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Original Article

Laccase production by a soil fungal isolate *Geotrichum Candidum* using response surface methodology based optimization a cost effective approach

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ABSTRACT

Laccases are multicopper enzymes produced by fungi with vast industrial applications. The current study focuses on isolating ,screening and optimization of nutritional parameters for the maximum production of laccase by a soil fungal isolate *Geotrichum candidum* .The influence of D(+) glucose ,$(NH4)_2 SO_4$, $KH_2 PO_4$, $MgSO_4$, $CaCl_2$, KCl , malt extract and pH on laccase production were optimized using statistical experimental design. Initially the fungus showed laccase activity of 160.73U/L in the submerged culture conditions. Laccase production increased by 1.57 folds by using the optimized medium to attain a final yield of 253.45 U/L. Further, the experimental value of laccase yield was close to the predicted values, indicating that the chosen method of optimization was less time consuming and a cost effective approach for effective laccase expression.

Key Words: *Geotrichum candidum,* Response Surface Methodology, laccase

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are blue multicopper oxidases that catalyze the oxidation of aromatic substrates concomitantly reducing molecular oxygen to water. Since its discovery in 1883 in the exudates of the Japanese lacquer tree *Rhus verniczfera*, this enzyme has presented a never ending story finding applications in various fields [1] and inviting much research interest attributed to its oxidative role. These enzymes display high oxidative property in phenolic and nonphenolic lignin related compounds. Apart from their use to degrade lignin in textile, paper and pulp industries, they find applications in food industry, bioremediation, teeth whitening, wine cork making, catalysts for manufacture of anticancer drugs, as ingredients in cosmetics, as biosensors for immunoassays, as a new biocatalyst in organic synthesis etc [2]The pharmaceutical potential of laccase is evident in its inhibitory effect on HIV 1 reverse transcriptase [3] synthesis of complex medical compounds [4] *insitu* generation of iodine (a disinfectant) to kill spores [5] fighting aceruloplasminemia [6] etc. The use of laccase in the synthesis of biofuel cells has also been studied [7].Thus studies on biosynthesis of laccases gain much relevance.

In nature laccases are often found in white rot fungi, higher plants, insects and bacteria [8] They have been isolated from Ascomyceteous, Deuteromycteous and Basidiomycetous fungi [9]. The fungal laccases are found to be superior to plant or bacterial counterparts contributed by their higher redox potential (up to+800 mV) [10] and low substrate specificity which allows them to oxidize a wide range of compounds without releasing toxic peroxide intermediates [11].They are naturally found to be involved in the removal of toxic phenols arising during lignin degradation [10], synthesis of dihydroxynaphthalene melanins against environmental stress [12] and fungal morphogenesis by catalysing the formation of extracellular pigments [13]. Laccase activity has also been found as a component of immune defense in the haemocytes of the red swamp crayfish [14] and to be an important virulence factor for the human pathogen *Cryptococcus neoformans* [15] Ceruplasmin (a mammalian plasma protein), ascorbate oxidases (plant proteins) and bilirubin oxidase are also members of this group [16] Large amounts of nitroaromatic compounds are released into the environment as a result of the manufacture and widespread use of explosives, pesticides, dyes, and parmaceuticals. The presence of 2,4,6-trinitrotoluene (TNT)in soils, groundwater, and

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surface water at sites where this explosive was formerly manufactured, loaded or demilitarized, represents a serious ecological problem worldwide. The potential of laccase for immobilizingTNT degradation metabolites into humic matrices has been demonstrated by a number of researchers [17] Enzymatic formulations can be gentler and easier to handle than present hair dyes [18]. Laccase has also been included in preparations for skin lightening [19].Recent patent literature has indicated that laccase may also be incorporated into chewing gum as an additive to counter halitosis [20]

Regardless of its wide utility in various fields, the industrial production of laccases is still facing many constraints to achieve its economized production. Utilizing the natural sources of laccases for industrial production of these enzymes could serve as a promising strategy. Media engineering and optimization of various physicochemical conditions of laccase fermentation by natural fungi using statistical experimental designs such as response surface methodology could greatly enhance the laccase yield. The conventional step wise optimization technique varying one independent variable while fixing other variables is found to be laborious and time consuming and consequently fails to provide information about the mutual interactions of the parameters [21] The fungal laccase expression in fermentation is influenced mainly by culture conditions such carbon source and its concentration, pH of the fermentation broth ,the presence of lignocellulose materials and nitrogen source The statistical optimization of medium components would be a rational and cost effective approach for effective laccase expression [22]

The present paper focuses on response surface methodology based optimization of laccase production by *Geotrichum candidum* .The chosen method of optimization was successful resulting in less time consumption and a cost effective approach for effective laccase expression.

