Int J Pharm Bio Sci 2014 Jan; 5(1): (B) 801 - 809

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

OPTIMIZATION OF CULTURE CONDITIONS AND MEDIA COMPONENTS FOR LACCASE PRODUCTION USING WOOD ROTTING FUNGAL ISOLATE

PRIYANKA PUSHKARAJ VARTAK AND DR. DEBJANI DASGUPTA*

Department of Biotechnology and Bioinformatics,
Padmashree Dr. D. Y. Patil University, Navi Mumbai, India.

ABSTRACT

The present work focuses on optimizing the culture conditions and media components for enhanced production of laccases using wood rotting fungal isolate. Parameters such as shaking or static conditions, agitation rate, pH and time for higher enzyme production were monitored. Medium for optimum laccase production was analysed using one factor at a time method and Taguchi orthogonal array method. One factor at a time method indicated that maltose, meat extract and ammonium sulphate were optimal carbon and nitrogen sources. 1% maltose, 0.750% meat extract, 0.124% ammonium sulphate $(NH_4)_2SO_4$ and 0.1% potassium phosphate (KH_2PO_4) are found to be optimum concentrations for laccase production by Taguchi method. Under these optimized conditions laccase production increased by 49%. Enzyme production using various inducers showed that 2,6-xylidine was the most effective inducer.

KEYWORDS : laccase, wheat bran, 2,6-xylidine, basidiomycetes

DR. DEBJANI DASGUPTA
Department of Biotechnology and Bioinformatics,
Padmashree Dr. D. Y. Patil University, Navi Mumbai, India.

INTRODUCTION

Laccases (phenol oxidases; E.C. 1.10.3.2.), also known as multicopper blue oxidases, belong to the oxidoreductase group of enzymes. Biochemically, they are glycoproteins carrying molecular mass between 50kDa and 130kDa [1]. Laccase has broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups. These enzymes were known to catalyze the oxidation of a wide range of phenolic compounds and aromatic amines [2]. Laccases are widely distributed in higher plants, bacteria, fungi, and insects. They have been isolated from Ascomycetes, Deuteromycetes and Basidiomycetes to which more than 60 fungal strains belong [3]. Most of the laccases characterized so far have been derived from fungi mainly from white rot basidiomycetes especially from *Phlebia radiata, Pleurotus ostreatus, Trametes hirsuta*, *Trametes versicolor, Phanerochaete chrysosporium, Theliophora terrstrus, Stereum ostrea, Lenzitis betulina* and *Pycnoporus* species [4]. In basidiomycetes, extracellular laccases are constitutively produced in small amounts and they are affected by many fermentation factors like culture conditions and media composition [5]. Culture conditions were optimized by monitoring shaking or static conditions, pH, agitation rate. Media components were optimized using one factor at a time (OFAT) and Taguchi orthogonal array matrix method [5]. The advantage of Taguchi method is that quantitative information can be extracted by only a few experiment trials. Taguchi method is one of the powerful optimization techniques and requires half the time required for RSM (Response Surface Methodology) [6,7]. It can be used to evaluate the relative importance of various factors and their concentrations on laccase production. The optimization of medium to reduce the time and cost of laccase production is the basic research in the industrial applications [8]. Hence the objective of the present study was to optimize the culture conditions for laccase production by wood rotting fungal isolate by applying OFAT and Taguchi orthogonal matrix method.

MATERIALS AND METHODS

(i) Fungal strain

The fungus used in this study was isolated from a live jujube (*Zizyphus mauritiana*) tree in suburbs of Mumbai. It was maintained on malt extract agar (MEA) plates grown at 30°C (± 4 °C) and stored at 4 °C. Fungal culture was periodically sub-cultured at every 30 days interval.

(ii) Laccase Production Medium

The laccase production medium had following composition : 4.5% (wt/vol) wheat bran, 1.5% yeast extract, 1% glucose, 0.25% NH_4Cl, 0.05% thiamine dichloride, 0.2% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.01% $CaCl_2$ and 0.05% KCl. The pH was adjusted to 5.0 by using NaOH or HCl. Incubation was carried out at 30°C (± 4 °C) on a rotary shaker (100 rpm) in 250 ml Erlenmeyer flask containing 100 ml of media, inoculated with one agar disk taken from active borders of MEA fungal cultures. Cultures were harvested after 7 days, filtered through whattman filter paper to remove the mycelia and centrifuged at 8,000 x g for 20 min and the enzyme activity determined.

