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Original Research Article

Isolation of Potential Laccase Producing Strain *Lenzites* sp. DK14 and Laccase Production Using Response Surface Methodology

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Keywords

Laccase, *Lenzites* sp DK 14, lignolytic, Response surface Methodology

A B S T R A C T

Laccase producing strain DK14 was isolated from rotting wood collected from campus of Dr Panjabrao Deshmukh Krishi Vidyapeeth, Akola. Strain has identified by partial DNA sequencing as *Lenzites sp*. and sequence was submitted to GeneBank under accession number KT210101. Response surface methodology was applied to optimize nutritional conditions for increased production of laccase by *Lenzites spp DK14*. Significant variables that affecting enzyme production were screened as malt extract, lignin and pH by using Plackett-Burman design. Central Composite Design was then applied and optimum conditions for laccase production were found to be 6% and 0.5% malt extract and lignin concentration respectively, pH of 4.32. As compared to unoptimized condition (33U/ml), 6.5 fold higher laccase yield obtained in optimized condition. The maximum laccase activity was achieved at 199.21 U/ml as observed values which agreed with the predicted values (189.5U/ml). These results suggested the predicted values are consistent and model can be use for the optimization of laccase production.

Introduction

Laccases are multi copper containing enzyme that belongs to oxidoreductase group. White rot fungi are most extensively investigated for laccase due to its wide substrate specificity towards aromatic compounds containing hydroxyl and amine groups. They catalyze oxidation of a number of organic and inorganic substrates such as mono-, di-, polyphenols, methoxyphenols, aromatic amines and ascorbate with parallel four-electron reduction of oxygen to water (Thurston, 1994; Sharma *et al.*, 2013). Due to this laccases are valuable for diverse uses in numerous biotechnological practices in paper pulping and food industries, decolourization of dyes and textile effluents, biosensors improvement, PAHs and other pollutant degradation (Sharma *et al.*, 2013; Couto and Herrera, 2006).

The optimal nutritional conditions are contributing to higher production of laccase. In previous studies (Zhaou *et al.*, 2010; Zhao *et al.*, 2013) single-factor experiments and orthogonal design experiments were performed to optimize of fermentation conditions of white-rot fungus, but none of them found to be efficient as Response

Surface Methodology (RSM). Statistical methods especially Plackett-Burman design and response surface methodology, were most extensively applied for developing, improving, optimizing processes and formulation of culture medium for bacteria and fungi (Didier *et al.,* 2007). RSM includes factorial design and regression analysis, that evaluates effective factor and builds models, then interaction and optimization of variables can be studied (De Coninck *et al.,* 2000; Puri *et al.,* 2002).

The white rot fungi *Lenzites* sp. has been widely studied for ligninolytic activity (Vartak and Gupta, 2015; Nagadesi and Arya, 2013; Buddolla *et al.,* 2008). In present study isolation, screening and molecular characterization of *Lenzites* sp. DK14 was carried out. Also laccase production was optimized with white rot fungus, *Lenzites* sp. DK14 using RSM under liquid static cultivation conditions.

Materials and Methods

Isolation and Screening of laccase producing fungi

Decaying wood samples were collected from campus of Nagarjun Medicinal Plants Garden, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. The exterior surface of the decaying wood sample was rinsed with double distilled water and sterilized by wiping with ethanol. A small piece of rotten wood was cut using a sterile scalpel and directly inoculated onto a potato dextrose agar (PDA) plate supplemented with 550 ppm streptomycin sulphate in order to inhibit the growth of bacteria. The plates were incubated at 30°C and monitored every day until mycelium developed and pure culture was obtained by repeated subculturing (Aneja 2005). Obtained pure cultures were then screened for its laccase production on solid media containing guaiacol (0.02%) as an indicator (Gupte *et al.,* 2007; Buddolla *et al.,* 2008) in malt extract agar (MEA) containing (g/l): Malt extract 20.0, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.5, $Ca(NO_3)_2.4H_2O$ 0.5, pH 5.4 at 30°C (Sharma *et al.,* 2013). Laccase positive reaction was observed based on the visualization of brown zones in the plates due to the oxidative polymerization of guaiacol by the laccase. Laccase production was further confirmed by RBBR dye decolourization at 0.025% in MEA plate. Clear zones in the plates are due to degradation of dye serves as positive reaction. The isolate was maintained as a slant in 2% malt extract agar at 40°C for further experiment.

