MOLEKUL
eISSN: 2503-0310

Articles
https://doi.org/10.20884/1.jm.2025.20.1.14472

Response Surface Methodology to Optimize Laccase Produced from *Pleurotus ostreatus* Using Corn Cob and Rice Bran as Substrate

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Received January 05, 2025; **Accepted** March 10, 2025; **Available online** March 20, 2025

ABSTRACT. Laccase is an industrially significant enzyme capable of oxidizing a broad range of phenolic compounds, making it valuable for applications such as bioremediation, textile dye decolorization, and pulp bleaching. Therefore, this study aimed to optimize laccase production from *Pleurotus ostreatus* using agricultural waste substrates corn cob (BJ), rice bran (DP), and their 1:1 mixture (BJ:DP) through Response Surface Methodology (RSM). Extracellular laccase was obtained by centrifuging the cultured medium, followed by ammonium sulfate precipitation and dialysis. The enzyme activity was quantified, and the fraction with the highest specific activity was analyzed using SDS-PAGE. Furthermore, Central Composite Design (CCD) was used to assess the effects of three independent variables, namely ABTS concentration (0.01–0.1 mM), incubation temperature (20–30 °C), and reaction time (20–30 minutes). The results showed that for BJ substrate, the highest enzyme activity (13.7 ± 0.05 U/mL) was observed in the 0–20% ammonium sulfate fraction, with specific activities of 76.25 ± 0.09 U/mg and 90.28 ± 0.03 U/mg in the 0–20% and 20–40% fractions, respectively. Conversely, DP substrate achieved a maximum specific activity of 209.67 ± 0.028 U/mg in the 20–40% fraction. The crude extract from BJ:DP mixture showed a high protein content (0.636 ± 0.006 mg/mL) but the specific activity was substantially lower (19.33 ± 0.003 U/mg). Based on RSM analysis, the optimal conditions were ABTS concentration of 0.05 mM, incubation temperature of 22.21 °C, and reaction time of 28.64 minutes, resulting in a predicted laccase activity of 13.99 U/mL.

Keywords: Corn cob, Laccase, *Pleurotus ostreatus*, Response Surface Methodology, Rice bran

INTRODUCTION

Laccase plays a major role as a leading biocatalyst and is a potent substitute for chemical-based deconstruction of lignocellulosic materials (Sigh et al., 2023). In general, laccase, manganese peroxidase (MnP), and cellulase are lignocellulolytic enzymes produced by microorganisms such as fungi. White oyster mushroom (*Pleurotus ostreatus*), as one of the fungi producing lignocellulolytic enzymes, shows extraordinary ability to degrade lignin effectively, making it an ideal candidate for environmentally friendly enzyme production (Nurfitri et al., 2021).

Corn cob (BJ) represents an exceptionally abundant agricultural waste with considerable potential as a source of lignocellulosic biomass, containing approximately 40–45% cellulose, 30–35% hemicellulose, and 10–20% lignin, while ash comprises over 60% silica with minor metallic elements (Hermansyah et al., 2022). Due to its high availability, low cost, and suitable composition, BJ is a promising substrate for the production of lignocellulolytic enzymes such as laccase, regarding the substantial waste generated from high corn production, which remains largely underutilized (Rani et al., 2024). Furthermore, lignocellulosic materials found in BJ, rice bran (DP), and empty oil palm fruit bunches have significant potential as renewable energy sources (Ojo & Abidemi, 2023). The presence of lignin often hampers the enzymatic hydrolysis of cellulose and hemicellulose, limiting the efficiency of enzyme production (Mujtaba et al., 2023). Despite these challenges, the current literature provides limited insights into optimized strategies for overcoming lignin-related barriers and enhancing laccase production from BJ. This underscores the critical importance of further study to advance both industrial enzyme applications and sustainable bioenergy development (Zhang et al., 2023; Song et al., 2020).

Response Surface Methodology (RSM) is a statistical method used to optimize complex processes with a multivariate method. This method helps in the evaluation of the influence of multiple factors and interactions on the target response through a smaller number of experiments compared to other optimization methods, thereby saving time and costs.

RSM has been widely used in various fields, including enzymatic activity tests (Klongdee & Klinkesorn, 2022).

There has been significant progress in the use of agricultural wastes for enzyme production. However, most studies relied on single-substrate systems, which may limit overall yield and efficiency. In response, this study introduces a dual-substrate strategy combining BJ and DP for laccase production by *P. ostreatus*. RSM was used for systematic process optimization, while protein content was assessed using SDS-PAGE analysis. This method aims to enhance enzyme yield while promoting sustainability and cost-efficiency, addressing critical gaps in current biotechnological applications. By using BJ waste and a mixture of DP as substrates, laccase production was optimized with high efficiency, simultaneously reducing agricultural waste. This effort not only provides a viable solution for waste management but also opens new avenues for bioindustrial applications, including the production of biofuels, chemicals, and other environmentally friendly products.