(iii) Extracellular Laccase activity

Laccase activity was determined using 2, 6 - dimethoxyphenol (DMOP) as a substrate. The reaction mixture contained 1 mM DMP in 50 mM sodium malonate (pH 4.5). The formation of 2, 2' 6, 6' dimethoxyphenoquinone (orange /brown color) at 30°C was followed spectrophotometrically at 468 nm wavelength, and laccase activity was calculated from the molar extinction co-efficient (ε) of 49.6 mM cm^{-1} [9]. One unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of 1 µmole of DMOP per minute [17].
All values are mean of duplicates.

(iv) Optimization of Culture conditions for Laccase production

a) Influence of Shaking or Static condition

Inorder to find optimal condition for laccase production both shaking (100 rpm, temperature 30°C ± 4°C) as well as static conditions (temperature 30°C ± 4°C) were monitored.

b) Influence of pH

Laccase production was carried out at different pH levels ranging from 4.5, 5.0, 5.5, 6.0 and optimum pH was determined.

c) Influence of agitation rate

Fungal culture medium was subjected to agitation at 100, 120 and 150 rpm (rotations per minute) and laccase activity was assessed.

d) Incubation time course study

To find the optimal time of incubation for the maximum laccase production, the culture medium was inoculated for a period of 10 days. The culture was harvested at every 1 day interval and laccase activity determined.

(v) Optimization of media components

Media components were optimized by using two methods as follows

(a) One factor at a time method (OFAT)

OFAT involvles changing the independent variable while fixing the others at certain levels [5]. Accordingly effect of different carbon sources like starch, fructose, maltose, glycerol, cellulose and sucrose was tested. The standard medium with glucose was used as control and activities compared. Influence of different organic nitrogen sources viz. meat extract, beef extract and peptone on laccase production was assessed. Various inorganic nitrogen sources investigated were ammonium sulphate ($(NH_4)_2SO_4$), diammonium orthophosphate and ammonium nitrate.

(b) Taguchi Orthogonal Matrix method

This method optimizes the concentrations of different factors and their impact on enzyme production. Four factors viz. maltose, meat extract, $(NH_4)_2SO_4$ and KH_2PO_4, selected by OFAT method, were taken for an orthogonal array design of $L_9(3^4)$, which was developed by MINITAB 15 software. Table 1 and 2 shows the design of $L_9(3^4)$ array with concentrations of different media components. Accordingly 9 set of experiments were performed and different laccase activities obtained were analysed with MINITAB 15 software.

Table 1
$L_9(3^4)$ orthogonal array design

Run	(A)Maltose	(B)Meat Extract	(C) $(NH_4)_2SO_4$	(D) KH_2PO_4	Laccase Activity (U/ml)
1	1	1	1	1	2.059
2	1	2	2	2	2.216
3	1	3	3	3	2.017
4	2	1	2	3	1.993
5	2	2	3	1	4.435
6	2	3	1	2	5.294
7	3	1	3	2	3.174
8	3	2	1	3	3.993
9	3	3	2	1	2.875

The results are reported as the averages of two values.

Table 2
Selected factors and assigned levels

Level	(A)Maltose (g/L)	(B)Meat Extract (g/L)	(C)$(NH_4)_2SO_4$ (g/L)	(D)KH_2PO_4 (g/L)
1	0.50	0.375	0.124	0.050
2	1.00	0.750	0.248	0.100
3	2.00	1.500	0.374	0.200

(vi)Incubation time course study on optimized medium

To verify whether the medium optimized by orthogonal array method was optimal, time course study was carried out for 10 days and laccase activity checked at every 1 day interval.

(vii) Effect of inducers on Laccase activity

Three inducers were investigated for their capacity to increase enzyme activity in fungal cultures: guaiacol, $CuSO_4$ and 2,6-xylidine. One control was used without the addition of any inducer. The compounds were sterilized by filtration using a Millipore membrane (0.2 µm) and added aseptically into the flasks. Seven days after inoculation the laccase activity was determined.

