Laccase and Lignin Peroxidase Production by *Cerrena unicolor* and *Loweporus lividus* in Solid-State Fermentation Using Agricultural Biomass as Substrate and its Application in Dye Degradation

Subhananda Russalamma Flanetraj,[a,*] Venci Candida Xavier James,[b] Rajakrishnan Rajagopal,[c] and Selvaraj Arokiyaraj [d]

** Corresponding author: drflanetrajmsu@gmail.com*

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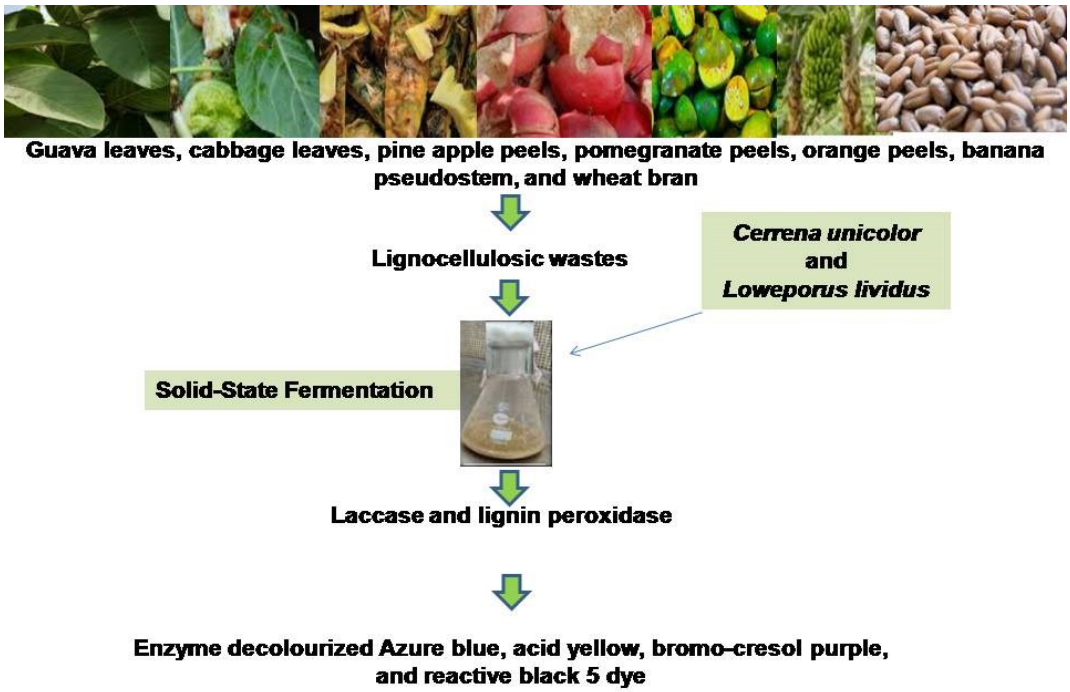
GRAPHICAL ABSTRACT



Laccase and Lignin Peroxidase Production by *Cerrena unicolor* and *Loweporus lividus* in Solid-State Fermentation Using Agricultural Biomass as Substrate and its Application in Dye Degradation

Subhananda Russalamma Flanetraj,[a,*] Venci Candida Xavier James,[b] Rajakrishnan Rajagopal,[c] and Selvaraj Arokiyaraj [d]

Lignocellulosic residues (guava leaves, cabbage leaves, pineapple peels, pomegranate peels, orange peels, banana pseudostem, and wheat bran) were used for the production of laccase (LaC) and lignin peroxidase (LiP) *via* solid-state fermentation (SSF) by fungi *(Cerrena unicolor* and *Loweporus lividus*). The results revealed that b*anana* pseudostems presented higher LaC and LiP activities (49.8±1.4 U/g and 7.8±0.24 U/g, respectively) than other agricultural residues did. Banana pseudostems presented increased amounts of lignin (21±0.28%), cellulose (42.8±0.92%), and hemicelluloses (22.3±0.14%), which stimulated enzyme production. The data revealed that glucose (a carbon source), ammonium sulfate (a nitrogen source), an inducer (polysorbate 80, 0.15%), a pH of 4.5, and a 60% moisture content were optimal for LaC and LiP production. Two-level full factorial designs revealed that the variables moisture, pH, polysorbate 80, and glucose significantly influenced LaC and LiP production ($p<0.001$). A central composite design was applied to optimize the medium components, and glucose and polysorbate 80 influenced LaC and LiP production. The optimized medium (4.82 pH, 0.13% polysorbate 80, and 0.57% glucose) improved LaC (151.9 U/g) and LiP (19.2 U/g) production. The crude enzyme was used to decolorize the dyes. The degradation rates of acid yellow, bromo-chloroform purple, and reactive black 5 were >82%.

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Keywords: Agriculture residues; Lignocellulosic wastes; Lignin peroxidase; Laccase; Decoloration

Contact information: a: Department of Zoology, Nesamony Memorial Christian College, Marthandam, Affiliated to Manonmaniam Sundaranar University, Tirunelveli, India. b: Department of Zoology, Holy Cross College (Autonomous) Nagercoil, Affiliated to Manonmaniam Sundaranar University, Tirunelveli, India; c: Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia; d: Department of Food Science & Biotechnology, Sejong University, Seoul 05006, South Korea

** Corresponding author: drflanetrajmsu@gmail.com*

INTRODUCTION

Fungi are the most important microorganisms responsible for the degradation of wood and plant biomass in the environment. In particular, specific types of basidiomycetes, *e.g.,* white-rot fungi, leaf-litter fungi, and brown-rot fungi, are able to completely degrade lignin (Martínez *et al*. 2005). They produce several extracellular oxidative biocatalysts, such as lignin peroxidase (LiP), laccase (LaC), and manganese peroxidase (MnP) (Tanaka

et al. 2009). Owing to their effective nonspecific mechanism, these compounds are helpful for several applications in the paper industry and for the degradation of several recalcitrant compounds. Among fungi, white-rot fungi have the potential to decompose lignin due to the synthesis capacity of ligninolytic enzymes. In addition, they produce cellulases, lignin-degrading enzymes, including three heme peroxidases, and versatile peroxidases, depending on the fungal strain, culture conditions and culture type (Thurston 1994). The reactions catalyzed by laccases and peroxidases are similar and are based on the reduction–oxidation mechanism. The other mechanisms of these enzymes involve electron oxidation, which generates radicals and facilitates the degradation of several aromatic amines and phenolic compounds (Bilal *et al*. 2022). Moreover, these biocatalysts have various mechanisms, especially their prosthetic groups, which are enzymes with greater redox potential than laccases do, and laccases use molecular oxygen (cosubstrates for catalysis), whereas peroxidases require hydrogen peroxide (H_2O_2) for catalysis (Leynaud Kieffer Curran *et al*. 2022). Lignin-modifying enzymes have the ability to catalyze inorganic and organic substrates, and these biocatalysts can be applied for the treatment of several environmental contaminants from agro-industrial waste, agricultural pesticides, insecticides, and the pharmaceutical and textile industries (Loi *et al*. 2021). Fungal laccases have the ability to oxidize lignin components, several chemicals, and xenobiotic substances, including textile dyes (Janusz *et al*. 2020; Suresh and Johney 2024; Benavides *et al*. 2024; Arumugam and and Uthandi 2024).

Laccase activity has been detected in several white-rot fungi, and microbial factories, especially fungi belonging to Deuteromycetes and Ascomycetes, are largely considered. The extracellular laccases were characterized from fungi such as *Fusarium solani* (Wu *et al*. 2010), *Lentinula edodes* (Nagai *et al*. 2002), *Leptosphaerulina chartarum* (Cruz-Vázquez *et al*. 2022), *Thermobifida fusca* (Mtibaà *et al*. 2018), and *Thielavia* sp. (Wang *et al*. 2017), which were isolated from various sources. Laccase is a useful enzyme that directly oxidizes phenolic lignin units, and the composition of phenolic lignin is 10% of the total polymer content (Bourbonnais and Paice 1990). Lignin peroxidase is a significant oxidizer of ligninase reported to date and has the potential to catalyze the oxidation of nonphenolic and phenolic compounds, aromatic ethers, aromatic amines, and polycyclic aromatic hydrocarbons (Martínez *et al*. 2009). Previously, LaC and MnP production was optimized *via* a statistical approach for white-rot fungi and other fungi, including *Trametes versicolor* (Tavares *et al*. 2006), *Pleurotus ostreatus* (Liu *et al*. 2009), *Bjerkandera adusta* (Seker *et al*. 2008), and *Panus tigrinus* (Quaratino *et al*. 2008). The combination of laccase and lignin peroxidase is more effective for lignin degradation than the individual activities of laccase and lignin peroxidase (Shi *et al*. 2021). Ligninolytic biocatalysts produced by white-rot fungi have several industrial applications; however, their high cost and low productivity are major limitations in the use of ligninolytic enzymes for environmental applications (Rivera-Hoyos *et al*. 2013). Therefore, the inexpensive production of ligninolytic enzymes is continuously being investigated. An improvement in the productivity of enzymes and an increased enzyme catalysis rate are needed for effective enzyme-catalyzed bioprocesses (Singh *et al*. 2023). Laccases are inducible enzymes, and certain chemicals have also been tested (*e.g*., ferulic acid, xylidine, pyrogallol, veratryl alcohol, and copper) to improve laccase production (Rancano *et al*. 2003).

