrum Filaments (Poland) was used to 3D print the carrier. Laccase from *Trametes versicolor* (EC 1.10.3.2, $\geq$ 0.5 U/mg), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 99%), and Bradford reagent were obtained from Sigma-Aldrich. 50 mM acetate buffer (pH 3–5), 50 mM phosphate buffer (pH 6–8), and 50 mM Tris-HCl (pH 8 and 9) were freshly prepared. All of the reagents were of analytical grade and were used directly without any further purification. Wastewater samples were kindly supplied by the local wastewater treatment plant.

2.2. Analytical techniques

Scanning electron microscopy (SEM), optical microscopy, thermogravimetric analysis (TGA), Fourier transform infrared spectroscopy (FTIR), zeta potential analysis and compressive strength were used to characterize the morphology, thermal stability, electrokinetic and mechanical properties of produced scaffolds, whereas gas chromatography coupled with mass spectroscopy (GC–MS) were applied for analysis of the mixtures after estrogens removal. A detailed description of this analytical technique is presented in the E-Supplementary data.

2.3. Fabrication of 3D polylactide scaffolds

3D polylactide scaffolds were prepared by the Fused Filament Fabrication (FFF) technique using Prusa i3MK3 3D printer with a 0.2 mm brass nozzle (Prusa Research, Czech Republic). Fusion 360 CAD software (ver. V.2.0.11680, Autodesk, USA) was used for 3D modeling. The PrusaSlicer software (ver. 2.2.0, Prusa Research, Czech Republic) was used for model slicing. Printing parameters were as follows: layer height 0.2 mm, extruder temperature 185 ℃, bed temperature 70 ℃,

retraction 2 mm, retraction speed 200 mm/s. Transparent plastic polylactide (PLA) (Spectrum Filaments, Poland) was used to produce the carrier.

2.4. Laccase immobilization, characterization, and optimization

For laccase immobilization, 3D PLA scaffold (ca. 100 mg) was added to 5 mL of the enzyme solution (5 mg/mL, pH 5) and mixed for 24 h at 100 rpm. Next, the PLA scaffolds were separated and washed with acetate buffer to remove unbound enzyme. The amount of immobilized enzyme (mg/g) was determined according to the Bradford assay (Bradford, 1976) based on spectrophotometric measurements (Jasco V-750, Japan) at the wavelength of 595 nm by considering: the differences in the amount of enzyme in the initial solution for immobilization, and the amount of the enzyme in solutions after immobilization and washing, as well as the mass of the support. Whereas enzyme loading (mg/cm^2) was determined by considering the amount of the immobilized enzyme, and the surface area of the scaffold. Laccase leakage (%) from the support was determined after 12 h of incubation and mixing (300 rpm) at 30 °C in 10 mL of acetate buffer (pH 5). The leakage was calculated by comparing the initial laccase content immobilized in 3D printed PLA scaffolds and the final laccase content determined by the Bradford assay after incubation.

Model reaction with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was used to characterize catalytic properties of the free and immobilized laccase. Briefly, 1 scaffold with immobilized laccase (ca. 20 mg) or the corresponding amount of free enzyme was added to 5 mL of 5 mM ABTS solution at pH 5 with 200 rpm mixing speed. The reaction was performed in darkness at 25 °C for 5 min and was followed spectrophotometrically at 420 nm. The enzyme activity recovery (%) of the immobilized laccase was defined as the activity of the immobilized laccase divided by the initial enzyme activity used for immobilization. Immobilized yield (%) was calculated based on the laccase activity in the solution before and after immobilization by considering the differences in the laccase activity used for immobilization and the activity of the enzyme in the supernatant and washing solutions.

The above-mentioned ABTS reaction was used to determine the effect of the pH (from 3 to 9) at 30 °C and the temperature (from 10 to 60 °C) at pH 5 on the activity of free and immobilized laccase. The highest obtained activity for both systems was defined as 100% relative activity.

Storage stability was determined using ABTS reaction by incubation of the free and immobilized laccase at 4 °C over 30 days. Thermal stability was examined by incubation of the free and immobilized laccase at pH 5, and temperature of 50 °C over 180 min. Samples were taken for ABTS reaction under optimal assay conditions at the specified time points. For storage and thermal stability tests, the initial value of the activity of the free or immobilized laccase was defined as 100%. The inactivation constant (k_D) and half-life ($t_{1/2}$) were evaluated based on the linear regression slope (E-Supplementary data).

The apparent kinetic parameters (Michaelis constant (K_m), and the maximum reaction rate (V_{max})), of the free and immobilized laccase, were defined based on the ABTS reaction (substrate concentration from 0.05 to 1 mM) under optimum assay conditions. The kinetic parameters were calculated by fitting the obtained data according to the Hanes–Woolf plot. All of the above-mentioned experiments were performed in triplicate and the results are presented as a mean value with standard deviation as error value.

Immobilization time, solution pH, and enzyme concentration were chosen for the optimization of immobilization conditions according to Box-Behnken's design. The model was quadratic with 12 independent experimental runs with three center points (E-Supplementary data). The parameters under optimization were immobilization yield, immobilized enzyme per unit mass of support, and enzyme activity recovery. These parameters were calculated as presented above, based on data from the preliminary experiments. For immobilization optimization, multiple linear regression (MLR) was used for the data analysis and the relative average predicted variance was 14.6.

2.5. Estrogens removal from wastewater

The wastewater used in this study was collected from the local wastewater treatment plant in the suburban area of Poznan Agglomeration (central-western Poland), which collects water from single-family houses and small farms. The samples were taken from primary wastewater, without initial purification or treatment. The collected wastewater is characterized by a nearly neutral pH (ca. 7.3), the conductivity of 2 mS/cm, and 150 mg/mL TOC. Further, the wastewater used in this study contained 356 ng/L of EE2 and 187 ng/L of E2.

