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Preliminary scale-up of fungal ligninolytic enzyme production in Fernbach flasks and application to the decolorization of synthetic dyes

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ABSTRACT

The use of enzymes has gained significant relevance due to their low ecological footprint and high efficiency across diverse industrial and biotechnological processes. Among them, ligninolytic enzymes such as laccase and manganese peroxidase stand out for their ability to degrade toxic compounds, including synthetic dyes. This study focuses on solid-state fermentation (SSF) as a novel and cost-effective approach for producing an enzymatic extract capable of decolorizing synthetic dyes used in the textile industry. Rice husk residue was employed as both support and substrate source, and the fungus *Trametes pubescens* was employed as the enzyme-producing organism. Statistical tools, including the Plackett-Burman design, factorial design, and central composite design, were applied to optimize the fermentation process in Erlenmeyer flasks, yielding an enzymatic activity of 20.9 U/gds. The process was subsequently scaled up 20-fold in Fernbach flasks, resulting in an enzymatic extract with an activity of 12 U/gds. Finally, decolorization assays using enzymatic extracts from both Erlenmeyer and Fernbach scales showed values above 85% for malachite green at 50 and 100 ppm, and 48% and 41% for brilliant blue at 50 and 100 ppm, respectively. These findings highlight that the enzymatic extract obtained through a sustainable methodology such as SSF represents a promising alternative for the treatment of highly persistent pollutants, such as synthetic dyes.

Keywords: ligninolytic enzymes, laccase production, white-rot fungi, synthetic dye decolorization, solid-state fermentation, process scale-up.

INTRODUCTION

In the present century, industrialization has increased substantially, becoming one of the main sources of environmental pollution, particularly in sectors such as agroindustry and textiles. The former generates significant impacts on soil and air quality due to the poor utilization and subsequent accumulation of waste generated during production processes. The textile industry, in turn, faces a major environmental challenge associated with the discharge of colored water during the dyeing stages and finishing stages, where highly polluting synthetic dyes are commonly used. Nevertheless, these dyes are more cost-effective than natural ones, which has led to their widespread use, reaching an estimated demand of approximately 10.000 tons in the textile industry alone (Gao et al., 2018; Moradihamedani, 2021).

The global production of synthetic dyes exceeds 70 million tons, and between 5% and 50% of dyeing solutions are not effectively utilized, ending up in wastewater streams. This results in approximately 200 billion liters of colored effluents annually, accounting for 17–20% of global water pollution (Dutta et al., 2024; Gao et al., 2018; Moradihamedani, 2021). In aquatic ecosystems, these compounds represent a severe environmental concern, as even at low concentrations, around 1 ppm, they can alter water bodies and reduce sunlight penetration required for photosynthesis,

thereby disrupting the trophic chain. Moreover, their high chemical stability allows them to persist in the environment and accumulate in aquatic systems (Drumond et al., 2013; Moradihamedani, 2021). In animals, exposure to these compounds can trigger the production of free radicals and oxidative stress, potentially causing damage to multiple organs (Dutta et al., 2024).

To address the problem of dye pollution, biotechnological developments offer significant alternatives for its treatment, enabling processes with lower sludge generation, reduced costs, and, above all, a more favorable environmental impact compared to traditional physical and chemical methods (Dutta et al., 2024; Vargas-Corredor and Pérez-Pérez, 2018). Solid-state fermentation (SSF) is considered a novel methodology that harnesses white-rot fungi and agricultural residues to produce value-added compounds, such as ligninolytic enzymes. This enzymatic complex has the ability to act on synthetic dyes through oxidative mechanisms that promote their decolorization (Chauhan and Choudhury, 2021). Furthermore, SSF presents several advantages over submerged fermentation, including the absence of free water, reduced operational costs, and increased efficiency in enzyme production (Krishna, 2005; Yafetto, 2022).

The main ligninolytic enzymes produced under SSF are laccases, manganese peroxidases, and lignin peroxidases, primarily obtained from white-rot fungi (WRF), which pose no risk to human or animal health (Córdoba and Cultid, 2015). Several WRF species have been evaluated using this methodology, such as *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Irpex lacteus*, and *Trametes pubescens*; the latter has proven to be a potential producer of ligninolytic enzymes in previous studies conducted by the research group. This fermentative process requires a substrate that serves both as a support and a nutrient source. For this purpose, different agricultural residues including rice husk, sugarcane bagasse, coffee husk, and corn cob have been evaluated, promoting their valorization and the generation of products with potential biotechnological applications (Mejía-Otálvaro et al., 2021; Merino-Restrepo et al., 2020).

In Colombia, large quantities of rice husk residues are generated. In 2024, more than 630,000 hectares were cultivated for rice production, yielding more than 3.5 million tons of rice and generating approximately 700,000 tons of rice husk (Fedearroz, 2024). Fortunately, in line with the current bioeconomy trend based on converting waste into productive inputs these agricultural residues have been successfully implemented as substrates in fermentative processes. For instance, Velásquez-Quintero et al. (2022) used corn cob as a substrate for SSF with *Pleurotus ostreatus*, achieving an enzymatic production of 52.2 U/gds ± 9.6 U/gds on the eighth day of cultivation. The subsequent application of the enzymatic extract to a 35 ppm Allura Red dye solution led to approximately 10% decolorization after six hours of exposure (Velásquez-Quintero et al., 2022).

Currently, one of the main uses of rice husk is directed toward the production of value-added products, such as biochar, silica, and bioenergy (Vijaya et al., 2025). In several of these processes, fermentation techniques are employed, where the rice husk undergoes hydrolysis, enabling the structural carbohydrates including cellulose, hemicellulose, and starch to be converted into fermentable sugars available for yeast metabolism (Chavan et al., 2024). Moreover, the carbohydrate content of rice husk has proven to be a promising substrate source for fungal growth and metabolic production. In this context, Mora López et al. achieved cellulase production on the 15th day of culture, obtaining enzymatic activities of 34.5 and 31.9 U/gds using *Trichoderma reesei* and *Aspergillus fumigatus*, respectively, with rice husk as substrate, confirming the potential of this agroindustrial residue for biotechnological applications (Osorio Echeverri et al., 2023).