The traditional single factor method of optimization is time consuming and inadequate for optimizing the variables and will not yield any solid outcome regarding the interactions between variables. Statistical methods are appropriate for optimizing the variables. Response surface methodology has been used for the optimization of the culture

medium for improved production of LaC and MnP and synthetic dye decolorization by *Trametes trogii* (Trupkin *et al*. 2003). A Plackett–Burman design was employed to evaluate the medium components, and central composite design (CCD) was used to determine the optimum concentrations of the significant variables (physical and nutrient factors) in *Aspergillus* sp. (Bhamare *et al*. 2018). Kumar *et al*. (2016) optimized laccase production, and the experimental design improved bioprocess design and optimized *Aspergillus flavus* culture conditions, such as temperature, pH, carbon and nitrogen sources. Agricultural residues are low-cost substrates used for the production of ligninolytic enzymes. These residues are second-generation substrates for enzyme production in solid-state fermentation (SSF). Agroindustrial residues provide nitrogen and carbon nutrients, which are potential substrates for laccase production in SSF. Oil palm frond parenchyma tissue has been used as a medium for laccase production *via* the white rot fungus *Pycnoporus sanguineus via* SSF. A tray bioreactor was used to optimize the factors *via* a statistical design (Kalaiyarasi *et al*. 2020). In general, the least expensive and most abundant lignocellulosic agrowastes are crop residues (wheat, rice or corn). Other major sources are sugarcane bagasse, apple pomace, sawdust, cotton stalks (Wang *et al*. 2019), wheat straw (Arora *et al*. 2002), lignocellulosic agricultural residues (Gomes *et al*. 2009), and agro-industrial wastes (Al-Ansari *et al*. 2020). Agricultural wastes contain carbon and nitrogen nutrients and are used to produce LaC and LiP in SSF. They are the least expensive and mainly composed of hemicelluloses, cellulose, and lignin. The valorization of agricultural wastes by the biosynthesis of low-cost enzymes under SSF is a promising approach (Arokiyaraj *et al*. 2024). White-rot fungi are involved in the valorization of various lignins and aromatic compounds because lignin and dyes have similar structures, and white-rot fungi degrade these substrates (Meng *et al*. 2020). The objective of the study was to use *Cerrena unicolor* and *Loweporus lividus* for the production of laccase and lignin peroxidase by utilizing agro-wastes. This study also aimed to analyze simultaneous production of these enzymes using the mixture culture of *C. unicolor* and *L. lividus* using banana pseudostems as a cheap substrate in solid state fermentation. The culture conditions were optimized and the crude enzyme was used to analyze the degradation potential of acid yellow, bromo-chloroform purple, and reactive black.

EXPERIMENTAL

Fungi and Culture Conditions

Cerrena unicolor MTCC 5159 and *Loweporus lividus* MTCC1178 are basidiomycete used for the production of laccase and lignin peroxidase. The fungi described were obtained from Microbial Type Culture Collection, Pune, India. They were maintained on agar slants, and potato dextrose agar (PDA) medium (Himedia Laboratories, India), respectively. The pH of the medium was adjusted to 6.0 using 1N HCl or 1N NaOH. They were inoculated in basal media (g/L) (yeast extract, 5; glucose, 10; $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 0.6; K_2HPO_4, 0.4; $MnSO_4 \cdot H_2O$, 0.05; $FeSO_4 \cdot 7H_2O$, 0.05; and $ZnSO_4 \cdot 7H_2O$, 0.001) and incubated for 8 days at 28±1 °C under static conditions. To propagate *C. unicolor* and *L. lividus*, they were grown individually on potato dextrose agar (PDA) plates and incubated for two weeks at 28±1 °C. After two weeks, the agar cylinders were cut and stored at 4 °C with 10% glycerol and used for propagation.

Fungal Biomass Assay

The fungi were inoculated in a 250 mL Erlenmeyer flask containing 100 mL of minimal medium (g/L) (glucose 10, KH_2PO_4 0.5, inositol 0.05, $CaCl_2$ 0.075, $FeCl_3$ 0.01, $MgSO_4$ 0.15, $MnSO_4$ 0.01; yeast extract 0.2, and peptone 2), inoculated with five agar discs (with mycelia), and incubated for two weeks at 28±1 °C under shaking (125 rpm) in an orbital shaker incubator. Five milliliters of culture was withdrawn every 48 h. The sampling procedure was performed every 48 h. The culture was filtered through Whatman no. 1 filter paper, and the biomass was collected. Furthermore, the cell-free extract was used as the source for extracellular enzymes. The collected samples were stored at −20 °C in 50 mL screw cap plastic vials (Hein *et al*. 2023).

Substrates

Lignocellulose residues, *i.e*., guava leaves, cabbage leaves, pineapple peels, pomegranate peels, orange peels, banana pseudostems, and wheat bran, were collected from Kanniyakumari, India. These lignocellulosic residues are cost effective and were washed, dried for 24 h at 80 ºC in an oven and ground mechanically using a mixer grinder. The final particle size was approximately 0.2 to 1.8 mm by sieving the particle using 1 to 2 mm sieve. These ready-to-use substrates were individually stored in airtight containers for SSF.

Analysis of Lignin, Hemicelluloses and Cellulose

The amounts of hemicellulose, cellulose, and lignin contents were analysed using the NREL method (Sluiter *et al.* 2008; Peng and Wu 2010; Yang *et al*. 2007). Briefly, 500 ± 15 mg of the sample was treated with 4 mL of sulphuric acid (72%) and was shaken vigorously. Subsequently, it was incubated for 60 min at 30 ± 1℃. It was transferred to a beaker and the volume was adjusted to 100 mL. It was sealed, filtered and the pH was adjusted between 1 and 3. The amount of sugar level was determined using a High-Performance Liquid Chromatography (Agilent Technologies, USA) and the content of hemicellulose, cellulose, and lignin were calculated.

Solid-State Fermentation

Five grams of the prepared substrate was individually added to 100 mL Erlenmeyer flasks. In each flask, 5 mL of water was added, and the mixture was mixed with a wooden stick. The mixture was sterilized for 30 min at 15 lbs in an autoclave. The mixture was cooled, and the substrates were inoculated with two cultures by adding 1.25 mL of the spore suspensions of *C. unicolor* and *L. lividus*. The culture flasks were subsequently maintained in an incubator at 28±1 °C for 7 to 8 days (El-Sheikh *et al*. 2020).

Enzyme Extraction

The experimental Erlenmeyer flasks were flooded with 50 mL of double distilled water and kept in a shaker incubator at 100 rpm for 30 min. The biomass was filtered through Whatman No. 1 filter paper, and the samples were stored in 50 mL plastic vials. The mixture was centrifuged at 5000×g for 10 min, and the clear supernatant was used for the enzyme assay (Vijayaraghavan *et al*. 2012).

Enzyme Assays

Laccase assay

The laccase activity of the extract was determined at 415 nm *via* the oxidation of 2,20-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (10 mM) in 0.1 M acetate buffer (pH 4.5) (Wang *et al*. 2014).

Lignin peroxidase assay

The LiP assay was evaluated by the development of veratraldehyde from veratryl alcohol (25 mM) in tartrate buffer (0.1 M, pH 3.0). The enzymatic reaction was initiated by adding 54 mM hydrogen peroxide to the reaction mixture, and the amount of veratraldehyde was measured at 310 nm.

