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Enhanced Thermostability of Laccase from *Myceliophthora thermophila* Through Conjugation with mPEG-SC

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Abstract: The search for more sustainable reaction conditions has been necessary to obtain more selective processes. In this context, laccases have gained great notoriety in recent years. However, these enzymes are unstable in organic solvents and have low thermal stability. Alternatively, conjugation with PEG (PEGylation) can be essential to overcome these problems. In this work, the commercial laccase from *Myceliophthora thermophila* (LacMT) was subjected to PEGylation with PEG functionalized as succinimidyl carbonate (mPEG-SC), followed by assessing its thermal stability and catalytic activity. Mono-PEGylated LacMT derivatives were obtained, with less than 50% of the enzyme remaining in its native form. In addition, 10% of the bi-PEGylated species was successfully obtained according to gel electrophoresis analysis. The PEGylated derivatives showed a significantly reduced ABTS oxidation activity (98 ± 3 U/mg) compared to the native LacMT (407 ± 9 U/mg) but higher than the control enzyme without PEGylation (51 ± 2 U/mg), demonstrating that the addition of activated PEG to the protein resulted in better protection against the harmful action of the pH change required in the process. PEGylated LacMT retained more than twice the initial activity of the native protein at 40 °C during 24 h. In addition, PEGylated LacMT exhibited kinetic changes, whereas the catalytic turnover rate (k_{cat}) of the PEGylated enzyme was reduced by 27% compared to the control. These findings are being reported for the first time. This sets precedents for constructing efficient catalytic systems involving laccases since no immobilized biocatalyst or commercial conjugate contains these proteins.

Keywords: laccase PEGylation; polyethylene glycol (PEG); mPEG-SC; PEGYlation; laccase

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1. Introduction

As global life expectancy continues to rise, the demand for food, energy, and consumer goods has grown, leading to unprecedented levels of industrialization and the emergence of new strategic sectors [1,2]. This rapid industrial growth has significant environmental consequences, including substantial waste generation without adequate treatment and pollution caused by large quantities of by-products [3–6]. To compound these problems, there is a finite supply of fossil fuels, underscoring the urgent need to develop innovative new protocols for synthesizing high-value-added compounds under more environmentally sustainable conditions. This change is essential to promote the circular economy and sustainable development [7,8].

In this context, enzymatic processes have gained significant attention due to their ability to catalyze reactions under mild conditions, specifically lower temperatures and

pressures. These processes are characterized by high selectivity, which minimizes the formation of by-products and reduces the need for extensive separation steps [9–12]. Laccases (EC 1.10.3.2; *p*-diphenol: oxidoreductases) have emerged as crucial biocatalysts among enzymes of significant industrial value. These multiple copper oxidoreductases, mainly involved in the biosynthesis and deconstruction of lignin in higher plants, have demonstrated the ability to oxidize various organic and inorganic substrates, including aromatic amines, phenolic compounds, and pharmaceuticals, while reducing molecular oxygen to water [13–16]. This remarkable versatility has led to their application in various industrial sectors, including the pulp and paper and food and beverage industries and in the remediation and removal of toxic compounds in industrial effluents in the pharmaceutical and textile sectors [17–20].

Laccases are ubiquitous in various organisms and microorganisms, with fungal laccases being the most widely studied due to their advantageous catalytic properties and favorable production conditions. Eukaryotic production systems for these enzymes allow efficient secretion into the extracellular environment, facilitating recovery and purification [21]. A notable example is laccase from *Myceliophthora thermophila* (LacMT), a monomeric protein with a molecular mass of 61.32 kDa. LacMT has demonstrated exceptional efficiency and selectivity in applications such as paper bleaching, dye and antibiotic degradation, beverage clarification, and tissue whitening [22–24]. However, despite its catalytic potential, LacMT has limitations in its free form, including low resistance to organic solvents, susceptibility to high shear forces, and reduced thermal stability [25]. To overcome these challenges, several studies have explored the immobilization of LacMT on solid and semi-solid supports, thus broadening the enzyme's industrial applications and expanding the types of reactors that can be used, with the main gains being more excellent operational stabilities, activity retention, and increased application possibilities [26–29]. However, the selection of suitable supports, the complexities of functionalization and various steps in developing complex protocols in some cases, the associated costs of reagents, and the risk of low yields continue to represent significant obstacles [30]. Laccases have been widely applied in photocatalytic reactions by our research group, where the homogeneous distribution of the enzyme in the medium is an essential factor in the success of the oxidation reaction [9,27]. In this process, laccases immobilized on solid supports threaten light distribution in the photoreactors. On the other hand, the direct incidence of light on the surface of the laccases can cause hyperexcitation and conformational changes, generating metastable intermediates with low or no activity. Thus, new ways of stabilizing laccases in aqueous environments are essential for constructing new catalytic systems.

An innovative approach, extensively applied to improve the properties of biopharmaceuticals, therapeutic proteins, and other enzymes, is conjugation with poly(ethylene glycol) (PEG) chains and known as PEGylation. This technique has gained momentum in the pharmaceutical and biopharmaceutical sectors. PEGylated active compounds have improved their pharmacodynamic properties, such as half-life time, renal clearance, and resistance to proteolytic degradation, favoring better therapeutic regimens. In addition, PEGylation can attenuate immune responses and increase thermal stability, thus improving bioavailability and stability during storage [31–35]. For industrially applicable enzymes, the main objectives of PEGylation are to increase thermal stability and enhance activity in the presence of organic solvents, which generally cause harmful effects on the protein, mainly the inversion of hydrophilic and hydrophobic domains. Thus, improving solubility in these systems is also required [36–38]. The interaction of enzymes with polyethylene glycol (PEG) can generate and stabilize structural modifications in proteins, allowing the resulting conjugates to remain in solution for single-use applications. In addition, the PEGylated derivative can be associated with enzyme immobilization techniques on solid supports, combining the advantages of both approaches to improve functionality [39–41].