Prior to the removal experiment, also test with laccase immobilized subjected to thermal inactivation (inactivation at 80 °C for 5 h) was performed to determine the estrogens removal by adsorption. Adsorption test and removal tests were performed at the same process conditions. Estrogen removal by adsorption was less than 5%. The removal of E2 and EE2 from real wastewater was evaluated using free laccase and laccase immobilized on 3D PLA scaffolds. 3D PLA scaffold with 20 mg of laccase or the corresponding amount of free laccase was added to 50 mL of the wastewater and mixed at 100 rpm in darkness for 24 h. The pH of the wastewater was 7.3 and the process was performed at ambient temperature (ca. 23 °C). Thereafter, the samples were collected for E2 and EE2 quantitative analyses using the GC–MS method described by Gunatilake et al. (2014) with modifications described by Machałowski et al. (2022) (E-Supplementary data). Based on these results, removal efficiency (%) was calculated as a difference in the initial and final concentrations of estrogens in the solution (E-supplemenetary materials). The reusability study was performed according to the above-presented methodology over 5 repeated cycles. After each cycle, the scaffold was slightly washed with acetate buffer and placed into a new wastewater solution.

3. Results and discussion

3.1. Characterization of PLA scaffolds

Results from this study show that desirable carrier structures can be accurately obtained by 3D printing. Cylindrical-shaped carriers were obtained with diameter and height of 4 mm and 7 mm, respectively, providing well-developed surface area (determined based on the data provided with the model design using Fusion 360 CAD software) of about 5.42 cm^2. A microscopic image of the carrier shows a polymer surface that is clear, solid, and free of air bubbles.

Three additional analyses – namely, thermal stability, electrokinetic properties, and mechanical studies – were conducted to provide information on the stability and endurance of the carrier. The degradation temperature of PLA is about 385 ℃ with 100% mass loss at around 700 ℃ due to the destruction of the PLA structure. On the other hand, it must be kept in mind, that the glass temperature of PLA is high (60–80 ℃) (Iannace et al., 2014), similar to the temperature of enzyme degradation. It means, that the low glass transition temperature of PLA does not affect the action of the enzyme and the carrier retains its shape. The isoelectric point of the carrier is at pH 3 and drops to more than −40 mV with the increasing alkaline nature of the solution.

The use of PLA for scaffold production provides the advantage to incorporate appropriate functional groups for enzyme immobilization. Among them, especially the carbonyl group, which signal at FTIR spectrum is presented at 1750 cm^{-1} (C = O stretching) is desirable for enzyme support materials as it allows the formation of PLA-laccase interactions (Zdarta et al., 2018). Thus it seems that PLA is an exceedingly promising scaffold material for laccase immobilization.

The obtained carrier had good mechanical strength, with a maximum strength of 10.7 MPa and nominal deformation at destruction equals 0.87 mm. Mechanical property decreased only slightly after

immobilization when maximum strength was 9.8 MPa and nominal deformation at the destruction of 0.70 mm. It can be explained by the partial hydration of the carrier to some extent. Nevertheless, the analysis presented prove, the suitable properties of the carrier for enzyme immobilization. All of these results are presented in E-supplementary data.

3.2. Immobilization optimization

Fig. 1 presents the distribution of process responses according to the quadratic model fitted to experimental data. Activity retention decreased with increasing pH whereas, longer immobilization time along with lower enzyme concentration resulted in an increase in activity retention (Fig. 1a). Similar relationships can be observed for

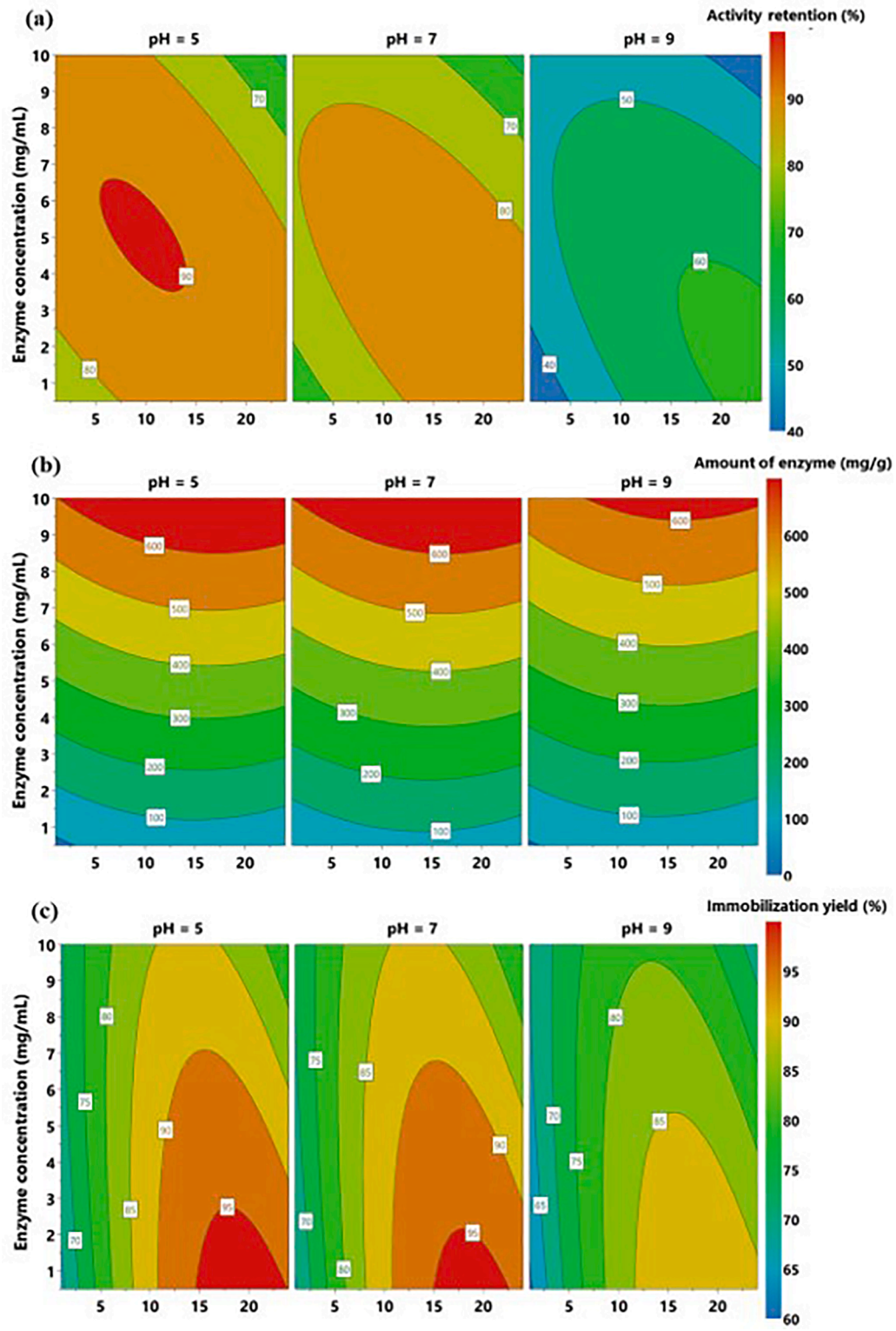

Fig. 1. Responses of the tested systems according to the adopted MLR model for enzyme activity retention (a), amount of immobilized enzyme (b) and immobilization yield (c).

immobilization yield. However, the impact of pH changes on immobilization yield is less discernible compared to activity retention (Fig. 1c).

