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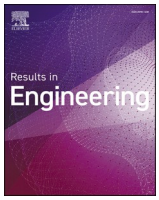

Natural mediators for indigo carmine dye removal with immobilized laccase in polyacrylic films

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ABSTRACT

This work presents a new polymeric material in the form of a film (F_{LAC}) containing immobilized laccase enzyme through diazo bonds, designed for the degradation of indigo carmine dye, which is highly toxic and commonly found in wastewater from the textile industry. The immobilization of the enzyme in the film was characterized by SEM, FT-IR, DSC, TGA and EDXS. The degradation of the dye by F_{LAC} initiates in the presence of a mediator due to the high redox potential of the dye. Six natural mediators (ferulic acid, syringaldazine, guaiacol, eugenol, thymol, and *p*-coumaric acid) were tested, and complete degradation of the dye was achieved in 180 min, with a mediator concentration of 1 ppm (syringaldazine) and a dye concentration of 10 ppm. A novelty in this study is the short exposure time of the dye-mediator solution to F_{LAC} (15 min) which allowed the degradation process to continue autonomously after the film was removed. Additionally, it was observed that the material was more effective in the presence of textile washing products, achieving over 99 % degradation in 40 min, surpassing its efficacy in distilled water. Regarding reusability, the material retained >90 % of its activity after five cycles of use and washing. Life Cycle Assessment (LCA) and Techno-Economic Analysis (TEA) identified key levers for techno-economic and environmental viability: shorten cycle time (surfactant media), maximise re-use and per-cycle volume/concentration, implement solvent recovery, and energy decarbonization thereby outlining a roadmap to sustainable scale-up.

1. Introduction

Classified as multicopper oxidases, laccases (EC 1.10.3.2) are broadly distributed among fungi, bacteria, plants, and certain insects. Their catalytic function involves oxidizing a wide spectrum of phenolic and non-phenolic substrates while reducing molecular oxygen to water. The catalytic mechanism relies on four copper atoms distributed across three binding sites: the type 1 copper site (T1), serving as the electron acceptor from the substrate, and a trinuclear cluster containing one type 2 (T2) and two type 3 (T3) coppers, which mediate oxygen reduction. Due to their high redox potential and broad substrate specificity, laccases play a crucial role in lignin degradation, stress response, and secondary metabolism. Their versatility has made them valuable tools in various biotechnological applications, including bioremediation, pulp and paper bleaching, wastewater treatment, biosensor development, and green chemistry [1].

The treatment of wastewater using laccase enzymes has been a subject of significant interest over the past decades [2]. This enzyme has been employed for the remediation of wastewater contaminated with antibiotics and pharmaceutical compounds [3], endocrine-disrupting phenolic compounds [4], and synthetic dyes [5]. In this study, we specifically focus on its application in the degradation of dyes present in wastewater, with particular emphasis on indigo carmine (Acid Blue 74), a widely used dye in the textile industry, cosmetics, the food industry, and biomedicine [6]. This dye is released into the environment through wastewater effluents, where it negatively impacts photosynthetic

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activity and exhibits toxicity toward aquatic organisms [7]. Furthermore, its complex aromatic structure and high molecular stability pose challenges for biodegradation [8].

Different methods have been used for the treatment of dyes like membrane technology, filtration [9], adsorption [10–12], or ion exchange [13], but most of them have shown some limitations like the high consumption of chemicals and energy, high costs of operation and maintenance and a high complexity [14]. In previous publications from our group, we have based the treatment of water contaminated with these dyes on extraction procedures of this nature [12]. However, as the redundancy suggests, these methods are limited to extracting the dye from the aqueous medium in which it is present; they do not eliminate it, merely remove it. If the goal is the degradation of these compounds or a combination of extraction and degradation, enzymatic treatment with enzymes such as laccases should be employed.

The biodegradation of these dyes through the laccase enzyme emerges as an economically and technically viable alternative that can be integrated with other technologies. The enzymatic process can occur in one or two stages. For instance, if the target species to be degraded (such as a dye) has a low redox potential and is therefore suitable for the enzyme, the enzyme can react directly with the dye and degrade it. In certain situations, laccases cannot directly oxidize substrates either because their redox potential is too high or because they are not accessible to the enzyme's active site [15], requiring the presence of a mediator [16].

Laccase mediators are low-molecular-weight compounds that undergo rapid oxidation by the enzyme and subsequently promote substrate degradation through indirect pathways. A wide range of mediators can be employed, including synthetic ones (e.g., ABTS, TEMPO, HBT) and natural ones (e.g., syringaldehyde, guaiacol, vanillin). Natural mediators, often phenolic compounds, are particularly attractive due to their low cost and non-toxic nature, leading to increasing interest in their application in recent years [8,17].

In addition to the use of mediators, another critical aspect to consider is the application of enzymes in their free form, which presents several limitations. These limitations include high costs in large-scale applications due to activity loss resulting from structural distortion under operating conditions, and difficult recovery and reuse [18]. A widely adopted strategy to overcome these challenges involves the immobilization of enzymes onto solid supports, such as silica [19], hydrogels [20], carbon nanotubes [21], iron oxide particles [22] or acrylic polymers [23,24]. Among the various types of supports used for laccase immobilization, synthetic polymers offer several advantages, including mechanical robustness, ease of handling, reusability, resistance to microbial degradation, biocompatibility, hydrophilicity, cost-effectiveness, and tuneable molecular structure and morphology [24]. Physical, chemical and combined methods have been investigated and developed using different supports. In contrast to physical approaches, chemical immobilization achieves enhanced stability as a result of the more robust bonds formed between the enzyme and the support [25].

In this context, we propose covalent immobilizing a commercial laccase from the fungus *Myceliophthora thermophila* onto a polyacrylic film support. The enzyme is immobilized chemically through azo bond formation mediated by the aniline functional groups of the polymeric support. This approach enhanced the degradation of indigo carmine with the assistance of natural phenolic mediators. This polymeric film is highly manageable and has been previously utilized by our research group with other enzymes, such as β-galactosidase (β-gal) [24], demonstrating excellent potential for efficient and user-friendly applications. A major advantage of this material is that it can be easily employed by simply immersing it in the indigo carmine contaminated medium and removing it upon completion of the process, eliminating the need for filtration steps for its recovery. Indeed, we have conducted experiments in which the material containing the immobilized catalyst is immersed for only a few minutes and then removed from the media. We have also tested the material in the presence of textile washing products, which significantly contributes to the accessibility and scalability of enzymatic degradation of this dye.

The proposed enzymatic degradation alternative is more environmentally friendly than the dye extraction techniques previously mentioned, as it not only permanently removes the dye, but also degrades it, enabling the direct reuse of the polymeric material. This also prevents the generation of secondary waste, making it a sustainable and efficient solution.

2. Experimental

2.1. Materials, instrumentation and methods

Comprehensive information on the chemicals, solvents, reagents, instrumentation, and experimental procedures employed in this work can be found in the Supporting Information (SI-Section S1).

2.2. Design and synthesis of polymer material with immobilized laccase

The acrylic polymer designed for laccase immobilization has also been previously employed as a support for other enzymes [24]. The polymeric film developed demonstrates high resistance to mechanical stress, making it suitable for a broad range of applications without delicate handling. It consists of VP (hydrophilic structural monomer, 45 mol%), MMA (hydrophobic structural monomer, 45 mol%), and SNH2 (functionalizing monomer, 10 mol%, required for enzyme immobilization), with ethylene glycol dimethacrylate incorporated as a cross-linker at 0.1 mol% of the total material. Synthesis was carried out via bulk radical polymerization at 60 °C overnight, employing AIBN as the radical initiator.

