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Original Paper

Response Surface Based Optimization of Laccase Production from Cerena Unicolor with Wheat Straw as a Substrate

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Laccase currently has a global market of more than 3 million USD, due to its redox potential, whose cost is determined by the raw material. Fungal laccase was produced from cheap agrowaste using a fungal source. Fungal isolation was done from microbes of decomposing cellulosic raw material. *Cerrena unicolor*was found to be the most promising fungus and identified using ITS based sequencing, phylogenetic analysis and morphological study. Among different cellulosic materials, wheat straw was a better raw material for fungal isolates in laccase production. The optimization of laccase production was done using wheat straw with one factor at a time to find optimaltemperature (30 °C), incubation period (14 days), moisture percent (80) and pH (5). Response surface method with central composite design using yeast extract as an organic source of nitrogen or ammonium sulphate as an inorganic source was compared for laccase production. Copper sulphate was used as an inducer. It gave a significant model with quadratic equation which gave nearly similar experimental and predicted laccase production values. A 6-fold change in laccase production was found after optimization. The purification product after SDS PAGE showed the isolated laccase to have a single polypeptide of around 64 kDa. The study found that copper acted as an inducer in the laccase production, where solid state fermentation using wheat straw was a compelling combination for the isolated fungi in laccase production. The isolated fungi have the potential to produce laccase as a result of different growth parameters and the use of different agricultural wastes.

Keywords: Laccase, RSM, CCD, optimization, OFAT, ABTS

1 Introduction

In the quest for sustainable solutions to the environmental and industrial challenges of our era, enzymes have emerged as remarkable biocatalysts, opening new avenues for bio-based technologies and processes. Among these, Laccases, with their diverse applications in waste degradation and recycling, play a pivotal role in addressing waste disposal issues, thus reducing the environmental burden. Laccase, as an enzyme, exhibits a remarkable capacity to facilitate the degradation of various compounds, whether they are phenolic or non-phenolic in nature. This ability extends to handling recalcitrant lignocellulosic waste materials, making laccase a versatile player in the realm of biocatalysis. India generates approximately 600 metric tons of agricultural waste, stemming from a variety of sources such as wheat straw, coconut husks, sugarcane residues, vegetable scraps, waste from the oil industry, and food byproducts (Chauhan & Jha, 2018). This extensive range of agricultural waste poses a substantial environmental challenge, primarily due to the absence of efficient waste management strategies. Exploring the potential of such organic waste as a nutrient source for laccase-producing microbes presents a cost-effective and sustainable alternative. This approach has gained increasing prominence as it aligns with the objective of utilizing agro-residue waste to produce laccase enzymes.

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Laccase production has been documented in many bacteri and fungi (Muthukumarasamy & Murugan, 2014; Rivera *et al*., 2013). A remarkable characteristic of fungal laccase is that it is released as a secondary metabolite, which makes it easy to be produced commercially (Margot *et al*., 2013). Very limited literature is available on the potential effects of the fungal laccases in their application in the bioremediation field. Certain fungal strains, like *Cerrena unicolor*, have demonstrated their potential for achieving maximum laccase production when cultivated in submerged or solid-state fermentation conditions (Janusz *et al*., 2012). This is primarily because such conditions offer a favorable environment for microbial growth and provide adequate nutrients. Consequently, the selection of specific fungal strains for laccase production in solid-state fermentation (SSF) processes using cost-effective substrates has garnered significant interest, particularly for large-scale applications. Current research efforts are primarily directed toward developing sustainable and economically viable technologies for laccase production using the SSF technique. The SSF approach is favored due to its efficient oxygen and nutrient availability within the culture medium. This fermentation mode offers several advantages, including precise control over various process parameters, scalability of production, enhanced product purity, and streamlined processing.

The conventional method of laccase production has involved the optimization of various process parameters (Chauhan *et al*., 2018). These include fine-tuning the environmental and nutritional composition of the growth medium, adjusting the incubation period, pH, temperature, and carefully selecting carbon (C) and nitrogen (N) sources, as these factors all have a significant impact on laccase production (Ghosh & Ghosh, 2017). To achieve maximum laccase production, an effective strategy involves optimizing the numerous combinations of these variables. Response surface methodology with central composite design (RSM-CCD) has proven to be a valuable tool in this regard. The goal is to enable the commercial-scale production of fungal laccase, and to achieve this, the use of organic residue waste as a substrate for laccase production holds promise. Such waste materials are rich sources of carbon, nitrogen, and phosphorus, which are essential for laccase synthesis.

This study focused on the screening and identification of laccase-producing fungi (*Cerrena unicolor* sourced from decomposed and organic wastes. Various organic carbon sources like rice bran, wheat bran, and rice straw were assessed for their suitability in laccase production under Solid State Fermentation (SSF). To maximize laccase production, a range of environmental and nutritional parameters were fine-tuned using the Central Composite Design-Response Surface Methodology (CCD-RSM). The laccase enzyme was partially purified through chromatography employing a Sephadex column (G-100), and its molecular weight was determined via SDS-PAGE. Furthermore, the enzyme's thermostability was investigated as part of this research.

The objectives of this research encompassed selecting the most promising fungal species and the optimal lignocellulosic support substrate for laccase production. Subsequently, the study aimed to determine the optimal conditions for Solid State Fermentation (SSF) using RSM with the selected fungus and substrate.

2 Material and methods

2.1 Isolation of laccase producing fungi

Decomposed tree stumps, leaf litter, biowaste from municipal compost, and fruiting bodies of mushrooms were used as samples for the isolation of the putative laccase-producing microbes using the serial dilution method (Terrón *et al*., 2004). The screening of laccase-producing fungi involved their growth on potato dextrose agar (PDA) (HiMedia, India) supplemented with 4 mM guaiacol as a specific substrate (Kiiskinen *et al*., 2004). The growth and development of the fungi were observed on PDA plates, focusing on the appearance of distinct characteristics. Red to brown precipitates were indicative of growth and laccase activity in plates containing guaiacol, further confirming the presence of laccase activity. Distinct colonies were selected for screening.