Analysis of Variables for LaC and LiP Production by Fungi

The banana pseudostem was used as the substrate for optimization studies unless otherwise stated. Approximately 5 g of substrate was mixed with an appropriate amount of carbon, nitrogen or inducer individually. To determine the effects of carbon sources on LaC and LiP production, a 1% (50 mg) carbon source (glucose, maltose, xylose, sucrose, and trehalose) was individually added, buffer (3.5 mL, acetate buffer, pH 4.5) was added, and the culture medium was sterilized. To determine the effects of nitrogen sources, yeast extract, beef extract, ammonium sulfate, ammonium nitrate, and casein were added individually. To determine the optimum concentration of Tween-80 (polysorbate 80), it was added to the solid medium at various concentrations (0.05 to 0.2%). To optimize the pH, the culture medium pH was maintained between 3.5 and 6.0, and the optimum moisture content was between 40 and 70%.

Screening of Medium Components *via* a Two-Level Full Factorial Design

The screening of culture medium components for LaC and LiP was carried out *via* 2^5 full factorial designs. Five medium components were used: ammonium sulfate (nitrogen), glucose (carbon), Tween 80 (polysorbate 80 surfactant), pH, and temperature (physical factors). High (+) and low (-) settings of each of these culture medium components were applied to make a combination of 32 experimental trials of the culture medium (Table 1). For the sterilized medium, 2.5% inoculum (v/w) was introduced, and a fermentation experiment was performed for eight days at 28±1 °C under static conditions.

Table 1. Analysis of Variables for LaC and LiP Production in SSF *via* Two-Level Full Factorial Design (2^5)

Factor	Name	Units	Low Actual	High Actual	Low Coded	High Coded
A	Moisture	%	60	80	-1	1
B	pH		3.5	5.5	-1	1
C	Polysorbate-80	%	0.5	1	-1	1
D	Glucose	%	0.1	1	-1	1
E	Ammonium sulfate	%	0.1	0.5	-1	1

Nutrient Source Interactions in the Central Composite Design

In this study, an experiment was performed to determine the effects of selected variable factors that can influence the biosynthesis of LaC and LiP *via* central composite

design and response surface methodology. The experimental runs were performed with varying ammonium sulfate and glucose concentrations, and Tween-80 was used as the inducer for enzyme production. The medium components corresponding to the actual levels and the experimental matrix are presented in Table 2. A total of 20 experiments were run for three variables at five different concentrations of ammonium sulfate, glucose, and Tween 80 (polysorbate 80). The pH and moisture content were excluded from this experiment because these variables had less impact than the other variables did, and the middle point was considered for this step of optimization (Alam *et al.* 2009). Experiments were performed at various ammonium sulfate, glucose, and polysorbate 80 concentrations, with a moisture level of 70%, and a pH of 5.5 and citrate buffer (pH 5.5, 0.1 M) were used to adjust the moisture and pH of the solid substrate. The design of the experiments was a combination of these three factors at five different levels; the values for LaC and LiP are given in Table 2.

Table 2. Low and High Concentrations of the Variables for Laccase and Lignin Peroxidase

Factor	Name	Units	Low Actual	High Actual	Low Coded	High Coded
A	pH		3.5	6	-1	1
B	Polysorbate-80	(%)	0.02	0.2	-1	1
C	Glucose	%	0.1	1	-1	1

The combination of these nutrient variables was used to determine the impacts of a particular nutrient on the production of LaC and LiP in the SSF. For interaction studies, experiments were performed in triplicate, and the average value of the triplicate findings was included. The relationships of the three selected independent variables (A, B, and C) and their estimated response (enzyme yield, Y) were calculated *via* the second-order polynomial equation. The statistical analysis was performed with Design Expert software (StatEase, USA). The levels of LaC and LiP in the medium were estimated *via* spectrophotometry.

Decolouration Properties

The decrease in the absorbance of the synthetic dyes azure blue (645 nm), acid yellow (404 nm), bromo-cresol purple (431 nm), and reactive black 5 (600 nm) was used to analyze the decolorization properties of LaC and LiP in the presence of the redox mediator 1-hydroxybenzotriazole (HBT). The dye solution was prepared at a 0.005% (w/v) concentration in acetate buffer (pH 4.5, 20 mM) with 1 mM HBT. The total volume of the solution was 2 mL, and the LaC and LiP concentrations were 10 U/mL. The decolorization efficiency was analyzed by measuring the decrease in absorbance with time (4 h, 8 h, 12 h, 16 h, 20 h, and 24 h). The enzyme was deactivated and incubated with dye, which was used as a negative control. The degradation (%) was calculated *via* the following formula.

$$\text{Decolorization (\%)} = (A_i - A_t)/A_i \times 100 \quad (1)$$

where A_i is the initial absorbance of the dye and A_t is the final absorbance of the dye after a specific time.

RESULTS AND DISCUSSION

Effects of Agroresidues on LaC and LiP Production

C. unicolor and *L. lividus* were cultured in substrates such as guava leaves, cabbage leaves, pineapple peels, pomegranate peels, orange peels, banana pseudostems, and wheat bran in the SSF. The synthesis of LaC and LiP was tested, and enzyme activity was assayed after 8 days. Banana pseudostems presented the highest LaC and LiP activities in fermented media (49.8±1.4 U/g and 7.8±0.24 U/g, respectively) (Fig. 1). Moreover, the other substrates also induced the production of LaC and LiP in the SSF. Similarly, several agricultural residues have been used for the production of LaC in SSF, and the yield varies on the basis of the type of substrate and microorganism (Risdianto *et al*. 2012; Chmelová *et al*. 2022). A lignogenic substrate was utilized by *Phanerochaete chrysosporium via* SSF, and lignin peroxidase production was reported (Asgher *et al*. 2006). Agro-industrial wastes such as wheat bran (Baker and Charlton 2023), cotton seed hull, corn cob, and rice straw (An *et al*. 2021) have been used for the production of laccases. LiP is produced by various white-rot fungi, including *Trametes trogii* and *Schizophyllum commune* IBL-06, *via* lignocellulolytic wastes, including banana stalks. The present findings support previous results on the utilization of banana stalks for LaC and LiP production (Irshad and Asgher 2011).

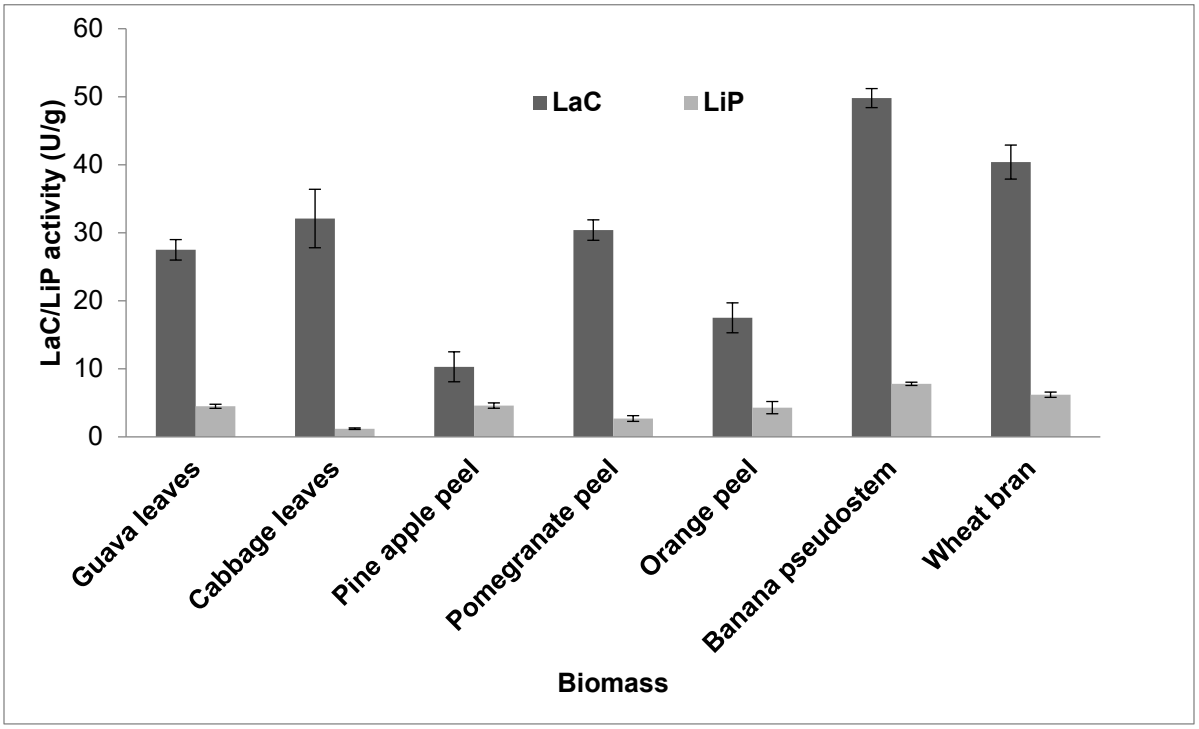


Fig. 1. Agricultural Residues for the production of LaC and LiP in Solid-state fermentation. Five grams of the prepared substrate was sterilized and inoculated into two cultures by adding 1.25 mL of the spore suspensions of *C. unicolor* and *L. lividus*. The culture flasks were subsequently incubated at 28±1 °C for 7 to 8 days.

