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Statistical Optimization and Partial Purification of Laccase from A Novel Fungal Strain *Peyronellaea pinodella* BL-3/4

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ABSTRACT

Peyronellaea pinodella BL-3/4, an ascomycete was isolated from the humus of municipal solid waste. The novelty regarding the present study is that, to date the isolated fungal strain has not been explored for laccase production and statistical optimization of medium parameters for enhanced laccase production. Efficient laccase production from this fungal strain was carried out by optimizing fermentation medium using the design of experiments through submerged fermentation. Initially, the medium components were screened using Plackett Burman design. A five-level-four factor central composite design was applied to statistically specify the effect of important process variables, namely glucose, orange peelings, peptone and copper sulphate. The significance of the factors and their interactions were verified by using the analysis of variance with 95% confidence level ($p<0.05$). Among the variable screened, orange peelings, glucose, peptone and copper sulfate were found significant in laccase production. The central composite design of response surface methodology revealed that the best combination of fermentation medium for maximum laccase production is 2% glucose, 1% orange peelings, 0.5% peptone and 0.001 mg% copper sulphate with maximum laccase production of151.5 U/mL. Statistical optimization leads to 2 fold higher laccase production than the unoptimized media in the present study. Purification by ammonium sulphate precipitation followed by dialysis and gel filtration chromatography leads to 17.5 fold purification with 14.1% yield of pure laccase. Purified enzyme was identified as 60 kDa monomeric protein by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The production of laccase by *P.pinodella* BL-3/4 was also confirmed by the evaluating presence of copper in the purified fraction. Presence of copper in structure of purified laccase was confirmed by UV-visible spectroscopy, atomic absorption spectroscopy and scanning electron microscopy coupled with energy dispersive X-ray analysis. Use of orange peelings as valuable substrate by *P. pinodella*, make the fungi a better candidate for large scale production of laccase as well as for bioremediation, when compared to all other reported fungi.

KEY WORDS: CENTRAL COMPOSITE DESIGN, LACCASE, PEYRONELLAEA PINODELLA, PLACKETT BURMAN DESIGN, STATISTICAL OPTIMIZATION.

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INTRODUCTION

The major structural component of all plant is a renewable organic material, lignocelluloses (Dashtban et al., 2009). Many industries like forestry, pulp and paper, agriculture, and food generates lignocellulosic waste during processing. Such wastes are also present in municipal solid waste (MSW), and animal wastes (Kim and Dale, 2004, Kalogo et al., 2007, Batista Meneses et al., 2020). Among all three different components of lignocelluloses, lignin is a natural heterogeneous biopolymer and highly recalcitrant in nature (Wong 2009, Anwar et al., 2014, Brenelli et al., 2018, Polo et al., 2020).

Due to complexity in structure, enzymes of most microorganisms are not able to degrade lignin. Ligninolytic enzymes are the group of enzymes that degrade lignin efficiently. Laccase (EC 1.10.3.2, para-diphenol: oxygen oxidoreductase) is one of the most important enzyme among group of ligninolytic enzymes. Having diversity in substrate specificity as well as catalytic active site of copper atom (Pointek et al., 2002), laccases non specifically catalyze oxidation of wide range of phenolic compounds, aromatic amines as well as non phenolic compounds with the four-electron reduction of molecular oxygen to water (Vishwanath et al., 2014, Jaber et al., 2017, Agrawal et al., 2018, Janusz et al., 2020).

The non specific catalytic ability makes laccase highly suitable biocatalysts for various Biotechnological applications. Such application includes effluents treatment and waste detoxification, food industry, paper and pulp industry, textile industry, synthetic chemistry, cosmetics, soil bioremediation, pesticide or insecticide degradation, organic synthesis, biosensor and analytical applications. Fungal laccases also play an important role in spore formation, pigment production, fruiting body formation, and plant pathogenesis (Sadhasivam et al., 2008). Laccase was first extracted and described by Yoshida (1883) from the sap of the Japanese lacquer tree, *Rhus vernicifera*. Laccases mostly been isolated and described from the white rot fungi, including *Trametes versicolor, Agaricus bisporus, Coriolus spp., Pleurotus ostreatus, Phlebia radiata, Pycnoporus cinnabarinus and Coprinus cinereus*. and few from the ascomycete group. Basidiomycetes are known laccase producers under both sub merged fermentation (SmF) (El-Batal et al., 2015) as well as solid state fermentation (SSF) (Patel and Gupte, 2016). Though SSF is preferred over SmF in terms of higher production yield, robust control of physical process parameters is difficult thus imposing problem in product recovery and scale up of laccase production.