Once FNH_2 was prepared, three sequential solid-phase reactions were performed to obtain the final laccase-immobilized material, as adapted from Vallejo-García et al. (2023) (Fig. 1) [24]: (i) formation of benzene diazonium salts at the -NH_2 pendant sites, producing FN_2^+; (ii) covalent immobilization of laccases onto FN_2^+ through diazo linkages, predominantly involving tyrosine and histidine residues [26], and (iii) blocking of residual N_2^+ groups with sodium azide, resulting in the FLAC film.

The main modification compared to the previously reported procedure concerns the reaction time in step (ii). Laccase immobilization via azo coupling was performed in 2.5 mL Eppendorf tubes containing one FN_2^+ disc and 0.75 mL of 10 U/mL enzyme solution, agitated on an orbital shaker at 25 °C for only 1 h, rather than the overnight reaction previously applied for β-gal. This modification was introduced with the aim of optimizing the material preparation time. Another notable modification was made in step (iii), corresponding to the blocking of excess diazonium benzene groups with azide. This step was also optimized by varying the incubation times (5, 10, 20, 40, and 60 min). It is important to note that the same enzymatic solution can be used for up to six discs with minimal loss of laccase activity.

2.3. Evaluation of immobilized laccase activity using a spectrophotometric method with ABTS

Immobilized laccase activity was determined via a spectrophotometric assay using ABTS. Each reaction contained a single F_{LAC} disc (6 mm diameter), 0.2 mL of 4 mM ABTS, and 0.1 M sodium acetate buffer (pH 4.0) in a total volume of 2.0 mL. The mixture was incubated at 30 °C on an orbital shaker at 130 rpm for 10 min. 0.1 mL of 50 % (w/v) trichloroacetic acid (TCA) was added to finish the reaction. The oxidation of ABTS was measured at 420 nm (ε=3.6 × 10^4 $M^{-1}cm^{-1}$) using a microplate reader (PowerWave XS2, BioTek) and 96-well plates. Laccase activity was reported as μg $ABTS^{\bullet+}/min \cdot cm^2$ for the immobilized enzyme. This assay was conducted to optimize the incubation times with azide, corresponding to step (iii) of the immobilization process

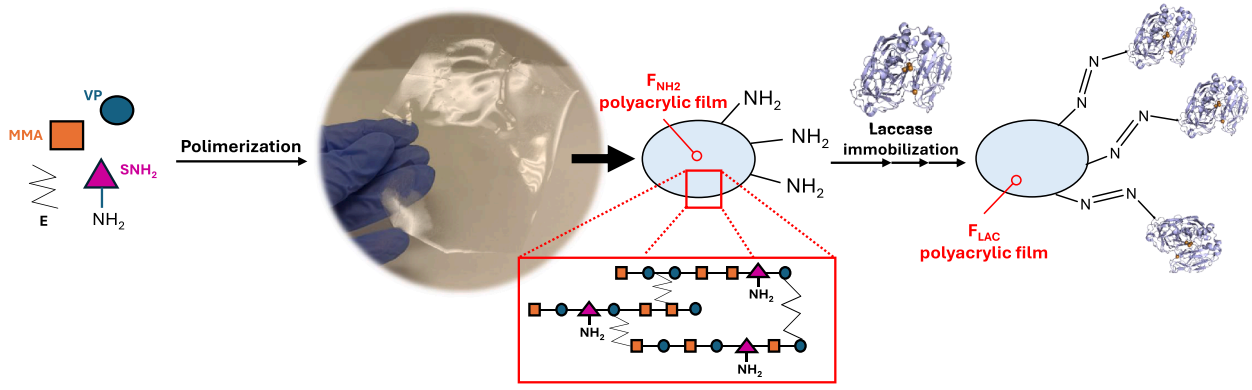


Fig. 1. Simplified diagram of the synthesis of the laccase polymeric film. First, the polymeric film (F_{NH2}) is synthesized through bulk radical polymerization of the monomers vinylpyrrolidone (VP, 45 mol%), methyl methacrylate (MMA, 45 mol%), 4-aminostyrene (SNH2, 10 mol%), and the cross-linking agent ethylene glycol dimethacrylate (E, 0.1 mol%). Then, laccase is immobilized through a series of intermediate steps, resulting in the final material (F_{LAC}).

described in the previous section. In addition, F_{LAC} was characterized as a function of pH, temperature, and substrate concentration (kinetic parameters) and compared with its free counterpart. These results were recently published by our research group [27].

2.4. Phenolic mediator screening for indigo carmine removal

The F_{LAC} polymeric material cannot directly degrade the indigo carmine dye and requires a mediator for the process to be successful. At the beginning of the study, several natural phenolic mediators (ferulic acid, *p*-coumaric acid, thymol, guaiacol, eugenol, and Syr) were tested for the degradation of indigo carmine. Initially, the mediators were dissolved in ethanol absolute to the desired concentration for solubility reasons. In UV–visible cuvettes, three F_{LAC} discs of 0.6 cm diameter were incubated in a final volume of 3 mL with 10 % ethanol, a final indigo carmine concentration of 10 ppm, and varying concentrations of the different mediators. The F_{LAC} discs were kept in the solution throughout the entire process. For all the mentioned mediators, a final concentration of 10 ppm was used, except for Syr, due to solubility issues; for this mediator, concentrations of 0.5, 1, and 2 ppm were used. Guaiacol and eugenol were also tested at 1 and 50 ppm.

The reaction was conducted under orbital shaking at 190 rpm and 30 °C and was monitored at different time intervals up to 300 min using UV/Vis spectrophotometer. To calculate dye degradation percentage, the following equation was employed:

$$\text{Indigo carmine degradation } (\%) = \frac{C_0 - C_F}{C_0} \times \ 100,$$

where C_0 and C_F, are the initial and final concentrations of the dye, calculated at A 610 nm.

For the mediator and concentration that exhibited the highest dye degradation efficiency (Syr at 1 ppm), an additional study was conducted. In this experiment, the polymeric material was removed after a short exposure time (15 min), allowing the reaction to proceed autonomously once the dye degradation had been initiated.

2.5. Reusability of F_{LAC}

Using the brief exposure strategy to the F_{LAC} material described in Section 2.4, a reutilization study was conducted with the mediator Syr at a concentration of 1 ppm. For this, the 6 mm diameter F_{LAC} discs were extracted from the reaction medium after 15 min and washed three times for 5 min each with distilled water under orbital agitation at 190 rpm. After washing, the material was reincubated with fresh dye and mediator. This process was repeated for five successive cycles, and the dye degradation efficacy was assessed in each cycle.

2.6. Interference assays in the presence of textile washing products

Given that this material is designed for easy and straightforward use by non-specialized individuals, as well as in domestic environments, we decided to evaluate the material's efficiency in common washing waters that may contain laundry products such as fabric softeners or detergents. This essay was conducted following the procedure described in Section 2.4, using Syr as a mediator at a concentration of 1 ppm, while maintaining F_{LAC} in the medium throughout the entire reaction. In this case, 10 μL of fabric softener and an additional 10 μL of laundry detergent were added to the medium, using tap water instead of distilled water.

