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STATASTICAL OPTIMIZATION OF MEDIUM FOR LACCASE PRODUCTION BY *RIGIDOPORUS SP.* DK4

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ABSTRACT

Due to enormousindustrial importance oflaccase enzyme,current study was focused on optimization of culture conditions for maximizinglaccase production by *Rigidoporus* sp. DK4 under submerged fermentation condition. Effect of carbon, nitrogen sources and inducers were evaluated by Plackett-Burman design. The factors that showed positive effect on laccase production were further optimized using central composite design of RSM. The multiple regression equation was used to optimize the values of carbon, nitrogen and inducer for maximum enzyme production.The optimized values were found to be 2%, 0.8% and 180 μM for maltose, ammonium nitrate and copper sulphate, respectively. The maximum enzyme activity of 30 UmL^{-1} obtained experimentally was very close to the predicted response, 29.315 U mL^{-1}. The most significant media composition enhanced the laccase production by nearly 4 fold as compared to un-optimized condition. Predicted mathematical model based on multiple regression was in close agreement with experimental observation.

INTRODUCTION

Laccase (EC 1.10.3.2) is copper containing enzyme that catalyzes the oxidation of variety of aromatic compounds including lignin and various phenolic compound with concurrent reduction of molecular oxygen to water(Xu, 1999). Due to such distinct ability, laccase production has achieved a lot of interest in industrial and environmental applications that includes textile-dye decolourization, biobleaching, biopulping, bioremediation of environmental pollutants, development of biosensors, biofuel cells, food and cosmetic manufacturing(Sharma, 2014; Kale *et al.*, 2014; Sharma *et al.*, 2013). Fungi especially basidiomycetes fungi are considered as main source of laccase production as compared to deuteromycetes and ascomycetes.

In the native state, many white rot fungi produces laccase in minute quantities and these enzymes may be sensitive to pH, temperature etc.That's why improvement of laccase production through optimization of nutrient medium is essential. In addition, reducing the costs of enzyme production by optimizing the fermentation medium is the basic research for industrial application (Das *et al.*, 2013; Souza *et al.*, 2006).

Response surface methodology (RSM) is widely used in the optimization of various industrially important microbiological, biochemical and biotechnological products such as chemicals and enzymes (Abeer *et al.*, 2016; Kumar *et al.*, 2015). RSM requires minimum experimentation and time, thus proving to be far more effective than the conventional methods of developing such products. Based on the principle of design of experiment, the methodology encompasses the use of various types of experimental designs, generation of polynomial equations, and mapping of the response over the experimental domain to determine the optimum product (Palvannan and Boopathy, 2005).

Laccase production by several white rot fungi (WRF), under submerged fermentation and solid state fermentation condition have been reported (Tripathi and Gupte, 2015; Sharma *et al.*, 2013). In order to meet industrial demands, optimization of cultural condition and nutritional parameter are mostly applied methods to get higher production of enzymes(Husain *et al.*, 2015; Das *et al.*, 2013). Several studies have been reported the optimized culture condition for laccase production (Patel and Gupte, 2016; Abeer *et al.*, 2016). Nutrient source, aromatic compounds and metal ions acts as inducersin microbial laccase production (Yang *et al.*, 2016; Stajic *et al.*, 2006).

The *Rigidoporus* sp. DK4 was isolated from dead Eukalyptus wood and was found to have excellent dye decolourizarion activity which could have potential industrial applications (Kale *et al.*, 2013). Therefore, the aim was to optimize media composition for increased production of laccases by white rot fungi *Rigidoporus* sp. DK4.

MATERIALS AND METHODS

Microorganism and chemicals

Strain DK4 isolated from decaying *Eucalyptus* wood that produces laccase was used in present study. It was identified by partial DNA sequencing to be closely related to *Rigidoporus* sp. The culture was maintained and periodically sub-cultured on malt extract agar (MEA) at 4±1°C.The 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sisco research laboratory, India and all other chemicals were of analytical grade.

Inoculum preparation

Strain DK4 was grown on MEA petri plates for 5-7 days at 29±1°C. Five disc (8mm) from margin of actively growing *Rigidoporus* sp.DK4 were used for inoculation of 100 mL of potato dextrose broth and kept under static condition for 6 days at 28°C (Sharma *et al.*, 2013). For the medium optimization studies 2% (v/v) of culture was used as inoculum for PBD and CCD experiments.