Scale up needs right choice of the nutritive substrate in the culture medium that significantly decreases the total production costs and reduces the time period for expression of enzyme. Carbon, nitrogen and copper sources are the main nutritional parameters that regulate the level of gene transcription for laccase expression. Strain improvement to obtain higher laccase yield by single parameter approach is simple but laborious and time consuming and often do not tell about interaction effects between the medium parameters. Statistical optimization by design of experiments (DOE) concepts is the only solution to search such key factors and study interaction between medium parameters in a very few experiments. Plackett-Burman design (PBD) [Plackett and Burman, 1946] is most widely used experimental design for initial screening of such significant factors from multiple nutritional parameters and optimizes only the positive and main effects on laccase production. The important and positive factors obtained from the screening experiments could be further optimized by employing response surface methodology (RSM) that enables the study of interaction effects between different variables.

Few reports are available on the statistical optimization of media components for the production of laccase in different fungal strain of division ascomycota i.e. *Trichoderma harzianum* strain (Gao et al, 2013, Bagewadi et al., 2017), *Aspergillus flavus* (Ghosh and Ghosh, 2017). Moreover, laccase production during dye degradation has only been reported from *Peyronellaea prosopidis* (Bankole et al., 2018). To the best of our knowledge there are no reports on laccase production and use of statistical approach for its optimization from novel fungal strain *Peyronellaea pinodella* BL-3/4. The main objective of the study is to statistically optimize laccase production by fungal strain *Peyronellaea pinodella* BL-3/4 using DOE concept.

MATERIAL AND METHODS

A newly isolated fungal strain *Peyronellaea pinodella* BL-3/4 (Gene bank Accession Number: KT833620) prescreened (using lignin model compounds) from the humus soil of composted MSW was used in this study. This genus of ascomycetes has not been explored for any enzyme production specifically laccase production. The fungal strain was grown and maintained on Mineral salt- glucose peptone (MS-GP) medium according to the method of Patel and Bhaskaran, (2020). Ground orange peelings were employed as support-substrates as it is the best source for improving laccase productivity during single parameter optimization studies performed with *Peyronellaea pinodella* BL-3/4. The enzyme production was performed in MS-GP medium supplemented with 0.5% orange peelings (Patel and Bhaskaran, 2020). The enzyme was extracted by filtering fermentation broth and the filtrate was used as the crude enzyme preparation. Extracellular laccase activity of crude enzyme preparation was determined spectrophotometrically with 2.5 mM 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (SIGMA) by method of Silva et al. (2007) and Patel and Bhaskaran, (2020).

Screening of factors by PBD is commonly employed to select significant factors in a production medium with lesser experimentations (Rajendran et al., 2007). For laccase optimization by Peyronellaea pinodella BL-3/4, factors considered for screening by PBD were

orange peelings (A), glucose (B), peptone (C), ammonium acetate (D), KH2PO4 (E), MgSO (F), CaCL2 (G) MnSO4(H) and CuSO4 (J). Selected factors were experimentally screened with 12 trials in triplicates at 2 stages, high (+1) and low (- 1) (Table 1). The laccase activity is mean of 3 independent experiments. PBD is based on the first-order polynomial model shown in equation 1.

$$Y = \beta 0 + \Sigma\, \beta i Xi \qquad (1)$$

Where Y is the response (laccase production U/mL), β0 is the model intercept, βi is the linear coefficient, and Xi is the level of the independent factor (i = A, B, C, D, E, F, G, H, J).

