Original Research Article

Optimizing Laccase Production from *Delonix regia* Pods by *Aspergillus carbonarius* F5 Using Response Surface Methodology and it's Dye Decolorization Potential

Abstract

Introduction: Laccase has been identified as a significant enzyme which possesses diverse interest in industrial sector due to its ability to degrade toxicants of various sources.

Aim: This present study screened and validate the best condition that could facilitate the maximum production laccase from *Aspergillus carbonarius* F5 under submerged fermentation, using *Delonix regia* pods as substrate for application in dye degradation.

Methodology: Potential laccase producing fungi were screened and characterized using 18s rRNA. Laccase production was further conducted on untreated and acid pretreated *Delonix regia* pods. Plackett-Burman design and Central Composite Design (CCD) of the Response Surface Methodology (RSM) was used to screen for various nutritional and environmental factors to improve the yield of the enzyme and then applied for dye decolonization studies.

Results: The fungal strain with the highest laccase activity was identified as *Aspergillus carbonarius* F5. The acid pretreated *Delonix regia* pods showed considerably high enzyme activity when compared with untreated. Moreover, variation of notable nutritional and environmental factors were detected to lead to increase in the production of laccase yield. The effect of input parameters such as incubation period, pH, temperature and $MgSO_4$ were documented as the major factors that could influence increase in laccase yield using Plackett-Burman design. Results obtained from the modelling of experiment using CCD-RSM shows that maximum laccase production of 8.04 U/ml was recorded at temperature 36 °C, pH 6, $MgSO_4.7H_2O$ at 0.2 (g/L), and 7 days which reveals a 8.14 –fold increase. The outcome of the investigation performed on dye decolorization suggests an 87.6 % and 62.6 % degradation on congo red and malachite green dyes respectively.

Conclusion: This study showed that *Delonix regia* pods could be a newer cost effective, ecofriendly substrate, suitable for optimization of laccase by *Aspergillus carbonarius* F5 most especially in decolorizing industrial dyes effectively.

Keywords: Laccase, *Delonix regia* pods, Optimization, Response Surface methodology, *Aspergillus carbonarius*F5, Dye decolorization.

1.0 INTRODUCTION

Laccases (p-benezediol: oxygen oxidoreductases; EC 1.10.3.2) are representative of the blue multicopper protein family that could catalyze oxidation of phenolic and non-phenolic compounds through generation of water from molecular oxygen reduction [1]. Laccases are ubiquitously distributed in wide range of biotic sources including plants [Rice, *Oryza sativa [2]*], bacteria [*Weissella viridescens* LB37, *Bacillus* sp. MSK-01] [3, 4], fungi [*Aspergillus* sp. Omeje, *Schizophyllum commune* Han 881] [5, 6], and insects [*Drosophila melanogasker*] [7]. However, they are predominantly found in fungi [8]. Laccase that are derived from fungal origin have attracted considerable attention over the years due to their ability to weaken all the wood cell wall components with the aid of their enzymatic systems [9].

The application of laccases have been documented in numerous sector such as food processing industry, pulp and paper industry, pharmaceutical sector, delignification of lignocellulosic waste, biodegradation in textile dye industry [10, 11]. Dyes are majorly used as colorants in food, pharmaceutical and textile industries. However, the presence of synthetic dye waste that a discarded into water body which are majorly generated from textile industries has been documented to pose a lot of environmental risk. This might be linked to the presence of undesirable and harmful compounds. Moreover, several strategies have been introduced that could be applied to overcome such environmental menace through the usage of laccase induced detoxification. Laccases have been found worthy in the degradation of phenolic substance present in dyes [5] and this biological detoxification of recalcitrant dyes is sustainable both ecologically and economically. Interestingly, different species or strains of beneficial microorganisms have been documented to possess capability in the production of laccase and dye degradation. Thus, searching for newer strain for laccase production is vital. Previous studies have demonstrated the effectiveness of laccase production for dye decolourization most especially from *Streptomyces ipomoeae* CECT 3341 [12], *Aspergillus* sp. [13] and *Peroneutypa scoparia* [14]. However, the production of laccase from *Aspergillus carbonarious* is scarcely reported.

Lignocellulosic biomass are versatile abundant bioresource that portends a lot of potentials most especially in green energy production as well as in the addition of values to diverse products, through various bioprocesses. In recent times, utilization of various lignocellulosic waste, including saw dust, rice husk and olive mill waste for enzyme production most especially from laccase have been reported which might be linked to its low-cost and

abundance [15-17]. However, evaluation of laccase activities from *Aspergillus carbonarious* on *Delonix regia* pods as a source of carbon have not been investigated. *Delonix regia* pods (flamboyant pods) is an abundant underuse biomass, growing on a flowering plant belonging to the pea family. The pods are oblong, woody, flat and approximately 55 cm. It is green and turns brown when matured [18]. The pods have been reported for use as bioabsorbent [19] and bioenergy [20]. The chemical composition of *Delonix regia* pods makes it an excellent biomass for the production of industrially important enzyme such as laccase.

Optimization of media composition is essential to enhance both the activity and titre of laccases. It is well proven that numerous micoorganisms differ in their cultural conditions and nutritional requirement for the production enzymes. Therefore, it is critical to study the effect of growth parameters on the organism of interest through factor optimization. Nambisan, (2018 carried out an optimization studies for laccase production through one factor at a time (OFAT) This techniques has been proven to be efficient in assessing the effect of each parameter (Variable) on laccase production when tested independently. However, it cannot assess the interactive effects of the parameters on laccase production. Statistical optimization studies by design of experiment using response surface methodology is an ideal modelling approach. It has been reported to portend the potential to improve production of enzymes.. In addition, it identifies significant factors from a preliminary screening studies that could increases the activities of the test microorganism. Thereafter, the combined interactive effect among the significant factors is directed towards enhancement of the enzyme production.

Therefore, this study intends to optimize the best process parameters that are suitable for maximum laccase production through the action locally isolated *Aspergillus carbonarius* F5 when cultivated on *Delonix regia* pods using Central Composite Design (CCD) of Response Surface Methodology (RSM) as well as to investigate the dye decolorization potential of its crude laccase.

2.0 MATERIALS AND METHODS

2.1 Sample collection and fungi isolation

Soil samples were obtained from five different locations within the University of Ilorin, Kwara State, Nigeria. The samples were transferred into sterile polythene bags and were labeled accordingly, which was transported to the laboratory for analysis. Appropriate

dilution were carried out and transferred on to potato dextrose agar (PDA) plate, followed by incubation at for 5 days at 30 °C. Pure cultures were obtained and stored at 4°C.

2.2 Screening for laccase producing fungi

2.2.1Plate screening method

The fungi isolates were screened on PDA medium supplemented with 0.02 % guaiacol and incubated at for 5 days at 30 °C (Adiveppa and Basappa, 2015). Culture plate showing dark brown coloration was selected and further tested for dye decolorization.