When considering how individual process variables affected the mass of immobilized enzyme, we can see significant differences from the parameters described earlier. The effects of both pH and immobilization time were virtually negligible. The only variable noticeably affecting the amount of immobilized enzyme was the concentration of enzyme in the solution used for immobilization (Fig. 1b).

Fig. 2 shows in further detail how individual process parameters affected the measured quantities. Assuming a confidence level of 0.05, one can confirm the observations made from Fig. 1. However, it is noted that in the case of immobilization yield, only the effect of process time is statistically significant. On the other hand, for the amount of immobilized enzyme, increasing the immobilization time is beneficial.

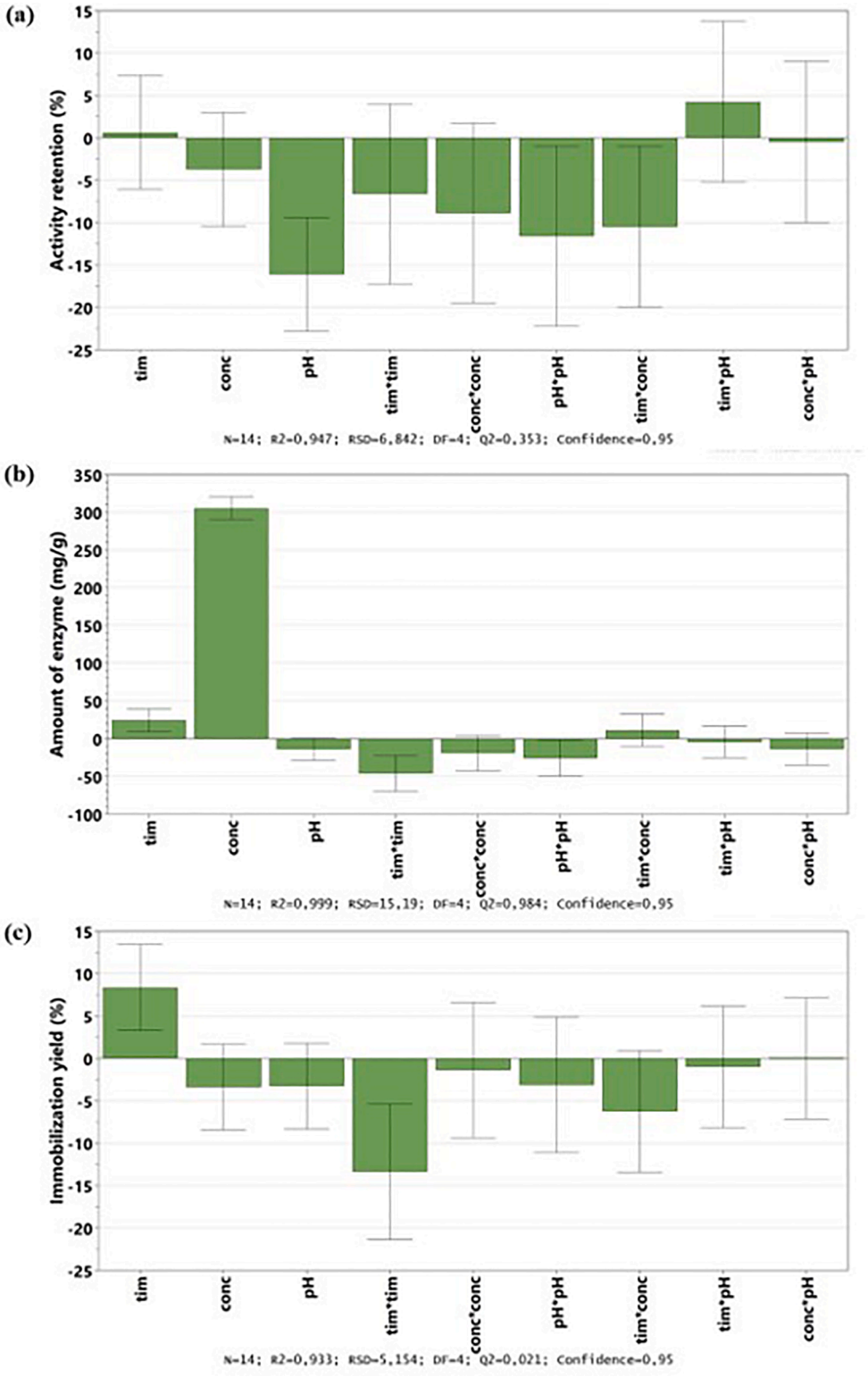

Fig. 2. Impact of particular optimization factors on the systems responses, the error bars show the standard deviation of the factor.

Confirmation of the degree of model fit is provided by the plot of the relationship of measured and predicted quantities (Fig. 2). The adopted model best describes the relationship of the amount of immobilized enzyme ($R^2 = 1.0$). The correlation coefficient for the value of activity retention was slightly lower ($R^2 = 0.95$). The weakest model describes the relationship between the variables and immobilization yield ($R^2 = 0.93$). The accuracy of the fit that was obtained in the adopted model is comparable to those reported in the literature. For example, Nair et al. (2013) obtained correlation coefficients ranging from 0.93 (for thermostability, as a measured parameter) to 0.99 (for immobilization yield) when analyzing the immobilization of laccase on a silica substrate. Maximum values of immobilization yield and activity retention were adopted as criteria for selecting optimal immobilization conditions. Based on the maximum values of immobilization yield and activity retention optimal conditions for enzyme immobilization on 3D PLA scaffolds were chosen which were pH 5, enzyme concentration 5 mg/mL and process duration 24 h. From the optimization data, these conditions should result in activity retention of 80.4%, immobilization yield 87.1% and amount of the immobilized enzyme equal to 349.2 mg/mL.