2.2 Screening of potent laccase producing fungi

Sterile medium containing glucose at 20 g/L, Peptone 5 g/L, Yeast extract 1 g/L , KCl 0.5 g/L, KH_2PO_4 at 1 g/L, $MgSO_4 \cdot 7H_2O$ at 0.5 g/L, $CuSO_4 \cdot 7H_2O$ at 0.25 g/L and pH 5.5 was inoculated with 1 ml of 10^6 CFU/mL concentration of spores (in 100 mL media) and kept at 25°C for 15 days on shaker incubator set at 100 rpm. Fungal mycelia was then filtered and filtrate collected for enzyme assay using standard ABTS method (Alcalde & Bulter, 2003). The fungi that yielded the maximum laccase activity was shortlisted and utilized in the optimization of laccase production.

2.3 Screening of Lignocellulosic material for laccase production using SSF

Rice husk, rice straw, wheat straw, wood chips, and coco peat were used as lignocellulosic materials for SSF. Prior to fermentation, the samples were cut into small bits of approximately 1 cm in length. No pre-treatment was done to any lignocellulosic material. The lignin, α-cellulose, and hemicellulose content of the materials was tested (Wang *et al*., 2016). The laccase production was screened for the best substrate selection (Alcalde & Bulter, 2003). 5 g of all the lignocellulosic substrate was moistened to 80% with media made up of 1 g/L yeast extract, 0.2 g/L ammonium sulfate, 0.002 g/L copper sulfate, 0.2 g/L magnesium sulfate, and 0.04 g/L calcium carbonate. This was incubated at 25 °C for 17 days. Laccase production was screened for all substrates by the ABTS assay.

2.4 Laccase Assay

The determination of laccase activity involved using 2,2'-azinobis(3-ethylbenzthiazoline 6-sulphonic acid) (ABTS) in 0.1 M sodium acetate buffer (pH 5). The oxidation of ABTS was assessed by measuring the increase in absorbance at 420 nm (A420), with an extinction coefficient of 36000 /M/cm utilizing a Spectrophotometer. One unit of enzyme activity (U) was defined as the amount of enzyme needed to oxidize 1 µmol of ABTS per minute.

$$\text{Enzyme}\left(\frac{\text{U}}{\text{mL}}\right) = \frac{\Delta\text{A420nm} \times \text{df}}{0.036 \text{ x } 0.02}$$

Where ΔA420nm is the change in absorbance per minute, df is the dilution factor, 0.036 is the molar extinction coefficient of ABTS radical cation, and 0.02 is the volume (in mL) of enzyme used.

2.5 Identification of isolates

The fungal identification was done using the rRNA and LSU gene sequences (Tekpinar & Kalmer, 2019). Identified fungi were deposited at the National Fungal Culture Collection of India (Agharkar Research Institute, Pune). Sequences obtained were aligned using ClustalW, and a neighbor-joining tree (NJ tree) was constructed to find the phylogeny of the isolates in MEGA-X. The homology analysis provided an assurance of the identification of an isolate when it showed significant sequence coverage and phylogenetic homology with already-registered partial genome sequences in the GenBank database (Kumar *et al*., 2018).

2.6 Optimization of Laccase Production by One Factor at a Time

The optimal physico-chemical parameters were primarily standardized using one factor at a time (Nambisan, 2018). 1 mL spore suspension containing 108 CFU inoculated in media (containing 5 g of wheat straw sieved through a 1.5 mm sieve, yeast extract 1 g/L, $(NH_4)_2SO_4$ 0.2 g/L, $MgSO_4$ 0.2 g/L, $CaCO_3$ 0.04 g/L, and $CuSO_4$ 0.002 g/L) and incubated at different temperatures (28.5, 29, 29.5, 30.0, 30.5, and 31 °C) for 14 days. The incubation period was optimized to a 17-day period at 30 °C. Moisture content was optimized using 40, 60, 80, and 100% moisture. pH ranged from 4.4 to 6.

2.7 Optimization of Laccase Production using RSM model

The optimization experiment was focused on comparing yeast extract (an organic source) and ammonium sulfate (an inorganic source) as nitrogen sources coupled with copper sulfate. The chosen substrate and fungi were employed to optimize the process parameters using two independent factors, namely yeast extract/ammonium sulfate and copper sulfate. The effects of each parameter were systematically studied using the Central Composite Design (CCD) of RSM from Design Expert Software version 7. The treatment combinations of the CCD were allocated in a single block, giving 13 runs in total for yeast extract as well as for ammonium sulfate tested with copper sulfate. The influence of each variable on laccase production was examined at five different levels (-α, -1, 0, +1, +α) using two factors. The temperature, pH, and moisture contents were used as found in the optimal values of OFAT optimization studies. Table 1 presents the actual values for each variable used in the optimization process. For the purpose of constructing a practical model, all analyses were based on the actual values obtained.

Based on a second-order equation, three-dimensional response surface plots were generated to visually depict the primary and interactive effects of the independent variables on the dependent ones. These plots were created by holding two of the independent variables at constant values, specifically the center points of the considered interval. Statistical analysis of the model was conducted using the 'Design Expert' software package (version 7.0.0, Stat-Ease Inc., Minneapolis, USA) (Vijayaraghavan & Vincent, 2014).

2.8 Enzyme Extraction

The enzyme production was done using the optimized parameters performed in RSM model. The sample was initially extracted by adding 50 mL of sodium acetate buffer (pH 5) and thoroughly shaking at 100 rpm for 2 hours. The sample was later filtered to remove any suspended materials. To this filtrate, precipitation was performed using cold acetone at the ratio of 1:1.5 and then centrifuged at 10000 rpm at 4°C. The enzyme activity was measured using ABTS assay.