Laccase assay

Laccase activity was evaluated by monitoring the rate of product formation due to the enzymatic oxidation of ABTS (Kale *et al.,* 2014). Oxidation of ABTS was monitored by an increase in absorbance at 420 nm (T420 = 36 000 $M^{-1}cm^{-1}$) at 30°C. One unit of laccase activity was defined as the amount of enzyme that oxidised 1 μmol of ABTS per min and expressed in U/mL.

Screening of factors using Plackett-Burman design

PBD was applied to investigate most significant factors affecting laccase production by *Rigidoporus* sp.DK4 (Plackett & Burman, 1946). A set of 12 experiment was conducted to evaluate 11 variables were screened including five carbon sources (sucrose, maltose, glucose, starch, fructose) at 0.1 and 2.5 % as (-) and (+) level, respectively two nitrogen sources (yeast extract, ammonium nitrate) at 0.1 and 2.5 % as (-) and (+) level three enzyme inducers like manganese sulphate, copper sulphate at 0.05 and 0.5 %,guaiacol and thiamine at 0 and 100 μM as (-) and (+) level were used for media optimization (Table 1). From the pareto chart analysis, the variables which show highly positive effect on each group were considered to have greater impact on laccase production (Pratheebaa *et al.*, 2013). Experimental PBD was based on the first order model:

$Y = b_0 + b_1X_1$ Eq. (1)

WhereYis the response (laccase production, U/mL), b_0 is the model intercept, and b_1 is the linear coefficient and X_1 is the level of independent variable. All experiments were carried out in duplicate and the average of laccase activities were considered as the response.

Response surface methodology

Preliminary screening of nutritional factor that influences laccase production were carried out by one factor at a time method using PBD. The factors like maltose, ammonium nitrate and copper sulphate were found to encourage laccase production. Further CCD was used to optimize concentrations of factors and to examine the interaction amongst most significant factors. The effect of each variables was studied at five levels (-±, -1, 0 +1, -±) with set of 20 experiment was performed in triplicates including 6 central points.The range of coded and uncoded variables was given in Table 3. The data were processed to attain eq. 2

Yi = β0+ βixi+βixii2 +βijxixj Eq. (2)

Where Yi is the predicted response, xixj are input variables,which influence response variable Y; β0 is the offset term; βi is the i^{th} linear coefficient; βii the i^{th} quadratic coefficient and βijthe ij^{th} interaction coefficient. Analysis of data and generation of responce graphs were performed using Design Expert (version 9.1) from Stat-Ease, Inc., Minneapolis, MN, USA.

RESULTS AND DISCUSSION

Plackett Burman design

Optimization medium using statistical designs have long-established to be a powerful and functional tool for biotechnological processes. Culture conditions might have an effect on the fungal physiology and expression of ligninolytic enzymes (Heinzkill and Schinner,1998; Galhaup *et al.,* 2002). So, PBD has applied in order to screen various nutritional factors affecting laccase production. Most significant factors were screened from pareto chart (Fig. 1).The larger the magnitude of T-test and smaller the P-values, higher is the significance of corresponding coefficient. So from P-Value obtained from regression analysis, three nutrients (out of eleven nutrient supplements studied) selected for further optimization of concentration.For fungal growth initiation and metabolism, carbon source is easily available form (Singh and Satyanarayana, 2006). Amongst various carbon source screened for their effect of laccase production maltose was showed significant effect than other carbon source.

Production of ligninolytic enzyme by wood-rotting basidiomycetes regulated by nature and concentration of nitrogen sources(Galhaup *et al.,* 2002) . From the nitrogen

Table 1: Variables showing medium components used in Plackett-Burman design

No.	Coded variables	Variables	High level (+)	Low level (-)
1	A	Sucrose (%)	1.5	0.1
2	B	Maltose (%)	1.5	0.1
3	C	Glucose (%)	1.5	0.1
4	D	Starch (%)	1.5	0.1
5	E	Fructose (%)	1.5	0.1
6	F	Yeast extract (%)	1.5	0.1
7	G	Magnesium Sulphate (%)	0.5	0.05
8	H	Copper sulphate (μM)	100	0
9	I	Ammonium nitrate (%)	0.5	0.05
10	J	Thiamine (%)	0.5	0.05
11	K	Guaiacol (μM)	100	0

