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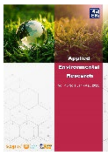

Research Article

An Optimal Culture Medium for Laccase Production and Sugar Cane Vinasse Biotreatment with *Trametes villosa* Using Plackett-Burman and Central Composite Designs

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Abstract

The optimal conditions for laccase production and vinasse biotreatment with a native strain of *Trametes villosa* were determined by a screening-optimization approach. Eleven factors including nutrient concentration, vinasse dilution (%v/v), inoculum volume, carbon to nitrogen ratio and initial pH, were investigated for their effects on laccase activity applying the Plackett-Burman screening design. The selected factors were optimized using a central composite design, and then evaluated on a vinasse biotreatment experiment. The factors that contributed the most to the enzymatic activity were the concentrations of $MgSO_4{\cdot}7H_2O$ (A), $FeSO_4{\cdot}7H_2O$ (B) and $CuSO_4{\cdot}5H_2O$ (C), alongside initial pH. After 10 days, laccase activity was 544.038 U L^{-1} for the following concentrations of A, B and C: 0.250 g L^{-1}, 0.020 mg L^{-1}, and 0.100 g L^{-1}, respectively. Vinasse biotreatment under optimized conditions resulted in 82.74%, 78% and 75.97% of phenol, color, and COD removal respectively, while final pH value was 6.90. These results showed that the native strain of *T. villosa* has a good potential for further research on laccase production and vinasse sustainable management.

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Introduction

Diverse species of white rot fungi have been studied in bench-top experiments to evaluate their potential for waste biotreatment and valorization. These experiments provide key information for the implementation of scalable solutions for solid biomass accumulation or wastewater management.

In this sense, Camacho-Morales et al. [1] evaluated the degradation of the herbicide Paraquat by strains of white rot fungi native to southern Mexico, relating this degradation to the production of laccase (EC 1.10.3.2) and Mn-peroxidase (EC 1.11.1.13). On the other hand, Junior et al. [2], used a strain of *Pleurotus sajor-caju* for the biotreatment of sugarcane vinasse, and purified the laccase enzyme obtained from the crude extracts. Likewise, different authors reported the biodegradation of lignin in agro-industrial wastes, enzymatic production, and waste recovery using various species of white-rot basidiomycetes [3–5].

These experiments involve fermentative processes that require the careful choice and control of factors such as pH, temperature, and composition of the nutrient medium. According to Singh et al. [6] and Ma et al. [7] optimizing these processes is essential for scaling up from the laboratory to pilot or industrial scale.

Different studies show the utility of surface respond methodologies (SRM) for optimizing the medium and fermentation conditions for time and material consumption [4, 8–10].

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Often, a screening experiment, helps to select the most influencing factors. Among the available screening designs are the Plackett-Burman and Taguchi designs. The former only considers the main effects, while the latter allows for the analysis of main effects and interactions. For example, Salihu et al. [11], applied the Plackett-Burman design to select the contributing factors in a study on lipase production by *Aspergillus niger*, while Muhammad et al. [12], applied the Taguchi analysis in optimizing the conditions for the production of a polypeptide with antibacterial properties from *Geobacillus pallidus*.

Sugarcane vinasse is the main liquid residue from the distillation process to obtain alcoholic beverages and has a high yield with respect to the final product. It is well known for its dark color, low pH, and high concentration of organic matter. If not effectively managed, it can cause severe damage to different ecosystems. [13–15]. Nevertheless, in the context of sustainable waste management, sugar cane vinasse should be viewed as a resource. Both biotreatment and generation of value-added products are interrelated approaches aimed to reduce its environmental impact [16–19].

Studies on vinasse biotreatment and valorization usually address the optimization of fermentation conditions or nutrient medium composition [8, 19], but a prior screening of key factors is not commonly reported. Since a culture broth with vinasse is a complex medium, we considered to perform a screening analysis before optimization. In this context, our first objective was to produce an optimal vinasse-containing medium for enzyme production by *Trametes villosa* using a screening-optimization approach. The second objective was to evaluate the biodegradation of vinasse in the optimized medium.