2.9 Laccase purification

The precipitated protein fraction was desalted through a dialysis membrane of pore size 150 to 200 µm, equilibrated with five mM Bis-Tris HCL buffer of pH 6.5 for 24 hours, in a two-liter flask on a magnetic stirrer. The purification of the protein was followed as per the method mentioned by Wood (1980). The primary purification was done using a DEAE-Sephadox A-50 column (Himedia) equilibrated with 5 mM BisTris-HCl buffer, pH 6.5, and the desalted protein solution was loaded into the column at a flow rate of 0.50 mL/min. Bound proteins were eluted with a 100-ml linear gradient of NaCl (0–0.5 M) in 5 mM Bis-Tris HCl buffer, pH 6.5, at the same flow rate. 1 ml of 20 fractions was collected. Protein concentration was estimated from the absorbance at 280 nm, and laccase activity was determined for each fraction using Lowrey's method. The putative protein fractions were clubbed and refiltered through a Sepharose gel filtration column, equilibrated using 0.1 M NaCl in 5 mM Bis-Tris-HCl buffer, pH 6.5, as eluent. A 1 ml sample was injected into each run, chromatographed at a flow rate of 0.1 ml/min, and collected in fractions of 0.5 ml. The fractions were assayed for laccase activity, and the active fractions were pooled for SDS-PAGE and percent yields. Laccase activity was determined spectrophotometrically by the ABTS assay.

2.10 SDS PAGE of purified fractions

Sample were resolved by SDS-PAGE according to a modified Laemmli method (Gallagher, 2006). Electrophoresis was performed in vertical mini-slab gel with a gel thickness of 0.75 mm and gel size 8 × 7 cm. The gels were composed of 10% resolving gel and 10% stacking gel and run at constant voltage of 150 volts for 50 min. 100 µL of sample was mixed with 50 µL of SDS PAGE dye respectively, from this 40 µL of each sample was loaded into separate wells of the gel. After electrophoretic separation, the gel was stained with Coomassie blue solution (0.01% Coomassie brilliant blue R250, 45% (v/v) methanol and 10% (v/v) glacial acetic acid) for 30 min at room temperature and subsequently destained in the destaining solution (50% (v/v) methanol and 2% (v/v) acetic acid) for 1hr.

2.11 Statistics

All tests were performed in triplicate. The mean values were used to find the standard error of mean and analysis of variance (at $p < 0.05$). The statistical calculations were performed using IBM SPSS ver. 24.

3 Results and discussion

3.1 Screening of potent Laccase-Producing Fungi

The laccase-producing fungi from different agricultural waste sources gave distinct reddish-brown oxidation zones around the colonies, in guaiacol media, indicating laccase production. Fungi exhibiting laccase activity were selected for further evaluation and subcultured on PDA medium. Among the laccase-positive fungi, one predominant fungus coded as LP2 was chosen for further investigation based on its initial higher laccase activity (fig 1). These screening results, based on color formation in the media, confirm the ability of fungi to produce laccase enzyme.

A total of 14 fungi were potential laccase producers. Five fungus isolates were screened for higher laccase production and coded as LP1 to LP5. Among those, LP 2 was found to be the highest laccase producer, with a laccase production of 96.7 ±0.3 U/mL, isolated from leaf litter. LP2 was a potent laccase producer from cheap substrates (Fig. 2).

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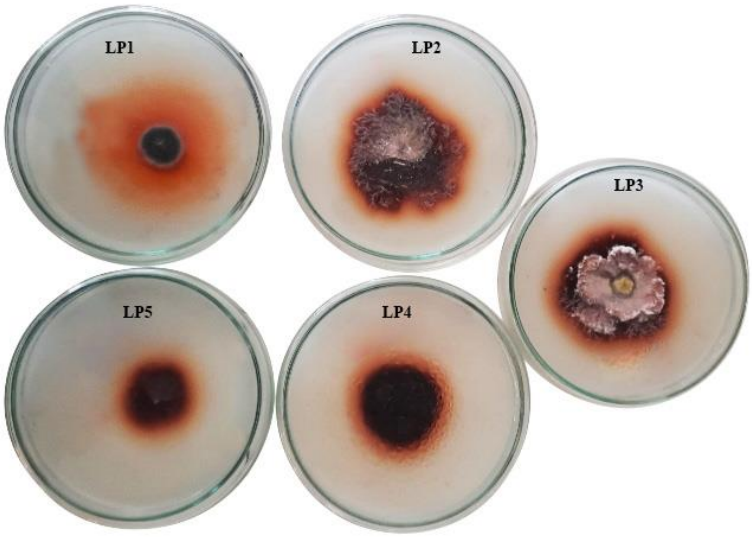

Fig. 1 Screening of laccase producing fungi using 0.16 mM guiacol media. LP2 gave the best growth and laccase production in the screening.

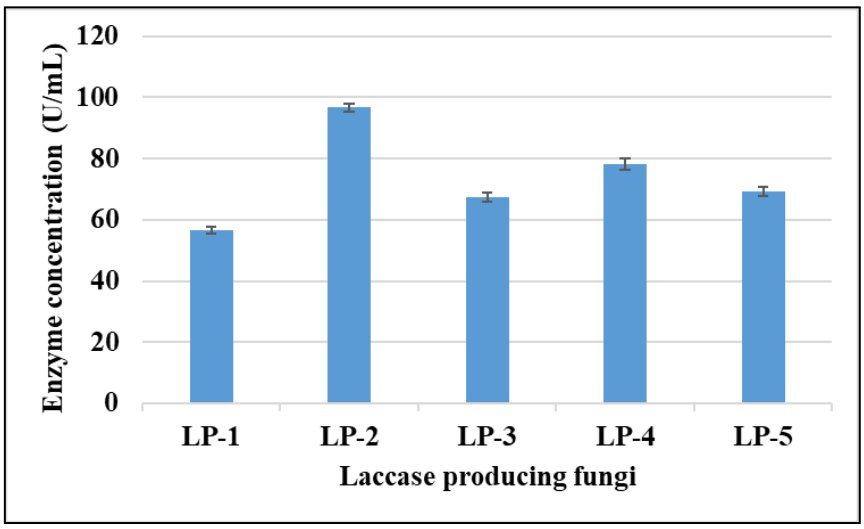

Fig. 2 Screening of fungal isolates based on the Laccase production.