The significant factors were identified by the analysis of the PBD experiments and their levels were further optimized by Central Composite Design under RSM. Each selected factor was studied at five different levels coded as $-\alpha$, -1, 0, $+1$, and $+\alpha$ in a total of ($\alpha = (2^4)^{1/4} = 2.000$) 30 runs, with two blocks (Bhamare et al., 2018). The laccase yield U/mL as the measured response (Y) was fitted by second-order polynomial equation 2.

$$Y = \beta o + \beta 1A + \beta 2B + \beta 3C + \beta 4D$$

$$+\beta 11A2 + \beta 22B2 + \beta 33C2 + \beta 44D2$$

$$+\beta 12AB + \beta 13AC + \beta 14AD + \beta 23BC + \beta 24BD + \beta 34CD \qquad (2)$$

Where, Y is the measured response (laccase production U/mL), A, B, C and D are independent factors, β1, β2, β3, β4, are linear coefficients, β 11, β 22, β 33, β 44 are quadratic coefficients and β 12, β 13, β 14, β 23, β 24, β 34 are cross product coefficients of the model.

This design was used to evaluate the main effects, interaction effects and quadratic effects to optimize the levels of parameters for enhancing laccase production. The fitted polynomial equation was expressed as three-dimensional response surface plots and counter plots to find the concentration of each factor for maximum laccase production (Sondhi and Saini, 2019). The statistical significance of the model terms was studied using analysis of variance (ANOVA). The significance of the model was assessed using Fisher's 'F' test and its corresponding probability 'p'. Design-Expert Design-Expert version 10.0.6.0 software Version 10.0.6.0, Stat-Ease, Minneapolis, USA. was used as a tool to design experiments of statistical optimization and all statistical analysis.

Purification of laccase was carried out by growing the fungal culture in statistically optimized medium. The fermentation broth was centrifuged at 3000 x g for 10 min at 4^0C for crude laccase preparation. Obtained supernatant was precipitated by ammonium sulphate in the range of 0-70% (w/v) at low temperature. Precipitated protein was dialyzed overnight with 0.1 M sodium acetate buffer using dialysis membrane of 10 kDa (Hi-Media Laboratories, India). Total protein content (method of Lowry et al., 1951) and laccase activity of the precipitated samples and dialyzed samples were determined according to method mention in laccase enzyme assay.

Concentrated dialyzed protein sample (1.5 mL) was applied to sephacryl s-100 HR (Amersham biosciences, USA) column (1.8x30 cm) pre-equilibrated with sodium phosphate buffer (pH 6.0). Protein was eluted with the same buffer having 0.15 M NaCl at a flow rate of 0.4 ml/min. A total of 30 fractions were collected and assayed for protein content and laccase activity. The purity of the laccase enzyme was confirmed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE) according to the method of Laemmli (1970). Prestained protein molecular weight marker (Genei, Bangalore, India) [Phosphorylase b (97.4 kD_a), bovine serum albumin (66 kDa), ovalbumin (43 kD_a), carbonic anhydrase (29 kDa), lacto globulin (18.4 kD_a)] was loaded along with crude, dialyzed and gel filtered protein samples to know the approximate molecular mass of laccase enzyme.

Protein bands on SDS-PAGE gels were stained with coomassie brilliant blue G-250 and compared with standard protein. The presence of Cu^{2+}, Zn^{2+}, Fe^{2+} and Mn^{2+} in purified laccase were determined and quantified by Atomic absorption spectroscopy (AAS) (SL 194; ELICO, India). Spectroscopic characterization (Schimadzu UV 1800) of purified laccase was performed to confirm type of Cu centers. The presence of Cu2+ in purified laccase was confirmed by scanning electron microscopy coupled with energy dispersive X-ray (SEM/EDAX) analysis (Model: ESEM EDAX XL-30; Philips, Netherlands).

