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ORIGINAL ARTICLE

Optimization of laccase production from *Aspergillus flavus* by design of experiment technique: Partial purification and characterization

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Laccase;
Aspergillus flavus;
Bromophenol blue;
ABTS;
Design of experiment

Abstract The present study describes isolation of laccase producing fungal strain and optimization of the process parameters by design of experiment (DOE) technique to achieve the maximum production of extracellular laccases by *Aspergillus flavus* obtained from natural habitat. Bromophenol blue dye and ABTS (2,2′-azinobis 3-ethyl-benzothiazoline-6-sulfonate) were used as substrates for the screening of laccase activity. Design expert 8.0.7.1 software was used to optimize culture conditions such as carbon source, nitrogen source, temperature and pH. Subsequently, optimization for inoculums size was also carried out. The optimization studies revealed that the laccase yield was highest when operated at the following conditions: carbon source – cellulose (8%), nitrogen source – peptone (2%), temperature – 35 °C, pH – 7 and inoculum of size 1.5 cm.

1. Introduction

Laccases (benzene diol: oxygen oxidoreductase, EC 1.10.3.2) are the most extensively studied group of enzymes among oxidases. Laccase was first described from the sap of the Japanese lacquer tree *Rhus vernicifera*. They belong to the family of blue multicopper oxidases, which catalyze the one-electron oxidation of four reducing-substrate molecules concomitant with the four-electron reduction of molecular oxygen to water [3]. Laccase has broad substrate specificity toward 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 [12].

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Laccases are ubiquitous in distribution. In fungi they are produced by *ascomycetes, basidiomycete* and *deutromycete*. These laccase producing fungi include *Trametes versicolor, Thermopsis villosa*, *Aspergillus* multicopper oxidases included in the ascomycete laccases cluster and have received little attention [19].

Owing to its vivid biotechnological applications, studies on laccase producing organisms have been intensified in the recent years and the optimization of laccase production from different microorganisms is being carried out by several groups [10,11,4]. The optimization of physico-chemical conditions generally involves variation in levels of one independent variable while fixing other variables at a certain level. This one factor at a time approach is laborious, time-consuming and often the interaction effects of the parameters are overlooked [17]. The methods for determining how to increase productivity and improve quality have changed from costly and time-consuming trial-and-error searches to the powerful, elegant, and cost-effective statistical methods under design of experiment approach.

Laccase expression in fungi is influenced by culture conditions such as nature and concentration of carbon and nitrogen sources, media composition, pH, temperature etc. In the present investigation a fungal strain of *Aspergillus flavus* has been isolated from natural habitat such as field soil samples, decaying wood, decaying wheat straw and waste water run offs from the textile dyeing shops and screened for laccase activity using ABTS and bromophenol blue as substrates. Time course of production of lacasse was performed. The maximum laccase production was obtained at 12th day of culture. Further the production medium was optimized for various parameters using DOE 8.0.7.1 software.

2. Materials and methods

2.1. Materials

The reagent grade chemicals potato dextrose agar, potato dextrose broth, Czapek Dox agar, Starch, Yeast extract, H_2PO_4, Na_2HPO_4, $MgSO_4$, $CaCl_2$, $FeSO_4$, $MnSO_4$, $ZnSO_4$, $CuSO_4$ were procured from Hi-Media, Mumbai. ABTS, bromophenol blue, ammonium sulfate, sodium malonate buffer (pH-4.5), Sephadex G-100 column, dialysis bag, NaCl, Folin–Ciocalteu's phenol reagent, gallic acid, sodium carbonate, ethanol, sodium acetate buffer were procured from CDH, HI-MEDIA and RANKEM.

2.2. Screening of fungi for laccase production

2.2.1. Isolation and identification of fungi

2.2.1.1. Bromophenol blue assay. The isolated fungus was screened for the degradation of bromophenol blue dye. The potato dextrose broth was supplemented with bromophenol-blue (0.2 g/l) and then the broth was inoculated with the mycelial disks and incubated at 27 °C for 3 days. Discoloration of the dye from the broth indicated the presence of lignolytic enzyme [19].