Against this background, the present work initially focused on the production of an enzymatic extract through SSF, employing rice husk as substrate and *Trametes pubescens*, aiming to evaluate its application in the decolorization of both anionic and cationic dyes. In a second stage, and with the purpose of validating the efficiency of this methodology, a scale-up prototype was carried out in Fernbach flasks, increasing the scale by a factor of 20 to assess the reproducibility of enzymatic activity in a system with higher fermentation capacity. The results were highly satisfactory, achieving decolorization percentages above 85% for malachite green dye. All evaluations were conducted using statistical tools. Furthermore, in *ex situ* decolorization assays, commercial laccase was used as a reference, revealing that the obtained enzymatic extract exhibited higher catalytic efficiency. In summary, the findings confirm that the SSF biological process

can be successfully scaled up while maintaining enzymatic activity, providing an efficient and environmentally friendly alternative for the treatment of colored effluents.

METHODOLOGY

Reagents and materials

Brilliant blue FCF (BB) and malachite green (MG) dyes were purchased from Colorquímica S.A (Colombia), and wheat bran was obtained from a local supermarket in Medellín, Colombia. Other analytical-grade reagents, including yeast extract, potato dextrose agar (PDA), bacteriological agar, and peptone, were acquired from Oxoid. Citric acid monohydrate, glacial acetic acid, and magnesium sulfate heptahydrate were supplied by Panreac. Sodium acetate trihydrate was obtained from Carlo Erba. Manganese sulfate monohydrate, malonic acid, and dibasic sodium malonate were acquired from Alfa Aesar. Hydrogen peroxide (30% w/w), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium dihydrogen phosphate, glucose, and disodium phosphate were obtained from Sigma-Aldrich.

Rice husk pretreatment

Rice husk was obtained from a local market in Medellín. It was ground using a microfine mill and sieved to obtain particle sizes between 300–700 μm. Subsequently, it was washed with distilled water, dried at 80 °C for 24 h, and stored in hermetic bags for subsequent trials. This particle size was selected because it proved to be the most suitable for this process according to previous studies conducted by our research group (Zuluaga Díaz et al., 2014).

Conservation and preinoculum of the fungal species

A *Trametes pubescens* was obtained from the Plant Tissue Cultivation Laboratory at the Universidad de Antioquia (Colombia). It was used and maintained under refrigeration at 4 °C on PDA medium. For preinoculum preparation, the fungus was transferred to an incubator at 28 °C one day prior. The preinoculum was prepared seven days before fermentation setup on wheat bran medium containing glucose 10 g/L, peptone 5 g/L, yeast extract 2 g/L, KH_2PO_4 0.1 g/L, $MgSO_4 \cdot 7H_2O$ 0.05 g/L, $MnSO_4 \cdot H_2O$ 0.076 g/L, wheat bran extract, and agar-agar 15 g/L.

Table 1. Conditions of categorical factors evaluated in the Plackett-Burman design

Factor	Low value	High value
Carbon source	Malt extract	Glucose
Nitrogen source	Yeast extract	Peptone
Inductor	Absence	Presence
C:N ratio	20:1	40:1
Moisture content	70%	80%
Temperature	20 °C	30 °C

Solid-state fermentation

Statistical design tools

A Plackett-Burman experimental design was initially applied to screen the most influential culture conditions affecting enzymatic extract production. Six independent factors were evaluated: carbon source, nitrogen source, inducer presence, C:N ratio, moisture content, and temperature. The low and high values assigned to each evaluated factor are presented in Table 1.

Carbon source concentrations were fixed at 18 g/L (malt extract) and 5 g/L (glucose), while nitrogen source concentrations were adjusted according to the C:N ratio established for each treatment. Fermentation was carried out for 11 days in 50 mL Erlenmeyer flasks containing 1 g of substrate and two mycelial discs (1 cm diameter) obtained from the peripheral zone of exponential growth of the pre-inoculum cultured on wheat bran medium. The 11-day period was selected based on previous kinetic results obtained by our research group under similar SSF conditions, where laccase production reached stable and high activity levels around day 11 (Velásquez-Quintero et al., 2022).

Continuous *in situ* pH monitoring was not performed due to the intrinsic technical constraints of SSF systems, where low water activity and matrix heterogeneity hinder the reliable use of conventional electrochemical probes. The development of non-invasive monitoring strategies could enable improved control of biochemical dynamics and enhance the robustness of environmentally oriented SSF processes in future (Kabir et al., 2025).

Subsequently, a 2^3 factorial design with central points was implemented to approach the optimal

conditions for enzymatic extract production, based on the most significant factors identified in the Plackett-Burman design. Glucose concentration (2, 5, and 8 g/L) was evaluated as the carbon source, and along with the moisture content at 65, 70, and 75%. Yeast extract was included as the nitrogen source at concentrations of 26, 53, and 80 g/L. Additionally, temperature was fixed at 25 °C and inducer absence was maintained, resulting in a total of 52 experimental runs. These experiments were conducted in 50 mL Erlenmeyer flasks containing 1 g of rice husk, inoculated with two mycelial discs, and incubated for 11 days in complete darkness.

Finally, to optimize the fermentation process, a central composite design (CCD) with five levels was performed, as summarized in Table 2, to evaluate the effects of carbon source, nitrogen source, and moisture content, as the most relevant factors for enzyme production. The experiments were conducted for 11 days in 50 mL Erlenmeyer flasks containing 1 g of rice husk and inoculated with two mycelial discs.