2.2.2 Dye decolorization method

The ability to decolorize dye in potato dextrose broth (PDB) supplemented with 1 mg/ml malachite green dye and 0.1 % congo red dye was tested on the fungi isolates. Ten millimeter (10 ml) of the broth was inoculated with 2 agar plugs of mycelia using 5 mm cork borer and incubated for 5 days at 30 °C. Tube containing PDB and dye without the fungi was regarded as the control. A discoloration of the dye from the broth indicated the presence of lignolytic enzyme [21].

2.3 Identification of fungal isolate

The laccase positive fungus was identified based on macroscopic and microscopic examination according to the method described by [22] and (Watanabe, 2010). For molecular identification, DNA extraction was performed giving the methods described by Girma, Rabbi [23]. To amplify the internal transcribed spacer (ITS) region of the rRNA gene, the forward primer (ITS4-TCCTCCGCTTATTGATATG) and (ITS5-GGAAGTAAAAGTCGTAACAAGG) reverse primer was used as described by [24]. The polymerase chain reaction (PCR) cocktail mixture comprised of 2.5 µl of 10x PCR buffer, 1 µl of 25 mM $MgCl_2$, 1 µl of DMSO, 2 µ l of 2.5 mMDNTPs, 0.1µ l of 5 u/l Taq DNA polymerase, and 3 µl of 10 ng/l DNA. The total reaction volume was increased to 250 µl with 13.4 µl of nuclease-free water. The PCR was executed using a Veriti thermal cycler (Applied biosystems) for 36 cycles of denaturation at 94 °C for 30 s after an initial denaturation at 94°C for 5 min. Annealing took place at 54°C for 30 seconds, followed by elongation at 72°C for 7 min and holding at 10°C. The PCR products were purified and the amplified fragments were visualized in a safe view-stained 1.5 % agarose electrophoresis gel. . Consequently, the purified PCR products were sequenced using an automated sequencer

(ABI genetic analyzer 3500, Applied Biosystems, USA). With the aid of the NCBI-BLAST-n search, the sequence were compared to those of other *Aspergillus* species available in the GenBank. Multiple-sequence alignments of parallel sequences was done using Seqtrace software (version 0.90). The evolutionary distances (Phylogenetic tree) were computed by neighbor joining method with bootstrap values of 1000 replica runs using MEGA 6.06.

2.4 Substrate collection and processing

Matured *Delonix regia* pods were collected within the campus of the University of Ilorin, Kwara State, Nigeria. The substrate were sun dried, crushed and milled to a particle size of 1-2mm. Acid catalyzed pretreatment of the substrate was carried out using dilute sulphuric acid at 1.5% v/v, substrate loading of 15 % w/v for 15 min. The pretreated substrate was filtered and subjected to repeated washing with distilled water. The residue was air dried at room temperature for use.

2.5 Time course for laccase production

Laccase production was carried out in a basal medium described by Jaber, Shah [25] with slight modification, containing :Yeast extract, 2g; Peptone, 2g; $CUSO_4$, 2.0mM; $MnSO_4$,1mg; $MgSO_4$. $7H_2O$, 0.2g; $FeSO_4$. $7H_2O$1mg; KH_2PO_4, 1 g; ZnSO40.1g; NaH_2PO_4, 0.5g; and $CaCl_2$ 0.2g in 1L distilled water pH 5.0. Two agar plugs (5 mm diameter) from a 5 day old fungi culture was inoculated into 250 ml Erlenmeyer flask containing 50 ml of the basal medium, 2 g pretreated and untreated *Delonix regia* pod. All flasks were incubated at 30 °C, under shaking at 150 rpm for 7 days. Samples were withdrawn at 24 h interval and centrifuged (centrifuge 80-2 Maxmill medical) at 5,000 rpm for 15 min at 4°C. The supernatant was obtained and used as crude enzyme for laccase assay.

2.6 Laccase Assay

Laccase activity was carried out in a reaction mixture containing 3 ml of 100 mM acetate buffer pH 5.0, 1ml of 10 mM guaiacol and 1ml crude enzyme. Enzyme blank containing 3 ml acetate buffer, 1 ml guaiacol and I ml sterile distilled water was also maintained. The intense brown colour observed after 10 min incubation at 30 °C was due to oxidation of guiacol by the laccase enzyme. Hence, confirming the laccase activity. The absorbance was read at 470 nm on spectrophotometer (VIS spectrophotometer S32A). One unit of laccase activity (U/ml)

was defined as the amount of enzyme catalyzing the 1 µmol of the substrate per min per ml under the specified assay condition. Enzyme activity was calculated as seen in equation 1:

$$Laccase\ activity\ (U/ml) = \frac{\Delta\ in\ absorbance/min \times 4 \times Vt \times DF}{€ \times VS}$$

Where; V_t = Final volume of reaction mixture (ml)

V_S = Sample volume (ml)

€ = Extinction coefficient of guaiacol (0.6740µ/M/cm)

4 = Derived from unit of definition

DF = Dilution factor (Adiveppa and Basappa, 2015). Equation 1

2.7 Selection of significant parameters by Plackett Burman (PB)

Based on literature search, 12 variables were selected as potential factors affecting laccase production. The parameters evaluated include 7 medium components: $MgSO_4.7H_2O$, peptone, yeast extract, $CaCl_2$, $CuSO_4$, $NaHPO_4$, substrate type and 5 physiochemical parameters including: pH, incubation period, inoculum size, temperature, and substrate concentration. A set of 40 experiment [duplicates at two stages high (+) and low (-1)] were designed using the PB design of the MINITAB 17 (Minitab LLC., Pennysylvania, USA) table 1. Plackett- Burman Design is based on the first-order polynomial model shown in equation 2

$\gamma = \beta_o + \varepsilon\beta_i X_i$

Where γ is the response (laccase production U/mL),

β_o is the model intercept

β_i is the linear coefficient and

X_i is the level of the independent factor (i = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) Equation 2

Table 1: Independent factors and levels for Placket-Burman design

Factors	Symbol code	Low level	High Level
		-1	1
Temperature	A	30	40
$MgSO_4.7H_2O$(g/l)	B	0.02	0.2
Inoculum size (mm)	C	3	5
$CaCl_2$ (g/L)	D	0.02	0.2
Substrate concentration (%)	E	3	5
$CUSO_4$ (mM)	F	2	4
pH	G	4	6
$NaHPO_4$ (g/l)	H	0.05	0.5
Incubation time (h)	I	24	120
Yeast extract (g/l)	J	1.0	2.0
Substrate type	K	Treated	Untreated
Peptone (g/l)	L	1.0	2.0

2.8 Optimization of laccase production using Response Surface Methodology (RSM)

The selected most significant independent variables including temperature (A), pH (B), $MgSO_4.7H_2O$ (C) and Incubation period (D) were further optimized using central composite design. These variables were evaluated at five coded levels –α, - 1, 0, +1 and +α. The ranges of variables studied with the coded and actual levels. Table 2 shows the ranges of variables studied with the coded and actual levels. A total of 50 experimental runs were created in duplicate, with 32 cube points, 2 center points, and 16 axial points. To determine the significance of the constructed model, the data obtained from laccase production were subjected to analysis of variance (ANOVA). In equation 3, the responses were fitted using the response surface regression approach with the second order polynomial equation

