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Research article

ANALYSIS OF FUNGAL CULTURES ISOLATED FROM ANAMALAI HILLS FOR LACCASE ENZYME PRODUCTION EFFECT ON DYE DECOLORIZATION, ANTIMICROBIAL ACTIVITY

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ABSTRACT: The productions of laccase enzyme from four Ascomycetes species were studied in detail by in silico and in vitro analyses. Initial screening of crude enzyme showed complete oxidation of ABTS and Guaiacol after 7 days of incubation. *Alternaria arborescence* showed maximum production of enzyme (800U/l) at 30^0 C in 4.5 pH followed by *Fusarium oxysporium* (JQ 950134) with 600 U/l at 45^0 C in 5 pH after 15 days of incubation. Enzymes were purified by ammonium sulfate precipitation, Enzymes were subjected to analyse antimicrobial and phytotoxicity study. All the laccase from four species shows zone of inhibition which was compared with the standard antibiotic disc kanamycin.
Key words: laccase, antimicrobial, phytotoxicity, Guaiacol, ABTS, Fusarium.

INTRODUCTION

About 10-15% of the dyestuffs used during dyeing process do not bind to the fibre and that the traditional textile finishing industry consumes about 100 litres of water to process about 1kg of textile fibre [14,4]. Several physicochemical methods ranging from coagulation/adsorption, ozonation, electrolysis, photocatalytic processes, reverse osmosis, advanced oxidation, membrane filtration, ion exchange and so on, have been employed in the treatment of dye containing wastewater to achieve decolorization, degradation and detoxification [12].However, the major disadvantages of the methods due to high energy consumption,very expensive, inability to remove recalcitrant azo dyes and/or their organic metabolitescompletely, generation of a significant amount of sludge, the use of chemicals which may cause secondary pollution problems. Environmental and public health impacts of effluent discharged from textile dye producing and textile dyes consuming industries to neighbouring water bodies and wastewater treatment systems are of serious concern, as a result of its large volume, colour and presence of chemicals used as raw materials, and the remaining unused chemicals during the process reaction [2]. The use of fungal enzymes in the diverse fields of biotechnological based industries has been increased in recent years. The search for efficient and green oxidation technologies has increased the interest in the use of enzymes to replace the conventional non biological methods. Among the different existing oxidant enzymes the fungal laccases (benzenediol:oxygen:oxidoreductases: EC 1.10.3.2) have been are of great interest since they have low substrate specificity; do not require the addition or synthesis of a low molecular weight cofactor ; more stable and utilize the enzyme in an immobilized state. Laccases are belonging to the group of oxidases also called as a blue copper oxidases or blue copper proteins. They are sometimes refers to as polyphenol oxidases (PPOs). They are extra cellular enzymes. Laccases catalyze the oxidation of a variety of phenolic compounds diamines and aromatic amines pigment formation, lignin degradation and detoxification [13]. Though the species fusarium is a pathogen of plants animal and humans. the Crude metabolites of Fungus Fusarium sp.,considerable antimicrobial activity against some clinically important microorganisms.[9,17].Some non pathogenic strains have been used as abiological control agent [11].Several *Fusarium* species isolated as endophytes have been reported to produce metabolites with antimicrobial and anticancer activity [17,3,7]. Mushroom metabolites are usually used as adaptogens and immunostimulants and they are now considered one of the most useful antitumor agents for clinical uses [6].

The use of inexpensive sources (industrial and agricultural wastes) for laccase enzyme production can is the need of the hour.

In the present study four fungal isolates have been taken to analyse their ability to produce laccase enzyme and dye Decolarization and Phytotoxicity and its antimicrobial activity.

MATERIALS AND METHODS

Collection screening and cultivation of the fungus

Soil samples were collected at Anamalai Hills Village Coimbatore District Tamil nadu India, Located at 3500 feet above the sea level coordinates Latitude : 10 22' 00" Longitude : 76 58' 00" Moderate rain fall region with a normal Temperature of 30^0 - 19^0 C .The site is covered by mixed trees, and shrubs, turn over of the organic matter in such soil is rapid.