We used a native strain of the fungus *T. villosa* (Sw.) Kreisel for the biotreatment of locally produced sugarcane vinasse. A culture medium was prepared by first selecting key factors with the Plackett-Burman screening design, followed by factor optimization through a central composite design. The response variable in these experiments was the activity of laccase (U L^{-1}); fungal laccases are extracellular enzymes with applications in different processes given their low specificity for the substrate, which take part in the biodegradation and detoxification of recalcitrant compounds [20–21]. Subsequently, vinasse biotreatment was conducted by applying the optimum levels of the selected factors. Biodegradation of vinasse was estimated by the changes in pH and chemical oxygen demand (COD), total phenols and color removal.

Material and methods

1) Biological material

A native strain of *Trametes villosa* (Sw.) Kreisel Polyporales (LG 72) was provided by the Herbarium of the Universidad Autonoma de Chiriqui, (UCH). Collection, isolation, identification, and conservation details appear in Caballero et al. [22].

2) Culture media

Basal medium for basidiomycetes was used according to Gutierrez et al. [23] with the following amounts (g L^{-1}): KCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, $FeSO_4 \cdot 7H_2O$, $ZnSO_4$, $CuSO_4 \cdot 5H_2O$ (0.01 of each), KH_2PO_4 1.0, Thiamine 0.001, Asparagine 2.0, Glucose 10 (J.T. Baker, Sigma Aldrich, USA) in 1 L of deionized water. For the in vitro cultures, we used a basal medium with 2% agar (Sigma Aldrich, USA) and 25% (v/v) vinasse. In both cases, the media were sterilized at 121 °C for 20 min. For screening and optimization experiments, fermentation was prepared in cell culture flasks, varying the amounts of the medium components according to the design detailed below. The inoculum was prepared from 7-day in vitro cultures with vinasse. The inoculum volume, the initial pH, and the dilution of vinasse were also evaluated in the screening experiment. Culture conditions such as temperature (33.6 °C), agitation speed (120 rpm), and light (alternate periods of 12 h), were established according to Caballero et al. [24], De Gracia and Navarro [25] and Santos [26].

3) Vinasse sampling

The samples were collected at the end of the distillation process in the Central Industrial Chiricana, S.A, cooled at room temperature, and stored in opaque plastic containers with screw caps after pH measurement. The samples were gravity filtered and centrifuged at 8000 rpm prior to chemical and enzymatic analyses.

4) Screening of key factors for laccase activity in vinasse medium

The screening included seven components of the basal culture medium, as well as the carbon to nitrogen ratio (C:N), the initial pH, the inoculum volume, and vinasse dilution (%v/v), for a total of 11 factors. Using a modified Plackett-Burman design, each variable was studied at three levels: low, medium, and high (Table 1); four central points were included (Table 2). The design is based on the following equation:

$$Y = \beta_0 + \Sigma\, \beta_i X_i + \varepsilon \qquad \text{(Eq. 1)}$$

Where, Y is the response variable (laccase activity U L^{-1}), β_0 is the intercept, βi is the regression coefficient ofthe independent factors orvariables, X_i is the factoror independent variable where i= 1,2,3…n and ε is the model error.

Table 1 Factors and levels in the Plackett-Burman screening design

Factor	Units	Code	Levels		
			Low (-1)	Medium (0)	High (+1)
KCl	g L^{-1}	A	0.50	1.0	1.5
$MgSO_4{\cdot}7H_2O$	g L^{-1}	B	0.50	1.0	1.5
$FeSO_4{\cdot}7H_2O$	g L^{-1}	C	0.01	0.02	0.03
$ZnSO_4$	g L^{-1}	D	0.01	0.02	0.03
$CuSO_4{\cdot}5H_2O$	g L^{-1}	E	0	1.00	2.00
KH_2PO_4	g L^{-1}	F	1.00	2.00	3.00
Thiamine	g L^{-1}	G	0.001	0.002	0.003
C:N ratio	-	H	10	20	30
Inoculum volume	m L^{-1}	J	10	20	30
Vinasse dilution	%	K	25	37.5	50
Initial pH	-	L	5	6	7

