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Article

Production of *Dichostereum sordulentum* Laccase and Its Entrapment in Lignocellulosic Biopolymers for Estrogen Biodegradation

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Abstract

The widespread presence of estrogenic pollutants in aquatic environments poses a significant threat to ecosystems and human health, necessitating the development of efficient and sustainable removal technologies. This study aimed to develop a cost-effective biocatalyst for estrogen biodegradation using a fungal laccase. The enzyme was produced by the native strain *Dichostereum sordulentum* under semi-solid-state fermentation conditions optimized using a statistical Design of Experiments. The design evaluated carbon sources (glucose/glycerol), nitrogen sources (peptone/urea), inoculum size, and *Eucalyptus dunnii* bark as a solid support/substrate. The resulting laccase was entrapped within a hydrogel made of lignocellulosic biopolymers derived from a second-generation bioethanol by-product. Maximum laccase production was achieved with a high concentration of peptone (12 g/L), a low amount of bark (below 2.8 g), 8.5 g/L glucose and 300 mg/flask of inoculum. The subsequent immobilized laccase achieved $98.8 \pm 0.5\%$ removal of ethinylestradiol, outperforming the soluble enzyme. Furthermore, the treatment reduced the estrogenic biological activity by more than 160-fold. These findings demonstrate that the developed biocatalyst not only valorizes an industrial by-product but also represents an effective and sustainable platform for mitigating hazardous estrogenic pollution in water.

Keywords: laccase; semi-solid-state fermentation; enzyme immobilization; endocrine disruptor; estrogen biodegradation

1. Introduction

The presence of pollutants in the environment, especially in water bodies, is a worldwide concert. Contaminants, such as pesticides, heavy metals, polycyclic aromatic hydrocarbons, microplastics, pharmaceuticals, and endocrine disruptors, are released into aquatic ecosystems through human activities such as agriculture, industry, and domestic waste disposal. These pollutants present serious risks to ecosystem health and human well-being due to their acute toxicity, bioaccumulation in organisms, and potential long-term effects [1,2].

Endocrine disruptors are particularly notable for their ability to interfere with the normal functioning of the endocrine system, even at low concentrations. These substances, whether natural

or synthetic, have complex effects on the environment and humans, contributing to reduced fertility, breast and prostate cancer, and causing feminization and hermaphroditism in aquatic animals [3]. Moreover, these compounds are particularly hazardous to vulnerable aquatic vertebrates, such as amphibians and fishes, and may lead to local species extinction at sub-lethal exposure levels [4]. Disruption of the endocrine system can occur in different ways depending on the pollutants' mechanism of action. Some of them can mimic a natural hormone or block its effect from certain receptors, others could have a direct impact on the endocrine system increasing or decreasing hormones production [5]. In humans, fetuses, children, and adolescents are at the highest risk of being affected. Endocrine disruptors are found in everyday products like canned goods, detergents, resins, plastics, pesticides, food, and cosmetics, and they reach the environment through various pathways, with wastewater treatment plants being a major point of entry, particularly for surface waters [3].

The European Commission has identified the estrogens 17 β -estradiol (E2) and 17 α -ethinylestradiol (EE2) as the most dangerous contaminants due to their endocrine-disrupting effects [6,7]. The use of synthetic estrogens in hormonal contraceptives, such as EE2, exacerbates their environmental impact. This compound shows higher estrogenic responses than the natural hormone E2, degrades more slowly, and persists longer in aquatic environments [8].

The presence of EE2 has been detected in surface waters at basin scale in Uruguay (concentrations between 0.13 and 45.51 $\mu\text{g/L}$), where oral contraceptives constitute the main method of contraception used [9,10].

Conventional wastewater treatment methods remove only a small fraction of these contaminants, proving ineffective. Advanced analytical techniques have enabled the detection of these compounds at trace concentrations in various matrices, including surface and deep waters, sediments, soil, wastewater, and irrigation water [11]. In response to these challenges, biodegradation emerges as a promising and sustainable alternative. This approach prevents the environmental release of estrogens, is non-invasive, utilizes renewable resources, and operates under mild conditions. Moreover, it is cost-effective and facilitates the transformation of the contaminants into less toxic or non-toxic compounds [12]. Enzymes present in the environment play an essential role as biological agents in wastewater treatment, as they have the potential to degrade and detoxify harmful compounds, contributing to environmental restoration [13]. In particular, fungal laccases can degrade endocrine disruptors, including natural estrogens (estrone, E2, estriol) and the synthetic estrogen EE2 [14–17]. However, laccases face industrial limitations due to their instability, difficulty in recovering the used free enzyme, and lack of reusability (Sheldon and Woodley 2018; Tikhonov et al. 2019; Wang et al. 2024). Thus, enzyme immobilization is an effective strategy to overcome these disadvantages. Among the different immobilization methods, one of the simplest techniques is the entrapment of the biocatalyst within a solid or gel matrix.

The present work further investigates the development of an active hydrogel for estrogen removal from water. This hydrogel has a laccase entrapped in a polymeric net of biopolymers derived from a lignocellulosic residue. The use of lignocellulosic materials to obtain the insoluble matrix represents a cost-effective and environmentally sustainable approach [18,19]. Laccases (benzenediol: oxygen oxidoreductases, EC1.10.3.2) belong to the multicopper oxidase family [20] and have been widely studied due to their ability to oxidize various phenolic compounds (e.g., phenols, polyphenols, and anilines) and their potential application in various processes, like endocrine disruptors degradation [14,15,21–26]. We have previously reported preliminary results concerning to EE2 removal by the laccase from a native strain of the basidiomycete *Dichostereum sordulentum* (DS), in both its soluble and immobilized forms [27]. A lignocellulosic material generated after second-generation (2G) bioethanol production was used as a source of biopolymers, mainly lignin, for hydrogel synthesis. To this end, using a 2G bioethanol by-product, we developed an eco-friendly method for laccase immobilization, based on lignin dissolution in ionic liquids (IL). These liquids are molten salts, behaving as green, non-volatile and thermally stable solvents [28]. After the solubilisation process, the mixture of biopolymers and IL was used to entrap laccase giving an active

hydrogel [29–31].

Prior to this study, DS laccase had only been obtained through submerged fermentation [32]. Laccase production from native microorganisms is particularly relevant, as it provides novel enzymes with unexplored potential. Ensuring an adequate production level is a key objective for enabling its practical application, and selecting an appropriate culture system is a crucial strategy to achieve this aim. In this study, semi-solid-state fermentation (SSSF) was chosen, as it utilizes an insoluble solid material as a support, mimicking the natural growth conditions of fungi. Additionally, this approach provides significant advantages over submerged fermentation in terms of cost-effectiveness and sustainability. Moreover, it adds value to agro-industrial residues as they are used as a solid support, preventing disposal and enhancing environmental and economic benefits [33,34]. In the present study, *Eucalyptus dunnii* bark was used as the support in an SSSF system and selected culture variables were analyzed to enhance laccase production by *D. sordulentum*, applying the statistical Design of Experiment (DOE) methodology.