RESULTS AND DISCUSSION

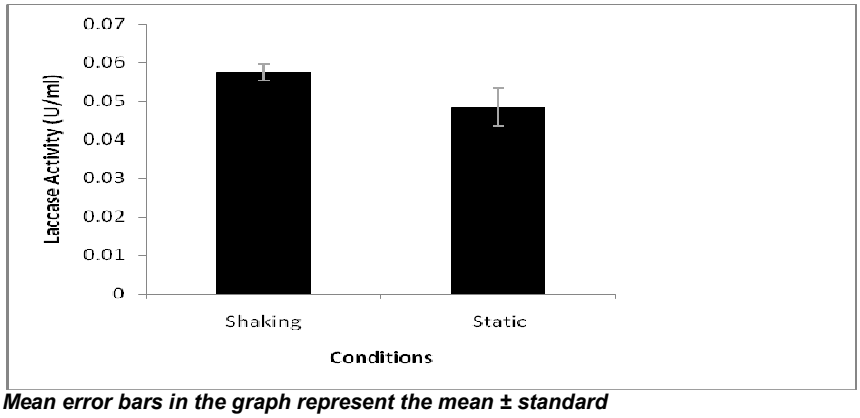
Laccase production in fungi is mainly influenced by culture conditions, the concentrations of different media components and the presence of inducers.

(i) Optimization of culture conditions

The laccase activity increased from 0.048 U/ml activity at static conditions to 0.057 U/ml on shaking conditons (Graph 1). Continuous shaking showed increase in activity possibly because of enhanced oxygen transfer. In stationary cultures, formation of mat at the surface of the medium results in oxygen limitations, which inhibits the oxidative enzymes [10]. Laccase production did not show variation over pH values from 4.5 to 6.0 (Graph 2). pH 5.0 was taken as optimum pH as, at this pH the standard laccase producer i.e *Trametes hirsuta* shows maximum laccase production [11]. Laccase activity shown by our fungal culture at pH 5.0 is 0.057 U/ml.