Table 2 Plackett-Burman design matrix and experimental results

Run	Coded levels											Response
	A	B	C	D	E	F	G	H	J	K	L	Activity (UL^{-1})
1	1	1	-1	-1	-1	1	-1	1	1	-1	1	110.385
2	1	-1	-1	-1	1	-1	1	1	-1	1	1	75.692
3	-1	1	1	1	-1	-1	-1	1	-1	1	1	57.400
4	1	-1	1	1	-1	1	1	1	-1	-1	-1	116.692
5	1	-1	1	1	1	-1	-1	-1	1	-1	1	29.015
6	0	0	0	0	0	0	0	0	0	0	0	*
7	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	155.169
8	1	1	-1	1	1	1	-1	-1	-1	1	-1	0.00
9	-1	-1	1	-1	1	1	-1	1	1	1	-1	2.523
10	0	0	0	0	0	0	0	0	0	0	0	*
11	0	0	0	0	0	0	0	0	0	0	0	3.785
12	-1	1	1	-1	1	1	1	-1	-1	-1	1	0.00
13	-1	1	-1	1	1	-1	1	1	1	-1	-1	1.262
14	0	0	0	0	0	0	0	0	0	0	0	16.400
15	-1	-1	-1	1	-1	1	1	-1	1	1	1	259.877
16	1	1	1	-1	-1	-1	1	-1	1	1	-1	0.00

Remark: *Value not included in the statistical analysis

To prepare the fermentation mixture, aliquots from stock solutions of each salt and thiamine were mixed in a total volume of 50 mL. Glucose and ammonium chloride served as carbon and nitrogen sources, respectively (Sigma Aldrich, USA). The addition of these sources was done by direct weighing. A Shimadzu model AUW220D precision analytical balance was used in all cases. The fermentation mixture was sterilized and inoculated as detailed before. The cell culture flasks were placed on a Shel Lab shaker incubator, model SI4, (Sheldon Manufacturing, USA) for 10 days. Thereafter, the content of each flask was vacuum filtered through Whatman 3 paper and stored in opaque plastic tubes with screw caps at 7 °C for laccase activity assay.

5) Optimization of key factors for laccase production in vinasse medium

A central composite design was used to optimize the key factors, using five levels of each (Table 3). A second order polynomial was proposed to explain the behavior of the response variable:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_{ii} + \Sigma\beta_{ij} X_i X_j + \varepsilon \qquad \text{(Eq. 2)}$$

Where, Y is the response variable (laccase activity U L^{-1}), β_0 is the intercept, β_i, β_{ii} and β_{ij} are the linear, quadratic and interaction coefficients respectively, of the factors or independent variables, X_i, and X_j are the factors or independent variables and ε is the model error.

Fermentation was prepared as described before. Non-significant factors were added in the proportion reported for the basal medium. Likewise, the following levels were fixed: C:N ratio (20:1), inoculum volume (20 mL) and vinasse dilution (25 % v/v); the initial pH was adjusted according to the screening design. After 10 days the samples were stored as previously detailed. The design matrix (Table 4) shows 20 randomized experimental runs with 6 replicates at central points.

6) Vinasse biotreatment

The medium was prepared as described for the optimization experiment. $MgSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$, and $CuSO_4{\cdot}5H_2O$ were added according to the optimization results. Fermentation conditions were established as described before. The total fermentation volume in each of three cell culture flasks was 50 mL. After 10 days, the contents were vacuum filtered through Whatman 3 paper for chemical and enzymatic analyses.

7) Chemical and enzymatic analyses

Phenols and COD analyses as well as the pH measurement were done according to standard procedures [27–28]. The percentage of decolorization after biotreatment was reported according to Vilar et al. [29], using a Thermo Scientific BiomateTM 6 spectrophotometer and a wavelength of 420 nm. This corresponds to the vinasse absorbance maximum after filtration and centrifugation.

Laccase activity (U L^{-1}) was obtained using syringaldazine 0.22 mM (Sigma Aldrich, USA), in a kinetic assay at (23–26 °C) with 100 mM phosphate buffer pH 6.5, measuring the change in absorbance at 530 nm (ε = 65 000 $M^{-1}cm^{-1}$) for 2 min with a Thermo Scientific BiomateTM 6 spectrophotometer. One unit of enzyme activity is defined as the amount of laccase needed to oxidize 1 μmol of syringaldazine per mL per min [30].