Analysis of Cellulose Materials

The lignin, hemicellulose and cellulose contents of the agroindustrial residues were analyzed, and the compositions are depicted in Table 3. In the tested agricultural biomass, cellulose (40 to 60%), hemicellulose (15 to 30%), and lignin (10 to 25%) are the major components, and the composition varies depending on the type of biomass being utilized for fermentation (Zhao *et al*. 2017). The amounts of hemicelluloses (11 to 34%), α-cellulose (15 to 35%), and lignin (17 to 35%) reported previously (Camarena-Tello *et al*. 2015) in guava tree pruning were similar to the present findings. Cabbage waste is rich in cellulose and hemicelluloses (Bamisaye *et al*. 2024), and a 31±0.49% cellulose content was detected in the pineapple peels, which was higher than that previously reported (Pereira *et al*. 2022). The amounts of lignin and hemicelluloses detected in pomegranate peel were similar to those reported in previous studies (Hasnaoui *et al*. 2014). The banana pseudostem used in this study contained 42.8±0.92% cellulose, and in an earlier report, 32.4 to 64.0% cellulose was reported (Pereira *et al*. 2022).

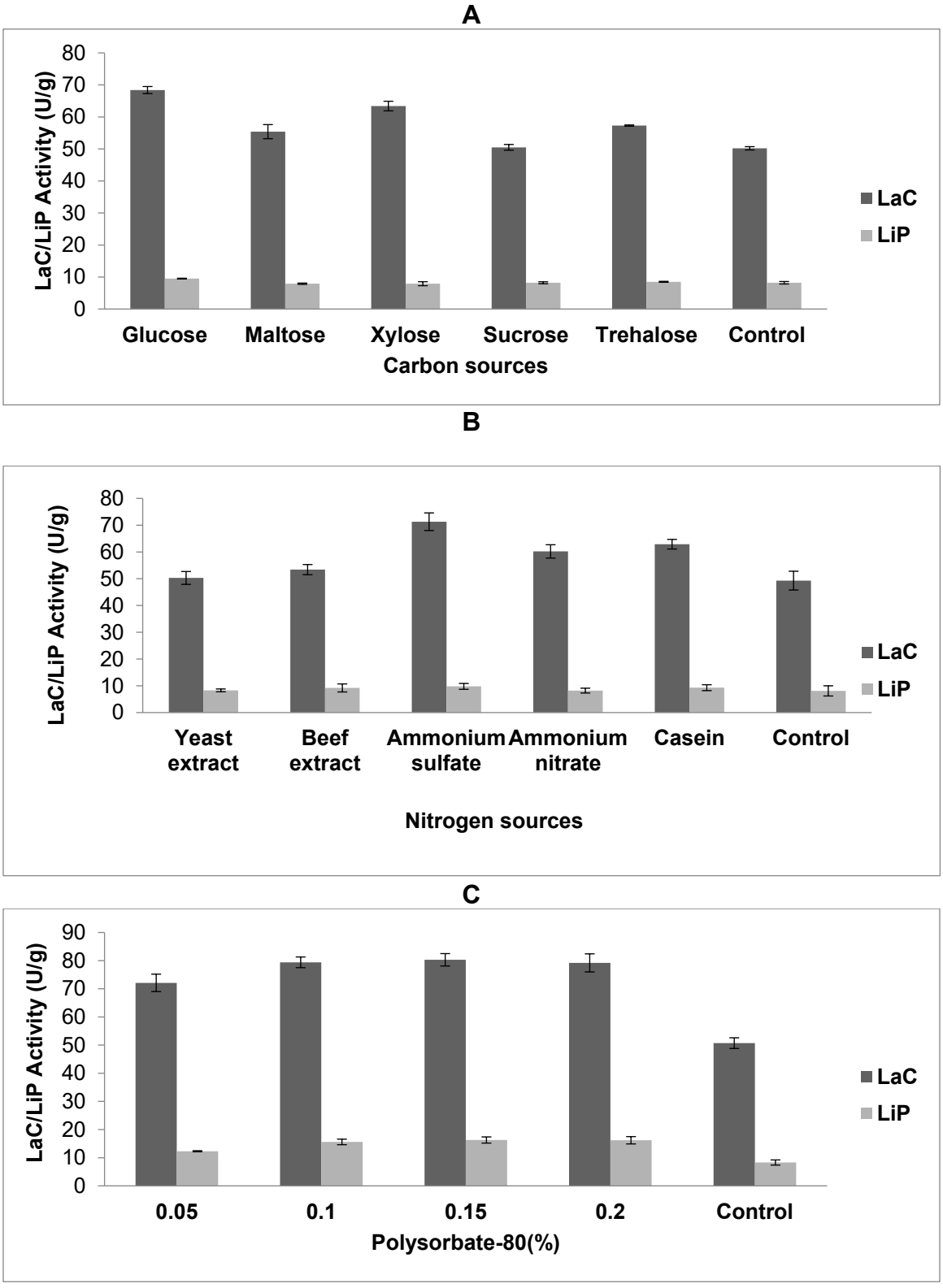
Table 3. Chemical Composition of Ligninocellulose Materials in Agricultural Residues

Agricultural Residues	Composition (%)		
	Lignin	Cellulose	Hemicellulose
Guava leaves	17.1±0.97	30.2±2.2	15.3±1.2
Cabbage leaves	12.5±1.59	29.3±0.19	18.2±0.19
Pineapple peel	12.4±2.8	31±0.49	21.4±0.19
Pomegranate peel	18±0.15	39.5±1.2	24.3±0.26
Orange peel	19.4±1.2	25.4±0.15	20.3±0.17
Banana pseudostem	21±0.28	42.8±0.92	22.3±0.14
Wheat bran	13.4±1.2	29.4±1.3	21±1.1

Optimization of Variables for LaC and LiP Production by Fungi in SSF

The data revealed that glucose (carbon source), ammonium sulfate (nitrogen source, inducer (polysorbate 80, 0.15%), pH (4.5) and moisture content (60%) were optimal in the one-variable-at-a-time approach (Fig. 2). The variation in the results highlights the importance of medium optimization in SSF. LaC and LiP production was influenced by these factors ($p<0.05$). The results of the carbon source experiments revealed the maximum production of LaC (68.4±2.2 U/g) and LiP (9.5±0.12 U/g) in the banana pseudostem medium supplemented with glucose (Fig. 2A). Similarly, nitrogen sources influenced enzyme production, and ammonium sulfate supplementation significantly improved the LaC and LiP yields (71.3±2.4 U/g and 9.8±1.1 U/g, respectively) (Fig. 2B). The results obtained with 0.15% polysorbate 80 supported the maximum production of LaC (80.3±3.1 U/g) and LiP (16.2±1.1 U/g) (Fig. 2C). White rot fungi reportedly synthesize many enzymes by utilizing carbon sources, nitrogen sources or inducers in the culture medium. The optimum pH improved growth and enzyme production in white-rot fungi (Xu *et al*. 2020). In this study, LaC and LiP production was high at pH 4.5 (Fig. 2D) and at 60% moisture content (Fig. 2E). In SSF, fungal growth occurs on the surface of the medium, and the fermentation process is governed by the available water content of the medium. The solid substrates have a greater surface area, which allows the growth of fungi. Microorganisms require optimal water for their metabolic activity, and lower and higher

water contents negatively influence enzyme production. In general, fungi require moisture contents between 50% and 65% (Wang *et al*. 2019; Arokiyaraj *et al*. 2024), which is consistent with the results of this study.