Erlenmeyer fermentation

Using the optimal values for yeast extract, glucose, and moisture identified by CCD for enzymatic extract production, fermentation was carried out in 50 mL Erlenmeyer flasks containing 1 g of rice husk, with a C:N ratio of 30:1 adjusted using yeast extract and glucose. Each flask was inoculated with two mycelial discs (1 cm diameter) collected from the peripheral region of the pre-inoculum plates. SSF was monitored for 11 days at 25 °C in complete darkness. All experiments were performed in triplicate.

Process scale-up in Fernbach flask

For the scale-up assays, fermentation was performed in 1800 mL Fernbach flasks containing 20 g of rice husk and a C:N ratio of 30:1, adjusted using yeast extract and glucose, while the concentrations of yeast extract, glucose, and moisture corresponded to the optimal values identified by the CCD. Each Fernbach flask was inoculated with 30 mycelial discs (1 cm diameter) collected from the peripheral region of the pre-inoculum plates. SSF was monitored for 11 days at 25 °C in complete darkness. All experiments were performed in triplicate.

Enzymatic extraction

Enzymatic activity was evaluated on day 11 of fermentation, based on previous studies using the SSF system. For each experiment, 10 mL of 50 mM sodium acetate buffer (pH 5.0) was added, and the sample was homogenized and agitated in an ice bath for 30 min. Subsequently, the liquid phase corresponding to the enzymatic extract was recovered and centrifuged at 4 °C for 10 min at 3000 rcf. The resulting supernatant was stored under refrigeration conditions until further analysis.

Enzymatic activity quantification

Enzymatic activity was quantified according to the International Union of Biochemistry, which defines one international unit (U) as the amount of enzyme required to oxidize 1.0 µmol of substrate per minute under the assay conditions. Enzymatic activities are expressed as units per gram of dry substrate (U/gds) of the residues employed.

- Laccase: Laccase activity was determined from the oxidation reaction with 2,2′-azino-bis-[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) used as substrate, with absorbance measured at 420 nm using 50 mM sodium acetate buffer (pH 4.0), according to Velásquez-Quintero, (2021). The reaction mixture consisted of 100 µL of enzymatic extract, 500 µL of ABTS solution (1.6 mM), and 1400 µL of buffer.
- Manganese peroxidase (MnP): MnP activity was determined using manganese sulfate as substrate in 0.1 M sodium malonate buffer (pH 4.0), in the presence of 50 mM hydrogen peroxide at 25 °C. Absorbance was recorded at a wavelength of 270 nm.

It should be noted that manganese peroxidase activity was measured on day 7 of fermentation, recording a maximum value of 0.4 U/gds. Due to

Table 2. Conditions of the central composite design

Factors	Axial low (-α)	Low (-1)	Central (0)	High (1)	Axial high (α)
Yeast extract	36.18	43	53	63	69.82
Glucose	3.32	4	5	6	6.68
Moisture content	66.64	68	70	72	73.36

the low activity levels observed, this enzyme was evaluated only at the initial stage of the study to confirm its presence within the enzymatic complex. Consequently, laccase quantification was prioritized, as this enzyme showed activities exceeding 20 U/gds in most experiments. Therefore, in the present study, the term "enzymatic extract" refers to the set of ligninolytic enzymes produced, with particular emphasis on laccase activity.

Ex-situ decolorization of cationic and anionic dyes

Two triphenylmethane dyes were evaluated: MG, a cationic dye, and BB, an anionic dye, at concentrations of 50 and 100 ppm. Sodium acetate buffer (pH 4.0) was used to maintain enzyme stability during the decolorization process. In the first assay, experiments were conducted in 50 mL Erlenmeyer flasks containing 40 mL of the dye solution, to which 10 mL of the enzymatic extract obtained from the optimized Erlenmeyer-scale process or from the Fernbach scale-up process was added. The flasks were incubated under constant agitation at 180 rpm for 100 h, with samples collected every 10 h under controlled temperature and complete darkness.

To assess the decolorization potential of the enzymatic extract, a second assay was carried out using commercial laccase from *Trametes versicolor* (Sigma Aldrich) under the same experimental conditions. The decolorization percentage (%D) was determined by measuring the initial and final dye concentrations by spectrophotometry at the maximum absorption wavelength of each dye and calculated using Equation 1, where Ci and Cf represent the initial and final concentrations, respectively:

$$\%D = \frac{Ci - Cf}{Ci} * 100 \quad (1)$$

Statistical analysis

All experiments were conducted in triplicate. Data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test to identify significant differences. All statistical analyses were performed using Statgraphics Centurion version 19.1.1 (x64, free evaluation edition). software.

RESULTS AND DISCUSSION

Statistical design tools

The results obtained from the Plackett-Burman design allowed the identification of moisture content, carbon source, and inducer presence as the factors having the greatest influence on ligninolytic enzyme production in the fermentation process under study. Moisture and carbon source showed a positive effect, whereas the inducer exhibited a negative effect, indicating that its absence favors enzyme production, as illustrated in Figure 1.

Among these factors, moisture content proved to be a critical parameter, requiring values above 65%. This finding is consistent with previous studies highlighting the key role of moisture in regulating fungal metabolism and nutrient availability in solid-state fermentation systems (Mahmood et al., 2022; Naik et al., 2022). Additionally, adequate moisture is essential for microbial development, particularly for filamentous fungi. In the case of WRF, for example, optimal growth has been reported over a broad moisture range, typically between 20 and 80% (Krishna, 2005; Manzoni et al., 2012).