EXPERIMENTAL SECTION

Tools and Materials

The tools used in this study were an autoclave (Tuttnauer), incubator (Thermo Scientific), freezer (LG), analytical ball balance (O'Haus), centrifuge (Beckman Coulter), and UV-Vis Spectrophotometer (Shimadzu), SDS-PAGE apparatus (Bio-Rad). The materials used were BJ, PDA (Himedia), strain *P. ostreatus* (F_0), corn kernels, DP, mushroom seeds *P. ostreatus*, sodium citrate buffer (Sigma-Aldrich), BSA (*bovine serum albumin*) (Sigma-Aldrich), and ABTS (*2,2'-azinobis-3-ethylbenzthiazolin-6-sulfonate*) (Sigma-Aldrich). Other materials used include $(NH_3)_2SO_4$ (Merck), cellophane membrane (Whatman), phosphate buffer (Merck), Folin-Ciocalteau reagent (Merck), Lowry's reagent (Merck), aquadest(Merck Millipore), sodium hydroxide (Sigma-Aldrich), sodium potassium tartrate (Sigma-Aldrich), Separating gel (10% or 15%) and stacking gel (4%) (Bio-Rad), loading buffer sample (Bio-Rad), Tris-Glycine-SDS buffer (Bio-Rad), and pH 8.3 Coomassie Brilliant Blue R-250 (Bio-Rad).

Sterilization and Preparation of PDA Media

Tools and materials were sterilized using an autoclave at a temperature of 121 °C and a pressure of 15 psi for 15 minutes. Subsequently, *P. ostreatus* (F_0) was cultured on PDA media using the modified method (Hamzah et al., 2022) for 7 days. The inoculum (F_1) seeds were prepared in glass bottles with sterilized corn seeds as substrate and incubated for 2 weeks at a temperature of 25-30 °C.

Substrate Preparation and Inoculation

BJ substrate and DP were soaked in a solution of $CaMg(CO_3)_2$ 1% for 24 hours, then sterilized, BJ and DP mixed with variations of BJ:DP (100:0, 0:100, and 50:50, w/w). The sterilized substrate was inoculated with an inoculum of *P. ostreatus* (F_1) and incubated at 25 -30 °C with a humidity of 70 -80%. The diameter of fungal colonies on PDA media was measured every day for 7 days and the length of mycelium on BJ and DP substrates for 25 days using calipers (Heirina et al., 2020).

Enzyme Extraction

The fungi were extracted using phosphate buffer (pH 7.2) with a ratio of 1:3 (w/v), and centrifuged at 5000 rpm for 15 minutes at 0-4°C (González-Blanco et al., 2021). The crude enzyme extract was purified by the salting out method using ammonium sulfate (saturation 0-20%, 20-40%, and 40-60%). Dialysis was carried out in 0.02 M phosphate buffer (pH 6.0) for 24 hours at 5-10 °C (Fadel et al., 2021).

Determination of Protein Content and Enzymatic Activity Test

Protein content in crude enzyme extract was measured by the Lowry method using the BSA standard with concentration made from graded dilution (1000-7.8125 ppm). Subsequently, the enzyme fraction of 0.1 mL was added with 5 mL of Lowry Reagent D (Copper reagent), shaken, and left for 10 minutes at room temperature. Approximately 0.5 mL of Lowry E (Folin-Ciocalteau reagent) was added followed by shaking and incubation for 30 minutes at room temperature. The absorbance of the sample was measured using a UV-vis spectrophotometer at a wavelength of 600-660 nm (Arunima & Verulkar, 2022).

Approximately 100 μL of enzyme extract was transferred into a test tube, and 1900 μL of 0.05 mM ABTS prepared in 0.1 M sodium acetate buffer (pH 4.5) was added. One laccase unit (U) is defined as the amount of enzyme required to oxidize 1 μmol ABTS per minute under assay conditions. The mixture was incubated at 25 °C for 25 minutes to allow oxidation of ABTS to a bluish-green radical compound. After incubation, absorbance was measured at a wavelength of 420 nm (Jeon & Lim, 2017).

SDS-PAGE Analysis

A separating gel with an appropriate concentration of 15%, depending on protein size was prepared. After the gel was split, a layer of stacking gel (4%) was added on top, and a comb was inserted to form the well. The sample protein was then mixed with loading buffer in a 1:1 ratio and heated using a water bath at 95 °C for 5 minutes to ensure the protein denaturation process. Once the gel was stacked, the comb was removed, and the gel was placed into the electrophoresis chamber. Running buffer was added to cover the gel and a protein sample of 10–20 μL was put into the prepared well, while the marker protein was placed into a separate well. Electrophoresis was carried out at a voltage of 80 V until the proteins passed through the gel buildup and continued at a voltage of 120 V to separate the proteins in the gel. After electrophoresis was complete, the gel was soaked in *Coomassie Brilliant Blue*

solution for 30 minutes to 1 hour for the staining process. The gel was then rinsed using a destaining solution until the protein bands were visible (Kurien & Scofield 2012).

Optimization Analysis of Response Surface Methodology (RSM)

RSM method with Central Composite Design (CCD) was used to evaluate and optimize the influence of independent variables on certain responses. In this study, three variables namely ABTS concentration (A) (0.01–0.1 mM), temperature (B) (20–30 °C), and reaction time (C) (20–30 min) were analyzed at three levels (lower, middle, top points) with $\alpha = 1$. The experimental design comprised 20 combinations, including repetitions at the center point to increase reliability. Data were analyzed using multivariate regression with Design-Expert 13 software to determine optimal parameters. All experiments were carried out in triplicate to ensure the accuracy of the results (Zhu et al., 2019).