After the entrapping process, the catalytic efficiency of the insoluble biocatalyst in degrading the endocrine disruptor EE2 was quantitatively assessed, using a highly sensitive analytical method. Additionally, the estrogenic activity of the treated solution was evaluated to determine the effectiveness of the enzymatic process in reducing toxicity, thus expanding the understanding of the biotechnological potential of the biocatalyst.

2. Results and Discussion

2.1. Optimization of Culture Medium for Laccase Production

Three sequential experimental designs were performed. Employing statistically-based experimental design methodologies offers significant advantages in process optimization, including reducing the number of assays, saving time and reagents, and enabling the analysis of both individual effects and interactions among independent variables [35]. In the first study, two experiments were carried out, one for each C source, applying the Multilevel categoric factorial design. In the Analysis of variance (**Tables 1 and 2**) values of "Prob>F" less than 0.0500 indicate model terms are significant. The residual error was decomposed in a pure error term and in a lack of fit term. The pure error was obtained from the replicates of experimental points. For both C sources, the model was validated with no significant lack of fit. In both cases the effect of the N source was significant, and when glucose was used, the interaction glucose-bark was also significant. This interaction, depicted in Figure 1a, prompted the subsequent experiment to further investigate the combined effect of both factors, aiming to maximize the obtained response. Enhanced laccase activity was observed when peptone was used as the nitrogen source (**Figure 1**).

Table 1. – ANOVA [Partial sum of squares -Type III], Multilevel categoric factorial design for Glucose.

Source	Sum of Squares	df	Mean Square	F Value	p- value Prob > F
Model	3.339E+008	6	5.566E+007	9.17	0.0021
A-Source of N	2.433E+008	1	2.433E+008	40.07	0.0001
B-Glucose	2.085E+007	1	2.085E+007	3.43	0.0969
C-Bark	1.150E+007	1	1.150E+007	1.89	0.2020
AB	7.373E+006	1	7.373E+006	1.21	0.2991
AC	9.213E+006	1	9.213E+006	1.52	0.2492
BC	4.172E+007	1	4.172E+007	6.87	0.0277
Residual	5.464E+007	9	6.071E+006		
Lack of Fit	1.141E+006	1	1.141E+006	0.17	0.6904
Pure Error	5.350E+007	8	6.688E+006		
Cor Total	3.886E+008	15			

Table 2. – ANOVA [Partial sum of squares -Type III], Multilevel categoric factorial design for Glycerol.

Source	Sum of Squares	df	Mean Square	F Value	p- value Prob > F
Model	2.768E+008	5	5.535E+007	4.07	0.0283
A-Source of N	1.739E+008	1	1.739E+008	12.78	0.0051
B-Glycerol	6.395E+006	1	6.395E+006	0.47	0.5087
C-Bark	5.839E+007	1	5.839E+007	4.29	0.0652
AB	1.666E+007	1	1.666E+007	1.22	0.2945
AC	2.143E+007	1	2.143E+007	1.57	0.2382
Residual	1.361E+008	10	1.361E+007		
Lack of Fit	1.066E+007	2	5.328E+006	0.34	0.7218
Pure Error	1.255E+008	8	1.568E+007		
Cor Total	4.129E+008	15			

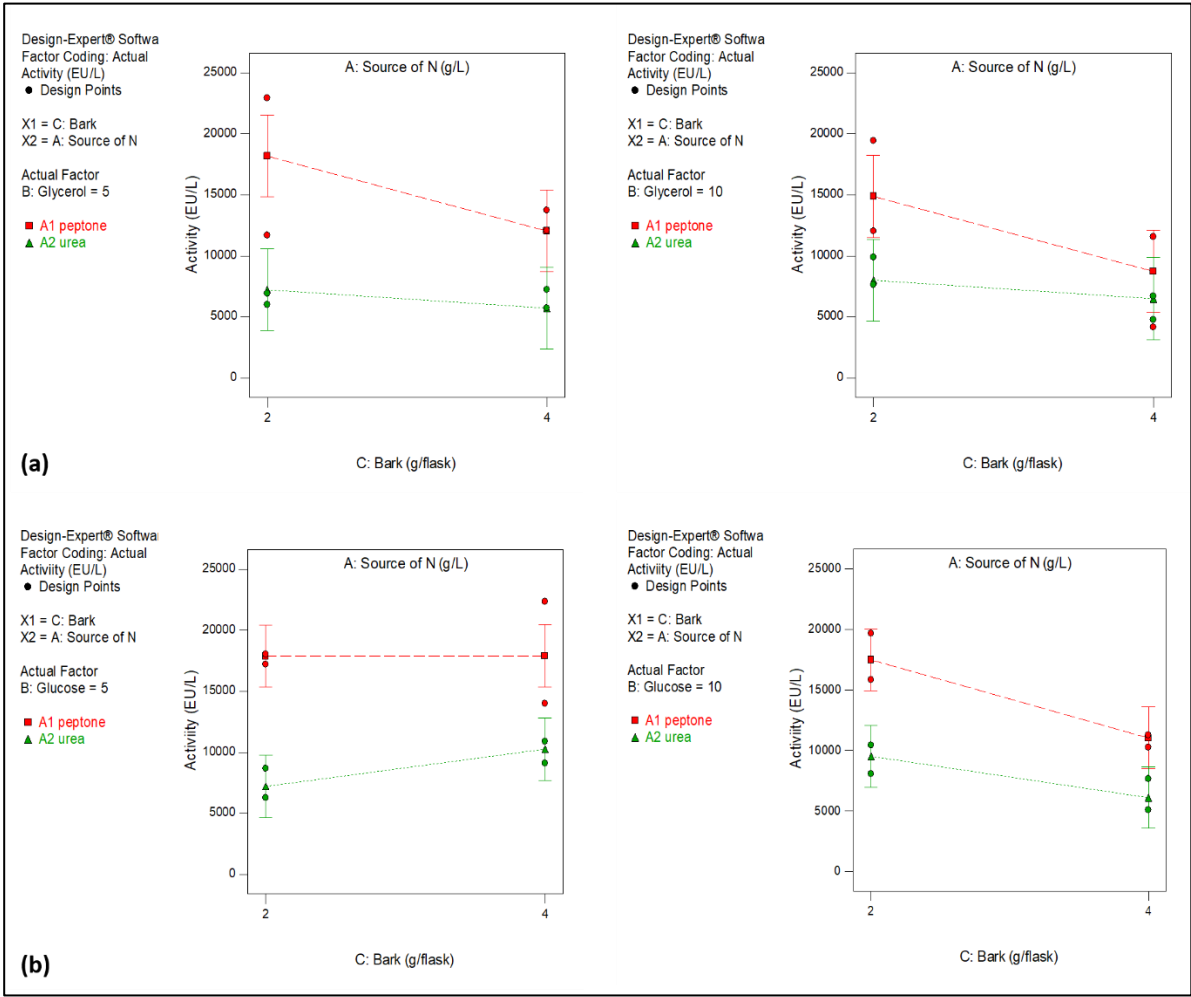


Figure 1. Multilevel categorical factorial design plot, showing the effect of N source, C source and bark on enzymatic activity for (a) glycerol and (b) glucose.