$\gamma = \beta_o + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D$ (Intercept and main effects) + $\beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2$

(interactions)$\beta_{12}AB + \beta_{13}BD + \beta_{14}BC + \beta_{15} AD + \beta_{23}AC + \beta_{24}CD$ (Quadratic effect) Equation 3

Table 2: Independent factors and levels for central composite design (CCD)

Independent Variable	Symbol code	Actual values of codes				
		+α	+1	0	-1	-α
Temperature	A	45	40	35	30	25
pH	B	7	6	5	4	3
$MgSO_4.7H_2O$	C	0.29	0.2	0.11	0.02	-0.07
Incubation time (h)	D	168	120	72	24	-24

2.9 Investigation of crude enzyme for dye decolorization

Two dyes including malachite green dye and congo red dye was chosen to initially test the dye degradation potential of the fungi. To begin with, the ability to degrade dye was checked on plate assay by platting the fungi on PDA media containing 0.05 % of each of the dyes. Crude laccase was produced at optimized condition obtained from the CCD-RSM with activity at 8.04 U/ml was used for this study. The ability of the crude laccase to degrade malachite green dye (1 mg/ml) and congo red dye (0.1 %) was further investigated. The absorbance of malachite green dye and congo red dye was measured at 610 nm and 560 nm respectively. Ten millimeters (10 ml) of the different dyes were dispensed in sterile tubes and sterilized at 121° C for 15 min. After autoclaving, 1 ml crude enzyme was added and incubated at 30 °C for 5 days at 100 rpm. The change in colour was assessed by the change in the absorbance measured after every 24 h [26]. Percentage decolorization was calculated using equation 4

$$\%\ Decolorization = \frac{Initial\ absorbance - Observed\ absorbance}{Initial\ absorbance} X\ 100 \qquad \text{Equation 4}$$

2.10 Statistical analysis

The data analysis was carried out by general linear model and regression analysis using MINITAB 17 software. Values reported are means of (n=3), ± SD.

3.0 RESULTS AND DISCUSSION

3.1 Laccase screening

The result obtained from the preliminary screening of diverse laccase producing fugal strains indicated that positive fungi on PDA-guaiacol plate developed a dark brown coloration at the reverse of the plate. Laccase is a highly active enzyme that is capable of oxidizing a wide variety of substrate. In this study, guaiacol was used as substrate because it allows rapid detection of results through colour change, gives reliable results and it's less expensive. According to Wong, Cheung [27], laccase of *Lentinula edodes* expressed from *Pitchia pastoris* exhibited a very high catalytic activity when assayed with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). However, its performance does not match the activity on green applications. On the hand it was revealed that guaicol showed much better association upon decolorization of dyes in comparison to ABTS and 2,6-dimethoxyphenol Wong, Cheung [27]. Complete degradation was observed when the fungus was further screened on broth medium containing malachite green and congo red dyes. This confirms that the fungus possesses ligninolytic properties. Argumedo-Delira, Gómez-Martínez [28], reported the decolorization of congo red dye and malachite green dye by *Trichoderma viride* and *Trichoderma virens* in broth media. They reported an absorption rate of 81.82 mg/g *T. virens biomass* with malachite green. Cultivation of *Aspergillus niger* in 500 g/L dyes resulted in a 100 % decolorization of different textile dyes [29]. According to Dexilin, Gowri Manogari [30] *Aspergillus flavus* showed capacity for acid blue and acid violet dyes decolorization at 96 and 97 %, respectively. However, Asses, Ayed [31] reported a 97 % decolorization of congo red dye by *Aspergillus niger* after 6 days cultivation in submerge fermentation.

3.2 Molecular analysis and phylogeny

The phylogenetic tree constructed through the neighbor joining method suggest a connection between *Aspergillus carbonarious* and *Aspergillus ibericus*. The fungus showed a 99 % similarity with *Aspergillus carbonarius* (Figure 1). *Aspergillus carbonarious* have been previously implicated in production of invertase and β-D-fructosyltransferase[32], biodegradation of polyethylene [33] and hydrocarbons [34]. Species of *Aspergillus a*re versatile and majority of them have been documented to play a significant role in lignocellulosic degradation through secretion of extracellular enzymes. However, to the best

of our literature search, there are little or no report on laccase production by *Aspergillus carbonarious.*

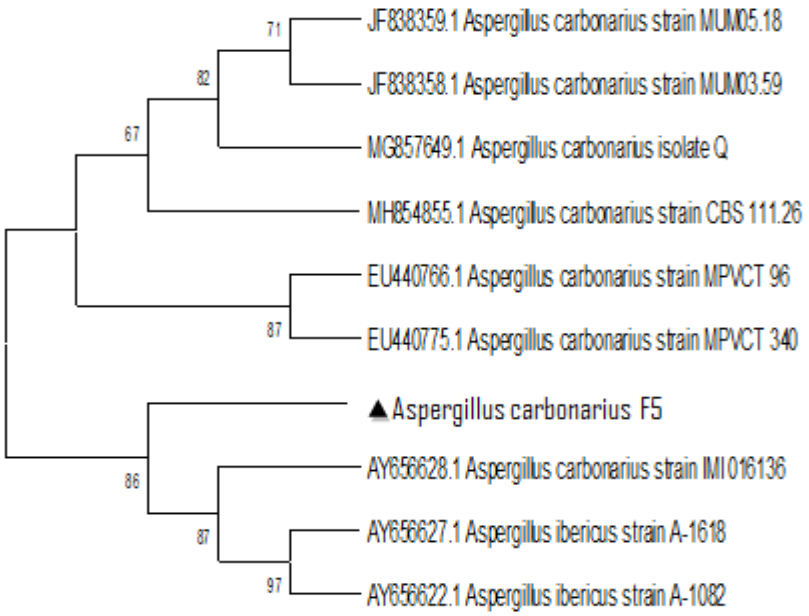

Figure 1- Phylogenetic relationship based on homology index for *Aspergillus carbonarius* F5

3.3 Fermentation and optimization

3.3.1 Pre- optimization

During this study, pretreated and untreated *Delonix regia* pods were used for laccase production in order to compare the enzyme yields. The selection of *Delonix regia* pods for laccase production was based on its high lignin content as laccase production by fungi was enhanced by the presence of lignin in the medium. Sugumaran, Susan [35], reported hemicellulose, cellulose and lignin content of *Delonix regia* pods to be 24.13%, 13.9%, 23.36 %, respectively. Interestingly, maximum laccase activity for both treated and untreated substrate were recorded at 0.97U/ml and 0.75U/ml respectively at 120 h with the treated substrate giving rise to relatively high laccase yield as compared to the untreated substrate (Figure 2). This could be due to the liberation of sugars from hemicellulose and cellulose fraction of the substrate during acid pretreatment, which makes it easily accessible by the

fungi. Interestingly, the fungus was able to degrade the untreated substrate to produce substantial amount of laccase. Also, a decrease in laccase activity for both substrates was observed at 144 to 168 h. Also, Sayyed, Bhamare [10]. reported a maximum yield in the level of laccase produced from *Aspergillus* sp. HB_RZ4 on the 8th day of fermentation