However, the literature on the PEGylation of LacMT is limited, indicating a significant gap in our understanding of how this modification could improve the catalytic properties of the enzyme in aqueous phase [42,43]. Therefore, this study aimed to investigate the PE-

Gylation of LacMT through conjugation with PEG functionalized as succinimidyl carbonate (mPEG-SC) and then evaluate its thermal stability and catalytic activity to establish a proof of concept for the construction of new aqueous phase catalytic systems for photocatalysis. Conjugation with mPEG-SC is relatively mild, modifying less than 5% of the free amino groups of LacMT [44–46]. This study focuses on understanding how adding a limited number of PEG chains influences the stability of LacMT. In contrast, studies such as that by López-Cruz and colleagues have explored the effects of extensive PEGylation of laccase, in which more than 50% of the protein's amino groups have been modified [47]. This research is promising for advancing the application of laccases in industrial processes, contributing to more sustainable practices, and addressing urgent environmental challenges.

2. Results and Discussion

2.1. Laccase Conjugation

2.1.1. Buffer Change

PEGylation is a well-established technique for increasing the thermal stability of therapeutic proteins and other enzymes. Due to the low reactivity and high strength of conventional PEG, we began this study by investigating the conjugation potential of laccase from *Myceliophthora thermophila* (LacMT) using PEG functionalized as succinimidyl carbonate (mPEG-SC) as an agent and then evaluating its effects on the thermal stability and residual activity of the conjugates obtained. Due to the electrophilic nature of this succinimidyl group, it was essential to adjust the pH of the biomolecule to an alkaline level to avoid protonation of electron-donating groups. Thus, the initial step involved transferring the protein to a borate buffer at pH 9.0 using gel filtration chromatography (Figure 1). This procedure ensured that the protein was maintained at the appropriate pH and removed components from the enzyme's commercial preparation that could potentially interfere with the PEGylation process. pH 9 was selected because a prior experiment demonstrated that PEGylation conducted at pH 8 resulted in over 80% of unconjugated protein. Laccase conjugation with succinimidyl carbonate-functionalized polyethylene glycol at pH values above 8 primarily occurs through the ε-amino groups of lysine residues [36]. The efficiency of this reaction in this pH range increases significantly with rising pH levels [37], likely attributable to the pKa of this amino group, which is approximately 10.5.

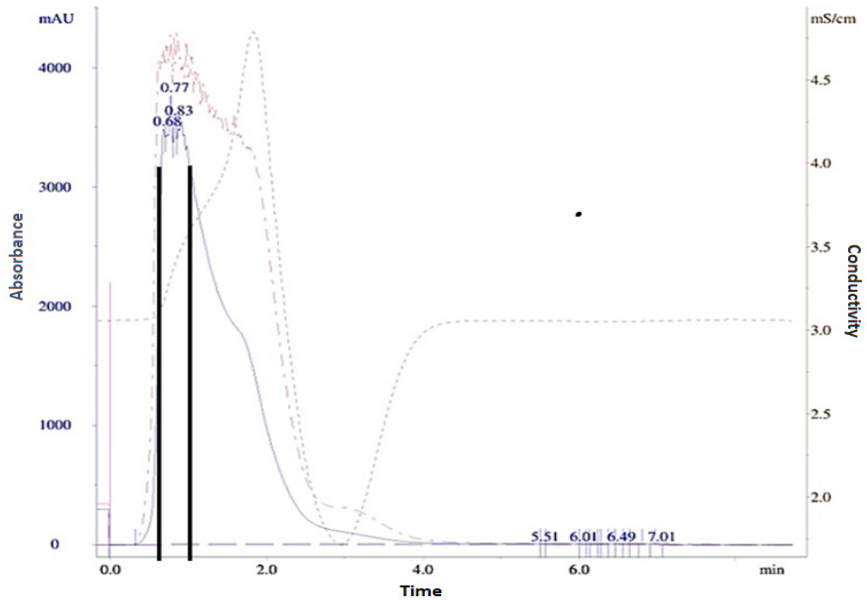

Figure 1. Buffer exchange was performed using chromatography with Sephadex G-25 (HiTrap Desalting column, 5 mL). The equilibration buffer consisted of 100 mM borate at pH 9, with a 5 mL/min flow rate. A sample volume of 2 mL was injected, and the collected fractions are indicated between the vertical black lines: continuous line, Abs280; dash-dotted line, Abs215; and dashed line, conductivity.

2.1.2. LacMT Conjugation

The relative proportions of the different protein species were quantified by analyzing the SDS-PAGE gel image (Figure 2) using ImageJ software, ver. 1.54g (https://imagej.net/ij/; accessed on 25 September 2024). The conjugation reaction demonstrated sufficient efficiency for the objectives of this study, with less than 50% of the enzyme remaining in its native form. Additionally, 10% of the bi-PEGylated species was successfully obtained. The high yield observed in this conjugation reaction is consistent with results reported by other researchers for PEG functionalization using succinimidyl carbonate [38]. In contrast, using other functional groups, such as aldehydes, in the PEGylation of laccases typically results in lower conjugation yields [39,40].

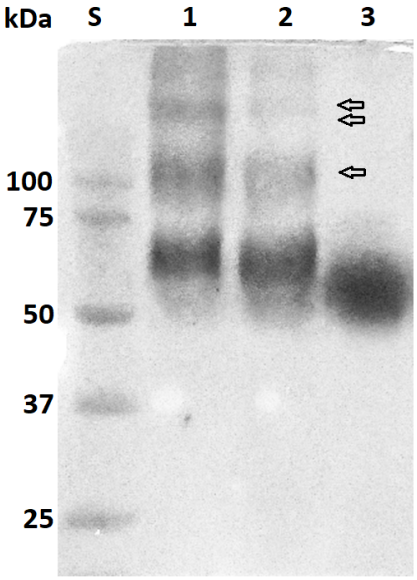

Figure 2. Electrophoretic analysis of LacMT PEG conjugation. The reaction mixture was diluted 1:1 with a sample buffer and subsequently separated on a 15% polyacrylamide gel: S, molecular weight standard; 1 and 2, conjugation reaction; and 3, native protein. monoPEGylated and biPEGylated conjugates are marked with one and two arrows, respectively.