3.3. Characteristics of biocatalytic systems

Based on immobilization optimization study it was determined that initial enzyme solution at pH 5 and 5 mg/mL concentration and 24 h of immobilization duration are optimal process parameters. At these conditions, 94.6% immobilization yield was achieved resulting in almost 200 mg of laccase immobilized per 1 g of PLA scaffolds and enzyme loading of 3.65 mg/cm^2 (Table 1). Optimization varies from the results obtained in real experiments performed at optimal conditions. That is acceptable as process optimization did not consider all dependencies occurring during the real processes (Bolivar et al., 2022). Nevertheless, real scenario results are even more promising, as less enzyme was deposited than predicted by the model, that resulted in higher activity recovery of the produced system (89.2%), as compared to modeling results (80.4%).

Results from this study show higher immobilized laccase activity than previous studies using PLA substrates. Zhang et al. (2021) deposited less than 0.5 mg of lipase per 1 g of the PLA scaffold. The high amount of the enzyme (around 200 mg/g) and high activity recovery (89.2%) in this study is directly related to the structure and morphology of the scaffold. Enzyme immobilization onto the surface of the scaffold supported by their open three-dimensional structure improves the easy flow of the reagents and direct access to the enzyme active sites. Moreover, the high surface-to-volume ratio and large surface area of the scaffold (5.42 cm^2) facilitates uniform distribution of the biomolecules that diminish enzyme aggregation and local overloading (Iark et al., 2019). These reduce diffusional limitations and avoid steric blockage of the enzyme active sites.

Effective enzyme immobilization in this study is supported by the kinetic data of the free and immobilized laccase (Table 1). Michaelis constant (K_m) of immobilized laccase was 15% higher, whereas maximum reaction rate (V_{max}) was 20% less in comparison to free laccase. These changes in the Michaelis constant and maximum reaction rate are small, which confirms that substrate flow and direct enzyme-substrate contact were only slightly affected upon immobilization (Lim et al., 2020; Gkantzou et al., 2022).

Table 1
Characterization of the immobilization process as well as biocatalytic characteristic of the free and immobilized laccase in terms of activity recovery and kinetic parameters. Results are presented as mean ± standard deviation from three replicate experiments.

Analyzed parameter	Biocatalyst	
	Free laccase	Immobilized laccase
Enzyme activity recovery	100%	89.2 ± 2.14%
Immobilization yield	–	94.6 ± 1.78%
Amount of immobilized enzyme	–	198 ± 4.95 mg/g
Enzyme loading	–	3.65 ± 0.09 mg/cm^2
Enzyme leaching	–	26 ± 0.78%
Michaelis constant (K_m)	0.59 ± 0.02 mM	0.66 ± 0.01 mM
Maximum reaction rate (V_{max})	0.089 ± 0.003 mM/s	0.071 ± 0.001 mM/s

In the characterization of an immobilized system, it is also important to determine changes in its activity over varying process conditions. The free and immobilized enzyme showed similar pH and temperature profiles with maximum activity at pH 5 and 30 °C (Fig. 3). This fact additionally confirms that performed immobilization provided no significant changes in enzyme structure. However, immobilized laccase presented significantly higher tolerance towards pH and temperature conditions than free enzyme. Over 60% activity was shown at pH from 3 to 7 and at temperatures from 20 to 50 °C. Further, even at pH 9 and 60 °C produced system showed around 30% activity, whereas catalytic properties decreased rapidly over varying process conditions. The improvement of catalytic properties under harsh conditions is related to the protective effect of the used PLA scaffolds and the stabilization of enzyme structure as well as its rigidization. Although presented immobilization is based on relatively weak adsorption interactions, mainly hydrogen bonds and electrostatic interactions, these forces are strong enough to tightly bound the enzyme and retain its structure, as reported also earlier (Jia et al., 2019). Moreover, in the structure of 3D PLA scaffolds, there are numerous carbonyl functional groups that are relatively close to each other. That might facilitate multiple enzyme binding, which provides additional enzyme stabilization. These facts make that the immobilized enzyme is less prone to inactivation triggered by process parameters (Rodrigues et al., 2021).

Improvement of pH and temperature profiles was reported earlier for instance for laccase immobilized using covalent attachment on commercial epoxy resin (Othman et al., 2023) as well as laccase absorbed and encapsulated in poly(l-lactic acid)–co-poly(ε-caprolactone) electrospun fibers (Zdarta et al., 2019a). In both cases, the relative activity of the immobilized enzyme was higher over the whole analyzed pH and temperature range. Although laccase stabilization upon immobilization is clear, the observed decrease in activity is affected by enzyme fragmentation or rearrangements of enzyme active sites at harsh conditions.

Thermal stability tests of free and immobilized laccase were performed to determine how exposure to elevated temperature (50 °C) affects its activity (Fig. 3c). After 3 h incubation, laccase immobilized on PLA scaffolds showed around 80% of its initial activity, whereas sharp drop activity of free laccase was observed. Moreover, after the corresponding incubation time activity retention reached around 20%. The significant improvement in the thermal stability of laccase after immobilization is related to the fact that PLA scaffold, which is thermally stable at 50 °C, might act as a heat absorber and protect laccase molecules against thermal fragmentation and heat inactivation (Ariaeenejad et al., 2022). Moreover, the shielding effect of the scaffold reduces aminoacids rearrangements in the biomolecule affected by long-term exposure to high temperatures. This is reflected by the inactivation constant value (k_D) and enzyme half-life ($t_{1/2}$) (E-Supplementary data). For free enzyme, these parameters were 0.0102 1/min and 68.2 min, respectively. For immobilized laccase, these parameters were improved by ten times, reaching values of 0.0009 1/min and 773.3 min, respectively, and clearly indicating improvement of laccase stability at elevated temperatures. In addition, laccase long-term stability (Fig. 3d) was significantly improved upon immobilization. After 20 days of storage immobilized laccase retained around 80% of the initial activity due to stabilization provided by the PLA scaffold and enzyme structure reinforcement (Zhang et al., 2020). By contrast, the drop in the activity of the free laccase was observed since the first day leading to the retention of 35% activity at the end of the test. Similarly to our results, an improvement of the storage stability of laccase immobilized via adsorption onto bimodal mesoporous Zr-MOF support by around 50%