Ten soil samples from different locations of the above mentioned place were collected. 1g of each samples were serially diluted upto 10^{-6} dilutions. Each dilutions were inoculated on Rose Bengal Agar Plate using spread plate technique; After 5 days of incubation 30 different individual colonies were selected aseptically and again inoculated in to a Petri plate containing B& K Agar medium which is composed of glucose 10g, peptone 2g, yeast extract 1g, agar 18 g and 4mM ABTS in 1l of 50 % sea water. (Atalla et al. 2010) Inoculated Plates were incubated at 25^0 C for 7 days. The Production of green colour in and around the fungal colony was considered as a positive reaction resulting from ABTS oxidation.

The diameter of colored zone, growth rate was measured in mm. Positive fungal Cultures were subcultred in slants for further research work.

Primary inoculum

The cultures in the slants were grown in altered basal medium [16] which consisted of (all in g/L) 0.2 KH_2PO_4, 0.5 $MgSO_4$, 0.5 $NH_4H_2PO_4$, 0.1 Yeast-extract and 10.0 glucose with 0.1% stock mineral medium containing (all in g/L) 1.4 $ZnSO_4.7H_2O$ and 1 $FeSO_4.7H_2O$ supplemented with sole carbon source glucose (l%, w/v), L-glutamic acid [0.07% (w/v); neutralized with KOH] as nitrogen source, and thiamin (0.2 mg 1-l) and biotin (0.02 mg 1-l) to satisfy vitamin requirements. These cultures were grown for 6 weeks, then the mycelium was removed, its dry weight was determined, and the laccase activity of the culture filtrate was measured. This was done duplicate for strain improvement.

Small-scale production

3 mL mycelial or conidial suspension got by mixing with 10 mL sterile distilled water was inoculated to 50 mL altered Basal medium and were left to grow in a shaking incubator at 150 r.p.m at 30°C for 12 days. After 4 days of growth period, 0.01 M of 2, 5 Xylidine (Sigma Co.) was added to the culture flask to induce the laccase synthesis. After the incubation period, the contents of the each flask were filtered through Whatmann filter paper No.1 and the filtrate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant thus obtained was treated as the enzyme extract for the study.

Enzyme Assay

The tubes were incubated at 37°C for 15 minutes. The blank contained substrate and crude enzyme which were inactivated by boiling. The optical densities of the tubes recorded for the amount of enzyme activity at 530 nm.

In order to optimize the culture condition the pH of the medium was adjusted to desired value by addition of either 0.1 M HCl or 2, 5 M NaOH to reach the proposed value. Laccase activity was estimated at pH 3- 11. To obtain pH 3, Glycine - HCl buffer (0.2M), for pH 4 and 5 acetate buffer at 0.2M concentration, for pH 6 and 7 phosphate buffer (0.2M), for pH 8 and 9 Glycine – NaOH buffer (0.2M) and carbonate- bicarbonate buffer (0.2M) for pH 10, 11 were used.

Temperature study

The effect of temperature on laccase activity was determined following the oxidation of Guaiacol at temperature of 20 to 50ºC. The incubation times of the conical flasks were done on various temperatures such as (20, 25, 30, 35, 40, 45, 50ºC).

Precipitation of Protein by ammonium sulfate

The crude fungal culture was filtered through Whatmann No 1 filter paper using a vacuum pump and the resulting filtrate was concentrated kept at 4º C. Proteins were precipitated by ammonium sulfate from the mycelia filtrate obtained after submerged culture fermentation. Solid ammonium sulfate was added slowly to the crude extract of each isolate to give 80% saturation and the solution was stirred gently for at least 1 hour at 4^0 C and then left to stand over night. The precipitate was collected by centrifugation at 8,000 g for 1 h at 4^0 C. The supernatants were discarded and the pellets were dissolved in Minimum amount of 5mM Bis tris (Bis – 2 – hydroxy ethyl) imino tris (hydroxy methyl) methane) HCL buffer pH 6.5.

Identification

DNA Isolation

Isolates were cultured on Sabouraud agar plates at 30°C for 48 hr. Colonies harvested using an inoculation loop and resuspend in 1 ml fungal saline(0.9% w/v NaCl) Centrifuged the cell pellets collected500 µl Lyticase solution added to the culture and incubate at 37°C for 30 min to produce spheroplasts. 10 U/ml Lyticase solution contains the mixture of 50 mM Tris, pH 7.5, 10 mM EDTA 28 mM ß-mercaptoethanol Centrifuged at 10,000 RPM for 10 min. Discard the supernatant the pellets resuspend in 180 µl Buffer ATL and 20 µl Proteinase K stock solution Incubate at 55°C for 15 min Elute the DNA once with 50 µl distilled water. Resulted DNA samples were amplified in PCR.