MATERIALS AND METHODS

Isolation, screening and identification of laccase producing fungi: Soil samples collected from different parts of Thiruvananthapuram district of Kerala (India), were enriched in Potato dextrose broth and different fungi were isolated on potato dextrose agar media plates after incubation at 25˚C for 2-3 days.Different fungal isolates were further screened for laccase production ability on PDA agar plates containing guaiacol as an indicator [23].High laccase yielding fungal isolate GC was selected and used for further study.

Primary level identification of the selected fungal isolate was done based on its morphological and staining properties [24].Further identification of the fungal isolate was done by 18S rDNA using U18S F: -5'- ACCTGGTTGATCCTGCCAG-3',
U18S R: - 5'-TGATCCTTCYGCAGGTTCAC-3' primers. Genomic DNA was isolated from the sample provided using Sigma Aldrich fungal DNA extraction Kit. The amplicon was further purified, sequenced and deposited in the NCBI Gene bank database.

Medium and culture conditions for submerged fermentation: The current study attempted to optimize laccase yield using basal liquid medium containing glucose as carbon source. The original composition and nutrients levels of the basal liquid medium consisted of (gL^{-1})(Qualigen chemicals) Glucose-15 gL^{-1},$(NH_4)_2SO_4$- 2.2 gL^{-1},KH_2PO_4 - 1.6 gL^{-1},$MgSO_4$- 0.3 gL^{-1},$CaCl_2$- 0.07 gL^{-1},KCl- 0.07 gL^{-1},Malt Extract- 5 gL^{-1},pH-7.Submerged fermentation (SmF) was carried out in 250ml Erlenmeyer flasks containing 100ml basal liquid medium and 0.1 mgL^{-1} $CuSO_4$ as inducer. A pre-inoculum was prepared using 13mm diameter plugs of mycelia discs of a 7 day old fungal culture and inoculated to PDA broth for 48 h to form the inoculums. The cultures were incubated at 25°C in a rotary shaker at 120 rpm and the crude enzyme was extracted on the ninth day. After fermentation the medium was centrifuged at 10000 rpm for 20 min at 4°C to yield the crude enzyme extract.

Enzymatic assay of laccase: The extent of laccase activity was measured using syringaldazine an indicator used to detect laccase enzyme. Laccase enzyme activity was conducted in a reaction mixture (3ml) containing 0.5 ml culture filtrate , 0.30 ml 0.2 mM syringaldazine and 2.20 ml of potassium phosphate buffer (pH 6.5) at 530 nm [25] The reaction mixture (3 ml) contained 0.5ml of culture filtrate which was diluted appropriately to get the absorbance in readable range. One unit of enzyme activity was defined as the amount of enzyme oxidizing 2µmol of syringaldazine per minute. The enzyme activity was expressed in enzyme units (U) per L (UL^{-1})

Optimization of laccase production: The optimization of laccase production was carried out using statistical design of experiments in two steps. The first step involved the screening of variables and the second step involved the optimization of significant variables. Plackett – Burman design, a widely used fractional factorial method was adopted for the screening of cultural and nutritional parameters influencing laccase production by *G.candidum* in submerged fermentation.