In SSF systems, moisture content is closely related to water activity, a thermodynamic parameter that represents the amount of free water available for microorganism growth and its interaction

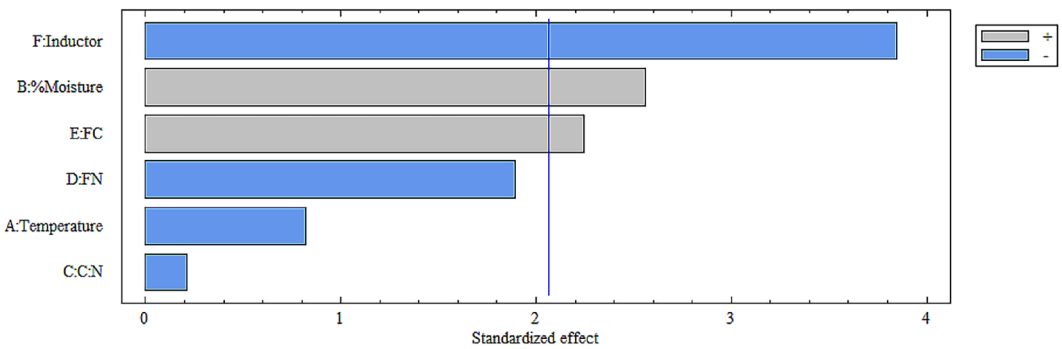

Figure 1. Pareto chart showing significant variables in enzyme production – Plackett-Burman design

with the solid substrate (Kumar et al., 2021). Water activity strongly influences microbial selectivity, favoring the growth of certain microorganisms over others. Therefore, low water activity generally limits bacterial contamination while favoring WRF growth, which enhances process efficiency and reduces operational costs by minimizing raw material use and wastewater generation.

Likewise, the carbon source had a significant effect on enzyme production, with glucose outperforming malt extract as the most effective substrate for promoting ligninolytic enzyme synthesis. This result agrees with previous reports indicating that the availability of readily assimilable carbon sources enhances the metabolic efficiency of WRF fungi and their capacity to produce extracellular enzymes (Chmelová et al., 2022).

Ultimately, the application of the Plackett–Burman design enabled a reduction in experimental complexity and allowed the identification of the most relevant factors for establishing optimal conditions for ligninolytic enzymes production under SSF conditions. Based on the results, a 2^3 factorial design with central points was subsequently carried out, considering glucose as the carbon source, moisture content, and yeast extract as the nitrogen source. The nitrogen source was included as the third factor in this design due to its well-documented influence on enzyme production in fermentation processes, as reported in previous studies (Merino-Restrepo et al., 2020; Šelo et al., 2021; Shet et al., 2022).

The results of the factorial design are shown in the Pareto diagram, Figure 2, which indicates that yeast extract had a statistically significant effect on enzyme production. This finding suggests that the inclusion of this nitrogen source positively modulates the expression of ligninolytic enzymes under solid-state fermentation conditions. The influence of yeast extract is consistent with previous studies reporting investigations that both the type and availability of nitrogen source play a key role in regulating ligninolytic enzyme synthesis by fungi (Tanruean et al., 2021; Velásquez-Quintero et al., 2022).

In contrast, glucose and moisture content were identified as non-significant factors for enzyme production, and no statistically significant interactions between the evaluated factors were observed. This outcome simplifies process management, as individual factors can be varied independently without compromising enzyme activity. As summarized in Table 3, the highest enzyme activities were observed in treatments 1, 4, 8, and 10, with an average activity of 18.51 U/gds. Notably, treatments 1, 4, and 8 shared a C:N ratio of 23.88, a carbon source concentration of 5 g/L, a nitrogen source concentration of 53 g/L, and a moisture content of 70%. These conditions were therefore selected for further optimization using a CCD, in accordance with the specifications presented in Table 2.

Accordingly, the 2^3 factorial design confirmed the relevance of yeast extract, together with glucose concentration and moisture content, as important factors for further optimization of enzyme production under SSF.

Following this initial screening stage, the optimization process culminated in the application of a CCD, which enabled the identification of optimal conditions for the three selected factors. CCD has been one of the most widely applied methodologies for enzyme optimization, as it allows the fitting of a second-order polynomial model within a sequential experimental framework (de Menezes et al., 2024).

Figure 3 shows the response surface plots obtained by varying glucose concentration, yeast extract concentration, and moisture content. In all cases, the optimal response zone is represented

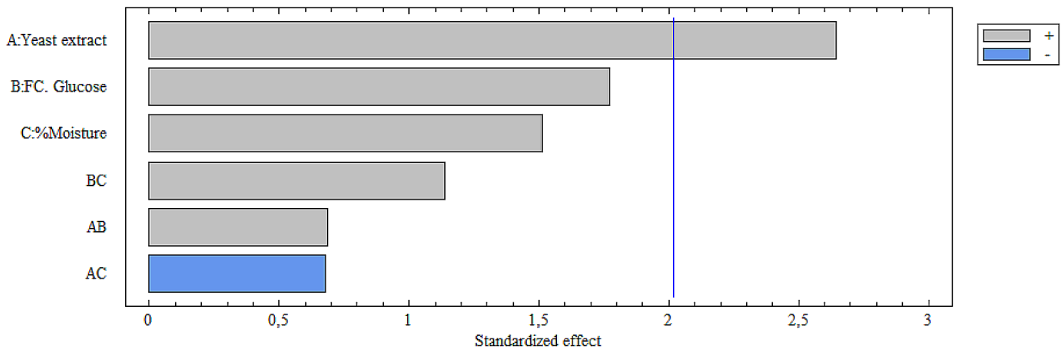

Figure 2. Pareto chart showing significant variables in enzyme production – 2^3 factorial design

Table 3. Enzymatic activity obtained under 2^3 factorial design conditions

Treatment	C:N Ratio	Glucose (g/L)	Yeast extract (g/L)	Moisture %	Enzymatic activity (U/gds)
1	23.88	5	53	70	$20.86^{a} \pm 6.3$
2	60.94	8	26	65	$1.05^{f} \pm 0.8$
3	60.15	2	26	65	$1.02^{f} \pm 0.19$
4	23.88	5	53	70	$16.91^{ab} \pm 6.5$
5	19.81	8	80	65	$10.74^{cd} \pm 4.7$
6	23.88	5	53	70	$10.80^{bcd} \pm 5.5$
7	12.49	2	80	75	$8.50^{de} \pm 2.1$
8	23.88	5	53	70	$20.19^{a} \pm 7.5$
9	39.25	8	26	75	$7.38^{def} \pm 1.63$
10	12.76	8	80	75	$16.07^{abc} \pm 1.5$
11	23.88	5	53	70	$10.43^{bcde} \pm 1.8$
12	19.55	2	80	65	$8.45^{de} \pm 1.7$
13	38.45	2	26	75	$4.79^{ef} \pm 1.2$