Table 3 Factors and levels in the central composite design

Factor	Units	Code	Levels				
			-1.6812	-1	0	1	+1.6812
$MgSO_4 \cdot 7H_2O$	g L^{-1}	A	0.079	0.250	0.50	0.75	0.920
$FeSO_4 \cdot 7H_2O$	mg L^{-1}	B	0.013	0.020	0.03	0.04	0.047
$CuSO_4 \cdot 5H_2O$	g L^{-1}	C	0.032	0.100	0.20	0.30	0.368

Table 4 Central composite design matrix and results

Run	Coded levels			Activity (U L^{-1})	
	A	B	C	Experimental	Predicted
1	0	0	0	397.385	412.432
2	1	1	1	462.038	472.896
3	-1	-1	1	370.577	365.824
4	0	0	0	468.346*	*
5	-1.6812	0	0	447.846	417.248
6	1	-1	-1	446.269	438.491
7	1	-1	1	335.885*	*
8	0	0	-1.6812	417.885*	*
9	1	1	-1	499.885	477.678
10	0	0	0	405.269	412.432
11	0	0	0	381.615	412.432
12	-1	1	1	380.038	354.913
13	0	0	1.6812	521.923*	*
14	1.6812	0	0	428.923	407.558
15	0	-1.6812	0	378.462	397.844
16	0	0	0	506.192*	*
17	-1	-1	-1	54 4.038	547.027
18	0	1.6812	0	402.115	426.513
19	0	0	0	435.231	412.432
20	-1	1	-1	342.192	361.288

Remark: *Value not included in the statistical analysis

8) Statistical analysis

The experimental design, statistical and graphic analysis were performed with the Design Expert® v 10.0.1.0 software (Stat Ease Inc. USA).

Results and discussion

1) Screening of key factors for laccase activity in vinasse medium

A variable transformation was applied to the proposed polynomial (Eq. 2). The resulting reduced model equation is shown in coded units (Eq. 3):

$$Y^{1/2} = +5.73 - 2.99B - 1.96C - 3.39E + 1.86L \quad \text{(Eq. 3)}$$

This model was statistically significant ($p<0.05$), the determination coefficient R^2, adjusted R^2, and predicted R^2 were 0.904 0.861, and 0.805, respectively. The lack of fit was not significant ($p>0.05$), and the adequate precision was greater than 4. From these values the model equation was good to explain the results.

Table 2 shows a wide variation on the enzymatic activity between the different combinations of factors. This agreed with various studies that reported wide ranges in the response at this stage of the analysis, supporting the need for further optimization [31–32]. The Plackett-Burman design is useful as a first screening since it only considers main effects. This is especially convenient to assess the influence of a considerable number of factors on the response variable [6, 33]. The highest laccase activity (259.88 U L^{-1}) was obtained with the combination of factors from experiment number 15 (Table 2).

Table 5 shows the analysis of the effects and the percentage contribution of each factor to the total sum of squares. All the factors were listed and those statistically significant ($p<0.05$) are shown in bold.

Table 5 Effect and contribution of factors on enzymatic activity

Factor	Effect	Sum of squares	Contribution (%)
A-KCl	-1.73	1.00	0.28
B-$MgSO_4$	**-5.97**	**107.10**	**30.07**
C-$FeSO_4$	**-9.93**	**46.23**	**12.98**
D-$ZnSO_4$	1.71	5.02	1.41
E-$CuSO_4$	**-6.78**	**137.81**	**38.69**
F-KH_2PO_4	-1.73	1.19	0.33
G-thiamine	1.91	0.05	0.01
H-C:N ratio	2.64	8.13	2.28
J-inoculum (%)	1.72	3.45	0.97
K-vinasse	1.60	0.22	0.06
L-pH	**3.72**	**41.52**	**11.66**

As can be seen, the concentration of copper, magnesium, and iron salts had a negative significant effect on laccase activity, which suggested that lower concentrations could be evaluated in further assays. On the contrary, the initial pH had positive significant effect showing that higher levels could be considered [34–35]. The concentrations of $CuSO_4{\cdot}5H_2O$ and $MgSO_4{\cdot}7H_2O$ showed the greatest contribution to the response. This means that major changes in enzymatic activity are expected in response to changes in the levels of these salts.