Figure 2 illustrates that each polyethylene glycol (PEG) chain increases by over 60 kDa in the apparent molecular mass of conjugated protein. Despite the PEG having a molecular weight of only 12 kDa, its strong interaction with water molecules significantly expands its hydrodynamic volume, rendering it much larger than that of a globular protein with an equivalent molecular mass. Similarly, Morgenstern et al. reported an increase of more than 50 kDa in the apparent molecular mass when lysozyme was conjugated with a 10 kDa PEG chain [41]. However, this phenomenon has been reported for LacMT for the first time.

2.2. Interactive Effects of Conjugation, Temperature, and pH on the Enzymatic Activity of LacMT

The impact of PEGylation on the catalytic activity of LacMT was studied, and the effect of pre-incubating the enzyme at various temperatures and performing the activity assay at different pH levels was investigated. The relationship between temperature and the catalytic activity of enzymes is complex. It can be influenced by several factors, one of the most significant being the kinetic effect, first described by Arrhenius, where temperature influences the movement and vibration of the protein affecting its three-dimensional conformation and leading to permanent or transient changes where the stability of active conformations can better recognize the substrate and perform catalysis [40,42]. On the other hand, the influence of pH on enzyme activity is a well-established phenomenon. The pH of the medium can significantly affect the ionization of various amino acid functional groups; therefore, significant pH changes can lead to global changes in the three-dimensional structure of the biomolecule subsequently affecting the solubility and catalytic activity of

the enzyme [43]. This study aimed to assess whether the conjugation of polyethylene glycol (PEG) chains alters the effects of pre-incubating the enzyme at temperatures that exceed the standard assay temperature of 25 °C. In addition, the activity assay was carried out at three different pH levels for each incubation temperature. This approach allowed us to investigate whether PEGylation modulates the structural changes induced by incubation at higher temperatures and the catalytic activity's dependence on the assay buffer's acidity.

A mixed-level factorial design was employed for this study (for more details, see the Supplementary Materials), incorporating three levels each for temperature (25, 40, and 55 °C) and pH (3, 4, and 5), along with two conditions for conjugation (native or PEGylated LacMT). The results (Figure 3) indicate that increasing temperature, pH, and PEGylation negatively impact the catalytic activity of laccase.

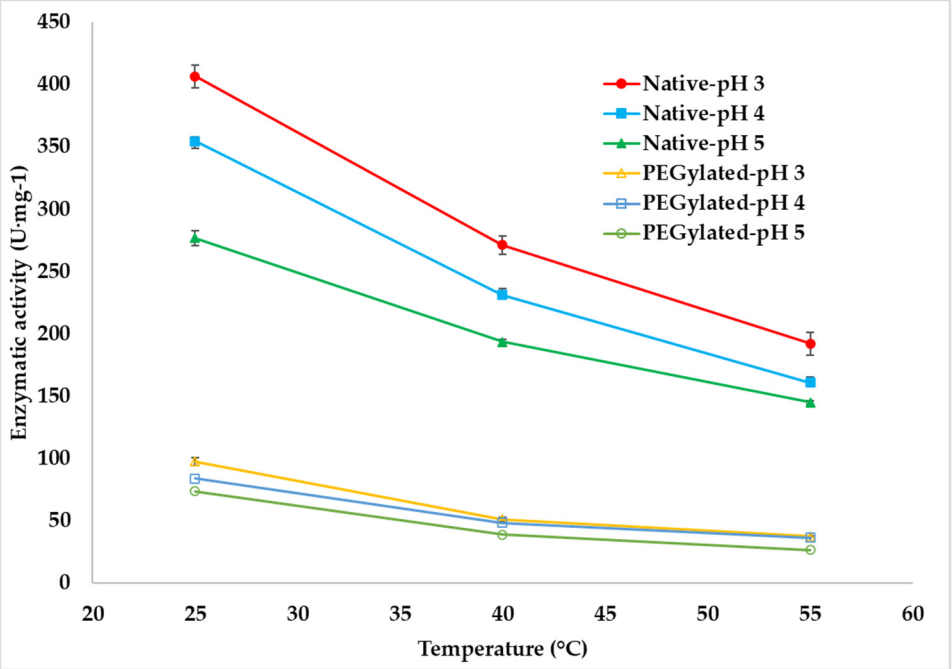

Figure 3. Enzymatic Activity at Different pH Values. Laccase LacMT samples, native (filled geometric shapes) and PEGylated (empty geometric shapes), were incubated at 25, 40, or 55 °C for one hour. After incubation, their ability to oxidize ABTS at specific pH values and 25 °C was measured.

Previous studies have indicated that temperature and pH can negatively affect enzyme activity. The structural changes resulting from high temperatures and variations in pH can influence the catalytic sites of proteins [42,43]. In addition, PEGylation can affect enzyme activity through two primary mechanisms: changes in the protein's tertiary structure due to polymer conjugation and protection of the PEG in the enzyme's active sites by steric hindrance. However, an analysis of the standardized Pareto chart (Figure 4) revealed that the combined effects of PEGylation with temperature or pH were positive (for more details, see the Supplementary Materials).

This suggests that PEG conjugation may be an essential ally in improving the resistance of LAcMT against the adverse effects of pre-incubation at elevated temperatures and variations in pH in the activity assay.

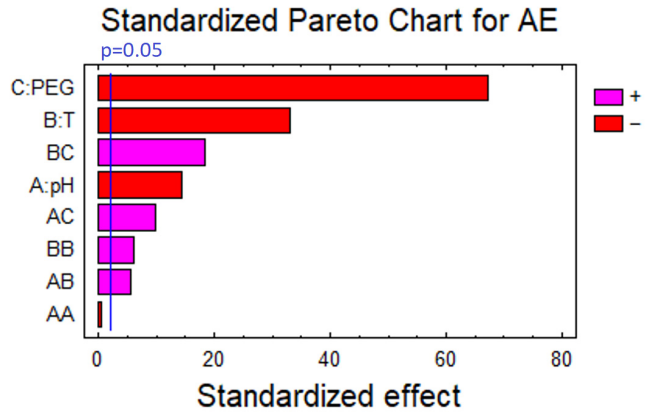

Figure 4. Pareto charts. The data presented in Figure 3 were analyzed using Statgraphics Centurion XVI software.