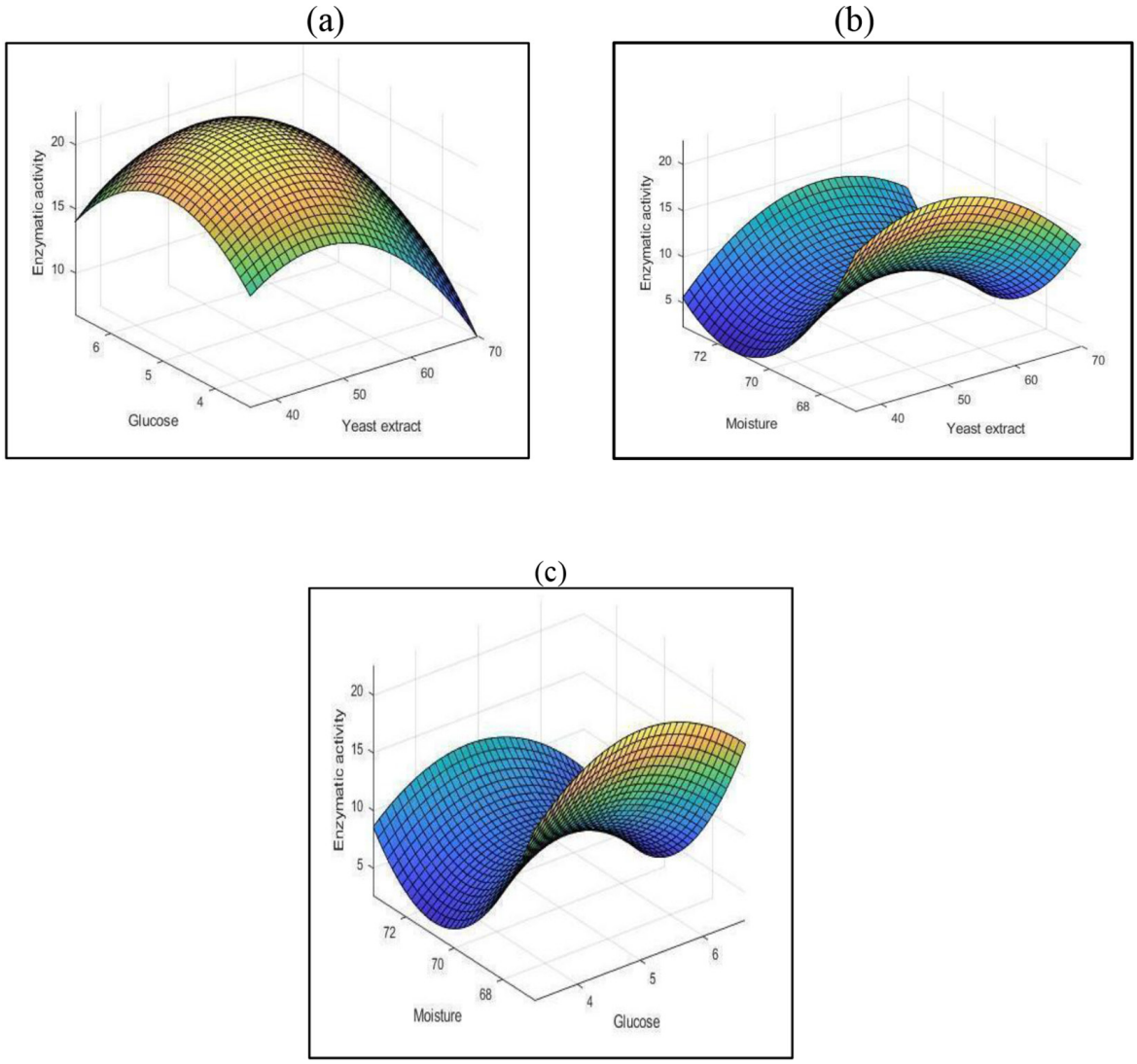

Figure 3. Central composite response surface at optimal condition for the enzymatic activity: (a) surface response with moisture fixed at optimum, (b) surface response with yeast extract fixed at optimum, (c) surface response with glucose fixed at optimum

by the orange-colored area, corresponding to the highest enzyme activity within the lower ranges of moisture, yeast extract, and glucose evaluated according to Table 2. Additionally, the curvature observed in Figure 3a confirms the presence of a maximum response, corresponding to an enzyme activity of 22.59 U/gds. Overall, the application of the CCD resulted in an increase of approximately 28% in enzyme activity compared to the average value obtained in the factorial design.

The analysis performed using Statgraphics indicates that a glucose concentration of 5.03 g/L, a yeast extract concentration of 48.42 g/L, and a moisture content of 66.64% resulted in a maximum enzyme activity of 22.59 U/gds. This response can be described by Equation 2, where X represents yeast extract concentration [g/L], Y represents glucose concentration [g/L], and Z represents moisture content [%].

$$Enzyme\ activity = 3931.51 - 1.55X + \\ + 22.27Y - 111.20Z - 0.02X^2 + \\ + 0.04XY + 0.05XZ - 1.74Y^2 - \\ - 0.10YZ + 0.77Z^2 \quad (2)$$

Thus, the fermentation process was conducted under the optimal conditions previously obtained through the CCD, using 50 mL Erlenmeyer flasks containing 1.0 g of rice husk substrate, achieving an enzyme activity of 20.91 U/gds, which was very close to the value predicted by the model. The difference between the value predicted by the CCD and the activity obtained during fermentation can be attributed to the complexity of the biological system, in which, in addition to the interaction between the fungus and the most significant factors, such as carbon source, nitrogen, and moisture content, other aspects may affect enzyme production, including pH value, temperature, and cofactors, among others. In this regard, Contato et al. reported a peak activity of 25,447 U/L at 60 °C, when evaluating temperature ranges from 35 to 70 °C for optimization of *Pleurotus pulmonarius* under SSF using orange residue as substrate after 12 days of fermentation. However, enzyme stability decreases at 60 °C after 15 minutes (Contato et al., 2020).