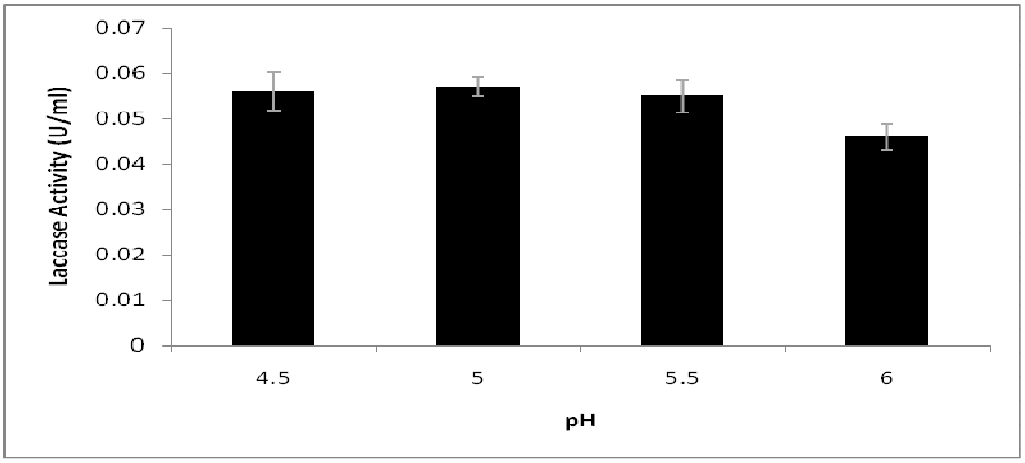
Graph 1

Effect of shaking and static conditions on laccase production



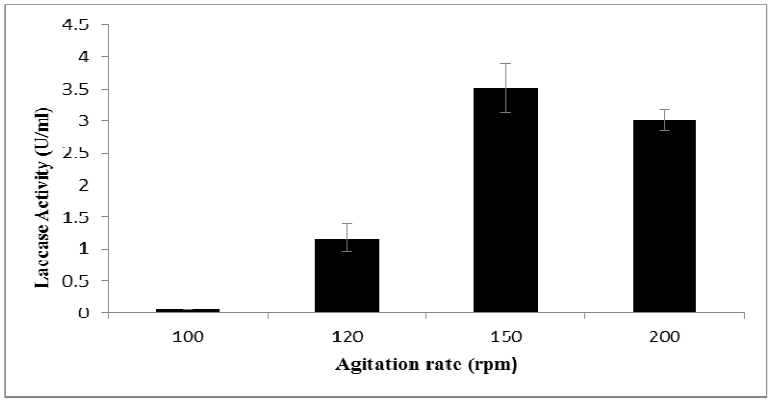
Mean error bars in the graph represent the mean ± standard error from the duplicate samples that were tested

Graph 2
Effect of pH on Laccase production

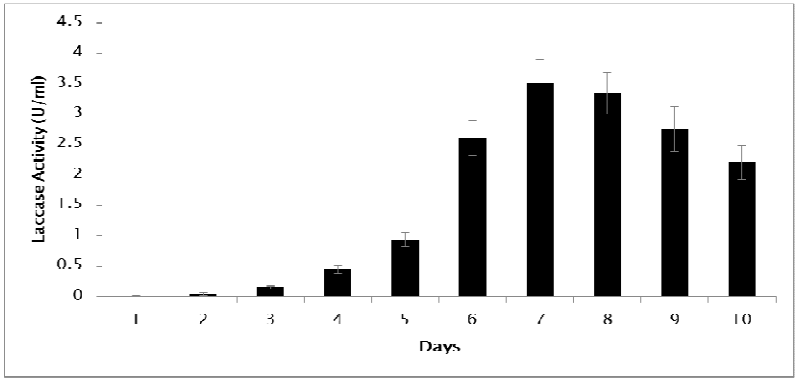


Optimum agitation rate was found to be 150 rpm which gave laccase activity of upto 3.519 U/ml (Graph 3). The time course study of laccase production was carried out under optimum culture conditions at pH 5.0 and 150 rpm. The maximum laccase activity was obtained on 7th day after which the activity declined (Graph 4).

Graph 3
Effect of Agitation rate on laccase production



Graph 4
Time course study for laccase production



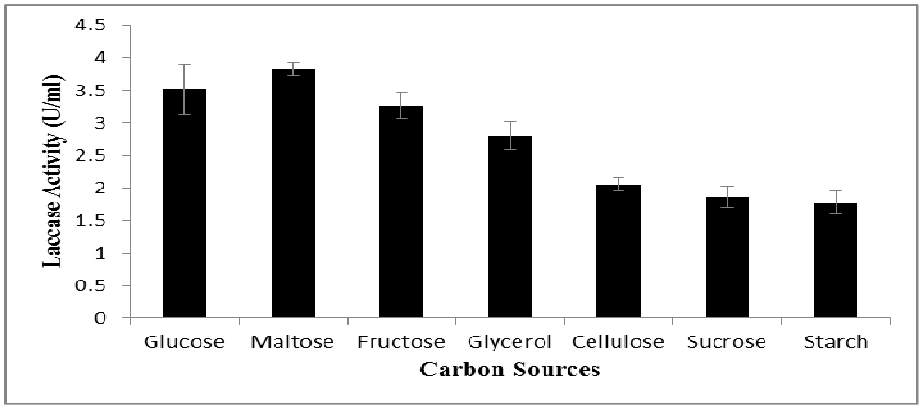
(ii) Optimization of media components for laccase production

(a) One Factor at a Time method (OFAT)

Six different carbon sources were tested with glucose as control. Maltose supported maximum laccase production at the activity of 3.826 U/ml (Graph 5). Generally, glucose is considered to be the best carbon source for production of enzymes [12]. In the present study, more laccase activity shown by the fungus using maltose instead of glucose as carbon source can be attributed to its preference. Other Basidiomycete species *Peniophora sp*. (NFCCI-2131) have also been reported to have similar preference for laccase production [13].