RESULTS AND DISCUSSION

The design matrix generated by design expert statistical software for the screening of variables and corresponding responses in terms of laccase enzyme yield is shown in Table 1. Highest laccase production (132.77 U/mL) was observed in a 9th run with a high level of glucose. Variation in laccase production among the different combinations occurred due to the influence of the factors at high and low levels as shown in Table 1. Parameters with statistically significant effects were identified using Fisher's test for ANOVA. ANOVA for laccase production indicated 'F-value' of 95.33, which implied that the model was appropriate. Model terms having 'Prob>F' values less than 0.05 are considered to be significant and Prob>F' values greater than 0.1 indicates the insignificant model terms (Niladevi et al., 2006, Sondhi and Saini, 2019).

Factors having a confidence level greater than 95% were considered to have a significant effect on the response and were selected for further studies. In present study glucose was found to be the most influencing factor ($p<0.0008$), followed by CuSO4 ($p<0.0013$), orange peelings ($p<0.0018$) and peptone ($p<0.0065$) in to the medium (Table 2). Positive effect of glucose and copper on laccase production has been reported recently

(Karp et al., 2015, Ghosh and Ghosh, 2017, Bhamare et al., 2018). Although ammonium acetate and $MnSO_4$ were significant model terms, they exerted a negative effect on laccase response. According to model KH_2PO_4, $MgSO_4$ and CaCl2 had no significant effect on laccase production as shown in Figure 1.

The first-order model was fitted to the experimental results with the following final equation 3 in terms of coded factors:

Laccase activity = +65.91 +9.97* A +13.00* B +6.35* C -6.42* D - 3.37* H +11.05 * J ..... (3)

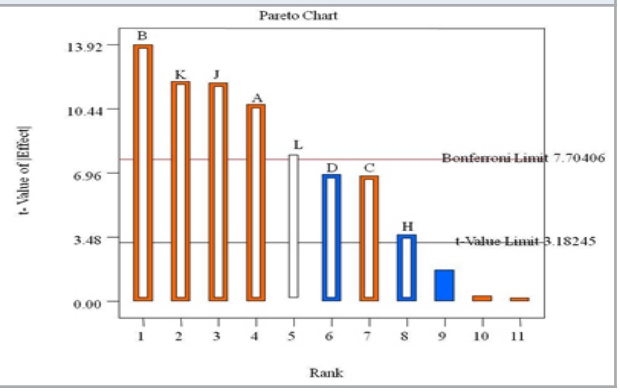
Where A, B, C, D, H and J are coded value of orange peelings, glucose, peptone, ammonium acetate, $MnSO_4$ and $CuSO_4$ respectively.

The positive effects of the four factors namely glucose, orange peelings, peptone and $CuSO_4$ on laccase production were studied using CCD of RSM to optimize their levels for maximum enzyme yield. The levels of other factors kept constant during experiments. Experimental study based on a CCD experimental design was performed according to Table 3 and 4. These factors and their levels were chosen based on the preliminary experiments. The 30 experimental trials reveal the different combinations of the factors. Maximum laccase response (125.0 U/mL) was obtained in run 30 having a maximum concentration of glucose (2.9%). Fisher's F test for the analysis of variance of data indicated that the model was highly significant with Prob>F' value of less than 0.0001 and F-value of 17.14 (Table 5). A not significant lack of fit showed that the quadratic model was valid for the present study. Among all factors and interactions considered in the experimental design, A, B, D^2 and AD were statistically significant at 95% confidence level. The value of R^2 (0.8903) indicated a good agreement between the experimental and predicted values of laccase yield.

Table 1. PBD matrix of nine variables (A-H and J) and two dummy variables (K and L) along with observed response.