On the other hand, Backes et al. conducted fermentation with *Trametes versicolor* and *Pycnoporus sanguineus* using pineapple residues, glucose, and yeast extract for 17 days, and evaluated the effect of farnesol as a cofactor on laccase production. Under these conditions, maximum activities of 77.88 U/gds and 130.9 U/gds were achieved, respectively, demonstrating the influence of cofactors on enhancing enzyme production (Backes et al., 2023). Consequently, the selection of an SSF system directly influences the enzymatic activity achieved, depending on the diversity of fungal species employed and the different conditions of the evaluated factors.

Process scale-up in Fernbach flask

After obtaining an enzymatic activity close to that predicted by the CCD in 50 mL Erlenmeyer flasks under optimal fermentation conditions, the scaling-up of the fermentative process was carried out using 20 g of rice husk in a 1800 mL Fernbach flask, under the conditions described in Process scale-up in Fernbach flask. Under these conditions, an enzymatic activity of 12 U/gds was obtained after 11 days of cultivation in darkness.

The decrease in enzymatic activity, approximately 46%, can be mainly attributed to complications arising during the scaling-up process, since changes in reactor geometry and the increase in substrate bed thickness affect SSF performance. For instance, aeration in the Fernbach flask is limited to the substrate surface, promoting CO_2 accumulation, which can lead to pH alterations in the medium. In addition, the absence of agitation aggravates this situation, negatively impacting heat transfer; consequently, the production of the metabolite of interest is compromised during process scaling (Chauhan and Choudhury, 2021).

Furthermore, the reduction in enzymatic activity may also be associated with factors such as fermentation time, initial inoculum, and the absence of inducers. For example, Jiménez et al. (2019) carried out a scaling-up process from 5.0 g of corn cob in Erlenmeyer flasks to a packed-bed bioreactor containing 10.0 g of substrate over 20 days and in the presence of $CuSO_4$ under controlled conditions (25 °C using a water bath). Under these conditions, degradation efficiencies of 84.40% and 88.70% were maintained for Allura Red and Tartrazine, respectively, in both the Erlenmeyer and bioreactor. (Jiménez et al., 2019). The authors reported that maximum enzymatic activity was reached on days 12 and 18, concluding that an increase in scale delays the time required to reach peak activity. In the present study, the fermentation monitoring period was not extended, and therefore, the fungi may not have fully developed all growth phases required to reach maximum enzyme production.

Similarly, other studies reported successful scaling-up from 500 mg of substrate in 50 mL Erlenmeyer flasks over 20 days to 200 g in a rotating drum bioreactor operated for 30 days under controlled aeration and agitation. In addition, the system included 0.5 mM $CuSO_4$ and a 10-day pre-inoculum in Fernbach flasks, achieving 75.74% degradation of red 40 on corn cob (Jaramillo et al., 2017). These results highlight the importance of extending fermentation time during scale-up, as well as apply strategies that allow better control of fermentation conditions in order to maintain optimal enzyme production and its application in dye degradation.

Ex situ decolorization of cationic and anionic dyes

Using the enzymatic extract obtained at a 1.0 g scale and that derived from the scale-up process in Fernbach flasks, where the substrate load was increased by 20 units, the enzymatic potential was evaluated for the decolorization of two synthetic dyes of triphenylmethane dyes with both cationic and anionic affinity, specifically malachite green and brilliant blue. These dyes were chosen due to their structural complexity, high chromophoric stability, and widespread use in industrial applications, which make them representative and challenging model pollutants. The molecular structures of these dyes are shown in Figure 4.

Figures 5 and 6 show the efficiency achieved by the enzymatic extract in the decolorization of each dye at the different concentrations evaluated (50 and 100 ppm). In each case, the decolorization obtained using a commercial laccase from *T. versicolor* is also included as a positive control. It is worth noting that these decolorization assays are conducted on a preliminary basis, with the aim of exploring one of the possible application pathways of the enzymatic extract associated with the treatment of colored effluent. Therefore, even more satisfactory results may be obtained through the optimization of the decolorization process. This work is currently under development within our research group.

First, MG exhibited the highest degree of decolorization at both 50 and 100 ppm, with efficiencies above 90% at the Erlenmeyer scale and above 86% at the Fernbach scale. The difference between these two concentrations was minimal, as MG decolorization efficiency decreases by only approximately 5% under identical fermentation times and similar cultivation conditions. This reduction can be directly associated with the scale-up process, since it is well established that process efficiency tends to decrease as system scale increases (Kumar et al., 2021). These results can be compared with the MG decolorization studies reported by Arunprasath et al. (2019), who obtained a decolorization efficiency of 96.9% at a concentration of 50 ppm using a *Lasiodiplodia* sp culture grown in MEB medium (yeast extract, malt extract, dextrose, and maltose) enriched with 2.5 ppm MG, at 30 °C and neutral pH. However, in the present investigation, decolorization was carried out using an enzymatic extract obtained from *Trametes pubescens,* which was brought into direct contact with the dye, rather than being applied through a fermentation process, representing a relevant methodological difference.