RESULTS AND DISCUSSION

Fungal Growth on PDA

The results showed that mycelium *P. ostreatus* had significant growth on PDA media for 7 days, with an increase in colony diameter from 0.8 cm at day 0, reaching 9 cm at day 7 **(Figure 1).** Measurements using two diagonals averaged ensure accurate and efficient results (Gwon et al., 2022). These growth curves provide an important guide for evaluating the effectiveness of media in supporting mycelium growth and can serve as a standard reference for comparing other media, including BJ-based substrates.

This growth pattern follows a logistic curve consisting of three phases. The lag phase (days 0 to 1) is characterized by slow initial growth (diameter 0.8-2.2 cm), indicating the adaptation of the mycelium to the medium. The log phase (days 1 to 5) reflects exponential growth with a significant increase in diameter every day (3.65-7.95 cm), showing the mycelium optimal ability to use nutrients (Jo et al., 2023). In the stationary phase (days 6 to 7), growth started to slow down approaching the physical limits of the medium (9 cm), which is most likely caused by limited space and nutrition (Argaw et al., 2023).

Observations of mycelium growth on BJ, a mixture of BJ:DP (1:1), and DP showed varying growth patterns over 25 days **(Figure 2**). Laccase production typically starts during the exponential (log) phase of growth and continues into the stationary phase.

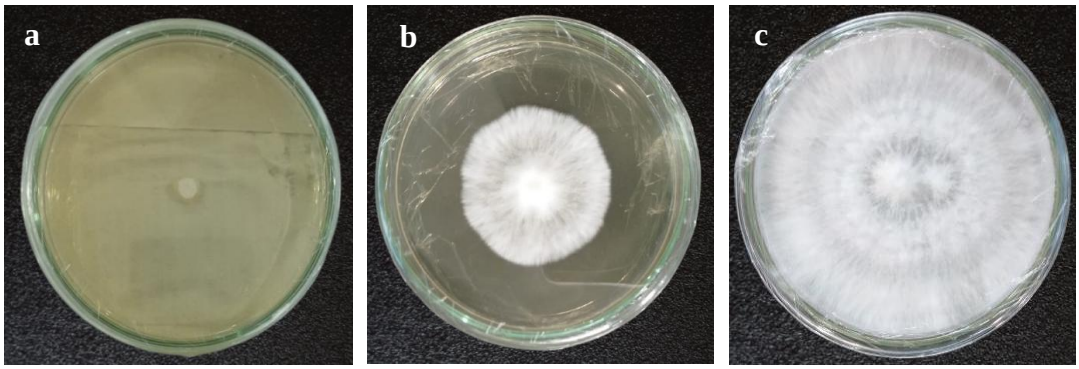

Figure 1. Growth of fungus *P. ostreatus* on PDA media: (**a**) 0 days, (**b**) 2 days, and (**c**) 3 days.

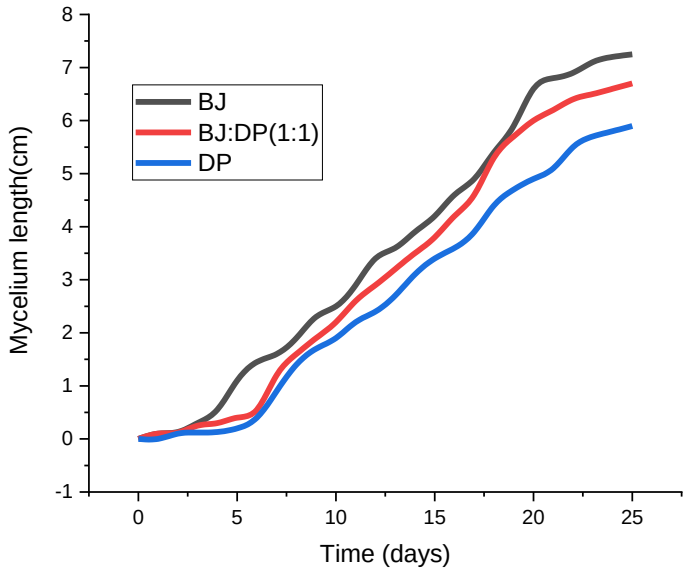

Figure 2. Fungal growth curve on corn cob (BJ) media, BJ:DP(1:1), rice bran (DP).

This is related to the function of laccase as a lignolytic enzyme which is often produced to support fungal growth on lignin-rich substrates (Brugnari et al., 2021). BJ substrate supports the best growth due to high lignocellulose content, an essential carbon source (Zou et al., 2020), followed by a BJ:DP (1:1) mixture which showed substrate synergy slightly slower. Conversely, DP media has the slowest growth, showing that DP as a sole substrate is less than optimal in providing the essential nutrients needed by mycelium in this condition (Adiandri et al., 2022). The mycelium growth pattern on the three substrates follows a logistic curve with a lag phase (days 0 to 2), a log phase with exponential growth (days 3 to 15, fastest at BJ and 50:50), and a stationary (after day 20) due to limited nutrition and space.