Laccase production and Determination of Laccase activity

Fungal strains selected in screening were further quantified for laccase production. 250 ml Erlenmeyer flasks containing 50 ml malt extract broth (MEB) was inoculated with 8 fungal discs (8 mm each) from peripheri of 5 days grown cultures at room temperature and for 7 days. Sterilized tween 80 was then added to each flask on 5^{th} day at 0.5% final concentration for induction of laccase production (Gupte *et al.,* 2007). For estimating laccase activity, the supernatant was separated from fungal mycelia and used as enzyme source.

Laccase activitiy in the sample was measured spectrophotometrically using 2, 2'- azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) as a substrate with an absorbance coefficient value of 36000/M/cm at 420nm by monitoring the rate of product (dark green colour) formation due to the enzymatic oxidation of ABTS. The reaction mixture consisted of 0.45 ml acetate buffer (0.1M, pH 4.5), 0.5

ml ABTS (50 mM) and 0.05 ml culture filtrate. One unit (U) of laccase activity was defined as the amount of enzyme oxidizing one micromole of substrate per min per ml (Tien *et al.,* 2008; Niku-Paavola *et al.,* 1988). All the experiments were performed in triplicate and the results are mean of the three values.

Identification of selected potential laccase producing basidiomycetes by partial DNA sequencing

Genomic DNA from the strain DK14 was isolated (Afzal *et al.,* 2010) and was used in PCR amplification. 18S rRNA sequences were amplified by using fungal universal primers ITS1 (5'TCCGTAGGTGAACCTGCGG3) and ITS4 (5'TCCTCCGCTTATTGATATGC3) primers for identification and characterization of fungi through PCR amplification and sequencing. The 500bp amplicon was gel eluted and subjected to sequencing. The sequencing results were assembled and compared with NCBI database. The phylogenetic tree was constructed by the minimum evolution method (Tamura *et al.,* 2011).

Culture condition and Optimization of Laccase production

Based on the preliminary screening experiments with Plackett-Burman Design (PBD), using one-at-a time approach to investigate the optimum level (data not shown), three identified independent variables, malt extract, lignin and pH were used for optimization of laccase production using RSM (Table 1).

RSM was employed to optimize the culture condition for laccase production and central composite design (CCD) was applied to optimize the selected variables. Each variable was studied at five different levels (-1.68, -1, 0, +1 and +1.68) and the variable ranges [(-)1–(+)1] were: malt extract (5 to 7%), lignin (0.4 to 0.6%) and pH (5 to 7). Total 20 experiments were planned using Design Expert-9 to execute CCD. Experimental variables and their actual and coded values in Central composite design were given in table 1. The polynomial equation for three variables is

$$Y= \beta_0 + \beta_1 A+ \beta_2 B+ \beta_3 C+ \beta_{11} A^2+ \beta_{22} B^2+ \beta_{33} C^2+ \beta_{12} AB+ \beta_{23} BC+ \beta_{13} AC$$

Where, Y is predicted production of Laccase (U/ml), β_0 is the intercept; β_1, β_2, β_3 are the linear coefficients, β_{11}, β_{22}, β_{33} are squared coefficients and β_{12}, β_{23}, β_{13} are the cross product coefficient. The coefficient of determination (R^2) expressed the quality of the fit of the quadratic model equation and graphical analyses with statistical significance (F-value) were carried out.

Results and Discussion

Forty one fungal isolates were screened for the formation of reddish brown zone in 0.02% guaiacol and clear zone in 0.02% RBBR dye containing MSM. These fungal isolates have shown reddish brown and clear zone formation in MSM that presented the particular microorganism having ability to oxidize lignin and related compounds due to production of lignolytic enzymes. The fungal isolates that were found positive in screening were estimated for laccase production under liquid static condition in MSM.

