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RESEARCH ARTICLE

STUDY THE EFFECT OF VARIOUS PHYSICAL AND BIOCHEMICAL PARAMETERS ON THE PRODUCTION OF LACCASE ENZYME PRODUCED FROM *ASPERGILLUS FLAVUS* AT *IN VITRO* CONDITIONS

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ABSTRACT

Laccase is a copper-containing polyphenol oxidase enzyme, which acts on a wide range of substrates. This enzyme is occur in many plant species and is widely distributed in fungi including wood-rotting fungi where it is often associated with lignin peroxidase, manganese dependent peroxidase, or both. Because of its importance in bioremediation, fungal cultures were screened for laccase production by plate testing method using the indicator compound guaiacol. The selected isolates of *Aspergillus flavus* were optimized for the production of Laccase grown on Glucose Asperagine agar medium. This exhibited the highest production on 5th day of incubation at pH= 5.5 and the optimum temperature for production was recorded at 30°C. The effect of metal ions on the activity of laccase production was also studied and observed that all the metal ions greatly inhibited the activity except Cu^{+2} and Ca^{+} ion. These two metal ions enhanced the activity of enzyme. Maximum inhibitory effect was observed in the presence of Zn^{+}, Mn^{+2}, and Cd^{+2} ions. To observe the effect of solvent systems on laccase production various solvent system were applied and found that, formic acid and acetaldehyde enhance the activity of enzyme but acetic acid showed maximum inhibitory effect. Whereas, β-mercaptoethanol and DTT did not affect the enzyme activity.

INTRODUCTION

Laccases are an interesting group of multi copper enzymes, defined in enzyme commission (EC) nomenclature as oxidoreductase, which oxidize diphenols and allied substances and use molecular oxygen as an electron acceptor. They are predominantly found in higher plants and fungi (Thurston, 1994; Mayer and Staples, 2002). In contrast to most enzymes, which are generally very substrate specific, laccases act on a surprisingly broad range of substrates, including diphenols, polyphenols, different substituted phenols, diamines, aromatic amines, benzenethiols and even some inorganic compounds such as iodine. When oxidized by a laccase, the reducing substrate loses a single electron and usually forms a free radical. The unstable radical may undergo further laccase-catalyzed oxidation or nonenzymatic reactions including hydration, disproportionation and polymerization. In laccase-mediated reactions, diphenolic compounds undergo a four-electron oxidation. During this reaction, Cu(II) is reduced to Cu(I). In the next step in the reaction, Cu(I) reduced molecular oxygen (O_2) to produce two molecules of water. During this reaction Cu(I) is oxidized back to Cu (II) thus completing the reaction cycle.

These enzymes received much attention of researchers in last decades due to their ability to oxidize both phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants and use molecular oxygen as an electron acceptor (Viswanath *et al*., 2008).

This makes these biocatalysts very useful for their application in several biotechnological processes. Such applications include the detoxification of industrial effluents, mostly from the paper and pulp, textile and petrochemical industries, use as a tool for medical diagnostics and as a bioremediation agent to clean up herbicides, pesticides and certain explosives in soil. Laccases are also used as cleaning agents for certain water purification systems, as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics. In addition, their capacity to remove xenobiotic substances and produce polymeric products makes them a useful tool for bioremediation purposes (Couto and Herrera, 2006).

Hence the present work reports the production of laccase enzyme by a local isolate of *Aspergillus flavus* and studied various parameters which affect the production of laccase enzymeat in *in vitro* conditions.

MATERIALS AND METHODS

For the present investigation, fungal cultures of *Aspergillus flavus* were provided by school of life sciences, Jaipur National University, Jaipur. One set of pure culture of isolate was maintained on potato dextrose agar at 28°C and other set was stored at 4°C in refrigerator for further use**.**

Screening of laccase producing fungi

As a screening method for detecting the ability of fungal strains to produce laccase enzyme, we adopted the compound degradating method. Screening was performed in Petri dishes with G.A.E. (Glucose Asperagine) agar medium (Dhoulib *et*

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al., 2005), the plates were incubated at 28°C for 7 days. Laccase activity was visualized on plates containing 0.02% guaiacol since laccase catalyzes the oxidative polymerization of guaiacol to form reddish brown zones in the medium (Coll *et al*., 1993).