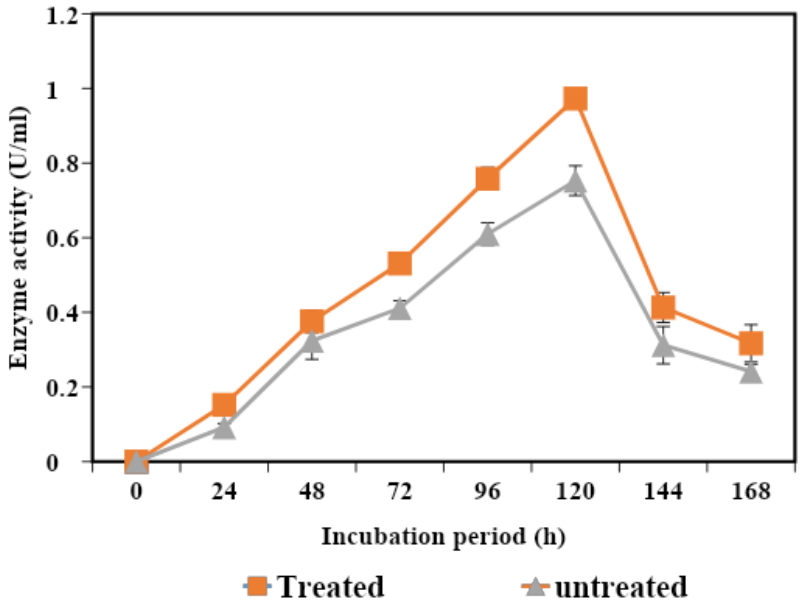

Figure 2 -Laccase production by *Aspergillus carbonarius* F5 grown on treated and untreated *Delonix regia* pods. Values are means of (n=3), ± SD. Error bars smaller than the symbol are not visible

3.3.2 Significant factors selection for F5 laccase production by PB design

The effects of medium components and physicochemical factors on laccase production by *Aspergillus carbonarius* F5 grown on treated and untreated *Delonix regia* pods carbon source were determined using PB design (Table 3). The parameters were selected based on available information reported in literature [36, 37]. It has been reported that the presence of diverse parameters such as carbon and nitrogen sources, inoculum size metal salts and substrate concentration could plays crucial role in laccase production. Also, the during this study the application of yeast extract and peptone were used in combination at different ratio as a source of nitrogen (Table 3). Maximum laccase yield was observed with run order 8. An enzyme yield of 3.14 U/mL was obtained from the treated substrate, while the untreated substrate gave rise to an enzyme yield of 3.04 U/mL (run order 5) with a combination of all the factors. Incubation period, pH, temperature and $MgSO_4.7H_2O$ have substantial effect on laccase yield as seen in (Table 4). According to Bakkiyaraj, Aravindan [36], the combination

of nitrogen sources enhances laccase production. However, Chhaya and Gupte [38], reported no significance on laccase production with the use of combination of peptone and yeast extract. Bagewadi, Mulla [37], carried out similar experiment using while screenning for medium components using the PB design, and reported yeast extract and copper sulfate as significant factors affecting laccase production. According to Mishra, Kumar [39], pH, temperature and $CuSO_4$ were found to contribute significantly to laccase production. The statistical significance model terms were studied using ANOVA. Fischer's test was used to assess the significance of the model which was compared to the corresponding p value. The ANOVA with $p<0.05$ indicate significant model terms. Incubation period, pH, temperature and $MgSO_4.7H_2O$ exert positive effect on laccasse yield. However, Yeast extract, peptone, $CaCl_2$, $CuSO_4$, $NaHPO_4$, inoculum size, substrate concentration and substrate type exhibited negative effects on laccasse yield. The coefficient of determination R^2, adjusted R^2 and predicted R^2 were 0.9085, 0.8987 and 0.8755 respectively showing correlation among the observed and predicted values. The first order linear model, as shown in equation 5, predicted the effect of the independent variables on laccase production.

$$Laccase\,(U/mL) = +\,2.373 - 0.0884\,A - 1.877\,B - 0.0781C + 1.741\,D - 0.0340\,E +$$
L Equation 5

The four significant factors were selected for further improvement in laccase production via RSM

Table 3: Plackett-Burman design matrix for screening of independent variables and the enzyme yield obtained

Run order	$MgSO_4.7H_2O$ (g/l)	Inoculum size (mm)	Substrate loading (%)	$CUSO_4$ (Mm)	pH	$NaHPO_4$ (g/l)	Incubation time (h)	Yeast extract (g/l)	Temperature (°C)	$CaCl_2$ (g/l)	Peptone (g/l)	Substrate type	Enzyme Yield (U/ml)
1	0.2	5	5	4	4	0.05	24	1	30	0.02	1	Untreated	0.96±0.007
2	0.2	3	5	4	4	0.5	120	1	40	0.02	1	Treated	1.04±0.007
3	0.2	3	3	4	6	0.5	120	1	30	0.2	2	Treated	2.98±0.140
4	0.2	5	3	4	6	0.05	120	2	40	0.02	1	Treated	1.64±0.070
5	0.02	3	3	4	4	0.5	120	2	30	0.2	1	Untreated	3.05±0.021
6	0.2	3	3	2	6	0.05	120	1	40	0.02	2	Untreated	1.35±0.040
7	0.2	5	3	2	6	0.5	24	2	40	0.2	1	Untreated	0.98±0.070
8	0.02	5	5	2	6	0.5	120	2	30	0.02	1	Treated	3.13±0.014
9	0.02	3	5	2	6	0.5	24	1	40	0.2	1	Treated	0.88±0.007
10	0.02	5	3	2	4	0.05	120	1	40	0.2	1	Untreated	1.02±0.007
11	0.02	5	5	4	6	0.5	24	1	40	0.02	2	Untreated	1.03±0.014
12	0.2	3	5	2	4	0.05	24	2	40	0.2	2	Treated	0.88±0.007
13	0.2	5	5	2	4	0.5	24	2	30	0.02	2	Treated	0.16±0.007
14	0.02	5	5	4	4	0.05	120	2	40	0.2	2	Treated	1.06±0.007
15	0.02	3	3	2	6	0.05	120	2	30	0.02	2	Untreated	2.40±0.007
16	0.2	3	3	4	6	0.05	24	2	30	0.2	1	Untreated	1.37±0.007
17	0.02	5	5	4	6	0.05	24	1	30	0.2	2	Untreated	2.33±0.007
18	0.02	3	3	4	4	0.5	24	1	40	0.02	2	Untreated	0.94±0.000
19	0.2	5	5	2	4	0.5	120	1	30	0.2	2	Untreated	2.20±0.021

20	0.02	3	3	2	4	0.05	24	1	30	0.02	1	Treated	1.08±0.007

Table 4: Analysis of variance for process optimization using Plackett-Burman design