2.2.2. ABTS plate screen assay

The isolates showing bromophenol blue degradation were further screened for lignolytic enzyme laccase, on Czapek-Dox agar. The substrate ABTS (1 mM) was amended with the basal medium in order to screen the presence of laccase. Plates containing lignolytic enzyme substrates were inoculated with a mycelial disk and incubated at 27 °C for five days. Blue-green oxidation zone around the mycelial colony indicated the presence of laccase [17].

2.2.3. Identification of fungus

The fungus used in this study was isolated from natural habitat such as decaying wood in the vicinity of Kurukshetra, Haryana, India. Decayed wood samples were first dipped in adequate amount of sterilized distilled water and agitated to form a solution. These samples were cultured on potato dextrose agar and screened for laccase production. The strain was identified at the Institute of Microbial Technology Chandigarh as *A. flavus* based on morphological, biochemical and physiological characterization. Morphological examination of the isolate on a plate revealed a clear white unraised lawn with green colored spores. This is a characteristic feature of *A. flavus*. The culture was periodically subcultured and maintained on potato dextrose agar plates grown at 27 °C and stored at 4 °C.

2.3. Production of laccase

The production of enzyme was carried out under stationary conditions. Disks taken from the active borders of PDA cultures were transferred to Erlenmeyer flasks (250 ml) containing 100 ml of the production medium having the following composition (in g/ml): starch (20), yeast extract (2.5), H_2PO_4 (1.0), Na_2HPO_4 (0.05), $MgSO_4$ (0.5), $CaCl_2$ (0.01), $FeSO_4$ (0.01), $MnSO_4$ (0.001), $ZnSO_4$ (0.001), $CuSO_4$ (0.002). The pH of the medium was adjusted to 5.5. Optimum time period of incubation was found by monitoring the enzyme activity in the broths of all the isolates till 14 days. The isolate which showed the highest enzyme activity was selected as the best isolate.

2.4. Analytical methods

2.4.1. Extracellular laccase assay

Extracellular laccase activity was measured spectrophotometrically with ABTS as a substrate. The reaction mixture contained 900 µl of 1 mM ABTS in 0.1 M sodium acetate buffer (pH 5.0) and 0.1 ml of enzyme. The reaction was monitored by measuring the change in A_{436} ($\epsilon = 2.9 \times 10^{-3}\ cm^{-1}\ \mu M^{-1}$) for 3 min. One unit enzyme activity was defined as the amount of enzyme that oxidizes 1 µ mole of ABTS per minute at 27 °C. The activities were expressed in IU/ml [17].

2.4.2. Estimation of protein

The concentration of protein was estimated with the Bradford's assay [2] and using bovine serum albumin BSA) as a standard.

2.5. Optimization of culture conditions for laccase production by *Aspergillus sp.*

2.5.1. Time course study

In order to find the optimal time of incubation for the maximum laccase production 250 ml production medium was

prepared in Erlenmeyer flask (500 ml) and autoclaved. To this two disks (1.5 cm in diameter) of a 5 day old culture was inoculated and incubated at 27 °C for a period of 14 days. The culture harvested at every 2 day interval was used to determine protein content and enzyme activity.

2.5.2. Optimization of media and culture conditions

For the purpose of optimizing the media and culture conditions for the best isolate design expert 8.0.7.1 software was used. Optimization was done for 5 carbon sources at 8% concentration viz.- glucose, fructose, cellulose, xylose and starch; 5 nitrogen sources at 2% concentration viz.- ammonium per sulfate, ammonium sulfate, ammonium chloride, peptone and yeast extract; 4 temperature levels viz.- 30 °C, 35 °C, 40 °C, 45 °C and 5 pH levels viz.- 4, 5, 6, 7, 8. This was done by replacing only the carbon and nitrogen source in the above mentioned production medium. With a view of dependence of utilization of nutrients on temperature settings were done to optimize while accounting for carbon sources interaction with temperature and nitrogen source interaction with temperature. The response of the experiment to be fed was set as enzyme activity in terms of IU/ml. Random combinations of levels of different factors (carbon source, nitrogen source, temperature, pH) were generated by the software. All these were executed experimentally in 500 ml flasks containing 250 ml of the production medium for the optimum time period of enzyme production. The results or enzyme activity for each run/combination was calculated and fed as response in the program. These were used to evaluate and analyze the results to find out the optimum combination of levels of different factors.