Enzyme extraction

Extraction of laccase was carried out in production medium supplemented with 0.02% guaiacol. Seven days old fungal culture was inoculated in above autoclaved 50 ml medium. The flasks were incubated at 28°C for 10 days. Crude enzyme extract was prepared from the culture supernatant which was centrifuged at 10,000 rpm for 10 min at 4°C. The crude extract was stored at -20°C for further use.

Enzyme activity

Enzyme activity was determined as per the protocol described by Das *et al.* (1997). The activity was assayed at 28 ±1°C by using 10 mM guaiacol in 100 mM acetate buffer (Gupta, 2009). The changes in absorbance of the reaction mixtures containing guaiacol was monitored at 470 nm (= 6,740 M^{-1} cm^{-1}) for 10 min of incubation. Enzyme activity is measured in IU/ml which is defined as the amount of enzyme catalyzing the production of one micromole of colored product per min per ml.

EFFECTS OF VARIOUS PARAMETERS ON THE ACTIVITY OF LACCASES ENZYME PRODUCTION

Effect of incubation time

The enzyme activity was observed from 1 to 10 days of incubation. 1.0 ml of crude enzyme along with 0.5 ml substrate and 3.0 ml buffer was incubated for 10 min at 28˚C as described above and laccase activity was observed spectrophotometrically against the control (Table 1).

Effect of temperature and ph

The effect of temperature on laccase activity was determined by recording the absorbance of
enzyme catalyzed reaction using guaiacol as substrate, incubated at different temperature. The reaction mixture was incubated for 10 min. Temperature at which enzyme showed maximum activity, was noted as optimum temperature of enzyme (Table 2).

The influence of pH on laccase activity was studied by recording the absorbance of enzyme catalyzed reaction at optimum temperature, using guaiacol as substrate dissolved in buffers of different pH (pH 3-9) and incubated at 28ºC for 10min and absorbance were recorded at 470nm.

The buffer systems used were citrate buffer (pH 2.0–3.5); acetate buffer (pH 4.0–5.5); phosphate buffer (pH 6.0–7.5); Tris–HCl buffer (pH 7.5– 9.0) (Table 3).

Effect of metal ions

The effect of metal ions on the activity of enzyme was studied to determine nature of active site of enzyme. During this study following salts of ions were taken at the rate of 50 mM/ml and 100 mM/ml of reaction mixture. The ions used were Na^{+} (NaCl), K^{+} (KCl), Mg^{+2} ($MgCl_2$), Ca^{+2} ($CaCl_2$), Mn^{+2} ($MnCl_2$), Co^{+2} ($CoCl_2$), Cu^{+2} ($CuSO_4$), Zn^{+2} ($ZnSO_4$), Cd^{+2} ($CdCl_2$) and Hg^{+2} ($HgCl_2$) (Table 4).

Effects of solvent systems

The effects of various solvents on enzyme activity were studied in order to find some valuable information about catalytic site of enzyme. Following solvent were added with reaction mixture at the rate of 50 mM/ml and 100 mM/ml concentration and centrifuged at 10,000 rpm for 10 min and incubated at 28˚C for 10 min. The solvent used were ethanol, formic acid, amyl alcohol, formaldehyde, toluene, aniline, acetic acid and acetaldehyde (Table 5).

Effects of inhibitors

The effect of several potential enzyme inhibitors such as EDTA, Sodium azide (NaN_3), DTT, SDS, β-mercaptoethanol and Urea on laccase activity was monitored. Inhibitors were added with reaction mixture at the rate of 50 mM and 100 mM per ml of mixture and the reaction mixture was incubated at 28˚C and the change in absorbance was measured spectrophotometrically at 470 nm (Table 6).

RESULTS AND DISCUSSION

The time course of laccase secretion and degradation of guaiacol by *Aspergillus flavus* in a glucose aspergine agar medium was determined by batch culture method. After complete incubation, extract was prepared from culture supernatant, which acts as crude extra cellular enzyme extract. The extracelluar laccase activity of crude enzyme extract was found to increase at initial growth and reached the maximum value of 124 IU/ml by 5th day of incubation after which it decreased slowly.

Table 1 Effect of incubation time on the activity of laccase enzyme production

S. No.	Incubation Time (Days)	Enzyme Activity (IU/ml)
1	2	74
2	3	88
3	4	110
4	5	172
5	6	76
6	7	72
7	8	56
8	9	48
9	10	22

pH is one of the the most important factors, which markedly influence enzyme activity. Extremely high and low pH values generally results in complete loss of activity for most of enzymes.