Table 2: Placket Burman Design variables and their laccase activity

Sr. No.	Variables (factors)											Laccase activity (U/mL)
	Xl	X2	X3	X4	X5	X6	X2	X8	X9	X10	X11	
1	-1	-1	1	-1	1	1	-1	1	1	1	-1	7.42
2	-1	-1	-1	1	-1	1	1	-1	1	1	1	5.42
3	-1	1	-1	1	1	-1	1	1	1	-1	-1	5.11
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	4.57
5	-1	1	1	-1	1	1	1	-1	-1	-1	1	8.42
6	-1	1	1	1	-1	-1	-1	1	-1	1	1	3.71
7	1	-1	1	1	1	-1	-1	-1	1	-1	1	8.85
8	1	-1	-1	-1	1	-1	1	1	-1	1	1	3.14
9	1	1	-1	-1	-1	1	-1	1	1	-1	1	6.57
10	1	1	-1	1	1	1	-1	-1	-1	1	-1	3.14
11	1	-1	1	1	-1	1	1	1	-1	-1	-1	6.28
12	1	1	1	-1	-1	-1	1	-1	1	1	-1	8.00

Table 3: Full factorial central composite design matrix and theirobserved responses for laccase production

Run	Coded Variables (A- Maltose, B- Ammonium silphate, C- Copper Sulphate			Laccase activity (U/ml)	
	A	B	C	Observed	Predicted
1	1.681	0	0	21.00	17.51
2	0	0	0	29.00	29.31
3	0	0	-1.681	16.20	17.82
4	1	-1	-1	18.30	20.72
5	0	1.681	0	17.49	17.40
6	-1	-1	1	18.00	21.00
7	1	1	-1	16.10	14.90
8	-1.681	0	0	24.10	25.04
9	0	-1.681	0	23.60	21.13
10	-1	-1	-1	24.80	22.74
11	0	0	0	29.30	29.31
12	-1	1	1	23.00	22.38
13	1	-1	1	12.45	14.70
14	0	0	0	29.80	29.31
15	0	0	1.681	21.00	16.81
16	0	0	0	29.30	29.31
17	0	0	0	30.00	29.31
18	1	1	1	11.60	15.44
19	-1	1	-1	18.00	17.56
20	0	0	0	28.00	29.31

sources tested (Fig. 1), ammonium nitrate showed positive effect, whereas yeast extract showed negative effect. Utilizable concentration of nitrogen in the medium regulates the ligninolytic enzymes. The ligninolytic enzyme production can be stimulated by low nitrogen level, while a high nitrogen level represses it (Kirk and Chang, 1990).Vikineswary *et al.,*2006 reported positive effect of nitrogen on growth and enzyme production by fungi. Also enhancement of fungal laccase production using nitrogen sufficient media was reported by Kaal *et al.,*1995. The results obtained in the present study further confirmed by abovefindings.The yield of ligninolytic enzyme increased by addition of the nutrient medium with an suitable inducer (Usha *et al.,* 2014, Patel *et al.,* 2009). The supplementation of an appropriate inducer to nutrient medium maximized the yield of ligninolytic enzyme. Aromatic compounds, such as, vertaryl alcohol, vanillic acid, 2,5 xylidine, ferulic acid and copper causes induction of laccase production by white rot fungi (Pratheebaa *et al.,* 2013, Hess *et al.,* 2002). Laccase inducers like copper sulphate has shown greater influence on laccase production as earlier reported by various researcher (Tychanowicz *et al.,* 2006, Palmieri *et al.,* 2001). In this study, copper sulphate showed high positive effect. It was reported in case of many white-rot species that level of laccase mRNA increases in copper containing nutrient medium, while the growth of the strain get supressed at higher concentration of copper (Collins & Dobson, 1997). According to Baldrian (2006) induction of laccase by copper sulpahte may be because of the involvement of defense mechanism and interaction between metals and fungi. Thus, maltose, ammonium nitrate and copper sulphate were identified as key components for laccase production.Difference in laccase activity in various runs of PBD experiments which call attention to the importance of optimization, and further optimization of concentration of major variable for higher production.