Therefore, these N and C sources were selected for the Full 2⁴ factorial design and the effect of their concentration was studied. Moreover, the effect of both the quantity of bark and the inoculum size were also analyzed. The Pareto analysis chart showed for this design that the most negative factor was the amount of bark, followed by the inoculum size (**Figure 2**). On the other hand, the amount of peptone had a positive effect on the expressed laccase activity. No effect of the glucose concentration was observed within the assessed range. However, the maximum activity values were obtained at the central points of the experiment, where the glucose concentration was 8.5 g/L. Therefore, this concentration was selected to continue the study. The model derived from this experiment was found to be significant (**Table 3**). Since the model presented curvature (for some factors there is no linear

relationship with the response), it was appropriate to perform a Central Composite design to optimize the process.

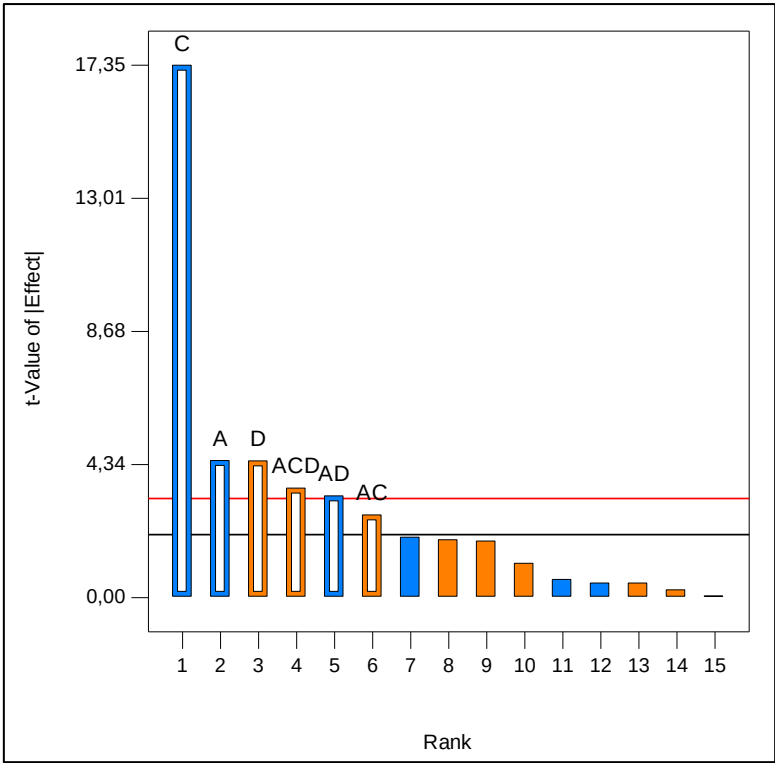


Figure 2. Pareto analysis chart (in orange factors with positive effects, in blue negative effects) for Full 2⁴ factorial design. A-Inoculum, C-Bark, D-Peptide. Bonferroni Limit 3.23131 (red line), t-Value Limit 2.05553 (black line).

Table 3. ANOVA of the Adjusted model for Full 2⁴ factorial design.

Source	Sum of Squares	df	Mean Square	F Value	p- value Prob > F
Model	1.769E+009	6	2.948E+008	58.68	< 0.0001
A-Inoculum	1.004E+008	1	1.004E+008	19.97	0.0001
C-Bark	1.513E+009	1	1.513E+009	301.04	< 0.0001
D-Peptide	9.977E+007	1	9.977E+007	19.86	0.0002
AC	3.649E+007	1	3.649E+007	7.26	0.0124
AD	5.522E+007	1	5.522E+007	10.99	0.0028
ACD	6.394E+007	1	6.394E+007	12.73	0.0015
Curvature	1.057E+009	1	1.057E+009	210.28	< 0.0001
Residual	1.256E+008	25	5.024E+006		
Lack of Fit	6.914E+007	9	7.682E+006	2.18	0.0837
Pure Error	5.647E+007	16	3.529E+006		

Cor Total

2.951E+009

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The Central Composite design has several advantages, among them, it allows a second-order polynomial model to be fitted to the data. This model can capture nonlinear relationships between the input factors and the response. It provides enough information to estimate the main effects, the interactions, and the curvature of the response surface, moreover, it requires fewer experiments than other designs [36]. The analysis of variance revealed that the amount of bark was the most critical factor, with significant interactions observed between it and both the N source and the inoculum (Table 4). Plots in Figure 3 provide a visualization of the interaction between the studied factors and their influence on laccase activity. Activity increased as the bark content decreased, reaching a maximum between 1.0 and 2.8 g per flask, independent of peptone concentration, using an inoculum of 300 mg/flask. As it can be observed in Figure 3c, maximum enzyme production was achieved using 2.5 g of bark per flask and an inoculum of 300 mg/flask.

The resulting final equation for this model, in terms of actual factors was: Activity (EU/L) = 24975.94187 + 305.90068 x Inoculum + 21489.68620 x Bark - 12850.66832 x Peptone - 9.42430 x Inoculum x Bark - 4.09822 x Inoculum x Peptone - 1399.42989 x Bark x Peptone - 0.41247 x Inoculum² - 1510.33822 x Bark² + 830.79382 x Peptone²

The production of laccase by *D. sordulentum* had been reported only once before, in a study on production of ligninolytic enzymes by a strain isolated in Uruguay [32]. In that work, a laccase activity of 6600 EU/L was achieved using a submerged fermentation system. Notably, for the same strain in the present study, semi-solid-state fermentation (SSSF) successfully improved laccase production nearly fourfold. This approach also enabled the valorization of a forestry industry residue through a culture method particularly well-suited for the growth of filamentous fungi. Eucalyptus bark is a residue generated in large quantities in Uruguay, where forestry is a major economic activity. Over one million hectares are dedicated to this industry, with Eucalyptus being the primary genus planted, and *E. dunnii* representing the second most important Eucalyptus species, accounting for 25% of the total forested area [37]. SSSF is a process based on solid-state culture with the addition of minimal free liquid to improve fermentation control and enhance fungal nutrient absorption [38,39]. In addition to allowing the use of a larger quantity of residue, SSSF presents lower operational costs due to the absence of stirring requirements and yields an extract with higher enzymatic activity in a reduced volume, compared to the submerged fermentation system.

Table 4. ANOVA for Response Surface Quadratic model [Partial sum of squares - Type III].