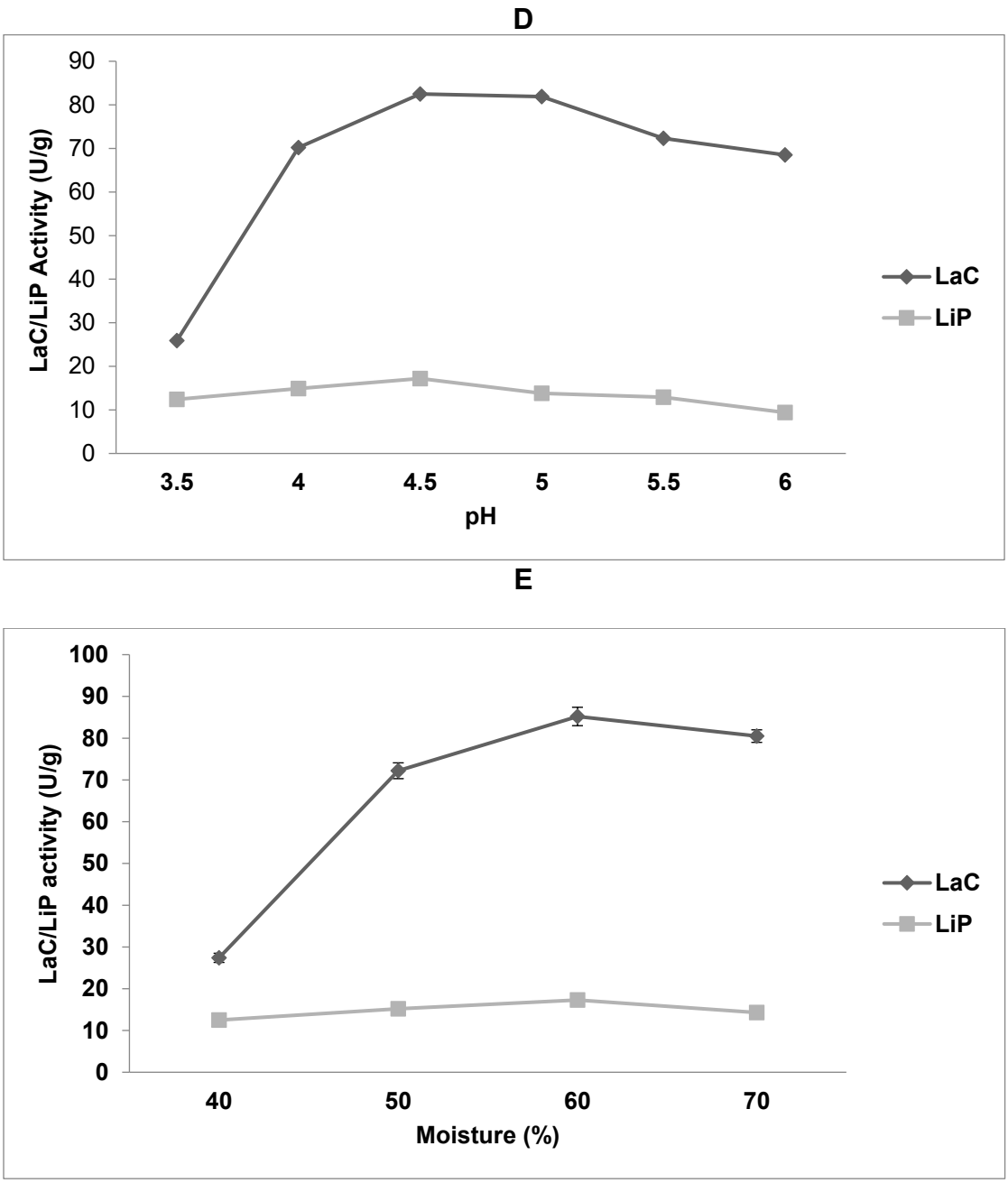


Fig. 2. Effects of Nutrient sources, inducers and physical parameters on laccase and lignin peroxidase production in Solid-state fermentation. (A) Carbon source, (B) Nitrogen source, (C) Tween-80, (D) pH, and (E) Moisture

Simultaneous Optimization of LaC and LiP Produced by Fungi

Response surface methodology was applied to optimize the synthesis of lignin-modifying LaC and LiP in the SSF. For LaC and LiP, the optimization experiment was performed *via* a statistical approach, and the enzyme activity and corresponding production rate were observed. The optimum factors for laccase production in one variable at a time approach were selected for statistical optimization for LaC and LiP production. The effects of nutrients (glucose, and ammonium sulfate), inducers (polysorbate 80), and physical parameters (moisture and pH) were chosen to assess the interaction effects of these selected

individual nutritional and cultural factors. This experiment was intended to overcome the poor reliability and achieve the most likely combinations of individual variables for improved production of enzymes on a large scale. The effective variables that directly affect the coproduction of LaC and LiP were screened *via* a two-level full factorial design. This design revealed that moisture, pH, polysorbate 80, and glucose concentration play significant roles in the coproduction of LaC and LiP (Table 4). Moreover, ammonium sulfate did not significantly influence these experimental results. Table 4 shows the influence of the selected medium components on LaC and LiP production. Among the tested process parameters, glucose significantly influenced LaC and LiP production. The model "F' value for LaC production was 24.57 and was statistically significant (Table 5). Similarly, the F value of "LiP" production was 33.72, and the model was significant ($p<0.0001$) (Table 6). The lignin content of the banana pseudostem was high, and the selected white rot fungi utilized glucose more easily. The concentration and nature of the source were the major factors influencing ligninolytic enzyme production by basidiomycetes. In this study, the supplemented ammonium sulfate had a positive effect on enzyme production; however, the effect was insignificant. In ligninolytic enzyme production, a low level of nitrogen stimulates enzyme secretion. The synthesis of laccases by white rot fungi was induced by the addition of polysorbate 80 to the medium. By the two-level full factorial design, a maximum activity of 148 U/g LaC and 15.2 U/g LiP was obtained.

Table 4. Production of Laccase and Lignin Peroxidase by Fungi in the Two-Level Full Factorial Design Experiment

Runs	Moisture (%)	pH	Polysorbate 80 (%)	Glucose (%)	A. sulphate (%)	LaC (U/g)	LiP (U/g)
1	35	3.5	0.02	0.1	0.5	12	1.9
2	65	5.5	0.2	0.1	0.1	48	5.1
3	35	3.5	0.02	1	0.1	85	6.2
4	35	5.5	0.2	0.1	0.5	48	5.1
5	65	5.5	0.2	1	0.1	201	2.94
6	65	3.5	0.2	1	0.5	85.3	9.1
7	35	5.5	0.2	1	0.1	89	9.2
8	35	5.5	0.02	1	0.1	72	7.3
9	65	5.5	0.2	1	0.5	110	11.5
10	35	3.5	0.2	1	0.5	20.4	2.09
11	65	5.5	0.02	1	0.5	120	12.9
12	65	3.5	0.2	0.1	0.1	65.4	7.1
13	35	3.5	0.02	1	0.5	15.2	1.62
14	35	3.5	0.2	1	0.1	72.5	8.1
15	65	5.5	0.2	0.1	0.5	78.5	6.9
16	35	3.5	0.2	0.1	0.5	50.6	5.2
17	35	5.5	0.02	1	0.5	75.3	8.3
18	35	3.5	0.02	0.1	0.1	19.3	2.4
19	65	5.5	0.02	0.1	0.1	74.2	7.5
20	35	5.5	0.2	0.1	0.1	43.2	5.1
21	35	5.5	0.02	0.1	0.5	61.4	6.3
22	65	3.5	0.02	1	0.1	43.5	5.3
23	65	3.5	0.2	0.1	0.5	41.5	5.4
24	65	3.5	0.02	0.1	0.5	12.3	2.5
25	65	3.5	0.02	1	0.5	61.5	8.9

26	65	3.5	0.02	0.1	0.1	6.3	0.42
27	65	3.5	0.2	1	0.1	64.3	8.2
28	35	5.5	0.02	0.1	0.1	89.5	9.1
29	35	3.5	0.2	0.1	0.1	72.5	7.3
30	65	5.5	0.02	1	0.1	24.3	2.5
31	35	5.5	0.2	1	0.5	148.3	15.2
32	65	5.5	0.02	0.1	0.5	69.5	9.2

Table 5. Analysis of Variance for the Production of Laccases *via* a Two-Level Full Factorial Design in Solid-State Fermentation