3.2 Identification of fungal isolate

Based on the screening strategy, *Cerrena unicolor* was found to have the best laccase production potential. The isolate was deposited in the national fungal culture collection of India, located at the Agharak Research Institute. The isolate has been registered with NFCCI with accession number 5146.

The phylogenetic tree was prepared by the neighbor-joining (N-J) method using MEGA X software. Here, a small subunit ribosomal RNA sequence was used to construct a phylogenetic tree for *Cerrena unicolor*. The result revealed that the LP2 strain was closely related to accession number MT720696.1, which was registered as *Cerrena unicolor*. The isolate has been registered in the NCBI GenBank with accession number OR713083.1.

3.3 Screening of lignocellulosic material for laccase production

Fungi generally grow over dead and decaying wood material in the wild. But it is very difficult for fungi to utilize the complex cellulosic material in the wood in the presence of lignin and hemicellulose. Despite the higher alpha-cellulose content in wood chips, the lignin and hemicellulose reduced the

potential of this raw material for faster fungal growth. Lignin, or lignocellulose itself, doesn't degrade more than 40% in solid-state fermentation (Adekunle, Zhang, *et al*., 2017). α-Cellulose is the preferred carbon source for fungal growth, but lignin reduces the growth rate, warranting the need for some other media as supplements in solid-state fermentation (Wang *et al*., 2019). There is enough evidence to ascertain that lignin in the agriwaste naturally induces laccase production (Mishra *et al*., 2017). There was a strong negative correlation (R2 = -0.89) between the lignin content and laccase produced by Cerenna unicolor. There was no significant effect of cellulose and hemicellulose on the laccase produced. The wheat straw was found to have the highest laccase activity among all the other cellulosic materials used. It can be said that lignin does have a considerable effect on the laccase content, though there is still a knowledge gap regarding optimizing the lignin content of the media for optimal laccase production. Figure 3 shows the chemical composition of the selected agricultural wastes.

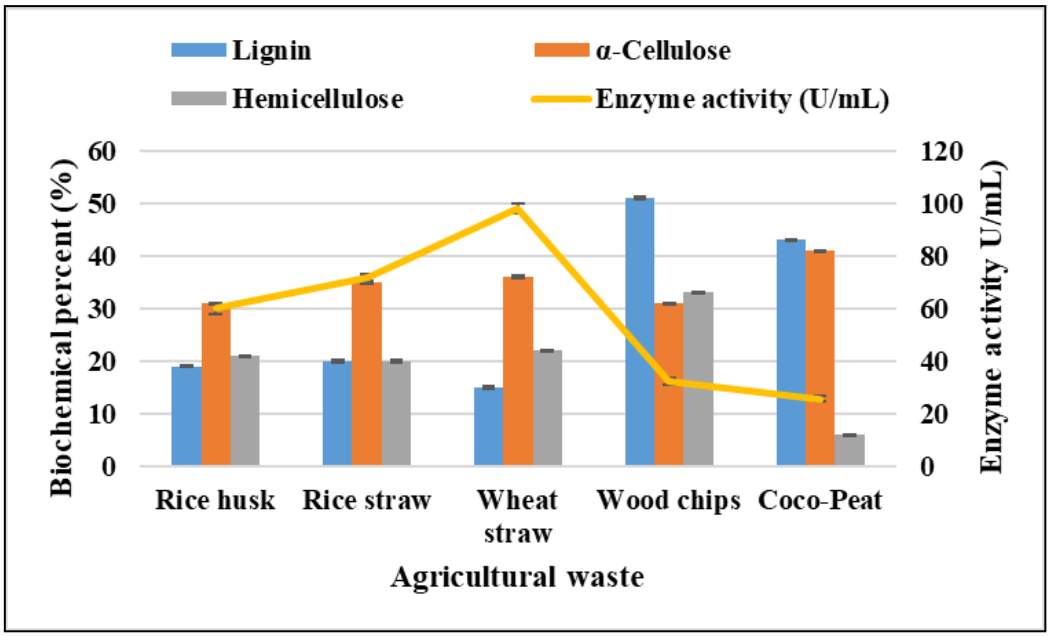

Fig. 3 Comparison of different agricultural wastes with respect to lignin, α-cellulose and hemicellulose content for laccase production.

3.4 Optimization of Laccase Production By One Factor at a Time

The critical determinant of success in any optimization process lies in the careful selection of appropriate cultural factors. Analysis of variance using post hoc Tukeys HSD showed that enzyme production at all temperatures was significantly different from laccase production. The significantly highest laccase production was obtained at 30 °C with about 101.5 ± 2.4 U/mL of laccase enzyme. This showed a 1.5-fold increase in the enzyme activity at 30 °C as compared to 20 or 40 °C. The incubation period showed a steady increase in laccase activity, with the highest enzyme produced on the 14th day of incubation (100.53 ± 3.1 U/mL). On the 14th day, there was no significant difference in enzyme activity. The moisture content of 80% was found to be optimal for laccase production. A similar moisture percentage (75%), has been found to be optimal for laccase production in similar growth conditions (Sharma & Murty, 2021). pH 5 gave the highest laccase production, which is generally seen in most of the laccase-producing fungi (Kolomytseva *et al*., 2017).