After determination of laccase activity, 6 isolates amongst 41 isolates, confirmed better activity out of which only DK14 produced highest laccase (Table 2). As a result, this isolate was chosen for further investigations.

The DNA of selected strain was isolated by C-TAB mini preparation method. High degree of variation even among closely related species makes wide use of ITS region in taxonomy and molecular phylogeny, so ITS sequencing was selected for identification of fungi. 18S rRNA gene sequence analysis for identification of fungi have been reported by several researchers (Gangoliya *et al.*, 2015; Jebapriya and Gnanadoss, 2014). ITS region in DNA amplified using ITS1 and ITS4 primers and the ITS sequencing for strain DK14 (Fig. 1) showed 99% identity at 100% query coverage with the *Lenzites* sp. BRFM 1079. In addition, Phylogenetic tree (Fig. 2) also attested that the strain DK14 is most probably *Lenzites* sp.

A response surface methodology allowed calculation of maximum production based on a few sets of experiments in which all the factors were varied within selected ranges. Response surface methodology permitted the calculation of maximum production.

It has been successfully applied in the optimization of medium compositions (Lee and Chen, 1997), conditions of enzymatic hydrolysis (Ma and Ooraikul, 1986) and fermentation processes (Rosi *et al.*, 1987; Sonia *et al.*, 2005). Each variable varies for a desired response represented at high and low levels. The data obtained from RSM on laccase production were subjected to the analysis of variance (ANOVA) given in Table 4. The results of RSM were used to fit a second-order polynomial equation that represents the behavior of the system.

$$Y= 197.80+11.84(A) -2.05 (B) -5.11 (C) - 4.36 (AB) +1.11 (AC) + 0.091(BC) -27.37 (A)^2-25.41(B)^2-5.98(C)^2$$

Where Y - Laccase activity (U/ml), A- Malt extract, B- Lignin, C- pH

The experimental variables, observed and predicted results of laccase production are shown in Table 3. It can be inferred that the experimental values were nearly significant with those predicted values. If the experimental results are well predicted by the model and estimated factors effects are real, calculated F values should be much higher than tabulated value.

A high F value and a very low probability ($P> F = 0.0001$) specify that the present model is in a good prediction of experimental results. The F value of model indicates that model was significant (Akhnazarova and Kafarov, 1982; Khuri and Cornell, 1987). After the analysis of variance (ANOVA), the regression equations provided the quantity of laccase enzyme produced as a function of the initial values of pH, malt extract and lignin.

The model is efficient and predicts better if the R^2 value is closure to 1.00.The coefficient of determination (R^2) was calculated as 0.9869 for laccase production and hence 98.69 % variability in the response could be explained by the model.

The R^2 value provided an assessment of variability in the observed response values, and could be explained by the investigational factors and their interactions.

The predicted R^2 (0.9010) was observed to be nearer to the adjusted R^2 (0.9751) for laccase yield, which shows number of terms in the model relative to the number of squares of points in the design (Akhnazarova and Kafarov, 1982; Khuri and Cornell, 1987; Yee and Blanch, 1993).

Higher value of the correlation coefficient suggests an excellent correlation between the independent variables (Cochran and Cox, 1957; Box and Wilson, 1951).

Table.1 Experimental variable and their actual and coded value in Central composite design

Levels	Malt extract % (A)	Lignin % (B)	pH (C)
-1.682	4.31	0.33	4.31
-1	5	0.4	5
0	6	0.5	6
+1	7.68	0.66	7.68
+ 1.682	7	0.6	7

Table.2 Screening of fungal isolates for lignolytic enzyme production and their laccase activity in MSM

SN	Isolates	Diameter of zone (mm) formation on screening media		Laccase activity (U/ml)
		Guaiacol (0.02%)	RBBR dye (0.02%)	
1	DK6	49	79	30.2±0.46
2	DK11	43	59	16.3±0.61
3	DK12	41	60	15.6±0.42
4	DK14	56	84	33.2±0.86
5	DK21	38	57	22.7±0.34
6	DK41	41	61	28.3±0.21
7	MTCC 543	42	61	26.3±0.30