Graph 5

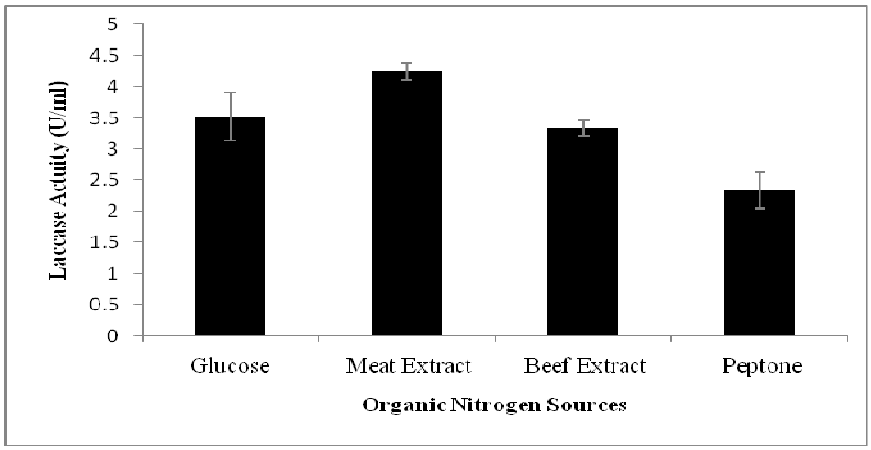
Effect of Carbon sources on Laccase production



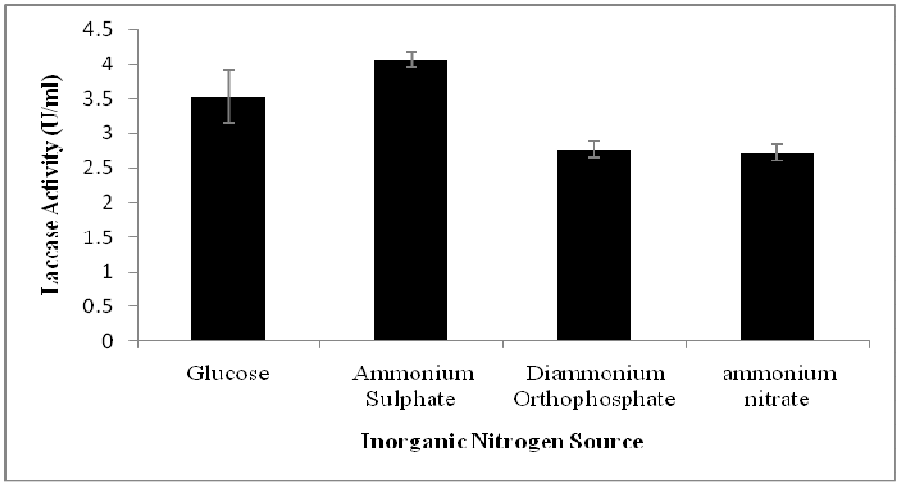
The influence of different organic and inorganic nitrogen sources were assessed keeping glucose as carbon source. Among different complex organic nitrogen sources used, meat extract gave high yields (4.250 U/ml) of laccase activity (Graph6) and from amongst various inorganic nitrogen sources $(NH_4)_2SO_4$ stimulated higher production of laccase (Graph 7). Kusum Dhakar and Anita Pandey also observed highest laccase activity in *Trametes hirsuta* (MTCC 11397) in a medium with ammonium sulphate as the nitrogen source [14]. Therefore by OFAT, appropriate carbon and nitrogen sources were selected.

Graph 6

Effect of Organic Nitrogen Source on Laccase Production



Graph 7
Effect of Inorganic Nitrogen Source on Laccase production



The orthogonal matrix method was to optimize concentrations of different media components for laccase production. $L_9(3^4)$ orthogonal array was selected for fungus with maltose, meat extract, $(NH_4)_2SO_4$ and KH_2PO_4 at three different concentrations. The laccase activities (Table 1) obtained from 9 set of experiments with selected factors and levels were analysed with Minitab 15 software. A response table (Table 3) is obtained by analyzing the laccase activities data. Rank and delta values obtained in this table show which factors have pronounced effect on laccase production. Delta values represented the order of effect of different factors on laccase production. The order of effect of the factors was Maltose>$(NH_4)_2SO_4$ > Meat Extract > KH_2PO_4. Maltose had stronger effect on laccase production. Figure 1 represents the main effect plots which are obtained by statistical analysis of experimental data i.e laccase activities. It depicted the influence of each factor at three levels (concentrations) on laccase production. By studying the main effects of each factor it was found that one level increases the mean value as compared to the other. From the results obtained in Figure 1 and Table 3, the optimal concentrations for four different media components are 1% maltose, 0.75% meat extract, 0.124% $(NH_4)_2SO_4$, 0.1% KH_2PO_4. The predicted value for laccase production using Minitab software was 5.294 U/ml. To verify whether the medium optimized by orthogonal array method was the best, time course study on optimized medium was carried out and activity obtained on 7th day was 6.900 U/ml.