Table 2 Effect of pH on the activity of laccase enzyme production

S. No.	pH	Enzyme Activity (IU/ml)
1	3.0	18
2	3.5	30
3	4.0	120
4	4.5	96
5	5.0	112
6	5.5	172
7	6.0	146
8	6.5	22
9	7.0	10
10	7.5	18
11	8.0	38
12	8.5	48
13	9.0	14

The fungus was able to indicate a maximum activity of 172 IU/ml at pH 5.5. The pH of the culture medium is critical for the growth, lignolytic enzymes production and xenobiotics degradation.

Table 3 Effect of temperature on the activity of laccase enzyme production

S. No.	Temperature (˚C)	Enzyme Activity (IU/ml)
1	4	22
2	20	34
3	25	54
4	30	172
5	35	24
6	40	10
7	45	30
8	50	24
9	55	20

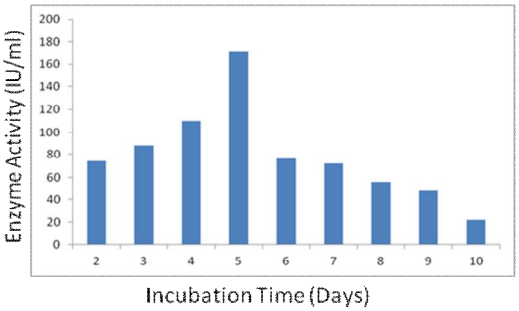

Figure 1 Effect of incubation time on the activity of laccase enzyme production

The influence of temperature on laccase production is related to the growth of the organisms. Hence the optimum temperature depends on whether the culture is mesophilic or thermophilic. Enzyme activity was found to be low at higher temperatures(40°C, 45°C, 50°C and 55°C) and increased with decrease in temperature reaching maximum of 172 IU/ml at 30°C. similarly, it was found that the optimal temperature for laccase production is between 25°C and 30°C (Pointing and Vrijmoed, 2000) and ,When cultivated fungi at temperatures higher than 30°C the activity of lignolytic enzymes was reduced.

Table 4 Effect of metal ions on the activity of laccase enzyme production

S. No.	Metal Ions	Enzyme Activity (IU/ml)	
		50 mM	100 mM
1	NaCl	102	88
2	KCl	68	74
3	$MgCl_2$	52	76
4	$CaCl_2$	172	206
5	$MnCl_2$	70	84
6	$CoCl_2$	64	28
7	$CuSo_4$	160	175
8	$ZnSo_4$	64	52
9	$CdCl_2$	54	38
10	$HgCl_2$	92	66

The interaction of metals with extracellular laccase was particularly important for the better understanding of the biotechnological processes of xenobiotic degradation. Therefore, the stability of laccase activity against several metal compounds was tested (Table 5).

Table 5 Effect of various solvent systems on the activity of laccase enzyme production

S. No.	Solvent	Enzyme Activity (IU/ml)
1	Ethanol	42
2	Formic Acid	74
3	Amylalcohol	76
4	Formeldehyde	28
5	Toluene	58
6	Aniline	10
7	Acetic Acid	34
8	Acetaldehyde	76

As reported from studies on the activity of enzyme at the concentration of 100 mM/ml of mixture, metal ions affected the enzyme activity. Supplementation of $CuSO_4$ at 100 mM concentration yielded high amounts of laccase (175 IU/ml) at an incubation period of 5 days. These findings are in agreement with previous reports showing that the addition of 100 mM $CuSO_4$ during the exponential growth phase of the fungus led to a remarkably increased laccase production (Galhaup *et al*., 2002; Rodriguez and Sanroman, 2005). Mg+2, Ca+ and Cu+2 enhanced the activity of enzyme whereas metal ions viz; Na+, Mn+2, Co+2, K+ and Cd+2 affected approximately 60-70% relatively activity , when the concentration was increased at 100 mM/ml of mixture all the ions greatly inhibited the activity except Ca+ and Cu+2.