Source	Squares	df	Square	Value	Prob > F
Model	8.302E+008	9	9.224E+007	49.49	0.0002
A-Inoculum	6.184E+006	1	6.184E+006	3.32	0.1282
B-Bark	8.426E+007	1	8.426E+007	45.20	0.0011
C-Peptone	1.538E+007	1	1.538E+007	8.25	0.0349
AB	3.291E+007	1	3.291E+007	17.66	0.0085
AC	1.107E+007	1	1.107E+007	5.94	0.0589
BC	7.258E+007	1	7.258E+007	38.94	0.0015
A ²	2.574E+007	1	2.574E+007	13.81	0.0138
B ²	1.661E+006	1	1.661E+006	0.89	0.3885
C ²	1.081E+006	1	1.081E+006	0.58	0.4806
Residual	9.320E+006	5	1.864E+006		
Lack of Fit	7.317E+006	2	3.659E+006	5.48	0.0996
Pure Error	2.003E+006	3	6.675E+005		
Cor Total	8.395E+008	14			

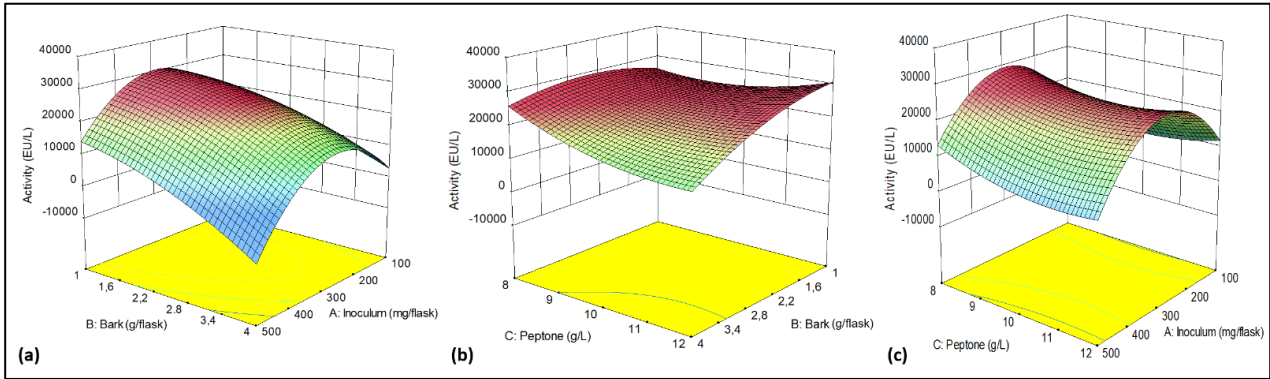


Figure 3. 3D graphs for Response Surface Quadratic model. a) Effect of Bark and Inoculum (Peptone conc. = 12 g/L). b) Effect of Bark and Peptone (Inoculum = 300 mg/flask). b) Effect of Inoculum and Peptone (Bark= 2.5 g/flask).

2.2. Evaluation of EE2 Removal by Soluble and Immobilized Laccase

The efficiency of EE2 removal was quantitatively compared between the soluble and immobilized forms of *D. sordulentum* laccase using UHPLC-MS/MS. This technique was selected for its superior sensitivity and low limits of detection (26.1-27.3 ng/L for EE2 [40,41]), essential for the accurate quantification of trace-level contaminants [42].

For the soluble enzyme, a clear dose-dependent response was observed (Table 5). Increasing the enzyme units from 0.1 to 0.3 significantly enhanced EE2 removal, but a further increase to 0.5 EU yielded only marginal improvement. This plateau suggests product-mediated inhibition, where reactive intermediates (e.g., free radicals, quinoid, or polymerized compounds) may interfere with laccase activity at higher conversion levels [43,44].

In contrast, the immobilized biocatalyst proved significantly more effective, achieving 98.8 ± 0.5% EE2 removal. This result not only confirms but also quantitatively validates our previous findings obtained with HPLC-UV, where we reported ~97% removal [27].

To elucidate the removal mechanism, the contribution of adsorption by the hydrogel matrix was investigated. The blank sample (B-EE2, hydrogels without entrapped enzyme) showed detectable decrease in EE2 concentration, suggesting analyte adsorption. This was confirmed by washing the hydrogels with AcOEt: from active hydrogels, 10.6 ± 5.7% of initial EE2 was recovered, versus ~20% from non-active hydrogels (B-EE2 W). These results demonstrate that while the blank hydrogel retains EE2 mainly through adsorption, the active hydrogel combines adsorption with enzymatic degradation, achieving more complete contaminant removal.

Table 5. Results of the UHPLC-MS/MS analysis.

Sample	EE2 recovered (%)
EE2	102.6 ± 7.1
R-SLac 0.1	27.2 ± 2.1
R-SLac 0.3	9.6 ± 1.0
R-SLac 0.5	7.1 ± 1.5
R-ILac	1.2 ± 0.5
B-EE2	3.0 ± 0.2
B-ILac	<LOD
SLac	<LOD
R-ILac W	10.6 ± 5.7
B-ILac W	<LOD
B-EE2 W	20.2 *

EE2 = Ethinylestradiol in buffer. R-SLac = Reaction with soluble laccase (0.1 to 0.5 EU). R-ILac = Reaction with immobilized laccase. B-EE2 = Blank of reaction using hydrogel beads without enzyme. B-ILac= Active hydrogel

beads in buffer without EE2. SLac = Laccase in buffer (not fortified). R-ILac W = Washes with ethyl acetate of active hydrogels previously used for estrogen degradation. B-ILac W = Washes with ethyl acetate of active hydrogels. B-EE2 W = Washes with ethyl acetate of B-EE2. Error shown represents standard deviation. * Unique sample.

The distinct EE2 recovery of active and blank hydrogels aligned with their performance in reducing estrogenic activity, as quantified by the Yeast Estrogen Screen (YES) assay (**Table 6**). This bioassay utilizes a genetically engineered yeast strain expressing the human estrogen receptor (hER), where estrogenic compounds induce β -galactosidase production, measured via CPRG substrate conversion [45].

The solution treated with active hydrogel beads exhibited a 160-fold reduction in overall estrogenicity compared to the untreated EE2, representing a significantly greater effect than the approximately 10-fold reductions observed for blank hydrogel (B-EE2) and free enzyme (R-SLac) controls.

Statistical analysis (one-way ANOVA, $F = 344.2841$, $p < 1.161 \times 10^{-10}$) confirmed significant differences among treatments. Tukey HSD post-hoc analysis verified that the active hydrogel (R-ILAC) yielded a significantly higher EC50 than all other samples. Together, the UHPLC-MS/MS and YES data provide complementary evidence that the immobilized biocatalyst not only removes EE2 but also drastically reduces its hormonal activity, generating transformation products with substantially lower estrogen receptor affinity. When compared to studies that have coupled chemical degradation with biological validation, our system – achieving a potency reduction greater than 99% – demonstrates exceptional performance, placing it among the most efficient approaches reported for enzymatic estrogen detoxification [14,46].

