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SCREENING, ISOLATION, AND PRODUCTION OF FUNGAL LACCASE FROM SAW MILL SOIL OF OSMANABAD.

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ABSTRACT The rainforest and wood decaying habitats are the main sources for the several laccase producers, which include plant, bacteria, fungi and actinomycetes. Among these, several fungal species having laccase producing ability, which mainly includes wood rooting fungi that are often associated with lignin peroxidase or manganese dependent peroxidase or both. In view of this, we screened the natural habitats like saw mill soil and nearby places of Osmanabad district of Maharashtra for isolation of potential fungal species having laccase producing abilities. A total 45 fungal strains were isolated and screened for laccase production on solid media like Czapak dox agar medium containing guaiacol, tannic acid, bromophenol blue, and ABTS. Out of that 24 isolates showed positive results and among these S19 was found to be a potent laccase producing ability. The S19 fungus sp. subcultured on Czapak dox agar slant and screened for laccase enzyme production on solid state fermentation using wheat bran. In conclusion, we isolated the most potent laccase producing S19 fungus sp. from local natural habitats. A total 11.35 U/L of laccase enzyme obtained under optimized solid state fermentation. The enzyme may find potential applications in degradation of xenobiotics, decolourization of dyes, pulp and paper industries, depolymerisation of lignin, pigment degradation, and several other industrial processes.

KEYWORDS : Fungi, Guaiacol, Laccase, ABTS.

INTRODUCTION

Fungi play an important role in human's life by involving in industry, agriculture, medicine, food industry, textiles, and bio-remediation. Several enzymes, penicillin, secondary metabolites and large number of by-products are obtained from fungi. Among the enzymes laccase producing fungi are largely evaluated and used for industrial production of enzymes. Laccase: (benzenediol oxygen oxidoreductases, EC 1.10.3.2) is an oxidoreductase group of enzyme and able oxidize various aromatic compounds [1, 2, 3, 19]. Laccase was discovered a century ago in Japanese lacquer tree *Rhus vernicifera* and subsequently reported in other several plants. The laccases are multicopper oxidizing group of enzymes also known as copper oxidases or blue copper proteins [4, 24]. Several microorganisms possess laccase producing abilities and widely distributed among several white rot fungi having ability to grow on plants decaying biomasses [18, 20]. Presence of laccase enzymes have been widely reported in fungi, bacteria, plants and insects. Among the laccase producing fungi white rot Basidiomyceteous fungi are extensively studied due to their inherent application in many industries. Many fungal laccase are belong to glycoproteious in nature with covalently-linked carbohydrate moiety (10-45%) having average molecular weight ranging from 50-100 kDa. The carbohydrate contents of the enzyme may provide stability to the fungal laccase [1, 7, 8, 9, 15].

The enzyme laccase is well known to degrade several phenolic aromatic amines and phenolic compounds. Laccase has been extensively studied group of enzyme for the degradation of xenobiotics, decolourization of textile dyes, several biotechnological processes, depolymerisation of lignin, pigment degradation, pulp and paper bleaching, biosensor for biofuel cells, and many more [8, 14, 17, 22, 23]. They are defined as oxidoreductases, which oxidizes diphenol and allied substances. Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water. The laccase oxidizes phenolic compound to generate molecular oxygen and subsequently reduce to form water molecule. The enzyme having ability to oxidize substrates like monophenols, diphenols, methoxy substituted compounds, aromatic and aliphatic amines in to free radicals [5, 6, 11, 12, 19].

In view of this, we screened the natural habitats like saw mill soil and nearby places of Osmanabad district of Maharashtra for isolation of potential fungal species having laccase producing abilities.

MATERIALAND METHODS

Collection of samples

Soil samples from saw mill area of Osmanabad district and nearby places were collected in sterile plastic bags. The samples were immediately transferred to laboratory and stored under cold chain until use.

