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Optimization of cultural conditions for enhanced production of laccase by *Aspergillus flavus* M*af* 0139

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ABSTRACT

Laccase enzyme has a range of biotechnological and industrial applications but its high cost hindered its use. Development of low-cost biotechnological process for laccase production has gained great attention. Present study is concerned with the enhanced production of laccase by *Aspergillus flavus* M*af* 0139. Five different cultural media were tested and best one was selected for laccase production. Different cultural conditions such as inoculum size, pH, temperature, incubation period, moisture content and inducer were studied. Medium components were further optimized by Plackett-Burman and central composite designs. Results showed that maximum enzyme activity (297±0.1 U/ml/min) was obtained by using AFM02 medium containing g/L: sawdust, 5.0; glucose, 5.0; peptone, 6.0; $MgSO_4.7H_2O$, 0.25; KCl, 0.5; KH_2PO_4, 0.025. Optimized conditions for maximum enzyme yield were inoculum size 2.5%, Temperature 25˚C, pH 4.5, incubation period 7 days and moisture content 60%. Eleven different inducers were evaluated but $FeSO_4.7H_2O$ showed maximum enzyme production (2156±0.57 U/mL/min). By using these statistical techniques more than 13 folds increase in enzyme yield (4022±0.25 U/mL/min) was observed. It is concluded that cultural conditions have great influence on enzyme production and enzyme yield could be enhanced by using advanced statistical techniques.

Keywords: Multicopper oxidase, lignocellulosic waste, Solid state fermentation, extracellular enzyme

INTRODUCTION

Laccases are blue multicopper oxidases having potential to oxidize various phenolic and diphenolic substances and reduce molecular oxygen to water (Arora & sharma, 2010; Songulashvili *et al*., 2016; Nguyen *et al*., 2016). Release of water as a byproduct increasse its significance for its use in various industrial applications as a 'green' catalyst (Surwase *et al*., 2016). Use of inexpensive raw materials in the form of agroindustrial or lignocellulosic waste (Arora & Sharma, 2010; Kumar *et al*., 2013; Daassi *et al*., 2016) for enzyme production make the whole process cost effective. Solid state fermentation is a better option for fungal cultivation as metabolite produced are more concentrated, economical purification procedures, easier product recovery and simple process (Pandey *et al*., 2000). One of the major advantage of solid state fermentation is high yield of enzyme (Ergun and Urek, 2017). Optimization of cultural conditions like inoculum size, pH, temperature, moisture content, incubation period play critical role in enzyme production. In contrast to an efficient but intricate and costly tool of bioengineering, addition of inducer either in the form of aromatic compounds and metal ions is supposed to be a simple and cost-effective approach to enhance enzyme yield (Levin *et al*., 2010; Baldarian & Gabriel, 2002; Hou *et al*., 2004). The optimization of medium components by one variable at a time is laborious and time consuming approach, often the effect of interaction between various components are overlooked. To overcome this problem advanced statistical experiments like Plackett Burman and Central Composite designs are effective to get information in minimum experimental runs (Yahya *et al*., 2016). Plackett-burman design is used to evaluate the effective components of medium and significant factors obtained after screening were further optimized for their concentration by central composite design. Although extensive data has been reported on the enhanced production of laccase by white rot fungi but only limited work is available on brown rot fungi. Present study is designed to optimize the physicochemical

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parameters to enhance the production of laccase enzyme from brown rot fungi.

MATERIALS AND METHODS

Organism

The strain *Aspergillus flavus* M*af* 0139 was previously isolated from decaying wood of *Mangifera indica.* Culture was maintained on freshly prepared potato dextrose agar (PDA) slants and stored in refrigerator at 4˚C.

Fermentation technique

The extracellular laccase production from *Aspergillus flavus* M*af* 0139 was carried out in shake flask using five different fermentation media. Chemical composition of media components expressed in g/L.