Several medium components expected to have vital effect on laccase production such as glucose-15 gL^{-1},$(NH_4)2SO_4$-2.2 gL^{-1}, KH_2PO_4 -1.6 gL^{-1}, $MgSO_4$-0.3 gL^{-1}, $CaCl_2$ - 0.07 gL^{-1},KCl-0.07 gL^{-1}, malt extract - 5 gL^{-1}, pH -7 were chosen as the experimental variables. The goal was to optimize the nutrient levels of the nutrients to improve the production of laccase in the submerged fermentation with $CuSO_4$ as an inducer to promote better expression of laccase activity. Each variable was studied at two different concentration levels representing high and low set points as shown in **Table 1**

In the experimental design using MINITAB *16 software, the nutrient levels were treated as quantitative factors where as $CuSO_4.5H_2O$ supplementation was studied as a qualitative factor. A full factorial design was used in the basal medium components. A total of 36 runs, including the three replicates, were performed in three experimental blocks. The runs were free from aliasing. The design runs also included six centre points which could be used to detect the presence of possible curvature in the response within the variable range tested. The significant variables were identified by the analysis of the Plackett and Burman experiments and their levels were further optimized for enhanced laccase production by employing a Central – composite design. The main effects for each of the studied factors on the response were analyzed using the software together with the effect of possible interactions between two different factors. The experiment was carried out in triplicate to estimate the experimental errors and to test for lack- of - fit of the data using the second degree polynomial model.

RESULTS AND DISCUSSION

Isolation, screening and identification of laccase producing fungi: Among the different fungal isolates obtained from the soil, the best laccase yielding isolate *G.candidum* was selected for further studies. 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. This potential strain expressed an average of 160.73 UL^{-1} laccase activity that was found to be the highest compared to the other fungal isolate with the initial nutrient media composition in broth culture.

The morphotaxonomic analysis indicated that *G.candidum* is an anamorph of 'imperfect fungi' belonging to the form-class Deuteromycetes but now it has been placed in Hemiascomycetes. Further 18S rDNA yielded a sequence **KJ814246 which** showed 99% similarity with other strains of *G. candidum*. Based on morphological, biochemical and 18S rDNA properties, the isolate was identified as *Geotrichum candidum.*

Optimization of laccase production: The Plackett - Burman (P-B) experimental design was applied to screen the key nutrient factors for the production of laccase enzyme by *G. candidum*. Statistical analysis was carried out using Analysis of variance (ANOVA) to check the adequacy of the test. The responses of laccase expression activity by *G.candidum* obtained for the various P-B experimental design combinations of the studied variables are shown in **Table 2.**

The first order polynomial equation for the predicted response 'Y' of laccase yield was given by the regression equation 1:

$Y = - 0.161 - 0.00029\ Glucose(X_1) + 0.0104\ (NH4)2SO4\ (X_2) + 0.0022\ KH2PO4\ (X_3) - 0.0087\ MgSO4\ (X_4) - 0.088\ CaCl2\ (X_5) - 0.169\ KCl(X_6) + 0.0287\ Malt\ Extract(X_7) + 0.0272\ pH(X_8)$

On analysis of regression coefficient of eight medium components malt extract, pH, $(NH_4)_2SO_4$, and KH_2PO_4 showed a positive effect on laccase production while glucose, $CaCl_2$ and KCl exerted a negative effect on the tested report. A regression analysis was done to examine the nature of relationship between the variables. So an estimation of the statistical correlation coefficient (r) was measured by correlation. Its value ranged from +1.0 to -1.0 indicating the strength of the relation (Co). The regression coefficient T values and P values of the factors were calculated for laccase production using the statistical software as shown in **table 3**.Based on the T statistics and p value, the variables malt extract and pH were found to significantly affect laccase production. Therefore these two variables were further selected for response surface based optimization.

After the main variables were identified, a steepest ascent experiment was carried out to investigate the central point of these variables for subsequent response surface design. Initial concentration of malt 4g/L and pH 6 were the central point of these levels in P-B design. A response surface was created using central composite design for these two factors, while the rest of the medium components were kept constant. Further optimization was performed using the response Optimizer function of MINI TAB *16 software. **Table 4** shows the observed and predicted responses for laccase production. Regression analysis of the data was performed for testing the adequacy of the proposed quadratic model and the following second-order polynomial equation was derived.

equation 2.