Source	Sum of Squares	df	Mean Square	F-Value	p value Prob > F
Model	51424.41	20	2571.22	24.57993	< 0.0001
A-Moisture	539.5613	1	539.5613	5.15801	0.0442
B-pH	12191.41	1	12191.41	116.5455	< 0.0001
C-Polysorbate-80	4930.245	1	4930.245	47.13135	< 0.0001
D-Glucose	7669.411	1	7669.411	73.31679	< 0.0001
AC	877.805	1	877.805	8.391498	0.0145
AD	552.7813	1	552.7813	5.284388	0.0421
AE	834.3613	1	834.3613	7.976191	0.0165
BD	798.0013	1	798.0013	7.628603	0.0185
BE	1247.501	1	1247.501	11.92566	0.0054
CD	1137.645	1	1137.645	10.87547	0.0071
ABE	1023.781	1	1023.781	9.786978	0.0096
ACE	2284.88	1	2284.88	21.84262	0.0007
BCD	7092.405	1	7092.405	67.80082	< 0.0001
BDE	316.2613	1	316.2613	3.023343	0.1099
CDE	557.78	1	557.78	5.332175	0.0414
ABCE	1383.38	1	1383.38	13.22461	0.0039
ABDE	2057.611	1	2057.611	19.67002	0.0010
ACDE	1866.605	1	1866.605	17.84407	0.0014
BCDE	2178	1	2178	20.82089	0.0008
ABCDE	1884.98	1	1884.98	18.01973	0.0014
Residual	1150.671	11	104.6065		
Cor Total	52575.08	31			

Table 6. Analysis of Variance for the Production of Lignin Peroxidase *via* a Two-Level Full Factorial Design in Solid-State Fermentation

Source	Sum of Squares	df	Mean Square	F-Value	p value Prob > F
Model	352.5311	18	19.58506	33.72411	< 0.0001
B-pH	56.2065	1	56.2065	96.7837	< 0.0001
C-Polysorbate-80	14.03175	1	14.03175	24.1617	0.0003
D-Glucose	33.68153	1	33.68153	57.99725	< 0.0001
E-Ammonium sulphate	10.52258	1	10.52258	18.11915	0.0009
AB	11.48403	1	11.48403	19.7747	0.0007
AE	41.2459	1	41.2459	71.02258	< 0.0001
BC	20.01863	1	20.01863	34.47069	< 0.0001
BE	38.21565	1	38.21565	65.8047	< 0.0001
DE	14.29788	1	14.29788	24.61995	0.0003
ABC	4.039903	1	4.039903	6.956433	0.0205

ABD	24.72803	1	24.72803	42.57995	< 0.0001
ACD	3.719628	1	3.719628	6.404941	0.0251
ACE	5.273128	1	5.273128	9.07996	0.0100
ADE	9.867903	1	9.867903	16.99184	0.0012
BCD	28.21883	1	28.21883	48.59086	< 0.0001
BCE	7.575778	1	7.575778	13.04496	0.0032
BDE	26.5174	1	26.5174	45.66113	<0.0001
ABCD	2.886003	1	2.886003	4.969497	0.0441
Residual	7.549666	13	0.580744		
Cor Total	360.0807	31			

Response Surface Methodology

To determine the optimum medium composition, the RSM methodology was selected as a statistical tool. As stated previously (Marraiki *et al*. 2020), to perform RSM analysis, a CCD design was selected because this method is flexible to five different levels, including negative and positive axial points with six central points. The experimental factors selected from two-level-full factorial designs were (A) pH, (B) polysorbate 80, and (C) glucose concentration. The individual and interactive effects of these three selected individual factors were analyzed at five different levels. Table 7 shows the full factorial CCD and their observed responses for LaC and LiP production in the SSF. A regression model with >0.9 was considered to indicate a significant correlation. Therefore, the R^2 value obtained in this study indicated a good fit between the predicted and observed responses, implying that the designed model was reliable for LaC and LiP production. The F test (178.9 and 26.14) showed high significance for the regression model for LaC and LiP. All of these considerations revealed the good adequacy of the designed CCD model. Tables 8 and 9 show the ANOVA results of the CCD for the production of LaC and LiP. The p values < 0.0001 indicate that the model terms were significant for LaC and LiP. Arokiyaraj *et al*. (2024) reported that the carbon source of the medium had a significant effect on fungal growth and LaC production. These previous investigations further confirmed the results obtained in this study. Response surface methodology has been widely used as a statistical tool for the improvement of enzymes. Yasmeen *et al*. (2013) used banana stalks and corn stover for the production of ligninolytic enzymes *via S. commune*. The optimized culture medium improved the production of laccase, manganese peroxidase, and lignin peroxidase. Gassara *et al*. (2010) optimized ligninolytic enzymes using agro-industrial wastes *via* response surface methodology. Brewery waste and apple pomace biomass were utilized as potential substrates for the production of laccase, lignin peroxidase and manganese peroxidase. Rubber tree sawdust has been utilized for the production of laccase and lignin peroxidase by *Trichoderma hamatum* (Arokiyaraj *et al*. 2024). Polysorbate 80 induced the production of LaC and LiP; however, glucose significantly influenced enzyme production. As depicted in Figs. 3 and 4, polysorbate 80 and glucose were more significant factors than the pH of the medium. Thus, among the selected variables, glucose and polysorbate 80 increased LaC and LiP production in the CCD design, followed by pH. However, pH had little effect on LaC and LiP production. An analysis of the response surface graph revealed that glucose had the greatest influence on the LaC and LiP enzymes. Glucose supplemented culture medium improved the production of ligninolytic enzyme in *Phanerochaete chrysosporium* in feeding experimental trials (Zhou *et al*. 2007) and 0.412% glucose significantly improved the production of LaC and LiP in saw dust medium in SSF (Arokiyaraj *et al*. 2024). Many recent works currently rely on statistical models for bioprocess optimization. These

statistical methods were used to determine the optimum factors and overall yield of enzymes (El Aty *et al*. 2014). The supplemented polysorbate 80 induced the production of LaC and LiP and this result was supported by previous studies (Usha *et al.* 2014; Teodoro *et al.* 2018). In this optimization method, the most promising factors affecting LaC and LiP were glucose and polysorbate 80. Similar increases in LaC and LiP production were reported by Patrick *et al*. (2010) and Chen *et al*. (2019). The predicted optimum LaC and LiP activity was 154.2 and 19.5 U/g and the experimental value was 156.3 U/g (LaC) and 20.2 U/g (LiP), respectively. The predicted optimum value was very close to the experimental value, which validated the designed experimental model.

Table 7. Central Composite Design Model for the Production of Laccase and Lignin Peroxidase

Run	pH	Polysorbate 80 (%)	Glucose (%)	LaC activity (U/g)	LiP activity (U/g)
1	3.5	0.02	1	71	11.4
2	3.5	0.02	0.1	2.4	0.39
3	4.75	0.11	0.55	139	15.7
4	3.5	0.2	1	53	9.4
5	4.75	-0.041	0.55	34	3.5
6	4.75	0.11	-0.20	42	3.9
7	6	0.2	0.1	23	2.9
8	4.75	0.11	0.55	129.5	13.6
9	4.75	0.11	1.3	110	12.5
10	2.64	0.11	0.55	10	1.4
11	4.75	0.26	0.55	44	4.9
12	6	0.02	1	41	0.52
13	4.75	0.11	0.55	145.8	18.3
14	4.75	0.11	0.55	151.9	19.2
15	4.75	0.11	0.55	143.9	18.4
16	6	0.2	1	45	5.2
17	6	0.02	0.1	0.2	1.4
18	4.75	0.11	0.55	146.9	19.1
19	3.5	0.2	0.1	19	2.1
20	6.85	0.11	0.55	3.1	0.52

Table 8. Analysis of Variance for the Production of Laccases *via* the Central Composite Design Model

Source	Sum of Squares	Df	Mean Square	F-Value	p value Prob > F
Model	60092.94	9	6676.993	178.9168	< 0.0001
A-pH	167.3341	1	167.3341	4.483887	0.0603
B-Polysorbate-80	130.5098	1	130.5098	3.497142	0.0910
C-Glucose	5730.948	1	5730.948	153.5666	< 0.0001
AB	99.405	1	99.405	2.663658	0.1337
AC	198.005	1	198.005	5.305744	0.0440
BC	356.445	1	356.445	9.551304	0.0114
A^2	34190.14	1	34190.14	916.1594	< 0.0001
B^2	19980.52	1	19980.52	535.3981	< 0.0001
C^2	8407.475	1	8407.475	225.2868	< 0.0001
Residual	373.1899	10	37.31899		
Lack of Fit	72.03653	5	14.40731	0.239202	0.9288
Pure Error	301.1533	5	60.23067		
Cor Total	60466.13	19			