Effect of PEGylation on LacMT Activity at Different pH Values

First, the enzymatic activity of PEGylated and native LacMT was evaluated at pH 3 and 25 °C. A control sample containing only non-PEGylated LacMT was subjected to identical buffer exchange processes and subsequently evaluated to discern whether the observed effects on enzyme activity were attributable to conjugation or the various buffer exchange steps. The results expressed in Table 1 indicate that commercial LacMT exhibited a significantly higher specific activity than the control sample and the PEGylated enzyme. This decrease in the enzymatic activity of the control sample can be attributed to the protein denaturation processes resulting from the rapid pH changes during buffer exchange via gel filtration chromatography [44]. On the other hand, the enzymatic activity of the conjugated protein was almost double that of the control sample.

Table 1. Enzymatic Activity at pH 3.0 and 25 °C.

Sample	Activity (U·mg^{-1})
LacMT	407 ± 9
PEGylated laccase	98 ± 3
Control	51 ± 2

Su et al. [40] studied the PEGylation of LacMT with mPEG-propionaldehyde (1:6 mole ratio) at pH 5.1, in which they reported that PEGylation increased the enzyme's catalytic activity. However, their study was aimed at polymerization activity rather than oxidation per se. In contrast, Mayolo-Deloisa and colleagues [39] observed a reduced enzymatic activity when they studied the PEGylation of laccase from *Trametis versicolor*, and their method was based on ABTS oxidation (the same assay used in this research). The conjugate studied by these authors was also based on PEG functionalized as an aldehyde and reacted at pH 5.1. Under these conditions, it is reported that PEGylation occurs mainly through the N-terminus of the protein [45]. At the same time, our study focused on conjugation through the ε-amino groups of the lysine residues. It is plausible to speculate that the enhanced enzymatic activity of the PEGylated protein compared to the control sample is due to the stabilization provided by the covalently linked PEG chains during pH fluctuations after the PEGylation reaction. However, more experiments are still needed to explore the confirmation of this hypothesis, as this is the first time this phenomenon has been reported for LacMT.

In a second experiment, enzyme activity was evaluated at three different pH values to assess the influence of PEGylation on pH-dependent catalytic activity. As shown in Table 2, the enzymatic activity decreased as the pH increased from 3 to 5, both for the native enzymes (commercial laccase and control) and for the PEGylated enzyme.

Table 2. Enzymatic activity at different pH levels relative to activity at pH 3.1 The average of the three replicates for each sample at pH 3 was defined as 100%.

	Relative Activity (%) 1		
pH	**LacMT**	**Control**	**PEGylated Laccase**
3	100 ± 2	100 ± 3	100 ± 3
4	87 ± 1	86 ± 2	86 ± 2
5	68 ± 1	71 ± 1	76 ± 1

However, significant differences in activity between the groups were observed at pH 5, as determined by analysis of variance (p = 0.0081) (for more information, see the Supplementary Materials). Although the enzymatic activity of the native enzyme was consistent between the two samples, the PEGylated enzyme showed higher activity, which was consistent with the results of an LSD multiple range test. Similar findings were reported by Mazlan et al. [46], who demonstrated that the immobilization of *Trametis versicolor* laccase on methacrylate-acrylate microspheres altered the pH dependence of the enzyme. This effect was attributed to the stabilization of the tertiary structure of the immobilized protein. In the present study, the conjugation of PEG chains similarly reduces the enzyme's sensitivity to pH variations, possibly through structural stabilization.

The impact of PEGylation on the kinetic parameters of the enzyme was investigated at pH 3 using ABTS as the substrate. The control and PEGylated enzymes exhibited typical Michaelis-Menten kinetics. The catalytic turnover rate (k_{cat}) of the PEGylated enzyme was reduced by 27% compared to the control (Table 3). This reduction may be attributed to alterations in the active site of certain positional isomers within the conjugated enzyme, as the conjugate comprises a mixture of positional isomers [37].

Table 3. Catalytic constant for LacMT, native and PEGylated.

Sample	V_{max} (μM min^{-1})	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} mM^{-1})
PEGylated Laccase	597	0.9	8.8×10^5	9.8×10^5
Control	8996	1.4	1.2×10^6	8.5×10^5

Conversely, PEGylation led to a nearly 40% decrease in the Michaelis constant (K_M) (Table 3), suggesting an enhanced enzyme affinity for the substrate. This phenomenon might be explained by the amphiphilic nature of PEG, which could facilitate the interaction between the enzyme and ABTS. Notably, the catalytic efficiency (k_{cat}/K_M) remained comparable between the two forms of the enzyme. Similar findings were reported by López-Cruz et al. for the extensive conjugation of LacMT [47]. The present study demonstrated that such effects on the kinetic parameters can be achieved through the conjugation of laccase with a minimal number of PEG chains.

2.3. Effect of PEGylation on the Thermal Stability of Laccase

The effect of PEGylation on thermal stability was evaluated by measuring the enzymatic activity of native and PEGylated laccase at 25 °C following incubation at 40 °C for 1, 2, 4, 6, and 24 h. The results (Figure 5) indicated that at the final time point, the PEGylated enzyme retained more than twice the initial activity of the native protein.

Several studies have reported that PEGylation enhances the thermal stability of proteins, including laccase [37,47,48]. Two primary mechanisms have been proposed to explain this effect: (i) steric hindrance that blocks degradation pathways through hydrophobic interactions and (ii) the creation of non-specific steric hindrance that disrupts intermolecular interactions contributing to thermal instability [49].