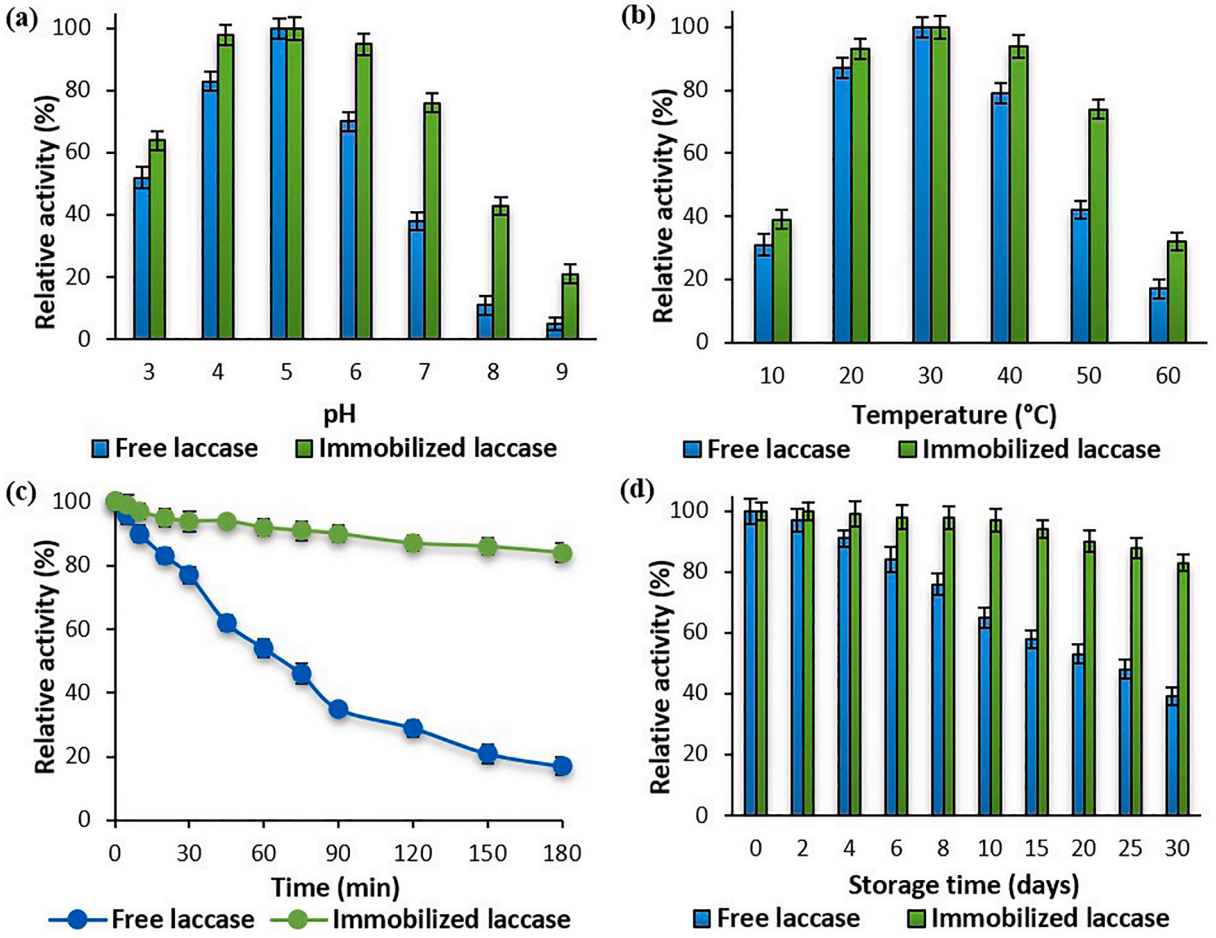

Fig. 3. Effect of pH (a) and temperature (b) on relative activity of the free laccase and laccase immobilized onto 3D polylactide scaffolds; thermal stability (c) and storage stability (d) of the free laccase and laccase immobilized onto 3D polylactide scaffolds. Results are mean ± standard deviation from three replicate experiments. The zero value indicate the factors of no impact on immobilization efficiency.

(as compared to the free enzyme) was also observed by Pang et al. (2016).

3.4. Estrogens removal

The produced 3D PLA scaffolds with immobilized laccase were evaluated for removing E2 and EE2 from wastewater and over repeated use to examine the reusability of the produced system. The wastewater in this study contained initially 356 ng/L of EE2 and 187 ng/L of E2. Their removal efficiency by laccase immobilized on 3D printed scaffolds was in the range of 35–40%, and was 10% higher than that by free laccase (Fig. 4a and Fig. 4b). This result shows that immobilized enzyme is less susceptible to inhibition and inactivation by the compounds present in the wastewater as compared to free counterpart (Feng et al., 2021).

Although the stability of laccase was improved upon immobilization and the PLA scaffolds provided the efficient flow of the solution and partially protect the enzyme against inhibition, removal efficiency achieved using real wastewater was significantly lower than in model solutions presented in the literature. From model solutions of micropollutants, over 90% removal rate of estrogens was reported using an enzymatic membrane reactor supported by the addition of commercial laccase and syringaldehyde (Nguyen et al., 2016a) as well as supported by the crude laccase (Nguyen et al., 2020). Also in laccase-based enzymatic bioreactor conversion supported by membrane distillation, 100% removal of E2 and EE2 was observed (Asif et al., 2017; Asif et al., 2018). Although available data showed a high removal rate of estrogens, these studies were performed on a batch scale or using small-scale reactors from model solutions of micropollutants. Moreover, their reaction time was significantly longer than in this study.

The lower removal rate of E2 and EE2 in this study indicates that the use of biocatalytic systems in real solutions encounters a series of limitations including the negative effect of wastewater matrix constituents, such as heavy metal ions, lipids, proteins, other organic and inorganic compounds as well as other insoluble compounds (Al-Maqdi et al., 2021). These factors negatively affect laccase activity leading to a lower removal rate. These stays in agreement with our recent findings, which showed that in the presence of a mixture of metal ions, salts, and surfactants, two times lower removal efficiency of E2 and EE2 was observed, as compared to model water solution (Zdarta et al., 2022b). Low concentration of targeted estrogens in the wastewater, the presence of interference, and the fact that enzyme might also interact with other organic compounds then estrogens also negatively affect the final removal rate. On the other hand, PLA scaffolds due to their sorption properties might act also as a sorbent for the wastewater matrix compounds (Zdarta et al., 2019b). This improves total removal efficiency due to sorption of micropollutants to the scaffolds. However, estrogen removal by adsorption in this study did not exceed 5%.