AFM01: Sucrose, 10; peptone, 5.0; NH_4NO_3, 1.0; $CaCl_2$, 1.0; KH_2PO_4, 1.0; $MgSO_4$, 1.0; $FeCl_3$, 1.0; $CoCl_2$, 1.0; $ZnCl_2$, 1.0; KCl, 1.0; $CuSO_4.7H_2O$, 1.0; $BaCl_2$, 1.0; $HgCl_2$, 1.0; $ZnSO_4$, 1.0

AFM02: Sawdust, 5.0; Glucose, 5.0; Peptone, 6.0; $MgSO_4.7H_2O$, 0.25; KCl, 0.5; KH_2PO_4, 0.025

AFM03: Glucose, 10.0; KH_2PO_4, 0.025; Peptone, 2.0; Potato dextrose broth, 24; $MgSO_4.7H_2O$, 0.5

AFM04: Glucose, 10.0; L. Histidine, 0.5; NaCl, 1.8; $NaNO_3$, 1.8; KCl, 0.5; $CuSO_4.7H_2O$, 7.0; $CaCl_2$, 0.5; $FeSO_4.7H_2O$, 0.05; Glycerol, 7.5; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5

AFM05: Rice bran, 5.0; Distilled water, 6.0Ml

These fermentation media were prepared in 250mL Erlenmeyer flasks. The flasks were autoclaved (HVA 110, JAPAN) for 15 min at 121 ˚C. The medium was allowed to cool at room temperature and then inoculated with 2.5mL of fungal spore suspension under aseptic conditions and kept for seven days in an incubator at 25±1˚C. After seven days of incubation the cultural broths of these five media were centrifuged (Hettic EBA 8S, GERMANY) at 6000 rpm for 15 minutes and the supernatant obtained was used for enzyme assay.

Different nutritional conditions and process parameters such as inoculum size (0.5-3%v/v) with interval of 0.5 units, temperature (20, 25, 30 and 35°C), incubation period (5, 7, 9, 11, 13 and 15 days), pH (3.5, 4.5, 5.5, 6.5 and 7.5), initial moisture content (10, 20, 40, 50, 60 and 70%) of the medium was established by adding salt solution before autoclaving the medium, effect of different inducers (Ethanol, methanol, isopropanol, acetone, Tween 80, $CaCl_2.2H_2O$, $NaNO_3$, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, glucose, $CuSO_4.7H_2O$, glycerine and Triton X and their concentrations at 1, 1.5 and 2% w/v were optimized to scale up the laccase production.

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Laccase enzyme assay

Laccase enzyme assay was performed according to the method of Khammuang & Sarnthima, (2007). The reaction mixture contained 0.5 ml of enzyme, 940 µl of 0.1 M sodium acetate buffer of pH 4.5 and 10µl of 10mM ABTS [(2, 2-azinobis (3-ethylbenzthiazoline-6-sulphonate) diammonium salt]. The reaction mixture was incubated at 30˚C in water bath for 10 minutes. The rate of ABTS oxidation was measured spectrophotometrically at 420nm.

Plackett-Burman Design

For screening of optimized medium (AFM02) components Plackett- Burman design was applied. This experiment was conducted in 12 trials. Optimized medium has six components and the effect of each component on laccase production was studied by missing (-) one or two components in each trial as given in Table I. Each trial was performed in 250 ml conical flask. Medium was inoculated with fungal spore suspension and kept at 25°C for 168 hrs. in static conditions.
Minitab version 18.0 was used for analyzing the data. All experiments were performed in triplicates and the mean of laccase production were used as response of experiment (dependent variable). Plackett Burman design is based on first order model as shown in equation 1.

$$Y = \beta_0 + \Sigma \beta i\, xi \text{ ---------- (1)}$$

Where,
Y= Dependent variable (enzyme activity)
$\beta_{0=}$ Model intercept
i= Variable number
βi= Variable estimated coefficient
xi= Independent variables

For determination of statistical significance of the model and significance of each term in equation, Student's t test was used to determine the significance of regression co-efficient. R^2 value evaluated the adequacy of the model. Variables having P value less than 0.1 had significant effect on enzyme production (Mathur *et al*., 2013; Yahya *et al*., 2016).

Central composite design

Central Composite design was applied to optimize the concentration of medium components selected in the placket Burman design. The variables used in this experiment are shown in Table III. Effect of increase or decrease in concentration of significant factors of medium components was determined by enzyme activity (dependent variable). Validity of the model was evaluated by R^2 value which is Coefficient of determination (Sharma *et al*., 2017).