2.5.3. Optimization of inoculum size

To determine optimum inoculum size the production was carried out in 500 ml Erlenmeyer flasks containing 250 ml of production medium. The flasks were inoculated with disks of different sizes viz.-1 cm, 1.5 cm, 2 cm, 2.5 cm, 3 cm. Final optimized production was carried out for an optimized time period, under optimized temperature and pH condition taking the optimized carbon and nitrogen sources and using the optimal inoculum size for inoculation. Enzyme activity was measured again from the filtered broth. Bradford's method was used for protein estimation.

2.5.4. Partial purification of enzyme

The filtered broth obtained from the final optimized production was subjected to ammonium sulfate precipitation (50 and 60% saturation) at 4 °C and the precipitate was collected by centrifugation. The precipitate was dissolved in 30 ml 0.1 M sodium acetate buffer (pH 5.0). Enzyme activity and the total protein content for the solution obtained was measured. An extensive buffer exchange was performed for 24 h using 0.1 M sodium acetate buffer (pH-5) in dialysis membrane of 12 kDa (cut-off). Enzyme activity and total protein content were determined for the dialysed enzyme extract.

2.5.5. Enzyme characterization

The temperature profile of the laccase was studied by measuring the activity in a range of 30–50 °C. The partially purified enzyme solution was incubated in 0.1 M sodium acetate buffer (pH 5.0) at different temperatures (30 °C, 35 °C, 40 °C, 45 °C, 50 °C) and the activity was determined with ABTS as substrate. The pH-dependence of the laccase activity was carried out at 25 °C using 1 mM ABTS in different buffer solutions, 1 M: glycine/HCl buffer (pH 3.0–3.5), sodium citrate buffer (pH 4.0–5.0) and sodium phosphate buffer (pH 6.0–8). The thermal stability of the enzyme was determined by following the oxidation of ABTS of 1 mM ABTS at optimum pH and temperature after pre-incubation of laccase at 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C and 55 °C. For pH stability, the enzyme extract was pre-incubated at room temperature in different buffers at pH 3.0–7.0. Aliquots were removed after one hour of incubation and assayed at optimum pH and temperature.

3. Results and dissusion

3.1. Screening of white rot fungi for laccase production

The fungus when screened for the production of laccase turned bromophenol blue in potato dextrose broth colorless, showing its complete degradation by a phenol-oxidase. The strain was confirmed for lacasse production on czapek dox medium amended with ABTS. The oxidation of ABTS was observed. The results are shown in Fig. 1.

ABTS is considered as a unique laccase substrate in the absence of hydrogen peroxidase; therefore, it is confirmed that the enzyme is a true laccase.

3.2. Optimization of culture conditions for laccase production by A. flavus

3.2.1. Time course study

The time course of laccase production by *A. flavus* was monitored. The maximum laccase production (17.39 IU/ml) was observed at 12th day culture. Fortina et al. (1996) reported

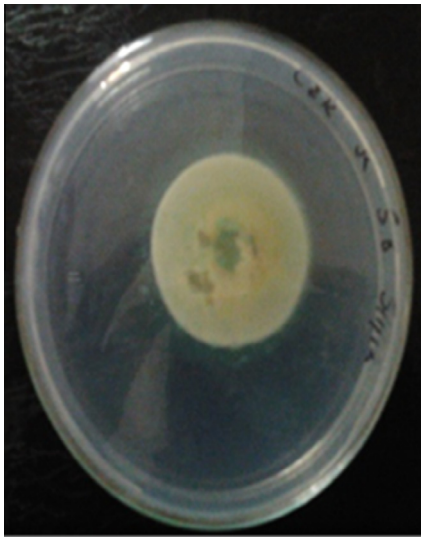

Figure 1 Screening of *Apergillus flavus* for laccase production on Czapek dox medium. The figure shows the result of primary screening. All the fungal strains isolated were screened on screening medium. Highest laccase producing strain of *Apergillus flavus* has shown the biggest zone of dye degradation.

that, *Botrytis cinerea* produced appreciable levels of laccase (9.8 IU/ml) in a brief period (5–7 days) and they stated that, some fungal species required a longer production time i.e. 12–30 days. With *Ganoderma* sp, the maximum laccase activity was recorded on 10th day of incubation [17]. It was reported that *Pleurotus florida* produced a high amount of laccase (4.60 IU/ml) in malt extract broth after 12 days under stationary conditions [17].