A culture medium requires the presence of these cations in adequate amounts. Fe^{+2} is part of hemo-proteins and enzymes; Mg^{+2}, an important intracellular cation, takes part in anabolic reactions, is part of the formation of ATP and a cofactor for some enzymes. Finally, Cu^{+2} is a transcription regulator and a cofactor for laccases [36]. The results mentioned above agree with these facts.

The relevance of Cu^{+2}, Mg^{+2} and Fe^{+2} salts on laccase activity has also been discussed in other studies. For instance, Zhou et al. [37] have reported the effect of Mg^{+2} of Cu^{+2} and Fe^{+2} as well as the sulfate anion on the activity and thermal stability of a commercial laccase obtained from the fungus *Trametes versicolor* in an experiment on the oxidation of guaiacol and treatment of lignocellulosic fibers. The positive effects with the adequate concentration of Cu^{+2} on laccase production in *Pleurotus sajor-caju* vinasse cultures have been studied by Junior et al. [2]. In a study conducted by An et al. [38] a positive effect of Cu^{+2} on laccase activity was seen in white rot fungi both alone and in cation mixtures; these authors reported that the presence of Cu^{+2} and Mn^{+2} had a positive effect on the activity enzymatic in *Pleurotus ostreatus* cultures.

The negative effect observed for copper, magnesium and iron salts can be related to the fact that in complex or undefined media, the requirement of some components may be reduced. In this case, the content (mg L^{-1}) of Cu, Fe and Mg in the crude vinasse measured by atomic absorption spectroscopy was 0.26, 29.21 and 1.18, respectively.

Factors such as C:N ratio and the vinasse dilution (%v/v) were not significant. This may indicate that these proportions were appropriate for fungal metabolism and laccase production. Future experiments should consider the availability of macro and micronutrients in the vinasse. By doing so, one can establish their concentrations in the screening design more accurately. Also, factor associations should be addressed for a deeper analysis of effects and contributions [12, 39].

With respect to vinasse, other studies also showed the ability of white rot fungi to grow and to biodegrade vinasse in different media formulations. For example, Ferreira et al. [40] reported variations in biomass production for white rot fungi cultivated on different vinasse dilutions. For *Pleurotus sajor-caju* in 100% v/v vinasse and in supplemented vinasse medium, high laccase activity was obtained around the ninth day of cultivation. High biomass production in 100% v/v vinasse supplemented with trace mineral solution was reported by Aguiar et al. [41] for different *Pleurotus* species.

2) Optimization of key factors for laccase production in vinasse medium

Following the results of the previous section, we optimized the concentration of the salts, but despite the significant effect of pH, it was not included in the analysis. The reason for this was the difficulty of measuring the pH in the established levels (Table 3). Since pH had a positive effect, its initial value was fixed at 6.5. In this sense, the work on Mycoremediation of vinasse with white rot fungi by Aragao et al. [8], Junior et al. [2] and Vilar et al. [29], used an initial pH around 6.0. The pH of the medium is important for growth and metabolism, since it affects enzyme production, cell permeability, and the availability of cofactors [36, 42].

A variable transformation resulted in the reduced polynomial equation, shown in coded units (Eq. 4).

Table 6 shows the statistical analysis for the reduced model equation. From these results the equation was considered adequate to explain the variation in enzymatic activity.

$$Y^2 = +1.71 \times 10^5 - 2{,}367.16\,A + 6{,}999.80\,B - 42{,}374.22\,C + 51{,}012.97AB + 40{,}106.37BC \qquad \text{(Eq. 4)}$$