Sequencing.

All the four fungal cultures were sequenced using the primers ITSF (5'TCCGTAGGTGAACCTGCG3') ITSR (5'TCCTCGGCTTATTGATATTGATATGC3'), Carried out by 16srRNA sequences. For tentative identification, fungal sequences were compared with NCBI database.

Decolarization of Textile Mill Effluents and Dyes

The culture filtrate with laccase activity was used as a source of enzymes to test its efficiency in decolorization in the efficiency in decolorization of effluents and dyes. This in brief was carried out by incubating the enzyme with effluents for 6 to 12 h at 45^0C in duplicates. The percentage of decolorization achieved was calculated with reference to the control samples that were not treated with enzyme.

Phytotoxicity Study

Phytotoxicity tests were conducted to assess the impact of textile effluents on vegetation and to explore the possible reuse of the treated solution in the irrigation of agriculture fields. Tests were carried out on Capsicum *annum* L plants which are most sensitive, fast growing and commonly used in Indian agriculture: seeds of each plant were sowed. A toxicity study was done by watering (5 mL) the seeds of each plant with Textile mill effluents and extracted enzyme sample. The control was run by watering the seeds with distilled water. The watering was done twice a day. Germination and length of shoot and root were recorded after 13 days.

Antimicrobial Activity

Microorganisms used in this study consisted of both gram positive and gram negative bacteria. Bacteria were grown and maintained on Luria – bertani (LB) slants. Inoculated agar slants were incubated at 37^0C.

Disc Diffusion Assay

The antibacterial activity assay was based on the disc diffusion assay using *Bacterial* cell suspension grown at 37^0 C in LB media. The exponentially growing bacteria (OD_{600} = 0.5, 10^{12} cfu/mL) were mixed with melted warm LB agar and pour in to the Petri dishes. Sterile paper discs were placed on the agar. Then the solution of enzyme was added to each disc. Disc with distilled water treated as control and 1 mg kanamycin discs were used as positive controls. The Plates were incubated at 37º C for 24 h. After the incubation period the zone of inhibition was measured.

RESULTS AND DISCUSSION

Out of 30 fungi isolated from forestry soil samples, 4 fungi showed positive reaction when grown in the presence of Guaiacol and ABTS. The edge of the fungal colonies showed the green to blue colour. Mycelial growth characteristics were measured at the 7th day of incubation period (Table 1). Under the culture condition in this study, laccase was the major ligninolytic enzyme, and activities of lignin peroxidase and manganese peroxide were not detectable. (Table 2) These results are in consistent with Hou *et al* [8] findings, who demonstrated that laccase was the only ligninolytic enzyme activity detected in the supernatant when the fungus was grown in liquid culture with or without shaking. The effect of pH, Temperature on enzyme activity were analysed.(Table 3) .All the four cultures show it maximum production of activity in Acidic pH with a temperature range between 35 – 45° C. Cultures were sequenced for its identification using ITSF and ITSR primers and were found that all the four sequences were belonging to Ascomycetes family. Culture C1 was found to be *Aspergillus niger*, C2 *Fusarium oxysporium,* C4 *Alternaria arborescence* and C6 *Penicillium marnefei*

For analysing the antimicrobial activity and phytotoxicity study enzyme from *Fusarium oxysporium* species have been used. The sequence of this culturis submitted to NCBI .Accession number JQ950134

500 µl culture supernatant were added to the dyes and Textile effluent A (TEA) with 10% final concentration. The day of addition of effluents to the pre grown cultures was considered as a day zero for all the color measurements. Decolarization of the TEA and dyes were monitored by changes in the absorbance. For Trypan blue 599 nm crytal violet 589 nm Congo red 486 nm were used. Percentage decolorization was calculated as the final concentration of the dye in the medium on day 0 was considered to be 100%. The extend of decrease of the spectrum area with respect to that of the control (0 day sample) after 6 and 12 hours. All the values are mean of two replicates Decolourization of effluents achieved with in 6- 12 hours by incubating the culture supernatant containing laccase enzyme (Table 4). How ever on incubation of the culture supernatant with textile effluent, reduction in color was seen in the initial 2 hour after which there was slow in further reduction in color. Addition of redox mediators and inducers may improve this situation.