3.2.2. Optimization of the media and culture conditions

Experiments were carried out according to the different combinations of the levels of factors generated by the Design Expert-8.0.7.1software. Optimum enzyme activity of 18.2 IU/ml was obtained with the experimentation of 26th run having the following factors- carbon source – xylose, nitrogen source–peptone, temperature – 35 °C and pH-8. The data fed for enzyme activities in the software as the responses of the practical execution of the runs, was used for the generation of different graphs for various factors. These graphs demonstrated the scenario displayed by the factors during the experiment. After analyzing the results and the graphs generated from the data, it was found that optimum activity of 27.30 IU/ml could be obtained under the following settings: carbon source – cellulose, nitrogen source – peptone, temperature – 35 °C, pH-7 as shown in Fig. 2.

Production carried out in 250 ml medium in 500 ml flask under these conditions resulted in an enzyme activity of 23.09 IU/ml. Srinivasan et al. [18] found that, the cellulose and lignocellulosic residues were considered to stimulate laccase production. The presence of water-soluble constituents from lignocellulose fractions of the lignocellulosic residues such as *p*-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol and other aromatic compounds might result in the increased laccase activities in comparison to the growth on glucose [15]. A similar contribution was expected by the water-soluble constituents from cellulose in this study.

Peptone was found to be the optimum nitrogen source for the production of laccase. Best enzyme production can be attributed to the fact that peptone provides the complete pool of amino acids required for enzyme synthesis [1]. Addition of peptone or ghee residue in Wheat bran formulated media lead to higher production of laccases [9].

The temperature of 35 °C was found to be the optimum for the laccase production. The optimum production of laccase by *Penicillium martensii NRC* 345 occurred at a temperature of 30 °C. Results from the study conducted by *Manimozhi* et al. (2012) showed that pH values of 5.5 were found to be the optimum for laccase activity from *Agaricus heterocystis*. The optimum pH and temperature for the activity of the enzyme produced was found to be 5.0 and 70–80 °C respectively.

By the use of this software (DOE 8.0.7.1), the optimum condition for the production of enzyme was predicted and upon its implementation an increase of 9.10 IU was achieved. On experimental validation of numerical and graphical optimization program within the tested range the optimal combination of the physicochemical factors for laccase production was obtained as cyanobacterial biomass, 2.03 g; groundnut shell, 8.26 g; pH, 5.42; temperature, 30.4 °C and moisture content, 70.30% (Kumar et al., 2011).

Production of enzyme for this combination of factor levels was further improved when 1.5 cm fungal disk was used for inoculation of the optimized medium. 300 ml of filtrate obtained from the 12 day old culture from this flask was used to determine enzyme activity which was recorded as 23.21 IU/ml with a protein concentration of 0.166 mg/ml. The optimal inoculum size for laccase production for *Penicillium martensii NRC* 345 was found to be five disks of 14 mm diameter each and a further increase in inoculum size reduced the level of enzyme formation