Table 6 Statistical analysis for the power transformed reduced model

Source	df	Sum of squares	Mean squares	F value	Prob>F
Model	5	2.703×10^{10}	5.405×10^{9}	12.26	0.0008
A-$MgSO_4$	1	6.507×10^{7}	6.507×10^{7}	0.1476	0.7098
B-$FeSO_4$	1	5.690×10^{8}	5.690×10^{8}	1.29	0.2854
C-$CuSO_4$	1	1.105×10^{10}	1.105×10^{10}	25.05	0.0007
AB	1	1.601×10^{10}	1.601×10^{10}	36.30	0.0002
BC	1	9.895×10^{9}	9.895×10^{9}	22.44	0.0011
Residual	9	3.969×10^{9}	4.410×10^{8}		
Lack of fit	6	2.949×10^{9}	4.914×10^{8}	1.44	0.4106
Pure error	3	1.021×10^{9}	3.402×10^{8}		
Corrected total	14	3.100×10^{10}			
R^2		0.872			
Adjusted R^2		0.801			
Predicted R^2		0.615			
Adequate precision		13.008			

Although the concentrations of $MgSO_4 \cdot 7H_2O$ (A) and $FeSO_4 \cdot 7H_2O$ (B) were not significant, these terms were kept in the model due to the significance of their interaction (AB). The $CuSO_4 \cdot 5H_2O$(C) concentration was significant as well as its interaction with $FeSO_4 \cdot 7H_2O$ (B) concentration. The factor C coefficient had a negative sign, which suggested that laccase activity should increase as its concentration decreases. Variable interactions had a positive effect, and the magnitude of the coefficients indicated a greater effect on the observed enzymatic activity.

The interaction between the factors and the optimal regions for response can be visualized with the graphical analysis (Figure 1). Figures 1a and 1b show surface and contour plots for the effect of varying concentrations of factors B and C, keeping factor A at its optimum. The plots clearly show a decrease in enzymatic activity with increasing levels of B and C. The highest predicted response was observed for low levels of both salts.

Figures 1c and 1d show surface and contour plots for the effect of varying concentrations of factors A and B, keeping factor C at its optimum. These plots show a complex effect of A and B on enzymatic activity, whereby high levels of B and low levels of A yield the lowest predicted enzymatic activity. Intuitively, the opposite is also shown low levels of B, and high levels of A yield a low enzymatic activity. The highest predicted response was observed for low levels of both salts.

Table 4 shows the experimental and prediction results. Based on these data, the combination of factors from experiment seventeen produced the highest response, with an experimental value of 544.038 U L^{-1} and a predicted value of 547.027 U L^{-1} for the following concentrations of the magnesium, iron, and copper salts: 0.250 g L^{-1}, 0.020 mg L^{-1}, and 0.100 g L^{-1}, respectively. As can be seen, a higher experimental laccase activity was achieved compared to the results from the Plackett-Burman design.

We have not found any screening or optimization studies on the effect of magnesium, iron, and copper salts on laccase production by white rot fungi in vinasse-containing media. This impedes comparing our results against prior findings.

Nevertheless, there are several reports about the effect of single or mixed cations on laccase production in basal or defined media. For example, Zhu et al. [43], found that yeast extract with $CuSO_4$, showed a strong effect on laccase production by *Pleurotus ostreatus*. The effect of copper as inducer of laccase activity was dose and time dependent. Arockiasamy et al. [44] applied the Plackett-Burman design to identify key factors for laccase production by a *Coriolus versicolor* strain, and further optimize the culture medium. These authors reported that $MgSO_4 \cdot 7H_2O$ had a significant effect on the enzymatic activity, compared to $CuSO_4 \cdot 5H_2O$.

Zheng et al. [45] studied the effect of several metal ions on the enzymatic activity of a novel laccase from a strain of *Trametes orientalis* using yeast glucose medium. Mn^{+2} was the best inducer compared to other cations such as Fe^{+2}, Mg^{+2}, and Cu^{+2}. Finally, An et al. [38] studied the effect of individual cations and cation mixtures in basal medium. These authors obtained better results for *Pleurotus ostreatus* with the addition of a mixture of Cu^{+2} and Mn^{+2}, whereas for *Flammulina velutipes* enzymatic activity was enhanced by the single addition of Cu^{+2}.