	Factor A	Factor B	Factor C	Factor D	Factor E	Factor F	Factor G	Factor H	Factor J	Factor K	Factor L	Response
Run	Orange Peel (gm%)	Glucose (gm%)	Peptone (gm%)	Ammonium Acetate (gm%)	KH_2PO_4 (gm%)	$MgSO_4$ (mg%)	$CaCl_2$ (mg%)	$MnSO_4$ (mg%)	$CuSO_4$ (mg%)	Dummy	Dummy	Laccase activity
1	0.2	1	0.5	0.1	0.05	0.5	0.5	0.2	0.25	1	1	59.16
2	0.2	0.5	0.5	0.05	0.1	1	0.5	0.2	0.5	1	-1	84.16
3	0.2	0.5	0.2	0.05	0.05	0.5	0.5	0.1	0.25	-1	-1	32.91
4	0.5	1	0.2	0.05	0.05	1	0.5	0.2	0.5	-1	1	79.58
5	0.5	0.5	0.2	0.05	0.1	0.5	1	0.2	0.25	1	1	50.41
6	0.5	0.5	0.5	0.1	0.05	1	1	0.2	0.25	-1	-1	43.33
7	0.2	0.5	0.2	0.1	0.05	1	1	0.1	0.5	1	1	46.66
8	0.5	1	0.2	0.1	0.1	1	0.5	0.1	0.25	1	-1	89.16
9	0.5	1	0.5	0.05	0.05	0.5	1	0.1	0.5	1	-1	132.77
10	0.2	1	0.2	0.1	0.1	0.5	1	0.2	0.5	-1	-1	58.6
11	0.5	0.5	0.5	0.1	0.1	0.5	0.5	0.1	0.5	-1	1	59.99
12	0.2	1	0.5	0.05	0.1	1	1	0.1	0.25	-1	1	54.16

Figure 1: Positive and negative effect of different factors on laccase production by *Peyronellaea pinodella* BL-3/4 as screened with a PBD.



To evaluate the main effects, interaction effects and quadratic effects of the selected factors on the laccase yield, second-order polynomial equation was derived equation 4:

Y= 58.8112 + 15.7583 A + 19.825 B + 1.39917 C + -4.15833 D + 2.60875 AB + -11.4312 AD + -0.18 BD + -0.9975 CD + -10.1603 D^2

Where Y is the predicted response and A, B, C, D are coded factors.

The equation can be used to make predictions about the response for given levels of each factor. A positive linear coefficient value for A and B indicates laccase production was increased with increased concentrations of orange peelings (up to 1%) and glucose (up to 2.9%)

as shown in Table 3 and 4. The response obtained at different level of orange peelings clearly indicate that growth and enzyme yield was high at high concentration of orange peelings (1%) and very low when orange peelings concentration was low (0.1%) or absent in the medium. However results of present study disagree with report of the Ire and Ahuekwe (2016) on use of 0.1% orange peelings for maximum laccase production by *Pleurotus ostreatus*. Moreover, laccase activity was high at high level of glucose (2.9%) and low when glucose concentration was low (0.2%) Ghosh and Ghosh (2017) studies have revealed that although glucose supports the highest specific growth rate, specific rate of laccase production was significantly reduced. This is not supported by the findings of the present study where maximum laccase activity was obtained at 2.9% glucose with good mycelial growth. Bhamare et al. (2018) reported less laccase production even at at the 9th day of incubation. More yield in less incubation period (4 days) indicated the metabolic potential of fungus and its suitability for cost efective and economized production of enzyme at industrial scale.

The interactive effect of various factors on laccase production by *Peyronellaea pinodella* BL-3/4 was investigated by plotting the contour plots and three-dimensional response surface curves against any two independent variables while keeping the third independent variable at the '0' level. Studying the interaction among two variables provides knowledge of the optimum concentration of individual factor for highest laccase yield. The interactive effect of response surface quadratic model reveals that interaction among factors AB, BD and CD are insignificant whereas AD is significant (Table 5). The response surface curve for interactive effect of AD is shown in Figure 2. The yield was found to be increasing with the increase in orange peelings (A) concentration with limited level of peptone (D). But increasing the concentration of peptone inhibits laccase production even in the presence of higher concentration of orange peelings.

Table 2.ANOVA table for selected factorial model in PBD.

Source	F Value	p-value Prob > F	
Model	95.33	0.0016	Significant
A-Orange Peel	113.89	0.0018	
B-Glucose	193.72	0.0008	
C-Peptone	46.30	0.0065	
D-Ammonium Acetate	47.32	0.0063	
H-$MnSO_4$	13.00	0.0366	
J-$CuSO_4$	140.08	0.0013	
K-Dummy 1	142.46	0.0013	
L-Dummy 2	65.90	0.0039	

The model R^2 Value: 0.9961, The Predicted R^2 Value: 0.9373 and the adjusted R2 value: 0.9856; Coefficient of Variance (CV): 4.91

Table 3. Central composite experiments design matrix (Block 1) for laccase production from *Peyronellaea pinodella* BL-3/4.