2.7. Life cycle assessment (LCA) study

LCA was carried out in accordance with ISO 14,040:2006 [28] utilizing SimaPro. Environmental impacts were quantified through the ReCiPe 2016 Endpoint (H) approach. The framework adhered to the standard LCA stages: (i) goal and scope definition, (ii) inventory analysis, (iii) impact assessment, and (iv) interpretation. Primary data were generated for the preparation and use of the F_{LAC} material and for the reference system selected from the literature, while background processes such as energy supply, transport, and auxiliary materials were modeled using data sets from the EcoInvent database. A comparative attributional LCA (ALCA) was performed to assess the environmental impacts associated with the use of two biocatalytic strategies for the degradation of 50 μg of indigo carmine dye.

The goal of this study is to compare the environmental impact of two alternative biocatalytic systems for the degradation of indigo carmine dye. The first is the novel film-shaped polymeric material containing laccase immobilized through diazo bonds (F_{LAC}), developed in this work, and the second is a reference solution based on laccase immobilized on chitosan–polyacrylic acid microspheres reported in the literature [29]. The comparison was established on the basis of an equivalent functional outcome, namely the degradation of a fixed amount of dye, and its ultimate purpose is to demonstrate that the material developed in this work was conceived with the intention of reducing environmental impacts. Although it is not feasible to include all immobilization strategies reported in the literature, the selected reference system was considered a relevant benchmark according to the criteria of the authors.

2.7.1. System boundaries

The system boundaries of this study comprise all processes directly related to the preparation and use of the biocatalysts required to achieve the functional unit. This unit was defined as the degradation of 50 μg of indigo carmine dye in aqueous medium. This definition provides a common and measurable basis to evaluate both alternatives and ensures that their environmental performance is compared on the same

functional outcome.

For both alternatives, this includes the synthesis of the polymeric support, the immobilization of the laccase enzyme, and the subsequent catalytic degradation process under the reported operational conditions. All chemical compounds were obtained from the Ecoinvent database; when an exact match was not available, a similar molecule was used as a proxy. Chitosan (75–85 % deacetylated) and N-(3-Dimethylamino-propyl)-N′-ethylcarbodiimide hydrochloride (EDC) were not considered in the modeling, as they could not be represented either in the database or through a suitable proxy.

2.7.2. Life cycle inventory

Comprehensive life cycle inventory (LCI) data, covering all inputs and outputs associated with the F_{LAC} biocatalyst and the reference system, are reported in the Supporting Information (SI-Section S2, Tables S1–S2). These data form the basis for the subsequent comparative environmental assessment.

2.8. Techno-Economic analysis (TEA) study

The analysis was conducted at laboratory scale for a system comprising three circular discs (6 mm diameter, radius $r = 0.3$ cm) operated in $V = 3$ mL of indigo carmine (IC, 10 ppm) at 30 °C and 190 rpm. Two functional units were used: (i) area-based performance ($L{\cdot}m^{-2}{\cdot}cycle^{-1}$ and $L{\cdot}m^{-2}{\cdot}h^{-1}$), and (ii) specific cost per mass treated (€/g of IC removed). Unless explicitly stated, cost items associated with the mediator (e.g., syringaldazine, Syr), detergent/softener, operational utilities of the bench test, and waste handling are excluded, to isolate the contribution of the film.

Three kinetic scenarios were defined at constant volume and area (3 discs, 3 mL); differences therefore reflect kinetics (mediator dose and medium composition): (i)Scenario 1 (baseline): Syr 1 ppm, target removal at 180 min, (ii) Scenario 2 (higher mediator dose): Syr 2 ppm, target removal at 90 min, (iii) Scenario 3 (laundry-type matrix): water + softener + detergent with Syr 1 ppm, target removal at 40 min. More details about equations, assumptions, and step-by-step calculations underlying the TEA are reported in the SI-Section S3.

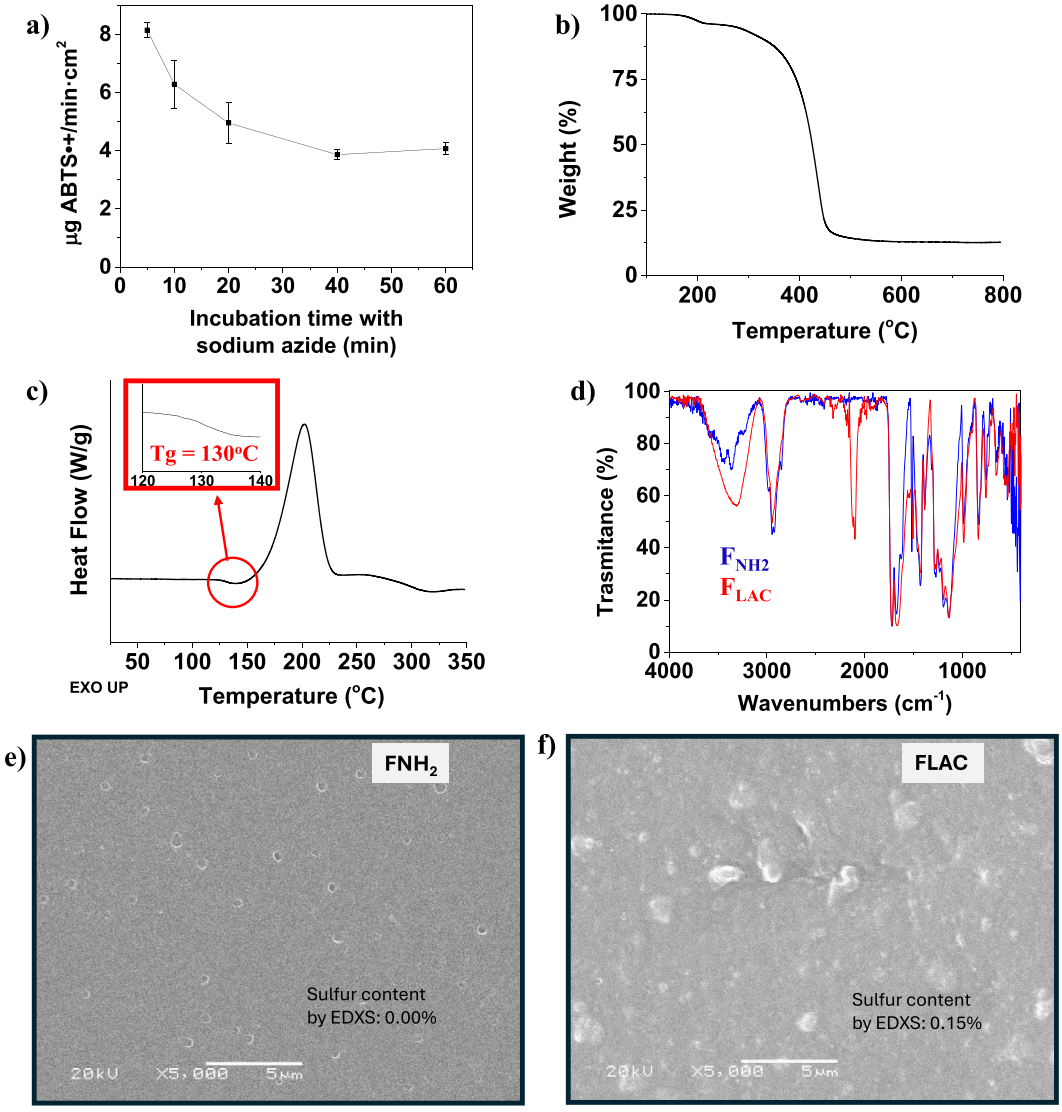


Fig. 2. Physicochemical characterization of the F_{LAC} material. a) Optimization of azide blocking times. b) Thermogravimetric analysis performed in a nitrogen atmosphere at a heating rate of 10 °C/min. c) DSC curve obtained with 10 mg of F_{LAC} at a heating rate of 20 °C/min in a nitrogen atmosphere. d) FT-IR spectrum. SEM micrographs of e) FNH_2 and f) F_{LAC}, showing sulfur content obtained through EDXS. The graph shows the ABTS activity (µg $ABTS^{\bullet+}$/min·cm^2) of F_{LAC} materials blocked for different periods with sodium azide.