Table.3 Results of optimization of laccase production by Central Composite design

Run order	Actual variable			Coded Variable			Laccase activity (U/ml)	
	A	B	C	A	B	C		
							Observed	Predicted
1	5.00	0.40	5.00	-1	-1	-1	126.12	131.2
2	7.00	0.40	5.00	1	-1	-1	157.00	161.4
3	5.00	0.60	5.00	-1	1	-1	132.00	135.6
4	7.00	0.60	5.00	1	1	-1	144.00	148.4
5	5.00	0.40	7.00	-1	-1	1	121.56	118.6
6	7.00	0.40	7.00	1	-1	1	155.43	153.2
7	5.00	0.60	7.00	-1	1	1	126.35	123.4
8	7.00	0.60	7.00	1	1	1	144.25	140.6
9	4.32	0.50	6.00	-1.682	0	0	101.45	100.5
10	7.68	0.50	6.00	1.682	0	0	141.31	140.3
11	6.00	0.33	6.00	0	-1.682	0	131.24	129.4
12	6.00	0.67	6.00	0	1.682	0	122.61	122.5
13	6.00	0.50	4.32	0	0	-1.682	199.21	189.5
14	6.00	0.50	7.68	0	0	1.682	164.54	172.3
15	6.00	0.50	6.00	0	0	0	197.50	197.8
16	6.00	0.50	6.00	0	0	0	197.35	197.8
17	6.00	0.50	6.00	0	0	0	197.45	197.8
18	6.00	0.50	6.00	0	0	0	199.00	197.8
19	6.00	0.50	6.00	0	0	0	198.10	197.8
20	6.00	0.50	6.00	0	0	0	197.04	197.8

Table.4 Analysis of Variance Table for Central Composite Design Model for Laccase Production

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	20800.17	9	2311.13	83.59	< 0.0001
A-ME	1914.24	1	1914.24	69.24	< 0.0001
B-Lignin	57.51	1	57.51	2.08	0.1798
C-pH	357.13	1	357.13	12.92	0.0049
AB	151.82	1	151.82	5.49	0.0411
AC	9.88	1	9.88	0.36	0.5633
BC	0.067	1	0.067	2.409E-003	0.9618
A^2	10794.40	1	10794.40	390.44	< 0.0001
B^2	9303.33	1	9303.33	336.51	< 0.0001
C^2	515.37	1	515.37	18.64	0.0015
Residual	276.47	10	27.65	-	
Lack of Fit	273.97	5	54.79	109.54	< 0.0001
Pure Error	2.50	5	0.50	-	-
Cor Total	21076.64	19		-	-

Mean=157.68, R^2=0.9869, R^2(adj)= 0.9751, R^2(pred)= 0.9010, CV= 3.33, Adequate precision= 26.17