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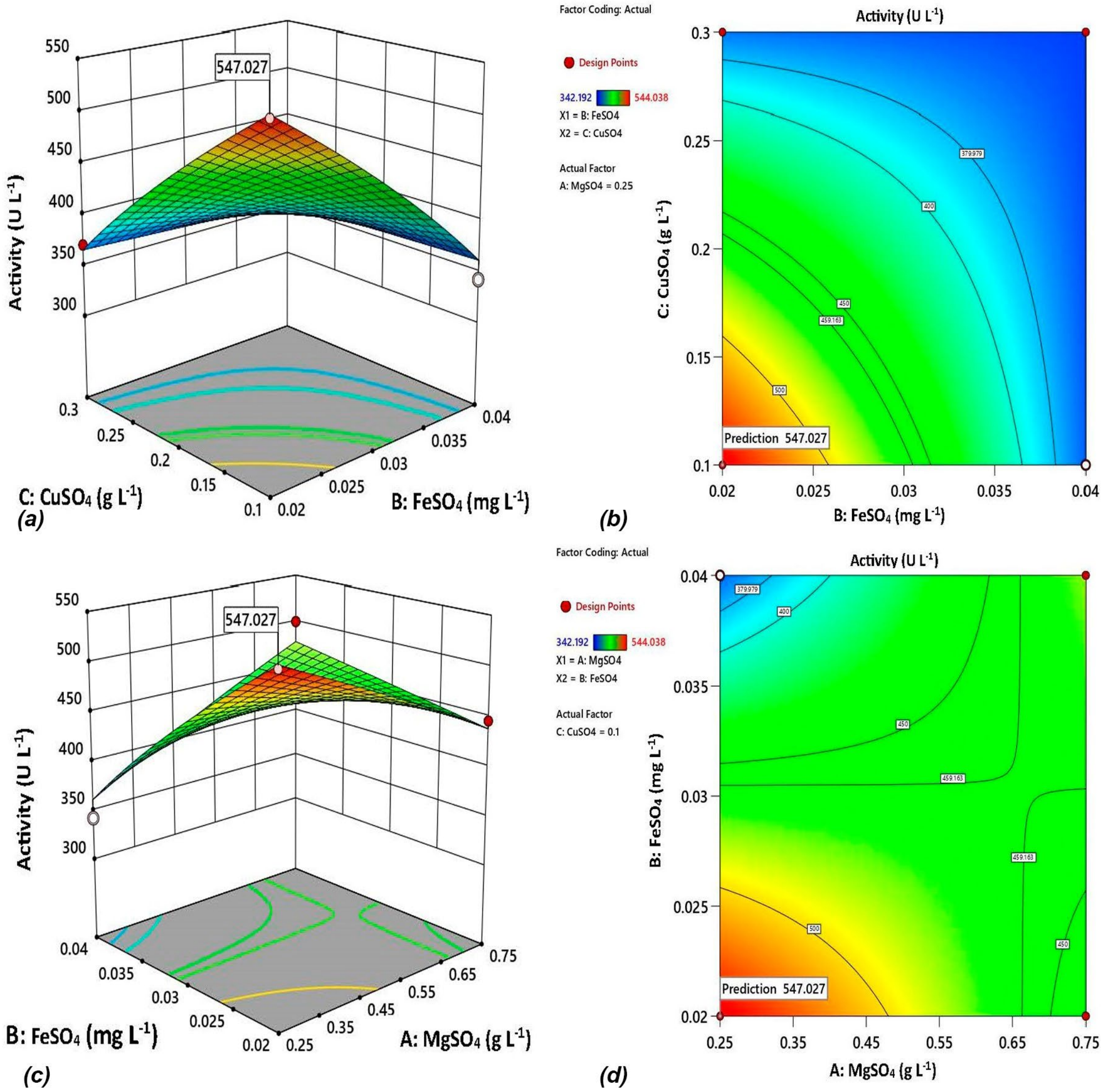

Figure 1 Surface (a) and contour (b) plots for laccase activity as a function of factors B and C, keeping factor A at its optimum concentration. Surface (c) and contour (d) plots for laccase activity as a function of factor B and A, keeping factor C at its optimum concentration.

3)Vinasse biotreatment

Fermentation was set up as described in other sections, adding the magnesium, iron, and copper salts according to the optimization results.