3. Results and discussion

3.1. Optimization of azide blocking times

As previously mentioned, our research group has utilized this material and immobilization method for the immobilization of other enzymes. One of the critical steps involves blocking the excess benzene diazonium groups after anchoring the enzyme, employing sodium azide. However, to reduce processing times and mitigate potential laccase inhibition caused by azide [30], an optimization of the azide-blocking step was performed. As illustrated in Fig. 2a, laccase activity decreases over time, reaching its minimum activity at 40 min and remaining stable until 60 min. Based on these observations, an azide incubation time of 5 min was selected, effectively optimizing the immobilization process without compromising laccase activity or the mechanical properties of the material.

3.2. Physicochemical characterization of F_{LAC}

Given that the material is designed for both industrial and domestic applications, it must exhibit good handling properties, which must be maintained under working conditions. These characteristics, perceivable by the end-user when handling the material, are closely related to its thermal properties. For instance, **thermogravimetric analysis (TGA)** (Fig. 2b) reports T_5 and T_{10} values of 275 °C and 330 °C, respectively. Likewise, **differential scanning calorimetry (DSC)** (Fig. 2c) reveals a glass transition temperature (T_g) of 130 °C. Both results confirm that the material meets the expected requirements under the intended operating conditions. Specifically, it does not undergo degradation or significant weight loss until temperatures exceed 250 °C and remains in a solid state below 130 °C (Tg). Regarding the immobilized enzyme, it is derived from a thermophilic organism with optimal activity at 80 °C. This ensures its proper functionality at typical temperatures used in the textile industry, where certain washing processes are conducted at 70 °C [31]. It is worth noting that the DSC curve exhibits a prominent exothermic peak at approximately 200 °C, attributed to the conversion of azide groups into nitrene groups. This transformation induces a highly exothermic curing process [32].

The FTIR analysis (Fig. 2d) of F_{LAC} revealed a broad band at 3300 cm^{-1}, attributed to the N–H stretching vibrations of the amide groups in laccase. Additionally, two $C = O$ vibration peaks were observed at approximately 1720 and 1660 cm^{-1}, corresponding to the stretching vibrations of carbonyl groups. The peak at 1720 cm^{-1} is attributed to the ester carbonyl ($C = O$) of MMA, while the peak at 1660 cm^{-1} corresponds to the amide carbonyl ($C = O$) of VP. However, the $C = O$ vibration of the amide I band in the protein may also contribute to the signal at 1660 cm^{-1}. Furthermore, two bands were identified around 650 and 550 cm^{-1}, which can be attributed to the metal centers in Cu–protein bonds. The band observed at 2900 cm^{-1} corresponds to the C–H stretching vibrations of the aliphatic groups in the polymer monomers and the enzyme, while the band at 2100 cm^{-1} is associated with the $N = N$ stretching vibration of the azide group. These results confirm the successful binding of the enzyme to the material and are consistent with previous studies conducted by our research group, which employed similar polymeric films [12,24].

The WSP value obtained for the F_{LAC} material (89.4 ± 4 %) is significantly different from that reported in previous studies for the same material but with a different immobilized enzyme (β-galactosidase (β-gal) enzyme, 139 ± 4 %) [24]. This difference could be attributed to variations in the two enzymes' structure, size, and hydrophilic nature. Laccase is a smaller enzyme (66.11 kDa) compared to β-gal (114 kDa) and has a GRAVY (General Hydropathy Average) value of −0.326, while β-gal has a GRAVY value of −0.479. Since more negative GRAVY values indicate greater hydrophilicity, this suggests that β-gal has a higher affinity for water, which would explain its higher WSP value. The GRAVY index values were calculated using the ProtParam tool from the ExPASy server [33].

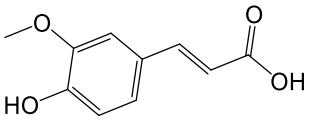
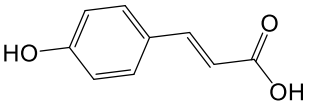
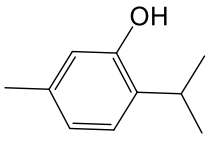
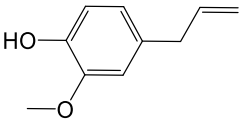
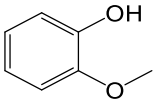
The SEM micrographs reveal increased roughness and the presence of particles in the enzyme-containing material (F_{LAC}), while the enzyme-free material (F_{NH2}) only exhibits small defects in the form of pores, caused by the release of dissolved gases in the monomers during polymerization, as well as defects inherent to the used mold (Figs. 2e and 2 f, respectively). This increased roughness in the enzyme-containing material has been observed previously in other studies [22,34]. Qualitatively, this allows for the identification of the enzyme on the material's surface. However, quantitative analysis was performed using EDXS, specifically through sulfur analysis. The presence of this element in the F_{LAC} material (and its absence in the F_{NH2} material) is indicative of enzyme immobilization, due to the cysteine and methionine residues present in the protein sequence [35]. In the case of the laccase enzyme, the calculated sulfur content is 0.15 %, a value consistent with that obtained from the EDXS analysis of the F_{LAC} material. This finding suggests that the enzyme is homogeneously distributed on the material's surface. Although this result supports the hypothesis that the enzyme is immobilized on F_{LAC}, it is essential to consider that EDXS analysis has a significant margin of error and provides information only about the material's surface, without assessing its internal composition.

3.3. Screening and evaluation of phenolic mediators for indigo carmine degradation

The catalytic efficiency and substrate versatility of laccase are increased by mediators, which operate predominantly via three pathways: (i) electron transfer, (ii) hydrogen atom transfer, and (iii) ionic mechanisms [36,37]. Natural phenolic compounds selected for indigo carmine degradation by F_{LAC} follow a reaction mechanism based on the generation of phenoxy radicals via hydrogen atom transfer [38,39]. The efficiency of these mediators in terms of speed and conversion yield depends on to the redox potential and stability of the formed phenoxy radicals. The nature of the substituent groups on the benzene ring determines this potential [40,41]. Table 1 shows the degradation percentages of indigo carmine at three different time points, using six different natural phenolic mediators, all at a final concentration of 10 ppm. The control film, without laccase, exhibited a slight reduction in dye concentration, likely due to the self-degradation of the dye or minor adsorption onto the film. However, this possible adsorption effect is negligible compared to the catalytic degradation achieved by the immobilized enzyme, confirming that the process is driven by enzymatic activity rather than adsorption. In mechanistic terms, the mediator first reaches the immobilized laccase at the film surface to be oxidized at the enzyme's active site. Once oxidized, it diffuses back into the solution and reacts with Indigo Carmine, so the degradation occurs mainly in the bulk phase [40,42,43]

Ferulic acid, *p*-coumaric acid, and thymol demonstrated low degradation percentages, remaining near 10 % after 300 min of reaction. In contrast, eugenol and guaiacol showed significantly higher degradation levels, reaching approximately 33 % and 85 %, respectively, in the same period. Notably, guaiacol achieved a remarkable 71 % degradation in just 180 min. In the literature, different results can be found with these mediators and other dyes. For example, with *p*-coumaric acid and the dye Reactive Black, the degradation reached about 10 % after 2 h at a concentration similar to that used in the present study, which is close to our findings. However, in that study, guaiacol did not lead to any noticeable decolorization [40,44]. In contrast, another work reported that guaiacol enabled up to 80 % decolorization of malachite green, although this required 24 h of reaction and a dye concentration of about 50 ppm. However, in that same study, p-coumaric acid and ferulic acid achieved much better results, exceeding 85 % degradation after 6 h [45]. Evidently, the conditions across these studies are not fully comparable, as they used different laccases, dyes, concentrations, and reaction times. To our knowledge, the present study is the first to combine *M. thermophila* laccase with these specific mediators and this particular

Table 1
Degradation of indigo carmine using the laccase film and different natural phenolic mediators. Three 6 mm diameter F_{LAC} discs were introduced into 3 mL of dye solution (10 ppm) and mediator (10 ppm) in 90:10 water-ethanol solution. The percentage of dye degradation at different time points (60, 180, 300 min) is shown.