$y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2$

y is the estimated response, b_0, a constant, b_i, coef-ficients for each term, and x_i, factors in coded values

The three dimensional response surface plots by SAS 9.2 analysis including ANNOVA depicted in **fig1** presented the effect of two variables on the production while the other six variables were held at zero level. Independent experimental runs (n =3) were set up to confirm the predicted optical response and levels of variables. The R^2 value of 96.22% and the adjusted R^2 value of 93.52% showed that the response surface model was highly reliable indicating a good correlation between observed and predicted responses. For a good statistical model R^2 value should be close to 1.0 where a value >0.75 indicates the aptness of the model. The current study succeeded in optimizing laccase production by *Geotrichum candidum* in a minimal medium containing glucose as carbon source. The two variables malt extract and pH played significant role in laccase production as detected by response surface analysis. The laccase yield was increased 1.57 fold to achieve a final yield of 253.45 U/L. Thus the optimized medium components of laccase production by *G.candidum* was formulated as depicted in **table 5**.

In the present work *Geotrichum candidum* was isolated from soil which is their natural habitat and is a soil borne fungi [26]. *Geotrichum candidum* are generally recognized as Safe (GRAS) , have been employed in cheese industry for many years [27] So working in a laboratory was found to be safe because of the GRAS status confirmed on the fungi.Laccase production in fungi is reported in Ascomycetes, Basidiomycetes and Deuteromycetes [28] . Most of the studies on fungal laccases concentrate around Basidiomycetes fungi mainly white rot fungi, but not much study is carried out on deuteromycetes. The dueteromycetes reported to produce laccase include *Myrothecium verrucaria* NF-05[29] , *Pestalotiopsis* sp [30] , *Paecilomyces inflatus* [31] .Here for the screening of the fungal isolate chromogen guaiacol was used. The chromogen guaiacol is a very sensitive substrate that allows rapid screening of fungal strains producing extracellular guaiacol oxidizing enzymes by means of a color reaction[32] . Laccase production by fungus is greatly dependent on its culture conditions [33] and generally the conditions for optimized fungal growth do not support optimized laccase production. Thus studies to identify the conditions favoring laccase production are quite essential. Generally fungus produces less amount of laccases but the addition of certain components promote or enhance laccase production. Such induction of laccase production could be achieved using low concentrations of Cu^{+2} to the cultivation media .The stimulatory effect of copper on laccase synthesis was also effective for several other basidiomycetes and hence could be used as a simple method to improve the production of this enzyme [34]. Hence the significant variables were identified by the analysis of the Plackett and Burman experiments and their levels were further optimized for enhanced laccase production by employing a Central – composite design. The optimization of parameters affecting laccase production by statistical experimental design (Plackett-Burman and response surface methodology) can eliminate the limitations of single-factor optimization process collectively [35] . This approach of statistical experimental designs for rapid screening of the fermentation variables was also emphasized by Zhang et al. [36] . The 3D response surface curve determines the optimum condition of each components for maximum response and their interaction effect when other variable was fixed at zero level. The integrations between the variables can be inferred from the shapes of response surface plots[37] Here the two variables malt extract and pH played significant role in laccase production as detected by response surface analysis. The laccase yield was increased 1.57 fold to achieve a final yield of 253.451 U/L. Thus the optimized medium components of laccase production by *G.candidum* was formulated. So the current study succeeded in optimizing laccase production in a minimal medium, containing glucose as a carbon source. However, laccase production by *Geotrichum candidum* is novel and thus gains much relevance. With increasing utility of laccase in industry, research interests and available patents [2] , each and every new information on this enzyme is considered noteworthy.

CONCLUSIONS

The present study have succeeded in optimizing laccase production by a soil fungal isolate *Geotrichum candidum* in a minimal media containing glucose as a carbon source.The statistical design of experiments was effective in improving laccase production in submerged fermentation .Further , the experimental values of the laccase yield was close to the predicted values , indicating that this method was less time consuming and cost effective for effective laccase expression without resorting to the addition of expensive or hazardous compounds or even elicitors like aromatic compounds . Moreover this work also depicts that soil is a natural medium for the growth of fungi which are excellent sources of valuable enzymes for industrial purposes.