In another study conducted by Abbas et al. (2024), laccase production from *Trametes pubescens* was optimized under SSF using sugarcane bagasse as substrate through a Box-Behnken design, yielding a maximum activity of 12.71 U/gds. The produced laccase was subsequently evaluated for the decolorization of a 0.01% MG solution (equivalent to 10 mg), achieving a decolorization of 73.68% after 24 h of treatment. In the present

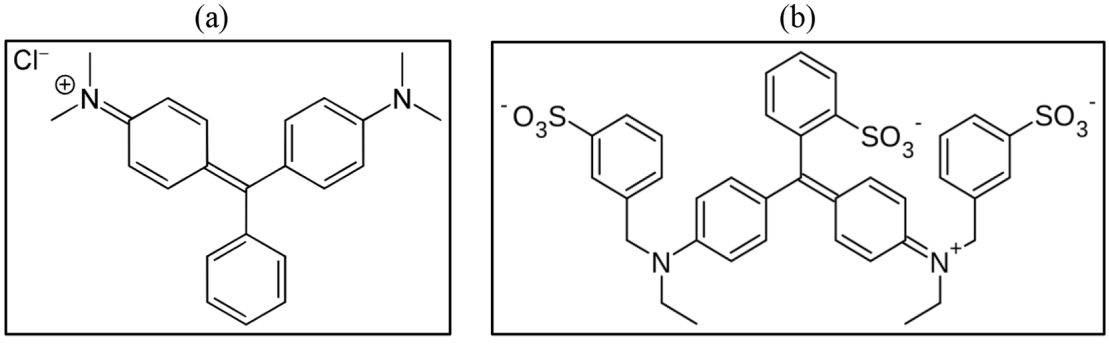

Figure 4. (a) Molecular structure of MG; (b) molecular structure of BB

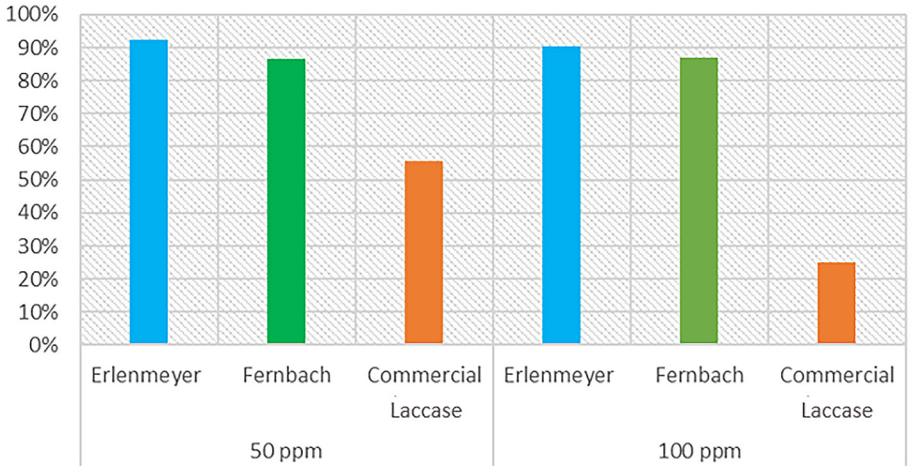

Figure 5. Decolorization percentage of MG at two SSF scales vs commercial laccase

study, a decolorization efficiency of 75.5% was achieved after 30 h using a dye concentration of 50 ppm. Unlike the study by Abbas et al. (2024), in which copper sulfate was employed as an inducer during fermentation, the results of the present work indicate that the enzymatic extract is capable of achieving satisfactory decolorization performance without the addition of inducers or enzymatic cofactors (Abbas et al., 2024).

Finally, it is relevant to mention that the MG decolorization values obtained using the commercial laccase were significantly lower than those achieved with the enzymatic extract produced by SSF, with decolorization efficiencies of 55% decolorization at 50 ppm and 24% at 100 ppm. In this way, the potential of the SSF biological process is highlighted, as well as the advantages of using crude enzymatic extract, which contains a set of enzymes and cofactors capable of acting synergistically to enhance the decolorization of synthetic dyes (Velásquez-Quintero et al., 2022).

In the case of BB dye, decolorization percentages of 56% and 50.5% were recorded at concentrations of 50 and 100 ppm, respectively, using the enzymatic extract obtained from Erlenmeyer flask fermentation. In contrast, when the enzymatic extract from the Fernbach scale was applied, decolorization exceeded 40% for both evaluated concentrations (50 and 100 ppm), representing an approximate difference of 13% compared with the smaller scale. Furthermore, at the scaled-up level, only a minimal variation of approximately 1% in decolorization efficiency was observed between the two concentrations, suggesting that scaling up SSF represents a promising and viable alternative, either at pilot scale or in industrial prototypes, for treating effluents contaminated with considerable concentrations of this dye.

In this regard, the study by Nabeela et al. (2023) reported a maximum decolorization of 91% for BB through an *in-situ* process using *Aspergillus fumigatus* at a dye concentration of 40

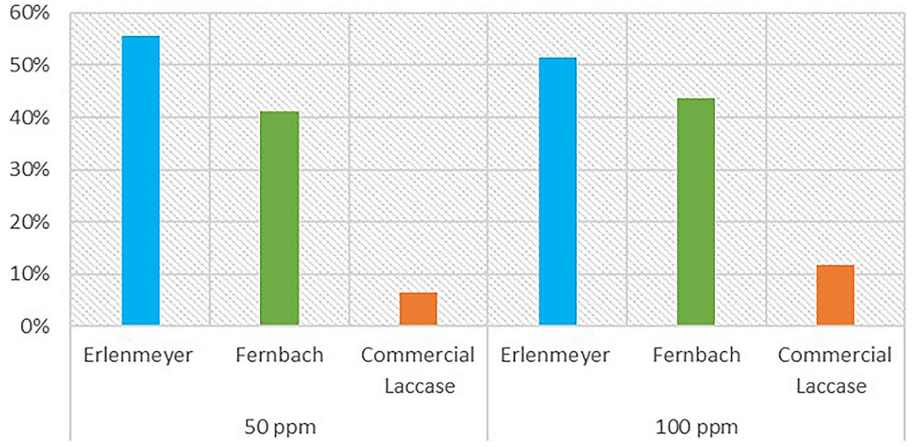

Figure 6. Decolorization percentage of BB at two SSF scales vs commercial laccase

ppm, with glucose as a carbon source after 5 days of incubation. However, when the BB concentration was increased to 100 ppm, the decolorization efficiency decreased to 60%. These results suggest that dye concentration is a determining factor in decolorization efficiency. Moreover, the decolorization values obtained at 100 ppm are comparable to those observed in the present study, suggesting a range of possibilities for BB removal using different fungal systems.