Statistical analysis

All results are the mean of triplicates and expressed as mean ± S.D using Microsoft excel 2010 and Minitab version 18.0.

Results and Discussion

Selection of Fermentation medium

Enzyme production varies according to the macro and micronutrients of the fermentation medium. To optimize best medium for laccase production by *Aspergillus flavus* M*af* 0139 five different fermentation media AFM01 (Vivekanandan *et al*., 2014), AFM02 (Daassi *et al*., 2014), AFM03 (Nadeem *et al*., 2014), AFM04 and AFM05 (Devi *et al*., 2012) were evaluated (Figure 1). It was observed that the basal medium (AFM02) having sawdust as a solid substrate gave maximum enzyme production (297±0.1 U/mL/min) whereas, the enzyme productivity in the other basal media i.e., AFM01, AFM03, AFM04 and AFM05 was 75±0.1, 19±0.15, 178±0.5, 155±0.2 U/mL/min, respectively. Our results are in close agreement with Daassi *et al*. (2016). Ado *et al*., 2018 also found sawdust as a best solid support for laccase production by *Trametes* sp.

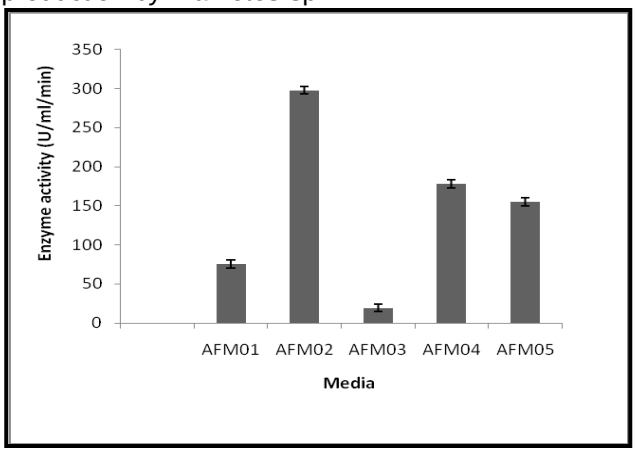

Fig. 1: Effect of cultural media on the production of laccase from *Aspergillus flavus* M*af* 0139

Size of inoculum

Effect of inoculum size on laccase production was studied ranging from 0.5 to 3.0 % (v/v). Results showed that maximum laccase productivity (670±0.15 U/mL/min) was achieved with 2.5% size of inoculum as shown in Figure 2. Further increase to 3.0% resulted in decrease of the enzyme yield (480±0.2). Vantamuri & Kaliwal, 2016 reported that highest laccase enzyme production (1.4 U/ml) was obtained when 2000µl of inoculum was added in 5g of rice bran. Enzyme yield decreased with the increase in inoculum size due to the accumulation of toxic metabolites and rapid utilization of substrate. These results are in accordance with Deb *et al*. (2013).

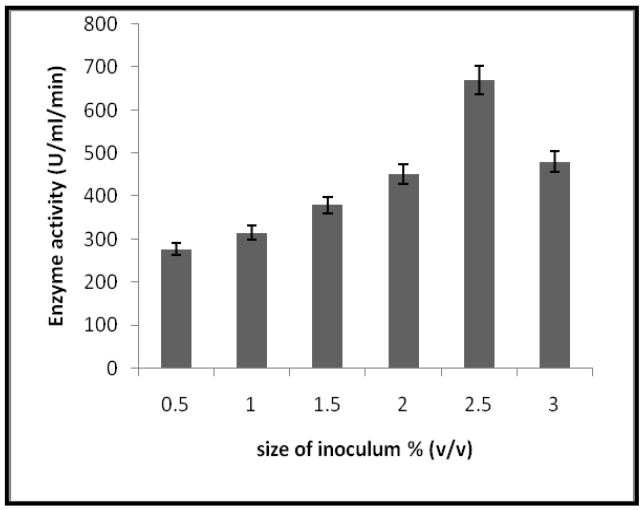

Fig. 2: Effect of inoculum size on the production of laccase from *Aspergillus flavus* M*af* 0139