Table 3
Response table for means

Levels	A (Maltose)	B (Meat Extract)	C ($(NH_4)_2SO_4$)	D (KH_2PO_4)
1	2.097	2.409	3.782	3.125
2	3.907	3.548	2.361	3.561
3	3.347	3.395	3.209	2.668
Delta	1.810	1.139	1.421	0.829
Rank	1	3	2	4

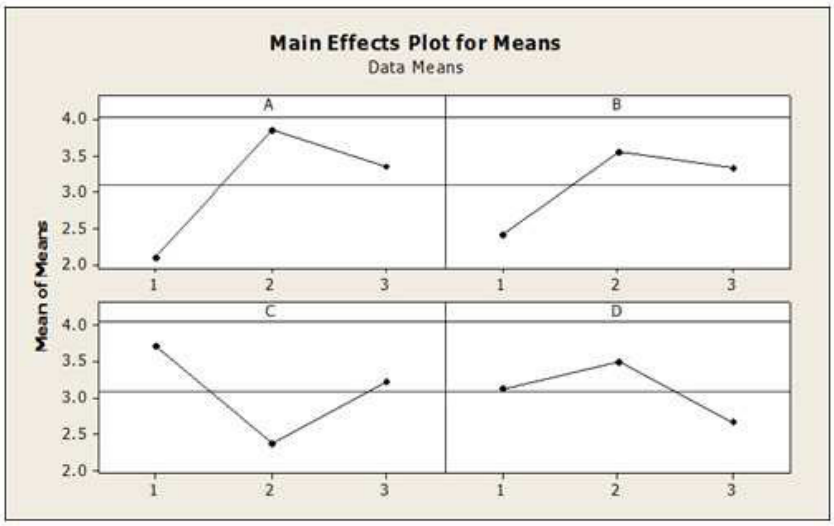


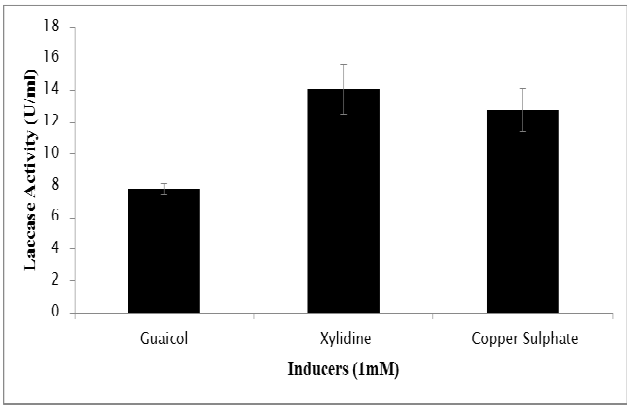
Figure 1
Main effect plots for laccase production by fungus.

Table 4
Optimized medium for laccase production by fungal isolate (pH 5.0, agitation rate 150 rpm, incubation period 7 days, temperature 30 ± 3° C)

Composition	Concentration (g/L)
Maltose	1
Meat Extract	0.75
$(NH_4)_2SO_4$	0.124
KH_2PO_4	0.1
Wheat Bran	4.5
Thiamine dichloride	0.05
$MgSO_4$	0.05
$CaCl_2$	0.01
KCl	0.05

Furthermore the laccase production was increased by application of various inducers. 2,6 - xylidine enhanced laccase production upto 14.119 U/ml (Graph 8). For optimization of laccase production in *P. ostreatus* 1804 [15] and *P. ostreatus* IMI 395545 [16], application of taguchi orthogonal method has been reported.