Table :1 Media components with high and low values

Medium components	Level (gL^{-1})		
	Low	Centre	High
Glucose	15	20	25
$(NH_4)_2SO_4$	1.6	1.9	2.2
KH_2PO_4	1.6	1.9	2.2
$MgSO_4$	0.3	0.5	0.7
$CaCl_2$	0.07	0.05	0.13
KCl	0.07	0.05	0.03
Malt extract	3	4	7
pH	5	6	7

Table 2: P-B design for screening significant variables for laccase expression activity by *G.candidum* (including replicates)

Runs	Glucose gL^{-1}	$(NH_4)_2SO_4$ gL^{-1}	KH_2PO_4 gL^{-1}	$MgSO_4$ gL^{-1}	$CaCl_2$ gL^{-1}	KCl gL^{-1}	Malt Extract gL^{-1}	pH	Laccase UL^{-1}
1	25	2.2	1.6	0.7	0.07	0.03	3	7	80.12
2	15	1.6	1.6	0.3	0.07	0.03	3	5	104.75
3	15	1.6	1.6	0.7	0.13	0.07	3	7	99.41
4	15	1.6	2.2	0.7	0.13	0.03	5	7	148.37
5	25	1.6	2.2	0.3	0.07	0.03	5	7	143.92
6	25	1.6	1.6	0.3	0.13	0.07	5	5	83.09
7	25	2.2	1.6	0.7	0.13	0.03	5	5	139.47
8	15	2.2	1.6	0.3	0.07	0.07	5	7	163.21
9	25	1.6	2.2	0.7	0.07	0.07	3	5	53.41
10	15	2.2	2.2	0.7	0.07	0.07	5	5	106.83
11	15	2.2	2.2	0.3	0.13	0.03	3	5	56.38
12	25	2.2	2.2	0.3	0.13	0.07	3	7	91.99
13	25	1.6	2.2	0.7	0.07	0.07	3	5	53.41
14	15	2.2	2.2	0.7	0.07	0.07	5	5	105.34
15	15	1.6	2.2	0.7	0.13	0.03	5	7	155.79
16	15	1.6	1.6	0.7	0.13	0.07	3	7	77.15
17	25	1.6	1.6	0.3	0.13	0.07	5	5	81.60
18	15	1.6	1.6	0.3	0.07	0.03	3	5	75.67
19	25	1.6	2.2	0.3	0.07	0.03	5	7	178.04
20	25	2.2	1.6	0.7	0.13	0.03	5	5	112.76
21	25	2.2	1.6	0.7	0.07	0.03	3	7	115.73
22	15	2.2	1.6	0.3	0.07	0.07	5	7	178.04
23	25	2.2	2.2	0.3	0.13	0.07	3	7	118.69
24	15	2.2	2.2	0.3	0.13	0.03	3	5	66.77
25	15	1.6	1.6	0.3	0.07	0.03	3	5	68.25
26	15	1.6	2.2	0.7	0.13	0.03	5	7	195.85
27	25	1.6	1.6	0.3	0.13	0.07	5	5	127.60
28	25	2.2	1.6	0.7	0.13	0.03	5	5	117.21
29	25	1.6	2.2	0.7	0.07	0.07	3	5	66.77
30	15	2.2	2.2	0.3	0.13	0.03	3	5	71.22
31	25	1.6	2.2	0.3	0.07	0.03	5	7	200.30
32	15	2.2	1.6	0.3	0.07	0.07	5	7	201.78
33	25	2.2	2.2	0.3	0.13	0.07	3	7	121.66
34	15	1.6	1.6	0.7	0.13	0.07	3	7	132.05
35	15	2.2	2.2	0.7	0.07	0.07	5	5	121.66
36	25	2.2	1.6	0.7	0.07	0.03	3	7	132.05

Table 3. Analysis of variance of Plackett Burman Design

Predictor	Coef	SE	T	P
Constant	-0.1609	0.0437	-3.68	0.001
Glucose	-0.0003	0.0007	-0.45	0.659
(NH4)2SO4	0.0104	0.0111	0.94	0.354
KH2PO4	0.0022	0.0111	0.20	0.844
MgSO4	-0.0086	0.0166	-0.52	0.607
CaCl2	-0.0879	0.1108	-0.79	0.434
KCl	-0.169	0.1661	-1.02	0.318
Malt Extract	0.0287	0.0033	8.63	<0.001
pH	0.0272	0.0033	8.19	<0.001