Isolation of fungi from soil

One gram of soil sample was dissolved in 100 ml of sterile distilled water and serially diluted up to 10^{-1} to 10^{-8} dilutions. 0.1 ml of each dilution was spread on sterile Czapak dox agar medium plate containing 0.02% Guaiacol and incubated at 30^{0}C. The Guaiacol containing plates were observed for development of reddish brown halo around isolated colonies up to seven days. After observation of zone the fungal colonies are isolated and repeatedly sub cultured until pure cultures were obtained. After isolation the pure culture it was stored at 2-8^{0}C on Czapak dox agar slants.

Screening for potent laccase producing strains

The indicator components like ABTS (2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (0.05% w/v), Bromophenol blue (0.05 w/v), Gallic acid (0.5% w/v) and Tannic acid (0.5% w/v) were mixed in sterile Czapak dox agar. The respective indicator agar plates inoculated with isolated fungal strains to identify potent laccase producing fungal strains. After incubation colonies showing green colored zones around colony for ABTS indicator, clear zone around bromophenol blue indicator, dark brown colored zone around colony for guaiacol, tannic acid and gallic acid indicator were picked up and identified as positive laccase producing fungal strains.

Production of fungal laccase

The solid state fermentation was carried out by using wheat bran obtained from local farm area of Osmanabad district. The solid state media contained 5.0g wheat bran and 5.0 ml of basal medium containing ingredients (g/L) (2.0g yeast extract, 2.0g Ammonium nitrate 0.8g KH_2PO_4, 0.75g K_2HPO_4, 0.5g $MgSO_4$. 0.002g $ZnSO_4$, 0.0005g $FeSO_4$, 0.05g $MnSO_4$, 0.006g $CaCl_2$). 5.0g of wheat bran weighed separately into six 250 ml Erlenmeyer flasks and moisten with 5.0 ml of the basal medium to wet the wheat bran, then sterilized at 121^{0}C for 15 min. After sterilization media flasks were inoculated with 2 ml of spore suspension prepared from a seven days old Czapek dox agar plate of the culture grown at 30°C. Sterile distilled water containing 0.1% (v/v) Tween 80 was used to prepare fungal spore suspension. Inoculated flasks were incubated at 30°C under static conditions for 6 days. Every day one flasks was removed and added

with 25 ml of 0.1M acetate buffer (pH 5.0) and filtered through muslin cloth by squeezing to extract sample up to six days. The extract was centrifuged at 10000 rpm for 10 min and clear supernatant was used as a crude enzyme for further studies.

Laccase assay

The laccase activity assay performed according to methodology of Rasera *et al.,* 2009 using, 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) ABTS as a substrate. The reaction mixture contained 1200µl of sodium acetate buffer (0.1 M, pH 5.0); 0.5mM 500µl of ABTS; 500µl of culture filtrate to obtain 2200 µl final volume. For blank, 500µl of water was added instead of crude enzyme sample. The mixture was then incubated for 3 min at 30^{0}C. The absorbance was measured immediately within 10 min at 420 nm with molar extinction coefficient [420] = 36,000M^{-1}. Laccase activity was defined as an enzyme that catalyses the conversion of 1 mole of ABTS per 10 min.

Laccase activity can be calculated by the formula

$$U/L = \frac{\Delta E \times Vt \times Df}{\times d \times Vs}$$

ΔE= absorbance at 420 nm

= extinction coefficient of ABTS = 36000 $M^{-1}gm^{-1}$

d = being the layer thickness [cm] in your cell that the light has to pass.

Vt = total volume of the sample

Vs= total volume of the enzyme stocks solution added in the ABTS stock solution

Df = Dilution factor

Optimization laccase production using various Agro waste and inducers:

The effect of various agro waste products (starch, rice bran, gram husk, and rice husk) on the laccase production were studied by substituting wheat bran at the concentration of 5.0gm in media. The effect of nitrogen was studied by replacing 2.0g of Ammonium nitrate (w/v) with various nitrogen sources (beef extract, yeast extract, urea, soy flour, and potassium nitrate) in the final basal medium. The laccase activity was assayed after 24, 48, 72, 96, 120, 144 h growth.