Response surface methodology

Central composite design was employed to optimized the concentration of major variable (maltose, ammonium nitrate, copper sulphate) on laccase production. The effect of each variables was studied at five levels (-±, -1, 0 +1, -±) with set of

Table 4: ANOVA for regression model

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value
Model	585.25	9	65.03	7.86	0.0017 [a]
A-Maltose	68.40	1	68.40	8.26	0.0165 [a]
B-Ammonium Nitrate	16.75	1	16.75	2.02	0.1853
C-Copper sulphate	1.22	1	1.22	0.15	0.7094
AB	0.20	1	0.20	0.024	0.8810
AC	9.14	1	9.14	1.10	0.3181
BC	21.62	1	21.62	2.61	0.1372
A^2	116.19	1	116.19	14.04	0.0038
B^2	181.44	1	181.44	21.92	0.0009 [a]
C^2	258.58	1	258.58	31.24	0.0002[a]
Residual	82.77	10	8.28		
Lack of Fit	80.27	5	16.05	32.20	0.0008 [a]
Pure Error	2.49	5	0.50		
Correlation Total	668.02	19			
R^2 – 0.87, Adj. R^2- 0.76, CV- 6.05%					

a- Significant

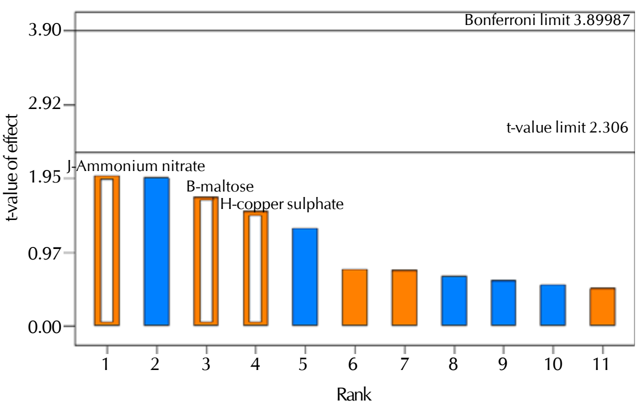

Figure 1: Pareto chart of effect of eleven factor on laccase production

20 experiment was performed in triplicates. Table 3 shows full factorial central composite design matrix and their observed responses for laccase production.

The determination coefficient (R^2) value of 0.87 indicates that the model was able to explain 87.61% of the variability in the response. Therefore, the present R^2 value revealed a great fit between the observed and predicted. Also the adjusted determination coefficient (adj. R^2= 0.76) was also high, indicating the high significance of model.ANOVA for the selected quadratic model showed that the model was significant with Model F=7.86 and P>F- value>0.0017. Thus the response of laccase production could be expressed in terms of following equation. Table 4 illustrates the ANOVA of the regression model. Several criteria were used to check goodness of fit of the model. Prob> F less than 0.05 indicates that the model terms are significant. In this case, maltose (the carbon source) had a significant effect on laccase yield (P<0.0001). Lack of fit F-value 32.20 implies that the lack of fit is significant. Adequate presicion measures signal to noise ratio. Ratio greater that 4 is desirable. In this study ratio of 7.18 indicates an adequate signal.In Table 4, it can be seen that the variables with higher effect were squared terms of ammonium nitrate concentration (X_2^2), chased by linear terms (X_2). Higher significance of the squared terms (X_2^2) over corresponding linear terms (X_1, X_2 and X_3) shows that the optimum values for enzyme production lies within the values chosen for experiment (Kaal *et al.,* 1995, Pratheebaa *etal.,* 2013). The interaction terms A*C & A*Bseems to be insignificant, which can be neglected from the model without affecting the goodness of the fit. Maltose was significant, whereas maltose, ammonium nitrate and copper sulphate interact insignificantly. The adjusted determination coefficient (R^2=0.76) was also satisfactory to confirm the significance of the model. Finally, the lower value of coefficient of variation (C=6.05%) shows that the experiments were precise and reliable.Data obtained in RSM were subjected to ANOVA and the results were used to fit a second order polynomialis given below.

$$La = 29.31-2.24*X_1-1.11*X_2-0.30*X_3-0.16*X_1X_2-1.07*X_1X_3+1.64*X_2X_3-2.84*X_1^2-3.55*X_2^2-4.24*X_3^2$$

Where X1 is maltose, X2 is ammonium nitrate and X3 is copper sulphate. 2D response plots was drawn based on the model equation to investigate interaction among the variables and determine the optimum concentration of each factor for maximum laccase production by DK14.