Block	Factor A Run	Factor B Orange Peel gm%	Factor C Glucose gm%	Factor D $CuSO_4$ mg%	Peptone gm%	Actual Response Laccase activity U/ml
Block 1	1	0.55	1.1	0.5625	0.55	52
Block 1	2	1	2	1	1	56
Block 1	3	1	2	0.125	1	51.24
Block 1	4	0.1	0.2	1	0.1	1.33
Block 1	5	0.1	2	0.125	0.1	27.49
Block 1	6	0.55	1.1	0.5625	0.55	50.83
Block 1	7	1	0.2	0.125	1	14.16
Block 1	8	1	0.2	1	0.1	45
Block 1	9	0.1	0.2	1	1	11.8
Block 1	10	0.1	2	1	0.1	35
Block 1	11	0.55	1.1	0.5625	0.55	53.74
Block 1	12	0.1	2	1	1	45
Block 1	13	1	0.2	1	1	17.49
Block 1	14	0.1	0.2	0.125	1	9
Block 1	15	0.1	2	0.125	1	45.41
Block 1	16	1	2	1	0.1	99
Block 1	17	0.1	0.2	0.125	0.1	8
Block 1	18	1	2	0.125	0.1	86.66
Block 1	19	0.55	1.1	0.5625	0.55	43.33
Block 1	20	1	0.2	0.125	0.1	48.74

This is in accordance with Hammel (1997), who confirmed that the ligninolytic enzymes are produced during the secondary metabolism under conditions of limited nitrogen. Optimum concentration of each factor was revealed by performing confirmation run in triplicates. Actual mean laccase activity of 151.5 U/mL was obtained with optimum concentration of orange peelings-1.0%, Glucose-2.0%, Peptone-0.5% and CuSO4-1.0 mg% against predicted laccase activity (139.0 U/mL) by design expert software. Extracellular laccase produced by Peyronellaea pinodella BL-3/4 was purified by ammonium sulphate precipitation followed by dialysis and gel filtration chromatography. Laccase purification at different steps is summarized in Table 5. Enzyme was purified to 6.01 fold with 85.1% yield after dialysis. Final purification with sephacryl s-100 HR gel filtration chromatography leads to 17.5 fold purification with 14.1% yield of pure laccase.

Table 4. Central composite experiments design matrix (Block 2) for laccase production from *Peyronellaea pinodella* BL-3/4.

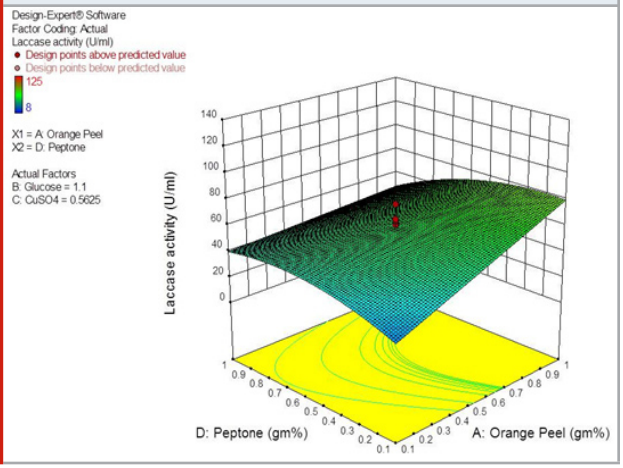
Block	Run	Factor A Orange Peel gm%	Factor B Glucose gm%	Factor C $CuSO_4$ mg%	Factor D Peptone gm%	Actual Response Laccase activity U/ml
Block 2	21	0.55	1.1	0.5625	0.55	76.66
Block 2	22	1.45	1.1	0.5625	0.55	99.16
Block 2	23	0.55	1.1	0.5625	-0.35	35.83
Block 2	24	-0.35	1.1	0.5625	0.55	39.19
Block 2	25	0.55	1.1	1.4375	0.55	64.16
Block 2	26	0.55	-0.7	0.5625	0.55	23.74
Block 2	27	0.55	1.1	-0.3125	0.55	75.83
Block 2	28	0.55	1.1	0.5625	0.55	64.99
Block 2	29	0.55	1.1	0.5625	1.45	24.99
Block 2	30	0.55	2.9	0.5625	0.55	125