Table 9. Analysis of Variance for the Production of Laccases *via* the Central Composite Design Model

Source	Sum of Squares	df	Mean Square	F-Value	p value Prob > F
Model	921.6579	9	102.4064	26.1416	< 0.0001
A-pH	15.9306	1	15.9306	4.066652	0.0714
B-Polysorbate-80	4.97713	1	4.97713	1.270527	0.2860
C-Glucose	85.61195	1	85.61195	21.85442	0.0009
AB	5.232613	1	5.232613	1.335745	0.2747
AC	35.65901	1	35.65901	9.102785	0.0130
BC	0.035112	1	0.035112	0.008963	0.9264
A^2	469.5051	1	469.5051	119.852	< 0.0001
B^2	299.9632	1	299.9632	76.57253	< 0.0001
C^2	142.8211	1	142.8211	36.45839	0.0001
Residual	39.17374	10	3.917374		
Lack of Fit	13.55374	5	2.710748	0.52903	0.7492
Pure Error	25.62	5	5.124		
Cor Total	960.8317	19			

A **B** **C**

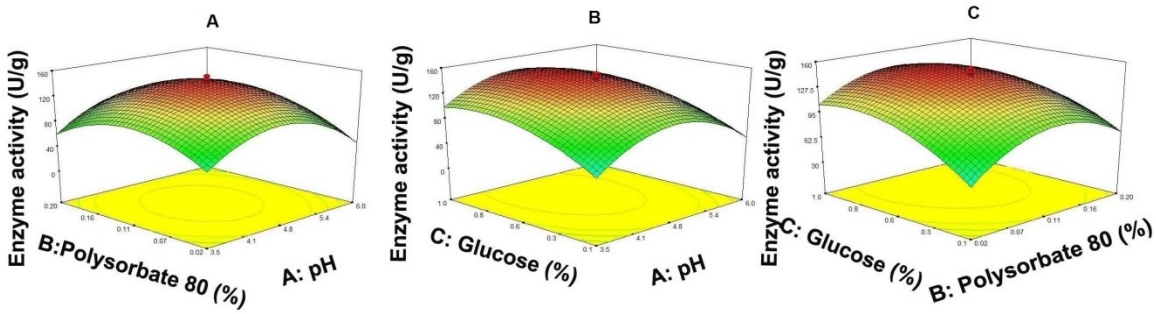


Fig. 3. Response surface model for Laccase production in Solid-state fermentation. (A) Interactive effects of polysorbate 80 and pH, (B) interactive effects of glucose and pH, and (C) interactive effects of glucose and polysorbate 80

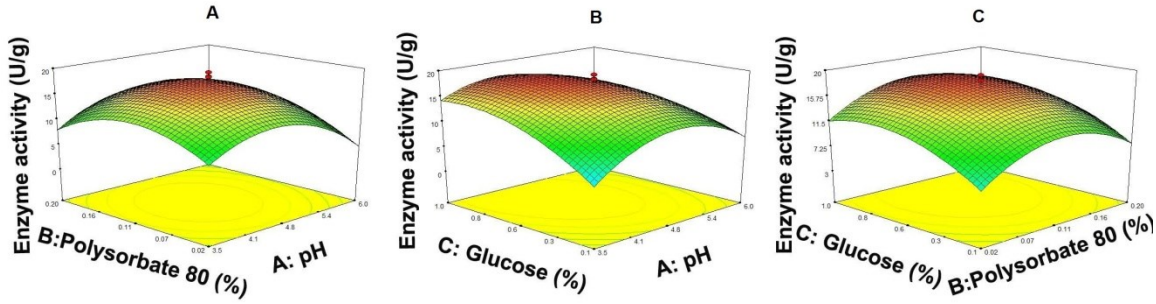
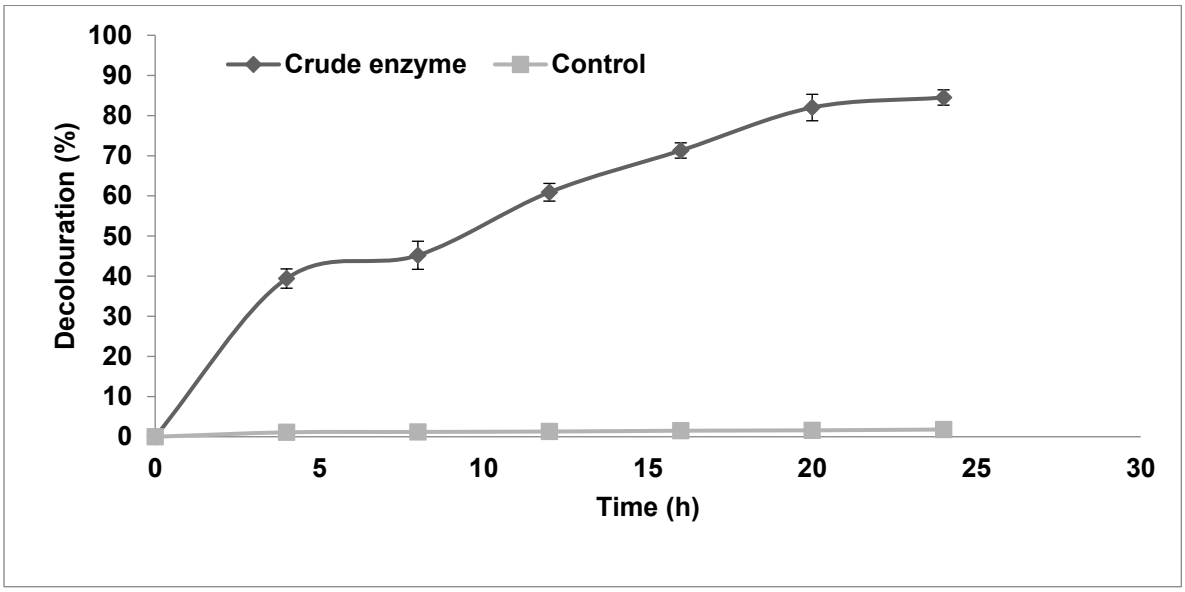


Fig. 4. Response surface model for Lignin peroxidase production in Solid-state fermentation. (A) Interactive effects of polysorbate 80 and pH, (B) interactive effects of glucose and pH, and (C) interactive effects of glucose and polysorbate 80

Degradation of Dyes by Ligninolytic Enzymes

The dye decolouration potential of LaC and LiP was analyzed *via* spectrophotometry. The results are expressed as percentages of degradation (Fig. 5). The selected azo dyes are widely used in the textile and dyeing industries. In this study, a redox (HBT) was not added in the initial experiments (data not shown), and later, HBT was added to improve the degradation performance of the dyes. The fungal enzymes degraded azure blue in a time-dependent manner, and maximum degradation was achieved after 24 h of treatment (84.5±1.9%) (Fig. 5A).

A


Crude enzyme
Control
Decolouration (%)
Time (h)
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10
20
30
40
50
60
70
80
90
100
0
5
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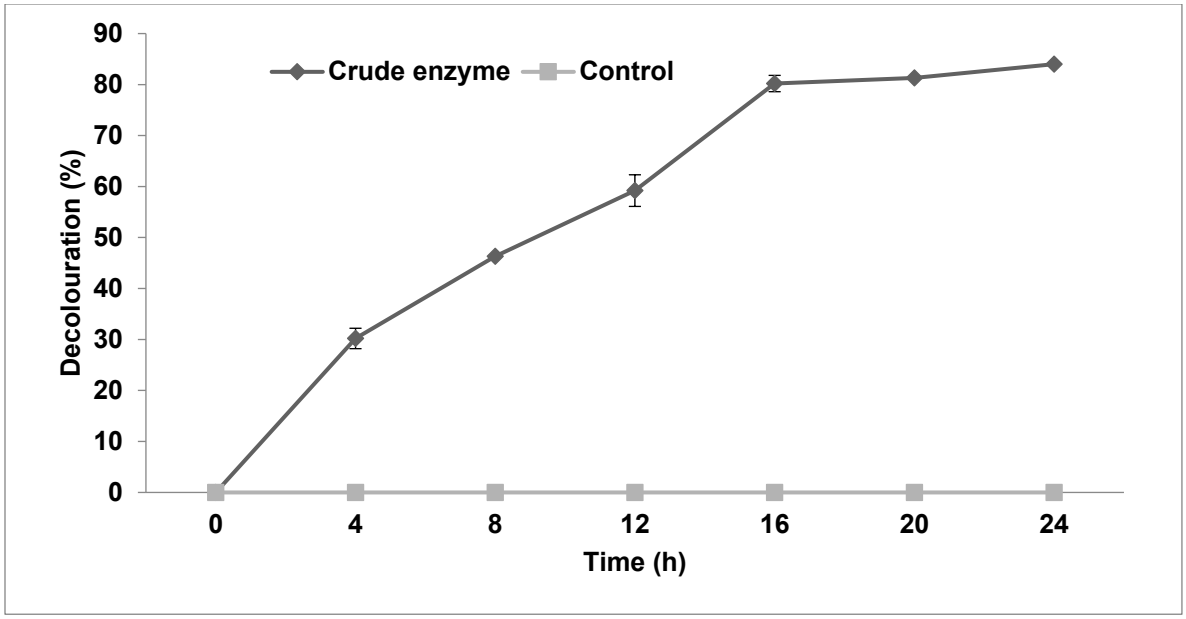
15
20
25
30