Table 7 lists the results for the chemical and enzymatic assays. Laccase activity was close to that obtained in the optimization experiment. Wastewater quality parameters improved after biotreatment. According to the technical regulation DGNTI-COPANIT 35-2019 [46], the pH of the medium after biotreatment is within the permissible limits for discharges of liquid effluents to receiving bodies of continental and maritime waters. This parameter is important because it alters the physicochemical properties of water bodies, which in turn has impacts on biota [47]. The pH at t_0 corresponds to the initial pH adjusted according to the chosen conditions. However, the pH of the crude vinasse was 4.59.

The maximum permissible limit for phenol in liquid effluents is 0.5 mg L^{-1} and the final phenol concentration in the fermentation medium was 9.7 mg L^{-1}. Although the permissible levels were not achieved, the biotreatment with crude extracts of *T. villosa* produced 82.74 % of phenol removal. For the COD, an important removal of 75.97 % was also obtained.

Table 7 Residual water quality parameters for vinasse samples

Parameter	**t_0**	**t_{10}**
Laccase activity (U L^{-1})	0	544.974
pH	6.50	6.90
DQO (mg L^{-1})	25,814±800	6,203±179
Total phenols (mg L^{-1})	56.2	9.7
% decolorization	-	78

Remark: t_0 centrifuged 25% v/v vinasse: t_{10} vinasse after biotreatment

Figure 2 shows culture flasks contents poured onto Petri dishes: t_0 corresponds to the centrifuged vinasse diluted 25% v/v; t_{10} corresponds to the fermentation medium after 10 days with 78% decolorization. Colored effluents may affect aquatic life by blocking the passage of light [47]. Melanoidins, phenols, caramels and furan derivatives are responsible for color in vinasse; moreover, they have an intrinsic toxicity and contribute to the organic load [48]. Therefore, *T. villosa* exhibited a good biodegradation ability in vinasse optimized medium.

Other vinasse biotreatment studies also showed the biodegradation ability of white rot fungi. For example, Vilar et al. [29] reported 97% decolorization and 50.6% reduction in COD after 15 days of fermentation and enzyme yields between 341 to 400 U L^{-1} after 12 days. They used a *Pleurotus sajor-caju* strain in two different vinasse-containing media at pH 6. Likewise, Ahmed et al. [49] reported a 43% decolorization, and 80% decrease of total phenolic compounds after 10 days of incubation with *Trametes* sp. in vinasse 10% (v/v).

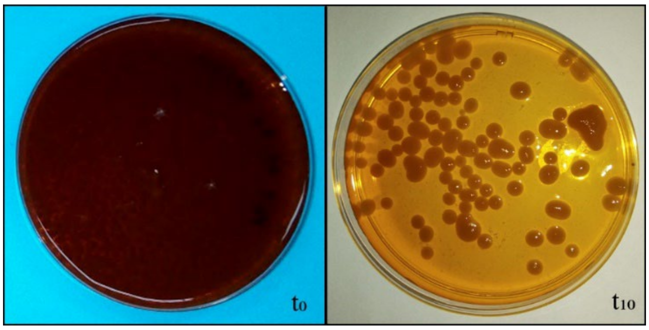

Figure 2 Pictures showing t_0 centrifuged 25% v/v vinasse and t_{10} medium after biotreatment.

It is recommended to complement this study with biodegradability assays. Biodegradability indexes are often expressed as the COD/BOD_5 ratio [50]. Usually, this index is used in decision-making for the combination of physical, chemical, or biological treatments [51]. According to Pire et al. [52] however, for effluents with a complex composition, BOD_5 may not reflect the biodegradability of a sample. Considering this idea, for vinasse complex media, we suggest the study of vinasse biodegradability through COD fractionation assays.

Conclusion

Optimization of fermentation parameters is crucial for the establishment of reproducible Mycoremediation or valorization protocols. In this sense, using a screening-optimization approach, we selected and optimized key factors for laccase production by T. villosa. This helped us define a suitable culture medium for vinasse biotreatment at the bench-top level. Overall, these conditions resulted in high laccase activity, and a high percentage of color, phenol, and COD removal.

The biotransformation capabilities of white rot fungi are well-recognized as an important aspect of an ecofriendly technology-development approach [53]. Thus, our work adds to the current understanding of these capabilities by providing baseline results for laccase production and vinasse biotreatment with *T. villosa*. We believe these results establish the potential of native *T. villosa* as a candidate for a sustainable management of vinasse.

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