Extraction and Fractionation

Enzyme extraction was carried out by mixing 30 g of fungus *P. ostreatus* and media with 90 mL of phosphate buffer pH 7.2 in a ratio of 1:3 (w/v), producing an initial supernatant of ±80 mL after the centrifugation process. Phosphate buffer at pH 7.2 was selected because it is close to the optimal physiological pH to maintain the stability and activity of lignocellulolytic enzymes (Umar & Ahmed, 2022).

Fractionation was carried out using ammonium sulfate in three stages (Table 1). At 0–20% saturation, proteins with high solubility started to precipitate, resulting in a fraction volume of 13–15.5 mL. Meanwhile, at 20–40% saturation, proteins with moderate solubility precipitated with a smaller volume fraction, namely 9–12 mL. At high supersaturation (40-60%), proteins with these characteristics remained soluble due to their small size. Substrates such as BJ and DP tend to produce more soluble proteins (Rivero et al., 2024). This precipitation process relies on a decrease in protein solubility due to salt ions competing with water molecules, causing the protein to partially denature and precipitate (Qing et al., 2022). The salting effect is mainly governed by the hydrophobic nature of the protein, with proteins having more hydrophobic surfaces tending to precipitate at lower to moderate salt concentrations, while more hydrophilic proteins remain in solution. Therefore, the initial supernatants obtained from BJ and DP substrates containing crude enzymes, small molecules, and residual buffers (Nguyen, Luong et al., 2020) are enriched with more soluble protein fractions.

BSA standard curve with the linear equation *(y = 0, 001x + 0,134*) describes the linear relationship between protein concentration (ppm) and absorbance (660 nm). The Lowry method was used due to the high sensitivity in detecting proteins through a chemical reaction between Cu^{2+} ions and peptide groups on the protein, followed by reduction of the *Folin-Ciocalteu* reagent by aromatic amino acid residues such as tyrosine and tryptophan (Shen & Chang-Hui, 2019).

As shown in Table 1, the BJ substrate yielded a crude extract with protein concentrations ranging from 444 to 470 ppm. The majority of the protein precipitating was in the 0–20% and 20–40% fractions. Conversely, BJ:DP (1:1) mixture showed the highest protein levels in the crude extract, at 631–644 ppm, reflecting the larger amount of protein solubilized in the substrate. The 0–20% and 20–40% fractions had significant protein levels, indicating that substrate combination increased protein complexity. There was no precipitate in the 40–60% fraction in all substrates, showing the main protein has high to moderate solubility (Table 1). The addition of ammonium sulfate to the protein solution causes protein precipitation through "salting out". Ammonium sulfate ions increase the ionic strength of the solution, reducing protein solubility, and leading to the precipitation of proteins with low solubility (Wingfield & Paul, 2016). Certain proteins that dissolve at higher ion concentrations may remain in the supernatant during dialysis, thereby reducing the total protein concentration measured after dialysis (Daugirdas & Jhon, 2018).

Table 1. Results of enzyme activity and specific activity of the enzyme laccase.

Substrate	Sample Type	Enzyme Activity(U/mL)	Protein Content(mg/mL)	Specific activity(U/mg)	Purification fold	% Yield
	crude extract (1)	11.56 ± 0.142	0.454 ± 0.013	25.43 ± 0.001	1	100
BJ	fraction 0-20%(2)	13.7 ± 0.05	0.179 ± 0.021	76.25 ± 0.09	2.99	118.51
	fraction 20-40%(3)	12.97 ± 0.09	0.143 ± 0.037	90.28 ± 0.03	3.55	112.19
	crude extract(4)	12.31 ± 0.15	0.636 ± 0.006	19.33 ± 0.003	1	100
BJ:DP(1:1)	fraction 0-20%(5)	12.3 ± 0.06	0.312 ± 0.035	39.46 ± 0.066	2.04	99.91
	fraction 20-40%(6)	12.96 ± 0.18	0.271 ± 0.02	47.82 ± 0.04	2.47	105.28
	crude extract(7)	12.34 ± 0.4	0.608 ± 0.04	20.3 ± 0.021	1	100
DP	fraction 0-20%(8)	13.48 ± 0.25	0.435 ± 0.03	30.97 ± 0.019	1.52	109.23
	fraction 20-40%(9)	13.35 ± 0.34	0.063 ± 0.008	209.67 ± 0.028	10.32	108.18

The Activity and Specific Activity of Laccase Enzyme

Laccase is an oxidase enzyme included in the lignocellulolytic enzyme group. It uses molecular oxygen as an electron acceptor to oxidize various phenolic and non-phenolic compounds, including lignin, organic dyes, and pollutants. This reaction produces free radicals which play a role in the degradation of complex molecules). On BJ substrate, the highest activity was found in the 0–20% fraction at 13.7 ± 0.05 U/mL **(Table 1)**, showing dominant active enzymatic protein. The mixture of BJ:DP (1:1) and DP substrate showed higher total protein content but the enzyme activities were significantly lower. This difference is likely due to the presence of large amounts of non-enzymatic proteins and inhibitory compounds such as polyphenols that can interfere with enzyme function. Polyphenols bind to the active site of the enzyme or cause conformational changes, thereby reducing catalytic efficiency (Adrar et al., 2019). Precipitation with ammonium sulfate proved effective in separating active laccase, with the 0–20% fraction being the main target for optimizing enzyme activity.