Source	Degree of freedom	Sum of squares	Mean squares	F- value	P- value
Model	12	22.8689	1.9057	11.68	0.000
Linear	12	22.8689	1.90574	11.68	0.000
Temperature (ºC)	1	7.8075	7.80749	47.86	0.000*
$MgSO_4.7H_2O$(g/l)	1	1.1411	1.14109	6.99	0.013*
Inoculum size (mm)	1	0.2440	0.24398	1.50	0.232
$CaCl_2$ (g/l)	1	0.9816	0.98157	6.02	0.051
Substrate concentration (%)	1	0.0464	0.04638	0.28	0.598
$CUSO_4$ (mM)	1	0.5978	0.59780	3.66	0.066
pH	1	3.0504	3.05035	18.70	0.000*
$NaHPO_4$	1	0.5072	0.50715	3.11	0.089
Incubation period	1	8.4309	8.43091	51.68	0.000*
Yeast extract	1	0.0543	0.05432	0.33	0.569
Substrate type	1	0.0058	0.00581	0.04	0.852
Peptone	1	0.0020	0.0202	0.01	0.912
Error	27	4.4050	0.16315		
Pure error	20	0.0074	0.00037		
Total	39	27.2739			

F= fichers function; *(*p<0.05) = probability; R2=0.9085; R2 adjusted=0.8987; R2 predicted 0.8755

3.3 3 Optimization of F5 laccase using CCD-RSM design

Statistical optimization of the significant factors affecting laccase production was necessary because the preliminary optimization could not provide information on the interactive effect of the factors responsible for higher laccase yield. It is important to note that the interaction of the factors are responsible for regulating fungal metabolism. Therefore, optimization of the significant factors using CCD-RSM was carried out to improve laccase production by *Aspsrgillus carbonarious* F5. A set of 4 factors (based on preliminary screening) and 50 runs was experimented. The results revealed variations in laccase yield due to the four independent factors maintained for the fermentation. Optimum laccase activity (5.05 U/ml) was obtained in run order 22 at 120 h of incubation while the lowest yield (0.71 U/ml) was obtained in run order 18 at 72 h incubation (Table 5). Laccase yield was increased by 5.20-folds after optimization. Senthivelan, Kanagaraj [40], also reported an increase in

laccase yield from 6.0U/ml to 7.9U/ml by *Penicillium chrysogenum* using central composite rotatable design. All the selected variables were statistically significant at 95% confidence level showing interactive effects among the variables (Table 6). Also, second order polynomial equation (regression equation) was generated to determine the main effects, interactive effects and quadratic effects of the significant parameters (pH, temperature, incubation period and $MgSO_4$) on laccase production Eq 6 using the Minitab software,.

$$Laccase\ U/mL = 16.76 - 0.124A - 5.07B + 6.57C - 0.0979D + 0.00003A2 + 0.536B2$$

Equation 6

The model generated was fitted into the regression equation. The final statistical significance of the significant factors were performed using the analysis of variance (ANOVA) (Table 6). The significance of the model was assessed using the probability "*p"* and Fichers "*F*" test. The adjusted R^2 and Co-efficient of determination R^2 predicted the statistical accuracy of the model. The p values < 0.05 indicated the significance of the model terms. From the table A, B, C, D, A^2, C^2, BC and CD were the significant model terms. The correlation coefficient (R^2) shows the coherency of the predicted and experimental values. From this study, the R^2 at 0.9269 indicated perfect correlation between the predicted and experimental values. The R^2 value 0.9269 indicates that the model could explain up to 92.69% of the variability of the response and 7.31% could not be explained by the model. The predicted R^2 (0.8976) is in reasonable agreement with the adjusted R^2 (0.9086).

Table 5: The RSM-CCD for significant factors affecting laccase production

Run order	$MgSO_4.7H_2O$ (g/l)	Temperature (°C)	pH	Incubation period (h)	Enzyme (U/ml)
1	0.2	30	4	120	2.56 ± 0.007
2	0.11	35	5	-24	0.78 ± 0.007
3	0.2	30	6	24	1.74 ± 0.007
4	0.2	40	6	24	1.47 ± 0.140
5	0.2	40	4	120	4.14 ± 0.037
6	0.02	40	6	24	1.70 ± 0.000
7	0.11	35	5	168	4.65 ± 0.007
8	-0.07	35	5	72	1.06 ± 0.000
9	0.11	35	5	72	1.20 ± 0.070
10	0.11	35	3	72	1.58 ± 0.000
11	0.29	35	5	72	1.57 ± 0.007
12	0.02	40	4	120	4.04 ± 0.000
13	0.02	40	4	24	1.34 ± 0.007
14	0.2	40	4	24	0.90 ± 0.000
15	0.2	40	6	120	4.60 ± 0.000
16	0.2	30	6	120	2.78 ± 0.000
17	0.2	30	4	24	1.68 ± 0.000
18	0.11	45	5	72	0.71 ± 0.000
19	0.02	30	4	120	2.38 ± 0.007
20	0.02	30	6	24	2.00 ± 0.007
21	0.11	35	7	72	4.78 ± 0.007
22	0.02	30	6	120	5.04 ± 0.140
23	0.02	40	6	120	4.09 ± 0.000
24	0.02	30	4	24	1.32 ± 0.000
25	0.11	25	5	72	1.41 ± 0.037

Table 6: Analysis of variance of response surface quadratic model for laccase production

Source	Degree of freedom	Sum of squares	Mean squares	F- value	P- value
Model	14	75.1075	5.3648	17.80	0.000
Linear	4	45.4001	11.3500	37.67	0.000
A- pH	1	6.5512	6.5512	21.4	0.000*
B- Temperature	1	2.6456	2.6456	8.78	0.005*
C- Incubation period	1	35.3960	35.3960	117.47	0.000*
D- $MgSO_4.7H_2O$	1	0.8073	0.8073	2.68	0.011*
Square	4	16.1987	4.0497	13.44	0.000
A^2	1	6.4843	6.4843	21.52	0.000*
B^2	1	0.0000	0.0000	0.00	0.995
C^2-	1	4.7636	4.7636	18.81	0.000*
D^2	1	0.1092	0.1092	0.36	0.551
2- way interaction	6	13.5087	2.2514	7.47	0.000
AB	1	0.0162	0.0162	0.05	0.818
AC	1	0.0873	0.0873	0.29	0.594
AD	1	0.0004	0.0004	0.00	0.970
BC	1	11.4924	11.4924	38.14	0.000*
BD	1	0.5949	0.5949	1.97	0.169
CD	1	1.3175	1.3175	4.37	0.044*
Error	35	10.5463	0.3013		
Pure error	25	1.2521	0.0501		
Total	49	86.6537			

R2= 0.9269; adjusted R2= 0.9086; predicted R20.8976 $p< 0.05$

Laccase yield through optimization on *Delonix regia* pods in the current study, is comparable to similar optimization studies (Table 7).