Obtained results on estrogens removal are promising. Nevertheless, to further evaluate the efficiency and economic viability of the system for micropollutant treatment, it is important to determine laccase reusability. The reusability study (Fig. 4c and Fig. 4d) shows that removal efficiency slightly (by around 5% per each cycle), but continuously decreased over multiple uses. A more pronounced drop was

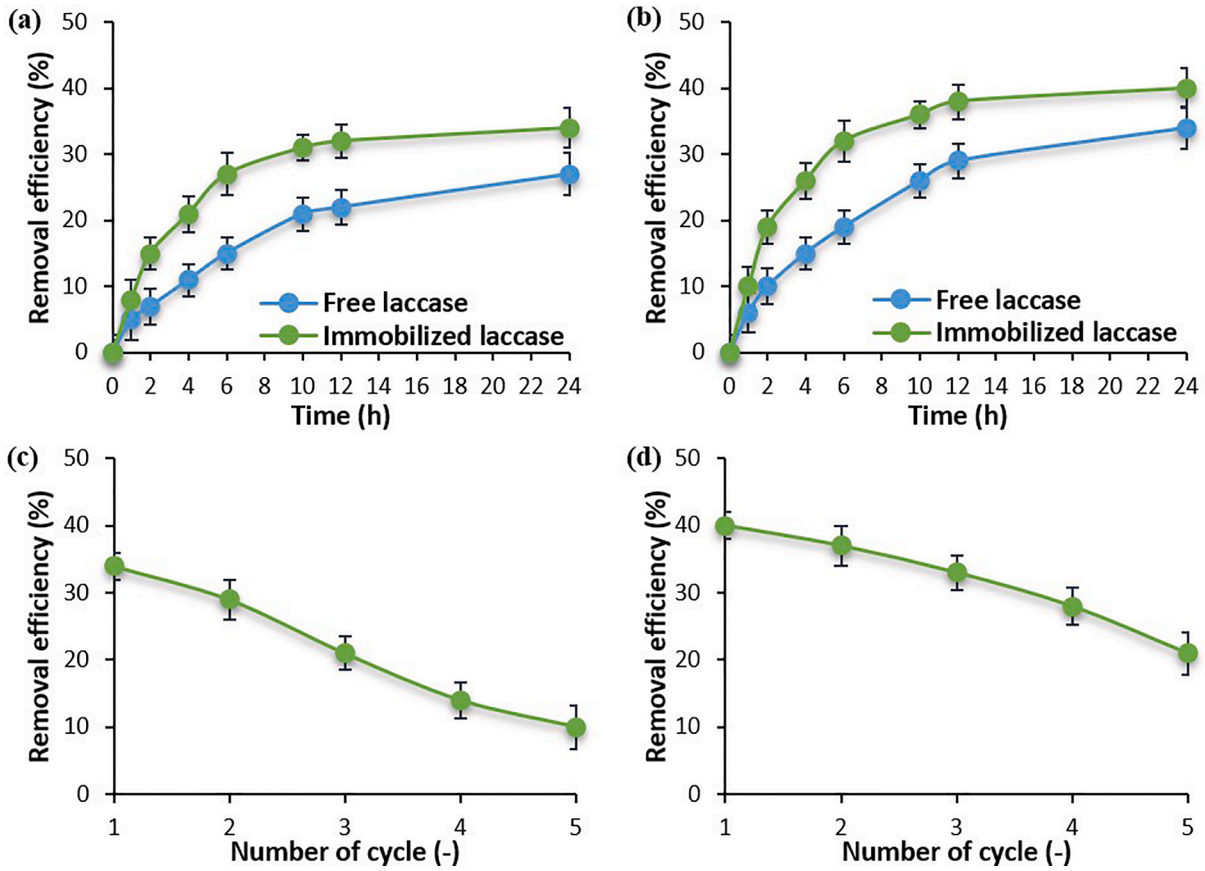

Fig. 4. Removal efficiency of 17β-estradiol (a) and 17α-ethynylestradiol (b) as well as reusability of the produced biocatalytic systems in removal of 17β-estradiol (c) and 17α-ethynylestradiol (d). Results are presented as mean ± standard deviation from three replicate experiments.

observed for E2 indicating that immobilized laccase has a higher affinity towards EE2 and its removal is less affected by repeated use. Moreover, based on visual observations and digital microscope images, no changes in the structure of the support after repeated use were observed indicating that 3D scaffolds are not degraded.

Once, the stability of the laccase has been improved during immobilization, its partial leakage as well as inhibition by both, wastewater ingredients and products of enzymatic conversion lead to a drop in removal efficiency over repeated use. Similar observations were also drawn by Nguyen et al. (2016b) who observed a drop in removal efficiency of selected micropollutants over repeated use, mainly due to enzyme inactivation, when packed-bed enzyme reactor with laccase immobilized onto activated carbon was applied for real wastewater treatment. Results from Fig. 4 suggest that produced systems are reusable and might be multiple use in the removal of both estrogens from real solutions. The proposed approach of enzyme immobilization is more cost-effective than free enzymes, as it was impossible to reuse native enzymes. Moreover, produced 3D scaffolds with immobilized enzyme are much more beneficial than free enzyme in terms of their recovery method, as might be easily separated from the post-reaction mixture by simple physical recovery, without a need for the use of advanced separation techniques, as in case of the native enzyme. In addition, process control when using 3D immobilized enzyme is also improved which additionally increases the usability of the proposed system. Finally, based on the previously reported data, partial mineralization of both estrogens should be considered as the main mechanism driving the removal process (Zdarta et al., 2022b).

Although the results presented in this study should be considered promising, further experiments are required, in which packed-bed or continuous reactor will be used. This will facilitate the transfer of the presented solution to a larger scale and will provide more information and economic feasibility of the proposed solution based on PLA scaffolds with immobilized laccase for the removal of various micropollutants from wastewater.

4. Conclusions

A novel technique of 3D printing of PLA scaffolding for laccase immobilization was presented. Optimal parameters including pH, enzyme concentration, and immobilization time were experimentally determined to achieve high activity recovery and significant improvement in chemical and thermal stability. The biocatalytic system proposed removed around 40% of E2, and EE2 from real municipal wastewater showing that the composition of the wastewater matrix strongly affects the removal rate. Further research is recommended to reduce the negative effect of inhibitory compounds on enzyme activity and improve laccase reusability. Of crucial importance is, however, the scaling up of this proposed approach for practical applications.