Table 5: Analysis of variance for response surface quadratic model

Source	F Value	p-value Prob > F	
Model	17.14	< 0.0001	significant
A-Orange Peel	43.68	< 0.0001*	
B-Glucose	69.13	< 0.0001*	
C-CuSO4	0.34	0.5643a	
D-Peptone	3.04	0.0973a	
AB	0.80	0.3829a	
AD	15.32	0.0009*	
BD	3.799E-003	0.9515a	
CD	0.12	0.7364a	
D2	21.79	0.0002*	
Lack of Fit	0.83	0.6517	not significant

R^2 = 0.8903; adjusted R2 = 0. 8384; Predicted R^2=0.7630; probability P *(P<0.05) corresponds to Significance; P^a corresponds to insignificance

Figure 2: Response surface curve showing the interactive effect of orange peel (A) and peptone (D) on laccase production.



AAS studies showed presence of copper in laccase active fraction (6.8 mg/mL) where as iron, zinc and manganese were absent. The type of copper catalytic centre was investigated spectrophotometrically by UV-Visible spectrum. The UV-Visible spectrum (Figure 4) shows presence of a shoulder at 330 nm. Shoulder at 330 nm indicates type III binuclear copper (Solomon et al., 1994, Solomon et al., 1996) having two electron accepting site, which is characteristic to the yellow laccases. Absorption peak around 600 nm confirms presence of type 1 copper, which is characteristic of blue laccases (Bertrand et al., 2002, Morozova et al., 2007, Madhavi and Lele 2009). Type III Cu exhibits a weak absorption at 600 nm (Palmieri et al., 1997). In present study, absence of peak around 600 nm (Figure 4) conferred absence of type 1 copper in purified laccase and

presence of type III copper in purified laccase. Evidence was also provided for instability of type I copper in all fungal laccases (Rogalski and leonowicz, 2004). Most of the laccases are blue containing four copper atoms per enzyme molecule. Reports of Giardina et al. (2009) suggests that formation of yellow laccase is due to altered oxidation state of active copper centre during binding of the lignin degradation aromatic products which in turn results in the reduction of type 1 copper and loss of the characteristic blue copper of laccase. SEM-EDAX analysis was performed to confirm presence of copper. Figure 5 shows scanning electron microscopic image of purified laccase. Peak at 8 KeV in SEM-EDAX spectrum confirmed presence of copper in structure of laccase (Figure 6).

Table 6. Purification of laccase from *Peyronellaea pinodella* BL-3/4.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude filtrate	13500	633	21.3	1	100
Ammonium sulfate precipitation and Dialysis	11491	89.7	128.1	6.01	85.1
Gel filtration chromatography	1900	0.85	2235.3	17.5	14.1

Figure 3: SDS-PAGE of purified laccase from *Peyronellaea pinodella* BL-3/4. Lane 1: Standard Protein molecular weight marker, Lane 2: Crude Laccase, Lane 3: Dialyzed Laccase and Lane 4: Purified Laccase

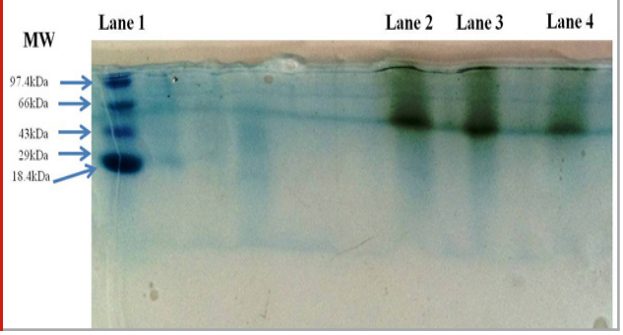


Figure 5: Scanning electron microscopy of purified laccase from *Peyronellaea pinodella* BL-3/4.