This assay was applied to evaluate the phytotoxicity of plant growing media based on the germination percentage of seeds. The germination percentage combines measurements of relative root and shoot elongation as both are sensitive to the presence of phytotoxic compounds (Table 5). Although several species have been traditionally used for evaluating phytotoxicity, there are no standardized seed species in use worldwide [10]

In antimicrobial activity (Table 6), the enzyme could inhibit both gram positive and gram negative bacteria and positive control kanamycin shows better zone of clearance. Compared to gram positive bacteria, gram negative bacteria shows low susceptibility due to the presence of lipopolysaccharides active as a protective barrier against it. [5]

In conclusion, the present study untreated dyeing effluents may cause serious environmental problems and health hazards. They are being discharged in water bodies and this water could be used for agriculture. Thus, it is of immediate concern to assess the phytotoxicity of the effluent before and after degradation. These results confirm that this enzyme might be useful in the textile dye effluent treatment, and in antimicrobial activity.

Table 1: Mycelial Growth Characteristics measured at 7th day of Cultivation

Selected fungal isolates	Colour Zone diameter (mm)	Fungal colony diameter (mm)
(C1) *Aspergillus niger*	20	17
(C2) *F. oxysporium*	21.5	29
(C4) *Alternaria arborescens*	18	20
(C6) *Penicillium mameffei*	22	18

* The values are mean of three replicates
* ND: not detected

Table 2: Quantitative estimation of Lignin-degrading enzymes production

selected fungal isolates	Laccase (IU/l)	Lignin peroxidase (U/ml)	Mn dependent peroxidase (U/ml)
(C1) *Aspergillus niger*	360	ND	ND
(C2)*Fusarium oxysporium*	600	ND	ND
(C4) *Alternaria arborescens*	800	ND	ND
(C6)*Penicillium marneffei*	500	ND	ND

Table 3: Effect of pH, Temperature and Enzyme activity

Culture Name	pH	Temp (^{O}C)	Enzyme Activity (IU/l)
C1	6	35	360
C2	5	45	600
C4	4.5	35	800
C6	6	40	500

Table 4: Decolorization of Dyes and Effluents using Enzyme

Dye & Effluent	% of Decolorization by the culture Supernatant	
	Hours	
	6	12
Trypan Blue (0.02%)	18	21
Crytal Violet (0.02%)	30	40
Congo Red (0.02%)	48	41
TEA (10%)	11	13

Table 5.Phytotoxicity Study

	% Of germinat	Plumule Length(cm)	Radical Length (cm)
Water	80	4.52 ±0.41	5.30 ± 0.10
TEA	30	0.83 ± 0.55	1.11 ± 0.46
Enzyme treat	80	3.27 ± 0.3*	2.10 ± 0.49

Phtotoxicity study in GreenChilli after 13 days of Growth .Values are mean of germinated seeds of three experiments, SEM, significantly different from the control (seeds germinated in water) at *$P < 0.05$ by one way analysis of variance (ANOVA) with Tukey-Kramer multiple comparison test.

Table 6: Antibacterial assessment by agar diffusion method

Organism Used	Zone of inhibition in cm	Kanamycin Disc	Control
Bacillus cereus	2.8	3.0±0	-
Bacillus subtilis	2.5±0.19	3.0±0	-
Pseudomonas aeroginosa	1.6 + 0.04	2.3±0	-
Salmonella typhi	1.8±0.11	2.3±0.5	-
Klebsiella pneumonia	1.8 ± 0.13	2.5±0.5	-
Escherichia coli	1.6±0.5	2.2±1.0	-
Shigella sp.,	1.8±1.1	2.0±0	-
Staphylococcus aureus	2.4±1.1	2.6±0	-
Streptococcus sp.,	2.1±1.1	2.8±0	-

(±) SD- standard deviation; (-) No inhibition Control – Disc with Water.

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