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CRediT authorship contribution statement

Agnieszka Rybarczyk: Conceptualization, Data curation, Formal analysis, Methodology, Investigation, Writing – original draft. **Wojciech Smułek:** Conceptualization, Methodology, Validation, Writing – original draft. **Adam Grzywaczyk:** Software, Investigation, Visualization, Writing – original draft. **Ewa Kaczorek:** Formal analysis, Writing – review & editing. **Teofil Jesionowski:** Supervision, Resources, Writing –

review & editing. **Long D. Nghiem:** Supervision, Writing – review & editing. **Jakub Zdarta:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.129144.

References

Al-Maqdi, K.A., Elmerhi, N., Athamneh, K., Bilal, M., Alzamly, A., Ashraf, S.S., Shah, I., 2021. Challenges and Recent Advances in Enzyme-Mediated Wastewater Remediation—A Review. J. Nanomater. 11 (11), 3124.

Ariaeenejad, S., Motamedi, E., Salekdeh, G.H., 2022. Highly efficient removal of dyes from wastewater using nanocellulose from quinoa husk as a carrier for immobilization of laccase. Bioresour. Technol. 349, 126833.

Asif, M.B., Hai, F.I., Kang, J., Merwe, J.P., Leusch, F.D.L., Yamamoto, K., Price, W.E., Nghiem, L.D., 2017. Degradation of Trace Organic Contaminants by a Membrane Distillation—Enzymatic Bioreactor. Appl. Sci. 7, 87.

Asif, M.B., Hai, F.I., Kang, J., Merwe, J.P., Leusch, F.D.L., Price, W.E., Nghiem, L.D., 2018. Biocatalytic degradation of pharmaceuticals, personal care products, industrial chemicals, steroid hormones and pesticides in a membrane distillation-enzymatic bioreactor. Bioresour. Technol. 247, 528–536.

Ayinla, Z.A., Ademakinwa, A.N., Agboola, F.K., 2022. Comparative modelling, molecular docking and immobilization studies on Rhizopus oryzae lipase: evaluation of potentials for fatty acid methyl esters synthesis. J. Biomol. Struct. Dyn. 2119279.

Blanco, I., 2020. The Use of Composite Materials in 3D Printing. J. Compos. Sci. 4 (2), 42.

Bolivar, J.M., Woodley, J.M., Fernandez-Lafuente, R., 2022. Is enzyme immobilization a mature discipline? Some critical considerations to capitalize on the benefits of immobilization. Chem. Soc. Rev. 51 (15), 6251–6290.

Boudrant, J., Woodley, J.M., Fernandez-Lafuente, R., 2020. Parameters necessary to define an immobilized enzyme preparation. Process Biochem. 90, 66–80.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1-2), 248–254.

Dong, C.-D., Tiwari, A., Anisha, G.S., Chen, C.-W., Singh, A., Haldar, D., Patel, A.K., Singhania, R.R., 2023. Laccase: A potential biocatalyst for pollutant degradation. Environ. Pollut. 319, 120999.

Feng, S., Hao Ngo, H., Guo, W., Woong Chang, S., Duc Nguyen, D., Cheng, D., Varjani, S., Lei, Z., Liu, Y.i., 2021. Roles and applications of enzymes for resistant pollutants removal in wastewater treatment. Bioresour. Technol. 335, 125278.

Gkantzou, E., Skonta, A., Tsakni, A., Polydera, A., Moschovas, D., Spyrou, K., Avgeropoulos, A., Gournis, D., Houhoula, D., Stamatis, H., 2022. 3D printed PLA enzyme microreactors: Characterization and application for the modification of bioactive compounds. J. Biotechnol. 350, 75–85.

Gonçalves, M.C.P., Kieckbusch, T.G., Perna, R.F., Fujimoto, J.T., Morales, S.A.V., Romanelli, J.P., 2019. Trends on enzyme immobilization researches based on bibliometric analysis. Process Biochem. 76, 95–110.

Gonsioroski, A., Mourikes, V.E., Flaws, J.A., 2020. Endocrine Disruptors in Water and Their Effects on the Reproductive System. Int. J. Mol. Sci. 21 (6), 1929.

Gunatilake, S.R., Clark, T.L., Rodriguez, J.M., Mlsna, T.E., 2014. Determination of five estrogens in wastewater using a comprehensive two-dimensional gas chromatograph. Anal. Methods 6 (15), 5652–5658.

Iannace, S., Sorrentino, L., Di Maio, E., 2014. Biomedical Foams for Tissue Engineering Applications. Elsevier LTD, Oxford.

Iark, D., Buzzo, A.J.D.R., Garcia, J.A.A., Côrrea, V.G., Helm, C.V., Corrêa, R.C.G., Peralta, R.A., Peralta Muniz Moreira, R.d.F., Bracht, A., Peralta, R.M., 2019. Enzymatic degradation and detoxification of azo dye Congo red by a new laccase from *Oudemansiella canarii*. Bioresour. Technol. 289, 121655.

Jia, Y., Chen, Y., Luo, J., Hu, Y., 2019. Immobilization of laccase onto meso-MIL-53(Al) via physical adsorption for the catalytic conversion of triclosan. Ecotoxicol. 184, 109670.

Koh, Y.K.K., Chiu, T.Y., Boobis, A., Cartmell, E., Scrimshaw, M.D., Lester, J.N., 2007. Treatment and removal strategies for estrogens from wastewater. Environ. Technol. 29 (3), 245–267.

Li, X., Su, Z., Luo, Y., Chen, X., Luo, J., Pinelo, M., 2022. Modelling of oligodextran production via an immobilized enzyme membrane reactor: Bioreaction-separation coupling mechanism. Sep. Purif. Technol. 282, 120024.

Lim, Y., Yu, J., Park, S., Kim, M., Chen, S., Bakri, N.A.B., Sabri, N.I.A.B.M., Bae, S., Kim, H.S., 2020. Development of biocatalysts immobilized on coal ash-derived Ni-zeolite for facilitating 4-chlorophenol degradation. Bioresour. Technol. 307, 123201.

Machałowski, T., Jankowska, K., Bachosz, K., Smułek, W., Ehrlich, H., Kaczkorek, E., Zdarta, J., Jesionowski, T., 2022. Biocatalytic System Made of 3D Chitin, Silica Nanopowder and Horseradish Peroxidase for the Removal of 17α-Ethinylestradiol: Determination of Process Efficiency and Degradation Mechanism. Molecules 27 (4), 1354.