B


Crude enzyme
Control
Decolouration (%)
Time (h)
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80
90
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8
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16
20
24

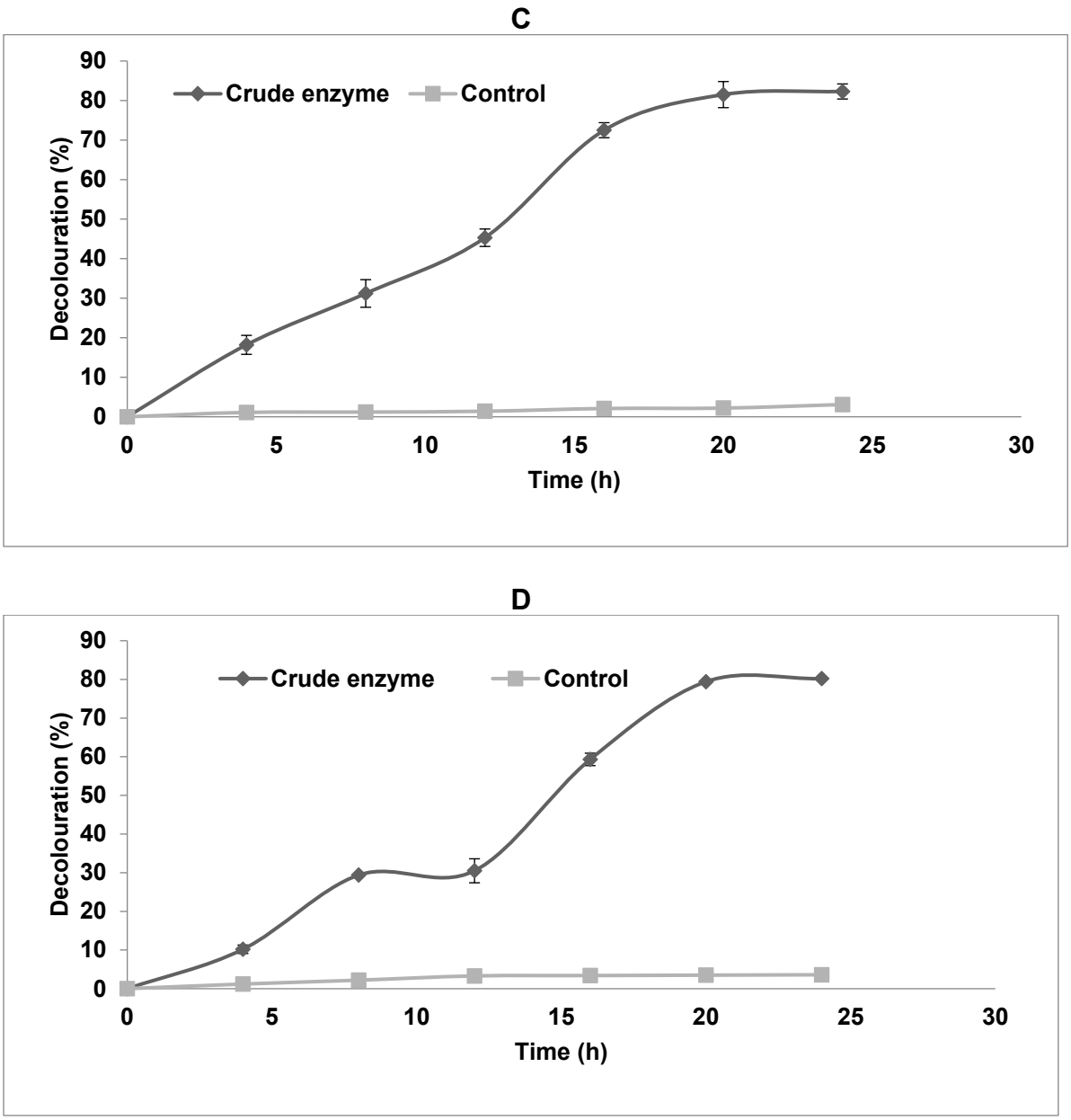

Fig. 5. Decolouration of Azo dye by the concomitant Ligninolytic enzymes produced in Solid-state fermentation. (A)-Azure Blue; (B)-Acid Yellow; (C)-Bromo Cresol Purple; and Reactive Black 5 (D). A decolorization experiment was performed for 24 h at the optimum pH for improved degradation of dyes at room temperature.

The degradation of acid yellow (Fig. 5B) and bromo-chloroform purple (Fig. 5C) was >82%, and that of reactive black 5 was comparatively lower than that of the other dyes (80.2±1.7%) (Fig. 5D). After 24 h of treatment, the degradation rate decreased (data not shown); hence, a degradation study was performed for up to 24 h. The main advantage of using laccases for dye degradation is that they are produced by utilizing agroindustrial residues in the SSF. Moreover, environmental factors influence dye degradation. The dye degradation potential of the ligninolytic enzymes prepared in this study was greater than that in previous reports. The recombinant laccases of *Pleurotus ostreatus* used previously for the discoloration of bromophenol blue, Remazol Bright Blue R, malachite green, and

methyl orange were 91%, 84%, 79%, and 73%, respectively (Zhuo *et al*. 2019). Fan *et al*. (2011) used *Trametes* sp. laccase and reported the discoloration of malachite green and bromophenol blue and achieved 97% and 90% removal of dyes, respectively. The application of mediators for dye discoloration has been reported previously. Similar to this study, *Coriolopsis gallica* strain BS9 synthesized laccases, and in the presence of a laccase mediator, HBT significantly improved azo-bond dye degradation (87%) (Zouari-Mechichi *et al*. 2024). In addition, a previous study revealed that immobilized laccase improved the degradation of the anionic dye Direct Red 23 and resulted in >70% degradation (Alsaiari *et al*. 2021). These earlier studies demonstrated the potential of ligninolytic enzymes for dye decolorization. Recently, fermented wheat bran was directly used as a ligninolytic enzyme source and achieved textile azo dye degradation (>70%) (Borham *et al*. 2023). Based on the present results, further studies will be performed to improve enzyme production in the bioreactor. The optimum parameters will be used to produce high enzyme titer for environmental applications. The amount of nutrient sources in the agroresidue are not uniform and may vary based on the season and environmental condition. Hence, nutrient analysis is required before SSF. Free LaC and LiP have limitations for environmental application and suitable support is required for enzyme immobilization.

CONCLUSIONS

1. Lignocellulosic residues (guava leaves, cabbage leaves, pineapple peels, pomegranate peels, orange peels, banana pseudostem, and wheat bran) were used for the production of laccase (LaC) and lignin peroxidase (LiP) using solid-state fermentation by fung*i* (*Cerrena unicolor* and *Loweporus lividus*). The optimum medium composition was 0.13% polysorbate 80, 0.57% glucose and pH 4.82. The present finding showed the use of agro-wastes for bioconversion into LaC and LiP using fungi in solid-state fermentation (SSF).
2. The optimized culture medium improved 2-fold enzyme production in comparison to unoptimized medium. The crude enzyme decolorized acid yellow, bromo-chloroform purple and reactive black 5. The degradation rates of acid yellow, bromo-chloroform purple, and reactive black 5 were more than 80%. Dye degradation was maximum within 24 h of treatment. Further, the good decolorizing capacity of LaC and LiP enzymes showed their broad prospects in wastewater treatment.

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