		Reaction time (min)		
		60	180	300
Structure	**Mediators**	**Dye degradation (%)**		
–	**None (control)**	0.81	0.44	1.58
	**Ferulic acid**	2.74	4.33	10.24
	***p*-Coumaric acid**	2.29	6.10	8.43
	**Thymol**	2.47	4.65	7.39
	**Eugenol**	8.84	28.51	32.73
	**Guaiacol**	27.06	70.99	85.12

dye.

These results can be explained by the fact that laccase activity on phenols increases when electron-donating groups, like methoxy, are present on the benzene ring, as these groups facilitate the phenol group's ability to lose electrons. This helps explain the higher degradation rates observed for eugenol and guaiacol. In contrast, although ferulic acid contains electron-donating methoxy groups, its degradation rates are lower because the carboxyl group acts as an electron-withdrawing group, counteracting the effect of the methoxy groups [38,40].

Due to solubility limitations, Syr could not be used at 10 ppm under the same reaction conditions (10 % ethanol). Consequently, it was tested at lower concentrations (0.5, 1, and 2 ppm). Fig. 3 shows the dye degradation achieved using these Syr concentrations over a 300-minute reaction period. Notably, even at the lowest concentration of 0.5 ppm, the results exceeded those obtained with the previously tested mediators at 10 ppm, achieving 31 % degradation at 60 min, 76 % at 180 min, and 92 % at 300 min. At 2 ppm, the time required to reach degradation above 90 % was significantly reduced, reaching this level in just 90 min. In comparison, at 1 ppm, the same degradation percentage was reached in 120 min. To achieve degradation rates above 99 %, 1 ppm required 160 min, while 2 ppm achieved this in only 120 min. These results indicate that Syr is the most efficient mediator in this study. As mentioned earlier, this efficiency is attributed to the multiple methoxy groups in Syr structure (Fig. 3), which not only facilitates the rapid oxidation of its phenolic groups but also enhances the stabilization of phenoxyl radicals. These findings are consistent with previous studies, where the most efficient mediators were syringaldehyde and acetosyringone, which share a very similar structure with Syr [44]. In other studies, the decolorization of indigo carmine in the presence of laccases from *Peniophora* sp. and *Bacillus velezensis* using syringaldehyde, showed efficiencies of 93 % and 90 %, after two and six hours, respectively [46,47]

Since eugenol and guaiacol were the mediators with the best results after Syr, a concentration of 1 ppm was used to compare their performance with Syr, and a higher concentration (50 ppm) was also tested (Table 2). At 1 ppm, eugenol showed very low degradation, reaching only about 11 % after 300 min, while guaiacol exhibited better performance, achieving nearly 43 % degradation in the same period. However, these results were significantly lower than those obtained with Syr at 1 ppm, which reached 50 % degradation in just 60 min. To achieve nearly 99 % dye degradation in 180 min with guaiacol, a concentration of 50 ppm was required. In contrast, under the same conditions, eugenol only reached approximately 31 % degradation in 180 min and nearly 50 % after 300 min. These results demonstrate that Syr at 1 ppm produced the best outcomes with the lowest mediator consumption, making it the preferred option for subsequent experiments.

Table 2
Degradation of indigo carmine using F_{LAC} and the best natural phenolic mediators (Syr, eugenol, and guaiacol). A 6 mm diameter F_{LAC} disc was introduced into 3 mL of dye solution (10 ppm) and mediator (1 and 50 ppm) in 90:10 water-ethanol medium. The percentage of dye degradation at different time points (60, 180, 300 min) is shown.

		Reaction time (min)		
	Concentration (ppm)	**60**	**180**	**300**
		Dye degradation (%)		
Eugenol	1	7.51	10.09	11.20
	50	11.84	30.91	48.69
Guaiacol	1	7.48	20.87	42.46
	50	53.73	98.48	98.68
Syr	1	38.82	99.8	99.99

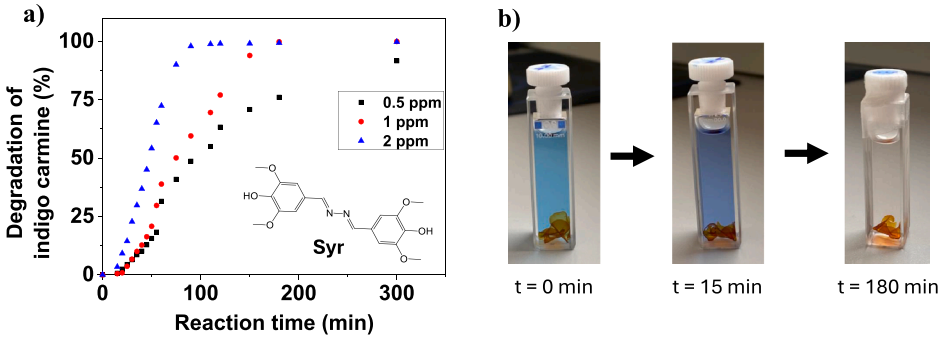


Fig. 3. a) Indigo carmine degradation using the laccase film and Syringaldazine (Syr) at three different concentrations (0.5, 1 and 2 ppm). b) Images at 0, 15 and 180 min of the Indigo Carmine degradation process using Syr (1 ppm).

3.4. Dye degradation assays with short exposure to the F_{LAC} polymeric film

One of the questions that arose during the research was the possibility that F_{LAC} acted solely as a degradation initiator, and that the system could then evolve independently. This scenario would involve an initial formation of the oxidized species of Syr by F_{LAC}, after which the presence of F_{LAC} in the media would become irrelevant. To investigate this hypothesis, once the peak absorbance of oxidized Syr was reached (15 min), as shown in Fig. 4a [48], the film was removed ("film out") and the subsequent dye degradation was compared with that observed when the films remained present throughout the reaction ("film"). As shown in Fig. 4b, the removal of the film resulted in a slower degradation rate; however, nearly 95 % dye degradation was achieved after 400 min.

It is worth mentioning that once the maximum absorbance of the oxidized form of Syr is reached, which acquires a pink coloration at 530 nm due to its oxidation by F_{LAC}, this absorbance gradually decreases over time (Fig. 4a), with a much more pronounced decrease when the film remains in the reaction. This is because the enzyme continues to promote oxidation or secondary reactions occur. Laccase may be catalyzing further transformation of the oxidized Syr, leading to the formation of more stable dimers or polymers of phenoxyl radicals, thereby accelerating the disappearance of the pink coloration [49].