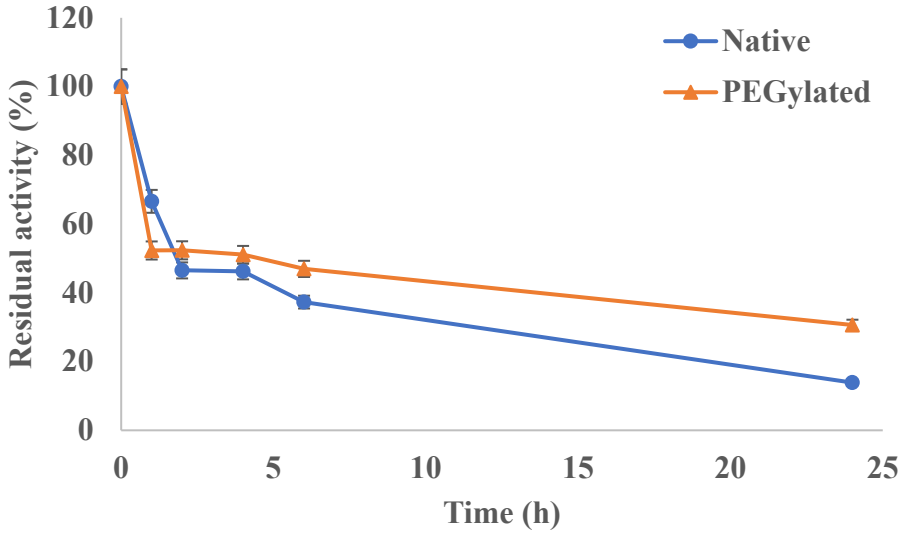

Figure 5. Thermal Stability of PEGylated Laccase. Laccase LacMT samples, native (circles) and PEGylated (triangles), were incubated at 40 °C. At designated time points, aliquots were taken, and the ability to oxidize ABTS at pH 3.0 and 25 °C was measured. Residual activity was expressed relative to the initial activity of each sample at time zero.

3. Materials and Methods

3.1. Materials

Laccase from *Myceliophthora thermophila* expressed in *Aspergillus oryzae* (Novozym 51003® laccase) and methoxy polyethylene glycol succinimidyl carbonate (mPEG-SC) with a molecular weight of 12 kDa were purchased from Sigma-Aldrich (Merck, Berlin, Germany). All other reagents used were of analytical grade.

3.2. PEGylation of LacMT

Initially, to perform the LacMT PEGylation reaction with mPEG-SC, it was necessary to change the pH of the sample since the commercial preparation is at pH 7. For this purpose, a buffer was changed to pH 9 so the protein's surface would be negatively charged and react with mPEG-SC. In addition, this buffer change made it possible to eliminate possible compounds from the commercial preparation that could interfere with the reaction. Size exclusion chromatography with a G25 column (HiTrap Desalting, 5 mL, Cytiva, Malborough, MA, USA) changed the buffer. The mobile phase was boric acid buffer pH 9 (100 Mm). Once the buffer was changed, 1 mL of enzyme extract was divided into aliquots in 1.5 mL centrifuge microtubes, and 12 kDa mPEG-SC was added at a molar ratio of 1:5 proteins/PEG in triplicates. The system was left to react at 4 °C overnight. Once the reaction was complete, the PEGylated and non-PEGylated enzymes were changed from pH 9 buffer to pH 7 buffer using 100 mM sodium phosphate pH 7 buffer. The PEGylated and non-PEGylated samples, as well as the control samples, were subjected to protein quantification, as well as enzymatic activity, which will be described below.

3.3. Assessment of Laccase Activity

The laccase activity of all samples was determined by monitoring the oxidation ABTS to $ABTS^{+\bullet}$ ratio at 420 nm using a ThermoScientific™ GENESYS™ 150 UV-Visible Spectrophotometer as described in previous studies [9]. To 100 microliters of the laccase solution, 1.7 mL of buffer at the desired pH and 200 µL of ABTS solution were added. The absorbance at 420 nm was monitored immediately at five-minute intervals, and the reaction was quenched with a concentrated acidic hydrochloric solution. One unit (U) of the laccase

activity was defined as the amount of laccase forming 1 µmol of $ABTS^{+\bullet}$ per minute and could be calculated using Equation (1):

$$Laccase\ activity\ (A)\ \left(U.mg^{-1}\right) = \frac{A \times 10^6 \times V}{\varepsilon \times t \times m} \quad (1)$$

where A is the absorbance of $ABTS^{+\cdot}$ at 420 nm (ε is the molar extinction coefficient of radical cation $ABTS^{+\bullet}$ at 420 nm = 36,000 $L.mol^{-1} \times cm$); V represents the total volume of the reaction (L); t is the reaction time (min); 10^6 is the conversion factor from M to µM; m is the amount of protein (mg).

When required, the laccase solution was incubated at specific temperatures in a thermal bath for one hour before the assay.

3.4. Determination of Kinetic Parameters

Kinetic parameters were determined using ABTS as the substrate, with concentrations ranging from 0.01 to 2.00 mM. Initial enzyme activity at each substrate concentration was measured as described in Section 3.3 under reaction conditions of pH 3 and a temperature of 25 °C. The Michaelis constant (K_M) and maximum reaction velocity (V_{max}) were calculated using the Michaelis–Menten equation:

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (2)$$

where V represents the reaction rate, and $[S]$ is the substrate concentration. The data were further analyzed using double-reciprocal plots (Lineweaver–Burk plots), and the linear model was fitted to the data using Microsoft Excel 2023.

3.5. Protein Quantification

Total protein concentration was determined by the colorimetric Bradford method (Bradford Reagent, Sigma-Aldrich, B-6916, Washington, DC, USA) [50]. The absorbance of the protein–Coomassie G-250 complex was measured at 595 nm using a plate reader spectrophotometer (BioTek Epoch 2, Winooski, VT, USA). An analytical standard curve was generated using bovine serum albumin at 1 to 10 µg/mL concentrations.