Table 6. Effective concentration 50 (EC50) of different treatment with three replicates (mean $\pm$ SD).

Sample	EC50 ($\mu\text{g/L}$)	Relative Potency
E2	0.047 $\pm$ 0.02	
EE2	0.028 $\pm$ 0.02	1,68
EE2 + soluble laccase (R-SLac 0.5)	0.391 $\pm$ 0.33	1,20 $\times 10^{-1}$
EE2 + hydrogel beads (B-EE2)	0.598 $\pm$ 0.13	7,90 $\times 10^{-2}$
EE2 + active hydrogel beads (R-ILac)	4.819 $\pm$ 0.22	9,75 $\times 10^{-3}$

* Relative Potency (RP) = EC50 (E2) / EC50 (test compound).

3. Materials and Methods

3.1. Chemicals

1-Butyl-3-methylimidazolium acetate (BmimAc) was purchased from IoLi-Tec Ionic Liquids Technologies GmbH Heilbronn, Germany. Ethinylestradiol and 2,6-dimethoxyphenol (DMP) were purchased from Sigma-Aldrich, St. Louis, MO, USA, and dimethyl sulfoxide from Carlo Erba (Milan, Italy).

3.2. Laccase Activity Assay

Laccase activity was determined using 2.0 mM DMP in 0.1M sodium acetate buffer pH 3.8. The reaction was monitored by measuring the formation of quinone at 477 nm ($\epsilon_{477} = 14.800 \text{ M}^{-1}\text{cm}^{-1}$) using a Shimadzu UV-1800 spectrophotometer. The reaction mixture was composed of 500 μL of DMP solution and 50 μL of laccase sample, prepared in the same buffer. One enzyme unit (EU) was defined as the amount of enzyme that catalyzed the appearance of 1 μmol of product per minute at 25 $^{\circ}\text{C}$ [27].

The immobilized enzyme activity was assayed by incubating active hydrogel beads with substrate under magnetic stirring (100 rpm). Supernatant aliquots were withdrawn from the reaction mixture at 30-second intervals and were returned afterward, keeping its volume unchanged. To

detect enzyme release, after measuring the immobilized activity, the hydrogels beads were removed, the filtrates incubated at room temperature, and variation in absorbance at 477 nm determined again.

3.3. Protein Determination

Protein content was quantified using the bicinchoninic acid (BCA) assay, bovine serum albumin was used as a standard [47]. The amount of immobilized protein was calculated as the difference between the total protein applied to the gel and the protein recovered in the collected supernatants and washes.

3.4. Laccase Production

A native strain of *Dichostereum sordulentum* (1488), isolated from Eucalyptus forests in Uruguay [48], was employed for laccase production under semi-solid-state fermentation (SSSF) conditions. It was grown in potato dextrose agar (PDA) at 28 °C for 7 days. A preculture in liquid medium was done, in cotton-plugged Erlenmeyer flasks (250 mL) containing 100 mL of malt extract 5%, Bactopeptone 1% and *Eucalyptus dunii* bark 0.25%. It was inoculated with five agar plugs (10 mm diameter) from PDA culture and incubated at 28 °C on a rotary shaker at 150 rpm for 7 days. At the end of the incubation, the preculture was homogenized and centrifuged, giving a fungal biomass pellet. Unless otherwise indicated, the inoculum was prepared by resuspending 100 mg of the pellet in 3.0 mL of sterile 0.1 M sodium phosphate buffer pH 6.0. The basal medium consisted of CuSO₄ 1 mM, KH₂PO₄ 2 g/L, MgSO₄·7H₂O 0.5 g/L, CaCl₂·2H₂O 0.1 g/L, in citrate-phosphate buffer 0.1 M, pH 5.0. *Eucalyptus dunii* bark was employed as the support-substrate (average granulometry 2 mm). The experiments for culture optimization were carried out in 250 mL flasks with 20 mL of liquid basal medium. They were incubated statically at 28 °C for 14 days. After that, 5 mL of 0.1M pH6.0 sodium phosphate buffer was added, shaken for 15 minutes, and centrifuged at 10,000 rpm for 10 min at 4 °C, laccase activity was measured in the supernatant.

3.5. Optimisation of Culture Medium for Laccase Production

Laccase production was optimized through a rational sequence of three experimental designs using the software Design Expert® version 10.0 by Stat-Ease, Inc. (Suite 480, Minneapolis, MN, USA) for the design and analysis. The figures corresponding to the experimental designs were also generated using this software. Laccase activity measured in the culture supernatant was the monitored response, expressed in EU/L. Raw experimental data for all the experimental designs are shown in the Appendix Supporting information.

3.5.1. Multilevel Categorical Model

This statistical model was used to evaluate the effect on laccase production of nitrogen (N) source (peptone or urea); carbon (C) source (glycerol or glucose) and bark. For the N source, a fixed concentration (10 g/L) was used while C source concentration and bark amount varied between 5 -10 g/L and 2 or 4 g per flask, respectively. These factors and ranges in which they were used were chosen based on literature and previous exploratory experiments. Two identical experimental designs were carried out, one for glucose and the other for glycerol, each one with 16 runs. The basal medium was supplemented with the C source, the N source and with *E. dunii* bark, as specified for each run in the design.

3.5.2. Full 2⁴ Factorial Design

Based on the results achieved with the Multilevel categorical model, Full 2⁴ factorial design was chosen to better adjust the levels of selected factors and assess a new factor to enhance laccase production. The new conditions were: inoculum (100-1000 mg/flask), glucose (2-15 g/L), bark (1-5 g/flask) and peptone (2-10 g/L). Four central points were used, and runs were performed in duplicate, so the new design required 36 runs.

3.5.3. Central Composite Design

In this statistical design the levels of the factors that showed a negative effect in the Factorial design were decreased: inoculum (100-500 mg/flask), bark (1-4 g/flask). On the other hand, peptone concentration was increased (8-12 g/L) and glucose concentration was set at 8.5 g/L. The model included 5 central points, generating a design of 19 runs.

3.6. Active Hydrogel Formation

Active hydrogel formation was performed under the optimized conditions previously reported [27].

The biopolymers were sourced from the solid residue generated during bioethanol production from *Eucalyptus* biomass. One gram of BmimAc was placed in a 20 mL vial and heated up to 100°C under gentle stirring with 0.6 mL of DMSO. Then, the lignocellulosic residue (175 mg) was added and stirred until dissolution. The mixture was cooled down to 40 °C, the lyophilized enzyme was added (30 EU), and the vial content was quickly transferred to a plastic syringe and dripped over 0.05 M pH 5.0 acetate buffer, giving beads of hydrogel. After removing the supernatant by filtration, the hydrogel beads were washed with the same buffer under gentle stirring conditions. Laccase activity was measured in supernatant, washes, and beads.