Table 7: Comparison of optimal laccase production on different carbon sources

Organism	Carbon Source	Fermentation type	Optimization method	Laccase yield (U/ml)	Fold increase	References
Aspergillus flavus PUF5	Ribbed gourd Peel	Submerged	CCD- RSM	15.96	4.60	Ghosh and Ghosh, 2017
Penicillium chrysogenum	Sucrose	Submerged	CCRD- RSM	7.9	1.32	Senthivelan *et al.* 2019
Pleurotus ostreatus	Glucose	Submerged	CCD- RSM	2.70	3.13	Pratheebaa *et al.* 2013
Aspergillus sp. HB_RZ4	Glucose	Submerged	CCD- RSM	9.03	1.24	Bhamare *et al.*2018
Pycnoporus sanguineus & *Beauvenia brongniartii*	Glucose	Submerged	CCD- RSM	2.08	2.50	Jimenez-Barrera *et al.* 2018
Aspergillus carbonarius F5	*Delonix regia* pods	Submerged	CCD- RSM	5.05	1.63	Present study

3.3.4 Analysis of variable interactions

The interactive 3D plot was determined to illustrate the interaction between the factors and to determine optimum concentration of specific factor for high laccase yield. The 3D plots shows the interactions between two varying factors while keeping the third factor at midpoint. Value for $p > 0.05$ for AB, AC, AD and BD shows insignificant interactions. From the plot, the highest laccase yield at 7.5 U/ml will occur when incubation period increased to 150 h (linear relation with laccase yield) and temperature of around 36 °C. Laccase activity was found to increase with longer incubation period, temperature around 36 °C increased laccase yield, further increase in temperature led to drastic decrease in laccase yield (Fig 3a). The incubation period increased with increase in laccase yield, and maximum yield of 6 U/ml was obtained when incubation period is maximum and $MgSO_4$ is around 0.2 g/L (Fig 3b). Lower concentration of $MgSO_4$ (0.2 g/L) induced the laccase synthesis. Higher concentration of $MgSO_4$ has toxic effect on the fungal growth, hence lower laccase activity was observed. Mesophilic temperature was sufficient for the fungi growth. Higher temperature was unfavorable and thus negatively affected laccase yield. Since longer incubation period was required, additional study was carried on time profiling to confirm the optimum predictions observed in the model. Laccase activity under optimized condition (0.2 g/L MgSO4,

temperature 36 °C and incubation period at 168 h) yielded 8.04 U/ml, compared to the predicted 7.5 U/ml.

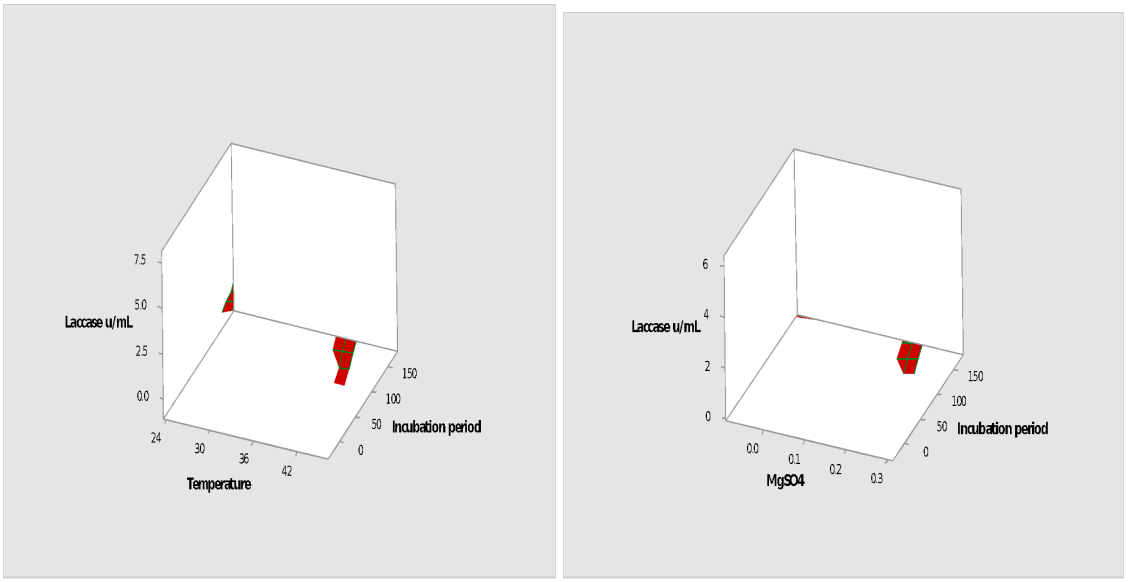

Figure 3 a-b: The 3-D response surface plots depicting interactions between independent varaibles. a temperature-incubation period and b MgSO4-incubation period affecting laccase yield (U/ml) by *Aspergillus carbonarius* F5

3.4 Dye decolorization by crude laccase

The crude laccase was evaluated for its ability to decolorize malachite green dye and congo red dye. Moreover, an increase in percentage decolorization was observed with longer incubation periods (120 h). Also, decolorization at 87.6% congo red was obtained, while 62.6 % decolorization was obtained for malachite green (Figure 5). This in agreement with the report of Wakil, Eyiolawi [26], where highest decolorization (65.84 %) of malachite green and 87.50 % decolorization of methyl red by purified laccase was achieved after 96 h. The decolorization of methyl orange by crude laccase of *Halopiger aswanensis* strain ABC_IITR was reported to be 55.2 % at 12 h of incubation [41].Tavares, Avelino [42] also reported azo dyes (RB220 and RB5) decolorization on the 6th and 10th day, respectively upon incubation with crude laccase, and obtained more than 90 % decolorization. However, Leo, Passari [43], reported decolorization of malachite green dye within the 1 h of incubation and congo red dye within 6 h with 68.61 % and 48.5 % decolorization, respectively.