This phenomenon could also help explain why, when the F_{LAC} films are removed after 15 min, the degradation of indigo carmine appears slower compared to when F_{LAC} remains throughout the reaction. It is possible that the oxidized form of Syr is not the only species mediating the degradation; other reactive species, generated by subsequent reactions on the initial oxidized form, could also be playing a significant role in the process.

3.5. Reusability of F_{LAC}

Reusability assays (Fig. 5a) showed that the F_{LAC} material retained nearly 90 % of its enzymatic activity after five rounds of use. By removing the materials after 15 min, it is possible to perform multiple dye treatment rounds in parallel, with only a 30-minute interval between each round (15 min of incubation + 15 min of washing). This strategy improves operational times, as it not only allows the reuse of the F_{LAC} material but also eliminates the need to keep it throughout the entire degradation process, limiting its presence to the first 15 min.

Previous studies with laccase-containing materials have reported similar results. Ruxandra-Leonties et al. reported a retention of 72 % of the activity of laccase immobilized in chitosan-polyacrylic microspheres after five cycles of indigo carmine degradation [29]. Similarly, Liu et al. achieved approximately 80 % activity retention under comparable conditions using laccase immobilized on silica nanoparticles [50]. In both cases, the laccase (or the materials containing it) remained present throughout the entire process. However, our study is, to the best of our knowledge, the first in which laccase-containing materials are removed right after the degradation reaction begins, allowing the process to continue autonomously in other vials while the system evolves without the presence of the material.

3.6. Impact of laundry detergents on indigo carmine degradation

Within the textile industry, Indigo Carmine is extensively utilized, with denim manufacturing representing its main area of application. During the dyeing process, washing with detergents and softeners is commonly applied. Since our material is designed for both domestic and industrial applications, we aimed to test it under conditions closely resembling real-world usage [6].

As shown in Fig. 5b, surprisingly, when the material acts in the presence of laundry detergent products, the dye degradation rate is significantly higher, reaching 100 % in less than an hour. In contrast, in the distilled water medium, this level of degradation is achieved in just over three hours. These results suggest an increase in the laccase activity in the presence of laundry products, which could be attributed to the surfactants present in these products. Surfactants may affect the conformation of laccase, making its active site more accessible to phenolic substrates. Furthermore, since Syr is poorly soluble in water, detergents could improve its solubility, making it more available to the enzyme [51]. This effect has also been reported in studies using other enzymes [52].

3.7. LCA study

The LCA followed ISO 14,040 using SimaPro and the ReCiPe 2016 Endpoint (H) method, with primary data for preparation and use of the diazo-bonded laccase film (F_{LAC}) and Ecoinvent datasets for background; proxies were used where exact matches were unavailable. The complete process trees for both systems (F_{LAC} and the literature benchmark with laccase on chitosan–polyacrylic acid microspheres [29]) are provided in the SI-Section S2, detailing the modelled flows of materials, energy and wastes to meet the functional unit (degradation of 50 μg indigo carmine in water). In the benchmark inventory, chitosan and EDC could not be represented in Ecoinvent nor by suitable proxies; this limitation is disclosed and their inclusion would increase the benchmark footprint (biopolymer and activator production), making our comparison conservative in favour of the benchmark.

As shown in Fig. 6, midpoint indicators were normalized against the benchmark (100 %). In categories such as climate change (human, terrestrial, and freshwater), photochemical ozone formation, particulate matter, terrestrial acidification, freshwater eutrophication, and human/ecotoxicity, FLAC values typically range between 40 % and 60 % of the reference. Sharper reductions are observed for fossil resource scarcity and water consumption (~30–40 % of the benchmark), consistent with lower solvent and electricity requirements per functional unit as seen in

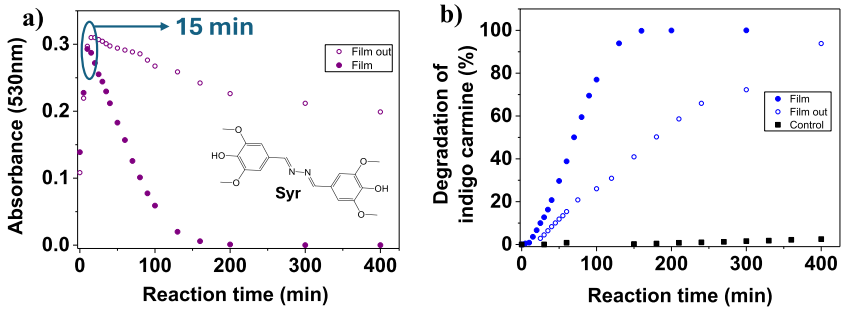


Fig. 4. a) Evolution of the oxidized Syr species monitored at 530 nm over time. The laccase film with Syr at 1 ppm was tested using two different methods: (i) keeping the material in the reaction throughout the entire process ("film") or (ii) removing the material after 15 min and allowing the reaction to proceed without it ("film out"). b) Degradation of indigo carmine using the laccase film and Syr at 1 ppm using the methods (i) and (ii). A control with the material lacking the enzyme is also included for comparison. Experimental conditions: 30 °C; 190 rpm, 10 ppm indigo carmine, 1 ppm Syr, 90:10 water-ethanol medium.

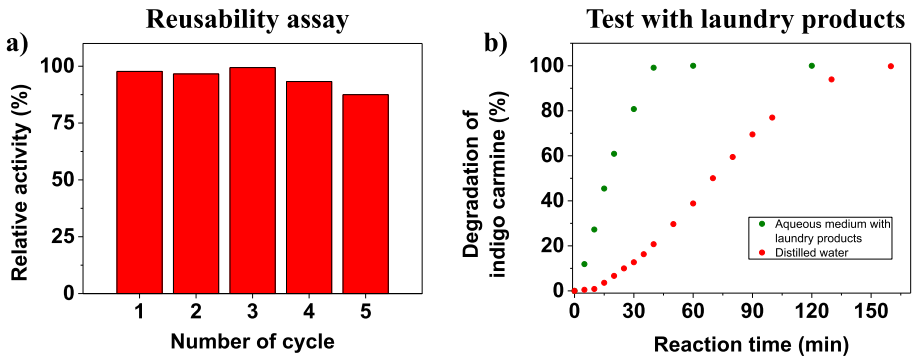


Fig. 5. a) Reusability assay of the laccase film. Experimental conditions: 30 °C, 190 rpm. Exposure time to F_{LAC}: 15 min; indigo carmine concentration: 10 ppm; Syr concentration: 1 ppm; total reaction time: 300 min. b) Degradation of indigo carmine using three F_{LAC} disks (6 mm diameter) in a simulated washing medium (3 mL distilled water, 10 μL fabric softener, 10 μL detergent), as well as in an aqueous medium (3 mL distilled water). Experimental conditions: 30 °C, 190 rpm. Exposure time to F_{LAC}: throughout the entire degradation process; Syr concentration: 1 ppm; indigo carmine concentration: 10 ppm.