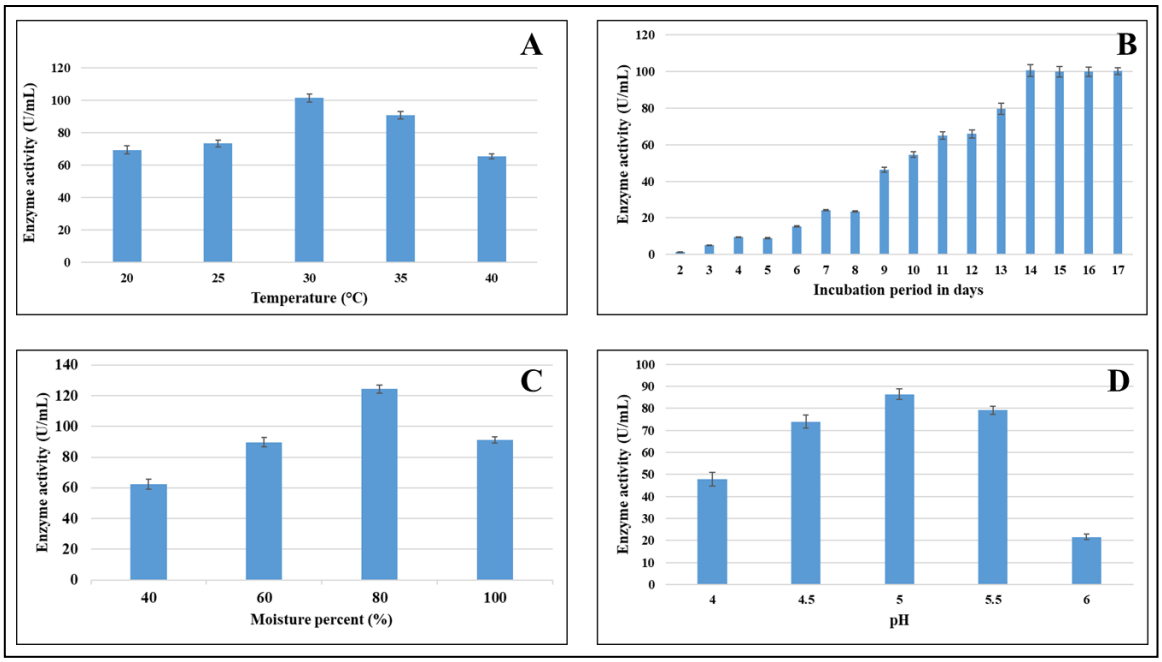

Fig. 2 Effect of different physicochemical parameters on laccase enzyme activity. A shows effect of temperature, B shows the effect of incubation period, C shows effect of moisture percent on the enzyme activity and D shows the effect of pH

3.5 Central composite design (CCD) model fitting and ANOVA

Table 1 presents the experimental domain, illustrating the defined levels for each selected variable, which in this case, corresponds to the media components. The experiments were conducted to establish the quadratic model with two independent variables in both the nitrogen source cases. The combinations of experiment are represented in table 2.

The correlation between the observed values and the quadratic polynomial model was found to be highly significant, indicating a robust representation of the actual relationship between the response variable (laccase activity) and the relevant factors. The varying laccase content, ranging from 75 to 110 U/mL, suggests that interactions among the yeast extract and copper sulfate had a more pronounced impact than individual factors in isolation. When compared to an inorganic source of nitrogen (ammonium sulfate), a comparatively lower response was seen, with laccase ranging from 45 to 86 U/mL.

The results were further subjected to standard analysis of variance (ANOVA), as shown in Table 3. The ANOVA for yeast extract revealed the model's high significance, with an F-value of 64.85, supported by a very low p-value (p model > F = 0.0001) according to Fisher's "F" test. Simultaneously, a relatively low coefficient of variation (CV = 2.68%) indicates the precision and reliability of the experimental procedures.

The determination coefficient (R2) of the model was 0.9789, explaining 97.89% of the variability in the response. Only 2.111% of the total variation remained unexplained by the model, underscoring the model's satisfactory fit to the experimental data. The analysis of the design demonstrated a high degree of agreement between predicted and experimental data, confirming the model's ability to accurately represent the relationships among the chosen factors.

Similar statistical readings were observed for ammonium sulfate as a nitrogen source, but with a comparatively lower magnitude (table 3). The model was significant with a non-significant lack of fit, both in the case of organic and inorganic nitrogen sources. The predictive equations for laccase production were found to be as follows:.

For yeast extract as nitrogen source, Laccase production = -200.45181 + 44.62665 * yeast extract +3.66220 * copper sulphate + 0.000000 * yeast extract * copper sulphate - 13.57500 * yeast extract^2 - 0.012306 * copper sulphate^2.

For Ammonium sulphate as substrate, laccase activity (U/mL) = +121.05398 + 74.36683 * Ammonium sulphate -1.47203 * copper sulphate -0.34524 * Ammonium sulphate * copper sulphate -4.05612 * Ammonium sulphate^2 + 6.68056E - 003 * copper sulphate^2

Table 1 Range of variables for CCD

	Unit	-1 Level	+1 Level	-alpha	+alpha
Yeast extract	g/L	0.5	2.5	0.086	2.914
Copper sulphate	µM/L	120	180	107.574	192.426
Ammonium sulphate	g/L	0.2	1.6	0.089	1.889

Table 2 Central composite design (CCD) for organic and inorganic nitrogen source with observed results

Yeast Extract (g/L)	Copper sulphate (µM/L)	Laccase production Experimental (U/mL)	Laccase production Predicted (U/mL)	Ammonium sulphate (g/L)	Copper sulphate (µM/L)	Laccase production (U/mL)	Laccase production Predicted (U/mL)
0.50	120.0	80.0	80.0	0.2	180.0	80.0	74.8
2.50	120.0	85.0	88.5	0.9	150.0	60.0	67.6
0.50	180.0	80.0	79.0	1.9	150.0	85.0	67.6
2.50	180.0	85.0	86.8	1.6	180.0	80.0	81.7
0.09	150.0	75.0	76.0	0.9	150.0	70.0	67.6
2.99	150.0	90.0	84.1	1.6	120.0	75.0	82.9
1.50	107.6	90.0	86.9	0.9	150.0	70.0	67.6
1.50	192.4	85.0	85.0	0.1	150.0	45.0	52.5
1.50	150.0	109.0	108.4	0.9	150.0	68.0	67.6
1.50	150.0	108.0	108.4	0.9	150.0	70.0	67.6
1.50	150.0	109.0	108.4	0.9	192.0	86.0	89.0
1.50	150.0	106.0	108.4	0.9	108.0	76.0	70.2
1.50	150.0	110.0	108.4	0.2	120.0	46.0	47.0