The high specific activity in the 20-40% fraction of DP shows that the optimal salt concentration for laccase precipitation is highly dependent on the nature of the substrate and the stability of the enzyme in the purification process (Mahmood et al., 2018). DP substrate produced the highest specific activity in the 20-40% fraction at 209.67 ± 0.028 U/mg (**Table 1**). The stability of activity in both fractions shows that laccase can maintain an active conformation during the precipitation process. This indicates that laccases from various substrates have moderate solubility, more suitable for precipitation at intermediate salt saturation levels (Yang et al., 2024). Ammonium sulfate precipitation successfully enriched enzymes from different substrates, as evidenced by the purification fold and yield data. In the crude extract of BJ substrate, with a specific activity of 25.43 U/mg, it was enriched to 76.25 U/mg and 90.28 U/mg in the 0–20% and 20–40% fractions. This corresponded to purification folds of approximately 2.99 and 3.55, with yields of 118.51% and 112.19%, respectively. Similarly, the mixture of BJ:DP (1:1) and DP substrate showed an increase in specific activity after purification. In particular, the 20–40% fraction of DP substrate achieved a significant purification fold of 10.32. Ammonium sulfate precipitation is the first step in enzyme purification that can lead to apparent recoveries of more than 100%. This phenomenon occurs because the purification process effectively removes inhibitors such as non-enzymatic proteins and small molecule inhibitors present in the crude extract. Removal of these inhibitors significantly increases the measured specific activity of the target enzyme, resulting in higher observed activity than the crude sample (Mahmoudi et al., 2020).

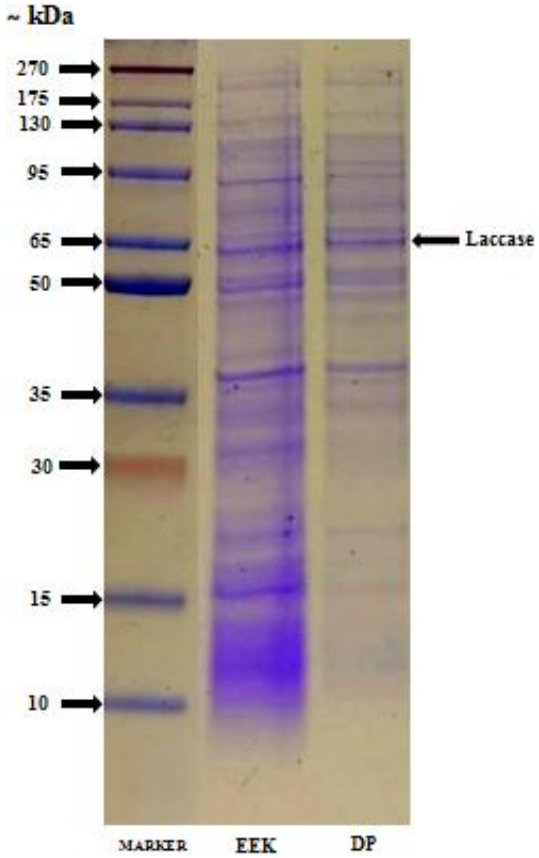

Figure 3. SDS-PAGE analysis of enzyme extracts using crude extract (EEK) and fraction rice bran 20-40% (DP). The left lane shows the molecular weight marker (kDa), Arrows indicate bands in the range of 65 kDa.

SDS-PAGE results showed a prominent band in the range of 60–70 kDa, which is in good agreement with previously reported molecular weights for fungi laccases (**Figure 3**). For example, studies on Agaricus species have consistently shown that laccase enzyme usually has a molecular mass of around 65 kDa (Ai et al., 2015; Arregui et al., 2019; Hao et al. 2021). Observation of similar bands on SDS-PAGE, compared to known Protein Markers provides strong evidence that the enzyme produced is laccase. Although SDS-PAGE is essentially a denaturation method that estimates protein size rather than confirming enzyme activity, the consistency of data with confirmed literature validates this method. For further confirmation, additional assays such as zymography or mass spectrometry can be performed. However, the agreement with previous reports provides strong support for the results.

Based on the results presented in **Table 1**, the specific activity of the enzyme increased after partial purification of each fraction. This indicates that the enzyme obtained has a higher level of purity. The fraction with the highest specific activity was DP 20-40%, with a value of 209.67 ± 0.028. This fraction has a lower protein concentration than the crude extract, due to the separation process which successfully removes non-enzyme proteins previously mixed with the enzyme (Zusfahair et al., 2018). The result is strengthened by the enzyme analysis using SDS-PAGE shown in **Figure 3**.

RSM Optimization Analysis Laccase Enzyme

RSM was used as a tool to optimize the production of laccase enzyme from the fungus *P. ostreatus* using BJ waste and DP as substrates. This method assisted in the evaluation of various variables, such as substrate concentration, temperature, and reaction time on DP substrate in the 20-40% fraction **(Table 2)**. This fraction was selected because it has the highest activity specific for finding areas optimal which supports increased enzyme activity. **Table 2** presents the results of laccase activity measurements conducted based on the Central Composite Design (CCD) experimental design with n = 3, where each experiment was replicated three times. The independent variables tested included ABTS concentration (mM), temperature (°C), and reaction time (minutes), while the dependent variable measured was laccase activity (U/mL).