In order to gain a better understanding of the effects of the variables on the production of laccase, the predicted model was presented as response surface graphs (Contour plot). Contour plots (Fig.2) shows the interaction between variables.Change in level of one variable influences the level of other variable for fixed level of enzyme production is known as interaction between variables.

The optimum level of nutrient medium for laccase production were 2% for maltose, 0.8% for ammonium nitrate and 180 μM for copper sulphate to obtain enzyme activity of 29.8 U mL^{-1} in addition to basal medium.An improvement in laccase production from 8.85 U mL^{-1} (PBD) to 29.8 U mL^{-1} was obtained after optimization using RSM. In a previous work, Palvannan and Sathish Kumar (2010), Pratheebaa *et al.,* 2013 have also optimized the production of laccase by *P. florida* and *P. osreatus* at maximum of 4.8 U mL^{-1} and 2.7 U ml^{-1}respectively using RSM. In another study, Shridhar *et al*, 2012 optimized laccase production under solid state fermentation using PBD (4.25 U gm^{-1}). In addition, Pointing and Vrijmoed (2000)reported maximum laccase production using

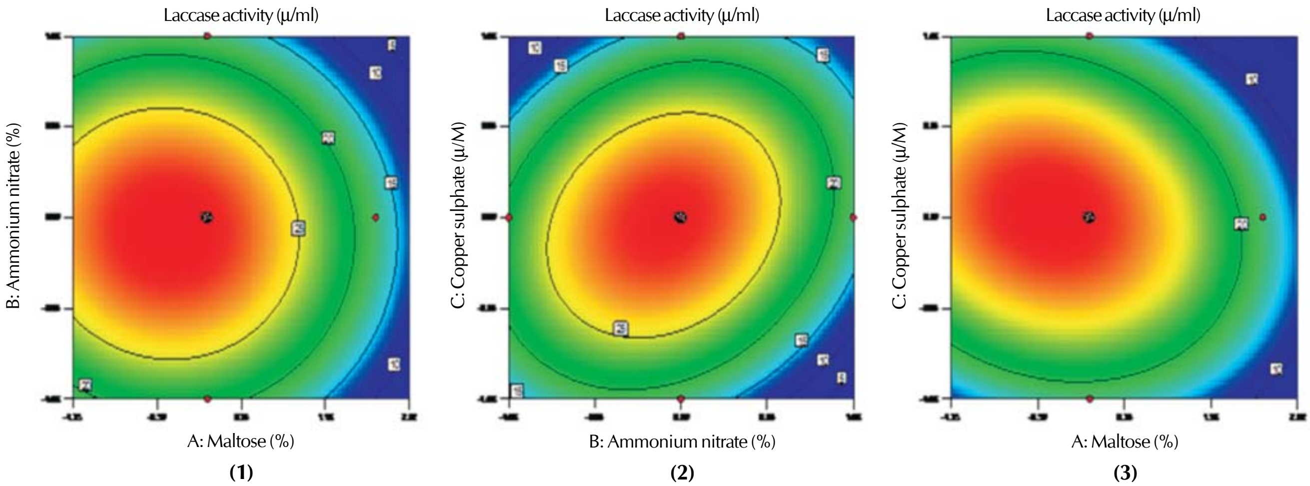

Figure 2: Response Surface Plots showing effect of (1) Maltose and Ammonium Nitrate, (2) Ammonium Nitrate and Copper Sulphate, (3) Maltose and Copper Sulphate

Pycnoporus sanguineus in medium containing high carbon and low nitrogen. Hence, it is clear from the present study that carbon, nitrogen and inducer when supplemented in properconcentration can serve as a absoluteblend of nutrient to get higher laccase production. In Fig. 2(2-3) an elliptical nature of the contour plot specifies that the interaction between the corresponding variable are significant, which shows that at low concentration of all variableslaccase activity increases. In Fig.2(1), the contour plots are not completely elliptical in nature. Accuracy of model was confirmed by validation experiment using software tool as the predicted and experimental value are in good agreement.

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