Table 3 ANOVA for Response Surface Quadratic Mode of Yeast Extract as Nitrogen source

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
For Yeast extract					
Model	2022.64	5	404.53	64.85	< 0.0001
A-yeast extract	121.78	1	121.78	19.52	0.0031
B-copper sulphate	6.25	1	6.25	1	0.3502
AB	0	1	0	0	1
A^2	1281.95	1	1282	205.5	< 0.0001
B^2	853.26	1	853.26	136.8	< 0.0001
Residual	43.67	7	6.24		
Lack of Fit	34.47	3	11.49	5	0.0771
Pure Error	9.2	4	2.3		
Cor Total	2066.31	12			
For Ammonium sulphate					
Model	1784.36	5	356.87117	9.51413	0.0050
A-yeast extract	915.25	1	915.24693	24.40033	0.0017
B-copper sulphate	353.01	1	353.01082	9.41121	0.0181
AB	210.25	1	210.25000	5.60523	0.0498
A^2	27.48	1	27.47935	0.73259	0.4204
B^2	251.48	1	251.47935	6.70440	0.0360
Residual	262.57	7	37.50961		
Lack of Fit	187.37	3	62.45575	3.32211	0.1382
Pure Error	75.20	4	18.80000		
Cor Total	2046.92	12			

For Yeast extract, R^2= 0.9789, C.V.= 2.68 %, *Significant at prob. > F < 0.0001.The Model F-value of 64.85 implies the model is significant. There is onlya 0.01% chance that a "Model F-Value" this large could occur due to noise. The "Lack of Fit F-value" of 5.00 implies there is a 7.71% chance that a "Lack of Fit F-value" this large could occur due to noise. For ammonium sulphate, R^2= 0.8717, C.V.= 8.73 %, Significant at prob. > F < 0.005.The Model F-value of 9.51 implies the model is significant. The "Lack of Fit F-value" of 3.32 implies there is a 13.82% chance that a "Lack of Fit F-value" this large could occur due to noise.

From the data in the model, it was evident that the linear coefficients and quadratic coefficients were highly significant, but the physical factors also played a greater role in the laccase production. Comparison of the observed and predicted values showed that Yeast extract provided a better model for laccase production. Ammonium sulphate was lesser effective in laccase production. It appears, nonetheless, that there is disagreement about how different bacteria' sources of nitrogen affect the release of laccase (Ire & Ahuekwe, 2016). Both organic and inorganic nitrogen sources were found to support increased laccase production by *Titanomyrma giganteum* but not growth, according to Patel and Gupte (2016). It has been suggested that yeast extract is a good supply of nitrogen for the synthesis of laccase, probably due to many other micronutrients along with nitrogen source.

To illustrate the interactions among the medium components and identify the optimal concentration of each component for enhanced laccase production, three-dimensional response surface graphs were generated. Fig. 5 illustrates the cumulative impact of yeast extract and $CuSO_4$ concentrations on laccase production. This is also shown with ammonium sulphate and $CuSO_4$. In this response surface plot, it is evident that laccase activity reached 110 U/mL when the production medium contained 1.5 g/L Yeast extract and 150 µM $CuSO_4$. Copper sulphate is generally required in lower concentrations (1-10 µM) in fungi for laccase production (Liu *et al*., 2009), but Cerrena genera is reported to generate laccase even at 0.75 mM (Mann *et al*., 2015). The concentration of yeast extract within the range was optimal and any further variation in the yeast extracts reduced the laccase production in this research.

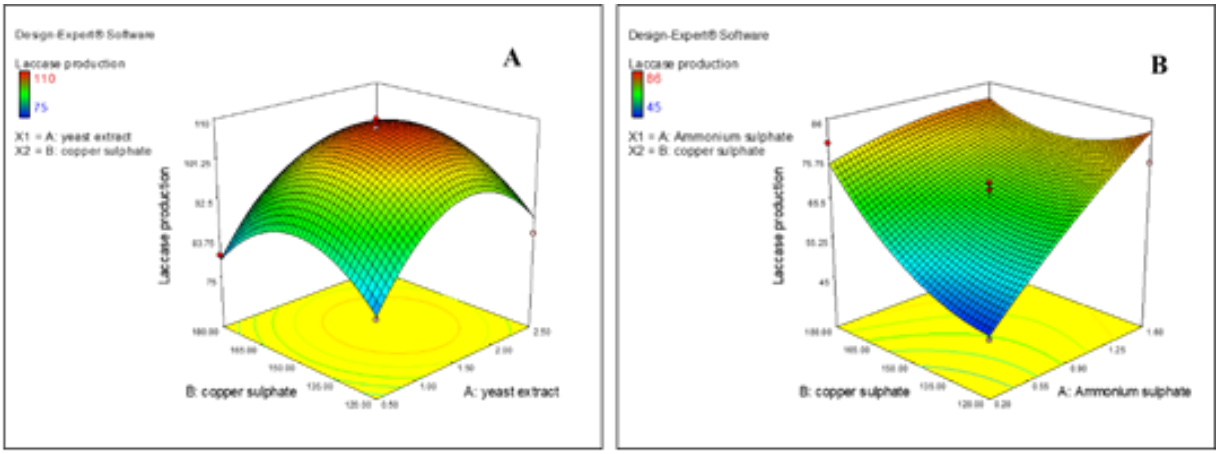

Fig. 5 Response surface plot showing the interactive effect of (A) yeast extract and (B) Ammonium sulphate on laccase production by *Cerrena unicolor*. Optimal laccase production was shown by yeast extract.