3.6. Analysis by Electrophoresis

Polyacrylamide gel electrophoresis [51] was used in this work to determine the profiles of PEGylated proteins, and concentrator and separator gels were prepared. The concentrating gel was made up of 2.1 mL of distilled water, 0.5 mL of 30% monomer solution, 0.38 mL of 1M Tris-base buffer—pH 6.8, 0.03 mL of 10% SDS, 0.03 mL of 10% ammonium persulphate (APS), and 0.003 mL of Temed (free radical stabilizer). The 12% separating gel was made up of 1.7 mL of distilled water, 2.0 mL of 30% monomer solution, 1.3 mL of 1M Tris-base—pH 8.8, 0.05 mL of 10% SDS, 0.05 mL of 10% APS, and 0.002 mL of Temed. For each sample to be analyzed, 15 µL of the sample and 15 µL of the sample application solution (1.25 mL of 1M Tris-base, 2 mL of 10% SDS, 0.5 mL of mercaptoethanol, 4 mL of 0.05% bromophenol blue, 1 mL of glycerol, and 1.25 mL of distilled water) were added to 250 µL microtubes. The samples were taken to a 95 °C bath for 10 min. A total of 20 µL of the sample was applied to each well. The vat (Bio-Rad, Hercules, CA, USA) was connected to the source, and the source was switched on at a voltage of 130 V. When the samples reached the bottom edge of the gel, the source was switched off. The gel was placed in a container, and the dye solution (Coomassie Brilliant Blue—CBB) was added. The gel was left in contact with the dye solution for an hour and a half. After this time, the dye solution was removed, and the bleach solution that had been in contact with the gel overnight was added. Densitometric analysis of the stained gels was conducted using ImageJ software, ver. 1.54g.

3.7. Thermal Stability

Samples (200 µL in 0.7 mL Eppendorf microtubes) of both conjugated and native enzymes were incubated in a water bath at 40 °C for 1, 2, 4, 6, and 24 h. After incubation, the samples reached room temperature, and enzymatic activity was measured as described in Section 3.3. Residual activity was calculated relative to the activity of the enzyme, whether PEGylated or not, without prior heat treatment.

4. Conclusions

In this work, we were able to demonstrate that our simple protocol for conjugating *Myceliophthora thermophila* laccase with mPEG-SC was able to improve its thermostability where, after 24 h, the PEGylated preparation retained more than twice the initial activity of the native protein at 40 °C. This stability is being reported for the first time. It sets precedents for constructing efficient catalytic systems involving laccases since no immobilized biocatalyst or commercial conjugate contains these proteins, which have the potential for application in various industrial sectors.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/catal14120887/s1: Table S1: Unprocessed data of specific enzymatic activity for the experimental design analysis, Table S2: Estimated effects for specific enzymatic activity, Table S3: Analysis of variance for specific enzymatic activity.

Author Contributions: L.L.O.G., conceptualization, methodology, software, validation, investigation, data curation, writing—original draft preparation; R.H.S.F., methodology, investigation, data curation, formal analysis; vs. methodology, software, validation, writing—original draft preparation; R.W., writing—review and editing, visualization, resources; J.R., conceptualization, resources, project administration, supervision, writing—original draft preparation, writing—review and editing, funding acquisition; I.I.J., conceptualization, resources, project administration, supervision, writing—original draft preparation, writing—review and editing, funding acquisition. V.S., conceptualization, validation, formal analysis, investigation. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The raw data supporting this article's conclusions will be made available by the authors of this article, and Supplementary Data can be made available upon request.

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Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Barbier, E.B.; Burgess, J.C. Economics of Water Scarcity and Efficiency. *Sustainability* **2024**, *16*, 8550. [CrossRef]
2. de S. Lira, K.R.; Zardini, R.T.; de Carvalho, M.C.C.; Wojcieszak, R.; Leite, S.G.F.; Itabaiana, I., Jr. Agroindustrial wastes as a support for the immobilization of lipase from Thermomyces lanuginosus: Synthesis of hexyl laurate. *Biomolecules* **2021**, *11*, 445. [CrossRef] [PubMed]
3. Agency, E.E. Global Average Air Temperature Anomalies. Available online: https://www.eea.europa.eu/data-and-maps/daviz/global-average-air-temperature-anomalies-6#tab-dashboard-01 (accessed on 28 August 2024).
4. Eurostat. Greenhouse Gas Emissions by Source Sector. Available online: https://ec.europa.eu/eurostat/databrowser/view/env_air_gge__custom_12545162/default/table?lang=en (accessed on 27 August 2024).
5. Kurkela, E.; Kurkela, M.; Frilund, C.; Hiltunen, I.; Rollins, B.; Steele, A. Flexible Hybrid Process for Combined Production of Heat, Power and Renewable Feedstock for Refineries: Managing seasonal energy supply and demand for heat and power in Europe. *Johns. Matthey Technol. Rev.* **2021**, *65*, 333–345. [CrossRef]
6. Nations, U. The Sustainable Development Goals Report 2023. Available online: https://unstats.un.org/sdgs/report/2023/The-Sustainable-Development-Goals-Report-2023.pdf (accessed on 18 September 2024).