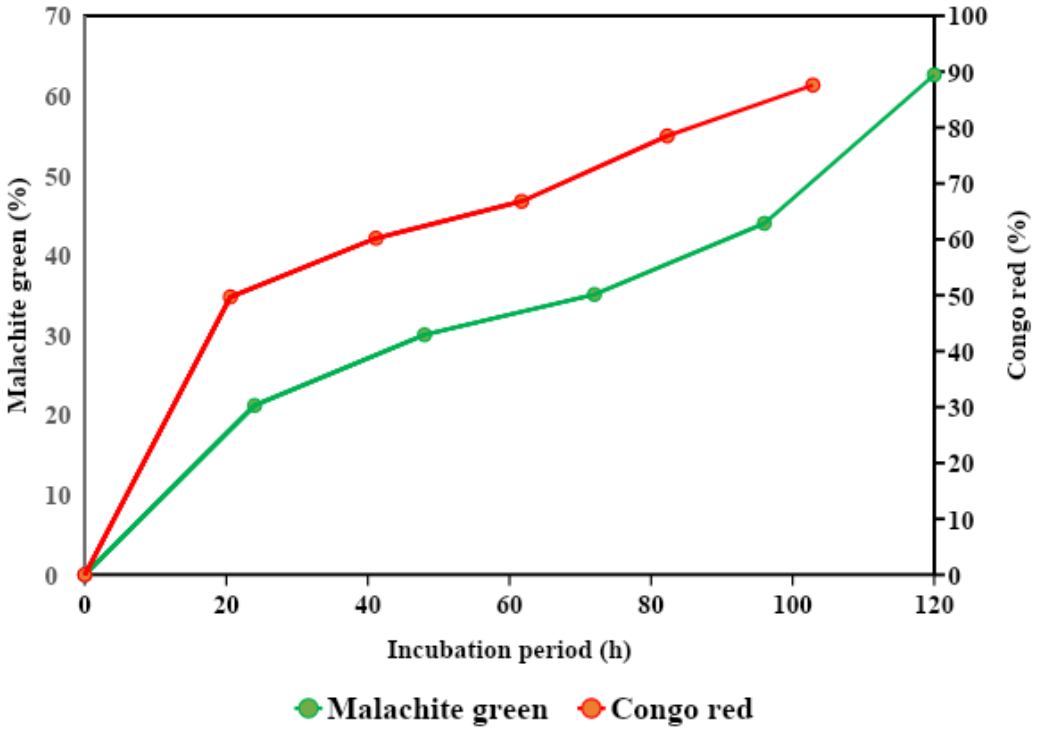

Figure 4 Decolorization of synthetic dyes by crude laccase obtained from *Aspergillus carbonarius* F5. Values are means of (n=3), ± SD. Error bars smaller than the symbol are not visible

4.0 CONCLUSION

In the current study, *Delonix regia* pods was established as a more cheaper, sustainable, effective and biodegradable ecofriendly substrate for laccase production when subjected to microbial biodegradation action of *Aspergillus carbonarius* F5 under SmF. The outcome documented from the statistical analysis using the PDB indicate temperature, pH, incubation period and $MgSO_4$ all have significant effect ($p<0.05$) on laccase production while a 8.26-fold increase in laccase yield and model accuracy was accomplished with CCD-RSM. Significant interactions were evident between incubation period-$MgSO_4$ and temperature-incubation period. Dye decolorization by the laccase was effective with higher percentage decolorization. Therefore, the study clearly demonstrates the potential of *Delonix regia* pods as carbon substrate for statistical-based optimization of laccase by F5 with

subsequent usage in dye decolorization. . Future studies will focus on purification of the enzyme for appropriate kinetic studies and characterization

Conflict of interest: Authors have declared no conflicting interest

References

[1] Casciello, C., Tonin, F., Berini, F., Fasoli, E., Marinelli, F., Pollegioni, L., et al. A valuable peroxidase activity from the novel species Nonomuraea gerenzanensis growing on alkali lignin. Biotechnology Reports. 2017, 13, 49-57.
[2] Huang, M.T., Lu, Y.C., Zhang, S., Luo, F., Yang, H. Rice (Oryza sativa) laccases involved in modification and detoxification of herbicides atrazine and isoproturon residues in plants. J. Agric. Food Chem. 2016, 64, 6397-406.
[3] Sondhi, S., Saini, K. Response surface based optimization of laccase production from Bacillus sp. MSK-01 using fruit juice waste as an effective substrate. Heliyon. 2019, 5, e01718.
[4] Nadaroglu, H., Mosber, G., Gungor, A.A., Adıguzel, G., Adiguzel, A. Biodegradation of some azo dyes from wastewater with laccase from Weissella viridescens LB37 immobilized on magnetic chitosan nanoparticles. Journal of Water Process Engineering. 2019, 31, 100866.
[5] Omeje, K.O., Nnolim, N.E., Ezema, B.O., Ozioko, J.N., Eze, S.O. Synthetic dyes decolorization potential of agroindustrial waste-derived thermo-active laccase from Aspergillus species. Biocatalysis and Agricultural Biotechnology. 2020, 29, 101800.
[6] Han, M.-L., Yang, J., Liu, Z.-Y., Wang, C.-R., Chen, S.-Y., Han, N., et al. Evaluation of Laccase Activities by Three Newly Isolated Fungal Species in Submerged Fermentation With Single or Mixed Lignocellulosic Wastes. Frontiers in Microbiology. 2021, 12.
[7] Clark, M., Tepper, K., Petroll, K., Kumar, S., Sunna, A., Maselko, M. Bioremediation of Industrial Pollutants by Insects Expressing a Fungal Laccase. ACS Synthetic Biology. 2021.
[8] Bassanini, I., Ferrandi, E.E., Riva, S., Monti, D. Biocatalysis with laccases: An updated overview. Catalysts. 2021, 11, 26.
[9] Ndochinwa, O., Amadi, O., Nwagu, T., Okpala, G., Nnamchi, C., Moneke, A. Screening of Laccase Producing Fungi Using Agro-Wastes under Different Cultural Conditions. Journal of Advances in Biology & Biotechnology. 2020, 44-57.
[10] Sayyed, R., Bhamare, H., Marraiki, N., Elgorban, A.M., Syed, A., El-Enshasy, H.A., et al. Tree bark scrape fungus: A potential source of laccase for application in bioremediation of non-textile dyes. Plos one. 2020, 15, e0229968.
[11] de Salas, F., Camarero, S. Fungal Laccases as Biocatalysts for Wide Range Applications. In: Encyclopedia of Mycology, Zaragoza, Ó., Casadevall, A. Eds., Elsevier, Oxford, 2021, pp. 233-46.
[12] Blánquez, A., Rodríguez, J., Brissos, V., Mendes, S., Martins, L.O., Ball, A.S., et al. Decolorization and detoxification of textile dyes using a versatile Streptomyces laccase-natural mediator system. Saudi Journal of Biological Sciences. 2019, 26, 913-20.
[13] Kang, Y., Xu, X., Pan, H., Tian, J., Tang, W., Liu, S. Decolorization of mordant yellow 1 using Aspergillus sp. TS-A CGMCC 12964 by biosorption and biodegradation. Bioengineered. 2018, 9, 222-32.
[14] Pandi, A., Marichetti Kuppuswami, G., Numbi Ramudu, K., Palanivel, S. A sustainable approach for degradation of leather dyes by a new fungal laccase. Journal of Cleaner Production. 2019, 211, 590-7.
[15] Yuliana, T., Komara, D., Saripudin, G., Subroto, E., Safitri, R. Potential of Lignocellulosic Waste for Laccase Production by Trametes versicolor under Submerged Fermentation. Pakistan Journal of Biological Sciences: PJBS. 2021, 24, 699-705.