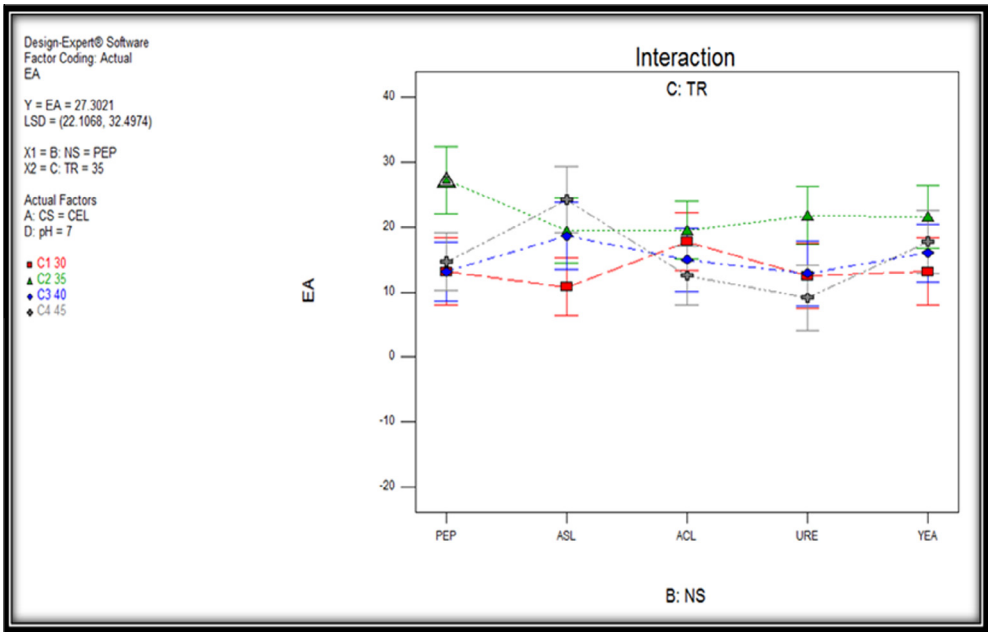

Figure 2 Settings for optimum enzyme production: carbon source – cellulose, nitrogen source – peptone, temperature – 35 °C, pH-7. The figure shows the interaction of temperature and nitrogen source. The best nitrogen source was peptone and optimum temperature was 35 °C. Carbon source was cellulose and pH as 7.0.

[4]. So, the final optimum conditions were given using Carbon source – cellulose (8%), Nitrogen source – peptone (2%), temperature – 35 °C, pH – 7 and inoculums of size 1.5 cm. (See Table 1).

3.3. Purification

Following ammonium sulfate precipitation the enzyme activity of the 30 ml buffer dissolved precipitate solution came out to be 25.66 IU/ml with a protein concentration of 0.048 mg/ml. The enzyme was purified to a concentration of 0.036 mg/ml with an enzyme activity of 30.57 IU/ml. The summary of purification results is given in Table 2. A total of 4.24-fold purification of crude enzyme extract was achieved.

Viswanath et al. [20] reported 70-fold purification of Laccase from *Stereum ostrea* using ammonium sulfate precipitation followed by Sephadex G-100 column chromatography. Laccase from fruiting bodies was purified by ammonium sulfate precipitation with 40–70% saturation and DEAE cellulose chromatography with a purification fold of 1.34 and 3.07 [8].

Table 1 Run sheet generated by the software and the fed response data (E.A.-IU/ml) after experimentation of the runs.