Table 2. Running results of laccase activity with CCD

Run	ABTS concentration (mM)	Temperature (°C)	Time reaction (min)	Actual Activity (U/mL)	Predicted Activity (U/mL)
1	0.01	20	20	3.88	3.93
2	0.1	20	20	9.25	9.19
3	0.055	20	25	13.5	13.51
4	0.01	20	30	3	2.99
5	0.1	20	30	7.3	7.4
6	0.055	25	20	14.2	14.32
7	0.055	25	25	15.9	16.02
8	0.055	25	25	16.1	16.02
9	0.055	25	25	16	16.02
10	0.055	25	25	16	16.02
11	0.01	25	25	6.35	6.33
12	0.1	25	25	13.3	13.29
13	0.055	25	25	16.2	16.02
14	0.055	25	25	15.8	16.02
15	0.055	25	30	16.1	16.02
16	0.01	30	20	2.4	2.38
17	0.1	30	20	11.3	11.29
18	0.055	30	25	15.6	15.61
19	0.01	30	30	5.1	5.09
20	0.1	30	30	13.2	13.15

*(n = 3)

Table 3. ANOVA for Quadratic model

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	481.19	9	53.47	398.36	< 0.0001	significant
A [ABTS] (mM)	113.03	1	113.03	842.17	< 0.0001	
B Temp (°C)	11.38	1	11.38	84.83	< 0.0001	
C reaction time (min)	1.35	1	1.35	10.04	0.01	
AB	6.72	1	6.72	50.04	< 0.0001	
AC	0.4371	1	0.4371	3.26	0.1013	
BC	6.9	1	6.9	51.42	< 0.0001	
A^2	112.7	1	112.7	839.74	< 0.0001	
B^2	7.73	1	7.73	57.61	< 0.0001	
C^2	3.19	1	3.19	23.76	0,0006	
Residual	1.34	10	0.1342			
Lack of Fit	1.01	5	0.2022	3.05	0.1232	not significant
Pure Error	0.3313	5	0.0663			
Cor Total	482.53	19				

The overall model (**Table 3**) demonstrated high robustness, as evidenced by an F-value of 398.36, indicating only a 0.01% probability that this result occurred due to random noise. In the analysis, all terms with p-values less than 0.05 were considered statistically significant. The results showed that factors A, B, and C, the interactions (AB and BC), and the quadratic terms (A^2, B^2, C^2) were all significant. Terms with p-values greater than 0.10 were considered non-significant, and when many terms were present aside from those required for hierarchical integrity, model reduction could potentially enhance performance. Moreover, the Lack of Fit F-value of 3.05 was not statistically significant relative to the pure error (with a p-value of 12.32%), indicating that the model adequately fits the data. These statistical results confirm that the model is both reliable and appropriate for predicting the response under study.

The predicted R^2 of 0.9834 is in excellent agreement with the adjusted R^2 of 0.9947, with the difference well below the acceptable threshold of 0.2. This strong concordance confirms that the model has strong predictive power. Furthermore, the Adeq Precision value, which measures the signal-to-noise ratio, is 53.643, well above the desired minimum value of 4. These statistics indicate that **(Table 4)** the model provides reliable and robust signals, making it very effective for navigating and optimizing in the design space. The following is a prediction model obtained to calculate the activity enzyme prediction.

$$\text{Activity (U/mL)} = -46.18 + 346.63(A) + 2.41(B) + 1.35(C) - 3161.39(A^2) - 0.043(C^2) - 0.067(B^2) + 4.07(AC) - 1.03(AB) + 0.03\,(BC) \quad \textbf{(1)}$$

Regression Equations **(1)** expressed in terms of actual factors are valuable for predicting responses at a given level, provided that the levels are entered using the original units. However, the equations should not be used to assess the relative impact of each factor. This is because the coefficients have been scaled according to the units of each factor, and the intercept does not necessarily represent the center of the design space. While effective for making predictions, caution should be exercised when interpreting the contribution of each factor based solely on the coefficient.

Table 4. ANOVA Fit Statistics

Source	Results
Std. Dev.	0.3664
Mean	11.54
C.V. %	3.18
R^2	0.9972
Adjusted R^2	0.9947
Predicted R^2	0.9834
Adeq Precision	53.643