S = 0.0199379 R-Sq = 84.3% R-Sq(adj) = 79.6%, P value cut off <0.005

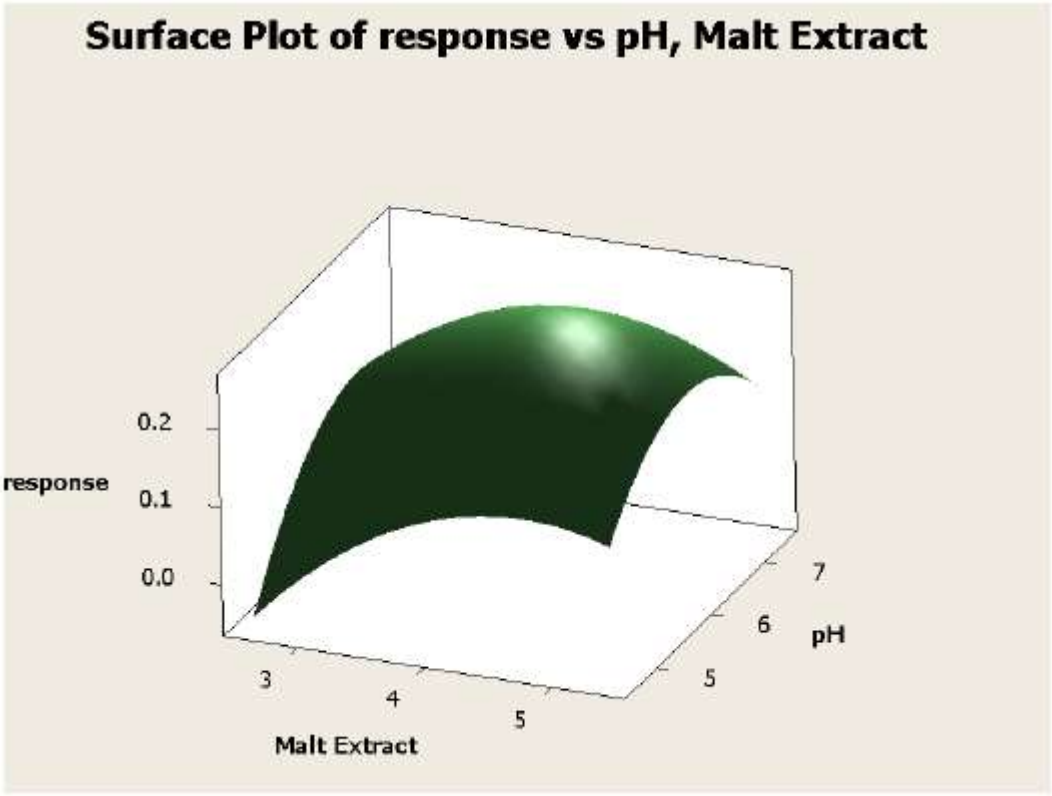
Table 4: Central Composite Design with observed and predicted values of laccase production by *G.candidum*

C1 (Malt extract)	C2 (pH)	Response observed UL^{-1}	Response predicted UL^{-1}	Residual
5	7	180.51	201.34	-20.83
4	6	253.71	244.20	9.51
5	5	171.31	180.83	-9.52
4	6	243.81	244.20	-0.39
2.59	6	106.31	118.61	-12.3
5.41	6	232.4	212.51	19.89
4	6	242.3	244.20	-1.9
4	6	242.9	244.20	-1.3
3	5	93.45	80.20	13.24
4	4.59	107.3	111.50	-4.2
4	6	238.3	244.20	-5.9
4	7.41	200.7	188.91	11.79
3	7	171.1	169.17	1.93

Table 5. Optimized media for laccase production by *G.candidum*

Optimized media Composition (g/L)	
Glucose	15
$(NH_4)_2SO_4$	2.2
KH_2PO_4	2.2
$MgSO_4$	0.3
$CaCl_2$	0.07
KCl	0.03
Malt Extract	4.39
pH	6.21

Figure1.Effect of malt extract and pH on laccase production by *G.candidum*



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