Std.	Run	Factor 1 A:CS	Factor 2 B:NS	Factor 3 C:TR	Factor 4 D:pH	Response 1 EA IU/ml
9	1	XYLOSE	URE	45	4	0
6	2	GLUCOSE	PEP	45	4	0
39	3	XYLOSE	URE	30	8	2.73
26	4	XYLOSE	ACL	45	6	0
32	5	FRUCTOSE	PEP	45	7	12.51
19	6	GLUCOSE	YEA	40	5	3.64
41	7	FRUCTOSE	ASL	35	8	9.1
28	8	GLUCOSE	ASL	40	7	10.69
15	9	GLUCOSE	YEA	35	5	7.85
7	10	STARCH	ASL	45	4	19.9
11	11	FRUCTOSE	URE	30	5	7.62
35	12	XYLOSE	YEA	45	7	12.4
3	13	GLUCOSE	URE	30	4	12.17
27	14	FRUCTOSE	YEA	45	6	7.74
22	15	GLUCOSE	ACL	35	6	7.74
21	16	XYLOSE	ASL	35	6	8.98
2	17	XYLOSE	ACL	30	4	10.46
12	18	GLUCOSE	YEA	30	5	7.96
5	19	XYLOSE	ACL	35	4	13.65
1	20	CELLULOSE	ASL	30	4	9.1
24	21	FRUCTOSE	YEA	35	6	10.35
16	22	CELLULOSE	PEP	40	5	6.82
25	23	CELLULOSE	PEP	45	6	8.76
43	24	XYLOSE	PEP	40	8	9.1
23	25	CELLULOSE	URE	35	6	17.63
40	26	XYLOSE	PEP	35	8	18.2
18	27	XYLOSE	URE	40	5	7.5
45	28	CELLULOSE	YEA	40	8	11.94
29	29	XYLOSE	ACL	40	7	14.1
33	30	GLUCOSE	ASL	45	7	12.74
4	31	STARCH	YEA	30	4	12.28
14	32	FRUCTOSE	URE	35	5	7.39
30	33	STARCH	URE	40	7	7.73
44	34	FRUCTOSE	ACL	40	8	7.96
37	35	STARCH	ASL	30	8	7.5
42	36	STARCH	YEA	35	8	7.39
34	37	STARCH	URE	45	7	7.96
13	38	CELLULOSE	ACL	35	5	11.94
17	39	STARCH	ASL	40	5	9.1
31	40	FRUCTOSE	YEA	40	7	11.14
36	41	FRUCTOSE	PEP	30	8	10.8
8	42	CELLULOSE	ACL	45	4	11.26
20	43	STARCH	PEP	35	6	12.4
38	44	CELLULOSE	ACL	30	8	13.65
10	45	STARCH	PEP	30	5	7.5

The content of table shows different combinations of carbon source, nitrogen source, temperature and pH which were generated by software (DOE 8.0.7.1). The experiments are performed using these combinations and results of experiments were filled as response in last column in the form of enzyme activity per ml.

Table 2 Purification index of laccase.

S. No.	Steps	Total recovery		Specific activity (IU/mg)	Purification fold	% Yield
		Protein (mg)	Enzyme (units)			
1	Filtration	34.8	6963	200.09	–	–
2	Ammonium sulfate	1.44	769.8	534.58	2.67	11.05
3	Dialysis	1.08	917.1	849.17	4.24	13.17

The table shows purification fold and percent yield. The enzyme was produced under optimized stationary conditions and then it was subjected to filtration and ammonium sulfate precipitation. Excess salt was removed through dialysis.

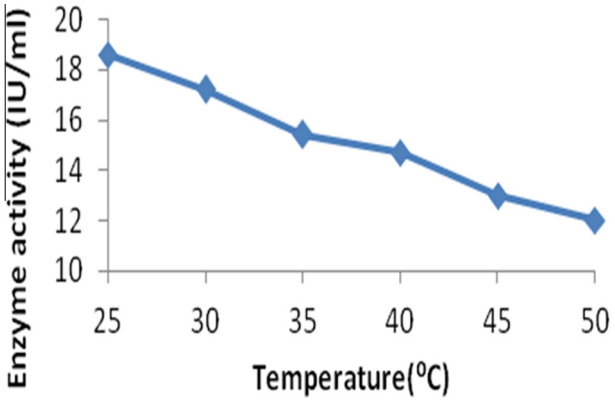

Figure 3 Laccase activity at different temperatures. The enzyme activity was determined at different temperatures by keeping all other conditions of enzyme assay constant. The optimum activity of 18.61 IU/ml was measured at 25 °C.

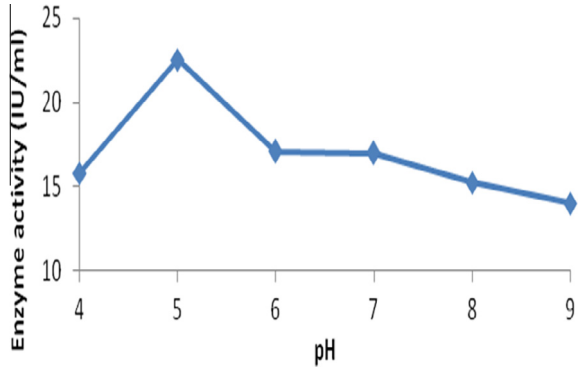

Figure 4 Laccase activity at different pH values. This figure is for pH optima of enzyme. Enzyme activity was assayed at different pH. The optimum pH of enzyme was found at 5. Other assay conditions such as incubation period, substrate concentration, was kept constant.