Effect of temperature

The production of laccase from *Aspergillus flavus* M*af* 0139 at 20-35˚C temperature range revealed that maximum enzyme production (890±0.2 U/ml/min) was achieved at 25˚C as shown in Figure 3. Increase in temperature to 30˚C resulted decrease in enzyme production (760±0.1U/mL/min) and significant decrease in enzyme production (192±0.15U/mL/min) was observed with increase in temperature to 35˚C. The increase in temperature decreases enzyme production because high temperature alters the composition of cell membrane and stimulates protein catabolism (Nadeem *et al*., 2014; Edae and Alemu, 2017). The trend of high laccase production at low temperature and decrease in production at high temperature was also reported by other workers (Adejoye and Fasidi, 2010; Patel *et al*., 2009; Ado *et al*., 2018).

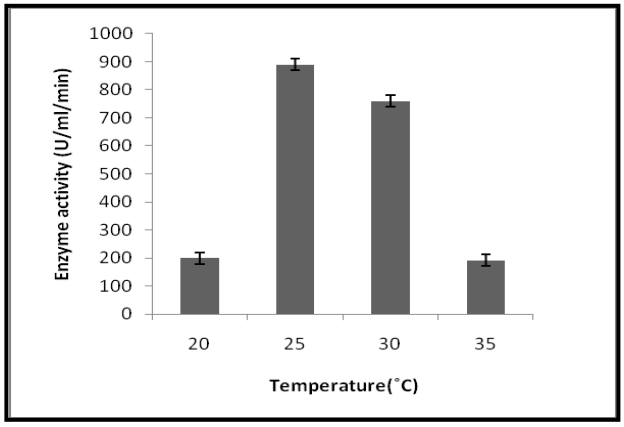

Fig. 3: Effect of temperature on the production of laccase from *Aspergillus flavus* M*af* 0139

Effect of pH

pH is an imperative factor and had great influence on enzyme production (Sivakumar *et al*., 2010). The fungus was cultivated at different pH ranging from 3.5-7.5. It was observed that laccase gave maximum enzyme production (896±0.1 U/mL/min) at pH 4.5 as shown in Figure 4. These results are in line with the Stoilova *et al*., (2010) who revealed that *Trametes versicolor* express maximum activity at pH 4.5. In another study, *Trametes* sp. gave maximum laccase production (2356 U/ml) at pH 5.0 (Ado *et al*., 2018). Further increase in pH decreases laccase production due to the fact that increase in pH is unfavourable for fungal growth. Increase or decrease in pH effected enzyme production due to changes in the structure of enzyme. Amount of soluble proteins and the fungal biomass gradually decreased with the increase in fermentation pH. These results are in accordance with other workers (Minussi *et al*., 2001; Yang *et al*., 2011).

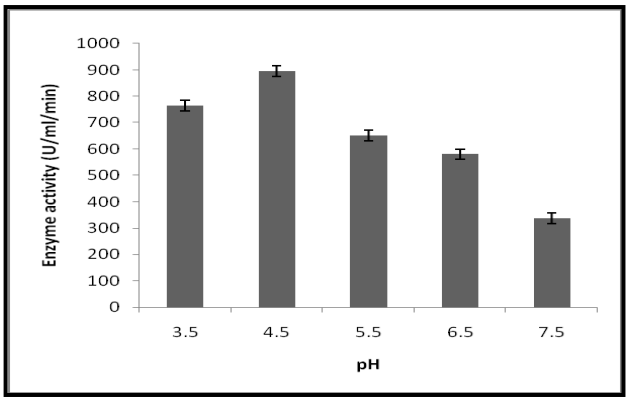

Fig. 4: Effect of pH on the production of laccase from *Aspergillus flavus* M*af* 0139