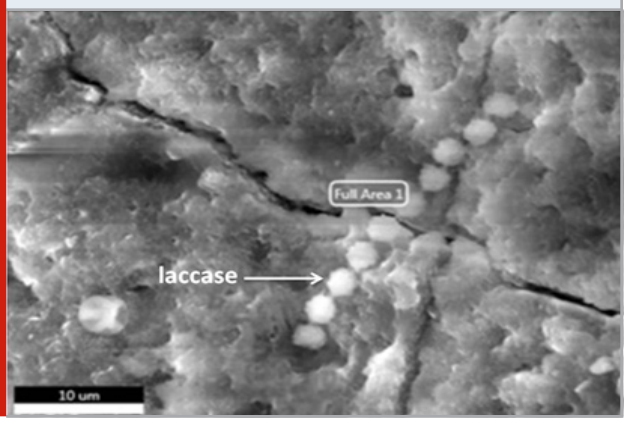


Figure 4: UV-Visible spectrum of purified laccase

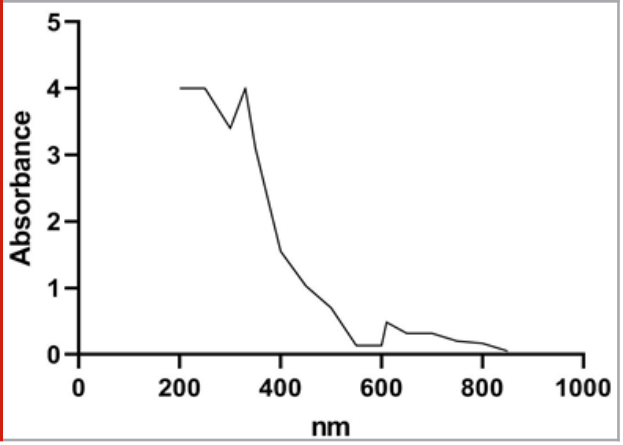
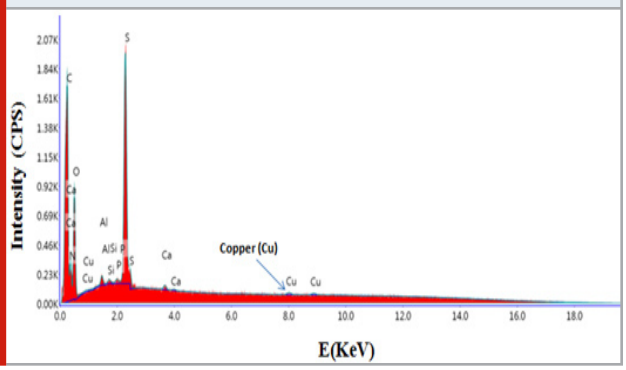


Figure 6: Energy dispersive X-ray (SEM/EDAX) spectrum showing presence of copper in purified laccase from *Peyronellaea pinodella* BL-3/4



CONCLUSION

The present study has explored the potential of Peyronellaea pinodella BL-3/4, a newly isolated ascomycetes to produce laccase under optimal medium components designed by statistical software through submerged fermentation. Statistical optimization has provided best combinations of medium components while considering interaction between medium components

studied. The usage of design expert software reduces the resources required and also saved time. Optimization leads to two fold increases in laccase production compared to control using orange peelings (1%) as a lignocellulosic substrate. Laccase yield of 14.1% was achieved in final purification with sephacryl s-100 HR gel filtration chromatography. The production of laccase by *Peyronellaea pinodella* BL-3/4 was also confirmed by the evaluating presence of copper in the purified fraction. SEM-EDAX analysis confirms the presence of copper in purified laccase. Further research on *Peyronellaea pinodella* BL-3/4 can be explored to scale up the laccase production for its vivid industrial applications.

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