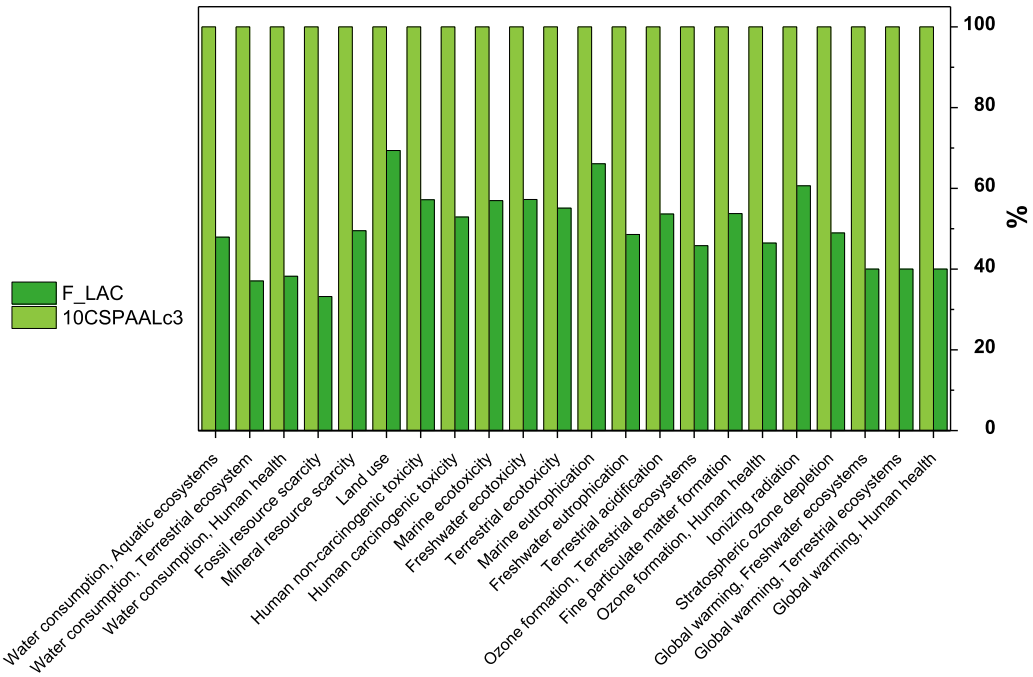


Fig. 6. Comparative environmental impacts of the F_{LAC} and reference biocatalysts (10CSPAALc3) for the degradation of 50 μg of indigo carmine grouped, by impact categories, calculated using the ReCiPe 2016 Endpoint (H) method.

the process trees for F_{LAC}. Hotspots for F_{LAC} are land use, marine eutrophication and ionizing radiation (≈60–70 %), mainly linked to the supply chains of vinyl/aromatic monomers (e.g., 4-aminostyrene, styrene, MMA) and the background electricity mix. In the benchmark, the process trees show more intensive solvent and electricity use, plus notable aqueous streams; as noted, excluding chitosan and EDC likely underestimates its true burden. Fig. 7 corroborates the trend at endpoint level, with ~55–70 % integrated reductions for F_{LAC}: ~45 % (Human health), ~43 % (Ecosystems) and ~31 % (Resources), the strongest improvement being in Resources.

The comparison is made with full respect to the benchmark study, which provides an effective and stable immobilisation route; our work advances the state of the art by showing that an alternative support (F_{LAC}) can improve environmental viability for the same function. Overall, guided by the process trees and the dominant categories, the results support F_{LAC}'s environmental promise and outline an eco-design pathway: (i) greener support chemistry (optimize/replace 4-aminostyrene/styrene and MMA; safer alternatives to azide blocking; >90 % solvent recovery), (ii) decarbonize the electricity background, and (iii)

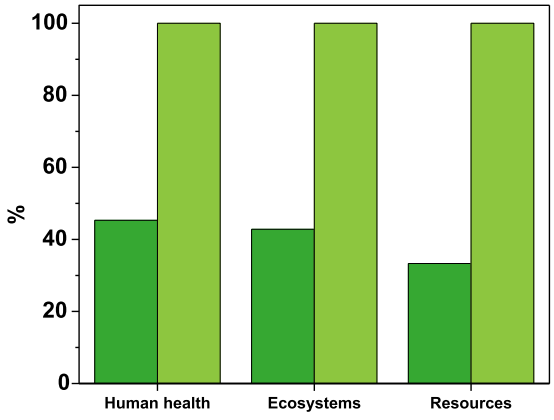


Fig. 7. Comparative environmental impacts of the F_{LAC} and reference biocatalysts (10CSPAALc3) for the degradation of 50 μg of indigo carmine, by damage categories, calculated using the ReCiPe 2016 Endpoint (H) method.

increase batch yield and effective re-use of the film to dilute impacts per functional unit. Given the attributional nature and bench scale with a very small functional load, values should be read as comparative signals; nevertheless, the consistent reductions across midpoints and endpoints indicate that FLAC offers substantive environmental advantages and a clear roadmap towards pilot scale.

3.8. TEA study

3.8.1. Area-based performance metric (unit-level)

Fig. 8a displays the area-normalised productivity as a function of cycle time for the three scenarios (3 discs, 3 mL; $\frac{V}{A_{tot}} = 35.37\ \mathrm{L{\cdot}m^{-2}{\cdot}cycle^{-1}}$). The second-order polynomial fit (red dashed line) captures the expected curvature from $\Pi_{A,t}\ \alpha\ t$ and is given by $y = 11.865\,t^2 - 61.254\,t + 88.74$. Within this framework, Scenario 3 ($t \approx 0.67$ h) reaches 53.05 $\mathrm{L{\cdot}m^{-2}{\cdot}h^{-1}}$, lying in the region of maximal slope (high marginal gain per additional time reduction) and very close to the empirical upper envelope over the measured window. Longer cycles deliver lower productivities: Scenario 2 (1.50 h) 23.58 $\mathrm{L{\cdot}m^{-2}{\cdot}h^{-1}}$, and Scenario 1 (3.00 h) 11.79 $\mathrm{L{\cdot}m^{-2}{\cdot}h^{-1}}$, consistent with the non-linear trend. Quantitatively, our improvement efforts deliver an ~350 % increase in area-normalised productivity relative to the baseline (11.79 → 53.05 $\mathrm{L{\cdot}m^{-2}{\cdot}h^{-1}}$; Scenario 1 → 3), attributable to optimising the surfactant-containing medium and the operational settings.

From a Techno-Economic Analysis (TEA) and sizing perspective, this representation confirms that shortening the cycle (e.g., via surfactants in the medium) is a more effective lever than increasing mediator dose: at 1 ppm Syr, Scenario 3 attains ~4.5 × the baseline productivity and ~2.25 × that at 2 ppm. The polynomial fit is empirical and, with three data points, reproduces the data exactly ($R^2 = 1$); therefore, extrapolation beyond 0.67–3.00 h should be treated with caution because the practical ceiling will be set by mass-transfer limitations and enzyme turnover, rather than by the polynomial intercept. However, the present laboratory results suggest the system is already operating close to its optimal productivity ceiling under the conditions studied. Taken together, the figure underscores that medium composition (presence of surfactants) is decisive for maximising area-normalised productivity and, consequently, for reducing the required area (associated CAPEX) and the operating time (time-dependent OPEX) per unit volume treated.