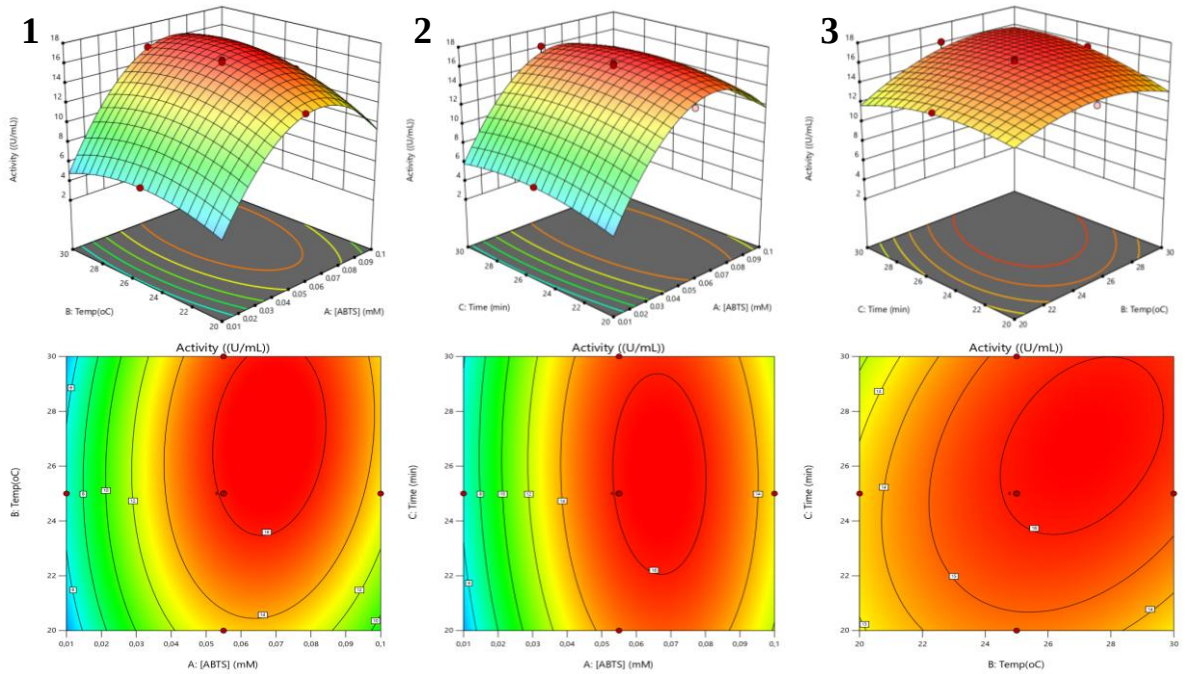

Figure 4. Response surface plot of the influence of interaction factors on the results of laccase activity. (**1**) interaction between temperature-ABTS concentration and enzyme activity. (**2**) interaction between ABTS concentration – reaction time and enzyme activity. (**3**) interaction between temperature-reaction time.

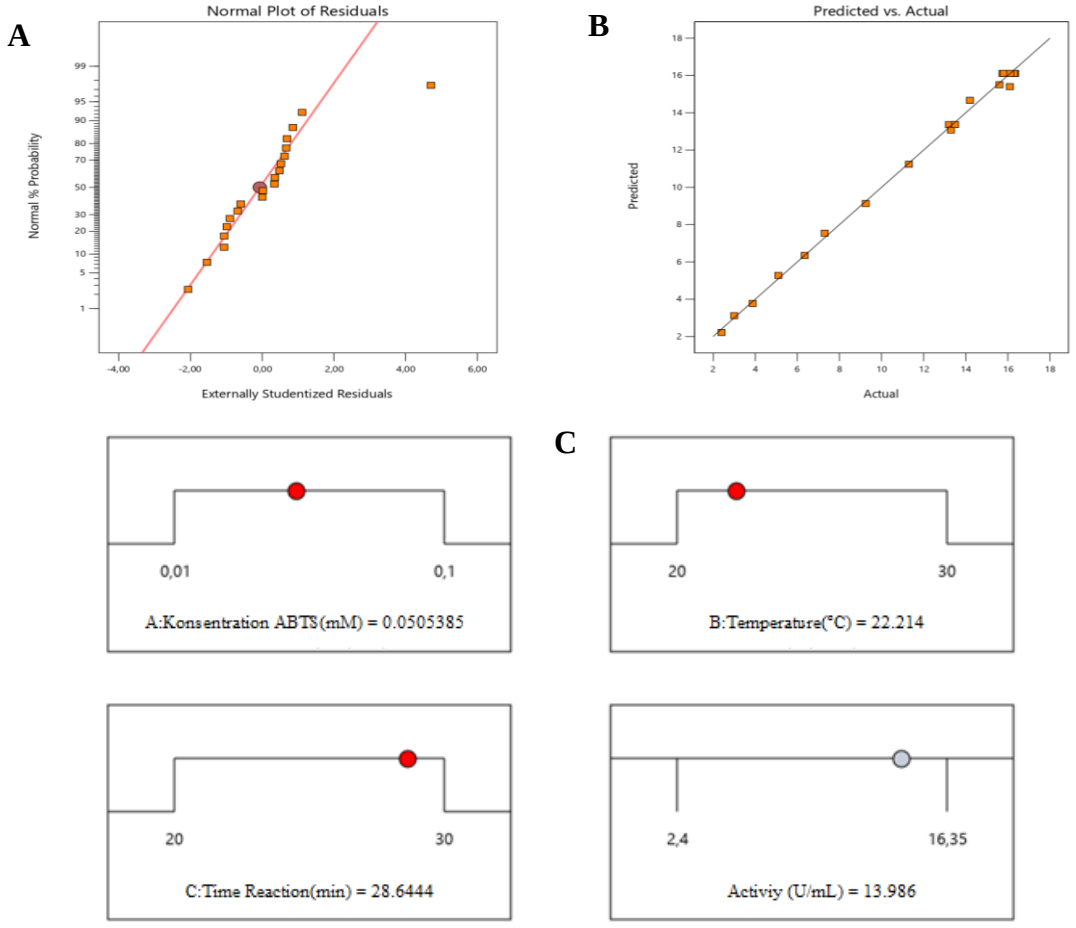

Figure 5. Interaction between variables (**A**) normal vs residual plot interaction (**B**) predicted vs actual interaction (**C**) The optimum condition results obtained by (Optimization Numerical Ramps).