3.7. Estrogen Biodegradation

Soluble laccase (SLac): A reaction mix (final volume = 5 mL) was prepared in 0.1 M sodium acetate buffer pH 5.0 containing aliquots of laccase (0.1, 0.3 and 0.5 EU) and ethinylestradiol (0.01 mg/mL). The mixture was gently agitated at 20°C for 24 h and the reaction was stopped by freezing the samples, followed by lyophilization. Immobilized laccase (ILac): Suction-dried aliquots of insoluble enzyme, containing 0.1 EU, were incubated with EE2, following the same protocol as for the soluble enzyme, except that the reaction was stopped by removing the hydrogel by filtration. Three blanks were analyzed: soluble laccase in buffer solution, supernatant from the incubated suspension of active hydrogel in buffer, and supernatant from the incubated suspension of hydrogel beads without enzyme in EE2 solution. Once the hydrogels (with or without enzyme) were separated from the supernatants, they were washed with ethyl acetate and these washes were also analysed.

Analytical: Samples were analyzed by Ultra-High Performance liquid chromatography coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometry detector (Waters Acquity, Applied Biosystems), (UHPLC-MS/MS), according to [41]. Lyophilized samples were redissolved in methanol (EE2, 25 mg/L) and centrifugated at 10,000 rpm during 10 min, giving the stock solutions. Aliquots of these solutions were diluted to achieve an EE2 concentration of 25 µg/L. 10 µL of a 1 mg/L standard solution of ethinylestradiol-d4 in methanol were added to the samples to get a final concentration of 10 µg/L. The samples were injected in the UHPLC-MS/MS system, under negative ionization mode, together with a calibration curve of analyte concentrations 0.52, 1.03, 5.15, 10.31, 25.77, 51.53 and 103.07 µg/L. The results were processed in the Analyst software.

3.8. Yeast Estrogen Screen (YES) Bioassay

To determine the effectiveness of laccase enzyme on estrogenicity reduction by degradation of EE2, a yeast strain kindly provided by Prof. Eduard Routledge (Brunel University) was used, and the assay developed in laboratories of CURE Maldonado. The assay procedure was according to the original protocol [49] following the adaptations of Bila et al. (2007). Briefly, the yeast stock stored at -20°C in a cryogenic tube (2 mL) with growth medium and glycerol (40%) was added to 10 mL of the growth medium and grew on an orbital shaker 48 h. 100 µL of culture were added for a new growth medium (10 mL) and grew on an orbital shaker for another 24 h. The assay medium was prepared by mixing 25 µL of the above solution, 25 mL of growth medium, and 250 µL of the Chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG, 10 mg/mL). The 17β-estradiol (E2) standard solution (54.48 µg/L) and the samples extracts were serially diluted in ethanol and 10 µL of

each dilution were transferred (in duplicate) into a 96-well cell culture flat bottom microtiter plate (Cellstar, Greiner bio-one) and allowed to evaporate until dryness under laminar flow cabinet. Then 200 μ L of the assay medium were seeded into each well and dilution series of E2 were used as a calibration curve. Plates were sealed with breathable masking tape and vigorously shaken on a plate shaker for 2 min. Then plates were incubated in darkness at 30 °C during 72 h for colour development and absorbance was read at 540 nm on a plate reader FLUOstar Optima (BMG). Estrogenic activity was calculated as E2 equivalents (E2-EQ) by interpolation from the E2 standard curves (ng/L). The Effective Concentration 50 (EC50) was obtained from the Hill's equation calculated from the sigmoidal curve generated by the serial dilution of the samples using R software and the drc package. ANOVA analyses were performed in order to determine a reduction on the estrogenic activity.

4. Conclusions and Future Work

Laccase from *Dichostereum sordulentum* was successfully produced through semi-solid-state fermentation using *Eucalyptus dun nii* bark, achieving a fourfold increase in enzymatic activity compared to the previous submerged fermentation system. When coupled with our previously developed immobilization approach—entrapping the enzyme in a biopolymeric net derived from a lignocellulosic bioethanol residue—the resulting biocatalyst proved highly effective. It achieved near-complete removal of 17 α -ethinylestradiol (EE2) at concentrations exceeding those typically found in contaminated water bodies [46] and drastically reduced the estrogenic activity of the treated solution, supporting its potential applicability for wastewater treatment processes.

This integrated process, which valorizes agricultural and industrial by-products, aligns with green chemistry principles by utilizing renewable resources and minimizing waste. Therefore, this technology represents a promising and sustainable approach for wastewater treatment that could provide added value to both the forestry industry and ethanol biorefineries. Future studies should focus on exploring biocatalyst reusability, testing its efficacy in authentic wastewater samples, and scaling up the application.

Supplementary Materials: Table S1a: Multilevel categoric factorial design for glycerol; Table S1b: Multilevel categoric factorial design for glucose; Table S2: Full Factorial Design; Table S3: Central Composite Design.