Effect of pH and temperature on laccase productivity

The optimum pH and temperature for maximum laccase production were studied by adjusting media pH (3, 4, 5, 6, 7 and 8) and effect of temperature was determined by incubating media flask at various temperatures (20, 30, 40, 50 and 60^{o}C). The laccase activity was assayed after 96 h growth under standard assay conditions.

Effect of inducers on enzyme production

The effect of various inducers (2, 5- xylidine, lignin, veratryl alcohol, and vanillin) were determined by assaying the optimized cultural media flask at different inducers to increase laccase activity

RESULTS AND DISCUSSION

Screening, isolation, identification, and production of enzyme

The present study was mainly targeted to screen laccase producing fungi from the different environmental samples from Osmanabad, India. A total 45 fungi were isolated by based on the results shown on Czapak dox agar lates containing ABTS, bromophenol blue, guaiacol, tannic acid and gallic acid indicator and their distinct morphology from the saw mill soil. The results of the screening tests are dipcted Table 1. All the isolated fungal strains were screened for laccase producing abilities on solid state fermentation media using wheat bran as the main lignolytic substrate.

Table 1: Fungal isolate screening result for laccase producers (+ indicates good laccase producers, + indicates moderate laccase producer and - indicates no laccase producer)

Strain	Indicators			
	Guaiacol	Tannic acid	ABTS	Brophinol blue
S1	+	+	+	+
S2	+	-	-	+
S3	+	-	+	-
S4	+	-	+	+
S5	+	-	-	+
S6	-	-	+	+
S7	+	-	+	-
S8	-	-	-	+
S9	+	-	-	-
S10	-	-	+	+
S11	+	+	+	+
S11	+	-	-	-
S12	+	-	-	+
S13	+	-	-	+
S14	+	-	-	-
S15	+	-	-	+
S16	+	-	-	+
S17	+	+	+	+
S18	+	+	+	+
S19	+	+	+	+
S20	+	-	-	+
S21	+	-	+	-
S22	+	-	-	+
S23	-	-	+	-
S24	+	-	-	+
S25	+	-	-	+
S26	+	-	-	+
S27	+	+	-	+
S28	+	-	-	+
S29	+	-	-	-
S30	+	-	-	+

S31	+	-	+	+
S32	+	-	-	-
S33	+	-	-	+
S34	+	-	-	+
S35	+	-	-	+
S36	+	-	-	-
S37	+	-	-	+
S38	+	-	+	+
S39	+	-	+	+
S40	+	-	-	-
S41	+	+	+	+
S42	+	-	+	+
S43	+	+	+	+
S44	+	-	+	+
S45	+	-	+	+

Several researchers reported ABTS and Guaiacol is considered to be more efficient screening indicator of laccase producer fungi as far as cost, time and method is concerned [10, 13, 21]. Tannic acid gave relatively weak positive reactions with many samples. However, the ability of most positive microbes to form brown color on tannic acid weakened during sub culturing of microbes. In view of this, the strains shown positive results on ABTS, Guaiacol and tannic acid plates are considered as laccase positive strains and subcultured on Czapak dox agar slants. Based on the screening results S1, S11, S17, S18, S19, S41, and S43 strains were selected for the further analysis of potent laccase producers.

The isolated strains are grown on solid state medium for the production of laccase under static condition. Among seven isolated strains S19 observed as the potent laccase producer with maximum 9.90 U/L laccase activities Table 2. The S19 fungal strain was then subjected for the primary identification by microscopic analysis and identified as *Aspergillus* sp.

Table 2: Time course study of Laccase production from isolated fungal strains.

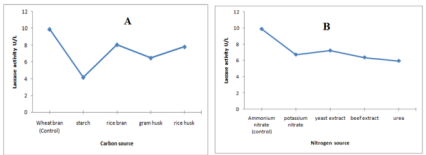
Hrs → / Strains ↓	24 (U/L)	48 (U/L)	72 (U/L)	96 (U/L)	120 (U/L)	144 (U/L)
S1	0.66	1.32	2.64	3.828	6.468	6.732
S11	0.528	2.244	3.564	4.62	4.62	5.412
S17	0.396	2.64	4.092	6.6	6.468	6.6
S18	0.66	3.96	4.092	5.28	6.6	6.6
S19	1.32	2.64	6.6	9.9	9.504	9.24
S41	0.264	0.792	0.396	6.468	7.92	5.94
S43	0.264	0.924	2.64	5.016	6.6	5.94

The selection of lignocellulosic substrate under solid-state fermentations is very important for the efficient and cost effective production of the ligninolytic enzymes. The maximum laccase activity was observed at 96 hr incubation and it was 9.90 U/L.