3.4. Enzyme characterization

3.4.1. Temperature optima and stability

Laccase activity was measured at different temperatures. Highest activity of 18.6 IU/ml was found at a lower temperature of 25 °C. The enzyme activity decreased proportionally with respect to increase in temperature (Fig. 3). The enzyme was found stable in a temperature range of 25–50 °C. At 25 °C enzyme has retained 91.4% of its initial activity after 1 h and had retained 86% even after 4 h. At 30 °C, though the enzyme had 97.7% of its initial activity after one hour it was significantly decreased to 75% after 4 h (Fig. 5).

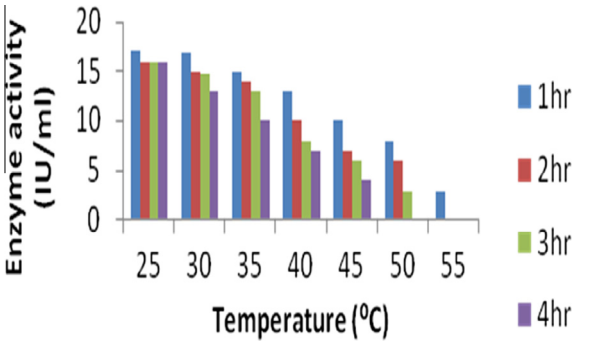

Figure 5 Thermostability of laccase. Enzyme was incubated at different temperature for 1 to 4 h and enzyme activity was assayed in percentage of initial activity. The enzyme assay conditions were remained same. The enzyme was found quite stable in a temperature range of 25–50 °C.

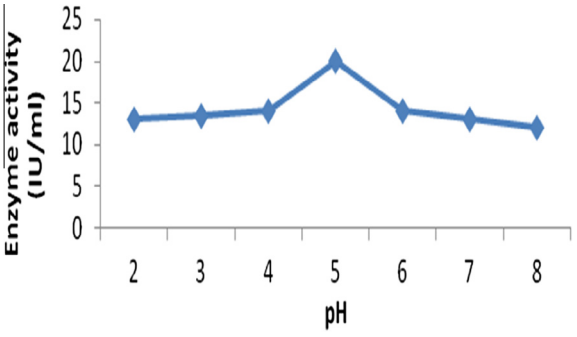

Figure 6 pH stability of laccase. The enzyme was incubated in different buffers having a pH range of 2–8. After one hour of incubation enzyme activity was determined. The assay condition were kept same for all enzyme assays. Enzyme was found stable in a pH range of 4–8.

3.4.2. The pH optima and stability

It was evident that the pH significantly influenced activity of laccase from *A. flavus*. At pH 4 enzyme activity of 15.81 IU/ml was observed. An optimum value of 22.54 IU/ml was measured at pH 5. This was followed by a steep decrease in

enzyme activity to 17 IU/ml at pH 6 (Fig. 4). Although pH optima may also depend on the substrate used [5], most laccases in other fungi also have an optimum pH of around 3 with ABTS as a substrate [13,6,14,16,7].

The enzyme was stable between pH 4 and 6 and maximum stable at pH 5. At 25 °C the enzyme had 91.4% of its initial activity after 1 h and retained 86.0% of its activity even after 4 h (Fig. 6). Thereby, the laccase under study was mesophilic in nature and was stable at its optimum temperature and pH.

4. Conclusion

In view of the results obtained, it can be concluded that the isolate was able to oxidize phenolic substrates such as guaiacol and ABTS. The optimization of various cultural and nutritional parameters for the production of laccase from *A. flavus* was done using DOE technology, where the interactions within the parameters were also accounted for. The usage of DOE technology revealed the optimum condition which increased the production by considerable amounts at the same time and saved resources and time. In future we are interested in exploiting harmless laccase mediators for various industrial applications. Further, high level production of laccase can be achieved by exploring the gene in a suitable host.

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