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References

1. Hejna, M.; Kapuścińska, D.; Aksmann, A. Pharmaceuticals in the Aquatic Environment: A Review on Eco-Toxicology and the Remediation Potential of Algae. *Int. J. Environ. Res. Public Health* **2022**, *19*.
2. Larsson, D.G.J. Pollution from Drug Manufacturing: Review and Perspectives. *Philos. Trans. R. Soc. B Biol. Sci.* **2014**, *369*, doi:10.1098/RSTB.2013.0571.
3. Moukhtari, F. El; Martín-Pozo, L.; Zafra-Gómez, A. Strategies Based on the Use of Microorganisms for the Elimination of Pollutants with Endocrine-Disrupting Activity in the Environment. *J. Environ. Chem. Eng.* **2023**, *11*, 109268, doi:10.1016/J.JECE.2023.109268.
4. Kidd, K.A.; Blanchfield, P.J.; Mills, K.H.; Palace, V.P.; Evans, R.E.; Lazorchak, J.M.; Flick, R.W. Collapse of a Fish Population after Exposure to a Synthetic Estrogen. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 8897–8901, doi:10.1073/pnas.0609568104.
5. EPA (United States Environmental Protection Agency) Overview of Endocrine Disruption Available online: <https://www.epa.gov/endocrine-disruption/overview-endocrine-disruption> (accessed on 8 May 2024).
6. Johnson, A.C.; Dumont, E.; Williams, R.J.; Oldenkamp, R.; Cisowska, I.; Sumpter, J.P. Do Concentrations of Ethinylestradiol, Estradiol, and Diclofenac in European Rivers Exceed Proposed EU Environmental Quality Standards? *Environ. Sci. Technol.* **2013**, *47*, 12297–12304, doi:10.1021/es4030035.
7. Yazdan, M.M.S.; Kumar, R.; Leung, S.W. The Environmental and Health Impacts of Steroids and Hormones in Wastewater Effluent, as Well as Existing Removal Technologies: A Review. *Ecologies* **2022**, *3*, 206–224, doi:10.3390/ecologies3020016.
8. Du, B.; Fan, G.; Yu, W.; Yang, S.; Zhou, J.; Luo, J. Occurrence and Risk Assessment of Steroid Estrogens in Environmental Water Samples: A Five-Year Worldwide Perspective. *Environ. Pollut.* **2020**, *267*, 115405, doi:10.1016/j.envpol.2020.115405.
9. Haakenstad, A.; Angelino, O.; S Irvine, C.M.; Bhutta, Z.A.; Bienhoff, K.; Bintz, C.; Causey, K.; Ashworth Dirac, M.; Fullman, N.; Gakidou, E.; et al. Measuring Contraceptive Method Mix, Prevalence, and Demand Satisfied by Age and Marital Status in 204 Countries and Territories, 1970–2019: A Systematic Analysis for the Global Burden of Disease Study 2019. *www.thelancet.com* **2022**, *400*, doi:10.1016/S0140-6736(22)00936-9.
10. Griffiero, L.; Alcántara-Durán, J.; Alonso, C.; Rodríguez-Gallego, L.; Moreno-González, D.; García-Reyes, J.F.; Molina-Díaz, A.; Pérez-Parada, A. Basin-Scale Monitoring and Risk Assessment of Emerging Contaminants in South American Atlantic Coastal Lagoons. *Sci. Total Environ.* **2019**, *697*, 134058, doi:10.1016/j.scitotenv.2019.134058.
11. Gomes, I.B.; Maillard, J.Y.; Simões, L.C.; Simões, M. Emerging Contaminants Affect the Microbiome of Water Systems—Strategies for Their Mitigation. *npj Clean Water* **2020**, *3*.
12. Dzionek, A.; Wojcieszńska, D.; Guzik, U. Natural Carriers in Bioremediation: A Review. *Electron. J. Biotechnol.* **2016**, *23*, 28–36, doi:10.1016/J.EJBT.2016.07.003.
13. Siddiqui, N.M.; Dahiya, P. Enzyme-Based Biodegradation of Toxic Environmental Pollutants. *Dev. Wastewater Treat. Res. Process. Microb. Degrad. Xenobiotics through Bact. Fungal Approach* **2022**, 311–333, doi:10.1016/B978-0-323-85839-7.00003-7.
14. Zdarta, J.; Nguyen, L.N.; Jankowska, K.; Jesionowski, T.; Nghiem, L.D. A Contemporary Review of Enzymatic Applications in the Remediation of Emerging Estrogenic Compounds. *Crit. Rev. Environ. Sci. Technol.* **2021**, *0*, 1–30, doi:10.1080/10643389.2021.1889283.
15. Macellaro, G.; Pezzella, C.; Cicatiello, P.; Sannia, G.; Piscitelli, A. Fungal Laccases Degradation of Endocrine Disrupting Compounds. *Biomed Res. Int.* **2014**, *2014*, 614038, doi:10.1155/2014/614038.
16. Lloret, L.; Eibes, G.; Moreira, M.T.; Feijoo, G.; Lema, J.M. Removal of Estrogenic Compounds from Filtered Secondary Wastewater Effluent in a Continuous Enzymatic Membrane Reactor. Identification of Biotransformation Products. *Environ. Sci. Technol.* **2013**, *47*, 4536–4543, doi:10.1021/es304783k.
17. Bilal, M.; Iqbal, H.M.N. Persistence and Impact of Steroidal Estrogens on the Environment and Their Laccase-Assisted Removal. *Sci. Total Environ.* **2019**, *690*, 447–459, doi:10.1016/j.scitotenv.2019.07.025.
18. Daronch, N.A.; Kelbert, M.; Pereira, C.S.; de Araújo, P.H.H.; de Oliveira, D. Elucidating the Choice for a Precise Matrix for Laccase Immobilization: A Review. *Chem. Eng. J.* **2020**, *397*, 125506.

19. 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, doi:10.1002/bit.28591.
20. Mate, D.M.; Alcalde, M. Laccase: A Multi-Purpose Biocatalyst at the Forefront of Biotechnology. *Microb. Biotechnol.* **2017**, *10*, 1457–1467, doi:10.1111/1751-7915.12422.
21. Brugnari, T.; Braga, D.M.; dos Santos, C.S.A.; Torres, B.H.C.; Modkovski, T.A.; Haminiuk, C.W.I.; Maciel, G.M. Laccases as Green and Versatile Biocatalysts: From Lab to Enzyme Market—an Overview. *Bioresour. Bioprocess.* **2021**, *8*, 1–29, doi:10.1186/s40643-021-00484-1.
22. Janusz, G.; Skwarek, E.; Pawlik, A. Potential of Laccase as a Tool for Biodegradation of Wastewater Micropollutants. *Water (Switzerland)* **2023**, *15*, 1–19, doi:10.3390/w15213770.
23. Chaudhary, S.; Singh, A.; Varma, A.; Porwal, S. Recent Advancements in Biotechnological Applications of Laccase as a Multifunctional Enzyme. *J. Pure Appl. Microbiol.* **2022**, *16*, 1479–1491, doi:10.22207/JIPAM.16.3.72.
24. Zofair, S.F.F.; Ahmad, S.; Hashmi, M.A.; Khan, S.H.; Khan, M.A.; Younus, H. Catalytic Roles, Immobilization and Management of Recalcitrant Environmental Pollutants by Laccases: Significance in Sustainable Green Chemistry. *J. Environ. Manage.* **2022**, *309*, 114676, doi:10.1016/j.jenvman.2022.114676.
25. Yu, S.; Cheng, J.; Li, K.; Zhang, S.; Dong, X.; Paizullakhanov, M.S.; Chen, D. Preparation and Application of Laccase-Immobilized Magnetic Biochar for Effective Degradation of Endocrine Disruptors: Efficiency and Mechanistic Analysis. *Int. J. Biol. Macromol.* **2025**, *305*, 141167, doi:10.1016/j.ijbiomac.2025.141167.
26. Rybarczyk, A.; Smulek, W.; Ejmont, A.; Goscianska, J.; Jesionowski, T.; Zdarta, J. The Role of Metal–Organic Framework (MOF) in Laccase Immobilization for Advanced Biocatalyst Formation for Use in Micropollutants Removal. *Environ. Pollut.* **2025**, *371*, 125954.
27. Vázquez, V.; Giorgi, V.; Bonfiglio, F.; Menéndez, P.; Gioia, L.; Ovsejevi, K. Lignocellulosic Residues from Bioethanol Production: A Novel Source of Biopolymers for Laccase Immobilization. *RSC Adv.* **2023**, *13*, 13463–13471, doi:10.1039/d3ra01520c.
28. Hasanov, I.; Raud, M.; Kikas, T. The Role of Ionic Liquids in the Lignin Separation from Lignocellulosic Biomass. *Energies* **2020**, *13*, 1–24, doi:10.3390/en13184864.
29. Meyer, J.; Meyer, L.E.; Kara, S. Enzyme Immobilization in Hydrogels: A Perfect Liaison for Efficient and Sustainable Biocatalysis. *Eng. Life Sci.* **2022**, *22*, 165–177, doi:10.1002/elsc.202100087.
30. Imam, H.T.; Marr, P.C.; Marr, A.C. Enzyme Entrapment, Biocatalyst Immobilization without Covalent Attachment. *Green Chem.* **2021**, *23*, 4980–5005, doi:10.1039/d1gc01852c.
31. Kim, M.H.; An, S.; Won, K.; Kim, H.J.; Lee, S.H. Entrapment of Enzymes into Cellulose-Biopolymer Composite Hydrogel Beads Using Biocompatible Ionic Liquid. *J. Mol. Catal. B Enzym.* **2012**, *75*, 68–72, doi:10.1016/j.molcatb.2011.11.011.
32. Botto, E.; D'annibale, A.; Petruccioli, M.; Galetta, A.; Martínez, S.; Bettucci, L.; Menéndez, P. Ligninolytic Enzymes Production by *Dichostereum sordulentum* Cultures in the Presence of Eucalyptus Bark as a Natural Laccase Stimulator. *J. Adv. Biotechnol.* **2015**, *5*, 478–484, doi:10.24297/jbt.v5i1.4848.
33. Pourkhanali, K.; Khayati, G.; Mizani, F.; Raouf, F. Isolation, Identification and Optimization of Enhanced Production of Laccase from *Galactomyces geotrichum* under Solid-State Fermentation. *Prep. Biochem. Biotechnol.* **2021**, *51*, 659–668, doi:10.1080/10826068.2020.1848867.
34. He, X.J.; Jiao, J.; Gai, Q.Y.; Fu, J.X.; Fu, Y.J.; Zhang, Z.Y.; Gao, J. Bioprocessing of Pigeon Pea Roots by a Novel Endophytic Fungus *Penicillium rubens* for the Improvement of Genistein Yield Using Semi-Solid-State Fermentation with Water. *Innov. Food Sci. Emerg. Technol.* **2023**, *90*, 103519, doi:10.1016/j.ifset.2023.103519.
35. Montgomery, D.C. *Design and Analysis of Experiments*; 8th ed.; John Wiley: New York, 2012;
36. Hadiyat, M.A.; Sopha, B.M.; Wibowo, B.S. Response Surface Methodology Using Observational Data: A Systematic Literature Review. *Appl. Sci.* **2022**, *12*, 10663, doi:10.3390/app122010663.
37. Dirección General Forestal MGAP *Superficie Forestal Del Uruguay 2022 (Bosques Plantados)*; Montevideo, Uruguay, 2022;