Effect of inducer

The addition of inducer is an effective approach to increase enzyme production. Different inducers such as ethanol, methanol, isopropanol, acetone, Tween 80, Triton X, $CaCl_2$, $MgSO_4$, $FeSO_4.7H_2O$, $CuSO_4.7H_2O$ and Glycerine were tested for their potential to induce laccase production (Figure 5). It was observed that in comparison to all other inducers, $FeSO_4.7H_2O$ gave maximum enzyme production of 1156±0.15 U/mL/min. whereas addition of acetone and copper sulphate as inducer showed medium productivity of 761±0.1and 684±0.3 respectively. According to Fonesca *et al*. (2010) laccase production was induced by iron and copper ions through post translational and translational regulation. It has been reported that expression of fungal laccases is regulated by Fe^{+2} at transcription level (Akpinar & Urek, 2017). In high affinity iron (Fe) uptake the reductive iron assimilation system plays important role in *Aspergillus*. This assimilation system consists of Ferric reductase, ferrooxidases and Fe permease (Hass, 2012; Blatzer *et al*., 2011; Bailao *et al*., 2015). Further increase in the production of enzyme was studied by the addition of inducer at different concentrations (1%, 1.5% and 2%) as shown in Figure 6. The optimum level of inducer concentration was found to be 1.5% (w/v) and the amount of enzyme produced was (2156± 0.2 U/ml/min). Further increase in the concentration of inducer to 2% reduced the laccase production (184± 0.5 U/ml/min).

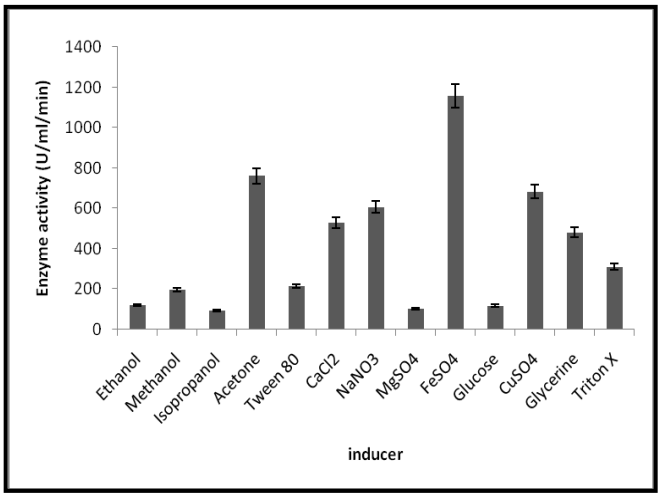

Fig. 5: Effect of different inducers on production of laccase from *Aspergillus flavus* M*af* 0139

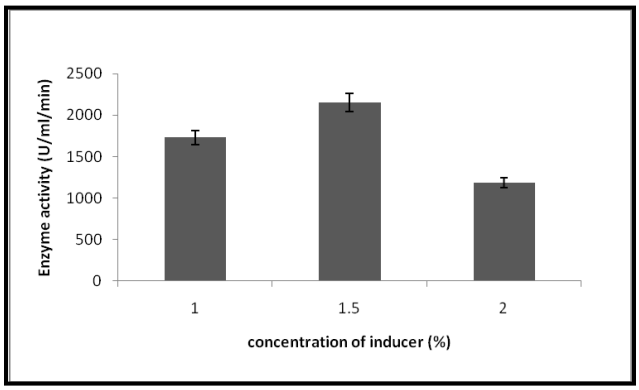

Fig. 6: Effect of concentration of inducer on production of laccase from *Aspergillus flavus* M*af* 0139

Effect of moisture content

In solid state fermentation initial moisture content played a vital role and affected significantly on laccase production and substrate utilization. Laccase produced by *Aspergillus flavus* M*af* 0139 was positively affected by high moisture content. Increase in moisture content from 30-60% remarkably increased laccase production. Maximum laccase production (2362±0.15 U/mL/min) was achieved at 60% moisture content as shown in Figure 7. However, increase in moisture content above 60% decreased laccase production (2318±0.3 U/mL/min) because porosity of the substrate was reduced due to increase in moisture content and also limiting oxygen transfer. In an earlier study it was reported that optimum moisture content was found to be 60% when the *Pleurotus ostreatus* was grown on wheat straw (Patel *et al*., 2009). Our results are consistent with Vantamuri & Kaliwal, 2016 who reported that 65% initial moisture content of the substrate (rice bran) was optimized for production of laccase by *Marasmius* sp.