Media optimization for laccase production

The S19 fungal strain showed maximum enzyme activity among all fungal strains. The media optimization study performed using various ingradients to find suitable media for maximum laccase production. The S19 strain showed maximum 9.90 U/L enzyme productivity in presence of wheat bran as a major carbon source and ammonium nitrate as the best nitrogen source (Fig. 1).

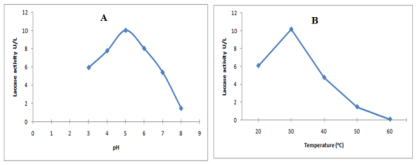
Fig.1. Effect of various carbon sources on the laccase production by S19 strain. Effect of various carbon and nitrogen sources on the laccase production by the S19 strain. (A) Demonstrate effect of carbon sources, whereas; (B) depicts the effect of nitrogen sources on enzyme production.



Effect of incubation temperature and pH on enzyme production.

The effect of pH and temperature on the productivity of laccase enzyme was studied using optimized media incubated at different pH and temperatures. The maximum 10.032 U/L laccase production was observed at pH 5.0 and 10.164 U/L laccase production obtained at temperature 30^0 C. The effect of pH and temperatures on laccase production was indicated in Fig. 2.

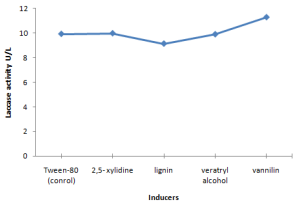
Fig.2. Effect of pH and temperature on growth and laccase production. (A) Demonstrate effect of pH on laccase production, whereas; (B) depicts the effect of temperature on enzyme production.



Effect of inducers on laccase production

The laccase enzyme producing ability of S19 strain increased in the presence of Tween-80 and vanillin as the inducers. A total 11.35 U/L of enzyme activity obtained after 96 hr incubation in optimized media (Fig. 3).

Fig.3. Effect of various enzyme inducers on the laccase production.



The wheat bran is an economical source of raw material and abundantly available at low cost agricultural by products as substrates for the laccase production. The strain S19 produced maximum enzyme in the optimized media at pH 5.0 at 30^0 C. The enzyme productivity of S19 strain induced in the presence of Tween-80 and vanillin. However several researchers reported various agricultural wastes may be used as a source of raw material for the production of laccase.

CONCLUSION

In the present study we isolated a total 45 fungal strains showed positive laccase activity on various indicators. Among these seven isolates were tested for production of laccase on solid state fermentation and out of that isolate no. S19 observed with significant laccase producing ability with 9.90 U/L. Further, the media was optimized for isolated strain to obtain maximum laccase production using various agricultural wastes and inducers. A optimized solid state media contained 5.0g wheat bran and 5.0 ml of basal medium containing ingredients (g/L) (2.0g yeast extract, 2.0g Ammonium nitrate 0.8g KH_2PO_4, 0.75g K_2HPO_4, 0.5g $MgSO_4$. 0.002g $ZnSO_4$,

0.0005g $FeSO_4$, 0.05g $MnSO_4$ 0.006g $CaCl_2$ 0.02 mM Tween-80, and vanillin 1.0 mM). Total 11.35 U/L of enzyme activity obtained after 96 hr incubation in optimized media. The laccase produced from S19 may find potential applications in various decolourizations of textile dyes, biotechnological processes, depolymerisation of lignin, pulp and paper bleaching industry and several other industrial processes. Further, a complete Strain identification, pilot scale enzyme production and characterization work is required to check the performance of the laccase produced from S19.

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