3.6 Partial purification of laccase enzyme

The quantification of the samples was done at every purification stage, to check the loss percentages of the enzyme during the purification process. The control was kept as a commercial laccase alongside the test samples for comparison. The crude dialysate, Ion exchange tubes and gel filtration were used along with ABTS method. Every time the purification was performed, a small portion of the laccase was lost. Finally, stock solution of 2754 U/ml concentration was prepared. To confirm the presence of laccase, guaiacol based quantification was also done.There was a significant difference in the laccase quantification done using two methods. This difference has been reported previously and high catalytic efficiency and high molar exticnction co-efficient of ABTS makes it sensitive for laccase quantification (Eichlerová *et al*., 2012).

Table 4 Comparison of laccase activity after purification of sample through guaiacol and ABTS method

Sample	ABTS Method OD at 436 nm	Activity U/mL	Guaiacol Method OD at 465 nm	Activity U/mL
Control	1.556	3112	1.898	759.2
Crude	0.252	504	0.401	160.4
Dialysate	0.668	1336	0.976	390.4
Ion exchange tube (6 & 7)	0.109	218	0.094	37.6
Ion exchange tube (13 to 15)	1.323	2646	1.623	649.2
Gel filtration tube (8 to 10)	1.377	2754	1.686	674.4

The purification of the laccase was confirmed by SDS PAGE to find whether the isolate was monomeric or polymeric. The fungal laccase in this research was found to be monomeric. There is a knowledge gap in relevance of polymeric nature of laccase on its activity. The best quality of separation was seen in the crude sample and test samples after SDS page. Monomeric protein was seen as a single distinct band in samples run through ion exchange chromatography. Figure 6 shows that the crude dialysate had greater contamination, which was later reduced in gel filtration tube 8 to 10. The ion exchange chromatography of pool 13 to 15 had greater purity as compared to pool 6 and 7. The control was found to be dimeric peptide but the isolated pure laccase was found to have a single peptide of 64 kDa. This was in liasion with many monomeric laccase found in general (Arregui *et al*., 2019).

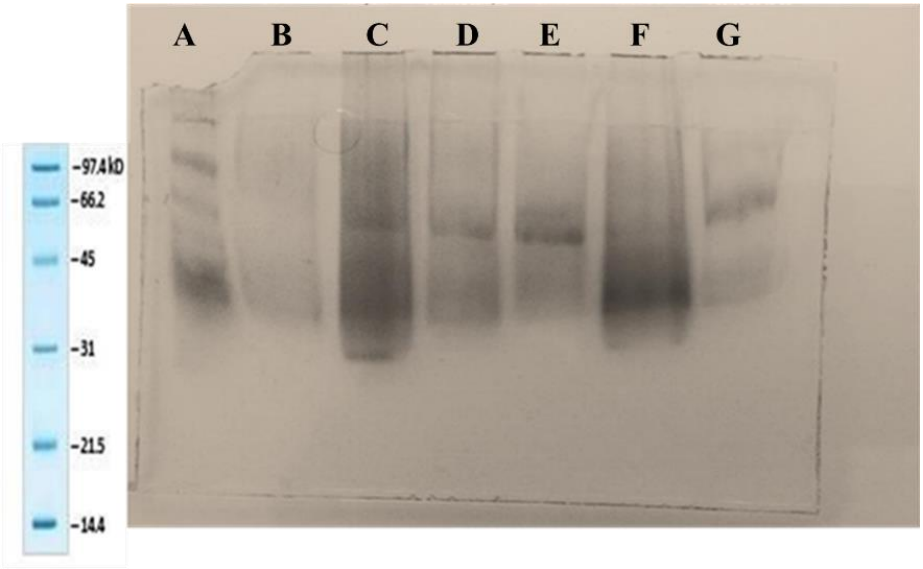

Fig. 6 Comparison of contamination at different purification stages of laccase. First lane is Marker with given molecular weights of bands, A lane is blank, B is the Crude dialysate, C is gel filtration purified sample, D is control, sixth and seventh are ion exchange purified samples of pool 6,7 and 13 o 15 respectively.

4 Conclusions

Cerrena unicolor has not been highly reported to be a candidate microbe in laccase production. It was isolated from agricultural waste. Genetic identification is a roust method for fungal identification unless there is sufficient ITS sequence data present in the GenBank database. The classical OFAT is a general method to identify individual factors affecting laccase production. But the central composite design (CCD) of response surface methodology (RSM) gave a better insight into the fungal response to raw material. Using the RSM model, more than 100 U/ml of enzyme was generated, which was also found to be very similar to the predicted value found in the statistical analysis. The research found that the *Cerrena unicolor* gave better growth in the presences of copper sulphate as inducer.

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References

Adekunle, A., Guo, C., & Liu, C.Z. (2017). Lignin-Enhanced Laccase Production from Trametes versicolor. Waste and Biomass Valorization, 8. https://doi.org/10.1007/s12649-016-9680-4

Adekunle, A., Zhang, C., Guo, C., & Liu, C.Z. (2017). Laccase Production from Trametes versicolor in Solid-State Fermentation of Steam-Exploded Pretreated Cornstalk. Waste and Biomass Valorization, 8. https://doi.org/10.1007/s12649-016-9562-9

Alcalde, M., & Bulter, T. (2003). Colorimetric assays for screening laccases. Directed Enzyme Evolution: Screening and Selection Methods, 193-201. https://doi.org/10.1385/1-59259-396-8:193

Arregui, L. *et al*. Laccases: structure, function, and potential application in water bioremediation. Microb Cell Fact 18, 200 (2019). https://doi.org/10.1186/s12934-019-1248-0

Aruna, S., Gomathi, M., & Gehitha, T. R. (2012). Screening, production and partial purification of laccase enzyme from white rot fungi. Indian Journal of Current research, 1 (2), 10-17.