38. Gioia, L.; Manta, C.; Ovsejevi, K.; Burgueño, J.; Menéndez, P.; Rodríguez-Couto, S. Enhancing Laccase Production by a Newly-Isolated Strain of *Pycnoporus Sanguineus* with High Potential for Dye Decolouration. *RSC Adv.* **2014**, *4*, 34096–34103, doi:10.1039/c4ra06039c.
39. Gupta, A.; Jana, A.K. Production of Laccase by Repeated Batch Semi-Solid Fermentation Using Wheat Straw as Substrate and Support for Fungal Growth. *Bioprocess Biosyst. Eng.* **2019**, *42*, 499–512, doi:10.1007/s00449-018-2053-6.
40. Ripollés, C.; Ibáñez, M.; Sancho, J. V.; López, F.J.; Hernández, F. Determination of 17 β -Estradiol and 17 α -Ethinylestradiol in Water at Sub-Ppt Levels by Liquid Chromatography Coupled to Tandem Mass Spectrometry. *Anal. Methods* **2014**, *6*, 5028–5037, doi:10.1039/c4ay00479e.
41. Turull, M.; Buttiglieri, G.; Vazquez, V.; Rodríguez-Mozaz, S.; Santos, L.H.M.L.M. Analytical Upgrade of a Methodology Based on UHPLC-MS/MS for the Analysis of Endocrine Disrupting Compounds in Greywater. *Water Emerg. Contam. Nanoplastics* **2023**, *2*, 17, doi:10.20517/wecn.2023.37.
42. Nahar, L.; Onder, A.; Sarker, S.D. A Review on the Recent Advances in HPLC, UHPLC and UPLC Analyses of Naturally Occurring Cannabinoids (2010–2019). *Phytochem. Anal.* **2020**, *31*, 413–457, doi:10.1002/pca.2906.
43. Auriol, M.; Filali-Meknassi, Y.; Tyagi, R.D.; Adams, C.D. Laccase-Catalyzed Conversion of Natural and Synthetic Hormones from a Municipal Wastewater. *Water Res.* **2007**, *41*, 3281–3288, doi:10.1016/j.watres.2007.05.008.
44. Pamidipati, S.; Ahmed, A. A First Report on Competitive Inhibition of Laccase Enzyme by Lignin Degradation Intermediates. *Folia Microbiol. (Praha)*. **2020**, *65*, 431–437, doi:10.1007/s12223-019-00765-5.
45. Beck, I.C.; Bruhn, R.; Gandrass, J. Analysis of Estrogenic Activity in Coastal Surface Waters of the Baltic Sea Using the Yeast Estrogen Screen. *Chemosphere* **2006**, *63*, 1870–1878, doi:10.1016/j.chemosphere.2005.10.022.
46. Zdarta, J.; Jankowska, K.; Strybel, U.; Marczak, Ł.; Nguyen, L.N.; Oleskiewicz-Popiel, P.; Jesionowski, T. Bioremoval of Estrogens by Laccase Immobilized onto Polyacrylonitrile/Polyethersulfone Material: Effect of Inhibitors and Mediators, Process Characterization and Catalytic Pathways Determination. *J. Hazard. Mater.* **2022**, *432*, doi:10.1016/j.jhazmat.2022.128688.
47. Smith, P.K.; Krohn, R.I.; Hermanson, G.T.; Mallia, A.K.; Gartner, F.H.; Provenzano, M.D.; Fujimoto, E.K.; Goeke, N.M.; Olson, B.J.; Klenk, D.C. Measurement of Protein Using Bicinchoninic Acid. *Anal. Biochem.* **1985**, *150*, 76–85, doi:10.1016/0003-2697(85)90442-7.
48. Martínez, S.; Nakasone, K.K. New Records of Interesting Corticioid Basidiomycota from Uruguay. *Check List* **2014**, *10*, 1237–1242, doi:10.15560/10.5.1237.
49. Routledge, E.J.; Sumpter, J.P. Estrogenic Activity of Surfactants and Some of Their Degradation Products Assessed Using a Recombinant Yeast Screen. *Environ. Toxicol. Chem.* **1996**, *15*, 241–248, doi:10.1897/1551-5028(1996)015<0241:EAOSAS>2.3.CO;2.

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