Using the commercial laccase, a low decolorization of BB was observed, with an average value close to 10% (8% and 12% at concentrations of 50 and 100 ppm, respectively). These results were considerably lower than those obtained with the enzymatic extract at both the Erlenmeyer and Fernbach scales, further confirming the potential of the enzymatic complex obtained through SSF. The lower efficiency observed with the commercial laccase may be associated with the absence of other enzymatic components or cofactors present in the crude extract, which can contribute synergistically to the decolorization process. Additionally, detailed technical information on the performance of commercial laccases in the decolorization of specific dyes is often limited or not publicly available, and comparative studies reported in the scientific literature remain scarce.

The efficiency observed in the decolorization of MG and BB can be partially attributed to their common chemical structure, characterized by the presence of the triphenylmethane unit. This structural feature may interact effectively with the active sites of the ligninolytic enzymes present in the analyzed extract, thereby favoring the decolorization process (Viswanath et al., 2014). In addition, the enzymatic extract contains, besides laccase and Mn-peroxidase, isoenzymes and other possible low-molecular-weight natural mediators, which could facilitate the approach and interaction between the enzyme and the contaminant molecule (Cañas and Camarero, 2010).

In the present study, dye degradation efficiency of the enzymatic extract was assessed based on laccase activity. This methodological approach was selected because laccase has consistently been reported to exhibit enhanced expression under growth conditions involving lignocellulosic substrates. In contrast, MnP requires more stringent conditions for optimal expression and catalytic activity, particularly the adequate availability of Mn^{2+} ions in the culture medium (Benavides et al., 2024). Moreover, ligninolytic enzyme production is strongly influenced by the fungal species employed. In this regard, Hultberg and Golovko (2024), using sawdust as a lignocellulosic substrate, demonstrated that white-rot fungi such as *Pleurotus ostreatus*, *Trametes versicolor*, and *Ganoderma lucidum* exhibited comparatively high laccase activities, whereas MnP activity remained significantly lower under identical fermentation conditions.

In contrast, decolorization mediated by commercial laccase was less efficient. Although a high performance might initially be expected due to its commercial origin, several studies have shown that purified laccases often require the addition of external mediators, such as ABTS or hydroxy benzotriazole, to become activated and promote dye degradation (Hilgers et al., 2018; Velásquez-Quintero et al., 2022). However, the implementation of such mediators leads to increased process costs. By contrast, the use of agricultural residues as supports for the production of ligninolytic enzymes through fermentative processes can enhance fungal metabolism, resulting in a complex enzymatic extract that, as demonstrated in the present study, offers advantages over commercial laccase in terms of both decolorization efficiency and economic feasibility.

The enzymatic extract obtained through SSF demonstrated a higher potential for decolorization of the evaluated dyes compared with purified commercial laccase, highlighting the relevance of producing ligninolytic enzymes using agricultural residues as support substrates. This approach could be further developed as a cost-effective and environmentally favorable alternative for the treatment of dye-contaminated industrial effluents. In addition, the residual biomass generated after fermentation exhibited characteristics suggesting that it could be further explored in future studies as part of complementary biomass valorization pathways.

From a process engineering perspective, the industrial implementation of SSF requires careful management of heat and mass transfer phenomena, particularly temperature gradients, moisture distribution, and oxygen availability, which are inherent to heterogeneous solid matrices. These factors may influence large-scale performance. However, advances in bioreactor configuration, aeration control, and monitoring strategies have progressively improved process robustness. In this context, the stable enzymatic production observed in preliminary scale-up assays supports the technical feasibility of further process development under optimized conditions.

CONCLUSIONS

This study demonstrates that SSF is a promising, sustainable, and cost-effective methodology for producing an enzymatic extract with high potential for the bioremediation of industrial effluents contaminated with dyes. The enzymatic complex was obtained through an optimized process using the fungus *Trametes pubescens* and rice husk as substrate. Process optimization, carried out through a Plackett-Burman design, followed by a 2^3 factorial design and a central composite surface design, allowed a maximum enzymatic activity of 20.9 U/gds under controlled conditions of moisture (66.64%), carbon source (glucose of 5.03 g/L), nitrogen source (yeast extract of 48.42 g/L) and incubation time (11 days) at 25 °C. Scaling up the process to 20 units in Fernbach flasks enabled validation of the methodology, although a 43% reduction in enzymatic activity was observed, attributed to limitations inherent to the scaling process.

The enzymatic extracts obtained at the Erlenmeyer and Fernbach scales exhibited high decolorization efficiency toward MG, exceeding 90% and 86%, respectively, at concentrations of 50 and 100 ppm. In the case of BB, the Erlenmeyer-scale extract showed superior performance, achieving average decolorization values of 53% and 42%. In all cases, the enzymatic extracts significantly outperformed commercial laccase, underscoring the functional importance of preserving extract integrity, likely related to the presence of isoenzymes and mediators. Overall, these findings position SSF as a viable and environmentally sustainable alternative for the treatment of textile effluents, promoting the valorization of agro-industrial residues for the production of efficient biocatalysts.

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