7. Kokkinomagoulos, E.; Kandylis, P. Grape pomace, an undervalued by-product: Industrial reutilization within a circular economy vision. *Rev. Environ. Sci. Bio/Technol.* **2023**, *22*, 739–773. [CrossRef]
8. Kurkela, E.; Kurkela, M.; Hiltunen, I. Production of synthesis gas from biomass residues by staged fixed-bed gasification-results from pilot test campaigns. *Chem. Eng. Trans.* **2021**, *86*, 7–12.
9. do Nascimento, M.A.; Haber, B.; Gomez, M.R.; Leão, R.A.; Pietrowski, M.; Zieliński, M.; de Souza, R.O.; Wojcieszak, R.; Itabaiana, I. Optimization of 5-hydroxymethylfurfural oxidation via photo-enzymatic cascade process. *Green Chem.* **2024**, *26*, 8211–8219. [CrossRef]
10. Serbent, M.P.; Magario, I.; Saux, C. Immobilizing white-rot fungi laccase: Toward bio-derived supports as a circular economy approach in organochlorine removal. *Biotechnol. Bioeng.* **2024**, *121*, 434–455. [CrossRef]
11. Sharma, R.; Malaviya, P. Ecosystem services and climate action from a circular bioeconomy perspective. *Renew. Sustain. Energy Rev.* **2023**, *175*, 113164. [CrossRef]
12. Tatta, E.R.; Imchen, M.; Moopantakath, J.; Kumavath, R. Bioprospecting of microbial enzymes: Current trends in industry and healthcare. *Appl. Microbiol. Biotechnol.* **2022**, *106*, 1813–1835. [CrossRef]
13. Aza, P.; Camarero, S. Fungal laccases: Fundamentals, engineering and classification update. *Biomolecules* **2023**, *13*, 1716. [CrossRef]
14. Pandey, D.; Daverey, A.; Dutta, K.; Arunachalam, K. Bioremoval of toxic malachite green from water through simultaneous decolorization and degradation using laccase immobilized biochar. *Chemosphere* **2022**, *297*, 134126. [CrossRef] [PubMed]
15. Rostami, A.; Abdelrasoul, A.; Shokri, Z.; Shirvandi, Z. Applications and mechanisms of free and immobilized laccase in detoxification of phenolic compounds—A review. *Korean J. Chem. Eng.* **2022**, *39*, 821–832. [CrossRef]
16. Singh, D.; Gupta, N. Microbial Laccase: A robust enzyme and its industrial applications. *Biologia* **2020**, *75*, 1183–1193. [CrossRef]
17. Ayodeji, F.D.; Shava, B.; Iqbal, H.M.; Ashraf, S.S.; Cui, J.; Franco, M.; Bilal, M. Biocatalytic versatilities and biotechnological prospects of laccase for a sustainable industry. *Catal. Lett.* **2023**, *153*, 1932–1956. [CrossRef]
18. Contato, A.G.; Inácio, F.D.; Brugnari, T.; de Araújo, C.A.V.; Maciel, G.M.; Haminiuk, C.W.I.; Peralta, R.M.; de Souza, C.G.M. Solid-state fermentation with orange waste: Optimization of Laccase production from Pleurotus pulmonarius CCB-20 and decolorization of synthetic dyes. *Acta Scientiarum. Biol. Sci.* **2020**, *42*, 1–9. [CrossRef]
19. Khatami, S.H.; Vakili, O.; Movahedpour, A.; Ghesmati, Z.; Ghasemi, H.; Taheri-Anganeh, M. Laccase: Various types and applications. *Biotechnol. Appl. Biochem.* **2022**, *69*, 2658–2672. [CrossRef]
20. Tiwari, A.; Chen, C.-W.; Haldar, D.; Patel, A.K.; Dong, C.-D.; Singhania, R.R. Laccase in biorefinery of lignocellulosic biomass. *Appl. Sci.* **2023**, *13*, 4673. [CrossRef]
21. Wang, C.; Jia, Y.; Luo, J.; Chen, B.; Pan, C. Characterization of thermostable recombinant laccase F from Trametes hirsuta and its application in delignification of rice straw. *Bioresour. Technol.* **2024**, *395*, 130382. [CrossRef]
22. García-Fuentevilla, L.; Domínguez, G.; Martín-Sampedro, R.; Hernández, M.; Arias, M.E.; Santos, J.I.; Ibarra, D.; Eugenio, M.E. Enzyme-Catalyzed Polymerization of Kraft Lignin from Eucalyptus globulus: Comparison of Bacterial and Fungal Laccases Efficacy. *Polymers* **2023**, *15*, 513. [CrossRef]
23. Gouveia, S.; Fernández-Costas, C.; Sanromán, M.; Moldes, D. Polymerisation of Kraft lignin from black liquors by laccase from *Myceliophthora thermophila*: Effect of operational conditions and black liquor origin. *Bioresour. Technol.* **2013**, *131*, 288–294. [CrossRef]
24. Su, J.; Wang, C.; Noro, J.; Cavaco-Paulo, A.; Silva, C.; Fu, J. Polymers from bamboo extracts produced by laccase. *Polymers* **2018**, *10*, 1141. [CrossRef] [PubMed]
25. Zerva, A.; Manos, N.; Vouyiouka, S.; Christakopoulos, P.; Topakas, E. Bioconversion of biomass-derived phenols catalyzed by *Myceliophthora thermophila* laccase. *Molecules* **2016**, *21*, 550. [CrossRef] [PubMed]
26. Brugnari, T.; Pereira, M.G.; Bubna, G.A.; de Freitas, E.N.; Contato, A.G.; Corrêa, R.C.G.; Castoldi, R.; de Souza, C.G.M.; de Moraes, M.d.L.T.; Bracht, A. A highly reusable MANAE-agarose-immobilized *Pleurotus ostreatus* laccase for degradation of bisphenol A. *Sci. Total Environ.* **2018**, *634*, 1346–1351. [CrossRef]
27. Dos Santos, P.M.; Baruque, J.R.; de Souza Lira, R.K.; Leite, S.G.; Do Nascimento, R.P.; Borges, C.P.; Wojcieszak, R.; Itabaiana, I., Jr. Corn cob as a green support for laccase immobilization—Application on decolorization of Remazol Brilliant Blue R. *Int. J. Mol. Sci.* **2022**, *23*, 9363. [CrossRef]
28. Nikpour, S.; Salehipour, M.; Rezaei, S.; Mogharabi-Manzari, M. Design and fabrication of magnetic cross-linked laccase aggregate using superparamagnetic metal-organic frameworks for phenol removal. *Biochem. Eng. J.* **2024**, *209*, 109385. [CrossRef]
29. Almulaiky, Y.Q.; Alkabli, J.; El-Shishtawy, R.M. Improving enzyme immobilization: A new carrier-based magnetic polymer for enhanced covalent binding of laccase enzyme. *Int. J. Biol. Macromol.* **2024**, *282*, 137362. [CrossRef]
30. Venkatraman, S.K.; Vijayakumar, N.; Bal, D.K.; Mishra, A.; Gupta, B.; Mishra, V.; Wysokowski, M.; Koppala, S.; Swamiappan, S. Degradation of environmentally harmful textile dye rhodamine B using silicate ceramic photocatalysts. *Inorg. Chem. Commun.* **2022**, *142*, 109674. [CrossRef]
31. Azeez, S.; Sathiyaseelan, A.; Venkatesan, K.; Wang, M.-H. Fabrication of Mupirocin-Loaded PEGylated Chitosan Nanoparticulate Films for Enhanced Wound Healing. *Int. J. Mol. Sci.* **2024**, *25*, 9188. [CrossRef]
32. Baldino, L.; Riccardi, D.; Reverchon, E. Production of PEGylated Vancomycin-Loaded Niosomes by a Continuous Supercritical CO2 Assisted Process. *Nanomaterials* **2024**, *14*, 846. [CrossRef]
33. De la Torre, B.G.; Albericio, F. The pharmaceutical industry in 2023: An analysis of FDA drug approvals from the perspective of molecules. *Molecules* **2024**, *29*, 585. [CrossRef]