[16] Melanouri, E.-M., Dedousi, M., Diamantopoulou, P. Cultivating Pleurotus ostreatus and Pleurotus eryngii mushroom strains on agro-industrial residues in solid-state fermentation. Part I: Screening for growth, endoglucanase, laccase and biomass production in the colonization phase. Carbon Resources Conversion. 2022, 5, 61-70.
[17] Pourkhanali, K., Khayati, G., Mizani, F., Raouf, F. Isolation, identification and optimization of enhanced production of laccase from Galactomyces geotrichum under solid-state fermentation. Preparative Biochemistry & Biotechnology. 2021, 51, 659-68.
[18] Fatmawaty, F., Astuti, H. Antimalarial activity of Delonix regia on mice with Plasmodium berghei. J. Nat. Prod. 2013, 6, 61-6.
[19] Babalola, B.M., Babalola, A.O., Adubiaro, H.O., Ayanda, O.S., Nelana, S.M., Naidoo, E.B. Application of waste Delonix regia pods and leaves for the sorption of Pb(II) ions from aqueous solution: kinetic and equilibrium studies. Water Quality Research Journal. 2019, 54, 278-89.
[20] Balogun, A.O., Adeleke, A.A., Ikubanni, P.P., Adegoke, S.O., Alayat, A.M., McDonald, A.G. Kinetics modeling, thermodynamics and thermal performance assessments of pyrolytic decomposition of Moringa oleifera husk and Delonix regia pod. Scientific Reports. 2021, 11, 13862.
[21] Zubbair, N.A.A., Ajao, A.T., Adeyemo, E.O., ADENIYI, O.D. Biodegradation of Malachite Green by White-Rot Fungus, Pleurotus Pulminarious. Egyptian Academic Journal of Biological Sciences, G. Microbiology. 2020, 12, 79-90.
[22] Senanayake, I., Rathnayaka, A., Marasinghe, D., Calabon, M., Gentekaki, E., Lee, H., et al. Morphological approaches in studying fungi: collection, examination, isolation, sporulation and preservation. Mycosphere. 2020, 11, 2678-754.
[23] Girma, G., Rabbi, I., Olanrewaju, A., Alaba, O., Kulakow, P., Alabi, T., et al. A manual for large-scale sample collection, preservation, tracking, DNA extraction, and variety identification analysis. A manual for large-scale sample collection, preservation, tracking, DNA extraction, and variety identification analysis. 2017.
[24] White, T.J., Bruns, T., Lee, S., Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications. 1990, 18, 315-22.
[25] Jaber, S.M., Shah, U.K.M., Asa'ari, A.Z.M., Ariff, A.B. Optimization of laccase production by locally isolated Trichoderma muroiana IS1037 using rubber wood dust as substrate. BioResources. 2017, 12, 3834-49.
[26] Wakil, S.M., Eyiolawi, S.A., Salawu, K.O., Onilude, A.A. Decolourization of synthetic dyes by laccase enzyme produced by Kluyveromyces dobzhanskii DW1 and Pichia manshurica DW2. African Journal of Biotechnology. 2019, 18, 1-11.
[27] Wong, K.-S., Cheung, M.-K., Au, C.-H., Kwan, H.-S. A novel Lentinula edodes laccase and its comparative enzymology suggest guaiacol-based laccase engineering for bioremediation. PLoS One. 2013, 8, e66426.
[28] Argumedo-Delira, R., Gómez-Martínez, M.J., Uribe-Kaffure, R. Trichoderma biomass as an alternative for removal of congo red and malachite green industrial dyes. Applied Sciences. 2021, 11, 448.
[29] Omar, S.A. Decolorization of different textile dyes by isolated Aspergillus niger. Journal of Environmental Science and Technology. 2016, 9, 149.
[30] Dexilin, M., Gowri Manogari, B., Kaliannan, T. Optimization of Culture Conditions for Enhanced Decolorization of Azo Dyes by Aspergillus flavus Isolated from Dye Contaminated Soil. In: Bioremediation and Green Technologies, Springer, 2021, pp. 143-54.
[31] Asses, N., Ayed, L., Hkiri, N., Hamdi, M. Congo Red Decolorization and Detoxification by Aspergillus niger: Removal Mechanisms and Dye Degradation Pathway. BioMed Research International. 2018, 2018, 3049686.
[32] Nascimento, G.C.d., Batista, R.D., Santos, C.C.A.d.A., Silva, E.M.d., de Paula, F.C., Mendes, D.B., et al. β-fructofuranosidase and β–D-fructosyltransferase from new Aspergillus carbonarius PC-4 strain isolated from canned peach syrup: effect of carbon and nitrogen sources on enzyme production. The Scientific World Journal. 2019, 2019.

[33] El-Sayed, M.T., Rabie, G.H., Hamed, E.A. Biodegradation of low-density polyethylene (LDPE) using the mixed culture of Aspergillus carbonarius and A. fumigates. Environ. Dev. Sustainability. 2021, 23, 14556-84.
[34] Sinha, M., Sørensen, A., Ahamed, A., Ahring, B.K. Production of hydrocarbons by Aspergillus carbonarius ITEM 5010. Fungal biology. 2015, 119, 274-82.
[35] Sugumaran, P., Susan, V.P., Ravichandran, P., Seshadri, S. Production and characterization of activated carbon from banana empty fruit bunch and Delonix regia fruit pod. Journal of Sustainable Energy & Environment. 2012, 3, 125-32.
[36] Bakkiyaraj, S., Aravindan, R., Arrivukkarasan, S., Viruthagiri, T. Lemon peelings: a potential substrate for laccase production by Trametes versicolor MTCC 138 in submerged fermentation. International Journal of Current Chemical Sciences. 2013, 2, 17-24.
[37] Bagewadi, Z.K., Mulla, S.I., Ninnekar, H.Z. Optimization of laccase production and its application in delignification of biomass. International Journal of Recycling of Organic Waste in Agriculture. 2017, 6, 351-65.
[38] Chhaya, U., Gupte, A. Optimization of media components for laccase production by litter dwelling fungal isolate Fusarium incarnatum LD-3. J. Basic Microbiol. 2010, 50, 43-51.
[39] Mishra, A., Kumar, S., Kumar, S. Application of Box-Benhken experimental design for optimization of laccase production by Coriolus versicolor MTCC138 in solid-state fermentation. 2008.
[40] Senthivelan, T., Kanagaraj, J., Panda, R.C., Narayani, T. Screening and production of a potential extracellular fungal laccase from Penicillium chrysogenum: Media optimization by response surface methodology (RSM) and central composite rotatable design (CCRD). Biotechnology Reports. 2019, 23, e00344.
[41] Chauhan, A.K., Choudhury, B. Synthetic dyes degradation using lignolytic enzymes produced from Halopiger aswanensis strain ABC_IITR by Solid State Fermentation. Chemosphere. 2021, 273, 129671.
[42] Tavares, M.F., Avelino, K.V., Araújo, N.L., Marim, R.A., Linde, G.A., Colauto, N.B., et al. Decolorization of azo and anthraquinone dyes by crude laccase produced by Lentinus crinitus in solid state cultivation. Braz. J. Microbiol. 2020, 51, 99-106.
[43] Leo, V.V., Passari, A.K., Muniraj, I.K., Uthandi, S., Hashem, A., Abd_Allah, E.F., et al. Elevated levels of laccase synthesis by Pleurotus pulmonarius BPSM10 and its potential as a dye decolorizing agent. Saudi journal of biological sciences. 2019, 26, 464-8.