Visualization of RSM analysis results using response surface plots (3D) and contour plots provides in-depth insight into the interaction between process variables on laccase enzyme activity. On the response surface plot **(Figure 4)**, the relationship between two variables was projected to observe the interactive influence on enzyme activity, with one other variable held constant. A curved surface indicates that the relationship between variables is non-linear, according to the selected quadratic model. Response surface peaks identify the optimal combination of process variables that results in maximum enzyme activity. This shows that the quadratic model is able to capture the complexity of interactions between variables.

The analysis shown in **Figure 5** provides a comprehensive overview of the statistical model, including verification of residual normality, the predictive ability of actual data, and determining the optimal conditions for achieving the highest laccase activity. **Figure 5A** (Normal Residual Plot) illustrates how well the residuals (differences between predicted and actual values) follow a normal distribution. The alignment of data points along the diagonal line indicates that the model assumption of normally distributed residuals is valid, supporting the reliability of the statistical analysis. **Figure 5B** (Predicted vs. Actual) compares the model-predicted values with the experimentally observed (actual) values. More importantly, points located close to the diagonal line reflect strong agreement between predictions and measurements, indicating that the model accurately captures the relationships among factors. **Figure 5C** (Optimization Numerical Ramps) presents the optimal conditions for each factor. Furthermore, RSM analysis showed that the optimal conditions were an ABTS concentration of 0.05 mM, an incubation temperature of 22.21°C, and a reaction time of 28.64 minutes, resulting in a predicted laccase activity of 13.99 U/mL. Each red marker indicates the level at which the factor should be set, and the corresponding final predicted activity (shown on the rightmost ramp) shows the maximum response achieved under these optimized conditions.

To verify the reliability of the optimum conditions generated by RSM in the production of laccase enzyme, three validation tests were carried out on the best-predicted parameters. This step aims to ensure that the obtained model is truly capable of consistently maximizing enzyme activity and can be replicated (Tišma et al., 2012). **Table 5** shows a comparison between the actual measured laccase activity and the values predicted by the model at optimum conditions. From three replications, the actual values ranged from 13.24 ± 0.05 to 13.88 ± 0.021 U/mL, while the model projected an activity of 13.986 U/mL. The small difference between the actual and predicted activities indicates that RSM model provides a good estimate, and confirms the proposed optimum conditions are truly capable of producing high laccase activity.

Table 6 shows the final confirmation results of RSM optimization for enzyme activity. Based on the results, the predicted mean and median for enzyme activity are both 13.986 U/mL, indicating that the model consistently predicts this value under optimized conditions.

The standard deviation of 0.366 indicates relatively low variability across three replicate experiments (n = 3), while the standard error of prediction (0.276939) reflects the accuracy of the estimate. More importantly, the 95% prediction interval (PI) ranges from 13.3689 to 14.603 U/mL, and the mean of the observed data is 13.5267 U/mL. This observed value falls in the predicted interval. The model predictions are considered accurate and reliable, validating the optimized conditions obtained from RSM (Burboa-Charisz et al., 2019). In general, these results represent the final stage of RSM analysis, indicating that the optimized parameters reliably maximize laccase activity, allowing the model to be used with confidence for further process development and scale-up.

Table 5. Prediction results of the laccase enzyme model.

Actual Activity (U/mL)	Predicted Activity (U/mL)
13.88 ± 0.021	13.986
13.24 ± 0.05	13.986
13.46 ± 0.034	13.986

Table 6. Results Post Analysis Confirmation.

Solution 1 of 100 Response	Predicted Mean	Predicted Median	Std Dev	n	SE Pred	95% PI low	Data Mean	95% PI high
Activiy	13.986	13.986	0.366351	3	0.276939	13.3689	13.5267	14.603

*Two-sided Confidence = 95%

CONCLUSIONS

In conclusion, this study successfully demonstrated that RSM through CCD could be effectively applied to optimize laccase production from *P. ostreatus* using agricultural waste substrates with BJ and DP substrates, as well as a mixture of both with a ratio of (1:1). The optimized conditions, ABTS concentration of 0.05 mM, incubation temperature of 22.21°C, and reaction time of 28.64 minutes, produced a predicted laccase activity of 13.99 U/mL. This was confirmed by three independent validation experiments with the results of the 95% prediction interval (PI) ranging from 13.3689 to 14.603 U/mL, and the average observed data at 13.5267 U/mL, which is in the predicted interval.

ACKNOWLEDGEMENTS

All the authors are thankful to Dept of Chemistry Faculty of Mathematics and Natural Sciences University of Sriwijaya for providing required facilities to carry out the work.

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