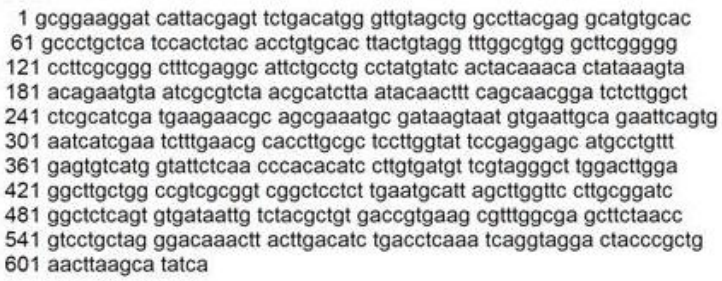

Fig 1 Partial DNA sequencing results of isolate DK14

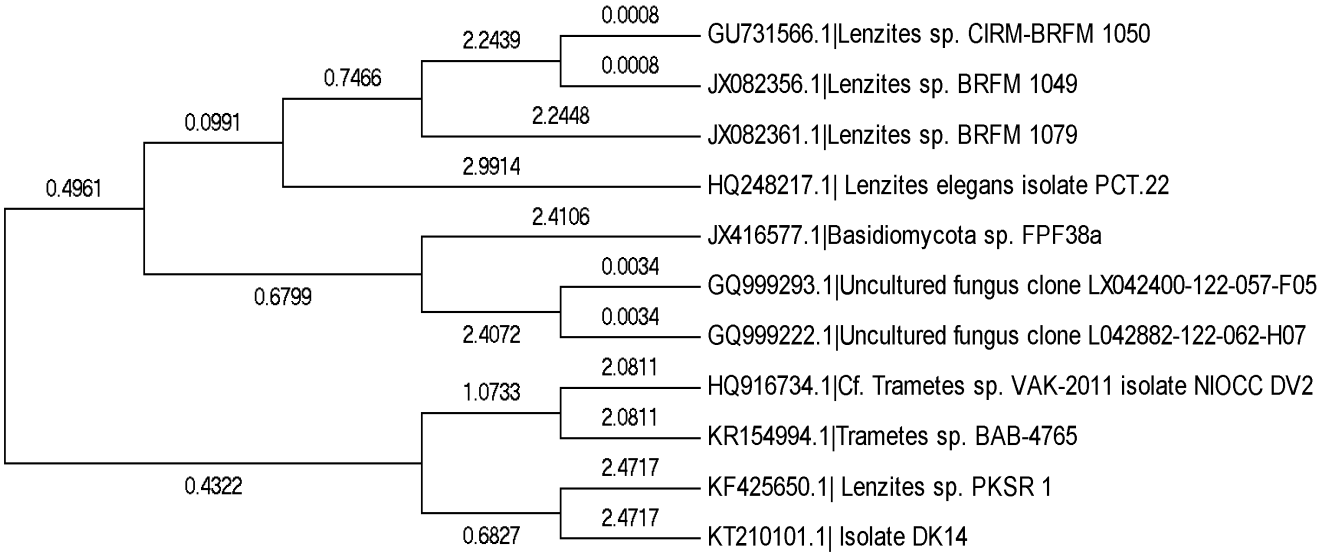

Fig 2 Phylogenetic tree of *Lenzites* sp. DK14 and their closest NCBI (BLAST) matches