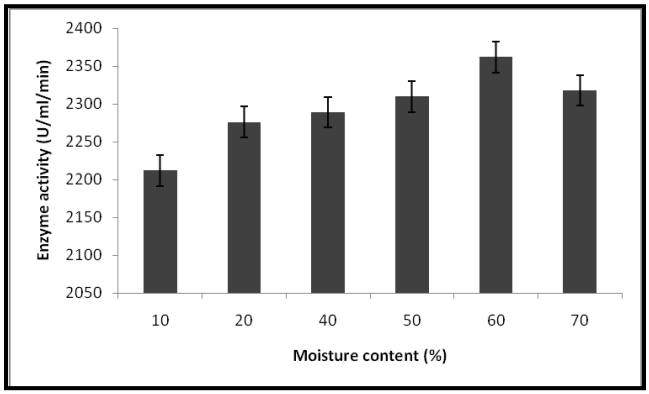

Fig. 7: Effect of moisture content on production of laccase from *Aspergillus flavus* M*af* 0139

Time course fermentation

To determine the effect of incubation period on laccase production by *Aspergillus flavus* M*af* 0139 shake flasks were incubated for different time intervals (5, 7, 9, 13, 15 days) at 25˚C. On 5th day of incubation, the amount of laccase was 2658±0.25 U/mL/min and increased (3217±0.12 U/mL/min) after 7 days of incubation (Figure 8). Further increase in the time course did not enhance enzyme production and resulted in significant decrease (1040±0.15 U/mL/min) after 15 days of incubation. In an earlier study, maximum laccase production in *Lentinus edodes* and *Ganoderma* sp. was also obtained on the 7th day of incubation (Sivakumar *et al*., 2010). According to Nadeem *et al*., 2014 sharp increase in laccase production by *P. ostreatus* was observed from 2nd to 6th day of incubation.

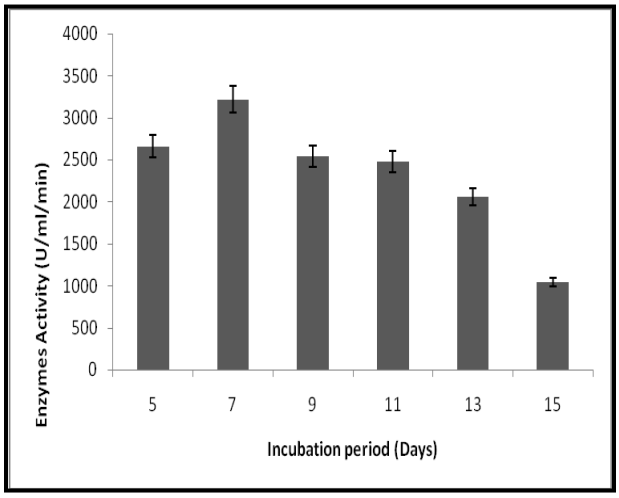

Fig. 8: Effect of time course on production of laccase from *Aspergillus flavus* M*af* 0139

Optimization of fermentation medium by Plackett- Burman and central composite design

Medium components for laccase production by *Aspergillus flavus* M*af* 0139 in solid state fermentation were optimized by Plackett-Burman and central composite design. Statistical approach helped us to enhance enzyme yield resulting in the reduction of production cost making the fermentation process cost effective (Kaur & Satyanarayana, 2005). The screening of multiple independent variables in a single experiment is the main advantage of Plackett-Burman design. Independent variables (medium components) including sawdust, peptone, glucose, KH_2PO_4, KCl and $MgSO_4.7H_2O$ were evaluated in six factors Plackett-Burman design (Table I) and result was that all these ingredients constituted positive effect

on the production of laccase having maximum activity 2675±0.1 U/ml/min. The effect of each variable at significant level was determined by student t test (Table II).

Further, concentration of medium components was optimized by central composite design as shown in Table III. The components of trial, 11 showed positive effects on the production of enzyme and enzyme yield was 4022±0.25 U/ml/min. Central composite design for optimization of medium concentration has also been reported by other workers for different fungal strains (Gao *et al*., 2013; Vivekanadan *et al*., 2014; Bagewadi *et al*., 2017). Analysis of variance was performed in order to validate the regression model (Table IV).