34. Makharadze, D.; Kantaria, T.; Yousef, I.; Del Valle, L.J.; Katsarava, R.; Puiggalí, J. PEGylated Micro/Nanoparticles Based on Biodegradable Poly (Ester Amides): Preparation and Study of the Core–Shell Structure by Synchrotron Radiation-Based FTIR Microspectroscopy and Electron Microscopy. *Int. J. Mol. Sci.* **2024**, *25*, 6999. [CrossRef] [PubMed]
35. Piao, M.; Zou, D.; Yang, Y.; Ren, X.; Qin, C.; Piao, Y. Multi-functional laccase immobilized hydrogel microparticles for efficient removal of bisphenol A. *Materials* **2019**, *12*, 704. [CrossRef] [PubMed]
36. Minten, I.J.; Abello, N.; Schooneveld-Bergmans, M.E.; van den Berg, M.A. Post-production modification of industrial enzymes. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 6215–6231. [CrossRef] [PubMed]
37. Ramon, J.; Saez, V.; Baez, R.; Aldana, R.; Hardy, E. PEGylated interferon-alpha2b: A branched 40K polyethylene glycol derivative. *Pharm. Res.* **2005**, *22*, 1374–1386. [CrossRef] [PubMed]
38. Moreno-Pérez, S.; Orrego, A.H.; Romero-Fernández, M.; Trobo-Maseda, L.; Mar-tins-DeOliveira, S.; Munilla, R.; Fernández-Lorente, G.; Guisan, J.M. Intense PEGylation of enzyme surfaces: Relevant stabilizing effects. In *Methods in Enzymology*; Academic Press: Cambridge, MA, USA, 2016; Volume 571, pp. 55–72.
39. Mayolo-Deloisa, K.; González-González, M.; Simental-Martínez, J.; Rito-Palomares, M. Aldehyde PEGylation of laccase from Trametes versicolor in route to increase its stability: Effect on enzymatic activity. *J. Mol. Recognit.* **2015**, *28*, 173–179. [CrossRef]
40. Su, J.; Noro, J.; Loureiro, A.; Martins, M.; Azoia, N.G.; Fu, J.; Wang, Q.; Silva, C.; Cavaco-Paulo, A. PEGylation greatly enhances laccase polymerase activity. *ChemCatChem* **2017**, *9*, 3888–3894. [CrossRef]
41. Morgenstern, J.; Baumann, P.; Brunner, C.; Hubbuch, J. Effect of PEG molecular weight and PEGylation degree on the physical stability of PEGylated lysozyme. *Int. J. Pharm.* **2017**, *519*, 408–417. [CrossRef]
42. Arcus, V.L.; Prentice, E.J.; Hobbs, J.K.; Mulholland, A.J.; Van der Kamp, M.W.; Pudney, C.R.; Parker, E.J.; Schipper, L.A. On the temperature dependence of enzyme-catalyzed rates. *Biochemistry* **2016**, *55*, 1681–1688. [CrossRef]
43. Bisswanger, H. Enzyme assays. *Perspect. Sci.* **2014**, *1*, 41–55. [CrossRef]
44. Baler, K.; Martín, O.A.; Carignano, M.A.; Ameer, G.; Vila, J.A.; Szleifer, I. Electrostatic unfolding and interactions of albumin driven by pH changes: A molecular dynamics study. *J. Phys. Chem. B* **2014**, *118*, 921–930. [CrossRef]
45. Behi, J.; Hassiki, R.; Said, N.B.; Bouhaouala-Zahar, B.; Benkhoud, M.L. Optimization of PEGylation reaction time and molar ratio of rhG-CSF toward increasing bioactive potency of monoPEGylated protein. *Int. J. Biol. Macromol.* **2018**, *109*, 888–895. [CrossRef] [PubMed]
46. Berka, R.M.; Grigoriev, I.V.; Otillar, R.; Salamov, A.; Grimwood, J.; Reid, I.; Ishmael, N.; John, T.; Darmond, C.; Moisan, M.-C.; et al. Comparative genomic analysis of the thermophilic biomass-degrading fungi Myceliophthora thermophila and Thielavia terrestris. *Nat. Biotechnol.* **2011**, *29*, 922–927. [CrossRef] [PubMed]
47. López-Cruz, J.; Viniegra-Gonzalez, G.; Hernández-Arana, A. Thermostability of native and pegylated myceliophthora t hermophila laccase in aqueous and mixed solvents. *Bioconjugate Chem.* **2006**, *17*, 1093–1098. [CrossRef] [PubMed]
48. Ramon, J.; Gonçalves, V.; Alvarenga, A.; Saez, V.; Nele, M.; Alves, T. Integrated Lab-Scale Process Combining Purification and PEGylation of l-Asparaginase from Zymomonas mobilis. *Ind. Eng. Chem. Res.* **2021**, *60*, 6932–6939. [CrossRef]
49. Hinds, K.D.; Kim, S.W. Effects of PEG conjugation on insulin properties. *Adv. Drug Deliv. Rev.* **2002**, *54*, 505–530. [CrossRef]
50. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
51. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685. [CrossRef]