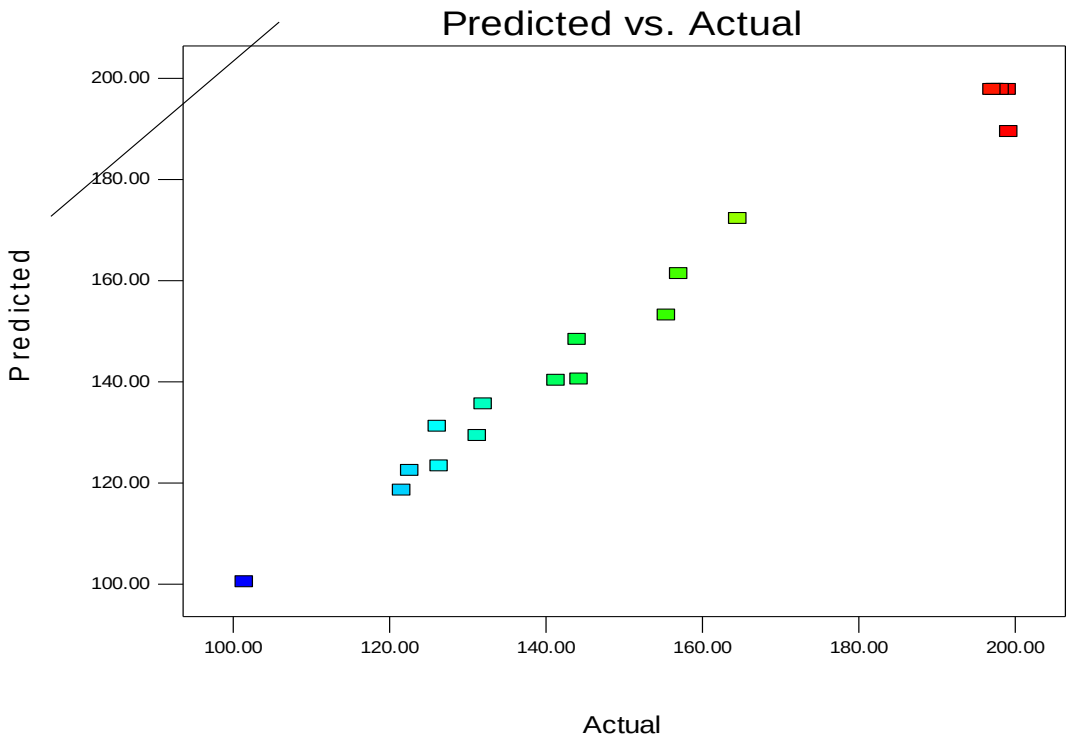

Fig 3 Predicted Vs Actual values for laccase response in *Lenzites* sp. DK14

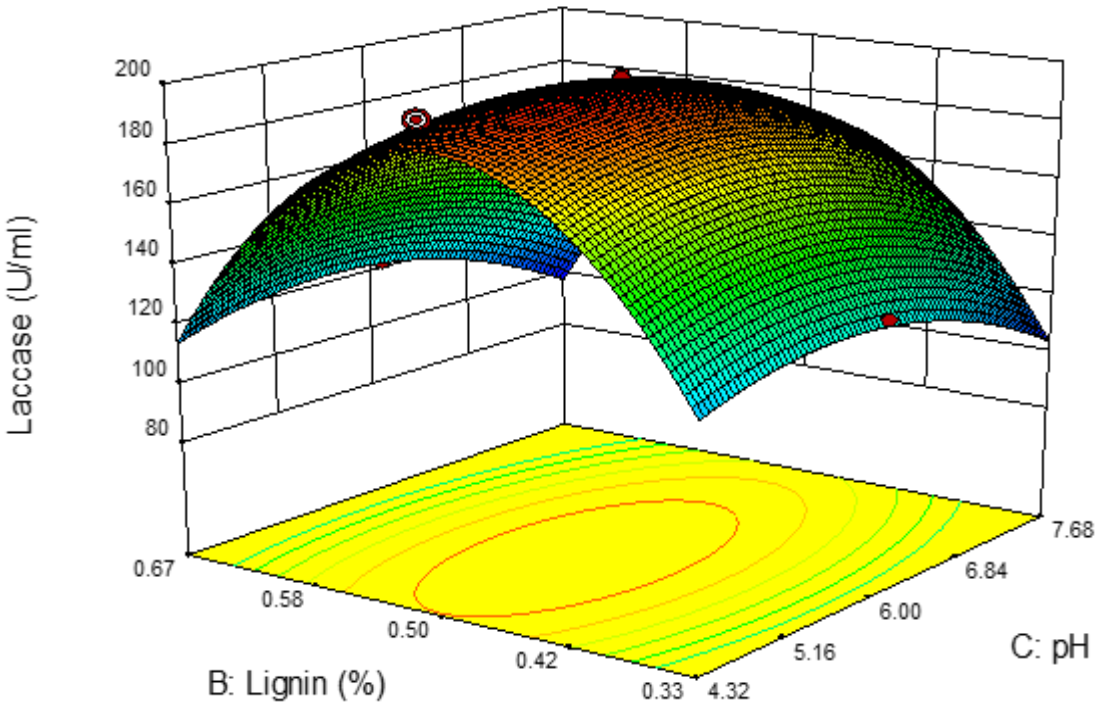

Fig 4 Response surface curve for Laccase production from *Lenzites* sp. DK14 showing interaction between Lignin & pH