Madhavan, A., Sindhu, R., Binod, P., Sukumaran, R.K., Pandey, A., 2017. Strategies for design of improved biocatalysts for industrial applications. Bioresour. Technol. 245, 1304–1313.

Nair, R.R., Demarche, P., Agathos, S.N., 2013. Formulation and characterization of an immobilized laccase biocatalyst and its application to eliminate organic micropollutants in wastewater. N. Biotechnol. 30 (6), 814–823.

Nguyen, L.N., Hai, F.I., Dosseto, A., Richardson, C., Price, W.E., Nghiem, L.D., 2016a. Continuous adsorption and biotransformation of micropollutants by granular activated carbon-bound laccase in a packed-bed enzyme reactor. Bioresour. Technol. 210, 108–116.

Nguyen, L.N., Merwe, J.P., Hai, F.I., Leusch, F.D.L., Kang, J., Price, W.E., Roddick, F., Magram, S.F., Nghiem, L.D., 2016b. Laccase–syringaldehyde-mediated degradation of trace organic contaminants in an enzymatic membrane reactor: Removal efficiency and effluent toxicity. Bioresour. Technol. 200, 477–484.

Nguyen, L.N., Vu, M.T., Johir, M.A.H., Pathak, N., Zdarta, J., Jesionowski, T., Semblante, G.U., Hai, F.I., Nguyen, H.K.D., Nghiem, L.D., 2020. A Novel Approach in Crude Enzyme Laccase Production and Application in Emerging Contaminant Bioremediation. Processes 8 (6), 648.

Othman, A.M., Sanromán, Á., Moldes, D., 2023. Laccase multi-point covalent immobilization: characterization, kinetics, and its hydrophobicity applications. Appl. Microbiol. Biotechnol. 107 (2-3), 719–733.

Pang, S., Wu, Y., Zhang, X., Li, B., Ouyang, J., Ding, M., 2016. Immobilization of laccase via adsorption onto bimodal mesoporous Zr-MOF. Process Biochem. 51 (2), 229–239.

Rodrigues, R.C., Berenguer-Murcia, Á., Carballares, D., Morellon-Sterling, R., Fernandez-Lafuente, R., 2021. Stabilization of enzymes via immobilization: Multipoint covalent attachment and other stabilization strategies. Biotechnol. Adv. 52, 107821.

Senćanski, M., Prodanović, R., Ristić, P., Balaž, A.M., Stanišić, M., Todorović, T., 2021. Modelling of catalytic activity and enzyme-MOF interactions using combined in silico approach. Mater. Res. Soc., 124.

Shao, Y., Liao, Z., Gao, B., He, B., 2022. Emerging 3D Printing Strategies for Enzyme Immobilization: Materials, Methods, and Applications. ACS Omega 7 (14), 11530–11543.

Singh, R., Souillard, G., Chassat, L., Gao, Y., Mulet, X., Doherty, C.M., 2020. Fabricating Bioactive 3D Metal-Organic Framework Devices. Adv. Sustain. Syst. 4 (12), 2000059.

Vu, H.P., Nguyen, L.N., Vu, M.T., Johir, M.A.H., McLaughlan, R., Nghiem, L.D., 2020. A comprehensive review on the framework to valorise lignocellulosic biomass as biorefinery feedstocks. Sci. Total. Environ. 743, 140630.

Wu, D., Lv, P., Feng, Q., Jiang, Y.u., Yang, H., Alfred, M., Wei, Q., 2022. Biomass-derived nanocellulose aerogel enable highly efficient immobilization of laccase for the degradation of organic pollutants. Bioresour. Technol. 356, 127311.

Yazdan, M.S., Kumar, R., Leung, S.W., 2022. The Environmental and Health Impacts of Steroids and Hormones in Wastewater Effluent, as Well as Existing Removal Technologies: A Review. Ecologies 3 (2), 206–224.

Ye, J., Chu, T., Chu, J., Gao, B., He, B., 2019. A Versatile Approach for Enzyme Immobilization Using Chemically Modified 3D-Printed Scaffolds. ACS Sustain. Chem. Eng. 7 (21), 18048–18054.

Zdarta, J., Meyer, A.S., Jesionowski, T., Pinelo, M., 2018. A General Overview of Support Materials for Enzyme Immobilization: Characteristics, Properties, Practical Utility. Catalyst 8, 92.

Zdarta, J., Jankowska, K., Wyszowska, M., Kijeńska-Gawrońska, E., Zgoła-Grześkowiak, A., Pinelo, M., Meyer, A.S., Moszyński, D., Jesionowski, T., 2019a. Robust biodegradation of naproxen and diclofenac by laccase immobilized using electrospun nanofibers with enhanced stability and reusability. Mater. Sci. Eng. C 103, 109789.

Zdarta, J., Meyer, A.S., Jesionowski, T., Pinelo, M., 2019b. Multi-faceted strategy based on enzyme immobilization with reactant adsorption and membrane technology for biocatalytic removal of pollutants: A critical review. Biotechnol. Adv. 37 (7), 107401.

Zdarta, J., Jankowska, K., Strybel, U., Marczak, Ł., Nguyen, L.N., Oleskowicz-Popiel, P., Jesionowski, T., 2022a. Bioremoval of estrogens by laccase immobilized onto polyacrylonitrile/polyethersulfone material: Effect of inhibitors and mediators, process characterization and catalytic pathways determination. J. Hazard. Mater. 432, 128688.

Zdarta, J., Jesionowski, T., Pinelo, M., Meyer, A.S., Iqbal, H.M.N., Bilal, M., Nguyen, L. N., Nghiem, L.D., 2022b. Free and immobilized biocatalysts for removing micropollutants from water and wastewater: Recent progress and challenges. Bioresour. Technol. 344, 126201.

Zhang, J., Gao, B., Lv, K., Kumissay, L., Wu, B., Chu, J., He, B., 2021. Specific immobilization of lipase on functionalized 3D printing scaffolds via enhanced hydrophobic interaction for efficient resolution of racemic 1-indanol. Biochem. Biophys. Res. Commun. 546, 111–117.

Zhang, C., You, S., Zhang, J., Qi, W., Su, R., He, Z., 2020. An effective in-situ method for laccase immobilization: Excellent activity, effective antibiotic removal rate and low potential ecological risk for degradation products. Bioresour. Technol. 308, 123271.