Table I: Plackett-Burman experiment of sawdust supplements assigned different levels of factors. Fermentation conditions: inoculums size: 2.5%, incubation time: 7days, temperature: 25˚C and medium AFM02

PLACKETT- BURMAN DESIGN							
Trials	**Sawdust (g/L)**	**Glucose (g/L)**	**KH_2PO_4 (g/L)**	**KCl (g/L)**	**$MgSO_4.7H_2O$ (g/L)**	**Peptone (g/L)**	**Enzyme Activity (U/mL/min)**
1	5	5	0.025	0.5	0.25	6	2675
2	-	5	0.025	0.5	0.25	6	64.1
3	5	-	0.025	0.5	0.25	6	2378
4	5	5	-	0.5	0.25	6	204
5	5	5	0.025	-	0.25	6	237
6	5	5	0.025	0.5	-	6	1731
7	5	5	0.025	0.5	0.25	-	36.9
8	5	-	0.025	0.5	-	6	1993
9	5	5	-	0.5	-	6	29.1
10	-	5	0.025	0.5	-	6	46.6
11	5	-	-	-	0.25	6	33
12	5	5	0.025	-	0.25	-	42.7

Table II: Regression analysis for the Plackett-Burman design.

Term	Coef	SE Coef	T-Value	P-Value
Intercept	28.16	2.89	9.73	0.001
Sawdust	-0.756	0.275	-2.74	0.052
Glucose	-0.396	0.143	-2.76	0.051
KH_2PO_4	-170.7	48.8	-3.50	0.025
KCl	-10.91	1.86	-5.88	0.004
$MgSO_4.7H_2O$	-23.72	2.47	-9.60	0.001
Peptone	-1.254	0.2033	-6.17	0.004

Coefficient of determination, r^2= 0.97

Table III: Central composite design for five sawdust supplements, assigned different levels of factors. Fermentation conditions: inoculums size: 2.5%, incubation time: 7 days, temperature: 25˚C and medium AFM02

CENTRAL COMPOSITE DESIGN							
Trials	**Sawdust (g/L)**	**Glucose (g/L)**	**KH_2PO_4 (g/L)**	**KCl (g/L)**	**$MgSO_4.7H_2O$ (g/L)**	**Peptone (g/L)**	**Enzyme activity (U/mL/min)**
1	5.0	5.0	0.025	0.50	0.25	6.0	1399
2	5.0	5.0	0.025	0.50	0.25	24	1608
3	5.0	2.5	0.025	0.50	0.25	6.0	1958
4	5.0	5.0	0.0125	0.50	0.25	6.0	752
5	5.0	5.0	0.6	0.25	0.25	6.0	1434.5
6	5.0	5.0	0.6	0.50	0.125	6.0	1976
7	2.5	5.0	0.6	0.50	0.25	2.5	1608
8	5.0	5.0	0.0125	0.50	0.25	6.0	1661
9	4.5	2.5	00125	0.50	0.25	6.0	1434
10	5.0	5.0	0.0125	0.1	0.25	6.0	119
11	5.0	5.0	0.125	0.05	0.25	6.0	4022

Table IV: Regression analysis for central composite design

Term	**Coef**	**SE Coef**	**T-Value**	**P-Value**
intercept	128.2	49.1	2.61	0.080
Sawdust	-12.03	5.06	-2.37	0.098
Glucose	0.476	0.988	0.48	0.663
KH_2PO_4	-118.2	55.3	-2.14	0.122
KCl	-11.82	5.25	-2.25	0.110
$gSO_4.7H_2O$	-232	104	-0.76	0.110
Peptone	-0.108	0.142	-0.44	0.503

Coefficient of determination, $r^2 = 0.84$

CONCLUSION

It is concluded that the isolated fungus *Aspergillus flavus* M*af* 0139 is an active laccase producer under solid state fermentation rather than submerged fermentation. The present study proved that sawdust, a cheap and inexpensive lignocellulosic waste, can be utilized for the production of valuable enzyme. Optimization of cultural conditions and use of an appropriate inducer enhanced laccase production and its hyperproduction could be achieved by optimization of medium constituents in minimum experimental runs using advanced statistical techniques.

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