3.8.2. Cost of solution per gram of dye removed (mediator-free)

This was the first step to compute the second indicator (See Table 3; Unit cost build-up per disc by process stage): the per-disc unit cost was decomposed exclusively into raw materials and was allocated by process stage (polymer synthesis, diazotization, enzyme immobilization, azide blocking). The following assumptions were made: (i) only materials costs were counted (energy, labour, utilities, waste handling and mediator were excluded); (ii) batch-to-unit allocation was set by the reported yields (150 discs for polymer synthesis, 10 for diazotisation, 6 for immobilization, 10 for azide blocking); (iii) distilled water was not costed (negligible/OSBL); and (iv) catalogue unit prices were used in €/g (or €/mL). The resulting total per-disc cost was €0.019, with polymer synthesis as the dominant contributor (€0.012; 64.33 %). Within that stage, aminostyrene (SNH_2) accounted for €0.010 per disc (57.16 % of the total) and was therefore identified as the primary raw-materials driver. The remaining contributions were diazotization 15.52 %, azide blocking 11.48 %, and enzyme immobilization 8.67 %. For the TEA, this breakdown indicates the main levers: batch yield should be increased (to reduce allocation per unit), the costly monomer (SNH_2) should be optimized or substituted, and procurement at scale should be pursued—measures expected to give the largest marginal reductions in €/g, particularly under multi-reuse operation and/or higher treatment throughput.

Table 3
Unit cost build-up per disc by process stage.

Stage	Cost per-disc (€)	Share of total (%)
Polymer synthesis	0.012153791	**64.33**
Diazotisation	0.002933000	15.52
Enzyme immobilisation	0.001637500	8.67
Azide blocking	0.002170000	11.48
Grand total per disc	**0.018894291 ~ 0.019**	100.00

Fig. 8b displays, as a function of re-use number N, the per-cycle efficiency (left axis) and the film's specific cost in €/g (right axis), computed with a fixed raw-materials cost for three discs $C = 0.056682873$ € and an IC mass per cycle of 3.0×10^{-5}. Over the first five uses, efficiency remains high (100–100–100–90–87 %), while the €/g decreases from 1889 to 396 (−79 %, ≈4.8 × improvement). This behaviour follows directly from the denominator (cumulative treated mass) increasing with N while the numerator remains constant. The analysis identifies the service life of the support (re-use) as a primary lever in the TEA: there is a natural operating window of $N = 3$–5 N in which the largest marginal reduction in cost is achieved with minimal efficiency loss. The end-of-life (EOL) definition—e.g., setting a minimum acceptable efficiency of 80 %—will determine the maximum N and hence the final €/g; with the observed profile, this threshold is not reached within five re-uses, and €/g continues to decline. Accordingly, extending service life and, in parallel, increasing the volume or concentration per cycle, emerge as critical strategies to enhance the techno-economic viability of the system.

When energy and labour are included in film fabrication, the total cost allocated to three discs is €10.21268 (energy: 0.682 kWh → €0.136; labour: €10.02; raw materials: €0.05668). Using this numerator, the specific cost is €340,422.76 per g (single use, 100 %), €341,104.97 per g

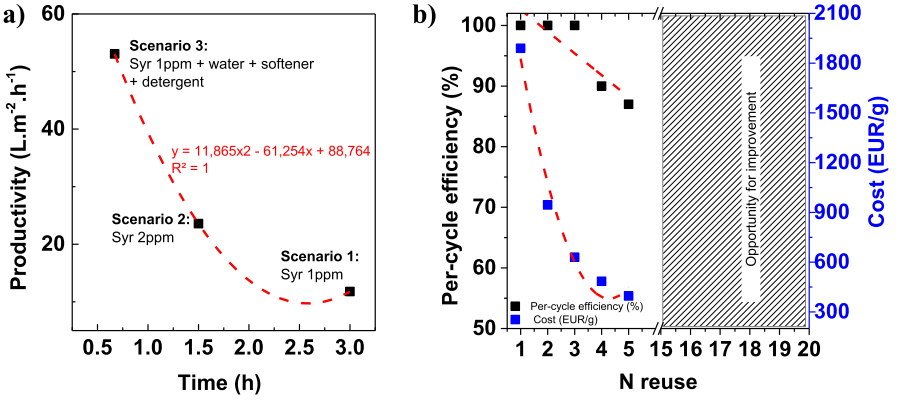


Fig. 8. a) Area productivity vs time; polynomial fit and near-ceiling performance in a surfactant medium. b) Effect of re-use number on per-cycle efficiency (left) and film specific cost (€/g, right); the hatched area denotes the opportunity for improvement.

(99.8 %), and €71,367.46 per g with five re-uses, see Table SI7 and SI8. Labour dominates the numerator, energy is secondary, and the raw-materials price has a marginal effect under these conditions. The absolute figures are large because the study sits at low TRL/bench scale, where (i) the mass treated per cycle is very small (30 μg), (ii) production is manual and batch-wise with high operator time, and (iii) fixed costs are allocated to few discs. The TEA therefore points to clear priorities: increase batch yield (more discs per polymerization), parallelise/automate wet-chemistry steps to reduce labour per unit, and extend service life (re-use); in parallel, increase volume or concentration per cycle and shorten cycle time in suitable media to grow the denominator and improve €/g. Overall, these results are not intended as definitive industrial costs but to expose the bottlenecks and chart the pathway so that, at pilot/demonstration scale, €/g falls substantially and the cost structure shifts away from labour towards inputs and equipment representative of scale-up.

4. Conclusions

This study presents a cost-effective and manageable immobilized laccase polymeric material (F_{LAC}) for efficient dye degradation, with significant potential for industrial and environmental applications. Syringaldazyne (Syr) was identified as the most efficient mediator, achieving high degradation rates even at minimal concentrations. The novelty of this work lies in the use of a film-shaped polymeric support that is robust, reusable, and easy to handle, combined with the exclusive application of natural mediators as a greener alternative to synthetic ones such as ABTS or HBT. Another important innovation is the brief exposure of the dye solution to FLAC for just 15 min, after which the degradation process continued autonomously even after removing the material. This short-exposure strategy, reported here for the first time to our knowledge, enhances efficiency and enables multiple reuse cycles. The system demonstrated excellent reusability, retaining over 90 % of its activity after several cycles, and its performance was notably enhanced in the presence of detergents and softeners, with complete dye degradation achieved in under an hour. These improvements under realistic washing conditions confirm the contribution of this work to bridging laboratory studies and real-world applications, offering a low-cost and sustainable solution for textile wastewater treatment. The unit-level TEA shows that shortening the cycle in surfactant media boosts area-normalized productivity by ~350 %, and re-use ($N \approx 3$–5) lowers €/g by ~5 ×; at bench scale, €/g is dominated by labour and aminostyrene, prioritizing higher batch yield and automation. The comparative A-LCA indicates that F_{LAC} achieves ~55–70 % reductions at the endpoint level (~40–60 % at midpoints) versus the literature benchmark, with hotspots in land use, marine eutrophication and ionizing radiation linked to monomers and the electricity mix. Together, both assessments identify the improvement levers: shorten the cycle, maximise re-use and per-cycle volume/concentration, increase batch yield and automate, manage the mediator, green the support chemistry and recover solvents, and decarbonise energy, defining a pathway to pilot with substantial reductions in €/g and in the environmental footprint.

Data availability

Open Data is available at https://riubu.ubu.es/handle/10259/5684 under the name "UBU-Polymers Research Group 21032024".

CRediT authorship contribution statement

J. Lucas VALLEJO-GARCÍA: Supervision, Writing – review & editing. **Miriam TRIGO-LÓPEZ:** Methodology, Writing – original draft, Writing – review & editing. **Saturnino IBEAS:** Formal analysis, Investigation, Methodology, Validation. **Félix C. GARCÍA:** Supervision, Writing – review & editing. **María D. BUSTO:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Karina C. NÚÑEZ-CARRERO:** Formal analysis, Software, Writing – review & editing. **Luis E. ALONSO-PASTOR:** Formal analysis, Software, Writing – review & editing. **Saúl VALLEJOS:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, 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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rineng.2025.107880.

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