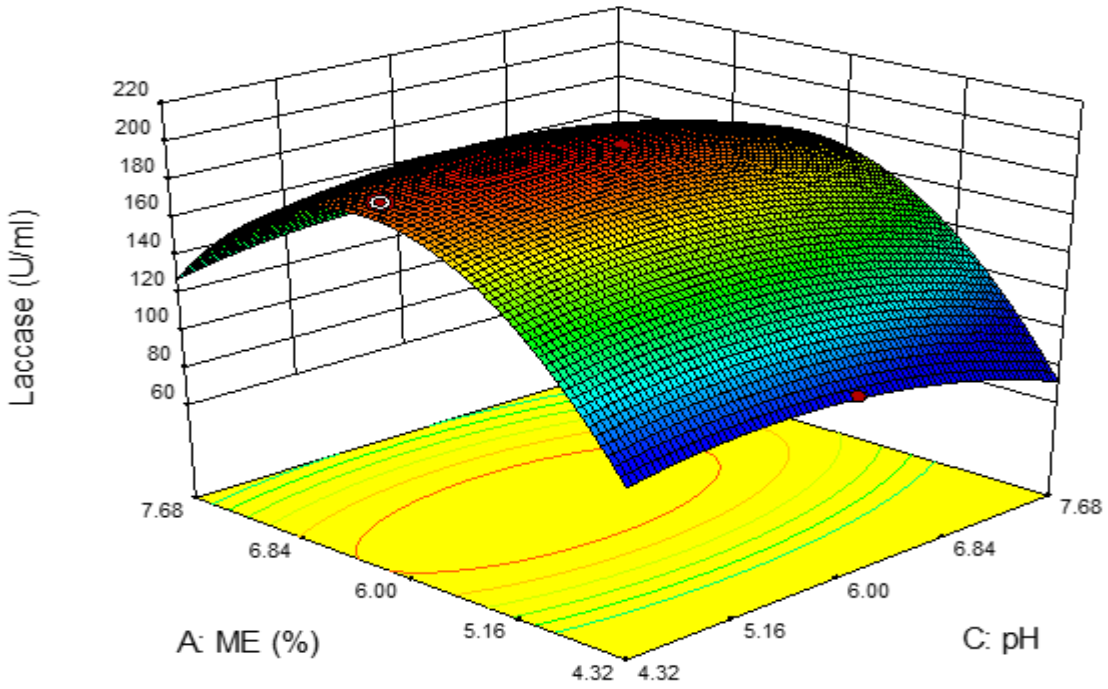

Fig 5 Response surface curve for Laccase production by *Lenzites* sp. DK14 showing interaction between Malt extract & pH

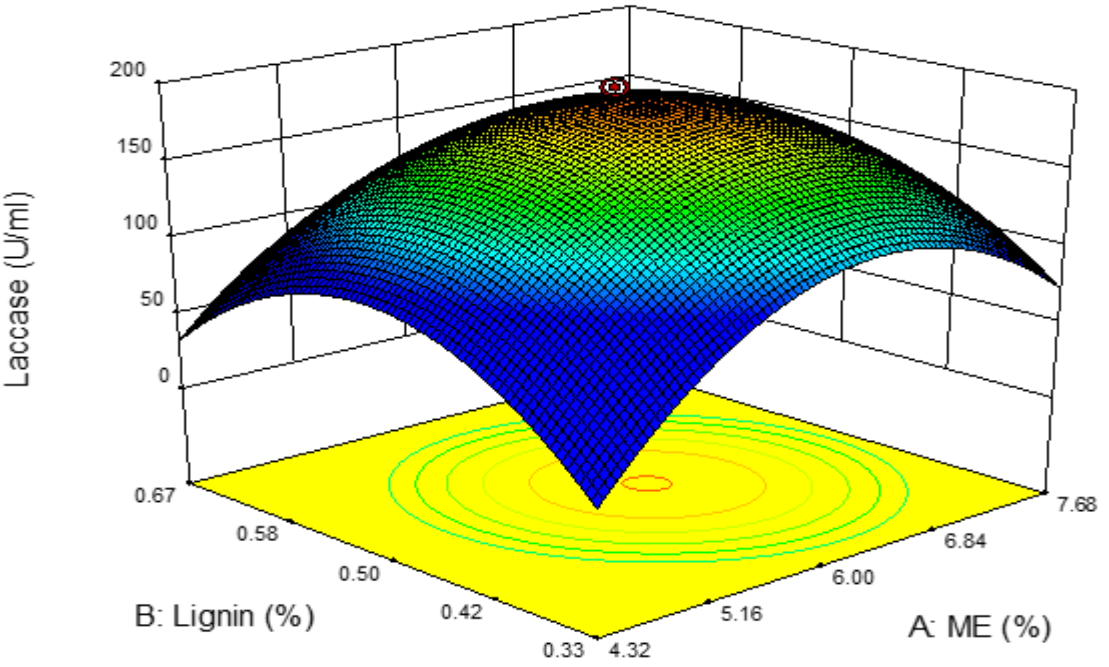

Fig 6 Response surface curve for Laccase production by *Lenzites* sp. DK14 showing interaction between Lignin & Malt extract