Graph 8
Effect of inducers on laccase production



CONCLUSION

It can be concluded from the results that application of one factor at a time method (OFAT) and Taguchi orthogonal method, in combination proved to be most conclusive in optimizing laccase production. The optimal concentrations for tested media components are 1% maltose, 0.750 % meat extract, 0.124% $(NH_4)_2SO_4$ and 0.1% KH_2PO_4 with shaking conditions, pH 5.0 and agitation rate 150 rpm as optimum culture conditions. The future course of this study would be to purify the laccase from this fungal isolate and investigate its decolorizing and degrading activity on various textile dyes.

REFERENCES

1. Morozova O. V., Shumakovich G. P., Gorbacheva M. A., Shleev S. V., and Yaropolov A. I., Blue laccases. Biochemistry, 72(10) : 1136–1150, (2007).
2. Kiiskinen, L.L., Viikari, L., and Kruus, K., Purification and characterisation of a novel laccase from the ascomycete *Melanocarpus albomyces*. Appl. Microbiol. Biotechnol., 59 : 198–204, (2002).
3. Gianfreda L., Xu F., and Bollag J. M., Laccases : a useful group of oxidoreductive enzymes. Bioremediation Journal, 3(1) : 1–25, (1999).
4. Desai S.S and Nityananda C., Microbial Laccases and their applications: A review. Asian J Biotechnol., 2 : 98-124, (2011).
5. Revankar, M.S. and Lele S.S., Synthetic dye decolorization by white rot fungus, *Ganoderma sp* WR-1. Bioresource Technol., 98 : 775-780, (2007).
6. Benyounis K. Y. and Olabi A. G., Optimization of different welding processes using statistical and numerical approaches - A reference guide. Adv. Eng. Soft., 39 : 483–496, (2008).
7. Aggarwal A., Singh H., Kumar P. and Singh M., Optimizing power consumption for CNC turned parts using response surface methodology and Taguchi's technique - A comparative analysis. J. Material. Pro. Technol., 200 : 373–384, (2008).
8. Pointing, S. B., Jones, E. B. G. and Vrijmoed, L. L. P., Optimization of laccase production by *Pycnoporus sangui-neusin* submerged liquid culture. Mycologia, 92: 139–44, (2000).
9. de Jong, E., de Vries F.P., Field J.A., Vander Zwan R.P. and de Bont J.A.M., Isolation and screening of basidiomycetes with high peroxidative activity. Mycol. Res., 96 : 1098-1104, (1992).
10. Swamy, J. and J.A. Ramsay, The evaluation of white rot fungi in the decolorisation of textile dyes. Enzyme Microb. Technol., 24 : 130-137, (1999).
11. Ali A. Taha Al Shaikly, Purification And Characterization of *Trametes hirsute DSMZ* No.5072 Laccase. Journal of Biotechnology Research Center, 3(1), (2009).
12. Piscitelli A., Giardina P., Lettera V., Pezzella C., Sannia G., and Faraco V., Induction and transcriptional regulation of laccases in fungi. Current Genomics, 12(2) :104–112, (2011).
13. Shiv Shankar and Shikha, Laccase Production and Enzymatic Modification of Lignin by a Novel *Peniophora sp*. Applied Biochemistry and Biotechnology, 166(4) : 1082-1094, (2012).
14. Kusum Dhakar and Anita Pandey, Laccase Production from a Temperature and pH Tolerant Fungal Strain of *Trametes hirsuta* (MTCC 11397). Enzyme Research, Volume 2013, Article ID 869062, 9 pages, (2013).
15. Prasad K. K., Mohan S. V., Rao R. S., Pati B. R. and Sarma P. N., Laccase production by *Pleurotus ostreatus* 1804: Optimization of submerged culture conditions by Taguchi DOE methodology. Biochem. Eng. J. 24 : 17–26, (2005).
16. Periasamy R. and Palvannan T., Optimization of laccase production by *Pleurotus ostreatus* IMI 395545 using the Taguchi DOE methodology. J. Basic Microbiol. 50 : 548–556, (2010).
17. Pahuja N., Singh G. H., Soni S. K., and Nandanwar H. S., Laccase from the Fungus *Simplicillium sp.* GSH 1: Effect of pH and temperature on enzyme activity and stability. Pharmacologyonline, 3: 1077-1084, (2011).