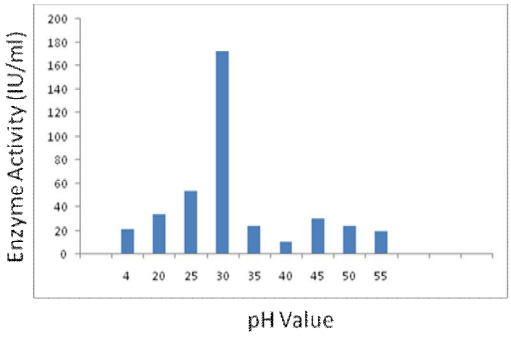

Table 6 Effect of various inhibitors on the activity of laccase enzyme production

S. No.	Inhibitors	Enzyme Activity (IU/ml)	
		50 mM	100 mM
1	EDTA	26	28
2	Sodium azide	10	8
3	DTT	68	70
4	SDS	6	4
5	Urea	42	54
6	β-mercaptoethanol	84	78

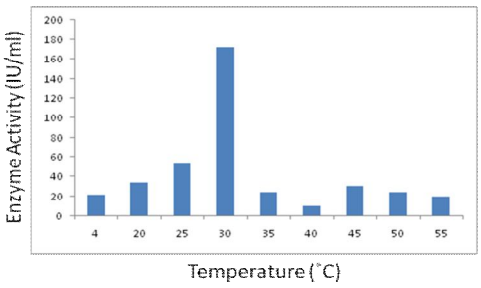

Figure 3 Effect of temperature on the activity of laccase enzyme production

Effect of a range of potent laccase inhibitors on the enzyme activity was tested with guaiacol as substrate. Addition of water miscible solvents such as ethanol caused a net decrease in enzyme activity. In general, organic solvents alter the pH of aqueous solution and there by affect the enzyme activity (Nowak *et al*., 2002). Laccase activity was completely inhibited by the common metalloenzyme inhibitor, sodium azide (NaN_3) at the concentration of 100 mM. It was reported that the binding of sodium azide to the types 2 and 3 copper sites affects internal electron transfer, thus inhibiting the activity of laccase (Ryan *et al*., 2003). EDTA which had moderately inhibited the purified laccase, At 100mM SDS concentration, complete activity was lost, hence it was much more effective denaturant. The Urea, β-mercaptoethanol and DTT were did not show any inhibitory effect.

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References

Coll, P. M., Abalos, J. M. F., Villanueva, J. R., Santamaria, R. and Perez, P. 1993. Purification and characterization Phenoloxidase (Laccase) from the Lignin-Degrading Basidiomycete PM1 (CECT 2971). Appl. Environ. Microbiol., 59: 2607-2613.

Couto, S. R. and Herrera, J. L. T. 2006. Industrial and biotechnological applications of laccases: A review. Biotechnol. Adv., 24: 500-513.

Dhoulib, A., Hamza, M., Zouari, H., Mechichi, T., Hamidi, R., Labat, M., Martinez, M. J. and Sayadi, S. 2005. Autochthonous fungal strains with high ligninolytic activities from Tunisian biotopes. Afr. J. Biotechnol., 4: 431-436.

Galhaup, C., Wagner, H., Hinterstoisser, B. and Haltrich, D. 2002. Increased production of laccase by the wood-degrading basidiomycete Trametes pubescens. Enzyme Microb Technol., 30: 529–536.

Gupta, 2009. Optimization of production and partial purification of laccase by *Phanerochaete chrysosporium* using submerged fermentation, International J of Microbiol Res, 1(2): 09-12.

Mayer, A. M. and Staples, R. C. 2002. Laccase: new functions for an old enzyme. Phytochemistry., 60: 551-565.

Pointing, S. B. and Vrijmoed, L. L. P. 2000. Decolorization of azo and triphenylmethane dyes by Pycnoporus sanguineus producing laccase as the sole phenoloxidase. World Journal of Microbiology and Biotechnology.,16(3): 317-318.

Rodakiewicz, N. J., Kasture, S. M., Dudek, B. and Haber, J. 2002. Effect of various water miscible solvents on enzymatic activity of fungal laccases. J Mol Catal B-Enzyme., 11: 1-11.

Rodriguez, C. S. and Sanroman, M. A. 2005. Coconut flesh: a novel raw material for laccase production by Trametes hirsuta under solid state conditions. Application to Lissamine Green B decolorization. J Food Eng., 71: 208–213.

Ryan, S., Schnitzhufer, W., Tzanov, T., Cavaco-Paulo, A. and Gubitz, G. M. 2003. An acid-stable laccase from Sclerotium rolfsii with potential for wool dye decolourization. Enzyme Microb Technol., 33: 766–770.

Thurston, C. 1994. The structure and function of fungal laccases. Microbiol., 140: 19-26.

Viswanath, B., Subhosh C. M., Pallavi, H. and Rajasekhar B. 2008. Screening and assessment of laccase producing fungi isolated from different environmental samples Afr J Biotech., 7 (8): 1129-1133

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