The aim of statistical analysis is to evaluate the experimental factors that produce signals which are large as compared to noise. Ample precision measuring the signal to noise ratio was observed as 26.17 for production of laccase. A signal to noise ratio higher than 4 is desirable. The relationship between the actual and predicted laccase activity (response) is shown in Fig. 3. A good fit of the model and a satisfactory correlation between actual and predicted values was demonstrated by the cluster of measurements near the diagonal line in the parity plot. The maximum response was obtained with ME-6%, Lignin- 0.5%, & pH-4.32% (199.21 U/ml) and the minimum response was obtained with ME-4.32%, Lignin- 0.5%, & pH-6% (101.45 U/ml). This demonstrates the fitness of model and its applicability to navigate the design space.

To study the interaction between different physiochemical parameters for optimal laccase production from *Lenzites* sp. DK14, 3D response surface curves were plotted. The plots were created by plotting the Laccase production (response) with the Z-axis against two independent variables while the other independent variables were placed at their O-level. At lignin concentration of 0.5% (w/v) and pH 4.32, an interaction among the two parameters i.e. pH and lignin produced a higher enzyme yield (Fig. 4). At the O-level of lignin, the response between pH and malt extract point out that a moderately acidic pH (4.32) was desirable (Fig. 5). When pH is kept at O-level and interaction response between malt extract and lignin is plotted (Fig 6), enhancement of laccase production was observed with increased malt extract concentration. Apart from this when lignin level increased from 0.4 to 0.5% (w/v), a linear increase in the laccase production was recorded. The experiments were designed with random levels of factors within and outside the design space to validate and confirm predictions. Validation was carried out under varying levels of malt extract, lignin and pH predicted by the model. As the experimental values were very close to the predicted values, it can be said that the model was validated successfully.

The positive effects of lignin on laccase production are already reported (Sharma *et al.,* 2013; Mansur *et al.,* 1997, it was proposed that, this increase in laccase production may be due to (a) synthesis of inducible laccase isozymes and (b) combinatorial effect of laccase and HOBT (a heterocyclic compound with N-OH moiety which can be oxidized by laccase to its nitroxide radical). These redicals act as proximal oxidant of lignin which depolymerizes lignin and give rise to phenolics (ferulic acid, vanillic acid, 3–4 dihydroxy benzoic acid) as well as non-phenolic structural polymers (Sharma *et al.,* 2013; Srebotnik *et al.,* 2000; Bourbonnais *et al.,* 1997). The phenolics produced then perform dual purpose of acting as substrate and also inducer for laccase biosynthesis. This observation is well supported by existing reports in literature regarding laccase induction. Fungal laccases usually have pH optima in the acidic range. Though the optimum pH for the oxidation of ABTS is less than 4.0, phenolic compounds like guaiacol, syringaldazine and di-methoxy phenol show higher values about 4.0–7.0 (Baldrian, 2006). It was reported that in the acidic pH range, the fungal laccases are more stable (Sharma *et al.,* 2013; Baldrian 2006). Similar results were reported by several authors (Sharma *et al.,* 2013; Arora and Gill, 2001), that demonstrated increased production of laccase due to malt extract, though the exact mechanism is not clear.

In general 6.5 fold increase in laccase yield was achieved after statistical optimization.

The study has confirmed the suitability of using statistical methods for improving laccase production by *Lenzites* sp. DK14

Present investigation demonstrated the laccase producing potential of locally isolated *Lenzites* sp. DK14 under liquid static condition. Significant increase in laccase production was achieved as a result of optimization of cultural condition using CCD in RSM. As compared to unoptimized condition, 6.5 fold higher laccase yield obtained in optimized condition.

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