Chauhan, P. S., Goradia, B., & Jha, B. (2018). Optimization and up scaling of ionic liquid tolerant and thermo-alkali stable laccase from a marine Staphylococcus arlettae S1-20 using tea waste. Journal of the Taiwan Institute of Chemical Engineers, 86, 1-8. https://doi.org/10.1016/j.jtice.2018.02.032

Chauhan, P. S., & Jha, B. (2018). Pilot scale production of extracellular thermo-alkali stable laccase from Pseudomonas sp. S2 using agro waste and its application in organophosphorous pesticides degradation. Journal of Chemical Technology & Biotechnology, 93(4), 1022-1030. https://doi.org/10.1002/jctb.5454

Eichlerová, I., Šnajdr, J., & Baldrian, P. (2012). Laccase activity in soils: considerations for the measurement of enzyme activity. Chemosphere, 88(10), 1154-1160.

Faulina, S. A. *et al*. (2020). The higher laccase enzyme producer, Cerrena sp. BMd. TA. 1, isolated from Gunung Rinjani National Park, West Nusa Tenggara, Indonesia. Biodiversitas Journal of Biological Diversity, 21(8). https://doi.org/10.13057/biodiv/d210853

Gallagher, S. R. (2006). One-dimensional SDS gel electrophoresis of proteins. Current protocols in immunology, 75(1), 8.4. 1-8.4. 37. https://doi.org/10.1002/0471142735.im0804s75

Ghosh, P., & Ghosh, U. (2017). Statistical optimization of laccase production by Aspergillus flavus PUF5 through submerged fermentation using agro-waste as cheap substrate. Acta Biologica Szegediensis, 61(1), 25-33.

Ire, F. S., & Ahuekwe, E. F. (2016). Production of fungal laccase using orange peelings as substrate by submerged static fermentation. British Microbiology Research Journal, 15(5), 1-19.

Janusz, G. *et al*. (2012). Cloning and characterization of a laccase gene from biotechnologically important basidiomycete *Cerrena unicolor*. https://doi.org/10.5109/22046

Kiiskinen, L. L., Rättö, M., & Kruus, K. (2004). Screening for novel laccase-producing microbes. Journal of applied microbiology, 97(3), 640-646. https://doi.org/10.1111/j.1365-2672.2004.02348.x

Kolomytseva, M. *et al*. (2017). Rapid identification of fungal laccases/oxidases with different pH-optimum. Process Biochemistry, 62, 174-183. https://doi.org/10.1016/j.procbio.2017.07.027

Kumar, S. *et al*. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular biology and evolution, 35(6), 1547. https://doi.org/ 10.1093/molbev/msy096

Liu, Z. *et al*. (2009). A newly isolated Paecilomyces sp. WSH-L07 for laccase production: isolation, identification, and production enhancement by complex inducement. Journal of Industrial Microbiology and Biotechnology, 36(10), 1315-1321.

Mann, J. *et al*. (2015). Use of olive mill wastewater as a suitable substrate for the production of laccase by Cerrena consors. International Biodeterioration & Biodegradation, 99, 138-145.

Margot, J. *et al*. (2013). Bacterial versus fungal laccase: potential for micropollutant degradation. AMB Express, 3(1), 63. https://doi.org/10.1186/2191-0855-3-63

Mishra, V. *et al*. (2017). Enhancement in multiple lignolytic enzymes production for optimized lignin degradation and selectivity in fungal pretreatment of sweet sorghum bagasse. Bioresour Technol, 236, 49-59. https://doi.org/10.1016/j.biortech.2017.03.148

Muthukumarasamy, N. P., & Murugan, S. (2014). Production, purification and application of bacterial laccase: a review. Biotechnology, 13(5), 196. https://doi.org/10.3923/biotech.2014.196.205

Nambisan, P. (2018). Data of optimization of laccase production by Marasmiellus palmivorus LA1 under solid state fermentation using one factor at a time method. Data in brief, 17, 1276-1282. https://doi.org/10.1016/j.dib.2018.02.011

Patel, H., & Gupte, A. (2016). Optimization of different culture conditions for enhanced laccase production and its purification from Tricholoma giganteum AGHP. Bioresources and Bioprocessing, 3, 1-10.

Rivera-Hoyos, C. M., Morales-Álvarez, E. D., Poutou-Piñales, R. A., Pedroza-Rodríguez, A. M., RodrÍguez-Vázquez, R., & Delgado-Boada, J. M. (2013). Fungal laccases. Fungal Biology Reviews, 27(3-4), 67-82.

Sharma, S., & Murty, D. S. (2021). Enhancement of Laccase Production by Optimizing the Cultural Conditions for Pleurotus sajor-caju in Solid-State Fermentation. Journal of Pure & Applied Microbiology, 15(2).https://doi.org/10.22207/JPAM.15.2.54

Tekpinar, A. D., & Kalmer, A. (2019). Utility of various molecular markers in fungal identification and phylogeny. Nova Hedwigia, 109(1-2), 187-224.

Terrón, M. C. *et al*. (2004). Structural close-related aromatic compounds have different effects on laccase activity and on lcc gene expression in the ligninolytic fungus Trametes sp. I-62. Fungal Genetics and Biology, 41(10), 954-962. https://doi.org/10.1016/j.fgb.2004.07.002

Vijayaraghavan, P., & Prakash Vincent, S. G. (2014). Medium optimization for the production of fibrinolytic enzyme by Paenibacillus sp. IND8 using response surface methodology. The Scientific World Journal, 2014. https://doi.org/10.1155/2014/276942

Wang, F. *et al*. (2019). Fungal Laccase Production from Lignocellulosic Agricultural Wastes by Solid-State Fermentation: A Review. Microorganisms, 7(12). https://doi.org/10.3390/microorganisms7120665

Wang, Z. *et al*. (2016). A comparison of chemical treatment methods for the preparation of rice husk cellulosic fibers. International Journal of Environmental & Agriculture Research, 2(1), 2454-1850. https://doi.org/10.1155/2014/276942

Wood, D. (1980). Production, purification and properties of extracellular laccase of Agaricus bisporus. Microbiology, 117(2), 327-338. https://doi.org/10.1099/00221287-117-2-327